PLASMA $\beta$-ENDORPHIN, THE MENSTRUAL CYCLE AND EXERCISE

By

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ABSTRACT

The underlying mechanism(s) of exercise-associated amenorrhea are not well understood. The discovery of β-endorphin, an endogenous opioid peptide that was shown to inhibit LH release during the menstrual cycle and become elevated during intense exercise, led to the postulate that this peptide may play a role in menstrual dysfunction observed in athletes.

The hypothesis for this thesis was that plasma β-endorphin concentrations are increased in vigorously exercising women with amenorrhea. At the time this hypothesis was generated, current radioimmunoassays to measure plasma β-endorphin were unreliable and insensitive. Therefore, the first objective was to develop a sensitive, specific and precise radioimmunoassay.

Two studies were then conducted to test this hypothesis. 1. Resting plasma β-endorphin concentrations were measured in non-exercising (n=10) and exercising (n=11) women during a regular menstrual cycle and in a group of exercising women with amenorrhea (n=11). Blood samples were obtained 3 times/week for one complete menstrual cycle or for 4 weeks in the amenorrheic subjects. Measurements were made of plasma β-endorphin, estradiol, progesterone, LH and FSH. 2. Plasma β-endorphin and LH concentrations were measured every 20 minutes for 8 hours in these 3 groups (n=3/group). Studies were conducted during the mid-follicular and mid-luteal phase of the cycle in ovulatory subjects.
Results from this investigation indicate that: 1. Significant increments of β-endorphin occur during the early follicular, early luteal and late luteal phase of the cycle in both non-exercising and exercising subjects, however the latter group demonstrate β-endorphin increments of greater magnitude during these times. Gonadal steroid modulation may be responsible for the early luteal phase rise of β-endorphin, but as yet the increases seen at the other times remain to be explained. 2. Plasma β-endorphin concentrations in amenorrheic athletes were similar to those observed in normal menstruating athletes, but no pattern was evident.

3. β-endorphin is released into the circulation in a pulsatile fashion. Pulse frequency appears to be reduced in the luteal phase of both non-exercising and exercising subjects, while amenorrheic subjects exhibit pulse frequency similar to that found in the follicular phase. Pulse amplitude did not vary between phases of sedentary or athletic subjects, but pulse amplitude was greater in the athletes. Amenorrheic athletes had greater pulse amplitudes when compared to the other 2 groups.

The significance of pulsatile secretion of β-endorphin is yet to be determined but the greater amplitude observed in amenorrheic athletes may suggest that LH secretion is being altered in this group. In conclusion, the hypothesis was confirmed that amenorrheic athletes have higher concentrations of β-endorphin and that these may play a role in exercise-associated amenorrhea.
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LIST OF ABBREVIATIONS USED

\( \alpha \)-MSH \( \alpha \)-melanocyte stimulating hormone
\( \beta \)-EP \( \beta \)-endorphin
\( \beta \)-LPH \( \beta \)-lipotropin
5-HT serotonin
ACTH adrenocorticotropic hormone
BSA bovine serum albumin
CLIP corticotropin-like intermediate-lobe peptide
CO\(_2\) carbon dioxide
CRF corticotropin releasing factor
CSF cerebrospinal spinal fluid
CV coefficient of variation
da dopamine
EDTA ethylene diaminetetraacetate
EF early follicular
EL early luteal
FSH follicle stimulating hormone
GH growth hormone
GnRH gonadotropin releasing hormone
H\(_2\)O water
HPLC high performance liquid chromatography
Leu-Enk leucine enkephalin
LF late follicular
LH luteinizing hormone
LL late luteal
MeOH methanol
Met-Enk methionine enkephalin
NaCl sodium chloride
NE norepinephrine
NEN New England Nuclear
O\(_2\) oxygen
PB phosphate buffer
PO\(_4\) phosphate
POMC proopiomelanocortin
PRL prolactin
RIA radioimmunoassay
RNA ribonucleic acid
S.E.M. standard error of the mean
TFA trifluoracetic acid
TSH thyroid stimulating hormone
\( V_0 \) void volume
cpm count per minute
dpm disintegrations per minute
min minute(s)
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CHAPTER ONE

1.1 INTRODUCTION

β-endorphin is an endogenous opioid peptide that has been implicated in several biological processes. Since its discovery, many investigators have examined the role of β-endorphin as a neuroendocrine modulator of anterior pituitary function, specifically its inhibition of luteinizing hormone (LH) throughout the normal menstrual cycle. The finding that vigorous exercise elevated plasma β-endorphin led to the hypothesis that β-endorphin may be partly responsible for exercise-associated amenorrhea.

1.2 DISCOVERY OF THE ENDOGENOUS OPIOID PEPTIDES

Discovery of the endogenous opioid peptides was the result of experiments designed to examine various biological and biochemical aspects of analgesia. Although the analgesic actions of exogenous opiates had been known for many years, it was not until 1957 that the neurotransmitter properties of morphine were demonstrated in guinea pig ileum (Paton 1957; Schaumann 1957). Further to this, attempts were undertaken to identify the mechanism or anatomical locus for analgesia. Akil et al (1978) demonstrated that electrical stimulation of the periaqueductal gray region in the rat brain induced rapid analgesia, yet was reversed by the opiate receptor antagonist naloxone.

Goldstein et al (1971) made the key observation that high affinity binding sites for morphine were present in subcellular fractions of mouse brain. These findings were confirmed by others and similar binding sites were identified using radiolabelled naloxone in guinea pig intestine and mammalian
brain (Pert & Snyder 1973).

With the discovery of specific, high affinity, reversible opiate receptor binding sites; an exciting search began for the endogenous ligand. Evidence for the existence of endogenous opioid peptides in the brain and cerebrospinal fluid was provided concurrently by Terenius & Wahlstrom (1974, 1975) and Hughes (1975). These investigators observed morphine-like substances present in these media that evoked contractions of the mouse vas deferens and guinea pig ileum which were antagonized by naloxone. Others identified morphine-like substances in bovine and porcine pituitary (Cox et al 1975; Teschemacher et al 1975).

Shortly thereafter, Hughes et al (1975) purified and characterized two opioid pentapeptides called Methionine-Enkephalin (Met-Enk) and Leucine-Enkephalin (Leu-Enk). (Enkephalin: Greek – in the brain). Amino acid sequences for the two enkephalins were identical except for the methionine residue in the position of leucine for Met-Enk. The sequences of the two peptides were H-Tyr-Gly-Gly-Phe-Met-OH for Met-Enk and H-Tyr-Gly-Gly-Phe-Leu-OH for Leu-Enk.

Hughes et al (1975) could not decide if their newly characterized pentapeptides were similar to the morphine-like substances described by Terenius and Wahlstrom (1974) and Pasternak et al (1975). But it was noted that the Met-Enk sequence was present as residues 61-65 in \( \beta \)-lipotropin \( \beta \)-LPH), a peptide isolated from the pituitary glands of sheep, pig and man. (Li 1964) In 1964, Li (1964) had inadvertently isolated this 91-residue peptide when isolating adrenocorticotropin hormone (ACTH) from sheep pituitary glands. The name \( \beta \)-LPH was given due to its biological property of adipolysis.

The discovery of \( \beta \)-endorphin seemed equally accidental as the discovery
of β-LPH. During the course of fractionation of camel pituitary, no β-LPH was found, however, a 31-amino acid peptide with little lipolytic activity was isolated (Li & Chung 1976). This peptide had significant opiate activity and was therefore named β-endorphin (Cox et al 1976; Li 1977). A molecule of similar size with opiate activity was obtained from porcine, ovine and human pituitaries (Chretien et al 1976; Bradbury et al 1976, Li et al 1977).

The concept that β-LPH was a precursor to other opioid peptides was examined by several investigators (Bradbury et al 1976; Chretien et al 1979). Mains et al (1977) provided overwhelming evidence that a family of related lipotropin-corticotropin peptides were derived from a common glycoprotein precursor with a molecular weight of 30,000, now called Proopiomelanocortin (POMC) (Chretien et al 1979).

Despite the presence of a Met-Enk sequence in β-LPH, there is no evidence that β-LPH acts as a precursor for this opioid. Neurons immunoreactive to β-endorphin, β-LPH, ACTH and α-Melanocyte-Stimulating hormone (α-MSH) have been localized in well-defined areas of the brain (Bloom et al 1977) and are clearly distinguished from the more diffuse enkephalin immunoreactive neurons (Watson et al 1978). In addition, specific lesions in the brain have been shown to differentially alter brain enkephalin levels without influencing β-LPH or β-endorphin levels (Akil et al 1978). It is therefore apparent that the brain contains totally separate opioid peptide systems.

Since these early developments, a host of endogenous opioid peptides have been isolated, purified and sequenced (Table 1.1). Largely due to recombinant DNA techniques, it is known that these opioid peptides belong to three genetically distinct peptide families designated Proopiomelanocortin
<table>
<thead>
<tr>
<th><strong>TABLE 1.1</strong></th>
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<tr>
<td><strong>LIST OF ENDOGENOUS OPIOID PEPTIDES DERIVED FROM THE THREE OPIOID PEPTIDE FAMILIES</strong></td>
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| **PROPOIOMELANOCORTIN** | B-lipotropin  
β-endorphin  
α-melanocyte stimulating hormone  
β-melanocyte stimulating hormone  
γ-melanocyte stimulating hormone  
ACTH  
CLIP |  |
| **PROENKEPHALIN A** | Methionine Enkephalin  
Leucine Enkephalin |  |
| **PRODYNNORPHIN**  
**(PROENKEPHALIN B)** | Dynorphin A  
Dynorphin B  
α-Neo-endorphin  
β-Neo-endorphin |  |
(POMC), ProEnkephalin A and ProEnkephalin B or ProDynorphin (Weber et al 1983) (Figure 1.1). Nakanishi et al (1979) were the first to report the complete nucleotide sequence for messenger RNA encoding for POMC. This was followed by Gübler et al (1982) for ProEnkephalin A and finally Kakidani et al (1982) reported the nucleotide sequence for messenger RNA encoding for ProDynorphin.

Peptides from the POMC molecule can be found in three major loci in nervous tissue. The prime site of production is the pituitary (Bloom et al 1977), where a small percent of the anterior lobe cells produce ACTH, β-LPH and β-endorphin. The intermediate lobe also produces POMC-related peptides (Akil et al 1979). The main group of cells in the brain to produce POMC peptides occur in the region of the arcuate nucleus where its fibers project widely to include many areas of the limbic system and brainstem (Krieger et al 1977). Synthesis of the POMC polypeptide occurs in several peripheral tissues including the placenta (Krieger 1982; Chen et al 1986), ovary (Lim et al 1983), gastrointestinal tract, lungs, reproductive tract of males and females (Krieger et al 1980; Eipper & Mains 1980) and the adrenal medulla (Evans et al 1983). Proteolytic processing of the POMC precursor appears to be tissue specific (Eipper & Mains 1980).

Since Goldstein et al (1971) identified stereospecific binding sites for the endogenous opioids, it is thought that opioid receptors are heterogeneous (Paterson et al 1983). The multiple forms of opioid receptors include μ (mu or morphine), K (Kappa), ε (epsilon), δ (delta) and σ (sigma) displaying different affinities for the various endogenous opioid ligands (Herz et al 1980; Paterson et al 1983). Studies reveal that β-endorphin shows some preference for the μ receptor but also affinity for δ and little affinity for K receptor (Herz 1984).
Figure 1.1 Schematic representation of the protein precursor structures of the three opioid peptide families. The double vertical lines represent dibasic amino acid cleavage sites. (Akil et al 1984)
In addition, studies using rat vas deferens, a tissue largely insensitive to morphine and enkephalin, reveals particular sensitivity to \( \beta \)-endorphin, i.e. the \( \epsilon \) receptor subtype (Herz 1984).

The heterogeneity of endogenous opioid ligands on one hand, and the multiplicity of opioid receptors on the other, leads to questions and potential confusion relating to specific ligands and the receptor with which they interact. The matching of ligand-and receptor is far from clear and has presented investigators with a challenge.

Utilizing the opiate receptor antagonist naloxone or its oral form, naltrexone, has often complicated the understanding of opioid function. Although naloxone is described as a pure receptor antagonist (Morley 1981; Pfeiffer & Herz 1984) having no agonist properties (Martin 1976), it is well-documented that naloxone has varying affinities for different opiate receptor subtypes. It has been shown that naloxone has a 10-20 fold greater affinity for the \( \mu \) vs. \( \epsilon \) receptor, is relatively insensitive to the K receptor and resistant to the \( \delta \) receptor (Yen et al 1985). There is also evidence that naloxone may, under certain circumstances, assume agonist properties and, at high concentrations, interact with other neurotransmitter receptors (Sawynok 1979).

A recent review by Howlett & Rees (1986) identifies another shortcoming of results derived with naloxone. The effects of naloxone are dose dependent. Low doses of naloxone interact with the \( \mu \) and \( \epsilon \) receptor while high doses of naloxone are required to interact with the \( \delta \) and K receptor. Dose-response effects of naloxone have not been documented. Consequently, it is difficult to interpret the results from those investigators using varying doses of naloxone. Furthermore, there are problems understanding the physiological
effects of opioids based on naloxone studies.

1.3 THE EFFECTS OF $\beta$-ENDORPHIN ON ANTERIOR PITUITARY HORMONES

The endogenous opioid peptides, in particular $\beta$-endorphin, have been associated with numerous physiological processes (Table 1.2). The neuroendocrine effects of $\beta$-endorphin have been examined extensively. Because of high concentrations of $\beta$-endorphin in the hypothalamus and pituitary, investigators have postulated an endocrine function for this peptide (Van Vugt et al 1980) and numerous reviews have been published on this topic (Bicknell 1985; Grossman & Rees 1983; Morley 1983). The results are often difficult to interpret due to different experimental designs, species variation, acute versus chronic administration of opiate agonists and varying dose of naloxone administered. Nevertheless, $\beta$-endorphin appears to have marked effects on some anterior pituitary hormones.

Intravenous or intraventricular injections of $\beta$-endorphin into both male and female rats consistently increase circulating growth hormone (GH) and prolactin (PRL) (Rivier et al 1977; Dupont et al 1977; Ragavan & Frantz 1981) while naloxone blocks these effects (Rivier et al 1977) or result in diminished concentrations of GH and PRL (Bruni et al 1977). It appears that $\beta$-endorphin has a definite role in modulating GH and PRL release in the rat, however, results in man are not as clear. Intravenous injection of $\beta$-endorphin into normal men and women results in no change to GH (Delitala et al 1981a; Morley et al 1980) but has varying effects on PRL concentrations (Mayer et al 1980; Reid et al 1981; Veldhuis et al 1981). Cetel et al (1983) reported
TABLE 1.2

PHYSIOLOGICAL PROCESSES ASSOCIATED WITH THE ENDOGENOUS OPIOID PEPTIDES

Analgesia (Guillemín 1978)
Cataplexy/Epilepsy (Frenk et al. 1978; Guillemín 1978)
Memory (DeWeid et al. 1978)
Thermoregulation (Holaday et al. 1978)
Appetite Control (Brands et al. 1979; Morley 1980; Morley & Levine 1980)
Cardiovascular Function (Lemaire et al. 1978)
Respiratory Function (McQueen 1983)
Glucoregulation (Feldman et al. 1983)
Immune System (Blalock 1985)
increases of PRL concentrations when naloxone was administered during the late follicular and mid-luteal phase of the menstrual cycle, but not during other phases. Most evidence does not implicate a physiological role for β-endorphin in regulating GH or PRL release.

Few have examined the effects of β-endorphin on thyroid stimulating hormone (TSH) secretion. Bruni et al (1977) and Meites et al (1979) report no change to TSH when naloxone doses are administered to rats. In humans, most indicate that naloxone produces no effect on TSH in normal men (Morley et al 1980; Delitala et al 1981a) however, Grossman et al (1981a) observed diminished TSH levels with a high dose of naloxone in an identical population.

High doses of naloxone enhance ACTH and cortisol concentrations in normal men (Volavka et al 1979; Morley et al 1980; Delitala et al 1981a) and women (Blankstein et al 1980), however, a low dose has no effect (Delitala et al 1982). Circadian variations in cortisol are unaltered by naloxone infusion (Grossman et al 1982a). Thus the role, if any, of β-endorphin in the normal physiological maintenance of ACTH and cortisol is unclear.

1.4 THE EFFECTS OF β-ENDORPHIN ON GONADOTROPIN SECRETION

Early investigations showed that exogenous alkaloid opiates, such as morphine, decrease LH (Barraclough et al 1955) and inhibit ovulation (Sawyer et al 1955) in female rats. These initial findings have been confirmed by others (Cicero et al 1977) and it has also been shown that the morphine-induced decrease of LH can be reversed with naloxone (Packman & Rothschild 1976; Bruni et al 1977; Sylvester et al 1980; Cicero et al 1979). It has also been demonstrated that morphine diminishes LH in female rhesus monkeys (Ferin et
The hypothesis that $\beta$-endorphin could alter gonadotropin release was supported by anatomical evidence showing $\beta$-endorphin neuronal cell bodies to be concentrated in the arcuate nucleus, an area known to be the site of gonadotropin-releasing hormone (GnRH) release and consequent gonadotropin control (Krieger et al 1980). Intraventricular administration of $\beta$-endorphin to rats (Kinoshita et al 1980; Leadem & Kalra 1985) and intravenously to normal men and women resulted in significant decreases in serum LH but little change to FSH (Reid et al 1981). In contrast, Gilbeau et al (1985) infused $\beta$-endorphin into male rhesus monkeys and reported no alteration in circulating LH. This author speculates that small sample size and low dose of $\beta$-endorphin may be responsible for these discrepant findings (Gilbeau et al 1985).

Most evidence supporting a role for $\beta$-endorphin and gonadotropin regulation has been derived from naloxone studies. Bruni et al (1977) were the first to show that intravenous naloxone resulted in significant LH increments, while FSH remained unaltered. Other investigators have confirmed these reports (Leiri et al 1979; Cicero et al 1979; Blank et al 1979). The work of Schulz and co-workers (1981) was novel and led to intriguing results. They microinjected antisera of $\beta$-endorphin and Met-Enk into the arcuate nucleus of female rats. Only the $\beta$-endorphin antisera caused increases in circulating LH, while Met-Enk was ineffective. This study gave strength to the hypothesis that $\beta$-endorphin was the specific endogenous opioid peptide responsible for altering LH.

Naloxone results in LH elevations in other species as well. Rabbits (Orstead & Spies 1987) and monkeys (Van Vugt et al 1983; Van Vugt et al 1984)
demonstrate similar LH increases in response to naloxone. Work by Van Vugt et al (1983) indicated that daily doses of naloxone increased LH only in the luteal phase of the menstrual cycle in rhesus monkeys and that long term administration of naloxone during the mid-luteal phase resulted in significant increases in the frequency of LH pulses with no accompanying change in amplitude (Van Vugt et al 1984). Naloxone administration to normal males yields similar results to those described above (Delitala et al 1981a; Moult et al 1981; Morley et al 1980; Veldhuis et al 1981).

