EFFECTS OF 10-WEEK UNDERFEEDING FOLLOWED BY *AD LIBITUM* FEEDING ON ESTROUS CYCLES AND HYPOTHALAMO-PITUITARY FUNCTION IN AGING RATS

By

Kathleen Lois Quigley

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ABSTRACT

EFFECTS OF 10-WEEK UNDERFEEDING FOLLOWED BY
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1) Chronic underfeeding for most of the life of rats and mice delays aging processes and increases life span. The objective of the first study was to determine the effects of only 10 weeks of 50% food restriction followed by 16 weeks of ad libitum feeding on estrous cycles in young and old rats. This underfeeding/refeeding regimen significantly delayed the decline in estrous cycles in the young rats and temporarily reinitiated cycles in the old rats.

2) To elucidate the mechanism(s) of the favorable effects on estrous cycles, positive feedback effects of ovarian hormones and the negative feedback effects of ovariectomy on LH and PRL secretion were assessed in young and older females. All rats were ovariectomized 10 days after refeeding. To assess positive feedback, rats were given estradiol followed by progesterone 2 or 4 weeks postovariectomy. The older previously underfed rats had higher LH peak values both 2 and 4 weeks postovariectomy

than young rats. Following the removal of negative feedback, the older previously half-fed rats showed higher serum LH 1, 2, and 3 weeks after ovariectomy than the young previously half-fed or full-fed rats. Underfeeding had no effect on PRL release. These results suggest that old rats develop increased sensitivity to ovarian hormone feedback due to underfeeding.

3) GH and thyroid hormone secretion were studied in young, middle-aged, and old male rats during underfeeding and after 5 days of refeeding. The decline in fT_3 and fT_4 with age and further decline during underfeeding paralleled the decline in GH. These results suggest thyroid hormones to be partially responsible for the GH reductions of aging.

4) These studies indicate than even short-term underfeeding begun in aging rats may produce beneficial effects on some body functions. These findings may have application in aging human subjects.

This dissertation is dedicated to my husband Frank and my sons, Darren and Todd.

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LIST OF ABBREVIATIONS

ACTH = adrenocorticotropin hormone

BW = body weight

CA = catecholamine

cAMP = cyclic adenosine monophosphate

CE = constant estrus

CNS = central nervous system

CRF = corticotropin-releasing factor

CV = coefficient of variation

DA = dopamine

EOP = endogenous opiate peptides

ff = full fed

FSH = follicle stimulating hormone

fT_4 = free T_4

fT_3 = free T_3

GH = growth hormone

GHRH = growth hormone releasing hormone

GnRH = gonadotropin-releasing hormone

5HT = serotonin

HHH = hypothalamic hypophyseal hormones

im = intramuscular

L-DOPA = L-dihydroxyphenylalanine

LH = luteinizing hormone

lsd = least significant differences

M = middle-aged

ME = median eminence

NE = norepinephrine

NS = nonsignificant

O = old

PRL = prolactin

RIA = radioimmunoassay

rT_3 = reverse T_3

sc = subcutaneous

SEM = standard error of mean

TRH = thyrotropin-releasing hormone

TSH = thyrotropin-stimulating hormone

TT_4 = total T_4

TT_3 = total T_3

Y = young

INTRODUCTION

Aging may be defined as a decline in body functions associated with a decrease in ability to adapt to the environment. While this distinguishes aging from development, the two processes are often similar and may likely operate along a continuum from conception, through maturity, to old age (Ordy, 1975). Manifestations of biological aging include increased probability of death and reduced behavioral, physiological, and biochemical adaptation to internal and external environmental challenges (Ordy, 1975). Although the capacity of every organ or organ system may or may not decline with age, some changing capacities may be observed only when subjected to environmental challenges or stresses. Many homeostatic control systems which continue to function normally and efficiently at rest may become less able to react to stress and perturbation and therefore become progressively impaired with age. This decline in adaptation with age may be associated with increased tissue and organ pathology and subsequent mortality of the organism. Factors implicated as causative mechanisms to aging range from defective macromolecules within the cells to altered neurohumoral feedback loops between organs/systems.

The idea that the endocrine system may be involved in aging processes began with Brown-Sequard in 1889 when, at the age of 72 years, he reported that he had succeeded in rejuvenating himself by injecting dog and guinea pig testicular extracts. These reports created interest in the relationship of hormones to aging and eventually led to the use of estrogens for treating post-menopausal symptoms in women and to use of androgens in treating some elderly males. The overriding importance of the brain, particularly the hypothalamus, in regulating pituitary and general endocrine activity began with Geoffrey Harris' work on the neural control of the pituitary (Harris, 1955). Later research established that changes in neuroendocrine activity are responsible for some of the decrements in body functions during aging in several animal species, particularly rats and mice. Changes in neuroendocrine function have been reported to be responsible for loss of estrous cycles in female rats, for development of mammary and pituitary tumors, for the decline in pulsatile GH secretion, and perhaps for the decrease in immune function (Meites et al., 1987). It has also been demonstrated that neuroendocrine intervention by direct manipulation of the hypothalamus or by use of central acting drugs can inhibit, halt, or even reverse some of the aging changes in body function in old rats. The rate of aging of many body organs and tissues and their capacity to respond to appropriate stimuli appear to be regulated primarily by the neuroendocrine system (Meites

et al., 1984, 1987). However, none of these neuroendocrine manipulations have yet been shown to increase life span of animals and thus influence all aging processes.

In 1934, McCay and Crowell reported that restricting food intake of rats markedly increased mean and maximum life spans compared with ad libitum-fed control animals. Dietary restricted animals were reported to be biologically younger than age-matched controls. Long-term caloric restriction in the rat or mouse retarded the rate of tail tendon collagen fiber aging, delayed the onset of puberty, prolonged reproductive life, delayed age-related changes in serum lipids, and in young rats appeared to delay development and maturation of neuroendocrine functions (Everitt 1973, 1982). It also has been shown to delay the age of onset of age-related disease and tumor development. Thus, food restriction appears to increase longevity by retarding general aging processes (Masoro, 1985).

The precise mechanisms by which dietary restriction retards aging remains unknown. In food restricted rats, retarded growth, maturation, and aging may be partly due to reduced secretion of pituitary and other hormones necessary for these processes. Mulinos and Pomerantz (1940) reported that food restriction depressed many pituitary functions producing "pseudohypophysectomy" in the rat. Hypophysectomy alone does not prolong life, but when combined with cortisone replacement inhibits the onset of some degenerative disease and prolongs life in the rat similar to the effects

of underfeeding (Everitt et al., 1980; Everitt, 1982). Later studies showed that food restriction depresses body growth as well as functions of the pituitary, thyroid, adrenal cortex, gonads, and to reduce their corresponding blood hormone concentrations. Hormones are believed to affect tissue development and aging by altering gene expression in the tissues (Adelman, 1976). The lack of hormone stimulation to target organs during underfeeding results in decreased organ function which may reduce "wear and tear." Reduced secretion of pituitary hormones in food restricted rats has been shown to be mediated primarily via the hypothalamus (Meites and Fiel, 1965; Campbell et al., 1977; Young, 1979; Finch, 1976).

Various chronic underfeeding regimens have been employed to affect reproductive aging. Most of these were initiated early in life and continued thereafter for long periods of time. Chronic underfeeding initiated at weaning in rats was shown to delay puberty (Glass et al., 1976; Kennedy and Mitra, 1963), increase reproductive life span (Merry and Holehan, 1979), and slow the rate of reproductive decline (Merry and Holehan, 1979). These studies were initiated early in life and continued for most of the life of the animals, and therefore it was of interest to determine the effects of a relatively short period of food restriction initiated during maturity or later in the life of rats. The purpose of the studies reported here was to determine the effects of only 10 weeks of underfeeding followed by ad

libitum feeding on estrous cycles, mammary tumors, and hypothalamo-pituitary function in aging rats.

Literature Review

I. Control of biological aging

The probability of death within a fixed period of time shows a dependency on the genome that is characteristic for each species (Fries, 1980). These systematic patterns of mortality are the basis of comparative and evolutionary gerontology (Kirkwood, 1985). The average life span of a species is also highly responsive to environmental conditions and tends to be substantially lower for animals in nature than for captive or protected animals. According to current views on molecular sources of aging, biological aging is genetically programmed and an extension of the DNA-directed ontogenetic sequences of early cell differentiation and growth (Strehler and Barrows, 1970; Strehler et al., 1971). However, the organism is environmentally modifiable in which the genotype is regulated by natural selection in evolution to adapt to conditions set by the environment. Thus, genetic and environmental factors interact to affect lifespan in the process of evolution. Genotypic environmental interactions on lifespan curves of animals of the same strain living in different environments have shown

that separation of genetic and environmental sources to lifespan even within a species may be quite arbitrary and artificial (Comfort, 1964; Strehler, 1967).

A. Definitions

1. Definition of aging

Lack of an agreed upon, universally accepted definition of aging previously was considered proof of the immaturity of gerontological science (Medawar 1955). Even today, "aging" is still only loosely defined and as a result confusion can easily arise when the term is used without proper qualification.

Aging may be defined as a loss of adaptation to the environment. While this clearly distinguishes aging from development, these two processes are often similar and likely operate along the same continuum from conception through maturity to old age. Two manifestations of biological aging include (a) increased probability of death and (b) reduced behavioral, physiological, and biochemical adaptation to internal and external environmental challenges (Ordy, 1975). Although the capacity of every organ or organ system may or may not decline progressively with time, a reduced capacity may be observed in numerous variables only when the organism is subjected to environmental challenges or various stress categories. Many homeostatic control systems, which continue to function normally and efficiently

at rest (basal state) may become less able to respond to stress or other perturbations, and become progressively impaired with time. This decline in adaptation with age may be associated with increasing mortality. Factors implicated as causative mechanisms range from defective macromolecules within cells to altered neurohormonal or immunological feedback loops between organs. Two aging concepts emerge: aging may originate from a single focus of cells, a "pacemaker," which triggers senescence, or aging may be an intrinsic property of all cells so that each cell may age autonomously (Ordy, 1975).

2. Longevity

It is necessary to carefully differentiate the terms "aging" and "longevity." Longevity refers to maximum life span for the species which is associated with aging but is not synonymous with aging of an organ or tissues which may begin relatively early in life. Longevity denotes merely how long the animal lives and is measured by mean and maximum life duration for a particular species (Kirkwood, 1985). Among mammalian species there is a significant difference in maximum life span, e.g., mouse and man. The phylogenetic range of maximum life spans is much greater than the range for individual life spans within a single species (Comfort, 1964). Since each species has its own characteristic DNA program, the most compelling evidence that maximum life span is genetically determined is the characteristic maximum life

span of each individual species (Comfort, 1964). Experimental evidence on animals demonstrates differences in longevity between strains and mutants. Genetic selection for long and short-lived strains has also been demonstrated (Clark, 1964).

3. Physiological aging--change in organ functions

Physiological aging refers to changes in organ function associated with increased age of the organism. Most of these changes are deteriorative in nature. Examples of these include age-related increases in serum cholesterol, reduced response to insulin, reduced number of dopamine (DA) receptors, reduced motor-behavioral capabilities, bone loss, reproductive decline, and decreases in immune function (Masoro, 1985). All tissues and cells of the body are involved. This physiological decline of body functions renders the organism with reduced capacity to adapt to environmental changes. These age-related changes in tissue function are not necessarily due to cell loss but rather to a decline in functional capacity of these cells due to intrinsic and/or extrinsic factors (Meites et al., 1987).

As biological systems become more complex, the methods by which changes in aging are manifested increases. Changes in basic molecules, cells, or organs which represent fundamental or basic aging processes, become increasingly difficult to monitor (Ordy, 1975). The more obvious changes with age, such as the physical appearance of the individual,

including changes in hair and skin, and in behavior are considered to be secondary to more basic changes.

4. Development of age-related diseases

A variety of disease processes are associated with the passage of time after maturity. These include chronic renal disease, tumors, myocardial degeneration, skeletal muscle degeneration, and skeletal disease (Everitt, 1982; Shock, 1977). In man, the majority of deaths have been attributed to a small number of diseases, including primarily: atherosclerosis, hypertension, and malignant neoplasms as the three current major causes of death (Kohn, 1971; Timiras, 1972). It is often considered that these develop secondarily to physiological aging in which the systems become more vulnerable to disease. Many theories have been proposed for the role of diseases in aging. It has been suggested that any decline in organ function with age may indicate the presence of some subtle or more recognizable disease process, or the disease-attributed decline may be superimposed on some negligible "normal" aging decline. It has also been proposed that if there is one, or a small number of basic aging processes that predispose all members of a population to all of the diseases that are age-dependent, then "aging" itself would constitute the 100% fatal "disease," Thus, the characteristic changes of a particular disease may be superimposed upon basic or so-called "normal" age changes even during senescence (Ordy,

1975). There is convincing evidence that beginning aging changes in an organ or tissue are associated with disease, although there is overwhelming evidence that accumulation of declining body functions renders individuals more susceptible to disease.

B. Different aging theories

The various theories of aging can be classified into genetic and non-genetic categories. Genetic theories emphasize genomic pre-programmed events which determine the species-specific life span. Non-genetic aging theories emphasize more causal factors such as environmental influences or accumulation of end products which induce chemical and structural changes that lead to cellular dysfunctions or death. Both genetic and non-genetic theories view changes in the DNA molecule or errors in transfer of information from DNA as resulting in a cascade of dysfunctions that lead to cellular senescence and death.

1. Genetic program for aging theory

The neuroendocrine system normally regulates or helps to regulate such important functions as reproduction, body and bone growth, protein, carbohydrate, fat, mineral and vitamin metabolism, thyroid function, adrenal cortical activity, hemapoesis, kidney and liver functions, the immune system (Makinodan, 1977; Meites et al., 1987). It appeared logical that the neuroendocrine system should have at least as

important a role in regulating body functions during later life as it has in controlling these functions during youth and maturity. Thus, the suggestion emerged of not only a genetic program for growth and development, but also a genetic program for the aging process.

2. Immune theory

The immune system protects the body in a highly specific manner against foreign invasion by viruses, bacteria, fungi, and neoplastic cells by seeking them out and destroying them. Thus, the immune system plays a major role not only in the preservation of life (Makinodan, 1977). The bone marrow and thymus are primary organs of the immune system and serve as the source of precursor cells. The spleen and lymph nodes are secondary organs of immunity and are the site of immunity initiation. According to current thinking, there are two distinct types of immunocompetent lymphocytes. The "B" cell develops in the bone marrow and is responsible for the humoral antibody response. "T" cells develop in the thymus are responsible for cell-mediated responses (delayed hypersensitivity, rejection of foreign tissues and organ grafts, immunity against tumor cell, etc.) (Makinodan, 1977). The first suggestion that normal immune functions may decline with age came from morphology studies showing decreases in the thymic lymphatic mass with age, beginning at the time of sexual maturity (Andrew, 1952).

The increasing susceptibility to disease and mortality with age have suggested that immunological reactions and tolerance may constitute fundamental mechanisms of aging (Walford, 1982). A general decline in immune competency occurs with aging, and this has been attributed to changes in functional capacity of both T and B cells populations (Segre and Segre, 1977; Freidman and Globerson, 1978a, 1978b). As normal immune functions decline with age, the incidence of infections, autoimmunity, and cancer increases (Walford, 1974; Chino et al., 1971; Mackay, 1972; Peterson and Makinodan, 1972; Smith et al., 1973). This age-related decline in immune function is believed to be an important contributor to senescence and to the development of chronic diseases and disorders (Burnet 1974).

3. Cellular theories

It is now known that normal cells derived from almost all vertebrate and many insect tissues can be cultivated in vitro for varying periods of time. Such cell populations divide for a finite number of generations and, after cessation of mitotic activity, the culture finally undergoes total degeneration (Hayflick, 1977). It follows, therefore, that if normal animal cells have only a limited capacity for division in cell culture, then manifestations of aging might very well have an intracellular basis. Subsequent to these studies, a report not only confirmed these findings but extended them significantly to show a decrement of Ø.2Ø

population doublings per year of donor life occurs in the cultured normal human fibroblasts (Martin et al., 1970). The replication of skin fibroblasts obtained from a nine-year-old boy with progeria, a condition associated with accelerated aging, were shown to be severely restricted (Goldstein, 1969, 1971). Other studies of cells in vivo suggest that functional and mitotic capacity decline with age (Daniel, 1977; Hayflick, 1977). Cellular theories suggest that aging of all organisms is the result of developmental changes within single cells. The concept of limited mitotic potential suggests expression of aging at the cellular level. However, functional and mitotic capacity of these cells (although reduced with age) persist longer than the life span of the organism (Daniel, 1977; Hayflick, 1977). This suggests that this may not be responsible for the general decline in physiological function (Meites et al., 1987).

4. DNA breaks and repair theory

There are two models concerning somatic mutation as a cause of aging. The first suggests that somatic mutation is a result of intrinsic mutagenesis at the time of division. The second suggests a time-dependent mutagenesis in resting cells (Sinex, 1974). DNA repair theory states that aging is associated with reduced ability to repair spontaneous DNA breaks which would allow mutations to accumulate. Many genes would then be altered leading to the production of

non-functional proteins. Cellular death would occur when the number of "non-functioning" proteins reaches a critical point leading to loss of functional capacity of the organism. It has been reported that fibroblasts from animals with longer life spans can better repair breaks in DNA than animals with shorter life spans (Kanugo, 1980). However, there is no evidence that the ability to repair DNA actually declines in normal aging animals (Tice, 1978).

Medvedev (1961) stated that errors during the transfer of information (e.g., transcription and translation) could induce formation of defective proteins that resulted in aging of cells and tissues. Strehler (1977) suggests that as cells age they lose their ability to translate genetic information from mRNAs. Improper formation of proteins may be produced by incorporation of an incorrect amino acid or synthesis of the wrong amino acid sequence. These alterations in proteins are believed to contribute to the age-associated decline in function, the development of age-related pathologies, and death. Fruit flies with experimentally induced errors had shorter life spans than normal fruit flies (Harrison and Holliday, 1967). With advancing age, tissues and cells were stated to contain enzymes that are defective, i.e., they change in heat lability, substrate specificity, or specific activity (Shock, 1977; Corsellis, 1975). However, studies on other enzymes and proteins have shown no changes with age (Kanugo, 1980; Van Keuren et al., 1983). Errors in amino acid

substitution have not been shown to occur to any significant degree with increasing age either *in vitro* or *in vivo* (Kanugo, 1980).

5. Free-radical and cross-linkage theories

The free radical theory of aging proposed by Harman (1984) states that aging originates from damage by free radicals which arise as end products of cellular metabolism and contain unpaired electrons that are highly reactive. Free radicals create cross links which deactivate functional molecules and promote other cellular damage as well. Although antioxidants inhibit formation of free radicals, they have not been shown to increase maximum life span when fed to animals (Meites et al., 1987). Agents produced during normal cellular metabolism, other than free radicals, may also produce cross-linking (Bjorksten, 1974). These include aldehydes, and molecules with one or more ionized groups which may irreversibly inactivate functional molecules. They apparently accumulate with advancing age. Data supporting the cross-linking theory of aging include age-related increases in insoluble collagen and decreases in extractable chromosomal proteins, both believed to be the result of cross-linking. However, cross-linking within DNA has only been demonstrated *in vitro*, not *in vivo* (Meites et al., 1984).

6. Lipofuscin

Accumulation of cellular debris (lipofuscin) is one of the most distinguishable age-related changes. Increases in lipofuscin in the cytoplasm are accompanied by a reduction in the number of mitochrondria, rough endoplasmic reticulum, simplification of the Golgi complex, and vacuolation of cytoplasm (Strehler, 1977). The origin of lipofuscin is unknown but is assumed to be derived from lysosomes or mitochondria and results from peroxidation of polyunsaturated lipids by free radicals. It is assumed that the large increase in lipofuscin leads to disruption of normal cellular function, but there is no clear evidence that accumulation of lipofuscin leads to age-related changes in enzymes, proteins, nucleotides, DNA, or cellular membranes (Meites et al., 1984). Because of its apparent universal occurrence in phylogeny (Timiras, 1972) and its progressive and irreversible accumulation in postmitotic cells, this process has been proposed as a "basic law" of cellular aging (Strehler and Barrows, 1970). It is not known whether lipofuscin accumulation is a fundamental part of the aging process per se or whether it is the result of various types of insult and injury to the animal.

7. Stress theory

Continuous exposure to stress is believed to increase physiological aging, accelerate the onset of age-related pathology, and shorten life in animals (Selye and Tuchweber,

1976). The effects of stress are mediated via the hypothalamo-pituitary-adrenocortical axis. The hormones secreted during stress (ACTH, corticosteroids, growth hormone (GH) in humans, and catecholamines), produce pathological lesions similar to those seen in old age. This theory applies to the hypothesis of "wear and "tear" and to auto-intoxication at the organismic level, and combines them with neuroendocrine theories of aging involving the hypothalamo-pituitary-adrenal axis (Meites et al., 1987). Long-term exposure to environmental stimuli may decrease and eventually exhaust the ability of organs to maintain homeostasis. According to Selye's "adaptation syndrome" theory, individuals are born with a fixed quantity of adaptive energy which is progressively reduced with each exposure to stress (Selye, 1946). Exposure to a variety of stresses is believed to lead first to activation of defense mechanisms necessary for survival (alarm reaction), followed by a period of enhanced adaptive capacity (stage of resistance), and terminating in a state of exhaustion. However, most of the present evidence shows no significant changes in basal adrenocorticotropin and adrenal cortical hormone secretion during aging, although some studies show a lower output of adrenal cortical hormones in response to stress in old relative to young animals (Riegel, 1983). Although a persistence of severe forms of stress may accelerate some aging parameters there is evidence in

rodents that mild stress may actually prolong life (Meites and Quadri, 1987).

C. The aging program

1. Genetic program--biological clock for growth and development

Many studies have described aging as an intrinsic process that is genetically determined. There is believed to be an orderly sequence of changes which appear to be programmed from the time of fertilization when the genetic code is laid down, until death. Physiological events are considered to be precisely timed during the developmental period, but not in the involutional period except for the menopause in women (Timiras 1972). Instead, there is a progressive decline in a large number of physiological parameters from age 25 to 80 years in man (Shock, 1977; Everitt, 1982). The sequence of change and rate of change appears to be clearly programmed and is presumably under genetic control. One theory proposes the existence of a biological clock somewhere in the brain, perhaps in the hypothalamus, which regulates body function; the clock mechanisms become disrupted with aging and lead to general deterioration of body functions. A related theory by Timiras (1983) is that aging is due to disruption of a "pacemaker" in the neuroendocrine system that controls the rhythmic release of hormones. Dilman (1971) proposed that the

neuroendocrine program of aging depends on intrinsic changes of the hypothalamus which elevates the threshold to feedback suppression. Gradual elevation of feedback threshold leads to increased stimulation of the pituitary which promotes growth and development. Continued threshold elevation throughout life causes alterations in reproduction, energy, and adaptation homeostasis which is maintained by compensatory oversecretion of hormones by appropriate endocrine glands. Hormone excess causes "diseases of compensation" which in man are age-related obesity, the climacteric state, reduced resistance to infection, and several varieties of cancer. Terminal diseases are thus caused by oversecretion of aging hormones.

2. Internal and external environmental factors

The rate at which programmed changes develop can be influenced by extrinsic factors such as food supply, environmental temperature, stress, and radiation. The hypothalamus and pituitary play an important role in mediating the effects of extrinsic factors on programmed aging. The brain acts as an interface between the environment and the organism in maintaining homeostasis. Endocrine secretion is regulated by the hypothalamus, and can change to meet the demands of the environment. Hormones help to maintain life in a hostile environment, but at the same time may accelerate aging (Everitt, 1976, 1982).

Further explanation of hormones and accelerated aging will be presented in a later section.

Studies of isolated cells in vitro have provided a method for studying the role of intrinsic factors in aging processes. However, one must keep in mind that cells do not exist in isolation and are influenced by contiguous cells. These effects may include paracrine influences, hormones, nutrients, products of metabolism, blood gases, acid-base balance, temperature alterations, hemodynamic changes, cell-to-cell communication, toxins, antibodies, as well as others (Meites et al., 1987). Many of these are subject to change by environmental influences which are mediated via afferent central nervous system (CNS) input. The homeostatic responses are then integrated through neuroendocrine mechanisms. The resultant changes in hormone secretion, particularly if chronic, can profoundly alter many body functions and influence aging processes (Meites et al., 1987).

D. Neuroendocrine system in control of aging processes

In highly organized animals, cellular function and survival are critically dependent on homeostatic and integrative mechanisms provided by the neuroendocrine and immune systems (Kohn 1971; Timiras 1972; Meites et al., 1987). Genomic expression in the "command" cells of homeostatic and integrative systems are important for determining when and where body aging changes will occur. Environmental influences also may contribute significantly

to aging of body functions, including those functions of the neuroendocrine system. The neuroendocrine system represents the main source of compensatory mechanisms which are known to become increasingly important during aging (Welford, 1965; Comfort, 1968; Ordy and Schjeide, 1973). A dysfunction that develops in one or more components of the neuroendocrine system may disrupt normal regulation of many body functions. Such dysfunctions may occur spontaneously at any stage of life, but appear to become more prevalent with advancing age. These neuroendocrine alterations result in a cascade of aging manifestations (Welford and Birren, 1965; Timiras, 1972; Meites, 1987).

1. Changes in hypothalamic neurotransmitters with age

Hypothalamic neurotransmitters are important in regulating the release of hypothalamic hypophyseal hormones (HHH) into portal vessels. Dopamine (DA), norepinephrine (NE), serotonin (5HT), and endogenous opiate peptides (EOP) appear to be the most important neurotransmitters for regulating release of HHH, although others may have a role. Their neurons are highly concentrated in the hypothalamus, particularly in the median eminence where they abut to the neurons containing HHH. The catecholamines (CAs), particularly DA and NE, modify the secretion of several pituitary hormones. NE stimulates the release of LH, FSH, GH, and TSH, whereas DA inhibits release of PRL and TSH but promotes release of GH (Meites et al., 1977; Weiner and

Ganong, 1978). Only DA has been shown to act directly on the pituitary to influence release of PRL whereas its effects on release of other pituitary hormones are exerted via the HHH (Meites and Sonntag, 1981).

CAs are reduced in the pituitary stalk portal vessels, in the hypothalamus, and in other brain areas in old male and female rats. Concentrations of NE were reduced in the medial basal hypothalamus and preoptic area, and anterior hypothalamus, but not in the ME or striatum (Simpkins et al., 1977). CA concentration in many brain areas also was observed to be reduced in elderly human subjects (Robinson et al., 1972).

The causes for the decline in CAs in the hypothalamus and other brain areas are not completely understood. There is evidence in rats and man that the number of neurons in the locus coeruleus, a major source of noradrenergic innervation to the hypothalamus and other brain areas, is reduced during aging (Simpkins, 1983). Peng (1983) reported a significant loss of neurons in the preoptic area, HHH, and arcuate nucleus of old rats. Cell loss in other areas of the aging brain also has been reported (Brody 1955; Corsellis 1976). Neuron loss in the brain normally occurs throughout life but their loss in the hypothalamus during aging may be particularly significant because of their neuroendocrine functions.

2. Changes in hypothalamic hypophysiotropic hormones with age

The HHH act directly on the pituitary to regulate secretion of pituitary hormones. Relatively little is yet known about possible changes in HHH synthesis and release during aging. There may be loss of some of these neurons with age as well as a reduction in the enzymes necessary for synthesis leading to decrease in certain HHH with age. The effects of HHH on the pituitary may be reduced due to loss of receptors or to other changes within the pituitary cells. Harman (1983) observed a progressive decrease with age in the ability of GnRH to elicit LH and FSH release in human male subjects.

Significantly lower GnRH was found in the hypothalamus of old constant estrous (Wise and Ratner, 1980) and anestrous rats (Steger et al., 1979). A decrease in GnRH concentration was also observed in the ME, arcuate nucleus, and organum vasculosum of the lamina terminalis in old male rats (Simpkins, 1983).

When median eminence tissue from old and young male rats was perfused in vitro, more somatostatin was released from tissues from old than from tissues from young rats (Sonntag et al., 1985). When a high dose of somatostatin antiserum was injected into old and young rats, a larger increase in GH release was observed in old than in young rats (Sonntag 1981), also indicating that more somatostatin is released by old than by young rats. This probably accounts for the lower

pulsatile GH secretion in old than in young rats (Sonntag et al., 1983).

Less TRH was reported to be present in the hypothalamus of old than young male Sprague-Dawley and Long-Evans male rats, but circulating TSH concentrations were reported to be reduced only in the Long-Evans strain (Perkary et al., 1984).

Most studies have shown no differences in circulating ACTH and adrenal glucocorticoid hormone levels between old and young animals or human subjects (Riegel, 1983). Synthetic CRF (1-41) was reported to induce less release of ACTH and corticosterone in vivo in old than in young male rats (Hylka et al., 1984). This may be related to the lower ACTH released in response to stress in older animals reported by some but not all investigators (Riegel, 1983).

3. Changes in responsiveness of endocrine glands and tissues to hormone release

During aging there may be alterations in cleavage of peptide hormones from their pro-hormones, and an increased incidence of polymorphic forms during development and with advancing age (Orgel, 1963). Other changes might involve the conversion of one hormone to another (e.g., T_4 to T_3) not only in the brain, but also in the liver and other tissues. Since the various hormonal forms have different biological potency, changes in hormone metabolism with age may explain altered target tissue responses to neuroendocrine stimuli.

There may also be altered tissue responsiveness due to receptor loss and intrinsic age-related changes in tissues, cells, and intracellular molecules (Timiras, 1983).

Hormone receptor concentrations for corticosteroids are reduced during aging in rats in cortical neuronal perikarya, adipocytes, and splenic leukocytes (Roth, 1974, 1975; Rosner and Cristofalo, 1978). Androgen receptor concentration and responsiveness are reduced in the rat during aging (Roy et al., 1974; Robinette and Mawhinney, 1977). Estrogen receptors in the rat and mouse uterus decline with advancing age (Holinka et al., 1975).

The role of post-receptor alterations must also be considered. In rat adipocytes, age changes in phosphodiesterase activity appear to contribute to the reduction in glucagon-stimulated lipolysis (Trabucchi et al., 1981). The age change in regulation of calcium mobilization appears to be responsible for impaired B-adrenergic control of contractile performance (Guarnieri et al., 1980). Another B-adrenergic response impaired during aging is the stimulation of adenyl cyclase in human lymphocytes (Krall et al., 1981a; Krall et al., 1981b). Data supporting at least a partial role of post-receptor defects in the age-related reduction in lymphocyte response comes from experiments in which young and old membrane preparations were mixed in the presence of guanyl nucleotides and Triton X-100. Age differences were observed in the kinetic and regulatory properties of adenylate

cyclase (Krall et al., 1981). Thus, age changes may occur in the catalytic subunits of adenylate cyclase or in the concentration of guanyl nucleotides requiring coupling factors. Age-related changes involving the cell membrane also occur for the glucose transport system of rat adipocytes. This becomes generally insensitive to both positive and negative regulation as age increases (Roth et al., 1979). The impairment in transport may explain the reduced inhibition of glucose oxidation seen upon exposure to glucocorticoids, which appears to be partly due to age-related receptor loss as well (Roth et al., 1976). In addition to loss of adipocyte glucagon receptors with increasing age, an increase in phosphodiesterase activity contributes to decreased lipolytic responsiveness (Livingston et al., 1974). The reduced ability of gonadotropin to stimulate testosterone production in aged rat Leydig cells seems to be due to changes in intracellular responsiveness to cAMP (Tsitouras et al., 1979). Although gonadotropin receptors decrease during aging, these appear to be of the "spare" type and generation of cAMP is not altered. However, if dibutyryl cAMP is administered to cells, the age-related impairment in testosterone production is still evident (Tsitouras et al., 1979; Chouknyiska, 1981). These results suggest a role of intracellular changes with age as sites for expression of aging phenomena in tissues and endocrine glands.

4. Effects of hypophysectomy

Hormones, by stimulating their target organs, accelerate the aging program of those organs and ultimately the whole body (Everitt, 1982). A number of investigators have suggested that pituitary hormones have a significant role in aging processes (Everitt, 1973; Denkla, 1974). Everitt et al. (1973) were first to demonstrate that hypophysectomy can retard certain age-related changes in body tissues. Collagen was shown to age more slowly in hypophysectomized rats than in normal controls (Olsen and Everitt, 1965). Studies in hypophysectomized animals have also revealed reduced proteinurea associated with common chronic kidney disease of laboratory rodents (Everitt and Cavanaugh, 1965), decreased aortic wall thickness and decreased numbers of tumors (Everitt et al., 1980). Hypophysectomy of Wistar rats in early life (age 70 days), in combination with cortisone replacement, was shown to substantially increase maximum life span (Everitt et al., 1980). Many of the observed changes following hypophysectomy resemble those of food restriction. Everitt et al. (1980) reported only one significant difference in both physiologic and pathologic changes following underfeeding and hypophysectomy: animals started at mid-life on food restriction showed no change in collagen aging, whereas hypophysectomized animals had diminished collagen aging. Jones and Krohn (1961) found that the loss of rat oocytes with age was greatly retarded by hypophysectomy. However, the decline in oocytes progressed

most rapidly shortly after birth, when gonadotropin stimulation is minimal.

5. Excess hormones and aging

In rats exposed to low temperatures there is activation of the hypothalamo-pituitary thyroid axis with increased TRH, TSH, T_3, T_4 release. In such cold-acclimated rats life span was diminished with premature onset of certain disease of old age such as nephritis and periarteritis (Johnson et al., 1963; Heroux et al., 1960). The hormonal mechanisms were complicated by concurrent hypersecretion of corticosteroids and CAs in these cold-adapted rats (Heroux, 1969). Thus, there was an associated hypersecretion of pituitary, thyroid, and adrenal cortical hormones with accelerated aging and shortened life span.

Proteinurea development during aging in hypophysectomized rats has been shown to be increased by chronic GH injections begun early in life. Proteinurea was significantly greater than in intact rats eating the same quantity of food (Everitt, 1982).

The hyperadrenocorticism of Cushing's disease has been associated with accelerated cardiovascular aging (Wexler, 1976) in the form of hyperlipidemia, hypertension, arteriosclerosis, myocardial infarction, and cerebrovascular accidents.

6. Reversibility of aging processes by neuroendocrine intervention

Studies in both humans and animals have suggested that deficiencies in brain aminergic transmitters occur with aging (Simpkins and Meites, 1977; Wise, 1983; Rogers and Bloom, 1983). L-dopa administration has been effective in reducing symptoms and slowing the progression of Parkinsons' disease. Also L-dopa and other drugs or hormones have been shown to reinitiate estrous cycles, inhibit growth of mammary and pituitary tumors, and elevate GH secretion and protein synthesis in old rats (Meites et al., 1987).

Early ovariectomy prevents the development of gliosis normally found in the arcuate nucleus in old CE rats (Brawer et al., 1980; Schipper et al., 1981) and to maintain the hypothalamic mechanism regulating reproductive capabilities far beyond those of intact rats. By addition of L-tyrosine to the diet starting at 7.5 months of age, Cooper and Walker (1979) observed a significant extension of the period of ovarian cyclicity in rats which was attributed to achievement of an appropriate CA to 5HT balance within the CNS. Administration of L-dopa or other drugs that increase CAs in the brain resulted in reinitiation of estrous cycles in old-noncycling female rats and increased testosterone secretion in old male rats (Clemens et al., 1969; Quadri et al., 1973a; Cooper et al., 1979). Restoration of rat prostate androgen receptors as well as androgen inducibility of enzymes by chronic androgen administration has been

reported (Pagano et al., 1981). The development of several age-related changes in rats is directly controlled by the neuroendocrine system, and probably are not the result of some general change in cellular function throughout the body (Meites et al., 1987).

E. Modulation of aging processes

1. Methods used to alter the rate of aging

The rate of aging of many body organs and tissues and their capacity to respond to appropriate stimulation appear to be determined primarily by the neuroendocrine system. Reduced function of some organs and tissues with age can be delayed or even reversed by neuroendocrine interventions.

Other methods have also been employed to alter the rate of aging. A wide range of environmental, chemical, hormonal, and pharmacological interventions have been examined for their ability to extend life span and to arrest aging processes in a variety of organisms. Methods employed include nutritional manipulation, lowering of body temperature, exercise, antioxidants, including vitamin E, neurotransmitter modulation, hypophysectomy, immunological interventions, and most recently, transplantation of neuronal tissue to specific brain areas (Schneider and Reed, 1985).

