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CALCIUM FLUX AND *IN VITRO* PROLACTIN SYNTHESIS AND RELEASE FROM THE ROSTRAL PARS DISTALIS OF COHO SALMON (*ONCORHYNCHUS KISUTCH*)

by

Donald John MacDonald

B.Sc., Simon Fraser University, 1977

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Examining Committee:

Chairman: Dr. Glen H. Geen

Dr. B. A. McKeown, Senior Supervisor

Dr. K. K. Nair

Dr. P. Belton

Dr. P. C. Oloffs, Professor,
Public Examiner

Date approved: Dec. 4, 1983

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ABSTRACT

An *in vitro* system for incubating the pituitary rostral pars distalis of coho salmon (*Oncorhynchus kisutch*) was used to examine the role of calcium in the synthesis and secretion of prolactin, as measured by ^{3}H-leucine incorporation. This *in vitro* system was shown to support linear release of prolactin and was used to demonstrate differences in ^{3}H-prolactin synthesis between males and females.

^{3}H-prolactin secretion was shown to be sensitive to Ca^{++} levels in the media. Maximum secretion occurred near the reported physiological levels for ionized Ca^{++} in salmon plasma.

The relationship between synthesis and release of prolactin and calcium influx was examined with ^{45}Ca media. High osmotic pressure medium, which inhibits prolactin secretion, showed that ^{3}H-prolactin accumulation in the tissue was directly associated with elevated ^{45}Ca. The synthetic cyclic nucleotide db-cAMP stimulated ^{3}H-prolactin synthesis and also showed a relationship between ^{3}H-prolactin accumulation and elevated tissue ^{45}Ca. The antipsychotic drug chlorpromazine decreased ^{45}Ca uptake while increasing ^{3}H-prolactin synthesis and secretion.

Efflux of ^{45}Ca increased in the rostral pars distalis treated with db-cAMP, indicating elevated intracellular calcium levels. It has been postulated that in response to a stimulus, an influx of calcium into the cell acts as a second messenger, causing a release of the cell's secretory product. No evidence was found for stimulus-secretion coupling in coho prolactin

cells.

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A. Introduction

It is a major physiological accomplishment that euryhaline fish can move from one environmental salinity to another. They have a large respiratory surface in direct contact with the environment that makes them particularly vulnerable to body water or electrolyte changes. Investigations of this osmoregulatory ability led to the discovery that prolactin is required for teleosts to survive in fresh water (Pickford and Phillips, 1959; see Clarke and Bern, 1980).

Two distinct mechanisms controlling the release of prolactin have been suggested in teleosts. The first is hypothalamic possibly via a hypophyseal portal system or a direct neurosecretory innervation. The second is a more direct mechanism in which the prolactin cells respond to electrolyte changes in the blood which are presumed to reflect environmental changes.

Evidence for hypothalamic control of prolactin secretion in teleosts was shown by transplantation studies (Ball and Baker, 1969); hypothalamic lesion studies (Peter and McKeown, 1974); and work with a variety of drugs, most notably using 6-hydroxydopamine decrease activity of adrenergic nerves (Zambrano et al., 1973). Dopamine has also been shown to inhibit in vitro release of prolactin (Higham and Ball, 1974). It has since been widely accepted that prolactin secretion in fish is primarily under dopaminergic inhibitory control, as is prolactin

release in higher vertebrates. However, up to sixteen natural peptides and their derivatives are also reported to influence prolactin secretion (see Fluchinger, 1982). Furthermore, release is modified by other hormones (Clarke and Bern, 1980), including possibly prolactin itself (Herbert et al., 1979).

In addition to hypothalamic control, it has been suggested for teleosts that certain characteristics of the water, such as osmotic pressure or calcium concentration, can affect blood electrolytes which in turn directly influence the activity of prolactin cells.

Sage (1965) suggested that prolactin cells respond directly to osmotic changes in the environment via changes in blood osmotic pressure. Further in-vitro work provided evidence that this might be an important control mechanism (Emmart et al., 1967; Sage, 1968; Ingleton et al., 1973; Zambrano et al., 1974). Pituitaries that were transplanted away from hypothalamic control also provided evidence that osmotic pressure may directly affect prolactin release (Leatherland and Ensor, 1973; Nagahama et al., 1975).

As an alternative to osmotic pressure, it has been proposed that blood calcium concentration directly effects release of prolactin (Wendellar Bonga et al., 1978; Wendellar Bonga and Vander Meij, 1980). They postulated that prolactin cells would respond to changes in the calcium levels in the blood, which reflect ambient environmental calcium concentrations; high in salt water and low in fresh water.

The possibility that blood calcium can affect prolactin release is interesting in view of the central role of calcium in stimulus secretion coupling (Douglas and Poisner, 1962, 1964.) It is suggested that stimulation of a secretory cell causes an influx of Ca^{++} which in turn causes the release of a hormone. If a similar series of events occurs with the teleost prolactin cell, calcium concentration of the surrounding waters might influence blood calcium concentrations ultimately determine the size of Ca^{++} influx into the cell, and thus the amount of prolactin release.

Prolactin Cells of Coho Salmon

As in other teleosts, the endocrine cells of the salmon pituitary are isolated in distinct regions. The rostral pars distalis (RPD) of immature salmon have been shown to contain three cell types (McKeown and Leatherland, 1973): (i) Adenocorticotrophic hormone (ACTH) secreting cells that border the neurohypophysis, (ii) non-secretory cells of unknown function and (iii) prolactin cells. The latter are arranged in follicles with a central lumen and are the predominant cell type of the RPD. Gonadotrophic cells also occur in this region but in immature fish these cells are not yet developed.

The follicular arrangement of the prolactin cells in isospondylid fish, such as salmonids and eels, is quite unique. Studies of the morphology of these prolactin cells (Cook and Van Overbeeke, 1969; Leatherland and McKeown, 1973; Schreibman et

al., 1973) show that they are elongated pyramidal cells with the apical portion bordering the follicular lumen. These cells are clearly polar with cilia projecting into the lumen, and the nucleus, endoplasmic reticulum, Golgi bodies and granules concentrated near the basal end. Mitochondria are more common near both the apical and the basal membranes. This increase in mitochondria might be associated with calcium regulation and/or the reported pinocytosis of the apical membrane and exocytosis of prolactin granules at the basal membrane.

The function of the non-secretory cells of the RPD is unknown, although they have numerous microvilli projecting into the follicular lumen. Microvilli are often associated with absorption. These non-secretory cells also have desmosomes connecting them to prolactin cells, commonly near the lumen.

Cellular Calcium

Historically, the importance of the calcium concentration in incubation media has been known since Ringer (1883). Too little calcium in the media caused the frog heart to stop beating in diastole, while too much calcium caused the heart to stop beating in a systolic or contracted state.

Possibly, cellular processing of environmental calcium was a trait selected for during early evolutionary periods, when the calcium concentrations of the oceans were steadily rising (see Rubin, 1982). Successful organisms developed relatively impermeable membranes and ion pumps to maintain low cytosolic

levels of calcium. Evidence that calcium can be toxic is seen in the fact that the death process for cells often includes an influx of external calcium. However whether or not this is a cause or effect is unknown.

This large calcium gradient, high outside the cell and low inside, could easily be adapted to a relatively fast intracellular message system. A "pulse" of calcium influx with the gradient could be turned off by very efficient calcium pumps that remove the calcium from the cytoplasm (see Rubin, 1982).

Thus, not only the toxicity of calcium but its role as a "second messenger" would require that the active ionized form of calcium in the cytoplasm be very carefully controlled at low levels.

The extrusion of calcium from the cytosol is an active process that maintains cytosolic levels in vertebrates at about 10^{-7} M against a 10^{-2} M concentration in extracellular fluid.

There are two well characterized pumps for extruding cellular calcium (see H.J. Schatzmann, 1982). The first is a (Ca^{++} and Mg^{++}) ATPase activated by calmodulin, an ubiquitous intracellular calcium receptor protein. The second is an exchange of intracellular calcium for extracellular sodium, making use of the already established sodium gradient. It is suggested that at high levels of calcium influx, the Na^{+}/Ca^{++} exchange is more important while the (Ca^{++} and Mg^{++}) ATPase may "fine tune" lower cytosolic calcium levels.

Four mechanisms for controlled calcium influx have been identified. Calcium may enter (i) via sodium channels (ii) voltage dependent calcium channels (iii) receptor regulated non-voltage dependent channels and (iv) Ca^{++}-triggered calcium influx, a gate sensitive to cytosolic calcium.

If plasma membrane processes determine the ultimate concentrations of calcium within a cell, temporary regulation of ionized cytosolic calcium can be affected by utilizing various compartments within the cell. These "pools" effectively isolate the calcium.

Four such intracellular pools are (i) a rapid exchange buffer pool within the cytoplasm (ii) the endoplasmic reticulum (iii) mitochondria and (iv) secretory vesicles. Depending on the cell type, each of these pools may have a unique capacity and velocity for calcium accumulation.

Since membrane bound secretory granules are derived from endoplasmic reticulum, it is perhaps not surprising that they have the ability to accumulate calcium. Despite the fact that these granules have a high calcium content, there is some debate as to whether this calcium is readily exchanged with the surrounding cytoplasm. Matthews (1979) reports that granules have a slow rate of calcium accumulation and exchange. However, there are cytological studies, using antimonate deposition to localize intracellular calcium, indicating that secretory granule membranes of prolactin cells lose calcium under conditions that stimulate release (Leuschen et al., 1981). This

high calcium content of the granules, which undergo exocytosis, may also be a method of eventually eliminating calcium from the cell.

Cell lipids and calcium

Calcium interaction with cell lipids can effect plasma membrane lipid composition and fluidity. Not only will calcium stabilize artificial membranes by binding to low affinity sites such as phospholipids (Saurheber et al., 1980), but it has been shown that calcium directly controls some lipid metabolizing enzymes.

Receptor effects on membrane composition were first described by Hokin and Hokin (1953; 1954; 1955) when it was noted that acetylcholine caused rapid phosphatidylinositol turnover.

Phosphatidylinositol is converted to inositol phosphate and 1,2-diacylglycerol by the enzyme phospholipase C, which requires a low concentration of calcium. This calcium requirement is so low that it has been argued that phospholipase C is not controlled by calcium, but rather that this phosphatidylinositol breakdown may be controlling the "gating" of calcium at the plasma membrane (Michell, 1975, 1982).

However, whether phosphatidylinositol breakdown is involved in calcium gating is not the only aspect of this metabolic pathway that is important in cellular activation. One of the products of phospholipase C is 1,2-diacylglycerol and in

unsaturated forms this greatly increases the activity of a recently discovered calcium sensitive protein kinase (see Takai et al., 1979). This enzyme has been called protein kinase C and may represent a new transmembrane control system. This enzyme is calcium dependent and has an absolute requirement for phospholipids, especially phosphatidylserine. It may be activated at cellular concentrations of calcium by 1,2-diacylglycerol or alternatively, without 1,2-diacylglycerol but under high cellular calcium levels. Thus 1,2-diacylglycerol may act as a "second messenger" between membrane activated phospholipase C and the intracellular protein kinase C that may regulate cellular processes.

A different phospholipase C, found in the cytosol, produces 1,2-diacylglycerol by a calcium dependent mechanism from polyphosphoinositols and this 1,2-diacylglycerol might enhance granular fusion, if it were incorporated into membranes. Thus, exocytosis of membrane bound granules may be enzymes mediated by calcium dependent lipid (see Allan and Michell, 1979).

Another effect of changing the composition of membranes may lead to an altering of the size of one of the internal calcium compartments. Phosphatidyinositol binds five calcium ions and any shift or enzyme hydrolysis could free some of this calcium (Kai and Hawthorne, 1979). Recently, this phosphatidyl shift has been suggested as a possible mechanism for the increase in cytosolic calcium seen in thyrotropin releasing hormone stimulated prolactin cells (Rebechi et al., 1982). Also, it has

been suggested that phosphatidic acid, produced by the phosphorylation of 1,2-diacylglycerol, may act as an intracellular ionophore (Tyson et al., 1977; Putney et al., 1980). As an ionophore, phosphatidic acid could free calcium from intracellular pools such as the endoplasmic reticulum.

