

6 Assessment of possible toxicity of foods derived from recombinant-DNA plants

Introduction

Risk assessment also takes into consideration the estimation and assessment of the level and frequency of intake of food from recombinant-DNA plants. This takes into account how frequently and to what extent the population would be exposed to newly expressed substances such as proteins, metabolites or endogenous compounds that are at altered levels in food due to the newly inserted gene (and/or other unintended effects resulting from genetic modification).

Conventional toxicological tests adopted from those originally developed for chemicals (i.e. food additives, pesticides and food contaminants) may be an appropriate approach to determining the safety of newly expressed substances. It is possible to determine the NOEL (no observed [adverse] effect level) of the new substance and subsequently the safety factor related to the level of exposure expected in the general population. Hence the safety factor is applied to derive the acceptable or tolerable daily intake. If such studies are to be undertaken, they should be designed according to the identity and biological function of the substances under consideration.

Conventional toxicology studies on the safety of whole foods are, however, not meaningful in practice because foods are complex mixtures of compounds characterized by wide variation in composition and nutritional value. Detecting any potential adverse effects and relating these conclusively to an individual characteristic of the food can therefore be extremely difficult. These difficulties in applying conventional toxicology approaches to recombinant-DNA plants have led to the development of the concept of substantial equivalence. This conceptual approach acknowledges that the goal of the assessment is not to establish absolute safety but to consider whether foods derived from recombinant-DNA plants are as safe as their traditional counterparts or not.

Conceptual approach to toxicity studies

The conceptual approach to the assessment of potential toxic properties of food involves biochemical characterization of the novel product from the inserted DNA element by *in vitro* digestibility studies, determination of the amino acid sequence similarity to known toxins, and acute oral toxicity studies based on an animal model. If on the basis of these studies a longer-term effect can be assumed then additional subchronic and chronic toxicity testing will be required. The *in vitro* digestibility studies are performed to determine the resistance of the novel product to acid, thus simulating the conditions in gastric and intestinal fluids. The sequence of the six amino terminal amino acids is compared with the amino terminal of the amino acid sequence of known toxins to determine their similarity. If the similarity is significant, it is possible that the novel product from the inserted gene is a toxin. The novel product is then subjected to subchronic toxicological studies to determine the safety factor for consumption relative to the exposure of the general population.



CODEX GUIDELINE PARAGRAPH 34. *In vitro* nucleic acid techniques enable the introduction of DNA that can result in the synthesis of new substances in plants. The new substances can be conventional components of plant foods such as proteins, fats, carbohydrates or vitamins which are novel in the context of that recombinant-DNA plant. New substances might also include new metabolites resulting from the activity of enzymes generated by the expression of the introduced DNA.

CODEX GUIDELINE PARAGRAPH 35. The safety assessment should take into account the chemical nature and function of the newly expressed substance and identify the concentration of the substance in the edible parts of the recombinant-DNA plant, including variations and mean values. Current dietary exposure and possible effects on population sub-groups should also be considered.

CODEX GUIDELINE PARAGRAPH 36. Information should be provided to ensure that genes coding for known toxins or anti-nutrients present in the donor organisms are not transferred to recombinant-DNA plants that do not normally express those toxic or anti-nutritious characteristics. This assurance is particularly important in cases where a recombinant-DNA plant is processed differently from a donor plant, since conventional food processing techniques associated with the donor organisms may deactivate, degrade or eliminate anti-nutrients or toxicants.

CODEX GUIDELINE PARAGRAPH 37. For the reasons described in Section 3, conventional toxicology studies may not be considered necessary where the substance or a closely

related substance has, taking into account its function and exposure, been consumed safely in food. In other cases, the use of appropriate conventional toxicology or other studies on the new substance may be necessary.

CODEX GUIDELINE PARAGRAPH 38. In the case of proteins, the assessment of potential toxicity should focus on amino acid sequence similarity between the protein and known protein toxins and anti-nutrients (e.g. protease inhibitors, lectins) as well as stability to heat or processing and to degradation in appropriate representative gastric and intestinal model systems. Appropriate oral toxicity studies¹⁵ may need to be carried out in cases where the protein present in the food is not similar to proteins that have previously been consumed safely in food, and taking into account its biological function in the plant where known.

