THE COMPARATIVE EFFECTS OF NATURALLY
OCCURRING, SYNTHETIC AND PLANT ESTROGENS
ON UTERINE METABOLISM

by

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ABSTRACT

Various estrogens biologically equivalent to 1.0 μg
estradiol-17β were administered intraperitoneally to immature
female rats to monitor the relative dose and time course effects
on water imbibition. Estradiol-17β, estriol and diethystil-
bestrol (DES) were the strongest estrogens in inducing tissue
edema. Plant estrogens, genistein and coumestrol, in pure
crystalline form were approximately 10^{-3} times as potent in
stimulating water imbibition as estradiol-17β.

The administration of estradiol-17β, genistein and coumes-
trol was shown to enhance the permeability of uterine vasculature
as indicated by the diffusion of intravenously infused India
ink.

Large doses (5.0 μg) of estradiol-17β were administered to
immature female rats to measure the rate of synthesis of RNA
and DNA in the uterus. It was observed that the uterine cell
cycle was significantly ($p < 0.025$) reduced after the administration
of estradiol-17β. Net accumulation of RNA and DNA was shown to
occur after 12 and 24 hours respectively.

The incorporation of $[5,6-^3H]$ uridine into the uterine
tissue was studied by administering natural, synthetic and plant
estrogens intraperitoneally to immature female rats. After a
six hour period there was a decreased uterine specific activity
of $[5,6-^3H]$ uridine in estrogen treated rats when compared to
control animals. Estradiol-17β produced the greatest reduction
in the specific activity of the hydrolyzed RNA while DES,
estriol and estradiol-17α produced relatively smaller reductions.

The reduction of specific activity six hours after in vivo pulsing with estradiol-17β was probably due to a dilution effect associated with changes occurring in cell permeability. Genistein and coumestrol also reduced the specific activity of $[5,6-^3H]$ uridine similar to that observed with estriol.

Extracts of alfalfa hay and soybean meal were analyzed quantitatively and qualitatively for plant estrogens. Short in vivo pulsing of 30 minutes of various estrogens was employed to determine the uptake of $[5,6-^3H]$ uridine by the uterine tissue. Estradiol-17β, purified genistein, alfalfa and soybean extracts were found to incorporate $[5,6-^3H]$ uridine at greater rates than control groups.

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INTRODUCTION

Following the publication by Jacob and Monod (1961) of

the operon model for regulating gene expression, the mechanism

of steroid hormone action has become one of the most popular

areas of research in endocrinology and animal regulatory

biology. In the last two decades an increasing amount of

evidence has accumulated suggesting that various hormones

regulate growth, differentiation and metabolic activity in tar-

get tissues through their effects on RNA metabolism. To date

there is an incredible amount of literature available on this

subject, however no unifying concept has emerged. The most

apparent sequence of events would be for a hormone to activate

or repress certain functionally linked genes and allow trans-

cription of new species of mRNA which would then code for

synthesis of specific proteins. In addition to this, there

appears to be a second function of most anabolic hormones, the

release of biogenic amines (i.e. histamine). Szego and Davis

(1967) reported that the earliest known effects of estrogens

were associated with the uterine cell membrane and histamine

release. Increases in 3;5' cyclic AMP in the uterine cell

membrane were observed 15 seconds after exposure to estradiol-

17β.

In addition to the naturally occurring estrogens, estro-

genic activity has been reported in many forages and other

plant materials (Guggolz et al. 1961; Beck, 1964; Allison

and Kitts, 1964). Bennett et al. (1946) first reported the

presence of phytoestrogens in subterranean clover (Trifolium

subterraneum).   Since that time plant estrogens have been re-
cognized as agents responsible for the infertility of grazing
stock.   Plant constituents which are regarded as estrogenic
and are responsible for many reported cases of infertility in
animals grazing estrogenic forages and pasture legumes are
the isoflavones, genistein, biochanin A, daidzein, equol and
formononetin (Bradbury and White, 1954; Cheng et al. 1953(b);
Cheng et al. 1954) and coumestrol (Cheng et al. 1953(a);
Bickoff et al. 1957).   The estrogenic potential present in
various plant sources is generally much weaker than naturally
occurring steroid or synthetic estrogens.   However, the large
amount of phytoestrogens ingested during grazing may elevate
the plasma levels of estrogen to a degree which would interfere
with reproductive processes.

The objectives of this study were to establish the base
lines regarding the time course and dose effects of estradiol-17β
and its various analogues on uterine tissue metabolism and
secondly to employ an in vitro bioassay which would elucidate
the initial metabolic events occurring in the cell after estro-
gen, phytoestrogen or plant extract administration.

REVIEW OF LITERATURE

1. Antiestrogens

The word "antiestrogen" is a widely used and grossly mis-
leading term used to describe compounds which exist naturally
(Cook and Kitts, 1964; Chow et al. 1972) or are produced syn-
thetically (Callantine et al. 1968; Davidson et al. 1968;
Humphrey, 1968) and which decrease the response of female
reproductive organs to estrogen administered or injested con-
comitantly. Estrogenicity of a compound is usually assessed
by gross, histologic and biochemical changes in the uterus,
cervix and vagina of ovariectomized animals. Difficulty in
defining the degree of estrogenicity arise as a result of dif-
ferent thresholds of the compound required for eliciting an
estrogenic response. The dose, mode of administration and
physiological status of the animal will govern the absorption
and retention of the hormone by a particular target organ.
These variables play significant roles in determining an estro-
genic response and therefore whether or not a compound is anti-
estrogenic.

Kato et al. (1968) and Eisinfeld (1970) reported that the
binding of estrogens to receptors in the hypothalamus and
pituitary as well as other target tissues constitutes an impor-
tant mechanism regulating reproduction. The mechanism whereby
antiestrogens inhibit biological responses to endogenous
estrogens involves the competition for specific macromolecular
components in the uterus (Wyss et al. 1968) pituitary and hypo-
thalamus (Kato and Villee, 1967); Leavitt et al. 1973).

Emmens et al. (1960) reported that steroids and nonsteroids are highly active when administered subcutaneously or when locally injected against estrogens. Nonsteroids which are well known antagonists of estrogens include DES and mesobutesterol while androgens and progestins are considered classical steroid antagonists.

The mode of action of antiestrogens is believed by many workers (Emmens et al. 1960; Kato et al. 1968; Newsome and Kitts, 1975) to entail a successful competition with the nuclear receptors as well as to cytosol receptors. They prevent the initiation of some relatively early events occurring in the cell which are the result of modifications in the transport of estradiol-17β through cellular and/or nuclear membranes (Emmens et al. 1960; Rochefort and Capony, 1972). Rochefort and Capony (1972) reported that the inhibition resulted from antiestrogens competing with estradiol-17β for binding sites in the target organ. This would be an example of competitive inhibition of the receptor site. Antiestrogens may also inhibit the receptor site noncompetitively by causing a reduction of the product formed or preventing uptake and retention of the hormone. Modification by antiestrogens of the metabolic steps subsequent to the initial hormonal stimulus may also be responsible in altering the physiological action of an estrogen (Rochefort and Capony, 1972). In this case antiestrogens would have a modifying effect on the secondary response of the estrogen, but no real effect on the primary action of the hormone.

These antiestrogens which reduce the concentration of estrogen at the site of the target tissues may do so by forming

a durable complex of low biological affinity with estrogenic binding sites (Callantine et al. 1968). Alternatively they can form a short-lived complex with the binding sites which are too transient to exert any biological effect such as growth stimulation (Rochefort and Capony, 1972). Nevertheless, the availability of receptor sites for circulating estrogens is reduced.

Antiestrogens inhibit the binding of estrogens to their receptor sites in varying degrees (Lee, 1974). One of the major factors governing the binding affinity of various antiestrogens is the structure of the individual compounds. Huggins and Jenson (1955) showed that maximum competition with ^{3}H estradiol-17β for receptor sites in the uterine cytosol depends strongly on the presence of phenolic hydroxyl groups located on the 3' position. They reported that substituents at the C-16 and C-17 position in the D-ring may also affect binding, and variation from the 17-hydroxyl group of estradiol resulted in reduced affinity. Shutt and Cox (1972) studied the mechanism of phytoestrogen action and reported that within the isoflavone series, the presence of a phenolic hydroxyl group in both rings A and B was associated with the highest relative binding affinity. The presence of an additional 5' hydroxyl group in genistein increased its affinity for binding. Upon methylation to biochanin A there was a sharp decrease in binding affinity. It appears from these reports that to be capable of competing with endogenous estrogens for similar protein subunits an antiestrogen must be structurally similar to the naturally occurring estrogens.

The degree of hydrophobic bonding is important in regard

to the binding affinities of various estrogens. Dimethylstil-
bestrol (DMS) and meso-butestrol differ from their more potent
counterparts DES and meso-hexoestrol only by the size of their
hydrophobic hydrocarbon chain.   The dose or circulatory level
of antiestrogens in the plasma may also have an effect on the
binding affinities.   Callantine et al. (1968) reported that a
maximum response of a hormone is characterized by saturated
receptor sites.   The degree of response is thus proportional
to the amount injected.   The steroid-protein interaction in
the cytoplasm is a random process and is a necessary step for
the formation of estrogen protein complex.   The presence of an
antiestrogen would result in the reduction of estrogen saturation.

Interference with various physiological events, associated
with reproductive failure due to the effect of numerous anti-
estrogens, has been documented (Engle et al. 1957; Leavitt and
Wright, 1965; Cox and Braden, 1974).   Kato et al. (1968)
reported that clomiphene, an antiestrogen possessing anti-
fertility characteristics suppressed the pituitary function and
gonadotrophin release.   Davidson et al. (1968) reported that
daily doses of clomiphene inhibited estradiol-17β and the
secretion of lutenizing hormone.   This was found to have an
effect on successful implantation which requires an optimum
concentration of estrogen as well as progesterone. Segal et al.
(1958) demonstrated that clomiphene was blastotoxic and pre-
vented implantation by its effect on the endometrium as well as
by damaging the zygote at biochemical levels.   Prasad et al.
(1965) suggested that most antiestrogens were not zygotoxic,
but they prevented implantation by their effects on the maternal

environment.

Carter et al. (1955) working with phytoestrogens demon-
strated that genistein when fed to mice at 0.02% of diet induced
the premature opening of the cervix and resulted in fewer litters
being born.   Similar studies regarding maternal environment
alterations, due to antiestrogens were reported by Finn (1966)
and Humphrey (1968).   Implantation and the induction of
deciduomata in intact or ovariectomized mice are strongly depend-
ent on optimum estrogen and progesterone levels.   Finn (1966)
demonstrated that decidualization is even more dose dependent
than implantation.

2.   Effect of Estrogen on Cell Metabolism

Estrogenic hormones exert an influence on the metabolism
of many target organs by binding effectively to intracellular
components.   There exists a strong, reversible association
between the circulatory hormone and its receptor site (Toft and
Gorski, 1966; Jenson et al. 1969; Means and O'Malley, 1972;
O'Malley and Schradier, 1976).   This association is the primary
step in uterotrophic processes and is governed by two main
criteria:

    1.   The uptake of the hormone is not saturated and is
       independent of any hyperphysiological level.

    2.   The retention of the hormone is saturated and is
       dependent on the dose whether or not it exceeds
       the physiological level.

Hamilton (1963) demonstrated that the rat uterus
possesses the capacity under estrogenic stimulus to synthesize

RNA _in_ _vivo_ and _in_ _vitro_ by mechanisms similar to those pre-
viously reported in the rat liver nuclei.   The sequence of
events following the administration of estradiol-17β is
initiated at the cellular level and progresses to the genome
level resulting in the stimulation of the synthesis of RNA,
protein and eventually DNA and cellular division (Fig. 1).

Two theories have been proposed by Gorski and Raker (1974)
to explain the mode of action of estrogens.   The "Domino
Theory" describes the early responses of estrogen (cytosol
binding and induced protein-RNA sythesis) which set off later
cellular events.   The "Sustained Output Theory" describes the
later events which are dependent on the continued presence of
estrogen in the nucleus and on the early events.   Gorski and
Raker (1974) demonstrated that both estradiol-17β and estriol
have the same effect on early responses (2-4 hours) of the rat
uterus.   However, at 18-24 hours estriol had little or no
effect, in contrast to the 300% increase over the control caused
by estradiol-17β.   These workers suggested that the presence
of an estrogen and the sustained output of some critical
factors seems to be essential for the full physiological effects
of estrogenic hormones.

The initial response of a hormone is its noncovalent
binding to a series of carrier proteins responsible for trans-
porting the hormone to the genome of its respective target organ.
Present in the eukaryotic organism are two types of proteins
which are important in the transport and binding of estradiol-17β.
Serum protein albumin is the principal binding protein involved
in the transport of the steroid to its target organ.   The
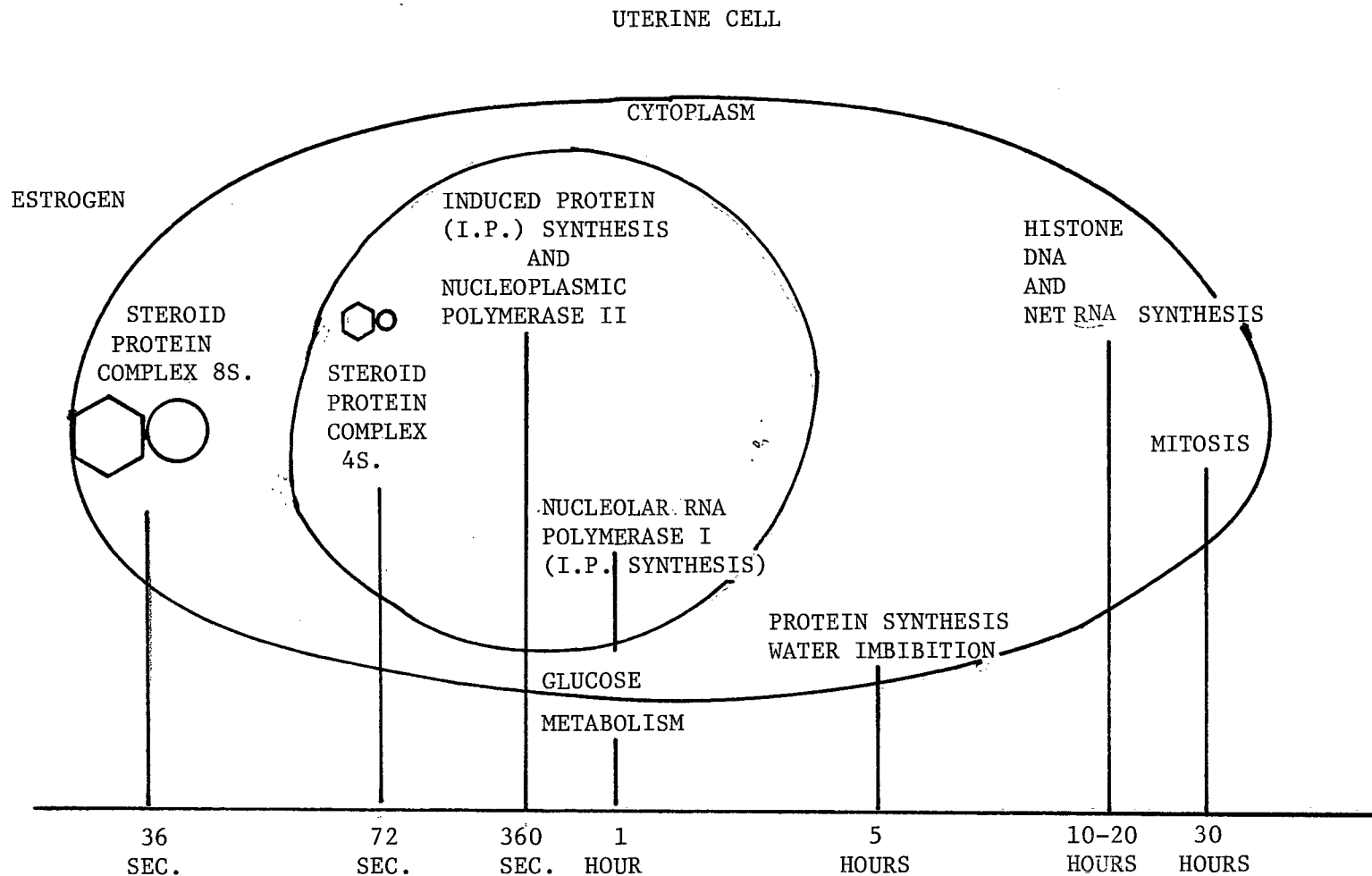
Fig. 1.   Temporal sequence of metabolic events in
Uteri of immature or ovariectomized rats
after in vivo administration of estrogen

circulating steroids are also present in a complex involving a glycoprotein with the carbohydrate moiety being glucuronic acid. This association with glucuronic acid is referred to as the conjugated form or inactive form of the hormone and cleavage is required before it can enter the cell (Jenson and DeSombre, 1973). The second binding protein involves the selective up-take of the steroid by the target organ (Chamness, 1972). Talwar et al. (1968) characterized the noncovalent linkage between the steroid and its specific carrier receptor in the target organ to be greater in its specificity than the serum albumin binding.

Target tissues of estradiol-17β possess receptor sites capable of recognizing and binding onto the hormone. The mech-anism by which steroids penetrate the cell membrane is hypothesized to be a facilitated diffusion (Katzenellenbogen and Gorski, 1975). After entry is made, the hormone is adsorbed by specific proteins possessing high affinities. The receptor protein binds estradiol-17β and DES with higher affinity and greater specifity than estradiol-17α , progesterone and testosterone (Terenius, 1969).The receptor, a term used to des-cribe a particular protein present in the tissue and whose inter-action with the steroid results in a hormone induced response, is a macromolecule with a minimum molecular weight of 100,000 (Gorski and Raker, 1974). Toft and Gorski (1966) using explicit sedimentation profiles of low salt and sucrose gradients, reported that the steroid hormone associates spontaneously with the extracellular protein to form a 8S (200,000 daltons) receptor -steroid complex. Higher salt concentrations and the

use of urea resulted in various slower sedimentation species.
They suggested that a smaller subunit, 4S (45,000 daltons) was
the native estrogen binding unit. O'Malley and Means (1974)
also reported that the 8S classical cytoplasmic estradiol-17β
receptor was estrogen specific and was transferred to the
nucleus where it stimulated RNA synthesis.

Jenson and DeSombre (1973) confirmed that cytoplasmic
binding was necessary for the transportation of the steroid to
the nucleus. The protein-steroid complex is very sensitive to
low temperatures (Puca et al. 1971), proteolytic enzymes, high
salt concentrations (Chamness, 1972) and specific inhibitors such
as various sulphydryl blocking reagents (King et al. 1971).
Raynaud et al. (1971), Solof et al. (1972) and Jenson et al.
(1969) established that the estradiol-17β receptor protein placed
under these conditions will dissociate and degrade to respective
4S subunits. If not interfered with, the 8S complex, possessing
an affinity for the nucleus will enter it in this form.

