Laboratory Animal Diets:
A Critical Part of Your In Vivo Research

Most all of us are aware that certain dietary choices can increase or decrease the likelihood of developing certain diseases. Our diets can also change our metabolism as well the levels of circulating factors (hormones, lipids, etc.) which may be markers for disease risk. What is often overlooked is the fact that these concepts also apply to laboratory animals, making diet a critical part of study design.

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Like humans, the animal's phenotype, ranging the full spectrum from good health to a disease state, is the result of the interaction between their genes and their environment. Since the genetic makeup of the animal is fixed, manipulating their phenotype generally means making subtle or drastic changes in their environment, which include diet, housing, and ambient temperature. For good and ill, environmental modifications can happen easily and sometimes occur without researchers' knowledge, leaving them scratching their heads and wondering why previous results were not repeated under what they thought were identical study conditions. Hence, control over environmental conditions is important to minimising data variability.

Nutritional science research during the 20th century has shown that diet is a powerful environmental tool capable of changing the phenotype of an animal. Diet-induced disease models rely on diet to drive the desired phenotype. Examples include diet-induced obesity, diabetes, dyslipidemia, hepatosteatosis, atherosclerosis, and hypertension, to name a few. Diet also plays an extremely important role even when it is not being used purposefully to develop a disease state. For one, diets fed during pregnancy and lactation can have long-term effects on the phenotype of the offspring. Additionally, diets fed during a toxicology study can affect how the test compound manifests its toxicological effects. Hence, conclusions drawn about the toxicology of a compound may vary depending on the type of diet fed during the study.

WE ARE ALL NUTRITIONISTS

Given the importance of diet on outcome, how should scientists make choices about what to feed? First, they should realise that since they are feeding an animal some type of diet, they should add "nutritional scientist" to their job description. It is now up to them to embrace this new title (or not). And, as all nutritional scientists know, it is in their best interest to be involved with and cognizant of the choice of diet fed to their research animals, as this may save innumerable headaches down the road. Secondly, they should know that while there is no perfect diet, some have real advantages over others.

REPORT, REPEAT, REVISE

When choosing a diet, one should ask three questions: Can I report it (can I tell others exactly what my animals were fed)? Can I repeat it (is there diet variability and will I be able to get the same results next year)? Can I revise it (as my hypotheses change, can I easily change the dietary components while keeping it otherwise matched to previous diets)? The answer should be "yes" to all three.

CEREAL BASED DIETS

Laboratory animal diets basically fall into two categories: chows and purified ingredient diets. Chow diets (Photo 1) have been used since the 1940s as the "background," "maintenance" or "control" diet in experiments. They are relatively inexpensive to produce and provide complete and adequate nutrition. Referred to as grain or cereal based, these diets typically contain ingredients such as ground corn, ground oats, alfalfa meal, soybean meal and ground wheat. Vitamins, minerals, and fat are added to ensure nutritional adequacy. Chow formulas are generally "closed" formulas, meaning that the exact amount of each ingredient added is kept secret by the manufacturer.

Photo 1: Example of a "cereal based diet"

An important point to remember is that each of the plant materials in chows contains many compounds, each inseparable from the next. Some of these are nutritive (protein, carbohydrate, fat, vitamin, minerals, and fibre) and some are non-nutritive (for example, plant derived compounds collectively termed phytochemicals) components. Because the nutritional content of these plant materials will naturally fluctuate with harvest location and across growing seasons, this means that the content of chow diets will vary from batch to batch.

For example, the soybean meal used in a chow today may not have the same percentage of protein (arguably the nutritional standard by which this ingredient is judged) as the soybean meal used six months ago. So when making a chow, one is left with two choices – to use the same amount of soybean meal every time the chow is made, or to account for nutritional differences by adding more of less soybean meal to "correct" for differences in the protein levels.

Actually, chows are made using both methods and each has disadvantages. If soybean meal levels are always kept constant, then the protein levels of the diet will vary with the protein levels of the soybean meal. With the second method, overall protein levels can be roughly maintained by varying the amount of soybean meal used in a particular batch of chow.

