PHD

Some aspects of oestrogen toxicity in the beagle dog and non-human primates.

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SOME ASPECTS OF OESTROGEN TOXICITY
IN THE BEAGLE DOG AND NON–HUMAN PRIMATES

submitted by
Peter Fenton Wadsworth
B.V.M.S., M.R.C.V.S.
for the degree of Ph.D.
of the University of Bath
1978

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Peter F. Wadsworth

Peter Fenton Wadsworth
To my wife MARGARET

and for Sarah, Benjamin and Richard
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SUMMARY
Experiments are reported on some aspects of oestrogen toxicity in the Beagle dog and the non-human primate.

Ethynyl estradiol produced in the dog, toxic changes in the reproductive system, blood and skin. Some females showed slight focal papilliform proliferation of the genital serosa.

Ethynyl estradiol induced in rhesus monkeys biochemical changes indicating hepatic dysfunction. No histological changes were detected in the liver by light microscopy, but by electron microscopy minor ultrastructural abnormalities were identified within hepatocytes. Bile flow increased when ethynyl estradiol was given to rhesus monkeys with an intact enterohepatic circulation. Ethynyl estradiol did not cause proliferative serosal lesions in female rhesus monkeys but hyperplasia of the fimbrial epithelium of the Fallopian tube was found.

Ethynyl estradiol had no apparent effect on lactation in rhesus monkeys, although there was evidence of oestrogen secretion through the milk. The effects of ethynyl estradiol in neonatal rhesus monkeys were similar to those in adults.

When given to pregnant rhesus monkeys during the period of organogenesis, ethynyl estradiol appeared to cause early embryonic death and abortion but no teratogenic effects were found in foetuses derived by caesarian section.

The combined hepatotoxicity of ethynyl estradiol and chenodeoxycholic acid in baboons was essentially similar to that of chenodeoxycholic acid alone.

High dose levels of diethylstilboestrol given to pregnant rhesus monkeys were apparently embryotoxic. Two infants were reared for 84 weeks; the female's reproductive tract was normal, but the male's prostate and seminal vesicles were small.

Interpretation of oestrogen tumorigenicity to dogs depends on establishing baseline background data. The spontaneous incidence of mammary tumours and dysplasias in forty nulliparous 8-year old female Beagle dogs is described.
PREFACE
Hormone is a Greek word meaning “I stir up or stimulate” and was first used by Bayliss and Starling in 1902. They defined a hormone as a chemical substance produced in one part of the body that diffuses or is transported to another area where it influences activity and tends to integrate component parts of the organism. This definition remains essentially unchanged. Hormones regulate specific processes and therefore it must be appreciated that an excess is as detrimental as a deficiency.

As early as 460—322 B.C. the writings of Hippocrates and Aristotle contain information that there might be internal control over some functions of the body. Aristotle wrote that ‘the excision of sows’ ovaries quenched their sexual appetite and stimulated growth”. However, only twentieth century endocrinology has established the association of the ovary with the female sex hormones, oestrogens and progestogens, and it was not until the second quarter of this century that these humoral substances started to be identified. It is therefore, a remarkable fact that in 1974 an estimated 50 million women in developed and developing countries around the world were taking oestrogens combined with progestogens, in the form of oral contraceptive pills, (Piowtrow and Lee, 1974).

The early history of oestrogenic hormones has been reviewed by G.W. Corner (1964). The initial advance was made by three investigators working independently who found that the way to isolate these hormones was by lipid extraction, (Iscovesco, 1908 ; Feliner, 1912 ; Herrman, 1915). Following on from this discovery Allen and Doisy (1923) developed a bio—assay for oestrogens based on a rat vaginal smear, enabling them to measure potency in terms of a physiological unit, the Allen—Doisy rat unit. All substances producing oestrus and vaginal cornification in spayed rats or mice are therefore said to be oestrogens. The last pioneering discoveries were made by the chemists Doisy (1930) and Butenandt (1930) who extracted the hormone oestrone in crystalline purity following Aschheim’s (1927) finding that large quantities are present in human urine during pregnancy. In 1933 Schwenk and Hildebrandt reduced oestrone to oestradiol.

Oestradiol is the principal natural hormone in women and is the hormone secreted by the ovary. Oestrogens are also formed in the adrenal cortex and placenta. Oestradiol exists in the body in equilibrium with the less biologically potent oestrogen, oestrone, which in turn is converted to oestriol for excretion by the kidneys. Oestradiol is metabolised largely by the liver where it is partly oxidised to inert compounds and partly converted to oestrone and oestriol (Fig. 1).

Oestradiol, oestrone and oestriol are steroid molecules derived from perhydrocyclopentanophenanthrene nucleus. Preparations of these and other naturally occurring oestrogens, such as oestradiol benzoate, together with semisynthetic derivatives including ethinyl estradiol (Fig. 2) and mestranol are now either extracted from human or mare’s urine or chemically synthesised for commercial use. Ethinyl estradiol was first synthesised in 1938 (Inhoffen et al) and is a potent orally active oestrogen. Commercially, ethinyl estradiol is prepared by the treatment of oestrone with potassium acetylide in liquid ammonia. Ethinyl estradiol is 19—nor—17α—pregna—1, 3, 5 (10)—trien—20—yne—3, 17β—diol. (British Pharmacopoeia, 1977). In Western Europe alone, sales in 1972 were estimated to have been not less than 100 kg. (International Agency for Research on Cancer, 1974).
FIGURE 1

FIGURE 2

FIGURE 3
Synthetic oestrogens were first described by Dodds et al in 1938. While the action of these synthetic oestrogens appears to be identical to that of the natural compounds, they are biochemically quite dissimilar, being derived from stilbene. Synthetic preparations include benzestrol, diethylstilboestrol (Fig. 3) and hexoestrol. Diethylstilboestrol is 3,4-di-(4-hydroxyphenyl)hex–3–ene (British Pharmacopoeia, 1977) and is prepared by converting anethole hydrobromide to an intermediate ether using sodamide in liquid ammonia and this is converted to diethylstilboestrol by heating with potassium hydroxide in ethylene glycol.

A. Therapeutic usage and adverse reactions in humans

Oestrogen preparations are, or have been, used for a wide variety of therapeutic reasons in women, the most important of which are:

1. Hormonal contraception
2. Treatment of menopausal disorders and hormone replacement therapy
3. Pregnancy diagnosis
4. Treatment of threatened and/or recurrent abortion

(1) Hormonal contraception

Although there are several different types of oral contraceptive regimens, most fall into two main categories: by far the most important is the combined pill, in which both oestrogen and progestogen are administered daily for three weeks out of four. The sequential pill contains oestrogens for the first 14 days of the cycle and progestogens for the following 6 days. Only two oestrogens ethinyl estradiol and mestranol are currently used in oral contraceptive pills but at least eight different types of progestogens are available. Ethinyl estradiol together with mestranol were included by chance in the first oral contraceptive formulations as contaminants of the first 19–norprogestins (norethynodrel and norethindrone). The original agents used in clinical trials contained either 150 or 60 μg of mestranol per tablet and these levels were adjusted to optimise menstrual bleeding patterns which result from cyclical activity (Helton and Goldzieher 1977). Originally, contraceptive properties were entirely attributed to the 19–norprogestins but Goldzieher et al (1975) have shown that they may act synergistically to inhibit ovulation at dosage rates which by themselves are insufficient for consistent ovulation inhibition.

Following the first large scale antifertility trials (Pincus, 1957; Pincus et al, 1958, 1959), oral contraceptives were first approved for prescription use in the United States in 1959, and in Britain in 1960. Oral contraceptive pills are the “medicine” most widely taken by healthy people; 850 million of these pills are consumed in a year in Britain alone.

Their mode of action is at the level of the hypothalamic–pituitary system. Inhibition of the release of gonadotrophic hormones by a negative feedback mechanism prevents ovulation, (Baird, 1976). The menstrual bleeding is caused by withdrawal of the oestrogen.
The dosage of ethinyl estradiol varies in different formulations. In 1969 the Committee on Safety of Drugs, predecessor of the Committee on Safety of Medicines, recommended a maximum dosage of 50 μg per day, although in some preparations the dosage is now as low as 30 μg (British Medical Journal, 1976 a).

Vessey and Doll (1976) have reviewed the adverse effects of oral contraceptives. It is well known that oral contraceptives cause minor side effects such as nausea, headache or breast tenderness, which in themselves are sufficient reason for cessation of treatment. However, there are more serious effects particularly with respect to cardiovascular disease (Beral and Kay, 1977; Vessey et al. 1977). Oral contraceptives can cause deep vein thrombosis, pulmonary embolism and certain types of cerebrovascular disease, especially thrombotic stroke (Royal College of General Practitioners, 1974). There is also strong evidence that they can cause acute myocardial infarction (Hennekens and MacMatton, 1977), and in some women may also produce malignant hypertension (Zech et al. 1975). Cholelithiasis is an important non-cardiovascular adverse effect (Lancet, 1973) and Bennion et al (1976) have reported that oral contraceptives may induce alterations in human gall bladder bile suggesting a biochemical basis for an increase in gall bladder disease. Similarly, cervical erosion also occurs more frequently in women taking oral contraceptives. An association has been suggested between oral contraceptives and hepatic neoplasia (British Medical Journal, 1975; Matiboubi and Schubik, 1976). An increased risk of admission to hospital for hepatitis has been reported by Morrison et al (1977).

The use of steroid contraceptives during the post-partum lactation period raises important questions about the effect on the amount and composition of milk and the risk that they may be transferred through the milk to the baby (Kessler and Standley, 1977).

Clear evidence has been found of impairment of fertility after discontinuation of oral contraceptives (Vessey et al. 1976).

To date, it is worthwhile noting that no obvious association exists between oral contraceptives and breast cancer (Vessey et al. 1975; British Medical Journal, 1976 a).

(2) Treatment of Menopausal Disorders and Hormone Replacement Therapy

The endocrine changes that occur after menopause involve a shift from the major oestrogen oestradiol of ovarian origin, to oestrone synthesised elsewhere in the body, particularly by the adrenal cortex. Oestrogens may also be continually produced in body fat from androgen precursors (McDonald and Longcope, 1971; Ryan, 1975).
Oestrogen lack after the menopause can be associated with vasomotor instability (hot flushes) and atrophy of the reproductive tract. These are well documented sequelae of a post-menopausal hormone deficiency and are responsive to low dose oestrogen therapy. Oestrogens may also be used prophylactically against osteoporosis and bone fractures (Lindsay et al, 1976; Horsman et al, 1977; Recker et al, 1977). The use of oestrogens for relief of menopausal disorders became popular in the middle 1960's, when oestrogens were also considered to help women to retain a youthful appearance. A leading proponent of this therapy has been a New York gynaecologist, Dr. R.A. Wilson. The number of women receiving oestrogens for treatment of menopausal disorders and hormone replacement therapy is unknown, but Zeil and Finkle (1975) note that dollar sales of oestrogens, particularly oestrone sulphate, quadrupled between 1962 and 1973. In the United Kingdom only a small percentage of post-menopausal women are receiving oestrogen replacement therapy, probably about 6% of those between 50 and 59 (Doll, et al, 1976). However, the life expectancy for women has risen markedly so that today 30% of women are over 50 years of age (Dalton, 1977).

The dosage of ethinyl estradiol used for treatment of menopausal disorders varies from 0.01 to 0.05 mg daily.

Recently published epidemiological surveys suggest an association between exogenous oestrogens and endometrial carcinoma (Smith et al, 1975; Zeil and Finkle, 1975; Quint, 1975; Mack et al, 1976). The results of these surveys were based on retrospective population analysis and so definite answers will therefore be only obtained from prospective studies with strict controls (Lancet, 1977). However, it is known that oestrogen stimulation, either endogenous or exogenous results in hyperplasia of the endometrium which in a small proportion of cases progresses to invasive carcinoma (Gusberg, 1967; 1976).

(3) Pregnancy diagnosis

Two preparations, both of which contain ethinyl estradiol combined with progestogens were formerly extensively used for pregnancy diagnosis; they were given once daily for two or three consecutive days and induced uterine bleeding if amenorrhoea was not caused by pregnancy (Lancet, 1974a). Pregnancy tests performed on urine have now superseded this form of pregnancy diagnosis.

The dosage of ethinyl estradiol in these preparations is 0.05 mg and 0.01 mg per tablet respectively for two or three days.
Mothers who take hormonal preparations containing oestrogens during the teratogenic period of pregnancy, i.e. from 34th to the 50th day from last menses, fall into two main groups. The first group comprises those women given hormonal pregnancy tests. The second group consists of those mothers who conceive while taking oral contraceptive pills, or who are already pregnant when they start to take them.

In establishing an epidemiological association between sex hormones and possible teratogenic effects it must, therefore, be first appreciated that this is not a homogenous group of mothers but a group who have taken widely differing hormonal preparations, some containing both oestrogens and progestogens, for differing periods of time and for different reasons. Whether such an association exists is unresolved. Possible teratogenic effects include limb reduction defects (Janerich et al, 1974), cardiovascular defects (Levy et al, 1973; Nora and Nora, 1973; Heinonen et al, 1977), Di George Syndrome (Nora and Nora, 1973) and the Vacteral syndrome (Nora and Nora, 1973). However, it is interesting to note that Yasuda and Miller (1975) found no association between oral contraceptive or other sex hormones and transposition of the great vessels.

(4) Treatment of threatened and or recurrent abortion

Diethylstilboestrol was first introduced by Smith for the prevention and treatment of complications of pregnancy in 1948. In the U.S.A. from 1948 till at least 1967, threatened abortion in particular was likely to be treated with diethylstilboestrol, often at a high dose level. Surveys indicate that in the U.S.A. 10,000 to 16,000 females born between 1960 and 1970 were exposed to diethylstilboestrol in utero (Lancet, 1974b). In England and Wales between 1940 to 1971, 7,500 pregnant women are likely to have been treated with diethylstilboestrol at various dose levels for threatened abortion (Kinlen et al. 1974).

In America, the total dosages ranged from 300 to 18,200 mg in an analysis of 170 registry cases (Herbst et al, 1974).

In 1970 Herbst and Scully described 6 cases of clear cell adenocarcinoma of the vagina in girls between 15 and 22 years. That such a rare tumour should appear in a group of young women suggested a special environmental influence. Investigation of the pregnancies (Herbst et al, 1971) showed a highly significant association with prenatal exposure to diethylstilboestrol from the first trimester of pregnancy. The risk of developing vaginal adenocarcinoma seems to be low in the exposed population and is estimated as 4 in 1,000 (Herbst et al, 1974), although in an analysis of 170 registry cases, recurrences developed in 37 of the patients and 24 of them died. Only one case has been reported in the United Kingdom (Monaghan and Sirisena, 1978). About two-thirds of the tumours arise in the vagina and one-third in the cervix. In a prospective study Herbst et al (1975) associated non-neoplastic changes, in particular cervical erosion and vaginal adenosis, with prenatal exposure to diethylstilboestrol.
B. Toxicological aspects

The first legislation controlling the production of drugs was the 1906 Pure Food and Drug Act U.S.A. Subsequently an incident in 1937 when deaths occurred following the administration of sulphanilamide elixir preparation containing an inappropriate solvent diethylene glycol promoted the 1938 legislation (U.S.A.), requiring new drugs to be tested for safety and Food and Drug Administration clearance before marketing. In Western Europe drug safety regulation was to follow the thalidomide tragedy in 1962. In the same year further legislation was enacted in the U.S.A. requiring a closer control of animal and clinical testing of new compounds and from this time it was required to show efficacy. Contraceptive steroids were in common use prior to the enactment of the legislation of 1962.

All toxicological studies are directed towards demonstrating a dose—response relationship. This concept is the only fundamental law of toxicology. The scope of suggested or obligatory testing procedures in animals varies according to the category of the substance and with the duration and intensity of exposure.

Rodents, Beagle dogs and non—human primates have been used as toxicological models in contraceptive development. The current requirements of the U.S. Food and Drug Administration for animal tests for contraceptives of the hormonal type, and also for progestogens and oestrogens for prolonged non—contraceptive use are shown in Table 1. (Owen and Briggs, 1976).

Similar requirements have been adopted by such countries as Sweden and Australia and other countries, notably the United Kingdom, also show great interest in whether carcinogenicity tests in other species apart from rodents have been conducted. The Medicines Act (1968, 1971) requires manufacturers to provide evidence of safety, efficacy and quality.

The conventional chronic long—term toxicity study remains a key feature in safety assessment. Animal studies are essentially comparative in that spontaneous and induced pathological manifestations are compared in control and test animals simultaneously. It is essential to determine whether an observation or finding is attributable to the test compound and, if so, whether its incidence is dose—related. Clinico—pathological findings can be categorised into three groups — the recognised common pathology of the species under investigation, changes predictable by virtue of the pharmacology of the compound, and manifestations of unusual and unpredicted responses.

Ideally metabolic data should be available before setting up long—term animal toxicity studies. The knowledge of the metabolism of synthetic and naturally occurring oestrogens is very meagre. The metabolic evidence to date indicates that, apart from the great apes, there appears to be no ideal species. (Diczfalusy, 1972; Fotherby, 1974; Cakin et al 1977).
### TABLE 1

**Animal toxicity studies for oestrogen and progestogen contraceptives**

<table>
<thead>
<tr>
<th>Clinical study</th>
<th>Animal toxicity study requirements (F.D.A.)</th>
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<tr>
<td>Phase I (limited to a few subjects for up to 10 days administration)</td>
<td>90-day studies in rats, dogs and monkeys.</td>
</tr>
<tr>
<td>Phase II (approximately 50 subjects for 3 menstrual cycles)</td>
<td>1-year studies in rats, dogs and monkeys.</td>
</tr>
<tr>
<td>Phase III (clinical trials)</td>
<td>2-year studies in rats, dogs and monkeys. Initiation of 7-year dog and 10-year monkey studies prior to start of Phase III.</td>
</tr>
<tr>
<td>New Drug Application</td>
<td>No further requirements but must include up-to-date progress reports on long-term dog and monkey studies.</td>
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The conventional procedure for a long term study is to have one control group and at least three groups of dosed animals. The lowest dose rate is usually comparable on a bodyweight basis with the intended human therapeutic dose or a small multiple thereof. The highest dose is intended to establish the target organs or systems for toxic effects, providing a so-called effect level and perhaps thereby giving clues as to countermeasures or antidotal treatment. The intermediate dose is a multiple of the proposed therapeutic dose and is intended to establish safety factors. During the studies, the animals are subjected to regular clinical examinations and bodyweight measurements, with food and water intake calculated. Blood and urine are subjected to a gamut of biochemical and cytological examinations. At termination, post mortem examination is accompanied by organ weight analysis and histopathological examination.

In studies designed to evaluate the safety of oral contraceptive steroids, dosage levels of 1, 10 and 25 times the anticipated human dosage level are used for dog studies, 1, 10 and 50 times for monkey studies, and for rodents 1, 10 and possibly 100 times are used. The use of the rat in carcinogenicity studies is generally accepted and a 2-year period coincides with the average life span (average mortality at 2 years 50%). The use of dogs and non-human primates in carcinogenicity studies of 7 and 10 year's duration respectively is more controversial. The life expectancy of a Beagle dog has been estimated as 12 years (Andersen and Rosenblatt, 1965). The life span of rhesus monkeys under experimental laboratory conditions is unknown.

The literature contains many accounts of the administration of oestrogens to various animal species. Despite the wide general use of oestrogens, there are few published toxicity studies in the common laboratory animals. These studies have been initiated to investigate some aspects of the toxicity of oestrogens in Beagle dogs and non-human primates.
CHAPTER ONE

THE ORAL TOXICITY OF ETHINYL ESTRADIOL
WHEN ADMINISTERED TO BEAGLE DOGS AT A
DOSE RATE OF 1 mg/kg/day FOR 28 WEEKS
INTRODUCTION

There have been several published accounts of the administration of natural or synthetic oestrogens to dogs, and their effects on the male and female reproductive tract, blood, bone marrow, and skin have been well documented. One of the more interesting features of long-term administration of oestrogens to dogs is their effect on the genital serosa. Mawdesley-Thomas and Sortwell (1968) reported that proliferative serosal changes, especially of the uterine serosa and mesometrium, occurred as a primary response to a novel steroidal oestrogen. O'Shea and Jabara (1971) made similar observations when diethylstilboestrol was administered to female dogs. In addition, they reported the occurrence of proliferative lesions in the epididymal serosa in male dogs. In order to find out whether ethinyl estradiol would induce proliferative changes of the genital serosa, the effects of long-term oral administration on the Beagle dog were studied.

MATERIALS AND METHODS

Seven pure-bred Beagle dogs (3 male and 4 female) were selected and housed singly in kennels. An eight-week period was allowed for preliminary investigations to take place and for the animals to adjust to their new environment. During this period one of the female dogs was ovariectomised. Details relating to the age and bodyweight of individual animals at the time dosing began are shown in Table 2.

All dogs were given a complete dry diet and water was freely available at all times. The ethinyl estradiol was diluted 1 to 10 with lactose and then administered in gelatine capsules once a day seven days a week at a dosage level of 1 mg ethinyl estradiol/kg (10 mg/kg/day of the mixture). Following adverse clinical findings, the experiment was terminated after 28 weeks' dosing.

Food and water consumption and any signs of ill health were monitored daily. Bodyweight was recorded at weekly intervals. Veterinary examinations and laboratory investigations were performed before the experimental period and then 4, 8, 12, 16, 20 and 26 weeks after dosing started. The haematological investigations comprised counts of total red and white cells, differential white cells, platelets and reticulocytes, and estimations of packed cell volume, haemoglobin, erythrocyte sedimentation rate and prothrombin index. The biochemical investigations comprised determination of plasma urea, plasma glucose, serum proteins (total and differential), serum alanine aminotransferase, serum alkaline phosphatase, serum bilirubin, sodium and potassium levels. Urine was examined qualitatively for specific gravity, pH, protein, reducing substances, glucose, ketones, bile pigments, urobilinogen and haemoglobin, and microscopically for sediment.
TABLE 2

Data relating to dogs at start of dosing

<table>
<thead>
<tr>
<th>Animal Number and Sex</th>
<th>Age (weeks)</th>
<th>Bodyweight (kg)</th>
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<tr>
<td>2♀</td>
<td>32</td>
<td>7.00</td>
</tr>
<tr>
<td>3♂</td>
<td>32</td>
<td>9.70</td>
</tr>
<tr>
<td>4♀ *</td>
<td>25</td>
<td>10.55</td>
</tr>
<tr>
<td>5♂</td>
<td>32</td>
<td>10.35</td>
</tr>
<tr>
<td>6♀</td>
<td>41</td>
<td>9.05</td>
</tr>
<tr>
<td>7♂</td>
<td>32</td>
<td>13.75</td>
</tr>
<tr>
<td>8♀</td>
<td>42</td>
<td>12.45</td>
</tr>
</tbody>
</table>

* ovariectomised
On completion of the dosing period the animals were killed, and immediately afterwards a full macroscopic examination was performed during which the adrenals, gonads, heart, kidneys, liver, lungs, pancreas, pituitary, prostate, spleen, thymus, thyroid and uterus were removed and weighed. Samples of these tissues and of the aorta, sternal bone marrow, cervix, colon, duodenum, epididymis, eyes, gall bladder, ileum, jejunum, cervical and mesenteric lymph nodes, individual mammary glands, oesophagus, skeletal muscle, sciatic nerve, skin, submandibular salivary gland, stomach, trachea, tongue, urinary bladder, urethra, ureters, vas deferens, vagina and vulva, with any macroscopic abnormality, were then placed in 10% buffered formalin fixative. The tissues were routinely processed in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin. Additional pieces of liver and kidney were placed in formol calcium, sectioned at 12 μm and stained for fat with Oil Red O.

RESULTS

Mortalities

Dog 29 was killed on humane grounds after 16 weeks’ dosing. Abnormal clinical signs noted during the three weeks before death included depression, inappetance and blood-stained faeces. With the exception of an absolute neutrophil leucocytosis the results of preterminal haematological and biochemical investigations were within the normal range, and no significant macroscopic or histological changes were found.