Once present in the nucleus, the steroid receptor protein
undergoes a change which sediments to a 5S complex after
extraction with a dilute potassium chloride solution. Williams
and Gorski (1971) and Jenson and DeSombre (1973) disclosed that
in living cells binding sites are bound to the nucleus. The
5S nuclear protein is found in the acid portion of the chromatin
and unlike the cytoplasmic receptor site, it differs in its size
and affinity from the steroid. Anderson et al. (1972) have con-
firmed the presence of a nuclear protein with experiments in-
volving tritiated estradiol-17β. They showed that within the
nucleus, the estrogen is bound to the nuclear receptor protein

which is smaller in size and greater in its affinity for the steroid than the cytoplasmic receptor protein.

The nuclear and cytoplasmic receptors have various immunological, chemical and physical properties in common. The protein moiety is an essential part of the binding properties in both receptors. After a period following the association with the cytoplasmic protein, the steroid-protein complex disappears from the cytosol and reappears in the nuclei with a smaller specificity but greater affinity. The nuclear protein receptors, termed "neo-receptors" are precursors of the cytoplasmic receptors. King et al. (1971) have proposed that it is the required ability of the nuclei to transform the cytoplasmic receptor to a form where it can then attach onto the chromatin. This transformation from cytoplasmic receptor to a nuclear receptor is also temperature dependent and sensitive to sulfhydryl blocking reagents. They also suggested that the chromatin receptor is possibly a histone, which would explain for its additive specificity. This hypothesis proposes that the structure of an acceptor is complimentary to the estradiol-17β receptor and the effect of an estrogen responsive cell would be determined by the position of the locus of the acceptor on the genome.

The concentration of cytoplasmic and nuclear receptor proteins appears to be a function of the circulating estrogen level in the plasma (Solof et al. 1972). After ovariectomy the acceptor property of the nuclear receptor is decreased. King et al. (1971) demonstrated that a reduction in 4S receptor sites as well as a decrease in the total amount of cytoplasmic binding sites occurred after ovariectomy or hypophysectomy was

performed. This would indicate that the number of cytoplasmic receptors and especially the 4S component is controlled by ovarian and pituitary hormones.

Ten minutes after the administration of an estrogenic compound a particular RNA is synthesized for a specific estrogen induced protein (IP) (Gorski et al. 1975). Observations made by Notides and Gorski (1966), Barnea and Gorski (1970) and Katzenellenbogen and Gorski (1972) have led to the concept that estrogens are responsible for the induction of the synthesis of a particular uterine protein. This protein fraction detectable after 40-60 minutes is essential for the increase in metabolic activities of the tissue. Purification of IP, has shown that it is very similar in characteristics to ovalbumin with a biological half life of nine hours. Ruh et al. (1973) demonstrated that the synthesis of IP can be regulated only by those compounds which possess estrogenic properties and which will bind to the uterine estrogen receptor proteins. Among several estrogenic compounds tested estradiol-17β, DES, estriol and estrone were effective in IP synthesis in that order. Progesterone and testosterone had no effect.

Recently Gorski et al. (1975) reported that the lag period of 40-60 minutes was the time necessary for mRNA to be processed and moved from the nucleus to the cytoplasm, where it associates with the polysomes, the site of protein synthesis. Estrogen is therefore considered to induce the synthesis of mRNA which in turn is essential for synthesis of an induced protein.

Though the synthesis of RNA as a primary event in early action of estrogens has been established, the mechanism by which

✗ estrogen influences RNA synthesis has been subject to question.

It is not clear whether the hormonal stimulation results from an

effect on the chromatin template activity (Barker and Warren,

1967), RNA polymerase activity (Maul and Hamilton, 1967), the

transport of RNA from the nucleus to the cytoplasm (Hamilton et

al. 1968), or a combination of all these factors.   DNA dependent

RNA polymerase enzymes have been isolated from the nucleolus and

the nucleoplasm of eukaryotic cells.   By the use of radio-

autography the sequential stimulation of a Mg^{++} dependent poly-

merase by 2.0 hours occurring in the nucleolus, and a Mn^{++}

dependent polymerase by 4.0 hours in the nucleoplasm was observed

in the ovariectomized rat (Maul and Hamilton 1967).   The success-

ive stimulation of these enzymes and the accelerated synthesis of

all types of RNA immediately following estrogen administration

provide evidence that increased rate of protein synthesis is

occurring in the uterus.   Greenman and Kenny (1964) demonstrated

that ovariectomy lowers the activity of the uterine ribosomes

and suggested that estrogens also have the ability to regulate

the capacity of the functional ribosomes to synthesize proteins

in addition to influencing the synthesis of ribosomes.

Several metabolic events have been shown to increase 1-2

hours after estradiol-17β administration.   Glucose, phospholipid

(Pepe and Yochim, 1971) and the activity of RNA polymerase I

(Noteboom and Gorski, 1963) are among the initial changes noticed

after a single estrogen injection.   Raynaud et al. (1971) were

the first workers to demonstrate an in vitro effect of estradiol-

17β on RNA synthesis in isolated uterine nuclei.   By incubating

calf uterine cytosol with estradiol-17β and adding isolated

nuclei they assayed for RNA polymerase activity and were able to demonstrate a successful interaction between the steroid and its specific receptor site. An increase in nuclear DNA dependent RNA polymerase activity was noted as well. Barker and Warren (1967) reported that a single intravenous injection of estradiol-17β to ovariectomized rats resulted in an increase in template of uterine chromatin for RNA synthesis which is tissue specific. The template capacity of liver and lung chromatin from the same animal was not elevated by estrogen.

In addition to being tissue specific, the effect was also steroid specific. After incubation in vitro for 12 hours, estradiol-17β enhanced the ability of uterine chromatin to serve as a template for DNA dependent RNA polymerase to a greater extent than estradiol-17β. Teng and Hamilton (1967) observed that estradiol-17β was bound to uterine chromatin in vivo as early as 2 minutes after its administration with maximal binding occurring after 8 hours. Twenty minutes after estrogen treatment Hamilton et al. (1968) noticed that the rate of nuclear RNA synthesis in vivo was as high as 500-600% of control. The activity of RNA polymerase was significantly elevated after one hour although an increase in uterine RNA content was not achieved until 8-12 hours (Meuller et al. 1958).Other reports (Billings et al. 1969(b) indicate that the total RNA and protein levels do not rise until 6 and 12 hours respectively. These series of events suggest that there exists a transfer of a part of newly synthesized RNA from the nucleus to the cytoplasm. This is in accordance with the increased accumulation of whole tissue RNA and protein between 2 and 24 hours without any

detectable increase in the RNA/DNA and protein/DNA ratios of the nuclei.

On the basis of histological evidence, Meuller et al. (1958) demonstrated that increased nucleolar size, greater abundancy of endoplasmic reticulum and increased basophilic character of the uterine cell cytoplasm were noticed 24 hours after estrogen administration and these were correlated with increased production and accumulation of RNA and enhanced protein synthesis. Hamilton (1963) reported that the uterus in a normal rat contains twice the amount of RNA and six times the quantity of protein than in the ovariectomized rat. The uterus obtained from a rat during estrus contained 0.3 to 6 times the amount of protein than during diestrus.

In addition to the effect on RNA and protein synthesis, estrogens have also been shown to stimulate many enzymes in the uterus (Pepe and Yochim, 1971; Hall, 1973). The increase in the activity of many enzymes is reflected in high incorporation of ^{14}C acetate and ^{14}C glucose into lipids (Noteboom and Gorski, 1963(a), ^{3}H uridine and ^{3}H-cytidine into RNA (Miller and Emmens, 1967; Billing et al. 1969(a)) and ^{14}C-glycine into protein (Meuller et al. 1958). Increases in phospholipid, glycogen and lactate metabolism in the rat endometrium have also been attributed to estradiol-17β (Walaas et al. 1952).

Estradiol-17β and its analogues are also associated with changes in the uterine membrane and histamine release. Lieberman et al. (1963) reported increased levels of 3',5'-cyclic AMP in the uterine membrane 15 seconds after the administration of estradiol-17β. Spaziani et al. (1958) demonstrated

that the release of histamine from the uterus was responsible
for imbibition of water which is one of the early effects of
estradiol-17β on the cell.   Hyperemia of uterine tissues is
detectable 1-2 hours after estrogen administration and fluid
uptake into the uterus reaches a maximum after 6 hours
(Astwood, 1938).   Increases in dry weight of uterine tissues
after estrogen administration are accounted for by increase in
protein content of the uterus first seen at approximately 18
hours (Billing et al. 1969(b)).   Increases in the rate of DNA
(Kaye et al. 1972) and histone synthesis (Williams and Gorski,
1971;   Anderson et al. 1972) begin at about 18 hours after
estrogen treatment.   This is followed by cellular division
which begins in the uterus at approximately 24 hours (Kaye
et al. 1972;   Lee, 1974).

3. Phytoestrogens

The effects of phytoestrogens on livestock reproduction
have been recognized for a long time (Bartlett et al. 1948; Ochi
et al. 1964;   Cayen et al. 1965;   Cox and Braden, 1974).   The
potential economic loss may be ascribed to their antagonism to
the biochemical and physiological responses to estrogen.   In
addition to estrogenic substances other compounds present in
certain species of plants have also been found to affect repro-
ductive processes.   For example, compounds extracted from the
ponderosa pine needles (Pinus ponderosa) are believed to be
responsible for weak calves at birth as well as abortion in
grazing animals in the western part of U.S.A. and Canada
(Cook and Kitts, 1964; Chow et al.1972; Stevenson et al. 1972).

The various factors influencing the presence of phyto-
estrogens and their effects on animal reproduction have been
studied by many workers.   The first report of estrogenic sub-
stances in plants was made in 1926 by Dohrn et al. Loewe et al.and
Fellner[1], who reported estrogenic characteristics from the
extracts of ovaries of the water rose and willow catkins.   Sub-
sequently many estrogen analogues in forages have been identified
and related to reproductive problems.   These include the sterols,
coumestrol and other coumestans (benzofuranocoumarins) and the
isoflavones, formononetin, biochanin A, daidzein, genistein and
the most recently isolated equol.

The presence of estrogenic compounds in forages was dis-
covered by Bennett et al. (1946), who reported on the estrogenic
activity in subterranean clover (Trifolium subterraneum).   Later
many estrogenic compounds have been identified from extracts of
British and American pasture plants, in particular red clover
(Trifolium pratenset), (Pope et al. 1953; Wong and Flux, 1962).
Bradbury and White (1951) isolated the isoflavone genistein from
subterranean clover and alfalfa, while Pope et al. (1953) iso-
lated another estrogenic isoflavone, biochanin A (5,7, dihydroxy
H-methoxy-isoflavone) from red clover.   Genistein has also been
isolated from soybean oil meal (Cheng et al. 1953(a)).   Pope
(1954) reported that genistein was not only present in soybean
meal but also occurred together with biochanin A and formono-
netin in both subterranean and red clovers.   The isoflavone,
equol, was first extracted and isolated by Marrian and Haselwood
(1932) from a phenolic fraction of ether-soluble.

([1] cited from Bradbury and White, 1954)

ESTRADIOL-17β.   ESTRONE   ESTRIOL

COUMESTROL

BIOCHANIN A   GENISTEIN

FORMONONETIN   DAIDZEIN   EQUOL
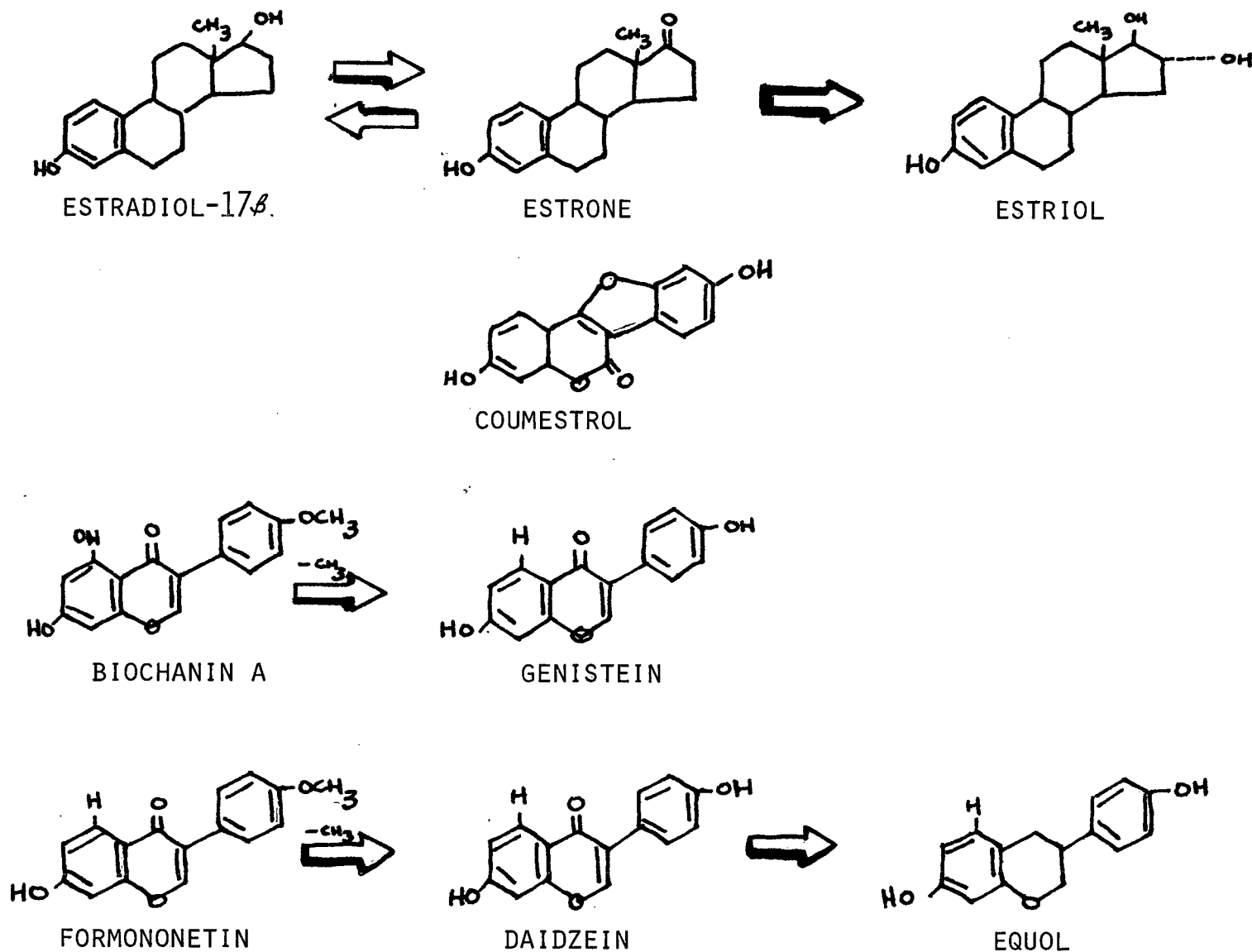
Fig. 2.   Structure and metabolism of estrogenic
compounds

extracts of pregnant mare's urine suggesting that this compound

may have originated from the metabolism of the ingested forage.

Since then it has been isolated from cattle and fowl urine

samples (Klyne and Wright, 1959; Cayen et al. 1965), the bio-

logical significance of which is discussed later.

The estrogenic activity of the isoflavones was demonstrated

by Chang et al. (1954) who fed them to immature mice and

measured the effect on uterine weight.   Results of preliminary

biological tests showed that genistein had an estrogenic activity

of approximately 10^{-5} times that of estrone.   Formononetin was

found to be inactive as assessed by the Allen-Doisy method which

is based on the estimation of the cornification of rat vagina.

The metabolism of various phytoestrogens by the rumen microbes

has been studied by many workers.   In a series of experiments

Nilsson (1961 a and b) and (1962) demonstrated that the rumen

microorganisms demethylated the comparatively weaker isoflavones,

formononetin and biochanin-A to yield more potent estrogenic

compounds such as daidzein and genistein (Fig. 2).   Braden

et al. (1967) found that in sheep the isoflavones, genistein,

biochanin A, and formononetin were all estrogenic when administered

intraruminally, but had only 5% of the activity when given

intramuscularly.   Biochanin A is methylated at the 4' position

on the C-ring and after microbial metabolism, the methyl group

is cleaved and genistein is produced (Braden et al. 1967; Cox

and Braden, 1974).   Formononetin which is also methylated at

the 4' position is converted to daidzein after similar action

of the microflora.   Daidzein in turn is metabolized to equol,

which is a very potent estrogen analogue and is believed to be

the chief plant constituent responsible for "Clover Disease"
(Lightfoot and Wroth, 1974). Coumestrol is found predominantly
in alfalfa. In the fowl it is degraded along pathways quite
different from those of genistein, despite the fact that the
biosynthetic pathway of coumestrol in the plant resembles that
of the isoflavones (Cayen et al. 1965).

Phytoestrogens have been reported to act like estradiol-17β
after their consumption and subsequent metabolism by the grazing
animal (Braden et al. 1967; Shutt et al. 1967; Lindner, 1967).
Endogenous estrogens circulate in a conjugate form which renders
them inactive. Phytoestrogens, after their metabolism and
absorption in the gut, are conjugated with glucuronic acid in
the liver and circulate in the plasma in the form of inactive
glucuronates. After their interaction with the receptor
proteins the covalent conjugation with glucuronic acid is split
by the action of glucuronidase (Cox and Braden, 1974). The
rate conjugation and the metabolism will govern the effect of
phytoestrogens on the reproductive performance of grazing
animals. Differences have been observed in the metabolism of
phytoestrogens between sheep and cattle. Braden et al. (1971)
reported that circulating phytoestrogens were more efficiently
conjugated in cattle than in sheep, rendering them less sus-
ceptible to the action of phytoestrogens.

Phytoestrogens are 10^{-3} to 10^{-5} times as estrogenic as
estradiol-17β in the laboratory mouse (Bradbury and White, 1951;
Carter et al.1955).However, with continuous grazing the
relatively high intake of phytoestrogens can induce significant
biological effects (Barret, 1961). Results on the degree of

estrogenicity of various plant compounds have been contradictory, primarily because of the errors associated with their low solubility in water. Relative estrogenic activities were investigated by Wong and Flux (1962) using the semi-quantitative mouse uterine weight assay method developed by Kitts et al. (1959). Shemesh et al. (1971) reported that approximately one unit weight of estradiol-17β was equivalent to 70 units of coumestrol and 175 units of genistein. The estrogenic potency of phytoestrogens is related to their binding affinity for macromolecular components located in the uterine cytosol. The methylated isoflavones, biochanin A and formononetin did not significantly inhibit estradiol-17β binding. This suggests that the free hydroxyl groups methylated in biochanin A and formononetin are essential for the effective interaction with the estrogen receptor.

