The manner by which laboratory animals are maintained can greatly influence the results of toxicologic bioassays. A balanced diet is the most complex mixture of organic and inorganic chemicals to which laboratory animals are exposed. Nutrition is important in these studies because the diet composition and the conditions of feeding can significantly affect the metabolism and activity of xenobiotic test substances and can change the results and reproducibility of long-term studies. Since laboratory rodents are the most common surrogate models used for human safety assessment, rodent bioassays must attempt to control as many potentially confounding experimental variables as possible, in order to precisely define dose-related and treatment changes with the smallest group sizes statistically possible.

The development of regulatory guidelines has been aimed at reducing intra- and interlaboratory variability and has succeeded in controlling many confounding environmental variables and eliminating many of the external stressors (Bhatt et al., 1986; Newberne et al., 1996) that modify an animal’s response to test substances. However, other variables remain uncontrolled. There has been a steady increase in study-to-study variability and a lack of reproducibility observed in these bioassays over the past 2 decades (Allaben et al., 1996; Keenan et al., 1998; Lang, 1991; Rao et al., 1990). The early onset and increased severity of certain spontaneous degenerative diseases and tumors has resulted in decreased survival and a loss of bioassay sensitivity to detect true treatment effects (Allaben et al., 1996; Fishbein, 1991; Hart et al., 1995; Keenan et al., 1992, 1994; Turturro et al., 1995). These adverse events have been the result of overnutrition leading to increased growth and body weight in most of the rodent strains and stocks currently used in these bioassays (Duffy et al., 1989; Haseman and Rao, 1992; Knight et al., 1998; Roe et al., 1995; Turturro et al., 1996).

While increased adult body weight has been influenced by breeder selection and the expected genetic drift seen in many animal populations, most of the adverse events leading to early mortality are clearly associated with a failure to control adult body weight due to the ad libitum (AL) overfeeding of otherwise nutritious food (Giknis et al., 1998; Keenan et al., 1998a, 1992). The most important dietary factor acting as an endocrine disrupter is excessive energy intake (calories) rather than any specific nutrient or contaminant in commercial rodent diets (Finch, 1990; Keenan et al., 1997; Kritchevsky, 1993; Newberne et al., 1996; Roe et al., 1995; Yu 1995). AL overfeeding induces early endocrine hypersecretion, hyperthrophy, hyperplasia, and metabolic disruption before endocrine tumors are observed (Han et al., 1998; Masoro, 1995; Masoro and Astdad, 1996, Masoro et al., 1992, 1991; Merry and Holehan, 1994). AL overfeeding of calories accelerates the onset time of spontaneous diet-related tumors, particularly those of the pituitary, pancreas, and target tissues such as the mammary gland (Keenan et al., 1994a, 1995b, 1996a). The current practice of AL overfeeding is the most poorly controlled variable in these bioassays, and it adversely affects every physiological process and anatomical structure to the molecular level (Finch, 1990; Hart et al., 1995; Masoro, 1995; Merry and Holehan, 1994; Sohal and Weindruch, 1996; Weindruch and Sohal, 1997; Yu, 1998).
The total amount of food (calories) fed per animal per day is the critical factor affecting their physiology, health, and longevity (Keenan et al., 1996b; Masoro, 1996, 1991; Weindruch and Walford, 1988; Yu, 1995). (Fig. 1).

The Adverse Effects of Ad Libitum (AL) Overfeeding

Even with the reduction or elimination of many intercurrent infectious diseases (Bhatt et al., 1986) and greatly improved laboratory animal housing and nutrition (Maynard et al., 1979; National Research Council, Subcommittee of Lab Animal Nutrition, 1995; NRC, Committee on Rodents, 1996; Purina Mills, 1996; Rogers et al., 1979), rodent survival in chronic 2-year bioassays has either declined or become highly variable during the 1980’s and 1990’s (Allaben et al., 1996; Haseman and Rao, 1992; Keenan et al., 1992, 1994a; Lang, 1991; Rao et al., 1990; Turturro et al., 1995, 1996). Sprague-Dawley (SD) rat survival has ranged from 7 to 80% (Keenan et al., 1994a, 1998; Knight et al., 1998). Similar declines in survival have been reported for Wistar rats, Fischer 344 (F344) rats, CD-1 mice, and B6C3F1 mice (Allaben et al., 1996; Haseman et al., 1992; Rao et al., 1990; Roe et al., 1995; Turturro et al., 1996). The main factor associated with this poor survival appears to be ad libitum (AL) overfeeding of calories (Fig. 1).

Suggestions that a genetically determined initial weanling body weight would be the best predictor of longevity was drawn from early correlations between weanling body weight and adult tumor incidence and from inverse correlations with survival (Ross, 1976). However, recent studies of SD rats did not show a correlation between weanling body weight and survival in either AL-fed or diet-restricted (DR) animals. Instead, it was the 12-month adult body weight following AL feeding that correlated best with 2-year survival (Laroque et al., 1997). Similar reports on F344 rats and B6C3F1 mice also indicate that initial body weight does not correlate with survival as well as the 12-month body weight (Turturro et al., 1995, 1996). These studies indicate that it is not initial body size, but the total adult food intake that is the most important factor affecting adult body weight and survival. In addition, studies of adult-onset DR in rats and mice indicate that those factors affecting initial body size, early growth, and metabolism are neither necessary nor sufficient to explain the adverse effects of AL overfeeding or the positive effects of DR on health and longevity (Masoro and Austad, 1996; Weindruch and Sohal, 1997; Weindruch and Walford, 1988).

