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Use of cultured fibroblasts to study effects of hormones and growth factors and to compare growth of cells to animal growth

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Use of cultured fibroblasts to study effects of hormones and

growth factors and to compare growth of cells to animal growth

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INTRODUCTION

Growth may be defined as an increase in cell size (hypertrophy) or an increase in cell number (hyperplasia). Early in life when an animal is rapidly growing and increasing in size, growth occurs by hyperplasia and hypertrophy. After birth, growth results primarily from hypertrophy and continual renewal of cells in some tissues. Factors such as diet, environment, genetics, age and sex influence animal growth. Various growth factors and hormones have been identified that are involved in regulating the growth processes of the animal.

The techniques currently used in animal science to study growth are vastly more complex than those used fifty years ago. At one time, the way to evaluate a nutrient or drug was to measure the animal response as an increase or decrease in weight. Now, there exists the capability to measure the response at the cellular or subcellular level rather than the entire animal.

Most studies of cell growth have involved human or rodent derived cells with very little research being done with cells from farm animals. Fibroblasts are relatively easy to grow and maintain in culture conditions. The response of these undifferentiated cells to serum, growth factors and hormones may be measured as a change in DNA or protein synthesis. Cells become quiescent in a serum-free medium, deficient of growth factors. Quiescent cells can be stimulated to grow by the addition of serum or growth factors to the medium.

The objectives of this work were to develop a technique for collecting skin tissue from farm animals; to demonstrate bovine

fibroblasts respond to serum, hormones and growth factors; and to evaluate the growth potential of bovine and porcine fibroblasts in culture as an indicator of animal growth. These objectives were accomplished by collecting skin tissue; growing fibroblasts; supplying various amounts of serum, hormones and growth factors to these cultured fibroblasts; and measuring the uptake of radiolabeled leucine, as an indicator of protein synthesis, and uptake of radiolabeled thymidine, as an indicator of DNA synthesis. To evaluate the use of cell culture as an indicator of animal growth, explants were cultured from cattle and pigs. Cell growth was measured as thymidine incorporation, leucine incorporation and measurement of cell outgrowth from the explant. Cell growth was compared to animal weights at various ages.

REVIEW OF LITERATURE

Cell Culture

Cell culture was first described at the turn of this century (Harrison, 1907). Cell culture provides a relatively.homogenous population that may be used to study a wide variety of effects in a controlled environment (pH, temperature, osmotic pressure, etc.).

Fibroblasts are nontransformed, undifferentiated cells that are easily grown and propagated in culture. Proliferative ability of fibroblasts is dependent on age, tissue source and species. While studying the longevity of skin fibroblasts from 100 donors, the number of cell doublings significantly decreased as donor age increased (Martin et al., 1970). Others have reported similar findings, that is, skin fibroblasts from young donors generally have a longer life span than those from older donors (Wharton, 1984; Rosner and Cristofalo, 1979).

The tissue source of the fibroblasts is also important. The replication rates of fibroblasts derived from skin and lungs of human fetuses were different, lung cultures grew more rapidly and had longer lifespans than skin cultures (Schneider et al., 1977).

Satellite cells are undifferentiated cells found in muscle that have also been studied in culture systems. Satellite cells are responsible for repair and growth in postnatal muscle and are the primary supplier of muscle fiber nuclei postnatally (Allen et al., 1979). Satellite cells from old rats initiate DNA synthesis at a slower rate than cells from young or fetal rats and cells from older animals form smaller colonies than cells from younger animals (Schultz and Lipton, 1982). There is a relationship

between growth rate of turkeys and the ability of their serum to promote growth of cultured satellite cells (Doumit et al., 1990). Serum from faster growing birds was more stimulatory to satellite cells than serum from slower growing birds. An age related decline was seen with hen serum but not with tom serum. Satellite cells from 15 week old tom turkeys increased relative cell number (ratio of nuclei to start of the experiment) more than did cells from younger males.

Cell cultures have been used to examine the effects of food deprivation on the ability of serum from fasted pigs to promote cell growth (White et al., 1988). Sera were collected from crossbred barrows before and after a five day fast. Addition of 2% serum from fasted animals to the medium decreased mitogenic activity of L6 myoblasts and the decrease varied from pig to pig, with individual pigs responding differently to the stress of fasting. This decrease was believed due to the presence of an unidentified inhibitor, since fasted serum inhibited proliferation in the presence of five percent sera from nonfasted pigs. Addition of serum collected after feeding was resumed, increased mitogenic activity of L6 myoblasts.

Growth factors and hormones have been identified as being involved in regulating growth of animals as well as cells in culture. Most of the research examining fibroblast growth has centered on the use of cells from human or rodent origin. These actively multiplying cells move repeatedly through a cell cycle consisting of four stages: 1) mitosis (M), 2) G_1 ("gap" between mitosis and DNA synthesis), 3) synthesis of DNA (S) and 4) G_2 ("gap" between DNA synthesis and mitosis). A variety of environmental

conditions can cause cells to become quiescent. This G_1-like state is typically designated G_0 (Ham, 1981).

Maintenance and growth of cells, with growth implying an increase in total mass and volume (hypertrophy) along with an increase in cell number (hyperplasia), necessitates the presence of proper nutrients, growth factors, and hormones to meet the requirements of cells. "Nutrients" refer to chemical substances that are taken into the cell and utilized in biosynthesis, energy metabolism or act as catalysts in these processes. "Growth factors" historically are used to refer to any chemical substances that promote cellular multiplication. In current usage, growth factors are non-nutritive substances that control proliferation of cells by interacting with specific cellular receptors. "Hormones" classically are defined as chemical substances produced in one area of the body and transported in the blood to another target location for their effect. "Growth requirement" refers to virtually anything that has a positive effect on cellular multiplication (Ham, 1981). Thus, animal cell cultures require the addition of nutrients, growth factors and hormones to the culture medium to allow progression through the cell cycle.

Serum is a very complex mixture, which has traditionally been used to provide these growth factors, hormones and nutrients. Serum is included during culture to supply the growth requirement needs of cells. More complex media have been developed to allow the survival of cells without serum by supplementing specific compounds, such as growth factors and hormones, to the medium. Much work has centered on the identification of growth promoting activities of various compounds, including peptides and steroids.

Peptides

Peptides may be growth factors, which possess stimulatory actions on the proliferation of cells by affecting cell growth and differentiation. Peptides may be hormones, examples include growth hormone (GH) and insulin. Thyroxine, although not a peptide, is an amino acid derivative and is discussed with the peptide hormones. Hormones are involved in regulating growth of the animal.

Growth Factors

Growth factors are classed as either competence or progression factors on their ability to affect the cell cycle. Platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) act as competence factors to allow quiescent cells to enter the cell cycle in the presence of plasma-derived factors (O'Keefe and Pledger, 1983). Progression factors, such as epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I), allow cells to traverse the cell cycle.

Epidermal Growth Factor   EGF was first isolated from mouse mandibular glands by Cohen (1962). It was later determined that urogastrone, a protein found in urine of pregnant women, and EGF were the same compound (Gregory, 1975). EGF is synthesized as a precursor of 1217 amino acids with repeating amino acid sequences. The active EGF is a single polypeptide chain consisting of 53 amino acids with a molecular weight of approximately 6000 (Cohen, 1962; Scott et al., 1983; Gray et al., 1983).

EGF has *in vivo* and *in vitro* biological actions. EGF stimulates the proliferation of epidermal and epithelial tissue in animals. Injection of

EGF into newborn mice results in earlier tooth eruption and eyelid opening than nontreated mice (Cohen, 1962). EGF stimulates proliferation of fibroblasts, keratinocytes and epithelial cells. EGF presence is required for approximately 8 hours to generate a mitogenic signal in cells maintained under culture conditions (Waterfield, 1989). In a mouse keratinocyte cell line dependent on EGF for growth, an increase in DNA synthesis occurred 6 hours after the addition of EGF, with maximal stimulation of DNA synthesis occurring approximately 20 hours after EGF addition (Zendegui et al., 1988). The results of adding EGF to a variety of cells from various species were reviewed with a slightly longer exposure time of 10 to 12 hours necessary to initiate DNA synthesis (Carpenter and Cohen, 1979). Similarly, EGF stimulated DNA synthesis in quiescent human fibroblasts eleven hours after addition (Westermark and Heldin, 1985).

Varying amounts of EGF have been reported to produce a response in cultured cells. EGF increased thymidine incorporation in density-arrested EL2 rat fibroblasts with peak stimulation occurring at 2 ng/mL in the presence of bovine serum albumin and transferrin (Liboi et al., 1987). EGF alone, however, did not enhance thymidine incorporation. In human foreskin fibroblasts, the addition of EGF (10 ng/mL) increased cell density 212% as compared with control cells (Kohase et al., 1987). Ten ng/mL EGF addition resulted in the greatest thymidine incorporation in mouse embryo fibroblasts (Mulder and Childress-Fields, 1990) and 5 ng/mL gave the greatest incorporation of thymidine by mouse keratinocytes (Ristow and Messmer, 1988). EGF in serum-free or serum containing medium was mitogenic for human skin cells (Hosokawa et al., 1986). However, adding EGF to cultures of fibroblasts from numerous adult humans gave inconsistent

responses to EGF: cells from some donors increased thymidine uptake, cells from other donors decreased thymidine uptake and cells from different donors were unresponsive to EGF treatment (Lechner et al., 1989). The variation among individuals was not correlated with donor age or sex.

An additive effect of EGF with other growth factors and hormones may occur. The combination of EGF and IGF-I resulted in greater thymidine incorporation than did either growth factor alone in quiescent mouse fibroblasts (Ristow and Messmer, 1988). The effects of insulin were additive with EGF for stimulating cell proliferation of human fibroblasts, but the combination was not as effective as the addition of five percent serum (Shipley et al., 1989).

Protein synthesis of cultured cells can be affected by EGF addition. Chick embryo epidermal explants subjected to EGF treatment had greater lysine uptake during 48 hours of culture compared with cells not exposed to EGF (Hoober and Cohen, 1967).

Insulin-like Growth Factors   In 1957, rat serum was reported to stimulate incorporation of ^{35}S into incubated rat cartilage and serum from hypophysectomized rats lacked this activity (Salmon and Daughaday, 1957). Plasma was shown to stimulate cartilage sulfation because of the presence of nonsuppressible insulin-like activity (NSILA; Hall and Uthne, 1972). Highly purified NSILA stimulated sulfate and thymidine incorporation into cartilage. Mitosis was stimulated in chick embryo fibroblasts maintained in serum-free medium for 48 hours by NSILA addition (Morell and Froesch, 1973; Zapf et al., 1978b). Two peptides, NSILA-I and NSILA-II, were isolated and shown to have growth promoting action in cartilage and produced insulin-like effects (Rinderknecht and Humbel, 1976). These

peptides were renamed, insulin-like growth factor-I (IGF-I) and -II (IGF-II), because of these insulin-like effects (Rinderknecht and Humbel, 1978).

These polypeptides, which are evolutionarily related to proinsulin, have been called various names, such as, "sulfation factor" (Salmon and Daughaday, 1957), "somatomedins" (Daughaday et al., 1972) and insulin-like growth factors (Rinderknecht and Humbel, 1978).

Somatomedin A and C and IGF-I have the same chemical structure (Svoboda et al., 1980; Klapper et al., 1983; Engberg et al., 1984). IGF-I is a basic peptide composed of seventy amino acid residues with a molecular weight of 7649 (Rinderknecht and Humbel, 1978). Use of the term, somatomedin has decreased because it implied GH regulation. IGF-I is regulated by GH but IGF-II is not (Baxter, 1986; Hall and Sara, 1983). In 1987, it was recommended that the term IGF replace somatomedin (Daughaday et al., 1987).

The liver is the major source of circulating IGF-I, but many other tissues possess the capability to synthesize IGF-I (D'Ercole et al., 1984). In humans, IGF-I plasma concentrations are low at birth (Gluckman et al., 1983), rise throughout childhood, increase markedly at puberty and decline post-puberty (Hall and Sara, 1984; Zapf et al., 1981a; and Zapf et al., 1981b). A similar pattern of IGF-I concentrations in plasma is seen in beef cattle (Plouzek and Trenkle, 1991b).

Both IGF-I and IGF-II circulate in the blood bound to large molecular weight binding proteins (BPs; Clemmons and Van Wyk, 1981c; Hintz, 1984; Hintz and Liu, 1977; Van Wyk and Underwood, 1978). Unbound IGF is not found in the blood in significant amounts. The BPs may be involved in regulation of binding of IGFs to cell surface receptors (Baxter and Martin,

1989) or the circulating IGF-I/IGF BPs may provide a storage reservoir and prevent rapid fluctuations in concentrations found circulating in the blood (Zapf et al., 1978a; Zapf et al, 1984; Daughaday, 1982). Originally, it was believed there were two IGF BP regulating the concentrations of free IGFs. Currently, three distinct BP (IGFBP-1, IGFBP-2, and IGFBP-3) have been fully identified with amino acid sequences determined. The report from the IGFBP workshop held in Vancouver, Canada, (June, 1989) proposed the adoption of specific nomenclature for the various IGFBPs, using a letter prefix to designate species specificity for that IGFBP (Ballard et al., 1990).

The presence of serum binding proteins is unusual for a peptide hormone. The lack of BP help to explain why 1 to 50 ng/mL of IGF-I in culture conditions is sufficient to stimulate biological activity (Zapf et al., 1981b), whereas, total serum concentrations of IGF-I range from 100 to 1000 ng/mL. Acute IGF administration does not mimic the normal physiological condition because of a marked increase in free IGF in circulation which is unusual in the animal. The plasma half-life of IGF is estimated to be approximately twenty-four hours when bound to BPs (Sara and Hall, 1990), but, free IGF has a half-life of approximately ten minutes (Zapf et al., 1981a) which is similar to insulin (Sara and Hall, 1990).

Insulin-like growth factors interact with cell surface receptors. Early work indicated IGF-I competed with insulin for receptor sites on isolated adipocytes, liver membranes and chondrocytes (Hintz et al., 1972; Marshall et al., 1974; and Zapf et al., 1978b). It was believed that the IGF receptor mediated the growth promoting action of IGF and insulin (Nissley et al., 1977), while the metabolic effects of IGF and insulin were

controlled by the insulin receptor (King et al., 1980; Van Wyk and Underwood, 1978). Later work has shown insulin stimulates growth via the insulin receptor and IGF stimulates glucose transport via the IGF receptor (Nissley and Rechler, 1984). Competitive binding studies examining IGF receptors have identified at least two types of IGF receptors: 1) type I receptors which bind IGF-I better than IGF-II with insulin having limited binding and 2) type II receptors which bind IGF-II better than IGF-I and no binding of insulin. There is limited ability of IGF-I and IGF-II to bind to the insulin receptor (Daughaday, 1982; Daughaday et al., 1981). The affinity of the insulin receptor for IGFs is approximately one hundred times lower than for insulin (Steele-Perkins et al., 1988). Insulin at supraphysiological concentrations (1 μg/mL) has been shown to bind to the IGF-I receptor (Van Wyk and Underwood, 1978). The type I IGF receptor has considerable sequence homology with the insulin receptor which allows insulin, when present in high concentrations, to bind to this receptor (Massague and Czech, 1982). There are differences in receptor affinity and receptor number for IGF-I in human skin derived fibroblasts (Clemmons et al., 1986). Cells from fetal-newborn donors had greater receptor affinity for IGF-I than did cells from either juveniles (3-14 years) or older persons (72-96 years), when grown at low culture densities. Increasing culture density decreased the receptor affinity for IGF-I in the newborns and juveniles. There was no change in receptor affinity at high or low cell density for cells from older persons. Cells from fetal donors possessed the lowest concentrations of receptors at low densities. The fetal-newborn group did not exhibit differences in receptor number in response to differing cell densities. High density culture conditions

reduced the receptor concentrations on cells from juveniles and older persons.

IGF-I can stimulate synthesis of nucleic acids and cell replication in primary chick embryo fibroblasts (Zapf et al., 1978b) and in human fibroblasts (Rechler et al., 1974). However, in these experiments, IGF-I was not as good as serum for stimulating cells. IGF-I stimulated satellite cell proliferation in serum-free medium (Allen and Rankin, 1990). Human IGF-I or porcine insulin stimulated the rate of DNA synthesis in chick satellite cells grown in medium containing .02% serum (Duclos et al., 1991). Maximal IGF-I response occurred at 50 ng/mL and a 1000 fold higher insulin amount was required to produce a similar effect. The insulin response resulted from binding to the type-I IGF receptor. The combination of insulin with IGF-I was not additive, supporting the theory that a single type of receptor was involved. IGF-I was weakly mitogenic for fibroblasts from adult humans grown in monolayers without serum (Conover et al., 1983). Human hypopituitary serum was not mitogenic. Combining IGF-I with .5% human hypopituitary serum (HHS) dramatically increased thymidine incorporation over IGF-I or serum alone. Dexamethasone added with IGF-I in the presence of .5% HHS increased number of fibroblast cells more than any of these compounds alone (Rosenfeld et al., 1984). IGF-I with .5% HHS increased thymidine incorporation 107% and cell replication 169% in human fibroblasts, as compared with cells grown in serum-free medium (Cook et al., 1988). IGF-I induced DNA synthesis, as measured by thymidine incorporation, in PDGF primed competent Balb/c 3T3 cells but not in quiescent cells (Kojima, 1990).

The effect of passage (dispersal of cells resulting from enzymatic

treatment or dilution for purposes of subculturing) number on binding of IGF-I has been examined (Conover et al., 1987a). IGF-I treatment in early (6-15 passage) and late passage (36-42 passage) human cells resulted in equivalent stimulation of thymidine incorporation.

IGF-I stimulated leucine incorporation in chick embryo fibroblasts (Zapf et al., 1981a) and in human fibroblasts (Conover et al., 1985a).

Hormones

Certain hormones have been shown to regulate growth of skeletal and soft tissues. These hormones react with receptors to cause growth stimulation and there are interactions between hormones.

Growth hormone   Both human and bovine GH are 191 amino acid single chain polypeptides containing two intrachain disulfide bridges (Butt, 1975). It was proposed that the growth promoting activity of GH was mediated through the actions of the insulin-like growth factors (Salmon and Daughaday, 1957).

