# EFFECT OF INDOMETHACIN (A PROSTAGLANDIN INHIBITOR) ON THE OVULATORY CYCLE, TOTAL PLASMA CALCIUM, AND EGG SHELL THICKNESS IN THE DOMESTIC LAYING HEN (GALLUS DOMESTICUS)

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ABSTRACT

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By

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Indomethacin (Indocin), 1-(p-chlorobenzoyl)-5-methoxy-2-methylimdole-3-acetic acid, a prostaglandin inhibitor, was administered to Single Combed White Leghorn laying hens to determine the effect of this drug on the reproductive cycle and total plasma calcium.

Hens, 18 to 20 months of age, with a 3 to 6 egg laying sequence, with 1 skip day and no history of soft-shelled or thin-shelled eggs, were individually caged and fed a commercial layer ration and water ad libitum. They were subjected to a 17.5 hr light and 6.5 hr dark photoperiod (lights off 2300 to 0530 hr), with times of oviposition being recorded.

Oral administration of indomethacin (50 mg/kg BW) given at 2300 hr the evening of the skip day in Experiment 1, significantly delayed the time of oviposition of the first egg in the sequence (5.0 to 17.5 hr), when 7 hens

were used as their own controls ($p < 0.01$). The second egg was prematurely expelled ($p < 0.01$), but no significant effect on time of oviposition of the third egg was observed. Luteinizing hormone (LH) release or ovulation was apparently not blocked by indomethacin as a second egg was laid by all birds. However, 2 of 7 birds did not lay a third egg.

The absorption and clearance of indomethacin from the plasma were determined at 50 and 100 mg/kg BW dosages, in Experiment 2. Indomethacin levels were determined by a spectrophotofluorometric technique. The drug was present in the plasma 1 hr after administration, reached maximum levels at 2 hr, steadily dropped to very low levels up to 18 hr and was cleared from plasma at 32 hr. The disappearance curves for the two-dose levels were found to be parallel up to 18 hr, after statistical analysis by split-plot method, and a dose-response effect was evident.

Luteinizing hormone was injected on the morning of the last egg in the sequence, in several experiments, in which each bird was its own control. This LH injection added another egg to the sequence, and before and after drug administration responses were observed.

Indomethacin (50 mg/kg BW), 1 hr before LH injection, caused a delay in oviposition ($p < 0.01$) of the last egg by 9.7 to 42.2 hr (Experiments 3 and 4). Most of these eggs had thicker egg shells, when compared to their own

control. Premature expulsion of the LH-induced egg was confounded with the amount of shell deposition that occurred. However, eggs which were expelled at the control time ($\pm$ 2 hr), were thinner than controls ($p < 0.01$). Total plasma calcium was lowered by approximately 30% from controls at 11 hr to at least 32 hr after indomethacin.

In Experiment 5, indomethacin (50 mg/kg BW) administered 1 hr after the LH injection, did not block ovulation in any of the 7 birds, as compared to their own control.

Two other experiments investigated the effect of indomethacin on the time of LH injection to the presence of a soft-shelled egg in the shell gland. In Experiment 6, a subcutaneous injection of prostaglandin $F_2$ (50 $\mu$g/kg BW) 7 hr after indomethacin, expelled the last egg just prior to ovulation of the next follicle. In Experiment 7, $PGF_{2\alpha}$ was used to prematurely expel the last egg in both the control and treatment period at 0900 to 1000 hr prior to LH injection. Birds were digitally palpated for the time of arrival of the soft-shelled egg after placebo or indomethacin at 15 min intervals. Following indomethacin administration, the time from LH injection to the appearance of a soft-shelled egg in the shell gland tended to be reduced (0.1 to 2.2 hr) in both experiments.

It is concluded that prostaglandins probably do not have an essential role in the processes of LH release or ovulation, as previously shown in mammals. However, these substances appear to have a role in the process of oviposition and may play a role in shell formation.

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to Lance and Christina

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# INTRODUCTION

In recent years, there has been a great deal of literature indicating a role for prostaglandins in several aspects of mammalian reproduction. The majority of this research deals with their smooth muscle stimulatory effect; although these biologically active fatty acids may also be acting as modulators of hormone actions.

Research suggesting a role for prostaglandins in the reproductive physiology of the bird is limited to just a few articles. These studies primarily deal with their possible role in ovum transport and oviposition (egglaying). There are no studies examining their possible role in gonadotropin release or the process of ovulation in the bird.

In addition, there is evidence that prostaglandins may play a role in calcium metabolism in mammals. Since the regulation of calcium in the bird is extremely important during the shell-forming phase of the ovulatory cycle, an investigation of the possible role of these substances in this phenomenon could be meaningful.

Several biologically active forms of prostaglandins are available for experimental and clinical investigation, as well as a large number of inhibitors and antagonists to

their biosynthesis.

In the following research, the prostaglandin synthetase inhibitor indomethacin was administered orally to laying hens to determine the effect of this drug on several events in the laying cycle and on total plasma calcium. Exogenous prostaglandin $F_{2\alpha}$ was also administered to overcome the indomethacin block of oviposition and as a tool to determine the effect of this inhibitor on ovum transport.

# REVIEW OF LITERATURE

## Indomethacin and Prostaglandins

Indomethacin (Indocin), 1-(p-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid, is one of the non-steroidal anti-inflammatory drugs which has been shown to block the synthesis of prostaglandins (Vane, 1971). This experimental evidence offered support to the concept that the therapeutic effects of aspirin-like drugs could be ascribed to such properties. Smith and Lands (1971) reported on the mechanism of this inhibition and have shown that indomethacin inhibits the deoxygenase enzyme of the sheep vesicular system in a time-dependent, concentration-dependent manner, irreversibly blocking the full activity of the synthetase system. Their work demonstrated that the block in biosynthesis occurs at a very early biochemical step, and thereby, shuts off the formation of all products--including all prostaglandins. The discovery that indomethacin and many other inhibitors interrupt the biosynthetic pathway at a specific point, has allowed them to be applied as useful tools to elucidate the physiological actions of prostaglandins.

Prostaglandins are 20-carbon carboxylic acids which

are formed enzymatically from polyunsaturated fatty acids. Pike (1972) has reviewed the chemistry of these biologically active fatty acids and many of the methods used in synthesizing them. In this discussion, he points out that these substances are found in the tissues of many species throughout the animal kingdom--coral to man.

## Possible Role of Prostaglandins in Several Aspects of Mammalian Reproduction

The role of prostaglandins in gonadotropin secretion has been postulated. Labhsetwar (1970) administered high doses of prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$) to pregnant rats and found a several fold increase in the amount of stored luteinizing hormone (LH) in the pituitary. Further work (Labhsetwar, 1971, 1972) showed that $PGF_{2\alpha}$ induced ovulation in pregnant rats and hamsters--the latter study showing a significant increase in LH in the blood after administration. Orcyzk and Behrman (1972) provided additional evidence for this possible role when they reported the blockage of ovulation by aspirin and indomethacin in the rat. In addition, Carlson et al. (1973) also observed an increase in serum LH after intracarotid infusion of $PGF_{2\alpha}$(6 $\mu$g/hr) during the luteal phase of cycling ewes. In contrast, Batta et al. (1974) presented data that suggest $PGF_{2\alpha}$, when given intracerebrally to unanesthetisized female rats, partially inhibits ovulation, whereas PGE, does not exert any effect. It is apparent from this work

as well as other examples, that the prostaglandins' actions vary greatly between species. The exact physiological role of prostaglandins in gonadotropin release, if any, is not clear (Goldberg et al., 1975). Some of these effects may be pharmacological and not physiological.

Armstrong and Grinwich (1972) concluded that prostaglandins were also involved in the process of ovulation, in that orally fed indomethacin was able to block the action of LH at the level of the ovary in the rat. Tsafriri et al. (1972, 1973) confirmed this block by indomethacin, showing that this drug does not interfere with LH release, but interferes with a late phase of the ovulatory cycle, in that it prevents follicular rupture and not ovum maturation in the rat. This indomethacin-induced ovulatory block was later shown to occur in mice (Lau et al., 1974). Grinwich et al. (1972) further demonstrated that LH-induced steroidogenesis was not interfered with in this indomethacin-blocked follicular rupture in the rabbit. More recently, prostaglandins E and $F_{2\alpha}$ have been shown to be involved with, and essential to, the normal process of ovulation in the rat and rabbit (Armstrong et al., 1974; Marsh et al., 1974). Marsh et al. (1974) reported that both LH and cyclic AMP increased prostaglandin synthesis in rabbit Graffian follicles in vitro.

One of the first, and possibly the most important biological activity of prostaglandins to be observed was the stimulation of smooth muscle (Kurzrok and Lieb, 1930). Bygdeman (1964) studied this activity further on human uterine smooth muscle, and this pharmacological principle is still being evaluated. Subsequent studies elucidating the role of prostaglandins in mammalian uterus and oviduct are indeed voluminous (for reviews see: Southern, 1972; Goldberg et al., 1975; Karim, 1975). The exact mechanism of action of prostaglandin smooth muscle stimulation is unknown. However, Carsten (1972, 1973) suggests that prostaglandins may be involved in the regulation of intracellular calcium transport in causing their effect.

In 1974, Csapo proposed that the mechanism of action of prostaglandins in inducing abortion (clinically or spontaneously) involves the immediately induced contraction of the myometrium followed by progesterone withdrawal, due to their luteolytic effect. Phariss and Wyngarden (1969) were the first to report the luteolytic activity of prostaglandins ($PGF_{2\alpha}$) in pseudopregnant rats. This phenomenon was later to be reported for the guinea pig (Blatchley and Donovan, 1969), rabbit (Phariss, 1970), sheep (McCracken et al., 1970), and hamster (Gutknecht et al., 1971; Labhsetwar, 1971). McCracken et al. (1971) demonstrated that tritiated $PGF_{2\alpha}$ may be transferred

directly from the uterine vein into the ovarian artery, which lie in close apposition in the sheep, and travel to the corpus luteum, where interference with progesterone synthesis occurs. As a result, the progesterone effect in maintaining a quiescent state of the uterus is overcome (Csapo, 1956). Hansel et al. (1973) demonstrated that estrogens are luteolytic in cows and ewes, and that this group of hormones is capable of stimulating prostaglandin synthesis in the uteri of cows and guinea pigs. In the sheep the onset of labor was preceded by an increased level of estrogen, and Caldwell et al. (1972) have shown that estradiol infusion into pregnant women at term produced an increase in uterine activity, but this was not sufficient to achieve delivery. During the 4 hr infusion periods, there were no changes in the plasma levels of progesterone or PGF. There are undoubtedly many between-species variations as to the effects of these biologically-active fatty acids.

Labhsetwar (1975) has discussed the possible role of prostaglandins in parturition and the general uterine physiology of laboratory animals, and suggests that data obtained by using various approaches strongly implicates prostaglandins in these areas. Ham et al. (1975) suggest that estrogen controls prostaglandin synthesis by regulating the prostaglandin synthetase complex in the uterus of

ovariectomized rats.

Karim and Hillier (1975) summarized the evidence implicating prostaglandins in the physiology of labor and spontaneous abortion in humans as being based on: "(a) Presence of prostaglandins in maternal blood obtained during labour; (b) Presence in intrauterine tissues and fluids; (c) The ability to stimulate pregnant human uterus; and (d) Delay in onset of labour and prolongation of active labour by prostaglandin antagonists and synthesis inhibitors." Because of these implications and findings, exogenous prostaglandins (particularly $PGF_{2\alpha}$ and $PGE_1$ ) are being used clinically as abortifacients, to terminate early and late stage pregnancies, as well as to enhance normal labor at term.

There appears to be a relationship between prostaglandins and oxytocin in their abortifacient effect. The uterotonic effect of oxytocin in humans is negligible during the first half of pregnancy, but it is markedly enhanced after exposure of the myometrium to prostaglandins (Brummer, 1971; Embry, 1971). A review of this relationship by Karim and Amy (1975) points out that prostaglandins and oxytocin appear to work synergistically when given to women in the second trimester of pregnancy. Sharma and Fitzpatrick (1974) demonstrated that relatively large doses of oxytocin (400-10,000 IU) administered as bolus intravenous injections, will elicit elevated

posterior vena caval PGF in estrogen-primed anestrous sheep, whereas, there was no effect on the untreated anestrous control animals. Roberts et al. (1974) have shown that the manipulation of the uterus in the sheep also caused PGF release. From these results, it is not clear, whether the effect of oxytocin in increasing PGF is a metabolic action or a stimulatory effect due to uterine contractions. The exact relationship between oxytocin and prostaglandins remains unclear. However, Novy et al. (1974) showed that indomethacin prolonged normal gestation in the rhesus monkey, but the uterus was still responsive to exogenous oxytocin.

Prostaglandins, with their potent smooth muscle stimulation activity, are also believed to be important in the mammalian oviduct. Spilman and Harper (1974) have reviewed the relationship of prostaglandins to oviduct motility and suggest a physiological role for prostaglandins in the passage of fertilized ova through the oviduct. In discussing this subject, Goldberg and Ramwell (1975) state: "In all species studied, PGE inhibited the spontaneous contractions of the fallopian tube while PGF stimulated tubal contractility." Prostaglandin treatment was either shown to retard or accelerate ova transport depending on individual species. This stimulatory or inhibitory effect of prostaglandins may also implicate them as having a functional role in sperm

motility and implantation. Lau et al. (1973) showed evidence for a role of endogenous prostaglandins in normal tubal-transport of ova, in that administration of indomethacin (225 μg/animal) to mice on day 2 of pregnancy, prevented implantation.

