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PROCEDURES FOR INVESTIGATING INTENTIONAL AND UNINTENTIONAL FOOD ADDITIVES

Report of a WHO Scientific Group

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WORLD HEALTH ORGANIZATION

GENEVA

1967

**WHO SCIENTIFIC GROUP ON PROCEDURES
FOR INVESTIGATING INTENTIONAL AND UNINTENTIONAL
FOOD ADDITIVES**

Geneva, 12-18 July 1966

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PROCEDURES FOR INVESTIGATING INTENTIONAL AND UNINTENTIONAL FOOD ADDITIVES

Report of a WHO Scientific Group

The WHO Scientific Group on Procedures for Investigating Intentional and Unintentional Food Additives met in Geneva from 12 to 18 July 1966. The meeting was opened by Dr J. Karefa-Smart, Assistant Director-General. Dr L. Golberg was unanimously elected Chairman of the Group and Professor R. Truhaut Vice-Chairman. Dr M. G. Allmark agreed to act as Rapporteur.

INTRODUCTION

The Scientific Group was convened on the recommendations made in the eighth¹ and ninth² reports of the Joint FAO/WHO Expert Committee on Food Additives. Its terms of reference were :

(1) to review, in the light of new scientific knowledge, the criteria used in establishing acceptable daily intakes, with the object of providing guidance to future Joint FAO/WHO Expert Committees on Food Additives and Joint Meetings of the FAO Working Party on Pesticide Residues and the WHO Expert Committee on Pesticide Residues;

(2) to suggest further studies on toxicological procedures used for the evaluation of intentional and unintentional food additives in order to establish their safety to the consumer.

¹ *Wld Hlth Org. techn. Rep. Ser.*, 1965, 309; *FAO Nutrition Meetings Report Series*, 1965, No.38.

² *Wld Hlth Org. techn. Rep. Ser.*, 1966, 339; *FAO Nutrition Meetings Report Series*, 1966, No. 40.

1. SCIENTIFIC BACKGROUND

During the last eleven years the Joint FAO/WHO Expert Committee on Food Additives¹ has prepared nine reports²⁻¹⁰ and three reports have resulted from the Joint FAO/WHO meetings on pesticide residues,¹¹⁻¹³ all of which are concerned with various aspects of the toxicology and the safety evaluation of chemicals that may be intentionally or unintentionally incorporated into food. A number of problems have been encountered in the course of the discussions that led to these reports. Furthermore, over the same period, significant advances have been made in many fields relevant to these problems.

Thus, many new and more sensitive methods of analysis have been devised and applied to the detection of minute amounts of chemicals in the environment. The detailed study of metabolism at the molecular level has been applied to many problems and this has special relevance to toxicology. Modification of substances in the course of their metabolism may significantly affect their toxicity; chemicals may alter enzyme activity and some substances may stimulate the production of metabolizing enzymes. Hence, for a full understanding of the effects of a chemical on biological systems it is necessary to have as much knowledge as possible

¹ The general term "food additives", as used in this report, is intended to apply to substances incorporated directly into foods, those arising indirectly from migration out of food-packaging materials, those present as pesticide residues, and any others resulting from the intentional or unintentional incorporation of chemicals into food.

² *Wld Hlth Org. techn. Rep. Ser.*, 1957, **129**; *FAO Nutrition Meetings Report Series*, 1957, No. 15.

³ *Wld Hlth Org. techn. Rep. Ser.*, 1958, **144**; *FAO Nutrition Meetings Report Series*, 1958, No. 17.

⁴ *Specifications for identity and purity of food additives. Vol. I Antimicrobial preservatives and antioxidants*, Rome, Food and Agriculture Organization of the United Nations, 1962.

⁵ *Specifications for identity and purity of food additives. Vol. II Food Colors*, Rome, Food and Agriculture Organization of the United Nations, 1963.

⁶ *Wld Hlth Org. techn. Rep. Ser.*, 1961, **220**; *FAO Nutrition Meetings Report Series*, 1961, No. 29.

⁷ *Wld Hlth Org. techn. Rep. Ser.*, 1962, **228**; *FAO Nutrition Meetings Report Series*, 1962, No. 31.

⁸ *Wld Hlth Org. techn. Rep. Ser.*, 1964, **281**; *FAO Nutrition Meetings Report Series*, 1964, No. 35.

⁹ *Wld Hlth Org. techn. Rep. Ser.*, 1965, **309**; *FAO Nutrition Meetings Report Series*, 1965, No. 38.

¹⁰ *Wld Hlth Org. techn. Rep. Ser.*, 1966, **339**; *FAO Nutrition Meetings Report Series*, 1966, No. 40.

¹¹ *Wld Hlth Org. techn. Rep. Ser.* 1962, **240**; *FAO Plant Production and Protection Division Report No. PL/1961/11*.

¹² *WHO/Food Add./23* (1964); *FAO Meeting Report No. PL/1963/13*.

¹³ *WHO/Food Add./26.65*; *FAO Meeting Report No. PL/1965/10*.

about the relationships between the chemical (and its derivatives) and the complex pattern of enzymes in living organisms.

Considerable advances have also been made in molecular biology, coupled with more detailed information on the structure and ultrastructure of cells and tissues; their relationship to function may now be interpreted in molecular terms. Various isolated fractions derived from cells and tissues can be subjected to detailed investigation. Electron microscopy and histochemistry are now commonly used in pathological and toxicological laboratories.

Important developments have occurred in the availability and quality of laboratory animals. Better genetic control, a wider range of species and the provision of animals in which common pathogens are controlled, or from which micro-organisms are completely eliminated, are also important to toxicologists.

These and other advances have resulted in the development of better methods of investigation in toxicology. It is now generally possible to study more precisely, or to follow in greater detail, the absorption, distribution, metabolism and elimination of a substance, to discover the modifications that may occur in the course of its passage through living systems, to investigate its effects on enzymes or morphology and to relate these observations to alterations in structure and function and to the signs and symptoms of toxicity. Thus, with increasing frequency, it should be possible to explain many toxicological phenomena in chemical and biochemical terms.

