CORTISOL, CORTISONE INTERCONVERSION IN THE HUMAN FETAL LUNG

by

MARK ABRAMOVITZ

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Medicine
Division of Experimental Medicine
McGill University, Montreal

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ABSTRACT

Since cortisol (F) can influence fetal lung maturation in late gestation, interconversion between cortisol and its inactive analogue cortisone (E), catalyzed by 11β-hydroxysteroid dehydrogenase (11-HSD) (E.C. 1.1.1.146), may be an important mechanism for controlling the level of F in the fetal lung parenchyma. The objects of this study were to: (1) resolve conflicting data; and (2) to study possible regulating factors of the 11-HSD. Human fetal lungs (HFL) of gestational ages 9-20 weeks were grown as monolayer cultures or as explants with $^3$H-F and $^3$H-E or $^{14}$C-E (0.01-17 ng/ml), for 6-16 days. Extracts of tissue culture medium were chromatographed to separate the steroids, and the per cent conversion was calculated. From the differences observed between explant and monolayer cultures it was concluded that: (1) the E to F activity observed in midgestational HFL monolayer cultures is an artefact of the culture system and is therefore not physiological, thus resolving the conflicting data; (2) F to E activity predominates in midgestational HFL and resides in undifferentiated epithelial cells; and (3) the 11-HSD in fibroblast-like cells is probably a different enzyme from the 11-HSD in HFL epithelial cells.
Résumé

Puisque le cortisol (F) peut influencer la maturation pulmonaire foetale pendant la période gestationnelle terminale, il est possible que l'interconversion entre le F et son analogue inactif, la cortison (E), (cette réaction étant catalysée par la 11β-hydroxystéroïde déhydrogénase (11-HSD) (E.C.1.1.1.146)) constitue un des mécanismes importants contrôlant le taux de F dans le parenchyme pulmonaire. Cette étude avait pour but de:

(1) résoudre le problème des données conflictuelles et; (2) d'étudier les facteurs régulateurs de la 11-HSD. Les tissus pulmonaires de foetus humains (TPFH) dont l'âge gestationnel variait entre 9 et 20 semaines ont été mis en cultures monostratifiées ou en explants avec du $^{3}$H-E et du $^{3}$H-E ou encore avec du $^{14}$C-E (0.01-17 ng/ml) pour une période de 6 à 16 jours. Des extraits du milieu de culture cellulaire furent chromatographiés afin de trier les stéroïdes et d'en calculer le pourcentage de conversion. En observant les différents résultats entre les cultures monostratifiées et les explants nous concluons que: (1) l'activité de conversion de E à F observée dans les cultures cellulaires monostratifiées de TPFH mi-gestationnels représente un artefact du milieu de culture et qu'en conséquence cette activité ne peut être considérée comme physiologique. Ainsi nous avons résolu le problème des données conflictuelles; (2) l'activité de conversion de F à E prédomine dans les TPFH mi-gestationnels et que cette conversion se fait via les cellules épithéliales non-différentiées; (3) que c'est probable que la 11-HSD des cellules 'fibroblaste-like' n'est pas une enzyme identique au 11-HSD des cellules de TPFH.
ACKNOWLEDGMENTS

Throughout this investigation the author has been privileged to benefit from the invaluable advice, criticism, and encouragement given by Dr. Beverley E. Pearson Murphy, his research director. Without her friendship and support this work would not have been possible.

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<tr>
<td>adrenocorticotropin</td>
<td>ACTH</td>
</tr>
<tr>
<td>centigrade</td>
<td>°C</td>
</tr>
<tr>
<td>centimeter</td>
<td>cm</td>
</tr>
<tr>
<td>chick embryo extract</td>
<td>CEE</td>
</tr>
<tr>
<td>corticosteroid binding globulin (transcortin)</td>
<td>CBC</td>
</tr>
<tr>
<td>corticosterone</td>
<td>B</td>
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<tr>
<td>cortisol</td>
<td>F</td>
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<tr>
<td>cortisone</td>
<td>E</td>
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<tr>
<td>counts per minute</td>
<td>cpm</td>
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<tr>
<td>11-dehydrocorticosterone</td>
<td>A</td>
</tr>
<tr>
<td>fetal bovine serum</td>
<td>FBS</td>
</tr>
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<td>gram(s)</td>
<td>g</td>
</tr>
<tr>
<td>hour(s)</td>
<td>hr</td>
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<tr>
<td>human chorionic gonadotropin</td>
<td>hCG</td>
</tr>
<tr>
<td>human fetal lung</td>
<td>HFL</td>
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<tr>
<td>human placental lactogen</td>
<td>hPr</td>
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<tr>
<td>human prolactin</td>
<td>hPl</td>
</tr>
<tr>
<td>hydroxy</td>
<td>OH</td>
</tr>
<tr>
<td>11β-hydroxysteroid dehydrogenase</td>
<td>11-HSD</td>
</tr>
<tr>
<td>intravenous(-ly)</td>
<td>iv</td>
</tr>
<tr>
<td>kilogram(s)</td>
<td>kg</td>
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<tr>
<td>light microscope</td>
<td>LM</td>
</tr>
<tr>
<td>liquid scintillation analyser</td>
<td>LSA</td>
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<tr>
<td>metabolic clearance rate</td>
<td>MCR</td>
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<tr>
<td>microcurie(s)</td>
<td>μCi</td>
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<td>%</td>
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<td>picogram(s)</td>
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<td>revolutions per minute</td>
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<tr>
<td>specific activity</td>
<td>SA</td>
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<td>standard deviation</td>
<td>SD</td>
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<td>tissue culture medium</td>
<td>TCM</td>
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<td>tetrahydrocortisol</td>
<td>THF</td>
</tr>
<tr>
<td>tetrahydrocortisone</td>
<td>THE</td>
</tr>
<tr>
<td>triiodothyronine</td>
<td>T₃</td>
</tr>
<tr>
<td>weight</td>
<td>wt</td>
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GLOSSARY OF TERMS

$^{14}$C. .......................................................... radioactive isotope of carbon, atomic weight 14

$^{3}$H. .......................................................... radioactive isotope of hydrogen, atomic weight 3

aldosterone ............................................. $11\beta,21$-dihydroxy-3,20-dioxopregn-4-en-18-al

allo-tetrahydrocortisol (alloTHF) .......... $3\alpha,11\beta,17\alpha,21$-tetrahydroxy-5$\alpha$-pregnan-20-one

allo-tetrahydrocortisone (alloTHE) ...... $3\alpha,17\alpha,21$-trihydroxy-5$\alpha$-pregnane-11,20-dione

androsterone ........................................ $3\alpha$-hydroxy-5$\alpha$-androstan-17-one

corticosterone (B) ......................... $11\beta,21$-dihydroxypregn-4-ene-3,20-dione (epi-B; 11$\alpha$)

cortisol (hydrocortisone) (F) ............ $11\beta,17\alpha,21$-trihydroxypregn-4-ene-3,20-dione (epi-F; 11$\alpha$)

cortisone (E) ........................................ $17\alpha,21$-dihydroxypregn-4-ene-3,11,20-trione

cortol .................................................. $3\alpha,11\beta,17\alpha,20\alpha,21$-pentahydroxy-5$\beta$-pregnane

$\beta$-cortol ........................................... $3\alpha,11\beta,17\alpha,20\beta,21$-pentahydroxy-5$\beta$-pregnane

cortolone ........................................... $3\alpha,17\alpha,20\alpha,21$-tetrahydroxy-5$\beta$-pregnan-11-one

$\beta$-cortolone ................................... $3\alpha,17\alpha,20\beta,21$-tetrahydroxy-5$\beta$-pregnan-11-one

11-dehydrocorticosterone (A) ............... $21$-hydroxypregn-4-ene-3,11,20-trione
dexamethasone ........................................... 9α-fluoro-16α-methyl-11β,17α,21-
trihydroxypregna-1,4-diene-3,20-
dione

dihydrocortisol (dihydro-F) ....................... 11β,17α,21-trihydroxy-5β-pregnane-
3,20-dione

20-dihydrocortisol (20-DH-F) ..................... 11β,17α,20β,21-tetrahydroxypreg-
(Reichstein's substance E) 4-ene-3-one

20-dihydrocortisone (20-DH-E) ................. 17α,20β,21-trihydroxypreg-n-4-ene-
(Reichstein's substance U) 3,11-dione

fluocinolone acetonide ......................... 6α,9α-difluoro-11β,16α,17,21-
tetrahydroxypregna-1,4-diene-3,20-
dione cyclic 16,17-acetol

11-keto-androstenedione ....................... androst-4-ene-3,11,17-trione
(adrenosterone)

11-keto-etiocholanolone ....................... 3α-hydroxyetiocholane-11,17-dione

11-OH-androstenedione ......................... 11β-hydroxyandrost-4-ene-3,17-
dione

11β-OH-androsterone ......................... 3α,11β-dihydroxyandrostane-17-one

11β-OH-etiocholanolone ....................... 3α,11β-dihydroxyetiocholan-17-one

prednisolone ........................................ 11β,17α,21-trihydroxypreg-n-1,4-
(epi-prednisolone; 11α) diene-3,20-dione

prednisone .......................................... 17α,21-dihydroxypreg-n-1,4-diene-3,
11,20-trione

progesterone ....................................... pregn-4-ene-3,20-dione

testosterone ........................................ 17β-hydroxyandrost-4-en-3-one

tetrahydrocortisol (THF) ....................... 3α,11β,17α,21-tetrahydroxy-5β-
pregn-20-one

tetrahyrocortisone (THE) ....................... 3α,17α,21-trihydroxy-5β-pregnane-
11,20-dione
CHAPTER I

HISTORICAL REVIEW
A. INTRODUCTION

Glucocorticoids play an important role in fetal organ differentiation and maturation. Specifically, these hormones affect fetal lung maturation and have been used successfully in the treatment of the Respiratory Distress Syndrome (RDS) in premature infants. Increasing corticosteroid levels towards term in several mammalian species, including the human fetus, have been observed. What causes this late elevation in corticosteroids in the fetus is not clearly understood. Both increasing adrenocortical production and changes in corticosteroid metabolism may be important.

Liggins (1976) has pointed out in his paper 'Adrenocortical-related maturational events in the Fetus' that "Nothing is known of the factors influencing the activity of 11β-hydroxysteroid dehydrogenase (11-HSD), the enzyme regulating cortisol-cortisone interconversion" (see Fig. 1-1) and, therefore, cortisol levels.

The following Historical Review attempts to be comprehensive in scope with respect to this enzyme. The initial sections of the review cover some early studies on the therapeutic and physiologic effects and metabolism of cortisol (F) and cortisone (E). 11-HSD activity was discovered as a consequence of these studies and was ascribed a regulatory role by some investigators, although the evidence was not impressive. In pregnancy the enzyme does appear to play a significant role in F regulation.

Subsequent sections of this thesis report findings on the activity and regulation of 11-HSD in the human fetal lung under various experimental conditions.
Fig. 1-1. The chemical structures and some general properties of $E$ and $F$. Oxidation (inactivation) indicates conversion of $F$ to $E$. Reduction (activation) indicates conversion of $E$ to $F$. 

$11\beta$HSD

- Binds strongly to human CBG
- Binds strongly to glucocorticoid receptors
- Biologically active

CORTISOL ($F$)

- Binds moderately to human CBG
- Binds poorly to glucocorticoid receptors
- Biologically inactive

CORTISONE ($E$)
B. PHYSIOLOGIC AND PHARMACOLOGIC EFFECTS OF CORTISOL AND CORTISONE

The importance of the human adrenal glands became evident in 1885 when Thomas Addison described a disease in which patients literally wasted away, caused by the destruction of their adrenal glands. In 1932 Cushing described a syndrome due to glucocorticoid excess. The physiological role of the adrenal glands began to emerge in the early 1900's. Carbohydrate metabolism and electrolyte regulation were associated with adrenal gland functions. In the 1930's some of the compounds in adrenal extracts were identified and synthesized. Deoxycorticosterone was synthesized in 1937 while corticosterone (B) was identified by Reichstein and associates, as well as Kendall and coworkers. F and E were partially synthesized in 1946. F emerged as the hormone responsible for glucocorticoid activity in adrenal extracts and was subsequently found to be the principal glucocorticoid secreted by the adrenal gland. E, a metabolite of F, was found to be biologically inert. (For excellent reviews on the history and actions of glucocorticoids and/or role of the adrenal glands see Monographs on Endocrinology V 12, 1979).

When E became available in the 1950's, it was immediately applied to the treatment of rheumatoid arthritis with encouraging initial success. It was shown to be a potent anti-inflammatory agent and was subsequently found to be efficacious in the treatment of a number of inflammatory diseases. F was soon found to be an even more effective anti-inflammatory agent than E. Thus, the physiologic and pharmacologic importance of F and E underscored the need to study the metabolism of these compounds in vivo and in vitro.
1. Cortisol Versus Cortisone as an Effective Therapeutic Agent

As therapeutic agents, F and E have been used in the treatment of rheumatoid arthritis, skin disorders and various other inflammatory conditions. When these compounds were administered orally or parenterally they both were found to be similarly effective. However, studies on the local effects of E and F injected into arthritic joints revealed that F was biologically active while E was not, although E could be activated upon conversion to F. The mode of action of glucocorticoids at the time remained unknown, and so reasons for differences in therapeutic effectiveness between E and F at the local level were difficult to understand.

(a) Rheumatoid arthritis. The first published report on the comparative effects of E and F as local anti-arthritic agents was by Hollander et al (1951). Injection of E acetate (25 mg) and E into the knee joint of 12 and 9 patients respectively did not result in any objective improvement as evaluated by the measurement of circumference, degree of tenderness, and range of motion of the knee joint. Additional information obtained by performing cell counts of the synovial fluid and recording the intra-articular temperature (shown to reflect changes in joint inflammation) concurred with the above results. F acetate (25 mg) injections resulted in significant improvement in all 178 studies on 51 patients. They also showed that F was beneficial in a number of other inflammatory diseases from various causes such as bursitis, gouty arthritis, osteoarthritis, disseminated lupus erythematosus, and rheumatoid arthritis with eye
inflammation. The results of Hollander and associates (1951) were substantiated by Ziff et al (1952) and by Dixon and Bywaters (1953). These latter authors concluded that the effect of F or E acetate was of a non-specific anti-inflammatory nature.

Orally, F was shown to be 50% more potent than either E or E acetate, even though F acetate was only equal to or less potent than E acetate (Boland, 1952). The author suggested that, because F acetate was much less soluble than F, this reduced its rate and degree of alimentary absorption. Intramuscular injections of F acetate were also found to be more slowly absorbed than E acetate, as assessed by clinical observations, erythrocyte sedimentation rate, and urinary metabolites (Ward et al., 1952).

Following intra-articular injection of 400 mg of F or E (synovial fluid collected following four 100 mg injections), several metabolites were detected, both more and less polar than the injected steroid in synovial fluid (Wilson et al., 1956). These authors found evidence of conversion of E to F in the synovial fluid of some patients but not in others. McEwen et al (1954) detected conversion of E to F within the synovial cavity following the intra-articular injection of 50 or 100 mg of E. Both E and F disappeared rapidly from the synovial cavity following injection.

Peterson et al (1959) injected ¹⁴C labeled E and F (0.1-5 mg) intra-articularly and followed their metabolism and disposition. They showed that ¹⁴C labeled E and F disappeared rapidly from the synovial cavity, but contrary to the results of Wilson et al (1956) and McEwen et al (1954), they did not observe any conversion of E to F by synovial tissue. They suggested that the ineffectiveness of E was due to the lack of conversion of E to F by the synovial tissue.
(b) **Dermatological disorders.** In dermatological therapy, similar to the local treatment of rheumatoid arthritis, topical application of F was shown to be effective (Sulzberger et al, 1953) while that of E was not (Goldman et al, 1952). It was further demonstrated that very little interconversion of E and F occurred (E was not confirmed as being a conversion product of F) in the dermis and apparently none in the epidermis (Malkinson et al, 1959). In vitro incubations of fresh human skin slices with F or E (40 ng/mg) resulted in approximately 10% of the steroid substrate undergoing metabolic alteration, with E being more readily metabolized than F. Hsia and Yu-Lee Hao (1967) reported that conversion of E to F by human skin would not contribute significantly to elevating F levels enough to be therapeutically effective.

(c) **Inflammatory diseases of the eye.** The experimental use of E in the treatment of inflammatory diseases of the eye was investigated by Steffensen and associates (1950). E was administered either intramuscularly or topically to seven patients. Both of these modes of treatment resulted in subjective and objective improvement occurring in all but one of the cases. These results showed that E was effective and were contrary to those for the topical treatment of the other inflammatory diseases with E.

A study done by Hamashige and Potts (1955) on the absorption of E and F by ocular structures revealed some very interesting results. $^{14}$C-E acetate was applied to the anterior corneal surface of rabbit eye. After 20 min, the tissue content of E acetate, E, and F was determined in various parts of the eye. Most of the radioactivity was confined to the cornea and aqueous humor, but none was detected in the lens, vitreous body or retina. Rapid degradation of E acetate occurred, resulting in 20 and 25% conversion to F in the cornea and aqueous humor, respectively. In view of the rapid conversion of E acetate to F, the authors questioned whether E was the therapeutic agent.
2. Effects of Methylated Corticosteroids

Although early studies on the effects of adrenal cortical hormones on muscle work (Ingle et al., 1953) and on the level of plasma amino acids in adrenalectomized and eviscerated rats (Bondy et al., 1954) showed that E was as effective as F, no consideration was given to possible transformations outside of the liver. Subsequent studies on various effects of F and E, administered systemically, showed that 20-50% more E was required to elicit a response equivalent to that obtained with F in various biological assay systems. The ability of E and F to suppress urinary 17-ketosteroids in humans, which reflects inhibition of ACTH secretion, was measured by Kupperman et al. (1955). F was shown to be more effective than E, even though the E to F dose ratio was 1.25. A study of the ability of corticoids to cause involution of the thymus gland in rats showed F to be 30 to 50% more potent than E in the thymus involution assay (Stephenson, 1956).

Studies of the metabolism and effects of the 2-methylcorticosteroids provided conclusive evidence that F, and analogues of F retaining an 11β-hydroxyl group, contained all of the biologic activity.

The relative potencies of various methylated and non-methylated corticosteroids on several metabolic parameters were studied by Liddle et al. (1956). In adrenalectomized dog, 2-methyl-F and 2-methyl-E had relative potassium-losing activity potencies of 1 and 0.009 respectively. F had a potency of only 0.04, and E was half as potent (0.02). Differences in metabolism in vitro between the methylated and non-methylated steroid were found in studies using rat liver. Active reduction of ring A did not occur with 2-methyl-F, but did with non-methylated corticoids. The authors suggested that 2-methylation of E resulted in loss of biologic activity.
Using a glycogen and an anti-inflammatory test in rats to assess the effects of 2-methylation on glucocorticoid activity, Dulin et al (1957) showed that 2-methyl-F was 3-5 times more potent than F, yet 2-methyl-E was 1.5-6 times less effective than E, which possessed half the activity of F. The reason for the discrepancy between potencies of 2-methyl-F and 2-methyl-E was explained in a subsequent paper by the same group of researchers (Glenn et al, 1957). They hypothesized that, if the 11-reduced form of the corticosteroid was biologically active and the 2-methyl-E was relatively inactive, then perhaps the conversion in vivo of 2-methyl-E to 2-methyl-F was slow as compared to the reduction of E to F. To study the question of conversion of the 2-methylated corticoids, rat liver microsomal fractions and cofactors were used. Very little 2-methyl-E was converted to 2-methyl-F (not enough to quantitate or identify) and therefore, they reasoned that the lack of anti-inflammatory or glycogenic activity of 2-methyl-E was due to its lack of conversion by the liver to 2-methyl-F.

Urinary metabolites of 2-methyl-E and 2-methyl-F were examined following their oral administration to male subjects by Bush and Mahesh (1958a). Most metabolites of 2-methyl-E were 11-keto analogues and only 10% were of the 11β-OH form. Similarly, 2-methyl-F administration gave rise to predominantly 11β-OH metabolites. In another study, when 50 mg of 2-methyl-E was given orally, 800 μg was excreted unchanged and 80 μg as 2-methyl-F; only 2-methyl-E was detected in plasma (Bush and Mahesh, 1957). This was in distinct contrast with E, which, when given orally, was in large measure converted to F in the body (Peterson et al, 1957).
3. Effects of Cortisol and Cortisone on Growth

(a) Connective tissue. A number of studies in the late 40's and early 50's were conducted on the effects of adrenal steroids on wound-healing (Ragan et al, 1949; Ragan et al, 1950; Baker and Whitaker, 1950), showing them to be inhibitory to the wound-healing process.

Alrich and colleagues (1951), studying the effects of ACTH and E on wound healing, showed that rats receiving daily 6 mg of E intramuscularly, had profoundly retarded healing of their standard wound (abdominal incision). They noted a delay in the proliferation of fibroblasts and the appearance of the usual cellular elements of repair of mesenchymal origin.

In the above studies on wound healing large doses of corticosteroids inhibited the wound healing process; however, when much smaller doses were employed there was no delay in wound healing and the tensile strength was similar to controls 20 days after wounding (Berliner and Nabors, 1967). Although the dose of corticosteroids applied to wounds in mouse and dog did suppress the inflammatory response and fibroblast proliferation, as well as decrease mucopolysaccharide production and fibrogenesis (collagen formation) initially, the hormones did not diminish the tensile strength of the matured wound (Berliner and Nabors, 1967). Epithelialization of the wound was not impaired (Berliner and Nabors, 1967), which had also been found earlier by Spain and associates (1950) for mice following E injections.

Since connective tissue was thought to be one of the principal targets of adrenal steroid action, many studies were undertaken to investigate the effects of corticosteroids on this and other tissues in culture.

Initial studies on the effects of E on tissue cultures were negative (Steen, 1951; Baldridge et al, 1951). Using the hanging drop culture
method, Steen (1951) studied 14 day old chick embryo heart muscle, lungs, and intestine cultured in 50% chick embryo extract (CEE) and 50% fowl plasma with the addition of 2–2,000 μg/ml of E acetate. He observed no inhibition of growth when 2–50 μg/ml of E was used. However, 500–2,000 μg/ml of E did inhibit growth somewhat, an observation which he attributed to a gross disturbance in the ionic balance of the culture medium, and from which he concluded that E's effects on wound healing were indirect in the living organism.

Baldridge and associates (1951) studied the effects of E on mesodermal tissue. Human leukocytes and chick embryo heart and thigh muscle were cultured on cover slips in a culture medium consisting of 33% each of chick plasma, human serum and CEE, and 50 μg/ml of E acetate. E had no inhibitory effect on leukocyte migration or on chick embryo fibroblast proliferation. These results therefore, did not show any direct effects of E on tissue cultures.

Fibroblast growth was not observed to be inhibited in explants of 9–10 day-old embryonic chick skin grown in 50% chick plasma and 50% CEE with 0.5 to 25 μg of E (Paff and Stewart, 1953).

In these three previous studies no attempt was made to quantitate the effects of E. Kaufman and colleagues (1953) measured the extent of migration of fibroblasts and thus were able to quantitate the effects of E and F on tissue cultures (plasma clot technique) of 16–19 day-old chick embryo hearts. The explants were grown in tissue culture medium consisting of chick plasma (33%), CEE (20%), rabbit serum (33%), and Hank's balanced salt solution (13%) with 5–100 μg/ml of E or F. Five μg/ml of F or 10 μg/ml of E added to two day-old cultures inhibited fibroblast migration by 11% and 13% respectively. Increasing concentrations of E and F resulted in increasingly more inhibition, although the greatest relative inhibition
occurred at the lowest concentrations employed. Inhibition, however, could not be detected between 2 and 4 days of culture, perhaps indicating that the cells adapted to the presence of the steroids. They ascribed the negative results in the previous studies regarding the effects of E on tissue cultures to a variation of methods, media (20% CEE versus 33% and 50% CEE), and the use of E unconjugated versus E acetate (Steen, 1951; Baldridge et al, 1952). They concluded that indeed the effects of E and F were exerted directly upon fibroblasts at the cellular level in vitro. In retrospect, this study also demonstrated the ability of chick embryo fibroblasts to convert E to F.

CEE was subsequently shown by Grossfeld and Ragan (1954) to be antagonistic to the effects of E and F. They studied fragments from 10-12 day-old chick embryo hearts as well as stomach and intestines using the hanging drop method of culture. CEE (20-50%) in chick amniotic fluid, used as culture medium, resulted in a great variation of the effects of F from one experiment to the next. When medium was made with chick plasma and amniotic fluid without CEE, 200-400 µg/ml of F produced easily reproducible results. E acetate was much less inhibitory to fibroblastic outgrowth than F. That some E acetate was converted to F was indicated by the slight inhibitory effect it had. F (250 µg/ml) inhibited the growth of fibroblasts from explants of stomach and intestine but had no inhibitory effect on growth of epithelial sheets, which was actually increased slightly. This effect on the epithelial cells was thought to be indirect due to the suppression of fibroblast growth. Grossfeld and Ragan (1954) found that removal of F from the medium resulted in complete recovery of growth equal to that of control cultures.
Growth of stock cultures of L strain fibroblasts (normal connective tissue of adult C3H strain mouse) in a nutrient mixture of horse serum (40%), CEE (20%) and Earle's saline (40%), was inhibited 58% by F (10 μg/ml) and 27% by E acetate (10 μg/ml), demonstrating that L strain fibroblasts were probably capable of converting E to F (Holden and Adams, 1957). F also caused changes in the morphology of the cells. The fibroblasts were less spindle-shaped and there was a definite shortening of their terminal processes; they also tended to cohere in sheets very similar to the appearance of epithelial cells in culture. When cells were studied over a 14 day period, 10 and 20 μg/ml of F were only inhibitory during the first 7 days, but surprisingly, from days 7-11 the growth rate was the same as for the control cells grown without steroid. One possible explanation is that the cells or a sub-population of cells were or became resistant to the inhibitory effects of F. Holden and Adams also showed that E or F were unable to influence or alter the mitotic index or 4 phases of mitosis of rat fibroblasts.

Variants of human uterine fibroblasts, discovered by Grosser and Swim (1958), were able to proliferate in medium containing up to 25 μg/ml of F while the original strain (U12-79) was inhibited by as little as 0.1 μg/ml of F or corticosterone (B). Many other steroids were also found to be inhibitory to growth of strain U12-79; estradiol, progesterone, testosterone and deoxycorticosterone (5 μg/ml), estriol, 11β-androstenedione and cholesterol (25 μg/ml). The reasons for the relative resistance of variants to steroids was and is not understood. Some researchers suggested that increased metabolism decreased the intracellular level of the steroid (see below), but this could not account fully for the increased resistance. It was also thought that perhaps the inability of the steroids to penetrate into the cells would render them resistant. However, Burton (1964) found no evidence for this.
Berliner (1964) found that L strain fibroblasts (primary cultures) grown in Eagle's minimum essential medium (MEM) supplemented with 10% calf serum was inhibited 90% by 10 μg/ml of F. Fluocinolone acetonide, at 0.1 μg/ml, was a potent inhibitor of fibroblast growth. This steroid was found to be poorly metabolized over a 48 hr period by L strain fibroblasts, which actively metabolized F, suggesting that the ability of fluocinolone acetonide to inhibit growth at lower concentrations was because it remained in its active form for a longer period of time than F.

Fibroblasts of Clone 929, strain L, were studied by Berliner and Ruhmann (Ruhmann and Berliner, 1965; Berliner and Ruhmann, 1966). The cells were grown in Eagle's MEM supplemented with 10% calf serum. F was able to inhibit growth at 0.1 μg/ml whereas in a previous study (Berliner, 1964) 10 times as much was necessary to inhibit growth; however, in the first study primary cultures were used, whereas in these two latter studies clones were utilized which may have accounted for the observed differences. E, 2-methyl-E, prednisone and 11-dehydrocorticosterone (A), even at 10 μg/ml, were not inhibitory to growth; if anything, E-treated cells' growth rate was accelerated over control levels. 2-Methyl-E was also somewhat stimulatory to growth. Clone 929, L strain, was shown to be incapable of interconverting F and E (Berliner and Ruhmann, 1966), although these cells actively converted E to 20-DH-E, 46%, and F to 20-DH-F, 25%, over a 48 hr period. This was in contrast to the L strain used by Holden and Adams (1957) which apparently could convert E to F, since E was about 50% as active as F in inhibiting growth.
(b) Leukemic tissue. Using an in vitro system for studying the lymphocytolytic effects of adrenal steroids, Gabourel and Aronow (1962) showed that a concentration as low as $10^{-7}$M (36 ng/ml) was effective in inhibiting 50% of mouse lymphoma (ML-388) cellular growth in culture. The 11-keto steroids E, A, and prednisone, were inactive in inhibiting ML-388 growth at concentrations at which F was inhibitory and were only effective at doses which were greater than $10^{-5}$ M (3.6 µg/ml) suggesting that the cells were incapable of converting the 11-keto to the active 11-OH compounds to any appreciable extent.

Fischer and Welch (1963) studied the effects of corticosteroids on the growth of mouse leukemia L5178Y cells in vivo and in vitro. They found that the steroidal structures which inhibited cell growth in culture were similar to those active in vivo, implying that the effects of these compounds were direct ones on the leukemic cells. In vitro, 20 ng/ml of F decreased by 50% the number of generations. Other steroids (9-bromo-11-keto-progesterone, E, 11-desoxycorticosterone, 11β-OH-progesterone, testosterone), at concentrations greater than 40 ng/ml, also inhibited growth in vitro, but they were all inactive in vivo. Thus, the mechanism by which steroids inhibit cellular growth in culture do not always reflect their in vivo activity.

E was the only exception to the above steroids listed in that E, at 350 times the concentration (7 µg/ml) of F, was ineffective in vitro yet was as effective as F in vivo indicating that conversion to F is a necessary step in the activation of E (carried out by the liver) and that the leukemic cells lacked any 11-HSD activity (i.e. conversion of E to F was not apparent).
4. Effects of Cortisol on the Inflammatory Response

Many studies on the anti-inflammatory effects of corticosteroids were performed by Dougherty and Berliner (and various associates) during the 50's, 60's and early 70's or the pre-steroid receptor era. Much of their attention was focused on the metabolism of these steroids by the cells involved in the inflammatory response.

It had been shown by Dougherty (1952) that adrenalectomized mice were severely compromised in resisting death from anaphylactic shock. Injection of E acetate restored protection to the adrenalectomized animal by inhibiting the destruction of fibroblasts and the number of invading polymorphonuclear leukocytes (PMN's) at the site of inflammation (Dougherty and Schneebeli, 1955) as opposed to affecting the antigen-antibody reaction. F was found to be 76 times more potent an antiphlogistic agent than E in mice. F was subsequently shown to be the most potent naturally occurring antiphlogistic agent followed in potency by E, B, and A. Connective tissue, which was involved in the inflammatory process as well as in wound healing, thus became the next target of investigation.