In summary, prostaglandins have been implicated as having a possible physiological role in mammalian reproduction by acting as hormone modulators and/or smooth muscle stimulators. The actions of these biologically active fatty acids may be at the level of the hypothalamus and pituitary (gonadotropin synthesis and release); the ovary (necessary for ovulation and luteolysis); the oviduct (causing motility in sperm and ovum transport); and the uterus (regulation of uterine contractions throughout the reproductive cycle, particularly during spontaneous abortion and normal parturition).

## Possible Role of Prostaglandins in Several Aspects of Avian Reproduction

Historically, the presence of endogenous prostaglandins and the actions of exogenous prostaglandins in avian species were limited to the domestic fowl (for review see Horton, 1971). Early studies revealed the presence of $PGE_2$ and $PGF_{2\alpha}$ in the central nervous system of adult fowl, and the administration of exogenous prostaglandins would cause such effects as sedation or temporary paralysis

in the chick. Prostaglandins were also shown to cause vasodilation and vasoconstriction in the cardiovascular system of the chick, presumably through their smooth muscle stimulatory effect on the blood vessels. More recently, Christ and Van Dorp (1972) demonstrated the presence of the prostaglandin synthetase enzyme system in the ovaries and oviduct of the hen and the seminal ducts and testis in the cock, as well as in many other non-reproductive avian tissues.

Hertelendy (1972) was the first investigator to report the smooth muscle stimulatory effect of prostaglandins in the reproductive system of birds. This study showed that intrauterine injection of prostaglandins $E_1$, $E_2$, $A_1$, $A_2$, $F_1$ and $F_{2\alpha}$ all induced premature egglaying (oviposition) in the coturnix (Japanese) quail. Of these prostaglandins, $PGE_1$, was the most potent--in very small amounts (5 ng per bird). This research also demonstrated that the avian uterus was capable of synthesizing endogenous prostaglandins in that endogenous precursors (arachdonic acid and phospholipase A) were able to cause premature oviposition--although the increase in PG's was not measured directly. The action of these precursors was blocked by intrauterine injections of indomethacin (0.1 mg), whereas twice this amount of inhibitor did not block the actions of exogenous $PGE_1$. Hertelendy (1973) extended this work to show that there is a possible relationship of

oxytocin and prostaglandins in oviposition, by reporting that oxytocin-induced oviposition was blocked by indomethacin and 5, 8, 11, 14 -eicosatetraynoic acid (another inhibitor of prostaglandin synthesis). However, these two inhibitors were unable to block the actions of $PGE_1$, in expulsion of the uterine contents, thereby suggesting a role for prostaglandins in spontaneous oviposition. Dibutyryl cyclic AMP, linoleic acid and human seminal plasma (shown to have large amounts of prostaglandins) were also shown to be capable of inducing oviposition in the quail, while administration of fowl, turkey, bull, boar, and stallion seminal plasma were ineffective agents (Hertelendy, 1974).

The hypothesis that prostaglandins have a role in oviposition was later extended to the domestic hen (Hertelendy et al., 1974). Prostaglandins $E_1$, $E_2$, and $F_{2\alpha}$ as well as their fatty acid precursor--arachidonic acid--all induced premature oviposition. Further observations showed that the hen was more responsive to $PGE_1$ 1-3 hours before the expected time of oviposition (hard-shelled egg in the shell gland) than 15-18 hours from this time (soft-shelled egg in shall gland) (Hertelendy et al., 1975). This effect, however, could not be attributed to lowered levels of the steroid hormone progesterone, as has been hypothesized in mammals, as the circulating levels of this hormone in the plasma were higher when there was a

hard-shelled egg in the uterus.

A possible role of prostaglandins in the physiology of the avian oviduct has also been suggested. Wechsung and Houvenaghel (1975 a, b) reported that $PGF_{2\alpha}$ and $PGE_2$ had a stimulatory effect on the muscle tone of circular uterine strips and that $PGF_{2\alpha}$ causes contractions of circular smooth muscle strips from other oviduct segments. These researchers later reported that $PGF_{2\alpha}$ provoked contractions of longitudinal tissue strips from uterine, vaginal and isthmus regions of the chicken oviduct (Wechsung and Houvenaghel, 1976). No response was induced in longitudinal tissue strips from the magnum and infundibulum. In contrast, $PGE_2$ induced contractions of circular and longitudinal uterine strips and circular strips from the isthmus and infundibulum. $PGE_2$ caused relaxation in both the circular and longitudinal vaginal strips. Circular magnum strips contracted to low doses of $PGE_2$ and relaxed to high levels, however, there was no response to this prostaglandin in longitudinal muscle strips from the isthmus, magnum and infundibulum. In vivo experiments by these researchers (Wechsung and Houvenaghel, 1975c) showed that intravenously administered $PGF_{2\alpha}$ and $PGE_2$ induced a dose-dependent pressure increase in the magnum and isthmus portion of the oviduct, in anesthetized laying hens as measured by a water-filled

balloon and pressure transducer. It was noted that by using $PGF_{2\alpha}$ and oxytocin as alternate intravenous injection--on a molar basis--that oxytocin was approximately 1.5 times more potent than $PGF_{2\alpha}$ in the blood. In addition to the above investigations, Verma et al. (1976) tested the effect of $PGE_1$, on oviduct segments from infundibulum, magnum, uterus, uterovaginal junction and the vagina of actively laying hens at preoviposition time by in vitro and in vivo methods. This study reported a maximum stimulatory response (contraction) from muscle strips from the infundibulum and a complete relaxation was recorded from vaginal strips. At the level of the uterovaginal junction, there was a difference in in vitro and in vivo studies--a mild prolonged inhibitory response in vitro and an increase in intraluminal pressure (contraction) in vivo. From this study, these researchers hypothesized that $PGE_1$ causes contraction of the uterus, while at the same time causes relaxation of the uterovaginal junction during normal oviposition.

Talo and Kekalainen (1976) approached the question of how ovum transport in the oviduct occurs by recording the electric activity of Japanese quail oviducts in vitro. In this study, six flexible suction electrodes were placed at various intervals along the oviduct and the electric activity was recorded as the ovum moved through this area. These researchers concluded that the oviduct

promotes its own transport in that as it progresses through the oviduct, it causes excitation of the smooth musculature. It was hypothesized that distension caused the ovum to initiate a positive feedback cycle in which the rate of transport is determined by maximum activity achieved behind the ovum and the steepness of the frequency gradient. Excitation was believed to be caused by the direct effects of mechanical distension and it was suggested that prostaglandins may be involved in this process in that stretch has been shown to enhance prostaglandin synthesis and release (Poyser et al., 1971). It should be pointed out, however, that prostaglandin levels have not been measured in the avian oviduct during transport.

The above research suggests that prostaglandins may play a physiological role in ovum transport and oviposition in the bird, however, no studies have investigated the possible role of these biologically-active fatty acids in gonadotropin release or the complex process of ovulation in these species. In addition, there have been no studies reporting the effect of inhibitors of prostaglandin synthesis on ovum transport (through the oviduct), or the effect of these compounds on other aspects of the ovulatory cycle.

## Prostaglandins and Calcium Metabolism

Another area of possible involvement of prostaglandins in the bird and the mammal is calcium and bone metabolism. This subject was reviewed by Dietrich and Raisz (1975). Briefly, it has been demonstrated that a wide variety of prostaglandins are capable of stimulating bone resorption in tissue culture, as determined by the release of previously incorporated $^{45}Ca$ from fetal rat long bones. $PGE_2$ was found to be the most potent in its action. The morphological effects of prostaglandins on bone were qualitatively similar to those produced by other resorbing agents (parathyroid hormone, active metabolites of vitamin D and osteoclast activating factor). Though the effects of prostaglandins on bone formation are not consistent, it appears that $PGE_1$ and $PGF_{1\alpha}$ stimulate, and possibly others are involved in amino acid uptake and protein synthesis. However, $PGE_2$ actually decreased collagen synthesis *in vitro*, suggesting that some prostaglandins may inhibit bone formation. This work suggested a hypercalcemic effect of prostaglandins. This was later shown to be the case in that prolonged intravenous infusion of $PGE_2$ was able to cause hypercalcemia in the rat. Administration of indomethacin to hypercalcemic mice and rabbits decreased serum calcium and $PGE_2$, as well as the $PGE_2$ content of bone tumors. These reviewers conclude that prostaglandins may play a role in bone resorption and formation, as well as

in pathological changes in bone even though their physiological role is unknown.

Considering the extremely high turnover rate of calcium in the domestic laying hen and the importance of calcium to egg shell formation (see following review of reproductive cycle of the hen), this researcher decided that plasma calcium levels should be determined after indomethacin administration.

## The Laying Cycle of the Domestic Hen

The "laying cycle" or "ovulatory cycle" of the domestic hen has been reviewed quite extensively (see General Bibliography). This description will deal with the general aspects of this cycle, with specific attention given to those areas that will be dealt with in the following research. Only specific reference will be made to publications whose detail are considered to be important to this study.

The "laying cycle" of the hen is relatively short in duration, lasting about 25-26 hr from the time of ovulation to the time of ovipositing (laying) of the egg. Hens will lay one or more eggs on consecutive days (sequence or clutch) and then end this daily phenomenon with one or more "skip" or "pause" days (on which no egg is laid), and then start a new sequence.

The endocrine control of the laying cycle is fairly

well understood, even though some aspects of it are still unclear. Egg production begins when a hen reaches sexual maturity and follicular growth begins--apparently due primarily to the release of follicle stimulating hormone (FSH) from the anterior pituitary lobe. There is a gradation in the maturation of these developing follicles in that several of varying sizes exist at one time and grow at different rates. The "egg formation phase" begins with the ovulation of the largest follicle (in most cases), which appears to be due to luteinizing hormone (LH)--being released from the anterior pituitary some 6 to 8 hr prior to this event. Follicular rupture is preceded by the formation of the "avascular-looking" stigmal area and apparent reduction in blood flow. It is possible that this sudden change in this preovulatory follicle is partially caused by prostaglandins via their smooth muscle stimulatory capabilities or other mechanisms, in that they have been previously shown to be necessary in the process of follicular rupture in several mammals (as discussed above).

Immediately after ovulation, the ovum is engulfed by the infundibulum (funnel) and spends the following periods of time in each portion of the oviduct (average figures from Warren and Scott, 1935): infundibulum, 0.3 hr; magnum, 2.9 hr; and isthmus, 1.5 hr. The average time reported by these researchers, for ovum transport down

the oviduct from the funnel to the shell gland, was 4.4 hr. After reaching the shell gland, the egg remains there during the shell forming process for a period of approximately 20.8 hr. These time periods would vary, depending on the length of the egg sequence, and other variations due to individual birds.

During the ovum's journey down the oviduct, the various layers of albumen are laid down in the magnum portion; the two shell membranes are formed and some water is added in the isthmic region; and in the shell gland (uterus), water and salts are added to the albumen before the deposition of the shell over the entire unit. In addition, the chalazae (paired twisted strands of albumen attached at opposite poles of the yolk, and parallel to the long axis) are formed by the mechanical twisting and segregation of the mucin fibers from the inner layer of albumen (General Bibliography, Sturkie, 1975). Apparently, the egg is rotated on its long axis in the isthmus and uterus to produce these strands.

The shell forming phase, during the egg's long duration in the uterus, is extremely complex and a great deal of information is known concerning it (see General Bibliography, Sturkie (1975)--for reviews and major articles). During this time, calcium carbonate deposition is the prevailing event. This deposition gradually increases

during the first 5 hr to approximately 300 mg/hr and this rate remains constant until about 2 hr before oviposition. Because of this high rate of deposition and the shell gland inability to store significant amounts of calcium, this ion must be constantly extracted from the blood--being derived from both the protein-bound and the diffusible calcium of the blood (Simkiss, 1967). Hodges (1970) showed that arteriovenous gradients in blood calcium can be detected between the sciatic artery and the inferior oviducal vein (draining part of shell gland) at approximately 2 hr after an egg enters the shell gland. This gradient ranged from 2-4 mg% between the fifth and the sixteenth hr of shell formation, and then decreased gradually. Sturkie (General Bibliography, 1975) points out that the process of shell formation withdraws an average of 100-150 mg calcium/hr from the blood of the laying hen, and that unless the concentration of calcium in the blood of the laying hen, and that unless the concentration of calcium in the blood were not continuously replenished through intestinal absorption and mobilization from bone, this concentration would be zero within 8-18 minutes.

