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Effects of Testosterone and Estradiol -17B On Growth Hormone Release From Dispersed Bovine Anterior Pituitary Cells In Vitro

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EFFECTS OF TESTOSTERONE AND ESTRADIOL -17B ON GROWTH HORMONE RELEASE FROM DISPERSED BOVINE ANTERIOR PITUITARY CELLS IN VITRO

BY

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ABSTRACT

Effects of Testosterone and Estradiol-17B on Growth Hormone Release from Dispersed Bovine Anterior Pituitary Cells In Vitro

By

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The effect of estradiol-17B (E) and Testosterone (T) on secretion of growth hormone (GH) by primary cultures of bovine adenohypophyseal (bAP) cells was studied. Dispersed bAP cells ($5X10^5$ cells/well) from either calves, heifers, steers or cows were incubated for 24, 48 or 72 h with E (10^{-10}, 10^{-8} M) or T (10^{-7}, 10^{-5} M). At 24 h the cells were challenged for 1 h with 1-44 NH_4 growth hormone-releasing hormone (GRF). Fungizone (2.5ug/ml) reduced ($P<.05$) GH released after 6 d of plating. Phenol red (45uM) had no effect ($P>.05$) on GH released into the media for up to 6 d after plating. Preincubation of bAP cells with either E or T did not affect ($P>.05$) basal GH released at 24, 48 or 72 h of treatment. Neither concentration of E and T (10^{-7}M) had an effect ($P>.05$) on GRF-induced GH release.

However, T (10^{-5}) increased ($P<.05$) the GH released in response to GRF challenge. These results indicate that E and T have no direct effect on basal GH release in vitro, but T enhances the effect of GRF on GH release.

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INTRODUCTION

Males of most vertebrate species, including humans, laboratory rodents (Wehrenberg et al., 1985) and ruminant animals (Irvine and Trenkle, 1971) are larger than females of the same species. Male compared with female cattle have slightly higher plasma concentrations of growth hormone (GH, Irvine and Trenkle, 1971). Sexual differences in pituitary content and release of GH first appear at the time of puberty (Hoeffler and Frawley, 1986). There are several lines of evidence to suggest that gonadal steroids, which increase at puberty, regulate this dimorphism (Davis et al., 1977). A number of studies had been conducted to examine the relationship of estradiol-17B (E) and testosterone (T) with secretion of GH (Cornin and Rogol, 1984; Jansson et al., 1985; Wehrenberg et al., 1985). Results of some of these studies have been contradictory (Davis et al, 1977). For instance, it has been reported that E treatment increased plasma GH in humans (Goldzieher et al., 1976), sheep and cattle (Preston, 1975), while others have failed to show any effect of E on plasma GH in cattle (Beck et al., 1976). Androgen treatment stimulates GH secretion in humans (Illig and Prader, 1970) and in cattle (Convey et al., 1971; Anfinson et al., 1975; Johke,

1984). Possible explanations for the conflicting results may be due to differences in animal species, age of animals, and method and time of sample collection. In addition, differences in GH secretory pattern between adult male and female animals may play a physiological role in the rate of somatic growth (Isgaard et al., 1988). It is well documented that males have more muscle and protein and less fat than females (Schanbacher et al., 1980). In addition GH is known to have an anabolic effect on protein metabolism in muscle and a lipolytic effect on fat (Cryer and Daughaday, 1977; Trenkle and Topel, 1978). The pulsatile pattern of GH secretion in males has larger amplitudes with a low base line in between pulses, while in females GH secretion exhibits more frequent episodes with low amplitudes and a high base line between episodes (Eden et al., 1987). These observations suggest that the difference in growth and composition between male and female animals is associated with the sex steroids and they may have a role in modulating the GH secretory pattern.

Understanding the endogenous secretory pattern, of estrogen and testosterone and their effects on GH release, is of both theoretical and practical importance in order to take advantage of any potential growth-promoting effects these steroids might have on stimulating GH secretion and

(or) synthesis. The overall objective of the present study was to determine whether testosterone and estrogen have direct effects, or modulate the effect of growth hormone releasing factor (GRF), on GH production from dispersed bovine anterior pituitary cells in a static in vitro incubation.

REVIEW OF LITERATURE

Growth is defined as the increase in the mass of an individual or the coordinated increase of all animal tissues over time (Beitz, 1985). The growth process involves interactions between nutrients, genotype, environment and the influence of a number of hormones on nutrient availability to cells and cell division (Spencer, 1986). Therefore, growth can be considered the most obvious long term process in animal production (Bauman et al., 1982). The primary goal of animal production is to supply high quality food for humans (Beitz, 1985). Thus, achieving maximum growth is a key objective in animal production (Young, 1987). Ruminants are important in human food production and animal ecology because of their unique digestive system for utilization of energy from plants with high cellulose content (Hood, 1982). Metabolic and endocrine manipulation is being used to improve the production of meat animals with the objective of attaining more protein and less fat deposition in their carcasses (Gopinath and Kitts, 1984). Implants of hormones (anabolic agents) have been widely used to improve the growth rate and the amount of meat produced from ruminants (Spencer and Garssen, 1983). These implants contain one or more of

the sex steroids. It is generally agreed that these anabolic agents improve growth rate and alter metabolism favorably towards increased protein production and for decreased fat. The mechanisms of these actions have not been fully elucidated (Gopinath and Kitts, 1984). It has been suggested that the anabolic steroids act by altering the concentration of endogenous hormones, such as growth hormone (Buttery et al., 1978).

Growth Hormone Secretion and Regulation

Growth Hormone Production and Clearance. Growth hormone (GH) is secreted by specific anterior pituitary cells, the somatotrophs (Daughaday et al., 1975). The somatotropic cells predominate in the lateral portion of the pars distalis (Dellmann, 1981). The somatotropic granules average about 300 to 400 nm in diameter, have affinity for acidophillic stains and stain immunohistochemically for GH (Banks, 1981). GH is the most abundant hormone of all the active principles of the human anterior pituitary (Murad and Haynes, 1985). GH is synthesized within the endoplasmic reticulum and the nascent hormone is packed into secretory granules within the Golgi apparatus of the cell (Phillips, 1987). Human GH is a single chain of 191 amino acids (Niall et al., 1971). It is a globular protein with a molecular weight of 22 Kdaltons,

isoelecteric point of pH 4.9, and stable in pH 7.0 solution at 100 C (Li, 1987). In nonlactating Holstein cows, the GH secretion rate is 19.1 mg/d, and the serum half-life ($t_{1/2}$) of bovine GH (bGH) is 22.5 min (Yousef et al., 1969). Gopinath and Kitts (1984) estimated the metabolic clearance rate of GH as 74.5 ml^{-1}.kg body $weight^{-1}.h^{-1}$ and secretion rate as 0.91 ug^{-1}.kg body $weight^{-1}.h^{-1}$ in steers. For calves, Trenkle (1976) reported the secretion rate as 2.6 ug^{-1}.kg body $weight^{-1}.h^{-1}$.

GH is secreted in a pulstile manner in all mammals studied (Martin et al., 1978); including bulls and steers (Anfinson et al., 1975); and lactating cows (Vasilatos and Wangsness, 1981; Moseley et al., 1982). The frequency and amplitude of GH secretory episodes are determined by the balance between the two main hypothalamic peptides (growth hormone-releasing and -inhibiting factors, which regulate the secretion of GH (Baile, 1985).

Factors Affecting GH Production. Several factors can alter GH concentrations in the blood such as age, sex, diet and metabolism, sleep, stress and exercise. Each of these factors will be discussed below.

Ontogenesis of GH: Factors that determine GH production (synthesis and release) in developing animals

are not fully understood (Walker et al., 1977). Pituitary responsiveness to the two hypothalamic peptides, growth hormone releasing factor (GRF) and somatotropin release-inhibiting factor (SRIF), and the presence of their receptors on somatotrophs are the major factors affecting GH secretion (Frohman et al., 1987). In cattle, circulating GH is higher in prepubertal calves than in pubertal animals (Renyaert et al., 1976; McCarthy et al., 1979; Johke et al., 1984). Somatotropic sensitivity to SRIF increases with age (Cuttler et al., 1986). The authors attributed the high plasma GH concentrations in young animals to a relative resistance to SRIF. In addition, GH released in response to GRF decreases with age (Johke et al., 1984).

Sex of Animal and GH Secretion. Sex of animal is known to influence concentration of GH in the blood (McCarthy et al., 1979). Jones et al.(1965) was the first group to suggest that GH concentrations differed between sexes. They used acrylamide gel electrophoresis of rat pituitary extracts to quantitate GH. Birge et al. (1967) demonstrated that anterior pituitaries from male rats contained more GH than those from female rats. It had been concluded that GH synthesis is greater in males as compared with pituitaries from females (McLeod et al., 1969; Burek and Frohman, 1970; Yamamoto et al., 1970). Using a reverse

hemolytic plaque assay, the individual somatotrophs of male pituitaries were shown to have greater secretory capacities than those of females (Hoeffler and Frawley, 1986). Meanwhile, it was concluded that serum GH concentrations of males are higher than those of females at all ages studied (Renaert et al., 1976).

Diet and Metabolic Substances and GH Secretion. The three major classes of metabolic substrates (carbohydrate, protein, and fat) affect GH secretion (Martin and Reichlin, 1987). GH concentration is negatively correlated with both energy and nitrogen intake (Waghorn et al., 1987). Also, increased fatty acids in the blood suppressed GH secretion (Imaki et al., 1985). In addition, insulin-induced hypoglycemia,as well as hypoglycemia from other causes, is a particularly potent stimulus to GH secretion (Murad and Haynes, 1985). Knopf et al. (1968) reported that the individual amino acids differ in their capacity to evoke release of GH; for instance, arginine is a potent stimulus of GH release. Metabolic status is postulated to alter the effect of exogenous GH injection on nutrient partitioning (Sechen et al., 1989).

Photoperiod and Sleep and GH Secretion: Exposure of animals to long daylights increased daily weight gain,

food intake and metabolic efficiency (Schanbacher and Crouse, 1980). Barenton et al. (1988) demonstrated in sheep that GH as well as prolactin could be involved in mediating the growth-promoting effect of increasing photoperiod . However, plasma GH and prolactin concentrations are affected differently by photoperiod. The authors suggested that photoperiodic stimulation of body weight in sheep is a consequence of light-induced GH secretion.

A major burst of GH secretion in man is associated with the onset of sleep, as well as the electroencephalographic (EEG) pattern of slow-wave sleep (Takahashi, 1968). These results were confirmed by Knip et al. (1987) who found that the episodes of high GH release in rats were usually associated with slow-wave sleep. Also, it had been demonstrated by the same group that GRF was secreted in a pulsatile manner; however, there was no association between GRF peaks and slow-wave sleep.

Stress and Exercise and GH Secretion. There is no doubt that a stress control mechanism operates in relation to the secretion of GH (Landon and Greenwood, 1968). A wide variety of stressful stimuli may cause an increase in the circulating concentrations of GH in a manner analogous to the release of adrenocorticotrophic hormone (ACTH) and the catecholamines (Brown and Reichlin, 1972). Martin and

Reichlin (1987) reported that physical and emotional stresses lead to an increase in GH secretion in humans. Also, moderate exercise may elevate plasma GH concentrations in man, independently from blood sugar concentrations (Roth, 1963).

Regulation of GH Secretion. Regulation of adenohypophyseal function is mediated mainly by hypothalamic regulatory peptides (Cryer and Daughaday, 1977). These peptides are transported from the hypothalamus to the anterior pituitary gland by the hypothalamo-adenohypophyseal-portal system, which originates from a capillary network in the median eminence (Murad and Haynes, 1985). The hypothalamic peptides are influenced by biogenic amines, which are transmitted from the central nervous system to the hypothalamus (Baile, 1981). Current evidence indicates that GH secretion, is under both stimulatory and inhibitory influences from hypothalamic and extra-hypothalamic sites (Reichlin, 1987). In addition, regulation of GH secretion is influence by the feedback mechanisms, as well as the neural signals, in regulating GH secretion (Tannenbaum, 1987). GRF and SRIF are the main hypothalamic peptides that regulate GH secretion (Jansson et al., 1987). SRIF withdrawal sets the timing of the episodic bursts of GH secretion (Kraicer et al., 1980),

while the magnitude of the bursts is set by the amount of GRF impinging upon the somatotrophs, before and during SRIF withdrawal (Kraicer et al., 1988).

Hypophysiotropic Hormones

Growth Hormone-Releasing Factor. GRF was isolated and identified from pancreatic tumors (Guillemin et al., 1982; Rivier et al., 1982). Several GRF peptides have been isolated from these tumors: hpGRF-44, hpGRF-40, and hpGRF-37 (Guillemin, 1984). The isolated peptides are the same major forms as those isolated from the human hypothalamus (Meister et al., 1987). Hypothalamic GRF isolated from other species differs from the human forms by at least three amino acids (Della-Fera et al., 1986). For instance, bovine GRF has been isolated and identified as a 44 amino acid peptide and differs from human forms in five amino acid residues (Esch, 1983). GRF is synthesized by cell bodies in the hypothalamus and transmitted through nerve endings to the median eminence where it is secreted into the hypophyseal portal circulation (Meister et al., 1987). The majority of the GRF-immunoreactive cell bodies were found in the arcuate nucleus and medial perifornical region of the lateral hypothalamus (Merchenthaler et al., 1984). The nerve fibers form a fan-like projection to the median

eminence where a dense accumulation of GRF-containing terminals are found (Martin and Reichlin, 1987). GRF has a high specificity for release of GH from dispersed pituitary cells (Guillemin et al., 1984). Plasma GH concentrations increased proportionally to the log dose of hpGRF-44 and reached a peak at 5 to 10 min (iv injection) and 15 min (sc injection), although the peak sc injection was 37% of that of iv injection (Johke et al., 1984). The first 29 residues of hpGRF possess all the information required for full in vitro activity (Rivier et al., 1982). In addition, the effect of all GRF peptides on GH release showed identical dose-response curves (Guillmin, 1984). GRF is believed to be the major physiological secretagogue of GH, however the precise mechanism of action has yet to be established (Brazeau et al., 1982b). To date, GRF treatment increases the intracellular Ca^{2+} concentration (Holl et al., 1987) and cyclic adenosine monophosphate (cAMP) synthesis and(or) stability (Tanner et al., 1988). GRF stimulates secretion, synthesis of GH, transcription of the GH gene, and proliferation of somatotrophs (Billestrup et al., 1987).

Somatotropin Release-Inhibiting Factor (SRIF). SRIF had been isolated and purified from ovine hypothalamic tissue as a 14-amino acid containing peptide (Brazeau et al., 1973). SRIF had been detected in the brain and other

tissues in two forms, one is a 14- and another is a 28-amino acid containing peptide (Benoit et al., 1982). SRIF-14 is less potent than SRIF-28 in its effect on GH-regulation (Patel, 1987). Somatostatin may be categorized as a neurohormone in pituitary regulation, whereas in the nervous system it may be described as a neurotransmitter or neuromodulator (Reichlin, 1983).

The SRIF molecule may inhibit some common cellular events in the pituitary and some other endocrine organs such as the pancreas, which results in inhibition of the release of some peptide hormones like insulin (Koeker et al., 1974). At the pituitary level, SRIF can inhibit basal release and GRF-stimulating effect of GH release (Wehrenberg et al., 1982). SRIF may be involved in short-loop feedback control of GH secretion (Martin and Reichlin, 1987). Incubation of hypothalamic tissue with GH (Berelowitz et al., 1981a) and SM-c (Berelowitz et al., 1981b) elevated the SRIF secretion into the media. SRIF reduces the calcium concentration in the somatotrophs (Thorner, 1987). He reported that concentration of calcium, amplitude and frequency of calcium spikes were associated with GH secretion . In addition, SRIF can reduce tissue cAMP (Borgeat et al., 1974) and it also inhibits release of stored and newly synthesized GH (Sheppard et al., 1979).