Dog 7d was also killed when found in extremis after 27 weeks’ dosing. With the exception of absolute neutrophil leucocytosis and elevated plasma urea, preterminal haematological and biochemical investigations were within the normal range. At post mortem examination the prostate was found to be massively enlarged with frank abscess formation within the parenchyma (Fig. 5). Bacteriological culture revealed a mixed growth of Proteus and Pseudomonas pyocyaneus). Significant histological findings included both acute prostatitis and cystitis. The prostatitis was associated with marked squamous metaplasia of acinar and ductular epithelium.

Clinical signs

In the male dogs, with the exception of dog 5d, the testes decreased in size and were soft on palpation. In dog 5d unilateral testicular and epididymal enlargement occurred. The prepuce of all male dogs became swollen and pendulous. In the female dogs vulval enlargement together with intermittent vaginal discharge occurred. The mammary glands of both male and female dogs were enlarged and the teats became pendulous. In addition, in dog 5d, a transitory cystic swelling was noted in one mammary gland. Varying degrees of alopecia were encountered in the skin of all animals with the exception of dog 29. The alopecia was usually bilateral, involving the lateral aspects of thorax, abdomen and thigh, and became extensive towards the end of the dosing period. Bodyweight, food intake and water consumption were not adversely affected.
Laboratory investigations

Apart from raised erythrocyte sedimentation rates in dog 5d after 20 and 26 weeks' dosing and in dog 7d after 26 weeks' dosing, red blood cell parameters remained within normal limits. A marked neutrophil leucocytosis was evident in all dogs after 4 weeks' dosing (Table 3 and Fig. 4). No other significant changes in white cell values were detected. Platelet counts varied throughout treatment, but were considered to be within the normal range. Prothrombin times were not prolonged.

Biochemical investigations did not reveal any abnormalities with the exception of a slightly elevated plasma urea in dog 5d after 20 and 26 weeks' dosing, and slightly elevated SAP values for dogs 4f and 6g after 26 weeks' dosing.

With the exception of minor changes which included traces of blood and protein in the urine of dogs 5d and 8g, the results of urinalysis were within the normal range.

Macroscopic findings

The prostates of all male dogs were massively enlarged (Fig. 5) with frank abscess formation within the parenchyma. On section of the enlarged left testis of dog 5d areas of necrosis were noted with associated widespread adhesions between the tunicas vaginalis and albuginea. The fimbriae of the Fallopian tubes of dogs 6f and 8g appeared enlarged and congested (Fig. 6). No other macroscopic abnormalities were observed that were attributable to treatment with ethinyl estradiol.

Organ weight analysis

The results of both absolute and relative organ weight analysis have been tabulated in Tables 4a and 4b respectively. The most significant finding was that the prostates of the male dogs weighed between 177 g and 406 g. Normal prostatic weight for untreated dogs of this age is between 5 g and 15 g. The group mean prostatic weight was 2.243% of the bodyweight. In addition a marked unilateral increase in testicular weight was found in dog 5d, in which the left testis weighed 27.3 g and the right testis weighed 9.5 g.
<table>
<thead>
<tr>
<th>Animal No./Sex</th>
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<th>8</th>
<th>12</th>
<th>16</th>
<th>20</th>
<th>26</th>
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<td>16.0</td>
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<tr>
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<td>13.6</td>
<td>11.4</td>
<td>11.9</td>
</tr>
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<td>14.1</td>
<td>16.0</td>
<td>14.4</td>
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<td></td>
<td>9.7</td>
<td>23.5</td>
<td>9.4</td>
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<td>14.6</td>
<td>15.7</td>
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<tr>
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<td>17.0</td>
<td>12.5</td>
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</tr>
<tr>
<td>Mean</td>
<td></td>
<td>7.6</td>
<td>26.2</td>
<td>9.6</td>
<td>14.6</td>
<td>15.1</td>
<td>12.6</td>
<td>10.1</td>
</tr>
</tbody>
</table>

Figure 4: Group mean absolute neutrophil leucocyte values (1,000/cmm)
Figure 5  Dog 7c
Macroscopic appearance of massively enlarged prostate (P).
Note also urinary bladder (U.B.)

Figure 6  Dog 6?
Macroscopic appearance of the enlarged Fallopian tubes (arrows)
Note also ovary (O) and uterus (U)
### TABLE 4a

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>Bodyweight</th>
<th>Pituitary mg</th>
<th>Heart</th>
<th>Lungs</th>
<th>Liver</th>
<th>Spleen</th>
<th>Pancreas</th>
<th>Thymus</th>
<th>Uterus/Prostate</th>
<th>Kidneys R</th>
<th>Kidneys L</th>
<th>Thymoids R</th>
<th>Thymoids L</th>
<th>Adrenals R</th>
<th>Adrenals L</th>
<th>Gonads R</th>
<th>Gonads L</th>
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<td>255.8</td>
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<td>3.9</td>
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<td>0.42</td>
<td>0.36</td>
<td>0.89</td>
<td>0.80</td>
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<td>–</td>
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<td>60</td>
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<td>0.90</td>
<td>1.06</td>
<td>0.70</td>
<td>0.61</td>
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<td>12700</td>
<td>–</td>
<td>96.4</td>
<td>105.4</td>
<td>377.2</td>
<td>108.0</td>
<td>29.9</td>
<td>12.3</td>
<td>17.30</td>
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<td>35.4</td>
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<td>101.2</td>
<td>422.7</td>
<td>25.4</td>
<td>30.7</td>
<td>2.6</td>
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<td>41.2</td>
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<td>9500</td>
<td>70</td>
<td>108.9</td>
<td>118.4</td>
<td>313.1</td>
<td>27.4</td>
<td>36.6</td>
<td>3.9</td>
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<td>25.0</td>
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<td>123.0</td>
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<td>1.09</td>
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— organ not weighed
### TABLE 4b
Relative organ weights (% bodyweight)

<table>
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<tr>
<th>Dog No.</th>
<th>Pituitary</th>
<th>Heart</th>
<th>Lungs</th>
<th>Liver</th>
<th>Spleen</th>
<th>Pancreas</th>
<th>Thymus</th>
<th>Uterus/Prostate</th>
<th>Kidneys</th>
<th>Thyroids</th>
<th>Adrenals</th>
<th>Gonads</th>
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<tr>
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<td>0.0124</td>
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<td>0.58</td>
<td>0.0087</td>
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</table>

Mean 0.00063 0.82 1.00 3.34 0.41 0.27 0.06 2.243f 0.66 0.0103 0.0147 0.1529f

– organ not weighed
Histological findings

The testes of all male dogs showed atrophy of seminiferous tubules together with inhibition of spermatogenesis (Fig. 7). The epididymides of dogs 3d and 5d showed atrophy of the ductular epithelium. In addition, acute orchitis and marked interstitial fibrosis of the epididymis were found in the markedly enlarged testis of dog 5d. Marked squamous metaplasia of the acinar and ductular epithelium with associated acute inflammatory cell infiltration, haemorrhage and necrosis was observed in the prostates of all male dogs (Fig. 8).

The morphological appearance of the ovaries of the entire female dogs (29, 69 and 89), was consistent with the state of anoestrus. These animals also showed varying degrees of hyperplasia of the fimbrial epithelium of the Fallopian tubes. In all female dogs, dilatation of endometrial glands in the uterus and hyperplasia together with cornification of the vaginal squamous epithelium was seen (Fig. 9). Slight focal papilliform proliferation of the uterine mesothelium was noted in dog 49 (Fig. 10–11) and of the Fallopian mesothelium in dogs 69 and 89.

In all male dogs, both gynaecomastia, characterised by hyperplasia of ducts and acini of mammary glands, and simple and papilliform cyst formation (Fig. 12) were evident. The majority of mammary glands in all treated female animals showed ductal hyperplasia with, in two dogs, simple and papilliform cyst formation; acini, however, were small and inactive. Focal areas of squamous metaplasia of the thyroid acinar epithelium were present in dogs 5d, 69, 7d and 89. Atrophy of the hair follicles and sebaceous glands was noted in the skin of all clinically affected animals. The morphology of the adrenals, pituitary and sternal bone marrow was considered to be within the normal range for dogs of this age group.

DISCUSSION

These results show that ethinyl estradiol at a dosage level of 1 mg/kg/day induces focal papilliform proliferation of the genital serosa in female dogs. This finding confirms previous observations made by Mawdesley—Thomas and Sortwell (1968) and O'Shea and Jabara (1971) that proliferative serosal changes may occur as a primary response to certain oestrogens in dogs. The pathogenesis of these proliferative lesions is obscure but they may be associated with the role oestrogens play in body defense. Oestrogens, either natural or synthetic, have been shown to be the strongest stimulant of the reticulo—endothelial system (Nicol and Ware, 1960; Nicol et al, 1961; Nicol et al, 1964) and tissue macrophages are widely distributed in the peritoneal mesothelium (Furth et al 1972). It is possible that such lesions may be due to oestrogenic stimulation of fixed macrophages within the genital serosa.

Jabara (1959; 1962a) and O'Shea and Jabara (1971), working with diethylstilboestrol, reported papillary proliferation of the ovarian mesothelium. Local invasion, and the presence of lesions considered to be metastases on non—genital serous membranes, suggested that these changes were attributable to malignant ovarian tumours. Hormone withdrawal (Jabara 1962a) led to regression of ovarian tumours with fibrosis and calcification of secondary growths, indicating that these tumours are hormone—dependant.
Figure 7  Dog 7d
Transverse section of testis showing diffuse inhibition of spermatogenesis within seminiferous tubules. Haematoxylin and eosin (x50).

Figure 8  Dog 7d
Transverse section of prostate showing squamous metaplasia (sm) of acinar epithelium. Note also prostatic stroma (st). Haematoxylin and eosin (x125).
Figure 9 Dog 69
Transverse section of the vagina showing hyperplasia and cornification of the squamous epithelium (→). Haematoxylin and eosin (x125).

Figure 10 Dog 49
Transverse section of uterine horn showing papilliform proliferation of the mesothelium (→). Note also myometrium (m). Haematoxylin and eosin (x 160).
Figure 11 Dog 49
Transverse section of uterine horn showing papilliform proliferation of the mesothelium (→). Note also myometrium (m).
Haematoxylin and eosin (x200).

Figure 12 Dog 56
Transverse section of mammary gland showing papilliform cyst formation (→).
Haematoxylin and eosin (x50).
In this experiment the ovarian mesothelium was unaffected by the administration of ethinyl estradiol. The proliferative lesions affecting the genital serosa were substantially less than those previously recorded, however, direct comparison is not possible due to differing dose rates and routes of administration. Nielsen et al (1976) have classified such lesions as "hormone dependent tumour like lesions".

In the female dogs, vulval enlargement, hyperplasia and cornification of the squamous vaginal epithelium, dilatation of the endometrial glands (Sokolowski et al 1973), hyperplasia of the fimbrial epithelium of the Fallopian tubes (Verhage et al 1973) and ductular hyperplasia of the mammary gland (Nelson and Kelly 1973) appear to be related to the normal physiological manifestations of endogenous oestrogens.

Other changes were encountered involving the testis, epididymus, prostate, skin and blood and these were essentially similar to those recorded in previous studies with oestrogens. Table 5 summarizes these physiological and toxicological manifestations of various oestrogens to dogs and compares them with the findings in this study.

ABSTRACT

Ethynyl estradiol was administered orally to a group of seven Beagle dogs at a level of 1 mg/kg/day. Two dogs died during the dosing period and the experiment was terminated after 28 weeks dosing.

Toxic changes were noted in the male and female reproductive system, blood and skin. Histologically, focal papilliform proliferation of the genital serosa was evident in a proportion of female dogs.
### TABLE 5

Summary of administration of oestrogens to dogs by previous authors

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<th>Tissue</th>
<th>Investigator</th>
<th>Oestrogen</th>
<th>Effect</th>
</tr>
</thead>
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<tr>
<td>Testis</td>
<td>Mulligan (1944)</td>
<td>Diethylstilboestrol</td>
<td>Inhibition of spermatogenesis and atrophy of seminiferous tubules.</td>
</tr>
<tr>
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<td>Mulligan &amp; Becker (1947)</td>
<td>Diethylstilboestrol</td>
<td>Inhibition of spermatogenesis and atrophy of seminiferous tubules.</td>
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<td>Berg (1958)</td>
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<td>Inhibition of spermatogenesis and atrophy of seminiferous tubules.</td>
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<td>Jabara (1962b)</td>
<td>Diethylstilboestrol</td>
<td>Testicular atrophy.</td>
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<td>Wadsworth</td>
<td>Ethinyl estradiol</td>
<td>Inhibition of spermatogenesis and atrophy of seminiferous tubules. Acute orchitis.</td>
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<td>Mulligan (1944)</td>
<td>Diethylstilboestrol</td>
<td>Atrophy of ductular epithelium.</td>
</tr>
<tr>
<td></td>
<td>Wadsworth</td>
<td>Ethinyl estradiol</td>
<td>Atrophy of ductular epithelium. Stromal fibrosis.</td>
</tr>
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<td>Prostate</td>
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<td>Oestrone</td>
<td>Arrest of secretion, epithelial hyperplasia, epithelial metaplasia and fibromuscular overgrowth.</td>
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<td>Huggins &amp; Clark (1940)</td>
<td>Diethylstilboestrol</td>
<td>Squamous epithelial metaplasia.</td>
</tr>
<tr>
<td></td>
<td>Mulligan (1944)</td>
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<td>Squamous epithelial metaplasia and abscess formation.</td>
</tr>
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<td>Diethylstilboestrol</td>
<td>Squamous epithelial metaplasia, atrophy of ducts and acini and abscess formation.</td>
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<td>Oestrogen</td>
<td>Effect</td>
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<td>Prostate (continued)</td>
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<td>Ethinyl estradiol</td>
<td>Squamous metaplasia of acinar and ductular epithelium. Abscess formation.</td>
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<td>Kunde et al (1930)</td>
<td>Estrin</td>
<td>Hyperplasia of the endometrial glands.</td>
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<td>Dilatation of endometrial glands and oedema of the endometrial stroma.</td>
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<td>Hyperplasia of the myometrium.</td>
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<td>Stilboestrol dipropionate or oestradiol monobenzoate</td>
<td>Oedema of the endometrial stroma.</td>
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<td>Jabara (1962b)</td>
<td>Diethylstilboestrol</td>
<td>Cystic hyperplasia, oedema of the endometrial stroma, endometrial atrophy, necrosis and pyometria.</td>
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<td>Proliferation of ducts and acini.</td>
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<td>Ethinyl estradiol</td>
<td>Gynaecomastia. ductal hyperplasia with simple and papilliform cyst formation.</td>
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<td>Investigator</td>
<td>Oestrogen</td>
<td>Effect</td>
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<td>-------------------------------</td>
<td>--------------------------</td>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Vagina</td>
<td>Mulligan et al (1943)</td>
<td>Estrone</td>
<td>Hyperplasia of the stratified squamous epithelium, oedema of the lamina propria.</td>
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<td>Mulligan (1947)</td>
<td>Diethylstilboestrol</td>
<td>Hyperplasia of the stratified squamous epithelium.</td>
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<tr>
<td></td>
<td>Wadsworth</td>
<td>Ethinyl estradiol</td>
<td>Hyperplasia and cornification of the squamous epithelium.</td>
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<td>Skin</td>
<td>Gardner &amp; de Vita (1940)</td>
<td>Estradiol benzoate</td>
<td>Failure of hair regrowth.</td>
</tr>
<tr>
<td></td>
<td>Mulligan (1943; 1944; 1947)</td>
<td>Diethylstilboestrol</td>
<td>Atrophy of hair follicle epithelium, atrophy of sebaceous glands.</td>
</tr>
<tr>
<td></td>
<td>Dow (1960)</td>
<td>Diethylstilboestrol</td>
<td>Atrophy of epidermis, hair follicles and sebaceous glands; fibrosis of the dermis.</td>
</tr>
<tr>
<td></td>
<td>Jabara (1962b)</td>
<td>Diethylstilboestrol</td>
<td>Atrophy of skin and hair follicles.</td>
</tr>
<tr>
<td></td>
<td>Wadsworth</td>
<td>Ethinyl estradiol</td>
<td>Atrophy of hair follicles and sebaceous glands.</td>
</tr>
<tr>
<td>Blood and bone marrow</td>
<td>Tyslowitz (1939)</td>
<td>Diethylstilboestrol</td>
<td>Agranulocytosis and anaemia.</td>
</tr>
<tr>
<td></td>
<td>Tyslowitz &amp; Dingemanse (1941)</td>
<td>Estrone, estradiol benzoate and diethylstilboestrol</td>
<td>Anaemia, thrombocytopenia, granulocytopenia following leucocytosis; haemorrhage.</td>
</tr>
<tr>
<td></td>
<td>Castrodale et al (1941)</td>
<td>Estradiol and diethylstilboestrol</td>
<td>Thrombocytopenia and haemorrhage; leucocytosis, followed by leucopenia; anaemia; hyperplasia followed by hypoplasia in the bone marrow.</td>
</tr>
</tbody>
</table>
TABLE 5
(continued)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Investigator</th>
<th>Oestrogen</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood and bone marrow</td>
<td>Crafts (1941; 1948)</td>
<td>Estradiol and diethylstilboestrol</td>
<td>Neutrophilia followed by neutropenia; anaemia.</td>
</tr>
<tr>
<td></td>
<td>Dougherty et al (1943)</td>
<td>Estrone and estradiol benzoate</td>
<td>Leucocytosis followed by neutropenia and agranulocytosis; normocytic anaemia; hyperplasia of the bone marrow.</td>
</tr>
<tr>
<td></td>
<td>Chiu (1974)</td>
<td>Estradiol cyclopentyl—proprionate</td>
<td>Thrombocytopenia; leucocytosis followed by leucopenia; anaemia; haemorrhage.</td>
</tr>
<tr>
<td></td>
<td>Wadsworth</td>
<td>Ethinyl estradiol</td>
<td>Neutrophil leucocytosis.</td>
</tr>
</tbody>
</table>
CHAPTER TWO

THE ORAL TOXICITY OF ETHINYL ESTRADIOL WHEN ADMINISTERED TO
RHEUS MONKEYS AT A DOSE RATE OF 500 \( \mu g/kg/\)week

FOR 26 WEEKS
INTRODUCTION

With the exception of the work of McClure and Graham (1973), there appears to be little recent information on the safety of oestrogens when administered to non-human primate species. McClure and Graham reported the occurrence of uterine mesotheliomas in 7 out of 10 squirrel monkeys (Saimiri sciurea) given high dosage levels of diethylstilboestrol by implantation. Extreme lesions were found in animals killed after 11 and 14 months of administration. In one animal, lesions were observed after only 5 months of treatment. After prolonged administration 2 animals developed extragenital serosal lesions involving the adrenals, spleen and mesentery. Graham (1978) noted similar lesions in the squirrel monkey following the administration of oestradiol benzoate.

In view of these observations, a preliminary toxicological evaluation of ethinyl estradiol was undertaken in the rhesus monkey (Macaca mulatta).

MATERIALS AND METHODS

Seven wild caught rhesus monkeys, 2 males, 2 entire females and 3 ovariectomised females, were housed separately in metal cages. A two-week period was allowed for preliminary investigations to take place and for the animals to adjust to their new environment. Details relating to individual animals and their bodyweight at the beginning of the dosing period are shown in Table 6.

All monkeys were fed a complete dry diet, supplemented by fresh fruit every second day and wholemeal bread each weekday. Water was freely available at all times. The ethinyl estradiol was dissolved in a solution of 2.5% ethyl alcohol in water, to give a concentration of 500 μg in 0.5 ml (0.0125% weight/volume). The solution was administered by gavage once per week at a dose level of 500 μg/kg. Monkey 4229 was dosed for 13 weeks and monkeys 625d, 6269 and 4239 were dosed for 26 weeks before being killed. Monkeys 627d, 6289 and 4219 were dosed for 26 weeks and retained for a recovery period of 12 weeks before being killed.

Food and water consumption, together with signs of ill health were monitored daily. Bodyweight was recorded twice weekly. Veterinary examinations and laboratory investigations were performed before dosing started, after 4, 8, 12, 16, 20 and 26 weeks' dosing, and after 4, 8 and 12 weeks' recovery. The haematological investigations comprised total red and white cell counts, differential white cell counts, platelet counts, reticulocyte counts, packed cell volume, haemoglobin, erythrocyte sedimentation rate, and prothrombin index. The biochemical investigations comprised measurement of plasma urea, plasma glucose, serum proteins (total and differential) serum alkaline phosphatase, serum alanine aminotransferase (SAIT), serum leucine aminopeptidase (LAP), serum bilirubin, sodium and potassium. Urine was examined qualitatively for specific gravity, pH, protein, total reducing substances, glucose, ketones, bile pigments, urobilinogen and blood pigments and microscopically for sediment.
## TABLE 6

Data relating to individual monkeys at the start of the dosing period

<table>
<thead>
<tr>
<th>Animal No./Sex</th>
<th>Bodyweight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>625♂</td>
<td>4.25</td>
</tr>
<tr>
<td>626♀</td>
<td>3.05</td>
</tr>
<tr>
<td>627♂</td>
<td>4.40</td>
</tr>
<tr>
<td>628♀</td>
<td>2.30</td>
</tr>
<tr>
<td>4219*</td>
<td>4.65</td>
</tr>
<tr>
<td>4229*</td>
<td>4.70</td>
</tr>
<tr>
<td>4239*</td>
<td>3.95</td>
</tr>
</tbody>
</table>

* Ovariectomised
On completion of the dosing period, the animals were killed and immediately afterwards a full macroscopic examination performed, during which the adrenals, brain, gonads, heart, kidneys, liver, lungs, pancreas, pituitary, prostate, spleen, thymus, thyroids and uterus were removed and weighed. Small portions of these tissues, together with aorta, sternal bone marrow, cervix, caecum, colon, duodenum, epididymis, Fallopian tubes, eyes, gall bladder, ileum, jejunum, cervical and mesenteric lymph nodes, mammary glands, oesophagus, skeletal muscle, sciatic nerve, skin, submandibular salivary gland, seminal vesicles, stomach, trachea, tongue, urinary bladder, vagina and vulva, together with any macroscopic abnormality, were placed in 10% buffered formalin fixative. The tissues were routinely processed in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin. Additional pieces of liver and kidney were placed in formol calcium, sectioned at 12 μm and stained for fat with Oil Red O.

RESULTS

Clinical signs

Development of the sex skin was seen in all animals from the second week of dosing onwards. When dosing was discontinued, this change gradually regressed, although extensive skin folding, was still apparent at the end of the recovery period, especially in the males. In the male animals there was a slight reduction in testicular size. Menstruation was suppressed in the entire females throughout the dosing period. During the recovery period, both the entire and ovariectomised females showed oestrogen withdrawal bleeding 19 days after the administration of the last dose of the test compound. This bleeding persisted in both animals for four days. During week 10 of the recovery period, the entire female had a menstrual flow of 3 days duration and 24 days later a further menstrual bleed was noted. No clinically detectable mammary development occurred in any male or female animals. Bodyweight, food intake and water consumption were not adversely affected.

Laboratory investigations

No significant changes were detected in the haematological parameters monitored.

The results of the SAIT values and LAP values are presented in Table 7 and the SAIT values are depicted graphically in Figure 13. These show a rise in enzyme levels which returned to baseline values during the recovery period. The other biochemical investigations were within the normal range.

