THE EFFECT OF GRADED DOSES OF HEPTACHLOR- A CHLORINATED HYDROCARBON PESTICIDE - ON THE REPRODUCTIVE PERFORMANCE OF ADULT FEMALE (SPRAGUE DAWLEY) RATS.

BY

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DECLARATION

I declare that the work presented in this thesis is my original work and has not been submitted in this or any other form for a degree in any other University.

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DEDICATION

This work is dedicated to my daughters Jacqueline Atieno and Annette Sarah Awuor. Seeing and being with them makes all my struggles worthwhile.

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ABSTRACT

Heptachlor is a pesticide for foliar, soil and structural (buildings) applications. It is particularly effective against termites. However heptachlor and related chlorinated hydrocarbons pose a special problem because they are not easily biodegradable and therefore affect the ecology of the environment. In addition, being persistent and highly lipophilic, these compounds tend to accumulate in body fat of humans and animals that ingest contaminated food. In the developed countries, USA in particular, the use of heptachlor is now restricted to underground termite control and few other non-food related applications. However, the insecticide is still rampantly used in several developing countries against not only termites but also other pests like the cotton ball weevil and various ants that adversely decrease food and other agricultural production. Notably heptachlor has been reported to cause interstitial fibrosis of the kidney, atrophy of the testes, fibrosis of the heart and polyarteritis, while high accumulation in the ecosystem are through to be responsible for the thinning of eggshell and reproductive failure of certain species of birds.

The effect of heptachlor on human reproductive health has not been adequately studied. Thus, apart from a few reports on its effect on litter size and mortality of neonates in laboratory rats and mice, there is little evidence of research undertaken to alucidate its effect on the mammalian reproductive system. This study was thus designed with the objective of investigating the effect of heptachlor on mammalian reproductive performance using the adult female laboratory rat as a model. To achieve this, adult (3-4 month old) normacyclic female Sprague Dawley rats were injected with corn oil (controls) or with various doses of 5 mg, 20mg, 25mg, 30mg and 40mg/kg body weight heptachlor in corn oil (experimental) every 24 hrs for up to 18 days after which

they were either mated or sacrificed. After the initial experiments, the higher dose treatments (25mg, 30mg and 40 mg/ kg bodyweight) were discontinued due to their lethal effect. For those that were mated, records were kept of their mating success (monitored by presence of sperms in vaginal smears), gestation length and litter size. Blood samples obtained from the sacrificed animals were assayed for progesterone, estradiol and some of the intermediate hormones namely pregnenolone, estrone, andostenedione 17α hdroxyprogesterone and dihydroepiandrosterone, by radioimmunoassay. Ovarian cells were isolated either from heptachlor treated rats or from untreated rats. These cells were incubated in minimum essential medium, MEM (Flow labs), either on their own, in the presence of gonadotrophins (LH and FSH), pregnenolone or androstenedione. Cells from untreated rats were incubated in MEM, either on their own or in the presence of heptachlor. A mixed population of ovarian cells or isolated follicular and luteal cells were used in these experiments. The incubation media were then analysed for the presence of the steroid hormones.

Results showed that heptachlor significantly depressed ($P<0.01$) the body weights of the treated rats, in a dose dependent manner, when these were compared with the controls. There was a significant delay ($P<0.001$) in mating in treated rats compared to controls. In addition mean gestation length was elongated by between 3 to 10 days ($P<0.05$) and mean litter size decreased in treated rats ($P<0.01$) compared to controls. These effects were more pronounced at the higher heptachlor dose of 20mg/kg body weight compared to the lower dose (5mg/kg) and controls. Heptachlor significantly depressed plasma levels of progesterone and estradiol. The degree of depression and the level of significance depended on the dose and ranged from $P<0.05$ at the lower heptachlor dose to $P<0.001$ at the higher heptachlor dose. The same effect was seen in the levels of the intermediate hormones of progesterone and

estradiol synthesis. In vitro production of the various hormones by ovarian cells from heptachlor-treated rats was lower than that of corn oil treated-controls, again the degree of suppression being dose dependent. This effect of heptachlor was demonstrated when the cells were incubated either in the presence or absence of gonadotrophins (LH and FSH) or in the presence or absence of substrate hormones (pregnenolone and androstenedione). Similar results were demonstrated in experiments where cells were taken from untreated rats and incubated with heptachlor. The effect of heptachlor on isolated follicular and luteal cells showed that heptachlor decreased hormone production by luteal cells more than follicular cells, the level of significance ranging from $P<0.05$ at the low heptachlor dose (0.06ng/well) to $P<0.01$ at the high heptachlor dose (0.6ng/well).

It was concluded that heptachlor impacts negatively on several reproductive parameters, for example, mating success, litter size, body weights, cyclicity and hormonal levels in the female Sprague Dawley rat. The negative effects of heptachlor seen in in-vivo studies were corroborated by the results of in-vitro experiments indicating that heptachlor at sub-lethal doses could still act at the cellular level to cause physiological changes. The results show that the effects of heptachlor on the reproductive parameters measured in this study were dose dependent, with more adverse effects being achieved due to the higher levels of the pesticide being administered. The fact that the secretion of several steroid hormones were depressed suggest that heptachlor may be affecting some pathway intermediate/s or activity of one or more enzymes involved in steroid biosynthesis.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

World attention is being focussed on the effect of human activities on the environment as it affects animal, plant life and in particular, human health. Several sectors have been implicated on the question of environmental degradation. One such sector is agriculture and its related industries where a lot of chemicals are used either as fertilizers, pesticides e,g herbicides and insecticides or as additives, preservatives, colourings and flavourings during processing and storage of the agricultural products. The positive effects of pesticides particularly the chlorinated hydrocarbons have been known since the early part of the 20th century when, during the second world war years, many of these pesticides especially dichlorodiphenyl-trichloroethane (DDT) were used liberally to save lives that would otherwise have been lost to insect borne diseases.

A lot of research has gone into evaluating the negative aspects of pesticides including the chlorinated hydrocarbons. Pesticides of various types can be found at all levels of the food chain originating from the environment especially the soil, from where they find their way into the human food chain. Early researchers established that most of these compounds were retained as residues in animal tissues especially the adipose tissue (Wassermann et al., 1972; 1974 a & b), excreted in milk of mammals, deposited in food crops as persistent residues and most disturbing, some of these compounds were capable of producing damage in certain tissues of animals

particularly rodents, when these animals were fed over a prolonged period with extremely low amounts in relation to the acute toxic doses (Radeleff, 1970). Apart from organochlorine pesticides, other compounds that are worth mentioning with regard to environmental pollution are chlorinated biphenyls. These are very persistent industrial contaminants which also readily accumulate in foodstuffs of animal origin (Tuinstra et al., 1981; Mugambi et al., 1989).

The effect of the environment on reproductive health is currently receiving a lot of attention. In 1991 the World Health Organization (WHO) organized, and co-sponsored with the Danish Government and other organizations, an international workshop devoted entirely to the 'Impact of the Environment on Reproductive Health.' From the proceedings of the workshop, published in the 101th. issue of Environmental Health Perspectives (suppliment 2) 1993, it is very apparent that the question of reproductive health as it is affected by the environment, needs to be looked into seriously. Indeed, mankind has introduced elements into the environment which either pollute or modify environmental conditions with potential negative effects on the health of human and other animals in general and reproductive health in particular, thus touching on the very survival of a species.

The concern about reproductive health and the environment is also critical if it is realised that the population of the world is growing, and this in turn may lead to further environmental pollution. A vicious circle is then established in which poverty, poor housing, hygiene and illiteracy interact concurrently with pollution of the environment to perpetuate the increased incidence of reproductive dysfuction to which women and children are particularly vulnerable. Where women form the majority of the agricultural workforce such as in Africa, they come into contact with uncontrolled use of pesticides and other agricultural pollutants which can concentrate in breast milk and have adverse effects on the mother and baby alike (Leke et al., 1993)

Men are not completely spared either as there is evidence suggesting that the concentration of sperms in semen of apparently normal men has been decreasing over the last 50 years (Giwercman et al., 1993). One cannot help linking this with the changes that have taken place in the environment over the same period of 50 years. Similar studies in women to determine potential changes in their reproductive capacity are lacking.

The reproductive system is particularly sensitive to adverse environmental conditions (Negro-Vilar, 1993). Every stage of the multi step process of reproduction can be disrupted by external environmental agents and may lead to increased risk of abortion, birth defects, growth retardation, functional defects and perinatal death. Physical hazards such as irradiation and chemical pollutants have direct effects on reproduction resulting in impaired fertility, high rate of abortions and abnormal pregnancies, (Shalet et al.,1976; Huang 1979; Critchley et al., 1992). Increasing levels of chemicals in breast milk has been documented, (Jensen and Slorach, 1990). Adverse health consequences have also been dramatically demonstrated following disasters such as occurred at the Chernobyl and Bhopal nuclear plants as reported by Kulakov et al. (1993). There is thus growing evidence that chemicals in the environment such as lead, methyl mercury, polychlorinated biphenyls (PCB) as well as alcohol are harzadous to human reproduction and development. Acute alcohol intoxication has been shown to decrease plasma testosterone levels in men by about 25% without affecting LH pulsatility suggesting a direct effect of alcohol on testicular function (Valemaki et al., 1990; Mendelson et al., 1977). Chronic alcoholism also reduces plasma testosterone levels and may also result in azoospermia and abnormal semen (Comhaire et al., 1987). Certain pharmaceuticals such as retinoids, valproic acids and cytostatic drugs, are proven examples of human developmental toxicants (Sullivan, 1993). Some chemicals in the environment, both natural and man made for example, chlorinated hydrocarbons (eg. heptachlor) have estrogenic

activity and there is growing concern that prenatal and postnatal exposure to these chemicals may permanently alter the reproductive system in ways that are inconsistent with normal functions (Dickerson et al., 1992).

It is difficult to assess the effect on human exposure to particular substances because multiple chemicals are frequently involved and these are superimposed on cultural and socioeconomic factors. Animal models therefore are, and will continue to be in the foreseable future, a major tool for predicting human developmental hazards in view of the great difficulties involved in the assessment of the same in humans. Comparisons with human data indicate that animal models are generally predictive of human responses. Indeed based on such studies, some of the chlorinated hydrocarbons namely, dieldrin, methoxychlor, lindane (gamma hexachlorocyclohexane), DDT, 1,1-dichloro-2,2-bis p-chlorophenyl ethylene (DDE) and heptachlor have been shown to have some toxicological effects on reproduction, at least in the rat and mouse (Mestitsova 1967; Odler, 1973; Good and Ware 1969; Virgo and Bellward 1973; 1975; 1977; Trapp *et al*:, 1984; Cummings & Gray, 1987, 1989; Johnson et al.,1988; Sircar & Lahiri, 1989; Uphouse & Williams, 1989).

However, studies done so far on heptachlor, regarding its effects on human health, and mammalian reproduction in particular, are scanty, inconsistent and inconclusive. In the human, although no adverse toxic effects of heptachlor have been reported in workers manufacturing or using heptachlor, the WHO (1984) report cautions that epidemiological studies are insufficient to judge the carcinogenic hazard of heptachlor for human. On the basis of this, WHO, Environmental Health Criteria for heptachlor recommends the following:

i) That figures relating to current production and use of heptachlor be made available.

ii) That more information on human exposure to heptachlor from sources such as breastmilk and application for termite control are required.

iii) That further research is required in order to properly assess the significance for man of the carcinogenic findings in mice.

iv) That continuing epidemiological studies be made on workers who have been exposed to heptachlor in the past.

Organochlorines are classified according to either their use e.g insecticides, nematocides and fungicides, or according to their chemical structure e.g chlorinated ethane, cyclodiene and chlorinated aromatic. Heptachlor, is chlorinated cyclodiene, along with endosulfan and chlordane.

Heptachlor is isolated from technical chlordane. It is a white crystalline solid with a mild camphor odour and was registered in the United States of America (USA) in 1952 as an agricultural and domestic insecticide. Heptachlor is used as a non-systemic stomach and contact insecticide, mainly in the control of termites and soil insects. It can be formulated into a powder for dressing seeds prior to planting, or emulsion for foliar, soil and structural applications against agricultural and household termites and insects. Although its use is now greatly restricted in most industrialised countries because of concerns over its environmental persistence and bio-accumulation potential, it is still finding its way into the developing countries. As a persistent organochlorine pesticide, heptachlor residues are detected in all components of the environment (WHO, 1984; Chuang et al., 1991). In historical use, heptachlor was applied directly to terrestrial systems while air and water were

secondarily contaminated via volatilization and land run-offs respectively. Within each environmental compartment, heptachlor undergoes a variety of metabolic and abiotic transformations (Fendick et al 1990). For instance, it can be transformed by microbiol action to other metabolites and several such metabolites have been found in soil, sludge and water. In biological systems, heptachlor is metabolised to its epoxide making this an important metabolic route and especially since heptachlorepoxide is comparably more toxic and more stable in biological systems than heptachlor. *In vivo* studies indicate that heptachlor epoxide is the predominant metabolite formed as a product of the mixed function oxidase system while 1-hydroxychlordene is the major soil metabolite (Fendick et. al., 1990).

Heptachlor is a fairly stable compound to light and moisture and it is not readily dehydrochlorinated (WHO, 1984). Volatilization is therefore the major mechanism of transport of topically applied heptachlor. Heptachlor is virtually insoluble in water but is lipid soluble. It is therefore not likely to penetrate into groundwater but contamination of surface water and sludge can occur. Maximal terrestrial residues coincide with temporal and spatial proximity to application. Peak residues are found in aquatic systems and these correlate with periods of maximum run off. The lipophilic nature of both heptachlor and heptachlor epoxide means that there is a significant bioaccumulation of the chemical in all lipid-type compartments in the environment (WHO, 1984; Chuang et al., 1991)

Exposure of the general public to heptachlor is thus mainly through residues in food. In countries like USA where the use of heptachlor for agriculture has been banned, these residues have decreased considerably over the years and exposures are generally far below the advised acceptable daily intake (WHO,1984). In areas where heptachlor is used, there may be some additional intake from volatilisation of sprayed heptachlor and from well-water. A significant source of heptachlor for infants is breast milk of exposed mothers in which the levels of heptachlor can be

considerably higher than those in dairy milk (Smith, 1982 a&b; WHO,1984; Chuang et al.,1991). In certain occupational exposures, heptachlor has been known to have exceeded the maximum allowable concentration (MAC).

Heptachlor has been reported as being one of the more toxic of the organochlorine pesticides (Radeleff, 1970). Hodge and Sterner (1956) however, classified the acute toxicity of heptachlor as moderate (acute oral LD_{50} for the rat is from 40 - 162 mg/kg body weight). WHO (1984) also classified the technical heptachlor (72-74% heptachlor) as moderately hazardous. Toxic symptoms are related to hyperexcitability of the central nervous system and include tremors and convulsions. Death will follow respiratory failure. At non-lethal acute exposures, heptachlor is hepatotoxic and in cases of prolonged exposures, proliferation of the smooth endoplasmic reticulum and induction of the mixed function oxidases in liver cells is one of the earliest indications. At high exposure levels, heptachlor can interfere with reproduction and the viability of offspring. In the latter cases cataracts were also observed in both parents and progeny in the rat (Mestitsova, 1987). Studies done so far have shown heptachlor to be toxic for aquatic life although its toxicity here is highly species variable. Marine crustacea and younger life stages of both fish and invertebrates are most sensitive (Eisler, 1969; WHO, 1984).

Studies have so far not shown indications of teratogenicity in rats, rabbits, chickens, and beagle dogs but there is evidence that both heptachlor and heptachlor epoxide are carcinogenic for mice and rats (WHO, 1984; Chuang et al., 1991)). Carcinogenicity studies of heptachlor and chlordane have been performed mostly on experimental animals. Whether heptachlor and chlordane also cause cancer in humans remains to be determined (Infante and Freeman, 1987). The information available on its toxicity for terrestrial species is thus insufficient. Radeleff (1970) has reported that heptachlor is capable of killing calves at oral doses as low as 2.5 mg/kg body weight given daily for 15 days. The toxic effects of heptachlor are not specific

for any one organ system, and although indications are that the liver and the central nervous system are the most significantly affected by heptachlor, its effects can also be seen in the reproductive, hematopoietic, immune and renal systems (Fendick et. al. 1990).

Considering that heptachlor is still being used in Africa, Asia and other developing countries against soil and plant insects, for example in the control of cotton ball weevil, termite control, and as seed dressing, (IARC, 1979; Peirano, 1980; WHO,1984), it is imperative that more studies be done on this compound to properly evaluate its toxic effects on human and livestock. Even in the USA where its use is now restricted to underground termite control and a few other nonfood applications, the compound still continue to enter that country's rivers and lakes as pollutants from other countries. (Chuang et al., 1991). Similar situation could be existing in Europe.

It is because of the scanty information on heptachlor that is currently available that this project was designed to study its possible effects on the mammalian reproductive system using the laboratory rat as a model.

1.1.1 Aim of the Study

The aim of this study was to look at the effects of graded doses of injectable heptachlor on the reproductive performance of the female rat. The study was carried out under the following specific objectives:-

1: To investigate the effect of heptachlor on body weight, ovulation, mating success, gestation period, and litter size.

2: To investigate the effect of heptachlor on plasma levels of estrogens and progesterone, during the estrous cycle.

3: To investigate by use of *in vitro* studies, the effect of heptachlor on steroidogenesis and steroid metabolism.

1.2 Literature Review

1.2.1 Environmental Exposures of Pesticides, with specific reference to Heptachlor

Organochlorine pesticides including heptachlor and related industrial waste compounds for example, polychlorinated biphenyls (PCB) are present in the environmental air, water, soil and food.

Air

Volatilization is the major mechanism of transport of topically applied heptachlor. In one study, (Taylor et al., 1976), 90% of heptachlor was volatilized from bare moist soil in 2 to 3 days following application. In another study, Peirano (1980) found that fields treated with technical heptachlor at 2.24 kg/ha gave rise to air concentrations around the field as high as as 244 ng/m^3, immediately following application. After three weeks, the concentrations still remained as high as 15.4 ng/m^3. Organochlorine insecticides have been used extensively for the past 35 years to reduce termite damage in dwelling places. The United States Environmental Protection Agency (USEPA) for instance estimates that chlordane and heptachlor have been used in 24 million homes in the United States (USA) (Savage, 1989). The pollution of air inside dwelling places is a growing concern in the USA because the population at risk includes the young and the aged. Such people are exposed to pollutants for up to 24 hours a day in the home as opposed to 8 hours a day for those exposed at the work place. As reported by Savage (1989), chlordane and heptachlor have been widely used as termicides and both have been implicated in causing serious health problems. Epstein and Ozonoff (1987) for instance reported 25 new cases of blood dyscrasia, including leukemia and thrombocytopenic purpura

following home termite treatment with the pesticides chlordane and heptachlor (C/H). The 25 cases according to Epstein and Ozonoff were consistent with 34 previously published cases which reported association of blood dyscrasia with C/H exposure. Further more, the newly reported leukemias were consistent with epidemiologic evidence of excess risk of leukemia and other cancers in C/H exposed populations and with the carcinogenic action of C/H in animals. The agricultural uses of heptachlor were phased out in the USA in 1978, largely on the grounds of 'emminent hazard' because of carcinogenesis, (Savage, 1989). However, Epstein and Ozonoff (1987) noted that, until the voluntary halt in production in July 1987, millions of homes in the USA had been treated with chlordane and heptachlor for the control of termites.

Water

Regarding heptachlor, the compound is quickly hydrolysed in water to form 1-hydroxychlordene, and this seems to be the major degradation pathway in moist soils. It has been shown that heptachlor epoxide is also metabolised to 1-hydroxychlordene (Harris & Miles, 1975).

Although heptachlor is not often found in surface waters it has nevertheless been detected at levels of 5-30 ng/litre in Canadian waters (IARC, 1974; Harris and Miles, 1975). According to the World Health Organization's Environmental Health Criteria No.38 on heptachlor, surveys carried out between 1967 and 1974 indicate that heptachlor and heptachlor epoxide were observed in chemical sewage sludges in Ontario Canada, in the water and sediment in the upper Great Lakes, in the major river basins, lakes, sediments and plant effluents in the USA and also in 18 different locations in Europe. The results of a survey conducted in the USA in the period 1958-1965 showed that heptachlor was present in 17% of the samples of drinking water studied. Sandhu et al. (1978) reported from similar surveys that heptachlor and

heptachlor epoxide were found in portable water supplies in rural areas of South Carolina in 45.5 and 63.6% of samples tested, with the range of residues varying from undetected to 44 ng/litre for heptachlor and from undetected to 87 ng/litre for heptachlor epoxide. Similar surveys have shown the presence of heptachlor residues in tap water from various places for example, Ottawa and Nova Scotia in Canada, and The Hague in the Netherlands (WHO, 1984). It is very likely that if similar studies were to be carried out in other parts of the world, particularly in the developing countries, heptachlor and its epoxide will be detected, among other pesticides, in the waters. Indeed Abdalla et al. (1990) performing surveillance studies on fish from several provinces referred to as governorates in Egypt, came up with results that indicated the presence of several pesticides in the surrounding waters. Analysing fish samples from Damietta govenorate for organochlorine pesticides, they found that beta-benzene hexachloride (BHC) and lindane were predominantly present at maximum levels of 435.30 and 59.00 µg/kg body weight respectively. Aldrin and o,p'-DDT were present at 34.27 and 734.10 µg/kg body weight respectively for samples from the Red Sea governorate while gamma-chlordane, p,p'-DDE and p,p'-DDT were present in samples from Ismailia governorate at 36.17, 234.4 and 57.19 µg/kg body weight respectively. Heptachlor was detected at 8.50µg/kg body weight in fish from Port Said whereas o,p'-DDE at 10.59 µg/kg were in fish samples from Suez governorate.

Soil

The half life of heptachlor in soil ranges between 9 months to 2 years when used at recommended agricultural rates. However, Nash & Harris (1973) reported the presence of the pesticide in the soil 16 years after the initial application. Although available data suggest that soil dissipation of heptachlor may be more rapid in tropical than temperate regions (Kathpal et al., 1983), it is evident that it has a

relatively long half life. In the soil, it is 1-hydroxychlordene which is once again the major residue while only small amounts of heptachlor epoxide and the hydroxy epoxide could be found (Harris & Miles, 1975). A survey conducted on crop soils in 37 states of the USA in 1971 revealed 1-hydroxychlordene residues in 4.9% of samples, while heptachlor epoxide was detected in 6.9% of samples (Carey et al., 1978).

Pesticides in food

Market basket surveys carried out by Johnson and Manske (1976) from 1972-73 in the USA showed maximum values for heptachlor epoxide ranging from trace to 2 mg/kg while Abbott et al. (1969), carrying out similar studies in the United Kingdom found levels in the total diet to be generally less than 0.5 mg/kg.

Within the framework of the joint FAO/WHO Food Contamination Monitoring Programme, the levels of heptachlor and heptachlor epoxide residues in various food items sampled in 1980-82 have been reported from Austria, Canada, Denmark, Guatemala, Japan, the Netherlands, and the USA. The median levels of the two compounds in the fat fraction of milk products ranged from 0 (not detected) in butter and cattle fat in Denmark to 13 ug/litre in cow's milk in Japan. Maximum levels of heptachlor and heptachlor epoxide found in milk and milk products in Ireland in 1971-72 were 62 and 21 ug/kg fat respectively (Downey et al.,1975). The presence of chlorinated hydrocarbon insecticide residues has also been reported in bovine milk (Steffey et al. 1984) and meat (Petterson et al., 1988). These workers found that while the levels of most compounds namely aldrin/dieldrin, (BHC) and DDT, decreased over the ten year study period (1972-1981), the levels of chlordane and heptachlor epoxide residues in milk did not decrease, confirming the persistent nature of these compounds.

In other food items the median levels ranged from 0 (not detected) in hen's eggs in Denmark to 4 ug/kg in fresh onions in Guatemala (WHO,1984). Henderson et al. (1969) demonstrated the presence of heptachlor and heptachlor epoxide in 32% of the 590 game fish samples analysed between 1967-68 in the USA. The concentration ranged from 0.01 to 8.33 mg/kg, while Misra et al. (1977) showed that potatoes grown in soils treated with heptachlor dust at 1.5 kg/ha were found to contain residues of heptachlor epoxide up to 151 days following application. Processing of the potatoes failed to reduce the heptachlor and heptachlor epoxide content below the maximum residue limit (0.1 mg/kg). All these studies serve to show the widespread distribution of pesticides and heptachlor (including heptachlor residues) in particular in the various food sources available to man.

Harradine and McDougall (1986) working in Australia, found heptachlor epoxide residues in meat that exceeded the maximum residue limit of 0.2 mg /kg, in fat of cattle grazed on land previously treated with heptachlor prior to planting potatoes or maize. The fields or paddocks had been treated 2 years previously with heptachlor at 1.1 kg/ha. Corrigan and Seneviratna (1990) on behalf of the Australian Quarantine and Inspection Service identified a number of pesticide residues in the Australian meat product exported to the USA in May 1987, despite several quality control procedures used by Australia to ensure the wholesomeness of the export meat. The pesticides involved were dieldrin and heptachlor. They attributed their findings to the persistence of organochlorines in soils, their illicit use or contamination of storage facilities. The other sources could have been animals grazing contaminated pastures, ingesting contaminated feed or held in contaminated yards over a period. All these sources of exposure led to bioaccumulation of the residues in the animals' adipose tissues which eventually exceeded maximum residue limit.

In a pilot project initiated to test an existing animal disease surveillance system for use in monitoring environmental pollutants and using bovine, serum samples from 53 Colorado beef ranches were analysed by Salman et al. (1990). They showed that 51% of the samples tested contained detectable amounts of 1 or more chlorinated hydrocarbon. Heptachlor, heptachlor epoxide, lindane, and oxychlordane were the insecticides detected most frequently in the bovine sera.

Wild birds are not, as a rule, a major source of food for humans although this position is changing in view of the growing interest in ostrich eggs and meat as sources of protein. Studies have shown that wild birds are also exposed to pesticides. Their exposure and consequent effects of such exposure emphasizes the danger to which poultry are exposed with regard to the same pesticides. Indeed Mugambi et al. (1989) found residues of several pesticides in eggs of domestic fowl (*Gallus domesticus*) from two locations Embu and Meru in Central Kenya. The pesticides most frequently found were DDT and its metabolites, dieldrin, lindane, HCH, endrin and aldrin.

The effects of heptachlor seed treatment on birds in the vicinity of the Umatilla National Wildlife Refuge, in Oregon and Washington states, were investigated from 1978-1981 (Blus et al., 1985). Heptachlor epoxide residues were detected in 35 out of 60 eggs from 60 different nests (one egg from each nest). The levels of the compound were particularly high (8-13 ug/g) in a few eggs of the black billed magpie, mallard and ringnecked pheasant. Blus et al. (1985) further reported that these residues were within the range that induced reproductive problems in other species in the area. Furthemore, diagnostically lethal residue levels (greater than or equal to 9 mg/g) of heptachlor epoxide were detected in the brains of nine birds (four species). Most of the avifauna in the area, according to Blus et al. (1985), contained residues of heptachlor epoxide and related compounds. On the other hand, lindane,

the replacement chemical for heptachlor, did not produce adverse effects in birds, and residues were not detected in either their eggs or brains.

Humans and other non-human species are thus exposed to heptachlor and other pesticides. Humans are exposed either by direct contact in the work places (occupational exposure), or by eating contaminated food or by handling for example, dressed seeds.

1.2.2 Pesticides in mammalian body tissues

The presense and or effects of pesticides in various animal body compartments including the reproductive system is well documented.

Presence in Serum

Mixtures of halogenated hydrocarbon insecticides and polychlorinated biphenyls (PCB) are routinely detected as residues in human adipose tissues, serum and milk. A survey of measured levels of selected organochlorine pesticide residues in human sera by Stehr-Green et al. (1988) indicated that levels increased with age with males having slightly higher levels than females. Consumption of eggs from home-raised hens contributed substantially to increased serum concentrations of trans-nonachlor, heptachlor epoxide and oxychlordane. Consumption of home grown root vegetables likewise contributed to increased serum concentrations of trans-nonachlor and oxychlordane.

Presence in adipose tissue

Halogenated organic compounds are highly lipophilic chemicals. Chronic exposure to the general population as already indicated, results mainly through the

food chain. Once in the body, these compounds tend to concentrate in body fat tissue.

Adeshina and Todd (1990) made a preliminary study of the occurrence and concentration of organochlorine compounds namely BHC, o,p'-DDE, p,p'-DDE, o,p'-DDT, p,p'-DDT, dieldrin, oxychlordane and heptachlor epoxide in the adipose tissues of residents of North Texas. These were persons who had no known occupational exposure to organochlorine pesticides, and yet the findings indicated a greater than 97% occurrence for each compound with the exception o,p'-DDE and o,p'-DDT each of which occurred in 54% of the population sampled. These results, according to the authors, indicate that for the banned compounds eg. DDT and its analogues, there is a decline in environmental exposure. However for oxychlordane and heptachlor epoxide whose uses are currently restricted but not proscribed the occurrence is still high.

Pesticides in Milk

Pesticide residues in human milk were first reported by Laug et al. (1951) who found that milk from normal healthy American women contained considerable amounts of the organochlorine insecticide DDT. Since then, DDT and other related organochlorine pesticides have been found in human milk throughout the world (Jensen, 1983; Slorach and Vaz, 1983). In Kenya, work by Kanja et al. (1986) demonstrated relatively high levels of DDT and its metabolite DDE in all the milk samples that were analysed although with great regional differences. Contamination of milk is not confined to human breast milk as the presence of chlorinated hydrocarbon insecticide residues in bovine milk was reported by Steffey et al. (1984). This study which was done over a period of ten years (1972-1981) indicated that, while the amount and levels of most compounds (aldrin, dieldrin, BHC and DDT) decreased over the ten year study period, the levels of chlordane and heptachlor

epoxide residues in milk did not decrease, suggesting their persistence. No report was found on contamination of milk from goats or camels.

Dogheim et al. (1991) assessed the contamination of human breast milk with organochlorine pesticide and PCB residues by monitoring these compounds in Egyptian food. They found that DDT complex was the most frequently found pesticide, followed by total hexachlorocyclohexane isomers. Heptachlor and heptachlor epoxide, dieldrin, hexachlorobenzene (HCB) and oxychlordane were also found although less frequently. Estimated dietary intakes (EDIs) of these contaminants by the breast-fed infants were compared to acceptable daily intakes (ADIs). EDIs of DDT complex, lindane (gamma-HCH), heptachlor and heptachlor epoxide and oxychlordane were below ADIs. Dieldrin EDI however exceeded the acceptable daily intake.

Milk is rich in fat and could therefore carry high concentrations of lipophilic pesticides (De Campos and Olszyna-Marzys, 1979) and thereby contribute significantly towards the daily intake of the pesticides by neonates. Studies by Siddiqui et al. (1981) do indeed show that the daily intake of pesticides through breast milk may be higher than that recommended by WHO.

1.2.2.1 Placental transfer of pesticides

Exposure to pesticides is not limited to neonates via breast milk as foetuses are also exposed to these compounds while *in utero*. The placenta serves as a barrier between mother and foetus for a large number of exogenous and endogenous substances. It contains considerable concentrations of fat, making it possible for the organ to function as a carrier for lipophilic xenobiotics including organochlorine pesticides (Rapport and Hall, 1968).

The transfer of organochlorine pesticides from mother to the developing foetus through the placenta has been described by Saxena et al. (1981) and Siddiqui and Saxena (1985). Their findings showed that the transfer was influenced by age and dietary habit of the mother. Siddiqui and Saxena (1985) reported that non-vegetarian mothers excreted relatively higher amounts of chlorinated pesticides compared to their vegetarian counterparts. They also showed that older mothers had a greater excretion of the pesticide through the placenta at the time of delivery than younger mothers. This suggests an accumulation of the pesticides within the body over the years.

In view of the role played by the placenta in the transfer of pesticides to the developing foetus, the evaluation of the importance of transplacental passage of chemical substances and their effect on the embryo or foetus is of special relevance. The passage of chemical substances (endogenous or exogenous) through the placenta depends on several factors. Among the most relevant of these are physical and chemical properties of the substances, their haemodynamics and pharmacokinetics in the mothers systems, placenta and embryonal circulation. These biological processes are time dependent and therefore the 'embryonal dose' depends not only on the exposure of the embryo but also on the level of the chemical substance in the mother's blood (Rosival et al., 1983).

Mammalian foetuses and newborns are thus being exposed to considerable quantities of pesticides and other toxic substances via the placenta and breast milk respectively (Eckenhausen, 1981). The possible effects of these substances on the outcome of pregnancy and child health are still matters for research.

1.2.2.2 Pesticides in ovaries and follicular fluid

The discovery of compounds with potential reproductive toxicity for example pesticides, in follicular fluid (Trapp et al., 1984) is of particular importance and concern. This is because the oocyte completes first maturation division before ovulation within the follicular fluid. Trapp et al.(1984) demonstrated the presence of considerable amounts of DDT in human follicular fluid. Considering that several investigations, based on animal models have shown that pesticides, especially hexachlorocyclohexane (HCH), dieldrin and DDT, have detrimental effects on plasma membranes of oocytes (Mattison, 1983), it is tempting to propose that oocytes could be damaged by the presence of such compounds, particularly during resumption of meiosis. This could in turn interfere with fertility. Preliminary observations by Trapp et al. (1984) indeed indicated a decreased cleavage rate of embryos from follicles with elevated pollutant concentrations. Since in their investigations, they used human follicular fluid and eggs, it is easy to suspect a possible disturbance of human reproductive performance as a consequence of these pollutants.

Hexachlorobenzene (HCB) is a persistent chlorinated organic compound. It has been detected in many tissues from a variety of species including ovary and ovarian follicular fluid (Jarrell et al., 1993a). The latter authors have demonstrated that HCB when administered in high doses causes destruction of ovarian primordial germ cells in association with systemic toxicity in non-human primates. Furthermore, HCB accumulated in a dose related manner in serum and other tissues. However, the accumulation did not cause any changes in the serum estradiol response to human menopausal gonadotrophin, oocyte maturation and fertilization in vitro, or early embryo cleavage rate. Jarrell et al. (1993a) suggested that since there were no changes in urinary porphyrin excretion, the mechanism of HCB ovotoxicity may be distinct from HCB induced cytochrome P450 dependent inhibition of

uroporphobilinogen decaboxylase in the liver, although such intraovarian metabolism cannot be excluded.

Other studies by Jarrell et al. (1993b), included looking at the extent of contamination of ovarian follicular fluid and serum samples by chlorinated organic compounds in women. Using samples from women undergoing *in-vitro* fertilization, their results indicated that five chlorinated organic compounds were frequently found in the two types of samples, these were alpha chlordane (ALCH), DDE, heptachlor epoxide, oxychlordane (OXCH), HCB, and PCB. The levels of these chemicals were however generally low, and did not seem to have any adverse biological effect on the rate of fertilization and the time of cleavage.

1.2.2.3 Presence of pesticides in other tissues of reproductive interest

Szymczynski and Waliszewski (1983) analysed testicular (man) biopsy material for residues of chlorinated pesticides namely HCB, (alpha, beta, gamma, and delta); epsilon-HCH isomers, heptachlor epoxide, DDE and DDT isomers. They reported that alpha and delta-HCH, and p,p'-DDE were found in nearly all samples analysed. The other compounds were also detected although in lower percentages, whereas o,p'-DDT and p,p'-DDT were not. However, the effect of these pesticides on testicular primordial germ cells were not reported.

Studies involving whole-body autoradiography of ^{14}C-labelled 3-methylsulphonyl-DDE (3- MeSO2-DDE) in female C57 BL mice (Lund et al.,1988), revealed a heavy accumulation of the pesticide in the adrenal cortex. Fairly high radioactivity appeared in the nasal mucosa and fat, while the labelling of the liver was intermediate. The tissue bound adrenal radioactivity was confined to the zona fasciculata leaving the other adrenal zones (namely zona glomerulosa and zona reticularis) devoid of bound material. Histopathological examination of the adrenals

revealed extensive vacuolation and necrosis of the zona fasciculata 1-12 days after single doses of upto 25 mg/kg. Degenerative changes were observed at 12.5 mg/kg. From these and other investigations, Lund et al. (1988) concluded that 3-MeSO2-DDE is a persistent environmental pollutant with unique ability to produce acute toxicity subsequent to metabolic activation in the adrenals. Considering that the adrenal cortex and zona fasciculata in particular is the region of adrenal cortico steroid synthesis, it is possible that a similar effect could occur in the other steroidogenic tissues namely the testes and the ovaries.

1.2.2.4 Relative toxicity of organochlorines

The dose related toxic effects of several of the organochlorine compounds on livestock have been studied. Dieldrin has been described as having a fairly high toxicity for domestic livestock (Radeleff, 1970). When used as a spray or dip, young dairy calves were poisoned by concentrations of 0.25% and over, lambs by 3%, cattle by 2% and sheep and goats by 4%. When administered orally, dieldrin produced poisoning in 2 week old (and less) dairy calves at 10 mg/kg, young pigs at 50 mg/kg and sheep and horses at 25 mg/kg (Radeleff, 1970). Dieldrin as a 5% solution in peanut oil can be injected subcutaneously. When this was done 6 times at a dosage of 25 mg/kg, each dose separated by four weeks, no poisoning was observed in cattle but at 50 mg/kg symptoms of poisoning were observed, including abnormal posture and spastic gait, continuous chewing, followed by convulsions, coma and death.

Methoxychlor is about one half as toxic as DDT and therefore the probability of its poisoning is rather remote. According to Radeleff (1970), water sprays containing 8% of methoxychlor were non-toxic for young calves and lambs. Concentrations of 1.5% applied every four days for 8 applications failed to produce poisoning in cattle, sheep, goats, hogs or horses. When administered orally doses of

500 or 1000 mg/kg produced intoxication in dairy calves but not death. Sheep were not poisoned by single oral doses of 1000 mg/kg.

Studies of the toxicity of hexachlorocyclohexane, commonly called benzene hexachloride (BHC), for various mammalian species have shown that its acute toxic effects are due to the gamma isomer i.e. lindane (gamma HCH), (Radeleff, 1970). Sprays or dips containing as little as 0.05% of the gamma isomer was found to kill dairy calves under 2 weeks of age whereas adult cattle were killed by 0.3%. Lambs three weeks of age were poisoned by 0.15%, young goats by 2 applications of 0.15% at four day intervals and sheep, hog and horses by 0.15% every four days for 8 applications. Emaciated animals were found to be more susceptible. For example emaciated ewes in lactation were found to be extremely susceptible to dips containing as little as 0.03% of lindane. When administered orally, lindane at 5 mg/kg was lethal for dairy calves under 2 weeks. Adult cattle were killed with doses of 25 mg/kg and higher. Sheep were slightly less susceptible than cattle being killed at doses of 50 mg/kg or more.

Gamma hexachlorocyclohexane (DDT) is not acutely toxic for most higher animals except at very high doses. Radeleff (1970) reported that although DDT was readily absorbed from all surfaces of the body, it was not possible to produce poisoning in young dairy calves, cattle, sheep, goats, hogs or horses with concentrations likely to be used, even through error. Young calves and lambs were sprayed with 8% suspensions of DDT without being poisoned. When used orally, the minimum toxic dose of DDT was 250 mg/kg for 2 week old dairy calves and 500 to 2,000 mg/kg for mature cattle. Minimum toxic dose for sheep was less than 500 mg/kg.

Heptachlor is one of the most toxic of the chlorinated hydrocarbons. The minimum toxic single oral dose was found to be between 15 and 25 mg/kg for dairy calves under 2 weeks old and between 25 and 50 mg/kg for sheep (Radeleff, 1970). When applied as a spray the minimum toxic concentrations for young calves was

between 0.25 and 0.5%. When administered orally, heptachlor appeared to have cumulative effects. Calves given 10 mg/kg daily dosage were affected by the second dose and died after the third. A calf given 5 mg/kg daily for 6 days and another given 2.5 mg/kg daily for fifteen days exhibited pronounced symptoms of poisoning and died a few hours after receiving their last respective dose. Poisoning was produced in young dairy calves with a single oral dose of 2.5 mg/kg while repeated daily doses at 1 mg/kg produced symptoms of poisoning in calves after 15 doses (Radeleff, 1970).

1.2.3 Normal Mammalian Female Reproductive Physiology: Possible target sites for reproductive aberrations by toxins

1.2.3.1 Reproductive organs

In the female, the success of the reproductive system is essentially dependent on the dual function of the ovary, namely, oogenesis (growth and development of the oocyte) and steroidogenesis (production of steroid hormones). These ovarian processes ensure the delivery of viable ova for fertilization and play a key role in establishing the appropriate environment for maintenance and development of the fertilised ovum. Throughout the menstrual/estrous cycle, the maintenance of normal ovarian morphologic and functional integrity is inextricably linked to the proper operation or function of the hypothalamo-pituitary-ovarian axis. Consequently, any agent interfering with the distribution of appropriate information along this neuroendocrine axis can disrupt, either directly or indirectly, ovarian function.

The basic functional unit of reproduction within the ovary is the follicle. A follicle is essentially composed of several key cellular constituents namely, the oocyte (germ cell), granulosa cells, forming layers around the oocyte, and the theca cells that

encircle the outermost layer of granulosa cells but are separated from them by a basement membrane.

In response to luteinizing hormone (LH), the selected or Graafian follicle raptures during ovulation, releasing the ovum into the fallopian tube. The remaining unselected follicles within the developing cohort undergo atresia (Greenwald and Terranova, 1988). Following ovulation, the remaining cellular components of the ruptured follicle, primarily the granulosa and theca cells, involute, establish increased vascularity and are transformed by the process of luteinization into progesterone secreting luteal cells. These luteal cells thus form a transient endocrine structure known as the corpus luteum in the place of the collapsed follicle.

Efforts to delineate the effects of chemical toxicants on the function of the ovarian follicle have met with numerous difficulties (Garospe and Reinhard, 1995). They attribute these difficulties to the constantly changing macro- and microenvironment of the cycling ovary, the morphological changes that the follicle undergoes during its maturation as well as biochemical alterations that take place in the follicle, during maturation. Such changes include steroid responsiveness and production, enzyme activation, sensitivity to gonadotrophins, and expression of various receptors (Richards et al., 1987). Alteration in any one or a combination of of these variables by chemical toxicants can result in impaired or complete reproductive failure. This possibility is further complicated by the fact that within the adult ovary, populations of follicles at different stages of development exist side by side, thereby allowing for differential follicular toxicity. Thus, reproductive outcome following exposure to a toxicant would be dependent on the specific type of follicle affected. For example, a toxic substance that affects Graafian follicles would result in immediate reproductive failure due to absence of viable oocytes. However, when the toxic agent is removed or metabolically cleared, fertility would be restored as early as the next cycle with the development of a new Graafian follicle.