Feedback Regulation

GH Autoregulation. GH itself might be capable of controlling its own signal via hypothalamic and (or) pituitary receptors (Martin and Reichlin, 1987). The concept of GH autoregulation was obtained by earlier studies that content or synthesis of pituitary GH was reduced by GH administration (Krulich and McCann, 1966; Muller and Pecile, 1966). Intravenous or intracerebroventricular injection of GH suppressed GH release independently from circulating SM-c (Abe et al., 1983). At the pituitary level, GH per se operates as a feedback inhibitor of basal GH release from bovine anterior pituitary cells in culture (Glenn, 1986). In addition, GH secretion from anterior pituitary cells in static in vitro incubation declines with time, while it was shown to increased spontaneously in a perfusion system (Lapp et al., 1987). Furthermore, GH may increase the pituitary SRIF receptor binding capacity (Kata-Kami, 1985). At the hypothalamic level, GH may stimulate SRIF release from incubated hypothalami in vitro (Berelowitz et al., 1981). Intercerebroventricular administration of GH increased SRIF released into the hypophyseal portal circulation (Chihara et al., 1981). It has been reported by Tannenbaum (1987) and Miki et al. (1988) that GH may decrease output of GRF concomitantly with increased release of SRIF.

Insulin-Like Growth Factor (IGF) Feedback control. IGF's are produced in many tissues and are believed to exert their action in a paracrine or autocrine fashion (Phillips, 1986). High concentrations of immunoreactive IGF-1 and IGF-2 have been observed in the brain and all three lobes of the pituitary (Valentino et al., 1988). Evidence has indicated that the IGF peptides exert feedback control on GH secretion by acting at both the pituitary and the hypothalamic levels (Berelowitz et al., 1981b). Basal and stimulated GH release from rat anterior pituitary cells in vitro can be inhibited by administration of IGF-1 (Berelowitz et al., 1981b; Brazeau et al, 1982a; Goodyer et al., 1984). Furthermore, transcription of GH mRNA in rats was suppressed by IGF treatment (Fagin et al., 1988) and even after GRF challenge (Yamashita and Melmed, 1986). Increased circulating concentrations of GH increased IGF-1 gene expression in rat pituitaries, which may result in inhibiting GH gene expression (Fagin et al., 1988). It has been suggested that IGF-1 gene expression in the pituitary appears to be dependent on the circulating, and not the local, pituitary GH concentration. At the hypothalamic level , IGF stimulates SRIF secretion from rat hypothalamic fragments in vitro (Berelowitz et al., 1981a). Surprisingly, Glenn (1986) found no effect of IGF-1 on

basal or GRF-stimulated GH release from bovine anterior pituitary cells in vitro, while GH was suppressed in cultures of rat anterior pituitariy cells.

Sexual Dimorphism of GH Secretion.

Males of most vertebrate species, including humans, laboratory rodents (Wehrenberg et al., 1985) and ruminant animals (Irvine and Trenkle, 1971) are larger than their female counterparts. Gonadal steroids, which constitute a fundamental difference between the sexes, might mediate their effects on growth through regulating GH synthesis and release (Cornin and Rogol, 1984; Jansson et al., 1985; Wehrenberg et al., 1985). In cattle, intact males compared with females have consistently higher plasma GH concentrations at all ages (Irvin and Trenkle, 1971; Reynaert et al., 1976). The difference in body weight gain between males and females becomes most pronounced near the time of puberty (Wehrenberg et al, 1985). Also, the difference in pituitary GH content (Birge et al., 1967) and release (McLeod et al., 1969; Yamamoto et al., 1970; Burek and Frohman, 1970) appears around time of puberty, when concentrations for males exceed those of females.

Plasma GH Patterns in Normal Males and Females. The episodic nature of GH secretion is influenced by animal age

and gonadal steroids (Eden et al., 1987). GH secretory patterns are different between males and females (Ohlson et al., 1977). GH secretion in adult males has been described as high intermittent episodes with low or undetectable concentrations in between episodes in rats (Tannenbaum and Martin, 1976), sheep (Davis et al., 1977), and cattle (Anfinson et al., 1975). These large surges of GH release are often multiphasic with two or three smaller spikes of GH within each surge (Millard et al., 1987). In adult females, GH secretion occurs in more frequent peaks with lower amplitudes than in males, while GH concentrations between pulses (baseline) are greater than in adult male rats (Saunders et al., 1976), pigs (Dubreuil et al., 1988), sheep (Davis and Borger, 1974), and cows (Vasilatos and Wangsness, 1981).

Influence of Androgens on Plasma GH Pattern. Differences in growth rate between intact and castrated males have been known for many years (Dubreuil et al., 1989). The endocrine basis for this difference in growth rate has logically been attributed to testosterone (Gortsema et al., 1974). However, the mechanism by which testosterone increases growth rate has not been fully explained (Anfinson et al., 1975). One possible explanation for the growth promoting effect of testosterone may be

through regulation of GH production (Jansson et al., 1985). It has been reported that increased testosterone concentrations are associated with increased circulating GH concentrations in human males (Illig and Prader, 1970) and in bulls (Convey et al, 1971). Gonadectomy of males resulted in partial feminization of the secretory pattern of GH in rats (Eden, 1987). In cattle, GH secretory spikes of bulls are greater in magnitude than in steers (Anfinson et al., 1975). Also, treatment of castrated male rats (Wehrenberg et al., 1985) and steers (Johke, 1983) with testosterone enhanced the pituitary GH response to GRF.

Influence of Estradiol-17B on Plasma GH Pattern. Estrogenic compounds improve growth rate and meat production in cattle and sheep (Gopinath and Kitts, 1984). However, the underlying mechanism has not been clarified (Simard et al., 1986), although it has been suggested that estrogenic compounds may exert their effect on growth through regulation of GH production (Jansson et al., 1983). Treatment with these compounds increases weight of the pituitary gland (Elias and Weiner, 1987) and increases numbers of acidophillic cells in the pituitary gland (Clegg and Cole, 1954). Moreover, administration of estradiol-17B increases plasma GH concentrations (Breier et al., 1988). Estrogen seems to influence GH secretion during adult life

by increasing basal GH concentrations and GH pulse frequency and by decreasing pulse amplitudes (Borger et al., 1973; Trenkle, 1976; Ohlson et al., 1977; Donaldson et al., 1981; Eden et al., 1987). It has been suggested that estrogen exerts a stimulatory effect on GH production via a direct action on the rat pituitary gland (Jansson et al., 1983; Simard et al., 1986. In addition, the increase in GH might be due to an increase in secretion rate, and not to a decrease in the clearance rate as suggested by Trenkle (1981). Moreover the estrogen-induced increase in GH has been reported to be independent of circulating somatomedin concentrations (Weidemann et al., 1976). At the level of the hypothalamus estrogen treatment may increase some of the biogenic amines such as dopamine, opioid peptides, serotonin, and acetylcholine (Kow and Pfaff, 1984; Elias and Weiner, 1987; Stumpf, 1988) which increase GH concentration in the blood (Martin and Reichlin, 1987; Armstrong et al., 1988).

Gonadal Steroids and GH-Regulation. Male anterior pituitaries are capable of secreting more GH than those of females (Evans et al., 1985). These differences were attributed to the secretory capacity of individual somatotrophs rather than to the difference in their number (Hoefler and Frawley, 1986). Also, it has been demonstrated

that the rate of GH synthesis by adult male pituitaries was greater than that of adult female pituitaries (Burek and Frohman, 1970). Individual somatotrophs in male pituitaries have a greater responsiveness and (or) sensitivity to GRF stimulation than those of females (Evans et al., 1985; Wehrenberg et al., 1985; Krieg et al., 1986). GRF induces cellular cyclic adenosine 3',5'-monophosphate (cAMP) accumulation and GH release in males to a greater extent than in females (Cronin and Rogol, 1984; Simard et al., 1986). Finally, it may be possible that the sex steroids exert an effect at the level of the hypothalamus to modulate neural signals, which may regulate the state of responsiveness of the somatotrophs (Evans et al., 1985).

MATERIALS AND METHODS

Hormones and Chemicals. Estradiol-17B, testosterone, 5a-Androstan-17B-OL-3-ONE, 5a-Androstane-3a,17B-Diol, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), sodium bicarbonate, penicillin and streptomycin solution, and collagenase type 1A, were purchased from Sigma Chemical Company, St. Louis, MO. Hanks' balanced salt solution (HBSS), Dulbecco's Modified Eagle Medium with low glucose (DMEM) with and without phenol red , fungizone, pancreatin 4X N.F., trypan blue stain 0.4 % , and newborn calf serum lot# 4705 were purchased from GIBCO Laboratories, Grand Island, NY. Growth hormone releasing hormone 1-44 was generously donated by The Upjohn Company, Kalamazoo, MI.

Preparation of Cells. Calf pituitaries were obtained from Wolverine Packing Company, Detroit, MI. Cow pituitaries were collected from Snyder's slaughter house, Fowler, MI. Steer and heifer pituitaries were obtained from the Michigan State University Meat Laboratory. Pituitaries were removed as aseptically as possible and kept in a cold sterile oxygenated Hank's Buffered Saline Solution (HBSS) until transferred to the laboratory. HBSS was supplemented with 10 mM HEPES, 10 U/ml penicillin, 10 ug/ml streptomycin and 2.5 ug/ml fungizone. All subsequent procedures were performed under sterile conditions.

Cell Dissociation and Culture. Bovine pituitary cells were prepared following the same techniques as reported by Vale et al. (1972) as modified by Miller et al. (1977) and Padmanabhan et al. (1978). Briefly, posterior pituitaries were removed and anterior pituitaries were sliced into 1 mm thick slices using a Staddie Riggs microtome. Anterior pituitary slices were suspended in HBSS containing 0.3 % collagenase, and the suspension was incubated at 37 C with gentle stirring. After 45 to 60 min the medium was replaced with HBSS containing 0.25 % pancreatin, and the incubation continued for 10 to 15 min. The resulting cell suspension was filtered through sterile gauze and the cells recovered by centrifugation of supernatant at 300 x g for 5 min. The average total cell yield was approximately 9.5 X 10^6 cells for each pituitary gland. Cells were suspended in culture medium (DMEM with phenol red). The medium was supplemented with penicillin (10 U/ml), streptomycin (10 ug/ml), HEPES (25mM), NaHCO3, and 10 % newborn calf serum, at pH 7.4. Fungizone (2.5 ug/ml) was added during the first 48 h only. Cell viability as judged by trypan blue exclusion was greater than 90 % in all experiments. Suspended cells were plated at a denisity of 4 to 6 X 10^5 cells/ml. Cells were plated in Corning 24-well treated dishes (25820-Corning, NY). Cultures were maintained at 37C in a humidified

atmosphere of 5 % CO_2, 95 % O_2; in a CO_2 incubator Model 3028, Forma Scientific (Marietta, OH). Medium was changed 48 h after plating, and then at 24-h intervals. All treatments were imposed 72 h after plating. Media were collected every 24 h and stored at -20 C until assayed for concentrations of GH.

Effect of Phenol Red and Fungizone. The effect of phenol red on media concentration of GH was tested in anterior pituitary cells obtained from calves. Cells were plated at $5X10^5$ cells.ml^{-1}.$well^{-1}$ using two 24-well dishes. Cells were incubated with 0, 10^{-10}, 10^{-8}, 10^{-6}M estradiol-17B (E) in the presence or absence of 45 uM phenol red. Treatments were started after 72 h of plating and continued for another 72 h with six replicate wells/treatment. Media were collected every 24 h and stored at -20 C until assayed for GH.

Fungizone (Fz) effects were examined in anterior pituitary cells from steers. Three dishes (24 wells/dish) with plating density of $4X10^5$ cells.ml^{-1}.$well^{-1}$ were incubated with 0 or 2.5ug/ml Fz. Treatments were started after 72 h of plating. Fz was present for another 72 h with 36 replicate wells/treatment. Media were collected at 24 h intervals and stored at -20 C until assayed for GH.

Incubation with Test Agents. Estradiol-17B (E) or testosterone (T) were dissolved in absolute ethyl alcohol. Concentrations of 10^{-10}, 10^{-8}M of E and 10^{-7}, 10^{-5}M of T were prepared, with a final concentration of 0.01% or less of alcohol. The effects of E or T were studied in anterior pituitary cells from either calves, heifers, steers or cows. Anterior pituitary cells from calves were plated at $5X10^5$ cells/well. Cells were incubated with respective steroids for 24 h with 24 replicate wells for each treatment. Each study was repeated in three independent experiments. After 24 h of incubation with the steroid, cells were washed four times with serum-free medium and then challenged with 10^{-8}M GRF for 1 h. All media were collected and stored at -20 C until assayed for GH.

Anterior pituitary cells from heifers, steers or cows were plated at $5X10^5$ cells/well. The steroid was present for 72 h with 12 replicate wells for each treatment. This study was repeated one more time. Media were collected at 24 h intervals and stored at -20 C until assayed for GH.

The effect of dihydrotestosterone (DHT) or 5a-Androstane-3a,17B-diol (3a-Diol) on GH release was studied using calf anterior pituitary cells. Cells ($5X10^5$) were incubated with T (10^{-7},10^{-5}M), DHT (10^{-8},10^{-6}M), 3A-Diol

(10^{-10},10^{-8}M) or control media. The respective steroids were present for 72 h with 12 replicate wells/treatment. Media were collected every 24 h and stored at -20 C until assayed for GH.

Effect of Cycloheximide. Cycloheximide (CHX) is an inhibitor of protein synthesis. Anterior pituitary cells obtained from heifers were plated at $5X10^5$ cells/ml and six dishes (24 wells/dish) were incubated with 0, 10 or 50 ug/ml CHX. Treatment was started after 72 h of plating and continued for another 72 h with 36 replicate wells for each treatment. Media were collected after each 24 h period and stored at -20 C until assayed for GH.

Determination of Intracellular GH. Intracellular GH was determined using a modification to the procedure described by Jones et al. (1965) and Birge et al. (1967). Briefly, immediately after dispersion, an aliquot of calf anterior pituitary cells ($5X10^5$ cells/ml) was washed three times with phosphate buffer (0.05M, pH 7) and then resuspended in the same buffer. Cell membranes were disrupted using a Branson Sonifier Model 350 (Branson Sonic Power Company, Danbury, CT). Sonification was performed for 15 s at 50 % duty cycle and an output of 4. Then the tubes were centrifuged at 1800 x g for 30 min and the supernatant was stored at -20 C until assayed for GH.

Bovine GH Radioimmunoassay. Bovine GH (bGH) was measured in duplicate by double antibody radioimmunoassay (Purchas et al., 1970). All reagents used in this study were kindly provided by Dr. H.A.Tucker (Department of Animal Science, Michigan State University). Briefly, bGH (NIH-GH) was labeled with ^{125}I on a Biogel P-60 column. The column yielded two radioactive peaks; the materials from the first peak (^{125}I-GH) was used in the assay. Rabbit anti-bovine gamma globulin (1st antibody, AB) was diluted 1:10 with rabbit control sera, which had been diluted 1:400 using phosphate buffer saline-EDTA (PBS-EDTA) 0.05M at pH 7. Sheep anti-rabbit gamma globulin (2nd AB) was diluted 1:15 using PBS-EDTA, 0.05M at pH 7. Standard tubes (one set /300 assay tubes) containing 0.1, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 ng standard NIH-GH were assayed with each experiment. Appropriate samples were prescreened and then all samples from one experiment were run in the same assay at relevant dilutions. Media were then incubated at 4 C with 1st AB for 24 h, followed by another 24 h incubation with the labeled hormone at 4 C. Finally, incubation was carried out for 72 h with the 2nd AB at the same temperature . Then all tubes were centrifuged at 1800 x g for 30 min and the supernatants were decanted. The tops of the tubes were washed and left

to dry overnight and then all tubes were counted in a gamma counter (Gamma Trac 1290, Tm Analytic, Elk Grove Village, Il) for 1 min/tube. Radioimmunoassay standard curves were obtained from a multiple regression equation with linear, quadratic and cubic components.