It is important to recognize that the changes in neuroendocrine activity during aging precede the associated alterations in functions of organs and tissues. However,

there may be limitations in the capacity of the neuro-endocrine system to maintain functional capacity of tissues and cells because of a progressive decline in peripheral cellular function that is independent of the neuroendocrine system. Whether estrous cycles can be maintained for the entire life span of rats, whether the appearance of mammary and pituitary tumors can be indefinitely postponed, and whether synthesis of protein can be persistently elevated remains to be determined.

2. Underfeeding is the only proven method to delay aging processes and increase life span in rodent species

The intervention that has most consistently been demonstrated to increase longevity and to affect the largest range of aging processes is food restriction. This has only been shown in rats, mice, and hamsters (McCay and Crowell, 1934; Barrows and Kokkonen, 1977). If is of interest that this intervention has been shown to be successful even if begun at mid-life. Many of the other interventions that have been reported to increase life span also produce weight loss, such as the combination of hypophysectomy and glucocorticoid hormones, or a tryptophan-deficient diet. Underfeeding has been shown to consistently increase longevity, delay the physiological decline in organ function, delay the onset of age-related diseases in rats and mice, and extend reproductive functions. Such animals often look and function like younger animals.

II. Undernutrition and Aging

A. Retardation of aging by food restriction

1. Historical background

Maximum life span of several rodent species (rats, mice, hamsters) can be extended by 50% or more by dietary restriction. McCay and Cromwell (1934) first reported that restricting the food intake of rats markedly increased longevity. This finding has been repeatedly confirmed in many subsequent studies. They found that rats were severely retarded in body growth by being fed a nutritionally adequate and balanced, but calorically deficient diet Postweaning rats fed a deficient diet had much longer mean and maximum life spans than control animals fed ad libitum.

Food restriction appears to influence longevity by retarding aging processes (Masoro 1985). This increase in mean and maximum survival times by dietary restriction has been demonstrated in diverse species including the protozoan Tokophyra (Rudzinska, 1962), rotifers (Fanestil and Barrows, 1965), Daphnia (Ingle et al., 1937), fish (Comfort, 1963), rats (McCay et al., 1935, 1943), and mice (Lee et al., 1956; Lane and Dickie, 1958; Silberberg et al., 1961) Only dietary restriction convincingly reduced mortality rates (Ross, 1972) and increased maximum survival times (Berg, 1960; Nolan, 1972) in homeotherms. Review of the evidence shows that old rodents on dietary restriction are

biologically younger than are age-matched controls (Silberbeg et al., 1961; Gerbase-DeLima et al., 1975; Comfort, 1963; Rudzinska, 1962); however, the precise mechanisms by which dietary restriction retards aging remains unknown.

2. Retardation of aging processes

Reduced food intake retards several physiological age changes. Long-term caloric restriction in the rat or mouse retards the rate of aging in tail tendon collagen fibers (Chvapil and Hruza, 1959; Giles and Everitt, 1967; Everitt, 1971), delays the age of onset of puberty (Merry and Holehan, 1979; Kennedy and Mitra, 1963; Glass et al., 1976), prolongs duration of reproductive life (Berg, 1960; Merry and Holehan, 1979; Carr et al., 1949), and delays age-related changes in serum lipids (Liepa et al., 1980). Food restriction in the young rat appears to delay the development and maturation of neuroendocrine functions (Segall et al., 1978). Caloric restriction has also been reported to decrease accumulation of liver collagen, and to increase both seminal vesicle weight and body growth period in response to growth hormone (GH) in rats (Hruza and Fabry 1957).

Biochemical studies have demonstrated that caloric restriction in rats partially delayed or prevented age-related (1) declines in lipolytic response of adipocytes to glucagon or epinephrine, (2) declines in post-absorptive

serum free fatty acid levels, (3) increases in post-absorptive cholesterol levels (Masoro et al 1980; Liepa et al. 1980), and (4) changes in isoproterenol-induced relaxation of rat aortic strips (Herlihy and Yu 1980). Moreover, calorically restricted rats do not show the same increases with age in serum triglyceride levels (Reaven and Reaven 1981a), serum insulin levels, or pancreatic islet pathology as sedentary control rats (Reaven and Reaven 1981b).

Berg and Simms (1960) hypothesized lack of excess body fat as a major causal factor in the increased longevity associated with food restriction. The view developed that there was a direct relationship between adiposity and mortality (Lew et al. 1979). However, Stuchlikova et al. (1975) found that food restriction for the first year of life in rats resulted in obesity when subsequently fed ad libitum; however, they lived longer than less obese rats fed ad libitum throughout life. Bertrand et al. (1980) reported no significant correlation between fat content and longevity in ad libitum-fed rats, but a positive correlation in food restricted rats between longevity and body fat content. Harrison et al. (1984) found that longevity of obese (ob/ob) mice was increased by food restriction and that it was significantly greater than the longevity of ad libitum-fed lean mice of the same strain, even though the former maintained a significantly greater fat content than the latter. These data suggest that it is unlikely that reduced

body fat content plays a significant role in the action of food restriction on aging processes.

3. Delay of age-related diseases and tumor development

Autopsy studies of food restricted rats revealed a decreased incidence of many diseases of old age (Berg 1976) such as chronic renal disease (nephroses, glomerulo-nephritis) tumors, periarteritis, myocardial degeneration, skeletal muscle degeneration, and skeletal disease. Dietary restriction delayed the age of onset of these diseases (Berg, 1976; Berg and Simms, 1960). Aging rats and mice develop numerous mammary and pituitary tumors (Russfield 1966). The incidence of mammary tumors may reach 80% in old female Sprague-Dawley rats and some mouse strains. Pituitary tumors also may occur in 50% or more of these rats and some strains of mice. Chronic dietary restriction has been shown to inhibit certain types of tumors, to decrease the frequency of neoplasms, and to delay the time of tumor appearance (Tannenbaum 1959; Ross 1976; Cheney et al. 1980; Yu et al. 1982).

In rats fed ad libitum, the incidence of tumors was proportional to the total caloric intake. In food restricted rats, the tumor risk over the life span of the animal was similar to that of ad libitum-fed rats over their shorter life span (Ross and Bras 1965; Ross et al. 1970). Caloric restriction does not affect all tumor types to an equal degree. Complex interrelationships exist among the level of

caloric restriction, protein intake, time of onset and duration of food restriction, tissue of origin, and type and degree of tumor malignancy (Ross and Bras 1973; Tucker 1979).

Pollard et al. (1984) reported that in rats the development of intestinal tumors, induced by the carcinogen methylazomethanol, can be inhibited by food restriction. Spontaneously hypertensive rats die of hypertension-related events when fed ad libitum; food restriction increases their longevity and death no longer results from hypertension-related events (Lloyd 1984). Diseases that shorten the life span of particular genotypes are retarded by food restriction. The progressive renal disease in NZBxN2W F mice, a result of autoimmune processes, is slowed by food restriction (Fernandes et al. 1978).

4. Influence of specific nutrients on aging

A reduced intake of protein, fat, or carbohydrates has only selective and limited effects on age-related physiological deterioration and disease. Yu et al. (1984) found that in rats restricting protein but not calories retarded the development of chronic nephropathy but did not influence the occurrence of neoplastic disease or the deterioration of most physiological systems. Davis et al. (1983) reported that protein restriction did not contribute to the survival promoting effects of calorie restriction. Reduced survival rates were noted in ad libitum-fed rats as

well as rats fed a diet low in both protein and calories. Renal function and survival were shown to be more sensitive to calories than quantity of protein ingested (Davis et al. 1983). High-fat diets have been shown to produce obesity and shorten life span (Stunkard 1983). Dietary obese rats have a shorter life span than rats on high carbohydrate diets even when the total calorie content of the diet was reduced.

The reduced intake of specific nutrients, unlike total food restriction, appears not to act on general aging processes, but rather to influence selectively specific physiological or pathological processes. Also, the design of some food restriction studies (Weindruch and Walford, 1982; Cheney et al., 1983; Yu et al., 1982, 1984) did not involve restriction of minerals or vitamins or both, which indicates that a decreased intake of these elements is not involved in the action of food restriction on aging.

Segall and Timiras (1976) have shown that feeding a diet deficient in an essential amino acid, tryptophan, can prolong the life span of rats and produce many other effects similar to caloric restriction including delayed fertility and reduced incidence of tumors. Rats on a tryptophan-deficient diet showed marked diminution in body weight gain. Thus, the observed effects of the tryptophan free diet may have been the result of simple caloric restriction.

The findings of Masoro et al. (1982) show that the lean body mass of rats is rapidly readjusted during food restriction so that caloric intake and the intake of other

nutrients per gram lean body mass are the same as those of ad libitum-fed rats. These results are contrary to the classical view (Sacher 1977) that food restriction acts by reducing the input of calories or another nutrient per unit of protoplasmic mass.

B. Initiation time and types of food restriction used

1. Food restriction initiated at weaning

Dietary restriction initiated at weaning in rodents (a 20-60% reduction in food intake) at 4 weeks of age was associated with a 10-300% increase in mean and maximum survival times (McCay et al., 1935; Ross, 1961; Tucker, 1979). In most studies on long-lived mouse or rat strains, maximum life spans for unrestricted animals ranged from 30-40 months vs. 40-50 months for rodents on weaning-initiated dietary restriction. Yu et al. (1982) studied male Fischer 344 rats on 60% food restriction beginning at 6 weeks of age. The mean life span was 32 months vs 23 months for ad libitum-fed controls and the maximum life span was 47 months vs 32 months for controls. Renal and other lesions occurred earlier in controls and progressed more rapidly. A similar longevity was observed by Merry and Holehan (1981) who imposed weaning-initiated 50% food restriction in male Sprague-Dawley rats.

In another study Merry et al. (1979) chronically underfed female rats from weaning by maintaining body weight

at 50% of control levels, and continued this dietary regimen for most of the life of the animals. This regimen produced similar anti-aging effects on reproductive function as that produced by the percentage food intake restriction methods. Nelson et al. (1985) restricted food intake of female C57BL/6J mice by alternating days of feeding and fasting for a 7-month period begun early in life. However, after several months the animals adapted by eating substantially more on the days of feeding to compensate for fasting days. At this time further dietary restrictions were imposed on the amount of food given in order to maintain adequate restriction of food intake. Similar anti-aging effects on reproduction were obtained here, too.

Immunologic aging is influenced by weaning-initiated dietary restriction. Female F hybrid mice exhibited a "younger" appearance of the thymus at 6 months of age than age-matched controls (Weindruch and Suffin, 1980). These mice had higher splenic T-cell proliferation induced by mitogens than ad libitum-fed controls (Weindruch et al., 1982). Caloric restriction begun after weaning resulted in improved immunological responses later in life to T and B cell mitogens, to sheep red blood cells, and to skin allografts, when compared with non-restricted age-matched controls (Walford et al., 1973/1974; Gerbase-DeLima et al., 1975). This improvement in immune competence in later life may be related to delayed maturation of the immune system

since food restriction depressed immunologic function prior to midlife (Gerbase-DeLima et al., 1975).

2. Food restriction initiated at adulthood

Compared with weaning-initiated dietary restriction, adult-initiated dietary restriction has been the subject of far fewer gerontologic studies. Initially it was believed that food restriction increased longevity by retarding growth and development (McCay et al., 1935), rather than by slowing the aging processes of mature adults. Barrows and Roeder (1965) reported that food restriction begun at 12 months of age in rats caused a small but significant increase in longevity. Subsequently, however, several reports have shown significant increases in longevity when food restriction was begun in adult life. Yu et al. (1984) reported that food restriction initiated in adult life was as effective in extending the life span of rats as that started at weaning. They also showed that food restriction limited to the early part of life (from 6 weeks to 6 months of age) had only a small effect on life span. These findings have focused attention on the probability that food restriction retards aging processes.

Cheney et al. (1983) reported that female B1ØC3F mice put on food restriction at 14 months of age lived 5-1Ø% longer (mean and maximum life spans) than controls. Mice on adult-initiated food restriction did not live as long as weaning-initiated food restricted mice. Tumor incidence was

reduced by adult-initiated food restriction. Thus, appropriate adult initiated food restriction can inhibit cancer and extend mean and maximum survival times (Weindruch, 1984).

Improved immune responsiveness has been reported when food restriction was initiated at 12 and 17 months in mice (Weindruch et al., 1979, 1982). Food restriction in midlife also produced a reduced occurrence of autoantibodies (Weindruch et al., 1982) and delayed development of immune-complex glomerulonephritis in strains of mice susceptible to "autoimmune" disease (Fernandes et al., 1976).

3. Severity of food restriction

Weindruch et al. (1986) utilized various severities of food restriction and determined beneficial effects on longevity, tumor incidence, and immune competence. They reported greater longevity associated with the increased severity of dietary restriction. The maximum life span of mice fed slightly restricted amounts of diet was 13% greater than that of ad libitum-fed controls. The maximum life span for the most severely restricted group was 51% greater than the ad libitum-fed controls. Mice restricted in both calorie and protein intake exhibited shorter mean and maximum life spans (about 5%) than mice fed the same number of calories of a high-protein diet. Beneficial effects on tumor patterns or on the age-related decline in T-lymphocyte proliferation were, like the effects on longevity, most striking in the

most severely restricted group. Gradual imposition of food restriction has been suggested by Weindruch and Walford (1982) to be more effective in obtaining prolongation of life.

Frisch (1972, 1980) suggested that a critical body weight or particular body composition triggers menarche in the human female. When the food intake of rats was reduced by 25% from weaning puberty was delayed and occurred at the same body weight and body length as in control animals (Wilen et al., 1978).

C. Mechanisms of action of food restriction

Before 1975 aging studies on dietary restricted rodents focused on its effects on longevity and disease incidence. More recently, dietary restriction studies have begun to emphasize mechanisms involved and have provided endocrinologic, immunologic, and biochemical insights. Yet the precise mechanism(s) by which dietary restriction influences aging remains unknown. This is most likely a result of the multitude of changes brought on by dietary restriction as well as the mysterious nature of biologic aging (Weindruch, 1984). Dietary restriction studies are yielding a clearer understanding of biologic changes associated with life span extension and may provide clues for ways to optimize the human diet.

1. Undernutrition and organ function

Undernutrition has beneficial effects on the function of various organs. Kidney function is retained better by dietary restriction, probably due to the reduction in protein intake, since beneficial effects to kidney function can be obtained by lowering only protein intake (Tucker et al. 1976; Johnson and Barrows 1980). Restriction in weight gain was effective in preventing the age-related increases in plasma insulin levels and triglyceride concentrations observed in sedentary rats fed ad libitum. These benefits were associated with retention of normal function and morphology of the endocrine pancreas (Reaven and Reaven, 1981a, 1981b). Chronic food restriction decreased brain serotonin levels (Segall et al., 1978) and retards the loss of striatal dopamine receptors in senescent rats (Levin et al., 1981). The maze learning ability of old restricted rats was found to be similar to that of young ad libitum-fed rats (Goodrick, 1984).

Chronic food restriction from weaning delayed thymus involution and the decline in T-cell immune response to mitogens, whereas it did not affect the B-cell response to mitogen (Gerbase-DeLima et al., 1975; Weindruch et al., 1979). It also delayed immunological maturation but immunological functions were maintained at a higher level in old mice (Walford et al., 1974).

Food restriction slowed gastric emptying, reduced vital capacity and other lung volumes, decreased both systolic and

diastolic blood pressure, lowered heart rate and GFR, decreased creatinine and urea clearance, and reduced muscular work capacity (Everitt, 1982). With less energy available from the diet, the body adapts by reducing the level of many organ functions. If wear and tear are significant factors in aging then a low level of organ function may be associated with increased life span of the organ (Pearl, 1928). It is not clear whether these decrements in organ function are due directly to a lack of nutrients at the tissue level, or indirectly by decreased secretion of pituitary and other hormones (Everitt, 1982).

2. Undernutrition and hypothalamo-pituitary function

In food-restricted rats, retarded growth, maturation, and aging may be partly due to reduced secretion of pituitary and other hormones which are necessary for these processes. In 1940 Mulinos and Pomerantz found that food restriction depressed many pituitary functions in the rat and described the state of undernutrition as a "pseudo-hypophysectomy." Later studies showed that food restriction not only depressed growth and endocrine functions of the pituitary, thyroid, adrenal cortex and gonads, but also reduced the corresponding blood hormone concentrations. Campbell et al. (1977) showed that either acute starvation (no food) for 7 days or severe chronic food restriction for 3 weeks (no food for 1 week followed by 1/4 ad libitum food intake for 2 weeks) significantly depressed circulating

concentrations of LH, TSH, GH, and PRL. FSH was depressed only in acutely starved rats. Thus, severe reductions in food intake resulted in reduced secretion of at least 5 anterior pituitary hormones. LH, FSH, TSH, and PRL were increased by hypothalamic releasing hormones (LHRH and TRH) in acutely starved and chronically food restricted rats. These results indicated that the reduced secretion of anterior pituitary hormones in food restricted rats is due primarily to reduced hypothalamic stimulation rather than to the inability of the pituitary to secrete hormones (Campbell et al., 1977).

There is evidence that tyrosine hydroxylase (TH), the rate-limiting enzyme for CA synthesis is decreased during food deprivation (Gibson et al., 1978). This may account for at least some of the reduced hormone concentrations observed during underfeeding since the CAs are involved in release of many hypothalamic hypophysiotropic hormones (Meites and Sonntag, 1981).

There is evidence that somatostatin may be involved in the suppression of GH, TSH, and PRL during starvation. Hugues et al. (1986) administered somatostatin antiserum to acutely starved rats. This resulted in a dose-dependent increase in plasma concentrations of GH, TSH, and PRL without changes in adenohypophysial somatostatin binding sites or binding kinetics.

EOP involvement in LH suppression during starvation has also been reported, since LH suppression can be reversed by

the opiate antagonists naloxone and naltrexone (Briski et al., 1984; Dyer et al., 1985).

3. Undernutrition, hypothalamo-pituitary function, and aging

It has been postulated (Everitt and Porter, 1973; Everitt et al., 1976) that the anti-aging action of food restriction is due to reduced secretion of an aging factor by the pituitary, since similar anti-aging actions of food restriction and hypophysectomy (with cortisone replacement therapy, only) retard collagen aging, inhibit the onset of certain diseases of old age, and prolong life in the rat (Everitt et al., 1980). Compared to intact full-fed controls, both hypophysectomized and food restricted rats are remarkably free of pathological lesions. Both procedures have similar depressing effects on body function such as heart rate, hemoglobin, white cell count, and creatinine excretion. However, the effects of hypophysectomy may be more severe than the effects of reduced food intake.

This anti-aging action of food restriction may have a hormonal rather than nutritional basis (Everitt 1982). Doubling the food intake of hypophysectomized rats by means of hypothalamic lesions does not increase the aging of tail tendon collagen fibers significantly nor increase proteinurea (Everitt, 1982). Proteinurea development in old age in hypophysectomized rats is increased by chronic GH injections begun early in life and the proteinurea is significantly

greater than in intact rats eating the same quantity of food (Everitt, 1982). Thyroidectomy retards collagen and renal aging but these anti-aging effects have been related to the lowered food intake since thyroxine does not increase collagen aging in food restricted thyroidectomized rats (Giles and Everitt, 1967).

Beneficial effects of early food restriction on aging and longevity have been proposed to be due to biochemical changes in the brain (Young, 1979), particularly the hypothalamus (Finch, 1976; Samorajski, 1977). Certain nutrients such as amino acids are necessary for neurotransmitter formation. A lack of tryptophan has a similar anti-aging action in the rat as caloric restriction (Segall, 1979), since the former also results in reduced food intake. Finch (1976) suggested physiological aging is due to changes in hypothalamic CA metabolism. Acute studies (Krieger et al., 1980) of food restricted rats showed a shift in the time of peak CA concentrations. However, in a chronic developmental study (Segall et al., 1978) food restriction failed to alter brain CA levels in female rats.

A model based on alterations in neurotransmitter metabolism in the hypothalamus resulting in reduced secretion of releasing hormones and hence pituitary and peripheral hormones has been proposed (Everitt, 1982). The decline in hormones then slowed tissue aging. In modification of this, a hypothalamic clock or center has been proposed whose effects depend on the actions of

neurotransmitters. The clock is thought to time the program of development and aging through peripheral hormones and neurosecretions. Hormones are believed to affect tissue development and aging by altering gene expression in the tissues (Adelman, 1976). Nutrients and metabolites also affect gene expression but the hormonal action is probably more important in higher animals (Maclean, 1976). The relative contributions of the direct aging action of nutrients and metabolites on tissue cells, and the indirect action via the hypothalamo-pituitary complex are unknown.

4. Overnutrition, hypothalamo-pituitary function, and aging

Overnutrition leading to overweight and obesity is associated with shortened life in man (Armstrong et al., 1951; Marks, 1960; Watanabe et al., 1968), the rat (Koletsky, 1975), and mouse (Lane et al., 1958). Life span is shortened due to increase mortality from degenerative diseases (Marks, 1960; Van Italie, 1979; Koletsky, 1975) of the cardiovascular system and kidneys, and from diabetes mellitus and liver disorders. The only evidence of accelerated physiological aging in obesity is the reported early onset of puberty in obese children (Bruch, 1941; Wolff, 1955) and the overfed rat (Kennedy et al., 1963).

In human subjects obesity is associated with elevated blood pressure, cardiac output (Alexander, 1963), and glomerular filtration (Gelman et al., 1972), and impaired

pulmonary function (Barrera et al., 1967), and reduced capacity for physical work (Dempsey, 1964). The major metabolic changes are increased blood glucose and triglyceride levels (Olefsky et al., 1975; Vaughan et al., 1980). There is also increased secretion of cortisol and insulin (Schteingart, 1965; Jackson and Mowat, 1970; Bagdade, 1968). Hypothalamic obesity in experimental animals is accompanied by hyperinsulinemia and reduced secretion of most pituitary and target gland hormones (Bray and York, 1970; Nosadini et al., 1980). Apart from the increased secretion of cortisol and presumably ACTH, there is no evidence of hypersecretion of pituitary hormones in the overnutrition of obesity. Hyperadrenocorticism of Cushing's disease is associated with accelerated cardiovascular aging (Wexler, 1976) in the form of hyperlipidemia, hypertension, arteriosclerosis, myocardial infarction, and cerebrovascular accidents. The extent to which the raised cortisol secretion in obese subjects contributes to cardiovascular pathophysiologic derangements is not known.

Activation of the hypothalamo-pituitary-thyroid axis in rats exposed to low temperatures produces a large rise in food intake (Hsieh and Ti, 1960; Johnson et al., 1964). In such cold-acclimated rats, life span is diminished by premature onset of certain diseases of old age such as nephritis and periarteritis (Johnson et al., 1963; Heroux and Campbell, 1960). The hormone mechanism is complicated by

the concurrent hypersecretion of corticosteroids and CA in cold-adapted rats (Heroux, 1969).

Thus, high food intake and hypersecretion of pituitary, thyroid, and adrenal hormones are associated with accelerated aging and shortened life span. However, the aging effects of high caloric food intake and hypersecretion of some hormones have not been separated, and further study is required.

5. Undernutrition effects on protein turnover and aging

A general characteristic of aging organisms is the progressive impairment of the ability of the organism to respond to stimuli. Because the responses to stimuli are largely protein-catalyzed reactions, changes in protein synthesis may play a role in the aging process. Ricketts et al. (1985) reported that protein synthesis by the kidney declined significantly with age and that this decline could be retarded by dietary restriction. The changes in kidney protein synthesis were inversely related to the changes in proteinurea that occurred with increasing age. Lewis et al. (1985), using 50% dietary restriction imposed at weaning, reported slowed whole body growth and retardation of the developmental decline in protein turnover. It was concluded that the dietary induced increase in longevity and retardation of aging processes was associated with enhanced protein turnover during the major portion of the life span of dietary restricted rats.

Lindell (1982) suggested that certain dietary regimens are capable of enhancing the syntheis of mRNA and probably also produce enhanced turnover of tissue protein. He proposed that the physiological "stress" produced by dietary restriction could enhance gene expression, and this may be a significant factor in maintenance of cellular homeostasis for a longer period.

III. Undernutrition and reproductive function in females

A. Control of reproductive function in the female rat

The neuroendocrine system is a complex system that consists of the brain/hypothalamus, pituitary, target glands (thyroid, adrenals, gonads), tissues directly controlled by the pituitary, and target glands, and other hormone-producing tissues. The hypothalamus contains neurons that secrete specific hypophysiotropic peptides that are released into the pituitary portal vessels to directly regulate pituitary hormone secretion, and neurotransmitters that modulate the release of the hypophysiotropic peptides into the portal vessels.

The functional interrelationship between the pituitary and hypothalamus has been firmly established. The first evidence of pituitary control by the CNS was discovered by Erdheim (1909), when he noted that gonadal atrophy was correlated with lesions of the hypothalamus. Aschner (1912)

later demonstrated that gonadal atrophy could be induced by placing a lesion in the anterior hypothalamus, while leaving the pituitary intact. Electrical stimulation of hypothalamic regions of the brain induced ovulation (Harris, 1937), and increased thyroid (Harris, 1948) and adrenal cortical secretion (De Groot and Harris, 1950). The effects of hypothalamic stimulation are specific since direct stimulation of the pituitary had no effect (Markee et al., 1946).

1. Neurotransmitters

Early work by Sawyer and colleagues (1947) showed that NE and epinephrine induced ovulation in rats and rabbits, whereas dibenamine, an adrenergic blocking agent, inhibited ovulation. Intraventricular infusion of NE was observed to induce release of gonadotrophic releasing hormone (GnRH) in the hypophysial portal circulation of the rat, and this is believed to be its mode of action. Reserpine, a drug which depletes hypothalamic stores of monoamines blocks ovulation (Barraclough and Sawyer, 1957). Both DA and NE are present in the hypothalamus and the concentrations of hypothalamic CA change in different physiological conditions associated with altered gonadotropin release (Kalra and Kalra, 1983). The rate of turnover of NE in the hypothalamus was accelerated following castration (Anton-Tay et al., 1969) and during proestrus (Simpkins et al, 1979). This suggests that increased release of CAs from synapses in the hypothalamus mediates gonadotropin release.

NE normally rises in the preoptic area, an important area for regulating cyclic GnRH release in the rat, just prior to ovulation (Simpkins, 1983). Under most physiological states, NE induces release of GnRH (Weiner and Ganong, 1978) although there is evidence that under some experimental conditions it may inhibit LH release (Sawyer and Clifton, 1980).

5-HT is involved in the rhythmic and phasic control of GnRH release (Walker, 1983). Electrical stimulation of midbrain raphe nucleus and the arcuate nucleus, which presumably elicited 5-HT release in the regions surrounding GnRH neurons in the medial basal hypothalamus, suppressed episodic LH secretion in ovariectomized rats, a response blocked by prior depletion of brain 5-HT levels or blockade of 5-HT receptors (Arendash and Gallow, 1978b; Gallo et al. 1977). Blockage of 5-HT reuptake by fluoxetine attenuated LH pulse amplitude (Rasmussen et al., 1981). Thus, 5-HT neurons have the potential to suppress LH release when called upon under various internal and external environmental challenges (Kalra and Kalra, 1983).

Much evidence suggests that the EOPs may be an integral component of the neural circuitry which exercises inhibitory control on GnRH secretion (Kalra, 1983). Morphine suppressed LH release in ovariectomized rats (Kalra, 1983; Sylvester et al., 1982). The site of action of EOP and opiate agonists is in the vicinity of GnRH neurons in the septal-preoptic-tuberal pathway (Kalra, 1981; Reymond et al., 1983) where

adrenergic neurons may be the putative loci for opiate receptors (Kalra, 1981; Kalra et al., 1982). Microinjection of morphine into the mesencephalic dorsal raphe nucleus of ovariectomized rats decreased pulsatile LH release (Johnson et al., 1982). Naloxone readily stimulates LH release in steroid-primed rats (Kalra et al., 1980; Sylvester et al., 1982). EOP are thought to exert an inhibitory effect on LH release throughout the estrous cycle (Kalra, 1983) the tone of which may vary in accordance with the pattern of circulatory ovarian steroids.

2. GnRH release

GnRH is a decapeptide that stimulates release of LH and FSH from the gonadotropic cells of the anterior pituitary (AP) in a number of species including man (Schalley et al., 1976). Administration of synthetic GnRH to females increases release of LH and FSH, stimulates follicular maturation, and results in ovulation. GnRH may have only a permissive role in the LH surge, with the major influence produced by steroid hormone alterations of pituitary sensitivity to GnRH action (Knobil et al., 1980).

GnRH has been localized in the retrochiasmatic, arcuate, and ME region, and in a prechiasmatic area that included the preoptic, anterior hypothalamic regions, and the organum vasculosum of the lamina terminalis. Immunoreactive GnRH in perikarya may represent a prohormone species of GnRH. Chromatography of hypothalamic extracts on Sephadex G-25

demonstrated three different molecular weight species of immunoreactive GnRH (Millar et al., 1977a) that do not represent aggregated or protein-bound GnRH. Estradiol implants for 2 weeks into ovariectomized female rats increased both the mature and high molecular weight forms of immunoreactive GnRH (Millar et al., 1977b). These data suggest the existence of a prohormone for GnRH that may be enzymatically cleaved into the mature form of GnRH, as it is transported down the axon terminal.

GnRH is secreted in a pulsatile fashion as are the gonadotropins, and the rate and amplitude of such pulses appear to be of paramount importance in determining the magnitude of the response of the pituitary. This releasing hormone also has a "self-priming" action on the pituitary which increases the amplitude of LH responses. It is generally accepted that the rate of endogenous GnRH release in conjunction with steroid feedback mechanisms controls the pituitary GnRH receptors (Kalra and Kalra, 1983).

3. Gonadotropin release

The amount of LH and FSH released by the pituitary is a function of both the concentration of GnRH applied to the gonadotrope and the pattern of its application. It was not until 1978 that *in vivo* studies in primates indicated that continuous exposure to GnRH led to a refractory pituitary while intermittent delivery of GnRH restored responsiveness (Conn, 1986). This explains why GnRH and its agonists can be

administered so as to provoke or inhibit gonadal function and why continuous exposure can actually evoke a functional castration. It is also possible to cure infertility in both men and women with GnRH deficiency. GnRH interacts with its plasma membrane receptor and causes receptor microaggregation. The activated receptor stimulates hydrolysis of inositol phospholipids and an influx of extracellular CA^{++}. The mobilization of CA^{++} activates calmodulin while diacylglycerol produced in the hydrolysis of inositol phospholipids activates protein kinase C (Conn, 1986). Calmodulin and protein kinase C synergistically alter cellular function to provoke gonadotropin (LH and FSH) release through a yet unidentified mechanism.

4. Sensitivity of hypothalamic feedback mechanisms and control

Hormones of the target glands can act back on the hypothalamus, pituitary, or both to help regulate secretion of hypothalamic and pituitary hormones (long-loop feedback). The pituitary hormones, particularly when secreted in large amounts can act on the hypothalamus to inhibit their own secretion (short-loop feedback). Administration of gonadal steroids to ovariectomized rats causes a decrease in gonadotropin levels in the blood (Ramirez and McCann, 1963; Ramirez et al., 1964).

Estrogen is the most potent steroid that inhibits LH: reductions in serum LH can be detected within 2 hours after

estrogen administration (Blake, 1977a). Progesterone, except in very large doses, has no effect in suppressing elevated gonadotropin concentrations after castration (McCann, 1962; Chen et al., 1977). Ovariectomy in female rats results in removal of the target organs for gonadotropins and results in increased release of gonadotropins (Ramirez and McCann, 1963; Gay and Midgley, 1969).

Similar results occur if female rats are exposed to long-term anti-estrogen administration (Docke, 1969). Ovariectomy also results in enlarged gonadotrophs in the pituitary. However, if the pituitary is removed and transplanted to other sites not adjacent to the hypothalamus, these enlarged cells do not develop (Hohlweg et al., 1932). These results suggest that regulation of gonadotropins is under tonic inhibition by the ovaries in female rats and is dependent upon hypothalamic input. This hypothalamo-pituitary-gonadal feedback loop can be either inhibitory or stimulatory in nature. These feedback effects of sex steroids are the major mechanisms controlling gonadotropin release.

The mature female shows a cyclical pattern of gonadotropin release and estrogen exerts both positive and negative feedback effects. The positive feedback action occurs at mid cycle when estrogen levels are high due to increased secretion from the maturing follicles. These relatively high levels of estrogen stimulate the preovulatory LH surge (positive feedback) and this surge is

responsible for rupture of the mature follicle and release of the ovum (ovulation) (Bennett and Whitehead, 1983). This positive feedback effect on LH secretion was first demonstrated by Hohlweg (1944) when he induced ovulation in prepubertal rats by administration of gonadal steroids.

The turnover rate of both hypothalamic DA and NE have been correlated with the differential effects of estrogen feedback. Most evidence now suggests that the preoptic area is the site where estrogen enhances GnRH release. GnRH release, as a result of electrical stimulation of the preoptic area, is enhanced in the presence of estrogen, but estrogen has no effect on GnRH release when electrical stimulation is applied to the medial basal hypothalamus (Sherwood et al., 1976). Estrogen increases the firing rate of hypothalamic and preoptic area neurons (Fink and Geffen, 1978). Progesterone is believed to shut off this estrogen-induced surge signal because progesterone reduced the firing rate of hypothalamic and preoptic neurons (Fink and Geffen, 1978). It is believed that this rise in blood progesterone is responsible for preventing a subsequent surge of LH the following day (Freeman et al., 1976; Blake, 1977a).

As progesterone gains dominance among the ovarian steroids, from the afternoon of estrus through noon of diestrus II, the pituitary response to exogenous GnRH gradually declines (Kalra and Kalra, 1974; Gabriel et al., 1983). As estrogen secretion increases, the pituitary response to GnRH also increased (Kalra and Kalra, 1974;

Cooper et al., 1973). This enhancement of LH release response to GnRH was observed to be directly related to the strength and exposure of the pituitary to estrogen during the ascending phase of estrogen secretion. Sustained estrogen action during this interval apparently increases the releasable stores of LH in the pituitary (Kalra and Kalra, 1974).

Despite a low correlation of hypothalamic GnRH and serum LH throughout the estrous cycle, the content of GnRH in the arcuate-ME region of the hypothalamus in male and female rats is influenced by the negative feedback effects of gonadal steroids under experimental conditions. Ovariectomy decreases hypothalamic GnRH content and increases pituitary secretion of gonadotropins, whereas estrogen reverses this effect. Progesterone acts synergistically with estrogen to increase GnRH content (Chen et al., 1977). Changes in GnRH content in the MBH occur in response to circulating steroids, basal levels of GnRH in rostral areas are not (Kobaysahi et al. 1978).

5. Control of pulsatile LH release

LH in ovariectomized rats is released in a pulsatile manner (Gay and Sheth 1972). The mechanism for this episodic secretion appears to be mediated by the hypothalamus and not the pituitary gland. Incubations of pituitaries have shown that the release of LH occurs in a non-pulsatile manner when the medium was perfused with constant concentrations of GnRH

(Osland et al., 1975). However, when GnRH was administered in a pulsatile manner, LH release also was pulsatile. Deafferentiation of the MBH in rats results in non-pulsatile LH release in ovariectomized rats (Blake and Sawyer, 1974; Arendash and Gallo, 1978a). Hypothalamic NE stimulates pulsatile LH release in ovariectomized rats. Drugs which block NE synthesis (Drouva and Gallo, 1976; Grodde and Schuiling, 1976) or block adrenergic receptors (Weick, 1977) inhibit pulsatile LH release.

Brain 5-HT is involved in suppression of pulsatile LH release in ovariectomized rats. Electrical stimulation of the midbrain dorsal raphe nucleus resulted in suppression of episodic LH secretion (Arendash and Gallo, 1978). When rats were pretreated with 5-HT synthesis inhibitors or 5-HT receptor blockers, stimulation of the dorsal raphe nucleus had no effect (Arendash and Gallo, 1978a). Administration of B-endorphin has also been demonstrated to inhibit pulsatile LH secretion in castrated rats (Konoshita et al., 1980).

6. Effects of environmental factors

The midbrain, forebrain and limbic system provide significant inputs to the hypothalamus which probably subserve the effects of external (visual, auditory, and olfactory) and internal factors on GnRH release. The limbic system conveys the olfactory signals, which are important in reproductive behavior in lower animals while light signals

could affect gonadotropin secretion via the direct retino-hypothalamic pathway (Bennett and Whitehead, 1983).

