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A TOXICOLOGICAL STUDY OF SOME CHEMICAL ACTIONS

ON THE TESTES OF LABORATORY RATS AND BEAGLE DOGS

submitted by

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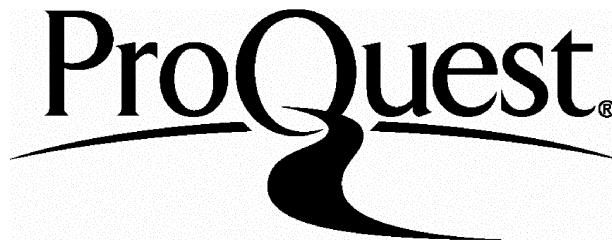
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To my parents

"... spiritus letales aut scrobibus emissi aut ipso loci

situ, mortiferi alibi volucribus tantum, ut Soracte vicino

urbi tractu, alibi praeter hominem ceteris, nonnumquam et

homini, ut in Sinuessano agro et Puteolano..."

Pliny (A.D.77)

CONTENTS

The testicular toxicity of several chemicals was investigated using laboratory rats and Beagle dogs. Testicular measurements, assays of circulating pituitary-testicular hormones, semen examinations and quantitative histometric studies of spermatogenesis were used, when appropriate, to complement the results of organ weight analyses and conventional histological examinations.

Variations in testicular function may be encountered as a result of differences due to species, strain, age, diurnal and seasonal rhythms, methods of sampling and spontaneous pathological changes in the male reproductive organs. These factors should be considered when assessing the importance of testicular changes found after administering large doses of chemicals to experimental animals.

Compounds were administered to rats and dogs which affected LH and prolactin concentrations as a result of pharmacological overdosage. Similar hormonal changes were detected for both species, but differing degrees of morphological change were encountered in the testes and secondary sex organs. The dog appeared to be more susceptible to effects on the hormonal control of spermatogenesis than was the rat. Hormonally mediated suppression of spermatogenesis was shown to be reversible within two spermatogenic cycles following withdrawal of the test compounds.

Toxicological assessment of some chemical actions on the seminiferous epithelium of rats and dogs was attempted. It was possible to obtain useful information for compounds having a primary antispermatogenic action, and the Beagle dog seems to be a suitable non-rodent species for the toxicological study of these compounds. The methods employed for these investigations were less useful for assessing the safety of domestic or agricultural chemicals, which affected the seminiferous epithelium when administered at excessive dosages only.

(i)

Almost 2000 years ago Pliny (77) observed that the toxic potential
of identical chemicals varied for different animal species. In
spite of this early observation,toxicologists have tended for
centuries almost exclusively to study the noxious effects of
xenobiotic substances. During the last few decades a change in
emphasis has begun and the modern toxicologist should aim to assess
the safety of chemicals in normal use. The demonstration of
toxicity following massive overdosage is probably of secondary
importance (Noel, 1970). Increasing awareness of the need for
consumer protection has prompted many governments to introduce
legislation intended to prevent hazardous exposure to chemicals.
A number of empirical animal toxicity studies must be performed,
normally in rodents and dogs or other suitable non-rodent species
(Noel, 1974). The experimental design and choice of animal species
for these studies is theoretically influenced by metabolic,
pharmacokinetic and pharmacological considerations (Burns, 1968;
Noel, 1970; Plaa, 1976). In practice these constraints are seldom
realised and, correspondingly, the predictive value of animal
toxicity data may be limited (Fletcher, 1978; Heywood, 1978).
Species difference in toxicology is not surprising when one
considers the diverse physical, behavioural, anatomical, physiolo-
gical, biochemical and genetic variations that occur in the animal
kingdom (Clarke, 1976). Although the need for consumer protection
is accepted, welfare groups also express concern about animal
experimentation. In an attempt to reconcile these points of view,
toxicologists should critically assess the scientific basis and
predictive limitations of their experiments before subjecting
animals to prolonged chemical overdosage.

The toxic effects of chemicals are often selective and tend to affect particular target organs or tissues, including the gonads. It is expected that 15% of married couples will be childless and that 30% of these cases are attributable to male reproductive failure (Walsh, 1979). The diagnosis, therapy and prognosis in cases of male infertility pose difficult problems for both human and veterinary clinicians (Yeates, 1978). Although the clinical manifestations of male reproductive disturbance are readily defined, it is possible to implicate a wide variety of aetiological factors. The role of chemicals in the pathogenesis of male gonadal disorders is largely undetermined. Conventional animal toxicity studies identify effects on male reproduction either as reduced pregnancy rates or increased intra-uterine losses during fertility or multigeneration studies (Jackson et al., 1961; Wright, 1978). Changes in the weight and histological appearance of the testes and secondary sex organs may become apparent during chronic toxicity tests (Heywood and James, 1978). Observations of this type merely draw attention to a response in the species tested and provide no information about the underlying disruption of physiological processes. Even if the mechanisms of toxic action are common to all species, extrapolation of toxicity data between species is possible only if the inter-species ability to adapt and respond to these common mechanisms is understood. Eliasson (1976) and also a recent editorial in the British Industrial Biological Research Association Bulletin (1977) have drawn attention to the fundamental lack of knowledge concerning chemically mediated testicular toxicity. Before considering how testicular function may be appropriately assessed during animal toxicity studies it, is pertinent to review existing knowledge of the ways in which chemicals may modify male gonadal activity. Most available data have been discovered during attempts to identify compounds suitable for use as male anti-fertility substances (Gomes, 1970; Jackson, 1969, 1972; de Kretser, 1976; Patanelli, 1975). More limited information concerning

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compounds with a non-andrological use is available from published

animal toxicity studies and retrospective epidemiological human

surveys (Lee and Dixon, 1978; Newburgh, 1975; Ribelin, 1963). The

most convenient classification is based on the primary disturbance

induced by the compound, but overlap does occur, and the categories

listed below are by no means mutually exclusive.

A. Effects on hypothalamic-pituitary-testicular relationships

The hormonal control of mammalian spermatogenesis depends on complex

interactions between hypothalamic releasing factors, pituitary

gonadotrophins and testicular androgens (Davidson and Bloch, 1969:

Greep, 1976; Kovacic, 1970; Steinberger, 1971). Prolactin also

appears to be important, although its precise actions have yet to be

defined (Bartke, 1976; Horrobin, 1977). The majority of investi-

gations have been performed with rodent species; more limited

information is available concerning man and domestic animals.

While a working hypothesis is available, it cannot be assumed that

species differences do not exist. A number of substances are known

to impair spermatogenesis by modifying the production or action of

male reproductive hormones. Table I lists some of the more well-

known examples.

i) Androgen antagonism

Anti-androgens prevent the uptake of androgens in target tissues

but do not affect androgen production. Androgens influence bone,

liver, kidney and skin physiology, as well as the gonads and second-

ary sex organs. Prolonged administration of anti-androgens may

therefore result in changes similar to those which occur following

castration (Neumann and Elger, 1966). Cyproterone acetate has been

extensively studied as a possible male fertility control agent

(Morse et al., 1973; Ott et al., 1972; Prasad et al., 1970).

TABLE I

Chemicals modifying hypothalamic-pituitary-testicular hormones

Mode of action	Generic class	Cited examples	Author(s)
Androgen antagonism	Synthetic steroids	Chlormadinone acetate	Gomes (1970)
		Cyproterone acetate	Neumann & Elger (1966)
		17α-Methyl-β-nortestosterone	Cummins & Orgebin-Crist (1974)
Gonadotrophin suppression	Analgesics	Heroin	Cicero et al. (1975a)
		Indomethacin	Saksena et al. (1975)
		Methadone	Thomas & Dombrosky (1975)
		Morphine	{ Cicero et al. (1977a,b) Muraki et al. (1978)
	Androgens	Danazol	Sherrins et al. (1971)
		Methyltestosterone	Briggs & Briggs (1974)
		Testosterone	Heller et al. (1950)
	Cannabinoids	Cannabis extract	Dixit et al. (1977)
		Δ^9-Tetrahydrocannabinol	Marks (1973)
	Histamine antagonists	Cimetidine	Van Thiel et al. (1979)
	Hydrazines	Methallibure	{ Hemsworth et al. (1968) Call & Barker (1967)
	'Inhibin'	Testis extract	Keogh et al. (1976) Reichert & Abou-Issa (1977)

TABLE I (continued)

Chemicals modifying hypothalamic-pituitary-testicular hormones

Mode of action	Generic class	Cited examples	Author(s)
Gonadotrophin suppression (continued)	Oestrogens	Clomiphene citrate	Kalra & Prasad (1967)
		Diethylstilboestrol	Noble (1938)
		Ethinyl oestradiol	McGinty & Djerassi (1958)
		17β-Oestradiol	Patanelli (1975)
	Progestogens	Medroxyprogesterone acetate	Heller et al. (1959)
		Megestrol acetate	Frick et al. (1976)
		Norethindrone	Coutinho & Melo (1973)
	'Releasing factors'	LH - RH	Coy et al. (1974)
Prolactin stimulation	Dopamine antagonists	Clomipramine	· Francis et al. (1976)
		Clozapine	Meltzer et al. (1975)
		Haloperidol	Rubin et al. (1976)
		Metoclopramide	Falaschi et al. (1978)
		Phenothiazine	Bohnet et al. (1976)
		Reserpine	Bartke (1976)
		Sulpiride	Debeljuk et al. (1975)
Testosterone synthesis inhibition	Hypocholesterol- aemics	Chlorcyclizine	Wong et al. (1972)
		Phenylmethyl- sulphonylfluoride	Takatori & Yamaoka (1979)
	Insecticides	DDT	Krause et al. (1975)
		Carbaryl	
		Dieldrin	
		Heptachlor	Thomas et al. (1978)
		Methoxychlor	
		Parathion	
		Hexachlorocyclohexane	Nigam et al. (1979)

Inhibition of spermatogenesis is achieved only at high dosages
which also affect libido and other androgen-dependent functions.

ii) Gonadotrophin suppression

Chemicals in this category affect the secretion and release of
hypothalamic or pituitary hormones. Many investigators have
attempted to utilise this action as a means of male fertility
control; compounds developed for non-andrological purposes have
also exerted similar effects.

Androgens suppress gonadotrophin secretion, but most preparations
are inactive by the oral route (Jackson, 1972). Methyltestosterone
is effective when given orally but induces hepatotoxic changes in
Beagle dogs (Heywood et al., 1977a) and irreversible testicular
atrophy in rats (Heywood et al., 1977b) at comparatively low
dosages.

Oestrogens are potent inhibitors of spermatogenesis. However,
prolonged administration of these compounds to dogs induced bone
marrow disorders, together with prostatic hypertrophy and metaplasia
(Wadsworth, 1979). Administration to rodents for their entire life
span may induce testicular tumours in mice and hepatic neoplasia
in rats (Bonser and Robson, 1940; Hooker et al., 1940). In man,
thrombo-embolic disorders are associated with oestrogen administra-
tion (Vessey and Doll, 1969).

Both the 19-norprogestogens and the 17α-acetoxyprogestogens can
suppress spermatogenesis, but they are not as active as the
oestrogens, and the 17α-acetoxyprogestogens are inactive when given
orally. Published toxicity studies with progestogens in male

laboratory animals are limited. However, the extragonadal

effects induced in female dogs included obesity, decreased values

relating to red blood cells, increased liver function enzyme

activity, hyperglycaemia and mucinous hyperplasia of the gall

bladder (Capel-Edwards et al., 1973; Rivett et al., 1977).

Hydrazine derivatives (e.g. methallibure) selectively suppress

gonadotrophin production in rats and dogs. These compounds,

together with the oestrogens and progestogens, also affect libido

and potency. Other reported adverse reactions following oestrogen

or progestogen usage by men include gynaecomastia, breast develop-

ment and tenderness, headaches and nausea (de Kretser, 1976).

Animal testicular extracts can exert anti-FSH properties (Keogh et

al., 1976; Reichert and Abou-Issa, 1977). While this may be a new

approach to the selective suppression of spermatogenesis, the

toxicological evaluation of these materials must include careful

assessment of the immune responses which will follow administration

of 'foreign' protein.

The hypothalamic releasing factors are polypeptide molecules, see review

(Crighton, 1973). Analogues of LH - RH have been synthesised (Coy

et al., 1974). However, both FSH and LH secretion are affected.

Depression of LH release produces undesirable loss of libido and

potency.

Compounds able to modify pituitary gonadotrophin output, but studied

for non-andrological reasons, include narcotic and peripheral

analgesics, cannabinoids and histamine receptor antagonists. A

significant proportion of the population takes narcotic and hall-

ucinogenic drugs indiscriminately and it is important to realise the

potential gonadal toxicity of these compounds. There is widespread

non-prescription usage of peripheral analgesics and Boyd (1970)
noted that many men admitted to hospital following analgesic abuse
had failed to father children.

The practice of repeatedly administering massive daily dosages to
animals can be expected to cause exaggerated pharmacological
reponses. Failure to explain and demonstrate the reversibility of
effects on reproductive hormones, such as those which occur following
high dosages of non-addictive narcotic analgesics to animals, means
that potentially useful drugs may fail to satisfy pre-clinical
toxicity regulations.

iii) Prolactin stimulation

A large number of tranquillisers, neuroleptic and psychotropic
drugs exert their pharmacological activity by inhibiting dopamine
synthesis or release. Bang and Gautvik (1977) have shown that
dopamine can inhibit prolactin release from pituitary cells. Over-
dosage with a dopamine blocking agent may therefore produce a state
of hyperprolactinaemia. In the human male, hyperprolactinaemia is
associated with decreased libido and testosterone levels and
testicular atrophy (Horrobin, 1977). Dopamine inhibitors often
have major clinical indications such as schizophrenia, alcoholism,
dyskinesias and depression syndromes. Assessment of hyperprolactin-
aemic potency may be an important factor in the animal safety
evaluation of such drugs.

iv) Testosterone synthesis inhibition

The Leydig cells are the major site of androgen synthesis. Drugs
which interfere with Leydig cell metabolism or block steroid
synthesis may decrease synthesis of testosterone and other related
androgens. The clinical manifestations of decreased androgen

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synthesis would be similar to those seen after administration of anti-androgens. Drugs with pharmacological actions on lipid and steroid metabolism, e.g. anorectics, hypocholesterolaemic agents and antihistamines,may induce phospholipidosis. Histologically, phospholipidosis is characterised by the presence of foamy macrophages and myeloid inclusion bodies at sites of steroid synthesis; the testes and epididymides are common sites for these lesions (Heywood and James, 1978).

Certain organochlorine insecticides are widely used for agri-cultural and horticultural purposes. The biodegradation of these compounds is slow, and appreciable quantities may accumulate in the environment. It has been shown that DDT and several other insecti-cides have induced fertility disorders in rodents and other wild life. The bulk of experimental evidence suggests that impaired testosterone metabolism occurs because these insecticides inhibit the binding of dihydrotestosterone to cytoplasmic receptors during testosterone synthesis, (Thomas et al., 1978).

B. Effects on the seminiferous epithelium

The seminiferous epithelium comprises the germinal cells and Sertoli cells. Cell renewal and division occurs continuously throughout adult male life. Characteristic associations between successive generations of germ cells (Clermont, 1962) and clockwork precision in the timing of cell divisions (Roosen-Runge, 1962) have been demonstrated for several mammalian species. The entry of substances into the germinal compartment of the testes is selectively regulated by a physiological 'blood-testis' barrier (Setchell and Waites, 1975). In addition to selective permeability, the presence of testicular detoxicating enzymes and efficient DNA repair mechanisms in pre-meiotic spermatogenic cells (Lee and Dixon, 1978) also influences

the toxic and mutagenic potential of chemicals. Table II lists some

chemicals known to exert cytotoxic actions on the seminiferous

epithelium. Selective chemical actions on the seminiferous epithel-

ium include alteration of blood flow and actions on mitotic and

meiotic stages of spermatogenesis. Ionising radiation attacks
                                    ʌ Dym and Clermont, 1970
dividing spermatogonia (Johnson and Newman, 1976), but resting

spermatogonia are unaffected; consequently transient infertility

occurs. Drugs such as aziridine derivatives, sulphonic acid esters,

nitrogen mustards, cyclophosphamide and trimethylphosphate also exert

this 'radiomimetic' effect. Many other substances, including anti-

biotics, anthelmintics, cyclamates, phthalate esters, organic

insecticides and metals also exert cytotoxic effects. The cell types

affected and the severity of damage often reflect the dose administe-

red. Cadmium has been shown to affect spermatogenesis by altering

testicular blood flow (Gunn et al., 1963).

The radiomimetic drugs have been studied extensively for their anti-

fertility effects. However, selective action cannot be obtained and

the toxic effects in other tissues, notably the bone marrow, preclude

long-term usage of these compounds. The diamines, nitrofurans,

thiopenes and nitro-aromatic compounds have all produced unacceptable

toxic reactions or side effects and cannot be considered to be suit-

able as male contraceptive agents. The indazole-carboxylic acids are

currently being investigated and extensive toxicity data are not yet

available. The data from animal studies concerning the testicular

damage due to cyclamates, other food additives and industrial chemi-

cals cause considerable debate. The changes are usually seen after

prolonged massive administration and, in most cases, the underlying

mechanisms of toxicity have yet to be defined. Human epidemiological

data show that 1,2-dibromo-3-chloro-propane is associated with

abnormal male fertility at production plants (Biava et al.,

1978; Sandifer et al., 1979; Whorton et al., 1977).

TABLE II

Chemicals acting on the seminiferous epithelium

Generic class	Cited examples	Author(s)
Alkylating agents	Aziridines	Hendry et al. (1951)
	Cyclophosphamide	Brittinger (1966)
	Hexamethylphosphoramide	Jackson & Craig (1966)
	Nitrogen mustards	Spitz (1948)
	Sulphonic acid esters	Jackson (1959)
Anaesthetics	Mepivacaine hydrochloride	Banhawy et al. (1977)
Antibiotics	Amphotericin B	Texter & Coffey (1969)
	Ethambutol	Trentini et al. (1974)
	Nitrofurans	Prior & Ferguson (1950)
Anti-cancer	Procarbazine	Sieber et al. (1978)
Anthelmintics	Carbon tetrachloride	de Toranzo et al. (1978)
	Hexachlorophene	Thorpe (1967)
Diamines	Dichloroacetyldiamines	Coulston et al. (1960)
Food additives	Cyclohexylamine	Mason & Thompson (1977)
	Metanil yellow	Singh & Khanna (1974)
Indazoles	5-Aminoindazole	Lobl & Porteus (1977)
	Indazole-carboxylic acids	Catanese et al. (1977)
Industrial solvents	Dibutyl phthalate	Cater et al. (1977)
Metals	Cadmium	Chiquoine (1964)
	Copper	Hoey (1966)
	Lead	Eyden et al. (1978)

TABLE II (continued)

Chemicals acting on the seminiferous epithelium

Generic class	Cited examples	Author(s)
Nitro-aromatic	Dinitropyrolles	King et al. (1963)
	Nitrofuranes	Jackson (1969)
	Nitrothiazoles	Jones et al. (1976)
Pesticides	1,2-Dibromo-3-chloro-propane	Biava et al. (1978)
		Sandifer et al. (1979)
		Whorton et al. (1977)
	Fluoroacetamide	Steinberger & Sud (1970)
Thiophenes	5 Chloro-2-acetyl-thiophene	Steinberger et al. (1956)
Methylxanthines	Caffeine	
	Theobromine	Friedman et al. (1979)
	Theophylline	
Vinca alkaloids	Vinblastine	Bustos-Obregon & Feito (1974)
	Vincristine	Cooke et al. (1978)

Torkelson et al. (1961) reported testicular damage as one of the toxic effects of this compound in several species of laboratory animal, and it was suggested that the maximum tolerated atmospheric concentration should be 1 ppm. This is a rare example of absolute correlation between empirical animal toxicity data and subsequent human experience.

C. Effects on extratesticular spermatozoa

After completion of testicular spermatogenesis, the formed spermatozoa are also vulnerable to chemical action during passage through the excurrent duct systems. Spermicidal and spermiostatic effects or interference with fertilising capacity have been demonstrated for a number of compounds (Table III). Mann (1964), although mainly using in vitro experiments, has demonstrated that spermicidal or spermiostatic actions follow disruption of spermatozoal carbohydrate metabolism and respiration, enzyme inhibition, sulphydryl binding and surface effects on sperm membranes. Many chemicals acting in this way have been used for local contraception in the form of creams and lotions, but most are unsuitable for parenteral administration. A number of synthetic proteinase inhibitors have been shown to inhibit sperm acrosin in vitro (Bhattacharyya et al., 1976). These substances cannot be tested in vivo until their effects on proteinases from other tissues, pancreatic trypsin for example, are known. Chlorohydrin and its analogues have been studied extensively for their selective glycolytic effect on epididymal spermatozoa when administered at low dosages (Mohri et al., 1975). The first analogues were found to be myelotoxic in primates and to produce severe testicular atrophy in rats at a small multiple of the clinically effective dosage. The most recent analogue, 1-amino-3-chloro-2-propanol hydrochloride, induced lesions in the medulla oblongata of rhesus monkeys (Heywood et al., 1978) and testicular atrophy and epididymal sperm granulomata in

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TABLE III

Chemicals affecting extratesticular spermatozoa

Generic class	Cited examples	Author(s)
Acrosin inhibitors	p-Nitrophenyl-p'-guanadino benzonate	Bhattacharyya et al. (1976)
	Diisopropyl fluorophosphate	Gilboa et al. (1973)
	Tosylamide phenylethyl chloromethyl ketone	Fritz et al. (1972)
	Tosyl-lysine chloromethyl ketone	Zaneveld et al. (1972)
Chlorohydrins	3-Chloro-1,2-propanediol	Ericsson & Youngdale (1970)
	1-Amino-3-chloro-2 propanol hydrochloride	Coppola & Saldarini (1974)
Chlorinated sugars	6 Chloro-6-deoxyglucose 6 Chloro-6-deoxyfructose	Ford & Waites (1978)

rats at doses of 50 mg/kg/day and above (James et al, 1978). Ford and

Waites (1978) have investigated the reversible contraceptive action

of some chlorinated sugars in rats. The results of studies on the

extragonadal toxicity of this group have not yet been published.

D. Effects of chemically induced nutritional disturbance

Present regulations for the safety assessment of food additives,

crop sprays, herbicides, insecticides, etc. require dietary

administration to dogs and rodents for up to two years. Addition

of large quantities of chemicals to the diet may restrict the

availability of some essential nutrients, or, alternatively, may

reduce palatability. The effects of restricted food intake on the

fertility of domestic animals were discussed by Reid (1949). Oishi

et al. (1979) studied the effects of food restriction on some common

toxicity characteristics in rats and noted that severe malnutrit-

ion prevented testicular growth. Much earlier, Mulinos and

Pomerantz (1941) showed that inanition atrophy of the rat testes

and secondary sex organs could be reversed by administration of

chorionic gonadotrophin. Dietary deficiency of the amino acid

valine resulted in decreased serum concentrations of FSH and LH in

female rats (Glass and Swerdloff, 1977). Leathem (1975) reviewed

the relevant literature and stated that essential fatty acid

deficiency, avitaminosis A and E, lack of biotin, pyridoxine and

thiamine and reduced availability of phosphorus, zinc, manganese and

calcium,can each produce adverse effects on spermatogenesis. The

same author concluded that the changes could be attributed to

effects either on testicular metabolism or on the production of

hypothalamic pituitary hormones. Immature animals are more prone to

nutritional disturbance of reproductive function than are adults.

This is important when one realises that most regulatory
authorities require long-term studies to incorporate the period
of active growth within the dosage schedule. Toxicological
investigations may also require overdosage with naturally occurring
substances. The observation that dietary excess of ethionine, for
example,(Goldberg et al., 1961) causes degenerative testicular
lesions,emphasises the need to consider the toxicological effects
of dietary excesses as well as those of deficiencies.

Within the existing framework of chronic and reproductive toxicity
testing it is sometimes difficult to assess the relevance of
adverse effects on the testes of laboratory animals, particularly
when the responses occur only after administration of abnormally
high dosages or as a result of impaired food intake. Understanding
of the pharmacological and physiological basis for the observed
changes may contribute significantly to the assessment of risk
attributable to the test chemicals. From the toxicological view-
point, the mechanisms of action, functional disturbances and
duration of the effect are important factors. Two major functional
disturbances are likely to arise: either infertility, or mutagenic
damage to spermatozoa. Bateman and Jackson (1974) have discussed
screening procedures for mutagenic hazards of chemicals exerting
antifertility effects and this aspect will not form a major theme
of this dissertation. The principal aim of the experiments
reported in the first section of this thesis was to assess the
feasibility of incorporating some clinical testicular function
tests and quantitative histological examinations within the frame-
work of existing chronic toxicity protocols. In the second and
third sections the value of these techniques in the toxicological
assessment of chemical effects on the hypothalamic-pituitary-
testicular axis and on the seminiferous epithelium is examined.

1. The assessment of testicular function

In clinical practice the diagnostic techniques available for the investigation of abnormal testicular function include detailed history, chromosomal analyses, endocrine assays, semen evaluation and histological examination of testicular biopsies (Adamopoulos et al., 1978; Chapman et al., 1979; Holdsworth et al., 1977; Nieschlag, 1979). Anamnesis includes in-depth surveys of previous fertility, contraceptive habits, intercurrent health or injury, libido and sexual behaviour. Genetically induced reproductive failure may be recognised by chromosomal examination. The functional integrity of the hypothalamic-pituitary-testicular axis can be assessed on the basis of circulating FSH, LH, prolactin and testosterone activities. Hormonal responsiveness in various stimulation or suppression tests is practicable. Such tests include the LH-RH stimulation test (de Kretser et al., 1975), the pituitary LH suppression and human chorionic gonadotrophin (HCG) stimulation tests (Holdsworth et al., 1977) and the metabolic clearance rate of LH (Pepperell et al., 1975). Aiman et al. (1979) have demonstrated the value of measuring the affinity of cytosolic receptor protein for dihydrotestosterone in genital skin fibroblast cultures as an index of androgen sensitivity. The potential of semen evaluation in the management of male infertility and the assessment of testicular hazards following occupational exposure to chemicals has been discussed by Eliasson (1978). The standard semen examinations include measurements of volume, viscosity and liquefaction together with spermatozoal motility, concentration and morphology. Biochemical investigations of semen can include estimation of acid phosphatase, citric acid, fructose, prostaglandins, zinc and magnesium. Crabbe (1977) considers that fumarase and diamine oxidase are the most reliable enzyme indices of fertility in human semen. Histological assessment of the seminiferous epithelium is normally limited to the examination of testicular

biopsies for the infertile human male (Hendricks et al., 1969; Rowley and Heller, 1966; Wong et al., 1973a, b). Qunatitative assessment of spermatogenesis (Holdsworth et al., 1977; de Kretser et al., 1974) and seminiferous tubule curvature (Averback and Wight, 1979) are useful adjuncts to subjective assessment of testicular biopsies. Application of some or all of the systematic testicular function tests already described enables andrologists to define lesions, to speculate on aetiological mechanisms and to prognosticate and institute appropriate therapeutic measures.

In the field of experimental animal pathology the range of available investigations can be extended (Neumann and Schenck, 1979) and includes testicular enzyme histochemistry (Blackshaw and Elkington, 1970; Cohen et al., 1976), blood-testis-barrier pharmacokinetics (Setchell and Main, 1978) and induction of testicular metabolising enzymes (Lee and Dixon, 1978). With regard to conventional laboratory animal safety evaluation studies, the inclusion of testicular function tests is subject to the constraint that investigations performed in the living animal should not exert any influence which might prejudice the overall objectives of the investigation. In practice, toxicological protocols are frequently limited to clinical observation, examination of body fluids and tissue biopsies, which complement a more extensive range of post-mortem studies.

The aim of the studies described in this chapter was to define characteristics of testicular function which can usefully be included in chronic animal toxicity protocols to clarify mechanisms of testicular toxicity and enhance the risk-benefit assessment of the data obtained.

1.1. Choice of animal models and parameters

The ultimate test of male fertility is the induction of pregnancy in receptive females. In the context of predictive animal safety evaluation the absence of effects in rodent breeding studies, and failure to induce gonadal changes following long-term administration to both rodent and non-rodent species, are normally considered to be satisfactory negative data for the assessment of potential human reproductive hazards. When positive findings occur, particularly after prolonged overdosage, it is desirable to determine the biological significance and potential reversibility of the observed effects. Serial mating procedures (Jackson et al., 1961; Wright, 1978) may clarify the situation. The availability, convenient size and reproductive physiology of the common laboratory rodent species facilitates the conduct of test-mating procedures. The non-rodent species, such as the Beagle dog and sub-human primates, take longer to attain sexual maturity, exhibit seasonal breeding rhythms, do not always breed successfully under laboratory conditions and have lengthy gestation periods. For these reasons, and the numbers of successful matings required for meaningful statistical appraisal, the assessment of testicular toxicity in non-rodent species presents serious practical difficulties. Assessment of male fertility based on testicular measurements, hormonal assays, and semen evaluation followed by detailed pathological examination,appears to offer attractive alternative possibilities (Heywood and James, 1978). Extension of these techniques to chronic toxicological evaluations in rodent species may provide explanations for apparent species differences in the severity and manifestations of testicular toxicity.

It should be stated at the outset that although all, or most, of these alternative techniques can be successfully applied to male rodents and dogs, the suitability of male laboratory macaques and baboons to these investigations is limited (Heywood and James, 1980). Male animals of these species do not attain sexual maturity before five years of age; the physical size and aggressive behaviour of mature male macaques and baboons presents problems with regard to housing, restraint, dosing and examination. The male rhesus (Macaca mulatta) is a seasonal breeder and despite maintenance of a constant laboratory environment, circulating reproductive hormone concentrations (Beck and Wuttke, 1979; Michael and Bonsall, 1977; Slob et al., 1979) and the histological appearance of the seminiferous epithelium (Richter et al., 1978) vary according to the time of year. Mahone and Dukelow (1979) were unable to demonstrate seasonal changes in body weight or the semen characteristics of cynomologous monkeys (Macaca fasicularis); however, seasonal variation in testicular volume was apparent. The importance of the latter observation with regard to the overall reproductive efficiency of laboratory-housed male cynomologous monkeys has yet to be determined. Rectal probe electro-ejaculation is the most feasible method of collecting semen from monkeys (Roussel and Austin, 1968). There are considerable variations in the quality of the semen collected by electro-ejaculation (van Pelt and Keyser, 1970) and, unless elaborate precautions are taken, the ejaculate rapidly coagulates (Fordney Settlage and Hendrickx, 1974). For these reasons the present studies were confined to rats and Beagle dogs.

1.2. Clinical Assessment

The procedures selected for study were testicular measurements,
assay of pituitary-testicular hormones, semen evaluation and
testicular biopsy. The principal aims were to establish ranges of
expected values for the various characteristics with particular
attention to methodology and age-related variations. A review of the
relevant literature indicated that, although considerable published
data were available for the laboratory rat, studies of the Beagle
dog were almost non-existent. Eight pure-bred male Beagles,
aged ten weeks, were selected from the colony maintained at
Huntingdon Research Centre. The animal management and housing was as
described in Appendices I (i) - (iii). These dogs were used to
monitor the onset of sexual maturity and to establish valid
testicular function criteria for young adult male Beagles. The
potential of testicular biopsy as an investigative toxicological
technique was assessed. Analysis of variance was performed on the
results from biopsied and non-biopsied dogs. Because no statistic-
ally significant differences (p > 0.05) were apparent, the pooled
data are presented in the following sub-sections. The results of
the canine investigations have been complemented by inclusion of
comparable data for rats abstracted from the literature available.

1.2.1. Testicular measurements

Although the embryology, gross anatomy and histology of the testes is
essentially similar for all mammals, marked species differences occur,
particularly in the position of the testes within the scrotum
(McKeever, 1970). The testes of the dog are well descended into a
thin-walled scrotum and the epididymides are easily located by
palpation: however, the scrotum of the rat is thickened and well
defined, the testes being carried high up and difficult to

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distinguish from the epididymides by palpation. The measurement of testicular dimensions is probably of value for the dog only.

The testes of eight growing Beagle dogs were measured and the cross-sectional area (mm^2) in a saggital plane calculated, see Appendix I (iv), at 13, 17 and 21 weeks of age and then at fortnightly intervals until 49 weeks and additionally at 53 and 60 weeks. The area of each testis, on each occasion, was averaged and mean values calculated. The body weight (kg) of each dog was also noted at the time of testicular measurement. Regression analyses were performed and linear correlations between body weight and testicular area were assessed.

The relationships found between testicular area and body weight are presented diagrammatically in Fig. 1.2.1.1. Statistically significant linear correlations ($p < 0.05$) were apparent between 19-31 weeks of age only. At all other times examined, testicular area appeared to vary independently of body weight. After 37 weeks there were no marked fluctuations in testicular area or body weight. The overall mean testicular area of the dogs between 37 and 60 weeks of age was 547 ± 27 (SEM) mm^2 per testis with a range of $456 - 746$ mm^2. The results are in agreement with the findings of Lützen et al. (1976) who recorded positive allometric growth of the Beagle testes between 7 and 180 days of age only.

1.2.2. Pituitary testicular hormones

Radioimmunoassay procedures for the determination of prolactin, follicle-stimulating hormone (FSH), luteinising hormone (LH) and testosterone are well established for the rat. Although prolactin, LH and testosterone assays are available for the dog, a validated canine FSH assay does not appear to have been described.

Body weight (kg)   Testicular area (mm^2)
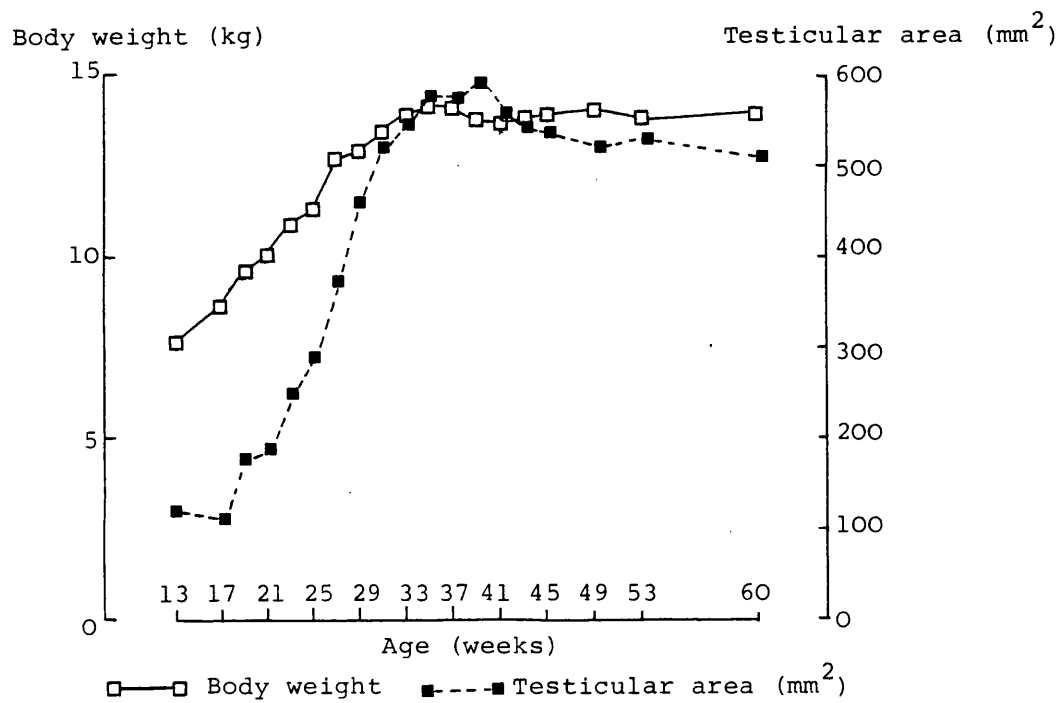
Fig. 1.2.1.1. Relationship between testicular area and body weight for growing male Beagles. Data are mean values obtained from 8 dogs on each occasion.

Plasma was obtained, Appendix I (v), from eight growing male
Beagles at monthly intervals from 13 to 45 weeks of age. The
samples were obtained between 10.00 and 12.00h on each occasion.
The plasma concentrations (ng/ml) of prolactin, LH and testosterone
were determined by appropriate radioimmunoassay techniques, Appendix
I (vi).

1.2.2.1. Prolactin

Only recently has prolactin been considered to be involved in male
reproduction. Under physiological conditions, prolactin synergises
with LH in the regulation of testosterone synthesis, secretion and
inhibition (Horrobin, 1977). Paradoxically, increased circulating
prolactin concentrations are associated with decreased androgen
synthesis and testicular atrophy.

For young adult male rats, aged 50-90 days, serum prolactin
concentrations between 20 - 40 ng/ml are expected (Table 1.2.2.1.).
Prolactin concentrations in sera from rats under 30 days old normally
measure less than 10 ng/ml (Négro-Vilar et al., 1973; Piacsek and
Goodspeed, 1978). Mattheij and Swarts (1978) confirmed the
existence of circadian variations in the plasma prolactin concen-
trations of adult male rats, aged 6 - 9 months. These authors
concluded that prolactin levels were most constant between 07.00
and 15.00h. Circulating prolactin concentrations of male rats, aged
22-30 months, show at least a three-fold increase in comparison with
rats aged 4-6 months (Riegle and Meites, 1976).

Euker et al. (1975) provided evidence that prolactin levels were
lower in serum from non-stressed decapitated rats than in serum
obtained by other more stressful sampling procedures.

Table 1.2.2.1. Circulating pituitary-testicular hormone concentrations of young adult male rats.

Hormone	Mean concentration (ng/ml)	Strain	Source
Prolactin	34	Holtzman	(4)
	20 - 40	Sprague-Dawley	(6)
Follicle-stimulating hormone (FSH)	500	Holtzman	(4)
	600 - 1200	Liverpool	(2)
	219 ± 55	Sprague-Dawley	(3,6)
	650		
Luteinising hormone (LH)	0.8 ± 0.2	Holtzman	(4)
	10 - 40	Liverpool	(2)
	3.7 ± 1.0	Sprague-Dawley	(3,6)
	35		
Testosterone	1.25 - 2.94	Inanovac	(1)
	2 - 6	Liverpool	(2)
	1.29 ± 0.56		
	3 - 5		
	2.2 - 11.9	Sprague-Dawley	(3,5,6)

Data sources: (1) Gupta et al. (1975)   (2) de Jong and Sharpe (1977)
(3) Lee et al. (1975)   (4) Négro-Vilar et al. (1973)
(5) Moger and Murphy (1977)   (6) Piacsek and Goodspeed (1978)

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Canine prolactin cannot be satisfactorily measured using

heterologous radioimmunoassay techniques. Homologous radioimmuno-

assays for canine prolactin have been described (Gräf et aL, 1977;

Knight et al., 1977). Published values for serum prolactin

concentrations of normal male dogs vary from 1-2 ng/ml (Gräf et al.,

1977) to 8.8 $\pm$ 0.8 ng/ml (Knight et al., 1977). No previously

published studies of prolactin concentration in growing or aged

dogs,or of the effects of stress, were found in the literature.

Measurements of plasma prolactin concentrations in samples obtained

at monthly intervals from eight Beagle dogs between the ages of

13 - 45 weeks (Fig. 1.2.2.1.) gave the highest values at 13 weeks

(2.4 - 5.8 ng/ml). Lower values were apparent at 17 and 21 weeks;

subsequently only minor variations in plasma prolactin concentrat-

ions occurred. From the pooled data obtained between 29 and 45

weeks of age,the results of this study suggest that prolactin

concentrations of the order of 1-2 ng/ml are to be expected in the

plasma of normal adult male Beagles.

1.2.2.2. Follicle-stimulating hormone (FSH)

signal

The stimulus for FSH secretion is thought to involve 'inhibin',

an inhibitory hormone produced in the seminiferous tubules

(Setchell et al., 1977). Although the precise role of FSH in

spermatogenesis remains controversial, rises in serum FSH follow

selective or massive depletion of the seminiferous epithelium

(Collins et al., 1978; Debeljuk et al., 1973).

Prolactin (ng/ml)
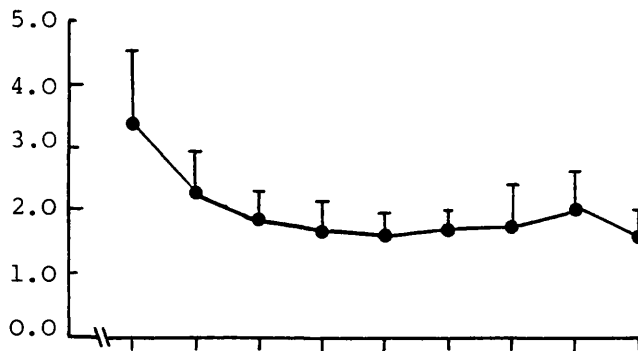
LH (ng/ml)

Testosterone (ng/ml)

Age (weeks)
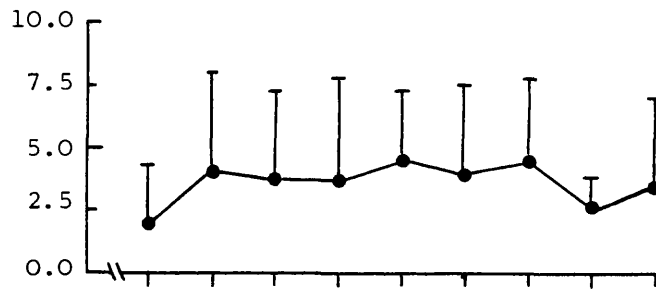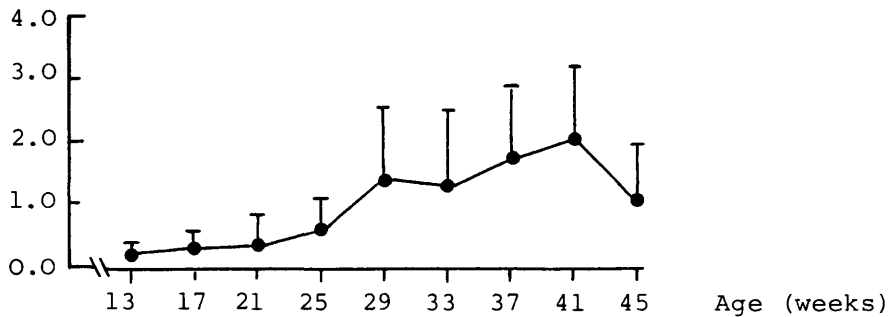
Fig. 1.2.2.1. Plasma hormone concentrations of growing male Beagles. Prolactin expressed as ng/ml of canine pituitary preparation CPA3/F4. LH expressed as ng/ml canine pituitary preparation LER-1685-1. Vertical lines represent 1 standard deviation of the mean.

In the male rat, FSH levels are high (660 $\pm$ 58 ng/ml) during the first five days of post-natal life (Lee et al., 1975), but subsequently fall to between 200-300 ng/ml. Elevated concentrations exceeding 300 ng/ml are apparent between 30 and 45 days of age, coinciding with the appearance of mature spermatozoa in the seminiferous tubules (de Jong and Sharpe, 1977; Lee et al., 1975; Negro-Vilar et al., 1973; Piacsek and Goodspeed, 1978). For young adult male rats, the published values for serum FSH concentration (Table 1.2.2.1.) appear to vary according to strain. For Sprague-Dawley rats, values between 200-600 ng/ml are expected.

As stated earlier, there appear to be no reports of a validated radioimmunoassay for canine FSH and, consequently, no data can be provided for FSH in the Beagle dog.

1.2.2.3. Luteinising hormone (LH)

The consensus of current scientific opinion is that LH regulates androgen synthesis and may be involved in spermatid maturation. Depletion of the seminiferous epithelium does not provoke increased LH secretion in the rat (Collins et al., 1978; Debeljuk et al., 1973), although Main et al., (1976) questioned this.

During immediate post-natal life the serum concentration of LH of male rats is high (>3 ng/ml) corresponding to the period of the regression of foetal Leydig cells (Lee et al., 1975). Lower circulating LH concentrations occur in male rats aged 10-35 days; peak concentrations occur from 35 days onwards, associated with the establishment of adult Leydig cell function (de Jong and Sharpe, 1977; Lee et al., 1975; Negro-Vilar et al., 1973; Piacsek and Goodspeed, 1978). There is considerable disagreement in the published values for the serum LH concentrations of young adult male rats

(Table 1.2.2.1.). Mock et al. (1975) studied seasonal variations

in serum LH concentrations of laboratory maintained Sprague-Dawley

rats and concluded that, although testicular function remained

constant throughout the year, seasonal effects on hormonal status

were apparent. Gray (1978) provided experimental evidence that the

concentration of LH in the circulation of male Long-Evans rats

declines with advancing of age. Reduced LH concentrations at 13,

17, 19 and 21 months of age differed significantly (p <0.05) from

those in rats aged 3 - 4 months.

