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## *Studies on the effect of oestrogen treatment on the lipid metabolism of the male chick (gallus domesticus)*

Steven Talbot

# STUDIES ON THE EFFECT OF OESTROGEN TREATMENT ON THE LIPID METABOLISM OF THE MALE CHICK (GALLUS DOMESTICUS)

by

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Graduate Society
University of Durham

## DECLARATION

I hereby declare that the work in this thesis is entirely my own
and that no part has previously been submitted for a degree in
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## ACKNOWLEDGEMENTS

## ABSTRACT

The aim of this study was to investigate the early ( $\leqslant 48$ h) changes in lipid metabolism occuring in the liver after 17$\beta$-oestradiol treatment (0.75 mg/100 g body wt.) of the male chick _in vivo_.

An increase in liver weight was observed from 13 - 61 h after oestrogen treatment while an oestrogen-induced increase in the total hepatic DNA content was observed from 37 - 61 h suggesting that the early ( <37 h) liver growth response involved cell hypertrophy where-as cell hyperplasia was involved in the later ( $\geqslant 37$ h) phase of the response. Oestrogen-induced increases in total liver triacylglycerol and phospholipid were observed from 7 h post-injection while an oestrogen-induced increase in total liver free fatty acid was not observed until after 26 hours.

Similarly, total triacylglycerol and phospholipid concentrations in the plasma increased progressively from 7 - 44 h after hormone treatment. Plasma levels of VLDL-triacylglycerol and VLDL-phospho-lipid in similarly-treated birds increased from 13 h and 6 h post-injection, respectively, involving an oestrogen-induced increase in the number of VLDL particles specifically enriched with phospholipid. An oestrogen-induced increase in total free fatty acids in the plasma was not observed at any time post-injection.

Analysis of the fatty acid compositions of liver triacylglycerol, VLDL-triacylglycerol and VLDL-phopsholipid, accumulating in oestrogen-treated chicks, revealed that the major oestrogen-induced change was an increase in the level of oleic acid, from 13 hours post-injection.

An oestrogen-induced increase in the hepatic activity of the key lipogenic enzyme acetyl-CoA carboxylase (E.C.6.4.1.2) was observed from 24 - 48 h post-injection, involving increased specific activity

both with respect to protein and DNA, and total organ activity.

The results from the study were correlated in an attempt to determine the early sequence ($\leqslant 48$ h) of oestrogen-induced changes in lipid metabolism.

# GLOSSARY

The abbreviations suggested by the Biochemical Journal (Policy of the Journal and Instructions to Authors; (1985) Biochem.J. 225, 1-26) have been adopted.  Other abbreviations are as follows:-

| | |
|---|---|
| ACC | acetyl-CoA carboxylase |
| c.AMP | adenosine 3',5'-phosphate |
| BSA | bovine serum albumin |
| FFA | free fatty acids |
| FLHS | fatty liver - haemorrhagic syndrome |
| FLKS | fatty liver and kidney syndrome |
| G.P.R. | general purpose reagent |
| HDL | high density lipoprotein(s) |
| LDL | low density lipoprotein(s) |
| PFS | particle-free supernatant |
| PHLA | post-heparin lipolytic activity |
| PL | phospholipid(s) |
| TCA | trichloroacetic acid |
| TG | triacylglycerol(s) |
| VLDL | very low density lipoproteins |
| VLDL-PL | phospholipid associated with very low density lipoprotein(s) |
| VLDL-TG | triacylglycerol associated with very low density lipoprotein(s) |

In addition, the following abbreviations have been adopted for fatty acids

| | |
|---|---|
| 12:0 | Lauric acid (Dodecanoic) |
| 14:0 | Myristic acid (Tetradecanoic) |
| 16:0 | Palmitic acid (Hexadecanoic) |
| 16:1 | Palmitoleic acid (cis-9-Hexadecenoic) |
| 18:0 | Stearic acid (Octadecanoic) |
| 18:1 | Oleic acid (cis-9-Octadecenoic) |
| 18:2 | Linoleic acid (cis, cis-9,12,-Octadecadienoic) |

α18:3    Linolenic acid (all cis-9,12,15-Octadecatrienoic)

γ18:3    Linolenic acid (all cis-6,9,12-Octadecatrienoic)

20:0    Arachidic acid (Eicosanoic)

20:1    cis-11-Eicosenoic acid

20:2    11,14-Eicosadienoic acid

20:3    all cis-8,11,14-Eicosatrienoic acid

20:4    Arachidonic acid (all cis-5,8,11,14-Eicosatetraenoic)

22:0    Behenic acid (Docosanoic)

22:4    7,10,13,16-Docosatetraenoic acid

22:6    4,7,10,13,16,19-Docosahexaenoic acid

24:0    Lignoceric acid (Tetracosanoic).

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# MATERIALS

All reagents were of analytical grade unless stated otherwise.
Materials were obtained from the following sources:-

Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K.
Diisopropyl ether


Alltech/Applied Science, Carnforth, Lancs., U.K.
10% Alltech CS-5, on a chromasorb WAW support (100 - 120 mesh)
G.L.C. fatty acid methyl ester standards (12:0 - 24:0)


Amersham International plc, Amersham, Bucks., U.K.
Sodium $[^{14}C]$ bicarbonate, aqueous solution


Analtech Inc., Newark, DE, U.S.A.
MN-300 Cellulose Uniplates, 250 micron layer


BDH Chemicals, Poole, Dorset, U.K.
Acetaldehyde (G.P.R.)
Ammonium molybdate
Bromocresol green
Diethyl ether
Dimethyldichlorosilane (laboratory reagent)
Diphenylcarbazide
Diphenylcarbazone (laboratory reagent)
Heptane
Hydrochloric acid
Kieselgel 60H (Merck)
Magnesium sulphate
Perchloric acid
Potassium bromide
Potassium chloride

(vi)

Potassium hydroxide
Sodium chloride
Sodium dihydrogen orthophosphate
Sodium hydroxide
Sulphuric acid
Zinc dibenzyldithiocarbamate (laboratory reagent)


Boehringer Corp. (London) Ltd., Lewes, East Sussex, U.K.
Citrate synthase
Dithiothreitol


B.O.C. Ltd., Vigo Lane, Birtley, Co. Durham, U.K.
Nitrogen
Air
Hydrogen


Fisons Scientific Apparatus, Loughborough, Leics., U.K.
Acetic acid, glacial
Acetic anhydride
Chloroform
Cupric sulphate
Diethyl ether, dried, distilled
2,5-Diphenyloxazole
Formic acid
Iodine
Light petroleum (b.p. 40° - 60°C)
Methanol
Potassium iodide
Potassium sodium tartrate
Trichloroacetic acid
Triton X-100
Xylene

Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.
1,4-Di(2-(5-phenyloxazolyl))benzene


JJ (Chromatography) Ltd., King's Lynn, Norfolk, U.K.
Glass g.l.c. columns, 2 m long, 2 mm internal diameter, 6 mm external diameter


Sigma Chemical Co., Fancy Road, Poole, Dorset, U.K.

Adenosine 5'-triphosphate (disodium salt, Grade I)

Alumina (activated)

Aspartic acid (monopotassium salt)

Boron trifluoride in methanol, 14% (w/v)

Bovine serum albumin (fatty acid poor)

Butylated hydroxytoluene

Citric acid (tri potassium salt)

Coenzyme A (sodium salt)

Deoxyribonucleic acid (sodium salt from calf thymus, Type I)

Dextran sulphate (sodium salt)

Diagnostic kit and Reagents for Colormetric Determination of Triacylglycerols (Technical Bulletin No. 405)

Diphenylamine

5,5'-Dithiobis(2-nitrobenzoic acid)

Ethylenediaminetetraacetic acid (disodium salt)

n-Hexane

Lipid standards (mono-, di- and triolein)

Magnesium chloride (4.9 M solution)

Malic acid (disolium salt)

Malonic acid (sodium salt)

17$\beta$-Oestradiol

Oleic acid

Oxaloacetic acid

Palmitic acid

L-$\alpha$-Phosphatidylcholine, dipalmitoyl

Potassium dihydrogen orthophosphate
Potassium hydrogen carbonate
Propane-1,2-diol
Silicic acid (100 - 300 mesh)
Sodium hydrogen carbonate
Triethanolamine
Tris (hydroxymethyl)aminomethane

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# CHAPTER 1

## GENERAL INTRODUCTION

## 1. Egg formation

The production of eggs by female oviparous vertebrates involves
dramatic changes in the composition of the blood and in the
metabolism of the liver and oviduct. The onset of egg-laying in the
domestic fowl and other oviparous vertebrates is associated with
alterations, both quantitative and qualitative, in blood lipids and
proteins (Laskowski, 1936; Vanstone et al., 1955; Dessauer & Fox,
1959; Schjeide et al., 1963; Follett & Redshaw, 1968). It is
generally agreed that proteins and lipids destined for the egg yolk
are synthesised in the liver, secreted into the blood and
transported to the ovary for incorporation into the developing
oocyte, whereas egg white components such as ovalbumin, conalbumin,
ovomucoid and lysozyme are synthesised by the oviduct (Oka &
Schimke, 1969; Palmiter & Wrenn, 1971; Griffin et al., 1984).

### (a) Changes associated with the liver

### (i) Proteins

The changes in protein metabolism that take place during the
process of vitellogenesis have been studied in the domestic fowl
and the African clawed toad, Xenopus laevis. At sexual maturity,
the liver of females synthesises large amounts of a glycolipo-
phosphoprotein which is secreted into the blood (Wallace & Jared,
1968; Ansari et al., 1971; Redshaw & Follett, 1971; Clemens, 1974;
Tata, 1978). This protein known as vitellogenin, is the plasma
precursor of the egg yolk proteins phosvitin and $\alpha$- and B-lipo-
vitellin (Mok et al., 1961; Bergink & Wallace, 1974; Gruber et al.,
1976; Tata, 1978). Like many proteins destined for secretion,
vitellogenin is synthesised on membrane-bound polysomes, passes
into the cisternae of the endoplasmic reticulum, and is transported
to the Golgi apparatus before being secreted from the cell. During

the passage to the Golgi apparatus, the newly-synthesised protein molecule is extensively chemically modified, the major post-translational modification being extensive phosphorylation, glycosylation and lipidation (Clemens, 1974; Follett & Redshaw, 1974; Tata, 1976, 1979; Shore & Tata, 1977; Wang & Williams, 1982). Chicken vitellogenin has a molecular weight of about 450,000 and is composed of 2 subunits, each having a molecular weight of 210,000 - 230,000, and it is suggested that each subunit consists of 2 phosvitin residues and 1 lipovitellin residue (Deeley et al., 1975; Gordon et al., 1977).

In the laying hen, vitellogenin present in the blood is taken up into developing oocytes by endocytosis (Griffin et al., 1984) and then split into its phosvitin and lipovitellin components within the oocyte (Redshaw & Follett, 1972). Phosvitins are glycosylated phosphoproteins, and 56% of their amino acids are serine (Clemens, 1974). Most of the serine residues are phosphorylated, which means that about 10% by weight of the phosvitins is phosphorus (Allerton & Perlmann, 1965; Christmann et al., 1977). The lipovitellins are also phosphoproteins but contain about 20% by weight of lipid (Tata, 1979). In addition, the lipovitellins are rich in methionine residues and about 4% of the amino acid residues are serine. The $\alpha$- and $\beta$-lipovitellins differ from each other in their phosphate content and in their ability to be dissociated into 2 subunits at alkaline pH (Wallace, 1965). It is not altogether clear whether the presence of multiple forms of phosvitins and lipovitellins is the result of polypeptide heterogeneity or of different degrees of lipidation, glycoslyation and phosphorylation. The lipovitellins contribute about 30% and phosvitin about 7% - 8% to the yolk protein (Gruber, 1972).

In addition to the changes in vitellogenin production, some of the

proteins that are deposited in the developing egg yolk are normally occurring plasma proteins which are made in the liver. During vitellogenesis the concentrations of these proteins in the plasma are altered. For example, in the domestic fowl, the concentrations of LDL and VLDL (Schjeide et al., 1963; Christie & Moore, 1972) and of various vitamin-binding proteins (Mandella et al., 1978) are increased, whilst the level of albumin appears to decrease (Schjeide et al., 1963), indicating that the stimulation of protein synthesis is specific rather than general in nature.

(ii) Lipids

In the rat and many other mammals, the adipose tissue and the liver are the major sites of lipogenesis, and the adipose tissue has been shown to be quantitatively more important than the liver in de novo fatty acid biosynthesis (Romsos & Leveille, 1974; Vernon, 1980; Pearce, 1983). In contrast, the liver has been shown to be the principal site of lipogenesis in avian species, including the domestic fowl (Leveille et al., 1968; O'Hea & Leveille, 1969; Brady et al., 1976). Although the adipose tissue of the chicken is capable of fatty acid synthesis (O'Hea & Leveille, 1968), the chicken liver has a far greater capacity for de novo fatty acid synthesis (Leveille et al., 1968; Goodridge, 1968d; Aprahamian et al., 1979). On the assumption that the liver and adipose tissue are the only lipogenic sites, chicken liver has been shown to contribute 90% - 95% to total de novo fatty acid synthesis (O'Hea & Leveille, 1969).

All plasma lipids, other than free fatty acids, are transported as part of macromolecular complexes called lipoproteins. The classification and nomenclature of these lipoproteins is based primarily on their electrophoretic mobility or on their rate of ultracentrifugal flotation in salt solutions (Jackson et al., 1976;

Chapman, 1980). Based on these criteria, the major vertebrate plasma lipoprotein fractions are (i) chylomicra, in which the lipid is of dietary origin, and 3 fractions in which most of the lipid is produced endogenously, namely, (ii) very low density lipoproteins (VLDL), (iii) low density lipoproteins (LDL) and (iv) high density lipoproteins (HDL). Plasma lipoproteins are similar in that their basic structure consists of a hydrophobic core, comprising mainly triacylglycerol and cholesteryl ester, surrounded by an amphipathic surface 'coat' of phospholipid, cholesterol and specific apoproteins. A major difference in the lipid metabolism of birds and mammals exists, however, in the absorption of exogenous lipid. In avian species, dietary lipid is absorbed via the portal system as large VLDL or 'portomicra' (Bensadoun & Rothfeld, 1972) rather than as chylomicra via the lymphatic system as typically occurs in mammals.

At the approach of lay in the female domestic fowl, the liver synthesises and secretes large amounts of lipoproteins, mainly VLDL, for transport to the yolk of the developing egg (Schjeide et al., 1963; Christie & Moore, 1972). Consequently, egg-laying domestic fowl exhibit hyperlipaemia, primarily due to increased plasma triacylglycerol levels associated predominantly with VLDL (Lorenz, 1954; Christie & Moore, 1972; Bergink et al., 1974). The liver has been shown to be the major site of VLDL synthesis (Haft et al., 1962; Jones et al., 1967) indicating, therefore, that most of the triacylglycerol is of hepatic origin.

As well as lipid being secreted into the blood in the form of lipoproteins for transport to the developing egg, much of the newly-synthesised hepatic lipid, at least initially, is used for the proliferation of the endoplasmic reticulum and Golgi apparatus in order to support the biosynthesis and export of large amounts of vitellogenin and other egg yolk proteins (Schjeide et al., 1963, 1974; Schjeide, 1967). Large amounts of hepatic lipid are also

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incorporated into the newly-synthesised vitellogenin molecule during the process of post-translational modification.

In contrast to the situation in laying domestic fowl, Xenopus laevis does not develop highly lipaemic plasma during the process of vitellogenesis (Follett & Redshaw, 1968; Wallace & Jared, 1968; Bergink et al., 1974). Xenopus laevis also differs from the domestic fowl in that the large amount of calcium observed in the blood of laying birds (Follett & Redshaw, 1968; Munday et al., 1968; Wallace, 1970; Clemens,1974) is not observed in the amphibian (Wallace & Jared, 1968), presumably since there is no egg shell production in this species.

That increased hepatic lipogenesis is a major and specific feature of vitellogenesis in the domestic fowl, and not simply part of a general increase in metabolic rate, has been indicated by the work of Pearce (1971) and Pearce & Balnave (1976). These studies showed that with the onset of laying, the specific activities of certain lipogenic enzymes were increased while the specific activities of a variety of gluconeogenic, glycolytic and amino acid metabolising enzymes were unchanged. Interestingly, in the same studies, a similar pattern of altered enzyme activities were observed in sexually immature pullets treated with exogenous oestrogen.

The physiological changes that occur at the approach of lay in female oviparous vertebrates are considered to arise as a result of the action of steroid hormones, notably oestrogen (Follett & Redshaw, 1968; Clemens, 1974), although a number of hormonal activities are almost certainly involved in the fine control. The importance of oestrogen in the induction of these changes in sexually mature domestic fowl has been emphasised by the fact that oestrogen treatment of male and immature females leads to hyper-lipaemia, a situation which mimics that observed in laying hens

(Lorenz, 1954; Schjeide et al., 1963; Heald & McLachlan, 1964; Clark, 1967; Bergink et al., 1974).

## (b) Changes associated with the oviduct

While the liver has been shown to synthesise and secrete protein and lipid for incorporation into the egg yolk, egg white components are synthesised and secreted by the oviduct (Oka & Schimke, 1969; Palmiter & Wrenn, 1971). The egg white contains over 40 individual proteins but the principal egg white proteins are, in order of decreasing percentage contribution, ovalbumin, conalbumin, ovomucoid and lysozyme (Aitken, 1971).

During the development of the female domestic fowl there is a substantial increase in the weight of the oviduct such that, at 16 weeks of age, the organ weighs, on average, 0.2 g whereas in the mature hen the organ weight is 40 - 60 g (Amin & Gilbert, 1970). It has been estimated that this weight increase represents a 20,000-fold increase in oviduct cell number after hatching (Palmiter, 1975). In the sexually active bird, the oviduct is a tortuous tube, approximately 80 cm in length, extending from the ovary to the cloaca. However, in sexually inactive birds, the oviduct is a fairly inconspicuous and narrow tube, approximately 14 - 19 cm in length (Aitken, 1971).

The oviduct of the egg-laying bird exhibits distinctive structural variations which allows its subdivision into 5 principal parts; infundibulum, magnum, isthmus, uterus (shell gland) and vagina. The magnum is responsible for the production and secretion of the egg-white proteins, and consists of a thick-walled tube with up to 90% of the cells being protein-secreting tubular gland cells (Aitken, 1971; Palmiter, 1975). Immediately prior to ovulation, the gland cells are distended with secretion droplets which are destined for incorporation into the developing egg (Aitken, 1971).

The increase in oviduct weight and the synthesis and secretion of egg-white proteins observed when the female domestic fowl reaches sexual maturity has been shown to be caused principally by oestrogen, since similar changes in oviduct structure and function have been mimicked in sexually immature birds following the administration of exogenous oestrogen. Substantial increases in oviduct weight have been observed in immature chicks after treatment with large doses of oestrogen (Hawkins & Heald, 1966; Aprahamian et al., 1979) and the oestrogen-induced increase in weight has been shown to involve both hypertrophy and hyperplasia (Pearce & Brown, 1971; Balnave & Pearce, 1974; Pearce & Balnave, 1975) and a massive proliferation of tubular gland cells (Kohler et al., 1969; Palmiter & Wrenn, 1971; Palmiter, 1972, 1975). Similarly, the administration of oestrogen to immature pullets results in a rapid induction of synthesis of the egg-white protein, ovalbumin by the tubular gland cells (Oka & Schimke, 1969; Palmiter et al., 1971; Palmiter, 1972).

While the studies using oestrogen-treated immature birds clearly implicate oestrogen as a major causative factor in the changes in oviduct structure and function in the female domestic fowl during sexual maturation, it is believed, however, that other sex steroids are also involved, to a lesser extent. This view is supported by evidence that the effect of exogenous oestrogen on the synthesis of egg-white proteins, by immature pullets, is modulated by exogenous progesterone and testosterone (Palmiter, 1973, 1975).

(c) Mechanism of action of oestrogen

In mature females of oviparous vertebrate species, the changes in hepatic lipid and protein production, and in oviduct physiology, occur constitutively in response to endogenous oestrogens secreted by the ovary. All steroid hormones are thought to act by combining

with specific receptor proteins in target cells to form a complex
that binds to chromatin in the nuclei, thereby switching on mRNA
synthesis from specific genes (King, 1984). A 'two-step' model of
steroid hormone intracellular action was put forward independently
by Gorski et al. (1968) and Jensen et al. (1968). This model is
based on studies of the uptake and binding of radioactive oestrogens
to receptors in reproductive tissues and according to the model,
oestradiol enters the target cell, binds to a specific cytoplasmic
receptor and then is translocated to the nucleus by a temperature-
dependent 'activation' process. This 'activation' of the steroid-
receptor complex was reflected both by a change in its sedimentation
coefficient from 4S to 5S and by its ability to bind to isolated
target cell nuclei. Therefore, in this 'two-step' model, cyto-
plasmic receptors can exist either steroid-filled or unfilled,
whereas nuclear receptors can exist only when steroid-filled.

A number of studies, however, have shown that unfilled receptors do
in fact exist in the nuclei of oestrogen target tissues
(Sonnenschein et al., 1976; Jungblut et al., 1978; Carlson & Gorki,
1980; Edwards et al., 1980; Clark et al., 1982) and thereby casting
some doubt upon the previously accepted 'two-step' model of
steroid action. In these studies careful attention was paid to
the purity of the nuclei, thereby ensuring no contamination of the
preparation with cytoplasmic receptors. Such observations led to
the hypothesis that in intact cells, unfilled receptors may exist
in a state of equilibrium between the cytoplasm and nucleus
(Sheridan et al., 1979). However, recent evidence suggests that
unfilled oestrogen receptors reside exclusively within the target
cell nucleus. Welshons et al. (1984) obtained cytoplast and
nucleoplast fractions from oestrogen receptor-rich cells, derived
from rat pituitary tumours, by a process of cytochalasin B-induced
enucleation. The cytoplasts were found to contain little oestrogen-
binding activity, with most of the unfilled receptor residing within

the nuclear fraction. The study of King & Greene (1984) also provides convincing evidence that oestrogen receptors may reside exclusively in target cell nuclei. These workers used immunocyto-chemical staining of a monoclonal antibody generated against the oestrogen receptor protein. Specific staining was found to be confined to the nucleus of a variety of oestrogen-sensitive tissues including human breast tumours and uterus, rabbit uterus, oviduct corpus luteum, mammary gland, pituitary and liver. There-fore, the distribution of oestrogen receptors as predicted by the studies of Welshons et al. (1984) and King & Greene (1984) has led to a 'one-step' model of oestrogen intracellular action (Clark, 1984). This latest model of steroid hormone action involves the entry of the hormone, in an unbound state, directly into the nucleus via the cytoplasm. Once inside the nucleus, the steroid hormone binds to specific receptor protein and forms a steroid-receptor complex which in turn can affect the transcription of specific genes.

Whilst the 'one-step' model of oestrogen intracellular action is the model most easily reconciled with the available evidence, a number of details still need to be sorted out, including the question of the location of filled and unfilled oestrogen receptors within the target cell nuclei (Clark, 1984).

2.   Oestrogen treatment of male and female oviparous vertebrates

Studies on male and immature female domestic fowl have shown that, after oestrogen administration, hepatic lipid and protein synthesis increase and certain lipids and proteins, including vitellogenin, VLDL and vitamin-binding proteins, accumulate in the blood in a manner which is qualitatively similar to the situation in females during the approach to lay (Lorenz, 1954; Bergink et al., 1973,

1974; Kudzma et al., 1973; Luskey et al., 1974; Chan et al., 1976, 1978). Furthermore, there does not appear to be a limit to the age at which the chicken liver will respond to oestrogen, since even the administration of oestrogen to chick embryos results in the production of plasma lipid and egg yolk proteins (Schjeide et al., 1960, 1980; Lazier, 1978; Elbrecht et al., 1981). Similar studies on male and immature female Xenopus laevis have produced similar observations (Follett & Redshaw, 1968; Follett et al., 1968; Munday et al., 1968; Smith et al., 1978b; Philipp & Shapiro, 1981). In consequence, male animals have been useful in the study of oestrogen-induced physiological changes which normally occur with the onset of egg-laying in the female, since no significant levels of endogenous oestrogen exist in such individuals and any changes observed can be assumed to be caused by exogenous oestrogen, after comparison with suitable controls. Immature females, however, are not as useful in this respect since the levels of endogenous oestrogen, and oestrogen receptors may increase during their development.

The induction of hepatic vitellogenin synthesis by exogenous oestrogen, in the domestic fowl and Xenopus laevis. is characterised by a lag period before vitellogenin is detectable. Steroid hormone action is normally preceded by a lag period, the length of which is dependent upon the hormone used, the target tissue and whether the target tissue has been previously exposed to the hormone. This effect has been observed in the livers of male oviparous vertebrates after oestrogen treatment, since secondary stimulation results in a shorter lag phase before vitellogenin is detectable, as compared with that obtained with primary stimulation (Bergink et al., 1973, 1974; Tata, 1976). In vivo studies in the cockerel have shown that, after a single oestrogen injection, vitellogenin first becomes evident in the blood from about 3 - 4 hours to about 20 hours later,

depending on the sensitivity of the detection assay (Greengard et al., 1964; Beuving & Gruber, 1971; Bergink et al., 1973) and increases in concentration to reach a peak after 3 - 4 days (Bergink et al., 1973, 1974). The dose level of oestrogen used has also been shown to affect both the magnitude and the time-course of the response (Wallace & Jared, 1968; Beuving & Gruber, 1971; Shapiro, 1982). A difference has been shown to exist between the time-courses of the responses of male Xenopus laevis and male domestic fowl. After a single injection of oestrogen, a peak in blood vitellogenin levels is observed more rapidly in cockerels than in the amphibians although, in both species, peak hepatic lipid production occurs earlier than the observed peak in blood vitellogenin content (Bergink et al., 1974; Tata, 1976).

Several workers have shown that oestrogen treatment of domestic fowl results in a dramatic increase in the plasma concentration of VLDL, with a lesser increase in LDL and a marked decrease in HDL (Annison, 1971; Luskey et al., 1974; Kudzma et al., 1979). All the lipoprotein fractions are enriched in triacylglycerol, whilst the proportion of cholesterol is decreased in all the fractions. Therefore, whilst the bulk of the plasma lipids constituting the lipaemia associated with oestrogen treatment of the domestic fowl are triacylglycerols (Kudzma et al., 1973, 1975, 1979; Luskey et al., 1974; Chan et al., 1976, 1977), the remaining hyperlipaemia is attributable to phospholipids and to a lesser increase in cholesterol. Chan et al. (1977) reported increases in plasma VLDL-protein, -phospholipid, -cholesterol and -triacylglycerol within 5 hours of administering 17B-oestradiol (1 mg) to young male chicks, reaching peak levels at 48 hours after injection before returning to control levels, 68 hours after treatment.

Xenopus laevis responds to oestrogen treatment in a similar way to

the domestic fowl, but this species differs from the domestic fowl in that the plasma remains transparent after oestrogen treatment, giving no indication of enhanced VLDL synthesis (Follett & Redshaw, 1968; Bergink et al., 1974). However, an increase in hepatic lipid synthesis, primarily of cholesterol and fatty acids, has been detected in the oestrogen-treated male and female Xenopus laevis, the bulk of the lipid being incorporated into intracellular membranes, although some of it is associated with the egg yolk proteins (Merry et al., 1973; Smith et al., 1978b; Philipp & Shapiro, 1981). The peak production of lipid in the oestrogen treated female Xenopus laevis occurs about 6 days after a single dose of 17$\beta$-oestradiol (1 mg.).

Vitellogenesis has also been induced by oestrogen in vitro, in cell and organ cultures of male Xenopus laevis liver (Wangh & Knowland, 1975; Wangh et al., 1979; Searle & Tata, 1981; Wangh & Schneider, 1982; Tenniswood et al., 1983) and embryonic and young chick liver (Carinci et al., 1976; Chan et al., 1977b). These studies have established that oestrogen is the sole inducer required for the synthesis of egg yolk proteins, that DNA synthesis does not appear to be essential for the initial stages of induction and that the action of the hormone upon the liver is direct.

As described above, liver cells of oviparous vertebrates respond to oestrogen with an increase in lipid production and the synthesis of specific proteins, including vitellogenin and apoprotein moieties of lipoproteins. In contrast, the oviduct of female oviparous vertebrates responds to oestrogen with an increase in growth and cell differentiation and the production of egg white proteins (Oka & Schimke, 1969; Palmiter & Wrenn, 1971) indicating that oestrogen stimulates the transcription of different genes in the two organs. Consequently, oestrogen-treated male domestic fowl and Xenopus laevis have proved to be valuable in the study of the early

cellular events in hormone action, particularly the role of
steroid receptors and the control of transcription and translation
(Tata, 1978). The induction of vitellogen synthesis in the
oestrogen-treated male animal is a very interesting phenomenon,
since it involves the activation of genes which are not normally
expressed significantly (Deeley et al., 1977; Blue & Williams,
1981). Following oestrogen treatment of male oviparous vertebrates,
a substantial increase in the hepatic synthesis of mRNA coding for
vitellogenin has been observed (Greengard et al., 1964; Beuving &
Gruber, 1971; Bos et al., 1972; Deeley et al., 1977).

The oestrogen-induced increase in hepatic VLDL production in male
domestic fowl has also been shown to be accompanied by increased
hepatic concentrations of mRNA coding for apo-VLDL-II, a specific
apoprotein moiety of oestrogen-induced plasma VLDL, indicating an
increased rate of transcription of the gene coding for apo-VLDL-II
(Chan et al., 1978, 1980; Codina-Salada et al., 1983). The
induction of apo-VLDL-II synthesis in the oestrogen-treated male
animal is similar to the induction of vitellogenin synthesis in
that the apo-VLDL-II gene is also not normally expressed to a
significant extent (Wiskocil et al., 1980).

A great deal of work has been done using birds and amphibians to
examine the transcriptional events and protein synthesis resulting
from oestrogen treatment. Similarly, a number of workers have
carried out investigations in order to increase our understanding
of the changes in lipid metabolism which underly the increased
lipid synthesis during oestrogen-induced vitellogenesis. Kudzma
et al. (1973) administered oestrogen to young chicks (sex
undetermined), in vivo, and observed an increased rate of de novo
fatty acid synthesis in liver slices, monitored by the incorporation of

$[1 - {}^{14}C]$ acetate into lipid. Similarly, Courtney (1984) measured increased de novo fatty acid synthesis in liver slices from oestrogen-treated immature male chicks as determined by the incorporation of $[1 - {}^{14}C]$ acetate and ${}^{3}H_2O$ into lipids. Furthermore, oestrogen treatment increased the rate of incorporation of preformed fatty acids into hepatic glycerolipids, in vivo (Kudzma et al., 1975), and in liver slices, in vitro (Courtney, 1984). Increases in the rate of de novo lipogenesis, monitored by the incorporation of ${}^{3}H_2O$ into hepatic glycerolipid in vivo, have also been observed in oestrogen-treated immature male turkeys (Dashti et al., 1983). A number of attempts have also been made to monitor any oestrogen-induced changes in hepatic lipogenic enzyme activities in male and immature female avian species. Oestrogen treatment of immature female domestic fowl has been shown to cause increased specific activity of hepatic ATP citrate lyase (E.C.4.1.3.8) (Balnave & Pearce, 1974; Pearce & Balnave, 1975). Similarly, an oestrogen-induced increase in the hepatic specific activity of 'malic' enzyme (malate dehydrogenase (decarboxylating; $NADP^+$), E.C.1.1.1.40) has been observed in immature female domestic fowl (Balnave & Pearce, 1974; Pearce & Balnave, 1975) and roosters (de Vellis & Schjeide, 1967). An increase in hepatic fatty acid synthetase activity has been reported in oestrogen-treated immature female (Aprahamian et al., 1979) and immature male (Courtney & Manning, 1984) domestic fowl. A rapid increase in the specific activity of hepatic acetyl-CoA carboxylase (E.C.6.4.1.2), within 3 hours after oestrogen treatment, has been reported in immature female Japanese quail (Pageaux et al., 1981).

A number of studies have also reported increases in the activities of enzymes involved in phospholipid and triacylglycerol biosynthesis in the liver of oestrogen-treated male and immature female domestic fowl. For instance, Vigo et al. (1981) reported increases in the

specific activities of hepatic choline kinase (E.C.2.7.1.32) and hepatic phosphatidylethanolamine-N-methyl-transferase (E.C.2.1.1.17) in oestrogen-treated male chicks. Coleman et al. (1977) detected increases in the total hepatic activities of fatty acid:CoA ligase (AMP-forming) (E.C.6.2.1.3), sn-glycerol 3-phosphate:acyl-CoA transferase (E.C.2.3.1.15) and diacylglycerol acyltransferase (E.C.2.3.1.20) in oestrogen-treated immature male and female birds.

The above reports of changes in hepatic lipogenic activity in oestrogen-treated birds are somewhat difficult to compare with each other since differences in the age and the sex of the birds used, and in the dose of oestrogen used exist between the studies. Therefore, at the moment, our understanding of the changes in lipid metabolism, which underly the oestrogen-induced increase in lipid production, is fragmentary and there is scope for a more coherent study in this field.

Although the administration of oestrogen to immature pullets and male domestic fowl cause qualitative changes in blood and hepatic lipids similar to those in laying hens, quantitative differences exist, suggesting that oestrogen administration does not entirely mimic the situation in the laying hen. Balnave (1971) has reported an over-reaction of immature pullets to oestrogen in that the resultant liver weights were greater than those in the laying hens. A substantial increase was also observed in the percentage of palmitic acid in the liver and blood lipids of the same oestrogen-treated birds, an increase not detected in laying hens. However, the mature hen was shown to have an increased capacity to retain dietary linoleic acid and this increase was not observed in the oestrogen-treated birds. Such differences could be explained by the fact that the physiological changes associated with sexual maturity in hens are not controlled solely by oestrogen, and other endocrine

secretions may exert an effect (Balnave, 1969; Balnave & Pearce, 1974). Furthermore, the egg-laying process itself may exert a significant effect since lipid is removed from the body when eggs are laid, an effect obviously not evident in oestrogen-treated immature birds.

3.   Medical implications of oestrogen-treated birds.

Severe hyperlipaemia is evident in a number of clinical conditions in man including uncontrolled diabetes (Bagdade et al., 1967), myxoedema (Fredrickson et al., 1963) and genetically determined hyperlipoproteinaemia (Porte et al., 1966). The incidence of cardiovascular disease has been shown to be linked with the levels of blood lipids (Barboriak et al., 1974). The domestic fowl has been a very useful experimental animal in the study of such medical conditions since oestrogen treatment leads to hyperlipaemia (Kudzma et al.,1973; Chan et al., 1976, 1978) and arteriosclerosis develops after prolonged oestrogen treatment or cholesterol feeding (Hillyard et al., 1956). In addition, the normal plasma levels of triacylglycerol, cholesterol and free fatty acid (Kudzma et al., 1973) and the lipid composition of the plasma lipoproteins (Kudzma et al., 1973; Chapman, 1980) of domestic fowl are similar to those of man.

Epidemiological studies have linked low plasma HDL and high VLDL and LDL levels with premature coronary arteriosclerosis (Miller & Miller, 1975; Blue et al., 1982). Women have been shown to have significantly higher levels of plasma HDL than men, at all ages after puberty, and lower levels of plasma VLDL and LDL than men in middle age (Heiss et al., 1980). It is interesting to note, therefore, that women have a reduced chance of suffering from arterio-sclerotic disease (Kissebah et al., 1973). These higher HDL levels

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have been attributed to endogenous oestrogen since treatment of men and women with exogenous oestrogen has resulted in elevated HDL levels (Barr, 1955; Schaefer et al., 1983). The slightly lower VLDL and LDL levels in women compared to men have been attributed to the higher progesterone levels in women (Schaefer et al., 1983).

Premenopausal women taking exogenous oestrogen, in the form of contraceptive treatment, have been shown to have elevated fasting plasma levels of lipids, mainly triacylglycerols, associated principally with an increase in VLDL levels and, to a lesser extent, increases in HDL and LDL levels (Sachs et al., 1969; Wynn et al., 1969; Schaefer et al., 1983). The hyperlipaemia in such women is qualitatively similar to the hyperlipaemia which develops in the oestrogen-treated fowl and, therefore, the oestrogen-treated fowl represents a model for use in studies of the hyperlipaemia evoked by oestrogen-containing oral contraceptives. Evidence has been presented which suggests that the altered plasma lipoprotein profiles of oestrogen-treated women increase their susceptibility to cardiovascular disease, such as myocardial infarction (Oliver, 1970) and cerebral vascular accidents (Vessey & Doll, 1969). Although the oestrogen present in oral contraceptives is believed to be responsible for the hyperlipaemia, the progestagen component, whichis a feature of most preparations, appears to be inert or antagonistic in this respect (Glueck et al., 1969; Kim & Kalkhoff, 1975; Chan et al., 1977).

The hyperlipaemia observed in both humans and domestic fowl treated with exogenous oestrogen could be caused by an increased rate of production of triacylglycerol-rich lipoproteins, especially VLDL, or a reduced removal of triacylglycerol from the plasma or a combination of the two. Studies on the oestrogen-treated fowl have shown that the increase in plasma VLDL levels is accompanied by

increased hepatic VLDL synthesis (Kudzma et al., 1975, 1979; Chan et al., 1976, 1978, 1980). Similarly, clinical investigations have indicated that the rise in plasma VLDL levels in women using oestrogen-containing contraceptives is accompanied by increases in the hepatic synthesis of apoprotein moieties of lipoproteins and triacylglycerol (Wynn et al., 1969; Kekki & Nikkilä, 1971; Kissebah et al., 1973; Schaefer et al., 1983; Gevers Leuven et al., 1984).

The clearance of triacylglycerol from the plasma occurs mainly through the action of lipoprotein lipase situated at the luminal surface of the endothelial cells of capillaries (Scow et al., 1976; Kompiang et al., 1976; Quinn et al., 1982). The intravenous injection of heparin causes the release of some or all of the enzyme in the blood. Several investigators have observed reduced post-heparin lipolytic activity (PHLA) in women receiving exogenous oestrogen in the form of contraceptives (Fabian et al., 1967; Ham & Rose, 1969; Hazzard et al., 1969; Rössner et al., 1971) suggesting that the hyperlipaemia may, in part, be due to reduced plasma triacylglycerol clearance. It has been suggested, however, that oestrogen treatment might lead to lipoprotein lipase becoming more resistant to release by heparin (Hazzard et al., 1972) and, therefore, any observed depression of PHLA need not necessarily reflect a reduction in triacylglycerol clearance capacity.

In comparison to the effect of oestrogen on PHLA in women, progesterone has been demonstrated to cause a decrease in plasma triacylglycerol levels with increased triacylglycerol clearance and increased PHLA (Glueck et al., 1969; Kissebah et al., 1973).

A number of studies have examined the ability of hyperlipaemic oestrogen-treated birds and laying birds to clear triacylglycerol from the plasma. Kelley et al. (1976) reported that the PHLA in

the plasma of laying turkeys was decreased in relation to non-laying birds, and oestrogen treatment of male turkeys caused a similar decrease. However, Dashti et al, (1983) suggested that the oestrogen treatment of male turkeys resulted in hyperlipaemia principally through an increased hepatic production of triacylglycerol-containing lipoproteins and that the observed decreased fractional turnover of triacylglycerol in the circulation was a secondary factor, resulting from the saturation of the removal mechanisms. Kudzma et al. (1975) arrived at similar conclusions from observations made on oestrogen-treated domestic fowl.

In this discussion of the relative roles of lipid synthesis and lipid plasma clearance in the development of hyperlipaemia it is of interest to note the findings of Griffin (1981). This worker performed studies on isolated lipoproteins and found that, although VLDL from immature hens were readily hydrolysed by lipoprotein lipase, those from laying hens were hydrolysed only to a limited extent. These results suggest that the hyperlipaemia of laying hens may be, in part, associated with impaired catabolism of triacylglycerol-rich VLDL by lipoprotein lipase. Furthermore, Griffin (1981, 1984) suggested that the limited susceptibility of laying hen VLDL to hydrolysis was almost certainly due to their low content of an unspecified lipoprotein lipase 'activator' apoprotein, the VLDL from male and immature female birds possessing a higher content of this 'activator' apoprotein.

4.   The purpose of the present study

Oestrogen-induced hyperlipaemia is well-documented in domestic fowl and it is generally accepted that increased hepatic synthesis and release of triacylglycerol-rich lipoproteins is a major causative factor (Luskey et al., 1974; Kudzma et al., 1975, 1979; Chan et al., 1976, 1978, 1980). However, the mechanism(s) responsible for the

oestrogen-dependent increase in triacylglycerol synthesis is
  unclear. Several studies have indicated that oestrogen
treatment of birds causes an increase in the activity of a number
of hepatic lipogenic enzymes (de Vellis & Schjeide, 1967; Balnave
& Pearce, 1974; Pearce & Balnave, 1975; Coleman et al., 1977;
Aprahamian et al., 1979; Vigo et al., 1981; Pageaux et al., 1981;
Courtney & Manning, 1984), together with an increase in plasma
triacylglycerol levels. However, many of these studies employed
relatively high levels of oestrogen, sometimes administered in
multiple doses, so the results may reflect a pharmacological
response, rather than a physiological response, to the treatment.
Furthermore, most investigators have examined the effects at
relatively long periods ( >24h) after oestrogen administration, and
so little is known about the events during the initial period of
the response.

Consequently, the aim of the present study was to study the early
changes in various parameters of lipid metabolism, in both the
liver and the blood of male birds which had received single intra-
muscular injections of oestrogen, in the hope of obtaining a greater
and more coherent understanding of the mechanism(s) involved in the
oestrogen-dependent stimulation of hepatic lipogenesis in birds.

# CHAPTER 2

CHANGES IN THE WEIGHT, SOLUBLE PROTEIN
CONTENT AND DNA CONTENT OF THE LIVER
OF THE MALE CHICK AFTER OESTROGEN
TREATMENT.

INTRODUCTION

1. Factors affecting avian liver morphology

(a) Effects of oestrogen

As the female domestic fowl approaches lay, the liver becomes
enlarged involving cellular hypertrophy and hyperplasia (Schjeide
et al.,1963; Pearce, 1971; Balnave, 1971). Laying hen liver, as
well as continuing with normal hepatic function, must synthesise
components of egg yolk and the hepatic parenchymal cells of such
birds are characterised by an extensive proliferation of the endo-
plasmic reticulum and Golgi apparatus (Schjeide et al.,1963;
Scheide, 1967). The liver cells produce large amounts of lipo-
proteins and yolk proteins which are secreted and then transported,
via the blood, to their site of deposition in the developing
oocyte (Schjeide et al., 1963; Heald & McLachlan, 1965). These
changes that occur in the sexually mature hen have also been reported
in chicks, non-laying hens and cockerels after the administration
of exogenous oestrogen (Lorenz, 1954; Schjeide et al, 1963; Bergink
et al.,1974; Chan et al., 1976). The results of these studies,
therefore, implicate endogenous oestrogen as the likely cause of the
changes in liver morphology as female domestic fowl approach lay.

Oestrogen treatment of male and immature female birds has been shown
to cause substantial increases in liver size and weight (Schjeide et
al., 1963, 1974; Hawkins & Heald, 1966; Balnave & Pearce, 1974;
Pearce & Balnave, 1976; Pearson & Butler, 1978). Balnave (1969)
treated 6 week-old male chicks with testosterone, progesterone or
oestrogen and found that oestrogen was the only hormone to cause an
increase in liver weight. An interesting observation was later
reported by Balnave (1971) involving an apparent 'over-reaction' of
the liver to exogenous oestrogen in immature female domestic fowl.

In this study 16-week old female chickens were treated with 5 mg
oestradiol dipropionate on alternate days, over a 7-day period
and the liver weights of these hormone-treated birds were similar
to those of heavier mature laying hens.

Other avian species have been shown to respond to oestrogen treatment
in a similar fashion to domestic fowl, in that increased liver weights
have been observed. Oestrogen treatment of 3 to 6 months old
Japanese quail (4.36 mg 17$\beta$-oestradiol/100 g body wt.) resulted in a
near doubling of liver weight 4 days after hormone administration
while after longer periods of time, the increase in liver weight
diminished until gradually the liver weight returned to values similar
to those of control birds (Gibbins & Robinson, 1982). Similarly,
treatment of 19 day-old male turkeys with a single dose of
diethylstilboestrol (40 mg/kg body wt.) resulted in increased liver
weight, both in absolute terms and in relation to the body weight,
at 24 hours, 48 hours and 72 hours after hormone injection maximum
stimulation of liver weight being observed 48 hours post-injection.
At this time, oestrogen-treated birds compared to control birds
exhibited a 1.28-fold increase in absolute liver weight and a
1.32-fold increase in liver weight as a proportion of body weight
(Dashti et al., 1983). It should be noted that this study on male
turkeys and the study on Japanese quail both used massive doses of
oestrogen.

It is interesting to note that, although oestrogen treatment of the
domestic fowl leads to an increase in liver weight, similar treatment
of chick embryos leads to a decrease in liver weight. Schjeide et al.
(1961) treated 15-day old chick embryos with large doses of oestrogen
(5 mg/bird) and observed an approximately 15% reduction in liver
weight after 3 days, compared with controls, whereas chicks oestrogen-
treated at hatching and sacrificed 3 days later had livers that
weighed 28% more than those from untreated chicks.

The dose level and the duration of the oestrogen treatment has been shown by a number of investigators to affect the extent of the liver weight response in domestic fowl. Balnave & Pearce (1974) treated immature female domestic fowl with 2 mg oestradiol dipropionate on alternate days over a 9-day period and showed that liver weight, expressed as a proportion of body weight, increased 1.6-fold over the first 4 days of hormone treatment, but the extent of the liver weight increase was depressed after 9 days of treatment. Similarly, Pearce & Balnave (1975) treated 4-week old pullets with single injections of oestradiol dipropionate and, 2 days later, they observed dose-dependent increases in liver weights up to 2 mg hormone/bird. With a dose of 4 mg hormone/bird, however, the extent of the observed increase in liver weight was depressed. Dose-related increases in liver weights of oestrogen-treated 6 to 7-week old pullets have been reported by Pearson & Butler (1978) who administered 5 doses of 0.25, 0.5 or 1 mg oestradiol dipropionate/ 100 g body weight at 3-day intervals, the birds being sacrificed 2 days after the last dose. Similar dose-related increases have been observed by Akiba et al. (1982) after implanting silastic tubes filled with oestradiol dipropionate subcutaneously in the necks of 2 to 3-week old male chicks. Different lengths of tube were used with release rates of 1 - 15.2 μg oestradiol/day and the birds were sacrificed 2 - 3 weeks after implantation.

Enlargement of the liver of oestrogen-treated domestic fowl and laying hens is considered to be predominantly due to hypertrophy as a result of water uptake and the accumulation of lipid (Scheide et al., 1963; Schjeide & Lai, 1970). There is an increase in the total hepatic lipid content, predominantly triacylglycerol, of both oestrogen-treated birds and hens at the onset of lay, which contributes to the increase in liver weight (Pearce, 1971, Pearce & Brown, 1972; Balnave & Pearce, 1974; Kuszma et al., 1975; Pearce & Balnave, 1975). Observations of the hepatocytes of such livers have

revealed a proliferation of the endoplasmic reticulum and Golgi apparatus together with an increase in the number of associated ribosomes and mitochondria (Schjeide et al., 1963; Skipper & Hamilton, 1977; Tarlow et al., 1977; Tata, 1979). The cisternae of the endoplasmic reticulum have also been shown to be dilated in appearance as they become increasingly active in the production of secretory proteins and become engorged with lipid droplets and nascent lipoproteins (Schjeide et al., 1963; Tarlow et al., 1977; Garber & Brasch, 1982).

As the liver enlarges it becomes paler in colour and fatty in appearance developing a softer, more fragile structure (Schjeide & Lai, 1970) and the increase in liver size and weight usually becomes obvious within 24 hours of oestrogen treatment (Schjeide & Lai, 1970; Balnave & Pearce, 1974). Hawkins and Heald (1966) measured the hepatic DNA content of 11-week old pullets, treated with 2 mg oestradiol benzoate on alternate days for 7 days and sacrificed on the 8th day, and showed that the DNA content per gram wet weight of liver was decreased compared with control birds. In the same study it was also reported that the DNA content of a unit wet weight of liver from immature pullets was approximately 1.4-fold greater than the corresponding value for the laying hen. This suggests that the increase in liver weight observed in hens approaching lay may occur by a similar mechanism to the increase observed in oestrogen-treated birds, in that cell expansion is a major causative factor of the weight gain. However, it has been estimated that, as well as an increase in cell volume, a proportion (~20%) of the liver parenchymal cells divide in response to oestrogen (Schjeide & Lai, 1970; van den Berg et al., 1974), and in addition, increased DNA synthesis, which is indicative of cell division, does occur (Jost et al., 1973). It is interesting to note that oestrogen-treatment of immature female birds has been shown to lead to an increase in

oviduct weight involving both hypertrophy and hyperplasia (Pearce & Brown, 1971; Balnave & Pearce, 1974; Pearce & Balnave, 1975) and in this respect, the responses of both the liver and oviduct to oestrogen are similar.

Changes in liver morphology and cytology in response to oestrogens, have been reported in other oviparous vertebrates, the changes being similar to those of the domestic fowl. Xenopus laevis has been shown to respond with an increase in liver size (Follett & Redshaw, 1968) and with a proliferation of the endoplasmic reticulum and Golgi apparatus (Follett et al., 1968; Lewis et al., 1976; Shapiro, 1982). Hepatocytes of the lizard, Uta stansburiana, have been shown to respond to oestrogen in a similar way and fat vacuoles become prominent in the cytoplasm (Schjeide & Lai, 1970). The adipose tissues of this species normally become enlarged before egg-laying and it is suggested that, in response to endogenous oestrogen, free fatty acids are released from adipocytes into the blood, and are carried to the liver where they are eventually utilised.

It is interesting to note that a slight increase in the liver weight of an oestrogen-treated viviparous vertebrate, the female rat, has also been reported, the response being somewhat similar to that in the domestic fowl in that cell expansion was primarily involved with hypertrophy of the endoplasmic reticulum (Song et al., 1969; Weinstein et al., 1979). However, it should be recognised that the oestrogen doses used in these mammalian studies were much lower than those used in studies involving domestic fowl.

(b) Effects of Prolactin

Certain other circumstances, apart from oestrogen treatment, have been found in which the avian liver responds with a change in weight

Of these, one of the best-documented is the liver enlargement and stimulation of hepatic lipogenesis associated with the action of prolactin. This pituitary hormone appears to be involved in bringing about the metabolic changes necessary for the deposition of fat reserves in migratory birds (Maier et al., 1969; Wheeland et al., 1976). Goodridge & Ball (1967b, 1967c) studied the response of the pigeon liver to prolactin, and observed that after a daily dose of 1 mg of hormone for 5 days the liver had nearly doubled in weight, although the increase in liver weight was apparent after only 1 day of prolactin treatment. These workers suggested that an uptake of water was involved in this increase in liver size, at least in the early stages of the response. Prolactin also causes the enlargement of the crop sac of the pigeon and the formation of crop milk. Goodridge & Ball (1967c) observed that starvation eliminated the increase in liver size caused by prolactin, but that the crop sac still responded. These investigators concluded that prolactin causes hyperphagia, which leads to an increase in body weights of specific organs including the liver, pancreas and intestine. Hyperphagia has been shown to cause an increase in liver weight in avian species, mainly as a result of the deposition of lipid (Shapira et al., 1978). It is of interest to note that increased food consumption has been observed in oestrogen-treated immature male and female domestic fowl (Pearce & Brown, 1971; Pearson & Butler, 1971).

(c) Effects of the diet and the pattern of food intake.

The liver weight of the domestic fowl has been shown to fluctuate according to the pattern of food intake and the composition of diet. Leveille (1969a) reported fluctuations in the liver weights of chicks which had been subjected to a 3-day fast followed by a period of re-feeding. During the fast the liver weight of the chick,

expressed as a percentage of body weight, decreased by approximately 30% whereas upon re-feeding for 2 days, the liver weight increased to a value greater than the control value.  After 3 days of re-feeding, however, the liver weight returned to the control value. During the 3-day fasting period the hepatic total lipid content (mg lipid/g liver) did not change significantly although the value was 10% lower than the corresponding value from fed birds.  The increase in liver weight after re-feeding correlated with an increase in hepatic total lipid content.

Meal-feeding of chicks has been shown to result in increased liver weights when compared to birds fed ad libitum (Muiruri et al., 1975). Moreover, 1 hour after allowing meal-fed chicks access to food, there was a significant increase in liver weight and it was suggested that this was primarily the result of increases in water and glycogen content and, to a lesser extent, in lipid content.

A diet rich in fructose caused a marked increase in liver weight and liver lipid content in 7 to 8-week old pullets (Pearce, 1970) and, presumably, this is related to the well-documented stimulation of hepatic lipogenesis by fructose (Hawkins & Heald, 1966;  Goodridge, 1973c).

In some species, including the domestic fowl, embryonic and newborn individuals have fatty livers, the lipid being derived from the lipid-rich yolk on which the embryo is nourished.  This phenomenon, however, is short-lived and the livers become normal a few days after hatching (Entenman et al., 1940; Lucas & Ridout, 1970).

(d) Fatty liver and kidney syndrome (FLKS) and fatty liver-haemorrhagic syndrome (FLHS)

FLKS and FLHS are two non-infectious diseases afflicting young chicks

and laying hens, respectively, and both cause enlargement of the
liver (Butler, 1976). Chicks suffering from FLKS have widespread
fatty infiltration of the tissues, particularly of the liver and
kidneys which become pale and enlarged (Wight & Siller, 1975).
Chicks suffering from FLKS are also hyperlipaemic, the plasma levels
of free fatty acids and triacylglycerol being high despite a
reduction in hepatic lipogenesis (Evans et al., 1975).

It is believed that the development of the symptoms of FLKS is
caused by a decreased uptake of lipid by adipose tissue as a result
of inhibition of lipoprotein lipase (Evans et al., 1975). This
enzyme is located on the luminal surface of endothelial cells of
capillaries in body tissues (Scow et al., 1976; Kompiang et al.,
1976) and is responsible for the hydrolysis of plasma triacylglycerol
leading to the release of free fatty acids which may be taken up by
adipocytes. Therefore, if this enzyme were to exhibit diminished
activity an increase in plasma lipid levels may ensue together with
an accumulation of fat in certain organs.

It has been demonstrated that FLKS can be caused by a combination of
nutritional factors, including low biotin, protein and fat levels in
the diet, leading to an impairment of hepatic gluconeogenesis
(Bannister, 1976; Whitehead et al., 1978). Susceptibility to the
disease seems to be caused by adverse environmental factors such as
stress or fasting, and death occurs as a result of hypoglycaemia
(Bannister et al., 1979).

Laying domestic fowl are affected by FLHS which manifests itself in
the accumulation of fat, of which most is triacylglycerol, in the
liver, kidneys and abdominal cavity. High plasma levels of free
fatty acids and triacylglycerol and reduced egg production are also
symptomatic of the disease (Butler, 1976). The internal structure
of the liver of a victim of FLHS is disrupted greatly by the increased

incidence of fat vacuoles in the cytoplasm of hepatocytes which
often leads to rupture of the cells, and by lysis of the reticulin
bands around the cells which results in structural weakness in the
liver. Furthermore, there are diffuse capillary haemorrhages,
blood clots, vascular breakdown, and regions of fibrosis and necrosis
(Butler, 1976; Jensen, 1979). In cases of FLHS, small haemorrhages
are found in the periphery of the liver and death is usually caused
by haemorrhage from the liver which ruptures the liver capsule.

Evidence suggests that the steatosis associated with FLHS is the
result of increased hepatic lipogenesis, and it seems probable that
the syndrome is caused by an excessive intake of carbohydrate,
leading to a positive energy balance, and results from an interplay
of dietary and environmental factors. Therefore, hens kept at high
environmental temperatures and which are unable to reduce their food
consumption have a greater susceptibility to FLHS. Another factor
implicated in the development of the disease is an abnormally high
level of endogenous oestrogen (Butler, 1976; Jensen, 1979). The
possible involvement of oestrogen in FLHS is supported by
observations that oestrogen treatment of domestic fowl results in
fatty liver and an increased incidence of hepatic haemorrhage
(Wolford & Polin, 1973; Pearson & Butler, 1978; Stake et al., 1981).

2.   Effects of oestrogens on extra-hepatic organs

Oestrogens have been shown to exert significant effects on the
morphology and metabolic activity of organs other than the liver,
notably the oviduct of avian species (Hawkins & Heald, 1966; Pearce
& Brown, 1971; Balnave & Pearce, 1974; Pearce & Balnave, 1975;
Aprahamian et al., 1979; Laugier et al., 1980), and the uterus
(Aizawa & Mueller, 1961; Martin, 1980; Thompson et al., 1983),
vagina (Martin, 1980) and mammary gland (Topper & Freeman, 1980) of
mammals.

Hawkins & Heald (1966) observed an approximately 25-fold increase in the oviduct weight of 11-week old chickens treated with 2 mg oestradiol benzoate on alternate days for 7 days. Similarly, Aprahamian et al. (1979) observed a 23-fold increase in oviduct weight in 1-month old chickens after treating each bird with 4 mg oestradiol benzoate/day for 6 days. Other investigators have observed similar increases in oviduct weight after treating immature pullets with oestrogen (Pearce & Brown, 1971; Balnave & Pearce, 1974; Pearce & Balnave, 1975). This increase in oviduct size is associated with both cell expansion and cell division, is accompanied by an increase in metabolic activity and stimulation of ovalbumin synthesis, and is a pre-requisite for the oviduct to respond to progesterone.

The uterus of mammals also responds to oestrogen treatment with an increase in size and metabolic activity (Thomson et al., 1983), the response involving a substantial increase in the number and size of cells and the water content of the uterus (Aizawa & Mueller, 1961). The mammalian vagina responds to oestrogen with an increase in the thickness of the epithelium resulting from an increase in the rate of cell division in the basal layers (Martin, 1980). Changes occurring in the structure and function of mammary gland during puberty and pregnancy appear to occur in response to several hormonal stimuli. Oestrogen, together with other hormones, is involved in the ductal growth which occurs during puberty, and lobuloalveolar growth which occurs during pregnancy. Hormone-induced ductal growth involves cell division giving rise to undifferentiated cells. Subsequently, during pregnancy, the inter-ductal spaces are filled with lobuloalveolar structures as a result of cell division and differentiation (Topper & Freeman, 1980).

In summary, oestrogen treatment of many species has been shown to cause substantial changes in the morphology of certain organs.

Therefore, in the context of a study of the effect of oestrogen on hepatic lipid metabolism of male domestic fowl, it was decided to carry out a preliminary investigation of the effects of oestrogen on liver morphology in similar birds. In the following account, details are presented of changes in liver weight, hepatic soluble protein content and hepatic DNA content at various times after 17$\beta$-oestradiol treatment. By a detailed analysis of the time-courses of changes in these parameters, it was hoped that an assessment could be made of the roles of cell expansion and cell division in any observed oestrogen-dependent liver growth. It was believed that information derived from this preliminary study might prove valuable in the interpretation of the results of subsequent investigations designed to follow the effect of oestrogen on various parameters of hepatic lipid metabolism.

