Components of the AIN-93 Diets as Improvements in the AIN-76A Diet[1,2]

Philip G. Reeves

U.S. Department of Agriculture, Agricultural Research Service, Grand Forks Human Nutrition Research Center, Grand Forks, ND 58202-9034

ABSTRACT The AIN-93 rodent diets were formulated to substitute for the previous version (AIN-76A) and to improve the performance of animals that consume them. They are called AIN-93G, formulated for growth, and AIN-93M, for maintenance. Major changes included substituting cornstarch for sucrose and soybean oil for corn oil and increasing the amount in order to supply both essential fatty acids (linoleic and linolenic). L-Cystine was substituted for DL-methionine to supplement the casein component. The mineral mix was reformulated to lower the amounts of phosphorus, manganese and chromium, to increase the amount of selenium, and to add molybdenum, silicon, fluoride, nickel, boron, lithium and vanadium. The amounts of vitamins E, K-1 and B-12 were increased over those in the AIN-76A vitamin mix. The AIN-93G diet contains 200 g of casein and 70 g of soybean oil/kg diet. The maintenance diet (AIN-93M) contains 140 g of casein and 40 g of soybean oil/kg diet. The 1993 diets have a better balance of essential nutrients than the 1976 diet and are better choices for studies with laboratory rodents. J. Nutr. 127: 838S–841S, 1997.

KEY WORDS: • *purified diets* • *nutrient requirements* • *rats* • *mice*

Diet is perhaps the most important factor in experimental animal nutrition. Where the mechanism of action of a particular nutrient is to be resolved, knowledge of the precise composition of the diet for the animal model to be used is of foremost importance. Laboratory animal diets are placed into three categories. One includes cereal-based, unrefined or nonpurified diets whose primary ingredients are provided from natural sources and which are classed as open or closed formula. The composition of the open formula is known and available to the potential user and must be constructed as specified. On the other hand, the composition of the closed formula diets is known only to the commercial manufacturer; thus these diets cannot be expected to have identical nutrient compositions from batch to batch.

Another category of laboratory animal diet is the purified diet. This diet is made of refined ingredients including isolated proteins, refined sugars and oils, and purified sources of vitamins and minerals. The third category is the chemically defined diet. These diets are made of chemically pure sources of amino acids, mono- or disaccharides, and purified fatty acids or triglycerides. Minerals are provided by reagent-grade chemicals, and the vitamins are of the highest purity.

For most studies in experimental animal nutrition, the purified or chemically defined diets are the most widely used. Although the major purified ingredients may contain small amounts of various micronutrients, resourceful manipulation of the ingredients will permit the formulation of diets adequately supplied with all known essential nutrients except the one in question.

In 1977 and 1980, the American Institute of Nutrition (AIN) published the formula of a purified diet for experimental rodents (AIN 1977 and 1980). For the most part, this diet (called AIN-76A) can be easily reformulated to be used in many different types of nutrition studies. As the diet was used over the years, however, numerous problems were discovered. In 1989, participants in an AIN workshop on laboratory animal diets came to the conclusion that the AIN-76A diet needed to be revised. In 1993, diet formulae that can be substituted for AIN-76A were published and given a general designation of AIN-93 (Reeves et al. 1993a).

The criteria used for the AIN-93 formulations were *1*) the diets can be made from purified ingredients, *2*) they conform to or exceed the nutrient requirements suggested by the National Research Council (NRC 1978 and 1995), *3*) they can be made with readily available ingredients at a reasonable cost, *4*) the compositions are consistent and reproducible, and *5*) the diets can be used over a wide range of applications.

The new diet comes in two versions: AIN-93G to be used during the early growth phase and during reproduction, and AIN-93M to be used during adult maintenance. **Table 1** gives the general formulae of these diets. **Table 2** gives the formulae of the mineral mixes for each diet, and **Table 3** gives the ingredient list for the vitamin mix to be used for either diet. In the reformulation of AIN-76A to AIN-93 diets, some major changes were made. These changes are highlighted below.

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[2] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

TABLE 1

Formulation of the AIN-93G diet for rapid growth and the AIN-93M diet for maintenance of rodents when casein is used as the protein source[1]

Ingredient	AIN-93G	AIN-93M
	g/kg diet	
Cornstarch	397.486	465.692
Casein (>85% protein)	200.000	140.000
Dextrinized cornstarch (90–94% tetrasaccharides)[2]	132.000	155.000
Sucrose	100.000	100.000
Soybean oil (no additives)	70.000	40.000
Fiber[3]	50.000	50.000
Mineral mix (AIN-93G-MX)	35.000	35.000
Vitamin mix (AIN-93-VX)	10.000	10.000
L-Cystine	3.000	1.800
Choline bitartrate (41.1% choline)[4]	2.500	2.500
tert-Butylhydroquinone (TBHQ), *mg*	14.0	8.0

[1] Values were taken from Reeves et al. (1993a).
[2] Dyetrose (Dyets, Bethlehem, PA) and Lo-Dex 10 (American Maize, Hammond, IN) meet these specifications. An equivalent product also may be used.
[3] Solka-Floc®, 200 FCC (FS&D Corp., St. Louis, MO) or its equivalent is recommended.
[4] Based on the molecular weight of the free base.