The correlations between adult body weight, obesity, and longevity have led many to extrapolate a mechanistic relationship between obesity and survival. However, different stocks of SD rats show large differences in adult obesity and body weight (Klinger et al., 1996; Knight et al., 1998), yet these stocks still suffer poor longevity if maintained by AL feeding (Keenan et al., 1998a, 1994b; Knight et al., 1998; Laroque et al., 1997). Moreover, studies of AL-fed F344 rats show no correlation between adult body weight and longevity, but DR-fed F344 rats show a positive correlation, with those with the heaviest body weight living the longest (Maeda et al., 1985; Masoro, 1996; Yu, 1995). Similar observations have been reported for naturally lean (C57BL/6J) and genetically obese (C57BL/6J ob/ob) mice (Harrison et al., 1987). These data call into question the conclusion that increased body fat content or obesity per se are causally related to decreased survival.

The adverse effects of AL overfeeding appear to result from long-term, low-intensity metabolic use of nutritional fuel when caloric intake is excessive. The use of oxygen in oxidative metabolism of nutrients results in free-radical production (Sohal and Weindruch, 1996; Weindruch, 1996; Weindruch and Sohal, 1997). Glucose, like other nonreducing sugars, undergoes a nonenzymatic reaction with amino groups of proteins called the glycation reaction (Masoro and Austad, 1992, 1991; Yu, 1995). Thus, nutritional fuel such as glucose can become reactive molecules in their own right. Studies of these basic metabolic processes have led to several hypotheses that implicate free radicals, glycation reactions, and/or Maillard reactions as causative factors in aging as a consequence of normal metabolism (Masoro, 1995; Masoro et al., 1996; Yu 1995). While oxidative free radical production is not reported to increase uniformly with age, the damage induced by oxidative metabolism such as lipid peroxidation does increase with age. In addition, the antioxidant defense systems are depleted with age, particularly under conditions of AL overfeeding (Masoro et al., 1995; Sohal and Weindruch, 1996; Weindruch and Sohal, 1997; Yu, 1995). Major repair functions, including DNA repair as well as to proteolytic and lipolytic enzymes that remove oxidatively damaged molecules, deteriorate most readily under AL feeding and are best maintained by a moderate DR regimen (Hart et al., 1995; Masoro, 1995; Masoro et al., 1996; Weindruch and Sohal, 1997). Glucose effectiveness and/or insulin sensitivity are both diminished over time in AL-fed rodents, and these are best maintained in moderate...
DR-fed animals (Duffy et al., 1989; Masoro, 1995; Masoro et al., 1992). AL-fed rats have higher levels of circulating plasma glucose and insulin than their moderately DR-fed counterparts (Masoro 1995; Masoro et al., 1992; Yu, 1995). AL-fed SD rats develop pancreatic islet cell hyperplasia and insulin hypersecretion that leads to islet cell damage and fibrosis prior to the first year of life (Keenan et al., 1994a, 1995a,b). These changes can lead to an increased incidence of islet cell tumors in the AL-fed animals, relative to their moderate DR-fed counterparts (Keenan et al., 1995b, 1998b). The beneficial effects of moderate DR on the control of plasma glucose levels and/or increased insulin efficiency may be a fundamental mechanism resulting in the efficient use of a potentially toxic fuel (glucose) at lower sustained concentrations, which are less damaging and endocrine disruptive over the animal’s lifespan (Duffy et al., 1989; Hart et al., 1995; Masoro 1995, 1991; Masoro and Austad, 1996, Masoro et al., 1992; Yu, 1995).