GH has both *in vivo* and *in vitro* activity. Feedlot steers receiving intramuscular injections of growth hormone have increased average daily gain and improved feed efficiency (Moseley et al., 1990). Fat content is reduced and protein content is increased in the carcass. Bulls and steers had highest IGF-I concentrations as compared with intact and ovariectomized heifers at 12 to 15 months of age (Plouzek and Trenkle 1991b) and also exhibited the highest average daily gain (Plouzek and Trenkle, 1991a). GH was higher in males than females. GH and insulin have been implicated in the production of IGF-I in cattle (Plouzek and Trenkle, 1991b).

GH stimulated human fibroblasts to undergo DNA synthesis in the

presence of platelet-poor plasma from a hypopituitary individual (Clemmons and Van Wyk, 1981b). GH has been shown to stimulate production of IGF-I in cultures of human skin and lung derived fibroblasts (Atkison et al., 1980; Clemmons et al., 1981a) and in rat fibroblasts from twenty-five day old rats but not from fetal rats (Adams et al., 1983).

The addition of GH (10 or 100 ng/mL) to serum-free medium did not alter thymidine incorporation of human fibroblasts (Cook et al., 1988). However, the addition of GH (10 ng/mL) with .5% human hypopituitary serum increased thymidine incorporation 127% and cell replication 172% compared with serum-free medium.

Insulin   Insulin has a molecular weight of 5734 and contains two chains, A (21 amino acids) and B (30 amino acids), which are linked through disulfide bonds (Guyton, 1968). Insulin has many functions, it enhances glucose metabolism, decreases blood glucose level, increases glycogen storage, and has a protein sparing effect in the animal.

The effects of insulin on growth have been studied in many types of cells, such as chick embryo fibroblasts (Temin, 1967; Temin, 1968), human fibroblasts (Hollenberg and Cuatrecasas, 1975), mouse 3T3 fibroblasts (De Asua et al., 1977a; De Asua et al., 1977b), rat liver cells (Leffert, 1974) and baby hamster kidney cells (De Asua et al., 1973).

In human breast cancer cells, insulin is a more potent mitogen than estradiol or EGF (Van der Burg et al., 1988). Using a combination of insulin with estradiol resulted in greater cellular proliferation than either hormone separately.

Insulin at levels from 10 to 100 μg/mL resulted in equal stimulation of thymidine incorporation by mouse embryo fibroblasts (Mulder and

Childress-Fields, 1990). However, response to insulin was not as great as seen from optimal concentrations of serum (Hollenberg and Cuatrecasas, 1975; De Asua et al., 1977a; De Asua et al., 1977b; Leffert, 1974).

Insulin in supraphysiological concentrations (10 μg/mL) enhanced epithelial cell proliferation in a small intestinal crypt cell line (Conteas et al., 1989). The presence of insulin with EGF increased the amount of EGF required to produce a proliferative response from 2 to 50 ng/mL.

Insulin addition increased incorporation of leucine into protein in chick embryo fibroblasts (Zapf et al., 1981a). Human fibroblasts also had increased leucine incorporation due to the presence of insulin in the serum-free medium (Conover et al., 1985a).

Thyroid hormones   Thyroxine (T_4) is an iodinated derivative of tyrosine and regulates metabolic processes in the animal. Triiodothyronine (T_3) has three atoms of iodine rather than four found in T_4. The effects of T_4 on animal growth have been well demonstrated. There is a highly significant curvilinear correlation between thyroid activity and growth rate in lambs, with growth being depressed in hypo- and hyper-thyroid lambs (Draper et al., 1968).

Female dwarf mice with pituitary glands that secreted very low amounts of GH, prolactin and thyroid stimulating hormone when injected with bovine GH and/or T_3, exhibited increased weight gain, tail length and liver cell size as compared with injection of saline (Fouchereau-Peron et al., 1981). The greatest response in tail length resulted from the combination of hormones; however, this was not statistically different from the two individual hormones. Dwarf mice given human GH and T_4 had increased

sulfate and thymidine incorporation into costal cartilage (Van Buul and Van den Brande, 1978). This was an additive response for the two hormones. The effect was age-dependent with greater incorporation seen in younger animals.

Hypopituitary dwarf mice have lower IGF serum levels than normal mice (Holder and Wallis, 1977). Treatment of dwarf mice with bovine GH or T_4 increased serum IGF level (primarily IGF-I) and increased growth. In neonatal and juvenile hypophysectomized rats, the combination of GH and T_4 restored growth to normal (Glasscock et al., 1991). T_4 replacement in the neonatal rat restored IGFBP-2 to normal levels. GH alone was effective in restoring IGF-I and -II to control values.

In cultured mice fibroblasts, T_4 bound to plasma membrane receptors with one-third the affinity of T_3 (Cheng, 1983). Cultured human kidney epithelial cells treated with T_3 and T_4 had increased thymidine uptake (Siegel and Tobias, 1966). T_4 stimulated an increase in cell number, whereas, T_3 stimulated protein synthesis and increased the number of nucleoli within the nucleus.

Thyroid hormones have been implicated in regulating EGF production (Hoath et al., 1983). Treatment of mice pups with T_3 elevated EGF levels, as measured by radioimmunoassay in neonatal skin and mandibular glands as compared with untreated pups. Cultured porcine aortic smooth muscle cells did not increase cellular replication following T_4 administration in serum-free medium or in medium containing platelet poor plasma supplemented with IGF-I following exposure to platelet derived growth factor (Clemmons, 1984).

Steroids

Steroid hormones contain the cyclopentanoperhydrophenanthrene nucleus and are classed on the basis of their effects. Examples of steroids include the glucocorticoids (such as hydrocortisone and dexamethasone), the mineralocorticoids (aldosterone) and the sex hormones (estrogen, progesterone and testosterone).

Glucocorticoids

Mixed results have been reported from the addition of glucocorticoids to cell cultures. Addition of hydrocortisone decreased DNA synthesis in chick embryo fibroblasts, but not in virus-transformed cells (Fodge and Rubin, 1975). Opposite results were observed in fetal lung fibroblasts, hydrocortisone consistently stimulated the growth of normal fetal lung fibroblasts and inhibited virus-transformed cells (Rosner and Cristofalo, 1979). Hydrocortisone increased cell proliferation in four lines of human fibroblasts derived from fetal lung and inhibited proliferation of one line (Kondo et al., 1985). Hydrocortisone stimulated cell proliferation in four lines of human skin fibroblasts and inhibited one cell line. There was no age dependent response to hydrocortisone by human skin fibroblasts, with age of donors ranging from 21 to 77 years. In contrast, earlier work had indicated fetal skin cultures were inhibited by glucocorticoid treatment; whereas, those from older donors reacted differently: five were stimulated, two were inhibited and four were unresponsive (Rosner and Cristofalo, 1979).

DNA synthesis was unaffected by hydrocortisone and insulin addition in early and late passage human fibroblasts (Germinario and McQuillan,

1985). These early and late passage cells were obtained from both young (average 20 years) and old (average 74 years) individuals.

An inhibition of amino acid incorporation was observed in rat and chick embryo fibroblasts from glucocorticoid addition (Murota et al., 1976; Baseman and Hayes, 1975). Others have seen a pronounced stimulation of amino acid uptake from glucocorticoid addition (Russell et al., 1982). Dexamethasone stimulated the uptake of α-aminoisobutyric acid, an amino acid analog, in cultured human skin fibroblasts (Hollenberg, 1977). Preincubating cells with dexamethasone, prior to growth factor addition was beneficial (Kaplowitz, 1987). Human fibroblasts derived from donors of various ages, pretreated with dexamethasone, increased uptake of α-aminoisobutyric acid and thymidine following IGF-I addition.

The growth promoting action of EGF was amplified by dexamethasone; whereas, dexamethasone alone did not increase human fibroblast density (Kohase et al., 1987). Dexamethasone enhanced the mitogenic action of EGF in human skin cells by approximately 50% (Baker et al., 1978). Mouse keratinocyte growth was inhibited by the presence of dexamethasone added with EGF (Zendegui et al., 1988).

Thymidine incorporation was enhanced by dexamethasone in human fibroblasts treated with IGF-I (Conover et al., 1985b) or growth hormone (Cook et al., 1988). Peak thymidine incorporation occurred 22 to 28 hours after IGF-I and dexamethasone addition (Conover et al., 1987a). Preincubation of dexamethasone with IGF-I gave a varied response between early and late passage human fibroblasts. Dexamethasone with IGF-I increased thymidine incorporation in early passage human fibroblasts (Conover et al., 1983; Conover et al., 1986; Conover et al., 1987a),

whereas, addition of dexamethasone to late passage cells did not enhance the effects of IGF-I (Conover et al., 1987a). Human lung fibroblasts also became less responsive to dexamethasone in the presence of insulin or EGF with increased age (Phillips et al., 1984).

Estrogen

Growth of rodents and cattle is affected by estrogen in different ways. Exogenous estrogen decreased the growth rate of rats (Heywood and Wadsworth, 1980). In mice, exposure to 640 ppb dietary estrogen for longer than ten weeks resulted in decreased body weights (Greenman and Delongchamp, 1979), whereas, a single subcutaneous injection of 2 mg/kg body weight estradiol (E_2) caused a significant increase in weight, cell count and protein and DNA content of liver in mice during a seven day experiment (Schwarzlose and Heim, 1970).

Exogenous estrogens improve weight gains of cattle (Burroughs et al., 1954; Brier et al., 1988). Cattle exhibit increased liver weight from exogenous estrogen treatment (Preston, 1975). The weight of the anterior pituitary, total amount of GH per animal and plasma insulin concentrations were also increased by exogenous estrogen administration (Trenkle and Burroughs, 1967). Steers treated with E_2 implants had increased IGF-I levels as compared with nonimplanted steers (Brier et al., 1988).

E_2 alone was slightly mitogenic in human breast cancer cells (Van der Burg et al., 1988). These cells were grown in medium containing inactivated fetal bovine serum. However, E_2 (10^{-9} M) with insulin (10 or 100 ng/mL) stimulated thymidine incorporation more than insulin alone. This response was not seen at high insulin concentrations, only suboptimal

insulin concentrations required the presence of E_2. IGF-I production may be induced by the presence of estrogen in human breast cancer cells (as reviewed by Dickson and Lippman, 1987).

Estrogen administration did not stimulate mitogenesis in chick embryo cells in serum-free medium supplemented with insulin (Baker et al., 1979). Similarly, the addition of E_2 with IGF-I and .5% human hypopituitary serum did not increase thymidine incorporation in adult human fibroblasts (Conover et al., 1983).

Testosterone

In steers or bulls, trenbolone acetate (TBA), a testosterone analog, did not increase average daily gain (Hunt et al., 1991). However, TBA with estrogen increased gain and feed intake in steers but not in bulls during the growing phase. Steers receiving a combination of TBA and estrogen had increased serum IGF-I levels as compared with nonimplanted controls.

Serum from rats treated with TBA, stimulated greater satellite cell proliferation than serum from untreated rats (Thompson et al., 1989). Satellite cells from TBA-treated rats were more responsive to IGF-I than cells from untreated rats. However, direct addition of TBA to cultured satellite cells had no effect on proliferation.

Testosterone did not have any mitogenic stimulation of chick embryo cells (Baker et al., 1979). Testosterone in the presence of IGF-I and .5% human hypopituitary serum did not increase thymidine incorporation as compared to IGF-I and human hypopituitary serum for adult human fibroblasts (Conover et al., 1983). Testosterone did not stimulate the uptake of the

amino acid analog, α-aminoisobutyric acid in human skin-derived fibroblasts (Hollenberg, 1977).

SECTION I.

CULTURE OF BOVINE FIBROBLASTS AND
THEIR RESPONSE TO SERUM AND HORMONES

INTRODUCTION

Fibroblasts are undifferentiated cells that are easily maintained and propagated in culture. Cells from one individual may be used in numerous experiments. Most research has centered on the use of human (Conover et al., 1987a; Conover et al., 1987b; Conover et al., 1989; Chen and Rabinovitch, 1990a; Chen and Rabinovitch, 1990b; Colige et al., 1990) and rodent-derived (Liboi et al., 1987) cells. Human samples are normally collected from the forearm (Cooper and Goldstein, 1973), the inner area of the upper arm (Sly and Grubb, 1979) or from genital skin (Griffin et al., 1976). Because of hair coat, size of animals and limited accessibility to different parts of the body, samples of skin are not easily obtained from cattle.

One objective of this research was to develop a culture system for bovine fibroblasts. A method is described for obtaining skin biopsies and propagating fibroblasts from the ears of cattle. A second objective was to study the effects of hormones on growth of bovine fibroblasts because cattle seem to respond differently to exogenous estrogen than do rodents (Brier et al., 1988; Heywood and Wadsworth, 1980).

MATERIALS AND METHODS

Obtaining a Skin Biopsy

<u>Materials</u>

Ear notcher, normally used for identifying baby pigs or calves

Sterile 100 mL glass bottle containing approximately 25 mL transport
   medium

<u>Methods</u>

The lower portion of the bovine ear was selected for the biopsy site,
with various sizes of animals sampled. The ear notcher cut a triangular
tissue sample approximately .5x.5x.5 cm in size. The tissue sample was
dropped into a sterile 100 mL screw capped glass bottle containing 25 mL
transport medium made up of Ham's F-12 medium (Sigma Chemical Co., St.
Louis, MO), prepared according to instructions, and contained 10% fetal
bovine serum (FBS; Gibco Laboratories, Grand Island, NY), $3x10^5$ U/L
penicillin (Sigma Chemical Co., St. Louis, MO), 300 mg/L streptomycin
sulfate (Sigma Chemical Co., St. Louis, MO) and $2.4x10^5$ U/L nystatin (Sigma
Chemical Co., St. Louis, MO). Tissue sections were plated upon return to
the laboratory or within twenty-four hours. When subsampling at a later
time, the bottle containing sample was placed in a 4 C refrigerator until
time of plating.

Establishment of the Cell Strain

<u>Materials</u>

60 mm sterile petri dish (Nunc, Roskilde, Denmark)

Stadie-Riggs Microtome (Thomas Scientific, Swedesboro, NJ)

100 mm sterile petri dish (Nunc, Roskilde, Denmark)

Scalpel with No. 10 blade, sterilized

Flat-tipped forceps, sterilized

Mouse-tooth forceps, sterilized

Plastic pipette tip (Rainin Instrument Co., Inc., Woburn, MA),
sterilized

18 mm round cover slips, No. 1 thickness (Fisher Scientific Co.,
Chicago, IL), sterilized

High vacuum grease (Dow Corning, Midland, MI), sterilized

70% alcohol

Ham's F-12 medium, containing 10% FBS, 1×10^5 U/L penicillin, 100 mg/L
streptomycin sulfate and 2.4×10^5 U/L nystatin. Medium was
prepared according to Sigma instructions with the addition of
FBS and antimicrobials.

Alcohol soaked cloth

Glass 5 mL pipette (Fisher Scientific Co., Chicago, IL)

Container for waste medium

Methods

The "plating" of a tissue biopsy sample was performed in a laminar-
flow hood to prevent bacterial contamination of the culture. The aim was
to slice the sample into small pieces to allow outgrowth of fibroblasts.

The medium and sample of tissue were poured from the glass bottle
into a sterile 100 mm petri dish. With flat-tipped forceps the sample was
placed on the base of the microtome rinsed with 70% alcohol. The epidermis

was sliced off and discarded. The remaining tissue was turned over and the other epidermal layer was removed and discarded. The remaining dermal sample was sliced to yield a tissue layer approximately .5 mm thick. Depending on the thickness of the remaining tissue, other slices of tissue were cut. Flat-tipped forceps rinsed in alcohol were used to gently place .5 mm layer of tissue in the sterile lid of the 100 mm petri dish. The scalpel, rinsed in alcohol, was used to cut 1 x 1 mm squares of tissue (.5 mm thick).

One piece of tissue was transferred on the scalpel blade tip to three dry 60 mm sterile petri dishes, two other pieces of tissue were placed within a semi-circular area to fit underneath a circular glass coverslip. A sterile plastic pipette tip was used to place a spot of sterile high vacuum silicone grease on the petri dish, near the tissue pieces. Mouse-toothed forceps were used to place a glass coverslip over the tissue pieces and silicone grease. The coverslip was gently pressed down onto the tissue pieces and silicone grease. The tissue pieces were located near the edge of the coverslip. The coverslip was used to hold the tissue pieces in place to allow outgrowth of cells. A small amount of medium was added near the edge of the coverslip using a sterile pipette. The medium flowed underneath the coverslip by capillary action. The remainder of the 5 mL of medium was added directly on top of the coverslip. Air bubbles were not allowed to remain underneath the coverslip. A permanent marker was used to label the dish with appropriate information, such as animal identification number and date.

The dishes were placed in a humidified, 5% carbon dioxide atmosphere incubator, maintained at 37 C. Dishes were left undisturbed for ten days

to allow outgrowth of cells. After ten days, dishes were examined with an inverted light microscope to determine if outgrowth or microbial contamination had occurred.

On the tenth day, the "spent" medium was removed and replaced with fresh medium. This was done in a laminar flow hood by carefully pouring off the old medium into a waste container. After pouring off medium, the edge of the dish was wiped with an alcohol soaked cloth to remove any medium that may have gotten on the outside of the dish. Five milliliters of fresh Ham's F-12 medium (same medium and technique as used in plating) was added to replace the "spent" medium. The medium was replaced every second day until outgrowth from the explant covered half of the dish. This occurred in approximately three weeks following plating.

