CHARACTERIZATION OF ESTROGEN AND GLUCOCORTICOID RECEPTORS, SKELETAL MUSCLE PROTEIN TURNOVER AND TISSUE GROWTH IN LAMBS TREATED WITH TRENBOLONE ACETATE AND ESTRADIOL

by

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(ABSTRACT)

A study was conducted to determine the effects of trenbolone acetate (TBA) and estradiol-17B (E2) implantation on the characteristics of the glucocorticoid and E2 receptor, skeletal muscle protein turnover and tissue growth. Twenty-four lambs were utilized. Trenbolone acetate did not affect ($P>.10$) degradation rates in the semitendinosus (ST) and triceps brachii (TB) muscles, the production of cortisol, adrenal weights and cytosolic glucocorticoid binding capcity (Bmax). Trenbolone acetate decreased synthesis rate of muscle protein ($P<.01$), the

percent of [^{3}H] dexamethasone binding in the nuclear fraction, Bmax and the disociation constant (Kd) of the cytosolic E2 receptor, only in the TB muscle. Deoxyribonucleic acid (DNA) of the TB was increased ($P<.05$) with TBA. Pituitary weights were decreased ($P<.005$) with TBA and increased ($P<.01$) with E2. Estradiol decreased ($P<.05$) Bmax of the cytosolic E2 receptor in the ST and decreased ($P<.05$) Bmax of the nuclear E2 receptor in the TB muscle. The TB muscle had greater ($P<.05$) synthesis rates than the ST and the protein:RNA ratio was decreased ($P<.05$) in the TB. The TB muscle had greater ($P<.005$) Bmax for the cytosolic glucocorticoid receptor.

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CHAPTER I

INTRODUCTION

Improvement in the efficiency of production of human food by animals is a principal goal of animal scientists. An understanding of the processes involved and the regulation of postnatal growth by anabolic agents is fundamental for securing effective approaches for improving production of meat animal species.

The research study reported herein involved treating lambs with two growth promoters, trenbolone acetate and estradiol. This study was undertaken to better understand the mechanism of anabolic agents in skeletal muscle. The growth of skeletal muscle was examined by estimating the synthesis and degradation of muscle protein, nucleic acids and glucocorticoid and estrogen receptors in semitendinosis and triceps brachii muscles. In addition serum cortisol was estimated.

CHAPTER II

Literature Review

Anabolic Agents and Animal Production. Growth is a complex biological phenomena involving interactions between hormonal, genetic, nutritional and metabolic factors. In the simplest of terms, growth can be defined as an increase in size. During the growth of an animal, the partitioning of nutrients to different tissues, varies considerably. Specific organs cease growing during the life of the animal, whereas, other organs grow, given the proper conditions, periodically or continually. The rate of muscle growth declines with advancing age and body weight gain at maturity is represented by the deposition of fat (Hammond, 1921; Palsson and Verges, 1952).

Although the genetic component is well recognized, the sex of the animal is also an important determinant of growth rate and carcass composition, presumably due to the secretion of the sex steroid hormones from the gonads. Bulls grow faster, require less feed per unit of gain and have a higher percentage of edible cuts in the carcass than steers (Field, 1971). However, castration of males improves carcass acceptability (Preston, 1975). The classic work of Bowman (1840) demonstrated that for some striated muscles, the fibers were larger in men than in women. A number of

animal studies have demonstrated that striated muscle growth and development is androgen dependent (Papanicolaou and Falk, 1938; Kochakian et al., 1948). Castration of male animals results in the regression of skeletal muscle but this reversal can be attenuated by androgen injection (Wainman and Shipounoff, 1941; Kochakian et al., 1956).

Estrogens influence growth of animals, ultimately in a manner similar to the androgens. Zondek and Marx (1939) were the first to observe increased fat content of the liver, lung and muscle of poultry as a result of injecting estradiol benzoate. Following the discovery that estrogenic compounds induce a hypertrophic condition, Lorenz (1943) implanted diethylstilbesterol, a compound developed by Dodds et al. (1938), in cockerels. Similar results were observed, with increased fat content of the breast, leg and liver. In the period 1950 to 1970, many experiments were conducted utilizing diethylstilbesterol and hexestrol (Preston et al., 1965; NAS, 1966). Diethylstilbesterol was first used to increase the gain of implanted heifers (Dinusson et al., 1948) and by 1953 had been shown to be orally active in sheep (Hale et al., 1953). Also, implantation of the natural estrogen, estradiol, gave comparable gain stimulation (Wright, 1961). Commercially estradiol is generally combined with progesterone or testosterone. Several reviews have been published regarding the effects of

various estrogens in stimulating growth of cattle and lambs (Gassner et al., 1958; Sykes et al., 1960; Trenkle, 1969).

Efficiency of feed use for gain is improved by estrogen administration. Feed intake is increased somewhat (Forrest and Sather, 1965), however, an increase in feed intake results in an increase in gain (Walletine et al., 1961). Dressing percentage is often decreased, reflecting a decrease in the amount of fat in the carcass, but changes in fat deposition in the form of marbling have been variable in response to estrogens (Preston, 1975). Because fat deposition tends to be decreased, water content of the carcass may also be increased; however on a fat free basis, no changes in water content were observed (Preston and Burroughs, 1958). Rib eye area and other measures of muscling generally show an increase with fat deposition generally showing a decrease (Preston and Burroughs, 1958).

Many experiments have been carried out to determine the importance of various production related factors in the growth response of cattle and lambs to estrogens. Usually a minimum level of protein is required, however increased dietary protein results in a higher gain response (Preston and Burroughs, 1958). Greater responses have been noted with higher energy rations (Struempler and Burroughs, 1959) than low to moderate energy rations.

Genetic background may alter the response to anabolic agents. Thompson et al. (1962) implanted steers from different sires with diethylstilbesterol and observed varying responses. In other work, no sire-diethylstilbesterol interactions were observed except for fat content of the longissimus muscle (Wilson et al., 1963). Clancy et al. (1986) reported that the Friesian breed displayed a greater percentage of red myofibers or aerobically functioning muscle than the Charolais Friesian cross when estrogen was administered. In sheep, growth in blackface wether lambs was stimulated to a greater extent by diethylstilbesterol than in whiteface wethers (Butcher and Raleigh, 1962).

Trenbolone acetate (TBA) is a trienic steroid, similar in structure to testosterone but 10 to 50 times more active than testosterone (Neuman, 1975). It is used alone in female cattle and in association with estrogens in male or castrated cattle and sheep. Neuman (1975) compared the pharmacological activity of TBA to that of testosterone, testosterone propionate and 19-nortestosterone in male rats. He observed the range of activity of TBA to be identical to that of 19-nortestosterone but with differences in intensity reaching 10 times that of testosterone propionate and up to 50 times of testosterone.

An average growth improvement in castrate male lambs of 18.3 and 15.0% was demonstrated by Szumowski and Grandadam (1976) resulting from implantation of 40 and 60 mg TBA. Grandadam et al. (1975) observed an increase of 26.6% in growth rate in 48 Southdown castrate lambs when a combination of TBA estradiol was implanted. Slightly lower rates of gain were seen when Sulieman et al. (1986) implanted 35 mg TBA + 5 mg estradiol (14% increase). Singh et al. (1983) demonstrated a 22% increase in rate of gain with a 35 mg TBA + 5 mg estradiol implant. However, not all reports have shown an increase in performance as demonstrated by Coelho et al. (1981) and Yasin and Galbraith (1981) in sheep and by Vernon and Buttery (1978a) in rats.

In veal calves the implantation of estradiol alone has been shown to produce only a moderate effect on weight gain and nitrogen balance while the combination estradiol/TBA proved to be very effective in male veal calves (Grandadam et al., 1975). Van Der Wal et al. (1975) demonstrated marked improvement in nitrogen balance when a 20 mg estradiol/140 mg TBA implant was administered to male veal calves.

Anabolics are not used on a large scale in older intact male cattle because of the endogenous secretion of testosterone (Van Der Wall and Berende, 1983). The general conclusion is that anabolic agents increase growth rate in intact males (Galbraith and Topps, 1981). However, there is

disagreement in the literature (Baker and Arthaud, 1972) possibly because of different dosages or incorrect estimates of growth parameters. Galbraith (1982) implanted 359 kg Friesian bulls with 45 mg hexoestrol, followed 21 d later with 300 mg TBA. He observed a significant improvement in live weight gain and feed conversion for TBA but not hexoestrol and reduction in plasma androgens concluding that TBA plus hexoestrol may have a more potent anabolic activity than the endogenous hormones of intact male cattle.

Experiments conducted with TBA in steers have demonstrated an improvement in weight gain compared to non-implanted animals. In three trials with a total of 1,190 steers, Roche and Davis (1978) obtained an improvement in gain with implantation of 300 mg TBA. Griffiths (1982) implanted 24 castrate male cattle with 300 mg TBA plus 36 mg resorcyclic acid lactone, over the live weight range 250 to 400 kg, at the initiation of the trial and again 65 d later. Live weight gain was greater for the implanted animals.

Chan and Heitzman (1975) investigated the nitrogen balance of heifers treated with TBA (300 mg) for 7 d. The treated heifers retained more nitrogen than the control animals, resulting in a 38% increase in daily gain and an increase in the number of animals ready for slaughter. Brown et al. (1982) also reported an improvement in performance with TBA in heifers.

Skeletal Muscle Protein Turnover

Turnover is a general term used to describe the process of renewal or replacement of a given substance. This process may either involve the production of newly formed material, with the disappearance of some material already present, or it may represent the exchange of material between two or more compartments.

The turnover rate was defined by Zilversmit (1960) as the rate at which a substance is turning over in a given compartment or metabolic pool. Similarly, Shipley and Clark (1972) define turnover rate as "the steady state quantity of tracer moving through the pool per unit time". These definitions are adequate for a system in the steady state. In a growing system, however, in which pool sizes are not constant it is necessary to be more specific in the definition. Reiner (1953) considered the turnover rate to be the molecules of a substance which are newly formed per unit time. Swick et al. (1956) on the other hand used separate turnover rates for both synthesis and degradation, but later Kolch (1962) concluded that turnover should only apply to the degradation process. The correct definition has not been generally agreed upon but the definition of Swick and coworkers is probably most appropriate.

Protein turnover considers a protein (either a single protein or a mixture) which is exchanging with a free amino acid pool. There appears to be uniformity of incorporation of amino acids from this free amino acid pool into protein. Simpson and Velick (1954) administered 5 different labeled amino acids to rabbits and isolated two proteins from muscle. The relative specific activity of the five amino acids was identical in the two proteins. Thus it can be inferred that the synthesis and breakdown of protein involves the passage of one amino acid into and out of protein. This means that the rate of incorporation of each amino acid into protein is directly proportional to its concentration in that protein.

Turnover rates are commonly expressed as fractional synthesis rates (k). This is the amount of material transferred in a unit time, as a percentage of the pool from which it is transferred. The rates of transfer of material from pool A to pool B or vice versa are designated as V_{AB} or V_{BA}. The amounts of material exchanging pools are designated M_A and M_B. Therefore, $K_{AB} = V_{AB}/M_A$ and $K_{BA} = V_{BA}/M_B$. In addition, in the steady state, when V_{AB} and V_{BA} are equal, $K_{AB} = (M_A/M_B)K_{BA}$. In a first order chemical reaction, the value of K is independent of the pool size; therefore it is referred to as a fractional rate constant

(Waterlow et al., 1978). The accepted viewpoint is that protein synthesis is zero order [(a fixed rate of synthesis per unit of DNA (DNA activity) and breakdown is first-order (a fractional rate; Swick, 1982). Protein turnover or a combination of synthesis and degradation can than be expressed conveniently when a combination of synthesis and degradation are applied, since they relate to the same pool (Milward et al., 1978). This assumes that neither pool is expanding or contracting (a steady state). Protein deposition or the relationship between synthesis and degradation can be expressed by the equation $K_s - K_g = K_d$, where K_s is the fractional synthesis rate, K_g the fractional degradation rate, and K_d the fractional rate of protein deposition (Milward et al., 1976; Scornik and Botbol, 1976).

Muscle Protein Synthesis. Postnatal growth of skeletal muscle occurs primarily by muscle cell hypertrophy because muscle fiber numbers do not increase dramatically postnatally. Growing skeletal muscles shows tremendous increases in DNA content which parallels with muscle protein accretion (Harbison et al., 1976; Trenkle et al., 1978). In muscle protein synthesis, extracellular amino acids can exchange with the free amino acid pool, inside the muscle cell. Free amino acids in cell particulate fractions may not be in equilibrium with those in the cytosol or in the immediate precursor pool for protein synthesis. Once amino

acids are in the cell and free, they are activated and converted to aminoacyl-tRNA complexes which are then joined together on messenger RNA to form protein molecules. Hider et al. (1971) have suggested that, in muscle, extracellular amino acids are preferentially incorporated into proteins. The formation of aminoacyl-tRNA may occur on, in or at the membrane. The generally accepted mechanism is that intracellular amino acids are directly involved in protein synthesis (Morgan et al., 1971; Li et al., 1973).

Regulation Of Protein Synthesis. Protein synthesis in animals is subject to both qualitative and quantitative regulation (Pain and Clemens, 1980). Qualitative regulation is important in determining the type of protein synthesized. This implies selective changes in the rates of synthesis of one or more particular proteins relative to that of other proteins within the cell. The regulation of the total amount or quantitative regulation is involved in variation of the protein mass of a tissue. In many developmental situations both kinds of control are exerted, with selective increases in specific proteins being generated against a general biochemical system of protein metabolism.

Since protein synthesis occurs on the ribosome, attention has been given to the ribosomal aspects of skeletal muscle protein synthesis. More than 80% of muscle RNA is ribosomal and this proportion appears to remain

constant, even in cases of protein depletion (Young, 1970). Henshaw et al. (1971) demonstrated a high correlation between the rate of in vivo muscle protein synthesis per unit of ribosomes and growth rate. However, parallel decreases in in vitro ribosomal activity and in vitro and in vivo rates of ^{14}C labeled amino acid uptake into muscle protein occur (Breuer and Florini, 1965) with progressive growth.

Peptide chain initiation appears to be rate limiting for translation in many cell types and the regulation of protein synthesis. In isolated skeletal muscles, the branched chain amino acids together or simply leucine alone stimulate protein synthesis (Fulks et al., 1975; Chua et al., 1980). The acceleration of protein synthesis by these amino acids occurs at the level of peptide chain initiation (Morgan, 1971). However, unlike most amino acids the branched chain amino acids are catabolized rapidly by muscle (Odessey and Goldberg, 1972; Goldberg and Chang, 1978). Some researchers indicate that aminoacylated tRNA may regulate levels of protein synthesis and degradation (Scornik et al., 1980). Tischler et al. (1982) were unable to prove that leucyl-tRNA regulates protein turnover in hindlimb skeletal muscle of the rat.

Relationship of RNA and DNA To Muscle Protein Synthesis. Content of muscle DNA corresponds directly with muscle protein accretion as tremendous increases in total skeletal muscle DNA occur (Harbison et al., 1976; Trenkle et al., 1978). Total muscle DNA is increased where breed or sex results in increased growth (Burleigh, 1980). Enesco and Puddy (1964) also reported an increase in total DNA with growth. In a review, Burleigh (1974) suggested that the number of muscle nuclei determines the mass of a given skeletal muscle. However, Dimarco et al. (1987) demonstrated postnatal growth in cattle may involve both hyperplasia and hypertrophy of muscle cells. Early postweaning growth was mainly hypertrophic as increases in protein/DNA occurred more than DNA content, up to 200 kg of body weight. At live weights of 350 kg, growth was due to DNA accumulation as well as cell enlargement, indicating both hyperplasia and hypertrophy. At weights above 350 kg, no increase in the protein/DNA ratio, and an accumulation of DNA, indicated a hyperplastic form of growth. No fractional synthesis rates (FSR) of muscle protein were reported nor was it pointed out that increased DNA content may result from non muscle nuclei (Waterlow and Garlick, 1978) and that the heavier animals may have had an increased fat content. Campion et al. (1981) reported the percentage of satellite cell nuclei in transverse sections of two muscles from pigs

decreased with age while myonuclei increased. Material presented by Black (1984) indicated that in lambs which had a body weight of 10 to 25 kg, muscle growth was largely hyperplastic, since no increase in the protein/DNA ratio was observed.

The concentration of DNA in skeletal muscle decreases during postnatal muscle growth. Harbison et al. (1976) reported that DNA concentrations in porcine skeletal muscle decreased by nearly 50% between 23 and 91 kg of body weight and remained constant between the live weights of 91 and 118 kg. Increase in body weight and size contribute to the decrease in the tissue concentration of DNA (Enesco and Puddy, 1964). Since muscle fibers are multinucleated, the term "muscle DNA unit" was defined by Cheek et al. (1971) as a volume of cytoplasm of a given muscle fiber, managed by a single nucleus. In strains of fast and slow growing and malnourished rats, Waterlow and Milward (1978) concluded that the number (not size) of DNA units was the determining factor in the size of a particular muscle. However, an inverse relationship between (FSR) of muscle protein and DNA unit size has been reported (Laurent et al., 1972) such that increases in FSR are seen with decreased DNA unit size. This was supported by Jones et al. (1986) as the protein/DNA ratio in the pectoralis major (PM) muscle of layers was greater with decreased FSR than the PM of broilers. Protein

synthesis decreases with a limited dietary protein intake. In relation to this, however, Graystone and Cheek (1969) reported that with a reduced calorie intake, DNA content was reduced, while the protein/DNA ratio was increased.

The concentration and total content of RNA in skeletal muscle are inversely related as the total content of RNA in muscleincreases during postnatal muscle growth while the concentration decreases (Harbison et al., 1976). However, Cheek (1968) demonstrated that the ratio of RNA concentration to protein concentration (RNA/protein) increases with age. The relationship of RNA concentration to protein synthesis can be described in terms of the capacity for protein synthesis (protein/RNA) and the activity of RNA, defined as the rate of protein synthesis per unit of RNA (Milward et al., 1973, 1976). Milward and Waterlow (1978) reported that the protein/RNA ratio is inversely related to FSR. However, Jones et al. (1986) suggested that there is RNA that is not active in protein synthesis since the protein/RNA ratio was similar for broilers and layers while FSR was the lowest for the layer. It can then be concluded that the FSR increases with age or that RNA activity (g protein synthesized/g RNA/day) falls with increasing age.

Content of muscle DNA and RNA corresponds directly with muscle protein accretion as tremendous increases in DNA and RNA occur. The protein/DNA ratio is inversely related with FSR such that when the muscle DNA unit size increases, dilution of muscle ribosomes occur which decreases muscle protein synthesis. The protein/RNA ratio is also inversely related to FSR since an increase in RNA would indicate a greater capability for protein synthesis.

Muscle Protein Degradation. The catabolism of skeletal muscle is a classic example of a homeorhetic coordination of body tissues necessary to support a physiological state. Catabolism of muscle protein is continually occurring and is essential for the maintenance of blood glucose concentrations via gluconeogenesis. In the fasting animal, once glycogen reserves become exhausted, the major source of glucogenic precursors from which glucose is synthesized, is muscle protein. In man, approximately 75 g of muscle protein per day are oxidized to satisfy the brain. If this was to continue for long, extreme muscle wasting would occur. As the brain switches to ketones as its energy source, the degradation of muscle protein falls to 25 g per day (Waterlow et al., 1978).

Two independent pathways of protein degradation have been proposed. A lysosomal dependent pathway in which vacuoles are formed by enclosure of cell constituents by

cell membranes. Autophagolysosomes are then formed by fusion of vacuoles with primary lysosomes. The proteins are subsequently hydrolyzed within the autophagolysosomes. The second degradative system involves direct proteolytic breakdown by cytosolic proteases and is limited by the ability of each protein to act as a substrate for proteolysis (Ballard, 1977).

The above discussion does not consider growth as a component of muscle protein degradation. In fact, protein breakdown is a very necessary component of growth (Milward et al., 1976). Milward and Waterlow (1978) suggested that increased rates of degradation accompany higher rates of growth. This conclusion was supported by several researchers who found increased rates of protein breakdown during rapid muscle growth (Sola et al., 1973; Turner and Garlick, 1974; Waterlow et al., 1978). However, Milward et al. (1978) reported that there are two types of protein degradation, an anabolic increase in degradation and an anabolic decrease. When protein degradation increases with increased growth, however, it appears to be a late response and it may follow an initial fall in degradation rate. These results presented by Milward et al. (1978) occurred after nutritional rehabilitation in rats. The authors suggested that this may be a result of a fall in proteinase activities in response to refeeding. This was in accord

with Young et al. (1971), that during rehabilitation of malnourished rats, muscle protein breakdown was decreased to zero. Milward et al. (1976) reported slow growing rats have a greater rate of protein breakdown than fast growing strains. This contrasts with other published results that suggest faster growing animals have higher degradation rates. Reports by Laurent et al. (1978) and Waterlow et al. (1978) are that a stretch induced factor may cause the increased breakdown rate, such that if bone lengths are the same, the muscle from faster growing animals will respond to less stretch imposition. Laurent et al. (1978) suggested that stretch may induce an increased transport or breakdown of amino acids since intracellular concentrations of free proline, alanine and serine were increased. In addition, Righetti et al. (1971) and Waterlow et al. (1978) suggest that amino acids derived from protein breakdown may be preferentially reincorporated into newly synthesized protein. However, this may not occur if slower growing rats had a decreased accretion rate.

Problems Associated With Measuring Protein Turnover. The measurement of turnover of proteins in vivo has been reviewed by Neuberger and Richards (1964), Waterlow (1964), Schimke (1970) and Waterlow (1970). The estimation of protein turnover is frequently based on the uptake or release of labeled amino acids (Waterlow, 1970). Injecting

a single dose of labeled amino acid into an animal is commonly used to study protein turnover in vivo. Theoretically, the isotope should enter the pool from which the protein is being synthesized and should leave the pool rather quickly, and should not reenter the pool at anytime following the initial isotope administration. The proportion of the labeled amino acid which is incorporated (synthesis rate) or the rate of disappearance (degradation rate) is the theory behind the measurement of protein turnover.

When an animal is injected with a single pulse dose of a isotopic amino acid, the specific activity in the blood rises rapidly and soon declines. As the amino acid is incorporated into protein, the specific activity in the blood declines at a slower rate compared to the time when the isotope was first injected. In a tissue in which the protein turnover is fast (liver) the specific activity rapidly increases as opposed to slower turnover rates (muscle) where the peak is much lower and occurs later. In these type of studies, it is imperative to measure the time course of the specific activity. This requires killing several animals at different periods of time. Different amino acids (depending on the particular protein being synthesized or degraded) as well as different animals may give disimilar specific activities so several time points

should be utilized (Waterlow et al., 1978). A problem associated with this method is that very large doses are needed such that the specific activity in the blood remains constant for longer periods of time. As the precursor amino acids get incorporated into protein, the specific activity difference between the blood and the tissue do not vary much, therefore minimizing the error in estimating the precursor specific activity (Waterlow et al., 1978).

The method of Scornik (1974) involves injecting single doses of a radioactive amino acid at varying concentrations and fixed specific activities into a group of animals. A graph of dose versus incorporation is then plotted and extrapolated to an infinite dose. The incorporation (synthesis) is then calculated from the incorporation at infinite dose and from the specific activity of the injected amino acid. A major assumption is that when the free amino acid pool is diluted with the large amount of isotopic amino acid, that the specific activity is equal to the injected specific activity. A problem with this procedure may be that the large dose of amino acids may influence the rate of synthesis. Scornik (1974) showed that the procedure did not affect the incorporation of 14C lysine. Large numbers of animals are required for this procedure since it can not be assumed that the line plotted will always be linear. There are instances when proteins can accumulate a labeled amino

acid, release this amino acid and then be reutilized by another tissue or the same tissue (Haverberg et al., 1975). This will lead to an underestimation of protein degradation. This can be partially overcome by using non-utilizable amino acids. Alternatively, by determining the breakdown rate, soon after injection of the isotopic amino acid or using shorter half-lifed isotopes, a better indication of the true degradation rate can be determined. Perry (1974) and Swick (1958) considered the fact that $^{14}CO_2$ is taken up by dicarboxylic acids. It follows that if ^{14}C carbonate is used, which labels primarily asparate and glutamate (Manchester and Young, 1959), that the problem of recycling of amino acids can be eliminated in the estimation of in vivo protein turnover.

The constant infusion of amino acids into an animal was first used by Loftfield and Harris (1956). Later Garlick et al. (1973) improved the constant infusion technique. The purpose of this technique is to produce a constant specific activity of the precursor. Rates of synthesis of protein in a number of tissues have been determined by this technique (Seta et al., 1973; Fern and Garlick, 1974). In tissue that has a slower rate of turnover, the time course for a constant specific activity may be considerable. A correction factor is usually applied which allows for the time required to achieve a constant specific activity. This

would involve either serial killings, longer infusion periods (which may promote more recycling of amino acids) or an estimate of the time course of maximum specific activity, which is then fitted into a mathematical formula (Waterlow et al., 1978).

Another method commonly used to estimate the synthetic and degradative capabilities of skeletal muscle protein is the in vitro method of Fulks et al. (1975). In this procedure, the incorporation of ^{14}C tyrosine into muscle protein is used to estimate protein syntheis. Tyrosine is commonly used because the amino acid has been shown to equilibrate rapidly between the medium and the intracellular fluid and that the free intracellular tyrosine pool is a valid measure of the precursor pool for protein synthesis (Li et al., 1973). Moreover, related studies have shown that ^{14}C tyrosine is similar to phenylalanine in that tyrosine is not oxidized to $^{14}CO_2$ (Odessey and Goldberg, 1972).