Arachidonic acid metabolites may have an important role in cell activation by either stimulating guanylate cyclase and/or prostaglandin synthesis. Arachidonic acid may be produced in the cell from two different pathways. It may result from the metabolism of 1,2-diacylglycerol or it may be released by the action of calcium-dependent phospholipase A^2 on phospholipids. Arachidonic acid may then enter either of two pathways that could affect cell activation. In one, lipooxygenases produce hydroxy fatty acids such as leukotrienes that have been suggested as activators of guanylate cyclase (see Takai et al., 1982). Alternatively, cycloxygenases can start a metabolic path resulting in prostaglandin synthesis. Prostaglandins have been suggested as stimulators of prolactin release (Ojeda et al., 1978). Thus calcium dependent Phospholipase A^2 increases the availability of free fatty acids such as arachidonic acid while the arachidonic acid metabolites, such as hydroxy fatty acids and prostaglandins, have been suggested as regulators of calcium translocation from intracellular pools.

To examine effects of calcium on prolactin secretion and its possible roles in the secretory process, an in vitro incubation method suitable for manipulation of calcium concentration was developed. A series of experiments was done showing the effects of different concentrations of Ca^{++} in the incubation medium on prolactin release. Influx and efflux of ^{45}Ca were studied to see how they relate to ^{3}H-prolactin synthesis and secretion.

B. Materials and Methods

Year old coho salmon (Oncorhynchus kisutch) were obtained from Capilano Hatchery, Pacific Biological Station, and a commercial fish farm in Indian Arm, B.C. They were maintained in a 760-liter tank using chlorine-free flow-through water at 8 C. They were fed commercial pellets (Oregon Moist) ad libitum under a 12 hr dark/ 12 hr light photoperiod. Fish size varied from 80 - 200 grams, although experimental and control groups were balanced for size of fish. Any sexually premature males (jacks) were not used.

The fish were decapitated and the top of the head then sliced off. The brain stem was pulled forward, lifting the brain out of its cavity and forward out of the way. Usually the hypophyseal stalk would break and the pituitary would have to be teased out of the sella turcica. The pituitary was placed in a small dissection tray containing non-radioactive incubation medium and the RPD teased away from the rest of the pituitary. If required, each RPD would then be divided into halves.

Serum-free, protein-free Puck's Saline A salts (Gibco) buffered at pH 7.3 with 0.1M N-2-Hydroxyethlpiperazine-N'-2-ethanesulfonic acid (Hepes) (Gibco) was chosen as the incubation medium (see Appendix). This avoided the problems of calcium phosphate precipitation, sequestering of ionic calcium by proteins, and competition by other divalent cations such as magnesium. Since Puck's Saline A is a calcium free medium, 53

mg/l Ca^{++} were added as calcium chloride. The osmotic pressure of this medium was 292 mOs/kg which is similar to measured plasma osmolarity values of fresh water maintained coho salmon (Brewer and McKeown, 1980). All incubations were done at room temperature (18-22 C), which is the reported optimum temperature for culturing tissue and cells of salmonids (Fryer et al., 1965). Newly synthesized prolactin was measured by ^{3}H-leucine incorporation (L-[4,5-^{3}H(N)]-leucine, Amersham) and calcium fluxes followed with $^{45}Calcium$ (Amersham).

Electrophoresis of incubation products was done with a 10% acid urea gel (Davis et al., 1972; McKeown et al., 1980) (see Appendix). The gel was photopolymerized with riboflavin phosphate and subjected to pre-electrophoresed overnight at 50V. Prior to sample application gel wells were cleaned of fluid extruded from the gels. The slab gel electrophoresis apparatus was constructed similar to the water cooled BioRad design ("Protean" Model). Power was supplied at constant voltage (Buchler Instruments) with the sample loaded gels first run at 100V for one hour and then at 200V, until the dye front started to leave the gel. The gels were stained for protein (Blakesley and Boezi, 1977) and the prolactin band oxidized (Packard combustor) and counted for tritium (Beckman, model LS 8000).

Prolactin was the major band in the gel. This prolactin band has been identified by Rf (0.27), molecular weight and immuno-reactivity to pollack prolactin antiserum (McKeown et al., 1980). Salmon growth hormone has similar molecular

structure and molecular weight to salmon prolactin, however purified salmon growth hormone has been shown to migrate independently of prolactin in this acid urea gel (Wagner and McKeown, unpublished). Purified salmon prolactin has an identical Rf in this gel (Pargher; pers. comm.).

Methods used for individual experiments are described in the following sections.

Release with Time

RPD's from three fish were incubated in 0.5 ml of medium containing L-[4,5-^{3}H(N)]-leucine (20 µCi/ml) in 10 ml round bottom flasks loosely covered with Parafilm and shaken gently.

The medium was changed at intervals between one and two hours. At the end of each incubation time, the medium was centrifuged to insure that free cells or bits of tissue would not contribute to medium counts. The centrifuged medium was precipitated overnight at 4° in 10% trichloroacetic acid (TCA) after the addition of bovine serum albumin (10 µg) so that the precipitate would give a visible pellet.

The tissue was carefully removed from the incubation flask and homogenized with a glass mortar and teflon pestle in 50 µl of nonradioactive incubation medium. Two 25 µl washes of the mortar and pestle brought the final tissue homogenate volume to 100 µl. This was also precipitated with 10% TCA.

The precipitated media and tissues were centrifuged and the supernatants discarded. The precipitated pellets were washed in

1 ml of diethyl ether and air dried. The pellets were then resuspended in 20 µl of sample buffer (see Appendix) and 5 µl of dye added (0.1% methyl green in glycerol). The sample was then applied to the electrophoresis gel.

Sex Differences

In these incubations, individual RPD's were placed in 25 µl of medium (20 µCi ^{3}H-leu) in a multiwell assay plate (Falcon 3084) for 1.5 hr. The medium was then pipetted off and placed on a square of Parafilm. Using a teflon toothpick, a drop (approx. 5 µl) of 0.1% methyl green in glycerol, acidified with concentrated acetic acid, was stirred in. Twenty µl were then applied directly to the electrophoresis well. The tissue was homogenized as described earlier. Twenty five µl of the final 100 µl homogenate volume were treated with acidified 0.1% methyl green in glycerol and applied directly to the gel. Electrophoresis and ^{3}H-prolactin determination were performed as described earlier.

Effects of Calcium Concentration in the Media

Eight groups of five RPD's were incubated in 0.5 ml of 0, 0+EDTA (0.01%), 7, 14, 27, 53, 105 and 424 mg/l Ca^{++} media in 2 ml snap top polyethylene vials. Groups were balanced for size and sex of fish. The incubations were terminated after two hours and the media and tissue homogenates precipitated in 10% TCA overnight. ^{3}H-prolactin was determined as described earlier.

The amount of prolactin was expressed as per cent of total secreted by all groups. The arc sine transformation of percent prolactin was examined using analysis of variance and Duncan's multiple range test.

Prolactin and ^{45}Calcium Influx

The initial experiment used whole RPD's from similar size and sex fish as controls. There were twelve incubations in this experiment of two whole RPD's each. Nine of the incubations were experimental and three were controls. The nine experimental preparations were three treatments with three different periods of exposure of 15, 38 and 67 minutes for each treatment. There was one control group for each time period.

Initially the groups were preincubated in 0.5 ml of media containing ^{3}H-leu (20 µCi/ml) before treatment exposure. After two hours, the preincubation medium was changed to 0.5 ml containing ^{3}H-leu (20 µCi/ml) and ^{45}Ca (10 µCi/ml). The experimental preparations contained either 6 mM gamma amino butyric acid (GABA), 6 mM dibutyrl cAMP or 5 µM dopamine. At the appropriate times one of each of the treatments and a control were terminated. The media were pipetted off and briefly centrifuged, decanted and precipitated overnight in TCA as described earlier. The tissues were rinsed for one minute in 0.5 ml of nonradioactive media, which was pipetted off and discarded. The tissue was then homogenized to a final volume of 250 µl, 50 µl of homogenate counted in 10 ml of Aquasol II for

^{45}Ca, and the remaining homogenate processed as previously described.

Since there seemed to be high variability between fish, a new series of experiments was run using paired hemi-RPD's, each using one half as its own control.

High Osmotic Media

Since it had previously been shown that high osmotic media inhibits prolactin secretion in teleosts, two experiments were conducted to investigate the relationship of high osmotic pressure medium (335 mOs/kg) to ^{45}Ca influx and ^{3}H-prolactin in tissue and media.

In the first experiment, five RPD's were divided and halves designated as either control or experimental. These were combined and preincubated for two hours in nonradioactive media (0.5 ml in polyethylene stoppered vials, gently shaken). The media was then changed and ^{3}H-leu (20 µCi/ml) and ^{45}Ca (10 µCi/ml) added. After 90 minutes, the media were removed and briefly centrifuged, decanted and TCA precipitated as described previously. The tissue was rinsed in non-radioactive media for one minute and then homogenized. Of the final homogenate volume of 250 µl, 20 µl were removed for ^{45}Ca counting (10 ml Aquasol II) and the remaining homogenate TCA precipitated and ^{3}H-prolactin determined as described earlier.

The second experiment was done on individual RPD halves (paired experimental + control) in a small volume of medium (25

µl) in a multicell assay plate as described previously.

The groups of hemi-RPD's of one male and two females were incubated in either experimental high osmotic pressure medium (335 mOs/kg) or normal control medium (298 mOs/kg) for one hour. The media were pipetted off and mixed with 5 µl of 0.1% methyl green in glycerol, acidified with concentrated acetic acid, and 20 µl applied directly to the gel well. The tissue was rinsed for one minute in 25 µl of non-radioactive medium, homogenized to a final volume of 100 µl; 20 µl counted for ^{45}Ca, 50 µl placed on Parafilm and mixed with 5 µl of acidified 0.1% methyl green in glycerol, 20 µl of this applied directly to the gel well for ^{3}H-prolactin determination.

db-cGMP

Five incubations of five hemi-RPD's (1/2 of each RPD as its own control) were used to examine the effect of 6 mM db-cGMP on ^{45}Ca influx and ^{3}H-prolactin in tissue and medium. The pituitaries were preincubated in 0.5 ml of media containing ^{3}H-leu (20 µCi/ml) in 2 ml stoppered polyethylene vials for two hours. The media were then changed and both ^{3}H-leu (20 µCi/ml) and ^{45}Ca (10 µCi/ml) added. After 90 minutes the incubations were terminated and the media and tissues ^{45}Ca and ^{3}H-prolactin quantificated, as described above.

db-cAMP

Three groups using paired hemi-RPD's, five halves per group, were pre-incubated for 90 minutes in 0.5 ml of ^{3}H-leu media (20 µCi/ml). The media were then changed to 0.5 ml containing ^{3}H-leu (20 µCi/ml) and ^{45}Ca (10 µCi/ml) for 55 minutes. The experimental media also contained 6 mM db-cAMP.

^{3}H-prolactin in the media and half of the final tissue homogenates volume of 500 µl were quantified as described above. 20 µl of tissue homogenates were counted for ^{45}Ca in 10 ml of Aquasol II.

An additional group using five paired hemi-RPD's was incubated following the above protocol except the pre-incubation was in non-radioactive medium for two hours and the treatment was for 90 minutes.

Chlorpromazine

Two different concentrations of chlorpromzaine were used to see if it would effect either ^{45}Ca influx or ^{3}H-prolactin in tissue and medium. In the first experiments, three incubations using five paired hemi-RPD's were pre-incubated in 0.5 ml of non-radioactive medium for two hours. The media were then changed, with 0.5 ml containing ^{3}H-leu (20 µCi/ml) and ^{45}Ca (10 µCi/ml). In addition, the experimental media contained 0.01 mM chlorpromazine (Rhone-Poulence). After 90 minutes the incubations were terminated and the medium and tissue

^{3}H-prolactin and tissue ^{45}Ca were determined as described earlier. A similar experiment using 0.1 mM chlorpromazine in two incubations was performed in the same manner.

cAMP and ^{45}Ca Efflux

Three groups of three hemi-BPD's each (one half of each BPD for control groups) were preincubated for two hours in incubation media containing ^{3}H-leu (20 µCi/ml) and ^{45}Ca (10 µCi/ml). The efflux washout was initiated by rinsing the tissue samples in a 10 mM Ethyleneglycol-bis-(B-aminoethyl ether)NNN'N'-tetraacetic acid (EGTA) (Sigma) buffer containing 11 mM calcium for five minutes, a buffer specifically designed to remove extracellularly bound $^{45}Ca^{++}$ (Aaronson et al., 1979). Media were then changed every 2-3 minutes with non-radioactive incubation media, with the first nine changes for the experimentals containing 6 mM db-cAMP. For the remaining eight changes both the experimentals and the controls received non-radioactive incubation media only. Media ^{45}Ca were expressed as percent of total efflux. The averaged arc sine transformed percent was examined using analysis of variance.