CARBOHYDRATES

The carbohydrate sources for the new diets are cornstarch and sucrose. Part of the starch component is composed of dextrinized cornstarch, which contains 90 to 94% tetrasaccharides. These sources of carbohydrate were chosen for various reasons. High dietary concentrations of sucrose, the carbohydrate source for the AIN-76A diet, can cause several complications in rodents. These include hyperlipidemia, hepatic lesions (Medinsky et al. 1982), enhancement of nephrocalcinosis (Bergstra et al. 1993) and increased copper requirement (Failla et al. 1988, Fields et al. 1983). Therefore, starch was chosen as the major source of carbohydrate for the AIN-93 diets. However, a diet with high starch will not pellet properly; therefore, a more water-soluble carbohydrate source must be substituted for a portion of the cornstarch. It was previously demonstrated that dextrinized starch would fill this requirement. Dextrinized starch is cornstarch that has been hydrolyzed to simpler sugars. Such products that contain greater than 90% tetrasaccharides seem to work best. However, making major changes in the amount of protein, fat or other components in the diet may require adjustments in the amount of dextrinized starch to make the diet pellet properly. A small amount of sucrose was added to the diet to provide sweetness and improve palatability, as well as to act as a dispersal medium for vitamins and minerals.

PROTEIN

There are numerous sources of protein that can be used in purified rodent diets. These include casein, isolated soybean protein, egg white solids, lactalbumin and wheat gluten. Each has its own limitation when used in animal diets (Reeves 1996). Casein was chosen as the source of protein for the AIN-93 diets because its amino acid composition is reasonably adequate, it is readily available, and the cost is low relative to the other sources.

The major limitation of casein is its shortage of sulfur amino acids, particularly cystine/cysteine. To meet the requirements for sulfur amino acids in the AIN-93 diets, the use of L-cystine is recommended. Most other experimental diets use DL-methionine instead of L-cystine. In those diets, it is assumed that the conversion of methionine to cysteine in vivo will be suffi-

TABLE 2

Minerals mixes that supply the recommended concentrations of minerals for the AIN-93G and AIN-93M diets[1]

Ingredient	AIN-93G-MX	AIN-93M-MX
	g or mg/kg mix	
Calcium carbonate anhydrous (40.04% Ca)	357.00	357.00
Potassium phosphate monobasic (22.76% P, 28.73% K)	196.00	250.00
Potassium citrate, tripotassium monohydrate (36.16% K)	70.78	28.00
Sodium chloride (39.34% Na, 60.66% Cl)	74.00	74.00
Potassium sulfate (44.87% K, 18.39% S)	46.60	46.60
Magnesium oxide (60.32% Mg)	24.00	24.00
Ferric citrate (16.5% Fe)	6.06	6.06
Zinc carbonate (52.14% Zn)	1.65	1.65
Sodium meta-silicate · $9H_2O$ (9.88% Si)	1.45	1.45
Manganous carbonate (47.79% Mn)	0.63	0.63
Cupric carbonate (57.47% Cu)	0.30	0.30
Chromium potassium sulfate · $12H_2O$ (10.42% Cr)	0.275	0.275
Boric acid (17.5% B), *mg*	81.5	81.5
Sodium fluoride (45.24% F), *mg*	63.5	63.5
Nickel carbonate (45% Ni), *mg*	31.8	31.8
Lithium chloride (16.38% Li), *mg*	17.4	17.4
Sodium selenate anhydrous (41.79% Se), *mg*	10.25	10.25
Potassium iodate (59.3% I), *mg*	10.0	10.0
Ammonium paramolybdate · $4H_2O$ (54.34% Mo), *mg*	7.95	7.95
Ammonium vanadate (43.55% V), *mg*	6.6	6.6
Powdered sucrose	221.026	209.806

[1] Values were taken from Reeves et al. (1993a).

