LIMITATIONS OF METABOLIC STUDIES

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INTRODUCTION

Metabolic studies are those related to the processes by which living cells or tissues undergo chemical change. In the context of nutrition, "metabolic studies" refer to the processes by which proteins, fats, carbohydrates, minerals, vitamins and water, of either exogenous or endogenous origin, participate in the chemical changes of an organism. In a narrower sense, and mainly for operational purposes, nutritional metabolic studies are usually defined as those that take place within the confines of a so-called *metabolic ward,* or those that define the fate of a nutrient ingested by a man or an animal. A variety of the latter are the *metabolic balance studies,* which measure and *compare* the amount of a nutrient that enters the body—usually by the gastrointestinal tract—and the amount that is excreted by that body.

This paper gives an overview of the characteristics of a metabolic ward and of various nutritional metabolic studies in humans. Emphasis is made on their applications, pitfalls and limitations.

The Metabolic Ward in Nutritional Studies

One of the major applications of a metabolic ward is research related to dietary studies and the consequences of eating —or of not eating— certain foods or nutrients. In contrast to experiments with isolated cells or subcellular fractions, man responds simultaneously to many factors in his environment, such as diet, climate, physical surroundings and emotional stimuli. These multifactorial responses are further influenced by circumstancial conditions such as exercise, feeding patterns, sleeping habits and behavioral changes. A meta-

ISBN 0-12-715950-9

bolic ward provides a suitable environment to control many of those factors and circumstances, thereby limiting the experimental variables to those of interest for a specific investigation. This allows a better evaluation and interpretation of the results.

Hodges (11) cited the work of James Lind in 1747 (16) as one of the earliest descriptions of a controlled dietary study, done in an environment that preceded by more than a century the establishment of metabolic wards. Twelve sailors on board of the British ship, "Salisbury", developed scurvy with "putrid gums, spots, lassitude and weakness of their knees". They were placed in an apartment for the sick in the ship's forehold, and received the same diet: water-gruel sweetened with sugar in the morning, fresh mutton broth, puddings and boiled biscuits for dinner, and for supper barley and raisins, rice and currents or sago and wine. Six different treatments were given to each of 2 patients: a quart of cider a day; 25 drops of "elixer vitriol" three times a day; two spoonfuls of vinegar three times a day; half a pint of sea water every day; two oranges and one lemon every day; or a purgative made of garlic, mustard-seed, raphon, balsam of Peru and gum myrrh, three times a day. The "most sudden and visible good effects" were perceived from the use of the oranges and lemons; one of the sailors who had taken them was fit for duty at the end of six days, and the other, who was the best recovered, was appointed nurse to the rest of the sick.

Facilities gradually developed in various hospitals and they evolved in this century from relatively simple research wards [e.g., 2] to elaborate, modern metabolic units. But these present-day metabolic wards, with all their sophisticated equipment and computerized facilities, still require the essential elements included in Lind's work aboard the "Salisbury", in order to conduct adequate research: a hypothesis to be tested, a group of individuals willing to participate as experimental subjects, a controlled environment, a uniform diet, well defined variable treatments, and an objetive evaluation of the results. Another essential requirement is a well-trained, highly-motivated staff. A metabolic ward is designed to standardize procedures, reduce the possibility of errors and keep a detailed record of the events that might influence the experimental subjects. All these require a strict discipline of the attending staff.

The metabolic ward must have adequate facilities to house the patients or healthy subjects, deal with therapeutic or emergency conditions, provide indoor-and outdoor-recreation and leisure activities, prepare and serve the diets, perform the experimental tests, handle and store biological specimens, and process or relay the specimens to the laboratory.

The attending staff must be well-trained, conscious about the importance of their work, highly motivated in its performance, tolerant and absolutely honest. They must be aware of the importance to establish a good rapport with the experimental subjects, to record all events meticulously, since even trivial details might later be significant, and to admit and report errors and departures from the experimental protocol.

All studies involving humans as experimental subjects, must be critically assessed by a committee of persons who are not related to the investigation, in order to protect the subjects, establish the safety or potential risk of the procedures and decide upon the relevance of the research in relation to the discomfort of the subjects. Guidelines for such committees, including the considerations of studies on children and mentally incapacitated persons, have been published.

It is essential that the persons who act as experimental subjects are willing to cooperate: an uncooperative subject can ruin any project, either by intent or neglect. These persons can be either patients who have a disease or condition of particular interest, or healthy volunteers. They should understand the purpose of the investigation, the nature of the procedures and the importance of the results. They should have a feeling of participation and must understand that their role in the project is of utmost importance. Children must be kept comfortable and happy at all times; whenever possible, the experimental procedures should be dealt as games and provide a pleasant experience.

