INVESTIGATIONS OF THE ACTION OF VITAMIN A AND BETA CAROTENE ON REPRODUCTIVE PERFORMANCE IN PIGS

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by

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PREFACE

The study presented in this thesis is original and was completed by the author while a full time student at the Victoria University of Technology, under the supervision of Drs R.G. Campbell and R.J. Fairclough.

I certify that the substance of this thesis has not already been submitted for any other degree and is not currently submitted for any other degree.

I certify that any assistance received in preparing this thesis and all sources used have been acknowledged in this thesis.

Jane Robertson

SUMMARY

The reproductive efficiency of the breeding sow is a major limitation to profitability in the pork industry. Investigations on the treatment of sows with retinol proprionate or retinol palmitate and their effect on reproductive function were undertaken. The objectives of this work were to investigate the transport and storage of this vitamin after injection, the mode of action of retinol on the ovary and the embryo, and the optimal timing of treatment with retinol to increase litter size in the sow.

The optimization of methods of extraction of beta carotene and retinol from plasma and tissue, the handling of samples to avoid degradation and quantitation by HPLC and spectrophotometry were investigated. It was found that an ethanol precipitation was adequate to extract retinol from plasma. Samples were separated by HPLC immediately after the precipitation. Beta carotene was extracted from plasma by first precipitating proteins with ethanol and then extracting with a 1: 1 mixture of diethyl ether and petroleum ether. Extracted samples were dried under a stream of Nitrogen and reconstituted for separation by HPLC. Both retinol and beta carotene were extracted from ovary and liver tissue using the same method. The main steps in this method were the homogenization of the sample in 1.15 % KCL, the precipitation of proteins from an aliquot of the homogenate with ethanol, followed by saponification using 1ON KOH at 70'C and then extraction with hexane. Extracts were collected and dried under a stream of Nitrogen and reconstituted for quantification. All extractions were carried out in subdued light to avoid degradation of samples.

The quantitation of retinol and beta carotene in plasma and retinol in tissue samples was carried out using reverse phase HPLC. For retinol in plasma, an isocratic elution of 47:47:6 (methanol: acetonitrile: chloroform) using a $C_{18.}$ reverse phase column with a UV-Vis detector set at 325 nm was used. The flow rate of the mobile phase was 1.0 ml/minute and retinol was eluted at 2.0 minutes, while

retinyl acetate, the internal standard appeared at 2.5 minutes. The same column type was used for the separation of beta carotene from plasma and a wavelength of 466 nm was used to detect beta carotene. An isocratic elution of 10:60:30 (methanol: acetonitrile: chloroform) at 1.3 ml/minute was used. The internal standard was beta-apo-8'-carotenal. Beta carotene was eluted at 4.2 minutes while the internal standard appeared at 2.5 minutes.

It was not possible in this thesis to optimize an assay which would satisfactorily separate retinol, retinyl acetate, beta carotene and beta-apo-8'-carotenal from tissues. The assay of retinol in extracts from tissues was carried out by reverse phase HPLC methods, using an isocratic elution of 95:5 (methanol: water) with the UV-Vis detector set at 350 nm.

Based on the quantitation of retinol and beta carotene in plasma and tissues, pharmacokinetic studies were carried out in multiparous sows to investigate the transport and storage of these agents using the optimized assays. In order to investigate the transport and storage of retinol in the sow after injection, three experiments were carried out. In the first experiment, catheterized multiparous sows (n=16) were injected intramuscularly with either 500,000 IU retinol proprionate (1 ml in Solutol) or 1 ml vehicle (Solutol). Blood samples were taken at 5 minutes before treatment, 5 minutes after treatment, 3, 6, 9 and 12 hours after treatment and then at intervals of 12 hours for a further 6 days. At 12 hours after treatment, animals treated with retinol proprionate had significantly higher plasma concentrations of retinol than control animals. Variation within treatment groups led to a second experiment, in which catheterized animals (n=8) were treated with either 1 ml of Solutol or remained untreated to investigate temporal changes in retinol over a 24 hour period. Blood samples taken at 30 minute intervals for 24 hours revealed that retinol concentrations are tightly controlled. There was no significant difference between treatment groups, indicating that Solutol did not cause changes in plasma concentrations of retinol. In a third experiment, multiparous sows (n=30) were injected intramuscularly with retinol roprionate

(500,000 1U, 1ml in Solutol) or 1 ml of Solutol and killed at 48 (n = 15), 72 (n = 10) or 96 hours after treatment (n = 5). Ovary and liver samples were collected at slaughter and the total retinol content in these tissues was quantitated. There was no significant difference between treated and control animals at any slaughter time for total retinol concentrations in the liver or the ovary.

The mechanisms of transport and storage of beta carotene was investigated in two experiments. In the first experiment catheterized multiparous sows (n = 18) were injected intramuscularly with either 250 ing beta carotene (in 6.5 mI Solutol) or 1 nil vehicle (Solutol). Blood samples were taken at 24 hours before treatment, 3, 6 and 12 hours after treatment and then at intervals of 12 hours for a further 5 days. At all times after treatment, beta carotene treated sows had significantly higher plasma concentrations of beta carotene than control animals. In a second experiment, multiparous sows (n=30) were injected intramuscularly with beta carotene (250 ing, in 6. 5 nil solutol) or 1 nil of Solutol and killed at 4 8 (n = 15), 72 (n = 10) or 96 hours after treatment (n = 5). Ovary and liver samples were collected at slaughter, the total retinol in 1 g of tissue was quantitated. There was no significant difference between treated and control animals at any slaughter time for total retinol concentrations in the liver or the ovary.

Retinol palmitate rather than retinol proprionate was used in two experiments to investigate the effects of retinol on follicle development. In the first experiment, carried out at Texas A&M University, 12 gilts were injected intramuscularly with either 1,000,000 IU retinol palmitate (1 mI in corn oil) or vehicle (1 ml corn oil) on day 16 of the oestrous cycle. Ovaries were surgically collected at day 18 of the cycle, follicles were measured and the follicular fluid was aspirated and assayed for oestradiol 17β and testosterone. In animals treated with retinol palmitate the follicles were significantly larger than those in untreated gilts, and the distribution of follicle sizes was skewed toward larger follicles in treated animals. Testosterone levels in follicular fluid of treated gilts were significantly higher than in control gilts, while there was no significant difference between retinol oestradiol

concentrations for either group. This was reflected in the follicular fluid oestradiol:testosterone ratio, which was significantly lower in treated than untreated gilts.

In the second experiment, carried out at Corowa NSW with identical treatments to the Texas experiment, follicles were smaller in both treated and untreated animals than those seen in the Texas experiment. There was a significant difference between gilts treated with retinol palmitate and controls in the distribution of follicle sizes. Follicles in the treated gilts were skewed towards the larger size classes. Both the follicular fluid oestradiol 17β and the testosterone concentrations in treated gilts were significantly higher in treated animals than untreated, and this was also reflected in the higher follicular fluid oestradiol:testosterone ratios in treated animals. These results suggest that retinol may play a major role in the development of the ovarian follicle.

In order to determine whether there was effect of retinol on expression of growth factors by developing embryos, an experiment was undertaken to study the effect of injecting retinol palmitate on the mRNA expression of IGF-1 by the embryo. Twelve gilts were injected with 1,000,000 IU units of retinol palmitate on day 16 of the oestrous cycle and a similar number of control gilts were injected with corn oil. Embryo's were collected between days 11 to 13 after the first mating. The RNA from the embryo's were extracted with Trizol, and subjected to Northern blotting using a 216 base pair IGF-1 probe. Results under stringent washing procedures indicated strong hybrization bands at 4 and 6.5 kb and weaker bands at 4 and 1.7kb. However there did not appear to be any marked differences in the expression of IGF-1 in retinol treated gilts compared to vehicle treated controls.

Optimization of the timing of treatment with retinol proprionate and/or treatment with folic acid was carried out at Corowa, NSW. Multiparous sows (n=850) were injected intramuscularly with retinol proprionate (500,000 IU retinol proprionate, 1 ml) on day 6 before mating (n = 135), on the day of mating (n = 135) or day

6 after mating (n = 137). Control animals were treated with 1 ml Solutol (vehicle, n= 133). The folic acid treatment was administered by daily supplementation of folic acid (5.32 mg/kg of the diet), which was fed from 6 days before mating until 35 days after mating (n = 155). A 6th group (n=155) was treated with retinol proprionate on day 6 before mating and fed the folic acid supplemented diet.

In the animals treated on day 6 before mating, the day of mating or on day 6 day after mating with retinol proprionate, there was no significant difference between treated and untreated sows for weaning to oestrus interval, farrowing rate, total piglets born, percentage of each litter born dead or number of piglets born alive. A comparison of the control sows with those treated with folic acid and those treated with folic acid and retinol proprionate on day 6 before mating revealed that the folic acid treatment significantly decreased the weaning to oestrus interval. However, there was no significant difference between the treatment groups for farrowing rate, total piglets born, percentage of each litter born dead and the number of piglets born alive.

These studies suggest that retinol palmitate is more efficacious in improving reproductive parameters than retinol proprionate. While retinol proprionate and beta carotene were detected in the plasma of treated sows, neither was increased in the of tissues. Retinol palmitate appeared to have a positive effect on the development of the follicle, possibly through the steroidogenesis pathway. However retinol did not seem to have an effect on the expression of IGF-1 in embryonic tissue although this effect must be viewed with caution as the numbers of embryo's were low. While the optimization trial did not bring about an increase in any reproductive parameters, the use of retinol palmitate in such a trial might improve litter size in the treated sow.

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For Cynthia,
who did not survive to see this work completed.

We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time.

T.S. Eliot

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General Introduction

The Industry

The Australian pig Industry is an important component of the Australian Agricultural Sector. Production of the pork industry at the farm gate is currently $700 million (Australian Pork Corporation). With an increasing export market as well as a strong domestic market, uniformity of product and year-round production are necessary to meet demand.

The Problem

One of the major constraints limiting profitability in the pork industry is lower than potential litter size. The average number of piglets born alive per litter in Australia is 10.5, ranging from 9.5 to 11.4. This compares favourably with the US average of 10.1 and the British average of 10.7 piglets per litter. The mean total piglets born per litter in the Australian herd is 11.3. However, simulations using AUSPIG have demonstrated that returns per sow can be increased by 55% if litter size were increased by 1 piglet per litter. Indeed, an increase of one piglet per litter is worth an extra $50 per annum, and based on a sow population of 300,000 this corresponds to an increased return of up to $15 million to the industry per year. The Australian pig industry is at present less competitive in the world market than it major competitors, the USA and Canada. The Australian industry spends $1.91 per kg carcass weight in production compared with $1.58 and $1.61 per kg carcass weight spent by the USA and Canada respectively (Campbell 1995). The most efficient way of lowering costs is to increase the number of piglets born alive per sow (Black 1994).

The Project

Recent studies in the USA have shown that injection of retinol or beta carotene is associated with an increase of 0.5-1.0 piglets born alive per litter, while

maintaining individual piglet weight (Coffey and Britt 1994, Chew et al 1991, Britt 1992). However, not all studies have shown a positive result. The variations in litter size in response to retinol or beta carotene may be due to differences in the retinol ester used, dose rates, timing of treatment and sow age.

The hypotheses investigated in these studies were:

(1) That injected retinol proprionate or beta carotene increase total litter size and improve other reproductive parameters such as weaning to oestrus interval and number of piglets born alive in the sow.

(2) That injected retinol proprionate or beta carotene will be detected in the plasma and stored in various tissues in the sow.

(3) That injected retinol palmitate has a direct or indirect effect on the development of ovarian follicles via advancement in steroidogenesis and/or expression of mRNA for growth factors such as IGF-1.

(4) That injected retinol palmitate has a direct effect on the development of the embryo via the expression of mRNA for growth factors such as IGF-1.

Chapter 1:
Reproduction in the sow

1.1 Introduction

In order to understand the problem of the failure to reach reproductive potential in the pig industry, this chapter outlines the major points of interest in the reproductive processes of the sow.

Failure to return to oestrus in multiparous sows (6% of culls) or failure to farrow for various reasons, including abortion and fertilization failure, in gilts and older sows (18% of culls) have been shown to contribute to the high culling rates (PRDC News 1993). Often in small and larger piggery units it is not economically viable to maintain non productive animals due to cost of feed and housing. Efficiency of reproduction in the sow can be increased by reducing the incidence of reproductive failure and/or increasing litter size. Overcoming reproductive failure and increasing litter size may increase the efficiency of many Australian piggeries, since an increase of one piglet per sow is worth an extra $50 per annum. Based on a sow population of approximately 130,000 in Australia this represents an increased return in the region of $6m to the industry per annum.

High embryo mortality, ranging from 20-45%, is a major cause of reproductive failure (Flint et al. 1982). In the gilt much of this loss occurs during the first 30 days of pregnancy. In the muliparous sow however, losses in the embryonic phase are lower and a higher proportion of losses occur at the fetal stage possibly due to limited uterine capacity (Pere et al. 1995). The physiological basis for the high rate of embryo loss is however, uncertain. Embryo wastage due to chromosomal aberrations and abnormal fertilizations, usually less than 10% of embryonic deaths, does not account for all embryonic loss. Recent studies by Lambert et al. (1991) and others have shown that most embryo loss occurs at day 11 of gestation, the time of elongation and attachment. Reproductive inefficiency may also be due to a variety of factors including diversity in oocyte development, follicle or embryo development, plane of

nutrition, hormone levels and general uterine environment and environmental conditions (Pope et al. 1990).

1.2 Follicular development

1.2.1 Summary of follicle recruitment and maturation

Primordial follicles are formed during fetal development in the pig. After the birth of the piglet no increase in the number of primordial follicles occurs. A detailed description of oogenesis and development is given by Wassarman and Albertini (1994). According to these authors the first stages of reproductive development the surface epithelium of the presumptive ovary becomes separated from the central cellular mass. This is followed by the proliferation of the ovarian cortex and the transformation of oogonia into oocytes. The oogonia enter the early stages of pre-meiosis before becoming separated from the mesenchyme by a single layer of differentiating follicle cells. Transformation of oogonia to oocytes continues until at least day 35 after birth. Meiosis is arrested at the diplotene stage presumably by autocrine and paracrine changes within the encapsulating primordial follicle. Most germ cells reach the diplotene stage by day 20 after birth. Female piglets are born with approximately one million primordial follicles (Anderson 1993).

Development of primordial follicles to primary and then graafian follicles occurs as early as day one post partum and continues throughout the reproductive life of the animal (Baker 1982). Although oocytes remain in the diplotene stage until just before ovulation, their diameter may increase threefold (Anderson 1993). In the sexually mature female, primordial follicles leave the non-growing pool by becoming primary follicles surrounded by a layer of flat epithelial or pre-granulosa cells. These cells become cuboidal and increase in number by mitosis. The developing follicle thus becomes two then three

layered. After a set number of divisions and a fourth layer, blood capillaries invade the cells around the follicle. These form a vascular coat or the theca interna. In turn, this is surrounded by the fibroblasts of the theca externa, the only source of nutrients for both the granulosa cells and the oocyte (Baker 1982). In the pig, thecal development is not consistently observed until late in the development of the preantral follicle, when an average of 11 layers of granulosa cells is present (Greenwald and Roy 1994). As development proceeds, fluid accumulates in the inter-cell spaces and gathers to form a single antrum (Figure 1.1, Baker 1982, Greenwald and Roy 1994).

The development from the primary follicle (oocyte surrounded by two layers of granulosa cells) to the small antral size of around 400μm takes 84 days in the pig and further growth to a 3mm follicle requires a further 14 days. Development to preovulatory size of about 8 mm requires a further five days (Greenwald and Roy 1994). The stimulus for recruitment into the follicular phase is uncertain. Recruitment is probably associated with adequate FSH level and an increase in episodic and basal LH secretion (Britt et al. 1985, Foxcroft and Hunter 1985).

Follicle recruitment occurs from a proliferating pool of about 50 follicles sized between 2 and 5mm in diameter (Anderson 1993), between days 14 and 16 of the oestrous cycle of the mature gilt (Anderson 1993, Foxcroft and Hunter 1985). The development of follicles recruited from this pool during the follicular phase and destined for ovulation is associated with the rapid atresia of smaller follicles. Replacement of these follicles from the proliferating pool is prevented until the next cycle (Clark et al. 1982, Grant et al. 1989). Further work has shown that the number of follicles greater than 6 mm in diameter in the early follicular phase is frequently lower than the expected ovulation rate. Further, a considerable difference between stages of both morphological and biochemical development of dominant follicles exists (Grant et al. 1989).

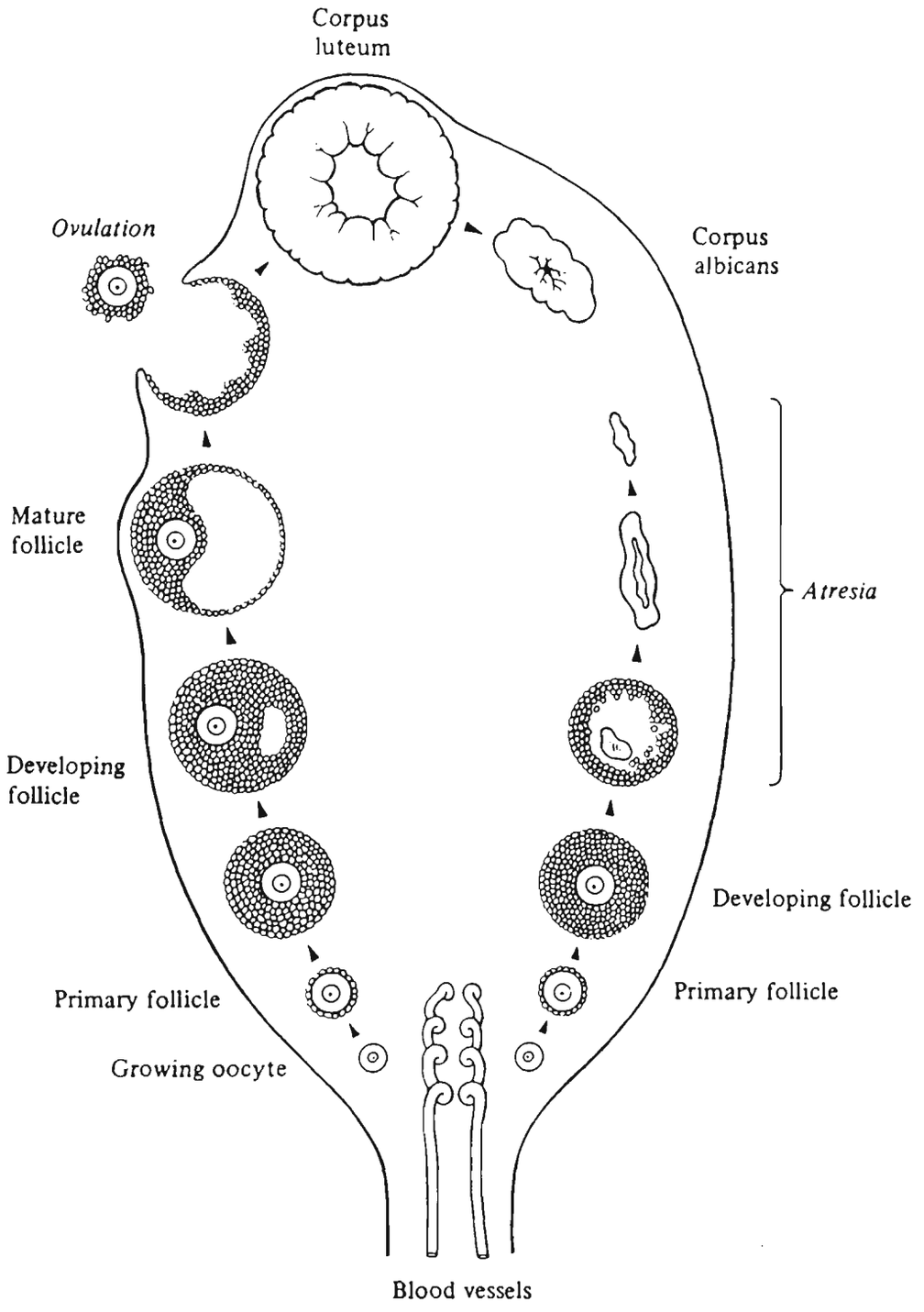


Figure 1.1 Stages of follicular development (From Baker 1982)

Xie et al. (1990) demonstrated that follicle development within gilts was skewed, that is, a small percentage of follicles nearing ovulation were less developed. Smaller selected follicles escape atresia but do not attain the maturity of the dominant follicles in the ovulatory batch. The number these follicles present at the time of recruitment which are sufficiently mature to respond to the preovulatory LH surge, therefore ultimately determines the ovulation rate (Hunter and Weisak 1990). Some immature follicles continue to develop past day 16 but the decline in aromatase activity by day 18 prevents the granulosa cells from synthesising oestradiol. Androgen precursor availability is also limited by low LH binding to both theca and granulosa cells. Lack of aromatase activity decreases the oestrogen availability within unselected follicles and therefore the full complement of LH and FSH receptors is never reached and follicles undergo atrophy (Foxcroft and Hunter 1985).

Several models have been proposed to offer a mechanism for the timing of recruitment of follicles. One is that the recruitment of follicles in an ovulation batch occurs at a set time when follicles selected are at markedly different stages of development. Alternately, the recruitment process may begin at day 14 or 15 and continue into the follicular phase, changing the hormonal stimulus for recruitment as the follicle matures (Foxcroft and Hunter 1985). Hunter et al. (1976) postulated that follicular recruitment must be completed by day 17 of the cycle since 91% of 5-6mm follicles marked between days 17 and 21 were represented by corpora lutea.

1.2.2 Steroidogenesis

The results of several studies on steroidogenesis in the pig support the "two cell, two gonadotrophin" theory (see reviews by Foxcroft and Hunter 1985 and Ainsworth et al. 1990). This hypothesis suggests that thecal cells under the influence of LH synthesize androgens, which are then transported to the granulosa cells where they serve as substrates for oestrogen synthesis under the

specific stimulation of FSH (Ainsworth et al. 1990, Gore-Langton and Armstrong 1994). Figure 1.2 shows a schematic representation of steroidogenesis according to this theory.

Gore-Langton and Armstrong (1994) extensively reviewed the current hypotheses for steroidogenesis. The model suggested by these authors is presented. Undifferentiated granulosa cells respond exclusively to FSH by a cAMP-dependent mechanism. This mechanism stimulates the enzyme activity required for the metabolism of cholesterol to progesterone and for the conversion of theca derived androgens to oestrogens. Steroid biosynthesis in the theca cells is stimulated exclusively by LH. As the follicle matures, plasma membrane receptors for LH and prolactin are acquired by the granulosa cells as a result of FSH action and stimulation by oestrogen. LH may initially contribute to granulosa cell differentiation by enhancing the various cAMP-dependent processes. A variety of steroidal influences on the activities of steroidogenic enzymes may come into play as the follicle becomes more steroidogenic. These effects may occur as the result of receptor-mediated actions of oestrogens, androgens and progesterone influencing responsiveness to FSH or LH. The apparent induction of the aromatase system is enhanced by androgenic and oestrogenic actions but is inhibited by progestins under certain circumstances. 5α-reduced metabolites of androgens may competitively inhibit the aromatase system while oestrogens may suppress 5α-reductase. While prolactin has been shown to prevent the induction of aromatase activity both *in vitro* and *in vivo* under certain conditions, its role in regulating oestrogen synthesis during the ovarian cycle is still uncertain. Androgens may also facilitate the synthesis of progesterone. After the LH surge, steroid production is briefly stimulated and then suppressed as FSH and LH receptors in granulosa and theca cells become desensitized. Transient increases of oestogen by granulosa cells causes inhibition of follicular oestrogen synthesis by the suppression of androgen biosynthesis in the theca cells. Progesterone production is restored subsequently and increases greatly as follicle cells

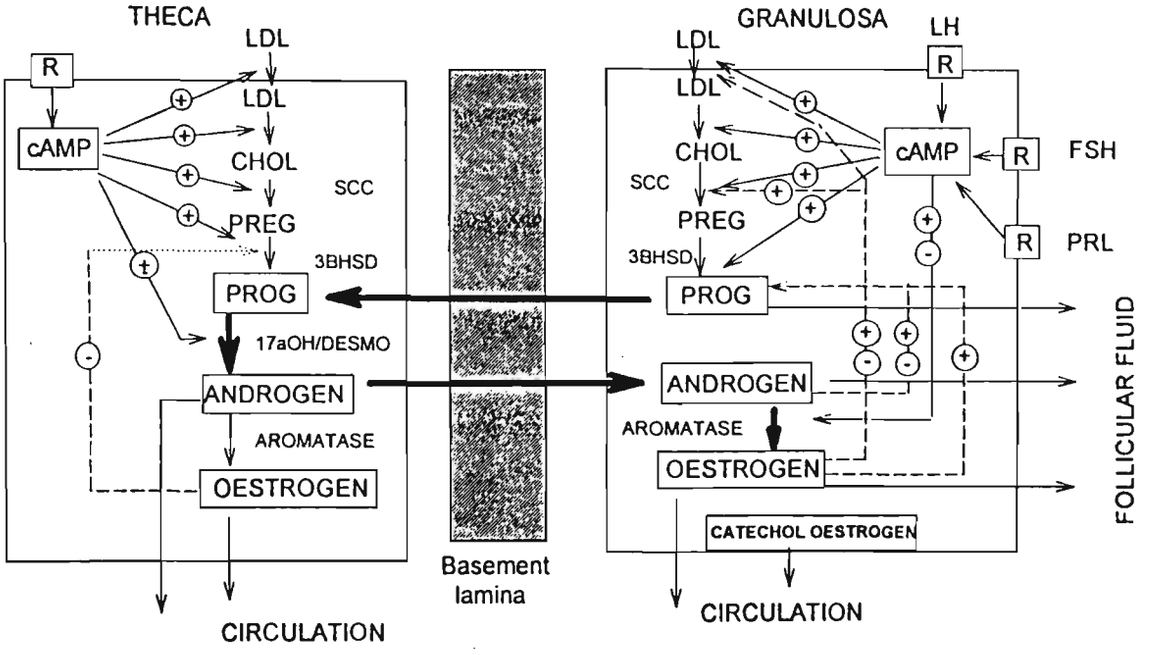


Figure 1.2. Principal sites of follicular steroidogenesis in the pig, diagram based on those from Gore-Langton and Armstrong (1994) and Ainsworth et al. (1990). Arrows pointing to the steroidogenic pathways indicate whether the enzyme is stimulated (⊕) or inhibited (⊖) by FSH or LH. Dotted lines represent the intrafollicular regulation of steroidogenesis by steroid hormones and stimulation or inhibition of the enzyme. Abbreviations: LDL=low density lipoproteins, CHOL=cholesterol, PREG=pregnenolone, PROG=progesterone, SCC=cholesterol side-chain cleavage enzymes, 3βHSD=3β-hydroxysteroid dehydrogenase, 17αOH/DESMO=17α-hydroxylase and $C_{17\text{-}20}$-desmolase, R=membrane-bound receptor for FSH, LH or prolactin.

luteinize. (Gore-Langton and Armstrong 1994).

The granulosa cells are the predominant sites of progesterone synthesis and are responsive to LH, FSH and prolactin. Pig granulosa cells lack the 17-α hydroxylase enzymes that synthesise androgens *de novo* from progesterone or pregnenolone (Conley et al. 1994). Oestrogen synthesis by these cells in culture has only been demonstrated in the presence of aromatizable androgens or when co-incubated with thecal tissues (Evans et al. 1981). FSH appears to bind only to granulosa cells (Nakano et al. 1977; Gore-Langton and Armstrong 1994) and the number of FSH receptors and the FSH-sensitive cAMP activity of granulosa cells stimulating the production of progesterone decreases as the follicle enlarges (Kolena and Channing 1971). *In vitro* work has shown that granulosa and thecal cells from large follicles contain more LH/hCG receptor sites than those in smaller follicles. The increase in LH/hCG binding sites by granulosa cells is associated with an increase in LH-stimulated cAMP production, accompanied by a decrease in FSH-stimulated cAMP production (Ainsworth et al. 1990). Increasing concentrations of the androgens, androstenedione and testosterone may contribute to the fall in response to FSH. According to Lischinsky et al. (1984) the addition of these androgens to cultures of granulosa cells from medium sized follicles inhibits FSH-stimulated progesterone synthesis, possibly by acting on the conversion of pregnenolone to progesterone. Ainsworth et al. (1990) assumed that this decreased responsiveness to FSH reflects a loss of FSH receptors.

Specific prolactin receptors have been isolated from pig granulosa cells. Rolland and Hammond (1975) showed that the specific binding to prolactin receptors is greater in cells of follicles less than 2mm in size than those of 6mm or greater. Prolactin has been shown to suppress *in vitro* progesterone production by granulosa cells from small follicles (<2 mm) but as the follicle matures, the action of prolactin on granulosa becomes stimulatory (Veldhuis et al. 1981). This may be one of the factors that is responsible for atrophy.

The main androgen produced by the theca tissue is androstenedione (Tsang et al. 1985). This androgen is transferred to the granulosa cells presumably by diffusion across the basement membrane. At the level of the basement membrane it is converted to testosterone and then aromatised to oestradiol under the specific stimulation of FSH. Unlike other species (eg. rat, cow, sheep; Heap et al. 1979) the cultured pig theca interna tissue produces oestradiol in similar quantities to granulosa cells (Evans et al. 1981, Foxcroft and Hunter 1985). However, Tsang et al. (1985) postulate that the thecal cells produce less oestrogen than the granulosa and oestrogen production is limited by aromatase activity rather than lack of androgens. These researchers suggest that the production of oestrogen by the granulosa cells *in vivo* increases significantly with follicle maturity. This is due to the increased number of granulosa cells per follicle, increasing availability of aromatase and the availability of thecal androgen. Foxcroft and Hunter (1985) suggest that both FSH and LH may activate aromatase synthesis in the thecal tissue because of the presence of the aromatase enzyme system. It has been reported that follicular fluid oestradiol concentrations increase with the advancing maturity of the follicle from day 17 to day 20 (Hunter et al. 1976). Oestradiol is thought to be involved in the induction of LH receptors and amplifies the stimulatory effects of FSH on steroid production by the pig granulosa cells (Veldhuis et al. 1982). After exposure to the LH/hCG surge there is a sudden decline in follicle oestrogen production due to the decrease in aromatase activity of the theca and granulosa cells (Tsang et al. 1985).

Oestrogens and androgens act in several ways on follicular granulosa cells and are critical to the developing follicle. There is increasing evidence that the interactions between granulosa and theca interna cells in follicular steroidogenesis are more complex than is suggested by the simple "two cell" theory (Conley et al. 1994, Ainsworth et al. 1990). *In vitro* studies have shown that androgens stimulate progesterone synthesis by granulosa cells. The available evidence suggests that androgens inhibit basal and FSH-stimulated

androgen production in the granulosa cell of the pig (Evans et al. 1981). Oestrogens have been shown to stimulate or inhibit progesterone production by pig granulosa *in vitro* depending on specific oestrogen treatment and the developmental stage of the cells.

1.2.3 Non-steroidal influence on the development of follicles

Follicular growth cannot be accounted for entirely by changing concentrations of gonadotrophins (Esbenshade et al. 1990, Tonetta and Di Zerega 1990). Tonetta and Di Zerega (1990) suggest that the developing responsiveness of follicles to stimulation by gonadotrophins is the result of changes in the production of, and alterations in, the sensitivity of the follicles to intra-ovarian paracrine and autocrine factors.

Factors that may influence the maturation of follicles include Inhibin, Activin, Follstatin, Follicle Regulating Protein and growth factors such as the Epidermal Growth Factor family and the Insulin-like Growth Factors. A brief description of each of these factors is given, and since IGF I is investigated in this work, a more detailed summary of its role in follicular development will be presented.

Briefly, inhibin is produced by the granulosa cells in the porcine follicle and increases during the follicular phase of development (Hasegawa et al. 1988). Its role appears to be the inhibition of FSH production, and therefore control of the FSH-stimulated cAMP cascade which controls steroidogenesis. Inhibin concentrations decrease at the time of the LH surge and then peak twice during the formation and lifetime of the corpus luteum indicating a role after follicular development (Tonetta and Di Zerega 1990). Activin occurs in 2 forms (activin and activin A) which are dimers of the β-subunit of inhibin (Ling et al. 1986). According to a review by Tonetta and Di Zerega (1990), these two peptides equally enhance the secretion of FSH in a dose dependent manner, without affecting LH production. These researchers state that activin has been shown to

enhance FSH-stimulated induction of LH receptors in the sheep but has little effect on progesterone secretion in the granulosa cells of this species. Receptors for the activins appear to be enhanced after gonadotrophin stimulation. However, the specific role for these proteins in follicular maturation is yet to be elucidated.

Follistatin is a single peptide chain found in porcine follicular fluid. Like inhibin it appears to suppress the release of FSH, but not LH (Ueno 1987, Tonetta and Di Zerega 1990), rather than completely inhibit FSH release like inhibin. When added to cultured pituitary cells with inhibin an additive effect is shown on the inhibition of the release of FSH. Studies have suggested that while GnRH regulates the synthesis and release of FSH from the pituitary, steroidal and non-steroidal factors such as follistatin, inhibin and the activins appear to act as secondary regulatory factors in the regulation of FSH (Tonetta and Di Zerega 1990).

Follicle regulatory protein (FRP) inhibits aromatase activity in the granulosa cells of the sow. FRP is secreted by the granulosa (but not theca) cells of small and medium follicles (Tonetta et al. 1988). Secretion of this peptide decreases as the follicle reaches preovulatory size, suggesting a link with increasing steroid production. Schreiber and Di Zerega (1986) suggest that FRP is able to modulate other FSH-responsive activities in the follicle such as induction of LH receptors, cAMP synthesis and 3βHSD, indicating that preovulatory follicles which are recruited for development are exposed to FSH early in development. In later work, Tonetta and Di Zerega (1990) reported that high concentrations of FSH in follicular fluid reduced the sensitivity of follicles to FRP, while early exposure of cells to FRP resulted in suppression of maturation.

The Epidermal Growth Factors are a family of growth factors including amphiregulin, TGFα (Transforming growth factor), EGF, and Heparin-Binding

EGF-like Growth Factor (Hammond et al. 1993). Epidermal growth factor is present in many tissues including theca cells and can stimulate the nuclear maturation of porcine oocytes (Ding and Foxcroft 1993). All four members of the EGF family are expressed in the porcine ovary, but their relative importance and mode of regulation are yet to be defined. One role for these peptides is thought to be in stimulating follicular growth, and in the selection and luteinization of follicles (Hammond 1993). In pig theca cells, EGF inhibits oestradiol production but does not affect secretion of progesterone or androgens (Caubo et al. 1989). EGF may therefore selectively modulate steroidogenesis in theca and granulosa cells as suggested by Tonetta and Di Zerega (1990).

1.2.3.1 Insulin-like growth factors (IGFs)

Insulin-like Growth Factors (IGFs) promote replication and differentiation in cultured cells. IGFs affect almost every aspect of granulosa cell development and function including: the enhancement of FSH-stimulated production of progestagen, oestrogen, cAMP and proteoglycans and LH receptor induction (Iwashita et al. 1994, Echternkamp et al. 1994, Hammond et al. 1993). Mondschein et al. (1989) have demonstrated that the concentration of IGF-I increases in the follicular fluid with follicle maturity. Hammond et al. (1994) showed in a series of cell culture experiments that IGF-I can serve as an autocrine amplifier of gonadotrophin action in those follicles developing the ability to express this growth factor. The same group have demonstrated that in immature porcine follicles, granulosa cells secrete IGF-I and that the levels of this peptide are enhanced by the presence of LH and FSH in a cAMP dependent manner (Hsu and Hammond 1987). These researchers reported that gonadotrophins, oestrogen and cAMP stimulated the secretion of immunoreactive IGF-I by ovarian cells. Further, strong evidence has been put forward demonstrating that in the presence of IGF- I, FSH, oestradiol and GH alone and in various combinations significantly stimulate progesterone production, but when a monoclonal antibody to IGF-I is introduced,

progesterone production is inhibited by approximately 50% (Mondschein et al. 1989). In the thecal cells IGF-I increases basal and gonadotrophin-induced secretion of progesterone and enhances LH-induced synthesis of androstenedione and testosterone (Caubo 1989). The androgens are necessary for the synthesis of oestrogens by the granulosa, but limit the production of oestrogen by the theca cells (Lischinsky et al. 1984). Therefore, IGF-I decreases secretion of oestradiol in cultured pig theca cells. Specific receptors for IGF-I have been demonstrated in pig theca and granulosa cells. FSH and FSH-stimulated steroids have been shown to increase the levels of IGF-I receptor. IGFs have been shown to affect lipoprotein binding, the processing of cholesterol and its esters, side-chain cleavage activity, aromatase, and 17α-hydroxylase (Hammond et al. 1993).

The level of IGF activity appears to be controlled by the IGF binding proteins (IGFBPs), of which 6 have been isolated. Of these, IGF binding proteins 2-5 have been isolated from follicular fluid and granulosa cells. The most abundant binding protein in these media is IGFBP 3 (Mondschein et al. 1991). It is interesting to note that concentrations of IGFBPs 2, 4 and 5 decrease with follicular growth and development (Howard and Ford 1992, Samaras et al. 1993). These researchers showed that as concentrations of oestrogen increase in the follicular fluid, concentrations of IGFBP-2 decrease. This suggests that IGFBPs, in particular IGFBP-2 play an important role in the selection of follicles for atresia (Hammond et al. 1994). Guthrie et al. (1995) demonstrated that the concentration of IGFBP-2 was three times higher in atretic than healthy follicles between 5 and 7 days after oestrus, associated with a significant decrease in oestradiol in the follicular fluid.

Hammond et al. (1993) have suggested a working model for the role of IGFBPs in the function of IGFs in the ovary. IGFs bound to IGFBPs are central to this model, providing a reserve of potentially active IGFs to the system, which can then be presented to the IGF-I receptors on the various target cells. The

presence of IGFs on receptors appears to positively feed back on the production of IGFs via the expression of mRNA. It seems that the IGFBPs themselves can bind to attachment sites on the cell or to mucopolysaccharides in the extracellular matrix. Post-translational modification of the IGFBPs by such steps as phosphorylation, glycosylation or proteolytic processing may modify the affinity for IGFs of these binding proteins and therefore the delivery of the IGFs to receptors.

On the basis of this and numerous other studies, Hammond et al. (1991) put forward the following model for the action of IGF- I in the development of the ovarian follicle: FSH and steroids stimulated by FSH increase the receptor levels for IGF-I; FSH and IGFs act synergistically to positively feed back on the cAMP-dependent steroidogenic cascade; IGF production is stimulated by gonadotrophins in granulosa cells in the presence of oestradiol; and FSH decreases the secretion of the inhibitory IGFBPs. However, much work is still required to clarify the role of IGF I in the development of the ovarian follicle.

1.2.4 Oocyte maturation

In the pig the ovarian follicle consists of a central germ cell or oocyte and somatic cells (granulosa and theca). When the antrum forms, two morphologically distinct populations of granulosa cells develop: mural granulosa cells lining the wall of the follicle and the cumulus granulosa cells that encompass the oocyte and form the cumulus cell-oocyte complex (COC). The cumulus cells are coupled by gap junctions to both the oocyte and the surrounding mural granulosa cells. Physical contact and intracellular communication between the oocyte and the somatic cells is necessary for the maturation, development and survival of the oocyte (Coskun et al. 1995). The two types of granulosa cells differ in their distribution of receptors and in their steroidogenic capabilities. In addition, each cell type shows a marked difference in the response to the LH surge. Gonadotrophins stimulate cumulus

granulosa cells to produce and secrete hyaluronic acid, which disperses or expands the cumulus cells. Mural granulosa cells do not undergo expansion but adopt a luteal role (Vanderhyden 1993).

The primary oocyte itself grows during follicular development. Oocyte diameter may increase threefold before the LH surge but the nucleus remains arrested at the diplotene stage of meiosis (Hirao et al.1994). On reaching maturation the oocyte either resumes meiosis in response to the LH surge or undergoes degeneration during the process of follicular atresia.

A detailed description of oocyte maturation is given by Baker (1982) and Wassarman and Albertini (1994). Briefly, at the LH surge the oocyte contains lampbrush chromosomes but the synthesis of RNA characteristic of the early stages of growth ceases. The chromosomes shorten and thicken with the lampbrush loops withdrawn. Chiasmata move along the chromosomes to become terminal threads resembling crosses and chains within the nucleus. These events occur at the germinal vesicle stage of meiosis and complete the prophase of the first meiotic division. Metaphase rapidly follows, in which the bivalents arrange themselves on microtubules at the equator of the meiotic spindle. During anaphase the bivalents move to opposite ends of the spindle, which makes a slow rotation through 90° causing the axis to become radially oriented. This rotation is completed by early telophase, with the repulsion of the two sets of chromosomes complete and the area of the spindle between them elongated. A non-equational division of the cytoplasm of the oocyte rapidly occurs in which the secondary oocyte receives most of the cytoplasm, while the minimum of the ooplasm is contained in the first polar body. Shortly after the extrusion of the first polar body the secondary oocyte undergoes the second meiotic division. Prophase is very short and the chromosomes condense to form a half-moon-shaped mass at the edge of the oocyte. Spindle microtubules appear beside the chromatin and the chromosomes arrange themselves on the metaphase plate. Metaphase II is a stage of arrested development, which

usually coincides with ovulation. Further meiotic maturation of the oocyte is dependent on penetration by a spermatozoon at fertilization.

The agents responsible for the inhibition or the resumption of meiosis are the subject of much discussion in the literature. The evidence for somatic cells being responsible for the inhibition of nuclear maturation is compelling. One hypothesis suggests that factors such as oocyte-maturation inhibitors accumulate in the follicular fluid, maintaining meiotic arrest (Tsafriri and Channing 1975). Other models are based on the inhibitory factor being produced by somatic cells and transferred to the oocyte via gap junctions. Control of the meiotic process then depends on either the production of the inhibitory factor or its transport (Mattioli et al. 1994). Meiosis resumes after the LH surge, which is thought to act on the somatic cells since no LH receptors appear on the oolemma. Mattioli et al. (1994) suggest that the LH surge causes the uncoupling of cells from the oocyte, freeing the germ cell to resume meiosis, presumably in the absence of the inhibitory factor. In this context, as LH uncouples the cumulus cells from the oocyte, the flow of cAMP (one of the proposed inhibitory signals) to the oocyte is interrupted.

1.3 Ovulation

Follicles in an ovulatory batch are often at different stages of development. The high concentrations of oestrogen in those preovulatory follicles which are destined to ovulate will therefore appear at different times in well developed compared with less developed follicles. The high concentrations of oestrogen in those preovulatory follicles which are destined to ovulate will therefore be expected to cause a positive feedback signal for the preovulatory surge of LH. Therefore, the oestrogenic activity of the most advanced follicles would be expected to set the timing of the LH surge and ovulation.

Xie et al. (1990) found that the patterns of maturation of follicles and ovulation

were skewed, ie a minority of follicles was smaller. The same researchers found that the chronological order of ovulation whether natural or artificially induced, parallelled the order of zygote development. If the later ovulating smaller follicles are destroyed by electrocautery, less developed low-protein embryos do not appear at day 11 (Pope et al. 1990). In contrast, Soede and Kemp (1993) did not find any relationship between duration of ovulation and embryo diversity when they examined ovulation and subsequent embryo development using transrectal ultrasonography, suggesting that other factors may influence embryo diversity.