However, this raises a new issue – in keeping dietary protein levels constant by changing the level of soybean meal, what has happened to the levels of non-nutritive components of that soybean meal? Soybean meal (and other plant-derived ingredients) contains many varied and interesting phytochemicals, numbering in the hundreds. A subclass of phytochemicals is the phytoestrogens. These phytoestrogens can bind to estrogen receptors in the animal and have either pro- or anti-estrogenic effects. Since the progression of disease states such as obesity, atherosclerosis and cancer can be affected by such estrogenic or antiestrogenic activity, it may be advisable to use a diet without phytoestrogens altogether. Secondly, if soybean meal levels are varied across batches to account for differences in protein levels, it follows that the levels of phytoestrogens will vary not only from chow to chow but also from batch to batch of the same chow. Such variability in phytoestrogens may translate into variability in data over time, leading to cost increases due to either repeating studies or having to use to larger numbers of animals per study. Neither of these outcomes is cost-efficient nor desirable.

Is it easy to report a chow? One can give the name of the chow being used, but is it really the same as what was fed last year, especially down to the non-nutritive components? Arguably, the answer is "no" given the variability in the ingredients used. Plus, since most chow formulas are closed, one can never truly know how much of each ingredient was used in this particular batch. Is it easy to repeat a chow? Using the same argument about ingredient variability, the answer here is also "no".

Can a chow diet be revised as research hypotheses change? Revisions can mean removing something from or adding something to a diet. Given that each plant ingredient in chow can contain a dozen (or more) nutrients, removing a nutrient from the chow is not possible. For example, one could not study the effects of a very low iron diet chow. There is just no way to remove the iron from any or all of the plant materials - it is like trying to remove the sugar from a baked apple pie.

This restricts chow revisions to additions. However, there are limitations here as well. As an example, let's examine high-fat diets. Given the increasing population of obese and diabetic people in Westernized cultures, research in these related areas has increased greatly in the last decade. Laboratory animals are fed high-fat diets in order to test the ability of therapeutic compounds to prevent or reverse obesity. While it is possible to make a high fat chow by mixing fat with powdered chow and either feeding it as such or pelleting the mixture, this should be done

Figure 1: Creating a high-fat chow can dilute the nutrient concentrations

Ingredient	Chow	Chow with 20% Fat
Chow (gm)	1000	800
Lard (gm)	0	200
Total	1000	1000
Gram %		
Protein	23	19
Carbohydrate	50	40
Fat	5	24
Kcal%		
Protein	28	17
Carbohydrate	60	36
Fat	12	48
Total	100	100

Figure 2: The AIN-76A Rodent Diet

Ingredient	gm	kcal
Casein	200	800
DL-Methionine	3	12
Corn Starch	150	600
Sucrose	500	2000
Cellulose	50	0
Corn Oil	50	450
Mineral Mix	35	0
Vitamin Mix	10	40
Choline Bitartrate	2	0
Total	1000	3902

with caution, because as fat is added, the nutrient concentrations in the chow are diluted (Figure 1).

In this example, 20% fat has been added to a chow (800 gm chow plus 200 gm lard). While this effectively increases the fat from 12% to 48% of calories, it has also diluted the level of protein from 28% to 17% of calories. Thus the protein calories and all other nutritive and non-nutritive components have been reduced by 40%. This can be problematic for two reasons. First, such overzealous addition of fat can dilute the diet enough as to make it protein deficient, clearly not the intention when studying the effects of a high-fat diet. Secondly, this dilution effect makes comparisons to the control diet (presumably the unmodified chow) difficult. Not only will the experimental group be eating a higher fat diet, but they will also be eating less protein, vitamins, minerals and fiber per calorie of food, relative to the control group. Hence when comparing data between the groups, it will be impossible to determine if differences in phenotype were due to changes in any one nutrient.