There have been many reports of profound effects on the growth, lactation and reproduction of animals grazing estrogenic forage plants or pastures (Bartlett et al. 1948; Pope, 1954; Sanger and Bell, 1960; Marshall, 1974). In many cases infertility is temporary in nature and recycling and conception return to normal five to six weeks after the consumption of the estrogenic material is stopped (Lightfoot and Wroth, 1974). Permanent infertility (clover disease) may result when ewes have been grazing estrogenic clover pastures for a number of years (Lightfoot and Wroth, 1974; Marshall, 1974). The magnitude of the effect of phytoestrogens depends on many environmental variables. Stage of maturity and defoliation (Kitts et al. 1959), trace mineral deficiency (Rossiter, 1970), route of

administration (Ostrovsky and Kitts, 1962), foliar disease (Saba
et al. 1972) and individual plant parts determine the amount of
estrogenic material present in forage and pasture plants.
Infertility resulting from the ingestion of estrogenic pastures
may be manifested in many forms (Sanger and Bell, 1960; Barrett,
1961; Lightfoot and Wroth, 1974). The effects of phytoestrogens
on sperm production and viability in rams have been shown to be
negligible (George and Turnbull, 1966). Lightfoot and Wroth
(1974) reported a reduction in the onset of estrus in ewes
grazing estrogenic pastures. Ovulation rates of ewes on similar
pastures were also reported to be reduced, thereby affecting the
normal length of the estrous cycle. Differences on ovum trans-
fer and the passage of the egg through the Fallopian tube for
successful fertilization have also been attributed to the effect
of phytoestrogens.

The establishment of a pool of sperm in the cervix immediate-
ly after mating is important for successful fertilization in sheep
(Restall and Wales, 1966; Smith, 1970). Lightfoot and Wroth
(1974) collected eggs from ewes fed estrogenic forages and found
that they had lower numbers of spermatozoa present on the zona
pellucida, which reduced the probability of fertilization.

No evidence has been established suggesting abnormalities
in the ovary, pituitary or adrenal cortex as a consequence of
grazing on estrogenic pastures. Obst and Semark (1970) reported
fluctuating levels of plasma progesterone due to functional
changes in the corpus luteum of animals grazing various clover
pastures. Leavitt and Wright (1965) reported that the principal
effect of phytoestrogens was to inhibit the release of gona-

dotrophic hormones by servomechanism on the hypothalamus.

A greater incidence of irregular estrous cycles in dairy cows fed estrogenic forages was reported by Alder (1965). Barrett (1961) found a correlation between fertility of the cow and size of macroscopic cysts seen at autopsy. Other changes observed in cattle grazing subterranean clover include the size of uterus and udder even in non-pregnant and nonlactating cows, and increased clinical incidence of swollen vulvas, cystic ovaries and hyperemic mucous membranes. Pathological conditions characterized by cystic glandular hyperplasia of the endometrium were also noticed in ewes. Variable degrees of hyperplasia in the stroma cells resembling lesions described by Novak and Richardson (1941) in post menopausal women were observed in the epithelial lining of the cystic glands. The epithelial lining was characterized as being flat and devoid of all mitotic activity.

The incidence of abortion following the ingestion of pine needles by cattle is not entirely known. The substance responsible for abortions in cattle, sheep or deer grazing pine needles is a teiterpene (Glycyrrhetmic acid). Chow et al. (1972) reported after examining the uterus of pregnant animals, consuming pine needles that fetal development had been arrested. The uteri were reported to be hyperemic or nodular and the fetus was in the process of reabsorption. Allen and Kitts (1961) and Cook and Kitts (1964) reported the presence of an agent in pine needles which suppressed growth of the uterus of immature mice and resulted in embryonic mortality. Aqueous extracts from yellow pine have been reported to possess estrogenic characteristics.

Attempts have been made to reduce the incidence of repro-
ductive disorders in animals consuming estrogenic plant
materials by altering the metabolism of phytoestrogens in animals
and by immunologic methods.   The former requires a better under-
standing of the chemical changes to these compounds by the action
of the rumen microorganisms and subsequent metabolism.   Cox
et al. (1972) reported that there is a covalent attachement of
antigenic protein with suitable phytoestrogens resulting in
haptens.   Sera obtained from sheep possessing antibodies against
various phytoestrogens reacted specifically with plant estrogens.
Antibody titres were reported to persist in sheep for about one
year, with no effect on the normal estrous cycle of the animal.

Experiment A : The Comparative Effects of Estrogens
               and Phytoestrogens on Water Imbibition
               and Hyperemia of the Rat Uterus.

Introduction

Estrogenic hormones exert an influence on the level of
metabolism in many target tissues. Most of these effects are
probably secondary phenomena resulting from an initial stimu-
lation of some key biochemical process. It is also possible
that estrogens exert more than one primary effect on many
cellular components in the target cell. In particular the
estrogen induced release of histamine by the uterus results in
estrogen receptor interaction and genome activation and may
therefore represent a separate hormonal action.

Stimulation of the uterine growth is a general character-
istic of estrogenic compounds which may differ quantitatively
in their capacity to promote growth of the female reproductive
tract. There are also marked species difference in response
to estrogens, some being more sensitive than others.

Standard estrogen assay techniques in laboratory animals
have been based on relatively late responses of the uterine
and vaginal tissues. Responses most frequently selected in
the past were the vaginal epithelial cornification and increased
tetrazolium reduction reactions (Martin, 1964). These
responses occur considerably later than certain vascular and
metabolic events and are also nonspecific to various estrogens.

The earlier six-hour water inbibition test, developed by
Astwood (1938) measures the accumulation of water in the uterus
during very initial stages of estrogen administration.

Spaziani et al. (1958) first reported that the accumulation of water in the uterus following estrogen administration was secondary to hyperemia and that increased permeability was the result of estrogen induced release of histamine.

The per cent of water in general reflects the physiological state of the uterus (Hinshaw, 1959). Finn and Martin (1973) studied the effect of estrogen on cell proliferation in both the glandular and luminal epithelial cells of mouse uterus and found that cell division in both types was stimulated in response to estrogen. However, the initial response to each was different. The luminal epithelium showed maximal mitosis after 24 hours in response to a single daily injection whereas the glands showed little response until 72 hours and required at least three daily injections for maximal response.

Kaye et al. (1972) reported an increase in cell division and maximum mitotic activity between 24 and 28 hours after a single dose of estrogen was given. The accelerated cellular division was more marked in the epithelium than the stroma of myometrium. These findings suggest that tissue growth is responsible for the notable increase in uterine dry weights of estrogen treated animals over control groups.

The object of the present series of experiments was to determine the mode of action of various estrogens with particular reference to the early biochemical mechanisms involved and the role of phytoestrogens if any, in modifying the same. As a preliminary step in this direction Experiment A was undertaken to test the potency of various estrogenic compounds and compare their time course effects on the basis of water inbibition

of immature rat uterine tissues.

Materials and Methods

Animals

Immature female rats (35-40 g), derived from Wistar stock
were purchased from UBC Colony and Woodlyn Laboratories, Guelph,
Ontario. They were housed in air conditioned quarters with
uniform exposure to 12 hours of light and 12 hours of darkness.
Animals had free access to pelleted food (Purina Laboratory
Chow) and water.

Materials

Estradiol-17β (estr-1,3,5(10)-triene-3,17β diol), Estriol
(estr-1,3,5(10)-triene 3,16 ,17β-triol), Estrone (estr-1,3,5
(10) triene 3-01-17-one), DES (α,α,-dimethyl-stilbene-4-4'-diol),
Estradiol 17α (estr-1,3,5,(10)-triene-3-17α diol), and
Testosterone (17β Hydroxy-4-androsten-3-one) were obtained from
Sigma Chemicals Co. The following plant estrogens were donated
by Dr. A.B. Beck of C.S.I.R.O., Western Australia, Dept. of
Agriculture: Formononetin (7-hydroxy-4'methoxyisoflavone),
Biochanin-A (4'methoxy-5-7-hydroxyisoflavone) and Coumestrol
(7-hydroxy-coumarono-coumarins). Plant extracts were made
from alfalfa by the method of Francis and Millington (1965).

Administration of estrogens and Plant estrogens

In Experiment A-I, eight groups of animals (4 animals/
group) with comparable body weights were given a single injection
of 1.0 mg of estradiol-17β intraperitoneally to study the time
course effect of estrogen on water imbibition by rat uterus
tissues. Experiments A-2 and A-3 were designed to study the
effect of varying concentrations of estradiol-17β and the
relative effects of various estrogens on water imbibition.

A stock solution of estradiol-17β was made by dissolving
5.0 mg in 2.0 ml of 95% ethanol. This was brought to a volume

of 5.0 ml with 0.9% NaCl to give a concentration of 100 µg/0.1 ml.
A 100 ul aliquot of this solution was made up to a volume of 10.0
ml with 0.9% NaCl to give an administration concentration of
1.0 µg/0.1 ml.   Solutions of DES, estriol and testosterone
were made in similar concentrations.   Estradiol-17α and estrone
were prepared in concentrations of 2.0 µg/0.1 ml and 6.0 ug/0.1
ml respectively.

The concentration of phytoestrogens was made to correspond
to estradiol-17β in terms of uterotrophic activity (Perel and
Lidner, 1970).   Coumestrol solutions were made by dissolving
3.0 mg of purified coumestrol in 10.0 ml of propylene glycol
giving a concentration of 90.0 µg /0.3 ml.   Genistein (8.0 mg)
was dissolved in 3.0 ml of propylene glycol to give a final
concentration of 0.8 mg/0.3 ml.   Formononetin (100 mg/ml) and
biochanin-A (100 mg/ml) stock solutions were made up in toluene.
An aliquot from both was taken and evaporated to dryness under
a stream of nitrogen.   The residue was dissolved in propylene
glycol to give a concentration of 100 mg/0.1 ml.

To test for uterotrophic activity the compounds were
injected intraperitoneally into immature female rats.   Control
animals were given intraperitoneal injections of 0.5% alcohol
in corresponding volumes of 0.9% NaCl or propylene glycol.
After appropriate time intervals the animals were sacrificed by
placing them in a jar filled with carbon dioxide.   Their uteri
were excised and stripped of adhering fat and mesentery.
Uterine tissues were blotted dry and weighed on a microprecision
torque balance.   Tissues were then transferred to an oven and
dried to a constant weight at 90°C for 12 hours.   The degree
of water imbibition was calculated from the per cent moisture.

All statistical analysis was done by Student's t-test.

Results

In Experiment A-I it was noticed that during the first few hours after estradiol-17β administration, water imbibition increased gradually, reaching a maximum of 40.6% over control levels after six hours (Fig. 4).   This was followed by a slight decrease in tissue moisture content between 12 and 24 hours.   Water imbibition was found to increase significantly ($P < 0.025$) again at 24 and 30 hours which corresponded with increases in uterine dry weights (Table 1).   Increases in moisture content noticed at 30 hours were, however, not as great as those reached at six hours.

Experiment A-2 was performed to study the increase in water imbibition at six hours in response to different doses of estradiol-17β .   It was noticed that increases in weight due to moisture content were significantly ($P < 0.025$) greater at lower doses (50-1000 ng) than in higher ones (5000-50,000 ng) (Table 2). Significant ($P < 0.025$) increases in uterine dry weights were noticed with increased doses of estrogen.

In Experiment A-3 estrogens were administered in various doses and were compared to the effect of estradiol-17β standards given in a dose of 1.0 μg intraperitoneally.   The results (Fig. 3, Table 3) show the relative effects of the steroid estrogens were in the order of estriol, estradiol-17β , DES, estradiol-17α and estrone.

The effects of plant estrogens on uterine water imbibition were compared to estradiol-17β and testosterone (1.0 μg I.P.)

standards.   Coumestrol and genistein displayed significant

(P ⩽ 0.025) effects over controls in regard to their degree of

water imbibition, but at much larger dosages (Table 3).

Formononetin and biochanin-A did not show any significant (P > 0.025)

increase in water imbibition.

Discussion

Results of Experiments A-1, 2, 3 indicate that the degree

of water imbibition varies with various estrogens.   The ability

of an estrogen to produce this effect is relatively constant,

governed mainly by the time and dose levels characteristic of

the estrogen.   Among the earliest known effects of estrogens

are those associated with the uterine membrane and histamine

release (Spazani et al. 1958;   Szego et al. 1967).

Experiment 1-A demonstrated that a single dose of estrogen

results in accumulation of fluid in uterine tissues which will

be at its maximum at six hours.   The per cent moisture reached

a maximum at this particular time.   This result is in accord-

ance with the observation of Hinshaw (1959) who reported that

the gain in weight during the first six hours was mainly due

to the accumulation of fluid in the lumen of the uterus.   The

decrease in per cent moisture content of the tissue after six

hours is probably due to partial reabsorption of fluid prior to

its entering into the uterine growth phase at 12-14 hours as

suggested by Hinshaw (1959).

In Experiment A-2 the amount of an estrogen administered

over and above 50 ng did not produce any further gain in wet

uterine weight or per cent moisture.   According to Hinshaw (1959)

the decreases in uterine weight which occurs six hours following a single large dose of estradiol-17β was not due to self inhibitory effects because greater uterine weights occurred three and four hours after estrogen administration.

In Experiment A-3 it was evident that water imbibition was a common response of all estrogens though their ability to produce such an effect may be affected by the time following the administration of estrogens and the dose.   From the data obtained in this experiment, estradiol-17β and estriol possessed similar abilities in producing this effect.   To obtain a 32% increase in uterine weight, estriol was the most effective, followed by estradiol-17β , DES, estradiol-17α , estrone and testosterone. This is in agreement with the findings of Szego and Roberts (1953) who reported that in 24-27 day old rats, the relative effectiveness of estrogens in promoting uterine water imbibition six hours after administration was in the order of estriol, estradiol-17β and estrone.

Among the phytoestrogens coumestrol and genistein were capable of bringing about an estrogenic response.   When administered intraperitoneally eight to ten times the concentration of genistein was required to elicit a response similar to that of coumestrol.

The use of uterine weight increases for the assay of phytoestrogen activity has yielded conflicting results in the past. Results obtained in Experiment A-3 are not in complete agreement with reports of phytoestrogen uterotrophic activity (Bickoff et al. 1962, Nilsson, 1962; Braden et al. 1967). The variables which must be taken into account when evaluating

such differences are the rate of absorption, rate of metabolism and method of administration of the estrogenic compounds. This has led to conflicting reports in the literature rendering the proper assessment of uterine weight bioassays difficult. In view of this it was considered desirable to undertake more accurate experiments which would describe the early events following the administration of estrogenic compounds.

Conclusions

These series of experiments were designed to establish an optimal time course and dose effect of estrogens for use in subsequent experiments. The purpose of increased imbibition of water due to estrogen is not entirely known, however it appears to be important due to its relatively early and dynamic effect.

A six hour time course proved to be optimal time after a standard 1.0 μg dose of estradiol-17β was given intraperitoneally. During this time and at this dose, it was found that the tissue reached its maximum ability to respond.

In Experiment A-3 results obtained (Fig. 3, Table 3) showed various estrogens have the capacity to induce water imbibition; however dose levels and time after dose were characteristic of particular estrogens.

From these results it may be concluded that some estrogens are more effective in enhancing water imbibition by the uterus while others are more active in the promotion of uterine growth. Estradiol-17β, the most prominent estrogen produced endogenously, is the strongest natural estrogen for growth and

water uptake by the uterus even at low doses.

Phytoestrogens also demonstrated some degree of estrogenic activity in regard to water imbibition, although it was felt that the carrying vehicle and route of administration were important criteria in influencing their response.

WET UTERINE WEIGHT (MG)
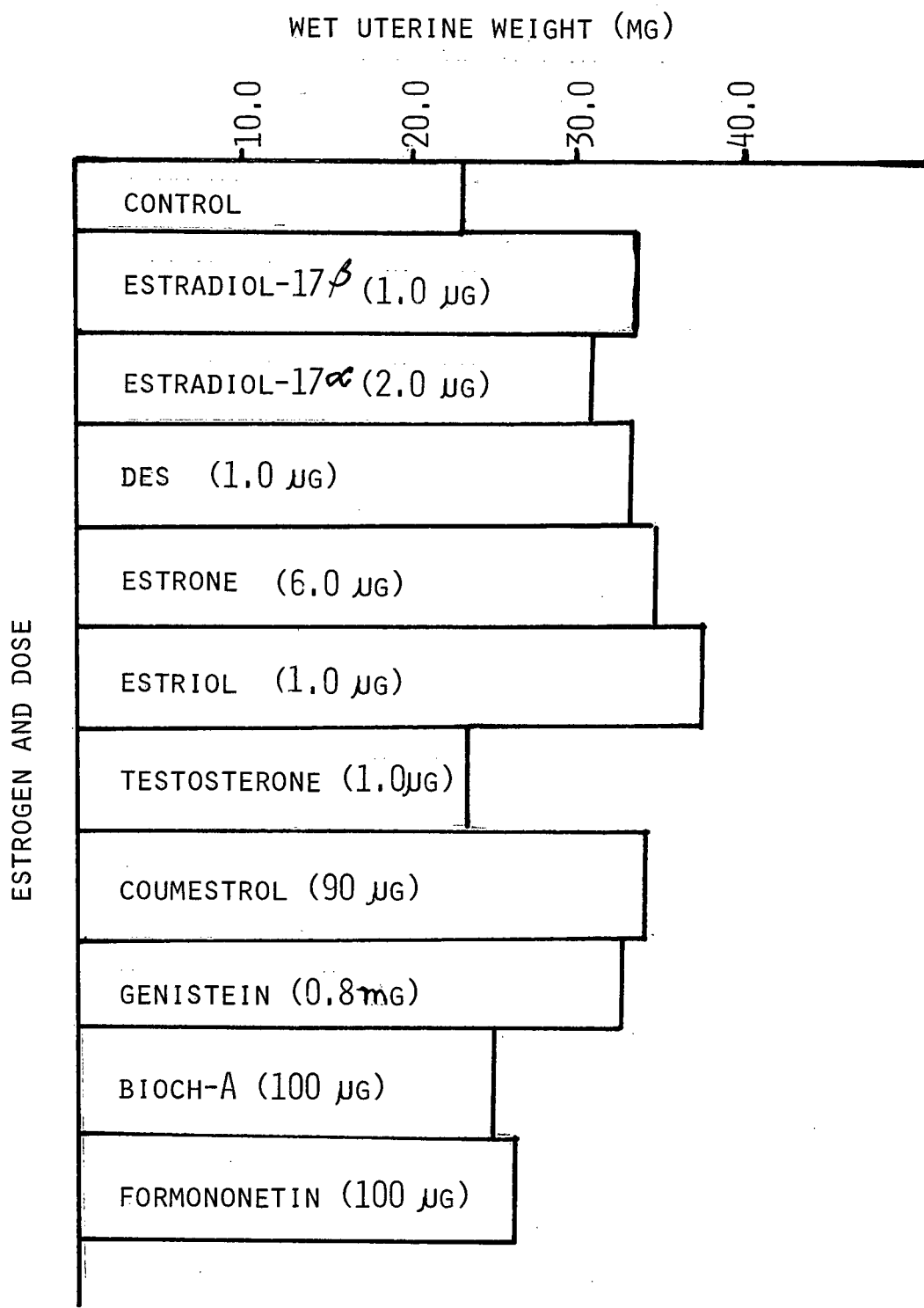
Fig. 3. Wet uterine weight six hours
         following estrogen administration
         (Expt. A-3)
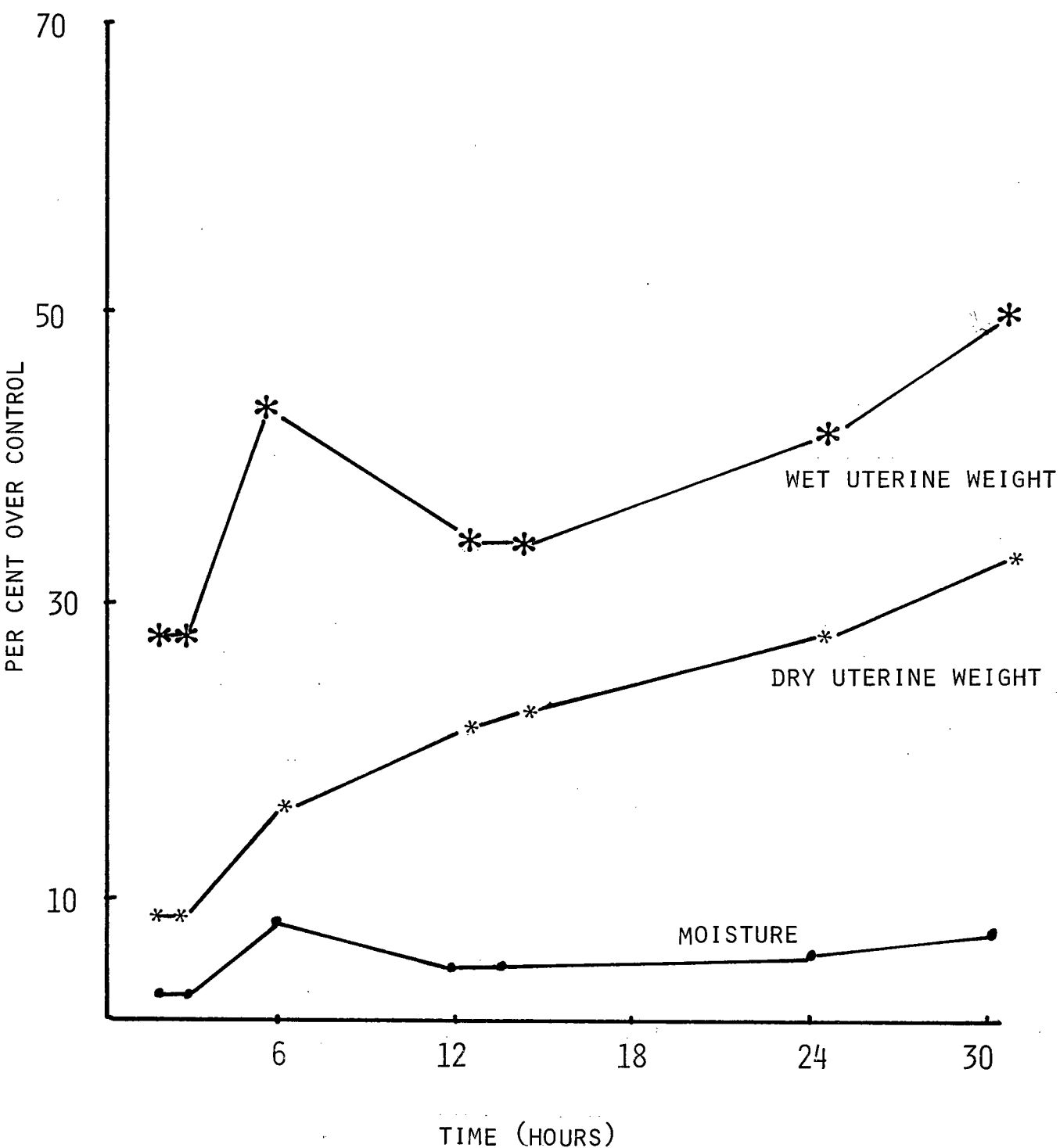
Fig. 4.   Effect of time on water imbibition in
          rat uterine tissues following adminis-
          tration of estradiol-17β (Expt. A-I)