TABLE 3

AIN-93-VX vitamin mix recommended for use with the AIN-93G and AIN-93M diet formulations[1]

Vitamin	Amount
	g/kg mix
Nicotinic acid	3.000
Ca pantothenate	1.600
Pyridoxine-HCl	0.700
Thiamin-HCl	0.600
Riboflavin	0.600
Folic acid	0.200
Biotin	0.020
Vitamin B-12 (cyanocobalamin) (0.1% in mannitol)	2.500
Vitamin E (all-*rac*-α-tocopheryl acetate)[2] (500 IU/g)	15.000
Vitamin A (all-*trans*-retinyl palmitate)[2] (500,000 IU/g)	0.800
Vitamin D-3 (cholecalciferol) (400,000 IU/g)	0.250
Vitamin K-1 (phylloquinone)	0.075
Powdered sucrose	974.655

[1] Values were taken from Reeves et al. (1993a).
[2] The gelatin-matrix form of these vitamins is recommended.

cient to prevent a deficiency of this amino acid. When casein is used at 200 g/kg and L-cystine at 3.0 g/kg diet, sufficient concentrations of each essential amino acid will provide the 1978 NRC requirements for growth (NRC 1978). One hundred forty grams of casein and 1.8 g of L-cystine in the AIN-93M diet will provide more than enough of the sulfur amino acids for maintenance of adult rodents, based on the 1978 NRC report.

The fourth revised edition of the NRC Nutrient Requirements of Laboratory Animals (NRC 1995) recommends an increase in the dietary concentration of many of the essential amino acids, including the sulfur amino acids. The estimated requirement for the sulfur amino acids was increased from 6.0 to 9.8 g/kg diet. At 200 g/kg AIN-93G diet, casein falls short of the recommended amounts of these amino acids. Even with the addition of L-cystine at 3.0 g/kg diet, it remains below the recommended amount for growth. To bring the AIN-93G diet up to the new requirement, an additional 1.6 g sulfur amino acid/kg diet will have to be added to the 3.0 g/kg of L-cystine already present. This can be done by using either L-cystine or L-methionine. However, L-methionine might be the amino acid of choice, because studies have shown that high concentrations of L-cystine in the diet of rats increase the chance of a negative interaction with copper metabolism and of lowering the copper status of animals, especially when copper is limiting (Kato et al. 1994).

Because it is sometimes necessary to change the composition of diets to meet experimental conditions, casein may not be the protein source of choice. The investigator should be aware, however, that the use of other protein sources will require alterations in the basic dietary ingredients. For more information about this point, see the reference by Reeves (1996), which describes how to manipulate the dietary ingredients to achieve a balanced diet when various sources of protein are substituted for casein in the AIN-93 diets.

FAT SOURCES

At least two fatty acids, linoleic [18:2(n-6)] and linolenic [18:3(n-3)], are dietary essentials. Bourre et al. (1989 and 1990) fed variable concentrations of linoleic and linolenic acids in the diet of rats and used tissue saturation of 20:4(n-6) and 22:6(n-3) as criteria for requirements. They found that 12 g linoleic acid and 2 g α-linolenic acid/kg diet were the minimal requirements for rats. Only soybean oil at 30 g/kg of diet as a single source of fat will provide the above requirements for linoleic and linolenic acids. However, in Bourre's work (1989 and 1990), 50 to 60 g of soybean oil equivalent was required to reach the plateau for maximal concentrations of the marker fatty acids in many tissues of growing rats. A 15% margin of safety was suggested for the AIN-93G diet, and the amount of soybean oil was set at 70 g/kg diet. This amount of fat is recommended for young growing rats and reproducing females. For maintenance of adult males and nonreproducing females, the recommended amount is 40 g soybean oil/kg diet. Studies by Lee et al. (1989) suggest that a ratio of (n-6):(n-3) of at least 5 and a polyunsaturate:saturate ratio of at least 2 are points of greatest influence on tissue lipids and eicosanoid production. Soybean oil provides an (n-6):(n-3) ratio of 7 and a polyunsaturate:saturate ratio of 4.

ANTIOXIDANTS

Highly polyunsaturated oils are subject to oxidation and must be protected (Fullerton et al. 1982, Warner et al. 1982). Because the sources of fatty acids in the AIN-93 diets are primarily unsaturated, it was recommended that the diet contain an antioxidant. Tertiary-butylhydroquinone (TBHQ) effectively prevents the oxidation of the highly polyunsaturated (n-3) fatty acids of fish oils (Fritsche and Johnston 1988, Gonzalez et al. 1992, Ke et al. 1977). Because soybean oil also contains (n-3) fatty acids, it was recommended that TBHQ be used in the AIN-93 diets at 200 mg/kg oil. If, however, the experimental design precludes the use of antioxidants, the diet may be protected from oxidation on a limited basis by storing it in a nitrogen atmosphere in the cold. Purified diets contain free copper and iron and other oxidizing components that could make them more prone to oxidation than a natural ingredient–based diet in which these metals are organically bound.