The results of the urinalysis showed no adverse effects.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Pre-dose</th>
<th>Number of weeks' dosing</th>
<th>Number of weeks' recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>SAIT</td>
<td>35</td>
<td>65</td>
<td>67</td>
</tr>
<tr>
<td>LAP</td>
<td>277</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 13
Group mean SAT (mU/ml) values

SAAT (mU/ml)

Weeks’ duration
0 2 4 6 8 10 12

Weeks’ recovery
0 2 4 6 8 10 12
Macroscopic findings

After 26 weeks' dosing, both the entire and ovariectomised female monkeys (6269 and 4239) showed bilateral enlargement and congestion of the Fallopian tubes adjacent to the ovary and extending along the dorsal aspect of the broad ligament (Fig. 14). This change was not present in the ovariectomised monkey killed after 13 weeks, nor in the two female monkeys maintained for the recovery period. There were no other macroscopic abnormalities that could be related to treatment.

Organ weight analysis

The results of both relative and absolute organ weight analysis were considered to be within the normal range.

Histological findings

Histological examination of the Fallopian tubes of monkeys 6269 and 4239 revealed bilateral hyperplasia of the fimbrial epithelium, together with congestion and oedema in the underlying lamina propria (Fig. 15). In addition, in these animals there was marked keratinisation and hyperplasia of the squamous vaginal and vulval epithelium. Slight ductal hyperplasia of the mammary gland was found in the male animals sacrificed after 26 weeks dosing. Histological examination of the livers showed no abnormalities that could be attributable to treatment with ethinyl estradiol.

DISCUSSION

In this study with the rhesus monkey, proliferative changes involving the peritoneal mesothelium were not induced. A number of other workers (Engle et al, 1943; Scott and Wharton, 1955; Geschickter and Hartman, 1959) have also given high levels of both steroidal and non-steroidal oestrogens for prolonged periods to rhesus monkeys without inducing this effect. The response of the squirrel monkey to oestrogens appears to differ from that of the rhesus monkey. This is probably a reflection of innate species variation and the relatively short duration of dosing in relationship to the relatively long life span of the rhesus monkey. Catchpole and van Wagenen (1975) and Hodgen et al (1977) record that menopause occurs approximately in the 25th year of life in the rhesus monkey.

The hyperplasia of the fimbrial epithelium of the Fallopian tubes seems to disappear with cessation of exposure to ethinyl estradiol, indicating this change to be hormone dependent. This change has also been recorded by Graham 1976.
Figure 14 Rhesus monkey 6269
Macroscopic appearance of the ovary (o) Fallopian tube (t) and uterus (u).
Arrow (→) indicates area of congestion and hyperplasia of the fimbriae.

Figure 15 Rhesus monkey 6269
Transverse section of the Fallopian tube showing hyperplasia of the fimbrial epithelium (→).
Haematoxylin and eosin (x160).
The increase in the liver enzyme values SAIT and LAP without definite pathological change indicates a functional disturbance in this organ. The results might be at variance with those of Goldzieher and Kraemer (1972) who stated that liver function tests did not alter when ethinyl estradiol was administered to rhesus monkeys for 36 months at dosage rates of 0.002, 0.01 and 0.05 mg/kg/day in a cycle of 21 days on, 7 days off.

The effects of oestrogen administration upon the sex skin, (Bachman et al, 1935; Zuckerman et al, 1938; Hisaw and Hisaw 1966), the vagina (Hisaw and Hisaw 1961) and the mammary gland (Gardner and van Wagenen, 1938; Folley et al, 1939) of rhesus monkeys have been documented. Oestrogen withdrawal bleeding has been used to compare the relative potency of natural and synthetic oestrogens (Eckstein et al, 1952; Eckstein et al, 1967; Hisaw et al, 1971; Schane et al, 1972).

ABSTRACT

Ethinyl estradiol was administered orally to a group of 7 rhesus monkeys at a dosage rate of 500 µg/kg/week for 26 weeks.

Biochemical investigations indicated a degree of hepatic dysfunction but histologically no morphological abnormalities were detected in the liver.

The only significant pathological change was hyperplasia of the fimbrial epithelium of the Fallopian tube which was hormone dependent.
CHAPTER THREE

THE ORAL TOXICITY OF ETHINYL ESTRADIOL WHEN ADMINISTERED TO RHESUS MONKEYS AT A DOSE RATE OF 100 \( \mu g/\text{kg/day} \) FOR 26 WEEKS
INTRODUCTION

The experiment previously reported showed that when ethinyl estradiol was administered orally to rhesus monkeys at a dose rate of 500 µg/kg/week liver enzyme values were raised but no changes in hepatocyte morphology were detected by light microscopy. The purpose of this experiment was to investigate further the effect of ethinyl estradiol on hepatocyte morphology by both light microscopy and electron microscopy.

MATERIALS AND METHODS

Six wild—caught adolescent female rhesus monkeys (Macaca mulatta) were selected and housed separately in metal cages. Two of these animals were maintained as undosed controls. A two—week period was allowed for preliminary investigations to take place and for the animals to adjust to their new environment. The bodyweight of individual animals at the time dosing commenced are shown in Table 8.

All monkeys were fed a complete dry diet, supplemented by fresh fruit every second day and wholemeal bread each weekday. Water was freely available at all times. The ethinyl estradiol was dissolved in a solution of 5.0% ethyl alcohol in water, to form a concentration of 0.0025 gm%. The ethinyl estradiol was administered by gavage once daily at a dose level of 100 µg/kg for 26 weeks.

Food consumption, together with clinical observations for signs of ill health, were monitored daily. Bodyweight was recorded at weekly intervals. Veterinary examinations were carried out before dosing started and after 24 weeks dosing. Laboratory investigations were performed before dosing commenced and then after 6, 12 and 24 weeks dosing. The haematological investigations comprised total red and white cell counts, differential white cell counts, platelet counts, reticulocyte counts, packed cell volume, haemoglobin, erythrocyte sedimentation rates and prothrombin time. The biochemical investigations comprised plasma urea, plasma glucose, serum protein (total and differential), serum alkaline phosphatase, serum alanine aminotransferase, serum leucine aminopeptidase, serum bilirubin, sodium and potassium.

On completion of the dosing period, the animals were killed and immediately afterwards a full macroscopic examination performed, during which the adrenals, brain, gonads, heart, kidneys, liver, lungs, pancreas, pituitary, spleen, thymus, thyroids and uterus were removed and weighed. Small portions of these tissues, together with aorta, sternal bone marrow, cervix, caecum, colon, duodenum, Fallopian tubes, eyes, gall bladder, ileum, jejunum, cervical and mesenteric lymph nodes, mammary glands, oesophagus, skeletal muscle, sciatic nerve, skin, submandibular salivary gland, stomach, trachea, tongue, urinary bladder, vagina and vulva, and any macroscopic abnormality, were placed in 10% buffered formalin fixative. The tissues were routinely processed in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin. Additional pieces of liver and kidney were placed in formol calcium, sectioned at 12 µm and stained for fat with Oil Red O.
### TABLE 8

Data relating to individual monkeys at commencement of dosing

<table>
<thead>
<tr>
<th>Dosage Group</th>
<th>Animal No./Sex</th>
<th>Bodyweight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undosed Control</td>
<td>525♀</td>
<td>3.35</td>
</tr>
<tr>
<td></td>
<td>526♀</td>
<td>4.15</td>
</tr>
<tr>
<td>100 μg ethinyl estradiol/kg/day</td>
<td>527♀</td>
<td>3.70</td>
</tr>
<tr>
<td></td>
<td>528♀</td>
<td>2.45</td>
</tr>
<tr>
<td></td>
<td>529♀</td>
<td>2.50</td>
</tr>
<tr>
<td></td>
<td>530♀</td>
<td>3.05</td>
</tr>
</tbody>
</table>
Liver samples were fixed in 4% glutaraldehyde buffered with 0.05M cacodylate to pH 7.3 at 4°C. Three 1 mm³ blocks of tissue from each animal were post-fixed in 1% osmium tetroxide buffered with 0.05M cacodylate to pH 7.3, dehydrated in alcohol and embedded in epoxy resin. 1 μm survey sections were cut and stained with 1% toluidine blue for examination with the light microscope. Silver/gold ultra-thin sections of representative areas were mounted on copper grids and subsequently stained with lead citrate and uranyl acetate. These sections were examined using a Philips EM 300 electron microscope operating at 60 kV. Selected areas were photographed on Kodak Fine Grain Positive 35 mm film and enlarged as necessary.

RESULTS

Clinical signs

Development of the sex skin together with vulval enlargement was seen in all treated animals from the second week of dosing onwards. In particular, the folding of the sex skin was progressive and towards the end of the dosing period it became extensive. There was suppression of menstruation in dosed animals whereas in the undosed controls menstruation, although not following a regular pattern, was readily observed. No mammary development could be detected in treated animals. Bodyweight and food consumption were not adversely affected.

Laboratory investigations

No significant changes were detected in the haematological parameters monitored.

Biochemical investigations showed that in animals receiving the ethinyl estradiol, the serum alanine aminotransferase values and the serum leucine aminopeptidase values were elevated during the dosing period. The results have been tabulated in Tables 9 and 10. In addition in monkeys 5289 and 5299 a slight decrease in the percentage serum albumin and a slight increase in percentage serum β globulins was found after 26 weeks dosing.

Macroscopic findings

The ovaries of the treated monkeys appeared small and inactive, whereas in the two control animals ovaries were enlarged and either mature Graafian follicles or corpora lutea were obvious indicating cyclical activity. The treated animals showed bilateral enlargement and congestion of the fimbriae of the Fallopian tubes which in the absence of ovarian activity in these animals was considered to be a physiological manifestation of the oestrogen administration. However, the degree of enlargement was no greater than that encountered in the untreated control monkeys with normal menstrual cycles.
### TABLE 9

**Group mean SAIT values (mU/ml)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dosage $\mu$g/kg/day</th>
<th>Weeks dosed</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>6</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>26</td>
<td>50</td>
<td>30</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>30</td>
<td>110</td>
<td>100</td>
<td>74</td>
</tr>
</tbody>
</table>

### TABLE 10

**Group mean LAP values (GR units)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dosage $\mu$g/kg/day</th>
<th>Weeks dosed</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>6</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>119</td>
<td>182</td>
<td>238</td>
<td>179</td>
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<tr>
<td>2</td>
<td>100</td>
<td>288</td>
<td>524</td>
<td>551</td>
<td>500</td>
</tr>
</tbody>
</table>
Organ weight analysis

The results of the relative and absolute organ weight analyses were considered to be within the normal range.

Histological findings

The morphological appearance of the livers of treated animals was essentially similar to that encountered in the control group. In the ovaries of the treated animals only small secondary follicles were apparent, whereas both corpora lutea and mature Graafian follicles were evident in the control monkeys. The fimbrial epithelium of the Fallopian tubes in treated monkeys showed bilateral hyperplasia. In the uteri of treated animals, slight dilatation of occasional endometrial glands was evident (Fig. 16). In the cervix occasional small focal areas of squamous epithelium were observed, whereas in the control animals only a columnar epithelium was noted (Fig. 17). The vaginal epithelium of treated animals was several cells thick and heavily cornified.

Ultrastructural findings

In the livers of two of the treated animals, occasional hepatocytes were seen to contain needle-shaped electron-lucent areas which were sometimes membrane-bound (Fig. 18). In addition, in one of these affected animals, occasional, mainly periportal hepatocytes showed intracisternal sequestration of the rough endoplasmic reticulum (Fig. 19).

No morphological abnormalities were detected in the livers of the other two treated animals, nor in the two controls.

DISCUSSION

The results confirm the previous observations that ethinyl estradiol may induce hepatic dysfunction in the rhesus monkey. Clinically, the SAIT and LAP values were found to be elevated throughout the dosing period. Although pathological changes were not encountered in the livers of treated animals with the light microscope, ultrastructural abnormalities were detected by means of transmission electron microscopy. However, the changes found in the hepatocytes were not considered to be sufficient to account for the increased SAIT and LAP values. The needle-shaped electron-lucent areas seen in the cytoplasm of occasional hepatocytes in two of the four treated animals could possibly represent a cleft remaining after a crystal had been lysed out during processing. Crystalline mitochondrial inclusions have been recorded in women on steroid therapy (Martinez-Manautou et al, 1970). While no definite evidence exists that crystals were present in this study, or that they were mitochondrial in origin, these cytoplasmic clefts were not apparent in the control animals and were considered to be related to the administration of the ethinyl estradiol.
Figure 16
Transverse section of uterus showing cystic dilatation of endometrial glands (g).
Note also endometrial stroma (s) and myometrium (m).
Haematoxylin and eosin (x50).

Figure 17
Transverse section of cervix showing focal areas of squamous epithelium (s).
Note also columnar epithelium (c). Haematoxylin and eosin (x200).
Figure 18
Hepatocyte containing cytoplasmic 'clefts' (→) adjacent to smooth endoplasmic reticulum and microbodies
Note also mitochondria (m), glycogen (g) and smooth endoplasmic reticulum (s) x 22,000.
Figure 19

Hepatocyte showing intracisternal sequestration of granular endoplasmic reticulum (→).

Note also nucleus (n), mitochondria (m) and glycogen granules (g) × 22,000.
Ghidoni (1967) also noted these cleft-like spaces in irradiated monkey hepatocytes, but was uncertain whether they were significant or whether some material had been lost during processing.

Intracisternal sequestration was noted in one of the four treated animals. The term intracisternal sequestration is used to describe a pattern of organisation of the rough endoplasmic reticulum where vesicular profiles of the rough endoplasmic reticulum are seen lying within dilated cisternae. It is thought that the primary stage of this process is the formation of papillary processes from the wall of a dilated cistern. Later such processes may be pinched off to form vesicular structures which come to lie free within the cisternae. The formation of papillary projections from ductal and surface epithelial has been linked with proliferative processes, and it could be that the above phenomenon noted in this study is a similar process and that it reflects a type of hypertrophy of the rough endoplasmic reticulum. This probably represents an increase in metabolic activity attributable to the administration of ethinyl estradiol.

The changes observed in the sex skin, ovaries, Fallopian tubes and vagina were essentially similar to those recorded in the previous study. Additional changes were noted in the uterus and cervix. In the uterus, cystic dilatation of endometrial glands following oestrogen administration has been recorded by Engle et al (1943), Iglesias and Lipschutz (1947), Geschickter and Hartman (1959) and McClure and Graham (1973).

In the cervix, Overholser and Allen (1933; 1935) first recorded squamous metaplasia following oestrogen administration. Many investigators (Engle and Smith 1935; Hisaw and Lendrum 1936; Zuckerman 1937; Engle et al 1943; Geschickter and Hartman 1959) made similar observations. Recent studies (Graham, 1970) have shown that this spread of stratified squamous epithelium throughout the endocervical canal is the result of stimulation of small foci of squamous cells occurring naturally at this site, thus questioning the theory that the squamous epithelium arose by metaplasia of columnar cells.

ABSTRACT

Ethinyl estradiol was administered orally to a group of 4 rhesus monkeys at a dose rate of 100 \( \mu g/kg/day \) for 26 weeks. Two other animals were maintained as undosed controls.

Biochemical investigations indicated a degree of hepatic dysfunction. Light microscopy showed no significant morphological changes in the livers. By electron microscopy occasional hepatocytes could be identified containing needle-shaped electron-lucent areas in two out of four treated animals only, one of which had periportal hepatocytes with intracisternal sequestration of the rough endoplasmic reticulum.
CHAPTER FOUR

THE EFFECTS OF ETHINYL ESTRADIOL, ADMINISTERED ORALLY

AT A DOSE RATE OF 100 μg/kg/day FOR 13 WEEKS,

ON BILIARY SECRETION AND COMPOSITION IN RhesUS MONKEYS

WITH AN INTACT ENTEROHEPATIC CIRCULATION.
INTRODUCTION

The use of non-human primates in the experimental study of the effects of oestrogens on bile composition and kinetics has only recently been described. The effects of the administration of single doses of oestradiol (Lynn et al, 1973a; Lynn, et al, 1973b) and of seven daily doses of oestrone (Lynn and Williams, 1975) to rhesus monkeys with an intact enterohepatic circulation have been reported. Other research workers (Javitt, et al, 1975a; Javitt et al, 1975b; Morrissey et al, 1977) have recorded the long-term effects of subcutaneous implants of ethinyl estradiol on bile acids using $^{14}$C–24 isotopes and bile cholesterol in baboons previously subjected to cholecystectomy. The purpose of this study was to investigate further the effects of ethinyl estradiol on bile flow and bile composition in rhesus monkeys with an intact enterohepatic circulation at a dose rate previously shown to induce changes in liver enzyme values.

MATERIALS AND METHODS

Three wild-caught mature female rhesus monkeys (Macaca mulatta) were allocated to the study (Table 11). They were fed a complete dry diet supplemented by fresh fruit every day and wholemeal bread each weekday. Water was freely available at all times.

The extrahepatic biliary circuit was surgically exteriorised to permit representative bile sampling with an electronic stream splitter. (Dowling et al., 1968; Campbell et al., 1971). Representative bile samples were taken on a 5% daily basis, i.e. one drop in twenty, and the remaining bile returned to the gastric antrum to maintain a virtually intact enterohepatic circulation (Fig. 20).

Throughout the study the animals were restrained in a chair to permit maximum freedom for the monkey, but to prevent interference with the biliary and gastric catheters (Fig. 21).

The animals were maintained for a three to four week period during which standard post-operative procedures were followed, and predose investigations were carried out. Once the animal was stabilised, ethinyl estradiol was administered at a dose level of 100 μg/kg/day for 13 weeks by intragastric intubation.

The volume of bile secreted during 24-hour periods was calculated from the volume of the 5% sample and, from this, the individual and group mean daily bile volumes per seven-day period were estimated. The pH and the viscosity of the bile samples were measured daily. The bile samples were analysed for total bile salt concentration, phospholipid concentration and cholesterol concentration. From these values the group mean daily secretion rates of bile salts, cholesterol and phospholipids, the group mean daily molar ratios of cholesterol, phospholipid and bile salts and the group mean daily cholesterol saturation index per seven-day period were calculated.
TABLE 11
Data relating to individual monkeys

<table>
<thead>
<tr>
<th>Animal</th>
<th>Bodyweight (kg)</th>
<th>Predose period (days)</th>
<th>Duration of dosing (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>4.50</td>
<td>29</td>
<td>96</td>
</tr>
<tr>
<td>D</td>
<td>4.55</td>
<td>26</td>
<td>94</td>
</tr>
<tr>
<td>E</td>
<td>4.20</td>
<td>25</td>
<td>94</td>
</tr>
</tbody>
</table>
MODEL FOR CONTROLLED INTERRUPTION OF E.H.C.

FIGURE 20 Schematic drawing of experimental model. When used as a sampling technique, every twentieth drop of bile was diverted, by means of the movable funnels (the black block) of the stream splitter, to a sampling tube. The remainder of the bile passed into a small cup, balanced in such a way that only a few drops of bile would close the switch and pump the bile back to the antral region of the stomach. (Campbell et al, 1971).

Figure 21 Photograph of the restraining chair, the sampling tubes and electronic stream splitter.
Haematological and biochemical investigations were carried out at weekly intervals. The haematological investigations comprised counts of total red and white cells and platelets and estimation of haemoglobin concentration and packed cell volume. The biochemical investigations comprised determination of serum alanine aminotransferase, serum aspartate aminotransferase, serum alkaline phosphatase, and serum leucine aminopeptidase levels, together with estimations of the serum bilirubin concentration.

On completion of the dosing period the animals were killed and immediately afterwards a full macroscopic post mortem examination performed, during which the adrenals, brain, gonads, heart, kidneys, lungs, liver, pancreas, pituitary, spleen, thymus, thyroids and uterus were removed and weighed. Small portions of or the whole of these tissues, together with samples of aorta, sternal bone marrow, cervix, caecum, colon, duodenum, Fallopian tubes, eyes, ileum, jejunum, cervical and mesenteric lymph nodes, mammary glands, oesophagus, skeletal muscle, sciatic nerve, skin, salivary gland, stomach, trachea, tongue, urinary bladder, vagina and vulva, together with any macroscopic abnormality were placed in 10% buffered formalin fixative. The tissues were routinely processed in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin. Additional pieces of liver and kidneys were placed in formol calcium, sectioned at 12 μm and stained for fat with Oil Red O.

RESULTS

Clinical observations

The administration of ethinyl estradiol produced clear changes in secondary sexual characteristics of all three monkeys with the result that at the end of the dosage period, the sex skin extended from the perineum along the abdomen and the thighs. With the exception of slight loss of bodyweight, particularly during the post—operative predose period, all three monkeys remained in good condition.

Laboratory investigations

The administration of ethinyl estradiol produced a marked increase in the individual and group mean daily bile volume. (Table 12 and Fig. 22). The viscosity and the pH of the bile samples were unaffected by treatment with ethinyl estradiol.

The results of the gall bladder bile analysis are shown in Table 13. The administration of ethinyl estradiol caused a marked fall in group mean concentration of cholesterol, bile salts, and phospholipid. However the group mean daily bile salt secretion rate was essentially unchanged while the daily secretion rates of cholesterol and phospholipid fell (Table 14). The results of the group mean molar ratios of cholesterol, bile salts and phospholipids together with the group mean cholesterol saturation index are shown in Table 15 . The group mean molar ratios of cholesterol and phospholipids decreased slightly whereas the group mean bile salt ratio increased slightly. The group mean cholesterol saturation index was essentially unchanged.
TABLE 12

Individual and group mean daily bile volume (ml) per 7-day period

<table>
<thead>
<tr>
<th>Weeks dosed</th>
<th>Individual mean daily bile volume (ml)</th>
<th>Group mean daily bile volume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>D</td>
</tr>
<tr>
<td>−1</td>
<td>137</td>
<td>133</td>
</tr>
<tr>
<td>0</td>
<td>148</td>
<td>142</td>
</tr>
<tr>
<td>1</td>
<td>116</td>
<td>149</td>
</tr>
<tr>
<td>2</td>
<td>151</td>
<td>148</td>
</tr>
<tr>
<td>3</td>
<td>149</td>
<td>149</td>
</tr>
<tr>
<td>4</td>
<td>173</td>
<td>153</td>
</tr>
<tr>
<td>5</td>
<td>190</td>
<td>169</td>
</tr>
<tr>
<td>6</td>
<td>170</td>
<td>168</td>
</tr>
<tr>
<td>7</td>
<td>162</td>
<td>139</td>
</tr>
<tr>
<td>8</td>
<td>180</td>
<td>65*</td>
</tr>
<tr>
<td>9</td>
<td>160</td>
<td>183</td>
</tr>
<tr>
<td>10</td>
<td>200</td>
<td>168</td>
</tr>
<tr>
<td>11</td>
<td>228</td>
<td>149</td>
</tr>
<tr>
<td>12</td>
<td>203</td>
<td>178</td>
</tr>
<tr>
<td>13</td>
<td>204</td>
<td>145</td>
</tr>
</tbody>
</table>

* Excluded from mean
FIGURE 22

Group mean bile volume (ml per day)
TABLE 13
The group mean daily molar concentration (mmol/L) of cholesterol bile salts and phospholipid per 7-day period

<table>
<thead>
<tr>
<th>Weeks dosed</th>
<th>Molar concentration (mmol/L)</th>
<th>Cholesterol</th>
<th>Bile Salts</th>
<th>Phospholipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td></td>
<td>1.36</td>
<td>52</td>
<td>10.1</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>1.23</td>
<td>51</td>
<td>11.1</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.90</td>
<td>41</td>
<td>5.4</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.85</td>
<td>42</td>
<td>5.8</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0.80</td>
<td>44</td>
<td>4.9</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.76</td>
<td>47</td>
<td>4.0</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>0.80</td>
<td>42</td>
<td>4.2</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>0.78</td>
<td>41</td>
<td>5.4</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>0.83</td>
<td>43</td>
<td>4.4</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>0.80</td>
<td>38</td>
<td>4.2</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>0.40</td>
<td>49</td>
<td>3.6</td>
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<tr>
<td>11</td>
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<td>0.54</td>
<td>38</td>
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<tr>
<td>12</td>
<td></td>
<td>0.43</td>
<td>42</td>
<td>4.4</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>0.60</td>
<td>44</td>
<td>5.2</td>
</tr>
</tbody>
</table>
The group mean daily cholesterol, bile salt and phospholipid secretion rate per 7—day period

<table>
<thead>
<tr>
<th>Weeks dosed</th>
<th>Daily secretion rate (mmoles)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cholesterol</td>
<td>Bile Salts</td>
</tr>
<tr>
<td>-1</td>
<td>0.18</td>
<td>6.9</td>
</tr>
<tr>
<td>0</td>
<td>0.17</td>
<td>7.2</td>
</tr>
<tr>
<td>1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>0.13</td>
<td>5.8</td>
</tr>
<tr>
<td>3</td>
<td>0.13</td>
<td>6.4</td>
</tr>
<tr>
<td>4</td>
<td>0.12</td>
<td>6.9</td>
</tr>
<tr>
<td>5</td>
<td>0.13</td>
<td>7.9</td>
</tr>
<tr>
<td>6</td>
<td>0.13</td>
<td>6.6</td>
</tr>
<tr>
<td>7</td>
<td>0.12</td>
<td>6.1</td>
</tr>
<tr>
<td>8</td>
<td>0.12</td>
<td>6.1</td>
</tr>
<tr>
<td>9</td>
<td>0.13</td>
<td>6.4</td>
</tr>
<tr>
<td>10</td>
<td>0.07</td>
<td>8.8</td>
</tr>
<tr>
<td>11</td>
<td>0.10</td>
<td>7.1</td>
</tr>
<tr>
<td>12</td>
<td>0.08</td>
<td>7.6</td>
</tr>
<tr>
<td>13</td>
<td>0.10</td>
<td>7.3</td>
</tr>
</tbody>
</table>
TABLE 15
The group mean daily molar ratios of cholesterol, bile salts and phospholipid and the group mean cholesterol saturation index per 7-day period

<table>
<thead>
<tr>
<th>Weeks dosed</th>
<th>Group mean molar ratios</th>
<th>Mean cholesterol saturation index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cholesterol</td>
<td>Bile Salts</td>
</tr>
<tr>
<td>-1</td>
<td>2.1</td>
<td>82</td>
</tr>
<tr>
<td>0</td>
<td>1.9</td>
<td>81</td>
</tr>
<tr>
<td>1</td>
<td>1.9</td>
<td>87</td>
</tr>
<tr>
<td>2</td>
<td>1.7</td>
<td>86</td>
</tr>
<tr>
<td>3</td>
<td>1.6</td>
<td>89</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
<td>91</td>
</tr>
<tr>
<td>5</td>
<td>1.8</td>
<td>89</td>
</tr>
<tr>
<td>6</td>
<td>1.7</td>
<td>87</td>
</tr>
<tr>
<td>7</td>
<td>1.8</td>
<td>89</td>
</tr>
<tr>
<td>8</td>
<td>1.9</td>
<td>89</td>
</tr>
<tr>
<td>9</td>
<td>0.8</td>
<td>93</td>
</tr>
<tr>
<td>10</td>
<td>1.5</td>
<td>90</td>
</tr>
<tr>
<td>11</td>
<td>1.0</td>
<td>89</td>
</tr>
<tr>
<td>12</td>
<td>1.2</td>
<td>88</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The results of the individual and group mean LAP values are given in Table 16. The group mean LAP levels became markedly raised after seven weeks' administration of the ethinyl estradiol, and this change persisted for the remainder of the dosage period (Fig. 23). The results of the other biochemical and haematological investigations were within the normal range.