Advantages of the Metabolic Ward

The main advantages are the collection of carefully controlled information and the reduction of interfering variables. The detailed and careful recording of all observations, whether part of the experimental protocol or due to unexpected circumstances, may give results of great importance in the short-and long-term. Important, and sometimes serendipitous, discoveries can result from well-kept records. For example, the occurrence of infections during the investigation of nutritional requirements can provide useful information about metabolic alterations of a nutrient during the disease, or about the change in requirements during and after the disease.

Limitations of Metabolic Ward Studies

One of the main limitations is the small number of subjects in most studies, usually due to high costs or to a scarcity of appropriate subjects. This can be compensated by the high precission, accurancy and number of metabolic measurements: we can sometimes learn as much from a large number of detailed observations obtained in a small number of persons, as from a small number of less rigorous observations in a large number of persons. But if the inter-individual variability is too large, it may not be possible to draw definite conclusions from a small number of subjects. Nevertheless, the results may suggest phenomena that can be tested or confirmed out of the metabolic ward with a larger number of individuals.

Another limitation may be the artificial conditions of the metabolic ward, as compared with the so-called "field" or "real life" environment. Before generalizing the conclusions drawn from a study, one must consider the characteristics within the metabolic ward that often contrast with those of the outside world, such as: 1) the hygienic environment with low infectious morbidity; the rigid scheduling of activities such as arising, eating meals, engaging in exerci and going to bed; 3) the homogeneity and monotony of the diet, sometimes eat in the same amounts day after day and divided into identical servings; and, 4) t confinement to the metabolic ward facilities. These limitations may be of little ir portance for studies such as the evaluation of a specific treatment for bed-ridd patients, but they may be of great importance for other investigations, such as t establishment of certain nutritional requirements for a population. In the latt case, some crucial field conditions may be mimicked in the metabolic ward l a programmed modification of schedules, provision of varied diets that can freely chosen by the subjects, and physical activities similar or equivalent those of the general population, including supervised walks and out-of-doo games. All these conditions must be under strict control of the investigato and quantitatively measured. This can be done, [e.g., 22], although it comp cates the design of the experimental protocol and taxes the efforts of the inve tigators and attending staff. However, some field conditions, such as po environmental hygiene or high risks of infection, cannot be mimicked for o vious ethical reasons and others cannot be technically reproduced.

The duration of studies that require longitudinal observations can also be limiting factor in the interpretation and generalization of the results. Lor term investigations are expensive and it is often difficult to find healthy sul jects who are willing to live in the metabolic ward for extended periods of tim Some researchers have succeeded in recruiting highly motivated individua who otherwise would be confined to prisons or orphanages. They must be prov ded with an environment at least as good as that of the institution from where th come, and there must be strict adherence to their human rights and privacy.

Metabolic Balance Studies

The name of these studies derives from the maxim that "input minus outp equals balance". They compare the amounts of a nutrient that enters the hu man body with the amounts of the nutrient or its metabolites that are excrete This can be expressed by the equation $B = I - E$, where B is balance, I is intal and E is excreta.

The usefulness and uncertainties of metabolic balance studies have been objec of discussions for many years, strongly expressed by critics and supporters alil [3, 9, 11, 12, 25], but are still the keystone of many nutritional investigations. D pending on the cooperation and reliability of the experimental subjects and on t nature and duration of the investigations, metabolic balance studies can be done o an inpatient or outpatient basis, although it is preferable to do them in a metabol ward. Subjects who are not confined to the ward can eat their meals there or ca perform the whole study in their home environment. However, the lack of superv sion may lead to errors, unless the experimental subject is highly motivated, unde stands perfectly the procedure and the investigation, and is honest and reliabl

Requirements and Sources of Error in Metabolic Balance Studies

Accurate balance calculations require precise weight or volume measurements of foods and body excreta, accurate timing of the beginning and end of the measurements, adequate homogenization and aliquoting of representative samples for analysis, and detailed recording of the conditions surrounding the experimental subjects. Most balance studies also require attaining a steady state, specially after introducing dietary changes.

The intake portion of the balance equation usually refers to food, but the ingestion, and parenteral administrations of drugs and other substances are also part of the intake. Foods must be prepared in a metabolic kitchen under standardized conditions, using sensitive and accurate weighing instruments and, whenever possible, the same batch of raw materials. When the nutrient being assayed is a trace mineral, special attention must be payed to the water and the utensils used to prepare and feed the meals; for example, tap water contains variable amounts of zinc and ironware can give off relatively large amounts of iron when cooking or storage conditions are favorable. Therefore, the water source and utensils must be standardized and, if necessary, the chemical contribution made to the foods must be assayed frequently.

