THE ROLE OF PROSTAGLANDINS DURING SEXUAL MATURATION, OVULATION AND SPERMIATION IN THE GOLDFISH, CARASSIUS AURATUS

BY

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ABSTRACT

The objective of this study was tc determine the involvement of prostaglandins in the sexual development of both male and female goldfish, Carassius auratus .

A chromatographic method was developed to separate the different prostaglandin groups. To standardize the procedure, extraction and separation recoveries were measured using tritiated-prostaglandins. Radioimmunoassay was used to measure the PGB1, PGE1 and $PGF2_{\alpha}$ in the plasma and gonad.

Initially, a seasonal study was undertaken to assess the importance of prostaglandins during sexual maturation. Samples of plasma and gonad were assayed monthly for prostaglandins (from December to March) from two groups of fish, one held under natural photoperiod and the other under long photoperiod (16L:8D). Although monthly variations occurred in all three prostaglandins examined, these changes did not correlate with changes in gonadal maturation.

Prostaglandins were then measured in serial plasma samples of non-gravid and ovulating female goldfish. Ovulation was induced in gravid fish by increasing the water temperature from 14° C to 20° C and by injecting human chorionic gonadotropin (HCG). It was found that:

1) PGF2 increased over 14 fold, 12 hours after the onset of ovulation (from pre-injection levels of 300 pg/ml to more than 4,000 pg/ml); however, this increase appeared to commence immediately after ovulation. There was no change in plasma $PGF2_{\alpha}$ levels in non-gravid control fish. The concentration of $PGF2_{\alpha}$ in the ovarian fluid was over 9,000

pg/ml.

2) PGE1 decreased almost three-fold between the time of HCG injection (an average of 10 hours before ovulation) and 24 hours later. The plasma PGE1 levels in the non-gravid females were up to 20 times less than the gravid ovulating females. The concentration of PGE1 in the ovarian fluid was 630 pg/ml.

3) PGB1 levels decreased in the plasma of non-gravid and ovulating goldfish, following HCG injection. The ovarian fluid contained 300 pg/ml of PGB1.

Parallel experiments were performed on male goldfish that were spermiating. There were no significant changes in plasma $PGF2_{\alpha}$ within 24 hours of HCG injection, whereas PGB1 decreased slightly (as for the females), and PGE1 increased significantly 10 hours after HCG injection.

The findings of this study suggest that PGF2 and PGE1 in the ovarian fluid are the agents controlling ovulation in the female goldfish and that corresponding levels in the blood contribute to other events associated with ovulation. The experiments on males indicate a possible role for PGE1 during spermiation; however, it is difficult to ascertain its precise involvement at present.

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Finally, I would like to dedicate my thesis to my parents, Mary and Ernest Bouffard, and I would like to thank them for their untiring support and encouragement throughout my studies.

1 GENERAL INTRODUCTION

1.1 HISTORICAL BACKGROUND

In 1930, Kurzrok and Lieb first described the physiolcgical actions of prostaglandins (PG). They had observed that human seminal fluid had an effect on isolated human uterine strips. Relaxation occurred in the uteri from fertile women, but stimulation occurred in the uterine strips from women with complete or long-standing infertility.

Goldblatt (1933) and Von Euler (1934, 1935, 1936, 1938, 1939) independently demonstrated the presence of an active principle in seminal plasma which stimulated smooth muscle and lowered blood pressure in rats. This substance reacted differently from other naturally occurring substances already known. This new compound was called prostaglandin, because of its occurrence in extracts from the prostrate and vesicular gland. However, Eliasson (1959) later found that the seminal vesicle was the main site of PG production, and nct the prostate.

In 1934, Von Euler found that the active principle in the seminal vesicle fluid could be extracted frcm an acid sclution with lipid solvents; however, it was also soluble in an alkaline medium. This simple purification step suggested that the principle was of an acid nature, and had properties similar to those of the fatty acids. At Von Euler's suggestion, Bergstrom (1949) confirmed the fatty acid nature of the prostaglandin, and realized that more than one unsaturated hydroxy-acid was present

in the sheep seminal fluid.

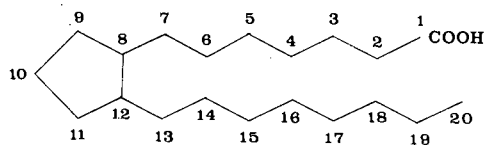
In 1957, prostaglandins were finally isolated in a crystalized form by Bergstromm and Sjovall. These substances were called PGF and PGE. Bergstromm's group went on to differentiate and isolate 13 other substances, all of which were based on the parent molecule, prostanoic acid. Subsequently large scale biosynthesis was successful, and prostaglandin research advanced rapidly into many areas of physiological, medical, pharmacological and biological inquiry.

1.2 NOMENCLATURE AND STRUCTURE

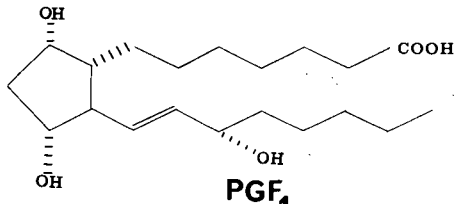
The term prostaglandin has remained in the literature although it is a misnomer. Variations on the term prostaglandin were introduced and include both natural compounds and synthetic analogues. The letters E and F were added to differentiate between the two compounds which separated out when an extraction was peformed with an ether phosphate buffer solution. The E substance was found in the ether fraction and the F in the phosphate buffer ("fosfat" in Swedish). The A and B designate the respective derivatives of an acid or basic treatment of PGE.

The stucture of the main prostaglandins and of the parent molecule, prostanoic acid, is give in Figure 1. The various prostaglandins differ only slighty in their structure, by modifications in two areas of the molecule - the side chains and the cyclopentane ring. The letters A, B, C, E or F indicate the changes in the cyclopentane ring, whereas the numeral subscript denotes the number and position of double bonds in the side

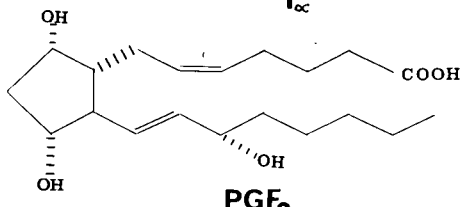
FIGURE 1: Molecular structure of various prostaglandins and their parent molecule, prostanoic acid.



PROSTANOIC ACID



$PGF_{1\alpha}$



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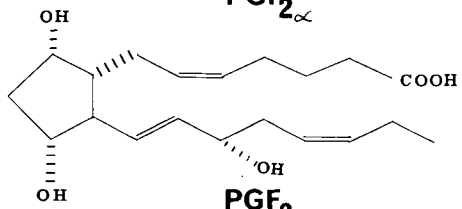
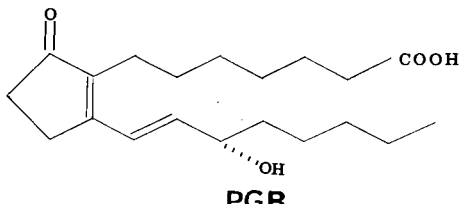
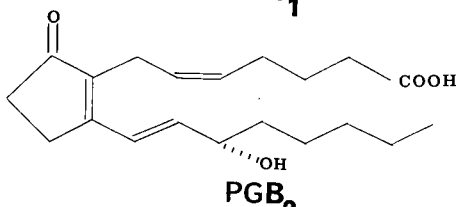


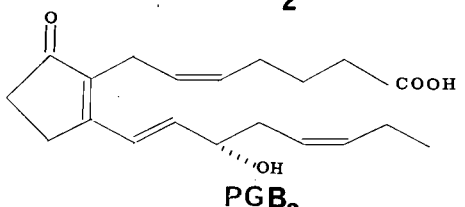
$PGF_{3\alpha}$



PGB_1



PGB_2



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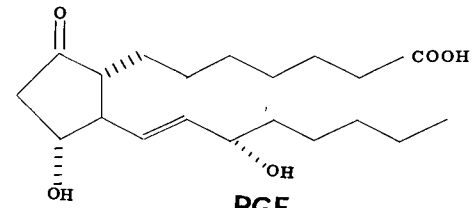
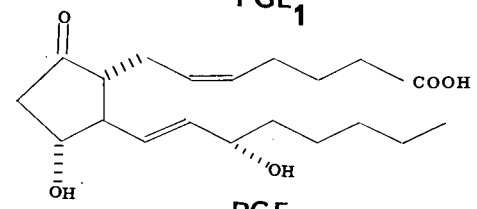


PGE_1



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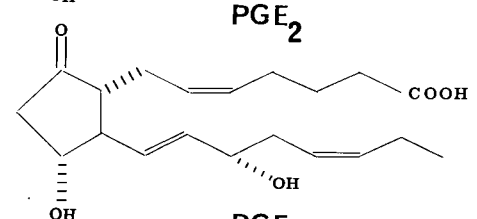

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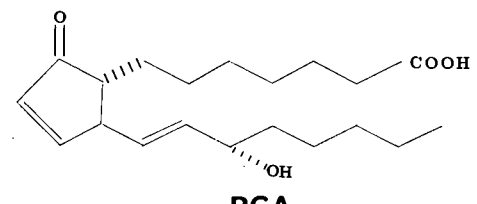

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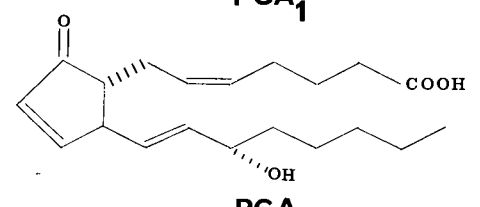

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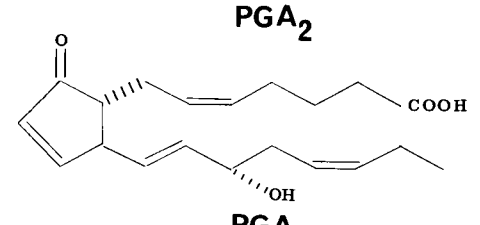


PGA_3

chain. The configuration of the naturally occurring prostaglandins is rigid. The A or B are only used when refering to PGF, and they designate the configuration at C 9; however, only the alpha series occurs naturally. There exists a number of derivatives and structural variants of prostaglandins, and these may be PG metabolites or synthetic compounds. In these cases, they are usually named after their closest structural relative, either the PG precursors, prostanoic acid, or the prostaglandins. Several of the PG precursors, such as the endoperoxides, the thromboxanes, and prostacyclin itself, are potent yet unstable compounds. However, their structure and nomenclature will not be outlined here.

1.3 OCCURRENCE AND RELEASE

Most of the work concerning prostaglandin synthesis has been done on mammals, and almost all tissues analysed have been shown to produce PGs. The first account of PGs in invertebrate tissues was in 1969, when Weinheimer and Spraggins reported two isomers of PGA2 in the gorgonian coral, *Plexaura homonalla*. Later, Light and Samuelsson (1972) demonstrated two more isomers of PGE2 in the same animal.

The first indication of PG production in non-mammalian vertebrates was demonstrated by Christ and Van Dorp (1972); these workers found PG synthetase activity in many animal tissues, including those of birds, fish and amphibians. In 1972, a general survey was performed by Bito who studied the uptake of labelled PGs in both invertebrate and vertebrate tissues. He

found that marine bivalves could accumulate tritiated-PG in most of their tissues and that other marine invertebrates could do so to a lesser extent. In addition, he showed that two species of elasmobranchs, and five marine teleosts could concentrate tritiated-PG in the choroid plexus and the liver. However, this study did not demonstrate that the tissues produced PG, but only that they could incoporate them into their systems.

There is much variation in the occurrence and concentration of a specific prostaglandin in different tissues. As a rule, the concentration is usually in the ng to pg range for each gram of tissue, or milliliter of fluid. One exception is seminal fluid, where the greatest variety of prostaglandins are found, and in the greatest concentration (approximatly 134ug/ml: Cenedella 1975).

1.4 BIOSYNTHESIS

The chemical resemblance of prostaglandins to arachidonic acid suggested that it could be a precursor of the prostaglandins. The first evidence that this was true came when VanDorp and Bergstromm (1964) found that homogenates of sheep vesicular gland incubated with arachidonic acid could generate PGE2. The enzyme complex concerned was called prostaglandin synthetase and appeared to be membrane-bound in the microsomal fraction of the cell. The synthetic pathway involved several steps and was controlled by a feedback mechanism.

Prostaglandin biosynthesis has several inhibitors notably acetyl salycic acid (asperin) and indomethacin. These inhibitors

appear to block the formation of cyclic prostaglandin derivatives at the enzyme level, rather than interfering with the availability of precursor acids.

Total chemical synthesis of PGs has been acheived for all the naturally occurring PG groups, and various "unnatural" sterioisomers have also been produced. Although biosynthesis has been used successfully to produce PGs, chemical synthesis is more efficient and also enables the preparation of PG analogues which have not been found in nature.

1.5 METABOLISM

The main sites of PG breakdown in the mammal are the lungs, liver, placenta, kidney, spleen and adipose tissue. Marazzy and Anderson (1974) described a 90 - 100% decrease of PGE and PGF after passage through the lung.

Although many enzymes are involved in PG inactivation, the main one is 15-hydroxy-PG-dehydrogenase. This enzyme has been found in the lung, as well as in most of the tissues mentioned above (Curtis-Prior 1976). As the name of the enzyme suggests, it catalyses the dehydrogenation of the hydroxyl group at the C-15 position thus greatly reducing the potency of the PG molecule. After this, several other enzymes continue the process of PG degradation.

1.6 MODE OF ACTION

Despite much research in this area, the precise mechanism of prostaglandin action is still far from clear. However, it is becoming more evident that PGs act at the molecular level via changes in the cyclonucleotides. Some of the evidence that both cyclic AMP and cyclic GMP are involved in the action of PGs will be outlined briefly.

Kuehl et al (1972) discovered that the PGEs seemed to be the most effective of the PGs in increasing cyclic AMP levels in the intact mouse ovary. This led to the discovery of membrane bound receptors in lipocytes which had a preferential affinity for PGEs (Kuehl and Humes 1972). The PGFs did not seem to stimulate cyclic AMP production to the same extent, and their affinity for the PGE receptors on the lipocytes was very weak. These findings led some investigators to look at the potential relationship between PGF and cyclic GMP. Powell et al (1974) discovered a PGF-sensitive receptor in ovine and human corpora lutea. Moreover, Dunham (1973) demonstrated an increase in the cyclic GMP/cyclic AMP ratio following application of $PGF2_{\alpha}$ to ovine and bovine veins, and a decrease following application of PGE2. These findings tie in well with observations of opposing actions of PGE and PGF on smooth muscle.

1.7 INVOLVEMENT OF PROSTAGLANDINS IN DIFFERENT AREAS

As stated previously, prostaglandins are found in most tissues, and their physiological actions are manifested in many areas. An overview of some of the research in the major areas

will be outlined here, and some aspects will be discussed in more detail.

1.7.1 Circulation

Prostaglandins seem to be involved in many aspects of circulatory homeostasis such as blood pressure, peripheral circulation and heart rate. Generally the PGEs decrease blood pressure in mammals; PGE1 is the most potent followed by PGE2 and PGE3. This decrease is brought about by peripheral vasodilation. However, PGE1 increases cardiac output, and raises coronary flow; this normally coincides with a fall in blood pressure. The PGEs have little or no effect on the rate and force of the isolated heart. However, they do seem to have a hypertensive effect in the kidney, and McGriff et al (1974) has suggested that PGE2, in particular, could regulate renal blood flow.

The PGFs are mainly concerned with non-vascular smooth muscle, although they do have some effect on the blood vessels. In genaral, when they do influence vascular muscle, they are vasoconstictors, but there is much species variation in these studies. Overall, the PGFs do not appear to play a major rcle in circulatory physiology.

1.7.2 Nervous tissue

Prostaglandins are present in the central nervous system, as well as in the cerebrospinal fluid, and PGF seems to predominate in both (Coceani and Wolf 1965). Many studies suggest that prostaglandins are implicated in both the central and peripheral nervous systems. The PGEs appear to be implicated in sympathetic neurotransmission (Hedqvist 1970) as well as in the mechanisms of thermoregulation and fever (Miltcn and Wendant 1970). Administration of PGEs to rats will increase body temperature very rapidly, and it is believed that the PGEs act as mediators of pyrogen-induced hyperthermia.

Both PGE1 and $PGF2_{\alpha}$ seem to affect the brain stem cardioregulatory centers (Kaplan et al, 1969), and they may also be involved with the regulation of the release of pituitary hormones (LeMaire et al 1974). PGE2 and $PGF2_{\alpha}$ are released peripherally following stimulation of both sympathetic and parasympathetic nerves, and the release appears to be post-synaptic (Hedqvist 1970).

1.7.3 Inflamation

Inflamation is a local tissue response to foreign stimuli, such as bateria or viruses, various chemicals or ultraviolet and infrared light. It may also be a component of the autoimmune reaction. Many factors are involved in inflamation and its associated symptoms, such as pain, oedema, erythema and heat. Histamine, bradykinin, serotonin, certain plasma glcbulins and PGs seem to play a role in the inflamatory process. Exactly how

they interact has not yet been elucidated.

There have been many instances were PGs have been identified after induced inflamation (Willis 1969; Piper and Vane 1969; Eakins et al 1972). PGE1 and PGF2$_\alpha$ have been identified in the inflamatory exudate during carrageenin-induced inflamation (Willis 1969). In addition, PGE1 and PGE2 have been associated with the pain and inflamation of arthritis. For this reason, aspirin and indomethacin, (PG inhibitors), are effective in relieving the symptoms of arthritic inflamation and pain.

The exact role of PGs during inflamation is not known. However, it has been postulated that they may potentiate vascular permeability by inducing leakage at the collecting venules (Kaley and Weiner 1971).

1.7.4 Prostaglandins in mammalian reproduction

The female reproductive cycle in mammals consists of an intricate series of endocrine steps leading from maturation of the oocyte(s) to ovulation. This cycle repeats itself if the egg(s) is not fertilized. However, if fertilization occurs and implantation is successful, another series of hormonal events takes place. The main organs involved in this cycle are the hypothalamus, pituitary, ovary and, to some extent, the adrenals. During pregnancy, the placenta takes over producton of most of the pregnancy-supporting hormones. The principal reproductive hormones are luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary, their respective releasing factors from the hypothalamus, and the steroids from

the ovary and the adrenals. When implantation occurs, chorionic gonadotropin is produced from the placenta and it maintains steroid production in the corpus luteum of the ovary.

The discovery of the involvement of prostaglandins in female reproduction has added a new dimension tc the understanding of this system. The importance of prostaglandins has been well documented in mammals, yet many questions remain unanswered, particularly in regard to their specific roles in normal reproductive physiology.

Prostaglandins have been identified in the reproductive organs and fluid of both male and female mammals. In the female, PGs have been found in the ovary, uterus, menstrual fluid and amniotic fluid. They seem to be involved in ovulation, luteolysis, menstruation, parturition and spontaneous abortion. They are present in the testis and accessory genital glands of male mammals, and human seminal fluid is the richest source of PGs yet known. They appear to be implicated in the processes of erection, ejaculation, sperm motility and morphology. The following discussion will attempt to cover some of the facets of PGs involvement in these major areas of male and female reproductive physiology.

1.7.4.1 Prostaglandins in female reproduction

A) ovulation

As the ovarian follicle matures, the PG levels within it increase (LeMaire et al 1975a). However, the exact relationship between PGs and oocyte maturation is not yet clear. Studies have shown that in the ovary, in the presence of PG inhibitors, both the growth and maturation of the follicle proceed normally, but that the follicles fail to rupture (Linder et al 1974). This suggested that PGs were not essential for oocyte maturatien but may be important in the release of the ovum. The necessity for PGs in the expulsion of the ovum was futher supported by the following findings: 1) PGEs and PGFs both increase after HCG injection in rabbits (LeMaire et al 1973), 2) indomethacin could block HCG induced ovulation (Yang 1973), 3) exogenous administration of $PGF2_{\alpha}$ could overcome the indomethacin block (Diaz-Infante et al 1974).

B) maintenance of the corpus luteum

Prostaglandins appear to play an important role in the control of the corpus luteum. However, the exact role is unclear. In vitro work indicates that PGs promote steroidogenesis in most cases. However, in vivo, they are luteolytic in most species. $PGF2_{\alpha}$ appears to be the most potent, althought PGE2 is also effective in promoting luteolysis.

Goldberg and Ramwell (1975) have discussed three different mechanisms for the luteolytic effects of the PGs. Their first proposal is that PGs interfere with the hormonal support of the corpus luteum. The second is that PGs could have a direct lytic

effect on the steroid producing cells in the ovary. Thirdly, they suggest that $PGF2_{\alpha}$ could induce hypoxia in the ovary by constricting the ovarian blood vessels. The studies so far have not delineated which, if any, of the above explanations is closest to reality. It is quite possible that the effects of PGs on the corpus luteum are species dependant, making it very difficult to ascertain if indeed any one mechanism applies.

C) motility of the reproductive tract

The smooth muscle stimulating properties of PGs would suggest that they could influence the motility of the reproductive tract. Indeed , it is well established that the PGEs inhibit spontaneous contractions of the fallopian tube, whereas PGFs are stimulatory. PGs could control tubal contractility and ova transport. This is very interesting, in light of the high concentration of PGs in the semen.

PGs have also been shown to affect uterine motility. The extent of stimulation is dependant on three factors:
1) the mode of administration, 2) the reproductive status of the person, and 3) which PG is given. In fact, PGs have been postulated to be involved in parturition, and have been used clinically to induce parturition and abortion.

1.7.4.2 Prostaglandins in males

Depite the abundance of PGs in the male reproductive system, relatively little research has been done in this area. A comprehensive review on PGs in male reproductive physiology has been written by Cenedella (1975).

PGs are present in relatively high concentration in the semen and in lower levels in the accessory reproductive tissues. In the seminal plasma of man, PGEs are predominant over the other prostaglandins, with an average concentration of 50 ug/ml. The PGF levels are lower at approximatly 7 ug/ml. The PGA and PGB are, in combination, about the same concentration as the PGEs. Bygdeman et al (1970) noted that seminal PGE levels were lower in men who were unaccountably infertile . It has been postulated that PGs could act internally or externally. Internally, PGs could influence sperm physiclogy, steroidogenesis, or the smooth muscle of the accessory sex glands. Externally, they could act upon the female reproductive tract, as was mentionned previously. Unfortunately, very few studies have dealt with these questions.

In males, PGs could play a role in regulating gcnadotropin release in the pituitary. Linder et al (1974) discussed the rapid increase in LH following the administration of $PGF2_{\alpha}$ to the male rat. PGs may also influence steroidogenesis in the male. Bartke et al (1973) demonstrated a significant decrease in testosterone levels following administration of PGs tc mice. PGE2 was also effective in inhibititing testosterone biosynthesis (Sakena et al 1973). As suggested in the female, the action of PGs may be due to a decrease in blood flow to the

testis.

The scarcity of information on the involvement of prostaglandins in the male reproductive system makes it very difficult to asses their potential importance at this time.

1.7.5 Prostaglandins in fish

Little research has been done on prostaglandins in non-mammalian animals. In view of the demonstrated importance of these substances in so many facets of physiology, it is likely that there is some phylogenetic overlap in their function in mammalian and non-mammalian systems.

However, some recent research on prostaglandins in fish has yielded very interesting results. Abramowitz and Chavin (1973) demonstrated that some of the PGs were very effective in eliciting melanocyte dispersion in the dermal melanophores of the goldfish, Carassius auratus L. They found that PGB1 and PGB2 produced the greatest response, followed by PGE1 and PGE2; however, $PGF1_{\alpha}$ and $PGF2_{\alpha}$ were only slightly effective. They suggested that the PGs were acting via the cyclic AMP system.

In that same year, Nomura, Ogata and Ito (1973), using various chromatographic techniques, were able to identify 3 prostaglandins, $PGF1_{\alpha}$, PGE1, and PGE2 in the testes of three species of fish: the flounder, Paralichthys olivaceus, the bluefin tuna, Thunnus thynnus and the chum salmon, Oncorhynchus keta . Two of these authors later reported the presence of PGE2 in the intestine of the shark, Triakis scyllia (Ogata and Nomura 1975). Peyraud-Waitzenegger et al (1975) also observed that PGE2

had both cardiovascular and ventilatory effects in the carp, Cyprinus carpio L., which they attributed to the activation of adenyl cyclase by PGE2.

The first research on reproduction concerning prostaglandins in fish was done be Stacey and Pandey (1975). They reported that indomethacin could block HCG-induced ovulation and spawning behaviour in the female goldfish, Carassius auratus . In addition, they found that excgenous administration of $PGF2_{\alpha}$, PGE1 or PGE2 could overcome the indomethacin block and induce ovulation and subsequent spawning. Their results suggested that prostaglandins could play a key role in ovulation in the goldfish.

This work was supported by the findings of Jalabert and Szollose (1975) who demonstrated that $PGF2_{\alpha}$ could induce in vitro ovulation of mature trout oocytes. However, they found that PGE2 had no visible effect on the system. In a review on in vitro oocyte maturation and ovulation in teleost fish, Jalabert (1975) proposed two sites for the action of $PGF2_{\alpha}$ on the oocyte: 1) the smooth muscle-like cells located in the theca and 2) non-muscle cells containing actin-like filaments. This proposition was made on the basis of observations by Szollose and Jalabert (unpublished data), that: 1) the response to $PGF2_{\alpha}$ was inhibited when calcium was absent in the incubation medium or when calcium influx was inhibited, and 2) that partial contraction occurred with $PGF2_{\alpha}$ after inhibition of smooth muscle. Recently Goetz and Theofan (1979) have completed a similar study using the perch, Perca flavescens . However, they reported that PGE1 and PGE2, as well as $PGF2_{\alpha}$ could stimulate in vitro ovulation of mature

oocytes, and that PGE2 was the most potent of the three.

The effect of prostaglandins on pituitary gonadotropin (GTH) release was studied by Peter and Billard (1976). They observed that injections of $PGF2_{\alpha}$ and PGE2 into third ventricle of the goldfish suppressed serum GTH secretion and that PGE1 had no effect. If indeed prostaglandins play a role in the initiation of ovulation, these findings would suggest that they would not act by influencing gonadotropin secretion prior to ovulation.

Singh and Singh (1977) reported that prostaglandins may affect thyroid activity in fish. They observed that administration of PGE1 and $PGF2_{\alpha}$ to the catfish, Heteropneustes fossilis , resulted in a reduction of thyroid activity which was coincedent with a decrease in TSH in the pituitary and an increase in TSH levels in the blood.

Kuo and Watanabe (1978) have investigated in vitro changes in cyclic AMP levels following the administration of PGE2 and $PGF2_{\alpha}$ to mature oocytes from the mullet, Mugil cephalus , both prostaglandins increased the level of cyclic AMP in the occyte, however, PGE2 was the most potent in this respect. This evidence suggests that the mode of action of the prostaglandins in fish is similar to that observed in mammals.

1.8 STATEMENT OF THE PROBLEM

Increasing information on prostaglandins in fish suggests that they are implicated in the process of gonadal development and/or final maturation. In an attempt to clarify this

relationship, the changes in three prostaglandins were measured in male and female goldfish, Carassius auratus, during three gonadal events: maturation, ovulation and spermiation. Prior to the present study, there was no evidence for the presence of prostaglandins in goldfish tissues. Prostaglandins have, however, been detected in the testis of some fish (Nomura Ogata and Ito 1973); although there has not even been an attempt to measure them in the ovaries.

2 GENERAL TECHNIQUES

2.1 FISH MAINTENANCE

Goldfish, Carassius auratus, of the common comet variety were purchased from Grassy Forks Fisheries Company, Martinsville, Indiana. The fish were kept in cutdoor aquaria, in flowing dechlorinated water, at 12-15° C and fed trout pellets two or three times a week ad libitum. Dead fish were removed immediately from the stock. Shipments of fish were kept fcr at least one month before experimentation.

2.2 REAGENTS AND GLASSWARE

Only spectroquality or redistilled reagents were used for extraction, chromatography and radioimmunoassay (RIA). Reagents and glassware were precooled to 5° C and were kept cn ice throughout the experiment. To avoid adherance of PGs to the glass, all glassware exposed for long periods to the tissues was siliconized (Siliclad, Clay Adams, N.Y.). Pipettes and micropipettes were not siliconized, as no difference was found in radioactivity after measuring ^{3}H-PGF2$_{\alpha}$ and ^{3}H-PGE2 with both siliconized and non-siliconized micropipettes. All glassware was presoaked in chromic acid or in a bacterioside-fungicide detergent (Mucosit, Mrez Co. Ltd.) before normal washing.

The formulae for the various experimental solutions are given in the Appendix. The phosphate buffer and the extraction solution were prepared just prior to use. The phosphate buffer

contained an antioxidant, p-aminosalicyclic acid, to reduce the breakdown of PGs.

2.3 COLLECTION AND EXTRACTION OF PROSTAGLANDINS

Fish were anesthetized in a 0.02% solution of tricane methane sulphonate (MS 222, Sandoz) and weighed pricr to sampling. The fish were handled in a moist paper towel to prevent excessive drying.