Statistical Analysis . In experiments conducted for 24 h, data were analyzed by a randomized complete block design to compare the effect of concentration of steroid with the control. In experiments in which the effect of time (72h) on GH production was studied data were analyzed by split plot design with repeat measurements (Gill,1978). The effect of GRF challenge on GH production was tested using an approximate t-test, which examines the ratio of GH released after GRF challenge for steroid treated and nontreated wells (Gill, personal communication).

RESULTS AND DISCUSSION

Media Composition and GH-Production

Hormone production by dispersed anterior pituitary cells in culture can be altered by a variety of nonhumoral factors, such as medium pH, buffer composition, and medium formulation (Wilfinger et al., 1979). In the present study pH was adjusted to 7.4 and maintained throughout the experiment by using the CO_2-$NaHCO_3$ buffer system, in addition to 25 mM HEPES buffer. It is well known that serum supplements (15 to 20 %) can correct medium deficiencies and enhance in vitro anterior pituitary hormone production (Wilfinger et al., 1979). However, serum components are highly variable and thus, become an important source of experimental error when used in high concentrations (Rayan, 1989). Ham (1974) indicated that serum supplements for primary cell cultures could be reduced by adjusting nutrient concentrations to optimum values. Under the experimental condition employed in the present experiments, all serum used was purchased as one lot of newborn calf serum (lot No. 4705, GIBCO, NY). Media, Dulbecco's Modified Eagles Medium (DMEM), were supplemented with 10% serum as described by Padmanabhan et al. (1978), Simard et al. (1986) and Rayan (1989). Under

these conditions the cells were able to maintain a normal performance for more than 17 d. Figure 1a shows the anterior pituitary cells in culture after 17 d of plating. It is apparent from this figure that the cells have a normal appearance figure 1b, even 17 d after plating. This suggested that the conditions used in this experiment were sufficient to maintain the bovine anterior pituitary cells in a normal performance throughout the experimental period of approximately 7 d.

Phenol Red. Phenol red, a pH indicator in tissue culture media, has estrogenic activity at concentrations of 15 to 45 uM (Berthois et al., 1986). The authors indicated that phenol red should be considered in any studies with estrogen and estrogen-responsive cells in cultures. Figure 2 illustrates the effect of 45 uM phenol red (concentration normally found in DMEM) on GH concentration found in the media after 24, 48, 72 h incubation of calf anterior pituitary cells in a static in vitro incubation. GH concentrations were not different ($P>0.05$) from controls (media without phenol red) at all periods studied. These results indicated that incubation of anterior pituitary cells with phenol red did not affect GH concentration in the media. The interaction between phenol red and various

Figure 1 Photomicrograph of calf anterior pituitary cells (A)in primary culture 17 d after plating stained with Mallory stain (magnification 7,560X). (B)histological section of normal calf anterior pituitary cells stained with Orange G stain (magnification 18,900X). Provided Courtesy of Dr. A.L. Stinson, Dept.of Anatomy.

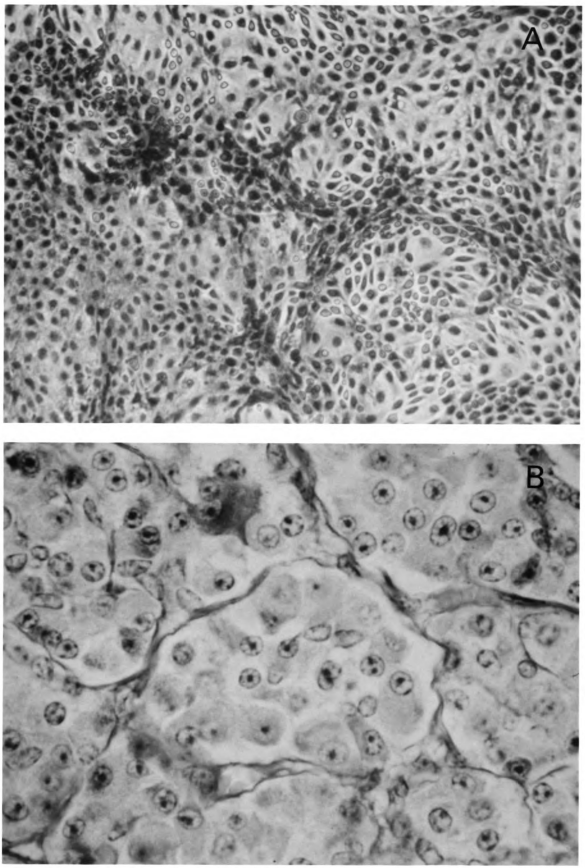
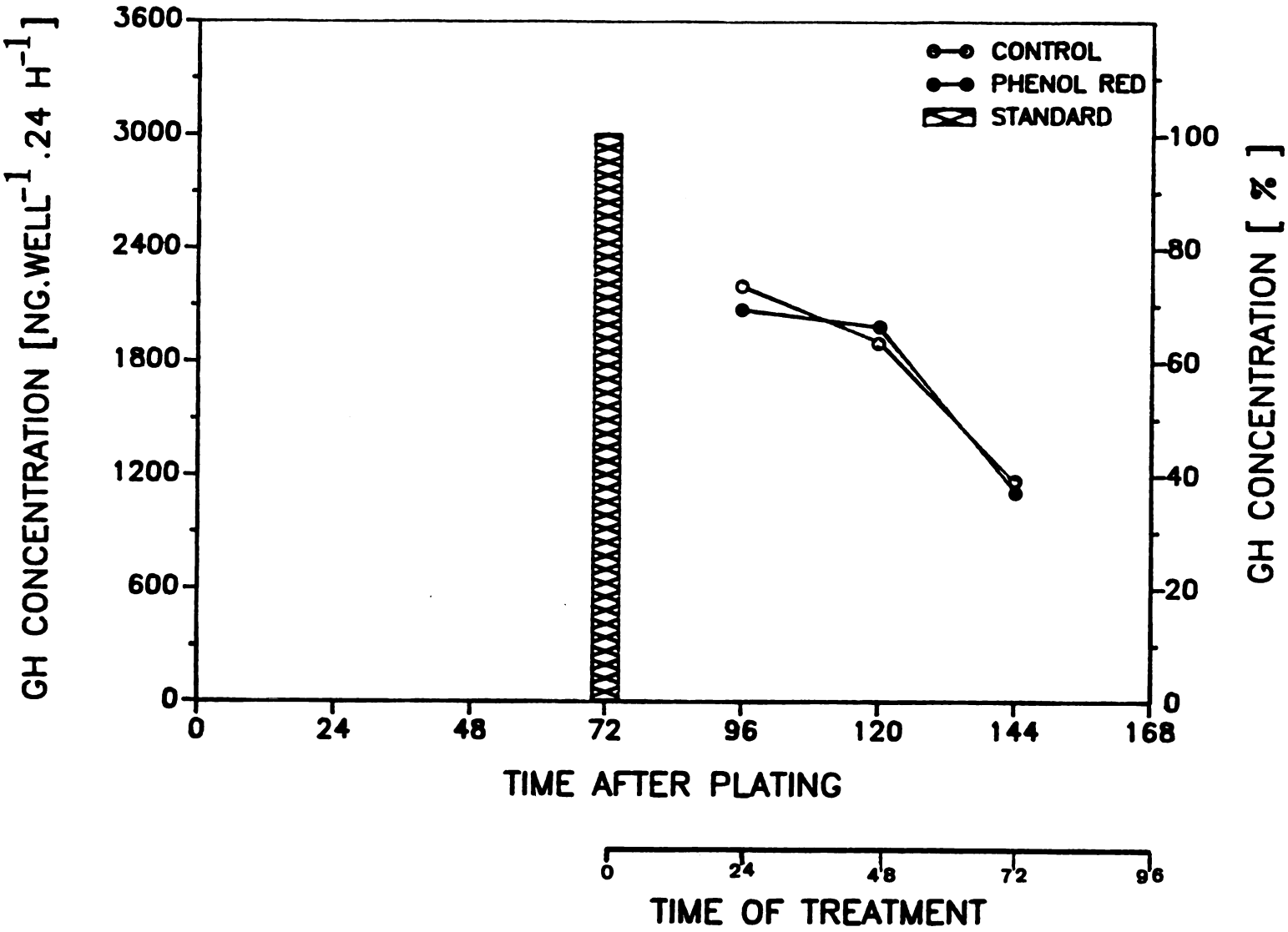

A
B

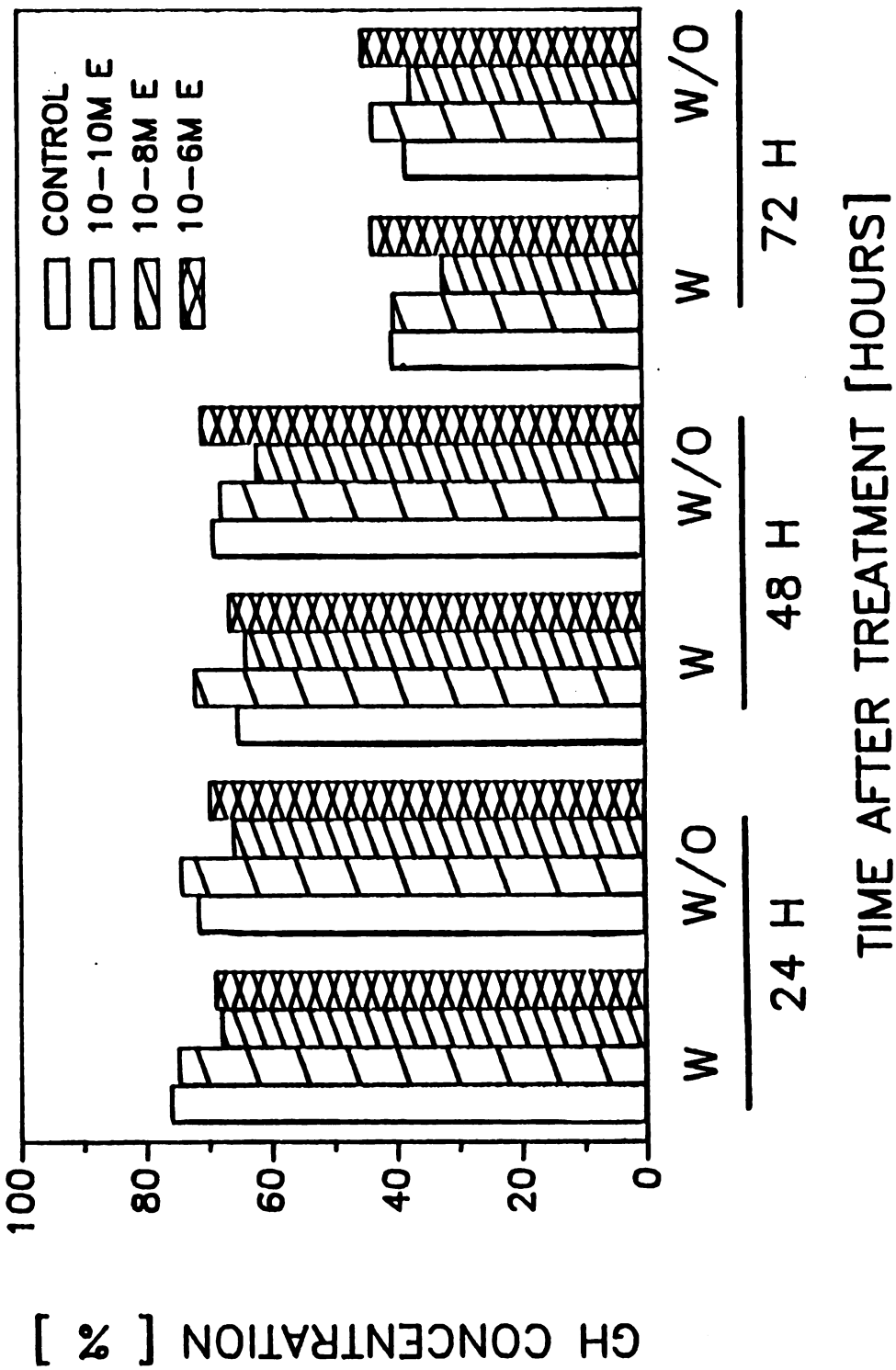
Figure 2 The effect of 45 uM phenol red on media GH concentration from calf anterior pituitary cells in culture. Media was first changed 48 h after plating and every 24 h thereafter. Media were collected each 24 h from each well. The bar at 72 h after plating is the average GH concentration from all wells at the time the treatment was imposed. GH concentration in the media ($ng.well^{-1}.24\ h^{-1}$) is shown on the left ordinate. GH concentration at 24, 48 and 72 h of treatment is expressed as a percentage of GH concentration at initiation of treatment (72 h after plating) and is shown on the right ordinate. Each point represents the mean of 6 replicate wells. Pooled standard error was 5 %.


GH CONCENTRATION [NG.WELL^{-1}.24 H^{-1}]
3600
3000
2400
1800
1200
600
0
CONTROL
PHENOL RED
STANDARD
100
80
60
40
20
0
GH CONCENTRATION [%]
0
24
48
72
96
120
144
168
TIME AFTER PLATING
0
24
48
72
96
TIME OF TREATMENT

concentrations of estrogen (10^{-10}, 10^{-8}, and 10^{-6}M) on media GH concentration from calf anterior pituitary cells in culture is presented in figure 3. No differences ($P>0.05$) were observed between any of the treatment combinations and the controls. These observations indicate that there were no interactions between phenol red and all concentrations of estrogen used on GH released from anterior pituitary cells of heifers in culture for up to 72 h after plating.