Table 1 : Effect of time on water imbibition by rat uterine tissues following administration of estradiol-17β (Expt. A-I)

Animal Characteristics

Time (hours) following administration of estradiol-17β	Number of Animals	Body Weight (g)	Uterine Wet Weight (mg)	Uterine Dry Weight (mg)	Moisture Content of Uterus (%)
Control (0)	4	37.6 ± 0.60	19.0 ± 0.01 (a)	4.0 ± 0.01 (a)	79.4 (a)
2	4	37.6 ± 0.90	22.0 ± 0.01 (b)	4.2 ± 0.01 (b)	80.1 (a)
3	4	37.5 ± 1.20	22.0 ± 0.02 (b)	4.2 ± 0.01 (b)	81.3 (b)
6	4	37.5 ± 0.70	32.1 ± 0.02 (c)	4.6 ± 0.01 (c)	85.2 (d)
12	4	37.4 ± 0.60	28.1 ± 0.01 (c)	5.0 ± 0.01 (d)	82.1 (b)
14	4	36.7 ± 1.20	28.0 ± 0.02 (c)	5.1 ± 0.01 (d)	81.0 (b)
24	4	37.5 ± 1.70	32.0 ± 0.02 (d)	5.5 ± 0.01 (e)	82.2 (b)
30	4	37.2 ± 0.60	37.0 ± 0.02 (e)	5.9 ± 0.02 (f)	83.7 (c)

(a,b,c,d,e,f) Means with different subscripts are significantly different ($P < 0.025$)

Table 2: Effect of dose on water imbibition by rat uterus tissues
following administration of estradiol-17β (Expt. A-2)

Animal Characteristics

Dosage (ng) of estradiol-17β injected	Number of Animals	Body Weight (g)	Uterine Wet Weight (mg)	Uterine Dry Weight (mg)	Moisture Content of Uterus (%)
Control (O)	4	37.6 ± 1.6	19.0 ± 1.10 (a)	4.0 ± 0.10 (a)	79.0 (a)
50	4	38.6 ± 2.1	32.6 ± 0.80 (b)	4.6 ± 0.10 (b)	86.0 (b)
100	4	41.6 ± 3.6	33.6 ± 0.40 (c)	4.8 ± 0.10 (c)	85.7 (b)
500	4	44.1 ± 2.1	33.1 ± 0.20 (b)	4.9 ± 0.05 (c)	85.2 (b)
1000	4	42.9 ± 1.8	33.8 ± 0.20 (c)	5.0 ± 0.10 (c)	85.2 (b)
5000	4	43.7 ± 1.2	32.8 ± 0.90 (b)	5.2 ± 0.05 (c)	84.2 (c)
50000	4	42.8 ± 1.1	32.9 ± 1.20 (b)	5.1 ± 0.01 (c)	84.2 (c)

(a,b,c) Means with different superscripts are significantly different (P ≤ 0.025)

Table 3 : Effects of steroid and plant estrogens on water imbibition by
rat uterine tissue six hours following administration (Expt. A-3)

Animal Characteristics

Estrogens injected and dose	Number of Animals	Body Weight (g)	Uterine Wet Weight (mg)	Uterine Dry Weight (mg)	Moisture Content of Uterus (%)
Contol (0 ug)	4	37.2 ± 1.30	23.1 ± 3.10 (a)	4.6 ± 0.10 (a)	79.8 (a)
Estradiol-17β (1.0 ug)	4	42.9 ± 0.50	33.8 ± 1.0 (c)	5.0 ± 0.40 (b)	85.2 (b)
Estradiol-17α (2.0 ug)	4	43.1 ± 1.30	30.5 ± 0.8 (b)	4.8 ± 0.10 (c)	83.9 (c)
DES (1.0 ug)	4	46.9 ± 0.80	33.1 ± 0.6 (c)	4.9 ± 0.10 (c)	85.0 (b)
Estrone (6.0 ug)	4	48.9 ± 1.00	34.1 ± 1.6 (c)	5.0 ± 0.20 (b)	85.1 (b)
Estriol (1.0 ug)	4	47.2 ± 1.00	36.9 ± 1.2 (d)	4.8 ± 0.10 (c)	85.6 (b)
Testosterone (1.0 ug)	4	36.5 ± 1.30	23.6 ± 1.1 (a)	4.7 ± 0.10 (c)	80.1 (a)
Coumestrol (90.0 ug)	4	43.9 ± 1.50	33.9 ± 1.2 (c)	4.6 ± 0.30 (a)	86.3 (b)
Genistein (0.8 mg)	4	47.1 ± 1.90	32.7 ± 1.5 (c)	4.7 ± 0.60 (c)	85.6 (b)
Biochanin A (100 ug)	4	48.2 ± 1.30	24.7 ± 1.5 (a)	4.8 ± 0.10 (c)	80.5 (a)
Formononetin (100 ug)	4	47.5 ± 1.90	25.3 ± 1.3 (a)	4.3 ± 0.70 (a)	82.8 (c)

(a,b,c,d) Means with different subscripts are significantly different (P $\lessgtr$ 0.025)

Experiment B : Effect of Estrogen on Hyperemia of
Uterine Tissue

Introduction

In Experiment B, changes in the uterine vasculature were
studied in immature female rats by introducing India ink into
the vascular system six hours after the administration of
estradiol-17β , genistein or coumestrol.

Materials and Methods

Immature female rats (25-30 g) were employed.   They were
maintained and housed as described in Experiment A.   Estradiol-
17β, genistein and coumestrol (1.0 µg, 80.0 mg and 90.0 µg
respectively) were administered intraperitoneally.   Animals
were prepared for perfusion by opening the thoracic wall and
exposing the thoracic aorta.   The esophagus was severed and all
connective tissue was removed.   Polyethylene tubing (0.47" x
0.67") was threaded into a Pasteur pipette which in turn was
fitted to an adaptor (Leur-Lok Tuohy-σ).   The other end of the
adaptor was attached to a 26 G needle which was inserted into
the artery.   A Polystaltic perfusion pump (Buchler Instr.)
was employed to infuse warm 0.9% saline (37°C, pH 7.2) at a
rate of 3.2 ml min^{-1} through the polyethylene tubing.   The
duration of the perfusion was determined by the appearance of
the viscera and usually lasted 3.5 minutes depending on the
size of the artery.   Caution was taken not to manipulate the
uterus too much during the perfusion.   Warm, undiluted India
ink (10-20 cm^3) was introduced into the tubing by means of a

hypodermic syringe.

Following the perfusion with India ink, the reproductive
tract was removed with a minimal amount of handling and was
rinsed in warm 0.9% normal saline. The uterine tissues were
excised to form open sigments close to the extra uterine plexus
and placed on microscope slides for an examination of the anti-
mesometrial region. Uteri were fixed with 10% formalin for
24 hours and then dehydrated successively with 50, 80, and 2 X
95% alcohol. Following fixation and dehydration, the tissues
were cleared with xylene for 5 minutes. The prepared slides
were observed under 100X power using a phase contrast micro-
scope.

Results

From Fig. 5 and 6 it may be noted that estradiol-17β
possessed the greatest ability to induce hyperemia in the
immature female rat uterus. Genistein and coumestrol also
enhanced uterine vascular permeability when compared to control
tissues. The degree of hyperemia induced by genistein and
coumestrol did not appear to be as distinct as estradiol-17β,
even though relatively larger doses were administered.

Discussion and Conclusions

The use of this technique has definite drawbacks in study-
ing estrogenic potency of various compounds. The difficulties
are associated with the manipulation of uterine tissues during
preparation and the consequent damage. To minimize these
disadvantages every effort was made to treat the specimens

the same way including the injection of saline at constant rate,
temperature and the preparation of sections with the same fixing
and clearing reagents.   Capillaries in any given region were
found to vary in diameter though almost all specimens were
filled with India ink.   Some appeared as exceedingly fine lines
under a magnification of 100X.

By means of visual comparison of various treatments with
controls, it was noted that the blood vessels of the uterus
reflect a gradient of response to estrogen concentration.
Capillaries around the uterine horns become an easily recogniz-
able feature of vascular architecture of the uterus as seen in
the injected specimens.   Genistein and coumestrol (0.8 mg and
90. ug, respectively) had some effect on the vascular permea-
bility of the uterine cell.   Perel and Lidner (1970) reported
similar responses occurring with hyperemia after the injection
of coumestrol and genistein (80 μg, 0.62 mg respectively).
These phytoestrogens did not provide that same magnitude of
response as did estradiol-17β (1.0 μg).
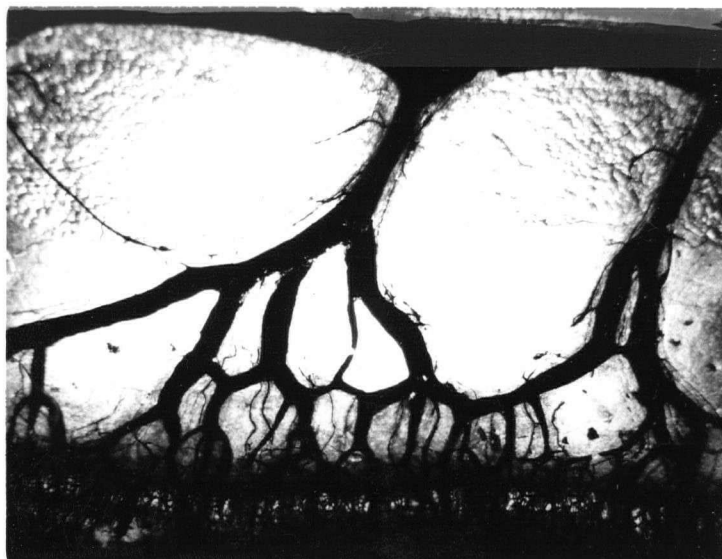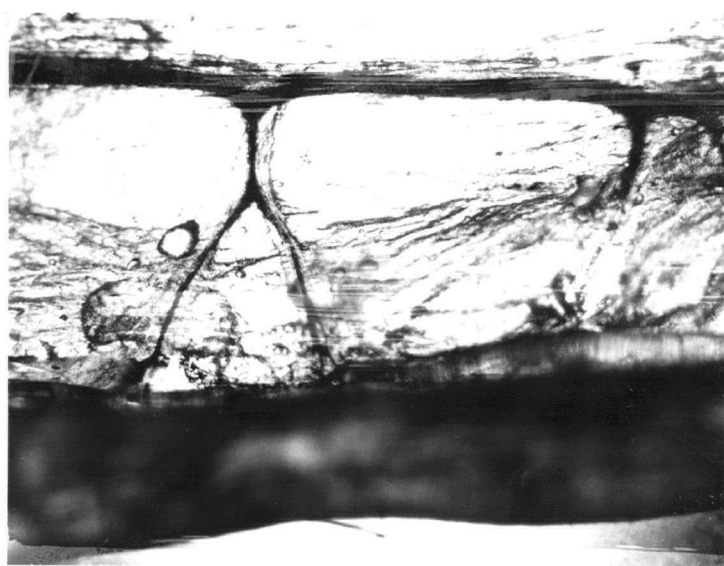
ESTRADIOL-17β

CONTROL

Fig. 5. The distribution of India ink in the uterine vasculature of control and estradiol-17β treated rats (Expt. B) x 100.
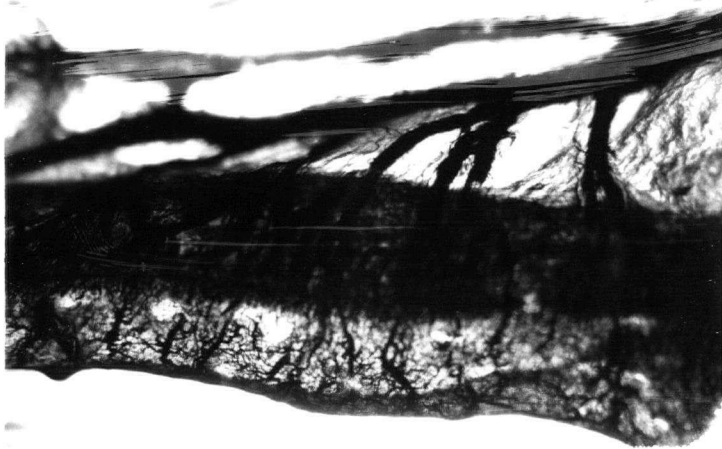
COUMESTROL
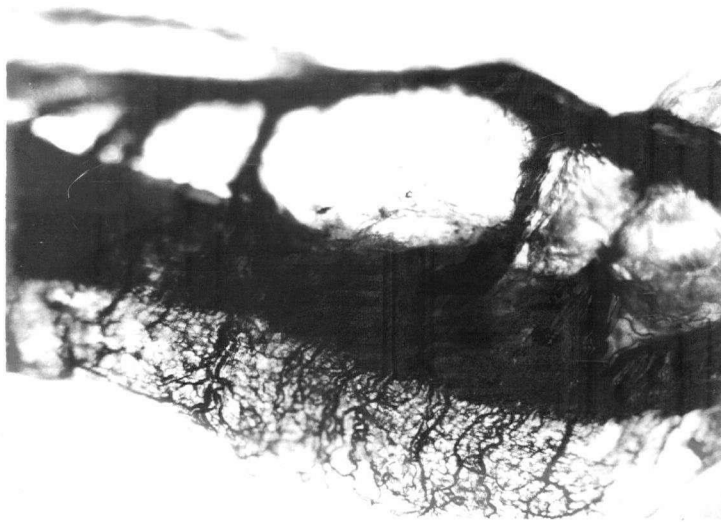
GENISTEIN

Fig. 6. The distribution of India ink in the
uterine vasculature of coumestrol and
genistein treated rats (Expt. B) x 100

Experiment C : Effects of Estradiol-17β on RNA and
DNA Synthesis in Immature Uterine Tissues.

Introduction

The effect of estrogenic hormones on RNA and DNA synthesis
in the mammalian uterus has been reviewed in detail by Means and
O'Malley (1972). In order to study the earliest detectable
response occurring in the uterine cell after the administration
of estrogens, attempts have been made to study the effects of
antibiotic inhibitors of RNA and DNA synthesis. As a result
of these studies a chronological sequence of biochemical events
occurring in vivo after the administration of estrogens has
been established (Gorski and Katzellenbogen, 1975).

From studies based on the incorporation of various labelled
precursors, the rate of RNA and DNA synthesis has been deter-
mined by many workers. Gorski and Nicolette (1963) reported
an increased rate in RNA synthesis occurring in the nucleus one
hour after estradiol-17β administration. Additional information
regarding the early action of estradiol-17β on RNA synthesis was
obtained by Noteboom and Gorski (1963(b)) who reported an increased
activity in the RNA polymerase enzyme as early as one to four
hours after estrogen administration. Although RNA polymerase
activity is significantly elevated one hour after the adminis-
tration of estrogen an increase in net uterine RNA content is
not achieved until 8-12 hours (Billing et al.1969(a)). The
newly synthesized RNA appears to represent all types of RNA,
but is primarily ribosomal RNA and may differ in composition
from that produced in the absence of estrogens as indicated by

DNA-RNA hybridization studies (Hahn et al. 1968). A conversion of ribosomes to polysomes occurs due to the entry of new messenger RNA into the cytoplasm.(Means and O'Malley, 1972). These polyribosomes synthesize a quantitatively different population of peptides in vitro which should reflect the earlier alterations in messenger RNA production. This sequence of events indicates that estrogen exerts its primary effect in the nucleus to promote selective gene activation and subsequent synthesis of new protein molecules on cytoplasmic polysomes.