The effects of naloxone on circulating FSH in normal men are conflicting. A bolus injection of naloxone or infusion schedules lasting several hours result in moderate FSH increments (Delitala et al 1981a; Moult et al 1981; Morley et al 1980). In contrast, a high dose of naloxone infused over 3 hours had no resulting effect on FSH (Delitala et al 1980). Other investigators found no naloxone-induced effect on FSH (Veldhuis et al 1981). The different responses of LH and FSH to naloxone in normal men are not easily explained, however, a similar dichotomy exists in normal women.

Quigley and Yen (1980) were the first to investigate the effects of naloxone infusion during various phases of the menstrual cycle. Naloxone infusions had no discernible effect on LH release during the early follicular phase, however, significant LH increments were observed during the late follicular and mid-luteal phases. Interestingly, it was noted that the pattern of LH increments were distinct. Naloxone administered during the late follicular phase resulted in slow, progressive increases in LH, while prompt and greater LH increments were evident during mid-luteal phase infusion. No significant changes in serum FSH levels were recorded during any of the 3 stages of the
menstrual cycle studied.

Quigley and Yen (1980) concluded that normal LH secretion is under chronic opioid inhibition, however, the degree of inhibition varies throughout the menstrual cycle. Opioid inhibition was at its highest during times of elevated gonadal steroid phases of the cycle. This finding is supported by the work of others (Lightman et al. 1981; Ropert et al. 1981). The modulation of \( \beta \)-endorphin by circulating steroids will be addressed in another section.

In contrast, Moult et al. (1981) and Grossman et al. (1981b) report significant increments of serum LH levels in response to high doses of naloxone administered during the early follicular phase, a time when others observed no effect of naloxone on LH (Quigley & Yen 1980). The different doses of naloxone used may explain these contradictory results.

The effects of naloxone on serum FSH concentration in normal women are not well understood. Some report an increase in FSH (Lightman et al. 1981; Moult et al. 1981; Morley et al. 1980) while others report no change to basal FSH levels (Quigley & Yen 1980; Veldhuis et al. 1981).

In summary, the literature agrees that exogenous and endogenous opiates promptly suppress LH in both animals and man. Naloxone increases serum LH, however, the effects on FSH are less clear. Naloxone increases both LH pulse frequency and amplitude in men and women, however women demonstrate a changing sensitivity to naloxone throughout the menstrual cycle.
1.5 GONADOTROPIN RELEASE AND $\beta$-ENDORPHIN: SITE OF ACTION

There is convincing evidence to indicate that $\beta$-endorphin and other endogenous opioids modulate the release of gonadotropins through interactions at the hypothalamus and not directly at the anterior pituitary (Van Vugt et al 1980; Howlett & Rees 1986). Sites outside the hypothalamus have been proposed as the loci of opioid action since hypothalamic GnRH neurons in the rat originate outside the hypothalamus in the preoptic area and amygdala (Parvizi & Ellendorf 1980), however, most of the literature indicates otherwise. Cicero et al (1977) report that morphine does not alter pituitary content of LH nor does it change the pituitary's response to exogenous GnRH, during in vitro and in vivo observations in rats. This was later supported by Cicero et al (1979) and others (Bhanot & Wilkinson 1983a) when they showed that naloxone had no effect on GnRH-stimulated release of LH by the anterior pituitary or on $\Delta$-dihydrotestosterone-induced suppression of LH release in vitro.

The exact site of $\beta$-endorphin action in the hypothalamus has yet to be determined, but some suggest a direct action on the median eminence GnRH terminals (Drouva et al 1981). This concept was supported by the elegant studies of Kalra (1981). Implants of naloxone were placed in discrete areas of rat hypothalami following which serum LH levels were measured. The median eminence-arcuate nucleus was the most successful implant area to result in LH increments.

Panerai et al (1983) arrived at similar conclusions when the use of peripherally acting naloxone derivatives (acting outside the blood brain barrier) resulted in significant LH increases. Further evidence that $\beta$-endorphin acts directly on the hypothalamus is provided by the finding that administration of a
GnRH antagonist in conjunction with naloxone prevented the naloxone-induced LH increment, thus indicating that opioids act by altering GnRH and not LH release from the pituitary (Cicero et al 1985; Blank & Roberts 1982).

Similar conclusions have been reached by those investigating opioid action on the hypothalamus in primates and man. Ferin et al (1982) administered morphine to pituitary stalk-sectioned monkeys receiving regular GnRH pulses and concluded that the LH responses to GnRH were not significantly altered by morphine. Investigators report that naloxone and/or opioid pretreatment do not alter the LH response to GnRH in human subjects (Moult et al 1981). Blankstein et al (1981) observed no LH response to naloxone in patients with GnRH deficiency despite a brisk response to an exogenous bolus of GnRH.

An elaborate experimental design by Rasmussen et al (1983) observed GnRH release from human fetal mediobasal hypothalamic tissue in an in vitro perfusion system under different conditions. The GnRH response to naloxone, in the presence or absence of $\beta$-endorphin, were recorded and the results convincingly demonstrate that a constant infusion of naloxone induced a sustained increase in GnRH which was promptly inhibited by a pulse of $\beta$-endorphin.

Many agree that $\beta$-endorphin modulates GnRH secretion by altering the release or synthesis of neurotransmitters regulating GnRH (Van Vugt et al 1980; Holaday 1979; Morley 1981), but it is not clear which neurotransmitter(s) are interacting with $\beta$-endorphin. Neurotransmitters that have been investigated include dopamine (DA), norepinephrine (NE) and serotonin (5-HT).

Dopamine has received much attention for its role in $\beta$-endorphin/GnRH
interactions. Investigators have shown that β-endorphin blocks the action of
DA-induced GnRH release from the mediobasal hypothalamus of male rats
(Rotsztejn et al 1978) and diminish hypothalamic DA turnover (Van Loon et al
input on the GnRH-LH system by measuring the LH response to a DA receptor
antagonist (metoclopramide) during different phases of the menstrual cycle in
normal women. Metoclopramide increased circulating LH in the mid-luteal phase
but had no observable effect in the early or late follicular phase subjects.
However, when DA was infused concomitantly with naloxone in the mid-luteal
phase subjects, the naloxone-induced increments of LH were completely
suppressed.

Recent work by Rasmussen et al (1983,1987) confirms the findings of
Ropert et al (1984): mediobasal hypothalami obtained from human specimens
were set-up in a perfusion system and measured for immunoreactive β-endorphin
release in response to DA and haloperidol (DA receptor antagonist). Dopamine
alone evoked a striking release of β-endorphin while haloperidol blocked this
effect. Phentolamine, a NE (α-adrenergic) antagonist did not have any effect
on β-endorphin release from human hypothalami. This finding is supported by

In contrast, Foresta et al (1985a, 1985b) present data to indicate that
DA is not involved in the naloxone-induced LH elevations and suggests that 5-
HT is. These results were arrived at when sulpiride (a DA antagonist) did not
change the LH response to naloxone, whereas fenfluramine (a 5-HT receptor
stimulant) blocked the expected LH rise to naloxone. Since dopamine or
domperidone (DA antagonist) infusions caused no change in circulating serum LH
(Delitala et al 1981b, 1981c), it was concluded that opioid peptide alteration of LH secretion involved different neurotransmitter mechanisms. Although they ruled out DA, NE and 5-HT were not examined. Those investigating neurotransmitter-opioid interactions in the rat model agree that DA is not involved in the LH increments observed with naloxone (Kalra 1981; Kalra & Simpkins 1981) and suggest that NE may be the mediator.

Neurotransmitter regulation of GnRH/LH release is complex and the evidence to date does not present a strong argument that any single neurotransmitter is responsible for β-endorphin modulation of GnRH secretion. Interpretation of the current literature is difficult since species variation between man and animal may exist and different neurotransmitter pathways may play roles of varying importance. Perhaps future research may find that more than one neurotransmitter is involved in this regulatory process. It is interesting to note that recent data from Barkan et al (1983, 1985) describe GnRH receptor concentrations being altered by opiate administration and that both DA and NE neurotransmitter systems may be involved. Hence, the inhibitory role of β-endorphin may not only involve neurotransmitter release and synthesis but control of receptor numbers as well.

1.6 THE NORMAL MENSTRUAL CYCLE AND β-ENDORPHIN

In light of the preceding evidence associating an inhibitory function of β-endorphin on LH release, it is not surprising that investigators have examined the role of β-endorphin throughout the normal menstrual cycle. The normal menstrual cycle is characterized by a repetitive, predictable change of gonadal steroid and gonadotropin events requiring coordinated feedback between the
hypothalamic-pituitary-ovarian axis (Everitt & Keverne 1986) (Figure 1.2). In particular, the pulsatile pattern of LH release throughout the menstrual cycle is not constant. Santen and Bardin (1973) demonstrate that LH pulse frequency and amplitude change from the follicular phase through the luteal phase. The follicular phase is characterized by LH pulses of high frequency and low amplitude while the luteal phase has LH pulses of reduced frequency yet greater amplitude. Similar findings have been reported by others (Backstrom et al 1982; Filicori et al 1986).

Questions have been posed as to whether β-endorphin might play a role in modulating these documented changes in LH pulse pattern throughout the human menstrual cycle. The opiate receptor antagonist naloxone has been the major tool used to investigate this hypothesis.

Most report that naloxone elevates LH concentrations above baseline in both non-human primate (Van Vugt et al 1983; Van Vugt et al 1984; Gosselin et al 1983; Ferin et al 1984) and human subjects (Blankstein et al 1981; Ropert et al 1981; Grossman et al 1981b; Quigley & Yen 1980; Petraglia et al 1986b; Moult et al 1981), however, the serum LH response varies depending upon the phase of the cycle when naloxone is administered (Quigley & Yen 1980; Blankstein et al 1981). Naloxone is reported to have its greatest influence on increasing plasma LH during the late follicular and mid-luteal phase of the cycle, while LH is unresponsive to naloxone during other times of the cycle (Quigley & Yen 1980; Ropert et al 1981; Petraglia et al 1986a; Blankstein et al 1981). In contrast, significant increases in plasma LH with infusions of high doses of naloxone during the early follicular phase, a time when others find no effect of naloxone on LH, has also been reported (Moult et al 1981; Grossman et al 1981b).
Figure 1.2  Patterns of hormone secretion throughout the normal menstrual cycle. (Everitt & Keverne 1986)
Because different investigators have used varying doses of naloxone, interpretation of the data is difficult and often confusing. For example, the use of high dose naloxone can result in: a) naloxone acquiring agonist properties (Sawynok et al 1979) and b) naloxone functioning at a different receptor subtype when compared to low dose naloxone (Howlett & Rees 1986). Thus firm conclusions cannot be made from studies which have used naloxone infusion alone.

1.7 GONADAL STEROID MODULATION OF β-ENDORPHIN'S INHIBITION OF GONADOTROPIN SECRETION: STUDIES WITH NALOXONE

Quigley and Yen (1980) were the first to postulate that endogenous opioid activity may be modulated by gonadal steroid concentrations throughout the normal menstrual cycle. Data in the rat model support this finding (Gabriel et al 1983; Sylvester et al 1982). Bhanot and Wilkinson (1983a, 1984) have shown that there is a reduction in the inhibitory effects of endogenous opioids on gonadotropin release following gonadectomy in male and female rats. Naloxone's ability to increase serum LH is restored when the ovariectomized rats are treated with combined estrogen and progesterone (Bhanot & Wilkinson 1984). In normal women, Steele and Judd (1986) demonstrated that the progesterone-induced decrease of LH pulse frequency (Soules et al 1984) can be blocked with naloxone.

Subjects deficient in endogenous gonadal steroids have provided an ideal model to examine the effects of estrogen and progesterone on the inhibitory role of β-endorphin on gonadotropin release but results are variable.Investigators have found that naloxone was unable to alter LH levels in
postmenopausal women, oophorectomized women and castrated men, but steroid replacement therapy restored the typical naloxone-induced LH increase (Casper & Alapin-Rubillovitz 1985; Foresta et al 1983; Melis et al 1984; Reid et al 1983).

Women with amenorrhea, due to a variety of etiologies (hypogonadotropic hypogonadism, polycystic ovarian disease, hyperprolactinemia and gonadectomized patients with testicular feminization) have been investigated. Nappi et al (1987) report that all patients with hypogonadotropic hypogonadism or secondary amenorrhea show no increase in LH after a naloxone infusion. Gonadotropin treatment restores the naloxone-induced LH response in the patients with secondary amenorrhea but is absent in the hypogonadotropic hypogonadism patients.

In contrast, Petraglia et al (1985) demonstrated a naloxone-induced LH increment in those women with hyperprolactinemia. Gonadectomized patients with testicular feminization undergoing estrogen-replacement therapy respond to naloxone with significant increments in LH, therefore Veldhuis et al (1985a) conclude that the inhibitory effects of opioids on gonadotropins can be revealed after exogenous estrogen administration. The evidence indicates that replacing ovarian steroid hormones may restore β-endorphin activity on the hypothalamic-pituitary-ovarian axis.

1.8 GONADAL STEROID MODULATION OF β-ENDORPHIN'S INHIBITION OF GONADOTROPIN SECRETION: STUDIES MEASURING β-ENDORPHIN

Direct measurements of β-endorphin in tissue or blood have been performed less frequently when examining the effects of circulating steroids on opioid inhibition. Studies that have measured β-endorphin in the periphery of
rats and monkey support the above findings derived by naloxone. A number of investigators report consistent changes in \( \beta \)-endorphin concentrations over the estrous cycle in the hypothalamus, anterior pituitary and hypophyseal portal plasma (Hulse et al. 1984; Ishizuka et al. 1982; Sarkar & Yen 1985). This suggests that the variations in \( \beta \)-endorphin content are likely due to the changing gonadal steroid environment (Sarkar & Yen 1985).

In addition to this, Sarkar and Yen (1985) noted a significantly lower concentration of \( \beta \)-endorphin in hypophyseal portal blood following ovariectomy but no change to anterior pituitary levels. Neuro-intermediate lobe concentrations of \( \beta \)-endorphin in rats were increased after ovariectomy, but concentrations were reversed by estradiol administration in a dose-related manner (Lim & Funder 1984). Wardlaw et al. (1982) observed no change in hypothalamic content of \( \beta \)-endorphin during the estrous cycle, but the concentration of this opioid dropped significantly after chronic estradiol treatment in ovariectomized rats. Progesterone partially blocked the estradiol-induced decrement, hence they concluded that estradiol stimulates the release of \( \beta \)-endorphin from the hypothalamus while the addition of progesterone promotes the synthesis of the peptide. The evidence gathered from the rat model strongly suggests that physiologic concentrations of estradiol and progesterone can alter the content of hypothalamic \( \beta \)-endorphin and ovarian steroids may be important regulators of this endogenous opioid peptide (Lim & Funder 1984; Wardlaw et al. 1982).

Studies performed in non-human primates have strengthened the hypothesis that \( \beta \)-endorphin is modulated by circulating gonadal steroids. Wehrenberg et al. (1982) report striking differences in \( \beta \)-endorphin
concentrations in hypophyseal portal blood throughout the menstrual cycle of rhesus and pig-tail monkeys. Ferin et al (1984) confirmed this finding but observed no change in peripheral venous β-endorphin. Greatest β-endorphin concentrations were detected when serum progesterone and/or estradiol were raised. By contrast, when ovarian steroid concentrations were low, such as at menstruation or after ovariectomy, β-endorphin levels were undetectable. When ovariectomized monkeys were treated with estrogen and progesterone, β-endorphin concentrations returned to detectable levels (Wardlaw et al 1982).

Surprisingly, amidst the many reports of β-endorphin’s varying influence on gonadotropin secretion at different times of the menstrual cycle, little work has been done to document β-endorphin’s variation in peripheral plasma. The anterior pituitary represents the most important source of β-endorphin in the periphery (Krieger et al 1980) while the arcuate nucleus and mediobasal hypothalamus represent the main source of β-endorphin in the brain (Watson et al 1978). This finding is based on the evidence that the source of β-endorphin in the brain is of a different origin from the anterior pituitary as demonstrated by the observation that concentrations of β-endorphin in the brain are unchanged following hypophysectomy in rats (Krieger et al 1980).

There has been some debate regarding the value of measuring plasma β-endorphin and whether these concentrations influence or reflect concentrations in the brain (Foley et al 1979; Nakao et al 1980). Some argue that circulating β-endorphin cannot affect sites in the brain through the blood brain barrier since intravenous injections of β-endorphin do not affect perception of pain or mood (Foley et al 1979). However, there is evidence to indicate that intravenous injections of β-endorphin can alter anterior pituitary hormone
secretion. Although intravenous β-endorphin may not affect sites within the brain and central nervous system to alter mood or pain perception, sites outside the blood brain barrier, such as the arcuate nucleus, are quite susceptible to the influence of circulating β-endorphin (Ferin et al 1984; Reid et al 1981).

β-endorphin has been considered impermeable to the blood brain barrier but a number of studies are emerging that are leading investigators to reconsider peptide permeability through the blood brain barrier (Banks & Kastin 1985; Pardridge 1986). The failure to show a correlation between plasma and cerebrospinal fluid (CSF) concentrations of β-endorphin or to show an increase in CSF concentrations following peripheral infusion of β-endorphin, does not rule out blood brain barrier penetration. The lack of correlation may be due to several factors: 1. variability in the assay procedure and limits of detection, 2. lag time between peripheral administration and central uptake due to moderate cerebrovascular permeability (Rapoport et al 1980) and 3. poor correlation between CSF sampled from lumbar spine and plasma concentrations (Nakao et al 1980) due to different peptide gradients within the CSF (Banks & Kastin 1985).

Similarly, a positive correlation of plasma and CSF concentrations of β-endorphin does not prove that the blood brain barrier has been penetrated. β-endorphin is known to be synthesized in areas on both sides of the blood brain barrier (Krieger et al 1980; Watson et al 1978) and may respond to similar stimuli, such as stress. Some investigators report that β-endorphin may cross the blood brain barrier (Merin et al 1980; Rapoport et al 1980; Houghten et al 1980).

Under differential experimental conditions, including stress of sufficient
intensity, certain molecules normally impermeable, can breach the blood brain barrier. McArthur (1985) cites an unpublished observation by Richard Bergland that rats injected intravenously with trypan blue and forced to swim to exhaustion were found to have their brains suffused with dye, whereas in non-exercised controls, no such phenomena occurred. While it cannot be assumed that circulating $\beta$-endorphin behaves in the same manner as the dye or that similar results would be observed in humans, Bergland's observation nevertheless suggests an example whereby exercise might facilitate the entry into the brain of compounds otherwise excluded.