A major difficulty in evaluating the degradative rate has been the reincorporation of amino acids released from protein (Goldberg and Dice, 1974). The in vitro technique of Fulks et al. (1975) eliminates this problem by the

addition of cyclohexamide to the incubation medium. Cyclohexamide has been shown to block synthesis of protein by inhibiting elongation preferentially (Stanners, 1966). A potential difficulty with this approach is that under certain conditions, cyclohexamide can reduce intracellular protein degradation (Hershko and Tomkins, 1971; Woodside and Mortimore, 1972).

Several other studies have dealt with the fact that the in vitro technique requires that the muscle be gently stretched as it is in vivo. This is needed to maximize the movement of amino acids, glucose and other substances present in the incubation buffer. Goldberg and Odessey (1974) and Goldberg et al. (1974) have observed a decrease in the rate of protein degradation in the stretched muscle in vitro. However, Turner and Garlick (1974) and Laurent et al. (1978) reported that stretching muscle in vivo increases the rate of protein degradation. Seider et al. (1980) demonstrated that, although concentrations of ATP and phosphocreatinine were less in unstretched muscle than in stretched muscle, rates of protein degradation were similar in these two groups.

Kipnis and Cori (1957) have demonstrated that cut muscle fibers have larger extracellular spaces, and higher glycogen concentrations (Gross et al., 1976). Seider et al. (1980)

reported that the rate of protein synthesis in vitro was lower in cut soleus and extensor digitorum longus muscles than in their respective uncut muscles. No differences were observed between cut and uncut muscles in degradation rate. The in vitro muscle biopsy technique requires that muscle fibers be cut. This is a potential problem in this particular assay.

Rates of protein synthesis in incubated rat muscles are lower than those measured in vivo (Goldspink et al., 1983; Maltin and Harris, 1985). Commonly, extrapolations of conclusions from whole muscle and stripped muscle preparations are seen. Depressed protein synthesis of muscle incubated in vitro has been reported, assumed to result from an oxygen deficiency in the central portion of the muscle. Rates of protein synthesis in central cores were lower than in peripheral areas, a distribution not seen in vivo (Maltin and Harris, 1985). Segal and Faulkner (1985) suggested that oxygen diffusion will only be adequate if the tissue does not exceed 1.2 mm in thickness. In addition, Maltin and Harris (1985) reported that tissue taken from an animal of higher metabolic rate, will have less oxygen diffusing into the central core of the muscle upon incubation in vitro.

The above techniques provide researchers with opportunities for the measurement of muscle protein turnover. Each technique, however has inherent problems that must be encountered. In vivo estimations of protein turnover require large doses of isotope such that a constant specific activity is maintained. This may lead to the problem of reutilization of amino acids which can underestimate the degradation rate. In vitro techniques require that muscle fibers be cut creating larger extracellular spaces. In addition, oxygen diffusion in an artificial system can certainly be impaired when muscle biopsy sizes are not kept at a minimum.

Effect of Hormones on Muscle Metabolism

Insulin. A major problem, once thought to be associated with insulin administration in isolated muscles, was the possibility that increased protein synthesis resulting from insulin might occur as a simple result of increased availability of energy (glucose) in the tissue. However, many investigators have shown that insulin stimulates uptake of amino acids in muscle (Manchestor and Young, 1960; Manchestor, 1970;) by stimulation of the A transport system. Following the work of Woll and Cavecchi (1967) that

ribosomes from muscle of diabetic rats were less active in protein synthesis, these researchers demonstrated that insulin works post-transcriptionally. Presumably, insulin administration has no effect on the amount of mRNA production (Fahmy and Leader, 1980). Contrary to this, Jefferson et al. (1972) found a greater availability of mRNA following insulin administration. A decreased rate of proteolysis in muscle has been shown to occur with insulin administration (Jefferson et al., 1977; Williams et al., 1980). However, not all proteases are affected by insulin as McElligott and Bird (1981) found that there was no effect of insulin treatment on the serine proteases. Insulin administration to cultured muscle cells stimulates protein synthesis (Jefferson et al., 1974), however, King et al. (1980) reported insulin cross-reacts with somatomedin receptors. The effects seen with insulin might be due to other factors beside strictly insulin.

The ability of a tissue to respond to insulin depends on the presence of specific insulin receptors located on the plasma membrane. The number of cell surface receptors depends on insulin concentration and duration of exposure (Gammeltoft, 1984). Thus high insulin concentrations result in a down regulation of receptors while the inverse results at low insulin concentrations. An indication that insulin

has specific receptors located in the muscle cell has not been demonstrated as yet in skeletal muscle.

Thyroid Hormones. It is known that thyroidectomy reduces growth rate in rats and the replacement of the hormone with physiological levels, returns the growth rate to normal levels. King and King (1976), Widner and Holloszy (1977) and Flaim et al. (1978), have all indicated an increase in muscle protein synthesis with thyroid hormone administration. However, Young and Munro (1978) observed a decrease in 3-methylhistidine production in thyroidectomized rats and with thyroxine replacement there was an increased rate of muscle protein breakdown. Since protein deposition increased, protein synthesis must be occurring at a faster rate than degradation.

The mechanism of action of thyroid hormones has not been completely elucidated but it is thought that the basic unit of thyroid hormone action is the T3-nuclear receptor complex. Oppenheimer (1979) suggested that the cytoplasmic T3 receptor cannot be ruled out but this system operates optimally at higher than physiological levels. Prolonged increases in T_3 blood levels have been shown to increase basal insulin levels. Godden and Weekes (1984) used the euglycaemic insulin clamp technique to investigate the effects of hyperthyroidism on the responsiveness of whole

body glucose clearance sensitivity to insulin. Glucose clearance was not elevated by thyroxine but the sensitivity to insulin was increased. They suggested that an increased insulin receptor number or affinity on target tissues, predominantly skeletal muscle augmented the sensitivity to insulin.

Growth Hormone. Growth hormone exerts a variety of effects on metabolism. The ability of exogenous growth hormone to stimulate protein accretion and reduce carcass lipid deposition is well established in growing livestock (Bauman, 1982; Etherton and Kensinger, 1984). The insulin-like effects of growth hormone injections and the subsequent lipolytic response was first demonstrated in sheep by Bassett and Wallace (1966). In man, elevated growth hormone levels antagonize the effects of insulin to stimulate extrahepatic glucose disposal, primarily as a result of a post receptor defect in insulin action (Bratusch-Marrain et al., 1984). Physiological levels of growth hormone when administered to cultured sheep hepatocytes, antagonize the insulin mediated effects on lipogenesis.

There are several reports that growth hormone is active in stimulating amino acid uptake in muscle (Kostyo and Engel, 1960; Kostyo and Nutting, 1973; Clemmens et al., 1981). These studies suggested that growth hormone was more

effective than somatomedin in stimulating this process. However Liberti and Miller (1978) and Maicig et al. (1979) discovered that some fragment of GH may contain somatomedin activity. The generally accepted mediator og GH or muscle protein synthesis is by hepatic or local generations of somatomedins, or insulin-like growth factors (IGF).

Glucocorticoids. Young (1976) and Rannels et al. (1978) reported corticosteroid hormones have a catabolic effect on skeletal muscle protein. Young (1980) in his review of the relationship between glucocorticoids and growth, reported 1) low doses of glucocorticoids inhibit cell replication (liver, heart, gastocnemius muscle and the kidney), 2) is probably not related to inhibition of GH secretion, 3) inhibit somatomedin production and antagonize somatomedin effects on target cells and 4) have a direct effect by binding to cytosolic receptor proteins. Several experiments have demonstrated a negative relationship between glucocorticoids and growth in cattle (Purchas et al., 1971; Trenkle and Topel, 1978). However, Purchas et al. (1980) reported no significant relationship, while Purchas (1973) reported a positive relationship. The measurement of total cortisol is probably not the physiologically active component as compared to free cortisol, as free is in equilibrium with transcortin and albumin (Sharpe et

al.,1985). Barnett and Star (1981) observed a negative correlation between growth rate and free cortisol, while with total cortisol, there was no significant correlation.

Protein synthesis is reduced in glucocorticoid treated muscles in vivo (Rannels and Jefferson, 1980; Odedra et al., 1983). In addition, McGrath and Goldspink (1982) reported a reduction in protein synthesis when glucocorticoids were administered to muscles in vitro. Baxter (1978) reported a reduction in DNA synthesis and Rannels and Jefferson (1980) observed a decrease in RNA activity. An increase in 60S and 40S ribosomal subunits was also seen in corticosterone treated rats, indicating that glucocorticoids are interfering with initiation steps in protein synthesis.

As reported previously, TBA has been shown to decrease both synthesis and degradation rates of muscle protein while increasing growth rate of animals. Presumably, degradation is decreased to a greater extent than synthesis. Tyrosine amino transferase (TAT) is an enzyme induced by glucocorticoids (Lin and Knox, 1957). Rodway and Galbraith (1979) and Thomas and Rodway (1983a) reported a decrease in TAT in the liver of TBA treated animals. In addition, Thomas and Rodway (1983a) reported a reduction in the response of the adrenal gland to an exogenous dose of ACTH when TBA was added to the media. The diurnal rhythm of

corticosterone has a reduced magnitude in TBA treated animals. Thomas and Rodway (1983a) repeated this work in rats, with similar results. In addition, studies were carried out with isolated adrenal cells in vitro. Trenbolone acetate reduced adrenal cell production of glucocorticoids although E2 and testosterone had similar effects (Thomas and Rodway, 1983b). Sharpe et al. (1984) reported decreased total cortisol in sheep but the biologically active component, free cortisol was increased.

The binding of dexamethasone (synthetic glucocorticoid) in the cytosol fraction appears to be reduced when TBA is administered to lambs (Sharpe et al., 1984), while zeranol had no effect on dexamethasone binding. Sharpe et al. (1984) suggested that TBA may be binding to glucocorticoid receptors, making them unavailable or undetectable. However, in a specificity assay, TBA had little affinity for the glucocorticoid receptor. In contrast, Mayer et al. (1975) reported androgens displace glucocorticoids from their receptors. In addition, Sinnett-Smith et al. (1983b) and Sinnett-Smith (1987) reported TBA decreases binding capacity of androgen receptors, however TBA did not effect the E2 binding capacity (Meyer and Rapp, 1985).

Trenbolone Acetate and Estradiol In Animal Growth

Metabolism of Steroids. The metabolism of endogenous steroids has been well established in man and domestic animals. This topic has been reviewed by Vermeulin (1976) and Velle (1976). Biodegradation of steroids normally produces biologically less active metabolites. In cattle, 17-alpha epimerization seems to be the major metabolic pathway of E2 (Martin, 1966; Velle, 1976) and occurs in the liver (Hoffman, 1979).

In general, most of the steroids are eliminated with the feces, where 60-90% of the metabolites are found in the free form, while the steroids excreted from the urine are predominatly conjugated (Velle, 1976). Estradiol-17-alpha glucopyranoside is the major estrogen metabolite in cattle (Poe et al., 1978) after implantation of E2.

Studies regarding the metabolism of TBA have dealt mainly with cattle. TBA is hydrolyzed to trenbolone and both trenbolone and its metabolites are rapidly excreted as the glucuronides and sulfates, mostly in the bile (Rico and Burgat-Sacaze, 1983). Phillips and Harwood (1982) and O'Keeffe (1984) reported that TBA is hydrolyzed to 17-B-OH trenbolone which is the major metabolite in muscle and fat,

while the epimer of trenbolone, 17-alpha-OH trenbolone is the major metabolite in liver and kidney.

Trenbolone Acetate and Muscle Protein Turnover.

Normally, rapidly growing muscle is accompanied with an increased turnover of muscle protein. As maximum body weight is reached, a higher percentage of the carcass is fat, and muscle protein turnover rates tend to decline. The regulation of protein turnover with anabolic agents is not entirely understood. Protein synthesis would appear to be a primary focal point, although, synthesis and degradation have both been reported to be affected by steroid hormones (Goldberg et al., 1974).

Vernon and Buttery (1976) using the continous infusion technique and the $^{14}CO_3$ method to measure synthesis and degradation respectively, in the rat, reported TBA (80 ug/100 g body weight) reduced the FSR as well as the FDR of mixed muscle protein. Weight gain was also increased. In males, mixed muscle proteins have a faster FSR than in females (Waterlow and Stephen, 1967) presumably due to testosterone. Therfore, the observed reduction in protein turnover is not consistent with significant androgenic activity. In castrate male rats, Vernon and Buttery (1978a) reported no differnece in growth rate between TBA treated and control rats. In this study TBA caused a

significant decline in urinary N^t-methylhistidine (3MH) while there was no difference in the 3MH content of the muscle in entire female rats. A correlation between muscle protein degradation and total cathepsin D activity in the perfused rat hemicorpus has been reported (Rannels and Jefferson, 1980). Sinnett-Smith (1983a) demonstrated a reduction in total cathepsin D activity in castrate male lambs after treatment with 140 mg TBA plus 20 mg E2. Sinnett-Smith et al. (1983a) also investigated the effect of TBA in ewe lambs and reported a reduction ($P<.10$) in FSR using the constant infusion technique when compared to control lambs. However, muscle intracellular free leucine concentration was not affected be TBA suggesting that increased growth rate is brought about by other means other than protein synthesis. Garlick et al. (1973) reported whole body protein turnover was not affected by TBA and Vernon and Buttery (1978b), reported that after day 1, there was no difference in FSR from TBA and control rat skeletal muscle. Griffiths (1982) implanted twenty-four castrate male cattle with 300 mg TBA plus 36 mg resorcylic acid lactone and demonstrated that excretion of 3MH was not different. Bohorov et al (1987), reported no significant change in FSR with 52.5 mg TBA plus 7.5 mg E2 in the longissimus dorsi, vastus lateralis and vastus intermedius

of wether lambs. Lobley et al (1985) implanted steers with 140 mg TBA plus 20 mg E2 and reported a decline in whole body protein synthesis at week 5 post-implant while amino acid oxidation was lower at weeks 2 and 5 compared to control animals. There was a slight decrease in 3MH elimination after implantation and was insufficient to account for the total improvement in growth rate. Lobely et al (1985), suggested that in other experiments where TBA decreased both synthesis and degradation, there was an increase in feed intake. This increased supply of nutrients may have caused an increase in muscle mass rather than a change in degradation rates compared to synthesis of muscle protein. Ballard and Francis (1983) reported that TBA had no effect at 1, 10 and 100 nm on protein breakdown in L6 myoblasts when the incubation period was 4 or 18 hours. There are discrepancies in the literature concerning the effect TBA has on muscle protein turnover. It is evident that TBA may affect both synthesis and degradation of muscle protein, however this may not be the case in the whole body.

Estrogens and Muscle Protein Turnover. Commonly, E2 is administered with TBA so definitive reports of E2 on protein turnover at the skeletal muscle tissue level are few and obscure. Sinnett-Smith et al (1983a) implanted ewe lambs

with 12 mg zeranol, a E2-like compound. They demonstrated that skeletal muscle homogenate (intracellular) free leucine concentrations were reduced slightly ($P<.10$) and liver homogenate free leucine was not altered. Protein FSR, when calculated from muscle homogenate was not different while there was a difference in FSR when calculated from plasma free leucine. Sinnett-Smith (1983a) reported that zeranol reduced cathepsin D activity although, total cathepsin D and plasma leucine flux was not effected. Roeder and Gunn (1987) reported no difference and Roeder et al. (1986) reported a decrease in protein synthesis with zeranol and E2 in tissue cultures however, zeranol decreased protein degradation (roeder and Gunn, 1987). No difference in protein degradation in muscle cultures, has been reported with E2 or DES (Powers and Florini, 1975; Ballard and Francis, 1983; Roeder et al., 1986). Stack and Gorski (1985) reported that a single injection of 16-alpha E2 caused only a transient stimulation of overall protein synthesis as compared to a single injection of 17-B E2 in the rat uterus, suggesting that the mitogenicity of estrogen may be related to its ability to maintain a prolonged

stimulation of protein synthesis.

DNA, RNA and Protein. With increasing FSR, total RNA content of skeletal muscle usually increases (Garlick et al., 1975). However, some reports with that TBA have different conclusions. Bohorov et al. (1987), reported no difference in protein content of the longissimus dorsi, vastus lateralis and vastus intermedius with TBA-E2 implantation in wether lambs. In addition, Knudsen and Max (1980) reported no increase in protein content of the levator ani muscle under conditions in which aromitization of testosterone (or injection of E2) gave substantial induction of glucose-6-phosphate dehydrogenase. Vernon and Buttery (1978b) reported TBA (800 ug/kg body weight) treatment caused an increase in skeletal muscle RNA content (1.36 mg versus 1.13 mg), and a reduction in RNA activity (FSR/RNA:Protein), 14 d after commencement of treatment. However, Milward et al. (1973) reported that a reduction in growth rate is consistent with decreased RNA activity. Sinnett-Smith et al. (1983a) demonstrated RNA concentration decreased ($P<.05$) with only DNA tending to decrease with TBA-E2 in wether lambs. Protein content was not altered. These researchers also investigated the effects of zeranol and TBA on DNA, RNA and protein of skeletal muscle and liver tissues in ewe lambs. Although no differences were

detected between TBA and zeranol both decreased RNA amd DNA concentrations from control lambs while protein content in muscle was not altered. Zeranol increased RNA activities while TBA had no effect. This report indicated that the decrease in DNA concentration after TBA or zeranol treatment may indicate that increased growth is due to hypertrophy rather than hyperplasia. However, treatment with testosterone (similar to TBA) has been reported to stimulate RNA synthesis in rat skeletal muscle (Breuer and Florini, 1966), implying a potential increase in protein synthesis, unlike TBA treatment. The anabolic agent nandrolone phenylpropionate (19, nor-17B-hydroxy-3-keto- androstene-17-phenyl-propionate), which is also very similar in structure to testosterone, increases rat skeletal protein synthesis and, to a lesser extent, increases muscle protein degradation (Dumelow et al., 1982). It becomes apparent that not all androgenic agents act in the same manner in their influence on RNA, DNA and protein metabolism.

Gland Growth and Growth Systems for Estradiol. The weight of the anterior pituitary (AP) seems to be consistently increased when estrogens are administered (Trenkle and Burroughs, 1967; Wiggins et al., 1979) This is due primarily to an increase in cell size, although cell numbers are also increased (Martin and Lamming, 1958).

Gonadotropin levels are sometimes decreased (Shroder and Hanshard, 1958; Burgess and Lamming, 1960), while adrenocorticotrophic hormone (ACTH) and thyrotropic hormone levels are unchanged (Clegg and Cole, 1954; Shroder and Hanshard, 1958). Protein, RNA and growth hormone (GH) concentrations in the AP appear unchanged, but the total amount of these substances is increased due to the increased weight of the gland (Hinds et al., 1959; Struempler and Burroughs, 1959).

Lorenz (1954) first proposed that increased GH secretion may mediate the effects of E2. This proposal was supported by the results Gee and Preston (1957). Increased AP activity appears to follow E2 treatment as previously reported. The mechanism that has been postulated is that E2 causes a release of GH releasing factor (GHRF) from the hypothalamus, which in turn stimulates a release of GH from the AP. This however, does not explain why in cattle and sheep, E2 stimulates growth, while in other animals (rodents), E2 is sometimes noneffective or inhibitory (Preston, 1975). Wright (1961) postulated that because ruminants utilize different substrates for energy (fatty acids) than do nonruminants (glucose), that the effects seen are quite different.

Hsu and Hammond (1987), investigated the capacity of E2 to stimulate Insulin-like Growth Factor-I (IGF-I) production in porcine granulosa cells in vitro. These researchers are the first to demonstrate that E2 stimulates IGF-I (1.26 times control value) at least in granulosa cells. Shulman et al. (1987) reported low doses of E2 increased IGF-I concentration in ovariectomized female rats, although the GH secretory response to GHRF was similar to control rats, in vivo. They suggested that the rise in IGF-I levels associated with E2 may not be mediated through a change in GH secretion. Fukata and Martin (1986) reported E2 showed no apparent influence on GHRF induced GH release or any significant influences on basal or somatostatin suppressed GH release in dispersed pituitary cells. Simard et al. (1986) while investigating the effects of E2 on the secretion of GH in rat AP cells in culture reported that E2 exerts a stimulatory effect on spontaneous and GHRF induced GH release as well as cellular GH content.

The weight of the adrenal gland is less consistently increased than is true for the AP when estrogens are administered (Burgess and Lamming, 1960; Preston and Burroughs, 1960). This is probably due to to an increase in the size of the adrenal cortex (Clegg and Cole, 1954). Shroder and Hansard (1958) reported increased adrenal

cholesterol levels when E2 was administered in lambs. Wiggins et al. (1979), reported no effect of zeranol on adrenal gland weight.

The effect of E2 on thyroid size has been quite variable, although the size is often decreased (Lamming, 1960; Preston et al., 1960). Increased follicular epithelial cell height has been reported in lambs administered DES (Davey et al., 1959; Burgess and Lamming, 1960). Wiggins et al. (1979) observed decreased thyroid gland weight when lambs were implanted with zeranol, however, Wiggins et al. (1976) observed increased thyroid gland weight. Gopinath and Kitts (1984) reported decreased tetraiodothyronine (T_4) levels in plasma of cattle when zeranol was implanted. Wiggins et al. (1979), postulated that the decreased T_4 in zeranol implanted lambs, could lower the basal metabolic rate and therefore would increase the proportion of nutrients available for growth. A direct effect of thyroid hormones on muscle protein accretion has not been demonstrated. However, King and King (1976) demonstrated that thyroid hormones injected into chicks (made hypothyroid by injecting propylthiouracil) had growth promoting effects in muscle and stimulated the formation of ribosomal RNA (King and King, 1978). It is generally accepted that thyroid gland weight is decreased with E2, and that if E2 does stimulate thyroid gland production of T_4 or affect on skeletal muscle protein accretion.

T_3, then these hormones, normally do not have a direct affect on skeletal muscle protein accretion.

Steroid Receptors

The concept that steroid hormone action is mediated by interaction with specific target tissue receptor proteins has become generally well accepted. The pioneering studies of Glascock and Hoekstra (1959) demonstrated the selective accumulation of tritium-labeled hexestrol by the reproductive organs of female goats and sheep. This initial study on selective E2 retention by known target tissues led to the proposal that steroid interaction with specfic macromolecular receptors mediates the hormonal response of target tissues (Glasser and Clark, 1975; Roberts et al., 1976).

Structure. When steroid depleted cells or tissues are homogenized, most of the steroid receptors are recovered in the cytosol fraction, usually in a 8S (250-300 kilo Daltons) form. This 8 S form consists of one ligand binding unit plus two 90 kDa non-hormone binding subunits. One of these non-hormone binding domains is a DNA binding unit. King (1986 a,b) reported that these characteristics are more convincing for the glucocorticoid and progesterone receptor, although the presence of the same 90 kDa protein in higher

molecular weight forms of E2 and androgen receptors (Renoir et al., 1986), suggests a common structure for all these receptor complexes.

Monospecific, polyclonal and monoclonal antibodies against E2 and glucocorticoid receptors have been developed. These have been used to indirectly identify their associated mRNA's and complementary DNA's (cDNA), which subsequently have detected the appropriate genomic sequences (King 1986a,b). The amino acid sequences have been predicted for glucocorticoid and E2 receptors (Danielson et al., 1986).

One of the two non-hormone binding subunits is a DNA binding domain. The steroid binding domain is at the carboxy terminal end of the protein while the DNA binding region being nearer to the amino terminus end. The second non-hormone binding subunit appears to be of different sizes for E2 and glucocorticoid receptors while the hormone and DNA binding domain are of similar size. The second non-hormone binding domain is of ill defined function (Carlstedt-Duke et al., 1982). The steroid binding domain is hydrophobic compared to the DNA subunit which is hydrophillic. Weinberger et al. (1986) reported that the DNA binding unit has loops, which they termed "Zinc Fingers" which attach to the major groove of chromatin DNA. These fingers may be generated by coordination of zinc with four

cysteine residues within each finger (Green and Chambion, 1987). Green and Chambion (1987) placed DNA coding for receptor proteins into lymphoma cells that are glucocorticoid resistant. They then replaced the DNA binding domain of the E2 receptor with the same domain of the glucocorticoid. The hybrid E2 receptor, upon binding to E2 behaved as a glucocorticoid. They suggested that the DNA binding domain determines specificity of function.

Models of Cytosol/Nuclear Receptor Distribution

Two Step Model. The hormone induced change in molecular form (8S to 5S) of the steroid receptor and its apparent cytosol-to-nuclear translocation led to the following hypothesis of a two step mechanism of steroid interaction within the cell proposed by Gorski et al. (1968) and Jensen et al. (1968): 1) The hormone associates spontaneously with an extranuclear receptor to form a 8S complex. 2) The 8S complex transfers the hormone to the nucleus by a temperature dependent process which consumes the 8S receptor. 3) Formation of the characteristic 5S complex in the nucleus requires the presence of the supernatant (cytosol) fraction to contain a steroid in the form of a 8S complex. These researchers suggested that the 8S complex

may be transformed into the nuclear 5S complex by cleavage of the 8S receptor molecule.

Equilibrium Model. This model developed by Williams and Gorski (1972) considers that the cytoplasmic and nuclear receptor forms exist in a rapid reversible equilibrium. Upon hormone occupancy, the interaction with components of the nucleus becomes thermodynamically favored. This concept was supported by Williams and Gorski (1972) in which they incubated receptors with a wide range of receptor saturation. They observed that the ratio of nuclear to cytoplasmic occupied receptors was constant at all saturation levels. A similar conclusion by Sheriden et al. (1979) involved the equilibrium of unbound nuclear receptors with unbound cytosolic receptors except that the distribution is defined by a distribution coefficient which reflects their partitioning as a function of the water volumes of each compartment.