Fungizone. Long term incubation with fungizone (Fz), an antifungal agent, reduced GH secretion from the pituitary cell line, GH_3 cells, (Lapp et al., 1987). Since this experiment dealt with a primary culture, the possibility for contamination exists even when great precaution is taken. Therefore, it was necessary to use antifungal agents to prevent fungal outgrowth. Fungizone is the most commonly used antifungal agent in tissue culture (Oosterom et al., 1983; Lapp et al., 1987). The apparent advantages of Fz have been: a) solubility in culture medium; b) stability in solutions at 37 C, and c) documented use with anterior pituitary cells in primary cultures (Foord et al., 1983; Oosterom et al., 1983; Lapp et al., 1987). In the present study the effect of preincubation with Fz for 24, 48 and 72 h on GH

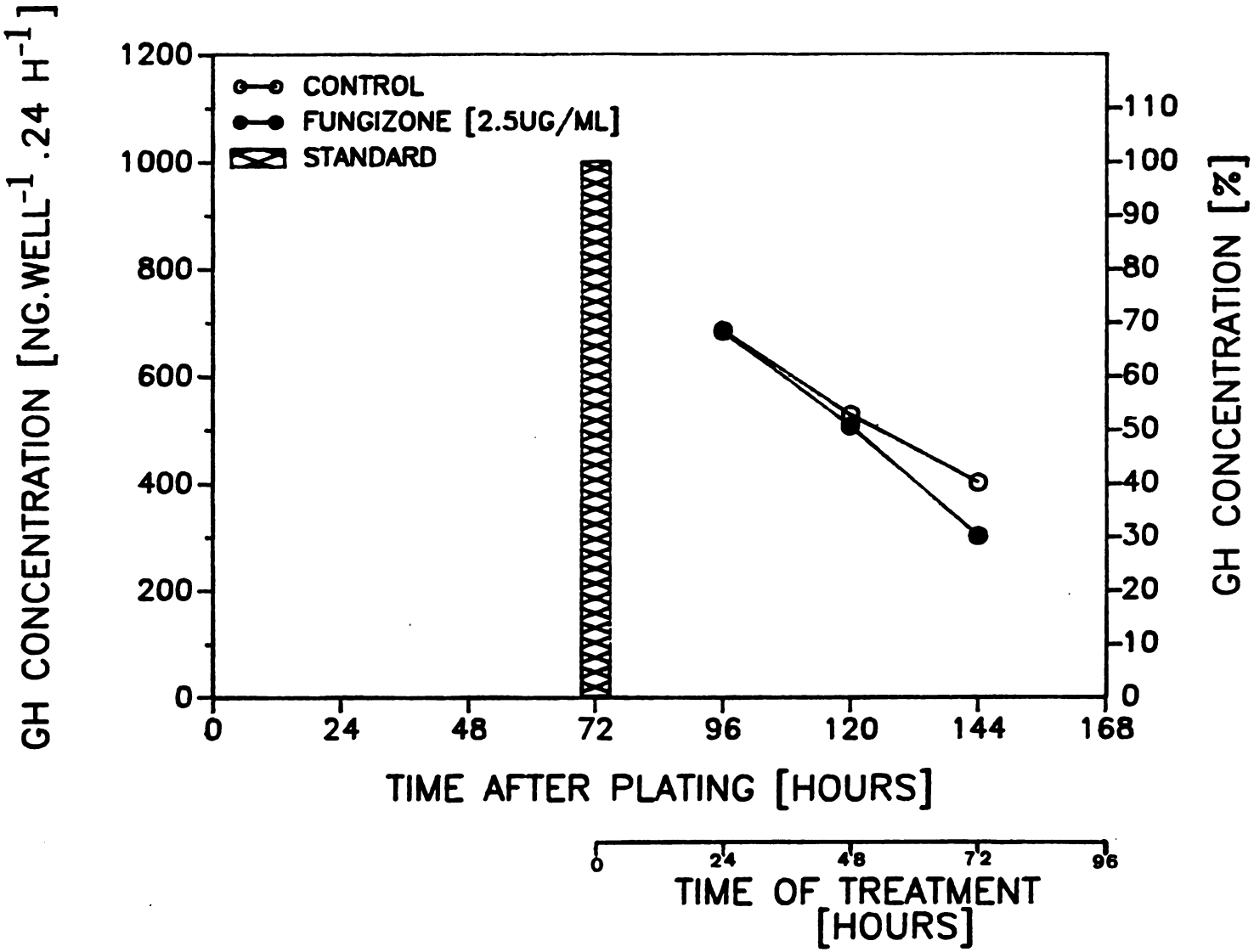
Figure 3 The effect of phenol red (45uM) and estrogen (10^{-10}, 10^{-8}, 10^{-6}M) on media GH concentration, expressed as a percentage of the GH concentration at initiation of treatment (72 h after plating), from calf anterior pituitary cells in culture. Each point represents the mean of 6 replicate wells. Pooled standard error was 4.7 %.


CONTROL
10-10M E
10-8M E
10-6M E
100
80
60
40
20
0
GH CONCENTRATION [%]
W
W/O
W
W/O
W
W/O
24 H
48 H
72 H
TIME AFTER TREATMENT [HOURS]

concentrations found in the media was observed. Figure 4 shows the effect of Fz (2.5 ug/ml, normal concentration used in cultured pituitary cells) on GH concentration from anterior pituitary cells of steers in culture. The data demonstrate no reduction ($P>0.5$) in GH concentration at 24 or 48 h after incubation with Fz. However, a reduction ($P<0.05$) of 25% in GH released was noted after 72 h of incubation with Fz. These results are in agreement with those of Lapp et al. (1987) who concluded that Fz is not an acceptable medium constituent for long term culture (more than 72 h) of somatotrophic cells. The data herein suggested that Fz may not be the best antifungal compound to be used in these tissue cultures. However , Fz can be used as an effective therapy, since the effect of Fz on GH secretion is reversible (Lapp et al., 1987). In the present study, Fz was added during the first 48 h only to prevent fungal outgrowth from contamination during pituitary collection and cell preparation.

Cycloheximide. Cycloheximide (CHX), an inhibitor of protein synthesis was investigated to determine whether cells in the present study and under the experimental conditions used were capable of synthesizing GH. Also of interest was whether the newly synthesized GH interferes with the amount of GH released. The effect of 10 and 50

Figure 4 The effect of Fungizone (2.5 ug/ml) on media GH concentration from steer anterior pituitary cells in culture. The bar at 72 h after plating is the average GH concentration in the media from all wells at the time the treatment was imposed. GH concentration in the media (ng. well^{-1}. 24 h^{-1}) is shown on the left ordinate. GH concentration at 24, 48 and 72 h of treatment is expressed as a percentage of GH concentration at initiation of treatment (72 h after plating) and is shown on the right ordinate. Each point represents the mean of 36 replicate wells. Pooled standard error was 2.3%.


GH CONCENTRATION [NG.WELL^{-1} .24 H^{-1}]
1200
1000
800
600
400
200
0
CONTROL
FUNGIZONE [2.5UG/ML]
STANDARD
0
24
48
72
96
120
144
168
110
100
90
80
70
60
50
40
30
20
10
0
GH CONCENTRATION [%]
TIME AFTER PLATING [HOURS]
0
24
48
72
96
TIME OF TREATMENT [HOURS]

ug/ml CHX on the percentage of GH concentration found in the media after incubation of anterior pituitary cells from heifers for 24, 48, 72 h is presented in figure 5. Incubation with CHX for 24 h had no effect ($P>0.05$) on GH concentration but decreased ($P<0.05$) GH concentration after 48 and 72 h of incubation with CHX. These data suggest that CHX (10 and 50 ug/ml) had no effect on GH released at 24 h of incubation; however, it may effect viability of cells after 48 h when all protein synthesis was inhibited.

Pattern of GH-Production In Vitro

Figure 6 presents the pattern of GH production from calf anterior pituitary cells in vitro. The GH production from somatotropic cells declined from 24 to 96 h after plating as GH concentration in the media was measured at 24 h intervals. Media concentration of GH at 72 h of plating was 2.6 ug/ml and a further decrease of 42% of the later concentration was observed 96 h after plating. In a preliminary study using the same experimental conditions in culture, GH production was measured in cow anterior pituitary cells. A continuous decrease in GH production was noted after 17 d of plating which was about 80% of the media concentration at 72 h after plating. Similar findings were reported by Hoeffler and Frawley (1986) for anterior

Figure 5 The effect of cycloheximide (10 or 50 ug/ml) on media GH concentration from heifer anterior pituitary cells in culture. The bar at 72 h after plating is the average GH concentration in the media from all wells at the time the treatment was imposed. GH concentration in the media (ng. well^{-1}. 24 h^{-1}) is shown on the left ordinate. GH concentration at 24, 48 and 72 h of treatment is expressed as a percentage of GH concentration at initiation of treatment (72 h after plating) and is shown on the right ordinate. Each point represents the mean of 36 replicate wells. Pooled standard error was 3.2%.

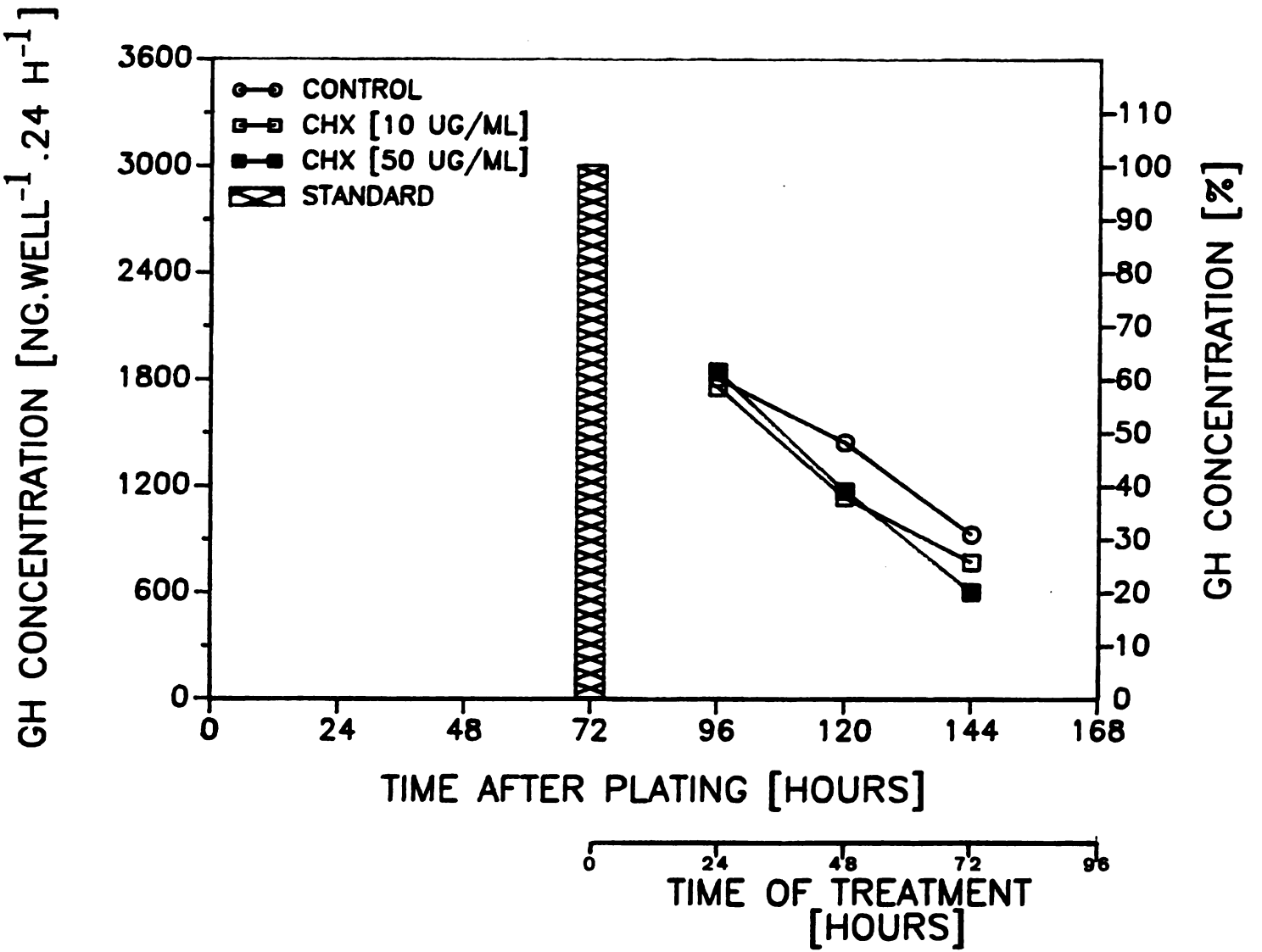
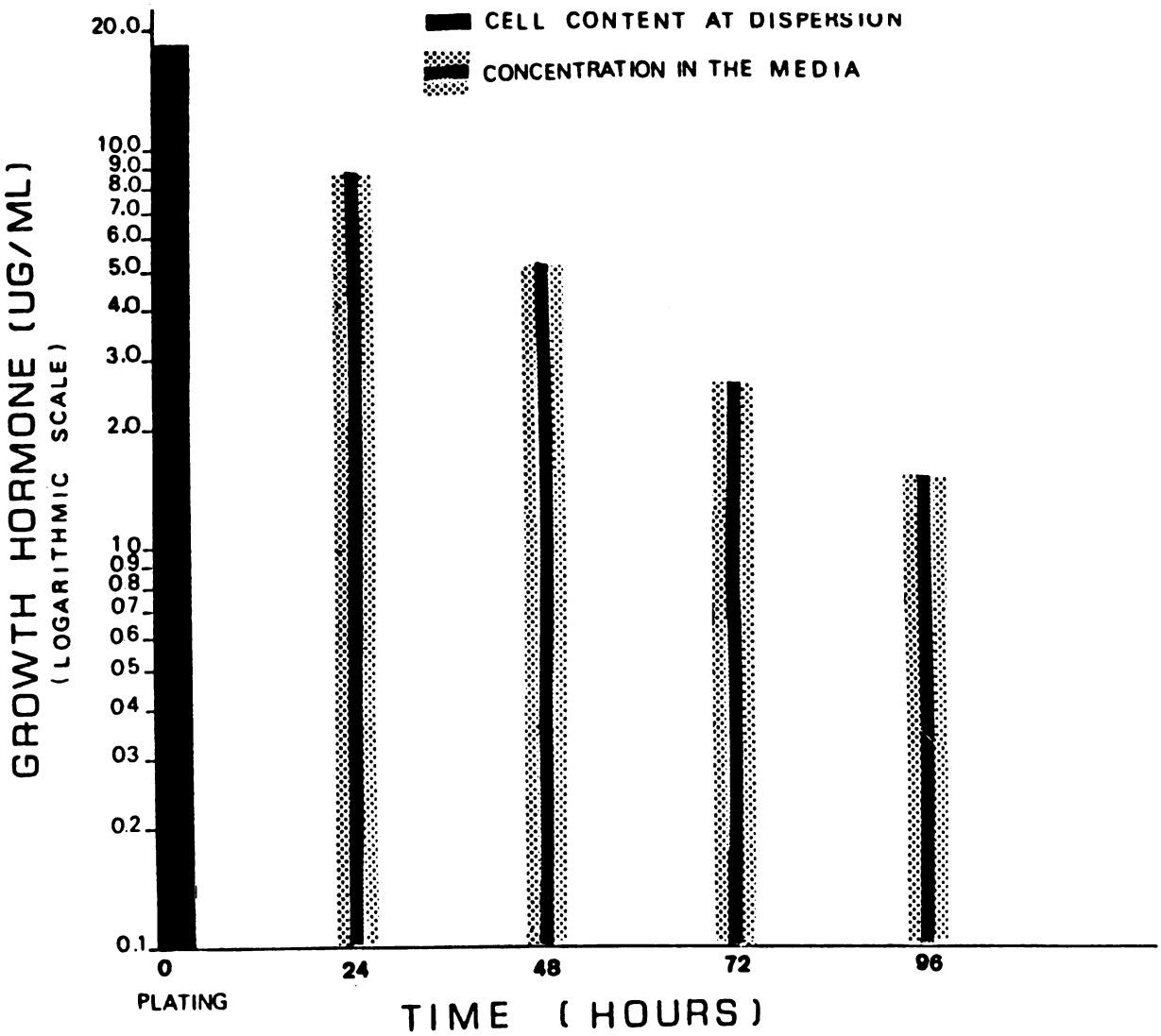

GH CONCENTRATION [NG.WELL^{-1}.24 H^{-1}]
3600
3000
2400
1800
1200
600
0
CONTROL
CHX [10 UG/ML]
CHX [50 UG/ML]
STANDARD
110
100
90
80
70
60
50
40
30
20
10
0
GH CONCENTRATION [%]
0
24
48
72
96
120
144
168
TIME AFTER PLATING [HOURS]
0
24
48
72
96
TIME OF TREATMENT [HOURS]

Figure 6 Pattern of GH released from calf anterior pituitary cells. The bar at 0 time is the average cell content of GH at plating. After plating, media were collected every 24 h from each well and assayed for GH concentration. Each bar represents the mean GH concentration (ug/ml) of 12 replicates.