"CAFETERIA" DIETS

It is worth spending a short time describing so-called 'cafeteria' diets and their shortcomings even though their use is diminishing due to a greater understanding of the diet choices available to researchers. The first published studies using 'cafeteria' diets in laboratory animal research appeared just over 30 years ago. As their name implies, these diets offer the animals a choice of foods found in supermarkets, including chocolate, nuts, cookies, peanut butter, cheese, salami, to name a few. It is not uncommon for the available food choices to change during the course of the study. To ensure nutritional adequacy, an appropriate laboratory chow diet is usually also offered to the animals.

It should be fairly evident that feeding such cafeteria diets by definition means that researchers will have very little control over what the animals eat. As a result, it will be impossible to accurately report their nutrient intake. Furthermore, this food self-selection model almost ensures that it will be impossible to repeat the study, given that another group of animals will likely choose a different combination of foods. Finally, as with chows, it is not possible to make subtle nutritional changes to the diet, since nutrients are being supplied by many dietary sources and because the animals are making different food choices every day.

As mentioned earlier, the use of cafeteria diets has thankfully declined in the past decade. However, although most nutrition-oriented journals do not (and should not) accept articles that use cafeteria diets, it is still possible to find such

papers (interestingly, most recently published papers using cafeteria diets are from labs in Europe). Twenty years ago, Dr. Barbara Moore made a convincing argument against the use of cafeteria diets in a well-written article whose message remains valid today[1].

PURIFIED INGREDIENT DIETS

Purified ingredient diets (Photo 2) were initially used by biochemists and nutritional scientists in their first major, shared endeavor of delineating the limited list of required nutrients - the simplest list of chemicals and molecules required in the diet for life versus death. Later, they studied the interaction between various nutrients and the influence of diet on more subtle quality of life (health and disease) issues, like diet and cancer, for example.

The idea behind purified diets is simple: each nutrient is supplied by a separate, purified ingredient. In the strictest sense of the terms, purified and semipurified diets differ in the types of ingredients used, though today the terms are generally used to mean the same thing. Purified ingredient diets can be (and we would argue should be) "open source" formulas, meaning that they are published and available to the scientific community. One note of caution on purified diet formulas – if a researcher is trying to repeat published data, they should make sure that the diet company they are using (if different from the one in the paper) is making the formula exactly as was reported. It is quite possible to find instances in which diets were made "to be similar to" published formulas, but in reality used different ingredients. Changes in ingredients can lead to changes in phenotype, something not helpful for the scientist trying to compare their data to what is in the literature.

In the early days of purified diet use, many research nutrition groups developed; each using their own favorite purified diet and usually using making them in house. For example, Vitamin A researchers developed separate and very distinct purified diet formulas from those studying Vitamin D or selenium or Vitamin E. Because of these differences, it became quite difficult to compare observations across these nutrient study disciplines, from lab group to lab group. Despite these differences, the formulas were generally well reported, allowing one group to know exactly what another group had fed their animals.

In the early 1970's, the American Institute of Nutrition (AIN) recognized that research nutritionists were traveling down these many separate tracks and also that other non-nutrition biologists were returning to the fold and using purified ingredient diets to study all aspects of health and disease. The AIN formed a committee and suggested that a simple purified ingredient diet be adopted for use as a "standard" purified diet by all biologists. The result of this collaboration was the AIN-76A rodent diet formula (Figure 2).

Photo 2: Example of a purified ingredient diet

In the AIN-76A rodent diet, the protein requirement is met by the milk protein casein, along with added methionine (to meet sulfur-containing amino acid requirements). Carbohydrates in this case are supplied by corn starch and sucrose, corn oil provides the fat, and cellulose supplies the fiber. Vitamin and mineral mixes specific to rodents are added to ensure adequacy. Each nutrient is supplied by a separate, purified ingredient. It is true that casein, for example contains trace levels of certain vitamins and will contain small amounts of some minerals. In general, this only becomes of importance when the goal of the experiment is to induce a deficiency state in one of those vitamins or minerals. In those cases, one can use alcohol-extracted casein (to remove the trace amounts of fat and fat-soluble vitamins) or individual amino acids (the literal links in the protein chain) to lower the background levels of these nutrients.