METHODS

1.  Animals

Day-old male Hi-Sex (strain-cross, White Leghorn) chicks were
obtained from a commercial hatchery and housed in a communal pen,
the environmental temperature being 19° - 21°C. During the first
10 days of life the birds were subjected to constant illumination
provided by a lamp in the pen which served to provide localised
heat. Subsequently, a fixed 9 hours/day photoperiod of artificial
dimmed red light was maintained (08.00 hours to 17.00 hours).
From 19 days of age the birds were housed in groups of 3 - 5 in
cages with raised wire floors. Throughout their lives, the chicks
were fed on Dalgety Spillers, 404 Gold-Start Crumbs/ACS.

In all experiments control chicks were injected intramuscularly
(pectoral muscle) with 0.1 ml propane-1,2-diol/100 g body weight. In
one set of experiments, oestrogen-treated birds received injections
of 17β-oestradiol in propane-1,2-diol (0.1 ml/100 g body wt.)
equivalent to one of the following doses; 0.25, 0.5, 0.75, 1.0,
1.25 mg hormone/100 g body weight. In a second set of experiments,
all oestrogen-treated birds were injected with 17β-oestradiol in
propane-1,2-diol (0.1 ml/100 g body wt.) equivalent to a dose of
0.75 mg hormone/100 g body weight.

2.  Preparation of particle-free supernatant from chick liver

Birds were weighed and then killed by decapitation between 11.00 and
13.00 hours in order to minimise differences caused by diurnal
effects and to ensure, as far as possible, similar prandial states.
Immediately after death, livers were rapidly excised and weighed. A
portion of each liver was weighed rapidly, then plunged into liquid

nitrogen and stored at -20°C for future DNA estimation.  Another
portion of liver (5 g) was homogenised using a teflon-glass
homogeniser with 2 volumes of ice-cold homogenisation buffer
consisting of 94 mM-potassium phosphate, 0.5 mM-EDTA, 0.2 mM-
dithiothreitol, 65 mM-potassium chloride and 5 mM-potassium
carbonate, at pH7.0.  The tissue was disrupted with 8 'up and down'
strokes of the rotating pestle.  Volumes of homogenates were
measured and they were then centrifuged at 100,000 $g$ ($r_{av.}$ 6.4 cm)
for 1 hour at 4°C in a M.S.E. Prepsin 50 centrifuge.  The super-
natantswere diluted with an ice-cold dilution buffer, consisting
of 50 mM-potassium phosphate, 0.1 mM-EDTA and 0.5 mM-dithiothreitol,
at pH7.0.  Aliquots of the diluted liver extracts were stored at
-20°C for future determination of soluble protein whilst the activity
of acetyl-CoA carboxylase (see Chapter 4) was determined in fresh
extracts.

3.  Determination of DNA

After storage for up to 14 days at -20°C, liver samples (0.8 - 1.0 g)
were thawed and then homogenised in 10 volumes (v/w) of 0.9% (w/v)
NaCl.  From duplicate samples (2 ml) of the homogenates, the DNA
was precipitated, washed and extracted using a slight modification
of the procedure of Prashad & Cutler (1976).  An equal volume of
cold IM-perchloric acid was added to an aliquot of homogenate and
stored on ice for 1 hour.  Following centrifugation at 6000 $g$ for
20 min. at 4°C, the supernatant was discarded and the pellet washed
2 times with aliquots (2 ml) of cold 0.5 M-perchloric acid.  DNA
was extracted from the washed pellet by resuspension in 0.5 M-
perchloric acid (2 ml), followed by heating at 70°C for 20 mins.
After centrifugation at 6000 $g$ for 20 mins. at 4°C, the supernatant
was collected and further DNA extracted from the pellets another 2
times.  The supernatants were combined and extracted DNA determined

by the method of Burton (1956) with slight modification. A 2 ml
aliquot of the DNA extract was mixed with 4 ml diphenylamine reagent,
prepared by dissolving 1.5 g diphenylamine in 100 ml glacial acetic
acid and 1.5 ml of conc. $H_2SO_4$, and 0.2 ml aqueous acetaldehyde
(1.6 mg/ml). Following incubation for 18 hours at 30°C, the
absorbance at 600 nm was measured in a Pye Unicam SP8-100 spectro-
photometer. Duplicate blank assays and assays using a standard DNA
solution were included. A stock standard DNA solution was prepared
by dissolving calf thymus DNA in 0.5 M-perchloric acid to give a
concentration of 0.2 mg/ml. The DNA was solubilised by heating at
70°C for 20 minutes and standards of a variety of concentrations
(0.025 - 0.2 mg/ml) were prepared by dilution with 0.5 M-perchloric
acid. The resultant DNA calibration curves indicated that the
assay was linear up to a total amount of 0.30 mg DNA.

4.   Determination of Protein.

After storage for up to 7 days at -20°C, the soluble protein
concentration of each liver extract was determined by the biuret
method described by Hübscher et al. (1965), with some modification.
The biuret reagent of Weichselbaum (1946) was modified as follows:
biuret reagent (200 ml) and 1 M-NaOH (160 ml) were mixed, potassium
iodide (4 g) was added and dissolved, and the solution was made up
to 1 litre with distilled water and stored at 4°C. Duplicate
protein samples were precipated with an equal volume of ice-cold
10% (w/v) trichloroacetic acid. The precipitate was pelleted by
centrifugation at 4°C and the pellet was dissolved in 1 ml of
0.1 M-NaOH after removal of the supernatant. The modified biuret
reagent (2 ml) was added and colour developed by incubation at 37°C
for 15 minutes. After cooling, water-saturated diethyl ether (3 ml)
was added to remove any lipids which often cause cloudiness. The
tubes were mixed, using a vortex mixer, and the phases separated by

subsequent low-speed centrifugation. The upper (ether) phase was removed and the absorbance of the lower layer measured at 555 nm using a Pye-Unicam SP8-100 spectrophotometer. Duplicate blank assays and assays using a standard aqueous solution of BSA (fatty acid poor) were included in each set of assays. Calibration curves indicated that the assay was linear up to 9 mg protein (BSA).

## 5.   Statistical analysis

Data were analysed statistically by Student's 't' test. Probability values (p) of 0.05 or less were taken to be significant. Standard errors are provided to show the degree of variance and levels of statistical significance are indicated.

RESULTS

1. The effect of varying doses of 17β-oestradiol on the weight and
   soluble protein content of male chick liver

Variations in the weight and soluble protein content of livers from
male chicks killed 48 hours after receiving a single intramuscular
injection of 17β-oestradiol (0 - 1.25 mg hormone/100 g body wt.) in
propane-1,2-diol are presented in Table 2.1.

At all dose levels the mean liver weight (as % of body wt.) for
oestrogen-treated birds was greater than the values for control and
untreated birds. Significant increases were observed over controls
at all doses except 0.25 mg 17β-oestradiol/100 g body weight.
Maximum liver weight (as % of body wt.) was observed with the
maximum dose levels of 1.25 mg hormone/100 g body weight, the value
for oestrogen-treated chicks being 1.84-fold greater than the value
for control chicks.

A dose-related decrease in the soluble protein content of unit wet
weight of liver was observed in oestrogen-treated birds when
compared to controls. Significant differences were observed between
the value for control birds and those for oestrogen-treated birds at
all dose levels except 0.25 mg hormone/100 g body weight and the
minimum soluble protein content/g liver was observed with the highest
dose of 1.25 mg hormone/100 g body weight, the value for oestrogen-
treated birds being 82% of the value for control birds.

Although the soluble protein content/g liver decreased upon oestrogen-
treatment, a dose-related increase in the total hepatic soluble
protein content was observed in the same birds. At all hormone levels
except 0.25 mg 17β-oestradiol/100 g body weight, significant

Table 2.1

The effect of varying doses of 17β-oestradiol on liver weight and the hepatic soluble protein content of birds killed 48 hours after injection.

Each oestrogen-treated bird received an intramuscular injection of 17β-oestradiol in propane-1,2-diol, equivalent to one of the following doses:-

0.25, 0.5, 0.75, 1.0, 1.25 mg 17β-oestradiol/100 g body weight

Control birds received an equivalent volume of propane-1,2-diol, alone.  Birds were sacrificed 48 hours after injection and liver extracts were obtained and assayed for soluble protein as described in the Methods section.

Values are the means ($\pm$ S.E.M.) of 5 - 8 birds.

Data were analysed statistically by Student's 't' test.  Levels of significance presented with oestrogen-treated values are with respect to control values.

* significant at $p < 0.05$

# significant at $p < 0.01$

¥ significant at $p < 0.001$

Birds were aged 28 - 34 days.

| | 17β-oestradiol (mg/100 g body wt.) | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | 0 UNT. | 0 CONT. | 0.25 | 0.50 | 0.75 | 1.00 | 1.25 |
| Number of birds | 8 | 8 | 6 | 6 | 5 | 6 | 6 |
| Body weight (g) | 324 ±17 | 303 ±20 | 294 ± 6 | 343 ±18 | 345 ±27 | 387 ±25 | 425 ±10 |
| Liver weight (as % body wt.) | 2.88 ±0.18 | 2.75 ±0.09 | 3.06 ±0.12 | ¥ 3.91 ±0.22 | ¥ 4.50 ±0.29 | ¥ 4.69 ±0.14 | ¥ 5.05 ±0.24 |
| Hepatic soluble protein (mg/g liver) | 81.0 ±1.3 | 81.9 ±1.7 | 79.9 ±1.0 | # 70.2 ±2.6 | * 72.9 ±3.0 | # 70.4 ±2.2 | ¥ 66.8 ±2.1 |
| Total hepatic soluble protein (mg/liver) | 747 ±47 | 677 ±48 | 720 ±43 | # 936 ±73 | ¥ 1113 ±87 | ¥ 1270 ±69 | ¥ 1433 ±83 |

increases in total hepatic soluble protein content were observed with respect to the value for control birds. Maximum total hepatic soluble protein was observed in birds injected with the highest dose of 1.25 mg hormone/100 g body weight, the value for oestrogen treated birds being 2.12-fold greater than the value for control birds.

2. The effect of a single intramuscular injection of 17β-oestradiol on the weight, DNA content and soluble protein content of male chick liver

Variations in the weight, DNA content and soluble protein content of livers from male chicks killed during the 61-hour period immediately following a single intramuscular injection of 17β-oestradiol (0.75 mg/100 g body wt.) are presented in Table 2.2 and Figures 2.1, 2.2 and 2.3. A dose of 0.75 mg hormone/100 g body weight was adopted because measurements of the hepatic activity of acetyl-CoA carboxylase (E.C. 6.4.1.2), a key lipogenic enzyme, in birds treated with different doses of oestrogen, indicated that optimal stimulation of activity occurred with this dose (see Chapter 4).

The mean liver weight of oestrogen-treated chicks was not significantly different from that of control chicks during the first 6 hours after injection (Table 2.2 and Fig. 2.1). At later times, however, the mean liver weight of oestrogen-treated birds increased significantly, compared to control values, reaching a maximum at 37 hours after injection, when the value in oestrogen-treated birds was 1.61-fold greater than the corresponding control value. After this time, the mean liver weight of oestrogen-treated birds decreased progressively until, at 61 hours after hormone treatment the value was 1.54-fold greater than the corresponding control value (Table 2.2 and Figure 2.1).

Table 2.2

The effect of a single intramuscular injection of 17β-oestradiol on the weight, DNA content and soluble protein content of male chick liver

Each oestrogen-treated bird received an intramuscular injection of 17β-oestradiol (0.75 mg/100 g body wt.) in propane-1,2-diol. Control birds received an equivalent volume of propane-1,2-diol alone. At the indicated times after injection birds were sacrificed, liver extracts prepared and assayed for DNA and soluble protein as described in the Methods section.

OE = values for oestrogen-treated birds

C = values for control birds

Values are the means ($\pm$ S.E.M.) of 8 - 19 birds.

Data were analysed statistically by Student's 't' test. Levels of statistical significance presented with values for oestrogen treated birds are with respect to the corresponding control value.

* significant at $p < 0.05$

# significant at $p < 0.01$

¥ significant at $p < 0.001$

The liver weight data, the hepatic DNA content data and the hepatic soluble protein content data are presented graphically in Fig. 2.1, Fig. 2.2 and Fig. 2.3, respectively.

Birds were aged 25 - 32 days.

Time after injection (h)

| | 0 | 3 OE | 3 C | 6 OE | 6 C | 13 OE | 13 C | 19 OE | 19 C | 24 OE | 24 C | 37 OE | 37 C | 48 OE | 48 C | 61 OE | 61 C |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Number of birds | 19 | 6 | 6 | 12 | 12 | 9 | 9 | 9 | 9 | 9 | 9 | 8 | 8 | 9 | 9 | 9 | 9 |
| Body weight (g) | 313 ±11 | 332 ±21 | 318 ±11 | 336 ±23 | 303 ±21 | 311 ±7 | 310 ±9 | 356 ±18 | 323 ±21 | 303 ±15 | 280 ±18 | 361 ±10 | 318 ±12 | 338 ±20 | 312 ±13 | 318 ±12 | 338 ±13 |
| Liver weight (as % body wt.) | 3.33 ±0.04 | 3.52 ±0.10 | 3.41 ±0.07 | 3.42 ±0.06 | 3.51 ±0.08 | 3.59 * ±0.08 | 3.29 ±0.08 | 4.11 ¥ ±0.09 | 3.20 ±0.10 | 4.78 ¥ ±0.18 | 3.23 ±0.06 | 5.53 ¥ ±0.10 | 3.44 ±0.15 | 5.11 ¥ ±0.23 | 3.26 ±0.06 | 4.94 ¥ ±0.16 | 3.21 ±0.07 |
| Total hepatic DNA (mg/liver) | 32.0 ±1.3 | 32.1 ±1.3 | 30.7 ±0.8 | 31.4 ±2.0 | 30.6 ±1.9 | 30.1 ±0.9 | 31.0 ±1.5 | 33.4 ±2.1 | 30.5 ±1.3 | 30.8 ±2.2 | 29.0 ±1.9 | 42.3 * ±2.4 | 30.6 ±2.6 | 40.3 # ±1.7 | 29.5 ±2.2 | 37.4 # ±1.6 | 31.4 ±1.1 |
| Hepatic DNA (mg/g liver) | 2.99 ±0.07 | 2.78 ±0.08 | 2.84 ±0.08 | 2.76 ±0.05 | 2.89 ±0.05 | 2.70 ¥ ±0.06 | 3.03 ±0.06 | 2.35 ¥ ±0.07 | 3.00 ±0.06 | 2.12 ¥ ±0.04 | 3.10 ±0.09 | 2.12 # ±0.08 | 2.83 ±0.12 | 2.37 # ±0.08 | 2.90 ±0.12 | 2.60 ±0.13 | 2.91 ±0.08 |
| Total hepatic soluble protein (mg/liver) | 787 ±28 | 837 ±56 | 810 ±33 | 795 ±47 | 743 ±44 | 760 ±34 | 791 ±32 | 943 * ±57 | 776 ±34 | 919 * ±65 | 706 ±52 | 1244 ¥ ±45 | 811 ±35 | 1186 ¥ ±51 | 751 ±38 | 1139 ¥ ±70 | 802 ±36 |
| Hepatic soluble protein (mg/g liver) | 75.8 ±1.1 | 71.9 ±2.0 | 75.0 ±1.9 | 70.0 ±0.9 | 70.5 ±1.5 | 67.8 ¥ ±1.1 | 77.5 ±1.1 | 65.4 # ±2.6 | 76.4 ±2.3 | 63.2 ¥ ±1.2 | 78.2 ±2.3 | 62.4 ¥ ±1.5 | 74.7 ±1.5 | 68.6 ±2.1 | 73.7 ±1.4 | 72.1 ±2.0 | 74.1 ±1.2 |
| Hepatic soluble protein (mg/mg DNA) | 25.6 ±0.7 | 26.1 ±1.3 | 26.4 ±0.7 | 25.4 ±0.5 | 24.4 ±0.6 | 25.3 ±0.8 | 25.6 ±0.6 | 28.6 ±1.5 | 25.6 ±1.2 | 29.9 ¥ ±0.5 | 25.3 ±0.7 | 30.4 ±1.4 | 27.4 ±1.4 | 29.5 # ±0.8 | 25.8 ±1.1 | 29.9 ±2.3 | 25.6 ±0.9 |

Figure 2.1

The effect of a single intramuscular injection of oestrogen on
the liver weight of male chicks

Consult the legend of Table 2.2 for details of experimental
procedure.

&#9650; value for untreated chicks

o values for oestrogen-treated chicks

&#9679; values for control chicks, injected with
   propane-1,2-diol, alone

The results are expressed as means ($\pm$ S.E.M.) of 8 - 19 birds.
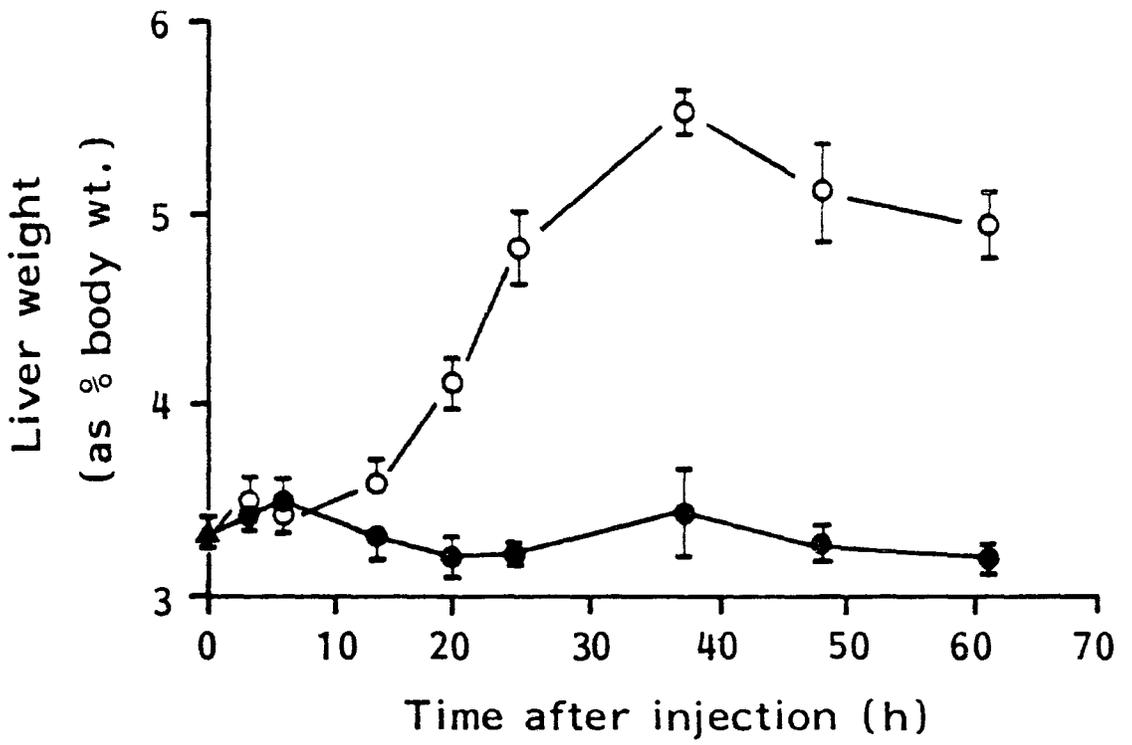
The mean total amount of DNA in the liver of oestrogen-treated chicks was not significantly different from that of control chicks during the first 24 hours after hormone administration (Table 2.2 and Fig. 2.2a). At later times (37 - 61 hours) however, the mean total hepatic DNA content was significantly greater than the corresponding control value such that, at 37 hours after injection, the value in oestrogen-treated birds was maximal, being 1.38-fold greater than the control value. After this time, the mean total hepatic DNA content of oestrogen-treated birds decreased progressively until at 61 hours after hormone treatment the value was 1.19-fold greater than the corresponding control value (Table 2.2 and Fig. 2.2a).

Expressions of the hepatic DNA content in terms of the amount of DNA/g liver revealed that during the first 6 hours after injection, the value in oestrogen-treated birds was not significantly different from that of control birds (Table 2.2 and Fig. 2.2b). At later times, however, the mean amount of DNA/g liver of oestrogen-treated birds decreased significantly, compared to control values, reaching lowest values at 24 hours and 37 hours after hormone administration, when the values in oestrogen-treated birds were 68% and 75% of the corresponding control values, respectively. Subsequent to 37 hours post-injection, the mean amount of DNA/ g liver in oestrogen-treated birds increased progressively until at 61 hours after hormone administration, the value was 89% of the corresponding control value and the two values were not significantly different (Table 2.2 and Fig. 2.2b).

Following oestrogen-treatment, the soluble protein content of liver from male chicks was determined (Table 2.2 and Fig. 2.3) and expressed as total soluble protein/liver (Fig. 2.3a), soluble protein/ g liver (Fig. 2.3b) and soluble protein/mg DNA (Fig. 2.3c), the

Figure 2.2

The effect of a single intramuscular injection of oestrogen
on the hepatic DNA content of male chicks.

Consult the legend of Table 2.2 for details of experimental
procedure.

▲ values for untreated chicks.

○ values for oestrogen-treated chicks.

● values for control chicks, injected with
propane 1,2-diol, alone.

The results are expressed as means ($\pm$ S.E.M.) of 8 - 19 birds.

(a) Total hepatic DNA content (mg/liver).

(b) Hepatic DNA content (mg/g liver).

a.

b.
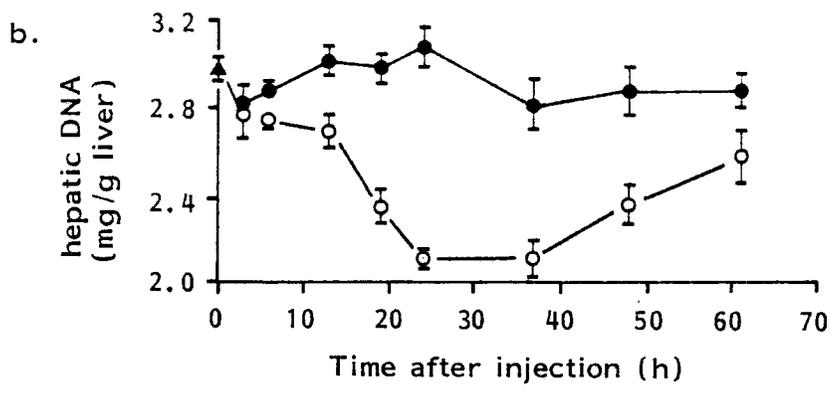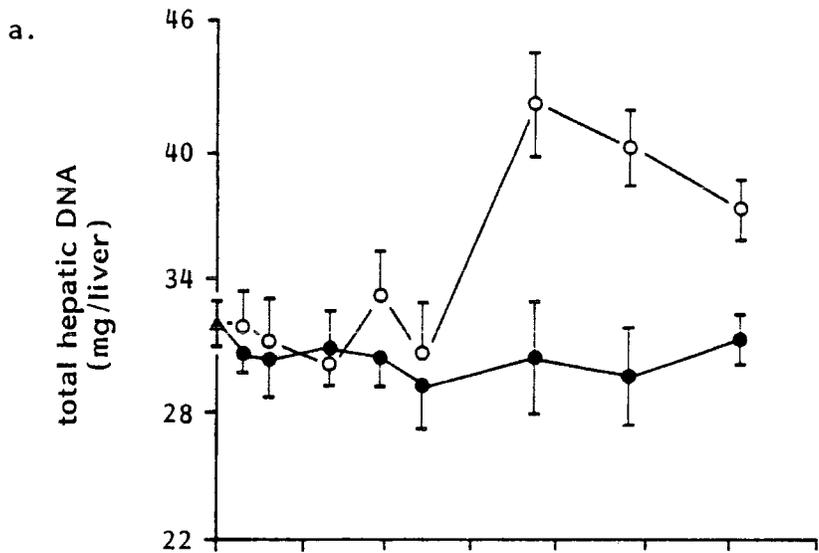
latter expression being used as an indication of the cellular content of soluble protein.

The mean total hepatic soluble protein content of oestrogen-treated chicks was not significantly different from that of control chicks during the first 13 hours after injection (Table 2.2 and Fig. 2.3a). At later times (24 - 61 hours), however, the mean total hepatic soluble protein content of oestrogen-treated birds increased significantly, compared to control values. A maximum value for oestrogen-treated birds was observed at 37 hours after injection, being 1.53-fold greater than the corresponding control value. After this time, the mean total hepatic soluble protein content of oestrogen-treated birds decreased slightly such that at 61 hours after hormone treatment, the value was 1.42-fold greater than the corresponding control value (Table 2.2 and Fig. 2.3a).

The mean soluble protein content/g liver of oestrogen-treated chicks was not significantly different from that of control chicks during the first 6 hours after injection (Table 2.2 and Fig. 2.3b). However, at later times the mean soluble protein content/g liver of oestrogen-treated birds decreased significantly, compared to control values reaching a minimum at 37 hours after hormone administration, when the value in oestrogen-treated birds was 83% of the corresponding control value. After this time, the mean soluble protein/g liver of oestrogen-treated birds increased progressively such that at 48 hours and 61 hours after hormone treatment the value was not significantly different from the corresponding control value (Table 2.2 and Fig. 2.3b).

During the first 13 hours after injection the mean hepatic soluble protein content/mg DNA of oestrogen-treated chicks was not significantly different from that of control chicks (Table 2.2 and

.

Figure 2.3


The effect of a single intramuscular injection of oestrogen
on the hepatic soluble protein content of male chicks.



Consult the legend of Table 2.2 for details of experimental
procedure.


▲ values for untreated chicks.
○ values for oestrogen-treated chicks.
● values for control chicks, injected with
propane-1,2-diol, alone.

The results are expressed as means (± S.E.M.) of 8 - 19 birds.


(a) Total hepatic soluble protein content (mg/liver).

(b) Hepatic soluble protein content (mg/g liver).

(c) Hepatic soluble protein content (mg/mg DNA).

a.

total hepatic soluble protein (mg/liver)

b.

hepatic soluble protein (mg/g liver)

c.

(mg/mg DNA)

Time after injection (h)
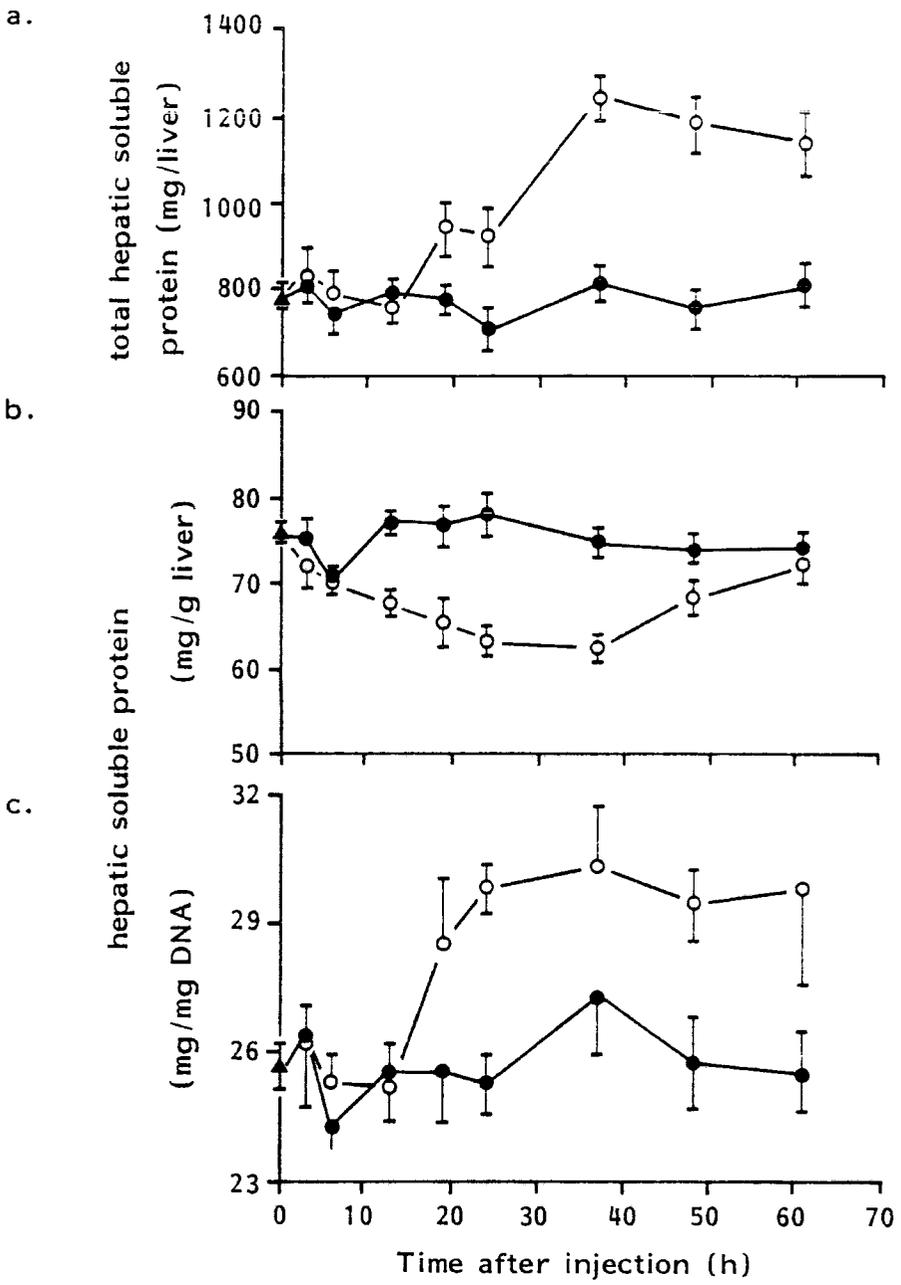
Fig. 2.3c). At later times (19 - 61 hours), however, the mean
hepatic soluble protein content/mg DNA of oestrogen-treated birds
increased, reaching a maximum at 37 hours after hormone
administration and only slightly decreasing subsequently. During
the same 19 - 61 hour post-injection period, the mean hepatic
soluble protein content/mg DNA of control birds remained at a level
similar to the value for untreated birds. Although the mean
hepatic soluble protein content/mg DNA of oestrogen-treated birds
was greater than the value for corresponding control birds during
the 19 - 61 hour post-injection period, only at the 24 hours and
48 hours time-points were the increases significant (Table 2.2 and
Fig. 2.3c).

DISCUSSION

1.  The effect of varying doses of oestrogen on the weight and soluble
    protein content of male chick liver

The data presented in Table 2.1 illustrates a dose-related increase
in the liver weight of oestrogen-treated male chicks over the dose
range 0.5 - 1.25 mg 17$\beta$-oestradiol/100 g body weight when the birds
were injected with a single dose of hormone and sacrificed 48 hours
later.  Similar dose-related trends in liver and oviduct weight were
observed by Pearce & Balnave (1975) who injected 4-week old pullets
with varying doses of oestradiol dipropionate (0.5 - 4 mg/bird) and
sacrificed the birds 48 hours after a single injection or 8 days
after the start of hormone treatment,during which the birds received
a hormone injection on alternate days.  After 2 days of oestrogen
treatment, oviduct weight (as % of body wt.) increased up to a dose
of 1 mg oestradiol dipropionate, and with the higher doses the
increase in weight was less.  After 8 days of oestrogen treatment,
oviduct weight increased progressively with increasing dose level.
Liver weight (as % of body wt.) after 2 days of oestrogen treatment
increased up to a dose of 2 mg oestradiol dipropionate and the
response was depressed with a dose of 4 mg.  A similar response
pattern was observed after 8 days of hormone treatment.  Balnave
(1971) observed an 'over-reaction' of immature female chickens to
exogenous oestrogen such that the liver weights were greater than
those of mature laying hens.  These observations suggest that large
oestrogen doses and extended times of oestrogen treatment may lead
to pharmacological effects rather than to a simulation of the
physiological situation existing in the laying hen.  A decrease in
liver weight after treatment with high oestradiol doses was not
observed in the present study, even though a similar range of
oestradiol dose levels was administered.  Taking into account the

different form of oestradiol used by these workers and the approximate body weights (250 g) of the birds used, Pearce & Balnave (1975) used a maximum dose of 1.13 mg oestradiol/100 g body weight which evoked a depressed liver weight increase when compared to that observed with a dose of 0.57 mg oestradiol/100 g body weight. In the present study, however, the maximum dose level was 1.25 mg oestradiol/100 body weight and the increase in liver weight observed at this level was greater than at all lower doses. This difference in dose levels in the two studies may be the result of sex differences, since male chicks were used throughout the present study, whilst Pearce & Balnave (1975) used immature females.

The suggestion that sex differences may be important in determining the nature of the liver growth response to varying doses of oestrogen is supported by the results of Courtney (1984), who treated 3 - 5-week old male chicks with varying doses of 17β-oestradiol (0.25 - 1.25 mg/100 g body wt.) and observed a dose-dependent increase in liver weight, the greatest response being observed with administration of the greatest dose. However, the results of Pearson & Butler (1978) clearly demonstrate the importance of considering the age and sex of the birds used, together with the dose and duration of hormone administration, when attempting to make comparisons between individual studies. Pearson & Butler (1978) administered 5 doses of 0.25, 0.5 or 1 mg oestradiol dipropionate/ 100 g body weight at 3-day intervals to 6- to 7-week old pullets and sacrificed the birds 48 hours after the last dose. A dose-dependent increase in liver weight was observed with the greatest increase being obtained with the greatest dose of hormone.

Table 2.1 shows that, as the liver weight increased with increasing hormone dose level, the soluble protein content of unit wet weight of liver decreased, suggesting that the increase in liver weight may be accompanied by cellular hypertrophy.

The dose-dependent increase in liver weight was accompanied by a
dose-dependent increase in the total soluble protein in the liver
such that at the maximum dose level (1.25 mg hormone/100 g body wt.)
both liver weight and total hepatic soluble protein content were
maximal. This suggests that the hormone-induced stimulation of
liver weight involves a stimulation of the rate of synthesis of
hepatic soluble protein and/or a decrease in the rate of degradation
of soluble protein.

That the oestrogen-induced increase in liver weight may be
accompanied by cellular hypertrophy is supported by the following,
more detailed analysis of changes in liver morphology occurring
during the 61-hour period immediately after injecting birds with a
dose of 0.75 mg 17β-oestradiol/100 g body weight.

2.  The effect of a single injection of 17β-oestradiol on the weight,
    DNA content and soluble protein content of male chick liver.

The administration of a single intramuscular injection of 17β-
oestradiol to male chicks was found to result in an increase in
liver weight, expressed as a percentage of body weight, compared to
control birds (Fig. 2.1). Initially, there was no significant
difference between the liver weight of hormone-treated and control
birds for the first 6 hours after injection. This was followed by
a progressive and substantial increase in the liver weight of
hormone-treated birds up to 37 hours after injection, followed by a
gradual decline in the liver weight of these birds. A number of
workers have obtained similar trends in liver weight, comb weight
and oviduct weight of immature female chickens after treatment with
gonadal hormones. For example, Balnave & Pearce (1974) injected
4-week old pullets with 2 mg oestradiol dipropionate on alternate
days for 9 days. No difference in liver weight, expressed as a
proportion of body weight, was observed between control and oestrogen–

treated birds during the first 12 hours after hormone administration. After 24 hours, however, and up to 4 days after the start of hormone treatment, the liver weight was found to progressively increase, although by the 9th day of treatment the liver growth response was considerably reduced, compared to controls. Treatment of 4-week old pullets with 2 mg testosterone propionate on alternate days over a period of 9 days caused a time-related increase in comb weight, expressed as a proportion of body weight, from the 2nd day until the 9th day. Both treatment with oestradiol dipropionate, as described above, and similar treatment of 4-week old pullets with 2 mg oestradiol dipropionate and 1 mg testosterone propionate together, caused a time-related increase in oviduct weight from 24 hours after hormone treatment until the 9th day of hormone treatment. On the 4th and 9th days of hormone treatment, the oviduct weights (as proportions of body weights) of the birds treated with oestradiol and testosterone were significantly greater than those of birds treated with oestradiol alone, representing an enhancement of oestrogen-induced oviduct growth by testosterone.

Pearce & Balnave (1975) also noted time-related increases in oviduct weight and liver weight of 4-week old pullets treated with varying doses of oestradiol dipropionate, in the range 0.5 - 4 mg hormone/ bird. The weights of individual organs were measured 2 days after a single injection of oestrogen, and 8 days after the start of hormone treatment, during which oestrogen was administered on alternate days. At all dose levels, the liver weight and the oviduct weight were greater after 8 days than after 2 days of hormone treatment.

Similar time-related increases in liver weight have been observed in oestrogen-treated birds of other species. Gibbins & Robinson (1982) treated 3- to 5-month old male Japanese quail with a single

injection of 4.36 mg oestradiol/100 g body weight, and observed a
near doubling of liver weight by the 4th day after treatment.
Subsequently, however, the extent of the response decreased.
Dashti et al. (1983) treated 19-day old male turkeys with a single
injection of diethylstilboestrol (4 mg/100 g body wt.) and observed
a time-related increase in liver weight expressed as a percentage
of body weight, up to 48 hours after injection, followed by a
diminished response at 72 hours after injection.

The DNA content of liver in the present study was used as an
indicator of cell number. Although somatic polyploidy has been
shown to be a common developmental feature of endocrine target tissue,
including vertebrate liver (Brasch, 1980), the assumption was made
that the DNA content of individual liver cells was constant. There-
fore, DNA content of liver expressed as mgDNA/whole liver (Fig. 2.2a)
was used as an indication of the number of cells/liver, whereas
expression of the hepatic DNA content as mgDNA/g liver (Fig. 2.2b)
was assumed to be indicative of individual liver cell mass.
Significant decreases in the DNA content of unit wet weight of liver,
with respect to control birds, were observed from 13 hours after
oestrogen treatment, reaching a minimum value at 24 - 37 hours after
injection. Subsequently, this value returned to the control value
61 hours post-injection (Table 2.2 and Fig. 2.2b). These results
indicate an increase in the mass of individual liver cells during
this period, reaching a maximum of 24 - 37 hours after hormone
treatment. Significant increases in the total hepatic DNA content
were observed in oestrogen-treated chicks, with respect to control
birds, at 37 - 61 hours after injection, the maximum value being
observed at 37 hours post-injection. After this time, the total DNA
content of oestrogen-treated male chick liver decreased somewhat.
(Table 2.2 and Fig. 2.2a). These results indicate an increase in
the number of cells per liver, with respect to controls from 37 - 61

hours after oestrogen treatment with a maximum number of cells apparent at 37 hours after hormone administration. Therefore, the changes in DNA content of unit wet weight of liver, and in the DNA content of whole liver suggest that both cellular hypertrophy, resulting in increased cell mass, and cellular hyperplasia are involved in the increase in liver weight in response to oestrogen treatment, although increased cell division does not appear to play a part during the early stages (0 - 24 hour post-injection) of the response. In addition, the decrease in the total DNA content of livers, and the increase in DNA content of unit wet weight of liver observed in oestrogen-treated birds from 37 - 61 hours after injection suggests that the decrease in liver weight observed during this period is accompanied by a decrease in the total number and the mass of individual liver cells.

A number of investigators have observed changes in the livers of oestrogen-treated domestic fowl which suggest that the response of the liver to the hormone involves both cellular hypertrophy and increased cell division. For example, van den Berg et al. (1974) treated 8- to 12-week old male birds with subcutaneous injections of oestradiol (2.5 mg/100 g body wt.) and observed that the total hepatic DNA content did not change significantly during the first 24 hours after injection. Subsequently, however, a sharp increase in the hepatic DNA content of oestrogen-treated birds was obtained such that 45 hours after hormone treatment the value was 1.37-fold greater than that for control birds. These results suggest that cell division may have played a part in the later stages (24 - 45 hours post-injection) of the liver's response to exogenous oestrogen. Schjeide & Lai (1970) administered large doses of oestrone (1 mg/ 100 g body wt./day) to approximately 8-week old male birds. From 2 - 4 days after the start of the treatment, electronmicrographic analysis of liver from these birds revealed an increase in cell volume, involving a proliferation of total and rough endoplasmic

reticulum, and evidence that approximately 20% of cells were dividing. Similarly, Schjeide et al. (1963) treated male domestic fowl with pharmacodynamic doses of oestrogen and observed that, 7 days after hormone treatment, the liver had increased 50% in total weight. Electronmicrographic analysis of the liver tissue from oestrogen-treated birds revealed increased cell division, many of the cells exhibiting highly developed and engorged endoplasmic reticulum.

In the present study the mean hepatic DNA content of untreated birds was 2.99 mg DNA/g liver and a minimum value of 2.12 mg DNA/g liver was observed 24 - 37 hours after oestrogen treatment. Similar changes in the levels of hepatic DNA in the liver of oestrogen-treated birds have been reported by other investigators. For example, Hawkins & Heald (1966) reported decreases in the DNA content of unit wet weight of liver from both oestrogen-treated female birds and hens as they came into lay. Treatment of 11-week old pullets with 2 mg oestradiol monobenzoate on alternate days for 7 days resulted in a reduction of approximately 33% in the DNA content of a unit weight of liver, from a value of 4.0 mg DNA/g liver to 2.7 mg DNA/g liver. In the same study, the hepatic DNA content of 9- to 16-week old immature hens was found to be 3.46 mg DNA/g liver whereas the mean value for 23- to 32-week old laying hens was 2.50 mg DNA/g liver. Courtney (1984), using 3- to 5-week old male chicks, determined a value of 3.16 mg DNA/g liver for control and untreated birds. Treatment of similar birds with a single injection of 17β-oestradiol (1 mg/100 g body wt.) resulted in a marked decrease in the DNA content of unit weight of liver during the 50-hour period immediately after injection, the lowest individual level of 1.79 mg DNA/g liver being recorded 27 hours post injection.

The possibility that cellular hypertrophy plays an important part in the liver growth response to oestrogen in the present study, is

supported by the following observations:-

(a)   the increase in liver weight was accompanied by a decrease in the
      soluble protein content of unit wet weight of liver, the liver
      weight reaching a maximum when the soluble protein/g liver value
      was at a minimum 37 hours post-injection, and

(b)   the total hepatic DNA content of oestrogen-treated chicks was not
      significantly different from that of control chicks during the first
      24 hours after injection.  As mentioned earlier, the cellular hyper-
      trophy observed by Schjeide et al.  (1963) and Schjeide & Lai (1970)
      in liver from 8-week old roosters treated for up to 7 days with
      oestrone (1 mg/100 g body wt./day) was shown to be caused, in part
      by a proliferation of endoplasmic reticulum.  However, these workers
      also showed that the oestrogen treatment led to the livers of the
      birds becoming more fragile and fatty in appearance and, therefore,
      concluded that the cellular hypertrophy also involved an uptake of
      water and lipid by the cells.  In the present study, a close analysis
      of the hepatic soluble protein data also suggests that enhanced
      water and lipid uptake may be involved, in part, in the liver
      response to oestrogen.  Expression of the hepatic soluble protein
      content as soluble protein/mg DNA was taken to be an indication of
      the cellular soluble protein levels.  This parameter did not
      significantly increase over controls until 24 hours after oestrogen
      treatment, suggesting that the increase in cell mass observed, at
      least at 13 hours and 19 hours after hormone administration, may
      have principally involved an increase in the water and lipid content.
      Such a possibility is further supported by the observation that, in
      the present study, the enlarged livers of oestrogen-treated birds
      were almost invariably pale in colour, and fragile.

Taken as a whole, the observations made during the 61-hour period

immediately following oestrogen treatment, in the present study, indicate that a time-related increase in liver size occurred, reaching a maximum 37 hours post-injection and that this involved an initial increase in liver cell mass, probably as a result of accumulating water and lipid, with a later increase in cell number.

# CHAPTER 3

QUALITATIVE AND QUANTITATIVE CHANGES
IN LIPIDS OF THE LIVER AND PLASMA
OF MALE CHICKS AFTER OESTROGEN TREATMENT

1.  Lipid transport in the blood

    Plasma lipids, other than free fatty acids, are transported as
    macromolecular lipoproteins which consist of a hydrophobic core,
    comprising mainly triacylglycerol and cholesteryl ester, surrounded
    by an amphipathic surface 'coat' of phospholipid, cholesterol and
    specific apoproteins. The apoproteins are important in maintaining
    lipoprotein structure and some have specific regulatory functions
    in lipid and lipoprotein metabolism. In mammals, for example,
    apo-C-II activates lipoprotein lipase (E.C. 3.1.1.34) (La Rosa et al.,
    1970; Havel et al.,1970) and apo-A-I activates lecithin:cholesterol
    acyltransferase (E.C. 2.3.1.43) (Fielding et al.,1972).

    Plasma lipoproteins represent a continuum of lipid-protein complexes
    in which the ratio of lipid to protein, and hence their density,
    varies (Gurr & James, 1980). In mammals, they can be divided into
    groups, according to their density, which include chylomicra, very
    low density lipoprotein (VLDL), low density lipoprotein (LDL) and
    high density lipoprotein (HDL). Chylomicra are involved in the
    transport of dietary lipid from the gut to the body tissues, while
    the latter three classes are involved in the transport of endogenous
    lipid. The principal features of the structure and function of
    human plasma lipoproteins are summarised in Table 3.1.

    In the domestic fowl, which lacks a functional intestinal lymphatic
    system, absorbed dietary lipid enters the portal system as large
    VLDL or 'portomicra' (Bensadoun & Rothfeld, 1972) rather than as
    chylomicra (and VLDL) via the lymphatic system as typically occurs
    in mammals.

    Chicken VLDL (density < 1.006 g/ml) consists predominantly of

.

Table 3.1

Principal features of the structure and function of the major classes of human plasma lipoproteins

| | Chylomicra | VLDL | LDL | HDL |
|---|---|---|---|---|
| Density (g/ml) | < 0.95 | 0.95 - 1.006 | 1.019 - 1.063 | 1.063 - 1.21 |
| Diameter (nm) | 75 - 1000 | 30 - 70 | 20 - 25 | 10 - 15 |
| Components (as % of dry wt.) | | | | |
| Protein | 1 - 2 | 10 | 25 | 50 |
| Triacylglycerol | 83 | 50 | 10 | 3 |
| Cholesterol and cholesteryl esters | 8 | 22 | 43 | 18 |
| Phospholipids | 7 | 18 | 22 | 29 |
| Major apoproteins | B<br>C-I, C-II, C-III | B<br>C-I, C-II, C-III<br>E | B | A-I, A-II |
| Minor apoproteins | A-I, A-II | A-I, A-II<br>D | | C-I, C-II, C-III<br>D<br>E |
| Function | Transport dietary triacyl-glycerol and cholesterol from intestines. | Transport triacyl-glycerol and cholesterol from the liver to various tissues. | Major cholesterol-transporting lipoproteins | Involved in reverse cholesterol transport from peripheral tissues to the liver. |

triacylglycerol (Yu et al.,1976) and is produced mainly in the liver and then exported via the blood to other tissues. The triacylglycerol undergoes lipolysis by the action of lipoprotein lipase, an enzyme located on the luminal surface of endothelial cells of capillaries in body tissues (Scow et al.,1976; Kompiang et al.,1976). The free fatty acids produced can enter the tissues or some can remain in the blood.

Chicken LDL (density 1.006 - 1.063 g/ml), in comparison with chicken VLDL, has a decreased content of triacylglycerol and increased proportions of other components (Yu et al.,1976). Chicken HDL (density 1.063 - 1.21 g/ml) is produced in the liver and consists mainly of protein with phospholipid being the next most abundant constituent (Kruski et al.,1975; Evans et al.,1977).

Although the lipoproteins have been presented above as distinct fractions, it must be remembered that within each fraction there is a range of densities, and that fractions are structurally and functionally inter-related, the whole being regarded as a dynamic system. The transfer of lipid and apoprotein components from one fraction to another has been shown to take place and the production of some lipoproteins relies on the use of catabolic products (remnants) of others. (For reviews see Smith et al.,1978a; Havel et al.,1980; Mahley et al.,1984). For example, LDL is formed from remnants of VLDL in many animals.

Free fatty acids are normally transported in the avian blood mainly bound to albumin (Butler, 1971) although they are capable of binding to other blood components such as triacylglycerol-rich lipoproteins, a fact which becomes important at the onset of egg-laying in hens, (Leclercq et al.,1979) when there is a large increase in the concentration of such lipoproteins.

2.    Alterations in the metabolism and transport of lipid in laying hens
and oestrogen-treated birds.

At the onset of egg-laying the liver assumes the role of synthesising
the lipids and proteins required by the developing egg (Heald &
Badman, 1963; Hawkins & Heald, 1966). Marked elevations in the
plasma levels of lipid and egg-yolk proteins are observed during the
fourteen days prior to the onset of laying, when the plasma lipid
level can rise to 10 - 14 g/100 ml plasma compared to 0.2 - 0.5 g/
100ml plasma in immature hens. At the time the first egg is laid
the level of total plasma lipid decreases to 1.5 - 3 g/100 ml (Heald
& Rookledge, 1964)

Walker et al. (1961) reported that 70% - 80% of the lipaemia was
caused by accumulating triacylglycerol, the remainder being
phospholipids together with a smaller quantity of cholesterol.
These large increases in lipids were shown to be predominantly
associated with VLDL and, to a lesser extent, LDL (Hillyard et al.,
1972; Chapman et al,,1977).

Oestrogen-treatment of male and immature female domestic fowl has
been shown to lead to an accumulation of plasma lipids and proteins
similar to that observed in laying hens and it is possible that
similar metabolic processes are involved (Lorenz, 1954; Schjeide
et al.,1963; Bergink et al,,1974; Chan et al.,1976). The bulk of
the plasma lipids constituting the lipaemia in oestrogen-treated
domestic fowl are triacylglycerols, predominantly in the form of
VLDL (Kudzma et al.,1973, 1975, 1979; Luskey et al.,1974; Chan et
al.,1976, 1977). It has been shown that oestrogen-treatment of
immature female domestic fowl results in a dramatic increase in the
plasma levels of VLDL (400-fold) with a lesser increase in LDL
(70-fold) and a marked decrease in HDL (Kudzma et al.,1979).
Similar alterations in lipoprotein levels have been observed at the

onset of egg-laying in hens involving an increase in the levels of VLDL (200-fold) and an approximately 50% decrease in HDL (Yu et al., 1976). The accumulation of triacylglycerol in the plasma of oestrogen-treated birds may be due to an increase in the rate of hepatic production of VLDL or a decrease in the removal of VLDL from the blood, or a combination of these effects. Oestrogen treatment has been shown to increase the rate of synthesis of VLDL in avian liver (Luskey et al.,1974; Kudzma et al.,1975). In addition, it has been reported that the fractional turnover rate of VLDL-triacylglycerol in oestrogen-treated hyperlipaemic birds was substantially less than that in untreated birds, despite the fact that the total turnover rate was nearly 50-fold greater (Kudzma et al.,1975). Furthermore, Bacon et al. (1978) noted a decreased rate of clearance of VLDL-triacylglycerol from the plasma of laying hens.

These studies suggest that oestrogen causes increased VLDL production in avian liver, leading to a massive increase in plasma VLDL levels. Under these circumstances, despite the reported increase in total turnover rate of plasma VLDL, the magnitude of this lipaemia appears to be so great that it exceeds the capacity of the organism to remove lipid from the plasma.

As well as changes in the quantities of lipoproteins in the plasma, egg-laying or oestrogen treatment has been shown to cause a number of compositional changes in the lipoprotein fractions. Elevated triacylglycerol and phospholipid and diminished cholesterol contents of VLDL in laying hens have been observed, when compared with non-laying birds (Yu et al.,1976; Evans et al.,1977). Griffin et al. (1984) suggested that such lipid compositional changes in VLDL in laying hens are primarily due to oestrogen-induced changes in the pattern of lipid synthesis. Kudzma et al. (1979) reported that LDL

and HDL in the plasma of oestrogen-treated sexually immature hens were enriched with triacylglycerol while the proportion of cholesterol in all lipoprotein fractions decreased.

With the onset of egg-laying the size of VLDL particles diminishes (Chapman et al.,1977) and the LDL particles are larger (Hillyard et al.,1972; Chapman et al.,1977) compared to non-laying birds. In oestrogen-treated non-laying hens Kudzma et al. (1979) found that lipoproteins of density less than 1.060 g/ml could not be grouped clearly into discrete VLDL and LDL fractions on the basis of discontinuous density distributions. Instead, these lipoproteins possessed a continuous spectrum of densities, up to 1.060 g/ml. Analysis of the apoprotein composition of these lipoproteins showed that plasma VLDL from non-laying birds contains at least six apoproteins whereas only two major apoproteins are present in VLDL from oestrogen-treated birds, a high molecular weight component. ($M_r$ 250,000) which also predominates in the untreated state, and a low molecular weight apoprotein ($M_r$ 7,000). The latter component is normally only present, if at all, in very small amounts in untreated birds and it has been called apo-VLDL-II by Jackson et al. (1977). Kudzma et al. (1979) have also shown that, in the untreated state, the apoprotein composition of VLDL and LDL were dissimilar whereas, after oestrogen treatment, they were indistinguishable. Furthermore, the lipid composition of VLDL and LDL were similar after oestrogen-treatment. These observations, therefore, suggest that in oestrogen-treated birds, the 'VLDL' and 'LDL' represent a single lipoprotein class, with essentially similar lipid and apoprotein composition, which exhibits a continuous spectrum of density, up to 1.060 g/ml.

Several investigators have demonstrated an increased production of the apoproteins of VLDL and LDL in oestrogen-treated birds (Chan et al.,1976, 1978, 1980; Kudzma et al.,1979; Lin & Chan, 1980). The

increase in apo-VLDL-II synthesis is due to increased concentration of hepatic mRNA coding for the apoprotein (Chan et al.,1978, 1980; Codina-Salada et al.,1983) which is translated on membrane-bound ribosomes before association with lipid, in the cisternae of endoplasmic reticulum and Golgi apparatus, and eventual release into the plasma (Lin & Chan, 1982; Higgins & Hutson, 1984).

The differences, both quantitative and qualitative, between plasma lipoproteins (mainly VLDL) from laying hens or oestrogen-treated birds and non-laying untreated birds are believed to be a reflection of the fact that the laying hen synthesises specialised lipoproteins (including vitellogenin) for transfer to the yolk in developing oocytes (Christie & Moore, 1971; Griffin et al.,1984). This is strongly supported by observations, amongst others that (a) the size distribution of plasma lipoproteins is similar to that of yolk lipoproteins (Bacon et al.,1973), (b) lipid and fatty acid compositions of plasma and yolk triacylglycerol-rich lipoproteins are similar (Christie & Moore, 1972) and (c) the structure of plasma apo-VLDL-II (Jackson et al.,1977) appears to be the same as that reported for apo-vitelline I of yolk (Dopheide & Inglis, 1976). Fujii et al. (1984) detected a marked alteration in the percentage contributions of particular fatty acids in both liver and plasma lipids of the hen when approaching lay. In particular, oleate (18:1) increased and decreases were observed in stearate (18:0) and arachidonate (20:4). The altered fatty acid compositions of the plasma and liver lipids of the laying hen were similar to that of yolk lipid suggesting that lipids of a particular composition are being transported to the developing egg. Furthermore, oestrogen-induced changes in the fatty acid composition of liver lipids in immature pullets have been reported which are similar, in some respects, to those occurring in the laying hen (Balnave & Pearce, 1975; Pearce & Balnave, 1975), with increases in the percentages of

palmitate (16:0), palmiteolate (16:1) and oleate (18:1) and decreases
in those of stearate (18:0) and linoleate (18:2). However, the
resultant fatty acid composition of liver lipids from treated birds
was seen to be intermediate between the untreated, immature and
the mature, egg-laying pattern (Pearce & Balnave, 1975), and the
percentage of palmitate (16:0) in plasma and liver lipids in
oestrogen-treated pullets was substantially greater than that in
the mature laying hen (Balnave, 1971). Clearly, oestrogen plays a
significant role in the changes observed in the fatty acid
compositions of lipids as the hen approaches lay. That such changes
may not be reproduced exactly by exogenous oestrogen administration
is to be expected since, undoubtedly, the physiological state of the
laying hen is not determined solely by oestrogen and other ovarian
and endocrine secretions may exert an influence (Balnave, 1969;
Balnave & Pearce, 1974). It must also be remembered that the egg-
laying process itself, involving the removal of lipid from the
body, may exert a significant physiological effect which is
obviously lacking in oestrogen-treated birds.

The liver is the main site of lipogenesis in avian species (Leveille
et al., 1968; O'Hea & Leveille, 1969; Brady et al., 1976) and is also
the main site of production of lipoproteins such as VLDL. Using
the technique of functional hepatectomy, Ranney & Chaikoff (1951)
demonstrated that the liver was responsible for the development of
lipaemia in the oestrogen-treated cockerel. After oestrogen
treatment of male and immature female birds, and at the onset of
lay in the mature hen, there is an increase in the total lipid
content of the liver, particularly triacylglycerols, which is
associated mainly with LDL and VLDL (Pearce, 1971; Pearce & Brown,
1971; Balnave & Pearce, 1974; Kudzma et al., 1975; Pearce & Balnave,
1975). In such conditions, there is a proliferation of the
endoplasmic reticulum and the Golgi apparatus together with an

increase in the number of associated ribosomes and mitochondria (Schjeide et al.,1963, 1974: Tarlow et al.,1977). Consequently, in the early stages of the transformation of hepatic activity induced by oestrogen treatment or egg-laying, much of the lipid and protein synthesised would appear to be used for membrane biosynthesis so increasing the cellular capacity for the subsequent production of proteins and lipids for export to the developing oocyte.

In most mammals, plasma free fatty acids are important precursors of plasma lipids which are manufactured in the liver (Havel, 1961). In the domestic fowl, however, the liver is a most active site of de novo fatty acid synthesis (Leveille et al.,1968). Attempts have been made to correlate changes in hepatic lipogenic activity, both from non-lipid precursors and pre-formed fatty acids, with the increased levels of liver and plasma lipids in the laying hen and oestrogen-treated bird. Studies using liver slices and in vivo techniques have shown that the liver of the oestrogen-treated bird has an enhanced capacity to synthesise lipids, de novo, from non-lipid precursors (Kudzma et al.,1973, 1975: Dashti et al.,1983) and Courtney (1984) has shown that in oestrogen-treated immature male chicks, the increase was apparent $3\frac{1}{2}$ - $7\frac{1}{4}$ hours after injection, although a statistically significant increase did not occur until 14 - 17 hours post-injection. However, studies of liver slices from laying hens have indicated a lower level of hepatic lipid synthesis de novo when compared to immature, non-laying, female birds (Weiss et al.,1967; Duncan, 1968, 1970). Investigations in vitro, designed to measure the capacity of the liver to incorporate pre-formed fatty acids into complex lipids, have shown increased hepatic capacities in both oestrogen-treated birds and laying hens (Hawkins & Heald, 1966; Duncan, 1970; Kudzma et al., 1975; Courtney, 1984).