In the mouse, as well as other species, approximately 90% of connective tissue is comprised of fibroblasts. Dougherty and colleagues showed that the local administration of $^{14}$C-F to an inflamed area resulted in its localization in fibroblasts and to a lesser extent in fat cells. Radiographs of loose connective tissue did not show any uptake of radioactivity by other cell types (mast cells, PMN's, eosinophils, lymphocytes, histiocytes) (Dougherty et al, 1956). F also caused the cytoplasmic processes of the fibroblasts to withdraw and to become spherical. The endothelial cells in inflamed connective tissue behaved in a similar
fashion to fibroblasts following F treatment (Dougherty et al, 1973). Cellular processes such as pinocytosis, phagocytosis, and motility of fibroblasts and endothelial cells, were stopped in vitro within minutes of hormone treatment. F has also been shown to maintain the fibres of the inflamed loose connective tissue, whereas, destruction of fibres occurred without hormone treatment (Dougherty et al, 1973). These changes were also observed in tissue cultured fibroblasts following F treatment (Holden and Adams, 1958; Berliner et al, 1967).

An interesting experiment was carried out by Dougherty and associates (1958) in which the metabolism of F was studied during inflammation. Ten adrenalectomized female mice were topically inflamed by purified egg albumin, administered subcutaneously, followed 6 hr later by the injection of $^{14}$C-F through the tail vein (31 μg). The inflammation subsided 100 min after the hormone had been injected although at this time very little radioactivity could be detected in the inflamed connective tissue. After 100 min, 20% of the total radioactivity in the blood was F, with conjugates of F (THF-glucuronide) and THF rising to 65% and 15% respectively by 20 min, remaining constant thereafter. Although more radioactivity could be detected in the inflamed tissue at any given time, this was not due to any specific mechanism for localizing F, but rather could be attributed to increased capillary permeability and edema formation in the areas of inflammation. In fact the same proportions of F and its metabolites were detected in normal and inflamed connective tissue.

The greatest amount of radioactivity in the inflamed connective tissue occurred 40 min after the injection of $^{14}$C-F and it amounted to 9 ng/mg of dry tissue; half of this was conjugates of F, leaving only 4.5 ng/mg of radioactive steroid including a small portion of metabolites which
was subsequently identified (Berliner and Dougherty, 1958a). Therefore, F was shown to enter the inflamed tissue in a non-specific manner and produce its anti-inflammatory effects even though a major portion of the steroid was metabolized and excreted. Of the 30 µg of 14C-F which had been originally injected into the tail vein, there was never more than 4.5 ng/mg of the dose at the site of inflammation, yet this was enough to inhibit the inflammatory response.

Dougherty suggested that F was able to inhibit the maximum development of any stage of inflammation and that it acted within the area of inflammation in a direct manner. These early studies on the therapeutic and physiologic effects of F and E showed that F was biologically active and E inactive. However, E had potential biologic activity since it could be converted to F in the body.

C. CORTISOL AND CORTISONE METABOLISM IN VIVO IN MAN

1. Cortisol and Cortisone Metabolism as Reflected in Urine

The first evidence for the conversion of E to F was obtained by Mason (1950). Following the administration of E acetate to 2 Addisonian patients, urine extracts indicated that reduction of the 11-keto to the 11-OH group had occurred. The possibility of endogenous F production was ruled out, since both patients had severe Addison's disease. Other steroids, now known to be metabolites of F were also discovered in the early 1950's by Lieberman and associates (1950; 1951). They identified THE, THF, as well as E and F in the urine of human subjects after the administration of ACTH. Mason's work was confirmed by Burton and colleagues (1953) who looked at corticosteroid excretion after parenteral administration of E acetate to 5 patients, 2 of which were Addisonian. Finding increased excretion of F, F,
THE, and THF, they surmised that non-adrenal tissues were responsible for the conversion of E to those other compounds. These authors further considered that conversion of E to F might be necessary for biologic potency, in view of the evidence from Hollander and associates (1951) which showed that E was without anti-rheumatic effect following its injection into the knee joint, while F was very effective (see above).

The group of Burstein, Savard and Dorfman studied the in vivo metabolism of several steroids in man. They showed that the urinary excretion pattern was the same whether E or F was administered orally (Burstein et al., 1953a; Burstein et al., 1953b). They isolated the compounds E, F, THE, THF and several 17-ketosteroids. This, therefore, was the first demonstration of the conversion of F to E in vivo. Another of their studies (Savard et al., 1953) indicated that the enzyme responsible for the interconversion of E and F had a wide substrate specificity which included 17-ketosteroids. Administration of adrenosterone to man yielded several metabolites including 11β-OH-androsterone and 11β-OH-etiocholanolone, clearly demonstrating the biological reduction of the 11-keto to the 11-OH group in these C-19 steroids.

Using both labelled and unlabelled E to study its metabolism in vivo in man, Peterson and associates (1957) were able to obtain information regarding transformation, absorption, and excretion rates of E and its
metabolites, and conversion of E to F. Following the infusion of a tracer quantity of \(^{14}\text{C}-\text{E}\) plus 200 mg of carrier E, 91% of the radioactivity could be accounted for through urinary excretion by the fourth day. Of this, 7% was excreted as unconjugated steroids (0.3%, as E and 0.6% as F) and another 55% could be extracted after treatment with bacterial \(\beta\)-glucuronidase. They only identified E, F, and THE and THF unconjugated, or conjugated to glucuronic acid. They surmised that other, as yet unidentified metabolites and sulfoconjugated metabolites, could account for the undetermined radioactivity.

Fukushima and coworkers (1960) studied in detail the metabolism of \(^{14}\text{C}-\text{F}\) in normal men. They found that 75% of the iv administered dose could be accounted for in the urine after the first day. Of this, 73% was extractable after ketodase treatment (neutral steroid extract, unconjugated plus conjugated) and approximately another 2.5% was extracted following rehydrolysis with \(\text{IN}\) acid which liberated the sulfoconjugated metabolites. Eleven metabolites (E, cortol, \(\beta\)-cortol, cortolone, \(\beta\)-cortolone, THF, alloTHF, THE, 11\(\beta\)-OH-etiocholanolone, 11\(\beta\)-OH-androsterone, 11\(\beta\)-keto-etiocholanolone) accounted for 90% of the neutral steroid extract. There was extensive conversion of the 11-OH to the 11-keto group. The ratio of 11-keto to 11-OH was 1.7-5 for the cortols and cortolones (18-33% of the neutral steroid fraction), and 0.9-2.1 for THE and THF (THE + THF + alloTHF = 45 to 50% of the neutral steroid fraction). The E:F ratio was about 1 (E + F about 2% of neutral steroid fraction). Kowarski et al (1969) also found that urinary metabolites reflected extensive although incomplete interconversion between E and F. They employed \(^{14}\text{C}-\text{F}\) and \(^{3}\text{H}-\text{E}\) as tracers in doses of 0.24 and 0.015 \(\mu\)g respectively as compared with 250 \(\mu\)g of \(^{14}\text{C}-\text{F}\) administered iv by Fukushima et al (1960).
2. Cortisol Metabolism in the Human Forearm In Vivo

The metabolism of F in vivo, by tissues of the forearm in a normal subject, was studied by Jenkins (1966). Ten μg of 14C-F was infused at a constant rate into the brachial artery for a period of 30 min with blood sampling done at 10 min intervals from a deep vein from both arms. Approximately 1% of 14C-F present in the blood of the infused arm was converted to E and to 20-DH-F. These metabolites were not found in the blood from the opposite arm.

A more detailed study on the uptake and metabolism of F by the human forearm in vivo was conducted by Asmal et al (1974). Infusion of 3H-F resulted in the conversion of a small fraction to a compound which was chromatographically similar to E. The use of 3H-F, with its higher specific activity than 14C-F, caused a minimal increase (1%) of F upon infusion which was a more physiological approach compared to the infusion of 14C-F. F was shown to penetrate into the tissues in a manner which could not be accounted for by transit time in the vascular bed alone.

The in vivo work of Jenkins (1966) and of Asmal et al (1974) on the metabolism of F by forearm tissue in humans showed that the tissue could only metabolize a very small fraction of F and the only metabolites that were detected were E and 20-OH-F.

3. Cortisol, Cortisone Interconversion in Blood

Extensive interconversion was reported to occur in blood by Bailey and West (1969) and by Dazord et al (1972). Bailey and West suggested that tissue, other than the liver, capable of effecting this interconversion, could control the level of the active hormone F either by converting E to F or by metabolizing F. By utilizing a constant infusion technique of both
$^{3}$H-F and $^{14}$C-E, Dazord et al (1972) were able to measure the interconversion of E and F in normal subjects. They calculated that 60% of the iv-infused $^{3}$H-F was converted to $^{3}$H-E and 85% of the infused $^{14}$C-E was converted to $^{14}$C-F. However, in the plasma the $^{3}$H-E:$^{3}$H-F ratio was much lower than the $^{14}$C-F:$^{14}$C-E ratio, indicating that the equilibrium of the reaction clearly favoured F. They also found evidence for secretion of E from the adrenals by measuring a higher concentration of E in the adrenal vein compared to the peripheral venous blood, but the concentration of E in the adrenal vein was 20-30 times less than that of F. Therefore, in man, interconversion of E and F occurs with the equilibrium in plasma favouring the conversion to F. Both Bailey and West (1969) and Dazord et al. (1972) suggested a role for the kidney in the oxidation of F to account for the discrepancy in the proportion of 11-OH:11-keto metabolites found in urine compared with E, F interconversion measured in plasma (see below).

4. Interconversion of Pharmacologic Doses of Corticosteroids

E (200 mg) given either orally or intramuscularly resulted in the conversion of 1/2 to 2/3 of E to F, as measured in plasma, 30 min later. Iv administration resulted in lower plasma levels of F presumably due to conversion of E to THE (Peterson et al, 1957). In the treatment of rheumatoid arthritis, E, as an anti-inflammatory agent, had been shown to be 2/3 as effective as F on a weight basis. These authors, therefore, speculated that most, if not all, of the potency of E resided in F following its conversion in the body.

Relatively few studies have been conducted on the in vivo conversion of pharmacologic doses of E to F in plasma. Jenkins (1967) addressed himself to this question in more detail than Peterson and colleagues (1957)
had, confirming their results completely and extending them. Nine normal subjects, 3 Addisonian patients, and 4 patients with liver disease, were given E, F, or prednisone orally ranging in dose from 25 to 200 mg. The results indicated that at lower doses, E was more efficiently converted to F, while at higher doses proportionally more E was converted to THE. Thus, administration of 100 mg of F resulted in F plasma levels similar to those after 200 mg of E. Prednisone was much more efficiently converted to prednisolone and plasma levels of prednisolone were not much different whether prednisone or prednisolone was administered. E could not be detected after F administration. Interestingly, the peak prednisolone plasma level after prednisolone administration was twice that of F following the same administered dose as F. Jenkins concluded that the above mentioned differences between the F and prednisolone plasma levels achieved were because following conversion of F to E the reduction of ring A occurred forming THE, but ring A of prednisone once formed could not be reduced (Sandberg and Slaunwhite, 1957). Thus, therapeutically, systemic administration of prednisone or prednisolone would produce equivalent results, E, on the other hand, would only be 1/2 to 2/3 as effective as F. The results of Peterson's group and those of Jenkins on E and F conversion in plasma, reflect the handling by the body of large amounts of these steroids, and F was not detected to be converted to E. Yet, in the physiological state, there is evidence of interconversion as seen in urine (Fukushima et al, 1960; Kowarski et al, 1969) and to a lesser degree in plasma (Bailey and West, 1969; Bazord et al, 1972).
D. 11-HSD ACTIVITY IN VITRO IN ANIMAL AND MAN

1. Liver

Schneider and Horstmann (1952) found that rat liver was capable of considerable metabolism of both E and F in terms of reduction of the conjugated unsaturated system (E to THE) and degradation of the side chain (E to 17-ketosteroids) although the products were not identified. A comparison of the ability of various rat tissues to metabolize E revealed that liver was the most active, then kidney, with relatively little activity in adrenal homogenates, diaphragm segments, heart slices or rectus mince. They found that liver or kidney slices were twice as active as their respective minces (equivalent wt) and the activity was not restored by adding cofactors NAD or NADH. They suggested that the physiological inactivation of the hormone E occurred through reduction to possibly THE, which was irreversible, followed by conjugation with glucuronic acid.

Fish, Hayano and Pincus (1953) were the first to report the conversion of E to F in vitro. Conversion of 30% E to F occurred in incubations of pig liver homogenates, at a tissue to steroid ratio of 200:1. Beef and rat liver homogenates were also effective in converting E to F. Conversion in the opposite direction was not studied. These authors also found compounds with a reduced C-20 ketone (carbonyl group), in agreement with the previous work by Schneider and Horstmann (1953). These results prompted a closer examination of steroid metabolism by the liver.

Hechter et al (1953) reported on the rapid metabolism of E and F in rat liver perfusions (corticosteroids were added at a concentration of 100 \(\mu g/ml\); rat liver wt 10.8-23.2 g). The disappearance of various functional groups of the corticosteroids was measured in order to assess their
metabolism, and it was found that the rat liver was capable of a tremendous amount of metabolism (100 μg/min/g initial rate). The authors hypothesized that if E and F possessed antiarthritic and thymolytic activity in vivo, yet were so efficiently metabolized by the liver in vitro, that metabolically transformed products may have also possessed the above activities. Hechter et al (1953) went on to say that "a simple conversion of cortisone to 17-hydroxycorticosterone (F) would not suffice to explain the oral activity of cortisone." In retrospect, however, such a conversion does indeed explain the therapeutic efficacy of E.

Several other investigators in 1953 showed that the liver was capable of converting E to F in vitro (Caspi et al, 1953; Eisenstein, 1953; Amelung et al, 1953a). Caspi et al (1953) isolated F after perfusion of rat liver with E. F was also identified following incubation of rat liver slices (Eisenstein, 1955) or incubations of rat, pork and beef liver homogenates (Amelung et al, 1953a) with E. This latter group also found microsomes to be active, although the whole homogenate was more active than the microsomes, suggesting that the enzyme activity resided in the structural elements of liver cells.

Hubener et al (1956) confirmed the work of Amelung et al (1953a) using rat liver homogenates incubated with E. They showed that part, if not most, of the enzyme activity was located in the microsomal fraction. They also suggested that the reaction was reversible, since, in an earlier experiment they had found conversion of F to E (Amelung et al, 1953b). Therefore, they named the enzyme 11β-hydroxydehydrogenase (the enzyme has subsequently been known as 11β-hydroxysteroid dehydrogenase, 11β-oxidoreductase or 11β-reductase). That the 11-HSD activity was reversible was also demonstrated by Hurlock and Talalay (1959). The 11-HSD activity of
male rat livers was localized in the microsomes and the enzyme showed dual nucleotide specificity for NAD(H) and NADP(H) which acted as cofactors (the words cofactor and coenzyme are of equivalent meaning and are used interchangeably).

Brown et al (1957) compared the ability of various rat tissues to metabolize F. While most tissues (kidney, G.I. tract, spleen, adrenal, and pituitary) could metabolize small but significant amounts of F, the liver was found to be the most active. The microsomal fraction could metabolize considerable amounts of F (see below).

Sex differences in 11-HSD activity in liver, as well as kidney and gonads in rat, were reported by Ghraf and associates (1975b). The ontogeny of the 11-HSD activity (F to E) was studied from day 15 of life onward. In the microsomal fraction of liver of both sexes it rose rapidly, peaking on day 30 and subsequently falling and leveling off in both males and females by day 60, with activity at least 3 times greater in males than in females.

Mouse liver minces metabolized $^{14}$C-E predominantly to F (69%) with lesser conversion to 20-DH-F (13%) and 20-DH-E (12%) (Sweat and Bryson, 1960). Addition of NADP pushed the reaction in favour of 20-DH-F (30%).

Substantial 11-HSD activity was shown to occur in rat, mouse and rabbit liver homogenates, incubated with $^{14}$C-F (0.4 μg/mg) (Koerner, 1966). The enzyme in the liver was associated with the microsomal fraction with NADP being the preferred cofactor, although NAD was also effective.

Extensive studies on 11-HSD activity in rat, guinea pig, rabbit and calf liver homogenates, and various preparations derived from them by ultracentrifugation, were carried out by Bush, Hunter and Meigs (1968). They looked at different substrates and at coenzyme requirements, as well as enzyme kinetics. In contradistinction to previous results they found
NADP(H) to be effective while NAD(H) was very poor as a coenzyme for the reaction. Reduction of the 11-ketosteroids E, A, 11-oxo-progesterone, and adrenosterone occurred at comparable initial velocities. The addition of a 2α-methyl group prevented the reduction of the 11-keto group. B was oxidized at the same rate as F, while 11β-OH-androsterone was rapidly oxidized in contrast to prednisolone which was converted to prednisone at 1/5 of the initial rate of F oxidation.

In human liver (Meigs and Engel, 1961), 11-HSD activity was also localized in the microsomal fraction. The enzyme reaction was found to be reversible, with NAD(H) or NADPH(H) being equally effective as cofactors.

Although these studies showed that the liver metabolized F, other tissues were involved as well. A report by Murphy and West (1964) in fact showed that when $^3$H-F (10 μg), taken by a volunteer, was converted to conjugated F metabolites (75.4% of the administered dose) little of the substrate metabolized by the liver was unchanged F.

2. Kidney

Cope and Hurlock (1954) speculated that the kidney might be involved in corticosteroid metabolism since the E:F ratio was high in the urine in comparison with blood. This turned out to be correct. The oxidation of F to a number of conversion products, including E, by mammalian kidney was first demonstrated by Ganis et al (1956). Bovine kidney minces (80 gm) were incubated with 500 mg of F of which 37% was recovered. Of this, 14% was converted to 20-OH-F and only 1% to E. Bovine kidney homogenate (10 ml) was also incubated with F (25 mg). A number of conversion products were identified, among them E, as well as 20-OH-F and 17-ketosteroids (11-keto-androstenedione and 11-OH-androstenedione).
When 100 µg of F (plus NADP as cofactor) was incubated with rat kidney slices or minces (1 g) conversion to E averaged 26%, to 20-DH-E, 35%, and to 20-DH-F only 11%, which accounted for 68% recovery (Mahesh and Ulrich, 1960). Under the same conditions homogenates were less active, converting F to E (21%), 20-DH-E (3%), and 20-DH-F (2%). Both NAD and NADP were effective as cofactors, in agreement with rat liver studies (Hurlock and Talalay, 1959). With E as substrate, a different metabolic picture emerged (Mahesh and Ulrich, 1960). In slices, minces, and homogenates E was mainly reduced at the 20 position to form 20-DH-E (55%), and to a much lesser extent to F (3%), and to 20-DH-F (4%). The nuclear fraction and microsomes were found to contain some of the 11-HSD activity. 11-HSD activity was highest in slices and minces and lowest in homogenates and subcellular fractions, which was thought to be due to the disruption of the cellular organization.

The ontogeny of 11-HSD activity was studied in rat kidney by Ghraf et al (1975a). Using 0.2 mg of cytoplasmic or microsomal fraction with 42 µg/ml of F (plus NAD as cofactor), enzyme activity increased in the microsomal fraction from day 15 and plateaued at day 30, remaining constant in males for the duration of the study (150 days), while decreasing in females. The 11-HSD activity was restricted to the microsomal fraction of the kidney.

11-HSD activity was shown to be fairly high in kidney homogenates of rat, mouse, and rabbit incubated with $^{14}$C-F (0.4 µg/mg) (NADP preferred cofactor) and was localized in the microsomal fraction (Koerner, 1966).

Jenkins (1966) studied F to E metabolism in human kidney tissue. One g slices were incubated with 1.6 µg of F or 0.4 µg of E. Of the 78.5% of the radioactivity which was recovered after incubation with $^{14}$C-F, 65.8%
was converted to E and only 11.3% and 4.4% to 20-DH-E and 20-DH-F respectively; only 17.3% of F remained unconverted. However, when the kidney slices were incubated with $^{14}$C-E (66% of the radioactivity was recovered), 87% was unchanged and 12% was reduced forming 20-DH-E with only minimal conversion to F (0.9%) (cofactors were not used in this incubation).

A study on renal oxidation of F in man in vivo (Hellman et al, 1971) using 11-$^3$H-F, showed that the maximum rate of renal oxidation was at least 10% of all the tritium oxidized. They suggested that the kidney plays an important role in the metabolism of F apart from its function in eliminating end-products.

Whether the species differences which exist between cow (Ganis et al, 1956), rat (Mahesh and Ulrich, 1960), and human kidney (Jenkins, 1966) in terms of F metabolism are physiological and/or experimental (due to different concentrations employed in the experiments: 6.25 mg/g, 100 µg/g, and 1.6 µg/g respectively) is difficult to determine. Certainly, in all species studied, the 11-HSD activity in the kidney clearly favoured the oxidative direction (F to E) with insignificant E to F conversion occurring.

3. Adrenal Gland

A study of conversion of B to A by the adrenal gland of various animal species revealed differences in 11-HSD activity (Fazekas et al, 1970). Adrenal homogenates were incubated with $^3$H-B (0.1 µg/mg) and no added cofactors. Homogenates from cat and rabbit converted 95% and 53% B to A respectively, while man and guinea pig oxidized 20% of B. Rat adrenal was relatively inactive converting only 8% of B to A. Rabbit adrenal homogenates incubated with $^3$H-A (0.4 µg/mg) were incapable of reducing A to B
unles NADPH was added (29% conversion); NADH was not effective (Kokai and Fazekas, 1968). Mouse and rat whole adrenals could convert A to B, albeit to a limited extent (less than 4%) (De Nicola et al, 1969). Fazekas and associates (1970) suggested that differences in 11-HSD activity in the adrenal of various species may be due to differences in the utilization of A as a substrate for further 18-hydroxylation which does not occur in rat or mouse adrenals (De Nicola et al, 1969) in vitro. Although interconversion can occur in rabbit adrenal homogenates with the addition of cofactors (Kokai and Fazekas, 1968), 11-HSD oxidative activity (B to A) in rabbit adrenal slices predominated, conversion of B to A was as high as 92% (Kokai and Fazekas, 1968) - and may be more indicative of in vivo activity.

4. Intestine

Incubations of large and small intestines of rat with F (0.6 μg/mg) were carried out by Mahesh and Ulrich (1960). Very little 11-HSD activity could be demonstrated, less than 1% conversion of F to E. However, in a subsequent study, 50% interconversion of F and E occurred in everted sacs of rat small and large intestine (Stahl and Tapley, 1963).

In human very little 11-HSD activity (E to F or F to E) was detected in minces of intestine in which tracer amounts of 3H-F or 3H-E had been added without the addition of cofactors (Murphy, 1981a).

5. Brain

Brain minces from male rats were capable of metabolizing F to 20-DH-F and E (Mahesh and Ulrich, 1960). However, Grosser and Bliss (1963) were the first to demonstrate the in vitro reversible conversion of 11-hydroxy-steroids in the cerebral cortex of adult male rats. Significant conversion
occurred in minces and homogenates incubated with E, F, or 11β-OH-androstenedione. Increased reaction rates were achieved by addition of NADP or NADPH. Their work was confirmed by Peterson et al (1965) for whole brain of young and adult rats. Homogenates and subcellular fractions were incubated with 14C-F, but reversibility was not tested. The enzyme reaction was shown to be NADP specific and oxidative activity, which was low at birth, increased rapidly in the developing rat brain, reaching a plateau at about 21 days. A more complete study of 14C-E and 14C-F metabolism by adult rat brain was undertaken by Sholiton et al (1965). Metabolism by male and female brain homogenates was examined separately although no differences were detected. F to E conversion averaged 17% with the reverse reaction averaging 22%. The major metabolite formed following incubation of rat brain homogenates with 14C-F was E, with little ring or side chain reduction occurring. Although F was the major metabolite formed after incubation with 14C-E there was significant (about 10%) conversion of 14C-E to THE.

Mahesh and Ulrich (1960) incubated 500 mg of rat brain mince with 300 μg of F (600 ng/mg) which resulted in F to E conversion of 0.04% while conversion of F to 20-DH-F was 0.4%, or ten times greater. In comparison, Sholiton's group (1965) used 14C-F and 14C-E in concentrations of 3.6 ng/mg and 25 ng/mg respectively (25 times and 10 times less than those employed by Mahesh and Ulrich), which resulted in F to E conversion of 17% and only 1-9% side chain reduced compounds (20-DH-E and 20-DH-F). These authors therefore explained the metabolic inactivity seen by Mahesh and Ulrich as being due to the differences in F substrate concentrations. These discrepancies in results are not confined to brain but occur as a general phenomenon in the study of steroid metabolism whereby differences in tissue
preparations—minces, homogenates, tissue slices, perfusions, and cellular fractions—and steroid concentrations (pharmacological versus physiological) have led to problems in the interpretation of results in terms of physiology.

Mouse brain tissue homogenates, fortified with NADP, also metabolized F, converting it to E, 11%, while B (the natural glucocorticoid of mice) was converted to A, 29% (Grosser, 1966). Very little other metabolism was detected. Brain homogenates from mice bearing glioma tumors were 3 times more active in converting F and B to E and A respectively, suggesting that glial cells may be involved in corticosteroid metabolism. Tumor tissue metabolism, however, may or may not be indicative of normal tissue metabolism. 11-HSD oxidative activity was found to be high in 14 day-old fetal mouse brain falling to low levels in 17 day-old fetuses (Tye et al, 1978a; also see 11-HSD Activity in Pregnancy).

14C-F metabolism by homogenates of developing baboon brain differed from that of the adult (Grosser and Axlerod, 1968) and indicated that changes occurred with fetal age. The most active region of the brain, with respect to 11-HSD activity, was the cerebellum during the third trimester (136 days gestation; term = 175 days). However, third trimester cortex, brain stem, and thalamus-hypothalamus also converted significant amounts of F to E. Second trimester and newborn 11-HSD activity was much lower compared with third trimester, although adult 11-HSD activity did increase somewhat. Murphy (1981a) detected conversion of F to E in midgestational human fetal cerebral cortex minces using tracer amounts of 3H-F. However, with the addition of substrate (10 μg/g tissue) to the incubations of cerebral cortex mince, 11-HSD activity fell to undetectable levels; no coenzymes had been added to the incubations.
11-HSD activity in adult lung of various species was detected by Koerner (1966). Homogenates of rat and mouse lung incubated with cofactor and $^{14}$C-F (0.4 µg/mg) contained high enzyme activity while those of pig, sheep, and beef had much lower 11-HSD activity. Only F and E were detected in incubations with $^{14}$C-F. The enzyme activity, conversion of E to F, could also be reversed when NADP was used instead of NADPH as cofactor. When $^{14}$C-E was incubated with homogenates of rat lung (83 ng/mg), 32% of the radioactivity was recovered as F, 16% as alloTHE with only minor amounts of 20-DH-E (2%) and other more polar metabolites, as well as 48% of unchanged E (Sowell et al, 1971). They postulated that the 11-HSD activity in the rat lung would be involved in steroid metabolism. Although 11-HSD activity was detected in sheep, beef, and pig, it was not of the magnitude of activity in mouse or rat. By homogenizing the rat lung tissue and adding cofactors, 11-HSD activity was shown to be reversible (Sowell et al, 1971). However, enzyme reactions in homogenates may or may not occur in intact tissue; for example, the formation of alloTHE by rat lung homogenates (Sowell et al, 1971) was not observed in perfused rat lung (Nicholas and Kim, 1975), adult rabbit (Brooks et al, 1977) or fetal lung (Torday et al, 1976) to any significant extent.

In perfusion studies of adult rat lungs, conversion of E to F was about 60%, while the reverse reaction, F to E, was about 20-25%. Thus, 11-HSD activity was predominantly reductive (Nicholas and Kim, 1975). Under similar experimental conditions adult guinea pig lung was virtually devoid of the ability to convert E to F, but the reverse reaction was not studied (Nicholas and Kim, 1975). Nicholas and Kim suggested that the lung could...
play an important role in maintaining F and E levels in plasma, especially since 100% of the cardiac output passes through the lungs. The suggestion could only be valid for rat lung if the lung is as efficient at converting A to B, the indigenous compounds in the rat. On the other hand, the guinea pig lung would not function in maintaining plasma F levels since the conversion of E to F in the lung was minimal. Therefore, species differences must be taken into account.

7. **Salivary Gland**

Homogenates of rat submandibular salivary gland incubated with $^{14}$C-F (25 ng/mg) contained 11-HSD activity converting F to E (Ferguson and MacPhee, 1975). NAD was a more effective cofactor than NADP and the optimal pH was found to be between pH 8.1 and pH 8.9.

Parotid tissue from dog was shown to be capable of oxidizing F (0.05 ng/mg) to E (Katz and Shannon, 1964).

8. **Muscle**

Metabolism of F and E by mouse muscle tissue was studied by Sweat and Bryson (1960). The major steroid product of F metabolism was E and the 11-HSD activity was NADP specific. The reverse reaction was also observed. However, the major conversion product following incubation of muscle tissue with $^{14}$C-E (2 ng/mg) was 20-DH-E, 37% conversion, with similar amounts of conversion to F and 20-DH-F, 23% and 20% respectively, without added cofactor. Addition of NADP favoured the conversion of E to 20-DH-E (65%) while the conversion to F and 20-DH-F decreased (10% and 15% respectively). This was in contrast to metabolism by liver tissue where E was mainly converted to F, 70%, and relatively minor amounts were converted to 20-DH-F.
(13%) or 20-DH-E (12%). NADP pushed the reaction towards 20-DH-F with no further increase in 20-DH-E.

Dog heart muscle incubated with $^3$H-E or $^3$H-F was to a small extent capable of interconverting the two steroids. F to E conversion was 8% while E to F conversion was only 5% (Kolanowski et al, 1981).

Human skeletal muscle mince was only capable of a limited amount of E to F or F to E conversion when incubated with radioactive tracers E and F (Murphy, 1981a).

9. Uterus

11-HSD activity was studied in the non-pregnant and pregnant uterus (Murphy, 1977b). Minced tissue (0.2 g) was incubated with $^3$H-F or $^3$H-E for 2 hr. In the non-pregnant uterus F to E conversion predominated, but in the pregnant uterus at term E to F conversion was the favoured direction. This increase in E to F conversion appears to be in part responsible for the increase in F in the uterus at term (Murphy, 1977b).

10. Gonads

11-HSD oxidative activity in testis homogenates of rat and rabbit was found by Koerner (1966) to be high (1/6 and 1/3 the activity found in liver homogenates of rat and rabbit respectively); in contrast 11-HSD activity in pig and sheep was at least 175 times less (F at 0.004-0.4 μg/mg were the concentrations used). The microsomal fraction was shown to contain the activity and NADP was the preferred cofactor. Reversibility was demonstrated for the reaction in the testis.

Although 11-HSD activity found in rat testis homogenates was comparable to that of kidney, very little activity could be detected in rat ovary (Chraf et al, 1975b). Ontogeny of 11-HSD activity in testis was
first detected on about day 30 and rose to reach a plateau by day 60 of life. Sex differences in 11-HSD activity also occurred in liver and kidney although these were not as pronounced as for testis and ovary (F was used as substrate, 1 mg/ml, and NAD as cofactor).