C. Results

Release With Time

Since there was virtually no difference in amount of quenching in the combustor oxidized samples, the results were expressed as counts per minute (CPM).

Fig. I shows that the release of prolactin was linear for up to four hours with only a small drop at about six hours.

Sex Difference

There is a strong correlation between the size of fish and the total ^{3}H prolactin unique for each sex of fish. Fig. 2 shows a correlation of r=0.98 for the females and a correlation of r=0.91 for the males, while a line fitted to all the points (male and female) has a correlation of only r=0.28 (not shown).

Effects of Media Calcium Concentration

The amount of ^{3}H-prolactin released as a percentage of total ^{3}H-prolactin is shown in Fig. 3. Maximum release occurred with 53 mg Ca^{++} /l media ($p< 0.001$). Calcium concentrations higher and lower resulted in less ^{3}H-prolactin secretion. At $p< 0.05$, EDTA group secreted less than all other groups except for zero calcium; 106 mg/l group secreted more than 7 and 424 mg/l groups.

Fig. 1. ^{3}H-prolactin secreted into the media over six hours, showing linearity over at least four hours.

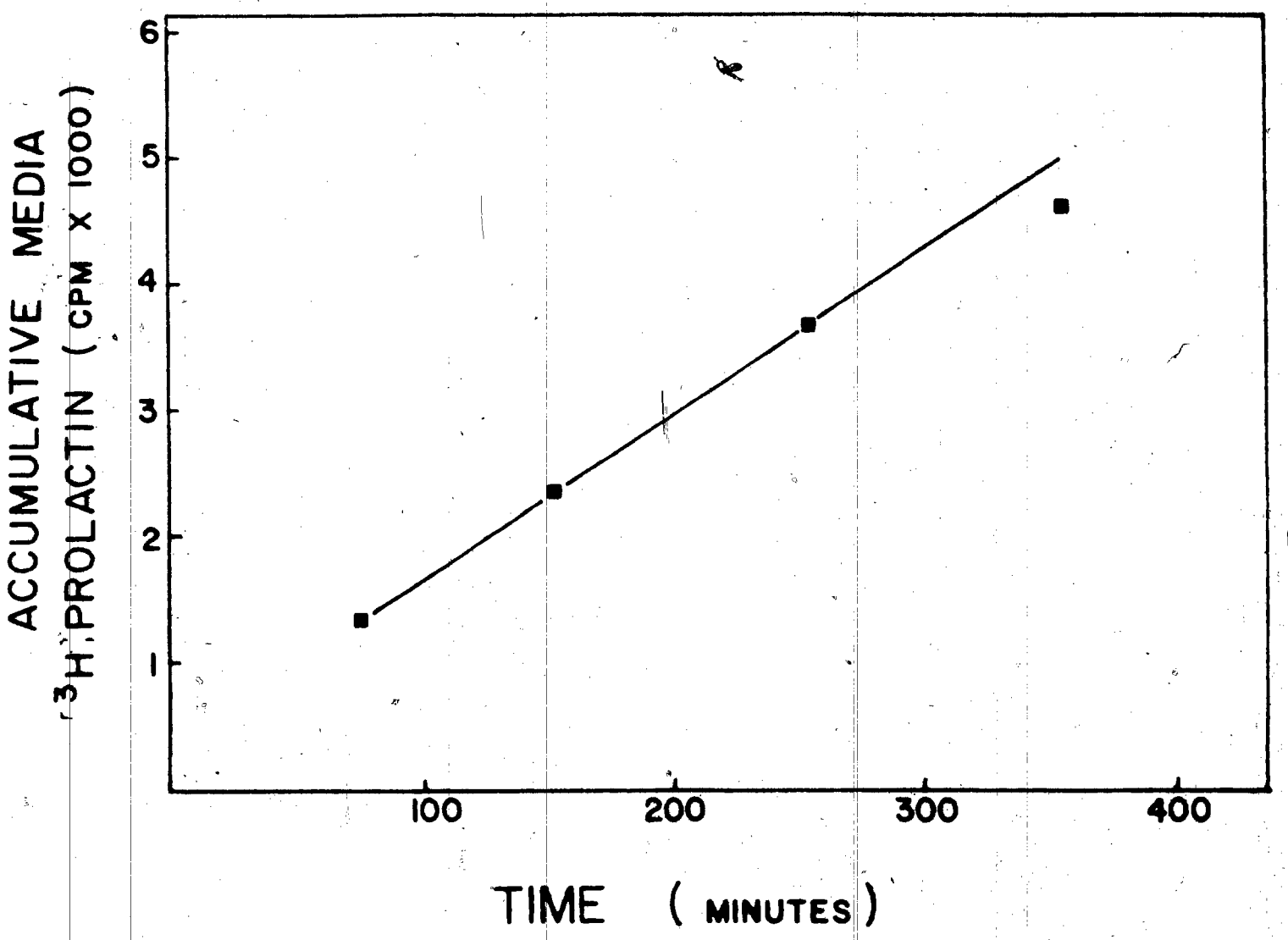

ACCUMULATIVE MEDIA
^{3}H-PROLACTIN (CPM X 1000)
6
5
4
3
2
1
100
200
300
400
TIME (MINUTES)

Fig. 2. Total ^{3}H-prolactin produced by individual coho rostral pars distalis compared to the weight of the fish. Best fitted lines indicate differences between males and females.

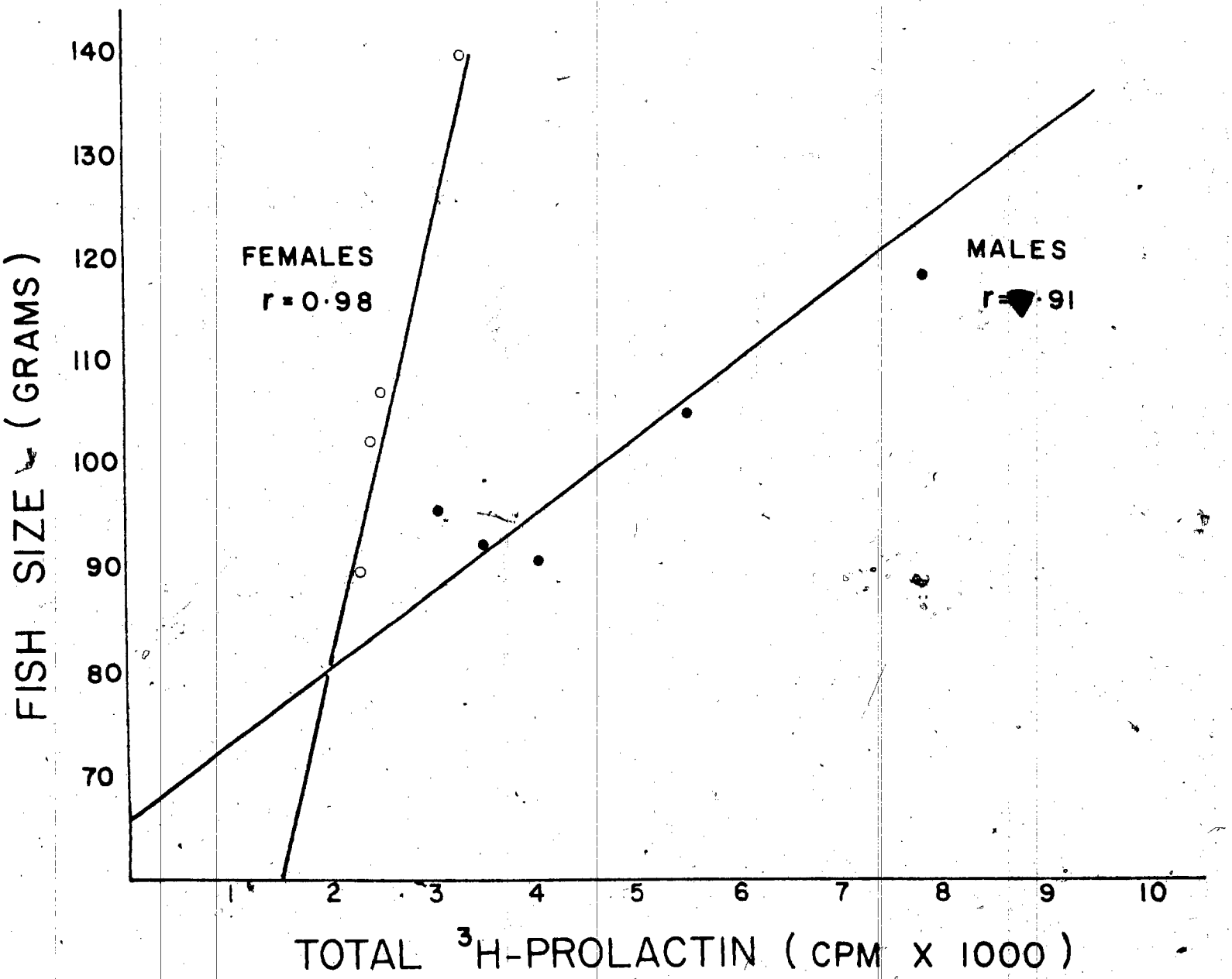

FISH SIZE (GRAMS)
140
130
120
110
100
90
80
70
FEMALES
r = 0·98
MALES
r = 0·91
1
2
3
4
5
6
7
8
9
10
TOTAL ³H-PROLACTIN (CPM X 1000)

Fig. 3. ^{3}H-prolactin released, expressed as a percentage of all groups, into media containing differing amounts of Ca^{++}. Significantly more ($p=0.001$) was secreted in 0.53 mg/l media. At $p=0.05$, EDTA group secreted less than all other groups except for zero Ca^{++}; 106 mg/l group secreted more than 7 and 424 mg/l groups.