The use of anti-ovine LH has been validated for the heterologous

radioimmunoassy of canine LH (Smith and McDonald, 1974). DePalatis

et al. (1978) measured the serum LH concentrations of healthy adult

male mongrel dogs at 20-minute intervals for 24 consecutive hours

and recorded episodic values of 0.2-12.0 ng/ml. Measurement of plasma LH

concentrations in eight young Beagle dogs at monthly intervals

between 13 - 45 weeks of age (Fig. 1.2.2.1.) indicated a wide

variation in LH concentration and, apart from lower values (<0.5 ng/

ml) at 13 weeks, no obvious trends were apparent. For young adult

male Beagles aged 29-45 weeks, plasma LH values varied between <0.5

- 11.6 ng/ml (Table 1.2.2.2.).

1.2.2.4. Testosterone

In man it is known that significant cross-reaction between

circulating androgens other than testosterone e.g. dihydrotestos-

terone and androstendione, requires elaborate extractive chromato-

graphic treatment of samples before radioimmunoassay. Tremblay

et al. (1972) studied the dynamics of plasma androgens in dogs,and

concluded that the major peripheral androgen is,in fact,testosterone

Hormone	Concentration (ng/ml)		
	Mean	SEM	Range
Prolactin	1.76	$\pm$ 0.16	1.1 - 3.3
Luteinising hormone (LH)	3.81	$\pm$ 0.78	<0.5 - 11.6
Testosterone	1.55	$\pm$ 0.38	0.2 - 4.1

Table 1.2.2.2. Circulating pituitary-testicular hormone concentrations of young adult male Beagles.

Pooled data based on 40 assays per hormone using plasma obtained from eight Beagles at monthly intervals from 29 until 45 weeks of age. Prolactin expressed as ng/ml of canine pituitary preparation CPA3/F4. LH expressed as ng/ml of canine pituitary preparation LER-1685-1.

Frankel and co-workers (1975) undertook extensive characterisation and physiological validation of a radioimmunoassay for plasma testosterone in the male rat. These workers developed an extraction procedure, excluding a chromatographic phase, which reduced cross-reaction with dihydrotestosterone to less than four per cent. This particular radioimmunoassay procedure has previously been used for the measurement of circulating canine testosterone (DePalatis et al., 1978).

For male rats, circulating testosterone concentration is consistently less than 0.5 ng/ml before the 25th day of post-partum existence (Gupta et al., 1975; de Jong and Sharpe, 1977; Lee et al., 1975; Moger, 1977). Subsequently, values tend to increase until about 70 days, when adult values are established, the expected range being shown in Table 1.2.2.1. Circadian variations in testosterone secretion are found in both immature and mature male rats (Bartke et al., 1973; Moger and Murphy, 1977), and seasonal variations in testosterone concentrations are also apparent (Mock et al., 1975). Aged Long-Evans rats developed a statistically significant reduction of circulating testosterone after 15 months of age; by 21 months the values obtained showed a three-fold diminution in comparison with contemporary male rats aged 3 - 4 months (Gray, 1978).

Previously recorded peripheral testosterone concentrations for normal adult mongrel dogs were 1.15 $\pm$ 0.46 ng/ml (Tremblay et al., 1972) and 0.4 - 6.0 ng/ml (DePalatis et al., 1978). Serial examinations of plama testosterone values in growing Beagle dogs (Fig. 1.2.2.1.) revealed marked age-related changes. At 13 weeks of age, values varied between 0.2 and 0.4 ng/ml, and successive increases were recorded between 13 and 29 weeks of age. After 29 weeks there were no major differences in the range of results obtained at each examination. The findings of this study agreed

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with previously published data and established a range of expected
plasma testosterone values of O.2 - 4.1 ng/ml for young adult
male Beagle dogs.

1.2.3. Semen examination

Semen collection using a 'teaser' bitch, artificial vagina or
digital manipulation is feasible for dogs. The most convenient
method is to use an artificial vagina (Harrop, 1954). There are
comparatively few reports of systematic semen evaluations performed
with rats. Electro-ejaculation of male rats is recorded, but the
applied voltage and frequency of stimulation are critical factors
(Birnbaum and Hall, 1961; Blandau and Jordan, 1941; Moore and
Gallagher, 1934; Scott and Dzuick, 1959). Long-term adverse
effects on subsequent growth and performance were apparent, follow-
ing electro-ejaculation of rats (Scott and Dzuick, 1959). Surgical
cannulation of the rat rete testis efferent ductules (Cooper and
Waites, 1974; Tuck et al., 1970) is not entirely satisfactory
because the spermatozoa are not exposed to the secondary sex
secretions produced in the epididymis, prostate and seminal
vesicles. Mason and Thompson (1977) described a procedure for the
collection and examination of epididymal spermatozoa during post-
mortem examination. In life, male rats, housed away from females
ejaculate spontaneously (Orbach, 1961). Vandenbergh (1971) suggests
that regular examination of penile smears may provide limited
information concerning testicular function. In the absence of a
satisfactory technique for collecting semen from rats, semen
examination was therefore restricted to Beagle dogs. Physical and
biochemical investigations are pertinent to the examination of dog
semen.

1.2.3.1. Physical examination of canine semen

The physical examination of semen includes assessment of ejaculate volume, motility and density together with determinations of sperm concentration and morphology. Normal values, including expected ranges of variation for these semen characteristics, are reported for healthy adult dogs of various breeds, including Beagles (Boucher et al., 1958; Harrop, 1955; Heywood and Sortwell, 1971).

Eight young Beagles were introduced to the use of an artificial vagina at 25 weeks of age. Once the dogs had produced ejaculates regular semen collections were made at fortnightly intervals until 45 weeks of age, also at 49 and 53 weeks with weekly collections between 61 and 66 weeks. The volume, density, motility, sperm concentration and morphology of the samples were assessed, see Appendix I (vii).

Although some of the dogs responded to stimulation with the artificial vagina at 29 weeks, no ejaculates were obtained before 33 weeks of age. The changes in the mean values for the selected characteristics at all the examinations made between 33 and 66 weeks are presented graphically in Fig. 1.2.3.1. The first ejaculates tended to be of low volume (1.6 $\pm$ 0.8 ml) and contained large numbers of dead (41.2 $\pm$ 17.8%) and morphologically abnormal (41.8 $\pm$ 8.2%) spermatozoa. The principal structural abnormalities observed were loose or split heads, cytoplasmic beads and coiled or short tails. Between 35 - 41 weeks of age, semen volume increased and the incidence of dead or abnormal spermatozoa decreased. The data obtained between 41 and 66 weeks were used to calculate expected values and variations for the selected semen parameters (Table 1.2.3.1.).
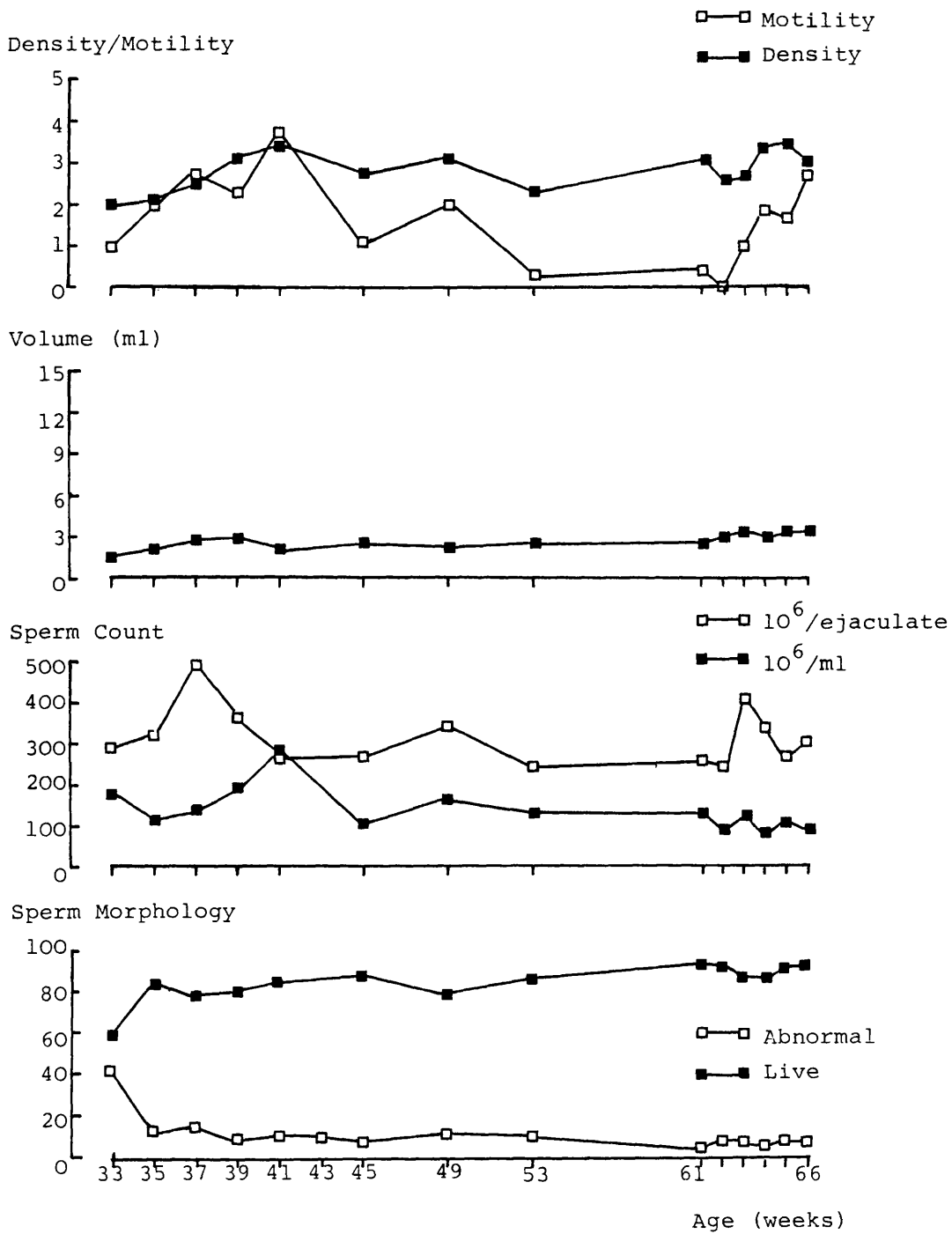
Fig. 1.2.3.1. Changes in the semen characteristics of Beagle
dogs between 33 and 66 weeks of age.

Semen characteristic	Values obtained for		
	Mean	SEM	Range
Volume (ml)	2.81	± 0.47	0.1 - 6.6
Density (0-5)	2.96	± 0.39	1 - 5
Motility (0-5)	1.45	± 1.08	0 - 5
Sperm count			
10^6/ml	132.6	± 55.0	21 - 810
10^6/ejaculate	293.5	± 53.1	81 - 794
% Sperm			
Live	87.9	± 3.8	72 - 99
Abnormal	7.8	± 2.3	1 - 18

Table 1.2.3.1. Pooled semen evaluation data (88 samples) following collections from 8 Beagle dogs on 11 occasions between 41 and 66 weeks of age.

Inspection of the results shows that considerable variations

occurred for all the parameters measured but most notably for the

ejaculate density, motility and sperm concentration.

1.2.3.2. Biochemical examination of canine semen

The accepted methods of assessing sperm concentration, morphology

and motility, although essentially quantitative, may be prone to

observer bias. The sperm concentration of canine ejaculates is

very variable and, because of the length of time elapsing from

start to completion of ejaculation, the often low values recorded

for sperm motility may not be a reliable index of semen quality.

Crabbe (1977) has suggested that determinations of enzyme

concentrations in human semen could form the basis of a rigorous

quantitative assay of semen quality. Parallelism between semen

characteristics and aminotransferase concentrations has been

investigated in the bull by Roussel and Stallcup (1961). The same

authors (1966) published data for the phosphatase activities of

bovine semen. Bartlett (1962b) reviewed the results of biochemical

studies of canine semen published before 1962; data concerning pH,

electrolyte and trace element composition, protein, carbohydrate

and amino-acid content were also described. The large volume of

the canine ejaculate is largely attributable to the third fraction

secreted by the prostate gland (Bartlett, 1962a; Harrop, 1955).

The presence of both alkaline and acid phosphatase was first

recorded by Huggins (1945). The presence of acid phosphatase in

canine prostatic secretion has been confirmed by Rosenkrantz and

Kirdani (1961). Boria et al. ₂ (1963) compared the phosphatase

activities of canine prostatic fluid obtained by pharmacological

and mechanical stimulation and found some evidence that these

enzymes may be useful indices of canine prostatic function.

Tenniswood et al. - (1978) concluded, on the basis of histochemical
studies, that acid phosphatase is an accurate biochemical marker
of prostate activity in the rat.

Semen was collected from eight Beagle dogs at weekly intervals from
61 to 66 weeks of age. The same dogs had been used for the
studies previously described, and were considered to be a
population of dogs with normal testicular function. The volume,
density, motility, sperm concentration and morphology of each
ejaculate was assessed, see Appendix I (vi). The ejaculates were
kept at room temperature for approximately 30 minutes and then
centrifuged at 2000 g for 10 minutes. The supernatant seminal
plasma (free from spermatozoa) was decanted. The remaining sperm
fractions were resuspended in Fison's phosphate buffer solution
(pH 7, sodium 53 mEq/litre, potassium 20.1 mEq/litre). Seminal
plasma and sperm fractions were stored deep-frozen (-20°C) until
biochemical examinations were made. Sodium and potassium ions,
alanine and aspartate aminotransferase and alkaline and acid
phosphatase concentrations were measured, see Appendix I (vi).

Mean values for each physical (Table 1.2.3.1.) and biochemical
(Table 1.2.3.2.) parameter were calculated. Statistical compari-
sons (Anovar) between the mean values for individual dogs and
pooled weekly data did not reveal any significant differences
($p > 0.05$). The following results are therefore based on the
pooled weekly data.

Each dog produced satisfactory ejaculates at every collection and
the physical characteristics of the ejaculates were all within
previously published ranges (Boucher et al., 1958; Harrop, 1955;
Heywood and Sortwell, 1971). The concentrations of all the bio-
chemical parameters examined in seminal plasma differed from those
found in sperm fractions. Soidum ion and acid and alkaline phos-
phatase concentrations were higher in seminal plasma but amino-
transferases were present in greater amounts in sperm fractions.

Physical characteristics	Mean	SEM	95% range
Volume (ml)	3.1	$\pm$ 0.3	2.8 – 3.4
Motility (0-5)	3.0	$\pm$ 0.3	2.2 – 3.9
Density (0-5)	1.5	$\pm$ 1.1	0.0 – 4.2
Sperm count			
10^6/ml	111	$\pm$ 22	57 – 164
10^6/ejaculate	301	$\pm$ 60	154 – 449
% Spermatozoa			
live	90	$\pm$ 2	84 – 95
abnormal	7	$\pm$ 1	3 – 10

Table 1.2.3.1. Physical characteristics of ejaculates used for biochemical studies of Beagle dog semen.

Biochemical characteristic	Mean	SEM	95% range
Seminal plasma			
Na^+ (mEq/l)	141	± 5	128 – 153
K^+ (mEq/l)	12.1	± 0.6	10.7 – 13.4
AlT (mU/ml)	12	± 6	0 – 27
AsT (mU/ml)	71	± 24	11 – 130
Alkaline phosphatase (mU/ml)	9705	± 1703	5532 – 13877
Acid phosphatase (mU/ml)	2012	± 543	903 – 3122
Sperm fractions			
Na^+ (mEq/l)	27	± 1	25 – 29
K^+ (mEq/l)	0.6	± 0.4	0.0 – 1.0
AlT (mU/ml)	180	± 35	95 – 265
AsT (mU/ml)	190	± 42	87 – 294
Alkaline phosphatase (mU/ml)	129	± 114	0 – 409
Acid phosphatase (mU/ml)	26	± 12	0 – 55

Table 1.2.3.2. Overall means for the results of biochemical examinations of 66 semen samples collected from Beagle dogs at weekly intervals from 61 to 66 weeks of age. AlT, Alanine aminotransferase (2.6.1.2.); AsT, Aspartate aminotransferase (2.6.1.1.). Cation concentrations in sperm fractions determined by difference.

The amount of alanine aminotransferase in seminal plasma was less

than one-tenth of that found in sperm fractions. Seminal plasma

was approximately one hundred times richer in phosphatases than

were sperm fractions. The sodium ion concentrations in seminal

plasma were similar to the values reported by Bartlett (1962b).

The values found for potassium ion concentration were higher than

those reported by Bartlett (1962b).

Regression analyses were performed to assess linear correlations

between the physical and biochemical characteristics of the

ejaculates (Table 1.2.3.3.). In order to reduce statistical ~~bias~~ *artefact*,

the mean weekly values were analysed in preference to individual

results from the same dogs. In consequence, the number of degrees

of freedom in the analysis were correspondingly reduced and

correlation coefficients less than 0.8 did not attain statistical

significance ($p > 0.05$).

Statistically significant ($p < 0.05$) linear correlations were found

between sperm concentration with alkaline phosphatase in sperm

fractions and potassium ions in seminal plasma. The percentage of

morphologically abnormal spermatozoa correlated with acid and

alkaline phosphatase in sperm fractions and aspartate amino-

transferase concentration in seminal plasma. Sperm motility was

correlated with potassium ion and aspartate aminotransferase

concentrations in seminal plasma. The visual density of ejaculates

showed correlations between potassium ion concentration in seminal

plasma and alanine aminotransferase in sperm fractions. Although

not attaining statistical significance, good correlations ($r \geq 0.5$)

were apparent for seminal plasma acid phosphatase and the volume,

density and motility of the ejaculates.

Correlation coefficient with physical characteristic

Biochemical characteristic	Volume (ml)	Density (0-5)	Motility (0-5)	Sperm count 10^6/ml	Sperm count 10^6/ejaculate	% spermatozoa live	% spermatozoa abnormal
a) Seminal plasma							
Na^+ (mEq/l)	- 0.5	- 0.3	- 0.7	0.1	- 0.2	- 0.5	0.0
K^+ (mEq/l)	0.2	0.8	- 0.3	- 0.8	- 0.7	0.1	- 0.1
AlT (mU/ml)	- 0.6	- 0.3	0.6	0.5	- 0.1	- 0.1	- 0.7
AsT (mU/ml)	- 0.2	0.7	0.8[a]	0.1	- 0.2	0.3	- 0.8
Alkaline phosphatase (mU/ml)	0.1	- 0.4	0.5	- 0.1	0.2	- 0.1	- 0.5
Acid phosphatase (mU/ml)	0.7	- 0.6	0.7	- 0.5	0.2	0.3	0.1
b) Sperm fraction							
Na^+ (mEq/l)	- 0.6	- 0.2	- 0.2	0.4	- 0.6	0.5	0.5
K^+ (mEq/l)	- 0.7	- 0.3	- 0.2	0.5	0.0	- 0.6	- 0.3
AlT (mU/ml)	0.2	0.8[a]	0.4	0.3	0.0	0.5	0.8[a]
AsT (mU/ml)	0.2	0.1	0.7	- 0.3	0.3	- 0.2	- 0.3
Alkaline phosphatase (mU/ml)	0.7	- 0.2	0.1	- 0.1	0.9[a]	- 0.5	0.5
Acid phosphatase (mU/ml)	- 0.6	- 0.5	- 0.1	- 0.1	0.5	- 0.5	0.9[a]

Table 1.2.3.3. Correlation coefficients between biochemical and physical characteristics of Beagle dog semen
[a] Statistically significant (p < 0.05) linear correlations; AlT, Alanine aminotransferase (2.6.1.2.); AsT, Aspartate aminotransferase (2.6.1.1.).

These observations provide some evidence that the concentrations of aspartate aminotransferase and acid phosphatase in seminal plasma may serve as biochemical indices of canine semen quality, particularly in respect of sperm morphology and motility and the quality of the prostatic secretion.

1.2.4. Testicular Biopsy

The idea of defining the onset and progress of histological changes in any tissue is attractive to all experimental pathologists. The surgical removal of small samples of tissue by means of appropriate biopsy techniques is both convenient and practical. For the purposes of toxicological investigation the inclusion of serial testicular biopsies in experimental protocols is acceptable only if representative samples of tissue can be obtained, and provided that the procedure does not itself induce any detrimental effects on testicular function.

The problems associated with testicular biopsy in the dog have been discussed in detail by several authors (Evans and Renton, 1973; Finco, 1974; Hadley, 1972; Harrop, 1966; Hunt et al., 1965; Larsen, 1977; Renton and Aughey, 1971). A variety of techniques are available (Larsen, 1977), including open excision and percutaneous needle-punch or aspiration biopsy. The risk of post-operative infection, haemorrhage or adhesions is possible with all methods. The significance and duration of slight post-operative changes is relatively unknown. Hunt et al. (1965) monitored semen production in Beagle dogs following excisional testicular biopsy; Galina (1971) has evaluated the effects of needle-punch biopsy in some farm animals. Both investigators reported initial adverse effects on semen characteristics but persistent effects were not apparent.

Martin and Richmond (1972) have described a procedure for the
repeated biopsy of rodent testes, but follow-up studies do not
appear to have been reported. Because it is easy to study
sufficiently large groups of rats to obtain whole testes at intervals
during the dosing period, testicular biopsy in the rat will not be
considered further.

The histopathological changes at 72h and 14 days after single
percutaneous testicular biopsy (Hendricks et al., 1969), were
assessed in a young male Beagle aged 28 weeks. Tissue was removed
under general anaesthesia ('Brietal', Elanco Ltd., administered
intravenously at a dose of 10 mg/kg using a 2.5% w/v solution). The
scrotal skin was cleaned with chlorhexidine gluconate solution
('Hibitane', ICI), followed by application of surgical spirit. An
incision (0.5 cm long) was made in the scrotal wall over the poster-
ior pole of the testis, taking care to avoid the epididymis, and
extended through the underlying dartos and tunica vaginalis. A
sterile Menghini biopsy needle (1.0 mm diameter x 40 mm length)
attached to a 5 ml syringe was inserted through the scrotal incision
and directed anteriorly into the body of the testis. A portion of
testicular tissue was removed by withdrawing the plunger of the
syringe to provide negative pressure. The wound was not sutured and
capillary haemorrhage was controlled by digital swab pressure. The
tissue obtained was preserved in Helly's fluid, see Appendix I (viii).
The procedure was repeated immediately for the contralateral testis.
   ‒ castrations
Hemiorchidectomies were performed at 72h and 14 days after biopsy:
each testis was fixed in buffered neutral formalin and paraffin wax
sections were examined after staining with haematoxylin and eosin,
see Appendix I (viii).

At 72h, post-biopsy haemorrhage, cellular degeneration and necrosis were obvious at the sampling site (Fig. 1.2.4.1.). The changes were confined to a small focal area and the majority of seminiferous tubules were considered to have a normal appearance. Sections from the testis obtained 14 days after biopsy (Fig. 1.2.4.1.) contained a single focus of atrophic seminiferous tubules with associated interstitial tissue fibrosis. In all other respects the sections appeared to be histologically normal.

In order to assess the value of serial percutaneous testicular biopsy and to identify possible adverse effects, four Beagle dogs were subjected to seven successive monthly testicular biopsies taken alternately from the right and left gonad between 29 and 53 weeks of age. Each biopsy specimen was processed through graded ethanol, and paraffin wax sections were stained with PAS, Appendix I (viii). The biopsies were examined to monitor changes in the seminiferous epithelium and to determine the number of tubules present in circular cross-section, suitable for quantitative analysis of spermatogenesis, see Appendix I (ix). The effects of repeated testicular biopsy were assessed by comparing testicular area, see Appendix I (vi), semen characteristics, see Appendix I (vii), plasma prolactin, LH and testosterone concentrations, see Appendix I (vi), with comparable data from four unoperated dogs maintained as contemporary controls. These observations were performed at monthly intervals starting when the dogs were 17 weeks old, although it was not possible to collect semen until the dogs were 33 weeks old. Semen collections and testicular measurements continued at monthly intervals until 53 weeks, with additional examinations 7 and 13 weeks after the last biopsy. Plasma hormone assays were not performed after 45 weeks.
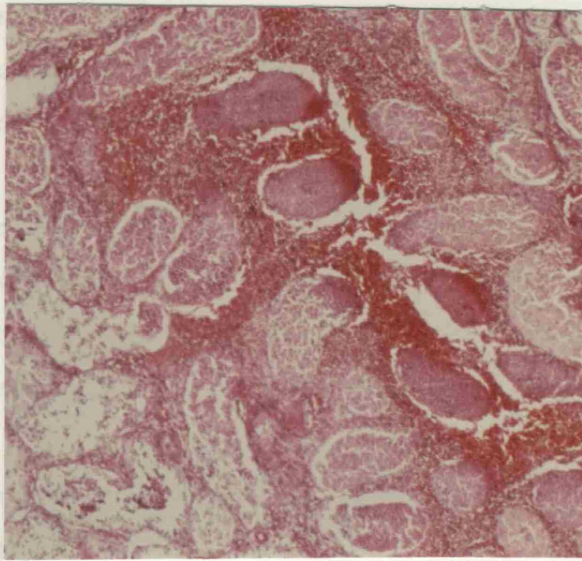
Haemorrhage and necrosis present 72h after testicular biopsy, H & E, x 125
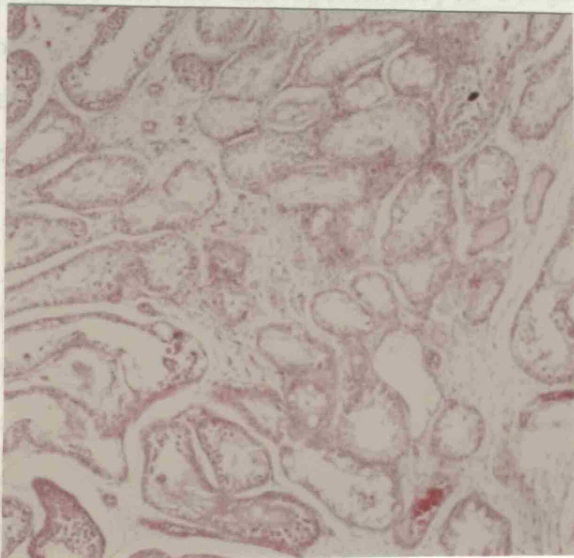
Interstitial fibrosis and tubular atrophy 14 days after testicular biopsy, H & E, x 125.

Fig. 1.2.4.1. Histological appearance of the testes from a Beagle dog 72h and 14 days after single bilateral testicular biopsy.

The technique proved satisfactory in that portions of tissue were
obtainable at each biopsy. Haemorrhage rarely occurred and was
readily controlled by gentle digital swab pressure. Minimal scrotal
swelling and erythema were sometimes seen up to 72h after biopsy.
After the first biopsy one dog developed clinical atrophy of the
biopsied gonad; however, the semen characteristics and hormonal
values relating to this dog remained within normal limits through-
out the investigation.

Although a large number of tubular sections were present in the
biopsy material it was difficult to find tubules in circular cross-
section (Table 1.2.4.1.). The earliest biopsies at 29 and 33 weeks
showed poor organisation of the seminiferous epithelium, and cells
were rarely apparent beyond the spermatocyte stage (Fig. 1.2.4.2.).
At 37 weeks the germinal epithelium was organised and all stages of
spermatogenesis, see Appendix I (ix), could be defined, although it
was rare to observe all eight stages in circular cross-sections from
an individual dog. It was not possible to undertake meaningful
assessment of differential germ cell counts at each stage of
spermatogenesis with increasing age. As a compromise, the number of
type B spermatogonia, primary spermatocytes, spermatids and Sertoli
cells were counted in all tubules found in circular cross-section,
regardless of the stage of spermatogenesis. Tubular diameters and
the mean diameter of ten nuclei of each cell type were measured,
Appendix I (ix). The crude cell counts were then mathematically
adjusted for differences in tubular and nuclear diameter
(Abercrombie, 1946; Swierstra and Foote, 1963). The ratio of the
total number of germ cells to Sertoli cells per tubule, designated
the Sertoli cell index, was then calculated. Quantitative differ-
ences in germ cell populations were apparent (Table 1.2.4.1.).

Mean age (weeks)	Number of tubules examined	Mean values obtained for					
		Tubule diameter (μm)	Differential cell counts				Sertoli cell index
			Type B spermatogonia	Primary spermatocytes	Spermatids	Sertoli cells	
29	18	154	10.1	13.2	8.4	8.4	3.77
33	16	155	16.9	19.7	32.1	9.6	7.16
37	18	182	11.1	18.5	46.6	7.3	10.44
41	20	179	11.7	19.5	57.1	5.9	14.97
45	9	160	12.2	16.1	50.1	6.3	12.44
49	20	166	15.0	18.8	60.1	6.5	14.45
53	20	154	10.8	18.2	44.8	5.7	12.95
Mean ± SEM from 41 to 53 weeks		165 ± 11	12.4 ± 1.8	18.2 ± 1.5	53.0 ± 6.9	6.1 ± 0.4	13.70 ± 1.20

Table 1.2.4.1. Results of quantitative histometric analysis of serial testicular biopsies obtained from 4 Beagle dogs. Crude cell counts were corrected for nuclear (Abercrombie, 1946) and tubular diameter (Swierstra and Foote, 1963).
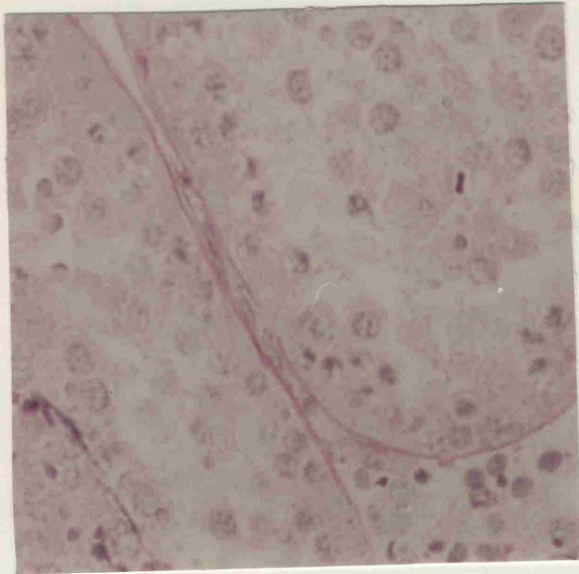
Age 29 weeks. Poor organisation
of germinal epithelium; few
spermatids present.
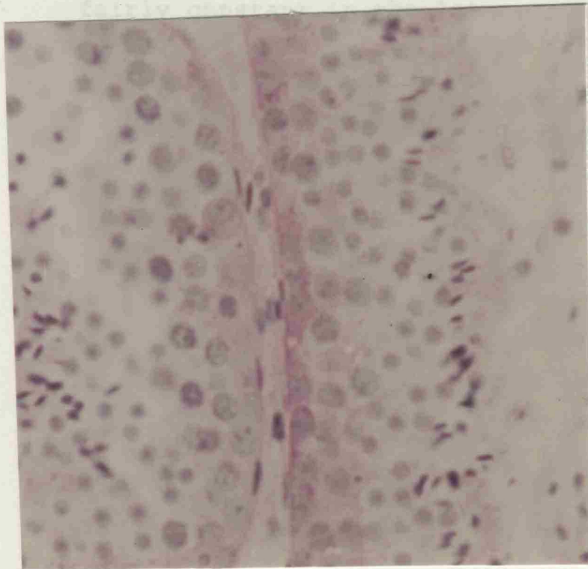
Age 33 weeks. Germinal epithe-
lium becoming organised; round
and elongated spermatids present.
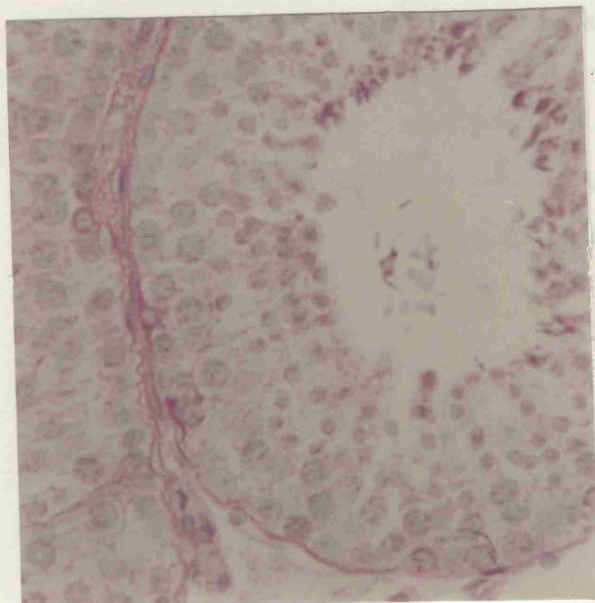
Age 37 weeks. Germinal epithe-
lium well differentiated and
organised, with mature spermat-
ids present.

Fig. 1.2.4.2. Examples of testicular biopsies obtained from
Beagle dogs; PAS, x 500.

At 29 weeks of age the numbers of primary spermatocytes and spermatids were much less than recorded subsequently. The number of primary spermatocytes was comparable at all examinations between 33 and 53 weeks. Spermatid numbers gradually increased at successive examinations until 41 weeks of age; subsequently the spermatid counts remained reasonably constant. The number of Sertoli cells found in tubules between 29 and 37 weeks decreased slightly but remained fairly constant thereafter. The proportion of germ cells to Sertoli cells increased between 29 and 41 weeks, but remained fairly constant in the later specimens.

The mean changes in testicular area are plotted in Fig. 1.2.4.3. The marginal reduction in group mean testicular area for biopsied dogs between 29 and 41 weeks is possibly accentuated by the onset of unilateral testicular atrophy for one dog. After the fifth biopsy (45 weeks) the group mean testicular areas were essentially comparable for biopsied and control dogs.

Differences between control and biopsied dogs in respect of plasma prolactin, LH and testosterone are shown in Fig. 1.2.4.4. The data were statistically analysed (Anovar) and no statistically significant differences were apparent $(p > 0.05)$. The maturational patterns for plasma prolactin and testosterone were similar for both groups of dogs. Although minor differences in LH and testosterone concentrations were sometimes apparent, all the values obtained were within published ranges for normal dogs (DePalatis et al., 1978; Tremblay et al., 1972).
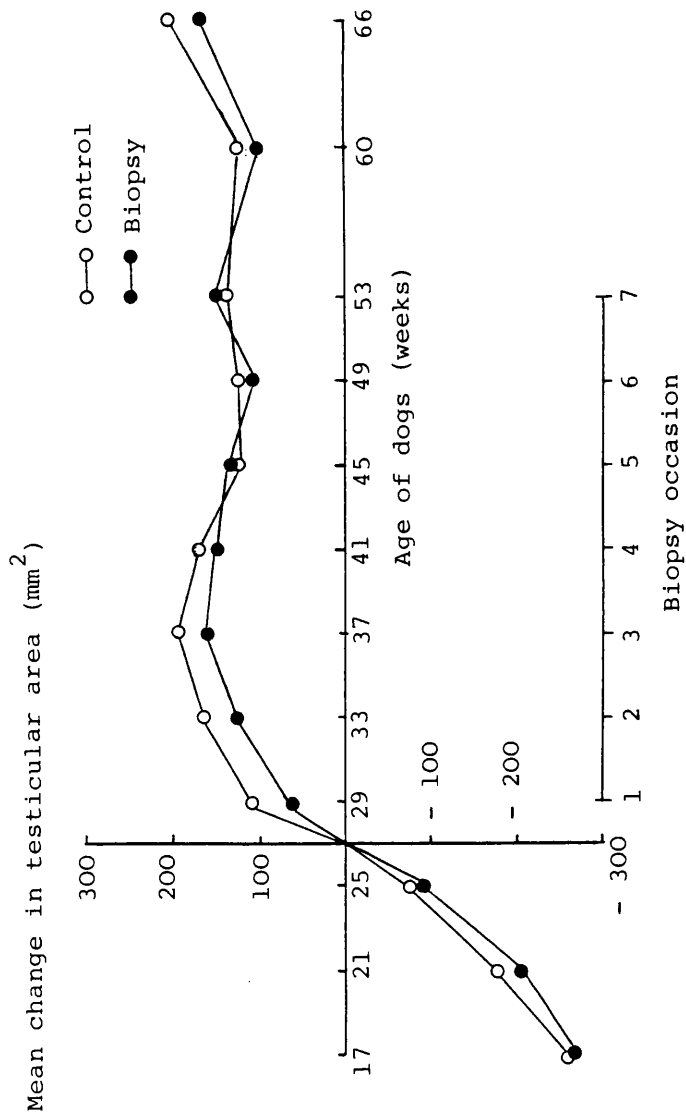
Fig. 1.2.4.3. Changes in mean testicular area relative to values at 27 weeks for Beagles subjected to serial percutaneous testicular biopsy at 7 successive monthly intervals. Control values were obtained from 4 unoperated contemporary controls.

Prolactin (ng/ml

Biopsy Period

4

3

2

1

0

Luteinising hormone (ng/ml)

10

8

6

4

2

0

Testosterone (ng/ml)

2.5

2.0

1.5

1.0

0.5

0.0

13   17   21   25   29   33   37   41   45

(1)   (2)   (3)   (4)   (5)

Age (weeks)
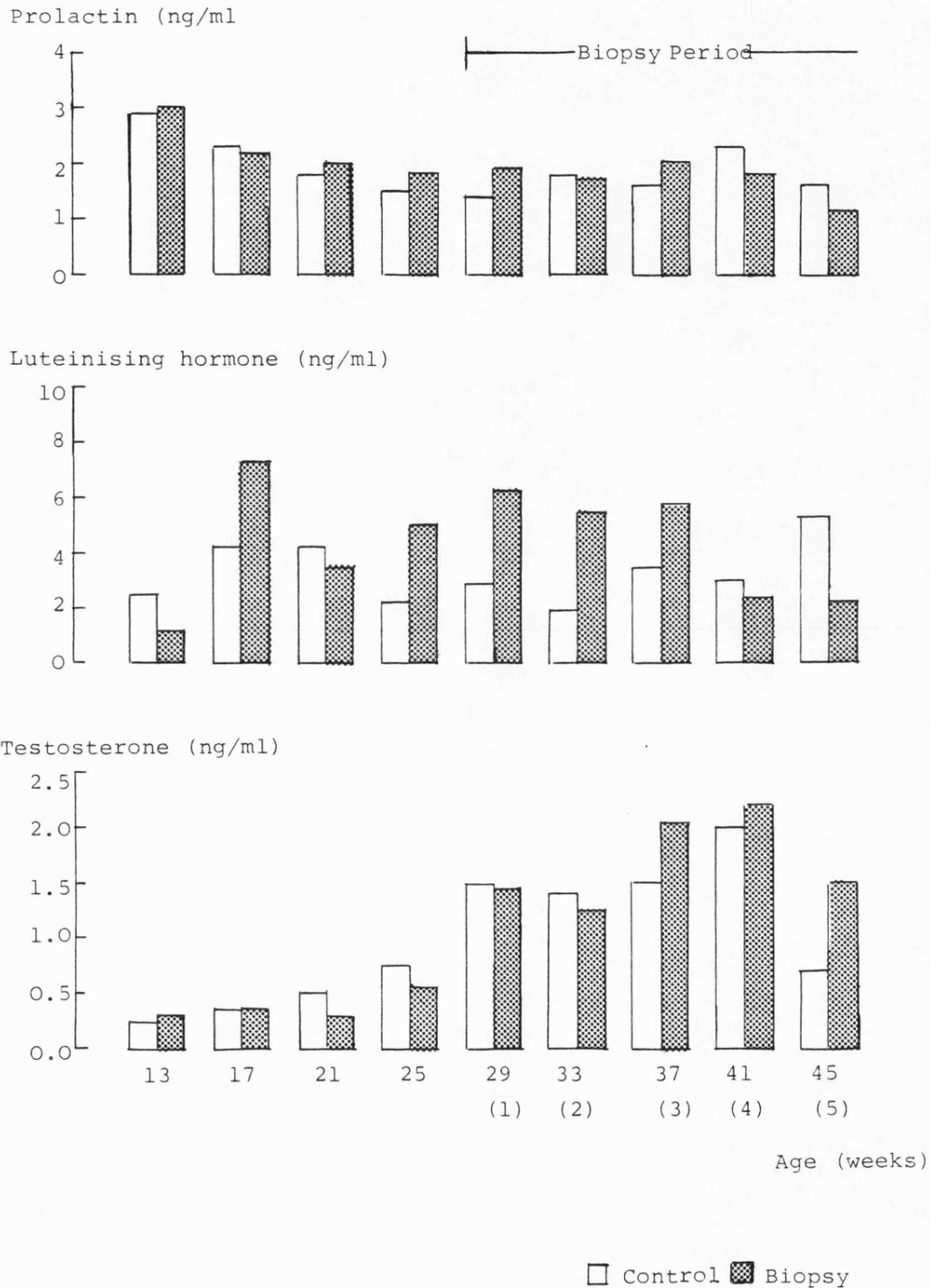
☐ Control ▨ Biopsy

Fig. 1.2.4.4. Comparisons of plasma prolactin, LH and testosterone concentrations in 4 Beagles subjected to repeated testicular biopsy with data from 4 Beagles maintained as contemporary controls. Prolactin expressed as ng/ml canine pituitary preparation CPA3/F4; LH expressed as ng/ml canine pituitary preparation LER-1685-1.

Table 1.2.4.2. summarises the semen examination data. No marked differences were found in the semen characteristics of either group of dogs. It was not possible to collect semen until after the second biopsy; on this occasion the numbers of spermatozoa per ml and the percentage of live forms was decreased in the biopsied dogs. Although reduced sperm counts were recorded for biopsied dogs at 37, 41 and 45 weeks the values still fell within accepted ranges (Boucher et al., 1958; Harrop, 1955; Heywood and Sortwell, 1971). There were no obvious persistent effects on any characteristic, and routine analysis of variance did not indicate any experimentally induced effects (p > 0.05).

Monitoring testicular measurements, circulating prolactin, LH and testosterone and semen characteristics failed to demonstrate any statistically significant differences caused by testicular biopsy. However, it should be remembered that severe clinical unilateral testicular atrophy was induced in one dog.

Biopsy occasion	Age (weeks)	Volume (ml)	Density (0-5)	Motility (0-5)	Sperm count 10^6/ml	Sperm count 10^6/ejaculate	Spermatozoa (%) Live	Spermatozoa (%) Abnormal
2	33	1.9 (1.6)	2.3 (2.0)	1.5 (1.0)	147 (254)	297 (278)	51 (85)	43 (37)
3	37	3.3 (2.4)	2.3 (2.3)	2.8 (2.5)	120 (132)	580 (390)	84 (70)	12 (15)
4	41	2.4 (1.5)	3.3 (3.5)	3.3 (4.0)	145 (425)	180 (266)	87 (83)	11 (9)
5	45	2.5 (2.5)	2.8 (2.7)	1.0 (1.3)	95 (125)	245 (287)	84 (94)	9 (4)
6	49	2.4 (2.5)	3.3 (3.0)	2.3 (1.7)	155 (155)	378 (295)	72 (90)	16 (7)
7	53	2.0 (3.0)	2.5 (2.0)	0.5 (0.0)	187 (70)	222 (283)	88 (87)	10 (12)
	60	2.9 (2.2)	3.0 (2.7)	1.3 (1.3)	147 (129)	320 (278)	87 (90)	4 (6)
	66	3.0 (4.5)	3.3 (2.7)	2.5 (0.3)	104 (85)	209 (423)	91 (93)	7 (8)

Table 1.2.4.2. Results of semen examinations from 4 Beagles following 7 consecutive testicular biopsies at monthly intervals, with additional examinations 7 and 13 weeks after the last biopsy. Figures in parentheses are the mean values obtained from 4 Beagles maintained as contemporary controls.