Taylor (1972) suggests that requirements for calcium may be a limiting factor in egg production. In this review, he points out that there may be a link between calcium metabolism and ovulation. This is supported by

the fact that pullets fed a diet deficient in calcium (0.2%) ceased laying after five or six eggs had been produced and had atretic follicles whereas control birds given the same diet and injected with chicken pituitary extract laid an egg a day for at least 5 days after the first injection and their ovaries contained many growing follicles (Taylor et al., 1962). Taylor (1972) suggests that these results are due to a lowered ionic calcium in the plasma causing a reduction in gonadotropin secretion and reduced follicular growth, which in turn, would reduce the rate of estrogen secretion and lower egg production by lowering the rate of yolk synthesis in the liver. In addition, this reviewer states that the necessity to secrete large amounts of calcium for shell formation in some way limits egg production. In defense of this position, he cites experiments by Lake and Gilbert (1964) in which a surgical thread was placed in the shell gland of laying hens. These researchers showed that this operation caused the bird to lay thin or soft shelled eggs due to premature expulsion from the shell gland and to ovulate at a higher rate than the unoperated control.

The above research is interesting in that the reason for the premature oviposition and subsequent higher rate in ovulation may be directly or indirectly related to prostaglandin synthesis. In the mammalian uterus,

Eckstein (1970) suggests that the presence of the foreign intrauterine contraceptive devices (IUCD) causes increased uterine and tubal motility via an increase in prostaglandin synthesis. If this were the case in the birds with the surgical thread in the uterus, then prostaglandins may be the possible mechanism of the premature expulsion. In addition, if prostaglandins are involved with calcium mobilization in birds, as previously suggested in mammals, then via this mechanism an increase in ovulation rate could come about. Another possibility is the direct effect that prostaglandins may have an ovulation in the bird. However, in addition to Lake and Gilbert's (1964) experiments, Koga (1965) showed that insertion of a glass catheter in the shell gland resulted in premature expulsion of eggs, but in contrast a cessation of lay was the outcome. To date, a role for prostaglandins in regulating these events has not been established.

The immediate causes of uterine contraction and oviposition are not completely understood. However, most reviewers agree that the posterior pituitary hormone arginine vasotocin may be the initiator of oviposition. In addition, as previously discussed, prostaglandins may play an important role as an intermediate or work synergistically with vasotocin to induce oviposition. Other

agents such as acetylcholine, nembutal, and uterine distension may also cause premature oviposition, but it appears that these effects are indirect.

# OBJECTIVES

The purpose of this study was to investigate the effect of the prostaglandin synthesis inhibitor indomethacin on total plasma calcium and the following reproductive phenomena of the Single Combed White Leghorn (S.C.W.L.) laying hen:

1) Ovulation - as determined by the presence or absence of a soft-shelled egg in the shell gland after LH injection
2) Ovum transport - indirectly, by determining the time from LH injection to the presence of a soft-shelled egg in the shell gland
3) Oviposition
4) Egg shell thickness.

# MATERIALS AND METHODS

## General Procedures and Comments

The following experiments were conducted in a sequential manner in order to elucidate a possible role for prostaglandins in the physiological regulation of several reproductive events in the laying cycle of the hen. Since levels of prostaglandins were not measured directly, the amounts of inhibitor (indomethacin) were adjusted to levels tolerated by the animals and those demonstrating a dramatic delay in time of oviposition in the test animals.

Seventy Single Combed White Leghorn (S.C.W.L.) laying hens of a commercial strain, 18 to 20 months of age, were randomly assigned to treatment groups. The hens were housed individually in wire cages within three-tiered portable laying battery units within a windowless room with constant temperature (22 $\pm$ 1°C) and forced air circulation. All hens had a 3 to 6 egg laying sequence, with a 1 skip day and no history of soft-shelled or thin-shelled eggs. A commercial layer ration and water were given ad libitum. A 17.5 hr light and 6.5 hr dark photoperiod was maintained in which lights were automatically

turned off at 2300 hr and were turned back on at 0530 hr. Under this lighting schedule, most eggs were laid between 1000 and 1600 hr. Times of oviposition were recorded individually at 30 min intervals between 0900 and 1700 hr each day. These records were started at least one month prior to the beginning of the treatment periods.

## Experimental Design and Procedures of Individual Experiments

### Experiment 1

#### Rationale

This experiment was conducted in order to test the effect of indomethacin on the ovulatory cycle and normal sequence of laying hens. Previous research has shown that this drug will delay oviposition. Time of oviposition, effect on subsequent ovulations and general characteristics of the eggs after a single bolus of the drug were recorded.

#### Procedure

Indomethacin (Merck, Sharp and Dome, Rahway, N.J.) was administered orally (50 mg/kg body weight (BW) in gelatin capsules (No. 0, Eli Lilly and Company, Indianapolis, Indiana) to 7 randomly assigned hens at 2300 hr (just before lights-out) on the evening of their skip day. This was done by extending the bird's neck, placing the capsule into the mouth, and flushing the bolus down the esophagus with water. All birds were palpated manually

to confirm the presence of a hard-shelled egg in the shell gland at this time. The subsequent effects on the reproductive cycle were then monitored for the first 3 eggs in the following laying sequence. The effect of the drug on ovulation was determined by digital palpation--i.e., the presence of a soft-shelled or hard-shelled egg in the shell gland after oviposition of the first egg in the sequence was noted. In addition, the effect of indomethacin on the time of oviposition of the first 3 eggs in the present sequence was compared to the average oviposition time of these eggs in the 4 previous egg sequences, respectively. The time of oviposition was monitored at 30 min intervals during the light hr only, in order that no disruption of the photoperiods of these birds and other birds within this room would occur. Those eggs (soft or hard-shelled), which were found the following morning, were recorded as being laid at 0215 hr (midway through the dark period). When lights came on at 0530 hr, all the treatment birds were palpated digitally for the presence of a hard-shelled or soft-shelled egg in the shell gland and this information was recorded. General observations of the effect of the drug on the birds and the eggs laid were noted.

### Statistical Analysis

Student's t-test for paired observations was used for the statistical analysis of data. The fact that previous research (Hertelendy, 1972, 1973) and the experimental results showed a delayed-oviposition effect of the drug, a one-tailed test was used.

## Experiment 2

### Rationale

As will be shown subsequently, the results from Experiment 1 indicated that considerable variation in response to orally fed indomethacin existed between individual birds. In an attempt to reduce this variation, feed was removed from the birds several hr before the bolus was given. Levels of indomethacin in the plasma were also determined over time, in order to establish a dose-response.

In addition, the administration of 100 mg/kg BW dose of indomethacin to one treatment group was decided upon, because the 50 mg/kg level in Experiment 1 had failed to block ovulation of the second egg of the sequence, and in 5 of 7 birds did not block the ovulation of the third egg of the sequence. It was felt that a higher level of the drug may cause blockage of gonadotropin release and/or ovulation, as previously shown in mammals.

## Part 1: Determination of Plasma Indomethacin by Serial Bleeding

### Procedure

Two groups of 7 laying hens each, were given a single bolus of indomethacin (as previously described in Experiment 1) at levels of 50 mg/kg BW and 100 mg/kg BW, respectively, at approximately 1100 hr. Feed was removed from the birds just before lights-out of the previous evening (2300 hr) and returned one hr after drug treatment.* Ten heparinized blood samples (4 ml each) were then taken from the wing vein of each bird at the following time periods after drug administration: 0 (just prior to dosing--used for control), 1, 2, 4, 6, 8, 10, 18, 24, and 32 hr. The collected heparinized blood was immediately centrifuged (2600 g) in a refrigerated centrifuge for 10 min. The plasma was then pipetted into 12 x 75 mm stoppered test tubes and refrigerated at 4°C until the time of assay for indomethacin content--within 72 hr of collection.

The plasma samples were assayed by a slightly modified spectrophotofluorometric technique previously described by Hucker et al. (1966). This procedure is as follows:

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*This procedure was used in all subsequent experiments, in both the control and treatment periods.

1) Two 1 ml samples of plasma were pipetted into two 40 ml glass-stoppered centrifuge tubes containing 2 ml of 0.5 M citrate buffer (pH 5.0) each.
2) To each tube, 25 ml of heptane containing 3% isoamyl alcohol were added and the tubes were shaken for 15 min.
3) The tubes were then centrifuged for 10 min at 2600 g and 20 ml of the organic phase (upper portion) were transferred into two 40 ml centrifuge tubes containing 5 ml of 0.1 N Na OH.
4) The tubes were shaken for 5 min and centrifuged at 3000 g for 15 min.
5) The upper organic phase was then carefully aspirated, and fractions of the bottom aqueous phase were transferred to a quartz cuvette for fluorescence reading, in triplicate. These readings were then averaged for final determination of levels of indomethacin.
6) The fluorescence was measured in an Aminco-Bowman spectrophotofluorometer (activation maximum, 295 mμ ; fluorescence maximum, 385 mμ uncorrected).
7) Standards (1 to 10 μg/ml) were made alkaline (0.1N NaOH) and read at the beginning, middle, and end of each recording period. These values were averaged and a standard curve plotted for each group of samples that were read at the same interval.

8) Recovery from pooled laying hen plasma with additions of indomethacin up to 50 μg/ml and refrigerated for 72 hr was 95.10 ± .65% (Means ± S.E.)--see Table 1.

Table 1. Percent recovery of indomethacin from pooled hen plasma.

| Amount Added (μg/ml) | | 5 | 10 | 25 | 50 |
|---|---|---|---|---|---|
| Sample No. | 1 | 92.00 | 96.67 | 97.80 | 94.42 |
| | 2 | 90.80 | 100.22 | 93.04 | 93.94 |
| | 3 | 92.00 | 96.67 | 94.64 | 93.94 |
| | 4 | 98.80 | 99.36 | 91.44 | 92.20 |
| | 5 | 96.20 | 99.36 | 94.64 | 93.94 |

Analysis of Data

Plasma indomethacin levels that were taken by repeat measurement were analyzed by a split-plot method previously described by Gill and Hafs (1971). In order to correct for heterogeneous variance and non-normal distribution, the individual plasma indomethacin values up through the 18 hr were transformed to their $\log_{10}$ equivalent (Gill, 1977) and analyzed by a computer using a split-plot analysis of variance program.

### Part 2: Determination of Plasma Indomethacin After One Sample Bleeding

#### Procedure

Serial bleeding and possible hemodilution may have had an effect on the levels of indomethacin in the blood over time. In order to test for this possible effect, two more randomly assigned groups of 7 birds each were given a single bolus of 100 mg/kg BW of indomethacin per bird, after taking a control sample of blood (4 ml) from the wing vein. An additional 4 ml sample was drawn from one group at 8 hr and the other group at 32 hr post treatment. These blood samples were treated in the same fashion as those in Part 1 of Experiment 2 in determining the indomethacin levels.

#### Analysis of Data

The values of indomethacin in plasma at 8 hr and 32 hr post-treatment for this experiment were compared to the two respective time period levels of the serially-bled birds in Part 1 of Experiment 2 by a t-test for differences (Gill, 1977).

## Experiment 3

#### Rationale

From the results of Experiment 1, as will be shown subsequently, it was noted that LH release or the subsequent ovulation of the second ovum in the sequence was not blocked by indomethacin. For the third ovum of the

sequence, 2 of 7 birds did not ovulate. This may indicate an effect of the drug on gonadotropin release or an effect on maturation of smaller follicles, or a failure of the oviduct to receive the ovum. The first egg of the sequence appeared to have a very thick shell after its delayed period in the shell gland, whereas the second and third eggs seemed to vary greatly in their egg shell thickness, as well as their time in the shell gland.

In order to avoid a possible effect of indomethacin on gonadotropin release, luteinizing hormone (LH) was injected on the morning of the day of the last egg of the hen's laying sequence, and thereby adding an additional egg onto the sequence. Total plasma calcium and egg shell thickness was also measured.

Procedure

Seven randomly assigned birds were subjected to a control period in which each bird was used as its own control, allowed to complete 1 or 2 normal sequences, and was then given indomethacin during the day of the last egg of this latter sequence. In the control period, each bird was given a placebo (empty gelatin capsule) between 0900 and 1000 hr followed by an intravenous (i.v.) injection of LH (Bovine-NIH-B8) into the wing vein, at the dosage of 60 μg/kg BW--1 hr after placebo.

The treatment period was conducted in the same

fashion as in the control period, except that the birds were given a 50 mg/kg BW dose of drug in a gelatin capsule instead of an empty capsule. Ovulation of the next ovum would occur approximately 8 hr after the LH injection--approximately 9 hr after drug administration.

The day of the last egg was determined by past laying records, and the presence of the egg in the shell gland was determined by digital palpation. The time of oviposition of the last egg of the sequence and LH induced egg were determined. These eggs were broken open, any internal abnormalities were noted, and the egg shells were rinsed clean of all albumen with cold water and allowed to dry for 48 hr at room temperature. The egg shell thickness was then measured, including the shell and shell membranes, with an Ames Thickness Measure and recorded to the nearest ten thousands of an inch.

One ml heparinized blood samples were taken from the wing vein at 11, 24, and 32 hr after placebo. These samples were centrifuged at 2600 g in a refrigerated centrifuge and the plasma was transferred to 12 x 75 mm glass test tubes with plastic stoppers which were frozen (-5°C) until analysis for total calcium--within 72 hr. The total calcium content was measured by a Corning Calcium Analyzer, Model 940, in duplicate, and the average value was recorded in mg%.

The same procedures as above were followed in the treatment period, with a 50 mg/kg BW dosage of indomethacin being administered 1 hr before the LH injection.