Stress affects GnRH and LH release. Evidence suggests that stressful stimuli promote an increase in activity of central opiatergic neurons (Madden et al., 1977). Acute stress exposure elicits an increase in LH (Ajika et al., 1972; Krulich et al., 1974), whereas chronic stress resulted in a reduction in LH below baseline values (Gray et al., 1978; Tache et al., 1976). It is believed that chronic stress impairs hypothalamic neuroendocrine stimulation of LH since pituitary responsiveness to exogenous GnRH is not altered by stress (DuRuisseau et al., 1979). Dietary restriction also inhibits GnRH and LH release (Holehan and Merry, 1985). More detailed information will be provided in subsequent sections.

B. Age-related reproductive decline

1. Manifestations of reproductive decline

Aging female rats usually show no evidence of cyclic surges of LH and FSH nor a rise in estrogen secretion every 4-5 days as is seen in normal cycling female rats. At the age of about 8-15 months, female rats progress from regular to irregular estrous cycles, then to constant estrus (CE) or pseudopregnancies of irregular length, and finally to an anestrous state (Huang et al., 1975). The CE state may last for many months and is characterized by ovaries with many

follicles but no evidence of ovulation or corpora lutea. The pseudopregnant state is characterized by ovaries with numerous corpora lutea that actively secrete progesterone. Rats between 2-3 years of age exhibit an anestrous state characterized by atrophic ovaries containing only small, undeveloped follicles, with little evidence of estrogen secretion as indicated by an infantile appearing uterus. In old male rats and mice, testosterone secretion is reduced and there is some evidence for a decline in spermatogenesis, but the capacity to reproduce may persist to almost the end of life (Harman and Talbert 1985). The major cause for the decline in reproductive functions in these animals appears to be faults that develop in the hypothalamus, although other components of the neuroendocrine system (pituitary, gonads, reproductive tract) also may develop deficiencies (Meites, 1982).

Although aged female rodents lose their reproductive capacity long before they reach the end of life, there is no evidence for increased gonadotropin secretion as in post-menopausal women. Aschheim (1976) reported the presence of deficiency cells in ovarian interstitial tissue whose anatomical appearance could be restored by LH injections. This indication of inadequate gonadotropin secretion in aged female rats is supported by direct measurements of LH. Old female rats have increased serum PRL but reduced LH concentrations (Huang et al., 1976; Shaar et al., 1975; Watkins et al., 1976). The increased incidence of mammary

and pituitary tumors in these animals (Russfield, 1966) is believed to be the result of high PRL secretion.

2. Changes in releasing factors and neurotransmitters with age

In aging female and male rats, the declines in the reproductive system are due mainly to a decrease in the ability of the hypothalamus to release GnRH apparently due to a decrease in activity of the hypothalamic CAs, particularly NE (Simpkins et al., 1977; Wise, 1983). In addition to a fall in hypothalamic NE activity, hypothalamic 5-HT levels in old rats may rise slightly or exhibit no change (Simpkins et al., 1977) resulting in an increase in the ratio of 5-HT to NE. This may be important since there is evidence that 5-HT is involved in the rhythmic and phasic control of GnRH release (Walker, 1983). Other neurotransmitters also may be involved in altering GnRH release with age.

CAs are reduced in the hypothalamus and other brain areas, as well as in pituitary stalk portal vessels in old male and female rats (Simpkins, 1983). Turnover rates, which provide a better index of activity than content of DA and NE in the medial basal hypothalamus and whole hypothalamis also were reduced in old female and male rats (Simpkins, 1977) and in middle-aged rats 8-12 mo of age (Wise, 1983). There is considerable evidence in rats and man that the number of neurons in the locus coerulus, a major source of noradrenergic innervation to the hypothalamus and other

brain areas, is reduced during aging (Simpkins, 1983). Peng (1983) also reported significant loss of neurons in the preoptic area, anterior hypothalamic area, and arcuate nucleus of old rats, but it was not indicated whether these were catecholaminergic or other neurotransmitter neurons. Simpkins et al. (1983) reported lower DA and DOPAC concentrations in the ME, MBH, and preoptic anterior hypothalamic area of 25-26-month-old rats in constant estrus than in the 3-4-month-old cycling rats.

Loss of CA neurons in old rats may be due in part to the persistent elevation of estrogen during the CE syndrome and possibly also the high PRL concentrations. Chronic estrogen treatment can increase the number of glial cells which are associated with neuronal loss in the arcuate nucleus (Brawer et al., 1978). Administration of high doses of estrogen can acutely lower DA and NE concentrations in the hypothalamus.

The persistent prolactinemia in old rats is believed to damage DA neurons in the arcuate nucleus and ME (Sarkar et al., 1982). A damaging effect on DA neuronal function has been observed in mature female rats after prolonged exposure to a transplanted pituitary tumor which secretes large amounts of PRL (Sarkar et al., 1983). A reduction in brain dopamine beta-hydroxylase, which converts DA to NE was reported in 16-20-month-old mice (Finch et al., 1984), as well as an increase in monoamine oxidase, the major enzymes that catabolize CAs and other monoamines. The mechanism responsible for these changes in activity of major enzymes

involved in regulating synthesis and metabolism of neurotransmitters remains to be determined.

Significantly lower GnRH was found in the hypothalamus of old CE (Wise and Ratner, 1980) and anestrous rats (Steger et al., 1979). A reduction in GnRH concentration also has been observed in the ME, arcuate nucleus, and organum vasculosum of the lamina terminalis in old male rats (Simpkins, 1983). In old rats no decline in GnRH content was observed after castration (Wise and Ratner, 1980). This may account for the widely reconfirmed observation that after castration, the rise in serum concentrations of LH and FSH is much smaller in old male and female rats than in young or mature rats. Long-lived white footed deer mice, which maintain normal estrous cycles through 3-4 years of age, had normal hypothalamic GnRH concentrations as well as normal blood LH values (Steger et al., 1980). Thus, the capacity to release GnRH, LH, and FSH is reduced and this is associated with loss of estrous cycles in females and reduction of testosterone secretion in males.

3. Changes in hypothalamic and target organ sensitivity to feedback and gonadotropin release

In addition to disruptions of normal hypothalamic function, there is evidence that the pituitary becomes less responsive to GnRH stimulation (Bruni et al., 1977; Sonntag et al., 1984), the gonads to gonadotropic stimulation and the reproductive tract to stimulation by gonadal hormones

(Harman and Talbert, 1985). The hypothalamus of old female rats also becomes less responsive to the positive feedback action of ovarian hormones on GnRH release (Meites, 1982), perhaps because of a reduction in estrogen receptors (Peng, 1983). The pituitary gonadotropic cells exhibit no decrease in GnRH receptors during aging, suggesting that events beyond GnRH-receptor binding are responsible for the decreased responsiveness of the pituitary to GnRH (Sonntag et al., 1984).

The positive feedback action of estrogen on LH release is reduced in middle-aged and old ovariectomized female rats compared with young ovariectomized female rats (Lu, 1983), which could be due in part to lower uptake by the hypothalamus of estrogen and to a reduction in estrogen receptors. The negative feedback action of estrogen and androgens on gonadotropin release also may be reduced in aging rats due to lower uptake by the hypothalamus. Castration is known to lead to a smaller rise in LH and FSH in old than in young male and female rats (Lu, 1983; Shaar et al., 1976). Thus, general decline in reproductive functions in aging rats is be due to a multiplicity of causes.

4. Reversal by neuroendocrine manipulations

The decrease in hypothalamic CA activity is thought to be mainly responsible for loss of estrous cycles in aging female rats and for the decline in testosterone secretion in

aging male rats. This is supported by the many demonstrations that administration of L-DOPA or other drugs that increase CAs in the brain, result in reinitiation of estrous cycles in old noncycling female rats and increase testosterone secretion in old male rats (Clemens et al., 1969; Quadri et al., 1973; Cooper et al., 1979). Daily feeding of L-DOPA to aging female rats prior to loss of estrous cycles prolongs the occurrence of regular estrous cycles when compared to control rats not given L-DOPA (Forman et al., 1980). Electrical stimulation of the medial preoptic area induced ovulation in old CE rats (Clemens et al., 1969), perhaps by increasing NE activity or by directly acting on GnRH neurons to promote GnRH and LH release. Administration of neuroleptic drugs to young rats is known to decrease hypothalamic CAs (Weiner and Ganong, 1978) and results in loss of estrous cycles and early development of mammary tumors (Welsch and Aylsworth, 1983). Chronic treatment of young ovariectomized rats or mice with estrogen also decreases hypothalamic CA activity (Weiner and Ganong, 1978) and results in increased PRL secretion and the development of mammary and pituitary tumors (Furth, 1975).

By addition of L-Tyrosine to the diet, Cooper and Walker (1979) reported a significant extension of ovarian cyclicity in rats. This treatment was suggested to result in an appropriate CA to 5-HT balance within the CNS is reduced during aging.

C. Undernutrition effects on reproductive function

Reproduction is an energy-consuming process. It is thus subject to inhibition both by food scarcity and by any condition that increases the body's other competing demands for energy. The mass of offspring of many female mammals is quite large relative to her own mass, yet her small size dictates high thermoregulatory costs, a paucity of fat reserves, and a continuing need to find and consume relatively large quantities of food (Millar, 1977). These characteristics make the reproductive effort of these small females exceptionally susceptible to food scarcity, particularly when it occurs in combination with low ambient temperature (Barnett, 1973; Marstellar and Lynch, 1983, Bronson, 1985; Perrigo and Bronson, 1985).

1. Changes in neurotransmitters, GnRH, and estrous cycles during underfeeding

It is well established that reduced food intake can decrease release of LH and other pituitary hormones (Campbell et al., 1977) and disruption of estrous cyclicity (Merry and Holehan, 1979). Since pituitary responsiveness to GnRH is not diminished by starvation (Campbell et al., 1977) these effects are believed to be mediated by an alteration in hypothalamic neuroendocrine function.

Reduced food intake in rats decreases hypothalamic GnRH secretion (Campbell et al., 1977; Everitt, 1982), LH release by the pituitary (Ratner et al., 1978; Everitt, 1982), and

ovarian function (Holehan and Merry, 1985) with cessation of cycles. In addition, it has been reported that underfeeding reduces brain and hypothalamic NE activity (Wurtman and Wurtman, 1983) which normally promotes GnRH release during the preovulatory phase of the estrous cycle in rats (Simpkins et al., 1979). Tyrosine hydroxylase, the rate-limiting enzyme for CA synthesis, decreases during food deprivation (Gibson et al., 1978).

Piacsek et al. (1967) induced an increase in gonadotropin release and activated the ovaries of underfed rats that had ceased to cycle by daily administration of epinephrine, a CA. Pirke and Spyra (1982), using rats starved for 5 days, reported that after only 2 days of food deprivation, NE and DA turnover in the basal hypothalamus was decreased. CA turnover was also reduced in the preoptic area and in the ME. However, injection of L-DOPA, the CA precursor, could not prevent the LH decline. The reduction in plasma LH was not influenced by DA or NE agonists. These authors suggested the decline in NE and/or DA turnover either may be too small to influence GnRH secretion during food deprivation or more likely another mechanism could be responsible for the reduced LH secretion. Bronson (1986) induced ovulation and estrous cycles in underfed, prepubertal rats by injecting GnRH in pulses. These effects were similar to those produced by refeeding.

Bronson (1986) suggested a close metabolic coupling between nutrient energy processing and the GnRH pulse

generator. The hypothalamus is believed to retain its ability to increase GnRH release during food deprivation, since the effects of underfeeding on gonadotropin hormone release can be reversed by appropriate neuroendocrine manipulation such as ovariectomy or GnRH injections.

Briski et al. (1984) reported that daily administration of naltrexone counteracted the inhibitory effects of food deprivation on LH release in rats, and Dyer et al. (1985) prevented inhibition of pulsatile LH release during fasting by naloxone administration. These reports suggest a role for EOPs in LH suppression during underfeeding.

2. Effect on reproductive decline

a. Delay onset of puberty

Reduction of dietary intake remains one of the most effective methods of modifying early development and the apparent rate of rodent senescence. Although animals fed a restricted diet live longer and exhibit fewer age-related diseases that affect control animals fed ad libitum, they remain sexually immature (Asdell and Crowell, 1935; Kennedy and Mitra, 1963). After dietary restriction for periods up to 1000 days, survivors were reported capable of resuming growth and achieving sexual maturity when returned to ad libitum feeding (McCay, 1942).

Chronic underfeeding initiated at weaning in rats delays puberty (Kennedy and Mitra, 1963; Glass et al., 1976),

increases the reproductive life span, and slows the rate of reproductive decline (Merry et al., 1979). Merry et al. (1979) chronically underfed rats from weaning by maintaining body weight at 50% of control levels and continued this dietary regimen for most of the life of the animals. Puberty was delayed and associated with high levels of serum estradiol-17-B and low levels of serum progesterone and FSH. Although puberty was delayed for as long as 180 days by restricted feeding, the onset of reproductive decline was also markedly delayed. Asdell et al. (1941) reported a delay of approximately 6 days between the opening of the vagina and the ability to conceive in rats fed ad libitum. This period may be greatly exaggerated in the dietary restricted animal. These results show that the chronic restriction of food intake delays but does not inhibit sexual maturation.

b. Extension of cycles and litter production

Once puberty is established in the dietary restricted animal, estrous cycles of 5 days duration ensue. There was no extension of estrous cycle length with increased age as exhibited by full-fed controls, and normal cyclicity continued to an age when full-fed animals demonstrated a reproductive decline. Merry et al. (1979) reported 80% of the animals were able to conceive and wean young at 510 days of age, whereas controls ceased to reproduce at approximately 450 days of age; 25% were able to breed at over 800 days of age. Merry et al. (1981) also reported that

underfeeding delayed the testosterone decline in aging male rats. Berg (1960) utilized only a 33% food reduction in rats and assessed fertility after returning rats to ad libitum feeding at 730-790 days of age: 67% were still fertile and able to reproduce, although litter size was small; none of the control rats of the same age were fertile. Carr et al. (1949) and Ball et al. (1947) obtained similar results in mice on a chronic 50% caloric-restricted diet begun at weaning. When returned to ad libitum feeding, the animals were still fertile at 21 months of age, whereas ad libitum-fed mice became sterile at 11-12 months of age. Nelson et al. (1985) restricted food intake of female 57BL/6J mice for a 7-month period, and observed a delay in the age-related loss of cycles and retardation of the rate of follicular depletion. In this particular mouse strain, follicular depletion is believed to largely account for cycle cessation. Segall and Timiras (1983) fed a tryptophan-deficient diet to Long-Evans rats which also resulted in reduced total food intake. Growth was suspended for 2 years and when returned to ad libitum feeding, these animals were able to reproduce even at 17-28 months of age.

IV. Underfeeding effects on GH release

A. GH actions

1. Sites of GH receptors

Binding studies with ^{125}I-labelled GH suggest that cellular receptors are located on the cell membrane and are widely distributed throughout the body. GH binds to liver and kidney in high concentrations as well as to spleen, pancreas, intestine, adrenal cortex, thymus, heart, and skeletal muscle. Cellular events following hormone binding to receptors in their target cells include alterations of transport of amino acids and other metabolites, induction of specific proteins, and nucleic acid synthesis (Sonntag et al., 1983).

2. Protein synthesis and growth

GH is the most important protein anabolic agent in the body and is essential for protein synthesis. GH promotes body and bone growth, liver, kidney and hematopoietic functions. It stimulates the thymus gland producing T cells and thymosin for antibody production (Sonntag et al., 1983). GH stimulates somatomedin-C release (Daughaday et al., 1974) and protein synthesis (Knobil and Hotchkiss, 1964) in many tissues. The most obvious action of GH is its marked growth promoting effect on bone, cartilage, and other tissues. This occurs as a result of the stimulation of protein and

collagen synthesis and the uptake and utilization of circulating amino acids by the tissues. GH is essential for maintaining linear bone growth and is mediated by the production of growth factors in the blood. The prime factors are peptide hormones, and somatomedins which are synthesized in the liver and kidneys (Daughaday et al., 1972). GH is the premier protein anabolic agent in the body and is essential for protein synthesis through the life span of the animal.

Hypophysectomy, which eliminates GH secretion, decreases amino acid transport and protein synthesis in diaphragm muscle (Riggs and Walker, 1960). GH replacement returned both amino acid transport and protein synthesis to levels observed in intact animals. Similar effects have been reported in vitro (Kostyo et al., 1959; Kipnis and Reiss, 1960). Thus, the effects of GH on protein synthesis may be the result of increased amino acid transport into cells (Noall et al., 1957; Riggs and Walker, 1960) stimulating the synthesis or activity of amino acid-transporting proteins (Kostyo, 1968; Hjalmarson, 1968) or possibly by increases in RNA translation (Kostyo and Rillema, 1971).

3. Effects on metabolism

GH has important roles in the metabolism of proteins, carbohydrates, and fats. The general protein anabolic effects result in maintaining a positive balance of nitrogen and phosphorus in the body, responses encouraged by insulin. The interactions between GH and insulin promote glucose up-

take and formation of glycogen and triglicerides (Rabinowitz et al., 1966, 1968). Chronic GH administration produces a diabetogenic effect. Blood glucose increases significantly, whereas tissue sensitivity to insulin is reduced (Engel and Kostyo, 1964; Weil, 1965). GH, together with the adrenal corticosteroid hormones, antagonizes the effects of insulin on glucose uptake and utilization by muscle and other tissues, and breaks down liver glycogen stores to increase blood glucose. The possible role of somatomedins in the metabolic actions of GH is unsettled. The diabetogenic effect of GH may be the result of increasing fatty acid metabolism, which inhibits glycolysis and glycogenesis (Weil, 1965). Acute GH treatment decreases fatty acid synthesis (Knobil and Hotchkiss, 1964), whereas chronic GH administration inhibits glyceride synthesis and decreases body fat accumulation (Goodman, 1963). The decrease in fat formation attributed to GH is partly the result of a decrease in glucose utilization and subsequent conversion to fatty acids (Goodman, 1969). GH also may act by mobilizing fatty acids (inhibiting re-esterification) and accelerating lipolysis (Goodman, 1969; Winkler et al., 1969).

Administration of GH produces hypercalcemia and hyperphosphatemia by increasing calcium and phosphate absorption from the intestine (Knobil and Hotchkiss, 1964; Root, 1972) and by promoting tubular resorption of calcium and phosphate by the kidney (Corvilain and Abramow, 1962). Unknown is

whether some of these effects are mediated through parathyroid hormone.

There is sufficient evidence for an important role for GH in immune function. Administration of antiserum against GH resulted in atrophy of the thymus and a wasting syndrome (Pierpaoli and Sorkin, 1968). GH increased immune function in immune-deficient dwarf mice (Pierpaoli et al., 1968) and increased DNA synthesis by the thymus and spleen of both hypophysectomized and intact rats (Pandian and Talwar, 1971).

4. Role of somatomedins

Many of the effects of GH are the result of its direct action on target tissues. However, all of the effects of GH cannot be attributed to a direct action on tissues. One of the most potent actions of GH *in vivo* is stimulation of bone growth as measured by enlargement of the epiphyseal plate cartilage or stimulation of chondroctin sulfate synthesis in cartilage. However, *in vitro* GH administration or serum from hypophysectomized rats was unable to stimulate chondroctin sulfate synthesis whereas serum from normal or hypophysectomized rats treated with GH produced a significant increase in chondroctin sulfate synthesis (Salmon and Daughaday, 1957). These observations led to the hypothesis that some of the actions of GH were mediated by other factors.

It has since been demonstrated that many actions of GH (Van Wyk and Underwood, 1975) including at least some of the its actions on muscle (Florini et al., 1977; Merrill et al.,

1977) are mediated by somatomedins. This family of hormones includes somatomedins A and C. Although somatomedins A and C differ in amino acid composition, they compete for the same receptors on many types of cells, and all exhibit the same general properties: (1) stimulation of $^{35}SO_4$ uptake in cartilage, (2) potent mitogenic action in vitro, (3) weak insulin-like activities, and (4) GH dependence (Florini et al., 1981). Somatomedins generally are synthesized in the liver and released in response to GH stimulation (McConeghey and Sledge, 1970; Hintz et al., 1972; Phillips et al., 1976; Moses et al., 1979; Schalch et al., 1979).

B. Hypothalamic control of GH release

1. Characteristics of release

GH secretion is regulated by stimulatory and inhibitory factors of hypothalamic origin. Like other anterior pituitary hormones, GH is secreted episodically. In all mammalian species so far studied, spontaneous episodes of GH secretion occur several times over a 24-hour period (Finkelstein et al., 1972; Tannenbaum and Martin, 1976; Davis et al., 1977; Steiner et al., 1978). Particularly in the adult male rat there is a striking regularity in the GH pulses which occur at 3-4 hour intervals and reach concentrations of several hundred ng/ml. Between the peaks, concentrations are low or undetectable (Tannenbaum et al., 1976). In contrast, female rats have a more continuous secretion (Saunders et al.,

1976; Eden, 1979). The intermittent secretion of GH is of importance for the biological effects of the hormone in peripheral tissues.

2. Hypothalamic regulation: GRF, somatostatin, neurotransmitters

There is considerable evidence for the existence of a hypothalamic GRF (Deuben and Meites, 1964; Martin, 1979; Meites and Sonntag, 1981). Bioassayable GRF is believed to be present in the ventromedial nucleus of the rat hypothalamus, since electrical stimulation of this area increases GH release whereas lesions reduce GH release and result in growth retardation (Frohman et al., 1968; Frohman and Bernardis, 1968). Neuronal cell bodies with immunoreactive GRF were found in the arcuate nucleus and ventromedial nucleus; the arcuate nucleus seems to be the site of origin of these fibers (Bloch et al., 1983, 1984).

The hypothalamus also inhibits GH secretion via the GH release-inhibiting factor, somatostatin (Brazeau et al., 1973). The highest somatostatin concentration in the CNS is present in the ME (Brownstein et al., 1975) and is also present in neuronal perikarya located in the periventricular preoptic/anterior hypothalamic area (Alpert et al., 1976). Electrical stimulation of the preoptic area inhibits GH secretion (Martin et al., 1978), whereas lesions of the medial preoptic area or interruption of the connections between this area and the ME result in the sustained rise in

plasma GH (Critchlow et al., 1978) and a decline in ME somatostatin concentration (Critchlow et al., 1978; Epelbaum et al., 1977). Low plasma GH between pulses in the male rat are probably due to somatostatin release. Somatostatin antibodies increase basal plasma GH in rats (Ferland et al., 1976; Terry and Martin, 1981; Wehrenberg et al., 1982). Lesions of the medial preptic area or hypothalamic deafferentiation deplete somatostatin in the ME, increase GH nadir, and cause more frequent bursts of GH secretion in the male rat (Willoughby et al., 1977; Willoughby and Martin, 1978).

High concentrations of monoamines are present in the hypothalamus including the ME, and pharmacological manipulations have profound effects on GH release (Martin et al., 1978). While the roles of DA and 5-HT in control of GH release are controversial (Martin et al., 1978; Eden et al., 1979; Baldin et al., 1980), NE and/or epinephrine exert a stimulatory influence via central receptors that is essential for the normal pulsatile secretion of GH. EOPs appear to facilitate GH release, probably by adrenergic stimulation to GRF release, and perhaps by reducing somatostatin release (Miki et al., 1984; Ferland et al., 1977; Chihara et al., 1978). However, GH secretion in normal male rats seems to be independent of EOP influence, since naloxone has no effect on basal GH secretion (Martin et al., 1979).

3. Role of environmental factors

Studies utilizing electrical stimulation indicate that extrahypothalamic structures such as the hippocampus and amygdala may be important for GH secretion (Martin et al., 1978). Environmental factors such as the light-dark cycle and stress have an effect on GH secretion, and indicate the presence of a supra-hypothalamic component in regulating GH secretion (Martin et al., 1978). The light-dark entrainment of the GH secretory pattern in the adult male rat (Tannenbaum et al., 1976) may be mediated via the retinohypothalamic tract in which visual information from the retina is projected to the suprachiasmatic nucleus in the anterior hypothalamus (Moore-Ede et al., 1983). Other factors such as thyroid, gonadal, adrenal cortical, and pancreatic hormones as well as other physiological stimuli such as exercise, sleep, and diet influence GH secretion, either by acting directly on the pituitary or acting via the hypothalamus. Insulin induced hypoglycemia and arginine administration both stimulate GH release through hypothalamic mechanisms. In the rat, stress depresses GH release (Krulich et al., 1974). Passive immunization with somatostatin antiserum prevents, at least partially, the stress-induced depression of plasma GH (Arimura et al., 1976; Terry et al., 1975).

4. Role of anterior pituitary

GH release from dispersed pituitary cells can be demonstrated approximately 30 sec after exposure to GRF (Brazeau

et al., 1982). GH releasing effect of GRF *in vitro* is blocked by both somatostatin-14 and somatostatin-28 in a typical non-competitive manner indicating that GRF and somatostatin act on different pituitary receptors (Brazeau et al., 1982; Vale et al., 1983). GRF stimulates the efflux of cAMP from pituitary cells *in vitro* in parallel to GH release (Brazeau et al., 1982), while somatostatin suppresses cAMP levels in the pituitary (Vale et al., 1975).

5. GH feedback regulation

GH is capable of regulating its own secretion through a short-loop feedback mechanism. The amplitude of endogenous GH pulses is suppressed after administration of rat or human GH into the lateral cerebral ventricle (Abe et al., 1983; Tannenbaum, 1980), suggesting a hypothalamic locus for GH autoregulation. Somatostatin seems to play a role in GH regulation. *In vitro* administration of GH results in a dose-dependent increase in somatostatin release from incubated rat hypothalami (Sheppard et al., 1978) and there is a dose-related increase in somatostatin in hypophyseal portal blood after intracerebroventricular administration of rat GH (Chihara et al., 1981). Somatomedins stimulate somatostatin release *in vitro* from rat hypothalami (Berelovitz et al., 1981) and exert a potent direct inhibitory effect on GRF stimulated GH release in primary cultures of rat adenohypophyseal cells (Berelowitz et al., 1981; Brazeau et al.,

1982) indicating a direct inhibitory effect of somatomedins on GH release at the pituitary level.

6. Role of other hormones in GH regulation

The thyroid hormones, thyroxine and triiodothyronine, are involved in the regulation of GH synthesis and release by the pituitary. In the rat, thyroidectomy reduces both serum and pituitary GH (Reichlin, 1966; Peake et al., 1973; Hervas et al., 1975), and abolishes the pulsatile release of GH (Takuchi et al., 1978). Thyroid hormone replacement in these animals increases plasma and pituitary GH within 24 hours (Hervas et al., 1975; Coiro et al., 1979). In humans the GH response to insulin-induced hypoglycemia or arginine infusion was impaired in the absence of normal thyroid hormone secretion. In addition, hypothyroid children exhibited a reduction in normal body growth (Iwatsubo et al., 1967; Mac Gillivray et al., 1968; Kato et al., 1969). Administration of thyroid hormone restored body growth and promoted GH release in response to various stimuli. Dupont et al. (1978) suggests they may increase activity of hypothalamic enzymes that metabolize somatostatin.

Steroid hormones are also known to influence GH secretion. The gonadal steroids estrogen and testosterone appear to potentiate GH secretion in both rat and man. Dickerman et al. (1971) suggested that estrogen stimulated GH release but more recent evidence suggests that estrogen does not have a major role in regulating pulsatile GH

release (Saunders et al., 1976). The pulsatile release of GH is initiated at puberty when circulating estrogen and testosterone are increasing (Finkelstein et al., 1972).

C. Changes in GH release with age

1. Changes in neurotransmitters controlling GH release with age

It appears well established that the content and turnover of hypothalamic CAs, DA and NE, are reduced in old rats, whereas the turnover of hypothalamic serotonin is increased (Huang et al., 1979; Simpkins et al., 1977). Old male rats have less capacity to release GH in response to clonidine, after CA depletion (Sonntag et al., 1981), and also have lower spontaneous GH pulses than young male rats. Sonntag et al. (1980) demonstrated in rats 18-20 months old reduced GH pulse amplitude, but similar nadir values to those in young rats. This decrease in pulse amplitude with age may be related to the observation that old male rats, in contrast to young gonadectomized and intact males, have lower hypothalamic CA concentration and turnover (Simpkins et al., 1977). Chronic administration of the CA precursor, L-DOPA, which is capable of increasing brain CA levels (Ng et al., 1970) restores high GH pulses in old male rats (Sonntag et al., 1982). L-DOPA administration to old rats also increases pituitary GH concentration in old rats to the same concentrations as in young rats.

That neurotransmitters are important in the control of GH release and the decline in hypothalamic content and turnover of some of these substances particularly the CAs suggests that they contribute to the decline in GH pulse amplitude. The attenuated increase in GH in old animals after clonidine, peribedil, and morphine administration, and the finding that L-DOPA restores pulsatile GH release without affecting the response to these drugs, suggests that other deficiences in the neuroendocrine system of old rats may also contribute to the reduction in GH pulses (Sonntag et al., 1982, 1983).

2. Changes in hypothalamic control of GH release with age

Hypothalamic somatostatin content is reduced in old male rats (Sonntag et al., 1980). Administration of anti-somatostatin serum into old rats can elicit greater increases in GH release in old than in young rats (Sonntag et al., 1981). In vivo injection of hpGRF to old rats resulted in about 50% of the plasma GH of that in young rats. In vitro release of GH after 20 minutes of incubation of anterior pituitary slices with hpGRF showed an equal GH response in both young and old rats. There are reports of diminished release of GH with age in man after L-DOPA (Bazarre et al., 1976), exercise (Bazarre et al., 1976), or insulin-induced hypoglycemia (Laron et al., 1970). The reduced capacity to increase GH in response to pharmacological stimuli

with age may be the result of an inadequate release of GRF, a failure of these drugs to reduce somatostatin secretion, or altered pituitary responsiveness to these hormones. It is also possible that these differences are the result of a deficiency in postsynaptic neurotransmitter receptors that has been reported to occur with aging (Govoni et al., 1978; Greenberg and Weiss, 1978; Maggi et al., 1979).

There are no direct studies on the regulation of GH feedback in aging animals or man. The reduction of GH in aging animals may be partially attributable to the inability of the hypothalamus to recognize diminished GH in plasma, and to respond by stimulating GRF release or by inhibiting release of somatostatin (Sonntag et al., 1983).

3. Manifestations of reduced GH with age

In aging animals and humans there is a reduction in protein synthesis, reduced glucose tolerance, often a decline in lean body mass, loss of bone, decline in immune function, and reduction in kidney and liver functions. All of these processes are normally influenced by GH to varying degrees, but little is known of their relation to the reduced GH secretion during aging. The binding of GH to mouse liver membranes is unchanged during aging (Sorrentino and Florini, 1976). In aging rats there is reduced skeletal response to GH administration (Savostin-Asling, 1980). GH corrects the reduced protein synthesis seen during aging in rat diaphragm muscle (Sonntag et al., 1983).

Somatomedins from youth (2-6 months) to middle age (15-18 months) show little or no decrease, but a significant decrease occurs (to 60-70% of young levels) in older rats (24-28 months) (Florini et al., 1981; Florini and Roberts, 1980). It has not been established whether this decrease can be attributed to age-related changes in the liver which is the primary source of circulating somatomedins (Daughaday, 1981), or results from a reduction in pulsatile GH secretion (Sonntag et al., 1980).

Studies which assessed the pulsatile release of GH in old male rats revealed a significant reduction in amplitude of these pulses as compared with those in young animals (Sonntag et al., 1980). There was no apparent change in the periodicity of these pulses or entrainment to the light-dark cycle. Nadir was not different but mean GH concentrations were approximately 3 times greater in young than in old animals. Since evidence for attenuation of the pulsatile release of GH during aging in rats and man has only recently been demonstrated, its relationship to decrements in function of many body tissues and organs is unknown. It would be of interest to determine whether return in old male rats of high amplitude pulsatile GH secretion by neuroendocrine intervention also can reverse some of the decrements in body functions seen in these old animals and influence longevity (Sonntag et al., 1983).

D. Underfeeding effects on GH release

1. Effects on GH pulses during underfeeding

The ultradian GH rhythm in the rat dramatically alters in response to prolonged food deprivation (Tannenbaum et al., 1979). A significant depression in amplitude of GH pulses was observed as early as 24 hours after food removal and both the amplitude and duration of GH secretory episodes declined progressively after 48 and 72 hours of food deprivation. Neither entrainment of the GH pulses to the light-dark cycle nor normal periodicity was evident in food deprived animals. Pituitary GH concentrations increased 48 and 114 hours after food deprivation (Tannenbaum et al., 1979). This suggests that the suppression of GH secretory episodes after 72 hours food deprivation is not due to the inability of the pituitary to synthesize GH but rather is the result of an alteration in the GH release mechanism. Using passive immunization with a specific antiserum to somatostatin, circulating somatostatin reversed the starvation-induced inhibition of GH suggesting an important role for somatostatin in the GH starvation response (Tannenbaum et al., 1978).

In many species, a positive correlation has been demonstrated between length of the growth period and life span (Asdell, 1946; Hammond and Marshall, 1952; Rockstein et al., 1977). Chronic food restriction, which increases the length of the growth period, increases longevity in rats and mice

(Northrop, 1917; McCay et al., 1935; Saxton, 1945; Lansing, 1948; Everitt 1959; Berg and Simms, 1960, 1961; Comfort, 1963, 1964; Miller and Payne, 1968). Food restriction in rats reduced GH secretion and other hormones. However, administration of GH to maintain body growth and to reverse some of the age-related changes in metabolism due to aging have met with only limited success. Several investigators claimed to have restored some components of metabolic functions by GH but others found no effects (Asling et al., 1952; Emerson, 1955; Everitt, 1959; Beck et al., 1960; Jelinkova and Hruza, 1964; Marelli, 1968; Root and Oski, 1969). These studies concluded that one aspect of aging is a decline in tissue responsiveness to GH which could involve a reduction in GH receptors in some body tissues.

2. Effects during refeeding

A rebound response was reported in rats allowed to refeed for 3 days after 72 hours food deprivation. This was evidenced by an increased number of GH secretory episodes and a shorter period of GH rhythms (Tannenbaum et al., 1979). These findings provide support for the view that the pituitaries of these animals contain adequate amounts of GH and appear to be hyper-responsive to stimuli regulating GH release. However, Campbell et al. (1977) reported after 7 days of refeeding GH concentrations increased but were still slightly below control concentrations. There have been no

reports of GH responses during refeeding in rats chronically underfed for longer periods.

Materials and Methods

I. Research animals

Animals used in all of the studies were male or female Sprague-Dawley rats obtained from Harlan Laboratories (Indianapolis, IN) and Sasco Laboratories (Oregon, WI). Upon arrival all animals were housed in individual metal mesh cages (18x18x24 cm) in temperature (22+_2°C) and light-controlled (14 hr light, 0500-1900 hr/10 hr dark) animal facilities. Rats were provided with ground Teklad Rat Chow (Harlan Sprague-Dawley, Winfield, IA) and tap water ad libitum for a 2-week acclimation period. Body weights were monitored weekly on all rats throughout the experimental periods. Young male and female rats were initially 4 months old. Old and middle-aged female rats were initially 10 months or 15-16 months old. Male middle-aged and old rats were initially 10 and 16 months old. Each age group was randomly divided into half-fed and control groups.

II. Underfeeding regimen

Determinations of daily normal food intake were made by recording the weight of food consumed by 15-20 representative rats from each age group during a 5-day period. Food was provided once each morning to the half-fed rats between 800-900 hr. Ad libitum-fed controls had food available constantly. Rats were half-fed for a 10-week period followed by ad libitum feeding for the remainder of the experiment.

III. Vaginal smears

To describe individual differences in reproductive status, vaginal cytology was measured daily in each female rat up to the time of ovariectomy or for the entire experimental period. Vaginal smears were made from a vaginal lavage collected daily from each rat between 800-1000 hr and examined soon thereafter under a light microscope. Determination of the proportion of cornified epithelial cells, nucleated epithelial cells, and leukocytes were estimated and classified as proestrus (predominantly nucleated cells), estrus (predominantly cornified free of leukocytes, or diestrus (predominantly leukocytes). The end of one cycle and the beginning of a subsequent cycle was defined by the transition from a leukocytic smear to one

characteristic of proestrus (>80% nucleated epithelial cells) when at least 4 days had passed since the previous transition. Thus all cycles were at least 4 days in length. A regular cycle was defined as a cycle 4-5 days in length. Irregular cycles were more than 5 days in length. Rats in constant estrus (CE) exhibited cornified vaginal smears for at least a 2-week period. Mean number of cycles per group were calculated per 2-week period. Rats were considered to have irregular cycles if they exhibited cycles of varying lengths. Two weeks before the beginning of underfeeding, the reproductive status of each rat was assessed from the daily vaginal smears.