Macroscopic findings

At post mortem examination, minimal focal adhesions between the liver capsule and parietal peritoneum at the wound site were discovered in monkeys B and E. In addition, a small raised subcapsular area of discolouration (15 x 15 mm) was noted in the liver of monkey E adjacent to the adhesions. The livers of monkeys B and D were diffusely pale. The Fallopian tubes adjacent to the ovaries of all three monkeys were found to be bilaterally enlarged and congested and the serosal surface of the uterus was diffusely congested. No other macroscopic observations that could be attributable to treatment were recorded.

Organ weight analysis

The absolute and relative organ weights were within the normal range for rhesus monkeys in this age group.

Histological findings

At histological examination the pale subcapsular area in the liver of monkey E was identified as an area of abscess formation. In the livers of monkeys B and D, minimal portal to portal fibrous tissue bridging with associated mononuclear cell infiltration was seen. In addition, in monkey D, a few small subcapsular areas of necrosis were evident. Small secondary follicles only were noted in the ovaries. There was bilateral hyperplasia and congestion of the fimbrial epithelium of the Fallopian tubes and, in the uterus, slight dilatation of endometrial glands was observed. The vaginal epithelium showed marked hyperplasia with associated keratinisation. No other significant histological changes were seen.

DISCUSSION

The administration of ethinyl estradiol at a dose rate of 100 μg/kg/day produced clear changes in bile flow and composition. A sustained rise in bile volume was recorded but, in spite of the fact that the bile salt concentration decreased, the bile salt secretion rate was unchanged. A reduction in both concentration and secretion rate of phospholipid and cholesterol were also noted, but the relative molar concentration of individual bile constituents was such that the overall cholesterol saturation index was unchanged. The metabolism of individual bile acids was not determined.
**TABLE 16**

Individual and group mean LAP values (GR units)

<table>
<thead>
<tr>
<th>Weeks dosed</th>
<th>Animal Number</th>
<th>Group Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>D</td>
</tr>
<tr>
<td>–1</td>
<td>178</td>
<td>249</td>
</tr>
<tr>
<td>0</td>
<td>253</td>
<td>209</td>
</tr>
<tr>
<td>1</td>
<td>270</td>
<td>206</td>
</tr>
<tr>
<td>2</td>
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<td>297</td>
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<td>4</td>
<td>715</td>
<td>579</td>
</tr>
<tr>
<td>5</td>
<td>574</td>
<td>259</td>
</tr>
<tr>
<td>6</td>
<td>686</td>
<td>524</td>
</tr>
<tr>
<td>7</td>
<td>1104</td>
<td>1542</td>
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<td>8</td>
<td>1078</td>
<td>1705</td>
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<td>9</td>
<td>907</td>
<td>2339</td>
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<td>10</td>
<td>1722</td>
<td>1955</td>
</tr>
<tr>
<td>11</td>
<td>1538</td>
<td>1417</td>
</tr>
<tr>
<td>12</td>
<td>2020</td>
<td>2114</td>
</tr>
<tr>
<td>13</td>
<td>1975</td>
<td>1770</td>
</tr>
</tbody>
</table>
FIGURE 23

Group mean LAP (GR units) values
Lynn et al., (1973b) and Lynn and Williams (1975) recorded that the administration of both oestriol and oestrone significantly reduced bile flow and bile salt secretion rate, but relatively short dosing periods were employed. Morrissey et al (1977) reported a significant decrease in biliary cholesterol concentration following the long-term administrations of ethinyl estradiol to baboons and this change was reversible following oestrogen withdrawal. Oestrone, however, increased bile secretion rate, producing bile that was supersaturated with cholesterol (Lynn and Williams, 1975).

With the exception of the morphological changes observed in the liver, the effects of the administration of ethinyl estradiol on secondary sexual characteristics, liver enzyme values and the reproductive tract were similar to those previously noted. The macroscopic and histological changes seen in the liver were probably associated with the surgical procedure and not the result of the administration of ethinyl estradiol.

**ABSTRACT**

Ethinyl estradiol was administered orally at a dosage rate of 100 µg/kg/day to a group of 3 rhesus monkeys with an intact enterohepatic circulation for 13 weeks.

The administration of the ethinyl estradiol produced changes in bile flow and composition. A sustained rise in bile volume was recorded but, in spite of the fact that bile salt concentration decreased, the bile salt secretion rate was unchanged. A reduction in both concentration and secretion rate of phospholipid and cholesterol were also noted, but the relative molar concentration of individual bile constituents was such that the overall cholesterol saturation index was unchanged. Biochemical investigations showed a rise in LAP values.
CHAPTER FIVE

THE ORAL TOXICITY OF ETHINYL ESTRADIOL
WHEN ADMINISTERED TO POST PARTUM
RHEUS MONKEYS AT A DOSE RATE OF
100 μg/kg/day FOR 12 WEEKS
INTRODUCTION

Bahgat et al (1975) administered a progestogen to lactating rhesus monkeys and recorded the appearance of the progestogen in the milk and its affect on lactation. An extensive literature survey has not brought to light any published account of the effects of oestrogens when administered during post partum lactation to simian primates. The following study was therefore designed to investigate this important aspect of developmental toxicology.

MATERIALS & METHODS

Four wild—caught female post partum rhesus monkeys (Macaca mulatta) were allocated to the study. Individual mothers and infants were housed in metal cages under standard laboratory conditions. One pair of mothers received 100 µg ethinyl estradiol per kg from day 5 post partum for 12 weeks. The other pair was undosed and acted as controls (Table 17). The drug was administered to the mothers by gavage. The mothers were fed a complete dry diet supplemented by fresh fruit every second day and wholemeal bread each weekday.

Food consumption of the mothers and signs of ill health in mothers or infants were monitored daily. Bodyweights of mothers and infants were recorded before dosing began and then at weekly intervals. Biochemical investigations of serum alanine aminotransferase and serum leucine amino peptidase levels of mothers and infants were carried out before dosing and then after 1, 3, 5, 7, 9 and 11 weeks’ dosing. The concentrations of ethinyl estradiol present in both serum samples from mother and infant and in milk samples from the dam were determined by radioimmunoassay before dosing started and then after 4, 8 and 12 weeks’ dosing. (Warren and Fotherby 1974). Milk sampling was facilitated by intravenous administration of 5 i.u. oxytocin.

On completion of the dosing period, the infant monkeys were killed and immediately afterwards a full macroscopic examination was made, during which the brain, pituitary, heart, lungs, liver, spleen, pancreas, thymus, prostate, uterus, kidneys, thyroids, adrenals, gonads and seminal vesicles were removed and weighed. Small portions of these tissues, together with aorta, trachea, lymph nodes, gall bladder, urinary bladder, salivary gland, tongue, oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, skin, skeletal muscle, sciatic nerve, eye, vagina and vulva, together with any macroscopic abnormality, were placed in 10% buffered formalin fixative. The tissues were routinely processed in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin. Additional pieces of liver and kidney were placed in formol calcium, sectioned at 12 µm and stained for fat with Oil Red O.

The mothers were maintained for a further 12 weeks’ observation period.
TABLE 17

Data relating to individual monkeys at the start of the dosage period

<table>
<thead>
<tr>
<th>Dosage group</th>
<th>Adult animal number</th>
<th>Sex of infant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undosed Control</td>
<td>202</td>
<td>♂</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>♀</td>
</tr>
<tr>
<td>100 µg ethinyl estradiol/kg/day</td>
<td>213</td>
<td>♂</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>♀</td>
</tr>
</tbody>
</table>
RESULTS

Clinical observations

The male infant of monkey 2139 showed slight reddening of the perineum from week 3 of dosing onwards. By week 9, reddening and oedema were considerable and in addition the mammary glands appeared prominent. These changes persisted until the animal was sacrificed at week 12 (Fig. 24—25). The female infant of monkey 109 showed slight reddening of the perineum through weeks 5, 7 and 8. The perineum and mammary glands of the untreated control infants were unaffected.

Post partum menses were not recorded in any of the animals during the experimental period. None of the treated females returned to menses during the 12 week recovery period and only one of the control animals returned to cycle 6 weeks after weaning.

Bodyweight

The weights of the infant monkeys are shown graphically in Figure 26. The small number of animals used precludes definite conclusions, but there is some indication that the infants of the dams receiving 100 pg ethinyl estradiol per kg per day showed only slightly slower growth rates than the animals in the control group. Although no attempt was made to measure milk yield, the fact that this difference in growth rates was marginal suggests that milk yield was not significantly depressed.

The weights of the mothers are shown graphically in Figure 27. Administration of ethinyl estradiol caused a slight loss of bodyweight when compared to the controls.

Biochemical investigations

Administration of ethinyl estradiol to the mothers had no adverse effect on the LAP and SAIT levels. Figure 28 shows the mean SAIT values of dosed adults and infants depicted graphically.

Serum and milk ethinyl estradiol concentrations

In the conjugated fraction of plasma, ethinyl estradiol was detected after 4 (400 pg/ml), 8 (200 pg/ml) and 12 weeks’ dosing (130 pg/ml) in adult monkey 109 only. All other values in the conjugative fraction were negative. Ethinyl estradiol was not detected in any of the milk samples nor in the freely extractable fraction of plasma, the lower level of sensitivity in this instance being about 200 pg/ml.
Figure 24 Male infant of monkey 213
Posterior aspect showing marked development of the sex skin

Figure 25 Male infant of monkey 213
Mammary gland and teats showing development
FIGURE 28
Mean SAIT (mU/ml) values: Adults and infants
Adults dosed 100 μg/kg/day ethinyl estradiol
Macroscopic findings

The mammary glands of the male infant of monkey 2139 were prominent and the teats pendulous. In this animal, the sex skin extended from the perineal region, medio—posteriorly to the knee, antero—dorsally to the sacral region and backwards along the tail. The sex skin was folded, reddened and appeared oedematous. The scrotal skin was erythematous. Macroscopic abnormalities were not observed in any of the other infants.

Organ weight analysis

The organ weights of the treated infant monkeys were comparable to those of the untreated control group.

Histological findings

Histological examination of the mammary gland of the male infant from monkey 2139 revealed slight ductal proliferation (Fig. 29). No other morphological abnormalities were encountered that could be attributed to treatment.

DISCUSSION

The results demonstrate that a physiological change may be induced in the infants of the mothers dosed with ethinyl estradiol indicating that the oestrogen is secreted through the milk. Bahgat et al (1975) showed by radioimmunoassay that progestogens may similarly appear in milk. It is of interest to note that the male rhesus monkey is born showing swelling and turgescence of the scrotum (Wislocki, 1933). This naturally occurring physiological effect would appear to be due to the markedly increased circulating levels of oestradiol and oestrone during the last few days of pregnancy in the rhesus monkey (Resko et al, 1975 ; Weiss et al, 1976 ; Challis et al 1977).

In contrast to previous observations, administration of ethinyl estradiol to post—partum rhesus monkeys failed to induce any changes in liver enzyme values. It is possible that the metabolism of ethinyl estradiol in the lactating animal may differ from that of the non—lactating animal. Weiss et al (1973) have observed that in the post—partum rhesus monkey, lactation prolongs the morphological and functional integrity of the corpus luteum of pregnancy.

In addition, the experiment showed that once lactation was established, the administration of ethinyl estradiol did not suppress lactation, nor did hormone withdrawal bleeding occur following cessation of treatment.
Figure 29  Male infant of monkey 213
Mammary gland showing ductal proliferation (→).
Note also stroma (st). Haematoxylin and eosin (x160).
ABSTRACT

Ethinyl estradiol was administered orally to two post-partum lactating rhesus monkeys at a dosage rate of 100 μg/kg/day for 12 weeks. Two other post-partum animals were maintained as undosed controls.

Clinical and histological changes were induced in the infants of the mothers dosed with ethinyl estradiol indicating that the oestrogen was secreted through the milk. Lactation did not appear to be suppressed. Following cessation of treatment, hormone withdrawal bleeding did not occur.
CHAPTER SIX

THE ORAL TOXICITY OF ETHINYL ESTRADIOL
WHEN ADMINISTERED TO NEONATAL
RHESUS MONKEYS AT A DOSE RATE OF
100 μg/kg/day FOR 13 WEEKS
INTRODUCTION

It has long been recognised that the immature animal is not merely a miniature of the mature. Recently we have begun to appreciate the practical importance of developmental toxicology (Done, 1964). Differences in drug toxicity, in drug distribution, in quantitative and qualitative sensitivity of target organs, in detoxification mechanisms and in excretory mechanisms such as efficiency of renal function might affect the toxicity of a compound in the neonate. The purpose of this study was to evaluate the oral toxicity of ethinyl estradiol in the neonatal rhesus monkey.

MATERIALS AND METHODS

Four neonatal rhesus monkeys (Macaca mulatta) aged not less than 3 weeks were allocated to the study. Two monkeys were given 100 μg ethinyl estradiol/kg/day by oral gavage for 13 weeks. The other two monkeys were not dosed and were maintained as untreated controls (Table 18). Throughout the study the monkeys were housed individually with their mothers in metal cages.

Clinical observations for signs of ill health were made daily. Bodyweight was recorded at weekly intervals. Haematological investigations comprising total red and white cell counts, differential white cell counts, haemoglobin, and packed cell volume were performed at monthly intervals. Biochemical investigations consisting of serum alanine aminotransferase (SAIT), serum leucine aminopeptidase (LAP) and serum aspartate aminotransferase (SA sT) were also carried out at monthly intervals.

On completion of the dosing period the animals were killed and immediately afterwards a full macroscopic examination was performed. The adrenals, brain, gonads, heart, kidneys, liver, lungs, pancreas, pituitary, prostate, spleen, thymus, thyroids and uterus were removed and weighed. Small portions or the whole of these tissues together with samples of aorta, sternal bone marrow, cervix, caecum, colon, duodenum, Fallopian tubes, eyes, gall bladder, ileum, jejunum, cervical and mesenteric lymph nodes, mammary glands, oesophagus, seminal vesicles, skeletal muscle, sciatic nerve, skin, submandibular salivary gland, stomach, trachea, tongue, urinary bladder, vagina and vulva were placed in 10% buffered formalin fixative. The tissues were routinely processed in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin. Additional pieces of liver and kidney were placed in formol calcium, sectioned at 12 μm and stained for fat with Oil Red O.
TABLE 18

Data relating to individual animals at commencement of dosing

<table>
<thead>
<tr>
<th>Animal No./Sex</th>
<th>Dosage Group</th>
<th>Age at start of dosing (days)</th>
</tr>
</thead>
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<tr>
<td>337♂</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>158♀</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>294♂</td>
<td>100</td>
<td>16</td>
</tr>
<tr>
<td>316♀</td>
<td>100</td>
<td>16</td>
</tr>
</tbody>
</table>
Liver samples were taken and fixed in 4% glutaraldehyde buffered with 0.05 M cacodylate to pH 7.3 at 4°C. Three 1 mm³ blocks of tissue from each animal were processed in 1% osmium tetroxide buffered with 0.05 M cacodylate to pH 7.3, dehydrated in alcohol and embedded in epoxy resin. Survey sections 1 μm thick were cut and stained with 1% toluidine blue for examination with the light microscope. Silver/gold ultra-thin sections of representative areas were mounted on copper grids and subsequently stained with lead citrate and uranyl acetate. These sections were examined using a Philips EM 300 electron microscope operating at 60 KV. Selected areas were photographed on Kodak Fine Grain Positive 35 mm film and enlarged as necessary.

RESULTS

Clinical signs

During the second week of dosing, slight stimulation of the sex skin was apparent in the treated animals. This change was progressive and, after 12 weeks, skin folding together with erythema was generalised. In addition, the teats of both treated monkeys became enlarged and pendulous. No adverse effects were observed in bodyweight gain in the treated animals.

Laboratory investigations

During the dosing period group mean LAP values increased in treated animals, whereas in the undosed controls, LAP values fell. This has been illustrated graphically in Figure 30. Significant differences in group mean SAsT and SAIT values were not observed. (Table 19). No significant haematological abnormalities were noted.

Macroscopic findings

The seminal vesicles of monkey 294d and the cervix, uterus and Fallopian tubes of monkey 316n were markedly enlarged in comparison to those of the control animals. The cervix and uterus of monkey 316n measured 15 mm and 10 mm in diameter respectively, whereas the cervix and uterus on monkey 158b both measured 1 mm in diameter only.
Figure 30

Group mean LAP (GR units) values

LAP (GR units)

100 µg/kg/day ethinyl estradiol

Control

Weeks dosed

0 4 8 12
TABLE 19
Group mean SAIT (mU/ml), SAS(T (mU/ml) and LAP (GR units) values

<table>
<thead>
<tr>
<th>µg/kg/day ethinyl estradiol</th>
<th>Weeks dosed</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>4</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>SAIT</td>
<td>34</td>
<td>35</td>
<td>24</td>
<td>28</td>
</tr>
<tr>
<td>100</td>
<td>14</td>
<td>20</td>
<td>23</td>
<td>37</td>
</tr>
<tr>
<td>SAS(T</td>
<td>54</td>
<td>51</td>
<td>34</td>
<td>36</td>
</tr>
<tr>
<td>100</td>
<td>38</td>
<td>48</td>
<td>44</td>
<td>66</td>
</tr>
<tr>
<td>LAP</td>
<td>635</td>
<td>360</td>
<td>282</td>
<td>263</td>
</tr>
<tr>
<td>100</td>
<td>581</td>
<td>437</td>
<td>661</td>
<td>881</td>
</tr>
</tbody>
</table>
Organ weight analysis

The seminal vesicles and the uterus of the treated animals were markedly enlarged, weighing 2.60 g and 1.19 g respectively (Table 20a and 20b). All other values were considered to be within the normal range.

Histological findings

No definite morphological abnormalities that could be attributed to treatment were observed in the livers of the two monkeys given ethinyl estradiol.

However, in the treated male animal oestrogenic effects were noted in the prostate, seminal vesicles and mammary glands. Moderate focal squamous metaplasia of both the prostatic acinar and ductular epithelium and of the transitional epithelium of the prostatic urethra was seen (Fig. 31). The seminal vesicles showed marked diffuse hyperplasia and in the mammary gland slight acinar hyperplasia with lobular formation was evident.

In the treated female monkey, oestrogenic effects were noted in the uterus, Fallopian tubes, cervix, vagina and mammary gland. Marked diffuse hyperplasia of the endometrium and myometrium was apparent in the uterus. In addition, one endometrial gland showed moderate cystic dilatation. The fimbrial epithelium of the Fallopian tubes was diffusely hyperplastic. The cervical mucosal epithelium showed diffuse hyperplasia and in some areas small foci of squamous epithelium were observed. In the vagina, marked diffuse hyperplasia and cornification of the stratified squamous epithelium were seen. The changes in the mammary glands were similar to those in the mammary glands of the treated male monkey.

Ultrastructural findings

The ultrastructure of the livers of the treated animals was essentially similar to that of the livers of the control group.
### TABLE 20a
**Absolute organ weights (g)**

<table>
<thead>
<tr>
<th>Animal No./Sex</th>
<th>μg/kg/day ethinyl estradiol</th>
<th>Bodyweight</th>
<th>Seminal vesicles</th>
<th>Uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td>337 δ</td>
<td>0</td>
<td>950</td>
<td>0.44</td>
<td>—</td>
</tr>
<tr>
<td>158 ⊘</td>
<td>0</td>
<td>1016</td>
<td>—</td>
<td>0.13</td>
</tr>
<tr>
<td>294 δ</td>
<td>100</td>
<td>1250</td>
<td>2.60</td>
<td>—</td>
</tr>
<tr>
<td>316 ⊘</td>
<td>100</td>
<td>878</td>
<td>—</td>
<td>1.19</td>
</tr>
</tbody>
</table>

### TABLE 20b
**Relative organ weights (% bodyweight)**

<table>
<thead>
<tr>
<th>Animal No./Sex</th>
<th>μg/kg/day ethinyl estradiol</th>
<th>Seminal vesicles</th>
<th>Uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td>337 δ</td>
<td>0</td>
<td>0.046</td>
<td>—</td>
</tr>
<tr>
<td>158 ⊘</td>
<td>0</td>
<td>—</td>
<td>0.013</td>
</tr>
<tr>
<td>294 δ</td>
<td>100</td>
<td>0.208</td>
<td>—</td>
</tr>
<tr>
<td>316 ⊘</td>
<td>100</td>
<td>—</td>
<td>0.0136</td>
</tr>
</tbody>
</table>
Figure 31 Rhesus monkey 294d

Transverse section of prostate showing focal squamous metaplasia of acinar epithelium (→).