CODEX GUIDELINE PARAGRAPH 39. Potential toxicity of non-protein substances that have not been safely consumed in food should be assessed on a case-by-case basis depending on the identity and biological function in the plant of the substance and dietary exposure. The type of studies to be performed may include studies on metabolism, toxicokinetics, sub-chronic toxicity, chronic toxicity/carcinogenicity, reproduction and development toxicity according to the traditional toxicological approach.

CODEX GUIDELINE PARAGRAPH 40. This may require the isolation of the new substance from the recombinant-DNA plant, or the synthesis or production of the substance from an alternative source, in which case the material should be shown to be biochemically, structurally, and functionally equivalent to that produced in the recombinant-DNA plant.

¹⁵ Guidelines for oral toxicity studies have been developed in international fora, for example, the OECD Guidelines for the Testing of Chemicals.

The conceptual approach to evaluating the toxicity of an introduced substance is described in Codex Guideline paragraphs 34–40.

Methods used to determine absence of toxicity

The requirements for and methods used to determine whether the new substance from the inserted gene is a toxin or not are described in the Codex Guideline paragraphs 34–4. Large amounts of purified protein expressed by the transgene are required for toxicity studies. The levels obtainable in plant tissue are generally not sufficient, and the proteins are therefore usually extracted from GM micro-organisms (such as *Escherichia coli*) engineered to express the protein in large amounts. In such cases, biochemical and functional equivalence of the bacterially derived version and the plant-expressed version must be demonstrated.

Animal feeding studies are usually performed to establish the absence of acute and subchronic toxicity. Animal feeding studies nevertheless have recognized limitations. It is important to realize that whereas carefully performed animal feeding studies demonstrating a lack of effect on selected physiological outcomes can be useful, the studies do not provide complete assurance of safety, because of the usual caveats with extrapolating results from other animals to humans. The results should be considered as “confirmatory” and “safety assuring”

CODEX GUIDELINE PARAGRAPH 10. The use of animal models for assessing toxicological endpoints is a major element in the risk assessment of many compounds such as pesticides. In most cases, however, the substance to be tested is well characterized, of known purity, of no particular nutritional value, and human exposure to it is generally low. It is therefore relatively straightforward to feed such compounds to animals at a range of doses some several orders of magnitude greater than the expected human exposure levels, in order to identify any potential adverse health effects of importance to humans. In this way, it is possible, in most cases, to estimate levels of exposure at which adverse effects are not observed and to set safe intake levels by the application of appropriate safety factors.

CODEX GUIDELINE PARAGRAPH 11. Animal studies cannot readily be applied to testing the risks associated with whole foods, which are complex mixtures of compounds, often characterized by a wide variation in composition and nutritional value. Due to their bulk and effect on satiety, they can usually only be fed to animals at low multiples of the amounts that might be present in the human diet. In addition, a key factor to consider in conducting animal studies on foods

is the nutritional value and balance of the diets used, in order to avoid the induction of adverse effects which are not related directly to the material itself. Detecting any potential adverse effects and relating these conclusively to an individual characteristic of the food can therefore be extremely difficult. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal studies could be requested on the whole foods. Another consideration in deciding the need for animal studies is whether it is appropriate to subject experimental animals to such a study if it is unlikely to give rise to meaningful information.

CODEX GUIDELINE PARAGRAPH 12. Due to the difficulties of applying traditional toxicological testing and risk assessment procedures to whole foods, a more focused approach is required for the safety assessment of foods derived from food plants, including recombinant-DNA plants. This has been addressed by the development of a multidisciplinary approach for assessing safety which takes into account both intended and unintended changes that may occur in the plant or in the foods derived from it, using the concept of substantial equivalence.

and are an additional component of the overall safety assessment in those circumstances in which they are warranted. The advantages and limitations of animal studies that must be taken into consideration in the determination of the safety of the foods derived from recombinant-DNA plants are discussed in the Codex Guideline paragraphs 10–12.

Feeding studies that use whole foods rather than isolated compounds may be appropriate when there are significant compositional changes in the food derived from recombinant-DNA plants; see Codex Guideline paragraph 53.