1.4 Embryonic Development

Several excellent reviews of embryonic development are available. Figure 1.3 shows the main stages of blastocyst formation (Baker 1982). According to Soede et al. (1995) and Findlay (1993) ovulation commences 35-36 hours after the onset of oestrus, but varies widely from 10 to 58 hours, in the sow and pro-nuclei-bearing oocytes appear in the oviducts 6-8 hours later. The embryo reaches the 4-cell stage at 30 hours post fertilization and does not develop further until it enters the uterus on day 3-4 after pregnancy. The zona pellucida degrades between days 6 and 8, followed by formation of the bilaminar blastocyst at day 10-11. At day 11 post coitum the corpora lutea of the cycle are "rescued" and thus become the corpora lutea of pregnancy. Following mesoderm formation, the ovoid sac elongates to a filamentous blastocyst. By this stage the embryos have positioned themselves along the uterine horns. Over a period of 3-4 days embryos increase in size from the ovoid sac (about 0.5-1 cm) to 100-200 cm in length. The blastocyst attaches to the endometrial epithelium at day 11 and secretion of specific endometrial proteins to nourish the conceptus begins (Roberts et al.1993, Bazer, 1992, Flint et al. 1982). The blastocyst begins to expand from an ovoid sac to a filamentous blastocyst at day 12-14. Many elongated blastocysts can be accommodated in each horn due to the overlaying folds of the pregnant endometrium.

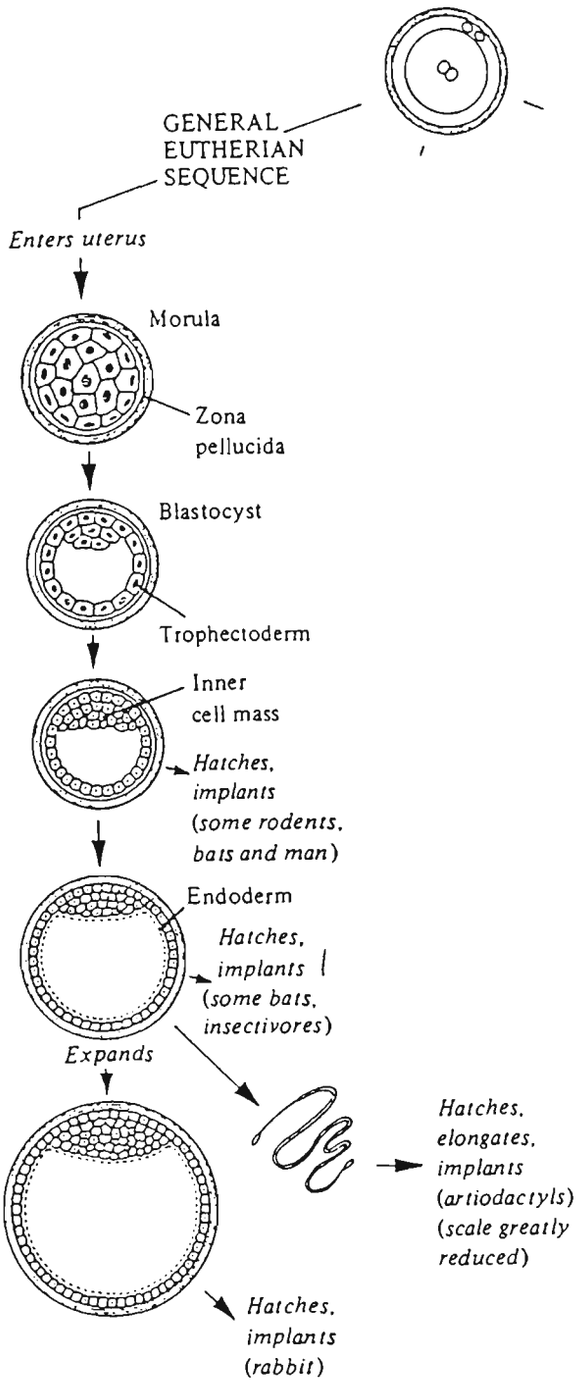


Figure 1.3 Stages of blastocyst development (From McLaren 1982)

According to the review by Findlay (1993), by day 14, local areas of apposition of the trophectoderm and epithelium of the endometrium can be distinguished, followed by the appearance of the trophoblastic areolae and progressively increasing areas of apposition. By days 18-22 microvillous interdigitation occurs between the surfaces of the trophectoderm and the endometrial epithelium and by day 24 chorio-allantoic fusion is complete. Therefore, definitive attachment occurs around days 18-22 in the pig. The outer trophoblastic layer of the blastocyst is non-invasive and no pronounced decidual reaction characterizing implantation in the stroma is seen as in rodents and primates.

1.4.1 Local mechanisms on the development of the embryo

Uterine secretions play an important role in the development of the embryo. The following section highlights several of the major growth factor families and a brief description of their actions in embryo development.

1.4.1.1 Insulin-like growth factors

Peak concentrations of IGF-I occur at day 12 of pregnancy with diminished but detectable levels in mid to late pregnancy (Simmen and Simmen 1990, Tavakkol et al. 1988). IGF-I is synthesised by the uterine endometrium and released into the lumen of the uterus where it comes into contact with the conceptus. This temporarily coincides with blastocyst oestrogen synthesis and elongation (Simmen and Simmen 1990). The signals for the synthesis of IGF-I have not been defined but it appears to respond to exogenous treatment with oestrogen or growth hormone in the rat (Simmen and Simmen 1990). Since plasma oestrogens remain low in concentration until day 16 of pregnancy, well after the peak of synthesis of IGF-I, blastocyst-derived oestrogen may trigger the release of IGF-1 rather than maternal sources (Simmen and Simmen 1990). It is possible therefore that a paracrine role for IGF-I in conceptus development exists.

IGF-II has long been considered a fetal growth factor although no specific function has yet been identified. The lowest concentration of IGF-II mRNA occurs in early pregnancy (pre-implantation) but a greater than 10-fold increase occurs by day 30 of pregnancy, declining again at day 90-110. Since the pattern for the appearance of IGF-II is not similar to that of oestrogen, this steroid is unlikely to be responsible for the observed changes in IGF-II.

The actions of the IGFs are mediated by IGF-I and IGF-II receptors in the plasma membranes of target cells. Binding proteins for the IGF family play an important role in determining the biological effect of IGF-I and IGF-II (Rutanen et al. 1988). These proteins inhibit or stimulate IGF bioactivity (Brewer et al. 1988). IGF-I and IGF-II may represent major mediators of uterine secretory activity in the pig.

1.4.1.2 Epidermal Growth Factor

The role of EGF in early pregnancy may be twofold. It may induce endometrial growth and differentiation to create a favourable growth environment for the foetus. A second possible role is that the conceptuses may use luminal EGF for their developmental needs. Labelled mouse EGF has been shown to bind to day-12 to 16 pig conceptuses (Letcher et al. 1989), suggesting a biological function for EGF in conceptus growth or development. The source of EGF is probably the uterine cells, implying an autocrine and paracrine mechanism of EGF in early pregnancy. The mechanism controlling EGF production in early pregnancy is unknown.

1.5 Embryo diversity in the pig.

Embryo diversity is most evident in the pig from early cleavage to somite formation (Pope et al. 1990) and the relationship between diversity and embryo mortality is complex. Wright and his co-workers (1982) recovered pig

blastocysts from sows on days 6-9 of pregnancy and showed that around 20% of embryos contained less protein than the average protein content of all embryos in the same uterus; however, viability was difficult to estimate by size alone. These apparent differences in stage of development suggest that the low-protein embryos may be less viable and more susceptible to changes in the uterine environment than the high-protein embryos.

Wilde and colleagues (1988) recovered day 7 blastocysts from gilts and segregated them into 3 groups, largest, intermediate and smallest. Four to five of each of the smallest and largest were transferred to opposite, but ligated uterine horns of synchronous (day 7) or asynchronous (day 6) recipients. By day 12, the originally smaller blastocysts were less viable after synchronous transfer than were their larger litter-mates. However, in the less advanced recipients, the smaller blastocysts survived as successfully as their larger litter mates. This suggests that less developed blastocysts are more susceptible to an advanced uterine environment than morphologically mature blastocysts (Pope et al. 1990). More recently Cassar et al. (1993) showed that male embryos grow faster than female embryos from hatching to the commencement of attachment. However, when data were analysed for survival rates at day 11 post-conception no significant difference was found between the survival of male and female embryos. This suggests that embryos need to be within a critical window of growth development before their survival is assured.

1.6 Uterine environment and embryo survival in the pig.

Well developed embryos appear to synthesise more oestradiol than do lesser developed litter mates. Pope et al. (1990) suggest that as a result, segments of the gravid uterus adjacent to the more developed embryos may become more advanced than sections next to the lesser developed embryos and thus grow more quickly. As embryonic synthesis of oestradiol increases, all portions of the uterus become exposed to oestradiol and the local uterine environment by the lesser developed embryos becomes more advanced. The entire uterine

environment then provides substances (such as proteins, growth factors, calcium and prostaglandins) which are far in advance of the needs of the less developed blastocysts and asynchrony occurs.

Oestrogens appear to stimulate the growth of the endometrium through a variety of mechanisms (see review by Findlay 1993). One of the factors which may be involved in mediating the effect of oestrogen on endometrial growth is EGF. This factor has well known mitogenic and differentiating effects and mRNA expression encoding IGF-1 has been reported in endometrial tissue of both the mouse and the rabbit. It has been shown that oestrogen can stimulate the production of EGF from mouse endometrial cells *in vitro* and influences the expression of the mEGF receptor in rodents. EGF is also found in the uterine fluid of both non-pregnant and pregnant sows, peaking at days 10 and 11 of pregnancy suggesting a role in embryo development (Diehl et al. 1993).

Chapter2:

The retinoids and reproduction

2.1 The Retinoids

Much work has been done to document the importance of the retinoids in regulating vision (Dowling and Weld 1960), growth (Zile and Cullum 1983, Schoene et al. 1988), reproduction (Thompson et al. 1964), and health (Chew 1987). Vitamin A is found in three forms: retinol, retinoic acid and retinal (Frickel 1984). The difference between these forms is in their chemical oxidation stage, but each form plays a different role in regulating physiological functions.

Retinol is the major form in which Vitamin A is transported and is stored in the liver in the esterified form. During absorption of Vitamin A from other animal sources, the esters are completely hydrolysed in the small intestine to free retinol, which is then transferred across the mucosal cell membrane. Once inside the mucosal cells the retinol becomes re-esterified with long-chain fatty acids, usually palmitic, stearic and the oleic. These esters are transported first to the lymph then to the blood (Lambert et al. 1985). Retinyl esters in excess of the body's immediate requirements are stored in the liver (Ball 1988a).

Retinaldehyde (retinal) is part of the dye molecule of rhodopsin, the visual protein in the retina, essential for normal night vision (Chew 1993, Ball 1988a). Retinoic acid is important in maintaining cellular differentiation. This form alone is unable to sustain aspects of male and female reproduction or vision (Zile and Cullum 1983, Ball 1988a). Preformed sources of vitamin A are more common in meat than vegetables or other plant sources (Chew 1993).

Plant sources of vitamin A contain the precursors to vitamin A, known as carotenoids. However, of more than 600 naturally occurring carotenoids (Straub 1987), less than 10% possess provitamin A activity, of which beta-carotene is the most biologically active. Beta carotene has a role in regulating immune function, specifically in increasing the number of helper T cells in mice and humans, and in promoting mitogen-based lymphocyte proliferation in rats and pigs (Chew 1987)

when administered orally. Brief and Chew (1988) and Coffey and Britt (1993) have demonstrated a function for beta carotene in the reproductive processes, possibly via a special antioxidative action (Burton and Ingold 1984).

While most animals can convert beta carotene to a retinoid form at the lining of the intestinal wall, efficiency of conversion varies between species. Further, large species differences occur in the ability to absorb beta carotene intact. Chew et al. (1984) found that carotenoids are only detected at very low concentrations in the peripheral circulation of the pig, in contrast with Parker's (1989) findings in cattle and humans. These species differences in the absorption and conversion of beta carotene to vitamin A may be due to the differences in the amount and activity of conversion enzymes, the presence or absence of transport proteins, or other factors.

2.1.1 Conversion of beta carotene to retinol or retinoic acid

Dietary beta carotene is converted to retinol by the action of enzymes in which molecular oxygen reacts across the central C-15,15' double bond to form peroxide (Ball 1988a). The peroxide cleaves to yield two molecules of retinaldehyde. A small proportion of the retinaldehyde is irreversibly oxidised to retinoic acid, while the remainder is reduced to retinol by means of enzyme action. This conversion occurs at the lining of the intestinal wall and at the liver of many species and in the liver alone in man (Cremin and Power 1985). An alternate pathway for conversion has been suggested by Schweiter and Isler (1967), in which the conversion of beta carotene to retinaldehyde is via stepwise conversion of the provitamin to form apocarotenoids such as beta-apo-8'-carotenal. Beta carotene is not stored in body tissues of the pig. However, it has been found in high concentrations in the ovary, in particular the corpus luteum of the cow (O'Fallon and Chew 1984) and in the tissues of other species (Schweigert 1990).

2.1.2 Biological activity and deficiency

It is well known that deficiency of vitamin A produces a range of adverse changes affecting the eyes, skin, nervous system, bones, mucous membranes and the reproductive system (Ball 1988a). Many signs of deficiency are attributable to the lack of differentiation of mucus-secreting cells in the membranes lining the eyes, respiratory tract, alimentary tract and urogenital tract. The effect of deficiency on these membranes is cessation of differentiation of membrane cells and loss of secretory function. The undifferentiated cells become flattened and multiply at an increased rate causing cells to pile up on one another and keratinisation occurs. In this condition the cells are very susceptible to infection. Reproductive changes include atrophy of the testes and male accessory glands and of the ovaries. Other signs of deficiency may be attributed to disturbed bone growth, impaired utilisation of feed, lack of antibody formation, night blindness and raised cerebro-spinal fluid pressure.

Typical symptoms of deficiency in animals include roughened hair (cattle) and scaly skin. Prolonged deficiency affects the eyes. In breeding animals deficiency may lead to infertility, absorption or stillbirths. Ganguly et al. (19971) showed that in retinol deficient female rats there was a reduction in progesterone and 20-α-hydroxypregn-4-ene-3-one in the ovarian venous blood at certain days of the cycle. It has been suggested that vitamin A deficient cows suffer lower conception rates due to the reduced synthesis of the sex hormones and cornification of the vaginal epithelium causing irregular oestrus (Ball 1988a). Deficiency of vitamin A in sows may be responsible for weak, dead, or malformed piglets (Palludan, 1975). Besides acting as a precursor to vitamin A, beta carotene plays a specific role in progesterone synthesis in cattle and is necessary for the maintenance of oestrous cycles (Ball 1988a). Defects in fetal development are attributable to necrosis of the placenta. In the male, continued deficiency leads to degeneration of the seminiferous tubules and a consequent reduction in semen volume and sperm count, and an increased proportion of malformed spermatozoa (Ball 1988a).

2.1.3 Vitamin A and Reproduction

Vitamin A is essential for the maintenance of reproductive function and fetal development (Thompson et al. 1964). Brief and Chew (1985) demonstrated that gilts injected with retinol at mating and weekly until weaning showed a decrease in embryo mortality. This resulted in more piglets per litter, which did not differ in piglet weight from those born to unsupplemented sows.

Characteristic symptoms of deficiency in pregnant sows include birth of weak, dead or deformed piglets. Vitamin A is thought to overcome these symptoms by influencing steroidogenesis and the uterine environment (Chew et al. 1982). Ganguly et al. (1971) showed that rats reared on a vitamin A deficient diet had a reduced ability to secrete progesterone. Later work by Talavera and Chew (1988) demonstrated that collagenase-dispersed porcine luteal cells showed increased production of progesterone when incubated in the presence of retinol and retinoic acid.

Retinol may directly influence the uterine environment and the development of the embryo. Alternately it may affect the uterine environment indirectly by affecting ovarian progesterone production (Chew 1993, Chew et al. 1982). Vitamin A status affects placental protein secretion. For example, Steele and Froseth (1980) found that in vitamin A deficient sows there were structural and compositional changes in placental glycosamineglycans. Other uterine proteins that may be affected by vitamin A levels (eg uteroferrin, the serpin superfamily and retinol binding protein) are reportedly influenced by progesterone, although data in this area are inconsistent (Chew et al. 1982, Roberts and Bazer 1980, 1988). These proteins are an important source of nutrition to the conceptus (Roberts and Bazer 1980) and are particularly important in the pig since the conceptus does not invade the uterine epithelium in the true sense of implantation. One of the uterine

proteins is uteroferrin, an iron-carrying purple glyco-protein secreted by the endometrium (Bazer 1975). A group of low molecular weight proteinase inhibitors with antiplasmin activity, a basic glycoprotein in the serpin superfamily of proteins and retinol binding protein also number among these proteins (Roberts et al. 1993).

Retinol-binding protein (RBP) and retinoic acid binding protein (RABP) have been isolated from the endometrium, ovary, testis and other tissues of the pig (Chytil et al. 1975, Adams et al. 1981). RBP is progesterone-induced and is present in uterine fluid during the luteal phase of the oestrous cycle (Adams et al. 1981). The pig conceptus also produces RBP throughout the peri-implantation period. Secretion may occur as early as day 10 of pregnancy (Harney et al. 1990, Trout et al. 1990). Retinol binding proteins are further discussed in following sections.

2.1.4 Mechanisms of action of retinoids.

Retinoids are fat soluble with an affinity with water-soluble retinoid-binding protein in plasma (Kanai et al. 1968), cytoplasm (Chytil and Ong 1984) and nucleus (Petkovich et al. 1987) of cells and uterus (Adams et al. 1981).

2.1.4.1 Follicular and oocyte development

Hormonal conditions are critical for the normal development of follicles and subsequent ovulation of oocytes. As suggested earlier, retinol may influence ovarian steroid production.

Bagavandoss and Midgely (1987) demonstrated that at a low dose of retinol or RA (10^{-9}M) in cultured granulosa cells enhanced the ability of FSH to induce LH receptors and stimulate cAMP production and therefore progesterone and oestrogen synthesis. RA did not stimulate progesterone production in cultured granulosa cells in the absence of LDLs. In the presence of lipoproteins, retinol or

RA increased progesterone production 3-fold by LH associated increases in lipoprotein receptors on the luteal cell surface. Bagavandoss and Midgely (1987) concluded that 3βHSD (the enzyme responsible for the oxidation of pregnenolone to progesterone) was not rate limiting and that the observed progesterone increase represented an increased availability of cholesterol to the pathway. However, at higher concentrations (10^{-6}M) retinoids inhibited the FSH effects on LH, reduced LH receptor levels, reduced the production of cAMP and therefore reduced steroid production.

Strong similarities exist in the action of retinol on steroidogenesis in the granulosa and theca cells in the female and the Leydig and sertoli cells in the male. Information from one cell model is likely to be useful in investigating the other.

It has been proposed by Galdieri and Lorenzo (1994) that retinol or retinoic acid at a concentration of 0.25μM (or $0.25x10^{-6}$) added to rat sertoli cells in culture decreases FSH-induced production of cAMP. A series of experiments carried out in the Galdieri laboratory suggested that retinol modulated the activity of the stimulatory component of adenylate cyclase. Since the aromatization of androgens to oestrogen is also actively regulated by FSH, a similar decrease in activity is attributable to the effects of retinol.

Lefevre et al. (1994) report that retinol and retinoic acid at concentrations of around 10^{-6}M induced a down-regulation of LH binding sites in rat K9 Leydig cells. This was time and dose dependent and may have been due to a reduction of the mRNAs for these binding sites. Given the time requirement for downgrading (25-35% repression over 48 hours) these authors suggest that the effect of retinol or retinoic acid is likely to be indirect. However, retinol and retinoic acid greatly enhanced P-450 17α mRNA levels (responsible for androgen synthesis) in a time and dose dependent manner. At the same time 3βHSD mRNA levels (the enzyme responsible for the oxidation of pregnenolone to progesterone) at first rose (at lower concentrations of retinol or retinoic acid), but at higher concentrations of

retinol or retinoic acid 3βHSD levels were repressed as P450 17α reached its peak.

2.1.4.2 Maturation of the oocyte

As discussed earlier, the maturation of the oocyte is an essential part of the ovulation process and this is marked *in vivo* by the resumption of meiosis. Mattioli et al. (1994) found in contrast to the material presented above, that a transient increase in the intracellular concentration of cAMP occurs in the first hours of maturation. This returns to basal levels at about the time of germinal vesicle breakdown. While it is generally held that cAMP is the oocyte maturation inhibitor, particularly in the rodent model, several groups have found an increase in cAMP in the oocyte at the resumption of meiosis in the rabbit (Yoshimura et al. 1992) and sheep (Moor and Heslop 1981). Mattioli et al. (1994) further suggested that pig oocytes can synthesize cAMP and that the production of this nucleotide is likely to be stimulated by a soluble factor which originates from LH-stimulated mural granulosa cells. Retinol and retinoic acid have been shown to increase LH, which stimulates progesterone production, in turn enhancing retinol-binding protein production, which resulted in an increase in cellular retinol availability. The increase in cAMP appears to occur with the progressive desensitization of granulosa cells to LH. This desensitization is likely to be caused by down regulation of LH receptors by the LH surge. Coskun et al. (1995) found that the porcine oocyte secretes a heat-stable, charcoal extractable molecule that inhibits FSH-induced steroid production by cumulus and mural granulosa cells.

If the suggestion that retinoic acid or retinol was released from the oocyte after being imported from the cytoplasm is correct, then it may occur via CRABPs and RABPs both inside and outside the oocyte. CRABPII is localized in the cytoplasmic compartment and is a small protein, of a size which should not exclude it from the nucleus. However, CRABP is not found in the nuclear compartment unlike CRABPII. It has been suggested that CRABP and CRABPII

act as a "sink" for retinoic acid, restricting access to the nuclear receptors (Boylan and Gudas 1991). The hypothesis may be supported by studies showing accelerated catabolism of retinoic acid when high levels of CRABP are present in retinoid-sensitive cells. This occurs despite a lower affinity of retinoic acid for CRABP compared with the nuclear receptor (Boylan and Gudas 1992). When the proposal of the RA sink and the increased catabolism of RA are put together, a mechanism allowing some cells (but not others) to be desensitized to the presence of RA is put in place. CRABP expression corresponds with peak times of differentiation, raising the possibility that its function is to relieve the suppression of differentiation pathways by RA.

2.1.4.3 Embryo survival

Adams et al. (1981) reported that the total amount of vitamin A secreted from the uterus increased in progesterone-treated pigs. These researchers suggested that retinol increased transport of nutrients across the uterine epithelium during pregnancy when blood progesterone was elevated. In addition, RBP may also have increased local transport of retinoids by RBP to the developing conceptus. The mechanism of cellular uptake of the retinoids is not fully understood. They may be taken up by target cells through a receptor-mediated mechanism, through nonspecific spontaneous transfer or through fluid-phase endocytosis (Blomhoff et al. 1991). Once inside the cell the retinoid is bound to cellular retinoid proteins. Cellular binding proteins for retinol, retinoic acid and retinaldehyde have been identified (Blomhoff et al. 1991) as well as nuclear retinoic acid receptors (Petkovich 1987) in at least three distinctive forms (Mangelsdorf et al. 1990). These receptors belong to a superfamily of hormone receptors that bind DNA. Two hypotheses for the role of cellular retinoid-binding proteins have been suggested (Chew 1993). The first is that cytoplasmic carrier proteins may serve to transport bound retinoids to sites of metabolism. The second is that the cytoplasmic proteins may serve to transport bound retinoids to the nucleus, where the retinoids (retinoic acid) but not the binding protein become bound to nuclear

retinoic acid receptors. Cytoplasmic carrier proteins play an important role in the movement of retinoids to the nucleus, thereby regulating the concentration of the retinoids. The nuclear retinoic acid receptors function as direct agents in gene regulation related to cell differentiation (Chew 1993).

Petkovich et al. (1987) and Dolle et al. (1989) have suggested that the mechanism of action for retinoic acid is similar to those described for steroid hormones and vitamin D3. Retinoids may directly affect embryo development by regulating cell differentiation and proliferation (Schindler 1986), transcription of specific genes (Chiocca et al. 1988) or they may act as a morphogen. Alternately retinoids may indirectly influence embryo development by regulating ovarian steroid production (Talavera and Chew 1988), immune cell function (Chew 1987) or interferon production (Blalock and Gifford 1976, 1977). All of these may affect the establishment and maintenance of pregnancy. Through these combined actions, retinoids may regulate follicular maturation, early embryo development and development of the conceptus (Chew 1993).

2.2 Beta carotene and reproduction

Given the assumption that the only action of beta carotene had in reproduction was to exist as a provitamin A, there has been little work on the effect of beta carotene itself on reproduction in the sow. However, studies in the cow have shown that beta carotene has an important role in controlling reproductive efficiency which is distinct from that of retinol. White-fat animals such as the pig have a low ability to absorb carotenoids in the small intestine. Studies on the possible role of beta carotene in the multiparous pig have shown lower embryo mortality, larger litter size and heavier litter weight at birth and weaning (Brief and Chew 1985, Coffey and Britt 1993). No treatment effect has been seen in gilts. Chew (1993) suggests that the effect of beta carotene is to decrease embryo mortality given that progesterone-induced uterine secretions have the potential to lower embryo death. Gilts injected with beta carotene on alternate days from mating until day 15 of

pregnancy had greater quantities of uterine-specific proteins than untreated gilts at day 15 (Chew et al. 1982). Talavera and Chew (1988) also showed that beta carotene stimulated a 10-fold increase in progesterone production in collagenase-dispersed porcine luteal cells after an incubation period of 24 hours in beta carotene. The response was several orders of magnitude higher than that observed in the presence of retinol and retinoic acid.

2.2.1 Mechanism of action of beta carotene

The mechanism of action of beta carotene in reproduction has not yet been elucidated. It may serve as an antioxidant against lipid peroxidation (Burton and Ingold 1984) and in so doing protect the uterine and ovarian steroidogenic cells from damage due to oxidation. Chew (1993) suggests that beta carotene may regulate nuclear events in target tissues. The uptake of beta carotene into subcellular components of blood lymphocytes but not blood neutrophils or erythrocytes implies the presence of a carotenoid carrier protein (Chew et al. 1991 a, b).

The hypotheses investigated in this thesis were:

(1) That injected retinol proprionate or beta carotene increase total litter size and improve other reproductive parameters such as weaning to oestrus interval and number of piglets born alive in the sow.

(2) That injected retinol proprionate or beta carotene will be detected in the plasma and stored in various tissues in the sow.

(3) That injected retinol palmitate has a direct or indirect effect on the development of ovarian follicles through an effect on steroidogenesis and/or expression of mRNA for growth factors such as IGF-I.

(4) That injected retinol palmitate has a direct effect on the development of the embryo via the expression of mRNA for growth factors such as IGF-I.

Chapter 3:
General Materials and Methods

The purpose of this chapter is to outline methods which were used in more than one experiment during the study of the effects of retinol on reproductive parameters of the sow. Methods which are unique to a single experiment in the results section are described in the relevant chapter. An outline of experiments carried out is shown in Figure 3.1.

3.1.1 Location of Experiments

Experiments were conducted over a period of three years (1994-1997), most trials being at Bunge Meat Industries (BMI), Corowa, in south-eastern New South Wales, 35°E,148° S. Some experiments were also carried out at Huntly, N.S.W.

Part of the experimentation for Chapter 7, the work investigating the effect of retinol on follicle development, was carried out at Texas Agriculture and Machinery University at College Station, Texas, United States of America, 30° 36' E, 97° 12' S.

3.1.2 Animals

Animals used in all experiments excepting those used in the USA were Large White x Landrace sows and gilts bred at Bunge Meat Industries, Corowa. For the component of Chapter 7, carried out in Texas, the gilts were Duroc x Hampshire x Yorkshire crosses. Specific details of age and breeding status of animals used in trials appear in the relevant chapters.

3.1.3 Husbandry

Bunge Meat Industries

Sows at BMI were housed in either single crates or in groups of up to 35 depending on reproductive status. Diet also varied according to the reproductive

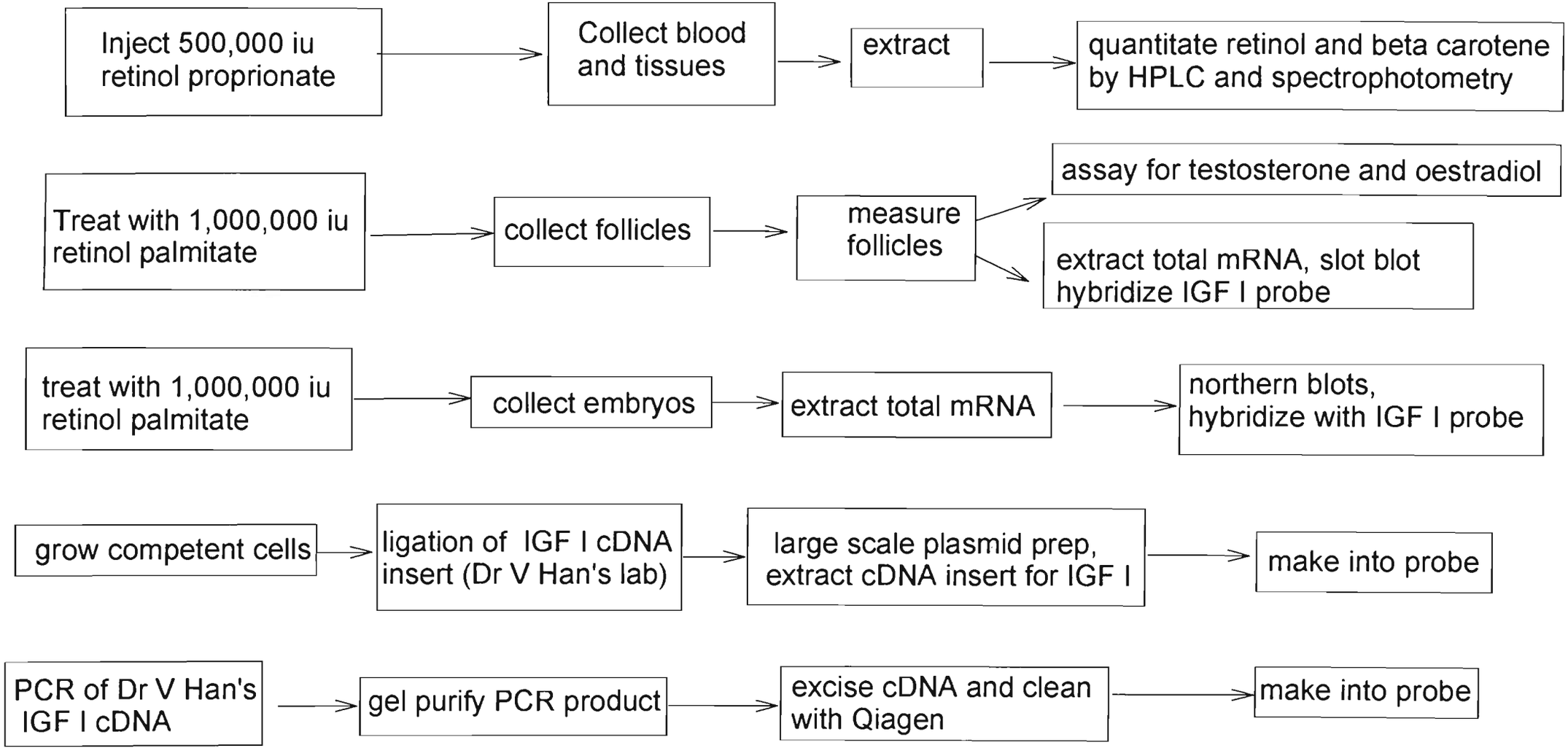


Figure 3.1 Outline of field and laboratory work carried out in this project

state of the sows. Examples of the lactation and dry sow diet are shown in Table 3.1. Pregnant and lactating sows were fed ad libitum while dry sows received 2.25 kg of feed per day.

Mating at BMI was carried out in the morning when sows were detected in heat by the back pressure test and then 24 hours later with a second boar to ensure fertilization.

Table 3.1. Composition of basal diets[a].

Dry sow feed		Gestation feed mix	
Wheat	40%	Wheat	45%
Barley	17.6%	Barley	10%
Millmix	30%	Peas	10%
Soya bean meal	4.6%	Millmix	14.2%
Meat meal	3.2%	Soya bean meal	10%
Water	1%	Meat meal	4.5%
Tallow-mixer	1.6%	Water	1%
Salt	0.25%	Tallow-mixer	3.2%
Limestone	1%	Salt	0.25%
Premix	0.7%	Limestone	1%
		Premix	0.72%
Dig. energy	13.0	Dig. energy	13.9
Crude protein	13.99%	Crude protein	17%
Lysine	0.77%	Lysine	0.78%
Calcium	0.9%	Calcium	1.0%
Phosphate	0.75%	Phosphate	0.68%

[a]The basal diets contained 15,000,000 iu vitamin A per tonne and 0.5 g/tonne folic acid. Additional folic acid was added to diets to attain desired final folate levels

for folic acid treated animals.

Texas A&M University

Gilts at Texas A&M were housed in groups of up to 20 and were fed 2.5 kg per day of the standard dry sow diet. Gilts in this trial were observed for oestrus daily in the presence of intact boars and the first day of oestrus was designated day zero. After gilts had experienced one or two normal oestrous cycles, they were randomly assigned to treatment groups.

3.2 Experimental techniques

All experimentation was carried out in accordance with the Animal Ethics act and was approved by the Animal Welfare committees at Bunge Meat Industries, Texas A & M and Victoria University of Technology. Embryos were collected at surgery so that the animals could be returned to the BMI breeding program. All equipment and instrumentation used for this body of work are described in Appendix B.

3.2.1 Surgery

Bunge Meat Industries

In preparation for surgical flushing of embryos at Corowa, gilts were fasted for 12 hours prior to surgery. Anaesthesia was induced with 5% Thiopentane and maintained with halothane. Surgery was conducted under aseptic conditions and gilts recovered in separate pens until able to stand. On the following morning animals were returned to group pens.

On the day of tissue collection in the trial at Texas A&M, gilts were transported to the surgery where anaesthesia was induced with sodium thiamylal and maintained with halothane. Surgery was conducted under aseptic conditions. Gilts recovered at the surgical facility overnight and were returned to the swine centre the following morning where they were housed in groups.

3.2.2 Jugular catheterization of sows via the ear

Sows were immobilized by nose rope in a restraining crate. A rubber band was placed around the base of the ear to raise the vein and the surface of the ear was swabbed with alcohol. A Jelco IV catheter placement unit (14 gauge) was used to enter the auricularis rostralis vein, after which the rubber band was removed. The needle was withdrawn and the Teflon sleeve left in the vein, through which 30-35 cm of clear vinyl tubing (internal diameter 1 mm, external diameter 1.5 mm) was inserted, leaving between 10 and 15 cm outside the ear. The tubing was taped to the ear at the point of insertion with non waterproof tape. A three-way stopcock was placed on the end of the tubing and this was confined in a bag taped to the back of the sow's neck. Indwelling catheters were flushed and kept patent with 1:20 sodium heparin: 0.9% normal sterile saline.

3.2.3 Blood sampling

Blood samples were taken from sows at either 12 hour or 30 minute intervals, as per the protocol of the trial being carried out (see specific chapters). The heparin saline in the catheter was withdrawn until blood entered the syringe. This first blood was discarded. The sample (10 ml) was then drawn into a fresh syringe coated with heparin:saline solution (1:20). Clean heparin saline was reintroduced to the catheter after the sample was taken. Blood samples were transferred to 10 ml amber tubes and placed in the dark until centrifugation, carried out as soon as

possible after collection. Plasma was collected into plain, 5 ml amber tubes and frozen at -20°C immediately pending analysis by HPLC.

Solutol HS 15 is the BASF registered name for Polyethylene glycol 660 hydroxy stearate. It is a non-ionic solubilizer for injection solutions. Solutol has high chemical stability and is recommended for aqueous parenteral preparations with vitamins A, D, E and K and a number of other lipophilic pharmaceutical active agents such as propanidid, miconazole and others (BASF Technical Information Sheet)

3.3 Laboratory Procedures: Extraction of retinol and beta carotene from plasma and tissue and HPLC

Development of extractions, HPLC and spectrophotometric methods and their optimization is detailed in Chapter 4 contained in part II of this volume.

3.4 Techniques used for RNA studies

All techniques detailed below with the exception of the Trizol extraction of embryos and granulosa cells are used directly from or slightly modified from Current Protocols in Molecular Biology, edited by Ausbel et al. (1996).

3.4.1 Total mRNA extraction from granulosa cells and embryos

Total mRNA was extracted from granulosa cells and embryos with Trizol ®, (Gibco Australia) using the protocol provided by Gibco. Four ml of Trizol was added to the cells. The mixture was triturated and vortexed until all cells were lysed and left to stand for 5 minutes. For embryos, the tissue was homogenized, then 4 ml of Trizol was added. The mixture was triturated and allowed to stand at room temperature for 5 minutes. Chloroform (200 μl) was added and the mixture was vortexed well and allowed to stand at room temperature for a further

5 minutes. The samples were then centrifuged at 4000 rpm for 10 minutes at 4°C and the upper phase was transferred to a new tube. An equal volume of isopropanol was added and the mixture was allowed to precipitate overnight at -20°C. Following centrifugation at maximum speed for 15 minutes at 4°C, the supernatant was decanted. The pellet was dried and resuspended in TE buffer. Samples were stored at -80°C pending the determination of concentration and purity of nucleic acids and slot blotting.

3.4.2 Total mRNA extraction from porcine liver tissue

The method used is slightly modified from that shown in Current Protocols in Molecular Biology (Ausbel et al. 1996). A known weight of tissue (0.5-1.0 g) was placed into a 50 ml RNase free falcon tube, to which 10 ml guanidium thiocyanate containing 78 μl/ml 2-mercaptoethanol was added. A Takmar Tissuemizer® was used to homogenize tissues in 4 bursts of 4-5 seconds. Samples were kept on ice throughout the procedure. One ml of 2M sodium acetate was added followed by 10 ml water-saturated phenol and the sample was mixed by gentle inversion. After the addition of 2 ml chloroform, the sample was vortexed for 10 seconds and cooled on ice for 15 minutes. Following centrifugation at 3,800 rpm for 20 minutes, the upper phase was transferred to a new 50 ml tube and 10 ml isopropanol was added and the contents of the tube were gently mixed. Samples were precipitated in the -20°C freezer for 2 hours then centrifuged at 3,800 rpm for 20 minutes. After the supernatant was decanted and the tube blotted on a clean paper towel, the pellet was resuspended in 2 ml TES buffer and transferred to a new 15 ml conical tube to which 2 ml chloroform was added. The mixture was vortexed and centrifuged at 3,800 rpm for 10 minutes then the upper phase was transferred to a new 15 ml conical tube and 200 μl of 2M sodium acetate and 2 ml isopropanol were added and mixed. The sample was stored at -20°C overnight to precipitate RNA.

Samples were centrifuged at 3,800 rpm for 30 minutes the following day, the

supernatant was removed, the tube was blotted dry and the pellet was washed in 1 ml of 70% ethanol. Samples were centrifuged at 3,800 rpm for a further 10 minutes, the supernatant decanted and the pellet was dried for 10-15 minutes. TE buffer (100-200 μl) was added to resuspend the pellet and the mixture was left at room temperature for 10 minutes, vortexed gently and stored at -80°C pending testing for concentration and purity.

3.4.4 Agarose Gel Elecrophoresis

Analysis of RNA was carried out in non-denaturing gels containing 0.8 -1.0 % agarose in 1 x TAE buffer (40mM Tris, 20mM sodium acetate, 2mM EDTA pH 7.4). Electrophoresis was carried out at 80 volts for the required time. RNA samples (usually 1-5 μg) were loaded after addition of loading buffer comprising 20 % Ficoll, 10mM EDTA and 0.001% bromophenol blue. Xylene Cyanol was omitted from the loading buffer as this has a tendency to obscure the flourescence of RNA bands if stained with ethidium bromide. Generally a third of total sample volume was added. Gels were stained with ethidium bromide (0.5mg/ml) after completion of electrophoresis.

3.4.5 Formaldehyde Gel Elecrophoresis

Denaturing formaldehyde gels were used to separate RNA species of different molecular weights for northern blot analysis. These gels contained 1.2% agarose, 0.6 M formaldehyde in 1 X MOPS buffer. Gels were allowed to set for several hours before use. Prior to loading, RNA samples (5 - 20 ug) were denatured at 65^{0}C in a total volume of 20 μl containing 1 x MOPS, 3.5μl formaldehyde and 10 μl formamide.

A small quantity (5 μl) of formaldehyde gel loading buffer was added before sample application. If visualisation of RNA samples was required, 1μl of ethidium bromide (100 μg/ ml) was also added before loading.

Electrophoresis was carried out at 60 volts for the appropriate time.

3.4.5 Downward blotting of RNA (for Northern blot)

A glass dish was lined with plastic wrap and narrow paper towels were stacked inside the dish to a height of 5-6 cm. The gel was checked under long-wave UV light and measured, then washed for 20 minutes in 200ml 10x SSC. The nylon membrane for the blot was cut to the same size as the gel and soaked in 20x SSC for 2 minutes. Three pieces of dry blotting paper cut to the size of the gel were placed on top of the paper towel stack, followed by piece of blotting paper wet with 20x SSC. The nylon membrane was placed on top, followed by the gel, face down. The surface of the gel was rolled with a sterile glass pipette with firm downward pressure to remove air bubbles. A piece of blotting paper wet with 20x SSC and cut to the same size as the gel was placed on top. A second glass dish lined with plastic wrap and filled with 20x SSC was placed alongside the dish containing the blotting stack. A long piece of blotting paper was soaked in the 20x SSC. One end of this blotting paper was placed on the top of the stack the other end remained in the SSC forming a wick. The blotting paper was held clear of the paper towel stack to ensure that the SSC passed through the gel. The stack was left to blot for 1.5 to 2 hours.

Northern hybridization

Prehybridization

The membrane was wet with 6x SSC and placed RNA side up in a petri dish. One ml of high SDS 50% formamide prehybridization solution containing 10 μg/ml yeast tRNA was added per 10 cm^2 of membrane (see appendix A). The dish was covered, sealed and placed in a hybridization oven at 42° -46°C for at least 2 hours, but usually overnight.

3.4.6 Labelling of cDNA probes

IGF-1 specific cDNA probes were radioactively labelled by nick translation using a commercial kit from Promega, Australia.

The double stranded cDNA fragment to be used as the probe (25-100 ng) was not denatured. One μl of the cDNA in water was transferred to a new microfuge tube and the following was added: 10 μl of an equal mixture of dGTP, dTTP and dATP, 5 μl of 10x nick translation buffer, 2.5-5 μl α ^{32}P-dCTP (70μCi at 2-3000 Ci/mM and 10 Ci/ml), DNA polymerase/DNase mixture and sterile distilled water to bring the sample volume to 50 μl. The reaction volume was centrifuged at 12,000 rpm for 5 seconds and incubated at room temperature for 1 hour. Stop solution (5 μl) was added to stop the reaction.

A Sephadex G-50 spin column was used to separate labelled products from unincorporated nucleotides. To prepare the column for the probe, a 1 ml syringe was plugged with a small amount of cotton wool and filled with G50 in TE. The column was placed in a 15 ml conical tube and centrifuged for 30 seconds to exclude air bubbles and 100 μl STE was added. After a further 30 seconds of centrifugation 100 μl of TE was added. The column was spun for 30 seconds and the supernatant in the conical tube was discarded. Before replacing the column a microfuge tube was placed in the bottom of the conical tube.

Immediately before the addition of the incubated probe to the column, 2 μl of 5 mg/ml tRNA was added, followed by 75 μl of 1 x TE. The probe was then added and the column was centrifuged for 30 seconds. Of the volume collected in the microfuge tube at the base of the column, 100 μl was transferred to a clean microfuge tube. TE (200 μl; 1x) was added and the probe was denatured for 10 minutes at 95°C, then placed on ice. The entire probe reaction volume was added to the prehybridization mixture the petri dish was resealed and replaced in the hybridization oven at the optimum temperature (42°- 46°C) overnight. When

using the 216 bp probe, purification from unincorporated nucleotides was found to be unnecessary.

3.4.7 Hybridization

The following day the hybridization solution was poured off and an equal volume of a low stringency wash (4 x SSC and 0.1% SDS or 2 x SSC + 0.1 % SDS) added. The membrane was incubated at room temperature for 2-15 minutes and given a second low stringency wash. Further washes were carried out under more stringent contions of lower salt and/or higher temperature to further reduce background adherence of the probe to the membrane and remove non-specific hybridization products. Firther details of washing procedure are given in chapter 8. When background levels of radioactivity were reduces sufficiently the membrane was blotted dry, wrapped in plastic film and exposed to x-ray film.