Propagation of Cells

Materials

    60 mm sterile petri dish

    Trypsin (Sigma Chemical Co., St. Louis, MO)

    Centrifuge tubes (sterile)

    Ham's F-12 medium

    Pasteur pipette (sterile)

    Parafilm "M" (American National Can, Greenwich, CT)

Methods

After growth had covered approximately half of the dish, cells were harvested and transferred to a new dish. In the laminar flow hood, medium was poured off and dish was rinsed with 1.5 mL trypsin (contains 2.5 g

trypsin/L dissolved in Hanks Balanced Salt Solution) to remove any
remaining medium. Two milliliters of fresh trypsin were added to the dish.
The dish was returned to the incubator for 13 minutes. After incubation
with trypsin, the dish was returned to the laminar flow hood and a pasteur
pipette was used to dislodge the coverslip. Cells were visible, trypsin
caused the cells to detach from the dish and assume a spherical shape.
Five milliliters of sterile Ham's F-12 medium was added to the cells, the
mixture was gently pipetted to disperse cells. The medium containing cells
was placed in a sterile centrifuge tube using a pasteur pipette. The tube
was covered with parafilm, because the centrifuge was located outside of
the laminar flow hood. The medium containing cells was centrifuged for one
minute at 20 x g to collect cells. The upper portion of medium was gently
pipetted off to prevent disturbance to the cells sedimented at the bottom
of the tube. Approximately 1 mL of medium was left in the tube, which
contained the cells. Approximately 4.5 mL fresh medium were added to the
tube, a pasteur pipette was used to gently disperse cells and cells were
transferred to a sterile 60 mm dish. Every second day, the medium was
replaced with 5 mL fresh Ham's F-12 solution which contained 10% FBS and
antimicrobials. After approximately five days, cells were ready for
replating. An inverted light microscope was used to determine if cells
were reaching confluency. Prior to confluency, cells were "trypsinized" as
previously described except cells were placed in a sterile 100 mm dish.
Every third day, the medium was replaced with 10 mL fresh Ham's F-12
solution containing 10% FBS and antimicrobials. After approximately a
week, cells were ready to be replated, prior to confluency. The medium was
poured off, the dish was rinsed with 2 mL trypsin, 3 mL fresh trypsin was

added and returned to the incubator for thirteen minutes. After incubation, 5 mL Ham's F-12 containing 10% FBS and antimicrobials was added to the dish. A pasteur pipette was used to transfer cells to a sterile centrifuge tube, the tube was covered with parafilm and centrifuged for one minute at 20 x g. A pasteur pipette was used to remove approximately 7 mL medium without disturbing cells in the bottom of the tube. Approximately 4.5 mL fresh Ham's F-12 containing 10% FBS and antimicrobials were added to the centrifuge tube and the mixture was gently pipetted to disperse cells evenly throughout the medium. Cells were divided evenly between two sterile 100 mm dishes. Ham's F-12 medium was added to the dishes to result in 10 mL volume. The medium was replaced with 10 mL fresh Ham's F-12 solution every third day and cells were divided prior to confluency. Cells were replated at approximately 10 day intervals.

<div align="center">Experimental Use of Fibroblasts</div>

<u>Materials</u>

Materials were purchased from the following sources: MCDB 201 medium, Ham's F-12 medium, penicillin, streptomycin sulfate, nystatin, trypsin and dexamethasone from Sigma Chemical Company (St. Louis, MO); leucine (14-C) and thymidine (3-H) from Amersham International (Amersham, UK); fetal bovine serum from Gibco Laboratories (Grand Island, NY); Universol ES from ICN Biochemicals, Inc. (Irvine, CA); petri dishes from Nunc (Roskilde, Denmark); testosterone, beta-estradiol and L-thyroxine from ICN Nutritional Biochemicals (Cleveland, OH); and insulin from Eli Lilly Inc. (Indianapolis, IN). GH was purified using the methods of Dellacha and

Sonnenberg (1964), Wallis and Dixon (1966) and Lorenson and Ellis (1975), as modified by J.L. Bobbitt (Eli Lilly Inc., Indianapolis, IN).

Methods

Cells were obtained and cultured as described above. Cells from third to tenth passage were used in the following experiments, with cell strains from different animals being utilized in different experiments. Cell count was approximated by use of a hemacytometer following treatment of dishes with trypsin. Cells were plated in triplicate 35 mm dishes containing 3 mL Ham's F-12 medium with 10% FBS and antimicrobials at 1.5 - 2.0 x 10^4 cells/mL. Forty-eight hours later the medium was changed to MCDB 201, prepared according to instructions with 1 x 10^5 U/L penicillin, 100 mg/L streptomycin sulfate, 2.4x10^5 U/L nystatin and containing 0, 1 or 10% FBS. Cells were not disturbed for 48 to 72 hours to allow the cells to become quiescent. Medium was then replaced with 3 mL MCDB 201 containing 14-C-leucine (approximately 90,000 dpm/dish, 57 mCi/mmol specific activity) and remained for the duration of the experiment. 3-H-thymidine (approximately 105,000 dpm/dish, 89 mCi/mmol specific activity) was added two hours prior to harvest of cells.

Hormone additions and final concentrations used were: control - no hormones (C), dexamethasone (D) 25 ng/mL, estradiol (E_2) .5 ng/mL, growth hormone (GH) 50 ng/mL, insulin (I) 2.5 ng/mL, testosterone (Tes) .5 ng/mL and thyroxine (T_4) 100 ng/mL. D, E_2 and Tes were dissolved in 95% ethanol, I was dissolved in pH 2.0 water and GH and T_4 were dissolved in MCDB 201 medium.

Cells were harvested 48 hours after addition of hormones. The medium

was aspirated from the dish and each dish was rinsed with MCDB 201 medium. To dissolve the cells, 1 mL of 3 N NaOH was added and incubated for one hour at room temperature. Dissolved cells were placed in a glass scintillation vial. The dish was rinsed with 1 mL deionized water followed with .5 mL 6 N HCl to bring pH of the dissolved cells in the vial to near neutral. Universol ES (ICN Biochemicals Inc., Irvine, CA) was added to the vial for liquid scintillation counting. Thymidine and leucine incorporation were measured as dpm per dish.

Statistical Analysis

Data were analyzed by analysis of variance using the General Linear Models procedure of SAS (SAS, 1989). Results were expressed as least squares means, with differences between means separated on the basis of least significant differences using the predicted difference option (SAS, 1989).

RESULTS

Examination with an inverted light microscope revealed fibroblasts were growing from the tissue explant. The fibroblasts surrounded the explant and were spindle shaped, approximately double in length as compared with width of the cell. The cells adhered to the surface of the dish with the cells at the outer fringes of the outgrowth being more disperse and randomly oriented than ones near the explant. With increasing cell number, orientation of the fibroblasts occurred with cells becoming parallel with each other. When a monolayer of fibroblasts covered the surface of the dish, confluency occurred and the cells ceased to grow. After cells were subjected to trypsin and replated, the cells became separated and randomly oriented. As cell numbers increased and they became more crowded, there was more orientation of cells in parallel rows.

Serum

The results from cells exposed to three levels of FBS for 48 hours are shown in Table 1. Cultured fibroblasts responded to FBS by increasing both thymidine and leucine incorporation. Results from cells harvested at one hour intervals from 24 to 48 hours after the addition of FBS to the medium are given in Figures 1 and 2. Thymidine and leucine incorporation were greatest by cells grown in the presence of 10% FBS ($P < .05$). Cells grown in medium lacking serum had the lowest thymidine and leucine incorporation.

Hormones

The results of addition of individual hormones to cultured

Table 1. Response of cultured bovine fibroblasts to 0, 1 or 10% FBS after
          48 hours

FBS	Thymidine		Leucine	
%	n	dpm±SE	n	dpm±SE
0	5	117 ± 24^a	7	176 ± 20^a
1	6	280 ± 22^b	6	1112 ± 21^b
10	6	818 ± 22^c	6	2525 ± 21^c

[a,b,c]Means without the same superscript within each column differ
(P < .05).

fibroblasts are given in Table 2. In serum-free medium, D or E_2 decreased
thymidine incorporation. I, T_4 or GH treated cells were not different
from C for thymidine incorporation in serum-free medium. The addition of
1% FBS overcame the depression in thymidine uptake resulting from the
addition of E_2. In the presence of 1% FBS, I increased thymidine
incorporation as compared with control cells. The addition of 1% FBS did
not overcome the depression in thymidine incorporation due to the addition
of D.

In serum-free medium, D or I decreased leucine incorporation as
compared with the control cells. GH, E_2 or T_4 treatment did not alter
leucine uptake. Adding 1% FBS did not prevent depression of leucine
incorporation caused by D. E_2 and GH depressed leucine incorporation,
whereas, addition of I was not different from the control cells. One
percent FBS with T_4 increased leucine incorporation.

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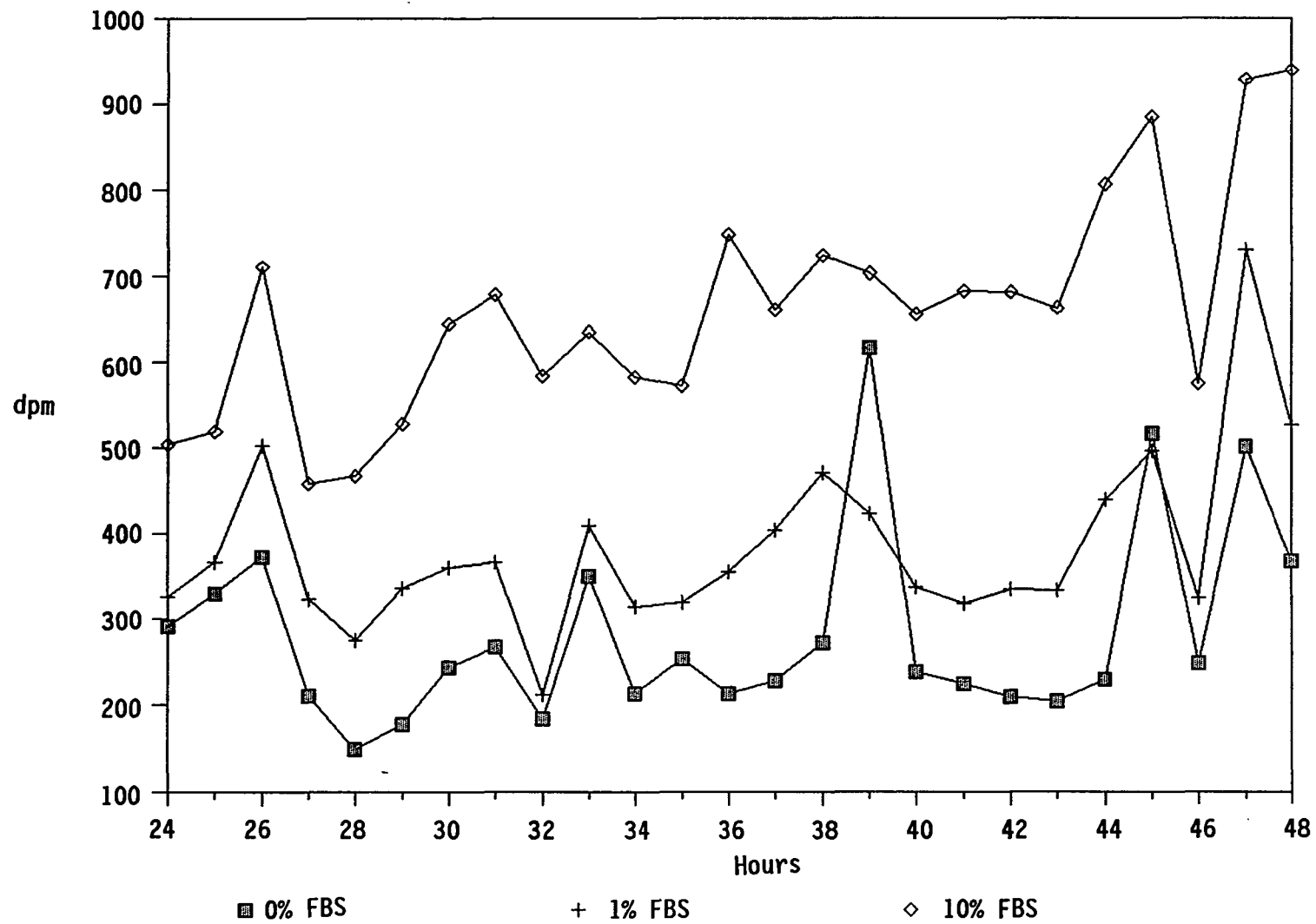
Figure 1.   Thymidine incorporation 24 to 48 hours after FBS addition

□ 0% FBS   + 1% FBS   ◇ 10% FBS
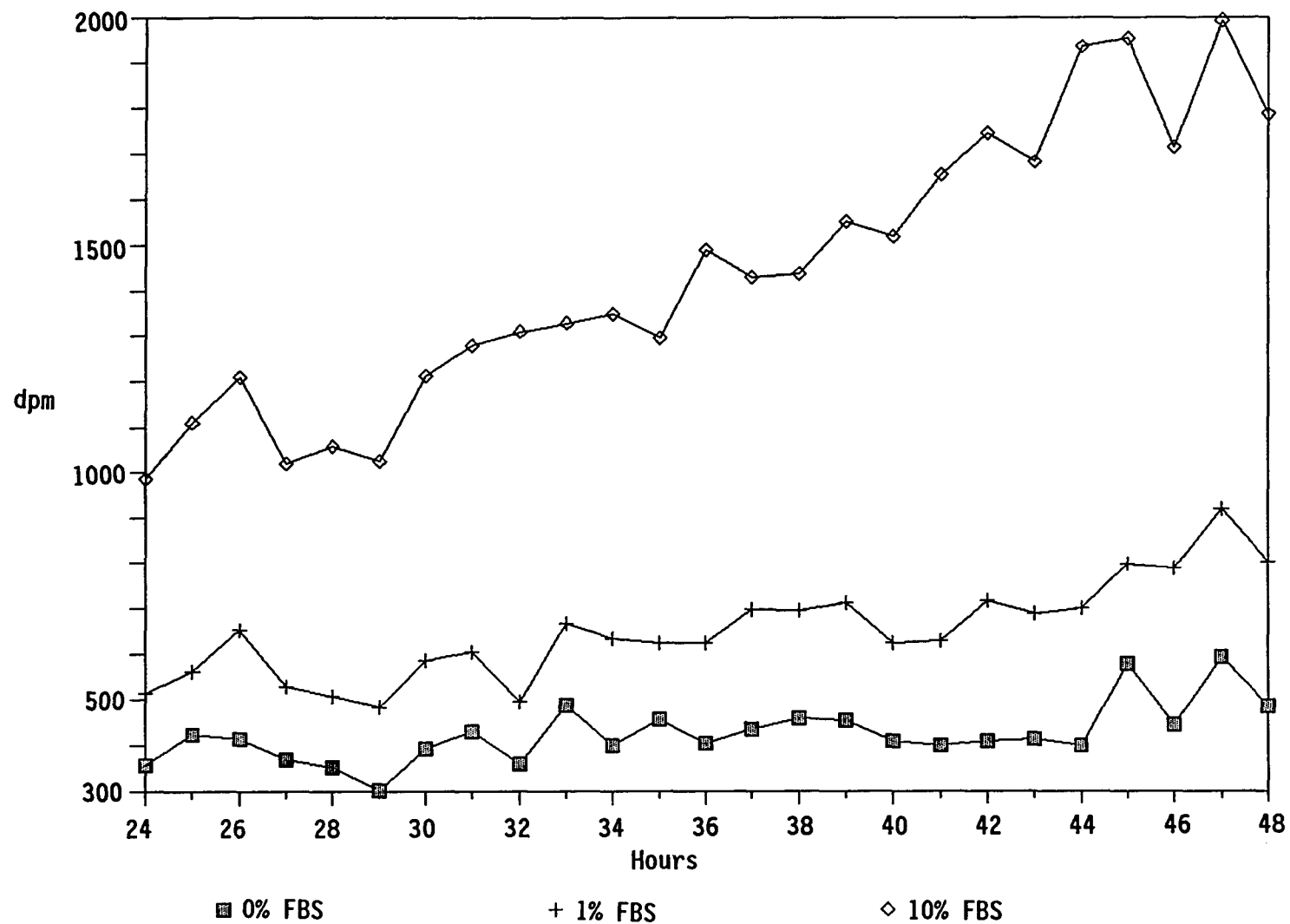
Figure 2. Leucine incorporation 24 to 48 hours after FBS addition

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Table 2. Bovine fibroblasts cultured for 48 hours in the presence of hormones and 0 or 1% FBS

	Thymidine				Leucine			
	0% FBS		1%		0% FBS		1%	
Treatment	n	dpm±SE	n	dpm±SE	n	dpm±SE	n	dpm±SE
C	20	485±27[a]	17	678±30[a]	17	1602±44[a]	16	3447±45[a]
Dex	7	356±46[b]	7	439±46[b]	5	1346±81[b]	6	2914±74[b]
E_2	7	358±46[b]	7	702±46[a,c]	6	1440±74[a,b]	6	3263±74[c]
GH	7	456±46[a,b]	7	710±46[a,c]	7	1584±69[a,c]	6	3266±74[c]
I	6	388±50[a,b]	6	796±50[c]	8	1446±64[b,c]	6	3365±74[a,c]
T_4	6	459±50[a,b]	4	792±61[a,c]	6	1454±74[a,b]	6	3679±74[d]

[a,b]Means without the same superscript within each column differ (P < .05).

Table 3. Response of cultured fibroblasts to hormones after 48 hours in 1% FBS

	Thymidine		Leucine	
Treatment	n	dpm±SE	n	dpm±SE
C	3	140±14[a]	3	1182±50[a]
I, E_2, GH	3	122±14[a]	3	919±50[b]
I, T_4, GH	3	119±14[a]	3	1063±50[a,b]
I	2	178±17[a,b]	2	1155±61[a,b]
Tes, E_2, GH, T_4	3	113±14[a]	3	1009±50[b]
T_4, GH	3	199±14[b]	3	1048±50[a,b]

[a,b]Means without the same superscript within each column differ (P < .05).

The cells grown in the presence of 1% FBS and treated with hormone combinations are given in Table 3. The addition of T_4 and GH increased thymidine uptake in cells cultured in 1% FBS. No combination of hormones or insulin alone increased leucine uptake.

The results from addition of combinations of hormones to cultures of fibroblasts derived from a different animal and grown with or without FBS are shown in Table 4. In serum-free medium, the combination of I, GH and E_2 stimulated thymidine uptake, but inhibited it in 1% FBS. T_4 and GH did not alter thymidine incorporation from that of the control cells in serum-free medium. In 1% FBS, thymidine incorporation was inhibited by T_4 and GH. No combination of hormones increased leucine incorporation in 0 or 1% FBS. In 1% FBS, all combinations of hormones except I, T_4 and GH inhibited leucine incorporation as compared with the control cells.

Table 4. Cultured bovine fibroblasts response to combinations of hormones after 48 hours in 0 or 1% FBS

		Thymidine			Leucine		
		0% FBS		1%	0% FBS		1%
Treatment	n	dpm±SE	n	dpm±SE	n	dpm±SE	n	dpm±SE
C	2	97 ± 23^a	3	270 ± 19^a	3	315 ± 24^a	3	451 ± 24^a
I, GH, E_2	2	248 ± 23^b	2	167 ± 23^b	2	$292\pm30^{a,b}$	3	310 ± 24^b
I, T_4, GH	3	$146\pm19^{a,b}$	3	250 ± 19^a	3	355 ± 24^a	3	$433\pm24^{a,b}$
I, T_4, GH, E_2	3	119 ± 19^a	3	$230\pm19^{a,b}$	3	$292\pm24^{a,b}$	3	368 ± 24^b
T_4, GH, Tes	3	95 ± 19^a	3	189 ± 19^b	3	241 ± 24^b	2	358 ± 30^b
T_4, GH	3	103 ± 19^a	3	144 ± 19^b	3	$251\pm24^{a,b}$	3	311 ± 24^b

[a,b]Means without the same superscript within each column differ (P < .05).