Under the influence of follicle stimulating (FSH), granulosa cells which bear receptors for pituitary gonadotrophins, produce estrogen. The estrogen in turn maintains mitotic support and promotes further differentiation of the granulosa cell itself. Estrogen is also necessary for the increase of crucial enzyme activities (ie. cholesterol side chain cleavage enzyme, aromatase, and prostaglandin synthetase), induce cellular expression of FSH and LH cell surface receptors, triger the onset of the preovulatory surge of LH and prevent premature biochemical luteinization. Therefore the development and differentiation of the granulosa cells are essential for the follicle to deliver a viable oocyte for fertilization. Following ovulation, granulosa cells are transformed into luteal cells secreting progesterone which is responsible for the growth and development of the endometrium in preparation for implantation of the blastocyst. Any agent therefore that disrupts the proper proliferation, differentiation and function of the granulosa cell, imposes a significant hazard to normal reproductive processes (Garospe and Reinhard, 1995). This has also been demonstrated by Treinen and Heindel (1992), when they showed that mono-(2 ethylhexyl) phthalate, the active metabolite of the reproductive toxin di-(ethylhexyl) phthalate, significantly inhibits granulosa cell function by suppressing FSH-stimulated cAMP accumulation.

In response to LH, theca cells convert progesterone to androstenedione through the enzyme 17α hydroxylase and C17-20 lyase present in the cell (Richards, 1980). It should be emphasised that according to Miller, 1988 the enzymes 17α-hydroxylase and C-17-20 lyase are the same enzyme, both enzymes being referred to as P450c17. Since granulosa cells lack these enzymes, they instead use the inherent FSH stimulated aromatase enzyme to convert the androgen substrate provided by the theca cells into estradiol (Richards, 1980).

There is thus a close and crucial interdependence between granulosa and theca cells. Indeed without the androgen substrate derived from the theca cells,

granulosa cells cannot produce estrogen in sufficient amounts to maintain viable follicles. It is therefore reasonable to postulate that any xenobiotic agent that prevents sythesis of androgens by the theca cells will result in demise of the growing follicle mostly through atresia.

As for the oocytes, any agent that impairs cell division, prevents cell migration, or directly damages oocytes *in utero*, leads to decreased fertility by reducing the population of quiescent oocytes from which crops of follicles will ultimately be recruited for growth and development. Unfortunately, very little is known about the direct effects of chemical toxins on germ cells. There is indication that the germ cells may have intrinsic abilities to metabolically transform toxic chemicals into reactive intermediates that either kill the cell immediately or exert more subtle damages through the induction of viable mutations (Garospe and Reinhard,1995). Nevertheless, several classes of xenobiotic agents have been shown to exert oocyte toxicity. For example, the polycyclic aromatic hydrocarbons (PAHs) can directly destroy resting primordial follicles once they have been processed into reactive intermediates by enzymes in the ovary (Mattison et al., 1983b). In addition PAHs can cause ovarian tumors or induce chromosomal aberrations in oocyte meiosis (Haney, 1985). Another class of compounds that can adversely affect the oocyte and follicular development is the antineoplastic agents. These substances according to Dobson and Felton (1983) have a demonstrated ability to destroy developing follicles as well as mutate preovulatory follicles.

The corpus luteum formed by the luteinization of granulosa and theca cells following ovulation, has the primary function of sythesizing progesterone in preparation for implantation of a fertilised ovum and for the subsequent development of the placenta and foetus (Rao and Gibori, 1987). The majority of the parenchyma of the corpus luteum consists of steroidogenic cells- the luteal cells. Support cells account for approximately 20% of the volume and include vascular elements

(endothelial cells and pericytes) as well as macrophages, smooth muscle cells and fibroblasts (Farin et al., 1986). Two morphologically and biochemically distinct steroidogenic luteal cell types have been identified in several species including ewe (Fitz et al., 1982), cow and pig (Lemon and Loir 1977), rat (Nelson et al., 1992), ovine (Hoyer and Niswender, 1986), rabbit (Hoyer et al ,1986), monkey (Hild-Petito et al., 1989) and human (Ohara et al., 1987). The most obvious difference between the two steroidogenic cell types is size, leading to their designation as large and small luteal cells.

The principal hormone that stimulates progesterone production by the corpus luteum is LH. There is evidence that unstimulated large luteal cells secrete progesterone at a higher rate (2-40 fold) than small luteal cells (Fitz et al., 1982; Lemon and Loir 1977; Nelson et al., 1992; Hild-Petito et al., 1989). Small luteal cells respond to maximally effective doses of LH with a large increase (up to 40-fold) in secretion of progesterone, while LH has little or no effect on large luteal cells (Fitz et al., 1982; Lemon and Loir, 1977). Interestingly though, a similar number of LH receptors has been observed on large and small luteal cells of normally cycling animals, for example ewe, (Harrison et al., 1987), cow (Chegini et al., 1991), and rat (Nelson et al., 1992). Large luteal cells produce over 80% of the progesterone secreted by the corpus luteum during the midluteal phase of the estrous cycle.

If pregnancy does not occur, it is essential that the corpus luteum regress, allowing initiation of a new reproductive cycle. Two processes are involved in the loss of luteal function at the end of the cycle. First, there is decreased secretion of progesterone, followed by loss of luteal tissue or lutoelysis. The mechanisms involved in luteal regression in the different species are complex and varied but evidence is accumulating to the effect that the antisteroidogenic actions of PGF_{2a} are mediated through activation of the PKC pathway while the luteolytic actions of PGF_{2a} are most likely to be manifested through the process of apoptosis, with increases in

concentrations of free intracellular calcium being the signal for induction of this process.

Normal pregnancy depends upon the early embryo signaling its presence to the maternal system in a process termed maternal recognition of pregnancy. Hormones involved in this signaling differs between species, as do the mechanisms by which these signals maintain the corpus luteum. Although successful maternal recognition of pregnancy is essential to the survival of an embryo and ultimately of a species, the mechanisms for maternal recognition of pregnancy do not appear to be well conserved during mammalian evolution (Niswender et al., 1994). Maintainance of the corpus luteum of pregnancy is due to a diverse group of hormones and mechanisms involved are focused on preventing regression of corpus luteum either by stimulating luteal function or by inhibiting PGF_{2a} secretion or action (Niswender et al., 1994).

Disruption of corpus luteum function results in inadequate endometrial development and maturation, low post ovulatory progesterone levels and shortened luteal phase length (Jones et al., 1974). Since the corpus luteum is derived from the developing follicles, any toxicant that disrupts normal follicular development, ovulatory mechanism or luteal phase endocrine events, can perturb corpus luteum function. The best recognized group of environmental contaminants capable of evoking luteal dysfunction through suppression of ovarian progesterone production are the estrogenic compounds namely DDT and Diethylstilbestrol (DES). By-products of cigarette smoke such as benzo(a)pyrene, a polycyclic aromatic hydrocarbon also inhibits corpus luteum formation (Millar et al., 1992). A report by Clark et al. (1974) indicates that the estrogen antagonist, clomiphene citrate, although used to induce ovulation, can also disrupt corpus luteum function.

1.2.3.2 Mechanisms of hormone action

Peptide hormones such as gonadotrophin releasing hormone (GnRH), LH, FSH, chorionic gonadotrophin(CG), prolactin, inhibin and activin are all involved in mammalian reproduction. They bring about biologic responses by binding to their respective cell surface receptors with high affinity and stereochemical specificity. Peptide hormones are hydrophilic in nature and are unable to pass through the plasma membrane like the hydrophobic steroid hormones, Peptide hormone receptors are integral membrane proteins having three main domains, i) extracellular, ii) transmembrane and iii) intracellular or cytoplasmic domain.

1.2.3.2.1 Signal Transduction by LH and FSH

1) Stimulatory G-Protein Activation and Generation of Second Messenger cyclic Adenosine Monophosphate (cAMP)

Increased intracellular formation of 3'5'-AMP (cyclic AMP) is thought to be one of the mechanisms by which a variety of protein hormones such as FSH, LH, chorionic gonadotrophins (CG), adrenocorticotrophic hormone (ACTH), thyroid stimulating hormone (TSH), insulin, vasopressin and glucagon, produce their biochemical effects (Dorrington, 1979 ; Kalimi and Dave, 1995). These hormones bind to specific receptors on the plasma membrane of target cells resulting in conformational changes, with activation of stimulatory G-protein (Gs). G-proteins are heterotrimeric (made up of three subunits, α,β,γ) and exist in inactive form when bound to GDP and active form when bound to GTP. In the absence of hormone, Gs-protein remains bound to GDP and thus remains inactive. The binding of hormone to their respective receptors and the subsequent formation of the hormone-receptor-complex results in exchange of GDP for GTP on the α subunit of the Gs-protein and

dissociation of Gsα-GTP from Gβγ. Binding of Gsα-GTP to adenylate cyclase causes the activation of adenylate cyclase, an enzyme found on the inner surface of the plasma membrane. Activated adenylate cyclase in turn causes the production of cAMP from ATP (Levitzki, 1988). Studies have shown that cAMP acts as the intracellular mediator of many actions of LH and FSH in the ovary and the testis (Dorrington, 1979, Levitzki, 1988).

Cyclic AMP produces diverse responses in different tissues some of which may involve the activation of protein kinase A (PKA). Cyclic AMP-binds to the regulatory subunit of PKA inducing conformational changes resulting in release of catalytic subunit and subsequent activation of PKA (Beebe et al., 1989; Magoffin, 1989). There is a widespread distribution of cAMP dependent protein kinases in mammalian tissues. The protein kinases function to transfer the phosphate from ATP to a substrate and consequently by this process of phosphorylation, the enzymes activate proteins important in the control of cell function. The precise role of Gβγ is not known. However it has been shown that Gβγ subunits contribute to the receptor recognition site. They may also directly regulate effectors such as enzyme phospholipase A2 and they help in anchoring the α subunit to the plasma membrane. (Freissmuth et al., 1989; Hollenberg, 1991).

2) Mechanism of action of FSH

Relatively little is known about the mechanism of action of FSH as compared to the other glycoproteins. From the information available (Dorrington, 1979; Minegish, et al.,1991; Leung and Steele, 1992; Kalimi and Dave, 1995), the following sequence of events has been proposed.

FSH binds to its target cells leading to activation of adenylate, which results in increases in the intracellular concentration of cAMP. Cyclic AMP then activates

protein kinases resulting in the phosphorylation of proteins. The phosphorylated proteins may then bring about their effects in three main ways:-

a) by influencing the transcription of DNA, and thus the synthesis of specific mRNAs which code for proteins required for the expression of the FSH effects,

b) by influencing cell function at the translocational level , and thus protein synthesis or

c) by acting directly on the rate limiting steps in the reactions involved.

One well known effect of FSH on cellular function is its stimulatory action on estradiol synthesis. In some of these studies, (Dorrington, 1979; Kalimi and Dave, 1995) report that cAMP derivatives could produce the same effects as FSH both qualitatively and quantitatively. It was noticed that a time lag existed before estradiol synthesis was stimulated by either FSH or cAMP derivatives. Subsequent studies, using inhibitors of RNA synthesis and protein synthesis indicated that a new mRNA synthesis and its subsequent translocation to provide a unique protein product was taking place during this lag period, the unique protein being essential for the manifestation of the FSH effect.

3) Mechanism of action of LH

The stimulation of steroidogenesis by LH in either the ovary or the testis has been extensively studied (Dorrington, 1979; Minegish, 1990; Kalimi and Dave, 1995). The synthesis of progesterone from cholesterol proceeds by the same pathway in the adrenal, the ovary and the testis. LH and ACTH accelerate steroidogenesis in their respective target tissues by influencing the conversion of cholesterol to pregnenolone, the rate limiting step in the biosynthetic pathway. Cyclic AMP is again implicated in these processes. Studies on the adrenal, ovary and testis showed

that cAMP could stimulate steroidogenesis in these tissues (Dorrigton, 1979; Baird, 1987; Leung and Steele, 1992; Kalimi and Dave, 1995). These studies also showed that LH rapidly increased the level of cAMP in gonadal tissues and that this effect preceded the increase in steroidogenesis. It is not clear however, how cAMP accelerates the conversion of cholesterol to pregnenolone. It has for example been suggested that cAMP may accelerate the reaction by increasing,

a) the level of the cofactor NADPH, or

b) the total amount of intracellular free cholesterol, or

c) the transport of cholesterol to the enzyme system in the mitochondrion responsible for cleavage of the cholesterol side-chain, or

d) the activity of the side-chain cleavage enzyme system.

Arguements and evidence for and against each of the above possibilities have been presented, but the overall and current concensus of opinion is that the primary action of LH (and cAMP) is on the transport of cholesterol into the mitochondria.

1.2.3.2.2 Signal Transduction by GnRH

Activation of G-Protein and Generation of Second Messengers Calcium and Phospholipids.

Cyclic AMP was for a long time accepted as the intracellular mediator of many actions of protein hormones. The last decade however has seen the emergence of another signal transduction mechanism - the phosphoinositides - calcium messenger system. This system is also activated by hormones as well as neurotransmitters and growth factors.

Binding of GnRH to its receptor results in activation of the membrane-bound enzyme phospholipase C through activation of heterotrimeric G-protein (Gq) as reported by Hawes and Conn (1993). The GDP-GTP exchange reaction, dissociation

of Gα–GTP, binding of Gα-GTP to phospholipase C and hydrolysis of Gα-GTP by GTPase are similar to that described for adenylate cyclase mechanism of hormone action. The activated phospholipase C then converts the plasma membrane phospholipid, phosphatidyl inositol 4,5-biphosphate (PIP2) to inositol 1,4,5-triphosphate (Ins 1,4,5-P3) and 1,2-diacylglycerol (DAG) (Davis et al., 1981; Davis et al., 1986; Dimino et al., 1987).

Ins(1,4,5)P3 binds to a receptor on the intracellular calcium storage sites, diffuses to the endoplasmic reticulum or a specified component of it (Rossier and Putney, 1991) and causes the release intracellular stored calcium into the cytoplasm. This release of intracellular calcium in some manner also signals the activation of calcium entry in a process known as capacitative calcium entry (Putney, 1990; Putney, 1992). The increase in Ca^{2+} is thus due to the release of stored intracellular Ca^{2+} and stimulated Ca^{2+} entry from extracellular space. The link therefore between phosphoinositide turnover and Ca^{2+} mobilization is inositol 1,4,5-triphosphate [Ins(1,4,5)P3] (Streb et al., 1983).

The calcium so released then act as the intracellular signal for the activation of various cellular proteins including calmodulin which in turn activates protein kinase C (PKC) and and other regulatory proteins. The formation of Ins (1,4,5)P3 and diacylglycerol (DAG) is the most important consequence of hormone-stimulated phosphoinositide turnover. The Ins (1,4,5)P3 produced during agonist stimulation is rapidly metabolised by dephosphorylation, either directly or after phosphorylation to Ins (1,3,4,5)P4 to produce various InsP2 and InsP isomers and eventually inositol (Balla and Catt, 1994). The potential signaling function of Ins (1,3,4,5)P4 in cell activation is still a matter of debate. However its most accepted role is in the regulation of Ca^{2+} influx (Putney and Bird, 1994). Other suggested roles include stimulation of Ca^{2+} sequestration (Hill et al., 1988) and activation of a protein phophatase (Zwiller et al., 1988).

Diacyl glycerol also stimulates the release of calcium and hence the activation of the enzyme protein kinase C (PKC) which consequently enhances the phosphorylation of various proteins (Hollenberg, 1991; Putney, 1992; Lefkowitz et al., 1988; Takai et al., 1979). Both Ins(1,4,5)P3 and DAG thus stimulate the release of calcium from the endoplasmic reticulum and activate PKC resulting in subsequent expression of biological actions. It has been demonstrated that for a full physiologic response, calcium mobilization and activation of PKC are necessary (Kalimi and Dave, 1995). The phosphorylation cascade ultimately leads to the transduction of hormonal signal from cytoplasm into nucleus by an undetermined pathway.

In oocytes and probably also in excitable cells, another potential calcium-mobilizing messenger is cyclic adenosine diphosphate ribose (cADP ribose). However there is yet little evidence that the levels of cADP ribose are regulated by hormones or other extracellular mediators (Balla and Catt, 1994).

1.2.3.2.3 Signal Transduction by activin, prolactin and inhibin

Activin binds to type I and type II receptors. The type II activin receptor belongs to the transmembrane protein serine/threorine kinases. Binding of activin to type II receptors results in autophosphorylation on serine/threonine residues within its cytoplasmic domain. Although the signal transduction pathway is still unclear, it is likely that phosphoserine/ phosphothreonine residues generated by intrinsic serine/threonine kinase may, in turn, allow the activated receptor to associate with other proteins involved in the activin-dependent signal transduction pathway (Kalimi and Dave, 1995). The mechanism of signal tranduction for prolactin and inhibin are largely uknown.

1.2.3.2.4 Steroidogenesis

i) Preamble

The steroid hormones comprise a large group of organic molecules all derived from a common sterol precursor, cholesterol. Cholesterol is a fat-soluble lipid synthesised from acetate in many tissues of the body. Because of its fat solubility (with limited solubility in water), it localises in the lipid regions of cell membranes where it is said to be responsible for the maintanance of the structural integrity of the plasma membrane of the cell. Indeed cholesterol has a widespread structural role in the body and is not merely a substrate for steroidogenesis.

In steroidogenic tissues, steroids can be produced primarily from acetate with cholesterol as the intermediate product. In most cases however, direct synthesis of steroids from cholesterol is the more common pathway. The cholesterol substrate utilised may be derived from one of two sources, either cell reserves or pools of esterified cholesterol or, the circulating cholesterol in the blood and tissue fluids. Due to its relatively poor solubility in water, the circulating cholesterol is consequently found complexed with carrier protein molecules such as albumin and high or low density lipoproteins (HDL, LDL). Cholesterol obtained from the various sources can then be utilised for steroid synthesis or can be incorporated into more cholesterol esters by acyl CoA cholesterol acyltransferase (ACAT) and stored as lipid droplets. Release of cholesterol from cholesterol esters is dependent on a neutral cholesterol esterase (also known as hormone-sensitive lipase). Activity of this enzyme is regulated by phosphorylation of two serine residues. Cyclic AMP-dependent protein kinase A (PKA) causes phosphorylation of one serine residue and activation of the enzyme, whereas Ca^{2+}/calmodulin-dependent protein kinase phosphorylates the other serine residue and prevents activation of the enzyme (Niswender et al ,1994).

The first and common step in the formation of all the major steroid hormones is the conversion of cholesterol to pregnenolone. This conversion is a

rate limiting step and is therefore a most important step in the biosynthesis of the subsequent hormones. Once formed the pregnenolone may either remain or be oxidized to progesterone by the microsomal (agranular endoplasmic reticulum) enzyme complex 3β hydroxysteroid dehydrogenase $\Delta^{4,5}$ isomerase which needs NAD^+ (Δ4 pathway). The biosynthesis of progesterone, the hormone that plays such a vital role in reproduction, is thus the simplest steroidogenic pathway. The rate limiting step for androgen formation is the mixed function oxidase P450$_{17\alpha}$-hydroxylase: c-17-20-lyase enzyme complex present in the microsomes and which requires NADPH and oxygen. The action of this enzyme complex converts pregnenolone to DHEA and progesterone which are in turn converted to androstenedione. In the presence of 17β hydroxysteroid dehydrogenase, which needs NADH, the androstenedione is reduced to testosterone. This reaction is said to be reversible and the same enzyme can oxidize testosterone to androstenedione. Androstenedione can then be aromatized to estrone with the later being converted estradiol.

Steroidogenesis is thus a complex series of events whose success may well depend on several factors both within and without the endocrine system itself and the unravelling of these complex processes has been the subject of study for many researchers.

ii) Regulation of estradiol production

In-vivo and *in-vitro* studies have provided valuable information and evidence to support the complexity and diversity of the processes involved. For instance, androgens which are themselves products of steroidogenesis, play a big role in ensuring the success of the steroidogenic processes by inducing the very enzymes that catalyse their metabolism. Indeed there is increasing evidence that the granulosa cells of ovarian follicles actively secrete 17β estradiol when

cultured in medium containing an aromatizable androgen.(Dorrington et al.,1975; Erickson and Hsueh, 1978). This ability of cultured granulosa cells to produce estradiol is even more enhanced by the presence of FSH in the culture medium (Dorrington et al.,1975) or by *in-vivo* injection of FSH before isolation of the granulosa cells for culture (Armstrong and Dorrington, 1976). The interpretation of these observations according to Daniel and Armstrong,1980, is that FSH stimulates the activity of the aromatase enzyme system while androgens serve as substrate This indeed is the case as evidenced by reports that FSH is able to induce aromatase activity in rat granulosa cells whether administered *in-vivo* (Erickson and Hsueh, 1978), or *in-vitro* (Gore-Langton and Dorrington, 1979; 1981; Daniel and Armstrong, 1980).

Similarly androgens enhance FSH stimulation of cAMP production *in-vivo* (Leung et al.,1979), and by granulosa cells *in-vitro* (Goff et al.,1979). Daniel and Armstrong (1980) provided further confirmation that granulosa cells are capable of aromatizing androgens into estrogens in the presence of FSH. They went further to stress, that androgens must be provided in the culture system since granulosa cells do not have the necessary enzymes to convert progesterone to androgen as reported also by Fortune and Armstrong (1978). The findings of Daniel and Armstrong (1980) confirmed their earlier suggestion that androgens do not only influence estrogen secretion by serving as substrates for the FSH-induced aromatase activity in granulosa cells, but they also participate in the mechanism by which FSH increases the activity of the aromatase enzyme. They further suggested that aromatase induced by FSH has a relatively short half-life in cells cultured without the continued presence of FSH. According to their findings, aromatase activity during the test period was found to begin declining after 6 hours in the presence of androgens (testosterone) alone and that the

decline could be prevented by addition of FSH to culture medium during the test period.

The androgens involved in enhancing the FSH induction of aromatase need not be aromatized to be effective but on the other hand, the aromatizable androgens eg. testosterone, are more effective than the non aromatizable androgens in enhancing the FSH induction of aromatase. Daniel and Armstrong (1980) continued to observe that further enhancement of the FSH-induced increase in aromatase activity could not be obtained by raising the concentration of either DHT or androsterone, suggesting that estradiol may play some role in regulating its own synthesis in cultured granulosa cells.

Androgens have also been shown to have stimulatory effects on progesterone production in cultured granulosa cells from pigs (Schomberg et.al., 1976) and in rats (Nimrod and Lindner, 1976). The stimulation of progesterone production by androgens could be similar to its effect on estradiol whereby it augments the effect of FSH on the production of these hormones (Armstrong and Dorrington, 1976). Similar studies, examining the mechanism of action of steroids on gonadotrophin stimulated steroidogenesis by granulosa cells in general, have led to the conclution that steroids regulate some step distal to cAMP production.

Progestins have also been reported to modulate FSH-stimulated steroidogenesis. However, and in contrast to the stimulatory action of androgens, progestins were found to inhibit both aromatase and enzymes involved in the production of progesterone and 20α–dihydroprogesterone (Shreiber et al., 1980). Furthermore, Shreiber et al. (1981), found that progestins inhibited cholera toxin, prostaglandin E2 (PGE2) and (Bu)2 cAMP-stimulated estrogen synthesis, but had no effect on the ability of FSH to stimulate cAMP production. A follow up study by Daniel and Armstrong (1984), was designed to examine the role of cAMP in the induction of aromatase activity by FSH, and to locate the site of action of androgens

in enhancing FSH-induced enzyme activity. The results of the study demonstrated that in addition to FSH, cholera toxin and PGE2 induced granulosa cell aromatase activity and that higher concentrations of these compounds were less potent than lower concentrations. Although testosterone increased the effects of cholera toxin and prostaglandin E_2 (PGE2) in addition to enhancing FSH-induced aromatase, it had no effect on the stimulatory actions of cAMP analogs. This, according to Daniel and Armstrong (1984) suggests that the stimulatory effect of androgen on aromatase activity occurs at a site before cAMP production.

iii) Regulation of progesterone production

Tanabe et al. (1992) examined the influence of testosterone , androstenedione, and dihydrotestosterone (DHT) on progesterone production by cultured porcine cells in the presence or absence of gonadotrophins. In the study they were able to demonstrate that androgens stimulated progesterone secretion by cultured porcine granulosa cells in the presence of LH. In contrast, androgens suppressed progesterone secretion in the presence of FSH. From their data they were able also to suggest that the action site of androgens might be the conversion of cholesterol to pregnenolone (cholesterol side chain cleavage enzyme, cytochrome P450scc) in terms of both stimulation and inhibition of progesterone secretion by androgens. Tanabe et al. (1992) also found that DHT, a non aromatizable androgen could also produce the same effect as testosteone, suggesting that it was unlikely that testosterone and androstenedione affected progesterone secretion after conversion to estrogen. This agrees with what Daniel and Armstrong (1980) demonstrated that the androgens involved in enhancing FSH induction of aromatase need not necessarily be aromatized to be effective.

There are however some conflicting reports on the effects of androgens on progesterone secretion by cultured granulosa cells in the presence or absence of

gonadotrophins. Hillier et al. (1977) for instance, reported that testosterone elicited increases in progesterone production by 2.4 - 11.0 times that in controls by isolated rat granulosa cells in the absence of gonadotrophins. Lucky et al. (1977), also reported an increase in progesterone production by 400 - 700% in the presence of testosterone and DHT more than in paired control cultures. On the other hand, Armstrong and Dorrington (1976) could not find any effects of testosterone and DHT on progesterone secretion by cultured rat granulosa cells, but they reported that the same quantity of testosterone and DHT caused a dramatic, 8 to 19 fold increase in progesterone secretion in the presence of FSH. Nimrod (1977 a & b), while doing similar studies found that androstenedione enhanced progesterone secretion with FSH, and that the step in the regulation of progestin biosynthesis might be distal to cAMP.

Utilising porcine granulosa cell cultures, Schomberg et al. (1976) and Haney and Schomberg (1978), reported that DHT stimulated progesterone secretion in the absence of gonadotrophins while the effects of testosterone were inconsistent. On the other hand, Evans et al. (1984) reported from his studies that 5 mM testosterone suppressed progesterone production by cultured porcine granulosa cells although the suppression was not significant. These same authors (Evans et al., 1984) also showed that both testosterone and androstenedione depressed FSH or (Bu)2-cAMP-stimulated progesterone secretion by cultured porcine granulosa cells, and that the action of these androgens was the suppression of the conversion of pregnenolone to progesterone as reported also by Lischinsky et al. (1983). Moon (1981) used cultured human granulosa cells and was able to show the ability of 0.5mM testosterone to stimulate progesterone production in the absence of gonadotrophins and that testosterone further stimulated progesterone secretion in the presence of FSH. Veldhuis et al. (1983) on the other hand, had contrasting results in that, they reported the suppression of progesterone secretion by DHT in human granulosa cell

cultures. Polan et al. (1986), also reported that testosterone and androstenedione decreased hCG stimulated progesterone production. The findings of Tanabe et al. (1992) seem to be relatively similar to those reported by Evans et al. (1984) and Lischinsky et al. (1983), the only significant difference being in the location of the site of action of androgen which Tanabe et al. (1992) reported as cholesterol side chain cleavage enzyme as opposed to 3β-hydroxysteroid dehydrogenase.

Small but sustained increases in serum LH activity are required by many species for the development of small antral follicles to the pre ovulatory stage (Lostroh and Johnson, 1966; Bogovich et al.,1981). Follicles developing in response to LH, not only produce large amounts of estradiol and progesterone (Fortune and Armstrong, 1978; Richard and Kersey, 1980; Richards and Bogovich, 1980; Bogovich et al., 1981), but also acquire an increased ability to produce androgens (Fortune and Armstrong, 1977; Carson et al., 1981). According to Richards and Bogovich (1980), and Bogovich and Richards (1984), this increase in follicular androgen synthesis is due in part to the stimulation of thecal 17α-hydroxylase and 17-20 desmolase which catalyse the conversion of progesterone to androstenedione.

Infertility in humans due to corpus luteum insufficiency is a common problem. Although it is well known that pituitary gonadotrophins play a central role in luteinizing ovarian granulosa cells and in the maintenance of the corpus luteum, the precise mechanism of the regulation of progesterone secretion by the corpus luteum has not been clarified. Recent studies have led to the proposal that local factors modulate the effects of gonadotrophins on corpus luteum function in a paracrine or an autocrine fashion, (Hsueh et al., 1984; Hsueh et al.,1989; Brann et al, 1995). The local factors reported so far are, sex steroids, growth factors, activin, inhibin, neurotransmitters, prostaglandins and luteinization stimulator(s) and/or inhibitor(s). The corpus luteum itself as well as interstitial tissue can secrete androgens (Dupon et al., 1973; Judd and Yen, 1973). At the periovulatory period and also during the luteal

phase, LH is dominant. It is therefore possible to deduce that androgens derived from the corpus luteum and/or the interstitial tissue in cooperation with pituitary LH may enforce the corpus luteum to produce more progesterone in order to ensure the implantation of fertilised ova. On the other hand, FSH prevails during the follicular phase. Androgens from the theca interna cells may not only serve as a precursor of ovarian estrogens but also as a suppressor of progesterone secretion by the follicles in order to prevent premature luteinization. Androgens have been thought to be atretogenic (Louvet et al.,1975). However, it is now clear that androgens play an important role in follicle maturation, luteinization, the maintenance of the corpus luteum, as well as the modulation of the effects of gonadotropins in terms of progesterone secretion by the granulosa cells.

There is evidence that luteal 3β-hydroxysteroid dehydrogenase (3β-HSD) mRNA and P450 side chain cleavage enzyme (P450scc) mRNA are differentially regulated during the estrous cycle. This has been shown for ovine and rat luteal cells (Kaynard et al., 1992). According to Ravindranath et al. (1992), removal of LH support in monkeys caused dramatic down-regulation of mRNA for both P450scc and 3β-HSD whereas in pregnant rat, it is the removal of prolactin support which caused decrease in luteal mRNA encoding for P450scc and the enzyme itself (Hickey et al., 1989). The mechanism by which LH stimulates secretion of progesterone from small luteal cells involves formation of cAMP, activation of protein kinase A (PKA) and subsequently increased progesterone production (Hoyer et al., 1986). Generation of cAMP and activation of the PKA system stimulates cholesterol esterase activity (Niswender et al., 1994) and may enhance transport of cholesterol to the inner mitochondrial membrane (Ghosh et al., 1987). Large and small luteal cells have similar amounts of PKA activity (Wiltbank et al., 1989) but treatment of large cells with cAMP, cholera toxin or LH does not enhance secretion of progesterone. On the other hand, activation of the protein kinase C (PKC) enzyme system with

phorbol 12 myristate-13 acetate (PMA) decreases progesterone production (Wiltbank et al., 1989). The identity of the factors that stimulate PKC activity in small cells is not known.

Receptors for steroid hormones are also present in most corpus luteum. Estrogen receptors have been localised in sheep (Glass et al., 1984) and rat (Richards, 1975). In rats and rabbits it is well established that estrogens are involved in maintaining and enhancing luteal progesterone secretion (Gibori et al.,1988; Keyes et al., 1983). Corpus luteum of monkeys contain androgen (Hild-Petito et al:, 1991) and progesterone receptors (Hild-Petito et al:, 1988) but their function has not been defined. While the positive effect of estradiol on progesterone production *in vivo* in corpus luteum from pregnant rats is clear, conflicting data have been reported *in vitro* . Nelson et al. (1992) reported no effect on basal progesterone production by estradiol, whereas Tekpetey and Armstrong (1991) reported an inhibitory effect of estradiol on progesterone secretion. The difference in these two findings according to Niswender et al (1994) could have been due the great variation in the dose of estradiol used.

iv) Possible target sites for reproductive toxins

The process of steroidogenesis, by which the various steroid hormones are synthesised comprises therefore of a number of biochemical pathways that are precisely regulated by various peptide even by steroid hormones themselves within the reproductive tissues. All of these steps are vulnerable to the actions of reproductive insult by toxins. As already sited, some chemicals, for example, alkalating agents damage the gonads and their function by directly acting on the germ cells, These will destroy the oocytes or spermatocytes by their chemical reactivity whereas other compounds will interfere directly with steroidogenic pathways and enzymes involved. Various synthetic hormone agonists and

antagonists can interfere with the normal reproductive processes because of their high affinity for the receptors and their structural similarity to the natural hormones (Khan-Dawood and Satyaswaroop, 1995). Other drugs and chemicals have been known to affect reproductive function indirectly either through their metabolic conversion to potentially toxic agents or by modulating endocrine activities at sites of hormone action on the gonads or the hypothalamo-pituitary axis (Mattison et al., 1983a).

Studies on reproductive toxins are however raught with uncertainty resulting from differential sensitivity of reproductive tissues from different species. A chemical that may be a reproductive toxin in the rodent species may turn out to be non-toxic in humans or other species, and vice-versa. The teratogenic effects of thalidomide are a classic example in this aspect. Although rodents were insensitive to the developmental toxicity of this drug, humans and non-human primates were highly sensitive to it (Shepard, 1986). The observed species differences has been attributed to differences in anatomic features, pharmacokinetics, hormonal regulation and detoxification mechanisms. (Khan-Dawood and Satyaswaroop, 1995). It is difficult to assess the effect on human exposure to particular substances because multiple chemicals are frequently involved and these are superimposed on cultural and socioeconomic factors. However, the rodent models used so far for the evaluation of potential toxins, have been informative and have provided valuable insights into the putative mechanisms involved, even though there have been serious concerns in recent past about the extrapolation of research findings from these experimental model systems to human situation. However, because of the impractibility of using humans for experimental trials, animal models are and will continue to be, in the foreseable future, a major tool for predicting human developmental hazards. Comparisons with human data indicate that animal models are generally predictive of human responses.

1.2.4 Effects of Xenobiotics on Reproduction

1.2.4.1 Effects of xenobiotics in general

Research has shown that chlorinated hydrocarbons as pollutants have a wide distribution in various tissues in animals and humans. These compounds are found in levels that raise concern as to their possible effects on the well being of man and in particular the young ones since they are exposed to the pollutants in utero and later through breastmilk. Experimental studies in animals and findings in humans have revealed that organochlorine insecticides are potent hepatic microsomal enzyme inducers, capable of quantitatively altering the response to some xenobiotics for example drugs and toxic compounds (Wassermann et al., 1974a). They may also alter the response to naturally occurring compounds in the animal's body for example, steroid hormones. They may also have some influence on certain endocrine and immunological homoeostatic processes. The exposure of the oocyte to pollutants for instance would have possible effects on meiosis, enzyme functions, membrane characteristics and steroid hormone metabolism (Trapp et al.,1984). The consequences of these effects on the outcome of pregnancy are still questions to be answered by research.

It has been shown by Johnson et al. (1988) that a large variety of compounds, including some of the chlorinated hydrocarbon pesticides for example O,P'-DDT can produce estrogenic responses. The fact that pesticides can mimic estrogen actions means that they could interact with estrogen receptors and interfere with the final genomic expressions attributed to estrogens. Estrogens are implicated in several processes in the female mammal such as ovulation, uterine growth, uterine water imbibition and initiation of embryo implantation. Interference with some, if not all of these processes could lead to abnormal pregnancy outcome.

Methoxychlor (MXC) has gestagenic activities and is metabolised to a compound that has been shown to exhibit estrogenic activity *in vivo* and *in vitro*. According to Cummings and Gray (1987; 1989), long term exposure of female rats to MXC results in reduced fertility due to inhibition of decidualization of the placenta. Sircar and Lahiri (1989) demonstrated that lindane when given orally to pregnant Swiss female mice at various stages of pregnancy resulted in failure of implantation, fetal resorption and fetal death. The insecticide besides being fetotoxic, appeared to cause steroid hormone deficiency and hence reproductive and fetal developmental failure. Work by Uphouse and Williams (1989) also demonstrated that treating regularly cycling adult female rats with lindane doses ranging from 10 to 50 mg/kg during diestrous disrupted their reproductive cycles. Work by Virgo and Bellward (1975; 1977) showed that dietary dieldrin affected offspring viability, maternal behaviour and milk production in the mouse.

Sager (1983) reported on the effect of PCB exposure during the postnatal period on subsequent reproductive capacity of adult male offspring. His results indicated that male rat offspring from treated dams were less successful in mating and reproducing than the control offspring. His findings support the hypothesis that exposure of males to PCB's during early postnatal development results in offspring with a hypoandrogenic condition that is detrimental to normal reproductive functioning in the adult.

As already mentioned, halogenated hydrocarbon insecticides and polychlorinated biphenyl mixtures are frequently detected in human adipose tissues, serum and milk. Based on average values observed in analytical studies, Gyorcos et al.(1985) prepared reconstituted halogenated hydrocarbon pesticides and PCB mixtures and administered these to immature male Wister rats, at dose levels which approximate the concentrations which would be absorbed by an infant suckling for 180 days (low dose level,L) and at three higher dose levels (2xL, 10xL and 100xL).

They found that the low dose level of 0.95 mg/kg for the pesticide and 0.45 mg/kg for the PCB or a mixture of the two in corn oil on day 1 and 3 did not significantly alter hepatic drug-metabolizing enzyme activities or elicit any observable pathological damage 6 days after the first exposure. In contrast, administration of the higher dose levels of the mixture elicited a dose dependent induction of several hepatic drug metabolizing enzymes. Despite the short duration of exposure, the rats treated with higher doses (10xL and 100xL) of these mixtures exhibited mild alterations in thyroid architecture, changes in hepatocellular nuclei including variations in chromatin distribution, vesiculation of larger nuclei and frequent appearance of pyknotic shrunken nuclei

Haake et al (1987) investigated the effects of organochlorine pesticides as inducers of testosterone and benzo[a]pyrene hydroxylases. Their results indicated that p,p'-DDE, phenobarbital, dieldrin, heptachlor, chlordane and toxaphene induced rat liver microsomes, exhibited increased formation of the 4,5-dihydrodiol, 6-quinone, 9-and 3-hydroxymetabolites of benzo[a]pyrene and the latter three compounds also induced an increase in the rate of formation of the 9,10-dihydrodiol metabolite. Lindane was inactive as an inducer of benzo[a]pyrene hydroxylase. With the exception of lindane, all the organochlorine pesticides induced testosterone 6α,7α and 6β hydroxylases. Campbell et al (1983) looked at the effect of some 22 organochlorine pesticides as inducers of hepatic drug-metabolising enzymes in the immature Wister rat. Among the 22 pesticides looked at were heptachlor and heptachlor epoxide. With the exception of HCB, all of the pesticides induced microsomal dimethylaminoanti-pyrine, N-dimethylase and aldrin epoxidase activities as well as increasing cytochrome P-450 content of microsomes.

1.2.4.2. Effects on reproductive physiology and hormone function

1.2.4.2.1 General effects

It is becoming increasingly evident that the exposure of an individual to environmental pollutants, toxicants and xenobiotics, can impose detrimental effects on reproductive function (Longo, 1980; Haney, 1985; Mattison, 1985). The physiologic events underlying and regulating reproductive processes such as gametogenesis, gamete transport, fertilization, implantation, pregnancy and parturition, are immensely complex, involving numerous systems of precisely programmed positive and negative hormonal feedback loops occurring in the hypothalamus , anterior pituitary gland, and the ovary. These components in turn interact primarily through the actions of the hypothalamic hormones namely, gonadotrophin-releasing hormone (GnRH), the pituitary gonadotrophins i.e. follicle stimulating hormone (FSH), and luteinizing hormone (LH), as well as the ovarian steroids namely, estrogen and progesterone. Because of this complexity the possible sites and actions of toxicants within the reproductive system are numerous (Garospe and Reinhard, 1995). Several studies have now demonstrated that exposure of the female to a xenobiotic agent during pregnancy may lead to adverse effects on the unborn and newborn (Sullivan and Barlow, 1979). Additionally, exposure of a mature female or male to a toxicant may result in interference with fertility potential (Sullivan and Barlow, 1979).

1.2.4.2.2 Effects of synthetic and non-synthetic steroidal agentson steroidogenesis

The belief that administration of potent estrogenic agents may protect against threatened abortions or may have beneficial effects on pregnancy, and the realization that the introduction of potent estrogens at inappropriate times and dose may

reversibly alter fertility in the female has led to the synthesis of a whole range of pharmaceutical agents; steroidal and non-steroidal.

1) Synthetic Steroid Compounds

Diethylstilbestrol (DES), a potent synthetic estrogen was synthesised in 1938 and used in the treatment of threatened spontaneous abortions in women as well as superovulation and/or synchronization of ovulation in cattle. Assuming that DES was a safe and effective estrogen, it was also prescribed routinely to women during normal pregnancy. Not long after, and as reported by Dieckmann et al. (1953), it was shown by clinical trials that DES was infact ineffective in preventing miscarriages or premature births. Instead, DES exposure has since been associated with abnormalities of both male and female reproductive tracts. A report by Herbst and Scully (1970) documented the presence of clear cell carcinoma of the vagina in six young women whose mothers had received DES during pregnancy, a rare occurrence in that group of women. The association of *in-utero* exposure to DES and the occurrence of this rare form of cancer in the female offspring of DES exposed women was confirmed by several other studies thereby raising concerns about its potential adverse effects (Greenwald et.al., 1971; Herbst et al., 1971). DES was banned in 1971 in the USA. Several authors (Korach et al., 1979; Metzler, 1976; Metzler and McLachlan, 1979; Radiger et.al., 1979; Barette et al., 1981), showed that DES in addition to being estrogenic, was also mutagenic and carcinogenic.

Despite the work done so far, the question of impaired fertility among DES daughters still remains unresolved. However, it is apparent that once pregnancy occurs, DES daughters appear to be at high risk for unfavourable pregnancy outcomes such as miscarriages, ectopic pregnancy, stillbirth, and premature births, (Kaufman et al.,1980).

In addition to DES, synthetic compounds of the triphenylethylene series have been widely reported to have both estrogenic and antiestrogenic effects on estrogen target tissues (Lerner et al., 1958; Harper and Walpole, 1967). A good example of a compound in this category is clomiphene. Clomiphene exhibits antifertility properties in animals although it is used repeatedly in hyperstimulation of the ovary in infertile women (Huppert, 1979). Tamoxifen is another widely used triphenylethylene derivative. Several authors have shown that tamoxifen has both estrogenic and anti estrogenic effects. It has for example, a potent antimammary tumor activity in women, (Jordan and Koerner, 1976), it is a partial estrogen agonist in rats, a true estrogen in the mouse (Harper and Walpole, 1967), and a complete estrogen antagonist in the chick oviduct. (Sutherland et al., 1977). Studies of the antiestrogenic actions of tamoxifen led to its being an effective drug for the treatment of breast carcinoma especially in the post-menopausal women. On the other hand studies by Khan-Dawood and Satyaswaroop (1995) showed that tamoxifen had no anti estrogenic effects on the growth of endometrial carcinomas; furthermore, it has growth promoting effects on these endometrial carcinomas.

2) Pesticides: Steroidal effects

Health effects in humans from long-term exposure to pesticides have been widely studied. An association has been reported on the exposure to 1,2- dibromo-3-chloropropane (DBCP) and the suppression of spermatogenesis and increased FSH and LH levels (Khan-Dawood and Satyaswaroop, 1995). The gonadotoxic effects appear to be reversible in moderately exposed individuals. Studies on the evaluation of the risk of other adverse effects on reproduction such as pregnancy outcome birth defects, or cancer in the offspring of parents exposed to pesticides have been inconsistent so far. A study by Wolff et al.(1993) reported a significant positive

association between levels of DDE, the major metabolite of DDT in stored blood and increased risk of cancer of the breast.