Affinity Model. This model suggests that the equilibrium between cytosolic and nuclear receptors is a function of the concentration of the receptors, their relative affinity for nuclear components and compartment volumes (Walters et al., 1981). Assuming that each receptor form has its own molecular properties, then this model treats each receptor species as well as their form

(unoccupied, occupied transformed) as distinct molecules. Relating a receptor species and its affinity for nuclear components as well as the total volume of the system to the distribution between soluable and nuclear complexed forms is an important part of this model. In association with this concept, unoccupied hormone receptors appear to have low affinity for nuclear components while occupied or transformed receptors do not readily solubilize from the nucleus. Therefore, this model treats these forms separately. This model also provides an explanation for the apparent decrease in affinity of nuclear receptors for nuclear components with increasing ionic buffer strengths (KCl).

Nuclear Model. This model assumes that the hydrophobic steroid molecules diffuse through the outer cell membrane and cytoplasm to the nucleus. In the nucleus the steroids bind to their respective receptors which are assumed to be bound with low affinity to some nuclear component (Sakai and Gorski, 1984). This nuclear component could be DNA, nuclear matrix or some chromatin protein (acceptor). As a result of the steroid-receptor complex formation, the receptor conformation changes and its affinity for nuclear components becomes much higher (Hansen and Gorski, 1985). In most cells, ligand free receptors, which are weakly associated

with nuclear components are washed out of the nucleus by homogenizing cells in hypotonic buffers and sediment as 4S complexes on high salt sucrose gradients (Jensen and DeSombre, 1973). The ability to solubilize receptors in low ionic strength buffers suggests a weak association with the nuclear components (Gorski, 1987). Gasc et al. (1984) reported immunocytochemical studies indicate the receptor is dispersed widely in the nucleus. There is no localization in the nucleolus although less appears in denser heterochromatin than in less dense enchromatin. Chromatin may be associated with a fibrillar ne work called the nuclear matrix or scaffold (Barrack and Coffey, 1982). Steroid receptors have been shown to be associated with the nuclear matrix after extracting over 90% of the DNA. Steroid receptor complexes also bind to the nuclear matrix in cell-free experiments (Barrack and Coffey, 1982).

Hormone Induced Differences in Cytosol/Nuclear Receptors

To determine if hormones induce changes in receptor numbers, the distinction between occupied and unoccupied receptors must be clear. If a hormone treatment such as E2 or TBA decreases or increases a particular receptor than the receptor may be bound by the particular hormone therefore, the exchange assay developed by Anderson et al. (1972a) will distinguish between occupied and unoccupied receptors.

Commonly, the direct binding assay with dextran coated charcoal (DCC) (Snochowski et al., 1980, 1981) can only determine receptor numbers that are unbound.

Most studies have described receptor compartmentalization phenomena after hormone injections (Mueller et al., 1972; Jensen and DeSombre, 1973). No information concerning receptor compartmentalization in skeletal muscle has been published therefore, work in uterine tissue will be discussed.

Stack and Gorski (1985) reported that a single injection of 16-alpha E2 (16AE2) causes a slight to no change in protein synthesis as compared to 17-B E2. Multiple injections of 16AE2 were needed to overcome a deficiency in protein synthesis 12 h post injection as does 1 injection of 17-B E2 12 h later. In addition, Stack and Gorski (1985) reported there are differing fates of receptors when bound to 16AE2 and 17-B E2. Receptors bound by 16AE2 disappear more rapidly from the nuclear form and appear just as rapidly in the cytosolic fraction. Receptors activated by 17-BE2 are retained in the nucleus 2 to 4 times longer. Kassis and Gorski (1981) confirmed these observations. Anderson et al. (1972b) reported that a small pool of E2 receptors that are retained in the nucleus for 6 h is a prerequisite for uterine growth. Giannopoulos and Gorski

(1971) reported that for E2 receptors in uterine organ culture, nuclear translocation was stoichiometrically related to the loss of receptors in the nucleus. Cidlowski and Muldoon (1974) observed an increase in E2 receptor numbers in the cytosol after E2 injection. Milgrom et al. (1970) reported E2 increases rat uterine receptor levels for progesterone and Panko et al. (1981) observed an increase in glucocorticoid receptors upon E2 administration. However, receptor numbers or occupancy does not always parallel growth. McEwen et al. (1982) reported a stimulation of E2 receptors in the hypothalamus and pituitary when E2 was absent. Tissue differences in E2 induction of E2 receptors was observed by Clark et al. (1982). Walters (1985) suggests that receptor regulation is a property of gene expression in the target tissue, rather than an inherent property of the receptor species and/or regulation of its expression.

Dependence on the type of assay used to estimate receptor numbers, is of utmost importance in defining the mechanism of action of growth promotion in animals. Decreased receptor numbers may mean that more of the receptors are bound and can not be detected by direct assay. However, Juliano and Stancell (1976) reported that the decrease in receptor numbers, 6 h after a single injection

of E2, was the same whether receptors were assayed byexchange methods after an injection of unlabeled hormone or by direct binding assay after injection of labeled hormone.

CHAPTER III

Materials and Methods

Design of Experiment. A 2 x 2 factorial design was employed in a 42 d experiment to study the mechanism of action of trenbolone acetate and estradiol. Data were obtained on 24 wether lambs of Coopworth x Dorset, Barbado x Dorset and Finn x Dorset breeding. Animals were grouped according to weight and breed and randomly assigned the following treatment combinations:

1). with or without 12 mg estradiol-17B via compudose implant.

2). with or without 60 mg trenbolone acetate implant.

Feeding, Weighing and Managerial Procedures. Each animal was accustomed to a rope halter and individually fed ad libitum twice daily, a complete diet of corn, soybean meal and ground grass hay. Unconsumed feed was removed, weighed and recorded for adjustment of total feed intake. Feed records were maintained daily. Grab samples for each new batch of feed were obtained during the trial and analyzed for protein (AOAC, 1980) dry matter, acid detergent

fiber and neutral detergent fiber (Goering and Van Soest, 1970) and phosphorous (P) (Fiske and Subbarrow, 1926). Calcium (Ca), potassium (K) and magnesium (Mg) contents were determined by wet ashing procedures using perchloric and nitric acid digestion and analysis by Perkin-Elmer 370 atomic absorption spectrophotomter. Each animal was weighed weekly on a portable scales. All animals were housed on expanded metal floors which was divided into two pens (.75 m^2/hd). Fresh water was provided at all times. The temperature of the room was maintained at 22 C with constant light (24 h).

Collection and Preparation of Samples

Slaughter procedures were conducted on 5 consecutive d at the end of the trial. Four animals were slaughtered each d for the first 3 d followed by six animals per d, the next 2 d. On a particular slaughter d, all animals within a weight group were killed. An animal from each treatment was killed on a particular d. On the fourth and fifth d of slaughter, four animals from one weight group and the next two heaviest animals from the remaining weight group were killed.

Blood Serum. Blood samples were collected weekly from all animals and at the end of the trial via jugular puncture. Samples were collected in a vacutainer test tube and allowed to stand at room temperature for 1 h and then for 6 h at 4 C. Serum filters were placed in each tube and serum was harvested by centrifugation at 2000 x g for 15 min. The serum was transferred by disposable pasteur pipettes into clean labeled, 7 ml scintillation vials and stored at -20 C until cortisol hormone assay was performed.

Semitendinosus and Triceps Brachii Muscles. Immediately after exsanguination of the animal, the pelt was removed. Alligator clamps (2.5 cm) connected by a stiff wire, were put in place (6 on the semitendinosus (ST) and 6 on the triceps brachii (TB) muscle) to provide continual tension on a portion (250 to 350 mg) of the ST and TB and to allow sampling for in vitro synthesis and degradation of muscle protein. The ST and TB muscles were selected because they are easily accessible in a short period of time (< 5 min) and provide a representative sample from the hind and front quarter, respectively. Immediately after muscle biopsies were obtained, each muscle was excised from the carcass, trimmed free of any adhering fat and weighed to the nearest .01 g. Muscles were immediately place in a polybag, labeled and placed in liquid nitrogen (N_2). Muscles were

transferred to a freezer and kept at -80 C until time of preparation.

To assure homogeneity, the muscles were pulverized with a hammer, transferred to a Bel-Art tissue mill and ground to a powder at -20 C, in a walk-in freezer. Dry ice was added to the grinding head to insure against heat resulting from the friction of grinding. Ground muscles were then transferred to a clean polybag and returned to the -80 C freezer until time of assay.

Glands and Organs. The head of the animal was severed at the atlas joint. With a hand saw, the skull cap was removed. Located under base of the brain, the pituitary was carefully removed with a scapel and forceps by severing the connective tissue surrounding the hypophysis. The whole pituitary gland was weighed to the nearest .01 g, placed in a polybag and frozen in liquid N_2 then transferred to -80 C freezer for storage.

At the base of the neck, just below and in front of the trachea, the thyroid gland was located and removed with a scapel and forceps. Care was taken to separate the parathyroid glands from the thyroid gland. Thyroid was weighed to the nearest .01 g, placed in a polybag and frozen in liquid N_2 then transferred to -80 C freezer for storage. Both adrenal glands were removed with a scapel and forceps

and weighed to the nearest .01 g. Adrenal glands were placed in a polybag, frozen in liquid N_2 then transferred to -80 C freezer for storage.

In addition, the liver and heart were collected. The heart was removed and weighed. The entire liver was also weighed and subsamples were taken and placed immediately in a polybag, frozen in liquid N_2 and stored at -80 C.

Laboratory Analysis

In Vitro Synthesis and Degradation of Muscle Protein.

The in vitro synthesis and degradation of muscle protein in the ST and TB muscles were estimated by the method of Fulks et al. (1975) as modified by Hentges et al. (1983). Upon removal from the carcass, the muscle samples (connected to the clamps) were immediately placed in a clean, 20 ml scintillation vial containing a preincubation media which consisted of oxygenated (95% O_2 : 5% CO_2), cold Krebs Ringer Buffer (KRB; table C.1), pH 7.4 (Umbreit et al., 1951), to slow post mortem changes. This preincubation media containing the muscle biopsy, was incubated at 37 C for 30 min which contained five times the highest levels of amino acids found in sheep plasma (table C.4) (Bergen, 1979; Wolfrom and Asplund, 1979; Moore et al., 1980; Young et al.,

1981), .3 mM chloramphenicol, 1.0 mM glucose, .5 IU/ml insulin, .29 mM sodium propionate and 1.3 mM sodium acetate (table C.3; Harmon et al., 1985). After the preincubation, the muscle samples (connected to clamp) were placed into their respective incubation media and kept at 37 C for 2.5 h for synthesis estimation and 3 h for degradation. Muscle was incubated in a shaker bath with 50 oscillations/min. The synthesis buffer contained the same ingredients as the preincubation buffer except, .3 uCi/ml ^{14}C-tyrosine (table C.3) was added. The synthesis media was oxygenated just before starting the incubation. Degradation incubation buffer contained the same ingredients as the preincubation buffer except that L-tyrosine was omitted and .5 mM cyclohexamide (table C.3) was added to block synthesis. The interstitial space of the muscles was estimated as described in a previous experiment by the addition of .03 uCi/ml ^{14}C inulin (table C.3) (Gore, 1985). Muscle samples used to determine blank values and determination of the amount of unlabeled tyrosine in the buffer, were homogenized immediately after the preincubation period and prepared for assay.

After completion of the incubations, all of the muscle samples were removed from their incubation vials, blotted dry, weighed and placed in a test tube containing an ice

cold mixture of 2.0 ml phosphate buffer and .5 ml 50% trichloroacetic acid (TCA). Muscles were then homogenized using a Tekmar Tissuemizer (Tekmar Products, Cincinnati, Ohio) in three, 5-10 sec bursts at high speed. The tip of the homogenizer was washed with 1.0 ml of 10% TCA with the washings being allowed to run into the tube with the homogenized tissue. Care was taken to assure that no muscle collected on the tip of the homogenizer. The homogenate was centrifuged at 2300 x g at 4 C for 15 min. The supernatant was decanted into a clean, 7 ml scintillation vial. The precipitate was then washed twice with the same volume of cold 10% TCA. The supernatant and the two washes were combined and stored at -80 C. The precipitate was resuspended in 1.0 ml of diethyl ether and centrifuged at 2300 X g at 4 C for 10 min. The supernatant was discarded and the pellet was allowed to dry at room temperature for 12 to 18 h.

Also at the end of the incubations, 2.0 ml of the incubation media were mixed with .5 ml of cold 50% TCA, vortexed to precipitate proteins and centrifuged at 2300 x g at 4 C for 15 min. The supernatant solution was decanted and stored in a clean, 7 ml scintillation vial at -80 C until time of assay for tyrosine.

Muscle protein synthesis was estimated by measuring the

incorporation of ^{14}C-tyrosine into muscle protein and interstitial space was estimated by incubating muscle in the presence of ^{14}C inulin. The synthesis and inulin pellets were prepared for liquid scintillation counting by digestion in 1.0 ml of Solulene 100 tissue solubilizer (Packard Instrument Co.) for 12 h at 50 C. One ml aliquots of solubilized tissue was counted in 10 ml of scintillation solution comprised of toluene containing 4 g of a 98:2 PPO/bis-MSB mixture/liter. Before counting, .15 ml of distilled water and .05 ml of glacial acetic acid were added to each scintillation vial and mixed with the scintillation fluid. For the acid soluable supernates from the synthesis and inulin samples, 1.0 ml of the supernate was combined with 10 ml of scintillation solution that contained toluene-triton X-100 (2:1) mixed with 6 g of a 98:2 PPO/bis-MSB mixture/liter. The samples were counted in a Beckman LS 7500 scintillation counter with quench corrections made by H-number.

Intracellular specific activity (the quantity of amino acids available for synthesis into protein) was calculated by subtracting the amount of tyrosine in the inulin space (interstitial) from the disintegrations per min (DPM) detected in the supernatant (washes) of the muscle pellet. This quantity was then divided by the blank (determined

flurometrically (Ambrose, 1974) as the amount of unlabeled tyrosine intracellular, before synthesis was measured) minus the amount of tyrosine in the inulin space. Inulin space was calculated by adding the inulin DPM detected in the solubilized tissue to the inulin DPM in the supernatant (combined washes of the muscle pellet) and dividing that figure by the amount of inulin DPM/ml of media after incubation. The actual synthesis rate of muscle protein was calculated by dividing the DPM of the solubilized tissue by the intracellular specific activity. This expression is the actual amount of muscle protein synthesis as a function of the ^{14}C-tyrosine available for synthesis. Mathematical formulas for the calculation of synthesis rates and inulin space may be seen in figure A.4 and A.5.

The degradation of muscle protein was estimated by measuring the release of tyrosine from the muscle tissue into the incubation media. Tyrosine was determined by the method of Ambrose (1974). Reagent blanks were prepared by placing .5 ml of .06 M TCA into clean screw cap culture tubes in triplicate. A .5 ml aliquot of sample was also pipetted into clean screw cap culture tubes. The standard curve was constructed by putting .1 ml to .5 ml of L-tyrosine working standard into a series of clean screw cap culture tubes and adjusting the volume to .5 ml with .06 M

TCA. After the blanks, standards and samples were dispensed into culture tubes, 1.0 ml of nitric acid reagent (table C.9) was added, the tubes capped, vortexed and then heated in a 85 C water bath for exactly 6 min then cooled in a 33 C water bath for 10 min. After cooling, the tubes were removed from the water bath and 5 ml of ethanol was added to each tube. The tubes were then inverted to mix the contents and returned to the 33 C water bath for 30 min. At the completion of incubation, the samples were removed from the water bath and read in a Turner Model 111 Fluormeter equipped with a constant temperature door at 33 C (G. K. Turner Associates, Palo Alto, Ca). The fluorometer setting was a IX aperature with a blue lamp, T-5 envelope (Turner No. 110-853) and a primary filter combination of 2A and 47B to result in a 436 nm activation wavelength. A No. 16 secondary filter (535 nm emission wavelength) in combination with a 10% neutral density filter was used on the secondary side. Before reading, all cuvets were wiped with laboratory tissue to remove any lint or dust particles that could contribute fluorescence. The fluorometer was zeroed with a black dummy cuvet. The readings were then taken with the average blank value being subtracted from each sample and standard reading. Degradation was calculated by adding the tyrosine content of the supernates (washings) of the muscle

pellet to the tyrosine content of the media (nmoles/mg muscle) and subtracting from that the tyrosine content of the supernate (washings) of the muscle pellet blank.

Verification Procedure for In Vitro Protein Turnover. An in vitro muscle protein synthesis and degradation procedure was conducted according to Fulks et al. (1975) as modified by Hentges et al. (1983). This procedure was the same as the previous procedure in this manuscript except that synthesis and degradation estimates were determined at different periods of incubation. The addition of either 1) 1.0 mM glucose, 2) 1.3 mM sodium acetate plus .29 mM sodium propionate without glucose or 3) a combination of 1.0 mM glucose 1.3 mM sodium acetate and .29 mM sodium propionate was added to the incubation media. Ninty muscle biopsies were taken from the left and right ST muscle of one animal. Incubation periods for the estimation of synthesis were as follows: .5, 1.5, 2.5, 3.5 and 4.5 h after the initial start of the incubation, after preincubation. Incubation periods for the estimation of degradation were as follows: 1.0, 2.0, 3.0, 4.0 and 5.0 h after the start of incubation. Estimates of the interstitial spaces in each muscle biopsy were taken from the previous procedure.

Preparation of Muscle Cytosol for Receptor Analysis. The preparation of cytosol for E2 and glucocorticoid

receptors was exactly the same. The cytosol/nuclear receptor binding assays were performed according to Snochowski et al. (1980, 1981). Fresh ground muscle was used in the receptor assays. Two to three g of ground muscle were placed in a clean, 50 ml centrifuge tube. To this, 14 ml of ice cold homogenization buffer was added. Homogenization buffer contained 5.0 mM Tris-HCl, pH 7.4 (23 C), 1 mM ethlyenediaminetetracetic acid (disodium) (EDTA), .1 mM dithioerythritol (freshly added) and 10% (v/v) glycerol (TEDG). TEDG buffer was stored in 2-liter plastic bottles until used. Samples were homogenized thoroughly with a Tekmar Tissumizer for periods of 5 s intervened by 30-s intervals of cooling. The centrifuge tube containing the muscle was kept in an ice bath (200 ml beaker) while the sample was being homogenized. Care was taken to assure that no muscle collected on the homogenizer tip. The homogenate was centrifuged at 1000 x g for 10 min at 4 C. The supernatant was then transferred (avoiding the lipid layer) to a clean 25 ml polyethylene centrifuge tube and centrifuged at 105,000 x g for 30 min at 4 C.

Preparation of Nuclear Extract. The pellet from the previous centrifugation (1000 x g) was used for the extraction of nuclear receptors. The pellet was washed twice with 12 ml of ice cold TEDG buffer and centrifuged

after each wash at 1000 x g for 5 min at 4 C. The pellet was extracted at 4 C using 14 ml of TEDG buffer containing .6 M KCl (Miller et al., 1985) and resuspended every 5 min for exactly 1 h at 4 C. Nuclear extract was centrifuged at 1000 x g for 10 min at 4 C. The supernatant was pipetted (avoiding the lipid layer) into a clean 25 ml polyethylene centrifuge tube and centrifuged at 105,000 X g for 30 min at 4 C.

Preparation of Total (Cytosolic and Nuclear) Receptors. Two to three g of ground muscle were homogenized directly into 14 ml ice cold TEDG buffer containing .6 M KCl. The homogenate was resuspended every 5 min for 1 h at 4 C. The homogenate was centrifuged at 1000 x g for 10 min at 4C. The supernatant was pipetted into a clean polyethylene centrifuge tube and centrifuged at 105,000 x g for 30 min at 4 C.

Binding Kinetics. The association of [6,7-^{3}H] estradiol-17B and [1,2,4 ^{3}H] dexamethasone to their respective receptors in the cytosol and nuclear fractions were determined. Aliquots of .2 ml cytosol or nuclear extract were incubated with .1 ml TEDG buffer solution of ^{3}H labeled ligand at a final concentration of 6.4 nM, either in the presence or absence of a 100-fold excess of unlabeled DES or dexamethasone for E2 and glucocorticoid receptors,

respectively. Incubations were carried out in duplicate at 0 to 4 C for varying periods of time (0, 1, 2, 4, 8, 16, 20 and 24 h) until terminated by the addition of .4 ml of dextran coated charcoal (DCC). The suspension was vortexed, incubated at 0 to 4 C for 20 min, followed by centrifugation at 1500 x g for 5 min at 4 C. Total amount of radioactivity was determined from duplicate samples, but in which the cytosol or nuclear extract and charcoal suspension was replaced with .6 ml TEDG buffer. Following centrifugation, aliquots of .5 ml of the supernatant were combined with 5.0 ml of scintillation fluid in clean, 7 ml scintillation vials. Radioactivity was determined via a Beckman Model J2-2 liquid scintillation counter. All vials were wiped with fabric softener to eliminate any counts that could arise from static electricity. Quenching was corrected by the sample channels ratios method.

Ligand Specificity. The specificities of [6,7 ^{3}H] estradiol-17B and [1,2,4 ^{3}H] dexamethasone binding sites were studied. One hundered ul of an ethanolic solution of each ligand competitor at 10 and 100 times the concentration of labeled ligand was added to a series of 12 x 75 mm borosilicate tubes. The ethanol was evaporated to dryness. An aliquot of .2 ml cytosol or nuclear extract was added to each tube. Also added to each tube was .1 ml TEDG buffer

solution of ^{3}H ligand at a final concentration of 6.4 nM. Incubations were carried out in duplicate at 4 C for 20 h. Ligand competitors were cortisol, corticosterone, estradiol-17B, testosterone, triamcinolone, progesterone, dexamethasone, diethylstilbesterol (DES) trenbolone acetate and trenbolone-17 alpha. Nonspecific binding was determined from incubations containing the competitor plus a 100-fold excess of the non-radioactive ligand. Unlabeled dexamethasone and DES were used to determine nonspecific binding for glucocorticoid and estradiol receptors, respectively. Termination of binding via DCC and radioactive counting was performed as described in the previous section. The total binding in the absence of the competing ligands was determined from incubations of .2 ml cytosol or nuclear extract and .1 ml of ^{3}H labeled ligand.

Receptor Quantitation. Aliquots of .2 ml cytosol or nuclear extract were placed in 12 x 75 mm borosilicate tubes containing .1 ml of TEDG buffer solution of [1,2,4 ^{3}H] dexamethasone or [6,7 ^{3}H] estradiol 17B at six final concentrations (.2, .4, .8, 1.6, 3.2 and 6.4 nM), either in the presence or absence of a 100-fold molar excess of unlabeled dexamethasone or DES. Incubations were carried out in duplicate at 4 C for 20 h until terminated with DCC. The radioactive counting techniques were described

previously in the binding kinetics section.

Cytosol and Nuclear Extract Protein Determination. The protein content was determined by the dye binding method of Bradford (1976). The assay involved the binding of Coomassie Brilliant Blue G-250 to protein to form a protein-dye complex.

Protein standards were prepared in triplicate using bovine serum albumin solution containing 10 to 100 ug of protein. Standards were made up in TEDG buffer. An aliquot of .1 ml of cytosol or nuclear extract was pipetted into 16 x 100 mm borosilicate tubes (in triplicate). Five ml of the commassie blue reagent was added to the tubes and vortexed. Absorbance of the standards, samples and blank tubes was determined at 595 nm on a Perkin Elmer Lambda 3-B Ultraviolet/Visable Spectrophotometer. The spectrophotometer was zeroed against blank solutions containing .1 ml TEDG buffer and 5 ml coomassie protein reagent.

Muscle Protein Determination. Protein was determined by the method of Bradford (1976) with modification. Digestion procedure was a modification of Munro and Fleck (1966). Exactly 1 g of powdered muscle was placed in a 50 ml Erlenmyer flask. Fifteen ml of 6 N NaOH were added to each flask. Standard preparation consisted of placing 1 g of

desiccated bovine serum albumin in a 50 ml Erlenmyer flask. Fifteen ml of 6 N NaOH were added to the flask. Marbles were placed on top of each flask and the flasks incubated at 70 C for 4 to 5 h or until all tissue was completely solubilized. Aliquots of 10 ul of digested sample were placed in 25 x 75 mm test tubes in triplicate. Ten ug to 200 ug of standard were placed in 16 X 100 mm test tubes in triplicate. Blanks consisted of .2 ml of 6 N NaOH in 5 ml of coomassie blue dye. All tubes were adjusted to .2 ml with 6 N NaOH. Coomassie blue dye was added to each sample and standard and vortexed. Absorbance was read on a Perkin Elmer Lambda 3-B Ultraviolet/Visable Spectrophotometer with the wavelength set at 595 nm.

Muscle Nucleic Acid Extraction. A modification of the method of Munro and Fleck (1966) was utilized to determine RNA and DNA of the ST and TB muscles.

Five g of frozen, powdered muscle was weighed into a clean, 100 ml beaker. Twenty-five ml of ice cold distilled, deionized water was added for a dilution factor of 1:5 (w/v). Tissue was homogenized using a Tekmar Tissuemizer for three 10-s bursts at high speed followed by 1-min cooling periods on ice between homogenizations. Homogenate was filtered through eight layers of cheesecloth into a clean, 20 ml scintillation vial. Cheesecloth was squeezed

and any additional filtrate was collected in the vial. Vials were capped and stored at -80 C until future assay.