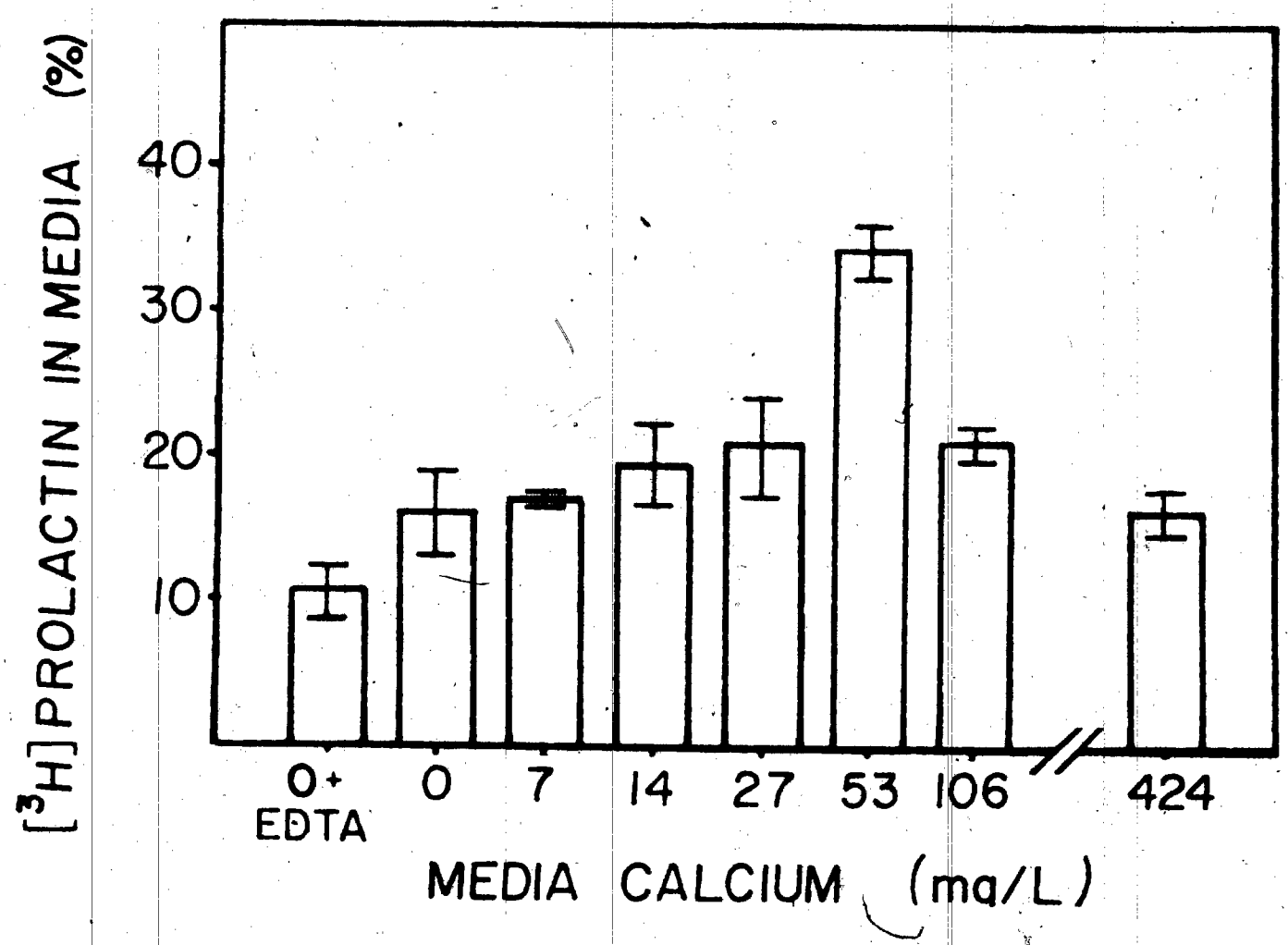

[3H]PROLACTIN IN MEDIA (%)
40
30
20
10
0+ EDTA
0
7
14
27
53
106
424
MEDIA CALCIUM (mg/L)

Fig. 4. ^{45}Ca tissue accumulation against tissue ^{3}H-prolactin. There was no relationship between ^{45}Ca tissue accumulation and ^{3}H-prolactin in media.

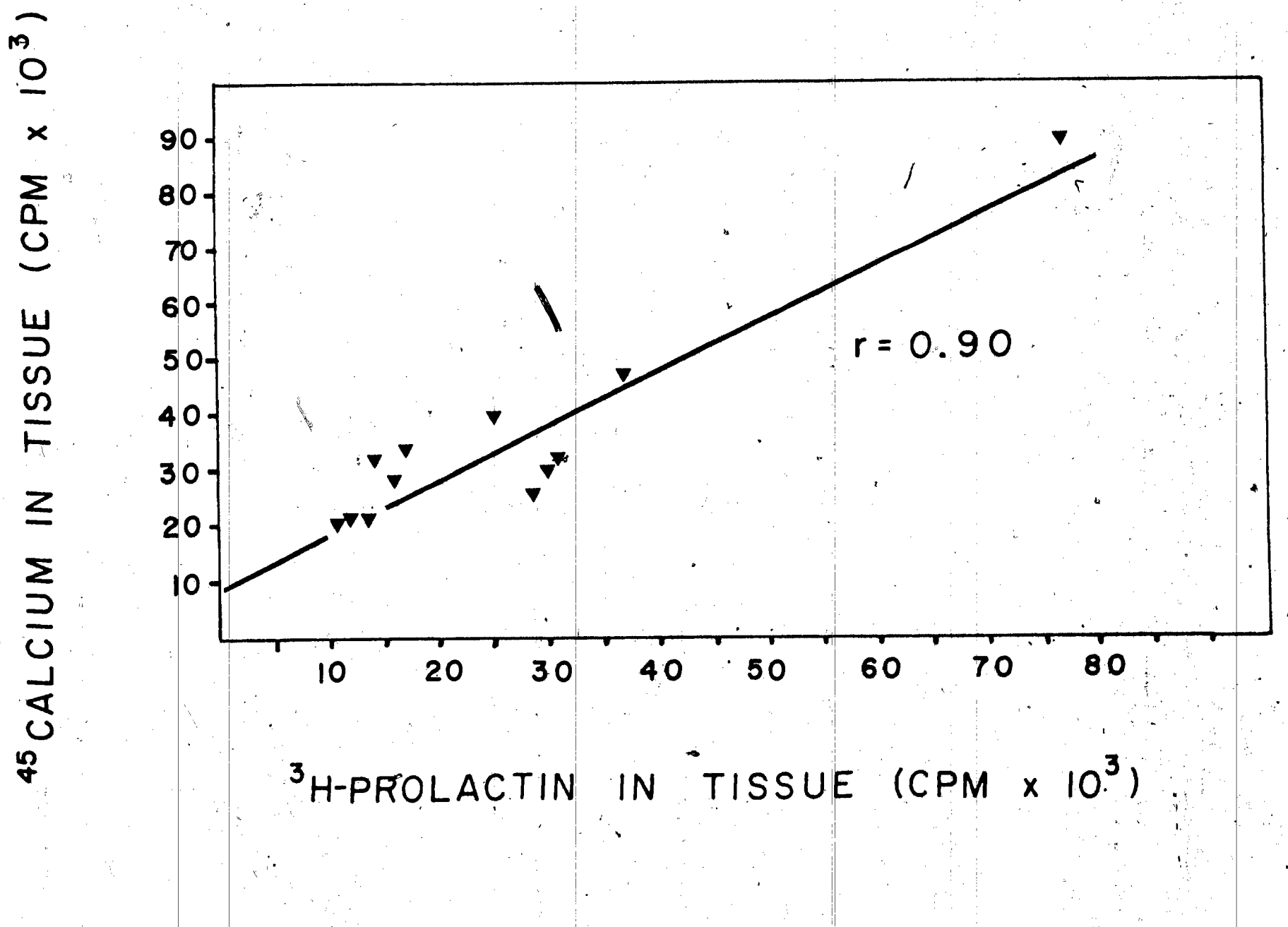

^{45}CALCIUM IN TISSUE (CPM x 10^3)
90
80
70
60
50
40
30
20
10
r = 0.90
10
20
30
40
50
60
70
80
^{3}H-PROLACTIN IN TISSUE (CPM x 10^3)

Fig. 5. Effects of high osmotic media on ^{3}H-prolactin in tissue (△) or media (X) against tissue ^{45}Ca accumulation. Experimental treatments expressed as per cent of total (experimental plus control), values over 50% indicate that the experimental groups were more active.

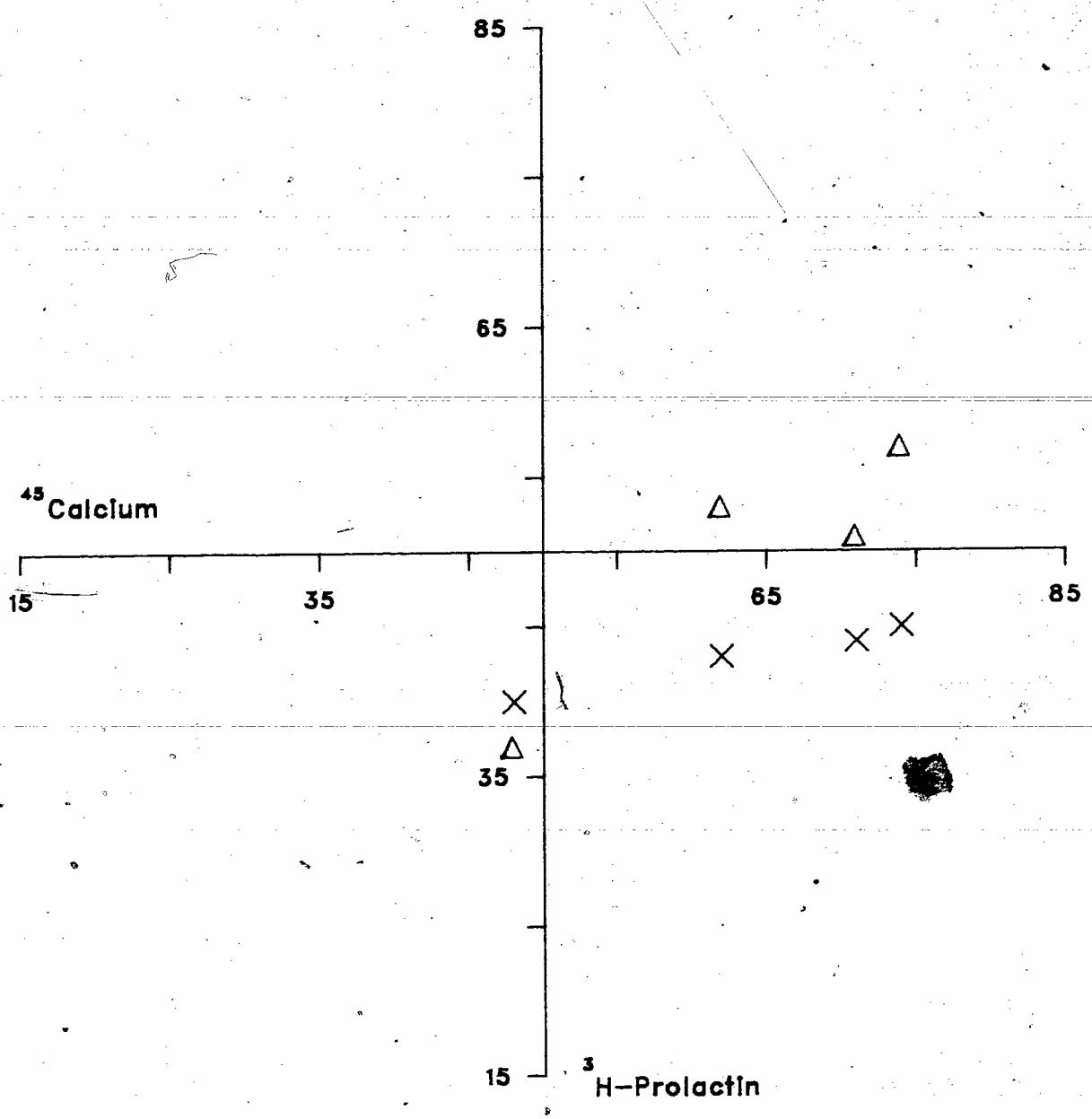

85
65
35
15
45Calcium
15
35
65
85
3H–Prolactin

Fig. 6. Effects of db-cGMP on ^{3}H-prolactin in tissue (△) or media (X) against tissue ^{45}Ca accumulation. Experimental treatments expressed as per cent of total (experimentals plus controls); values over 50% indicate the experimental groups were more active.