In an attempt to relate the presence in the body, of these organochlorine insecticides and polychlorinated biphenyls to reproductive problems, Bercovici et al., (1983) assessed the serum levels of these compounds in 17 women with recent missed abortions. The results indicated that 53% of the cases of recent missed abortions had PCB serum levels significantly higher than the control group. The mean PCB serum levels of women with former missed abortions was also significantly different from that of the control group. The percentage of total o,p'-DDT serum level as related to total DDT, showed an increase in the high PCB level/ recent missed abortion group and in the former missed abortion group, in comparison with the control group (36.18 and 48.98 versus 25.57, respectively). This lead the authors to postulate that, the fact that the former missed abortion group showed increased PCB serum levels similar to those found in the high PCB level/ recent missed abortion group, confirms the existence of an association between relatively high PCB serum levels and the occurrence of missed abortion, suggesing that PCB in high concentration may be the actual cause of fetal death and subsequent retention in the uterus.

3) Phytoestrogens

Phytoestrogens are naturally occurring compounds that have been identified in several plants as reported by Kaldas and Hughes (1989). Phytoestrogens have structural and functional similarities to gonadal estrogens and exhibit estrogenic activities. (Hughes, 1988). Since several dietary plants contain estrogen-mimicking agents, it is reasonable to assume that human exposure to these agents or compounds occurs regularly. Luckily, these compounds are 1000 to 10,000 fold less potent than

circulating estrogens and their effects are manifested by virtue of their long half-lives. One needs therefore a chronic exposure to be able to elicit an estrogen response.

4) Non-Steroidal Compounds

As has been mentioned earlier, gonadotrophic hormones seem to exert their effect via the activation of protein kinases. The phospholipid-sensitive and Ca^{2+} dependent protein kinase (protein kinase C) has been reported to be present almost universally in various phyla of the animal kingdom. (Lefkowitz and Caron, 1988; Hollenberg, 1991; Putney, 1992). Protein kinase C is known to be activated *in-vitro* and *in-vivo* by the tumor promoting phorbol esters, but not the non-tumor promoting phorbol esters. This seems to suggest that gonadal steroidogenesis could be modulated by tumor promoting phorbol esters. The tumor promoting esters apparently substitute for diacylglycerol. Phorbol esters, therefore provide a tool which in the presence of Ca^{2+}, renders protein kinase C amenable to manipulation in the intact cell. The most potent phorbol ester is phorbol 12-myristate-13-acetate (PMA). PMA has been shown by various authors to modulate (inhibit) the steroidogenic response of rat adrenocortical cells (Cutty et al.,1984), rat granulosa and Leydig cells (Welsh et al.,1984), and mouse Leydig cells (Mukhopadhyay et al.,1984). PMA also modulates the secretory response of various other endocrine cells. For example, it has been reported to stimulate insulin release from pancreatic islets of Langerhans (Virji et.al.,1978) and anterior pituitary hormones from the pituitary cells in culture (Osborne and Tashjian, 1981; Smith and Vale, 1980). In contrast PMA was reported by Brunswig et al.,(1986) to stimulate progesterone production by isolated bovine luteal cells in a dose and time related manner. Unlike hCG, the action of PMA appears not to require the generation of cAMP and that the site of action of PMA is located at a point between the cAMP formation and the production of pregnenolone in the mitochondria. Baum and Roseberg (1987) further

demonstrated the biphasic effect of PMA on steroidogenesis in rat luteal cells. Their findings showed that at lower concentrations, PMA inhibited LH-stimulated cAMP and progesterone production, whereas at higher concentrations of PMA, stimulated progesterone biosynthesis without a corresponding increase in cAMP. Sender and Roseberg (1987) concluded that the phorbol ester PMA mimic some of the luteolytic properties of prostaglandin PGF_{2a} in that it not only inhibits the luteal cAMP system but also induces lesions in the steroidogenic steps beyond the cAMP system. The most likely common denominator for these effects is protein kinase C which could affect both the cAMP system and steps in steroidogenesis.

All these reports on DES, a synthetic steroid; clomiphene, a synthetic estrogen; tamoxifen, an estrogen agonist/antagonist; phytoestrogens, naturally occurring estrogenic compounds identified in plants, and phorbol ester, a tumor promoting agent, demonstrate the possible means by which organochlorine pesticides may interfere, or even modulate steroidogenesis, thereby affecting reproductive performance. Indeed studies carried out by Moser and Smart (1989), showed that this was actually possible. They evaluated various chlorinated hydrocarbons for their ability to stimulate protein kinase C activity *in-vitro* . Out of the many chlorinated hydrocarbons they looked at, chlordane, kepone, toxaphene, heptachlor, PCB Aroclor 1254, aldrin, DDT, and lindane were the most potent stimulators of protein kinase C activity and out of these, chlordane was the most potent organochlorine pesticide. In the presence of calcium, chlordane stimulated mouse epidermal and hepatic protein kinase C as well as purified rat brain protein kinase C. Based on these findings, Moser and Smart (1989) concluded that a wide variety of chlorinated hydrocarbons, which are considered hepatic tumor promoters like heptachlor, do stimulate protein kinase C activity *in-vitro*.

Tumor promoters may also inhibit cell-cell communications resulting in disruption of cell membrane functions and regulated cell divisions. (Trosko et al.,

1982; Ruch et al., 1987; Chuang et al., 1991). Heptachlor has been shown to inhibit intracellular communication between cultured liver cells (Telang et al., 1982). Studies by Chuang et al.(1991) demonstrated that heptachlor induced human myeloblastosis leukemia ML-1 cells to undergo differentiation into monocyte- and macrophage-like cells and that there was similarity between heptachlor and other differentiation-inducing agents. The monocyte-macrophage cell type produced by heptachlor resembled that produced by 12-0-tetradecanoylphorbol-13-acetate (TPA), another tumor-promoting phorbol ester. It is therefore very likely that heptachlor and TPA have a common mode of action.

1.2.4.3. Effects of heptachlor

1.2.4.3.1 Reproductive effects of heptachlor

Long term exposure of rats to doses of either heptachlor or its epoxide exceeding 7 mg/kg body weight increased the mortality rate of the pups during the suckling period according to the WHO report (1984). In other studies, Witherup et al. (1976a) fed male and female rats exclusively on diets containing a mixture of heptachlor and heptachlor epoxide (3:1) at 0, 0.3, 3, or 7 mg/kg. The treated animals were allowed to reproduce throughout three succeeding generations. The results indicated that the number of pregnancies in the F1 and F2 generations were slightly reduced in the 0.3 mg/kg group, but not in the higher dose level groups. However their results also showed a slight increase in the mortality rate of the pups in the second and third week after birth in the 3 mg/kg group. Apart from these effects, the compound did not seem to have any statistically significant effect on the fertility of the progenators or the ability of the progeny to survive. In a repeat study by Witherup et al. (1976b), male and female rats were fed exclusively on diets containing heptachlor at 0, 0.3, 3, 6, or 10 mg/kg throughout three generations, and

again allowed to reproduce. The results obtained concurred with those of the previous study in that the mortality of the pups was increased. This however occurred only in the 10 mg/kg group during the second and third weeks after birth, and only in the 2nd generation. Cerey and Ruttkay-Nedecka (1971) and Ruttkay-Nedecka et al. (1972) fed rats with heptachlor at 1-10 mg/kg body weight per day for a period of a three generation reproductive study. They found that the number of resorptions was increased while the viability and lactation indices were lowered. In a joint report by Food and Agriculture Organization (FAO) and WHO (FAO/WHO 1967), heptachlor at 6.9 mg/kg body weight fed to rats for three generations before mating, was seen to cause not only cataracts in both the young rats and their parents, but also led to a decrease in litter size.

Crum et al. (1993), fed adult female minks with 6.25, 12.5 and 25.0 mg/kg of heptachlor. Females were then mated with males on the same dietary treatments. Whelping success rates were 67% for the control group, 83% for the 6.25 mg/kg group, 27% for the 12.5 mg/kg group and 0% for the 25 mg/kg group. Gestation length, litter size and birth weight of kits were not significantly affected by adult female consumption of 6.25 mg/kg heptachlor, while kits whelped by females on the 12.5 mg/kg diet weighed significantly less than control kits at birth. Survival of kits in the 12.5 mg/kg group from birth to three weeks of age was also adversely affected. At three and six weeks of age, kit body weights in both 6.25 and 12.5 mg/kg groups were significantly less than body weights of control kits.

Even more recently, Beard et al. (1995), while still investigating the mechanism of decreased fertility in mink, showed that lindane, an organochlorine pesticide, causes increased embryo mortality in these animals. They fed yearling minks with lindane treated diets (1mg/kg/day) from 6 weeks prior to mating until weaning, when the adults were killed. At this dose, lindane did not cause a

significant decrease in body weight, neither did it have any significant effect on the proportion of mink accepting to mate. However when all mink (mated and non-mated) were considered, lindane treatment was found to cause a significant decrease in the whelping rate ($P< 0.05$) and litter size ($P< 0.1$), thus agreeing with the findings of Crum et al. (1993) on whelping success but not on litter size, where the latter found no effect, (even though they worked with heptachlor and not lindane). The proportion of mated mink with implantation sites was not affected by lindane treatment, however the number of implantation sites per mink tended to be lower in the lindane treated group ($P< 0.1$). Beard et al. (1995) also noticed a tendency in the lindane treated minks to whelp later than control mink ($P< 0.01$). From their findings therefore, they concluded that the decreased level of fertility in mink exposed to lindane was primarily a result of embryo mortality post implantation, but pre-implantation loss also tended to be greater following lindane exposure.

No adverse effects of heptachlor were reported on rabbits , chickens or beagle dogs on which the compound was tested according to WHO (1984). However, Hickey and Anderson (1968) suggest that high concentrations of heptachlor accumulation in the ecosystem could be responsible for the thinning of eggshell and reproductive failure of certain species of birds.

1.2.4. 3.2 Some non-reproductive effects of heptachlor

Ogata et al. (1989) demonstrated the effect of heptachlor, heptachlor epoxide and chlordane, the compound from which heptachlor is synthesised, on oxidative phosphorilation in rat hepatic mitochondria. Their findings were that respiration rate, RCR and ADP/O ratio were affected by chlordane-related compounds. The degree of respiratory inhibition was in the descending order of trans-chlordane, cis-chlordane, heptachlor and heptachlor epoxide. Of all the respiratory parameters they

investigated, they found that state 3 respiration was the most sensitively inhibited by these compounds suggesting that the compounds inhibit energy transfer. Their results also showed that heptachlor epoxide had less effect on mitochondria than heptachlor. In similar studies, Meguro et al. (1990) found results which substantially supported those of Ogata et al. (1989) when they used heptachlor at a concentration of 50 mM with succinate as substrate. However, at 100 mM, heptachlor with succinate as substrate suppressed both state 3 and 4 respiration almost completely. The mode of inhibition of succinate oxidation by heptachlor appeared to be a non-competitive one.

Izushi and Ogata (1990) studied the toxicity of heptachlor on mice following oral and intraperitonial administration. The results of their study showed increased levels of serum alanine aminotransferase and decreased levels of serum cholinestrase activity. Serum creatine phosphokinase levels however increased significantly. They suggested that all these changes may be due to disruption of muscle membrane by chlordane, the compound from which heptachlor is isolated. The results also showed significant variations of serum lipid levels from controls and which according to them would not be surprising since heptachlor has a known effect on lipid metabolism. Lipid peroxide levels were also increased and this showed heptachlor's role in causing liver injury. Based on these findings, Izushi and Ogata (1990) suggest that the liver and muscle injuries attributed to heptachlor is as a result of the deterioration of membranes due to lipid peroxidation.

The cyclodiene pesticides have been reported to be non-genotoxic rodent hepato carcinogens. When these three compounds and several metabolites of endosulfan (endosulfan sulphate, endosulfan ether and endosulfan lactone) were examined for their effects on gap junctional intercellular communication (GJIC) by Ruch et al. (1990), some interesting results were obtained. Endosulfan ether and endosulfan sulphate inhibited rat and mouse hepatocyte GJIC in a dose -responsive

manner. Although endosulfan ether also affected rat hepatocyte GJIC, it had no effect on mouse hepatocytes. On the other hand endosulfan lactone did not affect rat or mouse hepatocytes GJIC. However chlordane and heptachlor inhibited both mouse and rat hepatocyte GJIC at concentrations of 50-200mM. What is more interesting is that concomitant treatment of the cells with inhibitors of cytochrome P450 monooxygenases (e g. carbon monoxide) did not alter the inhibition of GJIC by the cyclodienes. This seems to suggest that cytochrome P450 metabolism was not involved in the inhibitory mechanism (Ruch et al., 1990). What is not clear however from these reports is whether there is a relationship between GJIC and hepatic carcinogenicity caused by the pesticides.

Lummis et al. (1990) examined the actions of heptachlor and heptachlor epoxide on GABA receptors in insects and vertebrates and found that both heptachlor and heptachlor epoxide blocked functional GABA receptors. The block appeared to be non-competitive and was voltage-independent over the membrane potential range of 75 to 110 mV. Moreover there was no significant difference between the potencies of heptachlor and its epoxide in the functional assays for insect GABA receptors. These findings provide further evidence for inhibition of insect GABA receptors/Cl- channel by the cyclodiene class of insecticides such as heptachlor and heptachlor epoxide.

In another study Willett et al. (1989) investigated the possible effect of halogenated hydrocarbons, namely heptachlor ,PCBs, DDT and pentachlorophenol (PCP) on rumen microorganisms. Their argußment or rationale for carrying out the study was that, while exposed cattle may only show the effects of exposed at high dose levels of these compounds, rumen microorganisms however, may be affected at lower doses, thus possibly affecting the cows growth and milk production. The study involved determining whether the chlorinated hydrocarbons were metabolised during fermentation in an *in-vitro* system. Dry matter disappearance was used as the

index for determining microbial activity in the presence of the chemical. The results indicated that there was no difference in dry matter disappearance between controls and in all concentrations of PCBs , heptachlor and DDT. However, PCP significantly depressed the percent dry matter disappearance in 50 ppm and 100 ppm cultures to 45% and 30% at 24 hr and 70% and 50% at 48 hr compared to the controls. The rest of the other compounds had dry matter disappearance of approximately 50% and 80% at 24 hr and 48 hr period respectively.

1.2.4.3.3 Heptachlor and related compounds as neoplasm promoters

Williams and Numoto (1984), demonstrated that the organochlorine pesticides particularly heptachlor and chlordane were liver neoplasm promoters in mice. Their study involved exposing male B6C3F1 mice to diethylnitrosamine (DEN) for 14 weeks followed by 25 weeks on either control diet, or either chlordane or heptachlor. The results indicated that the mice on DEN followed by control diet developed a 40% incidence of liver neoplasms whereas those given chlordane or heptachlor after the DEN had approximately 80% incidence of liver neoplasms. Results with chlordane and heptachlor were similar to those obtained with DDT, a positive reference compound. Williams and Numoto (1984) also found that mice exposed to DEN developed neoplasms of the stomach and lung. However the incidences of these neoplams were not increased by chlordane, heptachlor or any other exposure. None of the chemicals given alone for the last 25 weeks of the study increased the incidence of liver neoplasms and none given before DEN produced a syncarcinogenic effect, hence the authors' conclusion that the organochlorine pesticides were liver neoplasm promoters and not inducers.

In a followup study, Numoto et al. (1985), looked at the relationships between the gross appearance, histologic types and cytochemical characteristics of hepatocellular neoplasms in mice given the liver carcinogen, diethylnitrosamine either

alone or followed by the organochlorine pesticides such as heptachlor, chlordane, and DDT, as promoting agents. Their findings showed that acidophilic neoplasms more often displayed increased activity of alkaline phosphatase than did basophilic neoplasms, and that the activities of glucose-6-phosphatase and adenosine triphosphatase were decreased in both acidophilic and basophilic neoplasms.

Heptachlor and its metabolite heptachlor epoxide are thus unequivocally carcinogenic in rats and mice. In addition to the reports by Williams and Numoto (1984) and Numoto et al. (1985), Reuber (1987) also found that these compounds did induce carcinomas of the liver, and that the results were highly significant. Reuber (1987) further reported the presence of neoplasms at other sites including endocrine and reproductive organs in rats and that both benign and malignant neoplasms were increased in heptachlor treated male rats and the testes were atrophied. Mice also developed thrombosis in the hepatic vein and the atria of the heart.

1.2.5. Metabolism and kinetics of Heptachlor

Heptachlor is readily absorbed following ingestion and skin contact and is transported throughout the body. Heptachlor epoxide, the most persistent metabolite, is rapidly formed and can be found in the body, mainly in adipose tissue. Heptachlor and other organochlorine pesticides exert their effects more effectively perhaps due to their persistence in body tissues. For this reason several workers have studied the clearance of these compouds from the body. Rozman (1984) demonstrated that phase II enzyme induction altered the kinetics of heptachlor dramatically, it reduced the half-life of heptachlor by about 3-fold and that this reduction of the half-life was reflected in correspondingly lowered adipose tissue and blood levels. Scheufler and Rozman (1984) also looked at the effect of phase II

enzyme induction on heptachlor clearance. They used the phase II enzyme inducer namely trans-stilbeneoxide (TSO) and administered it at 400 mg/kg i.p.on 4 consecutive days and studied its effect on the clearance of intravenously given [^{14}C] heptachlor (2 mg/kg) in male Sprague-Dawley rats. Their results indicated TSO significantly increased cumulative excretion of heptachlor derived material in the feces but had no effect on total urinary excretion. Pre-treatment of rats with TSO 4 days prior to heptachlor injection resulted in elevated fecal excretion of ^{14}C immediately on day one whereas the effect of the TSO was not apparent in rats given TSO either immediately after heptachlor injection or four days following heptachlor injection. However the effects of TSO became apparent after 2 days and reached its maximum 3 days after the start of TSO administration. At termination of experiments, tissue radioactivity was significantly lower in fat, kidney, brain, skin, heart and muscle but not in serum, erythrocytes, liver or spleen of TSO treated rats as compared to controls. The body burden calculated from the sum of ^{14}C remaining in tissues was reduced to about 27% of control values.

The elimination kinetics of organochlorine compounds namely DDE, lindane, aldrin, heptachlor, and heptachlor epoxide, from day 2 to day 10 of breast feeding was studied by Klein et al (1986) in 39 volunteers who had babies in the maternity department in a hospital setting. Whereas DDT was below level of detection, contamination with DDE was high upto 112.6 ng/g fat content on day 2. This level however decreased rapidly to 36.6 ng/g by day 3. On day 2 contamination with lindane, aldrin, heptachlor epoxide and heptachlor averaged 43.5, 23.5, 25.4 and 7.4 ng/g fat respectively and the levels showed a linear decrease on subsequent days in all cases except that of heptachlor for which no significant negative correlation with time could be demonstrated.

Heptachlor metabolism and clearance was also studied by Smith et al. (1989) in ovines. Feces, urine, blood, bile, and body tissues were analysed for ^{14}C activity

following administration of [^{14}C] heptachlor i.p, at a dose of 1.643mg/kg body weight. Results obtained indicated that radioactivity associated with heptachlor and/or its metabolites, eliminated into excreta (urine and feces) during a 21 day period amounted to between 34% to 36% of dose administered. Of this, 67% appeared in urine and 33% in feces. These results, according to Smith et al. (1989), and compared to their previous work on bovines indicated that ovines eliminated heptachlor much more rapidly than bovines.

CHAPTER TWO

2.0 THE EFFECT OF HEPTACHLOR ON MATING SUCCESS, GESTATION LENGTH, LITTER SIZE AND BODY WEIGHT

2.1 Introduction

Reports exist regarding the effect of organochlorine pesticides on reproductive parameters such as mating success, gestation length, litter size and even body weights of experimental animals. Organochlorines such as dieldrin, methoxychlor,lindane and DDT have been shown to adversely affect the litter size in rats and mice (Odler, 1973; Virgo and Bellward, 1977; Cummings and Gray, 1989; Sircar and Lahiri, 1989; Uphouse and Williams, 1989).

Aulerich et.al. (1990) fed mink (*Mustela vison*) with diets containing 0, 12.5, 25, 50, or 100 mg/kg active ingredient heptachlor (as technical grade formulation), for 28 days followed by a 7-day observation period to determine the toxicity of this insecticide. Their results indicated that diets containing 25 mg/kg or more heptachlor, resulted in a significant decrease in feed consumption, while 50 mg/kg or more heptachlor, caused a significant reduction in body weights. Mortality (37.5%) occured only in the group fed 100 mg/kg. This dose level also caused reduction in the relative weights of the spleen and kidney, and also increased relative weight of the adrenal glands when necropsied at the time of death or at the termination of the study.

Crum et al. (1993), doing similar studies, fed adult female minks with 6.25, 12.5 and 25.0 mg/kg of heptachlor. These dose levels resulted in significant reduction in feed consumption and body weights of the female mink. Mortality was 0% for the control group, 8% for the 6.25 mg/kg group, 67% for the 12.5 mg/kg group and 100%, for the 25.0 mg/kg group. Whelping success rates were affected in a dose related manner. Mink fed on the two higher heptachlor diets displayed clinical signs indicative of central nervous system impairment prior to death. Beard et al. (1995), also working with mink, showed that lindane in the diet of these animals resulted in increased embryo mortality. Data on the effect of heptachlor (one of the more toxic organochlorine pesticides) on various reproductive parameters are thus few and remain contradictory (Radeleff, 1970).

The aim of this experiment therefore was to establish the effect of heptachlor on mating success, gestation length, litter size and body weights of adult female rats. In these studies heptachlor administration was by subcutaneous injection. Heptachlor is a stomach and contact insecticide. Most investigators administer it orally. Considering that the compound is rapidly transformed into its epoxide within the liver, it is possible that the transformation may alter its ability to affect reproductive function. When given parenterally therefore, it may reach target organs and tissues in its unmetabolised form in high enough concentrations to show immediate effects.

2.2 Materials and Methods

Animals and Housing

Adult female rats (Sprague-Dawley, aged 3 to 4 months) were used in all the experiments. All the rats were caged in pairs in plastic bottomed rat cages with wire tops (45 cm x 28 cm x 21 cm) in all experiments. Male rats were kept in

the same room but in different cages. All rats had access to water and rodent pellets *ad libitum*
(obtained from Kenya Grain Growers Union, Nairobi). The bedding, consisting of wood shavings and sawdust, were changed every day. The animals were subjected to natural lighting conditions 12 :12 hr light: darkness, and average room temperatures of 23°C.

Chemicals

Analytical grade heptachlor (99.2%) was obtained from Bie and Barntsen Ltd, Sandbaekvej, Copenhagen, Denmark.

Methods

Control/baseline study

Ten 4-month old virgin female rats were used for this study. The animals were caged (2 per cage) to avoid overcrowding when they litter, and allowed plenty of water and rodent pellets. The animals were allowed ten days acclimatization before being mated. Male rats were then introduced into the female cages (2 per cage) after the 10 days.

Vaginal smears were taken from all the animals by swabbing daily from day 1 until pregnancy was suspected. Day of pregnancy and hence 1st day of gestation was taken to be the day spermatozoa were detected in the vaginal smears under the light microscope.

The females were monitored daily by weighing until they littered. The litter size as well as gestation length for all the animals was recorded.

Control study (saline)

Ten 4-month old virgin female rats were used for this study. The animals were separated (2 per cage) and allowed water and rodent pellets *ad libitum* . After a ten day period of acclimatization, the animals received subcutaneous injections of 250µl pysiological saline solution (0.85% NaCl) daily for a period of 14 days. The aim of this control study was to find the effect of injecting the animals every other day with a vehicle. Male rats, which were kept in the same room but in separate cages were then introduced into the female cages (2 per cage) after the 14 days.

Vaginal smears were taken from all the animals by swabbing daily from day 1 until pregnancy was suspected. Day of pregnancy and hence 1st day of gestation was taken to be the day spermatozoa were detected in the vaginal smears.

The females were monitored daily by weighing until they littered. The litter size as well as gestation length for all the animals was recorded.

Control study (corn oil)

The third control study was a repeat of the second control study except that the experimental animals (4 month old female rats) were injected subcutaneously with 250µl corn oil instead of physiological saline. Corn oil was used here as it was subsequently employed as the vehicle for heptachlor in the test animals.

The animals were injected daily for 14 days after which the males which were hitherto kept in same room but separate cages, were introduced into the

female cages (2 males per cage), and the rest of the exercise carried out as outlined above.

Experiment with heptachlor

Five groups of ten 4-month old virgin female rats were used for this study, one group at a time. The animals were caged (2 per cage) and allowed plenty of water and rodent pellets. The animals were monitored daily for ten days by vaginal swabs to ascertain cyclic normality before treatment. The animals were then injected subcutaneously with heptachlor in corn oil at a dose rate of 5 mg/Kg, and 20, 25, 30 and 40 mg/kg body weight for group one, two, three, four and five respectively, every 24hrs for 14 days that is the injections were done on days 1, 3, 5, 7, 9, 11, and13. This regime was adopted in order to minimize the effect of injections and wound development in the injected areas. The dose ranges were pilot experiments to ditermine dose response. Male rats (kept separately but in the same room) were then introduced into the female cages (2 per cage) after the 14 days treatment period. The rest of the exercise was carried out as outlined above.

It should be noted here that the several dose levels done here were necessary in order to establish the lowest effective dose and the highest non-lethal dose at which to operate.

2.3 Results

The results indicate that mating success, gestation lengths, and litter size were all affected by heptachlor.

Table 1 gives the results of the first control study using neither saline nor corn oil. All the females mated successfully within the first four days (with a mean ± SEM of 3.30 ± 0.25) of being paired with males. The mean ± SEM gestation length was 22.1 ± 0.23days and the mean litter size was 7.8 ± 0.70.

Table 2 shows the results of the second control study using saline. All the females mated successfully within the 1st four days (with a mean ± SEM of 3.20 ± 0.25) of being paired with males. The mean ± SEM gestation length was 22.6 ± 0.27 days and the mean litter size was 8.3 ± 0.87

The results of the second control study using corn oil are given in table 3. Again all the females mated successfully within the first five days (with a mean ± SEM of 3.60 ± 0 37) of being paired with males. Mean ± SEM gestation length was 22.7 ± 0.15 days while the mean ± SEM litter size was 6.9 ± 0.69.

The results of using heptachlor at 5 mg/kg body weight are given in Table 4. All the females mated successfully within the first four days (with a mean ± SEM of 3.00 ± 0.21) of being paired with males. The mean ± SEM gestation length was 23.00 ± 0.21 days and the mean ± SEM litter size was 6.00 ± 0.54.

Table 5 gives the results of using heptachlor at 20 mg/kg body weight. The day of mating ranged from day 3 to day 10 (mean ± SEM of 7.10 ± 0.71) with eight rats mating more than 6 days after the introduction of a male. Gestation length ranged from 23 to 32 days (mean ± SEM of 25.10 ± 0.91) while the mean ± SEM litter size was4.7 ± 0.40.

When heptachlor was used at 25 mg/kg body weight, only five of the original ten animals were still alive at the end of the treatment period and hence only five animals were paired with males (Table 6). These five animals however mated successfully within seven days (with a mean ± SEM of 5.20 ± 0.73) of being caged with the males. The mean ± SEM gestation length was 24.00 ± 0.54 days and the litter size was 5.80 ± 0.66 (mean ± SEM).

Results for doses 30 mg/kg and 40 mg/kg were not obtained for this particular experiment as all the animals died before they could be mated. For the 30 mg/kg group, 5 animals survived the treatment period but were all dead by day 18 (i.e. three days after end of treatment) whereas in the 40 mg/kg group all animals were dead by the 13th. day (i.e. a day before end of treatment).

The results obtained when the rats were injected with either pysiological saline or corn oil compare very well with the controls which were not injected, as there was no significant difference between them, suggesting that neither saline nor corn oil injections interfered with the normal reproductive cycles and behaviour. Corn oil was used as the vehicle for heptachlor. Comparisons are therefore made between heptachlor treated animals and corn oil-treated controls.

The results of injecting heptachlor at 5 mg/kg body weight seem to compare well with those obtained for the controls, suggesting that heptachlor at 5 mg/kg body weight has no marked effect on mating success or gestation length. There was however a significant reduction in litter size ($P<0.05$) between controls and heptachlor treated animals. Heptachlor at higher doses gave results that markedly deviated from those of controls, for instance the shortest gestation length (21 days) was observed in a saline treated control rat and the longest (32 days) in one animal treated with heptachlor at 20 mg/kg body weight. Animals treated with 20 mg/kg body weight of heptachlor had mean gestation length which were significantly different from those of corn oil-treated controls ($P<0.05$), but not significantly different from rats treated with heptachlor at 5 mg/kg body weight.

Litter sizes also varied with the concentration of heptachlor with high doses resulting in significantly smaller numbers of pups ($P<0.01$). Furthermore, the proportion of offspring still alive by weaning time was lower for rats treated with

20 and 25 mg/kg body weight than for the other experimental groups. The figures were however not statistically different.

Heptachlor at 20 mg/kg body also resulted in a significant increase ($P<0.001$) in the time it took for the animals to mate when compared to controls.

Figure 1 shows the variation in the weights of both the control and heptachlor treated animals. There is no marked loss of weight in the heptachlor treated non pregnant animals at 5 mg/kg, but the 20 mg/kg group showed a significant ($P< 0.05$) decrease in weight as the experiment continued. There is however remarkable drop in weight at the 25, 30 and 40 mg/kg dose levels. These results indicate that heptachlor had little effect on the weights of the experimental animals at low dose levels but had a marked effect at higher doses.

The effect of heptachlor on body weights of pregnant rats is given in Figure 2. The weights of all pregnant animals increased with age of gestation. However the weights of heptachlor treated animals were not significantly different from those of controls.

Table 1.

Mean weights, gestation length and litter size of control animals not injected with either saline or corn oil. All the females mated successfully within the 1st four days.

Animal	Mean Wt. (gm) ± SEM.	Day mated	Gestation length(days)	Litter size
1	230.90±5.90	4th day	22	5
2	236.12±3.64	3rd day	22	10
3	242.70±3.30	4th day	21	5
4	222.15±8.05	3rd day	23	9
5	210.15±10.00	2nd day	22	12
6	233.65±3.94	3rd day	23	8
7	208.26±10.76	4th day	22	8
8	223.11±8.36	2nd day	22	6
9	230.44±5.40	4th day	23	8
10	239.45±3.00	4th day	21	7
Mean ±SEM	227.77±3.70	3.30±0.25	22.1±0.23	7.8±0.70

Litter total 78

Males 40

Females 38

Numbers surviving until weaning 78 = 100%

Table 2.

Mean weights, gestation length and litter size of control animals using saline. All the females mated successfully within the 1st. four days after introducttion of males.

Animal	Mean. Wt. (gm) ± SEM.	Day mated	Gestation length(days)	Litter size
1	250.09±6.51	3rd day	22	13
2	239.20±6.99	3rd day	22	7
3	226.70±4.12	4th day	24	8 1 died
4	224.19±5.38	3rd day	23	10
5	243.68±3.48	4th day	23	9
6	243.62±3.94	3rd day	23	8
7	244.60±4.97	2nd day	23	3
8	246.01±4.83	2nd day	22	8
9	220.04±4.24	4th day	23	6
10	229.92±4.99	4th day	21	11
Mean ±SEM	236.81±3.35	3.20±0.25	22.6±0.27	8.3±0.87

Litter total 83

Males 40

Females 43

Number surviving up to weaning 82 = 99%

Table 3.

Mean weights, gestation length and litter size of control animals using corn oil. All the females mated successfully within the first 5 (five) days of being caged with males.

Animal	Mean. Wt. (gm) ± SEM.	Day mated	Gestation length (days)	Litter size
1	237.26±4.53	3rd day	23	7
2	224.90±5.40	2nd day	23	6
3	221.35±4.13	5th day	22	5
4	239.88±4.59	4th day	23	7
5	245.88±7.29	2nd day	23	9
6	221.02±6.96	4th day	22	8
7	216.74±4.45	3rd day	23	3
8	251.80±5.49	5th day	22	11
9	246.82±6.88	3rd day	23	7
10	226.17±4.20	5th day	23	6
Mean ±SEM	233.18± 3.99	3.60±0.37	22.7±0.15	6.9±0.69

Litter total 69

Males 29

Females 40

Number surviving up to weaning 69 = 100%

Table 4.

Mean weights, gestation length and litter size of experimental animals injected with heptachlor at 5 mg/Kg body weight. All the females mated successfully within the first 4 days of being caged with males.

Animal	Mean Wt. (gm) ± SEM.	Day mated	Gestation length (days)	Litter size
1	242.03±6.99	3rd day	23	5
2	235.88±10.90	3rd day	23	8
3	241.08±10.09	4th day	23	6
4	238.84±9.35	4th day	23	5
5	218.12±10.86	3rd day	24	5
6	220.84±7.48	2nd day	22	6
7	226.79±7.17	3rd day	24	5
8	244.41±9.87	2nd day	22	5
9	238.62±10.30	3rd day	23	5
10	214.33±5.22	3rd day	23	10
Mean ±SEM	232.09±3.50	3.00±0.21	23.00±0.21	6.00±0.54

Litter total	60	
Males	26	
Females	34	
Number surviving up to weaning	60	= 100%

Table 5.

Mean weights, gestation length and litter size of experimental animals injected with heptachlor at 20 mg/Kg body weight. The day of mating ranged from day 3 to day 10.

Animal	Mean Wt. (gm) ± SEM.	Day mated	Gestation length (days)	Litter size
1	207.07±6.72	4th day	23	4
2	208.48±9.42	7th day	23	6
3	233.52±4.15	8th day	23	4 one died
4	210.44±8.92	10th day	27	4
5	225.38±10.82	7th day	23	5
6	219.24±8.41	3rd day	32	6
7	220.56±4.64	8th day	25	3
8	203.40±7.91	10th day	27	7
9	228.87±10.07	7th day	24	4
10	230.08±4.66	7th day	24	4
Mean ±SEM	218.70±3.41	7.10±0.71	25.10 ±0.91	4.7±0.40

Litter total	50	
Males	28	
Females	22	
Number surviving up to weaning	47	= 94%
Males	26	
Females	21	

Table 6.

Mean weights, gestation length and litter size of heptachlor treated animals at 25 mg/Kg The five surviving animals mated successfully within seven days.

Animal	Ave. Wt. (gm) ± SEM.	Day mated	Gestation length (days)	Litter Size
1	Dead			
2	Dead			
3	244.73±7.50	4th day	23	5
4	Dead			
5	221.87±4.11	6th day	24	6 mother dead all pups dead
6	226.50±4.55	6th day	26	8 4 dead
7	231 93±5.58	3rd day	23	4
8	Dead			
9	241.90±6.92	7th day	24	6
10	Dead			
Mean ±SEM	233.39±4.37	5.20±0.73.	24.0±0.54	5.8±0.66

Litter total	29	
Males	16	
Females	13	
Number surviving up to weaning	19	= 65.5%
Males	9	
Females	10	

Figure 1.

The variation of the mean weights of both the control and heptachlor treated animals. The results indicate that heptachlor had little effect on the weights of the experimental animals at low dose levels but had a marked effect at doses above 20 mg/kg.

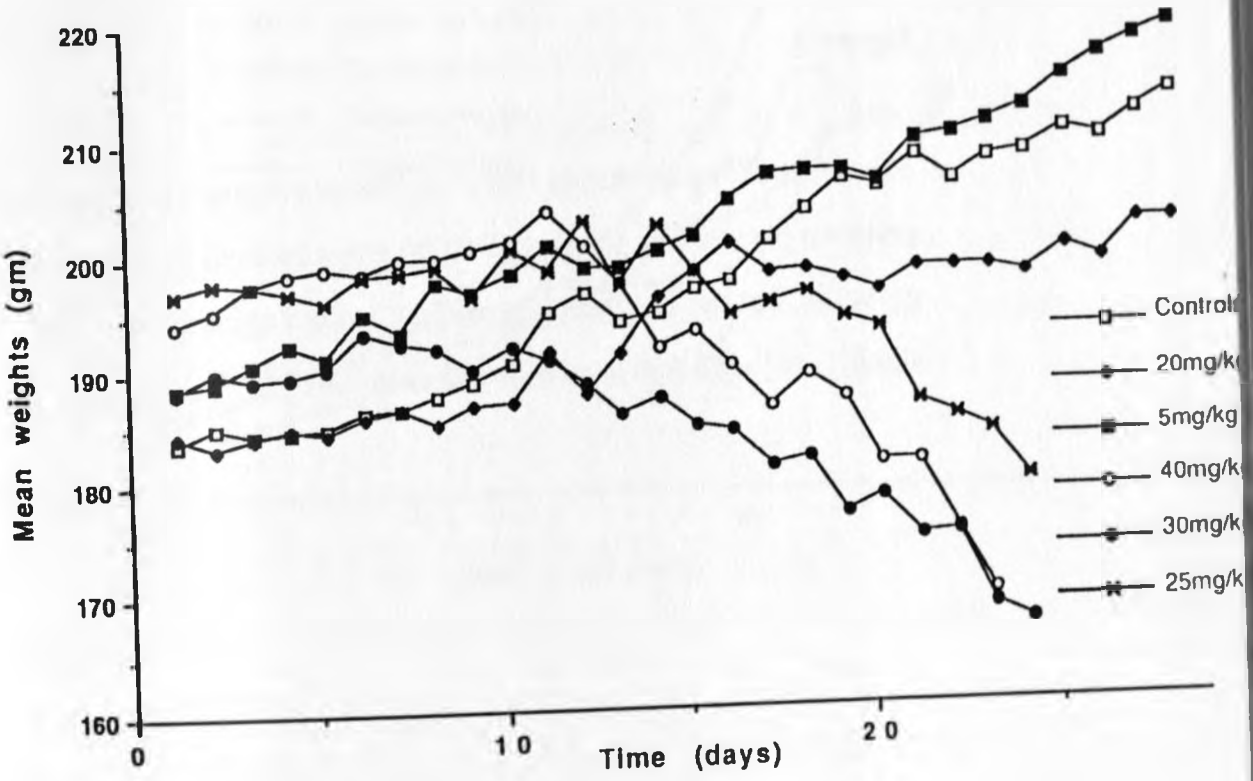
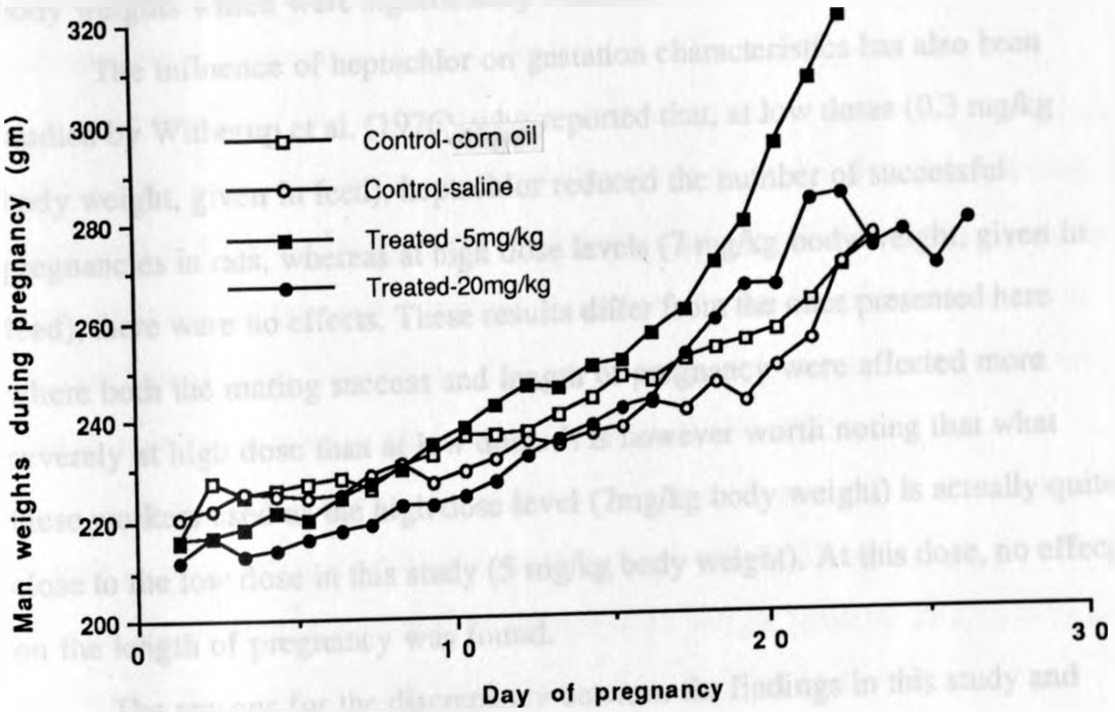

220
210
200
190
180
170
160
Mean weights (gm)
0
10
20
Time (days)
Control
20mg/k
5mg/kg
40mg/k
30mg/k
25mg/k

Figure 2.

The effect of heptachlor on body weights of pregnant rats throughout gestation period. The weights of all pregnant animals increased with age of gestation, but the weights of heptachlor treated animals were not significantly different from those of controls.


Man weights during pregnancy (gm)
320
300
280
260
240
220
200
0
10
20
30
Day of pregnancy
Control-corn oil
Control-saline
Treated -5mg/kg
Treated-20mg/kg

2.4 Discussion

The results obtained in this study show that heptachlor, when injected subcutaneously in female rats, has a dose-related influence on several reproductive parameters such as mating success which was decreased, gestation length which was lengthened, litter size which was significantly reduced and body weights which were significantly reduced.

The influence of heptachlor on gestation characteristics has also been studied by Witherup et al. (1976), who reported that, at low doses (0.3 mg/kg body weight, given in feed), heptachlor reduced the number of successful pregnancies in rats, whereas at high dose levels (7 mg/kg body weight, given in feed), there were no effects. These results differ from the ones presented here where both the mating success and length of pregnancy were affected more severely at high dose than at low dose. It is however worth noting that what these workers used as the high dose level (7mg/kg body weight) is actually quite close to the low dose in this study (5 mg/kg body weight). At this dose, no effect on the length of pregnancy was found.

The reasons for the discrepancy between the findings in this study and those of Witherup et al. (1976) cannot be explained. However, the rapid transformation of heptachlor to its metabolites when it passes through the liver after oral administration, may alter its ability to affect reproductive function. According to this hypothesis, it would appear that heptachlor when given parenterally, may reach target organs and tissues in its unmetabolised form in high concentrations. It is possible that, in its unmetabolised, heptachlor has more potent effects on reproductive parameters than its metabolites (Reuber, 1987). All these however, do not explain the fact that Witherup et al. (1976), found that heptachlor at a dose as low as 0.3 mg/kg body weight resulted in a decrease in the number of successful pregnancies.

Other studies in rats have shown that heptachlor interferes with pregnancy by causing foetal resorption (Cerey and Ruttkay-Nedecka, 1971; Ruttkay Nedecka et al., 1972), which may result in smaller litter sizes (Mestitzova, 1967; Odler, 1973; Oduma et al., 1995a), while comparable research in rabbits failed to demonstrate any heptachlor-related lesions during pregnancy (Wazeter et al., 1969). More recently, Beard et al. (1995), demonstrated that Lindane, causes increased embryo mortality in mink (Mustela vison).