Liquid formulas allow a better quantitation of intake, but they are less well accepted by older children and adults and they may result in a gastric emptying pattern different from that with solid foods, which might influence the digestion and absorption of certain nutrients. Each food component of a meal must be served in a separate container and the exact amount eaten by the subject must be assessed by differential weighing of the container before and after the meal. Children must be helped to eat, as needed. Losses through spillage or spitting must be determined by weighing tared clothes used to pick the spillage. Young children should wear tared clothes and bedding.

It is more accurate to weigh than to measure the urine voided in a large sized volumetric container; the exact volume can be calculated from the specific gravity. The beginning and end of the collection period should be timed after voluntary emptying of the bladder or, in small children, after spontaneous voiding. If the period is near 24 hours, mathematical corrections may be used to calculate the exact 24-hour output.

The deviation of fecal collection periods is usually determined by the appearance of a non-absorbable colored marker fed at the beginning and end of the period. The timing, however, may not be accurate in individuals with constipation or irregular bowel movements. Most investigators recommend collection periods of at least 7 days in adults to minimize the errors. Three or four days may be adequate for children who defecate more than once each day. A better, but more complex timing can be done by measuring the amount recovered in feces of a non-absorbable marker fed throughout the collection period in a constant proportion to the nutrient under assay. The marker must not interfere with gastrointestinal motility or chemical processes, and must progress along the gastrointestinal tract at the same rate as the nutrient being assayed.

Collections are more difficult in children, specially girls, who do not yet control urination and defecation. They are frequently confined to metabolic beds with collecting systems. The limited physical activity influences bowel movements and, if long enough, may influence the urinary excretion of some substances such as calcium. Consequently, inactivity must be reduced to a minimun, stimulating the children to move as much as possible in the metabolic bed, and allowing them to walk wearing self-adhesive urine collection bags after they defecate.

Vomitus is part of the excreta. Its weight can be quantified in a similar fashion as food spillage. Whenever possible, vomitus must be chemically analyzed to determine the exact amount of lost nutrient. If not, its nutrient concentration must be estimated from the food contents and the time of vomiting, relative to food intake. At best, this is an approximation and frequent or voluminous vomiting may invalidate the balance study.

Milk secretions must also be considered as "excreta" in lactating women.

Other losses of body constituents include visible and insensible sweat, desquamated skin, sputum, menstural bleeding, clinical blood sampling, etcetera. The role of these miscellaneous losses in balance studies was discussed by Calloway and coworkers [6]. They can sometimes account for large losses of nutrients (for example sodium excretion through sweat in cystic fibrosis or in hot environments). The miscellaneous losses of some nutrients can be calculated from data reported in the scientific literature.

Foods and excreta must be thoroughly homogenized to obtain truly representative aliquots for chemical analysis. When using mixed diets, it is best to analyze each food separately.

Some studies introduce a consistent bias in metabolic balance calculations. If it is assumed that all food is eaten, intake may be overestimated when the exact amount is not measured; excreta can be underestimated due to small losses in collection or neglect to include miscellaneous losses. The end-result is an overestimation of positive or an underestimation of negative balances.

Limitation of Metabolic Balance Studies

Some limitations of metabolic balance studies are universal but others are related to the proposed applications of the metabolic balance.

The limitations of metabolic ward studies apply to balance studies, but the duration of the balance period merits additional comments. Balance studies carried out during 3 to 7 days do not necessarily represent the long-term balance of a nutrient. In fact, the normal day-to-day variations in food intake end the short periods of augmented or decreased losses, suggest that days of negative and positive balance alternated to produce, in the long-rum, zero or nearzero balance in adults and a positive balance in growing children and pregnant women.

Another problem of short-term metabolic balance studies is the time required to reach a steady state after a dietary change. If the excreta are expected to indicate the metabolic fate of substances ingested under habitual conditions, the experimental subject must reach a new equilibrium after the transition from one pattern of intake to another. The duration of this equilibration period varies and may be long for some substances [17]. Furthermore, Sukhatme and Margen [21] postulated an autoregression process that serially correlates the fluctuations in nitrogen balance with the values of the previous days.