CELL CONTENT AT DISPERSION
CONCENTRATION IN THE MEDIA
GROWTH HORMONE (UG/ML)
(LOGARITHMIC SCALE)
20.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
10
09
08
07
06
05
04
03
0.2
0.1
0
PLATING
24
48
72
96
TIME (HOURS)

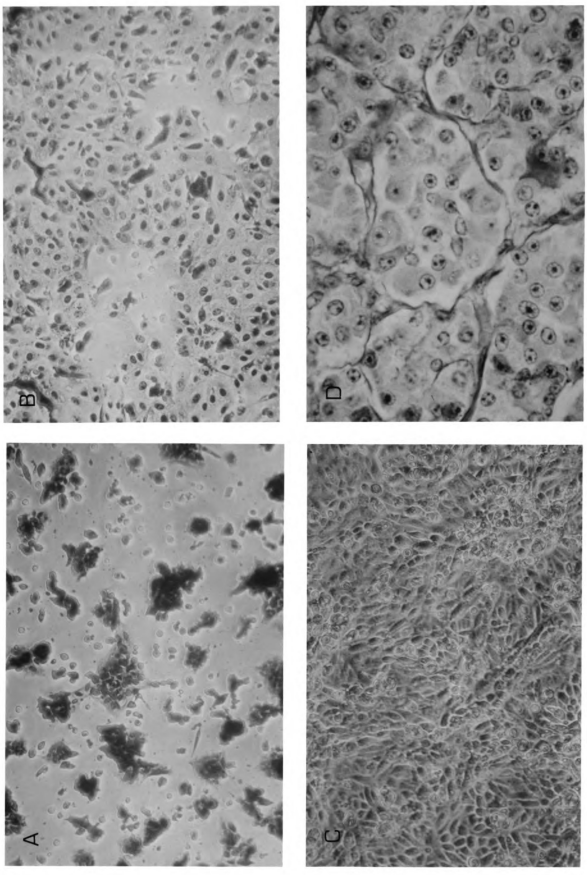
pituitary cells from rats in a static in vitro incubation experiment.

Several factors may contribute to the decline in the GH production namely: 1) cell viability may decrease with time, 2) separation of the anterior pituitary gland from the hypothalamic peptides (GRF and SRIF; Guillemin et al., 1984), and 3) the long term exposure of the somatotrophs to a high concentrations of GH which may negatively feedback on GH synthesis, release and stability (Lapp et al., 1987). These factors are described in the review of the literature. The data herein support the contention that GH production decreased with time up to 17 d in static in vitro incubation.

Cell Culture Architecture and Intercellular Communication

The importance of cell architecture and intercellular communication on function of the anterior pituitary cells in culture has been documented by Maertens and Denef (1987) and Baes and Denef (1987). Figure 7 shows the behavior of bovine anterior pituitary cells in culture. The dispersed cells start to reaggregate after 24 h of plating (figure 7a) and at 72 h the cells formed a monolayer and developed a colony-like formation (figure 7b). At 96 h the cell aggregates become more pronounced (figure 7c) and they stained with the acidophilic stains. This aggregation or

Figure 7 Photomicrograph of calf anterior pituitary cells in culture (A) 24 h after plating (3,700X), (B) 72 h after plating (3,700X), (C) 96 h after plating (3,700X) and (D) histological section of normal bovine anterior pituitary cells (9,400X). Provided courtesy of Dr. A.L. Stinson, Dept. of Anatomy.


A
B
C
D

colony formation appears to mimic the in vivo cellular arrangement of the bovine anterior pituitary gland (figure 7d). Referring to figure 1a the stained colonies are well defined and more visible.

The existence of intercellular signals, most probably of paracrine nature, between gonadotrophs and lactotrophs has been demonstrated by Denef et al. (1985). Moreover, Baes and Denef (1987) concluded that the interactions of somatotrophs with other cell types, modulate GH release; although the mechanism by which this interaction facilitates GH release is not fully understood. These latter authors suggested that the interaction could be explained through direct intracellular contacts or through secretion of paracrine factors. Ohlsson et al. (1988) mentioned that in a pure preparation of somatotrophs the response to GRF was greater in cells incubated for 3 d in culture than the fresh preparations, and this was due to cell conditioning and aggregation. Further studies are needed to investigate the mechanism by which the intercellular communication modulates GH release and (or) synthesis.

Selection of the Experimental Conditions to be Used

Based on the findings obtained in the present study which showed that: 1) GH release decreased with time, and

2) the culture cannot be used before 72 h of plating to ensure aggregation of cells and stability of the monolayer formation. In all subsequent experiments, the cells were left for 72 h after plating before any treatments were applied. Allowing the cells to aggregate also minimized the well to well variation.

Effect of Steroids on GH Release

The sex steroids, E and T, alter plasma growth hormone (GH) in vivo, Eden et al. (1987). However the mechanism, by which E and T affect GH production, is not fully understood. The main objective of the present study was to investigate whether E or T has a direct effect on GH release from bovine anterior pituitary cells in primary static in vitro culture.

Effect of E and T on anterior pituitary cells. In preliminary experiments, four different concentrations of E were used to determine the most effective concentration on GH released. Table 1 shows the effect of E concentration (10^{-12},10^{-11},10^{-10} and 10^{-8}M) on GH released from cow anterior pituitary cells in vitro. It is apparent from the table that none of the concentrations used altered GH release when compared with controls. In most previous studies with E and T greater than physiological

(Table 1) Effect of various concentrations of estradiol-17B on GH released ($ng.ml^{-1}.24\ h^{-1}$) from cow anterior pituitary cells in a static in vitro incubation[a]

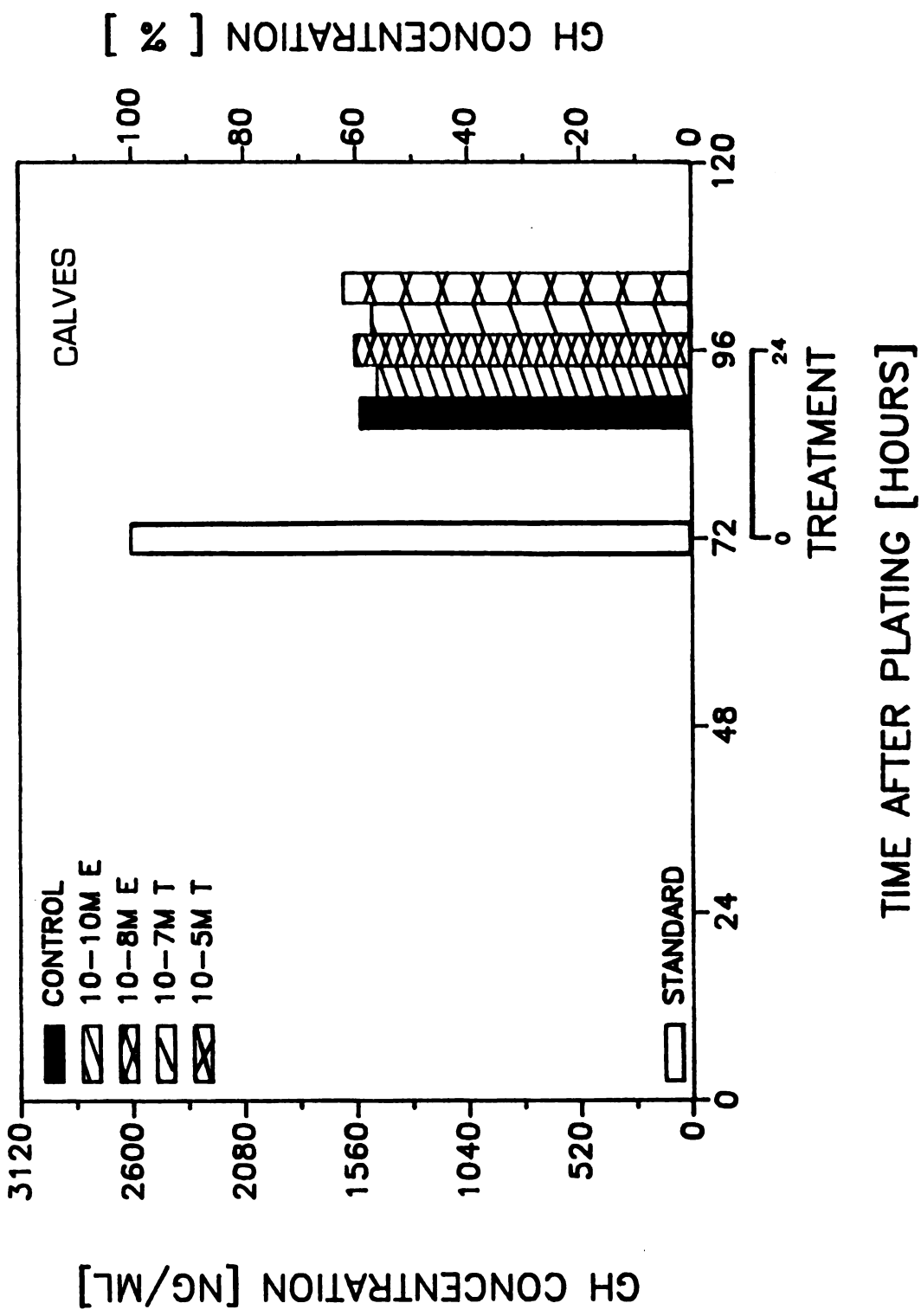
Length of treatment (h)	Estradiol-17B Concentration (M)					
	0	10^{-12}	10^{-11}	10^{-10}	10^{-8}	SEM[b]
24	384.3	383.9	371.6	359.7	379.9	27.7
48	260.8	186.1	169.0	220.2	303.3	24.2
72	136.9	109.5	145.0	164.5	157.0	12.4
% GH released after GRF Challenge after 72 h[c]	123.7	123.9	119.5	122.8	125.7	

a Values are means of 12 wells.
b Standard error of means.
c GRF (10^{-8}M) for 1 h, the percentage calculated as challenged vs unchallenged for each E concentration.

concentrations were used (Wehrenberg et al., 1985; Fukata and Martin 1986). In the present study, E as well as T were used at concentrations 10^2- and 10^4-fold greater than pubertal concentrations (i.e., 10^{-12}M for E and 10^{-9}M for T).

The Effect of 24 h Incubation with Sex Steroids. The effect of various concentrations of E and T on GH concentration (%) from calf anterior pituitary cells in primary culture is illustrated in figure 8. Preincubation with forementioned concentrations of E or T for 24 h had no effect ($P>0.05$) on GH concentration found in the media. This effect was confirmed in three independent replications utilizing cells from calf anterior pituitaries. These results are in agreement with the findings of Fukata and Martin (1986) and Wehrenberg et al. (1985). These authors concluded that neither E nor T showed any apparent influence on basal GH released by rat anterior pituitary cells in culture. Simard et al. (1986) demonstrated that incubation with physiological concentrations of E for 72 h increased basal GH release as well as cellular GH content in rat anterior pituitary cells in primary culture. However, this effect was not observed at 24 h of incubation. The data herein demonstrate clearly that 24 h incubation with the concentrations of these steroids used

Figure 8 The effect of estrogen (10^{-10}, 10^{-8} M) and testosterone (10^{-7}, 10^{-5} M) on media GH concentration from calf anterior pituitary cells in culture. The bar at 72 h after plating is the average GH concentration in the media from all wells at the time the treatment was imposed. GH concentration in the media (ng. $well^{-1}$. 24 h^{-1}) is shown on the left ordinate. GH concentration at 24, 48 and 72 h of treatment is expressed as a percentage of GH concentration at initiation of treatment (72 h after plating) and is shown on the right ordinate. Each bar represents the mean of 72 replicate wells. Pooled standard error was 4.2%.

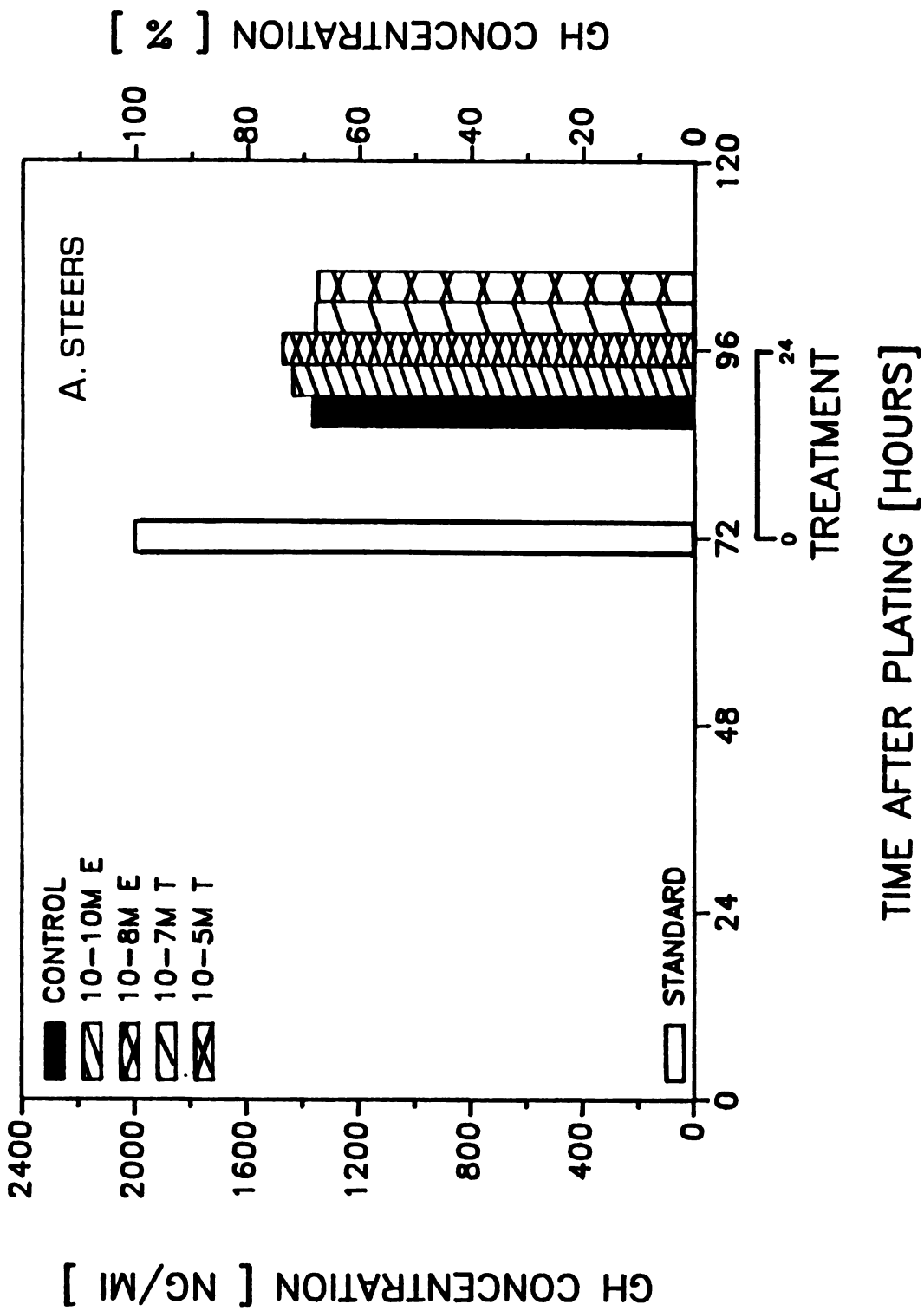

GH CONCENTRATION [%]
100
80
60
40
20
0
120
96
72
48
24
0
CALVES
CONTROL
10-10M E
10-8M E
10-7M T
10-5M T
STANDARD
24
0
TREATMENT
TIME AFTER PLATING [HOURS]
GH CONCENTRATION [NG/ML]
3120
2600
2080
1560
1040
520
0