At this point, it is of interest to note that elevated free fatty acid levels have been observed in the plasma of laying hens (Heald & Badman, 1963; Heald & Rookledge, 1964) and oestrogen-treated birds (Kudzma et al.,1973, 1975) when compared to non-laying, untreated birds. An understanding of the origin of the raised plasma free fatty acid concentrations is an important step in an evaluation of the relative roles of hepatic fatty acid synthesis de novo and hepatic lipogenesis from pre-formed fatty acids. The free fatty acids may have been released by the lipolysis of triacylglycerol in adipocytes or have been synthesised de novo at extra-hepatic sites. Another possibility, however, is that they may have been released from VLDL-triacylglycerol circulating in the blood, by the action of lipoprotein lipase in peripheral tissue. A detailed knowledge, therefore, of the time sequence of the increased plasma triacylglycerol content and the free fatty acid content of the plasma of laying hens and oestrogen-treated birds might provide information about the early events occurring in the elevated hepatic production of lipids. For example, if the increase in plasma free fatty acids occurs before the increase in plasma triacylglycerol this might suggest that the fatty aicds originate from extra-hepatic sources and are being transported to the liver for incorporation into complex lipids. Conversely, if the free fatty acid level increases after the appearance of increased levels of plasma triacylglycerol, this might imply that the liver was the site of origin of these fatty acids, implicating fatty acid synthesis de novo. Kudzma et al. (1973) reported that the rise in plasma free fatty acid levels did not occur until after the oestrogen-induced hypertriacylglycerolaemia in young chicks was well established and suggested, therefore, that the over-production of triacylglycerol by the liver was not dependent upon fatty acids arriving from extra-hepatic sources. It must be noted at this stage, however, that an increase in plasma free fatty acid levels

was observed in control birds. Pageaux et al. (1981), working with oestrogen-treated immature female quail, observed a decrease in plasma free fatty acid levels within one hour of injection, followed by a subsequent increase in concentration suggesting that initially, at least, the liver may have removed free fatty acids from the plasma.

The aim of the work presented in this chapter was to produce (a) a detailed account of the changes occurring in the levels of phospholipid, triacylglycerol and free fatty acids in the liver and blood of oestrogen-treated immature male chicks and (b) a description of the changes in fatty acid compositions of particular liver and plasma lipids in similarly treated birds. A detailed time-course of changes was determined in an effort to elucidate some of the physiological responses to oestrogen treatment and to correlate these changes with other observations in this thesis and elswehere.

METHODS

1. Animal housing

The birds were housed as described in Chapter 2, and used in
experiments when aged between 3 and 5 weeks. Oestrogen-treated
chicks were injected intramuscularly with 17β-oestradiol (0.75mg/
100 g body wt.) dissolved in propane-1,2-diol. Control birds
received an equivalent volume of propane-1,2-diol only.

2. Killing of animals/Preparation of plasma

Birds were killed between 11.00 hrs and 13.00 hrs in order to
minimise the effects of possible diurnal variations in behaviour
and physiology. The birds were weighed and then killed by
decapitation and the livers were quickly excised, weighed, rapidly
frozen in liquid nitrogen and then stored at -20°C for future assays.
Blood was collected from the severed necks of the birds into glass
scintillation vials which contained 1 ml of 127 mM-NaCl, 10 mg/ml
EDTA, pH 7.4. About 5 ml of blood was routinely collected and mixed
with the anti-coagulant to give a final concentration of about
2 mg EDTA/ml of blood. Plasma was separated by low-speed
centrifugation at 4°C, the volume measured and some aliquots were
stored frozen for future assays while other aliquots were used
immediately for the isolation of lipoproteins.

3. Isolation of a Very Low Density Lipoprotein fraction

Lipoproteins of a density less than 1.006 g/ml were isolated from
whole plasma by the ultracentrifugal flotation method described by
Havel et al.(1955). Aliquots of plasma (1 - 3 ml depending on the
experimental treatment of the bird) were carefully overlaid with

KBr (density = 1.006 g/ml), to give a final volume of 5 ml.
Following centrifugation at 125,000 g for 16 h at 4°C in a swing-out
rotor (r$_{av.}$ = 8.18 cm) lipoproteins of density less than 1.006 g/ml
were recovered from the top of centrifuge tubes using a pasteur
pipette. Random checks indicated that no triacylglycerol remained
in the clear infranatant. Lipid extractions were performed on the
VLDL (density <1.006 g/ml) fractions.

4.   Lipid extraction procedure

Lipids were extracted from whole plasma, VLDL fractions and from
liver by the rapid method described by Bligh & Dyer (1959). Whole
plasma was diluted with 154 mM-NaCl to an extent dependent upon the
experimental treatment of the bird. Diluted plasma (1.6 ml) was
mixed thoroughly with 6 ml of chloroform/methanol (1:2,v/v).
Subsequent additions of 2 ml chloroform and 2 ml distilled water
were each followed by thorough mixing and, following low-speed
centrifugation, the bottom phase, consisting of lipid dissolved in
chloroform, was removed and used for assays of free fatty acids,
phospholipid and triacylglycerol. Lipid extractions of VLDL
aqueous samples involved a similar procedure, the only difference
being that either 1.6 ml or 3.2 ml of aqueous sample were used.
Aliquots of the lipid extracts from VLDL were used subsequently in
lipid assay procedures and thin-layer chromatography.

Lipid extraction of liver involved the homogenisation of liver
samples and an aliquot of 154 mM-NaCl calculated to yield 2.4 ml
aqueous phase, with 9 ml of chloroform/methanol (1:2,v/v) using a
teflon-glass homogeniser. The tissue was disrupted with 10 'up and
down' strokes of the rotating pestle. The extraction was completed
by the addition of chloroform (3 ml) and water (3 ml). After mixing,
the phases were separated by centrifugation and aliquots of the

lipid extract were used in lipid assay procedures and thin-layer chromotography. In the production of lipid extracts which would be used for analysis of fatty acid composition, all solvents contained 0.005% (w/v) BHT. Lipid extracts not used immediately were stored under nitrogen, in sealed glass containers, in the dark at -20°C for a maximum of 14 days.

5.   Assay of triacylglycerol

Triacylglycerols were measured using a slight modification of the Sigma Diagnostic Kit procedure described in the Sigma Technical Bulletin No. 405 (1978). Aliquots of lipid extract, in chloroform, were transferred to vials and evaporated to dryness under a stream of nitrogen. Isopropanol (5 ml), distilled water (0.2 ml) and activated alumina (0.8 ± 0.2 g), an adsorbant used for the removal of interfering substances, were added and the vials shaken for 5 minutes. Blank and standard assays were included along with duplicate test assays. Standard assays contained 4.8 ml isopropanol, 0.2 ml distilled water and 0.2 ml triolein standard (300 mg triolein/ 100 ml isopropanol). Following low-speed centrifugation at 4°C, an aliquot (2 ml) of the clear supernatant was transferred to a clean test tube and 0.5 ml 1M-KOH was added with mixing. Each tube was incubated for 5 minutes at 60°C causing the release of glycerol by saponification of the triacylglycerol. Periodate solution (0.5 ml), prepared by dissolving 125 mg sodium m-periodate in 50 ml 2M-acetic acid, was added to the cooled contents of each tube, with mixing. After exactly 10 minutes at room temperature, during which time the glycerol was oxidised to formaldehyde, 3 ml of a chromogenic reagent was added, with mixing, and the tubes capped with aluminium foil and incubated for 30 minutes at 60°C. The chromogenic reagent was prepared by mixing 2M-ammonium acetate (20 ml), isopropanol (40 ml) and acetylacetone (0.15 ml). The prepared reagent was kept for at

least one day before use, since aging was required to obtain maximum colour in the final reaction in which yellow diacetyldihydrolutidine was formed.

$$Formaldehyde + NH_4^+ + Acetylacetone \longrightarrow Diacetyldihydrolutidine.$$

The absorbance of the cooled solution was measured at 410 nm and readings were completed within 20 minutes of the end of the incubation period.

A standard curve was prepared, using the triolein standard of 300 mg/100 ml isopropanol, and was linear up to 0.75 mg triacyl-glycerol. The chromogenic reagent and periodate solution were stored in the dark at 4°C and replaced each month.

6.  Assay of phospholipid

Phospholipid estimations were carried out by the method of Raheja et al (1973). Aliquots of lipid extract, in duplicate, were evaporated to dryness under a stream of nitrogen in thick-walled tubes. Duplicate blank and standard assays were included. Standard assays were performed by evaporating to dryness 1.0 ml of 0.17 mM-dipalmitoyl phosphatidycholine (chloroform solvent). Chloroform (0.5 ml) and chromogenic reagent (0.2 ml) were added and the contents mixed. Each tube was heated in a boiling water bath for exactly 3 minutes, then cooled and 3 ml of chloroform added. The contents were mixed thoroughly and centrifuged at low speed for 5 minutes at room temperature. The absorbance of the lower chloroform layer was measured at 716 nm. A standard curve was prepared using dipalmitoyl phosphatidylcholine (0.17 mM) in chloroform as a standard. The assay was linear in the range of 0 - 0.3 µ moles of phospholipid.

Preparation of the chromogenic reagent involved, firstly, dissolving
ammonium molybdate (8 g) in distilled water (60 ml) and, after
adding a few drops of concentrated hydrochloric acid to ensure
complete dissolution, this solution was used to prepare two further
solutions:

Solution 1

Redistilled mercury (5 g) was added to a mixture of 20 ml of
concentrated hydrochloric acid and 40 ml of the ammonium molybdate
solution.  The mixture was stirred on a magnetic stirrer for
45 minutes, then filtered to produce a red-brown filtrate.

Solution 2  (Acid molybdate)

This was prepared by carefully adding 100 ml of concentrated
sulphuric acid to 20 ml of the ammonium molybdate solution.

The final chromogenic reagent was prepared in a fume cupboard by
very carefully adding Solution 2 to Solution 1 with stirring at 0°C.
The resultant dark green solution (25 volumes) was mixed with
methanol (45 volumes), chloroform (5 volumes) and distilled water
(20 volumes).  The chromogenic reagent was stored in the dark at
4°C for 6 months, then replaced.

7.    Assay of free fatty acids

Total free fatty acids in plasma and liver samples and albumin-bound
free fatty acids in plasma were determined, using two separate
procedures.

(a) Total free fatty acids in plasma and liver

A modification of the method described by Antonis (1965) was used.
This method is based on the production of the copper salt of the
fatty acids in chloroform followed by estimation of the copper
content of the organic phase by reaction with zinc dibenzyldithio-
carbamate. Silicic acid (1.2 ± 0.1 g) was slurried with 7.5 ml
diisopropyl ether, in stoppered tubes. The ether had been passed
through a column of activated alumina just before use to remove
peroxides. Plasma (0.5 ml) was added and the mixture was well
mixed for 1.5 minutes. The silic acid adsorbant removed phospholipids,
which were a source of interference, without affecting the levels of
free fatty acids. Following low-speed centrifugation for 2 minutes
to precipitate the silic acid, 5 ml of the ether supernatant was
removed and transferred to glass centrifuge tubes which had
previously been 'siliconised' using dimethyldichlorosilane (0.5%,v/v)
in heptane. The ether was evaporated to dryness under a stream of
nitrogen and the lipid redissolved in 5 ml chloroform. 'Copper
reagent' (2.5 ml), consisting of 3.8% (w/v) copper sulphate,
pentahydrate, 0.45 M-triethanolamine and 0.05 M-acetic acid, in
final concentrations, was added and mixed thoroughly with the lipid
extracts. Each tube was then centrifuged at low speed for 10
minutes and care was taken to remove all of the excess upper aqueous
copper phase by aspiration with a pasteur pipette. A 3 ml aliquot
of the chloroform extract was placed into a clean tube and colour
was developed by the addition of 0.5 ml of zinc dibenzyldithio-
carbamate (0.3%, w/v) in chloroform. The absorbance of each tube
was measured, after 15 minutes at room temperature, at 440 nm.

A similar procedure was adopted for the measurement of free fatty
acids in liver samples, the only difference being that aliquots
(1.5 - 2.0 ml) of liver lipid extract in chloroform were evaporated
to dryness under a stream of nitrogen in stoppered tubes prior to

the addition of diisopropyl ether and silicic acid.

Standard curves were produced using 2 mM-sodium palmitate in Krebs
Ringer phosphate buffer, pH 7.4 (Dawson, 1969), containing 3% (w/v)
BSA (fatty acid-free). Assays were performed in duplicate, blank
assays and standard assays being included in each series of assays.
The sodium palmitate was prepared by the saponification of 25.6 mg
palmitic acid by 1.1 ml of 0.1 M-NaOH at 60°C prior to dilution to
50 ml with Krebs Ringer phosphate buffer, pH 7.4, containing 3% (w/v)
BSA, and was stored frozen. The 'copper reagent' and zinc
dibenzyldithiocarbamate (0.3%, w/v) in chloroform were stored in the
dark at 4°C and replaced every 2 months.

## (b) Albumin-bound free fatty acids

The levels of free fatty acids bound to albumin in the plasma were
determined using the method of Griffin & Mitchell (1984).
Triacylglycerol-rich lipoproteins were selectively precipitated
from aliquots (0.5 - 0.7 ml) of plasma in plastic minifuge tubes by
dilution with an equal volume of 0.2% (w/v) dextran sulphate in
200 mM-magnesium chloride, 10 mM-Tris/HCL, pH 7.4 (at 25°C). After
20 minutes at room temperature, the precipated lipoproteins were
pelleted by centrifugation at 1000 $g$ for 10 minutes. Highly
lipaemic plasma samples required 2 volumes of dextran sulphate/$Mg^{2+}$
reagent to cause complete sedimentation. Albumin-bound free fatty
acids in the supernatant were then assayed using the very sensitive
method of Itaya (1977). Aliquots (0.6 ml) of each supernatant were
mixed thoroughly, for 1.5 minutes, with 0.65 ml of 1M-sodium
phosphate, pH 6.5, and 5 ml chloroform in stoppered tubes. This
process removed phospholipids. After centrifugation at low speed
for 5 minutes, the chloroform phase was mixed with 2.5 ml of the
'copper reagent' (3.8% (w/v) $CuSO_4$, $5H_2O$, 0.45 M-triethanolamine,
0.05 M-acetic acid) in 'siliconised' glass centrifuge tubes. Excess

aqueous 'copper reagent' was removed by aspiration with a pasteur pipette and 3 ml of the lower chloroform was removed to a clean tube. Colour was developed by the addition of 0.75 ml of a 0.1% (w/v) solution of a mixture of diphenylcarbazone and diphenylcarbazide (5:95) in methanol. The absorbance was measured at 550 nm.

Standard curves were constructed using 0.2 mM-sodium palmitate in Krebs Ringer phosphate buffer, pH 7.4 (Dawson, 1969), containing 3% (w/v) BSA (fatty acid-free). Assays were performed in duplicate, blank assays and standard assays being included in each series of assays.

8. Assay of DNA

The DNA content of liver tissue was determined by the method described in Chapter 2.

9. Thin-layer chromatographic separation of lipids

Lipid extracts from VLDL fractions and liver samples were evaporated to dryness under nitrogen, dissolved in a small volume of chloroform, and aliquots (40 - 50 µl) applied as 2 cm 'streaks' to activated plates of Kieselgel 60 H. The solvent system used to separate lipid classes was light petroleum (b.p. 40° - 60°C)/diethyl ether/acetic acid (60:40:1, by vol.). Plates were run in chromatography tanks which had previously been 'flushed' with nitrogen just before use, and the solvent system contained 0.005% (w/v) BHT. These precautions were adopted to minimise oxidation of fatty acids. Authentic neutral lipid standards and oleic acid were run on the same plates. After development and drying, the separated lipid standards were visualised by exposure of the appropriate portion of each plate to iodine vapour. Silica gel was scraped off the non-exposed portions of the plates from those areas corresponding to the

positions of lipid standards. Scraped plates were later fully
exposed to iodine vapour to check that the correct areas had indeed
been scraped. The scrapings were collected into small bijou bottles,
flushed carefully with nitrogen and sealed tightly. Bottles were
stored in the dark at -20°C for up to 10 days before being used for
g.l.c. analysis.

10. Gas-liquid chromatography analysis of the fatty acid composition
    of lipid classes from VLDL and liver

(a) Preparation of fatty acid methyl esters.

Fatty acid methyl esters were prepared by a modification of the method
of Morrison & Smith (1964). For phospholipid samples, sufficient
boron trifluoride (14%, w/v) in methanol (approx. 0.3 ml) was added
to the bijou bottles to 'wet' the silica gel samples. 'Tuf-bond'
teflon seals were placed in the screw caps before the bottles were
sealed tightly and heated at 100°C for 15 minutes. For triacylglycerol
samples, methanolysis was achieved by the addition of 1 ml of a
mixture of boron trifluoride (14%, w/v) in methanol/hexane/methanol
(25:20:55, by vol.) to the silica gel samples followed by heating at
100°C for 30 minutes. After cooling the bottles, a stream of
nitrogen was passed over the samples to remove the bulk of the low
boiling point contaminants and to volatilise residual boron
trifluoride reagent. Fatty acid methyl esters were eluted from the
dry samples with n-hexane and the silica gel was removed by
filtering each suspension through a glass-wool plugged pasteur
pipettes. The filtrates were then evaporated, under a stream of
nitrogen, to minimal volumes (5 - 10 $\mu$l) and aliquots injected onto
the g.l.c. column.

(b) Gas-liquid chromatography of fatty acid methyl esters

Separation was carried out using a Shimadzu GC-9A Series gas

chromatograph. The glass columns used were 2 m long with a 2 mm internal diameter and 6 mm external diameter and were obtained from JJ (Chromatography) Ltd., King's Lynn. They were packed with a cyano-silicone stationary phase, 10% Alltech CS-5, on a chromasorb WAW 100 - 120 mesh support. Nitrogen was used as the carrier gas and the resolved components were detected by a flame ionisation combustion system (hydrogen/air). The detector was connected to a Trilab 2 computing integrator with graphics, supplied by Trivector Scientific Ltd., which calculated the area of individual peaks and the percentage contribution of each peak to the total peak area. Since peak area is directly related to the mass of resolved component, percentage area contributions were an indication of the percentage mass contribution of individual fatty acids to total fatty acid mass. The detector responded in direct proportion to the load of sample applied over the range $10^{-8}$ g to $10^{-4}$ g pentadecanoate methyl ester (Fig. 3.1).

A preliminary experiment, in which column efficiency (number of theoretical plates) and resolution of peaks were determined over a range of carrier gas flow rates, indicated that a flow rate of 50 ml/minute, measured at the inlet side of the column, was optimal (Fig. 3.1). The number of theoretical plates, which is a measure of the relative sharpness of a peak (Schupp, 1968), was calculated as follows:-

$$\text{No. theoretical plates} = 16 \left[ \frac{Rt}{W} \right]^2$$

where Rt = the retention time (in min.) of the resolved solute.
      W  = the peak width (in min.) at the base line cut by tangents
           of the slopes of the peak.

The resolution of two component peaks, palmitate (16:0) and

Figure 3.1

(a) The effect of carrier gas flow rate on the column efficiency
(no. of theoretical plates) and the resolution of separation
during gas-liquid chromatographic analysis.

Columns were packed as described in the Methods section and run
isothermally at 210°C.

Column efficiency at each carrier gas flow rate was calculated,
as described in the text, from data obtained after injecting
palmitate (16:0) methyl ester on to the column.  Resolution at
each carrier gas flow rate was calculated, as described in the
Methods section, from data obtained after injecting palmitate
(16:0) methyl ester and palmitoleate (16:1) methyl ester on to
the column.

● values for column efficiency.
○ values for resolution.

(b) Calibration curve of the magnitude of the g.l.c. detector
response with varying amounts of pentadecanoate (15:0) methyl
ester applied to the column.

Columns were packed as described in the Methods section and run
isothermally at 210°C.

For each particular mass of pentadecanoate (15:0) methyl ester
applied to the column, the magnitude of the detector response
was recorded.

Each value plotted represents the mean of 3 recordings.

a.



b.
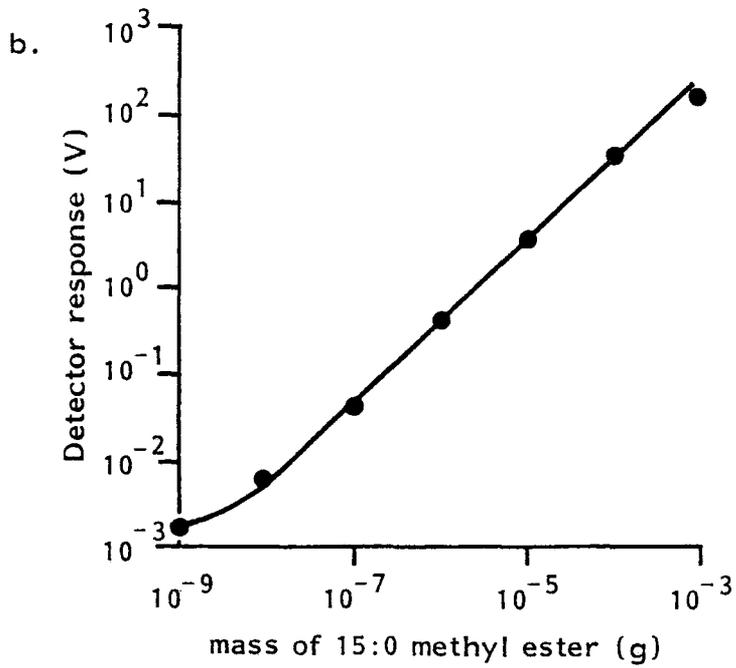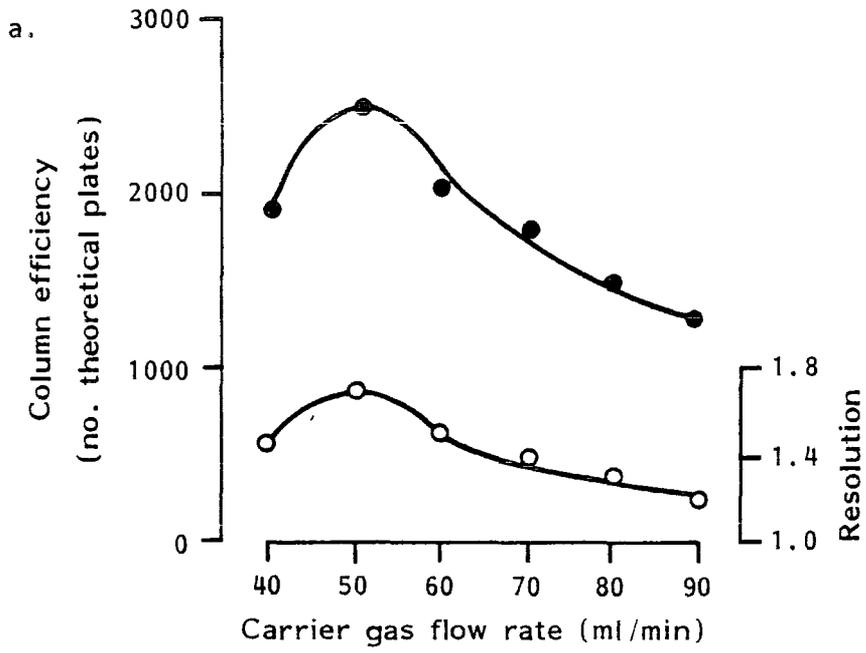
palmitoleate (16:1), was calculated using the equation:-

Resolution = $\dfrac{2\ \triangle Rt}{(W_1 + W_2)}$

where $\triangle Rt$ = the difference between the retention times (in min.)
of the two components
$W_1$ and $W_2$ are the peak widths (in min.) at the base line cut
by tangents of the slopes of the peak.

A carrier gas flow rate of 55 ml/minute was adopted, being a
compromise between the optimal flow rate (50 ml/minute) and higher
flow rates which reduce analysis time. The two columns (one
reference) were run in a temperature program, with one temperature
'ramp', as follows:-

Initial temperature = 210°C
Initial time         = 10 min.
Program rate         = 4°C/min.
Final temperature    = 250°C
Final time           = 7 min.

Peaks were identified by comparison of their retention times with
those of the following authentic fatty acid methyl ester standards:
12:0, 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1,
20:2, 20:3, 20:4, 22:0, 22:4, 22:6, 24:0.

11.   Statistical analysis

Data were expressed as the mean value ± standard error of the mean
and were analysed statistically by Student's 't' test, levels of
statistical significance being indicated where appropriate.
Probability values (p) of 0.05 or less were taken to be significant.

RESULTS

1. Changes in lipid levels in plasma following oestrogen treatment

The administration of 17β-oestradiol (0.75 mg/100 g body wt.) to
young male chicks caused a substantial and prolonged increase in the
concentration of triacylglycerol (Fig. 3.2a) and phospholipid
(Fig. 3.2b) in the plasma, when compared with control birds.
Significant increases in the concentration of triacylglycerol
($p < 0.001$) and phospholipid ($p < 0.05$) were first observed at 7 hours
following injection. At 4 hours after oestrogen treatment both
triacylglycerol and phospholipid levels were greater than control
values but these differences were not statistically significant.
From 7 - 44 hours after injection there were continuous increases in
triacylglycerols and phospholipids when compared to controls, such
that the greatest levels were observed 44 hours after injection. At
this time, 26.7-fold and 4.8-fold increases over corresponding control
values were observed for triacylglycerols and phospholipids
respectively.

The mean concentration of total free fatty acids in the plasma of
oestrogen-treated birds was significantly lower than the control
value ($p < 0.01$) at 2 hours after injection (Fig. 3.2c). At this time,
the control value was 2.06-fold greater than the oestrogen-treated
value and 2.00-fold greater than the untreated (zero-time) value while
the oestrogen-treated and untreated values were not significantly
different from each other. Free fatty acid concentrations in the
plasma of oestrogen-treated and control birds were not significantly
different at all time-points subsequent to 2 hours.

2. Changes in lipid levels of VLDL and in levels of albumin-bound free
   fatty acids in the plasma following oestrogen treatment

-81-

Table 3.2

The effect of a single dose of 17β-oestradiol (0.75 mg in propane-1,2-diol/100 g body wt.), or of propane-1,2-diol only, on the total plasma concentrations of triacylglycerol (TG), phospholipid (PL) and free fatty acids (FFA) at varying times after injection.

Each oestrogen-treated chick received a single intramuscular injection of 0.75 mg 17β-oestradiol in propane-1,2-diol/100 g body weight.  Control chicks received an equivalent volume of propane-1,2-diol only.  At the indicated times after injection, chicks were sacrificed and plasma collected as described in the Methods section.  The triacylglycerol, phospholipid and free fatty acid contents of the plasma were determined by the procedures described in the text.  Chicks were aged $3\frac{1}{2}$ - 5 weeks.

OE = values for oestrogen-treated chicks.

C = values for control chicks.

Values are the means ($\pm$ S.E.M.) of 3 - 8 birds; the number of birds involved in each group is given in parentheses.

Data were analysed statistically by Student's 't' test.  Levels of statistical significance indicated without parentheses are for oestrogen-treated values with respect to corresponding control values. Levels of statistical significance indicated within parentheses, are for that particular value with respect to the corresponding untreated value.

\* significant at $p < 0.05$

\# significant at $p < 0.01$

¥ significant at $p < 0.001$

The lipid concentration data are illustrated graphically in Fig. 3.2.

Time after injection (h)

| | 0 | 2 | | 4 | | 7 | | 11 | | 15 | | 20 | | 26 | | 34 | | 44 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | OE | C | OE | C | OE | C | OE | C | OE | C | OE | C | OE | C | OE | C | OE | C |
| TG in plasma (mg/100 ml) | 126 ±9 (8) | 124 ±5 (5) | 117 ±7 (5) | 165 ±16 (4) | 124 ±14 (4) | ¥ 300 ±8 (4) | 123 ±9 (4) | ¥ 430 ±22 (5) | 116 ±9 (4) | ¥ 654 ±21 (4) | 100 ±3 (4) | ¥ 977 ±50 (5) | 115 ±16 (5) | ¥ 1259 ±90 (5) | 119 ±9 (5) | ¥ 2445 ±182 (5) | 108 ±21 (4) | ¥ 2727 ±363 (5) | 102 ±2 (4) |
| PL in plasma (mM) | 3.51 ±0.28 (8) | 3.00 ±0.38 (5) | 3.67 ±0.49 (5) | 3.68 ±0.41 (4) | 2.97 ±0.65 (4) | * 5.03 ±0.70 (4) | 2.73 ±0.36 (4) | # 5.46 ±0.58 (5) | 2.86 ±0.35 (4) | * 6.57 ±1.11 (4) | 3.33 ±0.49 (4) | * 6.70 ±1.04 (5) | 2.78 ±0.19 (5) | ¥ 8.60 ±0.41 (5) | 3.12 ±0.14 (5) | ¥ 13.40 ±0.69 (5) | 3.44 ±0.34 (5) | # 14.24 ±1.93 (5) | 2.97 ±0.26 (4) |
| Total FFA in plasma (mM) | 0.34 ±0.05 (7) | # 0.33 ±0.08 (5) | (#) 0.68 ±0.09 (5) | 0.41 ±0.05 (5) | 0.47 ±0.13 (3) | 0.26 ±0.04 (4) | 0.35 ±0.04 (4) | 0.36 ±0.07 (5) | 0.41 ±0.05 (4) | 0.30 ±0.02 (4) | 0.46 ±0.06 (4) | 0.31 ±0.05 (5) | 0.36 ±0.08 (5) | 0.38 ±0.14 (4) | 0.34 ±0.02 (5) | 0.33 ±0.04 (5) | 0.41 ±0.04 (4) | 0.36 ±0.09 (4) | 0.53 ±0.14 (4) |
| Body wt. (g) | 327 ±12 (8) | 303 ±15 (5) | 317 ±20 (5) | 289 ±7 (5) | 299 ±15 (4) | 317 ±13 (4) | 310 ±4 (4) | 301 ±20 (5) | 338 ±14 (5) | 342 ±15 (4) | 339 ±2 (5) | 338 ±13 (5) | 301 ±12 (5) | 347 ±12 (5) | 316 ±21 (5) | 359 ±15 (5) | 318 ±21 (4) | 384 ±12 (5) | 364 ±15 (4) |

Figure 3.2

The effect of 17β-oestradiol in propane-1,2-diol, or of propane-
1,2-diol alone, on the total plasma concentrations of
triacylglycerol (TG), phospholipid (PL) and free fatty acids (FFA)
at varying times after injection.


Consult the legend of Table 3.2 for details of experimental
procedures.


        ▲ values for untreated birds.

        ○ values for oestrogen-treated birds.

        ● values for control birds, injected with
          propane-1,2-diol.


(a) Triacylglycerol (TG) in plasma (mg/100 ml)


(b) Phospholipid (PL) in plasma (mM)


(c) Total free fatty acids (FFA) in plasma (mM)

The results are expressed as means (± S.E.M.) of 3 - 8
observations.

Those values plotted without error bars indicate that the S.E.M.
was not sufficiently large to represent on the axes used.
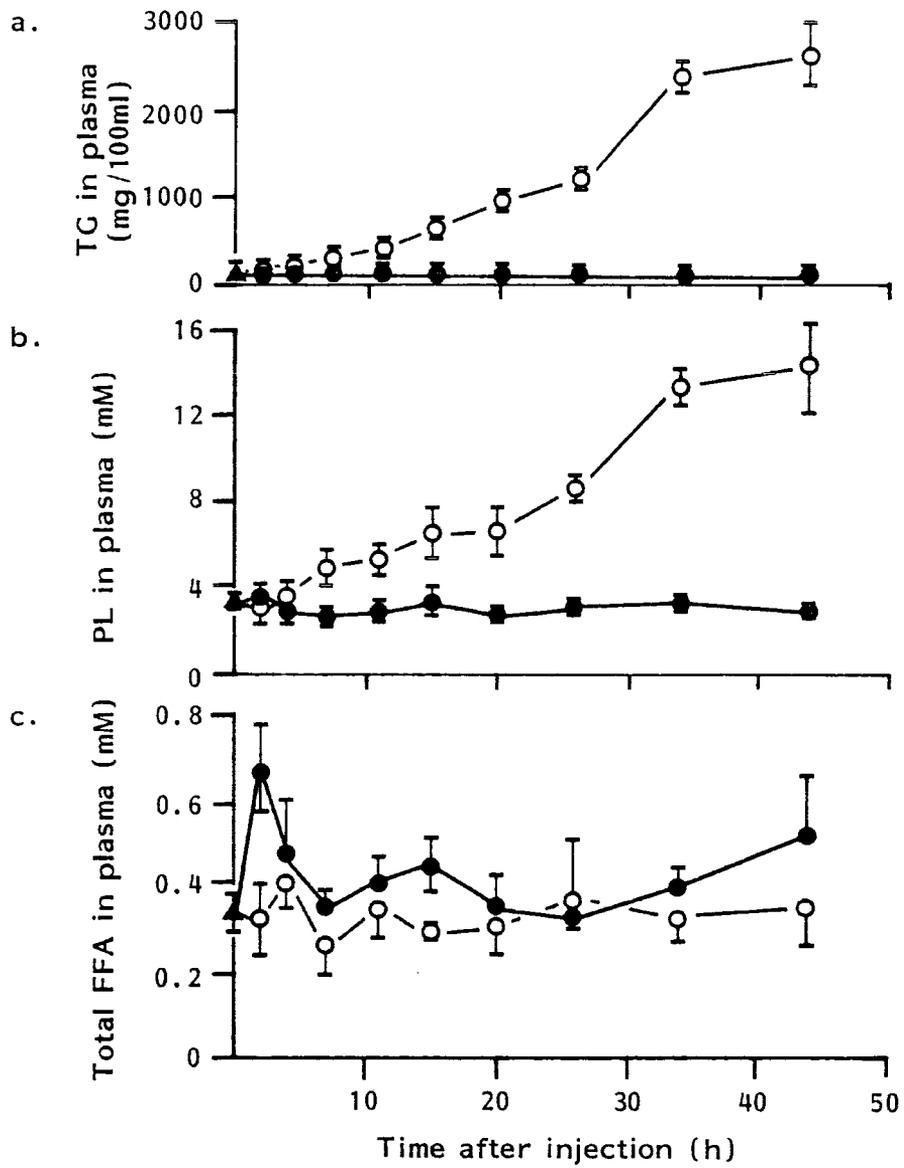
Variations in the concentrations of triacylglycerols and phospholipids associated only with the VLDL fraction following oestrogen treatment are presented in Fig. 3.3a and 3.3b. At 6 hours after injection the oestrogen-treated birds showed an increased VLDL-triacylglycerol concentration when compared with control birds but this increase was not significant. A significant increase ($p < 0.01$) in the concentration of VLDL-triacylglycerol was observed 13 hours after oestrogen treatment. From 13 - 48 hours after oestrogen treatment the VLDL-triacylglycerol concentrations were significantly greater than corresponding control values and a maximum increase (29.6-fold) was observed at 37 hours ($p < 0.001$). The mean concentration of VLDL-triacylglycerol in oestrogen-treated birds 48 hours after injection was slightly lower than that observed at 37 hours although there was no significant difference between the two values.

Significant increases ($p < 0.01$) in VLDL-phospholipid concentrations were observed in oestrogen-treated birds, compared to controls, at all time-points after injection. From 6 - 48 hours after oestrogen treatment there was a continuous increase in VLDL-phospholipid such that at 48 hours a 96.7-fold increase over controls was observed.

Following oestrogen administration the concentrations of albumin-bound free fatty acids were lower than corresponding control values at all time-points (Fig. 3.3c). Only at 24 hours following injection, however, was this decrease significant ($p < 0.01$), the oestrogen-treated value being 52% of the control value.

3. Changes in liver lipid levels following oestrogen treatment

(a) Triacylglycerols

The triacylglycerol content of liver tissue was expressed on a whole organ basis (mg triacylglycerol/liver) and on a 'cellular' basis

Table 3.3

The effect of a single dose of 17β-oestradiol (0.75mg in propane-1,2-diol/100 g body wt.), or of propane-1,2-diol only, on the concentrations of VLDL-triacylglycerol (VLDL-TG), VLDL-phospholipid (VLDL-PL) and albumin-bound free fatty acids (FFA) in the plasma of young chicks , at varying times after injection.

Each oestrogen-treated chick received a single intramuscular injection of 0.75 mg 17β-oestradiol in propane-1,2-diol/100 g body weight.  Control birds received an equivalent volume of propane-1,2-diol only.  At the indicated times after injection chicks were sacrificed and plasma collected.  Albumin-bound free fatty acids were assayed and VLDL isolated using procedures described in the Methods section.  Triacylglycerol and phospholipid contents of the VLDL fraction were determined, as described in the text.  Chicks were aged $3\frac{1}{2}$ - 5 weeks.

OE = values for oestrogen-treated chicks.

C = values for control chicks.

Values are the mean ($\pm$ S.E.M.) of 4 - 8 birds; the number of birds involved in each group is given in parentheses.

Data were analysed statistically by Student's 't' test.  Levels of statistical significance indicated without parentheses are for oestrogen-treated values with respect to corresponding control values. Levels of statistical significance indicated within parentheses, are for that particular value with respect to the corresponding untreated value.

# significant at $p < 0.01$

¥ significant at $p < 0.001$

Lipid concentration data are illustrated graphically in Fig. 3.3.

Time after injection (h)

| | 0 | 6 | | 13 | | 24 | | 37 | | 48 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | OE | C | OE | C | OE | C | OE | C | OE | C |
| VLDL-TG in plasma (mg/100 ml) | 63.8 ±6.9 (8) | 65.4 ±6.8 (6) | 60.6 ±8.8 (4) | # 190.9 ±25.5 (6) | 52.7 ±9.5 (4) | ¥ 651.0 ±58.7 (6) | 51.4 ±8.1 (6) | ¥ 1529 ±133 (6) | 51.8 ±16.9 (5) | # 1438 ±233 (6) | 50.6 ±6.4 (6) |
| VLDL-PL in plasma (mM) | 0.15 ±0.01 (8) | # 0.31 ±0.03 (6) | 0.17 ±0.02 (5) | # 1.23 ±0.20 (6) | 0.17 ±0.03 (6) | ¥ 3.87 ±0.37 (6) | 0.12 ±0.01 (6) | ¥ 8.23 ±0.70 (6) | 0.13 ±0.02 (6) | # 9.67 ±1.44 (6) | (#) 0.10 ±0.02 (6) |
| Albumin-bound FFA in plasma (mM) | 0.25 ±0.03 (4) | 0.15 ±0.03 (6) | 0.26 ±0.09 (4) | 0.15 ±0.02 (6) | 0.28 ±0.06 (6) | # 0.15 ±0.03 (6) | 0.29 ±0.03 (6) | 0.21 ±0.07 (5) | 0.31 ±0.06 (6) | 0.30 ±0.07 (6) | 0.35 ±0.09 (5) |
| Body wt. (g) | 320 ±7 (8) | 355 ±31 (6) | 350 ±29 (5) | 378 ±17 (6) | 368 ±11 (6) | 385 ±14 (6) | 330 ±30 (6) | 376 ±14 (6) | 363 ±10 (6) | 380 ±16 (6) | 367 ±9 (6) |

Figure 3.3

The effect of 17β-oestradiol in propane-1,2-diol, or of propane-
1,2-diol alone, on the concentrations of VLDL-triacylglycerol
(VLDL-TG), VLDL-phospholipid (VLDL-PL) and albumin-bound free
fatty acids (FFA) in the plasma of young chicks, at varying times
after injection.


Consult the legend of Table 3.3 for details of experimental
procedures.

     ▲ values for untreated birds.

     ○ values for oestrogen-treated birds.

     ● values for control birds, injected with
       propane-1,2-diol.

(a) VLDL-triacylglycerol (VLDL-TG) in plasma (mg/100 ml)


(b) VLDL-phospholipid (VLDL-PL) in plasma (mM)


(c) Albumin-bound free fatty acids (FFA) in plasma (mM)

The results are expressed as means (± S.E.M.) of 4 - 8
observations.

The values plotted without error bars indicate that the S.E.M.
was not sufficiently large to represent on the axes used.
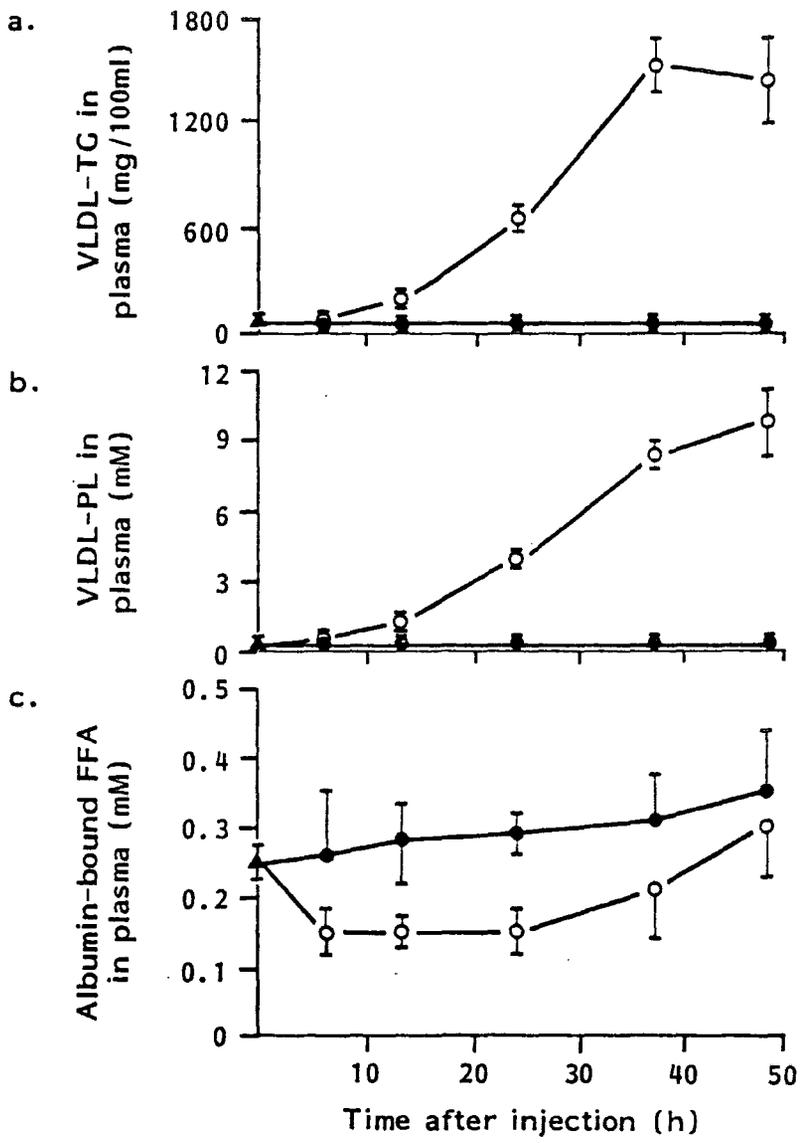
(mg triacylglycerol/mg DNA). After oestrogen treatment, elevations
in liver triacylglycerols, compared to controls, were observed at
all time-points. Significant increases, however, were first observed
at 7 hours ($p < 0.05$) when expressed on a whole organ basis (Fig. 3.4a)
and at 11 hours ($p < 0.01$) when expressed on a 'cellular' basis
(Fig. 3.4b). A build-up of triacylglycerol was observed up to 20
hours after oestrogen treatment. At this time there was a 6.6-fold
increase in the whole organ content and a 6.0-fold increase in the
'cellular' content of triacylglycerol in the liver suggesting that
the increase in liver triacylglycerol in the first 20 hours after
oestrogen treatment occurs in the absence of a significant amount of
cell division. At 26 hours after injection there was a decrease in
the mean level of triacylglycerol in the liver of oestrogen-treated
birds when compared to oestrogen-treated birds 20 hours after
injection but this decrease was not statistically significant.
Subsequently, there was a further increase in liver triacylglycerol
content such that, at 34 hours, a 23-fold increase in oestrogen-
treated birds compared to controls was observed when the results were
expressed on a whole organ basis (Fig. 3.4a) and a 15-fold increase
was observed when the results were expressed on a 'cellular' basis.
(Fig. 3.4b)

(b) Phospholipids

The effect of oestrogen treatment on the phospholipid content of chick
liver was determined and the results were expressed on a whole organ
basis ($\mu$mol phospholipid/liver; Fig. 3.5a) and on a 'cellular' basis
($\mu$mol phospholipid/mg DNA; Fig. 3.5b). The administration of
oestradiol- 17$\beta$ caused a substantial increase in the mean total
phospholipid content of chick liver, compared with control birds,
during the period 7 - 44 hours after injection (Fig. 3.5a). At each
time-point during this period, the difference between the values

Table 3.4

The effect of a single dose of 17β-oestradiol (0.75 mg in propane-1,2-diol/100 g body wt.), or of propane-1,2-diol only, on the liver content of triacylglycerol (TG), phospholipid (PL) and free fatty acids (FFA) and the liver weight at varying times after injection.

Each oestrogen-treated bird received a single intramuscular injection of 0.75 mg 17β-oestradiol in propane-1,2-diol/100 g body weight. Control chicks received an equivalent volume of propane-1,2-diol only. At the indicated times after injection, chicks were sacrificed and their livers removed and weighed. The triacylglycerol (TG), phospholipid (PL), free fatty acid (FFA) and DNA contents of the liver tissue were determined by the procedures described in the text. Chicks were aged $3\frac{1}{2}$ - 5 weeks.

OE = values for oestrogen-treated birds.

C = values for control birds.

Values are the mean ($\pm$ S.E.M.) of 2 - 8 birds; the number of birds involved in each group is given in parentheses.

Data were analysed statistically by Student's 't' test. Levels of statistical significance indicated without parentheses are for oestrogen-treated values with respect to corresponding control values. Levels of statistical significance indicated within parentheses are for that particular value with respect to the corresponding untreated value.

* significant at $p < 0.05$

# significant at $p < 0.01$

¥ significant at $p < 0.001$

The liver lipid content data are presented graphically in Fig. 3.4, Fig. 3.5 and Fig. 3.6. For body weights, consult Table 3.2.

Time after injection (h)

| | 0 | 2 OE | 2 C | 4 OE | 4 C | 7 OE | 7 C | 11 OE | 11 C | 15 OE | 15 C | 20 OE | 20 C | 26 OE | 26 C | 34 OE | 34 C | 44 OE | 44 C |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TG whole organ content (mg.) | 105 ± 7 (8) | 112±9 (5) | 82±15 (5) | 143±17 (2) | 118±2 (3) | * 288±32 (5) | 160±43 (5) | # 381±65 (5) | (*) 69±16 (5) | # 657±121 (5) | 79±26 (5) | * 737±170 (5) | 111±23 (5) | * 492±127 (4) | 124±12 (5) | # 1662±330 (5) | (#) 71±8 (4) | * 1362±405 (4) | 98±4 (4) |
| TG 'cellular' content (mgTG/mg DNA) | 3.1 ± 0.2 (8) | 3.7±0.4 (5) | 2.7±0.6 (5) | - | - | 8.2±1.0 (5) | 4.8±1.2 (5) | # 11.7±1.7 (5) | 2.2±0.5 (5) | * 17.5±3.7 (5) | 2.4±0.8 (5) | * 21.3±5.2 (5) | 3.6±0.7 (5) | * 14.1±2.5 (4) | 3.6±0.3 (5) | ¥ 32.8±2.7 (5) | (*) 2.2±0.2 (4) | * 27.3±6.6 (4) | 2.64±0.3 (4) |
| PL whole organ content (µmoles) | 418 ± 28 (8) | 419±33 (5) | 453±40 (5) | 454±40 (2) | 458±28 (3) | * 562±26 (5) | 478±22 (5) | 479±46 (5) | 458±35 (5) | * 573±35 (5) | 438±17 (5) | # 615±42 (5) | 393±30 (5) | # 617±41 (5) | 451±27 (5) | # 764±55 (5) | 418±29 (4) | # 746±36 (5) | 488±48 (4) |
| PL 'cellular' content (µmoles PL/mg DNA) | 12.2±0.6 (8) | 13.5±0.4 (5) | (*) 14.4±0.5 (5) | 14.9±0.5 (2) | 13.7±0.7 (3) | # 16.0±0.2 (5) | (#) 14.5±0.3 (5) | 14.8±0.5 (5) | 14.0±0.6 (5) | # 16.8±1.0 (5) | 13.0±0.5 (5) | ¥ 17.5±0.6 (5) | 13.1±0.5 (5) | # 15.9±0.6 (5) | 13.3±0.5 (5) | * 15.9±0.9 (5) | 13.0±0.2 (5) | * 15.5±0.8 (5) | 12.7±0.21 (4) |
| FFA whole organ content (µmoles) | 8.7 ± 1.2 (8) | 7.1±1.5 (5) | 8.0±1.2 (5) | 6.2±0.7 (2) | 6.2±1.4 (3) | 8.5±0.8 (5) | 8.8±1.1 (5) | 9.7±1.1 (5) | 9.5±0.8 (5) | 12.7±2.0 (5) | 8.3±1.3 (5) | 13.9±2.0 (5) | 9.8±1.0 (5) | # 10.6±0.6 (5) | 6.2±1.0 (5) | * 12.2±2.0 (5) | 6.0±0.3 (4) | * 14.0±1.3 (5) | 8.9±1.4 (4) |
| FFA 'cellular' content (µmoles FFA/mg DNA) | 0.25±0.03 (8) | 0.24±0.06 (5) | 0.26±0.03 (5) | - | - | 0.23±0.03 (5) | 0.27±0.04 (5) | 0.31±0.02 (5) | 0.29±0.03 (5) | 0.37±0.06 (5) | 0.24±0.03 (5) | 0.39±0.05 (5) | 0.33±0.02 (5) | * 0.28±0.02 (5) | 0.18±0.02 (5) | 0.25±0.04 (5) | 0.19±0.01 (4) | 0.29±0.01 (5) | 0.23±0.01 (4) |
| Liver weight (as % body wt.) | 3.30±0.10 (8) | 3.44±0.06 (5) | 3.40±0.11 (5) | 3.68±0.12 (5) | 3.78±0.06 (4) | 4.18±0.06 (4) | 3.78±0.24 (4) | # 3.82±0.16 (5) | 3.12±0.08 (5) | ¥ 4.18±0.12 (4) | 3.22±0.05 (5) | ¥ 4.48±0.07 (5) | 3.16±0.17 (5) | * 4.56±0.28 (5) | 3.58±0.10 (5) | ¥ 5.48±0.22 (5) | 3.18±0.08 (4) | ¥ 5.20±0.27 (5) | 3.18±0.15 (4) |

Figure 3.4

The effect of 17β-oestradiol in propane-1,2-diol, or of propane-1,2-diol alone, on the liver content of triacylglycerol at varying times after injections.


Consult the legend of Table 3.4 for details of experimental procedures.

        ▲ values for untreated birds.

        ○ values for oestrogen-treated birds.

        ● values for control birds, injected with propane-1,2-diol.


(a) Triacylglycerol (TG) content of liver (mg TG/liver)

(b) Triacylglycerol (TG) content of liver (mg TG/DNA)

The results are expressed as means ($\pm$ S.E.M.) of 2 - 8 observations.

Those values plotted without error bars indicate that the S.E.M. was not sufficiently large to represent on the axes used.

for oestrogen-treated and control birds was statistically significant ($p < 0.05$), with the exception of the values obtained at 11 hours after injection when the amount of phospholipid per liver declined in the oestrogen-treated chicks. The maximum value was observed at 34 hours after injection of oestrogen, when the total phospholipid content of chick liver was 1.8-fold greater than the control value.

When the levels of phospholipid in liver tissue were expressed on a 'cellular' basis ($\mu$mol. phospholipid/mg DNA) the mean values for oestrogen-treated birds were greater than those for control birds during the period 4 - 44 hours after injection (Fig. 3.5b). However, those differences at 4 hours and 11 hours after injection were not significant. A maximum amount of phospholipid/mg DNA was apparent 20 hours after oestrogen treatment, being 1.3-fold greater than the corresponding control value ($p < 0.001$).

## (c) Free fatty acids

The mean level of free fatty acids in liver, expressed both on a whole organ basis ($\mu$mol FFA/liver; Fig. 3.6a) and on a cellular basis ($\mu$mol FFA/mg DNA; Fig. 3.6b) was greater than the corresponding control values from 11 - 44 hours after oestrogen treatment. Significant increases ($p < 0.05$), however, were first observed after 26 hours when the oestrogen-treated levels of FFA/liver and FFA/mg DNA were 1.69-fold and 1.55-fold greater than the corresponding control values, respectively. Significant increases in the amount of FFA/liver were also observed 36 hours ($p < 0.05$) and 44 hours ($p < 0.05$) after oestrogen treatment, values being 2.04-fold and 1.58-fold greater than the corresponding control values, respectively (Fig. 3.6a). The elvation in the amount of FFA/mg DNA was only significant at 26 hours ($p < 0.05$) and 44 hours ($p < 0.05$) after oestrogen treatment, the value at the latter time-point being 1.26-fold greater than the corresponding control value.

Figure 3.5

The effect of 17β-oestradiol in propane-1,2-diol, or of propane-1,2-diol alone, on the liver content of phospholipid at varying times after injection.

Consult the legend of Table 3.4 for details of experimental procedures.

△ values for untreated birds.
○ values for oestrogen-treated birds.
● values for control birds, injected with propane-1,2-diol.

(a) The phospholipid (PL) content of liver (μmol PL/liver)

(b) The phospholipid (PL) content of liver (μmol PL/mg DNA)

The results are expressed as means ($\pm$ S.E.M.) of 2 - 8 observations.
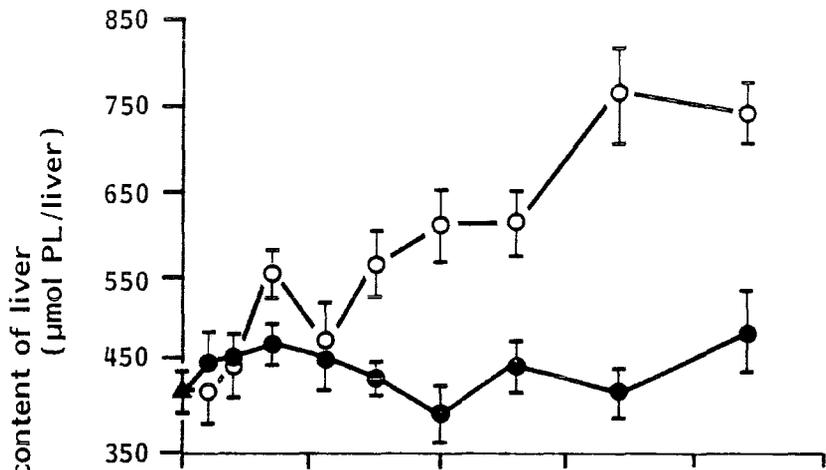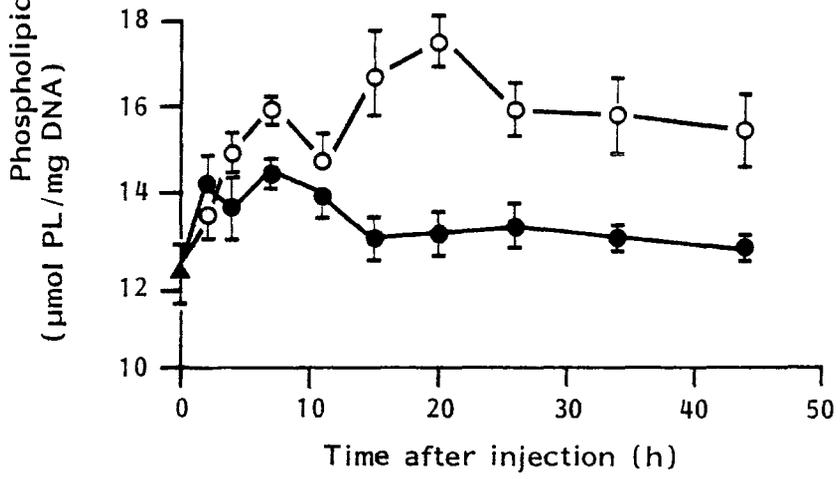
Figure 3.6

The effect of 17β-oestradiol in propane-1,2-diol, or of propane-1,2-diol alone, on the liver content of free fatty acids at varying times after injection.

Consult the legend of Table 3.4 for details of experimental procedures.

▲ values for untreated birds.
○ values for oestrogen-treated birds.
● values for control birds, injected with propane-1,2-diol.

(a) Free fatty acid (FFA) content of liver ($\mu$mol FFA/liver)

(b) Free fatty acid (FFA) content of liver ($\mu$mol FFA/mg DNA)

The results are expressed as means ($\pm$ S.E.M.) of 2 - 8 observations.

a.
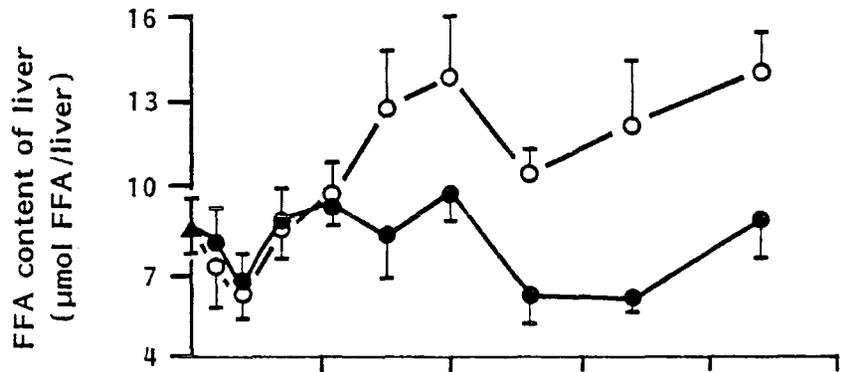
b.
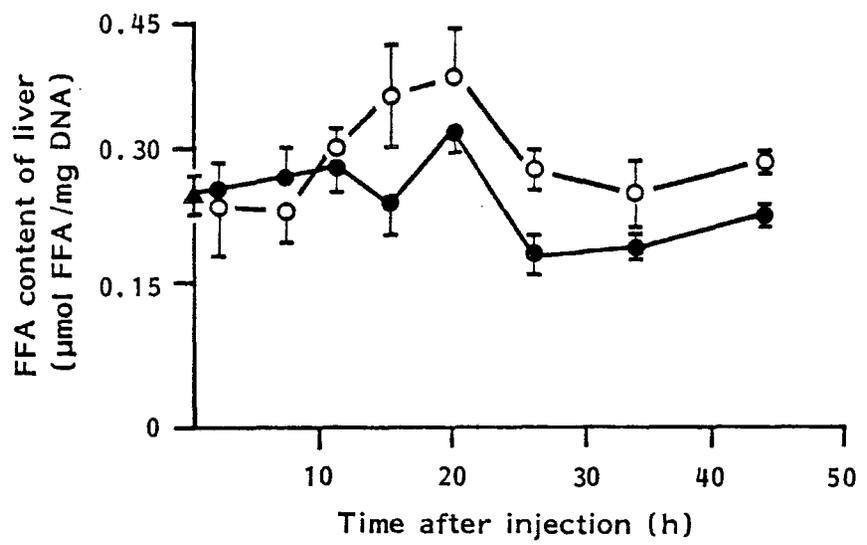
Variations in the liver weight (as % of body wt.) following oestrogen
treatment are presented in Table 3.4. The changes are similar to
those reported and discussed in Chapter 2.