2.3.1 Extraction from plasma

Blood was taken via the caudal vessel using a 3.0 ml disposable plastic syringe with a 23 or 25 gauge needle, depending on the size of the fish. The syringe contained 0.1 ml of a sodium heparin solution (heparin, sodium salt, Sigma #H-3125, Sigma, St-Louis, Mo) made in phosphate buffer saline (2 IU/ml) with p-aminosalicyclic acid (PBS-A). The blood was put into a siliconized test tube and centrifuged at 2500g, at 4° C for 10 minutes (Refrigerated-Automatic centrifuge, Servall Co.). The plasma was removed and measured with a disposable pipette, and placed in a 15 ml conical centrifuge tube containing 3.0 ml of petroleum ether; this removed the neutral lipids. After removal of the ether phase, 3.0 ml of an extraction solution (ethyl acetate: isopropanol:0.2N HCl, 3:3:1) was added to the plasma and mixed thoroughly. To this mixture, 3.0 ml of distilled water and 2.0 ml of ethyl acetate were added and mixed; the mixture was then centrifuged at the medium speed

setting (in an International Model HN IEC: International Equipment Company, Needham Heights, Mass.), at 4° C for 10 minutes . The organic layer was collected in a labelled test tube and kept on ice. An additionnal 3.0 ml of ethyl acetate was added to the centrifuge tube, mixed, and centrifuged as before. This second organic layer was combined with the first and evaporated under a stream of nitrogen at 40° C. The residue was covered with Parafilm (Parafilm, American Can Co., Dixie/Marathon, Greenwich, CT 06830), and kept at -30° C until the chromatography step.

2.3.2 Extraction from the gonad

The gonad was removed and weighed in cold PBS-A. A sample of approximatly one gram was dissected out quickly, and homogenized over ice in 1.0 ml PBS-A and 3.0 ml of the extraction solution given above (Thomas glass homogenising tube, #A30677, Philadelphia, Pa. and mechanized using a Cole-Parker motor, model R2R64, Cole-Parker Istruments and Equipment Co., Chicago, Il.). The homogenate was then added to 2.0 ml ethyl acetate and 3.0 ml distilled water in a 15 ml conical centrifuge tube, and processed in the same fashion as was described for plasma. Some of the remaining tissue was used for histology.

2.4 HISTOLOGY

Samples of the ovary and the testis were taken from each fish and fixed in either Bouin's solution, or in a solution of 2% gluteraldehyde with 1% formalin in PBS. Following fixation, the tissues were dehydrated and embedded in paraffin; and sectioned at 7um. The sections were stained with Mallory's Triple Stain or Hematoxylin-Eosin, for determination of sexual maturity.

2.5 COLUMN CHROMATOGRAPHY

The extracted samples contained a mixture of prostaglandins. Silicic acid chromatography was performed on all the samples in order to separate out the different PG groups from each sample. The basis for this separation on silicic acid is that the PGs have different polarities, and by changing the polarity of the eluting solvents it is possible to separate the three major PG groups. A modification of the Jaffe and Behrman (1974) technique was used, and will be described here. The efficiency of the extraction procedure, and of the column chromatography elution will be outlined in the section on Preliminary Work. The following modifications of the Jaffe and Behrman's method were used throughout these studies.

2.5.1 Preparation of the column

Silicic acid (Sil-A-200, Mesh 60-200, lot#115C-0185, Sigma, St-Louis, Mo.) was first suspended in distilled water and decanted several times to remove the fines. It was then dried at 115°C and maintained at this temperature until use.

A 0.5 g weight of silicic acid was placed in a round pouring device which consisted of a 20ml capacity glass vessel with a 1 cm hole on top and a 1.5 cm long spout at a 130 angle (see Figure 2b). The column consisted of a 9 cm siliccnized Pasteur pipette plugged with glass wool. A few drcps of benzene:ethyl acetate in a 6:4 volume ratio (refered to as B:EA) were added to the silicic acid to make a slurry. The spout fitted the top of the column and by adding B:EA dropwise to the top of the pouring device, the contents were easily transferred to the column. The column was then fitted with a piece of teflon tubing, making it possible to control the flow with a clamp.

The column was then washed with approximately five millilitres of B:EA. The silicic acid is a white powder which turns translucent blue when B:EA is added. It becomes more opaque when water or methanol are present. If the column was not blue after the first wash, then additionnal B:EA would be applied until the column became translucent.

2.5.2 Gradient elution apparatus

Two 100 ml graduated cylinders were adapted with spcuts at their bases (Figure 2a). Cylinder B had two spouts: the first spout connected with cylinder A by a small section of teflon

tubing; the second spout was the outlet for the eluting solvent. Clamps were used to control the flow between the two cylinders and the outflow.

Cylinder B contained 31 ml of B:EA, and cylinder A held a mixture of 15 ml methanol (M) and 15 ml B:EA. Both had magnetic stirring bars, and were held on a magnetic stirrer.

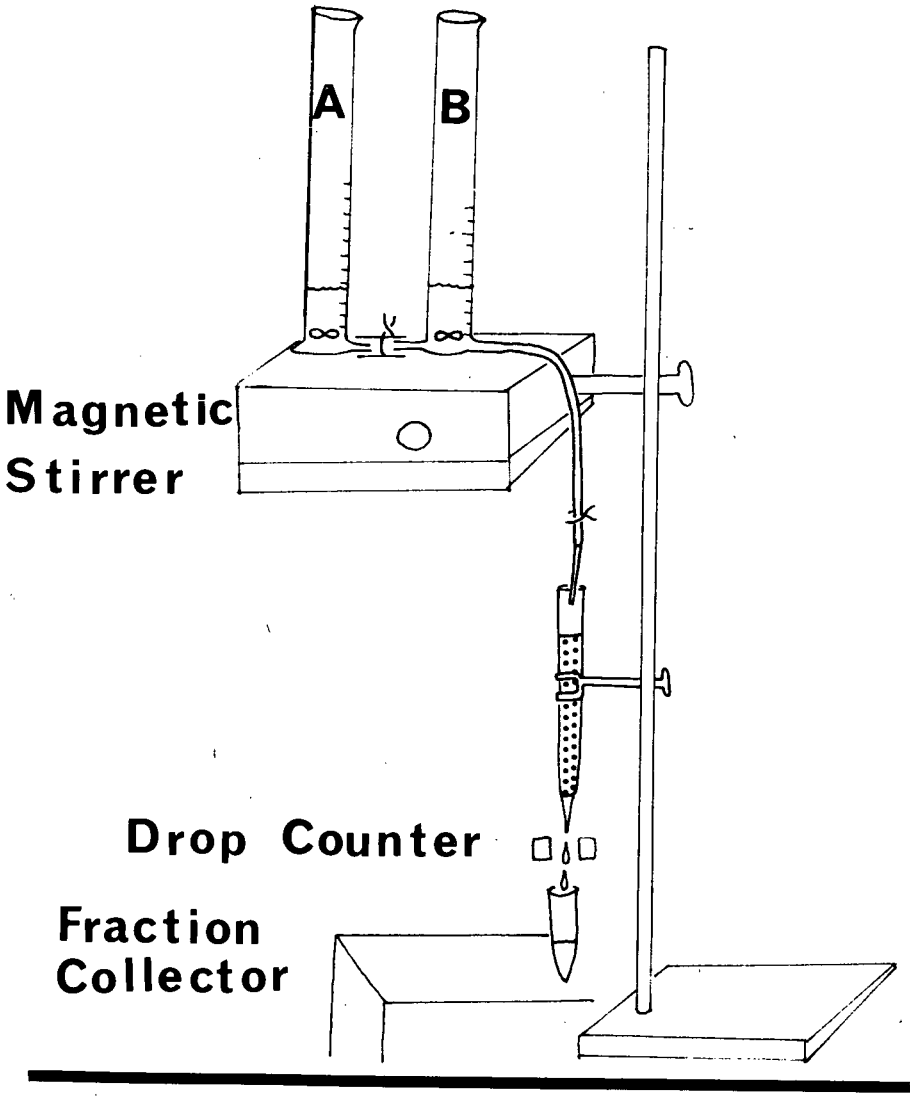
The sample was thawed on ice and then solubilized first in 0.2 ml of benzene:ethyl acetate:methanol (6:4:1), and a further 0.8 ml of B:EA was added to the sample and vortexed. The one ml of final extract was added to the top of the column and left to settle for a few seconds. The sides of the column were tapped gently to ensure an even interface. The sample was run onto the column and stopped quickly so as to prevent the column from running dry. 1.0 ml of B:EA was used to rinse the tube containing the sample. This 1.0 ml rinse was then gently placed on the column and the gradient elution started.

The gradient elution protocol was as follows: with the valve between the cylinders closed, 2.0 ml B:EA were delivered to the column. The valve between the cylinders was opened, and 3.0 ml of a mixture were delivered to the column, while the contents of the cylinders were allowed to mix. Whenever the valve was open, the magnetic stirrer was switched on to insure proper mixing of the two solutions. The valve was then closed and a further 7.0 ml were delivered to the column. The valve was opened for the final 6 ml of eluent. With this "on-off" gradient elution technique it was possible to control the methanol content in the outflow to the column.

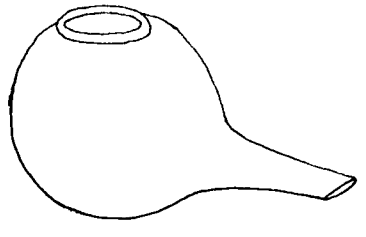
Eluates were collected with an automatic fraction collector

FIGURE 2: Chromatcgraphic apparatus.
a) gradient elution appararus.
b) glass pouring device for preparation of silicic acid columns.

a)



b)



(LKB, Ultrovac, Ltd.). The fractions were collected and measured by volume using a drop counter. The first four millilitres contained the PGB and PGA groups and the next eight millilitres contained the PGEs, whereas the final six millilitres eluted the PGF fraction. The fractions were evaporated tc dryness at 40 C under a stream of nitrogen, and then covered with Parafilm, and kept at -30° C until the assay.

2.6 RADIOIMMUNOASSAY

RIA kits for the measurement of prostaglandins $F_{2\alpha}$, B1 and E1 were obtained from Clinical Assays Inc., Cambridge, Mass. A list of the reagents included in the kit is given in the Appendix. The assay was performed in disposable pclypropylene tubes (Falcon #2053, 12x75 mm). Duplicates were carried out for both the standard curve and the unknown samples and a standard curve was done for each assay. The basic protocol outlined in the kit instructions was followed, except for a few steps which will be noted below.

2.6.1 Sample preparation

2.6.1.1 PGF

The samples were removed from the freezer and thawed in an ice bath. Then 1.4 ml of isogel tris buffer (ITB) were added to each tube by means of an equal volume dispenser (Repipet, Fisher

Scientific) and vortexed several times to ensure that the sample was completely dissolved in the buffer.

2.6.1.2 PGB

The same proceedure was followed as with $PGF2_{\alpha}$, except that only 1.0 ml of ITB was added to each tube.

2.6.1.3 PGE

An antibody toward PGE was not available. However, it was possible to convert PGE to PGB, and then measure PGE by using the PGB antibody. The conversion was performed as follows: 1.0 ml of ITB was added to all the tubes and and mixed with 0.1 ml of 1N NaOH. The tubes were placed in a boiling water bath for five minutes, removed, and then cooled in an ice bath. The pH was measured by a microelectrode (Fisher Scientific Ltd.) and adjusted to approximatly pH 7 using 0.1 ml of 1N glacial acetic acid. Because of the small volume involved, it was difficult to adjust the pH precisely, so a range of 6.8 to 7.8 was accepted.

2.6.2 Radioimmunoassay procedure

The reagents were thawed on ice and the antibody , tracer, standards, rabbit normal serum (RNS) and goat anti-rabbit serum (GARS) were reconstituted with ITB and kept on ice. Working standards were prepared by making a series of 3-fold dilutions starting with the stock standard. The final six solutions ranged

between 9.2 and 2400 PG per 100 ul for $PGF2_{\alpha}$ and 16.4 and 4000 PG per ul for PGB1.

The reagents were added to duplicate tubes fcllowing the protocol on page 178 in the Appendix for $PGF2_{\alpha}$ and on page 164 for PGE1 and PGB1 (the ITB was added by Repipet; the standards by disposable micropipettes; the unknowns by disposable glass pipettes; the tracer and antiserum by one or two ml glass pipettes). The tube was mixed gently after each addition (Vortex, Co Ltd). The tubes were then incubated in a water bath at 37° C for 1.5 hours, then 0.1 ml of RNS and 0.1 ml of GARS were added immediatly to each tube and vortexed. The tubes were covered with Saran wrap (Dow Chemicals of Canada Limited, Toronto, Ont.) and put in a plastic bag and incubated for 20-24 hours (this step precipitates the antibody-antigen complex). After the incubation, the tubes were centrifuged at 4000g, at 4° C for 50 minutes. The supernatant from tubes #1 and #2 (see Appendix) were decanted into separate scintillation vials; the radioactivity in these tubes is the Total Counts (TC). After decanting the supernatants from all the remaining tubes, into a waste container, the tubes were placed upside down in a rack, and lined with absorbant paper with a plastic backing. Each tube was carefully wiped with absorbant tissue twisted on the end of an applicator stick. The precipitate was disolved in 0.5 ml of 0.1N NaOH, then neutralized with 0.5 ml cf 0.1N HCl. This solution was decanted into a corresponding scintillation vial, and the tube was shaken gently to insure maximal transfer. A tilting dispenser was used to transfer 10 ml of scintillation fluid to each (LSC Cocktail, #3-4986, J.T. Baker chemicals,

Phillisburg, N.J., or NEF-948 Riafluor, New England Nuclear, Lachine, Quebec.) vial. The vials were left to equilibrate for one hour and then counted on the ISOCAP/300 liquid scintillation counter (Nuclear Chicago) for 10 minutes.

Two problems which can be encountered using RIA are the deterioration of reagents and the adsorption of prcteins and small molecules to glassware and plastics. The kit reagents were lyophylized, kept at -30° C and were used only once, immediately after reconstitution. Adsorption problems were avoided by adding gelatin to the buffer. This non-binding protein not only prevents adsorption but also seems to enhance antibody uptake (Murphy and Marvin, in press).

2.6.3 Calculations

The background count was the average counts per minute (cpm), left in tubes #1 and #2 after removal cf the supernatant. This value represented the trapping of radioactivity in the precipitate and was subtracted from the total cpm in each vial. Quench correction was performed by the Channels Ratio Method. This consisted of running a series of standard solutions with a known amount of radioactivity and various amounts of quenching (radioactivity in decomposition per minute or dpm). The efficiency of counting for the unknowns can be determined by the quench curve given as the A/B ratio (cpm in channel A / cpm in channel B) against the absolute efficiency of counting (total cpm/absolute dpm in the standard). The final radioactive value is in dpm, and is calculated by first subtracting the background

from each sample, and then dividing this value by its efficiency of counting.

Following the quench correction for each sample, the binding ratio can be calculated by dividing the dpms for the unknown sample by the average dpm in the total bound (TB) tubes, (#3 and #4). The standard curve is obtained by plotting the binding ratios for each concentration of standard against the logarithm of the corresponding concentration. The binding ratios of the unknowns were then calculated and the prostaglandin level for each sample defined for each tube, averaged for the duplicates, and corrected for dilution. The final values are given in pg per ml plasma or per g tissue. The mean, standard deviation, standard error and the coefficient of variartion were calculated for the different groups of animals and tissues by the use of an IBM 370 computer.

To assess the quality of the kit's reagents, the binding capacity was estimated by calculating the ratio of Total binding (tubes #3 & #4) over Total Counts (tubes #1 & #2). Any kit with a ratio under 0.3 was not used. The normal range for the ratio was 0.4 to 0.5.

3 PRELIMINARY WORK

3.1 INTRODUCTION

The development of an accurate and reproducible method for the analysis and identification of prostaglandins in fish tissue was crucial for this study. The established techniques for PG assay had been developed on mammalian tissue; therefore, it was essential to verify these procedures using fish tissue.

A selection of the most suitable separation and assay procedures was made following an analysis of the available methods. Therefore, prostaglandin methodology will be reviewed briefly. The verifications and modifications made on these procedures will be described in detail.

3.2 REVIEW OF METHODOLOGY

Two major preparatory steps are usually performed before prostaglandins can be measured in tissue. The first purification process is an extraction with organic solvents. Salmon and Karim (1976) outlined the three main reasons for organic extraction: the process increases the specificity of the analysis, concentrates the PGs (thereby increasing assay sensitivity), and removes substances such as protein, and lipids, which could interfere with the assay procedure. The second step is the separation of PGs into the three major groups: 1) PGA & PGB, 2) PGE and 3) PGF. Group separation will also increase the specificity of the analysis and further remove substances which

could interfere with the assay. Another advantage of separation, is that it enables the analysis of more than one PG from a single sample.

However, these steps will undoubtably be a source of some loss of PGs. It is therefore necessary to determine the extent of error by monitoring the recovery of labelled prostaglandins following extraction and separation. This is especially true in this study, as the recommended purification steps had been tested in mammals, rather than fish.

3.2.1 Extraction

Various extraction methods have been outlined in the literature. They are all based on the dual nature of the prostaglandin molecule, whereby PGs are soluble in both polar and organic solvents. The PG molecule is polar; therefore a neutral solvent system would not give a good yield and a basic solvent would ionize the carboxylic group on the prostaglandin. Either of these factors would decrease the extraction potential. To ensure a good recovery , it is necessary to acidify the aqueous phase of the extracton solution (pH 3 to 4). Once the PGs have been extracted in an acidic solution, they can easily be removed in an organic solvent and removed by evaporation prior to further purification.

3.2.2 Separation

Due to the relative polarities of the different prostaglandin groups, it is possible to separate them by controlling the polarity of the developing solvents. Chromatographic techniques are most frequently used for separating PGs. Column chromatography is used mainly for separation of PG groups while thin layer chromatography and reverse phase chromatography are used for both group and individual separation of PGs. Although it would have been desirable to have individual separation of PGs, the procedures involved are tedious and require a greater purification of the sample, and are therefore not practical on a large scale.

Column chromatography with silicic acid is the most widely used technique for prostaglandin separation. Salmon and Karim (1976) have pointed out some of its advantages: " it (silicic acid chromatography) can be used to separate out small amounts of PGs, gives low "blanks" in most assay systems and is relatively simple". It is also inexpensive and readily available. The one problem sometimes encountered during silicic acid chromatography, is poor reproducibility. However, this can be minimized by using the same batch of silicic acid (Kibby, Bronn and Minton, 1977), and by being consistent throughout the experiments.

3.2.3 Assay methods

Assay methods for prostaglandins have been the major setback in PG research. Until the last decade, the only reliable method for measuring PGs was the bioassay. With the increasing knowledge and interest in PG research, new assay techniques, such as radioimmunoassay (RIA) have been developed. In addition, other chemical assays have been adapted, and bioassays have been improved.

Each assay technique has some advantages over the other. It is therefore essential to weigh out the research problem against the limitations of the assay. During the description cf the different assay techniques, the reasons for choosing RIA for this study will be discussed.

3.2.3.1 Bioassay

Numerous bioassay systems have been developed based on the smooth muscle stimulating activity of the PGs, and isclated smooth muscle preparations are the most common bioassay techniques. Salmon and Karim (1976) reviewed the most frequently used methods, which included: gerbil colon, rat and hamster stomach and fundus, rabbit duodenum and jejunun, guinea pig ileum, chick rectum and the rat mesenteric vascular bed preparation. More exotic smooth muscle preparations, such as smooth muscle from the uterus and intestines cf the fruit bat and the goldfish, are outlined in Berstrom, Carlson and Weeks (1968).

In vivo preparations, such as blood pressure and blocd flow

assays, are sometimes used for measurement of PGs. They are especially sensitive to PGE and PGA; however, they are not as sensitive as the smooth muscle preparations. Parallel bioassays, using several tissues with varying sensitivities tc the different PGs, are used to identify various PGs from the same sample. Of the parallel bioassays, the blood bath technique is probably the most sophisticated (Vane 1969). It consists of superfusing heparinized blood continuously over isolated assay organs and then returning the blood to the animal. This technique offers information not only on the behaviour cf PGs over time, but also on the interaction of PGs with other substances.

The ionic composition of the medium, the presence of interfering substances in the sample, and the potential sensitization of PGs to themselves can all affect the action of PGs in the smooth muscle preparation. Purification of the sample must preceed PG bioassay to insure maximum specificity. Some problems can be overcome by the use of selective antagonists to interfering substances, such as smooth muscle activators. Futhermore, the addition of a PG antagonist would inhibit spontaneous generation of PGs during the assay.

Overall, PG bioassays are a sensitive and reliable method and in some cases, the results from bioassays have been confirmed by specific chemical analysis. However, bioassays on a large scale are not practical, even though many of the prcblems associated with them can be reduced to a minimum.

3.2.3.2 Chemical assays

Several chemical assays have been adapted for measuring PGs and have been reviewed by Salmon and Karim (1976). Enzyme analysis (Anggard 1971) and ultra-violet spectrophotometry (Shaw and Ramwell 1969) are both specific but have low quantitative abilities.

There are three gas-liquid chromatographic methods for measuring PGs: Flame-Ionization Detector, Electron Capture Detector and Mass Spectrophotometer. These techniques are all very sensitive, but require extensive sample preparation. Moreover, the instrumentation required is very expensive.

3.2.3.3 General basis of radioimmunoassay

Radioimmunoassay (RIA) is a competitive binding assay which relies on the stereospecific binding properties of a protein antibody. Enzymes, extracellular proteins (transins) and tissue receptors may also function as binding proteins in competitive binding assays. The first RIA was developed for insulin by Yalow and Berson in 1960. Since then many RIA's have been developed for metabolic substances in very low concentration. Clinically, they have been important in measuring low concentrations of protein and steroid hormones, vitamins, metabolic substances,blood proteins and some drugs.

The three subtances involved in the RIA reaction are the antigen, the labelled antigen and the specific antibody. Both the labelled and unlabelled antigen compete for binding sites on the antibody. The total amount of the two antigens should exceed

the number of binding sites on the antibody as the reaction equilibrium is reached when all the binding sites are filled. Following a suitable incubation period, the bound and unbound fractions are seperated and the percent binding determined.

If the concentration of the labelled antigen and the antibody are kept constant, the antibody-antigen complex of these two will be inversely proportional to the amount of unlabelled antigen. A standand curve is obtained by adding known amcunts of the unlabelled antigen to a constant mixture of the antibody and antigen, and then plotting the percent bound against the concentration of unlabelled antigen added (100% being antibody and labelled antigen only). The concentraticn of antigen in an unknown sample can be read from the standard curve by calculating the percent binding in the sample obtained under the same conditions.

A) radioimmunoassay of prostaglandins

Antibodies towards PGs were first described by Levine and Van Vunakis (1970). Because of the small size of the PG molecule, it is necessary to conjugate the PG with a protein in order to generate antibodies. The labelled markers are usually tritiated PGs. These markers are relatively stable and have a good affinity for the corresponding antibody. Some reseachers have been sucessful in iodonating a tyrosine methyl ester of $PGF2_{\alpha}$ (Levine and Van Vunakis 1970; and Ohki et al 1974). Although the iodinated PG has more specific activity than the tritiated form, it does not seem to possess the same antigenic

qualities and for this reason it is not commonly used. RIA for PGs offer many advantages over other methods for their measurement. One useful factor is the high sensitivity. Furthermore, the precision of the assay is insured by carefully monitoring the pipetting and the counting of radioactivity. They are also relatively rapid assays; once the experiment is set up, many samples can be processed simultaneously.

The major problems of RIA are cross-reactivity between the different PG groups and interfering substances. While organic extraction and separation does minimize these problems, cross-reactivity still decreases the specificity of the assay. Despite group separation of PGs, cross-reactivity within a PG group still occurs. Individual PGs cannot be measured precisely unless thin layer chromatography is performed as well . However, this is not done routinely, as it is much too tedious for a large number of samples, and in consequence, results are often quoted for PG groups, rather than individual PGs. Recently, it has been reported that silicic acid chomatography removes most of the non esterified fatty acids which were one of the main interfering substances in the assay (Gold and Edgar 1978).

RIA kits for the identification of PGs are available from Clinical Assays Inc. Details on the contents and cross-reactivety of the antibodies are given in the Appendix.

RIA was chosen as the assay method for this study because of its high sensitivity, and its adaptability for the rapid analysis of a large number of samples.

3.3 VERIFICATIONS AND MODIFICATIONS OF PG ANALYTICAL TECHNIQUES

The following discussion outlines all the steps taken to verify the extraction , separation and assay of PGs in fish tissue. Methods and results are described together for each of the above procedures. This description follows the approximate order in which the techniques were performed, and includes the reasons for modifications.

3.3.1 Extraction efficiency

3.3.1.1 Dilution of labelled prostaglandins

Tritium labelled PGs were purchased from New England Nuclear, Boston, Mass. The following PGs were solubilized in 70% ethanol and shipped on dry ice:

#482 PGE2 85,6,8,11,12,14,15-^{3}H(N)) lot# 932-108, specific activity 117 Ci/mmol, concentration of 0.025 mCi; 0.000075 in 0.25 ml.

#345 PGF2$_{\alpha}$ (9-^{3}H(N)) lot# 787-250, specific activity 9.2 Ci/mmol, concentration of 0.05 mCi; 0.0019mg in 0.5 ml.

The vials were stored at -70°C until use. A one hundred fold dilution was performed on the standards as follows: the PG standards were removed from the freezer and thawed on ice. Using a 100 ul Hamilton syringe, 200 ul of 70% ethanol was placed in a glass ampoule which had been previously siliconized and autoclaved. To this, 10 ul of ^{3}H-PG was added with a Hamilton syringe, and this was followed by another 750 ul of 70% ethanol,

(50 ul aliquots). Finally, a further 40 uml of 70% ethancl were added to make up a total of 1.0 ml per ampoule. The ampoules and stock solutions were then placed at -70°C (Harris deep freeze). The final concentrations of ^{3}H-PGs were:

^{3}H-PGE2; 3,000pg/ml (approximatly 24,000 dpm/ 10 ul)

^{3}H-PGF2$_{\alpha}$; -38,000 pg/ml (approximatly 20,000 dpm/10 ul). 1

It was known that PGE2 readily adsorbes to glass surfaces; therefore, the extent of this adsorption was tested by measuring duplicate 10 ul volumes of both ^{3}H-PGE2 and ^{3}H-PGF2$_{\alpha}$ using siliconized and non-siliconized micropipettes and a 50 ul Hamilton syringe. The final dpms in each aliquot were not sigificantly different from each other, for both PGs tested.

3.3.1.2 Extraction recovery

A 20 ul volume of either H-PGF or H-PGE was added to the sample at the homogenate stage, or directly to the plasma. This step was followed by the extraction procedures outlined in the general methods(Jaffe and Behrman, 1974). The radioactivity was measured in an aliquot of the final organic phase containing the extracted prostaglandins. The aliquot was placed in a scintillation vial, followed by 10 ml of scintillation fluid, then counted for 10 minutes in the ISOCAP. The percent recovery was calculated by dividing the dpms in the aliquot by the total dpms added, then multiplying by the ratio of the volume in the sample over the volume in the aliquot, and finally multiplying this value by 100. The average % yield ±SEM (# of samples) for ^{3}H-PGE2 , when added to the ovary homogenate was 86.5% ±2.9 (3)

and, for ^{3}H-PGF$_{2\alpha}$, 85.3% ±1.8 (4). When both tracers were added together, an average of 85.6% ±0.9 (5) of the radicactivity was recovered in the ovary homogenates, and 84.5% ±4.6 (3) in the plasma samples.

3.4 COLUMN CALIBRATION

3.4.1 Preliminary chromatography methods and results

Silicic acid (Sigma, SIL-A-200 Mesh 60-200 lot #115C-0185) was suspended in distilled water, left to settle for a few minutes, and then decanted. This was repeated several times to remove the fines. Removal of the fines ensures a better flow rate as well as decreasing the risk of blocking the column. The wet silicic acid was dried at 115 C for at least 24 hours, and kept at this temperature until use. This activation process is important since removing the free water from the silicic acid improves the adsorptive strength of the gel (Trueblocd and Malmberg 1949). To further increase the adsorptive characteristics, a prewash using B:EA was performed, prior to loading the column. If no water was present, the column would be a translucent blue. However, if the column was even partially opaque, more B:EA would be used to wash the cclunm, until the blue color was achieved.