Two, 2 ml aliquots of muscle homogenate were added to 16 X 100 mm test tubes and taken through extraction procedure. One ml of cold 1 N perchloric acid (PCA) was added to each tube. Each tube was allowed to incubate for 30 min while stirring intermittently with a small glass rod. Tubes were centrifuged for 15 min at 1400 x g. Supernatant was carefully pipetted with a clean pasteur pipet and discarded. The pellet was washed with 2.0 ml of ice cold .25 N PCA. Tubes were allowed to incubate for 30 min while stirring intermittently with a small glass rod. Tubes were centrifuged at 1400 x g for 15 min. Supernatant was carefully transferred and discarded. Two ml of .5 N PCA was added to each tube and the pellet was resuspended using a small glass rod. Tubes were incubated in 70 C water bath for 20 min while stirring intermittently. Tubes were then cooled in an ice bath for 10 min and centrifuged at 1400 x g for 15 min. Using a clean pasteur pipet, supernatant was carefully transferred into a clean, 7 ml scintillation vial. Pellet was resuspended in 2.0 ml of .5 N PCA. Tubes were incubated in 70 C water bath for 20 min while stirring intermittently. Tubes were cooled for 10 min and centrifuged at 2500 x g for 15 min. Using a clean pasteur

pipet, supernatant was carefully transferred to the same scintillation vial (supernates combined). Vials were capped and frozen at - 20 C for future RNA/DNA analysis.

Nucleic Acid Determination. Diphenylamine and acetaldehyde were utilized (Burton, 1956, 1968) to colorimetrically determine DNA concentration. The standard curve was constructed with .1 to 1.0 ml of working standard DNA solution. The source of DNA was calf thymus DNA. In duplicate, .25 ml of each unknown sample was pipetted into clean, 16 x 100 mm test tubes (four tubes per original tissue). The final volume of samples and standards was adjusted to 1.0 ml with .5 N PCA including at least three blanks of .5 N PCA. In the exhaust hood, 2.0 ml of diphenylamine reagent (including acetaldehyde) were pipetted into each test tube. Tubes were vortexed, covered with foil and incubated at 30 C for 16-20 h. Absorbance was determined on all tubes using a Perkin Elmer Lambda 3-B utaviolet/visable spectrophotometer at 600 nm.

RNA concentration was determined by a colorimetric procedure utilizing orcinol (Ceriotti, 1955). The standard curve was constructed with 4, 8, 16, 24, 80, 100, 200, 300, 400 and 600 ul of standard calf liver RNA solution. In duplicate, .25 ml of each unknown sample were pipetted into clean 16 x 100 test tubes (four tubes per original tissue).

The final volume of the standards and samples was adjusted to 1.0 ml with .5 N PCA including at least three blanks of .5 N PCA. In the exhaust hood, 2.0 ml of orcinol reagent were pipetted into each tube. Tubes were vortexed, capped with marbles and incubated in a boiling water bath for 30 min. All tubes were cooled in a water bath to room temperature. Absorbance was determined on all tubes using a Perkin Elmer Lambda 3-B ultraviolet/visable spectrophotometer at 670 nm.

Cortisol Radioimmunoassay. An Amerlex Cortisol Radioimmunoassay (Amersham Corp) Kit was utilized to determine cortisol concentration in serum. Five hundered ul of distilled water were added to each standard and allowed to dissolve at room temperature for 10 min. Standard concentrations were 0 to 60.9 ug/100 ml. Fifty ul of each standard and sample were pipetted into 12 x 75 mm borosilicate test tubes in duplicate. Two hundered ul ^{125}I Cortisol were pipetted into each tube. Following this, .2 ml of cortisol antibody suspension (sheep) was pipetted into each tube. All tubes were vortexed, covered with plastic and incubated in water bath at 37 C for 1 h. All tubes were centrifuged at 1500 x g for 15 min. Tubes were placed in decantation racks and the supernatnat was decanted. Tubes were then placed on a pad of absorbent

tissue and allowed to drain for 2-3 h. Radioactivity was determined in a Beckman gamma counter.

Statistical Analysis. Data were analyzed by least squares analysis of variance using the Statistical Analysis System (SAS, 1979) General Linear Model Procedures. Sources of variation were treatment, muscle, weight of the experimental units (animals), breed of the animals and all two-factor interactions.

Calculations of binding data from Scatchard plots (Scatchard, 1949) were conducted via the 1984 version of the Equilibrium Binding Data Analysis program (EBDA; McPherson, 1983).

Assay Variations. Cortisol analysis indicated an intra-assay coefficient of variation (CV) was 3.23%, with the r value equal of .93. All sample were analyzed with one standard curve so inter-assay coefficient of variation was not applicable.

Total muscle protein analysis indicated an intra-assay CV of 2.35. One standard curve was utilized for this assay also. The r value for the standard curve was .938.

Cytosolic and nuclear protein analysis indicated an intra-assay CV of 2.09 and an inter-assay CV of 2.43. The r value was .974.

CHAPTER IV

JOURNAL ARTICLE

MUSCLE PROTEIN TURNOVER AND TISSUE GROWTH IN SHEEP IMPLANTED WITH ESTRADIOL-17 BETA AND TRENBOLONE ACETATE

ABSTRACT

A study was conducted to determine the effects of trenbolone acetate (TBA) and estradiol-17B (E2) implantation on tissue growth and skeletal muscle protein synthesis and degradation. Twenty-four male castrate lambs (Barbados x Dorset, Coopworth x Dorset and Finn x Dorset) were grouped by weight, blocked by breed and alloted to 4 treatment combinations in a 2 x 2 factorial design. Treatments were: 1) with or without 60 mg TBA implant and 2) with or without 12 mg E2 implant. Lambs were killed at an average of 42 d following implantation. The semitendinosus (ST) and triceps brachii (TB) muscles were exposed and muscle biopsies were taken to measure in vitro protein synthesis and degradation. Muscle biopsy samples were incubated with [^{14}C] tyrosine for the estimation of synthesis rates. Degradation was estimated by the release of tyrosine in the presence of

cyclohexamide. Organ and gland weights were recorded. Implantation of TBA did not affect (P>.10) degradation rates, the production of cortisol or adrenal weights. Synthesis rate of muscle protein was decreased (P<.01) with TBA in the TB muscle and not affected (P>.10) in the ST. Estradiol did not affect synthesis or degradation rates of muscle protein. Deoxyribonucleic acid concentration of the TB was increased (P<.05) with TBA treatment. Ribonucleic acid (RNA) concentrations did not differ (P>.10) between treatments as well as muscles and protein:RNA ratio was lower (P<.05) in the ST muscle after TBA implantation. The TB muscle had greater (P<.05) synthesis rates than the ST muscle, and protein:RNA ratio was decreased (P<.05) in the TB muscle compared to the ST. Trenbolone acetate decreased (P<.005) pituitary weights while E2 increased (P<.01) pituitary weights.

KEY WORDS: Trenbolone Acetate, Estradiol, Protein Turnover, Muscles.

Introduction

Estrogens (E2) and androgens are used extensively to increase the productivity of animals. Increases in protein accretion and efficiency have been achieved in steers and wether sheep, presumably the result of the difference in synthesis and degradation of skeletal muscle protein.

Trenbolone acetate (TBA), an analogue of testosterone has been reported to stimulate muscle protein accretion. Various modes of action have been proposed. Vernon and Buttery (1976) and Sinnett-Smith et al. (1983) speculated that protein gain with TBA implantation results from either decreasing degradation or stmulating muscle protein synthesis. While other reports suggest TBA does little to modulate protein degradation either in vivo (Hayden et al., 1987) or in L6 myoblast cultures (Ballard and Francis, 1983).

Alternatively, trenbolone acetate may modulate protein metabolism by decreasing plasma glucocorticoid levels and adrenal production of corticosteroids, as observed in rats (Thomas and Rodway, 1983) and lambs (Singh et al., 1983). Young (1976) and Rannels and Jefferson (1980) reported corticosteroid hormones promote

catabolism of skeletal muscle protein. In addition, a reduction in muscle protein synthesis occurs when glucocorticoids are administered (McGrath and Goldspink, 1982).

Zondek and Marx (1939) were the first to observe anabolic effects in poultry following E2 injections. Estrogens administered in the forms of diethylstilbesterol, zeranol (an estrogen-like compound) or hexestrol also stimulate gain in lambs (Preston, 1975). In addition, Hayden et al. (1987) reported a tendency for E2 to stimulate muscle protein synthesis although Sinnett-Smith (1983) observed no increase in synthesis rates with zeranol administration. Results range from no alteration in muscle protein degradation in L6 myoblasts (Ballard and Francis, 1983; Roeder et al., 1986) to a reduced muscle cathepsin D activity in castrate lambs (Sinnett-Smith, 1983) when E2 compounds are implanted to improve growth rates.

In light of the variablity in findings from studies using either TBA or E2, this study was conducted to attempt to clarify some responses that TBA and E2 have on tissue growth, and synthesis and degradation of skeletal muscle protein in castrate lambs. Our goal was to provide some insight into the mechanism(s) whereby TBA

and E2 contribute to the improvement in efficiency of growth of lambs.

Materials and Methods

Twenty-four male castrate lambs of Barbado x Dorset, Coopworth x Dorset and Finn x Dorset were divided into weight groups, blocked by breed and alloted to four treatment combinations in a 2 x 2 factorial design. Treatments were as follows: 1). with or without 60 mg TBA and 2) with or without 12 mg E2 implant. All lambs were housed on expanded metal floors (.75 m^2/head) and individually fed a complete ration of concentrate and grass hay formulated to contain 16% crude protein and 80% TDN (table 1). Animals were weighed and blood samples collected (jugular puncture) weekly. Serum fractions were prepared by centrifugation at 2500 x g for 20 min and stored at -20 C until analysis. Lambs were killed at an average of 42 d following implantation. Muscle biopsys were taken from the semitendinosus (ST) and triceps brachii (TB) muscle and immediately placed in oxygenated (95% O_2-5% CO_2), ice-cold Krebs-Ringer bicarbonate, pH 7.4 (Umbreit et al., 1951), containing five times the highest level of amino acids found in

TABLE 1. COMPOSITION OF DIET[a]

Item	
Diet Ingredients, % as fed	
Corn, fan cracked	60.0
Soybean Meal, 44%	22.0
Orchardgrass Hay	9.4
Molasses	6.0
Mineral-premix[b]	2.0
Limestone	1.1
Selenium/Vitamin E premix[c]	.25
Chemical Composition	
Dry Matter, %	88.0
Crude Protein, %	16.3
Acid Detergent Fiber, %	9.0
Neutral Detergent Fiber, %	17.4
TDN, estimated, %	82.0
Phosphorus, %	.61
Calcium, %	.94
Magnesium, %	.22
Potassium, %	1.3

[a]All items on a dry matter basis except dry matter.
[b]Mixture contained a minimum of .2% Fe, .2% Mn, .35% Zn, .3% Cu, .07% I_2 and .25% NaCl.
[c]Premix contained approximately 16.5 mg/kg Se and 3630 IU/kg vitamin E.

sheep plasma (Bergen, 1979; Wolfrom and Asplund, 1979) and physiological levels of acetate and propionate (Harmon et al., 1985).

In vitro rates of protein syntheis and degradation were determined according to the method of Fulks et al. (1975) as modified by Hentges et al. (1983). Total tyrosine content of the incubation medium and muscle homogenates used for protein degradation determination, was assayed flurometrically (Ambrose, 1974), using a Turner fluorometer, Model 111, equipped with a constant temperature door. Synthesis of muscle protein was calculated as described by Fulks et al. (1975). The method of Hentges et al. (1983) was utilized to calculate degradation of muscle protein. Inulin space (.25 ul/mg of muscle) was determined, using the method of Fulks et al. (1975), to correct for any tyrosine in the interstitial space.

Following removal of the muscle biopsies, the ST and TB were removed from the carcass, weighed to the nearest .01 g, frozen in liquid nitrogen (N_2) and stored at -80 C. The muscles were ground to a fine powder using a Bel-Art tissue mill (Pequannock, NJ) at -20 C then restored at -80 C until analysis. Whole pituitaries, thyroid, liver, heart, spleen and both adrenal glands were excised

and weighed to the nearest .01 g. Muscle nucleic acid (RNA and DNA) extractions were performed as described by Munro and Fleck (1969), with modifications. Tissue was homogenized in ice cold distilled water using a Tekmar Tissuemizer for three, 10 s bursts at high speed followed by 1-min cooling periods on ice between homogenizations. Homogenates were filtered through eight layers of cheesecloth. Determinations of DNA concentrations were measured as described by Burton (1956) and RNA concentration was determined utilizing orcinol (Ceriotti, 1955). Muscle protein was determined by the method of Bradford (1976) with modification. One g of powdered muscle or 1 g bovine serum albumin was added to 15 ml of 6 N NaOH and incubated for 4 to 5 h at 70 C. After the addition of 5 ml coomassie blue reagent and .01 ml of sample, absorbance was read at 595 nm. Serum cortisol concentrations were quantitatively measured via an ^{125}I competitive single antibody radioimmunoassay (sensitivity to .1 ug/dl). Intraassay coefficient of variation was 3.2 percent. Data were analyzed by least-squares analysis of variance using the Statistical Analysis System (SAS, 1979) General Linear Model procedures. Sources of variation were treatment, block, muscle and all two-factor interactions.

Results and Discussion

The rate of gain, feed efficiency and feed intake data are shown in table 2. Estradiol and TBA did not increase overall growth rate. Final body weight was slightly increased with TBA but not statistically significant. Feed intake was increased ($P<.01$) by E2, while TBA did not change feed intake. Feed efficiency was not different with either treatment. A possible explanation for the apparent lack of response in measured performance criteria may lie in the handling and housing of the animals. Animals housed in confinement often times have reduced gain as well as intake. Typically, feed efficiency is very good for confinement raised animals, which is in fact, what was observed in our study. Animals fed similar types of diets, yet not under complete confinement exhibit feed efficiency values ranging from 5.0 - 8.0 kg dm/kg gain (McCarthy et al., 1987; Walls et al., 1988). Additionally, animals in our study were handled and restrained daily in order that individual feeding could be accomplished. This additional handling and the confinement arrangement could in part explain why weight gain was unaffected by

TABLE 2. GAIN, FEED EFFICIENCY AND FEED INTAKE OF LAMBS IMPLANTED WITH TRENBOLONE ACETATE AND ESTRADIOL[a]

Item	Trenbolone Acetate +	Trenbolone Acetate -	Estradiol +	Estradiol -	SE[b]
Initial weight, kg	25.3	24.5	25.1	24.7	.76
Final weight, kg	38.0	35.6	36.5	37.1	4.4
Weight gain, kg/d	.30	.31	.32	.29	.02
Intake, kg dm/d	.95	1.00	1.02[c]	.92[d]	.03[e]
Efficiency, kg dm/kg gain	3.60	3.68	3.73	3.62	.41

[a]Each value is a least square mean.
[b]Standard error of mean.
[cd]Values for estradiol in the same row differ (P<.01).
[e]Estradiol x weight interaction (P<.01).

treatment. Display of biochemical and physiological changes due to treatment may simply have been masked by environmental effects. Variability in the response of animals to the treatment may also result from different feeding periods, dosage of steroid or weight of animals at the time of administration of treatments. Galbraith and Geraghty (1982) and Peters et al. (1984) reported increases in gain when steers were fed for 3 to 4 mo following implantation of TBA plus hexestrol. However, Vernon and Buttery (1978), Coelho et al. (1981) and Yasin and Galbraith (1981) reported no increases in weight gain in castrate lambs previously implanted with TBA and/or estradiol.

The synthesis of muscle protein (table 3) in the TB muscle when TBA was implanted was decreased ($P<.01$) with no difference in the ST. Decreased in vivo synthesis rates have been reported in mixed muscles of lambs (Sinnett-Smith, 1983), and rats (Vernon and Buttery, 1976), following TBA administration. Our results indicate that this may be the situation in the TB muscle when TBA was implanted; however, a generalized response may not be observed across the entire muscle mass. Estradiol did not alter synthesis rates in either the ST and TB. Muscle did show variation in metabolism as the

TABLE 3. INVITRO SYNTHESIS AND DEGRADATION RATES OF MUSCLE PROTEIN AND COMPOSITION OF SEMITENDINOSUS AND TRICEPS BRACHII MUSCLES FROM TRENBOLONE ACETATE AND ESTRADIOL TREATED LAMBS[a]

Item	Muscle	Trenbolone Acetate +	Trenbolone Acetate -	Estradiol +	Estradiol -	SE[b]
Synthesis[cde]	ST	6.7	6.9	6.6	7.0	.76
	TB	9.4[f]	12.4[g]	10.9	10.9	.60
Degradation[c]	ST	46.7	43.3	46.7	43.3	6.7
	TB	50.0	46.7	53.3	40.0	10.0
Protein, mg/g	ST	214.8	229.9	221.9	222.8	11.2
wet tissue	TB	225.6	217.2	214.6	228.8	9.41
DNA, mg/g	ST	.13	.13	.14[h]	.12[i]	.009
wet tissue[dj]	TB	.14[f]	.10[g]	.12	.12	.007
RNA, mg/g	ST	.76	.60	.71	.64	.12
wet tissue	TB	.60	.51	.59	.53	.05
Protein:DNA	ST	1703.0	1840.0	1637.0[h]	1905.0[i]	123.7
	TB	1658.0	2080.0	1829.0	1909.0	160.4
Protein:RNA[de]	ST	310.2[k]	614.3[l]	425.6	498.9	94.5
	TB	383.8	444.2	379.1	448.8	53.75
RNA:DNA	ST	6.1	4.6	5.0	5.6	1.1
	TB	4.4	4.9	4.9	4.5	1.7

[a]Each value is a least square mean.
[b]Standard error of mean.
[c]Pmol·mg wet tissue^{-1}·h^{-1}.
[d]Muscles differ (P<.05).
[e]Trenbolone acetate x muscle interaction (P<.05).
[f,g]Values for each hormone in the same row differ (P<.01).
[h,i]Values for each hormone in the same row differ (P<.20).
[j]Trenbolone acetate and estradiol x muscle interaction (P<.05).
[k,l]Values for each hormone in the same row differ (P<.05).

TB muscle had greater ($P<.05$) muscle protein synthesis rates than the ST muscle. The TB muscle has less fast twitch, B type fibers, than does the ST muscle, which has a greater content of A type (intermediate) fibers in cattle (Suzuki et al., 1976). The slow twitch soleus is known to have a greater synthetic activity than the fast twitch extensor digitorum longus (EDL) muscle (Li and Goldberg, 1976; Skjaerlund et al., 1988). Red portions of the ST muscle also have elevated protein synthesis when compared to strips excised from the white portion of the same muscle (Mulvaney et al., 1983). Age may be a factor in altering rates of protein synthesis in that as the animal matures, protein synthesis declines (Young, 1974) as well as the activity of muscle microsomes and ribosomes (Srivastava, 1969).

No differences were noted in degradation of muscle protein with steroid treatment or muscle type (table 3). This concurs with Griffiths (1982) and Hayden et al. (1987) who reported no difference in degradation rates in cattle treated with TBA. Rats appear to respond differently to TBA since a reduction in urinary N-^{t} methylhistidine has been reported (Vernon and Buttery 1978a), as well as a reduced degradation rate (Vernon and Buttery, 1976). Our results indicate a 3.8- to 5.0-fold

greater degradation rate than synthesis rate across all treatment and muscles. However, protein degradation rates are increased and protein synthesis rates decrease when using an in vitro method compared to in vivo determinations (Goldspink et al., 1983). The reduced acute sensitivity of the in vitro method may not allow users to measure small differences. With muscle mass contributing the major portion of the empty body weight, minute changes in the balance of synthesis and degradation can affect accumulation of muscle proteins. Thus, TBA may be improving growth rate in lambs by modulating protein degradation; however, the in vitro muscle strip technique used in this study was unable to determine a difference.

Protein, RNA, DNA concentrations and ratios are shown in table 3. Protein concentration did not differ between muscles or treatments. Deoxyribonucleic acid concentration was greater ($P<.05$) in the TB muscle of TBA treated lambs and was greater ($P<.20$) in the ST muscle of E2 treated lambs (table 3). Presumably, postnatal growth of muscle occurs primarily by cell hypertrophy, however Di Marco et al. (1987) suggested postnatal growth in cattle may involve both hyperplasia and hypertrophy of muscle cells. Increased DNA content in muscle may also

result from non-muscle nuclei contribution (Enesco and Puddy, 1964) as well as satellite cell contributions. Campion et al. (1981) reported that the percentage of satellite cells in pig muscle decreased with age while myonuclei increased. Protein:DNA ratio was slightly ($P<.20$) lower in the ST muscle with E2 which implies increased protein synthesis (Laurent et al., 1978). However, no difference was observed with TBA treatment or between muscle types.

Ribonucleic acid concentrations did not differ between treatment or muscle types, although the protein:RNA ratio was lower ($P<.05$)in the ST muscle after TBA implantation (table 3). Protein:RNA ratios were consistently lower ($P<.05$) in the TB muscle when compared to ST. Milward and Waterlow (1978) reported that the protein:RNA ratio is inversely related with synthesis rates. Data from the present study would support this explanation since TB had the greater synthesis rate ($P<.05$) and a lower protein:RNA ratio ($P<.05$). It appears that the method to measure synthesis used in this study may not be sensitive enough to distinguish minute changes in rates. Although synthesis rates were not increased by treatment, the protein:RNA ratio was decreased with TBA in ST which implies that potential

changes in synthesis did exist.

Vernon and Buttery (1978b) reported an increase in RNA after TBA treatment and Cheek (1968) demonstrated that RNA concentration (RNA/protein) increases with age in rats. Although our results (table 3) demonstrate a slight RNA concentration increase with treatment, it was not statistically significant. Concentration of RNA may be reflecting the ribosome number and can be utilized as an index of the intensity of protein synthesis (Goldberg, 1967). Treatment with testosterone (similar to TBA) stimulated RNA synthesis in rat skeletal muscle (Breuer and Florini, 1966), implying increased protein synthesis, unlike TBA. Previous reports have indicated an additive effect of TBA plus E2 on growth rates in lambs (Grandadam et al., 1975b; Singh et al., 1983; Sulieman et al., 1986) We observed no TBA x E2 interactions for synthesis and degradation rates, protein:RNA ratio or protein:DNA ratio for ST and TB. A higher RNA:DNA ratio may indicate increased activity of DNA in RNA synthesis. Waterlow et al. (1978) reported RNA:DNA ratio is increased with higher synthesis rates of muscle protein. Data from the present study indicated no increase in RNA:DNA ratio, indicating no potential increase in synthesis of muscle proteins (table 3).

Trenbolone acetate and E2 may act by altering the production or release of endogenous hormones. Protein synthesis is reduced in glucocorticoid treated muscles in vivo (Rannels and Jefferson,1980) and in vitro (McGrath and Goldspink, 1982). Thomas and Rodway (1983c) reported TBA and E2 reduced adrenal cell production of glucocorticoids. While reduced total cortisol levels have been reported with TBA treatment (Thomas and Rodway, 1983a; Sharpe et al., 1984), and realizing the limitation of our sampling regime for cortisol, we observed peripheral cortisol levels to be only slightly depressed by TBA and slightly elevated by E2 (statistically non significant) (table 4). This observation was consistant throughout the sampling period. Adrenal weights (table 4) were also decreased slightly by TBA, suggesting that alteration of glucocorticoid production could possibly be due to tissue atrophy.

Pituitary gland weights (table 4) were increased ($P<.01$) with E2 but decreased with TBA ($P<.005$). The weight of the pituitary gland has been reported to increase with E2 administration (Trenkle and Burroughs, 1967; Wiggins et al., 1979). The theory that E2 has a direct effect on muscle growth can not be ruled out since E2 cytoplasmic receptors have been identified in skeletal

TABLE 4. TISSUE WEIGHTS AND SERUM CORTISOL LEVELS OF LAMBS IMPLANTED WITH TRENBOLONE ACETATE AND ESTRADIOL[a]

Item	Trenbolone Acetate +	Trenbolone Acetate -	Estradiol +	Estradiol -	SE[b]
Semitendinosus, g/kg BW[c]	2.64[d]	2.85[e]	2.77	2.72	.06
Triceps Brachii, g/kg BW	4.71	4.72	4.61	4.82	.16
Thyroid, g/kg BW	.99	1.3	1.4	.91	.48
Adrenal, mg/kg BW	92.0	101.0	94.0	98.9	9.6
Pituitary, mg/kg BW	15.3[f]	22.6[g]	21.7[h]	16.2[i]	1.3
Liver, g/kg BW	22.3	22.4	22.4	22.3	1.4
Heart, g/kg BW	4.51	4.78	4.31[h]	4.98[i]	.15
Spleen, g/kg BW	1.66	1.69	1.60	1.75	.11
Cortisol, ng/ml	3.55	4.39	4.56	3.38	.80

[a]Each value is a least square mean.
[b]Standard error of mean.
[c]Trenbolone acetate x estradiol interaction (P<.05).
[d,e]Values for each hormone in the same row differ (P<.05).
[f,g]Values for each hormone in the same row differ (P<.005).
[h,i]Values for each hormone in the same row differ (P<.01).

muscle (Meyer and Rapp, 1985). However, an alternative mode of action for growth enhancement by E2 involves the stimulated release of growth hormone releasing factors (GHRF) from the hypothalamus which in turn stimulates release of growth hormone (GH) from the pituitary. However, the opposite situation may occur with TBA treatment since pituitary weights were decreased ($P<.005$), an observation also noted by Galbraith et al. (1986). Trenbolone acetate may be affecting the size of the pituitary by acting as a negative feedback since it is known that concentrations of FSH and LH increase upon castration (Schanbacher, 1980).