3.4.8 Autoradiograhy

Autoradiography was performed with a single intensifying screen at -80^{0}C from 16-60 hours depending on the strength of the signal. The film used was Kodak, X-OMAT XAR5.
After the required exposure time the casette was removed from the freezer and allowed to warm to room temperature prior to development of the film. The film was developed manually, allowing 5 minutes in the developer, 1 minute in the stop bath and 3 minutes in the fixer. Finally the film was washed extensively undr running water and dried. (see Appendix 1).

3.4.9 Derivation and amplification of IGF-1 specific probes for hybridization analysis

Two IGF-1 probes were used in these experiments, the first was derived from a 540 base pair fragment, kindly donated by Dr. Victor Han and the second had a theoretical fragment length of 216 base pairs and was prepared during the course of this project using customised primers and RT-PCR (reverse transcriptase - polymerase chain reaction).

A. IGF-1 probe (540 base pairs)

A 540 base pair probe for IGF-1 was supplied by Victor Han as an insert in the phagemid p(Bluescript KS+) from Strategene. The insert was located between the BamH1 and Sac1 restriction sites in the. Due to difficulties initially encountered when trying to transform this plasmid into an *E.coli* host decision was made to adopt the following approach :-

1. Generate a workable quantity of the IGF-1 specific insert by PCR using the flanking T7 and T3 promotor sequences.
2. Use this PCR product for Northern Hybridization Analysis.
3. Re-clone this insert into p (Bluescript) for further use.

PCR Amplification

The following primers were used for amplification of the 540 base pair insert:-

T3 primer: 5' ATT AAC CCT CAC TAA AGG GA 3'

T7 primer: 5' TAA TAC GAC TCA CTA TAG GG 3'

PCR was carried out in a DNA Engine PTC-200 from MJ Research using sterile PCR tubes from Perkin Elmer. Reaction conditions and PCR cycling parameters

are shownin tables 3.2 and 3.3 respectively.

Table 3.2 Reaction conditions for PCR amplification of 540 base pair IGF-I fragment

Component	volume (μl)	Final Concentration
dH_2O		
10 X Amplification buffer	5	10mM Tris pH 8.3, 50mM KCl
10mM dNTP mix	1	0.2 mM
T7 primer (15.4μM)	2	0.6 mM
T3 primer (15.4μM)	2	0.6 mM
25mM $MgCl_2$	3	1.5 mM
template (250ng/ul)	1	500 ng/100 μl
Amplitaq DNA polymerase	0.25	2.5 units/ 100μl

The reaction (50 μl) was incubated for 5 minutes at 94^{0}C prior to addition of the Taq DNA polymerase.

Table 3.3 Cycling Parameters for PCR amplification of 540 base pair fragment

Cycling Parameters

Cycle	Denaturation	Annealing	Polymerization
First cycle	4 minutes at 95°C		
Subsequent cycles (n=25)	30 seconds at 94°C	45 seconds at 50°C	1 minute at 72°C
Last cycle			5 minutes at 72°C

The holding temperature at the end of the amplification was 4°C.

An aliquot of 5 μl was taken and run on a 1.5% TAE agarose gel for size determination. A further aliquot of the PCR product was run out on a 1.0% TAE agarose gel and the cDNA band of the appropriate size was excised and purified using a Qiaex II gel extraction kit. Final yield of purified cDNA was then subjected to gel electrophoresis for quantification with reference to the 100 base pair ladder from Promega. A 5 μl aliquot of this standard gives a distribution of bands each containg 50 ng DNA.
Procedures used for cloning of this product are detailed in sections 3.4.11 to 3.4.15.

B. IGF-1 Probe (216 base pairs

The second probe to be used in these experiments was prepared using RT-PCR (Reverse transcriptase - polymerase chain reaction).
Total porcine liver RNA was used as a template for RT-PCR, as this tissue is considerd to have high levels of IGF-1 expression.

PCR primers

Primer design was based on the published cDNA sequence for porcine IGF-1. (Tavakkol *et al.*, 1988).

Upstream primer : 5' TGC GGA GAC AGG GGC TTT TAT TTC 3'

Downstream primer : 5' ATG TAC TTC CTT CTG AGC CTT GGG 3'

First strand cDNA synthesis

First strand cDNA synthesis was performed in a 20μl reaction volume.

Total liver RNA (1μg) and 0.5 ng oligo $(dT)_{12\text{-}18}$ was denatured in a total volume of 115 μl at 70°C for 10 minutes. Following denaturation the mixture was quick chilled on ice, centrifuged and supplemented with the following components

Component	Volume	Final concentration
RNAsin (40 U/μl)	0.5 μl	1U/μl
5x First strand Buffer (BRL)	4.0 μl	50mM Tris pH 8.3 75mM KCl
DTT (0.1M)	0.5 μl	10 mM
dNTP (10mM)	1.0 μl	500 μM

Components were mixed and incubated at 42°C prior to the addition of 1 μl (200 units) Superscript™ II RNase H- Reverse Transcriptase (Gibco BRL). Following an incubation period of 50 minutes at 42° C the reaction was terminated by heating

to 70°C for 10 minutes. The reaction mix was used immediately for PCR or stored at -20°C. Control reactions omitting template or including template and omitting reverse transcriptase were also performed.

PCR amplification

As the target sequence was only 216 bp, no attempt was made to remove the template RNA prior to PCR amplification. A reaction volume of 50 μl was used and thermocycling was performed using the DNA Engine PTC-220 (MJ Research).

Table 3.4 The composition of the PCR reaction for the amplification of the 216 fragment pair.

Component	Volume	Final concentration
10x amplification buffer	5.0 μl	20mM Tris pH 8.4, 50mM KCl.
25 mM $MgCl_2$	3.0 μl	1.5 mM
10 mM dNTP mix	1.0 μl	200 μM
10 μM upstream primer	0.5 μl	0.1 μM
10 μM downstream primer	0.5 μl	0.1 μM
sterile water	38.5 μl	
first strand DNA	1.0 μl	
Taq DNA polymerase	0.5 μ	

Cycling parameters are shown in Table 3.5

Cycle	Denaturation	Annealing	Polymerization
First cycle	3 min at 94°C		
Subsequent cycles (n=36)	1 min at 94°C	1 min at 64°C	1 min at 72°C
Last cycle			5 min at 72°C

A holding temperature of 4^0C was used.

Analysis of PCR products was performed on a 2% agarose gel in 1 x TBE. The size of the cDNA present on the gel was consistent with the expected size of the cDNA for IGF I, approximately 216 bp. Purification of the cDNA band prior to radio labelling was performed by excising the appropriate band and using the Qiaex II extraction procedure according to the manufacturers instructions. Purification of the cDNA band prior to sequence analysis was performed directly from the PCR mix using the Wizard purification kit (Progen), according to the manufacturers instructions.

3.4.10 Oligonucleotide Synthesis

Oligonucleotides were synthesised using the PCR-Mate EP™ DNA synthesiser (Model 391) from Applied Biosystems. The 40nmol synthesis scale was used. Following synthesis , oligonucleotides were deprotected and cleaved from the solid support using concentrated ammonium hydroxide. Base protecting groups were removed by addition of fresh concentrated ammomium hydroxide and incubation overnight at 55^0C. Evaporation of the ammonia at 40^0C overnight the oligonucleotides were freeze dried and redissolved in sterile dH_20. Following addition of sodium acetate pH 5.2 to a concentration of 0.3M the oligonucleotides

were precipitated using isopropanol.

Following an ethanol wash oligonucleotides were dried, dissolved in sterile water and the concentration determined specrophotometrically. Oligonucleotides were stored as aliquots at -20^{0}C.

3.4.11 Large scale plasmid preparation

Three ml of overnight culture was added to sterile flasks containing 200 ml LB and 200 μl of the appropriate antibiotic. This mixture was grown overnight (approximately 14 hours) and bacterial cells were harvested by centrifugation at 4-6000 x g for 5 minutes the following morning. Optical density readings were taken to confirm bacterial growth. After centrifugation the supernatant was removed and the cells were resuspended in 8 ml ice-cold Solution A (900 mg glucose, 2.5 ml 1 M Tris (pH 7.5) and 2.0 ml 500 mM EDTA (pH 7.5) per 100 ml; autoclave). Lysozyme (25 mg/ml) made up in 1 ml solution A was also added at this time. The suspension was transferred to 2 Oak Ridge tubes (5 ml in each) and left at room temperature for 5 minutes. Freshly made 0.2 N NaOH:1% SDS (10 ml, at room temperature) was added, the solution was mixed by several sharp inversions and placed on ice for 10 minutes. Ice-cold Solution K (15 ml; 60 ml 5 M KOAc, 11.5 ml glacial acetic acid per 100 ml) was added and mixed using a digging motion with a glass pipette. After a 10 minute incubation on ice, samples were centrifuged at 15,000 rpm at 4°C for 20 minutes. Equal quantities of the supernatant were transferred to clean Oak Ridge tubes and 0.6 volumes of isopropanol were added. Tubes were kept at room temperature for 15 minutes and then centrifuged at 10,000 rpm for 30 minutes. The supernatant was decanted and the pellet was washed in 10 ml 70% alcohol and centrifuged for a further 10 minutes. After the supernatant was decanted, the pellet was dried and redissolved in 4 ml TE and stored overnight at 4°C. The following morning 4.4 g total of cesium chloride was added to each sample (2.2 ml per Oak Ridge tube) and mixed gently. Ethidium bromide solution (200μl) was added and the mixture was transferred to an ultracentrifuge tube, the remaining space being filled with a

mixture of 1:1 (w:v) cesium chloride:TE. Samples were centrifuged at 45,000 rpm overnight (18 hours) at room temperature and the plasmid band was recovered. The ethidium bromide was cleared by extraction with TE-saturated isoamyl alcohol until all pink colouration was gone. Plasmid DNA was then precipitated in 3 volumes of 70% ethanol overnight and centrifuged at maximum speed for 20 minutes at 4°C. The pellet was washed in 70% ethanol and centrifuged, dried and redissolved in TE and stored at -20° pending analysis for concentration and purity before further use.

3.4.12 Gel purification of plasmid inserts

The plasmids purified from the large scale preparations were used for this procedure. Five μg of each plasmid was mixed with 0.5 μl of RNase A, 2.0 μl of 10x reaction buffer and 1.0 μl of the restriction enzyme. The sample was then made up to a volume of 20 μl with nanopure water. Samples were incubated for 1-2 hours at 37°C and the reaction was stopped with 5.0μl of DNA loading dye. Samples were run on a 1% low melt agarose gel using 1xTAE as the running buffer. The appropriate band for each plasmid was excised from the gel under shortwave UV light.

3.4.13 Ligation of inserts to vectors

The excised gel bands were placed in separate microfuge tubes and heated to 65°C for 15 minutes. The volume in each tube was estimated. The 10x ligase buffer was warmed to room temperature. Microfuge tubes were transferred to a 37°C heating block for a further 15 minutes, during which time a small amount of DEPC water was also warmed to 37°C. The insert was mixed with the vector at a ratio of approximately 100:1, according to the amount of DNA estimated from visualizing the gel. Two μl of ATP was added and the volume made to 78.5 μl with the warmed water. Samples were cooled one at a time for 3-4 minutes according to the air temperature of the room. Nine μl of 10x ligase buffer was

added and mixed well. Immediately after this, 3.5 μl of the ligase was added and the sample mixed well. Microfuge tubes were then capped and covered with foil and left on the bench overnight.

3.4.14 Transformation

The products of the low melt ligation were heated to 65°C for 2 minutes and 200μl of TE was added. Competent cells were thawed on ice and 75 μl of these cells were added to 17x100 mm culture tubes. Ten μl of the low melt ligation product was added to the cells in the culture tube and were gently mixed. Samples were incubated on ice for at least 20 minutes and then heat shocked at 42°C for exactly 45 seconds. LB broth (400 μl) was added to each tube and all tubes were incubated at 37°C for 1 hour and then plated on LBA plates and incubated overnight at 37°C. Colonies were counted the following morning and single colonies were screened for inserts by alkaline lysis miniprep.

3.4.15 Alkaline lysis miniprep

Single cultures were removed from plates and grown overnight at 37°C in a shaking incubator in 5 ml of LB broth containing ampicillin. Culture (1.5 ml) was transferred to a microfuge tube and centrifuged at maximum speed for 30 seconds at room temperature. Supernatant was aspirated and the pellet was resuspended in 100 μl of ice-cold solution A. After incubating at room temperature for 5 minutes, 200 μl of freshly prepared 0.2 N NaOH:1% SDS (at room temperature) was added and samples were mixed by repeated sharp inversion and stored on ice for 5 minutes. Ice-cold solution K (150 ml) was added and mixed by simultaneous inversion and flicking of the tube. Samples were incubated on ice for a further 5 minutes and then centrifuged at room temperature for 5 minutes. The supernatant was transferred to a new microfuge tube and 2 volumes of 100% ethanol (at room temperature) was added. Samples were briefly vortexed and kept at room temperature for 2 minutes and centrifuged at maximum speed for 5 minutes. After

the supernatant was decanted, the pellet was dried and resuspended in 20 μl of distilled water. Between 0.5 and 5 μl of the suspension was used in a restriction reaction as described previously to screen for retained inserts. When an insert of the correct size was detected, glycerol stocks were made from the remaining overnight cultures, followed by a large-scale plasmid preparation of the culture.

3.4.16 Deoxyribonuclease treatment of RNA samples.

Deoxyribonuclease (DNase) treatment of samples was performed in a total volume of 10 μl for 15 minutes at room temperature. Reactions contained 1 μg RNA, a unit DNase I (Amplification grade; Gibco BRL) in 20 mM Tris HCl pH 8.4, 2 mM $MgCl_2$ and 50 mM KCl. Reactions were terminated by addition of 1 μl 25 mM EDTA followed by incubation at 65°C for 10 minutes. No detectable degeneration of RNA was observed.

3.4.17 Detection of IGF-I mRNA expression in embryo RNA samples by RT-PCR

RT-PCR was performed for detection of IGF I mRNA in the embryos from retinol and vehicle treated gilts. Conditions for first strand cDNA synthesis are essentially as described in section 3.4.9B except that 5 μl of template RNA (500 ng) was included in most experiments. Moreover gene specific downsteam primer (2 pMol) was used to prime first strand cDNA synthesis in place of oligo $(dT)_{12\text{-}18}$.

Conditions for PCR amplification are shown in Table 3.4.

To compare levels of IFG I mRNA in different samples, accumulation of the target PCR product with time was monitored.

PCR reactions (50 μl) were initiated with equal amounts of first strand reaction mix (1 μl). Aliquots of 10 μl of the PCR reaction were withdrawn for analysis after the appropriate number of cycles (usually 24, 28, 32, 36 and 40 cycles).

In order to remove samples, PCR cycling was 'paused' at the end of the 72°C extension step. Aliquots were removed and placed on ice. Following sample collection, reaction mixes were returned to the thermocycler, now at 92°C given 10 seconds to reach temperature and cycling was continued.

An alternate approach was to prepare a serial dilution of each first strand cDNA and to cycle for the appropriate number of cycles. This approach was used once with similar results, but was discontinued due to larger amounts of Taq polymerase required.

3.4.18 Sequence Analysis

Automated sequence analysis was performed at Monash University using an ABI Prism Sequencer (model 373).
Flourescence base cycle sequencing reactions were performed using the ABI Prism™ Dye termination cycle sequencing kit with Amplitaq DNA Polymerase, FS.

Sequencing on RT-PCR generated PCR products from porcine liver RNA or porcine embryo RNA was performed in a 20 μl reaction volume. This mixture contained 35 ng template DNA, 3.2 pmol upstream primer and 8 μl dye-terminator. PCR cycling parameters are shown in Table 3.5

Extension products were purified by ethanol precipitation after the addition of 3 μl 3M sodium acetate (pH 4.6). The precipitate was recovered by centrifugation at room temperature for 30 minutes and the pellet rinsed briefly with 70% ethanol. Ethanol was removed and the pellet transmitted on ice to the sequencing facilities at Monash University, Victoria Australia where samples were thoroughly dried before analysis.

Table 3.6 Cycle sequencing parameters.

Cycle	Denaturation	Annealing	Polymerization
First cycle	4 min at 96°C		
Subsequent cycles (n=25)	30 seconds at 96°C	15 seconds at 50°C	4 minutes at 60°C
Last cycle			4 minutes at 60°C

The holding temperature at the end of cycling was 4°C.

3.5 Statistical analysis

All data were evaluated using the Statistica program (Statsoft, Tulsa USA) or SPSS for Windows. Analyses utilized were analysis of variance (univariate, multivariate and repeated measures designs), Chisquare, Student's T test and Least Significant Difference. Specific analyses for experiments are detailed in each chapter.

Chapter 4:

The development of extraction and HPLC methods for retinol and beta carotene from plasma and tissues

4.1 Introduction

While several methods for the extraction of retinol and beta carotene have been reported, many lack a high degree of specificity, or are not designed for the extraction of animal products such as plasma and tissue. The methods that have been reported or used in various research projects involve colour fluorimetry, spectroscopy, column chromatography and thin layer chromatography (Chew et al. 1984, Britton 1985, and others). However, while many of these methods are suitable for identification and separation of carotenes and other compounds, they are not ideal for quantification. Many if these techniques seem to result in variable sample losses, require large sample sizes, are designed to extract retinol or beta carotene from food or vegetable sources, are time consuming and/or require considerable analytical skill (Catignani and Bieri 1983). A number of reports are also available on the separation of these vitamins by HPLC. The major advantages of HPLC separation of the retinoids include small sample size, rapid elution, accuracy and a low limit of detection (Bankson et al. 1986).

Recent HPLC methods for the quantitation of retinoids utilize reverse phase chromatography (Schweigert et al 1995, Chew et al 1991a, Catagnani and Bieri 1983) rather than normal phase (Napoli and Race 1988, Bankson et al. 1986), though both have been shown to be successful in the separation of the retinoids. Normal phase chromatography does not require a saponification step and has the advantage of requiring a simple extraction with a non-aqueous solvent to remove polar material. Since normal phase silica columns can tolerate relatively heavy loads of triglycerides and other non-polar material, the extract can be injected directly onto the column with no further purification (Ball 1988b). Esters of retinol have been successfully separated in plasma using normal phase chromatography with a non-saponifying extraction method (Bankson et al. 1986). However, given the quantity of fat present in animal tissue, saponification to remove fats from the extract seems necessary. According to Ball (1988b) quantification of carotenoids on silica columns is unreliable due to the catalysis of

on-column degradation of carotenoids due to strong interactions with active sites on the column. Reversed phase systems are generally preferred for the chromatography of the hydrocarbon carotenoids such as α and β carotene and are ideal for the separation of moderately polar compounds such as the fat soluble vitamins (Ball 1988b). Since both retinol and beta carotene were the compounds under investigation, for ease and convenience, a reverse phase system was selected for this study.

The aim of this work was to develop suitable methods for the extraction of retinol and beta carotene from plasma and tissues and to develop methods for quantifying levels of these vitamins in the extracts.

4.2 Materials and Methods

Reagents

Methanol, acetonitrile, chloroform and hexane were of liquid chromatography grade. Diethyl ether, petroleum ether and absolute ethanol were of reagent grade. Retinol and beta carotene stock solutions and the internal standards for the assays of these compounds were purchased from Kodak Fine Chemicals and Fluka (beta-apo-8'-carotenal). Note that 99.9% pure beta-apo-8'-carotenal is no longer available from Fluka, or from any other supplier in Australia. The beta-apo-8'-carotenal now available from Fluka (≤20% UV-Vis) requires purification before use.

Stock solutions of 100 μg/ml *all-trans* retinol and *all-trans* retinyl acetate were made up in methanol and stored in foil covered glass containers at -20°C. Beta carotene and beta-apo-8'-carotenal were made up to stock solutions of 100 μg/ml or 1000 μg/ml in chloroform and stored at -20°C . Standards for retinol and beta carotene of 0.1, 0.3, 0.5 and 1.0 μg/ml were made up in mobile phase. All stock and standard solutions were made up under yellow light to avoid degradation.

Standards and stock solutions were made up as required, the indicator of acceptance being the comparability of standard curves between runs.

Apparatus

Two identical Varian HPLC systems were used. These were controlled by one software package on a 486 computer, which was designed to allow for the simultaneous operation of both systems. The HPLC system consisted of a 9012 Solvent Delivery system, a 9100 Automatic Sampler and a 9050 UV Vis Detector from Varian, Texas, USA. The solvent delivery system featured a single reciprocating pump and was ported to accommodate 3 proportioning valves. The injection system on the automatic sampler was a Rheodyne automatic injector, equipped with a 50 μl sample loop. The UV-Vis detector was a single programmable wavelength, single channel detector, set at 325 nm to detect retinol and at 466 nm for beta carotene. The flow cell on the detector had a volume of 15 μl in an 8 mm path length. The signal output from both HPLC systems was integrated using the Varian Star Chromatography Software 4.01 package which was read through the ADC board of the 486 computer. Chart speed on chromatograms varied from 0.5 to 1.5 cm/minute. Other equipment used in extractions included a benchtop microfuge, a benchtop centrifuge, wrist action shaker, multitube vortex and a sample concentrator with heating block. All details of this equipment are shown in Appendix B.

The chromatography column used for assays was a Waters Resolve C_{18} reverse phase stainless steel column. The particle size was 5 μm, the pore size was 90Å and the column dimensions were 8 mm x 100 mm. The system was fitted with a Waters Resolve C_{18} guard column with a particle size of 10 μm and pore size of 90Å.

Samples

A plasma pool was collected from untreated sows for the development and optimization of extraction methods for retinol and beta carotene from plasma. A known amount of *all-trans* retinol or *all-trans* beta carotene and appropriate internal standard were added to the plasma and compared with blank plasma (to which no standard was added). Recoveries of these agents was calculated after the development of these methods of extraction in order to verify the efficiency of extraction. Details of these procedures appear in the results section.

Tissue samples (liver and ovaries) were collected from an abattoir and frozen at -80°C while the extraction method was being developed. A known amount of retinol or beta carotene and appropriate internal standard were added to homogenized tissue at the beginning of the extraction process. Various methods of extraction were tried and are detailed in the results section. Some methods used included a saponification step which reduced the fat content of the sample and cleaved retinol ester conjugates.

4.3 Results

While optimizing extractions and HPLC analysis, many methods were investigated, most of which are detailed below. The method finally used in each results chapter is boxed and highlighted.

4.3.1 Extraction of retinol from plasma.

The method used for retinol extraction from plasma was a single-step ethanol precipitation. The plasma was centrifuged at 2000 rpm to remove fibrin. Initially, duplicate samples of 990 μl were pipetted into microfuge tubes. Internal standard (10 μl, *all-trans* retinyl acetate, 100 μg/ml) was added and the sample was briefly vortexed. An aliquot of 200 μl was taken, to which was added 800 μl ethanol.

The sample was vortexed a second time and then centrifuged at 12,000 rpm for 5 minutes. The supernatant was then immediately separated by HPLC.

Recoveries were calculated in the following manner. Plasma was extracted in duplicate for retinol as above adding only internal standard. Further duplicates of plasma to which 50 μl of *all-trans* retinol (100 μg/ml) and 50 μl of the internal standard were added, were extracted as above with 800 μl of ethanol. The concentration of added retinol and retinyl acetate in each sample was 1.0 μg/ml.

To more efficiently use available plasma the amount extracted was reduced to 100 μl in duplicate, to which 100 μl of internal standard (1.0 μg/ml) was added. Ethanol (300 μl) containing 0.1% BHT was added, the sample was vortexed and centrifuged at 12,000 rpm in a benchtop microfuge. The supernatant was aspirated and analysed immediately by HPLC.

Method: Extraction of retinol from plamsa
100 μl plasma
100 μl retinyl acetate (1.0 μg/ml)
300 μl ethanol
vortex, centrifuge at 12,000 rpm

4.3.2 HPLC analysis of retinol in plasma

Initially a published method from Chew et al. (1991a) which used a mobile phase comprising Acetonitrile:Tetrahydrofuran: Water (50:38:12) was tried. The retention time of the all-trans retinol standard in ethanol using this method was 3.3 minutes, all-trans retinyl acetate eluted at 5.4 minutes and when retinol proprionate was added to samples, it appeared at 6.3 minutes. However, peak areas were much smaller than those achieved using the methanol:acetonitrile:chloroform mobile phase shown below, suggesting lower sensitivity of the former method.

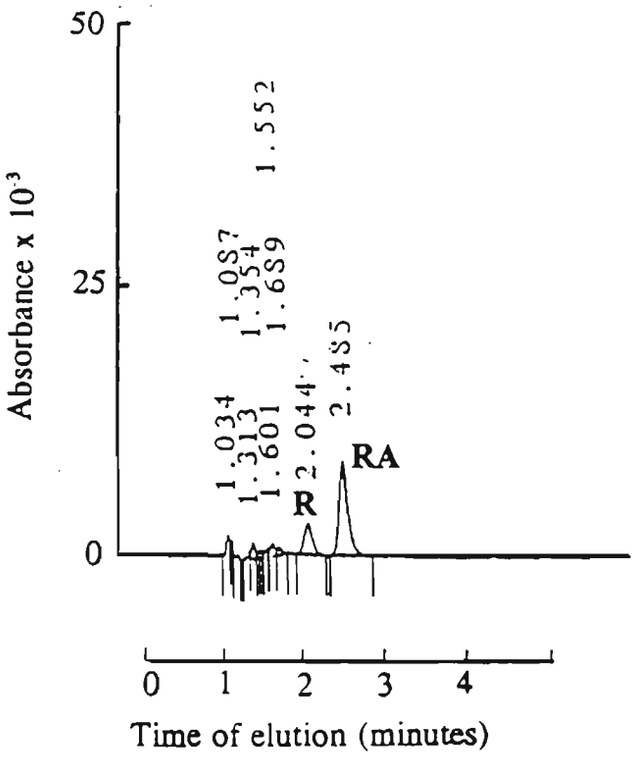


Figure 4.1. Chromatogram of the separation of Retinol (R) and Retinyl Acetate (RA) extracted from plasma using an isocratic elution of 47:47:6 (methanol:acetonitrile:chloroform) at a wavelength of 325 nm.

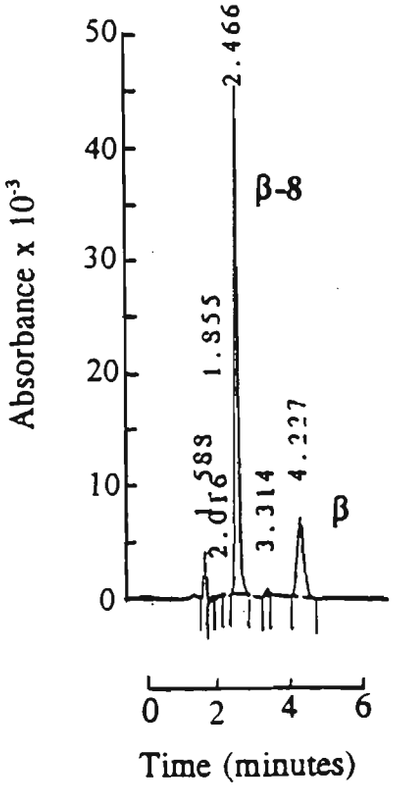


Figure 4.2 Chromatogram of the separation of beta carotene (β) and beta-apo-8'-carotenal(β8) from plasma using an isocratic elution of 10:60:30 (methanol:acetonitrile:chloroform) at a wavelength of 325 nm.

The mean peak area for the 1.0 μg/ml all-trans retinol standard in ethanol using the acetonitrile:tetrahydrofuran:water mobile phase was 40485$\pm$1331 units, while the peak area for the 1.0 μg/ml all-trans retinol standard in ethanol using the methanol:acetonitrile:chloroform solvent mixture was 194874$\pm$5434 units. The usage of the latter mobile phase increased the UV response of the method to 0.1 μg/ml.

The samples were separated using a method provided by Dr B.P. Chew (pers comm), using the following conditions:

HPLC analysis: Retinol in plasma
Mobile phase:47:47:6 Methanol: Acetonitrile: Chloroform (vol:vol:vol).
Flow rate: 1.0 ml per minute
Wavelength: 325 nm
Average elution time: retinol 2.0 mins; retinyl acetate 2.5 mins.
Sample size: 50 μl

No modifications to this method were required. Retinol eluted at approximately 2.0 minutes while retinyl acetate appeared at 2.5 minutes (Figure 4.1).

Assay drift for the analysis of retinol was monitored daily using the external standard *all-trans* retinol. The peak areas and calculated concentrations did not vary significantly over a period 3 months. The coefficient of variation between assays, calculated using the external standard of 1.0 μg/ml, was 7%.

The recovery of retinol from plasma was calculated using the formula below and a sample of recoveries is shown in Table 4.1. The adjusted retinol figure (R_{adj}) is calculated by subtracting the retinol value in unspiked plasma from the value in the spiked sample.

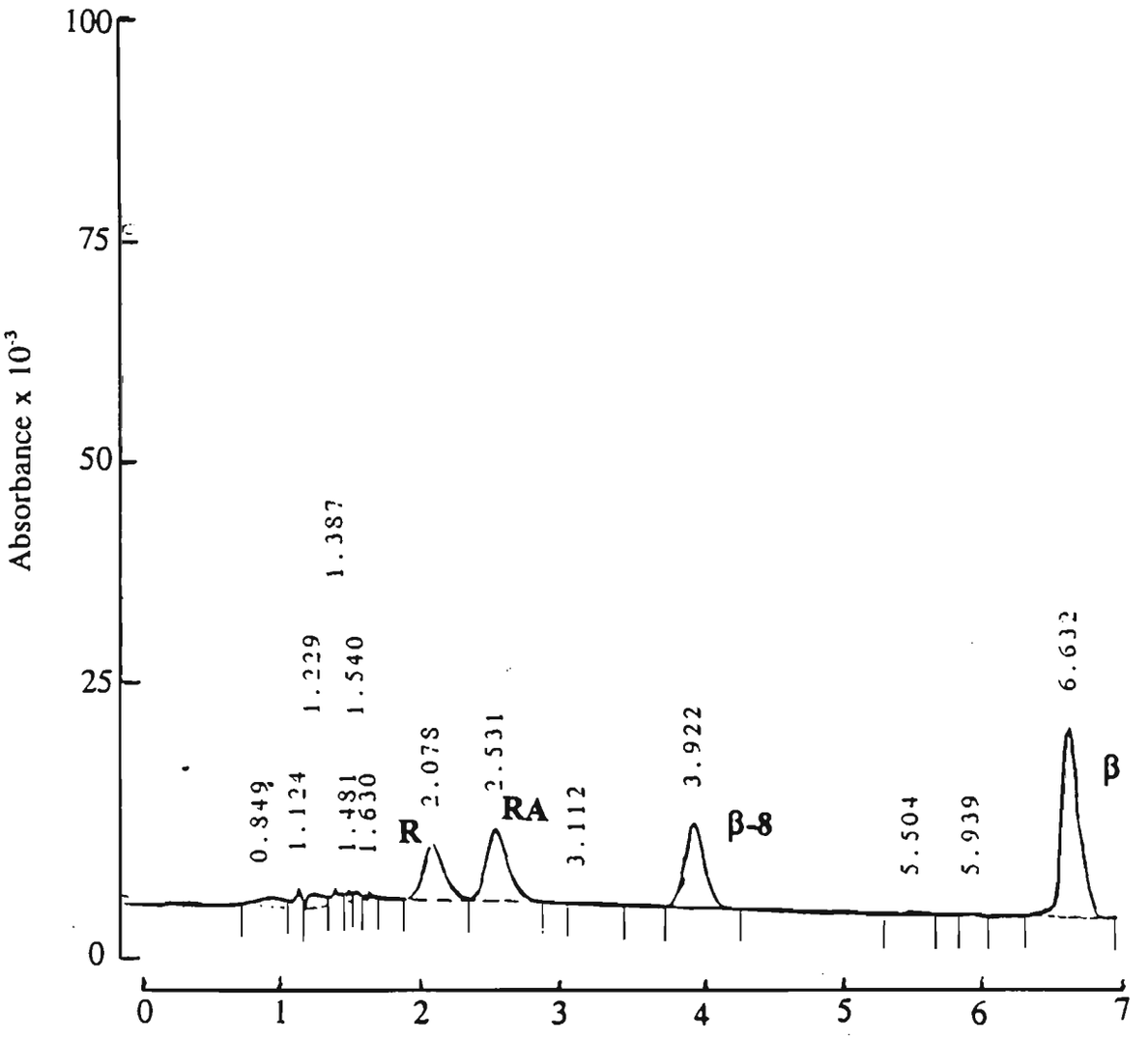


Figure 4.3 HPLC separation of a mixture of 1.0 μg/ml standards, Retinol (R), Retinyl acetate (RA), beta-apo-8'-carotenal (β8) and beta carotene (β) using a gradient elution of 47:47:6 (methanol:acetonitrile:chloroform) at 325 nm to 10:60:30 (methanol:acetonitrile:chloroform) at 460 nm.

$$\text{Concentration} = \frac{R_{adj} \times \text{Conc IS} \times \text{RRF}}{\text{RA}}$$

Where:

Radj = peak area of sample spiked with retinol-peak area of unspiked sample

Conc IS= Concentration of retinyl acetate (0.2 μg/ml as described above)

RRF= Relative Response Factor

= (peak area IS/conc IS x conc analyte/peak area analyte)

RA= Peak area of internal standard, retinyl acetate

Table 4.1 Recovery of retinol from plasma after the addition of 1.0 μg/ml *all-trans* retinol and retinyl acetate, the internal standard (RA) to plasma.

Peak area Retinol	Peak area Ret Acetate	Retinol adjusted	Conc (μg/ml)
41935	31902	39515	1.064
41802	34902	39382	0.968
40330	31974	37910	1.0193
44632	33882	42212	1.070
40761	30904	38341	1.066
		Mean recovery =103%	

Due to the use of a non-polar mobile phase and the undoubted deposition of fats and lipids, the column was regularly reconditioned, using the method provided by the manufacturer. Initially, a wash for 30 minutes with methanol at 1 ml/minute was carried out and this was graded up to 100% chloroform over a period of 30 minutes to 1 hour. The column was then washed with 100% chloroform for 10 minutes and then graded back to 100% methanol over 30 minutes. A wash for 1 hour with water returned the column to its original state.

4.3.3 Extraction of beta carotene from plasma

A technique provided by Dr B.P.Chew (pers comm) was used for the extraction of beta carotene from plasma. Plasma (500 μl) was pipetted in duplicate into glass extraction tubes and 100 μl of 1.0 μg/ml internal standard (beta-apo-8'-carotenal) was added. Samples were vortexed after the addition of 400 μl of ethanol containing 0.1% BHT. Petroleum ether: Diethyl ether (1:1; 3.0 ml) was then added and the samples were shaken vigorously on a wrist action shaker for 6 minutes. Tubes were then centrifuged for 10 minutes at 3000 rpm to separate the ethanol and ether phases. However, there appeared to be some variation in the proportion of ether available after shaking. Therefore it was decided to take an aliquot of equal volume (1 ml) from each tube in an attempt to reduce this variation. Samples were then dried at 45°C in a speedvac (the lowest temperature possible) and reconstituted in mobile phase for HPLC. However, variation between duplicates was considerable, possibly due to the degradation of beta carotene at these temperatures. In addition, the speedvac could not be placed in a fume hood, leading to ether vapours pervading the laboratory space. It was therefore decided to take the entire ether layer from samples and to dry the samples in a sample concentrator under a stream of nitrogen at 40°C. Dried samples were sealed and kept at -20°C until assayed by HPLC.

Method: extraction of beta carotene from plasma
500 μl plasma
100 μl internal standard
400 μl ethanol
Vortex
3.0 ml diethyl ether:petroleum ether (1:1)
Extraction time: 6 minutes
Centrifuge at 3000 rpm 10 minutes
Evaporate ether phase under nitrogen

4.3.4 HPLC analysis of beta carotene in plasma

Initially, analysis of beta carotene in plasma was attempted by the method provided by Chew (pers comm, section 4.3.1), which was suitable for the extraction of vitamins A, E and beta carotene. The solvent composition as outlined for the separation of retinol was 47:47:6 methanol: acetonitrile: chloroform at 1.0 ml/minute, with a wavelength of 466 nm. The sample size was 60 μl, injected onto a 50 μl loop. The mobile phase was not non-polar enough to elute beta carotene from the column, although the internal standard, beta-apo-8'-carotenal eluted satisfactorily. In a run time of 15 minutes there was no evidence of a beta carotene peak.

However, when the chloroform was reduced to 2% and the methanol and acetonitrile were each increased by 2% each, a non-integrated peak, presumably of beta carotene appeared at 18 minutes. In order to sharpen the peak, the flow rate was increased to 2.0 ml/min. The peak eluted at 13.6 minutes but was still not integrated by the software. Chloroform was returned to its original level of 6% and the flow rate of 2.0 ml/min was retained, giving a peak of beta carotene at 9.66 minutes. While this peak was integrated by the software, it was very wide.

Therefore, to sharpen the peak, the mobile phase was made more non-polar by increasing the proportion of chloroform to 10% and equally decreasing the methanol and acetonitrile to 45%, giving a final solvent composition of 45:45:10 (methanol:acetonitrile:chloroform). The flow rate was maintained at 2.0 ml/minute. This gave a peak at 7.0 minutes, but the peak was not integrated by the software. The non-polarity of the mobile phase was increased by the reducing methanol and increasing chloroform to 10:60:30 (methanol: acetonitrile:chloroform).

The final conditions which gave the best results for beta carotene and its internal standard, beta-apo-8'-carotenal were:

HPLC analysis: beta carotene in plasma
Mobile phase:10:60:30 methanol: acetonitrile: chloroform
Flow rate: 1.3 ml/minute
Wavelength: 466 nm
Elution time: beta carotene 4.2 mins; internal std 2.5 mins
Sample size: 50 μl

These conditions eluted the internal standard at approximately 2.5 minutes and beta carotene at 4.2 minutes (Figure 4.2). Beta carotene was undetectable at less than 0.3 μg/ml. The plasma samples from untreated sows to which no beta carotene had been added contained concentrations of less than 0.3 μg/ml beta carotene. Therefore, beta carotene in these samples was undetectable. These values were not deducted from the values from spiked samples.

The recovery of beta carotene from the plasma was calculated using the same formula as was used for the recovery of retinol from plasma (section 4.3.1). A sample of calculated recovery is shown in Table 4.2. Since beta carotene in the plasma of untreated pigs is undetectable by this method, no deduction of unspiked plasma was considered necessary.

4.3.5 The development of a gradient elution for the simultaneous assay of retinol and beta carotene from tissue samples.

Considerable time was spent in developing an assay to quantitate retinol and beta carotene in the same HPLC run. The results of this are shown in Table 4.3.

Table 4.2 Calculation of recovery of beta carotene after the addition of 1.0 μg/ml *all-trans* beta carotene (BC) and the internal standard (BCIS; beta apo-8'-carotenal) to plasma. (Formula for calculation of recovery from section 4.3.1)

Peak area BC	Peak area BCIS	Conc (μg/ml)
127712*	121031	1.036
108794*	101026	1.058
101908*	115018	0.871
31112	28843	1.059
28973	27650	0.998
50201	66869	0.783
48171	48104	0.900
20433	20826	0.964
	Mean recovery =95.8%	

* Plasma was spiked with 10 μg/ml beta carotene. In all other cases, plasma was spiked with 1.0 μg/ml beta carotene.

The mobile phase for each injected sample of 1.0 μg/ml *all trans* retinol, beta carotene or a mixture of the two standards (1:1) is shown. The retention times (RT) primarily for retinol, and its internal standard are shown. The separation of the internal standard and analyte peaks are shown in the last column. The time of the void volume in these assays was 2.0 to 2.2 minutes. It was found that using an isocratic system of methanol, acetonitrile and chloroform it was not possible to detect beta carotene without eluting retinol and its internal standard within the void volume.

The gradient and time of change of the gradient were further investigated as shown on Table 4.4. Various solvent compositions were tried in order to optimize the separation of retinol and retinyl acetate at 325 nm before the change to the more non-polar elution for the separation of beta carotene and beta-apo-8'-carotenal at

466 nm. The flow rate was increased at this point to sharpen the beta carotene peak and to reduce the retention time on the column. One of the problems encountered with the use of a gradient elution was that peaks could no be quantitated because of the changes in the wavelength, flow rate or the gradient.

Table 4.3 Mobile phases used to optimize a method for eluting retinol and beta carotene (BC) separately and from a mixture (1:1 retinol:beta carotene) in the same HPLC assay. Retention times (RT) are shown primarily for retinol and its internal standards (IS). Solvents used in the mobile phase were: (x% methanol: y% acetonitrile: z% chloroform).

Sample (μg/ml)	Solvent mixture MeOH:MeCN:CHCl$_3$	RT BC retinol	RT BC	RT RA	RT BCIS	Separation (mins)
BC	10:60:30		5.838			
retinol	10:60:30	2.155		2.298		0.143
retinol	15:70:15	2.230		2.404		0.173
retinol	10:78:12	2.393		2.595		0.199
retinol	10:80:10	2.571		2.757		0.236
retinol	20:70:10	2.415		2.658		0.246
retinol	5:80:15	2.460		2.503		0.07
retinol	20:65:15	2.282		2.458		0.279
retinol	25:60:15	2.272		2.470		0.25
mix	25:60:15	2.262		2.444		0.183
mix	30:55:15	2.241		2.435		0.194
mix	35:50:15	2.236		2.451		0.222
mix	40:45:15	2.218		2.449		0.237
gradient*	45:45:10 -10:60:30	2.389	6.710	2.680	3.54	0.287

*An initial gradient elution of methanol: acetonitrile: chloroform was as follows: 45:45:10 at 325 nm until 3 minutes to elute retinol and retinyl acetate; then 10:60:30 at 466 nm from 4.5 minutes until 10 minutes to separate beta carotene and its internal standard, beta-apo-8-carotenal.

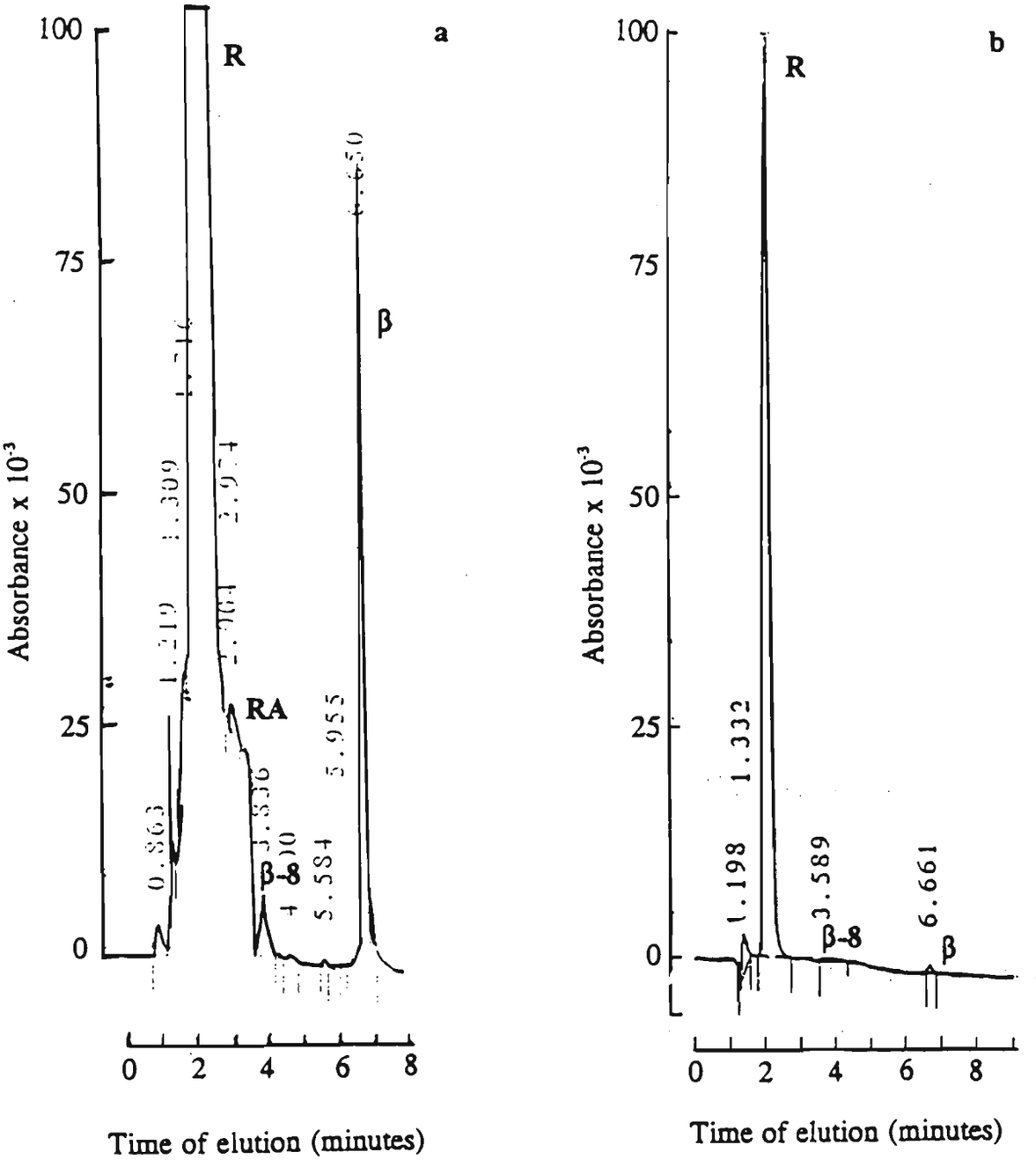


Figure 4.4 Chromatograms of Retinol (R), Retinyl Acetate (RA), beta-apo-8'-carotenal (β8) and beta carotene (β) in extracted liver sample. (a) undiluted sample, (b) sample dilution of 1:100.