DISCUSSION

To our knowledge, this is the first use of ear biopsies to obtain cells from cattle for culturing. The sample could be easily obtained within a few minutes while the animal was restrained in a head-gate without the administration of anesthetic. Animals could also be sampled again at later times.

Early attempts involving removal of hair (shaving with a razor or chemical hair remover) and sterilization (iodine surgical scrub and alcohol) prior to tissue biopsy were no more effective in preventing microbial contamination than increasing antibiotic concentration in the transport medium and use of a microtome to remove the epidermis. Rinsing the skin sample in a weak chlorine solution or passing sample through a flame prior to slicing did not reduce contamination compared with the method described. It is very important to maintain conditions as sterile as possible to prevent microbial contamination of the cultures. Viable tissue was obtained, which allowed outgrowth of fibroblasts. Resulting fibroblasts could be used in studies several months after obtaining sample from the animal, if the cells continued to grow and were replated.

MCDB 201 medium was developed to allow the growth of fibroblasts without the addition of serum. However, adding serum to this medium increased both thymidine and leucine uptake of bovine fibroblasts. Ten percent FBS dramatically increased leucine incorporation by bovine fibroblasts. FBS contains nutrients, hormones and growth factors to meet the growth requirements of fibroblasts.

In serum-free medium, no hormone by itself was mitogenic to bovine fibroblasts. The lack of response from GH addition in serum-free medium

was similar to findings with human fibroblasts (Cook et al., 1988). In cattle, GH increased rate of gain (Moseley et al., 1990) with IGF-I being credited for this growth promotion (Brier et al., 1988) because GH stimulates IGF-I production (Salmon and Daughaday, 1957). IGF-I has been shown to stimulate thymidine and leucine incorporation in human fibroblasts (Conover et al., 1983; Conover et al., 1985b).

It has been reported that glucocorticoids decreased the rate of progression of chick embryo fibroblasts through the G_1 phase of the cell cycle resulting in fewer cells synthesizing DNA at a given time (Fodge and Rubin, 1975). This same phenomenon may have occurred in the bovine fibroblasts, and would explain the resulting decrease in thymidine incorporation following D addition to the cells. The factors contained in 1% FBS were not sufficient to overcome the depressing effect of D on bovine fibroblasts. However, adding 10% FBS overcame the depression due to the addition of D (data not shown).

Exogenous estrogens improved weight gain of cattle (Burroughs et al., 1954; Brier et al., 1988). Animals treated with E_2 have increased liver (Preston, 1975) and anterior pituitary weights and circulating levels of GH and I (Trenkle and Burroughs, 1967). The bovine fibroblasts did not respond to E_2, which lends support to the theory that E_2 action may be mediated through GH and IGF-I (Plouzek and Trenkle, 1991b).

I alone did not increase thymidine incorporation in cells cultured in serum-free medium. The combination of I and 1% FBS resulted in increased thymidine incorporation as compared with C. Serum contains platelet derived growth factor that acts as a competence factor to allow cells to enter the cell cycle (O'Keefe and Pledger, 1983). Insulin present in high

concentrations can stimulate the type I IGF-I receptor (Massague and Czech, 1982) which would allow insulin to act as a progression factor and stimulate the cells to proceed through the cell cycle and increase synthesis of DNA. The I level used in this experiment was five to ten times greater than what is found in plasma of cattle and may have been acting as a progression factor (Plouzek and Trenkle, 1991b).

Treatment with T_4 increased weight gain and skeletal growth in neonatal hypophysectomized rats (Glasscock et al., 1991). The thyroid gland also regulates GH synthesis and therefore, IGF-I production. In cultured human epithelial cells, T_4 added to Eagle's minimum essential medium containing 10% FBS increased thymidine incorporation (Siegel and Tobias, 1966). Since T_4 did not stimulate thymidine incorporation without serum in bovine fibroblasts, it appears that T_4 acts indirectly in regulating fibroblast growth. The increased leucine incorporation following T_4 addition may have resulted from cellular production of a growth factor, such as epidermal growth factor (EGF) by bovine fibroblasts. Thyroid hormones have been implicated in regulating EGF production in neonatal skin (Hoath et al., 1983). EGF has been reported to increase protein synthesis in explants of chick embryo epidermis (Hoober and Cohen, 1967).

Combinations of hormones did not stimulate the uptake of thymidine consistently. In one experiment, T_4 and GH increased thymidine incorporation in the presence of 1% FBS (Table 3), in a second experiment it did not (Table 4). Cells used in these two experiments were from different animals of similar ages which might have been a factor.

The combination of I, GH and E_2 stimulated thymidine incorporation in

0% FBS. This anomaly is unexplained. In 0% FBS, none of these three hormones alone or with T_4 resulted in increased thymidine incorporation. In 1% FBS, thymidine incorporation was not stimulated by this combination of three hormones. In a human breast cancer cell line, the combination of I and E_2 increased DNA content per dish in medium supplemented with steroid-stripped serum with I being more mitogenic than E_2 (Van der Burg et al., 1988).

The addition of Tes with GH, E_2 and T_4 did not stimulate thymidine or leucine incorporation. It appears that these hormones may act via growth factors rather than by direct stimulation of cells to increase growth of animals.

CONCLUSIONS

Fibroblasts were cultured from the dermal tissue from the ear of cattle. These fibroblasts responded dramatically to the addition of one or ten percent FBS to the medium by increasing uptake of thymidine and leucine.

The hormones known to stimulate animal growth did not appear to be primary determinants of growth of these cells. The hormones had little or no effect on DNA and protein synthesis in medium without serum; however, factors contained in the serum modulated the response of bovine fibroblasts to hormones. In the animal, it appears that these hormones might function through increased production of growth factors rather than by a direct stimulation of cell growth.

SECTION II.

THE EFFECTS OF EGF AND IGF-I ON GROWTH

OF BOVINE FIBROBLASTS

INTRODUCTION

Various growth factors that stimulate cellular proliferation have been identified. Epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I) are two growth factors that have been shown to alter growth rate in cells of human and laboratory animals (Westermark and Heldin, 1985; Shipley et al., 1989; Conover et al., 1987b). Synthesis of nucleic acids is stimulated by EGF (Waterfield, 1989) and IGF-I (Furlanetto and DiCarlo, 1984) in human and rodent cells.

Bovine fibroblasts have not been extensively studied in cell culture and consequently their requirement for growth factors has not been characterized. Cattle respond to exogenous estrogen with increased weight gains (Brier et al., 1988), whereas, rodents exhibit decreased weight gains (Heywood and Wadsworth, 1980). Because cattle respond differently to exogenous hormones, it has been speculated that bovine cells may respond to growth factors in a different manner than human or rodent cells.

The purpose of these studies was three-fold. The objectives of Study I were to determine if IGF-I or EGF altered the growth of bovine fibroblasts and to determine the appropriate concentrations of the growth factors. The objectives of Study II were to determine if frequency of administration affected cell growth and if the combination of EGF and IGF-I increased cell growth more than either growth factor alone. The objectives of Study III were to determine if growth factors and hormones affected growth more than growth factors or hormones alone.

MATERIALS AND METHODS

Materials

Materials were purchased from the following sources: epidermal growth factor, MCDB 201 powdered medium, Ham's F-12 powdered medium, penicillin, streptomycin sulfate, nystatin, trypsin and dexamethasone from Sigma Chemical Company (St. Louis, MO); insulin-like growth factor-I from Imcera Bioproducts, Inc. (Terre Haute, IN); leucine (14-C) and thymidine (3-H) from Amersham International (Amersham, UK); fetal bovine serum (FBS) from Gibco Laboratories (Grand Island, NY); Universol ES from ICN Biochemicals, Inc. (Irvine, CA); petri dishes from Nunc (Roskilde, Denmark); Stadie-Riggs microtome from Thomas Scientific (Swedesboro, NJ); beta-estradiol and L-thyroxine from ICN Nutritional Biochemicals (Cleveland, OH); and insulin from Eli Lilly Inc. (Indianapolis, IN). GH was purified using the methods of Dellacha and Sonnenberg (1964), Wallis and Dixon (1966) and Lorenson and Ellis (1975) as modified by J.L. Bobbitt (Eli Lilly Inc., Indianapolis, IN).

Methods

Cells were cultured and propagated as described in Section I. EGF was added at 0, 2.5, 5.0, 7.5 or 10 ng/mL and IGF-I at 0, 25, 50, 75 or 100 ng/mL for Study I. In Study II, 5 ng/mL EGF and 50 ng/mL IGF-I alone and in combination were used. Additions of growth factors were made at twelve hour intervals at 0; 0 and 12; or 0, 12 and 24 hours. The combination of EGF and IGF-I was administered in the following manner: EGF was given at 0 hour and IGF-I was given at 0, 12 or 24 hours. In all comparisons, the final total quantities added were 5 ng/mL EGF and 50 ng/mL IGF-I. In Study

III, hormone additions and levels used: Control (C) - no hormones, dexamethasone (D) 25 ng/mL, estradiol (E_2) .5 ng/mL, growth hormone (GH) 50 ng/mL and thyroxine (T_4) 100 ng/mL. D and E_2 were dissolved in 95% ethanol. GH and T_4 were dissolved in MCDB 201 medium. Hormones were added at 0 hour. EGF and IGF-I were added at three times (0, 12 and 24 hours) to give a final concentration of 5 ng/mL EGF and 50 ng/mL IGF-I.

Cells were exposed to 14-C-leucine (approximately 80,000 dpm/dish, 57 mCi/mmol specific activity) for the duration of the experiments. The addition of 3-H-thymidine (100,000 dpm/dish, 89 mCi/mmol specific activity) was made two hours prior to harvest of cells.

Cells were harvested 48 or 96 hours after adding growth factors in Studies I and III. Cells were harvested 48 hours after the initial addition of growth factors in Study II.

To harvest cells, the medium was aspirated from the dish. Each dish was rinsed with fresh non-radioactive MCDB 201 medium. To dissolve the cells, 1 mL of 3 N NaOH was added and incubated for one hour at room temperature. Dissolved cells from the dish were transferred to a glass scintillation vial. The dish was rinsed with 1 mL deionized water and .5 mL 6 N HCl to bring pH of the dissolved cells in the vial to near neutral. Universol ES was added to the vial for scintillation counting. Thymidine and leucine incorporation were measured as disintegrations per minute (dpm) per dish.

Statistical Analysis

All experimental treatments were tested in triplicate within a study. Study I and II were repeated. The results are expressed as least squares

means, using General Linear Models procedure of SAS (SAS, 1989).
Differences between least squares means were separated on the basis of
least significant differences using the predicted difference option (SAS,
1989).

RESULTS

Study I

Addition of EGF to fibroblasts in MCDB 201 medium with 1% FBS resulted in significant stimulation of DNA synthesis (Table 1). At 48 hours, all additions of EGF increased thymidine incorporation, but only 5 ng/mL was significant. At 96 hours, maximum incorporation was obtained with 5 ng/mL EGF.

Table 1. The alteration in thymidine uptake by cultured bovine fibroblasts due to the addition of different levels of EGF in medium containing 1% FBS following 48 or 96 hours of exposure

Treatment	48 hours		96 hours	
ng/mL	n	dpm±SE	n	dpm±SE
0	6	408 ± 276^a	8	349 ± 226^a
2.5	6	$1063 \pm 276^{a,b}$	6	1482 ± 276^b
5.0	6	1285 ± 276^b	6	2526 ± 276^c
7.5	6	$1162 \pm 276^{a,b}$	6	1322 ± 276^b
10.0	6	$1085 \pm 276^{a,b}$	6	1232 ± 276^b

^{a,b,c}Means without the same superscript within each column differ (P < .05).

Addition of IGF-I in MCDB 201 with 1% FBS resulted in a nonsignificant increase in thymidine incorporation with increasing quantities of IGF-I at 48 hours and no definite trend at 96 hours (Table 2). Cells harvested at 96 hours had a lower level of thymidine incorporation per unit of time than cells harvested at 48 hours, indicating

a decrease in DNA synthesis as the culture aged. The reduced growth at 96 hours indicated that the growth requirement needs of the cells were not being met and that unidentified factors in the medium or serum may have been utilized by cells treated with IGF-I.

The effect of EGF on leucine incorporation is shown in Table 3. At 48 hours, cells exposed to 5 ng/mL EGF had the greatest leucine incorporation, while other levels of EGF were not different from the control cells. All levels of EGF increased leucine incorporation at 96 hours (P < .05).

The response of the cells to IGF-I is shown in Table 4. All additions of IGF-I except 25 ng/mL at 48 hours increased protein synthesis. Control cells did not have markedly greater leucine incorporation at 96 hours as compared with 48 hours. The amount of leucine incorporated per hour was less at 96 hours than for cells harvested at 48 hours. There appears to be a decrease in protein synthesis rate as the cultures aged.

Table 2. The alteration in thymidine uptake by cultured bovine fibroblasts due to the addition of different levels of IGF-I in medium containing 1% FBS following 48 or 96 hours of exposure

Treatment	48 hours		96 hours	
ng/mL	n	dpm±SE	n	dpm±SE
0	6	408±276	8	349±226
25	6	550±276	6	424±276
50	6	711±276	6	389±276
75	6	768±276	6	542±276
100	6	842±276	6	464±276

Table 3. The alteration in leucine uptake by cultured bovine fibroblasts
due to the addition of different levels of EGF in medium
containing 1% FBS following 48 or 96 hours of exposure

Treatment	48 hours		96 hours	
ng/mL	n	dpm±SE	n	dpm±SE
0	6	1470 ± 201^a	8	1898 ± 165^a
2.5	6	1786 ± 201^a	6	$3352 \pm 201^{b,c}$
5.0	6	2160 ± 201^b	6	$3032 \pm 201^{b,c}$
7.5	6	1871 ± 201^a	6	2903 ± 201^b
10.0	6	1695 ± 201^a	6	3468 ± 201^c

^{a,b,c}Means without the same superscript within each column differ
(P < .05).

Table 4. The alteration in leucine uptake by cultured bovine fibroblasts
due to the addition of different levels of IGF-I in medium
containing 1% FBS following 48 or 96 hours of exposure

Treatment	48 hours		96 hours	
ng/mL	n	dpm±SE	n	dpm±SE
0	6	1470 ± 201^a	8	1898 ± 165^a
25	6	$2017 \pm 201^{a,b}$	6	$3027 \pm 201^{b,c}$
50	6	2334 ± 201^b	6	2580 ± 201^b
75	6	2469 ± 201^b	6	$3056 \pm 201^{b,c}$
100	6	2458 ± 201^b	6	3589 ± 201^c

^{a,b,c}Means without the same superscript within each column differ
(P < .05).

Study II

It was determined in Study I that 5 ng/mL EGF resulted in optimum incorporation of leucine and thymidine uptake and was selected for use in the second study. For IGF-I, 50 ng/mL was selected based on a review of the literature and the response to IGF-I in Study I. It has been reported that making small additions of IGF-I at hourly intervals increased DNA synthesis in baby hamster kidney fibroblasts compared with a single addition at the beginning (Blum et al., 1989). Therefore, increasing the number of administration times for EGF and IGF-I was investigated.

The results of the cells grown in the presence of 1% FBS are given in Table 5. EGF or IGF-I administration increased thymidine uptake. The increase in thymidine incorporation was greater for the combination of EGF and IGF-I than EGF given at one or two times or IGF-I given at one, two or three times. EGF increased leucine uptake (P < .01), with greatest response seen when given at three times. IGF-I given at one time increased leucine incorporation more than EGF given at one time. IGF-I given at two or three times increased leucine incorporation more than EGF given at two or three times. The combination of EGF with IGF-I was not better than IGF-I given at two times.

The results from cells grown in serum-free medium are given in Table 6. EGF given at one time and EGF with IGF-I increased thymidine incorporation; whereas, IGF-I alone did not. Multiple doses of EGF or IGF-I were not effective. The combination of EGF and IGF-I increased thymidine incorporation. Adding EGF alone at one, two, or three times did not increase leucine incorporation. IGF-I increased leucine uptake as compared with control cells. IGF-I given at one time stimulated more leucine uptake

Table 5. Alteration in frequency of administration and combination of EGF and IGF-I on thymidine or leucine incorporation of cultured bovine fibroblasts in the presence of 1% FBS at 48 hours

		Thymidine		Leucine
Treatment	n	dpm±SE	n	dpm±SE
Control	6	229 ± 26^a	6	1491 ± 57^a
EGF/IGF-I	6	548 ± 26^c	6	2348 ± 57^e
EGF - 1 time	6	362 ± 26^b	6	$1548 \pm 57^{a,b}$
EGF - 2 times	6	342 ± 26^b	6	$1755 \pm 57^{b,c}$
EGF - 3 times	6	$459 \pm 26^{b,c}$	6	1826 ± 57^c
IGF-I - 1 time	6	361 ± 26^b	6	$1949 \pm 57^{c,d}$
IGF-I - 2 times	6	353 ± 26^b	6	$2140 \pm 57^{d,e}$
IGF-I - 3 times	6	336 ± 26^b	6	2091 ± 57^d

[a,b,c,d,e]Means without the same superscript within a column differ (P < .01).

Table 6. Alteration in frequency of administration and combination of EGF and IGF-I on thymidine or leucine incorporation of cultured bovine fibroblasts in the presence of 0% FBS at 48 hours

		Thymidine		Leucine
Treatment	n	dpm±SE	n	dpm±SE
Control	11	285 ± 24^a	10	523 ± 44^a
EGF/IGF-I	12	384 ± 21^b	11	918 ± 40^b
EGF - 1 time	9	362 ± 26^b	9	534 ± 47^a
EGF - 2 times	12	$331 \pm 21^{a,b}$	12	545 ± 38^a
EGF - 3 times	12	$329 \pm 21^{a,b}$	12	538 ± 38^a
IGF-I - 1 time	9	306 ± 26^a	9	1242 ± 47^c
IGF-I - 2 times	12	318 ± 21^a	12	713 ± 38^d
IGF-I - 3 times	12	279 ± 21^a	12	686 ± 38^d

[a,b,c,d]Means without the same superscript within a column differ (P < .05).

than did EGF given at one time. A similar response was seen with IGF-I given at two or three times.