1.3. Pathological Assessment

The pathological assessment of toxic effects in animals is usually based on organ weight analysis and descriptions of the macroscopic and microscopical appearance of tissues. Although emphasis is placed largely on comparison of data from test and concurrent control animals, knowledge of the spontaneous and age-related variations in the weight and structure of organs is necessary. This is especially true if small numbers of animals are studied. In the case of non-rodent studies, the group sizes rarely exceed five animals of each sex per group. A fortuitous distribution of normal variants among small numbers of subjects could lead to erroneous conclusions. In the following sections the findings of surveys undertaken to investigate the range of spontaneous variations in the weight and histological appearance of testes obtained from Sprague-Dawley rats and Beagle dogs maintained as untreated control animals for toxicity studies conducted at the Huntingdon Research Centre are described.

Although quantitative assessment of spermatogenesis is described in the literature, this approach has not been critically examined as an adjunct to the appraisal of testicular toxicity. The concept of quantitative testicular histomorphometry is introduced, with emphasis on the role this technique may have in defining sites or mechanisms of toxic actions on the testis.

1.3.1. Testicular Weights

Several investigators have published ranges for the organ weights of rats of various strains and ages (Schärer, 1977; Stevens, 1975; Trieb et al., 1976). Insufficient information was available to determine whether significant age-related changes in testicular

weight occurred for Sprague-Dawley rats. Data were abstracted from studies undertaken with CD strain Sprague-Dawley rats between 1972 and 1975. During this period 165 male rats were used as untreated controls for studies of 13, 26, 52 or 104 weeks' duration. The housing, feeding and management of the rats were as described in Appendices I (i) - (iii). At completion of the experimental periods the rats had been killed by carbon dioxide asphyxiation. The post-mortem procedures included weighing of the testes, free of fat. The data were classified according to the length of the experimental periods. Within each period the initial body weight, together with the arithmetic mean, standard deviation and ranges for final body weight and testicular weight were obtained.

The initial body weights of all the rats were in the range 90-180 g. The terminal body weight and testicular weight data are given in Table 1.3.1.1. Body weights increased according to the duration of the studies: rats maintained for 104 weeks were almost twice as heavy as rats maintained for 13 weeks. The mean testicular weights did not show appreciable age-related differences. Expression of the standard deviation as a percentage of the mean value gave higher values for studies of 52 or 104 weeks' duration (12% and 16% respectively) than after 13 or 26 weeks (8% and 4% respectively). The range of individual values increased with age, and after 52 or 104 weeks, at least 13% of the rats were found with testicular weights below the lowest values recorded in the 13- or 26-week groups. Thus, the variation in testicular weights appeared to increase with age, and this was principally due to some old rats, kept for more than 52 weeks, having smaller testes than rats maintained for a maximum of 26 weeks.

Study length (weeks)	Number of rats	Body weight (g)		Weight of testes (g)		
		Mean	SD	Mean	SD	Range
13	39	533	± 53	5.0	± 0.4	4.3 - 5.8
26	59	652	± 77	5.1	± 0.2	4.3 - 6.1
52	15	798	± 127	4.9	± 0.6	3.8 - 5.9
104	52	904	± 52	5.0	± 0.8	2.7 - 6.3

Table 1.3.1.1. Summary of body weight and testicular weights of Sprague-Dawley (CD strain) rats maintained as untreated controls for toxicity studies.

Ranges of normal Beagle dogs' organ weights have been described by Jackson and Cappiello (1964) and Lützen et al. (1976). The first-named authors studied biological variation in terms of relative organ weight only. Lützen and his co-workers consider this approach to be unsatisfactory and studied organ weights allometrically. The testes of Beagle dogs show positive allometric growth between 7 and 180 days of age only. This observation suggests that, if studies are started with dogs less than 180 days old, the dosing period not only includes the period of testicular growth, but, for 13-week studies, the dogs may be killed before the testes have reached adult size. In order to assess the impact of variations in testicular weight on shorter-term studies and also to determine whether testicular weight varies with age in adult dogs, a comparable survey to that already described in rats was instituted for Beagle dogs. Sufficient data were available for control dogs kept for 13-, 26-, 52- and 104-week experiments. Heywood et al. (1979) evaluated the carcinogenicity of toothpaste, containing chloroform, when given to Beagle dogs. No treatment-induced effects were apparent in male reproductive organs: data from the 48 male dogs used for this experiment were therefore included in the survey.

The population sample comprised 198 male Beagles aged from 37 weeks to 7.75 years at the time of examination. The data were classified according to the length of the experimental periods. Within each period the arithmetic mean, standard deviation and ranges were obtained for initial age and body weight together with terminal body weight and testicular weights. The percentage of dogs with testicular weights in various ranges was also determined for each category.

The findings of this survey are presented in Table 1.3.1.2. The
dogs used for 13-week studies were slightly older and heavier at
the start of experimentation. Judged on the basis of differences
in mean final body weight for studies of increasing duration it
was apparent that, after 13-week studies, a proportion of dogs were
killed before attaining full adult body weight. There were no
marked differences in mean testicular weight for dogs kept for 26-,
52- or 104-week observation periods. In comparison with these three
groups, dogs kept for 13-week periods had lower mean testicular
weight and, conversely, dogs kept for 377 weeks developed increased
mean testicular weight. Of the dogs kept for 13 weeks, 40% had
testes weighing less than 10 g. Over 50% of the dogs observed for
377 weeks had testes weighing more than 30 g. The majority of the
dogs in the 26-, 52- and 104-week categories had testes weighing
between 20 and 30 g.

These results suggested that a significant proportion of Beagle
dogs kept for 13-week studies (mean age 37 weeks when killed) can
be expected to have small testes weighing less than 10 g. Such a
finding is probably attributable to sexual immaturity and may pre-
clude meaningful assessment of toxic effects on the testes.

It is also apparent that a large number of dogs kept for long-term
(7-year) experiments can be expected to have higher testicular
weights than young adult Beagles.

1.3.2. Histopathological examination

The descriptive histopathology of the male reproductive tract of
laboratory rats and dogs has been reviewed by King (1978). More
detailed information with regard to the dog is available from
the works of Bloom (1954) and Jubb and Kennedy (1970).

Study duration (weeks)	Number examined	Initial mean age (weeks)	Final body weight (kg)	Weight of testes (g)	Percentage of dogs with testicular weight in range (g)			
					0 - 9.9	10 - 19.9	20 - 29.9	>30
13	42	24 ± 4	11.9 ± 1.8	20.7 ± 6.1	4.8	35.7	52.4	7.1
26	38	20 ± 2	15.6 ± 2.0	23.6 ± 5.0	0.0	26.3	63.2	10.5
52	41	21 ± 4	12.4 ± 2.0	25.0 ± 6.6	2.4	17.1	56.1	24.4
104	29	21 ± 2	12.6 ± 2.2	25.2 ± 5.9	3.4	13.8	58.6	24.2
377	48	20 ± 1	13.1 ± 2.0	29.7 ± 7.1	2.1	10.4	35.4	52.1

Table 1.3.1.2. Summary of initial age, final body weights and variations in testicular weights of Beagle dogs maintained for toxicity studies.

Hottendorf and Hirth (1974) reported the findings of a histological survey of the major organs of 1000 Beagle dogs aged 8 - 20 months. The findings in the testes included atrophic changes, giant-cell formation and epididymitis. In order to provide specific information concerning the spontaneous age-related variations in the histological characteristics of the rat and Beagle testes, a retrospective survey on populations of 165 Sprague-Dawley rats (CD strain) and 198 Beagle dogs was instituted. All the animals had been maintained at the Huntingdon Research Centre as previously described (1.3.1.). Paraffin wax sections (56°C) were cut at 5 μm and stained with haematoxylin and eosin for examination by light microscopy. The histological observations were collated according to the length of study and further classified according to the site and nature of the change. The classification of testicular tumours was based on the criteria of Nielsen and Lein (1974).

The findings of this survey are presented in Tables 1.3.2.1. and 1.3.2.2. Apart from a very low incidence of minor epididymal inflammatory cell infiltration, no changes were seen in the testes of rats maintained for periods of less than 52 weeks. The major changes seen in the testes of rats maintained for longer periods were varying degrees of lack of spermatogenesis. In up to 7% of rats maintained for 52 weeks, spermatogenesis did not proceed beyond the spermatocyte stages. A further 7% of rats in this group and almost 20% of those maintained for 104 weeks developed atrophy of the seminiferous epithelium, characterised by the absence of germinal cells in seminiferous tubules containing Sertoli cells only (Fig. 1.3.2.1.). Unilateral or bilateral cases occurred at similar frequency although the atrophic changes were almost invariably diffuse rather than focal. These findings were not surprising, because Gray (1978) has reported reduced circulating LH and testosterone concentrations in aged male rats. Ribelin (1963) has previously suggested that atrophy of the rat testis is a useful index of chemical toxicity. However, age-related spontaneous testicular atrophy must be considered.

Incidence of lesions (%)	Study duration (weeks)			
	13	26	52	104
Interstitial cell adenoma	-	-	-	1.9
Interstitial cell hyperplasia	-	-	-	1.9
Incomplete spermatogenesis	-	-	6.7	-
Atrophic germinal epithelium				
a) unilateral	-	-	6.7	9.6
b) bilateral	-	-	-	9.6
Spermatocoele granuloma	-	-	-	1.9
Epididymitis	2.6	1.7	-	-
Arteritic changes	-	-	-	17.3

Table 1.3.2.1. Incidence of testicular lesions observed in 165 Sprague-Dawley rats (CD strain) maintained as controls for toxicity studies.

% incidence of lesions

Study duration (weeks)

	13	26	52	104	377
Neoplasia					
Seminoma	–	–	–	–	2.1
Sertoli cell tumour	–	–	–	–	6.3
Interstitial cell tumour	–	–	–	–	6.3
Interstitial cell hyperplasia	–	–	–	–	8.3
Incomplete spermatogenesis	7.1	–	–	3.4	6.3
Arteritic changes	–	–	–	3.4	8.3
Cryptorchid testis	2.4	–	4.9	10.3	4.2
Spermatocoele granuloma	–	2.6	–	3.4	2.1
Epididymitis	2.4	2.6	4.9	6.9	6.3
Hyperplastic epididymal epithelium	–	–	–	6.8	76.0

Table 1.3.2.2. Incidence of spontaneous testicular lesions observed in 198 Beagle dogs maintained for toxicity studies.

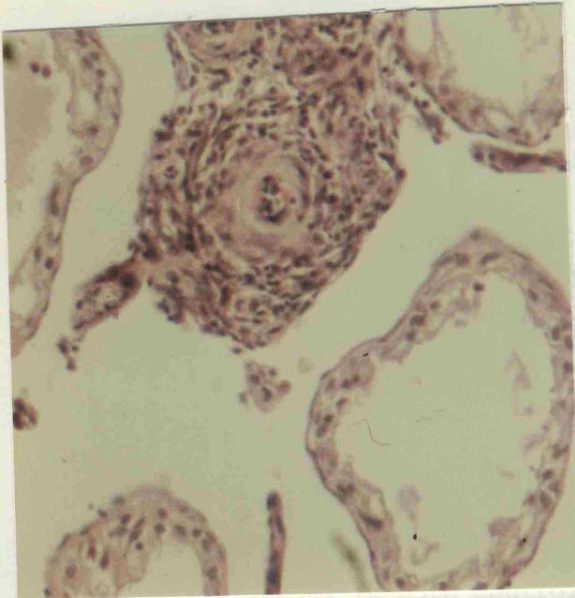Fig. 1.3.2.1. Rat testes showing atrophy of seminiferous tubules with periarteritic inflammatory cell infiltration. H & E, x 125.
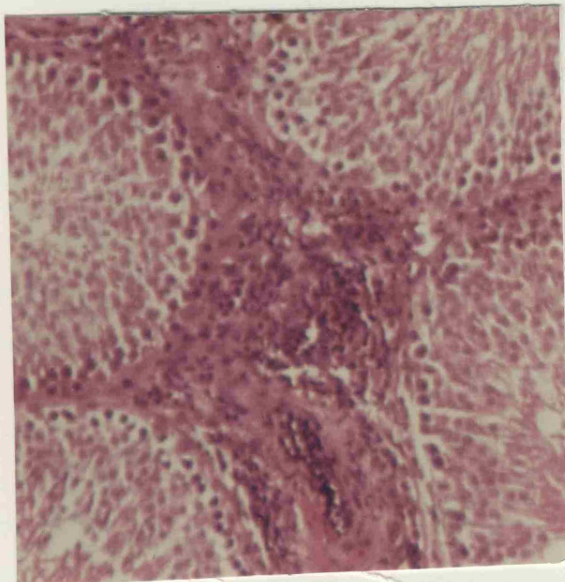

Fig. 1.3.2.2. Rat testes with normal seminiferous epithelium but marked inflammatory cell infiltration in and around blood vessels. H & E, x 125
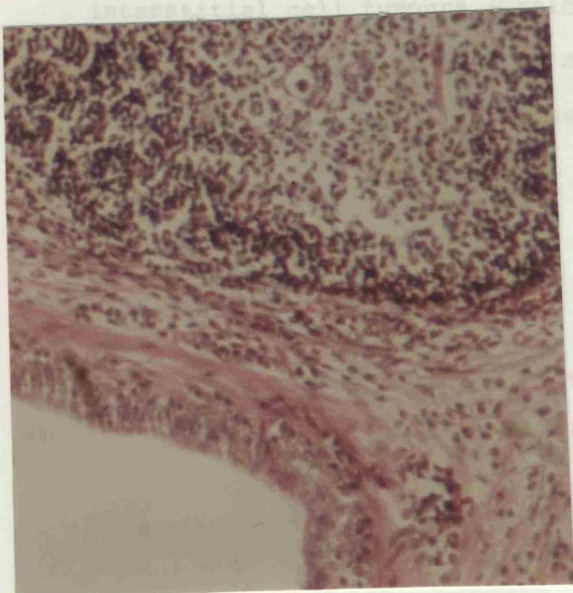

Fig. 1.3.2.3. Spermatocoele granuloma in rat epididymis, with extensive inflammatory cell infiltration of stromal zones. H & E, x 125.

A high incidence (over 17%) of inflammatory cell infiltrations into and around testicular blood vessels was found among rats maintained for 104 weeks (Fig. 1.3.2.2.). This result agrees with the findings of Yang (1965) who recorded a 14.6% incidence of polyarteritis in various organs of male and female rats aged 541 - 797 days. Lesions were observed in mesentery, pancreas, kidneys, stomach, intestine and testes. Although muscular arteries were most frequently involved, lesions were occasionally present in arterioles. The aetiological factors remain obscure. Yang (1965) observed similar lesions in both control and experimental animals, and drew attention to the resemblance to the human condition polyarteritis nodosa. The latter condition is considered to be based on auto-immune reactions.

Spontaneous testicular tumours occur at a low incidence among Sprague-Dawley rats (Gillmann et al., 1953; Russfield, 1967; Thompson et al., 1961). In the present survey, a very low incidence of less than 2% was recorded for neoplastic or hyperplastic changes in interstitial (Leydig) cells. It should be noted that this finding is in marked contrast to that in rats of the Rochester and Fischer strains, which develop a high incidence (60 - 70%) of interstitial cell tumours (Crain, 1958; Jacobs and Huseby, 1967). During the last 50 years there appears to have been only one report of a possible seminoma in a single rat of unspecified strain (Bullock and Curtis, 1930).

Spermatocoele granulomata (Fig. 1.3.2.3.) occurred at an incidence of approximately 3% among rats of varying ages. The development of these lesions probably represents a 'foreign body' reaction to 'non-self' protein following extravasation of spermatozoa into epididymal stroma (Glassy and Mostofi, 1956).

Although a low frequency of spermatocoele granulomata can be
expected, James et al. (1978) have reported the dose-dependent
induction of similar epididymal lesions in rats given high doses
of the male antifertility compound 1-amino-3-chloro-2-propanol
hydrochloride (CL 88263).

A variety of histological changes occurred in the Beagle dog testes
(Table 1.2.3.2.). Cryptorchid or retained testes were found at an
incidence of 3.82 - 4.36% irrespective of age. Most cases were
unilateral and inguinal, usually involving the right testis. The
histological appearance (Fig. 1.2.3.4.) was that of seminiferous
tubules lined by Sertoli and spermatogonial cells only. Retention
of the testes is considered to be a single autosomal recessive
trait which may not manifest itself before puberty (King, 1978).
A number of markedly age-specific changes were encountered in the
testes. Incomplete spermatogenesis, characterised by absence of
spermatids (Fig. 1.2.3.5.), was seen in 7% of dogs retained for 13-
week study periods. No cases of abnormal spermatogenesis,
excluding obvious cryptorchidism, were seen in dogs kept for 26 or
52 weeks. For studies of longer duration, a low but increasing
incidence of dogs showing regressive changes in the seminiferous
epithelium (Fig. 1.2.3.6.) was apparent. Inflammatory lesions
involving testicular blood vessels were not found in dogs kept for
52 weeks or less. Arteritic changes (Fig. 1.2.3.7.) were described
in dogs kept for 104 weeks or 377 weeks; the incidence (8.3%) was
highest for the oldest dogs. Arteritic lesions are found in a wide
range of tissues taken from domestic animals (Jubb and Kennedy,
1970). In the dog, testicular arteritis has been reported in dogs
infected with Brucella canis (Carmichael and Kenney, 1968).

Fig. 1.2.3.4. Cryptorchid Beagle
testis. Seminiferous tubules
contain Sertoli cells and sperm-
atogonia only. H & E, x 125.

Fig. 1.2.3.5. Immature Beagle
testes; note absence of elongated
spermatids. H & E, x 125.
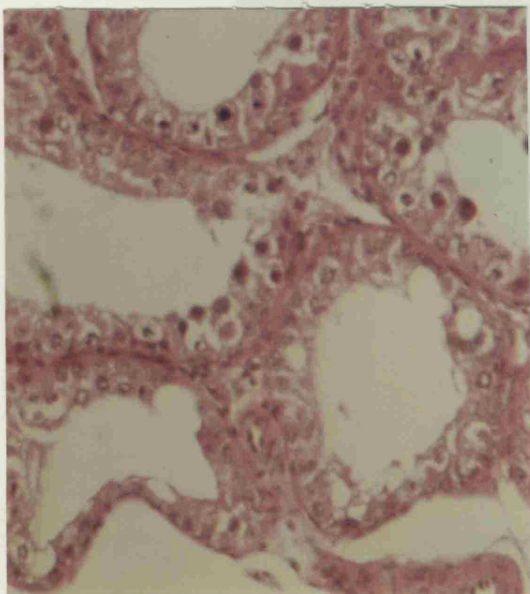

Fig. 1.2.3.6. Regression of
spermatogenesis seen in the testes
of an ageing Beagle. H & E, x
125.
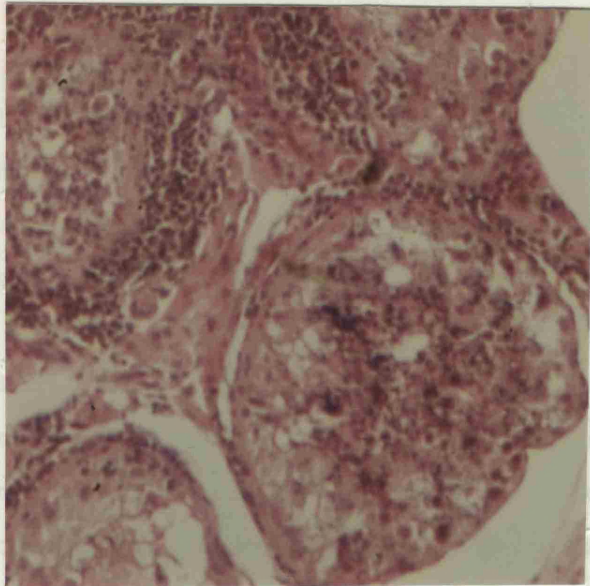
Fig. 1.2.3.7. Chronic inflammatory cell infiltration (arteritis) with disruption of spermatogenesis in an ageing Beagle. H & E, x 125.
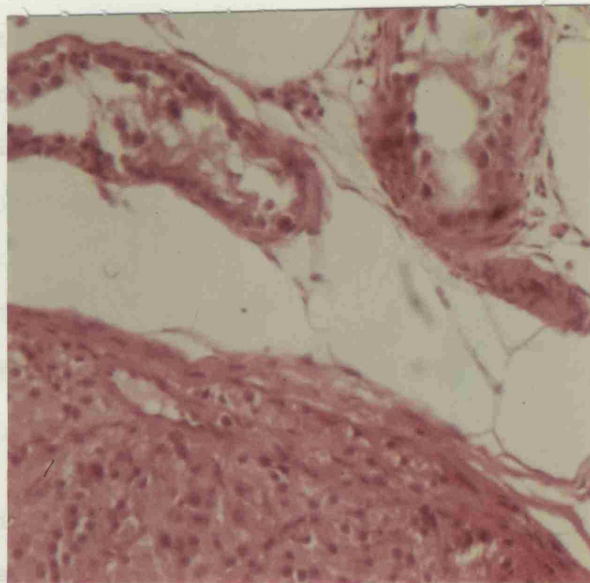
Fig. 1.2.3.8. Interstitial (Leydig) cell adenoma in testis of an ageing Beagle. Note atrophic seminiferous tubule adjacent to neoplasm. H & E, x 125.

Harcourt (1978) considers that autoimmune mechanisms are important in the pathogenesis of this disease. Testicular neoplasia or hyperplasia was found only among dogs kept for 377 weeks, but over 20% of these dogs were affected. Testicular neoplasms occurred in 5/48 dogs, comprising one case of unilateral seminoma, one case of bilateral interstitial cell adenoma (Fig. 1.2.3.8.) and three cases of unilateral Sertoli cell tumours, two of which developed contra-lateral interstitial cell adenomata. Two of the Sertoli cell tumours occurred in cryptorchid testes. Only two of the eight tumours were apparent at macroscopic post-mortem examination. Microscopical evidence of moderate to marked interstitial cell hyper-plasia was seen in 8.3% of the oldest dogs. Although non-rodent species are rarely used for carcinogenic evaluation of chemicals, the high spontaneous incidence of testicular neoplasms in Beagle dogs could complicate the assessment of chemical carcinogenesis involving the testis. The aetiological factors and endocrine implications of canine testicular tumours are well documented (Cotchin, 1960; Dow, 1962; Jones and Friedman, 1950; Reif et al., 1979; Scully and Coffin, 1952). Hooker et al. (1946) considered that the dog is an atypical mammal, in that the interstitial cells of the testes increase in size and vacuolation with advancing age, which probably represents a pre-neoplastic change.

Epithelial hyperplasia of the ductus epididymis was recorded for 76% of dogs maintained for 377 weeks and 6.8% kept for 104 weeks; younger age groups were not affected. The lesion (Fig. 1.2.3.9.) is characterised by the presence of epithelial bridges containing closely packed nuclei. The condition was first described by Mawdesley-Thomas and Urwin (1967), who included nutritional, hormonal and genetic factors among the possible causes.
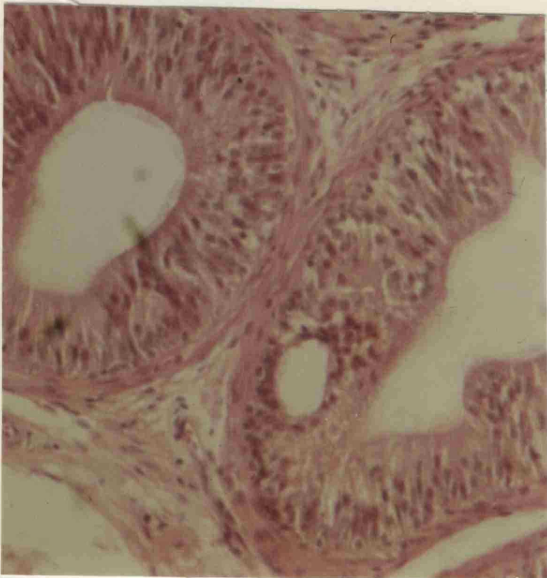
Fig. 1.2.3.9. Hyperplasia of epididymal epithelium of an ageing Beagle. H X E, x 125.
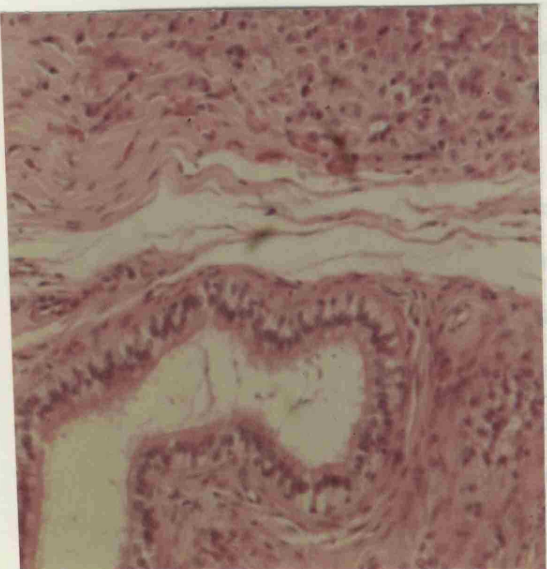
Fig. 1.2.3.10. Epididymal sperm-atocoele granuloma in a Beagle. H & E, x 125.
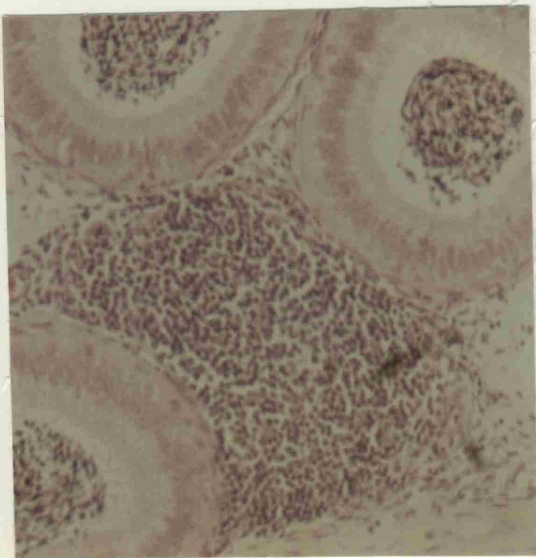
Fig. 1.2.3.11. Focal chronic inflam-matory cell infiltration in the epididymis of a Beagle dog. H & E, x 125.

Occasional epididymal spermatocoele granulomata were found for dogs
of variable age (Fig. 1.2.3.10.). The lesions are essentially
similar to those seen in the rat and probably occur as a result of
slight injury (Cronqvist, 1949; Friedman and Garske, 1949; Glassy
and Mostofi, 1956). Minor epididymal, focal chronic inflammatory
cell infiltrations (Fig. 1.2.3.11.) were found at a low incidence
in dogs of all ages, but occurred most frequently among the oldest
dogs.

1.3.3. Quantitative testicular histomorphometry

A number of well-defined spermatogenic cells can be identified in
histological sections of the testis. The status of spermatogenic
activity is normally assessed in a subjective manner, being
described as normal, reduced or suppressed. Counting of spermato-
gonia, spermatocytes and spermatids may be useful in determining
which particular cell types are affected when a selective toxic
effect on spermatogenesis is suspected (Heywood and James, 1978).
In order to undertake realistic quantitative assessment of spermato-
genic cell populations, the kinetics of the seminiferous epithelium
must be considered. Cell division proceeds in waves along the
longitudinal
horizontal axis of the seminiferous tubules. Clockwork precision
and synchronisation of the cell divisions results in constant and
predictable cellular associations. The time of appearance and
duration for each cell type is constant for each species. The
simplest available classification (Roosen-Runge, 1962) is based on
recognition of eight stages of spermatogenesis according to the
morphology and position of spermatids specifically associated with
earlier spermatogenic cells (Fig. 1.3.3.1.). Because
spermatogenesis is a continuous and dynamic process, not all cell
types are present in every tubular cross-section.

Detailed morphological descriptions of the cellular associations

found in the seminiferous tubules of both the rat and dog have been

published (Clermont and Perey, 1957a, b; Foote et al., 1972; Leblond

and Clermont, 1952a, b). The mechanism of stem cell (sperm-

atogonial) renewal is critical. Clermont (1962) undertook elaborate

mapping of spermatogonial populations in rat testes and subsequently

proposed a mathematical model of stem cell renewal for this species.

These investigations indicated that type A spermatogonia are

arranged in pairs which undergo three successive synchronous

mitoses. Of the 16 resulting cells, two become dormant for a time

and subsequently divide to form the next generation of type A

spermatogonia. The remaining 14 cells divide mitotically to form

intermediate type spermatogonia which undergo a further mitosis to

form type B spermatogonia. Mitotic division of type B spermatogonia

results in the formation of primary spermatocytes which undergo

meiotic division to produce spermatids. In the normal adult rat

the actual yields of spermatocytes and spermatids are lower than

expected, because of constant degeneration of some type A

spermatogonia and spermatocytes during cell division. Foote et al.

(1972) were unable to discriminate adequately between the various

kinds of canine spermatogonia to establish an accurate model of

stem cell renewal for the dog. While the spontaneous degeneration

of canine spermatogonia remains speculative, degeneration of sperm-

atocytes does not seem to occur in the dog. Counts of type B

spermatogonia, primary spermatocytes and spermatids in tubules

identified at appropriate stages of spermatogenesis may provide a

basis for quantitative assessment of spermatogenesis in both the

rat and dog. The appropriate stage of spermatogenesis for

counting a particular cell type should be selected on the basis

that a complete generation of these cells is present.

The expected frequency of tubules at each of the eight stages for

normal adult rats and dogs is given in Table 1.3.3.1. Inspection

of these data shows that the various stages occur at differing

frequencies, and species differences are also apparent. Fortunately,

the cellular associations at each stage remain reasonably constant

for all animal species studied so far (Fig. 1.3.3.1.). Type B

spermatogonia are present in greatest numbers at stage 7, divid-

ing to form preleptotene primary spermatocytes by stage 8. The

primary spermatocyte phase is of long duration and, in fact, a

second generation of primary spermatocytes is formed before the end

of the prolonged pachytene phase which extends from stage 5 until

stage 2. During stages 5 - 8, only pachytene primary spermato-

cytes are present. After the prolonged pachytene phase the primary

spermatocytes enter a short diakenesis (stage 3); the secondary

spermatocytes appear in stage 4 and undergo the second reduction

division becoming round (early spermatids) by stage 5. During

stages 5 to 1, round spermatids undergo changes in the acrosomal

and Golgi systems, and the process of elongation begins at stage 2 .

Only round spermatids are seen in the lumina of tubules at stage 1.

Mature (elongated) spermatids become spermatozoa at stage 8 when

they are found detached from Sertoli cells, lying free in the tubular

lumen.

Table 1.3.3.2. shows some published mean counts for type B

spermatogonia, pachytene primary spermatocytes, round (early)

spermatids and mature (elongated) spermatids found in tubules at

stages 7, 5 and 8 of the spermatogenic cycle of normal adult

rats and dogs (Clermont, 1962; Foote et al., 1972). Photomicro-

graphs of tubules found at stages 1, 5, 7 and 8 in the testes

of rats and dogs are depicted in Figs. 1.3.3.2. and 1.3.3.3.

Stage of spermatogenesis[1]	Mean frequency (%, $\pm$ SEM) of stages 1 to 8	
	Rat[2]	Dog[3]
1 (vᴜ-ıx)	9.9 $\pm$ 0.8	21.9 $\pm$ 0.5
2 (x-xı)	5.0 $\pm$ 0.5	12.7 $\pm$ 0.7
3 (xıı-xııı)	17.3 $\pm$ 1.4	2.8 $\pm$ 0.3
4 (xıv)	5.0 $\pm$ 0.2	11.5 $\pm$ 0.8
5 (ı-ııı)	22.3 $\pm$ 1.5	8.3 $\pm$ 0.5
6 (ıv-v)	9.6 $\pm$ 0.6	15.4 $\pm$ 0.8
7 (vı)	9.2 $\pm$ 0.9	13.3 $\pm$ 0.6
8 (vıı)	21.8 $\pm$ 0.5	14.0 $\pm$ 0.7

Table 1.3.3.1. Frequency of tubules at stages 1 to 8 of spermatogenesis per 100 circular tubular cross-sections of rat and dog testes.

1. Identification of stages based on Roosen-Runge (1962)
2. Data obtained from Leblond and Clermont (1952b)
3. Data obtained from Foote et al. (1972)

Roman numerals in parenthesis indicate equivalent stages in Clermont's classification of rat spermatogenesis.

Stage	1	2	3	4	5	6	7	8
Late spermatids								Sz
Early spermatids	Pa							
Old primary spermatocytes	Pa	Pa	D	S	Pa	Pa	Pa	Pa
Young primary spermatocytes	L	L	Z	Pa				Pr
Type B spermatogonia								
Type A spermatogonia								

Key

Pr	preleptotene
L	leptotene
Z	zygotene
Pa	pachytene
D	diplotene/diakinesis
S	secondary spermatocyte
Sz	spermatozoon

Fig 1.3.3.1. Diagrammatic representation of cellular associations found in the seminiferous epithelium. Based on the rat, Clermont (1962) and Roosen-Runge (1962)

Spermatogenic cell	Stage of spermatogenesis[1]	Number of cells counted per tubule	
		Rat[2]	Dog[3]
Spermatogonia (Type B)	7	31	10
Primary spermatocytes (pachytene)	5	69	42
Spermatids, round (early)	1	208	108
Spermatids, elongated (mature)	8	-	88

Table 1.3.3.2. Numbers of spermatogenic cells counted in circular cross-sections at specific stages of spermatogenesis for rats and dogs.

1. Identification of stages based on Roosen-Runge (1962)
2. Data obtained from Hemsworth et al. (1968)
3. Data obtained from Foote et al. (1972)

Stage 1. Early spermatids (E); pachytene primary spermatocytes (P); leptotene primary spermatocytes (L).

Stage 5. Late spermatids (S); early spermatids (E); pachytene primary spermatocytes (P).
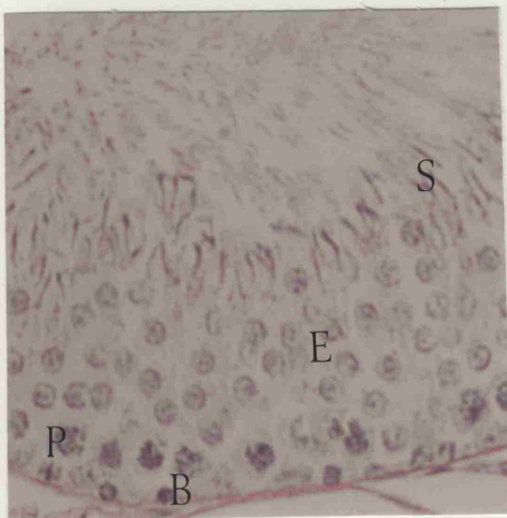
Stage 7. Late spermatids (S); early spermatids (E); pachytene primary spermatocytes (P); type B spermatogonia (B).
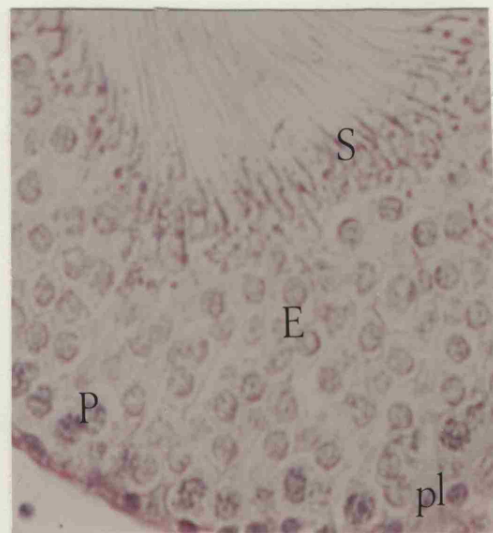
Stage 8. Late spermatids (S); early spermatids (E); pachytene primary spermatocytes (P); preleptotene primary spermatocytes (pl).

Fig. 1.3.3.2.   Photomicrographs of rat seminiferous tubules at stages 1, 5, 7 and 8. PAS, x 512
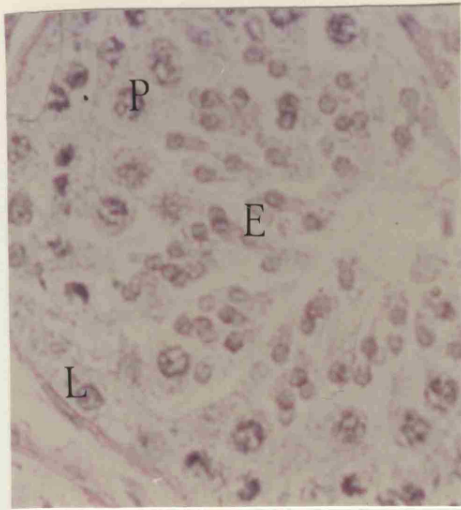
Stage 1. Early spermatids (E);
pachytene primary spermatocytes
(P); Leptotene primary
spermatocytes (L).
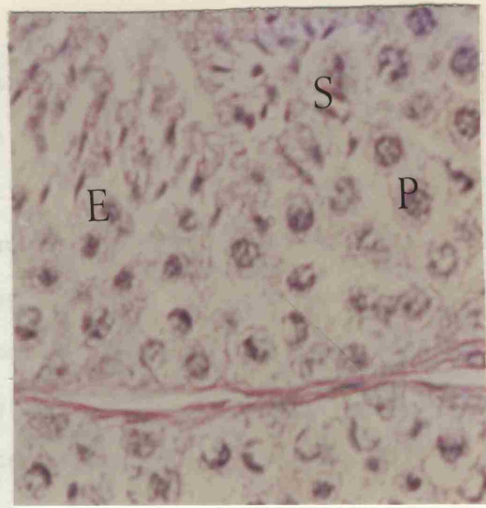
Stage 5. Late spermatids (S);
early spermatids (E); pachytene
primary spermatocytes (P).
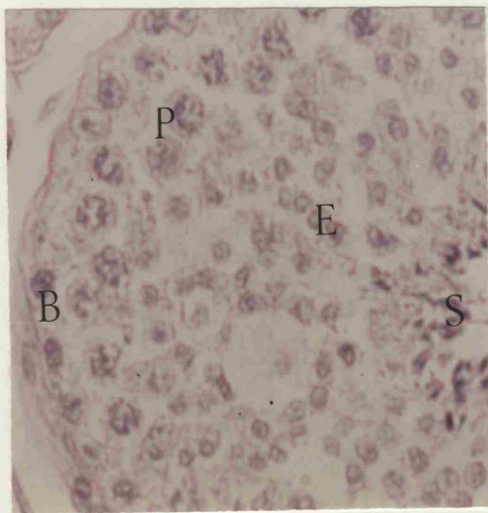
Stage 7. Late spermatids (S);
early spermatids (E); pachytene
primary spermatocytes (P); type
B spermatogonia (B).
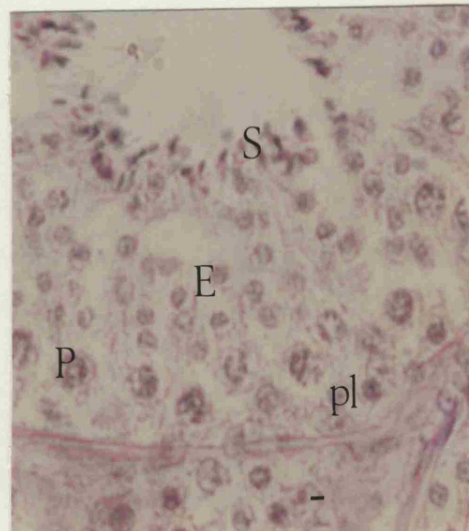
Stage 8. Late spermatids
(S); early spermatids (E);
pachytene primary spermatocytes
(P); preleptotene primary sperm-
atocytes (pl).

Fig. 1.2.3.3. Photomicrographs of canine seminiferous tubules at
stages 1, 5, 7 and 8. PAS, x 512.

Although a large number of substances have been found which induce changes in spermatogenesis, there are only a few reports of experimental studies utilising quantitative histomorphometric assessment of spermatogenesis. Hemsworth et al. (1968) made quantitative assessments of spermatogenesis in the testes of rats exposed to the pituitary inhibitor methallibure. Similar studies have also been undertaken for rats given triethylenemelamine and rabbits receiving amphotericin B (Steinberger, 1962; Swierstra et al., 1964). All these investigators were able to identify the target cell types affected and could at least infer the possible mechanisms of toxic action.

1.4. Discussion

The choice of animal model and characteristics selected for the predictive safety evaluation of chemicals exerting testicular toxicity is a challenging problem. The ideal criteria include demonstration of successful reproduction in animal species chosen on the basis of pharmacokinetic and metabolic similarity to man or the target species. The ultimate index of male fertility is the induction of successful pregnancy in receptive females. Although breeding studies are easily undertaken with rodent species, practical difficulties arise in the case of the larger non-rodent species. The problems to be overcome include seasonal breeding cycles, poor spontaneous conception rates, prolonged gestation periods and the large number of successful matings necessary for statistical analysis. The findings of the present studies offer possible alternative techniques to establish and elucidate mechanisms of testicular toxicity. Although these approaches are most useful for non-rodent studies, similar studies in rodent species may complement breeding performance data or provide a rational basis for evaluating differences in species response to the same chemical.

The measurement of testicular size, circulating pituitary-testicular hormones and semen examinations in the living animal can be undertaken without resorting to administration of diagnostic chemicals which may prejudice the overall toxicological objectives. Furthermore, the information obtained may usefully contribute to the assessment of histopathological observations in the dead animal. In practice, it seems that not all tests can be applied to all species of laboratory animal. The seasonal breeding rhythms and physical size of the common laboratory macaques and

baboons prevents serious consideration of these species except in the most specialised laboratories. It is also important to note that the present embargo on the export of rhesus macaques from their country of origin severely restricts the number of animals available to experimental scientists. It seems likely that, in the foreseeable future, the majority of studies will be restricted to laboratory rats and dogs. Consequently, the theoretical criteria of selecting animal species on the basis of physiological and metabolic suitability are set at nought. All the characteristics suggested are easily studied with dogs; however, the only useful observations applicable to the living rat are probably hormonal assays.

In order to interpret experimental data satisfactorily, background information concerning spontaneous and age-related variations of the measured parameters is vital. Considerable information of this type, concerning testicular function in the rat and dog, has become available as a result of the present work.

The measurement of testicular dimensions is a simple but reliable index of the rate of testicular growth in young dogs. Some variation in testicular dimensions is apparent for adult dogs, and the size of these organs does not appear to be related to current body weight. In order to assess the effect of chemical overdosage on testicular size, it seems necessary to establish predosing base-line data for individual dogs. Marked spontaneous fluctuations in testicular measurements are not expected to occur if young adult dogs are studied. The profile of circulating FSH, LH, testosterone and prolactin provides valuable information on hypothalamic-pituitary-testicular relationships. The pattern of chronological response may be expected to differ according to the site of chemical

action. The effects of chemicals which suppress pituitary
gonadotrophin secretion, inhibit hypothalamic control of prolactin
release or alter testosterone synthesis will be apparent at an early
stage and should precede any observable changes in the testicular
functions dependent on these hormones. Conversely, if the primary
sites of chemical action are the target tissues for these hormones,
delayed and progressive changes in circulating hormone concentrations
will follow as a result of the sensitive feed-back control mechanisms.
If the cells of the seminiferous epithelium are depleted, the
principal response is a compensatory rise in FSH (Collins et al.,
1978; Debeljuk et al., 1973). The lack of availability of a canine
FSH assay is a drawback in this respect.

When considering the results of hormonal investigations it is
important to consider any extraneous influences which may affect the
values obtained. Time of day, season of the year, age, species and
strain, or the stress of the sampling procedure, can all exert
measureable effects. The results of the plasma prolactin, LH and
testosterone determinations in growing male Beagles indicate that
the hormonal events coinciding with the onset of sexual maturation
in this species are, at least qualitatively, similar to the changes
observed in male rats (de Jong and Sharpe, 1977: Piacsek and
Goodspeed, 1978). Maturational changes in the concentration of male
reproductive hormones must be considered if rats aged less than 70
days or dogs under 29 weeks old are examined. There is sufficient
available evidence to show that the circulating hormonal profile
of male rats changes with age, particularly in respect of prolactin
(Reigle and Meites, 1976) and LH and testosterone (Gray, 1978).
There do not appear to be any published observations on the hormonal
status of aged male dogs.