4. Changes in the fatty acid composition of plasma and liver lipids
   following oestrogen treatment

Gas-liquid chromatographic analysis of the fatty acid composition of
lipid in the diet revealed that the predominant fatty acid was 16:0
(palmitate) (Fig. 3.7a). Other major constituents were 18:1 (oleate),
18:2 (linoleate) and 18:0 (stearate).

(a) Liver triacylglycerols

Changes in the molar ratio of unsaturated:saturated fatty acids in
liver triacylglycerols are presented in Table 3.5. This molar ratio
in untreated animals was 1.13 and, after oestrogen treatment, it
became significantly greater than the control values at 24 hours
($p < 0.001$), 37 hours ($p < 0.05$) and 48 hours ($p < 0.05$). In general,
the control values were similar to the untreated value, except at
24 hours after injection, when the control value was significantly
lower ($p < 0.01$) than the untreated value. (Table 3.5).

Details of the fatty acid composition of liver triacylglycerols are
shown in Table 3.6. In untreated animals, the predominant fatty acids
were 18:1 and 16:0, while 18:0, 18:2 and 16:1 (palmitoleate)
contributed to a lesser extent (Fig. 3.7b). After oestrogen treatment,
a number of marked changes occurred in the fatty acid composition of
liver triacylglycerols (Fig. 3.8). At 6 hours after injection,
the percentage contribution of 16:0 for oestrogen-treated birds was
significantly greater than that for control birds ($p < 0.05$).
Furthermore, values for oestrogen-treated and control birds at 6 hours
were higher than the corresponding value in untreated birds although

Table 3.5

The effect of a single dose of 17β-oestradiol in propane-1,2-diol, or of propane-1,2-diol only, on the molar ratio of unsaturated:saturated fatty acids in liver triacylglycerol, plasma VLDL-triacylglycerol and plasma VLDL-phospholipid at varying times after injection.

Each oestrogen-treated bird received a single intramuscular injection of 0.75 mg 17β-oestradiol in propane-1,2-diol/100 g body weight. Control birds received an equivalent volume of propane-1,2-diol only. At the indicated times after injection birds were sacrificed and VLDL isolated from the collected plasma, as described in the Methods section. Following lipid extraction procedures, triacylglycerol from the liver and VLDL, and phospholipid from the VLDL were subjected to g.l.c. analysis. Consult the Methods section for full details of the experimental procedures employed. Mean molar ratios were calculated for each lipid class from the percentage fatty acid compositions of individual birds. Birds were aged $3\frac{1}{2}$ - 5 weeks.

<div style="text-align:center">

OE = values for oestrogen-treated birds.

C = values for control birds.

</div>

Values are the means ($\pm$ S.E.M.) of 3 - 10 birds; the number of birds involved in each group is given in parentheses.

Data were analysed statistically by Student's 't' test. Levels of statistical significance indicated without parentheses are for oestrogen-treated values with respect to control values. Levels of statistical significance indicated within parentheses are for that particular value with respect to the corresponding untreated value.

<div style="text-align:center">

* significant at $p < 0.05$
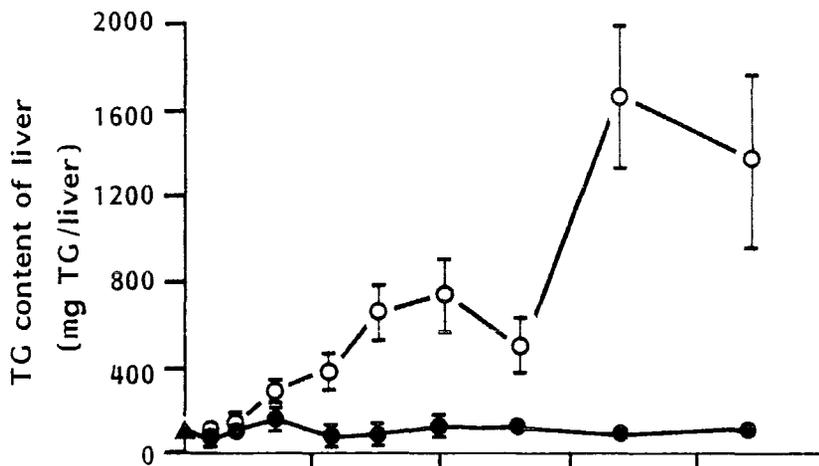
# significant at $p < 0.01$

¥ significant at $p < 0.001$     For body weights consult Table 3.3.

</div>

| | | Time after injection (h) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 6 | | 13 | | 24 | | 37 | | 48 | |
| | 0 | OE | C | OE | C | OE | C | OE | C | OE | C |
| Molar ratio in liver triacylglycerol | 1.13 ±0.12 (5) | 1.14 ±0.07 (6) | 1.28 ±0.05 (6) | 1.31 ±0.10 (6) | 1.14 ±0.04 (6) | ¥(*) 1.55 ±0.03 (6) | (#) 0.52 ±0.03 (6) | * 1.41 ±0.04 (6) | 1.18 ±0.07 (6) | *(#) 1.61 ±0.07 (5) | 1.26 ±0.12 (5) |
| Molar ratio in VLDL-triacylglycerol | 1.22 ±0.06 (8) | 1.36 ±0.06 (5) | (#) 1.57 ±0.08 (4) | (*) 1.50 ±0.07 (5) | 1.32 ±0.07 (6) | 1.26 ±0.12 (4) | 1.26 ±0.14 (6) | 1.38 ±0.11 (5) | 1.28 ±0.09 (5) | 1.34 ±0.10 (6) | (*) 1.51 ±0.07 (5) |
| Molar ratio in VLDL-phospholipid | 1.06 ±0.05 (10) | 1.06 ±0.03 (5) | 1.09 ±0.01 (3) | 1.18 ±0.05 (6) | 1.20 ±0.03 (6) | 1.04 ±0.06 (5) | 1.06 ±0.02 (7) | 1.11 ±0.09 (6) | 1.15 ±0.04 (6) | 1.09 ±0.03 (4) | 1.12 ±0.06 (5) |

Figure 3.7

The fatty acid composition of lipid in the chick diet, and of
liver triacylglycerol (liver-TG), VLDL-triacylglycerol (VLDL-TG)
and VLDL-phospholipid (VLDL-PL) in untreated chicks.

(a) Fatty acid composition of the chick diet (%, w/w).

Lipid was extracted from the chick diet by the procedure of
Bligh & Dyer (1959), as described in the Methods section. The
lipid extract was subjected to methanolysis and the resultant
fatty acid methyl esters analysed by gas-liquid chromatography,
as described in the Methods section.

Values are expressed as the means of 3 observations.

(b) Fatty acid composition of liver triacylglycerol(liver-TG;
%, w/w).

(c) Fatty acid composition of VLDL-triacylglycerol (VLDL-TG;
%, w/w).

(d) Fatty acid composition of VLDL-phospholipid (VLDL-PL; % w/w)

Consult the legend of Table 3.5 for details of the experimental
procedures employed in the analysis of the fatty acid composition
of liver-TG, VLDL-TG and VLDL-PL from untreated birds.

Values are expressed as the means of 5 - 10 observations.

Fatty acids are numbered as follows:

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|------|------|------|------|------|------|--------|--------|------|
| 14:0 | 16:0 | 16:1 | 18:0 | 18:1 | 18:2 | $\gamma$18:3 | $\alpha$18:3 | 20:0 |

| 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|------|------|------|------|------|------|------|------|
| 20:1 | 20:2 | 20:3 | 20:4 | 22:0 | 22:4 | 22:6 | 24:0 |

a. composition of diet (%, w/w)

b. composition of liver – TG (%, w/w)

c. composition of VLDL–TG (%, w/w)

d. composition of VLDL–PL (%, w/w)

Fatty acid
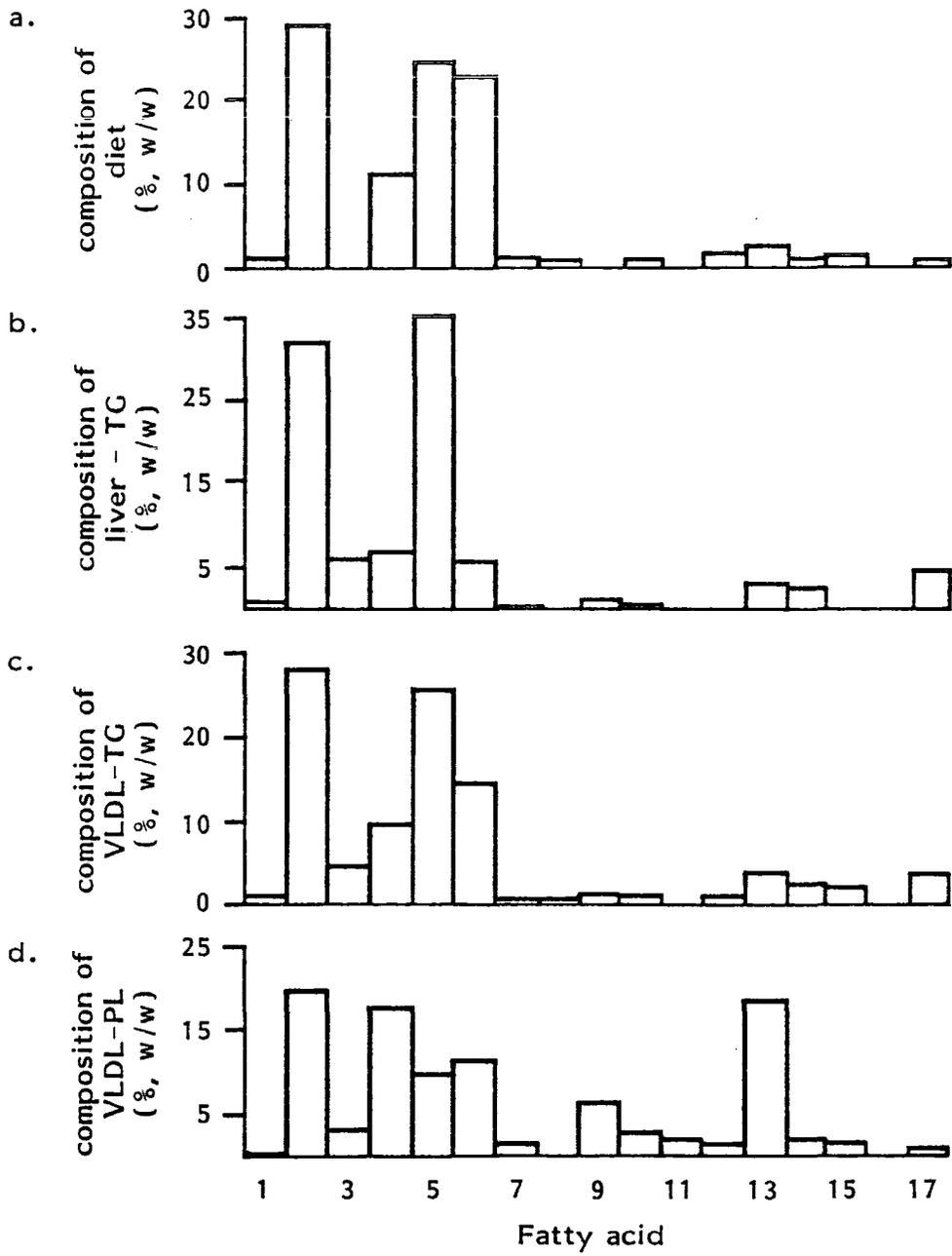
only the difference between oestrogen-treated and untreated birds was significant (p < 0.05). At 24 hours after injection, the percentage contribution of 16:0 for oestrogen-treated birds was significantly lower than control birds (p < 0.01). This may have been caused by the very high value for the control birds at this time (Fig. 3.8a), and further studies are required to determine whether this is a reproducible effect.

At 24 hours after injection the percentage contribution of 18:0 for oestrogen-treated birds was significantly lower than control birds (p < 0.01) and at this time, the control value was significantly higher (p < 0.01) than the corresponding untreated value (Fig. 3.8b). Similarly, the control values of 18:0 were significantly greater than the corresponding untreated value, at 13 hours (p < 0.05) and 37 hours (p < 0.05) after injection. However, no significant differences were observed between the 18:0 values for oestrogen-treated and control birds at times other than 24 hours after injection.

The levels of the principal saturated fatty acids, 16:0 and 18:0, and of the minor saturated fatty acid, 14:0 (Table 3.6), therefore, were all significantly increased in 24 hour control birds compared to all other control, treated and untreated values.

The percentage contribution of 18:1 was significantly greater in oestrogen-treated birds than control birds at 13 hours (p < 0.01), 24 hours (p < 0.001), 37 hours (p < 0.05) and 48 hours (p < 0.05) after injection (Fig. 3.8c). At 24 hours after oestrogen treatment, the extent of the increase in the percentage contribution of 18:1 in hormone-treated birds was enhanced by a significant decrease in the percentage contribution in control birds, relative to the untreated value (p < 0.001).

Oestrogen-treated values of 18:2 were significantly greater than the

Table 3.6

The effect of a single dose of 17β-oestradiol (0.75 mg in propane-1,2-diol/100 g body wt.), or of propane-1,2-diol only, on the fatty acid composition of liver triacylglycerol at varying times after injection.

Consult the legend of Table 3.5 for experimental details.

Values are the means ($\pm$ S.E.M.) of 5 or 6 birds; consult Table 3.5 for the number of birds involved in each group.

OE = values for oestrogen-treated birds

C = values for control birds.

Data were analysed statistically by Student's 't' test. Levels of statistical significance indicated without parentheses are for oestrogen-treated values with respect to corresponding control values. Levels of statistical significance indicated within parentheses are for that particular value with respect to the corresponding untreated value.

\* significant at $p < 0.05$

\# significant at $p < 0.01$

¥ significant at $p < 0.001$

For body weights, consult Table 3.3.

Some of these data are presented graphically in Fig. 3.7b and Fig. 3.8.

Time after injection (h)

| Fatty acid (%,w/w) | 0 | 6 | | 13 | | 24 | | 37 | | 48 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | OE | C | OE | C | OE | C | OE | C | OE | C |
| 14:0 | 0.8 ± 0.1 | 0.8 ± 0.1 | 0.6 ± 0.1 | # 0.9 ± 0.1 | 0.5 ± 0.1 | * (*) 0.4 ± 0.1 | (*) 4.5 ± 1.1 | 0.5 ± 0.1 | 0.7 ± 0.1 | (*) 0.3 ± 0.1 | (*) 0.4 ± 0.1 |
| 16:0 | 32.1 ± 2.5 | * (*) 39.0 ± 1.2 | 35.3 ± 0.5 | 33.1 ± 1.3 | 33.4 ± 1.3 | # 30.1 ± 0.8 | (*) 43.1 ± 2.5 | 31.1 ± 0.6 | 33.3 ± 1.4 | 27.3 ± 1.1 | 32.0 ± 3.1 |
| 16:1 | 6.0 ± 1.0 | 7.8 ± 0.3 | 7.7 ± 0.6 | 5.7 ± 0.5 | 5.5 ± 0.3 | 5.8 ± 0.5 | 5.5 ± 0.2 | 5.9 ± 0.5 | 5.0 ± 0.4 | 6.1 ± 0.7 | 7.2 ± 1.9 |
| 18:0 | 6.8 ± 1.0 | 7.2 ± 0.4 | 7.2 ± 0.6 | (*) 9.6 ± 0.8 | (*) 8.5 ± 0.4 | # 8.0 ± 0.5 | (#) 17.8 ± 2.1 | 8.7 ± 0.6 | (*) 9.0 ± 0.7 | 7.8 ± 0.6 | 7.2 ± 0.7 |
| 18:1 | 35.4 ± 1.6 | 39.2 ± 1.0 | (*) 40.1 ± 1.0 | # (*) 42.3 ± 2.0 | 34.4 ± 1.0 | ¥ (*) 40.7 ± 1.1 | (¥) 19.5 ± 1.9 | * (*) 41.3 ± 1.4 | 36.0 ± 1.5 | * 39.8 ± 1.1 | 34.7 ± 1.8 |
| 18:2 | 6.0 ± 1.1 | 4.4 ± 0.5 | 5.4 ± 0.7 | # 6.7 ± 0.6 | 4.4 ± 0.3 | ¥ 8.1 ± 0.5 | (*) 2.4 ± 0.2 | 7.4 ± 0.7 | 6.1 ± 0.4 | 6.2 ± 0.8 | 7.4 ± 0.4 |
| γ18:3 | 0.2 ± 0.1 | 0.03 ± 0.1 | 0.04 ± 0.1 | - | 0.9 ± 0.4 | 0.4 ± 0.2 | 0.4 ± 0.2 | 0.2 ± 0.1 | 0.5 ± 0.2 | 0.8 ± 0.2 | 0.4 ± 0.3 |
| α18:3 | 0.1 ± 0.1 | - | - | - | - | 0.1 ± 0.1 | - | 0.1 ± 0.1 | - | - | - |
| 20:0 | 1.0 ± 0.6 | * 0.03 ± 0.1 | 0.4 ± 0.1 | # 0.02 ± 0.1 | 1.1 ± 0.2 | 0.1 ± 0.1 | - | 0.2 ± 0.1 | 0.4 ± 0.2 | 0.6 ± 0.3 | 0.6 ± 0.2 |
| 20:1 | 0.6 ± 0.1 | (*) 0.2 ± 0.1 | (*) 0.2 ± 0.1 | 0.6 ± 0.2 | 1.1 ± 0.5 | 0.9 ± 0.5 | 0.2 ± 0.2 | 0.5 ± 0.1 | 0.6 ± 0.3 | 0.7 ± 0.3 | 0.9 ± 0.4 |
| 20:2 | - | - | - | 0.1 ± 0.1 | 0.5 ± 0.3 | - | - | - | - | - | - |
| 20:3 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.03 ± 0.1 | 0.1 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.1 ± 0.1 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.2 | 0.4 ± 0.2 |
| 20:4 | 3.2 ± 0.9 | * 1.1 ± 0.3 | 2.4 ± 0.4 | * 0.8 ± 0.3 | 4.3 ± 1.2 | 4.3 ± 0.9 | 5.5 ± 0.1 | 2.3 ± 0.2 | 3.9 ± 1.2 | 8.4 ± 2.1 | (*) 6.9 ± 1.3 |
| 22:0 | 2.6 ± 1.6 | - | 0.1 ± 0.1 | * - | 1.1 ± 0.4 | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.3 | 0.2 ± 0.2 | 0.5 ± 0.5 | 0.6 ± 0.3 |
| 22:4 | 0.1 ± 0.1 | - | - | - | 0.5 ± 0.3 | - | 0.1 ± 0.1 | 0.3 ± 0.2 | 0.1 ± 0.1 | - | 0.2 ± 0.1 |
| 22:6 | - | - | - | - | - | - | - • | - | - | - | - |
| 24:0 | 4.9 ± 3.2 | 0.1 ± 0.1 | 0.3 ± 0.2 | # 0.1 ± 0.1 | 2.4 ± 0.5 | 0.5 ± 0.2 | 0.5 ± 0.4 | 0.9 ± 0.3 | 1.0 ± 0.4 | 1.9 ± 1.0 | 1.1 ± 0.5 |

Figure 3.8


The effect of 17β-oestradiol in propane-1,2-diol, or of propane-1,2-diol alone, on the proportions of palmitate (16:0), stearate (18:0), oleate (18:1), and linoleate (18:2) in liver triacylglycerol, at varying times after injection.


Consult the legend of Table 3.5 for details of experimental procedures.

        ▲ values for untreated birds.
        ○ values for oestrogen-treated birds.
        ● values for control birds, injected with
          propane-1,2-diol.


(a) The proportion of 16:0 in liver triacylglycerol (%, w/w).

(b) The proportion of 18:0 in liver triacylglycerol (%, w/w).

The results are expressed as the means ($\pm$ S.E.M.) of 5 or 6 observations.

a.

b.
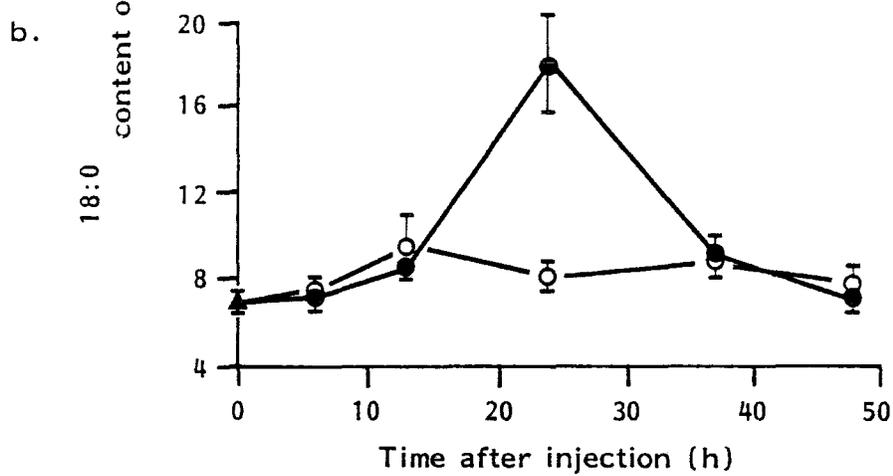
Fig. 3.8 - continued

▲ value for untreated birds.

○ values for oestrogen-treated birds.

● values for control birds, injected with
   propane-1,2-diol.

(c) The proportion of 18:1 in liver triacylglycerol (%, w/w).

(d) The proportion of 18:2 in liver triacylglycerol (%. w/w).

The results are expressed as the means ($\pm$ S.E.M.) of 5 or 6
observations.
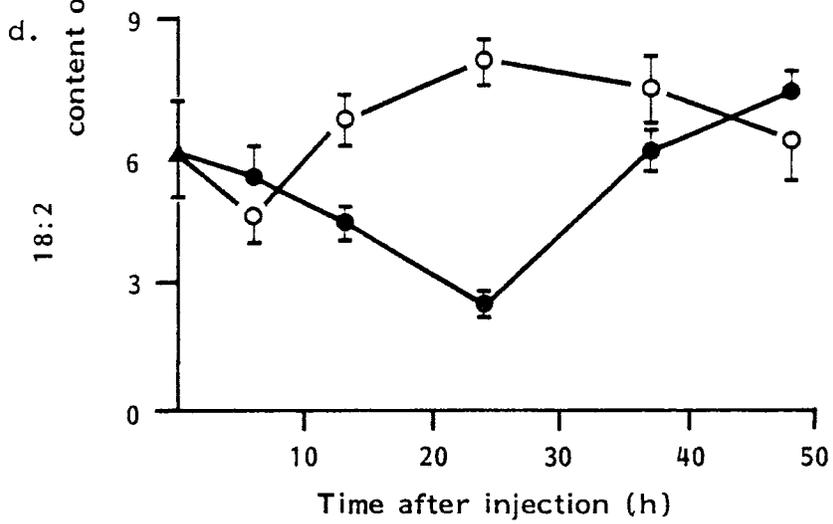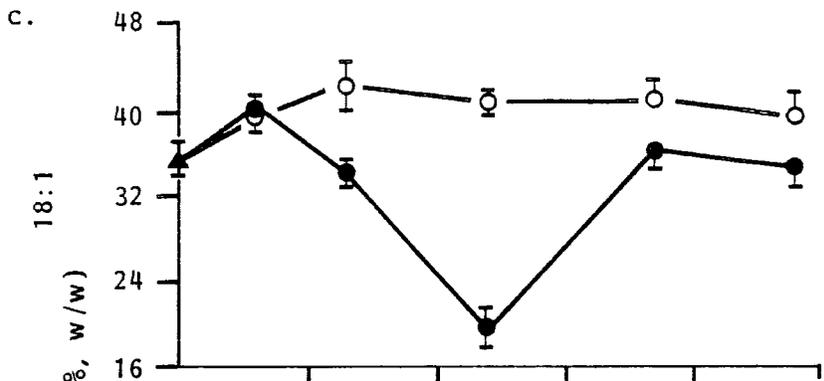
corresponding control values at 13 hours (p <0.01) and 24 hours
(p <0.01) after injection (Fig. 3.8d), partially because of decreases
in control values at this time; at 24 hours, the 18:2 control value was
significantly lower than the untreated value (p <0.05). From
0 - 24 hours after injection, there was a consistent decline in the
control values of 18:2, followed by a recovery at 37 hours and 48 hours.

Therefore, the levels of the unsaturated fatty acids, 18:1 and 18:2,
were significantly decreased in 24 hour control birds compared to all
other control, treated and untreated values.

The major effect of oestrogen treatment on the fatty acid composition
of liver triacylglycerol was to promote a substantial elevation in
the molar ratio of unsaturated:saturated fatty acids from 13 - 48
hours after injection (Table 3.5). This was caused largely by an
elevation of the levels of 18:1 and 18:2 accompanied by a decline in
the levels of 16:0 and 18:0 (Fig. 3.8).

(b) VLDL-triacylglycerols

Changes in the molar ratio of unsaturated:saturated fatty acids in
VLDL-triacylglycerols are presented in Table 3.5. The ratio in
untreated animals was 1.22 and after oestrogen treatment there were
no significant differences between hormone-treated and control birds.

Details of the fatty acid composition of VLDL-triacylglycerols are
shown in Table 3.7. In untreated birds, the predominant fatty acids
were 16:0, 18:1, 18:2 and 18:0, in decreasing order of percentage
contribution (Fig. 3.7c). After oestrogen treatment, no changes were
observed in the percentage contribution of 16:0 (Fig. 3.9a) during
the 48 hour post-injection period, whereas alterations were observed
in other fatty acids.

At 6 hours after injection, the level of 18:0 in oestrogen-treated and control birds were diminished in relation to the untreated value (Fig. 3.9b), the difference between the untreated and oestrogen-treated values being significant ($p < 0.01$). However, at no time after injection were there any significant differences between oestrogen-treated and control values of 18:0.

Oestrogen-treated values of 18:1 were significantly greater than the corresponding control values at 13 hours ($p < 0.001$), 24 hours ($p < 0.05$) and 37 hours ($p < 0.01$) after injection (Fig.3.9c). At 48 hours after injection, the oestrogen-treated value for 18:1 was greater than the control value, although the difference was not significant.

Conversely, oestrogen treatment caused a significant decrease in the percentage contribution of 18:2 in VLDL-triacylglycerol at 13 hours ($p < 0.01$), 24 hours ($p < 0.05$), 37 hours ($p < 0.01$) and 48 hours ($p < 0.001$) after injection, when compared to controls (Fig. 3.9d). Similarly, the percentage contribution of the minor component, 18:3 ($\alpha$-linolenate), was significantly lower than control values at 24 hours ($p < 0.05$) and 48 hours ($p < 0.05$) after oestrogen treatment (Table 3.7).

Therefore, the major effects of oestrogen treatment on the fatty acid composition of VLDL-triacylglycerol were to cause an elevation in the level of 18:1 from 13 - 48 hours after injection accompanied by a decrease in the levels of 18:2 (Fig. 3.9).

(c) VLDL-phospholipids

Changes in the molar ratio of unsaturated:saturated fatty acids in VLDL-phospholipids are presented in Table 3.5. This molar ratio in untreated birds was 1.06 and throughout the time-course of the study, there were no significant differences between the molar ratios of

Table 3.7

The effect of a single dose of 17β-oestradiol (0.75 mg in propane-1,2-diol/100 g body wt.), or of propane-1,2-diol only, on the fatty acid composition of VLDL-triacylglycerol at varying times after injection.

Consult the legend of Table 3.5 for experimental details.

Values are the means ($\pm$ S.E.M.) of 4 - 8 birds; consult Table 3.5 for the number of birds involved in each group.

OE = values for oestrogen-treated birds.

C = values for control birds.

Data were analysed statistically by Student's 't' test. Levels of statistical significance indicated without parentheses are for oestrogen-treated values with respect to corresponding control values. Levels of statistical significance indicated within parentheses are for that particular value with respect to the corresponding untreated value.

\* significant at $p < 0.05$

\# significant at $p < 0.01$

¥ significant at $p < 0.001$

For body weights, consult Table 3.3.

Some of these data are presented graphically in Fig. 3.7c and Fig. 3.9.

| Fatty acid (%,w/w) | 0 | 6 OE | 6 C | 13 OE | 13 C | 24 OE | 24 C | 37 OE | 37 C | 48 OE | 48 C |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 14:0 | 0.6 ± 0.2 | 0.7 ± 0.2 | 0.3 ± 0.1 | 0.4 ± 0.1 | 1.1 ± 0.4 | (*) 0.1 ± 0.1 | 2.6 ± 1.8 | 0.3 ± 0.2 | 0.8 ± 0.4 | 1.0 ± 0.2 | 0.8 ± 0.1 |
| 16:0 | 27.8 ± 1.5 | 26.2 ± 2.9 | 23.8 ± 3.2 | 28.6 ± 1.4 | 30.4 ± 0.5 | 29.3 ± 1.8 | 27.2 ± 0.5 | 28.6 ± 1.5 | 30.3 ± 1.0 | 26.3 ± 1.0 | 26.2 ± 1.1 |
| 16:1 | 4.3 ± 0.7 | 5.6 ± 0.8 | 4.9 ± 0.6 | 5.6 ± 0.2 | 4.9 ± 0.2 | 6.2 ± 1.6 | 3.7 ± 0.4 | 5.4 ± 0.6 | 5.0 ± 0.5 | 5.7 ± 0.5 | 4.7 ± 0.4 |
| 18:0 | 9.4 ± 0.6 | (#) 6.8 ± 0.3 | 7.7 ± 0.9 | 8.7 ± 0.2 | 8.2 ± 0.5 | 11.0 ± 0.4 | 9.2 ± 0.9 | 11.6 ± 1.1 | 8.8 ± 0.6 | 9.9 ± 1.2 | 9.5 ± 0.9 |
| 18:1 | 25.5 ± 2.1 | 28.1 ± 3.5 | 28.9 ± 2.3 | ¥ (¥) 38.0 ± 1.3 | 28.4 ± 1.2 | ★ (*) 35.3 ± 2.4 | 25.4 ± 1.8 | # (#) 35.9 ± 1.7 | 27.3 ± 1.1 | (*) 34.3 ± 3.0 | 29.2 ± 0.8 |
| 18:2 | 14.6 ± 1.3 | 13.7 ± 1.7 | 14.7 ± 1.4 | # (*) 11.4 ± 0.8 | 14.9 ± 0.6 | ★ (¥) 6.9 ± 0.7 | 17.2 ± 3.4 | # (¥) 7.6 ± 0.7 | 17.4 ± 1.3 | ¥ (¥) 7.2 ± 0.8 | 16.4 ± 1.0 |
| γ18:3 | 0.3 ± 0.2 | 1.2 ± 0.8 | 6.4 ± 0.4 | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.2 | 0.2 ± 0.2 | 0.9 ± 0.7 | 0.1 ± 0.1 | 0.7 ± 0.5 | 0.5 ± 0.3 |
| α18:3 | 0.4 ± 0.2 | 0.4 ± 0.2 | 0.4 ± 0.1 | 0.2 ± 0.1 | 0.5 ± 0.1 | ★ - | 0.8 ± 0.2 | - | 0.6 ± 0.3 | ★ - | 0.5 ± 0.1 |
| 20:0 | 1.2 ± 0.4 | 0.7 ± 0.2 | 1.2 ± 0.5 | 1.0 ± 0.3 | 1.8 ± 0.6 | 0.7 ± 0.3 | 1.9 ± 0.6 | 1.2 ± 0.7 | 2.4 ± 0.9 | 2.8 ± 1.3 | 2.0 ± 0.4 |
| 20:1 | 1.2 ± 0.3 | 2.0 ± 0.9 | 1.7 ± 0.8 | 2.7 ± 2.2 | 0.7 ± 0.2 | 0.7 ± 0.2 | 0.7 ± 0.3 | 0.4 ± 0.4 | 0.5 ± 0.2 | 0.9 ± 0.7 | 1.2 ± 0.3 |
| 20:2 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | - | - | 0.4 ± 0.4 | 0.7 ± 0.7 | 0.6 ± 0.6 | 0.04 ± 0.1 | - | - |
| 20:3 | 1.2 ± 0.4 | 2.5 ± 1.4 | 1.7 ± 1.5 | 0.2 ± 0.1 | 0.3 ± 0.2 | 1.4 ± 1.2 | 0.5 ± 0.3 | 1.5 ± 1.0 | 0.2 ± 0.2 | 0.9 ± 0.7 | 0.9 ± 0.3 |
| 20:4 | 3.8 ± 0.7 | (*) 1.1 ± 0.5 | 2.5 ± 1.3 | 3.2 ± 0.8 | 3.8 ± 0.8 | 2.5 ± 1.1 | 4.3 ± 0.9 | 4.6 ± 1.3 | 4.6 ± 0.8 | 5.1 ± 1.1 | 5.6 ± 0.8 |
| 22:0 | 2.4 ± 0.9 | 4.2 ± 2.5 | 2.7 ± 1.4 | 1.4 ± 1.3 | 0.7 ± 0.4 | 1.6 ± 1.0 | 1.2 ± 0.5 | 0.7 ± 0.5 | 1.0 ± 0.6 | 1.2 ± 0.7 | 0.6 ± 0.3 |
| 22:4 | 2.3 ± 0.8 | 2.2 ± 1.1 | 1.9 ± 0.9 | 0.6 ± 0.4 | 2.3 ± 1.4 | 1.6 ± 1.2 | 1.0 ± 0.6 | 0.6 ± 0.6 | (*) 0.3 ± 0.2 | 2.1 ± 0.7 | 1.1 ± 0.6 |
| 22:6 | 0.1 ± 0.1 | - | - | - | 0.3 ± 0.2 | - | 0.4 ± 0.4 | - | - | - | - |
| 24:0 | 3.8 ± 0.9 | 4.0 ± 1.3 | 3.1 ± 1.2 | (#) 0.2 ± 0.1 | (*) 1.1 ± 0.4 | 1.9 ± 1.9 | 3.1 ± 1.4 | (#) - | (*) 0.9 ± 0.4 | 2.0 ± 0.9 | (*) 0.9 ± 0.3 |

Figure 3.9


The effects of 17β-oestradiol in propane-1,2-diol, or of propane-1,2-diol alone, on the proportions of palmitate (16:0), stearate (18:0), oleate (18:1) and linoleate (18:2), in plasma VLDL-triacylglycerol, at varying times after injection.
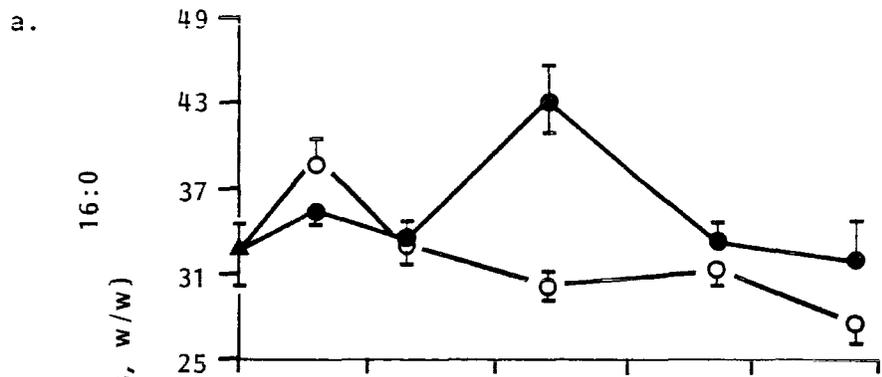

Consult the legend of Table 3.5 for details of experimental procedures.


▲ values for untreated birds.

○ values for oestrogen-treated birds.

● values for control birds, injected with propane-1,2-diol.


(a) The proportions of 16:0 in VLDL-triacylglycerol (%, w/w).

(b) The proportions of 18:0 in VLDL-triacylglycerol (%, w/w).

The results are expressed as the means (± S.E.M.) of 4 - 8 observations.

▲ values for untreated birds.

○ values for oestrogen-treated birds.

● values for control birds, injected with
   propane-1,2-diol.

(c) The proportion of 18:1 in VLDL-triacylglycerol (%, w/w).

(d) The proportion of 18:2 in VLDL-triacylglycerol (%, w/w).

The results are expressed as the means ($\pm$ S.E.M.) of 4 - 8
observations.
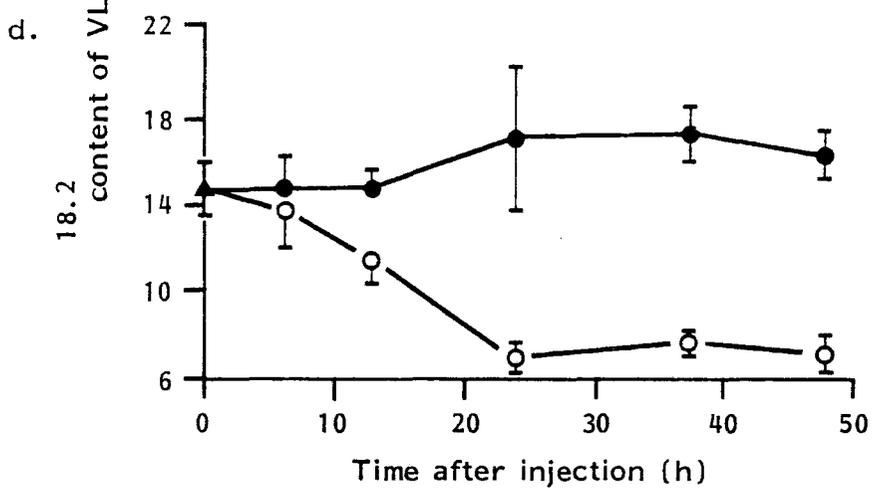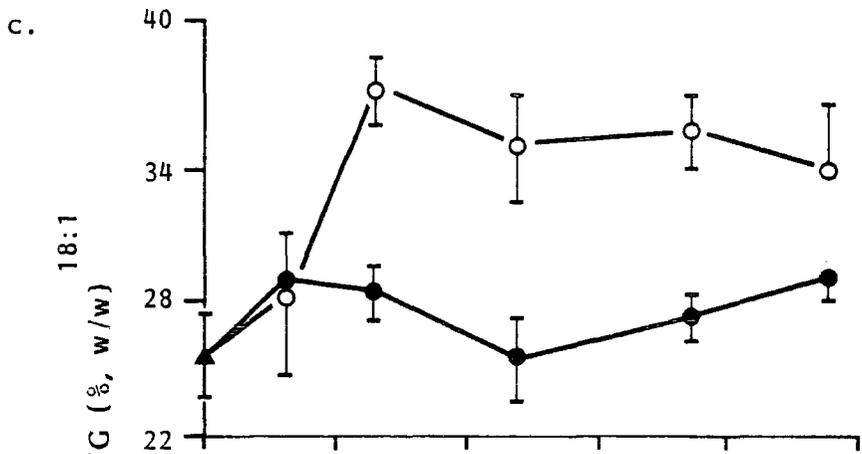
unsaturated:saturated fatty acids in the VLDL-phospholipid of
oestrogen-treated and control birds.

Details of the fatty acid composition of VLDL-phospholipids are shown
in Table 3.8. In untreated birds, the predominant fatty acids, in
order of percentage contribution, were 16:0, 20:4 (arachidonate),
18:0, 18:2 and 18:1 (Fig. 3.7d). After oestrogen treatment, a number
of marked changes occurred in the fatty acid composition of VLDL-
phospholipids (Fig. 3.10). Oestrogen-treated values of 16:0 were
significantly greater than the corresponding control values at 13
hours ($p < 0.01$), 24 hours ($p < 0.05$) and 37 hours ($p < 0.01$) after
injection (Fig. 3.10a).

The injection of propane-1,2-diol into control birds caused a
sustained decrease in the percentage contribution of 18:0 in VLDL-
phospholipid, when compared to untreated birds (Fig. 3.10b). In
addition, the administration of oestrogen caused a further decrease in
18:0 levels at 24 hours and 37 hours after injection. The decrease in
18:0 levels at 24 hours, 37 hours and 48 hours after hormone treatment
was not significant with respect to control birds, but it was
significant ($p < 0.05$) with respect to untreated birds.

Following oestrogen treatment there were significant increases, with
respect to controls, in the percentage contribution of 18:1 at 13 -
48 hours ($p < 0.05$) after injection (Fig. 3.10c).

The administration of oestrogen or propane-1,2-diol caused a transient
increase in the percentage contribution of 18:2 in VLDL-phospho-
lipids, when compared to untreated birds (Fig. 3.10d). The highest
level of 18:2 was observed at 6 hours after injection, then the level
decreased to that of the untreated value. Little difference was
observed between the oestrogen-treated and control birds, except at
37 hours after injection when the oestrogen-treated value was

Table 3.8

The effect of a single dose of 17β-oestradiol (0.75 mg in propane-1,2-diol/100 g body wt.), or of propane-1,2-diol only, on the fatty acid comosition of VLDL-phospholipid at varying times after injections.

Consult the legend of Table 3.5 for experimental details.

Values are the means ($\pm$ S.E.M.) of 3 - 10 birds; consult Table 3.5 for the number of birds involved in each group.

OE = values for oestrogen-treated birds.

C = values for control birds.

Data were analysed statistically by Student's 't' test.  Levels of statistical significance indicated without parentheses are for oestrogen-treated values with respect to corresponding control values. Levels of statistical significance indicated within parentheses are for that particular value with respect to the corresponding untreated value.

\* significant at $p < 0.05$

\# significant at $p < 0.01$

¥ significant at $p < 0.001$

For body weights, consult Table 3.3.

Some of these data are presented graphically in Fig. 3.7d and Fig. 3.10.

Time after injection (h)

| Fatty acid (%.w/w) | 0 | 6 OE | 6 C | 13 OE | 13 C | 24 OE | 24 C | 37 OE | 37 C | 48 OE | 48 C |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 14:0 | 0.1 ± 0.1 | 0.2 ± 0.2 | 0.2 ± 0.2 | * 0.4 ± 0.2 | (#) 0.9 ± 0.2 | 0.3 ± 0.1 | 0.2 ± 0.1 | (*) 1.0 ± 0.2 | (¥) 0.7 ± 0.1 | 0.3 ± 0.2 | 0.5 ± 0.5 |
| 16:0 | 19.6 ± 1.3 | 22.9 ± 0.9 | 17.6 ± 1.5 | # 22.0 ± 1.3 | 16.6 ± 0.7 | * 22.5 ± 2.1 | 16.6 ± 0.7 | # 21.2 ± 0.7 | 16.8 ± 0.7 | 19.1 ± 1.7 | 17.6 ± 1.2 |
| 16:1 | 3.0 ± 0.7 | 2.0 ± 0.3 | 2.4 ± 0.7 | 2.1 ± 0.4 | 3.7 ± 0.7 | 1.6 ± 0.4 | 1.6 ± 0.4 | 4.0 ± 0.6 | 2.7 ± 0.5 | 3.7 ± 0.9 | 4.0 ± 1.4 |
| 18:0 | 17.8 ± 0.9 | 17.4 ± 1.1 | 15.9 ± 0.9 | 15.5 ± 1.0 | 14.9 ± 1.4 | (#) 12.3 ± 1.2 | 15.1 ± 0.9 | (¥) 12.1 ± 0.8 | 15.0 ± 1.0 | (*) 12.7 ± 1.6 | (*) 12.5 ± 1.4 |
| 18:1 | 9.6 ± 0.9 | 13.0 ± 1.4 | 11.9 ± 1.8 | * (#) 14.7 ± 1.2 | 10.5 ± 0.6 | * 12.5 ± 1.3 | 7.9 ± 0.4 | * (*) 15.1 ± 1.8 | 9.3 ± 0.9 | * 13.5 ± 1.4 | 9.1 ± 0.8 |
| 18:2 | 11.5 ± 2.2 | (#) 19.3 ± 0.9 | (*) 17.8 ± 1.1 | 17.1 ± 1.7 | 13.5 ± 1.2 | 11.4 ± 1.6 | 14.5 ± 0.6 | * 11.8 ± 0.7 | 14.7 ± 1.0 | 10.4 ± 1.2 | 13.1 ± 2.4 |
| γ18:3 | 1.5 ± 0.8 | - | 0.1 ± 0.1 | - | 0.5 ± 0.2 | 0.2 ± 0.2 | 0.4 ± 0.2 | 0.4 ± 0.2 | 0.3 ± 0.2 | 0.6 ± 0.3 | 0.6 ± 0.4 |
| α18:3 | 0.1 ± 0.1 | - | - | - | 0.1 ± 0.1 | - | - | 0.2 ± 0.2 | 0.2 ± 0.2 | - | - |
| 20:0 | 6.6 ± 1.8 | 6.9 ± 1.4 | 12.0 ± 2.0 | # 6.4 ± 1.0 | 10.9 ± 1.0 | * 10.4 ± 0.9 | (#) 13.6 ± 0.9 | 9.6 ± 1.7 | 11.7 ± 1.6 | 10.3 ± 1.5 | (#) 12.9 ± 0.9 |
| 20:1 | 2.8 ± 1.6 | 0.2 ± 0.1 | - | 0.2 ± 0.1 | 0.2 ± 0.1 | 1.3 ± 1.3 | 0.4 ± 0.2 | 0.4 ± 0.1 | 0.5 ± 0.2 | 0.5 ± 0.5 | 1.4 ± 0.1 |
| 20:2 | 1.9 ± 0.7 | 1.3 ± 0.7 | (*) 0.1 ± 0.1 | 0.5 ± 0.2 | 0.6 ± 0.3 | 1.3 ± 0.9 | 0.7 ± 0.3 | 0.6 ± 0.2 | 0.5 ± 0.2 | 0.8 ± 0.5 | (*) 0.3 ± 0.2 |
| 20:3 | 1.3 ± 0.5 | * 0.9 ± 0.1 | 0.5 ± 0.1 | 0.7 ± 0.2 | 0.5 ± 0.3 | 1.0 ± 0.3 | 1.0 ± 0.2 | 0.9 ± 0.2 | 0.9 ± 0.1 | 1.1 ± 0.1 | 0.5 ± 0.1 |
| 20:4 | 18.5 ± 1.8 | 13.5 ± 1.7 | 18.5 ± 2.4 | # 15.7 ± 1.5 | (*) 24.1 ± 1.8 | * 19.2 ± 1.5 | (*) 23.9 ± 1.0 | 20.5 ± 2.6 | (*) 23.2 ± 1.3 | 19.7 ± 2.5 | 21.8 ± 2.1 |
| 22:0 | 2.1 ± 0.6 | (*) 0.6 ± 0.3 | 1.0 ± 0.1 | (*) 0.4 ± 0.1 | 1.1 ± 0.4 | 2.7 ± 1.5 | 1.7 ± 0.3 | 3.4 ± 2.2 | 1.7 ± 0.2 | 2.7 ± 0.6 | 1.8 ± 0.7 |
| 22:4 | 1.8 ± 0.7 | 0.3 ± 0.3 | 0.5 ± 0.5 | 2.7 ± 2.7 | 0.5 ± 0.5 | 1.3 ± 0.8 | 0.7 ± 0.3 | (*) - | 0.8 ± 0.6 | 1.1 ± 0.5 | 1.4 ± 0.8 |
| 22:6 | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.2 | 0.4 ± 0.2 | 0.04 ± 0.1 | 0.1 ± 0.1 | 0.4 ± 0.1 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.2 | 0.2 ± 0.2 |
| 24:0 | 1.0 ± 0.4 | 0.6 ± 0.3 | 1.3 ± 0.6 | 1.3 ± 1.2 | 1.0 ± 0.4 | 0.9 ± 0.5 | 1.5 ± 0.1 | 0.5 ± 0.2 | 0.8 ± 0.3 | 2.9 ± 1.2 | 2.3 ± 1.2 |

Figure 3.10


The effect of 17β-oestradiol in propane-1,2-diol, or of propane-
1,2-diol alone, on the proportions of palmitate (16:0), stearate
(18:0), oleate (18:1), linoleate (18:2) and arachidonate (20:4)
in plasma VLDL-phospholipid, at varying times after injection.


Consult the legend of Table 3.5 for details of experimental
procedures.


▲ values for untreated birds.

○ values for oestrogen-treated birds.

● values for control birds, injected with
   propane-1,2-diol.


(a) The proportion of 16:0 in VLDL-phospholipid (%, w/w).

(b) The proportion of 18:0 in VLDL-phospholipid (%, w/w).

(c) The proportion of 18:1 in VLDL-phospholipid (%, w/w).


The results are expressed as the means ($\pm$ S.E.M.) of 3 - 10
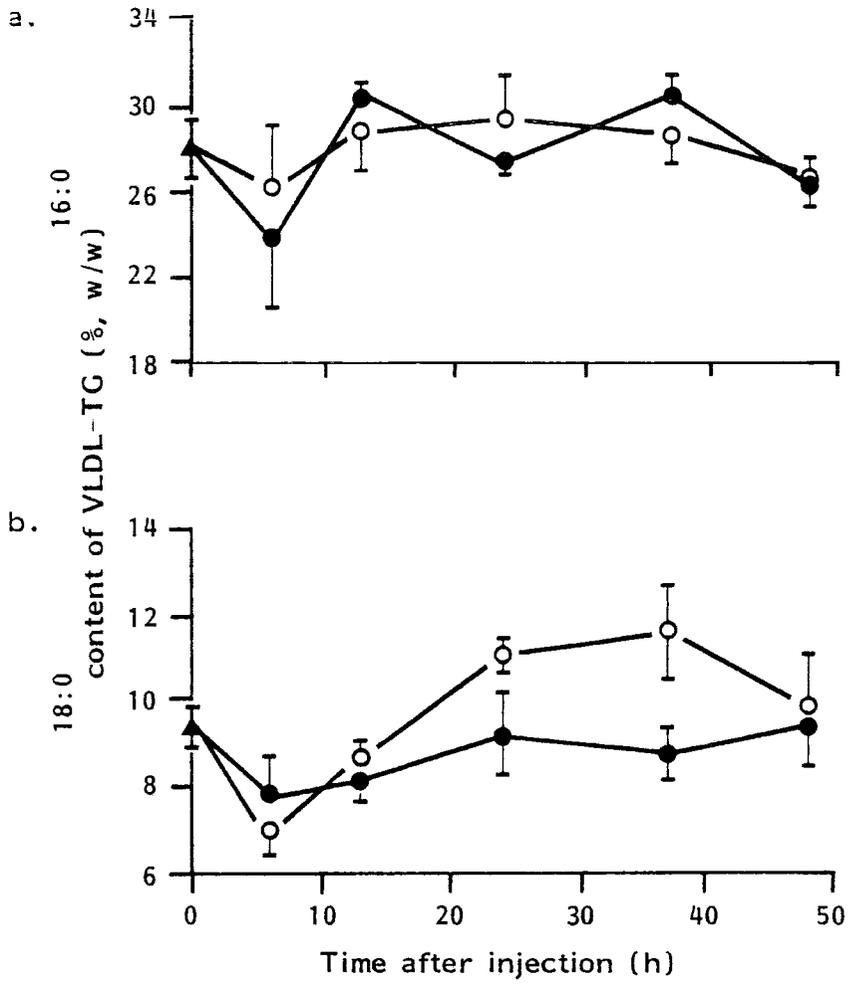observations.
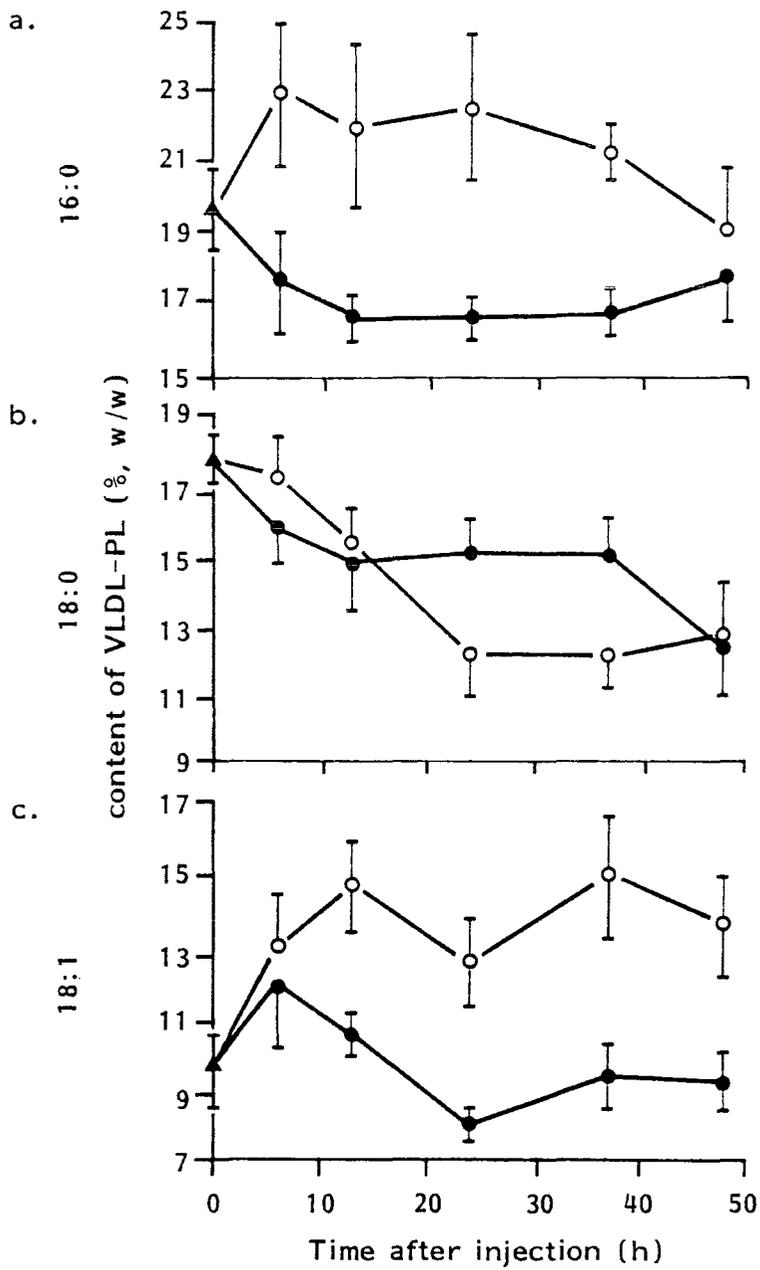
Fig. 3.10 /continued

▲ values for untreated birds.

◯ values for oestrogen-treated birds.

● values for control birds, injected with
   propane-1,2-diol.

(d) The proportion of 18:2 in VLDL-phospholipid (%, w/w).

(e) The proportion of 20:4 in VLDL-phospholipid (%, w/w).

The results are expressed as the means ($\pm$ S.E.M.) of 3 - 10
observations.
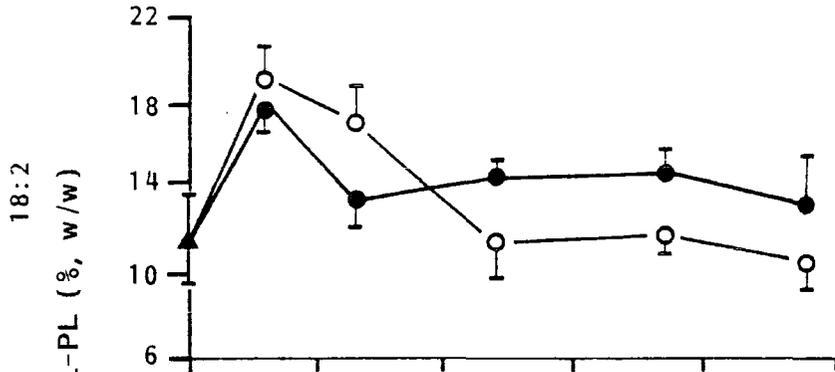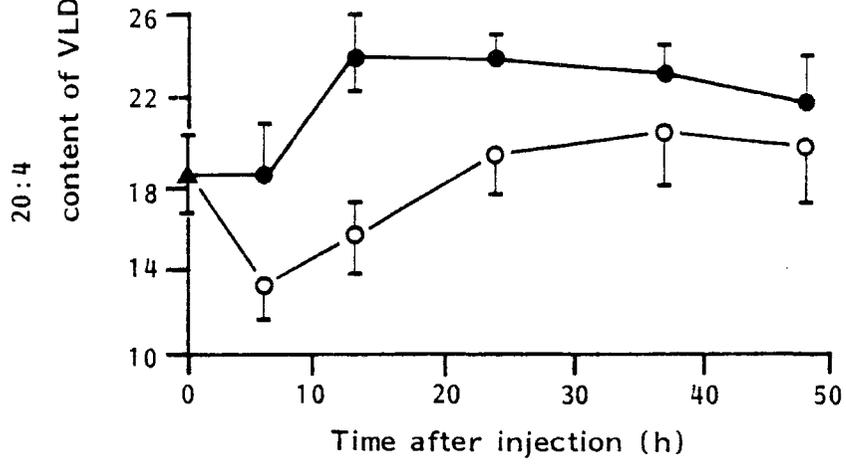
significantly lower than the control value (p < 0.05).

Oestrogen-treated values of 20:0 (arachidate) were significantly
lower than the corresponding control values at 13 hours (p < 0.01)
and 24 hours (p < 0.05) after injection (Table 3.8). At these times,
the control levels of 20:0 were greater than the untreated value, the
difference at 24 hours being significant (p < 0.01), and the
oestrogen-treated and untreated values were similar.

The changes in the levels of 20:4 (arachidonate) after oestrogen
treatment were similar to those observed in 20:0 in that the oestrogen
treated levels of 20:4 in VLDL-phospholipids were significantly lower
than control values at 13 hours (p < 0.01) and 24 hours (p < 0.05) after
injection (Fig. 3.10e). At these times, the control values were
significantly greater than the untreated value and the oestrogen-
treated and untreated values were similar.

In summary, therefore, the major effects of oestrogen treatment on
the fatty acid composition of VLDL-phospholipid was to cause an
elevation in the level of 16:0 from 13 - 37 hours after injection,
accompanied by an elevation in the level of 18:1 from 13 - 48 hours
after injection. These major alterations occurred together with
minor alterations in the levels of 18:0, 18:2 and 20:4 (Fig. 3.10).

DISCUSSION

1. Changes in lipid levels in plasma following oestrogen treatment

In untreated birds, the mean concentration of plasma triacylglycerol
was 126 mg/100 ml and this value is within the range of the rather
variable levels measured by other workers. Kudzma et al. (1975, 1979)
reported triacylglycerol levels of 56 - 89 mg/100 ml plasma and
37 mg/ml plasma in 850 - 1000 g chicks (sex undetermined) and
9 week old pullets, respectively. Hillyard et al. (1956) measured
a value of 187 mg/100 ml plasma in 2 month-old male birds and Chan
et al. (1977, 1978) recorded values of 150 mg/100 ml and 162 mg/100 ml
plasma, respectively in 3 week-old male chicks.