As the methods for the detection of changes in biological systems become more sensitive, the investigator needs to become more critical in his interpretation of the significance of the effects he observes. New approaches and new techniques make it possible to probe in depth into the nature of the earliest response of the organism to exposure to a chemical. The interpretation of observations is often difficult and is not made easier by the administration of high doses in studies on substances of relatively low toxic potential. There are also instances where adaptive changes occur. Since many toxicological decisions are based on the assessment of the highest dose level that causes no deleterious effect, the differentiation of adverse effects from other changes is crucial. Several examples of problems of this nature are mentioned elsewhere in this report.

Since these advances in scientific knowledge are being applied in the evaluation of safety, it is necessary to bring up to date the guidance given to all concerned in this field. It is equally essential that regulatory agencies should be aware of the importance and significance of such progress and should take appropriate action for the protection of the community.

The Group has given detailed consideration to a limited number of special procedures among the many involved in toxicology. Its general

conclusions are set out below. The remainder of this extensive field awaits future consideration.

2. GENERAL CONSIDERATIONS

The Group is in agreement with the general principles and approaches set out in the earlier reports. There are, however, a few points of interpretation that call for comment.

In the first report of the Joint FAO/WHO Expert Committee on Food Additives¹ it was suggested that "provision must be made for the admission with a minimum delay of properly tested food additives which are considered desirable". The Group feels that the permitted list should always remain open for additions resulting from technological development or changes depending on the progress of scientific knowledge.

The report stressed that "it would not be practicable for the responsible authorities to limit any group (of food additives) to a specific number, i.e., to insist that the inclusion in the list of a new additive requires the elimination of one already permitted". The Group is of the opinion that an increase in the number of food additives on a permitted list does not necessarily imply any over-all increase in additives used; the different additives are largely used as alternatives. From the point of view of food technology, the chemistry of food is highly complex and many different direct additives with generally similar actions may be needed to cope with a wide range of problems. From the toxicological point of view, there is less likelihood of long exposure to one chemical, or of high or cumulative dose levels being attained, if a wide range of substances is available for use. Similar considerations apply to pesticide residues.

It is desirable that national governments should maintain a check on the total intake of each food additive, based on national dietary surveys, to determine whether the total load in the diet approaches the acceptable daily intake. The Group was informed that the FAO/WHO Codex Alimentarius Commission will be using the data from such surveys to carry out this study on an international basis.

Toxicologically, it generally makes no difference how a chemical is distributed in the diet provided that the over-all content does not exceed the acceptable daily intake (ADI). In some instances, regulatory bodies may decide to recommend the use of a particular chemical in certain specified foods. If the levels proposed are likely to result in over-all amounts in the diet equal to the ADI, difficulties might arise from the presence of this chemical in other foods. A problem of this sort might

¹ *World Health Org. techn. Rep. Ser.*, 1957, 129; *FAO Nutrition Meetings Report Series* 1957, No. 15.

arise from background levels, spray drift or other causes; the Group considers it advisable to reserve some small proportion of the ADI to cover this.

It is well established that many food additives are altered by plants or animals or by reaction with foodstuffs, and that the products of such reactions are consumed. Efforts should be made to determine the ultimate fate of each such chemical. Safety-evaluation studies should take into account the safety of each compound formed, since in some cases the metabolites or degradation products may be more toxic than the original material. On the other hand, it may happen that these materials, as well as the original substance, are destroyed or eliminated during processing or cooking.

It has been stated that all intentional food additives should be subjected to individual toxicological investigation. This generalization needs modification along the following lines :

(a) Many additives may already be present in food or elsewhere in the environment. The background occurrence of the chemical must be taken into account in the evaluation of its safety.

(b) Some additives give rise to molecules already present in food in much greater amounts. If the biochemical evidence shows that the additive makes only a small contribution to existing metabolic pools from food components or in the tissues, there may be no need for detailed toxicological studies on it, provided that it conforms to adequate specifications. An example of a substance of this kind would be an ester of a sugar and a fatty acid that is completely hydrolysed in the intestinal lumen with the formation of two substances already present in the diet in much greater quantities.

(c) If a series of chemical analogues can be shown to give rise to the same main metabolic product and other compounds that are already present in the organism in greater quantities or that can be readily and safely metabolized, it may be sufficient to carry out toxicological studies on a suitable representative of the series.

(d) Toxicological information for the evaluation of safety is not always fully adequate and it is suggested that some substances, especially those that are urgently needed or are present in relatively minute amounts, might be given at least temporary clearance. Thus, the establishment of "temporary acceptable daily intakes" is recommended in situations in which a particular food additive would be useful, or may already be in use, and for which toxicological or other data are not fully adequate to permit an acceptable daily intake to be set by the normal procedure. The conditions that need to be satisfied before such a "temporary acceptable daily intake" is established are discussed later in this report (section 3.4.2).

In the case of substances that occur in minute quantities, a procedure for the establishment of an "administrative acceptable daily intake" is also discussed (section 3.4.3).

3. SPECIFIC PROBLEMS

3.1 Specifications

Adequate specifications for identity and purity should be available before toxicological work is initiated. Toxicologists and regulatory bodies need assurance that the material to be tested corresponds to that to be used in practice. Ideally, the specifications should be such as to define a material that will give reproducible biological results.

Specifications for food additives produced commercially should be broad enough to include all the variations in the composition of these additives that, according to current knowledge, do not significantly affect their biological properties. As an example, mono- and di-glycerides of edible fatty acids¹ were considered as coming under one specification for the purpose of toxicological evaluation. In any case, each such group of additives will have to be judged individually with respect to the limits of composition set out in the specifications.