The results of this study also indicate that pre-weaning survival rates were slightly reduced in the young of animals treated with high doses of heptachlor. Mestitzova (1967), reported similar results and suggested that this effect could be due to ingestion of heptachlor in milk. Apparently beagle dogs are not prone to this problem as it has been shown that pre-weaning pups survive relatively well even if their mothers are treated with heptachlor (IRDC, 1973).

Reports regarding the effects of heptachlor on body weights of female animals in several species still needs further clarification. In beagle dogs, for instance, dietary heptachlor did not change body weight patterns (IRDC, 1973). It is important to note that the latter studies have been severely critisized by Epstein (1990). In the mink however, significant decreases in weight were observed by Aulerich et al. (1990) and Crum et al. 1993). The data presented here indicate that heptachlor administered subcutaneously in rats caused a slowing down of increases in weight in non-pregnant but not in pregnant animals. It may be speculated here that during pregnancy, the rapid increases in uterine and foetal weights probably overshadowed the inhibitory effects of heptachlor on weight gain seen in non-pregnant rats. The difference in the effect of heptachlor on body weights of the different animals is difficult to explain but it is possible that the response to heptachlor is species-specific.

In conclusion, the results of this study suggest some disruptive effects of heptachlor on the rat reproductive system when the drug is injected subcutaneously into the animals.

CHAPTER THREE

3.0 THE EFFECT OF HEPTACHLOR ON ESTROUS CYCLES AND REPRODUCTIVE HORMONE PROFILES

3.1 Introduction

It has been shown in several studies (cited in previous Chapter) that chlorinated hydrocarbon pesticides and insecticides have toxicological effects on reproductive parameters such as litter size and litter viability in rats and mice. It was also shown more specifically in Chapter 2 that heptachlor lowers the litter size and litter viability in rats. However, the mechanisms by which these organochlorines interfere with reproductive parameters have not yet been elucidated. As indicated by Wassermann et al.(1974), Trapp et al. (1984) and Johnson et al. (1988), there are several possible mechanisms by which organochlorine pesticides may affect reproductive endocrinology. They can alter the response to reproductive steroids, they can affect enzyme functions or they can mimic reproductive hormones and thus interfere with their functions.

The objective of this experiment was to study the effect of heptachlor on estrous cycles and to relate this with progesterone and estradiol hormone profiles.

3.2 Materials and Methods

Animals and Housing

The animals used and conditions of their housing was the same as in Chapter 2.

Chemicals

Analytical grade heptachlor (99.2%) was obtained from Bie and Barntsen Ltd, Sandbaekvej. Reagents for radioimmunoassay (RIA) of progesterone and estrogen were provided by the WHO Matched Reagents.

Methods

Control /baseline study.

Ten 4 month old virgin female rats were used for this study. The animals were divided into two groups (5 per cage) for ease of handling, and allowed plenty of water and rodent pellets *ad libitum*. These females were not mated even though male rats were kept in the same room but in separate cages. The animals were monitored daily for the first 10 days for normal cycles before being put on a daily injection of 250 µl corn oil subcutaneously for 16 days. Vaginal wash samples were collected every day in saline solution from all the animals using a blunt tipped dropper. These were examined immediately for the specific cytological features that distinguish the four stages of the estrous cycle in the rat, namely diestrous, proestrous, estrous and metestrous. These findings were carefully recorded daily and for each female. Additionally, the animals were weighed daily.

At the end of the 16 day, the animals were bled by cardiac puncture and blood samples were collected into tube containing EDTA from the The blood sampling was extended into the 17th. day in order to obtain samples for each of the four estrous cycle stages.

After centrifugation, the plasma samples were collected and stored at -20°C until assayed for progesterone and estradiol by radioimmunoassay (RIA) in accordance with the methodology given in the WHO laboratory manual for RIA of reproductive hormones (WHO, 1989/90).

Experiment using heptachlor

Five groups of ten 4-month old virgin female rats were used for this study. The animals were separated (2 per cage) and allowed plenty of water and rodent pellets *ad libitum*. The animals were monitored daily for the first 10 days using vaginal smears to ascertain normal cyclicity. After this initial period they were injected subcutaneously with heptachlor in corn oil at a dose rate of 5, 20, 25, 30 and 40 mg/kg, body weight for Group I, II, III, IV and V respectively, every other day for 18 days. Vaginal wash samples were collected every day in saline solution from all the animals using a blunt tipped dropper. These were examined immediately for the specific cytological features that distinguish the four estrous stages in the rat cycle already mentioned for the controls. The findings were recorded for each female. These animals were also weighed daily.

At the end of the treatment period, blood samples were collected as already stated for the baseline control group. After centrifugation, the plasma samples were aliquoted and stored at -20°C. These were later assayed for progesterone and estradiol hormones by RIA (WHO laboratory Manual,1989/90)

3.3 Results

Figures 3, 4, 5, 6, 7 and 8, give the results of the cyclicity of the controls and heptachlor treated rats (dose 5, 20, 25, 30, and 40 mg/kg respectively) as monitored by vaginal cytology. It can be seen that all the control animals were having 4 day estrous cycles with the exception of a few instances where the previous stage seemed to extend into the next day. The results suggest that corn oil injections, neither blocked nor interfered with the estrous cycles of the rats. Heptachlor however seemed to intefere with the cycles as early as the fifth day of treatment with a marked disruption of the cycles towards the end of the treatment period for the 20, 25, 30, and 40 mg/kg dose groups. The low dose group (5 mg/kg body weight) shows minimal disruption of the cycles, indicating that the effect of heptachlor is dose dependent.

Figure 9 is the progesterone hormone profile of the estrous cycle of the control animals and experimental animals at 5, 20, 25, 30, mg/kg dose level. Plasma progesterone in corn oil-treated controls ranged from 16.2 ± 3.9 nmol/l during the estrous stage of the cycle to 5.1 ± 0.8 nmol/l during proestrous. These results indicate that heptachlor at all dose levels suppressed plasma progesterone with the most suppressive effects ($P<0.001$) being seen at diestrous and proestrous of all treated groups compared to controls. The effects of heptachlor on progesterone production during estrous ($P<0.01$) and metestrous ($P<0.001$ at the 20 mg and 25 mg/kg doses and $P<0.05$ at 30 mg/kg dose) stages were slightly less significant than for the other stages.

Figure 10 is the estradiol hormone profiles of the estrous cycle of both control and heptachlor treated animals at the same dose levels as above. Plasma estradiol in corn oil-treated control rats ranged from 741.7 ± 162.2 pmol/l at diestrous to 320.3 ± 59.9 pmol/l at metestrous. Heptachlor at all the dose levels

caused significant suppression in plasma estradiol during all stages of the estrous cycle. The greatest effects ($P<0.001$) were seen in the estrous and metestrous stages. Suppression of estradiol production was significant ($P<0.001$) at all dose levels of heptachlor during the estrous phase whereas at metestrous, 20 mg, 25 mg, 30 mg per kg body weight of heptachlor resulted in more significant suppression ($P<0.001$) than 5 mg per kg body weight of heptachlor. The inter-assay coefficients of variations ranged between 7-12% in all the assays.

It was noted also that in the groups of animals receiving 25 mg and 30 mg per kg body weight of heptachlor, both diestrous and proestrous stages could not be detected in the former, and no proestrous in the latter by the end of the treatment period. Therefore hormone values (both estradiol and progesterone) could not be determined.

The control/baseline studies formed the background upon which experiments with heptachlor were done. Deviations from these findings as seen during heptachlor treatment were therefore taken to be due to the heptachlor or its epoxide.

Figure 3.

Estrous cycle patterns in corn oil treated controls. The regular 4-day cyclical pattern is shown. D = diestrous, P = proestrous, E = estrous and M = metestrous.

ANIMAL
1
2
3
4
5
6
7
8
9
10
D
P
E
M
DAY
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
INJECT
D= Diestrous
P= Proestrous
E= Estrous
M= Metestrous
TREATMENT PERIOD
CONTROL PERIOD

Figure 4.

Estrous cycle patterns in heptachlor treated rats at dose 5 mg/kg body weight. The 4-day cyclical pattern is not significantly altered. D = diestrous, P = proestrous, E= estrous and M = metestrous.

ANIMAL
1
2
3
4
5
6
7
8
9
10
D P E M
DAY
1 2 3 4 5 6 7 8 9 10
INJECT
11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
D = Diestrous
P = Proestrous
E = Estrous
M = Metestrous
TREATMENT PERIOD
CONTROL PERIOD

Figure 5.

Estrous cycle patterns in heptachlor treated rats at dose 20 mg/kg body weight. The disrruption of the cyclical pattern is shown. D = diestrous, P = proestrous, E = estrous and M = metestrous.

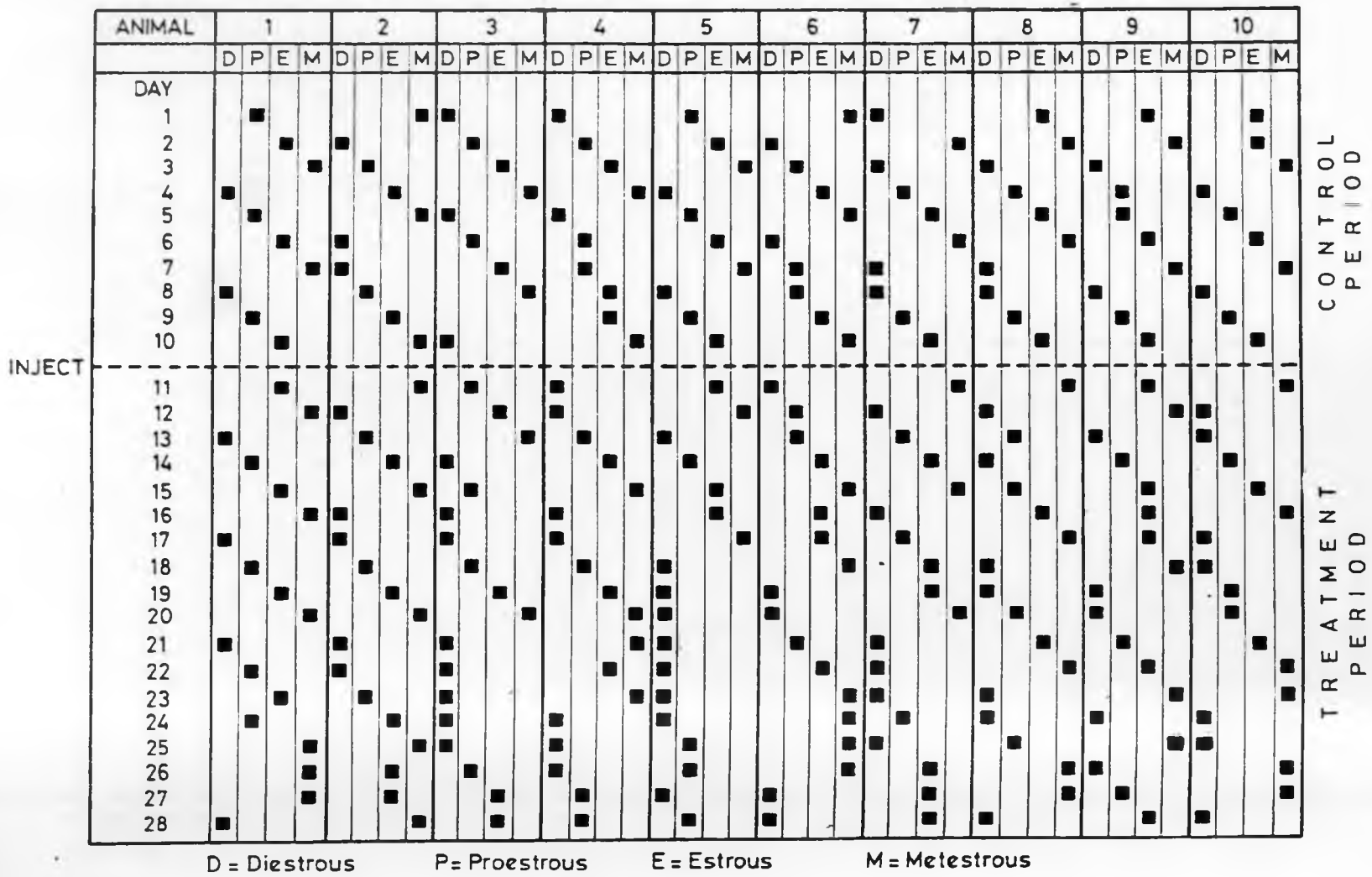

ANIMAL
1
2
3
4
5
6
7
8
9
10
D P E M
DAY
1
2
3
4
5
6
7
8
9
10
INJECT
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
CONTROL PERIOD
TREATMENT PERIOD
D = Diestrous
P= Proestrous
E = Estrous
M = Metestrous

Figure 6.

Estrous cycle patterns in heptachlor treated rats at dose 25 mg/kg body weight. The disrruption of the cyclical pattern is shown. D = diestrous, P = proestrous, E = estrous and M = metestrous.

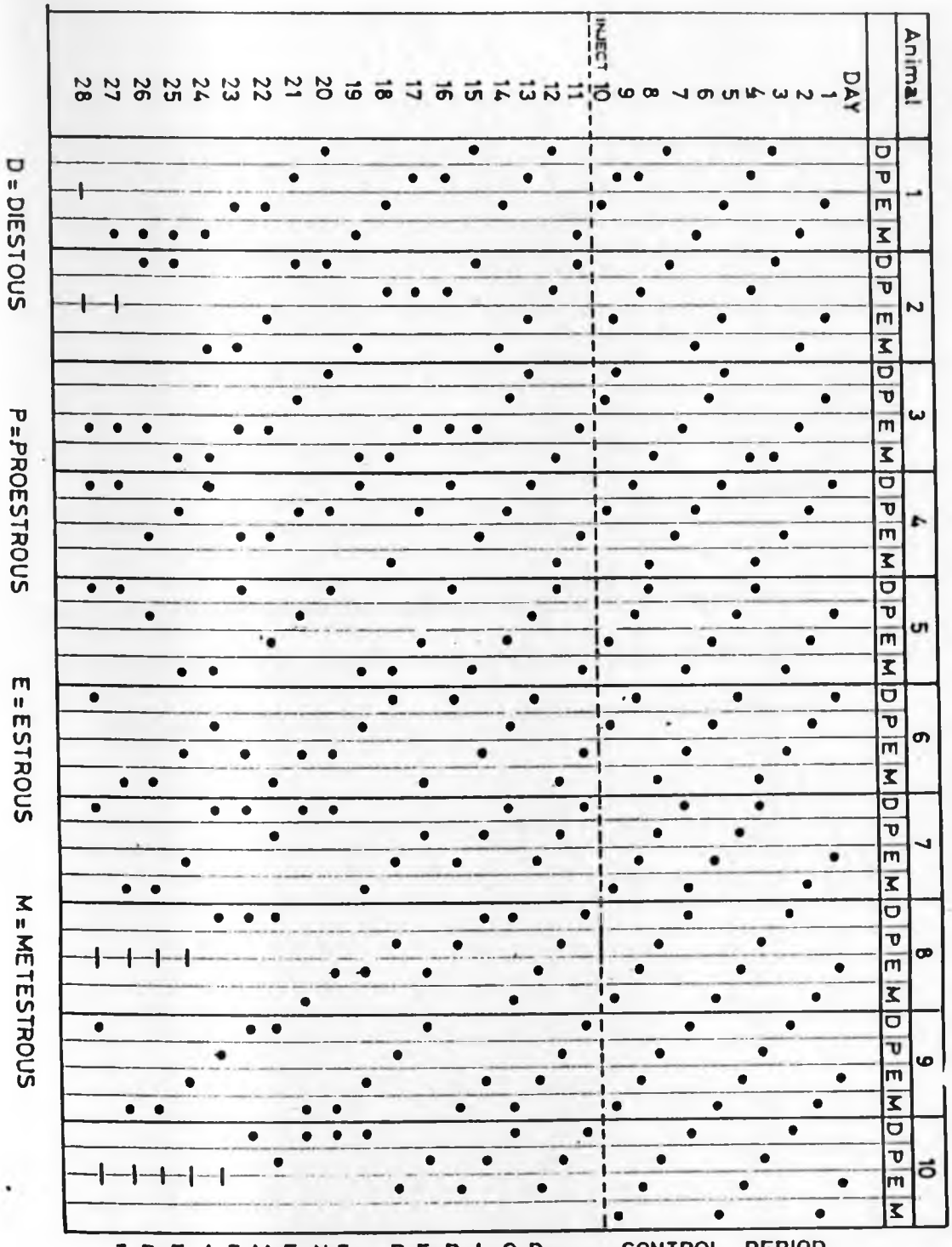

Animal
DAY
INJECT
D = DIESTOUS
P = PROESTROUS
E = ESTROUS
M = METESTROUS
TREATMENT PERIOD
CONTROL PERIOD

Figure 7.

Estrous cycle patterns of rats treated with heptachlor at dose 30 mg/kg body weight. Again the disrruption of the cyclical pattern is clearly seen. D = diestrous, P = proestrous, E = estrous and M = metestrous.

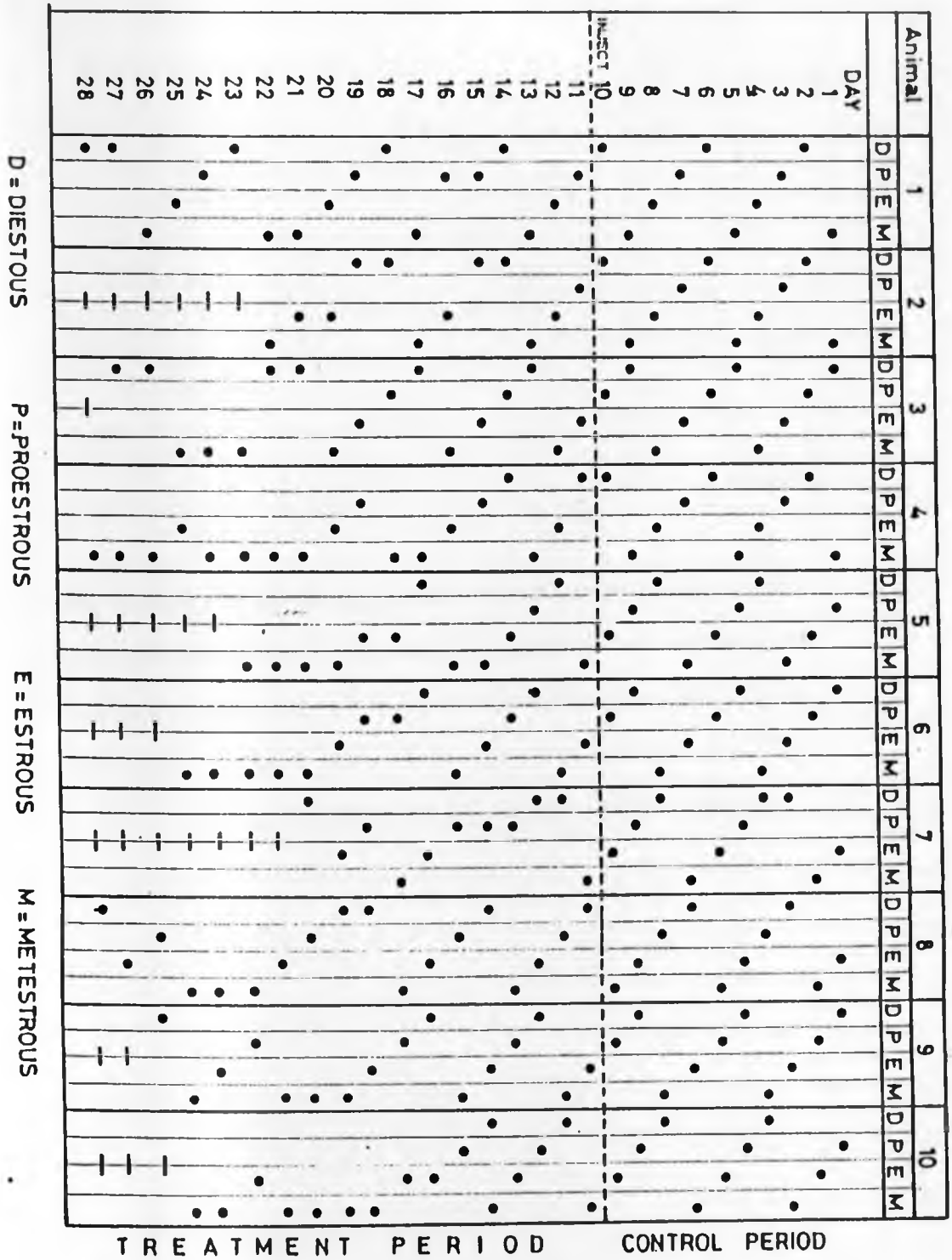

Animal
DAY
INJECT
D = DIESTOUS
P = PROESTROUS
E = ESTROUS
M = METESTROUS
TREATMENT PERIOD
CONTROL PERIOD

Figure 8.

Estrous cycle patterns in rats treated with heptachlor at dose 40 mg/kg body weight. The estrous cycle patterns are completely disrupted from as early as the fourth day of treatment. At this dose level mortality was also very high indicated by (-) against the cycle stage. D = diestrous, P = proestrous, E = estrous and M = metestrous.

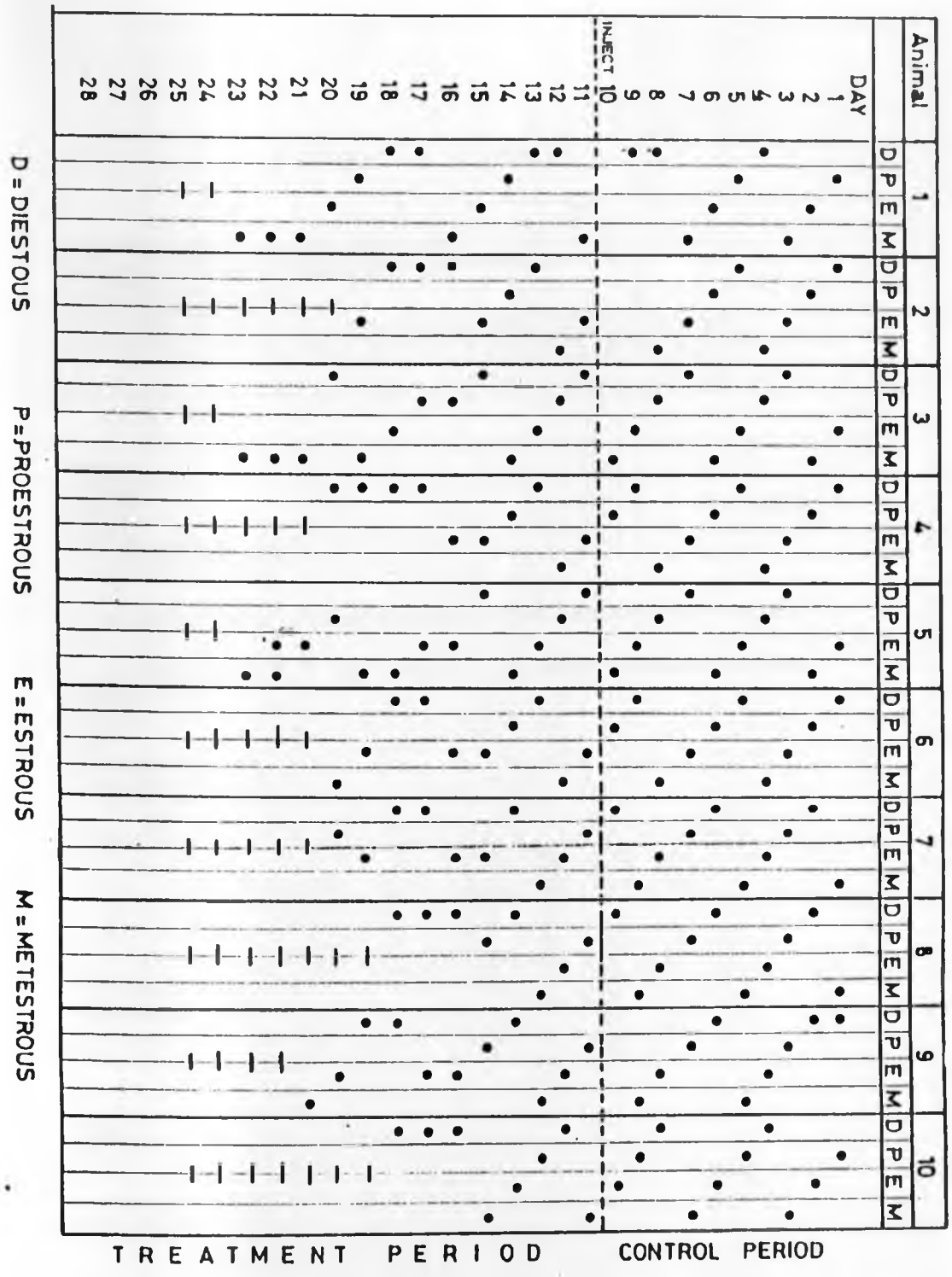

Animal
DAY
INJECT
D = DIESTOUS
P = PROESTROUS
E = ESTROUS
M = METESTROUS
TREATMENT PERIOD
CONTROL PERIOD

Figure 9.

Plasma progesterone levels in corn oil-treated control rats and rats treated with various concentrations of heptachlor. Values are means ± SEM.

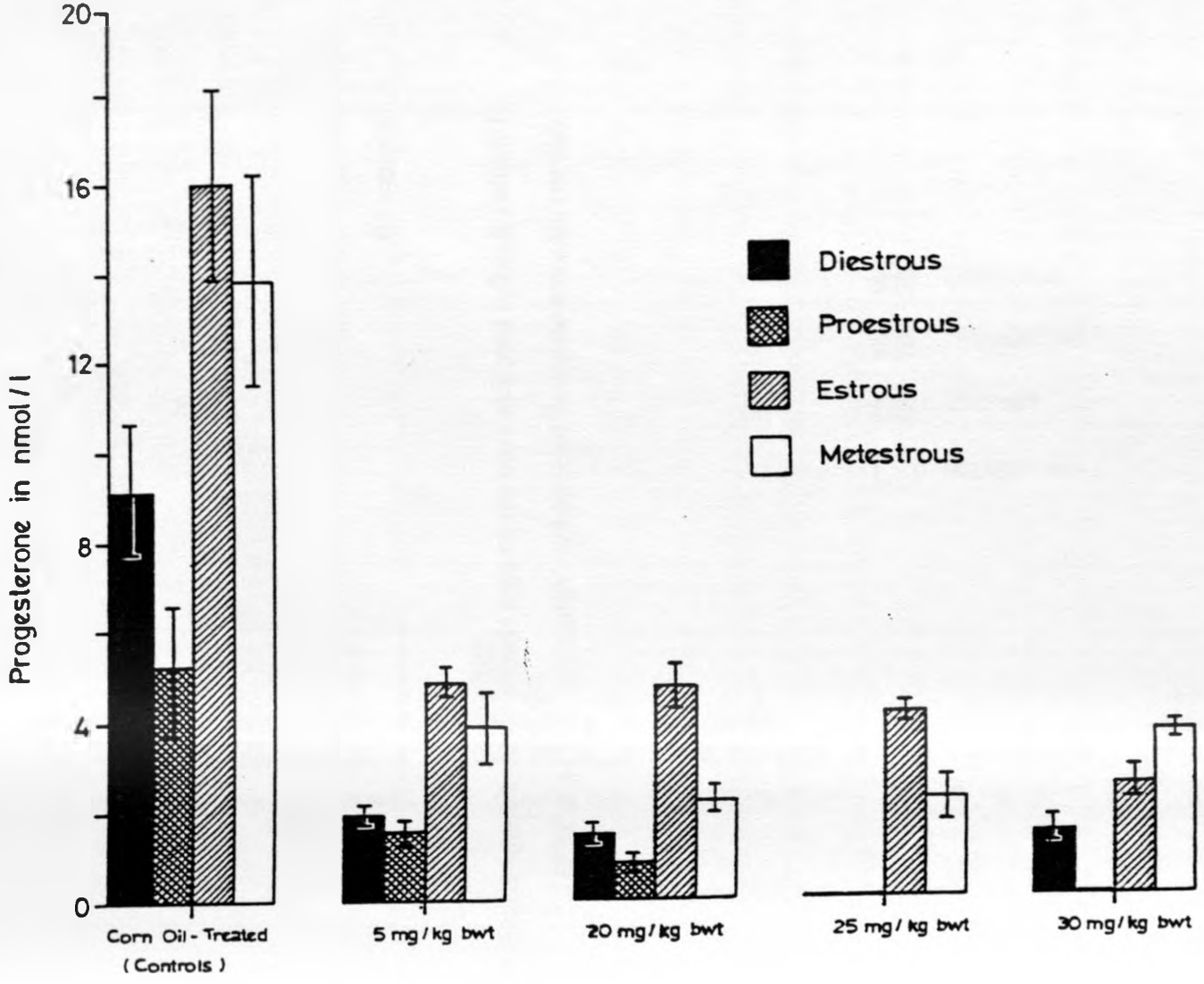
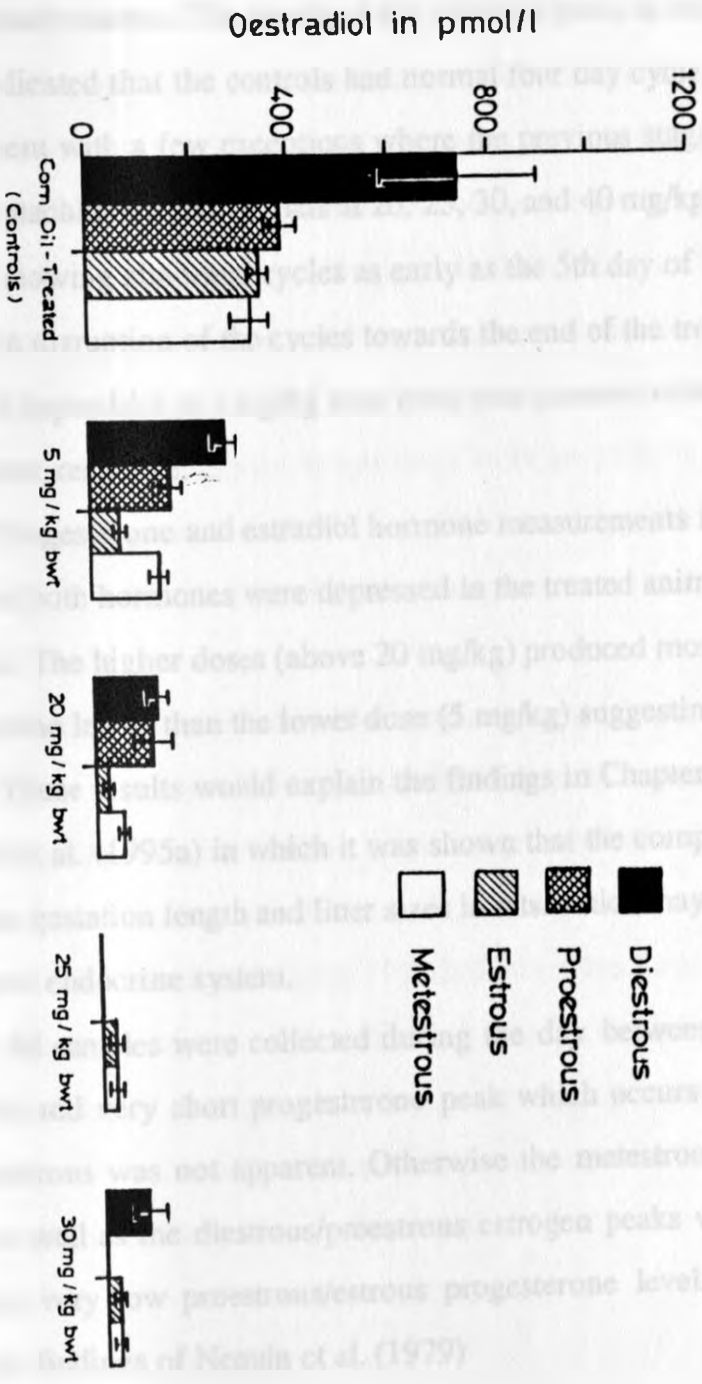

Progesterone in nmol / l
20
16
12
8
4
0
Diestrous
Proestrous
Estrous
Metestrous
Corn Oil - Treated
(Controls)
5 mg / kg bwt
20 mg / kg bwt
25 mg / kg bwt
30 mg / kg bwt

Figure 10.

Plasma estradiol levels in corn oil-treated control rats and rats treated with various concentrations of heptachlor. Values are means ± SEM.


Oestradiol in pmol/l
0
400
800
1200
Corn Oil-Treated (Controls)
5 mg/kg bwt
20 mg/kg bwt
25 mg/kg bwt
30 mg/kg bwt
Diestrous
Proestrous
Estrous
Metestrous

3.4 Discussion

The results of this study indicate that heptachlor disrupts estrous cycles in the rat and also depresses plasma progesterone and estradiol levels *in vivo*, in a dose related manner. The results of the cyclicity study as monitored by vaginal wash indicated that the controls had normal four day cycles throughout the experiment with a few exceptions where the previous stage extended to the next day. Heptachlor treated animals at 20, 25, 30, and 40 mg/kg dose levels. however started showing abnormal cycles as early as the 5th day of injection with the complete disruption of the cycles towards the end of the treatment period. The effect of heptachlor at 5 mg/kg dose level was however minimal, indicating a dose dependent response.

Progesterone and estradiol hormone measurements indicated that the levels of both hormones were depressed in the treated animals compared to the controls. The higher doses (above 20 mg/kg) produced more marked depression of hormone levels than the lower dose (5 mg/kg) suggesting again a dose related effect. These results would explain the findings in Chapter two and reported by Oduma et al. (1995a) in which it was shown that the compound affected body weights, gestation length and litter sizes in rats. which may be as a result of disrupted endocrine system.

All samples were collected during the day between 10.00 and 11.00 hrs. The first and very short progesterone peak which occurs during the second half of proestrous was not apparent. Otherwise the metestrous second progesterone peak as well as the diestrous/proestrous estrogen peaks were quite apparent, so was the very low proestrous/estrous progesterone levels. This is in agreement with the findings of Nequin et al. (1979)

Several researchers have investigated possible mechanisms by which organochlorines produce their toxic effects. Odler (1973) showed that heptachlor may affect ovarian function by interfering with the influence of gonadotrophins on ovarian cells thereby interfering with the steroid hormone synthesis. Virgo and Bellwood (1977) on the other hand reported that doses of dieldrin that caused offspring inviability in mice, also induced hepatic mixed-function oxidase system that metabolises progesterone. Other reports indicate that some of the pesticides are phenobarbital-type inducers of various enzyme systems and may thus enhance steroid hydroxylases (Trapp et al. 1984; Haake et al., 1987; Sirca and Lahiri, 1989). Furthermore, some organochlorines mimic estrogens (Johnson et al., 1988) or have gestagenic activities and are metabolised to compounds which exhibit estrogenic activities. (Cummings and Gray, 1987). Reports by Ogata et al. (1989) and Meguro et al. (1990) suggested that heptachlor probably caused some of its effects by inhibiting oxidative phosphorylation and electron transport in the cells.

As far as steroid metabolism and production are concerned, some authors have shown that organochlorines including heptachlor may inhibit gap junctional communication via mechanisms that involve cAMP (Ruch et al., 1990), and that they can also stimulate protein kinase-C activity in some tissues (Moser and Smart, 1989). Izushi and Ogata, (1990) on the other hand reported that heptachlor had damaging effects on cell membranes through its ability to cause lipid peroxidation. All these effects, if they were to occur in steroid producing tissues, would have adverse effects on steroid hormone production and could thus account for the effects seen in this study.

CHAPTER FOUR

4.0 EFFECT OF HEPTACHLOR ON IN-VITRO SECRETION OF PROGESTERONE AND ESTRADIOL BY MIXED OVARIAN CELLS IN THE PRESENCE OF GONADOTROPHINS AND PRECURSOR HORMONES

4.1 Introduction

Isolated ovarian cells or ovarian homogenates have been used by several investigators. (Daniel and Armstrong, 1980; Behrman et al., 1980; Dorflinger et al., 1984; Bogovich et al., 1986; Baum and Rosberg, 1987; Tanabe et al., 1992; Di Simone et al., 1993; Wiesak et al., 1994).

Follicles developing in response to LH not only produce large amounts of estradiol and progesterone (Lostroh and Johnson, 1966; Fortune and Armstrong, 1978; Richards and Bogovich, 1980; Richards and Kersey, 1980; Bogovich et al., 1981), but also acquire an increased ability to produce androgens (Fortune and Armstrong, 1977; Carson et al., 1981). This increase in follicular androgen synthesis is thought to be due in part to the stimulation of thecal 17α–hydroxylase and C17-20 lyase, which catalyze the conversion of progesterone to androstenedione (Richards and Bogovich, 1980; Bogovich and Richards, 1984; Bogovich et al., 1986). Although theca cells are the primary source of androstenedione in preovulatory follicles, both thecal and granulosa cells from preantral as well as antral follicles possess the ability to convert this substrate to testosterone and dihydrotestosterone (Bogovich and Richards, 1984).

The aim of this particular study was to investigate, in an *in-vitro* system, the *in vivo* findings in Chapter 3 that heptachlor can and does interfere with the production of progesterone and estradiol. The studies reported below were done to investigate the effect of heptachlor on the production of progesterone and estradiol in the presence or absence of gonadotrophins LH and FSH, and steroid hormone substrates namely, pregnenolone and androstenedione

4.2 Materials and Methods

Animals and Housing

The animals used and conditions of their housing was the same as in Chapter 2.

Chemicals

Minimum essential medium Eagle (modified) with Earle's salts (MEM) and Calf serum were obtained from Flow Labs, Irvine, UK. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) and Bovine Serum Albumen (BSA) were from WHO Matched Reagents Programme. Androstenedione and pregnenolone hormones were kindly donated by Professor Bernd Hoffmann, Ambulatorische und Gerburtschilfliche Veterinarklinik, Justus Liebig Universitat, Giessen, Germany.

Methods

Experiment 1. Long-time incubation

A modified mouse interstitial cell bioassay was also adapted for this procedure.

Ovarian cells (from both controls and heptachlor treated rats at the various dose levels), were isolated under sterile conditions. Sterile MEM containing 24 mM $NaHCO_3$ and five drops of combiotic (penicillin + streptomysin) was prepared. Sterile LP3 plastic tubes were three quarter filled with the medium and capped tightly with sterile caps and placed in the hood. A four month old female rat was anaesthetised using ether then thoroughly sterilised by wiping the whole body with 70% alcohol.and placed on a pre-sterilised tray. Using sterile dissecting instruments, the abdomen was quickly opened, the ovaries cut off from the fallopian tubes and placed in the LP3 tube containing the medium. The tube was immediately replaced in the hood.

Sterile petri dishes were prepared as follows:-

Petri dish 1 was left empty.

Petri dish 2 &3 contained 10 ml medium.

Petri dish 4 contained 10 ml medium + 0.1% collagenase in medium.

The tube containing ovaries was removed from the hood and the ovaries transferred into the first petri dish. The individual ovaries were picked up and transferred into the second petri dish washed slightly, then transfered to the third dish and washed again. They were finally transferred to the fourth dish where they were finely minced using sterile scissors and squashed with a pippet for 10 minutes until the tissue was well dispersed. The cell suspension was transferred

into sterile plastic tubes, capped and centrifuged at 4°C for 10 minutes at 1500 rpm (700xg). The supernatant was discarded and the pellet reconstituted in fresh medium containing foetal calf serum. Cell counts were taken using a haemocytometer and dilutions made to give $6x10^5$ cells per 100ml. Cells were then dispersed into culture plates (200 µl per well). The test compounds were prepared in phosphate buffered saline - bovine serum albumin (PBS-BSA) as follows. LH standard stock solution was 25 IU/L while FSH standard stock solution was 10 IU/L. pregnenolone, androstenedione and prolactin standard stock solutions were 100ng/ml each. 50µl of LH and FSH and 10µl (1ng/well) of pregnenolone, androstenedione, and prolactin were added to the wells, each compound in separate well. The cells were cultured at 37°C in an incubator (air circulated) for 72 hours. At the end of the first 24 hours 200 µl of the culture medium was harvested for hormonal analysis. The wells were then topped up with fresh serum free medium and the culture continued for another 24 hours. The harvesting was done as before and the culture procedure repeated once more. The harvested culture media were analysed for progesterone and estradiol.

Experiment 2. Short-time incubation

A modified mouse interstitial cell bioassay was adapted for this procedure.

Ovaries were dissected out of the sacrificed rats, under sterile conditions, trimmed and placed in ice cold sterile minimum essential medium (MEM), containing Earle's salts, sodium bicarbonate and calf serum and airated with CO_2:O_2 carbogen gas (5% CO2 and 95% Oxygen). The tissue was dispersed as previously stated and the mixture filtered through fine nylon mesh. The debri was discarded, and the isolated cells pre-incubated in plastic flasks in the same medium under CO_2:O_2, in a shaking water bath at 34^0C for 30 minutes. After the

incubation period the ovarian and luteal cell mixture was centifuged at 1000 rpm and the pellet resuspended in the same MEM medium and stirred slowly to disperse uniformly. Using trypan blue (vital stain), viable cells were quantified and a dilution made to achieve a concentration of $2.5x10^5$ viable cells/100 ml. (see Appendix 1).

Required doses of LH and FSH were prepared in PBS-BSA as previously stated. Triplicate aliquots of 50 µl of each gonadotrophin were separately placed in LP3 tubes. 200 µl of the cell suspension was then added to the tubes. Another set of tubes containing 200 µl cell suspension and 100 µl plain PBS-BSA were prepared also in triplicates. These tubes were used to determine basal secretion. Incubation was done under CO_2:O_2 carbogen (5:95%) gas in a shaking water bath at 37°C for 3 hours. The tubes were then centrifuged to precipitate the cells and the supernatant analysed by RIA for progesterone and estradiol secreted by the cells. These levels, obtained as a result of LH and FSH treatment were then converted to percent response when compared to those of basal secretion.which was designated 100%. Levels of less than 100% were regarded as negative response or inhibitory while those above 100% were taken as positive response or stimulatory. These *in vitro* studies were performed with cells from both control and heptachlor treated rats at various dose levels.

An additional test in which heptachlor was added *in vitro* to the incubation media at concentrations of 0.06 mM, 1.80 mM and 5.30 mM was also done. The heptachlor was dissolved in dimethylsulfoxide (DMSO) such that the total concentration of DMSO did not exceed 0.5% of the total incubation volume. The ovarian cells in this case were taken from rats which had not received heptachlor treatment. The cells were then incubated in the presence of either LH, FSH, prolactin or heptachlor at the given concentrations.

4.3 Results

The aim of the experiments 1 and 2 was to establish whether short or long incubation period would give maximum hormone yields . No increase in hormone secretion occured after the first 12 hours of incubation and the most active secretory phase lasted only about 3 hours upon comencement of incubation in both long and short incubations. The short incubation (3-6 hours), regime was therefore adopted for all future *in-vitro* work, although the results reported in this chapter are means of the two systems.