Male Beagles appear to respond readily to the use of the artificial vagina and no difficulties should be experienced in obtaining semen from young naive dogs which have not previously been used as breeding animals. The available evidence implies that adult semen characteristics are not fully established in Beagles less than 41 weeks old. The examination of semen quality for dogs aged between 41 and 66 weeks of age showed considerable variation in respect of ejaculate density, motility and sperm concentration. This is in agreement with the findings of other authors (Bartlett, 1962a; Boucher et al., 1958; Harrop, 1955; Heywood and Sortwell, 1971). This emphasises the need carefully to screen dogs selected for testicular function studies, during the predosing phase, in order to exclude dogs which consistently produce semen of poor quality. The assessment of sperm motility in canine ejaculates may be misleading, because zero scores are frequently recorded which may be attributable to cooling of the samples during the protracted collection time of up to 20 minutes. Changes in semen characteristics may reflect the mode of chemical action. The timing and appearance of changes in sperm concentration and morphology will differ if toxic action affects the cells of the seminiferous epithelium, the hormonal control of spermatid maturation or the production of epididymal secretions necessary for sperm capacitation. It is important to consider that, from the time of spermatogonial division until the appearance of spermatozoa in the ejaculate, four cycles of the seminiferous epithelium occur (Roosen-Runge, 1962). Consequently, there will be delays in the appearance of spermatozoal abnormalities following chemical insult to the seminiferous epithelium. The earlier the stage of spermatogenesis affected, the longer the latent period to the recognition of semen abnormalities. Rapid loss of libido, or marked changes in the volume of ejaculate, will be indicative of primary effects on androgenic hormones.

The limited data available for the biochemical examination of semen are insufficient to substantiate definite conclusions. There are some indications that useful information may be obtained from the determination of acid phosphatase and aspartate aminotransferase concentrations in seminal plasma. Further studies are needed to define the relationships between aspartate aminotransferase and sperm morphology and motility. There is evidence that acid phosphatase can serve as an index of prostate function, and this estimation can usefully be included where effects on the secretory activity of the prostate are suspected.

Testicular biopsy is not recommended for inclusion in routine toxicological studies. There is a low, but significant, risk of inducing testicular atrophy, which is unacceptable. While sufficient tissue can be obtained to allow subjective histological assessment, insufficient tubules in circular cross-section are obtained to perform detailed quantitative analysis. The serial testicular biopsies obtained from Beagles between 27 and 45 weeks of age have provided some useful information. Before 29 weeks, spermatogenesis did not proceed beyond the spermatocyte stage. The numbers of primary spermatocytes and spermatids increased between 29 and 37 weeks of age. The characteristic organisation of the seminiferous epithelium was not apparent before 37 weeks. It therefore seems that organised divisions of the canine seminiferous epithelium probably are not established earlier than 37 weeks post partum. This would appear to be the first reported investigation of the physiological onset and maintenance of canine spermatogenesis.

Interpretation of testicular weights should be based, not only on comparison with concurrent control animals, but also with regard to the age of the animals. The testicular weights of rats maintained for 13-week or 26-week studies showed remarkably little variation. The weight of the testes seems to decrease in ageing rats. Canine testicular weights showed considerably more variation than that found for rats. Testicular growth may not be complete in dogs of the age conventionally used for 13-week studies. Low testicular weights found for dogs of this age should be only cautiously attributed to chemical administration, particularly when small groups of dogs are studied. In marked contrast to the rat, the weight of the canine testes appears to increase in old age.

The principal spontaneous histological variation observed in rat testes was the development of inflammatory cell infiltrations, involving testicular blood vessels and atrophic changes in the seminiferous epithelium of aged rats. Approximately 20% of Sprague-Dawley rats maintained for 104 weeks' observation can be expected to develop these lesions. A much higher incidence of these changes would be necessary to recognise chemically induced lesions with certainty. The Sprague-Dawley rat appears to be unusual in that it shows a low incidence of spontaneous testicular neoplasms. No difficulty should be experienced, therefore, in recognising the carcinogenic effects of chemicals on the testes of rats of this strain. Incomplete testicular spermatogenesis was a feature of 7% of the dogs maintained for 13-week studies. This is a sufficiently high incidence to complicate the assessment of testicular effects in dogs studied before the attainment of sexual maturity. Arteritis and degeneration of the seminiferous epithelium occur in ageing dogs, but at a considerably lower incidence than among rats. Again in marked contrast to the Sprague-Dawley rat, spontaneous testicular neoplasia is a major feature of the older

dog. Although carcinogenicity studies are rarely undertaken with dogs, it is important to record the high rate of spontaneous testicular tumours in this species. Hyperplasia of the epithelium of the ductus epididymis was found in the majority of dogs exceeding seven years of age; however, no explanation can be offered for this phenomenon.

1.5. Abstract

Techniques, other than serial mating, for the assessment of testicular toxicity in the laboratory rat and Beagle are described. Function tests performed in live animals can complement post-mortem studies. Testicular measurements, pituitary-testicular hormone assays and semen examinations, together with testicular weights, histology and quantitative histometric analysis of sperm-atogenesis, provide useful information to identify sites of chemical action and so elucidate mechanisms of toxicity. The impact of spontaneous variations due to sexual maturation, ageing, circadian or seasonal rhythms and experimental techniques is discussed.

PART TWO

2. Studies of chemical action on the hypothalamic-pituitary-testicular axis

The testicular changes arising from chemical modulation of hypothalamic-pituitary hormones have been examined by many investigators as a possible method of male contraception. Natural and synthetic androgens, oestrogens and progestogens, hypothalamic releasing factors and compounds which compete for receptor sites mediating the neurohumoral feedback control and peripheral actions of male hormones, have been extensively studied. Toxicological appraisal of these compounds includes demonstration that the intended pharmacological action is readily reversible, assessment of potential teratogenic or genetic hazards, modifications in sexual activity and undesirable effects on extragonadal tissues. These aspects of testicular toxicity are also applicable to the safety evaluation of a wide variety of agents which, although developed for non-andrological indications, may modulate hypothalamic-pituitary hormones when repeatedly administered to experimental animals at excessive dosages. The roles of centrally active neurotransmitters and prostaglandins in the secretion of anterior pituitary hormones are becoming increasingly clear (Krulich, 1979; Sato et al., 1975). Modification of neurotransmitter substances forms the pharmacological basis for the therapeutic actions of many narcotics, sedatives, tranquillisers, mood-modifying drugs, bronchodilators and some cardiovascular agents. Inhibition of prostaglandin synthesis is an important property of non-steroidal anti-inflammatory drugs used in the treatment of arthritis. Although it is rare for these compounds to disrupt spermatogenesis when administered to animals at low multiples of the recommended therapeutic dosages, the effects of chronic overdosage must also be

studied. The currently accepted practice is to study the effects of

overdosage on the mating performance of rodents and to examine

changes in the weights and morphology of male reproductive organs

of rodents and non-rodents following prolonged exposure. Recent

experience at Huntingdon Research Centre has suggested that some

compounds,although not affecting the fertility of male rodents,

suppress spermatogenesis in Beagle dogs.

In view of these apparent differences in species sensitivity, some

experiments were undertaken to study the hypothalamic-pituitary-

testicular responses of rats and Beagle dogs, following overdosage

with compounds known to act on central neurotransmitters or to

inhibit prostaglandin synthesis.

2.1. Administration of morphine sulphate to rats for 9 weeks

Narcotic analgesic compounds are widely used in the symptomatic relief of severe pain. Many compounds such as morphine, heroin, methadone, levorphanol and pentazocine, which possess this pharmacological property, are based on the γ-phenyl-N-methyl piperidine configuration (Gero, 1954). The pharmacological mechanisms whereby these drugs induce analgesia are not clear, but inhibition of neuronal firing at opiate receptor sites occurs both in the central and peripheral nervous systems (Bradley et al., 1978; North and Williams, 1977). Social abuse of these drugs results in well-documented side effects in humans who become physically dependent on these drugs (Takemori, 1974). Modifications of male and female reproductive physiology in narcotic addicts have been described (Thomas et al., 1977). Decreased plasma testosterone concentrations, loss of libido, impotence, reduced ejaculate volume, and sperm motility defects have been correlated with heroin and methadone use in men (Cicero et al., 1975a; Mendelson et al., 1975). A number of investigators have attempted to define the effects of narcotic analgesic overdosage on the hypothalamic-pituitary-testicular axis and secondary sex organs of male rodents (Cicero et al., 1974, 1975b, 1976a; Thomas and Dombrosky, 1975).

Changes in the weight of the testes, prostate and seminal vesicles, and increased prostatic cyclic adenosine monophosphate (cAMP) activity, have been described. More recently, attention has been given to the effects of overdosage on hypothalamic luteinising hormone-releasing hormone (LH-RH) and pituitary secretion of LH (Cicero, 1977; Cicero et al., 1976b, 1977a, 1977b; Muraki et al., 1978). Morphine overdosage apparently inhibits release of LH-RH, so that LH is not released from the pituitary gland, and circulating concentrations of LH therefore decrease. This last effect is

correlated with the half-life of the drug and, following clearance of morphine, the circulating LH concentration reverts to normal levels but total 24-hour production is decreased. Follicle-stimulating hormone (FSH) production is not affected, but the decreased LH concentrations precede reductions in testosterone concentrations. The effects of narcotic analgesic overdosage on pituitary-testicular morphology have not, apparently, been assessed. An experiment was therefore performed with Sprague-Dawley rats in an attempt to correlate changes in pituitary-testicular morphology with the known effects of morphine overdosage on gonadotrophin and androgen secretion.

2.1.1. Experimental design

Thirty specific pathogen-free male rats (CD strain) aged 35 days were obtained, and maintained under standard conditions as described in Appendices I (i) and (ii). Morphine sulphate crystals B.P. were obtained (Boots Wholesale Chemists, Ltd., UK). The acute subcutaneous LD_{50} of morphine sulphate to rats is 229 $\pm$ 46 mg/kg (Chen, 1948). Fifty mg/kg/day was selected as a suitable dose to assess the chronic effects of morphine overdosage. Morphine sulphate was dissolved in sterile water for injection (5% w/v) and administered by subcutaneous injection at a volume of 0.1 ml/100 g to 15 rats for up to nine weeks so that each rat received 50 mg morphine sulphate/kg/day. The remaining 15 rats acted as controls and received daily injections of sterile water at 0.1 ml/100 g. Five rats from each group were killed after four or nine weeks' treatment; the remaining rats were allowed 13 weeks' recovery after receiving treatment for nine weeks.

The rats were examined several times daily for assessment of
clinical and behavioural abnormalities. Body weight changes and
food and water intake were monitored, see Appendix I (iii). Rats
which died prematurely were given complete post-mortem examinations
to establish factors contributing to death.

Serum was obtained, see Appendix I (v), from five rats per group,
4 h after dosing on day 1, during weeks 4 and 9, and after 13
weeks' recovery. Testosterone, FSH and LH concentrations were
measured in each serum sample, see Appendix I (vi). Terminal
examination of rats killed at the scheduled intervals included
weighing of the testes, seminal vesicles, prostate and pituitary
glands. Portions of these tissues were preserved and prepared for
histological examination, see Appendix I (viii). Measurements of
seminiferous tubule diameter, and counts of type B spermatogonia,
pachytene primary spermatocytes, early and late spermatids, and
Sertoli cells were made, see Appendix I (ix).

2.1.2. Results

There were three premature deaths: one control rat during week 7
of the recovery period and two rats which were given morphine, one
during the ninth week of dosing and one during the fourth week of
the recovery period. These deaths were attributable to chronic
respiratory disease.

Injection of morphine at 50 mg/kg/day induced marked sedation during
the first and second days of the dosing period; subsequent tolerance
developed rapidly. During the last two weeks of the dosing period,

the morphine-treated rats became irritable and aggressive during the later afternoon, presumably as a result of increasing physical dependence liability. Morphine-treated rats showed an overall 30% reduction of body weight gain in comparison with control rats over the nine-week treatment period (Fig. 2.1.2.1.). During the period following morphine withdrawal, the rate of change of body weight was essentially comparable for both groups of rats. Rats given morphine ate less than control rats during the first three weeks of dosing; this trend subsequently regressed (Fig. 2.1.2.1.). Reduction of food intake was also seen during the first week following withdrawal of morphine; there were no subsequent significant differences in food consumption among rats retained for 13 weeks' recovery. No differences were found between control and treated rats in respect of serum FSH concentration. Treatment with morphine sulphate at 50 mg/kg/day resulted in persistent reductions in the concentrations of LH and testosterone detectable in sera obtained 4 h after dosing (Table 2.1.2.1.). There was no evidence of a progressive reduction in hormone concentration as the period continued. This effect was completely reversed for rats examined 13 weeks after the final dose of morphine.

The effects of repeated morphine treatment on pituitary, testicular and secondary sex-organ weights are presented in Table 2.1.2.2. Because of the marked effects on body weight, analysis of covariance was undertaken with initial and final body weights as covariates. When significant interaction between body weight and treatment was apparent, organ weights were adjusted for differences in body weight before analysis of variance. No statistically significant effects (p >0.05) were found with regard to pituitary or testicular weights. Prostatic weights were reduced after four and nine weeks' treatment, but seminal vesicle weights were reduced after four weeks' treatment only.

Body weight (g)

Food intake (g/rat/week)
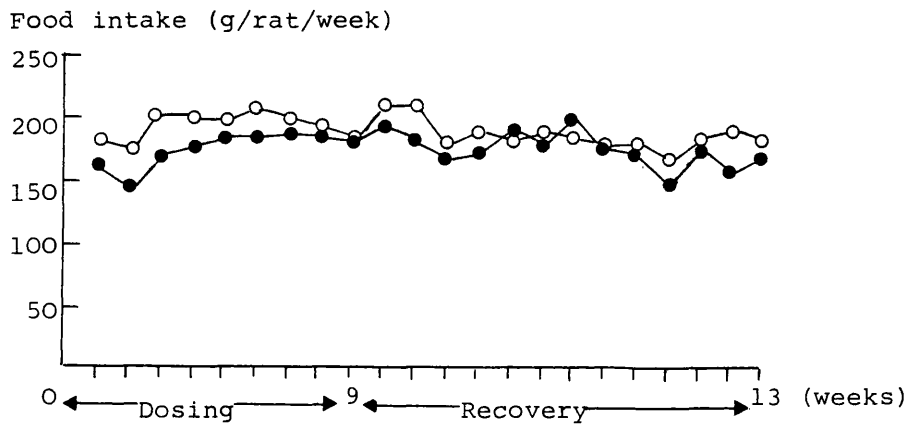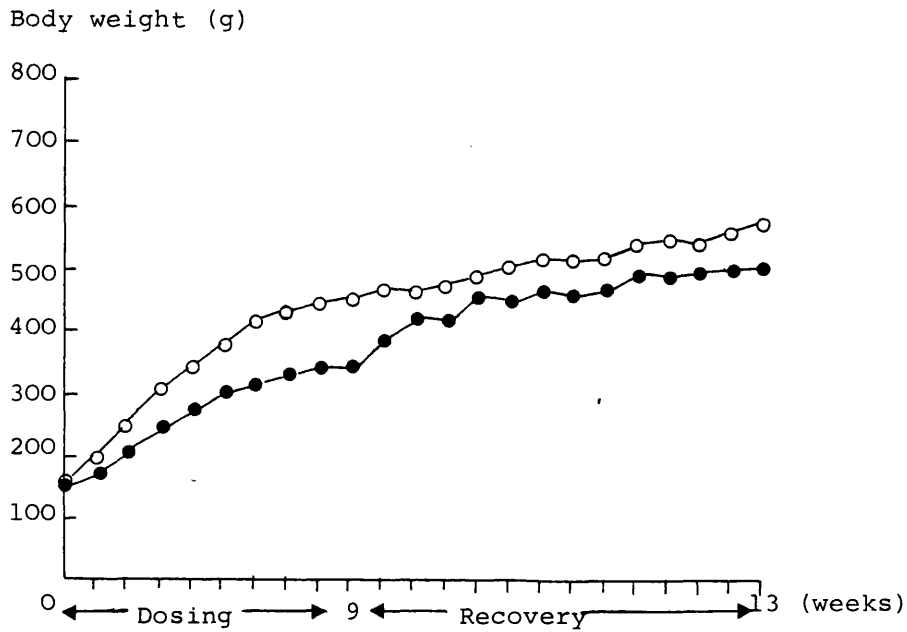
Fig. 2.1.2.1. Effects of morphine sulphate (50 mg/kg/day) on body weight and food intake when administered to male rats for nine weeks, followed by a 13-week recovery period. Controls (O); morphine sulphate (●).

Hormone	Treatment	Mean serum concentrations (ng/ml) after			
		Dosing			Withdrawal for
		1 day	4 weeks	9 weeks	13 weeks
LH	Controls	24 ± 31	14 ± 7	14 ± 15	52 ± 57
	Morphine	<5	<5	<5	43 ± 38
FSH	Controls	492 ± 159	284 ± 65	255 ± 67	235 ± 65
	Morphine	421 ± 177	389 ± 343	326 ± 65	278 ± 54
Testosterone	Controls	0.93 ± 0.21	0.58 ± 0.39	2.74 ± 1.43	0.65 ± 0.17
	Morphine	0.23 ± 0.06++	0.28 ± 0.18	0.60 ± 0.17+	1.15 ± 0.50

Table 2.1.2.1. Effects of morphine sulphate (50 mg/kg/day) on serum LH, FSH and testosterone concentrations when administered for nine weeks, followed by a 13-week recovery period.

Statistically significant differences from concurrent control data (ANOVAR): $p < 0.05$[+]; $p < 0.01$[++].

LH expressed as ng NIAMDD-LH-RP1/ml and FSH as ng NIAMDD-FSH-RP1/ml.

Examination	Treatment	Body weight (g)	Organ weights			
			Pituitary (mg)	Testes (g)	Prostate (g)	Seminal vesicle (g)
4 weeks' dosing	Controls	331 ± 28	9 ± 2	4.0 ± 0.2	0.5 ± 0.1	1.3 ± 0.4
	Morphine	266 ± 21[++]	12 ± 4	3.7 ± 0.2	0.3 ± 0.1[+]	0.8 ± 0.3[+]
9 weeks' dosing	Controls	450 ± 47	12 ± 2	4.7 ± 0.3	0.8 ± 0.3	1.6 ± 0.3
	Morphine	354 ± 49[+]	11 ± 2	4.4 ± 0.4	0.3 ± 0.1[+]	1.2 ± 0.3
13 weeks' recovery	Controls	534 ± 50	16 ± 3	5.3 ± 1.2	0.8 ± 0.4	2.1 ± 0.7
	Morphine	454 ± 38	13 ± 4	5.1 ± 0.5	0.7 ± 0.2	2.4 ± 0.3

Table 2.1.2.2. Body weights and pituitary, testes, prostate and seminal vesicle weights of rats killed after receiving morphine sulphate (50 mg/kg/day) for four or nine weeks, or after 13 weeks recovery. Statistically significant differences (ANOVAR) from concurrent control data: $p < 0.05$[+]; $p < 0.01$[++].

Differences in pituitary gonadotrophic cell morphology were
detected after four and nine weeks' morphine treatment (Fig.
2.1.2.2.). Cellular atrophy, nuclear pyknosis and condensation
of granules were thought to indicate a reduction in secretory
activity. Pituitary morphology was comparable for control and
previously dosed rats examined 13 weeks after experimental
treatment had stopped. No changes in cellular morphology of
prostates or seminal vesicles were detected, although decreased
eosinophilia of the secretions of these glands was apparent. All
stages of spermatogenesis were recognisable on histological
examination, but mature spermatids, detached in the lumina of
seminiferous tubules, were less obvious in testes obtained after
four and nine weeks' treatment with morphine sulphate (Fig. 2.1.
2.3.) than in the testes of control rats.

Quantitative histometric analysis of spermatogenesis gave further
evidence that administration of morphine sulphate affected
spermatid development (Table 2.1.2.3.). Statistically significant
($p < 0.05$) decreases in crude and corrected counts of early (stage
1) spermatids and late (stage 8) spermatids were recorded
after four and nine weeks' treatment. After nine weeks' treatment
significant decreases were also found for Sertoli cell counts,
pachytene primary spermatocytes and type B spermatogonia. These
differences had essentially regressed in rats examined after 13
weeks' recovery.

Control rat
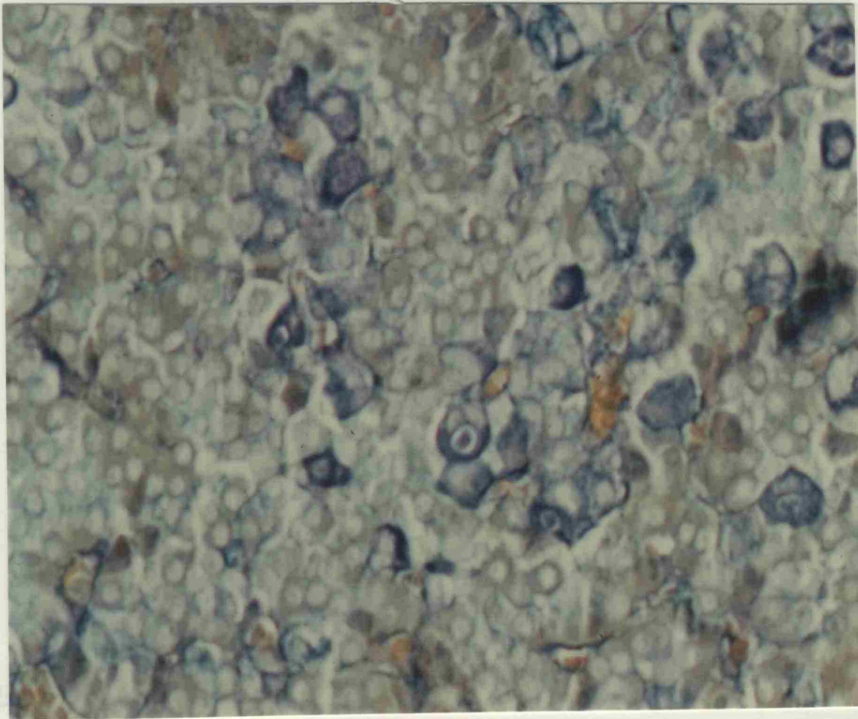
Morphine-treated rat
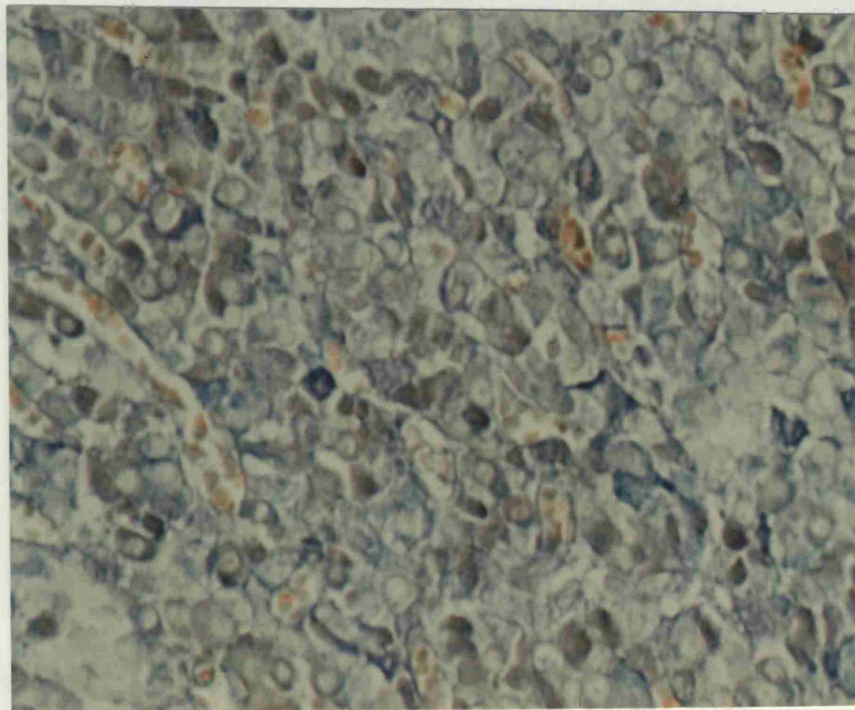
Fig. 2.1.2.2. Sections of anterior pituitary glands from a control

rat and a rat given morphine sulphate (50 mg/kg/day) for four weeks.

PAS-Alcian blue-Orange G: x 512.

Control rat
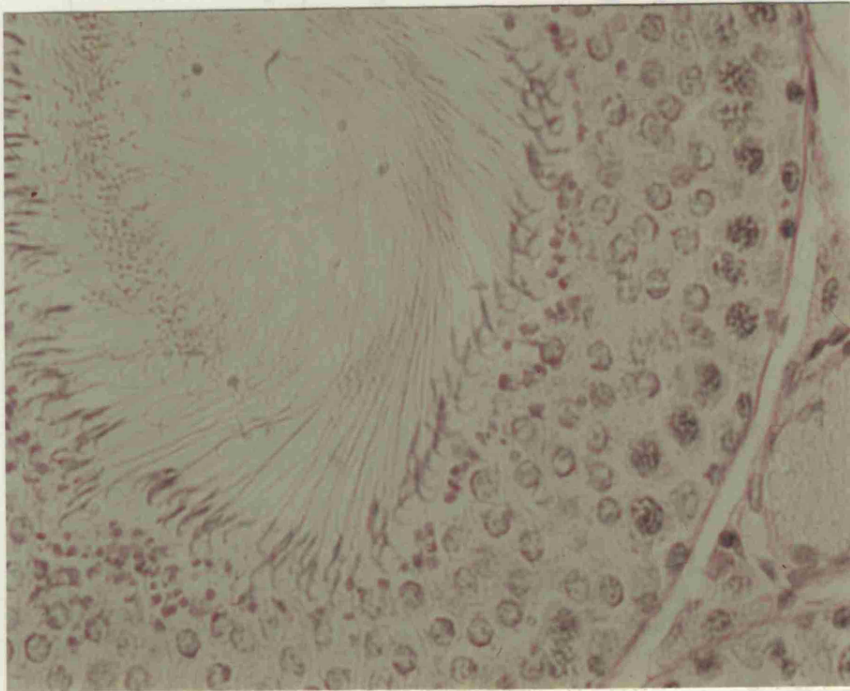
Morphine-treated rat
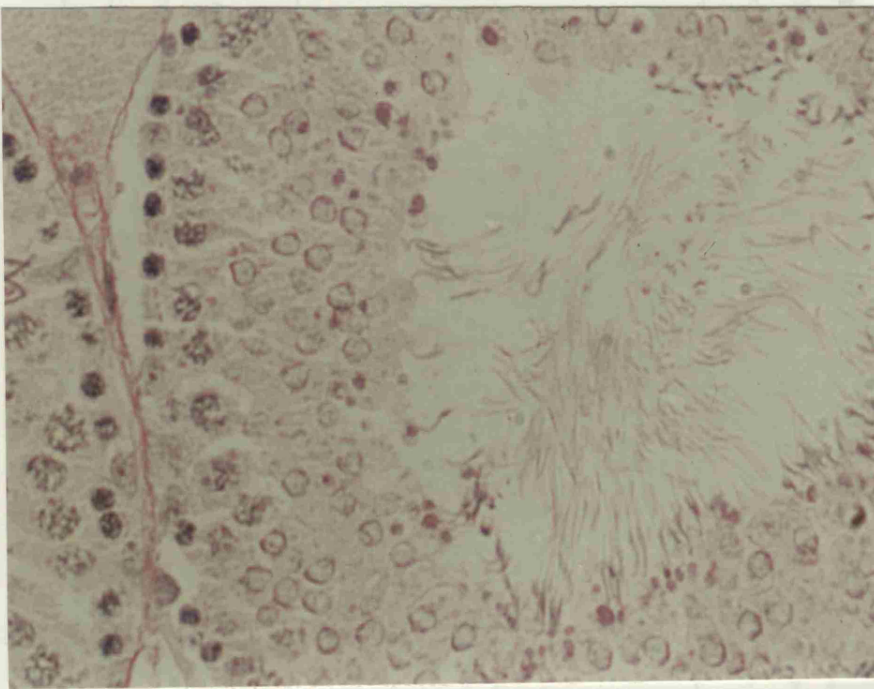
Fig. 2.1.2.3.   Seminiferous tubules at stage 8 of spermatogen-
esis of a control rat and a rat given morphine sulphate (50 mg/kg/
day) for nine weeks.   PAS: x 512.

Treatment	Mean values										
		Tubule diameter (µm)	Type B spermatogonia (stage 7)		Pachytene spermatocytes (stage 5)		Spermatids				Sertoli cells	
							Early (stage 1)		Late (stage 8)			
			Crude	Corrected	Crude	Corrected	Crude	Corrected	Crude	Corrected	Crude	Corrected
Controls		277 ± 13	35	15.5	63	20.0	173	67.3	150	64.1	30	16.8
Morphine sulphate (50 mg/kg/day)												
4 weeks' dosing		$255 \pm 11^{+++}$	31^{++}	14.2	58	19.4	150^{+}	61.1	95^{+++}	42.1	27^{++}	16.9
9 weeks' dosing		281 ± 15	28^{+++}	11.9	52^{++}	16.9	124^{+++}	47.3	91^{+++}	37.1	26^{++}	14.9
13 weeks' recovery		$302 \pm 7^{+++}$	32	12.8	63	18.9	183	65.3	153	60.0	29	15.7

Table 2.1.2.3. Quantitative assessment of spermatogenesis for male rats given morphine sulphate (50 mg/kg/day) for four or nine weeks, or after 13 weeks' recovery. Statistically significant differences from control data (ANOVAR): $p<0.05^{+}$; $p<0.01^{++}$; $p<0.001^{+++}$. N.B. Corrected counts not analysed statistically

2.1.3. Discussion

Decreases in circulating LH concentrations can be induced in rats, following overdosage with a variety of narcotic analgesic agents (Cicero, 1977). This effect can be correlated with pharmacological potency in a variety of in vitro test systems, including displacement of radiolabelled opiates from brain receptor homogenates, inhibition of guinea-pig ileum contractions, naloxone blockade and stereospecificity (Kosterlitz and Waterfield, 1975). Tolerance and physical dependence are important features of morphine abuse, but the available literature indicated that the hormonal modifications do not differ substantially between tolerant and non-tolerant rats. A constant dosage schedule was selected for this investigation because it was intended to assess the toxicological significance of simple narcotic overdosage rather than to mimic narcotic addiction. The dose of 50 mg/kg/day represents a 35- to 50-fold multiple of the recommended therapeutic dose (1-1.5 mg/kg). Although a degree of physical tolerance developed after repeated administration of 50 mg/kg/day, the hormonal effects after nine weeks' treatment were similar to those detected after one day's administration.

Macroscopic post-mortem examination, organ weight analysis and subjective histological examination are key features of conventional safety evaluation studies. Based on these criteria, the only conclusive evidence of treatment-related effects on male reproductive physiology were the differences detected in weight, without major morphological changes for the prostate glands and seminal vesicles. Specialised staining of pituitary gland sections provided some evidence of reversible modifications in the secretory activity of gonadotrophic cells. Although subjective assessment of testicular histology suggested a reduction in the numbers of mature spermatids among morphine-treated rats, all stages of spermatogenesis were present in the sections examined.

Quantitative assessment of testicular spermatogenesis revealed a degree of impairment in germ-cell differentiation. The earlier work of Clermont and Morgentaler (1955) demonstrated that spermatogenesis is not completely abolished in rats, following hypophysectomy. Although initial disruption occurs, a degree of residual spermatogenesis is restored within 25 days. Chowdhury (1979) provided evidence that, apart from stem-cell renewal and spermatid maturation, other phases of spermatogenesis in the rat are not entirely hormone-dependent. Administration of anti-LH serum for 14 days to 20-day-old rats reduced secondary sex-organ weights and the counts of pachytene spermatocytes and round spermatids (Madhwa Raj and Dym, 1976). It is possible to conclude that the reversible modifications of pituitary-testicular morphology detected in this investigation were consequences of reduced LH concentrations following morphine overdosage. Further experiments are necessary to determine whether these effects result in impaired breeding performance of male rats.

2.1.4. Abstract

Morphine sulphate was administered, by subcutaneous injection, to male rats at a dose of 50 mg/kg/day for up to nine weeks. Control rats were given injections of sterilised water (BP). Serum LH and testosterone concentrations and the weight and morphology of testes, pituitary glands and secondary sex organs were examined after four and nine weeks' morphine treatment and also 13 weeks after dosing stopped. Treatment with morphine decreased serum LH and testosterone concentrations and reduced secondary sex-organ weights. Differential staining techniques revealed modified secretory activity of pituitary gonadotrophic cells. All stages of spermatogenesis were found in testicular sections, but

quantitative reductions in spermatogenic cell populations were
found among morphine-treated rats. All the observed effects
were reversed within 13 weeks of drug withdrawal. These findings
are discussed in relation to existing knowledge of the hormonal
control of spermatogenesis in rats.

2.2. Administration of cogazocine lactate to Beagle dogs for 9 weeks

The effects of morphine overdosage in rats were studied in the previous experiment. A review of the literature did not provide any recorded evidence that the hypothalamic-pituitary-testicular effects of narcotic analgesic overdosage had been examined in dogs. Erratic responses to administration of therapeutic doses of morphine are common in dogs: consequently, prolonged morphine overdosage was considered to be impracticable for this species. In recent years, attention has been given to the development of morphine analogues which retain analgesic potency but have a reduced physical dependence liability. The benzmorphan derivatives which contain narcotic antagonist chains on the γ-phenyl-piperidine configuration show only minimal addictive properties. Karras and North (1979) consider that the beneficial pharmacological effects of narcotic analgesics are probably due to inhibition of neuronal firing or neurotransmitter release, whereas the development of physical dependence is likely to be associated with an effect on intracellular cyclic nucleotide metabolism. Because the effects of morphine on LH are probably due to inhibition of central neuronal pathways, overdosage with non-addictive narcotic analgesics may exert similar effects. Cogazocine lactate, 2-cyclobutyl methyl-5-ethyl-2'-hydroxy-9, 9-dimethyl-6,7-benzmorphan lactate, is a novel benzmorphan analgesic which is 80 times more potent in the rhesus monkey dental pulp stimulation test and 30 times more potent in the rat writhing test than morphine sulphate (unpublished data, Huntingdon Research Centre). Preliminary investigations showed that repeated overdosage of dogs with cogazocine lactate did not provoke the emesis, defaecation and hyperaesthesia sometimes induced with morphine in this species. This compound was

selected to study the testicular responses of dogs following overdosage with a narcotic analgesic. At the same time it was possible to determine whether suppression of circulating LH concentration is a common property of both addictive and non-addictive narcotic agents.

2.2.1. Experimental design

a) Four adolescent male Beagles were obtained; the husbandry and management were as described in Appendices I (i) and (ii). Cogazocine lactate, supplied by ACF Chemiefarma, Maarsen, The Netherlands, was prepared as a 0.667% (v/v) solution in 5% dextrose so that 0.15 ml of solution contained 1 mg cogazocine lactate. Each dog was weighed before receiving a single intramuscular injection of cogazocine lactate at a dose of 1 mg/kg. Serum was obtained from each dog, see Appendix I (v), immediately before dosing and 0.5, 1, 2, 4, 6 and 24 h after administration of the drug. The concentrations of LH and testosterone, see Appendix I (vi), were measured in each serum sample.

b) Six sexually mature male Beagles were also obtained and maintained under standard conditions of husbandry and management, see Appendices I (i) and (ii). These dogs were given daily intramuscular injections of cogazocine lactate (1 mg/kg/day) for nine weeks. Two dogs were killed for terminal studies after nine weeks' treatment. Two dogs were killed nine weeks after receiving the last dose of cogazocine lactate and the remaining two dogs were killed 18 weeks after the last dose. Body weight changes, food and water consumption were recorded, see Appendix I (iii). The

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testes of each dog were measured, see Appendix I (iv), before

treatment started, after 2, 4 and 9 weeks' dosing and during

weeks 4, 9, 13 and 18 of the recovery period. Semen samples were

collected, see Appendix I (vii) before treatment, after 1, 2, 4 and

8 weeks' dosing and at approximately monthly intervals during the

withdrawal phase.

LH and testosterone concentrations, see Appendix I (vi), were

measured in serum samples obtained 24 h after the preceding dose

of cogazocine lactate on days 2, 8, 15, 29 and 57 of the dosing

period and during weeks 8 and 17 of the withdrawal period. Terminal

examinations comprised complete post-mortem examinations, and weigh-

ing of testes, pituitaries and prostate glands, with preservation of

these organs for histological examination, see Appendix I (viii).

Counts of type B spermatogonia, pachytene spermatocytes, early

(round) spermatids, late (elongated) spermatids, Sertoli cells and

measurements of seminiferous tubule diameters were made, as

described in Appendix I (ix).

2.2.2. Results

The administration of a single dose of cogazocine lactate (1 mg/kg)

induced narcosis lasting approximately 4 h. However, the clinical

appearance of the dogs was normal 24 h later. Repeated daily

administration of 1 mg/kg produced the same effect during the

first five days. Subsequently, the severity decreased and, after

the second week, the only central effects were transient drowsiness

lasting about 30 minutes. During the first week of dosing, food

intake was reduced and all dogs lost weight (Fig. 2.2.2.1.).

Body weight (kg)

(weeks)

Food residue (g/dog/week)
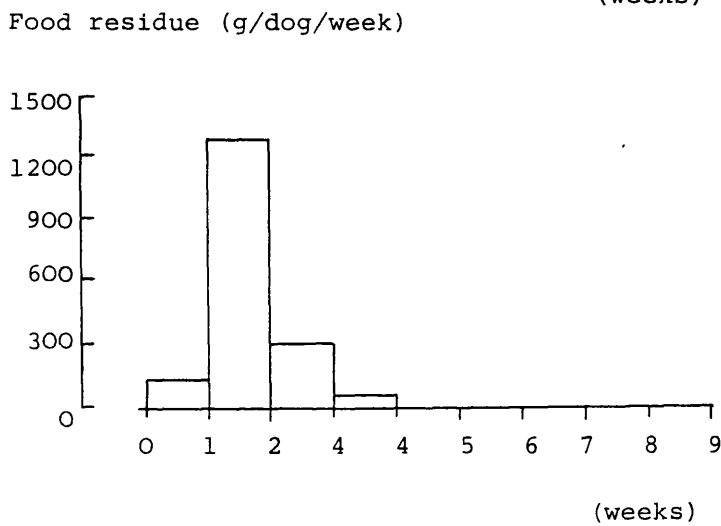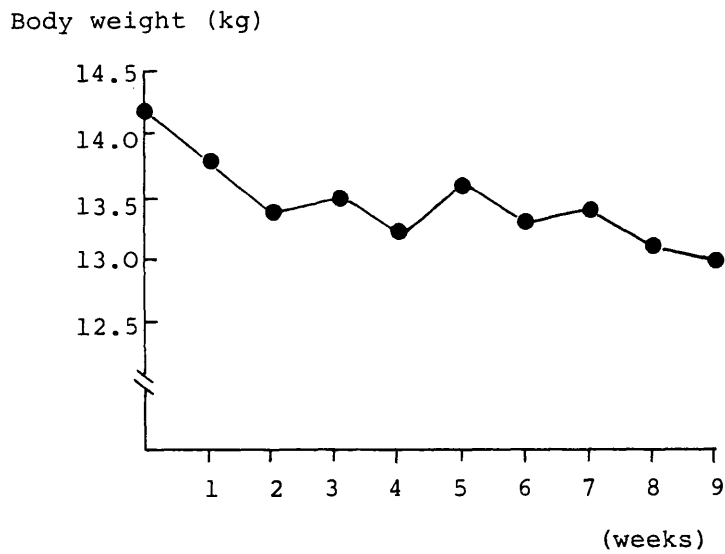
(weeks)

Fig. 2.2.2.1. Effects of cogazocine lactate on body weight
and food residue when administered to Beagle dogs at 1 mg/kg/
day for nine weeks.

Food intake subsequently improved but the body weight effect persisted. Following withdrawal of treatment, further weight loss occurred and body weight gains were not apparent until the tenth week after cessation of dosing. Eighteen weeks after withdrawal the body weights of the remaining two dogs were similar to those before treatment.

The acute effect of a single dose of cogazocine lactate (1 mg/kg) on serum LH and testosterone is shown in Fig. 2.2.2.2. The values obtained at 0 h were within the expected range of values for these hormones in young adult Beagles. After 0.5 h, LH values were decreased, although expected values for testosterone were still apparent. Serum concentrations of both hormones were depressed at 1, 2, 4 and 6 h after dosing. The serum of the same dogs showed normal LH and testosterone concentrations 24 h after administration of cogazocine lactate.

The mean changes in testicular area relative to the start of treatment are shown in Fig. 2.2.2.3. During the dosing period there was a progressive reduction in these areas, as shown by testicular measurement. Four weeks after cessation of dosing, recovery was apparent and the subsequent measurements did not differ appreciably from the pre-dose values. The results of hormonal assays, performed 24 h after the dose had been given, are shown in Table 2.2.2.1. Apart from reduced testosterone concentration 24 h after the first dose, the results were similar to, or slightly higher than, the baseline values. Before the start of treatment, all dogs produced satisfactory ejaculates.

Mean hormone concentration (ng/ml)
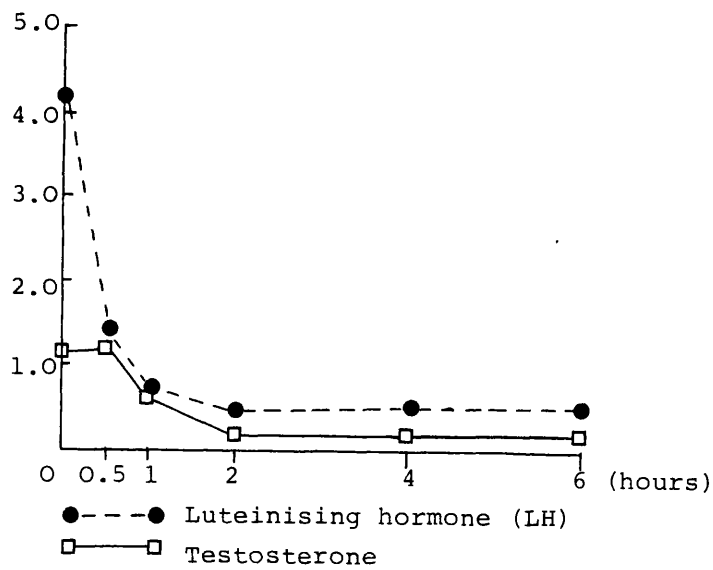
Fig. 2.2.2.2. The effects of a single dose of cogazocine
lactate (1 mg/kg) on serum LH and testosterone concentrations
of Beagle dogs. LH expressed as ng/ml canine pituitary
standard LER-1685-1. Lowest levels of detectability: LH <0.5
ng/ml; testosterone <0.2 ng/ml.
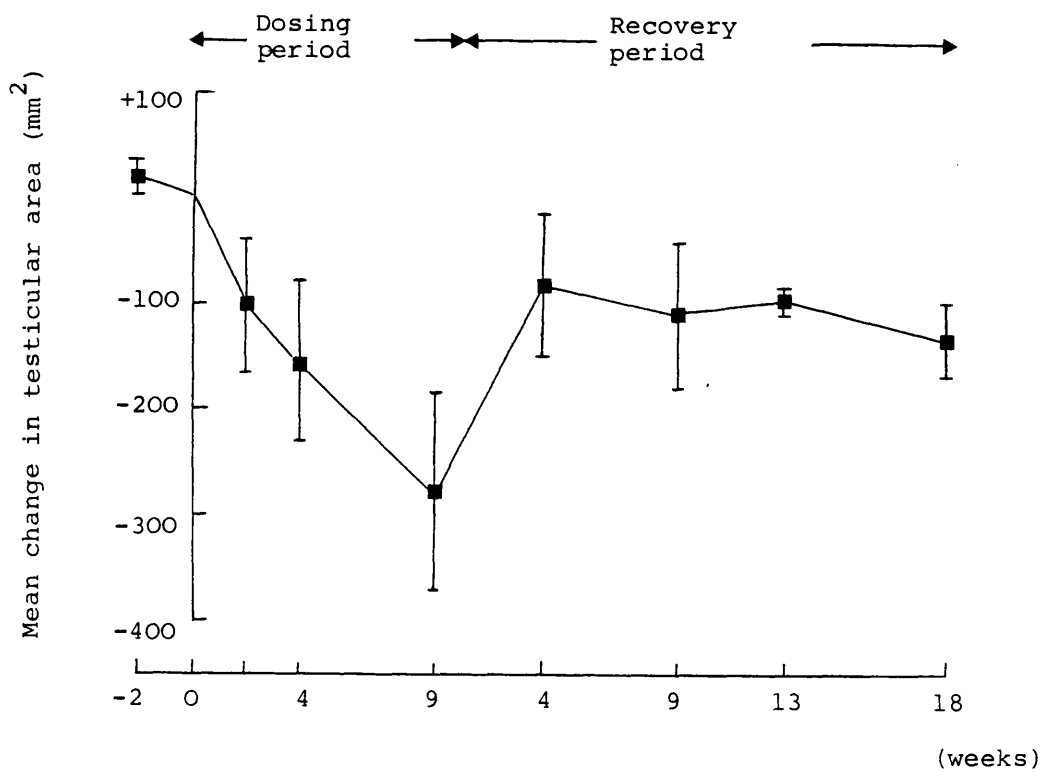
Fig. 2.2.2.3. Mean changes in testicular area, relative to the
start of dosing, for Beagle dogs given cogazocine lactate (1 mg/kg/
day) for nine weeks, followed by an 18-week recovery period.
Vertical lines represent $\pm$ standard deviation.