The time from the LH injection to the presence of a soft-shelled egg in the shell gland was determined by digital palpation, at 15 min intervals, during the control period and when the presence of hard-shelled egg did not prohibit the timing of this event in the treatment period.

Statistical Analysis

Each bird was used as its own control in this experiment. A t-test for paired comparisons was used for comparing differences in oviposition times, egg shell thickness, and total plasma calcium levels in the treatment period as compared to the control period.

## Experiment 4

### Rationale

In Experiment 3, several of the last eggs in the sequence were expelled during the dark hours (lights-out), as were a few of the soft-shelled LH-induced eggs. The times of these expulsions were determined by duplicating Experiment 3, and placing the hens in an adjacent room during both the control and treatment periods. This was done so that the birds could be monitored for time of oviposition without disruption of the other birds in their previous environment.

Procedure

Seven randomly selected hens were subjected to the same treatment as the hens in Experiment 3 and the same parameters were measured. However, this group was removed from their light-controlled caged environment and placed in new cages in another room during the dark cycle of the control and treatment periods and returned to their former cages after each period. These birds were then monitored for the time of oviposition at 30 min intervals throughout the dark portion of the photoperiod. The egg shell thickness of the last egg and LH-induced (additional) egg in both the control and treatment period were measured and compared, as in Experiment 3. Blood samples were taken for total plasma calcium determination at 11, 24, and 32 hr after placebo or indomethacin treatment, as previously described in Experiment 3.

Statistical Analysis

The t-test for paired comparisons was used for comparing differences in oviposition times, egg shell thickness, and total plasma calcium levels in the treatment period as compared to the control period, as was done in Experiment 3.

## Experiment 5

### Rationale

The previous experiments demonstrated no block in ovulation when indomethacin was given before LH release or LH injection. It is possible that LH is able to overcome any negative effect that indomethacin may have via reduction in prostaglandin synthesis. Indomethacin may also be clearing from the hen's tissues and blood before the ovulation process. Administering indomethacin after LH injection and closer to the time of ovulation may enhance a block of ovulation.

### Procedure

Seven randomly selected hens were injected with LH (60 $\mu$g/kg BW) between 0900 and 1000 hr and were given a placebo (empty gelatin capsule), 1 hr afterwards on the day of the last egg in the sequence. The birds were then monitored for effect on the subsequent ovulation induction and time of oviposition.

In the treatment period, indomethacin (50 mg/kg BW) was given in place of the empty capsule after each bird had completed one or two sequences after the control period.

Experiment 6

Rationale

In the previous experiments, indomethacin did not block LH-induced action when administered either 1 hr before or 1 hr after the LH injection. Because of the delay of the last egg in the sequence, it was impossible to determine the time from LH injection to the presence of a soft-shelled egg in this organ. In this experiment, $PGF_{2\alpha}$ (The Upjohn Company, Kalamazoo, Michigan) was used as a tool to induce oviposition of the last egg in the sequence, 6 hr after LH injection or 7 hr after indomethacin administration so that the effect of the time from LH to ovulation and time for ovum transport could be determined.

Procedure

Seven birds randomly assigned to this treatment group were used as their own controls, given placebo in the control period and indomethacin (50 mg/kg BW) in the treatment period between 0900 and 1000 hr on the day of the last egg of the sequence. One or 2 egglaying sequences lapsed between the control and treatment periods. Luteinizing hormone was given 1 hr after oral placebo/indomethacin treatment. Saline in the control period and $PGF_{2\alpha}$ (50 $\mu$g/kg BW) was administered subcutaneously in the treatment period, 6 hr after the LH injection or 7 hr after the placebo/indomethacin treatment. This procedure induced oviposition of the last egg of the sequence just before the

ovulation of the LH-induced ovum in the treatment period. The birds were then palpated at 15 min intervals from 9 hr after LH administration to the time of a soft-shelled egg in the shell gland. These times were recorded for the control and treatment period and compared statistically by paired-t analysis, as were egg shell thickness differences.

## Experiment 7

### Rationale

As will be presented, results from Experiment 6 suggest that indomethacin given 8 hr before ovulation (assuming 7 hr from LH injection to ovulation), had little effect on the time from LH injection to the entrance of a soft-shelled egg into the shell gland, even though there was a trend toward a reduction of this time after indomethacin. Indomethacin levels in the plasma at this time are, however, quite low (4 to 6 $\mu$g/ml plasma during transport period), as determined in Experiment 2.

### Procedure

In this present experiment, the last egg of the sequence was expelled from the shell gland (6-7 hr prematurely) between 0900 and 1000 hr on the day of the last egg of the sequence in both the control and treatment periods by an SC injection of $PGF_{2\alpha}$ (50 $\mu$g/kg BW). Luteinizing hormone was then injected 1 hr after the premature

expulsion (60 μg/kg BW), followed by placebo/indomethacin orally 3 hr after this injection. According to the plasma indomethacin data in Experiment 2, this would subject each bird to levels of approximately 30 μg/ml of indomethacin in the plasma just prior to ovulation and during the ovum transport period (5 μg/ml as ovum reaches the uterus). The birds were palpated at 15 min intervals beginning 8 hr after the LH injection for the presence of a soft-shelled egg in the shell gland and these times were recorded and compared between the control and treatment period.

# RESULTS

## Experiment 1

The data collected in Experiment 1 are tabulated in Table 2. Analysis of the data by the t-test for paired comparisons showed that time of oviposition of the first egg in the sequence of all birds was significantly different (delayed) from the average time of oviposition of the first egg of the four previous sequences ($p < 0.01$), whereas the second and third eggs of the same treatment sequence were not significantly delayed ($p > 0.2$) when compared to their respective average oviposition times of four previous sequences. In fact, 5 of 7 of the second eggs in the post-treatment sequence were prematurely expelled from the uterus, ranging from 6.9 to 15 hr ($p < 0.01$), whereas, 2 of 5 of the third eggs in the post-treatment sequence were prematurely expelled by 1.2 and 10.2 hr.

Six of the second eggs in this sequence were expelled as soft-shelled eggs; 5 of these prematurely and 1 was delayed by 7.5 hr (Bird No. 6) from the average time of the previous four sequences of this bird for this egg in the sequence.

The second egg of the treatment sequence of Bird

Table 2. Time of oviposition for the first 3 eggs in a sequence after indomethacin administration (50 mg/kg BW) as compared to the average time of oviposition in the four preceding sequences (expressed as differences).

| Bird No. | $C_1-\overline{C}_1$ (hr)[a] | $C_2-\overline{C}_2$ (hr)[b] | $C_3-\overline{C}_3$ (hr) |
|---|---|---|---|
| 1 | + 11.8 | - 9.2 | + 2.5 |
| 2 | + 12.3 | - 9.2 | + 0.8 |
| 3 | + 12.9 | - 6.9 | + 3.1 |
| 4 | + 21.8 | + 20.1 | N.D. |
| 5 | + 5.0 | - 7.8 | - 10.2 |
| 6 | + 17.5 | + 7.5 | N.D. |
| 7 | + 11.7 | - 15.0 | - 1.2 |
| Significance | $p < 0.01$ | N.S. | N.S. |

[a]Difference in oviposition time of indomethacin treated egg sequence ($C_1$ = first egg; $C_2$ = second egg; and $C_3$ = third egg) and the average time of oviposition of eggs of the four preceding sequences, i.e., $\overline{C}_1$, $\overline{C}_2$ and $\overline{C}_3$, respectively.

[b]Prematurely expelled ($p < 0.01$).

N.D. - No data--no egg laid and no soft-shelled egg after oviposition of second egg in sequence.

No. 4 was dramatically delayed by 20.1 hr. Despite this longer period in the shell gland, this egg was very thin-shelled and possessed an indentation in the side of the egg, giving this region a flat-type surface. An egg with this shape is sometimes referred to as a "truncated" egg. This indentation occurs when a soft-shelled egg enters the shell gland at the same time that a hard-shelled egg is present. The pressure of the uterine musculature upon this new soft-shelled egg causes an indentation in the shell membranes and the shell is laid down with the egg in this position with this shape solidifying, giving the shell an indented appearance. The second egg of the other birds of this treatment group, with the exception of Bird No. 5, also had a "truncated" appearance, even though there was little or no calcification of the shell on these eggs at the time of their expulsion. The lack of this irregular shape in the second egg of Bird No. 5 was probably due to the relatively short period of delay in oviposition of the first egg (5.0 hr). This normal shape indicated that the soft-shelled egg had not arrived or just arrived at the shell gland before the expulsion of the first egg (new soft-shelled egg in shell gland 5-6 hr after normal expulsion of the previous egg).

There was no ovulation or the ovum was not picked up by the fimbriae of the oviduct for the formation of the third egg in the treatment sequence in Birds No. 4 and 6.

These two birds also experienced a delay in oviposition time of the second egg of 20.1 hr and 7.5 hr respectively. The previous sequences of these 2 birds were 4 to 5 eggs in length.

The first, second, and third hard-shelled eggs in the treatment sequence were collected and broken out to check for any obvious visual abnormalities. The first eggs, which were held in the shell gland for an extended period of time, appeared to have very thick and chalky-looking shells. The second "truncated" eggs appeared to have relatively thin-shells, as compared to the first egg of this sequence and eggs of previous sequences. The third eggs of the sequence (total of 5) appeared to be normal as to shell-thickness, with the exception of Bird No. 5's egg, which was quite thin--probably due to its premature expulsion of 10.2 hr.

There were no abnormalities found in the interior of these eggs when they were broken out into Petri dishes and examined with the unaided eye.

## Experiment 2

### Part 1

The analysis of variance data are presented in Table 3. The f-statistic for treatments indicates that the levels of indomethacin in the plasma for the 50 mg/kg BW and 100 mg/kg BW are significantly different

Table 3. Split-plot analysis of variance for plasma levels of indomethacin at two orally administered levels.[1]

| Source of Variation | d.f. | Sum of Squares | Mean Squares | f-Statistic |
|---|---|---|---|---|
| Treatment (level of drug) | 1 | 1.978 | 1.978 | 8.447* |
| Animals Within Treatments | 12 | 2.811 | 0.234 | |
| Time | 6 | 24.85 | 4.142 | 34.23** |
| Treatment X Time | 6 | 0.499 | 0.083 | 0.688 |
| Residual Error | 72 | 8.711 | 0.1210 | |
| Total | 97 | 38.85 | | |

[1]Values are plasma indomethacin expressed in $\log_{10}$ of $\mu$g/ml of plasma.

*Significant at p 0.01.

**Significant at p < 0.01.

($p \simeq .01$). In addition, the two levels of drug are different in the plasma at the different sampling times ($p < 0.01$). However, there is no interaction between treatments and time, indicating that the curves for the two levels are essentially parallel and different at most points between the curves. Presented in Table 4 are the $\log_{10}$ transformed plasma indomethacin values for the two treatment levels as determined over time. The mean values over time for the 100 mg/kg BW level of drug ranged from 1.51 to 57.54 μg/ml plasma, whereas, the 50 mg/kg BW level range was from 1.10 to 38.01 μg/ml plasma.

The original unadjusted data is shown in Table 5 and represented graphically in Figure 1. These mean ± standard error (SE) values for the 100 mg/kg BW values ranged from 0.79 ± 0.25 to 62.4 ± 9.79 μg/ml plasma (mean ± SE) whereas, the 50 mg/kg BW dose values ranged from 0.44 ± 0.14 μg/ml to 48.0 ± 28.3 μg/ml (Table 5). Both plasma levels of indomethacin peaked at 2 hr after administration and both levels were at very low levels in the plasma 18 hr after oral dosing and almost completely cleared at 32 hr.