The levels of impurities that, according to current knowledge, are considered to be toxicologically significant and the methods for their determination must appear in the specifications. Tests for impurities such as lead, arsenic and heavy metals as a measure of good manufacturing practice should be maintained, unless and until a better measure becomes available. These tests are needed, irrespective of the high standards usually maintained in manufacture, in cases where inexperienced and less well-equipped manufacturers may produce food additives.

The Group was informed that in the eventual publication of the work on food additives and pesticide residues in the *Codex Alimentarius*, the foods in which these materials will be used and the levels of use will be indicated. Changes in usage of these materials will also be noted under the system of continuous revision that the *Codex Alimentarius* will undergo.

3.2 Methodology

3.2.1 *Appropriate species of test animals*

It is often stated that results obtained in the most sensitive species should take precedence in toxicological evaluation. The Group recom-

¹ *Wld Hlth Org. techn. Rep. Ser.*, 1964, 281; *FAO Nutrition Meetings Report Series*, 1964, No. 35.

mends that, wherever possible, the most appropriate species should be chosen for this purpose; this would be the species most similar to man with regard to its metabolic, biochemical and toxicological characteristics in relation to the substance under test.

3.2.2 *Investigation in human subjects*

Studies in experimental animals on the biological effects of chemicals that may be introduced into the environment have as one of their major objectives the prediction of any possible hazard to man. One of the greatest problems that arises in these studies is in the extrapolation of the data obtained from investigations in animals to the definition of safe levels of exposure in man. The purpose for which the chemical may eventually be used does not necessarily affect the nature of the investigational problems involved.

The prediction and prevention of possible toxic hazards to the community that might arise from the introduction of a chemical into the environment can be made more certain if information from meaningful studies in human subjects is available. Three particular aspects of toxicology require consideration in this connexion: first, the choice of the most appropriate animal species for investigations that aim at the prediction of human responses; secondly, the investigation of a reversible specific effect observed in the most sensitive animal species to determine whether it represents a significant hazard to man; thirdly, the study of effects specific to man.

Metabolic studies. There is a need at a relatively early stage to obtain information on the absorption, distribution, metabolism and elimination of the chemical in human subjects, since this makes it possible to compare this information with that obtained in various animal species and to choose the species that are most likely to have a high predictive value for human responses.

The problems that arise in connexion with such early human studies in the investigation of drug toxicity have been discussed in a recent report of a WHO Scientific Group.¹ The sooner, in the course of toxicological investigation, these studies at a low dosage level can be undertaken, the better; however, it is necessary to have adequate short-term toxicological information in several species before even low doses of a new chemical are administered to human subjects.^{1,2,3}

¹ *Wld Hlth Org. techn. Rep. Ser.*, 1966, 341.

² Hayes, W. J., jr (1965) *Research in pesticides*, New York, Academic Press.

³ Ad Hoc Subcommittee on Use of Human Subjects in Safety Evaluation, Food Protection Committee, Food and Nutrition Board (1965) *Some considerations in the use of human subjects in safety evaluation of pesticides and food chemicals*, Washington D.C., National Academy of Sciences—National Research Council (Publication 1270)

Confirmation of predicted safety margin. Chemicals intended for use as drugs are subjected to human pharmacological investigations and to clinical trials that must, of necessity, involve the use of biologically effective dosage levels. In the examination of other chemicals from a toxicological point of view, it is sometimes necessary to ascertain whether the safety margin predicted from animal data is valid. For this purpose it may be helpful to administer the chemical to human volunteers. It is emphasized that the following conditions should be fulfilled with regard to such a study :

(a) The chemical should have been fully studied in a range of experimental animals.

(b) There should be a clear need, in the public interest, for the study of some effect or effects in the human subject.

(c) The effect or effects studied should be reversible.

(d) The dose levels used should be based on full information of the toxicological properties of the substance in animals.

(e) The investigation should be terminated immediately the effect has been unequivocally demonstrated.

Effects specific to man. The Group considered the fact that, in the case of drugs, effects specific to man may be revealed during clinical trials or as a result of the reporting of adverse reactions after the drug is placed on the market.

In the case of other chemicals, it is not acceptable to study such effects by the use of volunteers. Toxicological studies can be made in those who are occupationally exposed to the chemical or in patients suffering from accidental poisoning. There is a need for more critical epidemiological and toxicological investigations in such situations. If unexpected effects apparently specific to man are observed, it is advisable to re-examine the evidence obtained earlier from animal studies to determine whether useful information in those investigations was missed or whether some different method of study might have been of greater predictive value.

Human volunteers. Ethical and legal problems may arise in connexion with the provision of volunteers for these investigations. Since the situation differs greatly from country to country, it should be left to the appropriate authorities in each country to decide any issues involved.

3.2.3 *Effect of age on toxicity*

The response of an animal to a particular substance may vary with age. In general, but not invariably, the young animal is more sensitive to the toxic effects of exposure to chemicals. The difference may arise from the presence of distinctive flora in the upper part of the bowel, a factor that accounts for the susceptibility of human babies to poisoning

by nitrates. In most cases, however, there exists an enzymic basis for the age difference in response to foreign compounds.

Many foreign substances are metabolized in the body by enzymes that occur in the endoplasmic reticulum of the liver cells. There is evidence that these enzymes may be poorly developed in newborn animals,^{1,2} possibly owing to lack of inducers.³ Here also there may be significant species differences. In the rat the activity of the group of enzymes commonly called "drug-metabolizing enzymes" and mainly studied in the liver is maximal at the age of about 30 days and thereafter declines to some extent. Glucuronyl transferase is deficient in many newborn animals, including man, but not in the rat.

Thus, the enzymes that metabolize foreign substances may be at a low level in newborn animals, but develop later. Occasionally, they may be found in the newborn of some species, but not of others. A few metabolizing enzymes may be present in the newborn of some species although enzyme activity is no longer manifest in the adult animal of the same species. In any case, the metabolizing enzymes may either enhance or diminish toxicity, depending upon the biological properties of the substrate and the various products, although in the main these enzymes act beneficially.