In humans, E to F and F to E conversion was detected in both males and females (Murphy, 1981a). Minced tissue incubated with tracer amounts of \(^{3}\text{H}-\text{F}\) or \(^{3}\text{H}-\text{E}\) for 2 hr interconverted E and F, approximately 50%.

11. Skin

Malkinson and coworkers (1959) studied the metabolism of unlabelled E and F by human skin. Human skin samples, obtained surgically, were incubated within 8-10 hr. If cadaver skin was used, incubations were not begun until 10-20 hr after death. Two hundred \(\mu\)g of F or E were incubated with 4-5 g of skin (40-50 ng/mg) for 6 hr. 11-HSD activity could not be detected in epidermis, although much less tissue was used. Dermis was found to be capable of converting about 10% of E to F. F was converted to a substance which possessed the same chromatographic mobility as E. However, upon further examination it was found to have a different sulfuric acid chromogen absorption curve from E and actually resembled the absorption curve for an unidentified compound isolated by McEwen et al (1954) from the synovial cavity following an injection of F. Although the substance remained unidentified, subsequent authors (Hsia and Yu-Lee Hao, 1966; Berliner and Ruhmann, 1966; Berliner, 1972), on quoting this paper, remarked that Malkinson et al (1959) had shown that skin was capable of carrying out reversible conversion of E and F, when in fact this was not the case. Unfortunately, most investigators neglected to study the metabolism of E by connective tissue or fibroblasts and only assessed the
metabolism of F. Malkinson et al's (1959) report was one of the few in which the metabolism of E by skin was evaluated (an exception was the paper by Hsia and Yu-Lee Hao, 1967) (see below).

Further studies on the metabolism of F by human skin were conducted by Hsia and associates (Hsia et al, 1965; Hsia and Yu-Lee Hao, 1966; Hsia and Yu-Lee Hao, 1967). Skin removed from the abdominal wall at autopsy 6-20 hr post-mortem was incubated for 5 hr as whole skin, dermis, or epidermis with 14C-F (23 ng/mg) and with or without cofactors (Hsia et al, 1965). NAD and NADH were found to be more effective than NADP and NADPH. Conversion of F to E by whole skin, dermis (connective tissue), and epidermis (epithelium) was not more than 7.2% when stimulated by coenzymes. Other metabolites included 20-DH-F and 20-DH-E, which never represented more than 2% of metabolism.

In a subsequent report by Hsia and Yu-Lee Hao (1966), in which 14C-F was incubated with human skin, they found that at most 10-15% of the labelled steroid was metabolized. A similar study of E metabolism by human skin was also carried out by Hsia and Yu-Lee Hao (1967). They showed that abdominal skin, incubated for 5 hr with 14C-E (11 ng/mg), formed F (1.2%), 20β-DH-E, and 20α-DH-E. The addition of the coenzyme NADPH increased the conversion of E to F from 1.2 to 6.7% while NADH was less effective. They suggested that the amount of conversion of E to F was minimal, although substrate levels were fairly high, and therefore, supported the evidence that topical application of E was without effect.

It is interesting to note that when 14C-F was incubated with human skin, NAD was the preferred coenzyme (Hsia et al, 1965), but when 14C-E was incubated with human skin, NADPH was the more effective coenzyme suggesting that perhaps 2 different 11-HSD enzymes reside in skin: one which catalyzes
the conversion of F to E, NAD dependent, and one which catalyzes the conversion of E to F, NADPH dependent.

Metabolism of F, to a small degree, does occur in skin; yet most of the attention has been focused on the fibroblasts of connective tissue, although it was shown by Hsia et al (1965) that epidermis was as active as dermis. Unfortunately, in this study the epidermis was not incubated separately so it is not known in which tissue E to F metabolism occurred or whether or not a different 11-HSD resides in dermal versus epidermal tissue.

12. Connective Tissue and Fibroblasts

Sweat and colleagues (1958) studied the metabolism of F by cultured uterine fibroblasts, strain U12-705, grown in medium 705 (5% CEE, 20% normal horse serum, and 75% solution 703). Tubes initially containing 5 x 10^4 - 1.2 x 10^5 cells were incubated with 3 μg/tube of 14C-F for 5 days, during which time the cell population increased 6-8 fold. Very little metabolism occurred: about 5% conversion of F to 20-DH-F and less to 20-DH-E. Conversion of F to E and 11β-OH-androstenedione were also possibilities, but positive identification of these compounds could not be made because of insufficient quantities. A more detailed study of F metabolism by uterine fibroblasts strain U12, was conducted by Grosser and coworkers (1962). Stock cultures of variants of strain U12-705 were propagated serially in roller tubes; variant U12-79 in medium 79 and the steroid resistant variant, U12-35, in medium 35 (medium 79 + 25 μg/ml F). On the fifth day the medium was removed from the tubes and replaced with medium 79 plus 0.3 or 0.9 μg/ml of 14C-F after which the cells were incubated for an additional 3 days. There was little metabolism when 0.9 μg/ml of F was used.
Most of the conversion that occurred was to 20-DH-F and 11β-OH-androstenedione. At 0.3 μg/ml of F, variant U12-35 more actively metabolized F to 11β-OH-androstenedione than U12-79. Both variants were relatively inactive in the oxidation of F as conversion of F to E was less than 2%. The authors concluded that the increased ability of U12-35 to metabolize F, when 0.3 μg/ml of 14C-F was used, could not account for all of the steroid resistance observed. Other mechanisms would have to be involved.

Uterine fibroblasts, strain U12-79, were also incapable of converting B to A (Berliner et al, 1960). Following 3 days of incubation of 3.4 x 10^7 cells with 0.3 μg/ml of 14C-F, conversion to only 20-DH-B (9.8%) was detected.

Mouse loose connective tissue metabolized F in a manner both qualitatively and quantitatively similar to human uterine fibroblasts (Berliner and Dougherty, 1958a; Sweat and Bryson, 1960). When pieces of tissue (0.3-0.6 g) were incubated with 14C-F (7.5 μg; 13^-25 ng/mg) for 4 hr (Berliner and Dougherty, 1958a) the metabolites included 20-OH-F (4%), E (3-5%) with dihydro-F, E, B and 11β-OH-androstenedione, as well as some unknown compounds together making up less than 4%. Sweat and Bryson (1960) incubated mouse connective tissue (0.2 g) for 2 hr with 14C-F (10 ng/mg). Without the addition of NADP to the incubation medium conversion of F to E could barely be detected, and only increased slightly with NADP (to 2%). NAD did not affect 11-HSD activity even though other enzymes were slightly inhibited by it.

Berliner and Ruhmann (1966) showed that fibroblasts of clone 929-L were incapable of interconverting E and F and thus, lacked an active 11-HSD system. The cells did convert F to 20-DH-F (25%) and E to 20-DH-E (46%) over a 2 day incubation period during which the cells were actively
growing. The authors stated that "although fibroblasts in connective tissue have an active 11β-OH dehydrogenase system, tissue culture fibroblasts do not manifest this enzymatic activity." Their own results for minces of connective tissue (Berliner and Dougherty, 1958a) and those of others using minces (Malkinson et al, 1959; Hsia et al, 1965; Hsia and Yu-Lee Hao, 1966) suggest rather that 11-HSD activity is minimal in both connective tissue and fibroblast cultures.

13. Lymphatic and Leukemic Tissue

(a) Lymphatic tissue. Studies conducted in the 1940's by various workers (Simpson et al, 1943; White and Dougherty, 1945; Valentine et al, 1948) on lymphoid tissues of rats showed that adrenocortical hormones influenced the distribution, mass, and functional activity of these tissues and played an important role in the overall physiology of lymphocytes.

Dougherty and associates had found that only the small (thought to be mature; see below) lymphocytes, in lymphatic organs, as well as in blood of rats and mice, were susceptible to lysis by increasing amounts of adrenocortical hormones (Dougherty and White, 1945) while the larger (thought to be immature) lymphocytes were relatively resistant. Mitosis of lymphocytes was also observed to be suppressed by adrenal hormones (Dougherty and White, 1945). Thus, F was thought to be the physiological regulatory hormone of lymphatic cell growth and differentiation, and it was postulated that F was ineffective in causing the destruction of lymphatic leukemic cells or 'immature' lymphocytes because of their ability to metabolize F to inactive metabolites (Dougherty, 1957).

The metabolism of F by the lymphatic organs of normal and F-treated female mice was studied by Dougherty and coworkers (1960a). Minces of thymi, lymph nodes and spleens from control and F-treated animals were
incubated for 3 hr with $^{14}$C-F (14 ng/mg). In control animals the % conversion of F to E was: thymus, 10.4; spleen, 17.3; and lymph node, 20.8. F treatment (1 mg F acetate for 9 days) caused a decrease in the size and weight of all the lymphatic tissues, as well as a profound reduction in the number of lymphocytes. F treatment also altered the amount of metabolic transformations which occurred. There was a slight increase in conversion in lymph node tissue from 20.8% to 25.2% and in the spleen conversion decreased slightly from 17.3 to 13.9%. The most striking change in conversion occurred in the thymus where conversion rose to 33.0% from 10.4%.

Following the F treatment period the cell population of the thymus had undergone considerable change. Most of the lymphocytes which survived were medullary lymphocytes. The authors suggested that the increase in 11-HSD activity in the thymus was due to a number of possibilities: (1) that the medullary lymphocytes more actively converted F to E and proportionately more of these lymphocytes were incubated from F-treated thymi; (2) thymi developed an adaptive enzyme; (3) lymphoid cells following F treatment contained more coenzymes, NAD, or NADP and thus had a greater 11-HSD activity or; (4) endodermally-derived reticular cells could participate in the reaction.

The thymus was subsequently shown to possess the ability to interconvert E and F (Dougherty et al, 1960b). Although conversion of F to E increased from 7% in controls to 22% in F-treated mice, E to F conversion rose only marginally from 7% to 8.5%. On the other hand, thymi from mice treated with T3 (0.25 μM for 4 days), which causes hyperplasia of lymphatic organs, converted F to E as in controls (7%) but E to F conversion was increased from 7% to 17%. Dougherty et al suggested that T3 made available
more coenzyme NADH and/or NADPH which stimulated the conversion of E to F in T₃-treated mice and that F worked in the opposite manner; it increased availability of the oxidized coenzyme. The authors proposed the following model for lymphocyte maturation: (1) lymphocytes contain a redox system (11-HSD) which depending on its functional state, due to either availability or concentration of oxidized and/or reduced coenzymes "determines the state of differentiation and the rates of proliferation and maturation of lymphocytes."; and therefore, (2) if F is oxidized, maturity will be delayed, but if conversion of E to F is enhanced, maturation will proceed and proliferation will be inhibited. Thus, the effects of T₃ and F were seen as being antagonistic, growth (T₃) versus involution (F). Whether the effects of F and T₃ on the thymus are related to their effects on 11-HSD activity in the tissue or indeed have anything to do with it is not known. Dougherty and his associates believed this to be the case, but it is now known that the medullary cells of the thymus are the mature cells (Claman, 1972) and not the immature ones as postulated by Dougherty and colleagues (Dougherty et al, 1960a; Dougherty et al, 1960b). The role of 11-HSD activity of thymic tissue, thought to be responsible for controlling F levels in lymphocytes and hence maturation of immature lymphocytes of the mouse thymus (Dougherty et al, 1960b), is now not clear at all.

Metabolism of F by normal lymphocytes could not be detected (Dougherty, 1957), while thymic mouse tissue could interconvert E and F, although it was not shown which cells were capable of what. It is possible that one or more 11-HSD enzymes reside in different cells of the thymus (epithelial tissue and thymocytes).

It has been shown that animals can be divided into glucocorticoid-sensitive and resistant species depending on whether or not the systemic
administration of steroids will cause a rapid involution of the thymus (Claman, 1972). Corticosteroid-sensitive species include mouse, rat, rabbit, and hamster; while man, monkey, parrot, and guinea pig are resistant species. Although the reasons for the differences in sensitivities is not well understood, much of the attention has more recently been focused on the glucocorticoid receptor (Lippman, 1979; Harris and Baxter, 1979).

(b) Lymphatic versus leukemic tissue. During the 1950's and 1960's a good deal of work was done on the metabolism of corticosteroids by lymphatic tissues and lymphatic and leukemic cells in an attempt to elucidate the mechanism of action of adrenocortical hormones on normal and malignant lymphoid tissue, and to determine why these tissues differed in their responses to these steroids.

Lymphatic leukemic cells in vitro from AK mice were able to convert 10-20% of F to E, 20-DH-F, and 2 unidentified zones of radioactivity (Berliner et al, 1956) which were subsequently identified (Dougherty, 1957). With NAD present as cofactor, the conversion was increased to 32.5%. When normal lymphatic tissue was incubated under the same conditions as leukemic tissue, metabolites could not be detected. This observation was subsequently shown to be both quantitative and qualitative (Dougherty et al, 1960a). The metabolites isolated from incubations with leukemic tissue were the same as those identified in connective tissue incubations (Berliner and Dougherty, 1958).

Osteosarcoma cells of beagle dogs metabolized 14C-F in a manner similar to leukemic cells from AK mice (Van Dooren and Dougherty, 1957). F was converted to E, 11β-OH-androstenedione and 20-DH-F, B, and 11-keto-androstenedione (tentatively identified). E was converted to A as well as
to an unidentified compound of similar polarity. Lymphatic leukemic cells taken from the buffy coat of blood from 3 patients also metabolized $^{14}$C-F in the same manner (Dougherty, 1957) as shown for AK mice.

It was also shown that F metabolism was increased in the spleens of chickens in which leukemia had been experimentally induced (Tuzson and Kertai, 1962). Normal spleens incubated with $^{14}$C-F (160 ng/mg) metabolized only 23% of it while spleens of erythroblastosis-afflicted chickens (leukemic) metabolized almost 60%.

Human peripheral leukocyte (polymorphs + lymphocytes) cultures metabolized $^{14}$C-F to a more polar compound which resembled THF (Forker et al, 1963). In the study of Forker and colleagues, 5,500 to 352,000 cells were incubated with 72 µg of F for 24 hr and conversion to the more polar compound ranged from 1.4-35.0%. Conversion was highest in leukocytes from patients with acute leukemia. However, the % conversion was no different for non-leukemic versus chronic leukemic patients.

Normal mouse thymus and lymphatic leukemic tissue were shown to interconvert E and F (Berliner and Dougherty, 1964). After a 3 hr incubation of 1 µM $^{14}$C-E and $^3$H-F with 300 mg of normal thymus or tumor tissue, tumor tissue was found to possess more oxidizing capacity (40% versus 30%) and less reducing capacity (14% versus 20%). This meant that more F would be inactivated by the tumor than by the thymus and so less biologically active molecules would be available for regulation of maturation and perhaps destruction of the malignant cells.

Burton (1964) tested the hypothesis put forth by Dougherty that tissues were sensitive or resistant to F depending on the degree to which F was metabolized by the various tissues. Burton did not find any difference in metabolism between F-sensitive (mouse thymus, rat thymus, mouse
lymphosarcoma P1798S, rhabdomyosarcoma P1798R, IRC leukemia-mouse) tissues. E was found to be the major metabolite following the incubation of cell suspensions, from the various tissues, with F. 11β-OH-androstenedione was also identified but in much smaller quantities than E. In sharp contrast to mouse thymus, which was capable of considerable conversion of F to E, rat thymus converted little if any F to E or any other metabolite.

Klein et al (1980) incubated for 17 hr lymphocyte suspensions, from patients with various diseases, with 3H-F and F (50 μg) and an NADPH generating system. They found that increased F metabolism by lymphocytes was not just associated with malignancy, but occurred with inflammatory diseases as well.

Dougherty and Berliner had proposed that cells became resistant to F because of their ability to metabolize F to other biologically inactive compounds. They also put forth the theory that those lymphocyte and lymphatic leukemic cells which were resistant to the action of F were so because of their enhanced 11-HSD activity (conversion of F to E), which reduced the intracellular levels of F. However, their own data, as well as the data of others (Burton, 1964), did not support such a proposal.

14. **Histochemical Distribution of 11-HSD**

Studies, mainly carried out in the Anatomy Department at the University of Glasgow, on the histochemical distribution of 11-HSD in various tissues of several species are described below. The method (Baillie et al, 1965) involved the freezing of tissue which was then sectioned on a cryostat and placed on dry glass slides. These slides were then incubated at 37°C in a phosphate buffer medium containing NAD or NADP, nicotinamide,
nitro blue tetrazolium, and ditetrazolium chloride with a steroid substrate, F or other 11-OH compounds, or E (0-1 mM). The slices were then microscopically examined for coloured formazan deposits.

(a) Liver. Liver 11-HSD activity occurred in the human fetus as well as adult mouse, rat, hamster, and guinea pig (Baillie et al, 1965a). Livers from male as opposed to female rats and guinea pigs possessed more 11-HSD activity, in agreement with the results of Koerner and Hellman (1964).

(b) Kidney. 11-HSD activity (F to E only) was found in the collecting tubules of the kidney in all mammalian species studied (Baillie et al, 1966). In mouse kidney, 11-HSD activity was detected from about the second week of age (Baillie et al, 1965). Only trace amounts of 11-HSD activity were found in midgestational human fetal kidneys. Murphy (1981a), however, did find that midgestational human fetal kidney minces were very active in converting F to E. This discrepancy points out that these histochemical distribution studies are qualitative rather than quantitative.

(c) Adrenal gland. Mouse fetal adrenal 11-HSD activity was detected on day 15 of gestation (Hart et al, 1966). In the post-natal female mouse adrenal most of the 11-HSD activity was confined to the zona fasciculata. 11-HSD activity was detected in midgestational human fetal adrenals in the fetal zone with trace amounts occurring in the definitive cortex (Hart et al, 1966). Adult adrenal 11-HSD activity was located in the zona fasciculata and zona reticularis, with none being detected in the zona glomerulosa. A comparative study of 11-HSD activity in adrenal cortex, incubated with F, was conducted on a number of species by Ferguson et al (1970). They found that some of the activity resided in the zona reticularis, with less activity in the zona fasciculata, while 11-HSD activity in
the zona glomerulosa was restricted to sheep and cattle. No 11-HSD activity was found in the zona glomerulosa of mouse, rat, guinea pig, rabbit, cat, dog, and pig. Thus, the zona fasciculata and reticularis, which are involved in synthesizing corticosteroids as well as sex hormones, could account for much of the 11-HSD activity in almost all species studied. Therefore, conversion of F to E could in part contribute to some of the E being secreted from the adrenals.

(d) Glandular tissue. 11-HSD activity was shown to occur in human Leydig cells using various substrates as well as in murine Leydig cells (Baillie et al., 1965b). In mouse Leydig cells 11-HSD activity increased steadily from birth to 10 weeks of age. Evidence for 11-HSD activity in the mouse ovary was also obtained. A histochemical study of 11-HSD activity in the rat submandibular gland revealed that it was localized in cells throughout the duct system (Hoyer and Moller, 1977). F and 11β-OH-androstenedione were equally utilized substrates and NAD was a more effective coenzyme than NADP. Whether the activity resided in one enzyme, two enzymes, or isoenzymes, could not be discerned from the study. No 11-HSD activity was detected in mast cells. Hoyer and Moller (1977) also demonstrated an increase in 11-HSD activity following a single pharmacologic dose of F; however, whether induction of the enzyme had occurred could not be determined.

E. EFFECTS OF HORMONES ON 11-HSD ACTIVITY

The metabolism of adrenal steroids was shown to be affected by various diseases and experimentally induced states in animals and man.
1. **In Animals**

Pretreatment of rats with F or T₃ (1 mg/day F, 10 mg T₃ for 2 weeks) resulted in an increase in F metabolism by liver slices which could be decreased by hypophysectomy or thyroidectomy (Brown et al, 1957). In these studies F was added to liver slices at a concentration of 3 μg/mg. Thus, the inordinate increase in the rats' concentration of F caused an increase in its metabolism by the liver, but if the concentration of F was reduced (as through hypophysectomy) metabolism was decreased. T₃ caused a general increase in metabolism, as measured by an increase in oxygen consumption and increased F metabolism. This could reflect an increase in the level of metabolizing enzymes and/or coenzymes in the liver. In this respect Huggins and Yao (1959) found that coenzyme-(NADH, NADPH) generating enzymes in the rat liver could be increased by T₃ treatment, or decreased following thyroidectomy or hypophysectomy. Female rats were shown to have levels of NADH- and NADPH-generating enzymes which were normally higher than in males.

11-HSD activity in liver homogenates from male rats was found to be greater than that from female rats (Koerner and Hellman, 1964), although this does not appear to be directly related to any effects of thyroxine, since the plasma thyroxine concentration is similar for males and females (4 μg/100 ml) (Kieffer et al, 1976).

Intraperitoneal administration of L-thyroxine (400 μg) for 7, 10, or 17 days, produced an increasingly greater decrease in 11-HSD activity in male rats in terms of F to E conversion; while 4 days of treatment was ineffective. The effect was similar when other substrates were used (B, allo-dihydro-F). Addition of L-thyroxine to liver homogenate incubations for 30 min had no effect on 11-HSD activity. Thyroxine treatment of male
and female rats had no effect on 11-HSD activity in kidney homogenates. In the other liver homogenates, from 10 to 30 days post-thyroidectomy, male rats had increased 11-HSD activity. However, livers from thyroidectomized animals were of much lower weight, thus minimizing the effect of increased 11-HSD activity per gram of liver. 11-HSD activity was also increased in liver homogenates of male or female rats when thyroxine was administered to either normal or adrenalectomized animals. In liver homogenates, oxidation (F to E) occurred three times as fast as reduction (E to F), yet for the indigenous corticoids (B and A in rats) the reduction of A occurred twice as fast as the oxidation of B. When the effect of a daily dose of thyroxine on 11-HSD activity was measured it was shown that 100 mg daily was necessary to produce a 50% decrease in 11-HSD activity.

Dougherty et al (1960b) had found that pretreatment of mice with T3 resulted in an increase in E to F conversion while F to E conversion was unchanged in their thymi without added cofactors. They suggested that availability of cofactors might be involved. Koerner and Hellman (1964) on the other hand had found that there was a decrease in 11-HSD activity, in liver homogenates although they were saturated with coenzyme, and further, that both directions of the reaction had been reduced. Therefore, they proposed that a likely explanation for the decrease in 11-HSD activity in their experiments was that an absolute decrease in the quantity of enzyme had occurred.

11-HSD activity in the kidney of adult male and female rats was found to exhibit sex differences. F to E conversion in the microsomal fractions of kidney homogenates was found to be twice as high in males as in females (Ghraf et al, 1975a). Both gonadectomy and/or hypophysectomy elevated 11-HSD activity to male levels. The 11-HSD was also shown to be an estrogen dependent enzyme, since the increased levels characteristic of
males occurred following castration in females, whereas the lower levels characteristic of females were found in males treated with estradiol.

Sex differences in 11-HSD activity were also shown to occur in liver of male and female rats. 11-HSD activity in the microsomal fraction of liver homogenates was almost five times greater in males than in females (Lax et al, 1978). Testosterone was found to maintain normal male 11-HSD activity levels in both gonadectomized males and females. Lax and his colleagues proposed that thyroid hormones, known to affect 11-HSD activity (Koerner and Hellman, 1964), may act by raising corticosteroid-binding globulin (CBG) levels, thereby decreasing free androgen levels which could affect 11-HSD activity in the liver. Hypophysectomy was shown to abolish the sex differences in 11-HSD activity between male and female rats. Therefore, the pituitary had a repressive influence on 11-HSD activity in females and also removed any effects of estradiol on the enzyme activity in males.

Preincubation of guinea pig liver, adrenal, and kidney slices with adrenosterone (300 μg) stimulated the conversion of F to E substantially (13-44%) (Yudaev and Filonova, 1966); the steroid had only minimal effect on the conversion of F to E by spleen, muscle, heart, lung, and testis (2.9-5.5%) which was very low to begin with. Pretreatment of guinea pigs with 10 mg of F acetate for 10 days had no effect on 11-HSD activity. The F treatment did seem, however, to stimulate the enzyme system of the liver and adrenal as there was a substantial loss of F from the incubation medium, presumably indicating increased metabolism of F into other compounds.

Seasonal variation in adrenal 11-HSD activity has been noted in the meadow vole (Unger et al, 1978). Using adrenal homogenates, incubated with 14C-progesterone (150 ng/mg) (which is converted to 14C-E), 11-HSD activity...
(B to A) was greatest in the late fall-winter and low or undetectable in early spring-summer. Unger and associates suggested that the increased 11-HSD activity may be related to the decreased adrenal activity and the absence of breeding seen during winter months although the events may not be causally related. Whether seasonality of 11-HSD activity in the adrenal occurs in other species remains unanswered.

2. In Man

Peterson's group (1957) found that in patients with liver disease F was metabolized at a slower rate than in control subjects. In patients with thyrotoxicosis, the rate of metabolism of both E and F was shown to be increased.

Peterson (1958) suggested that since plasma F levels were normal in patients with either myxedema or thyrotoxicosis, that there was a homeostatic mechanism, which operated through the liver-pituitary-adrenals in which increased secretion of F was balanced by increased metabolism, and decreased secretion of F was offset by a lowering of the metabolism of F by the liver. Thyroid hormone was shown by Hellman and coworkers (1961), in human studies, to cause a shift in the metabolism of F in terms of the oxidation-reduction state of the metabolites. The percentage of 11-keto metabolites (THE, cortolones) was increased in hyperthyroid patients or in subjects treated with T3.

Dazord and associates (1972) found that in hyperthyroid patients the conversion of E to F in plasma was greatly reduced while F to E conversion was not changed. Dazord et al suggested that the increase in THE seen in hyperthyroid patients (Hellman et al, 1961) was due to the decrease in E to F conversion and subsequent rise in 11-keto metabolites.
How thyroxine causes the decreased 11-HSD activity is not known. Daily administration of pharmacologic doses was required for more than 4 days for any effects to be seen (Koerner and Hellman, 1964), and thyroxine affects many enzyme activities (Brown et al, 1957; Peterson, 1957; Hellman et al, 1961).

F. EFFECTS OF INHIBITING 11-HSD ACTIVITY IN MICE AND RATS

Burton (1965) studied the effects of various inhibitors of 11-HSD activity, in mice and rats, on thymus involution and tumor growth. Epi-F, epi-prednisolone, and 11α-OH-progesterone were found to be effective competitive inhibitors, in concentrations up to 9 x F, of 11-HSD activity in all mouse tissues studied, which included kidney, thymus, liver, and mouse lymphosarcoma P1798. 11-HSD activity was assayed by incubating various tissues with 14C-F (2.5 ng/mg) for up to 3 hr. The highest activity was observed in kidney tissue of both mouse and rat (16-26% conversion/hr) with mouse lymphocytes and whole spleen from mouse next (7-9% conversion/hr), and followed by mouse thymocytes and reticular cells (3-4% conversion/hr). Very little, if any, activity was detected in rat thymocytes, reticular cells, lymphocytes, whole spleen, muscle (unless fortified with NADP) or mouse muscle (fortified or not).

When mice received injections of F and inhibitor twice, 4 hr apart, 20 hr following the last injection thymus weights were found to be decreased to a greater extent than if the inhibitor had not been used. Inhibitors alone had no effect on thymus weights. F or prednisolone combined with inhibitors injected daily for 5 days into mice bearing the steroid-resistant line of lymphosarcoma P1798 had no observable effect on tumor growth. Injection of 14C-F and inhibitor into mice, rats, and
adrenalectomized ovariectomized rats reduced the amount of conversion by the kidney as measured by the E:F ratio in the renal vein blood. After removal of the kidney, E could not be measured in blood taken from the aorta moments later thereby confirming the kidney as the site of F to E conversion, and also that the kidney contributed to plasma E levels in mice and rats. Burton commented that even though the conversion of F to E in thymocytes was much lower than in the kidney, the increased intracellular levels of F in mouse thymocytes created by the competitive inhibition of 11-HSD activity by several inhibitors caused an increase in the reduction of thymus weight. This was in distinct contrast to rat thymocytes in which 11-HSD activity was virtually nonexistent and in which the same inhibitors had no effect on increasing the reduction in thymus weight when injected with F.

Although lymphosarcoma cells had as much 11-HSD activity as mouse thymocytes, inhibition of the enzyme reaction had no effect on tumor growth. At the time Burton (1965) published his paper, hormone receptors were unknown. Resistance of tumors to steroids is due to a lack of receptors (Lippman, 1979), and therefore, even if F was increased intracellularly it could not have had any effect on tumor growth in vivo unless F receptors were present.

In another report (Burton and Turnell, 1968) the effects of the inhibitors 11-epi-F and 11α-OH-progesterone on the action of E on mouse thymus were also investigated. E (600 mg) alone or with inhibitor (11-epi-F, 4-8 mg; 11α-OH-progesterone, 3-6 mg) were injected twice 4 hr apart, with the last dose given 24 hr prior to sacrifice of the mice. While E reduced the ratio of thymus weight to body weight, with inhibitor, E was even more effective. Presumably E was converted to the active hormone F by
the liver, which then acted on the thymus and when inhibitor was added the conversion of F to E in the thymus was reduced resulting in more intracellular F. The authors suggested that the lack of effect of inhibitor on 11-HSD activity in the liver was because it contained a great deal of enzyme and the inhibitors could not prevent enough E from being converted to F. If this is the case, then small changes in glucocorticoid levels intracellularly could easily be controlled by various tissues with 11-HSD as the controller while the liver and kidney could control blood levels. Thus, although 11-HSD activity in the thymus was low (as it was in the lung, brain, spleen, and muscle), inhibition of its activity was enough to change the biological activity of F. How important this is in the adult is unknown. However, there is evidence to suggest that 11-HSD may be a crucial enzyme in controlling the intracellular levels of F in the fetus thereby affecting its maturation (Smith, 1978) (see section on 11-HSD Activity in Pregnancy).

G. 11-HSD Activity in Pregnancy

1. Mouse

Burton and Jeyes (1968) studied the conversion of B to A in mouse maternal blood and fetuses by injecting $^{14}$C-B into pregnant mice and removing the fetal tissues after 30 min. In maternal blood the conversion of B to A was never more than 3% while the conversion was 45% in fetuses of less than 5 mm crown rump length (CRL), 81% in fetuses of 5-15 mm CRL and dropping to 25% during the last days of gestation in fetuses greater than 15 mm CRL. Reduction of A to B was not detected in whole fetuses (3-5 mm CRL) with or without added coenzyme NADPH. Fetal liver (10-15 mm CRL) incubated with $^{14}$C-A (0.64 ng/mg) and/or NADPH was also inactive, but
fetal liver (greater than 20 mm CRL) at day 18-19 (term 21 days) formed 44% of B from A, which the authors suggested was probably responsible for the decreased reduction to 25% conversion in fetuses greater than 15 mm CRL. The placenta was also shown to be very active in converting B to A, more so than the fetus of 10-15 mm CRL.