The ethical aspects of and necessity for animal feeding studies are issues that must be continually reconsidered to avoid unnecessary animal suffering. The Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology in 2000 (*Safety aspects of genetically modified foods of plant origin*, Section 4.2, paragraph 4.2.2) provided a useful discussion of the need for animal studies (Box 6.1).

It is generally considered that a subchronic study in rodents of 90 days' duration is the minimum requirement to demonstrate the safety of repeated consumption of foods derived from recombinant-DNA plants in the diet. The Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology in 2000 (*Safety testing of food additives and contaminants and the long-term evaluation of foods produced by biotechnology*, page 4) provided a useful discussion of subchronic toxicity studies (summarized in Box 6.2).

The document produced by the United States Food and Drug Administration on the toxicological principles of the safety assessment of food ingredients (US FDA, 2003)

CODEX GUIDELINE PARAGRAPH 53. Some foods may require additional testing. For example, animal feeding studies may be warranted for foods derived from recombinant-DNA plants if changes in the bioavailability of nutrients are expected or if the composition is not comparable to conventional foods. Also, foods designed for health benefits may require specific nutritional, toxicological or other appropriate studies. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal studies could be requested on the whole foods.



Box 6.1. Need for animal studies (FAO/WHO, 2000)

If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, animal testing may be deemed necessary. This would particularly be the case if the food were expected to make a significant dietary contribution, if there is no history of consumption of the novel gene product or if the modification affects several metabolic pathways.

In the situation where the genetically modified food differs from the traditional counterpart by the presence of one or a few new genes and their products, it may be possible to isolate and study these in a manner analogous to conventional toxicity testing of food additives.

However it is essential to ensure that the material tested is biochemically and functionally equivalent to that produced in the genetically modified food. This provides the possibility of increasing the sensitivity of toxicity tests compared with that possible if the products of the genetically modified plants had been fed directly and avoids some of the artefacts that can occur in toxicity tests on whole foods. However, this strategy is only applicable if the preceding detailed analysis does not reveal significant changes other than those expected. Otherwise testing of the whole food may be required. When animal testing is conducted on the whole food, it should generally be on the food as consumed by humans. The type of animal study would need to be considered on a case by case basis. In addition to investigating potential toxicological effects, animal studies may also be required if the genetic modification directly or indirectly affects the content or bioavailability of nutrients.

Where toxicological studies are considered necessary to assess the safety of long term consumption of a food in the diet, it is generally considered that a sub-chronic study of 90-days duration is the minimum requirement to

demonstrate the safety of repeated consumption of a food in the diet. This may need to be preceded by a pilot study of short duration to ensure that the diet is palatable to the test species and that the levels of incorporation of the test article are appropriate, e.g. the control diet containing the equivalent level of the comparator does not produce effects, as a result of normal levels of natural toxicants present in traditional foods accepted as safe. The highest dose level used in any animal study should be the maximum achievable without causing nutritional imbalance while the lowest level used should be comparable to the anticipated human intake.

The need for additional toxicological tests should be considered on a case-by-case basis taking into account the results of the 90-day study and other studies. For example, proliferative changes in tissues during the 90-day study may indicate the need for a longer-term toxicity study.

Conventional toxicological tests are of limited value in assessing whole foods, including genetically modified foods. Based on the maximum levels of the whole food that can be incorporated into experimental diets as indicated previously, a margin of safety may be estimated based on the absence or nature of adverse effects and likely human exposure. Improved experimental designs should take into account the need for nutritionally adequate animal diets, avoiding some of the inappropriate testing of foods or products.

It has been suggested that the use of biomarkers of early effects might increase diagnostic value and sensitivity of toxicity tests on foods (Schilter *et al.*, 1996). However, it will be necessary not to confuse adaptive and toxic effects in applying this approach.

Box 6.2. Toxicological studies on foods produced by biotechnology (FAO/WHO, 2000)

When a food product of biotechnology differs from a traditional food in a few well defined characteristics, these may serve to focus the safety evaluation process and determine the tests required. The toxicological focus will be on the few well defined characteristics. It may be possible to isolate and study differences in one or a few new genes and their products in a manner analogous to conventional toxicity testing of food additives. The conventional toxicity testing of these new genes and their products is usually the standard 14-day subacute study (OECD, 1995: Guideline 407). A substance to be tested for toxicity is usually fed to rats in a standard 14-day subacute study at a level that would reflect a very large margin of safety. The NOEL would represent the maximum level that can be incorporated into experimental diets with no adverse effects, and this could be translated to the safety factor for human exposure to the product. Human studies should contribute to the evaluation

process, and might be conducted when the *in vivo* animal studies demonstrate no unexpected or irreversible effects¹⁶.