AL overconsumption of excessive calories is the most significant and complex nutritional determinant of chronic degenerative disease and cancer (Manson et al., 1995; McGinnis et al., 1993; Rogers et al., 1993; Rose, 1991; Rosenbaum et al., 1997). The multiple mechanisms by which AL overfeeding enhances tumorigenesis have been the subject of a number of reviews (Finch, 1990; Fishbein, 1991; Hart et al., 1995; Kurlfeld et al., 1989; Kritchevsky, 1993; McCoy et al., 1935; Pollard et al., 1985; Ross, 1976; Weindruch and Walford, 1988; Yu, 1995). Many of these adverse effects are a direct result of increased excessive growth of normal organs and the early development of neoplasia. In AL fed rodents, absolute organ weights (except brain weight) are generally increased proportional to caloric intake and body growth, but relative organ weights (as a percent of body weight) are generally reduced in AL fed and increased in DR-fed animals (Keenan, et al., 1994a, 1995a, 1998b). The excessive growth processes in endocrine-sensitive, metabolically-active organs of AL fed rodents are enhanced by the early and excessive secretion of growth-promoting hormones such as insulin, growth hormone, IGF-1, prolactin, and other mammatrophic hormones and by decreases in the growth controlling adrenal corticoids (Masoro, 1995; Merry and Holehan, 1994; Rogers et al., 1993). These growth effects have been particularly well documented in the liver, and are further complicated by alterations in the metabolism of carcinogens, other xenobiotics, and steroids in the AL fed animals (Fishbein, 1991; Hart et al., 1995; Masoro, 1995; Rogers et al., 1993). These metabolic and hormonal mechanisms are further augmented in AL fed rodents by increased oxidative free-radical damage to DNA, proteins, enzymes, and membranes, the loss of protective antioxidants, and damage to the antioxidant enzymes systems (Sohal and Weindruch, 1996; Weindruch and Sohal, 1997; Weindruch and Walford, 1988; Yu, 1995). In addition, nonenzymatic glycation reactions due to increased plasma glucose levels and/or decreased insulin sensitivity lead to further damage of essential enzymes, proteins, and DNA. These hormonal and metabolic effects result in excessive organ growth, especially in the metabolically active liver, kidneys, and endocrine organs. AL overfed rodents have increased cell division and DNA synthesis, spontaneous DNA adduct formation, and alterations in DNA repair, enhancing the probability of spontaneous tumorigenesis (Finch, 1990; Hart et al., 1995; Keenan et al., 1994b, 1995a; Yu, 1995). Also, they have decreased apoptosis of normal, aged, and preneoplastic cells that further increase the likelihood of an early spontaneous tumorigenic event (Grasl-Kraupp et al., 1994; James et al., 1994). AL overfed rodents have increased expression of tumor virus genes or protooncogenes, a decreased immune response, and an increase in autoimmune responses (Fishbein, 1991; Hart, 1995; Masoro, 1995; Rogers et al., 1993; Yu, 1995). These and other adverse effects have been observed with all diets tested, including semipurified and natural-product diets. There is no more effective preventative measure to avoid the adverse effects of AL feeding on the early onset of these spontaneous, degenerative metabolic diseases and their endocrine-disruptive consequences than by simple, moderate DR-feeding. It is remarkable that only laboratory rodents are currently maintained by ad libitum overfeeding, whereas as other laboratory animals such as dogs, rabbits, and primates are carefully fed measured amounts of feed, and to do otherwise with these species is considered poor veterinary and scientific practice (Lane et al., 1997; McDonald, 1997; National Research Council. 1996; Weindruch, 1996).

The Effects of Nutrients on the Rodent Bioassay

Over 40 nutrients are required in the diet of rodents, making water and diet the most complex mixture of exogenous organic and inorganic chemicals to which the animals are exposed. The diet needs to be controlled and optimized for the animal’s sex, physiological condition (i.e., maintenance vs. reproduction and lactation), and age because the diet’s composition and manner of feeding can change the animal’s physiology and metabolism and can alter the effects of test substances in experiments (Maynard et al., 1979; McDonald, 1997; National Research Council, Subcommittee of Lab Animal Nutrition 1995; National Research Council. Committee on Rodents 1996; Newberne et al., 1996; Rogers et al., 1979; Ross, 1976).

Historically, the principal indicators for dietary adequacy have been reproductive performance and growth. The use of AL feeding for pregnant and lactating animals is reasonable, because maximum nutritional intake is necessary if the goals of the experiment are maximum fecundity and lactation. The scientific basis for nutritional recommendations for growth and maintenance have been based on results from the growth curves of weaning rats as an indicator of optimal nutrition. Typically, the maximum growth rate (body weight) is compared in young, rapidly growing animals fed ad libitum diets with various concentrations of specific nutrients while keeping the concentrations of all other nutrients constant for a 1 or 2 month period. (Baker, 1986; Maynard, 1979; McDonald, 1997;
The most authoritative source of information on nutritional requirements of the rat is found in the most recent publication by the National Research Council (National Research Council, Subcommittee of Lab Animal Nutrition, 1995), which lists absolute minimal requirements for laboratory rodents and references the original literature supporting these recommendations. The recommendations are estimates of minimal nutrient levels required, with no margins of safety. Nutrient requirements change with sex, growth, and reproductive status. Growing rodents have greater protein and amino acid requirements than adults (Benevena et al., 1994). The requirements for reproduction and lactation are similar to those for growing animals, except for lactation energy requirements, which are much higher. Nutrient bioavailability must be considered, because nutrients found in natural feed ingredients are not 100% bioavailable to the rodent. In addition, there are various constituents in the diet, such as soluble and insoluble fibers, tannins, phytate, and lignin in the natural ingredients that can alter nutrient availability. Nutrient interactions occur, making some nutrients less bioavailable. The microbial status of the rodent is also a key factor when considering the proper levels of some of the water soluble vitamins, vitamin K and amino acids. Adjustments may also be required when formulating diets for pathogen-free or germ-free animals (Newberne et al., 1996).