The 95% confidence interval of the overall mean values (Table 4) are 0.97 ± 0.15 ($\log_{10}$) for the 50 mg/kg BW dose of indomethacin with a lower limit of 6.61 μg/ml and an upper limit of 13.18 μg/ml. This same

Table 4. $Log_{10}$ transformed plasma indomethacin levels (Mean ± SE) and the 95% confidence interval over time.[1]

| Time After Administration (hr) | Levels of Indomethacin (mg/kg BW) | |
|---|---|---|
| | 50 | 100 |
| 1 | 1.35 ± 0.18 (22.39) | 1.46 ± 0.18 (28.84) |
| 2 | 1.58 ± 0.18 (38.01) | 1.76 ± 0.18 (57.54) |
| 4 | 1.39 ± 0.18 (24.55) | 1.64 ± 0.18 (43.65) |
| 6 | 1.16 ± 0.18 (14.45) | 1.53 ± 0.18 (33.88) |
| 8 | 0.81 ± 0.18 (6.46) | 1.30 ± 0.18 (19.95) |
| 10 | 0.50 ± 0.18 (3.16) | 0.94 ± 0.18 (8.71) |
| 18 | 0.40 ± 0.18 (1.10) | 0.18 ± 0.18 (1.51) |
| Marginals-- (overall mean ± SE) | 0.97 ± 0.07 (9.33) | 1.26 ± 0.07 (18.20) |
| 95% confidence interval of overall mean values | 0.97 ± 0.15<br>0.82, 1.12 | 1.26 ± 0.15<br>1.11, 1.41 |
| antilog values | 6.61, 13.18 | 12.88, 25.70 |

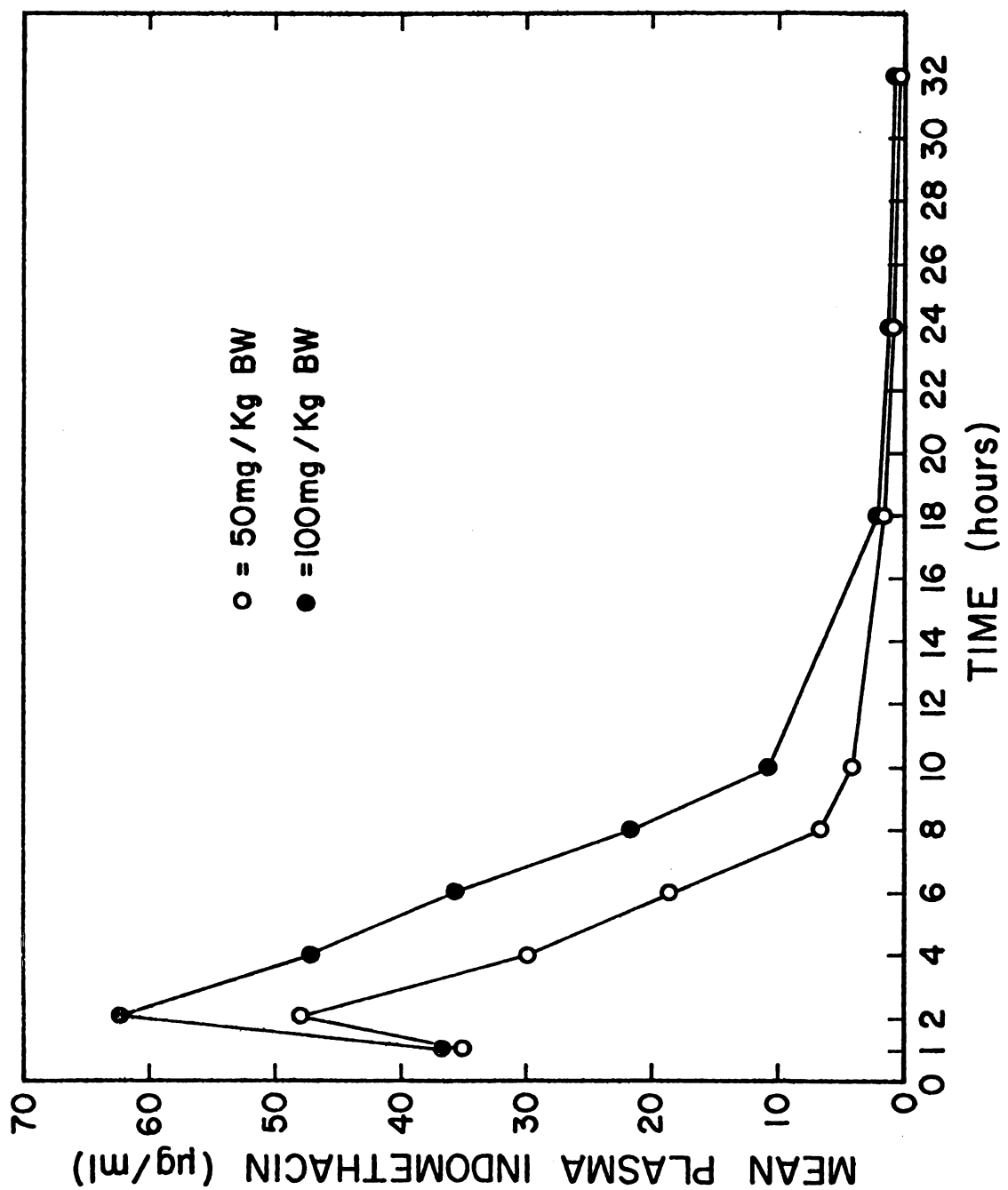
[1]Upper values in each cell are $log_{10}$ transformed mean ± SE values and the lower value is the antilog of the mean value (μg/ml plasma).

Table 5. Unadjusted plasma indomethacin levels (μg/ml) over time.

| Time After Administration (hr) | Levels of Indomethacin (mg/kg BW) | |
|---|---|---|
| | 50 | 100 |
| 1 | 35.3 ± 9.63[a] | 36.7 ± 7.87 |
| 2 | 48.0 ± 28.30 | 62.4 ± 9.79 |
| 4 | 30.0 ± 9.33 | 47.1 ± 7.40 |
| 6 | 18.8 ± 5.62 | 35.6 ± 4.87 |
| 8 | 6.9 ± 1.02 | 21.6 ± 3.02 |
| 10 | 4.2 ± 0.99 | 10.9 ± 1.78 |
| 18 | 1.6 ± 0.42 | 1.9 ± 0.46 |
| 24 | 0.8 ± 0.26 | 1.2 ± 0.42 |
| 32 | 0.4 ± 0.14 | 0.7 ± 0.25 |

[a]Mean ± SE.

Figure 1. Experiment 2--Plasma indomethacin levels over time, after a single oral dose.


MEAN PLASMA INDOMETHACIN (µg/ml)
70
60
50
40
30
20
10
0
0 1 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
TIME (hours)
o = 50mg / Kg BW
● =100mg / Kg BW

confidence interval for the 100 mg/kg BW dose is $1.26 \pm 0.15$ ($\log_{10}$) with a lower limit 12.88 $\mu$g/ml and an upper limit of 25.70 $\mu$g/ml.

Although the birds were subjected to treatment without regard to the presence or absence of a soft-shelled or hard-shelled egg in the shell gland, 3 birds of 14 given the 100 mg/kg BW oral dose with a hard-shelled egg in the shell gland at the time of dosing, laid very thin-shelled "truncated" eggs after the expulsion of the first delayed egg. When broken open, the interior of these eggs were normal looking, with the exception of there being no highly twisted chalazae at each pole of the yolks. This was not noted in "truncated" eggs from the birds given the 50 mg/kg BW dose.

Fifteen of the 21 birds given the 100 mg/kg dose of indomethacin died between 50 and 80 hr after treatment (4 of the serially-bled and 11 of the birds that were bled twice). For this reason, this level of drug was not used in any subsequent experiments.

Just prior to death, a 1 ml sample of heparinized blood was taken from the wing vein of 2 of the birds that were bled twice. These samples were centrifuged in a refrigerated centrifuge (2600 g) for 10 min and total plasma calcium was determined on a Corning Calcium Analyzer Model 940. The average values of duplicate reading for these 2 birds were 6.92 and 8.53% (normal values of

the laying hen being 20-30 mg% (Sturkie, 1976).

Soon after death, these 15 birds were autopsied. Upon examination, the internal organs and supporting tissue were spotted with an off-white granular precipitate, which was presumed to be uric acid, indicating kidney dysfunction. Indomethacin may have profound effects on the gastrointestinal tract and the kidney in that the prostaglandins, whose synthesis is inhibited by indomethacin have been shown to be necessary in the process of digestion via their smooth-muscle stimulating effect and have a role in normal kidney function. In addition, follicular atresia was evident throughout the ovaries of all 15 of these birds.

It was noted that at 2 to 4 hr or longer after oral dosing at both the 50 and 100 mg/kg BW levels, that the crops of the birds were greatly engorged with feed and water and that the amount of excreta deposited beneath the cages was markedly reduced as compared to pre-treatment observations.

Part 2

The t-test for differences revealed that there was no significant differences between the serially-bled and the birds that were bled either 8 or 32 hr after dosing at the 100 mg/kg BW level. The values for the serially-bled birds were 21.6 $\pm$ 3.02 $\mu$g/ml plasma (Mean $\pm$ SE) at the 8 hr bleeding and 0.7 $\pm$ 0.26 $\mu$g/ml at the 32 hr bleeding, as compared to 20.2 $\pm$ 5.24 $\mu$g/ml at 8 hr and

0.5 $\pm$ .31 $\mu$g/ml at 32 hr bleeding for the two groups that were bled at these time periods respectively, after a control bleeding.

Experiment 3

In Table 6 are shown the differences in the times of oviposition after indomethacin treatment. The last egg in the sequence was significantly delayed ($p < 0.01$) when compared to the average time of the last egg of the four previous sequences ($C_T'-C_T$). This time of delay, when compared to the time of oral indomethacin administration ranged from 16.5 to 28.3 hr. With the exception of 1 bird (No. 6 - 28.3 hr), all times of delayed oviposition from the time of drug were from 16 to 18 hr. The clearance curve in Experiment 2 (Figure 1) indicates that this is the approximate time that the drug had dropped to very low levels in the plasma. The LH-induced egg, which was added on to the sequence in both the control and treatment period was expelled prematurely ($p < 0.05$) in 6 of 7 birds in the treatment phase ($C_I'-C_I$). Three of these birds expelled these soft-shelled eggs with or shortly after the delayed hard-shelled eggs during the dark portion of the photoperiod. As in Experiment 1, the eggs found at 0530 hr (lights-on) were estimated to be expelled at 0215 hr. Therefore, the time from drug administration to the time of expulsion of the last delayed egg and the LH-induced egg were recorded as

Table 6. Difference in oviposition time of the last egg of the sequence and an additional LH-induced egg (hr) after indomethacin (50 mg/kg BW).

| Bird No. | $(C_T'-C_T)$ | $(C_T'-T_0)$ | $(C_I'-C_I)$ | $(C_I'-T_0)$ |
|---|---|---|---|---|
| 1 | + 10.0 | + 16.5 | - 17.1 | + 16.5 |
| 2 | + 9.8 | + 16.7 | - 17.7 | + 16.7 |
| 3 | + 11.4 | + 17.5 | + 1.2 | + 34.5 |
| 4 | + 11.4 | + 16.6 | - 1.0 | + 34.1 |
| 5 | + 12.4 | + 18.2 | - 15.6 | + 18.2 |
| 6 | + 22.4 | + 28.3 | - 0.4 | + 33.5 |
| 7 | + 9.7 | + 16.8 | - 1.1 | + 31.7 |
| Significance | $p < 0.01$ | | $p < 0.05$ | |

$C_T$ - average time of last egg in four previous sequences

$C_T'$ - time of last egg after indomethacin treatment

$C_I$ - time of LH-induced egg in control period

$C_I'$ - time of LH-induced egg after indomethacin

$T_0$ - time of indomethacin administration

Table 7. Egg shell thickness of the last egg in the sequence and the LH-induced egg after indomethacin (50 mg/kg BW).

| Bird No. | Egg Shell Thickness (inches) | |
|---|---|---|
| | $(C_T'-C_T)$ | $(C_I'-C_I)$ |
| 1 | + .0013 | - .0139 |
| 2 | + .0011 | - .0112 |
| 3 | + .0020 | - .0063[a] |
| 4 | + .0007 | - .0077[a] |
| 5 | - .0001 | - .0103 |
| 6 | - .0001 | - .0091[a] |
| 7 | - .0002 | - .0054[a] |
| Significance | $p < 0.05$ | $p < 0.01$ |

$C_T$ - last egg in sequence of control phase

$C_T'$ - last egg in sequence of treatment phase

$C_I$ - LH-induced egg of control phase

$C_I'$ - LH-induced egg of treatment phase

a - Oviposition time was only slightly premature or slightly delayed as compared to control period.

being the same time. All 3 of these soft-shelled eggs had a slightly "truncated" appearance, indicating that they had spent some time in the shell gland with the last egg of the sequence (blocked from expulsion from the shell gland by indomethacin). The other 4 LH-induced eggs also had this "truncated" appearance after indomethacin treatment. All of the birds responded to LH injection in both the control and treatment period, as determined by the presence of a soft-shelled egg in the shell gland. The time of the soft-shelled egg's arrival to the shell gland could not be determined due to presence of the delayed hard-shelled egg, as was the case during the control phase of this experiment. The time from LH to soft-shelled egg in the uterus ranged from 12.3 to 13.4 hr (Mean ± SE = 12.8 ± 0.14 hr) in the control period. Assuming that the time of transport down the oviduct is 4.4 hr, as previously determined by Warren and Scott (1935), then the average time from the LH injection to the time of ovulation for these birds is approximately 8.4 hr.

Egg shell thickness data for the last egg in the sequence and the additional LH-induced egg are shown in Table 7. The difference in shell thickness between the last egg in the treatment and control phase ($C_T'$-$C_T$) are significant at $p < 0.05$. Four of the last eggs in the

treatment phase had slightly thicker egg shells, whereas the 3 remaining eggs had slightly thinner egg shells.

The shell thickness of the LH-induced egg in the treatment phase was significantly different (thinner) from the control egg ($C_I'$-$C_I$). It should be noted that 3 of these eggs were prematurely expelled by a short interval (0.4 to 1.1 hr) and the other egg was laid 1.2 hr later than the control egg. When these 4 eggs were compared to their controls, they were also significantly thinner ($p < 0.01$).

Total plasma calcium levels after indomethacin are given in Table 8. The calcium levels were significantly reduced ($p < 0.01$) at 11, 24, and 32 hr after oral dosing with indomethacin.