The mean plasma concentration of phospholipid in untreated birds in
this study was 3.5 mM and this agrees with the value of 3 mM which
was measured by Hillyard et al. (1956) in 2 month-old cockerels.

Following oestrogen treatment, there was a concomitant increase in
phospholipid and triacylglycerol levels in plasma (Fig. 3.2).
Increases were observed in both parameters at the 4 hour time-point
although significant increases over controls were not observed until
7 hours after injection. Maximum levels of phospholipids and
triacylglycerols were measured 48 hours after oestrogen treatment.
The increase in triacylglycerols was the greater, however, being a
27-fold increase over the control value compared to the 4.8-fold
increase in phospholipids at 48 hours after injection. The observed
accumulation of these two lipid classes may be a reflection of an
increase in the rate of their release into the plasma, from the liver
and the gut, and/or a decrease in the rate of their removal from
this compartment. The rate of accumulation of both lipid classes
follows the same pattern, in that from 26 - 34 hours after injection
there was a high rate of accumulation whereas after 34 hours, the

rate slowed dramatically. The similar profile of appearance of phospholipid and triacylglycerol suggests that similar mechanisms may be involved in the accumulation of these lipid classes in the plasma.

Similar increases have been observed by other workers following oestrogen treatment of birds, although the responses were not directly comparable since the dose and nature of the oestrogen used, and the sex, age and species of the birds used were not the same in all studies.

Kudzma et al. (1973), after administering diethylstilboestrol of varying doses (0.1, 1.0 and 5.0 mg/day) for 18 days to 5 day-old chicks (sex undetermined) observed dose-dependent increases in plasma triacylglycerol levels such that concentrations of 1839, 10241 and 12371 mg/100 ml plasma were recorded for the 3 dose levels used, respectively, compared to a control value of 27 mg/100 ml plasma. The maximum mean plasma triacylglycerol concentration measured in oestrogen-treated birds in the present study was 2727 mg/100 ml plasma and this lies within the range of values measured by Kudzma et al. (1973).

Hillyard et al. (1956), using 2 month-old cockerels and administering 24 mg diethylstilboestrol at 2 month intervals, recorded plasma triacylglycerol and phospholipid concentrations of 8000 - 14000 mg/ 100 ml plasma and 27.54 mM, respectively, when the birds were 8 months old.

Chan et al. (1977) administered 17$\beta$-oestradiol (1 mg) to 3 week-old male chicks and observed increases in plasma triacylglycerols within 10 hours of injection reaching a maximum at 48 hours (1500 mg triacylglycerol/100 ml plasma) before returning to control levels at

72 hours after treatment. However, using a dose of 5 mg 17β-oestradiol, Chan et al., (1978) observed a decrease in plasma triacylglycerol concentration at 3 and 6 hours after injection and a subsequent increase, reaching a maximum (2780 mg/100 ml plasma) at 48 hours after injection. No such initial decrease was observed in the present study and this could be a reflection of the differing doses of 17β-oestradiol used in the two studies since Chan et al. (1978) also reported that the extent of the initial decrease was dose-dependent.

Dashti et al. (1983) administered diethylstilboestrol (40 mg/kg body wt.) in corn oil to 19 day-old male turkeys and measured plasma lipid levels 24, 48 and 72 hours after treatment. Maximum responses in triacylglycerol and phospholipid concentrations were observed at 48 hours after injection, the oestrogen-treated values being 55-fold and 3-fold greater than the control values, respectively.

Kudzma et al. (1975) administered 4 daily doses of diethylstilboestrol (2.0 mg/day) to 850 - 1000 g domestic fowl (sex undetermined) and observed an increased plasma triacylglycerol concentration of 4000 - 7000 mg/100 ml plasma. In a separate study, Kudzma et al. (1979) administered a total of 50 mg diethylstilboestrol over 10 days to 9 week-old pullets and observed an increased plasma triacylglycerol level of 7000 mg/100 ml plasma.

The isolation of a VLDL fraction in the present study included those large VLDL particles (portomicrons) produced at the gut and containing lipids of dietary origin. However, the portomicron fraction has been shown to provide only a negligible contribution to the lipaemia in oestrogen-treated birds (Kudzma et al., 1973) and the lipaemia has been shown to develop when food is withheld (Lorenz, 1954). Therefore, in this study, the assumption was made that the VLDL fraction from

oestrogen-treated birds consisted mainly of endogenous lipid.

In the present study, the concentrations of VLDL-triacylglycerol and
VLDL-phospholipid in the plasma of untreated birds were 64 mg/100 ml
plasma and 0.15 mM, respectively. Chan et al. (1976, 1977) and
Jackson et al. (1978) observed untreated VLDL-triacylglycerol and
VLDL-phospholipid levels of 75 mg/100 ml plasma and 0.08 mM,
respectively, in the plasma of untreated 3 week-old male chicks.
Kudzma et al. (1975, 1979), however, have reported somewhat lower
plasma concentrations of VLDL-triacylglycerol in 850 g - 1000 g birds
(sex undetermined) and 9 week-old pullets, values being 30 mg/100 ml
and 15 mg/100 ml plasma, respectively. These lower values could well
be explained by the differences in the sex and age of the birds used.

By comparing the total plasma concentrations of triacylglycerol and
phospholipid in untreated birds with the plasma concentrations of
VLDL-triacylglycerol and VLDL-phospholipid in similar birds, it can
be seen that 51% of plasma triacylglycerol and only 4.3% of plasma
phospholipid is associated with the VLDL fraction.   Kudzma et al.
(1979) and Chan et al. (1977) have shown that 41% and approximately
50% of plasma triacylglycerol, in untreated 9 week-old pullets and
3 week-old cockerels, respectively, is associated with VLDL.  Kruski
& Narayan (1972) determined the chemical composition of lipoprotein
fractions in immature male birds and found that phospholipid
compromised 23% and 24% of LDL and HDL, respectively, whereas 19% of
VLDL consisted of phospholipid.  Kudzma et al. (1979) have shown
that, in immature female birds, HDL comprises 92% of the total
lipoprotein fraction, the rest being LDL and a very small amount of
VLDL.  Therefore, in the untreated state, the low proportion of total
plasma phospholipid associated with VLDL (4.3%) in the present study,
is probably a reflection of the fact that the bulk of the plasma
phospholipid is associated with the large pool of HDL and, to a
lesser extent, LDL particles.  However, chemical analysis of VLDL and

HDL has revealed that HDL is only 2% triacylglycerol whereas VLDL
are triacylglycerol-rich, the triacylglycerol content being 29% of
the total, (Kruski & Narayan, 1972) and so the low quantity of VLDL
particles, in the untreated state, contribute about half of the
total plasma triacylglycerol content.

Oestrogen treatment leads to increases in the plasma concentrations
of VLDL-triacylglycerol and VLDL-phospholipid. However, a difference
was observed in the time after injection when the increases became
significant (Fig. 3.3). At 6 hours post-injection, the VLDL-
phospholipid concentration was significantly greater than the control,
representing a 1.8-fold increase, whereas at the same time, the
VLDL-triacylglycerol concentration was only 10% higher than the
control value and the difference was not significant. At 13 hours
post-injection, the VLDL-phospholipid and VLDL-triacylglycerol
concentrations were 7.2-fold and 3.6-fold greater, respectively, than
the corresponding control values, both increases being significant.
This suggests that, in the initial stages, there is a non-coordinate
increase in some VLDL components. It is interesting to note that
Chan et al. (1978, 1980) have observed non-coordinate responses in
the plasma of oestrogen-treated male chicks, an initial decrease in
total plasma triacylglycerol concentration taking place when the
concentration of plasma VLDL apoprotein was increasing.

Both VLDL-triacylglycerol and VLDL-phospholipid concentrations were
seen to increase in the post-injection period, maximum concentrations
being attained at 48 hours for VLDL-phospholipid and at 37 hours for
VLDL-triacylglycerol although this latter value was not significantly
different from the 48 hour value. At all time-points after oestrogen
treatment, the extent of the increase was greater for VLDL-
phospholipid than it was for VLDL-triacylglycerol, maximum stimulation
representing a 97-fold and a 30-fold increase, respectively.
Calculations of the ratio of VLDL-triacylglycerol:VLDL-phospholipid

reveal that at zero time this ratio was 425 whereas at 37 hours after injection the oestrogen-treated value was 186 compared to the control value of 397. These observations of the two time-courses suggest that in response to oestrogen there is (a) an increase in the number of VLDL particles in the plasma accounting for large increases in VLDL-lipids and (b) an early and consistent alteration in the composition of the VLDL such that the ratio of triacylglycerol: phospholipid decreases representing a specific enrichment of the phospholipid component. Since the VLDL fraction represents a continuum of particles, with respect to composition and density (Kudzma et al. 1979), an alteration of the triacylglycerol:phospholipid ratio in the fraction might involve an alteration in the density distribution of particles. Chapman et al. (1977) have reported a decrease in the size of VLDL particles and an increase in LDL particle size has been observed by Chapman et al. (1977) and Hillyard et al. (1972) when hens come into lay.

The increases in plasma VLDL components after oestrogen treatment are similar to those observed by other workers. Chan et al. (1977) reported increases in plasma VLDL-protein, -phospholipid, -cholesterol and -triacylglycerol within 5 hours of administering 17β-oestradiol (1 mg) to young male chicks, reaching peak levels at 48 hours after injection before returning to control levels 68 hours after treatment. Similarly, Chan et al. (1978, 1980) observed an increase in plasma apo-VLDL-II 6 hours after injecting 3 week-old cockerels with 17β-oestradiol (5 mg/kg body wt.) rising steeply thereafter to an 18-fold increase over the basal level, 48 hours after injection.

By comparing the time-courses of VLDL-associated lipids with those total lipid levels in plasma it should be possible to gain an indication of the importance of the changes in VLDL levels and composition in the development of the lipaemia following oestrogen

treatment. However, it must be recognised that any conclusions would be tentative, at best, since determinations of whole plasma lipids and VLDL-lipids were performed on two different groups of birds and at slightly differing time-points after hormone treatment. Such a comparison reveals that, over the 48 hour period, of the increase in total plasma triacylglycerol, only up to approximately 60% is comprised of VLDL-triacylglycerol. This suggests that a sizeable part of the developing hypertriacylglycerolaemia is accounted for by a concomitant increase in the levels of HDL and/or LDL fractions. Kudzma et al.(1979) reported that treatment of 9 week-old pullets with a total of 50 mg diethylstilboestrol, over 10 days, caused a 400-fold rise in the concentration of VLDL and a 70-fold rise in LDL. HDL levels dramatically decreased from representing 92% of total lipoproteins to only 1% upon oestrogen treatment. It was reported that the LDL fraction, as well as increasing in quantity (as measured by LDL-protein in plasma), had a greater proportion of associated triacylglycerol. Hence it seems likely that the remainder of the oestrogen-induced hypertriacylglycerolaemia observed in the present study, was caused by increased levels of LDL. It should be noted that, after oestrogen treatment the VLDL and LDL are compositionally similar, with respect to lipid and apoprotein content, and this has led to the suggestion that the two represent a single lipoprotein class in which the 'LDL' fraction represents remnant particles produced by hydrolytic modification of VLDL (Kudzma et al., 1979)

A comparison of the increases of plasma VLDL-phospholipid and total plasma phospholipid after oestrogen treatment shows that, over the 48 hour post-injection period, the VLDL fraction accounts for an increasing percentage of the total. At zero-time, the VLDL-phospholipid level represents 4.3% of the total plasma phospholipid level (mentioned earlier in the Discussion) whereas a comparison of the last time-points of the two studies (48 hours and 44 hours) indicates

a 68% contribution to the total plasma phospholipid level. Such an observation is consistent with the large decrease in HDL levels observed by Kudzma et al. (1979) after oestrogen treatment of birds, an increase in the number of VLDL particles and an enrichment of these particles in phospholipid, relative to triacylglycerol, discussed earlier.

Measurement of total free fatty acids in plasma of untreated birds gave a mean value of 0.34 mM, which compares well with values of 0.2 - 0.3 mM reported by previous investigators (Heald & Badman, 1963; Heald & Rookledge, 1964; Annison, 1971; Kudzma et al., 1973). The only significant difference observed between total plasma free fatty acid levels in oestrogen-treated and control birds was at 2 hours after injection (Fig. 3.2) when the oestrogen-treated value was similar to the untreated value and the control value was significantly higher than the untreated value. This suggests that a high control value was caused by the injection/handling procedure. Plasma free fatty acid levels have been shown to increase after handling immature chickens (Freeman & Manning, 1976), probably due to the release of free fatty acids from adipocytes under the influence of glucagon (Sitbon et al., 1980). Since oestrogen treatment did not evoke a similar increase after 2 hours, the treatment either overrides the release of free fatty acids from the adipocytes and/or causes the enhanced removal of free fatty acids from the circulation at this time-point. A similar significant decrease in plasma free fatty acids was observed by Pageaux et al. (1981) 1 hour after injecting 16-day old female quail with oestradiol benzoate (0.2 mg/kg body wt.) although increased levels were observed in the subsequent 23 hour period. It was suggested that, at least in the early stages, oestrogen treatment leads to the removal of fatty acids by the liver from the circulation.

The results of the present study, in which no significant increases

in plasma free fatty acid levels were observed after oestrogen
treatment, do not agree with previous reports of increased levels
in oestrogen-treated and laying birds. Plasma free fatty acid
levels of 1.4 mM have been reported in laying birds (Heald & Badman,
1963; Heald & Rookledge, 1964). Kudzma et al. (1973) administered
0.5 mg diethylstilboestrol/day to young chicks (sex undetermined)
for 6 days and observed significantly elevated plasma free fatty
acid levels after the second daily dose. Thereafter, oestrogen-
treated birds exhibited free fatty acid levels similar to control
birds, although both groups of birds had increasing free fatty acid
levels throughout the six-day period. Hypertriacylglycerolaemia
was apparent from 2 - 6 days in the experiment and it was suggested
that the over-production of triacylglycerol by the liver was not
dependent upon fatty acids arriving from extra-hepatic sources, since
elevated free fatty acid levels appeared after the hypertriacyl-
glycerolaemia. In the present study, plasma free fatty acid levels
were not elevated before triacylglycerol levels rose and, therefore,
it could be argued that the hypertriacylglycerolaemia may not be
dependent on hepatic lipogenesis involving pre-formed free fatty
acids arriving from extra-hepatic sources, suggesting that only
hepatic lipogenesis de novo is important, in the elevated triacyl-
glycerol levels, at least in the early stages. This argument,
however, is open to question since an increased flux of free fatty
acids from peripheral tissues to the liver, involving increased
release into the blood, increased uptake and an increased turnover of
free fatty acids, need not manifest itself with increased free fatty
acid levels in the plasma.

VLDL lipids are hydrolysed by lipoprotein lipase at the luminal
surface of endothelial cells in the capillaries (Scow et al., 1976),
causing the production of free fatty acids. In the present study,
however, even when the hypertriacylglycerolaemia was well-established
there were no increases in plasma free fatty acid levels and such an

observation might be explained by one or more of the following:-

(a)   Increasing amounts of VLDL-triacylglycerol were hydrolysed but the
      free fatty acids released were quickly removed from the
      circulation;

(b)   the increasing VLDL-triacylglycerol levels rapidly saturated
      lipoprotein lipase;

(c)   VLDL particles showed a reduced susceptibility to the hydrolytic
      action of lipoprotein lipase.  In support of this possibility,
      Griffin (1981) has shown that VLDL from laying hens are only
      hydrolysed to a limited extent by isolated lipoprotein lipase and
      has suggested that the VLDL have a low content of a lipoprotein
      lipase activator protein.

Following oestrogen treatment, there was a decrease in the mean
levels of plasma albumin-bound free fatty acids compared to controls
(Fig. 3.3) at all time-points after injection, although only at 24
hours post-injection was this decrease significant.  Comparison of
the time-courses of total plasma free fatty acids (Fig. 3.2c) and
albumin-bound free fatty acids (Fig. 3.3c) suggests that this
reduction is the result of a greater proportion of the total free
fatty acids being bound to triacylglycerol-rich lipoproteins after
oestrogen treatment.  This is consistent with the observations that
oestrogen treatment leads to an increase in plasma VLDL levels and
that in laying hens much of the plasma free fatty acids are associated
with the elevated levels of VLDL (Leclerq et al., 1979).  Griffin &
Mitchell (1984) reported that only approximately 5% of plasma free
fatty acids in laying hens was albumin-bound, representing
concentrations of 0.02 - 0.05 mM.  In the present study, a tentative
comparison of the concentrations of albumin-bound free fatty acids,
measured in one group of birds, and total free fatty acids in plasma,
measured in a different group of young male chicks, suggests that in

the untreated state, 74% of total plasma free fatty acids were
albumin-bound. A similar comparison of albumin-bound free fatty
acid levels 24 hours after oestrogen treatment and total free fatty
acid levels in plasma 26 hours after oestrogen treatment suggests
that 40% of total plasma free fatty acids were albumin-bound,
representing a concentration of 0.15 mM. The differences between
these figures and those reported by Griffin & Mitchell (1984) may
be due to differences in the age and sex of the birds.

2. Changes in the levels of liver lipids following oestrogen treatment

Liver lipid levels were expressed both on a whole liver and on a
'cellular' basis, the assumption being made that each cell contains
the same amount of DNA after oestrogen treatment. Significant
increases in the amount of triacylglycerol per 'cell' and per liver
were first observed 11 hours and 7 hours after hormone treatment,
respectively. At 7 hours after oestrogen administration, the
'cellular' content of triacylglycerol showed a 1.7-fold increase
over the control value but this increase was not statistically
significant. The liver triacylglycerol levels determined were a
reflection of the balance between (a) the rate of appearance of
triacylglycerol in the liver and (b) the rate of removal of
triacylglycerol from the liver, mainly in the form of lipoproteins.
It is likely that increased synthesis of triacylglycerol plays a
major role in the accumulation of liver lipids since the increase
in liver triacylglycerols accompanies the appearance and development
of hypertriacylglycerolaemia.

At 20 hours after oestrogen treatment, there was a 6.6-fold increase
in the whole organ content and a 6.0-fold increase in the 'cellular'
content of triacylglycerol in the liver, with respect to control
birds whereas maximum liver triacylglycerol levels were reached 34
hours after hormone treatment, representing 23-fold and 15-fold.

increases, over controls, in the whole organ and 'cellular' contents, respectively (Fig. 3.4). During the 44 hour post-injection period a consistent increase in mean liver weight, expressed as a percentage of body weight, was observed in oestrogen-treated birds when compared to control birds representing a 1.4-fold increase at 20 hours and a 1.7-fold increase at 34 hours. These observations, when considered together, suggest that the increase in liver weight and liver triacylglycerol in the first 20 hours after oestrogen treatment occurs in the absence of a substantial amount of cell division whereas at 34 hours after oestrogen treatment, the increases may be in part, due to an increase in liver cell division.

Dashti et al. (1983) administered diethylstilboestrol (40 mg/kg body wt.) to 19 day-old male turkeys and observed increased liver triacylglycerol levels, expressed as triacylglycerol per unit weight of liver, reaching a maximum 48 hours after injection, representing a 3.7-fold increase over controls.

Increases in liver phospholipid levels occurred at the same time as increases in liver triacylglycerol content, in the present study, significant increases being observed at 7 hours after oestrogen treatment in both the whole organ and 'cellular' phospholipid levels when compared to controls (Fig. 3.5). Concurrent increases were observed in liver and plasma phospholipid levels after hormone treatment suggesting that oestrogen administration led to coordinated increases in the hepatic synthesis and secretion of phospholipid. A difference was observed between the time-course of hepatic phospholipid accumulation after oestrogen treatment and the time-course of hepatic triacylglycerol accumulation in that maximum liver phospholipid content, on a 'cellular' basis, was observed at 20 hours after injection whereas the maximum triacylglycerol 'cellular' content of liver was observed at 34 hours. Following oestrogen treatment, phospholipid is needed for both lipoprotein synthesis and for

membrane proliferation in order to increase the synthetic capacity of hepatocytes, and therefore it is diffucult to gauge the significance of the difference in the hepatic accumulation of phospholipid and triacylglycerol expressed on a cellular basis. Expression of liver phospholipid levels on a whole organ basis shows that a maximum was attained 34 hours, rather than 20 hours, after oestrogen treatment and this may be due to an increase in cell division occurring in the period 20 - 34 hours post-injection.

Significant increases in the liver levels of free fatty acids, expressed on a whole organ basis, were observed at 26 hours, 34 hours and 44 hours after oestrogen treatment, compared to controls, although non-significant increases were apparent at the 15 hour and 20 hour time-points. Expression of the liver free fatty acid data on a 'cellular' basis showed significant increases, with respect to controls, at 26 hours and 44 hours after oestrogen treatment (Fig. 3.6). The extent of the significant increases were greater for the free fatty acid levels expressed on a whole organ basis than the levels expressed on a 'cellular' basis and this may be a reflection of the involvement of cell division in the observed increase in liver size following oestrogen treatment. For instance, at 34 hours post-injection, 1.3-fold and 2.0-fold increases were observed in the liver levels of free fatty acids, expressed on a cellular basis and a whole organ basis, respectively.

Hawkins & Heald (1966) reported a 2-fold increase in the free fatty acid pool in livers of hens when coming into lay and their value of 0.62 - 0.84 $\mu$moles/g wet liver for immature hens is in good agreement with that of 0.80 $\mu$moles/g wet liver measured in untreated birds in the present study.

Hepatic free fatty acids, measured in the present study, could have

arisen from a number of sources including uptake from the plasma
and fatty acid synthesis de novo from non-lipid precursors. The
observations of increasing hepatic lipid levels after oestrogen
treatment are consistent with other reports of variations in hepatic
lipogenesis and in levels of liver VLDL components after similar
treatment. Courtney (1984) administered 17β-oestradiol (1.0 mg/
100 g body wt.) to 3 - 5 week-old male chicks and measured lipogenic
activity in liver slices from birds killed at differing times after
injection. Significant increases in lipogenesis de novo, assayed
by measuring the incorporation of $^3H_2O$ into lipids, and lipogenesis
from pre-formed fatty acids, as measured by the incorporation of
$[9,10 - {}^3H]$ palmitate into lipids, were observed 14 - 44 hours after
oestrogen treatment reaching maximal 2.4-fold and 2.47-fold increases
at 40 - 44 hours, respectively. In both instances, the majority
of the recovered radioactivity was in the form of triacylglycerol
(approximately 90%). A slight, though not statistically significant,
increase in lipogenesis de novo was also observed $3\frac{1}{2}$ - $7\frac{1}{4}$ hours
after oestrogen treatment. Courtney & Manning (1984) reported
increased hepatic fatty acid synthetase activity, expressed as total
organ activity, in young male chicks 48 hours after receiving an
injection of 17β-oestradiol (0.75 mg/100 g body wt.), when compared
to control birds. Interestingly, in the same experiments, Courtney
(1984) and Courtney & Manning (1984) reported significant increases
in hepatic lipogenesis de novo and in the specific activity of
hepatic fatty acid synthetase in control birds, with respect to
untreated birds, up to 17 hours and up to 26 hours after injection
of vehicle, respectively. This is consistent with the measurements
of hepatic free fatty acid levels in the present study which
show that, from 15 - 20 hours after injection, both the oestrogen-
treated and control values increased, although the control values
were not significantly different to untreated values.

Chan et al. (1976) injected 3 week-old male chicks with diethyl-stilboestrol (2.5 mg) before measuring the rate of VLDL synthesis in liver slices, assayed by the incorporation of $[^3H]$ lysine into immunoprecipitated protein, from birds killed at differing times after treatment. An increase in VLDL synthesis was observed within 2 hours of injection and reached a maximum at the 24 hour time-point, representing a 6-fold increase over controls. In a similar experiment in which 17$\beta$-oestradiol (1 mg) was used, Chan et al. (1977) reported a maximal 4-fold increase in VLDL synthesis in liver slices from birds killed 17 hours after hormone treatment. Studies by Codina-Salada et al. (1983) supported these observations by demonstrating an increase in the number of molecules of mRNA coding for apo-VLDL-II in hepatocytes, in vivo, within 1 - 3 hours of injecting 5 week-old cockerels with 17$\beta$-oestradiol (5 mg/kg body wt.).

Increased liver lipid levels in oestrogen-treated young female birds have been reported which are similar to those in oestrogen-treated young cockerels. Balnave & Pearce (1974) reported no increase in liver lipid content, expressed as the weight of lipid per 100 g liver, of 4 week-old pullets in the first 12 hours after treatment with oestradiol dipropionate (2 mg). In a later experiment, however, the same treatment of similar birds led to an increase in liver lipid content (as % of liver wt.), from 4.69% in controls to 15.81% two days after injection (Pearce & Balnave, 1975). Pearce & Brown (1971) administered oestradiol dipropionate (15 mg), in the form of a subcutaneous implant, to immature pullets for one week causing an approximately 50% increase in levels of total liver lipids, expressed as weight of lipid/liver, when compared to controls. However, expression of the liver lipid content as the percentage of wet liver weight showed no statistically significant difference.

3.  Changes in the fatty acid composition of plasma and liver lipids
    following oestrogen treatment

The fatty acid composition of triacylglycerol isolated from the liver
and from the VLDL fraction of plasma lipoproteins of untreated birds
are similar, in that 16:0, 18:1, 18:0, 18:2 and 16:1 were found to
be the major components.  However, distinct differences are evident
in the percentage contributions of each major fatty acid component
of liver triacylglycerol and VLDL-triacylglycerol, the most obvious
being with respect to 18:2.  In liver triacylglycerol, 6% was
comprised of 18:2 whereas the figure was approximately 15% in VLDL-
triacylglycerol.  Similar differences between liver and plasma
lipids have been reported by other workers.  Balnave (1971) observed
that the percentage contributions of 18:2 to the fatty acid
compositions of total lipids from liver and blood, in immature
pullets, were 18% and 28%, respectively, and the percentage
contributions of 18:0 in the two tissues were 30% and 18%
respectively.  Balnave (1971) suggested that the differences observed
with the immature birds might have been physiologically important,
especially the greater levels of 18:2 observed in the blood.  Fujii
et al. (1984) also observed a much greater percentage of 18:2 in
the plasma total lipid of 21 day-old male chicks than the liver
total lipid, values of 13% amd 23% being reported, respectively.

A comparison of the fatty acid composition of total lipid in liver
and plasma cannot yield a great deal of information simply because
there are many liver lipids, such as membrane phospholipids, which
are not produced for export and hence there is no clear logical
relationshop between total lipids in the liver and those in the
circulation.  On the other hand, comparison of the fatty acid
composition of triacylglycerol in the liver and in the plasma is
more logical, since this lipid is produced largely for export.  One
reason for the differences observed in the triacylglycerol

compositions in the present study could be that, although VLDL-associated tricylglycerol is synthesised in the liver, only certain molecular species may be released into the plasma as lipoprotein. Christie & Moore (1972) proffered a similar explanation of the differences they observed in the lipid compositions of plasma and liver triacylglycerols and phospholipids in laying hens. On the other hand, lipoproteins in the plasma are susceptible to modification by enzymes such as lipoprotein lipase, which hydrolyses triacylglycerols, and lecithin:cholesterol acyltransferase which transfers unsaturated fatty acids from position 2 of phosphatidylcholine to cholesterol. Similar plasma lipoprotein metabolism, therefore, could also explain, in part, the observed differences in fatty acid composition of triacylglycerol from liver and VLDL. A further explanation is that the VLDL fraction isolated in the procedure adopted in the present study, and others, includes the portomicron VLDL particles whose triacylglycerol is composed of fatty acids of dietary origin. It is interesting to note that since the oestrogen-induced increase in VLDL-triacylglycerol in the plasma of chicks is due almost completely to the release of VLDL from the liver (Kudzma et al., 1973), the extent of any possible modification of the fatty acid composition of VLDL-triacylglycerol by the portomicron fraction would be greatly reduced after oestrogen treatment.

The fatty acid composition of VLDL-phospholipid in untreated chicks differs from that of VLDL-triacylglycerol mainly in that 20:4, a major component of animal phospholipids (Gurr & James, 1980), and 18:0 are present in larger proportions and that the proportions of 16:0, 16:1, 18:2 and especially 18:1 are decreased.

Although direct comparisons are not available, the fatty acid compositions of liver and VLDL-triacylglycerol and VLDL-phospholipid

from untreated birds in the present study are consistent with the literature. Fujii et al. (1984) reported that the major fatty acids of liver and plasma total lipids in 21 day-old male chicks were 16:0, 18:0, 18:1 and 18:2 with smaller contributions of 16:1 and 20:4.

The molar ratios of unsaturated/saturated fatty acids for liver triacylglycerol, VLDL-triacylglycerol and VLDL-phospholipid from untreated birds, in the present study, were 1.13, 1.22 and 1.06 respectively, indicating that in all cases there was a greater proportion of unsaturated fatty acids. Fujii et al. (1984) reported a molar ratio of 0.88 for total liver lipid in 21 day-old male chicks, the difference between their value and that of 1.13 for liver triacylglycerol in the present study probably being due to the difference in lipid classes studied and the greater sensitivity, in the present study, in the detection of polyenoic long chain fatty acids (20 carbon atoms and above).

Following oestrogen treatment, the proportion of 18:1 in liver-triacylglycerol was significantly greater than the proportion in control birds, the increase being observed from 13 - 48 hours after injection. This increase accompanies the observed accumulation of liver triacylglycerol, which has been discussed earlier. Other oestrogen-induced compositonal changes in liver triacylglycerol are not as persistent. Six hours after oestrogen treatment the proportion of 16:0 was significantly greater than both the corresponding control and untreated values. Dashti et al. (1983) after administering diethylstilboestrol (40 mg/kg nody wt.) to 19 day-old male turkeys and killing them 48 hours later, observed a significant increase in those liver triacylglycerols rich in 16-carbon fatty acids. Of these fatty acids, 16:0 is the major product of fatty acid synthesis in avian cells (Brodie et al., 1964), so the increased proportion of 16:0 observed in the present study might, therefore, be a reflection of increased fatty acid synthesis de novo.

One of the more obvious changes in liver triacylglycerol was observed 24 hours after injection, when control birds exhibited markedly different fatty acid composition to untreated birds. Significant increases, with respect to untreated birds, were observed in the saturated fatty acids 14:0, 16:0 and 18:0 whereas significant decreases were observed in the unsaturated fatty acids 18:1 and 18:2. These changes are reflected in the molar ratio of unsaturated:saturated fatty acids for liver triacylglycerol in control birds, 24 hours after injection, the value of 0.52 being significantly lower than that of 1.13 for untreated birds. The possibility exists that the markedly different fatty acid composition of liver triacylglycerols in control birds, 24 hours after injection, was the result of experimental error. However, the 'mean' fatty acid composition quoted for these birds was derived from analysis on 6 birds, each of which had distinctly altered liver triacylglycerol fatty acid compositions. A perhaps more likely explanation, therefore, is that the observed fatty acid composition of liver triacylglycerol in 24 hour control birds, was the result of the handling/injection procedure and/or a metabolic effect of the propane-1,2-diol injection vehicle.

Other changes in the fatty acid composition of liver triacylglycerol after oestrogen treatment include an elevation of the proportion of 18:2 at 13 hours and 24 hours after injection, when compared to control birds, although this may be due, in part, to a decrease in control values at this time. The proportions of 20:0 and 20:4 in liver triacylglycerol of oestrogen-treated birds decreased, compared to controls, at 6 hours and 13 hours after injection, although in each case, the oestrogen-treated value was not significantly different from the corresponding untreated value.

The overall effect of the compositional changes in liver

triacylglycerol following oestrogen treatment was, therefore, an increase in the proportion of unsaturated fatty acids at the expense of saturated fatty acids. This is reflected in the change in the molar ratio of unsaturated:saturated fatty acids which changed from 1.13 in the untreated state, to 1.61 at 48 hours after injection. Fujii et al. (1984) observed a similar increase in this ratio in total liver lipids from 21 day-old male chicks which had received 6 daily injections of 17$\beta$-oestradiol (20 mg/kg body wt.) the value rising from 0.88 to 1.32.

Following oestrogen treatment, an increase in the level of VLDL-triacylglycerol was apparent in the plasma of male chicks, the liver being responsible for the bulk of this increase. It is therefore interesting to note any similarities and differences between the fatty acid compositional changes observed in liver-triacylglycerols and plasma VLDL-triacylglycerol following oestrogen treatment. The increase in the proportion of 18:1 observed in liver triacylglycerol was also observed in VLDL-triacylglycerol, the increase being apparent from 13 - 48 hours post-injection. The significantly different composition of liver triacylglycerol in control birds 24 hours post-injection, however, was not observed in the VLDL-triacylglycerol of the same birds. Such differences between liver triacylglycerol and VLDL-triacylglycerol in non-lipaemic control birds can be explained by the fact that not all the molecular species of liver triacylglycerol might be released into the plasma and also that the composition of liver triacylglycerol released into the plasma as VLDL-triacylglycerol will be masked by the composition of the pre-existing pool of plasma VLDL-triacyl-glycerol. This last consideration will be especially important in oestrogen-treated hypertriacylglycerolaemic chicks with larger plasma VLDL-triacylglycerol pools and it is interesting to note that the slight variations in the levels of 16:0, 20:0 and 20:4 in liver triacylglycerol following oestrogen treatment were not apparent in

the VLDL-triacylglycerol of the same birds.

A major difference between the composition of liver triacylglycerol
and VLDL-triacylglycerol after oestrogen treatment is apparent when
the proportion of 18:2 is studied.  In VLDL-triacylglycerol, the
proportion of 18:2 is significantly reduced, with respect to control
and untreated birds and birds, at 13 - 48 hours after oestrogen
treatment, the reduction coinciding with elevated VLDL-triacyl-
glycerol levels.  Linoleate (18:2) is an essential fatty acid,
implying that it cannot be synthesised de novo by the animal and must
be obtained from the diet.  It is possible, therefore, that the
dietary intake of 18:2 may be insufficient to meet the increased
demand for fatty acid incorporation into triacylglycerols in the
liver, following oestrogen treatment, before being released into the
plasma.  A decrease in the low levels of $\alpha$-linolenate (18:3), an
essential fatty acid, in VLDL-triacylglycerol from 24 - 48 hours
after oestrogen treatment supports this explanation.  It is
interesting to note, however, that a reduction in 18:2 levels was not
observed in liver triacylglycerol of oestrogen-treated birds.

Similar reduction in the proportion of 18:2 in the plasma lipids of
oestrogen-treated birds have been reported by other workers.  Fujii
et al.  (1984) reported a decrease in the proportion of 18:2 in
total plasma lipids of 21 day-old oestrogen-treated cockerels, from
23% to 10%, and Balnave (1971) reported a similar decrease in 18:2
levels in the plasma lipids of oestrogen-treated 16 week-old
pullets, from 28% to 16%.  In both reports it was noted that the
proportion of 18:2 in the liver and plasma lipids of oestrogen-treated
birds were similar contrary to the situation in untreated birds,
where the proportion of 18:2 in plasma lipids was higher than that in
liver lipids.  These observations are consistent with the similar
fatty acid compositions of liver and VLDL-triacylglycerol from

oestrogen-treated birds in the present study. For example, 48 hours after treatment, the proportion of 18:2 in triacylglycerol from liver and VLDL were 6.2% and 7.2%, respectively. Such observations are probably a reflection of an increased hepatic synthesis of triacylglycerol for specific release into the plasma as VLDL-triacylglycerol.

The molar ratio of unsaturated:saturated fatty acids in VLDL-triacylglycerol following oestrogen treatment does not significantly differ from the control value throughout the 48 hour period and this is explained by the fact that the major changes involve an increase in one unsaturated fatty acid, 18:1, and a decrease in another unsaturated fatty acid, 18:2.

An increase in the proportion of 18:1 also occurred in VLDL-phospholipid, at 13 - 48 hours after oestrogen treatment and this change occurred as the level of VLDL-phospholipid in plasma increased. Lipiello et al. (1979) reported increased stearyl-CoA desaturase activity in crude microsomal extracts from the liver of male chickens (800 - 1200 g body wt.) which had been injected with 17$\beta$-oestradiol (3.5 mg/100 g body wt.). A 7-fold increase in the specific activity of the enzyme was observed in the 24-hour period following injection and the activity remained high through 48 hours before declining at 72 hours post-injection. Therefore, the increases in 18:1 in the plasma and liver lipids of oestrogen-treated birds in the present study may be due to an increase in hepatic stearyl-CoA desaturase activity within 13 hours of injection. Stearyl-CoA desaturase introduces a single double-bond into stearic acid (18:0) producing oleic acid (18:1). A decrease in the proportion of 18:0 in VLDL-phospholipid was evident 24 - 48 hours after oestrogen treatment, although, at each time-point, the decreased oestrogen-treated value was significantly lower than the untreated value and not significantly different from

the corresponding control value. No significant decreases in the
proportions of 18:0 in the liver and VLDL-triacylglycerol of
oestrogen-treated birds were detected. The changes in VLDL-
phospholipid composition after oestrogen treatment also differed
from the responses in triacylglycerol in that a persistent increase
in the proportion of 16:0 was observed in VLDL-phospholipid, the
increase over controls being significant from 13 - 37 hours after
oestrogen treatment. This increase could be a reflection of
increased hepatic fatty acid synthesis de novo leading to a large
supply of 16:0, the major product of fatty acid synthesis in avian
systems (Brodie et al., 1964), for incorporation into phospholipids.
In contrast, oestrogen treatment produced only a transient increase
in the proportion of 16:0 in liver triacylglycerol, at 6 hours post-
injection, but there was no oestrogen-induced increase in the
proportion of 16:0 in VLDL-triacylglycerol.

Contrary to the situation in VLDL-triacylglycerol, there was no
persistent decrease in the proportion of the essential fatty acid
18:2 in VLDL-phospholipid following oestrogen-treatment. However,
both oestrogen-treated and control birds exhibited greatly increased
18:2 proportions in VLDL-phospholipid at the 6 hour time-point,
compared to untreated birds, suggesting that this may be a 'stress'
effect evoked by the handling/injection procedure.

Decreases in 20:0 and 20:4 occurred in VLDL-phospholipid at 13 hours
and 24 hours after oestrogen treatment, with respect to controls.
However, in both cases the difference seemed to be due to an increase
in the corresponding control value, the oestrogen-treated values not
being significantly different to the untreated value. Since 20:4
is an essential fatty acid the diminished proportion of 20:4, in
VLDL-phospholipid after hormone treatment, may be a reflection of
an inability to match the increasing demand for fatty acids with a
specific supply of 20:4.

No change in the molar ratio of unsaturated:saturated fatty acids in VLDL-phospholipids was observed following oestrogen treatment, the increase in the proportion of the unsaturated fatty acid, 18:1 being balanced by an increase in the proportion of the saturated fatty acid, 16:0.

Following oestrogen treatment, the bulk of the VLDL-phospholipid· and VLDL-triacylglycerol is synthesised in the liver, involving the incorporation of fatty acids in the form of 'activated' fatty acyl-CoA thioesters. It has been shown that the biosynthetic pathways for the synthesis of phospholipid and triacylglycerol are very similar, in the initial stages, leading to the production of phosphatidic acid (and diacylglycerol). It is well documented, however, that there is a degree of specificity in the way fatty acids are incorporated into the developing acylglycerols. In general, phospholipid metabolism makes more extensive demands on a supply of unsaturated fatty acids than does triacylglycerol metabolism (Gurr & James, 1980). It has also been shown that in laying hens there is a degree of specificity in the positions occupied by fatty acids in triacylglycerol and phospholipid molecules (Christie & Moore, 1972). Stereospecific analysis of, for instance, plasma triacylglycerols revealed that 16:0 constituted over 70% of the fatty acids in position 1 of the glycerol moiety while position 2 was occupied largely by 18:1 and 18:2 and position 3 comprised over 70% 18:1 with most of the remaining saturated components. A similar analysis of phosphatidyl choline from the plasma of laying hens revealed that position 1 was occupied largely by saturated fatty acids and position 2 by unsaturated components.

It is obvious that the changes occurring in the composition of VLDL-phospholipid and VLDL-triacylglycerol after oestrogen treatment are similar in some respects, notably the increase in the proportion

of 18:1, and different in other respects, the proportion of 16:0 increasing in VLDL-phospholipid while remaining unchanged in VLDL-triacylglycerol. Such a situation might arise if oestrogen were to affect the size and composition of the hepatic fatty acyl-CoA pool, from which specific types of fatty acids were incorporated in triacylglycerols and phospholipids.

The changes in fatty acid composition of blood and liver lipids observed in the present study are generally in agreement with similar reports of compositional changes in lipids of oestrogen-treated male and female domestic fowl, although differences in the age and sex of the birds and dose of oestrogen used in each study may account for any slight differences in results. Balnave (1969) administered oestradiol dipropionate (10 mg) to 6 week-old male chicks over a 10 day period, involving 5 injections, and observed an altered liver lipid fatty acid composition consisting of increases in the proportions of 16:0, 16:1 and 18:1 and decreases in 18:0, 18:2. 20:3 and 20:4. The molar ratios of unsaturated:saturated fatty acids were reported to have increased from 0.79 to 0.94 and from 1.12 to 1.35 in two separate experiments.

In another report, Balnave (1971) administered oestradiol diopropionate (20 mg) to 16 week-old female birds over a 7 day period and observed increases in the proportions of 16:0 and 18:1 and decreases in 18:0 and 18:2 in plasma lipids. Similar changes were reported in the liver lipids of the birds although an increase in 16:1 was also observed.

Balnave & Pearce (1975) administered oestradiol dipropionate (2 mg) to 4 week-old female birds on alternate days over a period of 9 days. Birds were killed at 1, 2, 4 or 9 days after the commencement of hormone treatment. A study of the fatty acid composition of liver lipids during this period indicated increases in the proportions of

16:0, 16:1 and 18:1 and decreases in 18:0 and 18:2.

Fujii et al. (1984) administered 17β-oestradiol (20 mg/kg body wt.)
to 15 day-old male chicks over a period of 6 days. An increase in
the proportion of 18:1 and decreases in the proportions of 18:0,
18:2 and 20:4 were observed in both total plasma and total liver
lipids. In the same study, Fujii et al. (1984) observed increases
in the levels of serum oestradiol in maturing hens reaching peak
values a few days before the production of the first egg, which
occurred at an average age of 147 days. Changes in the composition
of liver and plasma lipids occurred concomitantly with the increase
in endogenous oestradiol, such that they closely resembled the
fatty acid composition of the yolk lipids from the same birds and
the compositions of liver and plasma lipids of the oestrogen-treated
cockerels.

In the present study, oestrogen treatment seemed to cause a shift
in the lipid metabolism of the male chicks similar, in many respects,
to the changes in lipid metabolism in laying hens, which involve
the production of large quantities of specific lipids for
incorporation into the developing eggs.

# CHAPTER 4

THE EFFECT OF OESTROGEN TREATMENT ON
THE ACTIVITY OF ACETYL-CoA CARBOXYLASE
IN MALE CHICK LIVER

## INTRODUCTION

1. <u>De novo</u> synthesis of long-chain saturated fatty acids

Changes in the rate of lipogenesis in a variety of animals are often accompanied by corresponding changes in the activities of lipogenic enzymes (Numa & Yamashita, 1974; Wakil <u>et al</u>., 1983). In birds, the major site of <u>de novo</u> lipogenesis is the liver (Goodridge & Ball, 1967a; O'Hea & Leveille, 1969) in contrast to the situation in many mammals, such as the rat, where adipose tissue is the major site, (Romsos & Leveille, 1974).

The <u>de novo</u> biosynthesis of long-chain saturated fatty acids is controlled by two cytosolic enzymes; acetyl-CoA carboxylase (acetyl-CoA:carbon-dioxide ligase (ADP-forming), E.C.6.4.1.2) and fatty acid synthetase (an enzyme system.)

Acetyl-CoA carboxylase catalyses the ATP-dependent carboxylation of acetyl-CoA to produce malonyl-CoA (reaction (1)), the activated donor of 2-carbon units in the process of long-chain fatty acid synthesis catalysed by fatty acid synthetase (reaction (2)).

$$\text{Acetyl-CoA} + HCO_3^- + ATP \underset{}{\overset{Mg^{2+}}{\rightleftharpoons}} \text{malonyl-CoA} + ADP + Pi \qquad (1)$$

$$\text{Acetyl-CoA} + 7 \text{ malonyl-CoA} + 14NADPH + 14H^+ \longrightarrow \text{palmitate} +$$
$$8CoA + 7CO_2 + 6H_2O + 14NADP^+ \qquad (2)$$
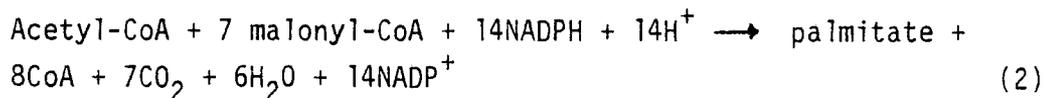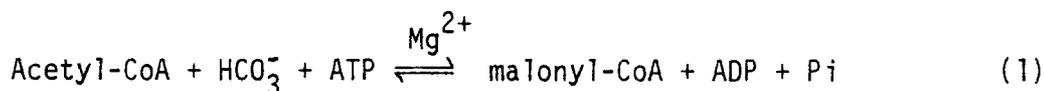
In many cells, the production of malonyl-CoA from acetyl-CoA is regarded as the rate-limiting step in <u>de novo</u> fatty acid synthesis (Vernon, 1980). This reaction occurs early in the fatty acid synthetic sequence and is the first committed step, malonyl-CoA having no other apparent metabolic alternative. Therefore, the

reaction catalysed by acetyl-CoA . carboxylase satisfies the
accepted criteria for a regulatory step in a biosynthetic pathway
(Monod et al., 1965).

The NADPH required in reaction (2) is usually produced by the action
of 'malic' enzyme (malate dehydrogenase (decarboxylating; NADP+),
E.C.1.1.1.40.), as shown in reaction (3), and/or the pentose phosphate
pathway enzymes, glucose 6-phosphate dehydrogenase (E.C.1.1.1.49)
and 6-phosphogluconate dehydrogenase (E.C.1.1.1.44), although it
has been suggested that the pentose phosphate pathway only plays a
minor role in birds (Goodridge, 1968a). This pathway is active in
the chick embryo but declines during incubation, being virtually
inactive at hatching, and in the adult bird (Pearce & Brown, 1971).

$$\text{L-malate} + \text{NADP}^+ \xrightarrow[\text{enzyme}]{\text{'malic'}} \text{pyruvate} + CO_2 + \text{NADPH} + H^+ \qquad (3)$$
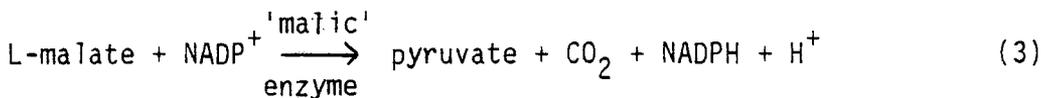
Acetyl-CoA, produced in the mitochondria from pyruvate is converted
to citrate which leaves the mitochondria and is cleaved in the
cytoplasm to yield acetyl-CoA by the action of ATP citrate lyase
(E.C.4.1.3.8):-

$$\text{citrate} + \text{ATP} + \text{CoA} \xrightarrow[\text{lyase}]{\text{ATP citrate}} \text{Acetyl-CoA} + \text{oxaloacetate} + \text{ADP} + \text{Pi}$$

Acetyl-CoA can also leave the mitochondrial matrix by conversion to
acetyl-carnitine which enters the cytoplasm and is re-converted to
acetyl-CoA.

The cytoplasmic acetyl-CoA can be used by acetyl-CoA carboxylase in
the production of malonyl-CoA (reaction (1)). Palmitic acid produced
by reactions (1) and (2) can be further elongated by mitochondrial
or microsomal enzyme systems, and/or converted to unsaturated fatty

acids by desaturation enzyme systems (Johnson et al., 1969). Fatty acids can then be used in the production of complex lipids such as triacylglycerols and phospholipids.

## 2. Acetyl-CoA carboxylase and its regulation

Acetyl-CoA carboxylase is located in the cytoplasm and electron-microscopic studies have shown that the enzyme exists as a large filamentous polymer with a molecular weight of 4 million to 11 million (Gregolin et al., 1966a; Kleinschmidt et al., 1969; Moss et al., 1972). Watkins et al. (1977) have provided indirect evidence for the occurrence of the filamentous polymeric form in vitro with cultured chicken liver cells using a 'digitonin-rapid-stop' technique. Digitonin perforates the plasma membrane making it permeable to cytosolic acetyl-CoA carboxylase at a rate inversely related to the cellular concentration of citrate and the apparent state of polymerisation. Acetyl-CoA carboxylase from animal species shows a requirement for citrate or isocitrate, the absence of which leads to inactivation (Martin & Vagelos, 1962; Matsuhashi et al., 1962; Waite & Wakil, 1962). Citrate activation of the crude or purified acetyl-CoA carboxylase is accompanied by polymerisation of the enzyme (Gregolin et al., 1966a, 1966b; Numa et al., 1966).

Studies on the inactive protomeric form of the enzyme are somewhat conflicting. Mackall & Lane (1978), using chicken liver acetyl-CoA carboxylase, suggested that a protomer consists of two non-identical subunits, both with molecular weights of approximately 230,000. However, more recently, Wada & Tanabe (1983) presented evidence suggesting that the chicken liver enzyme is composed of only one kind of subunit with a molecular weight of 220,000. All workers agree that the protomer contains one molecule of biotin, a prosthetic

group, which is covalently bound. It has also been shown that rat liver acetyl-CoA carboxylase consists of one kind of subunit with a molecular weight of 230,000 (Tanabe et al., 1975).

The reaction catalysed by acetyl-CoA carboxylase is believed to proceed in two steps as follows:-

$$E\text{-biotin} + ATP + HCO_3^- \underset{}{\overset{Mg^{2+}}{\rightleftharpoons}} E\text{-biotin} \sim CO_2 + ADP + Pi \qquad (1)$$

$$E\text{-biotin} \sim CO_2 + \text{acetyl-CoA} \rightleftharpoons E\text{-biotin} + \text{malonyl-CoA} \qquad (2)$$
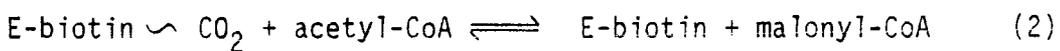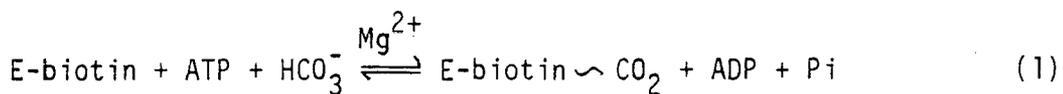
where E-biotin represents acetyl-CoA carboxylase.

The first step represents the carboxylation of the enzyme-bound biotin, and the second step the transfer of the 'activated' carboxyl group to acetyl-CoA.

It is generally accepted that the acetyl-CoA carboxylase step is the rate-limiting step in the biosynthesis of long-chain fatty acids in animal cells. The regulation of acetyl-CoA carboxylase is effected by short-term changes in the catalytic efficiency of a fixed mass of the enzyme and by long-term changes in the amount of the enzyme.

Two mechanisms are known to control the catalytic efficiency of the enzyme; allosteric modulation by metabolites and covalent modification of the enzyme involving phosphorylation and dephosphorylation. It is possible to regulate the amount of the enzyme by changes in the rate of synthesis and/or the rate of degradation of the enzyme. A brief description of these regulatory mechanisms is presented below, and further details can be obtained from several excellent reviews (Lane et al., 1974; Numa & Yamashita,

1974; Kim, 1983; Numa & Tanabe, 1984).

As mentioned earlier, acetyl-CoA carboxylase from animal tissues requires a hydroxy tricarboxylic acid allosteric effector for full catalytic activity, activation being accompanied by polymerisation of the enzyme (Gregolin et al., 1966a, 1966b; Numa et al., 1966). Many studies have been performed to establish whether or not citrate regulation is important in vivo. The concentration of citrate has been measured by a number of workers (Spencer & Lowenstein, 1967; Numa, 1974; Siess et al., 1978), both at the cellular and subcellular level, but difficulty has been encountered in carrying out these measurements. Siess et al. 1978) using a 'digitonin-rapid-stop' technique on liver cells have shown that 50% - 75% of the cellular citrate is cytoplasmic. This work contradicts the measurements of Tubbs & Garland (1964) and Leveille (1971), which suggested that the major fraction of citrate may be in the mitochondria. Given the difficulty in determining accurate sub-cellular citrate concentrations it is understandable that some workers state that the cytoplasmic concentration of citrate is close to the concentration required for half-maximal activation of acetyl-CoA carboxylase (Numa & Tanabe, 1984) whereas others state that the cytoplasmic citrate concentration, in vivo, is too low to produce any appreciable activation of the enzyme. Not surprisingly, different studies attempting to correlate cellular citrate concentration and the rate of fatty acid synthesis conflict (see Kim, 1983).

Another positive allosteric effector of acetyl-CoA carboxylase is CoA (Yeh & Kim, 1980; Yeh et al., 1981). The enzyme contains one CoA binding site per subunit and CoA binding is not affected by the presence of citrate. CoA activation of the enzyme is accompanied by polymerisation. However, if CoA activation is carried out with

an enzyme preparation which has never been exposed to citrate, CoA
activation occurs in the absence of polymerisation.

Long-chain fatty acyl-CoA, the end-product of fatty acid synthesis,
acts as a negative feedback allosteric effector of acetyl-CoA
carboxylase (Bortz & Lynen, 1963; Numa et al., 1965), being
competitive with respect to the activator, citrate, and non-
competitive with respect to the enzyme substrates.  Binding of
palmitoyl-CoA to the enzyme has been shown to accompany the adoption
of a protomeric enzyme form  (Ogiwara et al., 1978). Physiological
states which are known to depress hepatic fatty acid synthesis, such
as fasting (Lyon et al., 1952; Numa et al., 1970) and high-fat diets
(Hill et al., 1958), lead to an increased long-chain fatty acyl-CoA
concentration in the liver (Gruynn et al., 1972) suggesting that
fatty acyl-CoA inhibition is effective in vivo.

The immediate end-product of the acetyl-CoA carboxylase catalysed
reaction, malonyl-CoA, has also been shown to be a potent inhibitor
of the avian liver enzyme (Gregolin et al., 1966c).  Inhibition is
competitive with respect to acetyl-CoA and citrate, and the
malonyl-CoA has been shown to promote depolymerisation (Gregolin et
al., 1966c, 1968a).

Acetyl-CoA carboxylase has been shown to be regulated by a covalent
modification mechanism whereby the enzyme is inter-converted between
an inactive phosphorylated and an active dephosphorylated form
(Carlson & Kim, 1973).  The enzyme has been shown to become
disaggregated to protomeric and intermediate forms when
phosphorylated (Lee et al., 1973; Lee & Kim, 1978; Shiao et al.,
1981).  However, these disaggregated forms exhibit different
properties from those of the protomers formed in the absence of
citrate (Lee et al., 1973), in that full activity was not restored

when the phosphorylated protomeric form of the enzyme was incubated
with citrate. This indicates that the phosphorylated form of the
enzyme is intrinsically less active than the dephosphorylated form.

Lee & Kim (1979) showed that acetyl-CoA carboxylase from rat adipose
tissue can be covalently phosphorylated at two types of site, only
one of which leads to inactivation. This might explain the results
of Halestrap & Denton (1974) and Pekala et al. (1978) who concluded
that phosphorylation of the enzyme was of no physiological
significance.

Studies have indicated that the phosphorylated, inactivated forms of
the enzyme are more prone to the inhibitory effects of long-chain
fatty acyl-CoA than the dephosphorylated forms, whereas the
dephosphorylated forms require less citrate for maximum activation
(Carlson & Kim, 1974).

Specific endogenous acetyl-CoA carboxylase phosphatase (Krakower &
Kim, 1981) and acetyl-CoA carboxylase kinase (Shiao et al., 1981;
Lent & Kim, 1982) have been isolated, the kinase being c.AMP
independent. However, when various tissues are treated with c.AMP,
or dibutyryl c.AMP, acetyl-CoA carboxylase is inactivated (Allred
& Roehrig, 1972, 1973; Lee et al., 1973; Harris, 1975). The exact
site of c.AMP involvement in acetyl-CoA carboxylase activity is
unclear. Hardie & Guy (1980) and Brownsey & Hardie (1980) suggest
that the enzyme from rat mammary glands is phosphorylated and
inactivated by the catalytic subunit of c.AMP-dependent protein
kinase. Watkins et al. (1977) reported that, in the chicken liver
cell system, c.AMP affects the formation of cellular citrate which
in turn affects the polymeric status of the carboxylase.

It is now generally accepted that hormones such as glucagon and

and adrenaline, which raise intro-cellular levels of c.AMP, cause changes in the catalytic efficiency of acetyl-CoA carboxylase by means of covalent modification (see Kim, 1983). However, it is obvious that the control of lipogenesis, and acetyl-CoA carboxylase in particular, involves both allosteric and covalent modulation, the two fulfilling different yet complementary roles (Hardie, 1981).

In addition to the regulation by changes in the catalytic efficiency per enzyme molecule, the amount of acetyl-CoA carboxylase has been shown to alter in accord with the rate of fatty acid synthesis in a variety of dietary, hormonal, developmental and genetic conditions (Numa, 1974; Numa & Yamashita, 1974). For instance, studies have shown that the increase or decrease in the hepatic enzyme content in re-fed or diabetic rats is due solely to a corresponding change in the rate of synthesis of the enzyme, whereas the decrease in the enzyme content in fasted rats is due to both dimished synthesis and accelerated degradation of the enzyme (Nakanishi & Numa, 1970). The increase in enzyme content in the liver of growing chicks is caused by accelerated synthesis of the enzyme (Teraoka & Numa, 1975). Such changes in enzyme content reflect long-term adaptation to a particular condition whereas short-term changes are brought about by allosteric and/or covalent modulation.

3. The effect of diet on hepatic lipogenesis and lipogenic enzymes

In many animals, starvation or a high fat, low carbohydrate diet are accompanied by depressed hepatic lipogenesis and co-ordinate reduction in the activities of lipogenic enzymes, including glucose 6-phosphate dehydrogenase, ATP citrate lyase, 'malic' enzyme, acetyl-CoA carboxylase and fatty acid synthetase. This situation can be reversed by re-feeding following starvation or by feeding a low fat, high carbohydrate diet (Masoro, 1962; Numa & Yamashita,

1974; Romsos & Leveille, 1974). In this respect, birds are similar to rats although a difference does exist in the extent of the response to re-feeding after starvation. In birds, hepatic lipogenesis increases on re-feeding and the lipogenic enzyme activities return to normal or slightly above normal levels (Goodridge, 1968b; Muiruri et al., 1975; Shapira et al., 1979). However, in re-fed rats, there is a very high increase in lipogenic enzyme activity, including glucose 6-phosphate dehydrogenase, 'malic' enzyme and ATP citrate lyase, and hence lipogenesis, to levels well above normal rats. (Romsos & Leveille, 1974). This could imply that lipogenesis is more tightly controlled in avian liver than rat liver.