The National Research Council (NRC) recommends minimal dietary concentrations for protein required for growth, reproduction and maintenance of rats to be 15%, 15%, and 5% respectively (National Research Council, Subcommittee of Lab Animal Nutrition 1995; Rogers, 1993). However, these protein recommendations are based on studies with purified diets containing highly digestible proteins such as lactalbumin, and they are too low for rodents fed commercial diets composed of natural ingredients such as corn, wheat, and soybean meal because of the lower availability of the nutrients in natural feed ingredients. The protein requirement for adult rats fed diets composed of ingredients such as soybean meal is likely to be greater than 5% for maintenance but less than the 12% suggested for maximum growth in the NRC recommendations. Since most natural-product diets contain between 18 and 23% protein, the consumption of protein generally exceeds the amount necessary for maintenance of adult rats (Purina Mills 1996).

Modifying or limiting dietary protein intake has been reported to decrease renal disease without reducing body weight in F344 rats (Rao et al., 1993). Replacing casein with soybean as a source of dietary protein reduces the progression of chronic nephropathy in F344 rats when caloric intake and body weights are similar between groups (Iwasaki et al., 1988; Masoro et al., 1989). However, the restriction of dietary protein by 40% without the restriction of caloric intake had only minor effects on longevity, because few other aging processes are altered (Iwasaki et al., 1988; Masoro et al., 1989). Moreover, a 40% caloric restriction without protein restriction was as effective as caloric restriction with protein restriction in increasing F344 rat survival (Maeda et al., 1985). These studies show that AL fed Fischer 344 rats develop more severe renal disease than DR-fed F344 rats given 1.7 times the protein intake per unit body mass (Iwasaki et al., 1988, Masoro et al., 1989). Studies in SD rats and Wistar rats fed natural-product diets with reduced protein content have led to similar conclusions (Gumprecht et al., 1993; Keenan et al., 1994a, 1995a; Roe et al., 1995). These and other data indicate that controlling caloric intake is more important than protein intake in preventing renal disease and increasing the survival of rodents in long-term studies.

While no definitive carbohydrate requirements have been established for rodents, they need carbohydrates for successful growth, reproduction, and lactation. Carbohydrates such as sugars, starch, and fiber contribute to the gross energy in the diet. Most rodents do not have the enzyme systems needed to digest fiber; therefore, any caloric value obtained from fiber occurs as a result of bacterial fermentation in the cecum and colon with subsequent coprophagy by the animal. Purified diets that utilize starch, glucose, and sucrose can be utilized by rodents. However, some carbohydrates such as fructose (and sucrose as a source of fructose) can lead to metabolic abnormalities. Fructose and sucrose feeding will increase liver weight, lipid and glycogen content, and liver lipogenic enzyme activity, and will result in hypertriglyceridemia (National Research Council, Subcommittee of Laboratory Animal Nutrition 1995). Fructose feeding has also been associated with increased nephrocalcinosis due to alterations in urinary phosphorus and magnesium and lowering of urinary pH (Ritskes-Hoitinga et al., 1989, 1991; Stonard et al., 1984). These potential problems can be avoided by using carbohydrates derived from grain and grain byproducts such as corn, wheat, barley, oats, and wheat middlings.

Requirements for dietary fiber have not been demonstrated in rodents; however, fiber may be beneficial. The effects of fiber depend on its natural source and properties of solubility, viscosity, and fermentability. Fiber increases fecal bulk and the size and weight of the cecum and colon (Keenan et al., 1994a; National Research Council, Subcommittee of Lab Animal Nutrition 1995). Dietary fiber decreases gastrointestinal transit time, and fermentable fiber sources result in volatile fatty acid production, which is absorbed and used as an energy source. Increased dietary fiber increases the excretion of fecal nitrogen and decreases urinary nitrogen due to microbial fermentation and utilization of dietary protein.
Increasing dietary fiber with insoluble, largely indigestible fiber sources such as cellulose, oat hulls, wheat bran, and corn bran have been used in an attempt to control nutrient intake in rodents, but the results have been mixed in chronic bioassays. Dilution of dietary energy content requires as much as a 30–45% fiber intake to reduce energy consumption in rodents (Peterson et al., 1971a,b). Feeding a diet containing 16% crude fiber and 2.36 kcal/g diet resulted in SD rat’s compensating by eating 30% more food to maintain similar energy intake as those fed another diet containing 4% crude fiber and 3.1 kcal/g metabolizable energy. The net result of this dietary manipulation was minimal differences in a number of parameters, including longevity, when the two diets were fed AL (Keenan et al., 1994a,b, 1995a,b). These results call into question the utility of increased fiber content as a means of controlling caloric intake in rodents.

Dietary lipids are required in rodent diets as a source of essential fatty acids, a concentrated source of energy, and an aid in absorption of fat-soluble vitamins. Most commercial laboratory diets contain 4–6% dietary fat, and this level is adequate for growth and maintenance of rodents. It provides them with the required amounts of essential fatty acids in the omega 6 (primarily linoleic acid) and omega 3 (linolenic acid) groups. Higher concentrations of fat in rodent diets are of questionable benefit and have the potential of altering additional metabolic pathways and inducing pancreatic acinar cell tumors (Haseman and Rao, 1992; National Research Council, Subcommittee of Lab Animal Nutrition 1995).