IV. Blood sampling

Blood was collected by decapitation, by orbital sinus puncture under light ether anesthesia, or by a chronically implanted right atrial cannula. Cardiac cannulae were made from Silastic tubing having an inside diameter of 0.025" and an outside diameter of 0.047". The length of the tubing from the Silastic pad to the bevelled tip was 28-45 mm varying according to the size of the animal at the time of surgery. Saline (0.87% NaCl) filled cannulae were inserted into the right atrium of ether anesthetized animals via a small incision in the right external jugular vein. The cannula was secured in place by suturing above and below the

stabilization pad. The free end was passed underneath the skin to the back of the neck and exited 2 cm posterior to the base of the skull. The cannulae were flushed with heparinized (100 IU/ml) saline. Immediately after surgery the animals were injected IM with 0.2 ml penicillin (200,000 U/ml). Streptomycin sulfate powder was applied topically at the surgical site prior to closure.

Rats were allowed to recover for 2 days after ovariectomy, undisturbed in the animal rooms in their individual cages. Three days before sequential blood withdrawal, estradiol benzoate 80 ug/ml corn oil/kg BW was administered sc between 0800 and 0900 hr. On the day of the sequential blood draw, animals were moved to a room for blood collection and silastic tubing extensions with syringes were attached to the cannulas. Animals remained in their own individual cages during blood withdrawal. After a minimum of 2 hrs acclimation, progesterone 8 mg/ml corn oil/kg BW sc was injected in the positive feedback experiment (Experiment 2) and samples were withdrawn into heparinized syringes from freely moving, non-disturbed animals. In Experiment 3, blood samples were withdrawn every 30 minutes for a 5-hour period. The samples were immediately centrifuged, plasma separated and stored at -20^{o}C until assayed. The blood cells were resuspended in equal volume amounts of sterile saline and reinjected into the animal after the next blood withdrawal. Blood collected from animals by orbital sinus puncture was allowed to clot

overnight at 4°C and serum was centrifuged at 2500 rpm for 15 min. The serum was then frozen at -20°C until assayed.

Animals to be decapitated were moved to a different room and allowed to acclimate for at least 1 hr. When designated, each animal was removed from its cage and taken into another room for decapitation with the door closed, and thus completely isolated (sight, sound, and odor) from the remaining animals. Blood was allowed to clot at room temperature for 1-2 hr, centrifuged at 2500 rpm for 15 min. Serum was separated and frozen at -20°C until assayed.

Orbital sinus blood withdrawal was performed under light ether anesthesia between 800-900 hr. Animals were under ether for no more than 30 seconds. Pressure was applied to the eye after blood withdrawal to prevent excess blood loss.

V. Ovariectomy

All animals in the positive and negative feedback experiments (Experiment 2) were bilaterally ovariectomized under ether anesthesia during the second week of the refeeding period. Procaine penicillin 0.2 ml IM (200,000 U/ml) was given prophylactically immediately after surgery. Animals were then placed back into their own individual cages and allowed to recover undisturbed.

VI. Radioimmunoassay

Serum and plasma LH, prolactin (PRL), and GH were measured by RIAs. The assays were performed with RIA materials provided by the National Pituitary Agency of the NIADDK All hormones were assayed in duplicate by standard RIA (Niswender et al., 1968; 1969; Sonntag et al., 1980; Marshall and Odell, 1975). Iodination grade PRL, LH, and GH were radiolabeled by a modified lactoperoxidase-glucose oxidase method (Tower et al., 1977). Serum and plasma hormone concentrations were expressed in terms of NIADDK PRL-RP-3, rLH-RP-1, and rGH-RP-1. Only those volumes which gave hormone values corresponding to the linear portion of the standard curve were used. Hormone concentrations were expressed as the mean plus or minus standard error of the mean (SEM).

All thyroid hormones (TT_4, fT_4, TT_3, fT_3, and rT_3) were measured in trunk serum, employing reagents as stated in Experiment 3, using solid phase RIA methods and human serum as standards. Specific information about the sensitivity, precision, validity, and accuracy of each assay is included in Experiment 3.

VII. Drugs and endocrine manipulations

In the positive feedback experiment, estradiol benzoate (Sigma Chemical Co., St. Louis, MO) 80 ug/ml corn oil/kg BW was given sc. This was followed 3 days later by sc progesterone injection (Sigma Chemical Co., St. Louis, MO) 8 mg/ml corn oil/kg BW. Each steroid was mixed in corn oil and heated until in solution.

VIII. Mammary tumor measurements

Mammary tumors were palpated and measured at weekly intervals throughout the experimental period. Palpable mammary tumors were measured with a vernier caliper and the 2 largest perpendicular diameters were recorded and averaged. Weekly tumor measurements were totaled for each rat and expressed as the sum of average tumor diameter per rat.

IX. Statistical analysis

Hormonal data were analyzed by 2-way analysis of variance (ANOVA) using a randomized factorial design for age/feeding for non-repeating measures. When blood samples were collected sequentially over time, a split plot ANOVA was used (subject/feeding over time). These were followed by

Student-Newman-Keul's test and least significant differences (lsd) test for multiple range comparisons when significant differences between groups and/or over time were obtained. Differences were considered significant if $p < 0.05$. Specific intra- and interassay coefficients of variation were provided in the Materials and Methods section of each experiment.

X. Pulse analysis

GH pulses ocur an average of once every 3.3 hours in male rats (Tannenbaum and Martin, 1976), so cannulated blood samples were obtained every 30 min over a 5 hr period. Pulses were defined as a sequence of at least 2 increasing concentrations of hormone from nadir followed by the peak or pulse maximum. Peaks were at least 2 standard deviations above nadir. Further analyses are described in Experiment 3.

Experiment 1:
Rejuvenating Effects of 10-Week Underfeeding Period on Estrous Cycles in Young and Old Rats

I. Abstract

The effects of providing 50% of normal feed intake for 10 weeks followed by 16 weeks of ad libitum feeding on estrous cycles and mammary tumor incidence were studied in female rats initially 4 months and 15-16 months old. Initially all young rats exhibited regular or irregular estrous cycles and only about 41% of the old rats cycled regularly or irregularly; the remainder of the old rats did not cycle. During underfeeding, both the young and old rats lost body weight and ceased to cycle. After refeeding 100% of both young and old rats resumed cycling, the young rats for a much longer period than the old rats, and more of both groups continued to cycle than their ad libitum-fed controls. Upon refeeding, the young and old rats reached the body weights of the ad libitum-fed controls in about 3 weeks. Mammary tumors were initially present only in old rats and regressed during underfeeding; they rapidly reached control size upon refeeding. Plasma PRL declined during

underfeeding but rebounded to higher than control values upon refeeding in both young and old rats. In young but not in old rats, plasma LH levels fell during underfeeding but returned to control values upon refeeding. These results demonstrate that a relatively short period of underfeeding, followed by refeeding, can delay the decline in reproductive cycles in young rats and reinitiate estrous cycles in older rats. These effects appear to be mediated via the neuroendocrine system.

II. Introduction

Chronic dietary restriction is the only manipulation known to increase life span in rats and mice (Barrows and Kokkonen, 1977; McKay and Crowell, 1934). It inhibits development of aging manifestation in many body tissues (Everitt, 1982), including appearance of spontaneous tumors (Ross and Bras, 1965; Sacher, 1977; Tannenbaum, 1943; Tannenbaum, 1942; Weindruch et al., 1986), inhibits development of age-related diseases (Masoro, 1984; Weindruch et al., 1986), and delays the age-associated decline in protein turnover (Birchenall-Sparks et al., 1985; Lewis et al., 1985; Lindell, 1982). There is considerable evidence that reduced food intake produces a decrease in secretion of all pituitary hormones, a condition sometimes referred to as "pseudohypophysectomy" (Campbell et al., 1977; Everitt,

1982; Holehan and Merry, 1985). This probably constitutes the major mechanism by which underfeeding delays aging changes (Everitt et al., 1980). Hypophysectomy with cortisone replacement inhibits the onset of some degenerative diseases and prolongs life in the rat (Everitt, 1982; Everitt et al., 1980), somewhat similar to the effects of underfeeding.

Chronic underfeeding initiated at weaning in rats delays puberty (Glass et al., 1976; Kennedy and Mitra, 1963), increases the reproductive life span (Merry and Holehan, 1979), and slows the rate of reproductive decline (Merry and Holehan, 1979). Merry and Holehan (1979) chronically underfed rats beginning at weaning by maintaining body weight at 50% of controls, and continued this dietary regimen for most of the life of the animals. In these underfed rats, 80% were able to conceive and wean young at 510 days of age, whereas the controls ceased to reproduce at approximately 450 days of age; 25% were able to breed at over 800 days of age. Merry and Holehan (1981) also reported that underfeeding delayed the testosterone decline in aging male rats. Berg (1960) utilized only a 33% food reduction in rats and assessed fertility after returning rats to ad libitum feeding. At 730-790 days of age, 67% of these rats were still fertile and able to reproduce, although litter size was small; none of the control rats of the same age were fertile. Similar results were obtained in mice by Carr et al. (1949) and Ball et al. (1947) using a chronic 50%

caloric-restricted diet begun at weaning. When returned to ad libitum feeding, the animals were still fertile at 21 months of age, whereas ad libitum mice became sterile at 11-12 months of age. Nelson et al. (1985) restricted food intake of female C57BL/6J mice by alternating days of feeding and fasting for a 7-month period, resulting in delay in age-related loss of cycles and retardation of rate of follicular depletion. They believed that follicular depletion largely accounted for cessation of cycles in this strain of mice. Segall and Timiras (1976) fed a tryptophan-deficient diet to Long-Evans rats which resulted in body growth stasis for up to two years. When returned to ad libitum feeding, these animals were able to reproduce even at 17-28 months of age.

Since previous studies utilized dietary restrictions initiated early in life and continued for long periods of time, it was of interest to determine the effects of a relatively short period of food restriction initiated during maturity or middle age followed by unrestricted food intake. In the present study, female rats initially 4 months old or 15-16 months old were subjected to a 10-week period of food restriction followed by ad libitum feeding for 16 weeks. The effects on body weight, estrous cycles, and mammary tumor development and growth were determined, and serum LH and prolactin (PRL) were measured.

III. Materials and methods

A. Animals and treatments

Female Sprague-Dawley rats, 30 initially 4 months and 50 15-16 months old, were obtained from Sasco Laboratories (Oregon, WI). They were individually housed in metal cages and maintained in a room at a constant temperature of 22 $\pm$ 2°C and a light regimen of 14:10 hours daily (lights on at 0500 hr). Water was constantly available and they were fed ground rat feed obtained from Teklad Diet, Harlan S.D. Co., Winfield, IA.

Determination of daily food intake for each age group of rats was made by recording daily weights of food consumed during a 5-day period. Young and old rats were fed half (11 and 12 g/day/rat, respectively) the amount of food consumed daily by their ad libitum-fed controls. All rats were weighed once weekly throughout the 26-week study. The mammary glands were palpated once weekly for the presence of mammary tumors, and when found, the two largest diameters were measured with vernier calipers. The mean of the two diameters was used to calculate % change in tumor diameter. Vaginal smears were collected daily from all rats during the entire experimental period. Young rats were excluded if they did not exhibit at least one 4- or 5-day estrous cycle per 2-week period prior to initiation of the experiment. Old rats were considered to be cycling if they exhibited at least one regular or irregular cycle per 2-week period.

The young and old rats were randomly divided into half-fed and ad libitum-fed (controls) groups. Food was provided once each morning to the half-fed rats, whereas ad libitum-fed control animals had food available constantly. This regimen continued for a 10-week period, followed by 16 weeks of ad libitum feeding of the previously half-fed rats. The young rats, initially 4 months old, were 10.5 months old by the end of the 26-week experimental period. The rats initially 15-16 months old were 22-23 months old upon completion of the experiment.

B. Blood sample collection and radioimmunoassays

Blood samples for PRL radioimmunoassay (RIA) were obtained by orbital sinus puncture under light ether anesthesia prior to initiation of underfeeding, at the end of the 10-week underfeeding regimen, and two weeks after resumption of feeding of the previously half-fed rats. Trunk blood for LH RIA was collected from some of the rats from each group at the end of the 10th week of underfeeding and two weeks after resumption of ad libitum feeding.

LH and PRL were assayed in duplicate. Iodination grade LH and PRL were radiolabeled by a modified lactoperoxidase glucose-oxidase method (Tower et al., 1977). The iodinated hormones were purified on a 1.5 x 40 cm Sephacryl S-200 column equilibrated with 0.01 M phosphate buffered saline at pH 7.6. IgGSorb (Protein A, Enzyme Center, Malden, MA) was used to separate bound from free hormones.

Blood for PRL RIA was collected between 800-900 hr and for LH RIA between 1100-1200 hr to exclude the surge of prolactin and LH that normally occurs on the afternoon of proestrus. Blood was allowed to clot at room temperature, centrifuged at 2,500 rpm for 15 min, and the serum was stored at -20 ^{o}C until assayed. Serum LH and PRL levels were measured with RIA kits provided by the NIAMDD (Niswender et al., 1968; 1969). Each hormone was measured in a single assay. Intra-assay coefficient of variation for PRL and LH were 3.7% and 5.7%, respectively.

C. Statistics

The data were analyzed statistically by 2-way analysis of variance (ANOVA) using a split plot design subject/treatment over time for sequential samples and a randomized block design for trunk blood samples. These were followed by Student-Newman-Keuls multiple range comparisons when significant differences were observed. Differences were considered significant if $p < 0.05$. Plasma LH and PRL values were expressed as ng/ml in terms of NIAMDD-LH-PRL-1 and NIAMDD-PRL-RP-3.

IV. Results

A. Body weight

Both young and old underfed rats initially showed a marked loss of body weight during half-feeding, and then maintained their reduced body weight for the remainder of the 10-week period (Figure 1). The young rats showed a 28% body weight loss during underfeeding, whereas the old rats exhibited a 31% loss in body weight. Both the young and old rats reached ad libitum-fed control levels after 2-3 weeks of refeeding. Body weights of ad libitum-fed control rats remained essentially unchanged throughout the entire 26-week experimental period. Upon refeeding, young and old rats exhibited excessive feeding behavior with protruding abdomens, so the initial rapid body weight gain may reflect, at least in part, gut fill.

B. Estrous cycles

The effects of underfeeding/refeeding on estrous cycles are shown in Figures 2-4. All of the 28 young rats exhibited some regular estrous cycles and only 21 out of 51 (41%) of the old rats exhibited regular or irregular estrous cycles at the beginning of the experiment. The other old rats did not cycle and exhibited either a constant estrous state (28 rats, 55%) or pseudopregnancy (2 rats, 4%) at the beginning of the experiment. The young rats initially exhibited an approximately 3.3 fold higher mean number of regular and

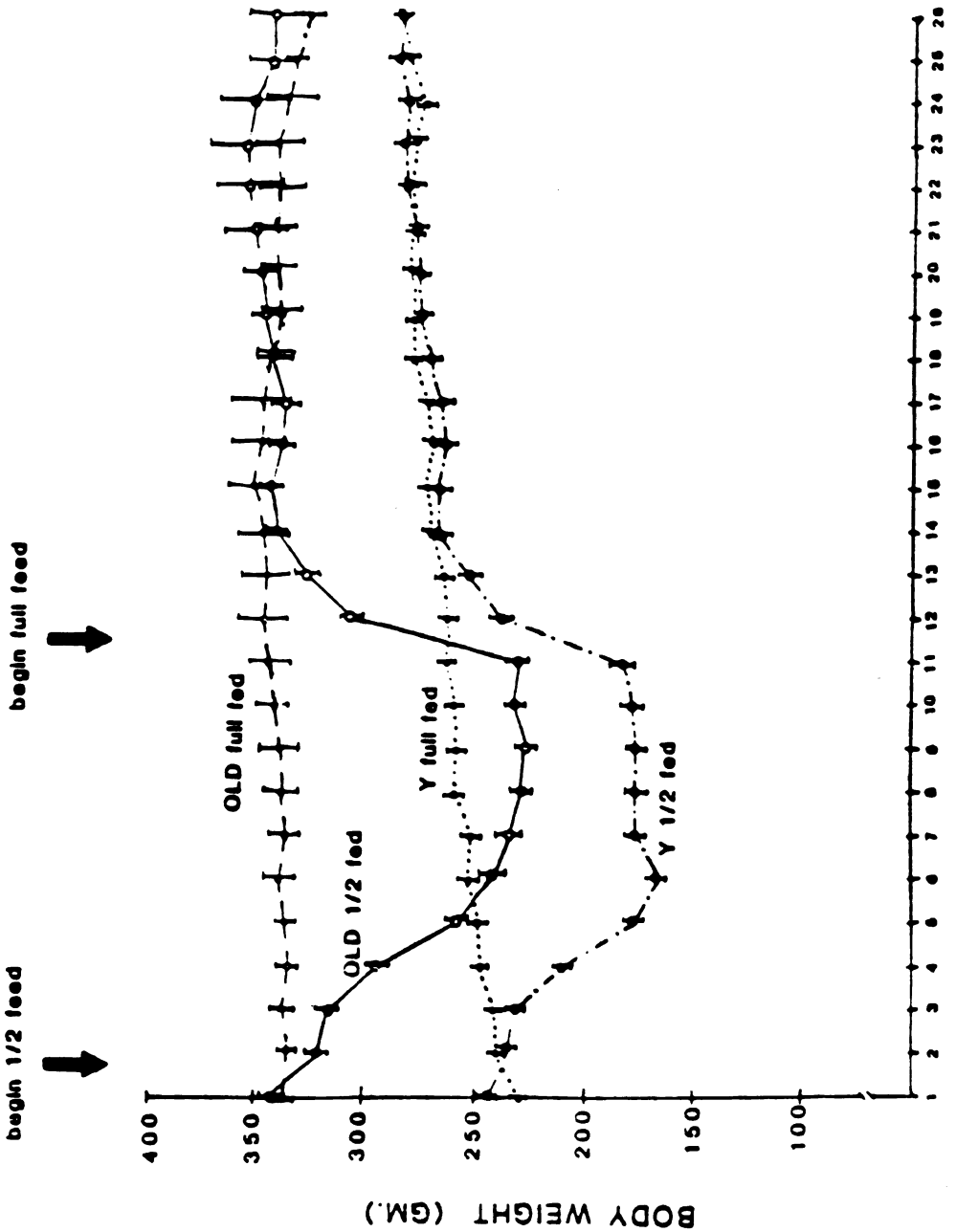

Figure 1. Effect of age and underfeeding/refeeding regimen on mean body weight. Arrows designate length of 50% food restriction for young and old rats. Period of underfeeding: weeks 1-10. Period of refeeding: weeks 11-26. Y = young.

irregular cycles than the old rats (Figure 2). The young rats initially exhibited a mean of 1.25 regular cycles per rat per 2-week period, whereas the old rats showed a mean of only 0.26 regular cycles per rat (Figure 3). None of the young or old rats showed regular estrous cycles after 5 weeks of underfeeding, and all young and old rats ceased to cycle completely by 6 weeks of underfeeding. At the end of the 10th week of underfeeding, 21% of the young, ad libitum-fed controls exhibited constant estrus.

Upon refeeding, 42% of the young rats exhibited proestrus followed by estrous vaginal smears by the third day; 31% of the old rats showed such vaginal smears by the fourth day and 69% by the fifth day of refeeding. About 75% of young and 100% of old rats were cycling by the end of the first week of refeeding. The young rats exhibited significantly ($p < .05$) more regular cycles during refeeding than before underfeeding was begun, and this was maintained for 6 weeks, after which time they returned to pre-underfeeding cycling levels. The old rats exhibited more regular and total cycles ($p < .05$) upon refeeding than prior to the underfeeding period, and this level was maintained for 6 weeks, after which time they exhibited a progressive decline to no regular cycles by the 16th week of refeeding (Figure 3). Both young and old previously underfed rats exhibited more regular cycles than ad libitum-fed controls during the refeeding period. The young and old ad libitum-fed controls showed a typical age-related decline in regular

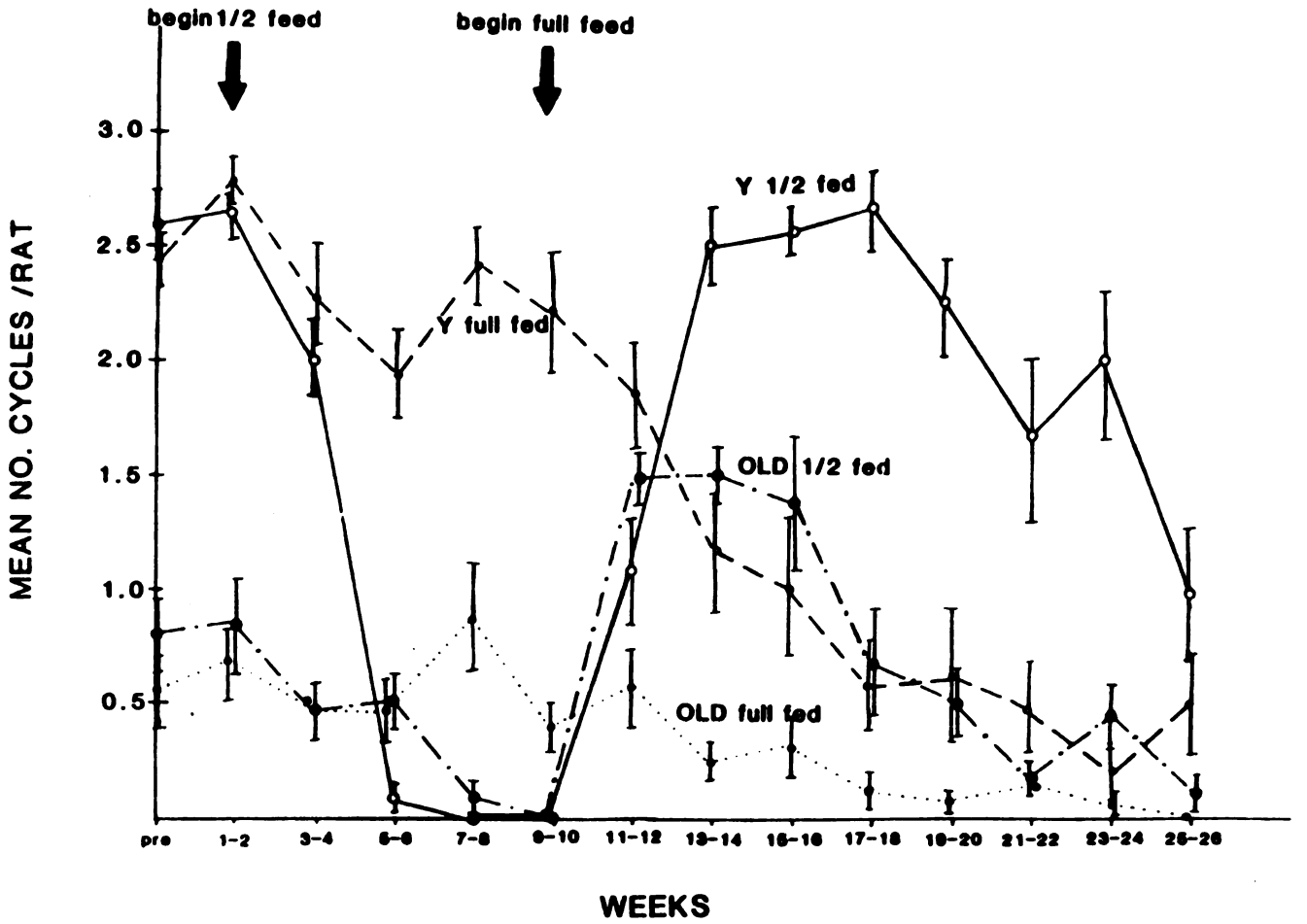

Figure 2. Effect of age and underfeeding/refeeding regimen on the total number of cycles (regular and irregular) per rat per 2-week period. Cycles include those 4-5 days in length (regular) and those of more than 5 days in length (irregular). Period of underfeeding: weeks 1-10; period of refeeding: weeks 11-26. Vertical bars represent SEM. Note decline in cycles over time exhibited by young and old full-fed groups. Y = young.

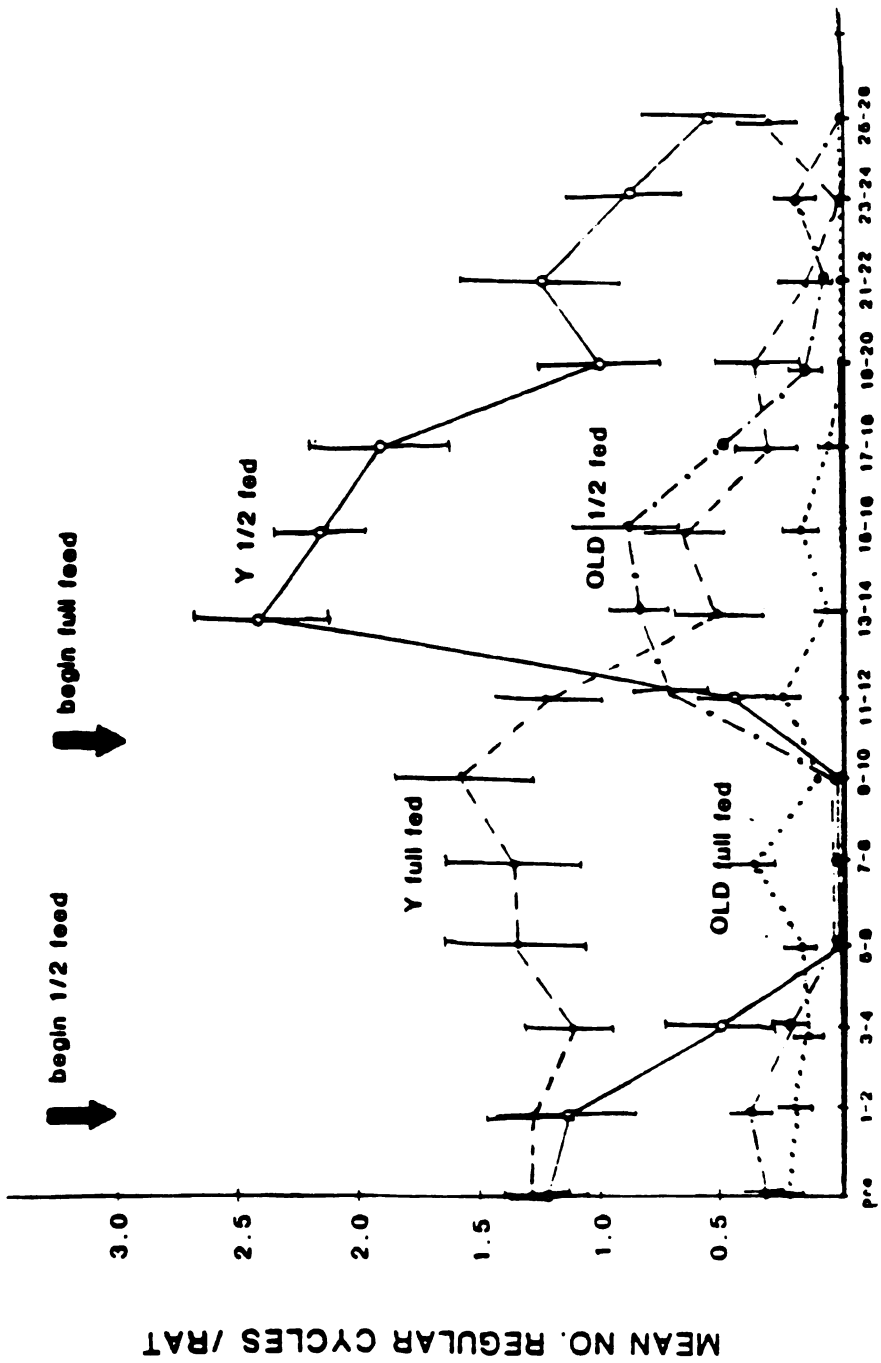

Figure 3. Effects of age and underfeeding/refeeding regimen on mean number of regular cycles per rat per 2-week period. Regular cycle defined as 4-5 days in length. Period of underfeeding: weeks 1-10; period of refeeding: weeks 11-26. Note that both young and old half-fed rats showed higher number of regular cycles upon refeeding than shown prior to underfeeding.

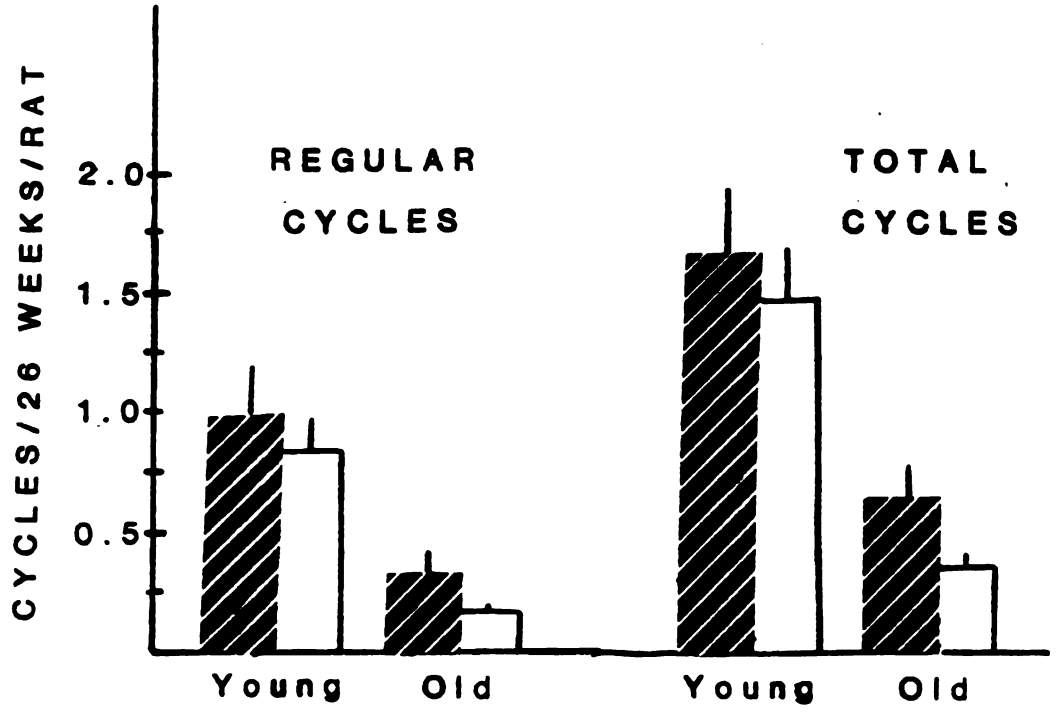

Figure 4. Comparison of mean numbers of regular or regular and irregular cycles exhibited over the entire 26-week experimental period between young and old rats. Regular cycles are defined as cycles of 4-5 days duration and total cycles as those 4-5 days and of > 5 days in length. Vertical bars represent SEM.

estrous cycles (Huang and Meites, 1975; Peng, 1983). None of the old ad libitum-fed rats exhibited regular estrous cycles by the 20th week of the experiment, whereas the young ad libitum-fed rats ceased to exhibit regular cycles by the 23rd week of the experiment. A few of the previously underfed young rats continued to show regular estrous cycles throughout the 16 weeks of refeeding.

When comparisons were made of total numbers of regular 4-5 day cycles and total cycles of all lengths over the entire 26-week experimental period, there were no significant differences between underfed and ad libitum-fed control rats, although the old previously underfed rats demonstrated a tendency toward more cycles than controls (Figure 4). The reductions in cycles during underfeeding appear to be compensated for during refeeding. However, there were significant ($p < .01$) differences between young and old groups in both regular and total cycles.

C. Mammary tumors

At the beginning of the experiment, none of the young rats had mammary tumors and none developed tumors during underfeeding. During the refeeding period one tumor appeared in the previously underfed young rats and two tumors developed in the young ad libitum-fed controls. In the older rats at the beginning of underfeeding there were initially 5 tumors and these regressed in size and no new tumors developed during underfeeding. During refeeding, 4 new

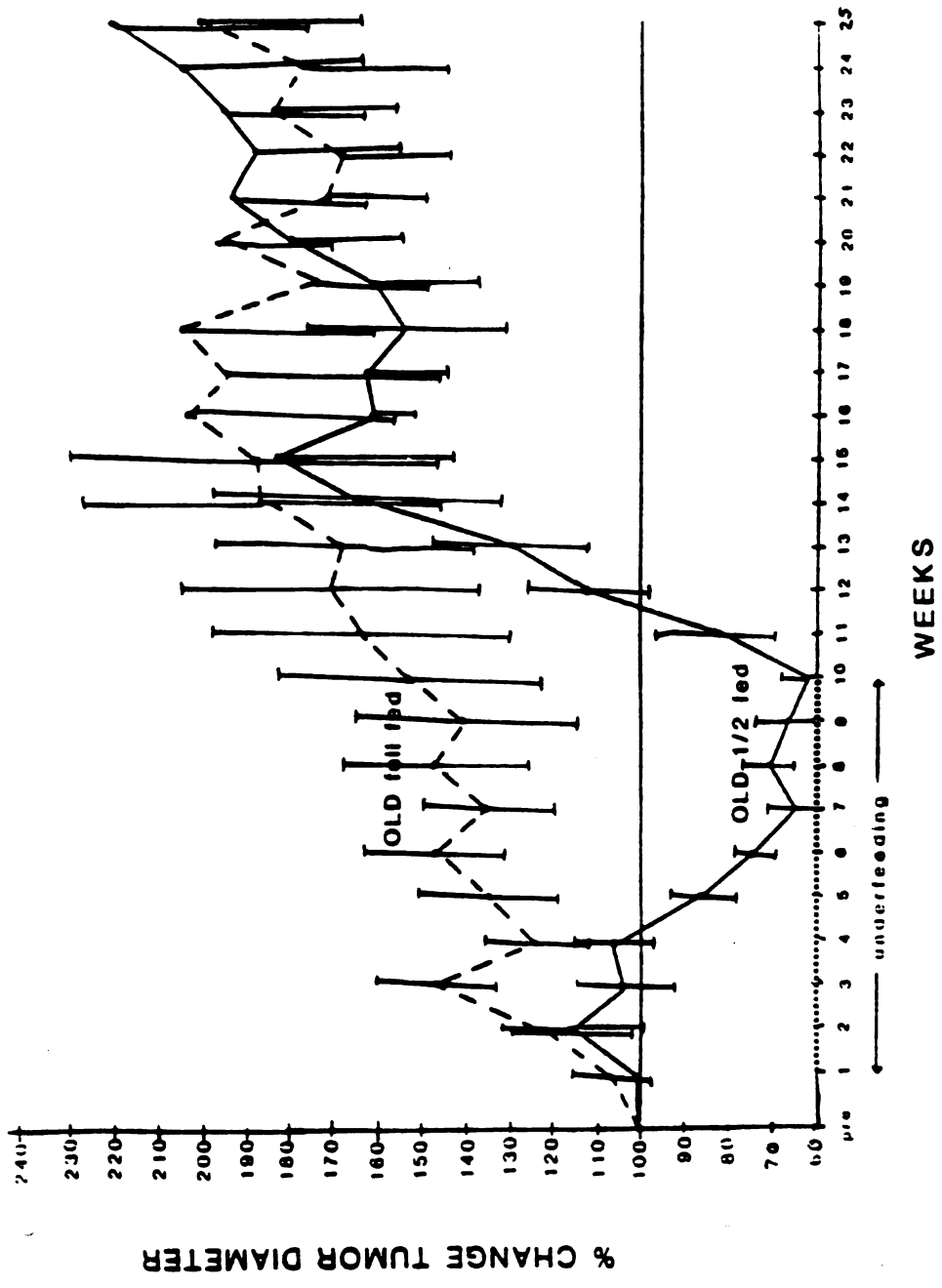

Figure 5. Effect of underfeeding/refeeding regimen on % change in mean mammary tumor diameter in old rats. Period of underfeeding: weeks 1-10; period of refeeding: weeks 11-26. Measurements are mean of two largest tumor diameters. All comparisons are with pre-experimental mean values.

tumors appeared. Old ad libitum-fed controls initially had a total of 3 tumors and 3 new tumors developed during the 10-week underfeeding period; this number increased to 10 during the ensuing 16 weeks. The previously half-fed old rats ended the 26-week experimental period with a total of 8 tumors and the ad libitum-fed control old rats ended with a total of 14 tumors. Two ad libitum-fed old rats died because their tumors became enlarged and infected during the course of the experiment and five tumors completely regressed. Two of the previously half-fed old rats also died from large, infected tumors on the 11th and 13th week of refeeding. The mammary tumors which regressed in the old rats during underfeeding reached control size within three weeks of refeeding (Figure 5).