FIBER

Although fiber is not considered a nutrient for rats and mice, it may provide some beneficial effects in regulating the microflora populations of the gut. The AIN-93 diets contain 50 g wood fiber/kg diet as the fiber source. The mineral content of fiber sources can vary, and iron seems to be the largest mineral contaminant. In general, the less purified sources contain the most mineral contaminants. The Solka Floc® (FS & D, St. Louis, MO) source of fiber recommended for the AIN-93 diets contains about 100 mg iron/kg. At 50 g/kg of the diet this could add a moderate amount of iron to the diet. Alphacel (ICN Biochemicals, Irvine, CA) contains only about 1.3 mg iron/kg, and Avicel cellulose fiber (FMC Corp., Philadelphia, PA) contains 1.6 mg iron/kg. Therefore, for nutrition studies of iron and other trace and ultratrace elements, the source of fiber should be a consideration.

VITAMINS

Table 3 lists the known essential vitamins for laboratory rodents and the amounts recommended for use in the AIN-93 diets. The vitamins are subject to oxidation and should be protected as much as possible during preparation and storage of the diet. Because riboflavin and vitamin K-1 are particularly vulnerable to degradation by light, the diets should be prepared in reduced light and stored in a dark cold room. There are interactions among some of the vitamins and trace elements. For example, a diet that is very high in vitamin E may suppress the gross signs of selenium deficiency in experimental animals. Diets with low amounts of vitamin D may affect the outcome of studies involving calcium metabolism. A vitamin B-12 deficiency anemia might be initially confused with symptoms of an iron deficiency.

MINERALS

Female rats are prone to develop kidney calcification when fed purified diets. This is especially true when the AIN-76A diet is fed. Female rats are very sensitive to the dietary Ca:P molar ratio, and this ratio in AIN-76A is too low to prevent the initiation of nephrocalcinosis. A Ca:P ratio of 1.3 or greater works well in the prevention of nephrocalcinosis in female rats when fed for 16 wk (Reeves et al. 1993b). If the calcium concentration in the diet is kept at 5.0 g/kg, then the phosphorus concentration should be 3.0 g/kg. Casein, the protein of choice for the AIN-93 diets, contains significant amounts of phosphorus; therefore, the phosphorus content of the mineral mix must be adjusted to obtain the correct dietary Ca:P molar ratio. The dietary calcium and phosphorus concentrations should not go below the requirements, however. Any change in the amount of dietary casein will require appropriate adjustments in the phosphorus concentration in the mineral mix.

Other changes in the mineral composition of the AIN-93 diets compared with the AIN-76A diet are noteworthy. The 1978 NRC requirement for manganese was 50 mg/kg diet; however, more recent evidence suggests that the requirement is much lower. The 1995 NRC lowered the manganese requirement to only 10 mg/kg diet, which is in agreement with the AIN-93G and AIN-93M diets. Although molybdenum is considered an essential mineral because of its involvement in the metabolism of sulfur compounds (Wang et al. 1992), the 1978 NRC did not have a requirement level, nor did the AIN-76A diet have molybdenum included in the mineral mix. The 1995 NRC recommends 150 μg molybdenum/kg diet, which is in line with the amount proposed for the AIN-93 diets. Chromium was listed in the 1978 NRC as a required mineral, but subsequent investigations could not corroborate earlier suggestions that chromium was essential for rats (Flatt et al. 1989, Holdsworth and Neville 1990); therefore, the 1995 NRC placed it in the "potentially beneficial nutrient" category until more conclusive evidence is found. Chromium is still included in the AIN-93 mineral mixes, but at a reduced amount compared with that in the AIN-76A diet. The selenium concentration in the diet was increased from 0.1 to 0.15 mg/kg, and the source of selenium was changed from selenite to selenate.

The ultratrace elements, other than selenium, molybdenum and iodine, represent a special class of proposed nutrients. When the formulae of the AIN-93 diets were proposed, it was recommended that a group of ultratrace elements be added to the diets. These included fluoride, boron, vanadium, nickel, lithium and silicon (regarded as a trace element). Although to date there is no convincing and/or repeatable evidence that these elements are essential for any specific metabolic process, studies show that they may interact with other nutrients under various conditions to give the appearance of beneficial effects (Nielsen 1987a, 1987b, 1991 and 1995, Seaborn et al. 1991). These elements are found in relative abundance in the natural ingredient diets, but their concentrations in purified diets are often very low. Therefore, minimal amounts were placed in the AIN-93 diets. It is in the best interest of the investigator to factor in and/or minimize fluctuations in the concentration of any dietary component.

PITFALLS

The preparation of a nutritionally balanced, palatable diet for laboratory animals requires a special appreciation for the nutrient requirement of the experimental animal model, how diets are constructed, and knowledge of the composition of the various ingredients that go to make up a diet. Although it is often tempting, these skills should not be the sole responsibility of the suppliers. For a more in-depth discussion of dietary ingredients, diet mixing and diet storage, see Reeves et al. (1993a and 1993b) and Reeves (1996).

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