The conversion of the 11β-OH-steroid to the 11-ketosteroid was examined in a number of other newborns (mouse, rat, hamster, guinea pig, rabbit, and cat) (Burton and Jeyes, 1968). In all species the conversion of the indigenous hormone in the blood to its 11-keto metabolite was similar, approximately 18%, suggesting a similar pattern of hormonal conversion.

Conversion of B to A in young mice revealed that 11-HSD activity increased from day 5-8 after birth and then gradually decreased to adult levels by 33 days, at which time conversion was 7% of B to A 30 min following the injection of 14C-B (Burton and Jeyes, 1968). The authors pointed out that the increase in non-protein bound B in pregnant mice between days 12 and 18 of gestation corresponded to the increase in B to A conversion in the fetus; thus the conversion of B to A offset the maternal increase in B, protecting the fetus from elevated levels of B. The evidence suggests that although corticosteroids cross the placental barrier, fetuses remain relatively resistant to the effects of corticosteroids administered to the mother (Jost, 1966; Pinsky and DiGeorge, 1965; Talwalker et al, 1961). Only relatively high doses of corticosteroids have any teratogenic effects on days 11-14 of gestation in the mouse fetus (Pinsky and DiGeorge, 1965). Human infants were also quite resistant to the effects of elevated maternal F levels (Kulin et al, 1966). Burton and Jeyes proposed that the data presented here are consistent with the concept of the 11β-hydroxysteroid dehydrogenase system acting as a protective mechanism, whereby
corticosteroid levels in the fetus are held to within certain values regardless of increases in the maternal circulation.” They further suggested that the decline in the A:B ratio to adult levels may reflect increasing CBG levels thereby decreasing the amount of non-protein bound B resulting in an apparent decreased B to A conversion in the adult mouse. Another possible contributing factor is that in the adult, 11-HSD activity is reduced (Burton and Jeyes, 1968) which would also lead to a decrease in the A:B ratio.

Further studies of 11-HSD activity in vivo and in vitro of various tissues in the non-pregnant and pregnant mouse were carried out by Burton and Turnell (1968). Kidney and liver 11-HSD activity was only matched by placental and fetal 11-HSD activity. Kidney and liver slices were much more active in converting B to A (kidney), and A to B (liver) than F and E, which is important to point out since B is the indigenous glucocorticoid of mouse and studies of F metabolism in mouse may be misleading.

Interestingly, only 11-epi-B was an effective competitive inhibitor of B to A conversion in the kidney while F to E conversion was inhibited by 11-epi-B, 11-epi-F, and 11α-acetoxyprogesterone. 11-HSD activity in vitro in kidneys from pregnant females was higher than in the kidneys from non-pregnant females (56.8% versus 46.1% A formed/100 mg/30 min from B). However, in vivo the opposite was true; the % A formed from B in the non-pregnant was much higher than in the pregnant female kidney (41.2 versus 19.5%). Removal of blood from the kidney using a saline injection had no effect on the conversion of B to A in the non-pregnant kidney but raised the % A formed in the pregnant kidney from 19.5 to 30.6%. Burton and Turnell suggested that increased transcortin levels in the pregnant mouse would increase the apparent concentration of B, and therefore, removal of
blood with saline raised the % A formed in the kidney, although not to levels found in the non-pregnant kidney. Some inhibition of the reaction may have also been caused by other unidentified steroids which are either of greater concentration in the pregnant versus non-pregnant animals or are only synthesized during pregnancy (Murphy, 1981b).

The injection of epi-B, an inhibitor of 11-HSD activity in the mouse (Burton and Turnell, 1968), increased liver glycogen in mice aged 8-14 days compared with untreated controls (Burton et al, 1970). The effect of inhibitor was believed to be due to the inhibition of metabolism of endogenous B. In newborns aged 5-25 days, there was a rise in % A formed from B in blood 20 min after the injection of $^{14}$C-B from 20 to 42%. Conversion of B to A in the adult was less than 5%. Eight days postnatally, liver glycogen levels fell from about 5 mg/g on day 5 to less than 1 mg/g, but rose steadily from day 15 onward reaching adult levels of 50 mg/g.

The rise of A in blood following the injection of $^{14}$C-B indicated that during the 8-14 postnatal days B was being inactivated. Therefore, any inhibition of this oxidative activity occurring in mice would contribute to increased levels of active hormone in the blood which would be able to affect glycogen levels in the liver. An equal weight of B was even more effective than inhibitor (epi-B) in raising liver glycogen. The inhibitor itself had no effect on liver glycogen in adult mice or when combined with B, perhaps indicating that additional stimulation of the liver by B was ineffective in the adult. Just before parturition liver glycogen reached 85.5 mg/g concomitant with a decrease in the fetus in the % A formed from B from 80% on day 15 to 20% on day 20. Burton and associates (1970) showed that when the mother was injected subcutaneously with B or F (4 mg) on day 15 of pregnancy, fetal liver glycogen was
increased 16 hr later from about 2 mg/g in controls to about 15 mg/g. The authors suggested that such a large dose may have been necessary, since at 15 days gestation a great deal of the B is inactivated on passage through the placenta and in the fetal tissues. It may also be that liver steroid receptor systems were not as yet fully developed, and therefore, although B and F increased liver glycogen levels very convincingly, the values were still far from liver glycogen levels at term. None the less, F and B do exert a significant effect. Non-protein bound B in maternal blood is known to rise 4-fold between days 12 and 18 of gestation and continues to rise throughout pregnancy. The 11-HSD activity in the fetus certainly has an effect on circulating B levels, which the authors suggest is related to liver glycogen levels in the fetus and newborn mouse. Reduction in oxidative 11-HSD activity near term in the fetus not only allows for increased circulating levels of active hormone, but would also increase intracellular levels of B in those cells whose 11-HSD oxidative activity falls or reductive activity rises (as in the fetal liver of mice). The authors concluded that "in the newborn, the enzyme (11-HSD) appears to play a modest but definite role in corticosteroid metabolism. In the fetus this role might be even more important, and conceivably other events occurring in late gestation might be related to changes in corticosteroid metabolism similar to those described here for glycogen." Glucocorticoids have been implicated in inducing a number of maturational events in the fetus (for summary see Liggins, 1976), and more and more evidence suggests that 11-HSD activity in the fetus plays a crucial role in the timing and maturation of the fetus (Smith, 1978; Murphy et al, 1974; Murphy, 1981a; Mitchell et al, 1981).
In fetal mice, Michaud and Burton (1977) have shown that even though the placenta extensively converts the active hormone B to its inactive metabolite A, the main site of inactivation may actually reside in the fetal head. They also showed that the fetal liver reduces A to B on day 14 of gestation, increasing on day 15, with or without NADPH, which they attributed to an increase in enzyme levels. Changes in reduction of inactive metabolite to active hormone, they suggested, could account for the increment of B in the fetal blood late in gestation, without any involvement of the fetal pituitary-adrenal-axis.

The fetal mouse brain of day 14 extensively inactivated B, but by day 17 of gestation very little inactivation occurred (Tye et al, 1978a). Decreased inactivation was found to coincide with decreased incorporation of $^{14}$C-leucine, $^3$H-uridine and $^3$H-thymidine, parameters which are a reflection of growth. At the same time, corticosteroid receptors in fetal mouse brain showed no increase on day 14 compared with gestational day-17 so that changes would most likely be mediated by hormone levels. Injection of dexamethasone on gestational day 13.5 resulted in values of the labelled substrates on day 14 normally found between days 15 and 18. The authors suggested that the experiments provided evidence for the control of B levels in the brain by 11-HSD activity since decreasing inactivation of B to A (Tye et al, 1978a) would result in elevated B levels in the brain with a concomitant rise in circulating B levels due to increasing activation by the fetal liver (Michaud and Burton, 1977).

Similar results for placenta were reported by the same group in terms of substrate incorporation which decreased from gestational day 14 to day 19 (Tye et al, 1978b). This coincided with increased reduction of A to
B by the liver, elevating the level of B in the blood entering the placenta from the fetus.

Interconversion of A and B was studied in various tissues of fetal mouse by Tye and Burton (1980b). The ratio of reduction (A to B) to dehydrogenation (B to A) was found to increase in all tissues from day 14 of gestation to day 19 by various degrees, suggesting that active hormonal levels in the tissue can be controlled by 11-HSD activity. Incorporation of leucine, uridine, and thymidine was found to be reduced in the liver, gut, lung, and heart during day 14 of gestation to day 19 (Tye and Burton, 1980a), as for placenta (Tye et al, 1978b) and for brain (Tye et al, 1978a). Injection of dexamethasone on gestational day 13.5 induced changes on day 14 normally seen between days 15 to 18. The authors interpreted their data as evidence for the important influence of corticosteroids on fetal development in the mouse.

2. Sheep

The distribution of a tracer amount of \(^3\text{H}-\text{F}\), injected into the amniotic fluid of fetal sheep, in various fluids and tissues was carried out by Carson and associates (1979). After 120 min following the injection the fetuses were removed for analysis. \(^3\text{H}-\text{F}\) disappeared from the amniotic fluid, with the subsequent appearance of \(^3\text{H}-\text{E}\), but \(^3\text{H}-\text{E}\) was never more than 7% of \(^3\text{H}-\text{F}\). The amount of \(^3\text{H}-\text{F}\) in the fetal blood reached equilibrium after 30 min and was the same in both the umbilical artery and vein. The concentration of \(^3\text{H}-\text{F}\) was lower in tracheal fluid than amniotic fluid and closer to that of blood, which suggested movement of F out of tracheal fluid, consistent with other evidence (Boddy and Dawes, 1975). A greater concentration of \(^3\text{H}-\text{E}\) in adrenal, kidney, pancreas, and lung than in blood could
be measured, suggesting that these tissues possessed 11-HSD activity. The greatest concentration of radioactivity was found in the membranes (amnion and chorioallantoic tissue) and it suggested that a possible route of amniotic F might be through the fetal membranes and membrane vasculature. Carson and colleagues commented that "the possibility of local regulation of biologically active glucocorticoid concentration, which may change with gestational age, is an attractive one and would constitute a further component in the regulation of the fetal steroid milieu."

3. Rhesus Monkey

Kittinger (1974) studied the production and transfer of F in the mother and near-term fetus of rhesus monkey (Macaca mulatta) by a constant infusion technique, in which $^{14}$C-F was infused into the mother, and $^3$H-F into the fetus. The ratio of plasma F levels of mother to fetus was 2 or 3 to 1. The plasma concentration of E in the mother was much lower than F, but in the fetus the reverse occurred. The rate of transfer of F from mother to fetus or fetus to mother was essentially the same, although the amount of F in the fetus calculated to come from the mother was 58% compared with only 4% F contributed from the fetus to the mother.

Kittinger suggested that the F concentration in fetal plasma was controlled by the conversion to E by placenta and fetal liver, since he found slices of these 2 tissues to be very active in converting F to E, THE, and THF (Kittinger, 1974, no details given). The slices also converted E to F, but no amounts were given. Other fetal tissues were not studied in regard to 11-HSD activity (although 11-HSD activity has been reported to occur in virtually all mammalian fetal tissues studied, including mouse (Burton and Jeyes, 1968), rat (Smith, 1978), sheep (Carson et al, 1979),
and human (Murphy, 1981a). The % of E in the fetal plasma of maternal origin was calculated to be 76% in the rhesus monkey (Kbitzinger, 1974), which was in agreement with the work of Murphy et al (1974), whose data suggested that a large fraction of maternal F was converted to E when crossing the human placenta. The fetal adrenal output of F in the rhesus did not appear to contribute to the maintenance of the maternal fetal plasma F ratios or concentrations which were identical in intact or decapitated rhesus fetuses (McNulty et al, 1973). Therefore, the control of F concentration differences between mother and fetus were independent of the control by the fetal hypothalamic-pituitary-axis. Kittinger (1974) suggested that F differences between mother and fetus involved placental metabolism and rates of transplacental passage of F in both directions.

A study similar to the one conducted by Kittinger (1974) was carried out on rhesus monkey by Mitchell and coworkers (1981). In this report values were expressed in terms of birth weight of the fetus, which was not done in the study of Kittinger. Results reported in this manner showed that the MCR was at least 7 times greater in 135-140 day-old fetuses (term, 168 days) than in adults and 3 times more than in infants 1 week-old. The production rate was also higher in fetuses and infants than in adults. The secretion rates were similar for fetus and mother. Sixty % of fetal F was transferred to the mother and only 5% of maternal F was transferred from the mother to the fetus. The transfer rates were similar in agreement with Kittinger's (1974) results. Only 9% of maternal F was derived from the fetus, but 44% of fetal F was derived from the mother. In terms of E in the fetal circulation, 30% originated from the fetus, and 72% from the mother, while in the maternal circulation only 9% was derived from the fetus, the remainder from the mother. This study points out a number of things. First
of all when taking fetal size into account, the transfer of fetal F to the mother represented more than 60%, yet only 5.5% of maternal secretion was transferred to the fetus. This 5.5% transfer actually accounted for more than 40% of the circulating F in the fetus, whereas, only 6.6% of maternal F originated from the fetus. Also, most of the E in the fetus (greater than 70%) was derived from conversion of maternal F on passage through the placenta, as well as by conversion of F in various other fetal tissues. Therefore, at least 2 factors were thought to contribute to the high MCR in the fetus: (1) the transfer of 60% of fetal F secretion to the mother; and (2) the extensive conversion of F to E by the fetus. The high fetal MCR may be an important determinant of plasma F levels which were much lower in the fetus (135-140 days; 70 ng/ml) compared with the infant (212 ng/ml) or adult (131 ng/ml). Mitchell and colleagues also suggested that F levels may be regulated by 11-HSD activity, since so much maternal F was found as E in the fetus. The results of Mitchell et al (1981) also suggested that amniotic fluid F may come from maternal or fetal E which is subsequently converted back to F, possibly by the membranes (Murphy, 1977a). This could also contribute to fetal F production, since amniotic fluid F is taken up into the fetal circulation. Mitchell et al (1980) proposed that factors which were thought to play a role in the regulation of F in the fetus included: (1) fetal adrenal secretion; (2) the transfer of F from mother to fetus; (3) the interconversion of F and E resulting from 11-HSD activity; and (4) a high MCR to which factors (2) and (3) contribute.

4. Baboon

Almost all of the studies on metabolism of F in baboons (Papio papio) has been conducted by Pepe and associates. They found little
difference in the metabolism of F in pregnant versus postpartum animals, and suggested that, since production rates of F were similar in pregnant and non-pregnant baboons, that the fetus did not contribute significantly to total F production during pregnancy (Pepe and Townsley, 1975) (previously shown in rhesus monkey, Kittinger, 1974; see above). They found, in fact, that the production rate of F was lower in newborns than in adult baboons (Pepe and Townsley, 1976), which has also been noted in human neonates (Kenny et al, 1966).

The only differences in F metabolism between pregnant and non-pregnant animals were: (1) the excretion of unconjugated F was increased; (2) the formation of highly polar metabolites was increased; (3) the production of glucuronoside conjugates was decreased; and (4) the F binding capacity was increased (Pepe and Townsley, 1974; Pepe and Townsley, 1975). Metabolic clearance rates of E and F were the same in pregnant (of various gestational ages) and non-pregnant baboons (Pepe et al, 1976). The transfer constant, ρ (interconversion of E and F), following the constant infusion of 3H-F and 14C-E, was in the non-pregnant 63% E to F and 42% F to E. In the pregnant animal both transfer constants increased; 76% for E to F and 54% for F to E (Pepe et al, 1976). Therefore, in both cases ρ favoured F formation. The transfer constant, ρ, in neonates was 18% E to F and 60% F to E (Pepe, 1979) favouring E production opposite to what was found for non-pregnant and pregnant animals (Pepe et al, 1976).

Further studies of the metabolism of F by newborn baboons revealed some interesting differences when compared with pregnant and postpartum baboons (Pepe and Townsley, 1976; Pepe, 1979). The % unconjugated steroids in the urine of newborns was 45%, compared with 25% in pregnant and postpartum baboons, with 2 unidentified polar metabolites making up 81%
of the unconjugated fraction in newborns and only 60% in pregnant and post-partum animals. The glucuronoside fraction was decreased in newborns with the polar metabolites accounting for 62% of the fraction compared with 28% in pregnant and postpartum animals (Pepe and Townsley, 1975; Pepe and Townsley, 1976). The decrease in conjugation in the newborn baboon has also been observed in humans (Giroud, 1971), as well as in fetal mice (Wong et al, 1975) and premature baboons (Pepe and Townsley, 1977).

The distribution of $^{14}$C-F in the blood and various tissues in premature and term baboon neonates was studied by Pepe and Townsley (1977). $^{14}$C-F (164 µg) was injected into a peripheral arm or leg vein of newborns and 30 min later 10 ml of blood were removed by heart puncture and the animals sacrificed. The tissues were removed and the metabolites of $^{14}$C-F were subsequently identified. The radioactivity was found to be distributed in the various neonatal tissues as follows: 10% in the liver, 4.3, 4.4, and 2.1% in the intestine, brain and lung respectively, with < 1% detected in blood, kidney, heart, spleen, skeletal muscle, and urine; the values were similar in the premature and 1 day-old neonates. There were slight differences in the 5-12 day-old neonates including an increase in the radioactivity in the intestine from 4.3 to 9.1% as well as an increase in the urine from 0.3 to 1.6%. Seventy-eight % of the dose was unconjugated, and 80% of this was identified as E and F in most tissues, with the exception of the liver in which the major metabolites were THF, THE, and unidentified highly polar compounds. These results were in agreement with those of Pasqualini et al (1970b) in which very little conjugation was detected in midgestational human fetuses. The concentration of radioactive E in kidney, heart, intestine, spleen, and lung was greater than that found in blood and remained unchanged in prematures on day 1 or in 5-12 day-old
neonates, suggesting that these tissues possessed 11-HSD activity, although uptake of E by the tissues cannot be excluded. The ratio of F to E was greater than 1 in the urine, yet in the kidney the ratio was much less than 1, indicating that much of the E was reabsorbed and not excreted. The authors therefore suggested that 11-HSD activity in extrahepatic tissues was an important mechanism in maintaining a low F:E ratio in the blood.

The metabolism of F in premature and term newborns was similar, and Pepe and Townsley (1977) suggested that changes in metabolism would be unlikely to account for an increase in fetal blood F concentrations prior to parturition, and proposed that increases in F could be contributed to by increased adrenal F production or maternal transfer of F to the fetus.

In an attempt to evaluate F adrenal production, adrenals from fetuses of various gestational ages and from 1 day-old and 5-12 day-old newborn baboons were homogenized and incubated for 1 hr with either tritiated pregnenolone, progesterone, or 11-deoxy-F and conversion to F was determined (Pepe et al, 1977). Fetal adrenal weight increased 4-fold from 100 days to 184 days gestation (term), which was mainly due to the development of the cortical fetal zone. Conversion of progesterone or deoxy-F to F decreased with gestational age, in terms of activity per mg of adrenal, but conversion of pregnenolone to F increased. However, in terms of total activity, the production of F from all 3 sources remained relatively unchanged with increasing gestational age. In the 1 day-old and 5-12 day-old newborns pregnenolone contributed 1.4 and 13.6% respectively to total F production compared with 2.7% in adrenals from 160-167 day-old fetuses. The authors therefore, suggested that increasing 3α-OH-steroid dehydrogenase-isomerase activity, coupled with increasing fetal weight, would allow for an increased utilization of pregnenolone and less dependence upon
placental progesterone for F adrenal production. But in this study, no increase in total activity of adrenal production of F with increasing gestational age could be noted from their results, so that increased F plasma levels may not be a consequence of only increased adrenal production in the baboon fetus near term.

5. Human

Pasqualini and coworkers (1970b) studied the distribution of $^3$H-F and $^{14}$C-E in two midgestational (18 and 20 weeks; 40 weeks term) human fetuses. They injected the labelled steroids into the umbilical vein of the intact feto-placental circulation 15 min prior to the interruption of gestation. The metabolites in the various fetal tissues and maternal urine were isolated and identified. Most of the radioactivity was taken up by the liver and residual fetal tissues (25%) with lesser amounts found in placenta (10%), brain (8.5%), and intestine (3%). Very little radioactivity was detected in adrenal, kidney, umbilical cord, or lung (1%). Conversion of E to F was not detected in the fetal organism; however, in all fetal tissues there was evidence for conversion of F to E. These results were confirmed and extended by Murphy (1981a). In vitro studies were done on F and E interconversion in various tissues of the human fetus as well as neonate, child, and adult. At midgestation 11-HSD activity in all tissues was predominantly oxidative; however, towards term 11-HSD activity (F to E) dropped off sharply, which would help to raise the F plasma levels. In the child and adult the 11-HSD activity resided mainly in the liver (reductive) and kidney (oxidative).

(a) Placenta. Adrenal type steroids in the human placenta were investigated by Berliner et al (1956). They identified THE, 20-DH-E,
aldosterone, F (4 ng/g), E (90 ng/g), and a number of other compounds. They put forth several hypotheses to explain the more than 20 times greater concentration of E than F in placenta, one of which included the possibility that conversion of F to E was occurring in placental tissue. This was confirmed by Osinski (1960). Homogenates of human placenta were incubated with various ^H-steroids (1 µg/g) for 30 min. F to E conversion was approximately 60% with NADP as coenzyme, which was more effective than NAD. B and 11β-OH-androstenedione were also readily oxidized. Reversibility was demonstrated although only 7% of E was converted to F; later studies, however, did not confirm the reversibility of the enzyme (see below). The membranes were also found to be a good source of 11-HSD activity.

Sybulski and Venning (1961) were able to demonstrate the conversion of F to E, but reduction of E to F in 2 of 3 placentae was not detected. This study and the one by Troen (1961) both suggested that the placenta did not produce adrenal steroids but rather was actively involved in their metabolism. 11-HSD activity was located in the microsomal fraction and both NAD and NADP were effective as coenzymes (Meigs and Engel, 1961). Reversibility was not demonstrated for the placental 11-HSD in their study. Interconversion of E and F was shown to occur in vivo in humans (Pasqualini et al, 1970b), but Pasqualini et al failed to separate the membranes from the placenta (personal communication); the membranes have subsequently been shown to readily reduce E to F (Murphy, 1977a) (see below). 11-HSD activity was found to be similar in placental homogenates whether the tissue was obtained after spontaneous or induced labor, or in the absence of labor, which suggested that parturition did not have any influence on 11-HSD activity (Lopez Bernal et al, 1982a). Placental homogenates were capable of oxidation; however, reduction of E was negligible, even in the presence of
NADPH (Lopez Bernal et al, 1980). These authors suggested that at term 11-HSD activity was not reversible in vitro and could only convert F to E. They also showed that the decidual 11-HSD was reversible and was different from the placental enzyme. The microsomal fraction contained the highest 11-HSD specific activity and the activity increased with increasing pH. Progesterone was found to be an effective inhibitor of 11-HSD oxidative activity in placental microsomes as was B and 11α-OH-progesterone (Lopez Bernal et al, 1980).

Murphy (1981b) found that the placental 11-HSD had a high degree of specificity for 11β-OH-progesterone and 11β-OH-pregnenolone and their 20α-OH analogues and suggested that these substrates were as good if not better substrates for the enzyme than F or B.

Beitins et al (1973) conducted a study on various parameters of F metabolism, including interconversion, in human pregnancy near term through a constant infusion of 1H-F and 3H-E and subsequent blood sampling from a maternal vein and cord vein. They calculated that almost 90% of fetal E originated from maternal F while only 25% of fetal F came from the mother, suggesting that conversion of F to E occurred during transplacental passage. A more direct indication of extensive placental conversion of F to E was provided by Murphy et al (1974) who showed that in early pregnancy in the human, most of the F injected into the mother was converted to E during placental transfer to the fetus. In another study by Blanford and Murphy (1977), F and prednisolone were shown to be extensively oxidized by midgestational and late pregnancy placental minces, 67.4% and 51.4% respectively. On the other hand, betamethasone and dexamethasone were only converted to their 11-keto metabolites to the extent of 7.1% and 1.8% respectively (the ability of the placenta to metabolize these various
steroids is of clinical interest since they are used in the antepartum glucocorticoid treatment of RDS in premature infants. Their findings conflicted with those of Levitz et al (1978) who showed that perfused human placenta could metabolize F, prednisolone, dexamethasone, and betamethasone quite extensively during placental transfer in a manner which was efficient and similar for all four steroids. Levitz et al surmised that the difference between perfused placenta and minces was that the perfusion system was a dynamic one, which allowed for the simultaneous addition of new substrate and removal of the product. Although their system may seem to better reflect the in vivo situation than placental minces, it is possible that by perfusing the placenta with a buffered solution, which washed out the normal placental constituents without replacing them, that the physiological situation was compromised.

The influence of binding proteins on placental clearance and F conversion has also been studied using the perfused placental system (Dancis et al, 1978) and minces (Murphy, 1979b). Dancis et al found that corticosteroid binding globulin (CBG) at a concentration enough to bind 50% of F had no effect on its clearance while albumin enough to bind 50% (association constant 1,000 fold less than CBG) lowered clearance by 30%. Albumin and CBG together only lowered clearance to that of albumin alone. F to E conversion was about 85% and this was not altered significantly by protein binding. They suggested that albumin may be more important in vivo than CBG in controlling the transfer rate of F to the fetus. On the other hand, Murphy (1979b) using minces and in one instance a placental culture found that CBG did play a role in retarding F entry into placental villi, yet albumin only decreased F to E conversion slightly, results which were opposite to those of Dancis et al (1978).
What is clear from all of the above studies is that the placenta is an effective barrier to the transfer of F from the mother to the fetus. Dancis and coworkers suggested that control of induction of fetal enzymes by F could be controlled by endogenous F levels without interference from maternal F due to the placental barrier (Beitins et al, 1973). Also, many fetal tissues could control intra-tissue levels of F by means of 11-HSD activity. Murphy et al (1974) suggested that interference by maternal F in the fetus could be prevented by placental metabolism which would allow the fetal pituitary-adrenal axis to develop, in addition to preventing any unwanted catabolic or growth retarding effects that free passage of maternal F to the fetus could have. Most other species are analogous to the human in the ability of the placenta to metabolize F including rhesus monkey (Mittinger, 1974), baboon (Pepe and Townsley, 1977), and mouse (Burton and Jeyes, 1968). In sheep the barrier is not a metabolic but a physical (anatomical) one to the movement of F across it (Beitins et al, 1970). In either case, whether metabolic or physical, the placenta appears to provide a critical mechanism in maintaining low F levels in the fetus in face of high maternal levels of F.

(b) Membranes (choriodecidua). 11-HSD activity (oxidative) in homogenates of human fetal membranes (fetal chorion plus maternal decidua or choriodecidua) was first noted by Osinski (1960). This finding was substantiated by Murphy (1977a) and by Tanswell and associates (1977), but contrary to the study by Osinski the activity was described as being predominantly reductive (E to F) in minces of choriodecidua from 20 weeks (gestational age) onward. Subsequent to this work, Lopez Bernal and colleagues (1980) suggested that the 11-HSD activity, which was reversible, resided in the maternal decidua since they could not detect any 11-HSD
activity in chorion (free of decidua) from dichorionic twins. Lopez Bernal et al. (1982a) have also reported recently that the direction of the reaction in human decidual tissue was dependent upon the tissue preparation. 11-HSD activity in homogenates was predominantly oxidative while that in minces was predominantly reductive. These findings have been confirmed in our laboratory (Murphy et al., 1982) for explant cultures of various preparations of choriodecidua. 11-HSD activity was not found in explant cultures of chorion from which the decidua had been scraped off and homogenization of decidual tissue altered the direction of conversion from E to F to F to E.

What physiologic role the enzyme plays is not known, however, it has been suggested that the conversion of E to F by the decidua provides the amniotic fluid with F as well as maintaining a high F:E ratio in the membranes (Murphy, 1977a; Tanswell et al., 1977) which could be involved in the prevention of fetal allograft rejection.

6. 11-HSD Activity in the Fetal Lung

(a) In rabbit and rat fetal lung. 11-HSD activity in rabbit fetal lung was first noted by Giannopoulos (1974). It was thought that if F played a major role in the maturation of the fetal lung, then conversion of circulating E, reaching the lung, to F could raise the intracellular levels of F so that its hormonal effects could be evoked. Injection of 0.2 μg 3H-F (3H-E was not injected) into 28 day-old rabbit fetuses (term, 31 days) and subsequent removal of the fetal lungs 20 min later, resulted in 1.0-1.4% of the radioactive dose reaching the lungs. Most of it was unchanged 3H-F, and only a minor amount (5-7%) of 3H-E was isolated. These in vivo results agreed with in vitro incubations of fragments of fetal lung with
In which only 10-14% of F was converted to E. On the other hand, $^3$H-E was converted to F, 64-71%. In terms of nuclear uptake and binding, whether the fetal lung was incubated with $^3$H-F or $^3$H-E, the same amount of uptake and binding in the nucleus occurred, and virtually all of the radioactivity was F, suggesting that conversion of E to F was a necessary step for uptake and binding to occur. Therefore, in late gestation, a rise in the concentration of F, as well as of E in the plasma, could contribute to increasing the intracellular levels of F in the lung through the action of the enzyme 11-HSD.

11-HSD activity has also been studied in serially propagated cell cultures derived from 20 and 28 day-old (gestational age) rabbit fetal lung (Smith and Giraud, 1975). Small petri dishes (30 mm), initially plated with 2 x 10^5 cells and allowed to reach confluence, were used in the determinations of 11-HSD activity. $^3$H-E and $^{14}$C-F were added to the culture medium in the dishes in equimolar amounts (22 μg) which were "exceedingly high." Twenty and 28 day-old (gestational age) fibroblast monolayer cultures converted E to F to the extent of 3.5% and 15.6% respectively. F to E oxidation was also detected; 0.5% for 20 day cultures and 0.1% for 28 day cultures. A mixed population of 28 day-old rabbit fetal lung cells, as monolayer cultures, was also shown to convert 21% E to F over a 6 hr incubation at a concentration of $^3$H-E of 2.5 x 10^{-6} M (0.9 μg/ml) in the culture medium (Torday et al, 1975). Phase contrast photomicrographs of these cells in culture revealed two cell types, fibroblast-like and epithelial-like, which increased proportionally in the presence of F (2 μg/ml) (Smith, et al, 1974). Cells (initially plated at 1-2 x 10^6 cells/ml), grown for 6 days without any steroids added to the tissue culture medium, and then incubated for 6 hr with $^3$H-E (0.9 μg/ml), converted 21% E to F. Cultures grown for 6 days with F (2 μg/ml) converted
even more E to F, 38%. 11-ketoprogesterone inhibited the enzyme activity and conversion of E to F was reduced to 3% along with a reduction of lecithin synthesis (Torday et al, 1975). 11-Ketoprogesterone alone had no effect on lecithin synthesis, but when F was added with the inhibitor no difference in lecithin levels was observed compared with cultures in which only F had been added. These results suggested that F was the active hormone and that E only became effective in stimulating lecithin synthesis following its conversion to F.