Examination	Serum LH (ng/ml)			Serum testosterone (ng/ml)		
	Mean	SD	Range	Mean	SD	Range
Predose (n=6)	2.6	± 2.2	0.5 – 7.1	1.9	± 1.8	0.2 – 5.1
Dosing period (n=6)						
Day 2	2.3	± 4.2	0.5 – 10.9	0.3	± 0.1	0.2 – 0.5
Day 8	6.0	± 8.7	0.5 – 23.4	1.8	± 1.3	0.4 – 3.5
Day 15	9.6	± 7.4	1.3 – 19.1	3.4	± 1.2	1.5 – 4.7
Day 29	3.6	± 3.1	0.8 – 9.3	2.4	± 2.1	0.3 – 4.6
Day 57	2.6	± 4.3	0.5 – 15.2	2.6	± 2.2	0.2 – 6.2
Recovery period						
Week 8 (n=4)	1.2	± 0.7	0.5 – 2.2	1.3	± 1.1	0.2 – 2.2
Week 17 (n=2)	5.4	± 1.9	4.0 – 6.7	6.9	± 0.2	6.7 – 7.0

Table 2.2.2.1. Semen LH and testosterone concentrations in Beagle dogs given cogazocine lactate (1 mg/kg/day) for nine weeks, followed by a recovery period of 18 weeks. During the dosing period, serum was obtained 24 h after the dose of cogazocine lactate. LH expressed as ng/ml canine pituitary standard LER-1685-1.

During the dosing period all dogs continued to show normal libido, although after seven days' dosing, only two dogs actually ejaculated. Most dogs subsequently produced ejaculates, but there was a marked reduction in its volume, density, motility and sperm concentration. A large number of dead and morphologically abnormal spermatozoa were present, the principal abnormalities being loose heads and coiled tails. Fig. 2.2.2.4. illustrates the progressive changes seen in volume of ejaculate and sperm characteristics. After withdrawal of the treatment, all the dogs readily produced ejaculates. One week after withdrawal, the percentage of dead or abnormal spermatozoa was less than that during the dosing period, but the lowest sperm counts were recorded at this time. Four weeks after withdrawal, and at all subsequent examinations, the semen characteristics assessed were all satisfactory. The selected organ weights of the dogs examined at the chosen intervals are given in Table 2.2.2.2. Apart from reduced prostatic weight following nine weeks' treatment and also nine weeks' withdrawal of the drug, the other organ weights were considered to be within the normal range of variation. Light-microscopic examination of the testes after nine weeks' treatment (Fig. 2.2.2.5.) revealed an obvious reduction in the number of elongated (late) spermatids in the lumina of the seminiferous tubules. Subjective assessment of testicular sections examined after nine and 18 weeks' withdrawal indicated normal morphology with mature spermatids present. Quantitative histo-metric analysis (Table 2.2.2.3.) revealed a statistically significant (p <0.01) reduction in the number of pachytene spermatocytes after nine weeks' treatment. The major effect was, however, a marked reduction in both early (round) and late (elongated) spermatids after nine weeks' treatment, but only in late spermatids after nine weeks' withdrawal. Sertoli cells appeared to be unaffected and the values for the germinal cell counts obtained after 18 weeks' withdrawal were similar to values for normal dogs, see Appendix I (ix).
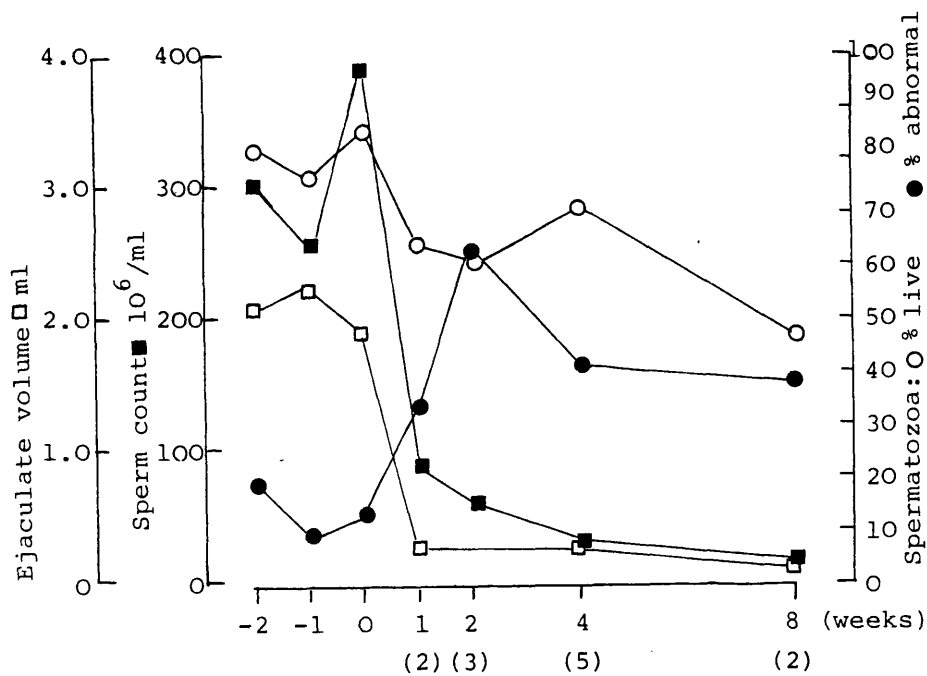
Fig. 2.2.2.4. Changes induced in ejaculate volume, sperm count and spermatozoal morphology by administering cogazocine lactate (1 mg/kg/day) to Beagle dogs for nine weeks. Numbers of dogs providing ejaculates, if less than six are indicated parenthetically.

Mean values obtained for

Duration of dosing	Body weight (g)		Pituitary (mg)	Testes (g)			Prostate (g)
	Initial	Final		Right	Left	Mean	
9 weeks	13.5	13.3	60	11.1	11.3	11.2	2.2
9 weeks' recovery	13.0	11.0	54	10.9	11.5	11.2	3.3
18 weeks' recovery	15.1	15.7	77	15.2	15.3	15.3	12.1

Table 2.2.2.2. Weights of testes, pituitary glands and prostates obtained from Beagle dogs after treatment with cogazocine lactate (1 mg/kg/day). Two dogs were examined on each occasion.

:112:
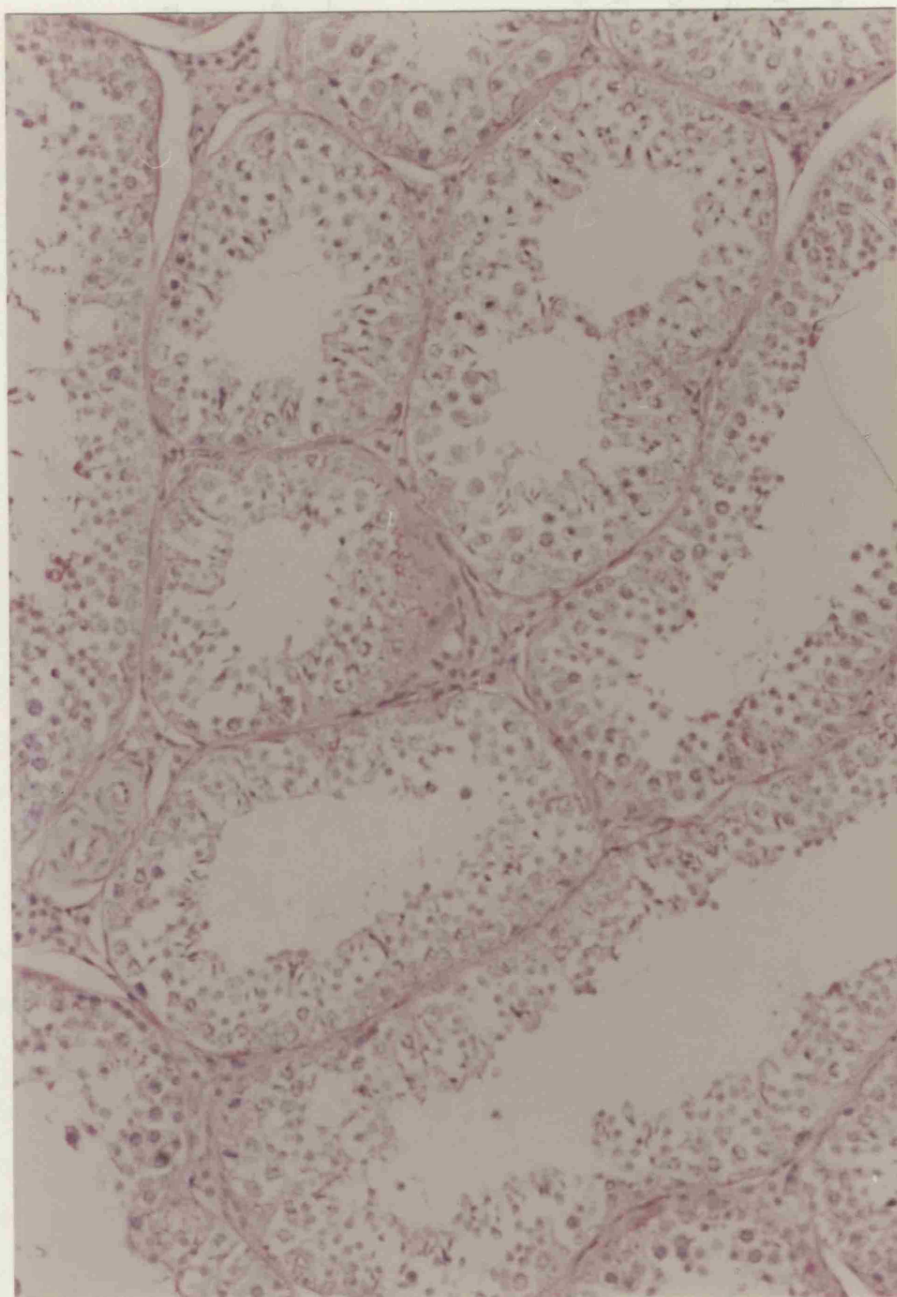
Fig. 2.2.2.5.   Section of a testis obtained from a Beagle dog given cogazocine lactate (1 mg/kg/day) for nine weeks, showing marked absence of mature spermatids.   PAS; x 200.

Treatment	Mean values for:										
	Tubule diameter (μm)	Type B spermatogonia (stage 7)		Pachytene spermatocytes (stage 5)		Spermatids Early (stage 1)		Late (stage 8)		Sertoli cells	
		Crude	Corrected	Crude	Corrected	Crude	Corrected	Crude	Corrected	Crude	Corrected
Cogazocine lactate (1.0 mg/kg/day)											
9 weeks' dosing	213 ± 9	15	7.4	34^{+++}	13.4	48^{+++}	29.2	31^{+++}	18.3	25	9.9
9 weeks' recovery	228 ± 19	16	7.4	47	17.3	110^{++}	60.8	69^{+++}	38.1	24	8.8
18 weeks' recovery	193 ± 7	19	10.3	52	22.6	136	88.8	126	82.3	23	10.0

Table 2.2.2.3. Quantitative assessment of spermatogenesis for Beagle dogs given cogazocine lactate (1 mg/kg/day) for nine weeks, followed by a recovery period of 18 weeks. Statistically significant differences(ANOVAR),with dogs allowed 18 weeks' withdrawal: $p<0.01^{++}$; $p<0.001^{+++}$.

After nine weeks' treatment, the prostate glands (Fig. 2.2.2.6.) showed predominance of interstitial stroma, and inactive acini lined by flattened epithelium with no evidence of secretory activity. Nine weeks after withdrawal the prostatic acini, although reduced, showed some evidence of secretory activity. Prostate morphology was considered to be normal after 18 weeks' withdrawal. Differential staining of the pituitary glands (Fig. 2.2.2.7.) revealed cellular atrophy, pyknosis and condensation of granules in gonadotrophic cells after nine weeks' treatment, but not after nine or 18 weeks' recovery.

2.2.3. Discussion

The effects of a single dose of cogazocine lactate (1 mg/kg) on serum LH and testosterone in the Beagle dog are similar to the effects observed following morphine administration to rats. Repeated administration of cogazocine lactate to dogs, at approximately 20 times the likely clinical dosage (0.05 mg/kg/day), induced reversible effects on semen characteristics, spermatid maturation and in the androgen-dependent prostate gland. The principal abnormalities found in semen were dead sperm, coiled tails and loose heads. Swanson and Boyd (1962) studied the factors associated with coiled-tail spermatozoa in the bull and found that variation of this type developed after migration to the cauda epididymis, and susceptibility increased towards the vas deferens. It was suggested that variations in the composition of the secondary sexual fluids governed the presence or absence of this abnormality. Although the evidence is circumstantial, the appearance of coiled-tail spermatozoa in ejaculates from dogs given cogazocine lactate could be due to the reduction of the prostate secretion (Harrop, 1955) as a result of testosterone depletion.
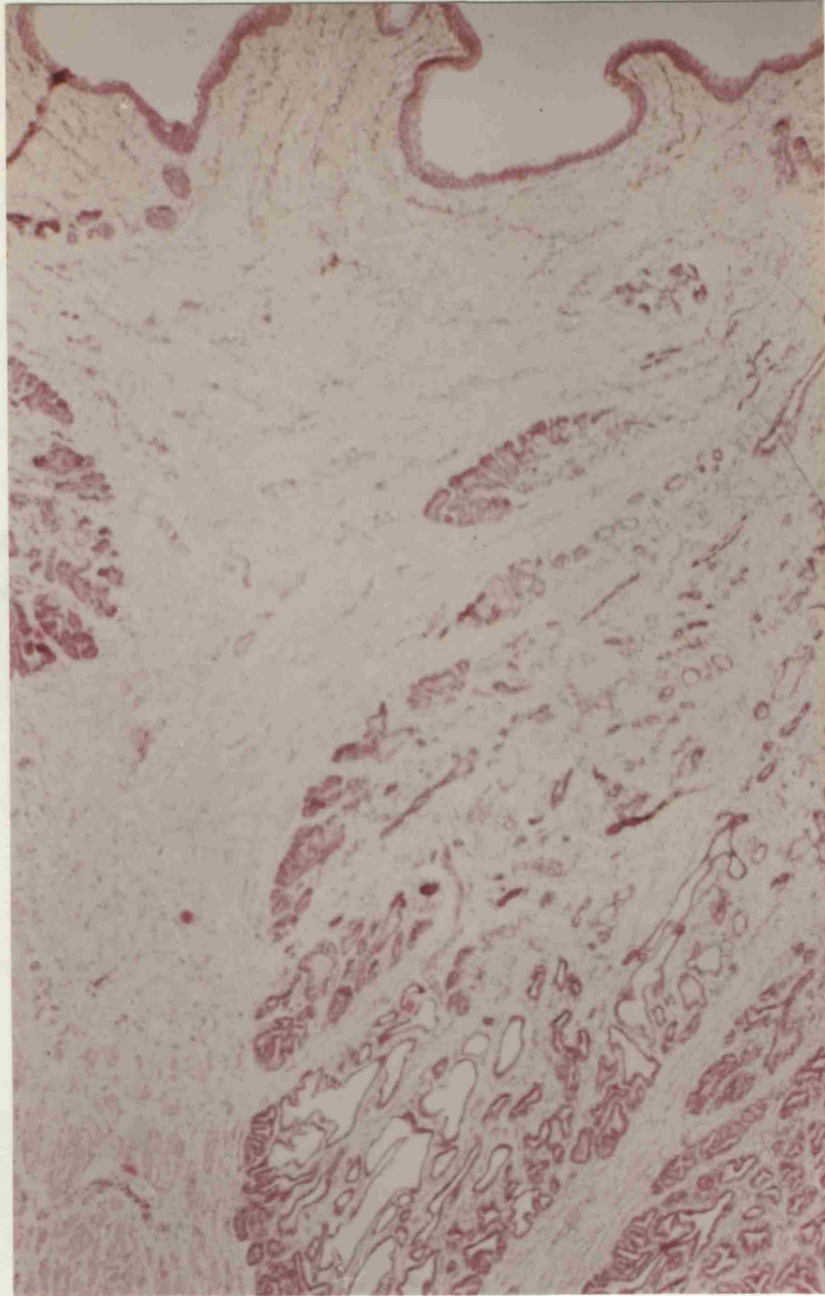
Fig. 2.2.2.6. Section of prostate gland obtained from a Beagle
dog after nine weeks' administration of cogazocine lactate (1 mg/
kg/day). Note atrophy of prostate acini and predominance of
interstitial stroma. H & E, x 50.

Appearance of pituitary gonadotrophs (blue cells) after nine weeks' treatment.
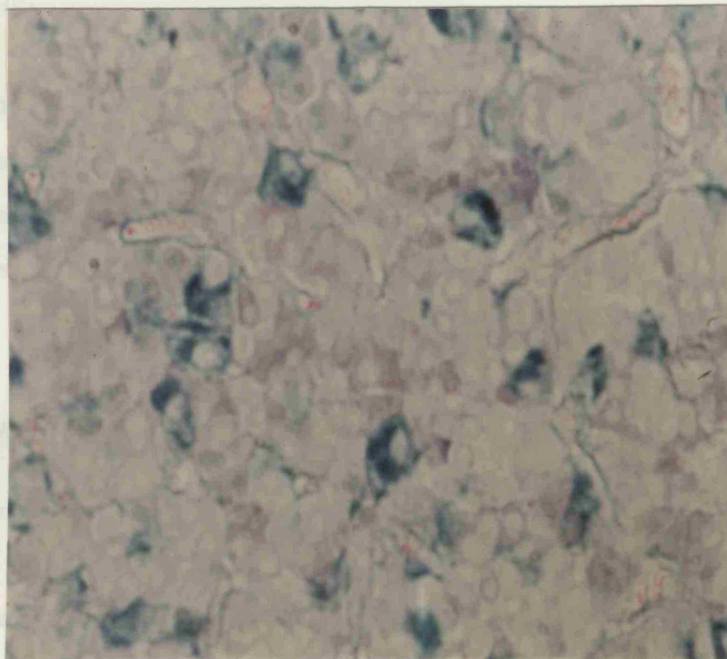
Appearance of pituitary gonadotrophs (blue cells) after 18 weeks' recovery.

Fig. 2.2.2.7. Histological sections of pituitary glands obtained from Beagle dogs after nine weeks' treatment with cogazocine lactate (1 mg/kg/day) and after 18 weeks' recovery. PAS-Alcian blue-Orange G; x 512.

In the dog, ablation of the posterior median eminence of the hypothalamus induces severe testicular and prostatic atrophy. Ablation of the anterior or median eminence results in reduced sperm concentration and motility, together with partial suppression of spermatogenesis (Davidson and Ganong, 1960). These observations, and the occurrence of altered staining properties of pituitary gonadotrophic cells after administering cogazocine lactate for nine weeks, support the hypothesis that the effects on the late stages of spermiogenesis, prostatic function and circulating testosterone concentrations are secondary to inhibition of LH release. The reversibility of the changes and the underlying biological mechanism do not suggest an unpredictable toxic hazard, but merely an exaggerated pharmacological response to repeated overdosage.

The similarity of the results obtained with morphine in rats and with cogazocine lactate in dogs suggests that similar effects may be induced in either species following overdosage with a variety of addictive or minimally addictive narcotic analgesic drugs.

2.2.4. Abstract

Acute single overdosage (1 mg/kg, i.m.) with the novel benzmorphan narcotic analgesic agent, cogazocine lactate, lowered serum LH and testosterone concentrations of Beagle dogs. The effect was rapidly reversible and persisted for less than 24 h after drug administration. Repeated administration (1 mg/kg, i.m.) induced reversible suppression of spermatid maturation with subsequent effects on semen characteristics. Examination of circulating LH and testosterone concentrations, together with pituitary and prostate morphology, suggested that the changes were due to transient inhibition of LH release.

It is probable that the underlying mechanism is an exaggerated

pharmacological response to repeated overdosage, common to many

potent narcotic analgesic agents.

2.3. Administration of Compound A to Beagle dogs for 9 weeks

Body (1970) drew attention to the association between male infertility and analgesic abuse. This author also described experimentally induced testicular atrophy in rats and guinea-pigs as a result of prolonged overdosage with aspirin, phenacetin or paracetamol. Inhibition of prostaglandin synthesis is an important property of aspirin-like drugs (Vane, 1971). Abbatiello et al., (1975) reported that administration of prostaglandin-inhibiting agents could modify the seminiferous epithelium of laboratory animals. Prostaglandins are thought to be mediators in the release of anterior pituitary hormones (Batta et al., 1974; Carlson et al., 1977; Ojeda et al., 1974; Sato et al., 1975). Prostaglandin synthesis occurs in mammalian testicular tissue (Ellis et al., Carpenter and Bray (1977).
1975). Prostaglandin concentrations are low in seminiferous tubules and interstitial tissue, but greater amounts are found in the epididymides and vas deferens (Johnson and Ellis, 1977). Seminal plasma is also rich in prostaglandin activity (Badr et al., 1975; Poulos et al., 1975; Sorgen and Glass, 1972; Voglmayr, 1973). Bartke et al. (1976) have demonstrated the role of prostaglandins in testicular synthesis of androgenic hormones. While the precise role of prostaglandins in male reproductive physiology remains debatable, it is apparent that continuous administration of high doses of prostaglandin inhibitors to experimental animals may be expected to modify testicular function.

Indomethacin and structurally similar compounds are important non-steroidal anti-inflammatory drugs, all of which are recognised inhibitors of prostaglandin synthesis. The preclinical safety evaluation of these agents in laboratory animals presents practical

difficulties. Rats and dogs tend to concentrate non-steroidal anti-inflammatory drugs in the enterohepatic circulation to a greater extent than humans (Duggan et al., 1975). The development of gastrointestinal, hepatic or renal disturbances often limits the magnitude and extent of exposure which can be achieved in laboratory species. Compound A, a structural analogue of indomethacin, is well tolerated by Beagle dogs. This provided an opportunity to assess the testicular responses following exaggerated prostaglandin inhibition in the Beagle dog.

2.3.1. Experimental design

Fifteen sexually mature Beagle dogs were obtained and were maintained under standard conditions, see Appendices I (i) and (ii). Compound A tablets were made available on condition that the source and structure of the active principle remained confidential. Three dogs were given compound A at a dose rate of 150 mg/kg/day and six dogs received 600 mg/kg/day. The doses are expressed in terms of active ingredient and correspond to 10 and 40 times the proposed therapeutic dosages. Six dogs were dosed with placebo tablets containing the same amount of excipient as the compound A formulation. Dosing continued on a daily basis for nine consecutive weeks.

Body weight changes, food and water consumption were recorded, see Appendix I (iii). The dogs were examined several times daily for clinical evidence of malreaction to the experimental treatment. The dogs given 150 mg/kg/day, three dogs given 600 mg/kg/day and three control dogs, were killed on completion of nine weeks' dosing.

All remaining dogs were killed 13 weeks after receiving the final dose of compound A or placebo tablets. The testes of each dog were measured, see Appendix I (iv), before treatment started, after two, four and eight weeks' treatment and also four, eight and 12 weeks after dosing stopped. LH and testosterone concentrations were measured, see Appendix I (vi), in serum samples obtained 4 h after dosing on day 3, during weeks 4 and 9 of the dosing period and also after 12 weeks' withdrawal of treatment. Semen samples were collected and evaluated, see Appendix I (vii), for each dog at the same intervals as the testes were measured. Acid phosphatase concentrations, see Appendix I (vii), were measured in seminal plasma obtained after centrifugation of each semen sample.

During the terminal studies each dog was given a complete post-mortem examination which included weighing and preservation of the testes, prostates and pituitary glands, see Appendix I (viii). Histological sections of the preserved organs were prepared for light-microscopical examination. Measurements of seminiferous tubule diameter and counts of type B spermatogonia, pachytene primary spermatocytes, early and late spermatids and Sertoli cells were made for each dog, see Appendix I (ix).

2.3.2. Results

Vomiting and salivation occurred among dogs given compound A at 600 mg/kg/day. The signs appeared up to one hour after dose administration: the frequency diminished as the treatment period progressed, and neither sign was seen after week 5. Body weight changes and appetite were not adversely affected. Dogs given 600 mg/kg/day drank significantly increased ($p < 0.001$) quantities of water, but excessive thirst was not apparent. Modifications in

testicular area, serum hormone concentrations and semen characteristics were seen among dogs given 600 mg/kg/day. Progressive reductions in the mean testicular area of these dogs attained statistical significance (Table 2.3.2.1.) after eight weeks' treatment. This effect was, at least partly, reversible after 13 weeks' withdrawal of compound A. Consistent reduction of circulating LH and testosterone concentrations were induced in dogs receiving 600 mg/kg/day (Fig. 2.3.2.1.; Table 2.3.2.2.). This trend had completely regressed by 12 weeks after the final dose of compound A. Treatment with 600 mg/kg/day was associated with decreased ejaculate volume and sperm concentration, thoughout the nine weeks of dosing (Fig. 2.3.2.2.; Table 2.3.2.3.). These trends were reversed following withdrawal of compound A treatment, but statistically significant (p <0.01) increases in the number of dead and abnormal spermatozoa were apparent after eight and 12 weeks' recovery in dogs previously given 600 mg/kg/day. The principal morphological abnormalities were coiled tails and loose or detached heads. Seminal plasma acid phosphatase concentrations were unaffected by the experimental procedure.

No difference in macroscopic appearance or weight were found at the terminal inspections of the pituitaries, testes and prostate glands. No treatment-related histological abnormality was found in the prostate gland of any dog. Differential staining of pituitary sections, confined to dogs given 600 mg/kg/day and concurrent controls examined after nine weeks' treatment, indicated an effect on gonadotrophic cells. Evidence of reduced secretory activity in these cells was characterised by cellular atrophy, nuclear pyknosis and condensation of granules.

Mean testicular area (mm^2) $\pm$ one standard deviation

Examination	Controls	Compound A 150 mg/kg/day	Compound A 600 mg/kg/day
Pre-treatment	611 $\pm$ 57	601 $\pm$ 76	596 $\pm$ 98
Dosing period			
2 weeks	623 $\pm$ 59	593 $\pm$ 52	566 $\pm$ 117
4 weeks	609 $\pm$ 63	606 $\pm$ 54	545 $\pm$ 64
8 weeks	617 $\pm$ 50	620 $\pm$ 57	509 $\pm$ 67[+]
Recovery period			
4 weeks	652 $\pm$ 95	—	530 $\pm$ 97
8 weeks	586 $\pm$ 16	—	559 $\pm$ 95
12 weeks	544 $\pm$ 101	—	533 $\pm$ 44

Table 2.3.2.1. Group mean testicular areas (mm^2) of Beagle dogs during nine weeks' treatment with compound A and during the 12 weeks after dosing was stopped. Statistically significant differences (ANOVAR) with concurrent control data: $p < 0.05$[+].

Serum LH (ng/ml)

Serum testosterone (ng/ml)

day 3   2 weeks 4 weeks 9 weeks 12 weeks'
                                    recovery

←——————— Dosing period ———————→
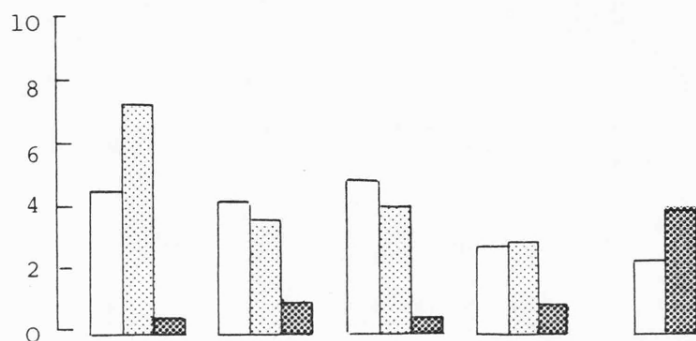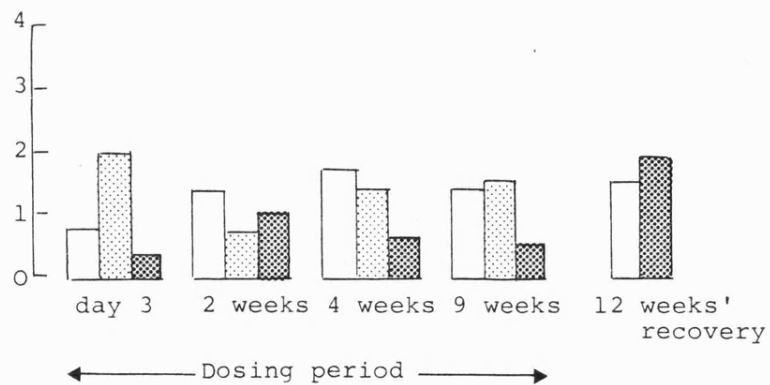
Fig. 2.3.2.1. Effect of compound A on serum LH and testosterone
concentrations of Beagle dogs. Controls: (☐); Compound A:
(▦) 150 mg/kg/day and (▨) 600 mg/kg/day.

Mean serum concentration (ng/ml)

Examination	LH Mean	S D	Range	Testosterone Mean	S D	Range
9 weeks' dosing						
Controls (n=6)	2.77	± 4.18	0.5 – 11.2	1.35	± 1.38	0.2 – 3.2
Compound A 150 mg/kg/day (n=6)	2.90	± 1.56	2.0 – 4.7	1.97	± 0.96	1.1 – 3.0
600 mg/kg/day (n=6)	0.90	± 0.62	0.5 – 2.1	0.48	± 0.21	0.2 – 0.8
13 weeks' recovery						
Controls (n=3)	4.17	± 2.35	2.3 – 6.8	1.50	± 1.10	0.4 – 2.6
Compound A ex 600 mg/kg/day (n=3)	5.90	± 4.33	1.0 – 9.2	1.93	± 0.65	1.3 – 2.6

Table 2.3.2.2. Comparison of serum LH and testosterone concentrations of control and treated dogs after nine weeks' administration of compound A and 12 weeks after dosing stopped. LH expressed as ng LER-1685-1/ml.
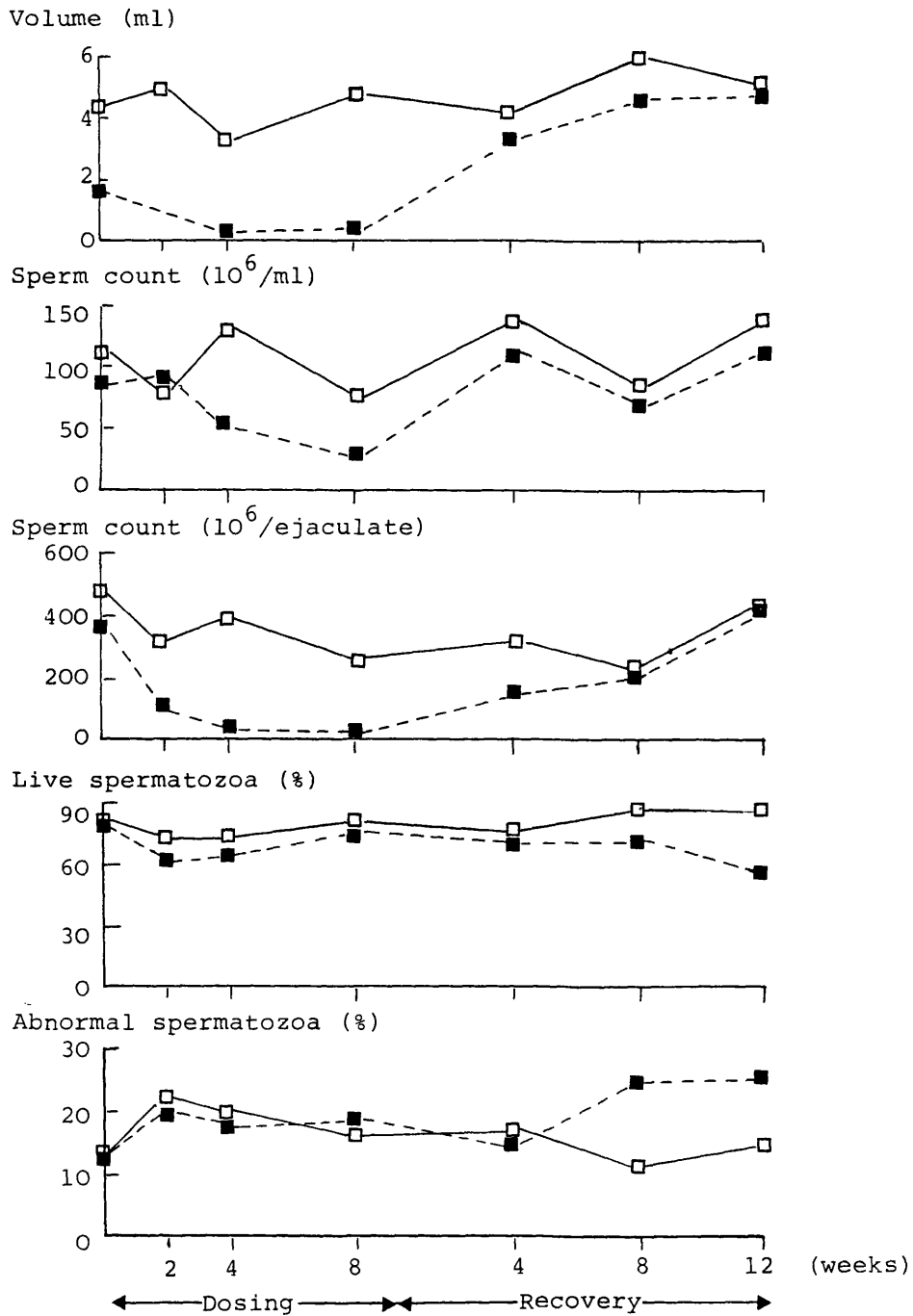
Fig. 2.3.2.2. Effect of compound A on semen characteristics of Beagle dogs. Controls: (□); Compound A 600 mg/kg/day (■).

Semen characteristic   Mean value $\pm$ one standard deviation

Semen characteristic	Controls		Compound A (600 mg/kg/day)	
	9 weeks' placebo (n=6)	13 weeks' recovery (n=3)	9 weeks' dosing (n=6)	13 weeks' recovery (n=3)
Volume (ml)	4.73 $\pm$ 2.15	4.93 $\pm$ 2.03	0.43 $\pm$ 0.57[++]	4.80 $\pm$ 0.57
Density (0-5)	2.7 $\pm$ 1.4	3.3 $\pm$ 0.6	1.8 $\pm$ 1.0	3.0 $\pm$ 1.0
Motility (0-5)	2.8 $\pm$ 1.5	1.7 $\pm$ 1.2	1.0 $\pm$ 1.4	2.0 $\pm$ 1.0
Sperm count				
10^6/ml	77.3 $\pm$ 67.5	137.7 $\pm$ 114.5	27.8 $\pm$ 39.2	115.7 $\pm$ 81.3
10^6/ejaculate	261.3 $\pm$ 120.4	542.3 $\pm$ 303.4	35.5 $\pm$ 60.7	447.0 $\pm$ 128.5
% spermatozoa				
live	82.0 $\pm$ 15.9	86.7 $\pm$ 5.6	82.0 $\pm$ 4.4	59.7[+] $\pm$ 11.6
abnormal	17.3 $\pm$ 15.0	14.7 $\pm$ 2.3	18.0 $\pm$ 3.0	25.0[+] $\pm$ 4.4

Table 2.3.2.3. Comparison of semen characteristics of Beagle dogs after nine weeks' administration of compound A or placebo tablets. Statistically significant differences with concurrent control data (ANOVAR): p <0.05[+]; p <0.01[++].

Histological examination of the testes of dogs which had received 600 mg/kg/day for nine weeks showed marked reduction in the number of mature spermatids present in the lumina of seminiferous tubules. Quantitative assessment of testicular spermatogenesis (Table 2.3.2.4) confirmed the subjective histological assessment. The numbers of all spermatogenic cells were conclusively reduced by nine weeks' treatment with compound A at a dose of 600 mg/kg/day. Following 13 weeks' withdrawal of compound A, the germinal cell counts of previously treated dogs were essentially comparable with the control data. Apparent increases in the Sertoli cell counts of dogs treated with compound A may have been artefactual consequences of decreased seminiferous tubule diameter (Clermont and Morgentaler, 1955), although after 13 weeks' withdrawal of clanobutin the seminiferous tubule diameters of dogs which had received the drug were comparable with control values.

2.3.3. Discussion

Previous investigators (Saksena et al., 1975) have studied the effects of repeated indomethacin overdosage on serum LH and testosterone, testicular and secondary sex-organ weight and male fertility in rats. The maximum tolerated daily dose was 2.5 mg indomethacin. At this dosage, serum LH and testosterone, and seminal vesicle weights were decreased, but testicular and prostatic weight and the ability to fertilise ova were unaffected.

Treatment	Mean value Tubule diameter (µm)	Type B spermatogonia (stage 7) Crude	Corrected	Pachytene spermatocytes (stage 5) Crude	Corrected	Spermatids Early (stage 1) Crude	Corrected	Late (stage 8) Crude	Corrected	Sertoli cells Crude	Corrected
Controls	229 ± 4	21	10.1	44	16.6	123	69.2	95	52.8	21	8.0
Compound A											
9 weeks' dosing											
150 mg/kg/day	$210 \pm 8^{++}$	19	9.8	46	19.2	110	67.3	89	53.3	20	8.2
600 mg/kg/day	$199 \pm 11^{+++}$	15^{++}	8.1	34	14.8	81^{+++}	52.5	53^{+++}	32.8	24	10.4
13 weeks' recovery											
ex 600 mg/kg/day	233 ± 12	22	9.7	44	16.3	126	68.7	117^{+}	62.3	27	9.4

Table 2.3.2.4. Quantitative assessment of spermatogenesis for Beagle dogs given compound A or placebo tablets for nine weeks, followed by a recovery period of 13 weeks. Statistically significant differences from control data (ANOVAR): $p < 0.05^{+}$; $p < 0.01^{++}$; $p < 0.001^{+++}$.

In addition to reducing serum LH and testosterone, treatment of dogs

with compound A at 600 mg/kg/day exerted anti-fertility effects as

evinced by decreased testicular area, ejaculate volume and sperm

concentration and also changes in testicular and pituitary morphol-

ogy. Two considerations are of merit in evaluating this apparent

discrepancy in species response. First, compound A overdosage is

well tolerated by dogs and it proved possible to administer a greater

multiple of the likely therapeutic dosage than would be possible

with indomethacin. Secondly, residual spermatogenesis can proceed, at a greatly

reduced rate, after hypophysectomy in the rat (Clermont and

Morgentaler, 1955) whereas hypophysectomy or ablation of the post-

erior median eminence invariably results in testicular atrophy in the

dog (Davidson and Ganong, 1960). The lack of effect of compound A

on prostate and testicular weights and on seminal plasma acid

phosphatase concentrations in Beagle dogs may appear to contradict

the observed effects on testicular size and morphology and semen

characteristics. Experimental data (Joseph and Siwela, 1976) exist

to show that suppression of prostaglandin synthesis may enhance the

sensitivity of the prostate to testosterone. It is possible, there-

fore, that adequate prostate function may be maintained in animals

with reduced endogenous testosterone concentrations due to prolonged

suppression of prostaglandin synthesis. A wide normal range is

found for the weight of Beagle dog testes, and serial testicular

measurements may be a more sensitive index of testicular toxicity

than comparison of testicular weights at terminal examination.

Although modification of serum LH and testosterone concentration

provides a basis for explaining primary biological mechanisms

involved in the genesis of abnormal testicular function, the

present data do not completely justify this conclusion. Although

a reduction in the number of mature spermatids found in semini-

ferous tubules may be a consequence of LH depletion (Steinberger,

1971) this does not readily explain the decreased counts of earlier

spermatogenic cells. Examination of the ratio between successive

:131:

generations of cell types counted in the germinal epithelium (Hemsworth et al., 1968) shows that pachytene spermatocytes and early spermatids were found at similar daughter-cell ratios in treated and control dogs. It may be inferred that reduction in germinal cell numbers occurred as a result of a drug effect on spermatogonial divisions. Jackson (1972) considers that drug-induced modifications in spermatogonia result from local action on the seminiferous epithelium. The persistence of morphological abnormalities in spermatozoa from dogs kept for recovery studies after receiving 600 mg compound A/kg/day cannot be explained from the available data. The effects of compound A overdosage on serum LH and testosterone were, however, reversed within 13 weeks of withdrawal of the drug.

This investigation supports the hypothesis that prolonged over-dosage with compounds belonging to a pharmacological class widely accepted as inhibitors of prostaglandin synthesis (Sorrentino et al., 1972) may modify pituitary-testicular physiology. The finding that administration of ten times the likely therapeutic dosage did not adversely affect testicular size, LH or testosterone, semen characteristics or testicular morphology, implies that testicular effects seen at higher dosages may be regarded as predictable responses to overdosage rather than indications of toxicity following normal use.

2.3.4. Abstract

Compound A, a prostaglandin inhibitor, was administered in tablet form to Beagle dogs at dosages of 150 or 600 mg/kg/day for nine weeks. Control dogs received placebo tablets containing the same amount of excipient as the compound A tablets. Treatment at 600 mg/kg/day was associated with decreased serum LH and testosterone

concentrations, reduced testicular size, ejaculate volume and sperm

concentration, lowered spermatogonial and mature spermatid counts in

seminiferous tubules and evidence of reduced secretory activity in

pituitary gonadotrophic cells. Recovery was assessed: although

hormonally mediated changes regressed, spermatozoal morphology was

persistently abnormal in dogs which had received 600 mg/kg/day.

The effects of exaggerated prostaglandin inhibition on pituitary-

testicular physiology are discussed, together with the toxicological

significance of these findings in relation to their clinical

application.

2.4.   Administration of Compound B to Beagle dogs (13 weeks) and to rats (median life span)

The role of prolactin in male reproductive physiology is becoming increasingly recognised (Horrobin, 1977). Experimental studies in rodents suggest a synergic role for prolactin at physiological concentrations, in testicular spermiogenesis and steroidogenesis (Bartke and Dalterio, 1976; Hafiez et al., 1972; Moger and Geschwind, 1972). Hyperprolactinaemia has been associated with infertility and hypogonadism in human males (Horrobin. 1977). Decreased circulating luteinising hormone (LH) and follicle-stimulating homone (FSH) concentrations are apparent in hyper-prolactinaemic male rodents and humans (McNeilly et al., 1978; Thorner et al., 1974). Testicular atrophy has been induced in rats by injection of prolactin-secreting pituitary tumour cells (Fang et aL, 1974). Ectopic pituitary transplantation with male rats and mice was used as a model to study hyperprolactinaemic modifi-cations of male ·reproductive behaviour (Svare et al., 1979). Rats appeared to be the more sensitive species, but both species exhibited longer latency in mounting females,intromission and ejaculation; however, fertility was not affected.

The release of prolactin from the pituitary gland is regulated by a negative neurohumoral hypothalamic feedback mechanism (Frantz, 1978). Modification of the neurotransmitter substance involved in this mechanism forms the neuropharmacological basis for the actions of many psychotropic drugs (Fjalland and Boeck, 1978). Enhanced prolactin release is a well-documented feature of dopaminergic blocking agents (Bang and Gautvik, 1977; Levin and Voogt, 1978; Macleod and Lehmeyer, 1974). Well-known drugs possessing this activity include clomipramine (Francis et al., 1976; Groom and Evans, 1977), clozapine (Meltzer et al., 1975), haloperidol (Quadri et al., 1978) metoclopramide (Carlson et al., 1973;

Falaschi et al., 1978; Fang et al., 1977), some phenothiazine derivatives (Bohnet et al., 1976) and sulpiride (Debeljuk et al., 1975; Mannisto et al., 1978). This biological activity may modify the pituitary-testicular axis of experimental animals subjected to prolonged overdosage with compounds belonging to this neuro-pharmacological category.