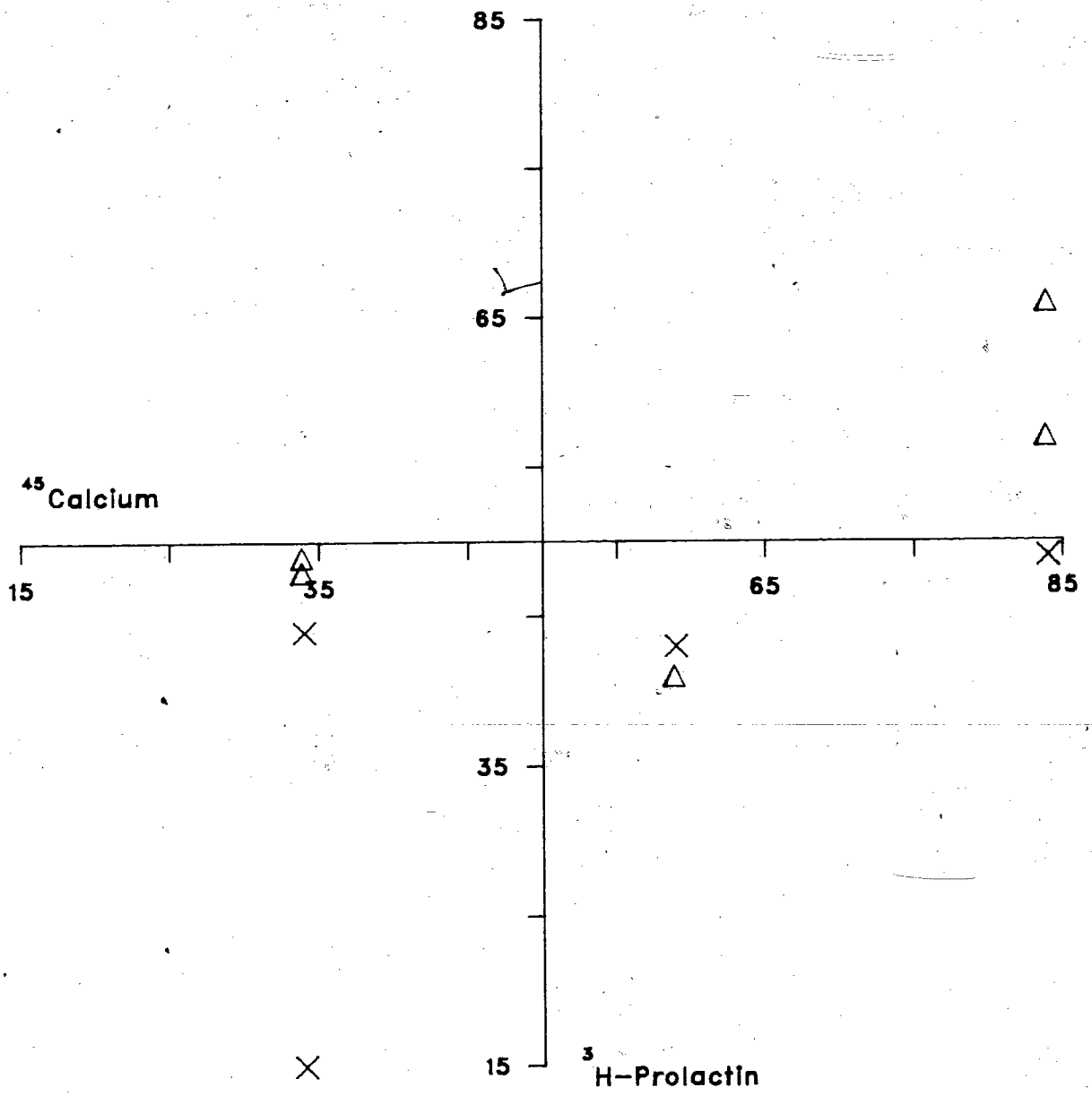

85
65
35
15
15
35
65
85
45Calcium
3H-Prolactin

Fig. 7. Effects of db-cAMP on ^{3}H-prolactin in tissue (△) or media (X) against tissue ^{45}Ca accumulation. Experimental treatments expressed as per cent of total (experimental plus control); values over 50% indicate that the experimental groups were more active.

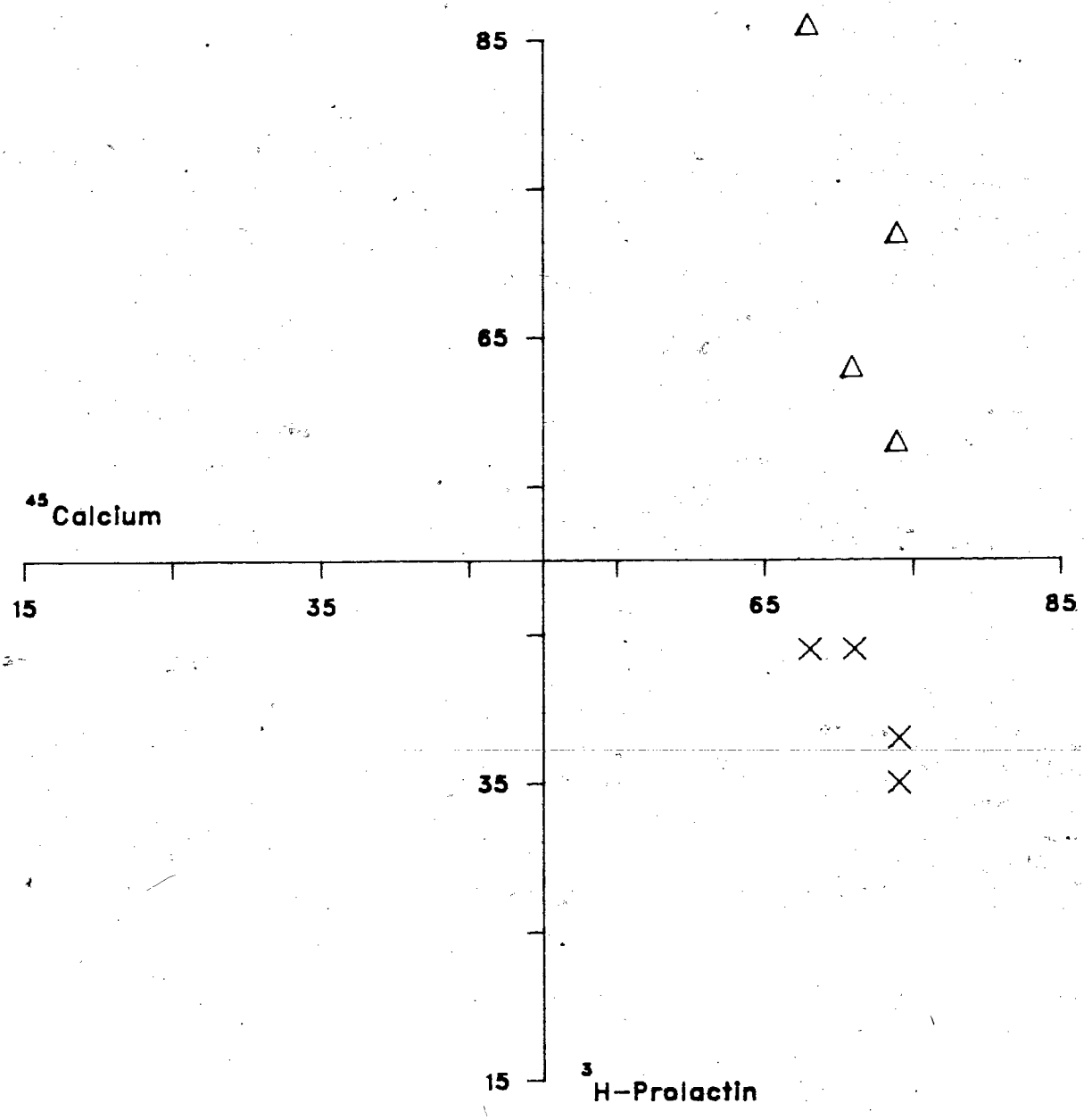

85
65
35
15
45Calcium
15
35
65
85
3H–Prolactin

Fig. 8. Effects of chlorpromazine on ^{3}H-prolactin in tissue (△) or media (X) against tissue ^{45}Ca accumulation. Experimental treatments expressed as per cent of total (experimental plus control); values over 50% indicated that the experimental groups were more active. Two incubations in 0.1 mM chlorpromazine show smallest net accumulation of ^{45}Ca; other chlorpromazine concentration 0.01 mM.

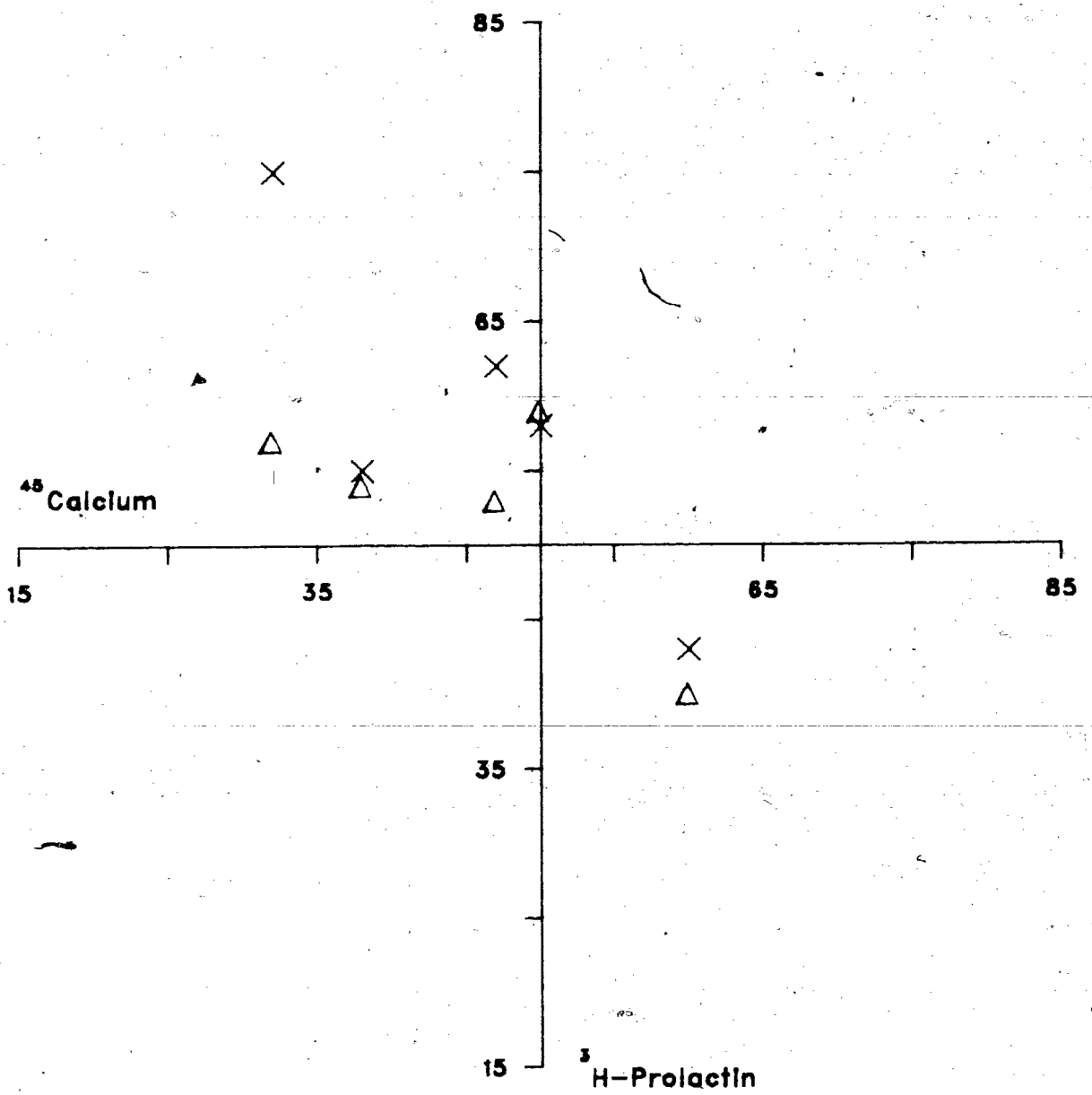
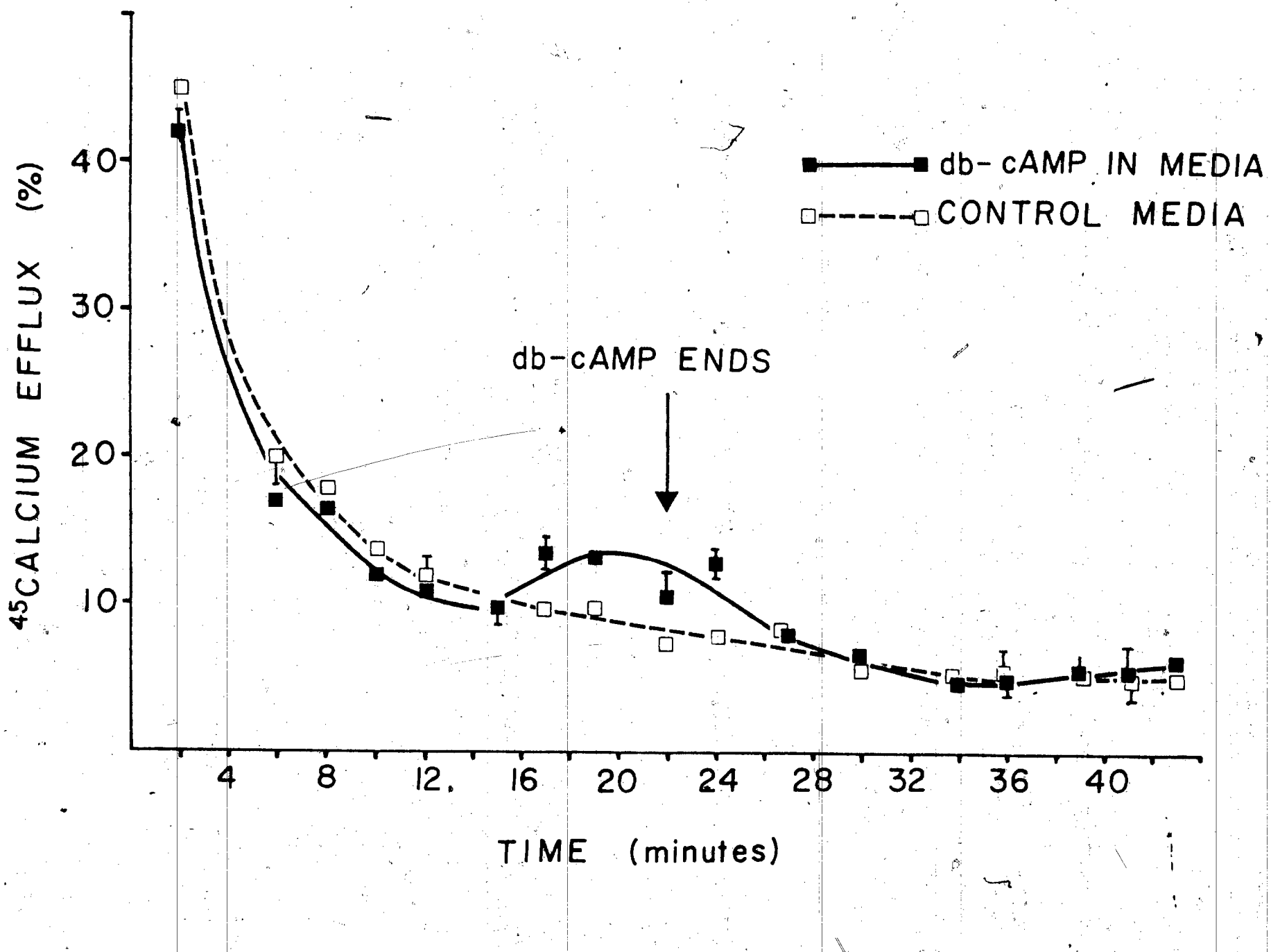