Vitamins and minerals are usually supplemented in commercial diets and are required to meet the animals’ needs for growth, reproduction, and maintenance. Certain factors need to be considered regarding sources of minerals, with phosphorus being a good example. Much of the phosphorus in natural-product diets is from plants in the form of phytate phosphorus, which is largely unavailable to the animal. Since the phosphorus requirements suggested by the NRC (National Research Council, Subcommittee of Lab Animal Nutrition, 1995) are based on highly bioavailable elemental phosphorus added to purified diets in natural-product diets, non-phytate phosphorus must be considered in formulating diets for rodents, rather than simple total dietary phosphorus (Maynard et al., 1979; Purina Mills, 1996). The ratio of dietary calcium to phosphorus is also a concern. When non-phytate phosphorus levels exceed the calcium levels in the diet, nephrocalcinosis occurs with mineral deposits at the corticomedullary-junction of the kidneys in rodents (Clapp et al., 1992; Rao et al., 1993; Stonard et al., 1984). This process can lead to renal hypertrophy and degenerative renal tubular changes, particularly in female rats. Generally, nephrocalcinosis is more common in rats fed purified diets, but may be avoided by a calcium to phosphorus molar ratio of 1.3, which equals a weight ratio of calcium to phosphorus of 1.68 to 1 (Clapp et al., 1982; National Research Council, Subcommittee of Lab Animal Nutrition 1995; Rao et al., 1993; Ritskes-Hoitinga et al., 1989; Stonard et al., 1984).

It has been speculated that food restriction may increase longevity by reducing the intake of toxic contaminants in the diet. However, this is unlikely, considering the broad variety of semipurified diets and natural-product diets that have been used in DR studies and the extensive analysis in testing for contaminants currently applied to most of these diets (National Research Council, Subcommittee of Lab Animal Nutrition 1995; Newberne et al., 1996; Purina Mills, 1996). The fact that DR-fed rodents consume approximately the same or slightly more food per gram/body weight as AL fed rodents means that DL-fed animals are exposed on a per gram/body weight basis to the same levels of both nutrients and contaminants as their AL fed counterparts (Keenan et al., 1994a, 1997; Masoro et al., 1991). Thus, research from many sources does not support the idea that decreased survival is due to an excess of specific nutrient or contaminant given to AL fed rodents. In contrast, all scientific evidence strongly points to total energy (calories) intake per animal as the main factor that accelerates aging and decreases survival in AL overfed rodents (Fig. 1).

**Energy Balance**

Animals eat to meet their energy needs. The energy content in food that is capable of being transformed by the body is called the metabolizable energy (ME). It is described for the laboratory rat by the equation: ME for maintenance (kcal/day) = 112 × body weight (kg)^0.75 (Maynard et al., 1979; National Research Council, Subcommittee of Lab Animal Nutrition, 1995). The ME requirements for most laboratory rodents are probably better estimates of the ME for growth rather than for adult maintenance (McDonald, 1997); however, the ME for growth is typically expressed as twice the ME for maintenance. In rats, the ME needs for reproduction are approximately 30% above the ME for maintenance up to the 16th day of gestation and 2.5 times maintenance in later gestation. Lactation requires 2 to 4 times the maintenance ME, depending upon the litter size (National Research Council, Subcommittee of Lab Animal Nutrition 1995). While controlling food intake by DR is appropriate for adult rodents on maintenance in toxicity and carcinogenicity studies, DR-feeding may not be indicated in pregnant or lactating dams or their rapidly growing offspring in reproductive toxicity studies.

Body weight appears to be physiologically regulated by balancing energy intake, expenditure, and storage. The “lipostatic theory” of body weight maintenance developed in the 1950’s and 1960’s proposed that a factor secreted from adipose tissue signaled the brain and thus regulated feeding behavior and body-fat mass. (Coleman and Ummel, 1969; Kennedy, 1953). More recently, studies of the leptin-deficient C57BL/6J ob/ob mouse have led to the development of a model of leptin-initiated energy balance that resembles the earlier theory (Banks et al., 1996, Flier et al., 1998; Friedman et al., 1998; Houseknect et al., 1998). It is now recognized that energy balance is the result of a complex, redundant, and highly
integrated neurohormonal system, which controls homeostasis by several hormones secreted in proportion to adiposity, including leptin and insulin and the targets in the central nervous system on which they act (Björntorp, 1997; Claycombe et al., 1998; Elmquist et al., 1997; Friedman et al., 1998; Woods et al., 1998). Anabolic pathways that stimulate food intake and weight gain include the hypothalamic neuropeptide Y (NPY) axis and catabolic pathways that reduce food intake and stimulate weight loss, including the hypothalamic melanocortin system. Insulin and leptin are hormones that are regulated by the adipose tissue, inhibit the anabolic pathways, and stimulate the central catabolic pathways through an expanding list of central nervous system signaling molecules. (Elmquist et al., 1997; Friedman et al., 1998; Flier et al., 1998; Woods et al., 1998).