Estradiol-17β was also reported to produce an increase in thymidine incorporation into DNA, 16 hours after estradiol-17β injection (Gorski and Raker, 1974). Increases in the rate of DNA and histone synthesis begin about 18 hours after estrogen treatment (Kaye et al. 1972). These events occur prior to cell division which begins in the uterus at approximately 24 hours after estradiol-17β administration (Meuller et al. 1958).

In view of the increases in uterine dry weights observed after the administration of estrogen in Experiment A, studies were initiated to determine the effects of estradiol-17β on overall nucleic acid metabolism in the uterine cell. The procedure for RNA and DNA extraction was standardized. This was followed by quantitative determination of the effects of estradiol-17β on RNA and DNA synthesis by the uterine tissues.

Materials and Methods

Animals

Immature female rats (40-50 g) were used for this study. They were housed and maintained as previously described under

Experiment A.

Materials

Estradiol-17β was obtained from Signa Chemicals. RNA (yeast, Sigma Chemicals) and s-RNA (A grade, Calbiochem) donated by Dr. B.C. Sung, Dept. of Neurological Sciences, UBC were used as RNA standards. Calf thymus DNA obtained from Mann Research Laboratories was used as DNA standards. Standard curves were obtained using s-RNA and calf thymus DNA in KOH-Trichloroacetic acid (TCA) and $HClO_4$ respectively.

Methods

1. Standardization of RNA and DNA Extraction Procedures

Initial experiments were performed to standardize the nucleic acid extraction procedures. This was accomplished by extracting RNA and DNA from increasing weights of uterine tissue as described below. Animals were sacrificed by exposing them to carbon dioxide in a sealed jar and uterine tissues were pooled in ice cold 0.9% NaCl. Wet tissue slices were randomly selected, blotted and weighed in quantities of 40.0, 50.0 and 100.0 mg respectively. Nucleic acid content was extracted by the methods of Schmidt-Thannhauser (1945) and Ceriotti (1955) with minor modifications as summarized in Appendix, Fig. A.

Three whole uteri were pooled in 4.0 ml of ice cold deionized water and homogenized using a tissue homogenizer (ASCO Industries) for 5 minutes. To the homogenate 7.0 ml of ice cold 10% TCA were added. The tubes were allowed to stand for 10 minutes on ice, centrifuged, and the precipitate resuspended and washed with 10 ml of ice cold 95% ethanol. Following centrifugation

the resulting precipitate was suspended in 2.0 ml of 1N KOH for
16 hours at 37°C.   After addition of 0.4 ml of 6N HCl, protein
and DNA were precipitated with cold 5% TCA.   RNA in the super-
natant fraction was estimated by reading the absorbance at
260 nm in an Unicam 800 spectrophotometer against an appropriate
KOH-TCA blank.   The precipitate of DNA and protein was suspended
in 2.5 ml of 10% $HClO_4$ and heated at 70-80°C for 25 minutes.
This material was centrifuged and the DNA in the supernatant
was measured at 260 nm using an appropriate $HClO_4$ blank.
Standard curves with yeast RNA or pure s-RNA and calf thymus
DNA were used to determine the nucleic acid content of the
extracts.   Analysis of data was done by Student's t-test.

2.   Time Course Effects of Estradiol-17β on Nucleic Acid
     Synthesis by the uterine tissue.

After standardizing the extraction procedure of RNA and
DNA, a time course effect of estradiol-17β on RNA and
DNA synthesis by the uterine tissue was studied.   Rats were
divided into groups of three animals and each animal was given
an intraperitoneal injection of 5 μg of estradiol-17β .   Control
animals received injections of equal volumes of 0.9% NaCl con-
taining 1% ethanol.   They were sacrificed after 0,6,12,24,48
and 72 hours by placing them in a jar of carbon dioxide.   RNA
and DNA were extracted from the uterine tissues to determine
the time when maximum effect of estrogen on nucleic acid syn-
thesis was manifested.

Results

1.   Standardization of RNA and DNA Extraction Procedures

Due to the impurities in the yeast RNA it was found neces-

sary to standardize this preparation against known solutions of purified s-RNA. The molar absorption coefficient of s-RNA was determined by dissolving it in 0.5 mg/ml of 0.1 M acetate buffer, pH 5.0. s-RNA in complete solution had a molar absorption coefficient of 18 $O.D._{260}$ x mg s-RNA^{-1}. After obtaining this value, it was possible to determine the concentration of the corresponding yeast RNA filtrate. Standard curves of RNA and DNA are given in Fig. 7.

The approximate content of RNA and DNA per uterus of immature rats was 112.0 ± 10 µg and 237 ± 11.0 µg respectively (Table 5). The RNA concentrations in 40.0, 50.0 and 100.0 mg of uterine tissues were 176.1 ± 2.1, 223.2 ± 1.9, 540.7 ± 2.5 µg respectively; corresponding values for DNA were 232 ± 11, 515 ± 9, 1023 ± 152 µg respectively (Fig. 8, Table 4).

2. Time Course Effect of Estradiol-17β on Nucleic Acid Synthesis by the uterine tissue

When estradiol-17β was administered to immature rats possessing equivalent body weights net RNA synthesis was seen after 12 hours (Table 5). Maximal RNA synthesis in the immature rat uterus was noticed 48 hours following a single injection of estradiol-17β. The concentration of RNA in the uterine tissue declined slightly between 48 and 72 hours, but it never reached its control level (Fig. 9).

The effect of estradiol-17β on the DNA content of the uterus showed a delayed response which lasted longer. Maximum increases in net uterine DNA did not occur until 48 hours after estrogen administration. Slight decreases in uterine DNA concentrations were noticed after 48 hours. A steady decrease

in the RNA/DNA rations was noted between 24 and 72 hours, but
the decrease in RNA/DNA ratios did not return to control
levels. All data was statistically analyzed by student's t-test.

Discussion

The levels of nucleic acid in the uterus of immature rats
are in agreement with the results obtained by Gorski and
Katzellenbogen (1975). They reported that concentrations of
RNA when expressed in terms of cellular RNA were comparatively
lower in the uterus of immature rats than in other tissues.
The RNA/DNA ratios in the immature rat uterus was 0.47 which
corresponds to the ratio of 0.4 reported by Gorski and
Katzellenbogen (1975). This is in contrast to ratios of 2.0
and 5.0 in liver and E-coli respectively.

Reports by Kaye et al. (1972) have revealed that the in-
corporation of (Me^{3}H) thymidine into DNA is dependent on age
and dose. In rats less than 15 days old, single injections of
estradiol-17β did not result in an increase in the uterine
weight, RNA content, or rate of DNA synthesis. Twenty day
old rats, on the other hand, showed significant increases in
the rate of RNA and DNA synthesis. Amounts of estradiol-17β
as low as 50 pg to 20 day old rats, weighing 33 grams were
also reported to increase the rate of DNA synthesis. With
this work in mind immature female rats were employed in this
study and a relatively large dose of estradiol-17β (5 μg) was
administered to elicit a definite response.

Results from this experiment indicate that the effects of
estradiol-17β on tissue growth are mediated by changes in

uterine RNA and DNA.   Hamilton et al. (1968) and Billing et al.
(1969(b)) reported that though no increase in the uterine RNA
content as measured by chemical methods was noticed over the
first seven hours, a significant increase was observed 12 hours
after administration of estradiol-17β.   Data from this experi-
ment is in accordance with these findings.   The increase in net
synthesis in the uterine tissue following administration of
estradiol-17β has been attributed to increased synthesis of
the enzyme RNA polymerase.

However, on the basis of the time lag noticed during the
early stages of estrogen administration and the changes in pre-
cursor pool sizes, Gorski and Katzellenbogen (1975) have
expressed doubts that RNA synthesis was greatly affected at
these early times.   They have suggested that the mode of action
of estrogens is more general in nature and requires to be sub-
stantiated by more experimental data.   They ascribed the
increased activity of various nuclear functions in response to
estrogen was dependent on the programming of the cell, i.e. the
production of specific mRNA and rRNA species.

Increases in the DNA content of the uterine tissue were
noticed to occur at a slower rate than the RNA content.   These
results agree with those of Meuller et al. (1958); Billing
et al. (1969(b)); Kaye et al. (1972), who reported that though
significant cellular hypertrophy occurred after six hours,
increases in cell number could be noticed only after 24 hours
following estrogen administration.   Similarly, Epifanova (1966)
using tritiated thymidine and autoradiographic techniques,
found increases in mitotic indexes of mouse uterine epithelium

42-24 hours following estrogen administration resulting in 1.5 fold shortening of the cell generation time.   This reduction of cell generation time occurred at the expense of the G_1 and S_1 stages which refer to the time of preparation for DNA synthesis and DNA replication respectively.

The time gap between the uptake of water and the maximum activity of the polysomes, particularly rRNA, the protein synthesizing organelles of the cells has been attributed by Gorski et al. (1975) to be the time required for the production of mRNA needed for the synthesis of induced protein (I.P.).

Conclusions

Experiment C was designed to standardize the method of RNA and DNA extraction from uterine tissues and determine the time course of the effect of estradiol-17β on the synthesis of RNA and DNA.   RNA and DNA concentrations in the immature female rat uterus were 112 ± 10 and 237 ± 21 μg respectively.

From the results of Experiments A, B and C it may be concluded that following estrogen administration to immature rats there is a distinct pattern of increased water retention at six hours, increased RNA synthesis at 12 hours and accelerated DNA metabolism and cellular proliferation at 24 hours.   RNA/DNA ratios which indicate cellular proliferation increased gradually after 12 hours.   Maximum cellular proliferation was noticed 24 hours after administration of estradiol-17β, indicating a levelling off of RNA production and an increase in DNA metabolism.
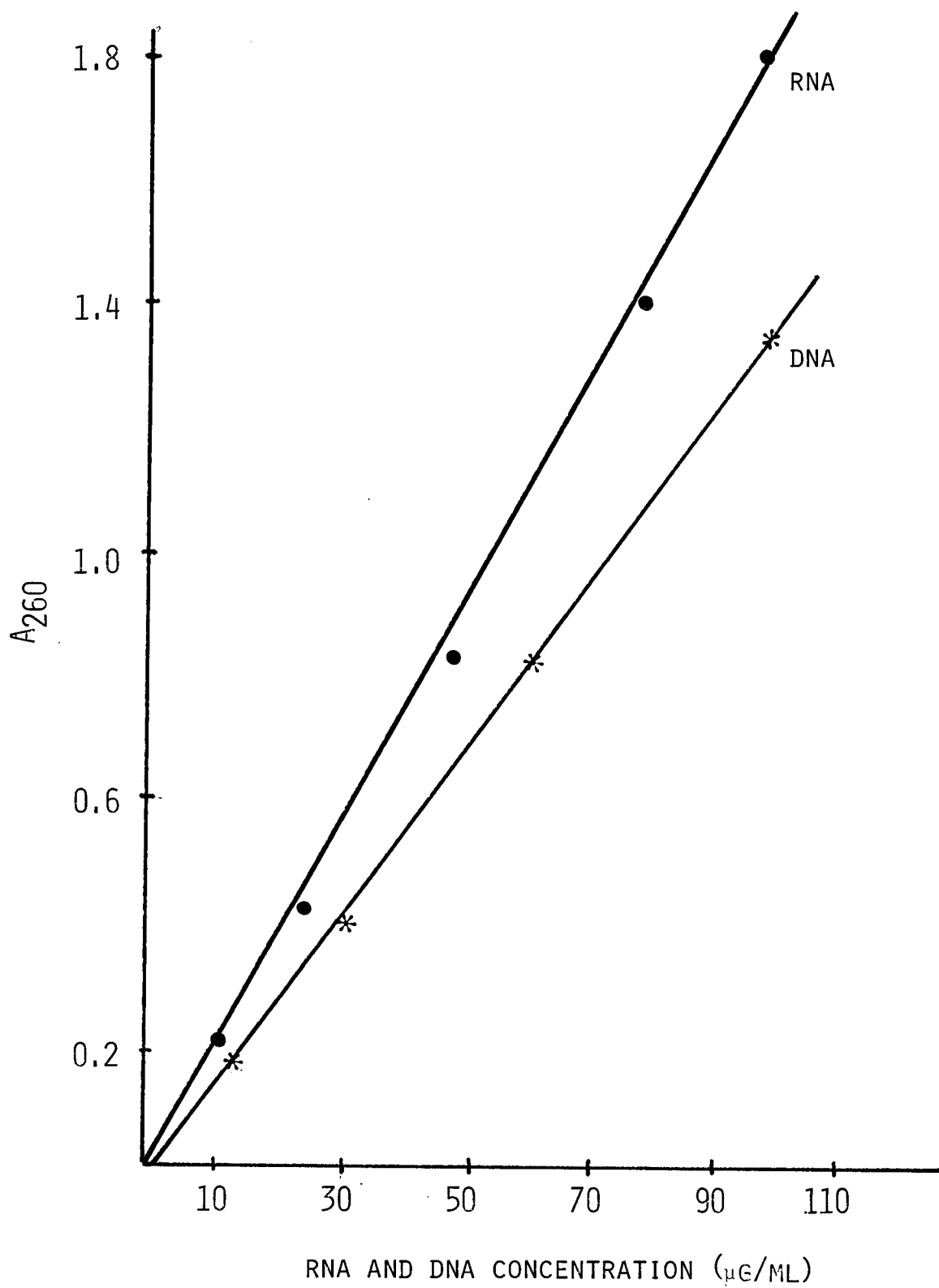
Fig. 7. Standard curves for RNA and DNA (Expt. C)
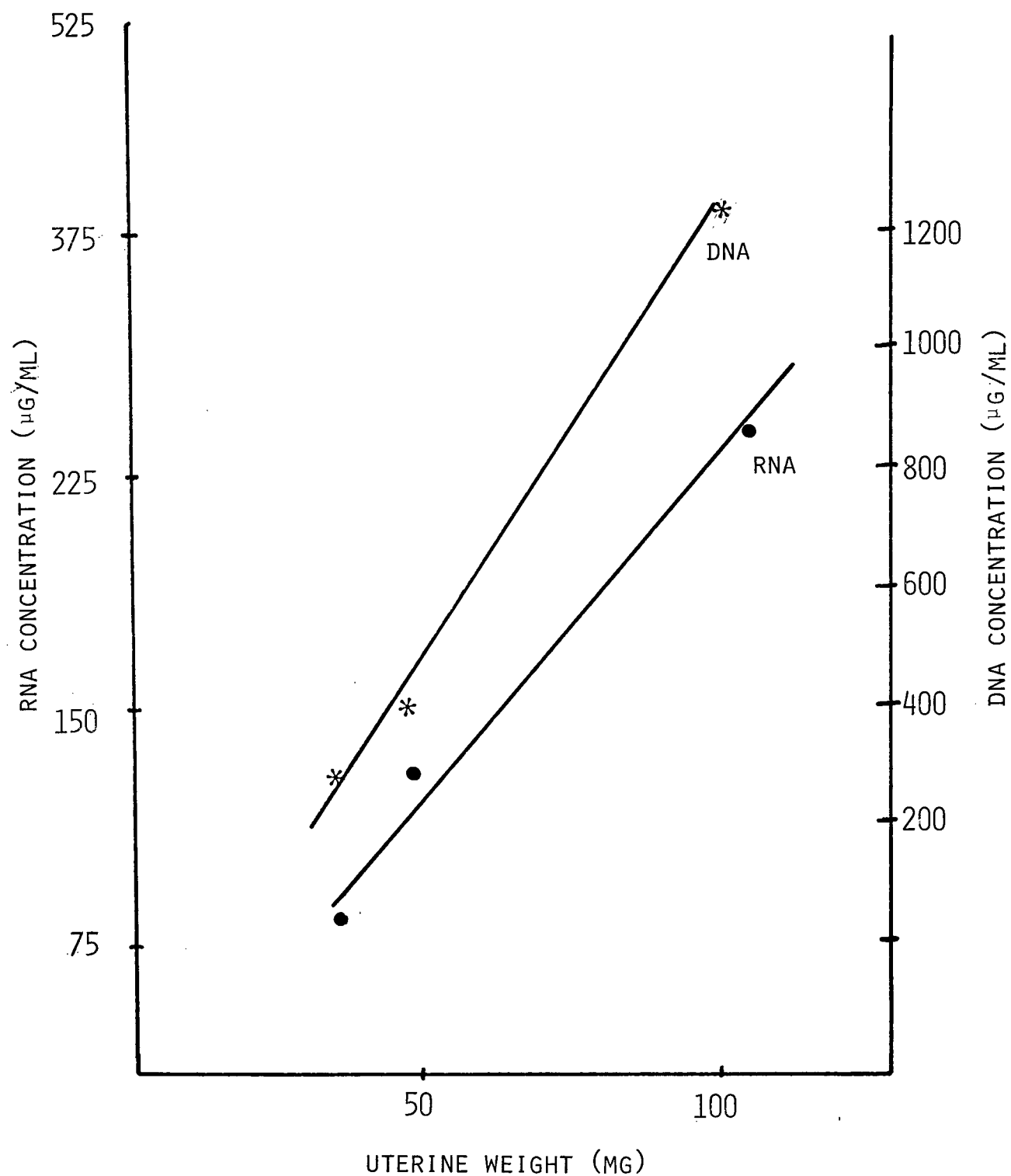
Fig. 8. RNA and DNA concentration in
homogenates of different immature
rat uterine weights (Expt. C)
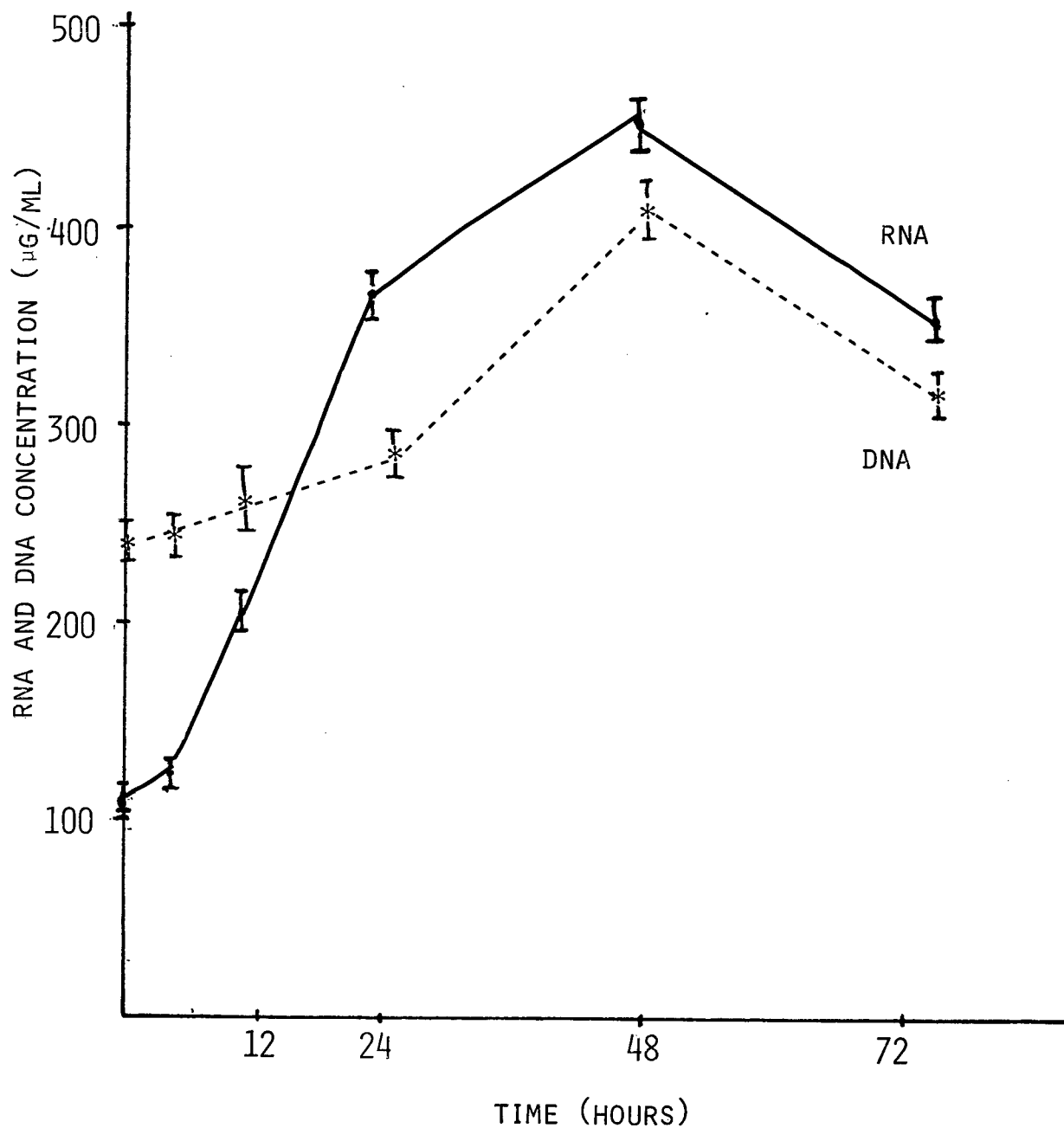
Fig. 9. Effect of time on RNA and DNA synthesis
         in rat uterine tissue following
         estrogen administration (Expt. C)

Table 4 : RNA and DNA concentrations in homogenates of
different immature rat uterine weights (Expt. C).