In order to study the effects of prolonged hyperprolactinaemia, Compound B, a novel neuroleptic derivative in the orthopramide 2-methoxybenzamide series of dopamine antagonists (Lanza et al., 1975), was administered to male rats and Beagle dogs.

2.4.1. Experimental design

Fifteen sexually mature male Beagles and 150 male Sprague-Dawley rats (CFY strain) weighing 80-120 g were obtained. The animals were maintained under standard conditions, see Appendix I (i) and (ii).

Compound B, supplied on condition that the source and structure of the active principle remained confidential, was administered in gelatine capsules to dogs at oral dosages of 30 mg/kg/day to three dogs, at 120 mg/kg/day to six dogs, and six control dogs were given empty gelatine capsules for 13 weeks. Three dogs given 120 mg/kg/ day and three control dogs were maintained for a recovery period of 13 weeks. Seventy-five rats were given compound B by oral gavage at a dose of 300 mg/kg/day, dissolved in distilled water. Seventy-five rats acted as controls, receiving distilled water alone. Five rats per group were killed after 13 or 52 weeks' treatment. Fifteen rats per group acted as satellite animals and were used solely for the provision of serum samples. Dosing of the remaining rats continued until the 20% survival point for each group.

Body weight changes, food and water consumption were recorded, see Appendix I (iii). The animals were observed several times daily for clinical evidence of malreaction to experimental treatment. Prolactin and testosterone concentrations were measured in serum samples, see Appendix I (iv), obtained from each dog before treatment started, 2 h after dosing during weeks 6 and 13, and after 13 weeks' recovery. Serum was also obtained from five rats per group, under ether anaesthesia, after 4, 13, 26, 52 and 78 weeks' dosing, for measurement of prolactin concentration. The testes of each dog were measured, see Appendix I (iv), before treatment started and subsequently at monthly intervals. Canine semen samples were collected and evaluated, see Appendix I (vii), before dosing commenced, after six and 13 weeks' treatment, and after six and 13 weeks' recovery.

Rats which died prematurely were given thorough post-mortem examinations, followed by preservation of the testes and pituitary glands. Terminal examinations of dogs killed at the scheduled intervals included weighing and preservation of the testes, prostate and pituitary glands. The testes and pituitary glands of rats killed after 13 and 52 weeks' treatment, or at the 20% survival point, were also weighed and preserved. All tissues were fixed in 10% buffered neutral formalin. Routine haematoxylin and eosin sections of all preserved organs were examined by conventional light microscopy, see Appendix I (viii). Measurements of seminiferous tubule diameter and counts of type B spermatogonia, pachytene primary spermatocytes, early and late spermatids and Sertoli cells were made, see Appendix I (ix), for all dogs and rats which attained the 20% survival point, but excluding rats which developed atrophy of the seminiferous epithelium.

2.4.2. Results

No detectable clinical signs or behavioural modifications were observed in rats given compound B. Pupillary constriction occurred in dogs receiving either dose level of compound B, but was apparent only during the first three weeks of the dosing period. Sedation, squinting, body tremors and occasional convulsions or vomiting were induced in dogs receiving 120 mg/kg/day. There were no persistent effects on body weight or food consumption. The water intake of rats was not affected by compound B, but dogs dosed at 120 mg/kg/day drank less water than control dogs during the treatment period. The 20% survival point was reached after 84 weeks' treatment of rats with 300 mg/kg/day: however, 20% of the control rats survived until week 113 of the investigation.

Prolactin and testosterone concentrations were affected at both 30 and 120 mg/kg/day in the canine study (Fig. 2.4.2.1.). Although the serum prolactin concentrations of dogs given compound B showed a five- to ten-fold increase in comparison with control data, a linear dose-response relationship was not apparent. A dose-related effect was not demonstrable with regard to the lower serum test-osterone concentrations found in the groups given compound B. The variations recorded in control rat serum prolactin concentrations followed the age-related pattern described by Riegle and Meites (1976). An overall three-fold increase in serum prolactin concen-trations was apparent for rats given compound B (Fig. 2.4.2.2.): differences between control and treated rats became more obvious as the dosing period progressed.

No significant variations were found in the testicular area of dogs acting as controls or receiving 30 mg compound B/kg/day.

Serum prolactin (ng/ml)

Serum testosterone (ng/ml)

Predose ◄───Dosing───► Recovery

Week 6   Week 13
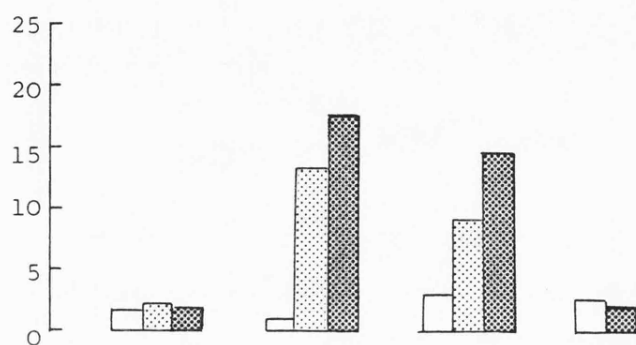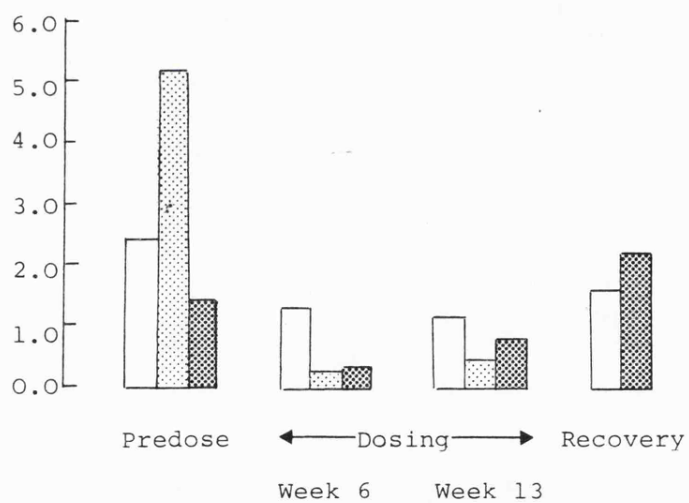
Fig. 2.4.2.1. Effects of compound B on serum prolactin and testosterone of Beagle dogs. Controls: (☐) Treated: (▨) 30 mg/kg/day; (▨) 120 mg/kg/day. Prolactin expressed as ng CPA3/F4/ml.

Serum prolactin (ng/ml)
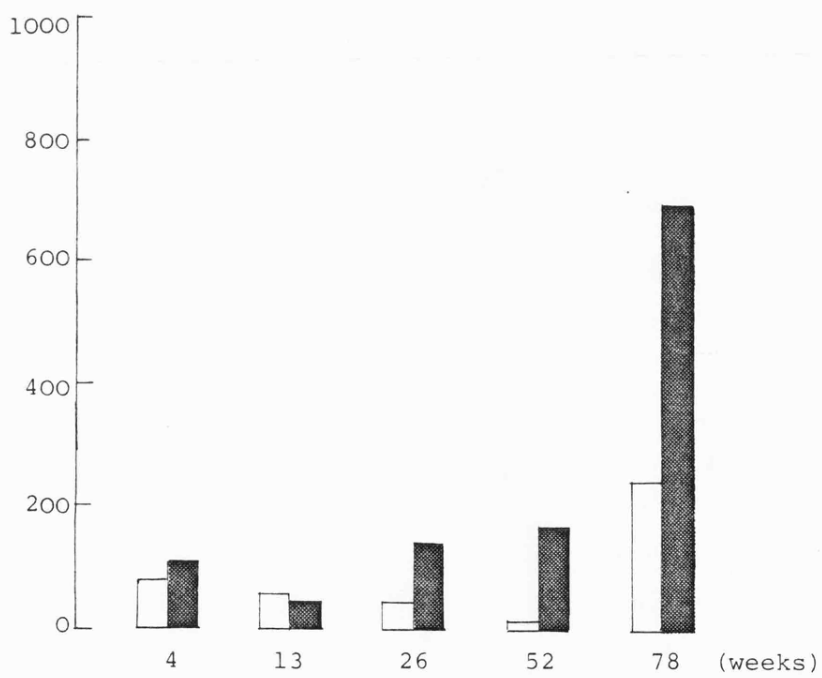
Fig. 2.4.2.2. Effects of compound B on serum prolactin concentration of rats. Controls: (☐); Treated: (▨) 300 mg/kg/day.

An overall 10% reduction of testicular area occurred during 13 weeks' treatment of dogs with 120 mg/kg/day, but this trend was completely reversed during the recovery period. Differences in ejaculate volume, sperm motility and morphology were found between treated and control dogs (Table 2.4.2.1.) after six and 13 weeks' dosing. There was an increase in the percentage of spermatozoa with proximal cytoplasmic beads, and decreased sperm motility at 30 and 120 mg/kg/day. Ejaculate volume was decreased and the number of dead spermatozoa increased, in dogs given 120 mg/kg/day. Although marked individual variations in sperm concentration occurred, there was no conclusive difference between control and treated dogs. After 13 weeks' withdrawal, the semen characteristics of dogs which had previously been given 120 mg/kg/ day were very similar to those of the concurrent control group.

Treatment of rats with 300 mg compound B/kg/day induced statistically significant increases in pituitary weights (Table 2.4.2.2.). The mean prostate weight of dogs given 120 mg/kg/day was significantly reduced after 13 weeks' treatment (Table 2.4.2.3.). No statistically significant differences were found in canine pituitary weights or in testicular weights for either species. Reduction of prostatic weight was not evident among dogs examined after the 13-week recovery period. There were no histologically detectable changes in the testes or pituitary glands of rats examined after 13 and 52 weeks' treatment. After 20% survival was attained, the cumulative incidences of pituitary chromophobe adenomata, chromophobe hyperplasia and cysts were 17%, 7% and 2% respectively among rats given compound B. The corresponding incidences among control rats were 24%, 7% and 7%. The incidence of testicular atrophy was 7% among rats given compound B and 24% among control rats. Arteritic lesions were present in the testes of 17% of control rats, but were not found in rats given compound B.

Treatment	Semen characteristics					
	Volume	Motility	Sperm count		% spermatozoa	
	(ml)	(0-5)	10^6/ml	10^6/ejaculate	live	abnormal
Predosing	3.2 ± 2.1	1.4 ± 1.6	186 ± 92	460 ± 244	85 ± 10	16 ± 20
13 weeks' dosing						
Controls	3.2	1.2	157	479	81	10
Compound B						
30 mg/kg/day	2.9	0.3	221	624	84	48[++]
120 mg/kg/day	0.9[+]	0.4	381	358	65	53[++]
13 weeks' recovery						
Controls	2.3	0.3	243	459	88	33
Compound B						
ex 120 mg/kg/day	2.4	1.0	264	734	80	21

Table 2.4.2.1. Effects on serum characteristics found in Beagle dogs given compound B for 13 weeks, followed by a 13-week recovery period. Statistically significant differences from concurrent control data (ANOVAR): $p < 0.05$[+]; $p < 0.01$[++].

Examination	Treatment	Mean values		
		Body weight (g)	Pituitary (mg)	Testes (g)
13 weeks	Controls	563 ± 92	14 ± 1	5.3 ± 0.6
	Compound B	543 ± 101	15 ± 1	4.8 ± 0.4
52 weeks	Controls	875 ± 89	15 ± 6	5.2 ± 0.3
	Compound B	772 ± 51	27 ± 8[+]	4.8 ± 0.8
20% survival	Controls (113 weeks)	791 ± 116	22 ± 18	4.7 ± 1.0
	Compound B (84 weeks)	739 ± 161	44 ± 27	4.7 ± 0.6

Table 2.4.2.2. Pituitary and testicular weights of rats given compound B (300 mg/kg/day).

Statistically significant differences (ANOVAR) with concurrent control data: p < 0.05[+].

Examination	Treatment	Mean values			
		Body weight (kg)	Pituitary (mg)	Prostate (g)	Testes (g)
13 weeks' dosing	Controls	12.4 ± 2.0	63 ± 15	7.3 ± 2.7	25.0 ± 4.0
	Compound B				
	30 mg/kg/day	13.9 ± 0.6	87 ± 12	6.1 ± 1.4	26.0 ± 3.7
	120 mg/kg/day	13.1 ± 1.5	80 ± 20	3.9 ± 1.2[+]	25.0 ± 4.6
13 weeks' recovery	Controls	13.1 ± 3.2	80 ± 10	8.1 ± 3.4	24.9 ± 1.9
	Compound B				
	ex 120 mg/kg/day	13.2 ± 2.1	70 ± 10	7.3 ± 2.2	29.1 ± 4.4

Table 2.4.2.3. Pituitary, prostatic and testicular weights of Beagles given compound B for 13 weeks, followed by a 13-week recovery period. Statistically significant differences (ANOVAR) with concurrent control data: $p < 0.05$[+].

The prostate glands of dogs examined after receiving 120 mg
compound B/kg/day for 13 weeks, showed evidence of acinar
epithelium and absence of secretion, with a relative increase in
stromal tissue. Considerable variations were noted for prostate
morphology among control and previously dosed dogs after the
13-week recovery period, but evidence of secretory activity was
seen in all the sections examined. All types of spermatogenic
cells were present in the seminiferous tubules of dogs at all
examinations. However, detached mature spermatozoa in the lumina
of seminiferous tubules appeared to be scanty in some dogs
examined after 13 weeks' treatment with compound B, at both dose
levels tested.

The results of quantitative assessment of canine testicular
spermatogenesis and a similar examination for rats attaining the
20% survival point, but excluding rats which developed testicular
atrophy, are presented in Tables 2.4.2.4. and 2.4.2.5.
Statistically significant reductions $(p < 0.05)$ in the counts of
late spermatids at both dosages, and also early spermatids in
dogs given 120 mg compound B/kg/day, were apparent after 13 weeks'
treatment. These effects were not apparent among dogs allowed
to recover for 13 weeks after the last dose of compound B. The
counts of pachytene primary spermatocytes, and of early and late
spermatids, were significantly lower $(p < 0.05)$ among compound B-
treated rats after 84 weeks' treatment than among control rats
examined after 113 weeks.

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Treatment	Tubule diameter (μm)	Type B spermatogonia (stage 7)		Pachytene spermatocytes (stage 5)		Spermatids Early (stage 1)		Late (stage 8)		Sertoli cells	
		Crude	Corrected	Crude	Corrected	Crude	Corrected	Crude	Corrected	Crude	Corrected
Controls	215 ± 12	16	8.3	42	16.8	101	59.6	84	49.1	24	9.3
Compound B											
13 weeks' dosing											
30 mg/kg/day	207 ± 17	14	7.1	36	14.7	87	53.6	55[+]	33.6	26	10.2
120 mg/kg/day	214 ± 26	13	6.6	36	14.4	83[+]	51.3	29[+++]	14.3	23	9.1
13 weeks' recovery											
ex 120 mg/kg/day	209 ± 35	13	6.8	41	16.6	91	54.1	84	47.0	25	9.6

Table 2.4.2.4. Quantitative assessment of spermatogenesis in Beagle dogs given compound B for 13 weeks, followed by a recovery period of 13 weeks. Statistically significant differences with control data (ANOVAR): $p < 0.05$[+]; $p < 0.001$[+++].

Treatment	Mean values										
	Tubule diameter (μm)	Type B spermatogonia (stage 7)		Pachytene spermatocytes (stage 5)		Spermatids Early (stage 1)		Late (stage 8)		Sertoli cells	
		Crude	Corrected	Crude	Corrected	Crude	Corrected	Crude	Corrected	Crude	Corrected
Controls (113 weeks)	279 $\pm$ 26	30	13.3	65	21.3	182	66.5	158	66.2	25	14.9
Compound B 300 mg/kg/day (84 weeks)	280 $\pm$ 10	33	14.2	59[+]	18.9	152[+++]	58.8	132[++]	55.1	28	16.1

Table 2.4.2.5. Quantitative assessment of spermatogenesis for rats attaining 20% survival while receiving compound B (300 mg/kg/day). Rats which developed atrophy of the seminiferous epithelium were excluded. Statistically significant differences (ANOVAR) from control rats: p<0.05[+]; p<0.01[++]; p<0.001[+++].

Discussion

The results of these investigations suggest that there are
differences in species-sensitivity to hyperprolactinaemia induced
with compound B. Physiological serum prolactin concentrations·in
young adult male Beagles vary from 1 to 3 ng/ml, whereas values of
30-90 ng/ml are recorded for male rats weighing 300 g (Debeljuk et
al., 1975). However, marked rises in peripheral prolactin
concentrations are induced by ether anaesthesia in rats (Simonel
et al., 1975). Furthermore, comparisons of inter-species varia-
tions are restricted by the marked degree of species-specificity
inherent in prolactin radioimmunoassays. The differences obtained
between the control and treated animals used for the present
investigations indicated that, although lower dose levels of
compound B were given to dogs, the relative degree of prolactin
stimulation was greater than that found in rats. The failure to
detect significant effects on testicular weight and morphology in
rats examined after 13 or 52 weeks' treatment concurs with
previous studies in rats showing a similar increase in serum
prolactin concentrations (McNeilly et al., 1978). Administration
of clomipramine to male rats at dosages of 50-100 mg/kg/day
induced testicular atrophy after 26 weeks' treatment, but measure-
ments of prolactin concentrations were not reported (Groom and
Evans, 1977). Administration of several benzamide derivatives to
female rats, for up to 38 days, increased the number of pituitary
cells, but pituitary weight was not affected (Lanza et al., 1975).
Pituitary weights were similarly unaffected in the rats used for
these experiments, after 13 weeks' dosing; however, marked increa-
ses were recorded after administering compound B for 52 or 84 weeks.
A high incidence of spontaneous testicular atrophy occurs in aged
Sprague-Dawley rats, and prolactin-dependent pituitary chromophobe
adenomata are common in several strains of laboratory rat (Ito et
al., 1972; Thompson and Hunt, 1963; Wolfe et al., 1938).

However, comparison of the incidence of pituitary chromophobe
adenomata or hyperplasia and of the quantitative reductions in
germinal cell counts found in rats after 84 weeks' compound B
treatment, with the data from control rats, may indicate an earlier
onset of these changes. Clarification of these phenomena requires
evaluation of effects induced by administration of compound B at dose
levels which do not decrease longevity in rats.

In contrast with the rodent data, compound B-induced-hyperprolactin-
aemia in Beagle dogs was conclusively associated with modified
testicular function. Effects on serum testosterone concentration,
ejaculate volume, spermatid maturation and prostate morphology
were apparent when control and treated dogs were compared. Although
some changes were induced at both dosages tested, reversibility
within 13 weeks of drug withdrawal was demonstrated. Although
there do not appear to be any specific reports of the role of
prolactin in canine testicular physiology, the responses obtained
in this investigation seem to be explicable entirely on the basis
of disruption of the synergistic role ascribed to prolactin in
testicular spermiogenesis and steroidogenesis (Bartke and Dalterio,
1976; Hafiez et al., 1972; Moger and Geschwind, 1972).

Although overdosage of experimental animals with various
psychotropic drugs has induced effects explicable in terms of
hyperprolactinaemia, there is a dearth of long-term follow-up
studies in man (Groom and Evans, 1977). Francis et al., (1976)
performed a cross-over study in patients treated with clomipramine
and amitriptyline, which failed to establish conclusive correlat-
ions between side effects and hyperprolactinaemia. It is not easy
to extrapolate risks on the basis of quantitatively differing
species-responses to hyperprolactinaemia induced by drug overdosage.

Further studies appear to be necessary to clarify the effects of long-term hyperprolactinaemia on the pituitary-testicular axis of different mammalian species.

2.4.4. Abstract

Hyperprolactinaemia was induced in male Sprague-Dawley rats and Beagle dogs by oral overdosage with compound B, a neuroleptic dopamine antagonist. Compound B was given to dogs for 13 weeks at dose levels of 30 or 120 mg/kg/day. Some dogs given 120 mg/kg/day were studied for a further 13 weeks after the last dose. Rats were given 300 mg compound B/kg/day until 20% survival was attained, with interim examinations after 13 and 52 weeks' treatment. Vehicle-dosed dogs and rats acted as control animals. Prolactin stimulation was more marked in dogs than in rats. Reversible changes in serum testosterone concentrations, ejaculate volume, spermatid maturation and prostate morphology induced in dogs were explicable in terms of disruption of the physiological synergism attributed to prolactin in testicular spermiogenesis and steroidogenesis. Failure to detect effects on the testicular weight and morphology of rats after 13 and 52 weeks' treatment concurred with published data. Decreased longevity was recorded among rats given 300 mg compound B/kg/day and there was possibly an earlier onset of naturally occurring age-related changes in the testes and pituitary glands of these rats. The toxicological significance of these observations is discussed.

PART THREE

3. Studies of chemical action on spermatogenic cells

In order to act directly on the cells of the seminiferous epithelium, chemicals must pass the 'blood-testis' barrier and subsequently modify cell structure or function (Fox and Fox, 1967). The precise nature of this physiological barrier is incompletely understood (Dym and Cavicchia, 1977, 1978; Setchell and Waites, 1975) and it is not possible to define concisely the physicochemical properties inherent in molecules possessing this biological activity (Lee and Dixon, 1978; Setchell and Main, 1978; Tuck et al., 1970; Waites, 1976; Waites and Einer-Jensen, 1974). The currently available techniques for the study of this aspect of testicular pathophysiology call for considerable technical expertise and facilities and therefore are not available in the majority of toxicological laboratories. While detailed understanding of primary causal mechanisms is scientifically desirable, the most urgent toxicological priority is to recognise those changes in testicular function and histomorphometry which can provide a rational basis for investigators to assess the safety, in normal use, of chemicals exerting this activity. This information may be useful in the toxicological evaluation of potential antispermatogenic agents, and also in explaining effects observed following the administration of unusually high doses of drugs, food additives and agricultural, industrial or household chemicals to laboratory animals. For these reasons, a series of experiments was performed with antispermatogenic compounds and some widely used chemicals, using rats and Beagle dogs.

3.1. Administration of lonidamine (AF 1890) to Beagle dogs for 9 weeks

Coulston et al. (1975) described the reversible inhibition of spermatogenesis, in rats and rhesus monkeys, induced by administration of 1-substituted indazole-3-carboxylic acids. Pharmacokinetic studies in rats (Catanese et al., 1977) suggested that selective action on spermatogenesis could be sustained by repeated gavage or dietary administration of doses corresponding to one-third of the effective single dose. The pharmacological and toxicological actions of 1-p-chlorobenzyl-1H-indazole-3-carboxylic acid (AF 1312/TS) were investigated with Long-Evans rats, CF1 mice, guinea-pigs, New Zealand White rabbits and cats (Silvestrini et al., 1975). The acute oral LD_{50} to rats varied between 966 and 1790 mg/kg. The compound was devoid of analgesic, anti-inflammatory, sedative or pressor activity. The lowest single oral dose showing antispermatogenic activity in rats was 200 mg/kg. Derangement of the seminiferous epithelium was characterised by degenerative changes in spermatids and spermatocytes and vacuolation of Sertoli cells. Because of the narrow ratio between antispermatogenic and acute toxic actions, further analogues were examined. Lonidamine, 1-$\left[(2,4\text{-dichlorophenyl})\text{methyl}\right]$-1H-indazole-3-carboxylic acid (AF1890) was found to be effective in inhibiting spermatogenesis of rats at 50 mg/kg (Coulston et al., 1975). Lobl and Mathews (1978) found that, at a dose of 50 mg/kg for five days, the antifertility effect could be confined to axoneme derangement and loss of fibre doublets in epididymal spermatozoa. The effect of lonidamine on circulating LH, FSH and testosterone concentrations of rhesus monkeys (Lobl et al., 1979) and rats (Lobl, 1979) has been investigated. Administration of antispermatogenic doses to rats increased FSH and decreased testosterone concentrations. With the rhesus monkey, antispermatogenic effects were variable and no conclusive changes in hormonal parameters were apparent. Heywood et al. (1980a) studied the sub-chronic toxicity of lonidamine in Sprague-Dawley rats and adolescent

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male rhesus monkeys. The only effect apparent in rats was on the hypothalamic-pituitary-gonadal axis: high doses in male rats induced testicular atrophy and increased pituitary weights; however, female rats developed suppression of ovarian activity and decreased pituitary weights. Variable effects were observed on spermatogenesis of rhesus monkeys, but the major manifestation of toxicity was renal tubular nephrosis at doses as low as 70 mg/kg/day. The renal effects were attributed to the rapid accumulation of the compound in proximal convoluted tubules. Since the pharmacokinetics in rhesus monkeys were possibly atypical, it seemed worth while to examine the effects of lonidamine in an alternative non-rodent species.

3.1.1. Experimental design

Four sexually mature male Beagles were obtained, the husbandry and management being as described in Appendices I (i) and (ii).

Body weight changes, food and water intake were recorded, see Appendix I (iii).

Lonidamine, supplied by the Istituto di Ricerca F. Angelini, Rome, Italy, was administered orally in gelatine capsules at a dose rate of 25 mg/kg/day for nine consecutive weeks. Two dogs were then killed for histopathological studies; the remaining two dogs were kept undosed for a further 13 weeks before histopathological evaluation.

Testes were measured, see Appendix I (vi) and semen examined, see Appendix I (vii) before treatment started, and during weeks 2, 4 and 8 of the dosing period and weeks 4, 8 and 12 of the withdrawal period. Na^+ and K^+ (mEq/l), alanine aminotransferase (E.C.2.6.1.2.), aspartate aminotransferase (E.C.2.6.1.1.) and acid phosphatase concentrations (mU/ml) were measured in seminal plasma on each occasion. Serum LH and testosterone concentrations, see Appendix I (vi) were determined after 8, 15, 29 and 57 days' dosing and again during weeks 4, 8 and 12 of the recovery phase. At the terminal examinations the testes, prostates and pituitaries were weighed, preserved and prepared for histological evaluation, see Appendix I (viii). Counts of type B spermatogonia, pachytene spermatocytes, early (round) spermatids, late (elongated) spermatids, Sertoli cells and measurements of seminiferous tubule diameters were made as described in Appendix I (ix).

3.1.2. Results

Body weight change, food intake and water consumption were unaffected by the experimental procedure. The mean changes in testicular area (mm^2) are depicted in Fig. 3.1.2.1. During the nine weeks of treatment a progressive reduction in testicular area was apparent. Statistically significant decreases ($p < 0.05$, paired 't'-test) in testicular area were recorded after two, four and eight weeks' dosing. Restoration of testicular size did not occur during the 13-week recovery period.

The findings of the hormonal assays are presented in Table 3.1.2.1. There were no marked differences in the mean values obtained for serum LH or testosterone at any of the selected sampling intervals.

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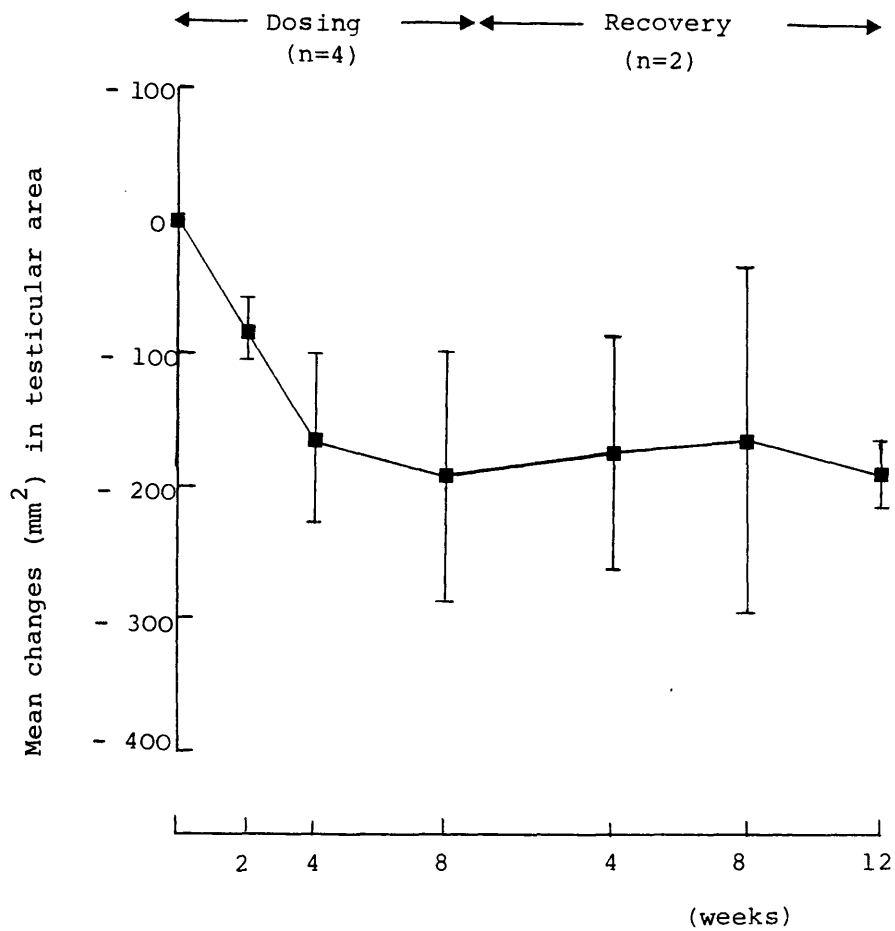
Fig. 3.1.2.1. Mean changes in testicular area for dogs given lonidamine (25 mg/kg/day). Vertical lines represent $\pm$ 1 standard deviation.

Mean concentration (ng/ml)

Examination	LH			Testosterone		
	Mean	SD	Range	Mean	SD	Range
Pre-dosing (n=4)	2.9	± 2.0	<0.5 - 5.1	2.0	± 1.6	0.5 - 4.2
Dosing period (n=4)						
Week 1	2.5	± 2.5	<0.5 - 6.1	1.9	± 1.3	1.3 - 3.6
Week 2	2.4	± 2.6	<0.5 - 6.2	1.1	± 0.9	0.2 - 1.9
Week 4	1.6	± 0.9	0.5 - 2.7	1.2	± 1.1	0.4 - 2.7
Week 8	3.0	± 2.7	2.8 - 7.5	2.4	± 0.8	1.6 - 3.4
Recovery period (n=2)						
Week 4	3.3	± 4.0	<0.5 - 6.0	1.6	± 0.6	1.1 - 2.0
Week 8	3.9	± 2.5	2.1 - 5.6	2.3	± 2.3	0.6 - 3.9
Week 12	2.9	± 3.2	0.6 - 5.1	3.2	± 2.3	1.6 - 4.8

Table 3.1.2.1. Results of hormonal determinations for Beagle dogs given lonidamine (25 mg/kg/day) for nine weeks followed by a 13-week recovery period. LH expressed as ng/ml canine pituitary standard LER-1685-1.

It is possibly noteworthy that, after eight weeks' treatment, the range of LH values was slightly increased in comparison with those obtained before dosing started.

The principal changes observed in the ejaculates (Fig. 3.1.2.2., Table 3.1.2.2.) were consistently decreased motility of spermatozoa, reduced sperm concentration and increased numbers of morphologically abnormal spermatozoa. Sperm counts were reduced after two weeks' treatment and oligospermia ($p < 0.05$, paired 't'-test) was apparent after four and eight weeks' treatment. The majority of spermatozoa were immotile after two, four and eight weeks' dosing and a high proportion of the spermatozoa present had coiled tails. These effects, although less marked, were still apparent after four weeks' withdrawal of lonidamine. After eight and 12 weeks' cessation of dosing, the semen characteristics examined were within the expected range of normal values. Seminal plasma biochemistry (Table 3.1.2.3.) was not affected by administration of lonidamine. It was not possible to establish correlations between seminal plasma aspartate aminotransferase concentrations and the effects on sperm concentration and morphology.

There were no treatment-induced differences in the weights of the testes, pituitary glands or prostates. No histological changes were evident in the pituitary or prostate glands. Histological changes were evident in the seminiferous tubules and epididymides after nine weeks' treatment (Figs. 3.1.2.3., 3.1.2.4.). Elongated spermatids were rarely found in seminiferous tubules and the numbers of round spermatids also appeared to be decreased. Spermatozoa were only occasionally seen in the lumen of the ductus epididymis, but round and multinucleate giant cells were often present. After 13 weeks' withdrawal of treatment, all stages of spermatogenesis were apparent in the seminiferous tubules, and mature spermatozoa were present in the ductus epididymis.
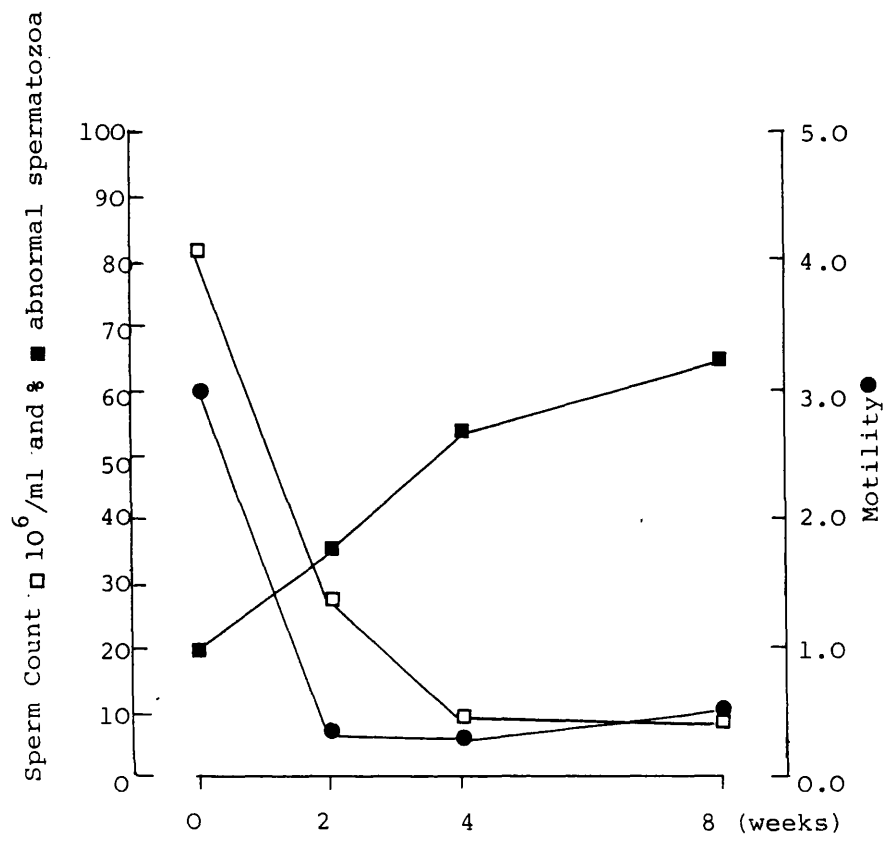
Fig. 3.1.2.2. Changes in semen motility, sperm concentration and morphology for dogs given lonidamine (25 mg/kg/day).

Semen characteristic	Before dosing (n=4)			After 8 weeks' dosing (n=4)			After 12 weeks' recovery (n=2)		
	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
Volume (ml)	3.1	± 2.0	1.8 - 6.0	2.9	± 0.8	1.8 - 3.8	3.9	± 0.4	3.6 - 4.1
Motility (0-5)	3.0	± 1.4	1.0 - 4.0	0.5[+]	± 0.6	0.0 - 1.0	2.5	± 0.7	4.0 - 5.0
Density (0-5)	3.0	± 0.8	2.0 - 4.0	2.0	± 0.0	2.0 - 2.0	4.5	± 0.7	2.0 - 3.0
Sperm count									
10^6/ml	82	± 25	57 - 112	8[+]	± 5	3 - 16	92	± 51	56 - 128
10^6/ejaculate	234	± 119	108 - 378	20[+]	± 9	8 - 29	364	± 228	202 - 502
% spermatozoa									
live	74	± 13	55 - 83	85	± 12	68 - 95	92	± 7	88 - 95
abnormal	20	± 1	19 - 22	65[++]	± 11	55 - 81	15	± 2	13 - 16

Table 3.1.2.2. Results of semen examinations for Beagle dogs given lonidamine (25 mg/kg/day) for nine weeks followed by a 13-week recovery period. Statistically significant differences (paired 't'-test) from pre-dose values, $p < 0.05$[+] $p < 0.01$[++]

Treatment	Seminal plasma biochemistry				
	Na$^+$	K$^+$	Acid phosphatase	AlT	AsT
	(mEq/l)	(mEq/l)	(mU/ml)	(mU/ml)	(mU/ml)
Predose	134 $\pm$ 14	10.9 $\pm$ 2.4	1112 $\pm$ 797	11 $\pm$ 4	31 $\pm$ 5
(n=4)	(114 - 143)	(7.6 - 13.2)	(256 - 2120)	(7 - 14)	(26 - 36)
Lonidamine					
9 weeks' dosing	142 $\pm$ 14	11.8 $\pm$ 3.0	1284 $\pm$ 653	14 $\pm$ 8	31 $\pm$ 3
(n=4)					
13 weeks' recovery	140 $\pm$ 1	11.7 $\pm$ 2.1	1560 $\pm$ 441	11 $\pm$ 0	45 $\pm$ 2[+]
(n=2)					

Table 3.1.2.3. Results of biochemical examination of seminal plasma for dogs given lonidamine (25 mg/ kg/day) for nine weeks followed by a recovery period of 13 weeks. AlT, alanine aminotransferase (2.6. 1.2); AsT, aspartate aminotrasferase (2.6.1.1). Statistically significant difference from pre-dose value (paired 't'-test) p < 0.05[+]. Pre-dose ranges are indicated parenthetically.
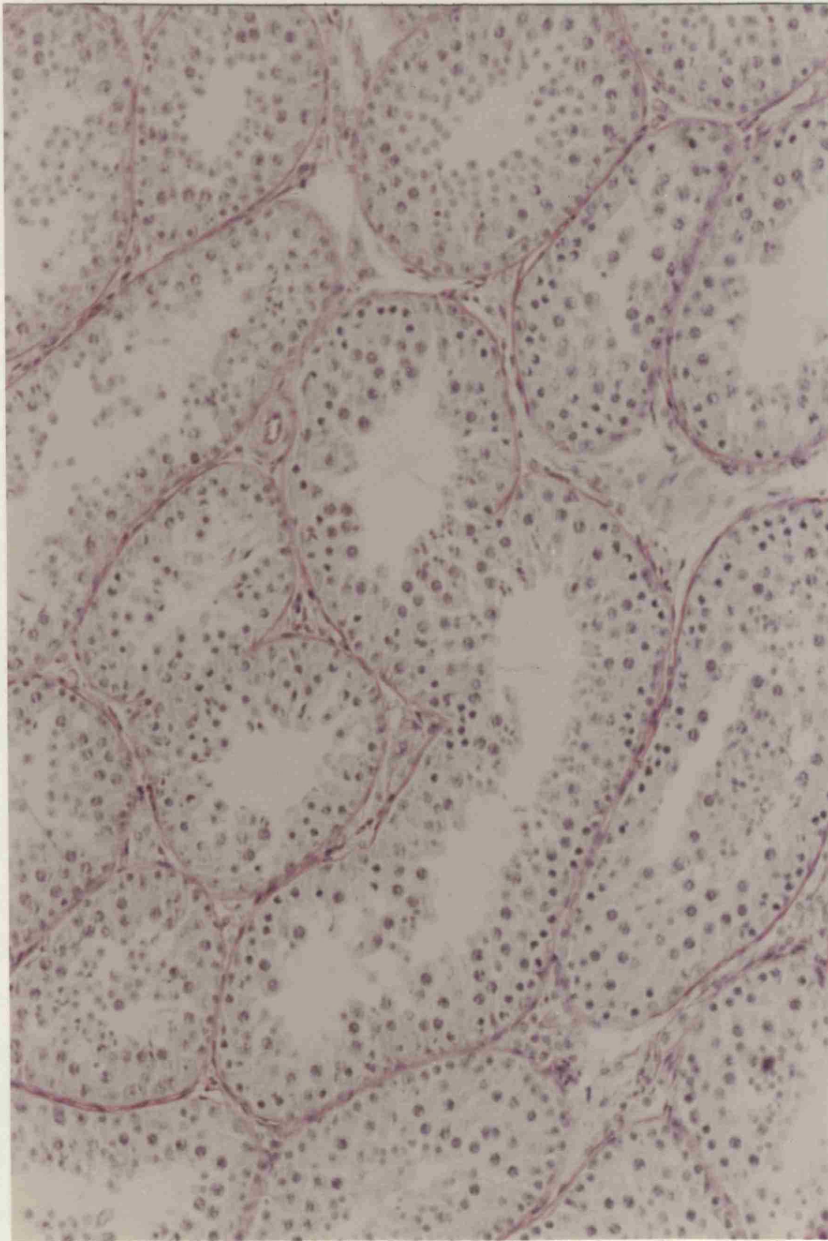
Fig. 3.1.2.3. Seminiferous epithelium of a Beagle dog given lonidamine (25 mg/kg/day) for nine weeks. Marked absence of spermatids, especially elongated forms. PAS, x 200.
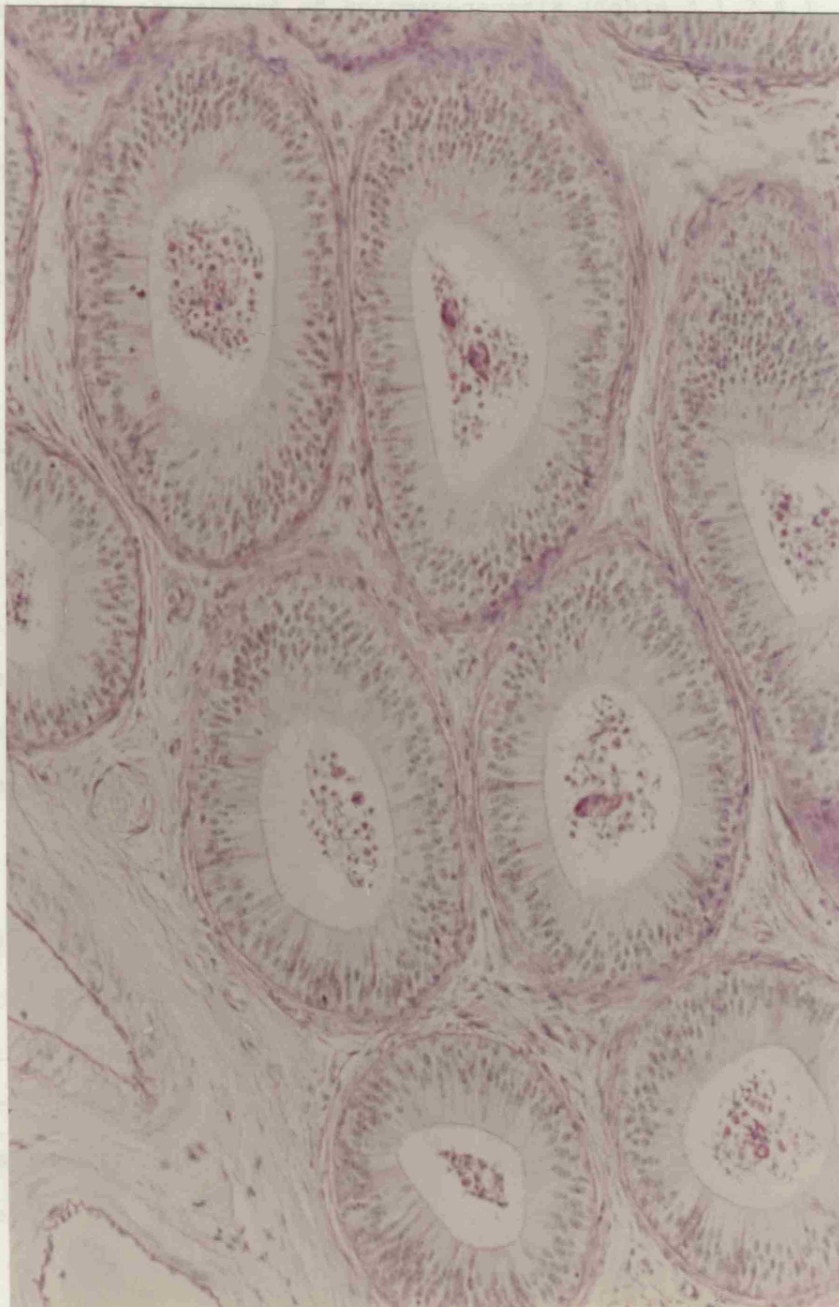
Fig. 3.1.2.4.   Ductus epididymis of a Beagle dog given lonidamine
(25 mg/kg/day) for nine weeks. Reduced numbers of spermatozoa
with round cells present in lumen. PAS, x 200.