Thyroid gland weights (table 4) were not different between steroid treatments although, E2 increased the mean weight by 35%. Wiggins et al. (1979) observed decreased thyroid gland weight and Donaldson et al. (1981) and Gopinath and Kitts (1984) reported decreased tetraiodothyronine (T_4) levels in plasma of animals implanted with zeranol, an estrogen-like compound. However, TBA plus E2 did not change triiodothyronine (T_3) and T_4 levels in plasma of wether lambs (Gopinath and Kitts, 1984).

Muscle weights are shown in table 4. The ST weight was decreased ($p<.05$) while the TB was not affected by TBA treatment. A lower weight of the ST with TBA indicated that the pattern of growth of the ST did not parallel body weight gains. Kochakian and Tillotson (1957) demonstrated that the muscle weights in the shoulder region of guinea pig are more sensitive to testosterone than are other muscles in the hind leg, suggesting that the ST muscle did not respond to endocrine therapy like the TB muscle. Estradiol did not change the weights of ST and TB but did decrease heart weights ($P<.01$). As might be predicted, the weight of the liver and spleen were unchanged by treatment (table 8). Other reports concur with this finding in steers (Heitzman et al., 1981), in wether lambs (Yasin and Galbraith, 1981; Coelho et al., 1981) and in rats (Vernon and Buttery, 1978).

Results from this study indicate that TBA decreased the protein:RNA ratio in the TB muscle implying that the capability for protein synthesis may be increased. Trenbolone acetate increased DNA concentration in TB muscle suggesting that it may be involved in modulating growth by satellite cell recruitment or hyperplasia of muscle cells, although the cause and effect relationship

has not been established. Neither treatment changed the measured degradation rate of skeletal muscle protein. In addition, we observed only a slight decrease in cortisol production as well as adrenal gland weight with TBA. Trenbolone acetate may be increasing growth by decreasing protein degradation. Androgenic hormones inhibit the binding of glucocoticoids to receptor sites (Mayer and Rosen, 1975; Waggoner et al., 1988) and this may be a mechanism by which TBA decreases protein degradation. Estradiol implantation increased pituitary gland weights which suggests that E2 may have an effect on pituitary hormone (GH) production or release. The mode of action of E2 in skeletal muscle probably has many facets. Estradiol may increase growth in animals by influencing GH release. Although E2 receptors have been identified in skeletal muscle, the effect of E2 addition to L6 myotube and myoblast cultures, is nonconclusive. The TB muscle had greater synthesis capabilities and a decreased protein:RNA ratio than did the ST muscle. This supports the suggestion that the TB muscle is more responsive to endocrine manipulation as first mentioned by Kochakian and Tillotson (1957).

CHAPTER V

JOURNAL ARTICLE

CHARACTERIZATION OF ESTROGEN AND GLUCOCORTICOID RECEPTORS IN SKELETAL MUSCLE OF LAMBS TREATED WITH ESTRADIOL-17 BETA AND TRENBOLONE ACETATE

ABSTRACT

A study was conducted to determine the effects of trenbolone acetate (TBA) and estradiol-17B (E2) implantation on the characteristics of the glucocorticoid and E2 receptor in skeletal muscle of sheep. Twenty-four male castrate lambs (Barbados x Dorset, Coopworth x Dorset and Finn x Dorset) were randomly divided into weight groups, blocked by breed and alloted to four treatment combinations in a 2 x 2 factorial design. Treatments were as follows: 1) with and without 60 mg TBA implant and 2) with and without 12 mg E2 implant. Lambs were killed at an average of 42 d following implantation. Trenbolone acetate or E2 did not affect cytosolic glucocorticoid binding capacity (Bmax) in the semitendinosis (ST) or triceps brachii (TB) muscles. Trenbolone acetate decreased ($P<.05$) the percent of [^{3}H]

dexamethasone binding in the nuclear fraction only in the TB muscle. The number of cytosolic E2 receptors and the Kd were decreased (P<.05) in the TB muscle by TBA. The cytosolic E2 binding capacity was decreased (P<.05) with E2 treatment in the ST muscle while E2 decreased (P<.05) the nuclear E2 receptor binding capacity in the TB muscle. The TB muscle had greater (P<.005) cytosolic glucocorticoid receptors compared to the ST muscle. All glucocorticoid hormones competed with [^{3}H] dexamethasone for cytosolic glucocorticoid receptor sites. Estradiol-17B competed for both cytosolic and nuclear E2 receptors and for cytosolic glucocorticoid receptors. The androgens, progesterone and diethylstilbesterol all showed some cross reactivity with [3H] dexamethasone binding to cytosolic glucocorticoid receptors. The androgens and progesterone had less affinity for the nuclear E2 receptor than the cytosolic E2 receptor.

KEY WORDS: Trenbolone Acetate, Estradiol Receptors, Glucocorticoid Receptors, Cytosol, Nuclear, Sheep.

Introduction

Effects of androgens (Snochowski et al., 1980) and estrogens (Meyer and Rapp, 1985) in skeletal muscle probably are mediated by cytosolic or nuclear receptors. These receptors upon binding to steroids, associate with specific DNA sequences, trigger transcription and hence the production of protein.

A model of steroid hormone receptor activation in the cytosol of the cell and the translocation to the nucleus was proposed by Jensen et al. (1968). However, reports using cell enucleation (Welshons et al., 1984) and immunocytochemical techniques (King and Greene, 1984) have suggested the cytosolic estrogen receptors are extraction artifacts. Using enucleated pituitary tumor derived cells, Welshons et al. (1985) reported that unoccupied receptors for glucocorticoids, estrogens and progesterone are nuclear. Recently, however, Wikstrom et al. (1987) suggested a strict localization of glucocorticoid receptors in the nucleus does not occur.

One theory of the action of anabolic steroids on protein metabolism in skeletal muscle suggests that decreased glucocorticoid binding to specific receptors reduces the catabolism of muscle protein (Mayer and

Rosen, 1975). In support, Singer et al. (1973) demonstrated that testosterone effectively competes with cortisol for binding sites in hepatic tissue. Implantation of trenbolone acetate, an analogue of testosterone, was also shown to decrease the number of cytosolic cortisol receptors in sheep skeletal muscle (Sharpe et al., 1984). However, trenbolone did not displace cortisol from its cytosolic receptor in vitro (Sharpe et al., 1984).

Additional knowledge of the mechanisms by which anabolic agents influence protein accretion would provide a basis for further developments in growth manipulation of meat animals. The objective of this study was to determine the effects of TBA and estradiol 17-B on binding capacity and affinity of the glucocorticoid and estrogen receptor in cytosolic and nuclear preparations of ovine skeletal muscle. A furthur objective was to compare these properties in fast and slow twitch types of skeletal muscle.

Materials and Methods

Twenty-four male castrate lambs (Barbados x Dorset, Coopworth x Dorset and Finn x Dorset) were randomly divided into weight groups, blocked by breed and alloted to four treatment combinations in a 2 x 2 factorial design. Treatment combinations were the following: 1) with or without implantation of 60 mg TBA and 2) with or without implantation of 12 mg estradiol-17B. All lambs were housed on expanded metal floors (.75 m^2/hd) and individually fed a complete diet containing corn, soybean meal and hay with proper mineral and vitamin supplementation (16% crude protein and 80% TDN; dry matter basis).

Lambs were killed at an average of 42 d following implantation. The semitendinosis (ST) and triceps brachii (TB) muscles were excised from the carcass, immediately frozen in liquid nitrogen and stored at -80 C. The muscles were subsequently ground to a fine powder at -20 C using a Bel-Art tissue mill (Pequannock, NJ) and returned to storage at -80 C until analysis (one to two mo) for glucocorticoid and E2 receptors.

Preparation of Cytosol and Nuclear Extract. The receptor binding assays were performed according to Snochowski et al. (1980, 1981) with minor modifications. Powdered muscle (2-3 g) was weighed and homogenized thoroughly in buffer (1:7, w/v) in an ice bath with a Tekmar Tissuemizer (Cinncinattii, OH) for periods (1 to 3) of 5 s intervened by 30-s intervals of cooling. Buffer was 5.0 mM Tris-HCl, pH 7.4 (23 C), 1 mM ethylenediaminetetracetic acid (EDTA), .1 mM dithioerythritol (added fresh daily) and 10% (v/v) glycerol (TEDG buffer). The homogenate was centrifuged at 1000 x g for 10 min at 4 C. The supernatant was withdrawn from beneath the lipid layer with a pasteur pipet and transferred to a centrifuge tube. To prepare a nuclear extract, the pellet was washed twice with 12 ml of TEDG buffer and centrifuged after each wash at 1000 x g for 5 min at 4 C. The washed pellet was then extracted at 4 C by suspending in 14 ml of TEDG buffer containing .6 M KCl (Miller et al., 1985). The sample was resuspended every 5 min during the 1 h extraction. The mixture was then centrifuged at 1000 x g for 10 min. The supernatant was pipetted (avoiding the lipid layer) into a clean centrifuge tube. The original supernatant and nuclear extracts were then centrifuged at 105,000 x g for 30 min

at 4 C. The supernatants from the cytosolic and nuclear fractions were subsequently used for receptor analysis, binding kinetics, ligand specificity testing and protein determinations.

Receptor Quantitation. Aliquots of .2 ml cytosol or nuclear extract were combined with .1 ml of TEDG buffer solution containing [1,2,4 ^{3}H] dexamethasone or [6,7 ^{3}H] estradiol 17-B at on of six final concentrations (.2, .4, .8, 1.6, 3.2 and 6.4 nM), either in the presence or absence of a 100-fold molar excess of unlabeled ligand, for glucocorticoid and estrogen receptor, respectively. Dexamethasone and diethylstilbesterol (DES) were utilized for the estimation of nonspecific binding for glucocorticoid and estrogen receptor experiments, respectively. Incubations were carried out in duplicate at 4 C for 20 h until terminated by the addition of .4 ml of charcoal suspension [.625% (w/v) of charcoal and .0625% (w/v) dextran in TEDG buffer (DCC)]. The suspension was vortexed, incubated at 4 C for 20 min and centrifuged at 1500 x g for 5 min at 4 C. Following centrifugation, aliquots of .5 ml of the supernatant were combined with 5.0 ml of scintillation fluid and radioactivity determined by liquid scintillation counting. Quenching was corrected for by the sample

channels ratio method. Specific binding was calculated from the difference in radioactivity between the incubations carried out in the absence and presence of unlabeled ligand. Total amount of radioactivity was determined from duplicate samples, in which the cytosol or nuclear extract and charcoal suspension were replaced with .6 ml TEDG buffer.

Binding Kinetics. Aliquots of .2 ml cytosol or nuclear extract were incubated with .1 ml TEDG buffer solution of [^{3}H] labeled ligand at a final concentration of 6.4 nM at 4 C, either in the presence or absence of a 100-fold molar excess of unlabeled dexamethasone or DES for glucocorticoid and estrogen receptors, respectively. Duplicate incubations were terminated after 1, 2, 4, 8, 16, 20 or 24 h by the addition of .4 ml DCC. Specific binding calculations and radioactive counting techniques were conducted as described previously.

Ligand Specificity. Aliquots of .1 ml of an ethanolic solution of each ligand competitor at 10 or 100 times the concentration of [^{3}H] labeled ligand were added to a series of 12 x 75 mm borosilcate tubes. The ethanol was evaporated to dryness and portions of .2 ml cytosol or nuclear extract were added to each tube. A .1 ml volume of TEDG buffer containing [1,2,4 ^{3}H] or [6,7 ^{3}H]

estradiol 17-B at a final concentration of 6.4 nM was added to duplicate tubes, and incubated for 20 h at 4 C. The total binding in the absence of the competing ligands was determined from incubations of .2 ml cytosol or nuclear extract and .1 ml of [^{3}H] labeled ligand. Termination of binding via DCC, radioactive counting and specfic binding calculations were performed as described.

Estimations of Protein and DNA. Protein in the cytosolic and nuclear fractions were measured according to Bradford (1976). Muscle DNA was extracted according to Munro and Fleck (1966) and colorimetrically determined according to Burton (1956).

Statistical Analysis. Data were analyzed by least squares analysis of variance using the Statistical Analysis System (SAS, 1979) General Linear Model procedures. Sources of variation were treatment, block and all two-factor interactions. Each muscle was analyzed independently.

Calculation of binding data from Scatchard plots (Scatchard, 1949) was carried out via the 1984 version of the Equilibrium Binding Data Analysis program (EBDA, McPherson, 1983). The program was obtained from the Biomedical Computing Technology Information Center, Vanderbilt Medical Center, Department of Radiology,

Nashville, TN.

Results

Figure 1. illustrates the time course of specific binding of [^{3}H] dexamethasone and [^{3}H] estradiol-17B to skeletal muscle. Maximal specific binding appeared to occur by approximately 8 h for both steroids and fractions. Nuclear glucocorticoid kinetics are not shown due to the extremely low binding capacity.

Figure 2. represents the concentration dependence of [^{3}H] dexamethasone and [^{3}H] estradiol-17B binding in skeletal muscle cytosol and [^{3}H] estradiol-17B binding in skeletal muscle nuclear extract. Nonspecific binding, considered as a high capacity, low affinity type binder, produced linear plots while total (nonspecific plus specific) binding had plots characteristic of a low capacity, high affinity type binder (Snochowski et al., 1980). These data were used for Scatchard analysis and production of the plots shown in figure 3. The Scatchard plots werelinear, indicative of a single class of binding sites for each ligand and fraction.

The apparent number of cytosolic [^{3}H] dexamethasone binding sites (Bmax) in the ST and TB muscle was not influenced by treatment with trenbolone acetate or

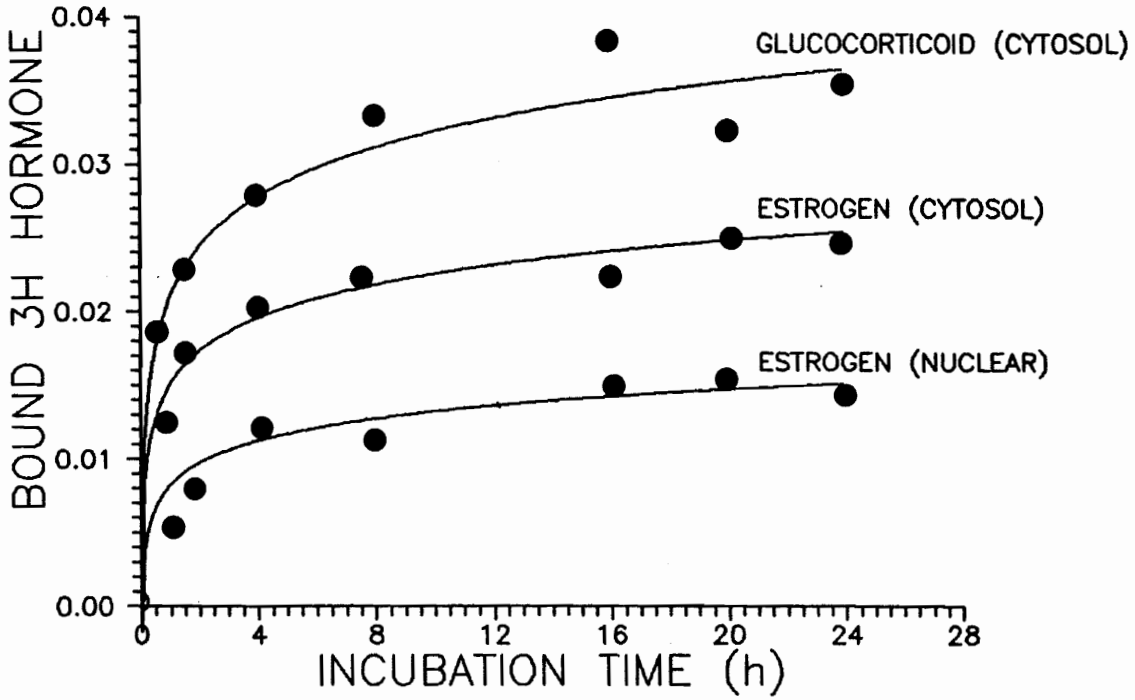

FIGURE 1. TIME COURSE OF SPECIFIC 1,2,4 3H DEXAMETHASONE AND 6,7 3H ESTRADIOL -17B BINDING TO SKELETAL MUSCLE CYTOSOL AND NUCLEAR EXTRACT. POINTS REPRESENT BOUND HORMONE AND ARE MEANS OF 8 DETERMINATIONS.

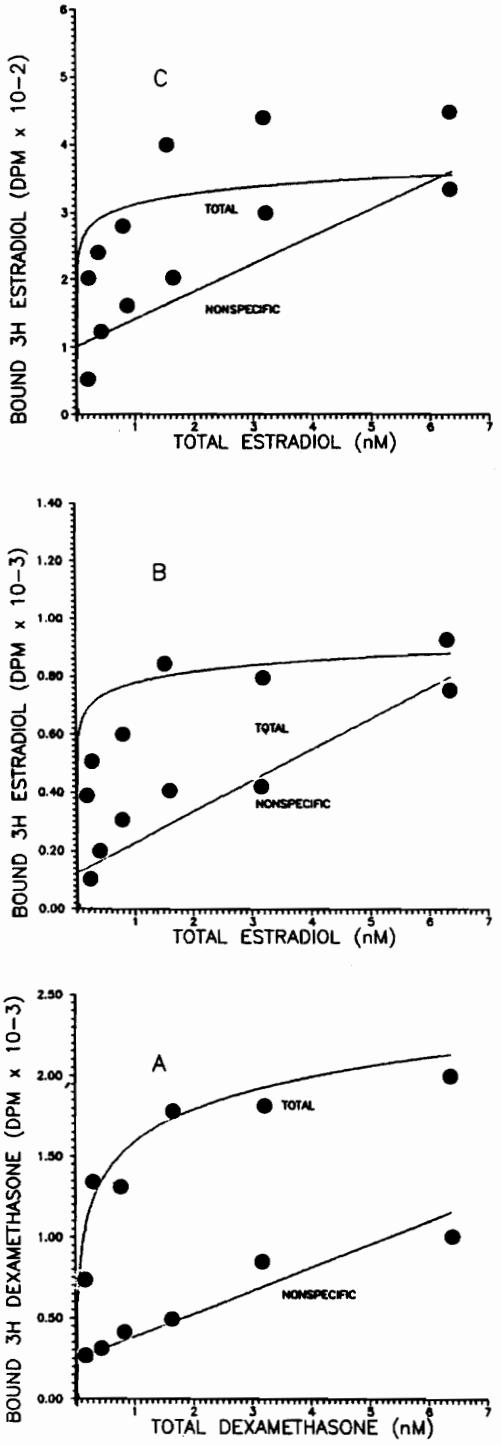

FIGURE 2. LIGAND CONCENTRATION DEPENDENCE OF CYTOSOLIC 1,2,4 3H DEXAMETHASONE (A) AND CYTOSOLIC (B) AND NUCLEAR (C) 6,7 3H ESTRADIOL-17B BINDING

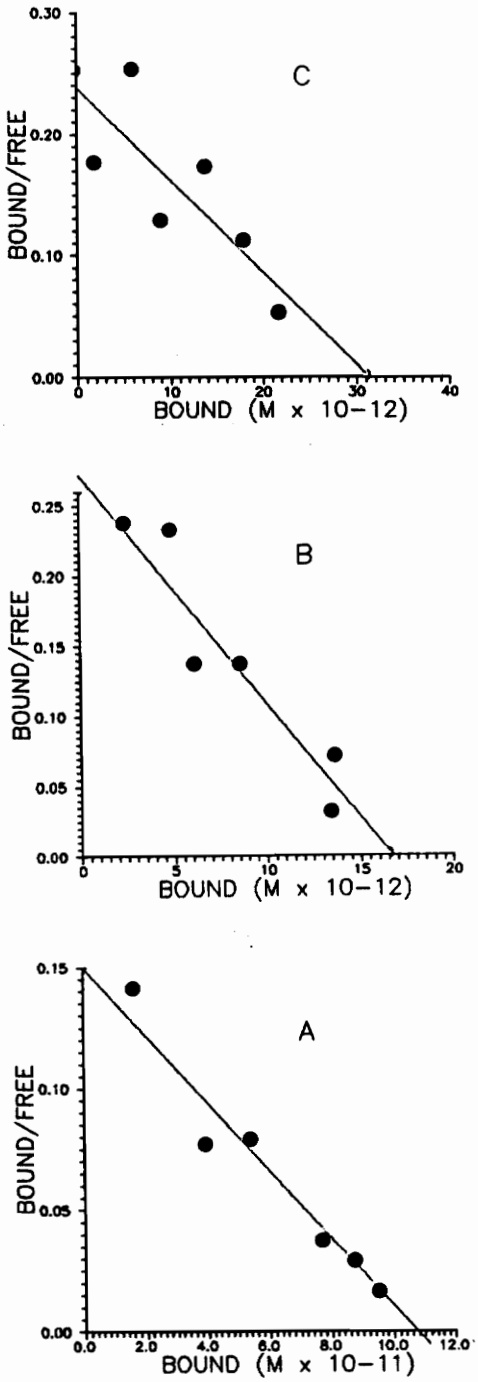

FIGURE 3. SCATCHARD PLOTS OF SPECIFICALLY BOUND 1,2,4 3H DEXAMETHASONE TO CYTOSOL (A) AND 6,7 3H ESTRADIOL-17B TO CYTOSOL (B) AND NUCLEAR EXTRACT (C).

estradiol (table 5). However, the TB muscle had more binding sites than the ST muscle when expressed on a tissue weight ($P<.005$), a tissue DNA ($P<.005$) or a cytosolic protein ($P<.05$) basis. The apparent equilibrium dissociation constant, Kd, was not altered by anabolic hormone treatments, however, in the TB muscle [^{3}H] dexamethasone had a greater ($P<.05$) affinity for its receptor. There were no treatment or muscle differences in cytosolic protein concentration; therefore, the apparent reduction in cytosolic glucocorticoid receptors in the ST muscle was not a consequence of a decrease in cellular protein.

Data for the nuclear glucocorticoid receptor (table 5), were expressed as the percent specific binding of total binding since receptor concentration was too low to be determined with a Scatchard analysis. Trenbolone acetate decreased the [^{3}H] dexamethasone binding only in the TB muscle. This occurred at final [^{3}H] dexamethasone concentrations of .2 nM ($P<.005$), 1.6 nM ($P<.10$) and 6.4 nM ($P<.05$).

The cytosolic estrogen receptor analysis of ST and TB muscles is given in table 6. Estradiol implantation decreased the apparent number of estrogen receptors in the ST when expressed on a tissue weight ($P<.05$) and

TABLE 5. CYTOSOLIC AND NUCLEAR GLUCOCORTICOID RECEPTOR ANALYSIS OF OVINE SEMITENDINOSUS AND TRICEPS BRACHII MUSCLES VIA [1,2,4 ^{3}H] DEXAMETHASONE IN TRENBOLONE ACETATE AND ESTRADIOL TREATED LAMBS[a,b]

Item		Trenbolone Acetate +	Trenbolone Acetate -	Estradiol +	Estradiol -	SE[c]
Cytosol						
Bmax, fmol/g tissue[d]						
	ST	74.2	73.4	63.9	83.7	19.1
	TB	88.7	120.5	97.4	111.8	24.5
Bmax, fmol/mg DNA[d]						
	ST	569.6	580.7	428.1	722.3	186.8
	TB	670.6	1155.5	824.9	1001.1	276.2
Bmax, fmol/protein[e]						
	ST	4.1	4.4	4.2	4.3	1.4
	TB	5.7	6.0	5.6	6.1	2.4
Kd, nmol[e]						
	ST	.64	.57	.65	.57	.14
	TB	.44	.39	.44	.39	.08
Protein, mg/g tissue						
	ST	19.8	21.4	18.7	22.4	3.7
	TB	23.9	20.5	22.0	22.3	4.2
Nuclear,						
Percent Specific[f] Binding						
.20 nM	ST	38.6	31.0	32.6	36.8	10.1
	TB	6.0[g]	46.1[h]	24.4	27.7	7.6
1.60 nM	ST	.59	2.0	2.6	0.0	1.1
	TB	0.0[i]	6.7[j]	3.8	2.9	2.4
6.40 nM	ST	21.5	23.4	23.0	21.9	3.6
	TB	18.0[k]	28.9[l]	23.6	23.4	3.5

[a] Each value is a least square mean.
[b] Data for cytosol from Scatchard analysis.
[c] Standard error of mean.
[d] Muscles differ (P<.005).
[e] Muscles differ (P<.05).
[f] Data for nuclear represents the percent specific binding of total binding.
[g,h] Values for trenbolone acetate within a row differ (P<.005).
[i,j] Values for trenbolone acetate within a row differ (P<.10).
[k,l] Values for trenbolone acetate differ (P<.05).

tissue DNA (P<.05) basis and tended to decrease the number of estradiol receptors when expressed on a cytosolic protein basis (P<.10). Treatment with trenbolone acetate decreased the number of estradiol receptors only in the TB muscle on a tissue weight (P<.05) and tissue DNA (P<.05) basis and tended to on a cytosolic protein basis (P<.10). The apparent dissociation constant was not different for the E2 receptor with estradiol implantation; however, trenbolone acetate decreased (P<.05) the Kd in the TB muscle. Cytosolic protein concentration of the ST or TB was not altered by treatment.