The column consisted of a Pasteur pipette which had been siliconized (Siliclad, Clay Adams, NY) and plugged with glass wool. 0.5 g of silicic acid was mixed intc two ml cf B:EA. The

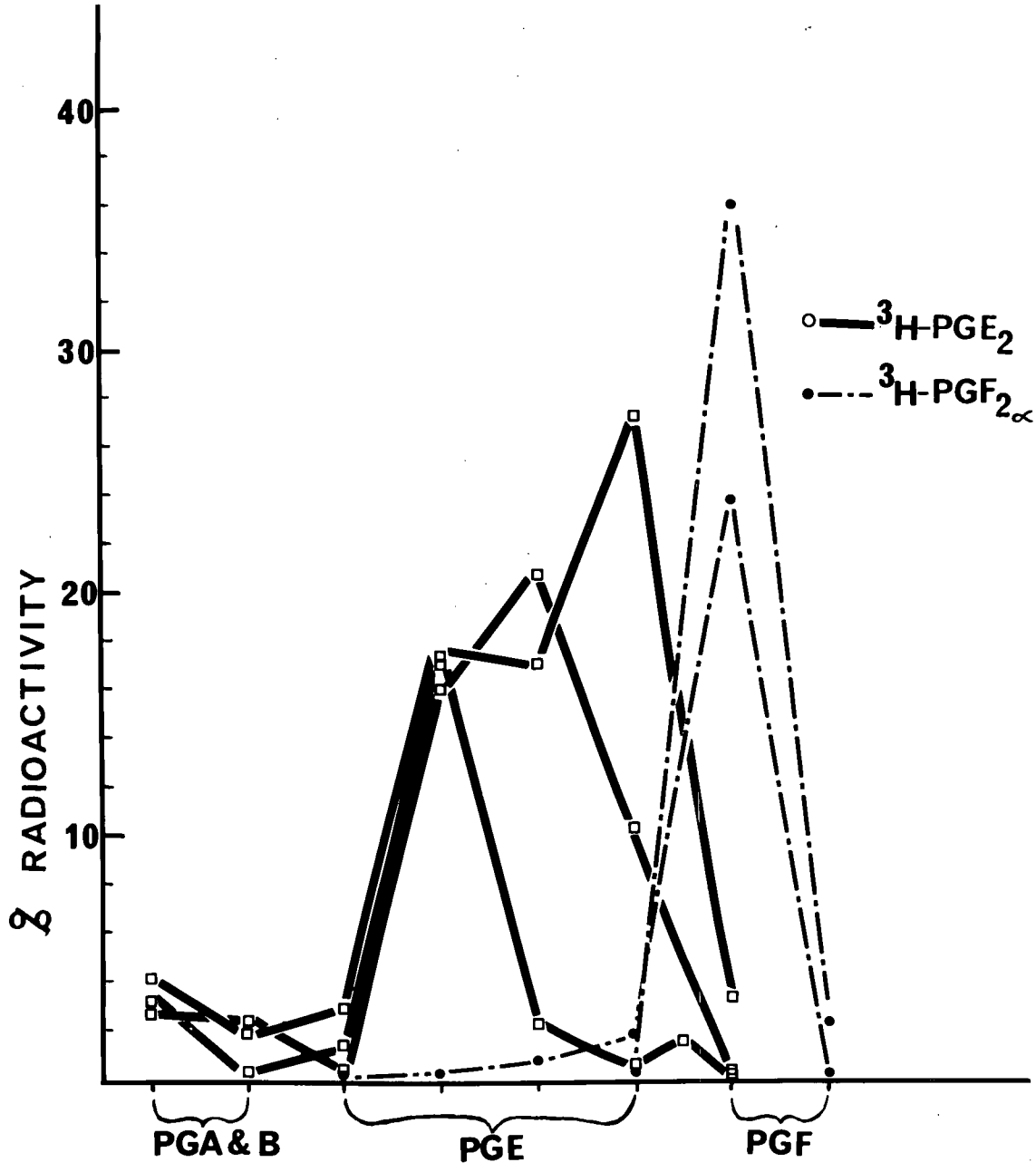
slurry was poured into the pipette, while adding more B:EA to the flask to ensure complete recovery. (Subsequently, a pouring device was made to facilitate this task.) The column was attached to a drop counter over the fraction-collector with teflon tubing. The flow from the column could be controlled by means of a clamp. The column was washed with five ml cf B:EA and clamped off. The sample was prepared by measuring either 10 or 20 ul of tritiated-PG in a test tube and blowing over it with N gas until it was evaporated. It was then suspended in 0.2 ml of a B:EA and methanol solution (6:4:1), and a further 0.8 ml of B:EA was added. An equivalent amount of the labelled PG was put into a scintillation vial in order to determine total dpms. The sample was loaded onto the column, which was clamped off after all the sample had run into the gel. Each different PG group was eluted off by adding the following solutions, according to the methods outlined in Jaffe and Berhman (1974):

- PGA and PGB fraction: 6 ml of benzene:ethyl acetate, 60:40 (B:EA).
- PGE fraction: 12 ml of benzene:ethyl acetate:methanol, 60:40:2.
- PGF fraction: 4 ml of benzene:ethyl acetate:methanol, 60:40:10. 1

the column was clamped off after each solvent had run through, and it was then opened for the next solvent. One millilitre samples were collected and transferred to a scintillation vial to be counted. The percent recoveries were calculated by dividing the dpms in the sample by the total dpms added x 100.

The results of these experiments are given in Figure 3. In

FIGURE 3: initial elution pattern of radioactive prostaglandins. Elution pattern obtained using the method of Jaffe and Berhgman (1974).
(note: each division on the abcissa represents one 3.0 ml fraction)


40
30
20
10
% RADIOACTIVITY
^{3}H-PGE$_2$
^{3}H-PGF$_{2\alpha}$
PGA & B
PGE
PGF

some cases, the results are satisfactory, but in others, they are not. In two columns, the resolution of PGE was not good, as the radioactivity was spread over a very wide area. Some technical problems were present in the system; these could have had a direct effect on the resolution of the prostaglandins. The clamping of the column (when changing solutions) could easily have distorted the bands by the production of differential flow at the interface of the glass. Also, it was difficult, when changing solutions, not to disturb the surface of the column. Finally, it is impossible, using this system, to maintain an even solvent flow, as the pressure head decreases during the column run.

3.4.1.1 Gradient elution

In an attempt to alleviate some of the above problems, and facilitate handling of the elution solvents, a gradient elution system was set up. Gradient elution is routinely used to separate lipid mixures (Hirsh and Ahrens 1958). Basically, gradient elution consists of gradually increasing the concentration of a solvent in an elution medium, thereby creating a concentration gradient which enables a group of compounds with slighty different solubilities to be separated. Bygdeman and Samuelsson (1966) described a continuous gradient method for separating the PG groups on a silicic acid column by increasing the concentration of ethyl acetate in benzene. In their study, the recovery and separation were very good; however, the number of trials was low: three for PGF and three

for PGE. Furthermore, their set-up was not practical for processing a large number of samples. To overcome the processing difficulties, and to maintain a high degree of efficiency, a smaller version of their system was designed. The elution pattern was modelled on Jaffe and Behrman's (1977) method of increasing the methanol concentration in B:EA. This procedure, which will be described, made it simple to determine the best elution sequence for the optimum resolution and separaticn of the PG groups.

A) apparatus

Silicic acid was prepared as described in the General Techniques. A diagram of the gradient elution apparatus is shown in Figure 2 . Two 100 ml glass graduated cylinders were adapted with spouts at their bases. Cylinder A had one outlet, and cylinder B had two outlets, each at 180 degrees to the other. A short section of silicon tubing (ID 1.35 mm, OD 3.35 mm, LKB #2030-962) joined the two cylinders. A 13-14 cm section of the same tubing, fitted with a 10 ul disposable pipette, was attached to the second outlet of cylinder B, and led directly to the column. The cylinders were held on fixed plastic bases, resting on a magnetic stirrer (Fllexa-Mix, Fisher Scientific). The stirrer was mounted on a support stand by means of a clamp. Solvent flow rates could be altered to maintain a constant pressure head on the column, by raising or lowering the stirrer. Magnetic stirring bars in each cylinder ensured complete mixing of the solvents. The column was held to a stand by means of a

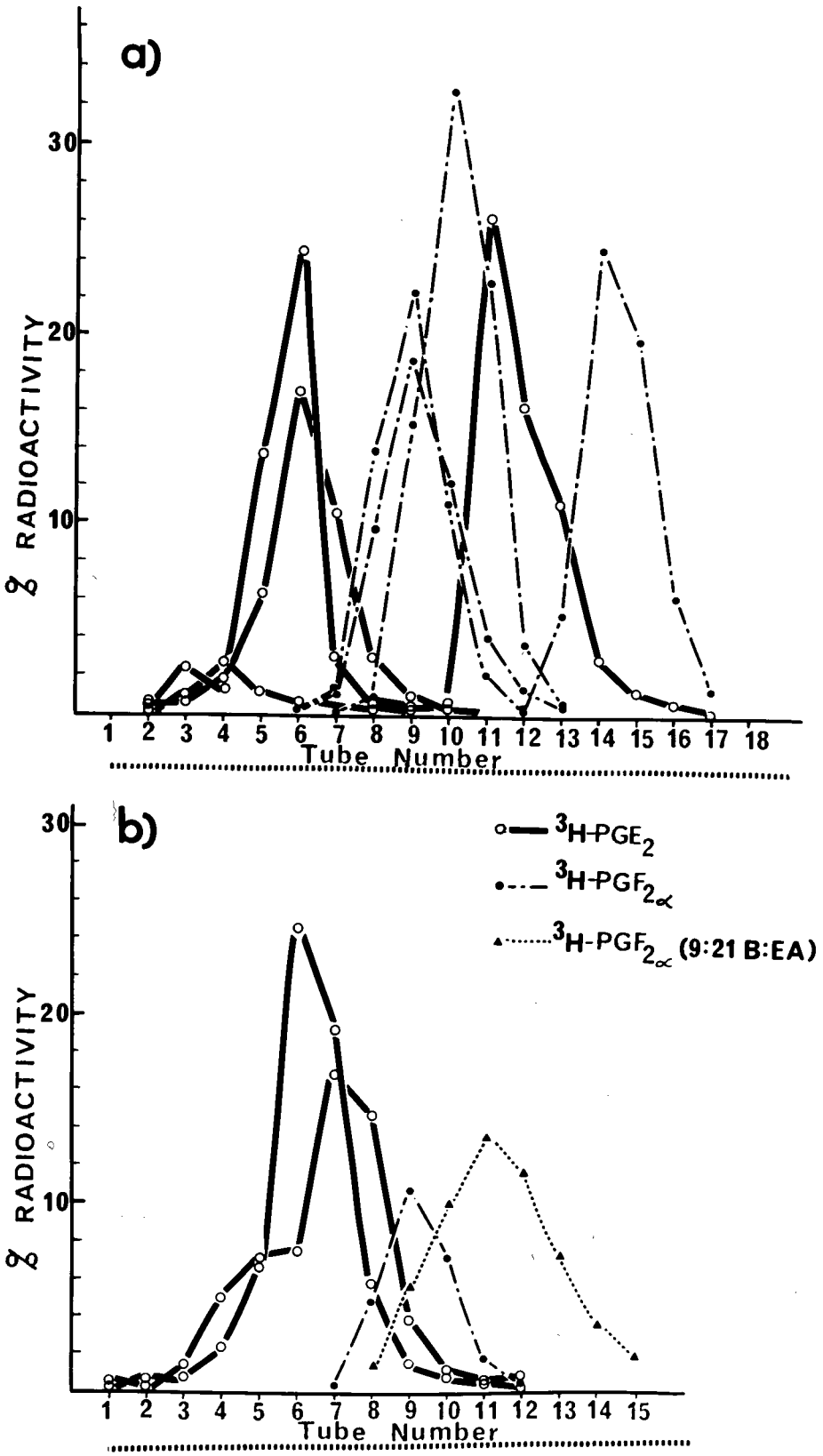
three-prong clamp over the fraction collector. A 5 ul disposable pipette was held between two pieces of silicon tubing, and was joined to the drop counter on the fraction collector. The small diameter of the pipette reduced the solvent outflow, thereby increasing the resolution by increasing the time the sample had to travel over the gel.

B) results of gradient elution of prostaglandins

Various solvent patterns were attempted with the gradient elution apparatus. Except for a few noted cases, the column bedding consisted of 0.5 g of silicic acid which had been prewashed with 5 ml of B:EA. 20 ul of one of the tritiated-PGS was evaporated under N suspended in 0.2 ml of B:EA and methanol (6:4:1). 0.8 ml of B:EA was then added, and the whole mixture was loaded on the column.

The results of the first gradient elution trials are given in Figure 4. In these experiments cylinder A contained 15 ml of methanol and 15 ml of B:EA, while cylinder B had 30 ml of B:EA. There is an obvious overlap between PGE and PGF in these columns. The PGFs came off much earlier than they did with Jaffe and Behrman's method. This could be due to the solvent becoming polar too rapidly, and eluting off the more polar PGFs along with the PGEs. However, when the methanol concentration was decreased to 9.0 ml in 21.0 ml of B:EA, the PGF peak was not shifted significantly, as can be seen in the column on the far right in Figure 4b. In an attempt to improve resolution by increasing column length, 0.6 g of silicic acid was used instead

FIGURE 4: elution pattern of radioactive prostaglandins using a continual gradient elution technique
a) with 0.5 g of silicic acid and 30.0 ml (B:EA):(methanol) into 30.0 ml B:EA
b) solid and slashed lines: with 0.6 g silicic acid and 30.0 ml 1:1 (B:EA):(methanol) into 30.0 ml B:EA
c) dotted lines: with 0.5 g of silicic acid and 30.0 ml 9:21 (B:EA):(methanol) into 30 ml B:EA.
(note: each tube contained 1.0 ml of eluate. The broken line below represents the time during which the gradient was on.)


a)
% RADIOACTIVITY
30
20
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Tube Number
b)
% RADIOACTIVITY
30
20
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Tube Number
^{3}H-PGE$_2$
^{3}H-PGF$_{2\alpha}$
^{3}H-PGF$_{2\alpha}$ (9:21 B:EA)

of 0.5 g. (three experiments). A continuous gradient was started with methanol:B:EA 1:1 in cylinder A and 30 ml B:EA in cylinder B: the results from these experiments are shown in Figure 4b. These results were not satisfactory, as the PGF and PGE peaks still overlapped. Therefore 0.5 g of silicic acid was used in all subsequent columns.

Some promising results were obtained in the elution pattern of two columns (Figure 5a). The elution of these two columns was started with 2.0 ml of B:EA, after which a continuous gradient was resumed with 30 ml of methanol:B:EA (1:1) in A, and 30 ml of B:EA in B: this slight change in elution shifted the PGF peak from the 8-9 position to the 11-12 position.

A few "on-off" gradients were attempted (Figure 5b). The cylinders contained the same solution as described previcusly. The "on-off" was accomplished by clamping the tubing between the cylinders with a microhemostat. The "on-off" settings are shown under the elution pattern for each column. The results from the three columns in Figure 5b were not satisfactory, as the PGF peak was still in the 8-9 position, directly over the PGE peak in every case. However, the PGF peak shown in Figure 5c had shifted to the 13-14 position, with very little overlap with PGE peak.

A satisfactory version of the elution pattern was finally found (Figure 6a). The cylinders contained the following: A had 30 ml of the methanol:B:EA (1:1) mixture and B had 32 ml of B:EA. The elution was started with 2.0 ml of B:EA, and then the gradient was turned on for the next 3.0 ml, turned off for 6.0 ml, and on again for the final 6.0 ml. The peaks for PGE fell in

FIGURE 5: Initial "on-off" gradient elution pattern of radioactive prostaglandins.
All columns had 0.5g of silicic acid and the gradient elution consisted of 30.0 of 1:1 (B:EA):(methanol) into 30.0 ml of B:EA.
a) gradient elution started after eluting the first 2.0 ml with B:EA
B) gradient was on for the first 7.0 ml, stopped for 5.0 ml, then started again for the final 4 ml.
C) gradient off for the first 2.0 ml, on for 4.0 ml, stopped for 4.0 ml, then on for the final 7.0 ml.

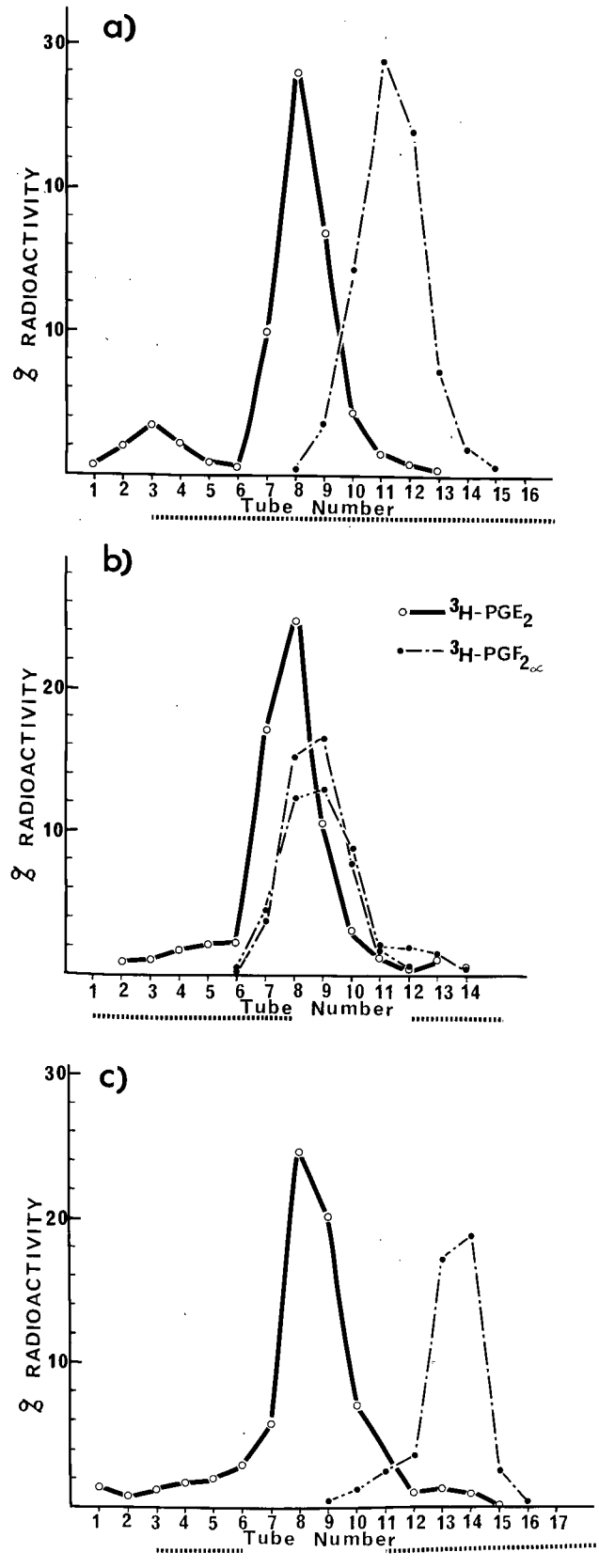
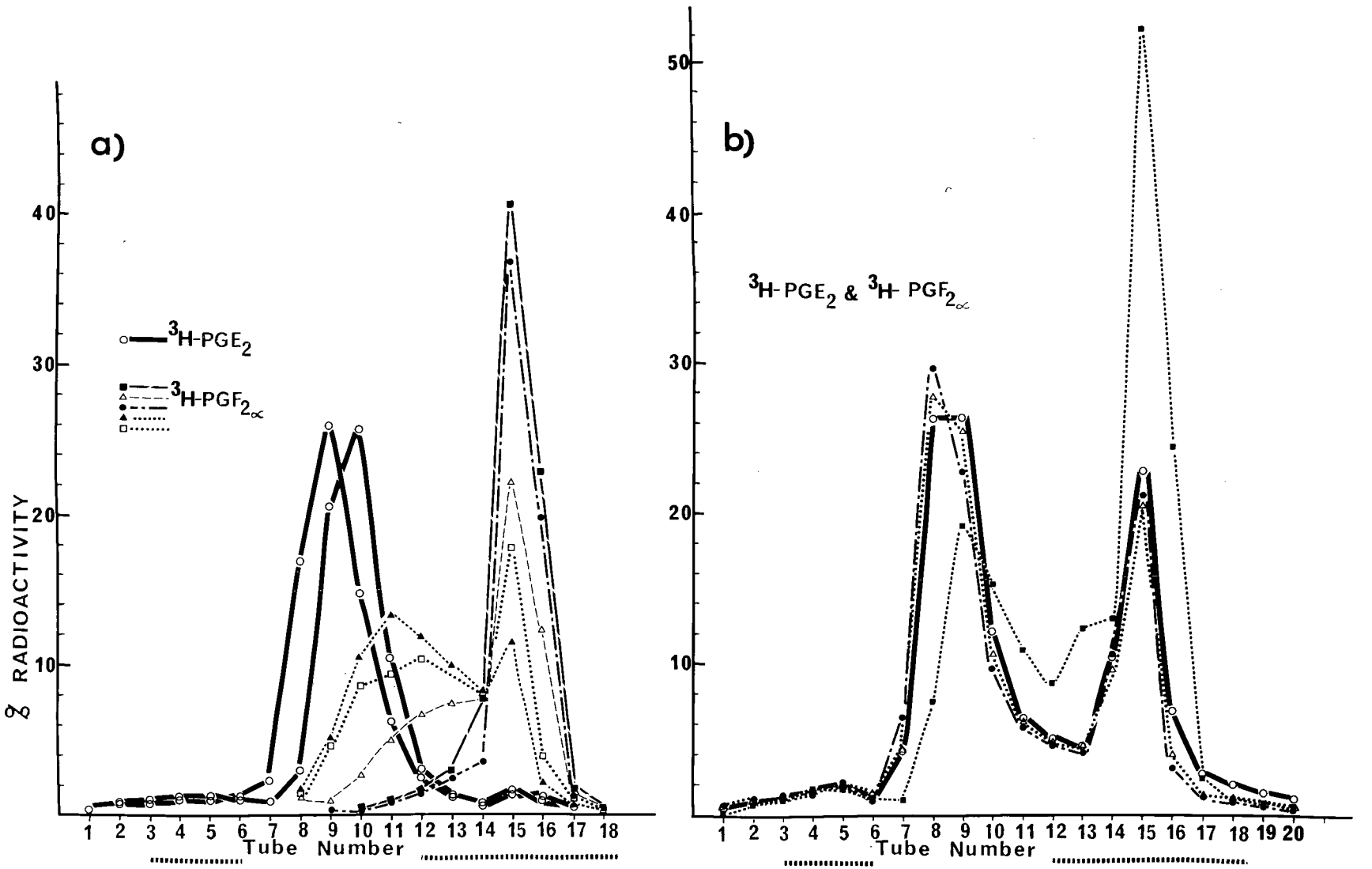

a)
% RADIOACTIVITY
30
10
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Tube Number
b)
^{3}H-PGE$_2$
^{3}H-PGF$_{2\alpha}$
% RADIOACTIVITY
20
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Tube Number
c)
% RADIOACTIVITY
30
20
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Tube Number

FIGURE 6: Successfull "on-off" gradient elution pattern of radioactive prostaglandins.
In all columns the gradient was off for the first 2.0 ml, on for the next 4.0 ml, off for 5.0 ml, then on for the final 6.0 ml.
a) with individual prostaglandins on a column.
b) with two prostaglandins together on a cclumn.


a)
b)
^{3}H-PGE$_2$
^{3}H-PGF$_{2\alpha}$
^{3}H-PGE$_2$ & ^{3}H- PGF$_{2\alpha}$
% RADIOACTIVITY
Tube Number
Tube Number

the 8 to 10 position, and the peaks for PGF had shifted over to the 14 to 16 position.

Under these same conditions, a combination of the two tracers was used on the columns shown in Figure 6b. The elution pattern of these columns correlate well with the pattern obtained with the single tracer experiments shown in Figure 6a. The bands of the double tracer experiments do not overlap, as the radioactivity in the 12 and 13 position is relatively low and the peaks are sharp. The percent recoveries are acceptable: 78.4% ±1.1 for PGE2, and 56.5% ±9.8 for PGF2$_{\alpha}$.

Although this elution pattern appeared to be the most suitable for separating out the PGE and PGF groups, it was necessary to test its performance using fish tissue. During these experiments, it was also possible to measure the effectiveness of recovery from the extraction procedure.

3.4.2 Verification of methods on fish tissue

Following the extraction recovery experiments with fish tissue, there was a sufficient amount of extract, containing tritiated PGs, to measure the column efficiency. A measured volume of this fluid was evaporated under N at 40° C and the residue was resuspended in 0.2 ml of benzene;ethyl acetate:methanol (6:4:1), and run on the column. To test the reproducibility of the separation pattern using fish tissue, the "on-off" gradient elution method was performed using the fish extracts. The elution pattern was followed exactly as described for columns #18 to #28; i.e., with 30 ml of methanol: B:EA (1:1)

in cylinder A and 32 ml of B:EA in cylinder B. The "on-off" gradient pattern used was as follows:

a) starting with 2.0 ml of B:EA from B with the gradient off

b) 3.0 ml with the gradient on

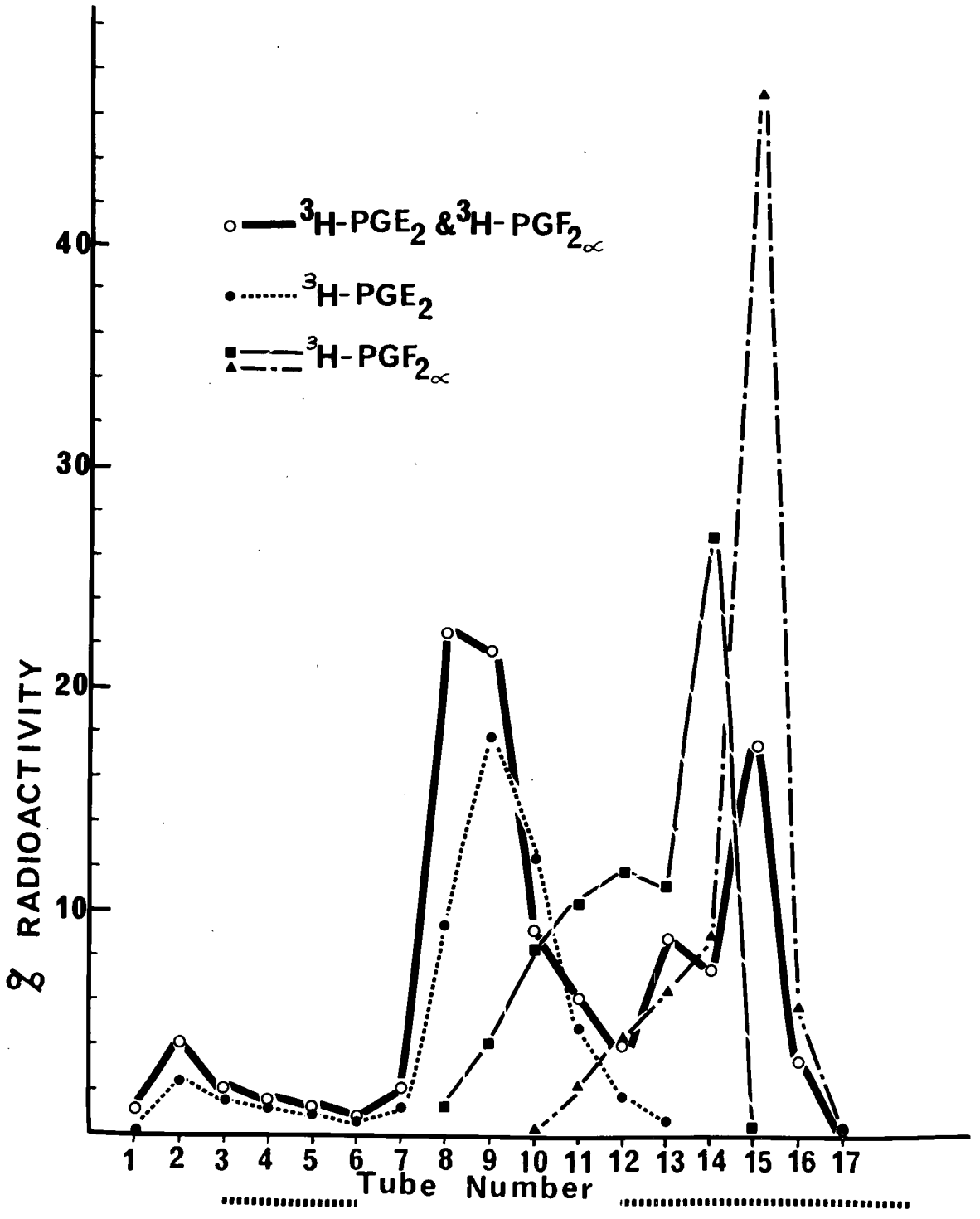
c) 6.0 ml with the gradient off

d) finishing with the final 6.0 ml with the gradient on.

The fractions were collected in one millilitre volumes. The results from these experiments are shown in Figure 7. The radioactive distribution correlates well with the pattern found when using tracer alone; however, the percent recovery was somewhat lower.

There were some problems which occurred initially, and could have decreased the efficiency of the column. In some instances, the column would plug after application of the sample. In these cases, the sample extract was usually flocculent and very deeply coloured. In an attempt to overcome this , a pinch of silicic acid was added to the sample prior to evaporation and permitted to settle for a few seconds. The liquid was removed, and placed in another test tube. The remaining silicic acid was washed once with B:EA, and this rinse was also added to the test tube. The final extract was evaporated to dryness and resuspended with 0.2 ml of the loading solutions. Presumably, the batch extraction with silicic acid could account for some of the loss of radioactivity. Fortunately, this cloudiness only occurred during these preliminary experiments, and it was therefore not necessary to repeat this step for any of the other samples.

FIGURE 7: gradient elution pattern of radioactive prostaglandins added to ovary homogenates.


^{3}H-PGE$_2$ &^{3}H-PGF$_{2\alpha}$
^{3}H-PGE$_2$
^{3}H-PGF$_{2\alpha}$
% RADIOACTIVITY
40
30
20
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Tube Number

To further maximize the transfer of PGs in the extract from the test tube to the column, the test tube was washed with 1.0 ml of B:EA after loading the extract on the column. This rinse was added to the column to start the elution (consequently cylinder B contained only 31 ml of B:EA). This protocol was performed for all subsequent samples.

There was some concern about using an acidic extraction solution for the initial extraction of PGs, as there was a possibility of some loss of PGE by conversion to PGB under acidic conditions. However, only a very small amount of radioactivity was found in the PGA and PGB fraction following extraction with ^{3}H-PGE2, and this loss was considered to be negligible.