The relationship between $\beta$-endorphin concentrations in peripheral blood and the one or more pools in the brain directly synthesized by peptidergic neurons has not been well-investigated. Evidence for a concomitant $\beta$-endorphin response by the pituitary and the central nervous system is, as yet, meager. Barta and Yashpal (1981) show that protracted swimming in cold water stimulates the discharge of $\beta$-endorphin from the rats pituitary into the periphery and raises the $\beta$-endorphin concentrations in such structures as the amygdala and hypothalamus, regions containing high concentrations of $\beta$-endorphin receptors. The limitation of these data is that they reflect tissue $\beta$-endorphin concentrations rather than turnover. Nevertheless, the data suggest a parallel increase in $\beta$-endorphin content of the two pools which seem to occur in association with exercise-induced stress. Therefore, it may be possible that an elevated plasma $\beta$-endorphin concentration may constitute a marker of brain arousal even though it may bear no quantitative relationship to brain $\beta$-endorphin secretion.
1.9 PLASMA $\beta$-ENDORPHIN CONCENTRATIONS: THE NORMAL MENSTRUAL CYCLE AND AMENORREA

When the work for this thesis was being initiated, there was only one publication where plasma $\beta$-endorphin had been measured during the menstrual cycle in humans (Vrbicky et al 1982). During the development of the methods for this thesis, to be described in Chapter 2, a few other papers have appeared and will be discussed in the final chapter.

The majority of cycles analyzed by Vrbicky et al (1982) had a preovulatory $\beta$-endorphin peak followed by a postovulatory drop of $\beta$-endorphin. A few cycles had a postovulatory peak or a postovulatory peak with sustained elevation of $\beta$-endorphin. Blood samples were not taken on a regular schedule and individual patterns of plasma $\beta$-endorphin were highly variable. Since these were the only available data describing plasma $\beta$-EP variation throughout the menstrual cycle, it became apparent that further work in this area was required and was the first question that needed to be answered.

Although there were few available studies, some investigators postulated that $\beta$-endorphin may be associated with menstrual dysfunction. The hypothesis that $\beta$-endorphin inhibits LH release (Quigley & Yen 1980) raised the possibility that, in certain cases, menstrual dysfunction may reflect abnormal $\beta$-endorphin activity. It was suggested that the absence of regular high and low periods of opioid activity may alter GnRH release and result in inappropriate gonadotropin ratios and interfere with normal ovarian development.

Studies in monkeys (Pohl et al 1983) and women (Marshall & Kelch 1985) have shown that altered GnRH frequency is deleterious to regular menstrual cycles. A sustained decrease in GnRH frequency, administered by a
continuous low frequency GnRH pulse regimen, in intact hypothalamic lesioned monkeys, results in a progressive decrease in follicular development and anovulation (Kahl et al 1983).

Clinical studies support the hypothesis for a role of β-endorphin in the etiology of some amenorrhea. Patients with hyperprolactinemia (Quigley et al 1980a; Lightman et al 1981) hypogonadotropic hypogonadism (Quigley et al 1980b) and polycystic ovarian disease (Lightman et al 1981) have been reported to increase LH in response to a naloxone infusion. This suggests that amenorrhea of different etiologies may be due, in part, to an increased inhibitory effect of endogenous opioids on GnRH and gonadotropin secretion. However, not all amenorrheic conditions can be explained by an elevated opioid inhibitory tone. Patients with amenorrhea not associated with hyperprolactinemia (Petraglia et al 1983) or due to weight-loss combined with low concentrations of LH, (Grossman et al 1982b; Baranowska et al 1984) were found to be unresponsive to naloxone.

Clinical studies measuring β-endorphin in the periphery have confirmed findings based on naloxone infusions that some forms of amenorrhea are accompanied by elevated β-endorphin activity. Aleem and McIntosh (1984) determined that women with polycystic ovarian disease had significantly higher concentrations of plasma β-endorphin when compared to a control group with ovulatory cycles. In another study, levels of β-endorphin in women with anovulatory cycles or primary amenorrhea showed no significant variation when measured over a span of 30 days. However, induction of ovulation with GnRH infusion or gonadotropin treatment resulted in elevations of β-endorphin comparable to the normal menstruating group (Petraglia et al 1985).
One of the more interesting types of amenorrhea that has been investigated with respect to its association with $\beta$-endorphin is exercise-related amenorrhea. Routine, strenuous exercise is now an accepted cause of menstrual dysfunction including delayed menarche, shortened luteal phase, primary and secondary amenorrhea (Baker 1981; Cumming & Rebar 1983; Carlberg et al 1983; Schwartz et al 1981; Warren 1980). Women's participation in recreational and competitive sport has increased dramatically in recent years. Russell et al (1984a) noted that the number of female participants in the New York Marathon has increased from 1 in 1970 to over 2500 in 1980.

The incidence of exercise-related amenorrhea has varied. Baker (1981) reviews the incidence of menstrual dysfunction and reports occurrences of amenorrhea from 0-50%. Historically, early reports were largely descriptive and described variable effects of exercise on the menstrual cycle (Baker 1981). Zaharieva et al (1965) investigated 66 athletes competing in the 1964 Tokyo Olympic games and documented one case of amenorrhea. Transient disruption of menstrual cycles was observed in competitive swimmers (Astrand et al 1963) and rowers (Erdelyi 1962) during intense training. Compared to the general population of 2.6% reported by Carlberg et al (1983), others have observed athletic amenorrhea in ranges of 19% (Glass et al 1987) to 43% (Feicht et al 1978). A higher incidence of amenorrhea occurs in endurance sports where competition is considered to be more stressful (Sanborn et al 1982). Amenorrhea was present amongst competitive swimmers, cyclists and runners, but was most prevalent in the latter group (Sanborn et al 1982).
1.10 FACTORS PREDISPOSING ATHLETES TO AMENORRHEA

The underlying mechanisms of exercise-associated amenorrhea are not well understood. A number of factors have been cited that may predispose the athlete to menstrual dysfunction. These are young age, nulliparity, history of menstrual dysfunction, intense training prior to menarche, diet, reduced body weight and/or percent body fat, physical stress and psychological stress (Baker 1981; Schwartz et al 1981) (Table 1.3). Many authors describe the presence of these characteristics among amenorrheic athletes, yet are vague about the mechanisms causing disruption of the hypothalamic-pituitary-ovarian axis.

Most studies investigating athletic amenorrhea are cross-sectional which is not the best study design to elicit cause-effect relationships between predisposing factors and menstrual dysfunction (Sackett et al 1985). A major weakness of cross-sectional studies is that subject recall is used to document menstrual history, training regimen, body composition changes and dietary habits. This method of gathering information is known to be inaccurate and unreliable (Sackett et al 1985).

A randomized prospective trial provides the soundest evidence that can evaluate a causal effect of intense training, reduced percent body fat or other related factors on menstrual regularity (Sackett et al 1985). Results from this type of trial have the greatest chance of being statistically significant (Sackett et al 1985). But few studies fulfill this criteria (Boyden et al 1983; Bullen et al 1985). Nevertheless, identification of factors predisposing athletes to menstrual dysfunction have assisted investigators in their direction of research.

Young, nulliparous women have been found to experience a greater frequency of secondary amenorrhea than older, multiparous women (Baker 1981).
<table>
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<th>TABLE 1.3</th>
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<td>FACTORS PREDISPOSING ATHLETES TO MENSTRUAL DYSFUNCTION</td>
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- Young age
- Nulliparity
- Prior menstrual dysfunction
- Begin training before onset of menarche
- Diet
- Low body weight &/or reduced body fat
- Physical stress
- Psychological stress
The incidence of amenorrhea among athletes is reported to be higher in those less than 30 years of age than in those over 30 years of age (Baker et al 1981; Glass et al 1987). In addition, multiparous women were less likely to be amenorrheic when compared to their nulliparous counterparts (Dale et al 1979; Baker et al 1981). Dale et al (1979) speculate that maturity of the hypothalamic-pituitary-ovarian axis, as indicated by pregnancy, may protect women from endocrine disruption.

Histories of previous menstrual dysfunction and delayed menarche are more commonly found in active amenorrheic than eumenorrheic women. Schwartz et al (1981) report that 54.4% of athletes with amenorrhea experienced prior menstrual dysfunction while only 15.5% of the normal menstruating athletes were in this category. In contrast, Speroff and Redwine (1980) observed amenorrheic runners had regular cycles prior to training.

Onset of vigorous training prior to menarche has also been identified as a factor predisposing athletes to amenorrhea (Frisch et al 1981; Warren 1980). Frisch et al (1981) observed that athletes training prior to menarche had a mean menarcheal age of 15.1 ± 0.5 years, whereas those commencing training post-menarche had a mean menarcheal age of 12.8 ± 0.2 years. Each year of training before menarche was reported to delay menarche by 5 months. In addition, the premenarche-trained athletes experienced more menstrual irregularity (61%) and amenorrhea (22%) while 60% of the post-menarche trained athletes had regular cycles.

Reduced protein content in the diet has been associated with athletic amenorrhea. Amenorrheic runners are reported to derive less of their daily calories in the form of proteins when compared to eumenorrheic runners.
(Carlberg et al 1983), even though their daily caloric intake was higher (Schwartz et al 1981). The mechanism(s) responsible for the altered reproductive hormonal pattern with decreased protein or caloric intake are as yet unknown. It has been noted that the hormonal changes that occur with acute exercise are similar to those observed with food restriction, suggesting that both stimuli create stress in an individual (Warren 1983) and disrupt the gonadal axis in a similar fashion. Studies on exercise-related amenorrhea in the presence of altered nutrition have been limited by the use of different protocols and lack of longitudinal analyses.

Exercise-induced amenorrhea has been strongly associated with the presence of low body weight and/or reduced percent body fat (Baker 1981). Reduced peripheral formation of estrogen (Baker 1981), diminished pituitary sensitivity to GnRH (Wentz 1980) and increased catecholestrogen concentration leading to reduced hypothalamic activity (Fishman et al 1975; Wentz 1980) have been suggested mechanisms for causing menstrual disturbances in a condition of low body weight and/or body fat.

The critical fat theory proposed by Frisch and McArthur (1974) is largely responsible for development of this hypothesis. According to these investigators, menarche and maintenance of menstrual cycles require a critical level of body fat for a particular height (Frisch 1971). It was determined that 17% body fat was required for menarche to occur and 22% for maintenance of menses (Frisch & McArthur 1974). These determinations were based on percentiles of total water/body weight percentiles, which were converted to percent body fat (Percent body fat = 100-(percent water +0.72).

The critical fat theory has been subject to criticism since the results
of Frisch and McArthur (1974) could not be duplicated (Trussell 1978) and most investigators do not observe a minimal percent body fat for maintenance of menses in athletic populations (Glass et al 1987; Sanborn et al 1987; Sinning & Little 1987).

The methods which measure body composition may also present problems when examining the relationship between reduced percent body fat and amenorrhea. Subcutaneous fat measurements provide useful comparisons of adiposity, but considerable error occurs when they are used in equations and are often inappropriate for use in athletes. Percent body fat equations are based on assumed densities of fat and non-fat compartments which are often substantially different in athletes compared to the normal population (Ross & Marfell-Jones 1982). Percent body fat measured by underwater weighing is the most widely accepted method for accurate body composition measurement (Behnke & Wilmore 1974) yet only one investigator has used this method to examine the relationship between athletic amenorrhea and percent body fat (Sanborn et al 1987). Interpreting results of those who have used subcutaneous measurements must be done with caution since this method may be subject to error.

A positive relationship between frequency of amenorrhea and quantity of exercise has been reported by some (Feicht et al 1978; Carliberg et al 1983), but not by others (Baker et al 1981; Glass et al 1987). Similarly, psychological stress, has been associated with reproductive dysfunction but results are inconclusive (Warren 1980, 1985). Psychological stress is difficult to measure and standardize and little work has been gathered in this area.

The literature has identified and examined a number of factors which
appear to make the athlete susceptible to menstrual dysfunction. There is little agreement about the relative influence of each factor, individually or in conjunction with others, in contributing to this condition. Cross-sectional study designs are poor at identifying cause-effect relationships between predisposing factors and athletic amenorrhea, therefore, little progress has been made at describing the underlying mechanism responsible for this phenomenon.

1.11 EXERCISE-ASSOCIATED AMENORRHEA AND $\beta$-ENDORPHIN

Even less is understood about the mechanism(s) causing menstrual dysfunction in the athlete, however, $\beta$-endorphin has been suggested as playing a role for two reasons:

1. $\beta$-endorphin has been shown to inhibit LH release in varying degrees throughout the menstrual cycle (Quigley & Fren 1980).

2. Circulating $\beta$-endorphin increases several fold in response to exercise (Fraioli et al 1980; Gambert et al 1981).

A number of investigators postulate that women involved in chronic exercise programs may be exposed to increased levels of $\beta$-endorphin for prolonged periods, hence reducing the release of gonadotropins and contributing to the onset of menstrual dysfunction (Carr et al 1981). The amenorrheic athlete is characterized by low gonadotropin and estrogen concentrations (Baker et al 1981; McArthur et al 1980) with an exaggerated LH response to exogenous GnRH (McArthur et al 1980), suggesting that amenorrhea is mediated at the hypothalamic level. This profile is in agreement with the hypothesis that $\beta$-endorphin modulates gonadotropin levels by altering GnRH release (Ferin et al 1984). If gonadotropin concentrations are being suppressed by chronically
elevated $\beta$-endorphin, then an increase in LH should be observed in response to naloxone. Unfortunately, little information has been gathered in this area of research. Naloxone has been reported to increase LH in some amenorrheic athletes (McArthur et al 1980), but have no effect in others (Cumming & Rebar 1983).

Most evidence supporting the $\beta$-endorphin/athletic amenorrhea hypothesis has been derived from experimental designs measuring the acute response of $\beta$-endorphin to exercise. Although the effects of acute bouts of exercise on plasma $\beta$-endorphin has been examined by many in men, few studies in women have been performed to test this hypothesis. Results from prospective studies indicate that:

1. $\beta$-endorphin increases in response to intense exercise (Bullen et al 1984; Howlett et al 1984).
2. Increments of $\beta$-endorphin appear to be enhanced with greater training intensities (Bullen et al 1984; Howlett et al 1984).
3. Menstrual disturbances may be associated with increased training volumes and increased circulating $\beta$-endorphin (Bullen et al 1984; Howlett et al 1984; Russell et al 1984b).

The review of the literature suggests that $\beta$-endorphin increases in response to exercise, but results must be interpreted with caution. Comparison between studies is limited due to differences in study design and a range of fitness within subjects. More importantly, the reported values of $\beta$-endorphin (resting or stimulated levels) vary widely. The radioimmunoassay (RIA) methods in which $\beta$-endorphin is measured in plasma may be responsible for the array of published results.
1.12 MEASUREMENT OF PLASMA β-ENDORPHIN

The introduction of RIA procedures by Yalow and Berson (1960) was an important advance in biological measurement. Since then, the criteria for a sound and accurate RIA have been well-established and are described by Chard (1987). These criteria include:

1. Sensitivity: defined as the minimal detection limit of an assay described in terms of concentration and should refer to the biological fluid for which the assay is intended. High sensitivity is often considered to be the hallmark of a good assay.

2. Specificity: defined as the degree to which an assay responds to substances other than that for which the assay was designed. Some groups of hormones are physicochemically similar, for example those derived from the precursor POMC. An antiserum may react with closely related materials and the degree to which it distinguishes between closely related molecules is referred to as specificity.

3. Precision: also referred to as reproducibility, is a measure of the variation observed between repeated determinations on the same sample. The aim of an RIA is to determine the true concentration of a ligand in a biological fluid and quality control parameters are often used to monitor the precision of an RIA. These parameters include within (intra-) and between (inter-) assay variation.

When this work was initiated, relatively few methods were described for the assay of plasma β-endorphin. Few investigators have taken into consideration the physical properties of β-endorphin which have made measurement of this peptide difficult. These properties include: 1. its
susceptibility to enzymatic degradation (Clement-Jones et al 1980), 2. low circulating concentrations and 3. presence of structurally related compounds (Eipper & Mains 1980).

To overcome these problems a number of procedures can be employed. Enzyme degradation has been minimized and protection of \( \beta \)-endorphin enhanced through the collection of blood samples in plastic or siliconized glassware (Angwin & Barchas 1982) with the addition of protease inhibitors, such as Trasylol (Cahill et al. 1983; Clement-Jones et al. 1980). In addition, losses of \( \beta \)-endorphin are reduced with the collection of plasma versus serum since serum has been shown to exhibit high losses of \( \beta \)-endorphin (Clement-Jones 1980). Low circulating concentrations of \( \beta \)-endorphin have been overcome by the application of several extraction techniques to concentrate \( \beta \)-endorphin and separate non-specific binding substances (Chard 1987). A major problem of the \( \beta \)-endorphin RIA has been the use of antisera having high degrees of cross-reactivity with other structurally related peptides, in particular, \( \beta \)-LPH which has insignificant opioid activity (Li 1964). Isolation of \( \beta \)-endorphin by chromatography has been used to reduce the interference of other peptides (Stenman et al. 1984).

Surprisingly few investigators have combined an appropriate method for blood collection with an extraction and chromatographic procedure (Fraioli et al 1980). In the absence of chromatography and the use of an antisera that cross-reacts 100% with \( \beta \)-LPH, a number of investigators have measured \( \beta \)-endorphin immunoreactivity, i.e., \( \beta \)-endorphin + \( \beta \)-LPH and their results may be suspect (Bullen et al 1984; Carr et al 1981; Farrell et al. 1982; Vrbicky et al. 1982). It is apparent that few investigators have satisfied the criteria of a good assay
and their results are difficult to interpret.

1.13 RATIONALE AND STATEMENT OF AIMS

The cause of menstrual dysfunction associated with vigorous exercise is as yet undetermined. Although a number of predisposing factors have been identified, the underlying mechanism(s) are not clear. The discovery of $\beta$-endorphin, an endogenous opioid peptide that was shown to inhibit LH release during the menstrual cycle and to become elevated during intense exercise, led to the postulate that this peptide may play a role in exercise-induced amenorrhea. However, present research in this area is limited.

Most evidence supporting an endocrine function for $\beta$-endorphin has been derived from indirect methods and few direct assessments have been made. The pattern of plasma $\beta$-endorphin throughout the normal menstrual cycle is poorly defined, hence results are difficult to interpret. Similarly, plasma $\beta$-endorphin has not been well-characterized in exercising women with normal or abnormal menstrual cycles. Close examination of the literature reveal a wide array of findings that may be due to inadequate $\beta$-endorphin RIA methods.

The purpose of this study was to provide greater insight into the relationship between plasma $\beta$-endorphin, the normal menstrual cycle and exercising women. The working hypothesis for this thesis was that plasma $\beta$-endorphin concentrations are elevated in vigorously exercising women with amenorrhea. In order to test this hypothesis, the following lines of investigation were undertaken:

1. To develop a sensitive, specific and precise RIA to measure $\beta$-endorphin in plasma.
2. To study the effect of exercise on plasma $\beta$-endorphin concentrations in amenorrheic subjects and those with regular menstrual cycles.

Specific questions examined were:

1. Are plasma $\beta$-endorphin concentrations constant or variable in non-exercising women throughout the menstrual cycle?

2. Do subjects who exercise vigorously have different concentrations of plasma $\beta$-endorphin throughout the menstrual cycle?

3. What are plasma $\beta$-endorphin concentrations in exercising women with amenorrhea and are they different from sedentary or trained menstruating subjects?

4. Is plasma $\beta$-endorphin released in a constant or variable (pulsatile) pattern in non-exercising and exercising normal menstruating women and in exercising women with amenorrhea?

