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Seminar on Protein Problems with
Particular Reference to Weaning Foods
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PAG GUIDELINE FOR HUMAN TESTING OF SUPPLEMENTARY FOOD MIXTURES[a]

(Incorporating the modifications suggested during the WHO Consultation on human testing of protein-rich foods, Geneva, 9-11 March 1970)

1. Applicability of the guideline

Tests for safety and suitability for human consumption, especially for feeding of infants and children, are essential in the development of protein-rich foods. When commonly used foods are newly processed to supply protein in a food mixture or when materials, not so far used as human food, are to be used as protein sources in a new food product, it is essential that certain preliminary steps be taken before human testing of the product. Some of these steps are outlined in PAG Guideline No. 6,[2] for pre-clinical testing of novel sources of protein.

Some preliminary steps are:

(a) Identification of the source of edible protein, the quantity available, and economic study of its development.

(b) Chemical evaluation of the quantity and quality of protein, if not already known, in each of the component foodstuffs from which protein-rich food mixtures are to be made

(c) Determination of the proportions of various components in the proposed mixture based on considerations of nutritional or other relevant factors. An evaluation of the probable price of the final product would be desirable at this stage, taking into account the costs of raw materials, processing, necessary packaging, storage conditions, shelf-life, commercialization, normal profits and all other elements which enter into the operation

(d) Measurements chemically and biologically, of the nutritive value of the mixture and evaluation of damage to protein or loss of nutrients available as a result of processing which may be necessary for industrial production, or consumption

(e) Assurance that it is free of harmful micro-organisms

(f) Tests indicating freedom from toxicity of the product and its components. Such toxicity may be due to the presence of intentional additives, of toxic substances naturally occurring or arising from mould infestation, or through the use of pesticides and fungicides. Qualitative and quantitative tests for determination of these compounds may be necessary, as well as animal tests for determination of acute and sub-acute toxicity The rules specified by the Food and Drug Administration, Washington, D C , United States of America, for acceptance of additives to common foods provide useful guidelines.[3] These include among others, full acute and chronic toxicity trials.

It is only when these steps have been satisfactorily accomplished that human tests should be considered. It is imperative, therefore, that all the requirements mentioned above be fully satisfied before planning an undertaking which will involve human subjects.

While there is no question as to the need for the clinical testing of really new sources of protein or of the consequences of new ways of processing protein concentrates, there is real danger that excessive and unnecessary testing of minor variations in the formulae using previously tested ingredients or processes could needlessly hamper progress in this field. It is therefore suggested that:

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[a] For the document issued by the PAG in 1966 on the same question see reference No. 1 at the end of the annex

Annex

(i) Proteins and processed protein concentrates previously not considered in the WHO/FAO/UNICEF programmes, or products previously considered but manufactured by new processes or by major changes in established processes, especially if these changes in processing raise any suspicions as to nutritional or toxicological properties of the product, must pass testing procedures of the type recommended in the proceedings of the Princeton Conference on Human protein requirements and their fulfillment in practice.[4]

(ii) Mixtures of well-known staples and protein sources which have already received favourable consideration should be accepted without insistence on clinical testing beyond acceptability and tolerance trials if there is no further processing which could cast a doubt on their safety. It would be advisable, however, to ascertain by animal experimentation, the nutritional value of the final product (PER or NPU).

(iii) In case of severe or unconventional processing of mixtures even of well-known staples and protein sources the products should be accepted for tests in man only after they have satisfied the necessary laboratory analysis and animal testing for protein quality. Clinical testing, although not mandatory in this case, may be helpful in ascertaining the value of some of these food mixtures in children's supplementary feeding.

Understanding of the technological steps involved in the processing will help to decide in which of these categories a food mixture should be classified.

2. Categories of tests

Human testing, as these observations will be termed, will fall into four main categories as follows.

(1) acceptability and tolerance tests,

(2) growth tests,

(3) nitrogen balance measurements,

(4) other criteria.

The actual type of tests to be carried out will be determined by considerations mentioned below. Careful clinical observations will, of course, be concurrent in all studies. One prerequisite is common to all, and that is full satisfactory information on items (a) to (e) above.

2.1 Preliminary acceptability and tolerance tests

It is possible that the foodstuffs from which a protein-rich food mixture is made have been individually in use as human food in one or more parts of the world. Processing of mixtures on an industrial scale, however, may affect not only their suitability for the groups for which they are intended, but also their palatability and acceptability Therefore, it may be necessary to determine the tolerance to the dosage level recommended for contributing significantly to protein needs. Under such conditions, "acceptability and tolerance" tests are indicated These tests should be carried out in an institution or in a closely observed sample of the population. If it is decided to have several such tests for a given product, at least one of them should take place in the country in which it is intended to introduce the protein-rich food mixture.