3.4.2.1 Radioimmunoassay of prostaglandins in fish tissues

To assess the amount of tissue necessary for the measurement of prostaglandins in fish tissue, several samples of ovary, testis and plasma were extracted and the PG fractions were separated on the column. These samples were then assayed for the three PGs by RIA. Approximately one gram or one milliliter of tissue was used, and except for the PGB, this quantity was sufficient for the detection of PGs. The mean PG values measured were as follows:

$PGF2_{\alpha}$ -plasma (four samples): 1165.4 pg/ml

-testis (two samples): 109.4 pg/g

-ovary (three samples): 226.4 pg/g

PGE1 -plasma (four samples): 112.5 pg/ml

-testis (two samples): 90.0 pg/g

-ovary (three samples): 0.0 pg/g

PGB1 -below threshold in all (nine) samples

From these findings, it was decided that a minimun cf one gram of ovary or testis would be used for PG measurement, and that plasma from two fish would be pooled, since cne ml did not contain sufficient PGB1 for detection.

In the course of the succeeding experiments, two other fish tissues, muscle and kidney, were assayed for PGs. The findings were as follows:

muscle :	$PGF2_{\alpha}$:	44.4 pg/g
	PGE1:	1370.0 pg/g
	PGB1:	72.8 pg/g
kidney :	$PGF2_{\alpha}$:	1481.1 pg/g
	PGE1:	5217.6 pg/g
	PGB1:	8.2 pg/g

This is the first record of prostaglandins in these tissues in Carassius auratus.

4 PROSTAGLANDINS DURING SEXUAL MATURATION; SEASONNAL CHANGES

4.1 INTRODUCTION

In mammals, prostaglandins have been shown to influence gonadotropin release (Ratner et al 1974; Carlson et al 1973; Sato et al 1974). Labhestwar (1972a, b) has suggested that prostaglandins affect steroid synthesis, as he found that $PGF2_{\alpha}$, and to a lesser extent PGE2, are luteolytic agents. Furthermore, $PGF2_{\alpha}$ was found to increase estradiol concentration in the ovary, whereas progesterone secretion dropped (Labhestwar, 1974). Because these hormones are essential for gonadal maturation in all animals, it seemed likely that PGs could function as a control of gonadal maturation. The following experiments were performed to observe any possible changes in PG levels in the gonad and plasma during maturation of male and female goldfish between late Fall and early Spring. Two experimental groups of male and female goldfish were set up: to accelerate the maturation process, one group was subjected to a long photoperiod regime (16L:8D), and the other was under natural photoperiod. Plasma and gonad samples were taken for PG measurement over this time period from both groups.

4.2 BACKGROUND REVIEW OF GONADAL DEVELOPMENT

In the female, the process of ovarian maturation has been extensively studied (Lam et al 1978) and the histolcgical development has been well documented (Yamamato and Yamazaki

1961). Recently, there has been more insight into endocrinological involvement and physical influences, such as temperature, photoperiod, or nutrition, which may be associated with the functionning of gonadal maturation.

The male reproductive system is not as well known . Although the histological changes which occur during maturation have been outlined (Hoar 1969), the histological and endocrinological events have not been as well researched as they have in the female. Some background on the histological and endocrinological changes in both females and males will be described below.

4.2.1 Changes in gonadal histology with development

4.2.1.1 The female system

In the female, oogonial proliferation is followed by a meiotic prophase in which oogonia are transformed into primary oocytes. The oocytes then go through two growth phases; the initial growth of the oocyte, pre-vitellogenesis, is followed by vitellogenesis, which involves the deposition of two kinds of yolk, yolk granules and yolk vesicles, into the oocyte. Yamamato and Yamazaki (1966) outlined the histological changes of the developing ovary in the goldfish, Carassius auratus, and they divided the pre-vitellogenic phase into three stages: chromatin nuclear stage (1), and early and late perinucleolar stages (2 & 3). They also divided the vitellogenesis phase into a further

seven stages: yolk vesicle (4), primary, secondary and tertiary yolky oocytes (5,6 & 7), migratory nucleus stage (8) prematuration (9) and ripe (10) stages. Atretic follicles are found during all stages of development but are predominant after the spawning season.

The rhythm of oocyte development in the goldfish is asynchronous. This type of development is distinguished by having oocytes at various stages in the ovary, and in the ability to spawn several times during a season. As the ovary matures, more of the later stages of oocytes are present; however, there are always some of the later stages present, even at full maturity.

4.2.1.2 The male system

In the male, spermatogenesis follows a similar but simpler pattern of development. The primary cells, or spermatogonia, go through mitotic proliferation to yield primary spermatocytes, followed by a meiotic division to give secondary spermatocytes, which further divide to give the spermatids, which, in turn, metamorphose into the spermatocytes, or motile sperm.

4.2.2 Endocrinological changes during gonadal development

4.2.2.1 The female system

Lam et al (1978) reviewed the endocrine control of reproduction in the female goldfish. In their summary they state that the pituitary appears to play a central role in most, if not all, of the stages and processes of cogenesis. Both the pituitary hormones and estrogens seem to be necessary during vitellogenesis. There are indications that estradiol mediates hepatic vitellogenin production, whereas the pituitary controls its incoporation into the oocytes (Jalabert 1967). Hurlburt (1977) showed that thyroid hormones influence vitellogenesis but only in conjunction with gonadotropin (GTH).

4.2.2.2 The male system

The endocrinological control of spermatogenesis has not been as extensively studied in the goldfish. However, the pituitary does play an essential role in the growth and function of the testis. Yamazaki and Donaldson (1968) showed that hypophysectomy inhibited testis growth and induced regression in the goldfish; however, injections of purified salmon pituitary gonadotropin could completely restore spermatogenesis in these fish.

4.2.3 Environmental influences on gonadal development

Environmental factors are also important in the reproductive processes of the goldfish; photoperiod and temperature have been investigated with respect to their effects

on oogenesis and on gonadotropin levels (Gillet et al 1978). Their studies indicate that high temperatures stimulate GTH secretion. However, the increase in GTH was not always correlated with an increase in gonadal development since high temperatures induced gonadal regression. They also reported that long photoperiod stimulated oogenesis in the winter.

4.3 METHODS

4.3.1 Fish maintenance and sampling

In mid-November, 1976, approximately 150 healthy fish, weighing between 60 and 100 g, were removed from the stock tank and divided into three groups. Two indoor tanks were set up with fluorescent lamps on a timing device set at 16 hours light and eight hours dark. One tank held 50 females and the other 50 males (however, there was some mixing in both tanks). A third tank under natural photoperiod held a mixture of both males and females. The males were differentiated by the presence of small tubercules on their front pectoral fins; these are absent in the female.

The fish were sampled in mid-December and then at four-week intervals in January, February and March. The natural-photoperiod fish were sampled at the same time as the long-photoperiod fish.

Blood and gonadal tissue was taken for PG measurement. The gonad was weighed for GSI and a sample was taken for histology.

The tissues were processed for the radioimmuncassay of PGF, PGE and PGB as outlined in the General Techniques.

4.3.2 Statistics

An analysis of variance (ANOVA) was perfcrmed cn the data from these experiments. A UBC program, the BMD 10V for unequal cell size, was used for the computation. The Newman-Kuels multiple range test (Zar 1974) was performed cn the different groups at a significance level of 0.05.

4.4 RESULTS FROM HISTOLOGY

Although the gono-somatic index (GSI) was recorded for each fish, this measurement was not found tc be a very sensitive reflection of sexual maturity and was not used as a parameter of comparison between fish.

4.4.1 Histological observations in the female

Histological examination of the gonad was done on all 45 females from both photoperiod groups sampled between December and March. In all the females, many stages of oogenesis were found in the ovarian sections, as would be expected with an asynchronous development. None of the last three stages of oocyte development (migratory nucleus, prematuratiCn and ripe oocytes) were present in any of the sections. The tertiary yolky oocyte stage was the most advanced stage observed in any month,

but only approximately one half of the ovaries had develcped to this stage. All the other stages were present in variable ratios. In addition, atretic follicles were cbserved in 35-45% of the fish taken in every month. There were no significant histological differences between the natural- and the long-photoperiod females, nor were there any differences in states of maturity between the months.

4.4.2 Histological observations in the male

The 45 males studied from both photoperiod groups were uniformly mature, as all the testicular sections showed seminiferous tubules which were very large and distended with spermatozoa. Furthermore, the male possessed small translucent tubercules (pearl organs) on the upper sides cf their pectoral fins; these secondary sex characteristics are an external indication that the fish is sexually mature.

4.5 RESULTS OF THE PROSTAGLANDIN MEASUREMENT DURING DEVELCPMENT IN THE FEMALE

A total of 45 fish were sampled between December and March. Eleven females from the long photoperiod group and three from the natural photoperiod group were sampled each month during the four month study period (except for February, where 12 females from the long photoperiod group were sampled). After spinning the blood for plasma, the plasma was pooled between two fish.

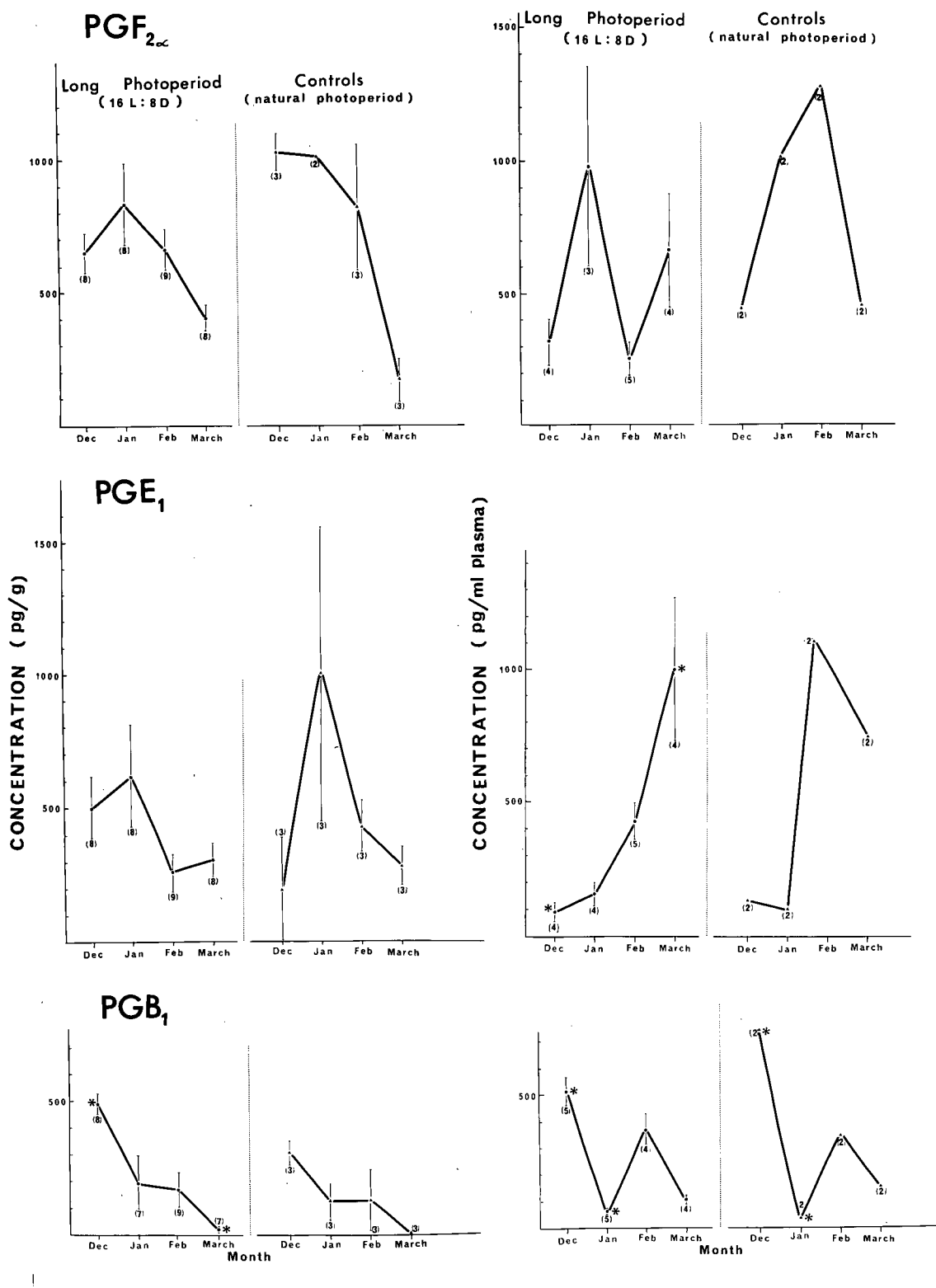
4.5.1 PGF2 levels in the ovary

The mean +SEM ovarian levels of $PGF2_{\alpha}$ in both of the long photoperiod females are given in Figure 8. The highest value (618.0 ±189.2 pg/ml) was found in January, and the lowest level (256.0 ±66.6 pg/ml) was found in February. No significant difference was found between the monthly levels of $PGF2_{\alpha}$. The mean ovarian levels in the natural photoperiod females are also given in Figure 8. There was a similar rise in the mean $PGF2_{\alpha}$ levels in January (1001.4 ±556.2 pg/ml). The lowest mean was, however, in March (277.8 ±74.3 pg/ml). Nevertheless, there was no significant difference between the means from each month. Furthermore, there was no significant difference between the $PGF2_{\alpha}$ levels in the natural- and long-photoperiod females.

4.5.2 PGF2 levels in the plasma

The mean ±SEM plasma $PGF2_{\alpha}$ levels in the long and natural photoperiod females are given in Figure 8 for each month. The levels ranged from 976.4 ±375.7 pg/ml in January, to 252.6 ±64.3 pg/ml in February, in the long-photoperiod fish. The natural-photoperiod group had a range of 438.9 pg/ml to 1282.0 pg/ml in February (note: there were only two samples in this group, after pooling the plasma). The analysis of variance showed there was no significant difference between the mean level of PGF2 in each month for both photoperiod groups. There was also no significant difference between the two photoperiod regimes.

FIGURE 8: Seasonal variations in prostaglandin levels in the female goldfish.
The mean ±SEM levels of $PGF2_{\alpha}$, PGE1 and PGB1 in the ovary (left hand side) and in the plasma (right hand side) of corresponding females during the four month study period. Starred points represent means which are significantly different from each other (Newman-Kuels, $p=0.05$).


PGF2α
Long Photoperiod (16 L:8D)
Controls (natural photoperiod)
Long Photoperiod (16 L:8D)
Controls (natural photoperiod)
PGE1
CONCENTRATION (pg/g)
CONCENTRATION (pg/ml Plasma)
PGB1
Dec
Jan
Feb
March
Month

4.5.3 PGE1 in the ovary

Mean ±SEM ovarian levels of PGE1 for the two photoperiod groups are given in Figure 8. The peak value of PGE1 in the long-photoperiod fish (834.0 ±155.5 pg/g) was found in January, and the lowest value (404.0 ±50.6 pg/g) was found in March. These levels were not significantly different from each other. The natural-photoperiod females had a peak level of PGE1 in December (1027.0 ±67.7 pg/g), falling to a low in March (171.0 ±82.9 pg/g). Although the decrease in mean levels is quite sharp, it is obscured by the high standad deviation, owing to a low sample size. There was no significant difference between long- and natural-photoperiod fish, but when an analysis of variance was done on the pooled monthly values of PGE1, the decrease observed in March was found to be significantly different ($p = 0.05$) from the other monthly values.

4.5.4 PGE1 levels in the plasma

Figure 8 gives the mean ±SEM PGE1 levels in plasma per month for the long- and the natural-photoperiod females. In the long photoperiod group, there was a significant rise in PGE1 levels, from 91.0 ±36.3 pg/ml in December to 1000.0 ±270.5 pg/ml in March. In the natural photoperiod animals, the lowest mean was in January, at 102.9 pg/ml, and the peak was in February, at 1108.3 pg/ml. There was no significant difference between the monthly levels of PGE1 in the natural photoperiod females. An analysis of variance indicated that the monthly means of the long- and natural-photoperiod animals were not significantly

different from each other.

4.5.5 PGB1 levels in the ovary

The mean ±SEM ovarian levels of PGB1 are given in Figure 8 for the long-photoperiod fish. There is a significant decrease in PGB1 from 490.0 ±38.2 pg/g in December, tc 20.0 ±7.2 pg/g in March. The PGB1 levels in the natural photoperiod fish, are also given in Figure 8 and show a similar pattern; however, there is no significant difference between any of these values.

There is no significant difference between the long-photoperiod and the natural-photoperiod animals. After pccling the values of these two groups, the analysis of variance indicated that all means except those in January and February, were significantly different from each other.

4.5.6 PGB1 levels in the plasma

Figure 8 gives the mean ±SEM plasma PGB1 levels fcr each month for long and natural photoperiod females. But the levels fluctuated in both groups, the peaks were found in December and February and the lower values in January and March. In the long-photoperiod group, the highest value was 387.9 ±73.6 pg/ml and the lowest was 74.7 pg/ml in March. In the natural photcperiod group, the peak was 543.3 pg/ml in December and 45.6 pg/ml in January. The fluctuations in PGB1 in either photoperiod group were not significantly different between each month. There was no significant difference between the photoperiod groups.

However, when all the photoperiod values are pooled, the decreases between December and January were found to be significantly different ($p=0.05$).

4.5.7 Comparison of the prostaglandin levels in the plasma and the ovary

An analysis of variance did not show a significant difference between monthly $PGF2_{\alpha}$ and PGB1 levels in the plasma and the gonad in both long- and natural-photoperiod fish, and in the pooled values. However, a significant difference was found in PGE1 levels between plasma and ovary in January, February and March, when the two photoperiod groups were pooled.

4.6 PROSTAGLANDIN MEASUREMENT IN THE PLASMA AND TESTIS DURING DEVELOPMENT IN THE MALE

A total of 45 males were sampled between December and March. Every month, eleven males were sampled from the long-photoperiod group and three from the natural-photoperiod group (except for December, when four males were sampled). The plasma from two samples was pooled in these experiments, as for the females.

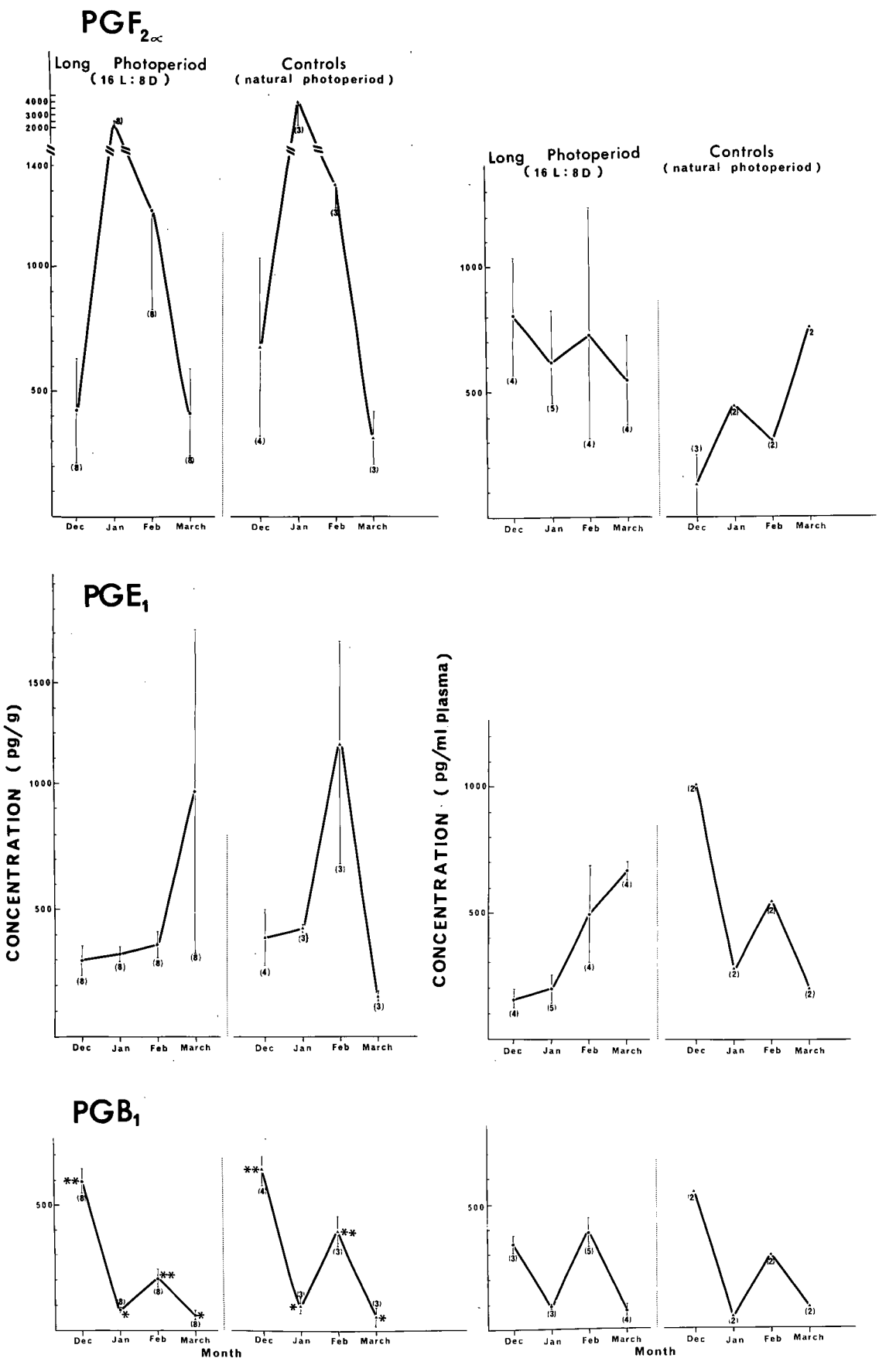
4.6.1 $PGF2_{\alpha}$ in the testis

The mean ±SEM monthly levels of $PGF2_{\alpha}$ in the testis of the goldfish are shown in Figure 9 for natural- and long-photoperiod males. There was no significant difference between the natural and long-photoperiod fish. Both groups had a peak $PGF2_{\alpha}$ level in January of 2135.2 ±837.0 pg/ml for the long-photoperiod group, and 4003.8 ±1799.9 pg/ml for the natural-photperiod group. These levels decreased five to 10 fold by March, to 418.6 ±174.0 pg/ml and 314.3 ±103.58 pg/ml, respectively. This increase in mean $PGF2_{\alpha}$ observed in January was not found to be significantly different from the other months, for both natural- and long-photoperiod fish. However, the mean pooled values of $PGF2_{\alpha}$ in January were significantly different from the other months.

4.6.2 $PGF2_{\alpha}$ levels in the plasma

The mean ±SEM plasma levels of $PGF2_{\alpha}$ in the males are given in Figure 9. These values did not show the large increase which was observed in the gonad. The range in plasma $PGF2_{\alpha}$ in the long-photoperiod fish was from 806.8 ±229.2 pg/ml in December, falling to 550.7 ±179.3 pg/ml in March. In the natural-photoperiod group, the highest level was in March, at 758.1 ±120.3 pg/ml, and the lowest was in December, at 112.9 pg/ml. There was no significant difference between the monthly values in both natural- and long-photoperiod, nor was there any difference between the two photoperiod groups. Futhermore, no significant differences were found after pooling the values.

FIGURE 9: Seasonal variations in prostaglandin levels in the male goldfish.
The mean ±SEM levels of $PGF2_{\alpha}$, PGE1 and PGB1 in the testis (left hand side) and in the plasma (right hand side) of corresponding males during the four month study period. Double starred points are means which were significantly different from single starred points (Newman-Kuels, $p=0.05$).


PGF2∝
Long Photoperiod (16 L:8D)
Controls (natural photoperiod)
Long Photoperiod (16 L:8D)
Controls (natural photoperiod)
PGE1
CONCENTRATION (pg/g)
CONCENTRATION (pg/ml plasma)
PGB1
Dec
Jan
Feb
March
Month

4.6.3 PGE1 levels in testis

Figure 9 gives the mean ±SEM testicular levels of PGE1 in males for each month. An increase in the mean levels of PGE1 occurs in March in the natural-photoperiod fish, and in February in the long-photoperiod fish (1161.8 ±484.6 pg/g and 970.0 ±646.0 pg/ml, respectively). However, these increases were not significantly different from the other months due tc the large standard deviation of the mean. There was no significant difference between the two photoperiod groups. The pooled monthly values of PGE1 observed in the testis were not significantly different from each other.

4.6.4 PGE1 levels in the plasma

Figure 9 shows the the mean ±SEM monthly PGE1 levels found in the male plasma. The levels for the long-photoperiod animals ranged from 160.2 ±37.1 pg/ml in December to 674.5 ±37.3 pg/ml in March. In the natural-photoperiod group, the highest mean was 1010.4 pg/ml in December, and the lowest was 275.8 pg/ml in February. There was no significant difference between the monthly values in either photoperiod group. The two photoperiod groups were not found to be significantly different frcm each other, and no significant difference was found between the monthly means of the pooled values.

4.6.5 PGB1 levels in the testis

Figure 9 shows the mean ±SEM testicular PGB1 levels for each month. The PGB1 levels in both the natural- and long-photoperiod fish decreased significantly between December and January, and again between February and March. Furthermore, the increase observed between January and February was also significant. The highest mean levels of PGB1 were 641.5 ±66.0 pg/ml and 594.9 ±50.5 pg/ml in the natural- and long-photoperiod groups, respectively. The lowest mean levels were 39.6 pg/ml in the natural-, and 62.4 ±18.0 pg/ml in the long-photoperiod groups. There was no significant difference in the monthly levels between the two photoperiod groups. When these values were pooled, the outcome was the same, in that all months were different from one another except January and March.

4.6.6 PGB1 levels in the plasma

The mean ±SEM plasma PGB1 levels in the males are given in Figure 9. The pattern of monthly changes paralleled those in the gonad. A significant decrease occurred between December and January in long-photoperiod and in natural-photoperiod fish. The highest levels were found in December and the means were 515.0 ±53.5 pg/ml in the long- and 745.1 pg/ml in the natural-photoperiod fish. The lowest levels occurred in January in both groups, and the levels were 62.4 ±10.9 pg/ml and 34.3 pg/ml in the long- and natural-photperiod groups, respectively. There was no significant difference between the two photoperiod groups. When the values were pooled, the increase in February was again

found to be significantly different from January and March.

4.6.7 Comparison of the prostaglandin levels in the plasma and the testis

There was no significant difference in the monthly values of PGE1 and PGB1 between the gonad and plasma in the male. However, after pooling the values of the two photoperiod groups, the January mean $PGF2_{\alpha}$ level in the gonad was found to be significantly higher than that of the plasma.

4.7 DISCUSSION OF PROSTAGLANDINS IN THE FEMALE

The results of this study do not clearly define the role of PGs during sexual maturation of the goldfish. At this point, the changes in monthly PG levels do not correlate with changes in gonadal maturity, nor does photoperiod appear to have a direct effect on any of the PG levels.

Due to the asynchronous nature of oogenesis in the goldfish and the very narrow range of ovarian states observed in this study, it is difficult to understand the changes which are occurring in PG levels over the period of study. It had been anticipated that the long photoperiod would accelerate gonadal maturity in the female, and that a definite polarity of ovarian states would result between December and March. However, this was not the case, as there was little difference in ovarian maturity, based on histology, between any of the months. In fact, that there was only a low ratio of tertiary oocytes in the

last month of sampling. Furthermore, photoperiod did not seem to affect PG levels, as there was no difference between the natural- and the long- photoperiod groups.

The PG levels of individual fish were investigated in order to see whether any of the extreme values belonged to fish which had similar ovarian characteristics. The PG concentration in fish which had a greater preponderance of atretic follicles was not significantly different from the mean value of that month. Ovarian levels of $PGF2_{\alpha}$ were found to have the greatest variation within a month. After looking at ovarian $PGF2_{\alpha}$ values in individual fish, it was observed that all the very high values of $PGF2_{\alpha}$ were measured in females whose ovaries had oocytes in the tertiary yolk stage. However, not all ovaries having tertiary oocytes had a high $PGF2_{\alpha}$ concentration. This observation suggested that the more mature ovary has a greater capacity for producing $PGF2_{\alpha}$ upon homogenization than the pre-vitellogenic or earlier stages of vitellogenesis. If this is so, the wide variation in $PGF2_{\alpha}$ concentration which was observed could be due to the relative quantity of tertiary oocytes homogenized in the sample.

The plasma $PGF2_{\alpha}$ and PGB1 concentrations correlated well with their corresponding values in the ovary, as no significant difference was found between the two tissues. However, between December and March there was a significant increase in mean plasma PGE1 in the female, with a concomitant decrease in mean ovarian PGE1. This is difficult to explain, as the gonadal state of the fish does not seem to correlate in any way with the PGE1 levels in the plasma or in the gonad. Although PGE1 does not

appear to have a direct role in reproduction, it may be acting indirectly by influencing the circulation of the ovary due to its vasodilatory capacity. This has, in fact, been suggested to be the case in some mammals (Smith et al 1967).

The pooled ovarian PGE1 and PGB1 levels showed a significant decrease in March, and the $PGF2_{\alpha}$ levels also decreased at this time, but not significantly. If, indeed, oogenesis is proceeding toward larger yolk-laden oocytes, it is possible that at this point the lipid pool in the ovary is being diverted toward oocyte production, rather than toward PG production.

4.8 DISCUSSION OF PROSTAGLANDINS IN THE MALE

In the males, the PG levels in the testis and in the plasma fluctuate over the sampling period. However, as in the females, these changes cannot be corrolated with sexual maturity, as no histological changes were observed in the testis over this period. All the male goldfish sampled were in a mature , prespawning reproductive state and, although there was a range in the GSI values of 2.26 to 5.46, there was no indication of a correlation between the GSI and the PG level. Furthermore, photoperiod did not seem to have an effect on PG levels in the male.