Rabbit fetal lungs at 26 days gestation, perfused with $^3$H-E or $^3$H-F after occlusion of the ductus arteriosus, causing shunting of 100% of the perfusate through the lungs, actively converted E to F with little conversion of F to E (Torday et al, 1976) in agreement with previous results (Giannopoulos, 1974). Fetal rabbit lung homogenates of various gestational ages, incubated for 2 hr with $^3$H-E (0.018-18 ng/ml) with or without cofactors, provided some interesting data as to the ontogeny of E to F 11-HSD activity: E to F conversion was not detected in day 21 fetal lung homogenates whether cofactor was added or not; it increased in homogenates from day 23 onward to day 29; and could be stimulated with the addition of cofactor. Unfortunately, F to E conversion was not investigated. In utero, when the ductus arteriosus is patent, only 10% of the cardiac output passes through the fetal lung. Torday and coworkers showed that the fetal rabbit lung near term was able to actively convert E to F, even at concentrations of E 40 times greater than that found in the circulation. In the fetal rabbit near term, F levels rise, while E remains fairly constant and slightly less than F (Barr et al, 1980).

Nicholas and colleagues (1978), studying 11-HSD activity in rabbit fetal lungs during the final ten days of gestation, reported similar
results to those of Torday et al (1976). Using minces of fetal lung, incubated with $^{14}$C-E or $^{3}$H-F (3.6 ng/mg), E to F conversion increased about 7 fold from day 20 to day 30; although contrary to the results of Torday et al's (1976) study (see above), 11-HSD activity was present on day 20, albeit low. The difference in results was probably due to the difference in tissue preparation; homogenates (Torday et al, 1976) versus minces (Nicholas et al, 1978). Choline incorporation into total lipids followed closely the increase in E to F conversion during gestation, as well as the retention of F in lung tissue following incubation with E. This suggested that F, increased by 11-HSD activity in the lung, could then stimulate synthesis of surfactant phospholipids in the fetal lung near term.

These studies have demonstrated that in vivo, and in vitro, rabbit fetal lung near term predominantly converts E to F and that this activity increases closer to term. Homogenates, however, of rabbit fetal lung 19, 24, and 30 days gestation, when incubated with $^{14}$C-F (6.6 ng/ml) and NADP as cofactor, progressively converted more F to E closer to term (Brooks et al, 1977) which does not agree with the above results for the in vivo situation. Thus homogenization of tissue, which destroys the normal cellular compartments, caused a change in direction of conversion from E to F to F to E. In the case of 11-HSD that change is usually in the oxidative direction for example as seen in human decidua (Lopez Bernal et al, 1980). Enzyme activities in homogenates, therefore, may not reflect the activity seen in vivo and may make physiological interpretations of results difficult. Conversion of F to E has been shown to occur in perfusion studies of adult rabbit lung, but conversion of E to F was not studied (Brooks et al, 1977). This seems to be a common problem in many studies on 11-HSD activity in which conversion in only one direction is considered.
11-HSD activity in 27 day-old rabbit fetal lung could not be stimulated by prior injection of 10 μg or 50 μg of dexamethasone into 25 day-old fetuses (Lugg and Nicholas, 1978). In a previous study, 11-HSD activity in midgestational HFL monolayer culture had been shown to be stimulated by F (Smith et al, 1973). Lugg and Nicholas suggested that the discrepancy in results may have been due to the differences between in vitro cultures versus in vivo experimental design, as well as perhaps differences between species. Lugg and Nicholas (1978) pointed out that cells in culture may not reflect their in vivo activity, since in one study after only one day in culture, type II alveolar cells differed histologically and biochemically from freshly isolated type II cells (Mason et al, 1977). They also raised the question as to whether dexamethasone was an appropriate substitute for the indigenous hormone.

In rat fetuses at 17 days gestational age, lungs converted more F to E than E to F. This was reversed in fetal lungs of 18, 19, 20, and 21 days gestation (22 days being term) in which the % of C-11 activation (net gain or loss of biologic activity, F being active, E inactive) increased and on day 21 was +48% (Smith, 1978). In the 21 day-old fetus though, A levels (ng/g fetus) were twice B levels. Injection of 11-ketoprogesterone (200 μg) to 17 day-old fetuses, which were removed on day 21, resulted in a decrease in C-11 activation to 10%, as well as a decrease in pulmonary saturated phosphatidylcholine in the lung. Smith suggested that the 11-HSD activity in the lung could elevate B levels in spite of overall higher A levels in the fetus. Also, A would act as a reserve for B, and the increasing C-11 activation in rat lung could act as the trigger for initiating the final stages of fetal rat lung maturation. In the mouse fetus there is a decrease in the conversion of B to A (inactivation) which Burton and Jeyes
(1968) attributed to increasing activation by the liver. However, the mouse fetal lung cannot be ruled out as a possible source of increasing activation, which may be similar to the rat and rabbit fetal lung in which activation of F, by increasing 11-HSD activity, occurs late in gestation (Smith, 1978; Nicholas et al, 1978; Torday et al, 1978). In the adult rat, the ratio of B to A is greater than 1, but in the fetus the opposite is true (Smith, 1978).

If only 10% of the blood flow passes through the lungs, then the development of an effective method of converting E to F could be an important mechanism for elevating F levels in the lung parenchyma to the point where the hormone can play a role in the maturation of the fetal lung near term.

(b) In human fetal lung (HFL). The metabolism of labelled steroids was studied in two midgestational human fetuses (Pasqualini et al, 1970b) (see above). There was no $^{14}$C-F found in any of the fetal tissues. In all fetal tissues, including lung, only $^3$H-E was recovered, indicating that 11-HSD activity in the fetus was oxidative (conversion of F to E) and no evidence for reductive activity was found. At midgestation, oxidative activity predominates in a number of other species and tissues including mouse (Burton and Jeyes, 1968), guinea pig liver (Pasqualini et al, 1970a), and rat lung (Smith, 1978).

Smith and associates (1973) were interested in studying the effects of F on the development and maturation of the human fetal lung and employed tissue culture techniques. They set up monolayer cultures of 10-20 week-old human fetal lung (HFL) and grew the cells in small flasks (25 cm$^2$) (initial plating was 5 x $10^5$ cells/flask) for a period of 5-10 days, at which time
confluence of the cultures was reached. Cultures, in which 2 µg/ml of F was added, reached confluence in 3 days, which Smith and colleagues suggested was due to the stimulation of cellular growth. Whether the cultures were or were not treated with F, at confluence the flasks contained the same amount of DNA, about 130 nM, indicating that at confluence cellular content was similar in all flasks. Cells, after various times in culture, were incubated for 12 hr with equimolar amounts of 3H-E and 14C-F (148 ng/ml). No significant conversion of F to E occurred at any time during the 10 days in culture. E to F conversion, which was 0 on day 0, increased with cellular growth and reached about 35% at confluence. Cells grown with 2 µg/ml F had increased 11-HSD activity, about 60% conversion at confluence. The results, in terms of 11-HSD activity, were opposite to the activity described in the human fetus by Pasqualini et al (1970b). The discrepancy in results in the work of Pasqualini et al, and that of Smith et al, was intriguing. Interestingly, cultures of a human tumor cell line (A549), possessing morphologic and biochemical features of the pulmonary alveolar type II cell, were found to be unable to convert E to F but could convert F to E following their incubation for 6 hours with equimolar amounts of 14C-F and 3H-E (36 ng/ml); albeit to a rather limited extent, only 5.2% (Smith, 1977). Whether this is indicative of normal type II cell activity is not known.

Studies of 11-HSD activity in human fetal lungs of various gestational ages and of infants revealed that enzyme activity decreased with gestational age and was variable in infants and children, although data was scarce for these latter categories (Murphy, 1978a). Lung minces were incubated for 2 hr with tracer amounts of 3H-F or 3H-E (40 pg). 11-HSD activity was highest in 11-16 week-old fetuses, falling to very low levels in spontaneous premature at 22-37 weeks. For 11-16 week-old fetal lungs, F
to E conversion was 54% and E to F was 2.1%; for 17-21 week fetal lungs, F to E conversion was 55% and E to F was 9.6%. 11-HSD activity was near 0 for prematures with E to F conversion being slightly higher than F to E, although this difference was not significant. The concentration (ng/g) ratio of F:E in the lung rose with gestational age, although in the serum the F to E ratio was higher than in the lung for midterm and premature infants. Thus, minces of midgestational HFL predominantly oxidized F to E (Murphy, 1978a) agreeing with the in vivo evidence of Pasqualini and co-workers (1970b), but directly contrary to the results of Smith et al (1973) who showed that 11-HSD activity in monolayer cultures of midgestational HFL was exclusively reductive (see Fig. 1-2). The reasons for this discrepancy and the regulation of 11-HSD activity became the objects of this thesis.

H. FETAL LUNG DEVELOPMENT

In order to interpret results of fetal lung studies, it is necessary to have some knowledge of fetal lung development which is briefly outlined below.

The entire epithelial structure of the fetal lung develops from the foregut, which is lined by endoderm, when the embryo is only 3 mm in length (during the fourth week of gestation).

The development of the fetal lung has been classically divided into three stages, the glandular, the canalicular and the terminal sac or alveolar period (Emery, 1969; Conen and Balis, 1969; Meyrick and Reid, 1977).

1. **Glandular Period** (up to 16 weeks in humans)

   During this phase, mesenchymal tissue surrounds epithelial lined tubes, the primitive bronchi and bronchioles, which by dichotomous
fig. I-2. Activation versus inactivation in midgestational HFL. Activation indicates % of labelled F in HFL lung derived from labelled E while inactivation indicates % of labelled E in HFL derived from labelled F.
branching generate other primitive bronchi and bronchioles. Capillaries are not in contact with the epithelial tubes. The pulmonary arterioles and arteries can be recognized and lymphatics are numerous, especially around arteries.

2. Canalicular Period (16 to 24 weeks in humans)

All primitive bronchi have developed, however there is still continued generation of primitive respiratory bronchioles from the primitive bronchi. There is some differentiation of epithelial cells into type I pneumonocytes during the formation of blood-air barriers. Connective tissue decreases relative to increased vascularization of the lung. The pulmonary arteries and lymphatic channels are well developed during this stage.

3. Alveolar Period (24 weeks onward in humans)

There is continued generation of primitive respiratory bronchioles as well as terminal air spaces (alveoli) with further differentiation of epithelial cells into type I and type II pneumonocytes. There are numerous blood-air barriers along with the thinning of septa. Surfactant, synthesized and secreted by type II pneumonocytes, becomes detectable during this period. At birth, however, only 8% of the adult number of alveoli (300 million attained by 8 years of age) are present (Meyrick and Reid, 1977).

4. Effects of Hormones on Fetal Lung Development

In experimental animal studies corticosteroids have been shown to stimulate maturation of various fetal organs, preparing the fetus for life after birth (Liggins, 1976; Ballard, 1979). Similar adrenocortical-related maturational events in the human fetus are not as well documented although evidence for them has been increasing.
A great deal of research has been carried out on the role that corticosteroids play in fetal lung maturation and most of the evidence suggests that glucocorticoids are critically involved in fetal lung development along with the involvement of other hormones as well (Ballard, 1979; Hitchcock, 1979; Hitchcock, 1980; Giannopoulou and Tulchinsky, 1979; Mendelson et al, 1981). These reviews (listed on the effects of glucocorticoids and various other hormones on fetal lung development) cite evidence for glucocorticoid effects assumed to be due to the direct action of the steroid on the target cell, in this case the type II pneumonocyte (i.e., stimulates the synthesis of an enzyme or enzymes involved in surfactant production in the type II cell, thereby increasing the level of surfactant in the lung). However, Smith (1979) proposed an indirect effect for the action of the hormone on fetal lung development: the hormone stimulates lung fibroblasts to synthesize a fibroblast pneumonocyte factor which could then act on the epithelial type II cells to synthesize more phosphatidylcholine, which in turn made up of an increased proportion of disaturated phosphatidylcholine (a major component of surfactant). Glucocorticoid receptors have been found in both fetal lung fibroblasts and type II cells (Ballard et al, 1978).
CHAPTER II

MATERIALS AND METHODS
A. MATERIALS

1. Tissue Culture Equipment

Edgegard laminar (horizontal) flow hood (Baker Co., Biddeford, Maine)
Incubator (National, Portland, Oregon)
Inverted microscope (Leitz Co., Midland, Ontario)
Low speed centrifuge (International, Boston, Mass.)
Alcohol burner
Glass bottles; 100 ml, 200 ml and 500 ml
Plastic centrifuge tubes (50 ml) with caps (Corning)
Pasteur pipettes (long; 23 cm) (Fisher Sci. Co.)
Millipore filters 0.22 µ (Millipore, Bedford Mass.)

Small plastic tissue culture dishes (60 x 15 mm), quartered petri dishes (100 x 15 mm), and small tissue culture flasks (25 cm²) were obtained from Falcon Plastics through Fisher Scientific Co. (Montreal, Canada).

Grids (2.5 x 2 cm) were cut from 40 mesh stainless steel which was obtained from Johnson Wireworks (Montreal, Canada).

2. Tissue Culture Reagents

Powdered Ham’s F-10 nutrient medium was obtained from Grand Island Biological Co. (GIBCO), Montreal. Fetal bovine serum (FBS) (virus screened, mycoplasma tested, and heat inactivated) was also purchased from GIBCO.

Antibiotics used were:

1) Amphotericin B (Fungizone; Squibb, 50 mg/vial)
2) Penicillin G (Crystapen; Glaxo Labs. 1 or 10 million I.U./vial)
3) Gentamycin Sulfate (Garamycin; Schering, 80 mg/vial)
Trypsin, 1:250 (Difco Laboratories, Detroit, MI.)
DNAase I from bovine pancreas; 2,000 Kunitz units/mg (Sigma Chemical Co.)

3. Hormones used in Tissue Culture Experiments

Unlabelled E and F (Sigma Chemical Co., St. Louis, MO.)
hCG (Sigma Chemical Co.)
hPr (NIH)
hPl (was a gift from Dr. H. Friesen)
Oxytocin (Syntocinon, Sandoz)
T3 (Tertroxin, Glaxo)
ACTH (Cortrosyn, Organon)
Estrone, estradiol, estriol, progesterone (Sigma Chemical Co.)
NAD, NADP, NADH, NADPH, (Sigma Chemical Co.)

4. Radioactive Compounds

[4-^14C]-F (SA 55 mCi/mmol), [1,2,6,7-(N)-^3H]-F (SA 98 Ci/mmol) and
[1,2-(N)-^3H]-E (SA 48 Ci/mmol) were purchased from New England Nuclear
Corp. (NEN) (Boston, MA.).

[4-^14C]-E was made by incubating 14C-F with fresh minced term placenta
(see methods section for details).

5. Column Chromatography Equipment and Materials

In initial experiments, automated separation of F and E was achieved
using a pre-existing setup in the laboratory (for details of equipment and
materials see Murphy and Diez D'Aux, 1975).

In subsequent separations, 10 ml pyrex burets from Fisher Scientific
Co. (Montreal, Canada) were used as columns. The column solvent was
methylene chloride:methanol 98:2 (all glass redistilled reagent grade,

6. Reagents for F Assays

Antibody to F (NEN, Boston, MA.)
Horse serum (obtained from a local farm)
Dog serum (obtained from Animal Research Center, McGill University)
Florisil, 100-200 mesh (Fisher Scientific Co., Montreal)
Gelatin (obtained from the local grocery store)

7. Counting of Radioactivity

All radioactive counting was done using a Philips Liquid Scintillation Analyser (LSA).

8. HFL Tissue

Lung tissue from human fetuses (9-20 weeks) was obtained at the time of therapeutic abortion by dilatation and evacuation (D and E) or hystereotomy, at the Montreal General Hospital (MGH) and was handled under sterile conditions.

B. STERILE TECHNIQUE

All culturing was done under sterile conditions. HFL tissue, once obtained, was placed in a sterile petri dish containing GIBCO 'A' solution, which had been previously sterilized using a millipore filter (0.22 μ). The dish was then placed in the horizontal laminar flow hood, which maintained a sterile environment within the hood area (Fig. II-1). All materials used (pipettes, pipette tips, bottles, filters, grids, scissors, forceps, etc.) in connection with tissue culture were gas or steam autoclaved at the Central Supply Room in the MGH. Sterile gloves and masks were also used to insure minimal possibility of contamination of the cultures.

Only during the initial experiments did some flasks become contaminated with bacterial or fungal infections and these flasks were immediately discarded upon detection of infection.
Fig. II-1. Photograph of the Edgeguard laminar flow hood and tissue culture set up used to change the TCM in flasks and/or culture dishes.
C. METHODS

1. Preparation of $^{14}$C-E

$^{14}$C-E is not available commercially, therefore, $^{14}$C-E was made routinely by incubating $^{14}$C-F with fresh minced term placenta, which converts F to E with no further metabolism (Murphy, 1978b). $^{14}$C-E was separated from other steroids using Sephadex LH-20 columns (40 x 0.9 cm). Steroids were eluted with methylene chloride:methanol (98:2) (Murphy, 1971), which was evaporated and replaced by ethanol, in which the $^{14}$C-E was stored at -20°C.

Results for a typical incubation (Fig. II-2) show that 2 g of placental mince in 3 ml of saline, containing approximately 9 x $10^6$ cpm of $^{14}$C-F, incubated for 2 hr at 37°C in a shaking water bath, converted 40% of $^{14}$C-F to $^{14}$C-E (3.4 x $10^6$ cpm). Only 6% of the total counts were not recovered as $^{14}$C-E or $^{14}$C-F from the column. The $^{14}$C-E was then used in subsequent experiments.

2. Preparation of Solutions for Tissue Culture

(a) GIBCO solution 'A'. (Earle's Balanced salt Solution 401, -Ca++, -Mg++, PO_4; pH 7.2)

For 1 l of 1x concentrated solution:

a) NaCl 6800 mg/l
b) KCl 400
c) glucose 1000
d) NaHCO_3 2200
e) phenol red 1.2

The reagents were dissolved in distilled water and the solution was sterilized by millipore filtration (0.22 µ filter). Antibiotics (Amphotericin B (5 µg/ml), Penicillin G (200 IU/ml), and Gentamycin Sulfate
Fig. II-2. Separation of $^{14}\text{C}-\text{E}$ and $^{14}\text{C}-\text{F}$ on a sephadex LH-20 column (length 40 cm, diameter 0.6 cm) following the incubation of $^{14}\text{C}-\text{F}$ with minced human placenta. $^{14}\text{C}-\text{E}$ eluted in the 36-42 ml fractions while $^{14}\text{C}-\text{F}$ eluted in the 58-68 ml fractions. Each bar represents the radioactive counts eluted in each fraction.
were then added and the solution was stored at 4°C for up to 6 months. The solution was used in the preparation of HFL monolayer cultures.

(b) 2% Versene solution for subculture.

a) versene (EDTA) 0.2 g/l
b) NaCl 8.0
c) KCl 0.2
d) Na$_2$HPO$_4$ 1.15
e) KH$_2$PO$_4$ 0.2
f) glucose 0.2
g) phenol red 1.2 mg/l

The reagents were dissolved in distilled water and the solution was sterilized by millipore filtration. Antibiotics were then added (as above) and the solution was stored at 4°C. For use in subculturing cells, 4 parts of the 2% versene solution were diluted with one part of (0.25%) trypsin solution.

(c) 0.25% Trypsin stock solution for HFL trypsinizations. 1,250 mg of trypsin was dissolved in 0.5 l of GIBCO Solution 'A' and left overnight at 4°C to dissolve. The solution was then millipore filtered. Antibiotics (as above) were then added and 50 ml aliquots were stored frozen at -20°C; these could be kept up to 6 months.

(d) DNAase for HFL culture preparations. 5 mg of DNase (2,000 units/mg) was weighed out and then dissolved in 5 ml of GIBCO solution 'A' making a 1 mg/ml solution. This solution was added as needed to the HFL cell dispersion. This solution was usually made up fresh for each experiment or was stored for no more than 1 month at 4°C.
3. Preparation of Tissue Culture Medium

Ham's F-10 powdered medium (for formulation see appendix I) was dissolved in 1 l of distilled water. Then, 1.2 g of NaHCO₃ was added and the pH of the solution was adjusted to 7.1 with 1N HCl. This was necessary since the pH of the solution prior to the addition of HCl was usually around pH 7.3-7.6. The solution was then millipore filtered (0.22 μ) (which resulted in an increase in the pH from 7.1 to approximately 7.3) into two 500 ml bottles. Fifty ml of fetal bovine serum (FBS) was then added to 450 ml of Ham's F-10. The medium was placed in an incubator overnight in order to test for sterility. The following day, antibiotics were added to the medium: Amphotericin B (5 μg/ml), Penicillin G (200 IU/ml), and Gentamycin Sulfate (40 μg/ml).

Thus, Ham's F-10 supplemented with 10% FBS and antibiotics constituted the tissue culture medium (TCM), which was used in all experiments in which HFL was cultured as monolayers or as explants.

In initial HFL monolayer culture experiments, conversion of F to E or E to F was studied per flask. Therefore, TCM was made up with tracer amounts (10-60 pg/ml) of ³H-F or ³H-E (4,000-10,000). One hundred ml of medium with radioactive tracer was made up by adding the required amount of tracer in absolute ethanol to a sterile bottle and evaporating the ethanol under a stream of air. Then 100 ml of TCM was added to the bottle and the steroids were allowed to dissolve with occasional gentle shaking. The TCM with the dissolved radioactive steroids was stored at room temperature in the dark. For each experiment, 100 ml of TCM lasted only 2 to 4 days, during which time steroid breakdown was minimal.
All other tissue culture experiments (the remaining monolayer culture experiments and all of the explant culture experiments) were carried out with TCM containing $^3$H-F and $^{14}$C-E added in equimolar amounts, 16.7-55.6 nM (6-20 ng/ml).

4. Monolayer Cultures

Tissue culture techniques were learned at the Montreal Childrens Hospital (MCH) under the supervision of Cheri Deal (a graduate student) and Drs. C. L. Branchaud and C. Goodyer.

The method of Smith and coworkers (Smith et al, 1973) was followed. The tissue was washed 2 or 3 times in GIBCO solution 'A' and then minced finely with scissors. The mince was added to about 15 ml of 0.25% trypsin solution with 5-10 drops DNAase (100-200 units) added to reduce tissue aggregation, and stirred gently at 37°C for 10-15 min using a Teflon-coated microspinbar. After the contents had settled, the supernatant was removed with a pasteur pipette and placed in a 50 ml plastic tube containing 10 ml of TCM to inactivate the trypsin. The cells were pelleted by centrifugation for 10 min at no more than 100 x g. The supernatant was discarded and the pellet was resuspended in TCM using a Pasteur pipette. After the remaining mince was subjected 2 more times to the above procedure, the contents of the 3 tubes were combined and centrifuged for 10 min at no more than 100 x g. The pellet was resuspended in TCM, and an approximate cell count was obtained using a hemocytometer. In initial experiments, the trypan blue dye exclusion test (cells with damaged membranes cannot exclude the dye and are thus seen as coloured blue when examined under the microscope) was used to show that approximately 90% of the cells were viable. Aliquots of 1 ml
containing 0.5 x 10⁶ cells were then plated in plastic culture flasks containing 3 ml of TCM for a final volume of 4 ml/flask. The flasks were flushed with 5% CO₂ and 95% air for 5 sec, capped tightly, and placed in an incubator at 37°C. Cultures were examined daily under the microscope. Media were collected and changed every 24 hr; approximately 1 ml was removed for processing, and the remainder was stored at -20°C.

5. Subculture of HFL Monolayers

For subculture, a 0.25% trypsin:2% versene solution (1:4) was used to lift the cells off the bottom of the flask. The monolayers were rinsed briefly with the trypsin-versene solution, and then 2 ml fresh trypsin-versene was added and left until the cells were observed under the microscope to have detached (about 5 min). Afterwards, they were pelleted by centrifugation at 100 x g, resuspended in TCM, and plated.

6. Explant Cultures

Lung tissue was placed in a culture dish with TCM and minced with scissors. Twelve pieces of lung (0.5-1 mm³) were placed on each stainless steel grid, and each grid was placed into a quadrant of a compartmented dish. Two ml of medium were added to each quadrant, and the dishes were placed in an incubator at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Cultures were examined daily under the microscope for outgrowth along the grid. Media were collected and changed every 24 hr; 1 ml was removed for processing, and the remainder was stored at -20°C.

7. Photography of Monolayer and Explant Cultures

Photomicrographs were made with a Nikon camera, loaded with Kodak film (400 ASA (B/W)), attached to a Nikon inverted microscope. One hundred times magnification was used for all photomicrographs. The photography was
done at the MCH under the supervision of Drs. C. L. Branchaud and C. Goodyer. The film was processed by the Audiovisual Department at the MCH.

8. Separation of E and F

Each 1 ml aliquot was extracted twice with 3 ml ethyl acetate, vortexed for 2 min, and then centrifuged to separate the upper ethyl acetate phase containing the extracted steroids from the lower aqueous phase. The combined extracts were evaporated, redissolved in 0.2 ml methylene chloride:methanol (98:2), and passed through a column (40 x 0.4 cm) of Sephadex LH-20 to separate the E and F fractions (Murphy, 1971).

The recovery of E and F was approximately 85%, indicating that little or no other metabolism occurred. Breakdown of the steroids to nonpolar compounds accounted for most of what was not recovered as E and F. The % conversion was calculated as counts per minute (cpm) of product x 100 divided by cpm of product plus substrate. The method is outlined in figure II-3. Conversion in the medium in the absence of cells was calculated and subtracted from the results unless stated otherwise.

Sephadex LH-20 has been shown to be very effective in separating unconjugated steroids, including E and F (Murphy, 1971). Separation of E and F in initial experiments, in which only tritiated E and F were being used, was done on 4 automated Sephadex LH-20 columns already existing in the laboratory (Murphy and Diez d'Aux, 1975). This only allowed for the processing of 4 samples at a time (automation of more columns was not feasible) which required 3 to 4 hr operating time.

Therefore, 10 columns, manually operated, which required the same amount of time to complete a run as the automated columns, were set up (Fig. II-4). In addition, with the use of $^3$H and $^{14}$C labelled steroids, it
Ethyl acetate extract of steroids (labelled and unlabelled) from TCM

Evaporate
Dissolve steroids in 0.2 ml column solvent (methylene chloride methanol) (98:2) and add sample to column

Glass Syringe (50 ml)
Hypodermic Needle (20)
Pyrex Buret (10 ml)
Sephadex LH-20
Length 40 cm
Diameter 0.4 cm
Teflon Stopcock
Plastic Vial

Evaporate
Add 2 ml of counting solution to each vial (Econofluor) and count in Philips LSA

Eluate (mLs)

% Conversion = \frac{\text{cpm product}}{\text{cpm product} - \text{substrate}} \times 100

Fig. II-3. Method for determining % conversion of radioactive E and F in TCM.
Fig. II-4. Photograph of the 10 sephadex LH-20 columns which were set up to separate labelled E and F in the TCM.
became possible to achieve in effect, 20 separations during one run of all ten columns, thereby greatly increasing the number of samples processed. Each run required 40 ml of solvent which took approximately 3 hr to run through, in which pressure was applied by means of a syringe attached to the top of each column, and flow rate could be controlled by turning the stopcock at the bottom of the column, thereby adjusting the opening (Fig. II-4). The flow rate was approximately 1 ml/5 min. As shown in figure II-5 and Table II-A, the separation of $^{3}H$-E and $^{3}H$-F was extremely consistent for all 10 columns.

Routine collection of fractions was carried out in the following manner:

<table>
<thead>
<tr>
<th>Fraction (ml)</th>
<th>Collected</th>
<th>Discarded</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-16</td>
<td></td>
<td></td>
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<td>16-18</td>
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<td>18-20</td>
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<td>20-22</td>
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<tr>
<td>22-26</td>
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<tr>
<td>26-28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28-40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Discarded</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Therefore, for each run, 7 fractions were collected which allowed for a good resolution of the separation of E and F. Collection of fraction 28-40 removed any remaining counts, thus preparing the column for each subsequent separation. Also, temperature of the room was kept at 20°C which has been shown to improve separation (Murphy and Diez d'Aux, 1975).

Since the columns were packed, over two years ago, separations of E and F have been more than satisfactory and virtually trouble free.

There was very little inter-sample variation as shown in Table II-B, in which, % conversion of E to F and F to E in representative samples was calculated in triplicate, and which had an average SD of approximately 2%. As can also be seen in Table II-B, storage of the samples for over a year did not affect the results to any noticeable degree.
Fig. II-5. Separation of $^3$H-F and $^3$H-E on 10 sephadex LH-20 columns (length 40 cm, diameter 0.4 cm). $^3$H-E eluted in the 10-16 ml fractions while $^3$H-F eluted in the 18-26 ml fractions. Each bar represents the radioactive counts in that fraction summed over 10 separations.
Table II-A. Separation of equal amounts of tracers $^3$H-E and $^3$H-F on 10 sephadex LH-20 columns.

<table>
<thead>
<tr>
<th>Fractions (ml)</th>
<th>0-4</th>
<th>4-6</th>
<th>6-8</th>
<th>8-10</th>
<th>10-12</th>
<th>12-14</th>
<th>14-16</th>
<th>16-18</th>
<th>18-22</th>
<th>22-26</th>
<th>Total (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column #1</td>
<td>120</td>
<td>533</td>
<td>251</td>
<td>273</td>
<td>657</td>
<td>3834</td>
<td>2260</td>
<td>278</td>
<td>3376</td>
<td>2680</td>
<td>14262</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
<td>532</td>
<td>317</td>
<td>323</td>
<td>1188</td>
<td>4851</td>
<td>1283</td>
<td>661</td>
<td>5736</td>
<td>1131</td>
<td>16077</td>
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<td>503</td>
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<td>3534</td>
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<td>10</td>
<td>68</td>
<td>586</td>
<td>320</td>
<td>381</td>
<td>1642</td>
<td>4834</td>
<td>611</td>
<td>560</td>
<td>6417</td>
<td>1120</td>
<td>16539</td>
</tr>
<tr>
<td>Total</td>
<td>681</td>
<td>5323</td>
<td>3077</td>
<td>3322</td>
<td>10179</td>
<td>41403</td>
<td>18924</td>
<td>4748</td>
<td>52403</td>
<td>19432</td>
<td>158492</td>
</tr>
</tbody>
</table>

Total cpm as E, 70,506
Total cpm as F, 71,835

Recovery as E and F was 90% of the total cpm from the columns.
Breakdown to non-polar substances was 7.4% of the total counts (the non-polar substances were collected in fractions 4-6, 6-8 and 8-10.)
TABLE II-B. Inter-sample variation of % conversion in triplicate samples from 11-week (gestational age) HFL monolayer subcultures.