Note also prostatic stroma (st) and acini (a). Haematoxylin and eosin (x 80.)
DISCUSSION

The results of the experiment show that ethinyl estradiol, when administered to neonatal rhesus monkeys, stimulated the development of the seminal vesicles in the male and of the uterus in the female. The development of the seminal vesicles was an unexpected change and suggests that in the rhesus monkey, the seminal vesicles and Müllerian ducts may have at least in part some common embryological origin. van Wagenen (1935) reported hyperplasia of the fibromuscular stroma of the seminal vesicles when “theelin” was administered to immature rhesus monkeys. Hisaw and Hisaw (1961) concluded that the outstanding effect of oestrogens on the uterus was that of growth, and so the development of the uterus in the neonate was predictable. The dependence of the female reproductive tract on ovarian hormones is strikingly demonstrated by the profound atrophy of the genital tract that follows surgical removal of the ovaries. The involuted uterus of a ovariectomised animal can be restored to its normal size in 2 or 3 weeks by daily injections of adequate amounts of oestrogens (Allen 1927; 1928).

The anabolic effects of oestrogens in other species have long been appreciated. In this study no appreciable difference was evident in the rate of growth of the treated animals compared with that of the controls. However, 13 weeks’ treatment may be insufficient for such differences to emerge.

The effects of oestrogens on the sex skin, the serum liver enzymes, the mammary glands, the Fallopian tubes, the cervix and the vagina in the neonate were essentially similar to those in adults. The effects of oestrogenic stimulation of the prostate of the castrated immature rhesus monkey have been described by Zuckerman (1938). The prostate greatly increased in size and while the epithelium of the true prostatic glands was unchanged, hyperplasia and metaplasia had taken place in the epithelium of the urethra, vagina masculina, terminal parts of the ejaculatory ducts and collecting ducts. van Wagenen (1935) also recorded squamous metaplasia of the epithelium of the colliculus seminalis and the bulbous and cavernous urethra following oestrogen administration.

ABSTRACT

Ethinyl estradiol was administered orally to two neonatal rhesus monkeys at a dosage rate of 100 μg/kg/day for 13 weeks. Two other neonatal rhesus monkeys were maintained as undosed controls.

With the exception of the stimulation of the development of the seminal vesicles in the male and of the uterus in the female, the changes induced by ethinyl estradiol were similar to those seen in adult rhesus monkeys.
CHAPTER SEVEN

A TERATOGENIC STUDY WITH ETHINYL ESTRADIOL WHEN ADMINISTERED TO RHESUS MONKEYS BY SUBCUTANEOUS INJECTION AT A DOSE RATE OF 50 μg/kg/day
INTRODUCTION

The experimental use of non-human primates to investigate abnormalities of intrauterine development essentially began with studies of thalidomide after the drug had been shown to be teratogenic in man. The sensitive period of organogenesis in the rhesus monkey, approximately from day 20 to day 45 of gestation (Wilson, 1971), compares well with that of man, which Lenz (1964) estimates to extend from day 20 to day 36 of gestation (34th to 50th day from last menses). Morris and van Wagenen (1966) administered oestradiol dipropionate to six monkeys at dosage rates from 4 mg — 25 mg/day and diethylstilboestrol at dosage rates from 1 mg — 250 mg/day to fourteen monkeys after implantation (days 18 — 167). Dosing was not confined to the period of organogenesis and they reported that no foetal abnormalities were observed nor was the incidence of abortion greater than that normally encountered in their primate colony.

With the exception of this report from Morris and van Wagenen, there would appear to be no other published studies of the teratogenicity of oestrogens in rhesus monkeys.

MATERIALS AND METHODS

Ten wild-caught female rhesus monkeys (Macaca mulatta) were selected and housed separately in metal cages. Following a period of acclimatisation, the animals were allocated to treatment groups. All monkeys were fed a complete dry diet, supplemented by fresh fruit every second day and wholemeal bread each week-day. Water was freely available at all times.

Clinical observations were made daily to determine the stage of the menstrual cycle of each animal. During the 10th, 11th and 12th days of the menstrual cycle (first day of menses being day 0 of the menstrual cycle and 11th day of the menstrual cycle being day 0 of gestation), each female was housed with a mature male rhesus monkey of proved fertility, to allow mating to take place. Pregnancy was determined by a chorionic gonadotrophin bio-assay (Mouse Uterine Test, Wilson et al, 1970) of a serum sample taken at day 17 of the gestation period and confirmed by rectal palpation of the uterus. Food intake was monitored daily and bodyweight recorded at weekly intervals.

The ethinyl estradiol was dissolved in a solution of 2½% ethyl alcohol in water, to form a concentration of 0.01 g% w/v. The ethinyl estradiol was then administered daily by subcutaneous injection at a dosage rate of 50 μg/kg. Monkey 1759 was dosed from day 23 to day 45 of gestation, monkey 2019 from day 29 to day 45, monkey 2149 from day 24 to day 45 and monkey 2159 from day 20 to day 45.
### TABLE 21

Data relating to individual animals at start of treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Animal No./Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 μg/ethinyl estradiol/kg/day</td>
<td>175♀</td>
</tr>
<tr>
<td></td>
<td></td>
<td>201♀</td>
</tr>
<tr>
<td></td>
<td></td>
<td>214♀</td>
</tr>
<tr>
<td></td>
<td></td>
<td>215♀</td>
</tr>
<tr>
<td>2</td>
<td>Undosed control</td>
<td>8♀</td>
</tr>
<tr>
<td></td>
<td></td>
<td>42♀</td>
</tr>
<tr>
<td></td>
<td></td>
<td>134♀</td>
</tr>
<tr>
<td></td>
<td></td>
<td>157♀</td>
</tr>
<tr>
<td></td>
<td></td>
<td>166♀</td>
</tr>
<tr>
<td></td>
<td></td>
<td>174♀</td>
</tr>
</tbody>
</table>

: 37 :
After the 100th day of gestation, a caesarian section was performed, during which the total conceptus was removed. The foetus was immediately killed and the weight of the entire conceptus, foetus, placenta and foetal fluids determined. The sex of the foetus and the placental type were noted. The foetus was then examined radiographically in two planes for skeletal observations using an A.F. Dean Mobile X-ray unit, operating at 42 kv and 300 MA for 0.24 seconds, with a Kodak Industrex C54 film. After an external examination a full macroscopic post mortem examination was performed, during which the adrenals, brain, gonads, heart, kidneys, liver, lungs, pancreas, pituitary, prostate, spleen, thymus, thyroids and uterus were removed and weighed. Small portions of these tissues were taken from the foetuses derived from treated mothers together with aorta, cervix, caecum, colon, duodenum, epididymis, Fallopian tubes, ileum, jejunum, cervical and mesenteric lymph nodes, mammary glands, placenta, oesophagus, skeletal muscle, sciatic nerve, skin, submandibular salivary gland, seminal vesicles, stomach, umbilical cord, trachea, tongue, urinary bladder, vagina and vulva, were placed in 10% buffered formalin fixative. The tissues were routinely processed in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin.

RESULTS

Maternal data

The extent and duration of vaginal bleeding varied considerably between monkeys (Fig. 32). However, vaginal bleeding generally was sporadic and appeared to be unrelated to treatment.

Two of the treated animals, 1759 and 2019, aborted after 54 days of gestation. There were no abortions amongst the control animals.

Analysis of bodyweight and food consumption data showed no obvious adverse effects.

Foetal data

The weights of the entire conceptus, foetus, placenta and amniotic fluids derived from treated animals were essentially comparable to those of the control group (Table 22). In addition all foetuses from both groups had double discoid placentae.

The absolute organ weights are presented in Table 23. Organ weights were essentially comparable in foetuses derived from both treated and control monkeys.
TABLE 22

Individual foetal and placental data

<table>
<thead>
<tr>
<th>Group</th>
<th>Foetus No.</th>
<th>Wt. of entire conceptus (g)</th>
<th>Foetus</th>
<th>Wt. (g)</th>
<th>Placenta</th>
<th>Amniotic fluid Wt. (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 50 μg/kg day</td>
<td>214</td>
<td>392</td>
<td>♂</td>
<td>168</td>
<td>Double 92</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>215</td>
<td>396</td>
<td>♀</td>
<td>134</td>
<td>Double 91</td>
<td>171</td>
</tr>
<tr>
<td>2 Undosed controls</td>
<td>8</td>
<td>283</td>
<td>♂</td>
<td>132</td>
<td>Double 50</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>318</td>
<td>♂</td>
<td>129</td>
<td>Double 76</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>134</td>
<td>313</td>
<td>♂</td>
<td>125</td>
<td>Double 66</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>157</td>
<td>276</td>
<td>♀</td>
<td>130</td>
<td>Double 61</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>166</td>
<td>267</td>
<td>♀</td>
<td>112</td>
<td>Double 54</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>174</td>
<td>340</td>
<td>♂</td>
<td>139</td>
<td>Double 78</td>
<td>107</td>
</tr>
<tr>
<td>Group</td>
<td>Foetus No.</td>
<td>Bodyweight</td>
<td>Brain</td>
<td>Heart</td>
<td>Lungs</td>
<td>Liver</td>
</tr>
<tr>
<td>-------------</td>
<td>------------</td>
<td>------------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 50 µg/kg/day</td>
<td>214</td>
<td>168</td>
<td>19.00</td>
<td>0.90</td>
<td>3.81</td>
<td>5.77</td>
</tr>
<tr>
<td></td>
<td>215</td>
<td>134</td>
<td>17.83</td>
<td>0.71</td>
<td>3.67</td>
<td>5.89</td>
</tr>
<tr>
<td>2 Undosed controls</td>
<td>8</td>
<td>132</td>
<td>15.94</td>
<td>0.89</td>
<td>3.02</td>
<td>4.24</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>129</td>
<td>15.21</td>
<td>0.62</td>
<td>3.04</td>
<td>3.81</td>
</tr>
<tr>
<td></td>
<td>134</td>
<td>125</td>
<td>14.38</td>
<td>0.72</td>
<td>3.19</td>
<td>4.93</td>
</tr>
<tr>
<td></td>
<td>157</td>
<td>130</td>
<td>16.22</td>
<td>0.80</td>
<td>3.20</td>
<td>4.10</td>
</tr>
<tr>
<td></td>
<td>166</td>
<td>112</td>
<td>13.98</td>
<td>0.66</td>
<td>2.92</td>
<td>4.02</td>
</tr>
<tr>
<td></td>
<td>174</td>
<td>139</td>
<td>15.62</td>
<td>0.86</td>
<td>2.78</td>
<td>4.63</td>
</tr>
</tbody>
</table>
Skeletal dimensions, vertebal and sternebral counts showed little variation between individuals with no treatment related trends. Fig. 33–34 illustrate the antero-posterior and lateral radiographs of the foetus derived from monkey 2149 and Fig. 35–36 of the foetus derived from monkey 2159.*

Macroscopic post mortem examination revealed no abnormalities in any foetus.

Histological examination of the foetuses from monkeys 2149 and 2159 showed no abnormality. Histological examination of aborted material from monkeys 1759 and 2019 gave positive confirmation of pregnancy.

DISCUSSION

The results of this investigation show that ethinyl estradiol has no apparent teratogenic effect when administered to rhesus monkeys at a dosage rate of 50 μg/kg/day during the period of organogenesis. However, further evaluation with higher dosage groups nearer the maximum tolerated dose for adult female monkeys is necessary for a full teratological screen. The possibility that ethinyl estradiol in the rhesus monkey is embryotoxic as manifested by early embryonic death and abortion cannot be excluded on the data available.

These results are not inconsistent with those reported by Morris and van Wagenen (1966) although the incidence of abortion in their study was no greater than that normally encountered in their primate colony.

ABSTRACT

Ethinyl estradiol was administered during the period of organogenesis to a group of four pregnant rhesus monkeys at a dosage level of 50 μg/kg/day. Six undosed pregnant monkeys were maintained as procedural controls. After the 100th day of gestation, caesarian sections were performed and the conceptus examined.

Two treated animals aborted indicating that ethinyl estradiol may cause early embryonic death at this dosage level. No teratogenic effects were evident in the two other treated foetuses derived by caesarian section.
Figure 33 Antero-posterior radiograph of foetus from monkey 214?

Figure 34 Lateral radiograph of foetus from monkey 214?
Figure 35. Antero—posterior radiograph of foetus from monkey 2159.

Figure 36. Lateral radiograph of foetus from monkey 2159.
CHAPTER EIGHT

THE ORAL TOXICITY OF ETHINYL ESTRADIOL
(500 µg/kg/week) AND CHENO DEOXYCHOLIC ACID
(45 mg/kg/day WHEN ADMINISTERED TO
BABOONS FOR 26 WEEKS
INTRODUCTION

Chenodeoxycholic acid, one of the primary bile acids, has been successful in dissolving human cholesterol gall-stones. The hepatotoxicity of chenodeoxycholic acid when administered orally to rhesus monkeys (Dyrszka et al., 1975; Dyrszka et al., 1976; Heywood and Sortwell 1977 (b); Fedorowski et al., 1977) and to baboons (Morrissey et al., 1975; Heywood and Sortwell 1977 (a)) is well established. Ethinyl estradiol when administered orally to rhesus monkeys also disturbs liver function. The purpose of the study therefore was to assess the combined oral toxicity of chenodeoxycholic acid and ethinyl estradiol. Female non-human primates were chosen because prevention and treatment of cholesterol gall-stones may be most necessary for patients during child-bearing years. Palmer and Heywood (1974) and McSherry et al (1976) have already recorded foetal toxicity of chenodeoxycholic acid both in rhesus monkeys and baboons respectively.

MATERIALS AND METHODS

Eight wild-caught female baboons (Papio anubis or Papio cynocephalus) were selected and housed separately in metal cages. A four-week period was allowed for preliminary investigations to take place and for the animals to adjust to their new environment. The monkeys were fed a complete dry diet, supplemented by fresh fruit every second day and wholemeal bread each weekday. Water was freely available at all times. The animals were divided into three groups. Two monkeys were given 500 μg/kg/week ethinyl estradiol together with 45 mg/kg/day chenodeoxycholic acid, three monkeys were given 45 mg/kg/day chenodeoxycholic acid and three monkeys were maintained as controls (Table 24).

Food and water consumption, together with clinical observations for signs of ill health, were monitored daily. Bodyweight was recorded at weekly intervals. Veterinary examinations were performed before dosing commenced and after 6, 12, 16 and 22 weeks' dosing. Biochemical investigations, comprising serum alanine amiontransferase, serum aspartate aminotransferase and serum leucine aminopeptidase, were carried out before dosing commenced and after 4, 8, 16 and 25 weeks' dosing for groups 1 and 2 and after 4, 8, 12, 16, 20, 24 and 26 weeks' dosing for group 3.

On completion of the dosing period, the animals were killed and immediately afterwards a full macroscopic examination performed, during which the adrenals, brain, gonads, heart, kidneys, liver, lungs, pituitary, spleen, thymus, thyroids and uterus were removed and weighed. Small portions of these tissues, together with aorta, sternal bone marrow, cervix, caecum, colon, duodenum, Fallopian tube, eyes, gall bladder, ileum, jejunum, cervical and mesenteric lymph nodes, mammary glands, oesophagus, skeletal muscle, sciatic nerve, skin, submandibular salivary gland, stomach, trachea, tongue, urinary bladder, vagina and vulva, together with any macroscopic abnormality, were placed in 10% buffered formalin fixative. The tissues were routinely processed in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin. Additional pieces of liver and kidney were placed in formol calcium, sectioned at 12 μm and stained for fat with Oil Red O.
TABLE 24
Data relating to individual animals at commencement of dosing

<table>
<thead>
<tr>
<th>Group</th>
<th>Dosage</th>
<th>Baboon No.</th>
<th>Bodyweight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>814</td>
<td>5.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>816</td>
<td>3.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>818</td>
<td>2.95</td>
</tr>
<tr>
<td>2</td>
<td>45 mg/kg/day chenodeoxycholic acid</td>
<td>832</td>
<td>4.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>834</td>
<td>4.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>836</td>
<td>2.85</td>
</tr>
<tr>
<td>3</td>
<td>45 mg/kg/day chenodeoxycholic acid + 500 μg/kg/week ethinyl estradiol.</td>
<td>863</td>
<td>3.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>864</td>
<td>4.65</td>
</tr>
</tbody>
</table>
RESULTS

Clinical signs

There was suppression of menstruation in both baboons in group 3 throughout the dosing period and vulval enlargement was seen in both animals from the eleventh week of dosing onwards. During the last two weeks of dosing, baboon 8639 became dull and lost condition slightly. There were no definite adverse signs that could be related to the administration of chenodeoxycholic acid alone. Bodyweight, food intake and water consumption were not affected.

Laboratory investigations

Group mean SAsT, SAIT and LAP values are presented in Table 25. After 4 week's dosing the mean LAP values were raised in group 2 and after 8 weeks dosing the mean LAP values in group 3 were raised. These changes persisted for the remainder of the dosing period. Both the mean SAsT and SAIT values in groups 2 and 3 showed transient increases after initiation of treatment but were not as consistently raised as were the mean LAP values.

Macroscopic findings

In baboons 8639 and 8649 there was bilateral enlargement and congestion of the Fallopian tubes adjacent to the ovary. In baboon 8639 the liver was pale and in baboon 8649 there were small areas of discolouration throughout the capsular surface of the liver. In baboon 8349 the liver was dark and the lobular appearance was accentuated. No other macroscopic abnormalities could be attributed to treatment.

Organ weight analysis

The relative and absolute organ weights were considered to be within the normal range and there were no group differences.

Histological findings

The livers of both baboons in group 3 showed minimal bile duct proliferation with occasional portal-to-portal bridging, and slight perportal chronic inflammatory cell infiltration (Fig. 38). Both animals showed bilateral hyperplasia of the fimbrial epithelium of the Fallopian tubes, and hyperplasia and cornification of the vaginal squamous epithelium. In baboon 8639 a small focus of squamous epithelium was evident in the cervical mucosal epithelium.
<table>
<thead>
<tr>
<th>Group</th>
<th>Enzyme</th>
<th>Precose</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>16</th>
<th>20</th>
<th>24</th>
<th>25</th>
<th>26</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SAIT</td>
<td>27</td>
<td>32</td>
<td>32</td>
<td>32</td>
<td>32</td>
<td>32</td>
<td>34</td>
<td>381</td>
<td>83</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>49</td>
<td>49</td>
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Figure 38 Baboon 863?
Liver showing bile duct hyperplasia with portal to portal bridging.
Haematoxylin and eosin (x50).
The livers of the baboons in group 2 showed minimal or moderate bile duct proliferation with portal to portal bridging, portal fibrosis and periportal mixed inflammatory cell infiltration.

No other morphological abnormalities were observed that were considered to be related to treatment.

DISCUSSION

The results show that the hepatotoxicity of both chenodeoxycholic acid (45 mg/kg/day) and ethinyl estradiol (500 µg/kg/week) in the baboon was essentially similar to that of chenodeoxycholic acid alone (45 mg/kg/day). Morrissey et al (1975) and Heywood and Sortwell (1977a) recorded significant increases in liver enzyme values associated with varying degrees of bile duct proliferation and periportal mononuclear cell infiltration when chenodeoxycholic acid was given to baboons at a dosage rate of 38 mg/kg/day and 45 mg/kg/day respectively. The ultrastructural changes in the liver associated with chenodeoxycholic acid treatment have been recorded by Dyrszka et al (1976). They described in addition to light microscopical findings, bile canalicular bleb formation and hypertrophy of the hepatocyte smooth endoplasmic reticulum. The toxicity of chenodeoxycholic acid is partly attributed to its metabolite, lithocholic acid. In the intestine, chenodeoxycholic acid undergoes 7α-dehydroxylation by bacterial action. Administration of chenodeoxycholic acid results in an increase in lithocholic acid in the bile (Dyrszka et al., 1975; Morrissey et al., 1975). Mosbach et al, (1975) and Fedorowski et al (1977) demonstrated that depression of bacterial action in the intestine by antibiotic therapy inhibited bacterial dehydroxylation and minimised liver damage. Hofman et al (1977) have reported that lithocholic acid in the chimpanzee is solely excreted in the bile in its conjugated form. This degree of sulphation was considered to resemble that of man and so they suggest that the chimpanzee may be a more suitable animal model for the toxicity of lithocholic acid than the rhesus monkey. Suzuki et al (1977) have also recorded that administration of chenodeoxycholic acid to squirrel monkeys did not alter the proportion of lithocholic acid in biliary bile acids and this was associated with a lack of hepatotoxicity when dosage levels were as high as 80 mg per kg for 52 weeks.

The effects of ethinyl estradiol on the fimbrial epithelium of the Fallopian tubes, the cervical epithelium and the vaginal epithelium were similar to those previously recorded.
ABSTRACT

Ethinyl estradiol (500 μg/kg/week) and chenodeoxycholic acid (45 mg/kg/day) were administered orally to two female baboons for 26 weeks, chenodeoxycholic acid (45 mg/kg/day) was administered orally to three female baboons for 26 weeks and three female baboons were maintained as undosed controls.

The results indicate that the combined hepatotoxicity of ethinyl estradiol and chenodeoxycholic acid was no greater than that induced by chenodeoxycholic acid alone.
CHAPTER NINE

THE PRENATAL ADMINISTRATION OF DIETHYLSTILBOESTROL TO RHESUS MONKEYS AT A DOSE RATE OF 1 mg/kg/day FOLLOWED BY A POSTNATAL RECOVERY PERIOD OF 84 WEEKS
INTRODUCTION

The experimental administration of androgens to pregnant rhesus monkeys at an appropriate stage of gestation — that is, from approximately the 40th to the 90th day of gestation, — has been shown to transform female foetuses into female pseudohermaphrodites (van Wagenen and Hamilton, 1943; van Wagenen, 1949; Wells and van Wagenen, 1949; Dantchakoff, 1950; Wells and van Wagenen, 1954; Young et al, 1964; Goy, 1966; Phoenix et al, 1967, and Goy, 1968). Apart from the report from Morris and van Wagenen (1966) who administered oestradiol and diethylstilboestrol to pregnant rhesus monkeys there appears to be no other published account of the administration of oestrogens to pregnant simian primates.

The rhesus monkey may be an appropriate animal model for the study of the foetotoxic effects of diethylstilboestrol because the biotransformation, the nature of the metabolites and the route of excretion of diethylstilboestrol closely resemble those of man (Metzler, et al, 1977).

MATERIALS AND METHODS

Four wild-caught female rhesus monkeys (Macaca mulatta) were selected and housed separately in metal cages. Clinical observations were made daily to determine the stage of the menstrual cycle of each animal. During the 10th, 11th and 12th days of the menstrual cycle (first day of menses being day 0 of the menstrual cycle and the 11th day of the menstrual cycle being day 0 of gestation), each female was housed with a mature male rhesus monkey of proved fertility to allow mating to take place (van Wagenen, 1945). Pregnancy was confirmed by rectal palpation of the uterus. Food intake was monitored daily and bodyweight recorded at weekly intervals. Diethylstilboestrol was administered daily by intramuscular injection at a dose level of 1 mg/kg from the 45th day of gestation until parturition, which in the rhesus monkey occurs at approximately the 168th day of gestation.

The infant rhesus monkeys were then maintained for a recovery period of 84 weeks. Clinical observations were made daily and bodyweight recorded every four weeks. When the infants weighed 1 kg (after 20 weeks) they were weaned; subsequently food consumption was monitored daily and bodyweight recorded at weekly intervals. Laboratory investigations were performed just before the animals were killed. The haematological investigations comprised counts of total red and white cells, differential white cells, platelets and reticulocytes, and estimations of packed cell volume, haemoglobin, prothrombin index and erythrocyte sedimentation rate. The biochemical investigations comprised determination of plasma urea, plasma glucose, serum proteins (total and differential) serum alanine aminotransferase, serum alkaline phosphatase, leucine aminopeptidase, serum aspartate aminotransferase, serum bilirubin, sodium and potassium levels.
On completion of the recovery period, the infant rhesus monkeys were killed, and immediately afterwards a full macroscopic post mortem examination was performed during which particular attention was paid to the reproductive tract. The adrenals, brain, heart, kidneys, liver, lungs, pancreas, pituitary, prostate, seminal vesicles, spleen, thymus and thyroids were removed and weighed. Small portions or the whole of these tissues together with samples of aorta, cervix, caecum, colon, duodenum, eyes, gonads, gall bladder, ileum, jejunum cervical and mesenteric lymph nodes, mammary glands, uterus, oesophagus, skeletal muscle, sciatic nerve, skin, submandibular salivary gland, stomach, trachea, tongue, urinary bladder, vagina, and vulva were placed in 10% buffered formalin fixative. The tissues were routinely processed in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin. Additional pieces of liver and kidney were placed in formol calcium, sectioned at 12 μm and stained for fat with Oil Red O.