These considerations must now be applied to the question of the possible presence of additives or trace contaminants in the diets of babies. It should be stressed that, in spite of the often considerable efforts made by manufacturers to avoid the presence of such contaminants in baby foods, the diet of babies is likely under present-day conditions to contain traces of pesticide and other residues. In addition, there are circumstances in which the benefit to the baby arising from the inclusion of some additive, for example a preservative, in its diet may greatly outweigh any possible hazard. Nevertheless, it is necessary to exercise great care in making such additions, keeping in mind the possible long duration of exposure.

The discussion presented above of species differences in response to the toxic effects of chemical compounds underlines the fact that data derived from such studies, or from other experiments with newborn or young animals, cannot be regarded as sufficient in themselves to permit the assessment of safety. It is particularly important to have a knowledge of the metabolism of the compound and to carry out other biochemical and toxicological studies in the most appropriate species in order to provide a firm basis for the evaluation of safety. However, pertinent information

¹ Hart, L. G., Adamson, R. H., Dixon, R. L. & Fouts, J. R. (1962) *J. Pharmacol. exp. Ther.*, **137**, 103.

² Kato, R., Vassanelli, P., Frontina, G. & Chiesara, E. (1964) *Biochem. Pharmacol.*, **13**, 1037.

³ Fouts, J. R. (1965) In: Robson, J. M., Sullivan, F. & Smith, R. L., ed., *Embryopathic activity of drugs*, London, Churchill.

derived from reproduction (multigeneration) studies provides some assurance on the safety of compounds that might be present in the diet of babies.

Since babies constitute a special population, close observation of epidemiology in this group is an important practical aspect of the evaluation of the effects of exposure. The need exists for further information on the development of enzyme systems in the human young, with particular emphasis on those enzymes responsible for dealing with foreign compounds.

Further problems may arise when aged animals are used for the evaluation of safety. It is better to carry out toxicity studies before the complications of senescence arise. It is clearly essential in all such investigations to record the age of the animals as one of the factors of major importance in the experimental design. More basic information is required on toxicity in aged as well as in young animals.

3.2.4 *Effect of nutritional state on toxicity*

Nutritional state can influence toxicity. The effects of starvation or more specific nutritional deficiencies on the biological response to different substances will vary widely. It has been shown, for example, that glycogen depletion in the liver may interfere with the production of metabolizing enzymes in mice and in guinea-pigs.^{1,2} Poor nutritional status does not necessarily increase susceptibility to toxic effects; for example, depression of metabolizing enzymes that give rise to toxic products may diminish susceptibility. The general effects of reduced food intake on biological responses have been reviewed by Friedman,³ and the effects of specific nutritional deficiencies on toxicity have been fully discussed by Hötzel.⁴ The relationship between nutritional status and carcinogenesis has been reviewed by Tannenbaum⁵ and discussed in the fifth report of the Joint FAO/WHO Expert Committee on Food Additives.⁶ Carcinogenic action tends to be diminished by calorie restriction and protein deficiency and in certain cases by specific nutritional deficiencies. The composition of the intestinal flora and such practices as coprophagy have an important influence on the nutritional status of experimental animals.

¹ Dixon, R. L., Shultice, R. W. & Fouts, J. R. (1960) *Proc. Soc. exp. Biol. (N.Y.)*, **103**, 333.

² Kato, R. & Gillette, J. R. (1965) *J. Pharmacol. exp. Ther.*, **150**, 279.

³ Friedman, L. (1966) *Fed. Proc.*, **25**, 137.

⁴ Hötzel, D. (1964) *Dtsch. med. Forsch.*, **2**, 105.

⁵ Tannenbaum, A. (1959) *Nutrition and cancer*. In: Homburger, F., ed., *The physiopathology of cancer*, 2nd ed., New York, Hoeber-Harper, p. 517.

⁶ *Wld Hlth Org. techn. Rep. Ser.*, 1961, **220**; *FAO Nutrition Meetings Report Series*, 1961, No. 29.

So far as toxicity studies are concerned, it is wise to maintain all the animals on a diet that is nutritionally adequate in every way, unless there is some specific reason for doing otherwise. It follows from the complexity of the effects of starvation or specific nutritional deficiencies in different species of animals that the indiscriminate use of undernutrition or dietary deficiencies might give rise to misleading results. A clear distinction must be drawn between investigations forming part of research projects and toxicological studies intended for safety evaluation. In applying the results of such safety tests, the state of nutrition of the individuals exposed is taken into account by means of appropriate adjustments in the margin of safety used in specific instances. Equally, such adjustments must make allowance for other relevant factors in the chemical environment to which the population under consideration is exposed.

Food additives may be used, or contamination of food with chemicals may occur, in countries in which malnutrition is widespread. The Group considers that further work is needed on the effects of various states of malnutrition or undernutrition on the toxicity manifested by chemical compounds.

Manipulation of the composition of the diet of experimental animals, either in an effort to simulate conditions of malnutrition in man or in the belief that such dietary modifications help to elicit latent toxic effects of the material under study, is not considered advisable in routine toxicological investigations intended for the evaluation of safety.

3.2.5 *Duration of toxicity tests in experimental animals*

For adequate interchange of information a precise description is more meaningful than such terms as "acute", "subacute", "short-term" and "chronic". Each report of an experiment should state in precise terms, in respect of both control and test animals, the species, sex, diet, route of administration and duration. The objective should be to define clearly all the known variables.

Scientific judgement is necessary in determining the duration of animal studies for the evaluation of an individual food additive. Where adequate biochemical and toxicological data on closely related chemicals are available, the objective becomes the detection of any deviation from the established pattern. This can usually be determined by intensive studies of a few months' duration when these are adequately designed and evaluated. Appropriate studies in humans add significantly to the adequacy of the data.