A danger to be avoided is that persons evaluating the food supplement may be swayed by their own "acceptability" criteria to influence the reaction of the recipient to the detriment of useful supplements Even young infants sense the emotional reaction of the mother or of other persons feeding them and may respond psychically by rejecting the food.

Annex

Mild disease processes, particularly infections, tend to reduce appetite and produce mild to moderate gastro-intestinal upsets, which could be interpreted as poor tolerance and acceptability. Because of these factors, it is suggested that a simultaneous negative control be run in these trials. Sequential periods with and without the test food may also be useful The total volume fed, the timing of the meals and the total intake should be kept consistent. If possible, test and control groups should be of similar age as well as have similar weights for their heights.

2.1.1 Number

Depending on the consistency of the results the number will vary. In any case, it is suggested that no less than 20, and preferably closer to 30, individuals be tested.

2.1 2 Age

The sample tested should consist of individuals of the age or ages for which the product is intended

2.1.3 Duration of feeding and observation

Occasionally upon the introduction of a new type of food a short period of apparent intolerance may occur, which is overcome in two or three days. If the diet is going to be unacceptable because of a tiring or boring taste, this is generally noted within the first weeks of the feeding trials It is therefore suggested that a period of at least four weeks be used before the clinical evaluation of protein quality or more extended tolerance tests are planned

2.1 4 Method of preparation

This should be either in the form of a suitably flavoured gruel or incorporated into a local recipe.

2.1 5 Level of protein feeding

That needed to supplement the diet to the levels recommended by the FAO/WHO report on protein requirements,[5] or preferably to cover more than 50 per cent. of the requirements of 97 per cent. of the population tested. An additional group fed the material ad libitum will provide information on maximum quantities acceptable per meal in the form supplied.

2.1.6 Level of caloric intake

Should be sufficient to maintain constant weight in adults or adequate weight gain in children.[6]

2.1.7 Observations to be made

Children should be left to feed themselves or should be helped by an attendant, but in this case care should be taken not to force the food on the child.

Refusal to eat the preparation is considered an indicator of poor palatability, provided that the trials take into careful consideration all of the potential interfering factors in this type of study. Even if acceptability is unsatisfactory as first tested, it may be possible to find an acceptable form of preparation by trial and error. This should be such as is practicable in homes and under conditions for which the food mixture is recommended.

Tolerance is judged by noting persistent gastro-intestinal upsets, such as loss of appetite, flatulence, vomiting, particularly delayed vomiting, undigested stool contents, diarrhoea or intestinal hurry. Where legumes are involved, it is important to note the extent of bloating and flatus production.

Annex

Other clinical manifestations such as allergic reactions should also be recorded if manifested.

Large-scale, three to six months, acceptability and marketing trials in selected future consumer groups should be started as soon as possible after this step Careful consideration should be given to the statistical design and evaluation of these trials.

When protein-rich foods are introduced to the family, observations should include the reaction of mothers to the products. It should be explained to them that although the food will contribute to the nutrition of all the members of the family, it has been prepared in a form which is of special value for the health and development of infants and young children

2.2 Growth tests

The principal methods used for the evaluation of protein quality in man are measurement of growth and of nitrogen balance. These represent alternate approaches, which will be selected depends upon many factors, such as the type of subject to be studied, the local conditions, the facilities and personnel available It is desirable but not essential that [illegible]y be done in the country of intended use. Complementary information may also be obtained by various types of measurements of blood chemistry and liver function. Both growth tests and nitrogen balance techniques should be carried out only in centres specialized in nutrition or allied disciplines. The use of tolerance and acceptability experiments to evaluate the effect on growth should be discouraged.

2.2.1 Growth

Trials in which growth is measured may be planned in many ways, depending upon the nature and age of the subjects to be studied, and the circumstances in each case. Therefore, only very broad guidelines can be laid down. Every trial should be planned on a sound statistical basis, so that the results can be accurately evaluated. Ideally, a closely matched control group should be studied.

2.2 2 Observations which may be made

The most commonly used index of growth is the rate of gain in body weight. Evaluation of protein value from the change in weight over a specified period on a given protein intake is analogous to the measurement of PER in animals, and is subject to the same criticisms, [illegible]t weight gain may not reflect accurately the change in lean body mass This difficulty [illegible] be circumvented in part by measurement of the urinary creatinine output over a timed period, since it is accepted that this provides an index of muscle mass Measurement of height or body length is of even greater significance, particularly in older children, because height is usually less variable than weight. However, since height increases more slowly, the measurements have to be made over a fairly long period. Supplementary information can be obtained by serial X-ray films of bone maturation and cortical thickness. The investigator should not neglect general observations such as the character of stools, amount of flatus, occurrence of allergic and other undesirable responses and general acceptability to mother and child.