Experiment 4

The last eggs in sequence were laid between 0200 and 0945 hr the following day (dark cycle to early lights-on) --4 of these being laid during the dark cycle at 0200, 0230, 0400, and 0430 hr. The other 3 birds oviposited their eggs at 0630, 0830, and 0930 hr. Bird No. 2 laid a soft-shelled egg approximately 15 min prior to laying the delayed last egg of the sequence. This bird was digitally palpated after discovery of the unplumped soft-shelled egg and the delayed last egg of the sequence was felt in the shell gland of the bird. This bird was checked

Table 8. Total plasma calcium levels (mg%) after indomethacin (50 mg/kg BW).

| Bird No. | Control | | | After Indomethacin[1] | | |
|---|---|---|---|---|---|---|
| | 11[a] | 24 | 32 | 11 | 24 | 32 |
| 1 | 24.71 | 21.92 | 22.04 | 13.40 | 11.71 | 12.79 |
| 2 | 26.40 | 25.16 | 28.59 | 22.31 | 21.58 | 21.56 |
| 3 | 22.56 | 22.13 | 21.10 | 19.36 | 17.81 | 20.01 |
| 4 | 17.53 | 17.10 | 18.42 | 10.59 | 8.40 | 9.73 |
| 5 | 31.52 | 24.92 | 29.31 | 19.15 | 20.02 | 20.22 |
| 6 | 21.22 | 20.99 | 24.61 | 15.13 | 16.74 | 16.40 |
| 7 | 25.22 | 27.84 | 29.43 | 22.71 | 18.43 | 21.56 |

[1]Significantly lower at all time periods ($p < 0.01$) as compared to controls.

[a]Hours after placebo/indomethacin administration.

following a 15 min interval and the hard-shelled egg had been oviposited.

The last eggs of the sequence were significantly delayed ($p < 0.01$) when compared to the mean value of the last eggs of the four previous laying sequences (Table 9). The last egg of the sequence of Bird No. 6 was delayed by 42.2 hr. The same bird was the only bird that did not respond to the LH injection (as determined by failure of the presence of a soft-shelled egg in the shell gland after the delayed egg was oviposited) and went out of production for more than 30 days post-treatment.

As previously shown in Experiment 3, the delayed eggs were oviposited after the drug was shown to be in low levels in the plasma (17-18 hr).

Egg shell thickness data for the last egg in the sequence and LH-induced egg are shown in Table 10. After the indomethacin treatment, the shells of the delayed last eggs in the sequence were thicker than controls ($p < 0.1$). All but 2 of these eggs were thicker than the pre-treatment control ($C_T'$-$C_T$).

The LH-induced egg was significantly thinner after indomethacin ($p < 0.01$) as compared to the control shell thickness ($C_I'$-$C_I$), even though 5 of these eggs were only slightly prematurely expelled or delayed by a short period

Table 9. Difference in oviposition time of the last egg of the sequence and an additional LH-induced egg (hr) after indomethacin (50 mg/kg BW).

| Bird No. | Time of Oviposition | | | |
|---|---|---|---|---|
| | $(C_T'-C_T)$* | $(C_T'-T_O)$ | $(C_I'-C_I)$ | $(C_I'-T_O)$ |
| 1 | + 12.8 | + 20.4 | + 0.2 | + 34.0 |
| 2 | + 12.7 | + 20.1 | - 13.2 | + 19.8 |
| 3 | + 10.8 | + 17.6 | + 2.3 | + 36.8 |
| 4 | + 11.2 | + 17.1 | + 0.2 | + 33.2 |
| 5 | + 14.5 | + 24.5 | - 1.6 | + 32.6 |
| 6 | + 42.2 | + 47.2 | N.D. | N.D. |
| 7 | + 15.1 | + 21.4 | - 1.8 | + 35.9 |

$C_T$ - average time of last egg in four previous sequences

$C_T'$ - time of last egg after indomethacin treatment

$C_I$ - time of LH-induced egg in control period

$C_I'$ - time of LH-induced egg after indomethacin

$T_O$ - time of indomethacin administration

* - time of oviposition significantly delayed ($p < 0.01$)

N.D. - No data - no ovulation from the LH injection during the treatment period.

Table 10. Egg shell thickness (inches) for the last egg in the sequence and the LH-induced egg after indomethacin (50 mg/kg BW).

| Bird No. | Egg Shell Thickness (EST) | |
|---|---|---|
| | $(C_T'-C_T)$ | $(C_I'-C_I)$ |
| 1 | - .0004 | - .0062[a] |
| 2 | + .0007 | - .0119 |
| 3 | .0000 | - .0061[a] |
| 4 | + .0011 | - .0019[a] |
| 5 | + .0003 | - .0082[a] |
| 6 | + .0005 | N.D. |
| 7 | + .0001 | - .0034[a] |
| Significance | $p < 0.1$ | $p < 0.01$ |

$C_T$ - EST of last egg in sequence of control period

$C_T'$ - EST of last egg in sequence of treatment period

$C_I$ - EST of LH-induced egg of control period

$C_I'$ - EST of LH-induced egg of treatment period

a - Oviposition time was only slightly premature or slightly delayed as compared to control period.

N.D. - No data--no response to the LH-injection as determined by no soft-shelled egg in the shell gland.

of time (Table 10 - ($C_I'$-$C_I$). When these 5 eggs were compared to their controls they were also thinner shelled ($p < 0.01$).

The differences in total plasma calcium levels are shown in Table 11 and demonstrate the same significant drop ($p < 0.01$) after indomethacin, as was the case in Experiment 3.

Experiment 5

Indomethacin (50 mg/kg BW) did not block the ovulation process when administered 1 hr after the LH injection. All 7 of the birds responded to the LH injection, as determined by the presence of a soft-shelled egg in the shell gland after oviposition of the delayed last egg.

Table 12 shows the results of delay or change in oviposition times for the last egg in the sequence and the LH-induced additional egg. The time of expulsion of the last egg in the sequence was significantly delayed ($p < 0.01$) when compared to the average time of expulsion of the last egg of the four previous sequences ($C_T'$-$C_T$). This egg was expelled from the shell gland between 12.2 to 22.6 hr (Mean = 15.8 hr) after drug administration--a time when drug is at low levels in the plasma, as was shown in the two previous experiments. After indomethacin treatment, all of the birds expelled the LH-induced egg prematurely as compared to controls ($C_I'$-$C_I$) and from

Table 11. Total plasma calcium levels (mg%) after oral dosing with indomethacin (50 mg/kg BW).

| Bird No. | Control | | | After Indomethacin[1] | | |
|---|---|---|---|---|---|---|
| | 11[a] | 24 | 32 | 11 | 24 | 32 |
| 1 | 31.05 | 29.40 | 30.70 | 19.98 | 14.74 | 18.65 |
| 2 | 26.20 | 24.78 | 24.85 | 17.30 | 9.34 | 5.48 |
| 3 | 24.51 | 24.21 | 23.89 | 16.23 | 15.41 | 14.10 |
| 4 | 21.38 | 20.59 | 21.84 | 17.91 | 8.11 | 10.54 |
| 5 | 23.78 | 23.85 | 22.52 | 16.18 | 18.54 | 19.21 |
| 6 | 18.07 | 17.28 | 17.84 | 14.95 | 13.91 | 11.27 |
| 7 | 19.54 | 18.85 | 19.72 | 15.72 | 12.84 | 17.74 |

[1]Significantly lower at all time periods ($p < 0.01$) as compared to controls.

[a]Hours after placebo/indomethacin administration.

Table 12. Oviposition time of the last egg of the sequence and an additional LH-induced egg (hr) after indomethacin (50 mg/kg BW).

| Bird No. | Time of Oviposition (hr) | | | |
|---|---|---|---|---|
| | $(C_T'-C_T)$ | $(C_T'-T_O)$ | $(C_I'-C_I)$ | $(C_I'-T_O)$ |
| 1* | + 11.5 | + 13.5 | - 16.1 | + 13.5[a] |
| 2 | + 11.2 | + 15.7 | - 3.1 | + 30.0 |
| 3 | + 10.4 | + 15.6 | - 16.2 | + 15.6[a] |
| 4 | + 7.9 | + 12.2 | - 18.4 | + 15.5[a] |
| 5 | + 9.3 | + 15.4 | - 0.2 | + 32.4 |
| 6 | + 15.4 | + 22.6 | - 8.9 | + 23.0[a] |
| 7 | + 9.1 | + 15.3 | - 1.8 | + 29.9 |
| Significance | $p < 0.01$ | | $p < 0.01$ | |

*Bird died about 30 hr after indomethacin administration.

[a]Soft-shelled eggs expelled prior to or shortly after the expulsion of the indomethacin delayed last egg.

$C_T$ - average time of last egg in four previous sequences

$C_T'$ - time of last egg after indomethacin treatment

$C_I$ - time of LH-induced egg in control period

$C_I'$ - time of LH-induced egg after indomethacin

$T_O$ - time of indomethacin administration.

15.5 to 32.4 hr (Mean = 22.8) after the drug administration ($C_I'$-$T_0$). Four of the birds expelled the LH-induced egg prior to, or shortly after the expulsion of the indomethacin-delayed last egg of the sequence. The time of expulsion of either of these eggs was estimated to be 0215 hr or midway through the dark cycle as in Experiments 1 and 3.

Differences in egg shell thickness in the control and treatment period for the delayed last egg and the LH-induced egg are tabulated in Table 13. The indomethacin-delayed last egg was significantly thicker than the controls ($p < 0.05$) even though 3 of these birds laid slightly thinner eggs (.0001 to .0002 in. thinner). All of the LH-induced eggs were prematurely expelled when compared to controls. This premature expulsion was confounded by the egg shell thickness data ($C_I'$-$C_I$), which showed that these egg shells were significantly thinner ($p < 0.01$) than controls. Three of these birds expelled these eggs a short time before their control oviposition time (Birds Nos. 2, 5, and 7 with 3.1, 0.2, and 1.8 hr premature expulsion, respectively). When these 3 eggs were compared to their control, they were also thinner ($p < 0.01$).

Bird No. 1 died about 30 hr after indomethacin treatment. Despite this lethal dose, ovulation after the LH injection in the treatment period was not blocked. Autopsy revealed the same basic internal abnormalities

Table 13. Egg shell thickness (inches) for the last egg in the sequence and the LH-induced egg after indomethacin (50 mg/kg BW).

| Bird No. | Egg Shell Thickness (EST) | |
|---|---|---|
| | $(C_T'-C_T)$ | $(C_I'-C_I)$ |
| 1 | + .0018 | - .0108 |
| 2 | + .0011 | - .0047 |
| 3 | - .0003 | - .0129 |
| 4 | - .0002 | - .0110 |
| 5 | + .0012 | - .0055 |
| 6 | - .0001 | - .0101 |
| 7 | + .0011 | - .0039 |
| Significance | $p < 0.05$ | $p < 0.01$ |

$C_T$ - EST of last egg in control period

$C_T'$ - EST of last egg in treatment period

$C_I$ - EST of LH-induced egg in control period

$C_I'$ - EST of LH-induced egg in treatment period

that were noted in the 15 birds dying from the 100 mg/kg BW dose in Experiment 2.

Experiment 6

In the control period, all 7 birds failed to respond to the subcutaneous (SC) saline injection, whereas, all birds expelled their eggs within 2.6 to 4.5 min after the SC injection of $PGF_{2\alpha}$ (Mean = 3.7 min). Table 14 shows that the indomethacin block of oviposition, shown in the previous experiments, was over-ridden by the SC injection of $PGF_{2\alpha}$. The first column ($C_T'-C_T$) shows that the significantly delayed egg ($p < 0.01$) was expelled by $PGF_{2\alpha}$ 0.1 to 1.2 hr after the expulsion time of the control. The second column ($C_I'-C_I$) indicates that 5 of 7 birds prematurely expelled the LH-induced egg after indomethacin treatment, despite the fact that there was no delayed egg in the shell gland (having been previously removed by the $PGF_{2\alpha}$ injection). These premature expulsions ranged from -0.4 to -17.5 hr from the control period's expulsion times. The 2 remaining birds slightly delayed their expulsion time by 0.3 and 0.5 hr as compared to their own control period.

All of the birds responded to the LH injection in both the control and treatment periods, as indicated by the presence of a soft-shelled egg in the shell gland. Compared in Table 15 is the time from the LH injection to

Table 14. Oviposition time of the last egg in laying sequence and the LH-induced egg after indomethacin (50 mg/kg BW).

| Bird No. | Oviposition Time (hr) | |
|---|---|---|
| | $(C_T'-C_T)$ | $(C_I'-C_I)$ |
| 1 | - 0.6 | + 0.3 |
| 2 | - 0.1 | - 10.8 |
| 3 | - 1.0 | - 17.4 |
| 4 | - 0.7 | - 17.5 |
| 5 | - 0.2 | - 0.4 |
| 6 | - 0.4 | + 0.5 |
| 7 | - 1.2 | - 2.9 |
| Significance | $p < 0.01$ | $p < 0.01$ |

$C_T$ - average time of last egg in four previous sequences

$C_T'$ - time of $PGF_{2\alpha}$ expelled egg from bird treated with indomethacin

$C_I$ - time of LH-induced egg in control period

$C_I'$ - time of oviposition of LH-induced egg after indomethacin

Table 15. Time from LH injection to time of soft-shelled egg in the shell gland.

| Bird No. | Time from LH injection to time of soft-shelled egg in shell gland (hr) | | |
|---|---|---|---|
| | Control (C) | Treatment (T) | C-T |
| 1 | 12.6 | 12.7 | + 0.1 |
| 2 | 11.5 | 13.3 | + 2.2 |
| 3 | 11.5 | 13.3 | - 1.8 |
| 4 | 9.1 | 8.4 | + 0.7 |
| 5 | 9.8 | 10.2 | - 0.4 |
| 6 | 12.0 | 11.8 | + 0.2 |
| 7 | 11.1 | 10.8 | + 0.3 |

the time of a soft-shelled egg in shell gland for both the control and treatment period. The difference (C-T) in times were not significant when analyzed by the t-test for paired comparisons, even though 5 of 7 of the soft-shelled eggs (after indomethacin) arrived at the shell gland sooner than the controls by 0.1 to 2.2 hr.