85
65
35
15
15
35
65
85
45Calcium
3H-Prolactin

Fig. 9. Efflux of ^{45}Ca, expressed as per cent remaining, over time. The experimental group received db-cAMP for the first eight media changes, after which both groups received control media. Significantly different ($p=0.05$) at 17, 19 and 24 minutes, standard errors less than 1% not shown.


db- cAMP IN MEDIA
CONTROL MEDIA
db-cAMP ENDS
^{45}CALCIUM EFFLUX (%)
40
30
20
10
4
8
12.
16
20
24
28
32
36
40
TIME (minutes)

The lowest levels of release were observed in groups containing 0.01% EDTA. Both 0 and the EDTA groups still showed some release of ^{3}H-prolactin.

There were no statistical differences in tissue content of labelled prolactin, except that 0.01% EDTA caused lower synthesis.

^{45}Ca Influx and ^{3}H-Prolactin

The experiment using whole RPD's from similar size and sex fish was of limited value because of an apparently high variability between RPD's of different fish, even if size and sex were accounted for. This is shown by the fact that the control tissue showed no pattern over the three times, either in media or tissue ^{3}H-prolactin.

However one relationship became apparent. If ^{45}Ca in all the tissues (including controls) were plotted against the ^{3}H-prolactin in all the tissues, a linear correlation ($r=0.90$) becomes evident (Fig. 4). No such correlation is found between ^{45}Ca in the tissue and ^{3}H-prolactin in the medium.

The experiments using paired hemi-RPD's provided more reliable results. The high osmotic media inhibited ^{3}H-prolactin release compared to controls in all incubations (Fig. 5) while three of the incubations showed ^{3}H-prolactin accumulation in the experimental tissue compared to controls. These three incubations also showed ^{45}Ca accumulation in the tissue.

Db-cGMP also seemed to inhibit release (Fig. 6). The relationship between tissue ^{3}H-prolactin and ^{45}Ca is also shown here: high tissue ^{3}H-prolactin compared to controls is accompanied by high tissue accumulation of ^{45}Ca. The inverse may not be true however, as one of these incubations shows a greater ^{45}Ca accumulation in the tissue compared to controls while there is less ^{3}H-prolactin than controls.

Db-cAMP also seemed to inhibit ^{3}H-prolactin release (Fig. 7) compared to controls. There was however an obvious stimulation of ^{3}H-prolactin tissue accumulation and again these tissues show a net accumulation of ^{45}Ca.

Chlorpromazine at both concentrations appeared to show a reverse pattern, (Fig. 8) with ^{3}H-prolactin in both the tissue and medium showing an inverse relationship to tissue ^{45}Ca net accumulation. This is also the only experimental treatment that caused ^{3}H-prolactin release compared to controls. The two incubations at the higher chlorpromazine concentration (0.1 mM) show the smallest net accumulation of ^{45}Ca in tissue compared to controls.

^{45}Ca Efflux and db-cAMP

The db-cAMP-treated tissue showed no differences compared to controls for the first fifteen minutes (Fig. 9), then there was an elevated ^{45}Ca efflux in the db-cAMP treated tissues that continued at least two minutes after the db-cAMP treatment stopped. At twenty seven minutes until the experiment was

terminated, there was again no difference between control and experimental efflux. Thus, this experiment indicates a "lag" period before db-cAMP causes efflux of ^{45}Ca and that this efflux then returns to control levels after db-cAMP treatment is stopped.

D. Discussion

^{3}H-prolactin release with time

These results show that the incubation medium chosen was satisfactory in that release was linear for up to at least four hours.

These results were in agreement with those of McKeown *et al.* (1980) who found linear release with time of ^{3}H-prolactin from the incubated RPD of the rainbow trout *Salmo gairdneri*, using a simple *Xenopus* Ringers, similar to Pucks saline A (see Appendix).

Tilley (unpublished 1980) did not find linear release with time for ^{3}H-prolactin from coho salmon RPD's. However, his *Xenopus* Ringers was of lower osmotic pressure (277 mOs/kg) than that observed by McKeown *et al.* (1980) and much less than the reported value of about 300 mOs/kg (Brewer and McKeown, 1980) for coho salmon plasma. Thus, this lower osmotic pressure could cause accelerated release and perhaps, due to depletion of different "pools" of prolactin within the cell, the release profile might change.

Also, variability is found in the prolactin secretion *in vitro* between individual fish and this could represent a source of error. This experiment avoids this possibility by changing the medium at intervals on the same group of RPD's.

Sex Differences

The males produced more ^{3}H-prolactin *in vitro* per gram of fish than the females. Production of ^{3}H-prolactin by females seems to be relatively independent of size, while the males show a much stronger relationship between size and total ^{3}H-prolactin. Since 17-β estradiol has been shown to be a potent stimulator of prolactin synthesis and release (see Labrie *et al.*, 1980; Buckman *et al.*, 1980), this might seem surprising.

Prolactin production is affected by 17-β estradiol at both the hypothalamic and pituitary levels. In the hypothalamus, both 17-β estradiol (Paul *et al.*, 1979) and progesterone (Cramer *et al.*, 1980) increase dopamine secretion. Olcese and de Vlaming (1979) show that both 17-β estradiol and progesterone inhibit hypothalamic monoamine oxidase, an enzyme that inactivates catacholamines such as dopamine.

At the pituitary level, 17-β estradiol has been shown to bind to cytoplasmic receptors and interact with the nucleus as expected in classic steroid theory (Hang *et al.*, 1978, Spona *et al.*, 1980). It is probably through this mechanism that estrogens cause increased synthesis of prolactin. Estrogen stimulation of prolactin synthesis has also been linked to increased levels of pituitary putrescine and spermidine *in vivo* (Gray *et al.*, 1980). These and other polyamines have been associated with *in vivo* and *in vitro* tissue growth, possibly interacting with nucleic acid regulation and protein synthesis and thus may explain the

stimulating effect of estrogen upon prolactin synthesis.

However, it is also reported that estrogen is involved with various membrane-mediated mechanisms such as receptors and granule processing. It has been reported that 17-β estradiol desensitizes prolactin cells to dopaminergic inhibition, possibly by altering dopamine receptor structure (Dufy et al., 1979) or number (Cronin et al., 1980). Membrane receptors for estrogens have been suggested (Pietras ans Szego, 1979), although a more general effect on membrane lipid composition or fluidity may be involved (Dufy et al., 1982).

Another membrane effect of 17-β estradiol is that of maintaining thyrotropin releasing hormone (TRH) receptors on prolactin cells (Piercy and Shin, 1980).

Prolactin granule membranes may also interact with estrogens. Dopamine has been shown to bind to prolactin granules, possibly inhibiting release, and estrogen treatment reduces this dopamine binding (Gudelsky et al., 1981). These workers have also shown that 17-β estradiol antagonizes dopamine's ability to stimulate lysosomal enzyme activity in the rat pituitary (Hansel et al., 1981), presumably decreasing granule crinophagy.

An indirect way that estrogens might influence prolactin production is by changing the plasma calcium levels. Pang and Balbontin (1978) have shown that estradiol elevates plasma calcium in the male killifish Fundulus heteroclitus while testosterone had no effect. Although the possibility that

estrogens can mediate prolactin production by altering plasma calcium is attractive, Copp and Ma (1980) do not find significant differences in ionized plasma calcium between male and female spawning salmon and there appears to be no difference in total plasma calcium between male and female salmon during their downstream migration (Parker, pers. comm.).

The possible role of 17-β estradiol as a regulator of prolactin fits well with the discovery of an extra-gonadal source of estrogens. While all vertebrates have the enzyme androgen aromatase present in their neural tissue, the sculpin Myoxocephalus octodecimspinosus has 100 to 1000 times the levels found in rats and rabbits (Callard et al., 1978) This enzyme transforms circulating androgens into estrones and estradiol. On a unit weight basis, this estrogen bio-synthetic pathway exceeds that of the gonads. While the pituitary aromatase activity seems relatively low, pituitary estrogen per unit weight was at least twelve times higher than that of other brain regions (Callard et al., 1981). The brain tissues were higher in estrone while the pituitary had a much greater proportion of estradiol, possibly reflecting differences in receptor binding affinities.

This central nervous system estrogen production varies with gender and season, but its role in juvenile salmonids is unknown.

However the object of this experiment was to determine if gender differences could account for some of the variability found in prolactin production between groups. It was considered

to be sufficient evidence and subsequently groups were not only balanced for size of fish but also sex.

^{3}H-prolactin and medium Ca^{++} levels

These experiments indicated that variation of medium calcium concentration affected the amount of prolactin released. Significantly more prolactin was released in the 53 mg/l Ca^{++} group. This is the group closest to the plasma concentration of 6.19 mg % ionized Ca^{++} reported by Copp and Ma (1980) for freshwater sockeye salmon Oncorhynchus nerka.

Consistent with stimulus-secretion coupling, I found that Ca^{++} free groups secreted less prolactin, although Moriarity and Leuschen (1981) attributed lack of response of prolactin cells in calcium free medium to loss of intracellular calcium and not the abscence of external calcium per se . However, I found that my calcium free group continued to secrete measureable amounts. This is likely due to endogenous interstitial calcium, as shown by the fact that 0.01% EDTA appeared to reduce secretion further. The higher concentrations of calcium, above physiological levels, caused reduced prolactin secretion. This effect of high calcium has been shown by Douglas and Poisner (1964) in the posterior pituitary, and more recently by Thorner et al. (1980) on prolactin secretion in rats and by Ray and Wallis (1982) in sheep.