2.2.3 Age and type of subjects

Since growth is faster and protein requirements are higher, the earlier the age, the greater are the advantages in using infants and young children, rather than older children, for measurements of protein quality. The children should be as normal as possible. Each investigator should determine the populations suitable for growth studies, but it is recommended that children who are frankly retarded in growth should not be studied because of great variabilities in responses. It is suggested that all children should be above the third percentile in height and should have weight for height above 95 per cent. of ideal, based on standards for well-nourished children such as those which have been published, inter alia, in Western Europe and the United States of America.

In testing a new protein, it is important to find out which is the lower age at which the protein supports adequate growth Consequently the age will vary, depending on the results obtained from the initial tests. Weaning foods should be tried first in children six months to one year. Special infant foods should be tested in younger infants.

2 2.4 Duration

The duration of the trial must depend upon the extent to which constant conditions can be achieved. With infants aged about one year, under close supervision in a hospital ward, a reasonably accurate measurement of growth rate can be obtained by daily weighing over a period of three to four weeks With somewhat older children, again under well-controlled conditions, e g in an orphanage, three to six months are necessary. Day care centres, orphanages and convalescent hospitals for children are likely to be convenient for such studies. It has been observed that children do not gain appreciable weight during the hottest months of the year, when temperatures reach 38°-40°C, so that short-term trials in such an environment should be avoided during these months. Similarly, epidemics of any infectious disease are likely to invalidate trials

2.2.5 Number of subjects

This will depend on the age and co-operativeness of the subjects, the duration of the trial, the extent to which infections and other interfering factors can be eliminated, and the adequacy of the controls. Valuable information may be obtained from as few as five infants per group in a well-controlled study in an institution.

2.2.6 Frequency of observations

In general, the greater the number of serial observations, the fewer the subjects required, and the shorter the necessary duration of the trial In studies on infants under hospital conditions, weights should be measured every day. In older children, weights should be measured at least every one to two weeks In infants length should be measured bi-weekly, but in older children height measurements at intervals of one to three months will be enough.[7]

2.2.7 Level of feeding

The trial will not be a true test of protein value if other elements in the diet are limiting. The diet must therefore supply adequate intake of calories (from fat and carbohydrates), and of vitamins and minerals

The total protein intake should at least conform to the recommended allowances of FAO/WHO.[5] The extent to which it may be higher than this depends upon the purposes of the trial - whether it is to determine the effect upon growth of a given protein supplement, or whether it is to find the minimum amount of a protein mixture which will support normal growth.

In general, the test protein should be the sole source of protein in the diet, for some special purposes it may be provided as a supplement to a natural diet. The control group should receive milk or egg as the source of protein with levels of protein and calories adjusted to be comparable.

2.3 Nitrogen balance measurements

The measurement of nitrogen balance in man is comparable to that of NPU in experimental animals.

Annex

2 3.1 Conditions

Staff and facilities must be adequate for the precise control of food intake, minimizing of cross-infections, complete collection of urine and faeces, and the necessary biochemical analyses. Experienced full-time personnel dedicated to the work are required for preparing and weighing the diets and giving close and continuous supervision to the subjects. Because of the constant and monotonous diets, special skill is needed to ensure that the intake is maintained throughout the period of observation.

2 3.2 Subjects

Measurement of N balance may be made on adults, children or infants. There are advantages in using as test subjects infants who are fully recovered from malnutrition, mainly because of their being accustomed to metabolic techniques Acutely and severely depleted subjects, as well as children recovering from malnutrition, are not suitable for such studies because another variable is introduced which complicates the interpretation of the results. It is also extremely important that the subjects have no infection. Even mild infections induce a stress response which increases urinary nitrogen loss

Most malnourished infants are not likely to reach a stage at which the tests can usefully be done until they have received optimum treatment for one to two months. Complete nutritional recovery must be estimated not only by normal weight for height and serum and blood biochemistries but also by adequate lean body mass for height, (refer to 2 4 3). It is difficult to specify an exact age range for such tests. Children six to thirty-six months are convenient subjects, but this does not exclude children outside this age range. In any case it is essential that the groups be carefully matched. This is necessary because with recovery from depletion nitrogen retention tends to fall The best plan is to use each subject as his own control, with consecutive tests on control and experimental diets. Because the subjects may vary in degree of depletion as the tests go on, the order of feeding should be varied.