Addition of LH to cells from corn oil treated control rats did not result in any significant increase in estradiol production ($P<0.1$), whereas FSH caused a significant increase ($P<0.05$). When ovarian cells from rats treated with heptachlor at 5 mg per kg body weight were incubated either on their own, or in the presence of LH or FSH, no significant effects were observed as compared with cells from corn oil-treated control rats (Fig.12). In incubations of cells from rats treated with heptachlor at 20 mg per kg body weight, comparisons between gonadotrophin free (but heptachlor-treated) samples, and gonadotrophin free (but corn oil-treated) controls showed no significant differences (Fig.12). However, when estradiol production in cells from corn oil-treated controls incubated in the presence of LH or FSH was compared with the corresponding heptachlor (20 mg/kg body weight) treated samples incubated in the presence of LH or FSH, significant suppression ($P<0.05$) in estradiol production was evident in the latter.

Results of incubating ovarian cells from heptachlor treated animals at 25 mg per kg body weight in the absence of gonadotrophins showed a significant suppression of estradiol production when these heptachlor treated samples were compared to corn oil-treated control samples ($P<0.05$). Furthermore at the same

dose of heptachlor, significant suppression was seen in the presence of LH ($P<0.05$) or FSH ($P<0.01$), (Fig.12)

Table 7 and Figure 11 show the levels of progesterone secreted during *in-vitro* treatment of the rat's ovarian cells (follicular and luteal) with both LH and FSH while Table 8 and Figure 12 show the levels of estradiol secreted during *in-vitro* treatment of the rat's ovarian cells with both LH and FSH. The results indicate that incubated ovarian cells produced both progesterone and estradiol, and that the levels of these hormones were modified by the addition of either LH or FSH.

Table 9 shows the levels of progesterone and estradiol secreted during *in vitro* treatment of the rat's ovarian cells with LH, FSH, prolactin and heptachlor. Heptachlor at all the three concentrations significantly depressed the production of both hormones ($P<0.05$ for progesterone and $P<0.001$ for estradiol). Both LH and FSH enhanced the production of the two hormones while prolactin was inhibitory. Table 10 and 11 show the results of *in- vitro* secretion of progesterone and estradiol by cells taken from animals treated with heptachlor at either 5 mg per kg body weight or 20 mg per kg body weight, and incubated in the presence of substrates namely androstenedione and pregnenolone. The results indicate slightly depressed progesterone (Table 10) and estradiol (Table 11) levels secreted by cells from heptachlor treated animals, not only at the basal level but even in the presence of the substrates, when these levels are compared to those of the control samples.

Table 7.

Progesterone (P4) levels secreted by rat ovarian cells following *in-vitro* challenge with FSH and LH. Percent response of cells from treated animals to the LH and FSH challenge as compard to cells from controls is shown. Experimental animals treated with heptachlor at 5 mg/kg, 20 mg/kg and 25 mg/kg.

	Control animals		Test animals (5mg/kg)		Test animals (20mg/kg)	
	P4(nmol/L)	% response	P4(nmol/L)	% response	P4(nmol/L)	% response
Basal secretion	2.10 ± 0.10	100	3.90 ± 0.70	195	1.50 ± 0.50	75
LH stimulation	3.43 ± 0.60	170	4.70 ± 0.70	235	1.70 ± 1.70	85
FSH stimulation	2.12 ± 0.40	105	3.30 ± 1.00	165	0.70 ± 0.20	35

	Test animals (25mg/kg)	
	P4(nmol/L)	% response
Basal secretion	1.10 ±0.20	55
LH stimulation	1.00 ± 0.30	50
FSH stimulation	0.70 ±0.10	35

Figure 11.

Production of progesterone by ovarian cells isolated from corn oil-treated control rats and test rats treated with varying doses of heptachlor. LH increased the production of progesterone by cells from corn oil-treated controls. Values are means ±sem (n=3 measurements).

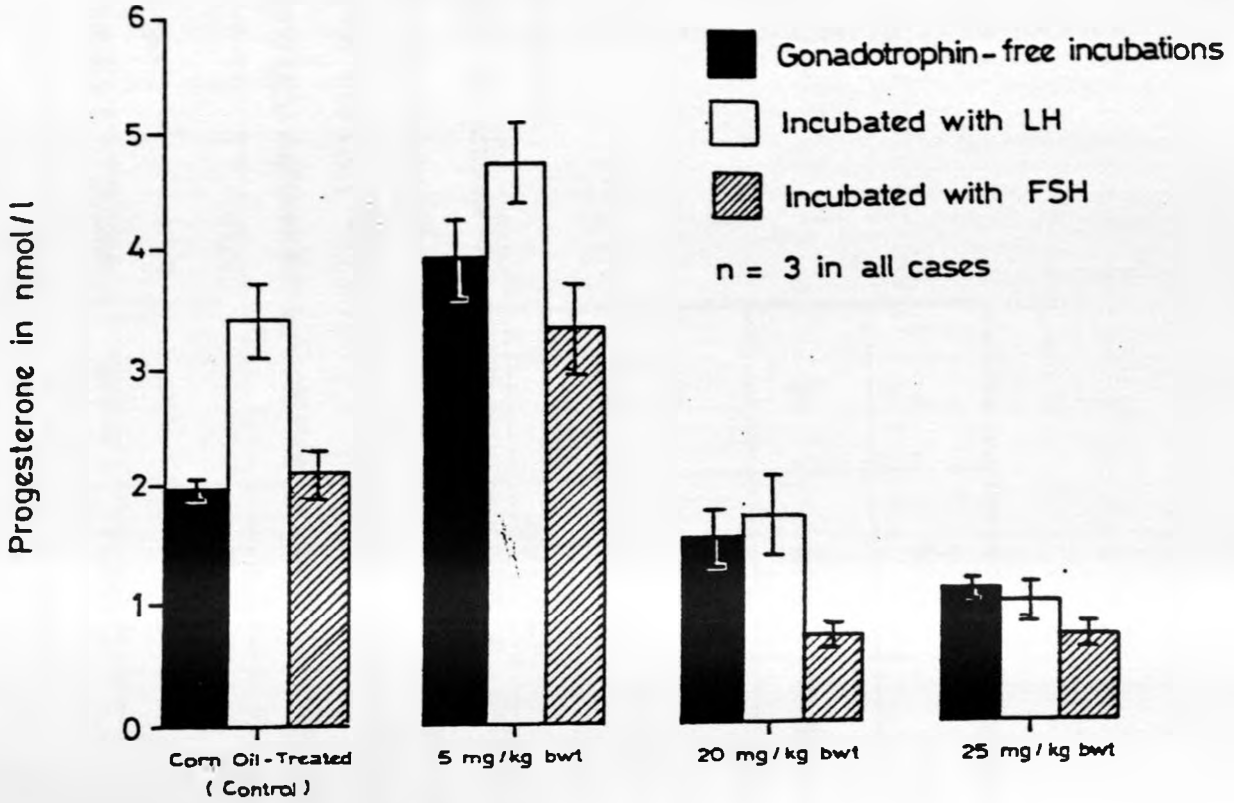

Progesterone in nmol/l
6
5
4
3
2
1
0
Gonadotrophin-free incubations
Incubated with LH
Incubated with FSH
n = 3 in all cases
Corn Oil-Treated
(Control)
5 mg/kg bwt
20 mg/kg bwt
25 mg/kg bwt

Table 8.

Estradiol (E2) levels secreted by rat ovarian cells following *in-vitro* challenge with FSH and LH. The percent response of cells from treated animals as compared to control cells, to LH and FSH challenge is shown. Experimental animals were treated with heptachlor at 5 mg/kg, 20 mg/kg and 25 mg/kg.

	Control animals		Test animals (5mg/kg)		Test animals (20mg/kg)	
	E2 (pmol/L)	% response	E2 (pmol/L)	% response	E2 (pmol/L)	% response
Basal secretion	186.00 ±16.50	100	211.00 ±32.50	113	113.00 ±9.30	61
LH stimulation	294.00 ±23.90	152	201.00 ±4.60	108	136.00 ±20.00	73
FSH stimulation	406.00 ±36.70	204	253.00 ±31.60	136	101.00 ±15.5	54

	Test animals (25mg/kg)	
	E2 (pmol/L)	% response
Basal secretion	99.00 ± 0.8	53
LH stimulation	112.70 ± 3.5	60
FSH stimulation	82.20 ± 3.1	44

Figure 12.

Production of estradiol by ovarian cells isolated from corn oil-treated control rats and test rats treated with varying doses of heptachlor, following *in-vitro* challenge with FSH and LH. Values are means ± sem (n=3 measurements).

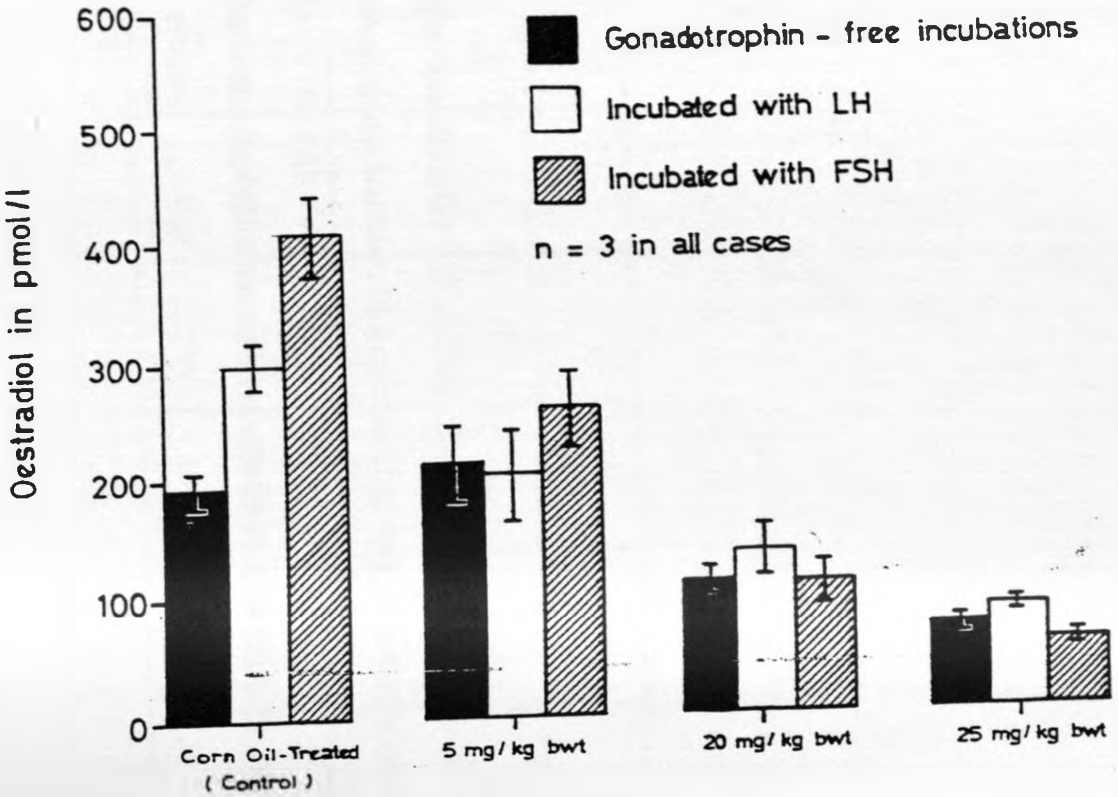

Oestradiol in pmol/l
600
500
400
300
200
100
0
Gonadotrophin - free incubations
Incubated with LH
Incubated with FSH
n = 3 in all cases
Corn Oil-Treated
(Control)
5 mg/kg bwt
20 mg/kg bwt
25 mg/kg bwt

Table 9.

Estradiol and progesterone secretory levels of ovarian cells from untreated rats following in-vitro challenge with FSH and LH and incubated in the presence of prolactin (prl) and heptachlor (hep) at various concentrations.

	Controls	LH 0.5mIU	FSH 0.4mIU	Prl .023mIU	Hep 1 0.06mM	Hep. 2 1.8mM	Hep. 3 5.3mM
Mean P4 ±SEM (nmol/l)	2.00 ±0.1	3.40 ±0.6	2.10 ±0.4	0.0	1.70 ±0.2	1.60 ±0.7	0.80 ±0.4
Mean E2 ±SEM (pmol/l)	177.7 ±2.60	386.7 ±3.70	210.0 ±3.00	146.0 ±2.00	149.2 ±3.39	156.0 ±2.47	110.0 ±3.80
n	6	3	3	4	5	3	4

Table 10.

Progesterone secretory levels of ovarian cells from heptachlor treated rats at either 5 mg/kg or 20 mg/kg incubated in the absence or presence of either androstenedione (andr) or pregnenolone (pregn) or both.

Heptachlor at 5 mg/kg				
P4(nmol/l±SEM	Basal secretion	+androstenedione	+pregnenolone	+andr and pregn
Control samples	5.6 ± 0.8	high	high	high
Test samples	8.5 ± 1.5	high	high	high
Heptachlor at 20 mg/kg				
P4(nmol/l±SEM	Basal secretion	+androstenedione	+pregnenolone	+andr and pregn
Control samples	6.3 ± 0.7	high	high	high
Test samples	3.9 ± 0.1	4.6±0.2	high	high
n	5	5	5	5

Table 11.

Estradiol secretory levels of ovarian cells from heptachlor treated rats at either 5 mg/kg and 20 mg/kg incubated in the absence or presence of either androstenedione (andr) or pregnenolone (pregn) or both.

Heptachlor at 5mg/kg				
E2(pmol/l±SEM	Basal secretion	+androstenedione	+pregnenolone	+andr and pregn
Control samples	194.5±12.5	414.0±39.3	262.5±37.6	537.5±74.0
Test samples	143.0±15.0	329.5±20.0	235.0±35.1	502.0±14.0
Heptachlor at 20mg/kg				
E2(pmol/l±SE)	Basal secretion	+androstenedione	+pregnenolone	+andr and pregn
Control samples	237.5±37.8	376.7±8.6	238.1±12.2	541.4±58.7
Test samples	106.0±23.2	202.3±5.7	155.8±0.9	350.0±6.0
n	5	5	5	5

4.4 Discussion

The results of this study clearly compliment the earlier ones (Chapter 3) in that they too show that heptachlor at high dose significantly depresses the production of progesterone and estradiol by ovarian cells *in- vitro.* With regard to progesterone, addition of FSH to ovarian cells from corn oil treated controls had no significant effect on progesterone production , whereas addition of LH resulted in increased production ($P<0.05$). Progesterone production in cells obtained from animals previously treated with heptachlor at 5 mg/kg body weight (and incubated in the absence of LH or FSH), was significantly ($P<0.01$) more than that produced by cells from corn oil treated controls. An increase was also observed in the same group of cells (i.e. from rats treated with heptachlor at 5 mg/kg), when they were incubated with LH or FSH (Fig. 11). However in this case, the increases were not significant when compared with those in cells from corn oil treated controls incubated with LH or FSH.

Ovarian cells from animals treated with heptachlor at the higher dose levels (20 mg and 25 mg/kg body weight) showed decreased production of progesterone, even in the presence of LH or FSH. These results indicate an inhibitory effect of heptachlor on the secretory capacity of the ovarian cells as shown by the marked negative response to LH and FSH treatment (Fig 11).

The levels of progesterone and estradiol produced following in-vitro treatment of ovarian cells with LH, FSH, prolactin and heptachlor (Table 9),also suggested an inhibitory effect of heptachlor on the secretory capacity of the cells, confirming further that heptachlor does have a negative effect on ovarian cells. In the presence androstenedione and pregnenolone, the effect of heptachlor on in vitro production of progesterone and estradiol (Table 10 and 11) was still apparent. Heptachlor at the higher dose level appeared to depress hormone

secretion more than at the lower dose level. It should be noted that the levels of progesterone measured in the presence of substrate hormones were mostly beyond detectable levels and could therefore not give conclusive results. For this reason, these tests were repeated and hormonal analysis done under different and improved conditions i.e. following HPLC separation. (see Chapter 6).

Although low doses seem to have a stimulatory effect on progesterone production. The fact that in animals treated with low heptachlor dose (5 mg/kg), *in-vitro* production of progesterone by isolated ovarian cells both in the presence and absence of LH or FSH was enhanced, is worth noting. Indeed this biphasic, dose-dependent response, in which low concentrations of a compound stimulate, while, high concentrations inhibit steroid hormone production *in-vitro* is not unique to this study. Similar effects have been reported to occur in other steroidogenic tissues when several compounds are tested *in-vitro* eg prolactin (Alila et al., 1987) and nordihydroguaiaretic acid (Wango et al., 1992). So far, no satisfactory explanation has been given as to the significance of such biphasic responses. However it should be pointed out here that these particular studies differ from the above cited investigations in that a mixed population of ovarian cells (follicular and luteal) was used as opposed to separated cell types used by the others. Secondly, pooled ovarian tissues from animals at different stages of the estrous cycle was used in this study.

CHAPTER FIVE

5.0 THE EFFECT OF HEPTACHLOR ON IN VITRO SECRETION OF PROGESTERONE AND ESTRADIOL BY ISOLATED GRANULOSA AND LUTEAL CELLS

5.1 Introduction

Previous *in-vitro* experiments (Chapter 4) established that heptachlor affects the secretion of progesterone and estradiol by isolated ovarian cells.

However these experiments were done using mixed ovarian cell preparations (ie. luteal, follicular and interstitial cells). The principal aim of this study was to find the effect of heptachlor on mammalian reproductive performance and thereby locate if possible, the site of action of the pesticide. It became necessary therefore to utilise isolated follicular and luteal cells separately.

It is generally accepted that the granulosa cells aromatise thecal androgens to estrogens during the follicular phase and that the corpus luteum secretes progesterone in the luteal phase. Estrogen is also secreted during the second half of the cycle and it is thought that this estrogen is derived from the corpus luteum (Schulster et al., 1976). Indeed *in-vitro* studies with the different ovarian tissues incubated with ^{14}C-acetate have shown that the isolated follicular cells will convert acetate mainly to estrogens and the corpus luteal cells on the other hand will convert the same mainly to progesterone (Schulster et al., 1976). In apes, human and perhaps the pig, estrogens are also formed in the corpus luteum. In the human female, estrogen production by the corpus luteum is as great as in the pre-ovulatory follicles. In other species including the rat, the corpus luteum is said to

produce only minute amounts of estradiol (Johnson and Everitt, 1988). Both hCG and LH stimulate the incorporation of ^{14}C-acetate into steroids in the follicle *in-vitro*. The stroma or interstitial tissue has been shown to form predominantly androstenedione, DHEA and testosterone plus small amounts of estrogens from acetate.

The aim of this experiment was to investigate the effect of heptachlor on the steroidogenic activities of the different cell types in the ovary. For this reason, isolated luteal or follicular cell preparations were incubated with either pregnenolone or androstenedione in the presence or absence of heptachlor.

5.2 Materials and methods

Animals and Housing

With regard to the experimental animals and housing conditions, a similar protocol as in Chapter 2 was used.

Chemicals

The chemicals used were the same as in Chapter 4.

Methods

Ovaries were isolated from mature normocyclic virgin rats (3 to 4 months old), under sterile conditions. Corpora lutea were dissected out from the ovaries under a dissecting microscope and placed in chilled culture medium (MEM). The remaining ovarian tissue comprising mainly follicular cells as well as interstitial cells was treated as follicle rich cell preparation and designated follicular cell preparation. Both the corpora lutea and the follicular cell preparations were separately finely minced using fine razor blade and the cells enzymatically

dispersed using collagenase as previously described. The cell preparations were then treated as previously described in Chapters 4 and 5 except that the incubations were carried out overnight (i.e 15 hrs) in order to ensure the incorporation of heptachlor. Although the 3 hour incubations used previously was adequate for the response to LH and FSH, longer time was necessary when heptachlor was given *in-vitro*.. The luteal cells were incubated with pregnenolone in the presence (experimental) or absence (control cells) of heptachlor after which progesterone and estradiol were measured in the incubation media. The follicular cells were incubated with either pregnenolone or androstenedione in the presence (experimental cells) or absence (control cells) of heptachlor and thereafter both progesterone and estradiol were measured in the incubation media. Heptachlor was used at two dose levels,(prepared using DMSO as in Chapter 4) The high level dose was 0.6ng/well while the low level dose was 0.06ng/well. Tubes containing dead cells were also included as controls for initial amounts, that is the amount of hormone present in the cell cytosol prior to the start of the incubations. Cells to be mortalised were aliquoted out of the pooled cell preparations into separate tubes, then placed in boiling water for ten minutes to kill them after which they were incubated in media alone. The amount of estradiol and progesterone in the media of the dead cells was analysed. The net hormone secreted during incubation by the control (challenged and unchallenged) as well as the experimental cells (challenged and unchallenged) was taken as the hormone level measured in the live cells minus the levels in the dead cells.

5.3 Results

Plate 1a shows a wet preparation of isolated corpus luteum. The tissue was placed on a slide and squashed by applying thumb pressure on the cover slip. Plate 1b is a light micrograph of a single corpus luteum. Plate 2 shows a wet preparations of follicles squashed to reveal oocytes. 2b is a higher magnification of 2a. Plate 3 is a wet preparation of a mature follicle magnified to show a mature oocyte.

Figures 13 to 16 show the results of analysing progesterone and estradiol in the incubation media of both isolated luteal and follicular cell preparations, in the presence or absence of precursor hormones. The test was repeated four times (each bar representing one test). Figure 13 (a,b&c) show the results of measuring progesterone in the incubation media in the absence of either pregnenolone or androstenedione in isolated luteal cells (Fig. 13a), follicular cell preparation (Fig. 13b) and their mean ± SEM (Fig. 13c) respectively. Heptachlor depressed the production of progesterone by both cell types. The effect was more significant on the luteal cells both at the low dose ($P < 0.005$) and at the high dose level ($P < 0.001$) than on the follicular cell preparation at the low dose ($P < 0.05$) and at the high dose level ($P < 0.05$).

Figure 14 (a,b&c) show the effect of heptachlor on progesterone production by luteal cells (Fig. 14a) and follicular cells (Fig. 14b) in the presence of pregnenolone. Figure 14c gives the mean progesterone secreted by the two cell types. The effect of heptachlor was more significant on the luteal cells both at the low heptachlor dose ($P < 0.05$) and at the high dose ($P < 0.02$) than on the follicular cells at the high dose.($P < 0.05$). Heptachlor at low dose had no significant effect on the follicular progesterone production in the presence of pregnenolone.

Figure 15 (a,b&c) give the results of analysing the incubation media for estradiol 17β, in the absence of substrate hormones. The effect of heptachlor on estradiol production by luteal cells (Fig.15a) was significant at the low dose ($P < 0.01$) and at the high dose.($P < 0.002$). On the other hand, the effect of heptachlor on estradiol production by follicular cells (Fig.15b) in the absence of substrate hormone was only slightly significant at the high dose ($P < 0.06$) but no significance at the low dose level. The mean of the four tests (Fig.15c) show however significantly higher levels of estradiol from follicular cells than from luteal cells in controls ($P < 0.05$), in the low heptachlor group ($P < 0.05$), and in the high heptachlor group ($P < 0.02$).

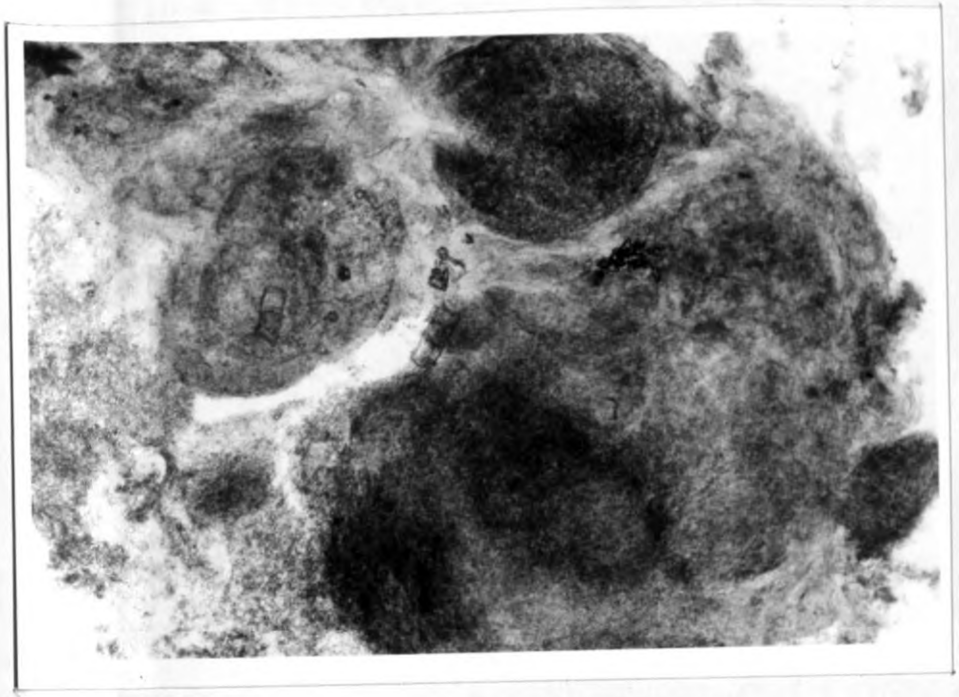
Figure 16 (a,b&c) give the results of the effect of heptachlor on estradiol production by luteal cells in the presence of pregnenolone (Fig. 16a) and by follicular cell preparation in the presence of androstenedione (Fig. 16b). Figure 16c gives the mean of the four tests. The results indicate significant decrease in estradiol production at the low dose ($P < 0.05$) and at the high heptachlor dose ($P < 0.02$) in the luteal cells. In the follicular cell preparation, the effect of heptachlor was significant at the low dose ($P < 0.05$) and at the high dose level ($P < 0.01$).

A summary of the response of the follicular and luteal cells in their production of estradiol and progesterone in the absence of heptachlor (control cells) and in the presence of either pregnenolone or androstenedione, is given in figure 17. These results indicate that luteal cells in the rat do produce appreciable amounts of estradiol. Luteal cells utilise pregnenolone better to produce progesterone whereas follicular cells utilise androstenedione better than luteal cells for the synthesis and production of estrogen.

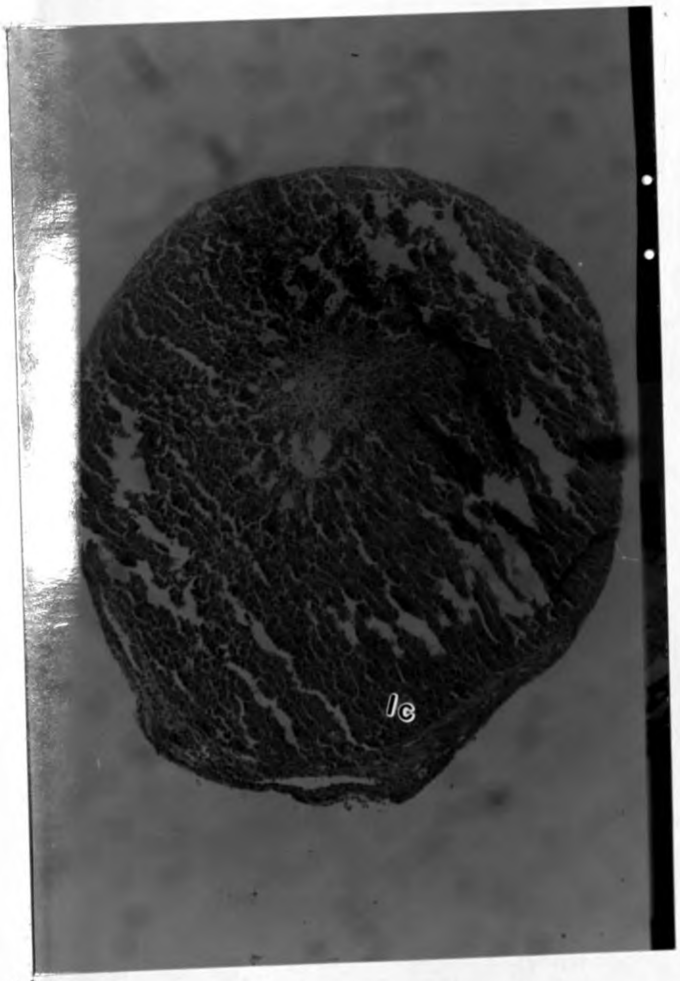
Plate 1.

a) Gross preparation of corpus luteum, isolated and squashed by pressing on the cover slip. Note the absence of difinitive luteinizing granulosa cells or oocyte (Mag. x 160).

b) Light micrograph of a histological section of a single corpus luteum. Luteal cells (lc) are clearly visible (Mag. x 75.6).



a)

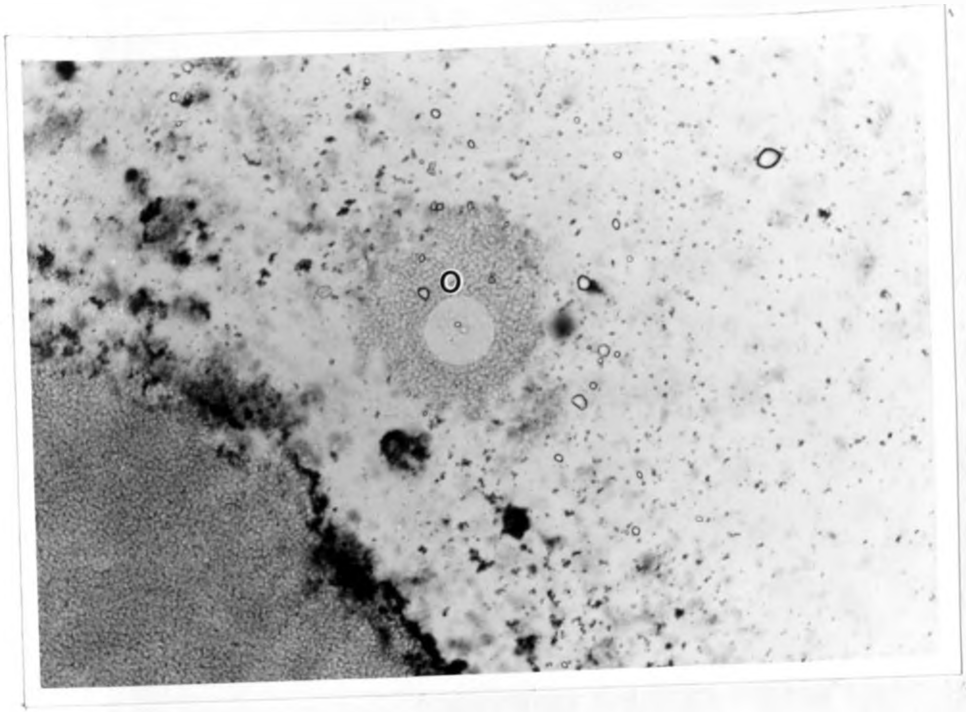


b)

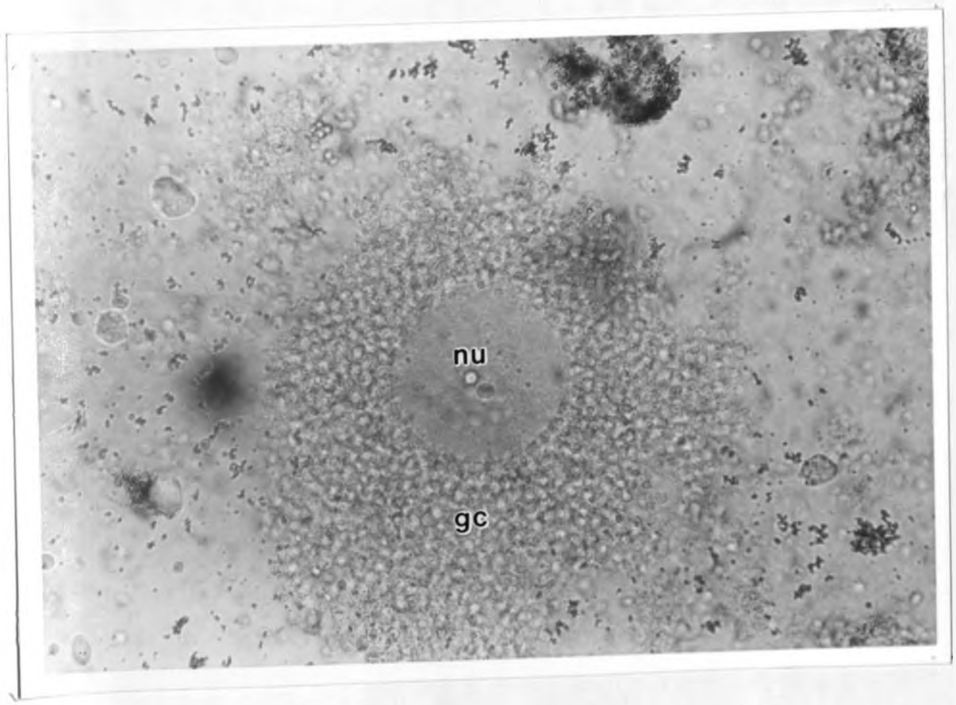
Plate 2.

a) Shows a wet preparation of a follicle isolated and squashed gently as previously explained, to reveal a well defined oocyte (o) surrounded by granulosa cells (Mag. x 160).

b) A magnification of 2a to show a well defined nucleus (nu) and granulosa cells (gc) (Mag x 400).



a)



b)

Plate 3.

A larger, more mature follicle, isolated and squashed as previously explained to reveal a mature oocyte with a well defined zona pellucida (zp) (Mag. x 400).

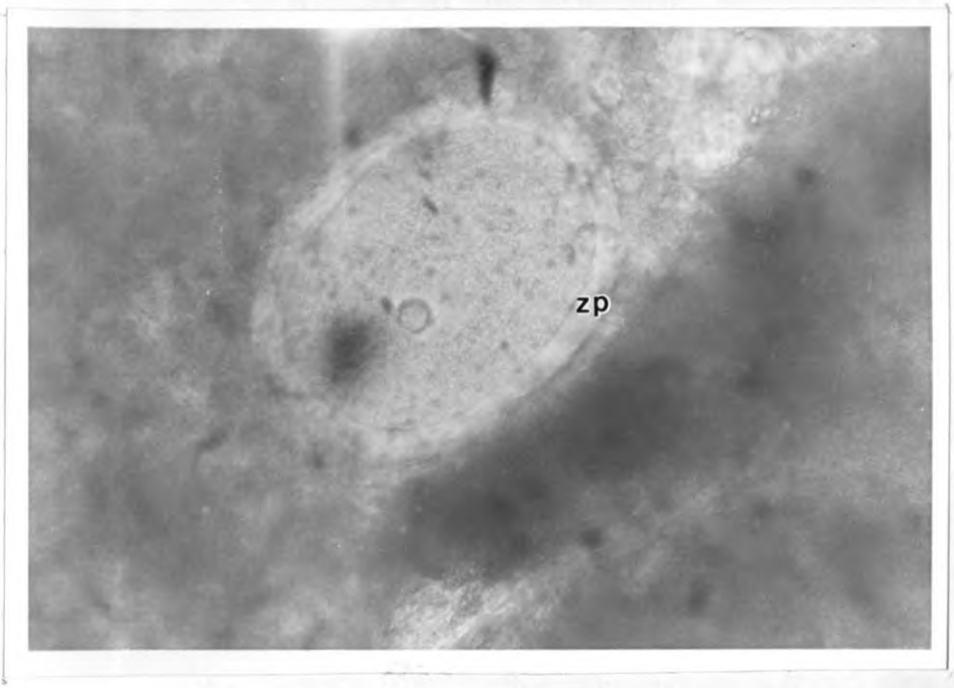

zp

Figure 13.

The levels of progesterone in isolated luteal cells (Fig. 13a) and follicular cell preparation (Fig. 13b) incubated in the absence (control cells) or presence (test cells) of heptachlor at a low dose and high dose. A total of four tests were done.

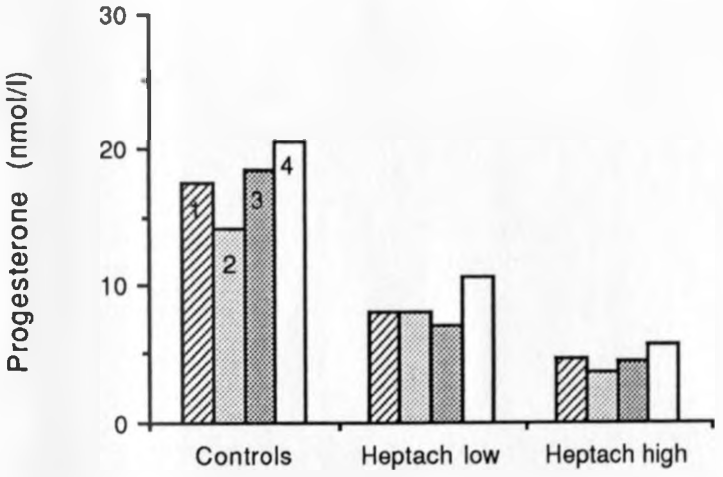


Fig. 13(a)

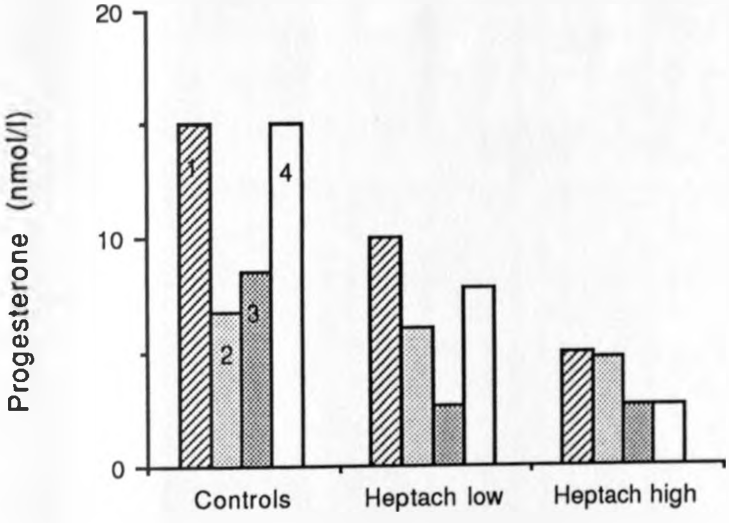


Fig. 13(b)

Figure 13c.

Mean plus SEM progesterone secreted by luteal versus follicular cell preparation in the absence of precursor hormone but in the presence of heptachlor.

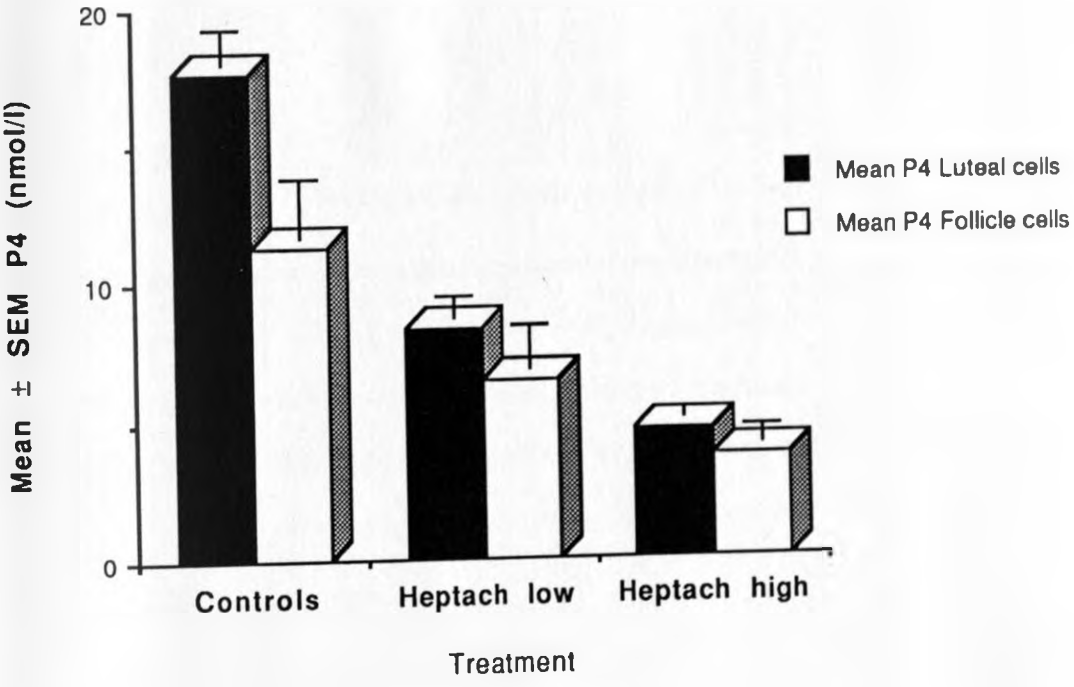

Mean ± SEM P4 (nmol/l)
20
10
0
Controls
Heptach low
Heptach high
Treatment
Mean P4 Luteal cells
Mean P4 Follicle cells

Figure 14.

Progesterone levels in the incubation media of luteal cell (Fig. 14a) and follicular cell preparation (Fig. 14b) in the presence of pregnenolone and incubated in the absence (control cells) or presence (test cells) of heptachlor at a low dose and high dose body weight). A total of four tests were done each represented by a bar.

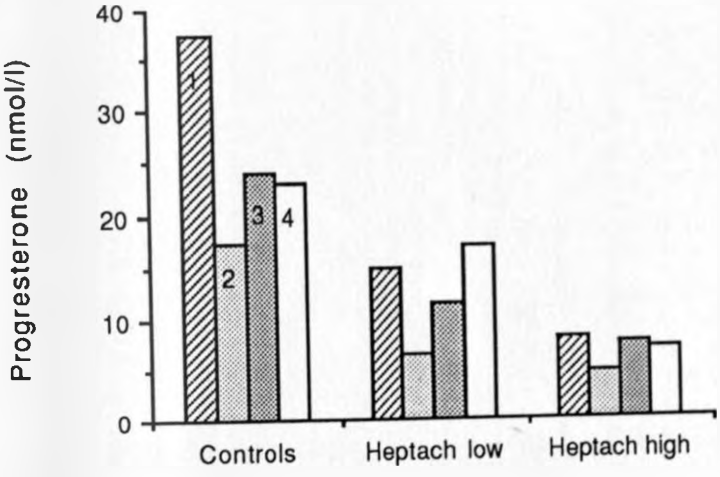


Fig. 14(a)

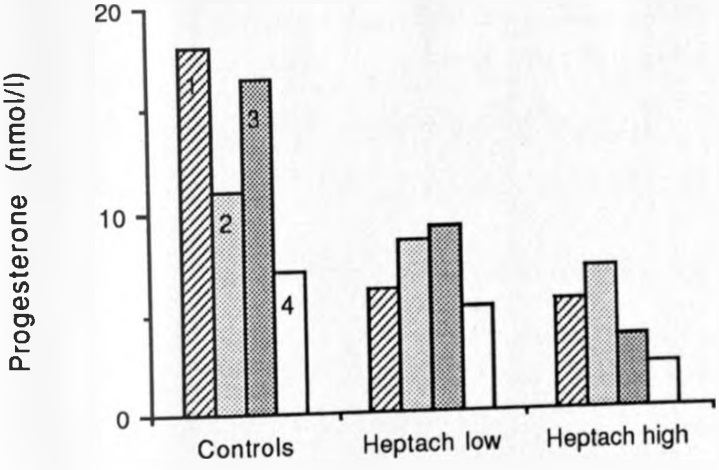


Fig. 14(b)

Figure 14c.