<table>
<thead>
<tr>
<th>Flask #</th>
<th>% conversion (E to F)</th>
<th>5-1</th>
<th>5-2</th>
<th>5-3</th>
<th>5-4</th>
<th>5-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-12-80*</td>
<td></td>
<td>42.0</td>
<td>49.5</td>
<td>46.0</td>
<td>42.6</td>
<td>50.0</td>
</tr>
<tr>
<td>10-7-82</td>
<td></td>
<td>45.6</td>
<td>52.9</td>
<td>48.9</td>
<td>42.4</td>
<td>53.7</td>
</tr>
<tr>
<td></td>
<td>mean</td>
<td>43.8</td>
<td>51.6</td>
<td>47.9</td>
<td>42.5</td>
<td>49.8</td>
</tr>
<tr>
<td></td>
<td>± SD</td>
<td>1.8</td>
<td>1.8</td>
<td>1.6</td>
<td>0.1</td>
<td>4.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Flask #</th>
<th>% conversion (F to E)</th>
<th>5-1</th>
<th>5-2</th>
<th>5-3</th>
<th>5-4</th>
<th>5-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-12-80*</td>
<td></td>
<td>10.7</td>
<td>13.3</td>
<td>8.1</td>
<td>9.1</td>
<td>8.6</td>
</tr>
<tr>
<td>10-7-82</td>
<td></td>
<td>12.1</td>
<td>12.5</td>
<td>11.5</td>
<td>11.1</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>mean</td>
<td>12.2</td>
<td>13.3</td>
<td>10.4</td>
<td>10.1</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td>± SD</td>
<td>2.8</td>
<td>0.8</td>
<td>2.0</td>
<td>1.4</td>
<td>2.5</td>
</tr>
</tbody>
</table>

* day on which samples were run on sephadex columns
9. Radioactive Counting

In the initial experiments, fractions were collected in test tubes, then transferred to glass counting vials (15 ml) and evaporated overnight. Then 2 drops of SnCl₂ (stannous chloride) and 10 ml of scintillation fluid (2,5-diphenyloxazole [PPO] 28 gm in 3.8 l toluene) were added to each vial, shaken, and placed into the Philips LSA for counting. The efficiency for \(^3\text{H}\) is approximately 50%, and for \(^14\text{C}\), 85%.

In all subsequent experiments fractions were collected directly into 4 ml plastic counting vials. The column solvent was evaporated and 2 ml of Econofluor (from NEN) was added to each vial for counting.

Dual label counting of \(^3\text{H}\) and \(^14\text{C}\) was achieved due to energy emission differences of the two isotopes (see appendix II for details).

LSA counting precision was assessed in a representative sample. As can be seen in Table II-C, the % F to E and F to E conversion, in which the counting was repeated 10 times, had SD's of less than ±1% conversion.

10. Identification of F in a HFL Monolayer Culture

It has been well established by several investigators, for various species, that in the fetal lung the major conversion product of E is F (Smith et al, 1973; Giannopoulos, 1974; Smith and Giroud, 1975; Torday et al, 1976; Nicholas et al 1978; Smith, 1978), and the major conversion product of F is E (Pasqualini et al, 1970b; Brooks et al, 1977; Murphy, 1978a).

The identification of F as the conversion product of E was, therefore, confirmed in one representative HFL monolayer culture experiment using the method of comparative competitive protein-binding assays (Murphy, 1973a).
TABLE II-C. Precision of scintillation counting by Philips LSA as assessed by calculating % conversion ten times in one sample of TCM from an 11-week (gestational age) HFL monolayer subculture.

<table>
<thead>
<tr>
<th></th>
<th>E to F</th>
<th>F to E</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>46.0</td>
<td>4.3</td>
</tr>
<tr>
<td>2.</td>
<td>46.8</td>
<td>4.0</td>
</tr>
<tr>
<td>3.</td>
<td>46.4</td>
<td>4.3</td>
</tr>
<tr>
<td>4.</td>
<td>47.0</td>
<td>3.7</td>
</tr>
<tr>
<td>5.</td>
<td>46.0</td>
<td>3.7</td>
</tr>
<tr>
<td>6.</td>
<td>47.5</td>
<td>4.0</td>
</tr>
<tr>
<td>7.</td>
<td>45.8</td>
<td>4.0</td>
</tr>
<tr>
<td>8.</td>
<td>45.8</td>
<td>3.6</td>
</tr>
<tr>
<td>9.</td>
<td>46.9</td>
<td>4.1</td>
</tr>
<tr>
<td>10.</td>
<td>45.6</td>
<td>4.0</td>
</tr>
</tbody>
</table>

mean ± SD | 46.4 ± 0.64 | 4.0 ± 0.24
In an experiment, in which 11-week old HFL was cultured, the net % conversion (% conversion of E to F minus that from F to E) reached 83% by 5 days in culture. Therefore, the concentration of F theoretically would have risen quite substantially, which would lend itself to easy confirmation by simply measuring the concentration of F in the TCM. This was done using horse and dog transcortin and an antiserum to F raised to a C³ hapten. The calculated F and observed F concentrations were in close agreement, confirming the identification of F as the conversion product of E (Table II-D).

11. Competitive Protein-Binding Assay Methods for F

(a) Radiotransinassay (RTA). The method below was first described by Dr. BEP Murphy (1964) (for details of the methods see Murphy, 1967). The following methods are, therefore, outlined in brief.

\(^3\)H-B (SA 50 Ci/mmol) was used as the tracer in all the assays. The protein tracer solution (PTS) was made by first adding \(2 \times 10^4\) cpm of \(^3\)H-B in ethanol to a 250 ml beaker and evaporating the ethanol. Then the serum (dog or horse) was added along with a 0.5% gelatin solution to the 100 ml mark in the beaker. The PTS was then mixed well by swirling and was ready to use. The standard curve for the assay was set up in duplicate with F in amounts of 0, 0.5, 1.0, 2.0, 4.0, and 6.0 ng/tube. The assay tubes received approximately 40 µl of TCM to be assayed, which was boiled for 2 min to inactivate the proteins in the TCM then 1 ml of PTS was added to each assay tube and the rack was shaken by hand gently for 1 min. The rack was then preincubated in a 45°C water bath for 5 min, after which it was incubated in a cold water bath (4°C) for an additional 45 min. With the rack still in the cold water bath 60 mg of Florisil was added to each assay
Table II-D. Identification of F as a product of E in monolayer culture.

<table>
<thead>
<tr>
<th>Day</th>
<th>Net conversion (%)*</th>
<th>Calculated F (ng)</th>
<th>Horse (ng)</th>
<th>Dog (ng)</th>
<th>Antibody (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>17</td>
<td>16</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>57</td>
<td>27</td>
<td>29</td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td>5</td>
<td>83</td>
<td>32</td>
<td>34</td>
<td>34</td>
<td>41</td>
</tr>
</tbody>
</table>

Each value is the mean of triplicate determinations.

*Net conversion = conversion of E to F minus that from F to E.
tube. Florisil was used to adsorb the free $^3$H-B. The rack was then shaken on an automatic shaker at high speed for exactly 1 min and allowed to stand for 4 min in the cold water bath to permit settling of the Florisil. Out of each assay tube, 0.5 ml was pipetted, being careful not to disturb the Florisil, and placed into a plastic vial along with 2 ml of Econofluor for radioactive counting in the LSA.

(b) Radioimmunoassay (RIA). The set-up for the RIA was slightly different from the RTA. Radioactive F was used as the tracer and approximately $10^4$ cpm were added to each assay tube. The standard curve was set up in duplicate with F in amounts of 0, 0.1, 0.2, 0.6, and 1.0 ng/tube to which the antibody solution was added. Fifteen µl of the TCM samples to be assayed were added to the tubes along with 0.5 ml of the antibody solution. The tubes were incubated for $\frac{1}{2}$ hr at room temperature, then centrifuged for 10 min at 3,000 rpm in a clinical centrifuge. The supernatant from each tube, which contained the unbound F, was decanted into a plastic vial and counted in the LSA.

12. Statistics

The statistical methods which were used included calculations of standard deviation and independent-samples t test. These are described by Colton (1974).
CHAPTER III

RESULTS
A. HFL MONOLAYER CULTURE EXPERIMENTS

1. Variability of E to F Conversion Between Monolayer Culture Experiments

In the control (untreated) flasks of all HFL monolayer culture experiments, initial conversion of E to F was minimal and only increased with cellular growth, reaching a plateau at about the same time the cultures became confluent. As can be seen in Table III-A the day on which the conversion of E to F began to increase varied from one experiment to the next although the time during which the increasing conversion occurred was similar in all experiments, i.e. 4 to 5 days. Consequently the day on which E to F conversion reached a plateau was different for each experiment. The % conversion reached at the plateau also differed from one experiment to another.

The data in Table III-A would suggest that neither HFL age nor initial plating concentration could account for the observed variability between experiments. A possible explanation is the FBS with which the TCM was supplemented. Each batch of FBS is made up of pools from bovine fetuses (6-9 months) and it cannot be assumed that different batches were similar in chemical and hormonal content (see Discussion).
Table III-A. Variability of results among HFL monolayer culture experiments.

<table>
<thead>
<tr>
<th>EXP #</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11 (Fig. III-1)</td>
<td>0.01</td>
<td>1.3 x 10^6</td>
<td>2-5</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>20 (Fig. III-2)</td>
<td>6.0</td>
<td>0.5</td>
<td>7-11</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>12 (Fig. III-3)</td>
<td>0.01</td>
<td>1.0</td>
<td>7-10</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>16 (Fig. III-4)</td>
<td>0.01</td>
<td>1.5</td>
<td>8-12</td>
<td>48</td>
</tr>
<tr>
<td>5</td>
<td>11 (Fig. III-5)</td>
<td>6.0</td>
<td>1.0</td>
<td>5-8</td>
<td>43</td>
</tr>
<tr>
<td>5</td>
<td>11 (Fig. III-5)*</td>
<td>6.0</td>
<td>1.0</td>
<td>4-8</td>
<td>78</td>
</tr>
<tr>
<td>6</td>
<td>12 (Fig. III-6)</td>
<td>6.0</td>
<td>1.0</td>
<td>5-8</td>
<td>50</td>
</tr>
<tr>
<td>7</td>
<td>11 (Fig. III-7)</td>
<td>17.0</td>
<td>0.7</td>
<td>2-5</td>
<td>86</td>
</tr>
</tbody>
</table>

A= HFL age (weeks).
B= substrate concentration (ng/ml).
C= initial plating density (cells/flask).
D= day of culture on which the % conversion of E to F began to increase until the day on which the % conversion of E to F reached a plateau.
E= % conversion (mean) in control flasks reached at the plateau.

* % conversion in flasks with placental medium (see text for details)
Another possible cause of the observed variability in results could have been differences in the plating efficiency (the % of cells initially plated which attach to the bottom of the flask) of the HFL cells. Plating efficiency can be influenced by a number of factors which could differ from one experiment to another related, for instance, to the trysinization procedure, the FBS used, or the tissue itself. Differences can be independent of the serum used since, in one experiment (Fig. III-7), some control flasks were plated in TCM which was supplemented with 10% human cord serum (HCS), and in these flasks the plating efficiency was observed to be similar to control flasks to which TCM plus 10% FBS had been added. However the cells failed to grow in TCM supplemented with HCS.

In experiment 5 (Fig. III-5), when control medium was mixed with medium from human term placental cell monolayer cultures (see below) the % conversion was increased when compared with the % conversion in control flasks. Growth was also observed to be stimulated. Since the placental medium contained factors, including hCG produced by the placental cells in culture the observed differences in results were perhaps due in part to the effects of one or more of these placental factors.
The variability between experiments made comparisons somewhat difficult so that each experiment was treated as a single unit and only general conclusions are drawn from the experiments as a group.

2. 11-HSD Activity in Monolayer Cultures

Figure III-1 shows results for 11-week HFL in monolayer culture. Two flasks contained $^3$H-E, and two flasks contained $^3$H-F at concentrations of 50 pg/flask. The concentrations of labelled steroids used were tracer amounts, $10^4$ times less than Smith et al (1973) used in their experiments (590 ng/flask). The flasks were initially plated with $1.3 \times 10^6$ cells/flask yet E to F conversion was minimal. Considerable F to E conversion was detected on day 2 of culture (30%), while E to F conversion was very low (5%). However, by day 6, F to E conversion had fallen to 10%, while E to F conversion had risen to about 20%, compared with 35% conversion found by Smith et al (1973).

In other experiments in which tracer amounts of E and F were used (Figs. III-3 and III-4), the labelled medium was not added to the cultures until some growth was apparent, which was not until 4 to 8 days in some cases.

In experiment 2 (Fig. III-2) 20-week HFL was cultured with 6 ng/ml of substrate E and F instead of tracer amounts. Similar E to F conversion results compared with experiment 1 (Fig. III-1) were found, but F to E conversion on day 1 was less than 5%, unlike the results for experiment 1 in which F to E conversion was 30% on day 2. This difference in results can
Fig. III-1. Conversion per 24 hr of $^3$H-E to $^3$H-F and of $^3$H-F to $^3$H-E by 11-week (gestational age) HFL monolayer cultures over 6 days. The substrate concentration was 50 pg/flask (12 pg/ml). Confluence occurred at 6 days (~10 mg cells). Each line represents conversion per one flask.
Fig. III-2. Conversion per 24 hr of $^{14}$C-E to $^{14}$C-F and $^3$H-F to $^3$H-E by 20-week (gestational age) HFL monolayer cultures over 14 days. The substrate concentration was 24 ng/flask (6 ng/ml). Confluence occurred at 10 days (~10 mg cells). Each line represents conversion per one flask (C denotes control flasks).
most probably be accounted for by the difference in substrate concentrations used when cell numbers were still low, and therefore, the approximately 500 times greater concentration used in the experiment (Fig. III-2) provided such an excess of substrate that the % conversion of F to E was undetectable.

3. Effects of Various Hormones on 11-HSD Activity in Monolayer Cultures

12-week HFL monolayer cultures were initially plated at 1.0 x 10^6 cells/flask. TCM, without tracer, was added for the first two days of culture. Starting from the third day, TCM with tracer, ^3^H-E or ^3^H-F, was added to the appropriate flasks. Hormones were added for three consecutive days starting on day 6 and the experiment was terminated on day 12 of culture. Figure III-3 shows results for the experiment. E to F conversion rose with growth of cells and reached 20-25% for control (untreated), hPr (250 ng/ml) and hPl (250 ng/ml) flasks at confluence (complete cellular coverage of the bottom surface of the flask as seen under the inverted LM). HCG (40 ng/ml) increased conversion significantly (p < 0.01 for hCG1 and hCG2 versus controls on days 9, 10, and 11) above controls on days 9, 10, and 11 averaging approximately 100% (Fig. III-3A). Thus effects of hCG were seen only after hormone treatment had ended. By 10 days in culture only the flasks to which hCG had been added were not completely confluent; thus, although hCG increased E to F conversion it did not seem to be associated with a general increase in growth and possibly may have slowed growth. This apparent effect, although observed in the subsequent experiment, was not studied further.

F to E conversion was low throughout the experiment, less than 10%, and was unaffected by any of the hormones; hCG, hPr or hPl (Fig. III-3B).
Fig. III-3. Conversion per 24 hr of \(^3\)H-G to \(^3\)H-F (A) and \(^3\)H-F to \(^3\)H-G (B) by 12-week (gestational age) HTL monolayer cultures in the absence and in the presence of hCG (40 ng/ml), hPr (250 ng/ml) and hPl (250 ng/ml) added to the cultures on days 6, 7 and 8. The substrate concentration was 48 pg/flask (12 pg/ml). Confluence occurred at 9 days (~10 mg cells). Each line represents conversion per one flask.
The results for the control flasks from this experiment were in agreement with those of Smith et al's (1973) in that E to F conversion rose with growth of the cells and F to E conversion was very low throughout the culture period.

In experiment 4 (Fig. III-4), 16-week HFL was cultured in flasks initially plated at $1.5 \times 10^6$ cells/flask. The cells grew very slowly and therefore, TCM with tracers was only added starting on day 5 until the end of the experiment (17 days). Test hormones were added beginning on day 10 for 4 consecutive days.

In the control, ACTH (300 pg/ml) or ACTH + hP1 (250 ng/ml) flasks, E to F conversion rose to 45-55% at confluence of the cultures. hCG (50 ng/ml) or hCG + ACTH treatment resulted in an increase ($p < 0.01$ for hCG or hCG + ACTH versus controls on days 14-17) in E to F conversion by approximately 20% above controls during the last few days of culture (Fig. III-4A). In this experiment, as in the last one, hormonal effects only occurred 2 or 3 days following the start of treatment and also, as in the previous experiment (Fig. III-3A), hCG treated cultures had not reached confluence by the end of the culture period while all others had, including those cultures treated with hCG + ACTH. Therefore, ACTH treatment seemed to counter the effects of hCG in terms of confluence of the cultures but not the increase in E to F conversion. ACTH + hP1 or ACTH alone had no effect on E to F conversion.

None of the hormones had any effect on F to E conversion (Fig. III-4B), which was low, as in the previous experiment, throughout the culture period.

In all of the following experiments, equimolar amounts of $^3$H-F and $^{14}$C-E (6-20 ng/ml) were added to each flask, which was comparable to the
Fig. III-4. Conversion per 24 hr of $^3$H-F to $^3$H-F (A) and $^3$H-F to $^3$H-E (B) by 16-week (gestational age) HFL monolayer cultures in the absence and in the presence of hCG (50 ng/ml), hPRL (250 ng/ml) and ACTH (300 pg/ml) added on days 10, 11, 12 and 13. The substrate concentration was 48 pg/flask (12 pg/ml). Each line represents conversion per one flask (C denotes control flasks).
concentrations of E and F found in cord blood of midterm human fetuses (Murphy, 1973b) and approximately a minimum of 600 times the tracer dose used in the previous experiments.

The effects of human term placental factors on enzyme activity in midgestational HFL cultures was studied in experiment 5 (Fig. III-5). Medium from term placental monolayer cultures (kindly provided by Drs. C. L. Branchaud and C. Goodyer; J Clin Endocrinol Metab 56:761, 1983) was mixed with fresh TCM (1:1) and added (placental medium) to HFL cultures. Cells (12-week HFL) were grown in either TCM (control medium) or placental medium from day 1 until the end of the culture period. The results (Fig. III-5A) show that E to F conversion was approximately 30% greater for cells grown in placental medium than in control medium ($p < 0.01$) for the last 3 days (8, 9, and 10) in culture. Also, cells grown in placental medium grew faster and reached confluence earlier.

The F to E conversion was increased only on days 5, 6 and 7, above control levels ($p < 0.01$) which corresponded to exponential growth of the cells.

The effects of increasing the substrate concentration of E and F on days 7, 8, 9, and 10 of culture was tested (Fig. III-6). F (2.5 μg/ml) stimulated E to F conversion by 20 to 25% (Fig. III-6A) while decreasing F to E to barely detectable levels (Fig. III-6B).

E (2.5 μg/ml), on the other hand, depressed E to F conversion while F to E (Fig. III-6A) conversion rose somewhat (Fig. III-6B).

4. 11-HSD Activity in Primary and Subcultured Monolayers

When 11-week HFL was put into culture (experiment 7; Fig. III-7) (initial plating was $7.0 \times 10^5$ cells/flask) growth of cells was very rapid
Fig. III-5. Conversion per 24 hr of $^{14}$C-E to $^{14}$C-F (A) and $^{3}$H-F to $^{3}$H-E (B) by 11-week (gestational age) HFL monolayer cultures in the absence and in the presence of placental medium (see text for details). The substrate concentration was 24 ng/flask (6 ng/ml). Each line represents conversion per one flask.
Fig. 111-6. Conversion per 24 hr of $^{14}C$-I to $^{14}C$-F (A) and $^{3}H$-I to $^{3}H$-E (B) by 12-week (gestational age) FPL monolayer cultures in the absence and presence of F (2.5 μg/ml) and E (2.5 μg/ml) added to the flasks on days 7, 8, 9 and 10. The substrate concentration was 24 ng/flask (6 μg/ml). Each line represents conversion per one flask (C, E and F denotes control, cortisol and corticosterone treated flasks respectively).
and the flasks were confluent by day 5 at which time E to F conversion reached its peak, 85-87% conversion (Fig. III-7), and remained constant for another 5 days. Over the next 10 days in culture, E to F conversion slowly fell to 52% while F to E conversion rose to over 40% by 20 days in culture (Fig. III-7). Meanwhile, the total % conversion (E to F and F to E conversion) remained constant throughout this time period.

All previous experiments were concluded when flasks had been confluent for at most 6 days, since cell degeneration occurs when confluent cultures are maintained for an extended period. In this experiment flasks were followed for 15 days after they were confluent and enzyme activity was affected.

Figure III-9 also shows that on the day that the flask was subcultured (flask 'C 2 in Fig. III-7), F to E conversion was up to 20% and E to F had fallen to 70%, but in the subcultured cells, the conversion of E to F and F to E was again similar to the results for the primary culture during the first 10 days. The increase in conversion of F to E coincided with the observed degeneration of the cells. O'Hare (1973) also showed that cellular damage affected 11-HSD activity (see Discussion). As the subcultures consisted only of viable cells the enzyme activity returned to the highest seen in the primary culture.

One flask was subcultured and split into 5 other flasks. Conversion of E to F was maintained and was even slightly higher (90%) (Fig. III-8) than in the primary culture. F to E conversion remained low, similar to the results for the primary culture (Fig. III-8).
Fig. III-7. Conversion per 24 hr of $^{14}$C-E to $^{14}$C-$\Gamma$ and of $^{3}$H-F to $^{3}$H-$\Theta$ by 11-week (gestational age) HFL monolayer cultures over 20 days. The substrate concentration was 68 ng/flask (17 ng/ml). Confluence occurred at 5 days (≈10 mg cells). Each line represents conversion per one flask (C represents control flasks).
Fig. III-8. Conversion per 24 hr of $^{14}$C-F to $^{14}$C-E and $^3$H-E to $^3$H-F by 11-week (gestational age) HFL primary and subculture monolayers over 5 and 9 days respectively. The substrate concentration was 68 ng/flask (17 ng/ml). I denotes the mean ± SD.
E to F conversion increased with the number of cells plated (Fig. III-9). Although in the primary culture in which $7.0 \times 10^5$ cells were plated, E to F conversion was only 7%. However, subcultured cells plated at $5.0 \times 10^5$ cells/flask converted over 30% E to F suggesting that perhaps:

1. enzyme induction had occurred with cellular growth;
2. cells converting E to F were only a small subpopulation of the originally plated cells which grew in culture and subsequently enzyme activity, in terms of E to F conversion, was expressed when the cell number had increased sufficiently;

or (3) that more cofactor was present in the subcultured cells.

By the second day, conversion of E to F in flasks plated with $2 \times 10^6$ cells, was near 90% (Fig. III-9), although the flasks were not yet confluent. In another subculture experiment of 14-week HFL, E to F conversion in the primary culture was about 50% at confluence; yet when subcultured, E to F conversion rose to about 85% (Fig. III-10) suggesting that perhaps when the cells were subcultured, a subpopulation of cells responsible for E to F conversion, became the dominant population in the subcultures and therefore, E to F conversion increased.

Figure III-11 shows that E to F conversion increased with time up to 12 hr, after which, no change in E to F conversion occurred when compared again within 24 hr. In fact by 6 hr 86% of the E to F conversion had occurred.
Fig. III-9. Conversion per 24 hr of $^{14}$C-E to $^{14}$C-F and $^{3}$H-F to $^{3}$H-F by 11-week (gestational age) MEF monolayer subcultures plated at various initial cell densities over 2 days. The substrate concentration was 68 ng/flask (17 ng/ml). Each bar represents conversion per one flask.
Fig. III-10. Conversion per 24 hr of $^{14}$C-E to $^{14}$C-F and $^{3}$H-I to $^{3}$H-I by 14-week (gestational age) H"{o}"l primary and subcultured monolayers. Substrate concentration was 20 ng/flask (5 ng/ml). Each bar represents conversion per one flask.
Fig. III-11. Conversion of $^{14}$C-E to $^{14}$C-F and $^{3}$H-F to $^{3}$H-F by confluent 11-week (gestational age) HFL monolayer subcultures incubated over various times. The substrate concentration was 68 nCi/flask (17 nCi/ml). Each bar represents conversion per one flask.
B. HFL EXPLANT CULTURE EXPERIMENTS

1. 11-HSD Activity in Explant Cultures

Since monolayer cultures of HFL at confluence were predominantly composed of fibroblast-like cells, a different experimental approach was undertaken in order to study the 11-HSD enzyme activity in relatively intact midgestational HFL. Therefore, E and F interconversion was studied in explant cultures of HFL, in which small pieces of HFL were incubated over a period of several days allowing for determinations of 11-HSD activity to be made on intact tissue versus the dispersed cells of the monolayer cultures.

Explant cultures of two 11-week HFLs converted F to E 25-30% for the first 7 days, with levels falling to 20% by day 14 (Fig. III-12 and Fig. III-13). E to F conversion was less than 5% on day 1, but subsequently rose to almost 20% by day 14, corresponding with outgrowth of fibroblast-like cells from the explants. Each dish contained 36 ng E and 36 ng F (12 ng/ml).

It was observed that some cells fell away from the explants on the grid to the bottom of the culture dishes and began growing as monolayers. The grids were removed to new dishes and the monolayers were allowed to continue growing. The monolayer so obtained from one lung converted E to F, reaching 20% by day 14 (Fig. III-13B), which paralleled the increase in E to F conversion seen in the explant cultures.
Fig. III-12. Conversion per 24 hr of $^{14}$C-E to $^{14}$C-F and of $^{3}$H-F to $^{3}$H-E by 11-week (gestational age) HFL explant cultures over 14 days. The substrate concentration was 36 ng/dish (12 ng/ml). Each line represents conversion by explants on a single grid (~5-10 mg tissue).
Fig. III-13 (A). Conversion per 24 hr of $^{14}$C-E to $^{14}$C-F and $^3$H-F to $^3$H-E by 11-week (gestational age) HDF explant cultures over 14 days. The substrate concentration was 36 ng/fkask (12 ng/ml). Each line represents conversion by explants on a single grid (~5-10 mg tissue).

(B). Conversion of these tracers by monolayers derived from the same explants. Each line represents conversion by monolayers in one dish.
2. Effects of Various Factors and Hormones on 11-HSD Activity in Explant Cultures

(a) Effects of low O₂. An experiment was carried out to test the effect of low O₂ (3%) on the enzyme activity of HFL explants, since in utero, HFL tissue is exposed to such low O₂ levels (Rudolph, 1974) and it was interesting to see if the enzyme activity could be affected. Three grids, in separate dishes, were placed in a jar which was flushed with 3% O₂, 97% N₂ and then placed in the incubator. The CO₂ in the jar was not monitored. Another 3 grids (controls) were placed in the incubator with an atmosphere of 5% CO₂ in air. No significant differences in F to E conversion were seen for the first 8 days in culture; although F to E conversion in explants cultured in low O₂ averaged 6% less than that seen in controls (p < 0.02) for the last 3 days in culture (9, 10, and 11) (Fig. III-14A).

Conversion of E to F was increased (p < 0.01) above controls in low O₂ cultures from day 7 onward (Fig. III-14B).

The variability, in terms of F to E conversion in the explants, was quite high, sometimes as much as 15% difference between grids. Yet, interestingly enough, the E to F variability from the same cultures was very low (Fig. III-14B). This was also found to be the case in all explant experiments, as can be seen in figure III-15, in which F to E variability between duplicate grids was high, but E to F conversion in duplicates was very consistent.

Marked outgrowth of cells onto the grids from these explants was observed in all dishes (see Fig. III-19 and Fig. III-20B).

(b) Effects of various hormones and placental medium. The following hormones were added to 13-week HFL explant cultures (Fig. III-15A): hCG (50 ng/ml), ACTH (25 ng/ml), hPr (250 ng/ml), hP1 (250 ng/ml), oxytocin
Fig. III-14. Conversion per 24 hr of $^3$H-F to $^3$H-E (A) and $^{14}$C-E to $^{14}$C-F (B) by 10-week (gestational age) HFL explant cultures in the presence of atmospheric $O_2$ levels (20%) and low $O_2$ (3%) over 11 days. The substrate concentration was 18 ng/dish (6 ng/ml). Each line represents conversion by explants on a single grid (~5-10 mg tissue).
Fig. III-15. Conversion per 24 hr of $^3$H-F to $^3$H-E and $^{14}$C-E to $^{14}$C-F by 13-week (gestational age) (A) and 12-week (gestational age) (B) HFL explant cultures in the absence and in the presence of various hormones ($E_1$, $E_2$, and $E_3$ denote estrone, estradiol and estriol respectively) and placent al medium. Hormones and placental medium were added from day 1 on. The substrate concentration was 12 ng/dish (6 ng/ml). Each line represents conversion by explants on a single grid (~5-10 mg tissue).
(5 mU/ml), T3 (10 ng/ml), and the following hormones and placental medium were added to 12-week HFL explant cultures (Fig. III-15B); estrone (10 ng/ml), estradiol (6 ng/ml), estriol (150 ng/ml), progesterone (400 ng/ml), and placental medium. None of these appeared to have any effect on F to E conversion and only placental medium had any effect on E to F conversion which was stimulatory (p < 0.01 for placental medium versus control cultures for days 3-5). hCG had no effect on conversion in explants, although it did stimulate E to F conversion in HFL monolayer cultures (Fig. III-3 and III-4).

The results of the effect of various hormones and treatments on E to F and F to E conversion by HFL monolayer and explant cultures are summarized in Table III-5. As can be seen, only E to F conversion was affected by the various factors. F to E conversion was not affected by any of the hormones or treatments.

C. 11-HSD ACTIVITY IN A CELL SUSPENSION, MINCES AND HOMOGENATES

When a cell suspension (4.0 x 10^6 cells) from a 13-week HFL was incubated for 2½ hr in 2 ml of TCM, containing tracer amounts of ^3H-E or ^3H-F (12 pg/ml), F to E conversion was 31% while E to F conversion was only 2.1% (Fig. III-16). Similarly when 11-week HFL minces (20-40 mg) were incubated for 2 hr in TCM containing equimolar amounts of ^3H-F and ^14C-E (12 ng/ml), F to E conversion was 17-40%, while E to F conversion was less than 2% (Fig. III-16). The results for conversion of E and F by HFL minces and a cell suspension were consistent with explant culture results.

The effects of cofactors on 11-HSD activity in HFL tissue was tested. When 11-week HFL homogenates (20 mg) were incubated in 2 ml of TCM
Table III-B. Summary of effects of various hormones and treatments on E to F and F to E activity in HFL cultures.