The egg shell thickness of the last egg of the sequence and the LH-induced egg in both the control and treatment period are tabulated as differences in Table 16. There is no significant difference between the thickness of the last egg in the sequence between the control and treatment period ($C_T'-C_T$). The difference in these thicknesses ranged from -.0038 to +.0012 in. However, there is a significant difference in the LH-induced eggs in that the $PGF_{2\alpha}$ and indomethacin treated egg in significantly thinner ($p < 0.01$). This thinness in egg shells appears to be partially due to premature expulsion of these eggs. However, the reduction in total plasma calcium may be also contributed to this phenomenon (as shown in Experiments 3 and 4).

Experiment 7

One of the 7 birds (No. 1) did not respond to the LH injection in the treatment period as it had in the control period two sequences prior to this time. The other 6 birds responded to the LH injection in both the control

Table 16. Egg shell thickness (inches) after $PGF_{2\alpha}$ and indomethacin (50 mg/kg BW).

| Bird No. | Egg Shell Thickness (EST) | |
|---|---|---|
| | $(C_T'-C_T)$ | $(C_I'-C_I)$ |
| 1 | + .0012 | - .0029 |
| 2 | - .0038 | - .0039 |
| 3 | .0000 | - .0101 |
| 4 | + .0002 | - .0113 |
| 5 | - .0004 | - .0016 |
| 6 | - .0003 | - .0031 |
| 7 | + .0001 | - .0044 |
| Significance | N.S. | $p < 0.01$ |

$C_T$ - EST of last egg of sequence in control period

$C_T'$ - EST of last egg of sequence in treatment period

$C_I$ - EST of LH-induced egg in the control period

$C_I'$ - EST of LH-induced egg in the treatment period

and treatment period, even though indomethacin was in relatively high levels in the plasma in the pre-ovulatory phase and prior to and during the ovulation process. The expulsion of the last egg in the sequence by $PGF_{2\alpha}$ in both the control and the treatment period ranged between 2.6 to 5.2 min after the SC injection of this prostaglandin (Mean $\pm$ SE = 4.0 $\pm$ .25 min).

After indomethacin, 5 of the 6 birds responding to the LH injection demonstrated a significant reduction in the time period required for the LH to cause ovulation and for this ovum to be transported to the shell gland ($p < 0.05$), as compared to pre-treatment (control) time periods (Table 17). These values ranged from 0.2 to 1.4 hr difference from controls. The other bird delayed the ovulation/transport of the treatment egg 0.1 hr.

The LH-induced egg was prematurely expelled in 4 of 6 birds which responded to the LH injection in the treatment period (Table 18). This premature expulsion of this egg was not significant ($p < 0.2$) when all 6 times were compared to the control by the t-test for paired comparisons. These eggs were expelled 24.8 to 31.5 hr after indomethacin treatment (Mean = 28.1 hr). Thus, considerably after the time when indomethacin in the plasma is at very low levels (18 hr). The time that these LH-induced eggs spent in the shell gland was slightly longer for controls than for the treatment period (Mean = 23.3 and 22.0 hr, respectively)

Table 17. Time from LH injection to the time of soft-shelled egg in the shell gland after indomethacin (50 mg/kg BW).

| Bird No. | Time from LH injection to time of soft-shelled egg in shell gland (hr) | | |
|---|---|---|---|
| | Control (C) | Treatment (T) | C-T* |
| 1 | 11.5 | N.D. | N.D. |
| 2 | 10.1 | 9.1 | - 1.0 |
| 3 | 9.3 | 7.9 | - 1.4 |
| 4 | 9.1 | 8.5 | - 0.6 |
| 5 | 10.3 | 9.5 | - 0.8 |
| 6 | 11.6 | 11.7 | + 0.1 |
| 7 | 8.4 | 8.2 | - 0.2 |

*Significant difference in LH to soft-shelled egg time ($p < 0.05$)

N.D. - No data--bird did not respond to LH injection in the treatment period.

Table 18. Time from soft-shelled egg in shell gland and oviposition of the LH-induced egg.

| Bird No. | Time (hr) | | | | |
|---|---|---|---|---|---|
| | $(C_I'-C_I)$ | $(C_I'-T_O)$ | $(C_I-SS_I)$ | $(C_I'-SS_I')$ | $(SG_I-SG_I)$ |
| 1 | N.D. | N.D. | N.D. | N.D. | N.D. |
| 2 | + 1.0 | 26.6 | 21.8 | 20.8 | - 1.0 |
| 3 | - 2.5 | 28.2 | 24.4 | 23.4 | - 1.0 |
| 4 | - 3.9 | 24.8 | 24.6 | 19.3 | - 5.3 |
| 5 | - 4.0 | 27.2 | 24.0 | 20.7 | - 3.3 |
| 6 | + 1.4 | 31.5 | 21.5 | 22.8 | + 1.3 |
| 7 | + 2.5 | 30.2 | 23.4 | 25.0 | + 1.6 |
| Sig. | $p < 0.2$ | | | | $p < 0.05$ |

$C_I$, $C_I$ - time of oviposition of LH-induced egg in control and treatment period, respectively

$SS_I$, $SS_I'$ - time of soft-shelled egg in shell gland for control and treatment period, respectively (from time of LH injection)

$SG_I$, $SG_I'$ - total time of the egg in the shell gland for control and treatment period, respectively

$T_O$ - time of indomethacin administration

N.D. - No data--bird did not respond to LH injection in the treatment period.

Table 19. Egg shell thickness (inches) for the last egg in the sequence and the LH-induced after indomethacin (50 mg/kg BW) and $PGF_{2\alpha}$.

| Bird No. | Egg Shell Thickness (EST) | |
|---|---|---|
| | $(C_T'-C_T)$ | $(C_I'-C_I)$ |
| 1 | - .0030 | N.D. |
| 2 | - .0227 | - .0075 |
| 3 | + .0003 | - .0043 |
| 4 | + .0013 | - .0069 |
| 5 | - .0003 | - .0057 |
| 6 | + .0003 | - .0033 |
| 7 | - .0003 | - .0036 |
| Significance | N.S. | $p < 0.01$ |

$C_T$ - EST of last egg in sequence in control period after premature expulsion by $PGF_{2\alpha}$

$C_T'$ - EST of last egg in sequence in treatment period after premature expulsion by $PGF_{2\alpha}$

$C_I$ - EST of LH-induced egg in control period

$C_I'$ - EST of LH-induced egg in treatment period

as shown in Table 18. Four of these eggs, after indomethacin, were in the shell gland for a shorter period than their controls ($SG_I'$-$SG_I$).

Differences in egg shell thickness for the last egg in the sequence and the LH-induced egg are presented in Table 19. The last egg in both the control and treatment period were prematurely expelled from the shell gland by an injection of $PGF_{2\alpha}$, as previously described. Column ($C_T'$-$C_T$) indicates that the differences in thicknesses between control and treatment as being different, but not significantly so. The second column ($C_I'$-$C_I$) indicates that the LH-induced egg was significantly thinner ($p < 0.01$) even though only 5 of these birds prematurely expelled these eggs by 0.2 to 1.4 hr.

# DISCUSSION

## Experiment 1

The results from Experiment 1 (Table 2) indicate that the oral administration of indomethacin (50 mg/kg BW) to laying hens, significantly delays the normal expulsion of the first egg of the sequence, when the dose was given 11 to 12 hr prior to the normal time of this event. The fact that indomethacin caused a delay in time of oviposition in the S.C.W.L. hen has been previously reported by Hertelendy *et al.* (1974). The fact that various prostaglandins were able to overcome this prostaglandins inhibitor's block of oviposition, suggests that prostaglandins may play an active role in the normal process of oviposition.

The effect of indomethacin or other prostaglandin inhibitors on the subsequent eggs in a sequence (ovulation and oviposition of) has not been previously reported. In this experiment, the ovulation of the second ovum in the sequence was not blocked by the single bolus of indomethacin--given approximately 4-5 hr before LH-release and 11-12 hr before ovulation of this ovum would have occurred. This observation is not in agreement with previous work reported in mammals, that inhibitors of prostaglandins (specifically indomethacin) will block the process of

ovulation and the belief by several researchers that prostaglandins may have an essential role in gonadotropin release and ovulation (see Review of Literature).

The time of oviposition of the second and third egg were not significantly delayed. Five of 7 of the second eggs were prematurely expelled by 6.9 to 15.0 hr. The fact that the first egg was laid, in most cases, during the dark portion of the photoperiod, and the time of the first egg and the second soft-shelled egg, were reported as being the same time (0215 hr) may cause these recorded expulsion times to be in error by several hours. However, the fact that 4 of 5 of these soft-shelled eggs had a "truncated" appearance indicates that these eggs spent some period of time in the shell gland with the first egg in the sequence. It is possible that these soft-shelled eggs were expelled from the shell gland at about the same time as the first eggs in the sequence.

The 2 birds which delayed the second egg in the sequence, as well as the first egg, also did not receive a third ovum in the shell gland for the production of a third egg--as could be predicted by their past laying records of 4 and 5 eggs per sequence. It was not determined whether ovulation had not occurred or that the fimbriae of the oviduct had failed to pick up the ovum after ovulation. The bird with the greatest delay of expulsion

(No. 4 - Table 1) of both the first and second egg (21.8 and 20.1 hr, respectively), also had a lower production rate (4 egg sequences) as compared to Bird No. 6 (5 egg sequences).

Of the 5 birds which a third egg in the treatment sequence, 3 of these eggs were delayed slightly from their normal time of expulsion--as compared to the average time of expulsion of the third egg in four previous control sequences. The 2 other birds expelled the third egg 1.2 and 10.2 hr prematurely.

The data for oviposition time from the second and third egg (Table 1) offer no real pattern or trend, in that time of expulsion of the eggs, were both premature and delayed. The fact that Birds No. 4 and 6 delayed the expulsion of the second egg, may have some relationship to the fact that no third ovum was ovulated or present in the oviduct. Ovulation of these ovum would have normally occurred about 30 min after the normal expulsion of the second egg. In the case of Bird No. 4, this ovulation would have occurred about 19.6 hr before the time the delayed second egg was oviposited. If we assumed a 4.4 hr time for transport of this ovum to the shell gland, this would place the egg in the shell gland 15.2 hr before the delayed second egg's expulsion. In the case of Bird No. 6, ovulation for the third egg would have been 7.0 hr before

the delayed expulsion of the second egg, and this ovum would have arrived at the shell gland 3.6 hr before the expulsion. The other 5 birds prematurely expelled the second egg by 9.6 ± 1.4 hr (Mean ± SE) or about 9 hr prior to the ovulation of the third ovum. At the time of the expulsion of the second egg, no soft-shelled eggs were found in the shell gland as determined by palpation. The exact time of arrival of the third egg to the shell gland was not determined.

If one assumes that no ovulation occurred for the third ovum in the sequence for Birds No. 4 and 6, there are several plausible explanations for this occurrence. The indomethacin, via inhibition of prostaglandin synthesis, may have inhibited the release of LHRH (luteinizing hormone releasing hormone) and LH for this ovulation. By this same mechanism, FSH (follicle stimulating hormone) release may be depressed, causing the lack in the maturation of developing follicles, preventing them from responding to LH. Another possibility is that indomethacin is interfering with normal levels of other hormones or factors (such as ion levels), that are necessary for the completion of the ovulation process. If ovulation did occur, then the egg was not received by the fimbriae of the oviduct, for transport to the shell gland.

Previous research has indicated that prostaglandins may be involved in calcium regulation in mammals (see Review of Literature). In this experiment, the eggs after

indomethacin appeared to have thinner egg shells (even though a considerable amount of time was spent in the shell gland), suggesting that this drug may have an effect on calcium levels, and by this mechanism, dictate the amount of shell that is deposited, and directly or indirectly dictate the time and amount of gonadotropin release. Some of these possibilities were looked into in other experiments within this research project.

Experiment 2

The results from Experiment 2 (Table 5 and Figure 1) indicated that indomethacin was found in the plasma within 1 hr was at maximum levels at 2 hr was at very low levels at 18 hr, and was essentially cleared in 32 hr after oral dosing at levels of 50 and 100 mg/kg BW.

The statistical analysis of these two curves suggests that they are parallel and essentially different at all sampling times up to 18 hr.

The reliability of these levels, due to the large amount of blood removal from repeat sampling (40 ml), was partially substantiated by the results in Part 2 of Experiment 2. The results indicate that there was no significant difference in the plasma indomethacin levels between two groups of birds which were bled twice when compared to the serially-bled birds at two different time periods, for the 100 mg/kg BW level.