A further problem was a fall in the baseline as the amount of chloroform was increased. Alterations of the timing of wavelength and gradient changes in some ways alleviated these problems. The final gradient elution that gave the most satisfactory results for the separation of retinol and beta carotene standards in the same run was as follows (Figure 4.3):

HPLC analysis: simultaneous separation of retinol and beta carotene
Time: 0:00 min 47:47:6 (methanol:acetonitrile:chloroform)
flow rate of 1.0 ml/minute, wavelength 325 nm
Time: 3:50 min 47:47:6 (methanol:acetonitrile:chloroform)
flow rate of 1.3 ml/minute, wavelength 325 nm
Time: 4.00 min 10:60:30 (methanol:acetonitrile:chloroform)
flow rate of 1.8 ml/minute, wavelength 466 nm.
Run time: 7.00 minutes
Sample size: 50 μl

However, when liver tissue samples were extracted and assayed using the optimum solvent elution conditions, the separation of retinol and beta carotene and their internal standards was still not satisfactory (Figure 4.4a). Retinol in the liver is very highly concentrated, while beta carotene concentrations are relatively low. Furthermore, the retinol and retinyl acetate were not completely separated, due to the large size of the retinol peak. When the sample concentration was high beta carotene could be easily separated and integrated. However, when the sample was diluted to reduce the retinol peak so that separation of retinol and its internal standard could be achieved, it was not possible to integrate the beta carotene and beta-apo-8'-carotenal peaks, because of the low levels in the sample (Figure 4.4b). Because of the difficulties in determining the concentrations of retinol and beta carotene simultaneously, it was decided to assay retinol and beta carotene separately.

Table 4.4. The effect of different solvent elution compositions retention times (RT) for retinol, retinyl acetate (RA), beta-apo-8'-carotenal (BCIS) and beta carotene (BC) in a single HPLC assay. Elution at the beginning of the run appears in column 1 (methanol:acetonitrile:chloroform). The time appearing in column 2 shows the new elution (methanol:acetonitrile:chloroform) and the time at which the new mixture of mobile phase was reached appears in cloumn 3 , the gradient programmed to occur over 30 seconds. Wavelength and flow rate were changed at 3.5 minutes and there was a re-equilibration time of 3.5 minutes between each run.

Solvent mix MeOH: MeCN: $CHCl_3$	Solvent mix/time MeOH:MeCN:$CHCl_3$	Time of gradient change	RT Retinol (min)	RT RA (min)	RT BC (min)	RT BCIS (min)
Run 1 45:45:10	10:60:30	4.0mins	2.318	2.533	9.80	3.509
Run 2 45:49:6	10:60:30	3.5	2.318	2.634	11.50	3.760
Run 3 47:47:6	20:60:10	3.0	2.315	2.625	7.242	3.691
Run 4 39:55:6	10:60:30	3.5	2.356	2.656	6.352	3.751
Run 5 55:39:6	10:60:30	3.5	2.319	2.657	6.280	3.684
Run 6 60:34:6	10:60:30	3.5	2.353	2.718	6.339	3.776
Run 7 60:37:3	10:60:30	3.5	2.477	2.869	6.622	4.099
Run 8 63:34:3	10:60:30	3.5	2.335	2.722	6.622	3.914
Run 9 55:42:3	10:60:30	3.5	2.466	2.837	6.587	4.058
Run 11 67:30:3	10:60:30	3.5	2.435	2.858	6.554	4.099
Run 12 75:20:5	10:60:30	3.5	2.475	2.936	8.269	4.209
Run 13 78:20:2	10:70:20	3.5	2.612	3.124	8.721	4.648

4.3.6 The extraction of retinol and beta carotene from tissues.

Initially, a method provided by the Analytical Reference Laboratory, a NATA accredited laboratory in South Melbourne, for the extraction of beta carotene from tissues was attempted. Since this method was designed for the extraction of total carotenes from plasma, it was modified for the extraction of total carotenes from tissues. A second method (Chew pers comm) specifically designed for the extraction of beta carotene and retinol from tissues was also tried. The results of both methods are shown below.

4.3.6.1 Method: Analytical Reference Laboratories: Extraction of total carotenes from tissues

Identical samples of liver tissue (1 g) were homogenized in 1 ml ethanol. Xylene (4 ml) was added and the mixture was shaken vigorously for 15 minutes, the liquid was decanted and centrifuged for 5 minutes at 3000 rpm. The xylene layer was then collected and the absorbance was read at 460 nm. Results and calculated concentrations are shown in Table 4.5. Concentrations of beta carotene were calculated using Beers Law as follows:

$$\text{Concentration of extraction } (\mu g/g) = \text{Dilution} \times \frac{\text{Absorbance}_{460} \times 10^6}{\text{Extinction coefficient} \times \text{cuvette width}}$$

The extinction coefficient for beta carotene (in ethanol) is 262000

A major problem with the extraction method was that the tissue was immiscible in the ethanol and did not disperse during the extraction. This made it difficult to remove the extract from the McCartney bottle in which it was homogenized. The liquid was therefore decanted and centrifuged, although the collection of all solvent from each extract could not be assured. Since unequal amount of solvent

from were recovered, rather than a consistent aliquot of homogenate, quantitative extraction of beta carotene was not possible using this method as illustrated in the inconsistent concentrations shown in Table 4.5.

Table 4.5 Concentration of beta carotene in liver tissue (1 g) extracted with xylene for 4 samples taken from the same liver.

Extract	Absorbance	Concentration (μg/g)
1	0.028	0.11
2	0.028	0.11
3	0.037	0.15
4	0.023	0.09

In order to reduce inconsistency in extractions, samples of liver tissue were homogenized in 5 ml 1.15% KCl instead of ethanol and the extraction carried out as above. A 1 ml aliquot of the homogenate was taken and added to 1 ml ethanol and 4 ml xylene and shaken vigorously for 15 minutes. When KCl was used rather than ethanol in the homogenization, the tissues were well dispersed in the homogenate. No decanting of the sample was required, the xylene layer was removed after centrifugation for 5 minutes at 3000 rpm and the absorbance was read at 460 nm. These figures are shown in Table 4.6. The absorbance was read at 460 nm, and is shown with the calculated concentrations on Table 4.6. In one of the samples the absorbance was increased due to interference by some of the ethanol/KCl layer collected with the xylene. This sample was discarded. However, using KCl for the initial homogenization step produced more consistent concentrations of beta carotene.

Table 4.6 Concentration of beta carotene from liver (1 g) without the addition of beta carotene to samples (1-3) and recovery of beta carotene after the addition of 5 μl of 1000 μg/ml beta carotene after homogenization (4-7) .

Extract	$Absorbance_{460}$	Concentration (μg/g)
1	0.010	0.19
2	0.009	0.17
3	0.010	0.19
4	0.242	4.83
5	0.321	6.41
6	0.240	4.79
7	0.293	5.85

In order to check the post-homogenization recovery of beta carotene using this method, 5 μg/ml beta carotene standard (1000 μg/ml) was added immediately after the homogenization of 1 g of tissue and the extraction done as described above. Absorbance and concentration of beta carotene are shown in Table 4.6. The recovery of added beta carotene was $109 \pm 6.8\%$.

Since the homogenization of tissues in 5 ml KCl was successful the amount of ethanol used in the original method was increased from 1 to 5 ml in an attempt to improve the reproducibility of the extraction method. During the extraction with the larger amount of ethanol, although no water was added, it became apparent that an aqueous component was required, presumably to prevent the fat in the liver tissue from entering the xylene layer. In this extraction, two layers formed, the larger, presumably ethanol layer on the bottom and a thin layer on top. The lower layer was yellow while the upper layer was clear. Both layers contained droplets of fat and absorbance was not taken on these samples.

Since it was unknown whether KCl would change the absorbance properties of

carotenes, it was decided to homogenize liver in 0.9% sterile saline and compare the outcome of extraction to tissue homogenized in KCl. Samples were spiked with 1.0 μg of beta carotene. A modified version of the ARL method of extraction was used as detailed above. Absorbance and concentration are shown below in Table 4.7

Table 4.7 Concentration of beta carotene from liver after homogenization with 0.9% saline or 1.15% KCl in untreated samples (extracts 1-4), or following the addition of a known amount of beta carotene and extraction with xylene (extracts 5-12).

Extract	$Absorbance_{460}$	Concentration (μg/g)
1 KCl	0.026	0.519
2 KCl	0.018	0.359
3 Saline	0.027	0.539
4 Saline	0.025	0.499
		mean 0.479
5 KCl	0.079	1.51
6 KCl	0.078	1.49
7 KCl	0.073	1.39
8 KCl	0.093	1.78
		mean 1.54$\pm$0.16
9 saline	0.102	1.95
10 saline	0.080	1.53
11 saline	0.077	1.47
12 saline	0.071	1.35
		mean 1.57$\pm$0.26

From these results there would appear that there is no adverse effect of KCl on the absorption properties of beta carotene in comparison with using 0.9% saline for

the homogenization. The recovery of beta carotene from samples to which 1.0 μg had been added was 156% using this method, suggesting the presence of compounds which absorb at 460 nm. In order to address this possibility a second extraction of the same liver tissue, to which no beta carotene was added, was carried out. Absorbances and concentrations of beta carotene are also shown in Table 4.7. When the concentration of beta carotene from blank samples was used as a correction factor for spiked samples, the recovery of beta carotene using this extraction method was 110%.

Since the ARL method did not include a saponification step, which hydrolyzes fatty acid chains it was decided to extract tissue samples using a method provided by Chew (pers comm). The details of the method are given below.

4.3.6.2 Method: Extraction of beta carotene and total retinol from tissues using the method described by Chew (pers comm)

Approximately 1 g of tissue was taken and the exact weight recorded. Each tissue sample was homogenized with 5 ml ice-cold 1.15% KCl using a Polytron homogenizer. However, a problem arising with this extraction method was incomplete homogenization of some tissues. The efficiency of the Polytron was compared to manual homogenization of tissue using a mortar and pestle, by comparing the homogeneity of tissues macerated by each piece of equipment in 5 ml KCl. By visual examination of the sample after homogenization, it was observed that although the Polytron could not completely break down the connective tissues, it was much more a more efficient tool for this task than the mortar and pestle. Therefore, in order to reduce the amount of unhomogenized tissue in the sample, the brief centrifugation of the homogenate was introduced at this point. Duplicate 1.0 ml aliquots of the homogenate for each tissue sample were transferred to glass extraction tubes. Ethanol containing 0.1% BHT (1.8 ml) and 100 μl of each internal standard (retinyl acetate and beta-apo-8'-carotenal; 100 μg/ml) were added. Samples were mixed well and incubated at 70°C for 5

minutes. Saponification of samples was the carried out by adding 1 ml 10N KOH and incubation for 30 minutes at 70°C. After cooling, 4 ml hexane was added and samples were vortexed for 5 minutes. Following centrifugation at 3000 rpm for 15 minutes, the hexane layer was transferred to a test tube. Each sample was extracted twice and the hexane containing the retinol and beta carotene were pooled and dried under a stream of nitrogen at 40°C. Samples were sealed and stored at -20°C until assay by HPLC.

Method: Extraction of retinol and beta carotene from tissue
1 g tissue
5 ml ice-cold KCl
homogenize
aliquot 1 ml
1.8 ml ethanol
100 μg/ml internal standard
incubate at 70°C 5 minutes
1 ml 10N KOH
incubate at 70°C 30 minutes
cool
4 ml hexane
vortex, centrifuge 3000 rpm 15 minutes
aspirate hexane layer
dry under nitrogen

Table 4.8 shows the absorbance of liver samples from untreated animals extracted using this method. As seen in Table 4.8 compounds absorbing at 460 nm were present in the liver of untreated pigs.

Table 4.8 Concentration of beta carotene in liver tissues with no added beta carotene.

Extract	$Absorbance_{460}$	Concentration ($\mu g/g$)
1	0.018	0.34
2	0.012	0.23
3	0.024	0.46
4	0.023	0.44

Total retinol was extracted from tissues using the same method. The recovery of retinol was calculated in the same way as for the extraction of retinol from plasma. That is, the concentration of a blank sample was subtracted from the concentration of a sample to which a known amount of retinol had been added. The recovery of retinol using the elution from Chew (pers comm) of 47:47:6 (methanol:acetonitrile:chlorofrom) was $106 \pm 11\%$ after the addition of 100 μl of 100 μg/ml each of retinyl acetate and *all-trans* retinol to the homogenized tissue (Table 4.9). By comparison, recovery with the method used by the Analytical Reference Laboratory (95:5 methanol:water) was $132 \pm 6\%$, higher than that of Chew.

Tissue samples from untreated animals contained 17.6 to 18.8 μg of retinol/g of tissue (wet weight) when assayed by the HPLC method used for the separation of retinol in plasma (47:47:6 methanol:acetonitrile: chloroform). The HPLC method used by the ARL to assay total retinol in tissues (95:5; methanol:water) gave corresponding levels of 21.6 to 26.4 μg of retinol/g tissue (wet weight), suggesting greater UV response of the retinol compounds to this mobile phase.

Table 4.9. Recovery of retinol from liver tissues, calculated as for Table 4.1. Samples were run using an elution of (1) 47:47:6 (methanol:chloroform:acetonitrile) or (2) 95:5 (methanol:water).

Sample	Concentration (μg/g)	Recovery (%)
1 elution 1	0.99	99.0
2 elution 1	1.15	114.9
		Mean 106%
5 elution 2	1.39	139.0
6 elution 2	1.23	123.0
7 elution 2	1.47	147.0
8 elution 2	1.19	119.0
		Mean 132%

4.3.7 The assay of beta carotene by spectrophotometry

The Analytical Reference Laboratory and other research groups have used spectroscopy to quantify beta carotene with some success. Due to difficulties with HPLC equipment on site, samples were out sourced to the Analytical Reference Laboratory where the spectrophotometric method was used to determine beta carotene concentrations in the liver and ovary of sows treated with beta carotene or vehicle. Beta carotene was extracted (with retinol) from tissues using the hexane extraction method from Chew (pers comm). However, since beta-apo-8'-carotenal, the internal standard, absorbs at the same wavelength as beta carotene it was not added to the extraction. Losses during extraction were monitored by the examination of samples extracted with a known amount of beta carotene.

Beta carotene concentration in standards was quantified by spectroscopy. The absorbances and calculated concentrations are presented on Figure 4.5. The calculated concentration of beta carotene however, was lower than expected. This

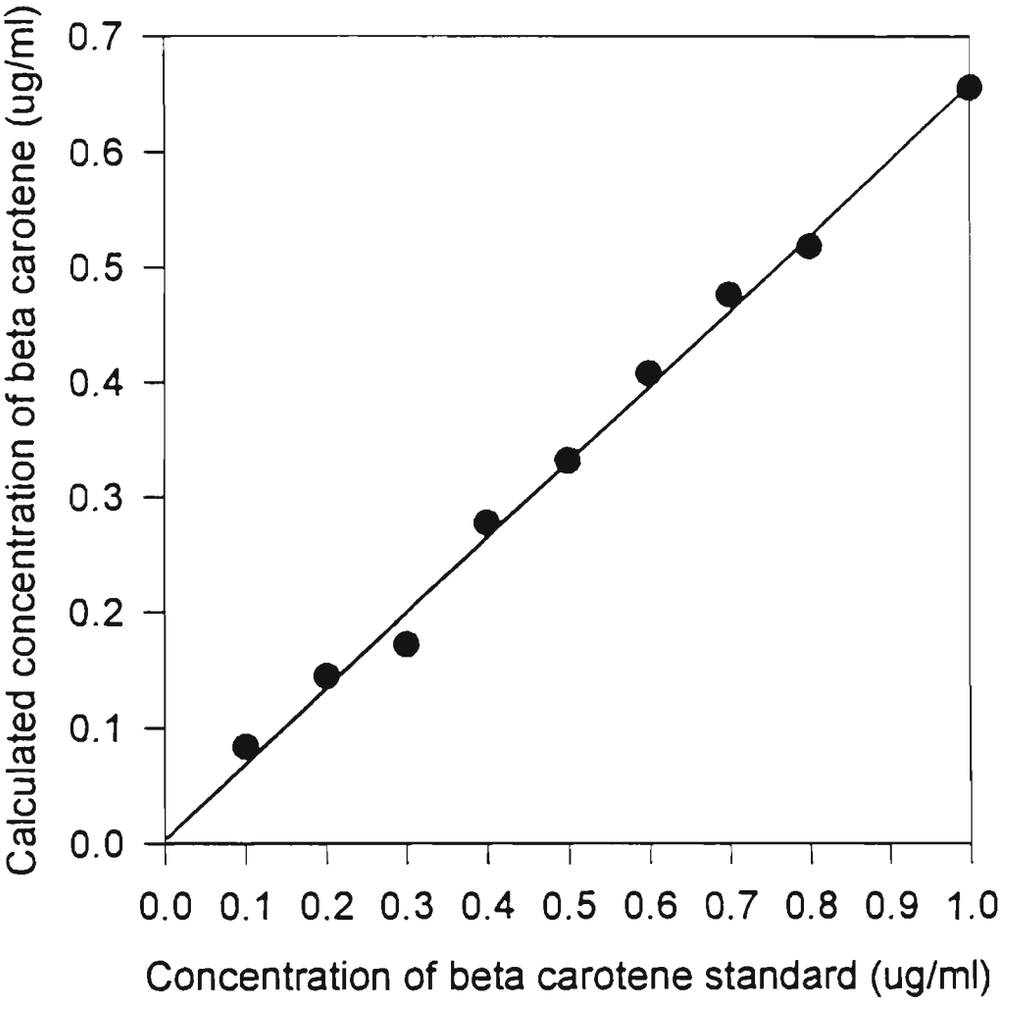


Figure 4.5 Concentration of beta carotene standards (ug/ml) vs calculated concentration of beta carotene using absorbance values.

may have been due to the initial purity of the compound (95%) and to some degradation of the standard. The concentration of beta carotene was calculated using Beers Law as follows:

$$\text{Concentration } (\mu g/ml) = \frac{\text{Absorbance}_{460} \times 10^6}{\text{Extinction coefficient} \times \text{width of cuvette}}$$

A sample liver tissue with no additions and a sample of liver tissue spiked with a known amount of beta carotene (5 μg/ml) were then extracted using the method provided by Chew (pers comm; section 4.3.6.2). The absorbance reading at 460 nm was used to calculate the concentration of beta carotene. This was compared with the concentration calculated from peak areas using HPLC. The HPLC conditions were as follows:

Mobile phase: 10:60:30 methanol:acetonitrile:chloroform

Flow rate: 1.3 ml/minute

Wavelength: 460 nm

Sample size: 60 μl injected onto a 50 μl loop

The results are shown in Table 4.10.

Table 4.10. Recovery of beta carotene in an untreated sample of liver compared with a sample spiked with 5 μg/ml beta carotene. Calculated concentration of beta carotene in the same sample assayed by HPLC.

Sample	HPLC BC Peak area	BC Ret time	conc (μg/ml)	Spectrophotometry absorbance	conc (μg/ml)
Blank 1.1	--	--	<0.3	0.015	0.29
Blank 1.2	--	--	<0.3	0.012	0.23
Spiked 2.1	137,549	2.373	5.299	0.234	4.47
Spiked 2.2	150,063	2.326	5.781	0.258	4.92
1.0 std	128,759	2.259	1.0	0.172	0.66
1.0 std	130,817	2.209	1.0		

The recovery of added beta carotene from a limited number of tissue samples using this method was 88.7% using spectrophotometric methods or 110.8% using HPLC separation of the extracted samples.

4.4 Discussion

Results of the experiments undertaken demonstrate that a simple precipitation of proteins from plasma with ethanol recovers close to 100% of retinol added to the plasma. Ethanol was used in this precipitation because it is a water miscible organic solvent, less polar than water, which attracts the non water-soluble retinol from the plasma and also denatures retinol binding protein and other blood-borne proteins. Because there is no stage at which samples were dried and reconstituted during this extraction, losses from this process were much reduced. The internal standard (retinyl acetate) was added to samples following homogenization to determine procedural losses. Retinyl acetate appears to be the internal standard of choice for retinol extractions (Chew et al 1991a, Bankson et al 1986, and others) indicating losses due to sample preparation and column losses, since it is completely separated from retinol during analysis.

Many methods of extraction of retinol from plasma include the addition of hexane to the ethanol, followed by centrifugation and removal of the hexane layer containing the retinol (Underwood et al. 1979, Bankson et al. 1986, Catignani and Bieri 1983 and others). Reported recoveries of retinol from plasma following the addition of hexane ranges from 94-108% (Catignani and Bieri 1983) to 84-96% (Bankson et al. 1986). These recoveries are comparable with those from the current work of 103%. Hexane is less polar than ethanol and therefore attracts the non-polar retinol from the ethanol layer. Triglycerides are removed from the sample in the saponification process (Ball 1988b). The hexane layer is then aspirated and subjected to HPLC. The hexane step was not included in the extraction of retinol from plasma in this work because the ethanol precipitation of

proteins alone was shown to be efficient in extracting retinol. The disadvantage of not adding hexane to the extraction was that lipids gathered on the column, necessitating repeated and frequent washing of the column.

The results of the beta carotene extraction from plasma show a recovery of close to 100% of beta carotene added to plasma. This extraction is a standard procedure and has been used as reported or only slightly modified, by many research groups (Chew et al. 1993, 1991a, 1984, Schweigert and Buchholz 1995 and others). Stahl et al. (1993), using an extraction which included 1 ml each of 2-propanol and dichloromethane with 6 ml of hexane achieved a beta carotene recovery of 90%, comparable with the recovery of beta carotene from the petroleum ether extraction used in the current work of 95.8%.

The first part of the extraction procedure in this study used ethanol. This extraction method involved the addition of ether as recommended by Chew (pers comm) which has the same effect as hexane as discussed above. Both diethyl and petroleum ether are less polar than ethanol and therefore preferentially partitioned the beta carotene from the ethanol layer to the ether phase. Samples were dried under a stream of nitrogen, a step at which most losses are assumed to occur. Since the recovery of beta carotene (added to plasma before extraction) was close to 100%, extraction losses from plasma in this study appeared to be minimal. The internal standard of choice for beta carotene was originally α-tocopherol acetate, a vitamin E standard, but beta-apo-8'-carotenal has been taken up by researchers such as Chew et al. (pers comm) due to its similarities to beta carotene, while offering complete separation by HPLC from beta carotene. The findings for this study have shown that beta-apo-8'-carotenal is an excellent internal standard since it is easily separated from beta carotene and appears to be stable during the extraction procedure.

Light has been shown to destabilize retinol and beta carotene. Egberg et al. (1977) showed that when all-trans retinol dissolved in ethanol was left exposed to white

light (a mixture of fluorescent lighting and natural sunlight) for 12 hours, 14% degradation occurred and 22% of the retinol was converted to the cis-isomer of retinol. Bankson et al. (1986) demonstrated that retinol, exposed to UV light for 30 minutes, was completely photo-oxidized. In the presence of light and high temperatures, carotenoids are also destroyed by acids or free halogens, forming cis-isomers. According to De Ritter and Purcell (1981) the primary site of attack is the 5,6 double bond or the in-chain double bonds, leading to chain cleavage or epoxide formation. Therefore, most extractions reported in the literature are carried out under yellow or dim light (Chew et al. 1993, 1991a, Catignani and Bieri 1983, Underwood et al. 1979) as was the present work.

Bankson et al. (1986), suggested that the extraction of retinol with ethanol and hexane was not recommended for tissues. However, the results of the extraction presented in this work and from that of other researchers (Chew et al. 1991a, Schweigert et al. 1995) demonstrate that these solvents extract retinol and beta carotene efficiently and repeatably from tissues. Chew et al. (1984) found concentrations of 16 μg/g retinol and 0.1 to 0.6 μg/g beta carotene in the liver of untreated pigs at slaughter, comparable with 17-26 μg/g retinol and 0.11 - 0.26 μg/g beta carotene from untreated pigs in the current study.

For the extraction of beta carotene, there appeared to be little difference in the amount of beta carotene from tissues of untreated animals extracted by the modified ARL method (homogenization followed by ethanol precipitation and xylene extraction) and the method recommended by Chew (pers comm) which involved homogenization, ethanol precipitation, saponification and then hexane extraction). The modified ARL extraction procedure gave a range of 0.19 to 0.53 μg/g while the method supplied by Chew (pers comm) gave 0.23 to 0.47 μg/g tissue wet weight. This may have been due to the similar polarities of hexane and xylene. These figures are within the range of those reported by Chew et al. (1984) using a hexane extraction with saponification.

A reverse phase chromatography system was selected for the separation of retinol and beta carotene because this system is ideal for the separation of non-polar to moderately polar compounds such as the fat soluble vitamins. According to Ball (1988b) the reverse phase system is more stable and reproducible than normal phase chromatography using silica or polar bonded phases for this group of compounds. Reverse phase chromatography systems are capable of separating isomers of beta carotene with little risk of degeneration of the compounds on the non-polar bonded phase. However, a strong, non-polar mobile phase containing little or no water is required to elute carotenoids (Ball 1988b). The reverse phase HPLC system with a non-aqueous solvent mixture or one containing only a small proportion of water has been used with success for the separation of beta carotene and the retinoids by a number of research groups (Chew et al. 1993, 1991a, 1984, Schweigert et al. 1995, Schweigert and Eisele 1995, Stahl et al 1993).

A comparison of two non-polar solvent mixtures was made in the selection of a HPLC mobile phase for the quantitation of retinol. When Chew et al.'s (1991a) published method using 50:38:12 (acetonitrile: tetrahydrofuran: water) as the mobile phase was used, peak areas of retinol standards were consistently smaller than for the 47:47:6 (methanol: acetonitrile: chloroform) method. It is possible that the absorbance qualities of the retinol were altered by the mobile phase, but since other research groups have been satisfied with the result using this mobile phase, this is unlikely. The latter method was preferred for the current work because the peaks eluted were sharp and the retention time was relatively short. Other groups have used a normal phase HPLC system (Bankson et al. 1986) but found that while the retinoids could be assayed by their system, beta carotene had no affinity for the normal phase column and was eluted with the void volume.

In the absence of a suitable mobile phase gradient for the separation of beta carotene and retinol and a consistently functional HPLC system, spectrophotometic quantitation of tissue samples for beta carotene was used to quantitate the levels of beta carotene in liver and ovarian tissues. This method has

long been an acceptable method of quantitating beta carotene (Britton 1985, Chew et al.1984, Johnston and Chew 1984, Coffey and Britt 1993). A comparison of HPLC analysis with spectrophotometric methods was carried out. When beta carotene samples extracted from tissue were analysed by HPLC a single peak was recorded at 450 nm, suggesting that at that wavelength, no other compound was present in quantities which could be detected by the method used. The concentrations calculated from the absorbance at 450 nm of the same samples on a spectrophotometer were in agreement with those calculated from peak area data provided by HPLC. It was therefore decided that the data provided by the ARL using spectrophotometric methods were comparable to data which could be attained using HPLC.

The limitations of the spectrophotometric method however, should be taken into account. Assay by spectrophotometry is not as specific as that provided by HPLC analysis, in that all compounds visible at the wavelength chosen are included in the absorbance figure. In comparison, the HPLC methods are able to separate different compounds visible at a particular wavelength by retention times specific to each compound on the stationary phase of the column. Given this limitation, it should be noted that extracted samples assayed by HPLC gave two peaks, these being beta carotene and the internal standard, suggesting that any other compounds present in the extract were in quantities below the detection limit of the UV detector and are therefore probably present in only low concentrations.

Further work could be done to refine the processes of extraction of retinol and beta carotene from tissues and plasma, particularly with a view to decreasing the amount of lipid in the extract. Further studies might include the additional development of an additional HPLC assay which allows simultaneous elution of retinol and beta carotene from plasma and tissue.

Chapter 5:

Retinol concentration in blood and selected tissues following treatment with retinol propionate

5.1 Introduction

Vitamin A has been shown to be essential in the regulation of vision, growth, reproduction and general health. It occurs in three naturally occurring forms, retinol, retinoic acid and retinal (Frickel 1984). Each form of retinol plays a unique role in the mammalian system. Retinoic acid is important in cellular differentiation, retinaldehyde is essential for the production of rhodopsin, the visual protein, while retinol is the vitamin A transport and storage form (Chew 1993). Retinoids are fat-soluble with an affinity with water-soluble retinoid-binding protein in plasma (Kanai et al. 1968), cytoplasm (Chytil and Ong 1984) and nucleus of cells (Petkovich et al. 1987) and uterus (Adams et al. 1981).

Brief and Chew (1985) examined the effects of feeding vitamin A on reproductive performance of gilts deficient in vitamin A. Weekly injection of vitamin A (12,300 IU) throughout gestation resulted in a significant increase in plasma vitamin A concentrations from 0.25 μg/ml before treatment to 1.0 μg/ml after 3 weeks of treatment. Treatment was associated with large improvements in reproductive traits such as decreased embryo mortality and consequent increase in litter size. There was no effect on neonatal piglet mortality and the overall result was an increase in the number of weaned pigs per litter.

In earlier work, Chew et al. (1984) determined the tissue levels of retinol after injection of vitamin A in the pig and cow. These workers found that retinol was deposited in the ovary (follicular fluid as well as corpora lutea) of cows and pigs. The liver was the primary site of storage in tissues and fluids measured in the cow, but in the pig less retinol was found in the liver than the plasma and follicular fluid. However, more recent work by Schweigert et al. (1995), who fed [^{3}H] retinol and [^{14}C] beta carotene to young pigs demonstrated that the primary site of storage of retinol was the liver, followed by kidney, duodenum, colon and then lung.

Most of the work carried out to date has been with retinol palmitate and retinol acetate. Little work using other esters has been published to date. An HPLC method was developed to separate retinol from extracted plasma for this study and is reported in Chapter 4. The aims of this experiment were to determine the plasma profile of retinol in mature pigs following an intramuscular injection of retinol proprionate, to determine the temporal changes in plasma retinol over a 24 hour period, and to determine the retinol levels in liver and ovarian tissue in mature sows after an intramuscular injection of 500,000 IU retinol proprionate.

5.2 Materials and Methods

Three experiments were carried out at the Research and Development Unit of Bunge Meat Industries at Corowa, NSW. Animals were slaughtered at the Hurstbridge Abattoir Pty Ltd, Hurstbridge.

Animals

Sows used for all experiments were the Bunge strain of Large-white x Landrace sows at 5th or 6th parity. Allocation to treatments took place at weaning for all experiments. Animals were fed the standard Bunge dry sow diet shown in Chapter 3, General Materials and Methods. This diet met or exceeded NRC requirements for all essential nutrients. Sows were fed once daily at 7:00 am and were allowed continuous access to water. Animals were housed indoors in single sow crates for experiments 1 and 2 and in groups for experiment 3.

Experiment 1 Plasma concentrations of retinol in sows treated wit retinol proprionate or vehicle

Sixteen freshly weaned sows were randomly allocated on the basis of stratified liveweight to one of two treatments in November 1993 at Bunge Meat Industries, Corowa. The average weights of sows allocated to group 1 and 2, respectively

were 234 ±21 kg and 231 ±25 kg. Twenty four hours before treatment indwelling catheters were introduced to the jugular veins of sows via the ear. Sows were housed in single dry crates and fed at 7:00am. Feed intake was observed and sows with low appetite were recorded. (Zanella and Mendl 1992; Chapter 3, General Materials and Methods).

Sows allocated to group 1 (n=8, control), were injected in the neck muscle with 1 ml of Solutol, the proprietary vehicle provided by BASF, Spain. Retinol proprionate (1 ml in Solutol, 500,000 IU, BASF Spain) was administered intramuscularly in the neck to the second group of sows (n=8). Treatments were administered commencing at 9:00am approximately 2 hours after feeding. Blood samples(10 ml) were taken at the following intervals: 16 hours before treatment, 5 minutes before treatment,% minutes after treatment, 3,6,9 and 12 hours after treatment and then at intervals of 12 hours for a further 6 days. Samples were collected into heparinized syringes and placed into amber tubes containing separation beads. Blood was centrifuged as soon as possible after collection and plasma was aspirated into 5 ml plain tubes and stored at -20°C until extracted and assayed for retinol by HPLC. Extraction techniques, HPLC conditions and their development are detailed in Chapter 4, but are briefly described below.

Retinol was extracted from plasma samples by ethanol precipitation. Ethanol (300 μl) was added to 100 μl of plasma and 100 μl of the internal standard, retinyl acetate (1.0 μg/ml). Samples were vortexed and centrifuged at 12,000 rpm for 5 minutes. The supernatant was assayed for retinol by HPLC immediately after extraction. An assay method provided by Dr B.P. Chew (pers. comm.) using a mobile phase of Methanol: Acetonitrile: Chloroform (47:47:6) was adopted with a flow rate of 1 ml per minute through a C-18 reverse phase column. Retinol was eluted at 1.97 minutes, followed by the internal standard, retinyl acetate at 2.36 minutes. The recovery of retinol using a spike of 1.0 μg/ml and calculated from 10 samples was 104±4.4%. The intra-assay coefficient of variation across all assays was 3% while the inter-assay coefficient was 7%. Peaks were positively

identified as being retinol by comparison with the retention time and peak area of external standards (All-trans retinol, Kodak) which were run daily. The lower limit of detection using the Chew assay was 0.1 μg/ml retinol. Samples for this and for all other assays (HPLC and spectroscopy) were analysed on the basis of random selection rather than on a treatment basis in order to ensure that there was no assay bias.

Experiment 2 Plasma concentrations of retinol in sows treated with retinol proprionate or vehicle.

Sixteen dry sows at 5th parity were randomly assigned to one of two treatments using a random number table in January 1995 at Bunge Meat Industries, Corowa. Mean weights of the two groups were similar. Group 1 (n=8) received an im injection of 1 ml vehicle (Solutol, BASF, Spain) in the neck,while a second group (n=8) were not treated. Twenty four hours prior to treatment indwelling catheters were placed in the jugular of each sow as for experiment 1. Sows were housed singly in dry crates. Blood samples of 10 ml were taken immediately before treatment beginning at 8:30am and then at intervals of 30 minutes after treatment for a period of 24 hours. Samples were collected into heparinized syringes, placed in amber tubes and centrifuged immediately after collection. Plasma was aspirated and stored at -20° pending extraction and assay for retinol by HPLC.

The same extraction and HPLC methods were used as for experiment 1 and peaks were positively identified as retinol by comparison with peak area and retention times for external standards. The intra-assay coefficient of variation across all assays was 9.4% while the intra-assay coefficient was 13.8%. In this assay, the retinol peak was eluted at 2.0 minutes while the retention time for retinyl acetate was 2.5 minutes.

Experiment 3 Tissue concentrations of retinol in the liver and ovary after treatment with retinol proprionate or vehicle

In the third experiment carried out in July 1994, 30 freshly weaned sows were randomly allocated using a random numbers table to one of two treatments. Mean weights of the two treatment groups were similar. Sows allocated to treatment 1 received an intramuscular injection of Solutol in the neck, while those allocated to treatment 2 were treated with an intramuscular injection of 500,000 IU retinol proprionate (1 ml). Sows were housed in groups and treated at Corowa and were transported by animal carrier to Hurstbridge 24 hours before slaughter, a distance of some 400 km. Animals were fasted and held overnight in group pens before slaughter the following morning. Sows from each treatment were slaughtered at 48, 72 or 96 hours after treatment. The number of sows allocated to each treatment and slaughter intervals is shown on Table 5.1.

Table 5.1. Numbers of sows allocated to treatments and slaughter times.

Treatment	48 hours	72 hours	96 hours	Total
Retinol	7	5	2	14
Control	8	5	3	16
Total	15	10	5	30

The entire gut was collected from the slaughter line and the liver and ovaries were removed and weighed. A sample of liver was taken from a uniform site (the left lower lobe) and liver and ovaries were snap-frozen in separate marked bags on dry ice. Samples were taken at 24 hours after treatment also, but due to inappropriate packaging, were not able to be processed. Frozen samples were transported to the lab and stored at -80°C until extracted and assayed for retinol. As for experiments 1 and 2, extraction procedures and HPLC conditions are detailed in Chapter 4, section 4.3.6.

Briefly, tissue samples (1 g) were homogenized in 5 ml 1.15% ice-cold KCl to

which was added 1.9 ml ethanol containing butylated hydroxy toluene and 100 μl of retinyl acetate (100 μg/ml). Samples were vortexed and then incubated at 70°C for 5 minutes. After the addition of 1 ml 10N KOH samples were incubated at 70°C for a further 30 minutes, allowed to cool and double extracted with 4 ml hexane. The hexane layers were aspirated and pooled and dried under a stream of nitrogen at 40°C. Samples were frozen at -20°C until assay by HPLC.

While extraction of retinol from tissue samples was carried out at Victoria University by the author, the assay of retinol concentrations by HPLC was undertaken by the Analytical Reference Laboratories in South Melbourne, using a method described in Chapter 4, section 4.3.6.2. The outsourcing of the retinol measurements was due to time constraints brought about by the continuing problems with the HPLC software at Victoria University. Recovery of retinol from 4 samples was 106$\pm$11%. The mobile phase used for the assays was Methanol:Water (95:5). The assays had linear regression values of 0.9982, 0.9999, 0.993 and 0.9999.

Calculations

Concentration of retinol in the plasma was calculated using the following formula, using the appropriate dilution factor:

$$\text{Concentration} = \frac{\text{Area(unknown)} \times \text{Amount(Internal standard)} \times \text{RRF(unknown)}}{\text{Area (Internal standard)}}$$

Where:

$$\text{RRF (Relative Response Factor)} = \frac{\text{Amount (External Std)}}{\text{Area(External Std)}} \times \frac{\text{Area(Internal Std)}}{\text{Amount(Internal Std)}}$$

For the daily calculation of the RRF, 60μl of the internal standard (1.0 μg/ml Retinyl Acetate) was injected and the peak area and retention time were recorded. A standard curve for all-trans Retinol was assayed by HPLC and the peak area for

1.0 μg/ml Retinol was utilized in the above equation.

Statistical analysis

Mean plasma concentrations of retinol were analysed by least squares analysis of variance (ANOVA) design for repeated measures and univariate ANOVA on the Statistica and SPSS packages. Concentrations of retinol in ovarian and liver tissue were compared using a least squares ANOVA. The ANOVAs for tissue concentrations were a two-way design. The dependent variable is the parameter which treatments may have affected. The covariate, where used, was a factor which in itself may not have directly influenced the dependent variable, but may have influenced changes in the dependent variable in conjunction with other factors. The analysis is designed to test whether there was a difference between the effects of selected factors such as treatment (eg vehicle or retinol or time of slaughter) on the dependent variable (plasma retinol concentrations).Where main effects were established significant differences between means were identified using the least significant differences test. For tables showing analysis of variation, see appendix 3.

5.3 Results

Experiment 1. Plasma concentrations of retinol in sows treated with retinol proprionate or vehicle.

The plasma concentrations of retinol in treated animals was significantly higher than that of untreated animals at 12 hours after treatment using a univariate ANOVA design ($P<0.05$, Figure 5.1). The mean plasma concentration in retinol treated animals tended to be higher from time 0 until 96 hours after treatment than in vehicle treated sows, but this difference was not significant. When data were analysed using a repeated measures design there was no significant difference

between retinol and vehicle treated animals for treatment or time. There was no difference between day and night plasma concentrations of retinol. Given the wide variation of retinol concentrations within animals (a sample of individual plasma profiles is shown in Figure 5.2 a and b) it was decided to monitor retinol concentrations in the plasma over a 24 hour period.

Experiment 2 . Plasma concentrations of retinol in untreated sows or sows treated with vehicle over a 24 hour period

Figure 5.3 shows the mean plasma concentrations of animals injected with solutol or untreated over a period of 24 hours. There was no significant difference between plasma concentrations of retinol in treated or untreated sows at any time during the sampling period ($P=0.89$). Mean plasma concentrations of retinol within each treatment group were shown to vary by 0.03 μg/ml over a 30 minute period. However, using a repeated measures design, there was a significant increase in plasma retinol concentrations over the sampling period ($P<0.001$). Individual plasma profiles are shown in Figure 5.4 a and b.

Experiment 3. Tissue concentrations of retinol in liver and ovary after treatment with retinol proprionate or vehicle.

The design of analysis of variance, results table and table of mean ($\pm$s.e.) concentrations of retinol in ovarian tissue are shown in Table 5.3.

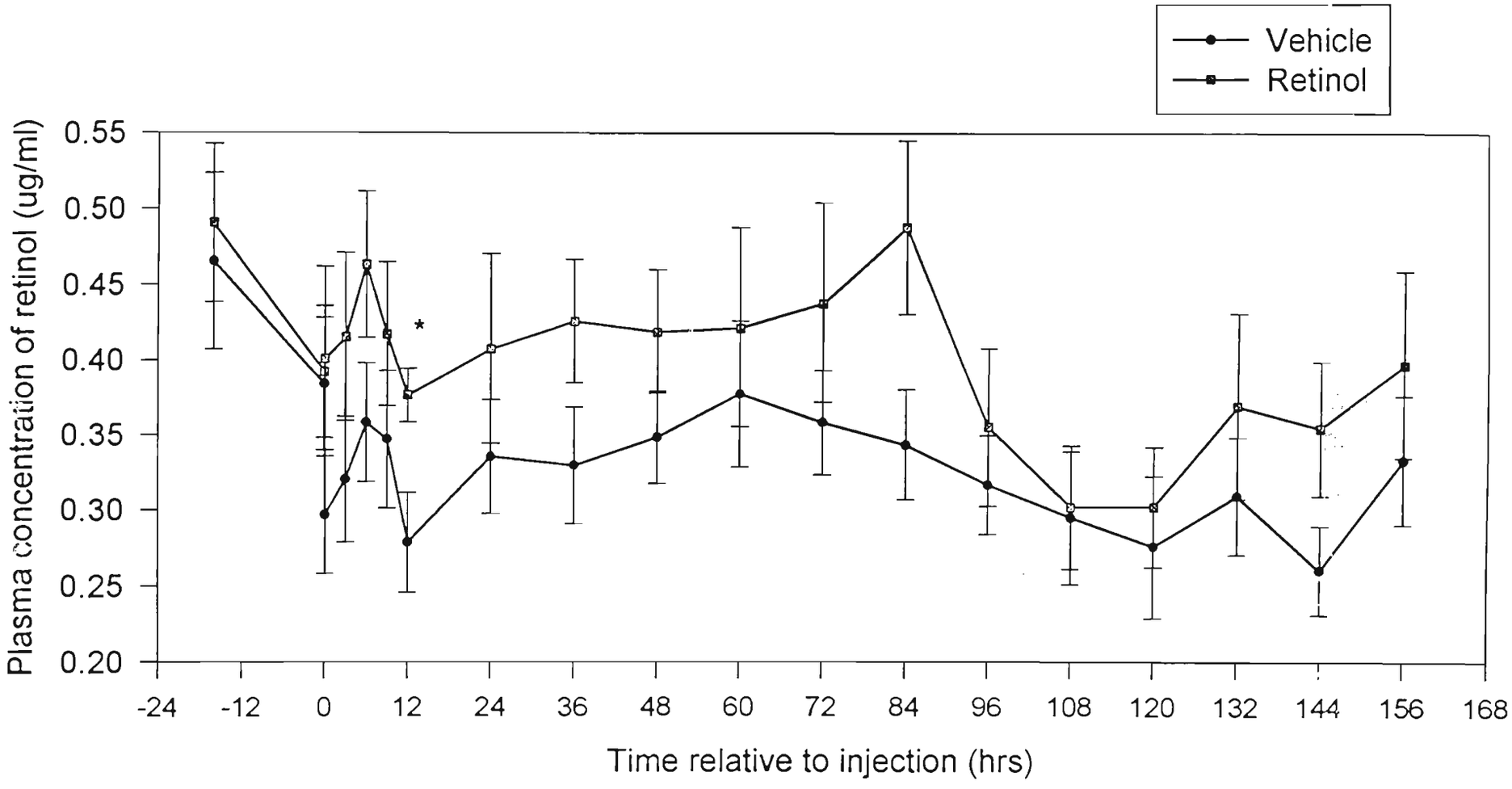


Figure 5.1 Mean concentration of retinol (ug/ml) in the plasma of sows after treatment with 500,000 IU retinol proprionate or 1 ml vehicle. (*) designates statistical significance.