Resting plasma $\beta$-endorphin concentrations were measured in non-exercising ($n=10$) and exercising ($n=11$) women during a regular menstrual cycle and in a group of exercising women with amenorrhea ($n=11$). Blood samples were obtained 3 times/week for the duration of one menstrual cycle beginning on day 1 or 2 of the cycle and continuing until day 1 or 2 of the subsequent cycle or for 4 weeks in the amenorrheic subjects. Measurements were made of plasma $\beta$-endorphin, estradiol, progesterone, LH and FSH. In addition, plasma $\beta$-endorphin and LH concentrations were measured at 20 minute intervals for 8 hours in these three groups of women ($n=3$/group). Studies were conducted during the mid-follicular and mid-luteal phase of the cycle in ovulatory subjects.
CHAPTER 2

MATERIALS AND METHODS

2:1 MATERIALS

2:1:1 Chemicals: Aprotinin, $\beta_h$-endorphin, florosil (100-200 mesh), silicic acid (100 mesh), talc powder, crude ACTH extract (porcine), Leu-Enk, Met-Enk, $\alpha$-endorphin, $\tau$-endorphin, Dynorphin, $\beta$-Lipotropin (88-91), $\alpha$-MSH (Sigma, St. Louis, MO), talc powder (Fisher Scientific, Pittsburgh, PA), florosil (100-200 mesh), Sephadex G50 Medium gel (Pharmacia, Dorval, Quebec), Bio-Gel P60 Fine gel (Bio-Rad Laboratories, Mississauga, Canada), $\beta_h$-endorphin (Bachem Inc., Torrance, CA), antisera to $\beta_h$-endorphin (Amersham Canada Ltd., Oakville, Ontario and Peninsula Laboratories Ltd., Belmont, CA and UCB Bioproducts, Cedarlane Laboratories Ltd., Hornby, Ontario), Goat-anti-rabbit-$\tau$-globulin (Calbiochem-Behring, Montreal; Quebec), ACTH$_h$(1-39) $\beta$-Lipotropin (1-91) (gift from National Institute of Health).

2:1:2 Isotopes and Iodinating Reagents: $^{125}$I $\beta$-endorphin (New England Nuclear, Dupont, Lachine, Quebec, Specific activity = 2200 Ci/mmmole), (3-$^{125}$Ijodotyrosyl) $\beta$-endorphin (Amersham Canada Ltd, Oakville, Ontario, Specific activity = 2000 Ci/mmmole), $^{3}$H $\beta$-endorphin (gift M. Wilkinson, Specific activity = 47 Ci/mmmole), $^{125}$I Na (New England Nuclear, Dupont, Lachine, Quebec), Iodo-Beads$^{TM}$ (Pierce Chemical Company, Rockford, Illinois).
2:1:3 **Radioimmunoassay Kits:** \( \beta \)-endorphin (Sera\-gen Inc., Boston, MA; Immuno Nuclear Corporation Stillwäter, MINN; New England Nuclear, Lachine, Quebec), LH and FSH (International Diagnostic Services Ltd, Scarborough, Ontario), estradiol and progesterone (Radioassay Systems Laboratories, Inc., Carson, CA).

2:1:4 **Other:** needles, syringes, 13x75 mm vacutainers (silicone-coated interior, 0.05 ml (7.5 mg) of 15% EDTA solution) (Becton-Dickinson, Rutherford, NJ), miniloop intermittent intravenous adapter (Medex Inc., Hilliard, OH), polypropylene tubes for plasma storage and radioimmunoassay: 2.0 ml microtubes, 13x100 mm (Sarstedt Canada Inc., St. Laurent, Quebec) 12x75 mm (Fisher Scientific, Pittsburgh, PA), chromatography columns and cartridges: Econo-Columns, 0.7x20 cm and 0.9x60 cm (Bio-Rad Lab, Mississauga, Ontario), Ultrasphere ODS, 4.6x150 mm and 4.6x250 mm (Beckman Instruments Canada Inc., Toronto, Ontario), Sep PakTM C18 reverse phase cartridges (Waters Associates, Milford, MA).

2:1:5 **Equipment:** Centrifuge (Damon/IEC PR-6000 and IEC B-20), Flash evaporator (Brinkmann Rotavapor-R), Lyophilization (Lab Con Co. Freeze Dry-5), Speed-Vac evaporator (Savant Instruments Inc. Hicksville, NY), \( \tau \)-counter, (Nuclear Chicago Automatic \( \tau \)-counting system 1185 Series and LKB Wallac 1261), RIA evaluation program, 1224 RIA CALC LM (LKB computer software).
2.2 METHODS

2.2.1 Development of a radiolmmunoassay procedure for plasma $\beta$-endorphin

The initial intent of this study was to develop a sensitive, specific and precise RIA to measure plasma $\beta$-endorphin. The following section describes those procedures examined to develop an optimized method which satisfies the criteria of a sound RIA.

2.2.1.1 Plasma Extraction: A number of plasma extraction procedures utilizing powdered adsorbants, Sep Pak™ C$_{18}$ cartridges and solvents were tested for their reproducibility and recovery of $[^{125}I] \beta$-endorphin. Methods requiring readily available materials without demonstrating batch to batch variation were evaluated.

2.2.1.1.1 Powdered adsorbants: Known amounts of radioactivity ($[^{125}I] \beta$-endorphin) were mixed with each milliliter of plasma to be extracted. Fifty to eighty mg of silicic acid, florosil or talc were added to the plasma preparation. The mixture was vortexed for 1-3 minutes and centrifuged at 800 x g for 10 minutes at 2-4°C. The supernatant was decanted and counted for radioactivity. The precipitate was washed with cold distilled deionized water and centrifuged as before. $[^{125}I] \beta$-endorphin was eluted from the adsorbant with 40% acetone in 0.1 N HCl by vortexing 5-15 minutes. The mixture was centrifuged as described and the supernatants were dried under $N_2$ or by flash evaporation. The dried sample was reconstituted in distilled deionized water and counted for radioactivity.
2:2:1.1 ii) Sep Pak™ C18 cartridges: Sep Pak™ C18 cartridges were activated according to manufacturer's instructions (2-20 ml MeOH, 5 ml H2O, 3 ml 0.1% trifluoroacetic acid, TFA). Plasma samples (1-3 ml) were mixed with known amounts of radioactivity ([125I] β-endorphin) and applied to the cartridge at the recommended flow rate (0.5 ml/minute). Cartridges were flushed with 2 ml H2O and samples were eluted with 70% MeOH on 80% acetonitrile in 0.1% TFA. Sep Pak™ eluants were reduced in volume by flash evaporation or by lyophilization, then reconstituted in H2O and counted for radioactivity.

2:2:1.1 iii) Solvent extraction: Plasma samples were combined with known amounts of radioactivity ([125I] β-endorphin). One milliliter of 0.1% acetic acid and 1.65 ml of acetonitrile were added to each ml of plasma. This mixture was vortexed for 20-30 seconds and centrifuged at 4500 x g for 10 minutes at 2-4°C. The supernatant was decanted off and dried by lyophilization or by evaporation using a Speed-Vac Evaporator. The remaining pellet and reconstituted samples were counted for radioactivity.

2:2:1.1 iv) Concentrating plasma extraction supernatants: To determine the optimal method for concentrating plasma extraction supernatants, samples dried by lyophilization, flash evaporator or by a Speed-Vac evaporator were reconstituted and applied to Sephadex G50 Medium gel columns (0.7x20 cm) and eluted with phosphate buffer (PB 0.02M PO4, 0.15M NaCl, 10 mM EDTA, 0.1% gelatin, 0.05% BSA, 0.01% Na-azide, pH 7.5). Fractions were counted for radioactivity.
2:2:1.2 Isolation of β-endorphin by chromatography: The presence of peptides structurally similar to β-endorphin, in particular β-LPH, may cause inaccurate estimations of β-endorphin in the final RIA. Several chromatographic procedures were examined with the intent of developing a reproducible method for isolating β-endorphin while maintaining high recoveries.

2:2:1.2 i) Gel chromatography: Columns containing Sephadex G50 Medium gel or Bio-Gel P60 Fine gel were prepared and packed according to the manufacturer’s instructions. Dry gels were swollen at room temperature for 3-4 hours in PB, degased by vacuum and packed into columns of various size (0.7x20 cm, 0.9x60 cm) and equilibrated with PB at 2-4°C. Void volume (V₀) of the columns was determined with the elution of Dextran Blue 2000. Aliquots of [¹²⁵I] β-LPH or [¹²⁵I] β-endorphin were applied to the top of the gel columns and eluted with PB at a flow rate of 2-3 ml/hr (0.9x60 cm) or 12-15 ml/hr (0.7x20 cm). Fractions of 0.7 ml (0.9x60 cm) or 0.3 ml (0.7x20 cm) were collected and counted for radioactivity. In addition, dried plasma extraction samples containing known amounts of radioactivity ([¹²⁵I] β-LPH or [¹²⁵I]β-endorphin) were reconstituted and applied to the gel columns as described above. Columns were run at 2-4°C or room temperature.

2:2:1.2 iii) High Performance Liquid Chromatography (HPLC): Reversed phase Ultrasphere ODS columns (4.6x150 mm, 4.6x250 mm) were used to isolate β-endorphin. Solvent A consisted of 0.155 M KCl adjusted to pH 2.2 with HCl and Solvent B was acetonitrile. Aliquots of [¹²⁵I] β-endorphin or β-endorphin standard (10-20 μg) were injected into the column. The columns were initially
equilibrated with Solvent A. Increasing concentrations of Solvent B were used to elute \( \beta \text{-endorphin} \) as follows: 0-12% B for 14 minutes, 12-17% B for 20 minutes, 17-26% B for 7 minutes and 26-32% B for 24 minutes at a flow rate of 1.0 ml/hr. Fractions were counted for radioactivity. The 4.6x250 mm column was also eluted with 20-26% B for 10 minutes, 26% B for 10 minutes, 26-32% B for 36 minutes, 32-50% B for 20 minutes at a similar flow rate.

2:2:1.3 Radioimmunoassay Procedures

2:2:1.3.1) Principles of a radioimmunoassay: The purpose of an RIA is to measure the concentration of a particular hormone in an unknown sample by comparison with standard solutions of the hormone. The mechanism of a classical RIA is often described as a competition between a radiolabelled hormone (\( H^* \)) and unlabelled hormone (\( H \)) for binding to a limited amount of antibody (\( Ab \)) (Figure 2.1).

\[
\begin{align*}
\text{\( H^* \)} & \quad \text{\( H \)} \\
\text{\( Ab \)} & \quad \text{\( HAb \)} \\
\text{\( + \)} & \quad \text{\( \leftrightarrow \)} \\
\end{align*}
\]

**Figure 2.1**

In an RIA, constant amounts of antibody and labelled hormone are reacted with a known aliquot of sample containing an unknown concentration of hormone. After equilibrium has been established, the percentage of total label which is bound will be inversely proportional to the amount of unlabelled
hormone present. The greater the amount of unlabelled hormone (H) present, the lower will be the amount of radiolabelled hormone combined to the antibody (*H.Ab). Radioactivity is measured in an RIA, therefore, the bound form of labelled hormone must be separated from that which is free. The amount of unlabelled hormone is expressed as a ratio of bound to free hormone. A standard curve, constructed by reacting increasing amounts of unlabelled hormone with fixed amounts of labelled hormone and antibody, permits the amount of hormone in unknown samples to be determined (Yalow & Berson 1960) (Figure 3.10). Determining the concentration of hormone in the unknown sample, the following equation is used:

\[
y = \frac{B - N}{B_0 - N} \times 100
\]

where "y" is the percent bound tracer in the unknown sample, "B" is the counts associated with the standard solution, "B_0" is the counts associated with the zero standard and "N" is the counts associated with the non-specific binding. "Y" is then compared to the standard curve and a corresponding concentration of hormone is determined as seen in Figure 3.10.

2:2:1.3 ii) Radioimmunoassay Kits: Kits were examined for purity and sensitivity. Pooled, blank plasma samples were spiked with varying amounts of β-endorphin (10-100 pg/ml) and processed according to manufacturer's instructions.
2:2:1.3 iii) Iodinations and Isotopes:

a) Chloramine-T. β-endorphin was iodinated by the Hunter and Greenwood method (1962). β-endorphin (2.5 μg) was mixed with Chloramine-T (50 μg) and Na[125I] for 30–45 seconds. The reaction was stopped with Na2-metabisulphite (100 μg). Iodinated β-endorphin was purified on Sephadex G50 Medium gel columns (0.7x20 cm), eluted with PB.

b) Iodo-Beads®. β-endorphin was iodinated with Iodo-Beads®, polystyrene beads covalently modified with an oxidizing agent (N-Chloro-benzenesulfonamide). Iodo-Beads® were washed with PB, dried on filter paper, added to a Na[125I] solution, capped and incubated for 5 minutes. β-endorphin (2.5 μg dissolved in 25 μl PB) was added to the Na[125I] solution and allowed to stand for 10 minutes. The reaction was stopped by pipetting the reaction mixture away from the beads. Iodinated β-endorphin was purified as described above. Specific activity of iodinated tracers were determined (dpm/M).

c) Commercially prepared isotopes: [125I] β-endorphin from Amersham and New England–Nuclear and [3H] β-endorphin from M. Wilkinson were applied to Sephadex G50 Medium gel columns (0.7x20 cm) and eluted as described above. [3H] β-endorphin was also analyzed on an HPLC Ultrasphere ODS (4.6 x 150 mm) column. Columns were eluted as described in section 2:2:1.2 i) and ii).

d) Tracers (iodinated and commercially purchased) were evaluated for binding with β-endorphin antiserum and the resulting displacement with increasing amounts of unlabeled β-endorphin was determined.
2.2:1.3 iv) Antisera to β-endorphin: Antibodies to β-endorphin were raised in rabbits using two methods.

1) Rabbits were injected in the dermis of the back at multiple sites with 5 mg crude ACTH (porcine) mixed with Freund's adjuvant (complete). They were boosted with 1.0 mg crude ACTH at monthly intervals. Sera containing antibodies were stored in 500 µl aliquots and kept frozen at -20°C.

2) Rabbits were injected subcutaneously with 100 µg β-endorphin mixed with Freund's adjuvant (complete). Initially they were boosted with 100 µg β-endorphin at biweekly intervals for 6 weeks, then were injected intravenously with 100 µg β-endorphin without Freund's and bled 3 days later to test for antibodies. Sera containing antibodies were stored in 500 µl aliquots and kept frozen at -20°C.

Antisera to β-endorphin from Amersham, Peninsula Laboratories and UCB Bioproducts were prepared according to manufacturer's instructions. Displacement binding curves were constructed using these antisera at various concentrations, radiolabelled β-endorphin and unlabelled β-endorphin (0-1000 pg).

2.2:1.3 v) Optimized β-endorphin radiolmmunoassay:

a) Extraction of plasma: Plasma samples (2 ml) were thawed once for analysis. One milliliter of 0.1 M acetic acid and 1.65 ml acetonitrile was added to each milliliter of plasma in a 13x100 polypropylene tube. Radiolabelled β-endorphin ([125I] β-endorphin) was added to each tube for purposes of calculating recovery (2500-3000 cpm/tube). The mixture was vortexed for 10-15 seconds and centrifuged at 4500 x g for 10 minutes at 2-4°C. The supernatant was decanted into a 13x100 polypropylene tube and evaporated to dryness.
b) Gel Chromatography: Dried plasma extracts were reconstituted in 400 µl distilled, deionized water. Sephadex G50 Medium gel columns were equilibrated at room temperature with PB. Dissolved plasma extraction samples were applied to the top of the gel bed, followed by a 400 µl rinse of the 13x100 polypropylene tube the sample was contained in. Samples were eluted with PB at a flow rate of 700-750 µl/minute. Column eluant containing β-endorphin (3-8 ml) was collected in a single 13x100 polypropylene tube and evaporated to dryness.

c) Radioimmunoassay: Dried column eluants were reconstituted in 400 µl distilled, deionized water. Day I, duplicates of 150 µl of samples were added to 12x75 mm polypropylene tubes. Unlabelled β-endorphin (100 µl) was made up at various dilutions (1.0-50 pg) to make up the standard curve. All samples and standard curve tubes had 20 µl of β-endorphin antisera added to them. The volume of each tube was adjusted with PB to 200 µl, vortexed and refrigerated for 24 hours. Day II, 100 µl of [125I] β-endorphin was added to all tubes (10,000-12,000 cpm), vortexed and refrigerated for 24 hours. Day III, a second antibody precipitation method was used to separate bound from free β-endorphin. Goat-anti-rabbit-γ-globulin (100 µl) and 200 µl 2% normal rabbit serum was added to all tubes (except Total tubes), vortexed and allowed to stand at room temperature for 3 hours. Tubes were centrifuged at 800 x g for 45 minutes at 2-4°C. Supernatant was aspirated and pellets counted in a multi-well γ-counter. Calculations for unknown samples were corrected for losses incurred during extraction and chromatography.
Characteristics of the β-endorphin radioimmunoassay (sensitivity, specificity and precision): Sensitivity: To determine the minimal detection limit of the assay, pooled blank plasma spiked with varying amounts of unlabelled β-endorphin (2.5-1000 pg/ml) was processed through the entire assay procedure (extraction, chromatography and final RIA). Samples were set up in multiples of 5 or 10 and analyzed in duplicate in the final assay. Final concentrations (determined automatically by RIA CALC LM, RIA evaluation program, see Section 2:2:1.3) were corrected for losses and the least concentration of unlabelled β-endorphin that could be distinguished from a sample containing no unlabelled ligand was measured.

Specificity: Interference by identifiable peptides which are physiocochemically similar to β-endorphin and may thus react directly with the antisera was assessed. The amount of cross-reactivity between antisera raised against crude ACTH and purchased from UCB Bioproducts was measured with the following peptides: α-endorphin, τ-endorphin, Met-Enk, Leu-Enk, α-MSH, dynorphin, β-LPH (88-91), ACTH₉ (1-39), and β₉-LPH (1-91).

Precision: The variation observed between repeated determinations on the same sample (reproducibility of the assay) was measured. Pooled blank plasma samples, spiked with varying amounts of unlabelled β-endorphin (2.5-1000 pg/ml), were assayed. Samples were set up in multiples of 5 or 10 and analyzed in duplicate in the final assay. Weekly samples obtained from male volunteers (n=5) were measured in triplicate as another means of observing reproducibility of the assay. Final concentrations were corrected for losses and within (intra-) and between (inter-) assay variation was assessed as the coefficient of variation (CV) of duplicate samples.
Coefficient of variation for duplicate samples = \[
\sqrt{\frac{\sum \left( \frac{d}{x} \times 100 \right)^2}{2N}}
\]

where "d" is the difference between duplicate estimates, "x" is the mean of duplicate samples and "N" is the number of duplicate estimates. Between assay variation was assessed as a coefficient of variation (CV) where the mean of quality control values (\( \bar{x} \)) is divided into the standard deviation (S.D.) and expressed as a percentage.

2.2.2 Menstrual Cycle Study

The intent of this study was to determine the levels of plasma \( \beta \)-endorphin during the menstrual cycle in non-exercising and exercising females and exercising females with amenorrhea.