Mean plus SEM progesterone secreted by luteal versus follicular cells in the presence of pregnenolone and heptachlor at low and high dose.

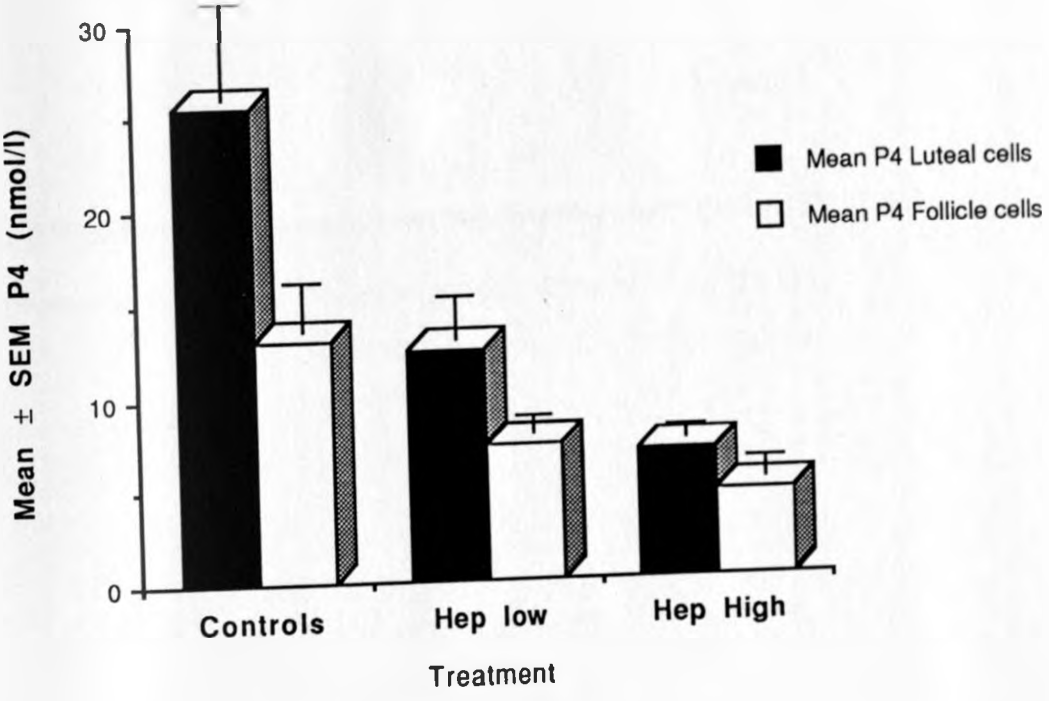

30
20
10
0
Mean ± SEM P4 (nmol/l)
Mean P4 Luteal cells
Mean P4 Follicle cells
Controls
Hep low
Hep High
Treatment

Figure 15.

The results of analysing the incubation media for estradiol 17β in the luteal cells (Fig. 15a) and follicular cells (Fig. 15b) in the absence of substrate hormones but in the presence of heptachlor.

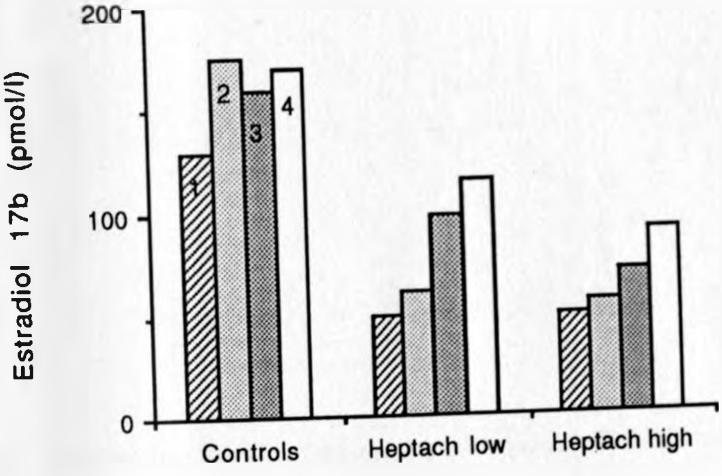


Fig. 15(a)

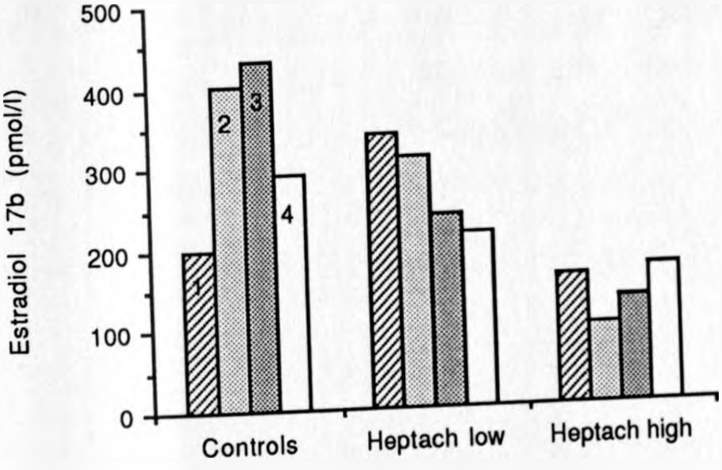


Fig. 15(b)

Figure 15c.

Mean plus SEM estradiol 17β secretion by both luteal and follicular cell preparations in the absence of precursor hormone. The cells were incubated in the presence of heptachlor at low and high dose.

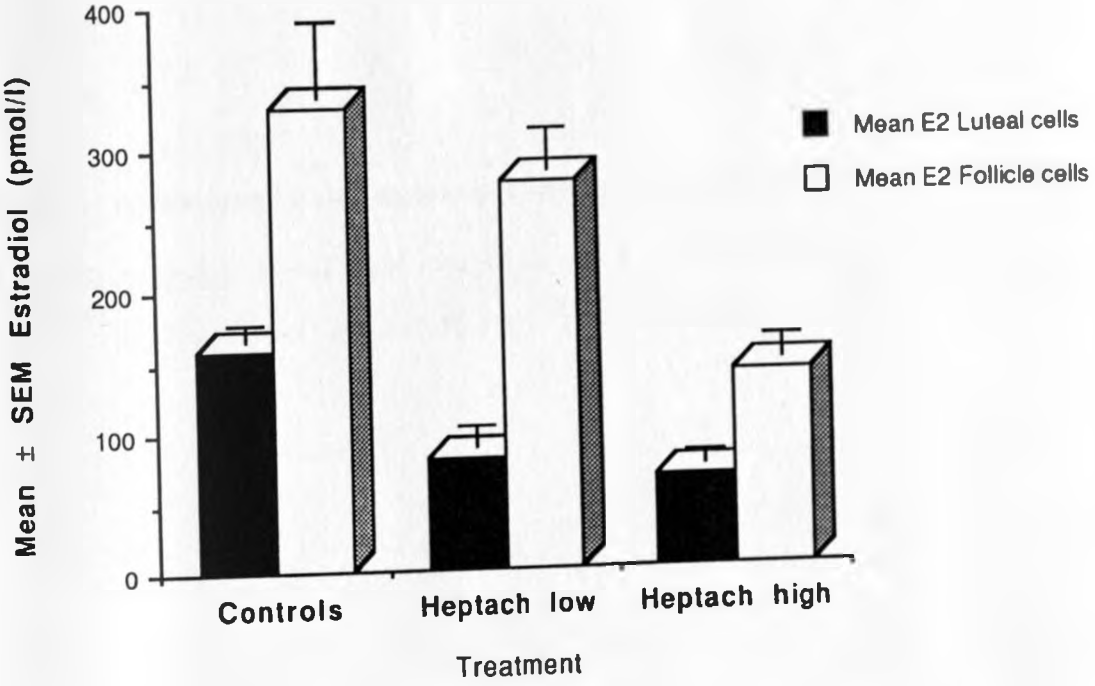

Mean ± SEM Estradiol (pmol/l)
400
300
200
100
0
Mean E2 Luteal cells
Mean E2 Follicle cells
Controls
Heptach low
Heptach high
Treatment

Figure 16.

The effect of heptachlor on estradiol 17β production in the presence of precursor hormone.and heptachlor in luteal cells (Fig. 16a) and in follicular cell preparation (Fig. 16b). Results of four tests each represented by a bar.

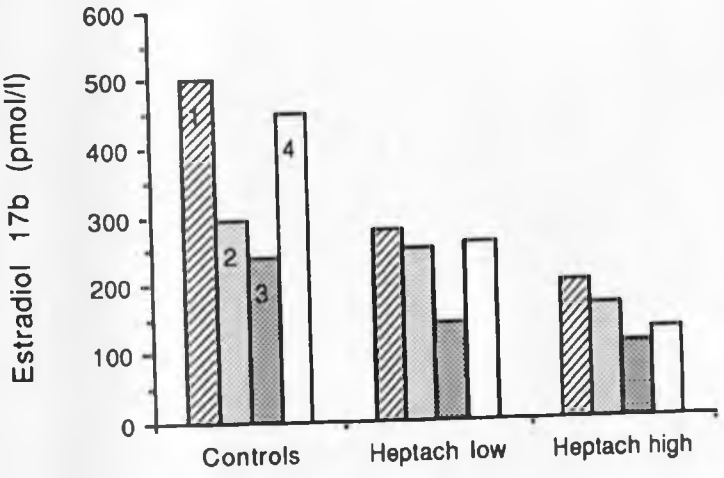


Fig. 16(a)

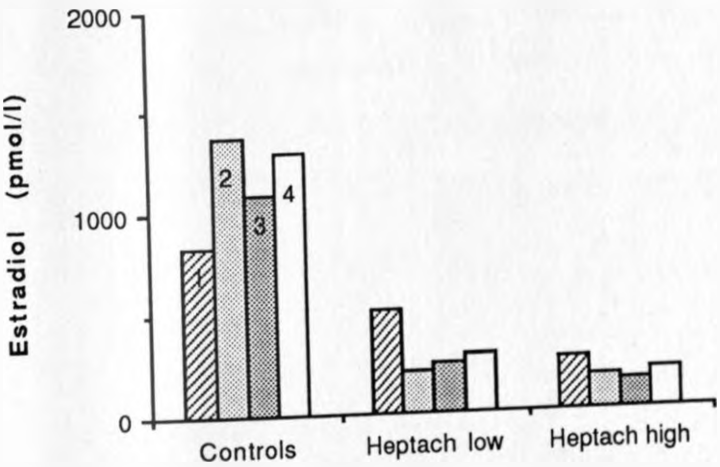


Fig 16(b)

Figure 16c.

Estradiol secretion (Mean plus SEM) by both luteal and follicular cell preparation in the presence of substrate hormones and heptachlor at both low and high dose.

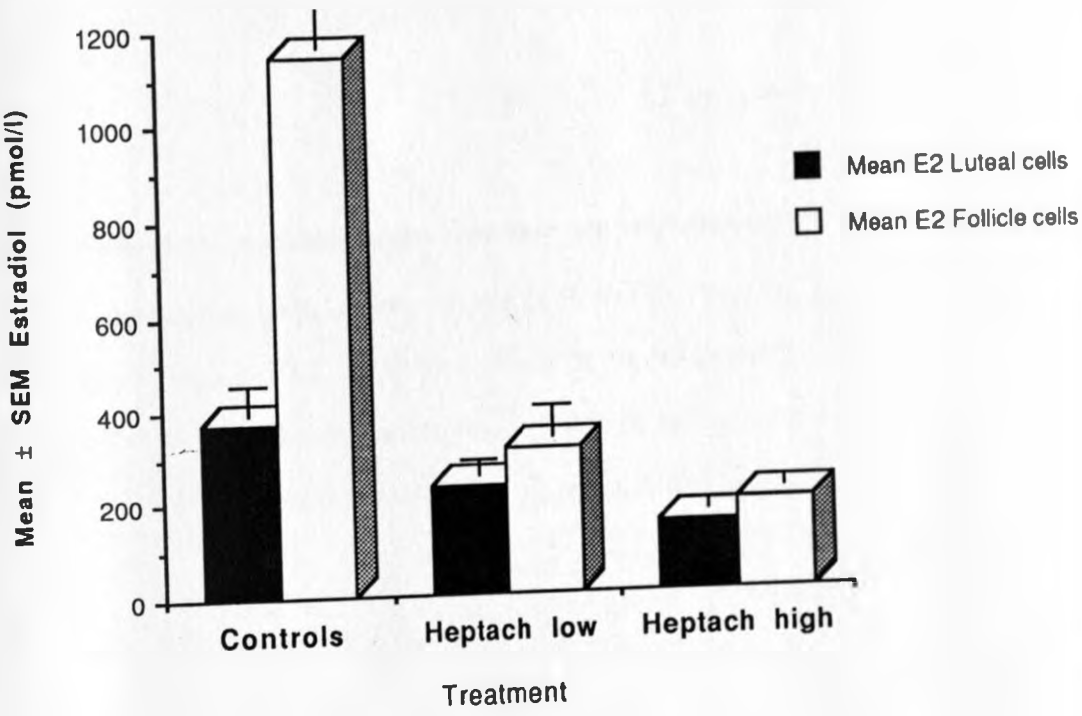
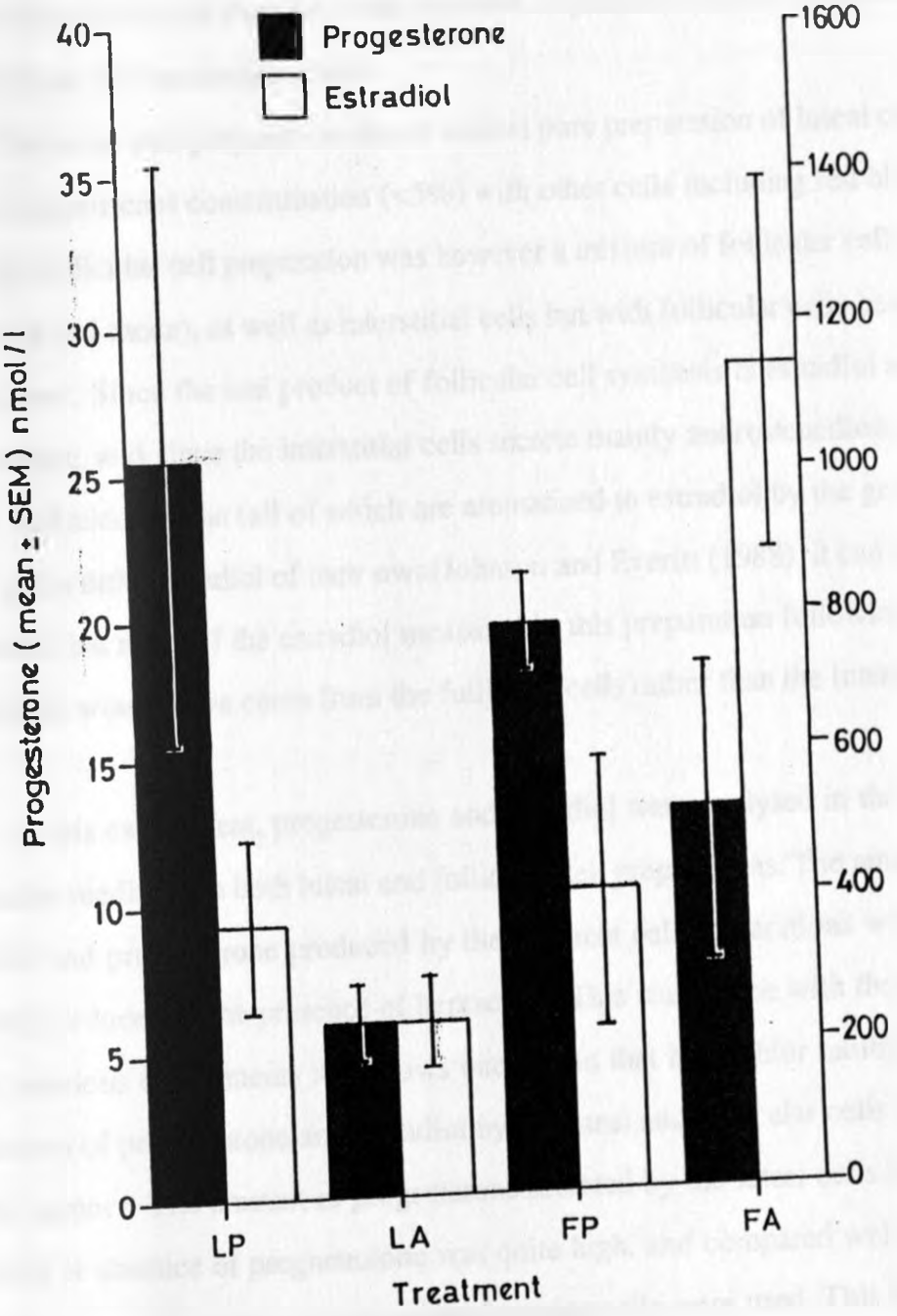

Mean ± SEM Estradiol (pmol/l)
1200
1000
800
600
400
200
0
Mean E2 Luteal cells
Mean E2 Follicle cells
Controls
Heptach low
Heptach high
Treatment

Figure 17.

Summary of the secretion of progesterone and estradiol by isolated control follicular and corpus luteal cells in the presence of either pregnenolone or androstenedione. LP = luteal cells + pregnenolone, LA = luteal cells + androstenedione, FP = follicular cells + pregnenolone, FA = follicular cells + androstenedione.


Progesterone
Estradiol
Progesterone (mean ± SEM) nmol / l
Estradiol (mean ± SEM) pmol / l
40
35
30
25
20
15
10
5
0
1600
1400
1200
1000
800
600
400
200
0
LP
LA
FP
FA
Treatment

5.4 Discussion

The results obtained in these experiments show that isolated luteal and follicular cells are capable of synthesising and secreting progesterone and estradiol in culture, and more so in the presence of precursor hormones namely pregnenolone and androstenedione.

The luteal cell preparation was an almost pure preparation of luteal cells with an insignificant contamination (<5%) with other cells including red blood cells. The follicular cell preparation was however a mixture of follicular cells (granulosa and theca), as well as interstitial cells but with follicular cells being predominant. Since the end product of follicular cell synthesis is estradiol and progesterone, and since the interstitial cells secrete mainly androstenedione, DHEA and testosterone (all of which are aromatized to estradiol by the granulosa cells), and a little estradiol of their own(Johnson and Everitt (1988), it can be concluded that most of the estradiol measured in this preparation following incubations would have come from the follicular cells rather than the interstitial cells.

In this experiment, progesterone and estradiol were analysed in the incubation media from both luteal and follicular cell preparations. The amount of estradiol and progesterone produced by the different cell preparations was markedly reduced in the presence of heptachlor. This was in line with the results of the previous experiments and shows once again that heptachlor inhibits basal production of progesterone and estradiol by rat luteal and follicular cells in a dose related manner. The amount of progesterone secreted by the luteal cells in the presence or absence of pregnenolone was quite high, and compared well with the amounts obtained previously when mixed ovarian cells were used. This is worth noting in view of what has been reported by Johnson and Everitt (1988) to the

effect that corpus luteum in the rat only become fully functional in producing progesterone during the luteal phase if the female mated at the time of ovulation. If on the other hand she failed to mate as is the case with the rats used in this experiment, then the corpora lutea are only transiently functional in producing progestagens. Thus they secret only a small amount of progesterone but mainly 20α hydroxyprogesterone. The results further indicate that appreciable amounts of progesterone was also secreted by the follicular cell preparation. This then would suggest that the high levels of progesterone found previously, especially following incubation with pregnenolone (Chapter 4) may be due to the fact that both follicular cells as well as luteal cells actively secret progesterone in the rat.

These results suggest that heptachlor affects more the production of progesterone by the luteal cells than the follicular cells. A possible explanation could be that since the enzymes involved in the synthesis of progesterone from pregnenolone are the same in both luteal and follicular cells then it would follow that if heptachlor affects these enzyme systems eg. 3β hydroxysteroid dehydrogenase and isomerase, then progesterone production would be curtailed in both cells.

As expected there was fairly large amounts of estradiol secreted by the follicular cell preparation and more so in the presence of androstenedione and even in the presence of pregnenolone. However, in this experiment, it was found that luteal cells also produced appreciable amounts of estradiol especially in the presence of substrate hormone pregnenolone. The estradiol from luteal cells was however significantly low ($P < 0.05$) compared to that produced by follicular cells, in the presence or absence of precursor hormones. This while it agrees with the findings in the human (Schulster et al., 1976) as well in the findings in, higher apes and pig (Johnson and Everitt, 1988), is however quite contrary to the observation made in the bovine, horse, monkey, sheep and rat (Johnson and

Everitt, 1988). According to these reports the corpus luteum in the rat only secrets trivial amounts of estradiol while the results in this study show that the amount of estradiol from luteal cells was substantial.

Statistical analysis suggest that the effect of heptachlor was more pronounced on the luteal cells than on the follicular cells. This being the case then, not only would it explain the low *in-vivo* levels of progesterone in the presence of heptachlor, found in the previous experiments, but also the low levels of estradiol as the progesterone is consequently metabolised to androgens which are then aromatized to estradiol. One would argue that follicular cells produce much more estradiol than luteal cells and that this should mask the effect of heptachlor on luteal estradiol. However these results show that heptachlor had less effect on follicular estradiol and since the corpus luteum produces little though appreciable amounts of this hormone, then the fall in its production seen in *in-vivo* experiments as well as *in-vitro* experiments with mixed ovarian cells could be due to the fall in progesterone production by either luteal cells or follicular cells both of which were significant. This would then agree with the earlier suggestion that since the enzymes involved in the production of progesterone from pregnenolone are the same in both luteal and follicular cells then heptachlor was affecting the activity of these enzymes in both cell types. If less progesterone is produced then less is available for metabolism into androgens and consequent aromatization into estradiol.

CHAPTER SIX

6.0 QUALITATIVE AND QUANTITATIVE ANALYSIS OF SELECTED STEROID COMPOUNDS IN PLASMA OF RATS TREATED WITH HEPTACHLOR: AN IN-VIVO AND IN-VITRO STUDY USING HPLC

6.1 Introduction

The findings in the preceding Chapters have shown that following treatment with heptachlor, either *in-vivo* or *in-vitro*, ovarian cells exhibited reduced responsiveness to either FSH or LH stimulation when these gonadotrophins were added to the incubation media (Oduma et al., 1995b). These experiments were followed by other experiments in which exogenous steroid precursors or substrates, namely pregnenolone and androstenedione, were provided and once again the level of progesterone and estradiol were lower in the media with cells from heptachlor treated animals than in controls. These effects of heptachlor appeared to be dose related (Chapter 4 and 5).

It was possible to deduce that heptachlor affected the synthesis of progesterone and estradiol in such a manner that the levels of both hormones were lowered in either plasma or culture media.

Experiments in this Chapter, therefore aimed at finding out whether the effect of heptachlor was due to its actions on the interaction between the gonadotrophins (FSH & LH) with their target cells (in this case ovarian cells) or whether the effect was due to interference with some of the stages during steroidogenesis, within the ovarian cells.

6.2 Materials and methods

Animals and Housing

The animals used and conditions of their housing was the same as in Chapter 2.

Chemicals

Hormones for cell incubation studies and Reagents for HPLC and radioimmunoassay (RIA) of progesterone, estrogen, androstenedione, dehydroepiandrosterone, 17α hydroxyprogesterone, pregnenolone and estrone were kindly provided by Professor Bernd Hoffmann of Ambulatorische und Gerburtschilfliche Veterinarklinik, Justus Liebig Universitat, Giessen, Germany.

Methods

Treatment and sampling

The experimental set up was the same as previously. The experimental animals were exposed to heptachlor and at the end of the treatment period, the animals were bled by cardiac puncture, the plasma collected and the ovaries excised immediately and prepared for *in-vitro* experiments. The *in-vitro* work involved incubating the ovarian tissue in the presence of pregnenolone and androstenedione as previously described for LH and FSH (Chapter 4). The harvested culture media and the plasma samples were stored at -20°C to await analysis.

Extraction and purification of hormones

The samples were each extracted with 3.0 ml toluene three times. the extract was evaporated to dryness under vacuum before being redissolved in 4.0

ml 35% methanol. The samples in 35% methanol were then submitted to reversed phase chromatography for further extraction and purification on octadecyl C18 cartridges (Bakerbond spe, Baker Chemicals, D-64521 GrobGerau). Preparation and calibration of the cartridge was as follows:- The column cartridge was first eluted with 1 ml of 100% methanol followed by 3.0 ml 35% methanol. The 4.0 ml sample was then placed on the column and the sample solvent allowed to run through leaving the steroids adsorbed onto the column. The adsorbed steroids were then eluted with 1.5 ml of 95% methanol. The steroid eluates were again evaporated to dryness and redissolved in 0.22 ml of 20% methanol. These samples were then submitted to separation on reversed phase HPLC using two solvent systems. The column system consisted of a pre -column (Li Chrospher, 30x3 mm, Merck, D- 64293 Darmstadt) and an RP 18-Lichrosorp, 150x3 mm column (Merck, D- 64293 Darmstadt).

Standardization of chromatograms and HPLC

Prior to subjecting the samples for separation the system was first standardized according to the retention times of the tritiated steroid standards. For each chromatogram, a total elution profile was established either by collecting fractions programmed according to peaks eluted or by collecting single 0.5 ml fractions and counting. A mixture of non tritiated standards of all the hormones to be investigated was first passed through the HPLC and standard peaks obtained. Tritiated standards were then eluted to confirm their peak positions against the standards. Appendix 1 is the elution profile for testosterone (T4) and progesterone (P4). Appendix 2 is the elution profile for androstenedione and pregnenolone (P5). Appendix 3 gives the elution profile for 17α

hydroxyprogesterone (17α OHP4) and estrone (E1) while appendix 4 gives the elution profile for estradiol17β and dehydroepiandrosterone (DHEA).

The mobile phase for the first solvent system for the separation of P5, OHP4, P4 and DHEA, was a mixture of methanol and distilled water. This was in the ratio of 50% methanol : 50% distilled water for the first 40 minutes followed by a ratio of 65:35 (methanol:water) for the next 40-70 minutes then 100:1 ratio (100% methanol) for the final 70- 79 minutes of the run (Fig. 18).

The mobile phase for the second solvent system for the separation of E1, E2 and androstenedione, consisted of a mixture of acetonitrile (ACN) and water in the ratio of 30:70 (ACN:water) for the first 44 minutes then a continuous gradient upto 100% ACN for the next 44-50 minutes followed by 100:1 (100% ACN) for the next 50-56 minutes and lastly 30:70 ACN:water for the last 56-70 minutes. This system was able to separate E1, E2β and androstenedione. (Fig 19). The column flow rate was 0.5ml/min in both systems.

The culture media and plasma samples collected following HPLC were analysed for the hormones isolated by RIA. Statistical analysis of the results was performed using the t-test.

6.3 Results

Figure 18 shows the HPLC elution profile for P5, 17α OHP4, DHEA and P4, run on the first solvent system (methanol:water). This system was able to separate the four hormones clearly. However E1, E2β, and androstenedione eluted confluently in this system and were thus collected as a pool to be separated in the second solvent system.

The pools of E1, E2, and androstenedione were first dried down and then redissolved in 0.22ml of 20% acetonitrile (ACN). O.1ml of this was submitted for

separation. Figure 19 is the HPLC elution profile for E1, E2, and androstenedione run on the second solvent system (water:ACN). This system was able to separate the three hormones.

Figures 20 to 26 represent the results of hormonal analysis in the plasma samples. The effect of heptachlor treatment on the levels of pregnenolone is shown in figure 20a & b. The levels of the hormone are higher in the treated animals at both low and high dose levels of heptachlor, during diestrous ($P< 0.005$) and at the higher dose level at metestrous stage ($P< 0.001$).

The levels of plasma dehydroepiandrosterone are significantly lower in heptachlor treated animals compared to controls (Fig. 21a&b) in a dose related manner with the higher heptachlor dose giving a larger decrease ($P< 0.05$ at and $P< 0.005$ at metestrous) at the dose of 20 mg heptachlor per kg body weight (Fig. 21b).

The effect of heptachlor on the synthesis of androstenedione follows much the same pattern (Fig. 22a&b) with the higher dose level of heptachlor resulting in a more significant decrease in androstenedione synthesis ($P< 0.001$) at estrous and at metestrous
($P< 0.05$), than at other stages of the cycle. There is an indication of an increase in androstenedione in the treated animals at the lower heptachlor dose and at the metestrus stage in particular, ($P< 0.005$) compared to controls at the same stage. (Fig. 22a).

The effect of heptachlor treatment on progesterone levels also follows the same pattern of a dose dependent decrease and confirms the results of the previous analysis (Fig. 23a&b). The fall in progesterone levels, although significant, ($P< 0.05$ at both estrous and metestrous) is however not as pronounced as previously found in the earlier experiments.

The levels of 17α hydroxyprogesterone are significantly lower in treated animals at 20 mg/kg heptachlor ($P< 0.001$, $P< 0.005$, $P< 0.05$, $P< 0.001$ at diestrous, proestrous, estrous and metestrous respectively), than in controls but a dose related dependency is not very apparent (Fig.24a&b), as fairly low levels of the hormone are seen in treated animals at the low dose of 5 mg/kg heptachlor ($P< 0.05$, $P< 0.05$, $P< 0.005$ at diestrous, estrous and metestrous respectively).

Figure 25 (a&b) gives the result of estrone levels in plasma. In this case, there is only a slight decrease in estrone levels following heptachlor treatment both at the lower dose (Fig. 25a) and at the higher dose (Fig. 25b), A significant decrease was however seen ($P< 0.05$) at diestrous and estrous for treated animals at 20 mg/kg and for those treated at 5 mg/kg at proestrous and metestrous ($P< 0.005$ and $P< 0.05$ respectively. The rest of the stages did not show significant differences in estrone levels.

Figure 26 shows the effect of heptachlor on the levels of estradiol 17β. The effect was significant ($P<0.05$, $P<0.001$,$P<0.01$ $P<0.01$) at diestrous, proestrous, estrous and metestrous stages respectively at the high dose, while at the low dose, it was significant ($P<0.05$, $P<0.05$ and $P<0.001$) at proestrous, estrous and metestrous respectively.

Figures 27 to 33 show the results of hormonal analysis in culture media samples following incubation of ovarian cells from either control animals or heptachlor treated animals with either androstenedione or pregnenolone or both.

In figure 27 the levels of pregnenolone in the culture media for cells at 5 mg/kg and 20 mg/kg (Fig. 27a &b) respectively are shown. Pregnenolone was only determined in the culture tubes into which exogenous pregnenolone had not been added except for the tubes which had both androstenedione and pregnenolone together. The results indicate higher levels of endogenous

pregnenolone at the lower heptachlor dose level than in the controls ($P< 0.01$). However levels of pregnenolone are significantly lower in heptachlor treated cells at both low and high heptachlor dose levels ($P< 0.001$) compared to controls.

Figure 28 gives the results of measuring DHEA in the culture media. The levels of DHEA are significantly lower at the higher dose of heptachlor in the presence of either androstenedione ($P< 0.005$) or both androstenedione and pregneneolone ($P< 0.05$).

Figure 29 shows the results of measuring androstenedione in the culture media. Androstenedione was only determined in those tubes having no exogenous androstenedione except for the tubes which had both androstenedione and pregnenolone together (column 4). The results indicate significantly lower levels of androstenedione secreted at the higher heptachlor dose ($P< 0.005$), compared to the lower dose, following incubation in the presence of pregnenolone.

Figure 30 shows the result of determining progesterone levels in the culture media. The secretion of progesterone is affected by heptachlor treatment with the higher heptachlor dose giving a more significant decrease ($P< 0.001$) in progesterone secretion compared to the lower heptachlor dose and the controls.

The results of assaying for 17α hydroxyprogesterone (OHP4) in the culture media is shown in figure 31. The levels of OHP4 are generally lower in the treated animals compared to controls and the decrease is more pronounced ($P< 0.001$) at the higher dose level of heptachlor (Fig. 31b) than at the lower dose level (Fig. 31a). There is also more OHP4 secreted following incubation with androstenedione alone or in combination with pregnenolone (column 2&4) but not with pregnenolone alone (column 3).

For estrone (Fig. 32) the results indicate significantly higher levels of estrone at the lower heptachlor dose (Fig. 32a) either in the control cells with no

exogenous hormone precursor ($P< 0.005$), or following incubation with androstenedione ($P<0.001$) and pregnenolone ($P< 0.001$). The results of measuring estradiol 17β (Fig. 33) indicate a significant decrease in its secretion at higher dose level compared to the lower dose ($P< 0.05$) and that more estradiol is secreted following incubation with androstenedione, either alone or in combination with pregnenolone (column 2&4), but not so much with pregnenolone alone.

Figure 18.

HPLC elution profile for P5, P4, OHP4 and DHEA run on the first solvent system (methanol:water). These four hormones separated clearly on this system. However, E1, E2, and androstenedione eluted confluently and had to be separated on another solvent system.

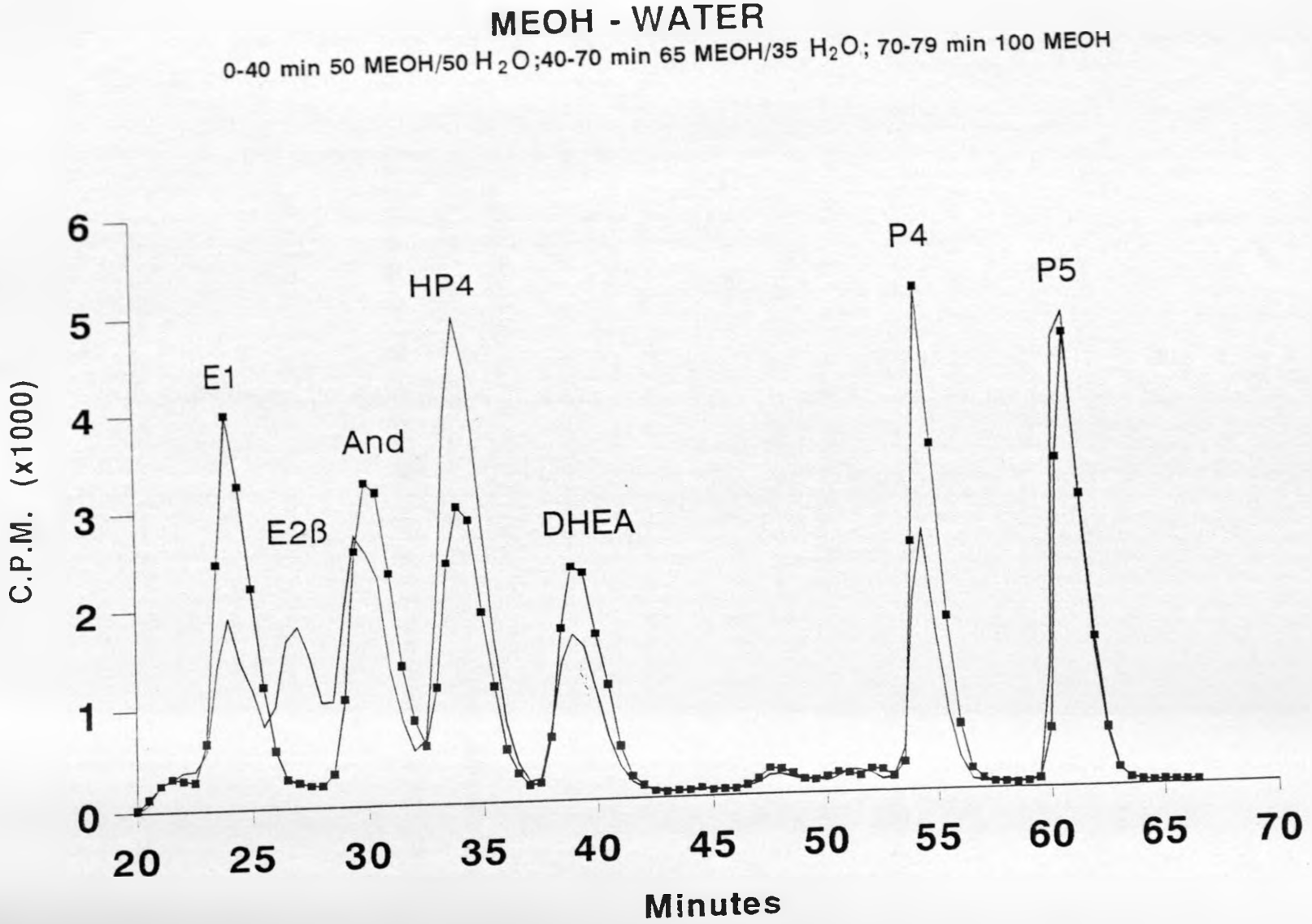
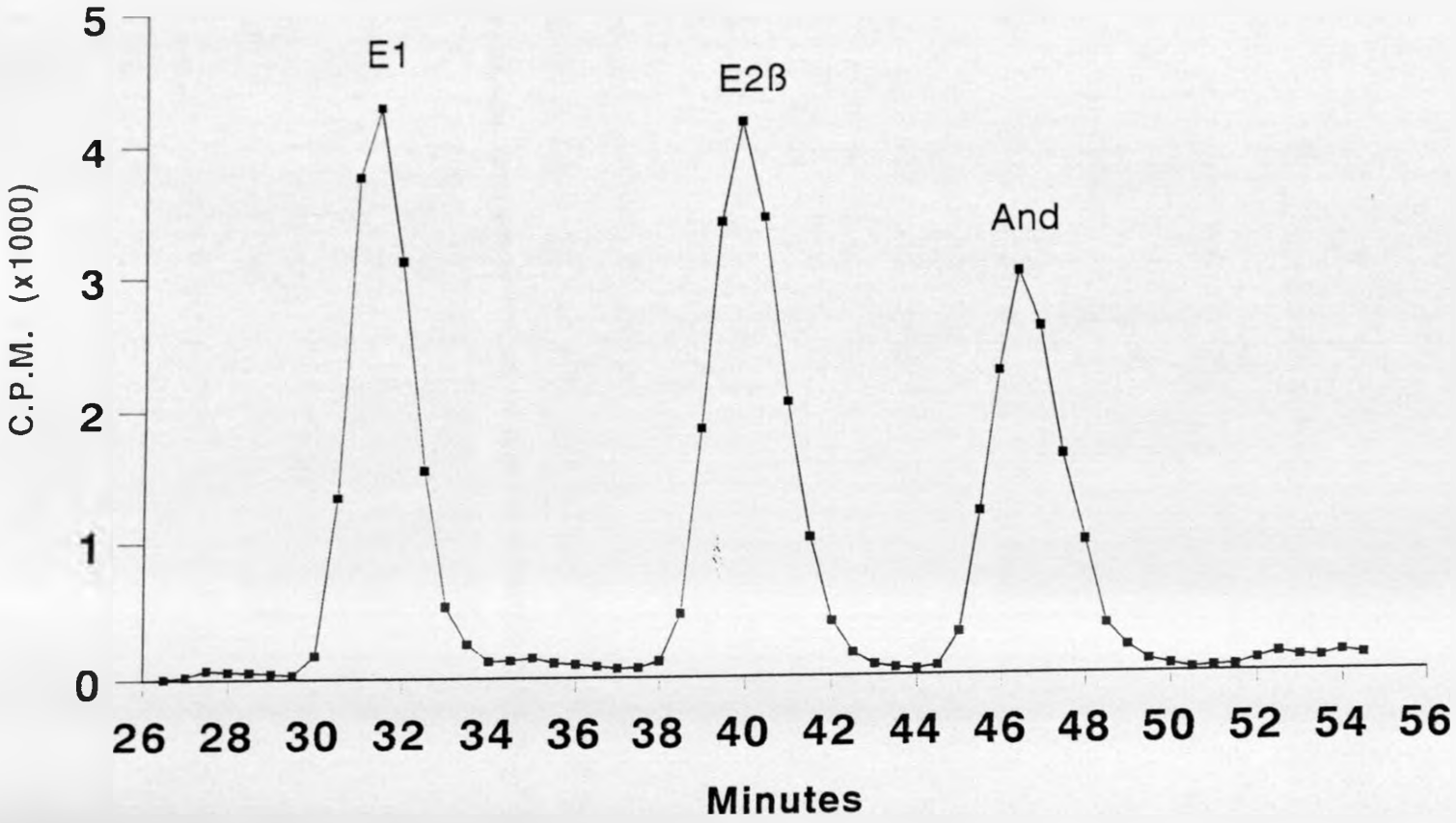

MEOH - WATER
0-40 min 50 MEOH/50 H2O;40-70 min 65 MEOH/35 H2O; 70-79 min 100 MEOH
C.P.M. (x1000)
Minutes
E1
E2ß
And
HP4
DHEA
P4
P5
0
1
2
3
4
5
6
20
25
30
35
40
45
50
55
60
65
70

Figure 19.

HPLC elution profile for E1, E2, and androstenedione ran on the second solvent system (acetonitrile:water). This system was able to separate the three hormones.

ACN - WATER

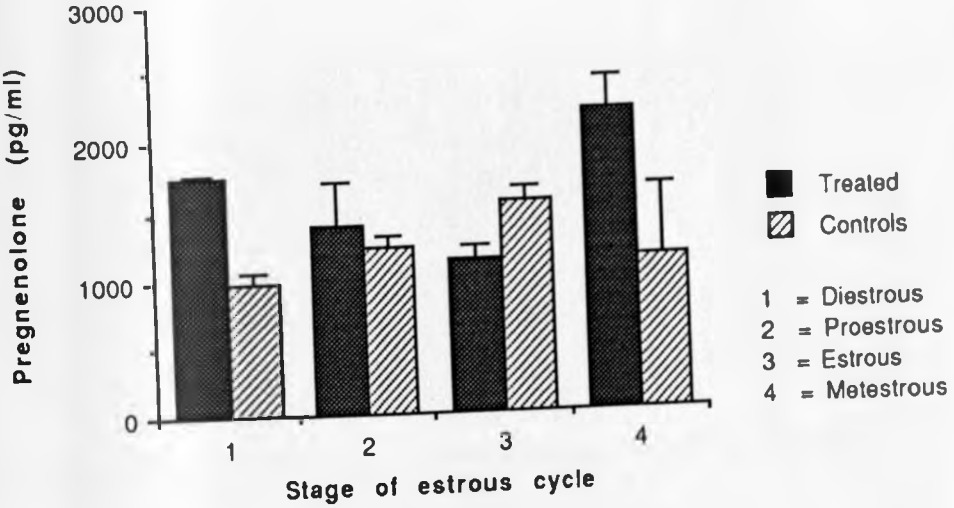
0-44 min 30 ACN/70 H_2O ;44-50 min Gradient n. 100 ACN;50-56 min 100 ACN;56-64 min 30 ACN;



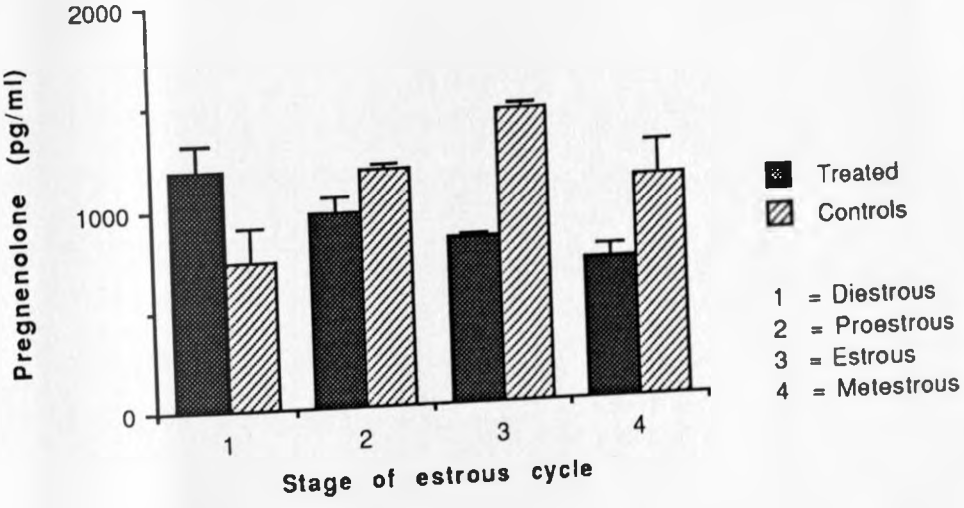
I-1961

Figure 20.