D. Hormone concentrations

Serum LH in the young underfed rats fell to approximately half of the values in young ad libitum-fed control rats ($p \leq .01$), and upon refeeding serum LH values rose to control levels (Table 1). The old underfed and ad libitum-fed rats showed no significant change in serum LH values during the underfeeding period, and serum LH values remained the same upon refeeding. Serum PRL was reduced below ad libitum-fed controls in both young and old rats during underfeeding (Figure 6). Upon refeeding, the previously underfed young and old rats exhibited a marked

Table 1. Mean LH (ng/ml) during last week of underfeeding and second week of refeeding.

Age/Diet	Underfeeding Week 10	Refeeding Week 12	p
Y 1/2 fed	27.2 ± 1.6	70.3 ± 8.7	<0.01
Y full fed	69.4 ± 8.3	66.8 ± 11.0	N.S.
old 1/2 fed	105.0 ± 16.7	86.7 ± 11.0	N.S.
old full fed	97.3 ± 8.9	100.6 ± 17.9	N.S.

Mean ± S.E.M. of all values

N.S. $p > 0.05$

Y = young

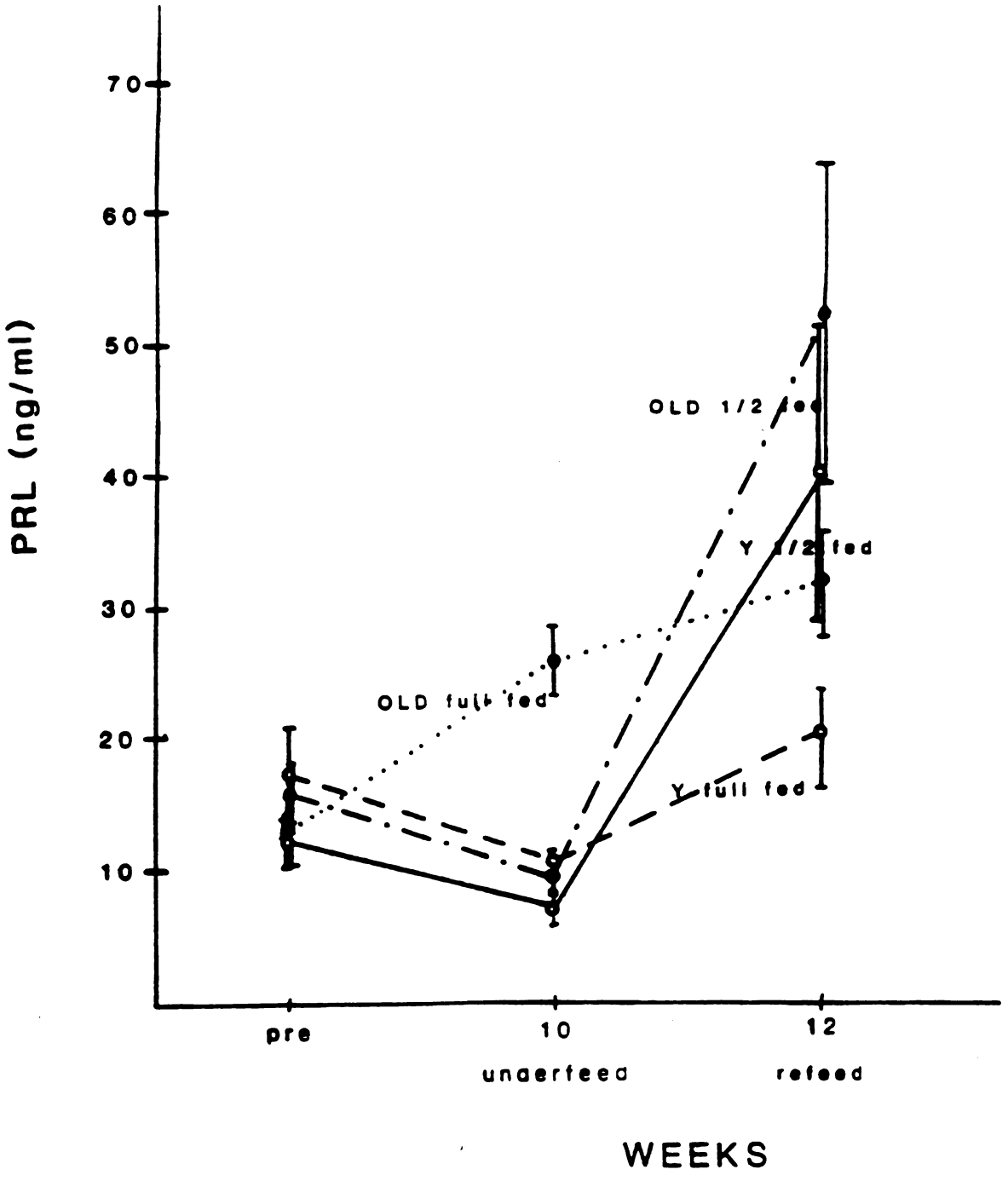

Figure 6. Effects of age and underfeeding/refeeding regimen on serum PRL. Blood samples were taken prior to experiment, during last week of underfeeding, and during second week of refeeding by orbital sinus puncture under light ether anesthesia. Note rebound effect during refeeding in young and old underfed groups.

and rapid rise in serum PRL approximately 2-fold greater than in ad libitum-fed controls.

V. Discussion

These results clearly show that 10 weeks of 50% food restriction, followed by 16 weeks of ad libitum feeding, was sufficient to extend the period of regular estrous cycles in young rats and induce temporary resumption of cycles in old rats. The young rats were initially 4 months old and reached 10.5 months old by the end of the experiment, an age when most female rats ceased to cycle. The older rats were initially 15-16 months old and reached 22-23 months old (old age) by termination of the experiment. The previously underfed young and old rats continued to cycle at a higher rate than their ad libitum-fed controls throughout the 16-week refeeding period. Sprague-Dawley rats have previously been reported to cease undergoing regular estrous cycles by 7 to 8 months of age, and to enter into a constant estrous state by 10-15 months of age (Huang and Meites, 1975). The underfeeding-refeeding regimen used here was more effective in promoting estrous cycles in the young than in the older rats upon refeeding, perhaps because they initially had a higher hypothalamo-pituitary-ovarian reserve capacity than the older rats.

In general, our results agree with those reported by Merry and Holehan (1979) and Berg (1960) in rats, and by

Carr et al. (1949), Ball et al. (1947), and Nelson et al. (1985) in mice. In all of the preceding studies in rats, underfeeding was usually begun at weaning and continued for most or all of the life span of the animals. Our study demonstrates that even a relatively short period of underfeeding (10 weeks) begun at 4 or 15-16 months of age, followed by ad libitum feeding, can still exert favorable effects on reproductive cycles, by increasing and prolonging the occurrence of regular cycles in the younger rats and by reinitiating estrous cycles in older rats after they had ceased to cycle. It appears that the young and old underfed rats are able to make up for the cycles lost during underfeeding when ad libitum feeding is reinstated.

Loss of estrous cycles during underfeeding is believed to be due to its decremental effects on hypothalamo-pituitary-ovarian function. Earlier work demonstrated that reduced food intake in rats results in decreased hypothalamic GnRH secretion (Campbell et al., 1977; Everitt, 1982), reduced LH release by the pituitary (Everitt, 1982; Ratner et al., 1978), and decreased ovarian function (Holehan and Merry, 1985). In addition, it has been reported that underfeeding decreases brain and hypothalamic norepinephrine (NE) activity (Wurtman and Wurtman, 1983), which normally promotes GnRH release during the preovulatory phase of the estrous cycle in rats (Simpkins et al., 1979). Prior to the normal preovulatory surge of LH, NE activity in the preoptic area, an area critically involved in GnRH

release in the rat, is increased (Wise et al., 1981). There is also evidence that tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine (CA) synthesis, is decreased during food deprivation (Gibson and Wurtman, 1978). Piacsek and Meites (1967) induced an increase in gonadotropin release and activated the ovaries of underfed rats that had ceased to cycle by daily administration of epinephrine, a catecholamine. Bronson (1986) induced ovulation and resumption of estrous cycles in underfed prepubertal rats by injecting GnRH in pulses. These effects appear to be similar to those produced by underfeeding/refeeding.

Bronson (1986) suggested a close metabolic coupling between nutrient/energy processing and the GnRH pulse generator. The hypothalamus is believed to retain its ability to increase GnRH release during food deprivation, since the effects of underfeeding on gonadotropic hormone release have been reversed by administering epinephrine in oil daily (Piacsek and Meites, 1967), by injecting GnRH in pulses (Bronson, 1986), or by ovariectomy (Howland, 1971; Howland and Ibrahim, 1973). Other pituitary hormones also are decreased during food restriction and their effects have also been shown to be mediated via the hypothalamus (Campbell et al., 1977; Tannenbaum et al., 1979), Briski et al. (1984) reported that daily administration of naltrexone counteracted the inhibitory effects of underfeeding on LH release for several days in rats, and Dyer et al. (1985)

prevented inhibition of pulsatile LH release during fasting by naloxone administration. These latter two reports suggest a role for endogenous opioids in LH suppression during underfeeding. Because reproduction is an energy-consuming process subject to inhibition by food scarcity and conditions that increase the body's other competing demands for energy, such as during lactation and pregnancy, acyclicity resulting from underfeeding may be viewed as a survival mechanism (Bronson and Marsteller, 1985).

In the present study, the young rats showed a significant fall in serum LH during underfeeding, and LH returned to controls after two weeks of refeeding. Serum LH values in the older rats did not appear to exhibit any significant change during underfeeding, in agreement with a report by Merry and Holehan (1981) who also found no significant change in LH release during dietary restriction in aging male rats. This may be related to the reduced responsiveness of the GnRH-LH mechanism in old rats to stimuli that normally release GnRH and LH. Thus ovariectomy in old rats resulted in a significantly smaller increase in release of LH than in young rats (Huang et al., 1979), and the positive feedback of estrogen or estrogen/progesterone on LH release was also lower in old than in young female rats (Huang et al., 1979).

Another factor that may have contributed to the favorable effects of underfeeding on subsequent cycling activity in our rats is the reduction in estrogen secretion

which presumably occurred as a result of cessation of estrous cycles. Studies by Aschheim (1976) in rats and by Felicio et al. (1983) in mice have demonstrated that chronic estrogen action by the ovaries hastens the reproductive decline in these animals. Ovariectomy of rats and mice early in life, followed many months later by transplantation of fresh ovaries, enabled these animals to undergo regular estrous cycles for many months beyond the period when intact controls had ceased to cycle. Brawer et al. (1978) demonstrated that chronic estrogen action results in damage to neurons in the arcuate nucleus and medial basal hypothalamus similar to damaged neurons observed in old anovulatory rats. Therefore, the probable reduction in estrogen secretion during underfeeding in our rats may have produced a sparing effect on the hypothalamic mechanisms regulating estrous cycles. This may also explain why the older animals exhibited reinitiation of cycles for a shorter period of time than the young rats, since they previously had been exposed to a longer period of damage to neurons in the hypothalamus than the younger rats. Neuroendocrine sensitivity and responsiveness to ovarian steroids may be heightened during underfeeding and counteract the reduction in estrogen and other hormone receptors reported in the hypothalamus of aging rats (Peng, 1983).

Reproductive aging in rats is associated with reduced CA and an increased serotonin (5-HT) to NE ratio in the hypothalamus (Huang et al., 1979; Simpkins et al., 1977). A

low-tryptophan diet was reported to extend fertility in aging female rats (Segall et al., 1983), perhaps by decreasing serotonin availability, by reducing food intake which resulted in loss of body weight, and perhaps by arresting depletion of neurons involved in regulating ovarian function. In young rats, 5-HT reuptake blockers produced constant estrus or pseudopregnancy by increasing 5-HT metabolism (Cooper, 1980). Drugs which increased central CA metabolism and decreased 5-HT reported to reinitiate regular estrous cycles in old constant estrous or irregularly cycling rats (Forman et al., 1980; Quadri et al., 1973). Therefore, underfeeding followed by ad libitum feeding may re-establish a normal CA/5-HT balance in aging rats and permit normal or supernormal GnRH release by the hypothalamus.

PRL and estrogen have been shown to be necessary for mammary tumor development and growth in rats, although PRL is generally considered to be the more important of the two hormones in rats (Meites, 1972). During underfeeding both of these hormones have been shown to be decreased (Leung et al., 1983; Sylvester et al., 1981). Our results, in agreement with others, demonstrate that underfeeding significantly inhibited development and growth of mammary tumors, and this is associated with a decrease in PRL secretion (Sylvester et al., 1981). It has been reported that inhibition of mammary tumor growth by underfeeding can be counteracted by administration of PRL and/or estrogen

(Leung et al., 1983). Although estrogen levels were not measured during underfeeding in our rats, it is reasonable to assume that estrogen secretion was reduced since estrous cycles ceased. Upon refeeding, the underfed rats in our study showed a rebound in PRL release, in agreement with results reported by Campbell et al. (1977). PRL reached significantly higher levels than in the ad libitum-fed controls in the young and old rats. This may at least partially explain the rapid regrowth of the mammary tumors to control size after three weeks of ad libitum feeding. The continuous rise in PRL levels during aging in female rats was associated with a progressive increase in development of spontaneous mammary tumors (Barrows and Kokkonen, 1977).

In conclusion, a relatively short period of underfeeding, initiated at 4 or 15-16 months of age, followed by refeeding, delayed the decline in reproductive cycles in young rats and temporarily reinitiated estrous cycles in old rats. Whether a relatively short period of reduced dietary intake would prove to be beneficial in aging human subjects remains to be demonstrated.

Experiment 2:
Effects of 10-Week Underfeeding Followed by Refeeding on LH Feedback Response to Ovarian Steroids and Ovariectomy in Old and Young Rats

I. Abstract

Young and older rats were subjected to a 10-week period of 50% normal food intake followed by ad libitum feeding, and the positive feedback effects of ovarian steroids and negative feedback after ovariectomy on LH and PRL release were measured. Female rats initially 4 and 10 months old were ovariectomized after 10 days of refeeding. Positive feedback was assessed 2 and 4 weeks postovariectomy. For this purpose, rats were given 80 µg/kg BW estradiol benzoate followed 3 days later by 8 mg/kg BW progesterone sc. Sequential blood samples were then collected via indwelling atrial cannulae for 8 hr for measurement of LH and PRL. Negative feedback was assessed by measurements of serum LH and PRL 1, 2, and 3 weeks postovariectomy.

The older previously half-fed rats had the highest LH responses to the ovarian steroids both at 2 and 4 weeks postovariectomy, above those of the young rats. The older

constant estrus ad libitum-fed controls had the lowest LH response 2 weeks postovariectomy, but by 4 weeks postovariectomy they exhibited LH values approaching those of the older half-fed rats. No differences were observed between young underfed and ad libitum-fed controls. Half-feeding followed by refeeding did not enhance the PRL rise in response to ovarian steroid administration or ovariectomy. The older previously half-fed rats also demonstrated a significantly higher LH response 1 week postovariectomy than all other groups and maintained this level for the entire 3 weeks. These observations are in sharp contrast to the previously reported effects of ovarian steroids and ovariectomy on LH release in young and old ad libitum-fed rats, in which old rats exhibited a lower LH rise than the young rats. These results are believed to partially explain why a relatively short period of underfeeding followed by refeeding can exert a temporary rejuvenating effect on the neuroendocrine mechanisms regulating estrous cycles in aging rats.

II. Introduction

Reproductive decline is one of the earliest symptoms of aging in the rat. Irregularities in estrous cycles are observed as early as 7-8 months of life, and loss of estrous cycles occurs by 10-15 months of age (Huang and Meites,

1975). It has been shown that feedback by gonadal steroids on gonadotropin release is reduced in aging rats (Meites et al., 1978). Thus, ovariectomy results in a significantly smaller LH rise in old than in young rats (Shaar et al., 1975), and the rise in circulating LH is delayed and prolonged in old rats (Lu et al., 1977). The positive feedback action of ovarian steroids on LH release also is reduced in aging rats. Thus, when young and old female rats were ovariectomized and given estrogen followed 3 days later by progesterone, the increase in plasma LH was significantly lower in old than in young animals (Lu et al., 1977; Huang et al, 1980).

Dietary restriction has been shown to increase the reproductive life span and slow the rate of reproductive decline in rats (Merry and Holehan, 1979). We recently reported that when female rats initially 4 or 15-16 months old were subjected to a 10-week period of half-normal food intake followed by ad libitum feeding for 16 weeks, loss of estrous cycles was delayed in the young rats and estrous cycles were temporarily reinstated in the old rats (Quigley et al., 1987). More of both the young and old underfed/refed rats continued to cycle as well as exhibit more regular estrous cycles than their ad libitum-fed control rats. It was of interest, therefore, to compare in female rats initially 4 and 10 months old the effects of a 10-week period of 50% normal food intake, followed by ad libitum feeding, on release of LH and PRL in response to the positive feedback

effects of estrogen followed by progesterone and the effect of negative feedback removal following ovariectomy.

III. Materials and methods

A. Animals and treatments

Sixty-one 4-month-old and 60 10-month-old female Sprague-Dawley rats were obtained from Sasco Laboratories (Oregon, WI). They were housed individually in metal cages and maintained in a room at a constant temperature of 22 ± 2°C under a light regimen of 14:10 hours daily (lights on at 0500 hr). All animals were allowed 1 week to acclimate to laboratory conditions and given tap water and ground Teklad Rat Diet (Harlan S.D. Co., Winfield, IA) ad libitum. Determinations of mean daily food intake were made by recording the weight of food consumed by representative rats from each age group during a 5-day period. Young and old rats were then randomly divided into half-fed and ad libitum-fed controls. The young and older half-fed rats received half (11.5 and 10 gm/day/rat, respectively) of the mean daily food intake for their control age group. Water was provided to all rats ad libitum.

Vaginal smears were collected daily from all rats after acclimatization, until the day of ovariectomy. Two weeks before the beginning of food restriction, estrous cycles in each rat were assessed. Rats which did not exhibit at least

one cycle during the two-week period were eliminated from the study. Regular cycles were 4-5 days in length, and irregular cycles were more than 5 days in length. Rats were considered to be in constant estrus (CE) when they exhibited continuous cornified vaginal smears for at least a 2-week period. All rats were weighed once weekly throughout the experimental period. Food was provided once each morning to the half-fed rats, whereas ad libitum-fed control animals had food available constantly. This regimen continued for a 10-week period followed by ad libitum feeding of the previously half-fed rats for the remainder of the experiment. The young rats, initially 4 months old, were 7-8 months old on the day of ovariectomy. The older rats, initially 10 months old, were 13-14 months old when ovariectomized. Because 14-16 weeks had elapsed since initiation of the experiment, the cycling status of older ad libitum-fed controls was carefully monitored and subsequently classified into CE or irregularly cycling rats prior to ovariectomy.

B. Surgery and blood sampling procedure

All rats were bilaterally ovariectomized under ether anesthesia after 10 days of refeeding. 0.2 ml procaine penicillin (200,000 U/ml) was injected i.m. and streptomycin sulfate (powder) was applied topically before suturing. All rats were divided into Group I or Group II after ovariectomy. Positive feedback was assessed in Group I 2

weeks after ovariectomy, and in Group II 4 weeks after ovariectomy. For this purpose, a silastic indwelling atrial cannula was implanted into each animal through the right external jugular vein under ether anesthesia 4 days before sequential blood collection. The free end of the cannula was brought out sc to the back of the neck. Animals were given 0.2 ml procaine penicillin i.m. (200,000 U/ml) immediately after surgery to prevent infection. Animals were brought to the experimental room 2.5 hrs before sequential blood collection. Silastic extension tubing was attached to the cannulae and the rats were left undisturbed for at least 2 hrs. Animals were allowed free access to food and water throughout the blood collection period.

Estradiol benzoate (Sigma Chemical Co., St. Louis, MO), 80 µg/ml corn oil/kg BW, was given sc to group I (2nd week postovariectomy). This was followed 3 days later by sc progesterone injection (Sigma Chemical Co., St. Louis, MO), 8 mg/ml corn oil/kg. BW. Blood samples (0.7 ml) were collected at the following times: prior to progesterone injection at 0930 hr, and 2, 4, 5, 6, 7, and 8 hr after progesterone administration. The blood samples were placed into heparinized syringes and centrifuged at 2,000 rpm for 5 min. Plasma was separated, and blood cells were resuspended in physiological saline (0.85% NaCl) and reinjected after the next blood withdrawal. The plasma was stored at -20°C for RIAs of PRL and LH. This same regimen was followed for group II, 4 weeks postovariectomy.

To assess negative feedback, blood samples for LH and PRL RIAs were collected by orbital sinus puncture under light ether anesthesia between 0800-0900 hr. Samples were obtained on the morning before ovariectomy and 1, 2, and 3 weeks postovariectomy from Group II. Blood was allowed to coagulate overnight at 4°C then centrifuged at 2500 rpm for 15 min. The serum was stored at -20°C until assayed. Group II was subsequently utilized for positive feedback assessment the following week.

C. Hormone assays

LH and PRL were assayed in duplicate by RIA. Reagents were provided by NIADDK. Iodination grade LH and PRL were radiolabeled by a modified lactoperoxidase-glucose oxidase method (Tower et al., 1977; Niswender et al., 1968; 1969). The iodinated hormones were purified on a 1.5 x 40 cm Sephacryl S-200 column equilibrated with 0.01 M phosphate buffered saline at pH 7.6. IgGsorb (Protein A, Enzyme Center, Malden, MA) was used to separate bound from free hormones. Each hormone was measured in a single assay for each experiment. Intra-assay coefficients of variation for the LH and PRL assays were 5.6% and 3.8%, respectively, in the positive feedback samples and 10.3% and 5.6% in the negative feedback samples. 50% inhibition of tracer binding for LH was 17.8 and 18.8 ng/tube; and for PRL 1.2 ng/tube.

D. Statistics

The data were analyzed by analysis of variance (ANOVA) using a split plot design (subject/treatment over time), followed by Student-Newman-Keuls test for multiple range comparisons when significant differences between groups were observed. Differences were considered significant if $p < 0.05$. Plasma LH and PRL values were expressed as mean $\pm$ SEM in nanograms of reference standards (NIADDK-LH-RP-1 and NIADDKPRL-RP-3, respectively).

IV. Results

A. Body weight

Both young and older rats demonstrated a significant ($p < .05$) loss of body weight during the first 4 weeks of underfeeding. The body weights then stabilized and remained at uniform levels the remainder of the 10-week underfeeding period (Figure 7). The young rats initially averaged 253.8 gm in body weight and declined to an average of 196.7 gm by the end of the underfeeding period (23% loss), and the older rats averaged 312.7 gm initially and declined to 193.6 gm at termination of underfeeding (38% loss). Upon refeeding, both young and older rats rapidly regained body weight and approached control values by the end of the 6th week of refeeding. Both young and old rats exhibited excessive feeding behavior with a protruding abdomen during the first week of

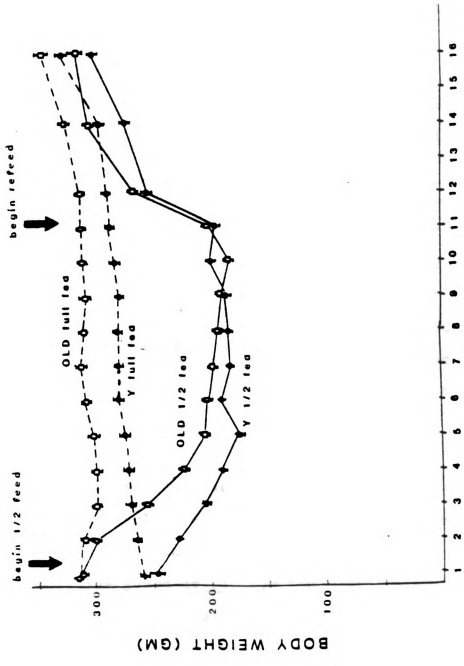

Figure 7. Effects of age and underfeeding/refeeding regimen on mean body weight. Arrows designate underfeeding period. Vertical bars represent SEM. Young half-fed (Y1/2fed), n = 30; young full-fed (Y full-fed), n = 30; old half-fed, n = 30; old full-fed, n = 31.

refeeding, which may account, at least in part, for the large increase in body weight so early after refeeding. Body weights of ad libitum-fed controls showed a slight but non-significant gain during the 16 weeks of the experiment.

B. Estrous cycles

The effects of underfeeding/refeeding on the total number of estrous cycles are shown in Figure 8. All rats exhibited regular 4- or 5-day cycles or irregular cycles of more than 5 days duration during the 2 weeks prior to underfeeding. None of the rats were CE. The young rats had significantly higher ($p < .05$) regular cycles and a greater total number of cycles of any length ($p < .05$) than the older rats during this period. In both young and older rats, regular cycles declined rapidly during underfeeding (Figure 9) . None of the young underfed rats exhibited regular cycles by the third week of underfeeding, and all ceased cycling by the 5th week of underfeeding. The older rats ceased cycling regularly by week 5 of underfeeding and ceased cycling entirely by week 7. After week 7 all underfed rats showed only anestrous vaginal smears.

The young ad libitum-fed controls showed a decline in mean number of regular and irregular cycles prior to ovariectomy, but none were CE. Similarly, the older ad libitum-fed rats demonstrated an age-related decline in both regular and irregular cycles during this period. A total of 18 out of 31 older ad libitum-fed rats (58%) were CE by the time of

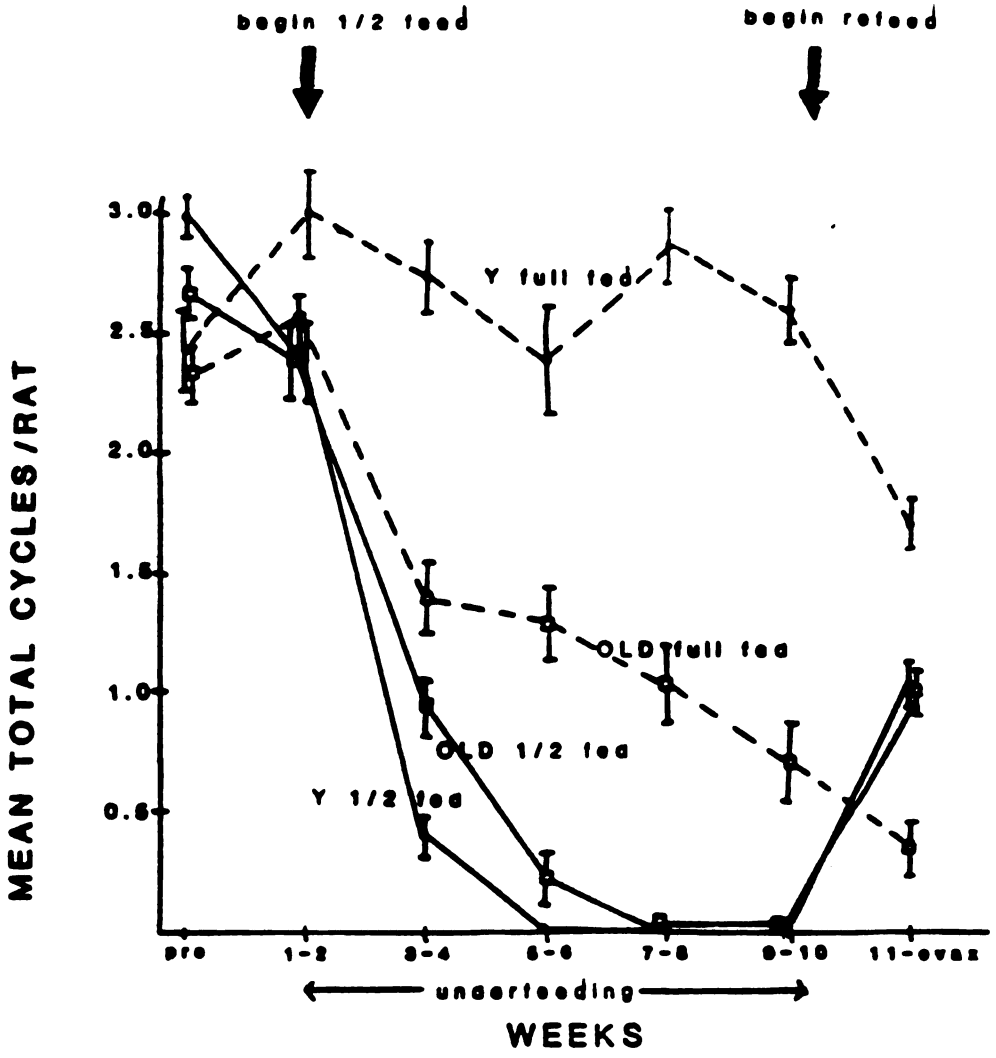

Figure 8. Effects of age and underfeeding/refeeding on mean total number of regular (4-5 day) and irregular (> 5 days) estrous cycles per rat. Arrows designate underfeeding period. Vertical bars represent SEM. Vaginal smears were collected up to the time of ovariectomy. Young half-fed (Y 1/2-fed, n = 30); Young full-fed (Y full-fed, n = 30); old half-fed, n = 31; old full-fed n = 30. Note decline in cycles with time in young and older full-fed controls.

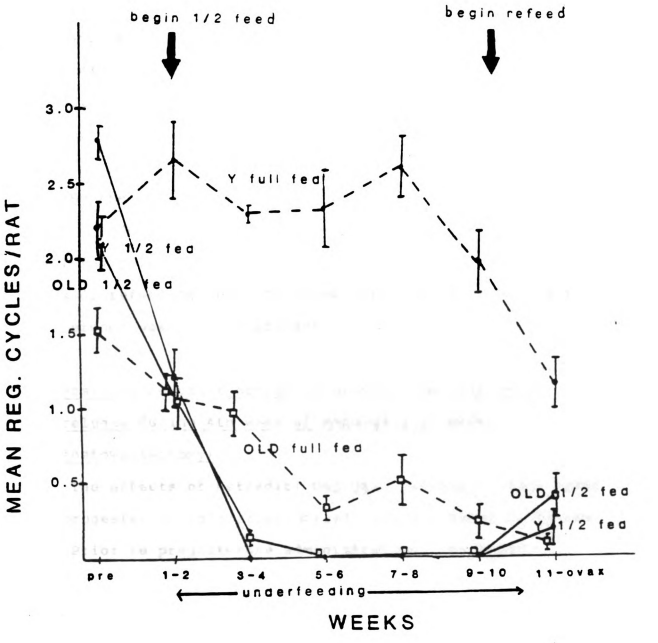

Figure 9. Effects of age and underfeeding/refeeding on mean number of regular (4-5 day) cycles per rat. Arrows designate underfeeding period. Vertical bars represent SEM. Vaginal smears were collected up to the time of ovariectomy. Young half-fed (Y 1/2-fed, n = 30); Young full-fed (Y full-fed, n = 30); old half-fed, n = 31; old full-fed n = 30. Note decline in cycles with time in young and older full-fed controls.

ovariectomy, and only 3 out of 31 (10%) had 4- or 5-day cycles intermixed with cycles of longer length. During the 10 days of refeeding before ovariectomy, 93% of the young and 90% of the older previously half-fed rats demonstrated proestrous followed by estrous vaginal smears. By the 5th day of refeeding 93% of the young and 85% of the older rats were in proestrus.

By the 2nd week of refeeding, 93% of the young and 90% of the older rats were exhibiting estrous cycles. The older half-fed rats had slightly more regular cycles during the 10 days of refeeding than the young half-fed rats, but this difference was not significant.

C. Positive feedback action of ovarian steroids on LH release during 4th week of refeeding (2 weeks postovariectomy)

The effects of estradiol benzoate followed 3 days later by progesterone in ovariectomized rats are shown in Figure 10. Prior to progesterone administration, the older previously half-fed rats had LH values 2-fold greater than the older CE and older irregularly cycling ad libitum-fed rats. At 5, 6, 7, and 8 hrs after progesterone injection, LH values in the older previously half-fed rats reached mean values higher than in all other groups. At 6 hr, the old previously underfed rats had LH values significantly ($p < .01$) above all groups. At 7 hr after progesterone, the old previously underfed rats had LH values significantly

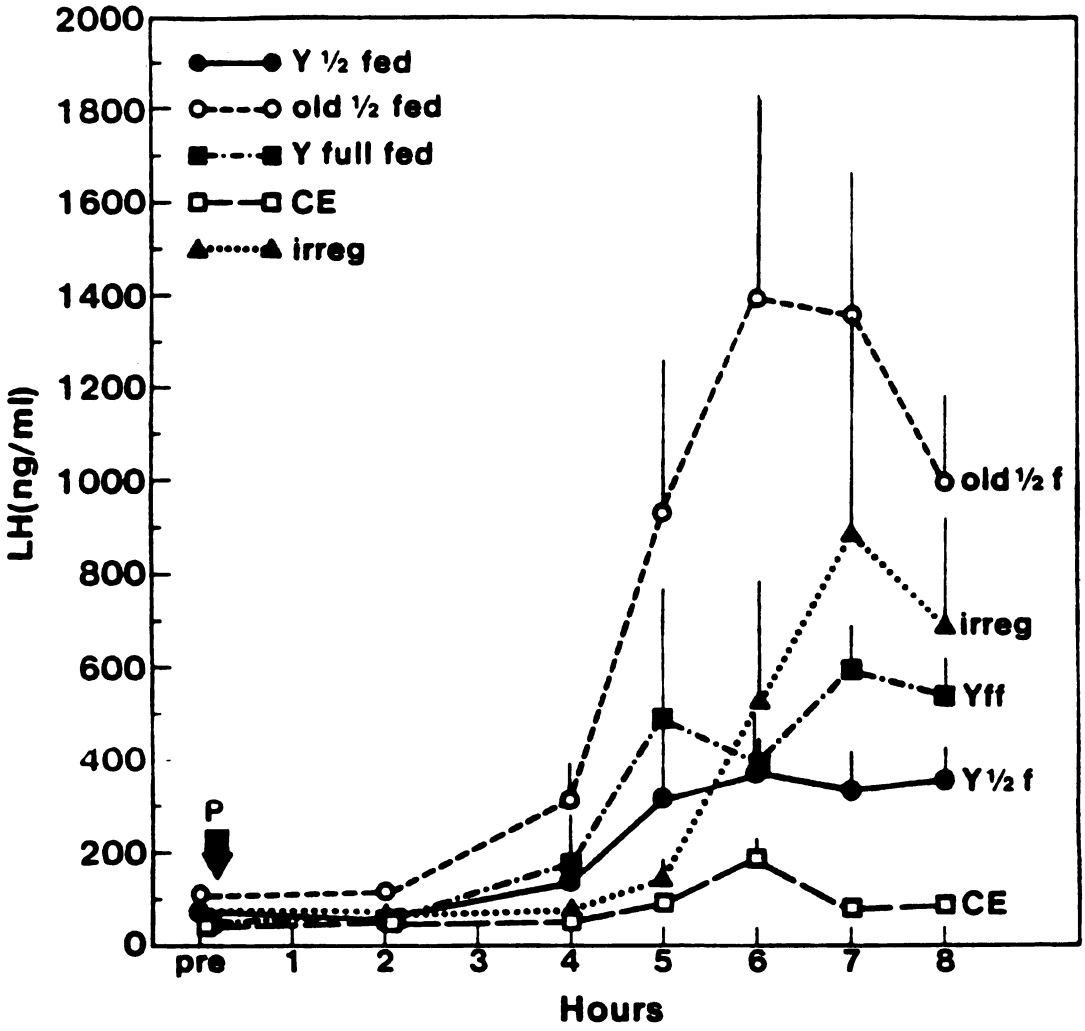

Figure 10. Effects of age and underfeeding/refeeding regimen on the positive feedback action of ovarian steroids on LH release during 4th week of refeeding (2 weeks postovariectomy). Old full-fed rats are distinguished according to estrous cycle status. Arrow designates time of progesterone administration. Note LH values of older previously half-fed rats and depressed response of older CE ad libitum-fed rats (mean ± SEM). Young half-fed (Y 1/2-fed, n = 12); young full-fed (Y full-fed, n = 13); older half-fed (old 1/2f, n = 8; constant estrous (CE) older ad libitum-fed, n = 9; irregularly cycling (irreg) older ad libitum-fed, n = 5.