2.3 3 Food

2.3.3 1 Calories, water, vitamins and minerals

The calories supplied must be equal in all balance periods (test and control) which are being compared, and should meet the level recommended by FAO/WHO [6] The proportion of fat to carbohydrate and the nature of the fat must be similar in groups which are being compared. Water, vitamins and minerals, including potassium and phosphorus, should also be fed in adequate constant amounts

2 3 3 2 Protein level

For tests of protein value the protein must be fed at a level or levels on the linear part of the curve of the nitrogen balance index.[5,8] This curve remains linear for some way into the region of positive nitrogen balance. In tests on human infants, it is undesirable to feed at maintenance level only. It is necessary to choose a level above maintenance that is enough to allow reasonable nitrogen retention and growth, but not so high that the efficiency of nitrogen utilization falls off so much that differences in biological value disappear. It has been shown[9] that in infants of about one year the average requirement for maintenance is 100 mg N/kg/day (in terms of cow's milk protein), and that almost all balances are positive at an intake of 130 mg N/kg/day. From the evidence available it seems that the total obligatory loss of urine and faeces per kg of body weight is only slightly higher in infants than in adults. It is recommended that for measurements of protein value the intake should not exceed an upper level of 300 mg N (i.e. slightly less than 2 g protein) per kg per day. If the clinical condition of the child justifies feeding at lower levels, e.g. 1-1.5 g protein/kg/day, difference in protein value will be shown still more clearly.

Annex

A test at one level within the limits just specified is adequate if the sole concern is the measurement of protein quality. However, a further practical question may arise: can a food which has a poor protein value produce adequate retention and growth if fed at a higher level, e.g. 3 g protein/kg? To answer this question, tests must obviously be made at whatever level is indicated by the measurement of biological value, but it should be clearly recognized that tests at such high levels are not reliable measures of protein quality.

2 3.4 Adaptation period

The number of days required for initial adaptation depends upon the age of the child and the magnitude of the change in quantity and quality of protein from that of the preceding diet. In infants a three-day adaptation period is generally sufficient, but in older children and adults five days or more may be needed The individual investigator should provide evidence that the adaptation period used under his conditions is adequate Subjects recovering from an infection may need one to two weeks before the nitrogen excretion is stabilized

2 3.5 Length of balance period

Collections should be obtained for a minimum of six days. Two three-day periods or, if defaecations are sufficiently frequent, three two-day periods represent a minimum study Where circumstances permit, balances can be conducted over a period of two to three months in such a way that six to nine balance periods of six days each may be obtained in a single child.

2.3.6 Digestibility

In boys, separate collection of urine and faeces will make possible measurement of apparent digestibility as well as of apparent biological value With many vegetable proteins digestibility is low, and the measurement of it is therefore important. In girls, even if faeces and urine cannot be adequately separated, it is still possible to measure the net protein utilization (NPU).

It is not generally necessary to measure the basal or "endogenous" urinary and faecal nitrogen loss, in order to estimate the true digestibility and biological value. For practical purposes it is probably accurate enough to use the figures published.[10] It is important, however, that the calculated N intake be verified by actual analyses of aliquots, since values from food composition tables do not give a sufficiently reliable estimate for the purpose.

2.3 7 Necessary precautions

In summary, nitrogen balance measurements will be useful, reliable and reproducible if the following precautions are observed.

(1) Calorie intake per kg is adequate and constant.

(2) Protein intake per kg is kept constant within a single trial.

(3) There are no complicating vitamin or mineral deficiencies.

(4) Water intake is controlled and excessive sweat loss avoided.

(5) No infections, even of seemingly mild degree, are present.

(6) Subjects are reasonably content and not psychologically disturbed

Subjects are in an adequate state of nutrition and receiving protein levels which
determination of protein quality.

Adequate adjustment periods are used

Meal times are standardized both between and within treatments.

Period(s) on the same diet are long enough to determine trends as well as the initial
to dietary change.

Food intake and collections of urine and faeces are obtained and measured accurately.

Other criteria

Serum albumin

In children recovering from malnutrition, changes in albumin concentration may give a
indication of protein quality. Measurements should be made periodically and the blood
should be taken at the same time in relation to meals. Standardization of laboratory
is desirable. Divergence of results for serum albumin and for growth and nitrogen
have been reported. In some cases this appears to be due to a rapid increase in
mass and blood volume concealing active albumin synthesis[11] unless total circulating
is measured Serum albumin also apparently behaves differently from other parameters
se to vegetable as compared to animal protein and is slow to decrease on some experi-
ots with low PER The significance of this difference requires further study.

Plasma amino acid and enzyme levels

Measurements of plasma amino acid levels and ratios, and of the concentrations of various
in the plasma have been proposed as useful criteria. These methods still require
evaluation.

Creatinine height index

It has recently been demonstrated that 24-hour creatinine excretion of a malnourished
child with that of a well-nourished child of the same height is a good measure of
tion of lean body mass due to malnutrition and its recovery with re-feeding [12]
used to evaluate the degree of recovery of lean body mass.

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