Study III

The results from combinations of hormones with growth factors are given in Tables 7 through 9. The combination of D with IGF-I did not affect thymidine incorporation (Table 7); whereas, the growth factors alone or in combination increased thymidine uptake at 48 hours. At 96 hours, D and IGF-I inhibited thymidine incorporation. The growth factors did not stimulate thymidine uptake at 96 hours. At 48 hours, leucine incorporation was increased by all treatments as compared with the controls. At 96 hours, D and IGF-I combination was not different from control cells but the

Table 7. The effect of hormones and growth factors on thymidine incorporation in bovine fibroblasts in 1% FBS at 48 or 96 hours

	Thymidine				Leucine			
	48 hours		96 hours		48 hours		96 hours	
Treatment	n	dpm$\pm$SE	n	dpm$\pm$SE	n	dpm$\pm$SE	n	dpm$\pm$SE
Control	3	344 ± 40^a	3	344 ± 40^a	3	2310 ± 114^a	3	2297 ± 114^a
D, IGF-I	3	$366\pm40^{a,b}$	3	210 ± 40^b	3	2688 ± 114^b	3	2319 ± 114^a
EGF	3	618 ± 40^c	3	$289\pm40^{a,b}$	3	$2815\pm114^{b,c}$	3	2897 ± 114^b
EGF, IGF-I	2	571 ± 48^c	3	427 ± 40^a	3	3453 ± 114^d	3	3786 ± 114^c
IGF-I	3	$487\pm40^{b,c}$	3	$310\pm40^{a,b}$	3	3108 ± 114^c	3	2978 ± 114^b

^{a,b,c}Means without the same superscript within a column differ (P < .05).

Table 8. The effect of hormones and growth factors on cultured bovine fibroblasts in 1% FBS at 48 hours

Treatment	n	Thymidine dpm±SE	n	Leucine dpm±SE
Control	3	365±29[a]	3	1389±64[a]
EGF	3	159±29[b,c]	3	1054±64[b]
E_2	3	163±29[b,c]	3	949±64[b]
E_2, EGF	3	157±29[b,c]	3	985±64[b]
E_2, IGF-I	3	235±29[b,c]	3	1544±64[c]
GH	3	204±29[b,c]	3	949±64[a,b]
GH, EGF	3	165±29[b,c]	3	993±64[b]
GH, IGF-I	3	186±29[b,c]	3	1682±64[c]
IGF-I	3	408±29[a]	3	2051±64[d]
T_4	3	167±29[b,c]	3	906±64[b]
T_4, EGF	3	174±29[b,c]	3	1058±64[b]
T_4, IGF-I	3	189±29[b,c]	3	1790±64[c]
T_4, GH, EGF	3	150±29[b]	3	920±64[b]
T_4, GH, IGF-I	3	185±29[c]	3	1670±64[c]
10% FBS	3	930±29[d]	3	2689±64[e]

[a,b,c,d,e]Means without the same superscript within a column differ (P < .05).

addition of growth factors alone or in combination increased leucine incorporation as compared with the control cells.

Ten percent serum increased thymidine incorporation, whereas, growth factors and/or hormones additions resulted in decreased thymidine uptake (Table 8). Leucine incorporation was increased by IGF-I alone or in combination with hormones. However, no combination of hormone and IGF-I increased leucine incorporation more than IGF-I alone. EGF addition, alone or in combination with hormones, decreased leucine incorporation. Hormones alone were unable to stimulate leucine or thymidine incorporation.

A similar experiment was performed using cells from a different animal of similar age and genetic background (Table 9). At 48 hours, EGF in combination with T_4 increased thymidine uptake. The combination of GH, T_4 and EGF increased thymidine uptake. However, this was not greater than T_4 and EGF. At 96 hours, EGF alone or T_4 with IGF-I increased thymidine uptake. At 48 hours, leucine incorporation was increased by IGF-I; IGF-I with E_2; IGF-I, T_4 and GH; or EGF, T_4 and GH. At 96 hours, cells treated with IGF-I except those treated with IGF-I, T_4 and GH had greater leucine incorporation than the control cells. EGF was not effective in stimulating leucine uptake except in combination with T_4.

Table 9. The effect of hormones and growth factors on thymidine and leucine incorporation in bovine fibroblasts in 1% serum at 48 or 96 hours

	Thymidine				Leucine			
	48 hours		96 hours		48 hours		96 hours	
Treatment	n	dpm±SE	n	dpm±SE	n	dpm±SE	n	dpm±SE
Control	3	237 ± 40^a	3	287 ± 40^a	3	1130 ± 83^a	3	1265 ± 83^a
EGF	3	$315\pm40^{a,b}$	3	327 ± 40^b	3	$1218\pm83^{a,b}$	3	$1410\pm83^{a,b}$
E_2, IGF-I	3	255 ± 40^a	3	249 ± 40^a	3	1477 ± 83^b	3	$1599\pm83^{b,c,d}$
GH, EGF	2	$318\pm48^{a,b}$	3	$311\pm40^{a,b}$	3	$1226\pm83^{a,b}$	3	$1426\pm83^{a,b}$
GH, IGF-I	3	270 ± 40^a	3	$294\pm40^{a,b}$	3	$1310\pm83^{a,b}$	3	$1519\pm83^{b,d}$
IGF-I	3	$339\pm40^{a,b}$	3	$342\pm40^{a,b}$	3	1417 ± 83^b	3	1765 ± 83^c
T_4, EGF	3	$403\pm40^{b,c}$	3	$305\pm40^{a,b}$	3	$1360\pm83^{a,b}$	3	$1528\pm83^{b,c,d}$
T_4, IGF-I	3	$301\pm40^{a,b}$	3	407 ± 40^b	3	$1360\pm83^{a,b}$	3	$1707\pm83^{c,d}$
T_4, GH, EGF	3	477 ± 40^c	3	281 ± 40^a	3	1481 ± 83^b	3	$1370\pm83^{a,b}$
T_4, GH, IGF-I	3	232 ± 40^a	3	$313\pm40^{a,b}$	3	1400 ± 83^b	3	$1477\pm83^{a,b,d}$

[a,b,c,d]Means without the same superscript within a column differ (P < .05).

DISCUSSION

Most of the research examining the effects of growth factors on fibroblasts has used cells of human or rodent origin. There are two types of factors (competence and progression) in serum to stimulate mitosis in cells (Pledger et al., 1978). EGF and IGF-I are progression factors which allow cells to exit G_1 and enter into S phase of the cell cycle (O'Keefe and Pledger, 1983). The bovine cells were grown in the presence of 0 or 1% FBS. In Section I, bovine cells responded to the presence of 1% FBS by increasing thymidine and leucine incorporation as compared with cells grown in serum-free medium. The bovine cell response to growth factors was modulated by the presence of FBS. Serum contains both competence and progression factors in an undefined mix, along with hormones and nutrients to regulate cellular growth. The addition of EGF or IGF-I with FBS may allow cells to proceed through the cell cycle, because these combinations may contain adequate amounts of competence and progression factors to stimulate cells to traverse the cell cycle.

EGF at 5 ng/mL increased DNA synthesis in these fibroblasts in all experiments except one. In these studies, the minimal time required for EGF presence to stimulate DNA synthesis in bovine fibroblasts was not examined. Some researchers have determined that DNA synthesis increased in human fibroblasts after exposure to EGF for eight hours (Westermark and Heldin, 1985). Others have reported that up to twelve hours exposure to EGF is required to increase DNA synthesis (Carpenter and Cohen, 1976). Maximal stimulation of DNA synthesis occurred approximately 20 hours after EGF addition in mouse keratinocytes (Zendegui et al., 1988). Chick embryo epidermis explants, grown in the absence of serum, had the greatest

thymidine uptake following 24 to 48 hours of EGF exposure (Bertsch and Marks, 1974).

EGF stimulated leucine incorporation in bovine fibroblasts in all experiments but one. Leucine incorporation was not dramatically different at 48 or 96 hours in these bovine fibroblasts. It is unknown why the cells in this one experiment (Table 8) decreased thymidine and leucine incorporation due to the addition of EGF. It has been reported that chick embryo fibroblasts increased protein synthesis two-fold during the first forty-eight hours of incubation with EGF as compared with control cells (Hoober and Cohen, 1967).

Bovine fibroblasts grown in 1% FBS did not respond to IGF-I by increasing the rate of thymidine incorporation in all experiments. In one experiment, IGF-I decreased thymidine uptake as compared with control cells; however, usually there was a nonsignificant or significant increase in thymidine uptake following IGF-I addition when cells were harvested at 48 or 96 hours. In human fibroblasts, peak thymidine incorporation was seen 22 to 28 hours after IGF-I addition (Conover et al., 1987b). Human fibroblasts exhibited increased thymidine uptake for approximately 40 hours following IGF-I addition. The peak incorporation time for IGF-I stimulation in bovine fibroblasts was not determined.

In the second study, the addition of all levels of IGF-I increased rate of DNA synthesis when cells were harvested 48 hours after initial addition of 50 ng/mL IGF-I. This is in the range of IGF-I (10-100 ng/mL) levels that have been reported to stimulate DNA and protein synthesis of human fibroblasts (Conover et al., 1985b).

Combining EGF with IGF-I enhanced thymidine and leucine uptake in

bovine fibroblasts compared to the control cells. Other research has shown that EGF and IGF-I work at different places within the cell cycle (O'Keefe and Pledger, 1983). EGF is required for cells to leave G_0, with EGF being required during the first 6 hours and IGF-I during the last 6 hours of G_0 to stimulate DNA synthesis. This would agree with the research showing EGF needs to be present for 8 hours to stimulate DNA synthesis (Westermark and Heldin, 1985). The bovine fibroblasts did not have a dramatic response to the combination of EGF/IGF-I. However, these bovine fibroblasts were grown in 1% FBS. There may have been adequate levels of EGF or IGF-I contained in the FBS to enable the cells to exit the G_0/G_1 phase of the cell cycle. In serum-free medium, the combination of EGF and IGF-I stimulated thymidine incorporation but this was not better than EGF given at one time.

In these studies, there was not seen a clearly mitogenic response to multiple administrations of IGF-I. Twelve hour intervals may have been too wide or fibroblasts do not respond in the same manner as hamster fibroblasts, which increased DNA synthesis in response to hourly additions of IGF-I (Blum et al., 1989).

In serum-free medium, only the combination of EGF with IGF-I or EGF given at one time increased thymidine uptake. There may not have been adequate EGF present at one time from the multiple doses to stimulate the cell receptors to allow increased DNA synthesis. The cells may have stopped in the first half of the G_0, where EGF is necessary to allow cellular progression through G_0/G_1 to S phase. IGF-I has been shown to be necessary in the second half of G_0. If the cells were in the first portion of G_0, the cells may not proceed through the rest of the cell cycle without EGF. This may explain why there were varied responses to the presence of

EGF or IGF-I in the different experiments. In some experiments there was increased thymidine uptake at 48 hours due to the addition of EGF to medium containing 1% FBS (Table 5); whereas, in others there was no increase (Table 9). Some cells did not increase thymidine incorporation due to the addition of IGF-I (Table 2); whereas, other cells grown in 1% FBS increased thymidine uptake (Table 5). Cells from different donor animals were used in these experiments. The cells from different donors did not respond to growth factors and/or hormones in the same manner. The cause of this variation is unknown at the present time. Similarly, response of human fibroblast cultures to EGF treatment ranged from increased to decreased thymidine uptake when cells from various human donors were tested (Lechner et al., 1989).

In 1% FBS, EGF, IGF-I or combination of these two growth factors increased protein synthesis by bovine fibroblasts. In 0% FBS, EGF alone was not effective in increasing leucine uptake. IGF-I was more stimulatory to leucine incorporation than EGF. IGF-I given at one time increased leucine uptake more than IGF-I given at multiple times in serum-free medium. It appears a threshold amount of IGF-I may be needed to stimulate protein synthesis.

In Section I, D inhibited thymidine and leucine uptake. The addition of IGF-I with D prevented this inhibition. However at 96 hours, IGF-I did not prevent the inhibition due to D. IGF-I may have a shorter half-life in the medium than did D.

Hormones combined with the growth factors did not increase leucine incorporation more than growth factors alone. The hormones did not appear to have direct control of protein synthesis in bovine fibroblasts. In

animals, hormones are probably stimulating growth factor production, be it endocrine, paracrine or autocrine to increase protein synthesis.

EGF and T_4 increased thymidine uptake in one experiment (Table 9); however, this was not seen in another experiment (Table 8). This increase in thymidine uptake was not greater than by cells given EGF alone (Table 9). The combination of EGF, T_4 and GH stimulated more thymidine incorporation than did EGF alone (Table 9); however, this combination inhibited thymidine incorporation in other cells (Table 8). It has been reported that T_4 was involved in regulating EGF production in neonatal skin (Hoath et al., 1983). If this were true in bovine fibroblasts, it seems there should have been a more consistent response to T_4. FBS was more effective than any combination of hormone and growth factors for stimulating thymidine and leucine uptake.

CONCLUSIONS

Bovine fibroblasts may respond to the addition of growth factors by increasing thymidine and leucine incorporation. However, cells from one animal decreased thymidine and leucine incorporation due to the addition of EGF or IGF-I to the medium. IGF-I addition to cultured bovine fibroblasts increased leucine uptake in all experiments, but IGF-I stimulated thymidine uptake in half of the experiments. EGF-I stimulated thymidine incorporation in some experiments; however, some cells did not respond to EGF. Cells from one animal decreased thymidine incorporation due to the addition of EGF to the medium. EGF stimulated, decreased or had no effect on leucine incorporation in bovine fibroblasts derived from different donor animals. More frequent administration of smaller amounts of EGF or IGF-I was not dramatically better than adding growth factors at one time. Ten percent serum was more effective in stimulating thymidine and leucine incorporation than IGF-I, EGF or a combination of a growth factor with hormones. Combinations of hormones with a growth factor did not result in a dramatic increase in thymidine or leucine incorporation in bovine fibroblasts.

Either EGF or IGF-I stimulated leucine incorporation in bovine fibroblasts, however, IGF-I appeared to be more stimulatory to protein synthesis than EGF.

SECTION III.

COMPARISON OF GROWTH OF BOVINE FIBROBLASTS

IN CULTURE WITH GROWTH OF DONOR ANIMALS

INTRODUCTION

Lean meat must be produced efficiently and economically, necessitating the identification of breeding stock possessing desirable genetic characteristics for producing this lean beef. The desire to accurately predict animal growth has intrigued animal scientists for years.

Some research has compared mature carcass weight with individual muscle weights (Orme et al., 1960; Butterfield, 1962). More sophisticated analytical assays allowed the examination of DNA and RNA content of dissected muscle to determine if differences existed among animals with different growth potentials (Trenkle et al., 1978).

Examination of blood concentrations for hormones (Trenkle and Topel, 1978; Verde and Trenkle, 1987; and Plouzek and Trenkle, 1991a) and growth factors (Plouzek and Trenkle, 1991b) allowed animals to be spared while measurements could be made. The next logical development was obtaining tissue biopsies for determining mRNA levels (Hannon et al., 1991) or culturing cells to study growth of cattle.

Animal growth, as measured by weight gain, represents an increase in cell number, an increase in cell size or a combination of these. It is possible to determine cellular incorporation of radiolabeled thymidine and leucine into DNA and protein as measures of cell growth by use of cell cultures. Mammalian skin fibroblasts can be easily cultured and maintained in a laboratory environment.

During the establishment of fibroblast cultures from different calves, an observation was made that outgrowth of cells from explants appeared to be related to growth rates of donor animals. The growth of fibroblast cultures from different breeds and ages of purebred and

crossbred cattle was studied to determine if there was a relationship between growth of cells in culture and growth of donor animals.

MATERIALS AND METHODS

Materials

Materials were purchased from the following sources: Ham's F-12 powdered medium, penicillin, streptomycin sulfate, nystatin and giemsa from Sigma Chemical Company (St. Louis, MO); leucine (14-C) and thymidine (3-H) from Amersham International (Amersham, UK); fetal bovine serum (FBS) from Gibco Laboratories (Grand Island, NY); Universol ES from ICN Biochemicals, Inc. (Irvine, CA); petri dishes from Nunc (Roskilde, Denmark); Stadie-Riggs Microtome from Thomas Scientific (Swedesboro, NJ); glass coverslips and methanol from Fisher Scientific (Chicago, IL); and high vacuum grease from Dow Corning (Midland, MI).

Methods

Five groups of animals were sampled from beef herds maintained at the Iowa State University Beef Nutrition (Charolais-Simmental crossbred cattle) and Teaching farms (purebred Simmental and Angus cattle). Eighty-seven animals were sampled over a two year period: Group I: Angus fall calves, sampled at approximately 3 mo; Group II: Charolais-Simmental crossbred calves, sampled within 1 mo of birth and again at approximately 5 mo; Group III: Angus and Simmental bulls sampled at 8 mo; Group IV: Charolais-Simmental crossbred calves sampled at 7 mo; and Group V: Angus cows, ranging in age from 3 to 11 years.

Samples of skin were collected via punch biopsy and plated as described in Section I, except one piece of tissue per animal was aseptically plated in three 35 mm petri dish in 3 mL sterile Ham's F-12 medium. Radiolabeled 14-C-leucine (approximately 50,000 dpm/dish, 57

mCi/mmol specific activity) was added at time of plating to the culture medium to estimate protein synthesis. One piece of tissue was plated in 5 mL of nonradioactive Ham's F-12 medium in triplicate 60 mm petri dishes with 2 x 2 mm grids etched in the bottom of the dish.

Dishes were left undisturbed for ten days in a humidified 5% carbon dioxide, 37 C incubator. On day ten, growth of fibroblasts from the explants was determined. Cells grown in gridded dishes were fixed with methanol and stained with giemsa. Number of grids containing fibroblasts was counted using an inverted light microscope.

Cells grown in non-gridded dishes containing radiolabeled leucine had 3-H-thymidine (approximately 160,000 dpm/dish, 89 mCi/mmol specific activity), added one hour prior to harvest for estimation of DNA synthesis. Radioactive medium was removed and plates were rinsed with nonradioactive medium. To dissolve the cells, 1 mL of 3 N NaOH was added and incubated for one hour at room temperature. Dissolved cells from the dish were placed in a glass scintillation vial. The dish was rinsed with 1 mL deionized water and .5 mL 6 N HCl to bring pH of dissolved cells in the vial to near neutral. Universol ES was added to the vial for liquid scintillation counting. Thymidine and leucine incorporation were measured as disintegrations per minute (dpm) per dish. Data on incorporation were normalized by calculating radioactivity incorporated as a percent of that added to the dishes.