A tiered approach to such studies should be adopted to investigate tolerance up to maximum levels of potential intake. The purpose is to have some confirmatory controlled clinical studies before getting into the greater complexities of general release. It is desirable that human studies are conducted as soon as possible within ethical constraints in order better to target animal studies and to avoid extensive but irrelevant animal studies. Observations from animal and human studies may reveal that the food is safe for its intended use, or may reveal unexpected indications that require more detailed investigation to confirm food safety.

¹⁶ Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology, Topic 6: Safety testing of food additives and contaminants and the long term evaluation of foods produced by biotechnology. 29 May–2 June 2000.

Box 6.3. Technical aspects of subchronic toxicity studies (FDA, 2003)*

Subchronic toxicity studies with rodents are generally conducted for between 90 days (3 months) and 12 months. Subchronic toxicity studies are generally used to help predict appropriate doses of the test substance for future chronic toxicity studies, to determine NOELs for some toxicology endpoints or to allow future long-term toxicity studies in rodents and non-rodents to be designed with special emphasis on identified target organs. They cannot be used to determine the carcinogenic potential of a test substance.

It is essential that all non-clinical laboratory studies are conducted according to the internationally recognized guidelines¹⁷ and good laboratory practice (GLP)¹⁸ regulations. Other factors that must be taken into consideration are discussed below.

Test animals

The care, maintenance and housing of laboratory animals must follow the guidelines in the *Guide for the care and use of laboratory animals*¹⁹.

The selection of species, strains and sex must take into consideration of test animals' general sensitivity. The responsiveness of particular organs and tissues of the test animals to the toxic substance to be tested must be considered when selecting rodent species, strains and substrains for toxicity studies. The selection of inbred, outbred or hybrid rodent strains for toxicity studies should be based upon the scientific questions to be answered. Moreover, the test animals should come from well characterized and healthy colonies, because recent information has suggested problems with the survivability of some strains of rats and test animals should be selected to achieve the recommended duration of the study.

The age of the test animals may result in variation in results. Testing should be conducted on young animals, and dosing should be commenced immediately after weaning, following an acclimation period of at least 5 days, and for rodents no later than 6–8 weeks of age.

An equal number of males and females of each species and strain should be used for the study. For subchronic toxicity studies, experimental and control groups should contain at least 20 rodents of each sex per group. These recommendations will help ensure that the number of animals that survive until the end of the study will be sufficient to permit a meaningful evaluation of toxicological effects.

The animals should be housed one per cage in order to address the following concerns.

If more than one animal is present in a cage, the feed efficiency (the relationship between feed consumed and body weight gained) cannot be determined with accuracy.

It is impossible to determine whether a decrease in body weight is due to decreased palatability or substance-mediated toxicity.

The organs and tissues from moribund and dead animals may be lost as a result of cannibalism if they are not individually caged.

The diet provided to the animals must be isocaloric and contain the same levels of nutrients (e.g. fibre and micronutrients) in both the treated and the control groups²⁰. Inadequately controlled

dietary variables may result in nutritional imbalances or caloric deprivation that could confound interpretation of the results of the toxicity study and alter the outcome and reproducibility of the studies.

The animals should be assigned to control and compound-treated groups in a stratified random manner; this will minimize bias and assure comparability of pertinent variables across treated and control groups (for example, mean body weight and body weight ranges). If other characteristics are to be used as the basis for randomization then that characterization should be described and justified. Animals in all groups should be placed in the study on the same day; if this is not possible because of the large number of animals in a study, animals may be placed in the study over several days. If recruitment to the study over several days is selected, a preselected portion of the control and experimental animals should be placed in the study on each day in order to maintain concurrence.

Experimental design

The animals should be exposed to the test substance on 7 days per week for a minimum of 90 consecutive days (3 months).

The route of administration of the test substance should be appropriate to the normal human exposure. A justification must be provided if alternative routes are used. Possible administration routes are described below.