It is possible that high calcium causes membrane immobilization since high calcium is reported to "freeze"

prolactin granules in exocytosis (Roubos and van der Wal-Divendal, 1980). It is also possible that calcium is having an electrical effect since prolactin cells are reported to have spontaneous action potentials (Brailes et al., 1977) and that teleost prolactin cells have a Ca^{++} component to their action potentials (Taraskevich and Douglas, 1978). It could be expected that one of the important factors influencing this calcium dependent electrical activity would be the extracellular Ca^{++} concentration.

In the rat (Thorner, 1980) and sheep (Ray & Wallis, 1982) it has been reported that prolactin release is dependent upon extracellular calcium concentration with maximum release at about 2 mM. However the media in the above studies contained proteins (serum or BSA) and thus the concentration of ionized calcium is not reported. Working with protein-free medium, White et al. (1981) report that prolactin secretion from neoplasic GH^3 is optimal at 0.2 mM calcium. Presumably, this calcium would be primarily in the active ionized form, while a large portion of the 2 mM calcium used in the protein-containing media would be sequestered and therefore inactive.

^{45}Ca Influx and ^3H-prolactin

The experimental treatments chosen in this experiment were dopamine, GABA and db-cAMP. However, the use of whole RPD's from similar fish as controls proved of limited value. There was a

high variability between controls that precluded obtaining useful data from the experimental treatments. This variability was reduced, but not sufficiently, by balancing control groups and experimental groups for size and sex of fish.

While this experiment using whole RPD controls from similar fish did not provide conclusive answers to the above suggested prolactin regulators, there was a definite relationship shown between ^{45}Ca accumulation in the tissue and ^{3}H-prolactin accumulation for all tissues, both experimental and control (Fig. 4).

If teleost prolactin cells utilize the granule release process as commonly described, increased granular production would likely increase the ^{45}Ca associated with this pool. As described earlier, prolactin granules accumulate calcium and thus ^{45}Ca accumulation could represent either an increase in size of the granular pool or increased turnover rate, which in the short term experiments would tend to fill a fixed size granular pool faster with ^{45}Ca. These explanations would not require an increase in cytosolic ionized calcium or an increase in calcium influx across the cell membrane, beyond homeostatic regulation of calcium. An alternative explanation is that there was an influx of calcium across the membrane and this was accommodated by uptake by various cellular calcium pools, such as granules, endoplasmic reticulum or mitochondria. In this explanation, an influx of calcium leads to ^{3}H-prolactin accumulation, either through increased synthesis, decreased

release or degradation (crinophagy). The third possibility is that calcium influx led to cytosolic accumulation. However, since free cytosolic calcium must be carefully regulated, it is doubtful that there is any long term increase.

The obvious variation in the above experiments made it necessary to use a different approach. Half of each RPD was used as a control, either singly or as part of a control group. Paired anterior hemi-pituitaires were used in studies of rats (Enjalbert et al., 1979; Ray & Wallis, 1981). Baker and Ingleton (1975) used paired hemi-pituitary glands to examine hormone secretion in Anguilla anguilla and Salmo gairdneri. The experimental treatments chosen were high osmotic pressure, db-cGMP, db-cAMP and chlorpromazine.

High Osmotic Pressure

As expected, high osmostic media inhibited ^{3}H-prolactin release. Studies employing Xiphophorus (Sage 1965), Anguilla anguilla , Poecilia latipinna (Ingleton et al., 1973), Tilapia mossambicus (Sarotherodon mossambica) (Zambrano et al., 1974) have shown that osmotic pressure can influence prolactin release in vitro. Preliminary work indicated that coho RPD's in vitro also were influenced by osmotic pressure (MacDonald, unpublished). This earlier work showed an inhibition of synthesis of ^{3}H-prolactin over a six day culture as opposed to the increased accumulation of ^{3}H-prolactin seen in the present

short-term incubation. However, short term incubations are reported to show an increase in tissue prolactin when inhibited by bromocriptine for the first four days, followed by a decline in ^{3}H-prolactin, possibly reflecting increased crinophagy (Dannies & Rudnick, 1980). Baker and Ingleton (1975) report that only newly synthesized prolactin (^{3}H-leucine incorporation) release was inhibited by high sodium concentration. However these experiments were done at 5° and 10° as opposed to 20° used in the present study and it is expected that Ca^{++} ATPases would be sensitive to temperature. Low temperature has been shown to affect prolactin release in two phases: a short term (2-7 min) Ca^{++} independent release and a long term (30-60 min) phase of release that seems to be associated with decreased Ca^{++} efflux (Milligan 1979, Milligan and Kraicer 1979). McKeown and Peter (1976) report that higher temperature caused increased prolactin release *in vivo* in the goldfish *Carassuis auratus*. Fryer *et al.* (1965) reported optimal growth of a salmon cell line at 20°.

Although high osmotic pressure inhibited release in all four of these incubations, ^{45}Ca tissue accumulation occurred only in the three incubations that had increased tissue ^{3}H-prolactin over controls. This is consistent with the first experiment showing a relationship between ^{3}H-prolactin and ^{45}Ca accumulation.

db-cGMP

The mechanism and physiological role of cGMP is still obscure, although apparently it neither parallels the role of cAMP as a second messenger according to Sutherland's criteria (Sutherland et al., 1968) nor antagonizes cAMP via a "duelistic" role as postulated by Goldberg (1975).

However, it has often been noted that cellular levels of cGMP seem to be associated with cellular calcium metabolism and Rasmussen (1981) suggested that a hormonally induced calcium influx leads to an increase in cytosolic cGMP which may increase both the amplitude of the calcium pulse and/or the sensitivity of the response to calcium. However, others have suggested that the action of cGMP parallels that of calcium. Rubin (1982) pointed out that physiological agonists of cGMP require whole cells, and fail to increase cGMP levels in broken cells, seeming to implicate intermediates. Michel (1975) pointed out an apparent relationship between phosphatidylinositol turnover and cGMP increase, in the absence of any increased cellular cAMP. Takai et al. (1982) supported this view that unsaturated fatty acid derivatives, with special emphasis on arachidonic acid, may link extracellular messengers to guanylate cyclase.

In these experiments there was no obvious effects of incubations with 6 mM db-cGMP, although the possibility of inhibited release is present. However, such large concentrations of exogenous cyclic nucleotide may interfere with cAMP

metabolism, since they may share the same phosphodiesterases (Cheung 1979).

The apparent increase in ^{45}Ca accumulation with an increase in tissue ^{3}H prolactin is also found in this treatment. However, the converse may not always be true as one incubation showed elevated tissue ^{45}Ca without elevated ^{3}H-prolactin.

db-cAMP

The first "second messenger" proposed by Sutherland et al. (1968) to link extracellular protein hormones to cellular response was cAMP. Adenylate cyclase regulation was recently reviewed by Swillens and Dumont (1980), cyclic nucleotide phosphodiasterase by Strada (1982) and cellular regulation by protein phosphorylation by Hofmann (1982).

There are many studies that implicate cAMP in regulation of prolactin production. However, it is not clear whether cAMP is primarily involved in synthesis or release, although it could be fundamental to both.

Nagasawa et al. (1972) reports that db-cAMP stimulates release of prolactin from rat pituitaries in vitro. However, the possibility that the primary effect of cAMP is to stimulate synthesis in rats has been suggested by Sundberg et al. (1976). They point out that TRH stimulates release without elevated cAMP and that cholera toxin elevates cAMP without increasing release. Thorner et al. (1980) found no increased release when endogenous cAMP was elevated by adenylate cyclase stimulation using

prostaglandin E and cholera toxin. However, db-cAMP did stimulate release. It was suggested that db-cAMP, as well as the phosphodiesterase inhibitor, theophylline, may mobilize intracellular calcium (also see Dunlop et al. 1981). Maurer (1982) showed that elevated cAMP increases prolactin mRNA and proposes that cAMP effects synthesis.

A db-cAMP stimulated release of prolactin has been reported in teleosts. McKeown et al. (1980) reports that 6 mM db-cAMP stimulated both synthesis and release in rainbow trout Salmo gairdneri. Grau et al. (1982) report a dose response for db-cAMP on prolactin release in tilapia (Sarotherodon mossambica). My results clearly show an accumulation of ^{3}H-prolactin in the tissue while ^{3}H-prolactin release to the medium is, if anything, inhibited by db-cAMP.

Two experimental differences, osmotic pressure and medium calcium concentrations, may account for the discrepencies between this work and the previously mentioned studies.

Osmotic pressure was different in the three studies. This present study was done at 298 mOs/kg, very close to the reported value for coho plasma (300 mOs/kg) (Brewer and McKeown 1980). Work done by Tilley (unpublished) confirmed the cAMP stimulated release reported by McKeown et al. (1980) However, both these studies were done at 287 mOs/kg or lower, and as discussed earlier, low osmotic pressure per se stimulates prolactin release in teleosts and could be "permissive" to release of cAMP stimulated synthesis. On the other hand, Grau et al. (1982) only

showed that db-cAMP relieves high osmotic inhibition of tilapia RPD's *in vitro*, rather than a direct stimulation. While it would be difficult to relate these effects into a single mechanism, osmotic pressure could influence cAMP stimulated release in a tonic manner, possibly even involving calcium.

As emphasized in current reviews (Rasmussen, 1981; Rubin, 1982), calcium and cAMP are internally linked in their roles as cellular regulators. Calcium can either stimulate or inhibit adenylate cyclase. Calcium can also stimulate calmodulin activated phosphodiesterases, thus degrading cAMP. Cyclic AMP can influence cytosolic Ca^{++} levels by (1) increasing uptake by sarcoplasmic reticulum or endoplasmic reticulum, (2) increased activity of the plasma membrane pump, (3) decreased permeability of the plasma membranes, (4) increased influx from an intracellular source (mitochondria), (5) increased binding affinity of calcium receptors for calcium and (6) altered binding of calcium receptor protein (calmodulin) to the membrane (Rasmussen, 1979).

In the db-cAMP incubations reported here, the medium Ca^{++} levels chosen (53 mg/l) were shown to be optimal for basal or unstimulated prolactin release and also are very near the physiological ionized calcium levels of salmon plasma. The studies of McKeown *et al.* (1980) and Tilley (unpublished) used higher levels of medium calcium (80.0 mg/l) as did Grau *et al.* (1982) (80.4 mg/l).

It is possible that the higher osmotic pressure and/or decreased medium calcium used in the present experiment could be inhibiting release in cAMP stimulated prolactin cells, and that at lower osmotic and/or higher calcium levels this cAMP stimulation could be translated into increased synthesis and release. The role that this high medium calcium could have in a cAMP stimulated cell is shown in vasopressin release. Matheson and Lederis (1980) report that vasopressin release from the neural pituitary lobe is coordinated by cAMP and calcium. Intracellular cAMP appears to regulate the sensitivity of the release mechanism to Ca^{++}. This cAMP dampening of secretory response may be overcome by increased concentrations of extracellular calcium in the medium, although extracellular calcium *per se* does not effect basal vassopressin secretion.

As mentioned earlier, cAMP and cGMP may share some phosphodiesterases and the large concentration of db-cAMP used here (6 mM) might interfere with cGMP metabolism. The db-cAMP concentrations of 6mM is the same as that used by McKeown *et al.* (1980) and in the same range as Grau *et al.* (1981). While these concentrations may seem high, as pointed out by Swennen and Denef (1982), tissue incubations would be expected to be less sensitive to treatments than would individual cells.

It has been suggested that db-cAMP could produce a "butyrate effect" as opposed to acting as an analogue of cAMP (Dannies *et al.*, 1976). However Maurer (1982) found no prolactin response to butyric acid in rats, and Grau *et al.* (1982) reports

no altered release for millimolar quantities of butyric acid for up to 20 hours, while the same concentration of db-cAMP produced dramatic elevations.