In the absence of such definitive data, or if there are reasons to suspect carcinogenic potential, longer-term studies must still be relied upon for reassurance. Recent advances in the quality of research animals, and particularly in the control of pathogens, have increased the life-span of

some strains of animals. In spite of this, feeding studies adequately designed and evaluated extending up to eighteen months in mice and two years in rats are still considered adequate to ensure a minimum safeguard in evaluating the carcinogenic potential of a chemical additive. In special cases it may be desirable to prolong the observations in these species.

If there are good reasons to doubt the relevance to man of the data obtained in rodent species — for example, if the metabolism of the additive in man is found to be significantly different from that in rodents — it may be desirable to carry out investigations of longer duration in other species. The design and conduct of reproduction and teratogenic studies should take into account placental and mammary transmission. In addition, the investigation of some potential toxic effects, particularly carcinogenicity, requires careful prolonged observation of the offspring.^{1,2} Detailed study of general appearance and behaviour, biochemical effects, metabolism and histopathology should be included and fully reported, both qualitatively and quantitatively.

Further research in these important areas should be encouraged.

3.2.6 *Enzyme studies*

The study of enzymes in relation to pharmacological and toxicological action has developed considerably in recent years. Increases or decreases in enzyme levels caused by foreign chemicals may be studied either in the blood or in the tissues. It has become more and more apparent that, among the mechanisms of action of toxic substances, those of a biochemical nature are of prime importance. In this connexion, the basic enzyme systems are certainly among the first sites of action to merit careful study, since their inhibition often constitutes the causal biochemical lesion that determines, at least in part, the nature of toxic effects. It is enough to recall, among other classical examples, the inhibiting effect of cyanides and sulfides on cytochrome oxidases, of the fluoride anion on phosphoenolpyruvase, of fluoroacetates on aconitase and of novobiocin on glucuronyltransferase to realize the importance of this approach to toxicological evaluation. Enzyme inhibition may explain the toxic phenomena found in routine tests in laboratory animals or in observations in man. It may also provide a basis for forecasting toxic effects by indicating the first steps in the process.

The difficulties are to select the right enzymes for study and the most significant sites (body fluids, tissues, cells or subcellular fractions) for the measurement of changes in enzyme activity brought about by the

¹ Druckrey, H., Ivanković, S. & Preussmann, R. (1966) *Nature (Lond.)*, **210**, 1378.

² Druckrey, H., Ivanković, S. & Preussmann, R. (1967) *Naturwissenschaften* (in press).

substance under test. This is probably why, in the field of food additives, this approach has been so little used.

Enzymes in blood. The part of this subject relating to decreased levels of cholinesterases in the blood is dealt with elsewhere in this report (section 3.3.2).

An increase in the level of certain enzymes in the blood may be indicative of tissue damage; from this point of view, transaminases and other intracellular enzymes have been studied. When tissue damage occurs, these enzymes may leak out of the cells and cause a significant increase in blood enzyme levels. Such changes have been extensively studied in clinical biochemical laboratories in relation to myocardial damage following infarction and, in this instance, the time relationships between the occurrence of myocardial damage and alterations in the blood enzyme levels are important. Alterations in glutamic-pyruvic transaminase and glutamic-oxaloacetic transaminase have been observed to follow liver damage. Damage to a number of other organs has also been associated with various changes in blood enzyme levels.¹⁻⁴ Changes of this sort may be useful in indicating that tissue damage has occurred without the need to sacrifice the animal. However, if the damage is caused gradually, enzymes may be lost from the tissue cells without causing a demonstrable change in blood enzyme levels.

Enzymes in the tissues. Some food additives produce their effects by enzyme inhibition. Thus, in moulds, sorbic acid inhibits a number of enzymes, including catalase and alcohol dehydrogenase and its fungistatic properties are probably related to this effect. However, in the animal body, metabolic degradation makes it impossible to administer enough sorbic acid by mouth to cause significant inhibition of dehydrogenase systems.⁵ Study of the effect of food additives on enzymes should be encouraged.

Apart from enzyme inhibition, the level of tissue enzymes may also give some indication of toxicity. As already mentioned, when cells are damaged, intracellular enzymes may enter the blood. The level of these released enzymes in the blood depends on the relative rates at which the enzymes leave the cell and are inactivated or otherwise eliminated. The acuteness and severity of the damage and the timing of changes in relation to damage are significant factors in determining the blood level. Loss

¹ Chirskey, M., Wolff, R. J. & Sherry, S. (1957) *Amer. J. med. Sci.*, **233**, 400.

² Koide, H. & Oda, T. (1959) *Clin. chim. Acta*, **4**, 554.

³ Somerville, R. L., Fleisher, G. A. & Dearing, W. M. (1960) *Gastroenterology*, **39**, 574.

⁴ Wroblewski, F. & La Due, J. S. (1956) *Ann. intern. Med.*, **45**, 801.

⁵ Melnick, D., Luckmann, F. H. & Gooding, C. M. (1954) *Food Res.*, **19**, 44.

from the tissues will lead to a diminished enzyme concentration in the cell, unless regeneration keeps pace with the rate of loss. Thus, the cellular enzymes may indicate either acute or chronic damage and measurement of these levels might prove useful in the differentiation of toxic and toxicologically irrelevant changes in cells and organs.

Another aspect of enzyme changes of toxicological interest is the induction of so-called drug-metabolizing enzymes, especially in the liver. Some substances cause a considerable increase in many of these enzymes, which are found in the microsomal fraction of liver cells. This increase enables the animal to metabolize greater amounts of the substrate and other substances. The products of metabolism may be more or less toxic than the original substance. If the products are less toxic, the tolerance of the animal to the original substance may greatly increase. However, not all substances induce metabolizing enzymes, and some may take several weeks before causing significant induction. Examples of the former are parathion and isopropanol and of the latter are carbaryl and, to a lesser extent, methoxychlor and TDE.¹ These biochemical changes may or may not be related to ultrastructural changes in hepatic cells, they are often quickly reversible and may represent adaptation of the cell to the administered chemical.