It is because these ingredients are refined materials, each containing one nutrient, (as opposed to the less refined chow ingredients) that allows research nutritionists to define the nutritional requirements of animals, by selectively removing one nutrient at a time from the diet. This also means that the possible modifications one can make to a purified ingredient diet are virtually limitless. This is also what continues to make purified diets powerful research tools and why so many scientists have turned to them in recent years.

First, purified diets are simple to report. For example, a paper may state that "rats were fed the AIN-76A diet for the entire study." The list of ingredients and their quantities can be easily and precisely described. Hence, researchers worldwide are able to duplicate the diet should they want to, or compare it to the diet they are using. And, since there is very little variation between batches of purified ingredients, the AIN-76A diet made today will be the same as the AIN-76A diet made a year from now. This repeatability of purified ingredient diets is clear-

ly advantageous during long-term experiments like toxicological studies, when variation in data over time may make interpreting the toxicity of the compound difficult.

REVISING PURIFIED DIETS

It is in diet modifications where purified ingredient diets most clearly illustrate their advantage over chow diets. For example, diets with high levels of sucrose (and no corn starch) have been formulated and used to study the development of insulin resistance. The fat source can be changed from coconut oil, to olive oil, to sunflower oil, to study the effects of changing the fat type from primarily saturated, to monounsaturated, to polyunsaturated fatty acids, respectively. As mentioned earlier, individual or multiple vitamins and minerals can be removed to study their deficiencies and to define requirements.

One key idea here is that when modifications are made, the remainder of the diet should be identical to the unmodified control diet. This makes comparisons across experimental groups easy to make, since only one diet component is changing at a time. This concept is quite simple to understand when it comes to removing or adding components that do not have caloric content – vitamins and minerals for example. So when vitamin B6 is removed from a diet, no calories are removed – just the vitamin. Hence the experimental and control diet are different only in presence or absence of this vitamin.

What about changing dietary components that contain calories – protein, carbohydrate, and fat? At this point, it is necessary to introduce a concept called the nutrient-to-calorie ratio. Not to be confused with the caloric density (the number of calories per gram of diet), this ratio compares the level of a particular nutrient (or nutrient group) per calorie of diet. Taking another look at the formula for the AIN-76A rodent diet (Figure 2) we see which ingredients have caloric content. Using the standard Atwater physiological fuel values of 4, 4, and 9 kilocalories (kcal) per gram for protein, carbohydrate, and fat, respectively, the 500 gm of sucrose, for example, contributes 2000 kcal to the diet. We now have the information we need to calculate the nutrient to calorie ratio for any nutrient. For example, this diet contains 10 gm of vitamin mix and 50 gm of cellulose per 3902 kcal.

Now that we calculate this ratio, why is it important? The answer lies in the fact that animals will for the most part, eat for calories, not weight of food, in an effort to consume the same amount of calories over the long term. This means that if an animal is used to eating a low-fat diet and they are switched to a higher-fat diet which (because fat is such an energy-dense nutrient) contains more kcal per gram of food, they will (after a period of adjustment) spontaneously eat fewer grams of food. They do this in order to continue eating the same number of calories (not grams) of food as they were when eating the low-fat diet. The reverse is true if switched from a high- to a low-fat diet. Similarly, rodents will eat more grams of food when the levels of dietary fiber (which has no caloric content) are increased, thereby lowering the caloric density of the diet. (In reality the ability to eat for calories does not always hold true – some species/strains will not regulate feeding and will overeat when exposed to a very high-fat diet for example).

Knowing that the animals will generally eat for calories explains why diets of different caloric densities (high- and low-fat diets for example) should be formulated to have similar nutrient to calorie ratios. This ensures that per calorie of food consumed (but not per gram), animals consuming diets of different caloric densities will receive the same absolute amount of nutrients (except those changed by design).

