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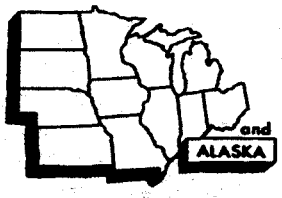
# Methods Used for Human Metabolic Studies in the North Central Region

University of Minnesota
Agricultural Experiment Station

NORTH CENTRAL REGIONAL PUBLICATION NO. 80




Agricultural Experiment Stations of Alaska, Illinois, Indiana, Iowa, Kansas, Michigan, Minnesota, Missouri, Nebraska, North Dakota, Ohio, South Dakota, Wisconsin, and the U. S. Department of Agriculture, cooperating

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# PREFACE

In 1935, research workers in human nutrition in the North Central Region decided that a study of a problem of mutual interest, i.e., the nutrition of the college woman, could best be approached by a cooperative endeavor. After two preliminary meetings, initiated by Iowa State College, representatives from five home economics departments associated with the agricultural experiment stations located in Iowa, Kansas, Minnesota, Ohio, and Wisconsin, met at Ames, Iowa, October 23-27, 1936. They outlined a master project, formulated a plan of organization, and drew up a statement of agreement. Later, Nebraska joined the group. The efforts of this group during the next ten years resulted in important publications relating to the nutritional status of the college-aged woman.

An expanded program of cooperative research on problems of a regional character was made possible by the Research and Marketing Act of 1946. In July 1947, a project dealing with the nutritional status and dietary needs of population groups in the North Central Region was formulated. Nine research groups located at the agricultural experiment stations of Illinois, Indiana, Iowa, Michigan, Minnesota, Missouri, Nebraska, South Dakota, and Wisconsin, elected to investigate problems relating to the older woman. Later, Oklahoma joined the group as a cooperating state.

Many of the research personnel involved were those who had participated in the previous project, thereby making it possible to utilize the earlier experiences of the group in assuring continuity of effort in extending balance studies to include eventually the entire life span of woman. Many of the procedures and laboratory methods which have evolved from the approximately 20 years of unique cooperative experience of those research nutritionists are presented in this publication.

# Methods Used for Human Metabolic Studies in the North Central Region

Metabolism studies with people are expensive from the standpoint of time, money, and personnel. It is important, therefore, that the research worker have clearly in mind the objectives toward which efforts are directed, how best to select subjects and methods, and how to evaluate data to achieve these objectives. The chief interest of the cooperators of the North Central Region in their study of the nutritional status and dietary needs of older people was in the utilization of the nutrients provided by the foods in the diets freely chosen by this age group. Dietary allowances need to be established eventually as a result of studies under these conditions rather than under those of controlled dietary intakes. This publication is concerned with the methodology used in the study of such subjects.

## Selection of Experimental Subjects

The objectives of the project will determine the age, sex, health, and socio-economic status of the experimental subjects. In selecting subjects for the study, the project leader must also consider such characteristics as the integrity, reliability, educational background, and the native ability of the individual. Often people are motivated to become experimental subjects because they feel satisfaction in participating in an activity which will benefit others. The investigator should foster this feeling by taking time to remind the subject of his indispensability and to praise him for his efforts so that he feels a justifiable pride in the important role he plays.

The subject should be given a thorough physical examination for assessing general health and nutritional status. This is essential to insure that the subject will not be endangered by the experiment and that the data secured will be usable. If an abnormal regimen is asked of the subject it is well to repeat the examination at the end of the experiment. This procedure affords protection to both the subject and the investigator.

Details of experimental procedures should be discussed with the subject if the knowledge will not adversely influence his cooperation. A clearly written statement about obligations to be met and directions to be followed in collecting food samples and excreta usually is appreciated by the subject.

Because metabolic processes may be affected by emotional stresses, it is important that the subject shall not be over-anxious or tense because of the new situation brought about by the study. A trial period in which everything with the exception of analytical work is done usually will clarify procedures and develop the self-confidence of the subject. The relationship between the subject and the project staff should be such that mistakes can be reported freely without implication of censure.

There should be a clear understanding about financial arrangements and other obligations assumed by each person connected with the project. The project leader should secure permission from the subject at the beginning of the experiment to use as desired any data obtained from the investigation.

## Selection of Laboratory Procedures

Much useful information about the nutrition of an individual or of a group may be obtained by measuring the intake of certain nutrients and by determining the amounts of selected excretory products derived from these nutrients. The procedures the laboratory may choose to use will depend upon many considerations, such as the equipment available and necessary, the skill of the laboratory personnel, the number of analyses involved, accuracy desired, size of samples available, duration of the experiment, and cost of the project. Because of these considerations no one procedure is best under all circumstances.

The procedures given are those which have been used successfully by nutrition research workers in the North Central Region in metabolism studies with people eating their regular self-selected diets. Directions are given for the collection, sampling, and preservation of food, feces, menses, and urine. Literature references which have been helpful in formulating these general procedures are given at the beginning of each section. Published methods that have proved satisfactory for the analysis of foods, feces, menses, and urine for amino acids, creatinine, energy, minerals, nitrogen, and vitamins are referred to specifically by literature citation at the end of the appropriate paragraph.