Quantitative assessment of spermatogenesis (Table 3.1.2.4.)

confirmed the histological impressions. After nine weeks' treatment

the decreased tubular diameter and reduced counts of pachytene

spermatocytes, early and late spermatids differed significantly

(p < 0.05) from the values determined for dogs examined after 13

weeks' withdrawal of lonidamine treatment.

3.1.3. Discussion

These data have provided useful information about the antispermato-

genic effects of lonidamine in Beagle dogs without recourse to

serial mating procedures. Daily dosing for nine consecutive weeks

at a dosage rate of 25 mg/kg/day induced obvious infertility

within 14 days, and the effects persisted for up to eight weeks

after cessation of dosing.

The results are compatible with the hypotheses of Coulston et al.

(1975) and Silvestrini et al. (1975), that the primary anti-

spermatogenic effect of indazole-carboxylic acids is an action

on the seminiferous epithelium and not the hormonal control of

spermatogenesis. Depletion of maturing spermatids may be expected

to provoke increased pituitary secretion of gonadotrophins

(Steinberger, 1971) via the hypothalamic feedback mechanisms. It

was not possible to examine FSH activity; however, the results of

the hormonal examinations which were undertaken provided no

evidence of anti-gonadotrophic or anti-androgenic effects.

Treatment	Mean values for										
	Tubule diameter (µm)	Germ cell counts								Sertoli cells	
		Type B spermatogonia (stage 7)		Pachytene spermatocytes (stage 5)		Spermatids					
						Early (stage 1)		Late (stage 8)			
		Crude	Corrected	Crude	Corrected	Crude	Corrected	Crude	Corrected	Crude	Corrected
Lonidamine (25 mg/kg/day)											
9 weeks' dosing	$179 \pm 8^{++}$	13	7.6	36^{+++}	16.9	42^{+++}	29.6	19^{+++}	13.3	22	10.3
13 weeks' recovery	214 ± 14	16	7.8	49	19.1	102	59.8	97	56.8	23	9.0

Table 3.1.2.4. Quantitative assessment of spermatogenesis for Beagle dogs given lonidamine (25 mg/kg/day) for nine weeks or after 13 weeks' withdrawal of treatment. Statistically significant differences, treated versus withdrawal dogs (ANOVAR), $p < 0.01^{++}$, $p < 0.001^{+++}$

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The results of the semen examinations, together with the histological findings, suggest that, in the absence of reduced gonadotrophic output, oligospermia was caused by cytotoxic effects on spermatid development. These results confirm previous findings in rats (Coulston et al., 1975) and rhesus monkeys (Lobl et al., 1979) following administration of substituted indazole-carboxylic acids.

Although the antispermatogenic effects of this compound in the dog appear to be essentially reversible, a number of points warrant further discussion. The area calculated from the gross dimensions of the testes, in the living animals, was apparently irreversibly decreased, although the weight of the testes at post-mortem examination was within the range of normal values. Caution is necessary in reconciling these observations, because the observed 30% decrease in testicular area followed mean decreases of less than 5 mm in testicular length and breadth. In view of the wide variations in weight of the Beagle testes, it is unlikely that reductions in testicular dimensions of this order would result in a detectable decrease in testicular weight.

At a dose of 25 mg/kg/day, obvious morphological changes in the seminiferous epithelium became apparent: in compounds not intended to act as contraceptives this is likely to be considered an undesirable toxic phenomenon. Although the observed histological changes were shown to be reversible, this was not so for changes in seminal plasma aspartate aminotransferase. The data do not permit assessment of mutagenic hazards during the induction of infertility or the restoration of normal spermatogenesis. The acceptability of using chemicals to act on dividing or differentiating spermatogenic cells remains, as yet, an unsolved toxicological question.

3.1.4. Abstract

Four sexually mature Beagle dogs were given lonidamine at 25 mg/kg/day, orally, for nine consecutive weeks. Two of the dogs were retained undosed for a further 13 weeks in order to assess recovery. Testicular dimensions, serum luteinising hormone (LH) and testosterone concentrations, together with semen characteristics, were assessed. The light-microscopic appearance of the testes was examined. Reduced testicular dimensions, oligospermia and coiled-tail immotile spermatozoa were evident after two weeks' dosing. There were no conclusive effects on serum LH or testosterone and testicular weights were within the normal range of variation. Light microscopy showed that spermatid development was arrested at the early round cell phases. Although testicular dimensions remained decreased, effects on the morphology of the seminiferous epithelium were reversed following 13 weeks' withdrawal of treatment.

3.2. Administration of 1-amino-3-chloro-2-propanol hydrochloride

(CL 88, 236) to rats for 12 weeks

A number of substituted chlorohydrin derivatives exert an

antifertility action in male rodents (Ericsson and Youngdale, 1970).

⍺-Chlorohydrin(3-chloro-1,2 propanediol) was used to inhibit

selectively the acquisition of fertilising capacity by rat

epididymal spermatozoa without disrupting spermatogenesis or

decreasing libido (Coppola, 1969). Development of this analogue

as a human male contraceptive ceased after toxic changes were found

in haemopoietic tissues of monkeys given comparatively low dosages

(Kirton et al., 1970; Setty et al., 1970). The search has contin-

ued, to identify a chlorohydrin derivative which retains selective

antifertility properties without concomitant toxicity in other

organ systems. Tsunoda and Chang (1976) reported that 1-amino-3-

chloro-2-propanol hydrochloride (CL 88, 236) effectively prevented

the acquisition of fertilising capacity by epididymal spermatozoa

of mature male Sprague-Dawley rats at an oral dose rate of 14.7 mg/

kg/day.

The chronic oral toxicity of CL 88, 236 has been studied in rhesus

monkeys at doses of 0, 50, 150 or 300 mg/kg/day (Heywood et al.,

1978) and in rats at doses of 0, 50, 250 or 500 mg/kg/day (James et

al., 1978). Acute neurotoxic lesions were induced in the medulla

oblongata of rhesus monkeys at all doses tested. Apart from

increases in kidney and liver weights without accompanying

histological changes, no toxic effects occurred in the extragonadal

tissues of rats given CL 88, 236. Although the possibility of

human usage is excluded by the neurotoxic effects seen in rhesus

monkeys, the effects of CL 88, 236 overdosage on the seminiferous

epithelium of rats has been studied as the basis of a toxicological model for the safety evaluation of future potential extra-testicular male contraceptive agents.

3.2.1. Experimental design

60 specific pathogen-free male Sprague-Dawley rats of the CFY strain were obtained and allocated to groups of 15 rats. The housing, management and husbandry of the rats is described in Appendices I (i) and (ii). Body weight changes, food and water intake were recorded, see Appendix I (iii). CL 88, 236 was supplied by Lederle Laboratories. Solutions were prepared daily in distilled water at concentrations of 1%, 5% or 10% w/v; these solutions were administered by gastric intubation at a standard dose volume of 0.5 ml/100 g body weight so that daily doses of 50, 250 or 500 mg/kg were given. The fourth group of rats acted as controls and were given distilled water at 0.5 ml/100 g body weight. Dosing was continued for 12 weeks, during which time any clinical abnormalities were recorded daily, and body weight and food intake were measured weekly. Premature deaths were fully investigated in order to establish factors contributing to death. All rats surviving the dosing period were killed by carbon dioxide asphyxiation and autopsied. The testes, epididymides and prostate of each rat were weighed and preserved. Histological sections of testes were stained with PAS; all the remaining tissues were stained with haematoxylin and eosin, see Appendix I (viii). A quantitative assessment of testicular spermatogenesis was undertaken, but rats which developed severe atrophy of seminiferous tubules and/or epididymal sperm granulomata were excluded from this examination. Counts of type B spermatogonia, pachytene spermatocytes, round (early) spermatids and elongated (late) spermatids, Sertoli cells and measurements of seminiferous tubule diameters were made as described in Appendix I (ix).

3.2.2. Results

One male rat receiving 500 mg/kg/day died during week 11, but a
conclusive cause of death could not be established. No adverse
signs were seen in the remaining rats, and food consumption was
unaffected by treatment with CL 88, 236. Rats given 500 mg/kg/day
gained less weight than control rats. The selected organ weight
data are presented in Table 3.2.2.1. Apart from a statistically
significant ($p < 0.001$) reduction in prostate weight for rats given
500 mg/kg/day there were no other differences between control and
treated rats. Oedema and/or yellow discoloration were apparent in
the epididymides of one rat given 50 mg/kg/day, two given 250 mg/
kg/day and nine given 500 mg/kg/day. The testes were considered to
be enlarged or flaccid in one control rat, six given 250 mg/kg/day
and 11 given 500 mg/kg/day. The macroscopic post-mortem examinat-
ions and histological observations are summarised in Table 3.2.2.2.
Epididymal sperm granulomata (Fig. 3.2.2.1.) were apparent at all
dosages and the x^2 test for linear trend with increasing dosage was
statistically significant ($p < 0.05$). Varying degrees of atrophy
of the seminiferous epithelium was found among all groups, including
controls. Unilateral small foci of atrophic seminiferous tubules
were evident in a single control rat and one given 50 mg/kg/day.

Bilateral foci of atrophic seminiferous tubules were seen in one
rat given 250 mg/kg/day and one given 500 mg/kg/day. One control
rat developed unilateral diffuse atrophy of seminiferous tubules.

Dose CL 88, 236 (15 rats/group)	Body weight change (g)	Absolute organ weights (g)		
		Testes	Epididymides	Prostate
Controls	394	3.4	1.4	1.0
50 mg/kg/day	401	3.5	1.5	1.0
250 mg/kg/day	392	3.5	1.5	1.0
500 mg/kg/day[a]	359^{+}	3.7	1.5	0.7^{+++}

Table 3.2.2.1. Body weight changes and testes, epididymal and prostatic weights of rats given CL 88, 236 for 12 weeks. Statistically significant differences from control data (ANOVAR), $p < 0.05^{+}$, $p < 0.001^{+++}$; [a], excludes animal dying during week 11.

Dose of CL 88, 236 (15 rats/group)	Number of rats with changes at							
	Macroscopic examination				Histological examination			
	Testes		Epididymides		Atrophic seminiferous epithelium		Ductus epididymis	
	Flaccid	Enlarged	Discoloured	Enlarged	No granuloma	Granuloma present	Sperm granuloma	Vacuolated epithelium
Controls	1	0	0	0	2	0	0	0
50 mg/kg/day	0	0	1	0	1	0	1	5
250 mg/kg/day	5	2	2	0	6	1	2	2
500 mg/kg/day[a]	6	5	9	2	4	5	9	1

Table 3.2.2.2. Summary of macroscopic and histological changes in the testes and epididymides of rats given CL 88, 236 for 12 weeks. [a], excludes rat dying during week 11.

Fig. 3.2.2.1.   Sperm granuloma seen in the ductus epididymis

of a rat given 250 mg/kg/day CL 88, 236 for 12 weeks.   H & E,

x 50.

Diffuse unilateral or bilateral atrophy of seminiferous tubules

(Fig. 3.2.2.2.) was not induced at 50 mg/kg/day but was apparent in

six out of 15 rats given 250 mg/kg/day and eight out of 14 rats

surviving treatment at 500 mg/kg/day. The incidence among the

latter two groups differed significantly from the control incidence

(Fishers' Exact test, two-tailed, $p < 0.05$). The occurrence of severe

atrophy of the seminiferous epithelium was not strongly correlated

with the presence of sperm granulomata. Although many rats given

CL 88, 236 developed vacuolation of epididymal epithelium, severe

atrophy of the seminiferous epithelium also occurred in the absence

of this lesion. The findings of quantitative assessment of test-

icular spermatogenesis in rats with all stages of spermatogenesis

recognisable in the majority of seminiferous tubules and absence of

sperm granulomata are tabulated in Table 3.2.2.3. The diameter of

seminiferous tubules was significantly decreased ($p < 0.05$) for rats

given 250 and 500 mg/kg/day. There were no statistically signifi-

cant differences ($p > 0.05$) in respect of tubule diameter or counts

of type B spermatogonia, pachytene spermatocytes, early or late

spermatids for rats given 50 mg/kg/day. Statistically significant

reductions in the counts of early (stage 1) and late (stage 8)

spermatids were apparent at 250 and 500 mg/kg/day. The pachytene

spermatocyte count of the single rat suitable for examination at

500 mg/kg/day was also decreased.

3.2.3. Discussion

The results of giving CL 88, 236 at 250 and 500 mg/kg/day are

similar to the epididymal lesions and atrophy of the germinal

epithelium previously induced with high dosages of ᴼ-chlorohydrin

(Cooper et al., 1974; Ericsson, 1970; Hoffer et al., 1973).
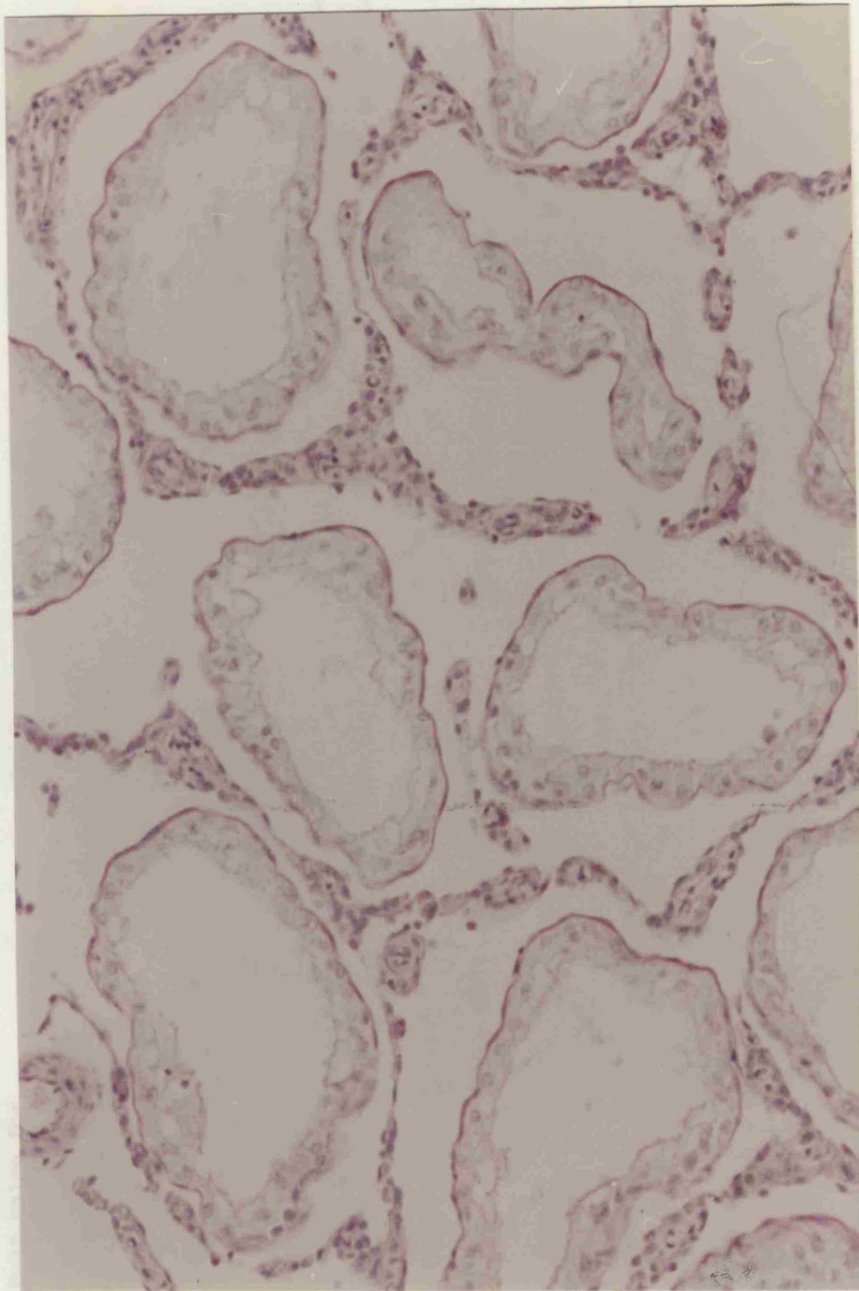
Fig 3.2.2.2. Total atrophy of seminiferous epithelium
observed in a rat given 250 mg/kg/day CL 88, 236 for 12
weeks. H & E, x 200.

Table 3.2.2.3. Quantitative assessment of testicular spermatogenesis for rats given CL 88, 236 for 12 weeks. Statistically significant differences (ANOVAR) from control data, $p < 0.05$[+], $p < 0.01$[++], $p < 0.001$[+++].

Dose CL 88, 236 (mg/kg/day)	Number of rats examined	Tubule diameter (µm)	Germinal cell counts									
			Type B spermatogonia (stage 7)		Pachytene spermatocytes (stage 5)		Spermatids				Sertoli cells	
							Early (stage 1)		Late (stage 8)			
			Crude	Corrected	Crude	Corrected	Crude	Corrected	Crude	Corrected	Crude	Corrected
Controls	13	298 ± 27	43	18.8	77	22.1	194	69.0	171	69.5	23	12.8
50	13	286 ± 35	43	18.6	79	23.6	203	75.3	165	69.8	22	12.7
250	7	257 ± 13[++]	43	20.7	73	24.2	153[+++]	63.1	136[+++]	64.0	23	14.7
500	1	265 ± 2[+]	40	18.6	62[++]	20.0	152[+]	60.8	103[+++]	47.0	22	13.7

It is, however, important to distinguish between the primary

mechanism of a selective antifertility action at low dosages and

the pathological changes induced following administration of

excessively large dosages. Studies of the influence of α-chlorohy-

drin on fluid reabsorption in the cauda epididymis (Wong and Yeung,

1977), necrosis of the epididymis (Gunn et al., 1970), epididymal

vasculature (Reijonen et al., 1975) and epididymal lipid and

prostaglandin content (Voglmayr, 1974), show that functional infer-

tility may be induced without structural alteration unless excessive

dosages are administered. Mohri et al. (1975) suggested that

α-chlorohydrin is phosphorylated on entry into spermatozoa and so

acts as a competitive inhibitor in the enzyme-mediated conversion of

glyceraldehyde-3-phosphate to 3-phosphoglycerate. Ford et al.

(1977) studied the effects of α-chlorohydrin enantiomers on triose

phosphate metabolism in the glycolytic pathway of ram testicular

spermatozoa and rat epididymal spermatozoa. Marked stereospecific

effects were apparent, but epididymal and testicular spermatozoa

showed qualitatively similar responses. However, a higher concen-

tration of enantiomer was needed to affect testicular than epididy-

mal spermatozoa. The results of this toxicological investigation

in the rat, using high dosages, do not conflict with these concepts.

The testicular histomorphometry of rats given CL 88, 236 at 50 mg/

kg/day was comparable with that of control rats. The quantitative

reduction in spermatogenic cells at the two higher dosages was

predictable on the basis of other investigators' results. The lack

of correlation between atrophy of the seminiferous epithelium and

epididymal lesions confirms the view that disruption of epididymal

physiology and testicular spermatogenesis in rats are independent

but predictable hazards of overdosage.

Although a dose of 50 mg/kg/day administered for 12 weeks did not induce deleterious changes in the seminiferous epithelium, epididymal changes were found. Even if the compound had not induced neurotoxic changes in primates (Heywood et al., 1978) the ratio between the minimal effective dose (14.7 mg/kg/day) and the lowest dose evoking signs of overdosage is unacceptably low.

3.2.4. Abstract

A quantitative histomorphometric assessment of testicular spermato-genesis was undertaken on testes obtained from rats which received 1-amino-3 chloro-2 propanol hydrochloride (CL 88, 236) at oral doses of 0, 50, 250 or 500 mg/kg/day for 12 weeks. Rats which developed epididymal sperm granulomata or severe atrophy of the germinal epithelium were excluded from quantitative examinations. Pathological changes in the epididymis and seminiferous epithelium were not strongly correlated. CL 88, 236 administered at 50 mg/kg/day was without effect on the histomorphometry of the seminiferous epithelium, although epididymal lesions occurred at this dose. At higher dosages a quantitative reduction in testicular spermatids was evident. It seems important to differentiate between the selective antifertility action of CL 88, 236 on the biochemistry of epididymal spermatozoa and the disruption of epididymal physiology and testicular spermatogenesis found at unusually high dosages.

3.3. Administration of cyclohexylamine to Beagle dogs and rats for nine weeks

Effects on testicular weight and histology have been associated with prolonged dietary administration of cyclamates to rats (Oser et al., 1975; Taylor and Friedman, 1974). Testicular changes occur only after administration of dietary concentrations of at least 3% for 12 months or longer. Such experimental treatment also induces gastrointestinal disturbances, reduced food intake and impaired body weight gain. Coulston et al. (1977) found that the testes of rhesus monkeys given cyclamate at an oral dose rate of 200 mg/kg/day for eight years, were histologically normal. While testicular effects have not been observed in short-term rodent or canine studies (Richards et al., 1951; Taylor et al., 1968) and testicular changes do not occur following chronic feeding at less than 3%, the safety in normal usage of cyclamates as food additives is a continuing focus for debate.

The metabolic fate of cyclamate has been studied in both man and animals (Golberg et al., 1969; Parekh et al., 1970; Renwick and Williams, 1972). Following continuous ingestion of cyclamate, up to 70% conversion to cyclohexylamine (CHA) occurs. The CHA formed is excreted in urine, mainly unchanged, with trace amounts of cyclohexanone, cyclohexanol and N-hydroxycyclohexylamine. The pharmacological characteristics of CHA include release of catecholamines, acetylcholine and histamine together with analgesic, sedative and tranquillising properties (Miyata et al., 1969). The compound is a strong base, irritant to skin and mucous membranes, with an oral LD_{50} value of 710 mg/kg for rats (Smyth et al., 1969).

The potential testicular toxicity and antifertility effects of CHA have been examined in rats (Gaunt et al., 1974; Green et al., 1972; Khera and Stoltz, 1970; Khera et al., 1971; Kroes et al., 1977; Mason and Thompson, 1977).

Testicular changes, reduced food intake and impaired weight gain are consistently reproducible at dosages of 288-528 mg/kg/day. At lower dosages of CHA, conflicting data exist: however, there is a definite 'no effect' dose at 30 mg/kg/day. The only effect observed in mating studies has been a slight increase in pre-implantation loss when high dosages are administered. Cattanach (1976) has reviewed the mutagenic studies undertaken with CHA and its metabolites. This author concluded that the effects observed in rodent breeding experiments could not be ascribed to dominant lethality. Furthermore, there was no evidence to implicate CHA as a mutagen in normal use. The mechanism underlying the testicular responses to overdosage with CHA remains a matter for speculation. Studies to examine the effects of massive dosages of CHA on pituitary-testicular hormones, semen characteristics and quantitative assessment of spermatogenesis were therefore undertaken with Beagle dogs and rats.

3.3.1. Experimental design

Four sexually mature Beagle dogs and 30 specific pathogen-free male Sprague-Dawley rats (CD strain) aged 35 days were obtained. The animal management, housing and husbandry were as described in Appendices I (i) and (ii). Body weight changes, food and water intake were recorded, see Appendix I (iii). CHA conforming to the specification of the British Standards Institution (British Standards Institution, 1968) was obtained from Fisons Limited, Loughborough, Leics., UK.

The 30 rats were allocated to an experimental or a control group, each of 15 rats, to comprise approximately equal initial group mean body weights. A 4.62% v/v suspension of CHA in corn oil was prepared once weekly and stored in airtight smoked glass containers. The CHA suspension was administered by oral gavage at a dosage volume of 0.5 ml/100 g so that each rat received 200 mg/kg CHA daily. Control rats were given corn oil at the same dosage volume. CHA was administered to all four dogs as a 3.47% v/v suspension in corn oil. Gastric irritation made it necessary to employ an incremental gavage regimen for the dogs. Initially the CHA suspension was administered to achieve oral doses of 75 mg/kg/day on days 1 and 2, of 75 mg/kg twice daily on days 3 and 4, of 150 mg/kg on days 5 and 6, then 150 mg/kg twice daily on subsequent days. After day 9 the dose was reduced to 125 mg/kg twice daily to avoid marked appetite suppression. Five rats from each group were killed after four and nine weeks' administration and two dogs after nine weeks' dosing. The remaining five rats per group and two dogs were maintained undosed for 13 weeks before terminal examination.

All animals were examined several times daily for evidence of clinical malreaction to CHA treatment. Rats that died during the investigation were autopsied in order to establish a possible cause of death. Testicular measurements and semen examinations of the dogs, see Appendices I (vi) and (vii), were made before starting treatment and again during weeks 4, 8 and 12 of the withdrawal period. The concentrations in seminal plasma of Na^+, K^+ (mEq/l), acid phosphatase, alanine aminotransferase (E.C. 2.6.1.2.) and aspartate aminotransferase (E.C. 2.6.1.1.) were determined (mU/ml) as described in Appendix I (vii).

Serum samples were obtained, see Appendix I (v), from five rats per group after one day and four and nine weeks' treatment and 13 weeks' withdrawal for determination of follicle-stimulating hormone (FSH), luteinising hormone (LH) and testosterone, see Appendix I (vi). Canine sera were examined for LH and testosterone concentrations, see Appendix I (vi), after one, two, four and eight weeks' treatment and during weeks four, eight and 12 of the withdrawal period.

Terminal procedures performed on up to five rats per group after four and nine weeks' dosing and 13 weeks withdrawal, and two dogs after nine weeks' dosing and 13 weeks' withdrawal, comprised full macroscopic post-mortem examination, together with weighing and preservation of pituitary, testes including epididymides, prostate and seminal vesicles (rats only). Histological preparations of the preserved organs were stained as described in Appendix I (viii).

Counts of type B spermatogonia, pachytene spermatocytes, round (early) and late (elongated) spermatids, Sertoli cells and measurements of seminiferous tubule diameters were made as described in Appendix I (ix).

3.3.2. Results

Two rats died during the investigation: one control rat during week 11 of the recovery period and one rat eight weeks after cessation of CHA treatment. In both cases death was attributable to chronic respiratory disease.

Clinical reactions to CHA administration were manifested in rats as reduced motor and grooming activity during the first week of treatment. Dogs given CHA vomited on up to 35% of the dosing occasions during the first four weeks; subsequently, vomiting occurred after approximately 12% of the dosing occasions. Vomiting occurred between five and 120 minutes after dosing. The dogs were frequently observed to be unusually quiet and tended to pass loose faeces.

Over the nine-week dosing period dogs showed a 10 % loss of body weight (Fig. 3.3.2.1.) and treated rats showed a 25 % suppression in the rate of weight gain in comparison with the control rats (Fig. 3.2.2.2.). The adverse effect on the body weight of dogs was completely reversed during the recovery period: however, rats which had received CHA still showed a 13% suppression in comparison with the corresponding control groups. Administration of CHA impaired appetite for both species: the dogs showed a 25% reduction (Fig. 3.3.2.1.) and rats a 15% reduction in food intake over the nine weeks of treatment (Fig. 3.3.2.2.). During the recovery period the dogs regained normal appetite, but previously treated rats continued to exhibit a 10% reduction in food consumption. Both species tended to drink slightly increased quantities of water during the dosing period.

Treatment with CHA did not significantly affect the serum LH or testosterone concentrations of dogs. All values were as expected for sexually mature Beagle dogs. Increased FSH and decreased testosterone concentrations attained statistical significance ($p < 0.05$) for rats given CHA for nine weeks (Table 3.3.2.1.).

Body weight (kg)
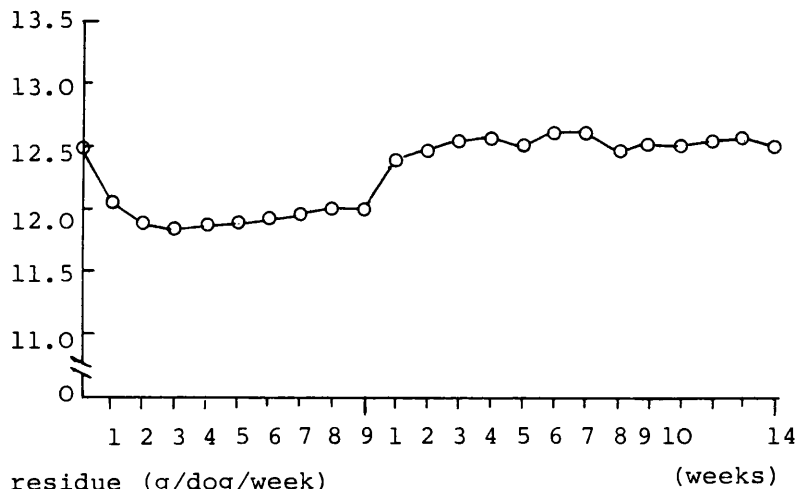
Food residue (g/dog/week)
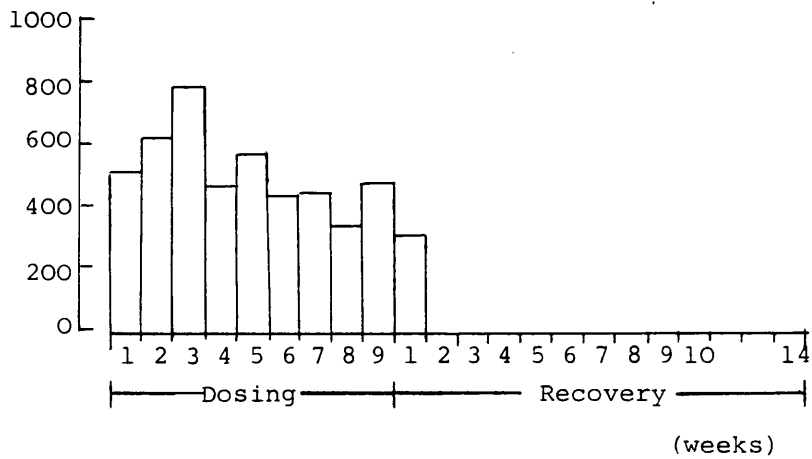
Fig 3.3.2.1. Effects of CHA on body weight and food residue when administered to Beagle dogs at 250 mg/kg/day for nine weeks followed by a 13-week recovery period.

Body weight (g)
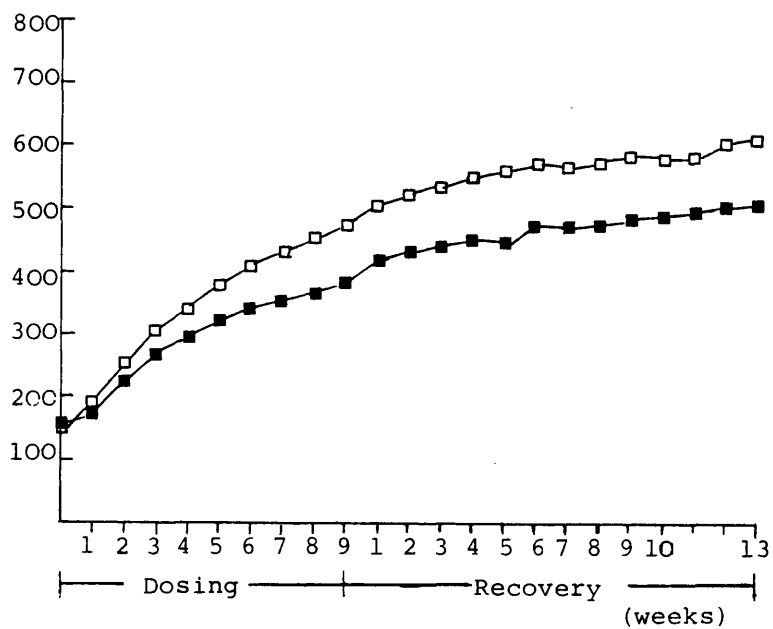
Food consumption (g/rat/week)
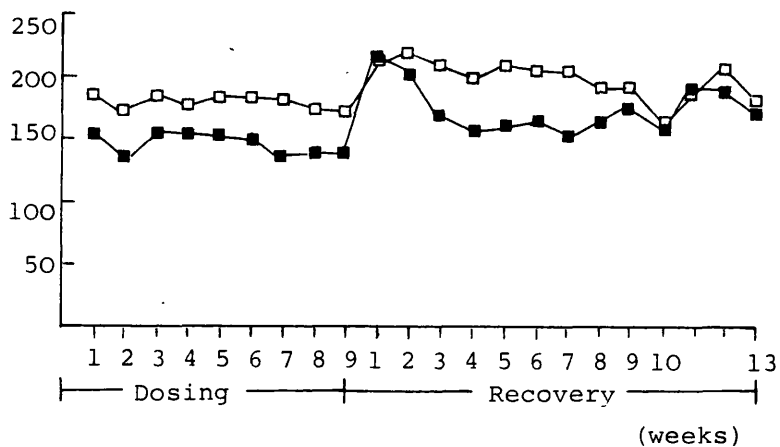
Fig. 3.3.2.2. Effect of CHA on body weight and food consumption when administered to rats at 200 mg/kg/day (■) for nine weeks followed by a 13-week recovery period in comparison with vehicle-dosed (□) control rats.

Examination	Treatment	Mean serum hormone concentration (ng/ml) $\pm$ S D		
		FSH	LH	Testosterone
2 days' dosing	Controls	357 ± 3	21 ± 27	0.78 ± 0.38
	CHA	367 ± 147	13 ± 8	1.57 ± 1.12
4 weeks' dosing	Controls	211 ± 53	10 ± 6	1.52 ± 0.44
	CHA	243 ± 55	< 5	1.42 ± 0.48
9 weeks' dosing	Controls	222 ± 55	14 ± 15	2.00 ± 0.36
	CHA	$358 \pm 91^+$	10 ± 29	0.76 ± 0.21
13 weeks' recovery	Controls	249 ± 32	35 ± 19	1.05 ± 0.52
	CHA	278 ± 136	41 ± 40	0.80 ± 0.32

Table 3.3.2.1. Results of serum FSH, LH and testosterone determinations for rats given cyclohexylamine (200 mg/kg/day) for nine weeks followed by a 13-week recovery period. Statistically significant difference from concurrent control data (ANOVAR) $p < 0.05^+$. LH expressed as ng NIAMDD-LH-RP1/ml and FSH as mg NIAMDD-FSH-RP1/ml.

Although not statistically significant, similar trends were apparent for rats examined 13 weeks after cessation of CHA treatment.

Slight reductions in the testicular measurements of dogs given CHA attained statistical significance ($p < 0.05$, paired 't'-test) after four and eight weeks' treatment. After eight weeks' CHA administration, decreased sperm counts and an increased percentage of abnormal (coiled-tail) spermatozoa were evident in canine ejaculates (Table 3.3.2.2.). Normal sperm counts were obtained at all examinations made during the recovery period. Increased numbers of abnormal spermatozoa were evident after four and eight weeks' withdrawal but not after 12 weeks. Administration of CHA did not modify the biochemical measurements made on seminal plasma samples. There were no correlations between seminal plasma enzyme concentrations and sperm morphology. The mean seminal plasma aspartate concentrations of each examination are depicted in Fig. 3.3.2.3. together with concurrent data for sperm concentration and morphology.

Organ weight data are summarised in Table 3.3.2.3. The pituitary, testicular and prostate weights of dogs examined after nine weeks' dosing were marginally lower than after 13 weeks' recovery. However, dogs in the first group examined were of lower body weight and all values were within expected ranges. Testicular and seminal vesicle weights of rats given CHA were consistently lower than those of control rats at all examinations. Analysis of covariance with initial and final body weight as covariates was performed with the rat data. No statistically significant differences ($p > 0.05$) were apparent.

Semen characteristic	Before dosing (n=4)			After 8 weeks' dosing (n=4)			After 12 weeks' recovery (n=2)		
	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
Volume (ml)	6.0	± 6.8	1.5 – 15.9	4.4	± 3.9	1.3 – 10.0	6.5	± 4.5	3.0 – 9.0
Motility (0–5)	2.0	± 0.6	1 – 4	2.0	± 1.4	0 – 3	2.0	± 1.4	1 – 3
Density (0–5)	2.5	± 1.4	2 – 3	2.5	± 0.6	2 – 3	3.5	± 0.7	3 – 4
Sperm count									
10^6/ml	67	± 48	18 – 125	29	± 11	19 – 42	106	± 49	71 – 104
10^6/ejaculate	241	± 140	59 – 286	119	± 81	25 – 220	563	± 314	423 – 703
% sperm									
live	79	± 13	68 – 97	85	± 3	82 – 88	87	± 6	83 – 91
abnormal	12	± 7	5 – 22	40	± 6[+]	31 – 46	9	± 4	6 – 11

Table 3.3.2.2. Effects of cyclohexylamine (250 mg/kg/day) on the semen characteristics of Beagle dogs.

Statistically significant difference (paired 't'-test) from pre-dose value, p < 0.05[+].
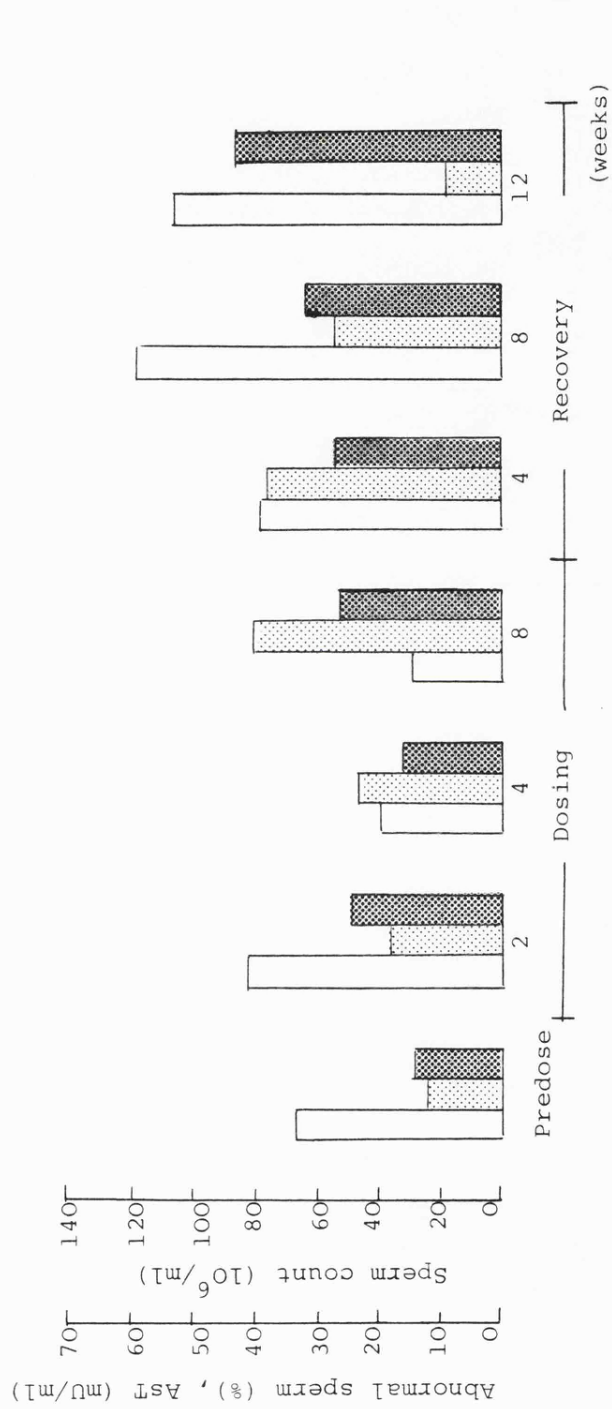
Fig. 3.3.2.3. Changes in sperm count (□), percentage abnormal sperm (▨) and seminal plasma aspartate aminotransferase (▧) for Beagle dogs given cyclohexylamine (250 mg/kg/day) for nine weeks followed by a 13-week recovery period.

Species	Treatment	Body weight (kg)		Mean organ weights (g)			
		Initial	Final	Pituitary (mg)	Testes	Prostate	Seminal vesicle
Dog	CHA (250 mg/kg/day)						
	9 weeks' dosing	12.0	10.6	68	18.7	7.2	–
	13 weeks' recovery	13.1	13.2	81	23.8	10.5	–
Rat	4 weeks' dosing						
	Controls	0.150	0.434	11	4.2	0.6	1.5
	CHA (200 mg/kg/day)	0.146	0.293^{+}	9	3.8	0.5	1.0
	9 weeks' dosing						
	Controls	0.146	0.460	11	4.8	0.7	1.4
	CHA (200 mg/kg/day)	0.152	0.375^{++}	11	4.5	0.8	1.2
	13 weeks' recovery						
	Controls	0.147	0.549	15	5.0	0.8	2.0
	Ex CHA (200 mg/kg/day)	0.152	0.501	10	4.0	0.8	1.8

Table 3.3.2.3. Organ weight data for Beagle dogs and rats given cyclohexylamine for nine weeks followed by a 13-week recovery period. Statistically significant differences for rats (ANOVAR) from concurrent control data, $p < 0.05^{+}$; $p < 0.01^{++}$

No histologically detectable lesions were found in any of the dogs' organs. One rat examined after 13 weeks' withdrawal of CHA showed diffuse bilateral testicular atrophy, the majority of seminiferous tubules being devoid of spermatogenic cells and containing syncytia of Sertoli cells. In this animal a few seminiferous tubules appeared morphologically normal (Fig. 3.3.2.4.).

Quantitative assessment of testicular spermatogenesis revealed some statistically significant decreases ($p < 0.05$) in the number of germ cells present in the seminiferous tubules of rats and dogs receiving CHA (Table 3.3.2.4.). After nine weeks' treatment, decreased counts of pachytene spermatocytes, early and late spermatids were apparent in the dog testes. The rat which developed testicular atrophy was excluded from the rat examinations. Significant reductions in the numbers of late spermatids were apparent for all other CHA-treated rats, both during the dosing and the withdrawal periods. After 13 weeks' withdrawal, rats previously given CHA were also found to have reduced numbers of pachytene spermatocytes and early spermatids. No statistically significant effects ($p > 0.05$) were obtained for counts of type B spermatogonia with either species.