Recall the problem with adding a fat source to a chow diet – the other nutrients were diluted down as the fat was added. Properly formulated purified ingredient diets avoid the dilution effect because the fat is not added "on top of" the other ingredients but rather replaces carbohydrate. We could choose to replace protein, but generally this is not done given the importance of having certain minimum and adequate levels of protein in the diet.

There are two conceivable ways to replace the carbohydrate with fat (as an example, see Figure 3). One way is to switch them on a gram for gram basis, which we argue is the wrong way. In the example, (using the AIN-76A diet as the starting point), 150 extra grams of corn oil were added while 150 gm of sucrose were removed. However,

Figure 3: Replacing Carbohydrates with Fat.

	AIN-76A RODENT DIET		WRONG Gram for Gram		RIGHT kcal for kcal	
Ingredient	gm%	kcal%	gm%	kcal%	gm%	kcal%
Protein	20.3	20.8	20.3	17.5	25.0	20.8
Carbohydrate	66.0	67.7	51.0	43.9	39.7	33.1
Fat	5.0	11.5	20.0	38.7	24.6	46.1
Total	91.3	100.0	91.3	100.0	89.3	100.0
kcal/gm	3.90		4.65		4.80	
Ingredient	gm	kcal	gm	kcal	gm	kcal
Casein	200	800	200	800	200	800
DL-Methionine	3	12	3	12	3	12
Corn Starch	150	600	150	600	150	600
Sucrose	500	2000	350	1400	162.5	650
Cellulose	50	0	50	0	50	0
Corn Oil	50	450	200	1800	200	1800
Mineral Mix	35	0	35	0	35	0
Vitamin Mix	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0
Total	1000	3902	1000	4652	812.5	3902

since fat is over twice as calorically dense as sucrose, this has changed the nutrient to calorie ratio for the high-fat diet as compared to the low-fat control diet (the AIN-76A). There are 10 gm of vitamin mix per 3902 kcal of AIN-76A and 10gm of vitamin mix per 4652 kcal of high-fat diet. Calculating per 3902 kcal for the high fat diet, this comes to 8.4 gm of vitamin mix per 3902 kcal. So when the animals of both groups consume the same number of calories as we expect they will, the high-fat group will be consuming proportionally fewer nutrients (except fat of course) than the low-fat group.

The solution to this is to substitute fat for carbohydrate on a calorie-for-calorie basis. Returning to our example, when we add 150 gm of fat, we are adding 1350 kcal, so we should remove 1350 kcal of sucrose (see last panel of Figure 3). Now, both the high- and low-fat diets have the same nutrient to calorie ratios - meaning that when both groups consume the same number of calories on a daily basis, they will be receiving the same amount of protein, vitamins, minerals and fiber. Hence, such calorie-for-calorie diet formulation limits the difference in the diets to fat and carbohydrate calories, so differences between the experimental groups can be attributed to the varying levels of just these two macronutrients.

YOUR ANIMALS ARE WHAT THEY EAT

When it comes to experimental design, it's important to realise that the diet is not "just the food." Rather, it's an important environmental study component that can and will affect the phenotype of the animals and therefore the variability of your data. When publishing their data, scientists should be strongly encouraged to describe their diets in the same detail they would use for how they performed a Northern blot or knocked out a gene of interest. This level of information will only help the reader to compare their own data or repeat the reported study. Recognize that if you are doing in vivo research, you are a nutritional scientist. While there is no perfect diet, you should be aware of advantages and limitations of the various diets available. Important to your decision should be the ability to report, repeat and revise your diet. Purified ingredient diets can be used to limit data variability due to diet and to simultaneously induce the desired phenotype. They also provide a clean, consistent background for short- or long-term studies. Importantly, purified ingredient diets are modifiable in just about any way and thus allow researchers to explore their hypotheses without limitation. Remember, you are what you eat (with genetic contributions in mind) and so are your lab animals.

Reference:

1. Moore, B.J. The cafeteria diet - an inappropriate tool for studies of thermogenesis. J. Nutr. 1987 Feb:117(2):227-31

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