4.  The effects of hormones on hepatic lipogenesis and lipogenic enzymes

(a) Effects of hormones other than gonadal hormones

Kompiang & Gibson (1976) have shown that relatively large doses of insulin increase hepatic lipogenic enzyme activity. Using a mono-layer culture of non-proliferating hepatocytes, Tarlow et al. (1977) observed high rates of fatty acid synthesis in the presence of insulin, the absence of insulin leading to a decline in lipogenesis. With respect to avian adipose tissue, insulin does not depress lipolysis (Heald et al., 1965) and only elevates lipogenesis slightly, if at all (Goodridge & Ball, 1966). This is in opposition to the effect of insulin in rats (Jungas & Ball, 1963; Goodridge, 1968c).

Glucagon appears to be the major avian lipolytic hormone, intravenous injection of glucagon into domestic fowl having been shown to cause a rapid increase in plasma free fatty acid levels (Heald et al., 1965). Fatty acid synthesis, studied in the chicken hepatocyte system of Tarlow et al. (1977) is inhibited by glucagon. In avian species, insulin seems to potentiate the stimulation of lipolysis

effected by glucagon in adipose tissue (Langslow & Hales, 1969) whereas in rat adipose tissue insulin is definitely anti-lipolytic (Jungas & Ball, 1963) and, therefore, antagonistic to glucagon. This difference in response to hormones is only one of a series of differences between birds and mammals in the endocrine control of carbohydrate and lipid metabolism (Pearce, 1977).

Catecholamines are activators of lipolysis in mammalian adipose tissue (Ball & Jungas, 1961) but are relatively insignificant in lipolysis in avian adipose tissue (Carlson et al., 1964; Langslow & Hales, 1969).

Thyroid hormones have been shown to stimulate rat hepatic lipogenesis, monitored by the incorporation of $\left[1 - {}^{14}C\right]$ acetyl-CoA into fatty acids, and the activity of the following lipogenic enzymes in rat liver and adipose tissue; acetyl-CoA carboxylase, fatty acid synthetase, ATP citrate lyase, 'malic' enzyme, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. In birds thyroxine has been shown to increase hepatic 'malic' enzyme activity when supplied with food and water ad libitum (Chandrabose & Bensadoun, 1971).

The development of birds is accompanied by major changes in nutritional and hormonal status which promote changes in hepatic lipogenesis (Pearce, 1980). In chicks, hatching marks a change from a high fat, low carbohydrate diet (egg yolk) to a low fat, high carbohydrate diet (cereal-based mash). This change is accompanied by a large increase in hepatic lipogenesis (Goodridge, 1968d) and in the activities of some lipogenic enzymes, including acetyl-CoA carboxylase, fatty acid synthetase, ATP citrate lyase and 'malic' enzyme (Felicioli & Gabrielli, 1967; Goodridge, 1968b, 1973b; Arinze & Mistry, 1970; Ryder, 1970; Joshi & Sidbury, 1975). The

emergence of hepatic lipogenic enzyme activities has been studied
with respect to possible hormonal influences.  Thyroid hormones
have been shown to increase the activities of acetyl-CoA carboxylase,
ATP citrate lyase, fatty acid synthetase and 'malic' enzyme in
cultured chick embryo hepatocytes (Goodridge et al., 1974; Goodridge
& Fischer, 1978).  Insulin, administered in vivo, has been
implicated in the stimulation of chick embryo hepatic fatty acid
synthetase activity (Joshi & Wakil, 1978).  The activities of
acetyl-CoA carboxylase, fatty acid synthetase and 'malic' enzyme
have all been shown to be stimulated by the in vivo treatment of
chick embryo liver tissue with insulin alone or in conjunction with
thyroid hormones (Goodridge & Adelman, 1976; Goodridge & Fischer,
1978; Joshi & Aranda, 1979).  It is apparent that there is an
intricate interplay of both hormonal and nutritional stimuli which
contribute to the changes occurring in lipogenic enzyme activities
during the early development of the chick liver.

Two other hormones which have been shown to affect avian lipogenesis
are prolactin and growth hormone which are responsible, in the
pigeon, for a food-dependent increase in hepatic lipogenesis and in
the activity of lipogenic enzymes, including 'malic' enzyme and ATP
citrate lyase (Goodridge & Ball, 1967b; 1967c).

## (b) Effects of gonadal hormones

A number of physiological changes are observed in the development of
young female chicks into sexually mature, egg-laying hens and changes
in the hepatic lipogenic activity have been well reported.  The
hepatic specific activities of the lipogenic enzymes, ATP citrate
lyase and 'malic' enzyme are relatively high in 4 - 7 week-old
female chickens.  However, these values subsequently decline with
age in the non-laying sexually immature female, then increase again

as the birds reach sexual maturity and come into lay (Pearce, 1971, 1972; Pearce & Brown, 1971). Thus, the laying hen has similar specific activities to those found in young birds (aged 4 - 7 weeks) whereas the non-laying sexually immature female bird has similar specific activities to those of the cockerel. These observations are in agreement with the work of Leveille (1969b) who found similar levels of hepatic lipogenesis in the laying hen and the young bird.

The administration of oestrogen to immature female birds results in biochemical and physiological changes similar to those found when the female comes into lay (Lorenz, 1954). With regard to changes in the lipogenic activity of female birds, short-term oestrogen treatment ($\leq$ 2 days, 1 - 2 mg oestradiol dipropionate/bird) resulted in increased specific activities of ATP citrate lyase and 'malic' enzyme in the livers of 4-week old pullets (Balnave & Pearce, 1974; Pearce & Balnave, 1975, 1976). Prolonged oestrogen treatment (4 mg $\beta$-oestradiol-3-benzoate/bird/day for 6 days) has also resulted in increased activities of oviduct and hepatic fatty acid synthetase in 1-month old pullets (Aprahamian et al., 1979). Furthermore, Pageaux et al. (1981), using 16-day old female quail, have reported a rapid increase in the specific activity of hepatic acetyl-CoA carboxylase within 3 hours after oestrogen treatment (0.2 mg oestradiol benzoate/kg body wt.), although this study appears to have lacked adequate controls.

Many workers have demonstrated that oestrogen treatment of male birds evokes changes in liver metabolism similar to those occurring when the female comes into lay (Chan et al., 1976; Tata, 1976; Shapiro, 1982). For example, an increase in the specific activity of 'malic' enzyme has been reported for the livers of oestrogen-treated roosters (de Vellis & Schjeide, 1967) and Lippiello et al. (1979) obtained increased stearyl-CoA desaturase specific activity

in rooster liver, up to 48 hours after birds had received a single injection of 17β-oestradiol (3.5 mg/100 g body wt.). Immature male chicks, treated with the synthetic oestrogen diethylstilboestrol, have been shown to exhibit increased specific activities of hepatic choline kinase (E.C.2.7.1.32) and hepatic phosphatidylethanolamine-N-methyltransferase (E.C.2.1.1.17), enzymes involved in phospholipid biosynthesis (Vigo et al., 1981). The increase in choline kinase activity was shown to be due to an increase in the amount of the enzyme (Paddon et al., 1982). Coleman et al. (1977), studying enzymes involved in triacylglycerol and phospholipid biosynthesis, detected increases in total hepatic activities of fatty acid:CoA ligase (AMP-forming) (E.C.6.2.1.3), sn-glycerol 3-phosphate: acyl-CoA acyltransferase (E.C.2.3.1.15) and diacylglycerol acyltransferase (E.C.2.3.1.20), in oestrogen-treated immature birds (male and female), without changes in their specific activities. The total hepatic activity of diacylglycerolcholine-phosphotransferase (E.C.2.7.8.2) was found to increase although the specific activity decreased..

Fatty acid synthetase can exist in a phosphorylated form, with reduced activity, and a dephosphorylated active form (Qureshi et al., 1975). In addition, it is activated by some phosphorylated sugars (Wakil et al., 1966) and inhibited by fatty acyl-CoA derivatives (Butterworth et al., 1966; Aprahamian et al., 1979), high concentrations of CoA (Stern et al., 1982) and malonyl-CoA (Plate et al., 1968; Cox & Hammes, 1983). Because of these characteristics (for reviews, see Numa & Yamashita, 1974; Volpe & Vagelos, 1976) it has been suggested that fatty acid synthetase may, in some circumstances, be the rate-limiting enzyme in fatty acid synthesis. For instance, Donaldson (1979) suggests that fatty acid synthetase plays an important role in the control of lipogenesis, in the chicken, when lipogenesis is minimal. Courtney & Manning (1984) have shown

that, following a single injection of 17β-oestradiol (0.75 mg/100 g
body wt.), immature male chicks exhibit increased total hepatic
fatty acid synthetase activity, compared to control birds, after
26 hours post-injection. Interestingly, these workers also reported
that an early increase ($\leq$ 26 h) in fatty acid synthetase activity
following oestrogen treatment is due to the injection procedure,
since a similar increase is observed in control birds.

The administration of exogenous oestrogen to immature birds, both
male and female, results in the increase in activity of many key
enzymes involved in hepatic lipogenesis. Furthermore, such
increases in lipogenic enzyme activity correlate very well with the
results from studies of oestrogen-treated birds in which the rate
of hepatic lipogenesis has been measured, either in vivo or in
vitro. For instance, the administration of exogenous oestrogen to
young chicks (sex undetermined), in vivo, increased the rate of
de novo fatty acid synthesis in liver slices, monitored by the
incorporation of $\begin{bmatrix} 1 - {}^{14}C \end{bmatrix}$ acetate (Kudzma et al., 1973). Similarly,
Courtney (1984) measured increased de novo fatty acid synthesis in
liver slices from oestrogen-treated immature male chicks, as
determined by the incorporation of $\begin{bmatrix} 1 - {}^{14}C \end{bmatrix}$ acetate and ${}^{3}H_2O$ into
lipids. Furthermore, oestrogen treatment increased the rate of
incorporation of preformed fatty acids into hepatic glycerolipids,
in vivo (Kudzma et al., 1975), and in liver slices, in vitro
(Courtney, 1984). Increases in the rate of de novo lipogenesis,
monitored by the incorporation of ${}^{3}H_2O$ into hepatic glycerolipid
in vivo, have also been observed in oestrogen-treated immature male
turkeys (Dashti et al., 1983).

While oestrogens have been shown to have a major involvement in the
physiological changes observed when the hen comes into lay (Lorenz,
1954), androgens and progestagens have also been shown to be

involved, to some extent (Balnave & Pearce, 1974). In particular, androgens exert significant influences on hepatic lipid metabolism in the domestic fowl. For example, testosterone treatment of 4-week old pullets (2 mg testosterone propionate/bird) caused increases in the specific activities of ATP citrate lyase and 'malic' enzyme in liver after 1 day of hormone treatment (Pearce & Balnave, 1973; Balnave & Pearce, 1974). However, Pearce (1977b) injected laying hens with a daily dose of testosterone propionate (1 mg/kg body wt.) for 4 days and observed significantly reduced specific activities of hepatic ATP citrate lyase and 'malic' enzyme 24 hours after the last injection. The apparently contradicting results in pullets and laying hens could be explained by differences in the age of animal used, in the hormone dose level used and in the duration of hormone treatment employed.

The involvement of progestagens in the regulation of lipogenesis is unclear, since progestagen treatment of domestic fowl has not been found to have marked effects on hepatic lipid metabolism, in general, or on hepatic lipogenic enzyme activities, in particular (Balnave, 1969; Balnave & Pearce, 1974).

In the light of the reports of increased activities of a number of lipogenic enzymes in oestrogen-treated birds, the aim of the present study was to investigate the effect of oestrogen treatment on hepatic acetyl-CoA carboxylase activity in immature male chicks. This enzyme is believed to be the rate-limiting enzyme in de novo fatty acid synthesis in many cases, and for this reason, the enzyme is worthy of detailed study in the liver of oestrogen-treated birds. Furthermore, attempts were to be made to correlate any changes in enzyme activity with changes in the profile of liver and plasma lipids, in an effort to determine the importance of acetyl-CoA carboxylase in the enhanced lipogenesis promoted by oestrogen treatment.

METHODS

1. Animals

(a) Housing

Birds were kept under the same conditions as reported in the Methods section of Chapter 2 and were used when aged between 3 - 5 weeks.

(b) Oestrogen treatment

Unless indicated otherwise, oestrogen-treated birds received an intramuscular injection of 17β-oestradiol (0.75 mg/100 g body wt.), dissolved in propane-1,2-diol (0.1 ml/100 g body wt.). Control birds received an equivalent volume of the propane-1,2-diol vehicle only.

(c) Sacrifice of birds

All birds were sacrificed between 11.00 and 13.00 hours to minimise any possible diurnal variation in the parameters under investigation. Birds were weighed and then killed by decapitation. The livers were removed quickly, weighed and rapidly chilled on ice. A portion of each liver was weighed and stored at -20°C for future DNA estimation.

2. Solutions

(a) Preparation and assay of acetyl-CoA

Acetyl-CoA was prepared by the method of Smith et al. (1966). Acetic anhydride was reacted with CoA in bicarbonate solution at 0° - 5°C, the final pH being adjusted to a value between pH 1 - 3.

The method of Chase (1967 was used to determine the concentration of acetyl-CoA. In this assay, given an excess of oxaloacetate and 5,5'-dithiobis(2-nitrobenzoic acid), the differences between the free thiol content of the preparation and the total thiol content after incubation with citrate synthase was used to estimate acetyl-CoA concentration. The production of 5-thio-nitrobenzoate was measured by following the increase in absorbance at 412 nm using a Pye Unicam SP8-100 spectrophotometer. Acetyl-CoA solutions (pH 1 - 3) were diluted to the required concentration (2 mM) with distilled water and then stored at -20°C. Fresh solutions were prepared every 3 weeks.

(b) Preparation and storage of stock sodium $[^{14}C]$ bicarbonate.

Sodium $[^{14}C]$ bicarbonate was mixed with non-radioactive sodium bicarbonate solution to produce a stock solution. The precise specific radioactivity of the stock solution was determined immediately after its preparation. Aliquots (50 - 100 $\mu$l) of this solution were diluted with non-radioactive sodium bicarbonate and 1 ml samples were mixed with 10 ml of xylene-Triton X-100(2:1, v/v) containing 2,5-diphenyloxazole (5.5 g/l) and 1,4-di(2-(5-phenyl-oxazolyl))benzene (0.1 g/l). Radioactivity was determined in a Packard 300CD Tri-Carb Liquid Scintillation Counter. Stock solutions of sodium $[^{14}C]$ bicarbonate, with a specific radioactivity of 0.22 - 0.38 $\mu$Ci per $\mu$mole, were stored at room temperature in a series of heat-sealed glass ampoules. Each ampoule contained sufficient sodium $[^{14}C]$ bicarbonate for 30 assays of acetyl-CoA carboxylase and, after opening, the contents were used immediately.

(c) Other solutions.

Stock solutions were prepared and stored as follows: IM-Tris/HCl of a variety of pH values (6.9 - 8.2 at 25°C), 0.2 M-magnesium chloride,

0.8 M-potassium citrate, 0.1 M-dithiothreitol, 0.65 M-potassium chloride, 10 mM-EDTA, pH7.0, and 0.2 M-potassium phosphate, pH7.0, were stored at 4°C and replaced at fortnightly intervals. The following solutions were stored at -20°C and replaced every 3 weeks; 8 mM-ATP, pH7.0, and 4.8 mg/ml BSA (fatty acid poor). Postassium bicarbonate (0.1 M) was prepared on the day of use. These solutions were mixed to prepare the various buffers and 'cocktails' on their day of use.

3.  Acetyl-CoA carboxylase

(a) Enzyme preparation

Since acetyl-CoA carboxylase is a soluble cytoplasmic enzyme, a particle-free supernatant (PFS) was prepared from each liver. Portions of the chilled livers were homogenised using a teflon-glass homogeniser with 2 volumes of ice-cold homogenisation buffer consisting of 94 mM-potassium phosphate, 0.5 mM-EDTA, 0.2 mM-dithiothreitol, 65 mM-potassium chloride and 5 mM-potassium bicarbonate, at pH7.0. The tissue was disrupted with 8 'up and down' strokes of the rotating pestle and care was taken not to cause foaming. Volumes of homogenates were measured and they were then centrifuged at 100,000 $g$ ($r_{av.}$ 6.4 cm) for 1 hour at 4°C in a M.S.E. Prepsin 50 centrifuge. The supernatants were diluted with an ice-cold dilution buffer, consisting of 50 mM-potassium phosphate, 0.1 mM-EDTA and 0.5 mM-dithiothreitol, at pH7.0, prior to assay for acetyl-CoA carboxylase.

(b) Assay of acetyl-CoA carboxylase activity

Enzyme activity was determined by following the rate of acetyl-CoA-dependent [$^{14}$C] bicarbonate incorporation into an acid-stable product.

All procedures were performed in a fume cupboard. Unless otherwise stated, the assay system contained, in a final volume of 0.4 ml, 100 mM-Tris/HCl (pH7.4 at 25°C), 8 mM-magnesium chloride, 11 mM-potassium citrate, 0.25 mM-acetyl-CoA, 0.6 mg/ml BSA (fatty acid poor), 3 mM-dithiothreitol, 1 mM-ATP, 25 mM-sodium [$^{14}$C] bicarbonate (0.22 - 0.38 $\mu$Ci/$\mu$mole) and an aliquot of the diluted PFS. The pH of the assay system, at the incubation temperature of 37°C, was pH7.05.

'Blank' assays contained all components except enzyme (or ATP or acetyl-CoA). Assays were performed in duplicate or triplicate at 37°C and were started by the addition of 100 $\mu$l of 'Start Cocktail' at 37°C, containing sodium [$^{14}$C] bicarbonate and ATP, to 300 $\mu$l of 'Assay Cocktail', containing the other components, which had been pre-incubated at 37°C for 15 minutes. The reactions were stopped after 1.5 minutes by the addition of 100 $\mu$l of 6 M-hydrochloric acid and the tubes were immediately stoppered. $^{14}CO_2$, released from non-incorporated [$^{14}$C] bicarbonate, was trapped by filter paper, dampened with sodium hydroxide solution, suspended from the stoppers. After 30 minutes, the contents of each tube were transferred to scintillation vials, then each tube was washed out with 1 ml of 0.5 M-sodium bicarbonate. The washings were added to the corresponding vials and the contents were dried down overnight at 50°C before being re-dissolved in 1 ml of distilled water and radio-activity measured as described previously.

One unit of acetyl-CoA carboxylase activity was defined, in this study, as the amount of enzyme required to catalyse the incorporation of 1 $\mu$mole of [$^{14}$C] bicarbonate into acid-stable product (malonyl-CoA) per minute at 37°C, under the assay conditions described.

4.   Protein determination

The protein content of each enzyme preparation was determined as described in the Methods section of Chapter 2.

5.   DNA determination

The DNA content of portions of liver from each bird was determined as described in the Methods section of Chapter 2.

6.   Statistical analysis

Data were analysed statistically by Student's 't' test.  Probability values (p) of 0.05 or less were taken to be significant.  Standard errors are provided to show the degree of variance and levels of statistical significance are indicated.

RESULTS

1. Characterisation of the procedure for the assay of acetyl-CoA carboxylase activity

(a) Loss of radioactivity from the 'Start Cocktail' and the 'assay cocktail' during the enzyme assay

Starting the acetyl-CoA carboxylase assay involved the addition of 100 $\mu$l of 'start cocktail', containing sodium $[^{14}C]$ bicarbonate and ATP, to a pre-incubated 'assay cocktail' that contained the liver extract. The 'start cocktail' was prepared just before it was needed by mixing stock solutions of sodium $[^{14}C]$ bicarbonate of known specific radioactivity with ATP solution. For the 5 minutes before its use the 'start cocktail' was placed in the 37°C water bath to thermally equilibrate. During this thermo-equilibration period, and during the 1.5 minute assay periods, it was known that there was likely to be a loss of carbon dioxide from the cocktails, leading to a reduction in bicarbonate concentration and radio-activity (Manning et al., 1976). Assuming that the loss of $^{14}CO_2$ occurred at the same rate as that of $^{12}CO_2$, the specific radio-activity would remain constant throughout. However, it was important to determine the extent of the loss of radioactivity, during the complete assay procedure, since this would be an indication of the reduction in the concentration of bicarbonate, one of the substrates of the reaction. Assays started later in the procedure would obviously receive a reduced amount of bicarbonate in comparison to those started earlier and it was necessary to ensure that the bicarbonate concentrations during each 1.5 minute assay were still optimal or near-optimal.

In order to quantify the loss of radioactivity from the 'start

cocktail' a number of 'start cocktails' were prepared and incubated
for up to 20 minutes at 37°C. Samples were removed at regular
intervals and, after dilution in non-radioactive sodium bicarbonate,
radioactivity was determined using liquid-scintillation counting as
described previously. Figure 4.1a shows the extent of the loss of
radioactivity from the 'start cocktail' during a typical experiment.
The 'start cocktail' was prepared at zero-time, stored on ice for
5 minutes and subsequently incubated at 37°C for 20 minutes. At the
end of the incubation, the level of radioactivity had reduced by 16%,
compared to the zero-time level. However, the loss of radioactivity
from the 'start cocktail' during the first 5-minute period, while
stored on ice, was negligible (< 1%).

The results of a similar experiment, designed to follow the loss of
radioactivity from an individual assay tube containing 100 $\mu$l of
'start cocktail' and 300 $\mu$l of 'assay cocktail' at 37°C, are
presented in Figure 4.1b. During the first 1.5 minutes of the
incubation period the radioactivity of the assay system (0.4 ml)
diminished by 5%, while at the end of 10 minutes the radioactivity
had reduced by 26%.

(b) Identification of the acid-stable products of the enzyme assay.

The acid-stable products of the assay were identified by thin-layer
chromatography, using the method of Myers & Huang (1969). Assays
were performed as described previously except the incubation time
was 3.5 minutes and the specific radioactivity of sodium $\left[ {}^{14}C \right]$
bicarbonate was 1.2 $\mu$Ci per $\mu$mole. These changes were adopted to
increase the amount of radioactive bicarbonate incorporated into
acid-stable products. After the reactions had been stopped,
precipated protein was removed by centrifugation and 5.25 $\mu$moles
of each of the following standards were added; citrate, malate,
aspartate and malonate. After drying down overnight at 50°C, the

Figure 4.1

The loss of radioactivity from 'start cocktail' held on ice or
at 37°C, and from assay incubations at 37°C.


(a) Loss of radioactivity from 'Start Cocktail'.

'Start cocktail' (2.5 ml) containing sodium $[^{14}C]$ bicarbonate
(40 mM) and ATP (8 mM), was prepared as described in the Methods
section.

The 'start cocktail' was kept on ice for 5 minutes and
subsequently incubated at 37°C for a further 20 minutes.
Samples (50 μl) were removed at regular intervals during the 25
minute period and radioactivity determined as described in the
text.


(b) Loss of radioactivity from an assay incubation.

'Start cocktail' (100 μl) was added to 300 μl 'assay cocktail',
as described in the Methods section, and was incubated at 37°C.
Final concentrations in the assay system were 60 mM-Tris/HCl
(pH 7.5 at 25°C), 0.2 mM-acetyl-CoA, 3 mM-dithiothreitol, 20 mM-
potassium citrate, 8 mM-magnesium chloride, 0.6 mg/ml BSA, 2 mM-
ATP and 10 mM-sodium $[^{14}C]$ bicarbonate. The assay system, however,
did not contain enzyme. Samples (25 μl) were removed during an
11-minute incubation period and radioactivity determined as
described in the text.

a.



Time after preparation of cocktail (minutes)

b.



Time of incubation (minutes)
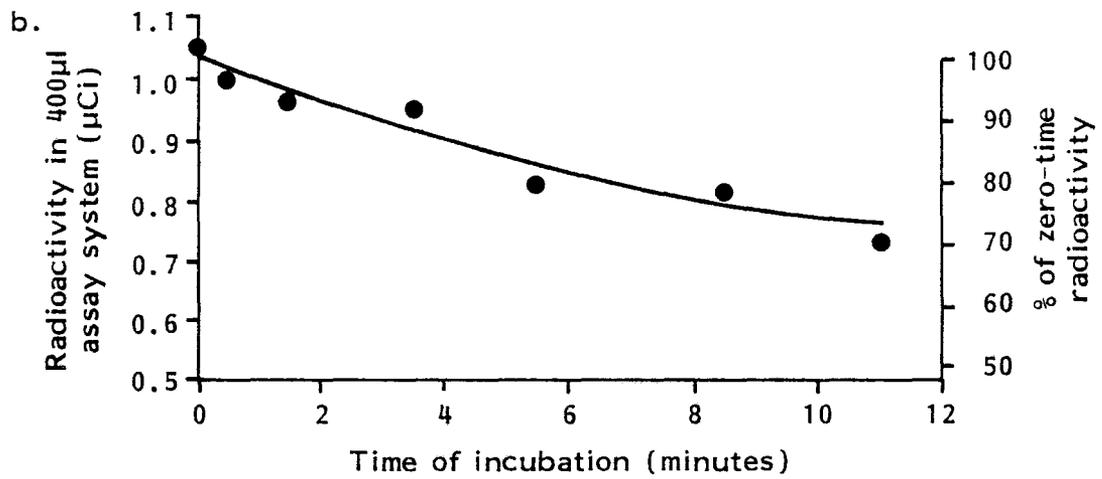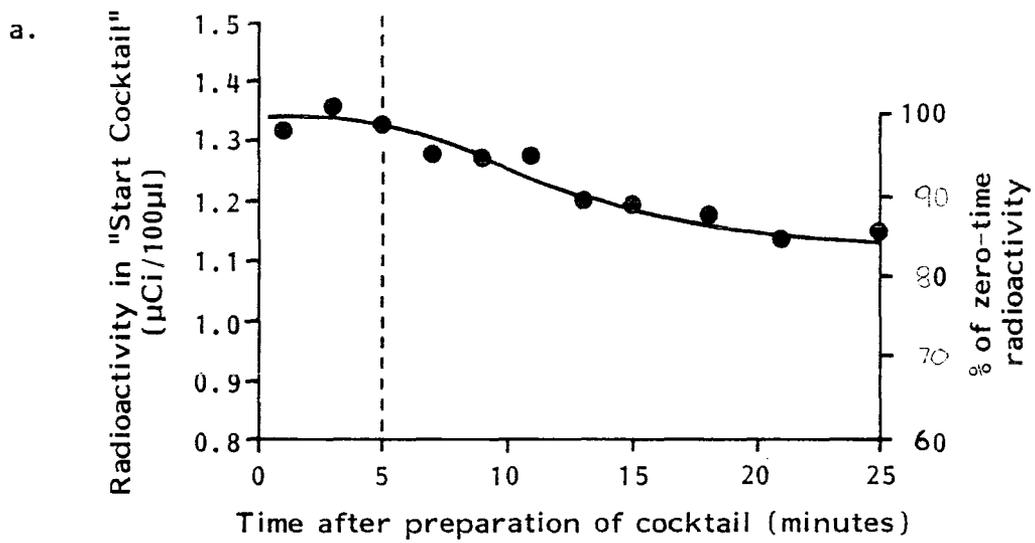
acid-stable products and standards were re-dissolved in 100 μl of distilled water. Aliquots (25μl) were measured for radioactivity and 6 ul aliquots were spotted on to a number of MN300 cellulose TLC plates of 250 μm thickness. Malonyl-CoA (0.15 μmol in 3μl) was spotted over each of the experimental samples. Mixtures of the standards were applied as spots to the rest of the plates before being developed in the following solvent mixture; diethyl ether/formic acid/water (7:2:1, by vol.). Once the plates had dried, 1 cm sections were scraped from the length of the experimental lanes, placed into scintillation vials and radioactivity determined as described previously. The positions of standards were identified by spraying the scraped plates with bromocresol green indicator (Myers & Huang, 1969).

The results of a typical thin-layer chromatographic analysis of the acid-stable products of the reaction are presented in Figure 4.2. Of the radioactivity applied to the plate, 96% was recovered in the scrapings of the developed plate, 61% being located in a spot which co-chromatographed with malonyl-CoA and 34.8% of the applied radioactivity being located in a spot which co-chromatographed with malonate.

The $R_F$ values of the standards were as follows:-

| Standard | $R_F$ Value |
| --- | --- |
| Malonate | 0.77 |
| Malate | 0.55 |
| Citrate | 0.39 |
| Aspartate | 0.27 |
| Malonyl-CoA | 0.07 |

It is assumed that the $[^{14}C]$ malonate is a degradation product,

Figure 4.2


Thin-layer chromotographic analysis of the acid-stable products
of the assay procedure for acetyl-CoA carboxylase.


Assay of chick liver acetyl-CoA carboxylase was performed using
the incubation procedure and conditions described in the Methods
section, with the exception that a 3.5 min. incubation period
was adopted and the sodium $\left[^{14}C\right]$ bicarbonate specific radio-
activity was 1.2 µCi/µmole.

After the reaction had been stopped by the addition of HCl (6 M),
the acid-stable products were subjected to t.l.c. analysis on
MN300 cellulose plates, as described in the text, using a solvent
mixture of diethyl ether/formic acid/water (7:2:1, by vol.).
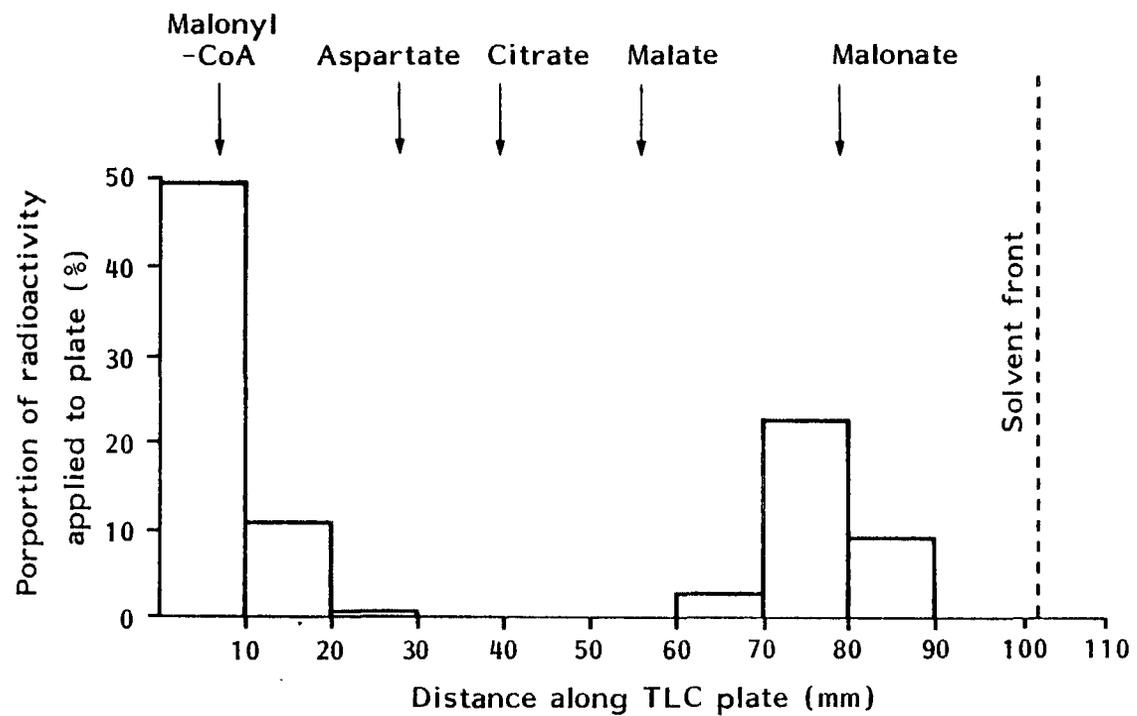
derived from $[^{14}C]$ malonyl-CoA as a result of the prolonged (over-
night) incubation at 50°C under acidic conditions. It appears,
therefore, that $[^{14}C]$ malonyl-CoA is the only major acid-stable
radioactive product formed under the present assay conditions,
allowing the amount of acid-stable radioactivity to be used as a
measure of the activity of acetyl-CoA carboxylase.

## (c) Establishment of optimum conditions for the assay of acetyl-CoA carboxylase

Optimum conditions for the assay of enzyme activity were determined
for enzyme preparations of untreated, control and oestrogen-treated
birds. Initially the conditions of Gregolin et al. (1968b) for the
assay of acetyl-CoA carboxylase activity from chicken liver were
adopted with slight modifications. The assay system contained 100mM-
Tris/HCl (pH7.5 at 25°C), 8 mM magnesium chloride, 2 mM-ATP, 20 mM-
potassium citrate, 0.2 mM-acetyl-CoA, 0.6 mg/ml-BSA, 20 mM-sodium
$[^{14}C]$ bicarbonate (0.2 µCi per µmole), and 3 mM-dithiothreitol in a
final volume 0.4 ml at 37°C. Each factor was varied in turn keeping
all other parameters constant. As each factor was optimised, this
value was inserted into the conditions used for subsequent
determinations. Unless stated otherwise, oestrogen-treated and
control birds were used 48 hours after receiving an injection.

When acetyl-CoA carboxylase activity was measured in liver extracts
from an untreated bird, the incorporation of $[^{14}C]$ bicarbonate into
acid-stable radioactive products was found to be linear with respect
to time only up to 1.5 minutes (Fig. 4.3). Time-course studies on
control and oestrogen-treated birds killed at 48 hours after
injection also showed linear incorporation up to 1.5 minutes
incubation time (Fig. 4.4). Similar results were obtained during
time-course studies on control and oestrogen-treated birds killed

Figure 4.3


The effect of assay incubation time on the activity of acetyl-CoA
carboxylase from the liver of an untreated male chick.


Enzyme assays were performed at 37°C as described in the text.
The enzyme was pre-incubated for 5 minutes at 37°C, in the
presence of citrate (27 mM), and then assayed using varying
incubation times in the presence of 100 mM-Tris/HCl (pH7.5 at
25°C), 8 mM-magnesium chloride, 20 mM-potassium citrate,
0.2 mM-acetyl-CoA, 3 mM-dithiothreitol, 0.6 mg/ml BSA, 2 mM-ATP,
and 10 mM sodium $\left[^{14}C\right]$ bicarbonate.


Assays were performed in duplicate.

Bird aged 27 days.

Figure 4.4

The effect of assay incubation time on the activity of acetyl-CoA
carboxylase from the livers of oestrogen-treated and control male
chicks.

Enzyme assays were performed at 37°C as described in the text.
The enzyme was pre-incubated for 15 minutes at 37°C, in the
presence of citrate (14.7 mM), and then assayed using varying
incubation times in the presence of 100 mM-Tris/HCl (pH7.4 at
25°C), 8 mM-magnesium chloride, 11 mM-potassium citrate, 0.25 mM-
acetyl-CoA,  3mM-dithiothreitol, 0.6 mg/ml BSA, 1 mM-ATP and
25 mM-sodium $[^{14}C]$ bicarbonate.

Assays were performed in duplicate.

Birds aged 25 days.

(a) Activity of acetyl-CoA carboxylase from liver of an
oestrogen-treated chick killed 48 hours after injection.

(b) Activity of acetyl-CoA carboxylase from liver of a control
chick killed 48 hours after injection.

The oestrogen-treated bird received a single intramuscular
injection of 17β-oestradiol (0.75 mg/100 g body wt.) in
propane-1,2-diol.  The control bird received an equivalent
volume of propane-1,2-diol.
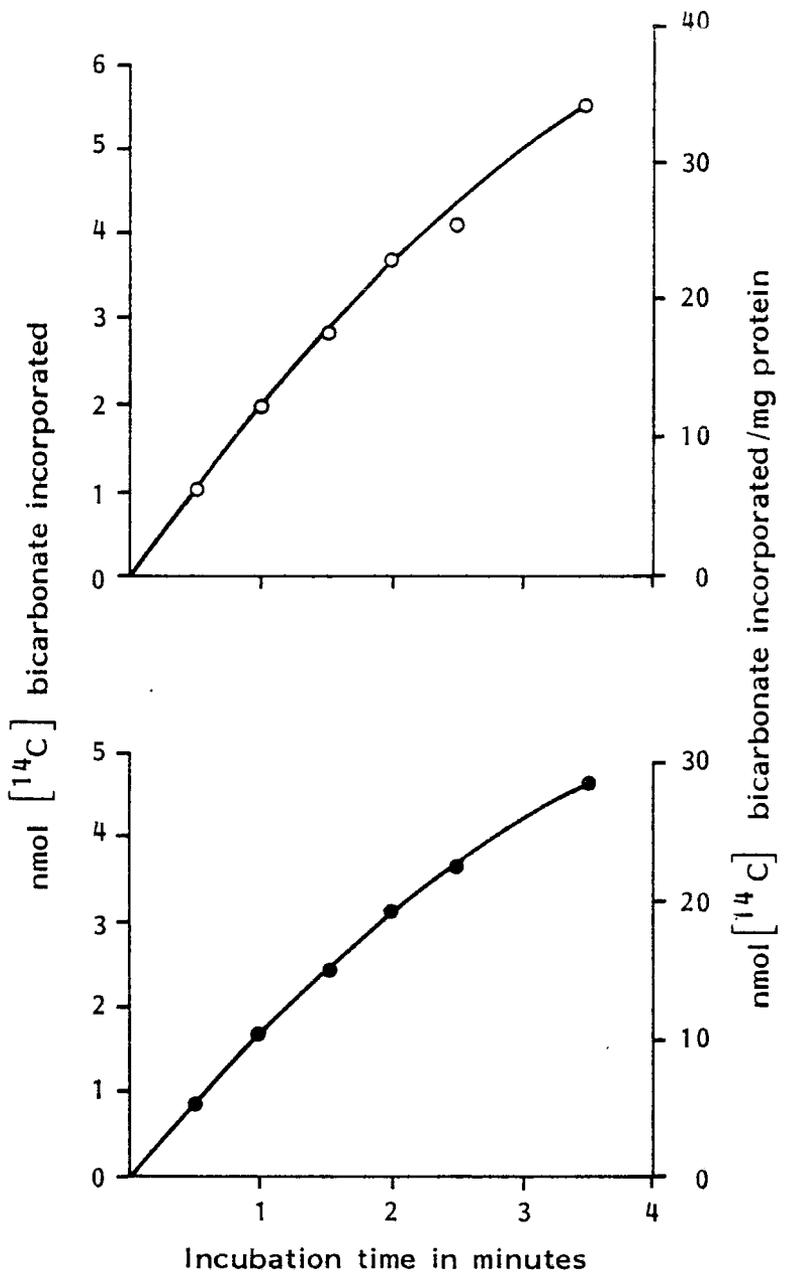
at 6 hours and 24 hours after injection. Consequently, all
subsequent assays were performed for 1.5 minutes.

The measured activity of the enzyme was linear with respect to
soluble protein up to 0.25 mg protein for untreated birds (Fig. 4.5).
Assays performed on control and oestrogen-treated birds killed at
48 hours after injection demonstrated linearity up to 0.18 mg
soluble protein per assay and 0.13 mg soluble protein per assay,
respectively. (Fig.4.6). Similar studies on control and oestrogen-
treated birds killed at 5 hours, 16 hours, 24 hours, 36 hours and
72 hours after injection also demonstrated linearity up to those
levels of soluble protein per assay reported for birds killed 48
hours after injection. Consequently, all subsequent assays were
performed using less than 0.20 mg protein for untreated birds, less
than 0.15 mg protein for control birds and less than 0.12 mg protein
for oestrogen-treated birds.

The optimum citrate concentration for the assay of the enzyme
extracted from untreated birds, as well as control and oestrogen-
treated birds killed 48 hours after injection, was found to be in
the range of 9 mM - 13 mM (Fig. 4.7). Furthermore, the results of
an experiment where the activity of the enzyme extracted from the
liver of an untreated bird was measured following pre-incubation of
the enzyme with citrate (14.7 mM) at 37°C for varying times
indicated that a pre-incubation time of 15 minutes was optimal
(Fig. 4.8) and this pre-incubation period was used in all subsequent
assays of the enzyme.

Figure 4.9a shows that acetyl-CoA concentrations of 0.22 mM -
0.38 mM produced optimum activity for the enzyme extracted from
untreated birds. Assays performed using enzyme derived from control
and oestrogen-treated birds, killed 48 hours after injection,

Figure 4.5


The effect of varying soluble protein content in the liver extract
on the activity of acetyl-CoA carboxylase from the liver of an
untreated male chick.


Enzyme assays were performed at 37°C as described in the text.
At each level of soluble protein, the enzyme extract was
pre-incubated for 5 minutes at 37°C in the presence of citrate
(27 mM), and then assayed in the presence of 100 mM-Tris/HCl
(pH7.5 at 25°C), 8 mM-magnesium chloride, 20 mM-potassium
citrate, 0.2 mM-acetyl-CoA, 3mM-dithiothreitol, 0.6 mg/ml BSA,
2 mM-ATP and 10 mM sodium $\left[^{14}C\right]$ bicarbonate.
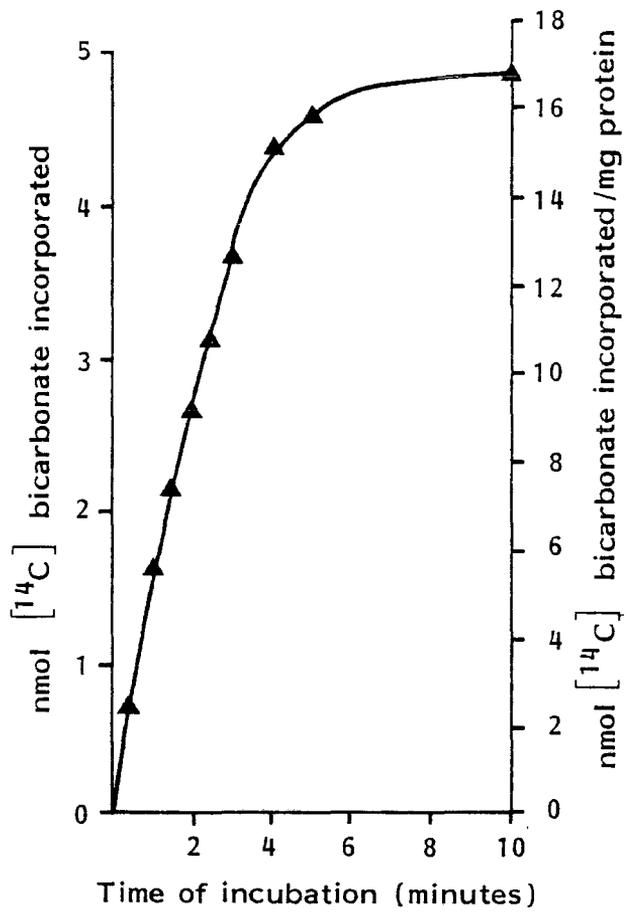
Assays were performed in duplicate.

Bird aged 29 days.

nmol [14C] bicarbonate incorporated/min
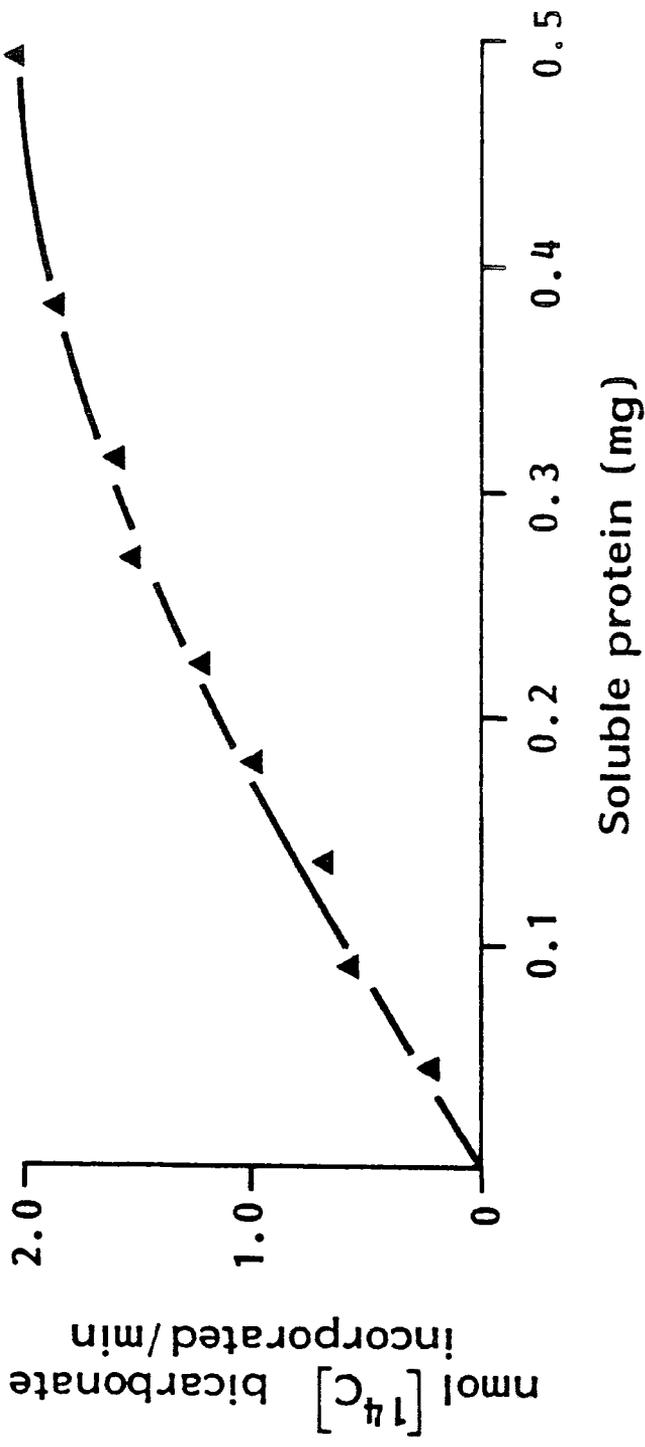
Soluble protein (mg)

Figure 4.6

The effect of varying soluble protein content in the liver
extract on the activity of acetyl-CoA carboxylase from the
livers of oestrogen-treated and control male chicks.

Enzyme assays were performed at 37°C as described in the text.
At each level of soluble protein, the enzyme extract was
pre-incubated for 15 minutes at 37°C, in the presence of
citrate (14.7 mM), and then assayed in the presence of 100 mM-
Tris/HCl (pH 7.4 at 25°C), 8 mM-magnesium chloride, 11 mM-
potassium citrate, 0.25 mM-acetyl-CoA, 3 mM-dithiothreitol,
0.6 mg/ml BSA, 1 mM-ATP and 25 mM-sodium $[^{14}C]$ bicarbonate.

Assays were performed in duplicate.

Birds aged 30 days.

(a) Activity of acetyl-CoA carboxylase from liver of an
oestrogen-treated chick killed 48 hours after injection.

(b) Activity of acetyl-CoA carboxylase from liver of a
control chick killed 48 hours after injection.

The oestrogen-treated bird received an intramuscular injection
of 17β-oestradiol (0.75 mg/100 g body wt.) in propane-1,2-diol.
The control bird received an equivalent volume of propane-
1,2-diol.

Figure 4.7

The effect of citrate concentration on the activity of
acetyl-CoA carboxylase from the livers of untreated,
oestrogen-treated and control male chicks.

Enzyme assays were performed at 37°C as described in the text.
The enzyme was pre-incubated for 15 minutes at 37°C in the
presence of citrate, and then assayed in the presence of
100 mM-Tris-HCl (pH7.4 at 25°C), 8 mM-magnesium chloride,
0 - 19 mM-potassium citrate, 0.25 mM-acetyl-CoA, 3 mM-
dithiothreitol, 0.6 mg/ml BSA, 1 mM-ATP and 25 mM-sodium $\left[^{14}C\right]$
bicarbonate.

Assays were performed in duplicate.

Birds aged 25 - 30 days.

(a) Activity of acetyl-CoA carboxylase from liver of an
untreated chick.

(b) Activity of acetyl-CoA carboxylase from liver of an
oestrogen-treated chick killed 48 hours after injection.

(c) Activity of acetyl-CoA carboxylase from liver of a
control chick killed 48 hours after injection.

The oestrogen-treated bird received a single intramuscular
injection of 17β-oestradiol (0.75 mg/100 g body wt.) in
propane-1,2-diol. The control bird received an equivalent
volume of propane-1,2-diol.
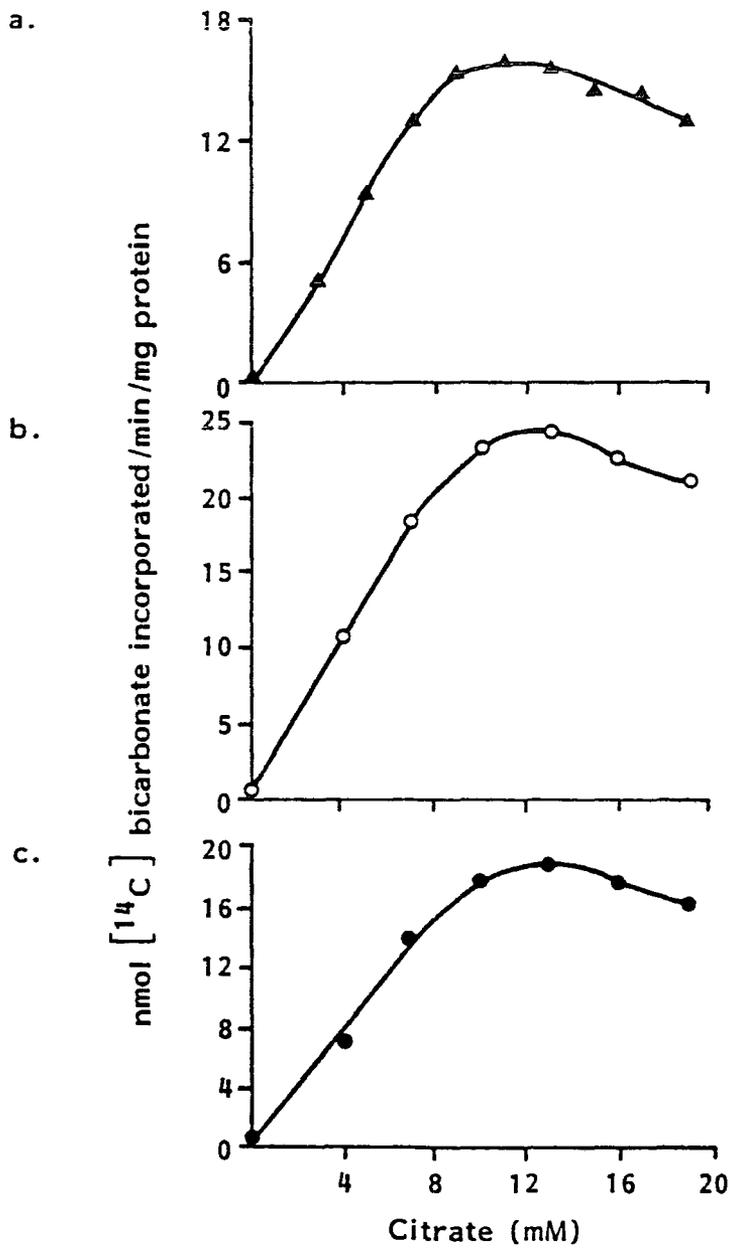
Figure 4.8


The effect of pre-incubation time, in the presence of citrate
(14.7 mM), on the activity of acetyl-CoA carboxylase from
the liver of an untreated male chick.


Enzyme assays were performed at 37°C as described in the text.
The enzyme was pre-incubated for 5 - 30 minutes at 37°C, in
the presence of citrate (14.7 mM), and then assayed in the
presence of 100 mM-Tris/HCl (pH7.5 at 25°C), 8 mM-magnesium
chloride, 11 mM-potassium citrate, 0.2 mM-acetyl-CoA,
3mM-dithiothreitol, 0.6 mg/ml BSA, 2 mM-ATP and 10 mM-sodium
$[^{14}C]$ bicarbonate.

Assays were performed in duplicate.

Bird aged 32 days.
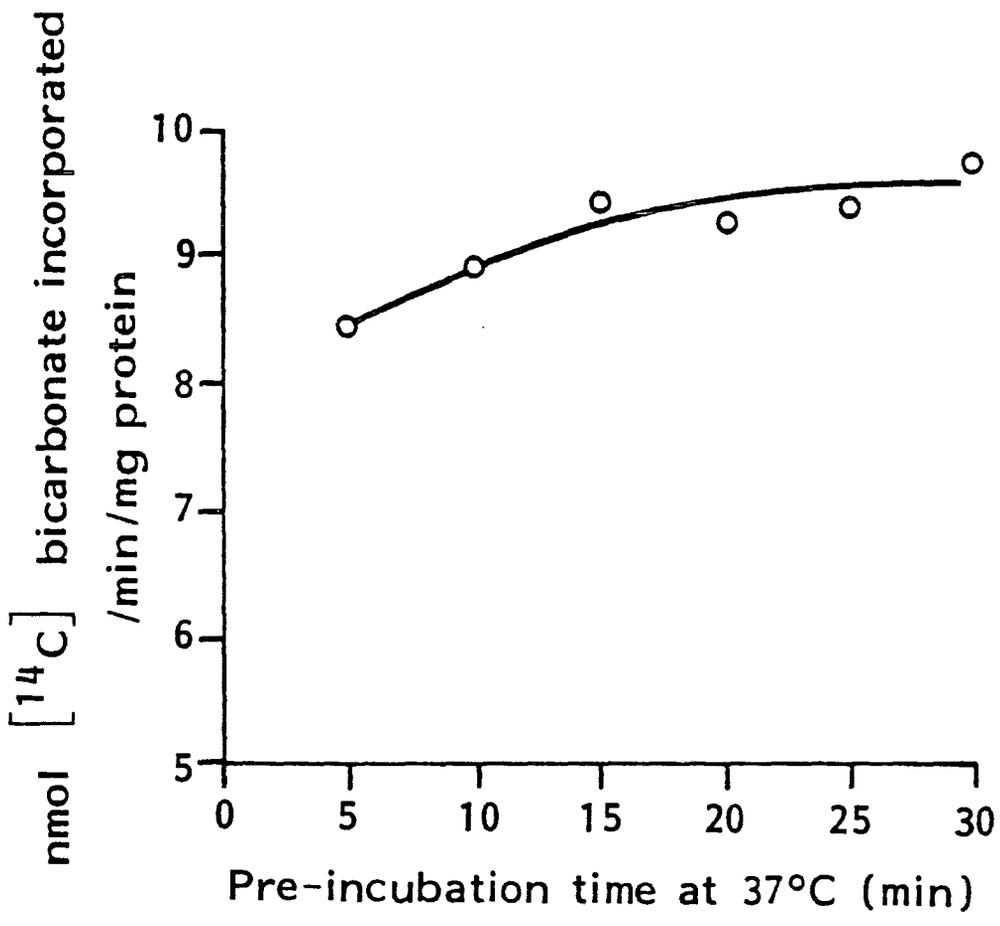
indicated optimum activity at acetyl-CoA concentrations above
0.25 mM (Fig. 4.9b & c) and, therefore, this concentration was
used in subsequent assays on all birds.

Optimum ATP concentrations for the assay of enzyme derived from
untreated, control and oestrogen-treated birds, at 48 hours after
injection were in the range of 0.8 mM - 1.2 mM (Fig. 4.10).
Greater ATP concentrations resulted in an inhibition of the enzyme
activity measured.  As a result, an ATP concentration of 1 mM was
used in subsequent assays on all birds.

Assays performed at various bicarbonate concentrations using enzyme
derived from untreated birds showed that maximal or near-maximal
enzyme activity was measured at concentrations of 25 mM - 35 mM
(Fig. 4.11a).  Similar results were obtained using enzyme extracted
from control and oestrogen-treated birds killed 48 hours after
injection, in that maximal or near-maximal enzyme activity was
measured at bicarbonate concentrations of 25 mM - 32 mM (Fig. 4.11b
& c.).  Consequently, a bicarbonate concentration of 25 mM was used
in all subsequent assays.

Assays performed at various magnesium chloride concentrations using
enzyme extracted from an untreated bird indicated peak enzyme
activity with magnesium concentrations of 7 mM - 9 mM (Fig. 4.12a).
As a result, a magnesium chloride concentration of 8 mM was used in
all subsequent assays.

An experiment in which the activity of enzyme, extracted from an
untreated bird, was determined at various pH values, revealed that
peak enzyme activity was measured over a range of pH6.7 to pH7.1,
measured at 37°C (Fig. 4.12b).  At greater pH values, the measured
enzyme activity was greatly reduced.  Consequently, a value of

Figure 4.9

The effect of acetyl-CoA concentration on the activity of
acetyl-CoA carboxylase from livers of untreated, oestrogen-
treated and control male chicks.

Enzyme assays were performed at 37°C as described in the text.
The enzyme was pre-incubated for 15 minutes at 37°C, in the
presence of citrate (14.6 mM), and then assayed in the
presence of 100 mM-Tris/HCl (pH 7.5 at 25°C), 8 mM-magnesium
chloride, 11 mM-potassium citrate, 0 - 0.38 mM-acetyl-CoA,
3 mM-dithiothreitol, 0.6 mg/ml BSA, 1 mM-ATP and 25 mM-sodium
$[^{14}C]$ bicarbonate.

Assays were performed in duplicate.

Birds aged 25 - 31 days.

(a) Activity of acetyl-CoA carboxylase from liver of an
untreated chick.

(b) Activity of acetyl-CoA carboxylase from liver of an
oestrogen-treated chick killed 48 hours after injection.

(c) Activity of acetyl-CoA carboxylase from liver of a
control chick killed 48 hours after injection.