The 100 mg/kg BW oral dose of indomethacin was not administered in any of the subsequent experiments, because 15 of 21 birds given this dose died within 50 to 80 hr after dosing. The exact cause of death was not completely substantiated, however, the fact that the total plasma calcium was extremely low just prior to death and the presence of an off-white precipitate in the internal organs, indicate that the low blood calcium and kidney dysfunction may have been contributing factors.

This toxic dose also caused follicular atresia in all 15 of these birds. This may indicate a reduction in the levels of the ovarian supporting hormones. However, the mechanisms of this level change via reduction in the appropriate levels of essential prostaglandins, would have to be verified by measuring these substances.

Experiments 3 and 4

Experiments 3 and 4 were different only in the fact that the birds in Experiment 4 were continuously checked at 30 min intervals during the dark portion of the photoperiod in the treatment phase of this experiment. For this reason, the results of these two experiments will be discussed simultaneously.

The last egg of the laying sequence in both experiments was quite dramatically delayed in its expulsion from the shell gland, after an oral dose of 50 mg/kg BW of

indomethacin--combined, the mean ± SE = 14.7 ± 2.28 hr (n = 14).

The average combined time of the difference from administration time to the time of delayed oviposition of this egg was 21.4 ± 3.10 hr (Mean ± SE; n = 14). This would indicate that the delayed expulsion occurred a few hours after the indomethacin in the plasma had reached a very low level (Figure 1). Assuming that this level of prostaglandin inhibitor is only expressing its delaying effect by way of prostaglandin reduction, then this recovery and subsequent expulsion of the delayed egg seems to offer some evidence that prostaglandins play an essential role in oviposition.

The expulsion time of the delayed last egg of the sequence and the soft-shelled LH-induced egg were estimated in Experiment 3; whereas, in Experiment 4, the hens were checked every 30 min during the dark portion of the photoperiod in the treatment period. This more precise timing of these expulsions of eggs during this period, may in part, have contributed to the differences in these mean recorded values. The mean ± SE delay for Experiment 3 was 12.4 ± 1.70 hr and in Experiment 4 this value was 17.0 ± 4.23 hr. It appears that the delay of the last egg's expulsion may have been poorly estimated in Experiment 3. However, one bird in Experiment 4 delayed the expulsion of this egg by

42.2 hr. If this value was not included, then the mean ± SE delay for Experiment 3 was 12.9 ± 0.70 hr.

Bird No. 6 in Experiment 4 did not respond to the LH injection after indomethacin, as it had in the previous control period. This response could be contributed to an interference by indomethacin in the maturation and rupture of the largest follicle, an anti-LH response of the bird due to antibody development after the LH injection in the control period, or a poor i.v. injection of the LH. The latter is highly unlikely due to caution taken to make consistently clean injections, but due to the positive response of the other 13 birds in these 2 experiments, under the same conditions, it is probably the best explanation.

The data in Tables 7 and 10 indicate that the egg shells are thicker than the controls for 9 of the 14 birds in these experiments ($p < 0.05$ for Experiment 3; $p < 0.01$ for Experiment 4). In the remaining eggs, there was no difference (0.000) to - .0004 inches difference in thinner egg shells. This indicates that the calcium deposition process was not completely stopped during this extended period of time in the shell gland (about 50-60% longer than normal). In contrast, all of the LH-induced eggs were thinner ($p < 0.01$) in the treatment period than they were in the LH-induced control period. The thinner shell

condition was partially confounded with premature expulsion of some of these eggs. However, 9 of 12 of these thinner treatment eggs were only slightly premature or slightly delayed when compared to their control's expulsion time. This would indicate that indomethacin was causing some disruption of the calcium deposition process.

The results from the total plasma calcium sample taken during the control and treatment period offer some evidence that there may be a reduced calcium deposition rate in the shell gland due to the lower amount of calcium available for this process.

In both experiments, the post-indomethacin total plasma calcium levels were significantly reduced as compared to pre-drug control levels, for all 14 birds.

The time periods for blood sampling (1 ml aliquots) were selected as 11, 24, and 32 hr after indomethacin, in accordance with the time of shell formation. Luteinizing hormone was injected 1 hr after the indomethacin bolus was given. Under these conditions, ovulation would occur at about 9 hr after the drug was given and the soft-shelled egg would arrive in the shell gland 4-5 hr afterwards, thus the egg would spend more than 20 hr undergoing shell formation. This would place the time of the first blood sampling at about 2-3 hr before the soft-shelled egg arrived in the shell gland, the second at about midway in the shell deposition process, and the third at a period after oviposition

and at a time when indomethacin was almost completely cleared in the blood.

For discussion and comparison purposes, the mean ± SE of all of the plasma control samples at each time period for both experiments was 23.51 ± 0.616 mg% calcium (n = 42) --see Table 8 and 11. The mean ± SE for all the treatment samples was 15.90 ± 0.664 mg% calcium (n = 42). This represents about a 30% reduction in the total plasma calcium after the oral indomethacin treatment.

The actual physiological events, which lead to this reduction in the total plasma calcium, were not determined in this study. However, several researchers have suggested that prostaglandins may play a role in the regulation of calcium in several mammals (Dietrich and Reiz, 1975). Indomethacin may cause lowered calcium by directly or indirectly lowering dietary calcium absorption in the gastrointestinal tract or lowering calcium mobilization from the medullary bone.

During the dark portion of the photoperiod, in the treatment phase of Experiment 4, it was noted that Bird No. 2 had expelled the LH-induced soft-shelled egg about 15 min prior to the expulsion of the delayed last egg in the sequence. This soft-shelled egg was not plumped out with water and salts from the shell gland and was therefore, quite small in size compared to a normal egg, thus allowing this egg to leave the shell gland through a

smaller opening. Digital palpation of this bird, immediately after finding this soft-shelled egg, revealed that the uterovaginal region was very taut, partially dilated, and unelastic. This condition would inhibit the expulsion of a normal hard-shelled egg. Verma et al. (1976) has hypothesized that the prostaglandin $PGE_1$ relaxes the smooth muscle in the uterovaginal region, while at the same time, causing the shell gland smooth muscles to contract and thereby expel the egg.

In addition, observation of birds in the dark and early lights-on portion of the photoperiod, after indomethacin treatment, revealed that all of the birds 1 to 2 hr prior to ovipositing the delayed last egg of the sequence, experienced quite rhythmic and deep abdominal contractions. It appeared that they were trying (unsuccessfully) to rid their shell gland of the delayed egg by use of their skeletal (voluntary) muscles. When observed closely, this phenomenon was also noticed in other birds given this level of indomethacin.

Experiment 5

Indomethacin (50 mg/kg BW), administered 1 hr after an LH injection, did not interfere with the physical process of ovulation of the LH stimulated follicle. Figure 1 indicates that the plasma levels are more than twice as high

at the time of ovulation (about 19 μg/ml) as the plasma levels when indomethacin was given 1 hr before the LH injection (about 7 μg/ml), as in the previous experiments.

The last egg in the sequence, which was in the shell gland at the time indomethacin was given, was delayed an average of 15.8 hr from their normal time of expulsion. As was shown in the previous experiments, this period of delay can be correlated with a time when indomethacin was at very low levels in the blood. Results from Experiment 3 and 4 indicate that this expulsion time occurred at a time when total plasma calcium levels were about 30% lower than their levels in the control period (11 to 32 hr after indomethacin). There is a possibility that the calcium levels in the blood may be dictating, in part, the period of time that an egg spends in the shell gland. This hypothesis can be supported, in part, by the fact that in this experiment, as well as in the previous, all or a large number of the LH-induced eggs were prematurely expelled after indomethacin treatment.

In this experiment, as well as the previous ones in which egg shell thickness was measured, premature expulsion of the LH-induced egg resulted in thinner egg shells. However, when one looks at individual birds which did not prematurely expel this egg (or only expelled this egg by a short period before the control time), these eggs were still significantly thinner than controls.

Experiment 6

The results of this experiment, indicate that the presence of a hard-shelled egg in the shell gland was not the reason that premature expulsion of the additional LH-induced egg was being prematurely expelled from the shell gland, as seen in the previous experiments. These data show that even with no indomethacin-delayed hard-shelled egg in the shell gland that 5 of 7 eggs were prematurely expelled (ranging from 0.4 to 17.5 hr) when compared to their own control oviposition time for this LH-induced egg. The thinner egg shell of the treatment LH-induced egg reflects this shorter period of time in the shell gland.

There appears to be some difference between the control and treatment periods (though not significant) as to the time from LH injection to the time that the soft-shelled egg (which was the result of this injection) arrived at the shell gland. Their was a trend toward an acceleration of these events in the treatment period (after $PGF_{2\alpha}$ and indomethacin) in that 5 of 7 of these birds reduced this period by 0.1 to 2.2 hr.

The significance of this trend toward acceleration could implicate prostaglandins in the regulation of these two events. However, when considering the total time period that we are dealing with (12-13 hr from LH injection

to the time of soft-shelled egg's arrival to the shell gland), one might be forced to conclude there, in fact, may be no difference at all in these time periods.

Experiment 7

The results of this experiment suggest that there was no blocking effect of indomethacin on the LH stimulated follicle, as verified in the previous experiments, even when given orally (50 mg/kg BW) 3 hr after an LH injection. This time of administration of indomethacin, subjected the animal to levels of 30 to 60 $\mu$g/ml of indomethacin in the plasma for 3 to 4 hr before the time of ovulation.

As was the case in Experiment 6, there was a trend in the data, suggesting that there was a reduction in the time period from the LH injection to the time of the arrival of this ovum in the shell gland. The difference was significant at $p < 0.05$. Four of 6 birds demonstrated an early arrival of the ovum to the shell gland when compared to the controls. However, this researcher is not convinced that this trend is the result of the treatment, due to the very short time periods involved and relatively small number of animals used. The literature indicates that prostaglandins possibly play a role in ovum transport in the S.C.W.L. hen and the quail. However, the effect of an inhibitor of prostaglandins (indomethacin) on this

large time period (LH to soft-shelled egg in the shell gland), cannot be conclusively drawn from these experimental data.

Four of the 6 birds responding to LH injection started a slight premature expulsion of the LH-induced egg after indomethacin. However, this premature expulsion is not significant ($p > 0.5$).

In Experiment 6, the birds demonstrated a more dramatic premature expulsion of the LH-induced egg during the treatment period in that 5 of 7 birds expelled their eggs prematurely ($p < 0.01$). There were two basic differences in the procedures of these experiments:

(1) The last egg of the sequence in both the control and treatment period were expelled by $PGF_{2\alpha}$ prior to LH and placebo/indomethacin treatment in this experiment, whereas, in Experiment 6, $PGF_{2\alpha}$ was used to remove the indomethacin blocked egg from the shell gland just prior to the expected ovulation from the LH-stimulated ovary.

(2) The level of indomethacin in the plasma at the time of this LH-induced soft-shelled egg was higher in Experiment 7 than Experiment 6 due to the time of administration of indomethacin in relationship to the LH injection.

The second difference (above) between the experiments, may serve to partially explain this premature expulsion. Indomethacin was given 1 hr before the LH injection, which (according to Figure 1) would indicate that there would be relatively low levels of indomethacin in the blood at the time of the earliest expulsion of these soft-shelled eggs.

In the case of Experiment 7, the indomethacin was given 3 hr after the LH injection. This fact would place the indomethacin levels at those of the 10 hr period on Figure 1 ( 4 $\mu$g/ml) instead of the 14 hr period ( 2.5 $\mu$g/ml). The fact that the calcium levels have been shown to be reduced at 11 hr and to remain so until 32 hr may also have some effect on the early expulsion of this soft-shelled and hard-shelled egg, as was previously discussed.

## SUMMARY

A. Seven experiments were conducted in which S.C.W.L. laying hens were administered indomethacin (orally) for the purpose of determining its effect on several reproductive parameters and total plasma calcium. The results were as follows:

1. The ovulation of a mature follicle was not blocked by indomethacin when given orally:
   a) One hr before or up to 3 hr after LH injection
   b) Eleven to 12 hr before time of normal ovulation.
2. Ovum tramsport (measured indirectly from time of LH injection to soft-shelled egg in the shell gland) is not dramatically effected by indomethacin when given 1 hr before or 3 hr after LH injection--though there is a trend toward a reduction in this time period.
3. Total plasma calcium is lowered (approximately 30%) by at least 11 hr after indomethacin and remains low for at least 32 hr.

4. Normal time of oviposition is delayed and the subsequent expulsion of this egg occurs at or after a time when indomethacin is at very low levels in the blood.
5. Egg shell thickness in eggs which are expelled at about the normal time of oviposition ($\pm$ 2 hr), is significantly reduced after indomethacin.

B. The conclusions drawn from these experiments with the domestic laying hen, using the prostaglandin inhibitor indomethacin, are as follows:

1. Prostaglandins probably do not have an essential role in the processes of LH release or ovulation, as previously shown in mammals.
2. Prostaglandins may play a role in shell formation by regulating the absorption, mobilization and excretion of calcium.
3. Prostaglandins appear to have a role in the process of oviposition, as previously reported.

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