The first four tables represent data collected from individual young animals with averages determined for groups, separated on basis of sex. Tables are arranged to include animal identification number, birth and later weights (3 mo, 5 mo, 6 mo, 7 mo, 8 mo or 11 mo), % thymidine

incorporation (THY), % leucine incorporation (LEU) and number of 2 x 2 mm squares containing fibroblasts (plate count, PLT CT).

The fifth table represents data from the cows. These animals were from the breeding herd. All animals were over two years of age. Animals were not weighed at time of sampling.

The sixth table contains correlation coefficients from multiple regression equations relating cell culture data and birth weight with animal weight and from linear regression equations relating birth weight to animal weight. Multiple regression equations were calculated using THY, LEU, PLT CT and birth weight (BIR WT, kg) as the independent variables and actual animal weight (WT, kg) at 3, 5, 6, 7, 8 or 11 mo as the dependent variable. Linear regression values were obtained by using BIR WT (kg) as the independent variable and WT (kg) as the dependent variable. Table 7 contains the multiple and linear regression coefficients for the regression equations.

To examine effect of age on THY, LEU or PLT CT, data were grouped according to age, with data from different sexes and breeds pooled. The age groups were 1, 3, 5, 7, 8 mo and greater than 24 mo.

Statistical Analysis

Data were analyzed by analysis of variance using General Linear Models or Regression procedures of SAS (SAS, 1989). Least squares means at the different ages were separated on the basis of least significant differences using the predicted difference option (SAS, 1989).

RESULTS

Cell culture data from Group I are shown in Table 1. THY values ranged from .85 to 2.11%, LEU from 6.51 to 16.13% and PLT CT 8.3 to 13.7, when these animals were approximately 3 mo.

The results from Group II are given in Table 2. THY values ranged from 1.31 to 4.95%, .53 to 1.35%; LEU values ranged from 5.27 to 15.44%, 1.35 to 4.39%; PLT CT values ranged from 9.7 to 18.7 and 0.0 to 13.3 at 1 mo and 5 mo, respectively.

Table 3 contains the results for Group III. THY values ranged from .66 to 2.19%, LEU from .4 to 2.85% and PLT CT from 3.5 to 11.5 at 8 mo.

The results from Group IV are given in Table 4. THY ranged from .49 to 1.69%, LEU from 1.61 to 3.27% and PLT CT from 6.0 to 14.0.

The average THY, LEU and PLT CT for Group V are shown in Table 5. THY ranged from .85% to 1.74% and LEU from 3.44 to 10.05% for the animals exceeding 24 mo. PLT CT was lower than those from the younger animals shown in the preceding tables, with a range of 1.0 to 7.0.

The correlation coefficients resulting from the multiple regression equations are shown in Table 6. Including cell culture data with birth weight improved the fit of the model, using cell culture data from 3 mo old animals and birth weight, to predict 6 mo weight of Group I heifers. Birth weight alone was not a good model for predicting 6 mo heifer weight and was not significant (P < .1). However for Group I bull calves, birth weight (P < .05) was a good model to predict 6 mo weight. Including cell culture data with birth weight was not significant (P < .1). For Group III Angus bulls, cell culture data from 8 mo old animals with birth weight was a good model for predicting 8 mo weight (P < .05). For Group IV, birth weight was

a good model for animal performance at both 7 and 11 mo (P < .05). The use of cell culture data with birth weight also provided a good model (P < .05) for predicting the performance of Group IV animals at 7 and 11 mo. In Group II, neither birth weight nor birth weight with cell culture data were good models for predicting animal weight at a future point in time.

Table 7 contains linear and multiple regression equation coefficients for the various groups of cattle. There were very different coefficients resulting for the regression equations.

Figure 1 represents the average THY and LEU for animals ranging in age from birth to maturity. Average THY was highest at 1 mo, decreased at 5 mo and increased slightly at 8 mo for the calves. The average THY for cows was not different from 3 to 8 mo calves. Average LEU increased from birth to 3 mo, and then leveled off between 5 and 8 mo. Cows had an increased LEU, but not as high as calves at 1 or 3 mo.

Figure 2 depicts PLT CT for the different ages. Calves at 1 mo of age exhibited the greatest average PLT CT. The extent of outgrowth of cells from the explants decreased as the animals aged.

Table 1. Group I, Angus bulls and heifers sampled at 3 mo to determine growth from the tissue explant and thymidine and leucine incorporation*

| ID | | BIR WT | 3 MO | 6 MO | PLT | THY | LEU |
| | | | WT | WT | | | |
No	SEX	kg	kg	kg	CT	%	%
A945	B	45.5	143.6	299.5	10.7	1.14	8.53
A947	B	44.5	142.7	299.5	10.7	1.67	10.51
A948	B	40.0	126.4	293.6	11.3	1.60	11.43
A943	B	43.6	124.5	291.4	9.3	1.36	10.66
A938	B	36.4	105.5	282.7	11.7	1.20	7.08
A941	B	40.9	130.0	275.9	8.3	1.78	8.58
A942	B	33.6	101.8	266.4	12.3	2.11	16.13
AVG		40.7	124.9	287.0	10.6	1.55	10.42
A940	H	35.5	131.8	310.9	9.0	1.77	9.73
A950	H	37.3	115.5	299.5	13.7	1.97	10.42
A951	H	36.4	104.5	294.1	8.3	1.46	11.47
A937	H	29.1	120.0	280.5	13.0	1.14	10.34
A944	H	35.5	95.5	261.4	12.7	1.51	11.14
A939	H	27.3	87.3	250.9	9.3	0.85	6.51
A949	H	34.5	85.5	239.5	11.7	1.31	7.70
AVG		33.6	105.7	276.7	11.1	1.43	9.62

*ID No is the animal ear tag number; Sex: B represents bull, H represents heifer; animals were weighed at 3 and 6 mo; PLT CT: number of squares containing cells; THY: % thymidine incorporation; and LEU: % leucine incorporation.

Table 2a. Group II crossbred bulls sampled at 1 and 5 mo to determine growth from the tissue explant and thymidine and leucine incorporation*

| ID | BIR | 1 Mo Sample | | | 5 Mo Sample | | | 5 MO WT | 7 MO WT | 11 MO WT |
| | | PLT | THY | LEU | PLT | THY | LEU | | | |
No	WT	CT	%	%	CT	%	%	kg	kg	kg
29	47.3	12.0	2.23	5.27	10.0	1.35	4.39	240.0	298.6	409.1
15	44.5	15.5	2.75	9.49	13.3	0.61	3.03	230.9	297.7	436.8
3	39.1	13.0	2.93	7.58	0.0	0.69	2.57	237.3	291.4	411.8
25	40.9	14.7	2.75	9.27	6.3	0.64	2.74	236.8	288.2	416.8
6	45.5	10.3	2.89	8.02	5.7	0.63	3.09	233.6	280.9	394.5
32	41.8	15.0	4.14	12.76	6.7	0.53	2.20	223.6	274.5	392.7
9	38.2	11.0	3.20	10.34	8.3	1.07	4.21	225.5	265.9	376.8
13	38.2	18.3	4.47	5.28	7.7	1.18	2.39	221.8	263.6	374.1
26	36.4	13.7	4.95	12.11	13.0	0.53	2.51	210.0	262.7	385.0
5	39.1	9.7	1.31	8.48	6.0	1.22	3.04	218.2	255.9	411.8
2	41.8	16.0	2.76	7.19	1.0	0.56	1.35	200.0	233.6	350.5
4	44.5	11.3	3.10	9.25	11.3	1.06	3.50	200.9	231.4	336.8
1	40.0	10.3	3.46	6.29	8.3	0.83	2.99	200.0	215.9	349.5
11	38.2	13.0	1.57	15.44	11.7	0.92	3.44	176.4	206.4	344.5
AVG	41.1	13.1	3.04	9.05	7.8	0.84	2.96	218.2	261.9	385.1

*ID No is the animal ear tag number; animals were weighed at 5, 7 and 11 mo; PLT CT: number of squares containing cells; THY: % thymidine incorporation; and LEU: % leucine incorporation.

Table 2b. Group II crossbred heifers sampled at 1 and 5 mo of age to determine growth from the tissue explant and thymidine and leucine incorporation*

| ID | BIR | 1 Mo Sample | | | 5 Mo Sample | | | 5 MO WT | 7 MO WT | 11 MO WT |
| | | PLT | THY | LEU | PLT | THY | LEU | | | |
No	WT	CT	%	%	CT	%	%	kg	kg	kg
21	46.4	14.7	2.51	8.66	8.0	0.55	2.36	230.0	296.4	404.1
35	35.5	12.0	2.48	9.45	7.0	0.74	3.14	197.3	257.3	354.5
18	45.5	17.0	4.82	14.17	11.7	0.84	3.18	202.3	253.6	345.9
10	35.5	10.3	3.82	11.45	8.0	1.23	3.80	193.6	246.4	340.0
23	41.8	16.0	2.58	7.50	10.3	0.59	2.19	200.5	240.9	354.1
20	40.9	14.0	3.02	10.49	8.3	0.68	1.65	198.6	235.5	351.8
41	37.3	16.7	3.97	9.63	8.3	0.72	3.79	172.3	226.4	313.6
31	40.9	13.7	2.76	6.03	11.3	0.82	3.27	185.5	222.3	348.6
7	45.5	13.7	2.95	7.72	9.3	1.14	4.07	178.2	206.4	350.0
16	37.3	9.7	4.74	14.22	6.7	0.78	3.17	179.1	205.0	300.5
37	49.1	13.0	2.38	8.74	8.7	0.93	3.75	160.9	194.5	306.4
40	36.4	18.7	3.76	8.63	6.3	0.74	3.10	151.8	184.5	317.3
AVG	41.0	14.1	3.32	9.72	8.7	0.81	3.12	187.5	230.7	340.6

*ID No is the animal ear tag number; animals were weighed at 5, 7 and 11 mo; PLT CT: number of squares containing cells; THY: % thymidine incorporation; and LEU: % leucine incorporation.

Table 3. Group III, Angus (A) and Simmental (Y) bulls sampled at 8 mo of age to determine thymidine and leucine incorporation and growth from the tissue explant*

ID No	BIR WT kg	THY %	LEU %	PLATE CT	8 MO WT kg
A912	44.6	1.65	0.54	8.5	374.6
A902	45.4	1.67	2.34	11.5	358.2
A914	40.9	1.36	2.32	11.0	329.1
A918	34.6	0.98	1.97	9.0	328.2
A927	44.6	2.19	2.55	6.7	322.7
A928	40.9	1.42	2.16	8.7	319.1
A911	37.3	0.66	1.45	8.5	315.4
A917	43.2	1.46	2.01	8.0	314.6
A924	41.8	1.10	2.41	8.5	310.0
AVG	41.5	1.39	1.97	8.9	330.2
Y903	50.0	1.70	1.75	5.3	385.4
Y928	48.2	2.12	2.23	4.0	362.7
Y905	42.7	1.14	2.17	10.5	353.6
Y925	46.4	1.00	0.40	3.5	345.4
Y926	47.3	1.23	2.20	8.5	344.6
Y920	45.4	1.67	2.32	6.7	341.8
Y927	44.6	1.36	2.85	7.5	324.6
Y924	50.0	1.33	1.36	7.3	323.6
Y909	40.0	1.23	1.89	8.5	313.6
AVG	46.1	1.42	1.91	6.9	343.9

*ID No is the animal ear tag number; animals were weighed at 8 mo; PLT CT: number of squares containing cells; THY: % thymidine incorporation; and LEU: % leucine incorporation.

Table 4. Group IV, crossbred heifers and steers sampled at 7 mo to determine thymidine and leucine incorporation and growth from the tissue explant*

ID No	SEX	BIR WT kg	THY %	LEU %	PLT CT	7 MO WT kg	11 MO WT kg
929	H	45.4	0.82	2.94	9.5	278.2	389.1
915	H	43.6	1.23	2.04	6.0	258.2	365.9
926	H	37.3	1.45	1.61	10.5	258.2	362.3
919	H	39.1	1.36	2.87	7.0	237.7	348.2
911	H	37.3	0.76	2.77	9.5	224.6	331.8
922	H	30.9	1.02	2.91	7.0	207.3	308.2
AVG	H	38.9	1.11	2.52	8.2	244.0	350.9
923	S	47.3	1.69	2.65	14.0	299.6	409.6
912	S	45.9	0.89	3.27	7.0	293.2	402.3
908	S	45.4	1.38	2.98	7.0	281.8	398.2
939	S	46.4	1.67	2.87	7.5	277.7	380.4
920	S	36.4	1.17	2.58	10.0	276.4	379.6
921	S	39.1	1.06	2.26	9.5	271.4	383.2
917	S	44.6	0.49	2.11	8.5	268.6	403.6
916	S	40.9	0.59	2.10	5.0	266.8	382.7
925	S	32.7	1.27	2.93	12.0	264.6	367.3
930	S	30.0	1.54	2.38	6.0	224.6	315.4
924	S	37.3	1.51	2.24	9.0	221.4	313.6
AVG	S	40.5	1.21	2.58	8.7	267.8	376.0

*ID No is the animal ear tag number; Sex: H for heifer and S for steer; animals were weighed at 7 and 11 mo; PLT CT: number of squares containing cells; THY: % thymidine incorporation; and LEU: % leucine incorporation.

Table 5. Group V, Angus cows sampled at ages exceeding 24 mo to determine growth from the tissue explant and thymidine and leucine incorporation*

ID No	PLT CT	THY %	LEU %
106	5.7	1.55	10.04
118	6.3	1.23	6.98
217	7.0	1.35	4.99
223	1.0	1.17	6.23
319	5.0	1.14	5.68
346	3.7	0.92	4.07
408	6.3	1.36	10.00
415	2.7	0.85	3.44
423	4.3	1.74	10.05
542	3.0	0.87	5.46
610	5.0	1.12	4.48
821	6.7	1.21	8.58
AVG	4.7	1.21	6.67

*ID No is the animal ear tag number; PLT CT: number of squares containing cells; THY: % thymidine incorporation; and LEU: % leucine incorporation.

Table 6. Correlation coefficients for multiple regression equations using birth weight with cell culture data and correlation coefficients for linear regression equations using birth weight to predict animal weight at different ages[a]

Group	n	Breed	Sex	Age at Sampling	Age at Pred. WT	Mult. Reg. r^2	BIR WT r^2
I	7	A	B	3	3	.97*	.58*
				3	6	.94	.64**
	7	A	H	3	3	.87	.06
				3	6	.96*	.20
II	14	C*S	B	1	7	.22	.13
				1	11	.08	.05
				5	5	.28	.13
				5	11	.10	.05
	12	C*S	H	1	7	.25	.01
				1	11	.30	.08
				5	5	.37	.04
				5	11	.33	.08
III	9	A	B	8	8	.91**	.19
	9	S	B	8	8	.38	.28
IV	11	C*S	S	7	7	.80**	.54**
				7	11	.84**	.59**
	6	C*S	H	7	7	.93	.79**
				7	11	.96	.84**

[a]Breed: Angus (A), Charolais (C), Simmental (S); Sex: bull (B) and heifer (H); Age at Sampling: mo of age at sampling time; Pred. Wt. Age: age of animal when weight was being predicted; Mult. Reg. r^2: correlation coefficient resulting from the multiple regression equation using cell measurements and birth wt; and BIR WT r^2: linear regression correlation coefficient for birth weight used to predict weight of animals.

**P < .05, *P < .1.

Table 7. Coefficients for linear regression equations using birth weight or multiple regression equations using birth weight and cell culture data to predict animal weight*

Group	Sex	TIME	Inter.	PL CT	THY	LEU	BIR WT
I	B	birth - 3 mo	71.07				1.50
		3 mo - 3 mo	122.93	-2.71	-6.14	-1.81	1.65
		birth - 6 mo	238.05				1.33
		3 mo - 6 mo	274.83	-1.81	-4.19	-1.40	1.44
I	H	birth - 3 mo	69.90				1.06
	·	3 mo - 3 mo	220.44	-2.98	82.34	4.96	-7.35
		birth - 6 mo	171.89				3.12
		3 mo - 6 mo	403.55	-6.68	113.05	8.04	-8.67
II	B	birth - 5 mo	134.25				2.04
		5 mo - 5 mo	148.56	-2.07	-13.67	10.13	1.64
		5 mo - 11 mo	322.91	-1.34	-32.31	12.15	1.56
		birth - 7 mo	127.10				3.28
		birth - 7 mo	95.69	2.04	4.05	-1.42	3.41
		birth - 11 mo	301.62				2.03
		birth - 11 mo	317.74	2.16	-4.94	-0.99	1.53
	H	birth - 5 mo	152.06				0.86
		5 mo - 5 mo	199.95	2.80	13.08	-18.20	0.23
		5 mo - 11 mo	326.32	2.53	18.04	-21.86	1.12
		birth - 7 mo	205.06				0.63
		birth - 7 mo	220.68	3.21	-36.29	12.09	-0.79
		birth - 11 mo	269.98				1.72
		birth - 11 mo	333.18	2.53	-26.32	4.83	0.29

*The linear regression equations are represented by birth - (3, 5, 6, 7 or 11 mo) to represent birth weight being used to predict 3, 5, 6, 7 or 11 mo weight, respectively, in the TIME column. The TIME column also represents the multiple regression equations using birth weight and cell culture data (THY, LEU, PLT CT and BIR WT) to predict weights at the time of sampling or later weights.

Table 7. Continued.