The oestrogen-treated chick received a single intramuscular
injection of 17β-oestradiol (0.75 mg/100 g body wt.) in
propane-1,2-diol. The control bird received an equivalent
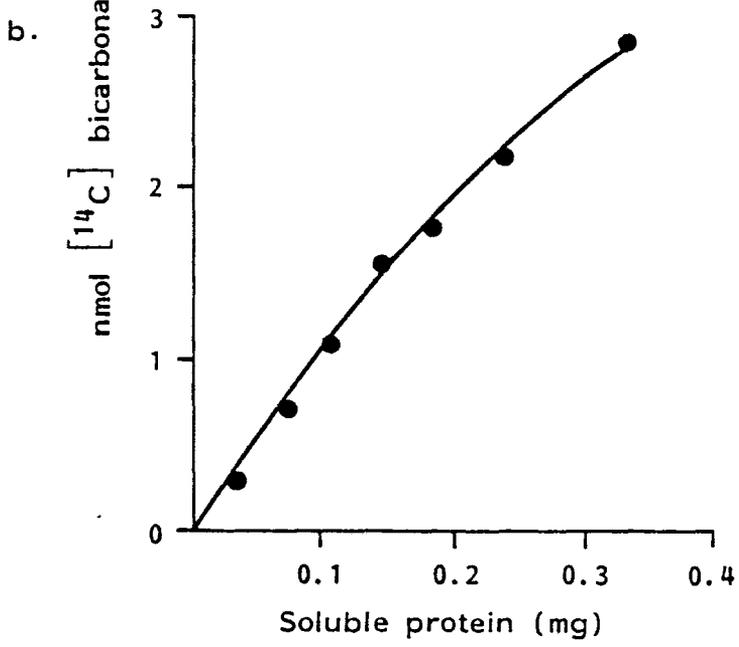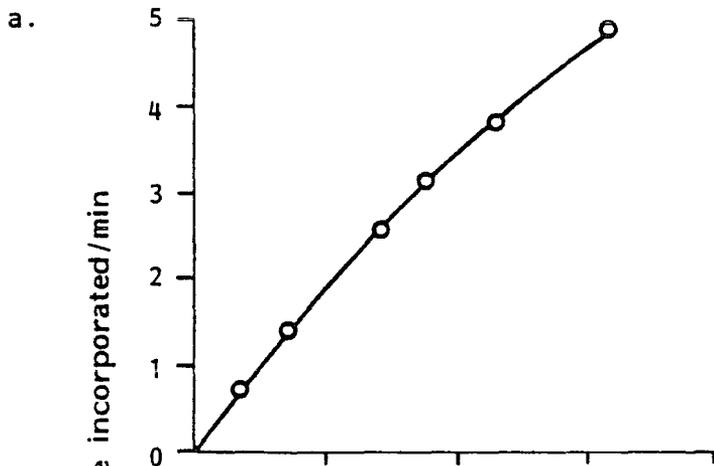volume of propane-1,2-diol.
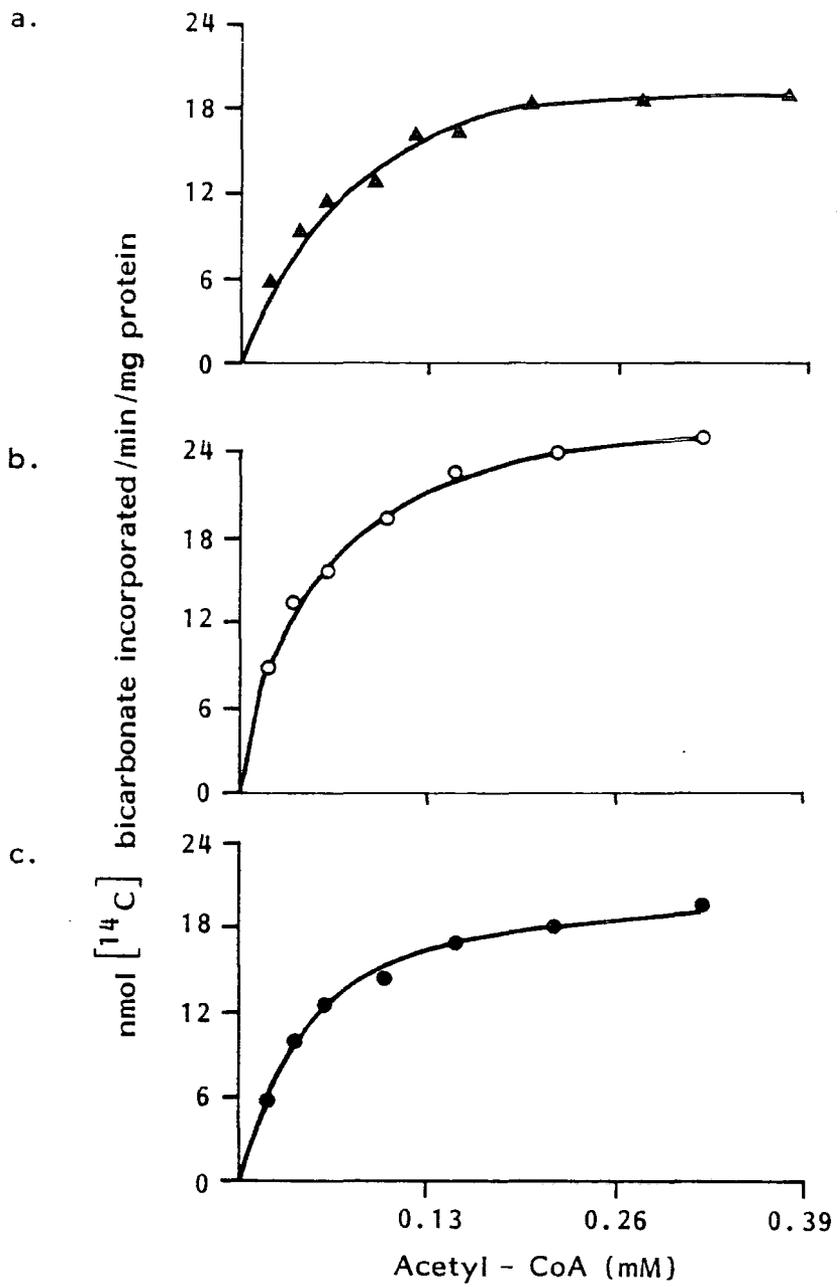
Figure 4.10

The effect of ATP concentration on the activity of acetyl-CoA
carboxylase from the livers of untreated, oestrogen-treated
and control chicks.

Enzyme assays were performed at 37°C as described in the text.
The enzyme was pre-incubated for 15 minutes at 37°C in the
presence of citrate (14.7 mM), and then assayed in the
presence of 100 mM-Tris/HCl (pH7.5 at 25°C), 8 mM-magnesium
chloride, 11 mM-potassium citrate, 0.25 mM-acetyl-CoA,
3 mM-dithiothreitol, 0.6 mg/ml BSA, 0 - 2.0 mM-ATP and 25 mM-
sodium $\left[^{14}C\right]$ bicarbonate.

Assays were performed in duplicate.

Birds aged 24 - 29 days.

(a) Activity of acetyl-CoA carboxylase from liver of an
untreated chick.

(b) Activity of acetyl-CoA carboxylase from liver of an
oestrogen-treated chick killed 48 hours after injection.

(c) Activity of acetyl-CoA carboxylase from liver of a
control chick killed 48 hours after injection.

The oestrogen-treated bird received a single intramuscular
injection of 17β-oestradiol (0.75 mg/100 g body wt.) in
propane-1,2-diol. The control bird received an equivalent
volume of propane-1,2-diol.
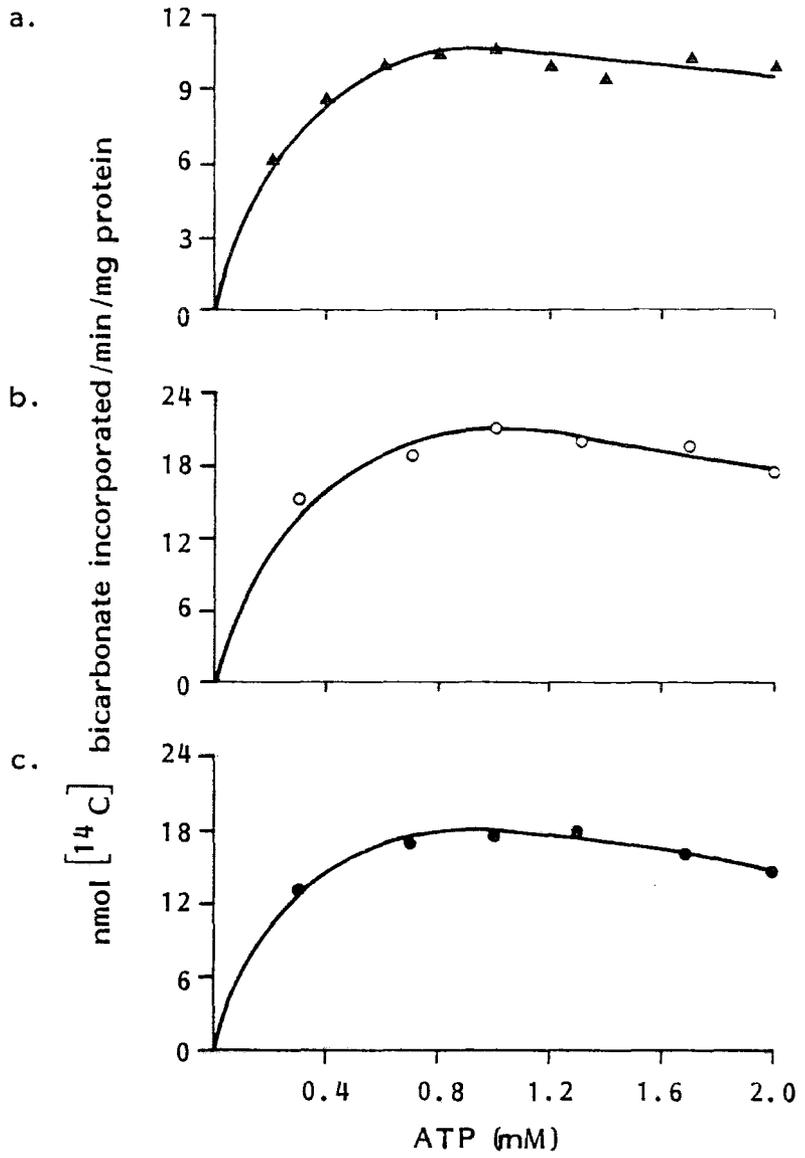
Figure 4.11

The effect of bicarbonate concentration on the activity of
acetyl-CoA carboxylase from the livers of untreated,
oestrogen-treated and control male chicks.

Enzyme assays were performed at 37°C as described in the text.
The enzyme was pre-incubated for 15 minutes at 37°C, in the
presence of citrate (14.7 mM), and then assayed in the
presence of 100 mM-Tris/HCl (pH7.4 at 25°C), 8 mM-magnesium
chloride, 11 mM-potassium citrate, 0.25 mM-acetyl-CoA, 3 mM-
dithiothreitol, 0.6 mg/ml BSA, 1 mM-ATP and 0 - 35 mM-sodium
$\left[ ^{14}C \right]$ bicarbonate.

Assays were performed in duplicate.

Birds aged 26 - 28 days.

(a) Activity of acetyl-CoA carboxylase from liver of an
untreated chick.

(b) Activity of acetyl-CoA carboxylase from liver of an
oestrogen-treated chick killed 48 hours after injection.

(c) Activity of acetyl-CoA carboxylase from liver of a
control chick killed 48 hours after injection.

The oestrogen-treated bird received a single intramuscular
injection of 17β-oestradiol (0.75 mg/100 g body wt.) in
propane-1,2-diol. The control bird received an equivalent
volume of propane-1,2-diol.
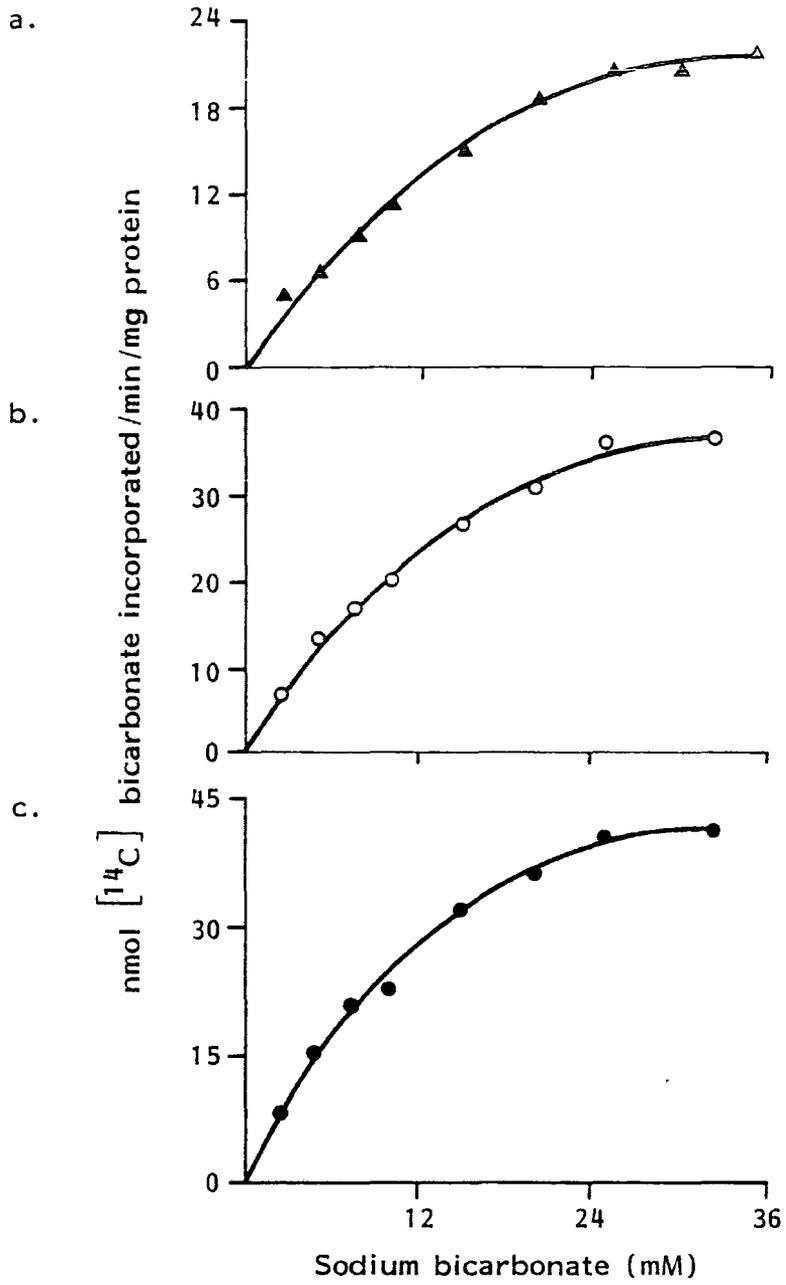
Figure 4.12

The effect of magnesium chloride concentration and of pH on
the activity of acetyl-CoA carboxylase from livers of
untreated birds.

(a)  Effect of magnesium chloride concentration.

Enzyme assays were performed at 37°C as described in the text.
The enzyme was pre-incubated at 37°C in the presence of
citrate (14.7 mM), and then assayed in the presence of 100 mM-
Tris/HCl (pH7.5 at 25°C), 0 - 18 mM-magnesium chloride, 11 mM-
potassium citrate, 0.25 mM-acetyl-CoA, 3 mM-dithiothreitol,
0.6 mg/ml BSA, 1 mM-ATP and 25 mM-sodium $\left[^{14}C\right]$ bicarbonate.

Assays were performed in duplicate.

Bird aged 31 days.

(b)  Effect of pH.

Enzyme assays were performed at 37°C as described in the text.
After pre-incubation at 37°C in the presence of citrate
(14.7 mM), the enzyme was assayed at varying pH values in the
presence of 100 mM-Tris/HCl (pH 6.4 - 8.2 at 25°C), 8 mM-
magnesium chloride, 11 mM-potassium citrate, 0.25 mM-acetyl-
CoA, 3 mM-dithiothreitol, 0.6 mg/ml BSA, 1 mM-ATP and 25 mM-
sodium $\left[^{14}C\right]$ bicarbonate.

Assays were performed in duplicate.

Bird aged 32 days.
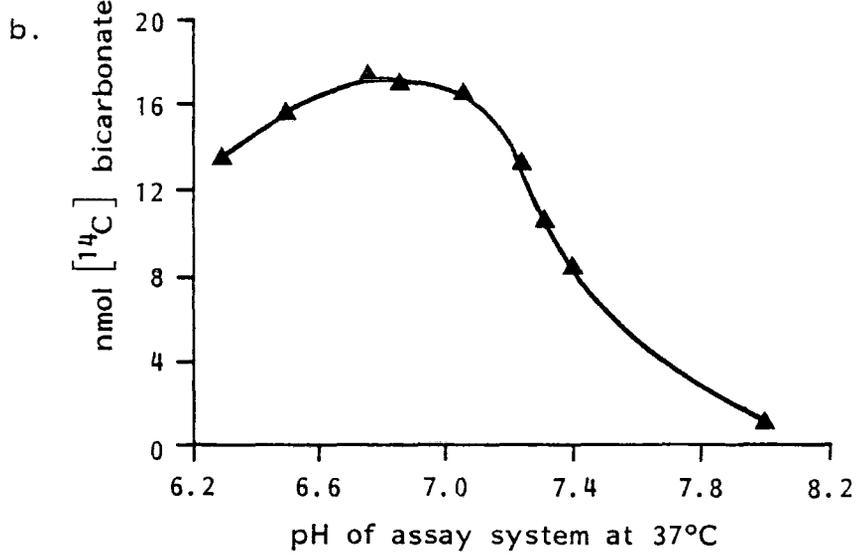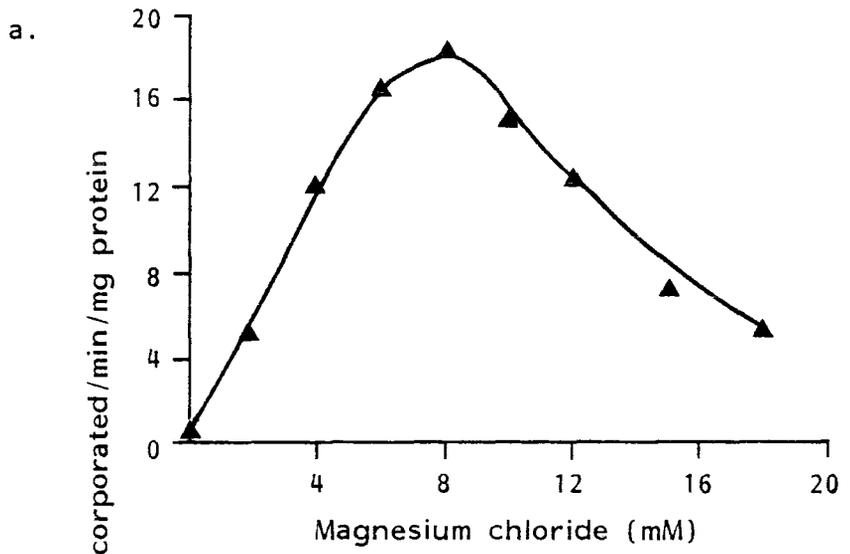
pH7.05 was adopted for subsequent assays, such a pH value being produced when a Tris/HCl buffer of pH7.4 at 25°C was used in the assay system.

In view of the results obtained in these preliminary experiments, all subsequent assays of acetyl-CoA carboxylase extracted from the livers of untreated, control and oestrogen-treated birds were performed at pH7.05 at 37°C with final concentrations of 100 mM-Tris/HCl, 8 mM-magnesium chloride, 11 mM-potassium citrate, 0.25 mM-acetyl-CoA, 1mM-ATP and 25 mM-sodium $[^{14}C]$ bicarbonate in a final volume of 0.4 ml. The enzyme preparations were pre-incubated in the presence of citrate for 15 minutes at 37°C, before the addition of 'start cocktail', and assay incubations were of 1.5 minutes duration.

2. <u>The effect of varying doses of 17$\beta$-oestradiol on the activity of hepatic acetyl-CoA carboxylase</u>

To study the effect of 17$\beta$-oestradiol on hepatic acetyl-CoA carboxylase activity it was necessary to establish an optimum dose of hormone. Each bird received a single intramuscular injection of 17$\beta$-oestradiol in propane-1,2-diol equivalent to one of the following doses; 0.25, 0.50, 0.75, 1.00, 1.25 mg 17$\beta$-oestradiol/ 100 g body weight. Control chicks received an equivalent volume of propane-1,2-diol only (0.1 ml/100 g body wt.). Chicks were killed 48 hours after injection and the enzyme activities in the high-speed supernatants prepared from the livers were measured.

The results of this experiment are presented in Table 4.1 and Figure 4.13, indicating an increase in the mean specific activity of acetyl-CoA carboxylase as a result of oestrogen treatment, reaching a peak at a dose of 0.75 mg hormone/100 g. body weight (Fig. 4.13a). At this dose the mean specific activity was 1.99-fold greater than the value for the control group of birds. Higher doses

Table 4.1

The effect of varying doses of 17β-oestradiol on liver weight, the soluble protein content of liver and the activity of hepatic acetyl-CoA carboxylase (ACC) 48 hours after injection.

Each oestrogen-treated bird received an intramuscular injection of 17β-oestradiol in propane-1,2-diol, equivalent to one of the following doses:-

0.25, 0.5, 0.75, 1.0, 1.25 mg 17β-oestradiol /100 g body weight.

Control birds received an equivalent volume of propane-1,2-diol, alone. Birds were sacrificed 48 hours after injection and liver enzyme preparations were obtained and assayed, in triplicate, for acetyl-CoA carboxylase as described in the Methods section. The protein content of the particle-free supernatant was assayed as described in the Methods section.

Values are the means ($\pm$ S.E.M.) of 5 - 8 birds.

One unit of acetyl-CoA carboxylase activity is defined as that amount of enzyme which catalyses the incorporation of 1.0 µmole $\left[^{14}C\right]$ bicarbonate into acid-stable products per min. under the assay conditions described.

Data were analysed statistically by Student's 't' test. Levels of significance presented with oestrogen-treated values are with respect to control values.

* significant at $p < 0.05$

# significant at $p < 0.01$

¥ significant at $p < 0.001$

The enzyme activity results are presented graphically in Figure 4.13.

Birds were aged 28 - 34 days.

Data for those parameters labelled '†' have been presented in Chapter 2, Table 2.1.

| | 0 Control | 0 Untreated | 0.25 | 0.50 | 0.75 | 1.00 | 1.25 |
|---|---|---|---|---|---|---|---|
| Specific activity of acetyl-CoA carboxylase (m units/mg soluble protein) | 11.87 ± 1.11 | 12.84 ± 1.23 | * 15.17 ± 0.93 | ¥ 20.84 ± 1.07 | ¥ 23.65 ± 2.36 | # 20.85 ± 1.79 | ¥ 20.22 ± 1.52 |
| Acetyl-CoA carboxylase activity (units/g liver) | 0.98 ± 0.11 | 1.04 ± 0.10 | 1.21 ± 0.07 | # 1.47 ± 0.09 | ¥ 1.68 ± 0.06 | * 1.42 ± 0.15 | * 1.35 ± 0.12 |
| Acetyl-CoA carboxylase activity (units/whole liver) | 8.16 ± 1.09 | 9.75 ± 1.27 | 10.86 ± 0.83 | ¥ 19.72 ± 2.35 | ¥ 28.60 ± 4.78 | ¥ 25.05 ± 2.61 | ¥ 29.54 ± 4.07 |
| † Body weight (g). | 303 ± 20 | 324 ± 17 | 294 ± 6 | 343 ± 18 | 345 ± 27 | 387 ± 25 | 425 ± 10 |
| † Soluble protein content (mg/g liver) | 81.9 ± 1.7 | 81.0 ± 1.3 | 79.9 ± 1.0 | # 70.2 ± 2.6 | * 72.9 ± 3.0 | # 70.4 ± 2.2 | ¥ 66.8 ± 2.1 |
| † Soluble protein content (mg/liver) | 677 ± 48 | 747 ± 47 | 720 ± 43 | # 936 ± 73 | ¥ 1113 ± 87 | ¥ 1270 ± 69 | ¥ 1433 ± 83 |
| ♣ Liver wt. (as % of body wt.) | 2.75 ± 0.09 | 2.88 ± 0.18 | 3.06 ± 0.12 | ¥ 3.91 ± 0.22 | ¥ 4.50 ± 0.29 | ¥ 4.69 ± 0.14 | ¥ 5.05 ± 0.24 |
| No. of birds | 8 | 8 | 6 | 6 | 5 | 6 | 6 |

of hormone ($\geq$ 1 mg/100 g body wt.) caused a slight decrease in the
mean specific activity of the enzyme. However, at all hormone doses
the mean specific activity was higher than the values for the
control and untreated groups. Statistical analysis of these results,
using Student's 't' test, indicated significant differences in
specific activity between control and oestrogen-treated birds at
all doses of hormone (Table 4.1). There was no significant difference
between control and untreated birds, with respect to the specific
activity of the enzyme. The mean specific activity of the enzyme
from birds treated with 0.75 mg hormone/100 g body weight did not
differ significantly from the specific activities of the enzyme from
those birds treated with 0.50, 1.00 and 1.25 mg hormone/100 g body
weight.

Expression of the data as mean enzyme activity per gram liver gave
a similar result (Fig. 4.13b). Maximum mean activity occurred with
a dose of 0.75 mg hormone/100 g body weight, being 1.71-fold
greater than the mean activity/g liver for the control group. All
doses, except that of 0.25 mg hormone/100 g body weight, produced
activities significantly different from the control value (Table
4.1). The mean activity/g liver at the dose of 0.75 mg hormone/
100 g body weight was significantly greater than the values obtained
for birds treated with 0.25 mg and 1.25 mg hormone/100 g body
weight, no significant difference being apparent with the other
dose levels (0.50 and 1.00 mg hormone/100 g body wt.)

Expression of enzyme activity as total organ activity revealed a
slightly different result (Fig. 4.13c). Maximum mean activity was
produced with a dose of 1.25 mg hormone/100 g body weight, being
3.62-fold greater than the mean control value, although it was only
slightly higher than the activity produced by the 0.75 mg hormone/
100 g body weight dose which was 3.5-fold greater than the control
value. All dose levels produced total organ activities significantly

Figure 4.13

The effect of varying doses of 17β-oestradiol on the activity
of hepatic acetyl-CoA carboxylase, 48 hours after injection.

Consult the legend of Table 4.1 for details of experimental
procedure.

▲ values for untreated chicks.

● values for control chicks, injected
with propane-1,2-diol.

○ values for oestrogen-treated chicks.

(a) Specific activity (nmol $[^{14}C]$ bicarbonate incorporated/
min./mg protein).

(b) Activity/g liver ($\mu$mol $[^{14}C]$ bicarbonate incorporated/
min./g liver).

(c) Total hepatic activity ($\mu$mol $[^{14}C]$ bicarbonate
incorporated/min./liver.

The results are expressed as means ($\pm$ S.E.M.) of 5 - 8
birds.
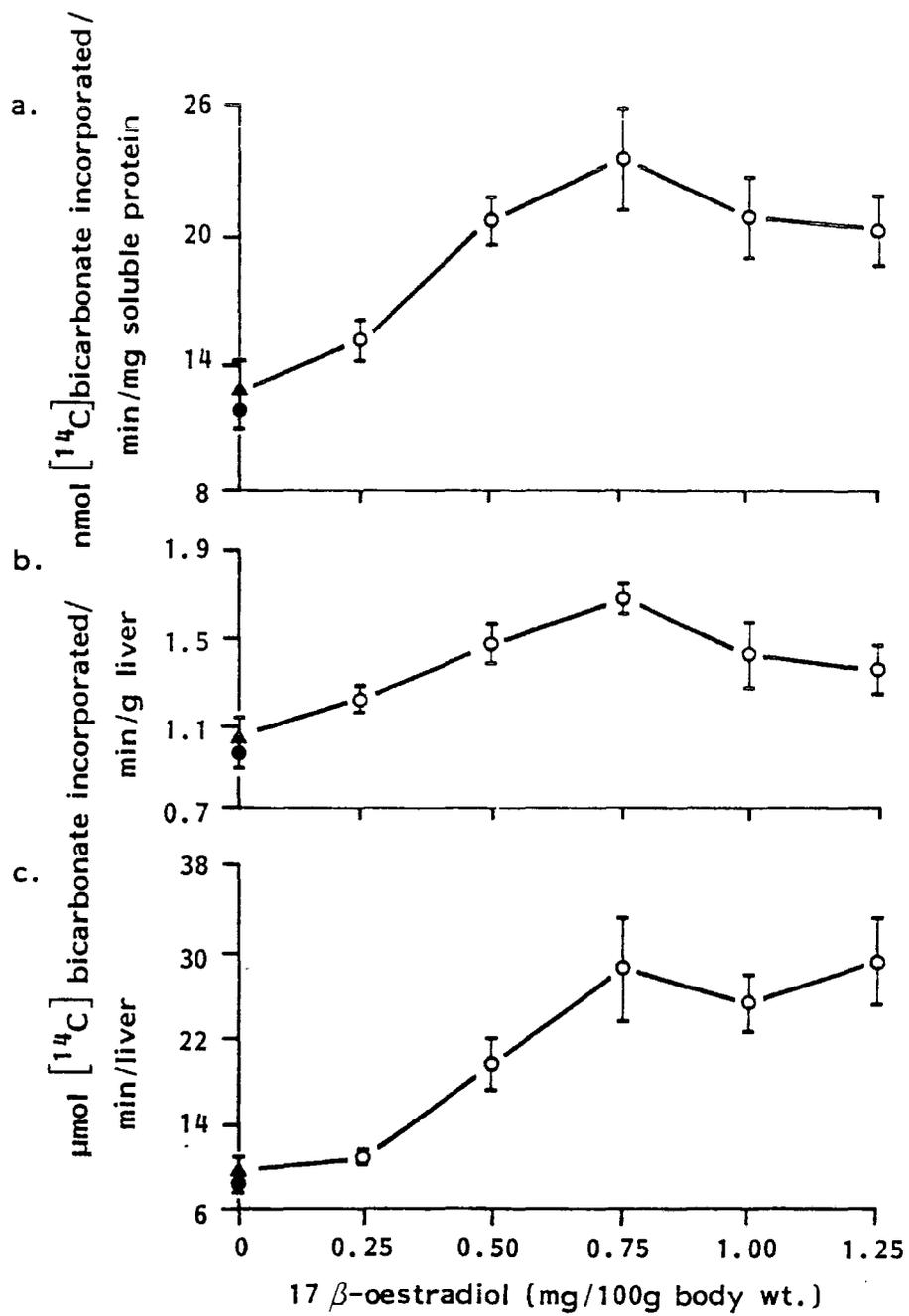
greater than the control value.  On the basis of these results, a
dose of 0.75 mg 17β-oestradiol/100 g body weight was selected for
future use.

Oestrogen treatment also caused a decrease in the mean soluble
protein content of a unit weight of liver (Table 4.1).  Significant
differences between control and oestrogen-treated values were
observed at all doses except that of 0.25 mg hormone/100 g body
weight.  The lowest soluble protein value, obtained with a dose of
1.25 mg hormone/100 g body weight, represented an 18% decrease in
in the value obtained for control chicks.

Liver weights, expressed as a percentage of body weight, increased
over the 17β-oestradiol dose range, a maximum mean value being
obtained with a dose of 1.25 mg hormone/100 g body weight (Table 4.1).
Despite a dose-dependent decrease in soluble protein/g liver, the
increase in liver weight after oestrogen treatment was accompanied
by a dose-dependent increase in total soluble protein in the liver
(Table 4.1) such that maximum total soluble protein content was
observed with a dose of 1.25 mg hormone/100 g body weight.

The variations in liver weight and soluble protein content of male
chicks treated with varying doses of 17β-oestradiol have been
reported and discussed in Chapter 2.

3.   The effect of a single intramuscular injection of 17β-oestradiol
     (0.75 mg/100 g body wt.) on the activity of hepatic acetyl-CoA
     carboxylase.

The results of an experiment designed to investigate the effect of
an intramuscular injection of 17β-oestradiol (0.75 mg/100 g body wt.)
on the activity of hepatic acetyl-CoA carboxylase at various times

after injection, are presented in Table 4.2 and Figure 4.14.
Hepatic enzyme activities were expressed as specific activities with
respect to soluble protein (Fig. 4.14a) and specific activities with
respect to DNA (Fig. 4.14b).

During the first 19 hours after injection the mean specific
activities, with respect to protein, of oestrogen-treated and
control birds were not significantly different (Fig. 4.14a).
However, during the 24 hour - 48 hour post-injection period the
specific activity, with respect to protein, of oestrogen-treated
birds increased in a time-dependent manner while the specific
activity of control birds remained at a level similar to the value
for untreated birds.  Maximum specific activity, with respect to
protein, was observed at 48 hours after oestrogen-treatment, the
value being 1.53-fold greater than the corresponding value for
control birds and 1.32-fold greater than the value for untreated
birds.  At 61 hours post-injection, the mean specific activities,
with respect to protein, of control and oestrogen-treated birds were
not significantly different.

The trends in mean specific activities, with respect to DNA, in
control and oestrogen-treated birds during the 61 hour post-
injection period (Fig. 4.14b) were similar to those for specific
activities with respect to protein.  During the first 19 hours
after injection the mean specific activities with respect to DNA,
of oestrogen-treated and control birds were not significantly
different.  However, the mean specific activities, with respect to
DNA, of oestrogen-treated birds at the 24-hour, 37-hour and 48-hour
post injection points were significantly greater than the
corresponding values for control birds which remained at a level
similar to the value for untreated birds.  Maximum mean specific
activity, with respect to DNA, was observed at 37 hours after

Table 4.2

The effect of a single dose of 17β-oestradiol (0.75 mg in propane-1,2-diol/100g body wt.), or of propane-1,2-diol alone, on the activity of hepatic acetyl-CoA carboxylase (ACC) and the hepatic soluble protein and DNA content, at varying times after injection.

Each oestrogen-treated bird received an intramuscular injection of 17β-oestradiol (0.75 mg/100 g body wt.) in propane-1,2-diol. Control birds received an equivalent volume of propane-1,2-diol alone. At the indicated times after injection birds were sacrificed, liver enzyme extracts prepared and assayed in triplicate for acetyl-CoA carboxylase as described in the Methods section. The protein content of the particle-free supernatant and the DNA content of liver samples were determined as described in the Methods section.

OE = values for oestrogen-treated birds. C = values for control birds.
Values are means ($\pm$ S.E.M.) of 6 - 19 birds.

One unit of acetyl-CoA carboxylase activity is defined as that amount of enzyme which catalyses the incorporation of 1.0 μmole $\left[ ^{14}C \right]$ bicarbonate into acid-stable products per min. under the assay conditions described.

Data were analysed statistically by Student's 't' test. Levels of statistical significance presented with values for oestrogen-treated birds are with respect to the corresponding control values.

* significant at $p < 0.05$
# significant at $p < 0.01$
¥ significant at $p < 0.001$

The enzyme activity results are presented graphically in Figure 4.14.
Birds were aged 25 - 32 days.
Data for those parameters labelled '‡' have been presented in Chapter 2, Table 2.2.

| | 0 | 3 OE | 3 C | 6 OE | 6 C | 13 OE | 13 C | 19 OE | 19 C | 24 OE | 24 C | 37 OE | 37 C | 48 OE | 48 C | 61 OE | 61 C |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Specific activity of ACC (m units/mg soluble protein) | 19.8 ±0.8 | 19.3 ±1.6 | 18.3 ±0.5 | 22.2 ±1.3 | 20.3 ±0.8 | 19.8 ±1.2 | 22.3 ±1.5 | 20.9 ±1.3 | 20.5 ±1.4 | 21.8* ±0.9 | 18.5 ±0.7 | 25.7 ±1.1 | 18.7 ±0.7 | 26.2 ±1.0 | ±17.1 1.1 | 22.2 ±1.7 | 18.9 ±0.8 |
| Specific activity of ACC (m units/mg DNA) | 497 ±29 | 509 ±54 | 484 ±19 | 563 ±35 | 497 ±25 | 502 ±36 | 576 ±48 | 603 ±58 | 514 ±22 | 649 ±28 | 468 ±22 | 790 ±84 | 496 ±45 | 772 ±34 | 444 ±36 | 714 ±117 | 494 ±36 |
| ACC activity (units/g liver) | 1.51 ±0.19 | 1.40 ±0.13 | 1.37 ±0.05 | 1.55 ±0.09 | 1.44 ±0.08 | 1.35* ±0.09 | 1.73 ±0.12 | 1.40 ±0.11 | 1.54 ±0.07 | 1.37 ±0.05 | 1.44 ±0.04 | 1.60 ±0.08 | 1.40 ±0.05 | 1.83 ±0.09 | 1.26 ±0.08 | 1.61 ±0.14 | 1.40 ±0.07 |
| ACC activity (units/whole liver) | 15.6 ±0.9 | 16.4 ±2.0 | 14.8 ±0.7 | 17.9 ±1.9 | 15.2 ±1.3 | 15.2 ±1.3 | 17.4 ±1.1 | 19.9 ±2.2 | 15.8 ±1.1 | 20.0 ±1.6 | 13.1 ±1.1 | 31.9 ±1.8 | 15.2 ±0.7 | 30.8 ±1.3 | 12.7 ±0.6 | 25.8 ±3.3 | 15.3 ±1.2 |
| ‡Body wt. (g) | 313 ±11 | 332 ±21 | 318 ±11 | 336 ±23 | 303 ±21 | 311 ±7 | 310 ±9 | 356 ±18 | 323 ±21 | 303 ±15 | 280 ±18 | 361 ±10 | 318 ±12 | 338 ±20 | 312 ±13 | 318 ±12 | 338 ±13 |
| ‡Liver wt. (as % of body wt.) | 3.33 ±0.04 | 3.52 ±0.10 | 3.41 ±0.07 | 3.42 ±0.06 | 3.51 ±0.08 | 3.59 ±0.08 | 3.29 ±0.08 | 4.11 ±0.09 | 3.20 ±0.10 | 4.78 ±0.18 | 3.23 ±0.06 | 5.53 ±0.10 | 3.44 ±0.15 | 5.11 ±0.23 | 3.26 ±0.06 | 4.94 ±0.16 | 3.21 ±0.07 |
| ‡DNA content (mg/liver) | 32.0 ±1.3 | 32.1 ±1.3 | 30.7 ±0.8 | 31.4 ±2.0 | 30.6 ±1.9 | 30.1 ±0.9 | 31.0 ±1.5 | 33.4 ±2.1 | 30.5 ±1.3 | 30.8 ±2.2 | 29.0 ±1.9 | 42.3 ±2.4 | 30.6 ±2.6 | 40.3 ±1.7 | 29.6 ±2.2 | 37.4 ±1.6 | 31.4 ±1.1 |
| ‡Soluble protein (mg/g liver) | 75.8 ±1.1 | 71.9 ±2.0 | 75.0 ±1.9 | 70.0 ±0.9 | 70.5 ±1.5 | 67.8 ±1.1 | 77.5 ±1.3 | 65.4 ±2.6 | 76.4 ±2.3 | 63.2 ±1.2 | 78.2 ±2.3 | 62.4 ±1.5 | 74.7 ±1.5 | 68.6 ±2.1 | 73.7 ±1.4 | 72.1 ±2.0 | 74.1 ±1.2 |
| ‡Soluble protein (mg/liver) | 787 ±28 | 837 ±56 | 810 ±33 | 795 ±47 | 743 ±44 | 760 ±34 | 791 ±32 | 943 ±57 | 776 ±34 | 919 ±65 | 706 ±52 | 1244 ±45 | 811 ±35 | 1186 ±51 | 751 ±38 | 1139 ±70 | 802 ±36 |
| ‡Soluble protein (mg/mg DNA) | 25.6 ±0.7 | 26.1 ±1.3 | 26.4 ±0.7 | 25.4 ±0.5 | 24.4 ±0.6 | 25.3 ±0.8 | 25.6 ±0.6 | 28.6 ±1.5 | 25.6 ±1.2 | 29.9 ±0.5 | 25.3 ±0.7 | 30.4 ±1.4 | 27.4 ±1.4 | 29.5 ±0.8 | 25.8 ±1.1 | 29.9 ±2.3 | 25.6 ±0.9 |
| ‡No. of birds | 19 | 6 | 6 | 12 | 12 | 9 | 9 | 9 | 9 | 9 | 9 | 8 | 8 | 9 | 9 | 9 | 9 |

oestrogen treatment when the oestrogen-treated value was 1.59-fold greater than the untreated value and the corresponding control value. It should be noted, however, that at 48 hours after injection, the mean specific activity, with respect to DNA, of oestrogen-treated birds was 1.55-fold greater than the value for untreated birds, but 1.74-fold greater than the value for the corresponding control birds. At 61 hours post-injection, while the mean specific activity, with respect to DNA, of oestrogen-treated birds was greater than the corresponding value for control birds, the two values were not significantly different.

When the results were expressed as activity per gram of liver, a slightly different picture emerged (Table 4.2). At 13 hours post-injection, the mean activity/g liver of control birds was significantly greater than the corresponding value for oestrogen-treated birds ($p < 0.05$). Subsequently the activity/g liver values for both oestrogen-treated and control birds were not significantly different until 48 hours after injection when the activity/g liver of oestrogen-treated birds reached a maximum and was 1.45-fold greater than the corresponding value for control birds. At 61 hours post-injection, the activity/g liver of control and oestrogen-treated birds were not significantly different.

Hepatic acetyl-CoA carboxylase activity was also expressed as total activity per whole liver (Fig. 4.14c). During the first 19-hour post-injection period, there was no significant difference between values for oestrogen-treated and control birds. However, from 24 hours to 61 hours after injection the mean total activities per liver for oestrogen-treated birds were significantly greater than the corresponding values for control birds. Peaks in total hepatic activity were observed at 37 hours post-injection when the value for oestrogen-treated birds was 2.10-fold greater than the control value

Figure 4.14

Hepatic acetyl-CoA carboxylase activity at varying times
after a single injection of 17B-oestradiol (0.75 mg/100 g
body wt.).

Consult the legend of Table 4.2 for details of experimental
procedure.

▲ values for untreated birds.
● values for control birds, injected
   with propane-1,2-diol.
○ values for oestrogen-treated birds.

(a) Specific activity, with respect to soluble protein
(nmol $[^{14}C]$ bicarbonate incorporated/min./mg protein).

(b) Specific activity, with respect to DNA (nmol $[^{14}C]$
bicarbonate incorporated/min./mg DNA).

(c) Total hepatic activity ($\mu$mol $[^{14}C]$ bicarbonate
incorporated/min./liver.

The results are expressed as the means ($\pm$ S.E.M.) of 6 - 19
birds.
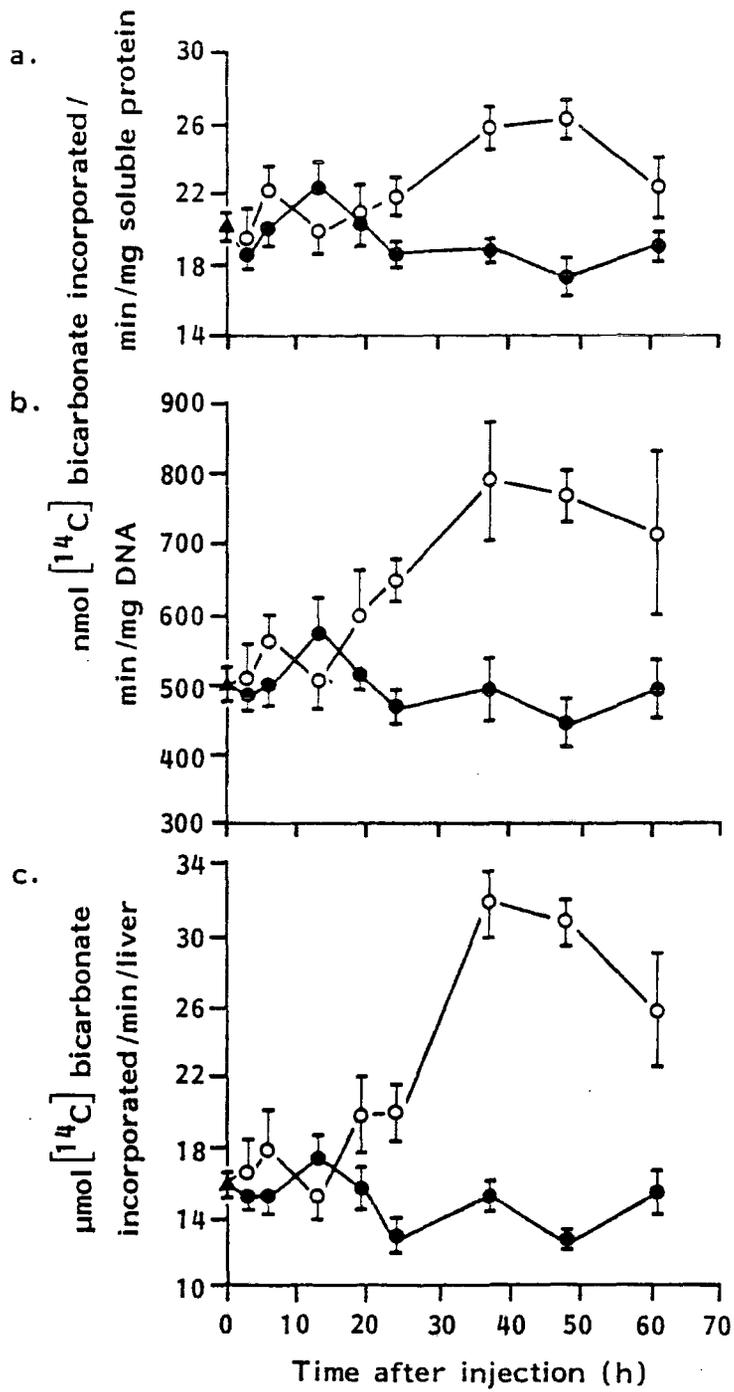
and 2.04-fold greater than the untreated value, and at 48 hours post-injection, when the oestrogen-treated value was 2.43-fold greater than the corresponding control value and 1.97-fold greater than the untreated value.

Throughout the time-course of the experiment (0h - 61h) the mean soluble protein content/g liver was lower for the oestrogen-treated group of birds than the corresponding control group (Table 4.2). However, only at 13 hours, 19 hours, 24 hours and 37 hours after treatment were statistically significant differences observed and a minimum value for oestrogen-treated birds was observed at 37 hours post-injection, the value being 82% of the untreated value.

Oestrogen-treatment also caused a time-related increase in liver weight, expressed as a percentage of body weight (Table 4.2). A peak in liver weight was observed at 37 hours after oestrogen treatment. The oestrogen-dependent increase in liver weight was accompanied by an increase in total soluble protein content per liver and total DNA content per liver, both parameters reaching a peak at 37 hours post-injection (Table 4.2). Measurements of the amount of hepatic soluble protein/mg DNA, a parameter which is indicative of the cellular soluble protein content, showed an increase over corresponding control values at 19 hours - 61 hours after oestrogen treatment, although significant differences were only observed at 24 hours and 48 hours after injection.

The variations in liver weight, soluble protein content and DNA content of male chicks treated with a single injection of 17$\beta$-oestradiol (0.75 mg/100 g body wt.) have been fully reported and discussed in Chapter 2.

DISCUSSION

1. Characterisation of the procedure for the assay of acetyl-CoA
   carboxylase

Assay of acetyl-CoA carboxylase involved the incubation of the
enzyme with $[^{14}C]$ bicarbonate, followed by the addition of HCl
and drying overnight at 50°C. Analysis, by thin-layer chroma-
tography, of the acidified dried reaction mixtures showed that the
radioactivity was incorporated into $[^{14}C]$ malonyl-CoA and $[^{14}C]$
malonate, the latter being assumed to be a degradation product of
the former. Less than 0.5% of the radioactivity detected on the
thin-layer plate was found to co-migrate with standards other than
these two. It can, therefore, be concluded that the assay employed
was specific for acetyl-CoA carboxylase and was not suspect to
interference from other enzymes present in the liver preparation.
It is important to establish this fact in the light of work presented
by Davies et al. (1982) which questioned the specificity of the
$[^{14}C]$ bicarbonate fixation assay for acetyl-CoA carboxylase. These
workers showed that, under certain assay conditions, the assay is
prone to interference by pyruvate carboxylase, a mitochondrial
enzyme with a requirement for acetyl-CoA which is an allosteric
effector of the enzyme. Davies et al. (1982) observed an acetyl-
CoA-dependent incorporation of $[^{14}C]$ bicarbonate into an acid-stable
product by a crude liver extract, from a fed rat. The extract was
prepared by homogenisation of the liver followed by centrifugation
at 27,000 g for 10 minutes and it was pre-incubated in the absence
of citrate at 37°C. Thin-layer chromatographic analysis of the end-
products of the assay showed the main reaction products to be
oxaloacetate, the product of the reaction catalysed by pyruvate
carboxylase, and malate, aspartate and citrate which may be produced
from oxaloacetate in crude enzyme preparations. However, under the

experimental conditions used in the present study, pyruvate carboxylase did not interfere with the assay of acetyl-CoA carboxylase for the following two reasons:

(a) Oxaloacetate did not survive the drying process at 55°C since Hohorst & Reim (1965) showed that recovery was less than 5% when oxaloacetate (up to 4 mM) was subjected to these conditions. For this reason, oxaloacetate was not included as one of the standards during the thin-layer chromatographic analysis in the present study.

(b) Radioactivity was not incorporated into malate, citrate or aspartate, which are conversion-products of oxaloacetate.

Reasons for the different assay end-products in the present study and that of Davies et al. (1982) could include (a) the use of a more careful homogenisation procedure in the present study, which minimised mitochondrial disruption, (b) the use of a less crude enzyme extract in the present study, the preparation of which involved a prolonged centrifugation step for 60 minutes at 100,000 g and the dilution of the particle-free supernatant, (c) the inclusion of a drying down process at 50°C overnight in the present study which destroyed any oxaloacetate and (d) the use of a citrate-activation assay procedure which 'fully' activated the acetyl-CoA carboxylase so reducing the effect of any possible interfering enzymes.

The particular assay conditions used by Davies et al. (1982), involving assay of a crude liver extract after pre-incubation at 37°C in the absence of citrate, have become widely used in recent years (Allred & Roehrig, 1978; Geelen et al., 1978; Witters et al., 1979) and have been believed to restore or preserve the in vivo activity of acetyl-CoA carboxylase, by a mechanism which is not fully

understood. While the results of Davies et al. (1982) have cast
some doubt on the specificity of the assay, under those particular
conditions, for acetyl-CoA carboxylase, it is interesting to note
that Allred & Goodson (1982) showed that assays performed on crude
rat liver extracts, pre-incubated at 37°C for 40 minutes in the
absence of citrate, incorporated radioactivity into malonyl-CoA
and malonic acid only.

Acetyl-CoA carboxylase from chick liver gave optimum activity when
assayed at pH7.05 at 37°C, with final concentrations of 100 mM-Tris/
HCl, 8 mM-magnesium chloride, 11 mM-potassium citrate, 0.25 mM-
acetyl-CoA, 1 mM-ATP, 3 mM-dithiothreitol, 0.6 mg/ml BSA and 25 mM-
sodium $[^{14}C]$ bicarbonate. It was found that oestrogen treatment of
chicks in vivo did not affect the optimum assay conditions of the
enzyme in vitro and all subsequent assays of the enzyme could be
performed under the same conditions, irrespective of the source of
the liver extract used. When arriving at an optimum bicarbonate
concentration of 25 mM it was necessary to use a value high enough
so that even allowing for unavoidable losses of $^{14}CO_2$ and $^{12}CO_2$
from the assay system, the bicarbonate concentration would still
allow the near-optimal assay of acetyl-CoA carboxylase. On the
other hand, the high cost of sodium $[^{14}C]$ bicarbonate precluded the
use of high concentrations of bicarbonate in the assay. The assay
procedure involved the addition of 100 µl of 'start cocktail',
containing sodium $[^{14}C]$ bicarbonate and ATP, to a pre-incubated
'assay cocktail' that contained the enzyme. Initial experiments
showed that freshly-prepared 'start cocktail', stored on ice for 5
minutes and then incubated at 37°C for 20 minutes, had lost 16% of
its radioactivity at the end of the 25 minutes period (Fig 4.1a).
Another source of loss of radioactivity, in the form of $^{14}CO_2$, was
from the assay itself during the 1.5 minute incubation period at
37°C after the addition of 'start cocktail'. Initial experiments
indicated that the radioactivity of the assay system diminished

by 5% during the 1.5 minute incubation.  In summary, therefore, the maximum loss of radioactivity for 'early' assays may be approximately 5% whereas the maximum loss for 'late' assays may be approximately 16% + 5% (21%).  Therefore, using a bicarbonate concentration of 25 mM, the minimum bicarbonate concentration of 'early' assays, allowing for $CO_2$ loss, would be approximately 23.75 mM whereas the minimum bicarbonate concentration of 'late' assays would be approximately 19.75 mM.  The effect of such reduction in bicarbonate concentration on the observed activity of acetyl-CoA carboxylase in liver extracts can be gauged by reference to Figure 4.11a, which is a plot of enzyme specific activity against bicarbonate concentration. A reduction of  bicarbonate concentration in the early assays, from 25 mM to 23.75 mM would not substantially affect the measured enzyme activity.  The possible reduction in bicarbonate concentration in the late assays, from 25 mM to 19.75 mM, however, could reduce the measured enzyme activities by up to approximately 8%. Consideration of these figures would suggest that, in the light of the predicted loss of $^{14}CO_2$ and $^{12}CO_2$, it would have been better to use a higher concentration of bicarbonate (30 mM, for example) to abolish the apparent reduction in enzyme activity in the 'late' assays.  However, the bicarbonate concentration of 25 mM was adopted as being the most suitable compromise between the need for a high bicarbonate concentration and the high cost of sodium $\left[ ^{14}C \right]$ bicarbonate.

The results of the experiment designed to monitor the loss of radioactivity in the form of $CO_2$ from the assay system cast some doubt upon the reliability of the acetyl-CoA carboxylase assay procedure adopted by some previous workers.  For instance, Gregolin et al. (1968b) used an assay procedure which involved an incubation period of 10 minutes, as opposed to the 1.5 minute period adopted in the present study.  Figure 4.1b, in the present study, indicates that an assay incubation period of 10 minutes at 37°C would lead to a 26% reduction in radioactivity and bicarbonate

concentration. Assuming that similar losses were evident in the 10 minute assay incubation of Gregolin et al. (1968b), the possibility exists that, without evidence to the contrary, substantial reductions in the measured enzyme activities could have been incurred.

All assay incubations, in the present study were performed for 1.5 minutes since assays on untreated, control and oestrogen-treated birds were all linear up to this point. When deciding how much soluble protein to use in a particular assay it was found that assays performed on liver extracts from oestrogen-treated birds showed a linear relationship between enzyme activity and the amount of soluble protein only up to 0.12 mg protein. This value was lower than the corresponding values for control and untreated birds and was indicative of a possible greater specific activity of the enzyme at certain times after oestrogen treatment.

The assay conditions adopted in the present study differ somewhat from those used by other workers to assay acetyl-CoA carboxylase from avian liver. Gregolin et al. (1968b) incubated the chicken liver enzyme for 10 minutes at 37°C in the presence of 60 mM-Tris/ HCl (pH7.5 at 25°C), 10 mM-potassium $\left[^{14}C\right]$ bicarbonate, 20mM-potassium DL-isocitrate, 3 mM-glutathione and 0.6 mg/ml BSA. Goodridge (1972) used essentially the same conditions as Gregolin et al. (1968b), dithiothreitol being used instead of glutathione. It has previously been reported that, unlike acetyl-CoA carboxylase from other sources, the chicken liver enzyme does not require preliminary incubation with citrate before assay (Gregolin et al., 1968b). However, in the present study, it was shown that pre-incubation of a liver extract with citrate at 37°C caused an increase in the acetyl-CoA carboxylase activity determined and, therefore, in subsequent assays a 15-minute pre-incubation

procedure was adopted. This citrate pre-incubation procedure has been shown to cause the conversion of the enzyme from the disaggregated protomeric form to the more catalytically active polymeric form (Gregolin et al., 1966a and 1966b; Numa et al., 1966). The assay conditions adopted in the present study also involved the pre-incubation of the enzyme with $Mg^{2+}$ in the absence of ATP. These conditions have been shown by a number of workers to lead to the activation of acetyl-CoA carboxylase in rat mammary gland extracts (McNeillie et al., 1981; McNeillie & Zammit, 1982) and rat adipose tissue extracts (Brownsey et al., 1979) by the activation of an endogenous phosphatase which leads to dephos-phorylation of the enzyme. Therefore, the in vitro assay used in the present study was designed to give an estimate of the total activity potential in the tissue since, in every assay, conditions were used to activate the enzyme as fully as possible. It is important to be aware of this fact when attempting to draw conclusions from the activity changes of the enzyme measured in vitro, since the optimum assay conditions may not exist in vivo.

Goodridge (1975) assayed acetyl-CoA carboxylase activity under optimum conditions and considered alterations in the activity measured to be indicative of alterations in the amount of enzyme protein present. This assumption can only hold if the optimum conditions fully activate all the enzyme protein, and the relationship should be established unequivocally with immunological techniques as performed by Silpananta & Goodridge (1971).

2. The effect of varying doses of 17β-oestradiol on the activity of hepatic acetyl-CoA carboxylase

When assaying the effect of an exogenous agent on enzyme activity it is necessary to establish a suitable time of exposure to the agent and a suitable dose of the agent.

Previous investigations into the effect of exogenous oestrogen on the activities of certain hepatic lipogenic enzymes in immature birds have demonstrated that substantial changes in enzyme activity occurred within 48 hours after oestrogen administration. For example, Pearce & Balnave (1975) reported marked changes in the activities of ATP citrate lyase and 'malic' enzyme in liver extracts from immature pullets within 48 hours after oestrogen treatment. Furthermore, these workers suggested that measurements during this period would be more indicative of physiological effects, and that pharmacological effects might predominate after longer periods of exposure to high levels of oestrogen. Similarly, Courtney & Manning (1984) observed increased activity of hepatic fatty acid synthetase in liver extracts from immature male chicks within 48 hours after oestrogen treatment. In the present study, there was an indication that there may be an increase in hepatic acetyl-CoA carboxylase activity at 48 hours after oestrogen treatment (Fig. 4.6).

In the light of these observations it was decided to carry out a dose-response experiment, involving the assay of acetyl-CoA carboxylase in liver extracts from male chicks at 48 hours after the administration of varying doses of oestrogen. Statistically significant increases in the mean specific activity of acetyl-CoA carboxylase in liver cell extracts prepared from oestrogen-treated birds were observed at each dose, within the range used, when compared with control and untreated birds. Maximum mean specific activity was obtained using a dose of 0.75 mg hormone/100 g body weight when the oestrogen-treated value was 1.99-fold greater than the value for control birds. Therefore, at this dose, a greater proportion of soluble cell protein exhibited acetyl-CoA carboxylase activity. This could be explained by a reduction in the amount of protein other than acetyl-CoA carboxylase. However, oestrogen has been reported to stimulate lipogenic enzyme activities

selectively (Pearce, 1971; Pearce & Balnave, 1976) and so a more
likely explanation is that the specific increase in acetyl-CoA
carboxylase activity was caused by changes in the catalytic
efficiency of the enzyme and/or changes in the amount of the enzyme.
Although maximum mean specific activity occurred at a dose of
0.75 mg hormone/100 g body weight, the value was not significantly
different from the slightly lower specific activities obtained with
doses of 0.50, 1.00 and 1.25 mg hormone/100 g body weight.