Wet weight of Uterus (mg)	RNA concentrations (ug)	DNA concentrations (ug)	RNA/DNA
40.0	176.1 ± 2.1	232.0 ± 11.0	0.75
50.0	223.3 ± 1.9	515.6 ± 9.03	0.43
100.0	540.7 ± 2.5	1023.6 ± 1.52	0.52

Table 5 : Effect of time on RNA and DNA synthesis
by rat uterine tissue (Expt. C)

Animal Charcteristics

Time (hours) following administration of estradiol-17β	Number of Animals	Body Weight (g)	Uterine Wet Weight (mg)	RNA concentration (ug/uterus)	DNA concentration (ug/uterus)	RNA/DNA
Control (0)	9	43 ± 2.0	25.7 ± 3.0 (a)	112 ± 10 (a)	237 ± 21 (a)	0.47
6	9	45 ± 3.0	31.1 ± 5.0 (b)	117 ± 12 (a)	232 ± 20 (a)	0.50
12	9	44 ± 2.5	44.5 ± 2.5 (c)	200 ± 19 (b)	253 ± 29 (a)	0.79
24	9	43 ± 3.0	66.1 ± 3.9 (d)	357 ± 37 (c)	271 ± 25 (a)	1.30
48	9	46 ± 1.5	75.3 ± 4.8 (e)	452 ± 29 (d)	402 ± 31 (c)	1.12
72	9	44 ± 1.8	47.2 ± 6.9 (c)	302 ± 19 (c)	309 ± 28 (b)	0.97

(a,b,c,d,e) Means with different subscripts are significantly different (P ≤ 0.025)

Experiment D : Effects of Estrogens and Phytoestrogens on the
             Incorporation of ^{3}H Uridine into RNA by the
             Uterus - Six Hour In Vivo Pulsing.

Introduction

A net synthesis of RNA by the uterus in response to estrogen
administration has been demonstrated in the previous experiment.
Other workers have also shown the incorporation of various pre-
cursors into different types of RNA (Hamilton et al. 1968;
Billing et al.1969(B)). However, isotopic tracer studies designed
to monitor the rate of uterine RNA synthesis have yielded con-
flicting results.   Billing et al. (1969(b)) allowed labelled
adenosine to equilibrate with the uterine adenosine nucleotide
pool prior to and for a limited time after estradiol-17β
administration, and noticed that incorporation of labelled
adenosine into uterine RNA increased only slightly during the
initial phase of the response and was not substantial until 5
hours.   On the contrary, Hamilton et al. (1968) have reported
that during a 10 minute pulse in vivo, 5-^{3}H uridine incorporation
into uterine nuclear RNA was maximal 20 minutes after estradiol-17β
administration.

The extent of isotope incorporation into RNA is governed
not only by the rate of RNA synthesis but also by the specific
activity associated with its nucleotide precursors at the time
of incorporation.   In regard to the uterus, estrogen treatment
has been shown to influence the specific activities of various
RNA precursors by increasing the vascularity (Szego, 1967), or
the permeability of precursors (Billing et al. 1969(b)) and
pool sizes (Mueller et al. 1958).   Gorski et al. (1975) have

drawn attention to the difficulties in drawing conclusions from experiments involving precursor incorporation.   Munns and Katzman (1971) employing an in vitro system of precursor incorporation preceded by an in vivo administration of estradiol-17β reported that L-[methyl-^{14}C] methionine was a useful precursor of methylated RNA's (i.e. tRNA and rRNA).   The apparent advantage of this label was its independence on fluctuating nucleotide precursor pool sizes.

Similar differences have not been encountered with thymidine labelled DNA.   Numerous workers have employed tritiated thymidine in both in vivo and in vitro assays (Kaye et al. 1972;   Carter et al. 1975).   Kaye et al. (1972) favored the use of in vitro systems due to greater incorporation and better recovery rates of ^{3}H-thymidine.

Recently Katzellenbogen and Gorski (1975) concluded from an extensive review of the methods employed by various workers that no clear cut evidence of early increases in the incorporation of precursors into RNA due to estradiol-17β treatment existed.

The purpose of the following experiments was to study in vitro the degree of tritiated uridine incorporation into RNA by the uterus following estrogen or phytoestrogen administration.

In view of data obtained in Experiments A, B and C regarding the estrogenic activity of various estrogens, Experiment D was initiated to study the comparative effects of steroid estrogens and phytoestrogens in inducing the incorporation of the isotopic nucleoside, uridine.

<center>Materials and Methods</center>

Animals

Immature female rats (40-50 g) were mostly used in this study.   In addition to these animals two groups of rats (60 g) were also used in a few experiments.   They were housed and maintained as described in Experiment A.

Materials

$[5,6-^3H]$ uridine (specific activity, 44.5 Ci/m mole was obtained from Amersham Searle.   TC medium 199, chemically defined biological medium was obtained from Difco Laboratories. It was supplemented with 1.0 mM glutamine (Difco) and 2% bovine albumin (Sigma).   Yeast RNA and calf thymus DNA were obtained from Sigma chemicals and Mann Research Lab. respectively.

Methods

<center>Administration of Estrogens <u>in vivo</u></center>

Immature female rats were given single injections of the steroid estrogens and phytoestrogens in doses mentioned in Experiment A.   Control animals received similar volumes of the test vehicles, alcohol-saline or propylene glycol.   The incorporation of ^3H-uridine by rat uteri was determined as described below.

<center>Incorporation of $[5,6-^3H]$ uridine by the uterus
<u>in vitro</u></center>

Animals were sacrificed six hours after the administration of estrogens by placing them in a sealed jar containing carbon dioxide.   The uterine tissues were removed and transferred to ice cold 0.9% NaCl.   Tissues were stripped of adhering fat and connective tissue and placed in a prewarmed (37°C) five ml

stoppered bottle containing 1.0 ml of TCM 199 and approximately 1.6 μCi of ^{3}H-uridine. Uterine tissues were incubated under an atmosphere of $95\%O_2-5\%CO_2$ for 1.0 hour in a water bath (37°C) at a shaking speed of one stroke per second.

After the incubation the media was removed and the uteri placed on dry ice to terminate the reaction. Whole uterine tissues were rinsed three times successively with ice cold distilled water to remove the extracellular radioactivity. This fraction representing tissue wash was pooled and an aliquot taken for determining the radioactivity. Duplicate aliquots were added to 10 ml of PCS scintillation fluid (Amersham Searle) and counted on an Isocap 300 liquid scintillation counter (Nuclear Chicago). The efficiency of the counting was 38-41% as determined by the channel ratic method. Tissues were blotted dry, weighed and stored at 20°C until RNA and DNA extraction procedures were carried out.

Determination of Specific Activity of RNA in Uterine Tissues

Three uteri from each group were pooled and tissue homogenates were prepared as described in Experiment C (Appendix Fig. A). The homogenates were first washed with 7.0 ml of cold 10% TCA. The resulting precipitate was resuspended in 7.0 ml of cold 5% TCA and the two washes pooled. Duplicate aliquots of the TCA acid soluble fraction were added to 10 ml of PCS and counted in the liquid scintillation counter. The efficiency of counting under these conditions was 33.5%. The radioactivity in this fraction represents the extent of precursor uptake which has not yet been incorporated into RNA. Anaylsis of data was done by Student's t-test.

Following the collection of the acid soluble fraction, the precipitate was resuspended in 10 ml of ice cold 95% ethanol to remove the greater portion of the fat soluble fraction. This was allowed to stand for 10 minutes then centrifuged at 3500 RPM on a desk centrifuge for five minutes. The resulting supernatant showed no radioactivity beyond background levels.

Following alkaline hydrolysis, the solutions were neutralized with HCl and protein and DNA were precipitated with cold TCA. Under these conditions RNA will be present in the form of mononucleotides in the supernatant which will be referred to as the hydrolyzed RNA fraction. RNA washes were pooled and duplicate 50 ul aliquots were taken for liquid scintillation counting. The efficiency of tritium counting under these conditions was 28-33%. The remaining portion of the fraction was used for quantitative determination by spectrophotometry. Specific activity of the RNA fractions was expressed as DPM per ug RNA.

DNA extracted in hot perchloric acid was also quantitated on the spectrophotometer and aliquots were taken to determine isotope radioactivity. No radioactivity significantly above background was recorded in this fraction.

The efficiency of the overall extraction procedure was checked by adding the radioactivity in individual fractions and expressing it as a per cent of total radioactivity administered.

Results

The extraction procedure used in Experiment D accounted for 85-90% recovery of the isotopically labelled uridine nucleoside.

In general the mean radioactivity in the hydrolyzed RNA
fractions was less in the estrogen treated animals than in the
controls (Table 6). The incorporation of tritiated uridine in
the RNA fraction obtained from the whole uterus was lower in the
estrogen treated rats (Table 7). The only exception was in the
case of estrone treated animals where the radioactivity in the
hydrolyzed RNA fraction was slightly higher than in the control.
The uptake of the radioactivity by the uteri of testosterone
treated rats resembled that of control rats.

When the radioactivity in the RNA fraction was expressed
per unit of uterine wet weight the uteri of estradiol-17β
treated rats had significantly ($P < 0.05$) lower incorporation of
the label than those of control rats (Table 7). In the rats
treated with DES, estriol and estradiol-17α the incorporation
of the label appeared to be lower than in the control and testos-
terone treated ones. The specific activity of RNA in the uteri
of rats treated with estradiol-17β was significantly ($P < 0.05$)
lower than in the control (Table 7). Similar trends in the
specific activity of RNA were observed in DES, estriol and
estradiol-17α treated rats. Estrone treated animals appeared
to show no difference from the controls.

In phytoestrogen treated groups (Table 7), coumestrol and
genistein treated rats incorporated $[5,6^{-3}H]$ uridine into the
hydrolyzed RNA fraction per uterus at levels higher than those
treated with estradiol-17β, estriol and DES, and at rates
similar to control and testosterone treated groups. Under the
influence of the weaker phytoestrogens, formononetin and
biochanin-A, the incorporation rate of $[5,6^{-3}H]$ uridine into

the hydrolyzed RNA fraction was similar to that observed with

the steroid estrogens.   When the radioactivity was expressed

in terms of unit uterine weight, coumestrol, genistein, biochanin-A

and formononetin induced a lesser incorporation of uridine than

testosterone and control treated groups.

The specific activity of the hydrolyzed RNA fraction in

phytoestrogen treated rats was generally lower than in the con-

trol and testosterone treated animals. Particularly the low

specific activity of RNA in genistein treated rats as compared

to the control was statistically significant ($P < 0.05$). Genistein

and coumestrol resembled estriol in their ability to stimulate

incorporation of the isotopic precursor.   Biochanin A and

formononetin were also lower than the control in their ability

to promote uptake of the label and resembled the trends observed

with DES and estradiol-17β in this respect.

Discussion

Experiment D was undertaken to compare the ability of dif-

ferent estrogens in enhancing the incorporation of an isotopic

precursor by the rat uterine tissue.   Having established in

Experiment A that maximum water imbibition occurred six hours

after estrogen administration this time interval was chosen in

this experiment to allow for maximal water imbibition and there-

fore greater permeability of the cell membrane to an exogenous

supply of the labelled nucleoside.

Though one would have expected an increase in the specific

activity of RNA after the administration of estrogens it was

surprising to find that both the steroid and phytoestrogens did

in fact reduce the specific activity after six hours.   Before

being incorporated into the uterine RNA, uridine must pass

through an uridine nucleotide pool, the size of which is ex-

panded by the action of estrogens as suggested by Mueller et al. (1958)

The reduction in the specific activity of RNA noticed six hours

after administration of estrogens may be ascribed to the

increased size of the nucleotide pool sufficient to reduce the

specific activity.   This is particularly noticeable in the case

of estradiol-17β and less so in DES and estriol.   The absence of

change in the specific activity of RNA in estrone treated rats

over the control suggests that it is a comparatively weak

estrogen and owes its biological activity solely to its con-

version to estradiol-17β (Terenius, 1969).   The higher specific

activity of RNA in rats treated with estradiol-17α than

estradiol-17β indicates the importance of configuration on hor-

monal effects.

Miller and Emmens (1967) observed an increase in the in-

corporation of labelled uridine in the mouse following

estradiol-17β, estriol and estrone treatment.   Munns and

Katzman (1971) found that exposure to estrogen in vivo even for

short periods enhanced the uptake of tritiated uridine by the

uterus in vitro.   If the duration between the administration

of estrogens and labelled uridine was too long a decrease in

the uptake of radioactivity by the uterine tissues was noticed.

To determine whether or not this was true, short in vivo

pulsing studies were attempted later on in Experiment E.

Comparable results on the effect of phytoestrogens on

uridine uptake by the rat uterus are not available in the

literature.   As already indicated the solubility of phyto-
estrogens in various solvents should be considered while assessing
their estrogenic potency.   In this study difficulty was exper-
ienced in dissolving coumestrol.

The low specific activity of RNA in the uteri of rats
treated with the phytoestrogens, biochanin A and formononetin is
of interest in the light of their relatively poor ability to
increase water imbibition as shown in Experiment A.   In par-
ticular the specific activity of RNA in rats treated with
biochanin A and formononetin was even lower than in those
treated with estradiol-17β and DES, two potent estrogens.   How-
ever, relatively heavier (60 g) rats were included in the group
treated with biochanin A and formononetin.   On the basis of the
increased uterine weight and RNA content it is possible that
these animals may have entered the early phase of the estrous
cycle and the circulating endogenous estrogens may have supple-
mented the action of the phytoestrogens.

Conclusions

This experiment was initiated to study the relative effects
of estrogenic steroids and phytoestrogens in terms of their
ability in vitro to stimulate incorporation of labelled uridine
in the RNA extracted from rat uteri six hours after administering
various estrogens in vivo.   Contrary to expectations data ob-
tained in this experiment showed that estrogen treated uteri
had a lower specific activity of RNA than control tissues.   It
is possible that the period of six hours following estrogen
administration was too long for the in vitro effects to be

manifested.   The low specific activity may also be due to the
increased size of the uterine nucleotide pool.   Decreasing
trends in the specific activity of RNA were noticed in the
order of DES $\leqslant$ estriol < estradiol-17α < estrone.   Estradiol-
17β (1.0 µg) and genistein (0.8 mg) produced a significant
(P$<$ 0.05) and (P $<$ 0.1) respectively decreasing effect on the
incorporation of $[5,6^{-3}H]$ uridine into the hydrolyzed RNA
fraction.   However, the specific activity of RNA in coumestrol
treated rats was not significantly lower than control animals.
Both genistein and coumestrol resembled estriol in their
capacity to promote the incorporation of labelled uridine.
In rats treated with biochanin A or formononetin the specific
activity of RNA in the uterus was very low which may be due to
the physiological state of the animal rather than to the true
estrogenic potential of these phytoestrogens.

Table 6 : The in vitro incorporation of radioactivity in different fractions of rat uteri, six hours following in vivo estrogen administration (Expt. D)

Distribution of radioactivity in different fractions (DPM)

Treatment	Groups of animals (n)	Medium and tissue wash	Cold TCA soluble fraction	Hydrolyzed RNA fraction	Recovery of administered label (%)
Control	4	2893886 ± 107,990	204531 ± 26,205	89,146 ± 14,780	88.1 ± 4.1
Estrone	1	2859439	283670	93,771	89.5
Estriol	1	2917317	245712	55,199	88.9
DES	1	2946730	284026	58,259	90.9
Estradiol-17α	1	2829438	280098	78,138	88.1
Estradiol-17β	4	2810321 ± 100,010	285316 ± 18520	54,299 ± 10,270	87.7 ± 3.5
Testosterone	1	2872281	220133	90,477	88.0
Genistein	3	2848505 ± 215,441	164578 ± 12,158	78,527 ± 10,290	85.4 ± 6.5
Coumestrol	2	2811235 ± 64,249	183639 ± 35,663	87,871 ± 2,543	85.2
Biochanin A	1	2919000	144268	67,419	86.5
Formononetin	1	2924519	116973	43,351	85.3

Table 7 : RNA content and specific activity of RNA extracted from
rat uteri six hours following estrogen administration (Expt. D)

Treatment	Mean Body Weight (g)	RNA content ug RNA / uterus	ug RNA / mg uterus	Radioactivity in hydrolyzed RNA DPM / uterus	DPM / mg uterine weight	DPM / ug RNA
Control	42.0 ± 8.3	134.0 ± 7.1	4.3 ± 0.2	29715 ± 4928	933.9 ± 1.5 (a)	6.9 ± 1.2 (a)
Estrone	41.0	134.8	3.9	31257	930.3	6.9
Estriol	39.4	133	3.9	18399	541.2	4.0
DES	46.8	143	3.8	19419	516.5	3.6
Estradiol-17β	38.3 ± 5.1	143.2 ± 3.2	4.1 ± 0.4	17659 ± 1583	501.7 ± 4419 (b)	3.5 ± 0.3 (c)
Estradiol-17α	49.1	161.0	5.2	26659	851.2	5.2
Testosterone	35.6	109.0	3.5	30159	985.4	9.0
Genistein	50.3 ± 3.7	156.5 ± 4.7	4.2 ± 0.1	26916 ± 1400	725.7 ± 106 (c)	4.6 ± 0.7 (b)
Coumestrol	50.3 ± 4.7	141.5 ± 0.9	3.5 ± 0.3	29306 ± 1498	741.9 ± 37.9 (c)	5.2 ± 0.3 (a)
Biochanin A	59.3	196.8	4.4	22473	510.7	2.5
Formononetin	59.7	176.5	3.9	14450	580.3	3.2

((a,b,c,d) Means with different subscripts are significantly different ($P < 0.05$).