There was a dramatic increase in the mean $PGF2_{\alpha}$ levels in the testis during January (Figure 9). A very large standard error of the mean is associated with these levels: the mean of the pooled values is 2644 pg/ml; yet half of the values are over

3000 pg/ml, and the other half are well below 1000pg/ml. There is no histological clue to explain what might be influencing PG synthesis in these fish. Clemens and Grant (1964) reported seasonal variation in the hydration of the testis of the goldfish. It is possible that changes in hydration may somehow be influencing PG levels, and that this may account for some of the variability in PG concentration in the testis. However, there are two arguments against this: first, that GSI, which is an indirect measure of gonadal water content, does not appear to reflect PG levels, and secondly, that only one of the prostaglandins, $PGF2_{\alpha}$, shows a high degree of variability. It is also possible that prostaglandins control circulatory changes that, in turn, are responsible for hydratuicn. However, there are no data to support this hypothesis at present.

The mean PG levels in the testis and in the plasma parallel each other, except for $PGF2_{\alpha}$ which is very high in the gonad in January and February, but stable in the plasma throughout the four month period. Nevertheless, these results suggest that plasma PGE1 and PGB1 could be used as a reflection of their concentration in the gonad; however, this would not be true for $PGF2_{\alpha}$, and it would be inadvisable to use plasma $PGF2_{\alpha}$ concentrations to assess the condition of the gonad.

4.9 CONCLUSION

The results of this study are inconclusive in regards to PG involvement in gonadal maturation. However, this is the first account of the presence of PG in the ovary, testis and plasma of

the goldfish, and certain observations suggest that the ovary is capable of producing a high concentration of $PGF2_{\alpha}$. It seems plausible that PGs do play a role in the sexual maturation of the goldfish, although these experiments did not demonstrate their precise involvement.

5 PROSTAGLANDINS DURING OVULATION IN THE GOLDFISH

5.1 INTRODUCTION

Prostaglandins have been suggested to be involved in ovulation in mammals (Clark et al 1978; LeMaire et al,1973), and indomethacin has been shown to block ovulation in goldfish (Stacey and Pandey 1975). The indomethacin block can be overcome in the goldfish by treatment with PGF2$_\alpha$,PGE1 and PGE2 (Stacey and Pandey 1975). Furthermore, Jalabert and Szollozi (1975) reported that PGF2$_\alpha$could stimulate in vitro ovulation of trout oocytes.

The experiments in previous sections of the present study have demonstrated the presence of prostaglandins in the plasma and gonads of male and female goldfish; however, they did not allow determination of the role of PGs in gonadal maturation. Therefore, the experiments outlined in this section were undertaken to study the possible influence of PGs in the final stages of oocyte maturation, and in ovulation in the female.

5.2 REVIEW OF THE MECHANISMS OF OVULATION

5.2.1 Histological changes of the oocyte

In the goldfish, oogenesis proceeds until the tertiary oocyte stage and the ovary, prior to ovulation, contains primarly oocytes at this stage of development. The maintenance

of these vitelogenic oocytes appears to be under the control of the pituitary (Yamazaki 1965; Stacey 1977; Hurlhurt 1977; Lam et al 1978).

Yamamato and Yamazaki (1967) outlined the following events which occur in the final maturation and ovulation of the goldfish oocytes:

1) There is an increased vasodilation in the follicular layer.

2) Germinal vesicle migration (GVM) takes place approximately five hours prior to ovulation. This consists of the migration of the nucleus to the animal pole of the oocyte.

3) Germinal vescicle breakdown (GVBD), or the degeneration of the membrane surrounding the nucleus, occurs.

4) A decrease in the number and size of the lysosomes present in the granulosa cells. (The lysosomes contain both proteases and carbohydrases.)

5) A degeneration of the microvilli which connect the oocyte with the granulosa (perhaps due to proteclytic enzymes (Jalabert 1976)).

6) An accumulation of fluid in the intracellular spaces between the theca externa and theca interna (due, perhaps, to the production of osmotically active substances by the carbohydrase enzymes).

7) These steps lead to follicle rupture and the active expulsion of the oocyte into the ovarian cavity (ovulation).

This series of events also occurs in other teleost fish;

Jalabert (1975) has described similar (*in vitro*) changes in the mature trout oocyte.

5.2.2 Endocrinological control of ovulation

The endocrine control of the final maturation and ovulation in teleosts has been studied by many authors (see reviews by Yamamato and Yamasaki 1967; Jalabert 1976; Lam *et al* 1978), and a brief description of some of the major controlling agents will be given here.

Although the pituitary plays a central role in coordinating the ovulatory process, other hormones such as the ovarian steroids, corticosteroids and prostaglandins have also been shown to be involved in the process. Several studies have demonstrated that substances which can stimulate gonadotropin (GTH) production, such as clomiphene citrate and certain luteinizing hormone-releasing hormones (LH-RH), can induce ovulation in intact goldfish (Pandey and Hoar 1972; Lam *et al* 1975; Lam *et al* 1976). Furthermore, administration of partially purified salmon gonadotropin (SG-G 100) restores ovulation in hypophysectomized goldfish (Yamasaki 1962; Yamasaki and Donaldson 1968). A gonadotropin surge has been demonstrated during ovulation in the goldfish (Breton *et al* 1972) and recently, Stacey *et al* (1979) were able to define the precise timing of this surge. They reported that GTH starts to increase approximately 10-12 hours prior to ovulation and remains elevated until ovulation, then decreases sharply. The pituitary appears to be controlled by environmental factors such as light

and temperature (Hontela and Peter 1978; Billard et al 1978).

Steroid hormones appear to play an important role at the time of ovulation in fish. Khoo (1974) claimed that progesterone was capable of inducing in vivo ovulation in goldfish at both 12 and 20 C. Jalabert (1976) reported that 17-α-hydroxy-20-β-dihydroprogesterone (17-α-OH20-β-P) was the most effective in promoting in vitro oocyte maturation in goldfish, rainbow trout and northern pike. However, this steroid could not induce ovulation in the goldfish at 12°C (Pandey, unpublished observations). However, it appears that 17-α-OH-20-β-P stimulates GVBD only after GVM has occurred and that GVM is under pituitary control (Jalabert et al 1977).

The corticosteroids seem to play an indirect role in ovulation in some fish. Certain corticostercids, particularly, 11-deoxycorticosterone and 11-deoxycortisol, are effective in inducing in vitro ovulation in the goldfish (Jalabert et al 1973; Jalabert 1976). The corticosteroid pathway could offer another control mechanism in ovulation which could be important in stress-related ovulation.

Research on the involvement of prostaglandins during ovulation in fish was described in the Introduction. However, a brief mention will be made here of some of these findings. Stacey and Pandey (1975) found that indomethacin, a prostaglandin inhibitor, could block HCG-induced ovulation in the goldfish, and that PGF2$_\alpha$, PGE1 and PGE2 could restore the response. Jalabert and Szollozi (1976) demonstrated that PGF2 was very effective in stimulating ovulation in mature oocytes of the rainbow trout. Jalabert (unpublished results, 1976) also

reported that the $PGF2_{\alpha}$ ovulatory response occurred with mature oocytes in the goldfish and northern pike. Recently, Goetz and Theofan (1979) have observed that PGE2, and not $PGF2_{\alpha}$ or PGE1, could induce *in vitro* ovulation of perch oocytes.

To further elucidate the role of prostaglandins during ovulation in fish, $PGF2_{\alpha}$, PGE1 and PGB1 were measured in the plasma of the goldfish during ovulation. The results of this experiment are reported below.

5.3 METHODS

5.3.1 Fish maintenance

A stock of approximately 150 fish weighing between 40 and 80 g each was purchased in April and kept in outdoor aquaria at ambient temperature (about 14°C). Feeding was increased to at least once a day to promote growth and development of the fish. Frozen brine shrimp was included in the diet, along with the trout pellets. Around mid-June it was evident that some of the females were becoming gravid, indicated by the distended apperance of the abdomen and an enlarged gonadopore.

5.3.2 Females

Ovulation was induced in gravid females by increasing the water temperature to 20°C, and injecting fish with human chorionic gonadotropin (HCG) as described by Stacey and Pandey

(1975). The advantage of this method is that ovulation is almost inevitable, as well as easily predicted. Fish will usually ovulate eight to 12 hours following HCG injection.

Selected fish were removed from the stock tank and groups of four to five were placed in 20 gallon tanks. The temperature of the water was approximately 14°C and imitation vegetation was made using strands of black plastic, to cover about 30% of the tank.

In late afternoon, (1600-1800 h) on the day prior to sampling, the water was turned off and a glass water heater was turned on, to gradually increase the temperature to 20°C. Serial blood samples were taken from each fish beginning the following morning. Between 0800 and 0900 h, a fish was anesthetized in MS222 (0.02% in water), weighed, and a blood sample of between 1.0 and 1.5 ml was taken. The fish was then injected intraperitonealy with a solution of HCG (Human Chorionic Gonadotropin, ,1000 IU/ml PBS; #CG-2, Sigma, St-Louis, Mo.) at 10 IU per g weight, and then returned to the tank. This procedure was repeated for the remaining fish. Individual fish were recognized by coloration or by fin clips. The blood was then processed as described previously. Starting at seven hours after HCG injection, the fish were checked every hour for ovulation. Ovulation was indicated by the occurrence of a stream of oocytes running freely from the ovipore when gentle pressure was applied to the abdomen.

At ovulation, and on the morning after, the fish were stripped of as many eggs as possible. They were collected in a polystyrene test tube and kept on ice. After a few minutes of

settling, some of the ovarian fluid was cc1lected and prccessed in the same fashion as the gonadal tissues for the PG assay.

Ovulation experiments were performed on three sets of fish. The preliminary work was done on Group I which consisted of six gravid females. Two or three blood samples were taken frcm each of these fish, the first prior to HCG injection and the second at ovulation. A third sample was taken from two of the six fish, on the morning following ovulation.

Group II was a small set of three females which had ovulated spontaneously by the morning, following the increase in water temperature. These fish were sampled twice, the first blood sample was taken immediately that morning, and the second 24 hours later, on the following morning. Unfortunately, it was impossible to assess the exact time of ovulaticn in these three fish; however, spontaneous ovulation usually occurred at 0400 - 0500 h, following an increase in water temperature (Stacey, personnal communication).

The third set of fish, Group III, consisted of 20 gravid female fish. Four blood samples were collected from these fish. As with Group I, the first sample was taken prior to HCG administration, the second sample was taken 6 hours later, and the third at ovulation. The fourth and final sample was taken on the morning following ovulation (24 hours after HCG injection).

Two fish from this set had not ovulated within the 24 hour period following HCG injection. Samples were alsc taken from these fish, at the described times. In these cases the third sample was usually taken about 15 to 16 hours after HCG administration. The mean time of ovulation of the remaining fish

was 10 hours after HCG injection.

5.3.3 Controls

An experiment was set up to check whether serial sampling affected PG levels. Blood samples were taken from five non-gravid females held in cold (14° C) running water. The time intervals were the same as for the ovulating fish; however, a 0.6% saline solution (0.01 ml/g) was injected instead of HCG. Thereafter, blood samples were taken in the same time sequence, and processed in the prescribed fashion.

5.3.4 Statistics

An analysis of variance was performed on all the data using the UBC ANOVAR program. The Newman-Keul multiple range test was performed on the different groups with a level of significance of 0.05. The paired t-test was used on the prelimenary data, where only two samples were taken from each fish; again a significance level of 0.05 was used. The statistics were computed on the IBM 370 at UBC.

5.4 RESULTS OF OVULATION EXPERIMENT

5.4.1 Prostaglandin levels in the ovarian fluid

The mean ±SEM levels of $PGF2_{\alpha}$ in the ovarian fluid was 9088 ±2306.9 pg/ml, that of PGE1 was 636.0 ±137.4 pg/ml and PGB1 was 303.1 ±64.2 pg/ml. It should be noted that in four of the eight samples, the level of $PGF2_{\alpha}$ was so high that its binding ratio was off the standard curve and that the highest value on the curve (2400 pg) was given to these samples. This was probably an underestimate of the true value of the $PGF2_{\alpha}$ concentration in these samples.

5.4.2 Prostaglandin levels in the plasma of ovulating and non-gravid females

5.4.2.1 $PGF2_{\alpha}$ levels in Group I fish

In the preparatory experiment, Group I, two blood samples were taken from each of six gravid females. The first was taken prior to HCG administration, and the second at ovulation. The mean ±SEM $PGF2_{\alpha}$ level before HCG injection was 532.6 ± 49.3 pg/ml, and had increased to 4322.1 ± 1221.7 pg/ml at ovulation. However, this increase was not shown to be significant (paired t-test), as the high mean was due to two very high values cut of the six samples. The samples taken from the two fish 24 hours after HCG injection had a mean $PGF2_{\alpha}$ level of 4778.0 pg/ml.

5.4.2.2 $PGF2_{\alpha}$ levels in Group II fish

In Group II fish, where three females had ovulated spontaneously by early morning, the $PGF2_{\alpha}$ levels in the plasma were very high. The mean values of $PGF2_{\alpha}$ in the first sample was 7754 pg/ml; however, this dropped to 885.2 pg/ml 24 hours later, after stripping the fish of eggs.

5.4.2.3 $PGF2_{\alpha}$ levels in Group III fish

The $PGF2_{\alpha}$ profile during ovulation is shown in Figure 10 for the fish from Group III, where four blood samples were taken. The mean ±SEM $PGF2_{\alpha}$ levels in the plasma taken in the morning following the increase in water temperature and prior to HCG injection was 317.4 ±30.3 pg/ml. There is nc significant change in the six hours following HCG administration; the mean plasma $PGF2_{\alpha}$ level at this time was 292.8 ±46.3 pg/ml. However, the mean $PGF2_{\alpha}$ did increase to 979.3 ±323.3 pg/ml at ovulation, when oocytes could be squeezed from the fish. This increase was not significantly different from the first two readings. The $PGF2_{\alpha}$ levels jumped to 4093.9 ±558.8 pg/ml 12 hours after ovulation. At this time, more eggs could be squeezed from the female, and generally there was a considerable volume of eggs. Twc HCG-injected fish did not ovulate within 24 hours. The $PGF2_{\alpha}$ in these individuals remained under 300 pg/ml in all plasma samples.

5.4.2.4 $PGF2_{\alpha}$ levels in Group IV fish

The mean ±SEM levels of $PGF2_{\alpha}$ in the non-gravid saline injected females, Group IV, are given in Figure 10 . There was a slight decrease in $PGF2_{\alpha}$ over 24 hours in these fish. The mean ±SEM in the first sample was 245.3 ±94.3 pg/ml and dropped to 94.6 ±41.9 pg/ml in the fourth sample. This decrease was not found to be significant.

5.4.2.5 PGE1 levels in Group I and Group II fish

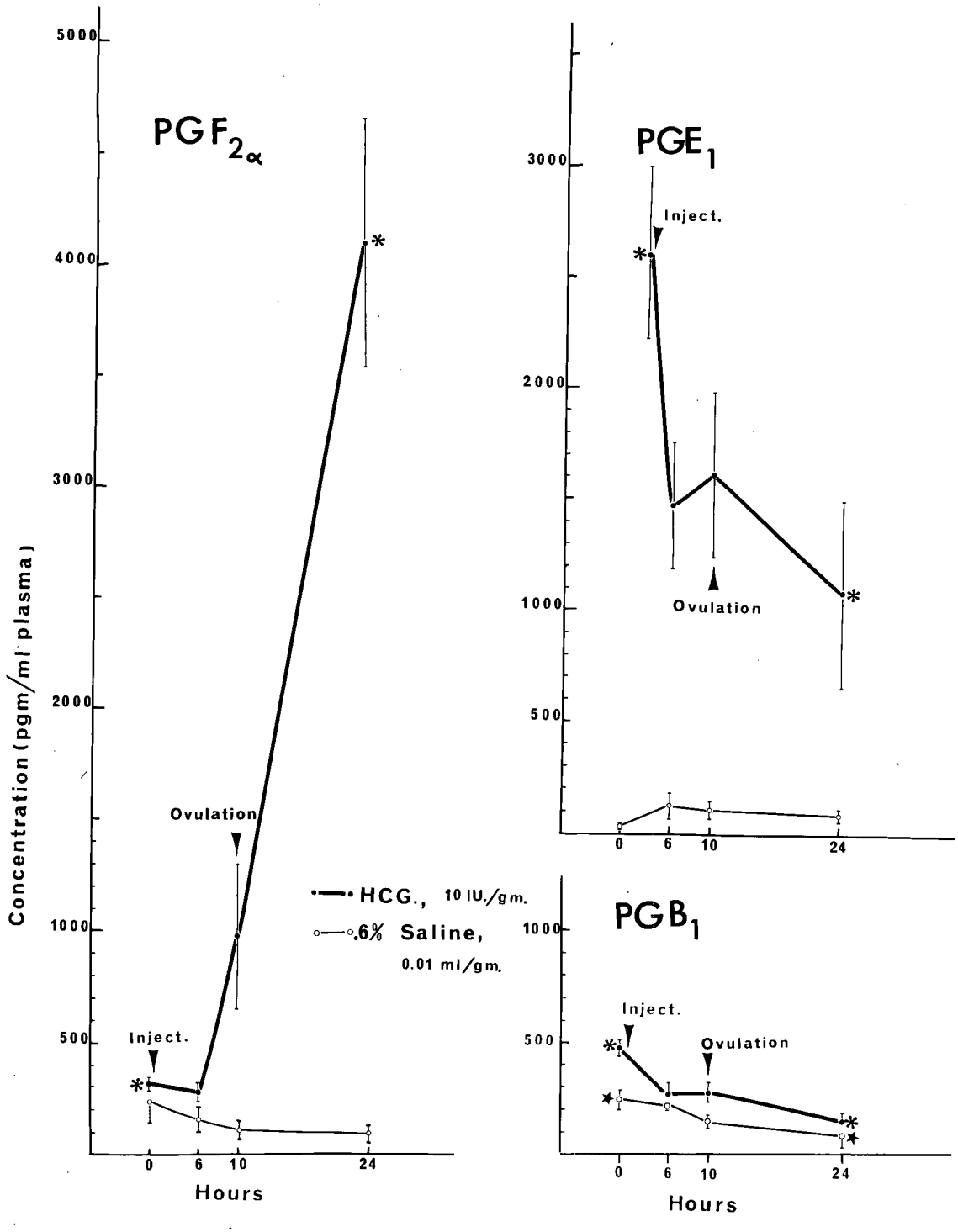
The six females in Group I had a PGE1 level of 7324.8 ±903.4 pg/ml prior to ovulation. This value decreased two fold, to 4402.2 ±1103.0 pg/ml at ovulation. The two females sampled at 24 hours after HCG injection had a mean PGE1 level of 2139.4 pg/ml.

A three fold decrease in PGE1 levels was found in the spontaneously ovulating fish, Group II, from 7187.6 pg/ml to 2100.3 pg/ml between the first sample taken after ovulation, and the sample taken 24 hours later.

5.4.2.6 PGE1 levels in Group III fish

The changes in plasma PGE1 are shown in Figure 10 . An overall decrease (two fold) was observed in PGE1 in the 24 hour period between HCG injection and 12 hours after ovulation in this group of fish. The highest levels were found prior to HCG administration , 2601.3 ±396.5 pg/ml, and decreased slightly to 1471.7 ±283.0 in the subsequent six hours. PGE1 leveled at

FIGURE 10: variations in prostaglandin levels during HCG-induced ovulation in the goldfish.
$PGF_{2\alpha}$, PGE1 and PGB1 in the female goldfish. The thick line represents changes which occurred in gravid females before and after HCG injection (10 IU//ml) and at ovulation (mean time 10 hours after HCG injection). The thin line represents changes which ocurred in non-gravid, saline-injected (0.6% saline, 0.01ml//g) females over the same time period. (note: means with a five point star are not significantly different from means with a six point star.)


PGF2α
PGE1
PGB1
Concentration (pgm/ml plasma)
5000
4000
3000
2000
1000
500
Inject.
Ovulation
HCG., 10 IU./gm.
.6% Saline, 0.01 ml/gm.
0
6
10
24
Hours

1607.1 ±370.2 pg/ml at ovulation, then decreased further to 1076.5 ±419.7 pg/ml 12 hours after ovulation. The decrease observed between the first and last samples was significant at the p=0.05 level.

5.4.2.7 PGE1 levels in Group IV fish

The mean PGE1 plasma levels in the non-gravid, saline-injected fish were very low in comparison to the ovulating fish. These levels ranged between 30.0 and 120.0 pg/ml and were 20 times lower than the highest mean in Group III. Figure 10 gives the changes in plasma PGE1 over the 24 hour sampling period. There was no significant change in PGE1 levels over this time.

5.4.2.8 PGB1 levels in Group I fish

In the preliminary study, a slight but insignificant decrease was found in the plasma PGB1 levels (800.0 ±96.5 to 550.4 ±89.8 pg/ml). Furthermore, there was no change in PGB1 in the plasma of the two fish sampled 12 hours after ovulation (679.0 pg/ml).

5.4.2.9 PGB1 levels in Group II fish

The PGB1 levels in the spontaneously-ovulating fish did not change between the first sample after ovulation and the second 24 hours later (806.6 ±135.5 to 806.6 ±299.5 pg/ml).

5.4.2.10 PGB1 levels in Group III fish

The mean PGB1 plasma levels in the fish from Group III are shown in Figure 10 . There was a significant decrease in PGB1 between the pre-injection sample and the second sample (470.5 ±39.5 to 266.3 ±45.8 pg/ml), and between the ovulatory sample and the last sample (270.1 ±42.6 to 147.7 ±34.4 pg/ml).

5.4.2.11 PGB1 levels in Group IV fish

There was a significant decrease between the first and final samples in the non-gravid, saline-injected females. The mean PGB1 levels in the non-gravid saline-injected females are shown in Figure 10 . There was a significant decrease in PGB1 between the first and final blood samples in this group.

5.5 DISCUSSION OF OVULATION

The above experiments strongly suggest that PGs play an important role in ovulation in the goldfish. Significant changes were found in $PGF2_{\alpha}$ and in PGE1 levels in the plasma. There was a significant decrease in PGE1 during the 24 hour sampling period. Furthermore, the levels of PGE1 in the gravid, HCG-primed females were over 20 times greater than in the non-gravid saline-injected females. These findings would suggest that PGE1 may be involved in final oocyte maturation, prior to ovulation. In the literature, it has often been shown that $PGF2_{\alpha}$ is the most influential of the prostaglandins at the time of ovulation in mammals (LeMaire *et al* 1975; Clark *et al* 1978). In the present

study, although there was an increase in $PGF2_{\alpha}$ at the time of ovulation, it does not seem likely that plasma $PGF2_{\alpha}$ was directly responsible for the induction of ovulation, as this rise occurred only after ovulation. However, the $PGF2_{\alpha}$ level is very high in the ovarian fluid, which bathes the oocytes, and might influence ovulation in the goldfish. The results from the saline-injection experiment demonstrated that repetitive sampling had no significant effect on either plasma $PGF2_{\alpha}$ or PGE1 in the goldfish. However, it does appear to have some effect on the plasma levels of PGB1 , as there was a significant decrease in PGB1 levels during the serial sampling of the non-gravid, saline-injected fish (Group IV). The relationship between the findings in the literature and those in the present study will be discussed in more detail in the General Discussion.

5.5.1 PGF2 during ovulation

Ovulation was checked every hour by gently massaging the abdomen of the fish. In nine out of 17 females in this group, only a few eggs had ovulated (less than 0.5ml). In these instances, the process of ovulation was considered to be just beginning. In the remaining eight fish, many eggs had ovulated when the blood sample was taken, and ovulation was considered to have taken place shortly before the check. The mean plasma $PGF2_{\alpha}$ concentration increased at ovulation, but this increase was not significant. The apparent increase was caused by a bias due to a few very high levels of $PGF2_{\alpha}$. It is interesting to note that the higher levels of $PGF2_{\alpha}$ were found in those fish which had

ovulated a greater volume of eggs. The range of $PGF2_{\alpha}$ values in the nine "just-ovulating" fish is 103 to 850.0 pg/ml with a mean ±SEM of 395.5 ± 72.1 pg/ml. The range of the $PGF2_{\alpha}$ levels in the other eight fish which had ovulated a greater volume of eggs is 332 to 5600 pg/ml with a mean ±SEM of 1808.9 ±638.5 pg/ml.

Since blood samples were taken at ovulation and 12 hours later, it is difficult to know exactly when $PGF2_{\alpha}$ started to increase in the blood and how rapidly it did so. It seems plausible from the above results that the increase in PG in the plasma is related to the volume of ovulated eggs in the ovarian cavity and that this increase in the plasma is very rapid.

Ovulated eggs were removed following blood sampling. This was done at the first observation of ovulation, and again the following morning, at which time the volume of eggs had greatly increased. On one occasion, many eggs had ovulated at the first ovulatory sampling, and the plasma $PGF2_{\alpha}$ levels in this fish at this time were 5600 pg/ml. The following morning, the plasma $PGF2_{\alpha}$ had decreased to 119.9 pg/ml, with very few eggs remaining in the ovarian cavity. A similar decrease in $PGF2_{\alpha}$ was observed following egg removal in the three spontaneously-ovulating fish. The mean ovulatory level in these fish was 7753.3 pg/ml. These samples were taken several hours after ovulation. Twelve hours later, the mean $PGF2_{\alpha}$ level had decreased to 885.2 pg/ml; again, this was after removal of many of the eggs. It would appear from these observations that the removal of the ovulated eggs leads to a decrease in $PGF2_{\alpha}$ in the plasma. These findings coupled with the presence of a very high concentration of $PGF2_{\alpha}$ in the ovarian fluid, would suggest that the ovary is the source of the $PGF2_{\alpha}$

found in the plasma. $PGF2_{\alpha}$ could be leaking from the ovarian fluid to the plasma after ovulation. $PGF2_{\alpha}$ could also be produced by the oviduct upon stretching. However, not all the oocytes are in this area after ovulation, as most of the ovulated eggs remain in the ovarian cavity, prior to spawning (Stacey, 1974).

The increase in $PGF2_{\alpha}$ cannot be attributed directly to the HCG injection, as plasma $PGF2_{\alpha}$ levels did not change in the two HCG-injected fish which did not ovulate. This further supports the hypothesis that the source of $PGF2_{\alpha}$ could be the ovarian fluid present in the ovarian cavity, and/or in the oviduct following ovulation.

5.5.2 PGE1 during ovulation

The PGE1 levels decrease in the plasma during the ovulation period. This decrease was observed in fish from Groups I, II and III, which contained gravid fish only. The fish from Groups I and II demonstrated a two to three times greater PGE1 concentation than the fish in Group III. There is no apparent reason for this discrepancy, as repetitive sampling of the saline-injected fish did not affect PGE1 levels. Moreover, of the two HCG-injected fish which did not ovulate, one showed a decrease in plasma PGE1, but the other showed no change.

There is a clear difference in the plasma PGE1 concentration between gravid and non-gravid females. The mean plasma PGE1 levels in the gravid females in the HCG-injection experiment are 10 to 30 times greater than the levels found in the non-gravid females used in the saline-injection experiment.

The high concentration of PGE1 in the gravid fish did not appear to be directly linked to ovulation, as the two gravid females which did not ovulate also had higher PGE1 levels than those in the saline group. This suggests that PGE1 may be a necessary component of the final phases of the maturation process, immediatly prior to ovulation.

The source of PGE1 is not clear; presumably it is synthesized in the ovary, as was suggested for $PGF2_{\alpha}$. However, PGE1 does not follow the same secretion pattern as $PGF2_{\alpha}$. $PGF2_{\alpha}$ increases during ovulation while PGE1 decreases, and the relative concentration of $PGF2_{\alpha}$ in the ovarian fluid is over 10 times that of PGE1 . The decrease observed in the plasma could be due to a shift in the precursor lipids toward the production of $PGF2_{\alpha}$.

5.5.3 PGB1 levels during ovulation

The plasma PGB1 levels decrease in both the ovulating fish and in the saline-injected fish. This suggests that repetitive blood sampling may deplete PGB1 in the plasma.

There is little difference between plasma PGB1 levels in the gravid fish in Group III and the non-gravid females from Group IV. However, fish in the prepatory experiments, Group I, and the spontaneously ovulating fish, Group II, exhibited plasma PGB1 levels which were two to three times higher than in fish from the other two groups.

The concentration of PGB1 in the ovarian fluid was the lowest of all the PGs measured in the female. Although PGB1 is

found in the plasma and the ovarian fluid during ovulation, there is no evidence that it is changing during this time. This would suggest that PGB1 does not play a significant role during ovulation.

The overall findings in these experiments suggest that both $PGF2_{\alpha}$ and PGE1 are important in the final maturation process, and at ovulation, in the goldfish. PGE1 may to be necessary prior to ovulation, as it is in a very high concentration in the plasma at this time, whereas $PGF2_{\alpha}$ seems to be more important at, or after, ovulation. There is no evidence that PGB1 influences ovulation or the final stages of oocyte maturation.

All three PGs are present in ovarian fluid, with PGF2 in the highest concentration. This suggests that the ovary is the main source of PGs . The implications of these results, together with the findings of other researchers, will be considered in the General Discussion.