The substance should be administered in the diet if the human exposure is likely to be through consumption of solid foods or a combination of solid and liquid foods. The animals should not be allowed to consume selectively either the basal diet or the test substance in the diet. Care must be taken to ensure that processes used during pelleting, such as heating, do not affect the test substance.

The test substance may be administered by dissolving in the drinking water. Alternatively, the test substance may be administered by encapsulation or oral intubation (gavage) if the human exposure is expected to be through daily ingestion of a single large dose instead of continual ingestion of small doses. Administration by gavage should be performed at approximately the same time each day, and the maximum volume of solution to be given by gavage in one dose should depend on the size of the test animal. In rodents, the volume should not exceed 1 ml/100 g body weight and for oily substances it should not exceed 0.4 ml/100g body weight. If the administered amount is to be divided into smaller doses, all must be administered within a 6-hour period.

Dose groups

At least three dose levels of the test substance should be used per sex (one dose level per group); however, ideally, four or five dose levels of the test substance should be used. A concurrent control group should be included. The appropriate dose levels for subchronic toxicity studies can be determined based on the information from acute and short-term toxicity studies.

(Continued)

¹⁷ OECD Guideline for the testing of chemicals, repeated dose 90-day oral toxicity study in rodents, 407, Sept. 1998.

¹⁸ OECD Principles of Good Laboratory Practice Directive 87/18/EEC, Directive 88/320/EEC.

¹⁹ National Research Council Institute of Laboratory Animal Resources. 1996. *Guide for the care and use of laboratory animals*. Washington, DC, National Academy Press.

²⁰ Nutrient requirements of laboratory animals, 4th Revised Edition, Subcommittee on Laboratory Animal Nutrition, Committee on Animal Nutrition, Board on Agriculture, National Research Council, 1995.

Box 6.3 (cont.)**Selection of treatment doses**

A minimum of three dose levels of the test substance and a concurrent control group should be used in toxicity studies. The three dose levels administered should follow the guidelines as follows:

- the high dose should be sufficiently high to induce a toxic response in the test animals;
- the intermediate dose should be sufficiently high to elicit minimal toxic effects in the test animals, such as alterations in enzyme levels or a slight decrease in body weight gain;
- the low dose should not induce toxic responses in the test animals.

Controls

A concurrent control group of test animals is required. The control group in dietary studies should be fed the basal diet.

The carrier or vehicle for the test substance should be given to control animals at a volume equal to the maximum volume of carrier or vehicle given to any dosed group of animals. Information on the toxicity of the carrier or vehicle should be available to ensure that it will not compromise the results of the study.

Observations and clinical tests:**observations of test animals**

Observations should be made of all animals at least once or twice a day throughout the study for general signs of pharmacological and toxicological effects, morbidity and mortality. The usual interval between observations should be at least 6 hours. Individual records should be maintained for each animal and the time of onset and characteristics and progression of any effects should be recorded, preferably using a scoring system. The clinical evaluations should not only assess the general pharmacological and toxicological effects but also neurological disorders, behavioural changes, autonomic dysfunction, and other signs of nervous system toxicity. The signs

noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and other evidence of autonomic activity. In addition, changes in posture and response to handling, as well as the presence of clonic or tonic seizures, stereotypes or bizarre behaviour should be recorded. The development of tumours should be recorded, particularly in long-term studies. During the course of a study, toxic and pharmacological signs may suggest the need for additional clinical tests or expanded post-mortem examinations.

Body weight and feed intake data

Test animals should be weighed at least once a week. Feed consumption (or water consumption if the test substance is administered in the drinking water) should be measured every week during a subchronic toxicity study.

Clinical testing

The following tests should be performed: ophthalmological examination, haematology profiles, clinical chemistry tests, urinalyses, neurotoxicity screening/testing and immunotoxicity studies.

Necropsy and microscopic examination

All test animals should be subjected to the following examinations: gross necropsy, measurement of organ weight, preparation of tissues for microscopic examination, microscopic evaluation, and histopathology of lymphoid organs.