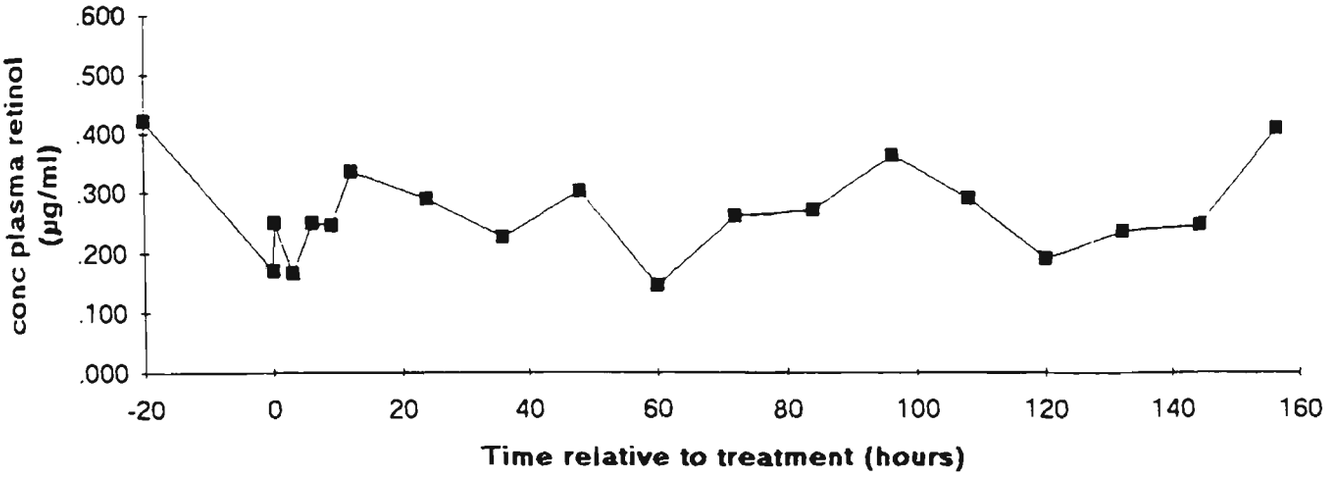


Fig. 5.2a Plasma concentrations of retinol in sow 9442 sampled at hourly intervals after treatment with vehicle only.

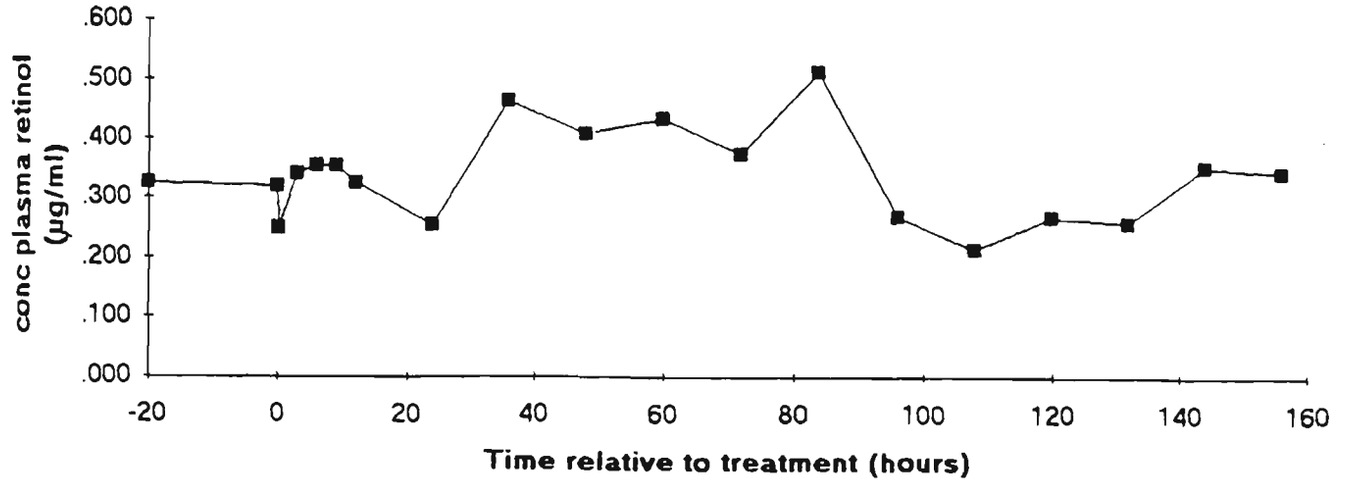


Fig 5.2b Plasma concentrations of retinol in sow 5217 sampled at hourly intervals after treatment with retinol proprionate.

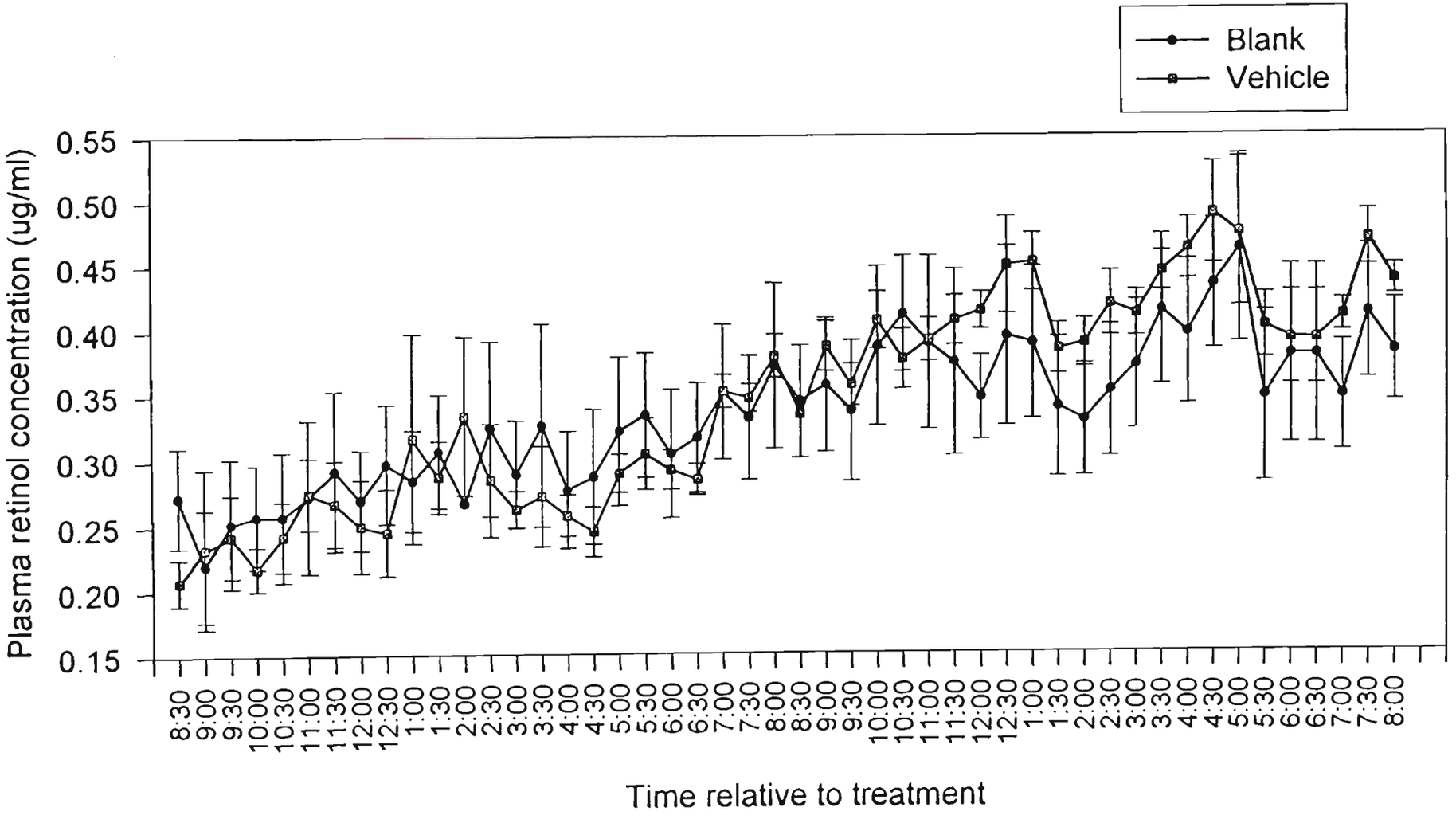


Figure 5.3 Mean retinol concentrations (ug/ml) in the plasma of sows after a single injection of 1 ml Solutol or no treatment over a 24 hour period.

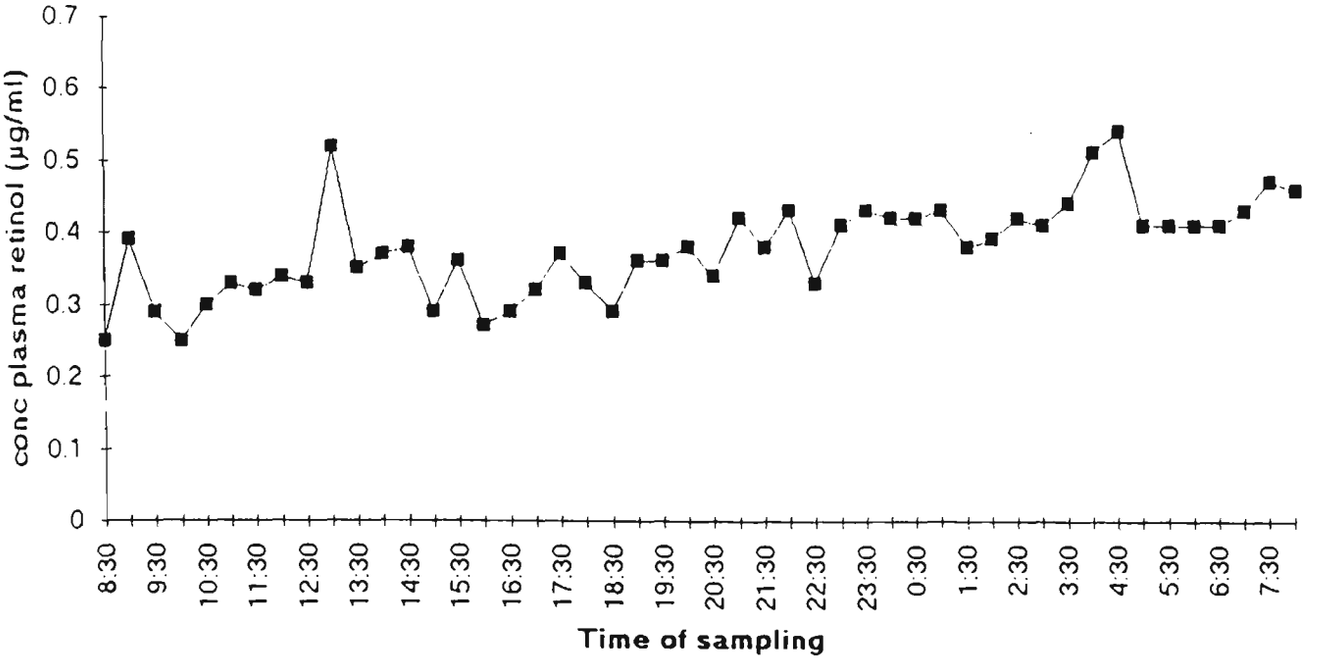


Fig. 5.4a Plasma concentrations of retinol in sow 6481 sampled after treatment with solutol.

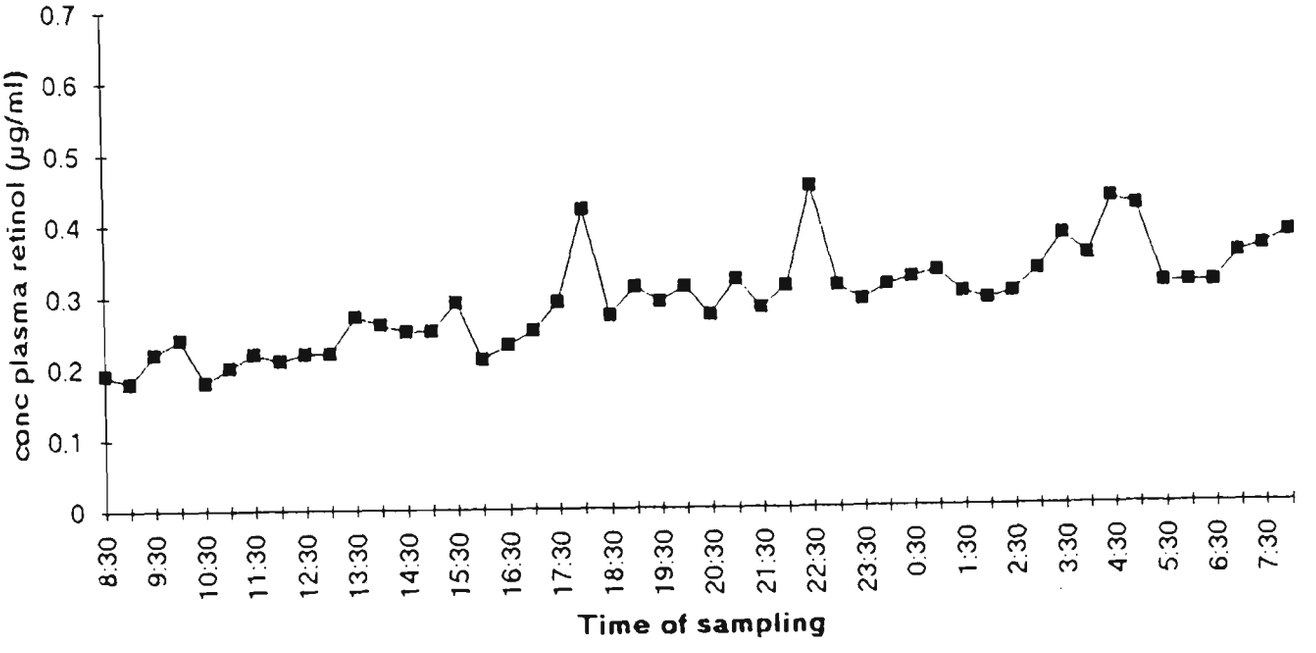


Fig. 5.4b Plasma concentration of retinol in sow 404 sampled after treatment with retinol.

Table 5.2. Sources of variance retinol concentrations in the ovary of animals treated with retinol or vehicle. The analysis is a two-way fixed effects analysis of variance with state of the ovary as a covariate. Treatments were retinol proprionate or vehicle and times of slaughter were 48, 72 and 96 hours after treatment.

Source	df	MS	F	P value
Treatment	1	3.9923	0.040925	0.842
Slt-time	2	201.1361	2.061869	0.151
Trt x Slt-	2	3.6824	0.037748	0.963
Error	22	97.55036		

There was no difference (Table 5.3) between the concentrations of retinol in the ovary between the retinol and vehicle treated animals. Since ovaries showed some variation in stage of development in follicles or corpora lutea, ovarian state was taken into account and used as a covariate. This factor alone did not contribute to significant difference ($p=0.237$).

Table 5.3 Retinol concentrations ($\mu g/g \pm s.e.$) in liver and ovarian tissues at 48, 72 or 96 hours after treatment with 500,000 IU retinol propionate or 1 ml vehicle

	Ovarian		Liver	
Time/Treatment	Retinol	Vehicle	Retinol	Vehicle
48 hours	31.7 ± 4.7	29.0 ± 4.3	204.0 ± 40.6	240.8 ± 44.8
72 hours	22.5 ± 3.3	23.2 ± 0.5	335.6 ± 58.6	331.2 ± 124.2
96 hours	22.0 ± 0.0	20.0 ± 4.2	238.0 ± 76.0	296.0 ± 4.0

Table 5.4 shows the statistical design and analysis of variance for retinol concentrations in the liver after treatment with retinol or vehicle. There was no significant difference between liver concentrations of retinol in the liver of animals treated with retinol or vehicle (Figure 5.3).

Table 5.4 Sources of variance in concentrations of retinol in the liver of animals treated with retinol or vehicle. The analysis is a two-way fixed effects analysis of variance. The treatments were retinol proprionate or vehicle and the slaughter times were 48, 72 or 96 hours after treatment.

Source	df	MS	F	P value
Treatment	1	4894.38	0.200008	0.658898
Slt-time	2	36913.75	1.508473	0.242338
Trt x Slt	2	1881.88	0.076903	0.926217
Error	23	24470.94		

Total liver concentration of retinol was calculated by multiplying the concentration per gram by the weight of the liver of each sow. Data are shown in Table 5.5. None of the compared data values were significantly different (P=0.784)

Table 5.5 Total liver concentration (mg) of retinol after treatment with 500,000 IU retinol proprionate or vehicle.

Treatment	48 hours	72 hours	96 hours
Retinol	525.4±117.6	846.15±330.5	456.4±19.6
Vehicle	567.5±94.6	920.2±170.4	475.6±63.0

5.4 Discussion

These studies suggest that there is considerable variation in plasma concentrations of retinol. Underwood et al. (1979) suggested that the homeostatic mechanisms of individual animals establish circulating plasma concentrations of retinol that can be regarded as normal for that individual. While acute or chronic deviations from this level may cause transitory or long term readjustment, the influence on this is only partly due to exogenous sources of vitamin A. Newly absorbed vitamin A is de-esterified by enzymes in the gut and transported by chylomicrons as retinol to the liver. Here retinol is de-estified and stored in parenchymal and non-parenchcmar stellate cells (Goodman, 1984). It is well known that retinol mobilization and transport are tightly regulated and controlled by the same processes that regulate the synthesis of retinol binding protein in the liver (Goodman 1984).

Intramuscular injection of 500,000 IU retinol proprionate in the current experiment resulted in increased plasma concentrations of retinol at 12 hours after treatment in Experiment 1 but was not different from the control group at any other time. When Schweigert et al.(1995) fed pigs aged 5 weeks with a single bolus of [^{3}H] retinol he found that plasma radioactivity reached its peak at 5-6 hours after injection and remained at these levels for at least 24 hours. Chew et al. (1984) found that plasma of gilts collected at random from a slaughter house had a concentration of 0.21 μg/ml total retinol, a figure comparable with retinol levels in the control group of the present study (0.27-0.5μg/ml). The range of the samples assayed by Chew et al.(1984) was between 0.13 and 0.43 μg/ml. The variation in the levels of retinol reported by these workers may have been due to age differences and the genetic component of the pigs used for the study. These researchers also commented that samples were assayed by spectrophotometry at 325 nm and correction for interference by beta carotene at this wavelength had to be made, assuming a constant contribution of 8% of the absorbance at 450 nm.

In later work in which Brief and Chew (1985) injected retinol deficient gilts with a combination of beta carotene and vitamin A weekly during pregnancy, plasma levels of retinol were significantly increased on the first day of treatment. Treated gilts exhibited plasma retinol concentrations of 1.0 μg/ml, approximately double the highest concentration detected in the current experiment. Gilts which were fed the same dose of vitamin A as that injected by Brief and Chew (1985) showed no difference in plasma concentrations of retinol from control animals. However, the gilts in this experiment were in a deficient condition and therefore the difference in nutritional status compared with the present experiment may have contributed to the lower increase in plasma retinol concentrations. In addition, weekly injection of 12,300 IU vitamin A would have retained plasma levels of retinol indefinitely.

The absence of an increase in retinol concentrations may have been due to one of several causes. Retinol proprionate may have been transferred to the lymph system, resulting in lower concentrations being detected in the plasma. However, due to the close association of these two systems, this explanation seems unlikely. In addition, under physiological conditions it has been shown that vitamin A is transported in the blood attached to retinol binding protein, chylomicrons (after ingestion of retinol) or lipoproteins (Schweigert et al. 1995). Another explanation is that the retinol may have remained in a pool in the subcutaneous fat and muscle at the site of injection, and released slowly over the period of sampling. Solutol, the proprietary vehicle supplied by BASF is known to be a slow release agent. This explanation seems possible since the retinol treated animals demonstrated a trend for higher plasma retinol concentrations than vehicle treated animals.

Alternately, since plasma samples were not saponified during the protein precipitation process, the possibility exists that the ester of retinol (proprionate) may not have been detected by the retinol assay used in this study. Schweigert and Schoon (1996) were able to separate various esters of retinol using an isocratic elution of methanol: acetonitrile:2-propanol (54:44:2) in a reverse phase system,

the 2-propanol serving to release the most strongly bound compounds from the non-polar stationary phase. The mobile phase used in the current experiment was isocratic and even less polar than the elution used by Schweigert and Schoon (1996). This mobile phase (methanol:acetonitrile:chloroform; 10:60:30) produced a single retinol peak at 1.97-2.0 minutes. Various alterations were made to the mobile phase to alter the polarity in order to release esters of retinol from the stationary phase of the column, or to separate esters eluted in a single peak. Neither increasing the concentration of chloroform within an isocratic method nor by gradient elution aided in producing any further peaks. We conclude that all esters of retinol were eluted in a single peak. Therefore it seems unlikely that the lack of a marked increase in plasma retinol levels in this study was due to a rise in retinol esters.

In the second experiment, in order to rule out any effect of the proprietary vehicle on the fluctuations in plasma retinol concentrations, gilts treated with 1 ml of Solutol were compared with untreated gilts. Plasma concentrations of retinol increased with time in both treatment groups. This may indicate a cortisol or adrenalin-related increase in retinol levels, possibly as a stress response. Further study in this area is clearly required. Plasma samples could be assayed for cortisol or adrenalin and these levels correlated with the concentrations of plasma retinol. However, the data presented illustrates the tight control of the mobilization and transport of retinol (Goodman 1984), as there was no difference between plasma retinol concentrations of the vehicle-treated and untreated sows. There was no significant evidence for an effect of timing of feeding or diurnal rythms in plasma concentration of retinol in this experiment.

When sows were injected with 500,000 IU retinol proprionate in experiment 3, concentrations of retinol in the liver and ovaries were not different to those in animals treated with the proprietary vehicle, even at 96 hours after the treatment. Schweigert et al. (1995) found that a single oral dose of [^{3}H] retinol in 5 week old pigs was stored within 24 hours primarily in the liver with small concentrations in

the kidney and small intestine, colon and lung. Chew et al. (1984) found approximately equal concentrations of total vitamin A in plasma (0.21 μg/ml) and follicular fluid (0.25 μg/ml) and 16 μg/g in the liver but much less retinol in the corpus luteum of gilts (1.2 μg/g). The current experiment demonstrated much higher levels of retinol in liver (240.7-331.2 μg/g) than plasma (0.27-0.5 μg/ml) or ovary (20.0-29.0 μg/g) in untreated sows, but this may be a reflection of the age of the sows. Retinol is a fat-soluble vitamin and when it is in excess to requirements it is stored primarily in the liver but also in the adipose tissues and to a lessor extent in blood cells and bone marrow, rather than excreted. Therefore, parity 5 sows (3 years of age) would be expected to behave much more stored vitamin A than gilts of 8 months. Retinol Binding Protein is present and associated with the regulated release of retinol from adipose tissues in the rat (Tsutsumi et al. 1992). Therefore, even in the event of increased catabolism of fat to maintain homeostatis is sows suffering from inappetite during oestrous would not be expected to cause the uncontrolled release of retinol into the plasma. Catabolism of fat and the controlled release of retinol from adipose stores may, however, have contributed to the fluctuations seen in plasma concentrations of retinol in earlier experiments.

Examination of ovarian tissue revealed no difference in concentrations of retinol between retinol and vehicle treated animals at any time after treatment. Since ovaries were at various stages of development at time of treatment, state of the ovary was used as a covariate, but even this was not a significant contributor to variation. It must be concluded from the present study that an injection of retinol proprionate into into the muscle has no immediate effect on the ovarian levels of vitamin A.

The lack of response in liver and ovarian storage of retinol after injection with retinol proprionate may have been due to several factors. Variation in the analysis may have been due to slight differences between assay runs. While the extraction of samples was as uniform as possible, not all extractions could be carried out on

the same day and in the same extraction run. Although samples were double-extracted with hexane, all beta carotene may not have been recovered. Similarly, all samples could not be assayed in the one HPLC run. Although the samples were assayed in a NATA accredited laboratory, there may have been slight differences between assay runs. Variation in the ability of individual animals to mobilize and utilize retinol may have contributed to the wide variation within treatment groups for plasma, ovary and liver measurements demonstrated in this experiment. The dose of retinol proprionate given to sows (500,000 IU) may have been inadequate to elicit a physiological response to treatment. The design of the experiment may have contributed to the variation within treatment groups. The random allocation of animals to treatment groups without reference to their basal storage levels of retinol may have reduced the possibility of achieving a significant difference in the tissue levels of retinol after treatment.

This study demonstrated that injected retinol proprionate caused a transient increase in plasma retinol at 12 hours after treatment. However there was no detectable increase in concentrations of retinol in the liver or ovary of treated sows.

Chapter 6:

Beta carotene concentrations in blood and selected tissues after treatment with beta carotene

6.1 Introduction

Improved reproductive performance has been reported after the dietary administration of beta carotene to dairy cows and heifers (Lotthammer 1979), horses (Kormann et al. 1989) and rabbits (Schweigert 1990). An improvement in litter size in pigs injected with beta carotene has been reported (Brief and Chew 1985, Coffey and Britt 1995).

Brief and Chew (1985) showed that weekly injection of beta carotene in crossbred gilts from day of mating, through farrowing until weaning resulted in lower embryo mortality, larger litter size and heavier litter weight than in untreated animals. Coffey and Britt (1989 and 1995) demonstrated a linear relationship between dose of beta-carotene (0-200mg) and litter size in multiparous sows. In contrast, no treatment effect was seen in primiparous animals given the same treatment.

There has been some discussion in the literature regarding the mode of action of beta carotene. However, little work has been done on the effect of beta carotene treatment on follicular development or ovulation rate. Chew et al. (1982) reported that although there was no significant increase in ovulation rate or ovarian weight in gilts injected with 16.4 mg of beta carotene, the number of corpora lutea present and the ovarian weight in treated pigs was higher than in controls. In yellow fat animals, which are able to take beta carotene up from the diet, beta carotene is stored in the ovary and is present in high concentrations in the corpus luteum, raising the possibility of a local role in ovarian function (Chew et al. 1991a, Schweigert et al. 1995).

Chew (1993) suggested that the action of beta carotene may be involved with the reduction of embryo mortality via the regulation of uterine secretions. As there are very few reports on the *in vivo* conversion of beta carotene to retinol or retinoic acid after injection, it would appear therefore that there is little evidence

to support the theory that beta carotene acts merely as a precursor to retinol in the reproductive processes. Indeed, Chew (1993), Coffey and Britt (1993) and Schweigert and Eisele (1990) suggest that beta carotene may have a unique function in the reproductive processes, independent of the action of retinol.

An extraction method and assay for the quantification of beta carotene in plasma and tissue were developed and are presented in Chapter 4. The aims of this study were to determine the plasma profile of beta carotene in mature sows after an intramuscular injection of 250 mg beta carotene and to determine beta carotene and retinol concentrations in ovarian and liver tissues in mature sows after intramuscular administration of 250 mg beta carotene.

6.2 Materials and Methods

Two experiments were carried out at the Research and Development Unit of Bunge Meat Industries at Corowa, NSW. Animals were slaughtered at the Hurstbridge Abattoir Pty Ltd, Hurstbridge Victoria.

Animals

Sows used for both experiments were the Bunge strain of Large-white x Landrace sows at 6th parity. Allocation to treatments for each experiment was carried out after weaning of litters from sows. Animals were fed the standard Bunge dry sow ration shown in Chapter 3, General Materials and Methods. The diet met or exceeded the NRC requirements for all essential nutrients. Sows were fed once daily between 7:00 and 7.10 am and had continuous access to water. Animals were housed indoors in single sow crates for experiment 1 and in groups for experiment 2.

Experiment 1 Plasma concentrations of beta carotene in sows treated with beta carotene or vehicle

Eighteen sows were randomly assigned on the basis of stratified liveweight at weaning to one of two treatments in October 1993 at Bunge Meat Industries, Corowa. The average weights of the sows allocated to groups 1 and 2 were 178±28 kg and 172±19 kg respectively. Indwelling catheters were placed in the jugular via the ear (Zanella and Mendl 1992; Chapter 3, General Materials and Methods) on the day before treatment.

Sows allocated to group 1 (n=9, control), were injected in the neck muscle with 1 ml of Solutol, the proprietary vehicle provided by BASF, Spain. Beta carotene (250 mg in 1 ml Solutol, BASF Spain) was administered intramuscularly in the neck to sows allocated to group 2 (n=9). Treatments were administered at 10.00 am. Blood samples of 10 ml were taken at the following intervals: 24 hours before treatment, 0.5minutes and 3, 6 and 12 hours after treatment, and at 12 hourly intervals for the following 5 days. Blood samples were collected into heparinized syringes and placed into amber tubes. Samples were centrifuged as soon as possible after collection and plasma was aspirated into 5 ml plain tubes and stored at -20°C until extracted and assayed for beta carotene by HPLC. Extraction techniques and HPLC conditions are detailed in Chapter 3, General Materials and Methods.

Briefly, duplicate samples of 500 μl of plasma was mixed with 100 μl of 1.0 μg/ml beta-apo-8'-carotenal (internal standard) and 400 μl of 95% ethanol containing butylated hydroxy toluene. A mixture of 1:1 petroleum ether: diethyl ether (3 ml) was added, the sample was shaken for 6 minutes and then centrifuged at 3000 rpm for 10 minutes. The upper phase was aspirated and dried under a stream of nitrogen at 40°C, then stored at -20°C until assay by HPLC. Samples were reconstituted in mobile phase. The separation of beta carotene from extracted plasma was carried out using a C_{18} reverse phase column at a wavelength of 466

nm. The mobile phase was 10:60:30 (methanol:acetonitrile:chloroform) at a flow rate of 1.3 ml/min. Beta carotene eluted at 3.9-4.2 minutes, using these conditions, and the internal standard appeared at 2.3-2.5 minutes. The recovery of beta carotene calculated from 8 samples was 89.4±13.6%. The inter-assay coefficient of variation across all samples was 4.9% while the intra-assay coefficient of variation was 7.5%. Peaks were positively identified by comparison with the retention time and peak area of external standards (All-trans beta carotene, Kodak) which were run daily. The lower limit of detection using this assay was 0.3 μg/ml beta carotene.

Experiment 2 Tissue concentrations of beta carotene in the liver and ovary after injection with beta carotene or vehicle.

In the second experiment carried out in July 1994, 35 freshly weaned sows were randomly allocated to one of two treatments using a random numbers table at Bunge Meat Industries, Corowa. Mean weights of the two groups were similar. Sows allocated to treatment 1 received an intramuscular injection of Solutol in the neck, while those allocated to treatment 2 were treated with an intramuscular injection of 250 mg beta carotene in 1 ml Solutol. Sows were housed in crates, treated at Corowa and were transported by animal carrier to Hurstbridge 24 hours before slaughter. Animals were fasted and held overnight in group pens before slaughter the following morning. Sows from each treatment were slaughtered at 48, 72 or 96 hours after treatment. The number of sows allocated to each treatment and slaughter intervals are shown in Table 6.1.

Table 6.1 Number of sows allocated to treatments and slaughter times.

Treatment	48 hours	72 hours	96 hours	Total
Beta carotene	9	5	5	19
Control	8	5	3	16
Total	17	10	8	35

As for the retinol experiment, the entire gut was collected from the slaughter line and the liver and ovaries were removed and weighed. A sub-sample of liver taken from a uniform site (the left lower lobe) and both ovaries were snap frozen in dry ice in separate bags and stored at -80°C until extracted for the beta carotene assay. Due to inappropriate packaging, samples taken at 24 hours after treatment could not be processed. Tissues were extracted using the hexane extraction method as detailed in Chapter 4 in section 4.3.6.

Briefly, tissue samples (1g) were homogenized in 5 ml 1.15% ice-cold KCl to which was added 1.9 ml ethanol containing butylated hydroxy toluene and 100 μl of retinyl acetate (100 μg/ml). Samples were vortexed and then incubated at 70°C for 5 minutes. After the addition of 1 ml 10N KOH samples were incubated at 70°C for a further 30 minutes, allowed to cool and double extracted with 4 ml hexane. The hexane layers were aspirated, pooled and dried under a stream of nitrogen at 40°C. Samples were frozen at -20°C until assayed by spectrophotometry. Dried samples were reconstituted in 1 ml methanol containing 5% chloroform. An aliquot of 0.5 ml was taken, to which 0.5 ml distilled water and 1 ml ethanol was added. Samples were then extracted with 4 ml xylene. The xylene phase was aspirated and assayed at 460 nm by spectrophotometry. The recovery of beta carotene calculated from a 4 samples was 92.8$\pm$7.6%. The inter-assay coefficient of variation across all samples was 1.5%. Development of the extraction and assay methods are detailed in Chapter 4, section 4.3.6.

Calculations

The concentration of beta carotene in the plasma was calculated using the formulae shown in Chapter 4, with the appropriate dilution factor. For the daily calculation of the RRF, 60 μl of the internal standard (1.0 μg/ml β-apo-8'-carotenal) was injected and the peak area and retention time were recorded. A standard curve for all-trans beta carotene was assayed by HPLC and the peak area for 1.0 μg/ml beta

carotene was used in the calculations.

Concentration of beta carotene in the tissue (μg/g tissue wet weight) was calculated using the following formula and taking into account the dilutions made:

$$\text{Concentration (mol/L)} = \frac{\text{Absorbance (460)}}{\text{Extinction coefficient x 1 cm}}$$

$$\text{Concentration } (\mu\text{g/ml}) = \text{Concentration (mol/L)} \times 10^3 \times \text{molecular weight}$$

Statistical analysis

Mean plasma concentrations of beta carotene were analysed by least squares analysis of variance (ANOVA) design for repeated measures using the Statistica package. Concentrations of beta carotene in ovarian and liver tissue were compared using a least squares ANOVA. The ANOVAs for tissue concentrations were a two-way design. The dependent variable is the parameter which treatment may affect. The covariate, where used, was a factor which in itself may not have directly influenced the dependent variable, but may have influenced the dependent variable in conjunction with other factors. The analysis is designed to test whether there was a significant difference between the effects of selected factors such as treatment (eg vehicle or retinol; time of slaughter) on the dependent variable. For the analysis of variance tables see appendix 3. Significant means were identified using the Least Significant Difference test. For analysis of variance tables, see appendix 3.

6.3 Results

Experiment 1. Plasma concentrations of beta carotene in sows treated with beta carotene or vehicle.

Beta carotene concentrations in the plasma of treated sows treated rose significantly ($P<0.001$) within 3 hours of treatment (Figure 6.1) and peaked at 36 hours after treatment. Levels fell to near baseline at 132 hours after treatment; however even after a further decrease at 144 hours after treatment there was still a significant difference in plasma concentrations of beta carotene between treated and untreated animals. Beta carotene was undetectable in the plasma of sows which were treated with the vehicle (Solutol) (Figure 6.1). Where the plasma beta-carotene concentration was undetectable, a value equal to the lower limit of the assay ($0.1\mu g/ml$) was assigned to the sample.

Experiment 2. Tissue concentrations of beta carotene in liver and ovary after injection with beta carotene or vehicle.

The design of the ANOVA, results and table of mean ($\pm$s.e.) concentrations of beta carotene in ovarian tissue are shown in Table 6.2 and Table 6.4a respectively.

Table 6.2. Sources of variance in beta carotene concentration in the ovaries of sows treated with beta carotene or vehicle. The analysis is a two-way fixed effects analysis of variance with state of the ovary as a covariate. Treatments were beta carotene or vehicle and the times of slaughter were 48, 72 and 96 hours after treatment.

Source	df	MS	F	P value
Treatment	1	0.430963	2.241141	0.146
Slt time	2	0.033919	0.176341	0.839
Treat x Slt	2	0.175746	0.913935	0.413
Error	28	0.192296		

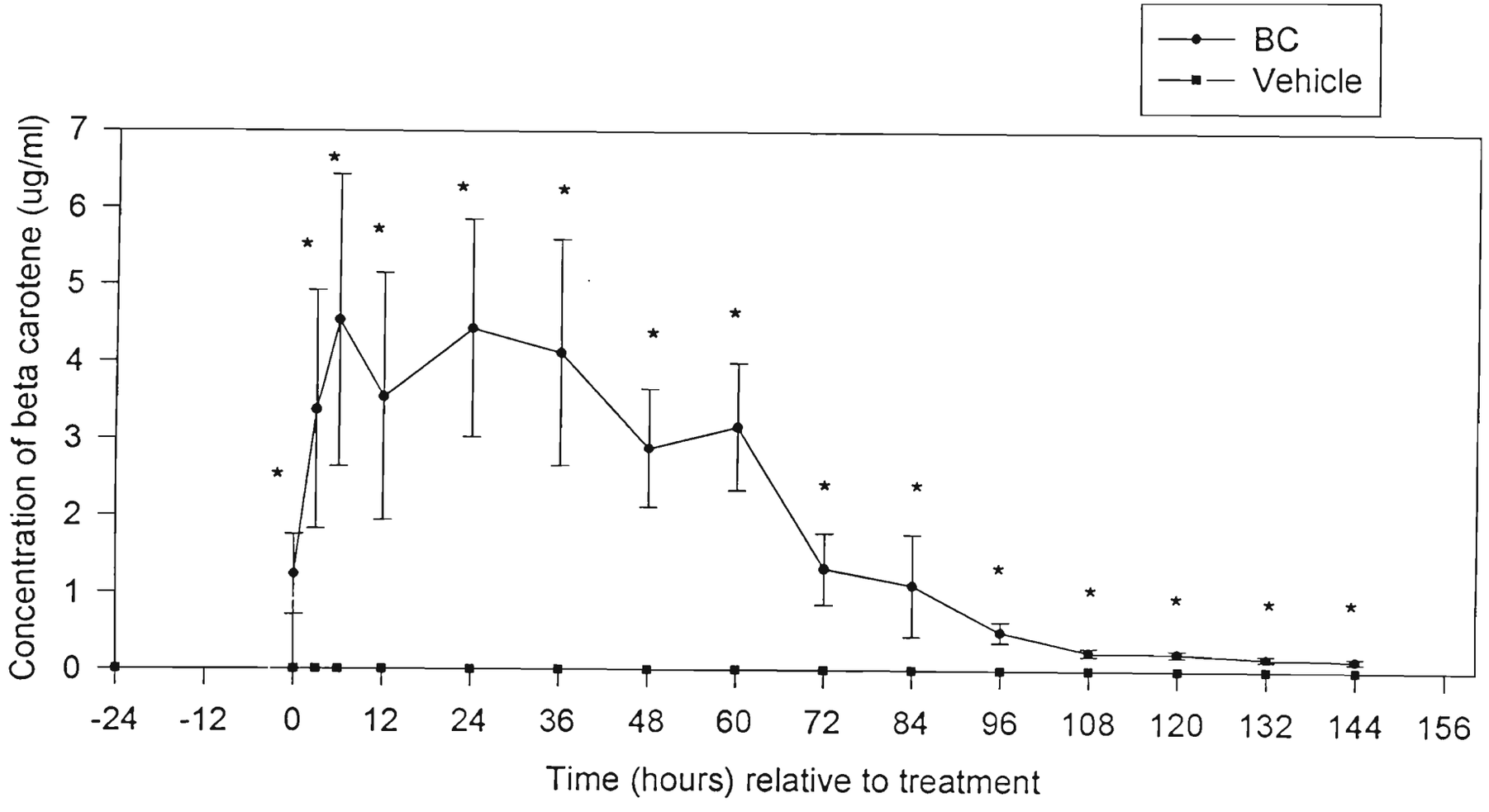


Figure 6.1 Mean plasma concentration of beta carotene (ug/ml) in sows after injection with 250 mg beta carotene or 1 ml vehicle. (*) designates statistical significance.

There was no significant difference in ovarian beta carotene concentrations between beta carotene and vehicle treated animals; or between beta carotene levels of treated and control animals at the different slaughter times (Table 6.4a). Mean concentrations of beta carotene ranged between 0.4 and 0.6 μg/g of tissue wet weight. As for Chapter 5, the presence or absence of CLs and/or follicles was used as a covariate in the statistical analysis, but state or reproductive status of the ovary in itself was not a significant contributor to the observed variation ($p=0.564$).

As beta carotene is a precursor to retinol, it was decided to measure retinol concentrations in the beta carotene-treated animals. There was no significant change in retinol concentrations after treatment with beta carotene ($P=0.2494$). The design and analysis of variance for concentrations of beta carotene in the liver after treatment with beta carotene or vehicle are shown in Table 6.3.

Table 6.3. Sources of variance in beta carotene concentration in the liver of sows treated with beta carotene or vehicle. The analysis is a two-way fixed effects analysis of variance. The treatments were beta carotene or vehicle and the slaughter times were 48, 72 and 96 hours after treatment.

Source	df	MS	F	P values
Treatment	1	0.309167	0.921552	0.345
Slt time	2	3.003942	8.954040	0.001
Treat x Slt	2	1.508391	4.496158	0.020
Error	28	0.335485		

Beta carotene concentrations in the liver tissue of treated animals did not significantly differ from liver samples in the untreated animals ($p=0.345$, Table 6.4b). However, there was a significant difference between beta carotene levels due to slaughter time ($P<0.001$), with levels of beta carotene (▲) being higher at 72 hours after treatment in both treated and untreated animals than at 48 or 96

hours after treatment. A significant interaction between treatment and time of slaughter ($P < 0.05$) is also shown in Table 6.4b.

Table 6.4a Beta carotene and retinol concentrations ($\mu g/g$; $\pm$ s.e.) in the ovarian tissue of sows at 48, 72 and 96 hours after treatment with 250mg beta carotene or 1ml vehicle.

Time (h)	Beta Carotene	BC Vehicle	Retinol	R Vehicle
48h	0.75 $\pm$ 0.4	0.64 $\pm$ 0.11	30.2 $\pm$ 4.6	29.0 $\pm$ 4.3
72h	0.94 $\pm$ 0.37	0.42 $\pm$ 0.10	20.8 $\pm$ 2.6	23.2 $\pm$ 0.5
96h	0.62 $\pm$ 0.09	0.53 $\pm$ 0.13	26.8 $\pm$ 12.9	20.0 $\pm$ 4.2

Table 6.4b Beta carotene and retinol concentrations ($\mu g/g$; $\pm$ s.e.) in the liver tissue of sows at 48, 72 or 96 hours after treatment with 250mg beta carotene or 1ml vehicle.