Pregnenolone analysis in plasma samples of controls and following treatment of female rats with heptachlor at either 5 mg/kg (Fig. 20a) or 20 mg/kg body weight.(Fig. 20b).


3000
2000
1000
0
Pregnenolone (pg/ml)
1
2
3
4
Stage of estrous cycle
Treated
Controls
1 = Diestrous
2 = Proestrous
3 = Estrous
4 = Metestrous

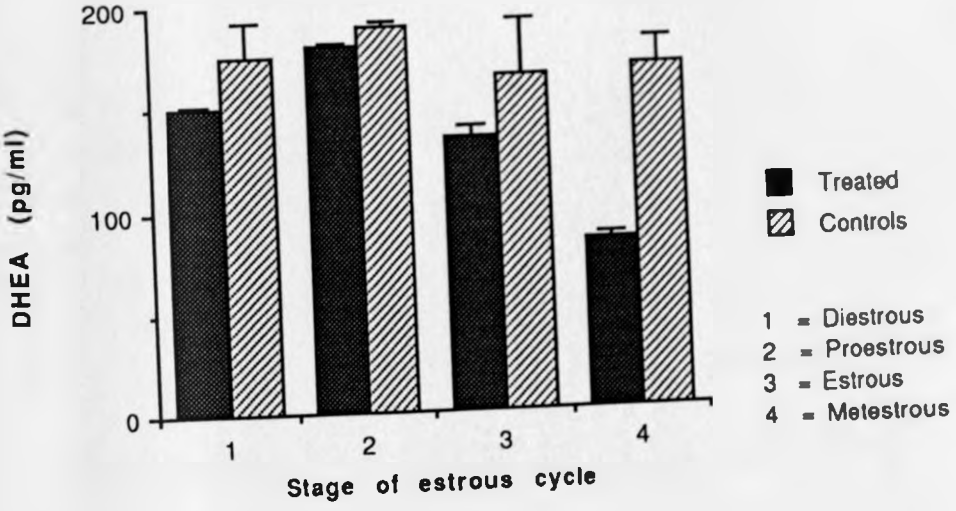
(a)


2000
1000
0
Pregnenolone (pg/ml)
1
2
3
4
Stage of estrous cycle
Treated
Controls
1 = Diestrous
2 = Proestrous
3 = Estrous
4 = Metestrous

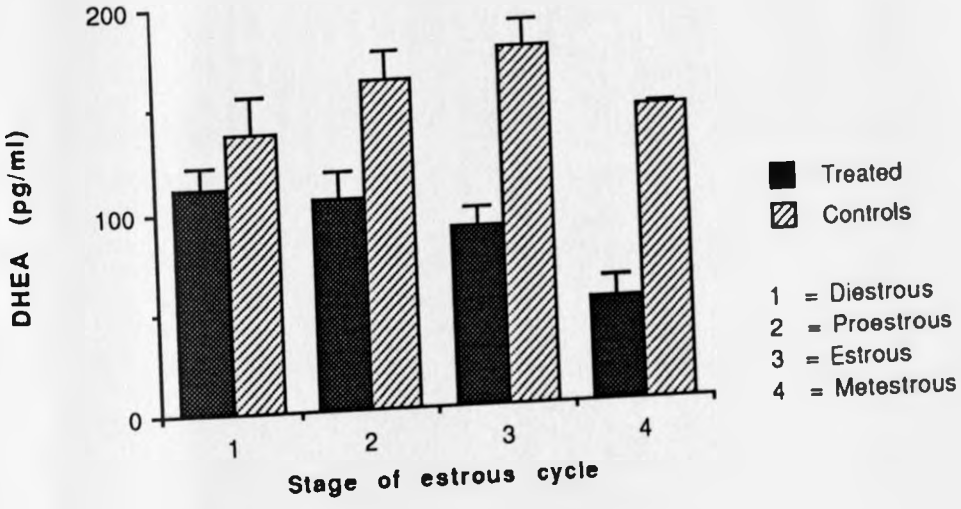
(b)

Figure 21.

Dehydroepiandrosterone (DHEA) analysis in plasma samples of controls and following treatment of female rats with heptachlor at either 5 mg/kg (Fig. 21a) or 20 mg/kg body weight.(Fig. 21b).


DHEA (pg/ml)
200
100
0
1
2
3
4
Stage of estrous cycle
Treated
Controls
1 = Diestrous
2 = Proestrous
3 = Estrous
4 = Metestrous

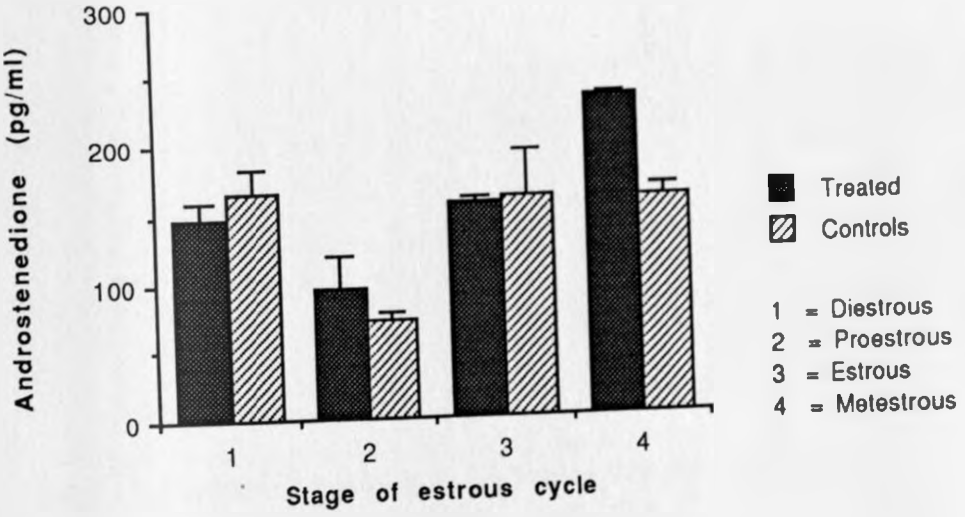
(a)


DHEA (pg/ml)
200
100
0
1
2
3
4
Stage of estrous cycle
Treated
Controls
1 = Diestrous
2 = Proestrous
3 = Estrous
4 = Metestrous

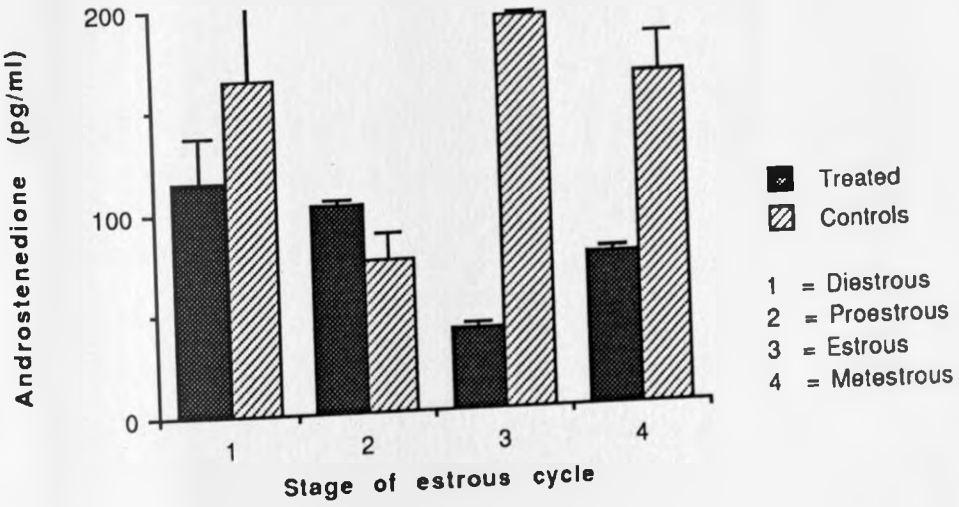
(b)

Figure 22.

Plasma androstenedione levels in controls and heptachlor treated animals at 5 mg/kg (Fig. 22a) and 20 mg/kg (Fig. 22b).


Androstenedione (pg/ml)
300
200
100
0
1
2
3
4
Stage of estrous cycle
Treated
Controls
1 = Diestrous
2 = Proestrous
3 = Estrous
4 = Metestrous

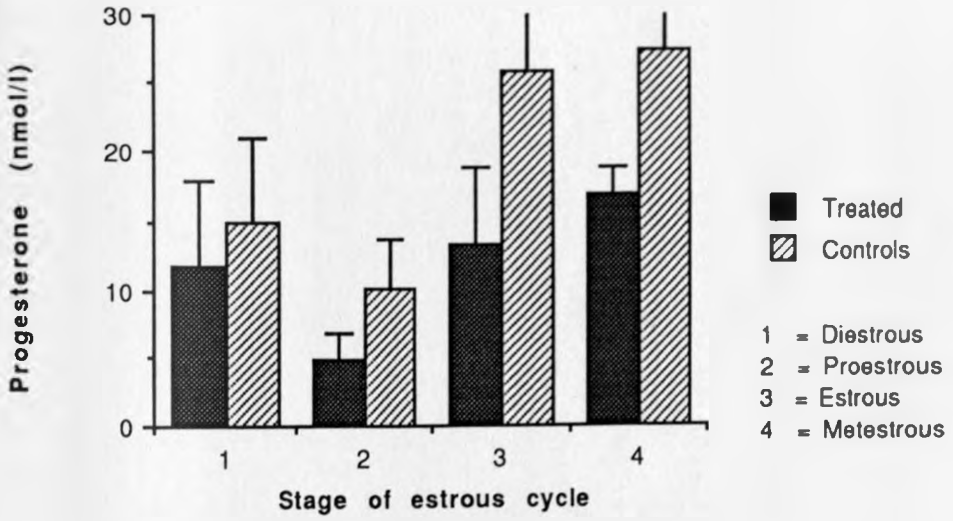
(a)


Androstenedione (pg/ml)
200
100
0
1
2
3
4
Stage of estrous cycle
Treated
Controls
1 = Diestrous
2 = Proestrous
3 = Estrous
4 = Metestrous

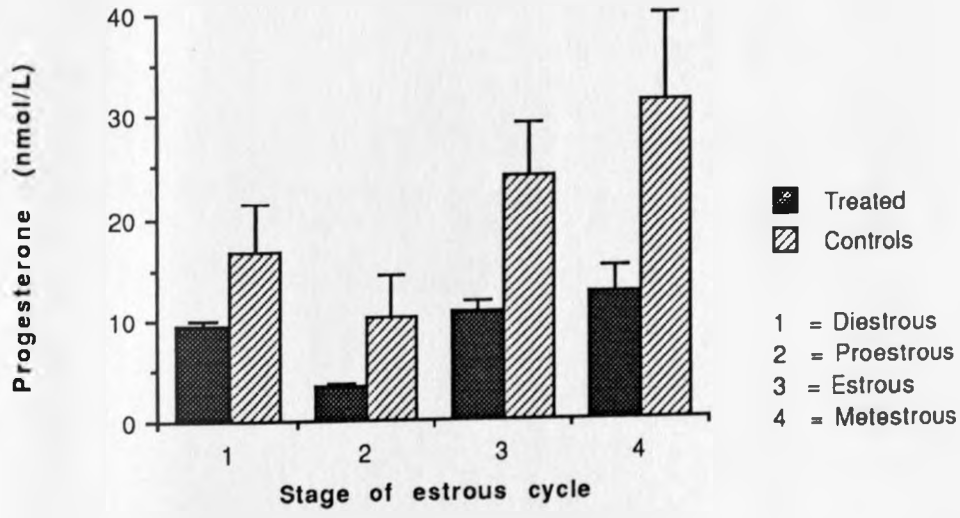
(b)

Figure 23.

Plasma progesterone levels in controls and heptachlor treated animals at 5 mg/kg (Fig. 23a) and 20 mg/kg (Fig. 23b).


Progesterone (nmol/l)
30
20
10
0
1
2
3
4
Stage of estrous cycle
Treated
Controls
1 = Diestrous
2 = Proestrous
3 = Estrous
4 = Metestrous

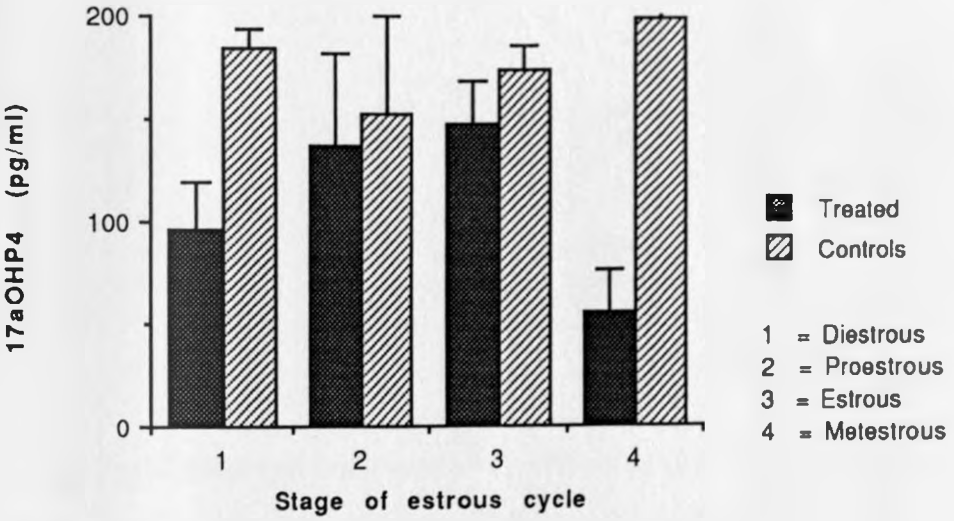
(a)


Progesterone (nmol/L)
40
30
20
10
0
1
2
3
4
Stage of estrous cycle
Treated
Controls
1 = Diestrous
2 = Proestrous
3 = Estrous
4 = Metestrous

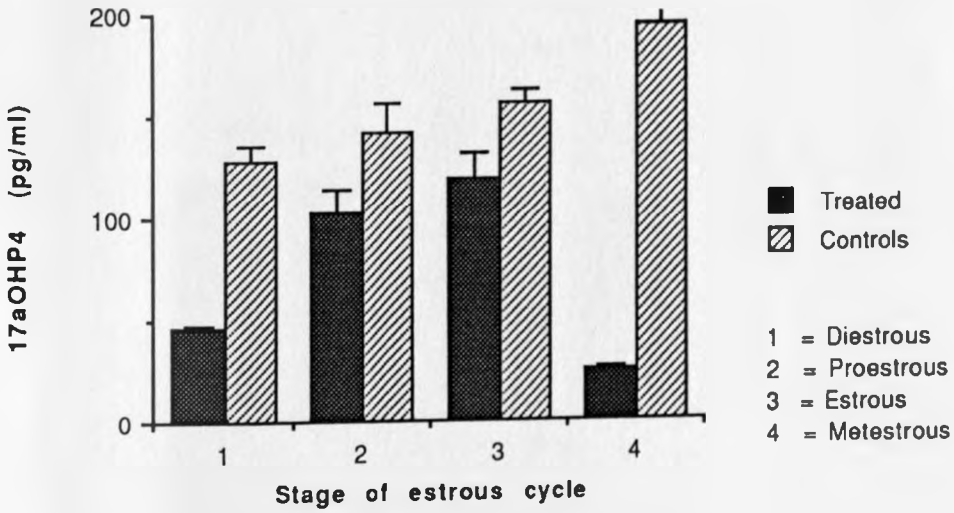
(b)

Figure 24.

Plasma levels of 17α hydroxyprogesterone (HP4) in controls and heptachlor treated animals at 5 mg/kg (Fig. 24a) and 20 mg/kg (Fig. 24b).



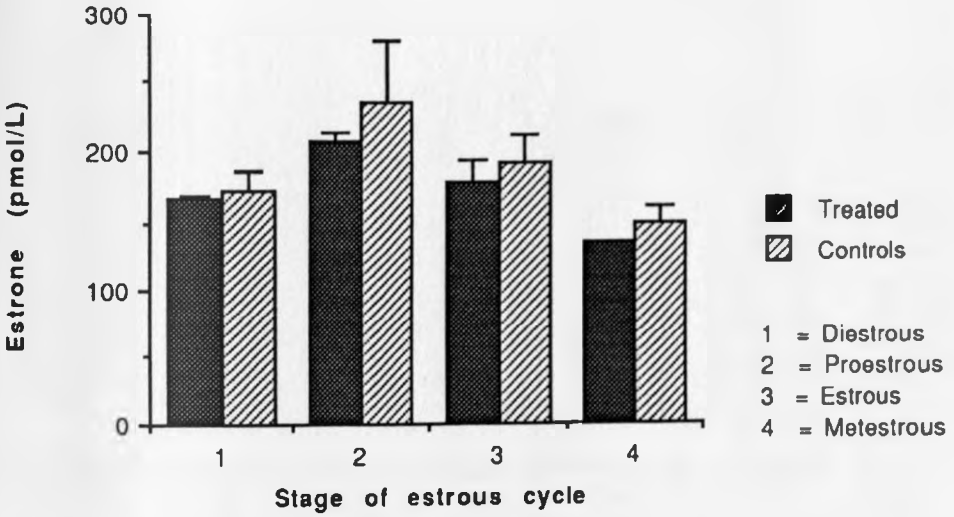
(a)



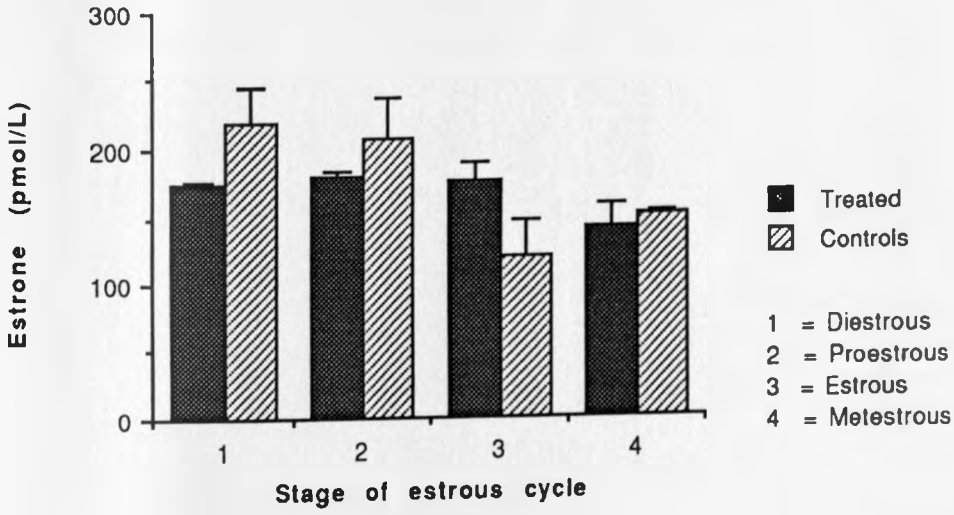
(b)

Figure 25.

Plasma levels of estrone in controls and heptachlor treated animals at 5 mg/kg (Fig. 25a) and 20 mg/kg (Fig. 25b).


300
200
100
0
Estrone (pmol/L)
1
2
3
4
Stage of estrous cycle
Treated
Controls
1 = Diestrous
2 = Proestrous
3 = Estrous
4 = Metestrous

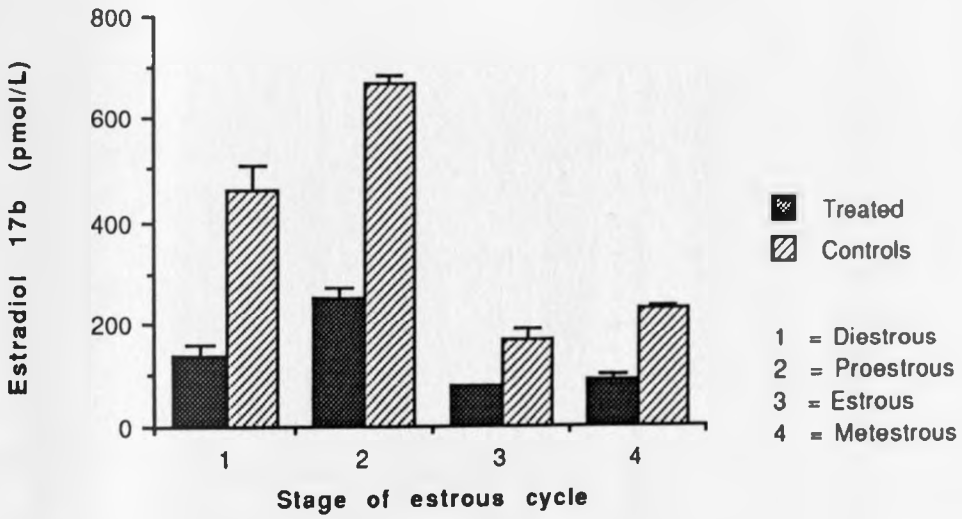
(a)


300
200
100
0
Estrone (pmol/L)
1
2
3
4
Stage of estrous cycle
Treated
Controls
1 = Diestrous
2 = Proestrous
3 = Estrous
4 = Metestrous

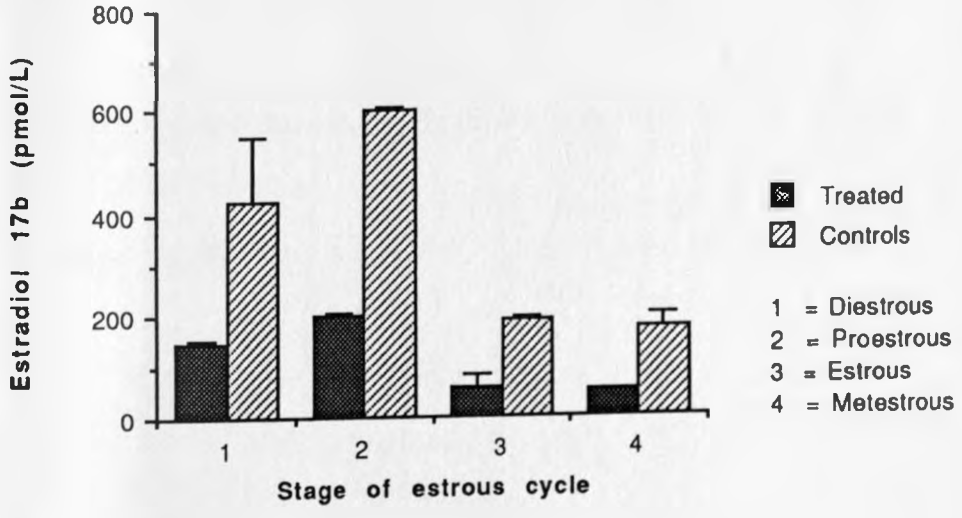
(b)

Figure 26.

Plasma levels of estradiol 17β in controls and heptachlor treated animals at 5 mg/kg (Fig. 26a) and 20 mg/kg (Fig. 26).


Estradiol 17b (pmol/L)
800
600
400
200
0
1
2
3
4
Stage of estrous cycle
Treated
Controls
1 = Diestrous
2 = Proestrous
3 = Estrous
4 = Metestrous

(a)


Estradiol 17b (pmol/L)
800
600
400
200
0
1
2
3
4
Stage of estrous cycle
Treated
Controls
1 = Diestrous
2 = Proestrous
3 = Estrous
4 = Metestrous

(b)

Figure 27.

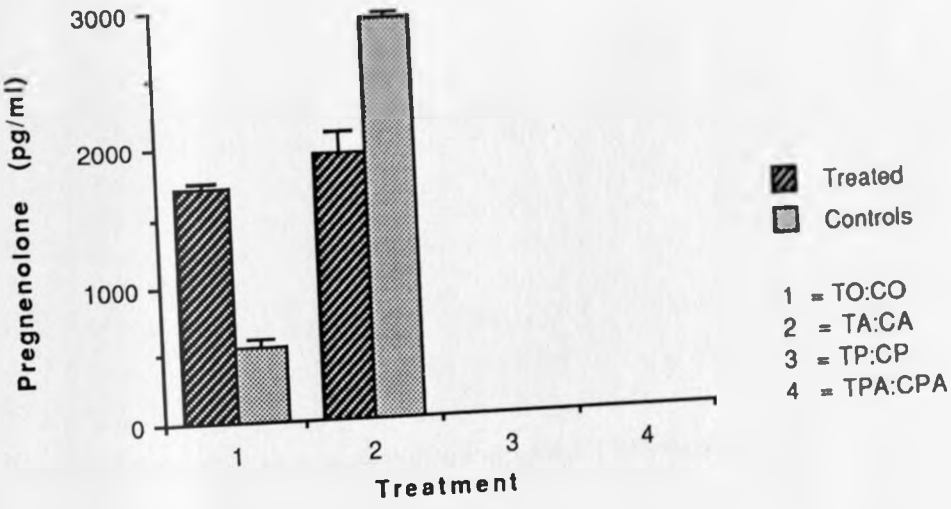
The levels of pregnenolone in the culture media of cells taken from control animals and animals treated with heptachlor at 5 mg/kg (Fig. 27a) and at 20 mg/kg (Fig. 27b).

TO:CO - Treated vs control cells incubated in culture medium alone

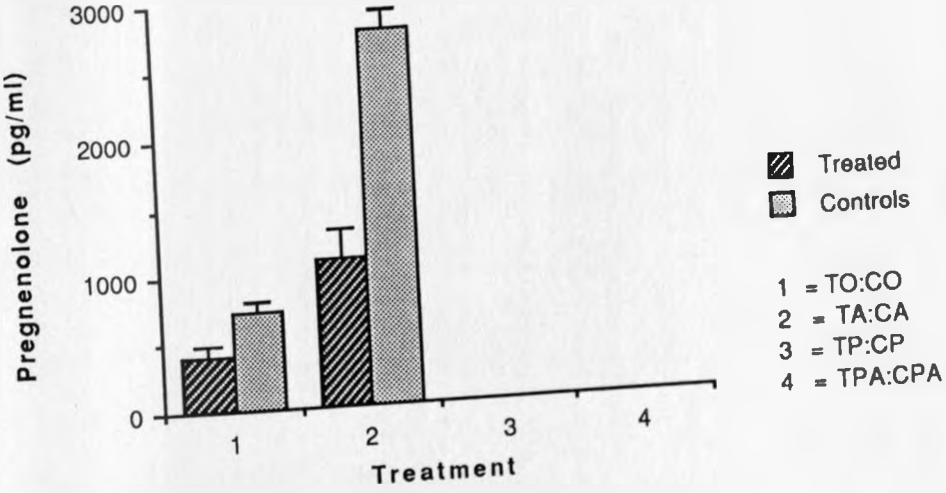
TA:CA - Treated vs control cells incubated with androstenedione

TP:CP - Treated vs control cells incubated with pregnenolone

TPA:CPA - Treated vs control cells incubated with both pregnenolone and androstenedione


3000
2000
1000
0
Pregnenolone (pg/ml)
1
2
3
4
Treatment
Treated
Controls
1 = TO:CO
2 = TA:CA
3 = TP:CP
4 = TPA:CPA

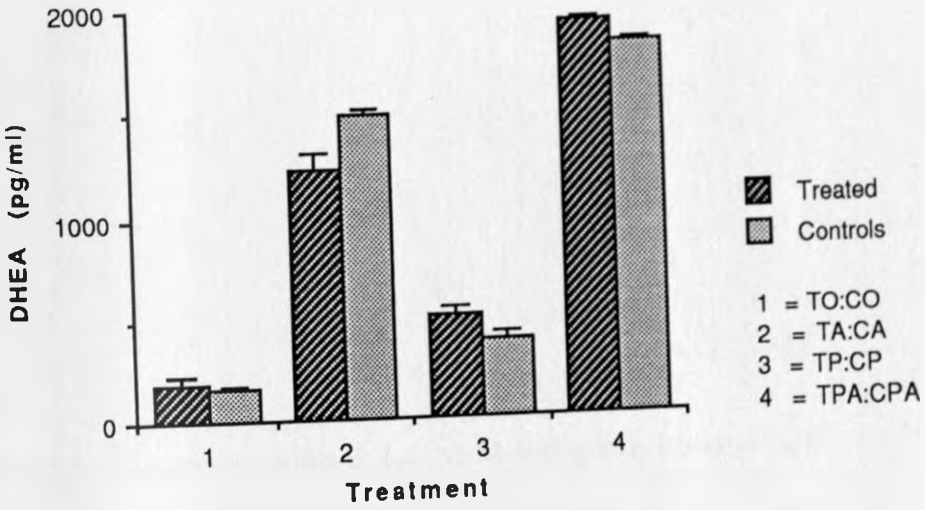
(a)


3000
2000
1000
0
Pregnenolone (pg/ml)
1
2
3
4
Treatment
Treated
Controls
1 = TO:CO
2 = TA:CA
3 = TP:CP
4 = TPA:CPA

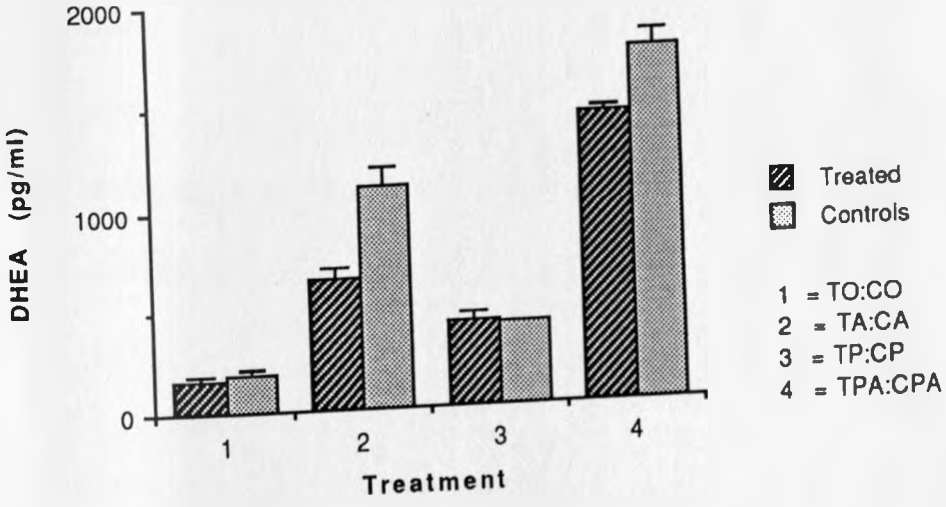
(b)

Figure 28.

The levels of DHEA in the culture media of cells taken from control animals and animals treated with heptachlor at 5 mg/kg (Fig. 28a) and at 20 mg/kg (Fig. 28b).


2000
1000
0
DHEA (pg/ml)
1
2
3
4
Treatment
Treated
Controls
1 = TO:CO
2 = TA:CA
3 = TP:CP
4 = TPA:CPA

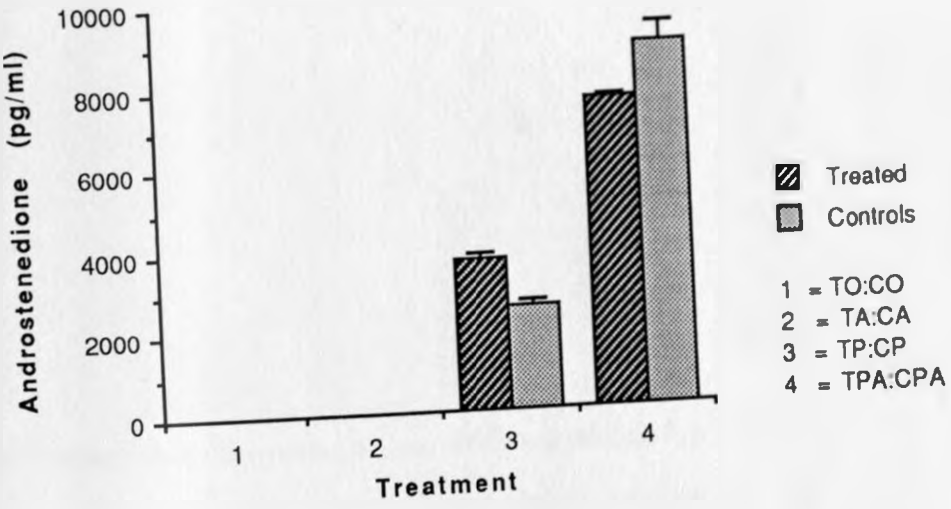
(a)


2000
1000
0
DHEA (pg/ml)
1
2
3
4
Treatment
Treated
Controls
1 = TO:CO
2 = TA:CA
3 = TP:CP
4 = TPA:CPA

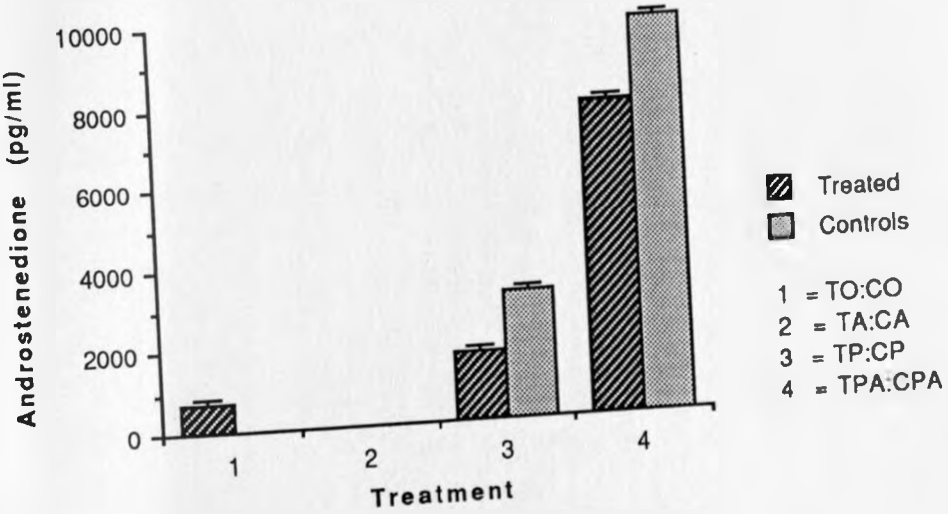
(b)

Figure 29.

The levels of androstenedione in the culture media of cells taken from control animals and animals treated with heptachlor at 5 mg/kg (Fig. 29a) and at 20 mg/kg (Fig. 29b). Androstenedione was not determined in tubes in which the hormone had been added exogenously, except where both androstenedione and pregnenolone was added.


Androstenedione (pg/ml)
10000
8000
6000
4000
2000
0
1
2
3
4
Treatment
Treated
Controls
1 = TO:CO
2 = TA:CA
3 = TP:CP
4 = TPA:CPA

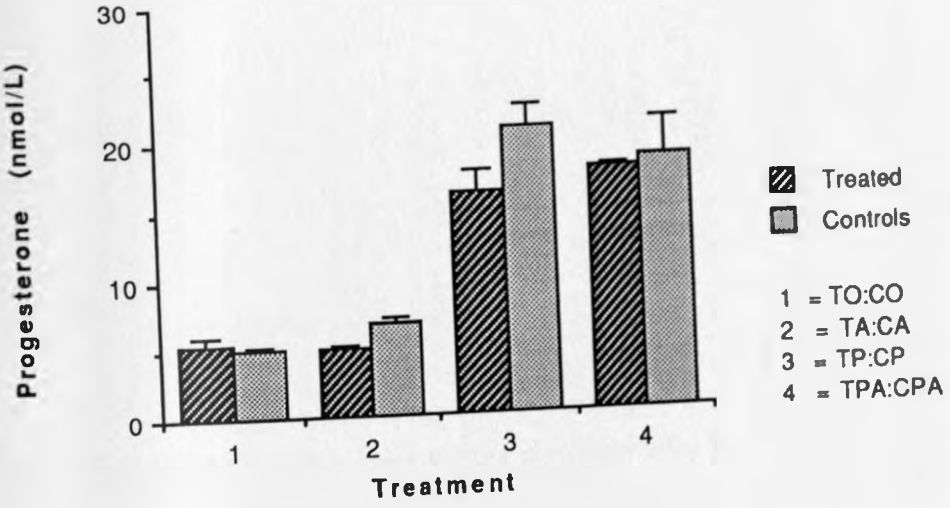
(a)


Androstenedione (pg/ml)
10000
8000
6000
4000
2000
0
1
2
3
4
Treatment
Treated
Controls
1 = TO:CO
2 = TA:CA
3 = TP:CP
4 = TPA:CPA

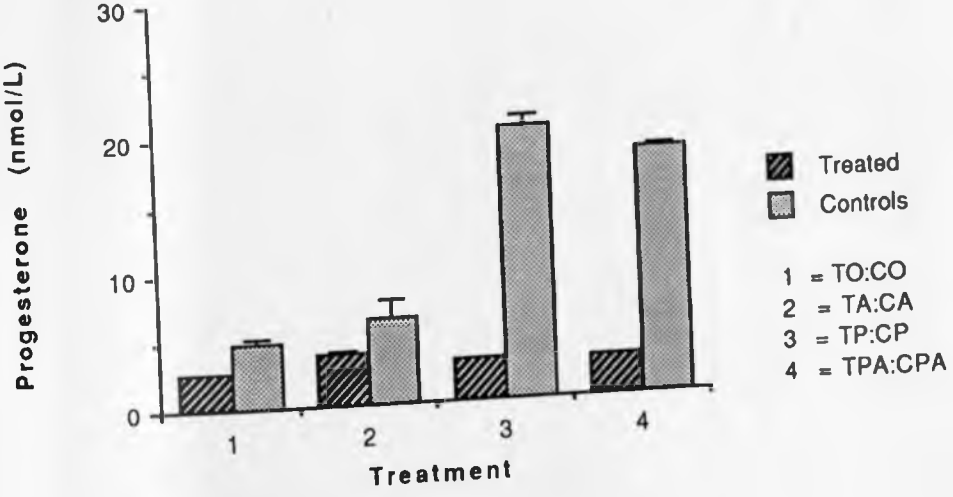
(b)

Figure 30.

The levels of progesterone in the culture media of cells taken from control animals and animals treated with heptachlor at 5 mg/kg (Fig. 30a) and at 20 mg/kg (Fig. 30b).


Progesterone (nmol/L)
30
20
10
0
1
2
3
4
Treatment
Treated
Controls
1 = TO:CO
2 = TA:CA
3 = TP:CP
4 = TPA:CPA

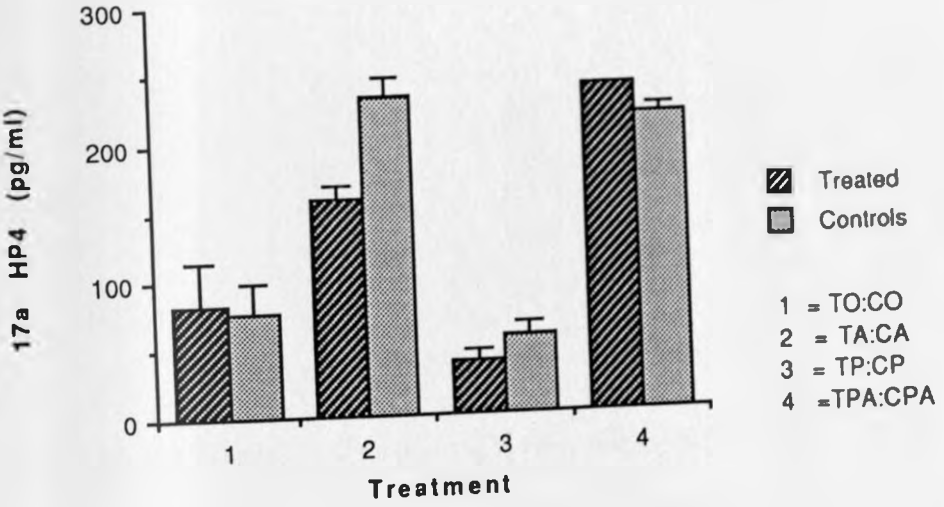
(a)


Progesterone (nmol/L)
30
20
10
0
1
2
3
4
Treatment
Treated
Controls
1 = TO:CO
2 = TA:CA
3 = TP:CP
4 = TPA:CPA

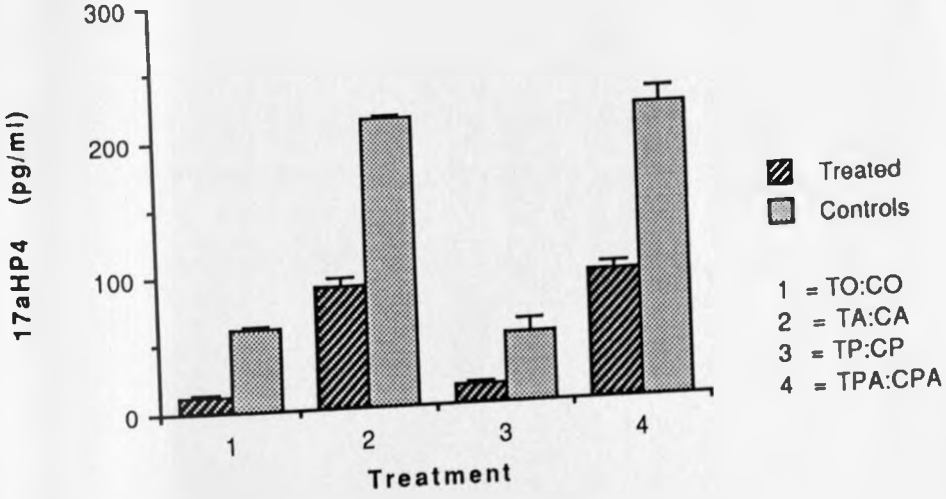
(b)

Figure 31.

The levels of 17α hydroxyprogesterone (HP4) in the culture media of cells taken from control animals and animals treated with heptachlor at 5 mg/kg (Fig. 31a) and at 20 mg/kg (Fig. 31b).