It has been proposed that body weight is maintained by establishing a “set point” at which body weight and fat mass are regulated (Keesey and Hirvonen, 1997). However, the set point is a range rather than a single value, and it is governed by various factors such as hormonal status, age, and genetics. It is possible to shift the point by a number of mechanisms (Friedman et al., 1998; Woods et al., 1998). The catabolic effects of leptin and insulin induce responses that lead to weight loss by loss of body-fat stores. However, most obese mammals, including humans, have increased plasma leptin and insulin levels except for the leptin-deficient, obese C57BL/6J ob/ob mouse. The leptin resistant, genetically obese Zucker rat (fa/fa, mutated at the leptin receptor gene) does not reduce food intake or weight when given insulin centrally. Thus, leptin and insulin act in complex ways that involve central nervous system peptide-signaling and other hormonal systems. Glucocorticoid hormones are implicated in energy balance by their effects on NPY. Adrenalectomy decreases the effects of fasting to increase food intake and NPY gene expression and enhances the ability of insulin and leptin to induce anorexia and weight loss. These effects are reversed by glucocorticoid treatment and indicate the glucocorticoids are endogenous antagonists of leptin and insulin in the control of energy balance (Woods et al., 1998), and other studies show the complex manner that genetic endogenous and exogenous factors controlling the hypothalamic-pituitary-adrenal-adipose axis could potentially result in changes in energy balance and the reestablishment of the “set-point” (Friedman et al., 1998; Flier et al., 1998; Keesey and Hirvonen, 1997; Woods et al., 1998).

The Effects of AL Overfeeding and Moderate DR on the Rodent Bioassay

Effects on Spontaneous Disease and Tumors

The adverse effects of AL overfeeding are the most complex set of factors accelerating the early onset of degenerative disease and spontaneous cancer in laboratory rodents. The mechanisms leading to the adverse effects of AL overfeeding involve the early disruption of multiple endocrine, metabolic, and growth regulatory pathways (Duffy et al., 1989; Finch, 1990; Han et al., 1998; Masoro, 1995, 1996; Merry and Holehan, 1994; Weindruch and Walford, 1988). The metabolic effects of overfeeding that lead to the early onset of cardiovascular and renal degenerative diseases are largely due to oxidative stress and the depletion of antioxidant defense mechanisms, and to spontaneous glycation injury due to increased plasma glucose levels and/or decreased insulin sensitivity (Masoro et al., 1992; Rogers et al., 1993; Weindruch and Sohal, 1997; Yu, 1995). These and other hormonal and metabolic effects lead to obesity, excessive organ growth (especially in the metabolically active liver, kidney, and endocrine organs), with increased cell proliferation and decreased normal apoptosis of damaged cells, thus increasing the likelihood of an early spontaneous tumorigenic event (Grasl-Kraupp et al., 1994; Hart et al., 1995; James et al., 1994; Keenan et al., 1995a; Kritchevsky, 1993). All of these spontaneous diseases induced by AL overfeeding can be delayed or prevented by moderate dietary restriction.

Effects on Toxicology and Experimental Carcinogenesis

It is generally accepted that major variables in experimental studies should be controlled. For example, infectious agents that act as enhancing factors in toxicity and cancer development are well documented, and they can be controlled by the use of specific pathogen-free animals and microbial monitoring (Bhatt et al., 1986; National Research Council, Subcommittee of Lab Animal Nutrition, 1995). Of the major non-genotoxic nutritional injuries, caloric overfeeding is an important enhancing factor that can best be controlled by moderate DR (Klurfeld et al., 1989; Kritchevsky 1993; Rogers et al., 1993). With AL feeding, there is wide interlaboratory variability in average food intake, body weight, and longevity even when using the same rodent stock and the same diet (Keenan et al., 1994a; Laroque et al., 1997; Turturro et al., 1995). These differences are due to uncontrolled laboratory factors such as unintentional restrictive feeders or housing that limits food intake under so called AL feeding conditions (Keenan et al., 1998a; 1994a, 1996a,b).

The major factor most frequently misinterpreted is the effect that AL overfeeding has on dose effects in toxicity and carcinogenicity studies. The same dose of a substance frequently results in different effects when tested in AL-fed versus DR-fed animals (Klurfeld et al., 1989; Kritchevsky 1993; Pollard et al., 1985). However, most studies of the effects of DR use arbitrarily selected doses or determine doses in young growing AL-fed animals and then test them in adult DR-fed animals (Klurfeld et al., 1989; Kritchevsky 1993; Newberne et al., 1996; Pollard et al., 1985; Rogers et al., 1993). The differences observed should not be surprising, because AL-fed animals have developed more diet-related, long-term metabolic injury and thus are less fit to handle the consequences of a xenobiotic load. The DR-fed animals are more resistant to long-term
metabolic injury and maintain their antioxidant and other protective mechanisms for a longer period than their AL-fed counterparts. A moderate level of DR in the SD rat does not significantly alter Phase I and Phase II drug metabolizing enzyme activities, fatty acyl coenzyme A oxidase activity, or the qualitative toxicological response to a number of pharmaceuticals given at maximum tolerated doses (Keenan et al., 1992, 1994b, 1996a). However, some quantitative differences in the maximum tolerated dose are seen between AL-overfed animals and moderately DR-fed animals. For that reason it is necessary to determine doses in the DR-fed model that will be used in the long-term bioassay (Keenan et al., 1996a).