Group	Sex	TIME	Inter.	PL CT	THY	LEU	BIR WT
III	B	birth - 8 mo	221.09				2.63
		8 mo - 8 mo	287.15	9.07	37.46	-27.26	-0.87
		birth - 8 mo	182.73				3.50
		8 mo - 8 mo	210.27	0.91	30.30	-6.84	2.12
IV	S	birth - 7 mo	143.94				3.06
		7 mo - 7 mo	99.64	3.44	-21.91	27.01	2.34
		birth - 11 mo	202.64				4.28
		7 mo - 11 mo	187.73	4.41	-42.16	23.21	3.48
	H	birth - 7 mo	72.69				4.40
		7 mo - 7 mo	24.85	4.33	19.25	-3.49	4.39
		birth - 11 mo	155.65				5.01
		7 mo - 11 mo	83.02	4.77	25.09	1.23	5.08
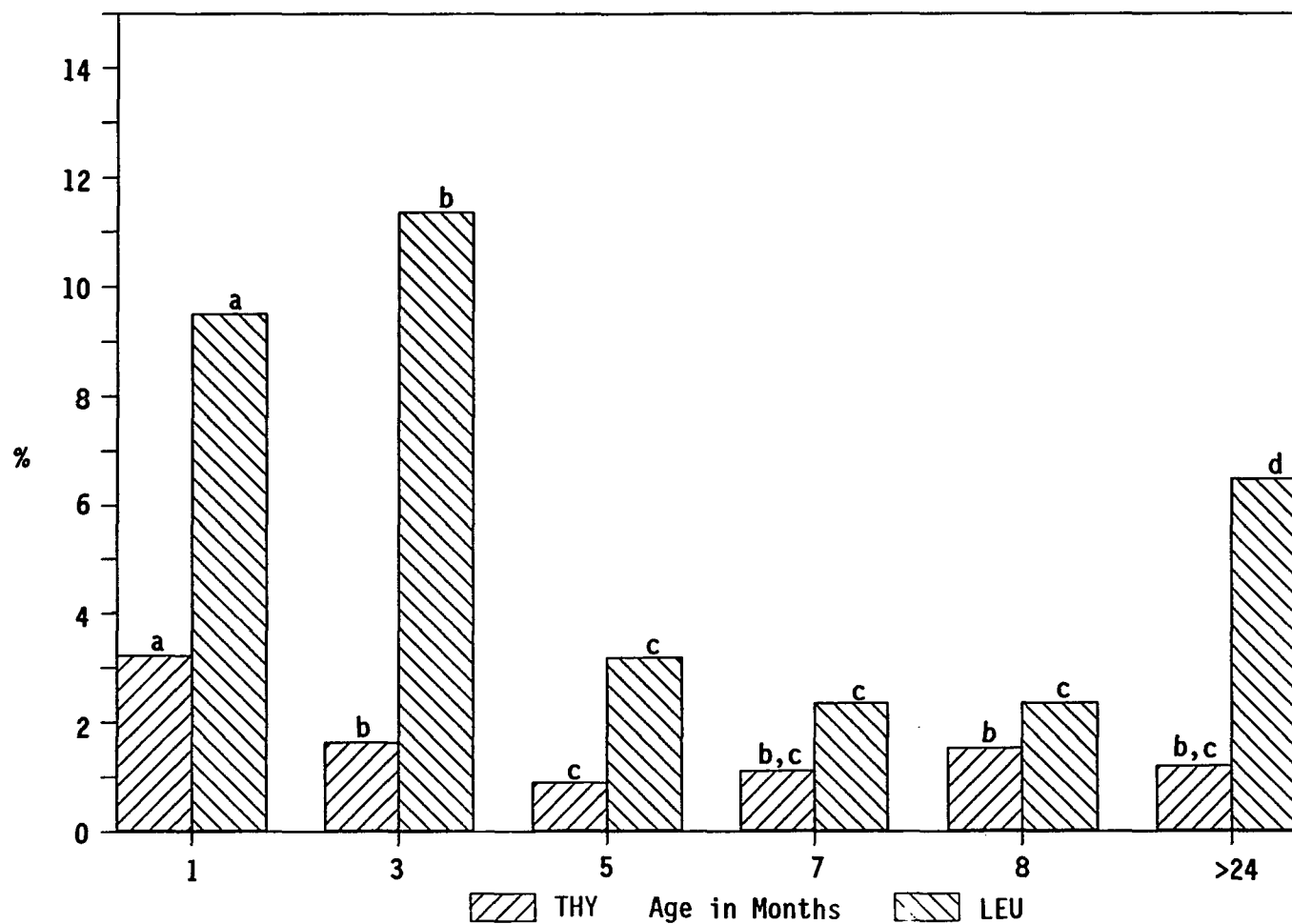
a,b,c,dBars with same stripes and different letters differ (P < .05).

Figure 1. Alteration in percent incorporation of thymidine and leucine of bovine fibroblasts as donors age increased

14 ── a

12 ── b

10 ──

8 ── c   c   c

PLT CT

6 ──

4 ── d

2 ──

0 ──
    1   3   5   7   8   >24

Age in Months
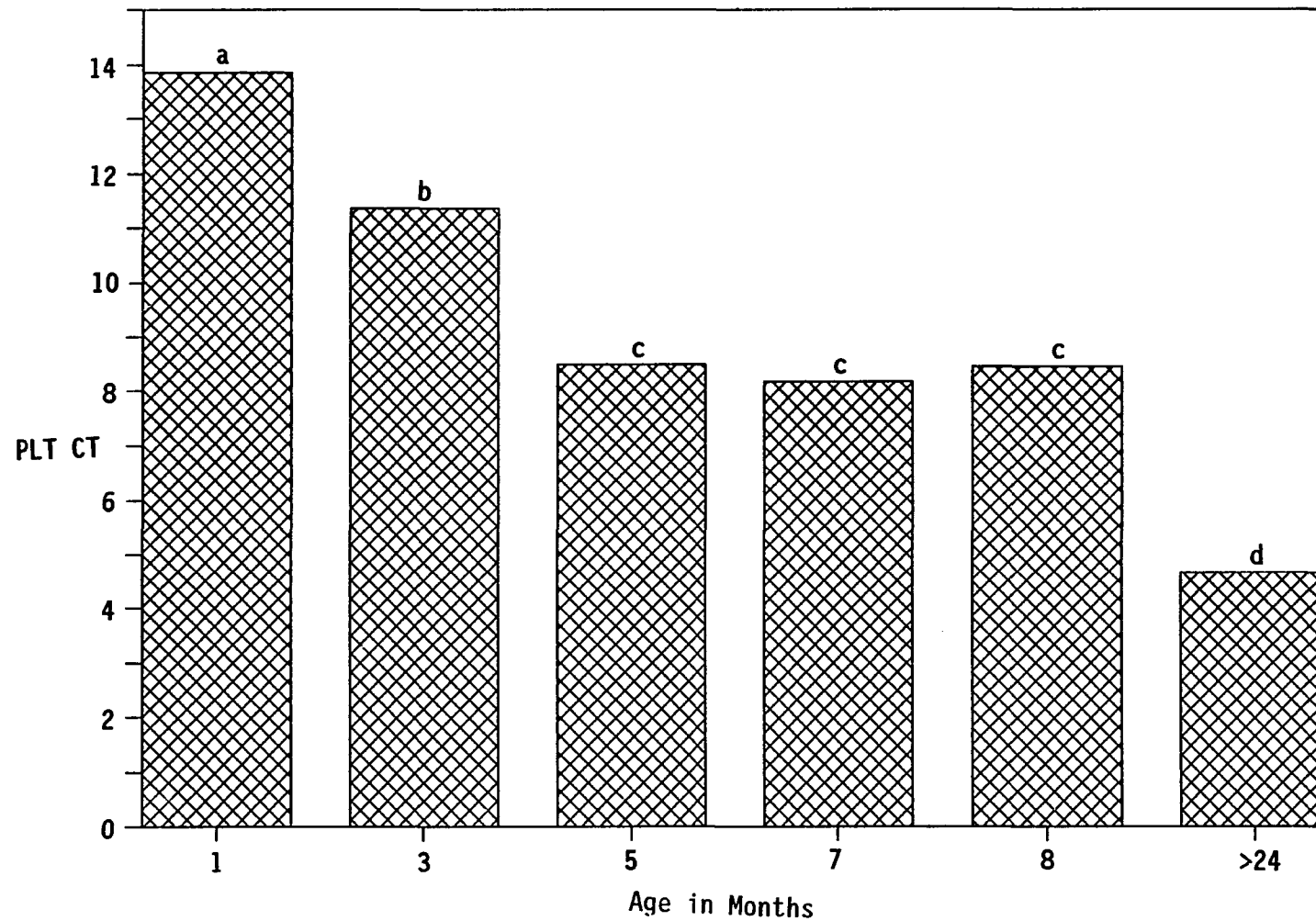
a,b,c,dBars with different letters differ (P < .05).

Figure 2. Alteration in outgrowth of bovine fibroblasts from tissue explants as donors age increased

DISCUSSION

The cell measurements and BIR WT or BIR WT alone were not consistent for predicting future animal performance in the different groups. The cell measurements and BIR WT resulted in high r^2 values indicating that multiple regression was a good model for these data. However, due to the small number of animals sampled, the r^2 values were not significant for the majority of the animals because of a lack of power for the statistical test. The predictive ability of the cell culture data with BIR WT was not dependent on breed or sex of the cattle.

Cells from calves, less than 1 mo, exhibited higher rates of THY than did older calves. If bovine fibroblasts do not increase cell DNA content with age, then young calves may have more cells traversing the cell cycle and fewer non-cycling cells or the cells may be proceeding through the cell cycle at a faster rate than cells from an older animal. DNA content of human fibroblasts did not change as a function of *in vitro* ageing and was not significantly affected by donor age (Schneider and Mitsui, 1976) and there was an increased number of non-cycling cells as donor age increased (Hasawaga et al., 1985).

Fibroblasts from young calves had the highest LEU indicating cells from calves were synthesizing more protein. Young calves are growing muscle tissue with very little adipose tissue being deposited. The increased leucine incorporation in the cows was unexpected and unexplained. These cows were pregnant, either late second trimester or early third trimester, which might have influenced LEU.

There was a linear relationship between animal age and PLT CT. Calves less than 1 mo of age had the highest growth rate relative to body

weight and the highest PLT CT. Mature cows had the lowest growth rate relative to body weight and the lowest PLT CT. This was similar to findings of Goldstein (1974) using skin from the base of the neck of juvenile and adult Galapagos turtles. The first appearance of fibroblasts from the young turtle primary explants occurred earlier than from older turtles. Cultured fibroblasts from young turtles incorporated more thymidine than did cells from older turtles. Conclusions were made that explants from older animals have a longer latent time prior to outgrowth and that outgrowth is less vigorous and from a smaller proportion of the tissue fragments in the older animals. An inverse relationship has been shown to exist between human donor age and the number of times cells may be subcultured prior to senescence, when cells no longer grow (Hayflick, 1965; Ohno, 1979; Phillips et al., 1984).

Young animals have rapid increase in body size that decreases as maturity is reached. Skeletal muscle growth in calves (110 to 360 kg) results from an increase in cellularity (number of nuclei per muscle fiber) and from an increase in cell size (Trenkle et al., 1978). In cattle exceeding 360 kg, muscle growth resulted from an increase in cell size. The measured cell parameters of bovine fibroblasts of dermal origin changed with age, suggesting that cultured fibroblasts retain the growth pattern of the donor animal. As maturity is reached, the animal enters a maintenance situation where body size remains fairly constant.

There was a wide range of variation in the values within animals in an age group and an overlap of values among age groups, indicating a large amount of variation in cells from different donors. Age of animals sampled ranged from a few days to over 10 years.

These results indicate that the potential for animal growth was expressed in cultured fibroblasts but the measurements of growth of the cells in culture did not always predict live weight at a future point in time. The use of cell culture data with birth weight provided a good model to predict weights of some groups but not for others.

There may be a specific protein, possibly a growth factor or enzyme, that would be more indicative of animal performance at a future point than measuring leucine incorporation by fibroblasts. If this protein is synthesized by fibroblasts, determination of the amount of this protein present in the cell culture will provide additional information for the beef producer to select animals that produce lean beef efficiently.

CONCLUSIONS

Cell culture data with birth weight provided a useful model to predict animal performance for some groups of cattle. In some cases, the high correlation coefficients for the multiple regression equations, using cell culture data and birth weight, were not significant due to the small numbers of animals sampled. A large group of young animals maintained in a similar environment need to be sampled at one time to help determine if cell cultures are indicative of future animal performance.

There was a linear relationship between outgrowth of cells from the explant and age of the animal. Fibroblast growth was greatest in explants from young calves and their growth occurs by increasing cellularity and cell size; whereas, growth in older animals occurs by increasing cell size. It appears that cultured fibroblasts may retain the ability to grow in a similar manner as the donor animal.

SECTION IV.

COMPARISON OF GROWTH OF PORCINE FIBROBLASTS
IN CULTURE WITH GROWTH OF DONOR ANIMALS

INTRODUCTION

Animal growth as measured by weight gain may represent an increase in cell number, an increase in cell size or a combination of these. Muscle DNA and RNA content and concentrations have been measured to determine if these are indicative of protein synthesis and lean muscle mass of the animal (Harbison et al., 1976). Blood concentrations of hormones (Trenkle and Topel, 1978; Verde and Trenkle, 1987; and Plouzek and Trenkle, 1991a) and growth factors (Plouzek and Trenkle, 1991b) have been related to animal growth. The next development was obtaining tissue biopsies for determining mRNA levels (Hannon et al., 1991) or culturing cells to study the effects on growth of hormones and growth factors in isolated cells.

During attempts to establish bovine cell cultures, an observation was made that the rate of fibroblast growth from tissue explants appeared to be related to donor animal growth. To determine if such a relationship exists, pigs were chosen for comparison of growth rate of fibroblasts in culture with growth rate of donor animals.

MATERIALS AND METHODS

Materials

Materials were purchased from the following sources: Ham's F-12
powdered medium, penicillin, streptomycin sulfate, nystatin and giemsa from
Sigma Chemical Company (St. Louis, MO); leucine (14-C) and thymidine (3-H)
from Amersham International (Amersham, UK); fetal bovine serum (FBS) from
Gibco Laboratories (Grand Island, NY); Universol ES from ICN Biochemicals,
Inc. (Irvine, CA); petri dishes from Nunc (Roskilde, Denmark); Stadie-Riggs
Microtome from Thomas Scientific (Swedesboro, NJ); glass coverslips and
methanol from Fisher Scientific (Chicago, IL); and high vacuum grease from
Dow Corning (Midland, MI).

Methods

Twelve litters of pigs from the Iowa State University Swine Teaching
Farm were utilized in this study. There were approximately 100 animals
sampled; however, data from 83 animals were used in this study. Data from
animals that died during the study were deleted. Animals were sampled at
near birth, approximately twenty-one days (21 d) and five months of age
(5 mo).

A sample of skin was taken from an ear via punch biopsy using an ear
notcher and plated in duplicate or triplicate as described in Section III,
with radiolabeled 14-C-leucine (65,000 dpm/dish, 57 mCi/mmol specific
activity) added at time of plating to the culture medium to estimate
protein synthesis. Radiolabeled 3-H-thymidine (180,000 dpm/dish, 89
mCi/mmol specific activity) was added one hour prior to harvest for
estimation of DNA synthesis. Thymidine and leucine incorporation were

measured as disintegrations per minute per dish. Data on incorporation were normalized by calculating radioactivity incorporated as a percent of that added to the dish.

The first three tables represent averages of data collected from groups of animals, separated on the basis of sex. Tables are arranged to include number of animals per group, weight at time of sampling, percent thymidine incorporation (THY), percent leucine incorporation (LEU) and number of 2 x 2 mm squares containing fibroblasts (plate count, PLT CT).

The correlation coefficients for the multiple regression equations calculated from the cell culture measurements are given in the fourth table. Multiple regression equations were calculated using THY, LEU and PLT CT as the independent variables and animal weight at birth, 21 d or 5 mo as the dependent variable. Cell culture data collected at birth were used to predict birth, 21 d or 5 mo weight. Twenty-one day cell culture data were used to predict 21 d weight or 5 mo weight. Five month cell culture data were used to predict 5 mo weight.

The coefficients of the multiple regression equations are given in the fifth table, using THY, LEU and PLT CT as the independent variables and WT, at birth, 21 d or 5 mo, as the dependent variable. There was a wide range of values from the intercept and coefficients for THY, LEU and PLT CT.

To examine differences related to age, data from different sexes and litters were pooled and sorted based on age: birth, 21 d and 5 mo.

Statistical Analysis

Data were analyzed by analysis of variance using the General Linear

Models and Regression procedures of SAS (SAS, 1989). Differences between least squares means at the different ages were separated on the basis of least significant differences using the predicted difference option (SAS, 1989).

RESULTS

The results from the samples taken at birth are given in Table 1. The animals were separated into groups based on sex: boars, barrows (castrated at approximately three weeks of age) and gilts. The averages for the animals at birth are: 2.2 to 2.5 kg body weight, .86 to .93% THY, 6.39 to 8.34% LEU and 12.8 to 13.5 PLT CT. Averages and ranges in values for animals separated into groups on the basis of sex are shown.

The results from samples taken at 21 d are given in Table 2. The increased number of animals reflects the addition of cell culture data from three litters that were lost due to failure of the incubator. The averages for the animals are: 6.8 to 7.0 kg body weight, 1.17 to 1.28% THY, 8.84 to 11.53% LEU and 9.3 to 10.8 PLT CT. The averages and range in values for the three groups of animals separated by sex are given.

The results from samples taken at 5 mo are given in Table 3. The averages for the animals are: 90.4 to 99.3 kg body weight, 1.08 to 1.17% THY, 6.15 to 6.59% LEU and 6.4 to 6.6 PLT CT. The averages and range in values for the three groups of animals separated by sex are given.

Table 4 contains the correlation coefficients for predicting animal weight from the cell measurements. For boars, the cell culture data collected at birth used to predict birth weight resulted in a high r^2 value ($r^2 = .73$) indicating the model fit the data. However, due to the small number of animals sampled, this was not significant due to a lack of power of the statistical test. Similarly, using cell culture data from the birth sample was not statistically significant in predicting 21 d boar performance even thought there was a high r^2 value ($r^2 = .79$). Cell culture data collected at birth was statistically significant for

Table 1. Cell culture data from pig samples collected at birth to determine thymidine and leucine incorporation and growth from the tissue explant*

SEX	n	Wt kg	THY %	LEU %	PLT CT No.
Boars, avg	7	2.2	.90	6.39	13.5
Range		1.5-3.6	.41-1.29	2.70-10.3	9.0-19.5
Barrows, avg	29	2.6	.86	8.34	12.8
Range		.9-4.1	.32-1.70	1.59-14.86	3.0-19.0
Gilts, avg	29	2.5	.93	7.90	13.8
Range		1.1-5.0	.30-1.80	1.90-12.88	4.5-21.3

*Average values for boars, barrows and gilts are given for weight at time of sampling, % thymidine and leucine incorporation by the explants and plate count, average number of squares containing cells. The range in values for the groups are listed.