($p < .01$) higher than young ad libitum-fed rats. However, old previously underfed rats were not significantly different than irregularly cycling ad libitum-fed rats at 7 and 8 hrs after progresterone administration.

There were no significant differences in the LH response to ovarian steroids among the young previously half-fed, young ad libitum-fed, and older irregularly cycling rats at any time period measured. Older ad libitum-fed CE rats demonstrated a minimal LH response to estrogen and progesterone. Six hours after progesterone administration, LH in the older ad libitum-fed CE rats was below 200 ng/ml, after which time LH reached baseline values. Note the early rise in LH exhibited by the old and young previously underfed and young ad libitum-fed rats at 4 hr after progesterone relative to the later rise exhibited in the CE and irregularly cycling ad libitum-fed rats at 5-6 hr after progesterone.

D. Positive feedback effects on LH release during 6th week of refeeding (4th week postovariectomy)

The LH rise induced by the estrogen-progesterone treatment in ovariectomized rats during the 6th week of refeeding is shown in Figure 11. Pre-progesterone LH values were not significantly different among the different groups. LH values in the older previously half-fed rats 5, 6, 7, and 8 hrs after progesterone administration were not significantly different than those of the old CE ad libitum-

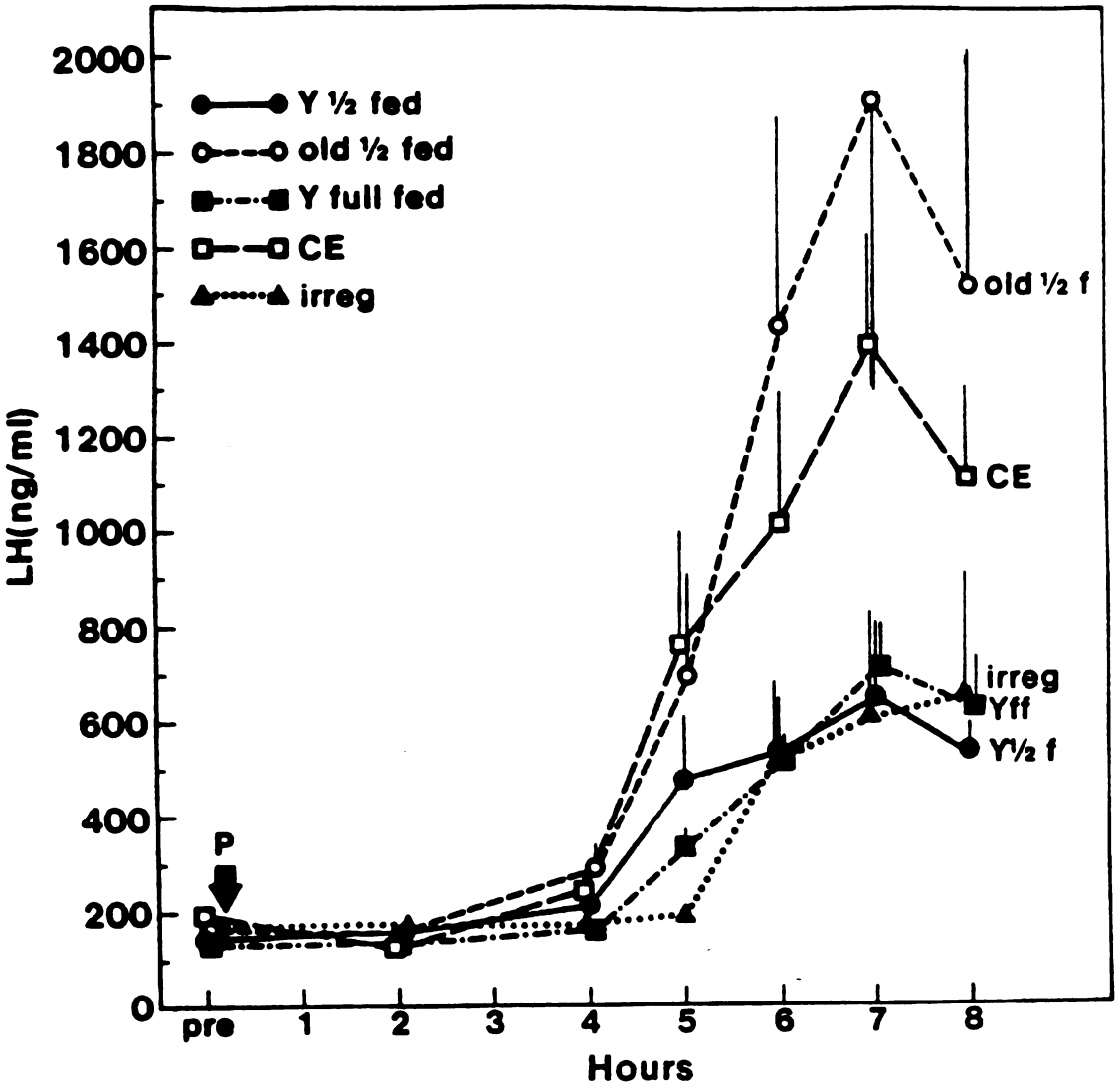

Figure 11. Effects of age and underfeeding/refeeding regimen on the positive feedback action of ovarian steroids on LH release during 6th week of refeeding (4th week postovariectomy). Arrow designates time of progesterone administration. Note the similarity in response of older previously half-fed rats and older CE rats and dramatic change in LH response of CE rats 4 weeks as compared to 2 weeks postovariectomy. Young half-fed (Y 1/2-fed, n = 12); young full-fed (Y full-fed, n = 14); older half-fed (old 1/2f, n = 10; constant estrous (CE) older ad libitum-fed, n = 7; irregularly cycling (irreg) older ad libitum-fed, n = 5. Mean ± SEM.

fed rats. At 6 and 7 hr after progesterone, the old previously underfed rats had significantly ($p < .05$) higher LH levels than the irregularly cycling ad libitum-fed young full-fed, and young previously underfed rats, but not significantly different than CE rats. The older previously half-fed rats had the highest mean peak LH levels 7 hrs after progesterone injection (1904.9 ng/ml), although these were not significantly greater than the peak LH mean values in the ad libitum-fed CE rats 7 hrs after progesterone administration (1386.1 ng/ml). The peak LH values in the older previously half-fed rats were approximately 36% greater at 4 weeks than at 2 weeks postovariectomy, but because of the large standard errors these differences were not significant. It is interesting that in the CE ad libitum-fed rats 2 weeks after ovariectomy, the mean peak values were below 200 ng/ml (Figure 10), whereas the mean peak LH values reached almost 1400 ng/ml, or about a 7-fold increase, 4 weeks after ovariectomy (Figure 11).

There were no significant differences in LH values between young previously half-fed and young ad libitum-fed rats after progesterone administration. Therefore, underfeeding had no effect on the LH feedback response in young animals. There were no significant differences in the LH rise in young previously half-fed, young ad libitum-fed, and older ad libitum-fed irregularly cycling rats. However, older ad libitum-fed irregularly cycling rats were slower in initiating an LH rise than young previously half-fed and ad

libitum-fed rats. Five hr after progesterone administration, the older ad libitum-fed irregularly cycling rats had significantly lower ($p < .05$) mean LH values (190.0 ng/ml) than the young previously half-fed rats (476.7 ng/ml).

E. Positive feedback effects of ovarian steroids on PRL secretion during the 4th and 6th week of refeeding (2nd and 4th week postovariectomy)

The PRL surge induced by estrogen followed 3 days later by progesterone in ovariectomized rats 2 weeks after ovariectomy is shown in Figure 12. Old previously half-fed, young previously half-fed, and young ad libitum-fed animals reached peak PRL values 4 hr after progesterone administration, whereas CE and irregularly cycling ad libitum-fed rats reached their peak PRL levels 5 and 7 hr after progesterone administration, respectively. The PRL responses in older previously half-fed rats were not significantly different than in young previously half-fed, young ad libitum-fed, or older irregularly cycling ad libitum-fed rats. Plasma PRL levels in CE ad libitum-fed rats at 4 and 5 hr after progesterone administration were significantly ($p < .05$) lower (59.7 and 91.0 ng/ml) than PRL values in the older previously underfed rats at these times (206.6 and 195.7 ng/ml), and tended to be lower than in all other groups during most periods measured.

During the 4th week after ovariectomy, the effects of estrogen followed 3 days later by progesterone on PRL

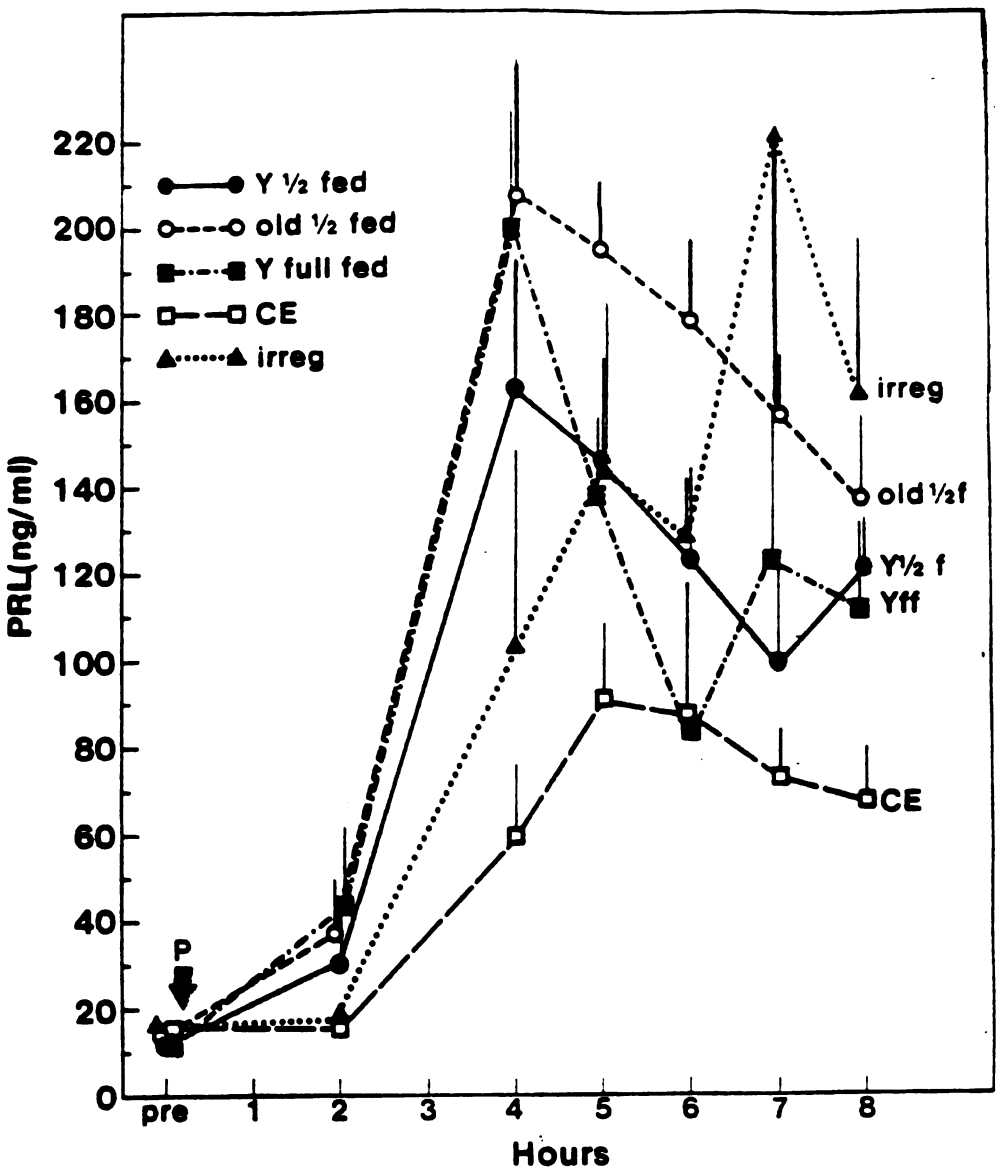

Figure 12. Effects of age and underfeeding/refeeding regimen on the positive feedback effects of ovarian steroids on PRL release during 4th week of refeeding (2nd week postovariectomy). Arrow designates time of progesterone administration. Young half-fed (Y1/2f), n = 12; young full-fed (Yff), n = 14; older half-fed (old 1/2f), n = 10; constant estrus (CE) older ad libitum-fed, n = 7; irregularly cycling (irreg) older ad libitum-fed, n = 5. Vertical bars represent SEM.

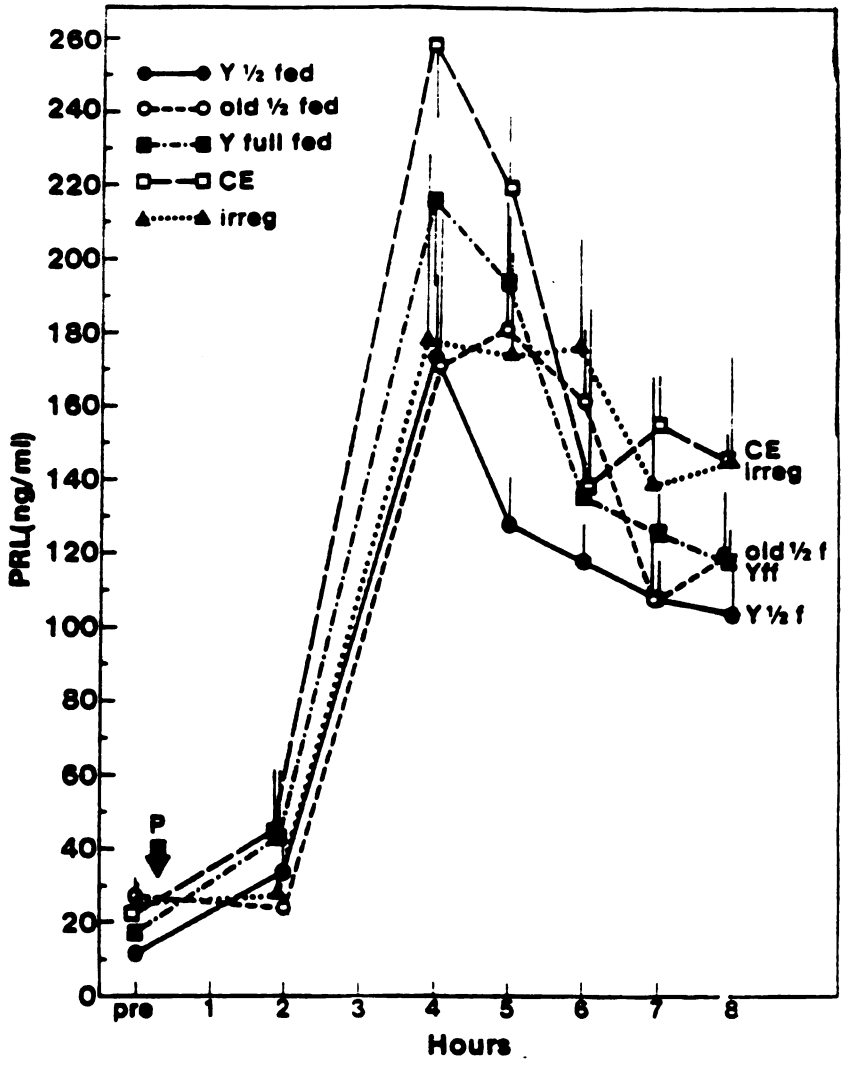

Figure 13. Effects of age and underfeeding/refeeding regimen on the positive feedback effects of ovarian steroids on PRL release during the 6th week of refeeding (4th week postovariectomy). Arrow designates time of progesterone administration. Note the differences in PRL response of old CE ad libitum-fed rats 2 and 4 weeks postovariectomy. Young half-fed (Y1/2f), n = 12; young full-fed (Yff), n = 14; older half-fed (old 1/2f), n = 10; constant estrus (CE) older ad libitum-fed, n = 7; irregularly cycling (irreg) older ad libitum-fed, n = 5. Mean ± SEM.

release also were determined (Figure 13). Pre-progresterone PRL levels in the older previously half-fed, irregularly cycling and CE ad libitum-fed older rats were more than 2-fold greater than in young previously half-fed and young ad libitum-fed animals. PRL values in the older CE ad libitum-fed rats 4 hr after progesterone administration were more than 4 times higher than the corresponding values 2 weeks postovariectomy (Figure 12), and were greater than in any other group. Underfeeding had no significant effect on the PRL positive feedback effects in both young and older rats.

F. Effect of ovariectomy (negative feedback removal) on LH release

Pre-ovariectomy serum LH in the older previously half-fed rats after 10 days of refeeding were approximately 33% higher than the older CE and irregularly cycling ad libitum-fed rats, although this difference was not significant. At 1 week postovariectomy, older previously half-fed rats demonstrated a 4.5 fold increase in serum LH values relative to preovariectomy values which was significantly greater ($p < .01$) than in any other group, including the young previously half-fed and young ad libitum-fed rats (Figure 14). The older previously half-fed rats reached maximal LH values before all other groups, and remained high for the 3 weeks after ovariectomy.

Mean serum LH values in young previously half-fed rats were higher, although not significantly, than in young

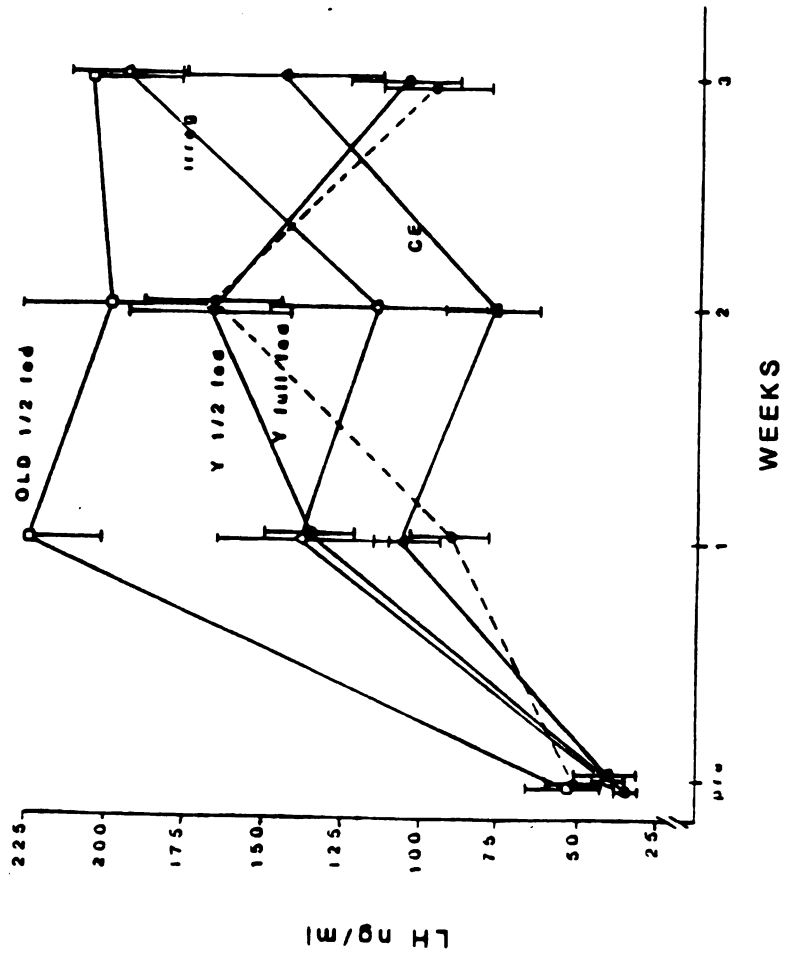

Figure 14. Effects of age and underfeeding/refeeding on serum LH levels 1, 2, and 3 weeks after ovariectomy. The rats were ovariectomized after 10 days of refeeding. Older ad libitum-fed rats are differentiated according to estrous cycle status: CE = constant estrus; irreg = irregularly cycling; pre = sample taken just prior to ovariectomy. Young half-fed (Y 1/2-fed), n = 13; young full-fed (Y full-fed), n = 15; old 1/2-fed, n = 10; CE, n = 9; irreg, n = 6. Vertical bars represent SEM.

ad libitum-fed controls 1 week postovariectomy, and at 2 and 3 weeks postovariectomy their LH concentrations were almost identical. Serum LH in both young half-fed and ad libitum-fed rats reached a peak at 2 weeks postovariectomy.

The LH response of the older ad libitum-fed CE and irregularly cycling rats paralleled each other for the 3-week postovariectomy period. Irregularly cycling rats had higher LH values than CE rats at 1, 2, and 3 weeks postovariectomy, although not significant. Both groups were not significantly different between 1 and 2 weeks postovariectomy. They showed a plateau until the 3rd week when LH levels attained peak levels. The peak LH values in both groups of older ad libitum-fed animals were delayed until the third week postovariectomy.

G. Effect of ovariectomy on PRL release

Serum PRL was measured just prior to ovariectomy, during the second week of refeeding, and 1, 2, and 3 weeks post-ovariectomy (Table 2). Young and older ad libitum-fed controls had significantly higher ($p < .05$) pre-ovariectomy PRL values than the young and older half-fed rats, with the older half-fed rats showing the lowest PRL values. At 1 week postovariectomy all groups showed a further decrease in PRL values except for the older half-fed group which remained essentially unchanged. PRL values among the young and older previously half-fed rats and full-fed controls were not significantly different 2 and 3 weeks postovariectomy.

Table 2. Mean serum PRL (ng/ml) in young and older rats after bilateral ovariectomy (mean ± SEM).

Group and treatment	2nd wk refeed, pre-ovax	3rd wk refeed, 1 wk post-ovax	4th wk refeed, 2 wks post-ovax	5th wk refeed, 3 wks post-ovax
Young 1/2-fed	8.4 ± 1.2 (6)	5.4 ± 1.7 (6)	11.7 ± 5.0 (6)	15.7 ± 3.8 (5)
Young full-fed	21.8 ± 5.8 (7)	4.5 ± 0.6*(8)	8.8 ± 1.1 (8)	11.4 ± 2.5 (7)
Old 1/2-fed	4.3 ± 1.1 (6)	5.0 ± 0.9 (6)	8.4 ± 1.6 (6)	15.0 ± 3.5 (6)
Old full-fed	13.1 ± 3.9 (7)	6.0 ± 1.0*(9)	12.6 ± 3.6 (8)	17.0 ± 1.7 (7)

* $p < 0.05$ relative to preovariectomy levels in same group
Number of animals in each group in parentheses

V. Discussion--positive feedback effects

These results show that a 10-week period of food restriction followed by ad libitum feeding produced a signficantly greater LH response to positive feedback by ovarian steroid challenge in the older than in the younger rats, but there were no significant differences in the PRL response. The greater LH response in the older previously underfed/refed rats was demonstrated both 2 and 4 weeks postovariectomy. This is in marked contrast to the previously reported positive feedback effects of ovarian steroids on LH release in young and old ad libitum-fed rats, in which ovariectomized old rats showed a much smaller LH increase than the young rats in response to ovarian hormones (Shaar et al., 1975). The present results are believed to at least partially explain why 10 weeks of underfeeding followed by refeeding resulted in temporary reinitiation of regular estrous cycles in old rats after they had ceased to cycle (Quigley et al., 1987). They do not explain why 10 weeks of underfeeding delayed loss of estrous cycles in the young rats (Quigley et al., 1987), since in the present study underfeeding/refeeding did not appear to enhance the LH response to ovarian hormones in the young rats. It is possible that short-term underfeeding/refeeding may have other effects favorable to reproductive functions in the young rats, including effects on the ovaries and reproductive tract.

Underfeeding in rats is known to result in reduced hypothalamic GnRH secretion (Campbell et al., 1971; Everitt, 1982), decreased LH release (Everitt, 1982; Ratner et al., 1978), lower ovarian function (Holehan and Merry, 1985), and cessation of cycles (Holehan and Merry, 1985; Ball et al., 1947). Since ovarian function is reduced and estrous cycles cease during underfeeding, estrogen secretion is reduced, resulting in less possible damage by estrogen on hypothalamic neurons involved in regulating reproductive cycles. Brawer et al. (1978) demonstrated that prolonged estrogen action resulted in injury to neurons in the arcuate nucleus and medial basal hypothalamus, similar to damage found in hypothalamic neurons of untreated old anovulatory rats. When rats or mice were ovariectomized early in life, followed many months later by transplantation of ovaries, these animals cycled far beyond the period when intact control animals had ceased to cycle (Aschheim, 1976; Felicio et al., 1983). This is believed to reflect the favorable effects on the neurons produced by estrogen removal. There is also the possibility that hypothalamic responsiveness to ovarian steroids may be heightened during underfeeding as a result of an increase in estrogen receptors induced by the decline in estrogen secretion. There is evidence for a decrease in hypothalamic estrogen receptors during aging in rats (Peng, 1983), and underfeeding/refeeding may inhibit this decline or even increase estrogen receptors, accounting

for the greater LH response to the ovarian steroids in the older half-fed rats upon refeeding.

Another possible cause for the increased LH release in response to estrogen/progesterone stimulation in the older underfed/refed rats may be due to altered pituitary sensitivity to GnRH action. Campbell et al. (1977) reported that mature underfed rats showed a greater LH response to GnRH administration than ad libitum-fed rats. In intact ad libitum-fed rats, no differences were found in GnRH receptors in the pituitaries of old and young rats (Sonntag et al., 1984), even though old rats respond less to GnRH administration than young rats (Watkins et al., 1975).

Lu et al. (1980) and Steger et al. (1980) demonstrated that the interval after ovariectomy was critical in determining the degree of the LH response to ovarian steroids in old CE rats. In their rats, ovarian steroids were less effective in producing an LH surge shortly after ovariectomy than after long-term ovariectomy. This observation is in agreement with the present results, since the older CE ad libitum-fed rats showed a much lower LH response to estrogen and progesterone at 2 than at 4 weeks after ovariectomy. Although estrogen is largely removed by ovariectomy, its effects on the CNS appear to persist. Lu et al. (1981) reported that old CE rats required a 4-5 week recovery period after ovariectomy in order to respond maximally to estrogen and progesterone on LH release. This response, however, was still below that in young rats. Our

old CE ad libitum-fed rats, 4 weeks after ovariectomy, demonstrated an LH rise approaching that of the older previously half-fed rats, and the LH rise was significantly greater than in the young rats. Lu et al. (1981) used older CE rats (18-19 months old) of the Long-Evans strain, and therefore their rats were exposed to estrogen for a longer period of time than our rats, and perhaps were subject to more neuronal damage. None of our older rats were in CE at the initiation of the experiment, although they began exhibiting CE vaginal smears during the period prior to ovariectomy. The 4-5 week interval after estrogen removal may have allowed the noradrenergic neurons time to recover from possible estrogen damage and increase their responsiveness to ovarian steroids. The striking similarity of the LH response to estrogen and progesterone of the older ad libitum-fed CE rats and old previously half-fed rats at 4 weeks postovariectomy suggests that a similar change in hypothalamic steroid sensitivity may have occurred at this time in both groups. It would be interesting to determine the duration of this LH response between these 2 groups.

The present results show that short-term underfeeding/refeeding had no significant effect on the PRL response to estrogen/progesterone administration in the young or older ovariectomized rats. This suggests that the mechanism controlling PRL release in response to ovarian steroids is unaffected by underfeeding. At both 2 and 4 weeks post-ovariectomy, there was a tendency for the older previously

half-fed rats to show a greater PRL response than in young rats, but the differences were not significant. Huang et al. (1980) reported no PRL rise after administration of estrogen followed by progesterone in old ad libitum-fed CE rats 2 weeks after ovariectomy, whereas we observed a small but significant PRL rise in our ad libitum-fed CE rats. However, Huang et al. (1980) used much older rats, 20-24 mo old, of the Long-Evans strain, which were therefore exposed to longer periods of estrogen action on hypothalamic neurons. Their rats may have required a longer recovery period to regain hypothalamo-pituitary sensitivity to ovarian steroids.

These results demonstrate that a short-term period of underfeeding, followed by refeeding, greatly enhanced the positive LH feedback sensitivity to ovarian steroids in aging rats, and provide some insight into the mechanism whereby this feeding regimen reinitiates estrous cycles in aging rats.

VI. Discussion--negative feedback effects

These results demonstrate that this underfeeding/ refeeding regimen also produces a greater LH response to ovariectomy in older than in similarly treated young rats during the entire 3-week postovariectomy period. LH values were not significantly different after ovariectomy in the

previously underfed young rats than in the young ad libitum-fed controls. These results are in marked contrast to the results reported after ovariectomy in ad libitum-fed old as compared with ad libitum-fed young rats, in which the old rats showed a smaller LH rise after ovariectomy than the young rats (Shaar et al., 1975). They suggest that a short period of underfeeding followed by refeeding can produce beneficial effects on hypothalamic negative feedback mechanisms controlling LH release in aging rats.

After ovariectomy, the rise in circulating LH was reported to be accompanied by increased norepinephrine (NE) turnover in the hypothalamus (Advis et al., 1980), increased concentrations of GnRH in pituitary portal blood (Sarkar and Fink, 1980), decreased hypothalamic content of GnRH (Kalra and Kalra, 1983), and increased numbers of pituitary GnRH receptors (Clayton and Catt, 1981). The reduced ability to release LH by old intact and ovariectomized rats has been attributed to the reduction in hypothalamic NE concentration and turnover (Simpkins et al., 1977; Huang et al., 1977), which renders rats less responsive to stimuli that normally promote LH release (Simpkins et al., 1977; Wilkes and Yen, 1981). However, 10 weeks of underfeeding followed by refeeding appears to significantly improve the responsiveness of the NE neurons to ovariectomy, perhaps by increasing gonadal steroid receptors, by increasing the sensitivity of GnRH neurons to NE, or by enhancing pituitary sensitivity to GnRH.

The precise mechanisms by which 10 weeks of underfeeding followed by refeeding results in a greater LH response to ovariectomy (and to the positive feedback action of ovarian hormones) in older than in younger female rats remains to be elucidated, but there are several possibilities. During underfeeding there is reduced brain and hypothalamic NE activity (Wurtman and Wurtman, 1983), accompanied by decreased tyrosine hydroxylase concentrations, the rate-limiting enzyme for CA synthesis (Gibson and Wurtman, 1978). These changes result in reduced GnRH release, decreased LH secretion, and cessation of estrous cycles (Gibson and Wurtman, 1978). The effects of starvation on LH suppression have been reported not to be reversed by administration of the CA precursor L-DOPA or by clonidine, a central-acting noradrenergic agonist (Pirke and Spyra, 1982). The endogenous opioid peptides (EOP) also may be involved in LH suppression during underfeeding, since administration of opioid antagonists (naloxone, naltrexone) counteract the inhibitory effects of starvation on LH release for at least a few days in rats (Briski et al., 1984; Dyer et al., 1985). Inhibition of LH release by EOP is mediated via NE neurons (Van Vugt et al., 1981; Kalra and Simpkins, 1981). Since reproduction is an energy-consuming process subject to inhibition by food scarcity, acyclicity resulting from underfeeding may be viewed as an adaptive mechanism. When the restraints of underfeeding to the GnRH pulse generator are removed by refeeding, EOP inhibition to LH release

apparently is removed, NE turnover is increased, and pulsatile LH release resumes (Bronson, 1986). Tyrosine hydroxylase may then become super-activated, perhaps more so in older than in younger rats, resulting in enhanced CA metabolism and heightened hypothalamic GnRH and LH release. Underfeeding/ refeeding may also re-establish a normal hypothalamic CA/5HT balance, reported to favorably influence LH release during the estrous cycle (Meites et al., 1987). The pituitary of older half-fed rats may also become more responsive to GnRH stimulation.

Underfeeding significantly reduced pre-ovariectomy PRL in both young and older rats, in agreement with previous reports (Campbell et al., 1977). In the ad libitum-fed rats, the higher PRL values in the older than in the young rats are in agreement with previous reports showing that old rats secrete more PRL than young rats (Clemens and Meites, 1971; Lu et al., 1979). Old female rats exhibit greater pituitary PRL content than young rats, and release more PRL when their pituitaries are incubated in vitro (Huang et al, 1976). Shaar et al. (1975) reported a greater fall in serum PRL 7 days after ovariectomy in old than in young rats. Our results show similar PRL concentrations in young and older rats 1, 2, and 3 weeks postovariectomy.

These results clearly demonstrate that underfeeding of only 10 weeks duration followed by refeeding can significantly enhance the LH response to ovariectomy in older rats more so than in younger rats, and are in sharp contrast

to the effects of ovariectomy on LH release in ad libitum-fed old and young rats after ovariectomy (Shaar et al., 1975). Further work will be necessary to determine precisely how underfeeding/refeeding increases the sensitivity of the hypothalamo-pituitary LH system to removal of ovarian hormones, or why older rats respond better than younger rats. Taken together, the present findings provide further evidence that a relatively short period of underfeeding followed by refeeding can improve neuroendocrine mechanisms regulating estrous cycles in aging rats.

Experiment 3:
Effects of 10-Week Underfeeding Followed by Refeeding on GH and Thyroid Hormone Secretion in Young, Middle-aged, and Old Male Rats

I. Abstract

Pulsatile GH release and thyroid hormone secretion have been shown to be significantly reduced in old rats. Chronic underfeeding and more recently only 10 weeks of underfeeding (Quigley et al., 1987) delayed some aging processes. The objective of this study was to determine the effects of 10 weeks of 50% food restriction followed by refeeding on pulsatile GH and thyroid hormone secretion in young, middle-aged, and old male rats. Sequential blood samples were collected every 30 min. for 5 hrs via indwelling atrial cannulae for measurement of mean and peak GH concentrations the 10th week of underfeeding and after 5 days of refeeding. Free and total T_3 and T_4 were measured in trunk serum. Ad libitum-fed middle-aged and old control rats showed a decline in mean and peak amplitude GH levels and a similar reduction in fT_3 and ft_4 levels relative to ad libitum-fed young rats. During underfeeding these values decreased in

all age groups, but were lower in the middle-aged and old rats than in the young rats. During refeeding all GH, T_3, and T_4 values returned to controls in all groups. Thus, the young rats exhibited a greater capacity to maintain GH and thyroid hormone levels during underfeeding and upon refeeding than the older rats. The declines in fT_3 and fT_4 levels with age and their further decline during underfeeding paralleled the decline in GH concentration. This suggests that thyroid hormones may be at least partially responsible for the reductions in GH secretion during aging. The reductions in GH and thyroid hormone secretion during underfeeding may have resulted in preserving the structural and functional integrity of body organs and tissues, thereby delaying some aging processes.

II. Introduction

Pulsatile growth hormone (GH) release was significantly decreased in old (18-20 mo) relative to young (4-5 mo) male rats. The frequency of the GH pulses did not change with age (Sonntag et al., 1980). Daily secretion of GH in old male rats was calculated to be less than half of that secreted by young male rats. In female rats, declines in GH pulses were reported as early as 11 months of age and are believed to account for the relatively early body growth stasis observed in mature female rats (Takahashi et al., 1987). Thyroid

hormone secretion is reduced in old rats (Huang et al., 1980), and it is possible that this is partly responsible for the reduction in GH secretion in old rats.

Chronic underfeeding delays aging processes, delays the onset of age-related diseases, and increases life span (McCay et al., 1935; Masaro, 1984). Underfeeding has also been shown to reduce GH (Campbell et al., 1977, Tannenbaum et al., 1979; Meites and Fiel, 1965) and thyroid hormone secretion (Glass et al., 1977). More recently, underfeeding for only 10 weeks followed by refeeding delayed the loss of estrous cycles in young rats, reinitiated estrous cycles in old rats (Quigley et al., 1987), and increased LH release in response to ovarian steroids in old rats. The effects of underfeeding followed by refeeding on other hormones remain to be determined in aging rats. It was of interest, therefore, to compare the effects of 10 weeks of 50% food restriction followed by ad libitum feeding on GH and thyroid hormone secretion in young, middle-aged, and old male rats. In the present study determinations of mean and pulsatile GH release and thyroid hormone levels were made in unanesthetized freely moving male rats during the underfeeding period, and after 5 days of refeeding.