300
200
100
0
17a HP4 (pg/ml)
1
2
3
4
Treatment
Treated
Controls
1 = TO:CO
2 = TA:CA
3 = TP:CP
4 =TPA:CPA

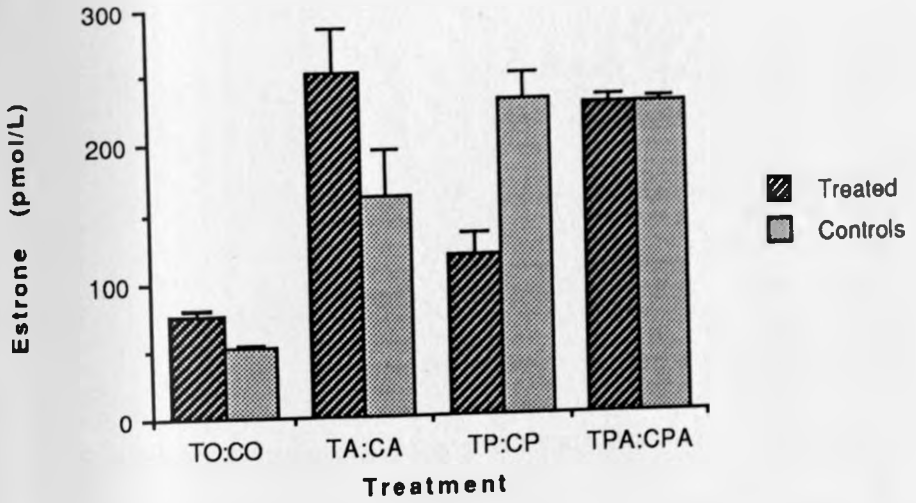
(a)


300
200
100
0
17aHP4 (pg/ml)
1
2
3
4
Treatment
Treated
Controls
1 = TO:CO
2 = TA:CA
3 = TP:CP
4 = TPA:CPA

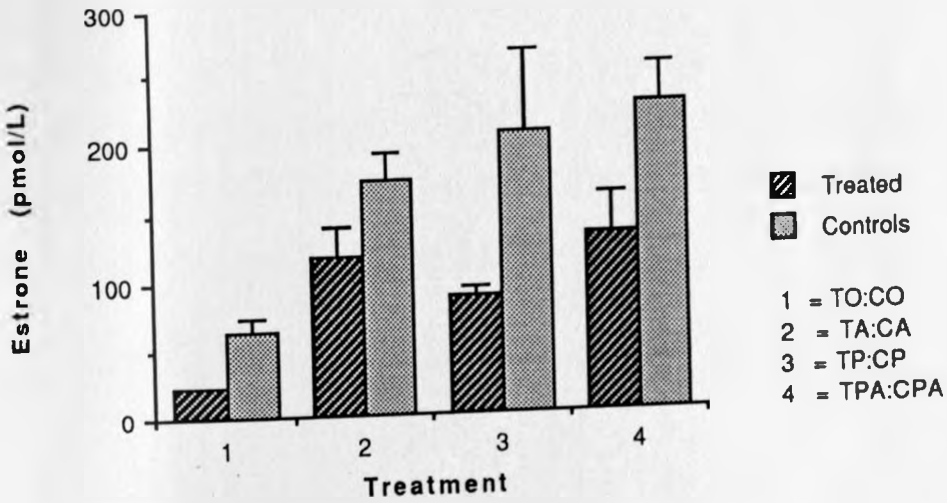
(b)

Figure 32.

The levels of estrone in the culture media of cells taken from control animals and animals treated with heptachlor at 5 mg/kg (Fig. 32a) and at 20 mg/kg (Fig. 32b).


300
200
100
0
Estrone (pmol/L)
TO:CO
TA:CA
TP:CP
TPA:CPA
Treatment
Treated
Controls

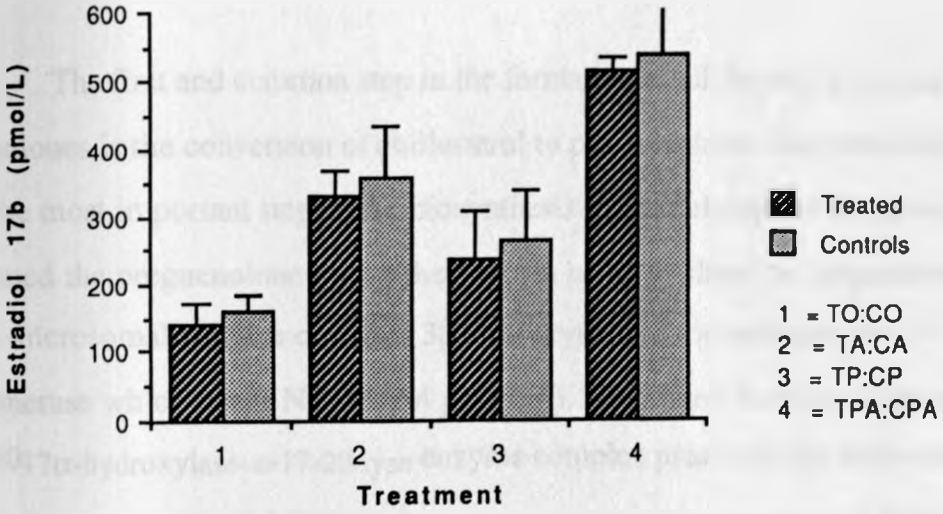
(a)


300
200
100
0
Estrone (pmol/L)
1
2
3
4
Treatment
Treated
Controls
1 = TO:CO
2 = TA:CA
3 = TP:CP
4 = TPA:CPA

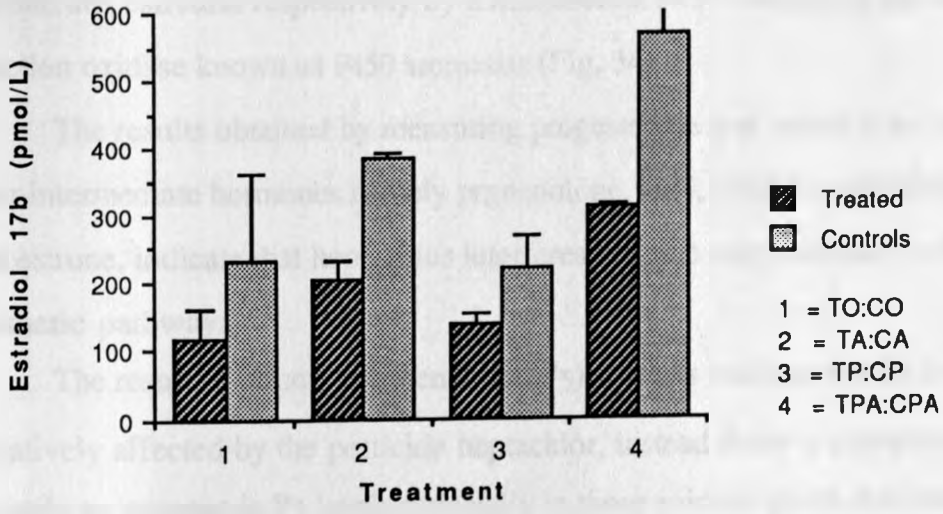
(b)

Figure 33.

The levels of estradiol 17β in the culture media of cells taken from control animals and animals treated with heptachlor at 5 mg/kg (Fig. 33a) and at 20 mg/kg (Fig. 33b).


Estradiol 17b (pmol/L)
600
500
400
300
200
100
0
1
2
3
4
Treatment
Treated
Controls
1 = TO:CO
2 = TA:CA
3 = TP:CP
4 = TPA:CPA

(a)


Estradiol 17b (pmol/L)
600
500
400
300
200
100
0
1
2
3
4
Treatment
Treated
Controls
1 = TO:CO
2 = TA:CA
3 = TP:CP
4 = TPA:CPA

(b)

6.4 Discussion

The first and common step in the formation of all the major steroid hormones is the conversion of cholesterol to pregnenolone. This rate limiting step is the most important step in the biosynthesis of the subsequent hormones. Once formed the pregnenolone may either remain or be oxidized to progesterone by the microsomal enzyme complex 3β hydroxysteroid dehydrogenase $\Delta^{4,5}$ isomerase which needs NAD+ (Δ4 pathway). The mixed function oxidase P450$_{17}$α-hydroxylase- c-17-20-lyse enzyme complex present in the microsomes and which requires also NADPH and oxygen, converts pregnenolone to DHEA and progesterone to androstenedione. In the presence of 17β hydroxysteroid dehydrogenase, which needs NADH, the androstenedione is reversibly reduced to testosterone. The androstenedione and testosterone can then be aromatized to estrone and estradiol respectively by a microsomal P450 containing the mixed function oxidase known as P450 aromatase (Fig. 34).

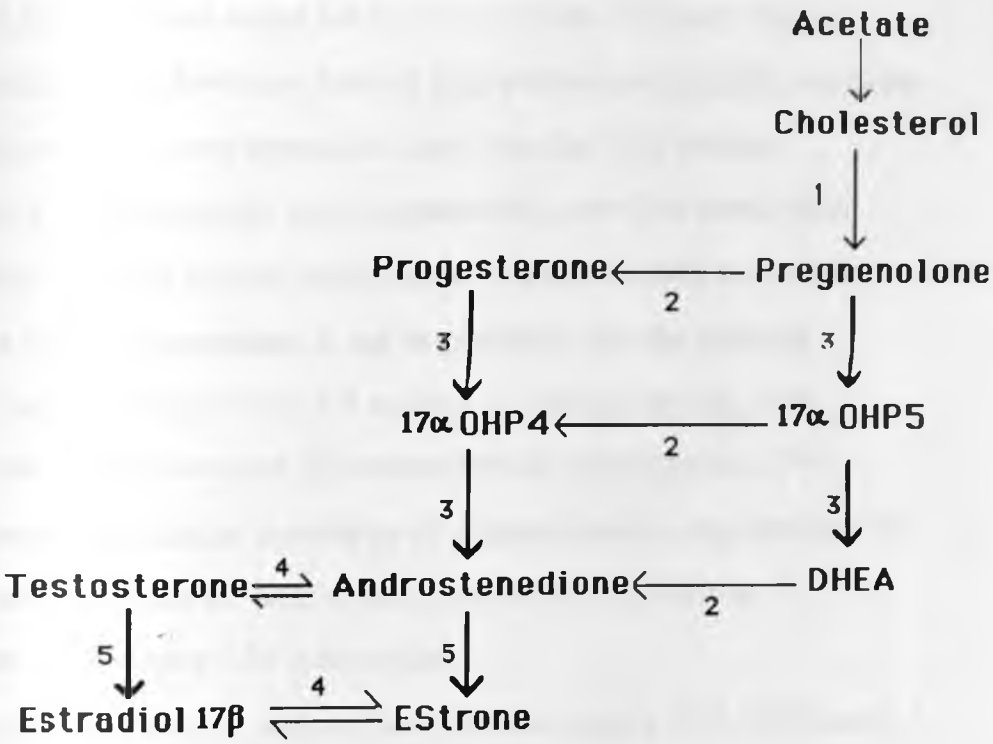
The results obtained by measuring progesterone and estradiol as well as their intermediate hormones namely prgnenolone, HP4, DHEA, androstenedione and estrone, indicate that heptachlor interferes in some way with some of these synthetic pathways.

The result of plasma pregnenolone (P_5) analysis indicate that its level is not negatively affected by the pesticide heptachlor, instead there is a tendency towards an increase in P_5 levels especially in those animals given the lower dose of the pesticide, and more so at the metestrus stage. However the P_5 levels are generally lower at the higher dose level of heptachlor than at the lower dose compared to
the controls.

The accumulation of pregnenolone in the latter may be as a result of several factors. Firstly, it could be that low levels of heptachlor act as inducers of enzymes responsible for *de novo* synthesis of pregnenolone from acetate (i.e. 20α desmolase). Secondly, the possibility that the same dose of heptachlor (5 mg/kg body weight), inhibits 3β HSD and 17α hydroxylase must be considered. Both the above actions would result in accumulation of P5 (see Fig. 34).

However it was noted that at low dose, in vitro production of pregnenolone by ovarian cells was also enhanced. Thus, it is proposed that at the latter dose there is selective induction/activation of 20α desmolase and 3β HSD and probably inhibition of 17α hydroxylase.

From pregnenolone onwards, the levels of every hormone along the two pathways falls significantly with increase in heptachlor dose except estrone whose fall is not significant. The most dramatic decreases in the plasma levels are those of progesterone and estradiol, which are significant even at the lower heptachlor dose. Progesterone is derived directly from pregnenolone as the first steroid after pregnenolone. Since the level of progesterone falls significantly even in the absence of a similar fall in pregnenolone, it can be postulated that the pesticide heptachlor affects, most drastically, the enzyme complex 3β-hydroxysteroid dehydrogenase $\Delta^{4,5}$ isomerase responsible for the conversion of pregnenolone to progesterone.



1 = 20α 22-27 desmolase

2 = 3 β hydroxysteroid dehydrogenase + isomerase

3 = 17α hydroxylase-C 17-20 lyase

4 = 17β hydroxysteroid dehydrogenase

5 = Aromatase

Fig. 34

From pregnenolone onwards, the levels of every hormone along the two pathways falls significantly with increase in heptachlor dose (5 mg/kg to 20mg/kg body wight) except estrone whose fall is not significant. The most dramatic decreases in the plasma levels are those of progesterone and estradiol, which are significant even at the lower heptachlor dose (5 mg/kg body weight). Progesterone is derived directly from pregnenolone as the first steroid after pregnenolone. Since the level of progesterone falls significantly even in the absence of a fall in pregnenolone, it can be postulated that the pesticide heptachlor (above the dose level of 5 mg/kg body weight) affects, most drastically, the enzyme complex 3β-hydroxysteroid dehydrogenase $\Delta^{4,5}$ isomerase responsible for the conversion of pregnenolone to progesterone. The possibility also exists that the latter doses accelerate the metabolism of progesterone by activating 17α hydroxylase.

The fall in the levels of intermediate hormones namely HP4, DHEA and androstenedione could then be explained by the decrease in their substrate hormones pregnenolone (at the 20 mg/kg dose level) and progesterone, or an increase in their metabolism. This explanation makes sense considering that the levels of DHEA and androstenedione, at the lower heptachlor dose are not significantly different from controls and that this could be due to the fact that the level of pregnenolone from where these two hormones are derived is higher at the lower heptachlor dose level.

At this point in the study therefore, all that can be proposed is that heptachlor affects the synthesis of several steroid hormones by ovarian cells. At a low dose of 5 mg/kg body weight, heptachlor seems to enhance the overall conversion of cholesterol to pregnenolone. However apart from this positive effect on pregnenolone, heptachlor affects the rest of the hormones in varied ways, and in a dose related manner.

The *in-vitro* experiments in which isolated ovarian cells were incubated with precursors, namely pregnenolone and androstenedione, were further attempts to find the precise effect of heptachlor on the ovarian steroidogenic pathways. The ovarian cells were isolated from adult rats previously treated with heptachlor as has been explained. The higher basal levels of pregnenolone in animals at the lower dose level of heptachlor is again apparent. However the high levels of pregnenolone following incubation with androstenedione, in both controls and treated animals at the lower heptachlor dose and controls at the higher dose (Fig. 27a&b), is not easy to explain at this stage. A possible explanation has been suggested by some authors, (Schomberg et al., 1976 and Nimrod and Lindner, 1976) who have implicated androgens in the control of granulosa cell activities, particularly in the regulation of ovarian steroidogenic pathways. They showed stimulatory effect of androgens on progesterone production in cultured granulosa cells from pig (Schomberg et al., 1976) and in rats (Nimrod and Lindner, 1976). The role of androgens is thought to be that they augment the effect of FSH on steroidogenesis. It is possible, therefore, that the presence of androstenedione in the culture media, enhanced the synthesis of pregnenolone and HP_4 both of which are progestins, the HP_4 coming perhaps from HP_5.

The results also indicate an increase in progesterone secretion following incubation with pregnenolone, alone or in combination with androstenedione (Fig. 30 column 3&4), but not in the presence of androstenedione alone (column 2). This increase in progesterone secretion is however only indicated in the control groups and at the lower heptachlor dose level (Fig. 30a) but not at the higher dose level (Fig. 30b).

Using the same argument as above for the role of androgens in the control of granulosa cells, one would expect the level of progesterone to be high in the

media of cells cultured in the presence of androstenedione. That this is not the case could mean that the conversion of pregnenolone to progesterone is interfered with in the presence of heptachlor. According to Tanabe et al. (1992), the action site of androgen is the conversion of cholesterol to pregnenolone. If androgens are going to stimulate the conversion of cholesterol to pregnenolone, which is clearly demonstrated by the results reported here (Fig. 27a&b), then the low levels of P_4 which is derived from P_5 could mean either a blockage of the enzyme responsible for the conversion in the heptachlor treated animals or that progesterone is formed but rapidly metabolised. The rest of the results suggest an overall detrimental effect of heptachlor on the ability of the ovarian cells to synthesis the rest of the steroid hormones from the two precursor or substrate hormones provided. The high levels of DHEA in cells cultured in the presence of androstenedione remain to be investigated.

The results in these *in-vitro* experiments indicate that the levels of HP_4 are affected more than those of DHEA. This would suggest that the P_5 - P4 - HP4 - androstenedione pathway ($\Delta 4$ pathway) was more prone to heptachlor interference than the P5 - HP5 - DHEA - androstenedione pathway ($\Delta 5$ pathway). This agrees with the earlier suggestion based on the results of plasma analysis that the conversion of pregnenolone to progesterone as a step, is mostly affected by heptachlor, followed by the androstenedione to estradiol step, i,e the P450 aromatase enzyme, and that the low levels of HP4 here are also due to low levels of progesterone. Furthermore, progestins have also been reported to modulate FSH-stimulated steroidogenesis. However, and in contrast to the stimulatory action of androgens, progestins were found to inhibit both aromatase and enzymes involved in the production of progesterone and 20α-dihydroprogesterone (Shreiber et al., 1980).

This could also explain (in addition to low levels of progesterone) the low levels of HP4 in cells cultured in the presence of P5 (Fig. 31a&b), and even though there is increased levels of P4 in the control cells, it is possible that the P4 measured would have been higher than recorded.

CHAPTER SEVEN

7.0 GENERAL DISCUSSIONS AND CONCLUSIONS

7.1 General discussion

Reproductive toxicity is the occurrence of adverse effects on the reproductive system that may result from exposure to agents from exogenous sources. The manifestation of such toxicity may include adverse effects on sexual maturation, gamete production and transport, cycle normality, sexual behaviour, fertility, gestation, parturition, lactation, pregnancy outcomes, premature reproductive senescence or modifications in other functions that are dependent on the integrity of the reproductive system. Reproductive toxicity may therefore take numerous forms and involve the reproductive system, the endocrine system and the nervous system.

Traditionally, reproductive toxicity testing has been apical in design, using methods that are intended to detect a broad spectrum of end points and alterations of the overall process of reproductive function. For example, in a multigeneration reproduction study, both males and females would be exposed, covering a period of the life cycle that coincide with major components of reproductive function. The parameters measured then would generally be outcomes of integrated function of the system components. Examples would include fertility, the ability to carry pregnancy to term, and the delivery and rearing of viable offspring. In this type of testing, the specific mechanism of toxicity receives little attention. Often, the degree of contribution of the female or male to the outcome is not determined, since both are treated simultaneously and

the effects that are measured are an assessment of the reproductive integrity of the mating pair (Kimmel et al., 1995).

Due to these shortcomings of the traditional approaches, specific target organ testing is now being encouraged to assess the effect of exposure on defined organ functions, using either *in-vivo* or *in-vitro* test systems. Testing adequately for reproductive toxicity requires coverage of a broad spectrum of potential effects (Kimmel et al., 1995). These can range from apical measures such as fertility and pregnancy outcomes, that require competence from partners of both sexes, to the examination of specific components of the reproductive process in one sex. This was the approach adopted in this study.

In the female, it has not been demonstrated that measurements of fertility and pregnancy outcomes are adequate to reflect the variety of adverse effects on the estrous cycle that can occur. This has been clearly shown in this study where it was found that female rats injected with high heptachlor dose (20 mg/kg body weight), were still capable of getting pregnant and producing viable offspring (be it in significantly less numbers) even in the presence of disrupted estrous cycles and hormonal imbalance.

Although rats or mice are not ideal models for evaluation of potential toxicity to the human menstrual cycle (eg. inadequate corpus luteum in the absence of fertilization), monitoring of the stages of the non pregnant rodent estrous cycle by vaginal smear cytology can detect agents that are potentially capable of causing adverse effects at the hypothalamic, pituitary or ovarian levels. This was also demonstrated by the results of this study where it was found the cycles of the treated females especially at the high dose level were disrupted as early as the fifth day of treatment.

Vaginal smear cytology is the most practical approach to monitor estrous cycle normality (Cooper et al., 1993). Abnormal patterns can show prolonged

proestrous or estrous, indicating interference with follicular maturation or with mechanism of ovulation, or prolonged diestrous, indicating failure of the endocrine mechanism initiating follicular development or pseudopregnancy.The cyclicity monitoring by vaginal smear cytology in this study (Oduma et al., 1995a) showed quite clearly that heptachlor too, an organochlorine pesticide, produced abnormal estrous cycle patterns showing mainly prolonged diestrous and metestrous and occasionally prolonged proestrous.

Despite extensive understanding of the physiologic events associated with fertilization, implantation and pregnancy in mammals, relatively little is known about xenobiotics that disrupt these processes. More importantly, of the handful agents that have clearly defined adverse effects on reproduction, the mechanisms by which they mediate these effects are poorly understood (Matt and Borzelleca, 1995). Pregnancy is a dynamic process with immense anatomic and physiologic changes that occur from fertilization to parturition. Any given agent may therefore have vastly different effects depending on the stage of embryo and fetal development. Toxicologic effects can also be species specific; that is, an agent may not show a detrimental effect during pregnancy in the rat but may show marked toxicologic effects in the human. Lastly, a given agent can be selectively toxic, in that it may be detrimental to the fetus while having no effects on the mother. Perhaps most illustrative of these concepts is the thalidomide tragedy (Schardein 1985). Failure to suspect thalidomide as a teratogen initially was due to fact that huge doses did not kill rats, and even a suicide attempt by ingestion of over 14 g was unsuccessful as reported by Schardein (1985). This apparent absence of toxicity in nonpregnant mammals thus led to the assumption that the embryo and fetus would also not be affected. Unfortunately, the reason thalidomide failed to show toxic effects in rats was its low solubility in rat blood. When this was corrected, the teratogenic effects were realised at low

concentrations (Schumacher et al., 1968). Heptachlor has a very low solubility in water and hence blood. The effect of this on experiments like the ones in this study needs consideration.

The male factor in toxicological effects in early pregnancy could come as a result of interference of sperm capacitation and acrosome reaction. Although there may be a variety of toxins that could block sperm capacitation, no tests are available to evaluate this. Sperm functional tests include a semen analysis to determine the concentration, motility and morphology of sperm. Any pertubations of these parameters are clearly a result of alterations in the male's physiologic processes (Matt and Borzelleca, 1995). It is well recognized that the human (and perhaps other mammals as well) cervical mucus plays a central role in sperm transport as well as capacitation (Lambert et al., 1985; Overstreet, 1983). The same could be true for other mammals. Since the status of cervical mucus is dependent on ovarian estrogen secretion, any toxin that disrupts the hypothalamic-pituitary ovarian axis, resulting in altered follicular development and estrogen secretion, would have profound effects on cervical mucus which would in turn impair both sperm capacitation and transport. This would subsequently interfere with fertilization resulting in smaller litter sizes, even in the absence of toxin-exposed males. Thus, the results in this study indicating reduced litter sizes may be explained by the latter.

Important to understanding the potential toxicologic insults that may occur in preimplantation embryos is that exposure must occur via uterine and tubal secretions, as the pre-embryo are freely floating at this stage of their development. The uterine and tubal concentrations of many compounds are similar to those within the maternal serum. However, some compounds eg. nicotine and DDT are reported to be at significantly higher concentrations in uterine fluid following maternal exposure (McLachlan et al., 1976). It is possible

that several other compounds including heptachlor, may also preferentially concentrate in the uterine fluid compartment. The possibility of this then interfering with implantation resulting in smaller litter sizes cannot be overlooked.

The placenta is a fetal organ of paramount importance for providing nutrient to the developing fetus. It is also an endocrine organ for the maintenance of pregnancy. The placenta thus has immense capability to manufacture and biotransform a host of compounds. In addition, the placenta plays a pivotal role in xenobiology for it acts as a potential barrier between the maternal and fetal compartment although it is becoming increasingly clear that most compounds easily traverse the placenta and enter fetal circulation. The placenta is also capable of metabolizing potential toxic substances into less detrimental or more detrimental compounds.

However, although the placenta is capable of actively transforming xenobiotic compounds into reactive intermediates (Matt and Borzelleca, 1995), the specific contribution that this may play in reproductive toxicology remains to be elucidated. The current thoughts are that the placenta plays a very minor role in inactivating xenobiotics. All the same, its potential to biotransform relatively inactive to active toxins requires further investigation (Matt and Borzelleca, 1995).

There is little information available on the potentially adverse effects of xenobiotics on lactation. This could be attributed in part to the difficulty in dissecting out a direct xenobiotic effect from that of maternal psychologic stress and decreased infant suckling behaviour. Still any substances that may alter the hormones that regulate lactation would possess potential effects on lactation (Matt and Borzelleca, 1995).In addition to the concerns of the effects of xenobiotics on the maintenance of lactation, there is increasing concern about the

transfer of substances in breast milk. The exposure of infants to xenobiotics via breast milk has been reviewed in Chapter one.

Xenobiotics may directly affect gonadol functions within the testis and ovary by altering the feedback mechanisms to the hypothalamus and pituitary. Alterations in the hypothalamic-pituitary portion of the axis may in turn impair the fertility potential. The role of pituitary gonadotrophins namely FSH and LH has also been reviewed in chapter one. Disruption of reproduction may result from damage by toxicants at the the level of the hypothalamus. Altering the pulsatile release of GnRH in either frequency or amplitude will impair its permissive action on the entire reproductive system by interfering with GnRH-induced levels of FSH and LH . Among such agents that can inhibit GnRH release are opioids and environmental toxins e.g. the insecticide chlordimeform. *In vitro* studies of this insecticide chlordimeform, which is still in use outside the United States, indicated that the agent may cause a lesion in the hypothalamic-pituitary-gonadal axis at the α-adrenergic receptor. In chlordimeform treated rats, in vitro secretion of GnRH by hypothalamic fragments after norepinephrine stimulation was drastically suppressed (Goldman et al., 1990).

Agents that act as antiandrogens or antiestrogens or those that mimic estrogens may also interrupt reproductive function by competing for androgen or estrogen receptors at the level of the hypothalamus. Several environmental agents posses estrogenic activity. The insecticides DDT, its derivative DDE, kepone, methoxychlor, as well as the industrial chemicals, the PCBs all compete for the the estrogen receptor (Dickerson et al., 1992). *In vivo* studies have shown that treatment of ovariectomized mice with these compounds resulted in both biphasic negative and positive feedback of LH secretion (Pasqualini et al., 1990). It is therefore possible that the results obtained in this study whereby heptachlor decreased the ovarian cells responsiveness to either LH or FSH challenge could

be as a result of its being estrogenic and therefore interfering with the activity of the gonadotrophins. A biphasic effect of heptachlor on gonadotrophin release was also found in this study where low levels of heptachlor resulted in an increase in FSH/LH-stimulated progesterone release while high levels of heptachlor were inhibitory. The environmental agent 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) also has anti estrogenic activity. Treatment with this polychlorinated hydrocarbon downregulated the estrogen receptor resulting in decreased levels of both the cytosolic and the nuclear receptors (DeVito et al., 1992).

In men, environmental estrogens have been suggested as a potential factor for the decline in sperm concentrations over the past 15 to 20 years according to Sharpe (1993). The mechanism of their action may be as potent inhibitors of sertoli cell replication in fetal or neonatal life or both and work via the feedback regulation of FSH secretion.

Tetrahydrocannabinol (THC), the lipid-soluble active ingredient of marijuana acts on the brain and gonadol tissues. In the brain it acts at the level of the hypothalamus resulting in inhibition of the midcycle LH surge and ovulation. At the level of the gonads, THC has a direct effect on steroidogenesis of the ovarian follicles (Asch et al., 1980). Several sites within the steroidogenic pathway are targeted by this compound including pregnenolone biosynthesis, 3β–hydroxysteroid dehydrogenase (Adashi et al., 1983) and FSH-stimulated cAMP accumulation (Treinen et al., 1993).

The results obtained in this study indicate that heptachlor's target site of action is after pregnenolone biosynthesis. The effect of the pesticide is biphasic in that at low dose it stimulates pregnenolone synthesis while at high dose it inhibits the biosynthesis of pregnenolone as well as several other hormone metabolites along the steroidogenic pathway suggesting inhibitory effect on 3β-

hydroxysteroid dehydrogenase and aromatase activity. This strongly supports what was found when progesterone was measured following incubation of the cells in the presence of gonadotrophins.

7.2 Conclusions

This study was set out to study the effect of heptachlor on reproduction in the female rat. From the literature and the general discussion, it is clear that reports on the effects of pesticides, and organochlorines in particular, on reproduction are scanty, and mostly inconclusive. Reports on the effects of heptachlor on reproduction are even more scarce. There is thus no conclusive report as yet on the effect of heptachlor and in particular its mode of action on reproduction.

This study sought to first of all establish the effect of heptachlor on general reproductive function by measuring its effect on parameters like cyclicity, mating success (ovulation), litter size and gestation length,.and secondly to find a link between these general aberrations with hormonal profiles and synthesis, using both *in vivo* and *in vitro* approaches.

At the end of the study, it was concluded that,

1) Heptachlor disrupts estrous cyclicity, blocks or delays ovulation, reduces the number of pups per litter, and prolongs gestation length. Concomitant with these, heptachlor lowers body weights of treated animals.

2) The level of end point.and potent reproductive hormones namely progesterone and estradiol were lowered in heptachlor treated animals compared to controls.

3) Heptachlor at both low (5mg/kg body weight) and high dose (20mg/kg body weight) lowers the levels of several progesterone and estradiol metabolite hormones with the exception of pregnenolone whose levels are increased at low dose and lowered at high dose, suggesting that heptachlor at low dose augments the action of desmolase enzyme, while at high dose, it is inhibitory to the enzyme.

4) Heptachlor at low and high doses (5mg/kg and 20 mg/kg body weight), lowered, in a dose dependent manner, the ability of isolated but mixed ovarian cells to respond to exogenously applied LH and FSH as measured by their ability to translate the LH and FSH challenge into progesterone and estradiol.

5) Heptachlor at low dose (5mg/kg body weight) and high dose (20mg/kg body weight), lowered (in a dose dependent manner) the ability of isolated but mixed ovarian cells to synthesise progesterone and estradiol as well as several of their metabolites from precursor hormones namely pregnenolone and androstenedione and the site of action of heptachlor is located after pregnenolone biosynthesis.

6) The effect of heptachlor on isolated luteal and follicular cells confirmed the results obtained with mixed ovarian cells except in addition, the effect of heptachlor was found to be more significant on luteal cells as opposed to follicular cells.

7) The results indicate that the enzyme system most likely to be affected is 3β hydroxysteroid dehydrogenase-P450.

This study has shown that heptachlor, has a direct effect on mammalian reproduction by interfering with the synthesis of progesterone and estradiol.These are the potent female reproductive hormones that help to regulate the reproductive process. The end result is a reproductive cycle in disarray.

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Appendix 1

VIABILITY AND PURITY OF OVARIAN CELLS.

Viability of cell preparations.

The viability of the cells were estimate by dye dilution method (Trypan Blue) as follows:

$$\% \text{ Viability} = \frac{\text{total viable cells (unstained)}}{\text{total cells (unstained and stained)}} \times 100$$

The viability ranged between 85% to 95%.

Purity of cell preparations.

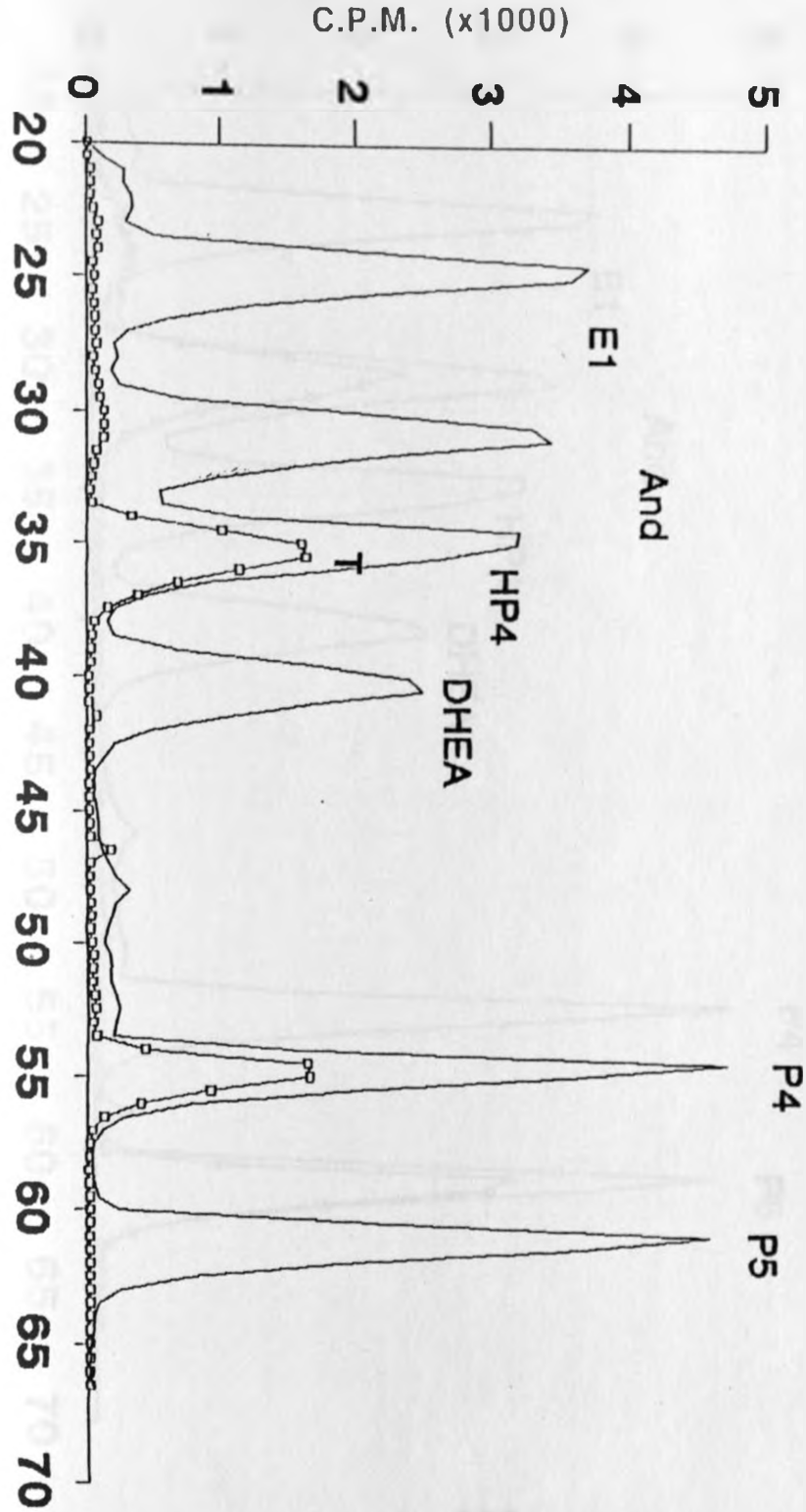
The purity was ditermined as follows:

$$\% \text{ purity} = \frac{\text{number of cells in question (granulosa etc)}}{\text{total number of cells}} \times 100$$

The purity ranged between 83% and 97%

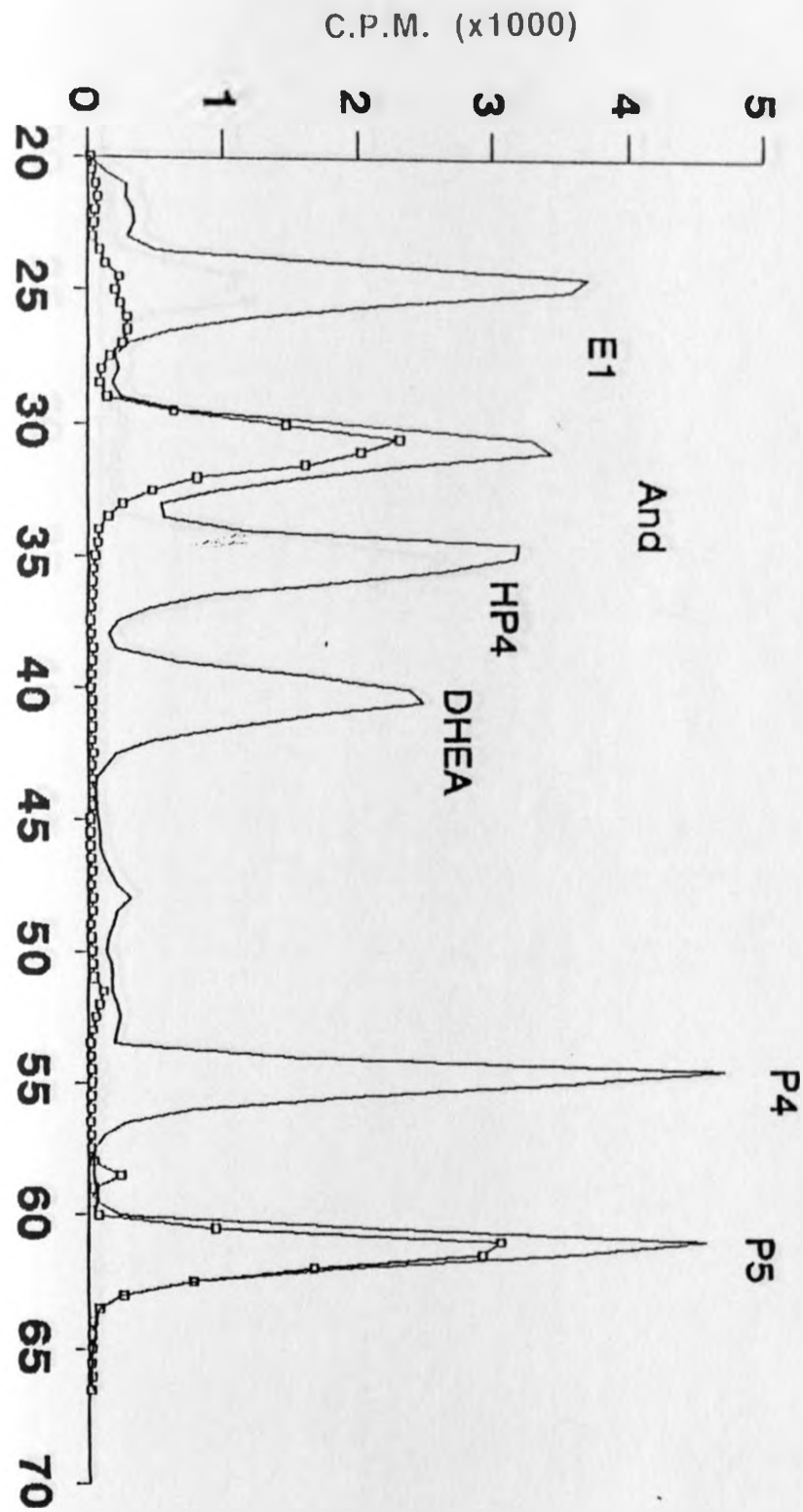
Appendix 2

HPLC calibration profile for testosterone and progesterone



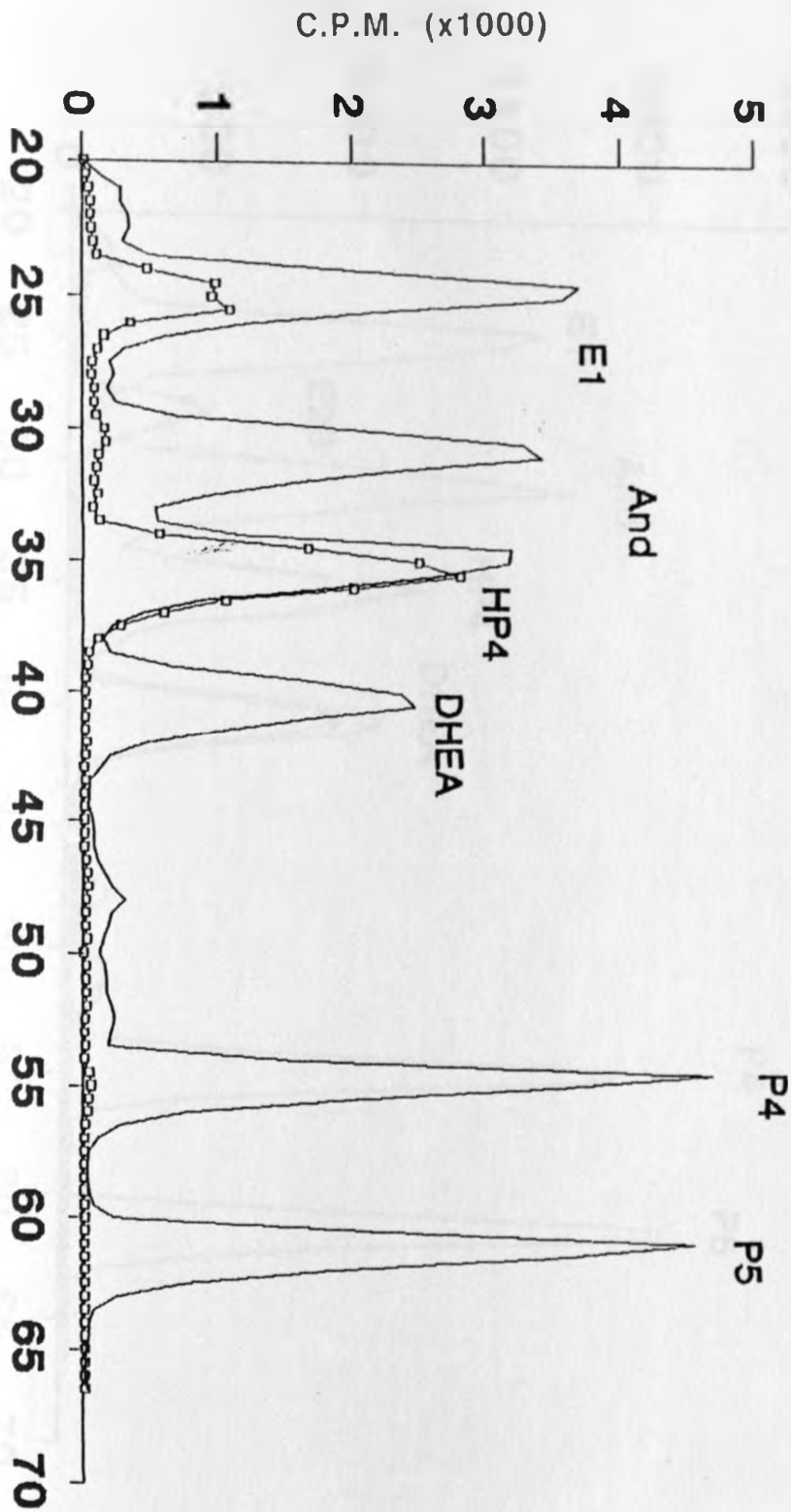
Appendix 3

HPLC calibration profile for androstenedione and pregnenolone



Appendix 4

HPLC calibration profile for estrone and 17α hydroxyprogesterone



HPLC calibration profilr for estradiol 17β and dihydroepiandrosterone