Investigation of these phenomena as a part of the biochemical and metabolic studies may be expected to contribute significantly to the understanding of the inter-relationships between the additive and other chemicals in the environment.

3.2.7 *Special studies*

3.2.7.1 *Mutagenicity.* Mutagenic action of chemical agents represents a problem since, although exposure to chemicals in the external environment is increasing, there is little information on their possible mutagenic action. Attention has been drawn to the genetic effect of chemicals in connexion with radiation hazards.²

This problem cannot be ignored, since it represents one of the potential risks from chemical exposure. However, insofar as food additives are concerned, this possible risk must always be considered in the context of toxicological hazards in general, including the possible mutagenic effects of food themselves. The Group stresses the difficulties of extrapolating experimental data on mutagenicity of chemicals obtained in bacterial systems, yeasts, or *Drosophila* to possible hazards of food additives in man; these special procedures commonly used to detect mutagenic

¹ Stein, W. H., Serrone, D.M. & Coulston, F. (1965) *Toxicol. appl. Pharmacol.*, 7, 499.

² *Wld Hlth Org. techn. Rep. Ser.*, 1962, 248.

activity cannot be recommended as part of the routine investigation of a food additive.

Further research in this field is needed.

3.2.7.2 Teratogenicity. By teratogenicity is meant a toxic effect on the embryo or foetus resulting in a congenital abnormality. The Group feels at present unable to recommend a specific test for the detection of teratogens, but reproduction studies currently recommended as part of routine investigations of food additives should reveal some teratogens. It is recognized that improved tests are required to provide additional safeguards. However, further research is needed to design such tests. Since much research in teratology is under way, it is important that investigators concerned with food additives should keep in close touch with advancing knowledge in this field.

3.3 Interpretation of findings

3.3.1 Decrease in rate of body-weight gain

This effect may be brought about by many factors, such as an alteration of water intake, increased water loss, an alteration of food or caloric intake or faulty utilization of absorbed nutrients. These various effects may be due to a toxic action of the substance under investigation or to causes that are not relevant to the assessment of toxic potential, as, for example, diarrhoea due to an osmotic effect at high dosage levels of the test substance or interference with the palatability of the diet by the presence of the test substance. The effects that are irrelevant to toxicity should be differentiated from true toxic effects by appropriate studies. A decrease in the rate of body-weight gain, accompanied by a corresponding reduction of food intake, should not be assumed to be caused by a palatability defect, since the reduction of food intake may be due to toxic anorexia. If a palatability defect is present, this may be disclosed by a preference test in which the diets fed to the control and experimental groups are compared.¹

3.3.2 Inhibition of cholinesterases.

Cholinesterases in both plasma and erythrocytes are markedly reduced by a number of substances, including many organophosphorus compounds used as pesticides. There is, however, poor correlation between the cholinesterase levels and the signs and symptoms of toxicity. Blood cholinesterase levels may be useful as an indication of exposure to a sub-

¹ Sharratt, M., Frazer, A. C. & Cutler, M. (1964) *Methodological studies in the assessment of the acceptability of a food additive.* In : *Proceedings of the First International Congress of Food Science and Technology*, New York, Gordon & Breach, p. 583.

stance with anticholinesterase activity, but not as an invariable guide to the degree of intoxication present or predicted. In general, lack of correlation between the activity of a particular enzyme, or the level of a chemical or one of its metabolites, at some specified site (for example, in blood) and the occurrence of toxic signs or symptoms may be due to the fact that the more significant change in activity or concentration is occurring at some other site (for example, at nerve endings). Thus, the changes being measured may correlate with changes at the more significant site only over a small part of the range. Alternatively, some other enzyme, chemical or metabolite may be more closely related to the toxic mechanism. Although changes in blood cholinesterase levels may be helpful in toxicological studies, it is important that further research should be done to relate the indices used as closely as possible to the biochemical changes concerned in bringing about the toxic effects. In this context, special attention should be paid to the method of estimating cholinesterases.

3.3.3 *Liver enlargement*

The occurrence of enlargement of the liver in the absence of other apparent changes in this organ has often been reported in toxicological studies. Customarily, hepatomegaly has been considered to indicate a pathological change, and this interpretation has been applied in establishing "no-effect" levels. It is reasonable to believe that this alteration may not always represent a pathological change and in some instances may, on investigation, be revealed to be a normal response to an increased work load. This seemingly logical contention requires substantiation. It is recommended that a detailed investigation of liver enlargement in toxicity tests be carried out (as set out below), including a study not only of the absolute weight of the liver (measured under standard conditions) but also of the relationship of the weight of the liver to body-weight, provided that the growth and condition of the animals justifies the calculation of the relative liver weight on this basis. If this is not the case, for example because of emaciation, liver weight may be related to the weight of the heart or brain for purposes of comparison with control groups.

In studies on food additives it is usual to administer the substance under investigation at several dose levels; some at least of these are far in excess of those that are ever likely to be administered to man. Such high dose levels of a metabolizable chemical substance must inevitably increase the load on the liver, if this organ plays any part in its metabolism. It is known that under these circumstances the endoplasmic reticulum in the liver cells frequently proliferates, elaborating more enzymes and thereby facilitating the metabolism of the compound. It is likely, therefore, that liver enlargement will often be observed in animal studies on the biological effects of new substances proposed for use as food additives.