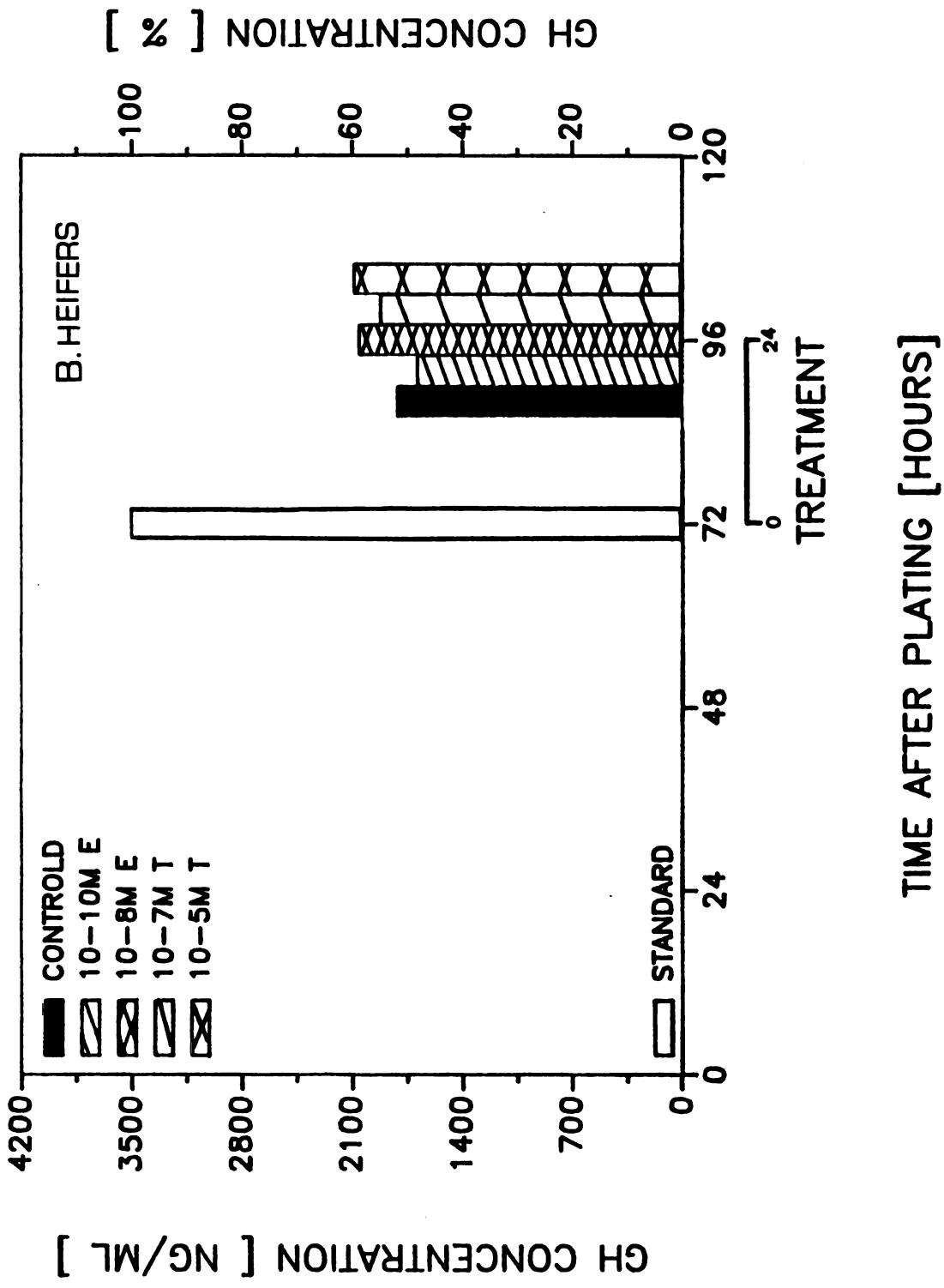
had no significant effect on basal GH released from calf anterior pituitary cells in a static in vitro culture.

Effect of preincubation with different concentrations of E or T for 24 h on GH concentration from cultured anterior pituitary cells isolated from steers and heifers is shown in figures 9a and 9b, respectively. It is apparent from the figure that neither E nor T had an effect on GH concentration ($P<0.05$). Each treatment was confirmed in two independent replications utilizing anterior pituitaries from heifers as well as steers. The initial GH concentration from anterior pituitaries of heifers was greater than those from steers (3534.4 ± 612.8 vs 2097.2 ± 231.7 ng/ml, respectively); however, the percentage of GH released from steer somatotrophs was greater than for somatotrophs from heifers (51.6 ± 3.1 vs 68.2 ± 2.9, respectively). These differences in GH release may be attributed to the secretory capacities of individual somatotrophs as well as the viability of different preparations (Hoeffler and Frawley, 1986).

Effect of 72 h Incubation with Sex Steroids Heifer anterior pituitary cells were used to study the effect of long term incubation (72 h) with gonadal steroids on GH concentration. Figure 10a illustrates the effect of different concentrations of E or T on GH concentration. No

Figure 9 The effect of estrogen (10^{-10}, 10^{-8} M) and testosterone (10^{-7}, 10^{-5} M) on media GH concentration from (A) steer anterior pituitary cells in culture and (B) heifer anterior pituitary cells in culture. The bar at 72 h after plating is the average GH concentration in the media from all wells at the time the treatment was imposed. GH concentration in the media (ng. well^{-1}. 24 h^{-1}) is shown on the left ordinate. GH concentration at 24, 48 and 72 h of treatment is expressed as a percentage of GH concentration at initiation of treatment (72 h after plating) and is shown on the right ordinate. Each bar represents the mean of 12 replicate wells. Pooled standard error was 4.4 % for heifers and 6.1 % for steers.

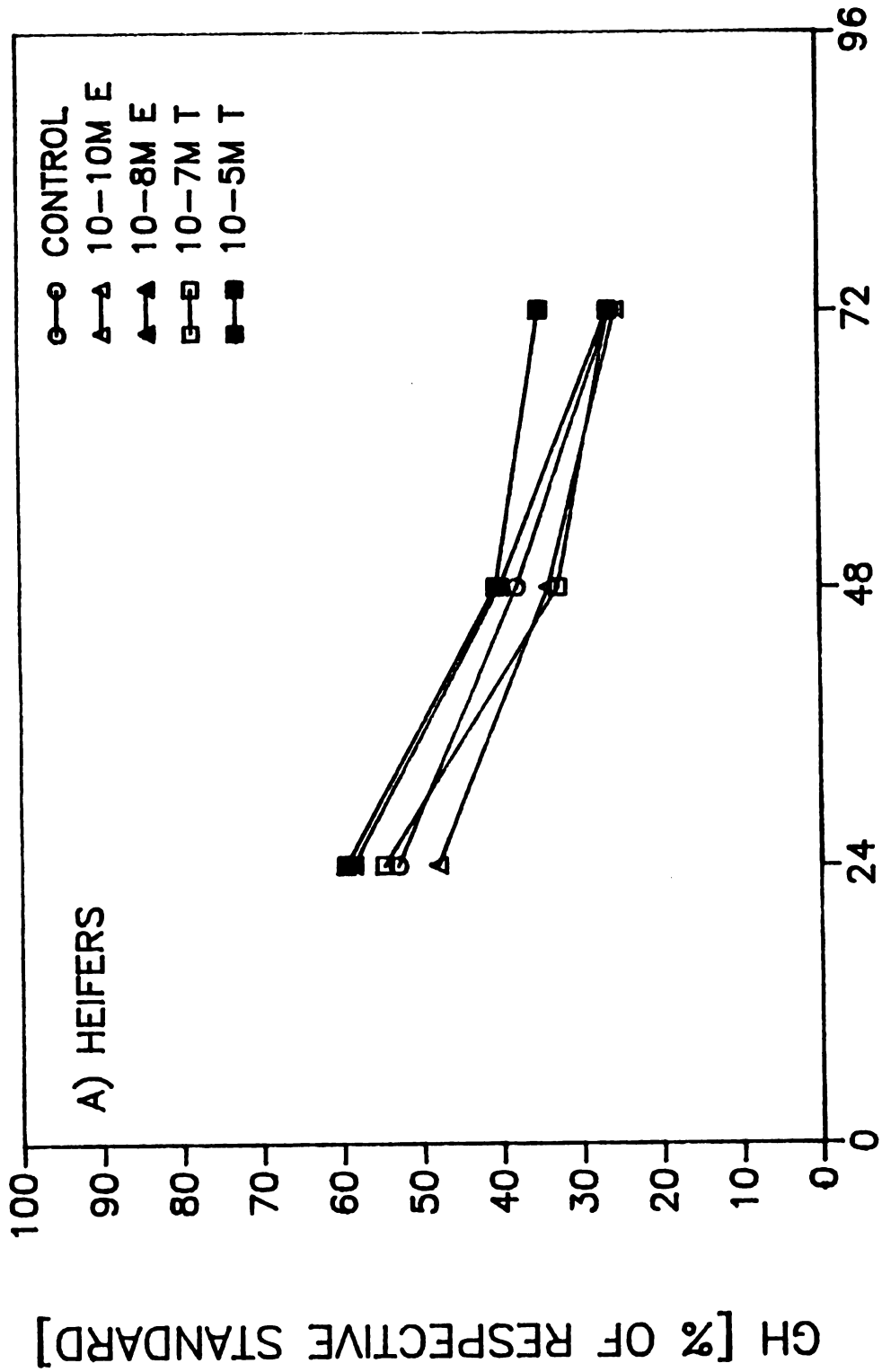

GH CONCENTRATION [%]
100
80
60
40
20
0
120
A. STEERS
96
24
0
TREATMENT
72
48
24
0
TIME AFTER PLATING [HOURS]
CONTROL
10-10M E
10-8M E
10-7M T
10-5M T
STANDARD
2400
2000
1600
1200
800
400
0
GH CONCENTRATION [NG/ML]

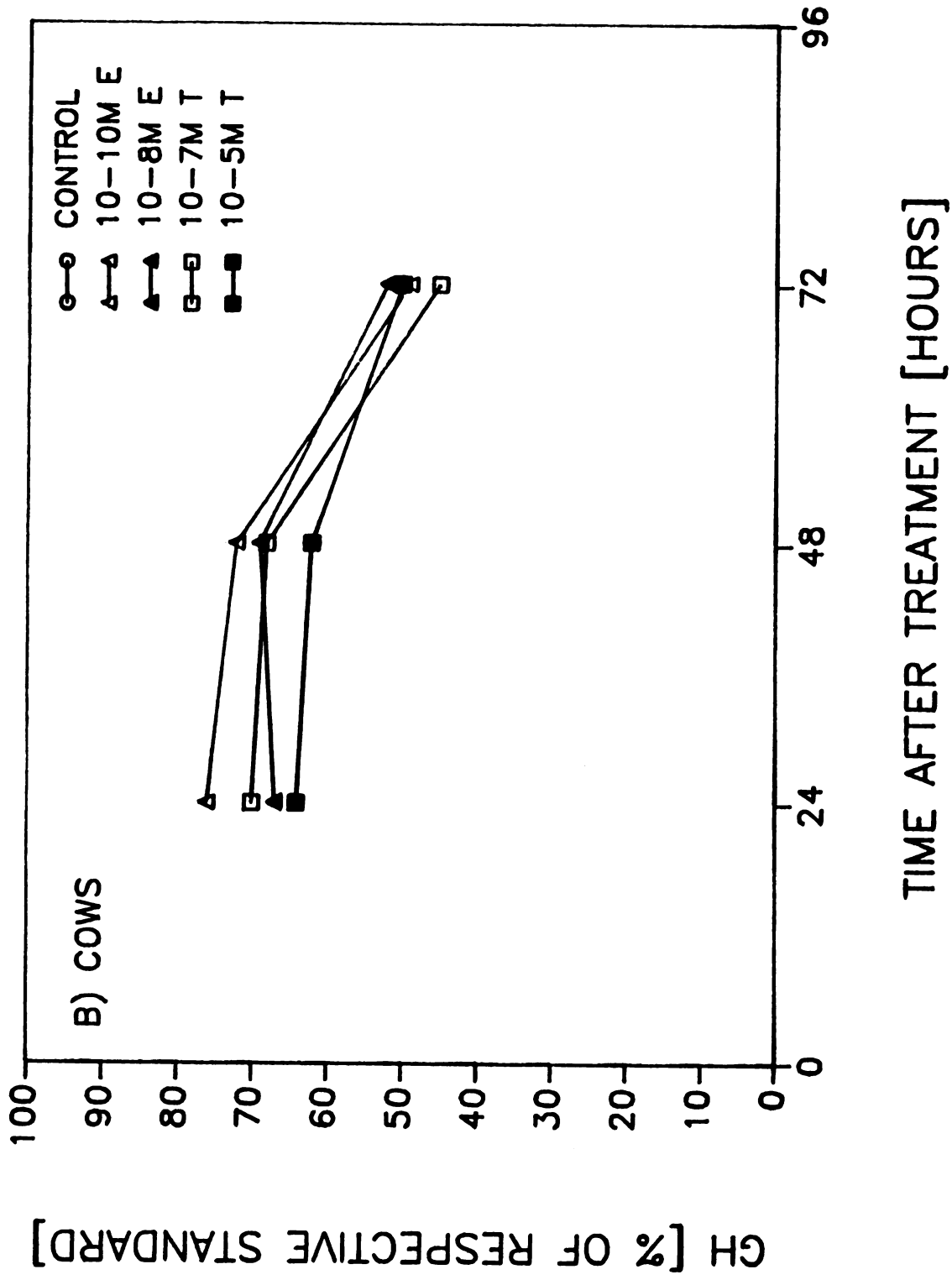

GH CONCENTRATION [%]
100
80
60
40
20
0
B.HEIFERS
CONTROLD
10-10M E
10-8M E
10-7M T
10-5M T
STANDARD
4200
3500
2800
2100
1400
700
0
0
24
48
72
96
120
TREATMENT
0
24
TIME AFTER PLATING [HOURS]
GH CONCENTRATION [NG/ML]

significant change ($P>0.05$) in GH concentration was noted after 24, 48 or 72 h of incubation with any of the mentioned concentrations of sex steroids. However, a significant ($P<0.05$) increase (33.6%) in GH release was noted after 72 h incubation with 10^{-5}M T. Using the same experimental conditions, cow anterior pituitary cells were studied. Figure 10b shows the effect of various concentrations of E and T on GH released from cow anterior pituitary cells in culture. No differences ($P>0.05$) were observed between treatments at any time period studied. These data agree with the work of Wehrenberg et al. (1985) who found no significant differences in GH release at 24, 48, and 72 h of incubation of rat somatotrophs at physiological concentrations for either E or T. In contrast Simard et al. (1986) reported a significant increase in GH released from rat anterior pituitary cells after 72 h of incubation with a physiological concentration of E. The present data suggest that neither E nor T had a direct effect on GH released from bovine anterior pituitary cells during static in vitro incubation.