6 SPERMIATION IN THE GOLDFISH

6.1 INTRODUCTION

6.1.1 Testicular changes during spermation

Spermiation in fish is defined as the release of mature sperm into the sperm ducts, and is due to a thinning of the semen (Clemens and Grant 1964). Spermiation usually begins early in the spawning season and the testis remains in this state during the entire spawning period.

Several authors (Yamamato and Yamazaki 1967; Yamasaki and Donaldson 1968b) have discussed the process of spermiation in the goldfish. Spermiation starts shortly after the last spawning season and is completed rapidly within one to two months. At the end of spermatogenesis, the spermatozoa are found in the lumen of the testicular lobules, where they are free from the Sertoli cells, and remain in this state throughout the spermiation period. Prior to spermiation, none of the lobules are connected to the seminal tubule. However, shortly before spermiation, long tubular lobules are formed as a result of breakage of many of the walls between the lobules; these lobules are then connected to the seminal tubules. Furthermore, the epithelial cells of the sperm ducts and the interstitial cells become hypertrophied at this time. There is also a marked hypertrophy of the blood capillaries in both the testicular lobules and the seminal tubules. In addition, gonadal hydration increases considerably,

and subsequently a fluidity of the milt develops.

Yamazaki and Donaldson (1968b) have linked the hypertrophy observed in the cells of the sperm ducts with the increase in seminal fluidity, and have suggested that these cells may be secreting fluid into the ducts. Yamamato and Yamazaki (1967), following histochemical observations, have attributed this increase in gonadal water content to an increase in the enzymatic breakdown of carbohydrate, which would favor water uptake by the tissues. Their histochemical and ultrastructural observations indicated that both the Sertoli cells and the sperm ducts are very active at this time.

Although the male is functionally mature from the onset of spermiation, Sanchez-Rodriguez et al (1978) have reported that there are changes in sperm volume and the number of sperm released over the spermiation period in the rainbow trout, Salmo gairdneri . It is likely that these changes also occur in the goldfish, and that fluctuations in testicular hydration would also occur over this period (Clemens and Grant 1965).

6.1.2 Endocrine control of spermiation

It has been well established that the pituitary plays a major role in the control of spermiation in fish (Clemens and Grant 1964,1965; Yamamato and Yamazaki 1967; Yamazaki and Donaldson 1968a, b,; Billard 1978; Billard et al 1978; Shanchez-Roriguez et al 1978). An increase in testicular hydration in the carp and goldfish, and seminal thinning in rainbow trout have been observed following injections of pituitary extracts

(Clemens and Grant 1964, 1965). Yamamato and Donaldson 1968a, b) have shown that spontaneous spermiation in goldfish does not occur following hypophysectomy, and that pituitary extracts and HCG could induce spermiation in both intact and hypophysectomized fish while LH and FSH were not effective. Crim et al (1975) have demonstrated that plasma gonadotropin levels, of several species of salmonids, are much higher at the end of spermatogenesis and during the spawning season than at any other time.

Yamazaki and Donaldson (1969) have also found histochemical evidence of 3-β-hydroxysteroid dehydrogenase in the interstitial cells of the goldfish. They observed that hypophysectomy reduced the activity of this enzyme, while administration of salmon gonadotropin (GTH) restored this activety. However, they found no change in enzyme activity during spermiation, despite findings that salmon-GTH induced hypertrophy of the interstitial cells and spermiation in hypophysectomized fish. They concluded that, although there appeared to be no correlation between 3-β-hydroxysteriod dehydrogenase and spermiation, there was an indication that GTH may influence steroid production which, in turn, could initiate spermiation.

Sanchez-Rodriguez et al (1978) have looked at weekly plasma gonadotropin and androgen levels in trout, over a 12 week period following the first indicatioan of spermiation. They reported that plasma GTH was high at the onset of spsermiation , decreased in the following 6 weeks, then started to increase, reaching maximum levels on the 12th week. Androgen levels followed a reverse pattern. From these results, they postulated

that the androgens could have a negative feedback on gonadotropin secretion. This hypothesis was also made following experiments by Billard, Richard and Breton (1977) and by Billard (1978). Their studies showed that administration of various androgens suppessed the increase in gonadotropin secreation which occurs in trout following castration, and that these responses were greatest during the spawning period.

Overall, the pituitary seems to contrcl the process of spermiation in fish. However, steroids also appear to be involved, and the exact functions of these hormones have yet to be elucidated.

6.1.2.1 Methods and results

In an attempt to observe the possible involvement of prostaglandins in spermiation in the goldfish, $PGF2_{\alpha}$, PGE1 and PGB1 levels were measured in the plasma of spermiating goldfish, over a 24 hour period following HCG administration. Four blood samples were taken from each of 10 spermiating males. The following procedure was patterned on the preceeding ovulation experiment: the water was warmed to 20°C and blood samples were taken the following morning. After this, HCG (Human Chorionic Gonadotropin 1,000 IU/ml PBS; Sigma, St-Louis) was injected at 10 IU/g, and blood samples were taken at six, 10 and 24 hours after HCG injection. The 10 hour sampling time was chosen as it was the mean time of ovulation in the females. Four blood samples were taken from each fish. Blood samples were processed as described previously and assayed for each prostaglandin. All

males were mature, as milt was produced when pressure was applied to the abdomen.

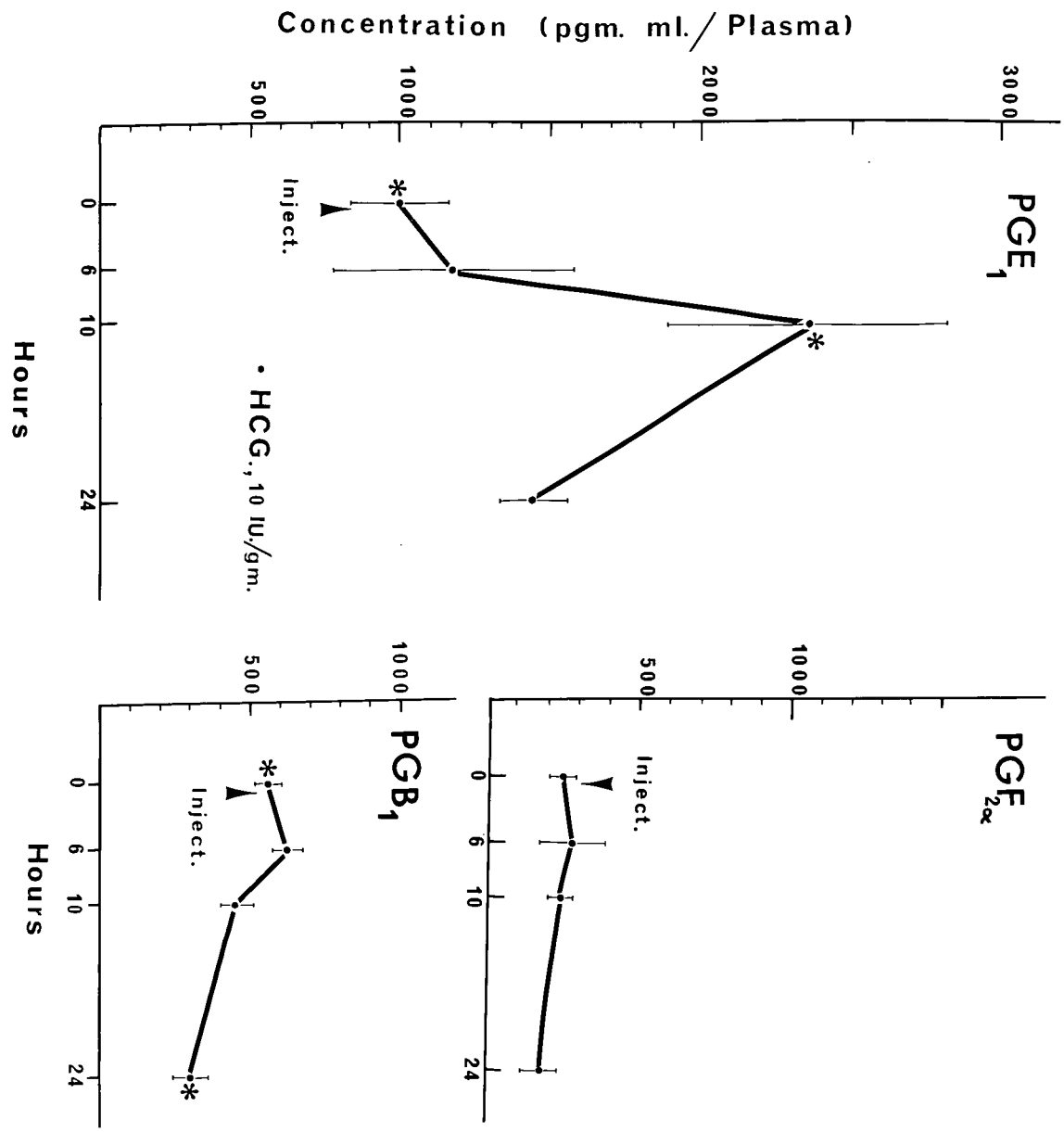
6.1.3 $PGF2_{\alpha}$, PGE1 and PGB1 levels in the male prior tc, and after, HCG injection

Figure 11 shows the profile of $PGF2_{\alpha}$ in the plasma of the male goldfish during the 24 hour period following HCG administration. The mean ±SEM of $PGF2_{\alpha}$ in the plasma sample prior to injection was 236.1 ±42.4 pg/ml. This level decreased slightly in the last sample to 172.9 ±63.1 pg/ml; there was, however, no significant difference between any of the samples.

The changes in the mean ±SEM in plasma PGE1 following HCG injection are given in Figure 11. There is a significant increase in the PGE1 level from the pre-injection sample (990.7 ±171.0 pg/ml) to the third sample (2351.4 ±470.0 pg/ml), taken ten hours later. The mean PGE1 level had decreased to 1432.1 ±117.1 pg/ml by the following morning; however, this decrease was not significant.

Figure 11 shows the changes observed in the mean ±SEM in plasma PGB1 in the same fish. There is a significant decrease between the pre-injection sample (561.1 ±45.6 pg/ml) and the third sample (452.2 ±52.9 pg/ml), taken ten hours later. A further decrease was observed in the fourth sample (298.0 ±64.5 pg/ml) the following morning, which is significantly different from the other three samples.

FIGURE 11 variations in prostaglandin levels follcwing HCG injection in male goldfish.
The mean ±SEM levels of $PGF2_{\alpha}$, PGE1 and PGB1 in the mature male goldfish before and after HCG injection (10 IU//ml). The starred points indicate means which were significantly different from each other (Newman-Kuels, p=0.05).


Concentration (pgm. ml./ Plasma)
3000
2000
1000
500
PGE1
Inject.
* HCG., 10 IU/gm.
Hours
0
6
10
24
PGF2α
PGB1

6.2 DISCUSSION

In the male goldfish, plasma levels of $PGF2_{\alpha}$ did not fluctuate in the 24-hour period following HCG administration. However, there were significant changes in PGE1 and PGB1 levels of the same fish. PGE1 increased more than two fold 10 hours after HCG injection, while in the same time period, PGB1 dropped significantly to approximately 20% of the pre-injection level. PGB1 had decreased a further 30% by the following morning. It is difficult, at this time, to correlate the changes in plasma PGE1 and PGB1 with changes in the reproductive status of the fish. The male goldfish used in this study were all fully mature and in a state of spermiation. For this reason, the changes in the prostaglandins cannot be directly attributed to the onset, or the induction, of spermiation, but may be regarded as changes due to an acute increase in gonadotropin in the fish.

These are the first findings which indicate that prostaglandins may be involved in the spermiation process in the goldfish. However, the function and mode of action of the prostaglandins in the reproductive systems of male fish still remains to be elucidated. The relationship between prostaglandins and other hormones involved in the spermiation process will be considered in the General Discussion.

7 GENERAL DISCUSSION

The experiments on seasonal variations in prostaglandins in the early part of this thesis demonstrated the presence of prostaglandins in the plasma and gonads of male and female goldfish; however, they did not establish their possible involvement in the early gonadal maturation of either sex. Nevertheless, there was an indication that ovaries having yolky oocytes are capable of a greater synthesis of $PGF2_{\alpha}$ than those in an earlier stage. The ovulation study suggested that PGE1 and $PGF2_{\alpha}$ are probably associated with the final maturation and ovulation of the goldfish oocyte. The high concentration of $PGF2_{\alpha}$ found in the ovarian fluid suggests that $PGF2_{\alpha}$ is very important at ovulation, or in the processes immediately following ovulation. In the males, however, significant changes were observed only in PGE1. PGB1 did not appear to be concerned with any of the reproductive processes investigated in either males or females. The present discussion will relate these results to the findings of other researchers. This will be followed by a model of the possible role of prostaglandins in goldfish reproduction, and an outline of some of the possible mechanisms of their action.

Although the seasonal study did not show any changes occurring in prostaglandin levels that could be attributed to gonadal maturation, this does not necessarily mean that prostaglandins are unimportant in this process. The fact that prostaglandins are present in the gonad and fluctuate during maturity suggests that they may be functioning in the overall maintenance of the gonad. It is possible that prostaglandins are

interacting with other hormones from the pituitary and the ovary and testis, during the development of the gonad of both male and female goldfish. However, in mammals, the involvement of prostaglandins in the reproductive system appears to be in the final stages of maturity, prior to ovulation. It is therefore not surprising to find a similar pattern occurring in fish. In both fish and mammals, the role of prostaglandins in spermiation is not clear, but there is a strong possibility that $PGF2_{\alpha}$ and PGE1 are very important in ovulation in both groups.

Stacey and Pandey (1975) demonstrated the necessity of prostaglandins in goldfish at ovulation. They showed that indomethacin, a prostaglandin inhibitor, could successfully block ovulation in HCG-primed goldfish and that PGE1, PGE2 and $PGF2_{\alpha}$ could overcome this block and restore ovulation. As the experiments dealt with here were based directly on the methods of Stacey and Pandey (1975), it is possible to make a close comparison between the two sets of findings. Their experiments showed that indomethacin was completely effective in blocking ovulation up to six hours following HCG injection. However, it was only partially effective at nine hours, and totally ineffective 12 hours after HCG administration. It appears that, in order to inhibit ovulation with indomethacin, it is necessary to administer it at least two to three hours prior to ovulation. They also found that indomethacin would continue to inhibit ovulation up to at least six days after its administration.

It appears from Stacey and Pandey's study that prostaglandin synthesis is essential for ovulation in the goldfish. They also demonstrated the importance of timing in the

administration of indomethacin and prostaglandins. The finding that indomethacin must be administered no later than two to three hours prior to ovulation, in order to effectively block ovulation, suggested two things: first, that prostaglandin synthesis increases shortly before ovulation; and secondly, that this specific prostaglandin production is associated with ovulation. In addition, they observed that prostaglandin replacement therapy was most effective when given close to the expected time of HCG-induced ovulation. This further supports the hypothesis that prostaglandin synthesis is crucial prior to ovulation. Furthermore, HCG-stimulated follicles were responsive to exogenous prostaglandins within a restricted period, as follicular sensitivity had decreased considerably by 15 hours after HCG administration.

The present study has demonstrated that levels of PGE1 and $PGF2_{\alpha}$ change significantly within the period of ovulation. The plasma $PGF2_{\alpha}$ levels in ovulating fish showed the most dramatic change; the $PGF2_{\alpha}$ levels had increased more than 14-fold after ovulation. The reverse occurred in PGE1 levels in the plasma, as PGE1 decreased by 2.5 times over the same period. These changes appeared to be related to the reproductive state of the fish. The plasma $PGF2_{\alpha}$ levels increased only in those fish which had ovulated; they did not increase in the two gravid females which did not ovulate following HCG injection. The plasma PGE1 levels were over 20-fold greater in the gravid, ovulating females than in the non-gravid, saline-injected females. Stacey and Pandey's (1975) study indicated that prostaglandin synthesis is most crucial just prior to ovulation. However, the timing cf the

prostaglandin changes in the blood suggests that plasma $PGF2_{\alpha}$ and PGE1 are not directly involved in inducing ovulation, as $PGF2_{\alpha}$ increased only after ovulation and PGE1 was at its highest concentration nine to 12 hours prior to ovulation. Nevertheless, these changes seem to be occurring due to an alteration in the reproductive state of the fish, and it is possible that prostaglandins in the blood are involved in other events in the ovulatory process, or in spawning.

PGE1 may indirectly influence hydration in the ovary by two means:

1) it may stimulate vasodilation in the ovary, thereby inducing the hypertrophy of the blocd capillaries in the follicular layer before the onset of ovulation, as observed by Yamamato and Yamazaki (1967)

2) it may also increase the vascular permeability (Kennedy 1979) which, in turn, would cause an increase in the uptake of water by the gonad.

An increase in water uptake by the ovary is one of the necessary steps leading to ovulation, and it is possible that PGE1 production is increased in order to alter the hydration of the ovary prior to ovulation. It also appears from the present study that HCG is not inducing the high levels of PGE1 observed in ovulating fish. However, these fish had been subjected to an increase in water temperature, from 14°C to 20°C, which may have stimulated PGE1 synthesis; alternatively, PGE1 may be in constant, high levels in gravid goldfish. This hypothesis is supported by evidence in the seasonal study which found that plasma levels of PGE1 in the female had increased significantly

from December to March. The mean PGE1 level in the plasma in March was about 1000 pg/ml, and was approaching that measured in the gravid females in the ovulation study (mean of 2600 pg/ml). The decrease in PGE1 observed during ovulation could be the result of a shift in prostaglandin synthesis toward the production of $PGF2_{\alpha}$.

$PGF2_{\alpha}$ increased in the plasma after ovulation, yet the exact time of this increase was difficult to determine. There was some indication that the increase was proportional to the quantity of oocytes ovulated, and that once the majority of ovulated oocytes had been removed, the $PGF2_{\alpha}$ levels decreased. There is some evidence in the literature on the possible functions of $PGF2_{\alpha}$ in the blood. Stacey (1977) found that $PGF2_{\alpha}$ was very effective in stimulating spawning behaviour in the female goldfish. Peter and Billard (1976) reported that $PGF2_{\alpha}$ decreased gonadotropin secretion in the pituitary. It seems plausible that the increase in $PGF2_{\alpha}$ following ovulation could be triggering spawning behaviour, and that the high levels of $PGF2_{\alpha}$ would have a negative feedback on gonadotropin secretion. These two hypotheses will be discussed in more detail later.

Although the prostaglandins in the plasma do not appear to be playing a direct role in ovulation, the ovarian fluid contained all three prostaglandins (PGB1, PGE1 and $PGF2_{\alpha}$), and these may be more directly involved in inducing ovulation.

Jalabert *et al* (1972) found that coelomic (ovarian) fluid collected from trout at ovulation, and used as an incubation fluid, was effective in inducing *in vitro* ovulation of mature trout oocytes. (GVM (germinal vesicle migration) and GVBD

(germinal vesicle breakdown) were completed.) Jalabert and Szollozi (1975) later demonstrated that $PGF2_{\alpha}$ at concentrations of 1.0 and 5.0 mg/ml could induce *in vitro* ovulation of mature trout follicles, but they found PGE2 to be ineffective at the same concentrations. Jalabert (1976) mentions in his review article that $PGF2_{\alpha}$ also promotes *in vitro* ovulation of goldfish oocytes. In view of the high concentration of $PGF2_{\alpha}$ present in goldfish ovarian fluid, it seems likely that $PGF2_{\alpha}$ could be responsible for stimulating the *in vitro* ovulation which Jalabert observed in the ovarian fluid medium.

In the present study, the concentration of $PGF2_{\alpha}$ in the ovarian fluid was over 9 ng/ml, and the concentration in the same incubation medium which induced 100% ovulation in trout oocytes in Jalabert and Szollozi's (1975) study was about 1000 times greater (1.0 mg/ml). However, doses as low as 0.15 to 0.30 mg/ml were effective in stimulating partial ovulation, yet these levels are still over 100 times greater than the concentration of $PGF2_{\alpha}$ measured in the ovarian fluid of the goldfish. Although the time required for 100% ovulation using 5.0 mg/ml PGF2 in the medium was 20 hours, ovulation usually started within an hour of $PGF2_{\alpha}$ addition and, in some cases, 50% ovulation had occurred within the initial three hours. The time lag of the response could be due to the time required for the oocytes to absorb $PGF2_{\alpha}$ from the medium or, perhaps, to small variations in maturity between oocyte preparations.

Jalabert and Szollozi (1975) reported that the follicles must be completely mature, i.e., GVM and GVBD must be completed, in order for $PGF2_{\alpha}$ to be effective, and that Ca^{++} and Mg^{++} are

also necessary for expulsion of the oocyte. From these data, they speculated that PGF2$_\alpha$ acts on the smooth muscle-like cell of the theca, thus stimulating contraction of the follicle and expulsion of the oocyte. Jalabert (1976) found that PGE2 was ineffective in stimulating in vitro ovulation. However, Stacey and Pandey (1975) showed that both PGE1 and PGE2 could induce in vivo ovulation in the goldfish, and Goetz and Theofan (1979) found that PGE1 and PGE2, as well as PGF2$_\alpha$, induced in vitro ovulation of the perch oocyte.

Goetz and Theofan (1979) reported that in vitro treatment of perch (Perca flavescens) oocytes with 17-alpha-hydroxy-20-beta-dihydroprogesterone at 15 C induced GVM and GVBD, and that ovulation usually occurred 33 hours later. This response was completely blocked for 48 hours when indomethacin was also added to the incubation medium. However, when PGF2$_\alpha$, PGE1 or PGE2 was added to the indomethacin-progesterone treated oocytes after a 35 hour incubation, the ovulation response was restored. Of the three prostaglandins tested, Goetz and Theofan found PGE2 to be the most effective; a dose of 9.8 ng/ml induced about the same percentage ovulation as would have occurred with the 17-α-OH-20-β-dihydroprogesterone without the indomethacin. A higher concentration (160 ng/ml) of PGE1 and PGF2 was needed to elicit a similar response. They therefore proposed that steroids (in particular, 17-α-hydroxy-20-β-dihydroprogesterone) initiate GVM and GVBD and stimulate prostaglandin production which, in turn, induces ovulation in the perch. The mean concentration of PGF2$_\alpha$ measured in the ovarian fluid of the goldfish was 9088 pg/ml, and that of PGE1 was about 15 times less, at 636 pg/ml. Goetz

and Theofan (1979) further observed that threshold responses of 1-5% ovulation could be obtained by adding 0.61 ng/ml of PGE1 or PGE2 to the incubation medium containing indomethacin; however, 9.8 ng/ml of $PGF2_{\alpha}$ was required to elicit a similar response. Although these lower concentrations of PGE1 and $PGF2_{\alpha}$ did not induce a significant ovulation response, they are very close to those measured in the ovarian fluid of the goldfish.

Breton et al (1972) reported an increase in serum GTH levels just prior to ovulation in the goldfish. Stacey et al (1979) have recently found that GTH starts to increase approximatly eight hours before ovulation in the goldfish, remains high throughout ovulation, then decreases rapidly shortly afterwards. Jalabert (1976) speculated that GTH is necessary in the final maturation of the fish oocyte. He stated that gonadotropin stimulates 17-α-OH-20-β-dihydroprogesterone progesterone synthesis which, in turn, triggers the later stages of intrafollicular maturation (migration of the nucleus to the animal pole, germinal vesicle breakdown and increase in intrafollicular fluid). As $PGF2_{\alpha}$ increases in the blood after ovulation, it is unlikely that $PGF2_{\alpha}$ influences synthesis of GTH or steroids prior to ovulation. However, PGE1 is at a very high concentration several hours prior to ovulation, which suggests that it could influence either (or both) of GTH and steroid synthesis. Unfortunately, the first PGE1 measurement was taken approximately 15 to 16 hours after the water temperature had been increased, and it is possible that endogenous GTH levels were already increasing by this stage. This makes it difficult to speculate, with any certainty, on the possible interaction of

PGE1 with other hormones.

Peter and Billard (1976) demonstrated that PGE2 and $PGF2_{\alpha}$ suppressed serum GTH when a dose of 2.0 ug was directly injected into the third ventricle of mature female goldfish. However, GTH levels increased slightly following a 2.0 ug dose of PGE1. Although lower doses of PGE1, PGE2 and $PGF2_{\alpha}$ were without effect, $PGF2_{\alpha}$ showed a tendency to decrease GTH at the lowest dose of 0.5 ug. They suggested that this effect was via the hypothalamus. It is possible that the increase in plasma $PGF2_{\alpha}$ observed after ovulation could have a negative feedback on GTH secretion. Therefore, $PGF2_{\alpha}$ could be the controlling agent which turns off the GTH secretion observed after ovualtion. This negative feedback from $PGF2_{\alpha}$ would be very important in fish which, like the goldfish, spawn several times during a season. A continual high level of GTH might induce all the mature eggs to ovulate at the same time, and successive spawnings would be impossible. It is interesting to note here that GTH levels remain high for several days in trout and in sockeye salmon, and that both of these are synchronous spawners (Crim et al 1975; Fostier et al 1978).

The involvement of prostaglandins in spawning behaviour of female goldfish was first established by Stacey (1976). An earlier study had shown that spawning behaviour could be induced in female goldfish by the injection of ovulated eggs (from a donor female) through the ovipore and into the ovarian lumen (Stacey and Liley 1974). Stacey (1976) then found that indomethacin (10 mg/g) could block this induced spawning behaviour when given either 10 hours prior to, or coincident

with, the injection of ovulated eggs. In addition, he disccvered that PGF2α (5 mg/g) was very effective in overcoming the indomethacin block and could restore spawning behaviour. The same dose of PGE1 and PGE2 was also tested and PGE2 was found to be only marginally effective. He also observed that the injection of PGF2$_\alpha$ alone could induce spawning behaviour in fish having vitellogenic oocytes, but that there was no response in fish which had been hypophesectomized three or four months earlier. However, when hypophysectomized fish were treated with salmon gonadotropin or pituitary extract for two weeks, they responded to either oocyte injection or PGF2$_\alpha$ administration. Replacement therapy using a mixture of steroids was ineffective. Although the steroids used did not restore the capability for spawning behaviour in hypophysectomized fish, Stacey and Liley's earlier work (1974) showed that 17-β- estradiol injection was effective in restoring the spawning response in intact fish having regressed ovaries. From the results of these two experiments, Stacey postulated that egg injection (or the presence of eggs in the ovarian lumen) stimulates the release of PGF2$_\alpha$ (perhaps via a direct stretch response or afferent stimulation) which, in turn, triggers spawning behaviour. He also stated that both pituitary hormones (e.g. gonadotropin) and steroids were essential for the complete series of events to take place. Again the time factor was shown to be important in this study, as administration of indomethacin could block egg-injection-induced spawning behaviour within minutes. However, spawning induced by PGF2$_\alpha$ alone could sometimes last up to two hours.

These observations are very interesting in light of the very high concentration of $PGF2_{\alpha}$ (over 9 ng/ml) in the ovarian fluid of the goldfish. In Stacey's (1976) study, ovarian fluid was injected along with the eggs and would be equivalent to injecting an exogenous source of $PGF2_{\alpha}$. This would not, however, refute the stretch hypothesis, as indomethacin could rapidly block spawning following egg injection, which suggests some de novo synthesis of prostaglandins. However, stretching alone cannot be the only stimulus which can elicit spawning, since artificial eggs were much less effective in inducing spawning (Stacey 1977). It appears that both ovarian fluid and stretching are contributing to the release or appearance of PGF2 at the time of ovulation. Spawning behaviour occurred following an artificial increase of $PGF2_{\alpha}$ (presumably in the circulatory system) when $PGF2_{\alpha}$ was injected intraperitoneally. This increase in $PGF2_{\alpha}$ levels can be compared to the increase which was observed in the plasma of the goldfish at ovulation. From these data, it is possible to surmize that the ovarian fluid is a major source of the $PGF2_{\alpha}$ found in the blood after ovulation, and that the sharp increase in $PGF2_{\alpha}$ could be controlling spawning behaviour. This is further supported by evidence that removal of all ovulated oocytes results in cessation of spawning (Stacey and Liley 1974), and that $PGF2_{\alpha}$ decreases in the blood following this removal.

The fact that hypophysectomized fish and fish having regressed ovaries would not respond to either egg injection or $PGF2_{\alpha}$ administration ties in with some of the observations from the seasonal experiments in the present study. As mentioned

previously, there was an indication that ovaries which had a greater proportion of tertiary oocytes had a greater capacity for $PGF2_{\alpha}$ synthesis, as the $PGF2_{\alpha}$ levels in several of these ovaries were very high (over one ng/g), compared to the other samples taken at the same time. It is possible that the fish with regressed ovaries, in Stacey's experiments, did not perform the spawning behaviour because the ovaries were unable to synthesize sufficient $PGF2_{\alpha}$ to trigger the response. In this event, the ovary would need to have vitellogenic oocytes present in order to produce a high secretion of $PGF2_{\alpha}$, and the pituitary hormones would be essential for $PGF2_{\alpha}$ synthesis because they would promote maturation and maintain vitellogenic oocytes. The steroids could influence prostaglandin synthesis by either direct stimulation, or by the maintenance of vitellcgenic oocytes.

Stacey's work indicated that prostaglandins were essential for spawning behaviour and that $PGF2_{\alpha}$ appeared to be the most influential. The results from my study support this hypothesis, and further suggest that the ovarian fluid is the source of $PGF2_{\alpha}$.

In the males, prostaglandins may be playing a role in sexual development; however, the results of this study did not delineate the precise function of prostaglandins during either maturation or spermiation. There was, however, a significant change in PGE1 levels following HCG injection in spermiating fish. It is difficult to relate these results to those of other researchers, as little work has been done in this area.