II. Materials and methods

A. Animals and feeding regimen

Forty-five young (initially 3-4 months old), 44 middle-aged (initially 11 months old), and 41 old (initially 15-16 months old) male Sprague-Dawley rats were obtained from Harlan Industries (Indianapolis, IN). The old animals were purchased as retired breeders at 10-12 months of age and maintained in our animal facilities until they reached 16 months of age. All animals were individually housed in metal mesh cages in a temperature-controlled room (22 $\pm$ 2^{o}C) on a 14:10 light/dark cycle. Water was available ad libitum and they were fed ground rat feed obtained from Teklad Rat Diet (Harlan S.D. Co., Winfield, IA). All animals were allowed at least 1 week to acclimate to laboratory conditions and given tap water and ground feed ad libitum.

Determinations of mean daily food intake were made by recording the weight of food consumed by 15-20 representative rats from the young, middle-aged, and old group during a 5-day period. Young, middle-aged, and old rats were randomly divided into half-fed and ad libitum-fed controls. The half-fed groups received half the mean food intake (11.1, 11.5, 14 gm/day/rat, respectively) of their ad libitum-fed controls. Food was provided once each morning to the half-fed rats, whereas ad libitum-fed control animals had food available constantly. This regimen continued for a 10-week period followed by ad libitum feeding of the

previously half-fed rats. All animals were weighed once weekly.

B. Surgery and blood sampling procedure

Animals were divided into 2 groups according to the period of blood withdrawal. All animals were cannulated under ether anesthesia 2 days prior to sequential blood collection. A silastic indwelling atrial cannula was implanted into each animal through the right external jugular vein (ID: 0.64 mm, OD: 1.19 mm). The free end of the cannula was brought underneath the skin to the back of the neck and exited 1 cm posterior to the base of the skull. Animals were given 0.2 ml procaine penicillin im (200,000 U/ml) and streptomycin sulfate (powder) was topically applied before suturing. Animals were brought to the experimental room daily for 3 days prior to blood collection for acclimation and remained in the room during the hours when sampling would occur. Animals were brought to the experimental room 2.5 hours before the actual blood collection. Silastic extension tubing was attached to the cannulae and the tubing was placed through the wire mesh top of the cages. The cannulae were rinsed with heparin (100 IU/ml) and the animals were left undisturbed for at least 2 hr. Blood samples (0.5 ml) were taken every 30 minutes for 5 hr, beginning at 1130 hrs, placed into heparinized syringes and centrifuged at 2,000 rpm for 5 min. Plasma was separated and blood cells were resuspended in saline (0.85% NaCl) and

reinjected after the next blood withdrawal. The plasma samples were immediately frozen at -20°C and stored until assayed for GH.

There were 2 blood sampling periods: the last week of underfeeding, week 10, and on the 5th day of refeeding, wk. 11. Three days after sequential blood withdrawals trunk blood was collected. The animals were moved to another room and allowed to acclimate for at least 1 hr. Each animal was then removed from its own cage and taken into the surgery room for decapitation completely isolated (sight, sound, odor) from the remaining animals. Blood was allowed to clot at room temperature for 1-2 hr, centrifuged at 2500 rpm for 15 min. Serum was separated and immediately frozen at -20°C until assayed for free T_3, total T_3, free T_4, total T_4, and reverse T_3.

C. Hormone assays

GH was measured by radioimmunoassay (RIA) with the reagents provided by NIADDK (Marshall and Odell, 1975; Sonntag et al., 1980). Iodination grade GH was radiolabeled by a modified lactoperoxidase-glucose oxidase method (Tower et al., 1977). The iodinated hormone was purified on a 1.5 x 40 cm Sephacryl S-200 column equilibrated with 0.01 M phosphate buffered saline at pH 7.6. IgGsorb (Protein A, Enzyme Center, Malden, MA) was used to separate bound from free hormones. Hormone concentrations were expressed in terms of NIADDK rGH-RP-1. GH assays were run in two volumes

using 10 µl and 40 µl of sample. Only those volumes which gave hormone values corresponding to the linear portion of the standard curve were used. The intra-assay coefficients of variation (CV) for GH ranged from 2.3%-9.6%. Interassay CV was 13.1%. 50% inhibition of tracer binding ranged from 1.29 to 1.4 ng/tube.

Total thyroxine (TT_4) was measured in trunk serum employing a Quanticoat^R T_4 kit provided by Kallestad Laboratories, Inc. (Austin, TX). Free T_4 (fT_4) was measured using reagents provided by Ciba Corning (Medfield, MA). For free T_3 (fT_3) and total T_3 (TT_3) assays, iodinated hormone was supplied by New England Nuclear (Boston, MA), antibody by Miles Laboratory (Naperville, IL). Wein standards (Succasunna, NJ) were used. The reverse T_3 (rT_3) assay utilized reagents from Serono Diagnostics (Norwell, MA).

TT_4 was measured by a solid phase RIA using human serum standards. All samples were run in one assay with an intra-assay CV of 5.5%. Samples were expressed in µg/dl according to human serum standards. Concentration at 50% bound was 4.76 µg/dl. Unknowns were parallel to the standard curve from 2.4 to 20.0 µg/dl using 50 µl serum. Normal male rat serum was diluted with saline or phosphate protein buffer and gave accurate parallel dilutions (90.6 to 113.5% of expected values). Assay sensitivity was 1.0 µg/dl. Cross-reactivity of antibody was reported as 49.33% for tetraiodothyroacetic acid, 2.78% for D-triiodothyronine,

2.27% for L-triiodothyronine, and < .01% for monoiodotyrosine, diiodotyrosine, and diiodothyronine.

TT_3 was measured by double antibody RIA. Inter- and intra-assay CVs were were 3.3% and 7.9%, respectively. Assay sensitivity at 90% B/Bo was 10 pg/ml. Samples were expressed in ng/ml according to human serum standards. Cross-reactivity at 50% T_3 antibody displacement was 0.14% L-thyronine, 24% triiodothyroacetic acid, and < 0.001% monoiodo-L-tyrosine and diiodo-L-tyrosine, Normal male rat serum was diluted with water, phosphate buffer protein and zero human serum to give accurate parallel dilutions (85.6 to 89% of expected values).

fT_3 RIA had inter- and intra-assay CVs of 4.9% and 8.9%, respectively. Assay sensitivity at 90% B/Bo was 0.30 pg/ml. Samples were expressed in pg/ml according to human serum standards. Cross-reactivity of T_3 antibody includes 0.64% by tetraiodothyronine, 0.17% by D-thyroxine, 0.13% by L-thyroxine, and < 0.01% by propylthiouracil, methimazole, diphenylhydantoin, diiodotyrosine, monoiodotyrosine, sodium salicylate, and aspirin. Normal male rat serum diluted with water, phosphate protein buffer, or zero human serum gave accurate parallel dilutions (72% to 96% of expected values).

fT_4 was measured by RIA with an inter- and intra-assay CV of 7.3% and 5.5%, respectively. Assay sensitivity at 90% B/Bo was 0.22 pg/ml. Samples were expressed in pg/ml according to human serum standards. Cross-reactivity of T_4 antibody includes 6.3% by L-triiodothyronine, 4.4% by D-

triiodothyronine, 2.4% by tetraiodothyroacetic acid, 1.3% by triiodothyroacetic acid, and < 0.1% by phenylbutazone, 5,5-diphenylhydantoin, monoiodotyrosine, sodium salicylate, aspirin, 6-N-propyl-2-thiouracil, and methimazole. Normal male rat serum diluted with water, phosphate protein buffer, or zero human serum gave accurate parallel dilutions (76% to 112% of expected values).

rT_3 RIA had an interassay CV of 2.9%. Assay sensitivity at 90% B/Bo was 60 pg/ml. Cross-reactivity of the rT_3 antiserum included 0.13% by L-thyroxine, 2.1% by 3,3'diiodothyronine, 0.084% by 3,5,3'triiodothyronine, 2.1% by 3,3'diodothyronine, and < 0.01% by 3,5,3'triiodothyroacetic acid, monoiodothyrosine, and diiodotyrosine. Samples were expressed in pg/ml according to human serum standards. When normal rat serum was diluted, values were below assay sensitivity.

D. GH pulse analysis

Since GH pulses occur every 3.3 hr in male rats (Tannenbaum and Martin, 1976), cannulated blood samples (0.5 ml) were obtained every 30 min over a 5 hr period. A pulse was defined as a sequence of at least 2 increasing hormone concentrations from nadir followed by a peak or pulse maximum. The pulse maximum was used to calculate the mean pulse amplitude for each animal. Peaks had to be at least 2 standard deviations above nadir. Mean peak amplitudes for each group were obtained by taking a mean of all pulse peaks

for every animal. Trough (nadir) levels were the minimum GH concentrations between peaks. Mean GH concentrations in animals were calculated by averaging GH concentrations over the entire 5 hr sampling period. Mean plasma GH concentrations for each group were also determined at each blood sampling time period.

E. Statistics

The data were analyzed statistically by two-way analysis of variance (ANOVA) using a split plot design of subject/treatment over time for repeated measures and a randomized block design for trunk samples. This was followed by Student-Newman-Keuls test or lsd test for multiple comparisons when differences were observed. Differences were considered significant if $p < 0.05$. Comparisons of the peak GH concentrations in individual animals during the sampling period were made using X^2 test. The maximum GH concentrations for each animal were classified and the resulting 6x5 contingency tables were analyzed (Steel and Torrie 1980). Subsequent individual group comparisons were made using 2x5 contingency tables.

IV. Results

A. Body weight

Young, middle-aged, and old underfed rats initially showed a marked loss of body weight during underfeeding, and then maintained reduced body weights with some variations for the remainder of the 10-week period (Figure 15). The young rats demonstrated a smaller decline in body weight during underfeeding than the middle-aged and old rats which tended to show a continuous decline in weight during the 10-week period. The young rats showed a 24% mean body weight loss compared with pre-underfeeding weights and approximately a 42% mean body weight loss relative to young ad libitum-fed controls. The young ad libitum-fed controls demonstrated a continuous body weight gain during the entire experimental period, attaining approximately a 1.5 fold increase during the total period of the study. The middle-aged rats showed a 29% mean body weight loss during underfeeding compared to pre-underfeeding weights. Middle-aged full-fed controls exhibited an 11% total weight gain. The old rats showed a 38% weight loss during underfeeding relative to pre-underfeeding weights. The old full-fed controls exhibited a small but insignificant weight gain of 4%. Thus the body growth rate in full-fed control groups exhibited an age-related decline in body growth rate.

Upon refeeding, all the underfed groups showed rapid body weight gains, although gut fill may at least partially

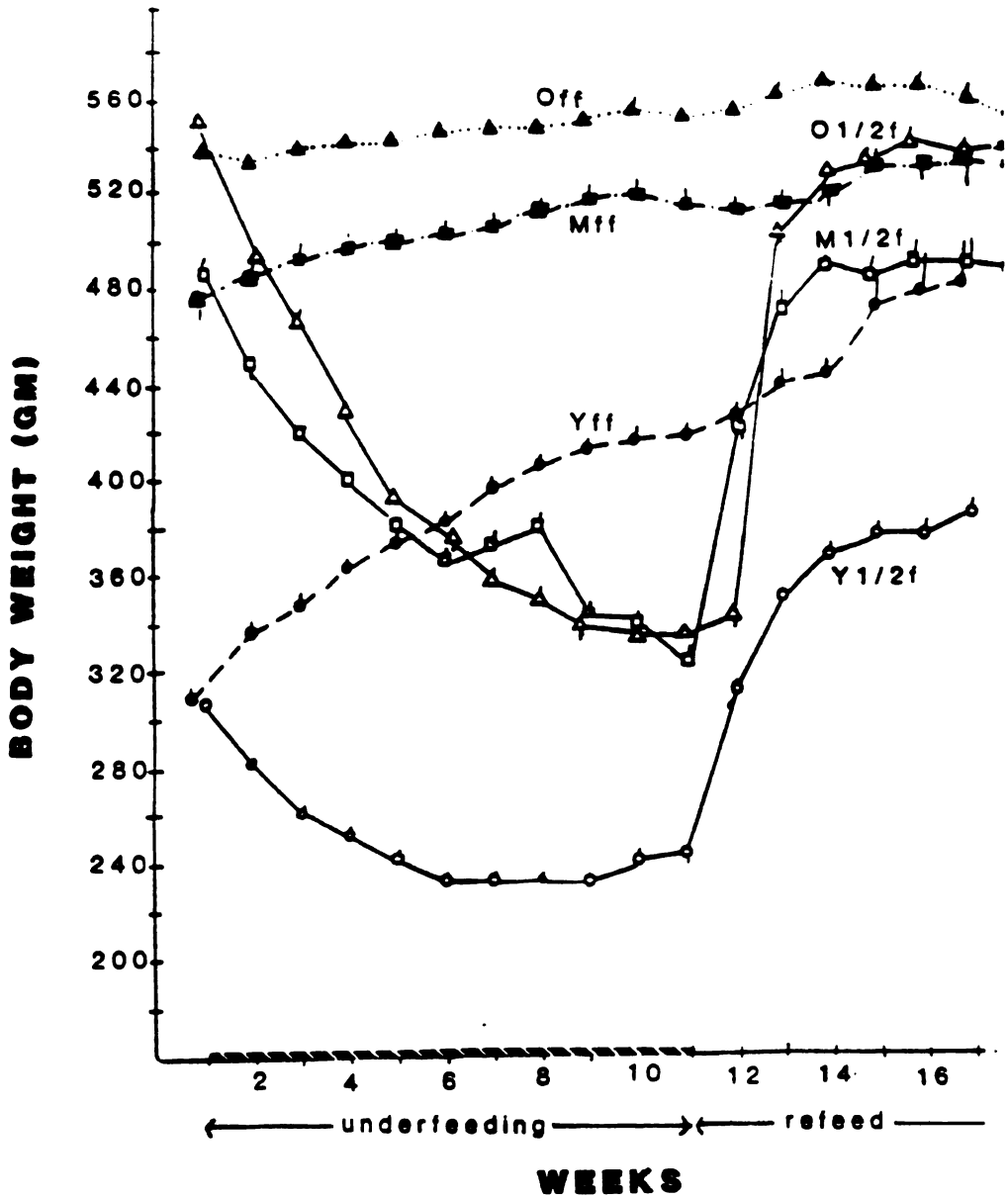

Figure 15. Effects of age and underfeeding/refeeding regimen on mean body weight. Period of underfeeding: weeks 1-10. Period of refeeding: weeks 11+. ○——○ Y1/2f = young half-fed; ●– – –● Yff = young full-fed controls; □——□ M1/2f = middle-aged half-fed; ●—·—● Mff = middle-aged full-fed controls; △——△ O1/2f = old half-fed; ▲···▲ Off = old full-fed controls. Open symbols designate underfed groups, closed symbols designate full-fed controls. Vertical bars represent SEM.

account for this large increase. Young rats regained pre-underfeeding weights in 1 week, the middle-aged in 3 weeks, and the old rats in 5 weeks. The young and middle-aged rats did not attain the body weights of the full-fed controls. The young previously underfed rats, although significantly below full-fed controls in body weight during refeeding, demonstrated similar rates of weight gain as middle-aged and old previously underfed rats.

B. GH pulses

The effects of underfeeding and 5 days of refeeding on mean GH concentrations over the 5 hr sampling period are shown in Figures 16-17. Young, middle-aged, and old rats all demonstrated absence of GH pulses during underfeeding. There were no significant differences among the 3 age groups during underfeeding. After 5 days of refeeding all groups exhibited pulsatile GH release. Young refed rats had mean GH values ranging from a nadir of 33 ng/ml to 234 ng/ml. Middle-aged refed rats also exhibited pulsatile GH release, with mean GH concentrations ranging from 53 to 174 ng/ml. Old refed rats had GH pulses ranging from 20 to 156 ng/ml. Mean GH concentrations of full-fed controls paralleled those of refeeding in contrast to those during underfeeding.

C. Mean GH concentrations

During underfeeding, young, middle-aged, and old half-fed rats had significantly lower mean GH than ad libitum-fed

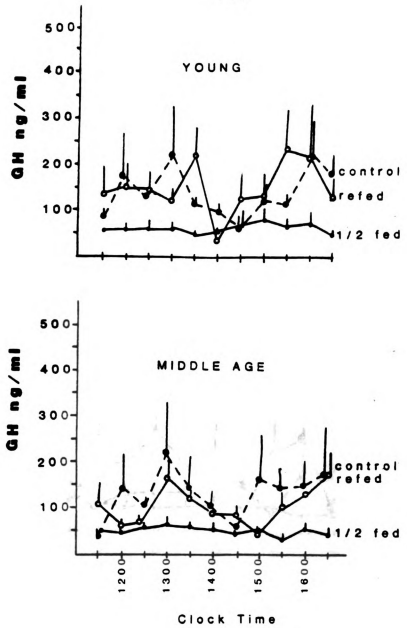

Figure 16. Effects of underfeeding and refeeding on mean GH concentrations over the entire 5 hr sampling period in young and middle-aged rats. Plasma samples were collected every 30 min beginning at 1130 hr. Vertical bars represent SEM. Young 1/2-fed, n = 13; young refed, n = 14; young full-fed controls, n =16; middle-aged 1/2-fed, n = 14; middle-aged refed, n = 13; middle-aged full-fed controls, n = 16. Note the absence of GH pulses in the underfed groups. Dashed bars represent full-fed controls. Open circles represent refed rats.

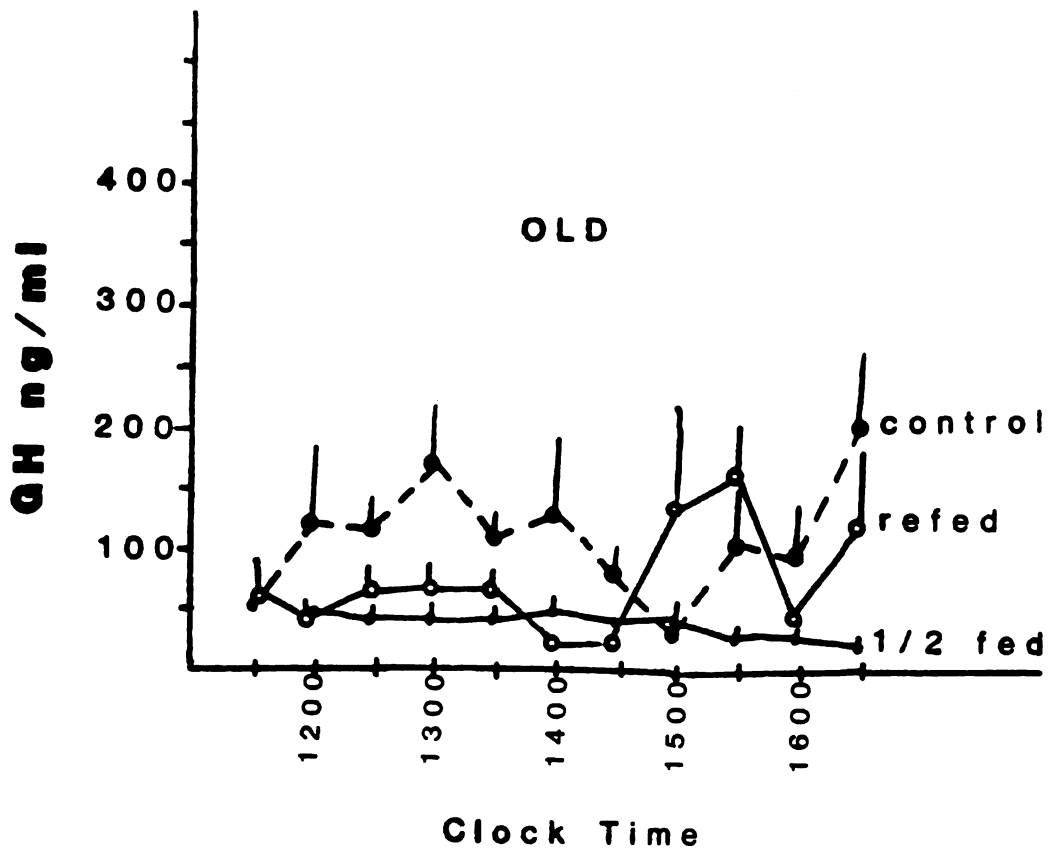

Figure 17. Effects of underfeeding and refeeding on mean GH concentrations over the entire 5 hr sampling period in old rats. Plasma samples were collected every 30 min beginning at 1130 hr. Vertical bars represent SEM. Old 1/2-fed, n = 12; old refed, n = 9; old full-fed controls, n = 14. Note the absence of GH pulses during underfeeding. Dashed bars represent full-fed controls. Open circles represent refed rats.

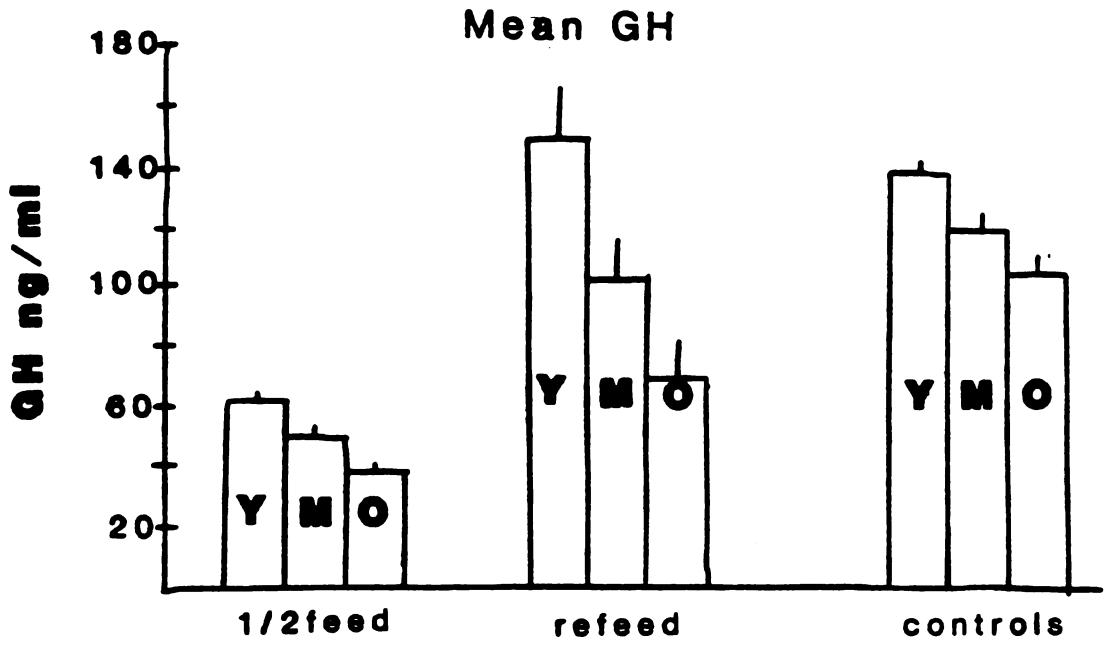

Figure 18. Effects of age and underfeeding/refeeding regimen on mean plasma GH concentrations. Cannulated blood samples were obtained the 10th week of underfeeding and the 5th day of refeeding. Vertical bars represent SEM. Y = young; M = middle-aged; O = old.

controls (Figure 18). Of these, the GH concentrations in the young rats were highest and levels in the old rats lowest, although not significant. The GH levels in the middle-aged rats were between these. Ad libitum-fed controls followed a pattern similar to that during underfeeding. Young rats had the highest mean GH levels, and the old rats the lowest. The GH concentrations in the young full-fed rats were significantly ($p < .02$) greater than old rats but were not significantly different from the middle-aged full-fed controls. Middle-aged full-fed rats showed greater mean GH values than old full-fed rats, but these differences were not significant.

By the 5th day of refeeding young, middle-aged, and old previously half-fed rats showed a rise in mean GH levels relative to underfeeding values. Young rats demonstrated more than a 2.4 fold increase in GH values, whereas the middle-aged and old rats exhibited a 2 and 1.78 fold increase, respectively. The young previously half-fed rats had significantly higher ($p < 0.01$) mean GH concentrations than the old previously half-fed rats, although the values were not significantly higher than in the middle-aged previously half-fed rats.

D. Mean GH peak amplitude

During underfeeding, all groups had reduced mean GH peak amplitudes relative to full-fed controls (Figure 19). The GH levels in the young half-fed rats were higher than in the

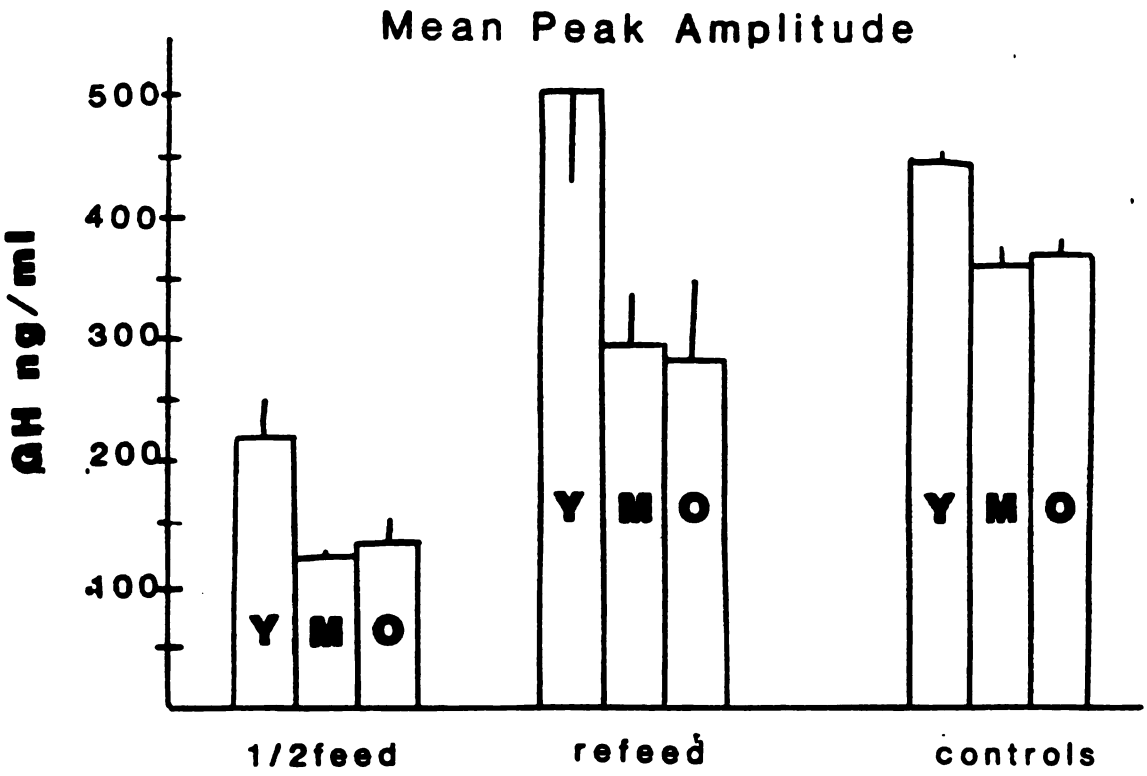

Figure 19. Effects of age and underfeeding/refeeding regimen on mean GH peak amplitude. Cannulated blood samples were obtained during the 10th week of underfeeding and the 5th day of refeeding. Vertical bars represent SEM. Y = young; M = middle-aged; O = old.

middle-aged and old half-fed groups. GH concentrations in the young half-fed rats were 47% below those in the young full-fed rats. GH values in the middle-aged half-fed rats were 57% below those in the middle-aged full-fed rats, and the old half-fed rats were 58% below old full-fed rats. Mean peak GH amplitudes of middle-aged half-fed rats were ($p < .02$) below those of middle-aged full-fed rats. The old half-fed rats showed GH peak amplitude values ($p < .05$) below those of old full-fed control rats.

Upon refeeding, the young previously half-fed rats exhibited more than a 2.2 fold increase in mean GH peak values over that during underfeeding ($p < .02$). Similarly, the middle-aged previously half-fed rats demonstrated a significant ($p < .02$) GH increase of more than 2.4 fold upon refeeding than during underfeeding. The old previously half-fed rats exhibited more than a 2 fold rise in GH amplitude levels over underfeeding values. Middle-aged and old previously half-fed rats exhibited similar mean GH peak amplitude patterns upon refeeding, but these were lower than in young half-fed rats. Thus, young, middle-aged, and old half-fed groups regained mean peak GH amplitude by the 5th day of refeeding and were not significantly different from their ad libitum-fed controls.

E. Individual GH maximum peaks

Maximum GH values for each animal during underfeeding and refeeding are shown in Table 3. During underfeeding

Table 3. Maximum GH concentrations for each animal during underfeeding and refeeding sampling periods.

	Group	n	<100	100 -300	301 -600	601- 800	>800	range
I wk. 10 during under-feed-ing	Yl/2fI	13	3	7	3	0	0	48.2 - 576.3
	MAl/2fI	14	6	8	0	0	0	42.3 - 176.6
	oldl/2fI	12	6	6	0	0	0	49.6 - 193.8
II 5th day refeed-ing	Yl/2fII	14	0	1	5	3	5	127.3 - 1829.6
	MAl/2fII	13	0	6	4	1	2	167.8 - 1106.5
	oldl/2fII	9	1	4	2	2	0	50.6 - 788.4
con-trols	Yff	16	0	6	4	2	4	155.5 - 1530.5
	MAff	16	0	6	7	1	2	156.9 - 930.0
	oldff	12	0	6	5	1	0	187.7 - 820.6

Yl/2f = young half-fed; Yff = young full-fed; MAl/2f = middle-aged half-fed; MAff = middle-aged full-fed; oldl/2f = old half-fed; oldff = old full-fed

there was a significant difference ($p < .001$) among the 3 age groups. In the young half-fed rats, maximum GH values ranged from 48.2 to 576.3 ng/ml, whereas the young full-fed rats ranged from 155.5 to 1067.4 ng GH/ml. Two out of 8 or 25% of young full-fed had GH peak values of >800 ng/ ml (1035.3 and 1067.4 ng/ml). Figure 20 shows the depressed ranges of middle-aged half-fed and old half-fed relative to their ad libitum-fed controls. Middle-aged half-fed and old half-fed showed significantly ($p < .01$) lower peak GH values than their full-fed controls during this underfeeding period.

By the 5th day of refeeding young and middle-aged rats showed GH maximum levels significantly greater ($p < .01$) than during underfeeding. Due to the large standard error, GH maximum levels were not significantly different during underfeeding and refeeding in the old half-fed rats. Young previously half-fed rats had a significantly higher ($p < .001$) GH peak distribution than young full-fed controls during the same time period. Five out of 14 or 36% of the young previously half-fed animals had GH peaks greater than 800 ng/ml. Middle-aged and old half-fed GH peak distributions were not significantly different than in their full-fed controls by the 5th day of refeeding (Table 3).

F. Free T_3 (fT_3)

During underfeeding, all age groups demonstrated significantly lower fT_3 concentrations than in full-fed

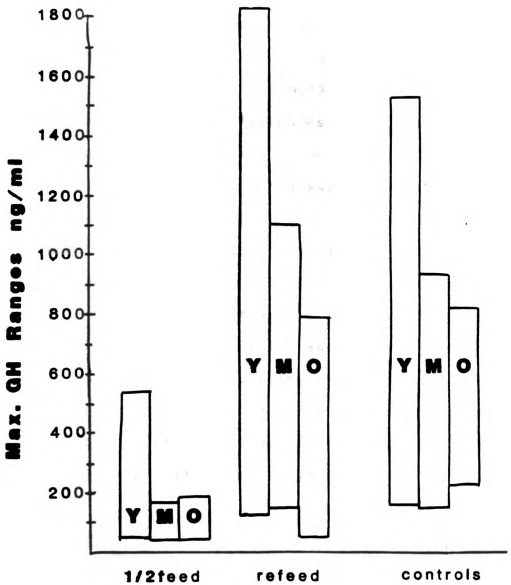

Figure 20. Effects of age and underfeeding/refeeding regimen on maximum GH ranges. Peak maximum from each animal was used in calculation of maximum ranges for each group. Cannulated blood samples were obtained the 10th week of underfeeding and the 5th day of refeeding. Y = young; M = middle-aged; O = old.

controls (Figure 21). The fT_3 concentrations in the young half-fed rats were 31% lower, the middle-aged rats 50% lower, and the old half-fed rats 80% lower than in the full-fed controls. Although fT_3 levels in all groups declined during underfeeding, the young rats had significantly ($p < .001$) higher fT_3 levels than the middle-aged and old rats. There were no significant differences in fT_3 values between middle-aged and old rats during underfeeding. Thus the decline in fT_3 during underfeeding was age-related.

During refeeding, the middle-aged and old rats demonstrated an increase in fT_3 levels over underfeeding values. The young previously underfed rats upon refeeding showed fT_3 levels significantly ($p < .001$) above old refed rats, but values not significantly above those in middle-aged refed rats. Among the full-fed controls, the old rats showed fT_3 levels lower than in the young or middle-aged rats. Thus the old rats had lower fT_3 values under all three conditions of this study than the young rats.

G. Total T_3 (TT_3)

During underfeeding all age groups demonstrated no significant change in TT_3 levels relative to values in full-fed controls (Figure 22). TT_3 levels in the young underfed rats were not significantly different than middle-aged underfed rats, but were significantly higher than in the old underfed group. During refeeding, the middle-aged and old rats showed no significant change in TT_4 from levels during

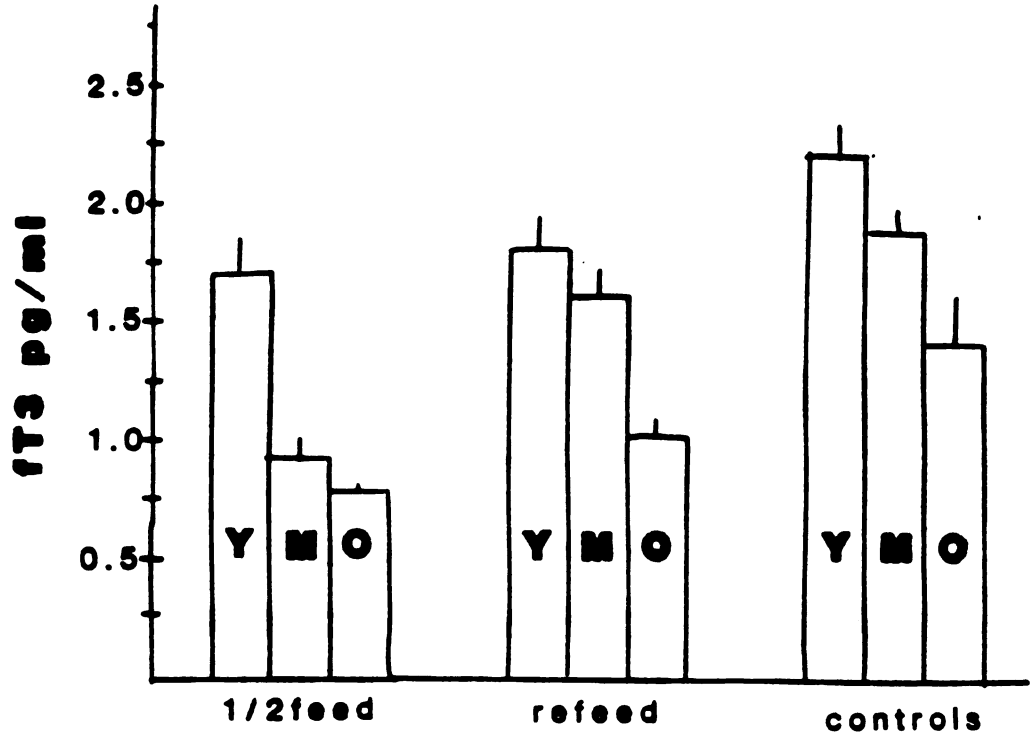

Figure 21. Effects of age and underfeeding/refeeding regimen on serum fT_3 concentrations. Trunk blood samples were obtained during the 10th week of underfeeding and the 5th day of refeeding. Note the age-related differences among all feeding regimens. Y = young, M = middle-aged; O = old. Vertical bars represent SEM.