RESULTS

Maternal data

Beginning at the 45th day of gestation, diethylstilboestrol was injected intramuscularly at a dosage rate of 1 mg/kg/day until termination. Two of the pregnant females, 459 and 509, aborted after 31 and 87 days of treatment respectively. The pregnant female 1779 gave birth to a male infant on the 168th day of gestation after receiving a total of 655 mg of diethylstilboestrol. The pregnant female 1859 gave birth to a female infant on the 169th day of gestation after receiving a total of 642 mg of diethylstilboestrol. The average gestation period of untreated control rhesus monkeys maintained at the Huntingdon Research Centre is 166.8 days with a range of 156—177 days (Heywood, 1974). Food consumption and bodyweight of monkeys 1779 and 1859 were not adversely affected.

Infant data

Both infants weighed 425 g at birth. The mean birth weight of rhesus monkeys maintained at the Huntingdon Research Centre is 440 ± 50 g (Heywood, 1974). The infant monkeys were maintained for a recovery period of 84 weeks. At birth, both infant monkeys showed slight folding and reddening of the sex skin. These changes persisted for 12 weeks in the male infant of monkey 1779 and for 10 weeks in the female infant of monkey 1859. Both infants were weaned at approximately 20 weeks of age when they weighed 1 kg. The bodyweight gain during the first year, while fluctuating, was similar to that normally encountered in untreated control animals at the Huntingdon Research Centre (Heywood, 1974).

With the exception of a slightly lower total serum protein level in the infant monkey 1779 and a slightly raised SAstT value in the infant of monkey 1859, the results of the preterminal laboratory investigations were within the normal range.
At post mortem examination, the male infant of monkey 1779 weighed 1.75 kg and the female infant of monkey 1859 weighed 2.20 kg. With the exception of the testes of the male infant which were found in the inguinal region, the external genitalia were normal in appearance. Internally, both the prostate and seminal vesicles were found to be poorly developed. The right and left seminal vesicles weighed only 0.148 g and 0.188 g respectively. The prostate was located by the examination of the prostatic urethra for the opening of the prostatic ducts and weighed 0.128 g. The combined prostate and seminal vesicle weight for animals weighing 1.71 kg ± 121 g (mean of 6) is normally 0.90 ± 0.14 g. In the female infant, the only abnormalities seen in macroscopic examination were prominent folds in the vaginal mucosa. The remaining relative and absolute organ weights were considered to be within the normal range for animals in this age group.

At histological examination the testes of the male infant were found to be immature, with spermatogenesis proceeding to the spermatocyte stage only. No morphological abnormalities were found in the prostate and seminal vesicles, the appearance of which was essentially similar to that encountered in immature male rhesus monkeys of this age group. The reproductive tract of the female infant was normal. Only small secondary follicles were evident in the ovaries and shallow endometrial glands were noted in the uterus. The cervix and vagina were lined by well developed columnar and stratified squamous epithelia respectively. No other morphological changes were seen that could be attributable to treatment with diethylstilboestrol.

**DISCUSSION**

The results indicate that prenatal exposure to diethylstilboestrol partially inhibits the normal development of the male accessory sex glands of rhesus monkeys. Further evaluation with larger groups of monkeys is necessary to confirm these preliminary investigations. Morris and van Wagenen (1966) reported that no foetal abnormalities were observed when diethylstilboestrol was administered to 14 pregnant macaques. The dosage rate and dosing period varied considerably, precluding valid comparison. In addition, a postnatal recovery period was not allowed.

Maternal plasma levels of steroidal oestrogens increase progressively during pregnancy, particularly during the last seven days (Challis et al, 1977) and are thought to be placental in origin. The steroid concentration of oestradiol in maternal plasma is higher than in amniotic fluid, although this relationship is reversed for oestrone, especially during the 20 days preceding delivery. Such changes are undoubtedly responsible for the swelling and turgescence of the scrotum seen at birth in male rhesus monkeys (Wislocki, 1933). In this experiment, diethystilboestrol had a marked effect on the sex skin of both infants, suggesting that the foetuses were exposed to high concentrations of diethylstilboestrol.
Our results have not ruled out the possibility that diethylstilboestrol in the rhesus monkey is embryotoxic, causing early embryonic death and abortion.

Histological evaluation of the vaginal and cervical mucosa in the female infant was unrewarding. In women exposed to diethylstilboestrol in utero non-neoplastic changes included cervical erosion and vaginal adenosis, the incidence of which is about 30% (Scully et al, 1974; Sherman et al, 1974). We do not know whether such non-neoplastic changes could be induced in female rhesus monkeys.

**ABSTRACT**

Diethyl stilboestrol was administered prenatally to a group of four pregnant rhesus monkeys from day 45 of gestation until parturition at a dose level of 1 mg/kg/day. Two treated monkeys aborted. Two infant monkeys, one male and one female, were born after approximately 168 days of gestation, their mothers having received a total of 655 mg and 642 mg of diethyl stilboestrol respectively. Both infants were allowed a recovery period of 84 weeks before being killed. During the first 10–12 weeks of the recovery period, evidence of physiological stimulation of the sex skin was apparent. At post mortem examination the prostate and seminal vesicles of the male infant were found to be small although no histological abnormalities were apparent. The reproductive tract of the female infant was normal.
CHAPTER TEN

THE INCIDENCE OF MAMMARY TUMOURS

IN A GROUP OF FORTY 8 YEAR OLD FEMALE

BEAGLE DOGS
INTRODUCTION

The value of the Beagle dog as a suitable animal model for the long-term toxicological evaluation of oral contraceptives has been questioned (Hill and Dumas, 1974; Drill, 1975). Such prospective studies were introduced in order to determine the effects that might result in women from the long-term usage of hormonal contraceptives, especially with regard to their carcinogenic potential (Berliner, 1974). Studies of seven years duration are mandatory in the United States of America, Sweden and Australia, while other countries, notably the United Kingdom, show great interest in whether such tests have been conducted. The spontaneous incidence of mammary tumours and dysplasias in the Beagle dog must be taken into account when assessing the results of these investigations and publication of such data has been encouraged (Owen and Briggs, 1976). The purpose of this study, therefore, is to record the spontaneous incidence of mammary tumours as determined by standard toxicological techniques using the International Histological Classification of Tumours of Domestic Animals (Hampe and Misdorp, 1974), in a group of 40 eight-year old nulliparous Beagle dogs.

MATERIALS AND METHODS

Forty pure bred Beagle bitches (Appleton strain) were housed individually from the age of 4-5 months under standard laboratory conditions at the Huntingdon Research Centre. They were fed a fixed amount of a complete dry diet, and water was available ad libitum. The dogs were the subject of a carcinogenicity experiment of 7½ years duration, details of which have been published (Heywood et al, 1977). Since treatment did not increase the incidence of mammary tumours, the data from both treated and control animals has been combined. Clinical examinations were made every six months, and just before the animals were killed. The animals were killed by injecting a lethal dose of pentobarbitone intravenously, and a detailed post mortem examination was immediately performed on each dog. The individual mammary glands on the left and right sides were dissected free as a unit with the attached covering skin. Representative sections of tissue were taken from all the major organs. These tissues, and any abnormalities detected on macroscopic examination were fixed in 10% buffered formalin for 4-8 weeks. After sections had been taken, through the teat, from each individual mammary gland, the tissues were processed in the usual way for microscopic examination by embedding in paraffin wax (M.P. 56°C), sectioning at 5 μm and staining with haematoxylin and eosin.
RESULTS

The average age of the Beagle dogs was 8 years and 2 weeks (range 8 years to 8 years and 7 weeks). A total of 417 mammary glands were examined, an average of 10.4 mammary glands per dog. Five palpable abnormalities of the mammary gland were found on clinical examination. After macroscopic and histological evaluation two benign mammary tumours (Table 26), a papillary complex cyst (Table 27), a fibroma and an incarcerated inguinal hernia were identified. The peri-canaliculare fibroadenoma (dog 928) and the papillary complex cyst were both detected when the dogs were 3 years of age. Over the 5-year observation period neither grew significantly. The papillary cystadenoma (dog 930) was detected when the dog was 7½ years of age.

Histological examination of each mammary gland revealed in addition to the tumours previously described three small benign mammary tumours (Table 26). These comprised an intracanaliculare fibroadenoma (dog 928), a complex adenoma (dog 950) and a tubular adenoma (dog 950). A total of 5 benign mammary tumours was therefore found in a total of 3 of the 40 dogs, making an incidence of 7.5%.

Dysplasias of the mammary gland were more frequent (Table 27). In addition to the papillary complex cyst previously described, areas of nodular hyperplasia and foci of regular proliferation of epithelial cells in ducts or acini (epitheliosis) were found at histological examination. A total of 11 areas of nodular hyperplasia was found in a total of 9 dogs, and foci of epitheliosis were seen in a total of 35 mammary glands in a total of 12 dogs.

DISCUSSION

There are many reports of the incidence of mammary tumours in dogs other than those maintained as laboratory animals, and these studies have recently been reviewed (Hamilton, 1974; Owen and Briggs, 1976). The difficulties of deriving and comparing such incidence rates (Hamilton, 1974) and the difficulties of extrapolating such information to the laboratory Beagle dog (Taylor et al, 1976) have been outlined.
### TABLE 26

Tumours of the mammary gland encountered in individual dogs

<table>
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<tr>
<th>Dog No.</th>
<th>Tumour type</th>
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<tr>
<td>928</td>
<td>* Pericanalicular fibroadenoma</td>
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<td></td>
<td>Intracanalicular fibroadenoma</td>
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<td>930</td>
<td>* Papillary cystadenoma</td>
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<td>950</td>
<td>Complex adenoma</td>
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<td>Tubular adenoma</td>
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* Detected at clinical examination

### TABLE 27

Incidence of dysplasias of the mammary gland

<table>
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<th>Dysplasia</th>
<th>Nos. of mammary glands affected</th>
<th>Nos. of dogs affected</th>
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<tr>
<td>* Papillary complex cyst</td>
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<tr>
<td>Areas of nodular hyperplasia</td>
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<td>9</td>
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<tr>
<td>Foci of regular proliferation of epithelial cells in ducts or acini</td>
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* Detected at clinical examination
There are few reports of the incidence of spontaneous mammary neoplasia in laboratory dogs. Age specific incidence rates for Beagle dogs have been derived from life-span studies (Andersen, 1965; Taylor et al, 1976), toxicological studies (Finkel and Berliner 1973; Owen and Briggs, 1976) and whole mount preparations of the mammary gland (Cameron and Faulkin, 1971; Warner, 1976). Andersen (1965) has stated that in a life-span study of 354 female Beagles clinical signs of mammary tumours had occurred in approximately 50% of these dogs by 6 to 8 years of age. Taylor et al (1976) have presented a more detailed account of the incidence of mammary neoplasia in the University of Utah Beagle dog colony. They reported that the incidence rate remained low through 7 years of age, and only 1 tumour was observed below the 5-year age class, a benign adenoma that appeared at 1.6 years of age. A sharp increase in the incidence rate occurred at approximately 8 years of age and remained high thereafter. They also noted that the first cancer occurred at 7 years of age.

Wyeth International (Owen and Briggs, 1976) have examined tissues from 50 normal female Beagle dogs, used in a control study of 7 years duration. A total of 36 mammary tumours were found in a total of 22 animals, 8 other animals showing areas of adenomatous hyperplasia within mammary tissue. All the tumours were benign, small in size and showed neither local invasion nor distant metastasis. Finkel and Berliner (1973) stated that in studies conducted by Food and Drug Administration and manufacturers of oral contraceptives, spontaneous incidence of mammary tumours in control animals averaged about 12% between 5 and 6 years. They also noted that during such studies nodules 1 to 2 mm in diameter were palpable and therefore tabulated.

Cameron and Faulkin (1971) using a whole mount technique have described hyperplastic and inflammatory nodules, many of which were microscopic, in the mammary glands of four untreated Beagle dogs. The dogs were aged from 7.6 to 8.5 years of age at post mortem. These nodules were studied histologically and a total of 654 atypical glandular nodules were identified, 94 of which were considered neoplastic (14% of the total lesions). Further classification of the neoplasms is not given. Warner (1976) also using whole mount preparations examined 39 Beagle bitches 6 months to 4 years of age. Dysplastic lesions occurred at 2–3 years of age at which time more than 50% of the females had dysplasias. At 3–4 years of age, a total of 1,795 dysplasias were found in 12 bitches. These lesions were 1–4 mm in diameter and not clinically palpable.

There would appear to be considerable variation in age specific incidence rate of mammary neoplasia in the Beagle dog. Some of these discrepancies are undoubtedly due to the widely different techniques employed in deriving such data and due to the fact that an International Histological Classification of Tumours of Domestic Animals has only recently been available. However, from these observations, it may be concluded that (a) malignant mammary tumours are very uncommon in Beagle dogs aged 8 years of age; (b) clinically palpable benign mammary tumours are present in only a small proportion of 8 year old dogs but this figure may be markedly increased by complete histological evaluation; (c) dysplasias of the mammary gland, the majority of which are microscopical, are very frequent in dogs of eight years of age.
ABSTRACT

The incidence of mammary neoplasia in a group of forty nulliparous 8 year old female Beagle dogs is described. The age specific incidence rates of mammary neoplasia in laboratory Beagle dogs are discussed.
CONCLUSION
A. Oral Contraceptives and adverse drug reactions

(i) Oral contraceptives are used by 50 million women in developed and developing countries around the world (Piowtrow and Lee, 1974), but only a few long-term follow-up studies are currently in progress. In the U.K. 23,000 women are taking part in a Royal College of General Practitioners Study (Lancet, 1977) and 17,000 women are participating in the Oxford Family Planning Association Study (Vessey et al., 1977). In these studies, “pill-takers” are considered as one group and no distinction is made between different oral contraceptive pills. Both groups of workers have found increased mortality from diseases of the circulatory system, confirming the results of retrospective studies.

Toxicity studies in non-human primates have failed to show any relationship between oestrogens and cardiovascular disease. The administration of ethinyl estradiol to rhesus monkeys at a dose rate of 0.050 mg/kg for 36 months (21 days on, 7 days off) produced no changes in the coagulation tests normally used in toxicity studies (Goldzieher and Kraemer, 1972). Cardiovascular disease was not encountered during a series of experiments with ethinyl estradiol (chapters 2–6). The administration of mestranol to rhesus monkeys for 84 months at a dosage rate of 0.1 mg/kg/day (21 days on, 7 days off) produced a mild decrease in haemoglobin and packed cell volume (Geil and Lamar, 1977). In a study in which ovariectomised baboons were given an atherogenic diet, treatment with ethinyl estradiol at doses equivalent to the human dose rate did not influence the development of atherosclerosis (McGill et al., 1977). Non-human primates would therefore appear to be unsuitable animal models for the investigation of the association between oestrogens and cardiovascular disease.

In contrast to non-human primates, the administration of oestrogens to the dog has been shown to cause marked changes in haemopoietic tissue, particularly bone marrow elements. Ethinyl estradiol at dosage levels of 5 mg/kg/day for up to 95 days produced an initial neutrophil leucocytosis followed by thrombocytopenia, leucopenia, haemorrhage and aplastic anaemia (Capel-Edwards et al., 1971). At a dose level of 1 mg/kg/day, ethinyl estradiol induced a neutrophil leucocytosis (chapter 1). The administration of mestranol to dogs for 77 months at a dose rate of 0.05 mg/kg/day (21 days on, 7 days off) induced a mild decrease in packed cell volume and haemoglobin (Geil and Lamar, 1977). The mechanism of action of oestrogens on haemopoietic tissue in the dog is unknown (Chiu, 1974). The initial neutrophil leucocytosis is probably associated with the role of oestrogens in uterine defence during oestrus (Rowson, et al, 1953; Broome, et al., 1959). The thrombocytopenia may possibly be associated with the diapedetic uterine bleeding which occurs during oestrus in the dog. These observations however, are not in keeping with the cardiovascular changes encountered in women.
The relationship between oral contraceptives and the liver has recently been reviewed (Metreau, et al, 1972). Both jaundice and changes in liver function tests have been reported in a small proportion of “pill—takers”. The jaundice usually occurs during the first three months and is both cholestatic and cytolytic in origin. Raised SAP and serum transaminase levels are often noted in association with increased serum bilirubin. Histological examination shows intrahepatic cholestasis with canalicular and hepatocellular bile stasis. Minor ultrastructural abnormalities are observed, including dilatation of canalliculi, smooth endoplasmic reticulum and mitochondria. Changes in liver function tests are more frequent than jaundice and include bromosulphalein retention and raised SAP and transaminase levels. Raised transaminase levels may disappear even if treatment is continued, suggesting that hepatocyte adaptation has taken place. The histological appearance of the liver is almost always normal but ultrastructural changes can be seen similar to those observed during jaundice.

The administration of ethinyl estradiol to rhesus monkeys at dose levels of either 500 μg/kg/wk or 100 μg/kg/day raised both LAP and SAIT levels. When dosing was discontinued these values fell to predose levels. The livers appeared to be normal histologically but ultrastructural changes were seen by means of transmission electron microscopy (chapters 2 and 3). No changes in liver enzyme values were observed when ethinyl estradiol was given to post partum lactating rhesus monkeys at similar dose rates suggesting that high levels of endogenous oestrogens during pregnancy may have altered the metabolism of ethinyl estradiol (chapter 5). These changes are similar in many respects to the changes in liver function tests noted in women using oral contraceptives and confirm that, as far as the liver is concerned, ethinyl estradiol is not highly cytolytic. However, the clinical, biochemical and morphological changes associated with jaundice were not evident nor was bile flow diminished (chapter 4). At dose rate of 0.050 mg/kg (21 days on, 7 days off) ethinyl estradiol did not change serum transaminase and isocitric dehydrogenase levels (Goldzieher and Kraemer, 1972). No changes consistent with liver dysfunction were observed when ethinyl estradiol was given to the dog at a dose level of 1 mg/kg/day (chapter 1). When ethinyl estradiol was given to female rats at a dose level of 250 μg/kg, serum alkaline phosphatase levels were increased (Gopinath et al, 1978). No abnormalities were seen histologically in the liver and it was concluded that the increased levels were at least in part from new synthesis by the liver.

Epidemiological investigations have shown that the incidence of gall bladder disease in women is increased by the use of oral contraceptives (Lancet, 1973). Other, more direct, evidence suggests that oral contraceptives produce changes in bile composition. Oral contraceptives have been shown to increase the cholesterol concentration of hepatic bile after cholecystectomy (Pertsemlidis, et al, 1974). A similar change was seen in gall bladder bile obtained by duodenal drainage from normal women (Bennion et al, 1976). Gall bladder bile was reported to be more saturated with cholesterol during contraceptive therapy than during the normal menstrual cycle and in addition, the proportion of chenodeoxycholic acid was reduced. Fundamental changes in bile composition, particularly in cholesterol solubility may therefore be a contributory factor in the aetiology of cholesterol gall—stones that are usually found int he gall bladder.
While spontaneous gall-stones in non-human primates are comparatively rare (van der Linden and Bergman, 1977) cholesterol gall-stones have been reported in baboons (Glen and McSherry, 1970; McSherry et al. 1971). In addition, analysis of the gall bladder bile of the rhesus monkey has shown that rhesus monkeys secrete a bile similar in composition to that of man (Campbell et al, 1971). Non-human primates have therefore been used to investigate the effects of ethinyl estradiol on bile flow and composition. Ethinyl estradiol caused a significant decrease in biliary cholesterol concentration in baboons subjected to cholecystopexy procedures, (Morissey et al, 1977). A decrease in both cholesterol concentration and secretion rate was observed in rhesus monkeys with an intact entero-hepatic circulation, but the relative molar concentrations were such that the overall cholesterol saturation index was unchanged (chapter 4). These observations are not in keeping with the effect of oral contraceptives on biliary cholesterol levels in women (Bennion et al. 1976). Furthermore, in the rhesus monkeys, bile salt concentrations decreased but the bile salt secretion rate was unchanged.

While the effects of oestrogens on flow and composition of bile in the dog has not been specifically investigated, an association between progestogens and changes in the gall bladder is well established. Long-term administration of progestogens may induce cystic mucinous hyperplasia of the gall bladder epithelium (Mawdesley-Thomas and Noel, 1967; Nelson and Kelly, 1976). In some cases the accumulation of mucoid material is sufficient to occlude the gall bladder lumen almost completely (Geil and Lamar, 1977). The significance and pathogenesis of such lesions remains uncertain.

Alterations in bile flow and bromosulphthalein (BSP) clearance have been recorded in the rat by Kreek et al (1969), who reported that 0.5 mg of ethinyl estradiol per day given to female rats for nine days reduced bile flow by 50% compared with the controls and delayed the appearance of BSP in the bile. A similar decrease in bile flow was shown by Heikel and Lathe (1970), who also reported that mestranol reduced bilirubin excretion. Davis and Kern (1976) showed bile acid synthesis to decrease by 50% after five days of ethinyl estradiol treatment at 5 mg/kg/day; the ethinyl estradiol did not alter biliary cholesterol excretion, and the bile contained less bile acid in relation to cholesterol. The hepatic bile acid concentration was not increased by ethinyl estradiol treatment.

The impairment of bile flow in rats might be due to an increase in cannicular permeability or be secondary to an impairment of the system responsible for the secretion of the bile salt non-dependent fraction of canalicular bile water. The results of Gumucio and Valdivieso (1971) indicate that the bile salt non-dependent fraction in the rat was the canalicular bile water and that the inhibitory effect of ethinyl estradiol on bile flow was exerted on the water secreted by the hepatocytes. Forker (1969), working with estrone administered to 250 g rats at the dosage level of 2.5 mg for seven days, demonstrated increased permeability of the biliary tree, as determined by an increase in the biliary clearance of sucrose and mannitol. Estrone reduced the clearance of BSP. He concluded that oestrogen cholestasis might involve enhanced diffusion of materials from bile and blood, in addition to inhibiting active transportation in the opposite direction.
Gallagher et al (1966), working with a variety of steroids including estradiol, estrone, stilbestrol and other synthetic steroid oestrogens, demonstrated impaired BSP metabolism in the rat; this effect extended to other steroids derived from the in vivo metabolic transformation of estradiol in man. In all rodent studies, jaundice has been absent from oestrogen induced cholestasis and this can probably be attributed to the low concentration of bilirubin and the high rate of bile flow characteristic to the rat.

(iv) Liver cell adenomas are rare in women. Recently a small but significant number of cases have been reported in young women who had taken oral contraceptives. (Christopherson and Mays, 1977). Most of these women had taken oral contraceptives for more than five years and although, the tumours were benign, there was a 21% fatality rate (Matiboubi and Shubik, 1976).

The effect of long-term administration of ethinyl estradiol on the liver of both non-human primates and dogs has not been recorded. Carcinogenicity studies with ethinyl estradiol alone have been carried out in both rats and mice (Committee on Safety of Medicines, 1972). Ethinyl estradiol increased the incidence of benign liver cell tumours in both male and female rats and produced malignant liver cell tumours in female rats.