Selecting doses in the DR-fed model not only reduces variability and background diseases, but also will allow higher doses of test substances to be tested, thus increasing drug and dose exposure and bioassay sensitivity. For example, examination of pharmaceutical candidates in AL-overfed and moderate DR-fed SD rats, in order to determine maximum tolerated doses (MTD’s), no-observable-effect level (NOEL) doses, and toxicokinetic parameters (area under the curve [AUC] and C\text{max}) have demonstrated that the DR-fed animals are better able to tolerate higher doses of pharmaceuticals because they are fundamentally healthier (Keenan et al., 1996a,b, 1998b). The high doses tested in these 3-month range-finding studies were better tolerated by the moderately DR-fed rats and the estimated MTD’s and NOEL’s were approximately 2- to 4-fold higher under moderate DR-feeding conditions. However, comparative toxicokinetic studies with these compounds demonstrates steady state systemic drug and/or metabolic exposures (AUC and C\text{max}) that were either equal to or higher in moderate DR-fed animals compared to their AL-fed counterparts (Keenan et al., 1996a, 1998b). These data indicate that the DR-fed rodent is a more appropriate model in which to study possible toxic effects of compounds, because these animals are better able to maintain their metabolic systems. They also have less confounding effects due to the early onset of degenerative disease in 3-month toxicity studies, than do AL-fed animals.

Since many studies show that severe DR dramatically delays both spontaneous tumors and those induced by a dose of carcinogen, concern has arisen over the potential loss of carcinogenesis sensitivity with moderate DR (Allaben et al., 1996; Turturro et al., 1995, 1996). However, many of these studies use an arbitrary dose or a dose selected in AL-fed animals. Many of these studies also use more severe food restriction than is proposed with moderate DR and may have shorter 5–12 month endpoints. Studies performed in this manner do not account for the long-term confounding effects of AL overfeeding on the pathogenesis of induced tumors. They also do not take into account the delay in tumor onset that is observed with severe DR (Klurfeld et al., 1989; Kritchevsky, 1993; Pollard et al., 1985). For example, with SD rats receiving moderate DR, the spontaneous tumor incidence observed at 2-years was in the same range as for AL-fed rats, but most of the DR-fed rats’ tumors were found incidentally at final necropsy rather than at an unscheduled early necropsy (Keenan et al., 1994a, 1995b, 1998b). Moderate DR appears to delay tumor onset time (approximately 16 weeks for spontaneous mammary gland tumors), but tumor progression does not appear to be altered when measured by tumor growth rate (Keenan et al., 1995b, 1996a). Moderate DR does result in a delay in the onset time of spontaneous endocrine and endocrine-related tumors, particularly those of the pituitary, the pancreatic islet cells, and the mammary gland in SD rats (Keenan et al., 1995b, 1996a). The same results are also seen in these and other sites in the F344 rat, as well as in other rodent species, stocks, and strains (Iwasaki et al., 1988; Masoro et al., 1991; McCay et al., 1935; Roe et al., 1995). Thus, moderate DR is an appropriate model for well-controlled toxicity and carcinogenicity studies if one gives careful consideration to using the model throughout the dose selection process, and recognizes that moderate DR will delay or prevent spontaneous background degenerative disease and tumors (noise) that might be confounding factors in detecting a true treatment effect (signal) of a test substance.

Effects on Statistical Sensitivity of the Bioassay

The statistical sensitivity (statistical power) of a rodent carcinogenicity study is strongly affected by survival, the incidence of tumors at terminal sacrifice, tumor onset times, and other factors (Keenan et al., 1994b; McKnight and Crowley 1984). Moderate DR improves survival, which allows more time for treatment-related tumors to become detectable and increases exposure times to the test substance. These effects tend to increase the signal to noise ratio and improve statistical sensitivity (Keenan et al., 1992, 1994b). However, marked DR can reduce tumor incidence or delay the time of tumor onset, which lowers sensitivity (Keenan et al., 1994a, 1995b). In addition, statistical power depends strongly on factors that modify the treatment effect at each dose: tumor incidence at final necropsy, the time of tumor onset, the tumor lethality, and the non-tumor mortality.

While the constituents of the diet can be tightly controlled under AL feeding, the total amount of food consumed (the dose) is not controlled. Thus, AL-fed animals vary considerably in body size, obesity, and general health. From a statistical point of view, the drawback to AL feeding is that the level of food consumption is a potential confounding factor impacting many aspects of the animal’s response to treatment. Moderate DR feeding prevents these adverse health effects and results in a uniform animal that simplifies study interpretation for both acute and chronic toxicity studies.

Group housing of multiple animals per cage is an undesirable way of controlling food intake for 3 reasons. First, it prevents the accurate measurement of food consumption in individual animals. Second, it can result in uneven food consumption, since a dominant animal may consume more than the others in the cage. Third, it is a less precise means of providing a test substance in the diet when an individual
animal’s food intake is unknown and uncontrolled. Since AL-fed and DR-fed animals consume the same amount of food per gram of body weight, dietary administration of compounds by moderate DR provides a highly accurate and precise means of delivering a known dose of both the diet and the test substance to the animal.