2.2.2.1 Protocol: A total of 32 subjects (19-38 years) took part in this study. All subjects were free from use of oral contraceptives or other medication known to interfere with menstrual regulation. Subjects were selected on a volunteer basis and interviewed for identification of selection criteria. Subjects were divided into 3 groups: Group A (eumenorrheic untrained) consisting of 10 subjects who reported 9 or more menses/year and did not participate in any formal exercise or activity program; Group B (eumenorrheic trained) consisting of 11 subjects who reported 9 or more menses/year and trained 6 or more
times/week and Group C (amenorrheic trained) consisting of 11 subjects who reported no menses in the last six months and trained 6 or more times/week. Trained subjects were members of varsity track, basketball or gymnastic teams and considered to be subjected to similar training intensities.

After explaining the purpose and procedures of the study and obtaining informed consent age, height, weight and menstrual history were recorded for each subject. Maximum oxygen uptake and percent body fat was determined and a diet questionnaire was administered to all subjects. Resting blood samples were drawn from subjects in Group A and B, Monday, Wednesday, Friday mornings between 8-10 a.m., commencing on day 1 or 2 of the menstrual cycle and continuing until day 1 or 2 of the subsequent cycle. For the training subjects, at least 12 hours had elapsed since completion of their last exercise bout. Cycles were confirmed to be ovulatory with luteal phase progesterone measurements. Blood samples were drawn from subjects in Group C as described above for a 4 week period. All samples were analyzed for β-endorphin, LH, FSH, estradiol and progesterone.

2:2:2.2 Blood Sampling: Twenty milliliters of venous blood was collected in 13x75 mm vacutainers and immediately placed on ice. Aprotonin (1000 KIU/ml blood) was added to each vacutainer, inverted 3 times gently to mix. Blood samples were centrifuged at 800 x g for 15 minutes at 2-4°C. Plasma aliquots were stored in 12x75 mm polypropylene tubes and frozen at -70°C until ready for analysis.
2:2:2.3 Hormonal Measurements:

a) Plasma $\beta$-endorphin: Samples from each subject were analyzed within the same assay as described in Section 2:2:1.3. The mean recovery of $\beta$-endorphin following extraction and chromatography was 78%. Within assay variation was 8% and between assay variation was 10%.

b) Plasma LH and FSH:
Gonadotropins were measured according to the RIA kit instructions provided by the manufacturers. All samples were analyzed in duplicate within the same assay. Within assay variation was 8% and 11% for LH and FSH, respectively.

c) Plasma estradiol and progesterone: Steroid hormones were measured according to the RIA kit instructions provided by the manufacturers. All samples were analyzed in duplicate within the same assay. Within assay variation was 10% and 11% for estradiol and progesterone, respectively.

2:2:2.4 Maximum oxygen uptake: Maximum oxygen uptake ($\dot{V}O_2_{max}$) was determined by the open circuit method during progressive exercise to exhaustion on a cycle ergometer at a time removed from blood sampling. The subject began exercising (Jaeger Ergotest, speed independent) at 50 watts and the power output was increased by 30-45 watts every 2 minutes until exhaustion (Thoden et al 1982). Heart rate was monitored continuously with a modified $V_s$ lead. Volume of expired gas delivered to a 6 litre gas mixing chamber was measured by a Validyne pneumotach. Expired gas concentrations were measured by means of a Rapox $O_2$ analyzer and a Hewlett-Packard $CO_2$ analyzer.
2:2:2.5 Percent body fat:

Percent body fat was estimated for each subject based on subcutaneous fat measures at 7 sites. Each site was measured in triplicate and the mean values were used to derive percent body fat (Yuhasz 1962).

2:2:3 Eight Hour Study

The intent of this study was to determine if β-endorphin is secreted in a pulsatile manner, similar to other hormones such as LH. In addition, the intent was to examine the pulsatile pattern of β-endorphin secretion in sedentary and trained females during the menstrual cycle and in amenorrheic trained females.

2:2:3.1 Protocol: Nine women participated in this second study. Three subjects were untrained eumenorrheic (Group A), 3 were trained eumenorrheic (Group B) and 3 were trained amenorrheic (Group C). Following a detailed explanation of the purpose of the study and its associated risks, informed consent was given by all subjects.

Resting blood samples were drawn from subjects by means of an indwelling venous catheter every 20 minutes for 8 hours. Subjects were ambulatory during the day, however, no exercise was permitted. Eating was not prohibited. Eumenorrheic subjects from Group A and B were sampled once during the follicular phase (day 9-11) and once during the luteal phase (day 23-25). Amenorrheic subjects from Group C were sampled at a time of their convenience. All samples were measured for β-endorphin and LH concentrations.
2:2:3.2 Blood Sampling: Blood was sampled through an 18 gauge teflon catheter which was inserted into an antecubital vein. An intravenous maintenance minilooop was attached to the catheter and taped to the forearm for ease of sampling. The catheter was kept patent with a 1-2 ml flush of isotonic saline-heparin (100 U Hepalen/ml of 0.9% sodium chloride). Indwelling catheters were inserted between 8:00 a.m. and 8:30 a.m. at which time a baseline sample was taken. Ten milliliter blood samples were drawn by plastic syringe every 20 minutes for 8 hours between 8:30 a.m. and 4:30 p.m. Blood was transferred immediately to chilled, 12x75 mm vacutainers containing Aprotonin (1000 KIU/ml blood). Vacutainers were capped and inverted three times gently to mix. Blood samples were centrifuged. Plasma aliquots (2-3 ml) were stored in 12x75 mm polypropylene tubes and frozen at -70°C until ready for analysis of β-endorphin and LH as described above.

2:2:4 Analysis of the Data

The questions being investigated will be restated and the analyses associated with each will be discussed. A description of results will be given for all questions followed by an appropriate statistical analysis, when necessary.

2:2:4.1 Questions regarding the Menstrual Cycle

1. Are plasma β-endorphin concentrations constant or variable in non-exercising women throughout the menstrual cycle (Group A)? Method: Increments of β-endorphin were identified throughout the menstrual cycle by means of the criteria developed by Ross et al (1983). This method is a modified version of Santen and Bardin (1973) pulse analysis used to discriminate
increments of LH above background noise of the assay. It selects criteria that would identify peaks reliably at all peptide levels. Briefly, all nadirs are identified. The concentration at the nadir was multiplied by 3.5 CV (criterion for an increment) to obtain the corresponding increment in hormone concentration. The value of 3.5 CV was the minimum factor determined to eliminate background noise of the assay and reduce the number of false peaks detected. An increment was defined as a rise in hormone concentration, relative to the preceding nadir, that exceeded this elevation. The dose-dependent intraassay CV applied to the menstrual cycle increment analysis was calculated from the CV of duplicates of the standard curve. Once increments were identified, a further analysis was applied to determine the significance of these increments. This was done by Student’s t-test (paired) whereby the concentration of β-endorphin at the nadir of a specific increment, occurring at a particular phase of the menstrual cycle, was compared with the concentration of β-endorphin at the peak of the same increment.

2. Do subjects who exercise vigorously have different concentrations of plasma β-endorphin throughout the menstrual cycle (Group B)?

Method: Increments of β-endorphin were determined as described above and the significance of these β-endorphin increments was determined by Student’s t-test (paired) as described above. Amplitude of the β-endorphin increments (peaks) at distinct times of the menstrual cycle was analyzed by Student’s t-test (unpaired) to determine significant differences between Group A and Group B.

3. What are plasma β-endorphin concentrations in exercising women with amenorrhea (Group C) and are they different from sedentary or trained menstruating subjects?
Method: Increments of $\beta$-endorphin were determined by the criteria of Ross et al (1983) and significance of these increments was determined by Student's t-test (paired) as described above. Amplitude of the $\beta$-endorphin peaks was analyzed by Student's t-test (unpaired) to determine significant differences between Group C with Group A and Group C with Group B.

2:2:4.2 Questions regarding the Eight Hour Study

1. Is plasma $\beta$-endorphin released in a constant or variable (pulsatile) pattern in non-exercising (Group A) and exercising (Group B), normal menstruating women and in exercising women with amenorrhea (Group C)?

Method: Pulse analysis, as previously described, was used to measure the pulsatile pattern of $\beta$-endorphin release. Subject numbers were small, therefore, no statistical analysis was performed on this portion of the data.
CHAPTER THREE

RESULTS

3:1 DEVELOPMENT OF THE RADIOIMMUNOASSAY PROCEDURE FOR PLASMA β-ENDORPHIN

3:1:1 Plasma Extraction: A number of plasma extraction methods were tested for reproducibility and efficiency of recovery using [¹²⁵I] β-endorphin as a marker.

3:1:1.1 Comparison of methods: Methods employing powdered adsorbants (silicic acid, Florosil and talc) resulted in repeatedly poor recoveries of labelled β-endorphin (18.5-43.8%) (Table 3.1). Silicic acid purchased from different sources demonstrated inconsistent and variable recoveries. Increasing the number of washes of silicic acid with 40% acetone in 0.1 N HCl, in an attempt to elute labelled β-endorphin, resulted in an approximate 10% increase in recovery (52.8%) but a wide range of recoveries were still apparent (45.4-56.8%) and the overall recovery was still low.

Results using Sep Pak™ C₁₈ cartridges for plasma extraction approached 80% recovery but batch-to-batch variation was quite high. Retention of the sample by the active surface of the packing in the cartridge was not consistent. Modifications in the activation procedures did not overcome these problems. However, no significant tracer breakdown occurs during this extraction. Chromatographic analysis of [¹²⁵I] β-endorphin following a Sep Pak extraction resulted in elution of a single major peak of radioactivity (Figure 3.1) corresponding in mobility of authentic β-endorphin. The solvent extraction method using 0.1M acetic acid and acetonitrile resulted in
<table>
<thead>
<tr>
<th>Plasma Extraction Methods</th>
<th>Number of trials</th>
<th>Recovery (% ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Powdered Adsorbants (source)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i) Talc (Sigma)</td>
<td>12</td>
<td>32.3 ± 6.6</td>
</tr>
<tr>
<td>ii) Talc (Fisher)</td>
<td>12</td>
<td>23.6 ± 1.5</td>
</tr>
<tr>
<td>iii) Florosil (Sigma)</td>
<td>12</td>
<td>18.5 ± 3.9</td>
</tr>
<tr>
<td>iv) Silicic Acid (Sigma)</td>
<td>15</td>
<td>35.5 ± 4.0</td>
</tr>
<tr>
<td>v) Silicic Acid (Fisher)</td>
<td>15</td>
<td>43.8 ± 11.6</td>
</tr>
<tr>
<td>Sep PAK C18 Cartridges (70% MeOH or 80% acetonitrile in 0.1% TFA)</td>
<td>10</td>
<td>61.6 ± 8.6</td>
</tr>
<tr>
<td>Solvent system (0.1M acetic acid + acetonitrile)</td>
<td>12</td>
<td>95.1 ± 1.2</td>
</tr>
</tbody>
</table>
Figure 3.1 Chromatographic analysis of $[^{125}I] \beta$-endorphin following extraction on Sep Pak$^\text{TM}$ C$_{18}$ cartridge on a 0.7 x 20 cm Sephadex G50 Medium gel column. Column recovery was 96%.
consistently high recoveries of radiolabelled \( \beta \)-endorphin (Table 3.1) and chromatographic analysis confirmed that this method of extraction did not alter \( \beta \)-endorphin (Figure 3.2).

3:1:1.2 Concentrating large volumes: Methods of reducing large volumes from plasma extractions were compared for their effects on the stability and recovery of \(^{125}\text{I}\) \( \beta \)-endorphin. Tracer alone (Control) applied to a Sephadex G50 Medium gel column resulted in a single, sharp peak (93% recovery) but concentration by flash evaporation resulted in a different profile (Figure 3.3). Damage to \( \beta \)-endorphin was apparent since the major peak represented only 66% of total radioactivity added to the plasma prior to extraction while the peak at Fraction 20-25 represented 27% of this total. Lyophilization resulted in an 87% recovery from the column with no apparent breakdown, however, residues reconstituted after this process were difficult to dissolve and resulted in varying flow rates which shifted the peak of \( \beta \)-endorphin to the right (Figure 3.3). Concentrating plasma extractions by Speed/Vac Evaporator resulted in 91% recovery of total radioactivity added to the initial sample and an elution profile similar to that of the tracer added alone (Control) (Figure 3.3).

3:1:2 Isolation of \( \beta \)-endorphin by Chromatography

Isolating \( \beta \)-endorphin and \( \beta \)-LPH by gel chromatography and HPLC was attempted. Aliquots of radioactive \( \beta \)-endorphin and \( \beta \)-LPH were applied individually to Sephadex G50 Medium gel columns (0.9 x 60 cm). Elution profiles reveal two distinct peaks (Figure 3.4). These profiles were reproducible when a combination of radioactive \( \beta \)-endorphin and \( \beta \)-LPH were applied to the
Figure 3.2 Chromatographic analysis of $^{125}$Iβ-endorphin added to plasma and extracted with 0.1M acetic acid and acetonitrile on a 0.7 x 20 cm Sephadex G50 Medium gel column. Column recovery was 93%.
Figure 3.3 Chromatographic analysis of $^{125}$I-endorphin added to plasma extraction supernatants and dried by various methods on a 0.7 x 20 cm Sephadex G50 Medium gel column. CTL=Control, FL EVAP=Flash Evaporator, LYOPH=lyophilization, S/V EVAP=Speed/Vac Evaporator.
Figure 3.4 Chromatographic analysis of $^{125}\text{I}$$\beta$-endorphin and $^{125}\text{I}$$\beta$-LPH on a 0.9 x 60 cm Sephadex G50 Medium gel column. Column recoveries for $\beta$-endorphin and $\beta$-LPH were 91% and 93%, respectively.
same gel column. Shorter columns (0.7 x 20 cm) packed with Sephadex G50
Medium gel were equally effective at isolating β-endorphin.

Chromatographic isolation of β-endorphin with HPLC was not successful.
Although ([125I]) β-endorphin eluted in a sharp peak from the Ultrasphere ODS
reverse phase column, increasing amounts of unlabelled β-endorphin (10-20 μg)
was never consistently detected with the solvent system used.

3:2

RADIOIMMUNOASSAY PROCEDURES

β:2:1 Radi immunoassay Kits

Plasma β-endorphin RIA kits were purchased from three manufacturers
and tested for sensitivity and reproducibility. Plasma extraction by affinity gel-
chromatography (Immuno Nuclear) resulted in variable recoveries. Results with
pooled plasma spiked with known amounts of unlabelled β-endorphin showed that
this method was incapable of detecting concentrations of β-endorphin below 20
pg/ml.

Silicic acid extraction (Seragen) has previously been described as
yielding poor and inconsistent recoveries. Final concentration determinations
with this kit were unable to detect levels of 50 pg/ml or less.

A direct serum assay was used with the β-endorphin kit from New
England Nuclear. A specific extraction procedure was not suggested and no
chromatography was applied despite an antisera displaying 50% cross-reactivity
with β-LPH. A charcoal method was used to separate bound from free ([125I]β-
endorphin but results indicated charcoal to be poor at absorbing free radioactive
β-endorphin leading to poor reproducibility.
3:2:2 **Iodinations and Isotopes**

Iodination methods using Chloramine-T or Iodo-Beads™ resulted in poor incorporation of $[^{125}I]$ into $\beta$-endorphin and specific activities were determined to be less than 10 Ci/m mole. Evaluation of $[^3H] \beta$-endorphin on Sephadex G50 Medium gel columns resulted in a single peak comparable to that seen with $[^{125}I] \beta$-endorphin (Figure 3.5). However, evaluation of $[^3H] \beta$-endorphin on HPLC column indicated the presence of a number of impurities. Chromatographic analysis of $[^{125}I] \beta$-endorphin from Amersham resulted in variable elution patterns (Figure 3.6). Under identical conditions the resulting peaks of two trials are vastly different. Chromatographic analysis of $[^{125}I] \beta$-endorphin from New England Nuclear on both gel (Figure 3.5) and HPLC columns resulted in single peaks of radioactivity corresponding to $\beta$-endorphin.

Displacement curves were used to compare binding sensitivities between $[^{125}I] \beta$-endorphin (Iodo-Bead™ method and NEN) and $[^3H] \beta$-endorphin (Figure 3.7). $[^{125}I] \beta$-endorphin prepared by the Iodo-Bead™ method was the least sensitive to changes in increasing concentrations of $\beta$-endorphin. $[^3H] \beta$-endorphin was moderately sensitive but $[^{125}I] \beta$-endorphin from NEN was the most sensitive to changes in $\beta$-endorphin concentration. Thus at 50% binding levels, the sensitivity of the NEN, $[^3H] \beta$-endorphin and Iodo-Bead™ tracers were 37, 320 and 2000 pg. respectively.

3:2:3 **Antisera to $\beta$-endorphin**

Rabbits immunized against crude ACTH showed high binding titers after 7 months of boosting. Final dilutions used in binding curves were 1:7500 or 1:15,000 resulting in a zero binding of 55% and 40%, respectively (Figures 3.8A
Figure 3.5 Chromatographic analysis of $[^3\text{H}]\beta$-endorphin and $[^{125}\text{I}]\beta$-endorphin on a 0.7 x 20 cm Sephadex G50 Medium gel column. Column recoveries for $[^3\text{H}]\beta$-endorphin and $[^{125}\text{I}]\beta$-endorphin were 92% and 91%, respectively.
Figure 3.6 Chromatographic analysis of $[^{125}]$β-endorphin (Amersham) on a 0.7 x 20 cm Sephadex G50 Medium gel column. Column recoveries for Trial 1 and 2 were 74% and 68%, respectively.
Figure 3.7 Radiolabelled \( \beta \)-endorphin of different sources was used to generate standard curves. The antiserum used was raised against crude ACTH (final dilution 1:7500). Iodo-Bead \( ^{125}I \)\( \beta \)-endorphin (\( \square \)), \( ^{3}H \)\( \beta \)-endorphin (\( \Diamond \)), New England Nuclear \( ^{125}I \)\( \beta \)-endorphin (\( \times \)).
Figure 3.8 Radiolabelled β-endorphin was used for the generation of standard curves with an antiserum raised against crude ACTH. Final dilution 1:7500 (A), 1:15,000 (B).
and 3.8B). Rabbits being immunized against β-endorphin were discontinued after 5 months of little improvement in binding titers.

Commercially purchased antisera from Amersham and Peninsula Laboratories, regardless of concentration used, displayed no binding with unlabelled and labelled β-endorphin, however, antisera from UCB Bioproducts displayed high affinity for unlabelled β-endorphin at low dilutions (Figure 3.9). Dilutions of 1:50,000, 1:100,000 and 1:250,000 were tested for sensitivity with unlabelled β-endorphin. A final dilution of 1:250,000 was highly sensitive for detecting changes in β-endorphin concentration between 5 and 20 pg (Figure 3.10).

3:2:4 Characteristics of the β-endorphin Radioimmunassay

Sensitivity: The least concentration of β-endorphin that could be distinguished from a sample containing no β-endorphin was 4 pg/ml.