Pathological or physiological alterations in metabolism can invalidate the comparisons of short-term balance studies. Infections and fever influence the subject's appetite and the intermediary metabolism of absorbed nutrients; there may be an increase in catabolism and augmented urinary losses of the nutrient or its metabolites. Periodic metabolic changes can influence balance data, as is the case with the fluctuations in urinary nitrogen during the mestrual cycle [14].

On the other hand, changes in physical activity or body composition can influence the results of long-term balance studies, and they should always be considered. Another potential problem with long-term balance studies is that small errors with a consistent bias (e.g., overlooking integumental losses) can have a large cumulative effect.

A major limitation of metabolic balance studies is that they only indicate the total net change of a substance in the body, but do not provide information about internal body distributions, turnover rates, pool sizes or intermediary metabolic fate of the substances. Furthermore, they do not indicate the amounts of endogenous substances excreted by urine or feces, nor of the bacterial contribution to fecal matter. The true absorption of a nutrient can be determined with the ingestion of isotopically labeled substances that can be differentiated from endogenous and bacterial products, or correcting from the calculations of fecal excretion with data from experiments done with a diet free of the nutrient.

The interpretation of metabolic balance data can be complicated by the yet unexplained phenomenon that high intakes of many substances produce apparently high retentions that are not confirmed by carcass analysis of animals nor by body composition measurements in humans [7, 9, 10, 13, 23]. The explanations offered for these findings include consistent errors in the measurement of losses, respiratory excretion of volatile substances and changes in body composition. None of them, however, are satisfactory and we must conclude that, for most nutrients, the assessment of retentions with high intakes give fallacious results.

Balance studies of some nutrients or of their metabolic results, can only be done if other methods are used simultaneously with the measurements of intake and excreta. Foremost among them is dietary energy balance. A very large component in the balance considerations is energy expenditure (Exp), such that the balance equation becomes: $B = I - E - Exp$. Energy expenditure must be measured by direct or indirect calorimetry, or estimated from heart rate or time-and-motion observations [1]. The errors inherent to these methods must be added to those of the metabolic balance technique.

Application of Metabolic Balance Studies

Metabolic balance data have useful applications in spite of their recogni limitations and technical uncertainties. Among them, the following can mentioned:

1. Definition of the minimal daily requirements of a nutrient.
2. Evaluation of changes in nutritional requirements due to physiologi conditions (e.g. age, pregnancy, lactation, exercise, genetic characteristics, fections, endocrine disorders, etc.)
3. Definition of the nutritional needs of hospitalized patients with acute chronic diseases, or with increased nutrient losses (e.g., burns, protrac diarrhea, trauma, prolonged immobilization, etc.).
4. Study of dietary, metabolic and environmental factors that influence sorption, excretion orretention of a nutrient.
5. Evaluation of long-term trends of change in body composition.
6. Complementary information for other metabolic, physiological or dia nostic studies (e.g., total energy requirements, malabsorption syndrome nutritional impact of environmental contamination, etc.).

Dietary energy metabolism

It is now accepted that energy requirements are determined by energy e penditure and not by energy intake [8]. Hence the importance of its mea urement, as well as that of total energy balance. Energy expenditure ca be measured by direct calorimentry, indirect calorimetry or physiologic measurements that correlate with oxygen uptake, such as heart rate monit ring. All these techniques have physiological or practical advantages an limitations.

Direct calorimetry is the most precise of the currently available methods. I main limitations are the costs of building a calorimetric chamber and the co finement of the experimental subject to such restricted quarters for the dur tion of the studies.

Indirect calorimetry is based on measurements of inhaled and exhaled oxyge and carbon dioxide, and on the energy equivalence of the oxygen consume The energy cost of various activities can thus be measured. The summation c the periods of time that a person spends in each activity, multiplied by the corre ponding energy costs, gives an estimate of total energy expenditure; these are th so-called time-and-motion studies [24]. Their main advantages are that they can b done in free-ranging individuals, either in a metabolic ward or in the field. Thei main limitations are: 1) when the energy cost of activities cannot be measured i the experimental subjects, it is necessary to rely on data published by other investi gators and to assume that they apply to our subjects; 2) it must be assumed tha the energy cost of an activity is constant throughout its performance, althoug the e may be wide variations in performance efficiency; 3) the activities must b accurately timed by an external observer who can observe only one subject at a time; 4) the presence of the observer may induce behavioral changes and modify the subject's habitual activities.

Heart-rate monitoring is based on its linear relationship with oxygen consumption [4]. Its main advantage is that the subject wears a heart-beat monitoring device and an observer need not be in the proximity. Its main limitations are: 1) the correlation between heart-rate and oxygen consumption must be assessed in each subject due to large inter-individual variability; 2) the correlation is linear within certain limits and is lost during rest periods; 3) frequent assessments of heart-rate must be made, since the correlation with oxygen consumption may vary at high and low levels of energy expenditure; 4) the correlation may be influenced by various physiological, pathological and environmental conditions.