Testosterone Metabolites. The present study showed no direct effect of T on GH release from anterior pituitary cells in culture. Similarly, Wehrenberg et al. (1985), Fukata and Martin (1986) and Simard et al. (1988)

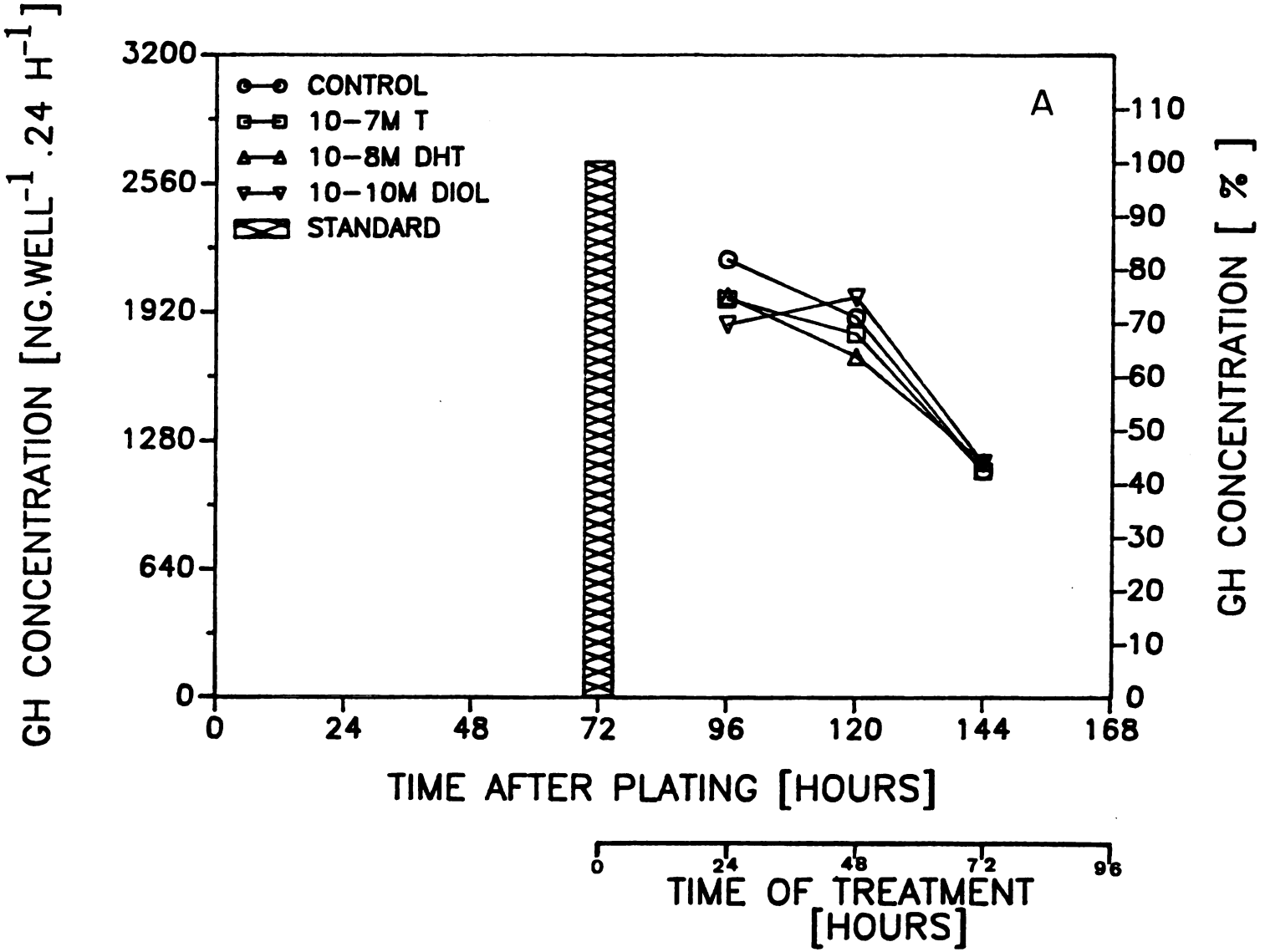
Figure 10 The effect of estrogen (10^{-10}, 10^{-8} M) and testosterone (10^{-7}, 10^{-5} M) on media GH concentrations from (A) heifer anterior pituitary cells in culture and (B) cow anterior pituitary cells in culture. GH concentration is expressed as a percentage of GH concentration at initiation of treatment (72 h after plating). Each bar represents the mean of 12 replicate wells. Pooled standard error was 5.8 % for heifers and 6.3 % for cows.

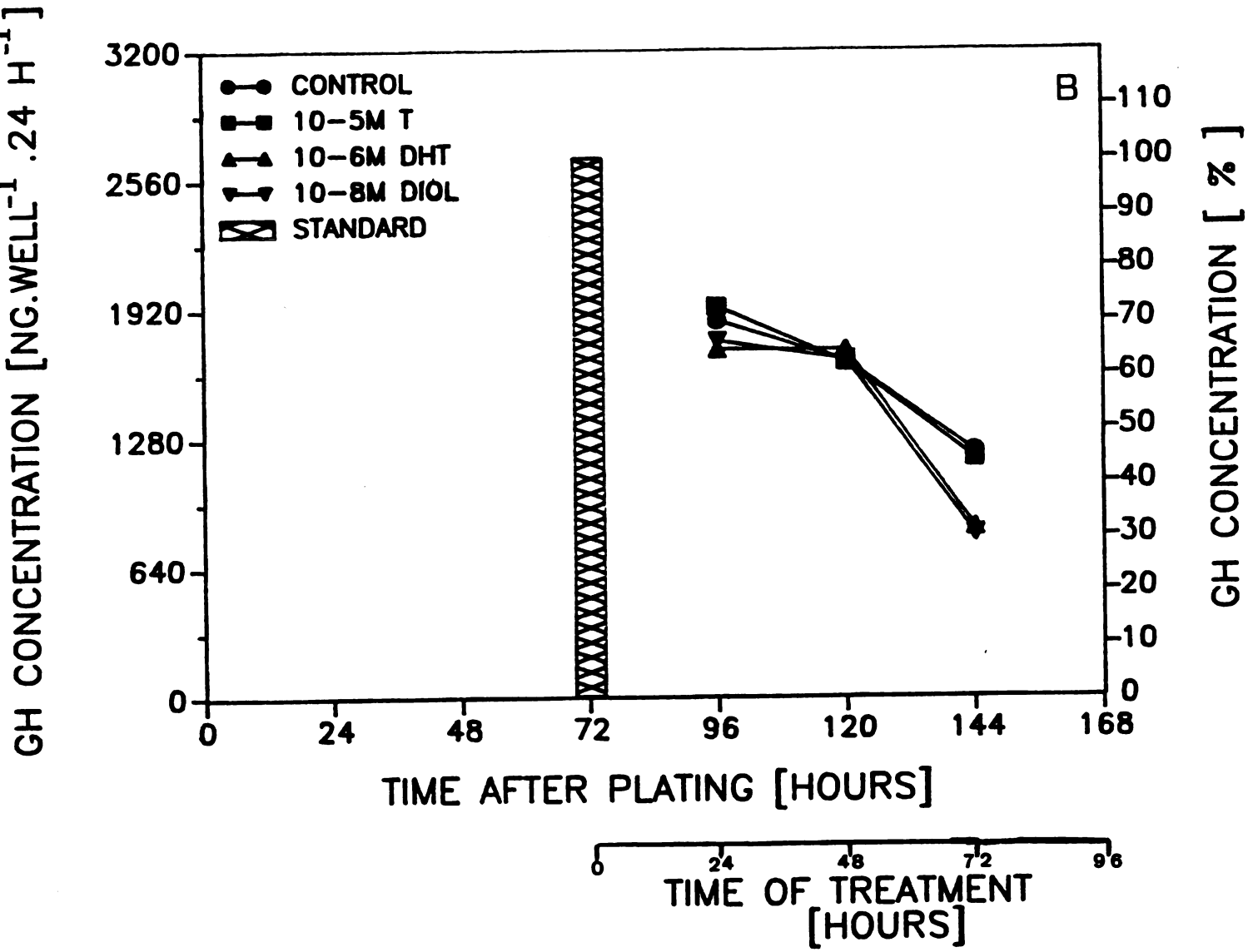

A) HEIFERS
CONTROL
10-10M E
10-8M E
10-7M T
10-5M T
GH [% OF RESPECTIVE STANDARD]
TIME AFTER TREATMENT [HOURS]
0
10
20
30
40
50
60
70
80
90
100
0
24
48
72
96


B) COWS
CONTROL
10-10M E
10-8M E
10-7M T
10-5M T
GH [% OF RESPECTIVE STANDARD]
TIME AFTER TREATMENT [HOURS]
0
10
20
30
40
50
60
70
80
90
100
0
24
48
72
96

also reported that T had no apparent influence on basal GH released by anterior pituitary cells in culture. Since the 5a-reduced metabolites act as intracellular mediators for many of the multiple actions that testosterone exerts on its target tissues (Martini,1982) study of the metabolites of testosterone is warranted. Moreover, the anterior pituitary cells (Cohen et al., 1980) and the hypothalamus (Sholiton et al., 1974) are able to metabolize T into 5a-androstane-17B-ol-3-one (dihydrotestosterone,DHT) and 5a-androstane-3a,17B-diol (3a-diol). Meanwhile, Aakvaag and Haug 1979 reported that GH_3 cells were able to convert T to E in culture. Therefore the effect of these 5a-reduced metabolites (DHT and 3a-diol) on GH released from anterior pituitary cells in culture was studied. In the present study anterior pituitary cells from calves were incubated with the following concentrations of T (10^{-5} and 10^{-7}M), DHT (10^{-6} and 10^{-8}M), and 3a-diol (10^{-8} and 10^{-10}M, respectively). Figure 11a and 11b show the results of 72 h of incubation with these steroids on GH concentration. Incubation with either the low or high concentrations of each of these steroids had no effect ($P>0.05$) on GH concentration at either 24, 48, or 72 h. However, incubation with the higher concentrations of DHT and 3a-diol reduced ($P<0.05$) media concentration of GH after 72 h

Figure 11 The effect of testosterone metabolites (dihydrotestosterone and 3a-diol) on media GH concentration from calf anterior pituitary cells in culture (A) the effect of low concentrations and (B) the effect of high concentrations. The bar at 72 h after plating is the average GH concentration in the media from all wells at the time the treatment was imposed. GH concentration in the media (ng. $well^{-1}$. 24 h^{-1}) is shown on the left ordinate. GH concentration at 24, 48 and 72 h of treatment is expressed as a percentage of GH concentration at initiation of treatment (72 h after plating) and is shown in th right ordinate. Each point represents the mean of 12 replicate wells. Pooled standard error was 4.2 % for low concentrations and 5.1% for high concentrations.
Testosterone (T), dihydrotestosterone (DHT), 5a-androstane-3a,17B-diol (3a-DIOL).


GH CONCENTRATION [NG.WELL^{-1}.24 H^{-1}]
3200
2560
1920
1280
640
0
CONTROL
10-7M T
10-8M DHT
10-10M DIOL
STANDARD
A
110
100
90
80
70
60
50
40
30
20
10
0
GH CONCENTRATION [%]
0
24
48
72
96
120
144
168
TIME AFTER PLATING [HOURS]
0
24
48
72
96
TIME OF TREATMENT [HOURS]


GH CONCENTRATION [NG.WELL^{-1} .24 H^{-1}]
3200
2560
1920
1280
640
0
CONTROL
10-5M T
10-6M DHT
10-8M DIOL
STANDARD
B
110
100
90
80
70
60
50
40
30
20
10
0
GH CONCENTRATION [%]
0
24
48
72
96
120
144
168
TIME AFTER PLATING [HOURS]
0
24
48
72
96
TIME OF TREATMENT [HOURS]

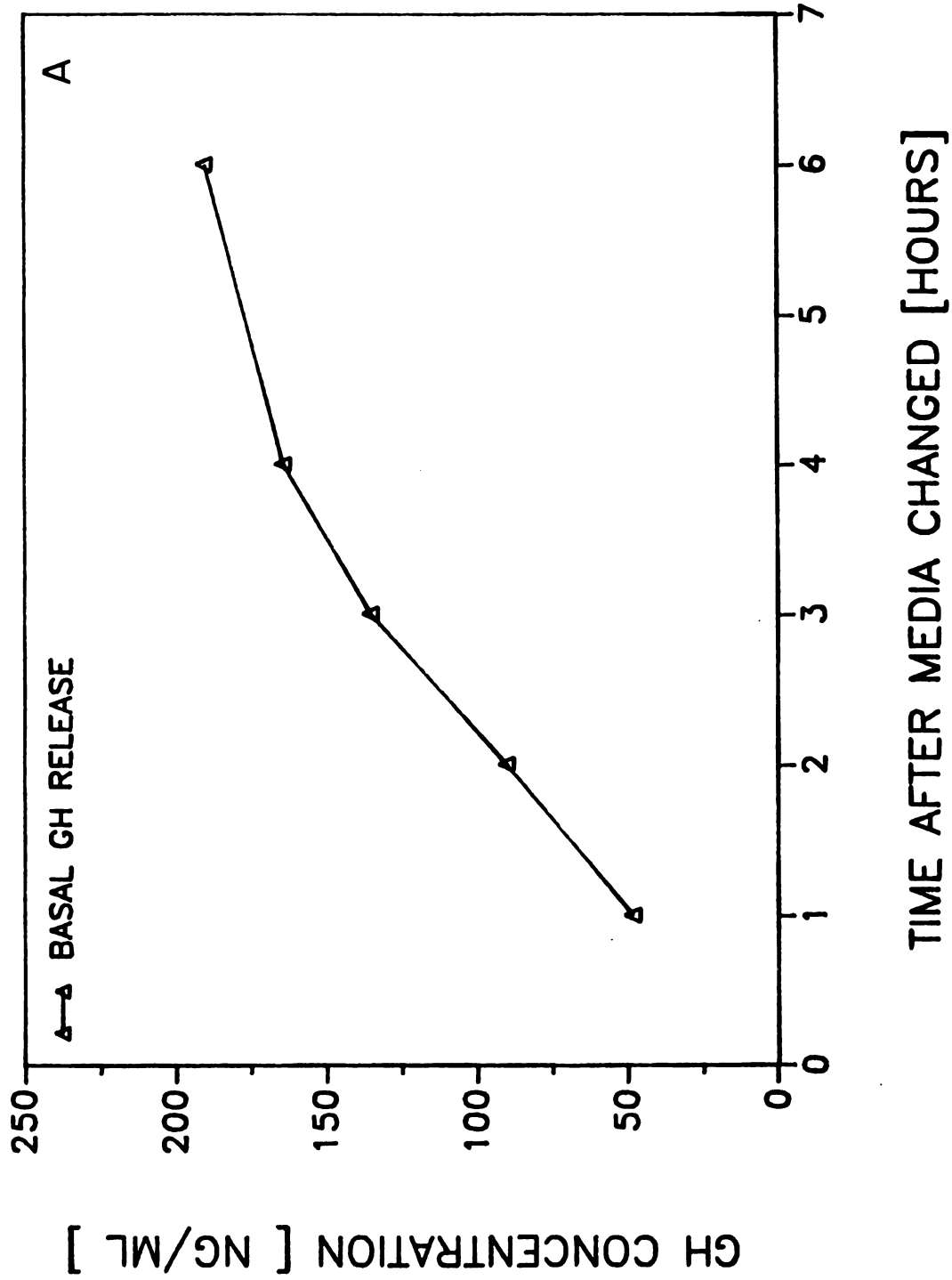
(33 and 35%, respectively). This decrease may be due to a decrease in GH released in the media, stability of GH and (or) viability of cells. These data agree with these of Fukata and Martin (1986) who used T and DHT at 10^{-7}M; and with Simard et al. (1988) who used 10^{-7}M T and 10^{-9}M DHT. They concluded that neither T nor DHT had an apparent influence on basal GH release. These results agree with the present study that neither T nor its metabolites (DHT and 3a-diol) had a direct effect on GH release from calf somatotropic cells in static in vitro incubation. However, these results do not exclude the possible role of testosterone metabolites in mediating the testosterone action on the anterior pituitary and (or) the hypothalamus in vivo.