(v) Women who use oral contraceptives have an increased rate of referral to hospital for treatment of cervical erosion (Royal College of General Practitions, 1974; Vessey et al, 1976). Whether oral contraceptives caused the erosions, or whether there was bias in diagnosis, is uncertain. While the gross anatomy of the cervical canal of the rhesus monkey is somewhat different from that of women (Davis and Schneider, 1975) the administration of ethinyl estradiol at a dose rate of 100 μg/kg/day induces histological changes in the cervical epithelium (chapter 3). Graham (1970) has shown that the spread of stratified squamous epithelium throughout the endocervical canal following treatment with oestrogens is the result of stimulation of foci of squamous cells present at this site. In rhesus monkeys therefore the cervical epithelium is a “target” tissue for oestrogens. Significant cervical changes have not been recorded when oestrogens have been administered to dogs.

(vi) In a recent review of fertility regulating methods, Kessler and Standley (1977) raised the question of whether hormonal contraceptives should be given to lactating women. Contraceptive steroids might affect the amount and composition of milk and might be transferred through the milk to the infant. In developing countries lactation performance is particularly important, as failure of breast feeding may lead to infant malnutrition. Since the introduction of oral contraceptives, physicians have suspected an inhibitory effect on lactation but the results of published reports on this subject are conflicting (Chopra, 1972). When ethinyl estradiol (0.3 mg daily) was given to healthy nursing mothers for five days after birth, milk yield was not affected but a significant increase in protein content of the milk was observed (Toaff et al, 1969). Milk yield was similarly unaffected when ethinyl estradiol was given to lactating women at a dose rate of 0.05 mg/day for 1 week (Borglin and Sandholm 1971).
A preliminary study on the effects of ethinyl estradiol given at a dose rate of 100 \( \mu g/kg/day \) to post partum lactating rhesus monkeys showed that ethinyl estradiol was secreted in the milk but at levels which were below the limits of detection of our assay methods. In addition, ethinyl estradiol did not appear to suppress established lactation (chapter 5). Physiological changes such as stimulation of the sex skin and mammary development were induced in the infants of mothers dosed with ethinyl estradiol; the toxicity of this compound in neonatal rhesus monkeys was therefore studied (chapter 6). At a dose rate of 100 \( \mu g/kg/day \), the oral toxicity of ethinyl estradiol to neonatal rhesus monkeys was essentially similar to that observed in the adult rhesus monkey. Considering the low dosage levels present in oral contraceptives, the transfer of ethinyl estradiol through milk would not appear to constitute a major health problem.

(vii) Both retrospective and prospective epidemiological studies have shown that the use of oral contraceptives is negatively associated with the appearance of benign lesions of the breast (Vessey, et al, 1972, Lancet, 1973; Vessey, et al, 1975; Vessey et al, 1976). When contraceptive steroids had been taken during the year prior to the diagnosis of breast cancer, the prognosis was not worsened (Spencer, et al, 1978). The results of such studies are reassuring but as most human carcinogens do not produce an effect until at least 10—15 years have elapsed from the time of exposure, final conclusions cannot be drawn (British Medical Journal, 1976a).

The requirement for the long-term administration of contraceptive steroids to Beagle dogs and rhesus monkeys was initiated in 1967 following the report of mammary nodule induction in dogs and monkeys by the progestogen ethynerone (Finkel and Berliner, 1973). Screening of all contraceptive steroids for carcinogenic potential, particularly as regards the mammary gland, became mandatory in such countries as the U.S.A. and Sweden. Finkel and Berliner (1973) reported that ethinyl estradiol when given to Beagle dogs at dose rates up to 25 \( \mu g/kg/day \) for five years did not influence the development of nodules in the mammary gland. Unfortunately, no further details are given. Geil and Lamar (1977) recorded that when mestranol was administered to Beagle dogs at a dosage of up to 0.05 mg/kg/day for 77 months, no more mammary nodules were observed than in control dogs. They also noted that when mestranol was given to rhesus monkeys at a dosage level of up, to 0.1 mg/kg/day for 84 months there was no increase in mammary nodules. It would, therefore, appear that neither ethinyl estradiol nor mestranol influence the development of mammary nodules in these species. In contrast, certain progestational compounds have a marked tumorigenic effect on the mammary gland of Beagle dogs (Finkel and Berliner, 1973; Geil and Lamar, 1977; Weikel and Nelson, 1977). Whether this is a species—specific response is uncertain. The spontaneous incidence of mammary tumours and dysplasias in the Beagle dog must be taken into account when assessing these investigations. Owen and Briggs (1976) have encouraged the publication of such data (chapter 10). Clinically palpable nodules are normally present in only a small proportion of Beagle dogs under 8 years of age but sub-gross pathological examination may show that there are a large number of dysplastic lesions in both the Beagle dog (Cameron and Faulkin, 1971) and the human breast (Wellings et al, 1975).
Carcinogenicity tests with ethinyl estradiol alone have been carried out in both mice and rats. Ethinyl estradiol increased the incidence of malignant mammary tumours in both male and female mice, but it was suggested that the strain of mice may have influenced the results (Committee on Safety of Medicines, 1972). Drill (1974) found that when either mestranol or ethinyl estradiol was given to male and female mice, the incidence of malignant mammary tumours was not affected. McKinney et al (1968) reported that the administration of ethinyl estradiol alone to rats for 104 weeks at a dose rate of 53 μg/kg/day did not increase the incidence of tumours in any tissues. A slight increase in malignant mammary tumours was recorded in both male and female rats by the Committee on Safety of Medicine (1972) following the administration of ethinyl estradiol alone. Drill (1974) reported that the treatment of rats with the oestrogens estradiol, estrone, ethinyl estradiol and mestranol did at times increase the incidence of mammary adenocarcinoma in rats. While this data should not be interpreted directly in terms of human risk, at the levels of human exposure no obvious health hazards are apparent.

(viii) The possibility that antenatal exposure to contraceptive steroids may be associated with meningomyelocele or hydrocephalus (Gal, et al, 1967), congenital limb reduction defects (Janerich, et al, 1974), cardiovascular birth defects (Levy, et al, 1973; Heinonen et al, 1977), Di George syndrome (Nora and Nora, 1973) or complex combinations of defects (Nora and Nora, 1975) has been recorded. Other studies (Smithells, 1965; Oakley, et al 1973; Mulvihill, et al, 1974; Yasuda and Miller, 1975) have revealed no suggestion of teratogenic action. Preparations taken during the teratogenic period include oral contraceptives, pregnancy test drugs and post-coital contraceptives.

There seem to be few published accounts of the experimental administration of oestrogens to non-human primates during the teratogenic period. A preliminary investigation with ethinyl estradiol when given to rhesus monkeys at a dose level of 50 μg/kg/day during the teratogenic period failed to show any teratogenic effect (chapter 7). Further work, not only with ethinyl estradiol, but also with other contraceptive steroids is required but may be hampered by the evidence that, at high dosage levels, oestrogens may cause early embryonic death and abortion.

B. Hormone Replacement Therapy and Endometrial Carcinoma

A possible association between oestrogens and endometrial carcinoma in women has long been suspected. These suspicions have been founded on sporadic examples of endometrial carcinoma that seem to be related to endogenous or exogenous oestrogens, and on epidemiological studies showing an apparent association between oestrogens used for hormone replacement therapy and endometrial cancer. (Ryan, 1975; Lancet, 1977).
Spontaneous tumours of the uterus are rare both in dogs and in non-human primates (Cotchin and Marchant, 1977). In addition, the long-term administration of oestrogens to these species has failed to induce endometrial carcinoma. Dogs and non-human primates do not seem to be suitable animal models for the induction of endometrial carcinomas by oestrogens. However, certain oestrogens, particularly diethylstilboestrol, are capable of inducing tumour-like hyperplasia of both the genital and non-genital serosa both in dogs (Mawdesley-Thomas and Sortwell, 1968; O'Shea and Jabara, 1971) and non-human primates (McClure and Graham, 1973) which in certain circumstances are indistinguishable from mesotheliomas. Ethinyl estradiol may also induce focal papilliform proliferation of the genital serosa but the extent and degree of this change is considerably less than with other oestrogens (chapter 1) and probably is of little toxicological significance.

The administration of ethinyl estradiol to mice produced malignant tumours of the uterine fundus and cervix in the high dosage group (Committee on Safety of Medicines, 1972) but no effects were apparent when ethinyl estradiol was given to rats (McKinney et al., 1968; Committee on Safety of Medicines, 1972). The fact that oestrogens may induce tumours of the uterus and cervix in mice has long been established. Cotchin and Marchant (1977) observed that the age at which experimental treatment begins is of importance and that as oestrogen administration leads to many untoward side effects, many animals die before such tumours may develop.

C. Prenatal exposure to diethylstilboestrol

An association between treatment of mothers with diethylstilboestrol for various complications of pregnancy and the development of vaginal and cervical adenocarcinomas in the female offspring has been well established. (Herbst and Scully, 1970; Herbst et al., 1971; Herbst et al., 1974). Non-neoplastic changes also found in female offspring include cervical erosion and vaginal adenosis (Scully, et al, 1974; Sherman et al, 1974; Staff and Mattingly, 1974; Herbst et al, 1975). Recently, adverse effects have also been found in male offspring (Bibbo et al, 1975; Bibbo et al, 1977). In contrast to these observations a 25-year follow-up study of the mothers exposed to diethylstilboestrol showed no increase in disease of the breast, uterus or ovary or other reproductive tract abnormality. (Bibbo et al, 1978).

Prenatal exposure of rhesus monkeys to diethylstilboestrol partially inhibits the normal development of the accessory sex glands of the male offspring (chapter 9). Abnormalities of the male reproductive tract have been reported in rats (Greene, et al, 1940), hamsters (Rustia and Shubik, 1976) and mice (Mc Lachlan et al, 1975; Nomura and Kanzaki, 1977) following prenatal administration of diethylstilboestrol. Whether non-neoplastic changes can be induced in the reproductive tract of female offspring of non-human primates has yet to be established.
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- Dr. S.J. Kennedy for assisting with the interpretation of the electron microscopy;

and Mrs. C. Kiberd for typing the thesis.
TECHNIQUES
### Haematology

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<th>Method</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocyte sedimentation rate (ESR)</td>
<td>Wintrobe</td>
<td>mm/1 hour</td>
</tr>
<tr>
<td>Packed cell volume (PCV)</td>
<td>Estimated by Technicon SMA4A or Hawksley Micro Centrifuge</td>
<td>%</td>
</tr>
<tr>
<td>Haemoglobin (Hb)</td>
<td>Estimated by Technicon SMA4A or Coulter Haemoglobinometer</td>
<td>g/100 ml blood (g%)</td>
</tr>
<tr>
<td>Red cell count (RBC)</td>
<td>Estimated by Technicon SMA4A</td>
<td>mill/cmm</td>
</tr>
<tr>
<td>Reticulocyte count (Retics)</td>
<td>Brilliant cresyl blue and new methylene blue</td>
<td>% red cells</td>
</tr>
<tr>
<td>Mean corpuscular haemoglobin concentration (MCHC)</td>
<td>MCHC = Hb (g%) x 100/PCV (% red cells)</td>
<td>%</td>
</tr>
<tr>
<td>Mean cell volume (MCV)</td>
<td>MCV = PCV (% red cells) x 10/RBC (mill/cmm)</td>
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<td>Total white cell count (WBC)</td>
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<td>Coulter Thrombo—Counter—C</td>
<td>1000/cmm</td>
</tr>
<tr>
<td>Prothrombin index (PTI)</td>
<td>Quick's one—stage method (using Simplastin)</td>
<td>% of mean control</td>
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### Biochemistry

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<td>Plasma urea</td>
<td>Technicon Autoanalyser method N 1—C (diacetyl monoxime)</td>
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<td>Plasma glucose</td>
<td>Technicon Autoanalyser method (glucose oxidase)</td>
<td>mg/100 ml (mg%)</td>
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<td>Investigation</td>
<td>Method</td>
<td>Units</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------------------------------------------------</td>
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</tr>
<tr>
<td>Total serum proteins</td>
<td>Technicon Autoanalyser method (Biuret)</td>
<td>g/100 ml (g%)</td>
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<td>Differential serum proteins</td>
<td>Electrophoretic breakdown of albumin a1, a2, β and γ globulins using a Millipore phoroslides staining with ponceau S.</td>
<td>g/100 ml (g%)</td>
</tr>
<tr>
<td>Serum alkaline phosphatase (SAP)</td>
<td>Technicon autoanalyser method (4-amino-phenazine)</td>
<td>King Armstrong (KA) units</td>
</tr>
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<td>Serum alanine aminotransferase (SAIT)</td>
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<td>Serum aspartate aminotransferase (SAsT)</td>
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<td>Serum leucine aminopeptidase</td>
<td>Sigma Technical Bulletin 251 Goldberg and Rutenberg (GR) units</td>
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<tr>
<td>Sodium (Na⁺)</td>
<td>Flame photometer (I.L.)</td>
<td>mEq/l</td>
</tr>
<tr>
<td>Potassium (K⁺)</td>
<td>Flame photometer (I.L.)</td>
<td>mEq/l</td>
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**Urinalysis**

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<td>Specific gravity</td>
<td>Pycnometer or refractometer against protein standards.</td>
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<tr>
<td>pH</td>
<td>pH meter</td>
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<tr>
<td>Protein</td>
<td>Sulphosalicylic acid test.</td>
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<td>Investigation</td>
<td>Method</td>
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<tr>
<td>-----------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>Reducing substances</td>
<td>Clinitest (Ames Co.)</td>
</tr>
<tr>
<td>Glucose</td>
<td>Clinistix (Ames Co.)</td>
</tr>
<tr>
<td>Ketones</td>
<td>Acetest (Ames Co.)</td>
</tr>
<tr>
<td>Bile pigments</td>
<td>Ictotest (Ames Co.)</td>
</tr>
<tr>
<td>Urobilinogen</td>
<td>Urobilistix</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>Haemastix (Ames Co.)</td>
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<tr>
<td>Microscopically for sediment</td>
<td>Centrifuged at 3000 rpm for 10 minutes</td>
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<table>
<thead>
<tr>
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<tr>
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<tr>
<td>Oil Red O</td>
<td>&quot;Histological Demonstration Techniques&quot; by H.C. Cook Butterworth and Co. Ltd 1974 p. 108 (Gomori's variant)</td>
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<table>
<thead>
<tr>
<th>Bile Assay</th>
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<td>Cholesterol concentration</td>
<td>Technicon Methodology AA 11–24.</td>
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Cholesterol saturation index (CSI) \[ \text{CSI} = \frac{\text{bile salt/cholesterol found}}{\text{bile salt/cholesterol at saturation}} \times 100 \]
### Expected Values for Dogs

<table>
<thead>
<tr>
<th>INVESTIGATION</th>
<th>RANGE</th>
<th>UNITS</th>
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<tbody>
<tr>
<td><strong>Haematology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESR</td>
<td>0 – 5</td>
<td>mm/hour.</td>
</tr>
<tr>
<td>PCV</td>
<td>35 – 60</td>
<td>%</td>
</tr>
<tr>
<td>Hb</td>
<td>10 – 19</td>
<td>g%</td>
</tr>
<tr>
<td>RBC</td>
<td>4 – 7.5</td>
<td>mill/cmm</td>
</tr>
<tr>
<td>WBC</td>
<td>6.0 – 25.0</td>
<td>1000/cmm</td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>&lt; 2.0</td>
<td>%</td>
</tr>
<tr>
<td>Platelets</td>
<td>150 – 450</td>
<td>1000/cmm</td>
</tr>
<tr>
<td>Prothrombin Index</td>
<td>85 – 110</td>
<td>%</td>
</tr>
<tr>
<td>MCV</td>
<td>70 – 100</td>
<td>cu.µ</td>
</tr>
<tr>
<td>MCHC</td>
<td>27 – 35</td>
<td>%</td>
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<td><strong>Biochemistry</strong></td>
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<tr>
<td>Urea</td>
<td>13 – 50</td>
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<tr>
<td>Glucose</td>
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</tr>
<tr>
<td>Total Protein</td>
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<td>g%</td>
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<tr>
<td>SAP</td>
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<td>KA units</td>
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<td>mU/ml</td>
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<tr>
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<tr>
<td>pH</td>
<td>5.5 – 7.5</td>
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</tr>
<tr>
<td>SG</td>
<td>&gt; 1035</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>&gt; 100</td>
<td>mg%</td>
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## Expected Values for Rhesus Monkeys

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<th>INVESTIGATION</th>
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<tr>
<td><strong>Haematology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCV</td>
<td>39 - 51</td>
<td>%</td>
</tr>
<tr>
<td>Hb</td>
<td>11.1 - 15.8</td>
<td>g%</td>
</tr>
<tr>
<td>RBC</td>
<td>4.5 - 6.6</td>
<td>mill/cmm</td>
</tr>
<tr>
<td>MCHC</td>
<td>25 - 30</td>
<td>%</td>
</tr>
<tr>
<td>MCV</td>
<td>69 - 93</td>
<td>cubic microns</td>
</tr>
<tr>
<td>WBC</td>
<td>3.4 - 22.2</td>
<td>1000/cmm</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0 - 11.4</td>
<td>1000/cmm</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.6 - 13.4</td>
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</tr>
<tr>
<td>Platelet count</td>
<td>190 - 510</td>
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<tr>
<td>Prothrombin index</td>
<td>92 - 107</td>
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<tr>
<td><strong>Biochemistry</strong></td>
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<td></td>
</tr>
<tr>
<td>Urea</td>
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<td>mg%</td>
</tr>
<tr>
<td>Glucose</td>
<td>64 - 114</td>
<td>mg%</td>
</tr>
<tr>
<td>Total protein</td>
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<td>g%</td>
</tr>
<tr>
<td>Albumin</td>
<td>3.5 - 5.4</td>
<td>g%</td>
</tr>
<tr>
<td>α1 globulin</td>
<td>0.1 - 0.5</td>
<td>g%</td>
</tr>
<tr>
<td>α2 globulin</td>
<td>0.2 - 1.0</td>
<td>g%</td>
</tr>
<tr>
<td>β globulin</td>
<td>0.9 - 2.1</td>
<td>g%</td>
</tr>
<tr>
<td>γ globulin</td>
<td>0 - 1.6</td>
<td>g%</td>
</tr>
<tr>
<td>SAP</td>
<td>33 - 144</td>
<td>KA Units</td>
</tr>
<tr>
<td>SAIT</td>
<td>0 - 58</td>
<td>mU/ml</td>
</tr>
<tr>
<td>SAsT</td>
<td>9 - 56</td>
<td>mU/ml</td>
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<tr>
<td>LAP</td>
<td>93 - 343</td>
<td>GR units</td>
</tr>
<tr>
<td>Sodium</td>
<td>136 - 159</td>
<td>mEq/l</td>
</tr>
<tr>
<td>Potassium</td>
<td>3.4 - 5.4</td>
<td>mEq/l</td>
</tr>
<tr>
<td><strong>Urinalysis</strong></td>
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<td></td>
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<tr>
<td>pH</td>
<td>6.7 - 9.1</td>
<td>–</td>
</tr>
<tr>
<td>Volume</td>
<td>0 - 69</td>
<td>ml</td>
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THE EFFECT OF ADMINISTRATION OF ETHINYL ESTRADIOL ON HEPATOCYTE MORPHOLOGY IN THE RhesUS MONKEY

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SUMMARY

Administration of ethinyl estradiol to the female rhesus monkey at a dosage level of 100 µg/kg/day for 26 weeks resulted in elevation of serum alanine aminotransferase (SALT) and LAP values. Light microscopy showed no morphological change, but by electron microscopy hepatocytes could be identified containing needle-shaped electronlucent areas in two out of four treated animals only, one of which had periportal hepatocytes with intracisternal sequestration of the endoplasmic reticulum.

INTRODUCTION

During the course of a preliminary study on the effect of ethinyl estradiol on the rhesus monkey, Wadsworth and Heywood [7] commented on a rise in liver enzyme values without histological change in the liver. It was decided to make further investigations with particular reference to hepatocyte morphology by electron microscopy.

MATERIALS AND METHODS

Ethinyl estradiol was administered daily to four young female rhesus monkeys at the dose level of 100 µg/kg. Two monkeys acted as undosed controls. Clinical signs, body weight and food consumption were monitored throughout the course of the study. Haematological investigations and biochemical tests, comprising estimation of plasma urea and glucose, serum protein (total and differential), serum alkaline phosphatase, serum alanine

Abbreviation: SALT, serum alanine aminotransferase.
aminotransferase, serum leucine aminopeptidase, serum bilirubin, sodium and potassium, were carried out before the start of dosing and after 6, 12 and 24 weeks' dosing. On completion of the study, the animals were killed and immediately afterwards a full macroscopic examination was made, during which the major organs were weighed and tissues preserved in 10% buffered formalin for subsequent sectioning and histological examination. In addition, liver samples were fixed in 4% glutaraldehyde buffered with 0.05 M cacodylate to pH 7.3 at 4°C. Three 1 mm³ blocks of tissue from each animal were post-fixed in 1% osmium tetroxide buffered with 0.5 M cacodylate to pH 7.3, dehydrated in alcohol and embedded in epoxy resin. 1 μm survey sections were cut and stained with 1% toluidine blue for examination with the light microscope. Silver/gold ultra-thin sections of representative areas were mounted on copper grids and subsequently stained with lead citrate and uranyl acetate. These sections were examined using Philips EM 300 operating at 60 kV. Selected areas were photographed on Kodak Fine Grain Positive 35 mm film and enlarged as necessary.

RESULTS

Development of the sex skin, together with vulval enlargement was seen in the four treated animals from the second week of dosing. The folding of the sex skin was progressive and towards the end of the dosing period extended over the lumbar region. There was suppression of menstruation in the dosed animals, whereas in the undosed controls menstruation, although not following a regular pattern, was obvious throughout the experimental period. Body weight and food consumption were not affected. No significant haematological changes were detected. There was an increase in SALT and LAP values (Table 1).

Post-mortem examination after 26 weeks' dosing showed the ovaries of the treated monkeys to be small and inactive, whereas in the two control animals the ovaries were enlarged and either mature graafian follicles or corpora lutea were evident. The treated animals showed bilateral enlargement and congestion of the fimbrium of the fallopian tubes. However, the degree of enlargement was no greater than that encountered in the untreated control monkeys with normal menstrual cycles. The results of the organ weight analysis showed the absolute and relative weights to be within our accepted normal range.

At histological examination, the morphological appearance of the livers of treated animals was essentially similar to that in the two control animals. In the ovaries of the treated animals, only small secondary follicles were apparent, whereas both corpora lutea and mature graafian follicles were evident in the control monkeys. The fimbrial epithelium of the fallopian tubes in the treated monkeys showed bilateral hyperplasia. In the uteri of the treated animals, slight dilatation of endometrial glands could sometimes be seen. In the cervix, sporadic small areas of squamous epithelium were observed,
TABLE I

GROUP MEAN SALT VALUES (mU/ml) AND LAP VALUES (G.R. UNITS)

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameter</th>
<th>Dose week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0  6  12  24</td>
</tr>
<tr>
<td>Control</td>
<td>SALT</td>
<td>26 50 30 24</td>
</tr>
<tr>
<td></td>
<td>LAP</td>
<td>119 182 238 179</td>
</tr>
<tr>
<td>Ethinyl estradiol</td>
<td>SALT</td>
<td>30 110 100 74</td>
</tr>
<tr>
<td>100 µg/kg/day</td>
<td>LAP</td>
<td>288 524 551 500</td>
</tr>
</tbody>
</table>

whereas in the control animals only columnar epithelium was noted. The vaginal epithelium of treated animals was several cells thick and heavily cornified.

By electron microscopy, needle-shaped electronlucent areas, which were sometimes bounded by a membrane were seen in a few hepatocytes (Fig. 1). This change was apparent in two of the treated animals. In addition, in one of these affected animals, some hepatocytes (mainly periportal) showed intracisternal sequestration of the rough endoplasmic reticulum (see Fig. 2). No morphological abnormalities were detected in the livers of the other two treated animals.