Nuclear estrogen receptor analysis in the ST and TB indicated that estradiol decreased the apparent number of binding sites but only in the TB muscle (table 6). This was observed regardless of how binding was expressed. Trenbolone acetate did not affect the number of [^{3}H] estradiol binding sites in the nuclear fraction. The TB muscle had a greater number of estradiol nuclear binding sites when expressed on a nuclear protein (P<.05) basis and tended to be higher on a tissue weight (P<.10) and tissue DNA (P<.10) basis. The apparent nuclear Kd and nuclear protein content were not affected by steroid treatment or muscle type.

TABLE 6. CYTOSOLIC AND NUCLEAR ESTROGEN RECEPTOR ANALYSIS OF OVINE SEMITENDINOSUS AND TRICEPS BRACHII MUSCLES VIA [6,7 ^{3}H] ESTRADIOL IN TRENBOLONE ACETATE AND ESTRADIOL TREATED LAMBS[a,b]

Item		Trenbolone Acetate +	Trenbolone Acetate -	Estradiol +	Estradiol -	SE[c]
Bmax, fmol/g tissue						
Cytosol[d]	ST	59.5	26.8	21.9[e]	64.4[f]	13.5
	TB	39.1[e]	73.7[f]	46.4	66.5	10.6
Nuclear[g]	ST	21.0	16.7	6.0	15.8	14.1
	TB	50.7	48.0	14.0[e]	84.8[f]	16.6
Bmax, fmol/mg DNA						
Cytosol[d]	ST	484.3	211.6	129.7[e]	566.2[f]	118.9
	TB	290.3[e]	673.0[f]	408.3	554.9	118.2
Nuclear[g]	ST	171.4	168.1	49.8	155.6	143.7
	TB	374.4	527.7	164.8[e]	737.3[f]	183.1
Bmax, fmol/mg protein						
Cytosol[d]	ST	1.9	.80	.70[g]	2.0[h]	.52
	TB	1.2[h]	2.3[i]	1.4	2.1	.35
Nuclear[l]	ST	.95	.90	.25	.82	.77
	TB	2.5	2.2	.63[j]	4.1[k]	.76
Kd, nmol						
Cytosol[l]	ST	1.8	1.4	1.2	1.9	.61
	TB	.39[e]	1.4[f]	.67	1.1	.29
Nuclear	ST	2.4	3.5	.20	3.0	3.7
	TB	4.4	4.7	2.1	7.0	3.3
Protein, mg/g tissue						
Cytosol	ST	32.1	33.6	32.7	33.1	1.3
	TB	33.0	32.5	33.4	32.0	.93
Nuclear	ST	22.4	21.5	23.0	21.0	1.9
	TB	20.7	20.5	21.0	20.3	1.0

[a]Each value is a least sguare mean.
[b]Data from Scatchard analysis.
[c]Standard error of mean.
[d]Trenbolone acetate x muscle interaction (P<.10).
[ef]Values for each hormone within a row differ (P<.05).
[g]Muscles differ (P<.10).
[hi]Values for each hormone within a row differ (P<.10).
[jk]Values for each hormone within a row differ (P<.01).
[l]Muscles differ (P<.05).

The results from the ligand specificity studies of cytosolic dexamethasone binding sites indicated there were no treatment or muscle effects on the ligand specificities; therefore, discussion will only evolve around cell location within muscle. The most effective competitor with [^{3}H] dexamethasone for the glucocorticoid receptor binding sites was triamcinolone followed by cortisol and corticosterone which had binding of 34.6% and 37.8% [^{3}H] dexamethasone binding remaining (table 7). Estradiol-17B exhibited cross reactivity with the glucocorticoid receptor, while all other competitors including the trenbolone acetate metabolite, trenbolone-17 alpha showed only little cross reactivity.

The strongest competitor with [^{3}H] estradiol-17B for the specific cytosolic estradiol receptor was estradiol-17B (7.1% binding remaining) at a 100-fold molar excess followed by DES (11.7%) (table 8). Dexamethasone (55.6%) and corticosterone (55.9%) showed moderate cross reactivity while other competitors exhibited slight cross reactivity (70.4% to 90.1% binding remaining) to the cytosolic estrogen receptor.

Table 8 illustrates the relative percentage of [^{3}H] estradiol-17B binding remaining in the nuclear fraction after the addition of various steroids. A similar

TABLE 7. EFFECT OF UNLABELED LIGANDS ON TOTAL BINDING OF [1,2,4 ^{3}H] DEXAMETHASONE IN OVINE SKELETAL MUSCLE CYTOSOL[a,b]

	% Binding			
	10X		100X	
Unlabeled ligand	Mean	SE	Mean	SE
Dexamethasone	55.1	8.5	21.7	20.9
Corticosterone	88.9	8.3	37.8	15.3
Cortisol	69.8	24.4	34.6	21.8
Triamcinolone	45.4	14.0	9.7	6.7
Estradiol-17, Beta	83.3	27.0	37.8	6.5
Diethylstilbesterol	86.9	17.6	67.2	14.9
Testosterone	92.9	13.0	73.5	22.0
Progesterone	85.8	18.8	72.6	21.1
Trenbolone Acetate	94.0	10.3	85.1	16.8
Trenbolone-17, Alpha	97.4	5.6	89.4	17.9

[a]Cytosol from the Semitendinosus and Triceps Brachii muscles.
[b]Each value is the mean of 8 determinations and represents the percentage of binding remaining at a 10- and 100-fold molar excess of competitor.

TABLE 8. EFFECT OF UNLABELED LIGANDS ON TOTAL BINDING OF [6,7 ^{3}H] ESTRADIOL-17 BETA IN OVINE SKELETAL MUSCLE CYTOSOL AND NUCLEAR EXTRACT[a,b]

	% BINDING			
	10X		100X	
Unlabeled ligand	Mean	SE	Mean	SE
Cytosolic				
Dexamethasone	85.3	16.1	55.6	18.4
Corticosterone	88.5	16.0	55.9	17.5
Cortisol	86.8	15.4	71.6	20.3
Triamcinolone	94.5	13.4	90.1	16.8
Estradiol-17, Beta	69.7	9.8	7.1	8.5
Diethylstilbesterol	29.5	16.7	11.7	10.9
Testosterone	90.6	13.0	75.2	15.7
Progesterone	97.1	7.0	77.7	9.6
Trenbolone Acetate	90.9	12.9	73.9	17.8
Trenbolone-17, Alpha	86.4	17.0	70.4	14.9
Nuclear				
Dexamethasone	85.5	19.6	87.6	27.7
Corticosterone	84.9	33.7	42.9	45.5
Cortisol	94.0	13.4	71.4	39.4
Triamcinolone	97.0	5.1	95.6	6.3
Estradiol-17, Beta	24.1	18.1	22.4	21.4
Diethylstilbesterol	97.1	5.8	24.3	28.0
Testosterone	100.0	0.0	96.7	6.7
Progesterone	100.0	0.0	100.0	0.0
Trenbolone Acetate	96.2	8.6	86.8	21.0
Trenbolone-17, Alpha	85.5	22.5	92.9	15.9

[a]Cytosolic and nuclear from the Semitendinosus and Triceps Brachii muscles.
[b]Each value is the mean of 8 determinations and represents the percentage of binding remaining at a 10- and 100-fold molar excess of competitor.

situation occurred as in the cytosolic fraction.

Discussion

The charcoal technique (Snowchowski et al., 1980, 1981) and the extraction of nuclear receptors with a high salt buffer (Miller et al., 1985) were used to quantitate and localize the unoccupied glucocorticoid and estrogen receptor in ovine skeletal muscle. This is the first report that has addressed the possible location of these steroid receptors in sheep skeletal muscle.

The anabolic agents, TBA (Sulieman et al., 1986) and estradiol (Preston, 1975) have been used to increase weight gains in sheep. Previous reports have suggested a variety of actions to explain TBA stimulation of weight gain in animals. Some studies have indicated that TBA acts on muscle by decreasing protein degradation (Vernon and Buttery, 1978), or by decreasing the binding of cortisol in the cytosol of the muscle cell (Sharpe et al., 1984), while other studies find no TBA link with the degradation of muscle protein (Hayden et al., 1987).

The data presented herein do little to support the contention of Sharpe et al. (1984) that TBA decreases the unoccupied glucocorticoid receptor in skeletal muscle cytosol. However, our data do indicate a decrease in the

[^{3}H] dexamethasone binding in the nuclear fraction of TB muscle after TBA implantation. This observation does not contradict the contention that TBA stimulates live weight gains in lambs by interfering with glucocorticoid induced muscle protein degradation. It does however, imply a different mechanism from that proposed earlier (Sharpe et al., 1984). The Kd of .4 nM to .6 nM for the cytosolic glucocorticoid receptor (depending on the muscle being evaluated), in our study is in agreement with values reported by Dubois and Almon (1984) in rat skeletal muscle and approximately one order of magnitude less than in porcine skeletal muscle (Snochowski, 1981). This variability in Kd values is probably due to differences in species. Dissociation constants are only apparent and may reflect differences in endogenous corticosteroid hormones. Dubois and Almon (1984) demonstrated that with the addition of corticosterone to the incubation media, the apparent Kd constant increased. Thus, sheep implanted with TBA and estradiol may have a decreased Kd for the glucocorticoid receptor as a result of the influence of endogenous corticosteroid hormones.

Table 5. illustrates that the TB muscle continually had a greater number of cytosolic glucocorticoid receptors. Suzuki et al. (1976) reported in cattle that

the TB muscle (caput longum) had fewer B type fibers (fast twitch) than did the ST muscle which had a greater number of A type fibers (intermediate). Shoji and Pennington (1977) and Dubois and Almon (1984) reported that the slow fiber soleus muscle has a two-fold higher concentration of glucocorticoid receptor sites than that of the fast fiber extensor digitorum muscle. However, Seene and Viru (1982) demonstrated that when rats were treated with the anabolic steroid, nandrolon, the fast twitch, white type muscle had elevated anticatabolic activity. Our findings indicate that the TB muscle has a greater number of unoccupied cytosolic glucocorticoid receptors and a lower dissociation constant when compared to ST. Adrenalectomized animals possess a greater number of unoccupied glucocorticoid receptors (Dubois and Almon, 1984). The greater Bmax and decreased dissociation constant for the TB muscle indicates less competition from endogenous cortisol in the cytosol (Dubois and Almon, 1984).

The estrogen receptor concentration was examined in the cytosolic and nuclear fractions after implantation of TBA and estradiol. The implantation of estradiol consistently decreased the unoccupied estradiol receptor number in the cytosol and nuclear fractions in both

muscles examined. A similar result was reported by Rabbii and Ganong (1976) and Dionne et al. (1979, 1980) when female rats were compared to ovariectomized rats. Exogenous estradiol may occupy the estradiol receptor making it undectectable in the assay used in the present study. Previous workers have suggested that steroid hormones upon binding to their receptors in the cytosol of the cell, translocate to the nucleus, offering a plausable explanation for the decreased number of receptor sites following estrogen treatment (Dionne, 1979, 1980). Our data also indicate that estrogen implantation decreased the receptor numbers in the nucleus, however a greater proportion of [^{3}H] estradiol binding occurred in the cytosol. Trenbolone acetate also decreased the number of unoccupied receptors, but only in the cytosol. These results agree with those of Meyer and Rapp (1985) who reported that TBA decreased the number of estrogen receptors in the cytosol of the skeletal muscle cell, unlike the report of Sinnett-Smith et al. (1983) which suggests that TBA does not affect the cytosolic estradiol binding sites.

Our observation that the TB muscle has greater concentrations of estradiol binding sites (per mg nuclear protein) in the nuclear fraction lends support to the

explanation that slower twitch muscles have greater steroid receptor numbers (Shoji and Pennington, 1977; Dubois and Almon, 1984). Since TBA, not estradiol, decreased the number of unoccupied cytosolic estrogen receptors only in the TB muscle, one can speculate that the TB muscle may be more responsive to estrogen (Kochakian, 1957) in maintaining a larger portion of the estrogen receptors in the nucleus. Moreover, the ST muscle had more of its unoccupied estrogen receptors in the cytosol than in the nuclear fraction when estradiol was present. These results parallel those of Stack and Gorski (1985) who reported the more potent, estradiol-17B maintained estradiol receptors in the nucleus longer than the less potent estradiol-16A.

The ligand specificity studies indicate that estradiol-17B competed with [^{3}H] estradiol for cytosolic and nuclear estrogen receptors demonstrating that an estrogen receptor was identified. In addition to identifying an estrogen receptor our data also implies testosterone, TBA and trenbolone-17A have less affinity for the nuclear than the cytosolic estrogen receptor. This is clearly evident for trenbolone-17A, as there was more than a 20% difference in the percentage of binding between extracts at 100-fold molar excess of trenbolone-

17A. Although trenbolone-17A is the major metabolite of TBA in the liver and not in muscle (O'Keeffe, 1984), it appears to be an effective competitor with [^{3}H] estradiol for binding sites in the cytosol, which implies that TBA derivatives bind more to the cytosolic rather than the nuclear estrogen receptor. Dube et al. (1976) and Dahlberg (1982) reported relative binding affinities for cortisol, progesterone and testosterone to cytosolic E2 receptors to be on the order of 5% to 12%. Our data produced slightly higher values in the cytosol for the estrogen receptor however, are comparable in the nuclear fraction. Aten et al. (1980) reported that dihydrotestosterone, progesterone or corticosterone did not compete with [^{3}H] estradiol in purified nuclei or cytosol of rats. Comparisons, however, must be approached with caution since studies by Dube et al. (1980) and Aten et al. (1980) utilized 10- and 30-fold molar excesses of competitor, respectively.

A glucocorticoid receptor appeared to be identified since triamcinolone (a fluorocorticoid derivative) and other glucocorticoid hormones competed effectively with [^{3}H] dexamethasone at 10 and 100-fold molar excesses in the binding to cytosolic receptors. Interestingly, our results also indicated that there was approximately a 25%

reduction in [^{3}H] dexamethasone binding capacity when diethylstilbesterol, testosterone, progesterone and approximately a 60% reduction when estradiol-17B were added at a 100-fold molar excess to the incubation media. Others have reported similar results when using estrogen, testosterone and progesterone as competitors for the cytosolic glucocorticoid receptor (Singer et al., 1973; Snochowski et al., 1980, 1981; Chrousos et al., 1983; Danhaive and Rousseau, 1986) . It has been suggested that some hormones inhibit glucocorticoid binding to receptors either by inducing conformational changes or by direct competition for binding sites, thus reducing the catabolic response (Mayer and Rosen, 1975). Results of the present study certainly indicate that competition for binding sites is a possible means for inhibition of glucocorticoid action. This observation was also noted by Waggoner et al. (1988) who reported that a low capacity glucocorticoid receptor pool which binds to several non-catabolic steroids may be one of the control mechanisms by which animals regulate protein turnover in skeletal muscle.

The presence of cytosolic glucocorticoid, and cytsolic and nuclear estrogen receptors in ovine skeletal muscle was demonstrated. A diminished glucocorticoid

receptor population in the nucleus compared to that of the estrogen receptors was observed, supporting findings of Wikstrom et al. (1987). Receptor binding characteristics suggest that TBA does not affect the cytosolic glucocortcoid receptor population but may have an affect on the binding of [^{3}H] dexamethasone in the nucleus of the cell. The TB muscle appears to have a greater number of the cytosolic glucocorticoid receptors as well as nuclear estrogen receptors than the ST muscle, which probably relates to the fiber type differences of the two muscles. In addition, E2 appears to occupy its receptor in the nucleus to a greater extent, in the more responsive TB muscle than TBA. Additional work must be conducted to determine the relationship between growth, receptor partitioning and fiber types in the skeletal muscles of animals used primarily for human consumption.

CHAPTER VI

SUMMARY

A study was conducted to determine the effects of trenbolone acetate (TBA) and estradiol-17B (E2) implantation on the characteristics of the glucocorticoid and E2 receptor and tissue growth and skeletal muscle protein synthesis and degradation.

Twenty-four male castrate lambs (Barbados x Dorset, Coopworth x Dorset and Finn x Dorset) were grouped into weight groups, blocked by breed and alloted to four treatment combinations in a 2 x 2 factorial design. Treatments were as follows: 1) with or without 60 mg TBA implant and 2) with or without 12 mg E2 implant. Lambs were killed at an average of 42 d following implantation. The semitendinosis (ST) and triceps brachii (TB) muscles were exposed and muscle biopsies were taken to measure in vitro protein synthesis and degradation. Receptor assays were conducted on the ST and TB. In addition, RNA, DNA and protein concentrations were determined on those muscles. Serum cortisol concentrations were also determined.

Estradiol and TBA did not increase overall growth rate. Final body weight was slightly increased with TBA. Feed

intake was increased (P<.01) by E2. Feed efficiency was not different with either treatment. A possible explanation for the apparent lack of response in measured performance criteria may lie in the handling and housing of the animals. Animals housed in confinement often times have reduced gain as well as intake. Typically, feed efficiency is very good for confinement animals, which is in fact, what was observed in this study. Additionally, animals in this study were handled and restrained daily in order that individual feeding could be accomplished. This additional handling could in part explain why weight gain was unaffected by treatment.

The synthesis of muscle protein in the TB muscle when TBA was implanted was decreased (P<.01) with no difference in the ST. However, decreased synthesis rates may not be observed across the entire muscle mass. Estradiol did not alter synthesis rates in either the ST and TB. Muscle did show variation in metabolism as the TB muscle had greater (P<.05) muscle protein synthesis rates than the ST muscle. Greater synthesis rates occur in muscles with less fast twitch fibers than muscles which are predominantly fast twitch. In addition, the TB muscle continually had greater (P<.05) unoccupied cytosolic glucocorticoid receptors. This is presumably due to a difference in the fiber types of the

two muscles. Glucocorticoids decrease synthesis rates of muscle proteins, therefore it would be reasonable to expect the TB muscle to have a greater synthesis rate when less of the TB muscle's glucocorticoid receptors are occupied. Moreover, protein:RNA ratios were consistently lower ($P<.05$) in the TB muscle when compared to the ST. This would also indicate greater capability for synthesis of muscle protein. It appears that the method to measure synthesis used in this study may not be sensitive enough to distinguish minute changes in rates. The ST weight was decreased ($P<.05$) while the TB was not effected by TBA treatment. A lower weight of the ST with TBA indicated that the pattern of growth of the ST did not parallel body weight gains. It may be that the TB muscle may be more responsive to endocrine therapy.

Protein concentrations did not differ between muscles or treatments. Deoxyribonucleic acid concentration was greater ($P<.05$) in the TB muscle of TBA treated lambs and was greater ($P<.20$) in the ST muscle of E2 treated lambs. Presumably, postnatal muscle growth occurs primarily by muscle cell hypertrophy. Increased DNA content may result from satellite cell recruitment and that 30% of the muscle nuclei may be contributed by non muscle cells.

Peripheral cortisol levels were only slightly depressed by TBA and slightly elevated by E2. This observation was

consistent throughout the sampling period. Adrenal weights were also decreased slightly by TBA, suggesting that alteration of glucocorticoid production could possibly be due to tissue atrophy.

Pituitary gland weights were increased ($P<.01$) with E2 but decreased with TBA ($P<.005$). A proposed mode of action for growth enhancement by E2 involves the stimulated release of growth hormone from the pituitary. However the opposite situation may occur with TBA. Trenbolone acetate may be affecting the size of the pituitary by acting as a negative feedback since the concentration of FSH and LH increase upon castration.

Trenbolone acetate did not effect cytosolic glucocorticoid receptor numbers however, TBA did decrease ($P<.05$) the binding of [^{3}H] dexamethasone in the nuclear fraction. This may be a different mechanism by which TBA decreases protein degrdation in vivo. Estradiol decreased ($P<.05$) the number of cytosolic E2 receptors in the ST and decreased ($P<.05$) the number of nuclear E2 receptors in the TB muscle.

All glucocorticoid hormones competed with [3H] dexamethasone, and E2 and diethylstilbesterol competed with [^{3}H] estradiol-17B for binding sites suggesting that a glucocorticoid and E2 receptor was identified.

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APPENDIX A

COMPOSITION OF REAGENTS FOR DNA AND RNA ANALYSIS

TABLE A.1 Orcinol Reagent

Make a stock solution of 0.80 g $FeCl_3 \cdot 6 H_2O$ in 250 ml concentrated HCl. Before each use, dissolve 1 g orcinol in 100 ml of stock solution.

TABLE A.2 Diphenylamine Reagent

Dissolve 1.5 g diphenylamine in 100 ml of glacial acetic acid and add 1.5 ml concentrated H_2SO_4. Store in dark room at room temperature.

TABLE A.3 Acetaldehyde Solution

Add 2 ml of acetaldehyde to 98 ml of deionized distilled water. Store at 4 C. On day of use add .1 ml of solution to diphenylamine reagent.

TABLE A.4 Standard DNA Solution

Dissolve 40 mg calf thymus DNA in 100 ml of 4.0 mM NaOH. From this, working standards are prepared by mixing a measured volume of stock solution with an equal volume of 1 N $HClO_4$ and heating at 70 C for 30 min.

TABLE A.5 Standard RNA Solution

Weigh out .0125 g highly polymerized calf liver RNA. Add RNA to 50 ml volumetric flask and bring to volume with .5 N $HClO_4$ for a concentration of 0.25 mg/ml.

APPENDIX B

COMPOSITION OF SOLUTIONS FOR RECEPTOR ASSAYS

TABLE B.1 Tris-HCl buffer (TEDG Buffer)

Place .758 g (MW=151.6) reagent grade Trizma-7.4 (5mM) (tris(hydromethyl)aminomethane and hydrochloride, pH 7.4, 23 C) in a 1 liter plastic bottle. Add .292 g (1 mM) Ethlyenediaminetetracetic acid (disodium, EDTA). Add fresh daily, .0154 g dithioerythritol (2,3-dihydroxy-1,4-dithiobutane). Add 100 ml glycerol (glycerin) (10% [v/v]) and dilute to volume with deionized distilled water. Store at 4 C.

TABLE B.2 Extraction Tris-HCl Buffer

Add 44.73 g KCl to buffer B.1. Dilute to 1 liter with deionized distilled water and store in platic 1 liter bottle at 4 C.

TABLE B.3 Charcoal Solution

Add .625 g of charcoal (activated, washed with HCl) to a 100 ml volumetric flask. Add .0625 g dextran (industrial grade) to this flask and bring to volume with buffer B.1.

TABLE B.4 [1,2,4 ^{3}H] Dexamethasone

Purchase 1 ml of stock solution (40 Ci/mmol) in ehtanol from Amersham Corporation. Dry down with evaporation and reconstitute to 4 ml with absolute ehtanol. Pipet 133.86 ul, place in 20 ml scintillation vial and evaporate. Dilute to 20 ml with buffer B.1. This solution = 38.4 nM [1,2,4 ^{3}H] dexamethasone.

TABLE B.5 [6,7 $^{3}H(N)$] Estradiol-17B

Purchase .25 ml of stock solution (60 Ci/mmol) in ethanol from New England Nuclear Corporation. Dry down and reconstitute to .25 ml. Pipet 69.1 ul and place in 50 ml glass bottle. Evaporate. Add 30 ml buffer B.1. This solution = 38.4 nM [6,7 ^{3}H] estradiol-17B.

TABLE B.6 Cold Steroids

1.	Cortisol.	384nM = 1.33 x 10^{-6} g/100 ml
	(MW = 346)	3840 nM = 1.33 x 10^{-5} g/100 ml
2.	Corticosterone.	384 nM = 1.392 x 10^{-6} g/100 ml
	(MW = 362.5)	3840 nM = 1.392 x 10-5 g/100 ml
3.	Estradiol-17B.	384 nM = 1.046 x 10^{-6} g/100 ml
	(MW = 272.4)	3840 nM = 1.046 x 10^{-5} g/100 ml
4.	Testosterone.	384 nM = 1.017 x 10^{-6} g/100 ml
	(MW = 288.4)	3840 nM = 1.017 x 10^{-5} g/100 ml
5.	Triamcinolone.	384 nM = 1.514 x 10^{-6} g/100 ml
	(MW = 394.4)	3840 nM = 1.514 x 10^{-5} g/100 ml
6.	Progesterone.	384 nM = 1.205 x 10^{-6} g/100 ml
	(MW = 314)	3840 nM = 1.205 x 10^{-5} g/100 ml
7.	Dexamethasone.	384 nM = 1.507 x 10^{-6} g/100 ml
	(MW = 392.5)	3840 nM = 1.507 x 10^{-5} g/100 ml
8.	Diethylstibesterol.	384 nM = 1.306 x 10^{-6} g/100 ml
	(MW = 268.4)	3840 nM = 1.306 x 10^{-5} g/100 ml
9.	Trenbolone Acetate.	384 nM = 1.996 x 10^{-6} g/100 ml
	(MW = 312.4)	3840 nM = 1.996 x 10^{-5} g/100 ml
10.	Trenbolone-17A.	384 nM = 1.038 x 10^{-6} g/100 ml
	(MW = 270.3)	3840 nM = 1.038 x 10^{-5} g/100 ml

Table B.7 Scintillation Coctails and Quench Corrections

All samples were counted in Scinti-Verse E. This is a standardized cocktail which contains primary and secondary solutes. The quench correction procedures consisted of glass scintillation vials each containing 43,663 DPM [1,2,6,7 $^{3}H(N)$]-Androst-4-ene-3, 17-dione ([^{3}H] Androstenedione). The vials also contained either 90, 80, 70, 60, 50, 40, 30, 20, 10 or 0 ul methylene chloride (HPLC grade) as a quenching agent. Each quenched sample was replicated 4 times. The sample channels ratio method was utilized to relate the counting efficiency of standards to the ratio of channel 2 (150-400) to channel 1 (0-150).