**HFL: MONOLAYERS**

<table>
<thead>
<tr>
<th>Increased E to F Conversion</th>
<th>No Effect on E to F or F to E Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>F (2.5 μg/ml)</td>
<td>ACTH (300 pg/ml)</td>
</tr>
<tr>
<td>hCG (50 ng/ml)</td>
<td>hPr (250 ng/ml)</td>
</tr>
<tr>
<td>placental medium</td>
<td>hPI (250 ng/ml)</td>
</tr>
</tbody>
</table>

**HFL: EXPLANTS**

<table>
<thead>
<tr>
<th>Increased E to F Conversion</th>
<th>No Effect on E to F or F to E Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>placental medium</td>
<td>ACTH (25 ng/ml)</td>
</tr>
<tr>
<td>low O₂ (3%)</td>
<td>hPr (250 ng/ml)</td>
</tr>
<tr>
<td></td>
<td>hPI (250 ng/ml)</td>
</tr>
<tr>
<td></td>
<td>estrone, estradiol, estriol</td>
</tr>
<tr>
<td></td>
<td>progesterone (10, 6, 150 ng/ml)</td>
</tr>
<tr>
<td></td>
<td>T₃ (10 ng/ml)</td>
</tr>
<tr>
<td></td>
<td>oxytocin (5 μU/ml)</td>
</tr>
<tr>
<td></td>
<td>hCG (50 ng/ml)</td>
</tr>
</tbody>
</table>
Fig. III-16. Conversion of $^3$H-F to $^3$H-E and $^{14}$C-E to $^{14}$C-F by 11-week (gestational age) HFL minces (substrate concentration was 12 ng/ml) and the conversion of $^3$H-E to $^3$H-F or $^3$H-F to $^3$H-E (substrate concentration was 12 pg/ml) by a 13-week (gestational age) HFL cell suspension during a 2 hr and a 2½ hr incubation at $37^\circ$C respectively.
Fig. III-17. Conversion of $^{3}$H-F to $^{3}$H-E by 13-week (gestational age) HFL homogenates incubated in the absence and in the presence of 1 mg of cofactor for 2 hr at 37°C. Conversion of $^{14}$C-E to $^{14}$C-F was not detected. The substrate concentration was 12 ng/ml. Each bar represents conversion per one homogenate incubation.
Table III-G. F-E interconversion in fetal lung homogenates in the presence of cofactors.

<table>
<thead>
<tr>
<th></th>
<th>F to E</th>
<th>E to F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>pg/mg.hr</td>
</tr>
<tr>
<td>Control</td>
<td>10.3</td>
<td>62.0</td>
</tr>
<tr>
<td></td>
<td>9.7</td>
<td>58.0</td>
</tr>
<tr>
<td>NAD</td>
<td>23.8</td>
<td>143.0</td>
</tr>
<tr>
<td></td>
<td>23.0</td>
<td>138.0</td>
</tr>
<tr>
<td>NADH</td>
<td>15.3</td>
<td>92.0</td>
</tr>
<tr>
<td></td>
<td>16.1</td>
<td>97.0</td>
</tr>
<tr>
<td>NADP</td>
<td>33.5</td>
<td>201.0</td>
</tr>
<tr>
<td></td>
<td>31.1</td>
<td>187.0</td>
</tr>
<tr>
<td>NADPH</td>
<td>16.1</td>
<td>97.0</td>
</tr>
<tr>
<td></td>
<td>17.5</td>
<td>105.0</td>
</tr>
</tbody>
</table>

Fresh fetal lung tissue was weighed and homogenized in culture medium (Ham's F-10 plus FBS). Tissue (20 mg) in 2 ml medium was incubated for 2 hr at 37°C in the presence and absence of 1 mg cofactor. Net conversion was calculated as observed conversion minus the conversion in medium without tissue added. 0; result not significantly different from zero.
containing equimolar amounts of $^3$H-F and $^{14}$C-E (12 ng/ml), in the absence and presence of NAD, NADH, NADP, and NADPH, conversion of F to E was stimulated by all four cofactors (Fig. III-17) (Table III-C), but E to F conversion was not observed, suggesting that the 11-HSD enzyme of HFL was not freely reversible.

D. MICROSCOPIC APPEARANCE OF HFL MONOLAYER AND EXPLANT CULTURES

Light microscopy of plated cells revealed two basic cell types: round (epithelial-like) and spindle shaped (fibroblast-like). After 7-10 days in monolayer culture, most of the cells appeared to be fibroblast-like (Fig. III-18). Subcultured cells were virtually composed entirely of fibroblast-like cells. When HFL explants attached to the bottom of flasks in monolayer cultures, fibroblast-like cells could be seen to grow out from the explants (Fig. III-19).

Explant cultures appeared to change little except for the growth of mesenchymal tissue at the edges, apparent after several days (Fig. III-20).

Histological preparations of cultured explant HFL tissue (kindly prepared by the Department of Pathology at the MGH) were examined. Two cell types could be detected, epithelial and mesenchymal. The relatively few epithelial cells were seen surrounded by mesenchymal tissue.
Fig. III-18. Photographs of a typical 11-week (gestational age) HFL monolayer culture showing growth over time. Note: at confluence (6 and 8 day-old cultures) fibroblast-like cells predominate (magnification 100x).
Fig. III-19 (A and B). Photographs of 11-week (gestational age) HFL monolayer cultures showing explants and typical fibroblast-like cellular (mesenchymal) outgrowth (magnification 100x).
Fig. III-20 (A and B). Photographs of typical 14-week (gestational age) HPL explants on steel grids. (A) Note "canals" (arrows) in explant, which are epithelial lined ducts of the glandular period. (B) Note mesenchymal outgrowth from explants along grid.
CHAPTER IV

DISCUSSION
A. INTRODUCTION

This research was begun in order to resolve the discrepancy between the observations of Smith and colleagues (1973) and those of Pasqualini and associates (1970b) and of Murphy (1978a) (see Historical Review; section G 6b). Smith's group had shown that monolayer cultures of midgestational HFL carried out exclusively the reduction of E to F. After the in vivo injection of labelled E and F into the umbilical vein of 2 fetuses, Pasqualini et al found no evidence for the conversion of E to F in any of the fetal tissues studied; on the other hand considerable conversion of F to E did occur. Murphy found that minces of midgestational HFL were capable of converting F to E with minimal conversion of E to F. Both the latter studies were short term in that fetal tissues were in contact with the labelled steroid for a short period of time, 15 min in the case of Pasqualini et al's study and 2 hr in Murphy's study. Smith et al conducted their experiments over a period of several days; consequently, each experimental design was quite different from the other, making comparisons of results difficult. We decided to approach the problem using tissue culture in an attempt to, first of all confirm the work of Smith et al for HFL cultures and then proceed to investigate possible factors which might play a role in regulating 11-HSD activity in HFL.

B. SUMMARY OF 11-HSD ACTIVITY IN MONOLAYER CULTURES

Monolayer cultures of HFL cells, gestational ages 11-20 weeks (glandular period), at confluence predominantly converted E to F 15-86% while F to E conversion was low (less than 10%), in accord with the results of Smith et al (1973). In one experiment (Fig. III-1), in which tracer levels of substrate (12 pg/ml; 4 ml of TCM/flask) were used, more F to E
than E to F conversion was detected for the first few days in monolayer culture, when the cell population was still low (1.3 x 10^6 cells/flask initially plated minus the number of cells which did not attach to the bottom of the flask during the first 24 hr and which were subsequently removed during the first change of TCM) and more representative of the initial mixed cell population (Fig. III-18). Similar percentages of F to E conversion during the first few days of culture were not detected in any other monolayer experiments probably because either: (1) determination of the % conversion was not done until after more than 3 days of culture; or (2) if it was determined on day 1 of culture the substrate concentration used (5-17 ng/ml), at least 500 times greater than in the first four experiments when tracer amounts were employed, was high enough to mask any F to E conversion which may have been occurring. Since Smith et al (1973) used 148 ng/ml of substrate E and F, it is not surprising that they could not detect any significant F to E conversion at any time in their cultures.

In all experiments, E to F conversion rose with cellular growth and plateaued at confluence, although in each experiment a different level of conversion was reached at confluence if one compares control flasks from one experiment to another (Table III-A). Why this occurred is not clear, although some possible explanations are discussed below.

Of the various hormones and factors added to HFL monolayer cultures only the hormones F and hCG along with placental medium were found to stimulate the conversion of E to F above control levels (Table III-B). HPI, hPr and ACTH did not affect the enzyme activity and also did not have any effect on the apparent growth of the cultures.
HCG, which stimulated E to F conversion, was observed to have a slight inhibitory effect on cellular growth, while placental medium was found to stimulate growth.

The addition of F to HFL monolayer cultures increased E to F conversion while decreasing the apparent F to E conversion. The cause of the decreased F to E conversion most likely reflected substrate overload, whereby competition of large amounts of unlabelled with labelled F for the enzyme lowered the amount of $^3$H-F converted to $^3$H-E. The reason for the increased E to F conversion is not clear. Smith and associates (1973) had shown that F (2 μg/ml) stimulated E to F conversion in midgestational HFL monolayers and suggested that F induced 11-HSD activity. F also stimulated growth, in terms of the time in which it took the cultures to reach confluence, although at confluence all flasks contained a similar amount of DNA (Smith et al, 1973). Perhaps a combination of the two explanations can best describe the observed increase in E to F conversion.

The effects of hCG, added during exponential growth, on conversion of E to F required 2 or 3 days to be detected following the start of hormone administration. Placental medium was added from the beginning of the culture period and the cells reached confluence at an earlier time, while E to F conversion also reached a plateau which was higher, in terms of % conversion, than in control flasks (Fig. III-5).

The results can be explained in at least two ways. It is possible that induction of the enzyme had occurred as a consequence of hCG or placental medium treatment, which may explain the time lag in onset of the effects in the case of hCG; however, it is also quite conceivable that
since growth of the cells was altered in placental medium treated flasks and possibly hCG treated flasks compared with control flasks, it resulted in a change of the cell population at confluence which affected the 11-HSD activity observed (see below).

C. SUMMARY OF 11-HSD ACTIVITY IN EXPLANTS

In the presence of 6-12 ng/ml substrate E and F, 10-13 week-old HFL explant cultures all converted F to E approximately 20-40%. E to F conversion was initially low (< 5%) during the first few days but then increased corresponding to outgrowth of mesenchymal cells from the explants (Figs. III-19 and III-20).

None of the hormones used (progesterone, the estrogens, hCG, hPr, hPl, ACTH, oxytocin, or T3) (see Table III-B), which were added at their physiologic concentrations found at midterm or term in the human fetus, or placental medium had any effect on the F to E activity in HFL explants, and low O2 only affected it to a minor degree on the last 3 days of culture (Fig. III-14A). Although hCG increased E to F conversion in monolayers, the hormone had no effect on explants. Why this is so is not clear. It may be that if the effects of hCG on 11-HSD activity were related to growth (see below), then it may have affected monolayer cultures of fibroblast-like cells differently than it would have explants of whole tissue.

Placental medium and low O2, on the other hand, did increase the E to F activity, which may have been due to increased mesenchymal outgrowth from the explants. Tanswell and Smith (1980) had found that HFL fibroblasts, in monolayer cultures, exposed to low O2 (1%) grew faster than HFL fibroblasts exposed to high O2 (20%).
It may also be that for explants grown in low O_2 the E to F conversion was greater (Fig. III-14B) due to differences in the CO_2 concentration which was kept constant in the control cultures, at 5%, but which was not controlled for in the low O_2 cultures.

D. IMPLICATIONS OF CELLULAR COMPOSITION OF MONOLAYER CULTURES

1. Relationship of Cell Type to 11-HSD Activity

In order to interpret the results from the HFL monolayer culture experiments in a meaningful way the cells involved must be characterized to some extent.

The HFL tissue used in experiments was from the glandular period of fetal lung development at which time the tissue is composed of mainly mesenchymal (undifferentiated and 'differentiated) and epithelial (undifferentiated) cells.

Light microscopy of young cultures revealed fibroblast-like (spindle shaped) and epithelial-like (polygonal shaped) (Fig. III-17) cells as the only distinguishable cell types. However, due to differences in the growth rates of different cell types in culture (which have been recognized for many years: Carrel and Ebelina, 1926; Swim and Parker, 1957), fibroblast-like cells quickly outgrew epithelial-like cells, and with the onset of exponential growth, which lasted for 3-4 days paralleling the increase in E to F conversion until the plateau (Table III-A), they became the dominant cell type (Fig. III-17). Following subculture of the cells virtually all were fibroblast-like.

Therefore, midgestational HFL fibroblast-like cells, derived from mesenchymal cells, accounted for all of the E to F enzyme activity in confluent monolayer cultures, and as shown for human (Tanswell and Smith,
1980) and rabbit (Smith and Giroud, 1975) fetal lung fibroblasts serially propagated (i.e. studies performed on subcultured cells). The E to F 11-HSD activity in confluent monolayer cultures of a so called "mixed" population of HFL cells (Smith et al, 1973) therefore, resides solely with the fibroblast-like cells which become the dominant cell type.

2. Relationship of Cell Type to Growth

The faster growth rate of fibroblast-like cells in culture is a problem which has been recognized in other systems as well. Katsuta et al (1957) studied the outgrowth from trypsinated 9 day-old chick embryo liver over a one week period. Although the outgrowth revealed a mixture of cell types, fibroblast-like cells and liver parenchymal cells, subculture yielded predominantly fibroblast-like cells, similar to the situation described for HFL in culture. Recently a similar problem arose in the study of prolactin release from monolayer cultures of human decidua cells (De Ziegler and Curpide, 1982). The cultures were predominantly fibroblastic in appearance and secreted insignificant amounts of hPr; however, when hPr producing cells were selected for, large amounts of hPr could then be detected in these cultures.

Smith and coworkers (1974) found that monolayer cultures of 20 and 28 day (term 31 days) rabbit fetal lung were different in that 20 day rabbit fetal lung cultures were virtually solely composed of fibroblast-like cells, which is again similar to the HFL monolayer culture situation, and the addition of F to the cultures (2 \( \mu \)g/ml) resulted in an increase in fibroblast-like cell growth. This was also shown for midgestational HFL cultures (Smith et al, 1973), and in both cases lung tissue was obtained from the glandular period of fetal lung development. Twenty-eight day
rabbit fetal lung (alveolar period) cultures were composed of a mixed population of cell types, fibroblast-like and epithelial-like, and the addition of F to the cultures (2 μg/ml) resulted in an apparent increase in the proportion of epithelial-like cells while total growth, as measured by DNA content per flask, had decreased (i.e. F inhibited the growth of 28 day fetal lung fibroblast-like cells) (Smith et al, 1974).

In the paper by Smith et al (1974) they remark in their discussion that "These observations suggest that cortisol has two separate effects on developing fetal lung cells, depending upon the age of the fetus (rabbit) at the time of exposure. It enhances cellular growth at the earlier gestations (20 days) studied, and slows growth near term (28 days)." It seems likely that these effects are "separate" because they are on different cell populations, fibroblast-like predominantly at 20 days, derived from mostly undifferentiated mesenchymal tissue, versus fibroblast-like and epithelial-like cells at 28 days derived from differentiated mesenchymal and epithelial tissue. Since fetal lung cultures from 20 day-old fetuses did not have any epithelial-like cells at confluence these cultures already do not reflect whole tissue. This is at least one reason why comparisons of the effects of F on growth between the two gestational ages of cultured cells would be difficult to interpret.

3. **Do the Fibroblast-like Cells Represent One or More Than One Population?**

The question that one must ask, in view of the fact that monolayer cultures of midgestational HFL are not representative of whole tissue, in that only fibroblast-like cells are present at confluence, therefore is: Do these fibroblast-like cells represent one population or subpopulations
of cells, and can this account for differences in the % conversion observed at confluence between experiments?

The data presented, along with other relevant papers, suggests that the fibroblast-like cells represent more than just a single population (ie. the mesenchymal cells).

At the time of plating of cells, cultures consist of some differentiatated and many undifferentiated mesenchymal cells which in culture are collectively known as fibroblast-like cells and therefore, the cultures already consist of at least 2 different populations of fibroblast-like cells.

The E to F activity resides in the fibroblast-like cells, but the undifferentiated mesenchymal cells which were originally plated can change and perhaps differentiate in culture. Undifferentiated mesenchymal cells in the developing lung differentiate into at least 7 different cell types (fibroblasts, interstitial cells, contractile cells, pericytes, lymphatic cells, and muscle cells) in the developed lung (Bradley et al, 1973). There is also evidence of heterogeneity within a population of cultured lung fibroblasts. Lung fibroblast cells in culture have been shown to have different life spans (Smith and Hayflick, 1974), sizes (Cristofalo and Kritchevsky, 1969), and interdivision times (Absher and Absher, 1976).

If only a small population of plated cells could convert E to F then this activity would be minimal until the cells were of sufficient number so that 11-HSD activity could be detected. If this subpopulation of cells grew by various degrees in each different monolayer experiment, then the 11-HSD activity would vary according to the percentage of those cells converting E to F which were present in the culture at confluence and any factor affecting this specific population of cells could therefore, influence the
11-HSD E to F activity. Possible factors which might alter the subpopulation cell numbers at confluence could include: (1) interaction between various cell types initially plated; and (2) perhaps different batches of fetal bovine serum (FBS), shown to have wide hormonal concentration fluctuations from one batch to the next (Honn et al., 1975), used in different monolayer culture experiments could influence the micro-population of cells in terms of growth.

Evidence for this hypothesis comes from experiments in which HFL fibroblast-like cells were subcultured. In one experiment it was found that $4.7 \times 10^5$ subcultured cells could convert E to F 30%, but $7.0 \times 10^5$ cells, in the original culture, converted E to F only 6% on day 2 (Fig. III-8). In another experiment, confluent primary cultures converted E to F only 55% while the confluent subculture converted E to F 90%. These observations suggest that at the substrate concentrations employed, when the primary cultures, consisting of mainly fibroblast-like cells at confluence, were subcultured, an increased proportion of those fibroblast-like cells capable of E to F conversion were plated and therefore, E to F conversion increased, as compared with the primary culture. The E to F converting fibroblast-like cell population in the subculture would have a chance to predominate due perhaps to its faster growth rate.

This difference in E to F activity seen in these HFL monolayer culture experiments could not be appreciated in the studies of Smith and coworkers since from one experiment to the next different substrate concentrations and incubation periods were used.

Data on fibroblast cultures from other tissues suggest that various strains can develop in culture. For example, two strains of uterine fibroblasts were isolated from a culture of uterine fibroblasts, U12-705
(Grosser et al, 1962), one which is F sensitive (growth is inhibited by F) and one F resistant and which can also grow in less stringently supplemented media; embryo extract is not required for growth as compared with U12-705, which requires both whole serum and embryo extract for in vitro growth. Therefore, within the population of uterine fibroblasts various strains were able to develop and grow depending on the medium (i.e. one strain could grow with 25 µg/ml of F and in the other strain growth was inhibited by as little as 0.1 µg/ml of F), and also behave differently in terms of metabolism of F (Grosser et al, 1962).

There is also some suggestion from the results of Ruhmann and Berliner (1965) that fibroblasts of clone 929-L may have developed into new strains of fibroblasts in culture when inhibited by various glucocorticoids. In some cases, growth rates of treated cells showed initial inhibition, but after 3 days of culture they were similar to controls. Thus, the various growth rates of different cells and/or the medium (environment) in which they grow may prefer one cell type over another, or may even induce changes in cells of one type (clones) which will behave differently biochemically and/or are different morphologically from the parent cell type.

The evidence presented does support the concept that a subpopulation of mesenchymally derived fibroblast-like cells in culture was responsible for the measurable E to F activity which was relatively low during the initial plating of cells. This would imply that effects on E to F conversion in monolayer cultures could be related to effects on growth rather than any direct effects on the enzyme per se.

The effects of the placental medium and hCG on E to F activity perhaps can be explained by the above hypothesis. For example, if
placental medium affected the growth of various populations of growing cells, thereby altering the composition of the culture so that the subpopulation of fibroblast-like cells in possession of 11-HSD activity was increased in relation to other subpopulations, then an increase in E to F activity would be observed.

D. EVIDENCE FOR TWO ENZYMES

1. Reversibility of 11-HSD Activity in Long Term Monolayer Cultures

   The 11-HSD activity of the fibroblast-like cells in monolayer cultures was predominantly E to F with some conversion of F to E; the direction clearly favouring reduction (activation). However, the reversibility of the enzyme activity was prominently underscored when after a lengthy culture period the conversion of F to E increased to over 40% which was balanced by decreasing E to F conversion (Fig. III-7). By this time cellular atrophy of the monolayer fibroblast-like cells had begun to occur. O'Hare (1973) had also found that cellular damage can affect 11-HSD activity (see below). Therefore, when the fibroblast-like cells were healthy and intact, E to F activity predominated with only a minimal amount of the back reaction, F to E conversion, usually less than 10%.

2. E to F Activity in Explants

   Explant cultures of midgestational HFL are composed of mainly undifferentiated mesenchymal and a much smaller proportion of undifferentiated epithelial cells of the glandular period of fetal lung development. During culture, peripheral growth of mesenchymal tissue along the grid occurs (Fig. III-20), while necrosis of tissue at the center of each piece takes place.
In explant cultures, F to E activity was present from day 1 and only decreased somewhat with time in culture. The E to F activity, as shown in figure III-13, was initially low (<5%) and increased with time in culture concomitantly with outgrowth of mesenchymal tissue. This activity was similar to that observed in the monolayer cultures of fibroblast-like cells which originated from cells from the explants (Fig. III-13B).

The E to F activity of explants therefore appears to be a consequence of the mesenchymal outgrowth from HFL explants (Fig. III-19 and III-20).

Subsequent studies of F to E and E to F conversion done in our laboratory (unpublished data) have shown that E to F conversion by 15 week (gestational age) human fetal kidney explants was less than 5% throughout the 5 day culture period in which mesenchymal outgrowth was virtually undetectable.

E to F 11-HSD activity, therefore, was present in fibroblast-like cells of monolayer cultures and in mesenchymal outgrowth from HFL explants. F to E 11-HSD activity, on the other hand, was present in explants. It was also found in HFL minces and a cell suspension (Fig. III-16); however, negligible amounts of E to F conversion were detected. These latter results are in agreement with those of Murphy's (1978a) in vitro studies and substantiate the findings of Pasqualini et al's (1970b) in vivo results.

3. Evidence for E to F and F to E Activity Residing in Different Cell Types

(a) From monolayer and explant experiments. In the first monolayer culture experiment (Fig. III-1) in which tracers were used, F to E conversion on day 2 was almost 30%, but E to F conversion was only 5%, when the
cell number was relatively small. Yet by the time the flasks were almost confluent, by day 6, E to F conversion was only near 20%. If the enzyme activity had simply reversed and resided in the same cell type then E to F activity should have been higher reflecting the increased activity of a reversible enzyme, especially since more cells were present near confluence. A more likely explanation of the results is that the activities resided in different cell types. As the cellular composition of the cultures changed with time so did the enzyme activities.

In explants of midgestational HFL, F to E activity did not decrease to any appreciable extent during the culture period and did not correspond to the E to F activity measured. Thus the initial F to E activity seen in midgestational HFL explants, minces or cell suspension did not reflect the reversal of E to F enzyme activity seen in the HFL monolayer cultured cells which were allowed to remain confluent for an extended period of time (Fig. III-7), but rather an activity of viable cells which was quite distinct and separate from the E to F activity of the HFL fibroblast-like cells in monolayer culture. It is most probable that these two activities in explants reside in different cell types; mesenchymal versus epithelial.

(b) From effects of various factors on 11-HSD activity. Conversion of E to F in monolayer cultures at confluence and/or explants was affected by hCG, placental medium and low $O_2$, but F to E activity of explants was unaffected (except for the slight effect of low $O_2$ on F to E activity near the end of the culture period; see above) (see Table III-B).

These results support the concept that the two activities reside in different cell types since only one activity was affected and not the other.
(c) **From variability of 11-HSD activity in explants.** While F to E activity was fairly variable from one grid to the next, E to F activity between the same grids was much less variable (Fig. III-15). Again this evidence suggests quite strongly, if not conclusively along with the evidence from all the other data (see above), that the two different enzyme activities reside in different cell types.

4. **Evidence that F to E Activity Resides in HFL Epithelial Tissue**

Although not conclusively proven, it seems likely that the undifferentiated epithelial cells of the midgestational HFL are responsible for F to E enzyme activity.

The most obvious explanation of why F to E activity is not detected in confluent monolayer cultures and subcultures of HFL is that F to E activity resides in the epithelial cells which are virtually absent from confluent monolayers and totally absent from subcultured monolayers. In explant cultures, the non-dividing epithelial cell population remains relatively intact (except for some necrosis) and therefore, F to E activity, which is detected, is very constant.

Smith (1977) has found that a human tumor cell line (A549), with morphological and biochemical features of the type II pneumocytes, could convert F to E 5.2% at 10^{-7} M of F substrate, but the conversion of E to F could not be detected, supporting the above thesis that the epithelial cells of the midgestational HFL explants are responsible for the F to E activity observed.

Evidence from the literature would seem to support the contention that in epithelial tissue, such as placenta, 11-HSD activity is predominantly oxidative (F to E) (Lopez Bernal et al, 1980). Epithelial cells from other tissues have also been found to possess 11-HSD activity which is
oxidative (F to E), such as parotid gland (Katz and Shannon, 1964), submandibular and salivary gland (Ferguson and MacPhee, 1975), and epididymis (McGadey et al, 1966), although the role played by the enzyme in these tissues is poorly understood.

5. Non-Reversibility of F to E Activity Found in Explants

If these two activities (F to E and E to F) reside in different cell types then there is a strong possibility as well that the two 11-HSD enzymes are different. The enzyme in fibroblast-like cells was shown to be reversible under certain conditions (see above). The F to E activity in HFL explants was tested for reversibility. The conversion of F to E observed in explants appears to be catalyzed by an enzyme which is not readily reversible. When cofactors were added to fetal lung homogenates only the oxidative reaction was increased (Table III-C), whereas enzymes catalyzing readily reversible reactions can be directed with the addition of cofactors.

These results support the proposal that the F to E activity observed in explants is catalyzed by an 11-HSD which is not readily reversible while the E to F activity observed in monolayers is catalyzed by a different 11-HSD which is reversible.

6. Summary of Characteristics of the Proposed Different 11-HSD Enzymes

The characteristics of the proposed two enzymes can be summarized as follows:

- **E to F Activity** (mesenchymal)
  - present in predominantly fibroblast-like cell cultures and subcultures composed of only fibroblast-like cells (perhaps in a subpopulation of fibroblast-like cells;
-corresponds to mesenchymal outgrowth from explants;
-in monolayers: affected by hCG, F, and placental medium;
-in explants: affected by low O_2 and placental medium;
-between grids very stable;
-is reversible.

**F to E Activity (epithelial)**
-disappears in monolayers when fibroblast-like cells take over;
-present in explants when tissue is composed of mainly (only) two types of tissue when examined histologically, epithelial and mesenchymal cells;
-not affected by any hormones, placental medium or low O_2;
-between grids quite variable;
-not readily reversible following the addition of cofactors.

The results from these explant experiments are in agreement with those of Murphy (1978a) and also substantiate the results of Pasqualini et al (1970b) in which no significant conversion of E to F could be assumed to have occurred in the midgestational HFL and are in direct contradistinction to the conclusions drawn by Smith et al (1973).

**F. IMPLICATION OF THESE FINDINGS WITH RESPECT TO THE FETAL LUNG**

1. **Fibroblast-like Cells of HFL Monolayer Cultures Versus In Vivo Activity.**

From the foregoing discussion it seems likely then that in the midgestational HFL in vivo, conversion of F to E is catalyzed by an 11-HSD enzyme, with affinity for both NAD and NADP, which is not readily reversible and resides in the undifferentiated epithelial cells. E to F activity, on the other hand, is minimal in vivo at midgestation, but in culture can become the main observable activity due to the growth of a subpopulation of fibroblast-like cells which is not representative of the in vivo situation. This activity has so far not been observed to any impressive extent in minces of HFL near term either; however, this activity
may be expressed in the adult lung (Murphy, unpublished observations; Aronson et al, personal communication).

The HFL fibroblast-like cells are derived from some differentiated and mainly undifferentiated mesenchymal cells which in culture can perhaps differentiate into various types of fibroblast-like cells (see above), and which may change into cell types which are artefacts of the culture system. In in vitro environments, in which the tissue structure is lost, the cell-cell interactions can change. The cells are also subjected to an artificial medium in which they must grow while anchoring themselves to the bottom of a plastic flask or petri dish.

This question of reliability of monolayer cultures has already been raised for cultures of type II alveolar cells (Mason et al, 1977) and correlations between in vitro and in vivo work is necessary for the correct interpretation of results.

Monolayer cultures of midgestational HFL or rabbit fetal lung are not representative of the fetal lung tissue. Although, for example, in two studies, F in high concentrations (2 μg/ml) was shown to stimulate growth of midgestational human (glandular period) (Smith et al, 1973) and rabbit (Smith et al, 1974) fetal lung fibroblast-like cells in monolayer cultures, this increased growth observed, very likely does not have any physiologic significance (see above).

F levels in the human fetus at midgestation are very low (10 ng/ml) (Murphy and Diez d'Aux, 1972) and since approximately only 10% of the fetal circulation passes through the lungs the F that reaches the lungs is being inactivated by the 11-HSD activity. At this stage of fetal lung development F would appear to be a hormone which is 'not wanted'. Near term, however, F
seems to play an important role in stimulating surfactant production, but not growth (see Historical Review; section H 4).

2. Possible Role of F to E Activity in the Fetal Lung

This dichotomy of enzyme location is apparent from this study in that within differently derived cells, one epithelial and one mesenchymal, in the midgestational HFL in culture there exist two different enzymes. However, in the midgestational HFL, as well as in other fetal tissues, in vivo the predominant enzyme activity present, from our results and those of Pasqualini et al (1970b) and Murphy (1978a) (see above), is the F to E activity. This activity is apparently decreased in newborns (Murphy, 1981a) and appears to be decreased towards term in HFL in vitro (Murphy, 1978a).

The present studies strongly suggest that the enzyme 11-HSD resides in undifferentiated epithelial cells of the midgestational HFL. Towards term the enzyme activity appears to decrease (Murphy, 1978a) and may be explained by the differentiation of the epithelial cells into type I and type II pneumonocytes and possibly other cells (Meyrick and Reid, 1977).

Thus, during most of pregnancy one important way in which a low F:E ratio in the circulation is maintained is through the action of high 11-HSD activity in the placenta, lung and kidney as well as lesser activity in many other fetal tissues (Murphy, 1981a). F is thought to be a critical hormone in affecting final maturational processes through enzyme induction (Rousseau and Baxter, 1979) in various fetal organs (Liggins, 1976; Ballard, 1979) near term, preparing the fetus for life after birth.

Through differentiation of the fetal lung tissue and decreasing 11-HSD activity, F levels in the fetal lung could slowly rise towards term. Since there is minimal circulation to the fetal lung, a decrease in the
conversion of F to E could elevate F within the lung parenchyma (target cells of F action) quite substantially and along with the action of other hormones (see Historical Review; section H 4) cause increased surfactant production and therefore, final maturation of the lung.