DISCUSSION

The results of this study confirm the previous observation by Wadsworth and Heywood [7] that ethinyl estradiol causes an increase in SALT and LAP values. Elevation of these enzyme levels had, in the past, been taken as an indication of liver dysfunction. Pathological changes could not be seen by light microscopy in the livers of treated animals, although ultrastructural abnormalities were detected by means of transmission electron microscopy in two of the four treated animals. The changes found in the hepatocytes were not considered to be sufficient to account for the increased SALT and LAP values. The needle-shaped electronlucent areas seen in the cytoplasm of the few hepatocytes in these two animals could possibly represent a cleft remaining after a crystal had been lysed out during processing. Crystalline mitochondrial inclusions have been recorded in women on steroid therapy [4]. While no definite evidence exists that crystals were present in this study, or that they were mitochondrial in origin, these cytoplasmic clefts were not apparent in the control animals and were considered to be related to the administration of the ethinyl estradiol. Ghidoni [2] also noted these cleft-like spaces in irradiated monkey hepatocytes, but could not determine their origin, or relate them to loss of material during processing.
Intracisternal sequestration was noted in one of the four treated animals. This term is used to describe a pattern of organisation of the rough endoplasmic reticulum where vesicular profiles of the latter are seen lying within dilated cisternae. It is thought that the primary stage of this process is the formation of papillary processes from the wall of a dilated cistern. Later, such processes may be pinched off to form vesicular structures which come to lie free within the cisternae. The formation of papillary projections from ductal and surface epithelia has been linked with proliferative processes and it could be that this phenomenon, noted in our study, is a similar process and that it reflects a type of hypertrophy of the rough endoplasmic reticulum. This probably represents an increase in metabolic activity caused by the administration of ethinyl estradiol.
The other changes observed involved the sex skin, ovaries, fallopian tubes, uterus, cervix and vagina. In the uterus, cystic dilatation of the endometrial glands has been recorded by Geschickter and Hartman [1] and McClure and Graham [5]. Overholser and Allen [6] first recorded squamous metaplasia in the cervix following estrogen administration. Studies by Graham [3] have shown that this spread of stratified squamous epithelium throughout the endocervical canal is the result of stimulation of small foci of squamous cells occurring naturally at this site, putting paid to the notion that the squamous epithelium arises by metaplasia of columnar cells.

Fig. 2. Hepatocyte showing intracisternal sequestration of granular endoplasmic reticulum. \( \times 22\,000 \).
REFERENCES

THE INCIDENCE OF MAMMARY TUMOURS IN A GROUP OF 40
8-YEAR-OLD FEMALE BEAGLE DOGS USED IN A TOXICITY STUDY

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SUMMARY

The incidence of mammary neoplasia in a group of 40 nulliparous 8-year-old female Beagle dogs is described. The age-specific incidence rates of mammary neoplasia in laboratory Beagle dogs shows considerable variation: malignant mammary tumours are very uncommon in 8-year-old Beagles; clinically palpable benign mammary tumours are present in only a small proportion of these dogs, but more are seen on histological examination; mammary gland dysplasias, although normally detectable only by microscopy, are prevalent in 8-year-old dogs.

INTRODUCTION

The value of the Beagle dog as a suitable animal model for the long-term toxicological evaluation of oral contraceptives has been questioned [4,9]. Prospective studies of this type were introduced in order to determine the possible effects in women of the long-term use of hormonal contraceptives, especially with regard to their carcinogenic potential [2]. Studies of 7 years' duration are mandatory in the United States of America, Sweden and Australia, while authorities in other countries, notably the United Kingdom, show great interest in whether such tests have been conducted. The spontaneous incidence of mammary tumours and dysplasias in the Beagle dog must be taken into account when assessing the results of these investigations, and publication of such data has been encouraged [10]. The purpose of this paper, therefore, is to record the spontaneous incidence of mammary tumours described by standard toxicological techniques using the International Histological Classification of Tumours of Domestic Animals [7], in a group of 40 8-year-old nulliparous Beagle dogs.
MATERIALS AND METHODS

40 pure-bred Beagle bitches (Appleton strain) were housed individually from the age of 4—5 months under standard laboratory conditions at the Huntingdon Research Centre. They were given a standard amount of a complete dry diet, and water was available ad libitum. The dogs were used in a carcinogenicity experiment of 7.5 years' duration, details of which have been published [8]. Table I presents the dosage groups, together with the findings. As the treatment did not increase the incidence of mammary tumours, the data from both treated and control animals have been combined. Clinical examinations were made every 6 months, and the animals were killed by intravenous injection of pentobarbitone: a detailed post-mortem examination was immediately performed on each dog. The individual mammary glands on the left and right sides were dissected free as a unit with the attached covering skin. Representative sections of tissue were taken from all major organs. These tissues, and any abnormalities detected on macroscopic examination, were fixed in 10% buffered formalin for 4—8 weeks. After sections had been taken through the teat from each individual mammary gland, the tissues were processed in the usual way for microscopic examination by embedding in paraffin wax (M.P. 56°C), sectioning at 5 μm and staining with haematoxylin and eosin.

<table>
<thead>
<tr>
<th>Dosage group</th>
<th>Animal no.</th>
<th>Macroscopic neoplasm</th>
<th>Histological classification</th>
<th>Total number female dogs per group</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 mg/kg/day chloroform in toothpaste</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>8</td>
</tr>
<tr>
<td>15 mg/kg/day chloroform in toothpaste</td>
<td>928</td>
<td>+</td>
<td>Pericanalicular fibroadenoma</td>
<td></td>
</tr>
<tr>
<td></td>
<td>928</td>
<td>—</td>
<td>Intracanalicular fibroadenoma 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>930</td>
<td>+</td>
<td>Papillary cystadenoma</td>
<td></td>
</tr>
<tr>
<td>Vehicle control</td>
<td>950</td>
<td>—</td>
<td>Tubular adenoma</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>950</td>
<td>—</td>
<td>Complex adenoma</td>
<td></td>
</tr>
<tr>
<td>Alternative non-chloroform toothpaste</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>7</td>
</tr>
<tr>
<td>Undosed control</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>5</td>
</tr>
</tbody>
</table>
RESULTS

The average age of the Beagle dogs was 8 years and 2 weeks (range 8 years to 8 years and 7 weeks). A total of 417 mammary glands was examined, an average of 10.4 per dog. Four palpable abnormalities of the mammary gland were found on clinical examination. After macroscopic and histological evaluation two benign mammary tumours, a pericanalicular fibroadenoma and a papillary cystadenoma (Table I), a papillary complex cyst (Table II) and a fibroma were identified. The pericanalicular fibroadenoma (dog 928) and the papillary complex cyst were both detected when the dogs were 3 years of age. Over the subsequent 5-year observation period neither grew significantly. The papillary cystadenoma (dog 930) was detected when the dog was 7.5 years of age.

Histological examination of each mammary gland showed, in addition to the palpable tumours previously described, three small benign mammary tumours (Table I). These consisted of an intracanalicular fibroadenoma (dog 928), a complex adenoma (dog 950) and a tubular adenoma (dog 950). Five benign mammary tumours were therefore found in three of the 40 dogs, an incidence of 7.5%.

Dysplasias of the mammary gland were more frequent (Table II). In addition to the papillary complex cyst previously described, areas of nodular hyperplasia and foci of regular proliferation of epithelial cells in ducts or acini (epitheliosis) were found on histological examination. Eleven areas of nodular hyperplasia were found in nine dogs altogether, and foci of epitheliosis were seen in 35 mammary glands in 12 dogs in all.

<p>| TABLE II |
| INCIDENCE OF DYSPLASIAS OF THE MAMMARY GLAND |</p>
<table>
<thead>
<tr>
<th>Dysplasia</th>
<th>Number of mammary glands affected</th>
<th>Number of dogs affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papillary complex cyst&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Areas of nodular hyperplasia</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Foci of regular proliferation of epithelial cells in ducts or acini</td>
<td>35</td>
<td>12</td>
</tr>
</tbody>
</table>

<sup>a</sup>Detected at clinical examination.

DISCUSSION

There are many reports of the incidence of mammary tumours in dogs other than those maintained as laboratory animals, and these studies have
recently been reviewed [6,10]. The difficulties of deriving and comparing such incidence rates [6] and the difficulties of extrapolating such information to the laboratory Beagle dog [11] have been outlined.

There are few reports of the incidence of spontaneous mammary neoplasia in laboratory dogs. Age-specific incidence rates for Beagle dogs have been derived from life-span studies [1,11], toxicological studies [5,10] and whole-mount preparations of the mammary gland [3,12]. Anderson [1] has stated that, in a life-span study of 354 female Beagles, clinical signs of mammary tumours had occurred in approximately 50% of these dogs by 6—8 years of age. Taylor et al. [11] have presented a more detailed account of the incidence of mammary neoplasia in the University of Utah Beagle dog colony. They reported that the incidence rate remained low through 7 years of age, and only one tumour was observed below the 5-year age class, a benign adenoma that appeared at 1.6 years of age. A sharp increase in the incidence rate occurred at approximately 8 years of age and remained high thereafter. They also noted that the first cancer occurred at 7 years of age. Scientists at Wyeth International [10] have examined tissues from 50 normal female Beagle dogs, used in a control study of 7 years' duration. A total of 36 mammary tumours was found in 22 animals, 8 other animals showing areas of adenomatous hyperplasia within mammary tissue. All the tumours were benign, small and showed neither local invasion nor distant metastasis. Finkel and Berliner [5] stated that, in studies conducted by the Food and Drug Administration and manufacturers of oral contraceptives, the average spontaneous incidence of mammary tumours in control animals was about 12% between 5 and 6 years. They also noted that, during such studies, nodules 1—2 mm in diameter were palpable and were therefore included in their statistics. Cameron and Faulkin [3], using a whole-mount technique for mammary glands described hyperplastic and inflammatory nodules, many of which were seen only under the microscope, in the mammary glands of untreated Beagle dogs which were 7.6—8.5 years old at post-mortem examination. These nodules were studied histologically and a total of 654 atypical glandular nodules was identified, 94 of which were considered to be neoplastic (14% of the total lesions). Further classification of the neoplasia was not given. Warner [12], also using whole-mount preparations, examined 39 Beagle bitches aged 6 months to 4 years. Dysplastic lesions occurred at 2—3 years of age at which time more than 50% of the females had dysplasias. At 3—4 years of age, a total of 1795 dysplasias was found in 12 bitches. These lesions were 1—4 mm in diameter and not clinically palpable.

There would appear to be considerable variation in the age-specific incidence rate of mammary neoplasia in the Beagle dog. Some of these discrepancies are undoubtedly attributable to the widely different techniques employed in deriving such data and also to the fact that an International Histological Classification of Tumours of Domestic Animals has only recently been available. However, from these observations in this present study, it may be concluded that (a) malignant mammary tumours are uncommon in Beagle
dogs aged 8 years; (b) clinically palpable benign mammary tumours are present in only a small proportion of 8-year-old dogs, but this figure may be markedly increased by complete histological evaluation; (c) dysplasias of the mammary gland, the majority of which are detectable only by microscopy, are very frequent in 8-year-old dogs.

REFERENCES

TREATMENT OF POST PARTUM LACTATING RHESUS MONKEYS WITH ETHINYL OESTRADIOL

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SUMMARY

The administration of ethinyl oestradiol to lactating rhesus monkeys had little effect on lactation. Physiological change characterised by stimulation of the sex skin and development of the mammary gland was induced in the infants suckled by the dosed mothers, although ethinyl oestradiol was not detected in the milk or serum of any of the infants.

INTRODUCTION

In a recent review of fertility-regulating methods, Kessler and Standley [3] raised the question of whether hormonal contraceptives should be given to lactating women. Contraceptive steroids might affect the amount and composition of the milk and might be transferred through the milk to the infant. Subhuman primates have not been used to investigate this problem, the only reference being that of Baghat et al. [1], who investigated the effects of 19-nortestosterone in lactating rhesus monkeys. This paper reports a preliminary study on the effects of ethinyl oestradiol in post partum lactating rhesus monkeys.

MATERIALS AND METHODS

Two post partum rhesus monkeys were allocated to receive 100 μg/kg/day of ethinyl oestradiol by gavage from day 5 post partum for 12 weeks. A further pair of post partum animals was undosed and acted as controls. The mothers were fed a complete dry diet, supplemented by fresh fruit and wholemeal bread. The body weights of the mothers and infants were recorded at weekly intervals.

Blood and milk samples were collected from the mothers, and blood samples were collected from the infants, at weekly intervals. To facilitate the collection of milk, 5 i.u. oxytocin was given i.v. immediately before collection. Abbreviations: LAP, leucine aminopeptidase; SAIT, serum alanine transaminase.
The blood samples collected after 1, 3, 5, 7, 9 and 11 weeks were assayed for SAIT and LAP. Ethinyl oestradiol in the milk and blood samples collected after 4, 8 and 12 weeks' dosing was estimated by radioimmunoassay, using the method described by Warren and Fotherby [5].

On completion of the dosing period the infant monkeys were killed and immediately afterwards subjected to full macroscopic examination, during which the brain, pituitary, heart, lungs, liver, spleen, pancreas, thymus, prostate, uterus, kidneys, thyroids, adrenals, gonads and seminal vesicles were removed and weighed. Small portions of these tissues, together with aorta, trachea, lymph nodes, gall bladder, urinary bladder, salivary gland, tongue, oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, skin, skeletal muscle, sciatic nerve, eye, vagina and vulva, together with any macroscopic abnormality, were placed in 10% buffered formalin fixative. The tissues were routinely processed in paraffin wax, sectioned at 5 \( \mu \text{m} \) and stained with haematoxylin and eosin. Additional pieces of liver and kidney were placed in formol calcium, sectioned at 12 \( \mu \text{m} \) and stained for fat with Oil Red O.

The mothers were maintained for a further 12 weeks' observation period.

RESULTS

The male infant suckled by the female dosed with ethinyl oestradiol showed slight reddening of the perineum from week 3 of dosing. By week 9 there was considerable reddening and oedema and mammary gland enlargement was occurring. This persisted until the animal was killed at week 12. The female infant suckled by the mother dosed with ethinyl oestradiol showed slight reddening of the perineum during weeks 5, 7 and 8. The perineum and the mammary glands of the untreated control infants were unaffected.

The body weights of the infant monkeys and their mothers are presented graphically (Figs. 1A and B). The small number of animals used precludes definite conclusions, but there is some indication that the infants suckled by the dams receiving 100 \( \mu \text{g} \) ethinyl oestradiol/day showed slower growth rates after 4 weeks' dosing than did the control infants. The administration of ethinyl oestradiol to the mothers caused a loss of body weight.

The results of the SAIT and LAP values show that the administration of ethinyl oestradiol to lactating rhesus monkeys failed to induce any changes in these liver enzymes (Fig. 2). Similarly, the liver enzymes of the infants were unaffected.

In the conjugated fragments of plasma, ethinyl oestradiol was detected after 4, 8 and 12 weeks' dosing in one adult female monkey only. The values were 400, 200 and 130 pg/ml respectively. Ethinyl oestradiol was not detected in any of the milk samples, although the lower level of sensitivity in this instance was about 200 pg/ml.

At post-mortem examination the mammary glands of the male infant suckled by the female dosed with ethinyl oestradiol were prominent and the teats pendulous. In this animal the sex skin extended from the perineal region
Fig. 1. Body weights of infant (A) and adult (B) monkeys. —, Control; ---, 100 $\mu$g/kg/day ethinyl oestradiol.
mediaposteriorly to the knee, anteriodorsally to the sacral regions and backwards along the tail. The sex skin was folded, reddened and oedematous. Histopathological examination of the mammary glands of this infant revealed slight ductal proliferation. Neither macroscopic nor morphological abnormalities were encountered in any of the other infants. Organ weight analyses of the treated infants were comparable to those of the untreated control infants.

During the experimental period post partum menses were not recorded in any of the mothers. Following weaning and the removal of the infants, one of the control animals returned to cycle after 6 weeks, but neither of the dosed females returned to menses following withdrawal of ethinyl oestradiol for the 12-week observation period.

DISCUSSION

The results of this study indicate that physiological change may occasionally be induced in the infants of mothers dosed with ethinyl oestradiol, indicating that the oestrogen is secreted in the milk. However, the levels secreted were below the limits of detection of our assay methods.

The male rhesus monkey is born showing swelling and turgescence of the scrotum [7]. This naturally occurring physiological effect appears to be caused by a marked increase in circulating levels of oestradiol and oestrone during the last days of pregnancy in the rhesus monkey [2,6].
In the infant in which marked physiological change was induced there was no evidence of the proliferative changes normally associated with oestrogen administration.

The experiment shows that once lactation is established in the rhesus monkey the administration of ethinyl oestradiol at high dosage levels does not suppress lactation. There was slight body weight loss in the mothers and marginal suppression of body weight gain in the suckling infants of the two treated dams.

The administration of ethinyl oestradiol to the non-lactating rhesus monkey has been associated with elevation of SAIT and LAP values [4]. However, no changes in these liver enzymes were recorded over the experimental period.

The results indicate that in the rhesus monkey the administration of daily doses of ethinyl oestradiol had little effect on lactating rhesus monkeys and their suckling infants. The extrapolation of experimental data to man is notoriously difficult, but this subhuman primate model suggests that the transfer of ethinyl oestradiol at the low dosage levels given in “the pill” does not constitute a major health hazard.

ACKNOWLEDGEMENT

The hormonal assays were carried out by Dr. K. Fotherby at the Postgraduate Medical School, Hammersmith Hospital, London, England.

REFERENCES

THE EFFECT OF PRENATAL EXPOSURE OF Rhesus MONKEYS (MACACA MULATTA) TO DIETHYLBESTROL

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SUMMARY

Diethylstilboestrol (DES) was administered parenterally to a group of four pregnant rhesus monkeys at the dose level of 1 mg/kg/day from day 45 of gestation until parturition. Two monkeys aborted, but two infant monkeys, one male and one female, were born. The infants were killed after an 84-week observation period. At post-mortem examination the prostate and seminal vesicles of the male infant were found to be small. The reproductive tract of the female infant was normal.

INTRODUCTION

The experimental administration of androgens to pregnant rhesus monkeys at an appropriate stage of gestation — that is, from approximately the 40th to 90th days of gestation — has been shown to transform female foetuses into female pseudohermaphrodites [10, 12—14]. Apart from the report from Morris and van Wagenen [5], who administered oestradiol and DES to pregnant rhesus monkeys, there appears to be no other published account of the administration of oestrogens to pregnant simian primates.

MATERIALS AND METHODS

Four wild-caught female rhesus monkeys (Macaca mulatta) were housed separately in metal cages. Clinical observations were made daily to determine the stage of the menstrual cycle of each animal. During the 10th, 11th and 12th days of the menstrual cycle (first day of menses being day 0 of the menstrual cycle and the 11th day of the menstrual cycle being day 0 of gestation), each female was housed with a mature male rhesus monkey of proved fertility to allow mating to take place [11]. Pregnancy was confirmed by

Abbreviations: AsT, aspartate aminotransferase; DES, diethylstilboestrol.
rectal palpation of the uterus. Food intake was monitored daily and body weight recorded at weekly intervals. DES was administered daily by intramuscular injection at a dose level of 1 mg/kg from the 45th day of gestation until parturition, which in the rhesus monkey occurs at approximately the 168th day of gestation.

The infant rhesus monkeys were then maintained for a period of 84 weeks. Clinical observations were made daily and body weight recorded every 4 weeks. When the infants weighed 1 kg (after 20 weeks) they were weaned. Subsequently, food consumption was monitored daily and body weight recorded at weekly intervals. Routine haematological and biochemical laboratory investigations were performed just before the animals were killed.

At the end of the 84-week observation period, the infant rhesus monkeys were killed and immediately afterwards a full macroscopic post-mortem examination was performed, during which particular attention was paid to the reproductive tract. Histological examination was carried out on the main organs and tissues.

RESULTS

Maternal data

Beginning at the 45th day of gestation, DES was injected intramuscularly at the dosage rate of 1 mg/kg until termination. Two of the pregnant females, 459 and 509, aborted after 31 and 87 days of treatment, respectively. The pregnant female 1779 gave birth to a male infant on the 168th day of gestation, after receiving a total of 655 mg of DES. The pregnant female 1859 gave birth to a female infant on the 169th day of gestation, after receiving a total of 642 mg of DES. The average gestation period of untreated control rhesus monkeys maintained at the Huntingdon Research Centre is 166.8 days with a range of 156–177 days [3]. Food consumption and body weight of monkeys 1779 and 1859 were not adversely affected.

Infant data

Both infants weighed 425 g at birth. The mean birth weight of rhesus monkeys maintained at the Huntingdon Research Centre is 440 ± 50 g [3]. The infant monkeys were maintained for an observation period of 84 weeks. At birth, both infant monkeys showed slight folding and reddening of the sex skin. These changes persisted for 12 weeks in the male infant of monkey 1779 and for 10 weeks in the female infant of monkey 1859. Both infants were weaned at approx. 20 weeks of age when they weighed 1 kg. The body weight gain during the first year, while fluctuating, was similar to that normally encountered in untreated control animals at the Huntingdon Research Centre.

With the exception of a slightly lower total serum protein level in the infant of monkey 1779 and a slightly raised serum AsT level in the infant of monkey 1859, the results of the preterminal laboratory investigations were within the normal range.
At post-mortem examination the male infant of monkey 177* weighed 1.75 kg and the female infant of monkey 185* weighed 2.20 kg. With the exception of the testes of the male infant, which were found in the inguinal region, the external genitalia were normal in appearance. Internally, both the prostate and seminal vesicles were found to be poorly developed. The right and left seminal vesicles weighed only 0.148 g and 0.188 g respectively. The prostate was located by examination of the prostatic urethra for the openings of the prostatic ducts and weighed 0.128 g. The combined prostate and seminal vesicle weight for animals weighing 1.71 kg ± 121 g (mean of 6) is normally 0.90 ± 0.14 g. In the female infant, the only abnormalities seen on macroscopic examination were prominent folds in the vaginal mucosa. The remaining relative and absolute organ weights were considered to be within the normal range for animals in this age group.

At histological examination the testes of the male infant were found to be immature, with spermatogenesis proceeding to the spermatocyte stage only. No morphological abnormalities were found in the prostate and seminal vesicles, the appearance of which was essentially similar to that encountered in immature male rhesus monkeys of this age. The reproductive tract of the female infant was normal. Only small secondary follicles were evident in the ovaries and shallow endometrial glands were noted in the uterus. The cervix and vagina were lined by well developed columnar and stratified squamous epithelia respectively. No other morphological changes were seen that could be attributable to treatment with DES.

DISCUSSION

The results indicate that prenatal exposure to DES partially inhibits the normal development of the male accessory sex glands of rhesus monkeys. Further evaluation with larger groups of monkeys is necessary to confirm these preliminary investigations. Morris and van Wagenen [5] reported that no foetal abnormalities were observed when DES was administered to 14 pregnant macaques. The dosage rate and dosing period varied considerably, precluding valid comparison. In addition, a postnatal recovery period was not allowed. Abnormalities of the male reproductive tract have been reported in rats [2], hamsters [7] and mice [4, 6] following prenatal administration of DES.

Maternal plasma levels of steroidal oestrogens increase progressively during pregnancy, particularly during the last 7 days [1] and are thought to be placental in origin. The steroid concentration of oestradiol in maternal plasma is higher than in the amniotic fluid, although this relationship is reversed for oestrone, especially during the 20 days preceding delivery. Such changes are undoubtedly responsible for the swelling and turgescence of the scrotum seen at birth in male rhesus monkeys [15]. In this experiment, DES had a marked effect on the sex skin of both infants, suggesting that the foetuses were exposed to high concentrations of DES.

Our results have not ruled out the possibility that DES in the rhesus monkey is embryotoxic, causing early embryonic death and abortion.
Histological examination of the vaginal and cervical mucosa in the female infant was unrewarding. In women exposed to DES in utero, non-neoplastic changes included cervical erosion and vaginal adenosis, the incidence of which is about 30% in the whole population exposed to DES [8, 9]. We do not yet know whether similar non-neoplastic changes could be induced in female rhesus monkeys.

REFERENCES