DR-fed animals can withstand a higher xenobiotic load and thus frequently achieve higher maximum tolerated doses of test substances as compared to AL-overfed animals (Keenan et al., 1996a,b). The use of high dose levels is frequently a key strategy in risk assessment, because the sample sizes in bioassays are usually too small to accurately predict small increases in risk. The statistical assumption is that a small increase in risk at therapeutic dose levels will translate at high dose levels into large increases in risk detectable with modest numbers of animals per group. DR-fed animals can tolerate substantially higher doses of test substances and so provide a more stringent test for safety in the bioassay.

In long-term studies, the cumulative adverse effects of AL overfeeding will manifest themselves in cardiovascular disease, progressive decline of kidney function, multiple endocrine organ dysfunctions, early onset of diet-related tumors, and ultimately higher mortality. These competing diet-related risks complicate study interpretation because many of the animals will not survive to study termination. This is a serious statistical drawback, because the animals that die are not exposed to the drug for the entire length of the study and many induced effects, particularly treatment-related tumors, will not become evident until the second year of the study. Decreases in survival imply decreases in study statistical power. Difficulty of interpretation is greatly increased when control groups have lower survival than the treated groups. In AL-fed studies, this is a frequent secondary effect of lowered food intake or utilization in the highest-dose groups compounded by the treatment toxicity. This problem can be avoided with moderate DR-feeding, since the controls and treated animals will all consume the same amount of feed.

The adverse effects of AL overfeeding in the statistical evaluation of data are seen most clearly in carcinogenicity studies where age adjustments are required because of low control group survival. The suitability of such adjustments for assessing carcinogenic risks becomes questionable when non-treatment-related mortality and tumor lethality are major confounding factors. With moderate DR, in addition to having fewer non-treatment-related deaths, certain tumors such as those of the pituitary or mammary gland, are less likely to be classified as the cause of death in DR-fed animals, and are more likely to be found incidentally at terminal necropsy (Keenan et al., 1995b, 1996b). Statistically, there is no completely satisfactory way to adjust tumor incidence for differential survival, because knowledge of tumor onset time is essential for the analysis and is unknown for most internal, non-palpable tumors (McKnight and Crowley, 1984). However, the discrepancy among competing methods of analysis is often greatly reduced if most of the tumors are known to be nonlethal at terminal necropsy.

Care must be taken not to restrict food intake too severely, because severe DR (i.e., 40–50% of AL) is known to markedly delay onset of certain tumor types. A delay in tumor onset is not a problem for statistical power, provided the tumors are still detected at the terminal necropsy, although they have not progressed far enough to kill the animal. The statistical power is lost, however, when treatment-related tumors are not detected by terminal necropsy.

From a purely statistical perspective, the issue is decreasing power due to delayed tumor onset time compared to increasing power due to improved survival and increased treatment exposure. In a few circumstances, we have directly calculated power under AL and DR feeding for a given hypothesized effect of treatment (Keenan et al., 1995b, 1996a). Mammary tumors are ideal for this purpose because they are common in many strains and stocks of rodents and because the time of onset can be approximated by initial palpation dates. Using incidence data obtained from AL-fed and moderate DR-fed SD rats in our laboratory, we found no change in statistical power for a treatment effect that multiplied the hazard function throughout the study period. However, increased statistical power was found with moderate DR-feeding when most of the treatment effects occurred late in the study (Keenan et al., 1995b, 1996a).

Conclusion

Toxicologists and pathologists need to recognize that uncontrolled AL overfeeding of calories to rodents is one of the most important determinant errors in the current bioassay. It significantly contributes to the high variability and poor reproducibility of these studies, limiting their usefulness in risk assessment (Hart et al., 1995; Newberne et al., 1996; Roe et al., 1995). The use of moderate DR provides a better-controlled animal model. DR-fed rodents will have a lower incidence or a delay in onset of spontaneous background diseases and tumors. Moderate DR has no adverse effects on physiology, metabolism, clinical biochemistry, or pathology and has the great advantage of reducing and delaying age-related, spontaneous renal, cardiac, and endocrine diseases. This operationally simple procedure can significantly improve the health and well-being of the animals, and it will increase their survival time, exposure time, and the statistical sensitivity of these bioassays to detect a true treatment effect (Keenan et al., 1996a,b, 1998b). We are not advocating a marked or severe dietary restriction, such as 40–50% of the maximal AL intake, as an appropriate method of controlling the bioassay. Rather, we are recommending a moderate DR regimen of 70–75% of the maximum unrestricted AL food-intake level to optimize the laboratory rodent’s health, reduce the individual and study-to-study variability, and provide a better model to determine dose responses for risk assessment. This operation is simple, nutri-
tionally intelligent, and a well-established scientific method that makes a great deal of humane sense for keeping laboratory animals healthy and conducting well controlled toxicity and carcinogenicity studies for human safety and risk assessment.

ACKNOWLEDGMENTS

We thank the following for their support and suggestions: D. L. Bokelman, M. J. van Zwieten, C. F. Hollander, M.-F. Hubert, S. Molon-Noblet, B. A. Mattson, D. G. Haught, C.-M. Hoe, C. McCoy, P. Duprat, and J. D. Burek. Thanks also to Ms. Regina Foy for preparation of this manuscript.

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