3.3.3. Discussion

The findings of these investigations are in agreement with previously published accounts of the effects on testicular function of over-dosage with CHA. The dosages used in rats (200 mg/kg/day) and dogs (250 mg/kg/day) quantitatively reduced testicular spermatogenesis in both species. The previously published 'no effect' level with regard to the rat testes is 30 mg/kg/day (Gaunt et al., 1974); the present dosages represent a six- to seven-fold increase and were chosen to attempt elucidation of mechanism of action rather than to confirm 'no effect' dosages.
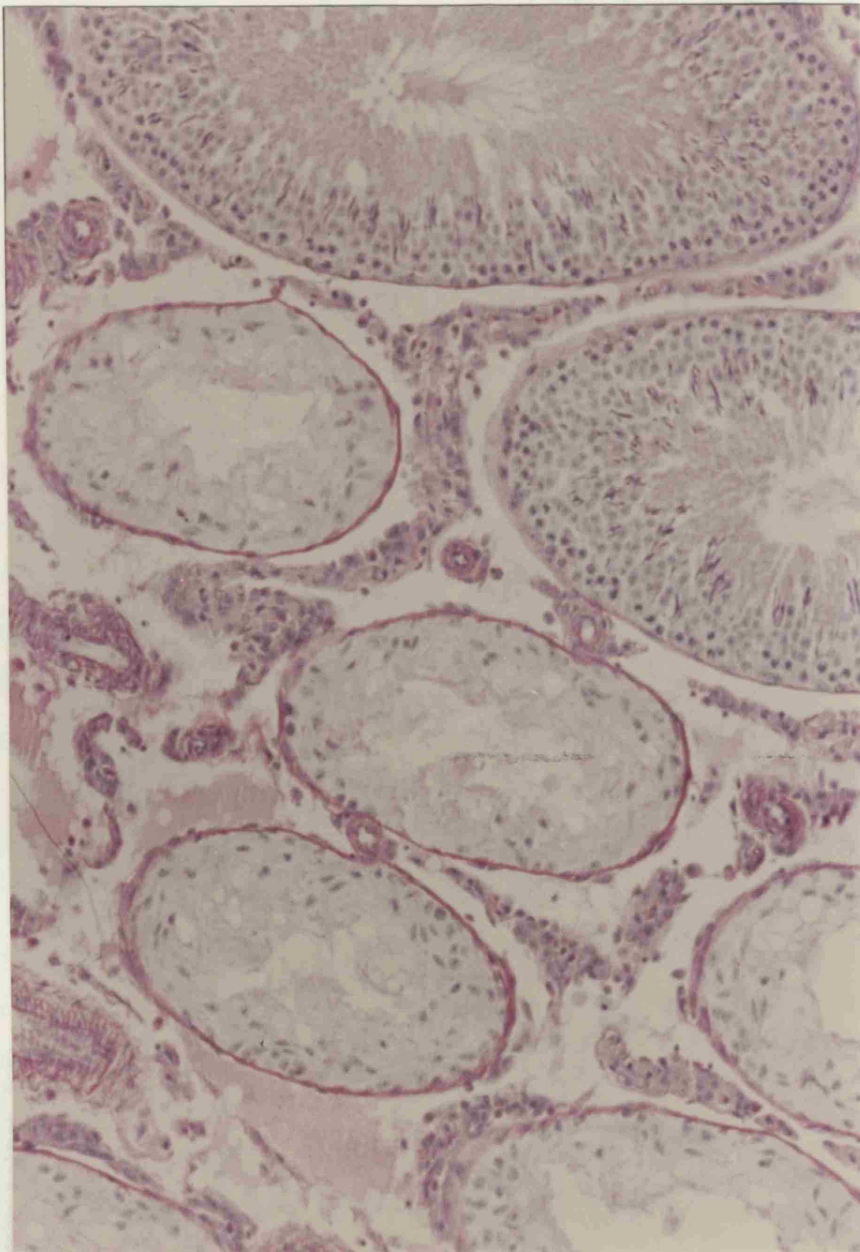
Fig. 3.3.2.4. Disruption of spermatogenesis in a rat examined 13
weeks after receiving cyclohexylamine (200 mg/kg/day) for nine
weeks. Note tubules showing severe atrophy of seminiferous
epithelium although some tubules show evidence of active sperm-
atogenesis. PAS, x 200.

Mean values for:

Species	Treatment	Tubule diameter (μm)	Germ cell counts									
			Type B spermatogonia (stage 7)		Pachytene spermatocytes (stage 5)		Spermatids				Sertoli cells	
							Early (stage 1)		Late (stage 8)			
			Crude	Corrected	Crude	Corrected	Crude	Corrected	Crude	Corrected	Crude	Corrected
Dog	CHA (250 mg/kg/day)											
	9 weeks' dosing	181 ± 11[++]	16	6.7	29[++]	13.5	68[+++]	47.3	61[+++]	42.5	24	11.1
	13 weeks' recovery	219 ± 7	19	9.1	39	15.0	104	59.8	92	52.9	29[+]	11.1
Rat	Controls	275 ± 18	37	16.6	68	21.1	209	80.6	177	77.9	20	12.0
	CHA (200 mg/kg/day)											
	4 weeks' dosing	250 ± 11	36	17.8	69	23.6	189[+]	80.2	133[+++]	64.6	20	13.2
	9 weeks' dosing	275 ± 24	38	17.1	75[+]	23.3	203	78.3	158[++]	69.5	21	12.6
	13 weeks' recovery[a]	271 ± 9	34	15.6	52[+++]	16.4	143[+++]	55.9	93[++]	41.5	20	12.1

Table 3.3.2.4. Quantitative assessment of spermatogenesis for rats and Beagle dogs given cyclohexylamine for nine weeks followed by a 13-week recovery period. Statistically significant (ANOVAR) differences from control versus treated rats and withdrawal versus dosed dogs $p < 0.05$[+], $p < 0.01$[++], $p < 0.001$[+++] ; a, excludes one rat with bilateral testicular atrophy.

Although effects on testicular function were apparent they cannot

be compared with the potent effects which may be produced with

hormonal steroids, antispermatogenic or cytotoxic agents (Gomes,

1970; Jackson, 1972; Patanelli, 1975). The observed effects of

CHA on the semen quality of dogs, the serum FSH and testosterone

concentrations of rats and the development of complete testicular

atrophy, albeit in one rat 13 weeks after cessation of CHA

administration, are circumstantial rather than conclusive evidence

of a testicular effect. However, the findings of the quantitative

assessments of testicular spermatogenesis indicate a subliminal

loss of differentiating spermatogenic cells. There is thus suppor-

tive evidence that large doses of CHA may partly suppress spermato-

genesis in both rats and dogs. This is also in agreement with the

finding (Gaunt et al., 1974) that rats remained fertile after CHA

treatment and no evidence of embryo or foetotoxic effects was

obtained. The single instance of testicular atrophy in this study

is difficult to explain; however, no effects were seen in the dogs

or remaining rats to suggest an effect on spermatogonial cells.

Furthermore, spontaneous testicular atrophy does occur in the

Sprague-Dawley rat. Either this was a spontaneous lesion or it

represents increased sensitivity to the effects of CHA in a single

rat.

Although obvious effects on body weight and food intake were

apparent, the testicular effects are probably not explicable simply

on this basis. The studies of Gaunt et al. (1974) and Mason and

Thompson (1977) included observations on pair-fed and paired-weight

rats. Both groups of investigators were of the opinion that

inanition per se did not explain the testicular effects of CHA, although interaction between inanition and chemical action could not be excluded. CHA administration to rats was shown to increase their basal metabolic rate, possibly as a result of sympathomimetic effects.

The hormonal responses of rats given CHA were characteristic reactions to depletion of the germinal epithelium (Collins et al., 1978; Debeljuk et al., 1973), and are therefore secondary responses and not the primary cause of the testicular effects. The possibility of direct action of CHA on the seminiferous epithelium cannot be excluded. Further investigations should be designed to assess the pharmacokinetics of CHA within the seminiferous epithelium compartment of the blood-testis barrier (Setchell and Main, 1978). The ability of CHA to induce metabolising enzymes within the testes (Lee and Dixon, 1978) is also worthy of investigation.

The animal toxicity data available show no evidence of testicular effects at dosages corresponding to a dietary CHA intake of 30 mg/kg/day, which is equivalent to 50 mg/kg/day of cyclamate. Human consumption of cyclamate is generally intermittent; CHA conversion does not always occur in man (Blumberg and Heaton, 1970; Glogner, 1970) and the proportion of cyclamate converted to CHA decreases with increasing cyclamate consumption (Litchfield and Swan, 1971). The results of these investigations using high oral dosages should not shed doubts on the safety of cyclamates in normal dietary use.

3.3.4. Abstract

Cyclohexylamine (CHA), the principal metabolite of cyclamate, was given by oral gavage to male rats (200 mg/kg/day) and male Beagles (250 mg/kg/day) for nine weeks. Some of these animals subsequently were maintained undosed for 13 weeks to assess recovery. CHA adversely affected body weight and food consumption in both species. Although a degree of tolerance developed, vomiting tended to occur after CHA administration to dogs. Serum FSH was increased and testosterone decreased in rats given CHA. No effects were found on the serum LH and testosterone levels in dogs, but reversible effects on sperm concentration and morphology were induced in this species. There were no statistically significant (p > 0.05) effects on the weight of the pituitaries, testes or secondary sex organs of either species. The only lesion detectable by conventional histological examination was focal atrophy of seminiferous tubules in one rat examined 13 weeks after cessation of CHA treatment. Quantitative assessment of testicular spermatogenesis showed that CHA administration reduced the counts of pachytene spermatocytes, and of early and late spermatids, in both species. These effects were apparently reversible in dogs but not in rats. The significance of these findings in relation to possible mechanisms of action and the assessment of safety in use of cyclamates is discussed.

3.4. Administration of hexachlorophene to Beagle dogs and rats for nine weeks

Hexachlorophene $\left[2,2'\text{-Methylene-bis} \ (3,4,6\text{-trichlorophenol}) \right]$, was patented in 1941. Since then, this chemical has found a variety of uses as an antibacterial or fungicidal agent in soaps, cosmetics and toiletries. Veterinary and agrochemical uses include the treatment of fascioliasis, and crop-spraying. The first major review of hexachlorophene toxicity was published by Gump in 1969. Kimbrough (1971) collated the available data relating to the symptoms of hexachlorophene toxicity in man. Accidental oral overdosage precipitates marked gastrointestinal upset and central nervous system derangement. The severity of the encephalopathy is dose-dependent and may be reversible. Hexachlorophene is readily absorbed by the dermal route; symptoms of dermal intoxication include skin rashes and clinical signs of central and peripheral neuropathy. The extent and potential reversibility of hexachlorophene encephalopathy has been studied in rats (Kimbrough and Gaines, 1971), and new-born primates (Lockhart and Simons, 1972). Weiss et al. (1978) demonstrated a long-lasting behavioural defect in rats allowed to recover from hexachlorophene intoxication. Hepatic damage has occurred in sheep dosed with hexachlorophene (Pugh and Crowley, 1966). Enzyme histochemical studies indicated that hepatic lesions were a direct toxic effect of hexachlorophene in the portal blood supply (Thorpe, 1967). Atrophy of the seminiferous epithelium has been described in rams and rats given single or short-duration repeated oral doses of hexachlorophene (Thorpe, 1967; 1969). The effect of chronic administration of hexachlorophene on the seminiferous epithelium at dosages below those inducing neurotoxic effects has not been described. A series of experiments were conducted with rats and dogs to examine this aspect of hexachlorophene toxicity.

3.4.1. Experimental design

Eight sexually mature Beagle dogs and 30 specific pathogen-free
Sprague-Dawley rats (CD strain) aged 35 days were obtained. The
animal management, housing and husbandry were as described in
Appendices I (i) and (ii). Body weight changes, food and water
consumption were monitored throughout the investigations, see
Appendix I (iii). Hexachlorophene powder of analytical quality
was obtained from the Sigma London, Chemical Company Ltd., Poole,
Dorset, UK. Administration of hexachlorophene to rats at 5 mg/kg/
day has previously been shown to be without neurotoxic effects
(Kimbrough, 1971). Hexachlorophene suspensions (0.1% v/v) were
prepared in corn oil and administered to 15 rats by oral gavage at a
dosage volume of 0.5 ml/100 g body weight for up to nine weeks so
that each rat received 5 mg/kg/day. Fifteen rats acted as controls
and were given corn oil at the same dosage volume. A suitable dose
for dogs was not apparent from the available literature; preliminary
studies were therefore necessary. Hexachlorophene powder was given
orally in gelatine capsules to dogs. A pair of dogs was given 15
mg/kg/day; a second pair received 3 mg/kg/day for 28 days, then the
dose was increased to 6 mg/kg/day for a further 14 days. On the
basis of these studies, four dogs were given 3 mg/kg/day for nine
weeks in order to assess effects on the seminiferous epithelium.

Testicular function was assessed in rats and dogs (3 mg/kg/day) which
received hexachlorophene for nine weeks, and in five rats per group
and two dogs which were retained undosed for 13 weeks after the last
dose of hexachlorophene. The dogs' testes were measured with
vernier calipers and semen was collected see Appendices I (vi) and

(vii), before dosing started, after two, four and eight weeks'
treatment and during weeks four, eight and 12 of the withdrawal
period. The semen characteristics and seminal plasma Na^+, K^+, acid
phosphatase, aspartate aminotransferase (E.C.2.6.1.1.) and alanine
aminotransferase (E.C.2.6.1.2.) concentrations were monitored, see
Appendix I (vii). Serum was obtained, see Appendix I (v), from dogs
after one, two, four and eight weeks' treatment and during weeks
four, eight and 12 of the withdrawal phase. Rat sera were obtained
from five rats per group after one days' dosing, four and nine weeks'
treatment and also after 13 weeks' withdrawal of hexachlorophene.
The concentration of LH and testosterone was determined in dog and
rat sera; FSH was also measured in rat sera, see Appendix I (vi).

Terminal investigation of dogs used in the preliminary studies was
restricted to macroscopic post-mortem examinations. Five rats per
group were killed after four and nine weeks' treatment and 13 weeks'
withdrawal. Two dogs given 3 mg/kg/day were examined after nine
weeks' dosing and the remaining two after 13 weeks' withdrawal.
In addition to macroscopic post-mortem examinations, the pituitaries,
testes and secondary sex organs of these animals were weighed and
preserved. Histological sections of the preserved organs were
prepared, see Appendix I (vii). Counts of type B spermatogonia,
pachytene spermatocytes, early (round) spermatids, late (elongated)
spermatids and Sertoli cells, together with measurements of
seminiferous tubule diameter were noted, see Appendix I (ix).

3.4.2. Results

Administration of hexachlorophene to dogs at 15 mg/kg/day produced
clinical encephalopathy and mucoid diarrhoea on the third day of
treatment. The dogs became prostrate and exhibited nystagmus,

opisthotonus and extensor rigidity. No macroscopic abnormalities were recognisable at post-mortem examination. No evidence of clinical malreaction was seen in dogs given 3 mg/kg/day for 28 days, but increasing the dose to 6 mg/kg/day induced posterior paresis and incoordination after a further 10 days. Again, no gross abnormalities were recognisable at post-mortem examination. Three rats died during the 13-week withdrawal period; of these, two rats previously had received hexachlorophene and one was a control. In all cases the death of rats was attributable to chronic respiratory disease.

No adverse effects on weight, food consumption or water intake occurred for dogs given 3 mg/kg/day, or among rats. There were no statistically significant differences (p > 0.05, ANOVAR) between the serum hormone concentrations of control and hexachlorophene-treated rats. The serum LH and testosterone concentrations of the dogs remained within the expected ranges for young adult Beagle dogs. The testicular measurements of the dogs remained essentially unaltered throughout the experimental period. Administration of hexachlorophene did not have any appreciable effects on the physical and biochemical parameters measured in the canine semen samples. No macroscopic post-mortem abnormalities, organ weight changes or lesions detectable by conventional light microscopy were found in the testes, pituitaries or secondary sex organs of either species.

Some statistically significant differences (p < 0.05) were found as a result of counting germinal cells in the seminiferous tubules of rats and dogs given hexachlorophene (Table 3.4.2.1.). Reduced numbers of type B spermatogonia were recorded for dogs after nine weeks' treatment although the counts of later cell types were as expected for normal dogs. Reduction of all cell types counted was apparent in the testes of rats examined after four weeks' treatment.

Species	Treatment	Mean values for										
		Tubule diameter (µm)	Germ cell counts				Spermatids				Sertoli cells	
			Type B spermatogonia (stage 7)		Pachytene spermatocytes (stage 5)		Early (stage 1)		Late (stage 8)			
			Crude	Corrected	Crude	Corrected	Crude	Corrected	Crude	Corrected	Crude	Corrected
Dog	Hexachlorophene (3 mg/kg/day)											
	9 weeks' dosing	$192 \pm 9^{+++}$	14^{+++}	7.7	44	19.2	111	72.8	93	61.0	13	5.7
	13 weeks' recovery	230 ± 9	24	11.0	44	17.2	116	63.5	92	50.4	17	9.3
Rat	Controls	275 ± 18	37	16.6	68	21.1	209	80.6	177	77.9	20	12.0
	Hexachlorophene (5 mg/kg/day)											
	4 weeks' dosing	257 ± 13	31^{+}	14.9	63^{++}	20.9	165^{+++}	68.1	152^{++}	71.6	20	12.8
	9 weeks' dosing	282 ± 16	42^{+}	18.4	68	20.6	196	73.7	179	76.8	20	11.7
	13 weeks' recovery	285 ± 7	36	15.6	69	20.7	189	70.3	184	78.1	20	11.5

Table 3.4.2.1. Quantitative assessment of spermatogenesis for rats and Beagle dogs given hexachlorophene for nine weeks followed by a recovery period of 13 weeks. Statistically significant differences (ANOVAR) treated versus withdrawal dogs and control versus treated rats $p < 0.05^{+}$; $p < 0.01^{++}$, $p < 0.001^{+++}$

A slightly increased count of type B spermatogonia was recorded in
rats after nine weeks' treatment. After 13 weeks' withdrawal of
hexachlorophene no effects were apparent on the cell counts
obtained from dog or rat testes.

3.4.3. Discussion

The object of these experiments was to determine whether hexa-
chlorophene can exert selective testicular toxicity at dosages
below those previously associated with neurotoxicity or hepato-
toxicity. The absence of clinical evidence of malreaction to
hexachlorophene dosing and the lack of adverse effects on body
weight or food and water intake suggests that the selected dosages
were appropriate for this purpose. Thorpe (1967, 1969) inferred
from his studies of the testicular toxicity of large doses of
hexachlorophene in sheep and rats that the lesions resulted from
cytotoxic effects on the seminiferous epithelium and, unless wide-
spread degeneration of spermatogonia occurred, then the lesions
should be reversible. In order to act directly on the seminiferous
epithelium, chemicals must penetrate the blood-testis barrier
(Waites, 1976). It is notable that several neurotoxic chemicals
which must be permeable to the blood-brain barrier can also affect
the seminiferous epithelium (Heywood et al., 1980b). The analogy
is not straightforward, because the principal barrier to the entry
of large molecules into brain tissue is at the level of the endo-
thelial junctions in the capillary wall, whereas similar junctions
have not been found in the testes (Fawcett et al., 1970). The
anatomical basis for the blood-testis barrier is found at the level
of the peritubular myoid cells and the tight junctions between
Sertoli cells (Dym and Fawcett, 1970). The blood-testis barrier is

resistant to temperature damage (Main and Waites, 1977) and remains

intact following hypophysectomy (Hagenäs et al., 1978). The

processes by which chemicals may penetrate the blood-testis barrier

have been discussed by Setchell and Main (1978). The blood-testis

barrier is most efficient in restricting chemical action on the

meiotic stages of spermatogenesis. Spermatogonia are enclosed by

the peritubular myoid cell system but not by the tight junctions

between Sertoli cells (Dym and Fawcett, 1970). Although the

susceptibility of spermatogenic cells to direct chemical action

will depend on dosage, tissue distribution and duration of treatment,

in purely anatomical terms the spermatogonia are most vulnerable.

The reduced number of type B spermatogonia observed in these

experiments may be explicable on this basis. As the maximum detec-

table change was observed after four and not nine weeks' treatment

of rats, the effects do not appear to be progressive and the

development of tolerance is a definite possibility. Calculation of

the ratio between successive spermatogenic cell generations for the

rats examined after four weeks indicates that the number of later

cell types was as would be expected from the reduced spermatogonial

count (Clermont, 1962). This is further evidence that the effects

of hexachlorophene administered at these dosages are probably

confined to spermatogonial cells. The discrimination between sperm-

atogonial cell populations of the dog is equivocal (Foote et al.,

1972) and inadvertent inclusion of Type A or intermediate spermato-

gonia may occur when quantifying canine type B spermatogonia. The

data obtained suggest that sufficient canine type B spermatogonia

were unaffected to allow spermatogenesis to proceed satisfactorily.

In conclusion, it appears that chronic exposure to subneurotoxic doses of hexachlorophene did not induce permanent or serious impairment of spermatogenesis. No clinical changes were detectable in pituitary-testicular relationships and the semen production of dogs was unaffected. The slight quantitative changes detected in the seminiferous epithelium were not progressive in nature and delayed effects were not apparent. While disruption of spermatogenesis may be one of the hazards of hexachlorophene overdosage, there is no evidence to suggest that this occurs at the concentrations normally used.

3.4.4. Abstract

Hexachlorophene was administered orally, at subneurotoxic dosages, to rats (5 mg/kg/day) and Beagle dogs (3 mg/kg/day) for nine weeks. A proportion of rats and dogs was observed for a further 13 weeks following cessation of dosing. The serum concentrations of pituitary gonadotrophins and testosterone were unaffected. No changes were induced in the testicular dimensions or semen characteristics of dogs. No macroscopic post-mortem abnormalities, organ weight differences or lesions detectable by conventional light microscopy were found in the testes, pituitaries or secondary sex organs. A transient reduction in the number of germ cells countable in cross-sections of seminiferous tubules was seen in rats after four weeks' treatment only. After nine weeks' treatment, reduced spermatogonial counts were recorded in canine seminiferous tubules; in other respects, spermatogenesis was proceeding normally. No delayed effects were apparent in either species. The possible mechanism of action and the role of the blood-testis barrier is discussed. It is concluded that repeated administration of hexachlorophene at subneurotoxic levels did not induce significant impairment of spermatogenesis in rats or dogs.

The primary objective in undertaking the work presented in this thesis was to provide a practical basis for the study of possible chemical testicular hazards using laboratory animal models. The current legislation for extensive toxicity testing of a large number of chemicals intended for human exposure must be considered, not only on the basis of scientific relevance, but also in terms of economic and logistic capability. The selection of appropriate animal models should be based on the similarity of their pharmaco-kinetic and metabolic characteristics to those of man, and on the ability to study breeding performance and the development of progeny following exposure to chemicals. In practice, safety evaluation studies are normally undertaken with laboratory rats, Beagle dogs or subhuman primates. Fertility and general reproduc-tive performance can be investigated in rats with comparative simplicity, but satisfactory data are unlikely to be obtained using subhuman primates or dogs, except in the most specialised labora-tories. The ready availability, simple restraint and handling, and lack of obvious seasonal breeding rhythms of the male Beagle make this species a possible alternative to the subhuman primates for the study of chemically induced changes in testicular size, semen characteristics and circulating pituitary-testicular hormone concentrations. The value of Beagle dogs for this type of experimental study would be enhanced by the development of a radioimmunoassay for canine FSH. It is possible to use clinical testicular function data as a guide to explaining the changes in testicular weight and morphology which are revealed by routine post-mortem studies.

Hormonal studies are useful for studies using rats, not only for clarification of mechanisms of testicular toxicity but also to reconcile apparent differences in species sensitivity with the

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testicular effects of the same chemical entity. Whichever species is studied, the sampling times for·hormonal examinations are crucial: unless the interaction between the pharmacokinetics of the test substances and the temporal effects on hormone concentrations are considered, misleading data may be obtained. Iatrogenic lesions may be caused by testicular biopsy and this technique is not recommended for routine toxicological studies with experimental animals. Quantitative histometric analyses of testicular spermatogenesis are useful to define the responses of the seminiferous epithelium but, in most instances, this technique merely reinforces the data provided by other less laborious techniques. Unless there is a specific need to study prepubertal or adolescent animals it is more appropriate to use sexually mature animals, in order to ensure that testicular function is adequate before administering the test substances. Awareness of the incidence and nature of spontaneous variations in testicular-pituitary physiology and pathology is necessary for a variety of reasons. Lifespan studies with rats are complicated by the high incidence of spontaneous testicular atrophy found in aged rats. The wide range of testicular weights found for Beagle dogs restricts the value of statistical comparisons when small numbers of dogs are studied. Although dogs are rarely used for lifespan studies, testicular tumours occur commonly among ageing Beagles.

The testicular effects of overdosage with several compounds able to modify centrally active neurotransmitter substances or prostaglandins have been examined. Although the rat and dog appeared to respond in a qualitatively similar manner to the chemical modulation of pituitary-testicular hormone relationships, differences between the two species were apparent in the observable effects on testicular and secondary sex-organ morphology and function. Depletion of LH and disruption of the physiological synergism between prolactin and LH induced reversible effects of spermatid maturation or

prostate secretion of the Beagle dog. In the absence of breeding

performance data, the effects on the testes of rats, induced by

overdosage with similar compounds, were more equivocal. Evidence

from the literature indicated that, although overdosage of rats

with indomethacin, an inhibitor of prostaglandin synthesis,

decreased LH and testosterone concentrations, the ability to

fertilise ova was unaffected. Repeated overdosage of Beagle dogs

with a prostaglandin inhibitor induced comparable hormonal effects,

which were correlated with suppression of spermatid maturation, but

prostate function remained adequate. In addition to hormonally

dependent retardation of spermiogenesis, changes in the counts of

earlier spermatogenic cells were considered to be a consequence of

inhibition of prostaglandin synthesis at the level of the semini-

ferous epithelium. No precise reasons have been determined for

these apparent quantitative differences in species response. The

physiology of canine spermatogenesis has not been studied exten-

sively and there may be, as yet, undiscovered differences between

the hormonal control of spermatogenesis in the rat and the dog.

The laboratory rat has an extraordinary ability to adapt to

sustained chemical overdosage and the observed differences may

simply reflect greater tolerance by rats, rather than intrinsic

differences in the physiology of the two species. In all cases,

the testicular effects of modified pituitary hormone secretion or

release were shown to be reversible. The time allotted for the

demonstration of reversibility was critical, because it seemed

unreasonable to expect restoration of normal spermatogenesis before

a complete spermatogenic cycle had elapsed after withdrawal of the

test compounds. However, as the deficits were considered to

represent disruption of physiological interactions, complete

reversibility within two spermatogenic cycles was an essential

criterion.

The nature of the pharmacological properties of the compounds studied for this aspect of testicular toxicity raises the question of whether the induced changes were truly toxic effects or simply manifestations of pharmacological overdosage. The assessment of safety in normal use depends on comparisons of the hormonal changes induced by small and large multiples of the recommended exposures, demonstration of 'no effect' levels and evidence of reversible effects following overdosage.

The assessment of toxicity arising from chemical actions on the seminiferous epithelium is less well defined. The antispermato-genic effects of potential male contraceptives can be studied in the rat and Beagle dog. The concepts of 'no effect' levels and safety margins change their toxicological definitions when this type of chemical activity is being considered. Safety assessment, in these cases, has to be assessed on the dual criteria of absence of toxic effects in extragonadal tissues, and careful character-isation of the pharmacological effects following prolonged over-dosage. The choice of dosage levels is paradoxical because selective action on the testes may not be possible with large multiples of the minimally effective dosages. Testicular function tests, such as those advocated in this work, could be usefully included in toxicological studies of potential male contraceptives with rats and Beagle dogs. The possibility of genetically damaged spermatozoa being able to fertilise ova, either during the induction of infertility or the restoration of fertility, is an important toxicological question. Currently, animal models are needed which could be used to predict such a hazard.

The most inconclusive aspect of this work relates to the studies of
those testicular hazards which may arise from the largely un-
controlled exposure of populations to industrial, agricultural or
domestic chemicals which affect the seminiferous epithelium of
experimental animals when given in excessively large amounts. It
becomes difficult to differentiate selective testicular effects
from secondary responses to systemic toxicity, disturbed metabolic
functions or dietary imbalance. Important features of the
investigations with hexachlorophene and cyclohexylamine were the
differences in systemic tolerance to these agents, shown by rats
and Beagle dogs. The experimental methods used here appear to
have limited application when studying this type of chemical
toxicity. The data obtained allows conjecture that the seminiferous
epithelium was accessible to these chemicals at the concentrations
used, but that the responses were incompletely characterised. When
slow progressive actions on the seminiferous epithelium are suspec-
ted, study of the roles of the physiological compartmentation of
the seminiferous epithelium, induction of metabolising enzymes and
covalent binding may be more rewarding. Estimates of the
distribution and clearance of chemicals within the seminiferous
tubules could be used to identify those threshold concentrations of
the test substances which are necessary for the onset of toxic
changes.

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ACKNOWLEDGMENTS

APPENDIX   I

Materials and Methods

(i) Animal Sources

Beagle dogs obtained from commercial suppliers (Beaulong Ltd.,
Harewood Park, Harewood End, Herefordshire; Balbeggie Kennels,
Thornton Road, Kirkcaldy, Fife), or the colony maintained at
Huntingdon Research Centre and Sprague-Dawley rats of the CD
(Charles River Ltd., Manston, Kent, U.K. or Wilmington, Mass.,
U.S.A.) and CFY (Anglia Laboratory Animals, Alconbury, Huntingdon,
Cambridgeshire) strains were used for the experiments. All
animals were allowed at least 10 days acclimatisation to our
laboratory environment before experimental treatment commenced.

(ii) Animal Management

Beagle dogs were housed individually in galvanised metal kennels.
Apart from vaccination against distemper, hepatitis and lepto-
spirosis and regular anthelmintic therapy ('Coöpane', Wellcome
Veterinary Products), no medication was permitted. Each dog was
offered 400 g Spratt's Dog Diet No. 2 (Spratt's Laboratory Services,
Barking, Essex) daily and tap water was freely available.

Rats were housed in barrier-maintained rooms and caged in fives in
suspended metal cages with wire mesh flooring. Room temperature
and relative humidity were maintained at $21 \pm 2^{o}C$ and $50 \pm 5\%$
respectively. Lighting was controlled to give alternate 12 h
periods (8 a.m. to 8 p.m. BST) of light and dark. Spratt's
Laboratory Rodent Diet No. 1 (Spratt's Laboratory Services, Barking,
Essex) and tap water were freely available.

(iii) Body Weight Change, Food and Water Intake

The body weight of all animals was determined at least once before
the start of experimental treatment. Throughout the experiments
body weight recordings were made at 7-day intervals. Food
consumption was measured daily for individual dogs but water intake
was measured on weekdays only. The cage mean water and food intake
of rats was measured once weekly.

(iv) Testicular measurements

Testicular measurements were recorded for the canine studies only.
The length (ℓ) and breadth (b) of each testis was measured in the
scrotum using vernier calipers. The area (mm^2) given by the
formula $\pi \ell b/4$ was used as an index of testicular size.

(v) Withdrawal of Blood

Blood samples were obtained from dogs by hypodermic puncture of
the jugular vein. Rats were bled by puncture of the orbital sinus
under deep ether anaesthesia. The blood was collected into lithium
heparin bottles (for plasma) or plain plastic vials (for serum) and
allowed to stand at room temperature for at least 20 minutes.
Following centrifugation at 5000 g for 10 minutes, the supernatant
plasma or serum was stored, deep-frozen (-20°C), pending further
examinations.

(vi) Hormonal Examination

Prolactin, luteinising hormone (LH), follicle-stimulating hormone
(FSH) and testosterone concentrations were measured in serum or
plasma by radioimmunoassay.

Canine samples were assayed for prolactin concentration using the
homologous double antibody method of Gräf, Friederich, Matthes and
Hasan (J. Endocr., (1977) 75, 93-103). Purified canine prolactin
CPA3/F4, supplied by Drs. El Etreby and Hasan, Endocrine Laborator-
ies, Schering AG, Berlin, was radio-iodinated using the lacto-
peroxidase procedure (Knight, Hamilton and Scanes, Acta Endocr.,
(1977) 85, 736-743). CPA3/F4 was also used as a standard, being
assayed in triplicate over the range 0.625 - 160 ng/ml. Test
samples were assayed in duplicate at a level of 200 μl. The
prolactin radioimmunoassays on samples from rats during median life-
span administration of Compound B were performed by Dr. C. Robyn,
Hospital Universitaire St. Pierre, Brussels.

Canine LH was assayed using the double antibody radioimmunoassay
devised by Niswender, Riechert, Midgley and Nalbondov (Endocrinology,
(1969) 84, 1166-1173) using Niswender's ovine LH antibody No. 15,
radio-iodinated ovine LH (LER-1056-C2) and a canine pituitary
reference standard (LER-1685-1; 0.025 NIH-LH-SI units/mg). The use
of anti-ovine LH antisera has been validated for canine LH assays
by Smith and McDonald (Endocrinology, (1974) 94, 404-412). The
buffer used for the dilution of unknown and standard samples was
0.1% gelatin in 0.01M phosphate-buffered saline, pH 7.6. Unknown
samples were assayed in duplicate at a volume of 100 μl; standards
were assayed in triplicate over the range 0.025 - 12.8 ng/tube
and contained 100 μl of pooled rhesus monkey serum per tube. The

second antibody (goat anti-rabbit gamma globulin) was used at 1:80 dilution in 0.01M phosphate-buffered saline, pH 7.6. The lowest limit of detectability of the assay was 0.5 ng/ml LH.

Rat LH and FSH were assayed by the method of Daane and Parlow, (Endocrinology (1971) 88, 653-663), using reagents and techniques obtained from the National Institute of Arthritis, Metabolism and Digestive Diseases, pituitary hormone distribution programme by courtesy of **Dr. A.F. Parlow,** Department of Obstetrics and Gynecology, Harbor General Hospital, University of California, 1000 West Carson Street, Torrance, California 90509. Testosterone concentrations were determined in rat and dog material essentially following the procedure of Frankel, Mock, Wright and Kamel (Steroids, (1975) 25, 73-98), but modified as follows. Testosterone was extracted using diethyl ether and petroleum ether (1:4 mixture); testosterone-11β-hemisuccinate was radio-iodinated by the 'chloramine T' procedure; separation of free and bound fractions was achieved using 50% saturated ammonium sulphate. The lowest level of detectability of the assay was 0.2 ng/ml testosterone.

(vii)   Semen examination

Semen was collected from dogs using the artificial vagina method described by Harrop (Br. Vet. J. (1954) 110, 194-196). The quality of the semen obtained was evaluated as described by Heywood and Sortwell (J. small Anim. Pract., (1971) 12, 343-346). The following characteristics were assessed:

Volume (ml)   - ejaculates were collected in graduated tubes;

Density (0-5)   - visual grading on a 0 - 5 scale;

Motility (0-5)   - examination of one drop of fresh semen at 100

                     x magnification;

                   0 = no motile spermatozoa

                   1 = approximately 20% of spermatozoa motile

                   2 = approximately 40% of spermatozoa motile

                   3 = approximately 60% of spermatozoa motile

                   4 = approximately 80% of spermatozoa motile

                   5 = virtually 100% of spermatozoa motile.

Sperm concentration   (10^6/ml or 10^6/ejaculate). 0.1 ml of

ejaculate was diluted in 2 ml 0.5% formol saline containing a trace

of eosin. 0.1 ml of the resulting diluent was placed in the well

of a modified Neubauer haemocytometer and the spermatozoa were

counted at x 250 magnification. Sperm concentration was expressed

both as millions per ml of ejaculate and as millions per total

ejaculate.

Sperm morphology   A drop of fresh semen was placed on a

microscope slide and mixed with a drop of 10% nigrosin followed by

mixing and spreading. The resultant smear was air-dried and

examined by oil immersion microscopy. Live spermatozoa are

unstained and dead spermatozoa stain pink. The percentage of live

or dead normal and live or dead abnormal spermatozoa was classified

by examination of five fields per smear. Air-dried nigrosin smears

do not constitute a permanent record, and when abnormalities were

apparent, air-dried unstained smears were fixed in 70% methanol and

stained with Giemsa stain (Watson, Vet. Rec., (1975) 97, 12-15), or

aniline blue and eosin (L. Cooke, personal communication) before

mounting in Canada balsam.

Limited biochemical examinations were performed on some semen samples. Ejaculates were kept at room temperature for approximately 30 minutes and were then centrifuged at 2000 g for 10 minutes. Supernatant seminal plasma was decanted off and the remaining sperm fractions were resuspended in Fisons' phosphate buffer (pH 7.0, Na^+ 53 mEq/l and K^+ 20.1 mEq/l). The samples were stored at $- 20^{\circ}C$ pending biochemical examination. Enzyme and electrolyte concentrations in either fraction were examined as follows. Na^+ and K^+ ion concentration was determined using an IL/343 flame photometer. Alanine aminotransferase (AlT 2.6.1.2.) and aspartate aminotransferase (AsT 2.6.1.1.) activities were estimated using a Union Carbide Centrifichem and Roche Diagnostics Test Kits (mU/ml). Acid and alkaline phosphatase concentrations were determined also using a Union Carbide Centrifichem and Smith Kline Instruments Test Kits (mU/ml).

(viii) Histological Preparation

Representative pieces of selected tissues were preserved for histopathological examination as follows: testes in formol saline solution for 24 h followed by post-fixation in buffered neutral formalin solution; pituitaries in mercuric chloride for 12 h followed by post-fixation in formol saline solution; prostate in buffered neutral formalin solution. The preparation and properties of these fixatives is described in 'Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology', Ed. L.G. Luna, 3rd Edition, McGraw-Hill Book Company, New York, (1960) pp 2-3. Samples of tissue obtained by percutaneous testicular biopsy were preserved in Helly's solution (Rowley and Heller, Fert. and Steril., (1966) 17, 177-186).

Following preservation and adequate fixation, the tissues were passed through graded ethanol solutions before being embedded in melted paraffin wax (56°C). Microtome sections cut at 5 μm were stained as follows. Testes were stained with the periodic acid schiff technique (PAS) for demonstration of glycogen in spermatids (McManus, Stain Techn., (1948) 23, 99-108). Differential staining of the anterior pituitary cells was achieved using the PAS-Alcian blue-Orange G procedure described by El Etreby and Tüshaus (Histochemie, (1973) 33, 121-127), Prostate sections were stained routinely with Harris' haematoxylin and eosin (Histopathology Laboratories, Armed Forces Institute of Pathology, Washington D.C., 20305).

(ix) Quantitative Testicular Histomorphometry

Quantitative histomorphometric analysis of spermatogenesis was performed using the histological sections prepared (Appendix I (vi)) from dog and rat testes. The cyclical activity of the seminiferous epithelium (Roosen-Runge, Ann. N.Y. Acad. Sci., (1952) 55, 574-584 and Biol. Rev., (1962) 37, 343-377) facilitates recognition of stages of spermatogenesis based on the position and morphology of spermatids associated with specific earlier cell types. The cycle of the seminiferous epithelium has been documented for a number of species including the dog (Foote, Swierstra and Hunt, Anat. Rec., (1972) 173, 341-351) and rat (Clermont and Perey, Rev. Canad. Bio., (1957) 16, 451-462). Although Clermont and Perey recognised 19 morphological phases of spermatid development for the rat, it is sufficient to define eight characteristic cellular associations in the rat and dog testis. The results presented in this thesis are based on adoption of the eight stage classification to facilitate species comparisons.

In the following description of the cells present in successive stages of spermatogenesis, the roman numerals in parenthesis indicate the corresponding stages in Clermont and Perey's more elaborate classification.

Stage 1 (VIII-IX)

Type A spermatogonia, leptotene young primary spermatocytes, pachytene old primary spermatocytes and round spermatids.

Stage 2 (X-XI)

Type A spermatogonia, leptotene young primary spermatocytes, pachytene old primary spermatocytes and elongating spermatids,

Stage 3 (XII-XIII)

Type A spermatogonia, zygotene young primary spermatocytes, diplotene old primary spermatocytes and elongating spermatids.

Stage 4 (XIV)

Type A spermatogonia, pachytene old primary spermatocytes, secondary spermatocytes and elongated spermatids attaching in groups to Sertoli cells.

Stage 5 (I-III)

Type A spermatogonia, pachytene old primary spermatocytes, round spermatids and elongated spermatids attached to Sertoli cells.

Stage 6 (IV-V)

Type A and B spermatogonia, pachytene old primary spermatocytes, round spermatids and elongated spermatids attached to Sertoli cells.

Stage 7 (VI)

Type A and B spermatogonia, pachytene old primary spermatocytes, round spermatids, elongated spermatids detaching from Sertoli cells and migrating towards tubule lumen.

Stage 8 (VII)

Type A spermatogonia, preleptotene young primary spermatocytes, pachytene old primary spermatocytes, round spermatids and tailed spermatids (spermatozoa) detached in lumen of tubule.

Tubules at stage 7, 5, 1 and 8 were selected for counting type B spermatogonia, old pachytene primary spermatocytes, early (round) spermatids and late (elongated) spermatids respectively. Only tubules in circular cross-section were examined; a minimum of three tubules at each stage was counted in each section (i.e. 24 tubules per animal). The diameter of each tubule (μm) was measured twice at right angles using an optical graticule (Graticules Ltd., Tonbridge, Kent, UK) and the mean value calculated. Sertoli cell counts were also recorded. The crude cell counts were corrected for differing nuclear (Abercrombie, Anat. Rec., (1946) $\underline{94}$, 239-247) and tubular (Swierstra and Foote, J. Reprod. Fert., (1963) $\underline{5}$, 309-322) diameters using the following formula:

$$T = C \times \frac{M}{N+M} \times \frac{A}{D} \text{ , where}$$

T = true count,   C = crude count,

M = thickness of section (μm),   N = average nuclear diameter (μm) of 10 nuclei of given cell type,   D = observed diameter (μm) of tubule,   A = average diameter (μm) of seminiferous tubules for that species.

Summary tables of the quantitative testicular histomorphometric
data obtained from the control rats and Beagle dogs used for these
studies follow overleaf.

1(x)   Statistical procedures

Measures of the distribution of the data were expressed as
arithmetic means, standard deviations and absolute or 95% ranges.
The experimental design and small number of animals used for some
of the investigations restricted the value of statistical
comparisons; however the results of statistical calculations were
included for completeness and to confirm biological trends. Tests
of statistical probability included analysis of variance (ANOVAR),
paired 't'-tests, linear regression analysis, Fisher's Exact test
and χ^2 test for trends in linear proportions (Snedecor, G.W.
and Cochran, G.W., Statistical Methods, Iowa State Press, 1968).
The use of ANOVAR was justified by testing for homogeneity of
variance (Bartlett's test) and calculating the variance (F) ratio.
When significant heterogeneity of variance occurred (p < 0.01),
data transformation did not stabilise variances and there were too
few observations to justify the use of non-parametric tests.
When experimental treatment affected body weight, organ weights
were analysed using analysis of covariance (Angervall, L. and
Carlström, E., J. Theoret. Biol., (1963) 4, 254-259).

Summary of quantitative testicular histomorphometric data recorded among control Beagle dogs

Parameter	Crude counts			Corrected counts		
	Mean	SD	95% range	Mean	SD	95% range
Tubule diameter (μm)	224	± 14	217 - 231	-	-	-
Type B spermatogonia (stage 7)	19	± 7	17 - 21	9.5	± 3.7	8.5 - 10.5
Pachytene spermatocytes (stage 5)	43	± 9	41 - 46	16.7	± 3.2	15.8 - 17.6
Early spermatids (stage 1)	115	± 27	107 - 122	65.9	± 14.2	62.0 - 69.7
Late spermatids (stage 8)	93	± 22	87 - 99	51.0	± 11.6	47.7 - 54.2
Sertoli cells	22	± 4	21 - 23	8.3	± 1.7	7.8 - 8.7

Data obtained by counting 13,634 nuclear points in 216 seminiferous tubules in 18 testicular sections from 12 young adult male Beagles.

Summary of quantitative testicular histomorphometric data recorded among control rats

Parameter	Crude counts			Corrected counts		
	Mean	SD	95% range	Mean	SD	95% range
Tubule diameter (μm)	285	± 21	278 – 292	–	–	–
Type B spermatogonia (stage 7)	38	± 9	37 – 40	16.2	± 3.8	15.5 – 16.9
Pachytene spermatocytes (stage 5)	69	± 12	67 – 71	22.5	± 5.9	21.4 – 23.6
Early spermatids (stage 1)	192	± 36	186 – 199	73.5	± 13.6	71.0 – 76.1
Late spermatids (stage 8)	164	± 29	159 – 170	69.4	± 15.0	66.7 – 72.1
Sertoli cells	24	± 6	23 – 25	13.3	± 4.7	12.5 – 14.1

Data obtained by counting 57,865 nuclear points in 492 seminiferous tubules in 41 testicular sections obtained from 41 adult male (age 17-29 weeks) Sprague-Dawley rats.

APPENDIX   II

Publications

1. Assessment of testicular toxicity in laboratory animals,
    R. Heywood and R.W. James, Environ. Health Persp., (1978) 24,
    73-80.

2. The oral toxicity of 1-amino-3-chloro-2-propanol hydrochloride
    (CL 88,236) in rats,
    R.W. James, R. Heywood, J. Colley and B. Hunter, Toxicology,
    (1978) 11, 235-243.

3. Serial percutaneous testicular biopsy in the Beagle dog,
    R.W. James, R. Heywood and D.J. Fowler, J. Small Anim. Pract.,
    (1979) 20, 219-228.

4. Biochemical observations on Beagle dog semen,
    R.W. James, R. Heywood and A.E. Street, Vet. Rec., (1979) 104,
    480-482.

5. Age-related variations in the testes and prostate of Beagle
    dogs,
    R.W. James and R. Heywood, Toxicology, (1979) 12, 273-279.

6. Age-related variations in the testes of Sprague-Dawley rats,
    R.W. James and R. Heywood, Toxicol. Lett., (1979) 4, 257-261.

7. Canine pituitary-testicular function in relation to toxicity
    testing,
    R.W. James, D. Crook and R. Heywood, Toxicology, (1979) 13,
    237-247.