APPENDIX C

COMPOSITION OF SOLUTIONS FOR IN VITRO PROTEIN TURNOVER

Table C.1 Krebs-Ringer Bicarbonate Buffer

1.	$NaCl$,	(MW = 58.44),	0.77 M,	44.99 g/l, 4.49 g/100 ml
2.	KCl,	(MW = 74.6),	0.77 M,	57.44 g/l, 5.74 g/100 ml
3.	$CaCl_2$,	(MW =147.02),	0.55 M,	80.86 g/l, 8.806 g/100 ml
4.	KH_2PO_4,	(MW =136.09),	0.77 M,	104.79 g/l, 10.48 g/100 ml
5.	$MgSO_4$,	(MW = 120.37),	0.77 M,	92.68 g/l, 9.27 g/100 ml
6.	$NaHCO_3$,	(MW = 84.01),	0.77 M,	64.69 g/l, 6.47 g/100 ml

These are 5X concentrations in the final medium diluted to 1 + 4.

Table C.2 Ratio of Stock Solutions For Krebs-Ringer Buffers

1.	NaCl	100.0 ml
2.	KCl	4.0 ml
3.	$CaCl_2$	3.0 ml
4.	KH_2PO_4	1.0 ml
5.	$MgSO_4$	1.0 ml
6.	$NaHCO_3$	21.0 ml

Concentrations is 5X and a total of 130 ml.

For Krebs-Ringer Phosphate buffer, omit solution # 4 and # 6 and add the remainder with deionized distilled water.

Table C.3 Other Solutions In Krebs-Ringer Buffers

1. Glucose, (MW = 180.16), 1 mM, .18 g/l.
2. Insulin, (From Bovine Pancreas), 26.2 IU/mg, .5 IU/ml, 1.908 mg/100ml.
3. Chloramphenicol, (Chloromycetin), (MW = 323.1), .3 mM, .09693 g/l.
4. Cyclohexamide, (MW = 281.4), .5 mM, .1407 g/l.
5. Na Propionate, (MW = 96.06), .29 mM, .02785 g/l
6. Na Acetate, (MW = 82.03), 1.3 mM, .1066 g/l.
7. L-[U-^{14}C] Tyrosine, 50 uCi/ml, 0.3 uCi/ml, 3.3 ml of

buffer in each vial, 20 ul [^{14}C] tyrosine per incubation vial.

8. [^{14}C] Inulin, 10 uCi/ml, .03 uCi/ml, 3.3 ml of buffer in each vial, 10 ul [^{14}C] inulin in each vial.

Table C.4 Amino Acids In Krebs-Ringer Buffers

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1. Lysine, (MW = 146.19), 10.4 mM, 74.1 mg/975 ml.
2. Methionine, (MW = 149.21), 3.4 mM, 24.8 mg/975 ml.
3. Cysteine, (MW = 121.16), 2.4 mM, 14.2 mg/975 ml.
4. Valine, (MW = 117.15), 20.0 mM, 114.3 mg/975 ml.
5. Isoleucine, (MW = 131.18), 8.8 mM, 56.3 mg/975 ml.
6. Leucine, (MW = 131.18), 19.0 mM, 95.9 mg/975 ml.
7. Phenylalanine, (MW = 165.19), 6.0 mM, 48.4 mg/975 ml.
8. Histidine, (MW = 155.16), 3.82 mM, 28.9 mg/975 ml.
9 Arginine, (MW = 174.20), 4.94 mM, 41.9 mg/975 ml.
10. Threonine, (MW = 119.12), 7.82 mM, 45.6 mg/975 ml.
11. Aspartate, (MW =133.10), 3.57 mM, 23.2 mg/975 ml.
12. Aspargine, (MW = 132.12), 18.0 mM, 23.2 mg/975 ml.
13. Serine, (MW = 105.09), 10.03 mM, 51.4 mg/975 ml.
14. Glutamate, (MW = 147.13), 6.14 mM, 44.1 mg/975 ml.
15. Proline, (MW = 115.13), 5.14 mM, 28.8, mg/975 ml.
16. Glycine, (MW = 80.11), 30.44 mM, 118.9 mg/975 ml.

17. Alanine, (MW = 89.09), 8.66 mM, 37.6 mg/975 ml.
18. Glutamine, (MW = 146.15), 30.93 mM, 44.1 mg/975 ml.
19. Trptophan, (MW = 204.23), 14.49 mM, 28.86 mg/975 ml.
20. Tyrosine, (MW = 181.19), 3.82 mM, 33.7 mg/975 ml.

Preincubation buffer and synthesis buffer contain all the ingredients in table C.2, C.3 and C.4 except for cyclohexamide. Preincubation buffer and degradation buffer did not contain [^{14}C] tyrosine. Specific samples contained [^{14}C] inulin. Degradation buffer contained cyclohexamide and no tyrosine. Amino acids are all at 5X concentration found in sheep plasma. After all ingredients were added, 95% O_2 - 5% CO_2 gas was added to all buffers. The pH was adjusted to 7.4 with .5 N NaOH. Buffers for verification experiment were the same as the buffers for the estimation experiment except that one buffer contained glucose and no Na propionate and Na acetate, another buffer contained no glucose with the addition of Na propionate and Na acetate and the final buffer contained all three ingredients. Concentrations were the same as in the estimation experiment.

Table C.5 2.5 M Nitric Acid

Place 156 ml of 16 M nitric acid in a 1 liter volumetric flask and dilute to one liter with H_2 that had been filtered through a .45 um millipore filter. Storage was at room temperature in a glass container.

Table C.6 Phosphoric Acid Reagent

Place 533 ml of 15 M phosphoric acid in a 1 liter volumetric flask and dilute to 1 liter with millipore-filtered water to result in an 8 M solution. Dissolve 100 g of $Na_2P_2O_7 \cdot 10$ H_2O (sodium pyrophosphate) in 500 ml of the 8 M phosphoric acid solution and dilute to 1 liter with millipore-filtered water. The solution was filtered once more with .45 um filters and stored at room temperature in a glass container.

Table C.7 Ethanol

Absolute ethanol was mixed with water to contain 5 ml of water per deciliter and filtered through .45 um millipore

filters and stored at room temperature in a glass bottle.

Table C.8 1-Nitroso-2-Napthol-Sodium Nitrite Reagent

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This reagent was prepared immediately before use. 1-nitroso-2-napthol (50 mg) and 345 mg of $NaNO_2$ were placed in a 50 ml beaker and 40 ml of 50 mM NaOH were added. The mixture was stirred with a glass rod untill dissolved and transferred quantitatively to a 100 ml volumetric flask. The glass rod and beaker were washed several times with 50 mM NaOH with the washings added to the volumetric. The volume was adjusted to to 100 ml with 50 mM NaOH. The solution was then shaken vigorously and filtered through a .45 um millipore filter and stored in the dark in a glass container covered with aluminum foil.

Table C.9 Nitric Acid Reagent

This reagent was prepared in glass immediately before use. One volume of nitric acid (2.5 M) was added to two volumes of the nitroso-napthol-sodium nitrite reagent. The mixture was stirred with a glass rod, covered, and allowed to stand for 20 min. After 20 min, 1.5 volumes of the phosphoric acid reagent were added and the mixture stirred again with a glass rod.

Table C.10 Trichloroacetic Acid (.6 M)

Place 98.10 g of TCA was diluted in 1 liter of H_2O to make a .6 M solution. The solution was then filtered and stored at 4 C in glass.

Table C.11 Trichloroacetic Acid (60 mM)

Place 100 ml of .6 M TCA in a 1 liter volumetric flask and dilute to volume with H_2O. Theis solution was then filtered with .45 um millipore filters and stored at 4 C in glass.

Table C.12 Primary stock and working L-Tyrosine Standards

A 50 ml/dl stock solution of L-tyrosine was prepared by placing 50 mg of L-tyrosine in a 50 ml beaker and adding 10 ml of filtered .6 M TCA plus 20 ml of filtered water. The tyrosine was then dissolved by stirring with a glass rod and transferred quantitatively to a 100 ml volumetric flask. The beaker and glass rod were rinsed several times with millipore-filtered water with the washings added to the volumetric flask. The solution was then diluted to 100 ml with millipore-filtered water. This solution was stored at 4 C in the glass volumetric flask for up to 5 d. A 1 mg/dl working standard was prepared fresh each day by transferring 1 ml of the stock standard to a 50 ml volumetric flask and diluted to volume with .6 M TCA. This solution was stored at 4 C in the glass volumetric flask.

Table C.13 Scintillation Coctails And Quench Corrections

All synthesis and inulin samples from the protein turnover assay were counted in toluene (Scintanalyzed). Primary solute was PPO (scintanalyzed, 2,5 diphenyloxazole, MW = 221.25, 3.92 g/l) . Secondary solute was Bis-MSB [(scintanalyzed) p-Bis(0-methylistyryl)benzene, MW = 310.44, .8 g/l]. For the acid soluable supernates from the synthesis and inulin samples, 500 ml trition X-100 (reagent grade, surfactant) was mixed with 1 liter toluene. Standards for quench correction were [^{14}C] toluene in scintillation solution of 4 g PPO and .05 g POPOP (p-bis[2-(5-phenyloxazolyl)]benzene) per liter of toluene. Radioactivity of the standards was .0198 uCi (4.4×10^4 DPM). Samples were corrected for quenching by the H number technique which is a microprocessor directed scan of the Compton pulse height distribution of the sample. Channel 1 was (0-397) and channel 2 was (397-655). The inflection point of the Compton edge of the sample is compared to that of the unquenched standard and the H number is the difference in pulse heights. Counting efficiency was plotted as a function of the H number and the efficiency of the samples was determined from this plot.

APPENDIX D

REAGENTS FOR PROTEIN DETERMINATION

Table D.1 Coomassie Brilliant Blue Dye (G-250)

Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol. To this solution 100 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 liter. Final concentrations in the reagent were 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid. The dye mixture was filtered through .45 um millipore filters.

APPENDIX E

TABLE E.1 INDIVIDUAL LAMB WEIGHT GAIN, FEED INTAKE AND FEED EFFICIENCY DATA

Tag No.	Breed[a]	Treatment[b]	Wt. (Kg)	Intake (Kg/d)	Gain[c]	Eff[d]
0	C	2	18.2	1.11	.24	4.6
1	C	3	21.8	1.29	.35	3.5
2	B	4	28.2	1.17	.27	4.4
3	M	4	27.3	1.22	.27	4.5
4	M	3	18.2	1.09	.26	4.2
5	B	2	28.2	1.32	.32	4.1
6	M	3	25.5	1.21	.33	3.7
7	C	4	28.2	1.25	.32	3.9
8	C	4	20.0	0.83	.22	3.7
9	C	1	26.4	1.17	.34	3.5
10	C	2	20.9	0.97	.36	2.7
11	C	4	21.8	0.96	.29	3.3
12	M	2	25.5	1.26	.30	4.3
13	B	1	29.1	1.06	.24	4.4
14	M	1	27.3	1.31	.42	3.1
15	M	1	24.5	1.35	.41	3.3
16	M	1	22.7	1.20	.32	3.8
17	M	2	24.5	1.19	.27	4.4
19	M	3	27.3	1.12	.26	4.3
20	C	1	21.8	1.20	.39	3.1
21	C	3	28.2	1.28	.35	3.6
22	C	2	26.4	1.18	.24	5.0
23	C	3	22.7	0.42	.00	---
24	M	4	22.7	0.93	.24	3.9

[a]Consists of Barbados = B, Coopworth = C and Mixed = M.
[b]Consists of trenbolone acetate plus estradiol = 1, control = 2, estradiol = 3 and trenbolone acetate = 4.
[c]kg/d.
[d]kg feed/kg gain.

TABLE E.2 SLAUGHTER MUSCLE, ORGAN AND GLAND WEIGHTS[a]

Tag No.	ST	Liver	Pituitary	Thyroid	Heart	Spleen	TB	Adrenal
0	2.6	25.3	20.3	0.82	4.9	1.9	4.5	87.3
1	2.7	27.4	29.4	0.80	4.9	2.0	4.6	101.5
2	2.5	21.6	11.5	1.30	4.7	1.9	4.8	111.0
3	2.7	22.8	14.8	0.94	5.3	1.8	5.4	131.5
4	2.7	28.8	32.0	0.65	4.8	1.7	4.5	105.0
5	2.7	21.5	15.9	0.92	5.3	1.7	4.8	104.2
6	2.9	23.0	20.7	0.90	4.2	1.7	4.8	130.2
7	2.6	20.6	14.8	0.67	5.2	1.6	4.8	148.0
8	2.4	25.5	18.7	0.78	5.1	1.8	4.4	81.0
9	2.5	22.0	17.8	0.73	4.8	1.8	4.9	110.0
10	2.6	23.1	22.5	0.70	5.1	1.7	4.9	108.4
11	2.8	24.1	18.3	0.69	4.8	1.6	4.5	60.3
12	2.7	19.9	16.8	1.00	4.2	1.5	4.7	99.0
13	2.8	20.5	18.2	1.10	2.9	1.6	3.9	57.8
14	2.7	20.7	18.4	0.73	4.4	1.2	5.1	111.3
15	2.4	23.4	15.7	1.20	4.7	1.7	5.2	114.7
16	2.6	24.6	17.9	0.92	5.2	1.4	4.1	92.6
17	2.8	22.6	16.8	0.84	5.3	1.9	4.4	99.3
19	2.8	20.2	17.3	0.93	4.7	2.2	4.9	171.3
20	2.4	22.1	11.0	0.74	4.5	1.4	4.9	104.6
21	3.1	21.9	15.7	0.78	5.1	1.7	5.4	83.6
22	2.9	20.8	14.0	0.61	4.6	1.5	5.1	111.4
23	2.9	14.7	26.6	5.30	4.9	1.3	5.7	126.9
24	3.2	22.0	12.9	0.77	4.8	1.8	5.4	75.0

[a]ST, liver, thyroid, heart spleen TB are expressed as g/kg BW. Pituitary and adrenal are expressed as mg/kg BW.

TABLE E.3 SERUM CORTISOL LEVELS[a]

Tag No.	Cortisol
0	2.07
1	5.50
2	0.90
3	1.70
4	6.80
5	2.60
6	5.40
7	3.90
8	3.70
9	6.10
10	1.70
11	6.00
12	4.70
13	0.80
14	2.90
15	2.30
16	3.50
17	6.40
19	8.70
20	10.50
21	1.80
22	6.60
23	0.47
24	0.45

[a]ng/ml serum

TABLE E.4 INVITRO SYNTHESIS AND DEGRADATION, DNA AND RNA[a]

Tag No.	Muscle	Synthesis	Degradation	DNA	RNA
0	ST	.025	.148	.10	0.45
	TB	.040	.117	.08	0.53
1	ST	.016	.204	.12	0.54
	TB	.027	.234	.08	0.41
2	ST	.012	.144	.12	0.56
	TB	.018	.085	.17	0.50
3	ST	.014	.146	.12	0.75
	TB	.018	.135	.12	0.49
4	ST	.018	.114	.12	0.39
	TB	.033	.132	.12	0.59
5	ST	.014	.130	.12	0.20
	TB	.024	.096	.12	0.46
6	ST	.023	.050	.10	0.87
	TB	.028	.076	.08	0.76
7	ST	.018	.087	.08	0.51
	TB	.023	.058	.13	0.74
8	ST	.034	.135	.12	1.17
	TB	.035	.177	.12	0.68
9	ST	.019	.223	.12	0.48
	TB	.023	.293	.15	0.62
10	ST	.019	.175	.12	0.39
	TB	.042	.200	.10	0.45
11	ST	.018	.165	.11	1.37
	TB	.025	.197	.14	0.51
12	ST	.015	.121	.08	1.06
	TB	.033	.093	.09	0.63
13	ST	.014	.226	.15	1.06
	TB	.030	.247	.13	0.74
14	ST	.013	.111	.20	0.51
	TB	.025	.073	.14	0.78
15	ST	.013	.059	.12	0.41
	TB	.020	.065	.12	0.68
16	ST	.022	.094	.12	0.49
	TB	.021	.122	.12	0.52
17	ST	.021	.096	.15	0.78
	TB	.034	.187	.12	0.62
19	ST	.017	.047	.12	0.56
	TB	.030	.065	.12	0.62
20	ST	.018	.097	.12	0.58
	TB	.022	.140	.16	0.50
21	ST	.017	.074	.12	0.40
	TB	.027	.104	.12	0.50
22	ST	.015	.108	.09	0.17
	TB	.024	.087	.12	0.19
23	ST	.015	.082	.16	0.90
	TB	.027	.056	.12	0.36
24	ST	.015	.097	.14	0.63
	TB	.021	.070	.12	0.56

[a]DNA and RNA expressed as mg/g tissue. Synthesis expressed as nmol · mg wet tissue^{-1}. 2.5 h^{-1}. Degradation expressed as nmol · mg wet tissue^{-1} · 3 h^{-1}.

TABLE E.5 PROTEIN, PROTEIN:DNA, PROTEIN:RNA AND RNA:DNA[a]

Tag No.	Muscle	Protein	Pro:DNA	Pro:RNA	RNA:DNA
0	ST	249.2	2492.0	553.8	4.5
	TB	204.9	2560.6	386.5	6.6
1	ST	165.4	1378.6	306.4	4.5
	TB	170.1	2125.9	414.8	5.1
2	ST	219.9	1832.7	392.7	4.7
	TB	233.6	1373.8	467.1	2.9
3	ST	238.8	1989.7	318.4	6.3
	TB	232.0	1933.6	473.5	4.1
4	ST	246.3	2052.5	631.5	3.3
	TB	222.4	1853.2	376.9	4.9
5	ST	228.6	1905.2	1143.1	1.7
	TB	232.7	1938.9	505.8	3.8
6	ST	209.1	2090.6	240.3	8.7
	TB	222.4	2779.9	292.6	9.5
7	ST	202.7	2533.5	397.4	6.7
	TB	212.5	1634.9	287.2	5.7
8	ST	219.3	1827.9	187.5	9.8
	TB	224.7	1872.6	330.5	5.7
9	ST	215.1	1792.9	448.2	4.0
	TB	167.2	1114.5	269.6	4.1
10	ST	205.1	1709.5	526.0	3.3
	TB	242.9	2429.7	539.9	4.5
11	ST	237.5	2158.8	173.3	12.5
	TB	241.8	1727.2	474.1	3.6
12	ST	161.5	2019.0	152.4	13.3
	TB	224.3	2491.9	355.9	7.0
13	ST	228.8	1525.1	215.8	7.1
	TB	223.9	1722.9	302.7	5.7
14	ST	228.3	1141.6	447.7	2.6
	TB	235.7	1683.7	302.2	5.6
15	ST	159.2	1326.7	388.3	3.4
	TB	229.8	1914.8	337.9	5.7
16	ST	213.1	1775.9	434.9	4.1
	TB	228.8	1906.3	439.9	4.3
17	ST	224.7	1498.1	288.1	5.2
	TB	217.0	1808.6	350.1	5.2
19	ST	231.8	1931.7	413.9	4.7
	TB	172.7	1438.9	278.5	5.2
20	ST	179.9	1499.4	310.2	4.8
	TB	210.5	1315.6	421.0	3.1
21	ST	224.1	1867.7	560.3	3.3
	TB	224.9	1874.4	449.8	4.2
22	ST	232.1	2578.9	1365.3	1.9
	TB	196.3	1635.8	1033.2	1.6
23	ST	237.9	1486.8	264.3	5.6
	TB	205.7	1714.4	571.5	3.0
24	ST	230.5	1646.5	365.9	4.5
	TB	233.5	1946.2	417.0	4.7

[a]Protein expressed as mg/g tissue.

TABLE E.6 CYTOSOLIC GLUCOCORTICOID AND CYTOSOLIC AND NUCLEAR ESTROGEN RECEPTORS[a]

Tag No.	Muscle	Glucocorticoid	Kd	Cytosolic Estrogen	Kd	Nuclear Estrogen	Kd
0	ST	12.60	0.51	1.90	1.60	2.30	2.40
	TB	14.70	0.36	----	----	4.80	9.20
1	ST	2.30	0.35	0.67	0.62	0.34	0.43
	TB	11.60	0.32	1.08	0.19	----	----
2	ST	3.20	0.64	3.30	3.90	----	----
	TB	0.80	0.24	0.93	0.15	7.40	12.30
3	ST	3.10	0.46	2.60	2.40	0.62	1.30
	TB	7.20	0.58	1.60	0.38	0.53	0.97
4	ST	1.80	0.85	0.84	0.38	0.24	0.19
	TB	5.80	0.24	1.10	0.08	1.50	9.10
5	ST	5.10	0.43	1.10	1.40	1.40	1.10
	TB	5.40	0.29	3.50	2.30	7.10	9.30
6	ST	1.10	0.31	0.31	0.45	----	----
	TB	4.50	0.85	3.30	0.86	0.03	1.00
7	ST	7.50	0.36	1.00	1.20	0.58	1.90
	TB	----	----	3.40	3.00	0.36	2.00
8	ST	4.10	0.79	1.20	0.30	0.35	1.20
	TB	11.90	0.40	2.10	0.36	----	----
9	ST	5.30	0.63	0.77	0.28	2.20	3.90
	TB	----	----	2.50	1.80	----	----
10	ST	0.38	0.67	0.33	1.40	0.39	5.80
	TB	3.10	0.24	2.70	2.30	----	----
11	ST	2.00	0.31	6.00	2.50	----	----
	TB	2.90	0.51	1.40	0.19	----	----
12	ST	3.20	0.49	2.30	1.90	1.40	14.00
	TB	10.10	0.61	1.40	0.11	1.70	1.10
13	ST	7.10	0.64	1.10	1.20	0.56	1.00
	TB	2.90	0.42	0.29	0.22	0.27	0.92
14	ST	2.20	0.58	0.42	1.40	0.36	1.50
	TB	8.40	0.31	1.30	1.20	1.50	3.00
15	ST	0.23	1.20	----	----	----	----
	TB	11.90	1.50	0.35	0.51	----	----
16	ST	5.30	0.36	1.02	0.04	----	----
	TB	2.20	0.44	0.89	1.10	2.60	2.50
17	ST	1.80	0.34	0.63	0.66	0.15	0.11
	TB	5.00	0.60	2.40	1.70	1.40	3.30
19	ST	1.00	0.37	0.36	1.20	----	----
	TB	2.20	0.30	0.51	0.63	0.40	2.60
20	ST	1.20	0.31	1.30	1.10	2.30	6.50
	TB	----	----	0.98	0.39	----	----
21	ST	0.58	1.00	0.33	2.00	----	----
	TB	5.80	0.50	0.84	0.10	2.80	11.00
22	ST	1.50	0.24	1.03	1.60	0.42	0.42
	TB	10.60	0.42	1.30	0.69	2.80	3.20
23	ST	3.50	1.10	1.20	6.60	----	----
	TB	0.79	0.43	1.70	1.30	1.00	1.90
24	ST	6.80	1.10	2.10	2.10	0.62	1.90
	TB	6.60	0.34	1.70	0.34	----	----

[a]Receptor number is expressed as fmol/mg protein. Kd expressed as nM.

TABLE E.7 [^{3}H] DEXAMETHASONE BINDING IN NUCLEAR EXTRACT[a]

Tag No.	Muscle	Concentration 0.2	0.4	0.8	1.6	3.2	6.4
0	ST	95.31	0.0	0.0	0.0	0.0	30.40
	TB	80.88	39.59	38.85	14.25	0.0	28.93
1	ST	0.0	8.23	17.60	0.0	0.0	20.80
	TB	77.39	0.0	18.86	15.08	0.0	34.73
2	ST	75.67	0.0	0.0	0.0	0.0	29.10
	TB	-----	-----	-----	-----	---	-----
3	ST	1.72	0.0	0.0	0.0	0.0	15.30
	TB	0.0	0.0	35.48	0.0	26.38	26.87
4	ST	63.96	0.0	4.37	0.0	0.0	26.53
	TB	8.75	0.0	0.0	0.0	0.0	10.96
5	ST	0.0	11.45	0.96	0.0	0.0	15.72
	TB	14.43	0.0	49.86	0.0	20.26	30.72
6	ST	46.08	33.12	0.0	0.0	0.0	29.73
	TB	47.55	61.75	0.23	0.0	0.0	33.74
7	ST	52.89	22.08	8.96	0.0	0.0	30.29
	TB	-----	-----	-----	---	-----	-----
8	ST	23.24	0.0	0.0	0.0	0.0	4.07
	TB	0.0	75.42	1.21	0.0	0.0	24.77
9	ST	36.34	0.0	0.0	5.98	0.0	12.65
	TB	1.95	92.19	0.0	0.0	0.0	32.36
10	ST	48.23	25.54	0.0	0.0	0.0	27.95
	TB	47.72	68.03	0.0	0.0	0.0	35.11
11	ST	-----	-----	-----	----	----	-----
	TB	0.0	0.0	0.0	0.0	0.0	0.0
12	ST	9.81	0.0	0.0	0.0	0.0	1.63
	TB	35.28	6.08	34.63	3.34	0.0	17.29
13	ST	-----	-----	-----	----	---	-----
	TB	0.0	19.75	0.0	0.0	0.0	19.71
14	ST	28.44	5.56	77.94	0.0	0.0	32.28
	TB	60.00	13.62	4.12	0.0	0.0	29.43
15	ST	75.68	0.0	0.0	0.0	16.61	32.05
	TB	10.52	0.0	0.0	0.0	0.0	7.43
16	ST	27.31	0.0	13.40	0.0	0.0	10.22
	TB	0.0	0.0	0.0	0.0	0.0	16.67
17	ST	0.0	0.0	0.0	0.0	0.0	14.51
	TB	67.30	29.09	0.0	16.87	0.0	35.40
19	ST	80.36	52.67	0.0	14.57	0.0	41.16
	TB	0.0	13.29	0.0	0.0	0.0	16.91
20	ST	0.0	0.0	0.0	0.0	0.0	3.36
	TB	0.0	20.35	0.0	0.0	0.0	18.11
21	ST	0.0	14.75	59.47	0.0	0.0	28.87
	TB	57.47	2.69	6.96	31.08	0.0	32.55
22	ST	27.20	14.23	18.29	0.0	0.0	25.37
	TB	86.97	58.62	0.0	0.0	0.0	40.93
23	ST	0.0	0.0	0.0	9.18	0.0	17.85
	TB	29.56	20.16	50.85	0.0	0.0	30.17
24	ST	64.39	30.65	67.77	0.0	0.0	43.43
	TB	0.0	0.0	0.0	0.0	0.0	10.21

[a]Concentrations are nM final concentrations. Data expressed as the percent of total binding constituting specific binding.