Specificity: The antisera raised against crude ACTH had 10% cross-reactivity with α- and τ-endorphin (Figure 3.11B) and greater than 100% cross-reactivity with β-LPH (Figure 3.11A) on a molar basis. No cross-reactivity (0%) with the other peptides (Leu-Enk, Met-Enk, dynorphin, β-LPH 88-91, α-MSH, ACTH₁₋₃₉) was observed. The antisera purchased from UCB Bioproducts had 20% cross-reactivity with β-LPH (Figure 3.12) on a molar basis and 0% with all other peptides measured (Leu-Enk, Met-Enk, dynorphin, β-LPH 88-91, α-MSH, α-endorphin, τ-endorphin, ACTH₁₋₃₉).

Precision: Within (intra-) assay variation was 8% and between (inter-) assay variation was 12%. Ten spiked samples of each concentration of β-endorphin were assessed by RIA and results are plotted in Figure 3.13. This
Figure 3.9 Radiolabelled β-endorphin was used for the generation of standard curves with the antiserum from UCB Bioproducts. Final dilution 1:50,000 (□), 1:100,000 (△), 1:250,000 (×).
Figure 3.10 Example of a β-endorphin standard curve generated with $[^{125}I]$$\beta$-endorphin. The percentage of tracer bound is approximately 50%; this corresponds to a value of 10 pg for the standards and this is therefore the concentration in the unknown.
Figure 3.11 Standard curves generated with $^{125}$I-β-endorphin and antiserum raised against crude ACTH (final dilution 1:7500). Cross-reactivity of β-LPH (A) with the antiserum was >100% while α- and γ-endorphin cross-reacted 10% on a molar basis (B).
Figure 3.12 Standard curves generated with $[^{125}I]$-endorphin and antiserum from UCB Bioproducts (final dilution 1:250,000). Cross-reactivity of $\beta$-LPH with the antiserum was 20% on a molar basis.
Figure 3.13 Precision of the β-endorphin radioimmunoassay. Each square represents the mean of 10 samples performed within the same assay (mean ± S.E.M.). r=0.98, p<0.01
figure illustrates the relationship between the amount of \( \beta \)-endorphin added to plasma and the amount detected at the end of the assay. Each point on the line represents the mean of 10 samples analyzed within the same assay. Reproducibility of the RIA was high for varying concentrations of \( \beta \)-endorphin \((r=0.98, \ p<0.01)\). Samples obtained from male volunteers were in the range of 10.6-21.6 pg/ml. Weekly variation of plasma \( \beta \)-endorphin within the same subject was less than 10%.

3:3  **MENSTRUAL CYCLE STUDY**

3:3:1  **General**

The physical characteristics for Groups A (normal, menstruating, non-exercising), B (normal menstruating, exercising) and C (amenorrheic, exercising) are listed in Table 3.2. The ages were 31.3 ± 2.3 years (mean ± S.E.M.), 25.1 ± 1.8 and 23.2 ± 1.5 for Groups A, B and C, respectively. There was no significant difference between the ages of Groups A and B or Groups B and C but Group C was significantly younger than Group A \( (p<0.01) \). There was no significant difference for height and weight among the three groups. Percent body fat was 24.4 ± 2.9% (mean ± S.E.M.), 17.7 ± 2.2 and 18.2 ± 1.7 for groups A, B and C, respectively, but these differences were not statistically significant. Age of menarche was 13.2 ± 0.3 years (mean ± S.E.M.), 13.6 ± 0.4 and 14.6 ± 0.5 for groups A, B and C, respectively. There was no significant difference between the age of menarche for Groups A and B or Groups B and C but Group C had a significantly older age of menarche than Group A \( (p<0.02) \). The length of the menstrual cycle for Group A \( (29.1 ± 1.2 \text{ days}) \) was not significantly different from Group B \( (30.6 ± 1.5 \text{ days}) \). No differences existed
<table>
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<th>GROUP</th>
<th>AGE (YRS)</th>
<th>HEIGHT (CM)</th>
<th>WEIGHT (KG)</th>
<th>AGE OF MENARCHE (YRS)</th>
<th>CYCLE LENGTH (DAYS)</th>
<th>TRAINING [TIMES/WK]</th>
<th>LENGTH OF ANEMORRHEA (YRS)</th>
<th>YEARS OF TRAINING</th>
<th>PERCENT BODY FAT (%)</th>
<th>MAXIMUM OXYGEN UPTAKE (ML/KG/MIN)</th>
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<td>21.3 (a)</td>
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<td>0</td>
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<td>(44.5-67.5)</td>
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<td>17.7</td>
<td>47.5 (c)</td>
</tr>
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<td>(26-39)</td>
<td>(6-9)</td>
<td>(2-16)</td>
<td>[1.3]</td>
<td>[2.2]</td>
<td>(40-61.4)</td>
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<td>C</td>
<td>25.2</td>
<td>165.5</td>
<td>61.1</td>
<td>14.6 (b)</td>
<td>N/A</td>
<td>7.7 (c)</td>
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<td>42.2 (c)</td>
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<td>(1-8)</td>
<td>(2-11)</td>
<td>(11.5-32.5)</td>
<td>[2.0]</td>
<td>(33.1-54.5)</td>
</tr>
</tbody>
</table>

* Mean value, [S.E.M.], (range).

a $p < 0.01$ (A>C)
b $p < 0.02$ (C>A)
c $p < 0.00001$ (B & C>A)
regarding training histories for Groups B and C (frequency of training and total years of training). Maximum oxygen uptake (ml/kg/min) was significantly higher in Groups B and C compared to A (p<0.00001) but no difference was observed between Groups B and C. Results from the diet questionnaire did not indicate any unusual nutritional practices for any subject. All subjects reported balanced diets and all subjects ate dairy and meat products.

3.3.2 **Plasma β-endorphin concentrations throughout the menstrual cycle**

Blood samples were obtained from non-exercising (Group A) and exercising (Group B), normal menstruating subjects 3 times/week for a complete menstrual cycle. The day of the mid-cycle increase of plasma LH was defined as LH 0. Sample points occurring before LH 0 were defined as representing the follicular phase while sample points occurring after LH 0 were defined as representing the luteal phase.

Plasma β-endorphin concentrations were variable throughout the menstrual cycle for Group A (Figure 3.14) or Group B (Figure 3.15). The individual pattern for β-endorphin was highly variable for each subject. Plasma β-endorphin increased from baseline levels at distinct times of the menstrual cycle. Table 3.3 indicates the number of subjects within Group A and B that had β-endorphin increments during the early follicular (EF), late follicular (LF), LH surge, early luteal (EL) and late luteal (LL) phases of the menstrual cycle. The range of days that β-endorphin elevations occurred were similar between Group A and B, except during the LF and EL phase. In Group A, increments of β-endorphin occurred during a limited number of days for the LF (d.-3 to -4)
<table>
<thead>
<tr>
<th>GROUP</th>
<th>EARLY FOLLICULAR</th>
<th>LATE FOLLICULAR</th>
<th>LH SURGE</th>
<th>EARLY LUTEAL</th>
<th>LATE LUTEAL</th>
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<tr>
<td>A (n=10)</td>
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<td>5 [-3 to -4]</td>
<td>5</td>
<td>5 [+1 to +4]</td>
<td>7 [+11 to +14]</td>
</tr>
<tr>
<td>B (n=11)</td>
<td>6 [-12 to -19]</td>
<td>6 [-2 to -7]</td>
<td>5</td>
<td>11 [+2 to +7]</td>
<td>5 [+12 to +14]</td>
</tr>
</tbody>
</table>

*The day of the mid-cycle increase of plasma LH was defined as LH 0. Range of days where peaks observed appear in square brackets.*
Figure 3.14 Plasma β-endorphin concentrations (mean ± S.E.M.) throughout the menstrual cycle for Group A.
Figure 3.15 Plasma β-endorphin concentrations (mean ± S.E.M.) throughout the menstrual cycle for Group B.
and EL (d+i to +4) phase whereas Group B had increments of β-endorphin over a wider range of days during the LF (d.-2 to -7) and EL (d.+2 to +7) phase of the cycle.

The range of β-endorphin concentrations for the follicular phase was 5.6-20 pg/ml for Group A and 7.3-31.9 pg/ml for Group B. The range of β-endorphin concentrations for the luteal phase was 5.4-18.7 pg/ml for Group A and 8.2-28.5 pg/ml for Group B.

Significant β-endorphin increments are illustrated in Figure 3.16 for Group A and Figure 3.17 for Group B. The points where concentrations were significantly higher than the previous nadir are indicated. Group A had significant β-endorphin increments during the EF (p<0.02), EL (p<0.05) and LL phases (p<0.02). Group B had significant β-endorphin increments at the same times as Group A: EF (p<0.003), EL (p<0.00001), LL (p<0.04).

The amplitude of a given phase of the menstrual cycle is defined as the difference between the highest and lowest β-endorphin concentrations measured during that phase. These amplitudes were compared by Student’s t-test (unpaired) for Group A and B. All peak β-endorphin amplitudes are greater in Group B than in Group A (Figure 3.18), however, only those amplitudes at the EF (p<0.01) and EL (p<0.03) phase are statistically significant. Peak amplitudes of β-endorphin for all three groups and number of days trained/week were compared and a significant correlation was observed (p<0.05).
Figure 3.16 The lowest (✓) and highest (◯) concentrations of plasma β-endorphin (mean ± S.E.M.) of peaks observed during distinct times of the menstrual cycle for Group A. EF=early follicular (d.-10 to -6), LF=late follicular (d.-4 to -3), LH surge (d.0), EL=early luteal (d.+1 to +4), LL=late luteal (d.+11 to +14).

* p<0.02    ** p<0.05
Figure 3.17 The lowest (○) and highest (★★) concentrations of plasma β-endorphin (mean ± S.E.M.) of peaks observed during distinct times of the menstrual cycle for Group B. EF=early follicular (d.-19 to -12), LF=late follicular (d.-7 to -2), LH surge (d.0), EL=early luteal (d.+2 to +7), LL=late luteal (+12 to +14).

★★p<0.003 ★★p<0.00001 ★p<0.04
Figure 3.18 The amplitude of β-endorphin peaks (mean ± S.E.M.) are compared between Group A, Group B and Group C. Group B was significantly greater than Group A at EF (*** p<0.01) and EL (**** p<0.03). Group C was significantly greater than Group A at LF (▵ p<0.001) and LL (◆ p<0.03). Group B (EF) was significantly greater than Group C (□ p<0.03).
3:3:3 Plasma β-endorphin concentrations in exercising amenorrheic subjects

Blood samples were obtained from training subjects with amenorrhea (Group C) 3 times/week for 4 weeks. The pattern of plasma β-endorphin concentrations throughout the 4 weeks for Group C is shown in Figure 3.19. The range of plasma β-endorphin concentrations for Group C was 6.9-39.1 pg/ml. These concentrations were not constant throughout the 4 weeks for most subjects (8/11). The individual pattern of β-endorphin was highly variable for 8 subjects while 3 subjects had β-endorphin patterns displaying little variation throughout the sampling time (6.9-11.7 pg/ml). The 8 subjects had large variations marked by greater β-endorphin elevations from baseline (7.4-39.1 pg/ml). The increments of β-endorphin observed during the 4 weeks were not significant (Figure 3.20).

The amplitude of β-endorphin increments (mean of all increments) in Group C was compared to the amplitudes in Group A and Group B (Figure 3.18). The amplitude for Group C was significantly greater than the LF (p<0.001) and LL (p<0.03) amplitude for Group A and significantly less than the EF (p<0.03) amplitude for Group B.

3:3:4 Other Endocrine Measurements

Concentrations of LH, FSH, estradiol and progesterone (mean ± S.E.M.) throughout the menstrual cycle are illustrated in Figure 3.21 for Group A and Figure 3.22 for Group B. Concentrations of LH at the mid-cycle were within the normal ranges observed for ovulatory subjects (30-140 mIU/ml) in both A and B groups. Progesterone measurements confirmed ovulation in all subjects.
Figure 3.19 Plasma β-endorphin concentrations (mean ± S.E.M.) throughout the 4 week sampling period for Group C.
Figure 3.20 The lowest (■) and highest (☑) concentrations of plasma β-endorphin (mean ± S.E.M.) of peaks observed during the 4 week sampling of Group C.
Figure 3.21 Plasma LH, FSH (A), estradiol and progesterone (B) concentrations (mean ± S.E.M.) throughout the menstrual cycle for Group A.
Figure 3.22. Plasma LH, FSH (A), estradiol and progesterone (B) concentrations (mean ± S.E.M.) throughout the menstrual cycle for Group B.
from groups A and B as indicated by the mid-luteal rise in excess of 4 ng/ml. Group B had lower progesterone concentrations throughout the luteal phase than Group A. Estradiol concentrations displayed the expected pattern of a large late follicular rise and a moderate luteal rise in subjects from Group A and B. Concomitant increases of gonadotropin or steroid hormone concentrations and β-endorphin were not apparent at any time of the menstrual cycle except the early luteal phase for Group A and B.

Concentrations of LH, FSH, estradiol and progesterone (mean ± S.E.M.) in Group C confirmed the lack of ovulation in these subjects. Gonadotropin and estradiol levels were low and unchanging at all times of sampling: LH (1.0-18.8 mIU/ml), FSH (5.7-23.4 mIU/ml), estradiol (less than 70 pg/ml). Progesterone concentrations were less than 1.5 ng/ml in all subjects.

3:4  **EIGHT HOUR STUDY**

3:4:1 **General**

The physical characteristics for Groups A, B and C are listed in Table 3.4. The ages were 33.3 ± 1.5 years (mean ± S.E.M.), 32.0 ± 2.1 and 22.7 ± 2.3 for Groups A, B and C, respectively. There was no significant difference between the ages of Group A and B or Group B and C but Group C was significantly younger than Group A (p<0.05). There was no significant difference for height, weight or percent body fat within all three groups. Age of menarche was 12.8 ± 0.6, 13.7 ± 0.3 and 15.0 ± 0.6 years for Groups A, B and C, respectively and no significant difference was found. Length of the menstrual cycle for Group A (28.3 ± 0.9 days) was not significantly different from Group B (31.7 ± 4.3 days). Neither the training frequency nor total years
<table>
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<th>GROUP</th>
<th>AGE (YRS)</th>
<th>HEIGHT (CM)</th>
<th>WEIGHT (KG)</th>
<th>AGE OF MENARCHE (YRS)</th>
<th>CYCLE LENGTH (DAYS)</th>
<th>TRAINING (TIMES/WK)</th>
<th>LENGTH OF AMENORRHEA (YRS)</th>
<th>YEARS OF TRAINING (YRS)</th>
<th>PERCENT BODY FAT (%)</th>
<th>MAXIMUM OXYGEN UPTAKE (ML/KG/MIN)</th>
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<td>(12.3-25.3)</td>
<td>(3.6)</td>
<td>(3.6)</td>
<td>(4.4)</td>
</tr>
<tr>
<td>B</td>
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<td>13.7</td>
<td>11.7</td>
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<td>52.3 (b)</td>
</tr>
<tr>
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<td>(11)</td>
<td>(9.8-16.6)</td>
<td>(2.7)</td>
<td>(2.2)</td>
<td>(4.9)</td>
</tr>
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<td>63.5</td>
<td>15</td>
<td>N/A</td>
<td>8 (b)</td>
<td>4</td>
<td>6</td>
<td>17</td>
<td>57 (b)</td>
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<td>(14-3-20)</td>
<td>(48-63.2)</td>
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* Mean value, [S.E.M.], (Range).

a p < 0.05 (A>C)
b p < 0.05 (B & C>A)
of training was significantly different between Groups B and C. Groups B and C had significantly higher (p < 0.05) maximum oxygen uptakes (ml/kg/min) compared to Group A, but no difference was observed between Groups B and C.

3:4.2 Plasma β-endorphin concentrations during an eight hour period

Blood samples were obtained at 20 minute intervals for 8 hours from Groups A, B and C (n=3/group). The range of β-endorphin concentrations for the follicular phase was 5.5-21.1 pg/ml for Group A and 5.6-15.0 pg/ml for Group B. The range of β-endorphin concentrations for the luteal phase was 4.4-15.3 pg/ml for Group A and 6.4-15.0 pg/ml for Group B. Amenorrheic subjects from Group C had plasma β-endorphin in the range of 6.0-21.0 pg/ml.

Pulse frequency of β-endorphin was found to be variable for each group. Group A had one subject with 5 pulses during the 8 hours (1 pulse/96 min) in both the follicular and luteal phase of the cycle while the two other subjects had 4 pulses (1 pulse/120 min) in the follicular and 3 or 2 pulses (1 pulse/160 min or 1 pulse/240 min) in the luteal phase during the 8 hour study. Plasma β-endorphin concentrations over 8 hours in Subject 1 from Group A is shown in Figure 3.23 as an example. All three subjects in Group B had 4 pulses (1 pulse/120 min) in the follicular and 3 pulses (1 pulse/160 min) in the luteal phase during the 8 hour study. Mean β-endorphin concentrations were similar between the follicular phase and luteal phase of the study in both Group A and B. Plasma β-endorphin concentrations over 8 hours in Subject 4 from Group B is shown in Figure 3.24 as an example. Two amenorrheic subjects from Group C had 4 pulses (1 pulse/120 min) during the 8 hours while the third
Figure 3.23 Plasma β-endorphin concentrations over 8 hours in Subject 1 from Group A. Follicular (d.10) (A), Luteal (d.24) (B). Asterisks (*) indicate a β-endorphin pulse (criterion of Ross et al 1983). Results from Subject 2 and 3 from Group A are in Appendix 1.
Figure 3.24 Plasma β-endorphin concentrations over 8 hours in Subject 4 from Group B. Follicular (d.11) (A), Luteal (d.25) (B). Asterisks (*) indicate a β-endorphin pulse (criterion of Ross et al 1983). Results from Subject 5 and 6 from Group B are in Appendix 2.
subject had 5 pulses (1 pulse/96 min). Plasma $\beta$-endorphin concentrations over 8 hours in Subject 7 from Group C is shown in Figure 3.25 as an example of the pattern seen in these amenorrheic subjects.

Pulse amplitude (mean $\pm$ S.E.M.) of Group A was similar in both the follicular ($2.3 \pm 0.6$ pg/ml) and luteal ($2.3 \pm 0.8$ pg/ml) phase (Figure 3.26). Group B also had similar amplitudes in both the follicular ($3.0 \pm 0.3$ pg/ml) and luteal ($3.0 \pm 0.8$ pg/ml) phase and these were greater than those of Group A (Figure 3.26). The pulse amplitude for Group C ($3.5 \pm 1.1$ pg/ml) was greater than any amplitude of Group A and B but statistical significance was not reached (Figure 3.26).

3:4:3 Plasma LH concentrations during an eight hour period

The range of LH concentrations for the follicular phase was 2.6-20.6 mIU/ml for Group A and 4.7-19.0 mIU/ml for Group B. The range of LH concentrations for the luteal phase was 1.0-12.8 mIU/ml for Group A and 1.3-15.2 mIU/ml for Group B. Amenorrheic subjects from Group C had plasma LH in the range of 2.4-21.9 mIU/ml.