To evaluate fully the significance of such findings the following studies are recommended :

- (1) Liver morphology; ultrastructural studies.
- (2) Detailed investigation of the relationship to the dose and to the time of development of hepatomegaly during feeding experiments.
- (3) Reversibility of liver enlargement on continuing dosage and on cessation of administration of the compound.
- (4) Additional criteria of liver response and the relationship of these to dose and liver enlargement. Such criteria may include the activities of microsomal-processing (drug-metabolizing) enzymes in the liver and of glucose-6-phosphatase or other indicators of changes taking place within the liver in response to exposure to the test compound.¹

3.3.4 *Local sarcomas*

The significance of the occurrence of sarcomas following subcutaneous injection has been discussed in the fifth report of the Joint FAO/WHO Expert Committee on Food Additives.² In that report it was concluded that in certain experiments sarcomas were a result of the physical characteristics of the test material. Further research was recommended. As a result of developments since that time it is now recommended :

(a) that for the *routine* testing of food additives and contaminants, the subcutaneous injection test should be considered inappropriate unless special conditions, such as lack of absorption from the gastrointestinal tract under conditions of routine feeding to experimental animals, demand additional studies;

(b) that the occurrence of a local sarcoma following subcutaneous injection of food additives or contaminants should not, alone, be considered significant evidence of a carcinogenic hazard; such a finding, however, indicates the desirability of a thorough study for systemic manifestations of carcinogenicity by other parenteral or further specific oral investigations.

3.4 Decision and regulation

3.4.1 *Margin of safety*

Some margin of safety is necessary for the extrapolation of the maximum dietary level causing no effect in experimental animals to the acceptable

¹ Golberg, L. (1966) *Proc. Europ. Soc. Study Drug Tox.*, 7, 171.

² *Wld Hlth Org. techn. Rep. Ser.*, 1961, 220; *FAO Nutrition Meetings Report Series*, 1961, No. 29.

dietary intake in man. An arbitrary factor of 100 has been widely accepted and this figure was recommended by the Joint FAO/WHO Expert Committee on Food Additives in its second report.¹ In practice the margin of safety has varied from 10-fold to 500-fold, based on the scope and comprehensiveness of the data available. The following points are relevant to the establishment of this margin of safety :

(1) *The "no-effect" level.* There would appear to be a need to consider more closely what "no-effect" means. The obvious intention is that the maximum dietary level that causes no *deleterious* effect should be taken for extrapolation to give the acceptable dietary intake in man. It may be difficult, however, to know whether an effect observed is deleterious or not. For example, diarrhoea may be a toxic effect, or it may be due to the osmotic effect often associated with a high dose level of the test substance; decrease of weight gain may be due to toxic anorexia or to a loss of palatability of the diet. If effects of a physical nature (such as osmosis) or other effects (such as a palatability defect or stimulation of the metabolizing enzymes in the liver) resulting from a high dose level of the test substance, but unrelated to its toxic action, are included with toxic effects in selecting the "no-effect" level, it is reasonable to apply a lower margin of safety than that required for an unequivocal toxic effect.

In the absence of adequate evidence to the contrary, it should be assumed that any effect observed is a toxic effect. The onus for establishing that an effect observed is not a toxic one must rest on the investigator. Such features as reversibility and the differentiation of effects at lower dose levels may assist in distinguishing between physiological and pathological phenomena; research aimed at elucidating problems of this nature should be encouraged.

(2) *Variation of margin of safety.* It is not necessary to demand the rigid application of an arbitrary figure. The grounds upon which a different figure might be applied can be considered as follows :

(a) *Possible grounds for increasing the margin of safety*

The Joint FAO/WHO Expert Committee in its sixth report² provided a greater margin of safety for any country that did not have expert advice on food technology and food standards. What was considered as an unconditional acceptance zone was usually based on a safety margin of 100; in many instances a margin of 200 was used.

There may be other reasons for using a greater margin of safety; a case in point is an additive for which an adequate amount of toxicological

¹ *Wld Hlth Org. techn. Rep. Ser.*, 1958, 144; *FAO Nutrition Meetings Report Series*, 1958, No. 17.

² *Wld Hlth Org. techn. Rep. Ser.*, 1962, 228; *FAO Nutrition Meetings Report Series*, 1962, No. 31.

data is not available. The Group have taken cognizance of the situation and have recommended the establishment of temporary acceptable daily intakes (section 3.4.2). In these cases a larger margin of safety should be employed and all such temporary acceptable daily intakes should be considered as conditional.

Another example that may be cited is when the food additive is proposed for use in food items that show wide variations in daily intake, perhaps for climatic reasons, such as ice cream or soft drinks. Again, some foods may be particularly popular with children and this may be thought to justify an increase in the safety margin.

(b) Possible grounds for decreasing the margin of safety

The magnitude of the margin of safety to be applied is technically a factor of the adequacy of available toxicological data. If an intentional food additive is a beneficial constituent of the diet or is a normal body constituent, this may provide grounds for a lower safety margin. It would not be feasible to apply a 100-times safety margin to many common food additives, e.g., sodium chloride. There are also many substances, some of which may be used as food additives, that are known to be well tolerated by man at certain dose levels. The relevance of human data is discussed elsewhere in this report (section 3.2.2). Valid human data should take precedence over predictions arrived at by extrapolation from animal studies and may make it possible to apply a lower margin of safety.

When pertinent biological data (such as acute and long-term toxicity, biochemical reactions and histopathology) reveal a uniform species response, and when the most sensitive criterion of effect is clear-cut and the effect is reversible, then a materially reduced margin of safety can and should be applied to the "no-effect" level. Another example is the situation where the "no-effect" level of a product is based on cholinesterase inhibition or adaptive liver enlargement. In these cases the margin of safety may be reduced substantially below the usual 100-fold margin of safety, provided that the additional biological data are satisfactory. In no case, however, should this factor be employed to justify the use of amounts of the additive in excess of that required for the indicated purpose.

The margin of safety to be applied to the "no toxic effect" level in the process of extrapolation from animal data to human exposure is fundamental for deriving values such as the acceptable daily intake and tolerances. It is important, therefore, that all details of the animal data and probable exposure be carefully evaluated. Continued research in this area is to be encouraged. Exploratory research in additional animal species, new techniques and new biological systems may yield data unique in character that are of research value but that should not necessarily

be used to determine the "no toxic effect" level. Only when such data become recognized as significant should they be prime factors of evaluation.