When the results were expressed as enzyme activity/g liver, all
doses of oestrogen caused an increase in activity with respect to
controls.  With the exception of the 0.25 mg hormone/100 g body
weight dose, these increases were significantly different from the
control value.  The maximum activity/g liver was obtained, once
again, with the 0.75 mg hormone/100 g body weight dose.  Although
the changes in activity/g liver and specific activity were
qualitatively similar over the range of doses, there was an obvious
quantitative difference which can be illustrated by a comparison
of the two activities at the 0.75 mg hormone/100 g body weight dose.
The mean activity/g liver at this dose was 1.71-fold greater than
the corresponding control value∗.For each dose level the
                                  ∧
percentage increase over controls was greater when the results were
expressed as specific activities.  This difference is a result of
the changes which were observed to occur in the amount of soluble
protein/g liver after oestrogen treatment (see Table 4.1).  There
was a dose-dependent decrease in the amount of soluble protein/
g liver such that, at the 0.75 mg hormone/100 g body weight dose,
the oestrogen-treated value was 10% lower than the control value,
the two values being significantly different.  These decreases,
which are thought to be caused by uptake of water by the liver
leading to cell hypertrophy (see Chapter 2), mean that the extent
of any increase in specific activity caused by oestrogen treatment

∗   .....whereas the mean specific activity was 1.99-fold greater
    than the corresponding control value.

would be somewhat masked when the same results were expressed in terms of activity/g liver.

When the results were expressed as the total hepatic activity of acetyl-CoA carboxylase it was found that the oestrogen-dependent increase in enzyme activity was increased further. For example, at a dose of 0.75 mg hormone/100 g body weight, the mean total hepatic activity was 3.5-fold greater than the control value (Fig. 4.13c) while the mean specific activity was 1.99-fold greater than the control value (Fig. 4.13a). Consequently, it is clear that the dose-dependent stimulation of total hepatic activity in liver weight (Table 4.1), is accompanied by a more moderate increase in total soluble protein, leading to a less dramatic increase in the specific activity of the enzyme with respect to protein. The increase in liver weight may be due partially to an uptake of water but it also represents increased cell growth with concomitant protein synthesis and increased cell division (see Chapter 2).

An interesting feature of the total hepatic activity changes was that the maximum value was obtained with a dose of 1.25 mg hormone/ 100 g body weight, although it was not significantly different from the value obtained with 0.75 mg hormone/100 g body weight (Fig. 4.13c). On the other hand, the mean specific activity of the enzyme decreased at high doses of oestrogen (Fig. 4.13a). This suggests that high doses of 17$\beta$- oestradiol, within the range 0.75 mg to 1.25 mg/100 g body weight, maintain the total hepatic activity of acetyl-CoA carboxylase at a relatively constant level, but this is accompanied by a dose-dependent increase in other types of soluble protein, causing a corresponding dose-dependent decrease in the mean specific activity of the enzyme.

Previous workers have found some variation in the optimum dose of

oestrogen required to elevate the specific activity of different lipogenic enzymes in chick liver. Courtney (1984) reported that maximum specific activity of fatty acid synthetase, assayed in liver extracts from immature male chicks at 48 hours after oestrogen treatment, was obtained using a dose of 0.75 mg 17$\beta$-oestradiol/ 100 g body weight. This is in excellent agreement with the optimum dose reported in the present study on acetyl-CoA carboxylase. In contrast, Pearce & Balnave (1975) reported lower optimum doses of oestrogen when they measured the effect of oestrogen on the specific activity of ATP citrate lyase and 'malic' enzyme in liver extracts from immature pullets. These workers used oestradiol dipropionate and measured enzyme activities 48 hours after treatment. After correcting their reported doses for body weight and quantity of oestradiol, the maximum specific activities were obtained with approximate doses of 0.57 mg oestradiol/100 g body weight for ATP citrate lyase and 0.28 mg oestradiol/100 g body weight for 'malic' enzyme. These differences suggest that different lipogenic enzymes may have different susceptibilities to oestrogen treatment. Another possible explanation of the lower optimum oestrogen doses reported by Pearce & Balnave (1975) may be that the immature pullets (4 weeks old) used by these workers could have possessed low levels of endogenous oestrogen and/or an enhanced ability to respond to oestrogen treatment through differences in hepatic oestrogen receptors.

3.  The effect of a single intramuscular injection of 17$\beta$-oestradiol (0.75 mg/100 g body wt.) on the activity of hepatic acetyl-CoA carboxylase

In an attempt to clarify the role of acetyl-CoA carboxylase in the initial stages of the oestrogen-dependent stimulation of lipogenesis in avian liver (see Chapter 3), it was decided to assay the activity

of this enzyme in liver extracts from immature male chicks at
various times after oestrogen treatment. It was shown that, during
the first 19 hours after injection, there was no significant
variation in the specific activity in oestrogen-treated birds, and
no significant difference between the specific activity in
oestrogen-treated and corresponding control birds. These
observations, however, do not preclude possible changes of specific
activity in vivo, over this initial 19-hour period, brought about
by modulation of the catalytic efficiency of the enzyme and this
possibility will be discussed later.

From 24 hours - 48 hours after oestrogen treatment there was a
significant increase in the specific activity of acetyl-CoA
carboxylase in liver extracts from oestrogen-treated birds, with
respect to control birds, such that, at 48 hours after injection,
the oestrogen-treated value was 1.53-fold greater than the
corresponding control value. This elevation could be brought about
by one or more of the following events: (a) a decrease in soluble
cell protein other than acetyl-CoA carboxylase, (b) an increase in
the amount of acetyl-CoA carboxylase protein, (c) an increase in
the catalytic efficiency of acetyl-CoA carboxylase protein. Over
the same period of time, 24 hours - 48 hours after injection, there
was a significant increase in the amount of protein/mg DNA
(Table 4.2) in oestrogen-treated birds with respect to controls.
Therefore, the possibility exists that the increase in specific
activity may have been brought about, at least in part, by an
increase in the amount of acetyl-CoA carboxylase through alterations
in the rates of synthesis and/or degradation of enzyme protein,
although such a statement cannot be asserted without direct
measurement of acetyl-CoA carboxylase protein.

After 61 hours post injection, the specific activities of oestrogen

treated and control birds were not significantly different, implying that a decreasing proportion of soluble protein exhibited acetyl-CoA carboxylase activity in oestrogen-treated birds after 61 hours, when compared with similar birds at 48 hours after injection. Therefore, a decrease in the synthesis and/or an increase in the degradation of acetyl-CoA carboxylase could play a part in the reduction in specific activity of oestrogen-treated birds after 48 hours. A reduction in the catalytic efficiency of acetyl-CoA carboxylase protein and an accumulation of proteins other than acetyl-CoA carboxylase are factors which could also be involved.

The results were also expressed as activity of acetyl-CoA carboxylase per mg DNA (Fig. 4.14b). This specific activity, with respect to DNA, can be used as an indication of the enzyme activity per cell and variations in this parameter, over the 61 hour period following oestrogen treatment were qualitatively similar to the corresponding variations in specific activity, with respect to soluble protein, discussed above. Maximum specific activity, with respect to DNA, was observed at 37 hours - 48 hours after oestrogen treatment (Fig. 4.14b). Furthermore, the extent of the increase in specific activity, with respect to DNA, from 24 hours - 48 hours after oestrogen treatment was greater than the corresponding increase in specific activity with respect to soluble protein. This implies that, over this time period, not only was there an increase in the amount of soluble protein exhibiting acetyl-CoA carboxylase activity but there was also a concomitant increase in the amount of soluble protein per cell. Analysis of the variation in the amount of soluble protein/mg DNA shows that a significant increase was observed 24 hours - 48 hours after oestrogen treatment compared to controls (Table 4.2).

From Table 4.2 it can be seen that the amount of soluble protein/g liver decreased upon oestrogen treatment such that from 13 hours - 37 hours after injection, the oestrogen-treated values were significantly lower than corresponding control values. This observation represents an oestrogen-dependent cellular hypertrophy in which water and lipid uptake by cells may play a part (see Chapter 2). Therefore, expression of acetyl-CoA carboxylase activity in terms of enzyme activity/g liver results in a slightly different variation in activity, over the 61-hour period following oestrogen injection, compared to that of specific activity; only at the 48 hour post-injection point was the enzyme activity/g liver for oestrogen-treated birds significantly greater than the corresponding value for control birds.

Following oestrogen treatment significant increases in liver weight (as % of body wt.) were observed at all times from 13 hours - 61 hours after injection. The greatest increase in liver size was apparent at 37 hours - 48 hours after injection, the oestrogen-treated values at these two time-points being 1.60-fold greater and 1.57-fold greater than the corresponding control values, respectively. Initially, the increase in liver weight is due to uptake of water leading to cellular hypertrophy but, later, there is also a significant increase in total soluble protein (at 19 hours) and total DNA (at 37 hours) which suggests that cellular hyperplasia contributes to organ growth in the later stages of this response. Expression of acetyl-CoA carboxylase activity in terms of total hepatic activity revealed maximum activities after 37 hours and 48 hours, when the oestrogen-treated values were 2.11-fold greater and 2.44-fold greater than the corresponding control values, respectively.

Comparison of the data presented in Table 4.3 suggests that the hormonal stimulation of the total hepatic activity of acetyl-CoA

Table 4.3

The stimulation of hepatic acetyl-CoA carboxylase, liver weight and hepatic DNA content in birds treated with 17β-oestradiol (0.75 mg/100 g body wt.) with respect to control birds.

Consult the legend of Table 4.2 for experimental details. Data presented in this Table were obtained by direct comparison of the mean values of each parameter for corresponding oestrogen-treated and control birds, at each of the indicated post-injection times.

| Parameter | Oestrogen-dependent stimulation relative to control. (%) | | | |
|---|---|---|---|---|
| | 24h | 37h | 48h | 61h |
| Total hepatic activity of ACC | 153 | 210 | 243 | 169 |
| Specific activity of ACC with respect to DNA | 139 | 159 | 174 | 145 |
| Liver weight (as % body wt.) | 148 | 160 | 157 | 154 |
| Total DNA content of liver | 106 | 138 | 136 | 119 |

carboxylase is due to an increase in the enzyme activity per cell, as represented by the increase in specific activity with respect to DNA, accompanied by an increase in the number of cells in the liver, as represented by the increase in the total DNA content of liver.

At the 61 hour post-injection point, the oestrogen-treated mean total hepatic activity of acetyl-CoA carboxylase was significantly greater than the corresponding control value. At the same time, specific activities, both with respect to DNA and protein, of oestrogen-treated and control birds were not significantly different.

In recent years several other workers have reported changes in the activity of lipogenic enzymes in avian liver after oestrogen treatment. Courtney (1984) found that oestrogen administration (0.75 mg 17β-oestradiol/100 g body wt.) to immature male chicks caused an increase in specific activity and total hepatic activity of fatty acid synthetase, when compared to control birds, between 26 hours and 48 hours after hormone treatment. However, the increases observed were 1.29-fold and 1.76-fold, respectively, which are smaller than the corresponding increases in acetyl-CoA carboxylase activity reported in the present study. Furthermore, Courtney (1984) detected an increase in fatty acid synthetase activity within the first 26 hours following oestrogen treatment and this increase was also found to occur in the corresponding control birds. A similar increase in acetyl-CoA carboxylase evoked by the handling/injection procedure was not observed in the present study.

Pageaux et al. (1981) reported a rapid increase in hepatic acetyl-CoA carboxylase activity after treating 16-day old female quail with a single injection of oestradiol benzoate (0.2 mg/kg body wt.). A

peak in the specific activity of acetyl-CoA carboxylase was observed
3 hours after injection, and the oestrogen-treated values at 3 hours
and 6 hours after injection were significantly higher than the
'control' value. No difference between the specific activity of
acetyl-CoA carboxylase in oestrogen treated birds and 'control'
birds was observed at 24 hours after injection. The results of the
present study, in which an increase in acetyl-CoA carboxylase
specific activity, with respect to controls, was only observed after
24 hours following oestrogen injection, are in sharp contrast to the
results of Pageaux et al. (1981). Such a difference might be
explained by the fact that these workers used female birds whereas
male birds were used in the present study. Pageaux et al. (1981)
also used birds of a different species, Japanese quail, and the
hormone dose level of 0.2 mg oestradiol benzoate/kg body weight
was very low compared to the dose level used in the present study
and with the dose levels used by other workers (Pearce & Balnave,
1975; Aprahamian et al., 1979). Furthermore, it seems that the
'control' birds used by Pageaux et al. (1981) were injected with
the olive oil vehicle just before death and, therefore, adequate
controls were not provided for each post-injection time-point. It
is possible, therefore, that the early peak in acetyl-CoA carboxylase
activity observed by these workers could have been a stress response.

Philipp & Shapiro (1981) administered 17$\beta$-oestradiol to male
xenopus laevis (0.4 mg/100 g body wt.) at 0 hour and 24 hours, and
measured acetyl-CoA carboxylase,fatty acid synthetase and HMG-CoA
reductase activities in liver extracts from animals killed up to 14
days post-injection. Oestrogen treatment resulted in increased
hepatic fatty acid synthesis, monitored by the incorporation of
$\left[1 - {}^{14}C\right]$ acetate into fatty acids in liver cubes, and an increase
in acetyl-CoA carboxylase activity per unit weight of liver.
These increases were correlated and reached maxima on the sixth
day after hormone administration. A later increase in the rate of

fatty acid synthesis (8 - 13 days) occurred without a concomitant increase in acetyl-CoA carboxylase activity. No change in fatty acid synthetase activity was observed during the study although HMG-CoA reductase, the major regulatory enzyme in cholesterol and isoprenoid synthesis, greatly increased in activity and reached a peak 5 days after hormone treatment. However, once again, firm conclusions cannot be drawn from the published data about the action of oestrogen on these enzyme activities since no control values were presented for comparison.

Aprahamian et al. (1979) examined hepatic fatty acid synthetase activity after treating 1-month old pullets with oestrogen. A prolonged oestrogen treatment regime was adopted involving injections of 4 mg β-oestradiol-3-benzoate/bird/day for 6 consecutive days. Hepatic fatty acid synthetase activity increased as a result, such that a 3-fold increase in total hepatic activity and a 2-fold increase in specific activity was observed. However, these changes in fatty acid synthetase activity occurred after prolonged exposure to high levels of oestrogen and may represent a pharmacological rather than a physiological response to the hormone. Furthermore, short-term changes in enzyme activity were not monitored, so this study does not help to resolve the initial sequence of events after hormone administration.

A number of investigators have examined the effect of oestrogen treatment on the lipogenic capacity of avian liver, in vivo, and in vitro, and on the level of lipids in avian liver and/or blood. Dashti et al. (1983) administered single injections of diethyl-stilboestrol (40 mg/kg body wt.) to 19-day old male turkeys. Oestrogen treatment resulted in a 55-fold increase in plasma triacylglycerol concentration and a 3-fold increase in plasma phospholipid concentration at 48 hours after injection. Increases

were also observed in the hepatic triglyceride content at the same time after injection. The elevation of plasma and liver lipids over control values was maximal at 48 hours post-injection, although elevation was apparent and significant after 24 hours. Tritiated water incorporation studies revealed that increased hepatic fatty acid synthesis was a primary and major causative factor in the development of the oestrogen-induced hyperglyceridaemia. However, this study did not report any early changes (< 24 hours) in lipogenesis and so throws little light on the sequence of events in the initial response to oestrogen administration.

Kudzma et al. (1975), after administering 8 daily doses of diethyl-stilboestrol (0.5 mg/bird/day) to 1-week old chicks (sex undetermined) observed an 86-fold increase in the incorporation of $[^{14}C]$ acetate into triacylglycerols associated with VLDL. This increase in lipogenesis, in vivo, was in agreement with earlier work by Kudzma et al. (1973), who reported increased lipogenesis from $[^{14}C]$ acetate by liver slices from chicks (sex undetermined) which had received 0.1 mg diethylstilboestrol/day for 18 days. However, since both of these studies involved prolonged exposure to high levels of hormone, the results may represent a pharmacological rather than a physiological response to oestrogen.

In an effort to examine the early events in oestrogen-stimulated lipogenesis in avian liver, Courtney (1984) measured the incorporation of $^3H_2O$ into lipids in liver slices taken from male chicks at various times after injection of 17β-oestradiol (1 mg/100 g body wt.). A significant increase in hepatic lipogenesis was reported 14 hours after oestrogen treatment, and the maximum stimulation was observed 40 - 43 hours after injection of hormone.

In the present study, it has been demonstrated that oestrogen

treatment caused a significant increase in lipid levels in the liver and plasma of male chicks within 7 hours (Chapter 3), but that significant increases in hepatic acetyl-CoA carboxylase activity did not occur until 24 hours after injection. This suggests that the initial phase (0 - 24 h) of the oestrogen-dependent stimulation of hepatic lipogenesis is not due to an increase in the activity of this enzyme in avian liver. In the later phase (> 24h) of the response, however, it is likely that the increase in hepatic acetyl-CoA carboxylase activity contributes to the enhanced rate of hepatic lipogenesis. It is important to realise that this interpretation is based on the assay of this enzyme under optimum conditions in vitro, and that these optimum conditions may not exist in vivo. In the cell, therefore, it is possible that changes in the activity of acetyl-CoA carboxylase may occur during the initial phase of the response as a result of allosteric modulation, covalent modification of the enzyme and/or changes in substrate availability. In consequence, acetyl-CoA carboxylase is not precluded from involvement in the changes in levels of de novo lipogenesis and hepatic and plasma lipids which occur within 24 hours after oestrogen treatment.

Recent work in which the activity of hepatic acetyl-CoA carboxylase in chicks has varied rapidly in response to sudden nutritional alterations has shown that the enzyme undergoes polymer/protomer inter-conversion (Ashcraft et al., 1980) and changes in phosphorylation state (Clarke, 1983) during the short term, in vivo, which affects the catalytic efficiency. Clarke & Salati (1985) suggest that during periods of food deprivation, increases in the concentration of long-chain fatty acyl-CoA esters in the liver favour depolymerisation of the enzyme. This is accompanied by elevations in plasma glucagon levels, inducing phosphorylation of the enzyme, which facilitates acyl-CoA binding and promotes depolymerisation. In this state, cellular c.AMP levels will be raised and this has

been shown by Watkins et al. (1977) to reduce the level of citrate in cultured chicken hepatocytes, thus reducing the level of a positive allosteric effector of the enzyme.

It is feasible that oestrogen treatment may either directly or indirectly, cause similar short-term variations in the regulatory metabolites affecting acetyl-CoA carboxylase. In this light, it is interesting to note that there are reports of steroid hormones affecting the activity of adenylate cyclase, and so altering c.AMP levels in human endometrium cells (Bergamini, 1985), in Xenopus oocytes (Finidori-Lepicard et al., 1981), in the uterine cervix of neonatal and immature mice (Kvinnsland, 1980) and in rat uterus (Szego & Davis, 1969; Francavilla et al., 1979).

The present study clearly demonstrates an oestrogen-induced increase in hepatic acetyl-CoA carboxylase activity from 24 - 48 hours after hormone treatment and it is likely that this increased enzyme activity contributes to the enhanced liver and plasma lipid levels in similarly treated birds. However, while the present study provides no evidence for the involvement of enhanced acetyl-CoA carboxylase activity in the changes in lipid levels occurring within 24 hours of hormone treatment, further work needs to be carried out to study the changes in activity of the enzyme, in vivo, during this initial phase of the response to oestrogen.

# CHAPTER 5

GENERAL DISCUSSION

1. The use of oestrogen-treated birds as a model for studying vitellogenesis in the domestic fowl

As the sexually-mature female domestic fowl approaches lay significant increases in the plasma vitellogenin and lipids, associated particularly with VLDL, are observed which are thought to be due principally to an oestrogen-dependent stimulation of lipid and protein biosynthesis in the liver. A similar situation can be evoked in male or sexually-immature female birds by treatment with exogenous oestrogen in vivo. Such hormone-treated birds provide a convenient model system for the investigation of the molecular events involved in these changes. Male birds have proved particularly useful since they lack endogenous oestrogen and they have been widely used in the study of the oestrogen-induced stimulation of hepatic VLDL biosynthesis (Kudzma et al., 1973, 1975, 1979; Luskey et al., 1974; Chan et al., 1976, 1977a, 1978, 1980) and hepatic vitellogenin biosynthesis (Bergink et al., 1973, 1974; Beuving & Gruber, 1971).

The administration of oestrogen to male and immature female birds, however, cannot be expected to completely reproduce the state of lipid metabolism existing in the laying hen since, undoubtedly, the physiological state of the latter is under the influence of a number of internal secretions as well as oestrogen. A number of differences in lipid metabolism between oestrogen-treated and laying birds have been reported, although such differences may be attributed to the high pharmacological doses of exogenous oestrogen applied. For example, Balnave (1971) observed heavier livers and higher liver and plasma levels of fatty acids for immature pullets treated with oestrogen than for mature laying hens. Significant increases in the percentage of 16:0 in plasma and liver fatty acids have been observed in oestrogen-treated pullets but not for laying

hens, suggesting that oestrogen treatment alone may result in an abnormal metabolism of 16:0 (Balnave, 1971; Paddon et al., 1982). The retention of dietary linoleic acid is increased at the onset of sexual maturity in the hen (Balnave, 1971) whereas the administration of exogenous oestrogen to immature pullets has no effect on dietary linoleic acid retention (Jackson et al., 1971).

Some of these differences between observations in oestrogen-treated immature birds and laying hens are probably a result of the egg-laying process. In the mature hen, lipid is continually removed from the body in the egg-laying process. In oestrogen-treated male and immature female birds, the accumulation of lipid and egg-yolk proteins in the liver and plasma might be expected to exert feed-back inhibition on their hepatic syntheses. Therefore, the events that occur at short intervals after oestrogen treatment of birds are probably more indicative of the situation in the laying hen.

However, the differences between oestrogen-treated birds and laying hens mentioned above are considered to be relatively minor, and the administration of oestrogen to immature male and female birds and cockerels remains a useful model for the study of the molecular events involved in avian vitellogenesis. The investigations carried out in the course of the present study were designed to establish changes in various parameters of lipid metabolism in the male chick during the 48-hour period immediately after oestrogen treatment.

2.    Summary of results obtained in the present study

The main features of the response of male chicks to single intra-muscular injections of oestrogen (0.75 mg 17$\beta$-oestradiol/100 g body wt.), observed in the present study, are summarised below.

(a)  Quantitative changes in plasma.

Total triacylglycerol concentration in the plasma of the oestrogen-treated male chick became significantly greater than the control value at 7 hours post-injection ($p < 0.001$), and increased progressively up to 44 hours after injection.  The highest elevation of total plasma triacylglycerol represented a 27-fold increase in concentration compared with control levels.

VLDL-triacylglycerol concentration in the plasma of oestrogen-treated birds became significantly greater than the control value at 13 hours post-injection ($p < 0.01$) and increased progressively thereafter.  Maximum plasma VLDL-triacylglycerol concentration was observed at 37 hours after oestrogen treatment and represented a 30-fold increase compared to control levels.

The total phospholipid concentration in the plasma of oestrogen-treated male chicks became significantly greater than the control value at 7 hours post-injection ($p < 0.05$) and increased progressively up to 44 hours after injection when the value in oestrogen-treated birds was 4.8-fold greater than the corresponding control value.

VLDL-phospholipid levels in the plasma became significantly greater than the control value at 6 hours after oestrogen treatment ($p < 0.01$) and increased substantially and progressively thereafter such that, at 48 hours post-injection, the oestrogen-treated value was 97-fold greater than the corresponding control value.

Total free fatty acid (FFA) concentration in the plasma of control chicks, treated with propane-1,2-diol alone, was significantly greater than the value in oestrogen-treated birds at 2 hours after

injection (p< 0.01).  Subsequently, up to 44 hours post-injection, there was no significant difference between the total FFA concentration in the plasma of oestrogen-treated birds and control birds.

Following oestrogen treatment of male chicks, the concentration of albumin-bound FFA in the plasma was lower than the corresponding control value from 6 - 48 hours post-injection, although only at 24 hours post-injection was the difference significant (p <0.001), the control value being 2.00-fold greater than the value in oestrogen-treated birds.

(b)  Quantitative changes in liver

(i) Weight, DNA content and soluble protein content

Liver weight increased with time after oestrogen treatment and maximum liver weight was observed at 37 hours post-injection when the mean liver weight of oestrogen-treated birds was 1.61-fold greater than the value for corresponding control chicks injected with propane-1,2-diol alone.  Similarly, a dose-related increase in liver weight was observed 48 hours after a single injection of 17β-oestradiol over a dose range of 0 - 1.25 mg/100 g body weight.

An increase in the total hepatic DNA content accompanied the oestrogen-induced increase in liver weight during the 37- to 61-hour post-injection period, and reached a maximum value at 37 hours post-injection, representing a 1.38-fold stimulation over control birds.  However, following oestrogen treatment a significant decrease in the amount of DNA/g liver was observed reaching a minimum value at 24 - 37 hours post injection.

Coincident with the increase in liver weight after oestrogen treatment was an increase in the total hepatic soluble protein content, reaching a maximum at 37 hours post-injection. Expression of the hepatic soluble protein content in terms of amount of soluble protein/mg DNA also revealed an increase after oestrogen treatment reaching a maximum at 37 hours post-injection, indicating an increase in the amount of soluble protein per cell. However, the increase in liver weight was accompanied by a decrease in the amount of soluble protein/g liver reaching a minimum value at 37 hours post-injection.

(ii) Triacylglycerol, phospholipid and free fatty acid content

Total triacylglycerol content of the livers of oestrogen-treated male chicks became significantly greater than the control value at 7 hours post-injection ($p < 0.05$). Subsequently, there was a progressive increase in the total hepatic triacylglycerol content of oestrogen-treated birds such that, at 34 hours post-injection, the value was 23-fold greater than the control value.

The 'cellular' triacylglycerol content of the liver, expressed as mg triacylglycerol/mg DNA became significantly elevated in oestrogen-treated birds, compared to controls, at 11 hours post-injection ($p < 0.01$), and increased subsequently such that, at 34 hours post-injection, the value was 15-fold greater than the corresponding control value.

Total phospholipid content of the livers of oestrogen-treated male chicks became significantly greater than the control value at 7 hours post-injection ($p < 0.05$), and increased subsequently such that, at 34 hours post-injection, the total hepatic phospholipid content in oestrogen-treated birds was maximal, representing a 1.8-fold increase over control birds.

The 'cellular' phospholipid content of the liver, expressed as µmol phospholipid/mg DNA, became significantly elevated in oestrogen-treated birds, compared to controls, at 7 hours post-injection ($p < 0.01$) and increased subsequently such that, at 20 hours post-injection, the value in oestrogen-treated birds was maximal, representing a 1.3-fold increase over control birds.

Total FFA content of the livers of oestrogen-treated male chicks became significantly greater than the control value at 26 hours post-injection ($p < 0.01$) and increased gradually up to 44 hours after injection. The highest elevation in total hepatic FFA content represented a 1.6-fold increase compared with control levels.

The 'cellular' FFA content of the liver, expressed as umol FFA/mg DNA, was significantly elevated in oestrogen-treated birds, compared to controls, at 26 hours and 44 hours post-injection ($p < 0.05$), the value at the latter time being 1.3-fold greater than the corresponding control value.

(iii) Acetyl-CoA carboxylase activity

Treatment of chicks with 0.75 mg 17β-oestradiol/100 g body weight caused an increase in the hepatic specific activity (with respect to protein) of hepatic acetyl-CoA carboxylase from 24 - 48 hours after injection. Maximum specific activity, with respect to protein, was observed 48 hours after oestrogen treatment, representing a a 1.53-fold increase over controls.

Oestrogen treatment also caused an increase in the hepatic specific activity, with respect to DNA, of the enzyme from 24 - 48 hours post-injection and maximum activity was observed after 37 - 48 hours,

representing a 1.59-fold and a 1.74-fold increase over controls, at the two individual times, respectively.

Expression of acetyl-CoA carboxylase activity in terms of total hepatic activity revealed a similar increase in enzyme activity from 24 - 61 hours after injection. Maximum total hepatic enzyme activity was observed after 37 - 48 hours, representing a 2.10-fold and a 2.43-fold increase over controls, at the two individual times.

During the first 19 hours after injection, no significant differences were observed between the hepatic acetyl-CoA carboxylase activity of oestrogen-treated and of control birds, irrespective of the manner of expression of enzyme activity.

(c) Qualitative changes in liver and plasma lipids

(i) Liver triacylglycerols

In untreated birds, the predominant fatty acids in liver triacylglycerols were 18:1 and 16:0, while 18:0, 18:2 and 16:1 contributed to a lesser extent. The major effect of oestrogen treatment on the fatty acid composition of liver triacylglycerol was to promote a substantial elevation in the molar ratio of unsaturated:saturated fatty acids from 13 - 48 hours after injection. This was caused largely by an elevation of the levels of 18:1 and 18:2 accompanied by a decline in the levels of 16:0 and 18:0.

(ii) VLDL-triacylglycerols

In untreated birds, the predominant fatty acids were 16:0, 18:1,

18:2 and 18:0 in decreasing order of percentage contribution. The major effect of oestrogen treatment on the fatty acid composition of VLDL-triacylglycerol was to cause an elevation in the level of 18:1 from 13 - 48 hours after injection accompanied by a decrease in the levels of 18:2.

(iii) VLDL-phospholipids

In untreated birds, the predominant fatty acids, in decreasing order of percentage contribution, were 16:0, 20:4, 18.0, 18:2 and 18:1. The major effects of oestrogen treatment on the fatty acid composition of VLDL-phospholipid was to cause an elevation in the level of 16:0 from 13 - 37 hours after injection, accompanied by an elevation in the level of 18:1 from 13 - 48 hours after injection.

3.  Sequence and correlation of oestrogen-induced changes in the lipid metabolism of avian liver

A number of workers have studied the effects of oestrogen treatment on the lipid metabolism of the domestic fowl. However, this work has been somewhat fragmentary and no clear understanding of the sequence of events in the initial stages of the response to exogenous oestrogen has been forthcoming. An understanding of the sequence of the changes in lipid metabolism that occur after oestrogen treatment of the male chick is important if the significance of these events is to be more fully appreciated.

The present study, therefore, was designed to produce detailed time-courses of the oestrogen-induced changes in various parameters of lipid metabolism. Since all the results were obtained from male birds, of similar age, which had been reared in identical

conditions and treated with the same dose of oestrogen, the present
study affords an opportunity for a greater understanding of the
sequence of events.  Furthermore, the results of the present study
can be correlated with the results of Courtney (1984) and Courtney
& Manning (1984) who treated 3- to 5-week old male chicks with
doses of oestrogen similar to those employed in the present study,
and produced time-courses of other parameters of lipid metabolism.

For the purposes of clarity and ease of comparison, Figure 5.1 and
Tables 5.1 and 5.2 present the time-courses of changes in various
parameters, in oestrogen-treated birds, observed in the present
study and in the studies of Courtney (1984) and Courtney & Manning
(1984).

In Chapter 1 of the present study, evidence was presented which
suggested that both cellular hypertrophy and cellular hyperplasia
may be involved in oestrogen-induced liver growth.  The fact that
there was no significant difference in the total hepatic DNA
content of oestrogen-treated and control birds during the first
24 hours after treatment, at a time when the liver weight of
oestrogen-treated birds had increased substantially, suggests that
the initial increase in liver weight was due to cellular hypertrophy.
Subsequently, an increase in the total hepatic DNA content
accompanied the oestrogen-induced increase in liver weight from
37 - 61 hours after injection, reaching a maximum value at 37 hours
when the total hepatic DNA content was 1.38-fold greater than that
in control chicks.  This suggests that the increase in liver weight
in the later stages of the response ( $\geqslant 37h$) was accompanied by
cellular hyperplasia.  Examination of the time-courses of the
changes in liver weight (Fig. 5.1a) and total hepatic triacyl-
glycerol content (Fig. 5.1d) suggest that the liver growth response
is, in part, due to an increase in the lipid content of liver.

Figure 5.1 (Overleaf)

Compilation of various time-courses of physiological changes in male chicks treated with oestrogen.

Each time course has previously been presented in the present study or in the studies of Courtney (1984) and/or Courtney & Manning (1984). In all cases, 3- to 5-week old male chicks were treated with 0.75 mg 17B-oestradiol/100 g body wt.

    ▲ = values for untreated birds.

    ○ = values for oestrogen-treated birds.

    ● = values for control birds.

Values are means ($\pm$ S.E.M.) of 2 - 19 birds.


(a) Effect of oestrogen on liver weight (as % body wt.)

Consult the legend of Fig. 2.1, in the present study, for details of experimental procedure.


(b) Effect of oestrogen on the total hepatic activity of acetyl-CoA carboxylase.

Consult the legend of Fig. 4.14c, in the present study, for details of experimental procedure.


(c) Effect of oestrogen on the total hepatic activity of fatty acid synthetase (F.A.S.)

Consult the legend of Fig. 1, in the study of Courtney & Manning (1984) for details of experimental procedure.


(d) Effect of oestrogen on total hepatic triacylglycerol content.

Consult the legend of Fig. 3.4a, in the present study, for details of experimental procedure.


(e) Effect of oestrogen on total plasma triacylglycerol level.

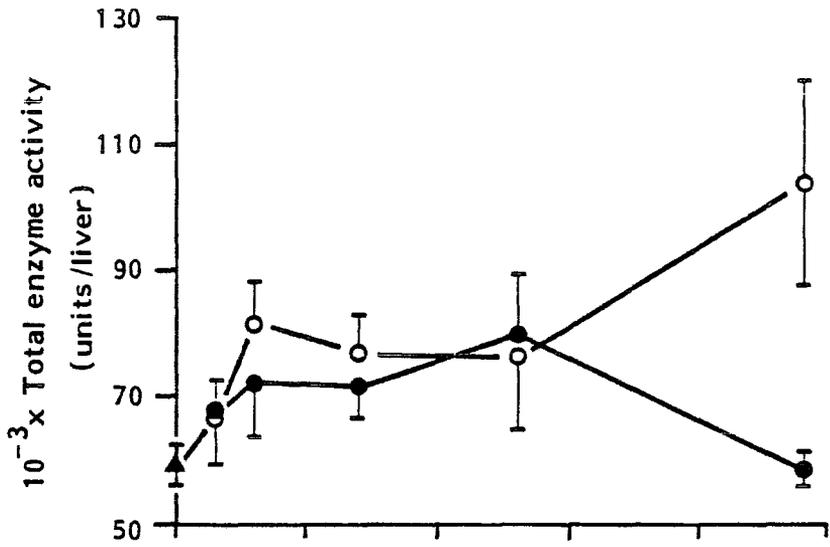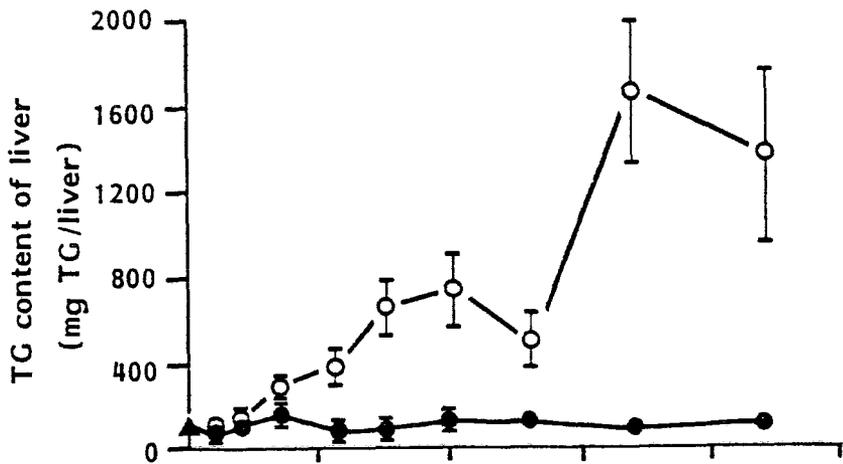Consult the legend of Fig. 3.2a, in the present study, for details of experimental procedure.

a.

Liver weight (as % body wt.)

Time after injection (h)

b.

$\mu$mol $[^{14}C]$ bicarbonate incorporated/min/liver

Time after injection (h)
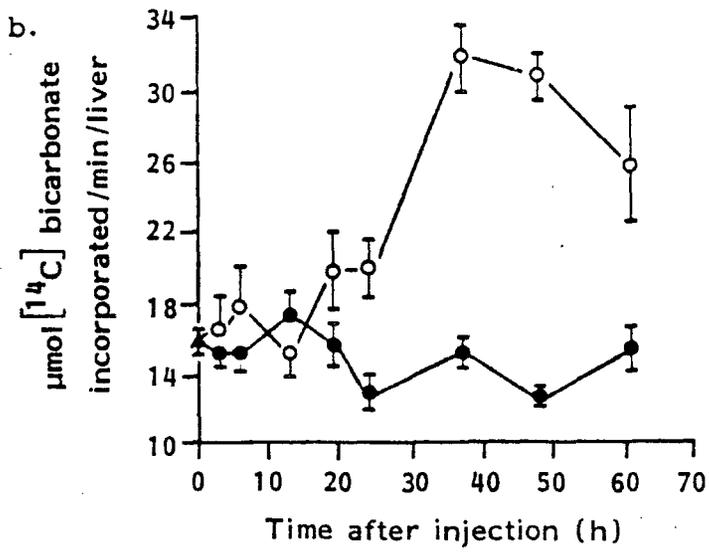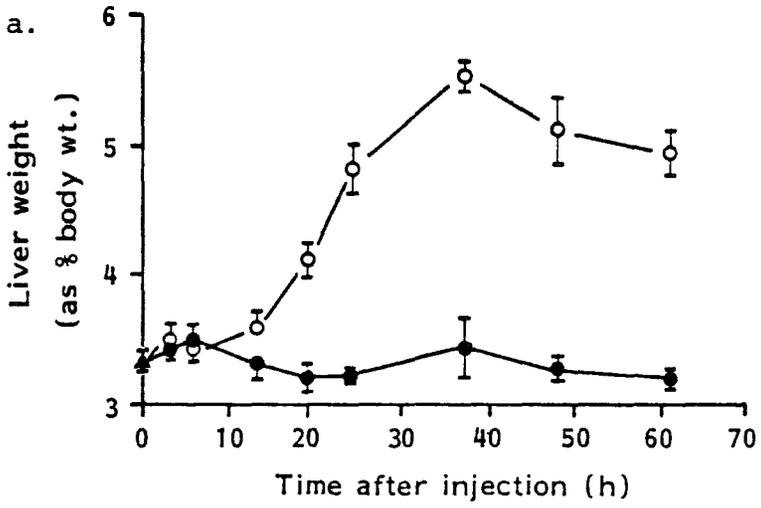
c.

d.

e.
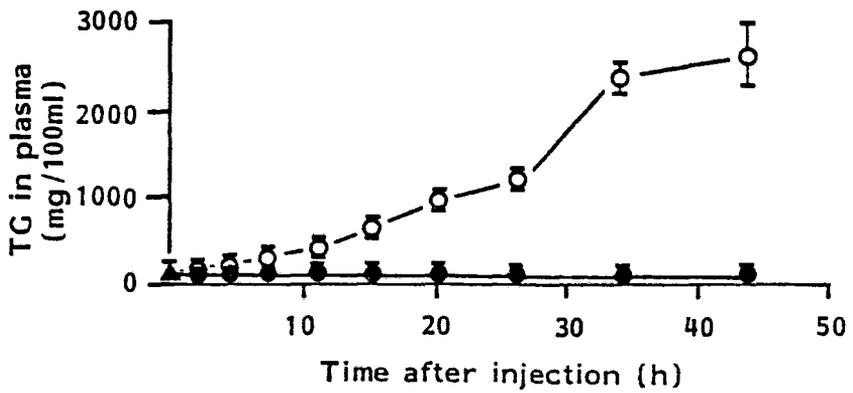
Increases in total hepatic phospholipid content accompanied the increase in total hepatic triacylglycerol (see Chapter 3). It therefore seems likely that the cellular hypertrophy involved increases in the cellular lipid content following oestrogen treatment. This suggestion is further supported by the time-courses of the oestrogen-induced elevation of hepatic triacylglycerol/mg DNA (Fig. 3.4b) and hepatic phospholipid/mg DNA (Fig. 3.5b) which revealed increases after 11 hours and 7 hours post-injection, respectively.

An oestrogen-induced hyperlipaemia was evident from 7 hours after oestrogen treatment of male chicks and involved an increase in the total concentration of triacylglycerol in the plasma (Fig. 5.1e) together with an increase in plasma phospholipid levels (Fig. 3.2b). A number of workers had previously reported that much of the increase in plasma lipids in oestrogen-treated domestic fowl are associated with VLDL (Chan et al., 1977, 1978. 1980; Kudzma et al., 1979). In the present study, oestrogen-induced increases in VLDL-triacylglycerol and VLDL-phospholipid were observed in the plasma of male chicks from 13 hours and 6 hours post-injection, respectively, and both lipid levels increased progressively with time (Fig. 3.3). A closer analysis of these two time-courses, however, revealed that at all times after oestrogen treatment, the extent of the increase was greater for VLDL-phospholipid than it was for VLDL-triacyl-glycerol. Calculations of the ratio of VLDL-triacylglycerol:VLDL-phospholipid showed that, at zero-time, this ratio was 425 whereas at 37 hours post-injection, the oestrogen-treated value was 186 compared to the control value of 397. Therefore, this comparison suggests that the hormone promotes (a) an increase in the number of VLDL particles in the plasma accounting for large increases in VLDL-lipids and (b) an early and consistent alteration in the composition of VLDL representing a specific enrichment of the phospholipid

component with respect to triacylglycerol. Furthermore, the observed decrease in the proportion of total plasma free fatty acids which are albumin-bound, from 6 - 48 hours post injection, is presumably a reflection of an increased proportion of the total plasma free fatty acid pool binding to the increasing number of triacylglycerol-rich lipoproptein particles.

The results of the present study also clearly show that oestrogen treatment of male chicks, as well as causing a time-related increase in liver and plasma lipids, also caused significant changes in the fatty acid composition of these lipids (Chapter 3). In liver triacylglycerol (Fig.3.8), VLDL-triacylglycerol (Fig. 3.9) and VLDL-phospholipid (Fig. 3.10) the predominant change was that of an elevation in the percentage contribution of 18:1 from 13 - 48 hours after oestrogen treatment. Lipiello et al. (1979) reported increased stearyl-CoA desaturase activity in crude microsomal extracts from the liver of male chickens treated with oestrogen (3.5 mg 17β-oestradiol/100 g body wt.). A 7-fold increase in the specific activity of the enzyme was observed in the 24-hour period following injection and the activity remained high through 48 hours before declining. Therefore, the increases in 18:1 in the plasma and liver lipids of oestrogen-treated birds in the present study may be due to an increase in hepatic stearyl-CoA desaturase activity within 13 hours of injection.

As mentioned previously, an accumulation of lipid in the liver and plasma of male chicks occurs in response to oestrogen treatment (Fig. 5.1d & e). The accumulation of these glycerolipids might be caused by an increased rate of hepatic de novo fatty acid synthesis and/or an increase in the rate of hepatic complex lipid formation from pre-formed fatty acids arriving from extra-hepatic sources. Courtney (1984) attempted to evaluate the importance of de novo

lipogenesis and lipogenesis from pre-formed fatty acids in the liver
of 3- to 5-week old male chicks which had been injected with a
single dose of 17β-oestradiol (1.0 mg/100 g body wt.). Hepatic
de novo fatty acid synthesis was monitored by measurement of the
incorporation of $^3H_2O$ into lipids in liver slices taken from male
chicks at various times after hormone injection, while complex lipid
formation from pre-formed fatty acid was monitored by measurement of
the incorporation of $[9,10 - ^3H]$ palmitate into triacylglycerol in
liver slices from similar birds. The results of these studies are
presented in Table 5.1 and Table 5.2 respectively. This study showed
total hepatic capacity for de novo fatty acid synthesis and for the
synthesis of triacylglycerol from pre-formed fatty acid were
stimulated in a roughly parallel manner during the first 44 hours
after oestrogen treatment. For example, at 14 - 17 hours after
hormone treatment, the total hepatic capacity for de novo fatty acid
synthesis was stimulated by 1.75-fold and this increased to 2.40-
fold stimulation at 40¼ - 43¾ hours post-injection (Table 5.1).
Similarly, at 14½ - 18½ hours after hormone treatment, the total
hepatic capacity for triacylglycerol synthesis from exogenous $[^3H]$
palmitate was stimulated by 1.61-fold, and this increased to 2.47-
fold stimulation at 40½ - 44 hours after injection (Table 5.2). No
significant stimulation in either process was evident at approximately
3½ - 7½ hours after oestrogen treatment.

These results suggest that oestrogen treatment has a general
stimulatory effect on lipogenesis in chick liver, causing a sustained
and roughly parallel increase in both de novo fatty acid synthesis
and complex lipid synthesis from pre-formed fatty acid, and that
this stimulation first becomes apparent within the 8 - 14 hour post-
injection period.

It should be noted that this study of Courtney (1984) employed a

Table 5.1

The incorporation of tritium from $^3H_2O$ into total lipid by liver slices from control and oestrogen-treated male chicks at varying times after injection.

| Incorporation of tritium from $^3H_2O$ into total lipid. | Untreated | Time after injection (h) | | | | |
| --- | --- | --- | --- | --- | --- | --- |
| | | $3\frac{1}{2} - 7\frac{1}{2}$ | 14 - 17 | $19 - 21\frac{1}{2}$ | $25\frac{1}{2} - 28\frac{1}{2}$ | $40\frac{1}{4} - 43\frac{1}{4}$ |
| µg atoms H/liver/min.  OE | 16.5 ± 2.2 (8) | 28.3 ± 1.5 (6) | 46.5 ± 5.1 (6) # | 40.0 ± 3.9 (6) # | 48.4 ± 3.7 (7) # | 53.5 ± 9.0 (6) # |
| C | | 26.8 ± 4.2 (6) | 26.6 ± 2.9 | 20.3 ± 2.6 (6) | 25.5 ± 5.7 (7) | 22.3 ± 2.1 (6) |
| Fold stimulation | | 1.06 | 1.75 | 1.97 | 1.90 | 2.40 |

These data are taken, with permission, from the study of Courtney (1984).  Male chicks aged 3 to 5 weeks were treated with a single injection of 1.0 mg 17β-oestradiol/100 g body weight.

OE = values for oestrogen-treated chicks; C= values for control chicks.

Consult the legend of Table 10 in the study of Courtney (1984) for details of experimental procedure.

Values are means (± S.E.M.) of 6 - 8 birds.  The number of birds in each group is given in parentheses.

Data were analysed statistically by Student's 't' test, and levels of statistical significance are presented with respect to corresponding control birds.

# significant at $p < 0.01$

Table 5.2

The incorporation of 0.65 mM-$[9, 10 - {}^3H]$ palmitate into triacylglycerol by liver slices from control and oestrogen-treated male chicks at varying times after injection.

| Incorporation of $[9, 10 - {}^3H]$ palmitate | | Untreated | Time after injection (h) | | | | |
|---|---|---|---|---|---|---|---|
| | | | $3\frac{1}{4}$ – 8 | $14\frac{1}{2}$ – $18\frac{1}{2}$ | 19 – $22\frac{1}{4}$ | 28 – $31\frac{3}{4}$ | $40\frac{1}{2}$ – 44 |
| nmol palmitate/ liver/min. | OE | 190 ± 19 (6) | 198 ± 16 (6) | # 230 ± 20 (6) | ¥ 278 ± 16 (6) | ¥ 382 ± 40 (6) | ¥ 390 ± 46 (6) |
| | C | | 202 ± 12 (6) | 143 ± 15 (6) | 171 ± 15 (6) | 169 ± 20 (5) | 158 ± 13 (6) |
| Fold stimulation | | | 0.98 | 1.61 | 1.63 | 2.26 | 2.47 |

These data are taken, with permission, from the study of Courtney (1984). Male chicks aged 3 to 5 weeks were treated with a single injection of 1.0 mg 17β-oestradio/100 g body weight.

OE = values for oestrogen-treated chicks; C = values for control chicks.

Consult the legend of Table 11 in the study of Courtney (1984) for details of experimental procedure.

Values are means (± S.E.M.) of 5 or 6 birds, the number of birds in each group is given in parentheses.

Data were analysed statistically by Student's 't' test, and levels of statistical significance are presented with respect to corresponding control birds.

# significant at $p < 0.01$

¥ significant at $p < 0.001$

hormone dose of 1.0 mg 17β-oestradiol/100 body weight whereas in the
present study a dose of 0.75 mg 17β-oestradiol/100 g body weight was
used. Therefore, the temporal changes in liver and plasma lipid
(Fig. 5.1d & e), in the present study, and the temporal changes in
hepatic lipogenic capacity (Tables 5.1 & 5.2) are not directly
comparable. Nevertheless, a tentative comparison of these parameters
suggests that, from 14 hours post-injection onwards, the oestrogen-
induced increases in hepatic de novo fatty acid synthesis and hepatic
lipogenesis from pre-formed fatty acids may account for the    served
increase in liver and plasma lipids. However, significant increases
in plasma and liver lipids were apparent as early as 7 hours post-
injection whereas significant increases in hepatic lipogenic
capacity were not evident until 14 hours post-injection. It should
be noted that the rate of de novo lipogenesis, as monitored by the
incorporation of $^3H_2O$ into lipids, might be slightly increased
during the 3½- to 7¼-hour period following oestrogen treatment
(Table 5.1) and, hence, could contribute to the early increases in
plasma triacylglycerol. Unfortunately, in this study of Courtney
(1984), no measurements were taken of hepatic de novo lipogenesis
or lipogenesis from pre-formed fatty acids during the 7- to 14-hour
period after hormone treatment.

In the present study, the hepatic activity of acetyl-CoA carboxylase
in oestrogen-treated birds was determined in vitro (Chapter 4) and
the temporal change in total hepatic enzyme activity is presented in
Fig. 5.1b. This enzyme is the first committed step in de novo fatty
acid synthesis and is considered to be the rate-limiting step in
animal cells. Oestrogen treatment led to a significant increase in
total hepatic acetyl-CoA carboxylase activity from 24 - 61 hours
post-injection with maximum activity being observed after 37 - 48
hours (Fig. 5.1b). This oestrogen-induced increase in total organ
activity was, in part, due to significant increases in the hepatic

specific activity of the enzyme, both with respect to DNA and to
protein, subsequent to 24 hours post-injection.  Since a significant
oestrogen-induced increase in hepatic acetyl-CoA carboxylase did
not occur until 24 hours post-injection, it seems likely that the
initial phase (0 - 24 hours) of the oestrogen-dependent stimulation
of hepatic lipogenesis and accumulation of liver and plasma lipid
is not due to an increase in the activity of this key lipogenic
enzyme in the liver.  In the later phase of the response (> 24h),
however, it is likely that the increase in hepatic acetyl-CoA
carboxylase activity contributes to the enhanced rate of hepatic
lipogenesis and the resultant accumulation of lipid in the liver
and plasma.

Similarly, Courtney (1984) and Courtney & Manning (1984) determined
the activity of hepatic fatty acid synthetase, in vitro, in 3- to
5-week old male chicks which had previously been treated with a
single injection of 0.75 mg 17β-oestradiol/100 g body weight.  The
temporal change in total hepatic fatty acid synthetase activity
following oestrogen treatment is presented in Fig. 5.1c.  No
significant increase in total enzyme activity was observed in
oestrogen-treated birds, compared to control birds, during the
first 26 hours after injection.  However, an  early increase in
total organ enzyme activity was observed in both oestrogen-treated
and control birds, compared to untreated birds, and it was assumed
that this increase was a result of the handling/injection procedure.
Therefore, the temporal change in hepatic fatty acid synthetase
activity after oestrogen treatment (Fig. 5.1c) is consistent with
the results for acetyl-CoA carboxylase in similar birds (Fig. 5.1b),
in that no increase in the activity of either enzyme was observed
in the first 24 hours after injection.  Subsequently (26 - 48 h),
a significant oestrogen-induced increase was observed in total
hepatic fatty acid synthetase activity, and this is consistent with

the results for hepatic acetyl-CoA carboxylase.

These results suggest that, during the first 24 hours after oestrogen treatment, there is an accumulation of lipid in the liver and plasma and an increase in hepatic de novo lipogenesis but there is apparently no concomitant increase in the activity of the two key lipogenic enzymes, acetyl-CoA carboxylase and fatty acid synthetase. It is important to realise, however, that this interpretation is based on the assay of these two enzymes under optimum conditions in vitro, and that these optimum conditions may not exist in vivo. In the cell, therefore, it is possible that changes in the activity of acetyl-CoA carboxylase may occur during the initial phase of the response to oestrogen as a result of allosteric modulation, covalent modification of the enzyme and/or changes in substrate availability. Similarly, fatty acid synthetase may be susceptible to in vivo activity changes, in the first 24 hours after oestrogen treatment, as a result of changes in the levels of regulatory metabolites and/or changes in substrate availability. In consequence, acetyl-CoA carboxylase and fatty acid synthetase are not precluded absolutely from involvement in the changes in levels of de novo lipogenesis and hepatic and plasma lipids which occur within 24 hours after oestrogen treatment. It should also be borne in mind that the possibility exists, until shown otherwise, that the initial phase of the oestrogen-induced increase in hepatic de novo lipogenesis may involve an increase in the activity of other lipogenic enzymes, such as 'malic' enzyme and ATP citrate lyase.

Therefore, in the 48 hours following oestrogen treatment of male chicks, the changes in the lipid metabolism can be summarised as follows:-

(a) An accumulation of triacylglycerol and phospholipid in liver and

plasma from 7 hours post injection.

(b)   The majority of accumulating glycerolipids in plasma are associated
      with VLDL.  VLDL particles become progressively enriched with
      phospholipid and become more numerous.

(c)   A lesser proportion of plasma free fatty acids are albumin-bound,
      presumably as a result of an increased proportion of the total
      plasma free fatty acid pool binding to the increasing number of
      triacylglycerol-rich lipoprotein particles.

(d)   The increase in liver lipid content is accompanied by an increase
      in liver weight involving both cellular hypertrophy and cellular
      hyperplasia, the initial stages of the response (0 - 24 h)
      principally involving hypertrophy.

(e)   The accumulating plasma and liver lipids exhibit altered fatty acid
      compositions, the major change being an increase in the proportion
      of 18:1.

(f)   An early increase ( < 18 h) in de novo hepatic lipogenesis and
      hepatic lipogenesis from pre-formed fatty acids.  However, increases
      in hepatic acetyl-CoA carboxylase and hepatic fatty acid synthetase
      activities, as determined in vitro under optimal conditions, were
      only observed during the later stages ($\geq$ 24 h) of the response.

4.    Suggestions for further work.

      (a) In vivo measurement of hepatic lipogenesis

      Greater understanding of the state of lipogenesis in the oestrogen-

treated male chick could be achieved by in vivo studies, involving
the simultaneous determination of the initial rates of incorporation
of injected $^3H_2O$ and $^{14}C$-labelled palmitate into liver lipids at
various times after oestrogen treatment. This would give more
information about the relative importance of de novo lipogenesis
and of lipogenesis from pre-formed fatty acids in vivo.

(b)  Further studies on lipogenic enzyme activities.

One of the most striking features of the present study is the lack
of an increase in the hepatic activity of the key lipogenic enzyme,
acetyl-CoA carboxylase, within the first 24 hours immediately
following oestrogen treatment of male chicks, even though an
increase in hepatic and plasma lipid was evident during the same
period. The enzyme activity was assayed in vitro under optimum
conditions and it is generally accepted that this gives an
indication of the total catalytic activity of the enzyme rather than
the activity that actually exists in vivo. As described in
Chapter 4, the activity of acetyl-CoA carboxylase is regulated both
by allosteric modulation and by covalent modification involving
phosphorylation and dephosphorylation. An obvious field of study,
therefore, would be to determine the hepatic level of the positive
allosteric effector, citrate, at various times after oestrogen
treatment, but particularly during the first 24 hours post-injection.
Furthermore, since acetyl-CoA carboxylase is a cytosolic enzyme,
determinations of the citrate concentration in sub-cellular
compartments could be extremely useful. Such sub-cellular
determinations would necessarily involve the rapid separation of
cytoplasm and mitochondria from liver cells followed by rapid
quenching with perchloric acid and subsequent measurement of citrate
in both fractions. Watkins et al. (1977) have attempted such
determinations of cytoplasmic citrate concentration in cultured
chicken hepatocytes, using a 'digitonin-rapid-stop' technique. This

process involved the incubation of the cells at an optimal digitonin concentration which resulted in a rapid disruption of the plasma membrane whilst the mitochondrial membranes were left intact. A similar 'digitonin-rapid-stop' procedure could be adopted in an attempt to study any possible changes in cytoplasmic citrate concentration of liver cells from male chicks, shortly after ($\leqslant$24h) oestrogen treatment. However, the adoption of this procedure would also require the prior isolation of hepatocytes from the oestrogen-treated and control birds at varying times after injection.

In recent years, it has become accepted practice in investigations of hormonal or nutritional effects on acetyl-CoA carboxylase, to measure enzyme activity in crude liver preparations which have been pre-incubated at 37°C in the absence of citrate (Allred & Roehrig, 1978; Geelen et al., 1978; Witters et al., 1979). This form of assay involving the process of 'heat activation' is believed to restore or preserve the in vivo activity of acetyl-CoA carboxylase by a mechanism which, although not fully understood, is believed to involve restoration or preservation of the dephosphorylation/ phosphorylation state of the enzyme in vivo (Allred & Roehrig, 1978). Therefore, the results of the present study could be supplemented by the measurement of activity of hepatic acetyl-CoA carboxylase, particularly during the first 24 hours after oestrogen treatment, using the 'heat-activation' procedure.

The possibility exists that the oestrogen-induced increase in de novo hepatic lipogenesis and liver and plasma lipid levels during the first 24 hours after oestrogen treatment, in particular, may involve an increase in the activity of lipogenic enzymes other than acetyl-CoA carboxylase and fatty acid synthetase. Therefore, the results of the present study, and of the studies of Courtney (1984) and Courtney & Manning (1984) could be augmented by the

determination of the hepatic activity of 'malic' enzyme and ATP citrate lyase during the 48-hour period immediately following the treatment of male 3- to 5-week old chicks with 0.75 mg 17B-oestradiol/100 g body weight. Previous studies of the activity of these two hepatic enzymes performed at short intervals after oestrogen treatment have mainly used immature female domestic fowl (Pearce & Balnave, 1973, 1975; Balnave & Pearce, 1974).

The increase in acetyl-CoA carboxylase specific activity reported in the present study, subsequent to 24 hours after oestrogen treatment, and any possible similar changes in other lipogenic enzymes in similarly-treated birds, could be further characterised by the following procedure:-

(a) purification of the enzyme and preparation of a monospecific antiserum,

(b) use of the monospecific antiserum to monitor changes in the amount of enzyme protein to distinguish between changes in the catalytic efficiency of a fixed amount of enzyme protein and changes in the quantity of enzyme protein.

Furthermore, the turnover of lipogenic enzymes could be studies in primary hepatocyte cultures, maintained in the presence or absence of oestrogen. The rates of synthesis of specific lipogenic enzymes could be quantified, after the incubation of the cells with isotopically-labelled amino acids, by disruption of the cells and isolation of the specific lipogenic enzymes by immuno-adsorption techniques. The rates of degradation of specific enzymes could also be quantified in a similar way. After incubation of the cells with an isotopically-labelled amino acid, cells could be placed into culture medium containing non-radioactive amino acids and, at various times during this 'chase' period, specific isotopically-labelled lipogenic enzymes could be isolated by immuno-adsorption and quantified.

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