Yamamato and Yamazaki (1968b) found that salmon

gonadotropin (s-GTH) and HCG were effective in inducing spermiation in hypophysectomized male goldfish. This suggests that the action of HCG on the gonadal tissues is similar to that of s-GTH. It is therefore reasonable to compare the results from the present study with findings of Clemens and Grant (1964) and Yamamoto and Donaldson (1968a, b; 1969) who used s-GTH in most of their work.

Clemens and Grant (1964) showed that the pituitary influenced testicular hydration in carp. The fluidity cf the semen of these fish increased to a total of 14-15% in 24 hours, following injections of pituitary extracts. However, a sharp rise of 12% hydration occurred during the initial 11 hours of the experiment. It is interesting to note that, in the present study, the time of this rise in water ccntent in the testis coincided with the peak in plasma PGE1 measured in the male goldfish 10 hours after HCG injection. This suggests that PGE1 may be influencing the hydration of the testis during spermination.

Further evidence, from Smith et al (1967) and Kennedy (1979), supports the hypothesis that PGE1 may somehcw be affecting the water content of the testis. In light cf the vasodilator qualities of PGE1 (Smith et al 1967), it is possible that PGE1 mediates the hypertrophy of the blcod vessels in the lobule walls during spermiation, as observed by Yamamoto and Yamazaki (1967). In addition, it is also possible that PGE1 influences the uptake of water by the testis, because PGE1 has been shown to increase vascular permeability (Kennedy 1979).

To my knowledge, there are no studies on the short-term

effects of gonadotropin administration on androgen synthesis to compare with the present prostaglandin study. Furthermore, the function of steroids in spermiation is not clearly understood, thus making it difficult to determine the relationship between prostaglandins and steroids in spermiation in fish.

The function of PGB1 in the male is virtually unknown. The decrease in plasma PGB1 noted in the male, was also observed in all the female groups, as described in the sections on ovulation. It is possible that the decrease in PGB1 levels is due either to a shift toward the synthesis of PGE1, or simply to a steady depletion of PGB1 in the blood.

PROPOSED MODELS OF THE FUNCTION OF PROSTAGLANDINS DURING OVULATION AND SPERMIATION

The following model for the function of PGF2 and PGE1 during ovulation in the goldfish is proposed on the basis of the data from this study and the relationship between prostaglandins and other hormones involved in ovulation, as discussed previously. At present, this model is very speculative.

It appears that in the goldfish, $PGF2_{\alpha}$ is the most influential at the time of ovulation. PGE1 also seems to be playing an important role; however, its function is less easily defined. Figure 12 shows the proposed mode of involvement of prostaglandins in the events preceding, during and following ovulation. The high levels of PGE1 observed in the plasma up to 12-15 hours prior to ovulation increase the hydration of the ovary firstly, by increasing vasodilation in the ovary and secondly, by increasing the permeability of blood capillaries in the follicular layer. PGE1 also stimulates the synthesis of

gonadotropin. (Peter and Billard (1976) found PGE1 to increase GTH secretion slightly.) at approximately the same time, gonadotropin secretion, which starts to increase several hours prior to ovulation (Stacey et al 1979), stimulates (particularly 17-α-OH-20-β-didydroprogesterone) which, in turn, promotes the final stages of oocyte maturation (GVM and GVBD).

These events promote the production of PGF2 by:

1) breakage of the tissues (such as the microvilli attacking the oocytes) as the follicle swells

2) stretching of the smooth muscle-like cells in the theca (Jalabert and Szollozi 1975) and

3) direct stimulation of PGF2$_\alpha$ synthesis by steroids or GTH.

Once PGF2$_\alpha$ (or the PGF2$_\alpha$/PGE1 ratio) reaches a sufficiently high concentration in the ovarian fluid, PGF2$_\alpha$ and/or PGE1 stimulates ovulation by:

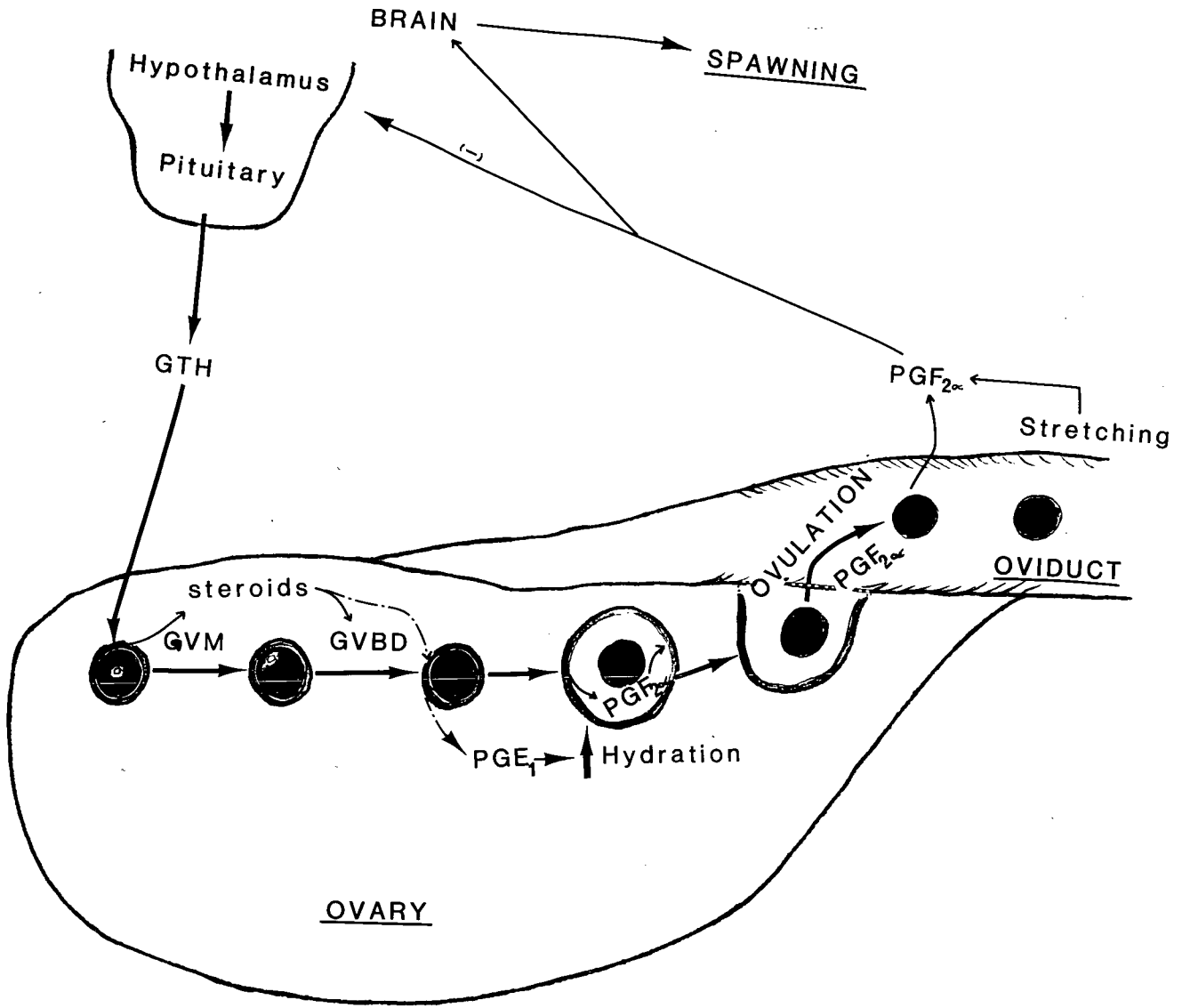
1) somehow influencing the protease enzymes which are contributing to the breakdown of the microvilli and the follicle

2) promoting the contraction of the follicle by stimulating the smooth muscle-like cells in the theca (most likely caused by PGF2$_\alpha$)

3) by further increasing the hydration of the follicle until it bursts and releases the oocyte

These mechanisms of action of prostaglandins are mediated by the stimulation of the cyclic AMP or GMP system. (This has already been suggested to occur in fish (Kuc and Watanabe 1978) as well as in mammals (Le Maire et al 1976b).)

FIGURE 12: Schematic representation of the proposed model of the role of prostaglandins in ovulation in the goldfish. GTH (gonadotropin), GVM (germinal vesicle migration) and GVBD (geminal vesicle breakdown).


BRAIN
SPAWNING
Hypothalamus
Pituitary
(−)
GTH
PGF2α
Stretching
OVULATION
PGF2α
OVIDUCT
steroids
GVM
GVBD
PGF2α
PGE1
Hydration
OVARY

Once ovulation has taken place, $PGF2_{\alpha}$ from the ovarian fluid is released into the circulatory system where it induces spawning behaviour and feeds back on the pituitary to decrease gonadotropin secretion. (It is necessary for gonadotropin secretion to decrease for repetitive spawning to take place; otherwise the oocytes would all ovulate at once.)

In the males, the function of prostaglandins is less clear; however, some suggestions can be made on the possible function, mainly of PGE1, during spermiation. The main action of PGE1 appears to be in influencing the hydration of the testis in spermiating fish. Figure 13 shows some of the possible modes of involvement of prostaglandins in spermiation.

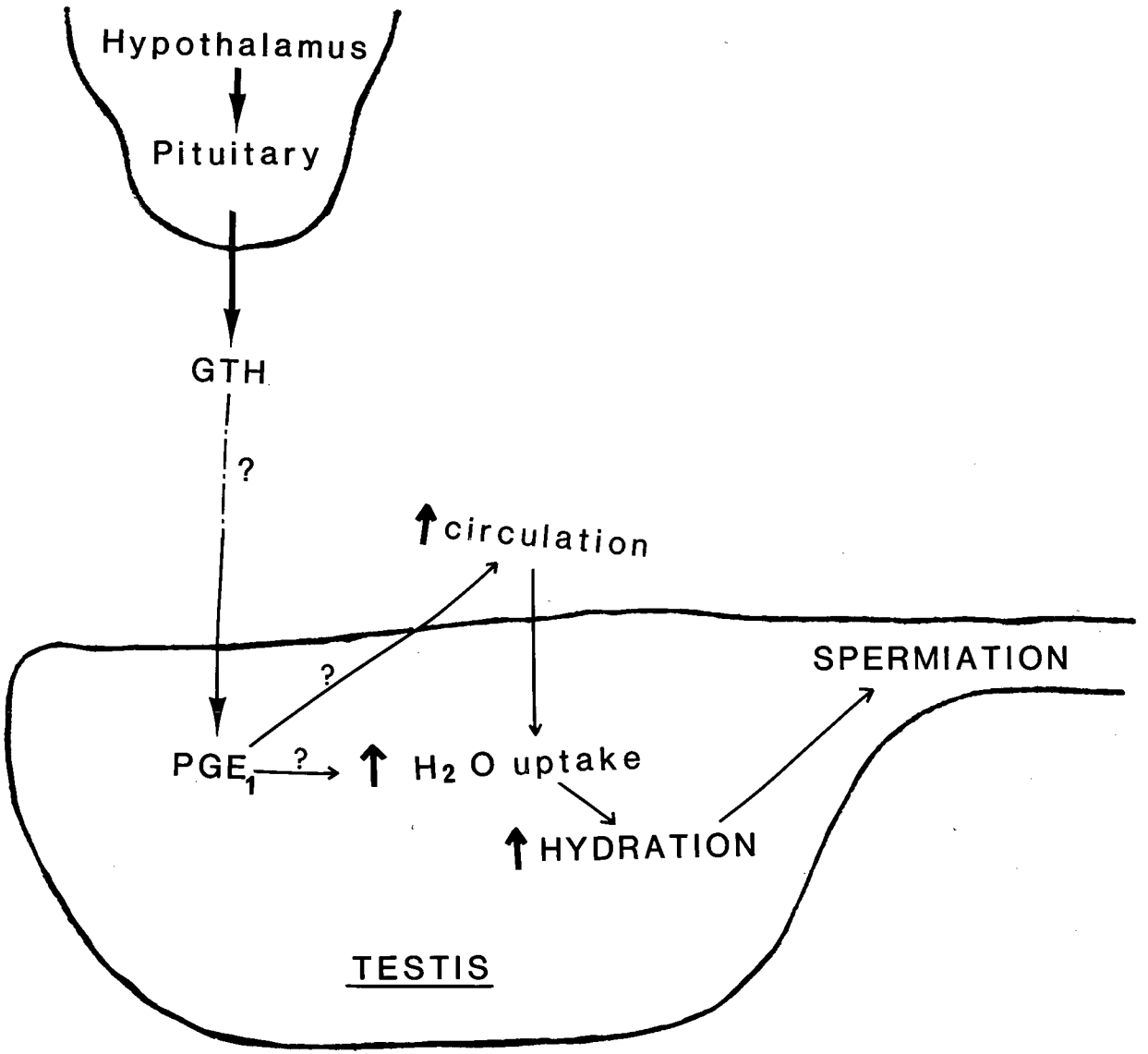
Gonadotropin is involved in the initiation of spermiation and may also affect hydration of the testis. (The water content of the testis is increased following administration of GTH, Yamazaki and Donaldson (1968a).) Gonadotropin stimulates PGE1 synthesis which, in turn, increases the permeability of the blood vessels therin. The testis is then able to increase its water content considerably (an essential step in the spermiation process).

PGE1 may then have some feedback effect on the pituitary, although it is difficult to speculate on its exact mode of influence, owing to a distinct lack of studies in this area.

Obviously, there is a definite need for further research on the involvement of prostaglandins in reproduction in fish. Specifically, the following areas should receive immediate consideration:

1) the involvement of the PGE series in testicular

FIGURE 13: Schematic representation of the proposed model of the role of prostaglandins in spermiation in the goldfish.


Hypothalamus
Pituitary
GTH
?
↑circulation
?
SPERMIATION
PGE1 ? ↑ H2 O uptake
↑HYDRATION
TESTIS

hydration,

2) the interaction of the prostaglandins with other hormones invloved in spermatogenesis and spermiation,

3) the exact site of prostaglandin synthesis in the ovary,

4) controlled observation of the influence of prostaglandins of the E series on gonadal hydration, as well as their influence on enzyme systems important during ovulation,

5) the interaction of prostaglandins with other hormones, particularly the steroids and the pituitary hormones,

6) the effect of the high concentration of $PGF2_{\alpha}$ in the ovarian fluid on the oocytes, themselves.

Prostaglandins are important components of reproductive physiology, and therefore should be researched more extensively to understand more fully the mechanisms of gonadal development, ovulation and spermiation in fish.

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APPENDIX

Phosphate Buffer Saline (PBS-A)

-to make 100 ml of PBS-A, 0.25 g of NaCl and 0.05 g cf p-aminosalisyclic acid were dissolved in 80 ml of a solution of 0.06N Na HPO , and was adjusted to a pH of 7.4 by the addition of approximatly 20 ml of 0.06N KH PO (this solution was added dropwise as pH 7 was approached).

Clinical Assays, Inc.
237 Binney Street, Cambridge, Massachusetts 02142 (617) 492-2526

CA-501

^{3}H PROSTAGLANDIN E RADIOIMMUNOASSAY KIT

For the quantitative measurement of

Prostaglandin A and E in serum or tissue extracts

NOTE: STORE THIS KIT AT -20° C AFTER REMOVING THE TWO VIALS OF TRIS BUFFER CONCENTRATE

February 14, 1975

Radioimmunoassays

Radioactive material — Not for Human Use — Introduction into Foods, Beverages, Cosmetics, Drugs, or Medicinals or into Products Manufactured for Commercial Distribution is Prohibited — Exempt Quantities Should not be Combined.

CLINICAL ASSAYS
Division of Travenol Laboratories, Inc.

PRECAUTIONARY INSTRUCTIONS

The user shall store the by-product material until used in the original shipping container or in a container providing equivalent radiation protection.

The following precautions should be observed in handling radioactive material:

1. Handling should preclude any pipetting by mouth.
2. There should be no smoking or eating while radioactive materials are being handled.
3. Hands should be covered with rubber gloves during and thoroughly washed after handling of radioactive materials.
4. Spills should be wiped up quickly and thoroughly and the contaminated materials added to radioactive waste matter.
5. Water soluble waste radioactive material can be disposed of into the sanitary sewage system, if the concentration, after dilution with the laboratory discharge, does not exceed 4×10^{-2} microcuries per liter (^{125}I) or 10 microcuries per liter (^{3}H), based on a daily average of effluent. Whenever possible, however, disposal of radioactive material should be made through a licensed disposal service.

LICENSING REQUIREMENTS

The procurement of radioactive material in this kit is exempt from NRC or Agreement State licensing requirements.

INTRODUCTION

The Clinical Assays, Inc., Prostaglandin E (PGE) Radioimmunoassay Kit offers a sensitive method of measuring prostaglandin content inplasma and tissues by measuring the competitive binding of ^{3}H labelled prostaglandin and unlabelled prostaglandin with antibody to prostaglandin. The kit includes all the reagents required in the procedure.

Assay of the PGE content is performed by measuring the amount of Prostaglandin B_1 (PGB_1) or Prostaglandin B_2 (PGB_2) obtained after conversion of PGE_1 to PGB_1 or PGE_2 to PGB_2 by alkaline treatment. An anti-PGB_1 rabbit serum is used for the radioimmunoassay.

Separation of prostaglandin bound to antibody and free prostaglandin is achieved by precipitating the bound prostaglandin with a second antibody. After centrifugation, the bound radioactivity is measured in a liquid scintillation counter.

To eliminate protein interference in the assay, a protein denaturation and/or extraction step is performed on the sample prior to assaying. Alternatively, extraction, followed by column separation of the various prostaglandins, may be employed (see page 5).

A standard curve is prepared for each group of samples by incubating known amounts of prostaglandins. Total time for the assay is approximately 2 working days.

This protocol is a modification of the procedures of Levine (1) and Gutierrez-Cernosek (2).

The assay measures both PGE_1 and PGE_2, since both are converted to PGB_1 and PGB_2 respectively, by the alkaline treatment. The cross-reactivity curves on page 13 indicate that PGB_2 cross-reacts 17% with the anti-PGB_1 serum at the 50% binding level.

If quantification of PGE_1 and PGE_2 levels is desired, it is possible to estimate the relative proportions of PGE_1 and PGE_2 present by converting these prostaglandins to $PGF_{1\alpha}$ and $PGF_{1\beta}$ and to $PGF_{2\alpha}$ and $PGF_{2\beta}$ respectively by a sodium borohydride reduction (3). The assay of $F_1\alpha$ and $F_2\alpha$ levels can then be performed with the highly specific antisera of the Clinical Assays Kits, CA-502 and CA-503, to obtain the relative proportions of the E_1 and E_2 originally present. This ratio can then be used in conjunction with the known cross-reactivity of PGB_2 with the anti-PGB_1 serum supplied to calculate the original PGE_1 and PGE_2 levels.

PGA_1 and PGA_2 are also converted to their respective PGB isomers by alkaline treatment. An assay of the plasma extract with the PGB_1 antiserum, with no alkaline treatment, will measure PGA_1 at about 14% cross-reactivity, which can then be deducted as equivalent B_1 from the assay of the alkaline-treated extract.

REAGENTS

This is the first commercially available kit for the determination of Prostaglandin levels by radioimmunoassay. The radioimmunoassay kit contains sufficient reagents for 200 assay tubes, including 5 standard curves. The reagents provided are:

		Reconstituted Volume
1 vial	Prostaglandin B_1 Antiserum, lyophilized	10 ml
1 vial	Prostaglandin Standard, lyophilized	1 ml
2 vials	Rabbit Normal Serum, lyophilized	10 ml each
2 vials	Goat Anti-Rabbit Serum, 10 ml each	
1 vial	^3H labelled Prostaglandin B_1 (2μCi), lyophilized	10 ml
2 vials	Trizma-NaCl Concentrate, preadjusted to pH 7.4, 10 ml each	300 ml each
1 vial	Gelatin (3g)	

The reconstituted reagents must be stored frozen at -10°C to -20°C. Prostaglandins are stable substances and the kit may be thawed and frozen repeatedly without affecting the performance of the kit reagents.

REAGENTS REQUIRED BUT NOT PROVIDED IN KIT

Glacial Acetic Acid
0.1N Sodium hydroxide, hydrochloric acid solutions

SUGGESTED APPARATUS

Precision Pipets: 1.0, 0.6, 0.4, 0.1, 0.05 ml
Cornwall 1 ml Repeating Syringe
Vortex Mixer
Centrifuge
Liquid Scintillation Counter
Water Bath (37°C) and Ice Bath
pH Meter
Dialysis Apparatus (Optional)

SUGGESTED DISPOSABLE MATERIALS

Polypropylene tubes, Falcon No. 2053, 12x75 mm or equivalent
Liquid Scintillation vials
Scintillation Fluid, CA-702 or equivalent
2 ml Calibrated tubes (Fisher 10-212-5B) or equivalent

REAGENT PREPARATION

It is recommended that distilled water be used to reconstitute lyophilized reagents and to prepare all the necessary reagents required in this assay.

A. Tris Buffer, Reagent A

Add: Contents of one vial of Tris Concentrate to 250 ml distilled water. Bring volume to 300 ml with water.
Storage: Keep refrigerated.
Stability: Two months.

B. Isogel Tris Working Buffer, Reagent B

Add: 0.10g Gelatin to 100 ml Reagent A. Heat and stir until the gelatin is dissolved. Cool the solution.
Adjust: pH to 7.4 if necessary, with 0.1N sodium hydroxide or 0.1N hydrochloric acid.
Storage: Keep refrigerated.
Stability: Prepare fresh every two or three days.

C. Prostaglandin Standard

Add: 1 ml water to the contents of the standard vial. Mix gently and avoid foaming.
Storage: Must be kept frozen.
Stability: Minimum three months.

D. Prostaglandin Antiserum

Add: 10 ml Reagent B to the contents of the antiserum vial. Mix gently and avoid foaming.
Storage: Must be kept frozen.
Stability: Minimum three months.

E. ^{3}H Prostaglandin

Add: 10 ml Reagent B to the contents of the ^{3}H Prostaglandin vial. Mix gently and avoid foaming.
Storage: Must be kept frozen.
Stability: Minimum three months.

F. Rabbit Normal Serum

Add: 10 ml Reagent B to the contents of a Rabbit Normal Serum vial. Mix gently and avoid foaming.
Storage: Must be kept frozen.
Stability: Minimum three months.

PGE TO PGB CONVERSION

The extract is made up to 2.0 ml in a calibrated tube with the isogel tris buffer (Reagent B). Place 1.0 ml of this solution in a 2 ml screw cap vial and add 0.1 ml of 1N sodium hydroxide (the pH should be adjusted to 12.5 to 12.9 using a pH meter with micro-electrodes). Screw the cap tightly, mix well and place in a boiling water bath for 5 minutes. Cool and adjust the pH to approximately 7.4 with 0.1 ml of 1N glacial acetic acid. Note the total volume of the PGB containing solution for later use in the calculation.

ASSAY PROCEDURE

Prior to use, thaw the vials containing the prostaglandin antibody, prostaglandin standard, labelled prostaglandin, assay samples, normal rabbit serum and goat anti-rabbit serum on ice. Throughout the procedure keep all reagents on ice.

The assay procedure includes the preparation of a standard curve where known amounts of prostaglandin are used to compete with a fixed amount of labelled prostaglandin in binding to a fixed amount of prostaglandin antibody. This standard curve is then used to determine the prostaglandin content of the assay samples from the binding obtained with each sample.

The assays are performed in disposable plastic tubes and each standard curve or assay point is carried out in duplicate. A standard curve with 6 points to cover the range in PGB_1 of 8 pg to 2 ng is used. The reagents in the kit are provided in the dilutions required to obtain the optimum sensitivity in the radioimmunoassay.

PREPARATION OF THE STANDARD CURVE

The PGB_1 prostaglandin standard is furnished at a concentration of 40 ng/ml in isogel tris buffer, Reagent B. Prior to use in the assay a serial dilution in the range of 1/1 to 1/243 should be prepared. The serial dilutions can be stored frozen and used as required to prepare the standard curves.

PREPARATION OF THE STANDARD DILUTIONS

1. Into 5 tubes marked 1/3, 1/9, 1/27, 1/81 & 1/243 pipet 0.6 ml of Reagent B.

2. The standard supplied is considered the 1/1 dilution. Pipet 0.3 ml of 1/1 standard into the 1/3 tube. Mix thoroughly.

3. Now take 0.3 ml of the solution in tube 1/3 and add it to the tube marked 1/9. Mix thoroughly. Take 0.3 ml of the solution in tube 1/9 and add it to the tube marked 1/27. Mix thoroughly. Repeat this serial dilution to the last tube, 1/243.

7.

The dilution concentrations are as follows, per 0.05 ml:

1/1	2000 pg	1/27	74 pg
1/3	667 pg	1/81	25 pg
1/9	222 pg	1/243	8.2 pg

These dilutions of standard should be kept frozen with the remainder of the kit, and are sufficient for five complete standard curves.

PREPARATION OF THE STANDARD CURVE AND ASSAY SAMPLES

1. Into sixteen tubes numbered 1-16 pipet 1.0 ml of isogeltris buffer. In addition, place an extra 0.1 ml in tubes 1 and 2. Tubes 1 and 2 are background controls and contain no antiserum or inhibitor (standard). Tubes 3 and 4 are binding controls and contain no inhibitors.

   Add 0.6 ml of the isogel tris buffer to the sample assay tubes, beginning with tube 17. Each sample is run in duplicate.

2. To tubes 5 and 6 add 0.05 ml of 1/1 standard; mix well. To tubes 7 and 8 add 0.05 ml of 1/3 standard. To tubes 9 and 10 add 0.05 ml of 1/9 standard. To tubes 13 and 14 add 0.05 ml of 1/81 standard. To tubes 15 and 16 add 0.05 ml of 1/243 standard.

3. Add 0.4 ml of each alkaline-treated sample extract to duplicate tubes, beginning with tube 17.

4. Add 0.05 ml of ^{3}H labelled prostaglandin to all tubes.

5. Stopper tubes 1 and 2. Incubation time is counted from the addition of antiserum. Add 0.05 ml of antiserum beginning with tube 3. Mix thoroughly on a vortex agitator.

6. Incubate the tubes for not less than one hour in a water bath at 37°C.

7. After incubation, add 0.1 ml of Normal Rabbit Serum and 0.1 ml of Goat Anti-Rabbit Serum to all tubes. Mix thoroughly and incubate 18-20 hours at 4°C. This incubation may be extended, if greater binding is desired.

At this point the tubes contain the following reagents:

Tube	Tris Buffer (ml)	Inhibitor (ml)	Tracer (ml)	Prostaglandin Antiserum (ml)	Rabbit Normal Serum (ml)	Goat Anti Rabbit Serum (ml)
1,2	1.1	--	0.05	--	0.1	0.1
3,4	1.0	--	0.05	0.05	0.1	0.1
5-16	1.0	0.05	0.05	0.05	0.1	0.1
17-18 etc.	0.6	0.40	0.05	0.05	0.1	0.1

8. After this incubation, centrifuge the tubes at 4°C for 30 minutes at 1600 G.

9. Decant the supernatant from tubes 1 and 2 into two scintillation vials marked T_1 and T_2.

10. Line the bottom of a suitable test tube rack with paper towels or other absorbent material. After decanting the supernatant from each tube into a waste container, place it upside down on this rack to drain. In a few minutes, dry the inside of each tube carefully with a folded strip of filter paper or other suitable material, taking care not to touch the precipitate at the bottom.

11. Add 1.0 ml NaOH (0.1N) to each tube, including tubes 1 and 2, mixing on a vortex mixer, to dissolve the precipitate. Decant the solution into a similarly numbered scintillation vial, hitting the rims of the tube and vial together firmly to insure maximal transfer.

12. Add 10 ml of scintillation fluid (CA-702) to each vial, including T_1 and T_2. Mix thoroughly.

13. Count each vial for 2 to 5 minutes. Vials T_1 and T_2 should contain 7-10,000 CPM.

9.

CALCULATIONS

BKG Background counts. The average counts in vials 1 and 2.

T Total counts. The average counts in vials T_1 and T_2.

T_c Corrected Total counts. $T_c = T - BKG$.

B Total bound. The average counts in vials 3 and 4, or the geltris control.

B_c Corrected Total bound. $B_c = B - BKG$. These counts represent the total amount of radioisotope that has been bound by the antibody. In the standard curve it is defined as 100% binding.

CPM_n Tube counts. The uncorrected counts for a tube n.

1. Quench Correction

Beta particle detection in a liquid scintillation counter depends on the optical characteristics of the sample. The color or transparency of the sample affect the efficiency with which beta radiation can be detected. Each sample may quench differently. Since the samples have been extracted, the incubation solutions are likely to have similar optical characteristics and probably will not require a quench correction.

Quench correction can be performed by using an Automatic External Standard as prescribed by the scintillation counter manufacturer. Quench correction can also be performed by using an internal standard. After counting each vial, add 0.01 ml of tracer (or labelled prostaglandin) to each vial, including total vials T_1 and T_2. Mix well and count again. The efficiency normalized counts are computed using the average counts for the total vials (T') and the new counts for the vial (CPM') as follows:

$$\text{Normalized CPM} = CPM_n \times \frac{T' - T}{CPM'_n - CPM_n}$$

Normalized CPM should be used for all calculations.