It may be concluded that the 100-fold margin of safety is a useful general guide; it should not be applied rigidly. If, however, a different margin of safety is used, the basis for changing the 100-fold margin should conform to the principles outlined above.

3.4.2 *Establishment of temporary acceptable daily intakes*

The Group approves in principle the establishment of temporary ADIs for those food additives that would be useful and those that are already in use but for which data may not be fully adequate by current standards. It is recommended that such temporary ADIs be used as a basis for the establishment of temporary tolerances only if the following specific conditions are rigidly adhered to :

- (1) Each chemical additive must be considered on its merits.
- (2) The temporary ADI must be established only for a specific and definite period, namely, 3-5 years.
- (3) In setting a temporary ADI, the additional biochemical and toxicological data required for the eventual establishment of an ADI must be clearly stated. The additional requirements must be justified as being essential for the protection of the consumer.
- (4) A review of the original and new data must be carried out before the expiration of the provisional period.

3.4.3 *Establishment of administrative acceptable daily intakes for pesticide residues*

The Group discussed various classes of food additives and concluded that in the case of some pesticides it may be advisable to allocate an "administrative ADI". This figure is intended to enable those concerned to establish a finite tolerance or "negligible level" in each case. The following factors especially should be taken into consideration :

- (1) The nature of the substance.
- (2) The magnitude of the residue and the availability of suitable analytical methods for control purposes.
- (3) The adequacy of toxicological and biochemical data for the purpose of establishing an administrative ADI.
- (4) The absence of specific justification requiring additional data.

4. SUMMARY AND CONCLUSIONS

Specifications

Adequate specifications for identity and purity should be available before toxicological work is undertaken. They should be broad enough to include all the variations in composition that do not significantly affect the biological properties of the additives and they should include methods for the determination of toxicologically significant impurities; tests for "good manufacturing practice" should be maintained.

Test animals

Data from the most appropriate, rather than the most sensitive, species should take precedence for toxicological evaluation.

Investigation in human subjects

It is desirable and in many cases necessary to study the metabolic fate and effects of food additives in man. Such investigations, which should be carefully planned and controlled, form a valuable part of the evaluation of safety.

Effect of age on toxicity

In general, the young animal is more sensitive to the toxic effects of exposure to chemicals, but this is not invariably so. Useful information may be obtained from studies in newborn or young animals, from reproduction studies and from biochemical studies.

Effect of nutritional state on toxicity

Because of the complexity of the relationships between nutritional state and toxicity, at present the evaluation of safety is best carried out by using healthy animals on adequate, balanced diets.

Duration of toxicity tests in experimental animals

Flexibility of approach is essential in deciding the duration of tests necessary to establish that a compound is safe. In certain circumstances, tests carried out over a few months may suffice for the purpose of detecting any deviation from the established pattern in a group of closely related chemicals.

Enzyme studies

The effects of food additives on basic enzyme systems, as a part of biochemical and metabolic studies, may be expected to contribute signi-

ificantly to the understanding of the inter-relationships between the additive and other chemicals in the environment.

Mutagenicity

At present no specific tests can be recommended for the assessment of mutagenic risk, but some safeguard is provided by multigeneration studies.

Teratogenicity

At present no specific tests can be recommended for the detection of teratogens, but some safeguard is provided by multigeneration studies.

Decrease in rate of body-weight gain

The observation of decreased rate of body-weight gain requires further study to differentiate between toxicologically relevant and irrelevant effects. A preference test may distinguish between palatability defect and toxic anorexia.

Inhibition of cholinesterases

Blood cholinesterase levels may be useful as an indication of exposure to a substance with anticholinesterase activity, but not as an invariable guide to the degree of intoxication.

Liver enlargement

Some additional studies are recommended in order to evaluate the significance of liver enlargement.

Local sarcomas

The significance of the occurrence of local sarcomas following subcutaneous injection is discussed in section 3.3.4.

Margin of safety

The factor of 100 is generally considered as a suitable margin of safety, but under certain conditions it may be increased or decreased.

Establishment of temporary acceptable daily intakes

Provided that a number of conditions are fulfilled, temporary acceptable daily intakes may be established for food additives.

Establishment of administrative acceptable daily intakes for pesticide residues

Provided that a number of factors are taken into consideration, administrative acceptable daily intakes may be established for pesticide residues.

Further studies

The Group considered that further research is especially needed on the following questions :

1. The development of enzyme systems in the human young, with particular emphasis on those enzymes responsible for dealing with foreign chemicals. This information is essential in assessing the safety of additives in baby food.
2. The effects of various states of malnutrition or undernutrition on the toxicity of food additives. Knowledge in this field is useful in selecting additives in countries in which malnutrition is widespread.
3. The duration of long-term toxicity tests in non-rodent species. Although there is general agreement on the duration of toxicity tests in rodents, further research is needed to define the appropriate duration of such tests in non-rodent species.
4. Inter-relationship between food additives and other chemicals in the environment. Information on this matter is important, since the toxicity of a food additive may be enhanced or diminished by other chemicals in the environment.
5. The mutagenic hazard of food additives. More work on this problem is needed to permit the design of more meaningful tests for assessing the potential risks of mutagenic changes due to food additives.
6. The teratogenic hazard of food additives. More knowledge is needed to improve the reproduction studies generally adopted at the present, and to design other more appropriate tests.
7. The relative physiological significance of cholinesterase inhibition at different sites in the organism. Information on this subject is useful in assessing the relative toxicity of compounds with anticholinesterase activity.
8. Criteria to be used in distinguishing biological effects that are toxicologically significant from those that are not. Such criteria are needed in the proper planning of toxicological investigation.
9. Exploratory research in the use of additional animal species, the development of new techniques and the modification of biological systems, with a view to improving the predictive value of the experimental results obtained in animals.

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