Experiment E : Effect of Estrogens and Phytoestrogens
            on the Incorporation of Tritiated
            Uridine Into RNA - Short in vivo Pulsing

Introduction

The very low concentration of phytoestrogens in plant
material and their weak estrogenic potency have made it
difficult to study their biological effects. The problem is
further complicated by the difficulties associated with various
extraction procedures which do not permit quantitative recovery
of the plant estrogens. Consequently the development of
suitable bioassays which could be employed to test their
estrogenic potency and to provide knowledge on their mode of
action has been hampered. Recently the introduction of competi-
tive protein binding techniques, which are sensitive enough to
measure the biological activity of very small quantities of
phytoestrogens has partly overcome the limitations of bioassays.
The sensitivity of bioassay procedures has also been greatly
increased by the development of techniques in which the incor-
poration of a labelled precursor by uterine cellular organelles
in vitro is determined quantitatively after the administration
of estrogenic compounds in vivo.

The results of Experiment D have indicated that the in
vitro administration of tritiated uridine to the uterine tissue
six hours after injection of estrogens in vivo has not resulted
in large incorporation of the label as expected. It is possible
that the six hour duration after in vivo administration of
estrogens was too long to observe the early effects produced in
the tissues.

Miller (1964) reported that reduction of tetrazolium salts occurred as early as 28 minutes after injection of estradiol-17β and suggested that this short duration is critical in observing the early effects of estrogens. Shutt (1967) employed a similar technique as an index of the biological effect of genistein. Subsequently isotopic precursors have been widely used with different degrees of success in studying the early action of estrogen. Munns and Katzman (1971) observed that the extent of incorporation of labelled precursors was high when the level was administered 30 minutes after injection of estradiol-17β. In the light of these experiments this experiment was undertaken to study the very early effects occurring in the uterine cell after a brief exposure to different estrogens using the basic technique of Munns and Katzman (1971). Phytoestrogens were first extracted from plant materials. The early effects which these extracts produced on the uterine tissue were then compared with those caused by estradiol-17β.

Estrogenic activity of certain plant materials has been attributed to the presence of nonsteroidal phytoestrogens capable of competing with estradiol-17β for specific binding sites located in the uterine cytosol. This study presents the results of qualitative and quanitative determinations of various phytoestrogens and their ability for the uterine binding protein.

Materials and Methods

Materials

First cuttings of orchard grass hay (<u>Dactylus glomerata</u>)

and alfalfa hay (Medicago sativa) were obtained from Agassiz

Research Station.   Soyabean (Glycine max) meal was obtained from

Buckerfields.   [2,4,6,7(n)$^{-3}$H] estradiol-17β (specific activity,

96 Ci/mmole) was obtained from Amersham/Searle.   Dextran T-40

was obtained from Pharmacia Chemicals and activated charcoal

from Sigma.

Methods

Extraction and Identification of Phytoestrogens

Phytoestrogens were extracted from 2.0 g samples of oven

dried ground orchard grass hay, alfalfa hay and soyabean meal by

treating them successively with absolute alcohol and peroxide-

free ether (Francis and Millington 1965).   The final extract

was concentrated to dryness under a stream of nitrogen and

absolute alcohol was added.   Samples were then stored at -20°C

until ready for use.   The efficiency of extraction was checked

by adding known concentrations of genistein to those samples and

by calculating the per cent recovery.

Qualitative determination of phytoestrogens was done on two

directional thin layer chromatography using G-25 silica gel

plates (D.C. Fertigplatten-Macheray-Nagel Co.).   The plates

were activated at 100°C for one hour prior to use and 10-20 μl

aliquots of plant extracts were placed on each plate.   Fifty

microliter aliquots of formononetin, genistein, coumestrol and

biochanin-A dissolved in alcohol were used as standards.   The

developing solvent systems used were chloroform-methanol

(91:9,V/V) in the first direction and ammonia saturated chloro-

form-methanol (91:9,V/V) in the second.   The plates were

allowed to dry at room temperature for approximately 15 minutes

before running them in the second direction. Developed chroma-
tograms were observed under ultraviolet light for the detection
of formononetin, daidzein and coumestrol. Identification of
these compounds was made by comparing their Rf values with
standards and by their visual characteristics as seen under
ultraviolet light. Areas representing the phytoestrogens were
circled with a pencil and the plates transferred to a fume hood
where they were sprayed with a solution of cold 1.0N sulphanillic
acid containing $10\%Na_2CO_3$ and 4.5% $NaNO_2$ for the detection of
the nonfluorescent compounds, genistein, biochanin-A and equol.
The former two compounds were identified by comparing their Rf
values with known standards.

Quantitative Determination of Phytoestrogens

The competitive protein binding assay of Korenman(1968)
was employed to determine the concentration of phytoestrogens
quantitatively. Uterine cytosol was obtained from a six day
pregnant rabbit and homogenized at 4°C in three volumes of Tris
buffer (pH,4.0 W/V) using a Waring blender (Sorvall). The homo-
genate was centrifuged at 7000 g for 15 minutes at 0°C. The super-
natant fraction was removed and recentrifuged (Beckman, ultra-
centrifuge L5-65) at 100,000 g for 90 minutes at 0°C. Immedi-
ately following ultracentrifugation the second supernatant fraction
containing the cytosol proteins was collected by Pasteur pipette
and stored in liquid nitrogen until ready for use.

Standard curves for competitive protein binding assays
were obtained using different concentrations of purified
genistein. Aliquots of increasing concentration of purified
genistein were added to test tubes and dried under a stream of
nitrogen. To each sample were added successively 100 μl of

Tris buffer, pH 8.0, 50 µl of ^{3}H-estradiol-17β and 100 µl of

rabbit uterine cytosol. The tubes were mixed after the

addition of the uterine cytosol, samples were allowed to stand

at room temperature for 30 minutes at the end of which 1.0 ml

of dextran coated charcoal was added to each tube. The tubes

were then mixed, kept at 4°C for 15 minutes and centrifuged on

a desk centriguge. The supernatant was collected with a

Pasteur pipette and an aliquot taken for the determination of

radioactivity. Based on the competition for binding sites

between estradiol-17β and the added genistein the radioactivity

in the supernatant will be directly proportional to the added

genistein.

Plant extracts were dried in a stream of nitrogen and the

concentration of total phytoestrogens was measured by the compe-

titive protein binding assay and the values expressed in terms

of estrogenic affinity of genistein.

Results

Qualitative analysis of orchard grass hay extracts on two

dimensional thin layer chromatography disclosed no identifiable

spots corresponding to phytoestrogens. The Rf values of puri-

fied phytoestrogens standards are given in Table 8. In the

alfalfa hay extracts spots corresponding to coumestrol, formo-

nonetin and genistein were observed (Table 8). Although no

standards of equol were used a weak spot corresponding

to the Rf value of equol was noticed in the alfalfa extract.

Both genistein and daidzein were present in the soyabean

extract (Fig. 10).

The standard curve for the competitive protein binding

assay of purified genistein is shown in Fig. 11. When known

quantities of genistein was added to orchard grass hay, the

extraction procedure gave a recovery of 59% as determined by the

competitive protein binding assay.

The concentration of phytoestrogens in the orchard grass

hay extract was very low and equivalent to 1.5-2.0 µg genistein

units per 2.0 g of the sample. Alfalfa hay and soyabean meal

extracts contained much larger concentrations of phytoestrogens

equivalent to 70 and 126 µg respectively of genistein units.

Discussion

The presence of estrogenic activity in forages has been

reviewed by many researchers working in this field (Bickoff et

al. 1957; Kohler and Bickoff, 1961). Beck et al. (1964) reported the

isolation and subsequent identification of various plant estro-

gens by thin layer chromatography. The plant constituents

currently believed to contribute to estrogenic action are the

isoflavones, genistein, biochanin-A, formononetin, daidzein and

equol as well as the coumarin derivative, coumestrol. The

isolation and identification of coumestrol, formononetin and

genistein in the alfalfa extract in this study support the

findings of Kohler and Bickoff (1961) who reported that coume-

strol accounts for the majority of the biological activity in

alfalfa while the isoflavones are responsible for the remaining

activity. The identification of genistein in soyabean meal

extract also agrees with Cheng et al. (1953(b) who reported high

levels of genistein in soyabean meal.

Phytoestrogens have since been reported to bind on specific

uterine proteins (Shutt and Cox, 1972) and act in various areas of cell metabolism (Noteboom and Gorski, 1963)(a).

It was also evident that sufficient estrogenic activity was present in various plant extracts strong enough to displace estradiol-17β from uterine binding proteins. Though no evidence for the presence of phytoestrogens in orchard grass hay could be detected by the TLC method, it was possible to detect very small quantities of phytoestrogen activity when the sensitive competitive protein binding assay method was used. It is logical to expect that following the initial binding of the phytoestrogen to uterine cytosol proteins additional estrogen mediated responses would occur sequentially later. To test this hypothesis it was decided to undertake further studies involving the in vitro incorporation of label nucleoside by the uterine tissue after a brief in vivo exposure to estrogens.

Conclusions

In Experiment E the quantitative and qualitative determination of the phytoestrogen content of various forages was made. Qualitative studies of the alfalfa extract revealed the presence of coumestrol, genistein and formononetin. From visual observations of the chromatograms from alfalfa extracts, the isoflavones appeared to be in a much smaller quantity than coumestrol. In the soyabean extract, genistein and daidzein were isolated and although no standards for daidzein were available, identification could be made from its characteristic fluorescence under ultraviolet light. According to the visual observations made from the soyabean meal chromatograms,

genistein appeared to be in greater concentration than daidzein. Qualitative examination of the orchard grass hay extract did not disclose any spot corresponding to phytoestrogens.

Quantitatively, estrogenic activity was observed in the genistein spiked hay and alfalfa hay and soyabean meal extracts. A very small amount of estrogenic activity (1.5-2.0 µg) per 2.0 g of sample was recorded in the orchard grass hay extract. Alfalfa hay and soyabean meal extracts possessed a substantial amount of activity of 70 µg and 126 µg respectively.
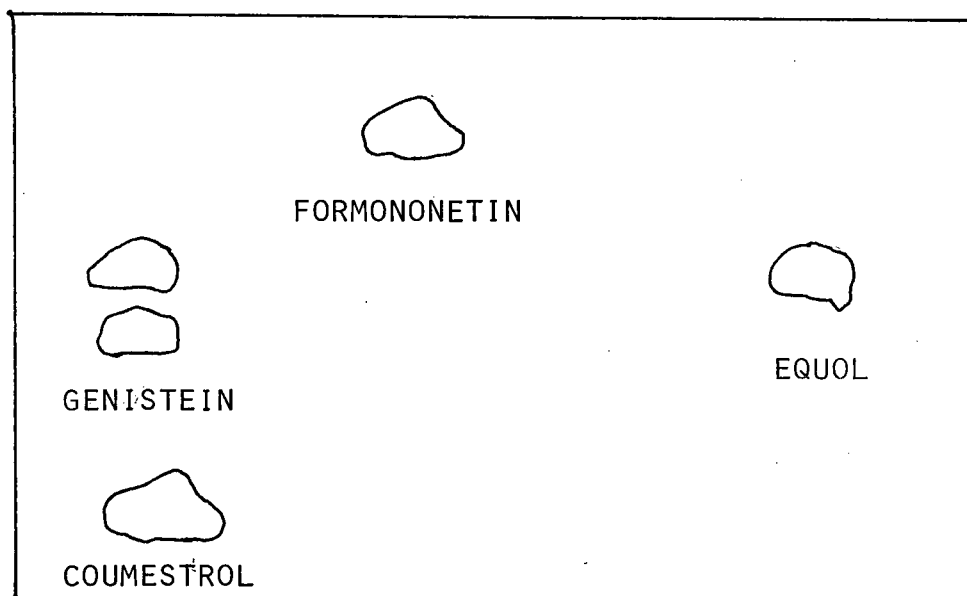
ALFALFA HAY (MEDICAGO SATIVA)
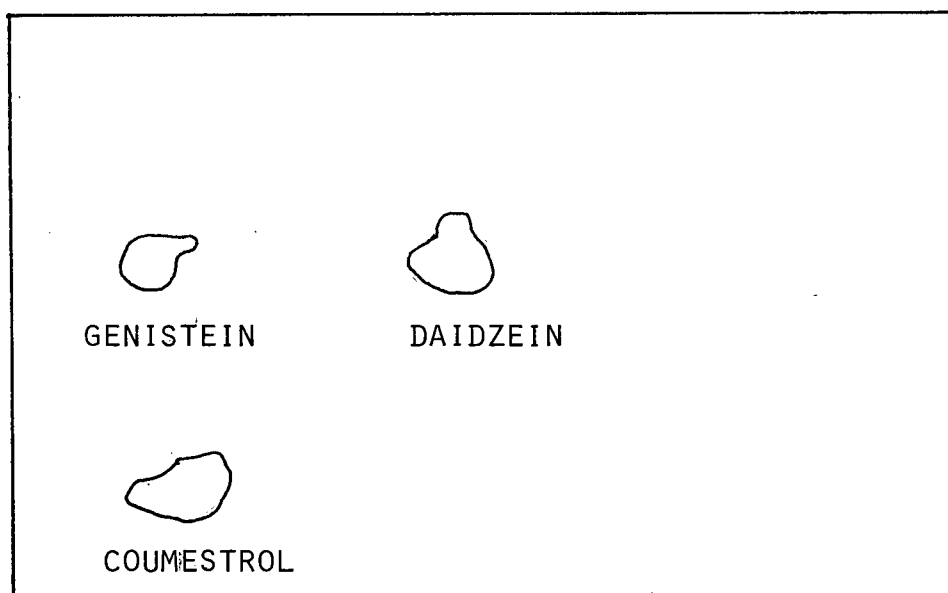
SOYBEAN MEAL (GLYCINE MAX)

Fig. 10.   Qualitative examination of phytoestrogen
content by thin layer chromatography

A = Chloroform-methanol (91:9 v/v)

B = Ammonia Saturated Chloroform-methanol (91:9 v/v)

Table 8 : Rf values of standard phytoestrogens and
those obtained from plant extracts as
measured by Thin Layer Chromatography (Expt. E)

Sample Used	Coumestrol	Genistein	Formononetin	Daidzein	Equol	Biochanin A
Standard	0.41 (0.85)	0.58 (0.91)	0.74 (0.74)	-		0.76 (0.79)
Alfalfa Hay	0.47 (0.87)	0.56 (0.94)	0.70 (0.82)	-	0.62 (0.53)	-
Soybean Meal	0.51 (0.83)	0.63 (0.89)	-	0.54 (0.14)	-	-
Orchard Grass Hay	-	-	-	-	-	-

- Figures outside brackets correspond to Rf values in first solvent

- Figures within brackets correspond to Rf values in second solvent

Experiment F : Early Effects of Estrogens and Plant
              Extracts on the Incorporation of Tritiated
              Uridine Into RNA by Uterine Tissue.

Introduction

In the light of quantitative and qualitative determination
of phytoestrogen activity in plant extracts made in Experiment
E further efforts were made to obtain information on the mode
of action of estrogenic compounds.   Based on the competition
for binding sites between estradiol-17β and the various phyto-
estrogens it was felt that additional experiments designed
specifically to study the uptake of a radioactive nucleoside
into the RNA fraction by uterine tissues would be informative in
defining the mode of action of estrogenic compounds in various
plant extracts.   In Experiment D the six hour duration between
the in vivo administration of estrogen and in vitro uptake of
labelled nucleoside by the uterine tissue resulted in a lower
specific activity of RNA.   Therefore it was decided to shorten
the duration following estrogen administration to 30 minutes
before undertaking in vitro uptake studies.

Materials and Methods

Animals

Immature female rats (33-43 g) were used in this study.
They were housed and maintained as described in Experiment A.

Materials

Plant extracts of orchard grass hay, alfalfa hay and soya-
bean meal were prepared as described in Experiment E.   Addi-

tional materials required for in vitro studies were the same as described in Experiment D.

Methods

Administration of Estrogens in vivo

Immature female rats were given single injections of estradiol-17β, genistein, alfalfa hay extract and soyabean meal extract in doses of 1.0 μg, 60.0 μg, 14.5 μg and 40.0 μg respectively. Estradiol-17β, genistein and soyabean meal extract administered intraperitoneally in 0.2 ml of aqueous 1% ethanol. Alfalfa hay extract was administered intraperitoneally in 0.2 ml of 50% propylene glycol. Control animals received similar volumes of 1% ethanol in saline.

Incorporation of $[5,6 \text{ }^{-3}H]$ Uridine By Uterus In Vitro

Animals were sacrificed 30 minutes after the administration of estrogens by placing them in a sealed jar containing CO_2. The whole uteri were removed within one minute, freed of fat and connective tissue and placed in ice cold isotonic saline. Two to three groups per treatment containing three whole uteri per group were gently blotted and transferred to prewarmed (37°C) 5 ml incubation vials containing 2.5 ml of TCM 199, 10.0 μCi of ^3H-uridine and 1.0 mM glutamine. Uterine tissues were incubated for 1.0 hour at 37°C in a water bath at a shaking speed of one stroke per second under an atmosphere of 95% O_2-5% CO_2.

After incubation the medium was removed by Pasteur pipette and 0.5 ml of 0.6 N $HClO_4$ was added to terminate the reaction. Whole uterine tissues were rinsed with ice cold distilled water

to remove extracellular radioactivity. This fraction
representing tissue wash was pooled and processed as described
in Experiment D. Tissues were blotted, weighed and stored at
$-20^{\circ}C$ until RNA and DNA extractions were carried out. The
radioactivity in the cellular fraction was determined as
described under Experiment D. Recovery rates of the isotope
were also made to determine the efficiency of the extraction
procedure. All results were analysed statistically by the
students' t-test.