Time (h)	Beta carotene	BC Vehicle	Retinol	R Vehicle
48h	0.99 $\pm$ 0.24	1.23 $\pm$ 0.19	332.7 $\pm$ 46.4	240.7 $\pm$ 40.1
72h	2.67 $\pm$ 0.47	2.00 $\pm$ 0.16	291.6 $\pm$ 99.0	331.2 $\pm$ 58.6
96h	1.31 $\pm$ 0.15	1.67 $\pm$ 0.05	275.6 $\pm$ 70.3	296.0 $\pm$75.9

There was no significant difference in the retinol concentrations in the liver after treatment with beta carotene ($P=0.854$). Retinol concentrations fell slightly in beta carotene treated animals in contrast with the peak of beta carotene at 72 hours

after treatment (Table 6.4b). There is no evidence to suggest that injected beta carotene was converted to retinol in the liver.

6.4 Discussion

The profile of plasma concentrations of beta carotene shown in Figure 6.1 peaked at 4.5 μg/ml at 3 hours after treatment, flattened until 36 hours after treatment and then began to fall. Chew et al. (1991a) injected 3 to 4 month old female pigs with 10 and 40 mg (between 0.18 and 0.73 mg/kg) of beta carotene and found an almost immediate plasma increase in beta carotene, peaking at 0.8 μg/ml at 12 hours after injection and then declining by 48 hours after treatment. The difference between the levels of beta carotene found in the present study where beta carotene was injected at a dose of 0.71 mg/kg and the data presented by Chew et al. (1991a) may be attributed to the higher dose used in the current study compared to the dose used by these workers and possibly the difference in the age of the animals used in each study. We selected the dose of 250mg based on the work of Coffey and Britt (1989) in which they showed a linear relationship between dose of beta carotene(0-200 mg) and litter size. Schweigert et al. (1995) showed a similar profile in 5 week old female pigs using a single oral dose of [^{14}C] beta carotene. These authors showed that the beta carotene level in the plasma was elevated within 1 hour of administration and peaked at between 5 and 6 hours after treatment. Results from the current experiment gave somewhat similar plasma profile to those shown by Schweigert and Eisele (1990) in dairy cows. In that study, cows were injected with a single dose of beta carotene (500 mg intramuscularly). Under these conditions beta carotene concentrations peaked at between 26 and 42 hours. These researchers also showed that beta carotene is transported in the plasma and lymph by lipoproteins. Later work by Chew et al. (1993) demonstrated that after feeding beta carotene to 3 to 4-month-old bull calves, there was an increase in total Low Density Lipoprotein-beta carotene complexes. Beta carotene was also associated with High Density Lipoproteins (the

usual beta carotene carrier) and Very Low Density Lipoproteins, but to a lesser extent. The lipoproteins are considered to be important in follicular and corpus luteum development because in association with retinol, they have been shown to increase progesterone in luteal cells (Bagavandoss and Midgely 1987). Given the association of beta carotene with lipoproteins shown by Schweigert and Eisele (1990) and Chew et al. (1993), there may be a role for beta carotene in the process of follicular and corpus luteum development also. This is particularly interesting in the light of the findings of Talavera and Chew (1988), who demonstrated that beta carotene increased the production of progesterone by cultured porcine corpus luteum tissue to a greater degree than retinol or retinoic acid.

However, the results of the tissue extraction after treatment with beta carotene presented here did not support the hypothesis that beta carotene has a role in the development of the follicle or the corpus luteum. O'Fallon and Chew (1984) showed that beta carotene forms an intergral component of bovine luteal cells and is responsible for the yellow colour of the corpus luteum (yellow body). This was not evident in the sow. There was no corresponding statistically significant increase in ovary concentrations of beta carotene after treatment with this provitamin. When reproductive status of the ovary (ie whether follicles or corpora lutea were present on the ovary) was used as a covariate in the analysis of data, no significant effects were observed.

In the current experiment the mean ovarian concentrations of beta carotene in 3 year old untreated sows at 48, 72 and 96 hours after treatment were 0.6, 0.4 and 0.5 μg/g wet weight respectively. Chew et al. (1984) noted that the mean concentration of beta carotene in the corpus luteum of abattoir material from untreated gilts was 0.1 μg/g of tissue, wet weight ranging from 0 to 0.6 μg/g wet weight. In the current experiment ovarian tissue incorporating connective tissue between the corpora lutea of follicles was used rather than corpora lutea only. Since samples were taken from the outer ovary, it is unlikely that ovarian cortex

was included. However, site of sampling and the age and breed of the animals sampled may have contributed to the difference in beta carotene concentrations found in the two experiments. As pigs are thought to take up beta carotene from the gut only poorly, an 8 month-old gilt is unlikely to have accumulated quantities of beta carotene in any tissue. This may account for the lower concentrations of beta carotene in the corpora lutea of gilts reported by Chew et al.(1984) compared to figures reported in the current experiment.

The extraction technique used by Chew et al. (1984) is similar to that used for this work except for the saponification step, which was included in the present study. Separation by alumina column chromatography may have resulted in the lower recovery of beta carotene from both tissues and plasma in the study of Chew et al (1984). Britton (1985) reported that alumina strongly retains some carotenoid compounds and may chemically react with retained compounds, changing their spectrophotometric qualities. This may have reduced the levels of beta carotene in tissues and plasma in the study of Chew et al. (1984). However, the possibility exists that by not separating extracts with the use of column chromatography prior to spectrophotometry in the present study, compounds other than beta carotene which absorb at the same wavelength may be present, resulting in higher values. However, this possibility is partly discounted by preliminary HPLC data which showed a single peak of beta carotene from tissue extracted by the same method. A comparison was made between spectroscopic measurements and separation by HPLC in a limited number of samples and these showed comparable results, although the HPLC method gave slightly higher results than the spectrophotometric method (Chapter 4, Table 4.12).

When liver retinol concentrations were measured in the present experiment, there was little evidence that any conversion of beta carotene to retinol had taken place. There was no difference between retinol concentrations reported in this experiment and those reported in Chapter 5 (Figure 5.5b). Schweigert et al. (1995) also observed that there was little conversion of beta carotene to retinol after feeding

a bolus of [^{14}C] beta carotene. However, it was interesting to note the fall in retinol concentrations in the ovary at 72 hours after treatment with beta carotene in the present study contrasting with the increase in beta carotene. It is tempting to suggest that retinol was reduced in response to higher than normal concentrations of beta carotene in these tissues as occurs in plasma (Chew et al., 1991a). However, further work is clearly required in this area.

This study was unable to demonstrate a significant difference in liver concentrations of beta carotene between beta carotene treated animals and controls despite increased plasma concentrations after treatment. When Schweigert et al. (1995) fed a bolus of [^{14}C] beta carotene to 5-week-old pigs, they found that a significant proportion of the beta carotene could be detected in the colon and lungs. This suggests that beta carotene can be absorbed by the gut in pigs, an observation which is confirmed by the presence of beta carotene in the liver of untreated sows in the present study. Most of the [^{14}C] beta carotene in Schweigert et als' (1995) work was found in the lung, followed by liver, colon, duodenum and kidney. However, in all tissues with the exception of the colon and lung, the [^{14}C] beta carotene became undetectable within 24 hours of administration. The lack of response in the liver storage of beta carotene in the present experiment may have been due to several factors. Choice of sampling times may have limited the information gained from this experiment. Schweigert et al. (1995) showed that [14C] beta carotene is undetectable after 24 hours, while the first reported sampling time in this experiment was 48 hours. Had the samples taken at 24 hours after treatment been suitable for extraction, an effect of treatment in animals injected with beta carotene may have been seen. The possibility of extraneous absorbers in the spectrophotometric assay, contributing to assay variation exists, although since all animals and all samples were treated in the same way, a degree of uniformity of extraneous absorbers might be expected across samples.

This study demonstrates that injected beta carotene is detectable in the plasma as early as 3 hours after injection. However, a corresponding increase in tissue

levels of beta carotene could not be demonstrated. If further studies were carried out in this area, an experiment which established pre-treatment liver and/or ovarian concentrations of beta carotene similar to that suggested for retinol in Chapter 5 could be designed. Serial samples at this and other times, beginning at the time of injection could be collected under anaesthesia by biopsy techniques. The separation of beta carotene extracted from tissue samples would be carried out by HPLC to more accurately quantitate beta carotene. Such a study might clarify the transport and storage of beta carotene in liver, ovary and possibly other tissues after injection.

Chapter 7:

The effect of retinol on ovarian follicle size and follicular hormone concentrations in the gilt.

7.1. Introduction

Reproductive efficiency is measured by the number of piglets produced per sow per year. One of the main constraints to this reproductive efficiency in the pig is the discrepancy between the number of piglets born alive and the number of ovulations. A further constraint is the lower litter size in the gilt at first parity in comparison with the multiparous sow.

Previous studies indicate that there may be a direct influence of retinol on the uterine environment and the development of the embryo and foetus. Brief and Chew (1985) showed that gilts injected with retinol at mating and then weekly until weaning showed a decrease in embryo mortality and an increase in the number of piglets per litter. Birth weight did not differ from those born to unsupplemented sows. Britt (1992) and Coffey and Britt (1993) demonstrated an increase in litter size in animals treated with a single injection of retinol 5-6 days before mating and suggested that the impact of retinol is in the first 2 weeks of pregnancy. The effect of retinol also appeared to overcome the detrimental effects of high energy diets fed during this time (Britt 1992).

There is also suggestion of an indirect influence of retinol on the ovarian production of progesterone. Retinol or vitamin A has been linked with increased production of progesterone by luteal tissue *in vitro* (Talavera and Chew 1988). Further work has been carried out by Trout et al. (1992) who showed a dramatic increase in retinol binding protein at day 11 of pregnancy. This appeared to be a response to a cascade of hormone production following the production of oestrogen by the embryo. These workers proposed that oestrogen acts at the levels of the corpus luteum to promote the increased production of progesterone. Day 11 corresponds with the onset of the critical period of elongation of the embryo and the recognition of pregnant status in the sow (Findlay 1993).

One aspect of the mechanism of action of retinol which has not been closely examined is its possible effect on folliculogenesis. Britt et al. 1985) showed that the timing of injection of retinol was crucial in increasing litter size in the gilt. If the treatment was given too late and missed the recruitment stage of the follicles then there was no effect on litter size in the subsequent pregnancy. This work suggests that not only is there an effect on the embryo at the stage of elongation and implantation, but also on the follicle itself. The present study was undertaken to investigate the effect of retinol on follicle size distribution and on follicular development. This was monitored through the measurement of the concentrations of the steroid hormones oestradiol and testosterone and retinol binding protein in the follicular fluid.

7.2. Materials and Methods

Locations and experimental animals

Two experiments were carried out for this study. Experiment 1 was located at Texas A&M University, Texas, USA in June-July 1995, while experiment 2 was carried out at Bunge Meat Industries, Corowa, Australia in June 1996. Animals used for experiment 1 in Texas were Duroc x Hampshire x Yorkshire gilts at first cycle. Those used in the Australian experiment were Large white x Landrace gilts at first oestrus.

Experiment 1 Ovarian follicle size and hormonal status of follicular fluid in gilts treated with retinol palmitate or vehicle.

Twelve group-housed gilts were detected in heat by the means of back pressure testing and the daily introduction of an intact boar to the pen. Gilts were not permitted to mate. Gilts were recorded for 2 oestrous cycles before allocation to treatments. Day of standing oestrus was designated day 0 of the cycle and cyclic gilts were randomly allocated to either a treated or control group. At

day 16 after ovulation the control gilts received 1 ml of corn oil intramuscularly in the neck region, while the treated group received an im injection of 1 ml retinol palmitate (1,000,000 IU, Sigma, Missouri, USA; R3375) reconstituted in corn oil. Treatments were administered between 7:00 and 9:00 am. Throughout the trial, gilts were fed a standard corn-based dry sow diet. At day 18 of the cycle ovaries were surgically collected and immediately placed in ice cold Hams F12 medium (Sigma, Missouri, USA; D2906) for transportation to the laboratory. Follicles were isolated, the diameter of those measuring greater than 4mm was recorded and the folliclar fluid was aspirated with a fine needle and collected separately into microfuge tubes for each follicle. A small number of follicles burst during isolation and were discarded from further analysis. This was immediately frozen for the later measurement of testosterone and oestradiol.

Experiment 2 Ovarian follicle size and hormonal status of follicular fluid in gilts treated with retinol palmitate or vehicle in Corowa, NSW.

Experiment 2 comprised 24 Large white x Landrace gilts, 12 randomly allocated to each of the two treatments. Cycling gilts were group housed throughout the trial and fed the standard wheat-based dry sow ration. Heat checks took place in the middle of the day using the back pressure test and a vasectomized boar. Day of oestrus was designated as day 0 of the cycle. Retinol palmitate reconstituted in 1 ml of corn oil was administered intramuscularly at 1,000,000 IU per ml while the control animals received 1 ml of corn oil. Injections were given as for experiment 1 between 7:00 and 9:00 am on day 16 of the cycle. The gilts were slaughtered on day 19 of the cycle and the ovaries were collected from the slaughter line. All visible follicles were measured *in situ* and the follicular fluid from the 12 largest was aspirated into individual microfuge tubes for each follicle for oestradiol and testosterone assay. Steroid hormones were measured as for experiment 1.

Radioimmunoassay of oestrogen and testosterone

Follicular fluid samples were diluted 1:100 in Phosphate Buffered Saline for oestrogen and 1:50 for testosterone and were assayed without extraction. All samples were assayed in duplicate. Testosterone concentrations were determined by a coated tube assay (DSL4000 Testosterone, Diagnostics Systems Laboratories, Texas, USA) using an [^{125}I]-labelled tracer. Tables 7.1 and 7.2 show cross reactivity of the testosterone and oestradiol respectively provided in the DSL kits.

Table 7.1 Cross reactivity of testerone

	Cross reactivity with testosterone (%)
	100
	5.8
	4.2
	2.3
	1.9
	0.92
10-hydroxyandrostenedione	0.86
	0.5

Table 7.2 Cross reactivity of oestradiol with 17β

	Cross reactivity with testosterone (%)
	100
	5.8
	4.2
	2.3
	1.9
	0.92
10-hydroxyandrostenedione	0.86
	0.5

Oestradiol was determined by a double antibody assay (DSL4400 Oestradiol; Diagnostic Systems Laboratories, Texas, USA) using an [^{125}I]-labelled tracer. Cross reactivity for the oestradiol kit was as follows: 17β-Oestradiol 100%, Equilenin 6.1%, Oestrone 3.4%, 17-β-Oestradiol-3-Glucuronide 1.8%, Equilin 0.84% and Oestriol 0.75%. All other tested compounds had less than 0.5% cross reactivity. Inter-assay coefficients of variation for experiments 1 and 2 were 14.7 and 18.1%, respectively. Intra-assay coefficients of variation were 7.3% and 3.4%, respectively. Assay sensitivity reported by DSL was 4.7 pg/ml.

Retinol binding protein in follicular fluid from experiment 1 was assayed at the Clay Center Nebraska using a previously described RIA method (Vallet 1994). Intra- and inter-assay coefficients of variation were 11.3 and 2.9% respectively.

Statistical analysis

Distribution of Follicle size classes were analysed by chi square using Fishers P

test while the comparison of hormone concentrations was carried out by least squares analysis of variance with follicle size as a covariate. Differences in retinol binding protein concentrations were examined by analysis of variance. When appropriate significant means were isolated using a post-hoc least squares means test. For analysis of variance tables see appendix 3.

7.3. Results

Experiment 1 Ovarian follicle size and hormonal status of follicular fluid in gilts treated with retinol palmitate or vehicle..

The results of experiment 1 carried out at College Station Texas, indicated that there was a significant increase in the size of the largest follicles in gilts treated with retinol ($P<0.05$; Table 7.2). This effect was due to a decrease in the number of follicles in the medium size ranking (4-6 mm) and an increase in the number of follicles in the large class (>6mm) in the treated group ($P<0.01$, $\chi^2 = 7.14$; Table 7.3).

Table 7.3. Number of follicles in the size classes 0-3 mm, 4-6 mm and >6mm (*s indicate the level of statistical significance. * 0.05, **0.01, *** 0.001)

Treatment	0-3 mm	4-6 mm	> 6 mm Treat
Exp 1 control	na	12	25
Exp 1 retinol	na	4 **	47 **
Exp 2 control	48	161	41
Exp 2 retinol	20 **	192 **	38 ns

When the concentrations of oestradiol and testosterone were measured in the fluid of the 12 largest follicles of each gilt, the E_2:T_4 ratio was higher in control animals than in those treated with retinol ($p<0.01$; Table 7.4). No significant difference was found between the oestradiol concentrations of follicles from

treated and control animals, but testosterone was significantly higher in treated than control animals ($p<0.001$).

Retinol binding protein (RBP) was significantly higher ($p<0.01$) in retinol treated animals (n=4) than in controls (n=6). The follicular fluid of control animals had a mean concentration of 34.1 ± 0.98 μg/ml RBP (mean$\pm$s.e.), while the follicular fluid of retinol treated animals contained 39.1 ± 1.27 μg/ml RBP.

Experiment 2 Ovarian follicle size and hormonal status of follicular fluid in gilts treated with retinol palmitate or vehicle.

The results of the second experiment indicated that all follicles were smaller than for experiment 1. While analysis of variance revealed no significant difference in mean follicle size (Table 7.4), the distribution of follicles was again significantly different. However, in this experiment there were more medium (4-6 mm) and less small follicles (>3mm) in the treated animals ($P<0.01, \chi^2=8.67$, Table 7.3) than in controls.

Table 7.4. Mean follicle size (mm), oestradiol and testosterone concentrations (ng/ml) and E_2:T_4 ratio in follicular fluid, of the largest 12 follicles dissected (Expt 1) or observed (Expt 2) on both ovaries.

Treatment	Size (mm)	E_2	T_4	E_2:T_4
Exp 1 control	7.4±0.2	268.4±42.5	109.3±12.4	2.3±0.3
Exp 1 retinol	8.3±0.2	299.9±44.4	196.1±18.9	1.4±0.2
Significance	*	ns	***	**
Exp 2 control	5.9±0.1	134.6±10.4	106.5±9.8	1.6±0.1
Exp 2 retinol	5.8±0.1	222.1±12.5	159.9±11.9	2.4±0.3
Significance	ns	**	**	*

n.s., *,**,***; effect of treatment; not significant and $p<0.05$, 0.01, and 0.001 respectively.

Oestradiol and testosterone concentrations were significantly higher in treated animals than untreated ($P<0.01$, Table 7.4) as was the oestradiol:testosterone ratio ($P<0.05$). Follicles seen in experiment 2 were smaller than those in experiment 1 and less hormonally developed, consistent with follicles that are approximately 1 day earlier in stage of development.

7.4. Discussion

The results of this work suggest that retinol, given at the time of follicular recruitment, caused significant changes in follicle size distribution, steroid production and retinol binding protein concentrations in ovarian follicles in gilts. The follicles of retinol treated animals showed a shift in distribution from medium to large in the first experiment and from small to medium in the second experiment. Several factors may have contributed to the observed responses to retinol. Untreated Duroc x Hampshire x Yorkshire gilts in the Texas trial had follicles which were larger than the average size reported in the literature for gilts of approximately the same age and at the same day of the cycle. Grant etl. (1989) reported a mean follicle size of 4.91 ± 0.5 mm at day 18 of the cycle in 80 kg large-white x landrace gilts, compared with a mean follicle size in the Texan gilts of 7.4 ± 0.21 mm. Large white x Landrace gilts from experiment 2 at Corowa had a mean follicle size of 5.8 ± 0.13 mm. The figures for day 20 of the cycle reported by Grant et al. (1989) of 5.5 ± 0.5 mm were more comparable to the follicle sizes found in the current experiment.

Grant et al. (1989) investigated levels of the steroids in the follicular fluid of 511 follicles. The oestradiol concentration at day 18 of the cycle reported by these authors was comparable with the concentration reported in the untreated gilts from experiment 2 in the current work (110.6 ± 89.8, Grant et al. (1989); 134.6 ± 10.4 ng/ml, experiment 2). Follicles from untreated gilts in experiment 1 contained considerably higher concentrations of oestradiol than those the reported by Grant et al. (1989), but the follicles were much larger in these gilts.

When compared with the results of large-white x landrace gilts at day 20 of the cycle, these levels of oestradiol were more comparable (235.5±89.9 and 268.4±42.5 ng/ml respectively). Testosterone levels for both experiments were higher than the levels reported by Grant et al. (1989) of 55.8 ng/ml cf 109.3±12.3 ng/ml in experiment 1 and 106.5±9.8 ng/ml in experiment 2, but similar to levels reported for gilts with a follicle size of 7.5 mm (115.5±30.9, Grant et al. 1989).

The E_2:T_4 ratios of untreated animals from the current experiments (2.31±0.3 and 1.62±0.12) were comparable to those of Grant et al. (1989) of 1.98. These authors demonstrated an E_2:T_4 ratio and follicle size pattern which rose from 0.95 and 3.99 mm at day 16, through a concentration of 1.98 and 4.91 mm at day 18. A peak of 3.11 (and 5.50 mm) was reached at day 20, followed by a fall in the E_2:T_4 ratio to 2.99 by day 21, the anticipated time of the LH surge, but an increase in follicle diameter to 8.8 mm. It is interesting to note the similarity in steroidogenic development suggested by the data presented in the current experiments and those reported by Grant et al. (1989). Both the E_2:T_4 ratio and the mean follicle diameter shown for untreated gilts in experiment 1 is similar to those of the day 20 gilts reported by Grant et al. (1989), while the gilts treated with retinol show an E_2:T_4 ratio representative of that near the LH surge. These data are supported by the findings in the second experiment in which untreated gilts at day 18 had a similar E_2:T_4 ratio and follicle size as gilts compared to those reported by Grant et al. (1989) at day 18, while retinol treated gilts displayed a steroid ratio and follicle diameter comparable with those reported for gilts at day 20 of the cycle (Grant et al. 1989). The data presented suggest that retinol may play a specific role in the development of the porcine ovarian follicle by influencing steroidogenesis. This hypothesis is supported by the finding that the follicular fluid of retinol treated animals in experiment 1 contained higher concentrations of RBP than control animals, suggesting higher associated levels of retinol in the follicular fluid.

Follicular growth and development is thought to occur under the influence of FSH (Baker 1982). Bagavandoss and Midgley (1988) demonstrated in cultured rat granulosa cells that a low dose of retinol enhanced the ability of FSH to induce LH receptors and to stimulate cAMP synthesis. In low concentrations, retinol has also been shown to enhance the production of enzymes responsible for the conversion of androgens to oestrogen and the oxidization of pregnenolone to progesterone in mouse K9 sertoli cells (Lefevre et al.1994). Through this possible mechanism, progesterone and oestrogen production is increased. However, in higher concentrations the action of retinol appears to be biphasic and antagonizes FSH, decreasing FSH-induced production of cAMP (Galdieri et al. 1994). Since the aromatization of androgens to oestradiol is also actively regulated by FSH, there is a similar decrease in activity attributable to the effects of retinol, which may also account for the lower oestradiol concentrations in more mature follicles in treated animals.

Further work is required to clarify the role of retinol in follicular maturation. Specifically, a porcine granulosa cell culture system could be established in the same way as that described by Bagavandoss and Midgely (1988). An increase in oestrogen in response to retinol or retinoic acid added to the culture at a concentration of 10^{-9}M would confirm the *in vivo* response to this vitamin. The FSH and cAMP response to the addition of retinol or retinoic acid to this culture system would suggest the effect of retinol at this level of the steroidogenesis cascade, already demonstrated in a rat granulosa cell culture system by Bagavandoss and Midgely (1988). An increase in the synthesis of 3βHSD and androgens would indicate an effect of retinol or retinoic acid at a point further down the steroidogenesis pathway, and correspond with the findings of Galdieri et al. (1994) in rat sertoli cells and Lefevre et al. (1994) in mouse K9 sertoli cells.

Chapter 8:

Retinol and mRNA for IGF-I during embryo development

8. Introduction

In-vitro studies using porcine ovarian tissue have demonstrated that insulin-like growth factor stimulates cell proliferation and enhances gonadotrophin-stimulated steroidogenesis (Echternkamp et al. 1994). In Chapter 7 of this thesis, treatment of gilts with retinol palmitate was associated with advancement in the development of ovarian follicles, with gonadotrophin-stimulated steroidogenesis being the possible site of action of retinol. Samaras et al. (1994), hypothesized that the IGF system acts as a local amplification mechanism for gonadotrophin action through a number of interlocking mechanisms. These include gonadotrophin induction of local IGF secretion, interactions between IGF and the gonadotrophins in steroidogenesis and gonadotrophin inhibition of the secretion of binding proteins inhibiting the action of IGFs (Samaras et al. 1994).

The IGFs are also implicated in the control and differentiation of the uterus in preparation for the implantation of the embryo in the sow and are thought to promote conceptus growth and/or differentiation (Simmen et al. 1992). According to Simmen and Simmen (1990) and Tavakkol et al. (1988), IGF-1 concentrations peak at day 12 of pregnancy, notably at the same time as retinol binding proteins and retinol peak during pregnancy. Both of these events coincide with blastocyst oestrogen synthesis and elongation (Simmen and Simmen 1990). Since plasma oestrogens remain at low concentrations until around day 16 of pregnancy, these researchers have suggested that blastocyst derived oestrogen may trigger the release of IGF-1 and that there may be a paracrine role for IGF-1 in conceptus development.

While there is little evidence to suggest that there is a link between retinol and IGF-I concentrations in the development of the embryo, the coincident appearance of these two compounds in uterine fluids at the time of elongation may suggest that the presence of retinol enhances the production of oestrogen in the embryo in a similar manner to that seen in the ovarian follicle. The increase in oestrogen

originating from the embryo may then act as a stimulant to increase IGF-1 production.

The aim of this part of the project was to investigate whether IGF-1 expression in porcine embryos is influenced by treatment of the gilt with retinol, prior to ovulation.

8.2 Materials and Methods

This experiment was carried out at the Research and Development Unit at Bunge Meat Industries, Corowa in November 1996.

Animals

Twenty four Large-white x Landrace gilts aged approximately 8 months were selected at first standing oestrus for participation in this experiment. Behavioural oestrus was detected by the back pressure testing method in the presence of an entire boar, twice daily. Animals were fed the standard Bunge gilt developer ration (meeting or exceeding the requirements of the NRC). Gilts were group housed, fed *ad libitum* and had continuous access to water.

Treatments

Gilts were randomly allocated to one of two treatments at the time of oestrus. Group 1 (n=12 gilts) were given an intramuscular injection of 1,000,000 IU retinol palmitate (1 ml in corn oil) on day 16 of the oestrous cycle following first recorded oestrus. The second group of gilts received an im injection of 1 ml corn oil on the same day. Injections were given at approximately 10:00 am. At the following standing oestrus gilts were artificially inseminated on the morning of oestrus and repeat inseminated on the following morning, according to standard procedures currently operating at Bunge Meat. As gilts were not synchronized,

they came into oestrus and were mated over a period of 6 days. Of the 24 gilts selected 18 were mated and 16 were pregnant at the time of surgical recovery of the embryos (n=7 retinol; n=9 control).

Collection and extraction of embryos

Surgical collection of embryos was carried out on the 7th, 11 th and 12th of November, so that the majority of the sows were at day 12 of pregnancy (n=11), and a small proportion were at day 11 (n=3) or day 13 (n=4). Embryos were surgically collected so that valuable breedibg animals could be returned to the Bunge Meat Industries breeding program. The anaesthesia for the surgery is detailed in Chapter 3, General Materials and Methods.

A single 12 cm incision was made in the ventral midline, about 2 thirds of the way between the sternum and the pubis and was continued through the fat and connective tissue layers into the peritoneal cavity. The uterus was exteriorized and the oviducts located. The uterus was clamped at the bifurcation. A small glass tube with a flared end was threaded into the uterus via a small incision at the ovarian end and the uterine horn was flushed with 50 ml sterile 0.7% saline from the uterine end via a blunt 14 gauge needle inserted through the uterine wall. A non-scrubbed assistant held the jar into which the solution was flushed. After both uterine horns were flushed all tubing was removed, incisions were stitched and the uterus was carefully returned to the abdomen. The body wall and peritoneum were sutured with number 2 Vicryl in a horizontal mattress pattern. The fat layers were closed with a continuous suture of number 2 Vicryl and the skin was closed with 3 or 4 horizontal mattress sutures in the same material. Sows were allowed to recover from the surgery overnight.

Embryos were placed on ice immediately after collection, filtered through a Sureflush nylon embryo filter (Pacific Vet) and washed into a 15 ml RNAse free tube with diethl pyrocarbonate (DEPC) treated water. After brief centrifugation at

low speed, the water was decanted and any remaining free water was aspirated. Trizol extraction of the embryos was carried out as detailed in Chapter 3 and RNA was stored under ethanol at -80° C. Due to the distance between the point of surgical collection of embryos and the appropriate laboratory facilities to complete the extraction using Trizol, samples were stored in a -80° C freezer under isopropanol until transported on ice to the laboratory in Melbourne. The extraction was completed at our laboratory at the Victoria University of Technology, Werribee campus. Following quantification of RNA by spectrophotometric methods, aliquots of a known amount (usually 20μg) were stored at -80° C under ethanol for northern blotting, or in water at -80° C. Details of the methods for northern blotting, amplification of the cDNA, the production of the cDNA probe and the hybridization technique are provided in Chapter 3, General Materials and Methods.

8.3 Results

As shown in Table 8.1 there was no significant difference between the retinol treated and the control animals for ovulation rate (12.8 ± 0.57 retinol; 14.12 ± 0.718 vehicle; $P = 0.16333$). The RNA from most samples was intact as judged by denaturing and non-denaturing gel electrophoresis (see Figures 8.1 and 8.2). The accuracy of concentrations measured by spectrophotometric methods was confirmed by non-denaturing gel electrophoresis, an example of which is shown in Figure 8.1. Yields of RNA differed widely and in some cases were too low for Northern blot analysis. The following representatives of treated and control samples were therefore chosen for further analysis. Vehicle: 5449, 5297, 5628 and 5367; Retinol treated: 5571, 5687, 5428.

The yield of RNA from each extract was dependent on the amount of embryonic tissue collected at surgery. Due to variation in the amounts of tissue recovered from each gilt, it was not possible to detect any correlation between retinol

treatment and the amount of RNA recovered at extraction.

IGF-1 probe preparation

A plasmid containing an IGF-1 specific insert of approximately 540 base pairs was generously provided by Dr. Victor Han's laboratory. Working quantities of this fragment were produced by PCR using the T3 and T7 promoter sequences, as described in Chapter 3, Materials and Methods. The fragment was also successfully re-cloned into p (Bluescript KS +) using *E. coli* TG2 competent cells as the host strain.
However, the identity of this probe could not be confirmed by sequence analysis and high non-specific binding to ribosomal RNA was found to be a problem during Northern hybridization. Therefore use of this probe was discontinued in favour of the RT-PCR-derived 216 base-pair probe.

A second probe was successfully prepared by RT-PCR using IGF-1 specific primers. The size of the fragment was consistent with the theoretical value of 216 base pairs and the identity of this probe was successfully confirmed by automated sequence analysis (Figure 8.0a)

Table 8.1 Number of corpora lutea (CLs) present in each gilt and total mRNA recovered from either expanded or elongated porcine blastocysts at day 11, 12 or 13 of pregnancy (DOP) using the Tri-Reagent, Trizol.

CLs (ID)	Treat	DOP	Blastocyst state	RNA (μg)	260:280 ratio	RNA quality
13 (5444)	vehicle	13	elongated	24.0	1.75	Good
17 (5269)	vehicle	12	elongated	20.0	1.71	Good
14 (5297)	vehicle	12	elongated	60.0	1.71	Good
15 (5285)	vehicle	12	elongated	<10	1.68	Degraded
15 (5687)	retinol	13	elongated	290	1.66	Good
11 (5628)	vehicle	12	elongated	80.0	1.72	Good
14 (5428)	retinol	12	elongated	40.0	1.70	Good
15 (5571)	retinol	11	expanded	25.0	1.81	Good
12 (5367)	vehicle	12	elongated	100	1.69	Good
15 (5697)	vehicle	12	expanded	60.0	1.65	Degraded
16 (5279)	vehicle	12	elongated	<10	n/a	Good
(5835)	retinol		expanded	<10	1.82	Good
(5438)	retinol		1 embryo	<10	1.88	Good
(5414)	retinol		1 embryo	<10	2.00	Degraded
(5278)	vehicle		expanded	<10	n/a	Good
(5299)	retinol		expanded	<10	n/a	Good

Figure 8.0(a)
Partial Sequence Alignment of 216 base pair IGF-1 probe prepared from porcine liver RNA (lower sequence) with the published IGF-1 cDNA sequence (upper sequence).

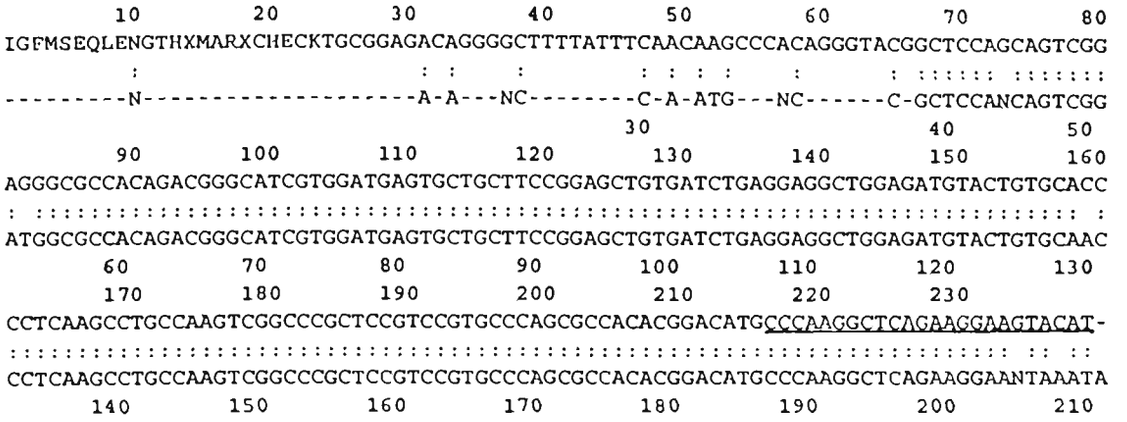
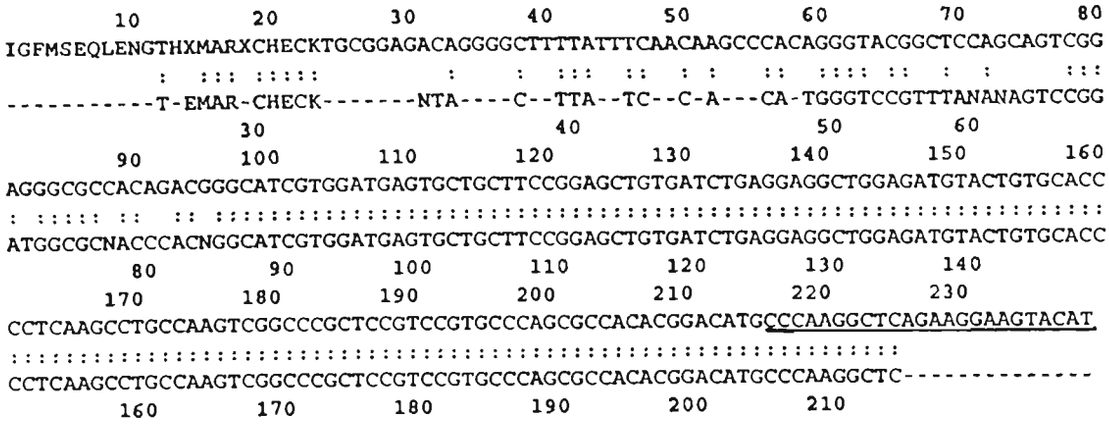


Figure 8.0 (b)
Partial Sequence alignment of a cDNA fragment produced by RT-PCR from porcine embryo RNA (lower sequence) with published IGF-1 cDNA sequence upper sequence).



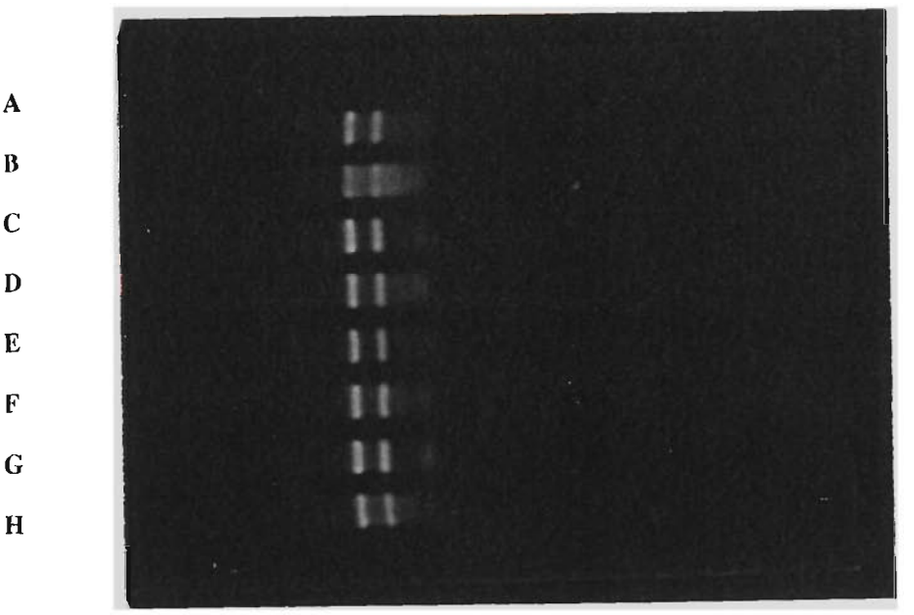
: identical bases.
The upstream primer was used for sequence determination. The sequence used to design the downstream primer is underlined.
Sequence alignment was performed using a local alignment program of the Australian National Genomic Information Service (ANGIS).

Figure 8.1

Example of embryo total RNA samples analysed by non-denaturing electrophoresis in a 1% agarose gel using a modified 1 x TAE buffer and stained with ethidium bromide.

Figure 8.1

Example of embryo total RNA samples analysed by non-denaturing electrophoresis in a 1% agarose gel using a modified 1x TAE buffer and stained with ethidium bromide.



Equal loadings of 1.5µg total RNA were applied to each lane from embryos 5297 (A), 5285 (B), 5628 (C), 5449 (D), 5269 (E), 5571 (F), 5428 (G) and 5687(H).

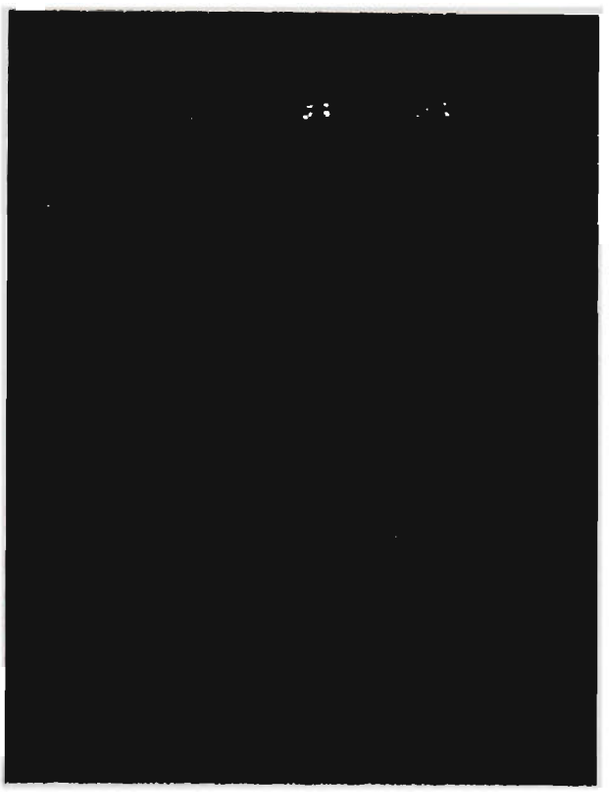
Figure 8.2

Formaldehyde gel electrophoresis of total RNA from embryos treated with 1,000,000 units of retinol palmitate (lanes A-C) and corn oil (lanes D-F).

Figure 8.2

Formaldehyde gel electrophoresis of total RNA from embryos treated with 1,000,000 units retinol palmitate (lanes A-C) and corn oil (lanes D-E).

A B C D E F



20 µg total RNA was ethanol precipitated, washed with 70% ethanol and heat denatured prior to gel electrophoresis. RNA samples were from animal :
A (5428), B (5687), C (5571), D (5367), E (5628), F (5297).

Northern Blotting

Northern blotting was found to be most successful when carried out at a hybridization temperature of 42° C. Washing procedures varied in their stringency, and were found to have an influence on the RNA species detected. The following conditions were found to be optimal for reducing background radioactivity and non-specific binding of the 216 base pair IGF-1 probe:

5 minutes at room temperature in 2 x SSC + 0.1 % SDS (2 washes)
5 minutes at room temperature in 0.2 x SSC + 0.1 % SDS (2 washes)
15 minutes at 42° C in 0.2 x SSC + 0.1 % SDS (2 washes)
15 minutes at 65° C in 0.1 x SSC + 0.1 % SDS (2 washes)

Figures 8.3 and 8.4 show replicate examples of Northern blots comparing IGF-1 mRNA levels from embryos of treated and non-treated animals, using the above washing procedure. Single stranded RNA markers were used to determine the molecular weight of these RNA species at approximately 4.0 and 6.5 kb. A faint band was also seen running just ahead of the 955 base RNA standard. This can be seen most clearly in Figure 8.3. No major difference was seen between the IGF-1 expression patterns of treated and untreated animals.

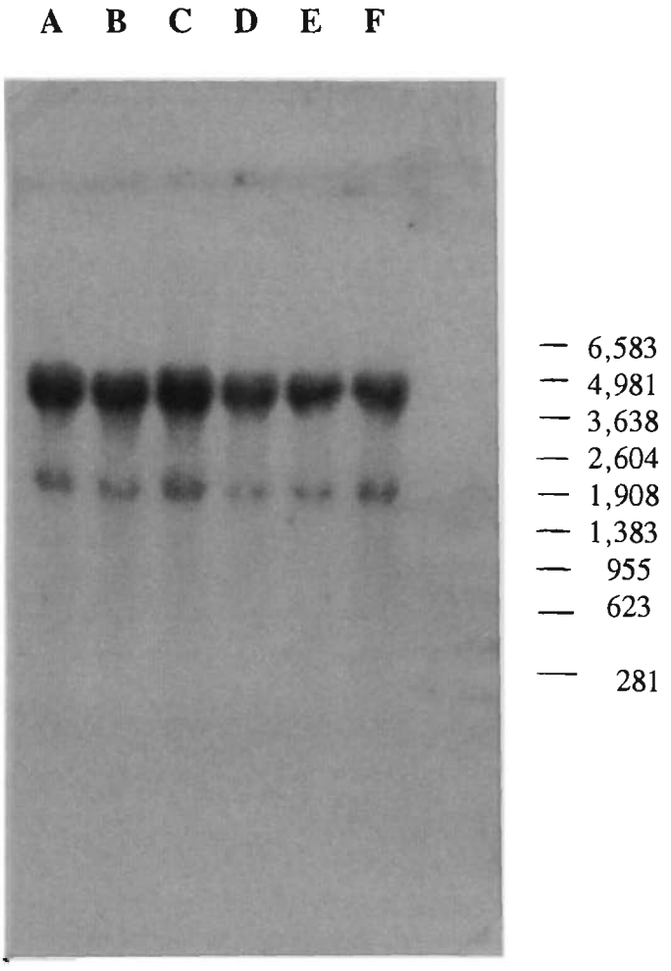
Figure 8.2b shows an example of Northern hybridization carried out at 42° C but washed using a less stringent protocol. Washes were carried out to a maximum stringency of 0.1 x SSC + 0.1 % SDS at room temperature (two washes 15 minutes each). In this experiment the 4.0kb transcript was present with a second band clearly visible with a molecular weight of approximately 1.7kb.