Water with a double isotope label of ^{2}H and ^{18}O has been recently used to assess energy expenditure in man [18]. It is based on assessments of body composition, CO_2 production and respiratory quotient. It seems to be a promising method for metabolic ward and field applications, but it must be further tested and validated. One of its main current limitations are the high costs of the doubly-labeled water and the analysis of the stable isotopes.

Hydrogen Breath Test

This is another useful test in nutritional metabolic studies. It is based on the observation that certain bacteria of the normal human colonic flora will ferment unabsorbed carbohydrate with evolution of hydrogen gas [5, 15]. The gas is absorbed into the blood by the colonic mucosa, and excreted through the lungs. Thus, an increase in the concentration of exhaled H_2 is stoichiometrically related to the amount of unabsorbed carbohydrate. Its major applications are in the study of intestinal carbohydrate absorption, diagnosis of lactose malabsorption, study of gastrointestinal transit time, and diagnosis of bacterial overgrowth of the small intestine. The method, however, has a series of pit falls and limitations that must be considered in its application and in the interpretation of its results. The Table, adapted from studies and reviews by Solomons [19, 20] summarizes the main pitfalls and limitations of the method.

SUMMARY

Metabolic studies are essential for nutritional research. However, all techniques have pitfalls and limitations that must be taken into account to apply them correctly and, more important, to interpret their results adequately. We have discussed in detail the limitations related to the metabolic ward and metabolic balance studies, and commented briefly on other techniques frequently used in our metabolic ward and field studies. The potential, limitations and applications of other methods are discussed in other sections of this Workshop.

LIMITATIONS AND PITFALLS IN THE APPLICATION AND INTERPRETATION OF THE HYDROGEN BREATH TEST*

Idiopathic absence of appropriate flora
Consequence: No H_2 response to nonabsorbed carbohydrate

Use of oral antibiotics prior to the hydrogen breath test
Consequence: Reduction in the mass of fermenting bacteria in the colon

Cigarette smoking during the hydrogen breath test
Consequence: Abrupt increase in breath H_2 concentration not related to carbohydrate

Active diarrhea at the time of the hydrogen breath test
Consequence: Reduced H_2 response to a given amount of nonabsorbed carbohydrate

Sleeping during the hydrogen breath test
Consequence: Increased concentration of breath H_2

Incorporation of the test carbohydrate into a fiber-containing meal
Consequence: Excess of excretion of breath H_2 not related to malabsorption of the test carbohydrate

Acidic colonic pH due to recent fermentation of carbohydrates
Consequence: Reduced H_2 production (pH optimum. 7.2)

Use of enemas prior to the hydrogen breath test
Consequence: Wash-out of fermenting flora

Delayed gastric emptying
Consequence: Alterations in the kinetic characteristics of the H_2 response curve

Hyperventilation or crying during collection of breath specimen
Consequence: Dilution of pulmonary H_2 with air of the anatomic dead space.

**Adapted from Solomons, NW, references 19 and 20.*

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DISCUSSION[1]

Sonya Connor asked if the hydrogen breath test might be useful in the measurement of dietary fiber intake. Torún was cautious in his response pointing out that the efficacy depended upon the type of fiber and the type of intestinal flora. He thought it might be adequate as a semiquantitative measurement in certain defined situations. Solomons concurred, mentioning studies by Eastwood et al. in which hydrogen levels were very slow to increase when high levels of hemicellulose and pectins were fed. The ingestion of other hydrogen producing carbohydrates would complicate this further. Scrimshaw raised the question of psychological as well as infectious factors complicating the interpretation of metabolic studies. He noted that when students were used for long-term balance studies, exams or other periods of stress caused them to deviate from the stable pattern they had shown before and afterward. Other examples were offered as well. He emphasized the importance of having the staff versed in the study so that unrecorded food intake was eliminated. He emphasized that whenever an outlier is detected, it is vital to be certain that this was not merely an error in the study. Harper commented that changes in pool size are measured by balance procedures and that with some additional studies determining the original pool size, a relationship between pool size and the development of symptoms can be made.

In: Genetic factors in nutrition. Eds.: Antonio Velásquez & Héctor Bourges. New York, Academic Press, Inc., 1984. p.361-372.

[1]*Summary of the discussion prepared by S. Cederbaum.*