Table 2. Cell culture data from samples collected at 21 d to determine thymidine and leucine incorporation and growth from the tissue explant*

SEX	n	Wt kg	THY %	LEU %	PLT CT No.
Boars, avg	8	6.9	1.17	8.84	9.3
Range		3.4-11.1	.53-1.63	5.50-11.43	6.0-13.0
Barrows, avg	33	6.8	1.21	11.53	10.7
Range		3.2-9.3	.69-2.14	2.58-23.82	5.0-18.5
Gilts, avg	42	7.0	1.28	9.40	10.8
Range		3.0-13.2	.53-3.43	3.10-17.90	1.0-20.5

*Average values for boars, barrows and gilts are given for weight at time of sampling, % thymidine and leucine incorporation by the explants and plate count, average number of squares containing cells. The range in values for the groups are listed.

Table 3. Cell culture data from pig samples collected at 5 mo to determine thymidine and leucine incorporation and growth of cells from the tissue explant*

SEX	n	Wt kg	THY %	LEU %	PLT CT No.
Boars, avg	8	99.2	1.17	6.21	6.5
Range		82.7-136.4	.43-1.94	2.48-8.74	3.0-11.3
Barrows, avg	33	93.2	1.08	6.59	6.4
Range		72.7-122.7	.48-2.16	2.33-15.08	3.0-14.3
Gilts, avg	42	90.4	1.12	6.15	6.6
Range		62.7-117.3	.22-2.09	1.45-12.56	0.0-11.0

*Average values for boars, barrows and gilts are given for weight at time of sampling, % thymidine and leucine incorporation by the explants and plate count, average number of squares containing cells. The range in values for the groups are listed.

predicting boar performance at 5 mo (r^2 = .89) as was 21 d cell culture data for predicting 21 d performance (r^2 = .78). For the barrows and gilts, cell culture data were not a good model to predict animal performance. Table 5 contains the coefficients of the multiple regression equations for predicting animal performance for the three groups of animals separated on the basis of sex.

Figure 1 represents the average THY and LEU for the pigs from birth to 5 mo. Average THY was lowest at birth and increased at 21 d. THY was not different between 21 d and 5 mo (P < .01). Average LEU increased from birth to 21 d and decreased at 5 mo (P < .01).

Figure 2 depicts PLT CT for the three ages. Pig samples collected at birth exhibited the greatest growth from the explant. The extent of cell growth from the explant decreased as animals aged.

Table 4. Correlation coefficients for the multiple regression equations using cell culture data to predict animal weight at different ages[a]

SEX	n	Age at Sampling	Age at Pred. Wt	r^2
Boars	7	birth	birth	.73
	7	birth	21 d	.79
	7	birth	5 mo	.89*
	8	21 d	21 d	.78*
	8	21 d	5 mo	.64
	8	5 mo	5 mo	.71
Barrows	29	birth	birth	.21
	29	birth	21 d	.12
	29	birth	5 mo	.18
	33	21 d	21 d	.17
	33	21 d	5 mo	.02
	33	5 mo	5 mo	.37
Gilts	29	birth	birth	.29
	29	birth	21 d	.07
	29	birth	5 mo	.08
	42	21 d	21 d	.15
	42	21 d	5 mo	.11
	42	5 mo	5 mo	.11

[a]Correlation coefficients for samples collected at birth to predict birth weight, 21 d weight or 5 mo weight; 21 d samples were used to predict 21 d weight and 5 mo weight; and 5 mo cell culture data were used to predict 5 mo weight.
*$P < .1$.

Table 5. Coefficients for the multiple regression equations using cell culture data to predict animal weight*

Sample Time	Age WT	Inter.	THY	LEU	PLT CT
Boars					
birth	birth	2.55	-0.80	0.12	-0.02
birth	21 d	5.46	1.04	0.12	-0.06
birth	5 mo	80.54	7.91	1.27	-0.33
21 d	21 d	5.25	1.54	0.05	-0.09
21 d	5 mo	92.18	2.64	0.07	-0.28
5 mo	5 mo	118.03	-13.67	-0.97	-0.58
Barrows					
birth	birth	2.04	.62	0.16	-0.11
birth	21 d	7.72	1.81	0.50	-0.43
birth	5 mo	94.57	3.59	7.06	-3.29
21 d	21 d	8.80	4.12	-0.83	0.07
21 d	5 mo	138.94	21.87	-5.60	-1.70
5 mo	5 mo	117.28	19.33	-6.28	-0.26
Gilts					
birth	birth	2.92	-1.08	0.12	-0.04
birth	21 d	7.65	-0.17	0.03	-0.11
birth	5 mo	80.09	0.41	1.26	0.06
21 d	21 d	7.75	-0.45	0.13	-0.13
21 d	5 mo	84.17	0.52	1.16	-0.50
5 mo	5 mo	74.27	9.85	-0.16	0.92

*Sample Time indicates when samples were collected and Age WT was the weight of the animal used as the dependent variable.

14

12

10

8

%

6

4

2

0

Birth   21 d   5 mo

AGE

a

a

b

b

b

a

▓▓▓ THY   ⊠⊠⊠ LEU
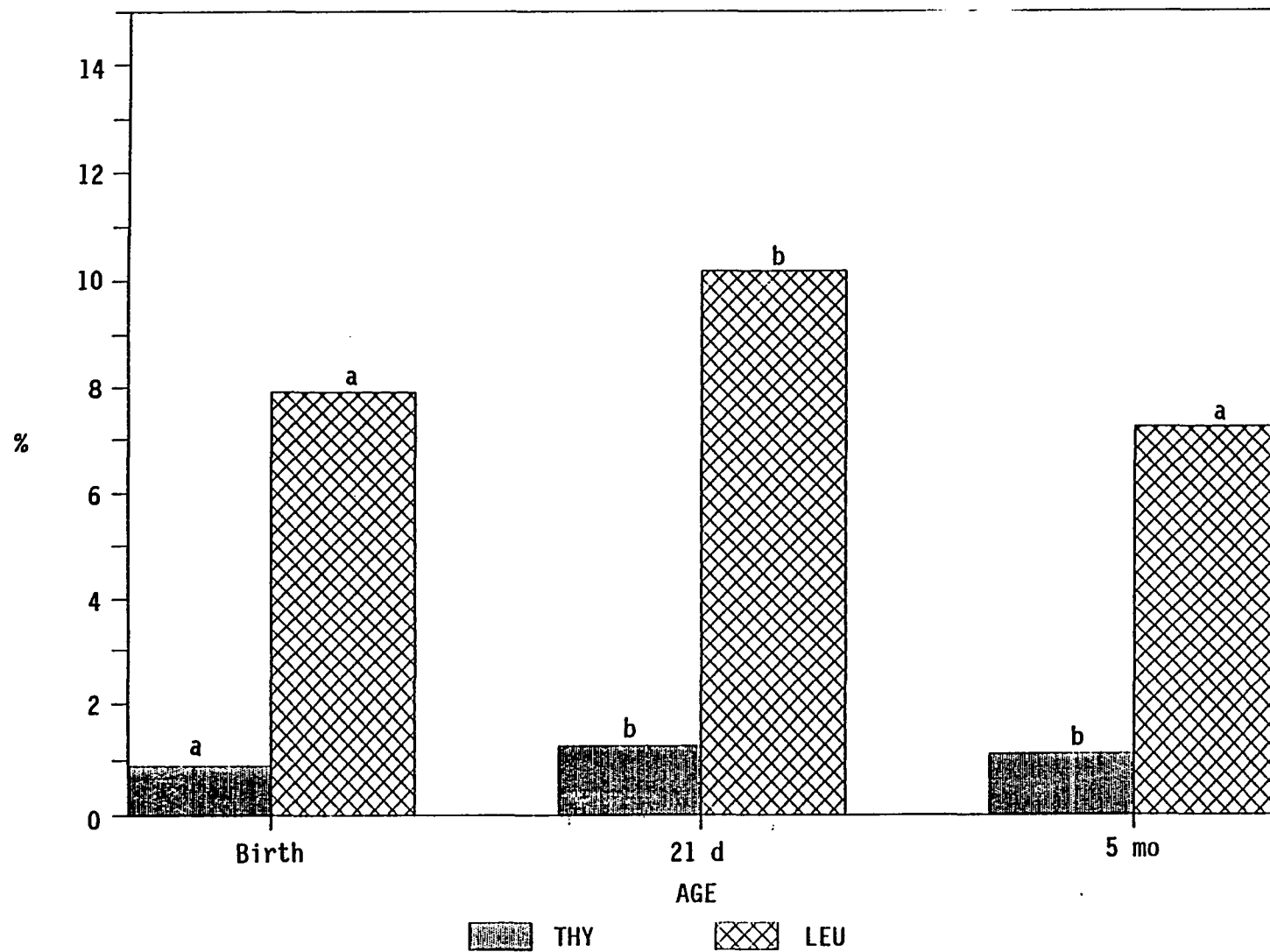
a,b,cBars with same stripes and different letters differ (P < .01).

Figure 1. Alteration in percent incorporation of thymidine and leucine of porcine fibroblasts as donors age increased
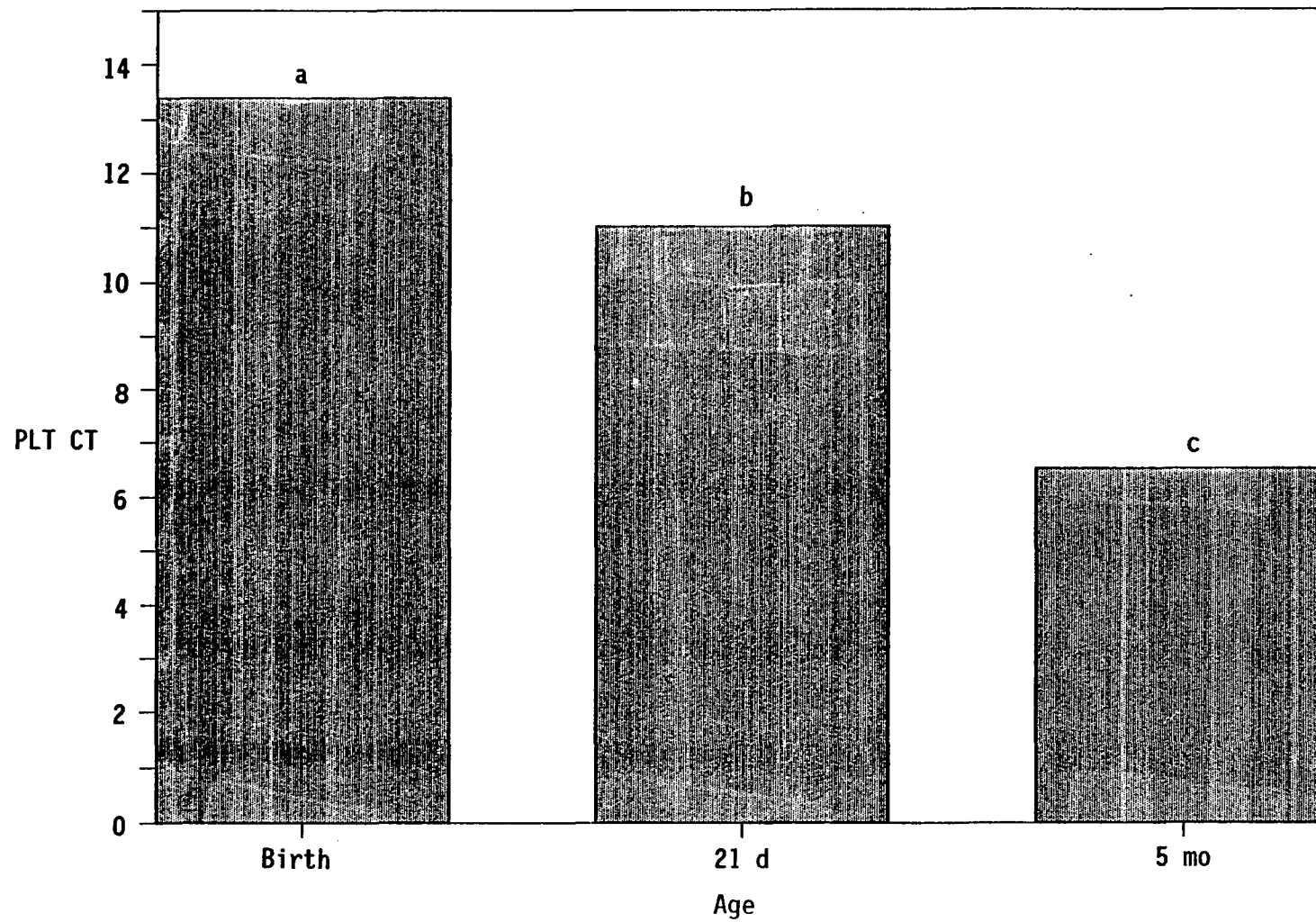
a,b,cBars with different letters differ (P < .01).

Figure 2. Alteration in outgrowth of porcine fibroblasts from tissue explants as donors age increased

DISCUSSION

The cell measurements were not consistent in the ability to predict future animal performance in the different age and sex groups. The cell measurements resulted in high r^2 values indicating that multiple regression was a good model for the boar data. However, due to the small number of boars sampled, the r^2 values were not significant for the majority of the animals because of a lack of power for the statistical test.

Cells from young pigs exhibited lower rates of THY than did cells from older pigs. If porcine fibroblasts do not increase cell DNA content with age, young pigs may have fewer cells traversing the cell cycle than cells from older pigs. DNA content of human fibroblasts did not change as a function of *in vitro* ageing and was not significantly affected by donor age (Schneider and Mitsui, 1976) and there was an increased number of non-cycling cells as donor age increased (Hasawaga et al., 1985).

Fibroblasts from pigs sampled at 21 d had the highest LEU, young pigs were synthesizing more protein than were 5 mo old animals. Young pigs are growing muscle tissue with very little adipose tissue being deposited; whereas, the 5 mo old animals are depositing both muscle and adipose tissues.

There was a linear relationship between animal age and PLT CT. Pigs at birth had the highest growth rate relative to body weight and the highest PLT CT. This was similar to findings of Goldstein (1974) using skin from the base of the neck of juvenile and adult Galapagos turtles. The first appearance of fibroblasts from the young turtle primary explants occurred earlier than from older turtles. Cultured fibroblasts from young turtles incorporated more thymidine than did cells from older turtles.

Conclusions were made that explants from older animals have a longer latent time prior to outgrowth and that outgrowth is less vigorous and from a smaller proportion of the tissue fragments in the older animals. An inverse relationship has been shown to exist between human donor age and the number of times cells may be subcultured prior to senescence, when cells no longer grow (Hayflick, 1965; Ohno, 1979; Phillips et al., 1984).

Young animals exhibit rapid increases in body size early in life and have decreased rate of growth as maturity is reached. The measured cell parameters of porcine fibroblasts of dermal origin changed with age, suggesting that cultured fibroblasts retain the growth pattern of the donor animal. As maturity is reached, the animal enters a maintenance situation where body size remains fairly constant.

There was a wide range of variation in the values within animals in an age group and an overlap of values among age groups, indicating a large amount of variation in cells from different donors. Age of animals sampled ranged from birth to five months. There were purebred spotted, duroc, and Yorkshire and crossbred animals included in this study. Some animals may reach maturity at lighter weights than others due to the differences in the genetic pool sampled. At the same age, animals could be at different points on their physiological growth curves.

These results indicate that the potential for animal growth was expressed in cultured fibroblasts but the measurements of growth of the cells in culture did not always predict live weight at a future point in time. There may be a specific protein, possibly a growth factor or enzyme, that would be more indicative of animal performance at a future point than measuring leucine incorporation by fibroblasts. If this protein is

synthesized by fibroblasts, determination of the amount of this protein present in the cell culture will provide additional information for the pork producer to select animals that produce lean pork efficiently.

CONCLUSIONS

The cell culture measurements used in this research were not good predictors of future animal weights. There was a linear relationship between age and outgrowth of fibroblasts from the explant. Explants from younger pigs exhibited more fibroblast growth than was seen from explants collected from animals at older ages. Because young animals have the highest growth relative to body weight, the greater growth of cells from explants of young animals may be indicative of growth potential.

Animals have the most rapid growth relative to body weight early in life. Thymidine incorporation increased as pigs aged from birth to 21 d and explants from animals at 21 d incorporated more leucine than did samples from animals at birth or 5 mo. Following birth, most of this soft tissue growth early in life is muscle. It appears that cultured fibroblasts may retain the ability to grow in a similar manner as the donor animal.

GENERAL SUMMARY

The research presented in this dissertation has utilized cells collected from farm animals. Much data have accumulated on the response of cultured fibroblasts, derived from humans or rodent tissue, to hormones and growth factors.

In Section I, the methods used to collect and propagate bovine fibroblasts were identified. The ears of cattle and pigs provided a readily accessible site for biopsy. Skin samples were collected from animals of various sizes and from the same animal at different times and were propagated and subcultured. Cells collected and grown in this manner responded to the addition of serum to the medium by increasing thymidine and leucine incorporation. The response of bovine fibroblasts to hormones that are known to regulate animal growth was examined. Without serum, growth hormone, insulin or thyroxine did not affect DNA synthesis. The addition of dexamethasone or estradiol was inhibitory. Addition of insulin to medium containing one percent fetal bovine serum increased thymidine incorporation in two of four experiments. Dexamethasone inhibited thymidine incorporation in the presence of one percent serum, but not in ten percent serum. The inhibition of leucine incorporation by the presence of insulin was unexpected. Estradiol alone did not stimulate leucine incorporation in cultured bovine fibroblasts. Thyroxine given in the presence of one percent serum stimulated leucine incorporation but thyroxine was not stimulatory by itself. Thyroxine required the presence of some unidentified compound contained in serum to elicit an increase in leucine uptake. None of the hormones alone or in combination seemed to be potent regulators of fibroblast growth.

Bovine fibroblasts derived from dermal tissue responded to the addition of growth factors by increasing thymidine and leucine incorporation. Similar levels of IGF-I were required by bovine cells as in humans fibroblasts to stimulate a growth response. EGF also increased uptake of thymidine and leucine by bovine fibroblasts. Combinations of hormones and growth factors were not better than growth factors alone. The addition of ten percent serum stimulated more thymidine or leucine incorporation than any combination of hormone and growth factors used.

The measurements on the growth of fibroblasts presented in Sections III and IV for predicting animal weight at a specific point in the future were not reliable or extremely accurate. The bovine or porcine fibroblasts from animals of different ages exhibited a wide variation in response to similar culture conditions. Outgrowth of fibroblasts from the explants of bovine or porcine tissue decreased as animal age increased. This decrease in outgrowth from the explant as animals age may be related to the decrease in growth rate relative to body weight as the animal approaches maturity.

Fibroblasts were a useful model for measuring the effects of hormones or growth factors but measurements of thymidine incorporation, leucine incorporation and fibroblast growth from the explants are not good predictors of animal growth.

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