TABLE E.8 TOTAL (CYTOSOLIC AND NUCLEAR) [^{3}H] DEXAMETHASONE BINDING (DPM)

		Trenbolone Acetate					
		+			-		
Conc (nM)	Muscle	Total	NS	Counts	Total	NS	Counts
.20	ST	55.7	39.4	3827	970.9	76.0	3680.9
.40		86.7	111.4	7708	193.6	185.2	7195.8
.80		176.4	197.9	14709	488.9	325.5	15110.1
1.6		571.9	436.7	29519	501.1	715.8	30398.2
3.2		848.4	836.2	53553	1573.9	1505.9	81597.9
6.4		2068.6	1878.6	111959	5919.2	4378.5	129010.4
.20	TB	123.7	11.1	3109	132.1	21.4	4107.2
.40		35.6	41.6	6601	152.9	111.3	7957.2
.80		133.9	106.1	14601	195.5	194.4	15845.3
1.6		313.4	254.5	28032	373.7	480.9	31107.8
3.2		420.1	501.6	57050	749.0	870.9	61972.3
6.4		1017.4	1485.7	119272	1553.4	1958.4	130284.7
		Estradiol					
		+			-		
.20	ST	74.4	38.3	3587	952.2	77.1	3341.2
.40		120.4	137.1	7508	159.9	159.5	7396.4
.80		337.9	203.8	14071	327.3	319.6	15747.9
1.6		524.3	481.7	28964	548.8	670.8	30952.9
3.2		1309.6	1122.3	76750	1244.9	1411.6	60824
6.4		2209.9	1863.4	96814	3923.3	3136.2	128057.6
.20	TB	104.7	9.2	3854	151.1	23.3	3362.8
.40		86.9	33.5	7462	101.7	119.4	7095.9
.80		94.4	87.6	15303	235.0	212.9	15143.6
1.6		247.2	214.1	29551	439.9	521.2	29589.1
3.2		514.1	475.7	59836	654.9	896.8	59186.4
6.4		1040.4	1159.1	127404	1530.4	2285.0	122153.1

TABLE E.9 VERIFICATION OF SYNTHESIS AND DEGRADATION PROCEDURE[a]

Time	Buffer	Synthesis	Degradation
1	AP	.0017	.1579
	G	.0030	.2534
	GAP	.0014	.1972
2	AP	.0053	.1837
	G	.0121	.1597
	GAP	.0051	.2089
3	AP	.0015	.1739
	G	.0142	.1785
	GAP	.0071	.1364
4	AP	.0112	.2154
	G	.0148	.1654
	GAP	.0203	.2314
5	AP	.0243	.2572
	G	.0164	.1451
	GAP	.0178	.2031

[a]AP = acetate plus propionate, G = glucose, GAP = glucose plus acetate plus propionate. Synthesis was determined at .5, 1.5, 2.5, 3.5 and 4.5 h and expressed as nmol · mg wet tissue^{-1}. Degradation was determined at 1, 2, 3, 4, and 5 h. and expressed as nmol · mg wet tissue^{-1}.

APPENDIX F

PROTEIN TURNOVER IN SKELETAL MUSCLE INCUBATED WITH VOLATILE FATTY ACIDS AND GLUCOSE

ABSTRACT

Glucose increased ($p<.05$) protein synthesis compared to either acetate plus propionate (AP) or glucose, acetate and propionate (GAP) at .5 h. Substrate did not effect synthesis rate at any other time period. There were no substrate differences in degradation of skeletal muscle protein. Acetate and propionate appear to maintain or increase protein synthesis when muscle is incubated for longer periods of time. Short incubation times need not contain acetate and propionate.

INTRODUCTION

In fed ruminants, acetate passes through the liver and into the general circulation, where it is a major source of energy for resting muscle and is the precursor for fatty acid synthesis in adipose tissue. In the post-absorbtive state, glycogenolysis can supplement gluconeogenesis, which falls as the concentration of propionate in the portal blood declines. At the same time, lipolysis in adipose tissue increases and plasma free fatty acid concentration increases. These fatty acids are taken up by muscle and used as the primary energy source in those tissues.

Acetate diminishes glucose uptake, lactate release and glucose oxidation in the rat soleus muscle (Maizels et al., 1977). Glucose by itself has been reported to promote protein synthesis in isolated rat diaphragm (Woll and Krahl, 1959). In addition, glucose inhibits protein degradation and stimulates incorporation of [^{3}H] tyrosine into protein but in the absence of insulin, has no effect on skeletal muscle protein synthesis (Fulks et al., 1975).

The hypothesis that glucose, acetate and propionate may influence protein turnover in isolated skeletal muscle strips was explored.

Materials and Methods

Muscle samples were excised from the right and left semitendinosis muscle of one intact male lamb and immediately placed in oxygenated (95% O_2 - 5% CO_2), ice cold Krebs-Ringer bicarbonate, pH 7.4 containing five times the highest level of all amino acids found in sheep plasma and .5 IU insulin. The approximate time period between death and sample incubation was 10-15 min. Treatments were as follows: 1) 1 mM glucose (G), 2) 1.3 mM acetate and .29 mM propionate (AP) and 3) 1 mM glucose, 1.3 mM acetate and .29 mM propionate (GAP).

In vitro rates of protein synthesis were estimated by determining tissue [^{14}C] tyrosine uptake. Rate of degradation was estimated using cyclohexamide to block synthesis. Total tyrosine content of the incubation media and muscle homogenates used for protein degradation determination, was assayed fluorometrically, using a Turner fluorometer, equipped with a constant temperature door. Interstitial space (.25 ul/mg muscle) of the muscles was determined utilizing [^{14}C] inulin.

RESULTS AND DISCUSSION

Figure F.1. illustrates the time course of [^{14}C] tyrosine incorporation into skeletal muscle protein. Glucose increased ($p<.05$) protein synthesis compared to either AP or GAP at .5 h. There were no significant substrate differences in synthesis rate at any other specific time period, although AP and GAP demonstrated increases ($p<.05$) in synthesis rate with time. It appears that AP may inhibit glucose stimulation of protein synthesis at relatively short periods of incubation however, AP exhibited low synthesis rate as well indicating some direct involvement with the synthesis mechanism. At relatively long periods of incubation (> 1.5 h), synthesis rates did not change when glucose was added to the incubation media. Previous research has indicated the perfusion of the rat heart with free fatty acids, acetoacetate or 3-hydroxybutyrate decreased the uptake of glucose (Maizels et al., 1977). Our results indicate this relationship in skeletal muscle protein synthesis. Acetate and propionate appear to maintain protein synthesis when muscle tissue is incubated for long periods of time, possibly after glucose reserves are depleted. It has long been known that free

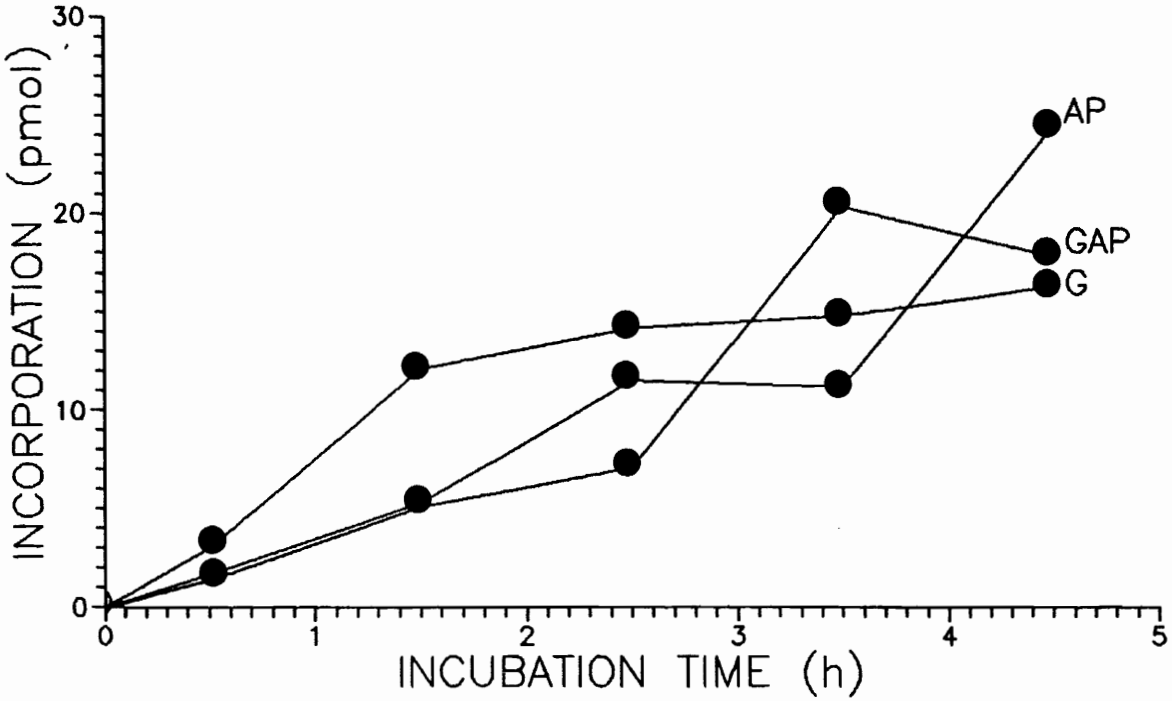

FIGURE F.1. INCORPORATION OF 14C TYROSINE INTO MUSCLE PROTEIN AS EFFECTED BY BUFFER COMPOSITION AND TIME.

fatty acids and ketone bodies replace glucose as a fuel in starvation and diabetes and may be doing so in an extended incubation period.

Table F.1. shows the effect of time and buffer on the degradation of muscle protein. There was a slight increase in degradation rate with G at .5 h while when AP was added to the buffer, a slight increase in degradation occurred at .5 h. There were no differences ($p>.10$) in degradation rates with time.

CONCLUSION

Previous research has indicated that acetate may decrease the utilization of glucose in skeletal muscle in vitro (Maizel et al., 1977)s. It is not clear whether this occurred in our system, since we did not measure specific enzymes involved in glucose metabolsim. However, with the addition of acetate, propionate and glucose to the incubation media, protein synthesis was less than when glucose alone was the substrate. Glucose appeared to stimulate synthesis of muscle protein, but at relatively short periods of incubation time. At longer periods of incubation AP appears to maintain protein synthesis, possibly when glucose reserves become depleted. Analysis of protein degradation indicated that substrate had no effect,

TABLE F.1 EFFECT OF TIME AND BUFFER COMPOSITION ON THE IN VITRO DEGRADATION OF SKELETAL MUSCLE PROTEIN[a]

Buffer	Time, (h)	Degradation[b]	SE[c]
Glucose	1	.25	.05
	2	.16	.05
	3	.18	.05
	4	.17	.05
	5	.15	.05
Acetate	1	.16	.05
+	2	.18	.05
Propionate	3	.17	.05
	4	.22	.05
	5	.26	.05
Glucose	1	.20	.05
+	2	.21	.05
Acetate	3	.14	.05
+	4	.23	.05
Propionate	5	.20	.05

[a] Each value is the mean of 3 determinations.
[b] Nmol· mg wet tissue^{-1}.
[c] Standard error of mean.

contradicting previous research suggesting that glucose inhibits skeletal muscle protein degradation (Fulks et al., 1975). The in vitro determination of muscle protein turnover is relatively less expensive and complicated than in vivo determinations. Commonly, with the in vitro procedure, degradation rates are greater and synthesis rates are less then those observed with in vivo techniques. Therefore, small changes in degradation rate that may result from treatments, are masked by the fact that incubated muscle tissue is in a state of nitrogen imbalance. Results from this study indicate that the addition of acetate and propionate to incubation buffers may be beneficial when incubating muscle tissue for longer periods of time in vitro. With shorter incubation times (.5 - 1.0 h), glucose appears to be a mandatory substrate when incubating ruminant skeletal muscle in vitro.

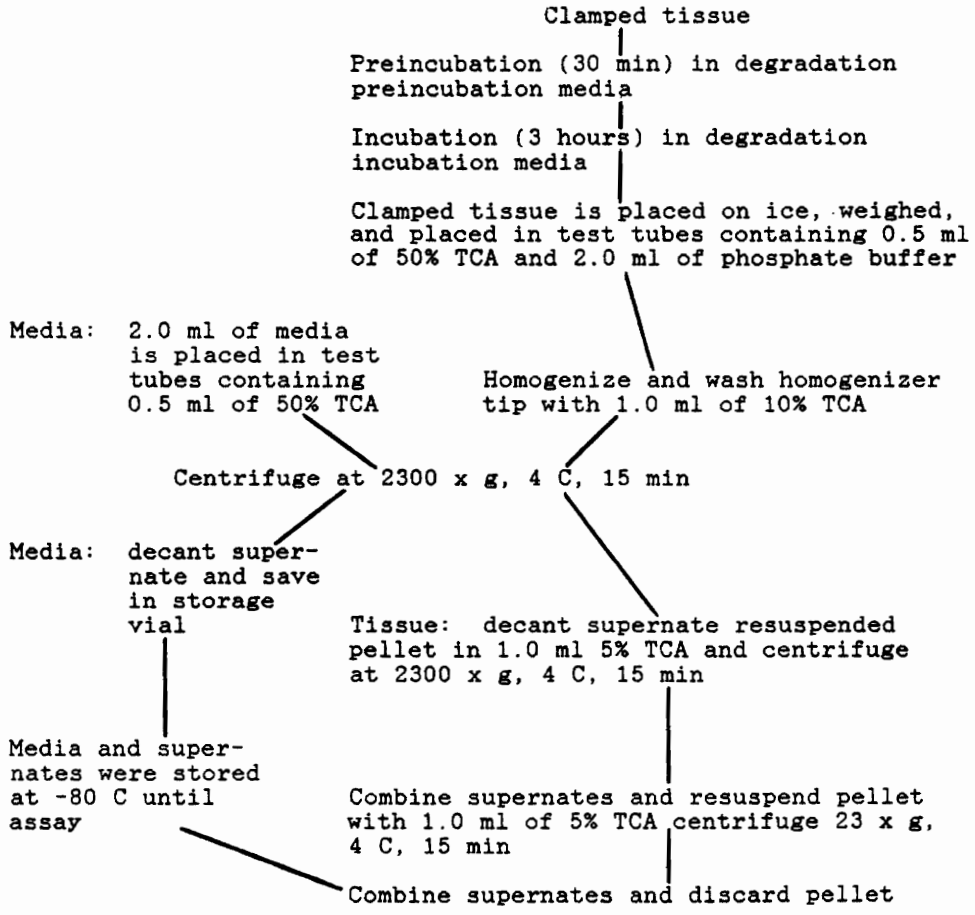

FIGURE A.1 SCHEMATIC OF DEGRADATION PROCEDURE

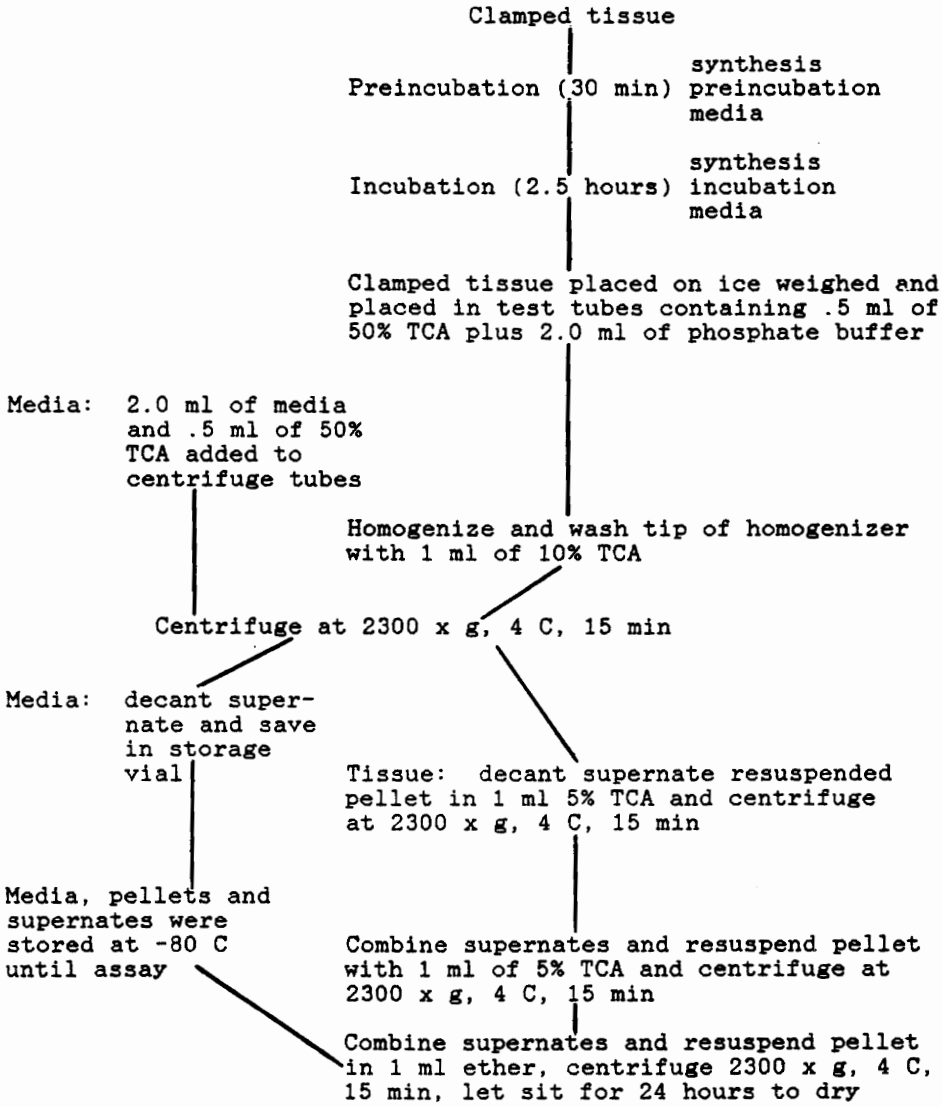

FIGURE A.2 SCHEMATIC OF SYNTHESIS AND INULIN PROCEDURE

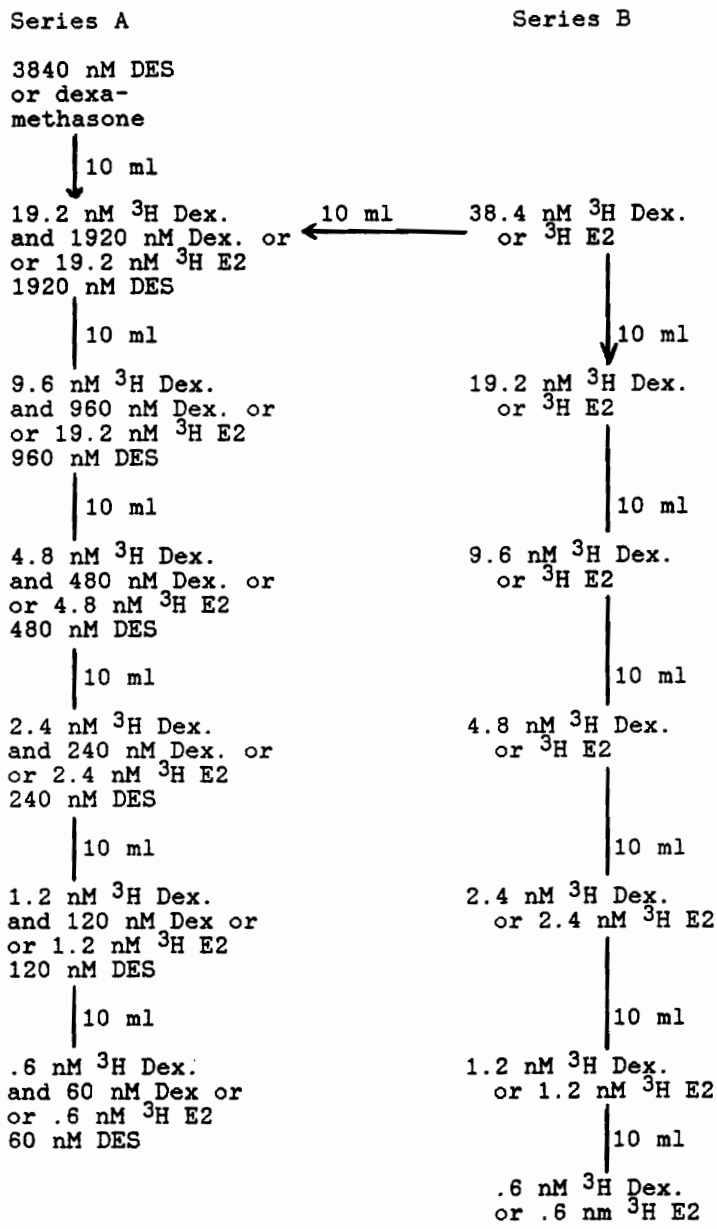

FIGURE A.3 SCHEMATIC OF DILUTIONS FOR RECEPTOR ASSAYS

$$\frac{\text{Amount of tyrosine in acid soluable supernate of the tissue (ug/ml)} + \text{Amount of tyrosine in acid soluable supernate of the incubation media (ug/ml)}}{\text{mg tissue}} - \frac{\text{Amount of tyrosine in acid soluable supernate of blank preincubation media (ug/ml)}}{\text{mg tissue}}$$

= ug tyrosine released/mg tissue for the time of incubation

Inulin:

$$\frac{\text{DPM in inulin pellet + DPM of supernate and combined washes of inulin pellet}}{\text{DPM/ml in the media after incubation}} \div \text{mg tissue}$$

= ul/mg tissue space that is taken up by inulin

FIGURE A.4 CALCULATION OF DEGRADATION RATE

$$\text{Intracellular Specific Activity (DPM/nmole)} = \frac{\underbrace{\text{Total TCA Soluabl DPM/mg tissue}}_{A} - \underbrace{\text{Inulin space ul/mg}}_{B} \times \text{DPM/ ul medium}}{\underbrace{\text{Total TCA Soluable Tyrosine of the blank (nmole/mg)}}_{C} - \underbrace{\text{Inulin space ul/mg}}_{B} \times \underbrace{\text{nmole Tyrosine/ul medium}}_{D}}$$

A: Total TCA Soluable DPM of the Synthesis Supernate:

DPM/ml Supernate x Total Volume of Supernate = Total TCA Soluable DPM of the Synthesis Supernate

Total TCA Soluable DPM of the Synthesis Supernate/mg of tissue sample = Total TCA Soluable DPM/mg tissue

B: Inulin Space of the Synthesis Media

Total DPM in the Synthesis Incubation Media After Incubation (DPM/ml)/1000 ul = DPM/ul Synthesis Media

DPM/ul of Synthesis Media x .252 ul of Inulin Space = DPM that represents Inulin Space

FIGURE A.5 CALCULATION OF SYNTHESIS RATE

C: TCA Soluable Tyrosine in the Blank Tissue

ug Tyrosine/ml of Supernate of the Blank Tissue Pellet x Total Volume of supernate = Total TCA Soluable Tyrosine for the Blank Tissue

Total TCA Soluable Tyrosine for the Blank Tissue/mg Tissue = TCA Soluable Tyrosine of the Blank/mg Tissue

TCA Soluable Tyrosine of the Blank/mg Tissue ÷ .181 = nmole of Tyrosine/ mg tissue

D: Inulin Space of the Soluable Blank

Total TCA Soluable Tyrosine in Degradation Incubation Media After Incubation ÷ 6 = ug/ml Tyrosine Released Over 30 min (the length of the preincubation)

ug/ml Tyrosine Released in Preincubation ÷ .181 ug/nmole = nmole/ml Tyrosine Released

nmole/ml Tyrosine Released ÷ 1000 ul = nmole/ul Tyrosine Released

nmole/ul Tyrosine Released x .252 ul = Inulin Space of the Acid Soluable Blank

$$\text{Synthesis} = \frac{\text{DPM/mg of Tissue for the Synthesis Pellet}}{\text{Intracellular Specific Activity DPM/nmole}} \quad \text{nmole/mg of Tyrosine Incorporated Over the Incubation Period}$$

FIGURE A.5

VITA

Randall S. Frey was born the son of Kathryn E. and A. Dale Frey on February 19, 1961 in Wauseon, Ohio. He has four brothers: William (1943-1961), Gene, David and Steven. He graduated from Pettisville High School in the year 1979. He enrolled in an Agricultural Business program at Northwest Technical College, Archbold, Ohio and received the A.A.B. degree in June of 1983. From there, Mr. Frey traveled to The Ohio State University and received the B.S. degree under the supervision of Dr. J.H. Cline. He initiated his graduate studies at Virginia Polytechnic Institute and State University on September, 1985.

He is member of the American Society of Animal Science.