**Reference: US FDA. 2003. Toxicological principles for the safety assessment of food ingredients: Red Book 2000, November 2003. IV.C.4a. Subchronic toxicity studies with rodents. Food and Drug Administration, Center for Food Safety and Applied Nutrition, Department of Health and Human Services.*

may also be a useful source for the technical aspects of subchronic toxicity studies (summarized in Box 6.3).

Chronic toxicity studies

Chronic toxicity studies involve long-term administration of the test substance, usually in the diet or drinking water, and sometimes by gavage. Chronic toxicity studies are designed to detect possible cumulative effects on target organ(s) in a dose–response dependent manner. The need for long-term chronic toxicity studies should be addressed on a case-by-case basis, and only when the results of the 90-day or other feeding studies indicate the need to consider toxicity from a longer term-perspective.

Quality assurance

It is very important that the organizational process and the conditions under which laboratory studies are planned, performed, monitored, recorded and reported are conducted according to the principles of good laboratory practice (GLP)¹⁸. The principles of GLP must be applied to

¹⁸ See page 28.

testing of chemicals to generate data on their properties and/or their safety for human health or the environment. In toxicology studies, it is essential to be certain that the data used to estimate safety are of a quality that is acceptable to all parties. It is also important in toxicology studies to establish the relationship between the changes in physiological parameters measured and the dose levels of the tested compound to which animals are exposed. Hence, good quality data are of the utmost importance and lead to accurate interpretation of the toxicity and estimation of the NOEL of the tested compound. From this interpretation, the safety factor can be established by estimating the maximum levels to which the human population can be exposed without observed adverse effects on health. Moreover, any observed differences between treated and untreated animals in the physiological parameters measured in animal experiments must be analysed statistically to establish the confidence limits of these differences.

References

- Doerfler, W. 2000. *Foreign DANN in mammalian systems*. Wennheim, Germany, Wiley-VCH. 181 pp.
- FAO/WHO. 2000. *Safety aspects of genetically modified foods of plant origin*. Joint FAO/WHO Expert Consultation on foods derived from biotechnology, 29 May–2 June 2000, Geneva, Switzerland. <ftp://ftp.fao.org/docrep/nonfao/ae584e/ae584e00.pdf>
- FAO/WHO. 2000. *Safety testing of food additives and contaminants and the long-term evaluation of foods produced by biotechnology*. Topic 6. Joint FAO/WHO Expert Consultation on foods derived from biotechnology, 29 May–2 June 2000, Geneva, Switzerland. <ftp://ftp.fao.org/es/esn/food/Bio-08.pdf>
- OECD. 1995. *Guideline for the testing of chemicals, Guideline 407. Repeated dose 28-day oral toxicity study in rodents*. Paris, Organization for Economic Co-operation and Development. <http://www.oecd.org/dataoecd/50/18/37478478.pdf>
- OECD. 1998. *OECD series on principles of good laboratory practice and compliance monitoring number 1*. ENV/MC/CHEM(98)17. Paris, Organization for Economic Co-operation and Development. [http://www.olis.oecd.org/olis/1998doc.nsf/LinkTo/env-mc-chem\(98\)17](http://www.olis.oecd.org/olis/1998doc.nsf/LinkTo/env-mc-chem(98)17)
- OECD. 2000. *Report of the task force for the safety of novel foods and feeds*. C(2000)86/ADD1. Paris, Organization for Economic Co-operation and Development. [http://www.olis.oecd.org/olis/2000doc.nsf/LinkTo/C\(2000\)86-ADD1](http://www.olis.oecd.org/olis/2000doc.nsf/LinkTo/C(2000)86-ADD1)
- Schilter, B., Holzhäuser, D., Cavin, C. & Huggett, A.C. 1996. An integrated in vivo and in vitro strategy to improve food safety evaluation. *Trends Food Sci. Technol.*, 7: 327–332.
- US FDA. 2003. *Toxicological principles for the safety assessment of food ingredients: Red book 2000, November 2003. IV.C.4a. Subchronic toxicity studies with rodents*. Washington DC, USA, United States Food and Drug Administration, Center for Food Safety and Applied Nutrition, Department of Health and Human Services.
- US National Research Council. 1995. *Nutrient requirements of laboratory animals*, 4th Revised Edition. Washington DC, USA, Subcommittee on Laboratory Animal Nutrition, Committee on Animal Nutrition, Board of Agriculture ●