A biphasic response to Ca^{++} by adenylate cyclase has been noted in several cell free preparations (Mahaffee and Otjes, 1980, Piascik et al., 1980). Low levels of Ca^{++} stimulate adenylate cyclase while higher concentrations inhibit this enzyme. Deery (1975) reports that adenylate cyclase in the RPD of the goldfish, Carassius auratus, is inhibited in vitro by Ca^{++} in the medium, with maximum inhibition occurring between 275 and 550 mg/l Ca^{++} Thus, it is possible that the higher Ca^{++} levels of McKeown et al. (1980) and Grau et al. (1981) could have inhibited endogenous cAMP production.

Chlorpromazine

Antipsychotic phenothiazines such as chlorpromazine have been shown to elevate serum prolactin levels in vivo (Ben-David et al., 1971; Lu et al., 1970; Takahashi et al., 1979) and in vitro (Clement-Cournier et al., 1977; Heindel and Clement-Cournier, 1981). However, the mechanism of action of chlorpromazine and other phenothiazines is not yet clear.

Chlorpromazine is a lipophillic drug that has many reported actions that could influence prolactin production. It has been reported as a dopamine receptor antagonist (Horne and Synder, 1971) although this has been questioned (Meltzer et al., 1979; Le Pur et al., 1980). Chlorpromazine is also a potent inhibitor

of the ubiquitous calcium receptor protein calmodulin (Levin & Weiss 1976) but there is some doubt as to its selectivity and effectiveness in whole cell treatments (Roufogalis, 1981; Feinstein & Hadjian, 1982).

This present work shows that chlorpromazine can increase ^{3}H-prolactin in both tissue and medium while decreasing ^{45}Ca accumulation. If this decrease in tissue ^{45}Ca represents decreased calcium influx, it would be difficult to attribute increased ^{3}H-prolactin release to a calmodulin effect, since Ca^{++} activates calmodulin. However, there is considerable evidence that intracellular calcium rearrangements without net influx can cause release of TSH (Gershengorn, 1980; Fleckman et al., 1981) and prolactin (Moriarty and Leuschen, 1981). The phenothyiazine trifluoperazine has been suggested to cause intracellular Ca^{++} release in platelets (Feinstein and Hadjian, 1982), and Dhalla et al. (1980) suggest that chlorpromazine inhibits microsomal Ca^{++} binding and uptake, probably by inhibiting Ca^{++}-ATPases. However, a mechanism of action for elevated cytosolic calcium is difficult to envision since both calmodulin and protein kinase C (Takai, 1979) are also inhibited by chlorpromazine. Since calcium efflux from chlorpromazine-treated tissues was not measured, it is possible that massive release of intracellular calcium is "flushing out" any ^{45}Ca influx.

Even if the three commonly described protein kinases are considered, it is difficult to assign them a stimulatory role in

chlorpromazine stimulated prolactin release. Protein kinase C, stimulated by calcium and phospholipid, is inhibited by chlorpromazine. Db-cAMP and db-cGMP presumably would stimulate protein kinases A and G respectively; however, as described earlier, there was no increased release with these treatments, although db-cAMP clearly increased synthesis. Moreover, chlorpromazine might be expected to inhibit the phospholipases (Feinstein and Hadjian, 1982) necessary to generate the lipid catabolic products thought to be necessary for guanylate cyclase activity (see cGMP discussion).

If no intracellular mechanism can readily account for chlorpromazine effects, perhaps membrane reactions are causing increased ^{3}H-prolactin release.

Prostaglandins have been suggested to release prolactin (Betteridge, 1980) but the phospholipase A^2 necessary to generate prostaglandins is reported to be inhibited by phenothiazines (Feinstein and Hadjian, 1982).

Another possibility is that the membrane lipid composition is changing due to chlorpromazine sensitive lipid metabolizing enzymes (Allan and Michell, 1975; Brindley and Bowley, 1975; Bowley et al., 1977; Sturton and Brindley, 1977; Plantavid et al., 1981). The net effect in lipid composition in response to chlorpromazine would be an accumulation of phosphatidate, a decrease in the number of neutral lipids such as triglycerides and phosphotidylcholine and an increase in the acidic phospholipids such as phosphatidylinositol. These

chlorpromazine-induced lipid composition changes in membranes may be important in modifying the calcium binding ability of the membrane and possibly the secretory activity of the membrane.

Membrane "deactivation" by calcium displacement from plasma membrane phospholipids is the suggested mechanism of action of local anaesthetics (see Papahadjopoules, 1972). These local anaesthetics work by interacting with acid phospholipids such as phosphatidylinositol, rather than neutral phospholipids such as phosphatidylcholine, and stabilize the membrane, increasing electrical resistance and raising membrane potential (Feinstein, 1968).

Membrane "activation" associated with increased membrane calcium binding is found in prolactin secreting GH cells stimulated with TRH (Leushen et al., 1983). Prolactin secretion stimulated by depolarizing concentrations of K^+ also show an increase in plasma membrane calcium (Leuschen et al., 1981).

Thus, chlorpromazine may modify membrane calcium binding characteristics, aided perhaps by a redirection of de novo phospholipid synthesis (Allen and Michell, 1975), although the lower ^{45}Ca accumulation of chlorpromazine-treated tissue seen in the present experiment seems to argue against a "high calcium" state of the chlorpromazine-activated cell. However, this lower ^{45}Ca accumulation of chlorpromazine treated tissue could be a result of altered ion flux rather than lowered ion binding.

There are at least four suggested mechanisms for calcium gates that involve phosphatidylinositol turnover (Michell, 1975,

Thompson Jr., 1980; Michell, 1982; Axelrod and Hirata, 1982) and, as described above, the enzyme reactions associated with acidic phospholipids are sensitive to chlorpromazine. Evidence that a phenothiazine can inhibit a Ca^{++} gate is provided by Fleckman et al. (1981) who report that trifluoperazine blocks K^{+}-induced Ca^{++} influx in TSH cells.

As cautioned by Sturton and Brindley (1977), drugs such as chlorpromazine interact with acidic phospholipids and therefore, any enzyme involved with these lipids are potential target sites, and any effects may depend on which enzyme is rate limiting and this may vary with tissue and physiological conditions.

^{45}Ca efflux and db-cAMP

Preliminary washing out of ^{45}Ca loaded RPD's showed that there was a very large (>50%) amount of ^{45}Ca that was lost in the first ten minutes of washing, most of this presumably extracellularly bound. Moriarity (1980) reported kinetic analysis to suggest three Ca^{++} compartments in rat anterior pituitary slices. The fastest of these was bound in the extracellular matrix. The second compartment was intracellular and the third, characteristic of mitochondria or microsomes. In the present study, soaking the tissues for five minutes in EGTA-Ca buffer (Aaronson et al., 1979) seemed to remove most of the superficially bound Ca^{++}, although the rapid loss between two and six minutes showed that there was still some easily

removed ^{45}Ca present. (Fig. 9)

Yancey et al. (1980) report that antimonate depositions examined by EM show that db-cAMP caused lower mitochondrial calcium levels after 30 minutes. But such studies cannot answer whether this is a direct or indirect effect of db-cAMP and whether this is an inhibition of mitochondrial uptake or stimulated efflux.

However, studies using mammalian liver, mammalian kidney or fly salivary gland tissue all show that cAMP causes prompt efflux of Ca^{++} (Rasmussen, 1981). He made the point that a small efflux of Ca^{++} from either the mitochondria or the endoplasmic reticulum would cause cytosolic levels to rise and that a rise in cytosolic Ca^{++} concentration per se increased an influx of calcium into some cells. That this "Ca^{++} trigger" mechanism of elevating cytosolic ionized levels is necessary for prolactin release would contradict several recent studies that have shown that while extracellular Ca^{++} is necessary for release, a large Ca^{++} influx need not occur (Moriarty and Leuschen, 1981; Eto et al., 1974; Milligan and Kraicer, 1979; Gershengorn, 1981).

Using a calcium containing buffer for efflux washing as I have done does not indicate whether db-cAMP causes efflux independently of an influx. The ^{45}Ca efflux response could be calcium displaced by a large cold influx. Influx studies (see Influx Section) show that there is at least some cadcium accumulation associated with db-cAMP.

E. General Discussion

Using radioisotopes, I find little evidence of stimulus-secretion coupling in teleost prolactin cells at physiological concentrations of ionized calcium in the medium. However this does not discount a role for calcium in prolactin secretion. As Triggle (1980) pointed out, there is a practical division of calcium affects between membrane related and intracellular. Intracellular affects might involve a regulatory protein such as a kinase or calmodulin, possibly regulating cyclic nucleotide metabolism. Membrane calcium might regulate such things as membrane charge, membrane fluidity, and modulation of ion channels and pumps.

There is considerable evidence, both mathematical and experimental, that there is a distinct "domain" for calcium influx that is limited to the immediate proximity of the plasma membrane (see Matthews, 1979). Thus an influx of calcium can effectively elevate cytosolic levels near the membrane, and as calcium diffuses further into the cytoplasm, it is rapidly sequestered. This restricted "domain" is even more significant when the calcium source is transient, such as the discrete spikes of electrical activity of prolactin cells.

This rapid attenuation by the cytoplasm of a calcium signal becomes even more complicated for polar cells (see Rasmussen, 1979) such as secretory cells that are stimulated at one end and

secrete at the other. As discussed earlier (see Introduction) prolactin cells seem to be distinctly polar.

Various possible mechanisms have been suggested for preventing calcium signal loss, among these is calcium "canals" or passages in the cell relatively free of sequestering processes (Matthews, 1979) or possibly a "second messenger" such as Na^+ or Cl^- that cause release of calcium from an intracellular source (see Rasmussen, 1979). Nevertheless, using a simple version of stimulus-secretion coupling, it would be difficult to elevate cytosolic calcium.

On the other hand there is evidence that calcium interaction does occur at the membrane level in stimulated and secreting prolactin cells (see preceeding antimonate discussion). It has been proposed that calcium may diminish the electrostatic repulsion between plasma membrane and secretory granule membrane (Dean, 1975), and this association of calcium and secretory granules close to the plasma membrane has been reported (Schechter, 1976; Cramer et al., 1978). However granules from stimulated prolactin cells are reported to have dramatically reduced levels of membrane calcium (Leuschen et al., 1981). This granule membrane effect may be related to the reports of receptors for regulating factors on prolactin granules (Dular and La Bella, 1977; Hansel et al., 1981). Thus, the interactions between chlorpromazine and membrane lipids (see chlorpromazine - Discussion) could be mimicking physiological events in prolactin secretion rather than interacting with

intracellular components of cellular activation that are regulated by calcium.

F. Conclusion

Flux studies, such as this present one, are only one aspect of the role of calcium in cell activation and cannot provide a complete picture since they are complicated by calcium pools, turnover rates and response times. Until comprehensive studies allow the intersection of several "lines" of evidence at the same time, these complex coordinated calcium responses will not be linked satisfactorily. For example, in response to drugs and/or natural regulators, prolactin cells could be examined by EM and antimonate procedures to identify calcium pools, ^{45}Ca and fluorescent probes can be used to estimate turn over of calcium in these pools, and electrophysiology and membrane probes can be used to examine membrane electrical activity.

In summary, I have shown that although prolactin cells are sensitive to extracellular calcium, stimulus-secretion coupling does not necessarily operate in teleost prolactin cells, at least in a direct manner. I have shown that ^{45}Ca accumulation is not necessarily related to increased secretion and that increased secretion does not necessarily require a calcium influx. Although this latter effect is due to "non-physiological" chlorpromazine, it does emphasize the importance of membrane events on prolactin release.

APPENDIX

Puck's Saline A

KCl	0.40	g/L
NaCl	8.00	g/L
$NaHCO_3$	0.35	g/L
Glucose	1.00	g/L
phenol red	0.005	g/L

Acid Electrophoresis Gel

Solution 1:

43.2% (v/v) glacial acetic acid and 4% (w/v) TEMED (N,N,N',N'-tetramethyl-ethylenediamine)

Solution 2:

28.0% (w/v) acrylamide and 0.735% (w/v) N,N-methylene-bis

Solution 3:

10 M urea

1 part solution 1 : 3 parts solution 2 : 4 parts solution three

gels polymerized with 200 µl of riboflavin phosphate (0.16% w/v).

Sample Buffer

0.9 M acetic acid

5.0 M urea

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