Figure 8.2B

Northern blot analysis of porcine embryo RNA showing the effect of using less stringent post hybridization washing procedures.

Figure 8.2 B

Northern blot analysis of porcine embryo RNA showing the effect of using less stringent post hybridization washing procedures.



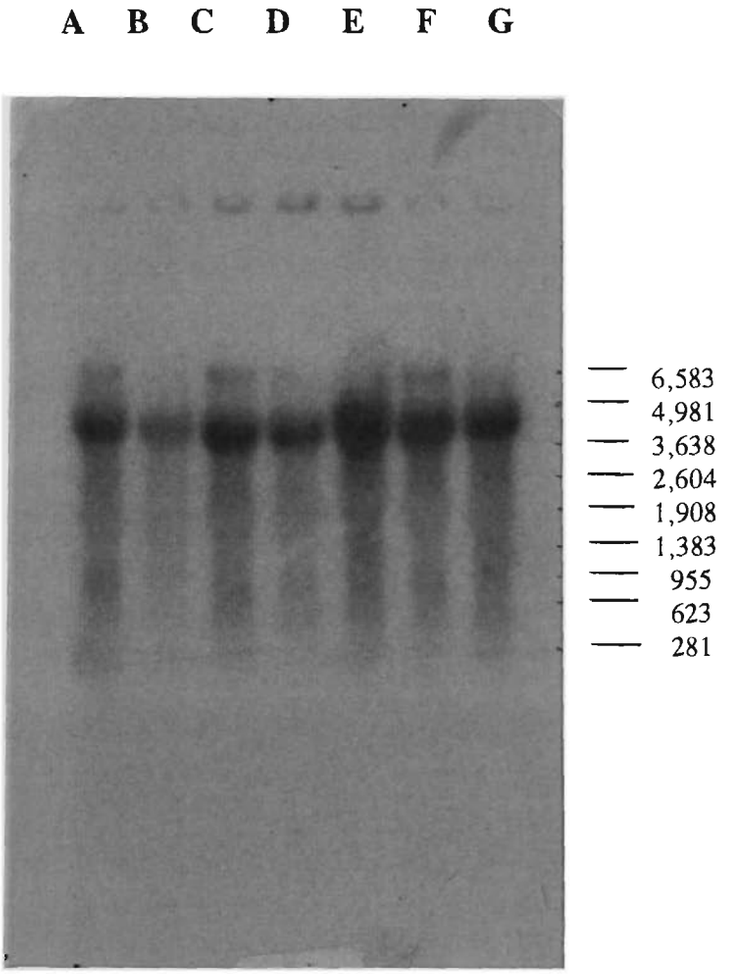
20 μg total RNA was ethanol precipitated, washed with 70% ethanol and heat denatured prior to gel electrophoresis. The formaldehyde gel used for Northern blotting is shown in Figure 8.2. Hybridization was carried out at 42^{0}C and washing was performed to a maximum stringency of 0.1% SSC + 0.1%SDS at room temperature.
RNA samples were from retinol treated animals :A (5428), B (5687), C (5571), and control animals: D(5367), E (5628), F (5297).

Figure 8.3

Northern blot analysis of total RNA samples from embryos of retinol treated animals (lanes E-G) and untreated animals (lanes A-D).

Figure 8.3

Northern blot analysis of total RNA samples from embryos of retinol treated animals (lanes E-G) and untreated animals (lanes A - D).



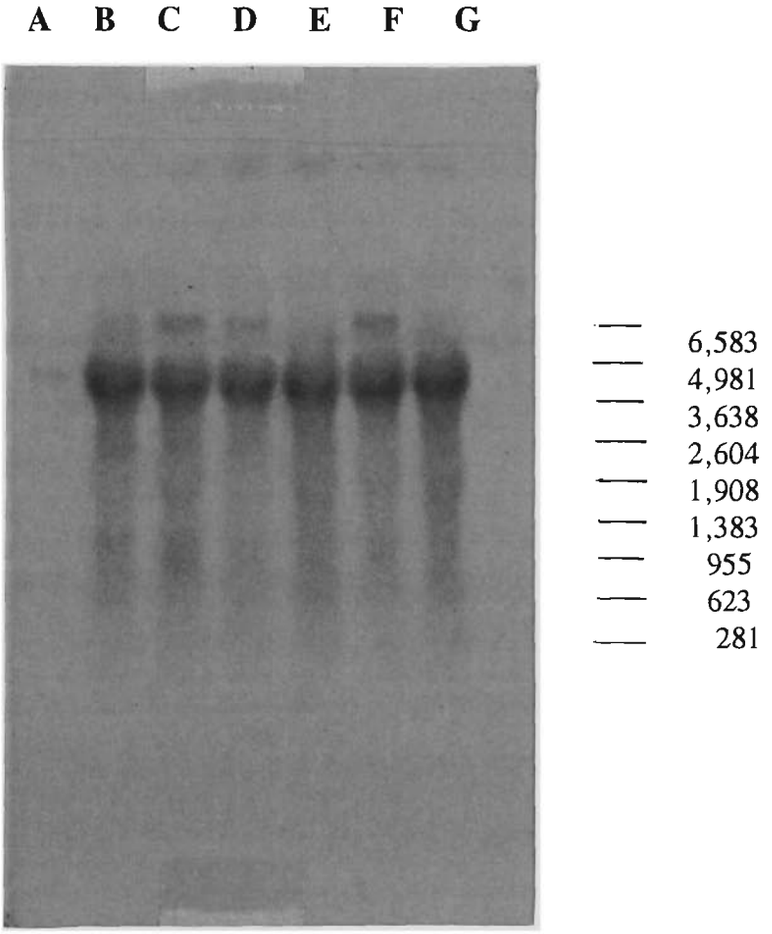
20 μg total RNA was ethanol precipitated, washed with 70 % ethanol and heat denatured prior to electrophoresis on a denaturing formaldehyde gel. The Northern blot was hybridized to the 216 base pair IGF-1 probe at a temperature of 42^0 C. Embryo RNA samples were from animal 5449 (A), 5367 (B), 5297 (C), 5628 (D), 5687 (E), 5428 (F) and 5687 (G).

Figure 8.4

Northern blot analysis of total RNA samples from of retinol treated animals (lanes E-G) and untreated animals (lanes A-D).

Figure 8.4

Northern blot analysis of total RNA samples from embryos of retinol treated animals (lanes E-G) and untreated animals (lanes A - D).



20 μg total RNA was ethanol precipitated, washed with 70 % ethanol and heat denatured prior to electrophoresis on a denaturing formaldehyde gel. The Northern blot was hybridized to the 216 base pair IGF-1 probe at a temperature of 42^0 C. Embryo RNA samples were from animal 5449 (A), 5367 (B), 5297 (C), 5628 (D), 5687 (E), 5428 (F) and 5687 (G).Loading of lane A was incorrect.

RT-PCR Analysis

RT-PCR was used to confirm the presence of IGF-I mRNA in the various RNA samples and to look for obvious changes in levels of expression.

When RT-PCR is used to monitor mRNA expression, it is important to ensure that PCR products obtained are dependent on reverse transcriptase activity and not derived from contaminating genomic DNA. Early experiments showed a low level of reverse transcriptase - independent product formation after 40 cycles of PCR, when using liver RNA as a template. Therefore all embryo RNA samples were routinely treated with DNAse when used for RT-PCR analysis. Figure 8.5 shows that after DNase treatment, generation of PCR products specific for IGF-1 only occurred after conversion of the RNA strand to cDNA. The identity of the major RT-PCR product from embryo RNA of gilt 5687 was confirmed to be IGF 1-specific by automated sequence analysis (Figure 8.0b). Thus under the conditions used here RT-PCR appeared to give a valid test for the present of IGF-1 mRNA.

Preliminary experiments also indicated that a 10-fold increase of primer to template ratio during first strand cDNA synthesis did not increase specific PCR product formation, and in fact only resulted in a higher background of non-specific products. This suggests that the primer concentration is non-limiting, a prerequisite when using RT-PCR to look at variations in the level of IGF-1 expression.

Figure 8.6 shows an example of RT-PCR analysis using IGF-1 specific primers performed over a time-course, using identical mounts of template RNA during first strand synthesis. No product formation was detected in the absence of reverse transcription indicating that mRNA transcription is being detected. Using RT-PCR analysis IGF-1 mRNA was detected in all samples. However no difference was seen in the rate of PCR product formation using RNA from treated and untreated animals. This experiment was repeated twice with similar results.

Figure 8.5

RT-PCR Analysis of porcine embryo and liver RNA samples showing the PCR product formation is dependent on reverse transcriptase activity.

Figure 8.5

RT-PCR Analysis of porcine embryo and liver RNA samples showing that PCR product formation is dependent on reverse transcriptase activity.

Lane A : PCR products from liver RNA including reverse transcription.
Lane B : PCR products from liver RNA without reverse transcription.
Lane C : PCR products from embryo RNA including reverse transcription.
Lane D : PCR products from embryo RNA without reverse transcription.
Lane E : PCR Marker - 1000, 750, 500, 300, 150 and 50 base pairs.

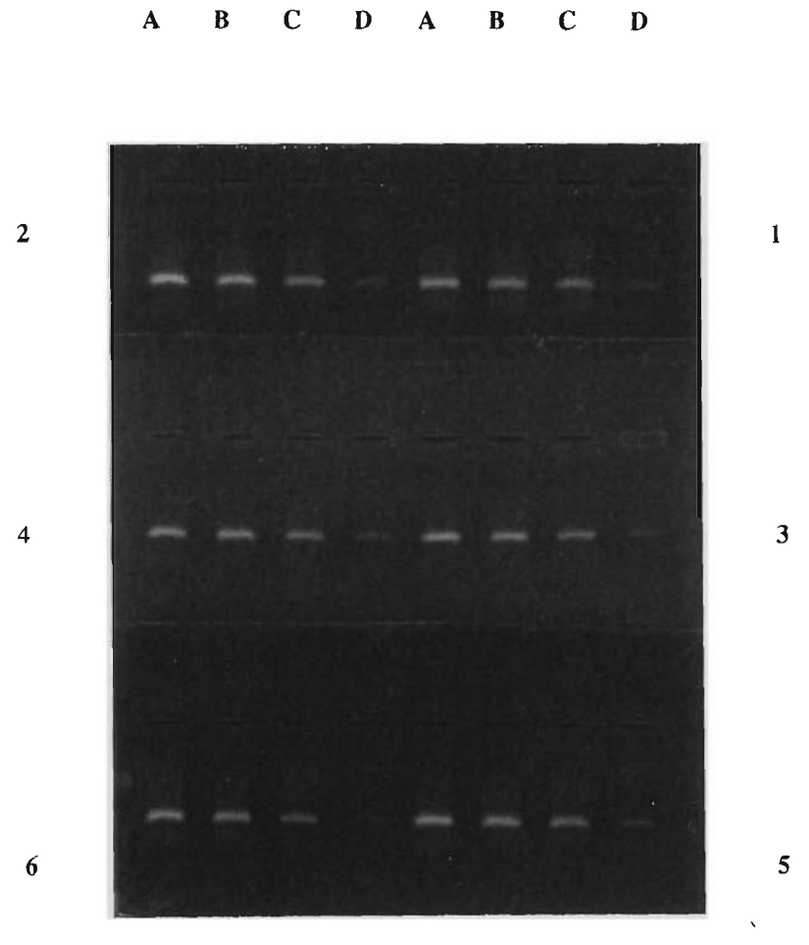
All RNA samples were treated with DNase prior to RT-PCR analysis.

Figure 8.6

RT-PCR analysis of IGF-1 mRNA expression in embryos treated with 1,000,000 units retinol palmitate and those treated with corn oil.

Figure 8.6

RT-PCR Analysis of IGF-1 mRNA expression in embryos of pigs treated with 1,000,000 units retinol palmitate and those treated with corn oil.



10ul samples of each PCR reaction were analysed after 40 cycles (A), 36 cycles (B), 32 cycles (C), and 28 cycles (D).

RNA was used from embryos of the following control animals:
5449 (set 1), 5628 (set 2), 5297 (set 3).
RNA was used from embryos of the following retinol treated animals:
5571 (set 4), 5428 (set 5), 5687 (set 6).

8.4 Discussion

Gadsby et al. (1996) using porcine corpus luteum tissue, reported a prominent IGF-1 mRNA transcript of 6.7 kb and a second less prominent transcript of 0.9 kb. Gadsby et al. carried out these experiments using poly (A)+ mRNA and included post hybridization washes of high stringency (two washes at 60° C in 0.1 x SSPE). Samaras et al. (1993) also detected these transcripts using both total and poly (A)+ RNA from porcine ovarian tissue, but also reported transcripts of 4.5 and 1.8 kb. The precise hybridization and washing conditions in these experiments were not defined.

In the present study bands of comparable sizes to all four transcripts were detected. However, the detection of each was dependent on the experimental conditions used, particularly the stringency of the post hybridization washes. Under less stringent washing conditions, with overnight autoradiography bands of approximately 4.0 kb and 1.8 kb were clearly visible as shown in Figure 8.2b. Under more stringent post hybridization washing conditions the 1.8 kb transcript was no longer clearly visible and the relative amounts of the 4.0 kb and the 6.5 kb transcripts had clearly changed. A transcript of approximately 0.9 kb was also visible but was not well defined. Moreover alignment of the autoradiograph shown on Figure 8.2b with the relevant formaldehyde agarose gel (Figure 8.2) showed the close proximity of the 4.0 kb and 1.8 kb bands to the 28s and 18s ribosomal RNA species. While the validity of the 6.5 kb and 0.9 kb transcripts is not doubted, the 4.5 kb and 1.8 kb transcripts give cause for concern and should be investigated further.

RT-PCR can also be used as a method for investigating gene transcription and has been used in this study as an alternative approach for investigating levels IGF-1 expression. This technique has the advantage of requiring very small amounts of starting RNA (less than 1 μg) and does not require the use of hazardous radioisotopes. In this study RT-PCR has been successfully used to detect IGF-1 in

RNA samples taken from both retinol treated and untreated embryos.

In this study the time course of IGF-1 specific PCR product formation has been considered as reflection of the level of mRNA present in the sample. A similar approach was successfully used by Culligan-Bove et al. (1993) to show variation in Vascular endothelial Growth Factor (VEGF) mRNA levels as a result of oestrogen stimulation in the uterus, although in this case dilution of the RNA was used as the variable rather than cycle number.

In these experiments every care has been taken to ensure that identical levels of starting RNA have been used for RT-PCR, by a combination of agarose gel electrophoresis and spectrophotometric methods. However the precision of these techniques is the limiting factor when using this RT-PCR in this way and therefore it is probable that small changes in IGF-1 expression may not have been detected using this method. The detection of precise changes in levels of gene transcription could more accurately be measured by inclusion of a competitive internal standard and requires further investigation.

In this study, no evidence was found to suggest that treatment with retinol palmitate increased IGF-1 mRNA expression in the embryos of treated gilts, compared to vehicle treated animals. In the ovarian follicle, IGF-1 has been shown to enhance steroid-stimulated steroidogenesis (Echternkamp et al. 1994) and that ovarian levels of this peptide are stimulated in response to the presence of LH and FSH in a cAMP-dependent manner (Hsu and Hammond 1987). Simmen et al. (1992) established that IGF- I mRNA in the endometrium and luminal fluid of pregnant gilts increases between days 8 and 12 of pregnancy and declines by day 14 of pregnancy. These authors also suggest that the elongation of the embryo and the consequent oestrogen concentrations in the uterine fluid originating from the embryo is coincident with the oestrogen-stimulated release of IGF-1 from the endometrium. A similar mode of action has been postulated for retinol, at least in the cultured granulosa cell. Bagavandoss and Midgely (1987) demonstrated that a

low dose of retinol or retinoic acid enhanced the ability of FSH to induce LH receptors and stimulate cAMP production and therefore progesterone and oestrogen. Further, a large increase in uterine concentrations of retinol in the pig is implied by the marked increase in retinol binding protein between days 12 and 13 of pregnancy (Trout et al. 1990). The coincidence of mode of action and timing of increase between these two agents suggests the possibility of additive or synergistic effects of retinol and IGF-1.

While IGF-1 plays an important role in the development of the embryo (Simmen and Simmen 1990), it seems likely that IGF binding proteins might act to modulate the effects of IGF-1. In a study using cows, de la Sota et al. (1996) found that treatments with steroids such as oestrogen did not change the concentrations of IGF-1 in the cell, but that concentrations of the IGF binding proteins were increased in response to the treatments. Echternkamp et al. (1994) postulate that IGFBPs, particularly in the ovarian follicle, modulate the activity of IGFs via autocrine and Paracrine means, while Frost et al. (1993) suggest that endometrial IGFBP-1 inhibits the IGF-1 stimulated synthesis of DNA, possibly providing a modulating effect. Simmen et al. (1992) found that RNA expression of IGFBP-1 in the endometrium of the cyclic sow was highest at oestrus, coinciding with the timing of the highest oestrogen : progesterone ratio. As the concentration of oestrogen during early pregnancy is elevated in the sow and endometrial expression of mRNA for IGFBP-2 is increased (Geisert et al. 1982), it would be interesting to investigate this relationship in the contextg of pregnancy, and in particular to investigate the expression of mRNA for IGFBP in the embryo. The modulatory effect of the IGFBPs therefore add a complicating factor to the suggestion of synergism between retinol and IGF-1.

While the majority of litters were retrieved as a clump of embryos yielding high quality RNA, in some cases less than the entire litter was recovered. In these cases the embryos were damaged and yielded little or degraded RNA. In order to

overcome this difficulty, a larger sample of gilts could be used. If further work were to be carried out in this area, the current experiment could be repeated with a larger number of animals, and all animals would be at the same day of pregnancy. The collection of embryos could be carried out after hysterectomy, using a large volume of saline, ensuring the collection of all embryos present, and inflicting less damage on embryonic tissues during collection. In addition to the measurement of mRNA for IGF-1, tissues could be analysed for the presence of mRNA for the IGF binding proteins, IGFBP-2 in particular. Further studies could be carried out in a culture system, where retinol, IGF-1 or a mixture of the two was added to culture media containing embryonic tissue, which would presumably contain the IGF type 1 receptor and the retinol receptor. Maternal plasma FSH, embryonic cAMP and oestrogen concentrations in response to the treatments could be measured, as well as expression of mRNA for IGFBPs.

Chapter 9:
The optimization of timing and treatment with retinol and/or folic acid on litter size of multiparous sows

9.1. Introduction

Embryo losses in pigs are thought to be due to a variety of factors such as diversity in the stage of oocyte, follicular and embryo development, plane of nutrition, hormonal levels and uterine environment as well as environmental conditions (Pope et al., 1990). The average litter size in commercial piggeries in Australia is around 10.5 piglets per sow (Australian Pig Industry Handbook). However, there is a wide variation in litter size among individual piggeries with values ranging from 9.5 to 11.5. One of the possible causes of low litter size in some piggeries may be high embryo mortality rates. A number of studies have indicated that embryo mortality is relatively high in pigs with figures ranging from 20-45% (Hunter 1980, Flint et al 1982).

Previous studies undertaken by Coffey and Britt (1993) have shown that an injection of -carotene or retinol palmitate increased the litter size by up to 0.6 piglets per sow. Brief and Chew (1985) also noted a reduction in the embryonic mortality rate after injection of vitamin A or -carotene in vitamin A depleted gilts. Evidence suggests that the effect of retinol or -carotene may be upon the embryo at the critical transitional period of the formation of the filamentous stage embryo from the blastocyst at about day 11 of pregnancy (Pope et al., 1990, Trout et al., 1992, Lambert et al., 1991). However, since treatment with retinol before ovulation appears to be effective in increasing litter size in the pregnancy immediately following, it is possible that the retinol may act on the follicle at the time of recruitment. Retinol may alter hormonal or other conditions in the developing follicle and/or enhance oocyte viability rendering the freshly fertilized oocyte less susceptible to mortality. Given that the action of retinol and retinoic acid is in cell differentiation neither of these sites of action is unreasonable.

Maternal folate deficiency in the early weeks of gestation may increase embryonic death and fetal resorption (women; Hibbard 1975, guinea pig; Habibzadeh et al.,1986) indicating that adequate levels of folic acid appear to be essential in maintaining embryo survival. Folic acid, a B-complex vitamin is essential for the synthesis of purines and pyrimidines and nucleic acid synthesis in the conceptus and in processes controlling rapid cellular division (Huennekens 1968; Herbert and Das 1976; Davis and Nichol 1988).

While much field work has been carried out to clarify the effects of folic acid, conflicting results have been published. Matte et al., (1984b) demonstrated that a series of 10 intramuscular injections of 15 mg folic acid prior to breeding and during early gestation resulted in an increase in the number of live pigs born per litter. Parenteral administration of folic acid (25 mg) at breeding and seven days later increased prolificity of multiparous sows by 10 to 15% (Friendship and Wilson 1991). Tremblay et al., (1989) suggest that improvement in litter size is most likely due to an increase in embryo survival during the first 30 days of gestation. Matte et al., (1984a) reported that sow serum folate concentration decreases markedly during gestation. Further investigations by Matte et al., (1984b) and others (Tremblay et al., 1986 and Harper et al., 1992c) have supported this observation. In contrast other investigations have reported that folic acid supplementation during gestation and lactation either in the diet (Harper et al.,1992c; Harper et al.,1993) or following parenteral administration (Gannon and Leibholz 1988) has no effect on the litter size, piglet weight, litter weight at birth or the weaning to return to oestrus interval. A recent study by Harper et al., (1994) showed that inclusion of folic acid in the diet had a pronounced effect in attenuating decreased serum folate concentration during gestation but was without benefit to reproductive performance.

Studies of both retinol and folic acid have produced equivocal results. While litter size appears to increase in association with retinol palmitate treatment, little work has been reported using other esters of retinol nor has a great deal of

work been published on the timing of treatment. The aim of these experiments was to examine the effects of the timing of parenteral treatment with retinol proprionate and the effects of feeding folic acid with or without an additional injection of retinol proprionate on the reproductive efficiency of the multiparous sow. The reproductive parameters measured as indicators of reproductive efficiency were: wean to oestrus interval, percentage of sows farrowing, the total number of piglets born per litter, the distribution of litter sizes, the percentage of each litter born dead and the percentage of each litter born alive.

9.2. Materials and Methods

Animals and Treatment

Weaned multiparous Large White x Landrace sows (N=850) were randomly allocated on the basis of parity to one of six treatments over a period of approximately 4 weeks. Numbers of each parity allocated to treatments are shown in Table 9.1. Treatments were as follows: Group 1 (n=133) was the control, receiving 1 ml of Solutol, the proprietary vehicle (BASF, Spain) day before weaning; group 2 (n=135) were injected with 500,000 iu (approximately 275 mg) retinol proprionate (RP; BASF, Spain) the day before weaning; group 3 (n=135) were injected with 500,000 iu RP at day of mating; while those allocated to group 4 (n=137) were treated with 500,000 iu RP 6 days after mating. Sows were fed the normal lactational diet (Chapter 3, General Materials and Methods) containing 15 million IU vitamin A/ tonne (approximately 8.25 mg/kg) and 0.5 ppm folic acid (approximately 0.5 mg/kg) ad libitum until day of mating and then 2.25 kg per head per day of the gestational diet containing the same levels of vitamin A and folic acid until farrowing. Treatment group 5 (n=155) received 13.3 mg folic acid in 2.25 kg of feed (approximately 5.9 ppm) plus ad lib normal lactational feed supplemented with the standard levels of folic acid and vitamin A for the period from day of treatment until mating. After

mating sows were fed the normal gestational feed supplemented with folic acid (13.3mg in 2.25 kg of feed) until 35 days after mating. Sows in treatment 6 (n=155) had the same feed regime as for treatment 5 but were also injected with 500,000 iu RP on the day before weaning. General feed formulations are shown in Chapter 3, General Materials and Methods.

Mating

All sows were checked for signs of oestrus twice daily and mated morning and night during their first standing oestrus after weaning and again 24 hours after the first mating. Animals were housed in group pens for the first 15 weeks of gestation, then in individual crates until farrowing. The interval between weaning and oestrus of mating for this trial was recorded (wean to oestrus interval). Percentage of sows farrowing, litter size, total born, number born alive, and number born dead were also recorded.

Table 9.1. Numbers of animals allocated to treatments on the basis of parity (par) and totals. RP designates retinol proprionate while FA refers to folic acid.

Treatment	Par 2	Par 3	Par 4	Par 5	Par 6	Par 7
Control	24	38	27	13	31	133
RP day before weaning	25	33	29	13	35	135
RP day of mating	23	41	24	17	30	135
RP 6 days after mating	24	35	25	19	34	137
FA	27	39	32	17	40	155
FA+RP day before wean	26	42	30	17	40	155
Total	149	228	167	96	210	850

Statistical analysis

The weaning to oestrus interval prior to mating for this trial, percentage of mated sows farrowing (farrowing rate), total piglets born, piglets born alive and percentage of each litter born dead were analysed by least squares analysis of variance using the statistical analysis program Statistica (Statsoft, USA). Independent variables in the model included treatment, parity and the interaction between these variables. Percentage of sows farrowing, total piglets born, piglets born alive and percentage of piglets born dead were analysed with wean to oestrus interval as a covariate. Means contributing to significant differences were identified by post hoc least significant difference test. Distribution of litter size was analysed by chi square using Minitab. Data were analysed in two ways: (a) to compare timing of treatment with retinol only and (b) to compare the folic acid treatments with the control group only. In each analysis, where parity did not contribute to significant difference, parity data were pooled within treatment and analysed for treatment only.

9.3. Results

Timing of treatment with Retinol Proprionate

The distribution data for litter size indicate a significant effect of treatment. Treatment with retinol proprionate on the day before weaning reduced the number of small litters 7 piglets in comparison to all other treatment groups while increasing the number of litters comprising 8-9, 10-11 and 12-13 piglets in comparison to controls ($P<0.01$; Figure9.1.1, $^2=35.09$, $df=15$; appendix 3). An increase in the number of litters of the 8-9 piglet range was associated with all retinol treatments compared to controls.

A summary of means for weaning to oestrus interval, farrowing rate and litter

parameters is given in Table 9.2. There was no significant difference in the weaning to oestrus interval for either timing of treatment (P=0.504) or for low parity sows compared with high parity sows (P=0.223; Figure 9.2.1). Since treatments 3 and 4 were given at or after oestrus they were not expected to influence this interval and were excluded from the analysis. When parities within the control treatment and Retinol proprionate on the day before weaning were pooled and data were analysed for treatment alone, Retinol proprionate on the day before weaning treatment neared significance (P=0.074).

Neither parity nor treatment alone significantly affected the percentage of sows farrowing in an analysis of covariance with WRI as the covariate (P= 0.587 and 0.290 respectively). There was a trend toward an interaction between parity and treatment (P=0.059) brought about by a lower farrowing rate in mainly parity 5 animals for treatments 2 and 3 (Figure 9.3.1). Given the large number of animals used in this trial, this interaction is probably meaningful.

Total piglets born was not affected by timing of treatment with retinol (P=0.553) but as expected, lower parity sows produced smaller litters (P<0.05, Figure 9.4.1). The percentage of stillborn piglets was not affected by treatment (P=0.873) but was significantly increased with parity (P<0.01, Figure9.5.1). Piglets born alive was affected by neither parity (P=0.389) nor timing of treatment (P=0.758; Figure 9.6.1). There were no significant parity by treatment interactions for any production parameter examined (Appendix 3).

Titles of figures overleaf.

Figure 9.1.1. Litter size distribution (number of litters) and number of sows not farrowing for sows treated with vehicle (control) day before weaning, retinol proprionate day before weaning (RP-W), retinol proprionate on day of mating (RP0) or retinol proprionate 6 days after mating (RP+6).

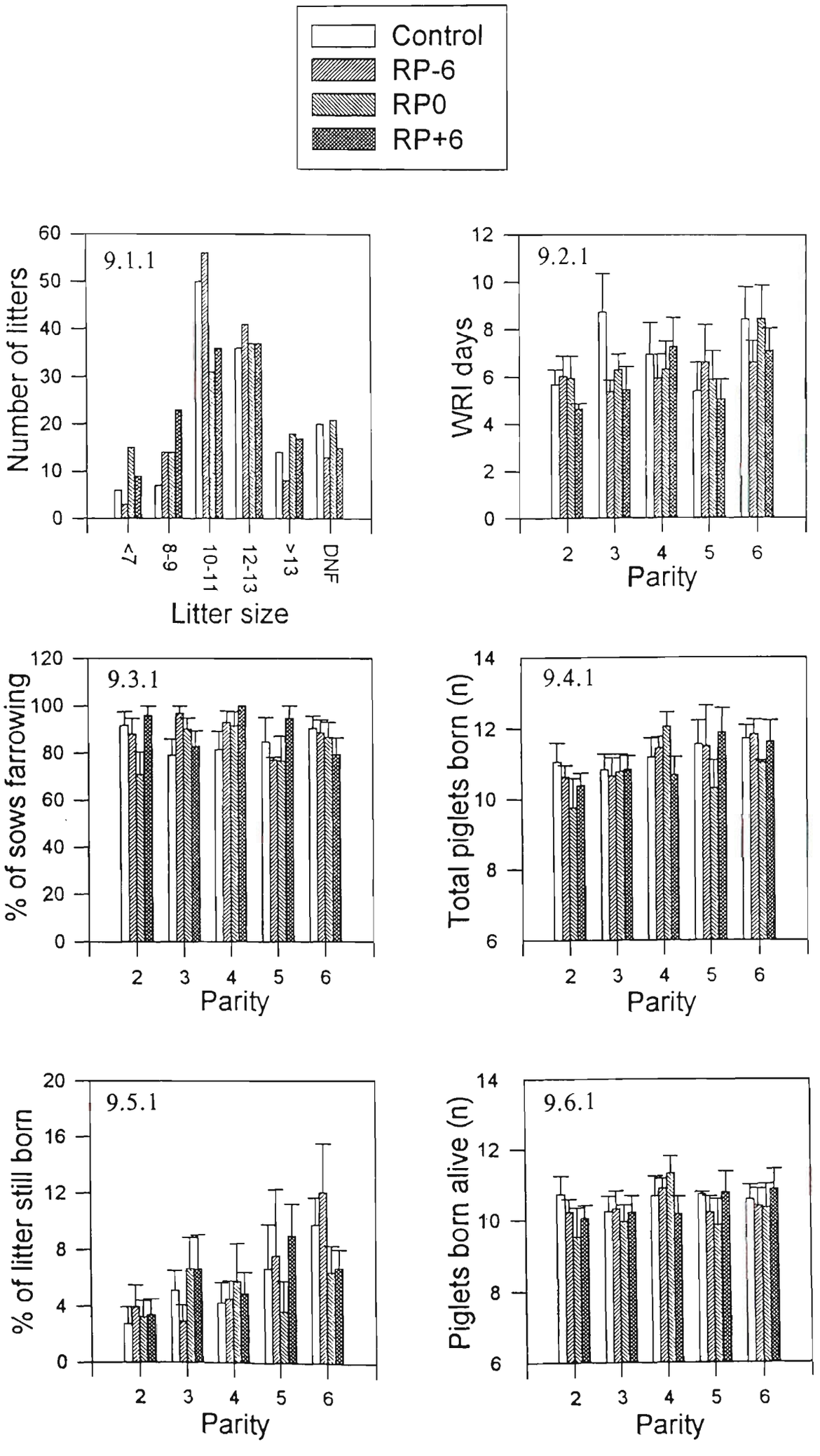
Figure 9.2.1. Mean (±s.e.) weaning to return to oestrus interval (WRI) for sows of parities 2 to 6 treated with vehicle (control) day before weaning, retinol proprionate day before weaning, retinol proprionate on day of mating or retinol proprionate 6 days after mating.

Figure 9.3.1. Mean (±s.e.) percentage of sows farrowing for parities 2 to 6 after treatment with vehicle (control) day before weaning, retinol proprionate day before weaning, retinol proprionate on day of mating or retinol proprionate 6 days after mating.

Figure 9.4.1 Mean (±s.e.) total piglets born per litter for parities 2 to 6 after treatment with vehicle (control) day before weaning, retinol proprionate day before weaning, retinol proprionate on day of mating or retinol proprionate 6 days after mating.

Figure 9.5.1 Mean (±s.e.) percentage of each litter stillborn for parities 2 to 6 after treatment with vehicle (control) day before weaning, retinol proprionate day before weaning, retinol proprionate on day of mating or retinol proprionate 6 days after mating.

Figure 9.6.1. Mean (±s.e.) number of piglets born alive per litter for parities 2 to 6 after treatment with vehicle (control) day before weaning, retinol proprionate day before weaning, retinol proprionate on day of mating or retinol proprionate 6 days after mating.


Control
RP-6
RP0
RP+6
9.1.1
Number of litters
60
50
40
30
20
10
0
<7
8-9
10-11
12-13
>13
DNF
Litter size
9.2.1
WRI days
12
10
8
6
4
2
0
2
3
4
5
6
Parity
9.3.1
% of sows farrowing
120
100
80
60
40
20
0
2
3
4
5
6
Parity
9.4.1
Total piglets born (n)
14
12
10
8
6
2
3
4
5
6
Parity
9.5.1
% of litter still born
20
16
12
8
4
0
2
3
4
5
6
Parity
9.6.1
Piglets born alive (n)
14
12
10
8
6
2
3
4
5
6
Parity

Titles of figures overleaf.

Figure 9.1.2. Litter size distribution (number of litters) and number of sows not farrowing for sows treated with vehicle (control) day before weaning, fed a diet supplemented with folic acid (FA) or fed the folic acid diet and treated with retinol proprionate day before weaning (FA+RP-W).

Figure 9.2.2. Mean (±s.e.) weaning to return to oestrus interval (WRI) for sows of parities 2 to 6 treated with vehicle (control) day before weaning, fed the FA supplemented diet or fed the FA diet and treated with retinol proprionate day before weaning.

Figure 9.3.2. Mean (±s.e.) percentage of sows farrowing for parities 2 to 6 treated with vehicle (control) day before weaning, fed the FA supplemented diet or fed the FA diet and treated with retinol proprionate day before weaning.

Figure 9.4.2. Mean (±s.e.) total piglets born per litter for parities 2 to 6 treated with vehicle (control) day before weaning, fed the FA supplemented diet or fed the FA diet and treated with retinol proprionate day before weaning (FA+RP-6).

Figure 9.5.2. Mean (±s.e.) percentage of each litter stillborn for parities 2 to 6 treated with vehicle (control) day before weaning, fed the FA supplemented diet or fed the FA diet and treated with retinol proprionate day before weaning (FA+RP-6).

Figure 9.6.2. Mean (±s.e.) number of piglets born alive per litter for parities 2 to 6 with vehicle (control) day before weaning, fed the FA supplemented diet or fed the FA diet and treated with retinol proprionate day before weaning.

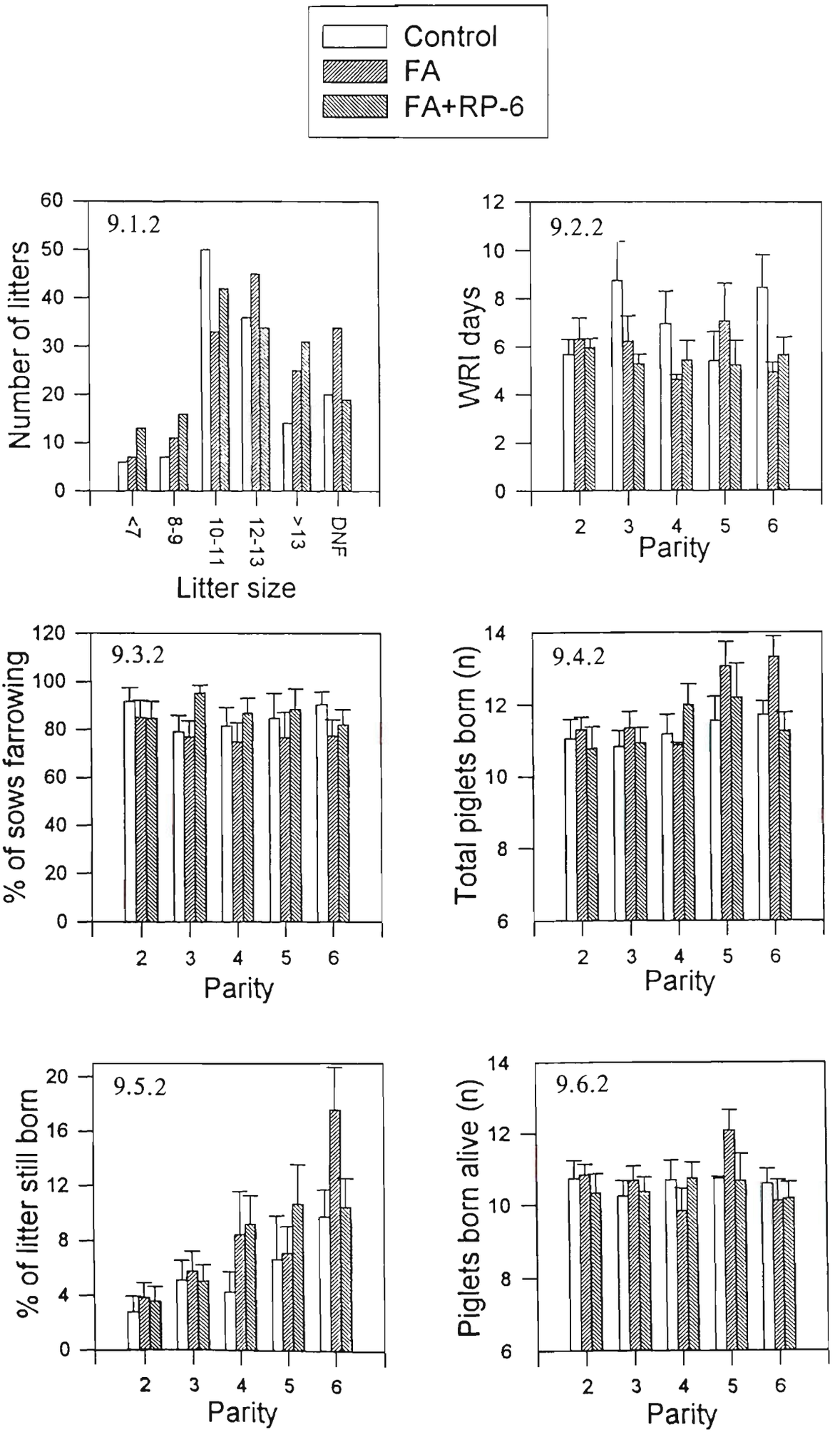

Control
FA
FA+RP-6
9.1.2
Number of litters
60
50
40
30
20
10
0
<7
8-9
10-11
12-13
>13
DNF
Litter size
9.2.2
WRI days
12
10
8
6
4
2
0
2
3
4
5
6
Parity
9.3.2
% of sows farrowing
120
100
80
60
40
20
0
2
3
4
5
6
Parity
9.4.2
Total piglets born (n)
14
12
10
8
6
2
3
4
5
6
Parity
9.5.2
% of litter still born
20
16
12
8
4
0
2
3
4
5
6
Parity
9.6.2
Piglets born alive (n)
14
12
10
8
6
2
3
4
5
6
Parity

Table 9.2 Mean (±s.e.) weaning to return to oestrus interval (WRI), farrowing rate, total piglets born, percentage of litter stillborn and piglets born alive in response to treatments with retinol proprionate and/or folic acid (parities pooled within treatment, all 6 treatments included in analysis of variance).

Time relative to mating	n	WRI	Farrow rate (%)	Total born (n)	Stillborn (%)	Born alive (n)
Control	133	7.4±0.65a	85.0±3.11a	11.2±0.22a	5.8±0.81a	10.6±0.22a
RP day before weaning	135	6.1±0.41b	90.0±2.50a	11.2±0.22a	6.2±1.12a	10.4±0.21a
RP day of mating	136	6.7±0.48	84.0±3.11a	11.0±0.28a	5.6±1.03a	10.2±0.27a
RP 6 days after mating	137	6.0±0.43	89.0±2.67a	11.1±0.23a	6.0±0.82a	10.4±0.22a
FA	155	5.7±0.37b	78.0±3.33a	11.7±0.25a	9.1±1.21b	10.5±0.23a
FA+RP day before wean	155	5.5±0.30b	88.0±2.64a	11.4±0.26a	7.6±0.84a	10.4±0.22a

[b] significantly different to [a] for treatment; Significant difference at the 5% level.

Treatment with folic acid and/or retinol proprionate

There was a significant difference between folic acid treated- and the control groups when litter size distribution data were analysed by chi square ($P=0.015$, $^{2}=22.23$, $df=10$; Figure 9.1.2, Appendix 3). Sows treated with folic acid alone produced more litters in the 8-9, 12-13 and especially the >13 piglet range than control animals. Litters in the 10-11 piglet range were decreased in these sows. Notably, the number of sows which did not farrow in the group fed the folic acid diet alone trended toward an increase ($P=0.062$) when compared with control animals. When sows were given retinol proprionate in addition to the folic acid supplemented feed, there was an increase in the number of litters in the <7, 8-9 and >13 size range but not the 10-11 size range. The number of sows which did not farrow was not significantly different to the control levels when retinol proprionate treatment was administered with the folic acid supplemented feed.

The folic acid treatment alone was associated with a significant decrease in the WRI ($P<0.01$; Figure 9.2.2, Appendix 3) when parities were pooled within treatment for the analysis. In sows fed the folic acid supplemented diet alone from the day before weaning until 35 days after conception, there was no significant difference between the farrowing rate for parity ($P=0.889$; Figure 9.3.2). When parity was therefore removed from the analysis, there was a trend toward a decrease in farrowing rate ($P=0.062$; Table 9.2). There was a trend toward a significant effect of parity on total piglets born ($P=0.077$, Figure9.4.2, Appendix 3) but no effect of treatment ($P=0.553$).

Parity but not treatment was a significant contributor to the percentage of stillborn piglets in all treatments ($P<0.01$; Figure 9.5.2). The number of piglets born alive per litter of sows treated with folic acid or folic acid and retinol did not vary from the control ($P=0.734$), nor did parity have a significant effect ($P=0.437$; Figure 9.6.2, Appendix 3).

7.4. Discussion

The finding of the study on the timing of treatment with retinol proprionate indicated that there was no significant difference between the control and any treatment group for total born, percentage stillborn or number born alive when retinol proprionate was used at any time relative to mating. These results are in contrast with the positive results when retinol palmitate was used, reported by Coffey and Britt (1993) and Brief and Chew (1985). The lack of positive effect in this experiment may be due to the ester of retinol used. As described previously in Chapter 5, when 500,000 iu of retinol proprionate was given intramuscularly there was no detectable increase in retinol in the plasma. This may have been because the vehicle in which the retinol was suspended was a slow release agent. Due to the time requirement for this experiment (approximately 20 weeks), it was begun before the results of the plasma trial were obtained.

In contrast to the effect of retinol proprionate on other production data, the distribution of litter size was significantly affected by retinol treatment. Results of the current experiments and the work of others lead us to hypothesize that one of the effects of retinol given on the day before weaning is at the level of the ovary, specifically in the development of the follicle and/or the corpus luteum. As shown in chapter 8, follicular development appears to be advanced in association with treatment with retinol palmitate at on the day before weaning. Further, in a previous study, Talavera and Chew (1988) were able to demonstrate an increase in progesterone production in cultured cells of corpus luteum on the addition of retinol or retinoic acid in the presence of LH. While there may be other factors contributing to the effect, these results taken together with other work suggests that the primary action of retinol is on the ovary. Further work is required todevelop this hypothesis.

Another component of the effect of retinol given at weaning may be on the embryo or the uterine environment as suggested by Britt (1992), Brief and Chew (1985) and other workers. This is suggested in the current study by the increase in litter sizes

from the 7 piglet class to the 8-9 through to 12-13 piglet per litter class, but not for the >13 piglets per litter class in this treatment group. However, this was not borne out by the litter size or piglets born alive data in this experiment. This may have been due to an increase in the number of piglets in the 7-8 size range compared to controls but a decrease in the number of litters in the >13 size range in comparison to controls. Therefore, though litter size appeared to be skewed away from the smallest class, there was a corresponding decrease in the litters falling in the largest size class also. Britt (1992) and Roberts and Bazer (1980) suggest that uterine histotroph is increased in response to treatment with retinol. Treatment on day of mating or 6 days after mating with retinol proprionate may have precipitated this effect and therefore may have contributed to the shift in litter size through this means.