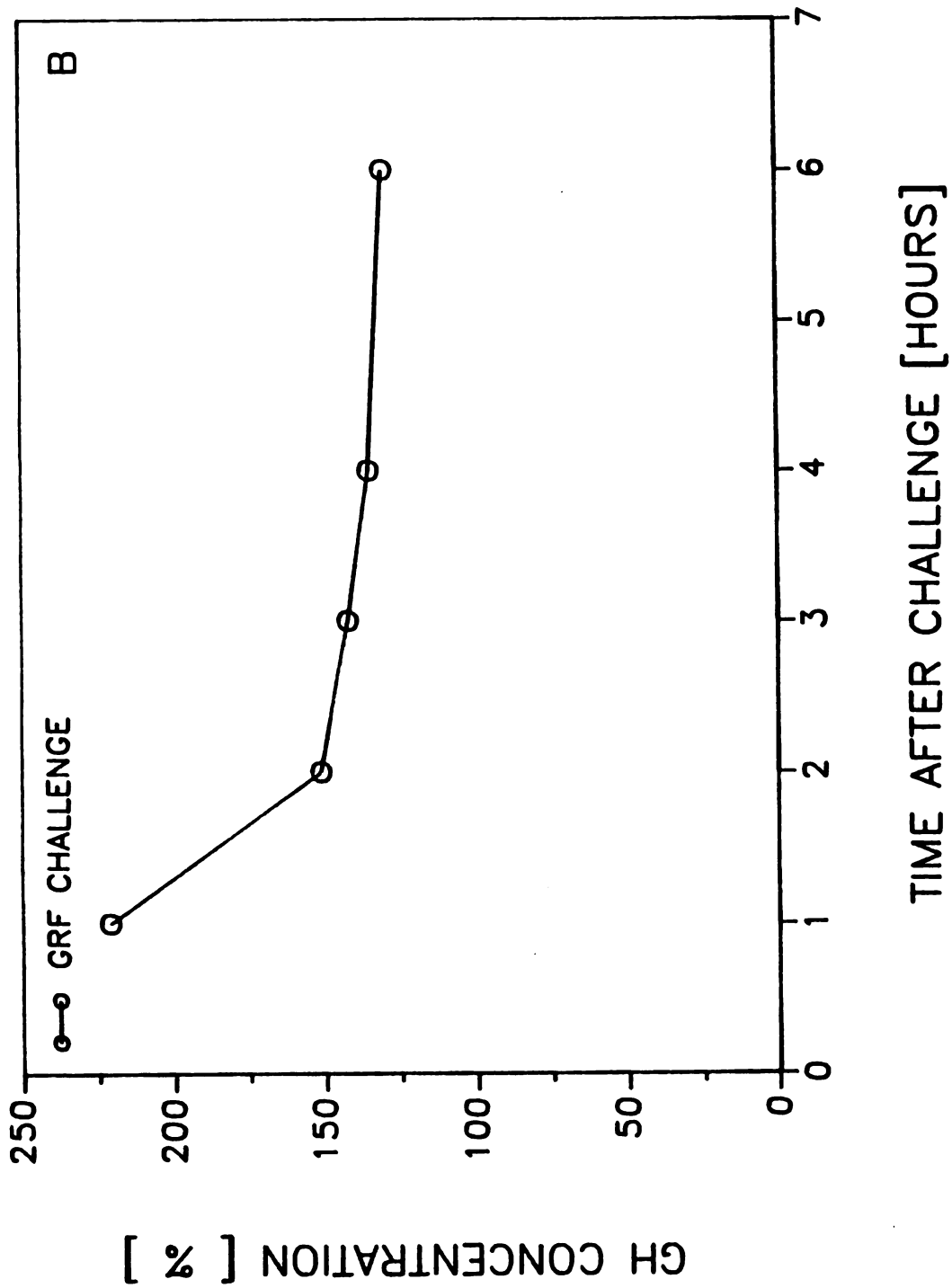
Effect of Growth Hormone Releasing Factor (GRF) on GH Release.

GRF is a potent stimulator of GH production (synthesis and release) in somatotropic cells (Kraicer et al., 1988). In addition, GRF (10^{-12} to 10^{-8} M) stimulates GH release in a dose dependent manner (Blanchard et al, 1988). In the present study GRF was used at a concentration of 10^{-8} M. Frohman et al.(1986) observed that in tissue culture medium GRF(1-44)-NH_2 disappeared rapidly ($t_{1/2}$= 63 min). Thus, a time course experiment was conducted to test

the effect of GH released in response to GRF. Figure 12a shows basal GH concentration found in the media during 6-h period. It was noted that the GH release rate was not sustained during the 6 h period in a static in vitro incubation. These observation was in agreement with Cornin et al. (1984) who concluded that GH release rate increases in a perfusion system while it was not sustained in a static in vitro incubation. Figure 12b shows the effect of GRF(1-44)-NH_2 (10^{-8}M) on GH concentration found in the media after 1 h challenge of calf anterior pituitary cells. GRF increased GH concentration by 221 % after 1 h, while it was increased by 151, 145, 138 and 134 % after 2, 3, 4, and 6 h, respectively. This reduction in the percentage of GH released after GRF challenge with time, is in agreement with the data of Brazeau et al. (1982), Borges et al. (1983) and Cornin et al. (1984) who concluded that GH release rate was not sustained during continuous stimulation with GRF in a perfusion system while intermittent pulses of GRF do not exhaust the responsiveness of dispersed rat anterior pituitary cells. Based on these observations and since it was observed previously that GRF treatment ranged from 1 to 4 h (Cuttler et al., 1986; Glenn, 1986; Blanchard et al., 1988; Kraicer et al., 1988), 1 h was chosen for the GRF challenge time in the present study.

Figure 12 A) Basal GH concentration (ng/ml) over time, B) The effect of 1-44-NH_4 growth hormone-releasing factor (10^{-8} M) on media GH concentration from calf anterior pituitary cells in culture. GH concentration is expressed as a percentage of the basal GH concentration. Each point represents the mean of 12 replicate wells.


A
BASAL GH RELEASE
250
200
150
100
50
0
0
1
2
3
4
5
6
7
GH CONCENTRATION [NG/ML]
TIME AFTER MEDIA CHANGED [HOURS]


B
GRF CHALLENGE
250
200
150
100
50
0
0
1
2
3
4
5
6
7
GH CONCENTRATION [%]
TIME AFTER CHALLENGE [HOURS]

GRF Challenge After 24 h Incubation with Sex Steroids.

The existence of sexual dimorphism in the pattern of GH secretion in several mammalian species suggested a role of sex steroids in GH secretion (Jansson et al, 1985). In the present study, incubation with sex steroids showed no direct influence on basal GH released in vitro. Thus, it was necessary to determine whether E or T may modulate the effect of GRF on GH released from anterior pituitary cells in culture. Table 2 show the effect of GRF (10^{-8}M) challenge for 1 h on GH concentration in the media from anterior pituitary cells, that were preincubated with different concentrations of E or T. The data indicate that the GRF challenge increased average GH concentration in control wells from 20.4±1.8 to 50.9±3.6 ng/ml. The percentage change in GH concentration of challenged vs unchallenged in all steroid treated and control wells is shown in figure 13. Incubation with 10^{-10}, 10^{-8}M E or 10^{-7}M T for 24 h did not affect ($P>0.05$) the percentage (15.0±4.9, 3.5±1.1 and 14.7±4.7 ng/ml, respectively). However, GRF increased ($P<0.05$) the percentage by 38.2 % when incubated with 10^{-5}M T.

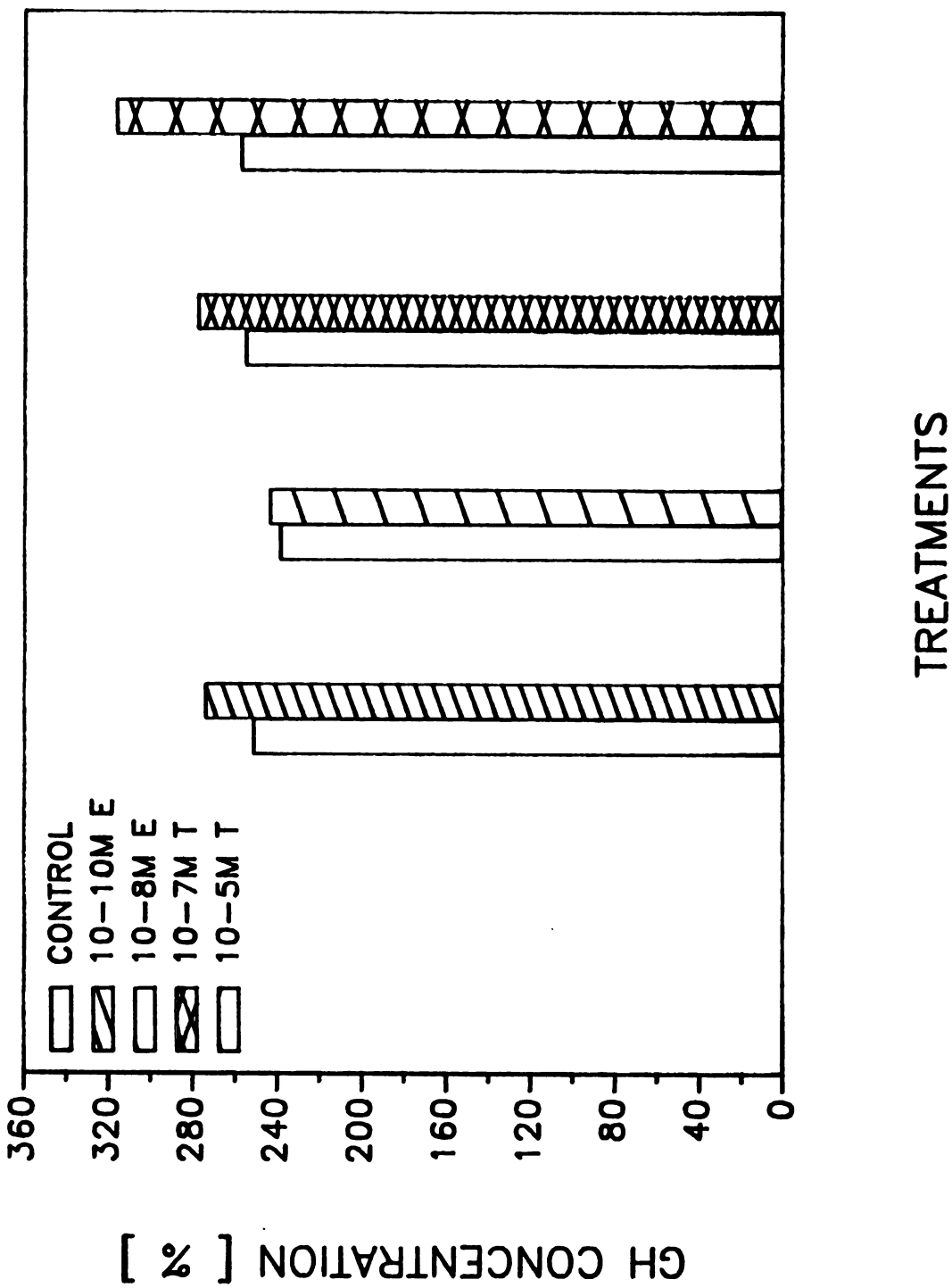
The nonsignificant effect of GRF following incubation with E is in agreement with data reported by Wehrenberg et al. (1985) and Fukata and Martin (1986) for rats. These

(Table 2) Effect of GRF challenge on GH released from cells incubated with different concentrations of estradiol-17B or testosterone for 24 h[a,b]

Treatment		GRF Unchallenged	GRF Challenged	% ± SEM[c]
Testosterone				
0		19.1±1.8	48.6±4.4	254.5 ±14.6
10^{-7}	M	17.2±1.2	47.7±4.6	277.3 ±15.4
0		18.8±1.4	48.3±3.6	256.9 ±12.4
10^{-5}	M	20.6±1.8	65.1±4.8	316.0*±17.1
Estrogen				
0		22.9±1.9	57.5±5.7	251.1 ±13.9
10^{-10}	M	20.9±1.6	57.2±4.8	273.7 ±14.4
0		20.6±2.1	49.1±4.0	238.3 ±12.5
10^{-8}	M	19.0±2.3	46.2±2.5	243.2 ±13.1

a GRF (10^{-8} M) challenge for 1 h.
b Values are expressed as mean (ng/ml) of 24 wells ± SEM.
c (Challenged / unchallenged) % ± SEM.
* Significantly different from controls (P<.05).

Figure 13 The effect of 1-44-NH_4 growth hormone-releasing factor (10^{-8} M) challenge for 1 h on media GH concentration (% of controls) from calf anterior pituitary cells which were preincubated with estrogen (10^{-10}, 10^{-8}M) and testosterone (10^{-7}, 10^{-5} M) for 24 h. Each bar represent the mean of 12 replicate wells.


CONTROL
10-10M E
10-8M E
10-7M T
10-5M T
360
320
280
240
200
160
120
80
40
0
GH CONCENTRATION [%]
TREATMENTS

authors demonstrated that incubation of rat anterior pituitary cells for 24 h with E (10^{-6} to 10^{-8}M) showed no apparent influence on GRF-induced GH release. In contrast, Simard et al. (1986, 1988) concluded that preincubation of rat anterior pituitary cells with E (10^{-10}M) for 72 h exerts a stimulatory effect on GRF-induced GH release. The data herein show that incubation of calf anterior pituitary cells with neither concentration of E affected GH release following GRF challenge. The increase in GH concentrations as a result of implantation with the estrogenic compounds in vivo (Breier et al., 1988) may be due to indirect mechanisms. For instance, E treatment can increase GH by altering serum somatomedins activity (Wiedemann, 1976). This may have a direct effect on GH production at the pituitary level (Yamashita and Melmed, 1986; Fagin et al., 1988) and (or) indirect effect at the hypothalamic level (Berelowitz et al., 1981a; Reichlin, 1983). Moreover, E can affect some biogenic amines in the brain (Stumpf, 1988; Kow and Pfaff, 1984) which has been reported to alter GH concentration in the blood (Martin and Reichlin, 1987; Armstrong et al, 1988).

In the present study, incubation with (10^{-5}M) T for 24 h enhanced the GRF-stimulated GH release in vitro. However, T (10^{-7}M) had no effect on GRF-induced GH release.

Wehrenberg et al. (1985) and Fukata and Martin (1986) observed no effect of the in vitro incubation with T (10^{-6} to 10^{-8} M) for 24 h on GRF-induced GH release in rats. The stimulating effect of T on GH production in vivo (Wehrenberg et al., 1985) may be, in part, due to modulating the somatotrophic response to GRF, which had been demonstrated in vitro in this study. The results of the present study agree with those of Cronin and Rogol (1984), Evans et al. (1985) and Ohlsson et al. (1987) who suggested that testicular androgen secretion increases the pituitary GH release in response to GRF in a static in vitro incubation.

An additional observation worthy of comment is the difference in the GH released, from the same number of cells, between experiments. In heifers and calves the maximum media GH concentration for 24 h was in the range of 3000 ng/ml, while it was in the range of 2000 and 500 ng/ml in steers and cows, respectively. This observation agrees with data reported by Hoeffler and Frawley (1986) for rats, who concluded that the sexual differences in GH release are attributable to the secretory capacities of individual somatotrophs rather than to differences in the number of somatotrophic cells. In any case, it is conceivable that the GH secretion and the factors that alter GH production

are regulated mainly by long term changes in hypothalamic release of GRF and SRIF as well as sex steroids which may modulate their synthesis or release at the level of the hypothalamus.

SUMMARY AND CONCLUSIONS

The effect of estradiol-17B, testosterone and testosterone metabolites on growth hormone released from bovine anterior pituitary cells in a static in vitro incubation were studied. Anterior pituitary cells from either calves, heifers, steers or cows were dispersed and plated at $5X10^5$ cells/ml in Corning 24-well dishes. Cells were allowed to attach for 48 h in a humidified atmosphere in a CO_2 incubator at 37 C. All treatments were started after 72 h of plating and media were collected at 24 h intervals. Effects of some media components on GH were studied. Phenol red , a pH indicator which has been documented to have estrogenic activity in tissue cultures, had no influence ($P>0.05$) on GH released. Also, there was no interaction ($P>0.05$) between phenol red and added estrogen (10^{-10}, 10^{-8}, 10^{-6} M) on GH released. Fungizone, an antifungal agent reduced ($P<0.05$) GH released (25%) after 72 h of incubation.

Estradiol-17B (10^{-10}, 10^{-8} M) or testosterone (10^{-7}, 10^{-5}M) was added to the cells for 24, 48 or 72 h. Neither estrogen nor testosterone had an effect ($P>0.05$) on the basal GH release from any of the pituitary cells irrespective of source (calves, heifers, steers, or cows).

Testosterone metabolites, DHT (10^{-6}, 10^{-8}M) and 3a-Diol (10^{-8}, 10^{-10}M), had no effect (P>0.05) on basal GH released at 24, 48 or 72 h, however the high concentrations of testosterone metabolites caused a decrease (P<0.05) in GH release. Calf anterior pituitary cells were also challenged with GRF (10^{-8} M) for 1 h. Preincubation with estrogen had no effect on GRF-induced GH release; however, preincubation with testosterone (10^{-5}M) increased (P<0.05) the GH released (38.2%) as a result of the GRF challenge. In conclusion, estradiol-17B and testosterone had no direct effect on basal GH released from bovine anterior pituitary cells in a static in vitro incubation. However, pretreatment with testosterone (10^{-5}M) increased the GRF induced GH release from calf anterior pituitary cells in culture.

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