Two subjects from Group A had 5 pulses during the 8 hour period (1 pulse/96 min), and one subject had 4 pulses (1 pulse/120 min) in the follicular phase of the study. All subjects in Group A had 3 pulses/8 hours (1 pulse/160 min) in the luteal phase. Plasma LH concentrations over 8 hours in Subject 1 from Group A is shown in Figure 3.27. During the follicular phase, 2 subjects in Group B had 4 pulses (1 pulse/120 min) and one subject had 2 pulses (1 pulse/240 min). During the luteal phase, 2 subjects from Group B had 3 pulses (1 pulse/160 min) and one subject had 2 pulses (1 pulse/240 min). Plasma LH
Figure 3.25 Plasma β-endorphin concentrations over 8 hours in Subject 7 from Group C. Asterisks (*) indicate a β-endorphin pulse (criterion of Ross et al 1983). Results from Subject 8 and 9 from Group C are in Appendix 3.
Figure 3.26 The amplitude of β-endorphin pulses (mean ± S.E.M.) over 8 hours for Group A ([]), Group B (XXX) and Group C (\). FOL=follicular, LUT=luteal, AMEN=amenorrhea.
Figure 3.27 Plasma LH concentrations over 8 hours in Subject 1 from Group A. Follicular (d.10) (A), Luteal (d.24) (B). Asterisks (★) indicate an LH pulse (criterion of Ross et al 1983). Results of Subject 2 and 3 from Group A are in Appendix 4.
concentrations over 8 hours in Subject 4 from Group B is shown in Figure 3.28. Mean LH concentrations (± S.E.M.) were higher in the follicular phase (Group A=10.6 ± 0.3, Group B=12.0 ± 0.4 mIU/ml) than the luteal phase (Group A=7.0 ± 0.5, Group B=7.4 ± 0.4 mIU/ml) for all subjects in Group A and B. In Group C, 2 subjects had 2 LH pulses and 1 subject had 3 pulses during the 8 hour study. Plasma LH concentrations over 8 hours in Subject 7 is shown in Figure 3.29. Mean LH concentrations of subjects from Group C (10.0 ± 0.6 mIU/ml) were similar to those for the follicular phase of Group A.

LH pulse amplitudes (mean ± S.E.M.) were greater in the luteal phase (Group A=4.7 ± 1.0, Group B=5.2 ± 0.7 mIU/ml) than in the follicular phase (Group A=3.6 ± 1.2, Group B=3.9 ± 0.7 mIU/ml) for both Group A and B. Group C had greater pulse amplitudes (7.5 ± 2.8 mIU/ml) than Group A and B at any time of the menstrual cycle (Figure 3.30).
Figure 3.28 Plasma LH concentrations over 8 hours in Subject 4 from Group B. Follicular (d.11) (A), Luteal (d.25) (B). Asterisks (*) indicate an LH pulse (criterion of Ross et al 1983). Results of Subjects 5 and 6 from Group B are in Appendix 5.
Figure 3.29 Plasma LH concentrations over 8 hours in Subject 7 from Group C. Asterisks (*) indicate an LH pulse (criterion of Ross et al 1983). Results from Subject 8 and 9 from Group C are in Appendix 6.
Figure 3.30 The amplitude of LH pulses (mean ± S.E.M.) over 8 hours for Group A ( ), Group B ( ) and Group C ( ). FOL=follicular, LUT=luteal, AMEN=amenorrhea.
CHAPTER FOUR

DISCUSSION

4.1 PLASMA $\beta$-ENDORPHIN RADIOIMMUNOASSAY

The endogenous opioid peptide, $\beta$-endorphin, has been shown to inhibit LH release throughout the normal menstrual cycle. It is also known that vigorous exercise elevates plasma $\beta$-endorphin. Consequently, it has been postulated that chronic elevations in $\beta$-endorphin may be partly responsible for menstrual dysfunction in athletes, in particular amenorrhea.

The intent of this investigation was to examine plasma $\beta$-endorphin concentrations in women with normal menstrual cycles and in exercising females. Although there is some data available regarding this area, a major problem has been the reliability of measurement for plasma $\beta$-endorphin. Consequently, reports of plasma $\beta$-endorphin concentrations throughout the menstrual cycle, or in response to exercise, are often contradictory. Because of this, an evaluation was done of those measurement techniques described in the literature. Those procedures thought to yield reliable and reproducible results were used to develop a sensitive, specific and precise RIA to measure plasma $\beta$-endorphin. Questions were then posed about plasma $\beta$-endorphin throughout the menstrual cycle in non-exercising and exercising women and in exercising women with amenorrhea.

The optimized method for measuring plasma $\beta$-endorphin developed for this thesis satisfies the criteria of an accurate and valid RIA. The procedures of this RIA accounted for the physical properties of $\beta$-endorphin that have made it difficult to measure this peptide.
Blood was collected in the presence of protease inhibitors to prevent breakdown of peptides. Plasma samples were extracted with solvents followed by gel chromatography that clearly separated \( \beta \)-LPH from \( \beta \)-endorphin (Figure 3.4) and increased sensitivity of the assay. Both extraction and chromatography procedures resulted in high recoveries of \( \beta \)-endorphin. Radiolabelled \( \beta \)-endorphin of high specific activity was used in the final RIA with an antiserum of moderate cross-reactivity with \( \beta \)-LPH (Figure 3.12). Sensitivity of the RIA was determined to be 4 pg/ml and precision was within the range recommended by Chard (1987) for a reproducible and reliable RIA.

Since completion of this work, a number of \( \beta \)-endorphin RIA methods have been published, yet few satisfy the criteria of a good assay. Commercially available kits are used by many (de Meirlier et al 1986; Goldfarb et al 1987; Hatfield et al 1987; McMurray et al 1987; Vrbicky et al 1982) but results from this study found these kits to be unreliable and insensitive.

When collecting blood samples, it was noted that few have taken the precaution to use plastic (polypropylene/polyethylene) or siliconized glassware and protease inhibitors (Farrell et al 1987; Laatikainen et al 1985a; Oltras et al 1987; Petraglia et al 1986b; Rahkila et al 1987; Viswanathan et al 1987). Many investigators extract plasma with silicic acid (Elias et al 1986; Farrell et al 1982, 1987; Gambert et al 1981; Petraglia et al 1986b; Viswanathan et al, 1987) or with Sep Pak\textsuperscript{TM}.\textsubscript{C}_{18} cartridges (Adams et al 1987; Rahkila et al 1987) but these procedures were not used for the optimized method since they resulted in poor recovery and reproducibility. The solvent extraction, resulting in high recovery, is used only by one group of investigators at this time (Laatikainen et al 1985a,b).
Although attempts have been made to raise specific antisera to increase the specificity of the assay (Farrell et al. 1987), more commonly the solution has been to employ chromatographic separation of $\beta$-endorphin from structurally similar peptides (Laatikainen et al. 1985a,b; Petraglia et al. 1986a,b). Chromatographic isolation of $\beta$-endorphin is clearly absent from most methods described to measure plasma $\beta$-endorphin.

In summary, surprisingly few investigators have combined an appropriate method for blood collection with an extraction and chromatographic procedure (Fraioli et al. 1980; Laatikainen et al. 1985a; Petraglia 1986b). Consequently, the findings of others are suspect.

4.2 PLASMA $\beta$-ENDORPHIN VARIATION

With the development of a sensitive, specific and precise $\beta$-endorphin RIA, it became possible to document patterns of $\beta$-endorphin throughout the normal menstrual cycle. Previously this has not been clearly defined in sedentary women. Subjects from this study were not randomly selected, but selected from a pooled group. Due to this study design, causal statements about the relationship between plasma $\beta$-endorphin, the menstrual cycle and athletic amenorrhea cannot be made. However, results from this study are representative of the different groups recruited.

A number of papers describe variation of $\beta$-endorphin in the menstrual cycle, but their use of RIA kits or less than adequate procedures make their results suspect (Janal et al. 1984; Tang et al. 1987; Vrbicky et al. 1982). In those instances where a reliable RIA was used, however, variations in $\beta$-endorphin have been documented (Laatikainen et al. 1985a; Petraglia et al. 1986b). Results
of the present study confirm the variation of plasma $\beta$-endorphin.

Results from this study observed normal menstruating, non-exercising women (Group A) to have significant peaks at the early follicular, early luteal and late luteal phase of the cycle, ranging in concentration of 5–20 pg/ml. This is in agreement with Laatikainen et al (1985a) who describe a significant rise of $\beta$-endorphin during the early luteal (d.0 to +1) and late luteal -early follicular (end of cycle to beginning of next) phase of the cycle. Concentrations reported by Laatikainen et al (1985a) were also similar to the findings of the present study.

Petraglia et al (1986b) also report a moderate rise during the early luteal phase (d.+3 to +4), but in contrast report significant $\beta$-endorphin increments during the preovulatory phase (d.-1 to -4). A number of subjects from Group A had increases in $\beta$-endorphin during the preovulatory phase, however, these were not significant. Daily blood samples were not obtained in this study and may have missed the $\beta$-endorphin elevations reported by Petraglia et al (1986b). Concentrations reported by Petraglia et al (1986b) were 30–40% higher than those found in the present study and by Laatikainen et al (1985a) but a different RIA technique may explain the different levels.

The present study found the highest levels of $\beta$-endorphin to occur in the early follicular phase as opposed to the preovulatory (Petraglia et al 1986b) and postovulatory phase (Laatikainen et al 1985a) reported in the other 2 studies. Differences in amplitude may be due to the $\beta$-endorphin RIA used and the frequency of blood sampling.

Although there is general agreement about menstrual cycle variation of $\beta$-endorphin and limited agreement about the location of $\beta$-endorphin increments,
the reason for changes in plasma $\beta$-endorphin are still unclear. Ovulatory pain was suggested by Laatikainen et al (1985a) to explain the postovulatory rise, however, it is unlikely for this pain to last for more than 24 hours as indicated in their paper. Similarly, the finding of increased $\beta$-endorphin at the onset of menstruation is explained as pain-related (Laatikainen et al 1985a). There are no earlier data on the relationship between menstrual pain and $\beta$-endorphin. Pain and $\beta$-endorphin has been investigated during labour where extremely high concentrations of $\beta$-endorphin have been reported (Pancheri et al 1984; Raisanen et al 1984; Wardlaw & Frantz 1979) however, the link between pain and the menstrual cycle is yet to be determined.

Investigators are in agreement that ovarian steroids may influence $\beta$-endorphin production. Quigley and Yen (1980) were the first to postulate that endogenous opioid activity may vary throughout the normal menstrual cycle due to the changing gonadal steroid environment. Most studies in support of this hypothesis (Blankstein et al 1981; Steele and Judd 1986; Soules et al 1984) have used naltrexone to confirm their theories. As previously documented, little work has been done on measuring peripheral $\beta$-endorphin changes in response to different gonadal steroid milieus. Direct measurements of $\beta$-endorphin in hypophyseal portal blood of non-human primates are found to vary in presence of circulating steroids. Greatest concentrations of $\beta$-endorphin were detected when progesterone and/or estradiol were raised (Ferin et al 1984; Wehrenberg et al 1982).

By contrast, $\beta$-endorphin levels were undetectable when ovarian steroid concentrations were low, such as at menstruation. It has been suggested that ovarian steroids modulate GnRH release through $\beta$-endorphin, whereby increased
opioid activity in the luteal phase diminishes GnRH pulse activity to permit FSH a preferential effect on the follicle (Van Vugt et al 1984). An early luteal increment of \( \beta \)-endorphin, observed in subjects from Group A, could be explained by the presence of increased progesterone and estradiol, but does not explain the elevations during the late luteal and early follicular phase of the cycle, a time when these steroid concentrations are diminished. Factors other than circulating steroids may influence \( \beta \)-endorphin concentrations and further work in this area is recommended to clarify the variation of \( \beta \)-endorphin throughout the menstrual cycle.

Results from the present study indicate that concentrations of \( \beta \)-endorphin in exercising women were generally higher than those of non-exercising women, a finding consistent with other reports. This trend of higher \( \beta \)-endorphin levels was persistent throughout the menstrual cycle and has not been previously documented. Significant \( \beta \)-endorphin increments were also determined to be at the same phases of the menstrual cycle for the exercising population as in the non-exercising population (EF, EL, and LL). Hence, the pattern of \( \beta \)-endorphin variation for menstruating women is similar, except the exercising group had higher levels of \( \beta \)-endorphin throughout the cycle. In addition, the exercising women displayed greater amplitudes of \( \beta \)-endorphin during these specific phases (Figure 3.18). This finding suggests that a regular routine of vigorous exercise stimulates \( \beta \)-endorphin not only in response to an acute bout of exercise, but that these levels remain elevated during times of rest. The time required for exercise-stimulated concentrations of \( \beta \)-endorphin to return to baseline are not known, therefore it is not known whether the resting levels in the trained subjects represent a true chronic effect or simply
residual effects from the last training session.

Trained women have also been noted to have higher resting levels of other exercise-responsive hormones such as cortisol (Villaneuva et al 1986). This is not surprising since exercise has been shown to stimulate the release of both ACTH and β-endorphin (Fraioli et al 1980), two hormones derived from the same POMC precursor (Guillemin et al 1977).

Significant increments of β-endorphin occurred during the early luteal phase for Group A (d.+1 to +4) and in Group B (d.+2 to +7). It is noted that Group B had EL increments over a wider range of days than Group A. The presence of gonadal steroids in the luteal phase has previously been suggested as an explanation for this β-endorphin increment (Steele and Judd 1986; Soules et al 1984) however, the disparity in days of peak onset is not clear. This difference cannot be explained due to difference in cycle length since both groups had similar lengths of menstrual cycle.

A similar situation occurred during the late follicular phase. Although the increment of β-endorphin was not significant for Group A or B during this time, Group B was noted to display a β-endorphin elevation over a wider range of days (d.-2 to -7) compared to Group A (d.-3 to -4). A lower level of estradiol cannot explain this discrepancy in Group B since estradiol concentrations were similar for both groups. Daily blood sampling might elucidate the differences in timing of peaks between an exercise and sedentary group of women.

Results from the present study indicate that concentrations of β-endorphin in exercising amenorrheic subjects were higher than those in non-exercising menstruating subjects, however, similar to those exercising subjects
with normal menstrual cycles. This is strengthened by the finding that a significant correlation was observed between the number of days trained/week and peak amplitude of \( \beta \)-endorphin (p<0.05). These findings are in agreement with those of Laatikainen et al (1986) who report significantly higher \( \beta \)-endorphin concentrations in exercising women with amenorrhea compared to normal menstruating, non-exercising women. A shortcoming of the Laatikainen et al (1986) study is that they did not compare these levels with normal menstruating exercising women as done in the present study. To establish the role of \( \beta \)-endorphin or another abnormality in causing athletic amenorrhea, a comparison of amenorrheic and eumenorrheic athletes is required to exclude those changes resulting from exercise training which are unrelated to the development of amenorrhea.

In addition, the present study found the pattern of \( \beta \)-endorphin release over the 4 weeks was not constant for most amenorrheic subjects. There was no single profile that could describe all subjects but 8 of 11 subjects displayed highly variable concentrations while the other 3 displayed low, unchanging levels. Hence, \( \beta \)-endorphin concentrations observed in exercising, menstruating women were not distinguishable from exercising, amenorrheic women.

The absence of a regular pattern of \( \beta \)-endorphin in amenorrheic athletes could not be due to differences in training or fitness level since these variables were not significantly different from Group B. Age of menarche, although significantly older in Group C than A (p<0.02), could not explain the lack of \( \beta \)-endorphin pattern since age of menarche was similar to Group B and age of menarche for all three groups was well within the normal range for onset of menarche.
Lack of maturity of the hypothalamic-pituitary-ovarian axis may be partly responsible for the irregular pattern of β-endorphin observed in Group C (Dale et al 1979). Maturation of the ovarian axis during puberty is characterized by a shift in hypothalamic sensitivity to gonadal steroids that occurs with puberty (Grumbach 1980). This shift in sensitivity may be slow in developing in subjects who are physically active during the years of pubertal maturation. Subjects from Group B had been experiencing normal menstrual cycles for 6 or more years while most subjects from Group C had 5 or less years of regular cycles before their onset of amenorrhea.

This difference in years of exposure to adult patterns of hormonal feedback may be associated with the irregular β-endorphin pattern observed in Group C. In addition, experiments in rats suggest that the tonic inhibitory control exerted by endogenous opiates on LH secretion is decreased with sexual maturation (Bhanot & Wilkinson 1983b). This finding suggests that endogenous opiates may play a role in pubertal maturation and physically active pre- or peri-pubertal girls may experience delays in maturation due to, in part, exercise-induced elevations of β-endorphin.

Percent body fat was also similar for all three groups and cannot explain the difference in pattern of β-endorphin. This study does indicate that exercising women regardless of menstrual status, have higher circulating β-endorphin than non-exercising women. However, there were 3 subjects from Group C that did not have higher β-endorphin levels. The differences observed in these 3 subjects could not be due to frequency of training per week, number of years training nor length of amenorrhea since all three of the subjects were similar to the rest of the amenorrheic group. It is not clear why these
amenorrheic athletes had such different profiles.

Concentrations and pattern of release of other hormones in amenorrheic athletes have been reported to vary. Basal serum gonadotropin levels have been found to be normal (Chang et al. 1986; Ronkainen et al. 1985; Veldhuis et al. 1985b) or lower (Loucks & Horvath 1984; McArthur et al. 1980) than concentrations observed during the early follicular phase of a normal cycle. Repetitive measurements of hormone secretion on different occasions in the same subject have rarely been made. However, they may reveal the degree of individual variation that exists among specific populations.

An interesting finding described by Crowley et al. (1985) was that abnormalities of GnRH-induced gonadotropin secretion can change within a given patient over time. A female with hypogonadotropic hypogonadism was studied on three occasions and her LH secretory pattern differed markedly each time. Amplitude and frequency of LH were different on each examination, resembling patterns commonly seen among ovulatory women at different phases of the cycle. Yet no spontaneous ovulation or corpus luteum formed and the amenorrhea persisted. Although this patient was not an exercise-related case of amenorrhea, this example serves to demonstrate the variability which can occur both within and between subjects with amenorrhea.

Investigating an exercising amenorrheic population cross-sectionally may not be the best way to reveal causal factors leading to the menstrual dysfunction. The ideal model to adopt would incorporate a randomized, prospective study observing normal menstruating, sedentary women through a rigorous, long-term exercise program and measure hormonal responses at frequent intervals. Although such a design would be difficult, the results may
yield long-awaited answers regarding exercise and menstrual regularity.

Few studies have been longitudinal (Boyden et al 1983; Bullen et al 1984, 1985) and random selection of subjects has not been performed. The major benefits of random selection is the removal of bias and the results have the greatest chance of being statistically significant (Sackett et al 1985). It may be difficult to design the ideal study since some propose that mature female athletes have been directed to sport at an early age. Malina et al (1978) suggests that delayed menarche makes girls more likely to take up and excel at athletics because the physique of late maturing girls, characteristically longer-legged and narrow hipped, would be advantageous. Malina et al (1978) also propose that early maturing girls may be socialized away from sports competition.