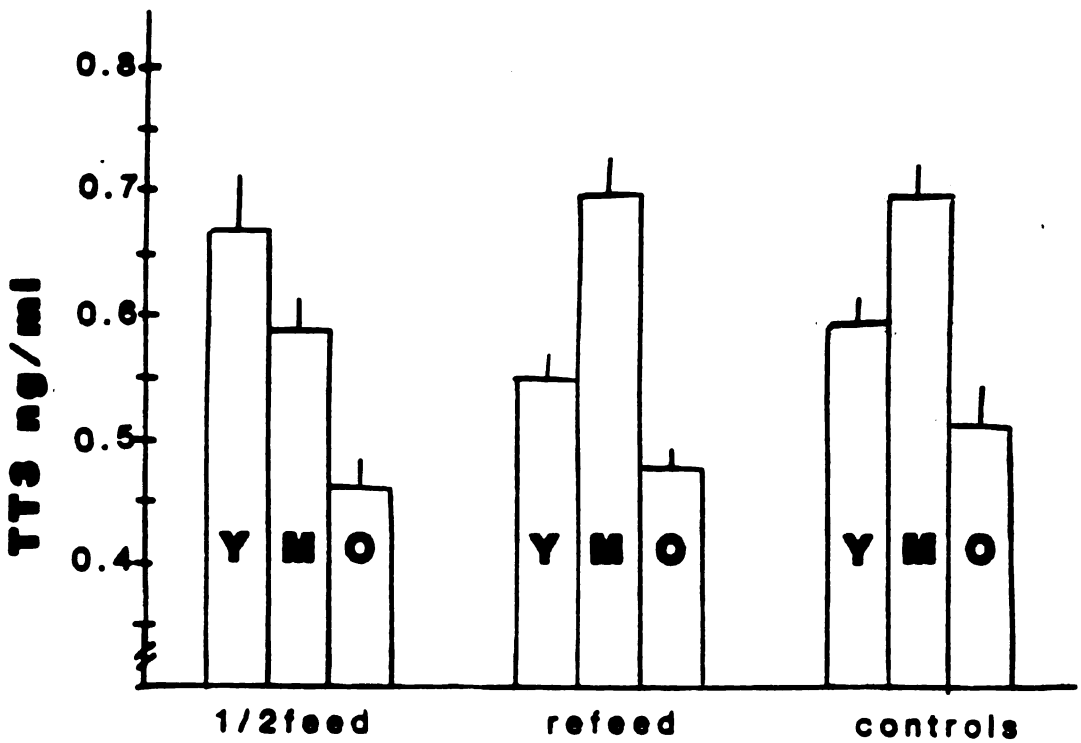

Figure 22. Effects of age and underfeeding/refeeding regimen on serum TT_3 concentrations. Trunk blood samples were obtained during the 10th week of underfeeding and the 5th day of refeeding. Vertical bars represent SEM. Y = young, M = middle-aged; O = old.

underfeeding, but the young rats showed some reduction as compared with underfeeding levels. Young rats upon refeeding showed TT_3 concentrations not significantly different than young full-fed controls. In the full-fed rats, there were no significant differences in TT_3 levels between the young and middle-aged rats, but the old rats showed values lower than the other two age groups. The values in the middle-aged full-fed controls were significantly higher ($p < .05$) than in the old full-fed controls.

H. Free T_4 (fT_4)

During underfeeding, all age groups showed significantly lower fT_4 levels than in full-fed controls (Figure 23). The values for the young underfed rats were 39% lower, the middle-aged 84% lower, and the old rats 65% lower than full-fed controls. The young underfed rats had significantly higher ($p < .001$) fT_4 levels than middle-aged or old rats during underfeeding. The middle-aged underfed rats showed mean fT_4 levels slightly above those of the old underfed rats, but these differences were not significant.

After refeeding, all age groups had fT_4 concentrations significantly greater than that during underfeeding. The fT_4 levels of the young refed rats were significantly ($p< .01$) above those of the middle-aged and old refed rats. The fT_4 levels in the young, middle-aged, and old refed rats were not significantly different from their respective full-fed controls. The mean fT_4 levels in the full-fed controls

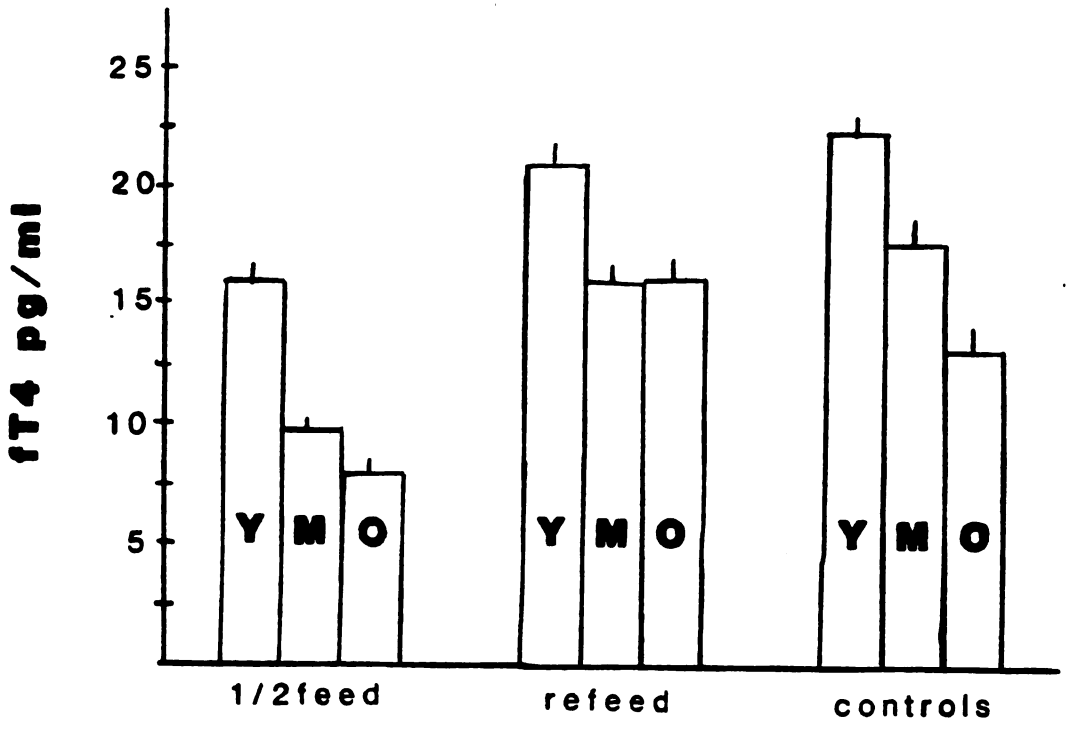

Figure 23. Effects of age and underfeeding/refeeding regimen on serum fT_4 concentrations. Trunk blood samples were obtained the 10th week of underfeeding and the 5th day of refeeding. Vertical bars represent SEM. Y = young; M = middle-aged; O = old. Note age-related suppression in all feeding regimens.

showed an age-related decline, the young rats showing significantly ($p < .01$) higher fT_4 levels than middle-aged rats, which in turn were significantly ($p < .01$) higher than in the old full-fed rats.

I. Total T_4 (TT_4)

During underfeeding the young rats had higher ($p < .01$) TT_4 concentrations than middle-aged and old rats (Figure 24). Upon refeeding, these values did not change significantly in any of the three age groups. In the full-fed controls, the TT_4 levels were not significantly different from those in the refed rats. TT_4 levels in the young full-fed controls were not significantly different from middle-aged rats, but higher than in the old controls. TT_4 levels in the middle-aged full-fed controls were significantly above those of the old full-fed controls.

J. Reverse T_3 (rT_3)

During underfeeding, the young rats exhibited a 72% greater rT_3 ($p < .01$) than in young full-fed controls (Figure 25). The rT_3 values in the young rats were significantly greater than in the middle-aged and old underfed rats. The rT_3 concentrations of middle-aged and old rats were not significantly different from each other even though mean rT_3 concentrations in the middle-aged rats were lower than in the old rats during underfeeding. The old underfed rats exhibited no significant change in rT_3 levels

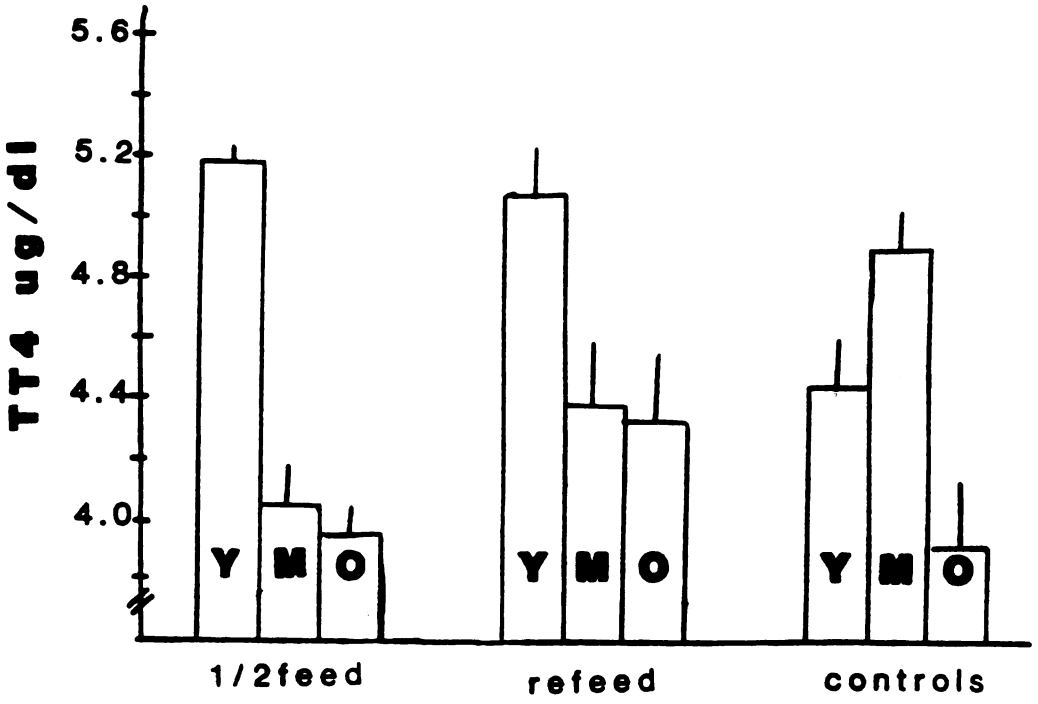

Figure 24. Effects of age and underfeeding/refeeding regimen on serum TT_4 concentrations. Trunk blood samples were obtained the 10th week of underfeeding and the 5th day of refeeding. Y = young; M = middle-aged; O = old. Vertical bars represent SEM.

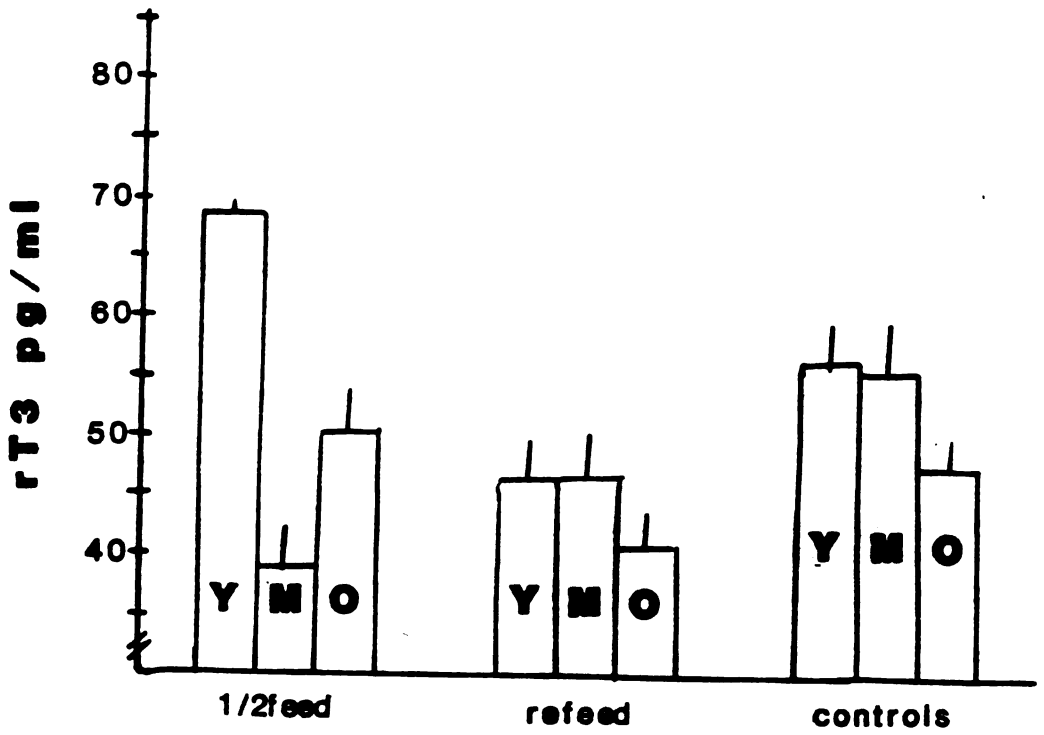

Figure 25. Effects of age and underfeeding/refeeding regimen on serum rT_3 levels. Trunk blood samples were obtained the 10th week of underfeeding and after 5 days of refeeding. Y = young; M = middle-aged; O = old. Vertical bars represent SEM.

when compared with full-fed controls. Thus only the young rats increased rT_3 levels in response to underfeeding. Upon refeeding there was a decrease in rT_3 levels in the young rats, but these were not significantly different from the full-fed controls.

There were also no significant differences in middle-aged and old rats during underfeeding or at refeeding. No significant differences were seen among the 3 full-fed control groups.

K. Free T_3/GH

The relationships of fT_3 levels to mean GH concentrations during underfeeding, at refeeding, and in full-fed controls are shown in Figure 26. A similar slope is exhibited between middle-aged and old rats during underfeeding and refeeding and in full-fed controls. However, the young rats show little or no relationship between fT_3 and GH values during the different feeding regimens, suggesting a greater ability to maintain fT_3 levels despite changes in food intake. Thus, a relationship is seen between fT_3 and GH levels only in the middle-aged and old rats.

V. Discussion

These results show a decline in mean circulating GH levels associated with a similar decrease in fT_3 and fT_4

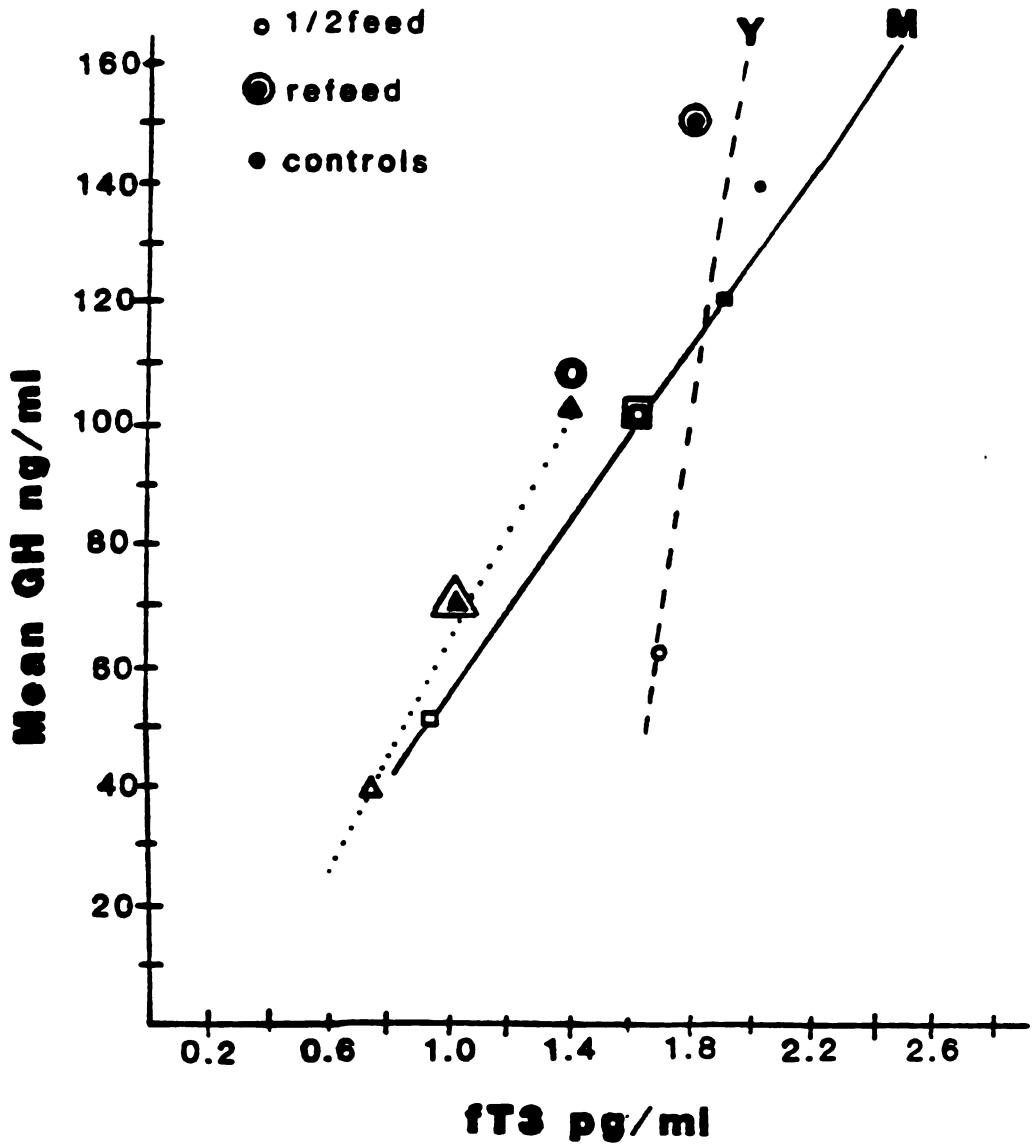

Figure 26. Relationship of mean GH and fT_3 concentrations during underfeeding, refeeding, and in full-fed controls among young, middle-aged, and old rats. Open symbols designate underfed rats, dark symbols as full-fed controls, framed symbols as refeed groups. Y = young; M = middle-aged; O = old. Circles = young; squares = middle-aged; triangles = old.

levels in full-fed, middle-aged, and old rats relative to full-fed young rats. During underfeeding, these values decreased in all age groups, but were lower in the middle-aged and old rats than in the young rats. Upon refeeding all GH and T_3 and T_4 values returned to control levels in all age groups. Thus, the young rats exhibited a greater capacity to maintain GH and thyroid hormone levels during underfeeding than the middle-aged and old rats.

The decline in mean GH concentrations and GH peak amplitudes with age in full-fed rats shown in the present study are in agreement with previous reports (Sonntag et al., 1980; Takahashi et al., 1987). Our data show similar reductions in peak and mean GH concentrations in middle-aged and old male rats, suggesting that this age-related decline in male rats begins at an earlier age than previously reported by Sonntag et al. (1980) who demonstrated a decline in mean and peak GH in male rats 18-20 months old. Our results show a decline in GH secretion even in 12-month-old male rats. The decline in GH amplitude in old rats was associated with a reduction in pituitary GH content and increased release of hypothalamic somatostatin (Sonntag et al., 1980, 1981).

Both dopamine and norepinephrine stimulate GH secretion in rats (Meites et al., 1977; Meites and Sonntag, 1981), presumably by promoting release of GHRH or by decreasing release of somatostatin, or both. Since CA metabolism is decreased in the hypothalamus of old male and female rats

(Simpkins et al., 1977), the reduced amplitude of pulsatile GH secretion may be the result, at least in part, of reduced hypothalamic CA activity. L-DOPA administration for 8 days to old male rats increases the amplitude of GH pulses and elevate mean plasma GH secretion in old male rats to the same levels present in young male rats (Sonntag et al., 1982). The secretion of GH is controlled both by somatostatin and GHRH, and the release of these in turn are modulated by several neurotransmitters, particularly CAs (Martin, 1979; Meites et al., 1977). Both diminished and unaltered *in vitro* pituitary responsiveness to GHRH have been reported in old as compared with young rats (Sonntag et al., 1983; Ceda et al., 1986). The *in vivo* response to GHRH administration in old male rats is also conflicting (Sonntag et al., 1983; Ceda et al., 1986; Wehrenberg and Ling, 1983) and therefore the effect of GHRH on GH release at the level of the pituitary in aging rats remains to be clarified.

The present results demonstrate a marked decrease in mean plasma GH concentrations and peak amplitude in response to chronic food restriction and are in agreement with other workers (Tannenbaum et al., 1979; Campbell et al., 1977) who showed that underfeeding reduced the secretion of GH pulses in young rats. Tannenbaum et al. (1979) also showed that starvation inhibited pulsatile GH release in young mature rats. Furthermore, these results show that the decline in GH secretion during chronic underfeeding is greater in middle-aged and old rats than in young rats.

Tannenbaum et al. (1978) reported that somatostatin has a physiologically important role in GH suppression during underfeeding, since administration of somatostatin antiserum was able to prevent the decline in GH amplitude during 1-3 days of starvation in young rats. Whether somatostatin is involved in the decline in GH secretion in middle-aged and old rats remains to be determined. Somatostatin antiserum increases GH equally or to a greater extent in old than in young rats, suggesting that aging rats release more somatostatin or the pituitary is more sensitive to its inhibitory effects (Sonntag et al., 1981).

The known regulators of GH gene transcription in the pituitary include thyroid hormone (T_3) (Solomon and Greep, 1959; Coiro et al., 1979), GH-RH (Barinaga et al., 1983; Gick et al., 1984), insulin (Yamashita and Melmed, 1986), somatomedin (Silva and Larsen, 1977), and glucocorticoids (Spindler et al., 1982). The largest effects on GH mRNA levels are induced by T_3. Martin et al. (1985) reported that thyroid hormones were necessary to maintain both pulsatile and induced GH secretion in unanesthetized rats. Our results show a decline in fT_3 and fT_4 concentrations with age, and a further decline during underfeeding, suggesting that the thyroid hormone levels may be at least partially responsible for the decrease in GH secretion.

Crew et al. (1987) suggested that the age-related decline in pituitary GH content is due to a decline in the level of GH mRNA. Since T_3 is vital to GH synthesis and

subsequent release by the pituitary, and T_4 can be converted to T_3, the age-related decline in the levels of both fT_3 and fT_4 in the full-fed controls and during underfeeding, is believed to be reflected in the age-related decline in GH secretion. Characteristic age-related changes have been reported in thyroid structure and function (Valueva and Verzhikovskaya, 1977), including proliferation of connective tissue with loss of cellular elements, a decrease in thyroid I^{131} uptake, and decline in oxidative processes. Thus serum T_3 and T_4 levels in old rats are reduced as well as T_4 binding capacity of serum proteins (Huang et al., 1980; Valueva and Verzhikovskaya, 1977). In agreement with these reports, the present results also show an age-related decline in serum fT_3 and fT_4 levels in middle-aged and old full-fed controls, and during underfeeding. Since thyroid hormones are necessary for GH pulsatile release and synthesis, our data suggest that the changes in available thyroid hormone during aging and underfeeding contribute, at least in part, to the decline observed in GH secretion during underfeeding in all age groups, and to the further decline in aging rats.

Our results show a reduction in fT_3 levels relative to full-fed controls in young rats during chronic underfeeding, in agreement with other reports (Glass et al., 1986), and show furthermore that this also occurs in middle-aged and old rats. Protein deficiency and starvation alter serum binding of thyroid hormones (Young et al., 1982, 1985).

Since underfeeding affects the distribution of thyroid hormones among the protein bound and free fractions, often even without much alteration in the total fraction, total serum T_3 may not be a good reflection of thyroid status (Glass et al., 1986). Changes in T_3 and T_4 binding to proteins have been reported during moderate underfeeding. Glass et al. (1986) reported a progressive decline in % fT_3 after 81 days of underfeeding. Our results are in agreement, since this decline occurred in young rats as well as in the middle-aged and old rats during underfeeding. The middle-aged and old rats showed lower % fT_3/TT_3 than the young rats during underfeeding as well as during refeeding, and in full-fed controls. This is believed to be relevant to the amount of thyroid hormones available for target site interactions. With aging there may be a concurrent change in thyroid binding protein affinities and kinetics. Reductions in TT_4 content and T_4 binding capacity of serum proteins have been reported (Valueva and Verzhikovskaya, 1977; Huang et al., 1980).

During periods of fasting and caloric deprivation, increased levels of reverse T_3 (rT_3), an inactive metabolite of T_4 and reduced fT_3 levels, have been reported in human subjects and animals. rT_3 inhibits T_4 to T_3 conversion by the liver in a dose-dependent manner (Chopra et al., 1978). Refeeding restored rT_3 and T_3 levels to normal (Chopra et al., 1978). Our results in young rats appear to be in agreement, since reductions in fT_3 and increases in rT_3

occurred during underfeeding which were then restored to normal levels upon refeeding. Inhibiting T_4 to T_3 conversion in conditions where catabolism is overactive may have survival significance. Thus metabolic homeostasis in catabolic states is achieved by inhibition of a non-catabolic hormone (T_4) to an intensely catabolic hormone (T_3). Food-restricted rats show considerable energy conservation, exhibited primarily by a reduction in the energy required for daily life maintenance, with a resultant survival benefit to the animal (Hill et al., 1986). The lack of change in rT_3 levels during underfeeding in the middle-aged and old rats suggests reduced sensitivity with age to factors regulating T_4 metabolism.

During refeeding, fT_3, fT_4, mean GH concentrations, and peak GH amplitude levels returned to control values in all age groups. Tannenbaum et al. (1979) reported a rebound response in GH secretory episodes in young rats after only 3 days of refeeding following 72 hours of starvation, and suggested that the pituitary gland of fasted rats contains adequate amounts of GH and is hyperresponsive to stimuli regulating GH release. Thus when the nutritional restraints to GH release are removed, the pituitary is able to adequately synthesize and release GH in a relatively short period. The changes we observed among the 3 age groups at refeeding suggest age-related alterations in the ability to regain normal GH secretion. The mechanisms are believed to be hypothalamic in origin, since pituitaries of old rats apparently can

secrete similar amounts of GH *in vitro* in response to GHRH (1-44) as the pituitaries of young rats (Sonntag et al., 1983).

Underfeeding slows growth and development in young animals, and has anti-aging effects in adult animals, including delays in the development of age-related diseases and progression of aging processes, as well as increased life span. In the neonate mammal and larval amphibian, thyroid hormones act to promote differentiation and proliferative growth according to genetically programmed periods required for maturation of individual tissues. Thus thyroid hormones have a definite role in growth and development of the organism and progression of the aging program. Underfeeding produces a state of reduced thyroid hormone secretion which may significantly contribute to the delay in aging processes. Thyroidectomy retards collagen and renal aging (Giles and Everitt, 1967). Thus the reduction in active thyroid hormone during underfeeding may have an important anti-aging role.

In contrast to the delay in reproductive aging we reported by an underfeeding/refeeding regimen in aging female rats (see first 2 Experiments), the present results indicate that such a regimen does not exert any favorable effects on GH pulsatile or thyroid hormone secretion in aging male rats. Although conditions for producing favorable effects on pulsatile GH and thyroid hormone secretion in aging rats may not have been optimal in this study, the results suggest that the beneficial effects of

underfeeding/refeeding on gonadotropic hormone secretion may not extend to other hormones. However, the reduction in GH and thyroid hormone secretion during underfeeding may exert a preserving effect on body organs and tissues and thus delay aging processes. Since GH and thyroid hormones are known to influence growth and function of many body organs and tissues, including the kidneys, liver, immune system, brain, etc., the reduction in secretion of these hormones during underfeeding may have helped to preserve their structure and function.

General Discussion

I. Effects of 10 weeks of underfeeding/refeeding on estrous cycles and LH secretion

Underfeeding begun relatively early in life and extending throughout most of the life span of the rat and mouse delays the onset of puberty and reproductive functions and extends these functions into later life (Merry and Holehan, 1979; Glass et al., 1976; Kennedy and Mitra, 1963). The work reported here shows that even a 10-week period of 50% food intake followed by ad libitum feeding can prolong estrous cyclicity in young rats and temporarily reinitiate cycles in old rats (Quigley et al., 1987). At least part of the mechanism by which underfeeding exerts these effects is by increasing the sensitivity of the hypothalamo-pituitary system, as indicated by the enhanced release of LH in response to both the positive and negative feedback effects of estradiol and progesterone.

There is evidence that underfeeding reduces the work of the hypothalamic neurons producing CAs and GnRH, as indicated by the reductions reported in hypothalamic CAs (Wurtman and Wurtman, 1983) and GnRH (Campbell et al., 1977;

Everitt, 1982). Underfeeding may therefore extend the life span of the neurons enabling them to function for greater periods of time. In addition, it is probable that underfeeding, resulting in cessation of estrous cycles, has a sparing effect on hypothalamic neuronal damage from the amounts of estrogen normally secreted during estrous cycles or in the CE state of older rats (Aschheim, 1983; Finch et al., 1984). Thus, there is less damage and less activity for the neurons involved in maintaining estrous cycles. Further work is needed to elucidate the mechanisms involved, which could include assessment of structural changes in the preoptic and medial basal hypothalamus. For example, it would be of interest to compare steroid receptor density and hypothalamic distribution of CA and GnRH neurons during underfeeding and upon refeeding in young and old rats. Are there changes in dendritic and glial processes, and in neuronal proximities which could affect subsequent gonadotropin release?

Administration of L-dopa or other drugs that increase hypothalamic CAs reinitiates estrous cycles in old rats (Huang et al., 1976b; Quadri et al., 1973; Cooper et al., 1979). It is possible that when underfed rats are refed, CAs in the hypothalamus as well as GnRH are increased above the levels of these two substances normally present in old rats. Although this remains to be proven, this is suggested by the increased release of LH in response to estradiol followed by progesterone administration.

In addition to the above, it is also possible that part of the favorable action of underfeeding on estrous cycles in aging rats is exerted directly on the pituitary. In fact, underfed rats are more sensitive to administration of GnRH, as demonstrated by greater release of LH (Campbell et al., 1977). The pituitaries of old ad libitum-fed rats show decreased LH response to GnRH administration (Bruni et al., 1977), but underfeeding may counteract the age-related effect of GnRH on LH release. It is also possible that when ovarian hormones are administered to previously underfed old rats, as was done in Experiment 2, this further enhances the responsiveness of the pituitary to the endogenous GnRH released.

There is evidence that GnRH receptors on the pituitary of ad libitum-fed old rats do not change relative to young rats (Sonntag et al., 1984; Steger et al., 1980). However, it is possible that underfeeding does change the number of GnRH receptors on the pituitary of old rats. Obviously this remains to be assessed. Also, changes may occur in LH mRNA levels in the pituitary during aging, underfeeding, and refeeding. These remain to be studied.

In addition to changes in hypothalamic and pituitary function during aging and underfeeding, there are also changes at the level of the gonads. During aging the ovaries undergo structural and functional changes, including a progressive loss of follicles and ova. This could change the capacity of the ovaries (and testes) to respond to

gonadotropic hormone administration with adequate secretion of gonadal hormones. Indeed, there is evidence that release of steroid hormones in response to gonadotropic hormone administration declines with age (Harman and Talbert, 1985). This may be due in part to a reduction in gonadotropic receptors in the gonads, and also to a decrease in functional thecal and granulosal cells and steroidogenic capabilities. There is also evidence for a reduced response of the reproductive tract of old rats to the action of gonadal hormones. Roth (1984) reported that the uterus of old rats contains fewer estrogen receptors than young rats, and is less responsive to estrogen administration. Underfeeding may also alter the responsiveness of the gonads to gonadotropic hormones, and the reproductive tract to gonadal hormones. This remains to be studied.

It may be possible to maintain estrous cycles in aging rats to the end of their life span by neuroendocrine intervention. Although the structural and functional capacity of the hypothalamus, pituitary, ovaries, and reproductive tract normally decline with age in the rat, the dysfunctions that develop appear to be reversible. Perhaps a combination of underfeeding/refeeding and/or administration of drugs to increase hypothalamic CAs would permit maintenance of estrous cycles to the end of life. Unlike postmenopausal women and some strains of mice, not all follicles and ova disappear in the ovaries of the aging female rat, and therefore follicular development and

ovulation should be possible. Obviously, this remains to be tested, but the results thus far achieved by various neuroendocrine interventions appear to be promising. Insofar as the male rat is concerned, the evidence indicates that healthy old males may produce offspring practically to the end of life (Harman and Talbert, 1985). In this respect, healthy old male rats resemble some healthy old human male subjects who have been reported to mate successfully with younger wives and produce offspring (Harman, 1983).

II. Effects of 10 weeks of underfeeding/refeeding on GH and thyroid hormone secretion in the male rat

In contrast to the anti-aging effects of 10 weeks of underfeeding followed by refeeding on reproductive function in the female rat, the effects on pulsatile GH and thyroid hormone secretion in the male rat are much less dramatic. This regimen of underfeeding/refeeding produced no increase in pulsatile GH or thyroid hormone secretion in young, middle-aged, and old males. However, the effects of underfeeding on pulsatile GH and thyroid hormone secretion differed in the 3 age groups just as they differed under the ad libitum feeding regimen. Under ad libitum feeding, the middle-aged and old rats showed lower GH and thyroid hormone secretory patterns than the young rats, in agreement with previous reports showing lower GH and thyroid hormone

secretion in older than in younger rats (Sonntag et al., 1980; Meites et al., 1987). During underfeeding, these same differences were manifest, with middle-aged and old rats exhibiting lower GH and thyroid hormone concentrations than young rats. Similar trends in the 3 age groups were exhibited upon refeeding.

Although there was no apparent increase in GH or thyroid hormone induced by the underfeeding/refeeding regimen followed in the study presented here, it is possible that under different underfeeding/refeeding regimens, such an increase might have been observed. It is also possible that the anti-aging effects of underfeeding may be produced precisely because there is a reduction in GH and thyroid hormone secretion, as well as in other pituitary and target gland hormones. When one considers the importance of GH and thyroid hormones, the maintenance of protein, carbohydrate and fat metabolism, and for bone, kidney, liver, pancreatic, gastrointestinal, and other body functions, it is possible that a temporary reduction in activity by the tissue involved may preserve and extend their functional capacities. Since chronic underfeeding has been shown to delay the progressive decline in protein turnover associated with aging (Lewis et al., 1985; Merry et al., 1987; Lindell, 1982), it would be of interest to measure relative rates of protein turnover in GH target tissues during underfeeding and after refeeding among rats of various ages. Do 10 weeks of underfeeding affect the decline of protein turnover in old rats?

Is the decline in thyroid hormone secretion during aging responsible to any extent for the age-related decrease in GH secretion? This is unknown at present but can be tested by administering physiological doses of T_3 or T_4 to old rats to bring circulating levels of these hormones to levels similar to those in young rats, and see whether this would increase GH secretion. Evidence has already been mentioned that thyroid hormones are essential to maintain normal GH secretion (Martin et al., 1985; Hervas et al., 1975) and that GH secretion declines during hypothyroidism (Reichlin, 1966; Hervas et al., 1975). Administration of drugs that increase hypothalamic NE activity may be another method to increase thyroid function during aging, since NE is the major neurotransmitter that promotes TSH release (Weiner and Ganong, 1978). It is interesting that in old rats, although thyroid hormone secretion declines, blood levels of TSH remain normal. The molecular form of TSH secreted during aging has low biological activity (Klug and Adelman, 1977), but this remains to be confirmed. In any case, there is ample evidence that thyroid hormone secretion is decreased in old rats.

III. Conclusions

What is the relevance of the research reported here on the questions of why aging occurs and whether body functions

can be improved in aging individuals by relatively short periods of underfeeding? The favorable effects of underfeeding on many body functions and on life span in rodent species appear to be produced primarily via reductions in neuroendocrine function. This results in reducing "wear and tear" on the neurons in the hypothalamus and on the cells of the endocrine tissues, resulting in decreased activity by the many organs and tissues the neuroendocrine system controls. Underfeeding may also enhance the ability of the neuroendocrine cells to respond to stimuli.

Can the benefits of underfeeding on body functions and longevity in rodent species be extended to aging human subjects? Obviously, this is a difficult problem to test, particularly as it relates to longevity. However, the effects of relatively short periods of reduced food intake, of the order of a few months or even for longer periods, could be tested on a number of body functions in elderly individuals, e.g., on heart, kidney, liver, brain, and immune function. Which types of individuals would most likely benefit from such a regimen? It would seem most likely that obese and overweight individuals would benefit the most from such a regimen. Would a relatively short period of dietary restriction be of benefit to individuals with pathology or disease? There is considerable evidence that reduced food intake results in inhibition of tumor development and regression of some existing tumors in experimental animals (Tannenbaum, 1943). Therefore, an

underfeeding regimen may benefit some elderly human subjects with tumors. Would a relatively short term of underfeeding improve immune function and enable older persons to more readily combat infectious agents? These possibilities can be explored, but it is first necessary to make the medical community as well as laymen aware of the findings in animals species and their potential significance for human subjects.

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