Results

The per cent of total radioactivity which was recovered in
the different fractions amounted to 79-91% (Table 9). There
was a slightly greater incorporation of tritiated uridine in
the hydrolyzed RNA fraction in uteri of estradiol-17β and phyto-
estrogen treated rats than in control animals. This apparent
increase was however not significant ($P < 0.05$) when analyzed by
the students' t-test. The radioactivity in the hydrolyzed RNA
fraction when expressed per unit of uterine wet weight was signi-
ficantly ($P < 0.05$) higher in the uteri of estradiol-17β and pure
genistein treated rats than controls (Table 10). Alfalfa hay
and soyabean meal extracts also increased the incorporation of
the labelled precursor into the hydrolyzed RNA fraction per unit
of uterine wet weight, however this increase was not significant.
The specific activity of RNA in the estradiol-17β and phyto-
estrogen treated rat uteri was significantly ($P < 0.05$) greater
than the controls (Table 10). The alfalfa and soyabean extracts
also significantly ($P < 0.05$) increased the specific activity of
RNA in the immature female rat uteri when compared to control

animals (Table 10).

Discussion

Having established in Experiment E that different phyto-
estrogens were present in alfalfa hay and soyabean, and that
they were capable of binding onto specific uterine proteins, Ex-
periment F was initiated to determine whether or not they were
potent enough to enhance the early incorporation of labelled
uridine into RNA of uterine tissues.   To determine this short
in vivo pulsing of the estrogenic compound in question was em-
ployed.

The increased uptake of the labelled uridine observed in
this experiment is in agreement with that reported by Munns and
Katzman (1971).   It is particularly noteworthy that the estra-
diol-17β or phytoestrogen treated rat uteri had a higher specific
activity of RNA unlike in Experiment D where they had a lower
specific activity than the controls (Tables 6 and 9).   Increases
in $[5, 6\ ^{-3}H]$ uridine incorporation into RNA during the early
phase of estrogen and phytoestrogen action may be attributed to
the increased permeability of the uterine cell membrane.
Further, during the relatively long duration after in vivo estra-
diol-17β administration in Experiment D, there may be an
increased influx of endogenous supply of nucleosides which would
cause a reduction in the uterine specific activity as suggested
by Munns and Katzman (1971).   Hence the duration time of 30
minutes chosen for in vivo pulsing with estradiol-17β and
various phytoestrogens in Experiment F appeared to result in
higher specific activities of RNA.

The doses of various estrogenic compounds administered were
chosen on the basis of the estrogenic activity determined by
competitive protein binding assays.   Despite the fact that
these compounds were administered in different quantities this
did not appear to influence the specific activity of the incor-
porated radioactive precursor.   When the phytoestrogen activity
of the extracts was expressed in terms of genistein units,
alfalfa and soyabean extracts (14.5 ug and 40.0 µg respectively)
were similar to estradiol-17β (1.0 µg) and genistein (60.0 µg)
in their ability to enhance incorporation of tritiated uridine.

Estradiol-17β has been reported to increase the activity of
DNA dependent RNA polymerase enzyme (Maul and Hamilton 1967).
Although no experiments were done to determine whether or not
various phytoestrogens are capable of increasing polymerase
activity, it seems reasonable that the greater uridine incorpora-
tion observed in the hydrolyzed RNA fraction may be due to
greater activity of this enzyme.   More experimental data is
required before conclusions can be made regarding phytoestrogen
induced polymerase activity.

Based on the effects on early events occurring in cellular
metabolism due to a single injection of phytoestrogens obtained
from a small quantity of plant material it is interesting to
speculate the magnitude of effects which could be produced in
grazing animals injesting large quantities of phytoestrogens.

Conclusions

In Experiment F the objective was to examine the estrogenic
potential of different forages by establishing a bioassay useful

in studying an estrogen induced response, namely the incorporation of a specific isotopic precursor.

Exposure of uterine tissue to estrogens for 30 minutes *in vivo* was sufficient to study the initial effects of estradiol-17β and phytoestrogens. Significantly greater ($P < 0.05$) differences were obtained between estrogen treated rats and control animals in regard to incorporation of tritiated uridine into the hydrolyzed RNA fraction of the uterine cell. It is probable that a shorter pulsing period and exposure of uterine tissues to larger quantities (10 μCi) of isotopic precursor than were used in Experiment D enabled the initial effects of estradiol-17β and phytoestrogens to be observed.

More experimental data regarding the specific mode of action of phytoestrogens on uterine cell metabolism (i.e. increased polymerase activity) are required before conclusions regarding phytoestrogen mediated RNA synthesis can be made.

Fig. 11.   Competitive Binding Assay of
            Phytoestrogens (Expt. E)

Increasing concentrations of
genistein were added to 100 μl
tris buffer;   50 μl ^{3}H Estradiol-17β
and 100 μl pregnant rabbit uterine
cytosol to determine the Genistein
Standard Curve.

Figure showing semi-logarithmic plot of RADIOACTIVITY OF ^3H ESTRADIOL-17 IN SUPERNATANT (CPM) $\times 10^3$ versus GENISTEIN ADDED TO ASSAY TUBE (NG), with data points labeled ORCHARD GRASS HAY, ALFALFA HAY, and SOYBEAN MEAL.
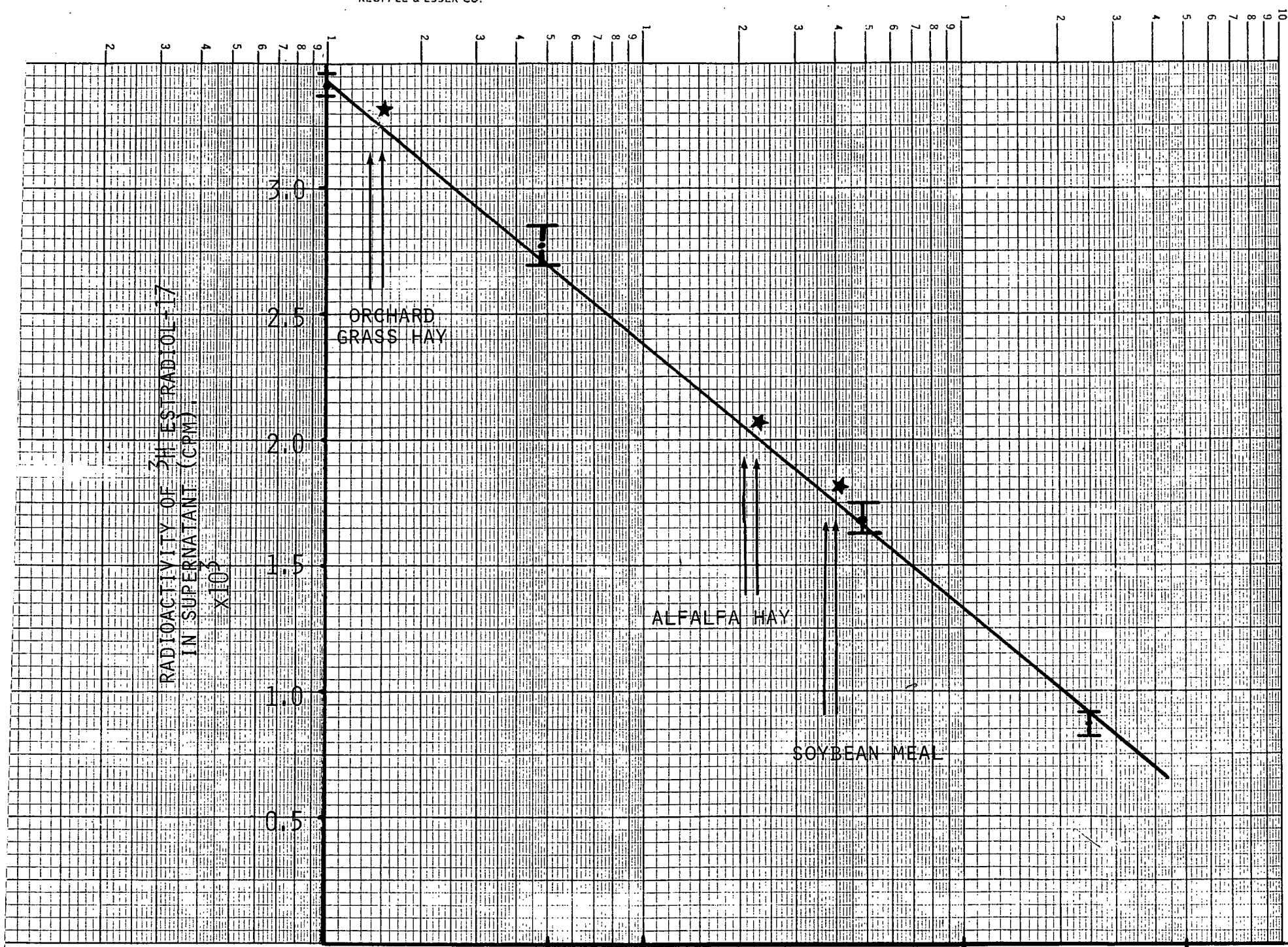
Table 9 : The in vitro incorporation of radioactivity in different
fractions of rat uteri, 30 minutes following in vivo
estrogen administration (Expt. F)

Distribution of radioactivity in different fractions (DPM)

Treatment	Groups of Animals	Medium and tissue wash	Cold TCA soluble fraction	Hydrolyzed RNA fraction	Recovery of administered label %
Control	2	22692616 ± 66,578	2248490 ± 179538	929984 ± 82521	91 ± 2%
Estradiol-17 β	2	22158070 ± 321857	2507680 ± 194458	1186117 ± 22805	91 ± 1%
Genistein	3	18844267 ± 232915	2672025 ± 508445	1033235 ± 17193	79 ± 1%
Alfalfa hay extract	2	19836720 ± 105723	2351821 ± 51510	1178381 ± 32684	82 ± 1%
Soyabean meal extract	2	20929731 ± 148367	2484358 ± 313876	1128354 ± 31858	86 ± 3%

Table 10 : Incorporation of $[5,6 {}^{-3}H]$ uridine into the hydrolyzed
RNA fraction of immature female rat uteri 30 minutes
following estrogen administration

Radioactivity in hydrolyzed RNA

Treatment	Groups of Animals (n)	$\dfrac{DPM}{uterus}$	$\dfrac{DPM}{mg\ uterine\ weight}$	$\dfrac{DPM}{ug\ RNA}$
Control	2	309,994 ± 27,507	8,732 ± 774 (a)	69.3 ± 6.2 (a)
Estradiol-17β	2	395,372 ± 10,750	11,526 ± 221 (b)	92.5 ± 1.4 (b)
Genistein	3	329,411 ± 17,477	11,124 ± 802 (b)	89.2 ± 6.2 (b)
Alfalfa extract	2	392,793 ± 10,894	11,385 ± 315 (a)	91.1 ± 2.5 (b)
Soyabean extract	2	376,118 ± 10,619	11,062 ± 220 (a)	87.8 ± 2.5 (b)

(a,b)   Means with different subscripts are significantly different ($P < 0.05$)

GENERAL CONCLUSIONS

Estradiol-17β, the major circulating estrogen in majority of mammalian species was chosen as the standard with which other estrogens were compared. Initial experiments involving dose levels and sequential time intervals following estradiol-17β administration were designed to establish baseline values regarding target organ metabolism.

Six hours following the intraperitoneal administration of a single injection of estradiol-17β (1.0 µg) maximal tissue edema was observed. The ability of other steroid, synthetic and plant estrogens to produce similar water imbibition was also studied. Estriol and DES were equivalent to estradiol-17β in inducing water imbibition. The plant estrogens, genistein and coumestrol were capable of inducing tissue edema however they were only 10^{-3} times as potent as estradiol-17β in doing so. Administration of estradiol-17β, genistein and coumestrol were shown to enhance uterine vascular permeability six hours after their administration as indicated by India ink perfusion experiments.

Studies on the metabolism of RNA and DNA were carried out by experiments designed to monitor the synthesis of nucleic acids following estradiol-17β administration. It was found that estradiol-17β significantly shortened the uterine cell cycle. Net accumulation of RNA and DNA was noticed to occur 12 and 24 hours respectively following the administration of estradiol-17β.

It was felt that these series of data would be useful in future isotopic precursor experiments. The first labelled nucleoside experiment employed the data obtained from earlier

experiments involving a six hour (in vivo) pulsing period of estradiol-17β or its equivalent followed by a one hour in vitro exposure to a labelled precursor. The estrogen treated groups displayed lower specific activities than control groups. With estradiol-17β having the lowest specific activity, it was felt that the data actually signified those compounds which possessed the greatest affinity to alter the cell membrane and by doing so resulted in the dilution of the tracer compound by similar endogenous precursors. Therefore the lower the specific activity, the greater the dilution of the tracer and the stronger the estrogen. Estradiol-17β significantly reduced the specific activity of a labelled precursor. Plant estrogens, genistein and coumestrol also showed reductions in the specific activities of the labelled precursor, and were similar to the trend noticed with estriol. The physiological status of the animals was seen to be an important factor as the results of bio-chanin-A and formononetin were misleading due to the suspected onset of estrus of these treated animals.

The early action of estradiol-17β, genistein and various plant extracts was studied in the final experiment aimed at looking at the comparative ability of each to incorporate an isotopic precursor. Quantitative and qualitative determinations of estrogenic activity of the plant extracts were made prior to employing a short in vivo pulsing method used to prevent the dilution of the radioactive tracer. From these studies, estradiol-17β, genistein, alfalfa hay and soyabean meal extracts contained sufficient estrogenic activity to incorporate uridine

into the RNA fraction of the uterus.   Additional experimental
data however is required before the complete mode of action of
various phytoestrogens in stimulating RNA synthesis and in turn
protein synthesis can be established.

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APPENDIX

Three Whole Uterine Tissues

- Homogenize in 2.0 ml ice cold
  demineralized water for approx.
  5 minutes
- add 7.0 ml ice cold 10% TCA
- stand 10 minutes and centrifuge

Supernatant	Pellet
- TCA, acid Soluble Fraction	resuspend in 95% ETOH at 0°C

Centrifuge

Pellet	Supernatant

Pellet

- add 2.0 ml 1 N KOH
- stand for 16 hrs., 37°C
- add 0.4 ml 6 N HCl
- add 5% TCA at 0°C
- mix and place in ice for 10 minutes
- centrifuge

Pellet	Supernatant
	- Hydrolyzed RNA Fraction
	- read at 260 nM

Pellet

- add 2.5 ml 10% $HClO_4$
- heat to 70-85°C for 25 min.
- Cool - stand for 10 min.
- Centrifuge

Pellet	Supernatant
Protein	- DNA Fraction
- hydrolyze in 2.0 ml NaOH	- read at 260 nM

Appendix Fig. A.   Nucleic Acid Extraction Procedure

2.0 Oven Dried Sample of Plant Materials

1. grind, add 3.0 ml 0.01 M HCl
   stand 20 minutes

2. add 3.0 ml Abs. ethanol

Reflux 10 minutes

Decant

Liquid Phase 1	Residue
	Reflux 10 ml ETOH
	Decant

Liquid Phase 2	Residue
- pool phases 1 & 2	- discard
- reduce volume	
- filter into extraction tubes	

Filtrate	Residue
- add 15 ml Distilled Ligroine Ether	- discard
- sit 10 min. at 45° angle	
- suction off ether	
- add 10 ml peroxide free ethyl ether - shake 20 min.	
Freeze in Dry Ice	

Liquid Phase 1	Solid
- collect ether and evaporate under N_2 (40°)	- add 10 ml peroxide free ethyl ether
	Refreeze

| Liquid phase 2	Solid phase
- add to Liquid phase 1	- discard
- evaporate under N_2	
add 0.5 ml Absolute Ethanol wait 20 min. and store at 4°C.	

Appendix Fig. B.   Extraction of Phytoestrogens
From Plant Materials
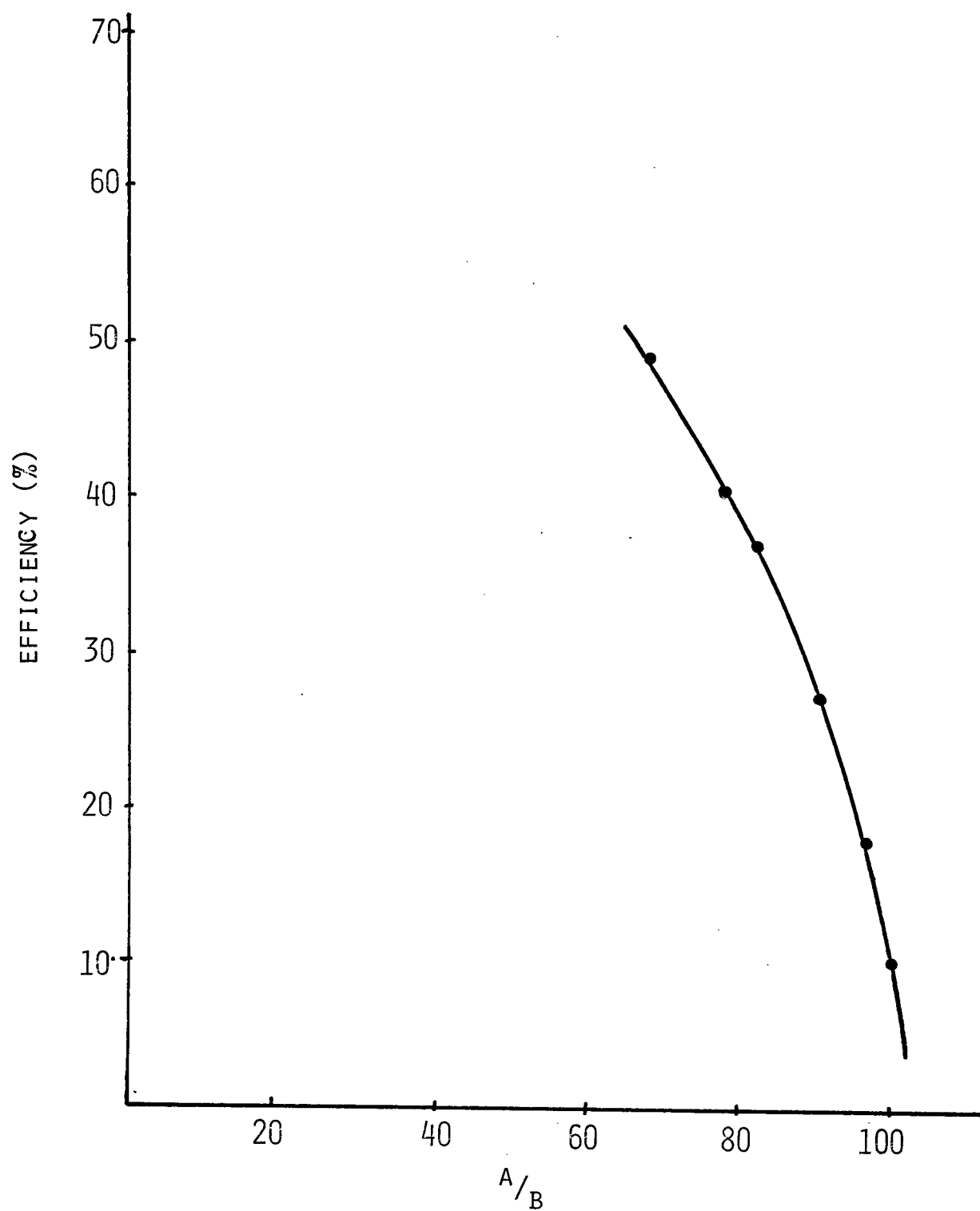
Appendix Fig. C.   Quench Correction Curve for
Tritium