If treatment with retinol on the day before weaning was expected to have its most immediate effect on the development of follicles, the weaning to oestrus interval and total piglets born would be the most likely reproductive parameters to be affected. The almost significant shortening of WRI in sows treated with retinol proprionate on the day before weaning may have been brought about by changes in steroid hormones produced in the follicle in response to retinol treatment. Retinol treatment at weaning may have the effect of shortening and almost synchronizing return to oestrus. As shown in Chapter 8 the retinol treatment induces higher levels of oestradiol in follicular fluid in gilts treated than in that of untreated animals. This may be via increased production of FSH-induced levels of cAMP in treated sows (Bagavandoss and Midgely 1987) leading to increased oestrogen production.

The percentage of stillborn piglets increased with parity though was not affected by treatment with retinol. Since the proportion of litters stillborn is an indication of late foetal death, other factors such as uterine crowding or nutrition may have contributed to this figure.

Further work could be carried out using retinol palmitate at the treatment times used in this trial. Plasma FSH and LH could be measured in the days prior to ovulation

to ascertain whether there is an earlier increase in the levels of these hormones in animals treated at weaning. Plasma concentrations of retinol and progesterone could be monitored for the first 15 days after treatment and uterine histotroph and embryos could be sampled.

The findings in this experiment of significant effects on litter size distribution, farrowing rate and weaning to return to oestrus interval in folic acid treated sows differed significantly from those attained by other researchers. Animals supplemented with folic acid demonstrated a shift toward larger litter sizes (12-13 and >13) in comparison with control fed animals. However, a significantly larger proportion of animals failed to farrow in the animals given folic acid alone when compared to controls. This is difficult to explain in the light of positive results gained by Matte and Girard 1989, Tremblay et al. 1989 and Lindemann et al. 1993.

Farrowing rate was significantly reduced in animals fed folic acid alone, as suggested by the litter distribution data. However, when folic acid was fed in conjunction with an injection of retinol proprionate on the day before weaning, the farrowing rate was returned to control levels. This finding suggests that there may be a toxic effect of high doses of folic acid. However, this hypothesis cannot be validated since ovulation rates and early embryo mortality were not measured. Thaler et al. (1989) reported lower farrowing rates in sows fed on a diet supplemented with 6.62 mg/kg folic acid (72.5 cf 76.5% respectively) however this difference was not significant. Thaler's work also showed that sows fed with higher levels of folic acid (6.62 mg/kg, compared to our dose rate of 5.5 mg/kg) produced smaller litters and more stillborn piglets than those fed at lower rates, although the number of piglets per litter from treated sows was still higher than the controls. These workers suggested that the early embryo may be highly sensitive to nutrient concentrations and that the level of folic acid or its metabolites may have been imbalanced or at worst, toxic to the embryo on reaching a threshold level.

The NRC (1979) has suggested a requirement of 0.6 mg folic acid/kg diet for all classes of swine based on the folacin levels found in commonly used feed. In 1988 the requirement for folic acid in swine diets was estimated at 0.3 ppm, considerably less than the 6 ppm fed in this trial. Because this vitamin is a water soluble compound, it is generally regarded as non toxic, though some toxic responses at very high doses (250 mg/kg body weight) have been recorded in the rat (Hommes and Obbens 1973).

The interval between weaning and oestrus was significantly shortened in sows fed the folic acid diet alone compared to the controls. The addition of retinol proprionate to the treatment to the folic acid treatment also resulted in a significantly shorter WRI than controls, suggesting that the effect was due to folic acid. Folic acid treatment in other experiments has resulted in either no effect on wean to oestrus interval (Lindemann and Kornegay 1989) or has lengthened this interval (Harper et al. 1994). In general however, these effects reflect treatment with folic acid at the previous mating without treatment through lactation in association with a larger litter size rather than treatment during the current weaning. Weaning through to oestrous has been identified as a critical time when a decrease of up to 30% of plasma folate levels occurs in untreated animals, from high concentrations of serum folate at the end of lactation with a further fall of 20% between days 30 and 60 of pregnancy (Matte et al. 1984a).

The total number of piglets born increased with parity as expected. Animals at parity 6 fed the folic acid supplemented diet had a significantly higher litter size than other parities, suggesting a high ovulation rate and less embryo death. This partially supports the suggestion of Tremblay et al. (1989) and others that treatment with folic acid is most efficacious in animals with a higher ovulation rate, noting that a higher ovulation rate is expected in higher parity animals such as parities 5 and 6. It should be noted however, that ovulation rate was not measured in our experiments. Serum levels of folic acid would be expected to fall between ovulation and implantation due to its increased utilization in high cell turnover for the development of the uterus and

embryonic membranes (Matte et al 1984b, Lindemann 1993). According to Matte et al. (1984b) however, under the conditions of normal ovulation rate, folic acid supplementation would have no effect on embryo mortality or therefore on litter size. It is only when uterine conditions prevent an increase in embryo mortality that litter size is increased.

The higher total litter size in parity 6 animals was associated with an increased percentage of piglets born dead. This percentage seemed to be increased in association with the folic acid supplementation, but when fed in conjunction with the retinol proprionate, the percentage of each litter born dead was reduced to control levels. This finding is difficult to explain given that the treatment with retinol occurs some 4 months earlier. It could hardly be expected that a transient increase in circulating retinol and a small increase in stored levels could have such far-reaching consequences as preventing the death of foetuses near term.

Further work is clearly required to clarify the function of both folic acid and retinol based on further development of hypotheses resulting from the experiments reported. Optimal dose rates and timing of treatment are critical in order to attain consistent results from treatment.

Chapter 10

Conclusions and further work

The aims of these studies were to investigate the mode of action of retinol after injection in increasing litter size in the sow. Parameters investigated included the transport and storage of retinol and its effect on the ovary and the embryo. This work was carried out in order to find the optimal time of administration of retinol for an increase in litter size in the sow.

Methods for the extraction of retinol and beta carotene from plasma and tissue and their quantification were investigated. Retinol was extracted efficiently from plasma by means of an ethanol precipitation, excluding the hexane or ether extraction usually employed for the fat-soluble vitamins such as beta carotene. While the efficiency of this extraction method was high, the use of a normal phase HPLC system for the separation of retinol may have been preferable for the separation of retinol from the extract, given the ability of the normal phase column to deal with the high lipid component of such an extraction method. The use of a reverse phase HPLC system in this work was satisfactory for the separation of retinol from the extract, but resulted in difficulties with the accumulation of lipids on the column. Another solution to this problem may have been an additional extraction step with hexane which would have allowed the separation of retinol with no such problems on the reverse phase HPLC system.

No adjustments to the extraction procedure were required for the extraction of beta carotene from plasma. However, a more non-polar solvent composition than that used for the separation of retinol from plasma was developed to separate the beta carotene from the extract and give a sharper peak and a short elution time.

Retinol and beta carotene were extracted efficiently from tissues using the hexane extraction including the saponification of samples commonly used for the fat-soluble vitamins. A xylene extraction which did not include saponification was investigated, but was not found to be as suitable for the tissue samples as the extraction technique adopted. Reverse phase HPLC was used to separate retinol from tissue extracts, while spectrophotometric methods were utilized for the quantification of beta

carotene from tissues. The post-homogenization recovery of beta carotene from tissue samples was close to 100%, but may have included other compounds which absorb at 460 nm. Ideally, beta carotene in tissue samples would be separated from extracts by HPLC in further studies. Further work in the development of methods could include the separation by HPLC of retinol and beta carotene in the same tissue sample extract.

The quantification of retinol and beta carotene in the plasma and tissues of multiparous sows after treatment with retinol proprionate or vehicle; or beta carotene or vehicle was carried out using the extraction and HPLC or spectrophotometric methods developed.

After the injection of 500,000 IU retinol proprionate or 1 ml of vehicle, a significantly higher plasma concentration of retinol was demonstrated in retinol treated animals at 12 hours after treatment than those treated with vehicle. Retinol concentrations were consistently higher (though not significantly so) in retinol treated animals than in vehicle-treated sows until 84 hours after treatment. However, a large degree of variation in mean plasma retinol concentrations within treatment groups was evident. In order to investigate whether the variation seen in this trial was within the normal range, the temporal changes in the plasma profile of retinol in untreated sows or sows treated with vehicle was carried out. In an experiment which involved taking blood samples at intervals of 30 minutes for a period of 24 hours, no significant difference between vehicle-treated or untreated sows was demonstrated although there was an overall increase in plasma retinol concentrations with time. It was not clear as to the cause of this rise. Further trials carried out in this area might include treatment with retinol proprionate dissolved in corn oil, and corn oil as the vehicle treatment. A comparison of plasma profiles of sows treated with retinol proprionate dissolved in corn oil and that of sows treated with retinol palmitate dissolved in corn oil could be made, since the palmitate ester is more commonly found in the plasma.

Beta carotene concentrations rose significantly in animals treated with 250 mg beta

carotene 3 hours after treatment in comparison to vehicle treated animals and remained significantly higher than those of vehicle-treated animals for the 7 day period of sampling. This was not reflected in tissue levels, after treatment with 250 mg of beta carotene or vehicle. A similar experimental design as suggested for further work with retinol which established pre-treatment tissue levels of beta carotene and then post-treatment levels in the same animals might reveal an increase in stored levels of beta carotene and/or the conversion of beta carotene to retinol in the liver.

When gilts were treated with 1,000,000 IU of retinol palmitate or vehicle and ovarian follicles were collected, an effect on follicle size and development was seen in treated animals. It would appear that retinol palmitate acts to advance steroidogenesis in the follicles. These findings require further clarification, however. Further work using a tissue culture system of porcine granulosa cells could be utilized to investigate the mode of action of retinol palmitate on steroidogenesis. Cultured porcine granulosa cells treated with retinol palmitate in low concentrations (eg 10^{-9} M) should respond with an increase in oestrogen production, corresponding with the findings of this study. Measurement of the concentrations of the androgens and 3βHSD would indicate that whether effect of retinol was at a point further down the steroidogenesis pathway.

When gilts were treated with 1,000,000 IU of retinol palmitate or vehicle, were artificially inseminated and embryos were collected at days 11-13 of pregnancy, an effect of retinol was not seen on the mRNA concentrations for IGF I. Ovulation rate was not increased in the treated gilts. In further studies, a larger number of animals could be treated and embryos could all be collected on a single day, eg day 12 of pregnancy.

The optimization of timing of treatment with vehicle or retinol proprionate with or without the addition of folic acid to the diet was investigated. No difference was seen in the measured reproductive parameters of sows treated with 500,000 IU of retinol

proprionate or 1 ml of vehicle for treatment at 6 days before weaning, day of mating or treatment at 6 days after mating and those treated with vehicle. In sows fed a diet supplemented with folic acid (5.9 mg/kg of feed) the weaning to oestrus interval was reduced. No other significant effects were seen in comparison with vehicle treated animals. A trial investigating the optimal timing of treatment with retinol palmitate could be carried out in future work.

These studies suggest that retinol palmitate is more efficacious in improving reproductive parameters than retinol proprionate. While retinol and beta carotene were detected in the plasma of treated sows, neither was increased in the tissues. Retinol palmitate appeared to have a positive effect on the development of the follicle, possibly through the steroidogenesis pathway. While the optimization trial did not bring about an increase in any reproductive parameters, the use of retinol palmitate in such a trial might improve litter size in the treated sow.

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Appendix 1

Buffers and solutions

Adenosine triphosphate (ATP) 0.1 M pH 7.0

60 mg ATP

0.8 ml H_2O

Adjust pH with 0.1 N NaOH

Make to volume to 1 ml with dH_2O

Store at -70°C

Ammonium acetate 10 M

770 g ammonium acetate

800 ml H_20 then make to 1 L

Filter sterilize

Blocking solution (100 ml)

10 g DIG blocking reagent (Boehringer Mannheim)

100 mM maleic acid buffer

Heat to dissolve but do not boil. (Causes coagulation)

Autoclave at 100°C for 10 minutes

$CaCl_2$ 1 M

54 g $CaCl_2.6\ H_2O$

200 ml deionized dH_2O

Filter sterilize

Store at -20°C

Denhardts Solution 50x

5 g Ficoll (type 400, Pharmacia)

5 g ployvinylpyrrolidone

5 g BSA (Fraction V; Sigma)

H_2O to 500 ml

DEPC water

0.1% DEPC

dH_2O

Shake vigorously incubate for several hours at 37°C

Autoclave

EDTA 0.5 M pH 8.0

186.1 g disodium ethylenediamineterta-acetate.$2H_2O$

800 ml dH_2O

Adjust pH while stirring to 8.0 with NaOH

Autoclave

Ethidium Bromide (10 mg/ml)

1 g Ethidium bromide

100 ml dH_2O

Stir several hours

Wrap in foil store at room temperature

Formaldehyde gel-loading buffer

50% glycerol

1 mM EDTA (pH 8.0)

0.25% bromophenol blue

0.25% xylene cyanol FF

Formaldehyde gel running buffer 5x

0.1 M MOPS (pH 7.0)

40 mM sodium acetate

5 mM EDTA (pH 8.0)

Guanidinium thiocyanate (GTE)

50 g guanidinium thiocyanate (M_r=118.1)

10 ml 1M Tris.Cl (pH 7.5)

DEPC H_2O to 100 ml

Wrap in foil and refrigerate

IPTG

2 g Isopropylthio-β-D-galactoside (mw=238.3)

8 ml dH_2O

Adjust volume to 10 ml

Filter sterilize

Store at -20°C

Lysozyme

10 mg/ml lysozyme

10 mM Tris.Cl (pH 8.0)

Maleic acid buffer

150 mM NaCl

100 mM maleic acid

$MgCl_2$ 1 M

203.3 g $MgCl_2$. 6 H_2O

800 ml dH_2O

Adjust volume to 1 L

Autoclave

MOPS buffer (10x)

41.8 g MOPS

16.6 ml 3M sodium acetate

20 ml 0.5 M EDTA

800 ml DEPC water

20 ml 5N NaOH (to bring pH to 7.0)

bring up to 1 L

NaCl 5M

292.2 g NaCl

800 ml H_2O

Adjust volume to 1 L, autoclave

NaOH:SDS solution

0.2 N NaOH

1% SDS

Potassium acetate (Solution K)

60 ml 5M potassium acetate

11.5 ml glacial acetic acid

Adjust volume to 100 ml with dH_2O

Potassium acetate 1 M pH 7.5

9.82 g potassium acetate

90 ml d H_2O

Adjust Ph with 2 M acetic acid

Adjust volume to 100 ml

Store at -20°C

Prehybridization solution for cDNA probe to DNA

5 x Denhardts solution

0.5% w/v SDS

Prehybridization solution for cDNA probe to RNA (High SDS 50% formamide buffer)

100 ml formamide

33.2 ml 30x SSC

10 ml 1M sodium phosphate (pH 7.0)

40 ml blocking solution

2 ml 10% N-laurylsarcosine

2 μl yeast tRNA (10 μg/ml)

14 g SDS

to 200 ml

Store at -20°C

SDS 10%

100 g Sodium dodecyl sulphate

900 ml dH_2O

Heat to 68°C

Adjust pH to 7.2 with conc HCl

Adjust volume to 1 L

Sodium acetate 3 M (pH 5.2 or 7.0)

408.1 g sodium acetate.$3H_2O$

800 ml dH_2O

Adjust pH to 5.2 with glacial acetic acid

Adjust pH to 7.0 with dilute acetic acid

Adjust volume to 1 L with dH_2O

Autoclave

Sodium phosphate buffer (pH 7.0)

68.4 ml 1M Na_2HPO_4

31.6 ml 1M NaH_2PO_4

Solution A

900 mg glucose

2.5 ml 1 M Tris.Cl (pH 8.0)

2.0 ml 500 mM EDTA (pH 8.0)

Autoclave, refrigerate

SSC 20x

175.3 g NaCl

88.2 g sodium citrate

800 ml dH_2O

Adjust pH to 7.0 with 10N NaOH

Adjust volume to 1 L

Autoclave.

SSPE 20 x

3 M NaCl

0.2M $NaH_2PO_4.H_2O$

20mM EDTA pH 7.4.

STE Buffer

1.0 M NaCl

10 mM Tris.Cl (pH 8.0)

1 mM EDTA (pH 8.0)

STET Buffer

0.1 M NaCl

10 mM Tris.Cl (pH 8.0)

1 mM EDTA (pH 8.0)

5% Triton X-100

TAE buffer

400mM Tris pH7.4

200mM sodium acetate

20mM EDTA (pH8.0)

TE Buffer pH 7.4

10 mM Tris.Cl (pH 7.4)

1 mM EDTA (pH 8.0)

TES buffer

0.3028 g Trizma

0.931 g EDTA

1.25 g SDS

DEPC water to 250 ml

Tris 1M

121.1 g Tris base

800 ml H_2O

Adjust pH as follows when solution is at room temperature:

pH	HCl
7.4	70 ml
7.6	60 ml
8.0	42 ml

Tris acetate buffer (TAE) concentration 50x

242 g Tris base

100 ml 0.5 M EDTA (pH 8.0)

Make to 1 L with dH_2O

Water saturated phenol

100 g phenol crystals

dH_2O

Heat to 60-65°C

Aspirate upper water phase

Refrigerate

X-ray Developer

400ml developer concentrate (AGVA GEVART)

2 litres dH_2O

X-ray Film Stop

3% Acetic acid

X-ray film fix bath

600 ml fixer concentrate (AGVA GEVART)

MEDIA AND GELS

Agarose gel 1.5% for DNA

0.75 g glucose

50 ml 1x TAE buffer

10 μl ethedium bromide

LB agar

5 g tryptone

2.5 g yeast extract

2.5 g NaCl

0.5 ml 1 N NaCl

7.5 g agar (bacteriological)

Make up to 500 ml with dH_2O

Autoclave cool to approx 60°C

Add 1 μl/ml antibiotic if required

Makes about 20 plates

LB broth

5 g tryptone

2.5 g yeats extract

2.5 g NaCl

0.5 ml 1 N NaCl

Make up to 500 ml with dH_2O

Autoclave

When cool add 1 μl/ml antibiotic if required

Appendix 2

Instrumentation and equipment

Blender bags

Centrifuge J2-HS
Beckman Industries Australia Ltd
Unit 16, 170 Forester Rd
Mt Waverly Vic 3149

Dry Block Heater
Ratek Instruments Australia
PO Box 131
Mitcham Vic 3155

Eppendorf tubes (1 ml)
Elkay Products Inc
Supplier: Pathtech Diagnostics
PO Box 175
Balwyn Vic 3103

G100 Electrophoresis tank and fittings
Amrad Pharmacia Biotech
34 Wadhurst Dve
Boronia Vic 3155

Griffen Flask Shaker (wrist action)

HPLC column C-18 Reverse Phase (3.9 mm x 150 mm; Resolve; Millipore)
Waters Millipore Australia Pty Ltd
4 Harper St
Abbotsford Vic 3067

Hybond N+ Nucleic acid transfer membrane

Amersham Australia Pty Ltd

PO Box 99

North Ryde NSW 2113

Kimwipes Fine Grade Cat # 4103

Kimberly Clark

PO Box 343

Milsons Point NSW 2061

Liquid Nitrogen

Liquid Nitrogen Services (Vic) Pty Ltd

PO Box 1216

Frankston Vic 3199

Microfuge Eppendorf Centrifuge 54515C

Supplier Crown Scientific Pty Ltd,

PO Box 101

1-3 Florence St

Burwood Vic 3125

Microfuge Microspin 24 Sorvall Instruments Dupont.

Supplier FSE Pty Ltd ACN 004 396 954

468 St Kilda Rd

Melbourne Vic 3004

Multitube vortex VWR

Supplier: Proscience Pty Ltd

7 Keysborough Ave

Keysborough Vic 1373

Needles hypodermic 19G 1½ TW 1.00 x 38 mm

Terumo Corporation

Supplier: Pathtech Diagnostics

PO Box 175

Balwyn Vic 3103

Nitrogen Industrial and High Purity Grades

Dry Ice

BOC Gases ACN 000 029 729

90 Bell St, PO Box 43

Preston Vic 3072

Nytran-Plus Nylon Membrane

Pasteur pipettes Cat# BILB 150

Selby Scientific

PO Box 276

Acacia Ridge Qld 4110

Photographics

Prossper Photographics

149 Nelson Place

Williamstown Vic 3016

Screw top open hole vial caps 8 mm Alltech Cat # 16-000698-00

Screw cap vials 12x32 mm (amber) Alltech Cat # 95194

Alltech Associates

1354B Malvern Rd

Malvern Vic 3144

Syringes 1 ml Cat # , 5 ml Cat # , 10 ml Cat #

Terumo Corporation

Supplier: Pathtech Diagnostics

PO Box 175

Balwyn Vic 3103

Techne Dry Block DB-C and Techne Sample Concentrator

Supplier: Proscience Pty Ltd

7 Keysborough Ave

Keysborough Vic 3173

Varian 9100 Autosampler

Varian 9012 Pump

Varian 9050 UV-Vis Detector

Varian Star 4.0 software

Varian Australia Pty Ltd

679 Springvale Rd

Mulgrave Vic 3170

Water bath

Ratek Instruments Australia

PO Box 131

Mitcham Vic 3155

Whatman Chromatography Paper 3mm Chr 46x57 cm Cat # 3030917

Supplier: LabSupply

PO Box 688

Mulgrave Nth Vic 3170

Chemicals

α ^{32}P Deoxycytidine 5'-Triphosphate

Bresatec ACN 070 039 913

PO Box 11

Rundle Mall

Adelaide SA 5000

Acetic acid Glacial AnalaR Cat # 945040

Merck Pty Ltd ACN 005 063 791

Supplier: Crown Scientific

PO Box 101

Burwood Vic 3125

ATP Adenosine 5'-Triphosphate Disodium Salt Cat # A 6144

Sigma Aldrich Pty Ltd

Unit 2, 14 Anella Ave

Castle Hill NSW 2154

Agar Bacteriological (Agar No 1) Code 11, Cat # LP011F

Unipath Ltd, Basingstoke, Hampshire, England

Supplier: Oxoid Australia Pty Ltd

104 Northern Rd

Western Heidelberg Vic 3081

All-trans Retinol Palmitate Type IV: Synthetic, sealed ampule Cat # R3375

All-trans Retinol Palmitate Type VI: Synthetic, crystalline dipersed in gel matrix Cat # R3625

Sigma Aldrich Pty Ltd

Unit 2, 14 Anella Ave

Castle Hill NSW 2154

All-trans Retinyl Acetate MW=328.5 Kodak Fine Chemicals Cat # 127-47-9 (No longer available)

All-trans Retinol MW=286.46 Kodak Fine Chemicals Cat # 68-26-8 (No longer available)

Ammonium Acetate Molecular Biology Grade MW=77.08 Cat # A 1542
Sigma Aldrich Pty Ltd
Unit 2, 14 Anella Ave
Castle Hill NSW 2154

Ampicillin D [-]-α-Aminobenylpenicillin MW=371.4 Cat # A 6144
Sigma Aldrich Pty Ltd
Unit 2, 14 Anella Ave
Castle Hill NSW 2154

Bovine Serum Albumin Fraction V Laboratory Reagent Cat # 100 018
Boehringer Mannheim Pty Ltd
353 Whitehorse Rd
Nunawading Vic 3131

Butylated Hydroxytoluene MW=220.3
Calbiochem-Novabiochem
PO Box 140
Alexandria NSW 2015

Chloroform HPLC Grade BDH

Merck Pty Ltd ACN 005 063 791

Supplier: Crown Scientific

PO Box 101

Burwood Vic 3125

Chloroform HPLC Grade BDH

Merck Pty Ltd ACN 005 063 791

Supplier: Crown Scientific

PO Box 101

Burwood Vic 3125

Diethyl Ether BDH MW=74.12 AnalaR, Product # 10094

Merck Pty Ltd ACN 005 0630791

Supplier: Crown Scientific

PO Box 101

Burwood Vic 3125

DEPC Diethyl Pyrocarbonate MW=162.1 Cat # D 5758

Sigma Aldrich Pty Ltd

Unit 2, 14 Anella Ave

Castle Hill NSW 2154

DNA Grade Agarose (Molecular Biology Grade) Cat # 200-0011

Progen Industries Ltd

2806 Ipswich Rd

Darra Qld 4076

EcoR1 Restriction Nuclease and Buffer Cat # 101S

Pst1 Restriction Nuclease and NEBuffer 3 Cat # 140S

New England Biolabs

Supplier: Genesearch Pty Ltd

14 Technology Drive

Arundel, Qld 4214

Ethanol Absolute

CSR

Supplier: Yarraville Distillery

PO Box 83

Yarraville Vic 3013

Ethedium Bromide MW=394.3 Cat # E 8751

Sigma Aldrich Pty Ltd

Unit 2, 14 Anella Ave

Castle Hill NSW 2154

Ethylenediaminetetra acetic acid Disodium Salt: Dihydrate MW= 372.2 Cat # E 4884

Sigma Aldrich Pty Ltd

Unit 2, 14 Anella Ave

Castle Hill NSW 2154

Ficoll 400

Amrad Pharmacia Biotech

34 Wadhurst Dve

Boronia Vic 3155

Formaldehyde MW=30.03 37% Solution ACS Reagent Cat # F 1268

Sigma Aldrich Pty Ltd

Unit 2, 14 Anella Ave

Castle Hill NSW 2154

Formamide MW=45.04 Cat # F 7503

Sigma Aldrich Pty Ltd

Unit 2, 14 Anella Ave

Castle Hill NSW 2154

D-Glucose BDH MW=180.16 Cat # 28450

Merck Pty Ltd ACN 005 063 791

Supplier: Crown Scientific

PO Box 101

Burwood Vic 3125

Glycerol BDH General Purpose Reagent Product # 28454

Merck Pty Ltd ACN 005 063 791

Supplier: Crown Scientific

PO Box 101

Burwood Vic 3125

Guanidinium Thiocyanate

High prime DNA Labelling Kit Cat # 1585 584

Promega Corporation Australia

PO Box 168

Annandale, NSW 2038

Isoamyl Alcohol BDH AnalaR Product # 1003832

Merck Pty Ltd ACN 005 063 791

Supplier: Crown Scientific

PO Box 101

Burwood Vic 3125

Isopropanol (2-Propanol) MW=60.10 Cat # I 9516

Sigma Aldrich Pty Ltd

Unit 2, 14 Anella Ave

Castle Hill NSW 2154

IPTG Isopropyl-β-D-Thiogalactopyranosid MW=238.3

Boehringer Mannheim Pty Ltd

353 Whitehorse Rd

Nunawading Vic 3131

Low Melt Agarose Molecular Biology Grade Cat # 200 0030

Progen Industries Ltd

2806 Ipswich Rd

Darra Qld 4076

Lysozyme Cat # 1585657

Boehringer Mannheim Pty Ltd

353 Whitehorse Rd

Nunawading Vic 3131

Magnesium Chloride BDH MW=246.47 Product # 29096

Merck Pty Ltd ACN 005 063 791

Supplier: Crown Scientific

PO Box 101

Burwood Vic 3125

Magnesium Sulphate BDH AnalaR MW=246.47 Product # 10151

Merck Pty Ltd ACN 005 063 791

Supplier: Crown Scientific

PO Box 101

Burwood Vic 3125

2-Mercaptoethanol (2-Hydroxyethylmercaptan; β-mercaptoethanol) MW=78.13

Cat # M 6250

Sigma Aldrich Pty Ltd

Unit 2, 14 Anella Ave

Castle Hill NSW 2154

Methanol HPLC Grade BDH

Merck Pty Ltd ACN 005 063 791

Supplier: Crown Scientific

PO Box 101

Burwood Vic 3125

MOPS (3[N-Morpholino]propane sulphonic acid) MW=231.2 Cat # M 9381

Sigma Aldrich Pty Ltd

Unit 2, 14 Anella Ave

Castle Hill NSW 2154

n-Hexane HPLC Grade (95%) Unichrom

Ajax Chemicals

Supplier:Crown Scientific

PO Box 101

Burwood Vic 3125

Petroleum Ether BDH AnalaR, Product # 10179

Merck Pty Ltd ACN 005 0630791

Supplier: Crown Scientific

PO Box 101

Burwood Vic 3125

Phenol

Supplier: Novachem Pty Ltd

50 Garden St

South Yarra Vic 3141

Polyvinylpirrolidine (Molecular Biology Reagent) MW=360,000

Sigma Aldrich Pty Ltd

Unit 2, 14 Anella Ave

Castle Hill NSW 2154

Potassium Acetate MW=98.24 Cat # P 1147

Sigma Aldrich Pty Ltd

Unit 2, 14 Anella Ave

Castle Hill NSW 2154

Potassium Hydroxide BDH MW=56.11 AnalaR, Product # 10210

Merck Pty Ltd ACN 005 0630791

Supplier: Crown Scientific

PO Box 101

Burwood Vic 3125

Potassium Chloride BDH MW=74.55, Merck (Laboratory Reagent) Product # 29594

Merck Pty Ltd ACN 005 0630791

Supplier: Crown Scientific

PO Box 101

Burwood Vic 3125

Retinol Proprionate

BASF Australia Ltd

6 Australia Ave

Australia Centre,

Homebush NSW 2140

RNAse Ribonuclease I Bovine Pancreas

Amrad Pharmacia Biotech

34 Wadhurst Dve

Boronia Vic 3155

Salmon Sperm DNA 1 ml (10 mg/ml)

Gibco BRL

Life Technologies Pty Ltd

PO Box 4296

Mulgrave Vic 3170

Sephadex G50

Amrad Pharmacia Biotech

34 Wadhurst Dve

Boronia Vic 3155

Sodium Acetate BDH AnalaR MW=82.03 Product # 10236

Merck Pty Ltd ACN 005 0630791

Supplier: Crown Scientific

PO Box 101

Burwood Vic 3125

Sodium Chloride BDH MW=58.44 AnalaR, Product #10241

Merck Pty Ltd ACN 005 0630791

Supplier: Crown Scientific

PO Box 101

Burwood Vic 3125

Sodium Chloride 0.9% Intravenous Infusion Drip Cat # AHB 1324

Baxter

Sodium Hydroxide MW=40.00 Cat # 10236

Ajax Chemicals

Supplier: Crown Scientific

PO Box 101

Burwood Vic 3125

Sodium Lauryl Sulphate (SDS) MW=288.4 Cat # L4509

Sigma Aldrich Pty Ltd

Unit 2, 14 Anella Ave

Castle Hill NSW 2154

Sodium Citrate BDH MW=294.10 AnalaR, Product # 10242

Merck Pty Ltd ACN 005 0630791

Supplier: Crown Scientific

PO Box 101

Burwood Vic 3125

tRNA Cat # 15401 011

Gibco BRL

Life Technologies Pty Ltd

PO Box 4296

Mulgrave Vic 3170

Tetracycline Hydrochloride MW=480.0 Cat # T 3383

Sigma Aldrich Pty Ltd

Unit 2, 14 Anella Ave

Castle Hill NSW 2154

Testosterone Radioimmunoassay kit Cat # DSL 4000

Estradiol Radioimmunoassay kit Cat # DSL C4400

Diagnostic Systems Laboratories

445 Medical Center Bvd

Webster Texas 77598 USA

Trans-β-apo-8-carotenal >99% (TLC) FSE Cat # 73212 (No longer available)

Fluka Biochemika

Unit 2, 14 Anella Ave

Castle Hill NSW 2154

Trans-β-apo-8-carotenal >20% (UV/Vis) Cat # 10829

Fluka Biochemika

Unit 2, 14 Anella Ave

Castle Hill NSW 2154

Trans-β-carotene Type 1: Synthetic MW= 536.9 Cat # 7235-40-7

Sigma Aldrich Pty Ltd

Unit 2, 14 Anella Ave

Castle Hill NSW 2154

Trans-β-carotene

Kodak Fine Chemicals

Tris Cl BDH MW=121.14 AnalaR, Product # 103157P

Merck Pty Ltd ACN 005 0630791

Supplier: Crown Scientific

PO Box 101

Burwood Vic 3125

Tris Cl MW=157.61 Cat # 103130.18

ICN Biochemicals Australasia Pty Ltd

PO Box 187, Unit 12

167 Prospect Highway

Seven Hills NSW 2147

Triton X-100 t-octylphenoxypolyethoxyethanol Cat # X 100
Sigma Aldrich Pty Ltd
Unit 2, 14 Anella Ave
Castle Hill NSW 2154

Trizol Cat # 301 431 8585
Gibco BRL
Life Technologies
PO Box 4296
Mulgrave Vic 3170

Tryptone Code L42 Oxoid Australia, Cat # LP042B
Unipath Ltd, Basingstoke, Hapmshire, England
Supplier: Oxoid Australia Pty Ltd
104 Northern Rd
Western Heidelberg Vic 3081

Yeast Extract Code L21,
Unipath Ltd, Basingstoke, Hampshire, England
Supplier: Oxoid Australia Pty Ltd
104 Northern Rd
Western Heidelberg Vic 3081

X-ray Film X AR 5 (43x35) Cat # 1651512
Integrated Sciences
1401 Burke Rd
East Kew Vic 3102

Appendix 3.
Analysis of variance tables and chi square data.

Plasma concentrations of retinol **12 hours** after treatment with retinol proprionate or vehicle. (Univariate analysis)

Source	df	MS	F	P value
Treatment	1	0.038514	6.800796	0.020660
Error	14	0.005663		

Plasma concentrations of retinol **84 hours** after treatment with retinol proprionate or vehicle. (Univariate analysis)

Source	df	MS	F	P value
Treatment	1	0.083377	4.538658	0.051354
Error	14	0.018370		

Plasma concentrations of retinol after treatment with retinol proprionate or vehicle. (Repeated measures design).

Source	df	MS	F	P value
Treatment	1	0.194274	1.560237	0.232112
Time	18	0.094183	1.528272	0.080416
Treat x time	18	0.049270	0.799490	0.700392
Error	252	0.061627		
	14 (treat)	0.12452 (trt)		

Total liver concentration of retinol after treatment with retinol proprionate or vehice.

Source	df	MS	F	P value
Treatment	1	103867E5	0.077218	0.783956
Slt time	2	103867E5	2.814872	0.083725
Treat x slt	2	122103E4	0.09078	0.990968
Error	20	134511E5		

Plasma concentrations of retinol after treatment with vehicle an in untreated animals over a 24 hour period. (Repeated measures design).

Source	df	MS	F	P value
Treatment	1	0.010965	0.05641	0.820168
Time	47	0.035104	20.01226	0.001
Treat x time	47	0.003130	1.78430	0.002355
Error	282	0.001754		
	6 (Treat)	0.19439 (trt)		

The effect of ovarian state on the concentration of retinol in ovary of sows treated with retinol proprionate or vehicle.

Source	df	MS	F	P value
Treatment	1	0.0000	0.000004	0.998
State	1	0.09329	1.470457	0.2366
Trt x state	1	0.009279	0.146251	0.7054
Error	25	0.063443		

Plasma concentrations of beta carotene after treatment with beta carotene or vehicle. (Repeated measures design).

Source	df	MS	F	P value
Treatment	1	199.5262	12.09616	0.003694
Time	15	13.2738	4.37544	0.001
Treat x time	15	13.2738	4.27544	0.001
Error	210	3.03371		
	14 (Treat)	16.495 (trt)		

The effect of ovarian state on the concentration of retinol in ovary of sows treated with beta carotene or vehicle.

Source	df	MS	F	P value
Treat	1	0.71153	0.970042	0.33229
State	1	0.059972	0.3399	0.564104
Trt x state	1	0.364864	2.06793	0.160445
Error	31	0.176439		

Chapter 7. The effect of retinol on ovarian follicle size and follicular hormone concentrations in the gilt.

Chi square analysis of ovarian follicle size classes in gilts after treatment with retinol palmitate or corn oil. (Texas)

Class	Treated	Control	CPR	chisq	heterogeneity	Fishers P
4-6 mm	4	12	0.2	7.14	7.14	0.0014
>7mm	47	25	5.6	7.14	1.58	0.0014

Chi square analysis of ovarian follicle size classes in gilts after treatment with retinol palmitate or corn oil. (Corowa)

Class	Treated	Control	CPR	chisq	heterogeneity	P value
<4mm	20	48	0.4	12.41	11.53	0.001
4-6 mm	192	161	1.8	8.67	2.72	0.01
>6 mm	38	41	0.9	0.06	0.11	0.06

Concentrations of retinol binding protein in follicular fluid after treatment with retinol palmitate or corn oil.

Source	df	MS	F	P value
Treatment	1	513.8563	9.774677	0.0024
Error	85	52.57016		

Concentrations of oestrogen in ovarian follicular fluid after treatment with retinol palmitate or corn oil (Texas).

Source	df	MS	F	P value
Treatment	1	21067.01	0.258710	0.612357
Error	83	81430.87		

Concentrations of testosterone in ovarian follicular fluid after treatment with retinol palmitate or corn oil (Texas).

Source	df	MS	F	P value
Treatment	1	170137.4	14.95142	0.000215
Error	85	11379.35		

Oestrogen:testosterone ratio in ovarian follicular fluid after treatment with retinol palmitate or corn oil (Texas).

Source	df	MS	F	P value
Treatment	1	19.09968	8.221591	0.005246
Error	83	2.323113		

Ovarian follicle size in gilts after treatment with retinol palmitate or corn oil (Texas).

Source	df	MS	F	P value
Treatment	1	16.4538	9.833398	0.00248
Error	75	1.673257		

Concentration of oestrogen in ovarian follicular fluid after treatment with retinol palmitate or corn oil (Corowa).

Source	df	MS	F	P value
Treatment	1	488946.4	32.28989	0.001
Error	233	15142.4		

Concentration of testosterone in ovarian follicular fluid after treatment with retinol palmitate or corn oil (Corowa).

Source	df	MS	F	P value
Treatment	1	173828.2	13.03209	0.001
Error	242	13338.47		

Oestrogen:testosterone ratio in ovarian follicular fluid after treatment with retinol palmitate or corn oil (Corowa).

Source	df	MS	F	P value
Treatment	1	31.62099	5.625153	0.018
Error	229	5.621356		

Ovarian follicle size in gilts after treatment with retinol palmitate or corn oil (Corowa).

Source	df	MS	F	P value
Treatment	1	0.383778	0.183854	0.668460
Error	244	2.087402		

Total piglets born after treatment of sows with retinol proprionate 6 days before mating, on day of mating, 6 days after mating or treated with vehicle.

Source	df	MS	F	P value
Treatment	3	4.72708	0.699502	0.552730
Parity	4	19.16855	2.836518	0.024090
Treat x parity	12	5.15786	0.763249	0.688578
Error	452	6.757773		

Total piglets born after treatment of sows with a diet supplemented with folic acid, retinol proprionate 6 days before mating in conjunction with the folic acid-supplemented diet or treated with vehicle.

Source	df	MS	F	P value
Treatment	2	7.41486	1.007978	0.365998
Parity	4	15.61452	2.122642	0.077532
Treat x parity	8	5.60294	0.761665	0.636873
Error	355	7.356174		

Percentage of each litter stillborn after treatment of sows with retinol proprionate 6 days before mating, on day of mating, 6 days after mating or treated with vehicle.

Source	df	MS	F	P value
Treatment	3	24.5039	0.233460	0.873060
Parity	4	400.2846	3.813698	0.004627
Treat x parity	12	96.3810	0.918267	0.528327
Error	452	104.9597		

Percentage of each litter stillborn after treatment of sows with a diet supplemented with folic acid, retinol proprionate 6 days before mating in conjunction with the folic acid- supplemented diet or treated with vehicle.

Source	df	MS	F	P value
Treatment	2	225.1008	2.120016	0.12157
Parity	4	994.0560	9.362091	0.00000
Treat x parity	8	128.9587	1.214543	0.289226
Error	355	106.1788		

Number of piglets born alive per litter after treatment of sows with retinol proprionate 6 days before mating, on day of mating, 6 days after mating or treated with vehicle.

Source	df	MS	F	P value
Treatment	3	2.569273	0.392870	0.758195
Parity	4	6.764664	1.034390	0.388982
Treat x parity	12	3.330516	0.509272	0.909007
Error	452	6.539762		

Number of piglets born alive per litter after treatment of sows with a diet supplemented with folic acid, retinol proprionate 6 days before mating in conjunction with the folic acid- supplemented diet or treated with vehicle.

Source	df	MS	F	P value
Treatment	2	1.915754	0.308426	0.734799
Parity	4	5.872541	0.945447	0.437689
Treat x parity	8	4.682029	0.753781	0.643877
Error	355	6.211389		

Percentage of sows farrowing after treatment of sows with retinol proprionate 6 days before mating, on day of mating, 6 days after mating or treated with vehicle.

Source	df	MS	F	P value
Treatment	3	0.137638	1.251243	0.290485
Parity	4	0.077820	0.707454	0.587083
Treat x parity	12	0.189513	1.722831	0.058738
Error	521	0.110001		

Percentage of sows farrowing after treatment of sows with a diet supplemented with folic acid, retinol proprionate 6 days before mating in conjunction with the folic acid- supplemented diet or treated with vehicle.

Source	df	MS	F	P value
Treatment	3	0.137638	1.251243	0.290485
Parity	4	0.077820	0.707454	0.587083
Treat x parity	12	0.189513	1.722831	0.058738
Error	521	0.110001		

Weaning to oestrus intervals in sows after treatment with retinol proprionate 6 days before mating, on day of mating, 6 days after mating or treated with vehicle.

Source	df	MS	F	P value
Treatment	3	31.13681	0.926154	0.427842
Parity	4	72.34910	2.152000	0.073267
Treat x parity	12	21.36955	0.635630	0.812244
Error	521	33.61946		

Weaning to oestrus intervals in sows after treatment of sows with a diet supplemented with folic acid, retinol proprionate 6 days before mating in conjunction with the folic acid- supplemented diet or treated with vehicle.

Source	df	MS	F	P value
Treatment	2	81.22831	2.791208	0.062464
Parity	4	17.34118	0.595886	0.665796
Treat x parity	8	36.83675	1.265803	0.259569
Error	428	29.10149		

Chisquare analysis of litter size distribution after treatment with retinol proprionate at day before weaning, day of mating, 6 days after mating or treatment with vehicle.

Expected counts are printed below observed counts.

Treatment	<7	8-9	10-11	12-13	>13	DNF	Total
Vehicle	6	7	50	36	14	20	133
	8.11	14.26	42.53	37.12	14.01	16.96	
RP-6	3	14	56	41	8	13	135
	8.23	14.47	43.17	37.68	14.22	17.22	
RP0	15	14	31	37	17	15	137
	8.30	14.58	43.49	37.96	14.33	17.35	
RP+6	9	23	36	37	17	15	137
	8.36	14.69	43.81	38.24	14.43	17.47	
Total	33	58	173	151	57	69	541

Chisq = 0.550+3.695+1.312+0.034+0.000+0.544+
3.328+0.015+3.813+0.292+2.723+1.033+
5.418+0.023+3.587+0.024+0.940+0.770+
0.050+4.704+1.392+0.040+0.456+0.350=35.095

df=15, p=0.003

Chisquare analysis of litter size distribution after treatment with a folic acid supplemented diet, the supplemented diet plus retinol proprionate at day before weaning, or treatment with vehicle. Expected counts are printed below observed counts.

Treatment	<7	8-9	10-11	12-13	>13	DNF	Total
Control	6	7	50	36	14	20	133
	7.81	10.21	37.53	34.53	21.02	21.92	
FA	7	11	33	45	25	34	155
	9.10	11.90	43.7	40.24	24.49	25.54	
FA+RP-6	13	16	42	34	31	19	155
	9.10	11.90	43.74	40.24	24.49	25.54	
Total	26	34	125	115	70	73	443

Chisq= 0.418+1.008+4.145+0.063+2.342+0.168+
0.483+0.068+2.635+0.564+0.011+2.801+
1.674+1.416+0.069+0.967+1.729+1.675=22.235

df=10, P=0.015