F in the adult is involved in homeostasis, a role completely different from that which it appears to play in the fetus. In the adult, levels of F are controlled through the adrenal-pituitary-hypothalamic axis; however, the relative autonomy of the fetus in terms of F levels in the circulation, is mainly achieved through the conversion of F to E by the placenta and other fetal tissues. The enzyme (11-HSD) would, therefore, be involved in fetal development and maturation through its control of F levels, but in the adult the same and/or other 11-HSD enzymes would be involved in metabolism of F.

3. Importance of 11-HSD Activity in Other Species

The 11-HSD inactivation activity appears to be important in a number of species (see Historical Review; section C) and plays a similar role to that seen in the human fetus, although differences are to be expected since gestational lengths are different and rapid development of various organs must occur in those short-gestation species.

As can be seen in Table IV-A the appearance of surfactant may occur only a couple of days prior to parturition in short-gestation length species (rat, rabbit) as compared with sheep or human in which surfactant appears weeks before parturition. It seems reasonable to assume that if F is involved in stimulating surfactant production, which in the lung must rise rapidly in short-gestation length species in order to insure that at parturition the lungs will be mature, then F must also be at the necessary
Table IV-A. Timing of various stages of fetal lung development in several species.

<table>
<thead>
<tr>
<th>Period of fetal lung development</th>
<th>Human</th>
<th>Sheep</th>
<th>Rabbit</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glandular</td>
<td>+112</td>
<td>+95</td>
<td>+24</td>
<td>+18</td>
</tr>
<tr>
<td>Canalicual</td>
<td>112-168</td>
<td>95-120</td>
<td>24-27</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>*160</td>
<td>120</td>
<td>27</td>
<td>19</td>
</tr>
<tr>
<td>Alveolar</td>
<td>168+</td>
<td>120+</td>
<td>27+</td>
<td>20+</td>
</tr>
</tbody>
</table>

*Term of gestation in days.

*Approximate day on which surfactant is detected (Meyrick and Reid, 1977).
levels within the target cells. In rabbit and rat it appears that a rapid rise of F and/or B in the lung does occur because E to F 11-HSD activity increases dramatically in those species while F to E conversion falls.

In the rabbit (term, 31 days) at 26 (Torday et al, 1976) and 28 days (Giannopoulos, 1974) gestation, both E to F and F to E conversion were shown to occur, with E to F conversion at term being substantial and the reverse direction minimal. Fetal rat lung (Smith, 1978) appears to behave in a fashion similar to that of fetal rabbit lung, with net conversion (B to A minus A to B) being B to A (the corresponding analogues of F and E respectively in this species), inactivation, occurring at 17 days gestation, changing to net activation, A to B conversion predominating, by 18 days (term, 21 days). The authors of these papers assumed that one 11-HSD enzyme was responsible for the interconversion of E and F or A and B. The increase in F or B levels in the lung of those species may actually involve a decrease in F to E or B to A conversion, carried out possibly by an epithelial 11-HSD, near term and the increase in E to F activity catalyzed by a different 11-HSD, perhaps in fibroblast cells differentiated from mesenchymal tissue, which would convert E to F or A to B. In longer gestational length species, such as sheep and primates, a reduction in the conversion of F to E would be all that was required in order to elevate F levels in the lung over a longer period of time with perhaps a later onset of 11-HSD activity in lung fibroblasts, in that induction prior to parturition would be unnecessary.

While the above is speculative what is relatively certain is that at midterm in all animal species studied, F to E conversion predominates in all tissues studied and would seem to indicate that this aspect of F metabolism is an important regulator of F levels in the fetus; however, in
the adult 11-HSD activity is variable in different species. Clear differences in corticosteroid metabolism have been observed; for example between adult rat and guinea pig lung, where rat lung has been shown to convert E to F, yet in the same system, guinea pig lung was inactive (Nicholas and Kim, 1975).

4. Differentiation and Fetal Lung Development

In the fetal lamb, at least, it appears that differentiation of the lung as marked by surfactant appearance occurs in face of constant F production and plasma levels (Meischer et al, 1975). This suggests that the stimulus for the release of surfactant into the alveolus is not a rise in F levels, although the rate of the differentiation process can be increased by corticosteroid administration during the last trimester of gestation (Platzker et al, 1975). In fact the action of corticosteroids is thought to involve effects on rates rather than initiation of reactions (Granner, 1979). Therefore, although F may stimulate increased surfactant production it is not involved in the onset of surfactant synthesis. None of the hormones used in the present study had any effect on the F to E activity.

To repeat a quote from Liggins (1976), he wrote "Nothing is known about the factors which influence the 11β-hydroxysteroid dehydrogenase the enzyme which interconverts cortisol and cortisone." The question, however, may not be what factors control the enzyme, but rather what factors control differentiation of the fetal lung. If the enzyme, which resides in undifferentiated fetal lung epithelium, and/or the activity were to disappear upon differentiation of the glandular epithelial cells to type I and type II pneumocytes, then the timing of differentiation of the epithelial cells would act as the trigger for the gradual increase in F
levels near term and the increase in the synthesis of surfactant so as to allow for the maturation of the lung in time for parturition and more importantly for life outside the uterus.

In man, our results (Murphy, 1978a) and those of Pasqualini et al (1970b) suggest that HFL at midgestation is only capable of converting F to E. Only after birth was net activation in human lung observed (Murphy, 1978a), and then of only minor degree. Thus it seems likely that in the human, rising intracellular fetal lung F concentrations are achieved by a combination of rising blood F levels and decreasing inactivation of F by maturing epithelial cells.

G. IMPLICATIONS OF THESE FINDINGS WITH RESPECT TO

11-HSD ACTIVITY IN GENERAL

1. 11-HSD Reversibility and Physiology in Various Tissues

In the previous discussion evidence was presented for the existence of two different enzymes in cultures of midgestational HFL. One 11-HSD enzyme residing in lung epithelial cells, which is 'irreversible' and another residing in fibroblast-like cells found in confluent HFL monolayer cultures and is reversible.

Reversibility of an enzyme reaction, however, does not mean that in vivo the enzyme reaction is readily reversible in that one direction of a reaction may be greatly favoured over the reverse reaction. In the present case other factors must also be taken into account when discussing oxidation-reduction reactions. The standard oxidation-reduction potential (redox potential) of a conjugate redox pair (an electron donor and its conjugate electron acceptor) determines the tendency of a reducing agent to lose its electrons (or an oxidizing agent to gain electrons). The redox potential
for the 11-hydroxy-ketone pair of F and E favours the formation of E since the 11β-hydroxyl group is relatively unstable. The concentration of cofactor can also influence the final equilibrium, and a reaction favouring the oxidized direction can in theory be altered to favour the reduced direction if enough of the reduced cofactor is made available. Of course the affinity of the substrate for the enzyme must also be considered along with other factors.

11-HSD activity in the fibroblast-like cells clearly favours the reductive (activation) direction with the back reaction, for the most part, remaining minimal. The conversion of F to E became quite substantial, however, when the monolayer cultures were maintained for an extended period allowing time for cellular damage to occur. O'Hare (1973) has shown that the conversion of B to A by damaged monolayers of rat adrenocortical cells was greatly increased over intact monolayers. He suggested that the increase in the 11-HSD activity may have been related to the "breakdown of subcellular localizations of enzyme, substrate or cofactors, and/or their leakage from the cells." It may be that in damaged cells the levels of the cofactors, NADH and/or NADPH, are not maintained in their reduced state and increasing levels of NAD and/or NADP would favour the reverse reaction. Also, the redox potential may play a greater role when enzyme systems are torn apart disrupting coupled enzyme reactions and allowing them to occur 'freely'. Nicholas and Lugg (1982) have shown that in adult rat lung a reversible 11-HSD would favour the F to E direction if cofactors, reduced and oxidized, were present in equal amounts (see below).

In a similar manner enzyme activity in homogenates of tissue, in which cellular disruption has occurred, may differ from whole cells and/or intact tissue. One striking example of the difference in enzyme activity in
homogenates compared with intact tissues has been found in human maternal decidua (see Historical Review; section G 5b). It has been shown that E to F conversion predominates in explant cultures of term maternal decidua (Murphy et al, 1982) as well as in minces (Murphy, 1977a; Lopez Bernal et al, 1982b) with very little F to E activity being detected. However, when homogenates of this tissue were made, F to E activity was favoured and there was less E to F conversion (Murphy et al, 1982). Turnbull’s group has recently reported this similar finding (Lopez Bernal et al, 1982b). Considering that the level of F is higher in decidua than E (Murphy, 1977a; Lopez Bernal et al, 1982b), the in vivo 11-HSD activity direction is E to F conversion and not F to E as seen in homogenates.

Homogenization of tissue probably alters the cofactor environment and also may allow the reaction to proceed in the direction favoured by the redox potential of E and F (see above).

Another example of altered enzyme activity in homogenates versus intact tissue is in lung. In near term rabbit fetal lung (29, 30 day gestational age) the 11-HSD activity was predominantly reductive (E to F) as shown for minces (Giannopoulos, 1974; Nicholas et al, 1978) and perfused lung (Torday et al, 1976); however, in homogenates of near term rabbit fetal lung, incubated with added cofactor, NADP, conversion of F to E was found to be about 60% (Brooks et al, 1977). They also found that perfused adult rabbit lung could actively convert F to E, thus, the authors state in their discussion that “While F is converted to E by adult rabbit lung, the amount of conversion seems to be maximal in fetal lungs at 30 days (gestation), just prior to birth.” This conclusion is directly opposite to the other work cited above and is a good illustration of how the wrong conclusions can be drawn for enzyme activity in homogenized tissue even if, as in
the above case, the enzyme activity in perfused adult rabbit lung was similar, which may have led them to misinterpret their data.

The 11-HSD enzyme discussed in the previous study was clearly reversible, but the direction of the reaction favoured was dependent upon the experimental design (tissue preparation and incubation conditions). In vivo, in the rabbit fetal lung at term, one direction predominates (E to F) and from a physiological standpoint, the direction is important.

Adult rat lung converted E to F, as shown by perfusion studies (Nicholas and Lugg, 1982) and only minimally converted F to E (Nicholas and Kim, 1975). However, lung homogenates and microsomes, in the presence of NADP, readily converted F to E (oxidation) (Nicholas and Lugg, 1982), perhaps indicating that cofactor levels could easily influence 11-HSD activity. The authors suggested that in view of a very active pentose-phosphate shunt in the lung (Tierney et al, 1973; Massaro et al, 1971), NADPH production would be important in maintaining the predominantly reductive activity observed in the perfusion studies. Nicholas and Lugg (1982) found that in fact the Km value for E was 5 times the Km for F, therefore, clearly favouring oxidation. Although 11-HSD, in the rat lung is readily reversible it favours the reductive direction in the perfusion studies and probably in vivo.

Most studies of liver 11-HSD activity have been done on homogenates (see Table IV-B) incubated with either E or F and only rarely both. The liver 11-HSD is reversible but in vivo E to F conversion predominates. The effectiveness of E as a drug is due to its conversion to F by the liver. A study by Murphy and West (1964) showed that most of the F was not metabolized by the liver but rather was first metabolized in extrahepatic tissues to other compounds before reaching the liver. Thus, although F to E
### Table IV-B. 11-HSD activity in various tissues of different species.

<table>
<thead>
<tr>
<th>Tissue (Species)</th>
<th>Incubation preparation</th>
<th>Substrate</th>
<th>11-HSD activity detected</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver (pig)</td>
<td>*H</td>
<td>E</td>
<td>E to F</td>
<td>Fish et al, 1953</td>
</tr>
<tr>
<td>(beef)</td>
<td>H</td>
<td>E</td>
<td>E to F</td>
<td></td>
</tr>
<tr>
<td>(rat)</td>
<td>H</td>
<td>E</td>
<td>E to F</td>
<td>Caspi et al, 1953</td>
</tr>
<tr>
<td>(rat)</td>
<td>perfusates</td>
<td>E</td>
<td>E to F</td>
<td>Amelung et al, 1953</td>
</tr>
<tr>
<td>(rat, beef)</td>
<td>H</td>
<td>E</td>
<td>E to F</td>
<td>Eisenstein, 1953</td>
</tr>
<tr>
<td>(rat)</td>
<td>microsomes</td>
<td>E</td>
<td>E to F</td>
<td>Hubener et al, 1956</td>
</tr>
<tr>
<td>(rat)</td>
<td>slices</td>
<td>E</td>
<td>E to F</td>
<td></td>
</tr>
<tr>
<td>(rat, guinea pig, rabbit, calf)</td>
<td>H</td>
<td>E, F</td>
<td>E to F, F to E</td>
<td>Hurlock and Talalay, 1959</td>
</tr>
<tr>
<td>(human)</td>
<td>H</td>
<td>F + NAD</td>
<td>F to E</td>
<td>Meigs and Engel, 1961</td>
</tr>
<tr>
<td>(human)</td>
<td>H</td>
<td>E + NADPH</td>
<td>E to F</td>
<td></td>
</tr>
<tr>
<td>Kidney (rat)</td>
<td>H</td>
<td>E</td>
<td>no conversion</td>
<td>Fish et al, 1953</td>
</tr>
<tr>
<td>(pig)</td>
<td>H</td>
<td>E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(bovine)</td>
<td>minces</td>
<td>F + NAD</td>
<td>F to E</td>
<td>Ganis et al, 1956</td>
</tr>
<tr>
<td>(rat)</td>
<td>H, minces and slices</td>
<td>F ± NADP</td>
<td>F to E (24%)</td>
<td>Mahesh and Ulrich, 1960</td>
</tr>
<tr>
<td>(many species)</td>
<td>histochemical</td>
<td>F</td>
<td>F to E</td>
<td>Baillie et al, 1966</td>
</tr>
<tr>
<td>(human)</td>
<td>slices</td>
<td>E, F</td>
<td>E to F (high)</td>
<td>Jenkins, 1966</td>
</tr>
<tr>
<td>(human)</td>
<td>in vivo</td>
<td>F</td>
<td>F to E (&lt; 1%)</td>
<td>Hellman et al, 1971</td>
</tr>
<tr>
<td>(human, fetal)</td>
<td>minces</td>
<td>F</td>
<td>F to E (high)</td>
<td>Murphy, 1981a</td>
</tr>
<tr>
<td>(human)</td>
<td></td>
<td>E</td>
<td>E to F (&lt; 6%)</td>
<td></td>
</tr>
</tbody>
</table>

* Homogenates
Table IV-B. Cont'd

<table>
<thead>
<tr>
<th>Tissue (Species)</th>
<th>Incubation preparation</th>
<th>Substrate</th>
<th>11-HSD activity detected</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal (rabbit)</td>
<td>*H</td>
<td>A, B</td>
<td>A to B, B to A</td>
<td>Kokai and Fazekas, 1968</td>
</tr>
<tr>
<td>Intestine (rat)</td>
<td>minces</td>
<td>E, F</td>
<td>E to F, F to E</td>
<td>Stahl and Tapley, 1963</td>
</tr>
<tr>
<td>Uterine fibroblasts (human) U12 35, U12 79</td>
<td>cells</td>
<td>F</td>
<td>F to E</td>
<td>Grosser et al, 1958</td>
</tr>
<tr>
<td>Uterine fibroblasts (human) U12 79</td>
<td>cells</td>
<td>B</td>
<td>no conversion</td>
<td>Berliner et al, 1960</td>
</tr>
<tr>
<td>Connective tissue (mouse)</td>
<td>minces</td>
<td>F</td>
<td>F to E</td>
<td>Berliner and Dougherty, 1958a</td>
</tr>
<tr>
<td>Skin (human)</td>
<td>slices</td>
<td>E</td>
<td>E to F</td>
<td>Malkinson et al, 1959</td>
</tr>
<tr>
<td></td>
<td>minces</td>
<td>F</td>
<td>F to E</td>
<td>Hsia and Hao, 1966</td>
</tr>
<tr>
<td></td>
<td>minces</td>
<td>E</td>
<td>E to F</td>
<td>Hsia and Hao, 1967</td>
</tr>
<tr>
<td>Brain (rat) (baboon)</td>
<td>minces</td>
<td>E, F</td>
<td>E to F, F to E</td>
<td>Sholiton et al, 1965</td>
</tr>
<tr>
<td></td>
<td></td>
<td>**</td>
<td></td>
<td>Crosser and Axelrod, 1968</td>
</tr>
<tr>
<td>Lung (rat) (rabbit, fetal)</td>
<td>perfusion</td>
<td>E</td>
<td>E to F (high)</td>
<td>Nicholas and Kim, 1975</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>F to F (low)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>minces</td>
<td>E</td>
<td>E to F</td>
<td>Nicholas et al, 1978</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>F to E</td>
<td>Brooks et al, 1977</td>
</tr>
<tr>
<td>Epididymis (hamster)</td>
<td>histochemical</td>
<td>F</td>
<td>F to E</td>
<td>McGadey et al, 1966</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>no conversion</td>
<td></td>
</tr>
<tr>
<td>Salivary gland ducts (rat)</td>
<td>histochemical</td>
<td>F</td>
<td>F to E</td>
<td>Ferguson, 1967</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>no conversion</td>
<td></td>
</tr>
</tbody>
</table>

* Homogenates
Table IV-B. Cont'd

<table>
<thead>
<tr>
<th>Tissue (Species)</th>
<th>Incubation preparation</th>
<th>Substrate</th>
<th>11-HSD activity detected</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placenta (various mammals)</td>
<td>histochemical</td>
<td>F</td>
<td>F to E</td>
<td>Ferguson and Christie, 1967</td>
</tr>
<tr>
<td>(human)</td>
<td>*H</td>
<td>F + NADH</td>
<td>F to E (66%)</td>
<td>Osinski, 1960</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>E + NADPH</td>
<td>E to F (7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>minces</td>
<td>F</td>
<td>F to E</td>
<td>Sybulski and Velling, 1960</td>
</tr>
<tr>
<td></td>
<td>slices</td>
<td>F</td>
<td>F to E</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>E</td>
<td>no conversion</td>
<td>Meigs and Engel, 1961</td>
</tr>
<tr>
<td></td>
<td>minces</td>
<td>E</td>
<td>no conversion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>explants</td>
<td>E</td>
<td>E to F</td>
<td></td>
</tr>
<tr>
<td>Decidua (human)</td>
<td>in vivo</td>
<td>E, F</td>
<td>E to F, F to E</td>
<td>Pasqualini et al, 1970a</td>
</tr>
<tr>
<td></td>
<td>in vivo</td>
<td>F</td>
<td>F to E</td>
<td>Murphy, 1974</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>F</td>
<td>F to E</td>
<td>Bernal et al, 1980</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>E + NADPH</td>
<td>no conversion</td>
<td></td>
</tr>
</tbody>
</table>

* Homogenates
conversion occurs in homogenates, it is rather unlikely that this plays any role in hepatic physiology.

Many studies on 11-HSD activity are done unfortunately with only one direction studied perhaps under the assumption that the direction chosen to be studied is the important one, and also, that only one enzyme is involved although more than one may be depending upon the tissue under study. Unless both directions are studied, a good deal of misinterpretation of data can be made. For example, if conversion of E to F is 50% and conversion of F to E is 30%, than overall (net) conversion is only 20%. Also, if one were to study only F to E conversion one would erroneously conclude that 30% of F was being inactivated when in fact 20% of E was being activated to F which makes quite a difference. Another problem with the enzyme studies in general is that from one laboratory to the next the enzyme activity is studied under various conditions which makes comparisons of results very difficult. If, for example, too much substrate is used (as was the case in many older studies in which detection methods were poor which necessitated the use of large amounts of substrate), one may conclude that no activity is present in a certain tissue, yet with low, perhaps physiological substrate levels, the activity would be present.

2. 11-HSD Activity in Mesenchymal Versus Epithelial Tissue

In tissues such as liver, thymus, brain, skin, intestine, adrenal, and decidua of various species the reversibility of 11-HSD could be clearly demonstrated (see Table IV-B). Many researchers have therefore accepted that 11-HSD is a reversible enzyme; however, careful examination of the literature on 11-HSD activity suggests otherwise. For tissues such as
kidney, epididymis, salivary glands and placenta the 11-HSD enzyme activity appears to be largely irreversible; F to E being the favoured direction.

Fish et al (1953) could not detect any conversion of E to F in kidney homogenates of rat or pig, yet F to E activity was subsequently shown to be high (Ganis et al, 1956; Hellman et al, 1971). Attempts to reverse the activity using homogenates, minces, and slices with and without cofactors resulted in E to F conversion of only 3% (Mahesh and Ulrich, 1960); while in another study of human kidney slices the E to F conversion was less than 1% (Jenkins, 1966). 11-HSD activity in the kidney of human and many other species was found to be located in the collecting tubules (Baillie et al, 1966) and in no case was 11-HSD activity detected when E was used as substrate instead of F. In other localization studies (see Historical Review; section D 14), 11-HSD activity in epididymis or ducts of salivary glands was not detected when E was used as a substrate.

Placental F to E activity in a number of species is very important in maintaining low F levels in the fetus (see Historical Review; section G 5a). The only evidence that the enzyme is reversible comes from work done in 1960 by Osinski (see Table IV-B). Placental homogenates, incubated with E and fortified with cofactor, converted E to F, 7%, compared with 66% conversion of F to E when incubated with F (and NADP as cofactor). Contrary results have recently been reported by Lopez Bernal et al (1980). They failed to detect any conversion of E to F in placental homogenates even when fortified with NADPH. It is possible that the E to F activity measured by Osinski reflected contamination of the placental tissue with decidua, which possesses a good deal of E to F activity (Murphy, 1977a; Lopez-Bernal et al, 1980; Murphy et al, 1982) and which may not have been removed. Pasqualini et al (1970b) did detect E and F interconversion in human
placenta, however, the membranes had not been removed from the placenta (personal communication) and would explain the E to F conversion detected.

The above data, therefore, taken together strongly suggest that there are at least two different 11-HSD enzymes, one which in vivo converts E to F but is reversible under appropriate conditions (for example, as homogenates with or without the addition of cofactors) and one which converts F to E in vivo and is not readily reversible in vitro.

Another interesting aspect of the 11-HSD's is that the F to E enzyme appears to reside in epithelial derived tissues (glandular-like tissue: placenta, kidney collecting ducts, salivary gland, and epididymis) while the other resides in mesenchymally derived tissue (fibroblasts, connective tissue) (see Table IV-C). Table IV-C lists F to E activity which resides in epithelial derived tissue.

Some studies in which the issue of enzyme location was never considered, suggest that the activity resided in mesenchymal and/or epithelial tissue (i.e. one enzyme or two). Some possible intra-tissue locations of 11-HSD activity are presented in Table IV-D. For example, evidence of two different 11-HSD enzymes in skin comes from work done by Hsia et al (1965). They found that in human skin, NADH was more effective as a coenzyme in E to F conversion while NADP was more effective as a coenzyme in F to E conversion. Intact dermal tissue, mesenchymally derived, may be responsible for E to F conversion, while the epidermis, epithelially derived, converts F to E. This may also be the case for other tissues as well (see Table IV-D). Thus in effect two different 11-HSD's can exist; one mesenchymally derived and one epithelially derived (see Table IV-E), similar to the situation described for explant cultures of HFL.
Table IV-C. ll-HSD activity in epithelial cells of various tissues.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>placenta (human)</td>
<td>F to E</td>
<td>Osinski, 1960; Murphy et al, 1974; Lopez Bernal, 1980</td>
</tr>
<tr>
<td>Fetal lung (human)</td>
<td>F to E</td>
<td>Present study</td>
</tr>
<tr>
<td>Fetal brain (mouse)</td>
<td>F to E</td>
<td>Tye et al, 1978a</td>
</tr>
<tr>
<td>Kidney (various species)</td>
<td>F to E</td>
<td>Baillie et al, 1966</td>
</tr>
<tr>
<td>Parotid gland (dog)</td>
<td>F to E</td>
<td>Katz and Shannon, 1964</td>
</tr>
<tr>
<td>Salivary and submandibular gland (rat)</td>
<td>F to E</td>
<td>Ferguson and MacPhee, 1975</td>
</tr>
<tr>
<td>Epididymis (hamster)</td>
<td>F to E</td>
<td>McGadey et al, 1966</td>
</tr>
</tbody>
</table>
Table IV-D. Postulated intra-tissue locations of 11-HSD activity.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Postulated site</th>
<th>Tissue type</th>
<th>11-HSD activity</th>
<th>Reference (indirect evidence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>skin</td>
<td>epidermis</td>
<td>epithelial</td>
<td>F to E</td>
<td>Hsia et al, 1965</td>
</tr>
<tr>
<td></td>
<td>dermis</td>
<td>mesenchymal</td>
<td>E to F</td>
<td></td>
</tr>
<tr>
<td>liver</td>
<td>*RES</td>
<td>mesenchymal</td>
<td>E to F</td>
<td>Berliner and Dougherty, 1960</td>
</tr>
<tr>
<td>thymus</td>
<td>parenchyma</td>
<td>epithelial</td>
<td>F to E</td>
<td>Dougherty et al, 1960a</td>
</tr>
<tr>
<td></td>
<td>thymocytes</td>
<td>mesenchymal</td>
<td>E to F</td>
<td></td>
</tr>
<tr>
<td>decidua</td>
<td>connective</td>
<td>mesenchymal</td>
<td>E to F</td>
<td>Lopez Bernal et al; 1982b</td>
</tr>
</tbody>
</table>

*RES = reticulo endothelial system
Table IV-E. Characteristics of 11-HSD enzymes.

<table>
<thead>
<tr>
<th>Origin:</th>
<th>Mesenchymal</th>
<th>Epithelial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of Activity:</td>
<td>E to F activity</td>
<td>F to E activity</td>
</tr>
<tr>
<td></td>
<td>readibly reversible in homogenized tissue by the addition of cofactors or can be altered by tissue preparation such as homogenization</td>
<td>not readily reversible</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ie. cellular disruption)</td>
</tr>
</tbody>
</table>
CHAPTER V.

CLAIMS TO ORIGINAL RESEARCH
CLAIMS TO ORIGINAL RESEARCH

1. This was the first investigation of 11β-hydroxysteroid dehydrogenase (11-HSD) activity in human fetal lung (HFL) explant cultures.

2. This was also the first evidence that HFL 11-HSD activity, described in the literature, comprises at least two different enzymes.

3. These were the first studies of the effects of various hormones on 11-HSD activity in monolayer and explant cultures.

4. These were also the first studies of the effects of low O₂ on 11-HSD activity in HFL explant cultures.

5. These studies resolved the discrepant results in the literature regarding the direction of 11-HSD activity in midterm HFL.
CHAPTER VI

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REFERENCES


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APPENDIX I

POWDER FORMULATION OF HAM'S F-10
POWDER FORMULATION OF HAM'S F-10

The following formulation of Ham's F-10 nutrient mixture is taken from the Gibco catalogue 1978/1979.

<table>
<thead>
<tr>
<th>Inorganic salts</th>
<th>mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂·2H₂O</td>
<td>33.29 (anhydrous)</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.0025</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>0.834</td>
</tr>
<tr>
<td>KCl</td>
<td>285.0</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>83.0</td>
</tr>
<tr>
<td>MgSO₄ (anhydrous)</td>
<td>74.64</td>
</tr>
<tr>
<td>NaCl</td>
<td>7400.0</td>
</tr>
<tr>
<td>Na₂HPO₄ (anhydrous)</td>
<td>153.7</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>0.0288</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Other components</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1100.0</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>4.68 (Na salt)</td>
</tr>
<tr>
<td>Lipoic acid</td>
<td>0.2</td>
</tr>
<tr>
<td>Phenol red</td>
<td>1.2</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>110.0</td>
</tr>
<tr>
<td>Thymidine</td>
<td>0.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Amino acids</th>
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</tr>
</thead>
<tbody>
<tr>
<td>L-alanine</td>
<td>9.0</td>
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<tr>
<td>L-arginine HCl</td>
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<td>L-asparagine·H₂O</td>
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<td>L-aspartic acid</td>
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<td>L-cysteine</td>
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<tr>
<td>L-glutamic acid</td>
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<td>L-glutamine</td>
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<tr>
<td>Glycine</td>
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<td>L-histidine HCl·H₂O</td>
<td>23.0</td>
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<tr>
<td>L-isoleucine</td>
<td>2.6</td>
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<tr>
<td>L-leucine</td>
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<td>L-lysine HCl</td>
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<td>L-methionine</td>
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<td>L-phenylalanine</td>
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<tr>
<td>L-proline</td>
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<td>L-serine</td>
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<tr>
<td>L-threonine</td>
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<tr>
<td>L-tryptophan</td>
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<tr>
<td>L-tyrosine (Disodium salt)</td>
<td>2.62</td>
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<tr>
<td>L-valine</td>
<td>3.5</td>
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</table>

<table>
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<tr>
<th>Vitamins</th>
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<tr>
<td>Biotin</td>
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<tr>
<td>D-Ca pantothenate</td>
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<td>Choline chloride</td>
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<td>Folic acid</td>
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<td>i-Insitol</td>
<td>0.541</td>
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<tr>
<td>Niacinamide</td>
<td>0.615</td>
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</table>
Pyridoxine HCl .............................................. 0.206
Riboflavin ...................................................... 0.376
Thiamine HCl .................................................. 1.000
Vitamin B₁₂ ..................................................... 1.360

NaHCO₃, 1200 mg/l, was added to the medium following the addition of distilled water.
APPENDIX II

DUAL LABEL COUNTING OF $^3$H AND $^{14}$C
Dual label counting of \( ^3\)H and \( ^{14}\)C

Dual label counting of \( \beta \)-emitters, \( ^3\)H (\( W_{\text{max}} = 18.6 \text{ keV} \)) and \( ^{14}\)C (\( W_{\text{max}} = 156.7 \text{ keV} \)) is possible because their maximum beta-particle energies (\( W_{\text{max}} \)) are sufficiently different so that interference of the \( ^3\)H spectrum in the channel of the higher energy isotope, \( ^{14}\)C, can be eliminated. Therefore, counts in the higher energy channel results from only the higher energy isotope and direct calculation (without correction for the lower isotope's counts) of the higher energy isotope, \( ^{14}\)C, is feasible. In order to count the cpm's of the lower energy isotope, \( ^3\)H, one must subtract the interference cpm's of the higher energy isotope \( ^{14}\)C.

Dual label counting of \( ^3\)H-F and \( ^{14}\)C-E on the LSA was done as follows:

When \( ^{14}\)C was being counted the LSA was set to count 68% of \( ^{14}\)C which allowed 1% of \( ^3\)H to be counted as well, therefore, there was no appreciable interference from \( ^3\)H. To count \( ^3\)H it was not possible to get rid of \( ^{14}\)C counts, therefore, when 65% of \( ^3\)H was counted 7-8% of \( ^{14}\)C was counted as well. Therefore, 7-8% (of 100%) of \( ^{14}\)C was counted for each sample of \( ^3\)H being counted and was subtracted from the \( ^3\)H counts. Standards of \( ^3\)H and \( ^{14}\)C were always counted as references with each set of samples to determine the percentage of \( ^{14}\)C.

In most experiments approximately twice as many \( ^3\)H counts were added as \( ^{14}\)C in order that the number of \( ^{14}\)C counts subtracted was a small fraction of the \( ^3\)H counts.