4.3 PULSATILE SECRETION OF β-ENDORPHIN AND LH

Investigators have postulated that characterization of pulse patterns of endogenous hormones may lead to a better understanding of the regulation of these hormones and the cause of certain pathophysiological conditions (Urban et al 1988). A variety of peak-detection methodologies are available and have been recently reviewed (Urban et al 1988). The ideal properties of a peak-detection method includes objectivity, validity and simplicity. The modified Santen & Bardin pulse detection method developed by Ross et al (1983) used in this study satisfies those criteria.

Application of statistical analysis was inappropriate for such small group numbers, therefore, results are presented in a descriptive nature only. Nevertheless, some trends were seen. Pulsatile secretion of β-endorphin was
seen in both the follicular and luteal phase of the cycle with 2 of 3 non-exercising subjects displaying decreased frequency in the luteal phase. Pulse interval increased from 120 minutes in the follicular phase to 160-240 minutes in the luteal phase while amplitude of the pulses between phases were not different. It is difficult to interpret differences in pulse frequency and similarity of pulse amplitude between menstrual phases because of small sample size. Moreover, blood sampling at 20 minute intervals is not the optimal frequency when analyzing pituitary pulse characteristics (Urban et al 1988) and consequently this study may have missed detecting some pulse features, such as differences in amplitude. More frequent sampling was precluded however, due to the need for relatively large plasma volume required for the assay. Despite this, the finding of \( \beta \)-endorphin pulses lends support to further detailed work in this area. Increased frequency of sampling (5-10 minute intervals) at different times of the cycle would provide information about pulse variation and amplitude during different levels of gonadal steroid stimulation.

The pulsatile secretion of \( \beta \)-endorphin has not been previously reported in women. However, pituitary release of ACTH is known to be pulsatile (Gibbs et al 1983; Donald 1980; Gallagher et al 1973) and its secretion to be concomitant with \( \beta \)-endorphin (Guillemis et al 1977) hence it is reasonable to expect the release of \( \beta \)-endorphin to be pulsatile. Detailed analysis of ACTH pulsatile secretion in humans has not been done, but results from Gallagher et al (1973) suggest that the frequency of ACTH pulses is slightly higher than that of \( \beta \)-endorphin pulses noted in this study. However, the study by Gallagher et al (1973) examined only one male subject and provided no details as to how an ACTH pulse was detected. Further work in this area is needed to evaluate the
relationship between pulsatile release of ACTH and β-endorphin. In addition to this, corticotropin-releasing factor (CRF) has been identified as the likely releasing factor for both ACTH and β-endorphin (Gibbs et al 1983; Jackson et al 1984; Vale et al 1981). The relationship between this hypothalamic releasing factor and its control of ACTH and β-endorphin release from the anterior pituitary may resemble the well-documented regulation of gonadotropin secretion by GnRH (Marshall & Kelch 1986) whereby the ACTH and β-endorphin pulses reflect the hypothalamic secretion of CRF.

The intent of measuring plasma β-endorphin over 8 hours was to establish the presence of pulsatile secretions and observe any variation between the follicular and luteal phase of the menstrual cycle. There are no data describing the interactions between CRF and circulating gonadal steroids making analysis and discussion of the current results difficult. At best there are data to show that CRF infusion produced a progressive and significant decrease in both LH and FSH in female rhesus monkeys. The addition of naloxone to CRF prevented the CRF-induced suppression of gonadotropin release, therefore it has been concluded that the CRF inhibitory action on gonadotropin is mediated by endogenous opioid peptides (Gindoff et al 1987). Further work in this area may lead to findings that support the role of endogenous opioids and menstrual regulation. These findings indicate a potential interaction between CRF and GnRH (Gindoff et al 1987).

All exercising subjects from Group B demonstrated reduced β-endorphin pulse frequency in the luteal phase (1 pulse/160 min). The pulse amplitude between the follicular and luteal phase was similar but of greater magnitude than the amplitude of the non-exercising group. Higher plasma concentrations
were observed throughout the menstrual cycle in this group as well. Again, a similar pattern is seen between the exercising and non-exercising groups, but the exercising groups had increased concentrations of \( \beta \)-endorphin.

Increased CRF secretion in response to stress has been documented in rats (Rivier et al. 1986), but evidence that exercise stimulates CRF is lacking. No direct evidence of this hypersecretion is available since secretion into the hypophyseal portal system is inaccessible. Measurement of CRF in the periphery would be difficult due to systemic dilution. However, increased ACTH secretion has been reported in exercising women (Villaneuva et al. 1986) as has \( \beta \)-endorphin (Carr et al. 1981; Howlett et al. 1984). Other evidence that the hypothalamic-pituitary-adrenal axis is stimulated as a result of exercise includes the finding that trained athletes have higher circulating levels of cortisol (Luger et al. 1987; Villaneuva et al. 1986). This indirect evidence suggests that CRF is stimulated by exercise.

A \( \beta \)-endorphin pulse interval of 96-120 minutes was observed in exercising women with amenorrhea. This frequency was similar to that seen in menstruating women during the follicular phase, but the amplitude of these pulses was greater than either group. Elevated \( \beta \)-endorphin has been identified as a potential factor in causing exercise-related amenorrhea by modulating gonadotropin release at the level of the hypothalamus (Bullen et al. 1984; Ferin et al. 1984). This 8 hour study supports these findings that exercising women have constantly higher concentrations of \( \beta \)-endorphin compared to non-exercising women. In addition, \( \beta \)-endorphin concentrations in exercising women throughout the menstrual cycle were similar to those levels of \( \beta \)-endorphin observed in amenorrheic athletes over 4 weeks but the pulsatile release of \( \beta \)-
endorphin was marked by a greater amplitude in the amenorrheic athletes. This finding cannot be attributed to inter-group differences in weight, percent body fat or training volume, since they did not differ. Results from this study suggest that amenorrheic athletes experience elevated opioid activity compared to their menstruating peers and that this may contribute to their amenorrheic status by diminishing output of GnRH at the hypothalamic level.

Others have postulated that highly trained athletes experience increased CRF secretion and mild hypercortisolism and may explain the hypothalamic hypogonadism observed in young female athletes (Warren 1980). Hypercortisolism alone cannot explain menstrual dysfunction since significantly elevated urinary cortisol levels have been found in both amenorrheic and eumenorrheic runners (Villaneuva et al 1986). However, results from Rivier et al (1986) suggest that endogenous CRF partially mediates stress-induced inhibition of LH in rats that CRF acts to inhibit GnRH secretion into the hypophyseal portal circulation. These results are consistent with those of Gindoff et al (1987) that CRF produced decreases in gonadotropin secretion that were blocked with naloxone.

There is an abundance of data describing the characteristics of pulsatile LH secretion in normal menstruating, non-exercising women. Early studies by Santen and Bardin (1973) showed that LH is released into the circulation in a series of pulses. Elegant work performed in primates have developed the concept that pulsatile LH secretion requires pulsatile GnRH secretion and that without the pulsatile mode of GnRH release, gonadotropin secretion and ovarian function are disrupted (Knobil 1980).

Furthermore, gonadal steroids affect GnRH pulse frequency and are thus
important regulators of gonadotropin secretion. Frequency and amplitude of LH secretion is modified by the prevailing steroid environment such that pulse frequency is at its highest during the follicular phase and pulse amplitude is at its highest in the luteal phase (Filicori et al 1986; Reame et al 1984). Results from the current study were in agreement with those of others reporting menstruating, non-exercising women with increased LH frequency in the follicular phase and increased amplitude in the luteal phase.

In this present study, exercising menstruating women were found to have diminished LH pulse frequency but no difference in amplitude compared to non-exercising women. These findings are in agreement with Cumming et al (1985) who observed defects in pulsatile LH release in normal menstruating runners reporting diminished LH frequency. A similar number of LH pulses were observed between the current study and that of Cumming et al (1985). In contrast, Cumming et al (1985) reported diminished LH pulse amplitude in the runners when compared to sedentary controls. Differences in amplitude were not detected in the present study but may be explained by increased frequency of sampling by these investigators.

Despite reduced frequency, exercising women from this study still displayed the general pattern of LH pulsatile secretion with increased frequency in the follicular phase and increased amplitude in the luteal phase. These data are compatible with the concept that vigorous exercise exerts an inhibitory effect on the hypothalamic-pituitary axis. These changes could be associated with minor variations in menstrual function which have been described in athletes who continue to menstruate (Prior et al 1982; Shangold et al 1979).

Amenorrheic athletes from this study were observed to have reduced LH
pulse frequency when compared to either non-exercising or exercising menstruating subjects. Amplitude of LH pulses were greater than those of menstruating women during either follicular or luteal phase. Veldhuis et al (1985b) also reported reduced frequency of LH secretion but was not associated with a decline in amplitude when compared to sedentary controls. Varying results among amenorrheic women are not uncommon (Crowley et al 1985) and these results should be interpreted with caution since between subject and within subject variation may exist. The pattern of the LH pulsatile activity, as seen with other forms of menstrual dysfunction, may vary among amenorrheic athletes (Wentz 1974). Reduced LH frequency may be due to elevated β-endorphin (Veldhuis et al 1985b) acting on the hypothalamus to decrease GnRH output (Ferin et al 1984).

4.4 EXERCISE-ASSOCIATED AMENORRHEA IN GENERAL

This thesis has addressed specific questions about the relationship between plasma β-endorphin, the normal menstrual cycle and exercising women. Elevated endogenous opioid activity has been associated with menstrual dysfunction and exercise, but additional predisposing factors have been cited (Baker 1981; Schwartz et al 1981). In this final section, the broader scope of exercise-associated amenorrhea will be discussed and a rationale presented for future research.

Investigating athletic amenorrhea has been difficult due to the number of variables that may influence menstrual regularity. The regulation of hormonal secretions in the female is complicated by a routine of vigorous exercise. The interplay between hormones influencing ovarian function in the
athlete has been difficult to describe and a single factor responsible for amenorrhea has yet to be identified.

The amenorrhea observed in the athletes in this study could not be explained by reduced percent body fat or weight, delayed age of menarche, large training volume or restricted dietary intake. These factors, however, are not to be discounted. Several investigators have identified these characteristics in amenorrheic athletes (Frisch et al 1980; Feicht et al 1978) but as yet, how these attributes contribute to menstrual dysfunction is not known. Progress has been made in identifying an endocrine profile for amenorrheic athletes (Veldhuis et al 1985b; Loucks and Horvath 1984) but the underlying cause of altered hormonal secretion is not clear.

Other exercise-responsive hormones have been examined for their role in inducing reproductive changes. The finding that female runners show a rise in serum prolactin after acute exercise, but sedentary women do not (Chang et al 1986) raised the possibility that amenorrhea in runners developed due to intermittent hyperprolactinemia. Boyden et al (1982) supported this theory when results showed that thyrotropin-releasing hormone-induced prolactin release increased as intensity of exercise increased. Prolactin responses to acute exercise in amenorrheic and eumenorrheic runners, however, are not different (Chang et al 1986; Loucks and Horvath 1984; Yahiroyo et al 1986) and basal serum prolactin does not differ between the two groups (Chang et al 1986; Veldhuis et al 1985b; Yahiroyo et al 1987). Although exercise induces prolactin output, the similarity of response in menstruating and non-menstruating runners suggests that hyperprolactinemia by itself does not cause amenorrhea in athletes.

Elevated testosterone levels or exercise-induced increases of
testosterone have led some to suggest that excess androgens cause amenorrhea. In opposition to this, normal levels of resting testosterone and no change in serum testosterone after exercise in amenorrheic runners have been found (Loucks and Horvath 1984; Yahiro et al 1987). Although hyperandrogenism has been associated with other amenorrheas, such as polycystic ovarian disease, its link with exercise-related amenorrhea is not supported.

Recently, studies have described an interesting relationship between the hypothalamic-pituitary-ovarian axis and the hypothalamic-pituitary-adrenal axis in athletes. Strenuous exercise activates the hypothalamic-pituitary-adrenal axis and the resulting increases in ACTH and cortisol secretion are well-documented (Farrell et al 1987). Mild hypercortisolism has been observed in women runners (Villaneuva et al 1986). The interaction between the adrenal axis and ovarian axis is poorly understood but a number of animal and human studies have revealed some intriguing results. Chronic treatment of female mice with ACTH arrests pubertal maturation (Christian 1964) and inhibits ovulation (Jarret 1965). Long term treatment with glucocorticoids inhibits GnRH-induced LH release (Sakahura et al 1975). Pulsatile secretion of LH is abolished with CRF infusion but this effect is blocked with the addition of naloxone (Gindoff et al 1987) suggesting mediation by endogenous opioids. Interactions between the adrenal and ovarian axis at the hypothalamic level may be enhanced in those who exercise vigorously.

A number of predisposing factors and hormonal responses to exercise are implicated in menstrual dysfunction. A single factor or mechanism is as yet unidentified, but at present, a "threshold" theory may best describe the occurrence of exercise-associated amenorrhea. Strenuous exercise presents to
the athlete stress in a variety of forms and each athlete will respond differently. Individual athletes may have different sensitivities to exercise-induced hormonal changes and lead to different manifestations of menstrual abnormalities. Individual responses to training volume or intensity may also differ among athletes. The role of stress is difficult to evaluate since it is a very subjective phenomenon. It has been observed that amenorrhea occurs more frequently in stressed athletes as determined by personality profiles (Schwartz et al. 1981), but research in this area is limited.

4.5 FUTURE RESEARCH

Interpreting the current body of literature is not easy. Results are frequently confusing and comparisons difficult to make due to vastly different approaches to examining exercise-related amenorrhea. Different methods and protocols account for most of the dissimilarities. Acute versus chronic hormonal response to varying types of exercise regimens is frequently measured but few results are compatible. A number of shortcomings in these methods have been identified: phase of the menstrual cycle not identified, time of day not reported, infrequent blood sampling and failure to measure a wide range of hormones to allow comparison of patterns of change of related groups of hormones. Attention to these details may facilitate interpretation of future research. A randomized, prospective study design would provide the soundest evidence regarding the effect of intense exercise on reproductive function.

Further research in the area of exercise-associated amenorrhea is strongly recommended. The consequences of long term amenorrhea in athletes was at one time not thought to be serious (Rebar & Cumming 1981), but recent
studies have found diminished bone density in young hypoestrogenic women comparable to postmenopausal women (Cann et al 1984; Drinkwater et al 1984). A hallmark study by Drinkwater et al (1984) found that amenorrheic runners (mean age of 27.0 years) had lumbar bone density scores comparable to women 52 years of age. Warren et al (1986) documented a higher incidence of scoliosis and stress fractures among young amenorrheic ballet dancers compared to their eumenorrheic peers. The implications of these findings for these athletes are far-reaching. Resumption of menses in amenorrheic athletes has been shown to reduce the rate of bone loss but concerns are expressed for the suspended activity of bone mineralization that would normally be occurring in young developing females but is retarded by hypoestrogenic states (Drinkwater et al 1986). An improved understanding of the underlying mechanisms surrounding athletic amenorrhea may assist in the treatment of this population and diminish the occurrence of negative consequences.
4.6 CONCLUSIONS

Results from this investigation have shown a variation of plasma \( \beta \)-endorphin in normal menstruating women. Significant increments of \( \beta \)-endorphin occur during the early follicular, early luteal and late luteal phase of the cycle in both sedentary and athletic females, however, active women demonstrate \( \beta \)-endorphin increments of greater magnitude during these times. Gonadal steroid modulation may be responsible for the early luteal phase rise of \( \beta \)-endorphin, but as yet the increases seen at the early follicular and late luteal phase remain to be explained. A highly variable pattern of \( \beta \)-endorphin release was observed in amenorrheic athletes with concentrations similar to those found in eumenorrheic athletes. Exercise alone cannot explain the absence of the pattern of \( \beta \)-endorphin secretion in amenorrheic athletes since the level of exercise was similar to the eumenorrheic athletes.

The second part of this study indicates that \( \beta \)-endorphin is released into the circulation in a pulsatile fashion. Sample size was small, therefore, results were presented descriptively. Pulse frequency of \( \beta \)-endorphin appears to be reduced in the luteal phase of both sedentary and exercising females, while amenorrheic athletes exhibited pulse frequency similar to that found in the follicular phase. Pulse amplitude did not vary between the follicular and luteal phase of sedentary women, nor did it vary between phases of athletic women, but amplitude of \( \beta \)-endorphin pulses in the athletes was greater than the sedentary women. Amenorrheic athletes had pulse amplitudes greater than either sedentary or athletic menstruating females. The significance of pulsatile secretion of \( \beta \)-endorphin is yet to be determined but the greater amplitude of \( \beta \)-endorphin pulses observed in amenorrheic athletes may suggest that
hypothalamic regulation of GnRH release and consequent gonadotropin secretion is being interrupted or altered in this group.

Combined results suggest that β-endorphin concentrations are elevated in athletic females, regardless of menstrual status. Increased concentrations of β-endorphin alone cannot explain the presence of amenorrhea in these athletes, however, the more subtle features of β-endorphin release require more detailed examination. The small numbers investigated in this study did not permit detailed statistical analysis of the results but preliminary findings support the current theory that chronic exercise stimulates β-endorphin release and may play a role in exercise-associated menstrual dysfunction. Long-term investigations should be conducted to delineate the reasons for and consequences of altered reproductive function associated with exercise.
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APPENDIX 1.1 Plasma β-endorphin concentrations over 8 hours in Subject 2 from Group A. Follicular (d.11) (A), Luteal (d.23) (B). Asterisks (*) indicate a β-endorphin pulse.
APPENDIX 1.2 Plasma β-endorphin concentrations over 8 hours in Subject 3 from Group A. Follicular (d.9) (A), Luteal (d.22) (B). Asterisks (*) indicate a β-endorphin pulse.
APPENDIX 2.1 Plasma β-endorphin concentrations over 8 hours in Subject 5 from Group B. Follicular (d.12) (A), Luteal (d.25) (B). Asterisks (*) indicate a β-endorphin pulse.
APPENDIX 2.2 Plasma ß-endorphin concentrations over 8 hours in Subject 6 from Group B. Follicular (d.11) (A), Luteal (d.25) (B). Asterisks (*) indicate a ß-endorphin pulse.
APPENDIX 3 Plasma β-endorphin concentrations over 8 hours in Subject 8 (A) and 9 (B) from Group C. Asterisks (※) indicate a β-endorphin pulse.
APPENDIX 4.1 Plasma LH concentrations over 8 hours in Subject 2 from Group A. Follicular (d.11) (A), Luteal (d.23) (B). Asterisks (*) indicate an LH pulse.
APPENDIX 4.2 Plasma LH concentrations over 8 hours in Subject 3 from Group A. Follicular (d.9) (A), Luteal (d.22) (B). Asterisks (*) indicate an LH pulse.
APPENDIX 5.1 Plasma LH concentrations over 8 hours in Subject 5 from Group B. Follicular (d.12) (A), Luteal (d.25) (B). Asterisks (*) indicate an LH pulse.
APPENDIX 5.2 Plasma LH concentrations over 8 hours in Subject 6 from Group B. Follicular (d.11) (A), Luteal (d.25) (B). Asterisks (*) indicate an LH pulse.
APPENDIX 6  Plasma LH concentrations over 8 hours in Subject 8 (A) and 9 (B) from Group C. Asterisks (*) indicate an LH pulse.