2. Standard Curve

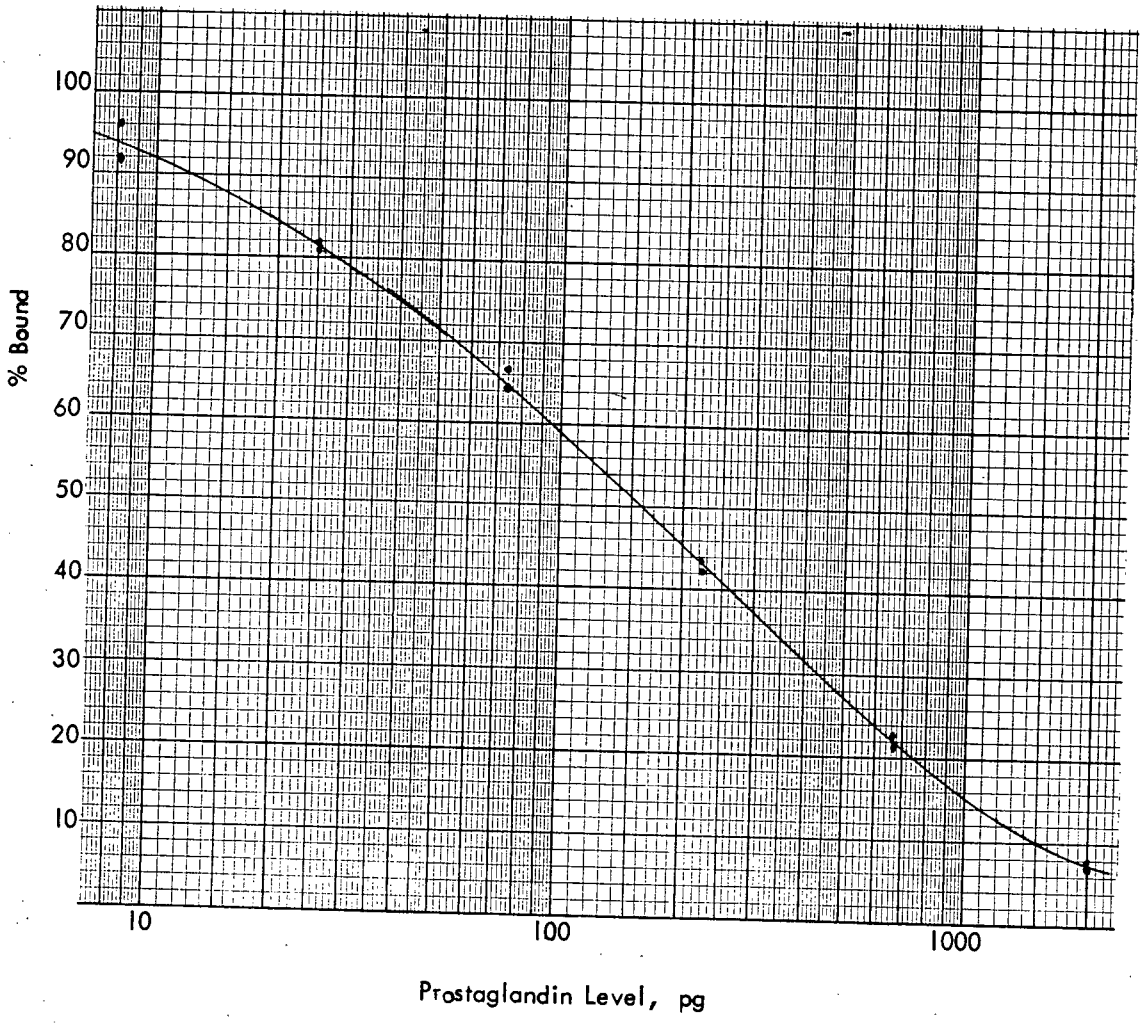
The standard curve is obtained by plotting the percent binding for each concentration of inhibitor.

The percent bound is

$$B_n = \frac{CPM_n - BKG}{B_c}$$

10

Figure I. A TYPICAL
PROSTAGLANDIN STANDARD CURVE



11.

A typical standard curve is shown in Figure 1. The percent binding will range from about 98% at 8.2 pg to 9% at 2.0 ng. To improve accuracy, tubes having binding higher than 95% should be repeated at a higher concentration of extract and tubes having binding below 10% should be repeated at a lower concentration of extract.

3. Kit Binding Capacity

The reagents have been selected to provide a constant binding over a long period of time. A noticeable change or trend in the percent of total binding should be considered an indication that either kit reagents or prepared reagents have deteriorated. Total binding should be above 30%.

$$\% \text{ TOTAL BINDING} = \frac{B_c}{T_c} \times 100$$

4. Sample Assay

After determining the amount of prostaglandin present in the extract aliquot used in the assay, it is necessary to convert this amount to a concentration of the original sample.

Correction factors include:

a. The amount of sample originally used in the extraction procedure.

b. An extraction efficiency factor.

c. A dilution factor for the fraction of extract used as inhibitor in the assay.

COMMENTS

Dialysis Efficiency

In multiple experiments performed in our laboratories, the dialysis efficiency has been shown to approach 46% after 10 hours of continuous agitation of the cells. Due to evaporation and other losses, the actual recovery of material has, in general, been less and varies from experiment to experiment. We have found it helpful in our laboratories to include with every dialysis experiment a set of cells containing tracer material. An equal amount of tracer is pipetted at the same time into a scintillation vial, held as a control and defined as 100% in computing dialysis efficiency.

Similarly, other extraction methods may be checked for extraction efficiency by adding tracer to the sample before extraction. After the extraction, and just prior to the radioimmunoassay, an aliquot is removed and counted to give the appropriate extraction efficiency factor.

Dialysis Apparatus

The dialysis step recommended in this procedure may be performed using a Karush-type Chamber assembly, such as those available from Bellco Glass, P.O. Box B, 340 Edrudo Road, Vineland, New Jersey, 08360 (cat. no. 3213, 1 ml capacity). To obtain the efficiency characteristics indicated above, this type of cell requires continuous agitation.

Semi-permeable cellulose membranes are available from a variety of sources. We have found Fisher Dialyzer Tubing (catalog no. 8-667C) to be satisfactory. This tubing must be treated before use by boiling about 5 feet of it in 500-1000 ml of distilled water containing a pinch of EDTA and sodium bicarbonate, until the tubing is soft and odorless. This usually requires three passes. Once the tubing is ready, it may be stored at 4°C under distilled water until used.

Antibody Cross Reactivity

The anti-PGB_1 antiserum used in this kit cross-reacts with PGA_1, PGA_2, PGB_2, PGE_1, PGE_2, $PGF_1\alpha$ and $PGF_2\alpha$. A set of Inhibition Curves is shown on Figure 2 for anti-PGB_1 antiserum.

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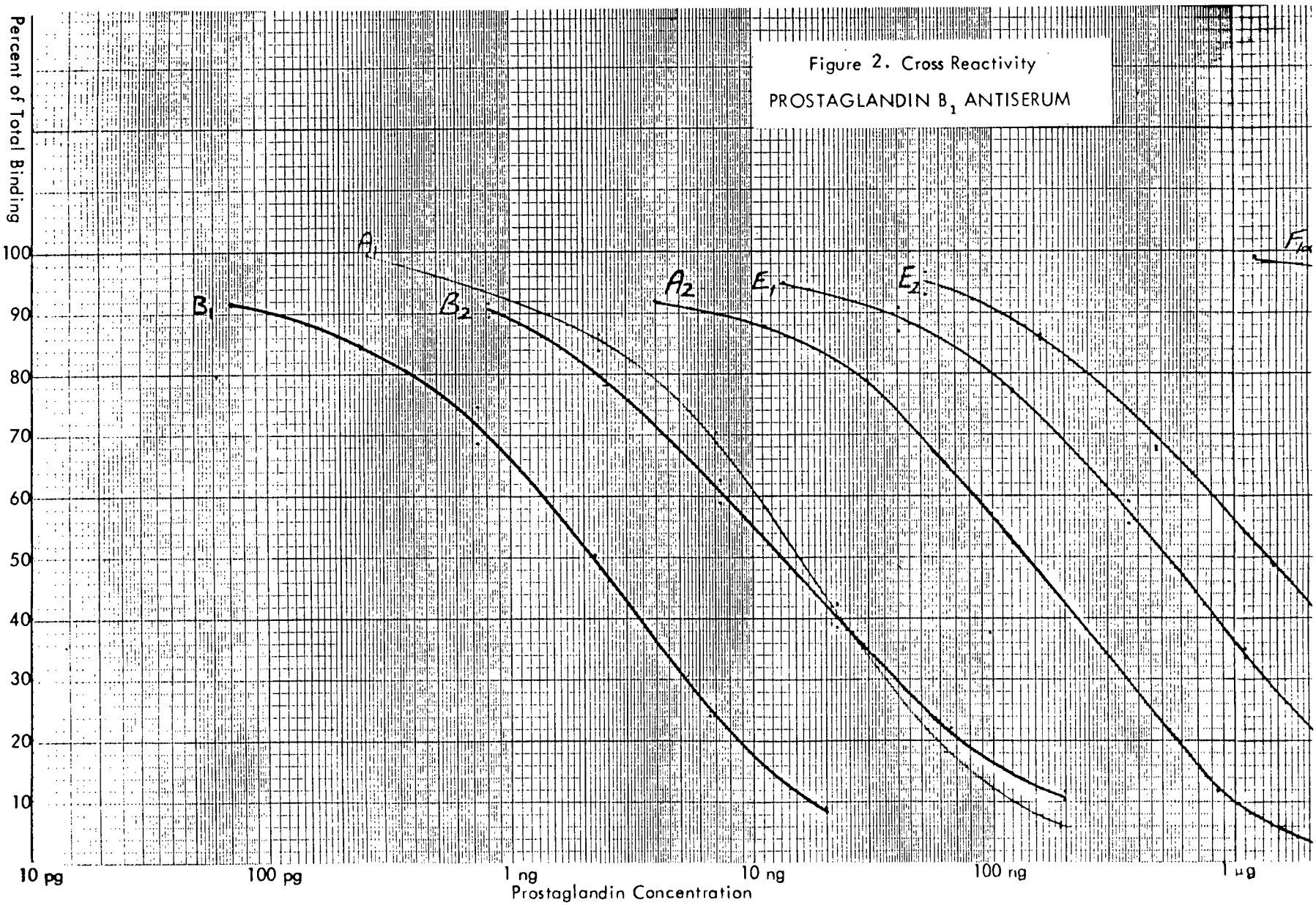
Figure 2. Cross Reactivity

PROSTAGLANDIN B_1 ANTISERUM



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^{3}H PROSTAGLANDIN $F_{2}\alpha$ RADIOIMMUNOASSAY KIT

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LICENSING REQUIREMENTS

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INTRODUCTION

The Clinical Assays, Inc., Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) Radioimmunoassay Kit offers a sensitive method of measuring prostaglandin content in serum or plasma by measuring the competitive binding of ^{3}H labeled prostaglandin and unlabeled prostaglandin with antibody to prostaglandin. The kit includes all the radioimmunoassay reagents required in the procedure.

Separation of prostaglandin bound to antibody and free prostaglandin is achieved by precipitating the bound prostaglandin with a second antibody binding reaction. After centrifugation, the bound radioactivity is measured in a scintillation counter.

To eliminate protein interference in the assay, a protein denaturation and/or extraction step is performed on the sample prior to assaying. Alternatively, extraction, followed by column separation of the various prostaglandins, may be employed.

A standard curve is prepared for each group of samples by incubating known amounts of prostaglandins. Total time for the assay is approximately two working days.

This protocol is a modification of the procedures of Jaffe (1), Levine (2), Caldwell (3), and Hickler (4).

Through the introduction of a new sensitive anti-$PGF_{2\alpha}$ serum and a very high specific activity ^{3}H prostaglandin $F_{2\alpha}$, (February, 1975) the assay can now detect as low as 10 pg of inhibitor. Wherever possible, a sample size should be selected which will direct the assay to a more sensitive portion of the standard curve.

REAGENTS

This is the first commercially available kit for the determination of prostaglandin levels by radioimmunoassay. The radioimmunoassay kit contains sufficient reagents to perform 200 assays including 5 standard curves. The reagents provided are:

		Final Volume
1 vial	Prostaglandin Antiserum, lyophilized	10 ml
1 vial	Prostaglandin Standard, lyophilized	5 ml
2 vials	Rabbit Normal Serum, lyophilized	10 ml, each
2 vials	Goat Anti-Rabbit Serum	10 ml, each
1 vial	^{3}H labeled Prostaglandin $F_{2}\alpha$ (2μCi), lyophilized	10 ml
2 vials	Tris Buffer Concentrate, 10 ml each	300 ml, each
1 vial	Gelatin (3g)	

The reconstituted reagents must be stored frozen at -10°C to -20°C. Prostaglandins are stable substances and the kit may be thawed and frozen repeatedly without affecting the performance of the kit reagents. The buffer solution is stored at 4°C.

REAGENTS REQUIRED BUT NOT PROVIDED IN KIT

0.1N Sodium hydroxide and hydrochloric acid solutions

SUGGESTED APPARATUS

Precision pipets: 1.0, 0.5, 0.1, 0.05 ml
Vortex Mixer
Centrifuge
Cornwall 1 ml Repeating Syringe
Liquid Scintillation Counter
Water Bath (37°C) and Ice Bath
pH Meter
Dialysis apparatus (optional)

SUGGESTED DISPOSABLE MATERIALS

Polypropylene tubes, Falcon No. 2053, 12x75 mm or equivalent
Liquid scintillation vials
Scintillation Fluid, CA-702 or equivalent
Dialysis membrane, Fisher 8-667C or equivalent

REAGENT PREPARATION

It is recommended that distilled water be used to reconstitute lyophilized reagents and to prepare all the accessory reagents required in this assay.

A. Tris Buffer, Reagent A
- Contains: The Tris Buffer Concentrate contains Trizma (0.36g) and sodium chloride (2.5g) in 10 ml water (30xconcentrate), preadjusted to pH 7.4.
- Add: Contents of 1 vial of tris concentrate to 250 ml distilled water. Bring volume to 300 ml with water.
- Storage: Keep refrigerated.
- Stability: Two months

B. Isogel Tris Working Buffer, Reagent B
- Add: 0.10g Gelatin to 100 ml Reagent A. Heat and stir until the gelatin is dissolved. Cool the solution.
- Adjust: pH to 7.4 if necessary, with 0.1N sodium hydroxide or 0.1N hydrochloric acid.
- Storage: Keep refrigerated.
- Stability: Prepare fresh every two or three days.

C. Prostaglandin Standard, 24 ng/ml
- Add: 5 ml Reagent B to the contents of the standard vial. Mix gently to avoid foaming.
- Storage: Must be kept frozen.
- Stability: Minimum three months

D. Prostaglandin Antiserum
- Add: 10 ml Reagent B to the contents of the antiserum vial. Mix gently to avoid foaming.
- Storage: Must be kept frozen.
- Stability: Minimum three months

E. ^{3}H Prostaglandin (2μCi)
- Add: 10 ml Reagent B to the contents of the ^{3}H Prostaglandin vial. Mix gently to avoid foaming.
- Storage: Must be kept frozen.
- Stability: Minimum three months.

F. Rabbit Normal Serum
- Add: 10 ml Reagent B to the contents of a Rabbit Normal Serum vial. Mix gently to avoid foaming.
- Storage: Must be kept frozen.
- Stability: Minimum three months.

G. Goat Anti-Rabbit Serum
- Contains: 10 ml Goat Anti-Rabbit Serum with preservative
- Storage: Must be kept frozen
- Stability: Minimum three months

EXTRACTION OF PROSTAGLANDINS

Plasma samples are recommended for the measurement of prostaglandins in blood. However, the presence of platelets in plasma has been shown to be responsible for the generation of PGE during storage of the sample both at 4°C and under freezing conditions (5). To avoid spuriously high values, plasma samples should be frozen and analyzed within a week.

Since proteins bind prostaglandins in competition with antibodies, it is necessary to extract the prostaglandins from plasma and tissues. With any extraction, the recovery efficiency should be monitored by adding several thousand CPM of the ^{3}H $PGF_{2\alpha}$ to representative samples.

Some typical extraction methods are summarized below:

1. Extraction of 1.0 ml sample aliquot (or more if available) with 3 ml petroleum ether to remove neutral lipids. After removal of the ether phase, add 3.0 ml of an ethyl acetate: isopropanol: 0.2N HCl (3:3:1; v/v/v) solution. Vortex for 15 seconds twice, and add 2.0 ml of ethyl acetate and 3.0 ml water. After mixing, the phases are separated by centrifugation. The organic phase (3 out of 3.5 ml) is transferred to a polypropylene test tube and dried at 55°C in an air stream (6). The residue may require additional cleanup before it is suitable for the radioimmunoassay. A silicic acid column separation is indicated, if separation of the prostaglandins into PGA-PGB, PGE, and PGF fractions is desired (see page 24, ref. (6)). Alternatively, dialysis of the extract may be performed for further purification without separating the PG fractions (see pp. 11, 12 of this protocol).

2. Extraction of 5 ml plasma with 10 ml methylal: alcohol (3:1, v/v). After filtering the precipitate, and evaporating the filtrate, the residue is dissolved in isogel tris buffer and dialyzed overnight (7). (Methylal is available from Fisher Scientific Co., Cat. No. M-222).

3. Tissue samples should be processed immediately. They are homogenized in a mixture of 1.0 ml of a phosphate buffer saline and 3.0 ml of the above ethyl acetate: isopropanol: HCl extraction solution in a mechanical homogenizer. The samples are then processed as in 1. above: 2.0 ml ethyl acetate and 3.0 ml water are added, mixed and the organic phase separated and dried (6). Another protocol for the extraction of tissues by successive partitioning in ethyl acetate, n-hexane, and aqueous methanol may be found in (8).

4. Samples of biologic fluids, such as aqueous humor and cerebrospinal fluid, which contain small amounts of protein, can be measured directly without extraction.

The dilutions concentration are as follows, per 100 microliters:

1/1	2400 pg	1/27	88.9 pg
1/3	800 pg	1/81	29.6 pg
1/9	267 pg	1/243	9.2 pg

These dilutions of standard should be kept frozen with the remainder of the kit, and are sufficient for five complete standard curves.

PREPARATION OF THE STANDARD CURVE AND ASSAY SAMPLES

1. Into sixteen tubes numbered 1-16 pipet 0.5 ml of isogeltris buffer. In addition, place an extra 0.1 ml in tubes 1 and 2. Tubes 1 and 2 are background controls and contain no antiserum or inhibitor (standard). Tubes 3 and 4 are binding controls and contain no inhibitors.

   Add the appropriate volume of isogeltris buffer to tubes 17 and up. The total volume of Reagent B and sample extract should be 0.6 ml. The sample size is the volume which is estimated to contain sufficient $PGF_{2\alpha}$ so that the level falls on a sensitive part of the standard curve. If the $PGF_{2\alpha}$ level cannot be estimated, then two different size assay samples may be taken with the appropriate adjustment made in the size of the volume of the buffer solution.

2. To tubes 5 and 6 add 0.1 ml of 1/1 standard; mix well. To tubes 7 and 8 add 0.1 ml of 1/3 standard. To tubes 9 and 10 add 0.1 ml of 1/9 standard. To tubes 13 and 14 add 0.1 ml of 1/81 standard. To tubes 15 and 16 add 0.1 ml of 1/243 standard.

3. Add the appropriate volume of sample extract in Reagent B solution to duplicate tubes, beginning with tube 17.

4. Add 0.05 ml of ^3H labelled prostaglandin to all tubes.

5. Stopper tubes 1 and 2. Incubation time is counted from the addition of antiserum. Add 0.05 ml of antiserum beginning with tube 3. Mix thoroughly on a vortex agitator.

6. Incubate the tubes for not less than one hour in a water bath at 37°C.

7. After incubation, add 0.1 ml of Normal Rabbit Serum and 0.1 ml of Goat Anti-Rabbit Serum to all tubes. Mix thoroughly and incubate 18-20 hours at 4°C. This incubation may be extended, if greater binding is desired.

At this point the tubes contain the following reagents:

Tube	Isogeltris Buffer (ml)	Inhibitor (ml)	Tracer (ml)	Prostaglandin Antiserum (ml)	Rabbit Normal Serum (ml)	Goat Anti Rabbit Serum (ml)
1,2	0.6	--	0.05	--	0.1	0.1
3,4	0.5	--	0.05	0.05	0.1	0.1
5-16	0.5	0.1	0.05	0.05	0.1	0.1
17-18 etc.	Total volume 0.6 ml See Text		0.05	0.05	0.1	0.1

8. After this incubation, centrifuge the tubes at 4°C for 30 minutes at 1600 G.

9. Decant the supernatant from tubes 1 and 2 into two scintillation vials marked T_1 and T_2.

10. Line the bottom of a suitable test tube rack with paper towels or other absorbent material. After decanting the supernatant from each tube into a waste container, place it upside down on this rack to drain. In a few minutes, dry the inside of each tube carefully with a folded strip of filter paper or other suitable material, taking care not to touch the precipitate at the bottom.[c]

11. Add 1.0 ml NaOH (0.1N) to each tube, including tubes 1 and 2, mixing on a vortex mixer, to dissolve the precipitate. Decant the solution into a similarly numbered scintillation vial, hitting the rims of the tube and vial together firmly to insure maximal transfer.

12. Add 10 ml of scintillation fluid (CA-702) to each vial, including T_1 and T_2. Mix thoroughly.

13. Count each vial for 2 to 5 minutes. Vials T_1 and T_2 should contain 6-8,000 CPM.

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CALCULATIONS

BKG Background counts. The average counts in vials 1 and 2.

T Total counts. The average counts in vials T_1 and T_2.

T_c Corrected Total counts. $T_c = T - BKG$.

B Total bound. The average counts in vials 3 and 4, or the geltris control.

B_c Corrected Total bound. $B_c = B - BKG$. These counts represent the total amount of radioisotope that has been bound by the antibody. In the standard curve it is defined as 100% binding.

CPM_n Tube counts. The uncorrected counts for a tube n.

1. Quench Correction

Beta particle detection in a liquid scintillation counter depends on the optical characteristics of the sample. The color or transparency of the sample affect the efficiency with which beta radiation can be detected. Each sample may quench differently. Since the samples have been extracted, the incubation solutions are likely to have similar optical characteristics and probably will not require a quench correction.

Quench correction can be performed by using an Automatic External Standard as prescribed by the scintillation counter manufacturer. Quench correction can also be performed by using an internal standard. After counting each vial, add 0.01 ml of tracer (or labelled prostaglandin) to each vial, including total vials T_1 and T_2. Mix well and count again. The efficiency normalized counts are computed using the average counts for the total vials (T') and the new counts for the vial (CPM') as follows:

$$\text{Normalized CPM} = CPM_n \times \frac{T' - T}{CPM'_n - CPM_n}$$

Normalized CPM should be used for all calculations.

2. Standard Curve

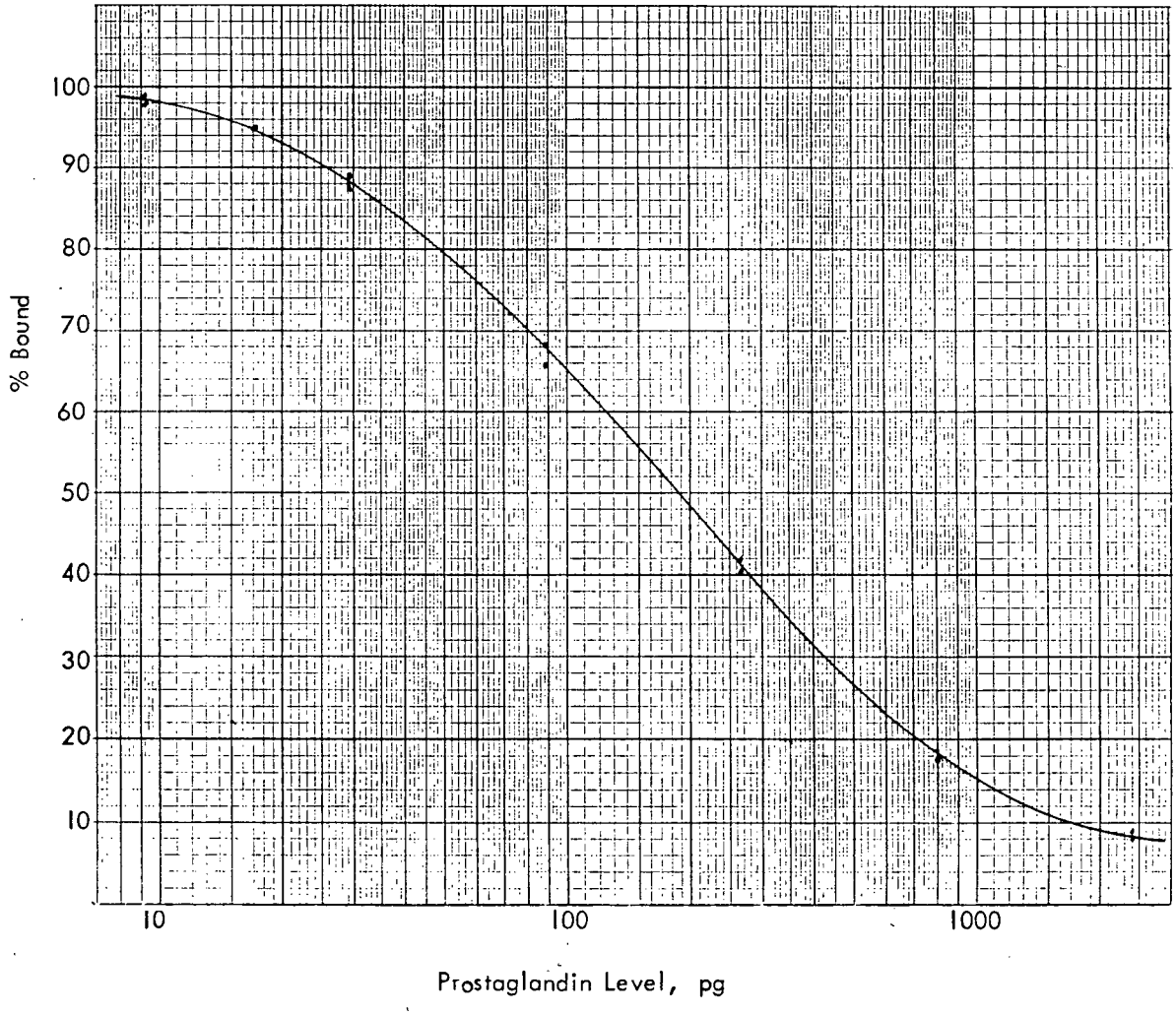
The standard curve is obtained by plotting the percent binding for each concentration of inhibitor.

The percent bound is

$$B_n = \frac{CPM_n - BKG}{B_c}$$

A typical standard curve is shown in Figure 1. The percent binding will range from about 98% at 9.4 pg to 9% at 2.4 ng. To improve accuracy, tubes having binding higher than 95% should be repeated at a higher concentration of extract and tubes having binding below 10% should be repeated at a lower concentration of extract.

Figure 1. A TYPICAL
PROSTAGLANDIN STANDARD CURVE



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3. Kit Binding Capacity

The reagents have been selected to provide a constant binding over a long period of time. A noticeable change or trend in the percent of total binding should be considered an indication that either kit reagents or prepared reagents have deteriorated. Total binding should be above 30 %.

$$\% \text{ TOTAL BINDING} = \frac{B_c}{T_c} \times 100$$

4. Sample Assay

After determining the amount of prostaglandin present in the extract aliquot used in the assay, it is necessary to convert this amount to a concentration of the original sample.

Correction factors include:

a. The amount of sample originally used in the extraction procedure.

b. An extraction efficiency factor.

c. A dilution factor for the fraction of extract used as inhibitor in the assay.

COMMENTS

Dialysis Efficiency

In multiple experiments performed in our laboratories, the dialysis efficiency has been shown to approach 46% after 10 hours of continuous agitation of the cells. Due to evaporation and other losses, the actual recovery of material has, in general, been less and varies from experiment to experiment. We have found it helpful in our laboratories to include with every dialysis experiment a set of cells containing tracer material. An equal amount of tracer is pipetted at the same time into a scintillation vial, held as a control and defined as 100% in computing dialysis efficiency.

Similarly, other extraction methods may be checked for extraction efficiency by adding tracer to the sample before extraction. After the extraction, and just prior to the radioimmunoassay, an aliquot is removed and counted to give the appropriate extraction efficiency factor.

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Dialysis Apparatus

The dialysis step recommended in this procedure may be performed using a Karush-type Chamber assembly, such as those available from Bellco Glass, P.O. Box B, 340 Edrudo Road, Vineland, New Jersey 08360 (Cat. No. 3213, 1 ml capacity). To obtain the efficiency characteristics indicated above, this type of cell requires continuous agitation.

Semi-permeable cellulose membranes are available from a variety of sources. We have found Fisher Dialyzer Tubing (Catalog No. 8-667C) to be satisfactory. This tubing must be treated before use by boiling about 5 feet of it in 500-1000 ml of distilled water containing a pinch of EDTA and sodium bicarbonate, until the tubing is soft and odorless. This usually requires three passes. Once the tubing is ready, it may be stored at 4° C under distilled water until used.

Antibody Cross-Reactivity

The anti-$PGF_{2\alpha}$ serum in this kit cross-reacts significantly with $PGF_{1\alpha}$ and should, therefore, not be used to differentiate between $PGF_{2\alpha}$ and $PGF_{1\alpha}$. At 50% binding the cross-reaction with PGE_1 and E_2 is 1:5000, and with PGA, and A_2 less than 1:10,000. 13,14-Dihydro-15-ketoprostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$ metabolite) cross-reacts 0.3%.

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