## General Directions

REFERENCES: A.O.A.C. (1940, 1950); Association of Vitamin Chemists (1947, 1951); Consolazio *et al.* (1951); Ohlson *et al.* (1952); Peters and Van Slyke (1932)

Unless otherwise stated, all reagents conform in purity to Recommended Specifications for Analytical Reagent Chemicals of the American Chemical Society; acids are concentrated; and water, in addition to being distilled, is specially purified if that is deemed necessary. All temperatures are centigrade.

**For storing samples, wide-mouthed French-square glass bottles with plastic screw caps are most satisfactory because they store compactly, although pharmaceutical bottles or round glass bottles, wide- or narrow-mouthed, can be used. Amber-colored bottles are** used for storing samples when analyses for light-sensitive nutrients are to be made. Plastic screw caps are inert to most chemicals used to preserve samples, although in the presence of toluene it is well to use a cardboard interliner. Metal screw caps rust easily and one should take precautions to see that the samples are not contaminated. Usually a piece of cellophane or aluminum foil placed over the mouth of the jar before the cap is put on will give protection. Corks should be coated with paraffin to prevent loss by evaporation.

Adhesive tape, "Labelon," or gummed labels are satisfactory for labeling sample bottles. Ink should not be used on the labels unless the label is coated to prevent moisture from smearing the ink. Typewritten or penciled labels are satisfactory. It is well to coat gummed labels with paraffin to prevent their drying and falling off the bottles.

## COLLECTION, SAMPLING, AND PRESERVATION OF FOOD

**The following equipment and supplies should be provided for the subject by the laboratory when conducting studies of food consumption in the home:**

Market basket for supplies and samples, 2 for each subject
Dietetic scale with rotating dial permitting cumulative weighings, capacity 500 gm; dial divisions, 1 or 2 gm
Waxed or other appropriately coated paper squares or cellophane, 4" × 4" and 6" × 6"
Plastic plate, diameter 10"
Stenographer's spiral notebook, or bound record book suitably ruled, or mimeographed record sheets
Lead pencil
Wax glass-marking pencil

Suitable containers in which to save food samples (Sample bottles, Mason jars, waxed paper cartons, plastic bags, or plastic freezer dishes are satisfactory. Tight-fitting lids should be provided for all containers which will be used for liquid foods.)

**Written instructions given to the subject**

The persons who are making this study wish an accurate picture of your food intake. They want you to live and eat as usual. In order that your diet remain the same as usual, try not to let weighing the food influence the kind or amount that you eat or that you serve to the family group. If your food intake is to be controlled by the laboratory, special directions as to what is to be eaten will be given to you.

1. Record of all the foods eaten

Weigh everything which you eat or drink except water, coffee, or tea unless specifically directed to do so. If cream or sugar is used in the latter two beverages be sure to record the weight of each used. Follow the plan given here for keeping a record. Describe the method that was used in processing the fruits or vegetables, e.g., "canned peas," "frozen broccoli," or "dried figs." Indicate the method of preparation, e.g., "mashed potatoes," "baked banana," or "roast beef."

List foods eaten under the following titles: breakfast, morning snack, lunch, afternoon snack, dinner, evening snack.

*Illustration*

| DINNER | |
|---|---|
| *Food* | *Weight in grams* |
| Ground round steak—broiled | 75 |
| Mashed potatoes | 113 |
| Milk gravy | 21 |
| Frozen peas (cooked) | 54 |
| with butter | 4 |
| Salad | |
| Lettuce | 15 |
| Carrots | 15 |
| Cabbage | 30 |
| Mayonnaise | 12 |
| Vanilla ice cream | 40 |
| Chocolate cookie—plain wafer | 10 |
| Coffee | 150 |
| Cream, light | 10 |
| Sugar | 5 |

| EVENING SNACK | |
|---|---|
| *Food* | *Weight in grams* |
| Buttered popcorn | 50 |

To record second helpings add the weight of the second serving to that of the original weight, e.g. mashed potatoes 113 + 25.

To record uneaten food, subtract the weight of that left from the original weight, e.g. mashed potatoes 113 − 20.

To record waste, such as bones, fat, orange rinds, egg shells, etc., weigh the refuse and subtract from original weight, e.g. egg 60 − 10. Any fat left uneaten from meat should be weighed and specially noted on the record.

2. Suggestions for weighing foods for serving

Foods should be weighed in the form in which they are served, e.g. boiled, baked, broiled, steamed, or raw. Inedible parts, such as bones, rinds, or egg shells, may be removed before weighing, or those parts of servings not eaten may be weighed after the meal, subtracting them from the original weights recorded for the food. If vegetables are to be eaten creamed or buttered, weigh the portion of plain cooked vegetable and add a weighed amount of butter or cream sauce to it.

Place the dietetic scales on a stable, level shelf or table. Move them by holding the body of the scales and not the platform. Use care in placing articles on or removing them from the platform of the scales. In reading any weight the observer's eyes should be opposite the pointer of the scale.

After putting the waxed paper or plastic plate on which food is to be placed on the platform, rotate the dial so that the 0 is directly under the pointer. Place the food on the paper or plate and record its weight. If additional portions of food are to be weighed on the same plate, reset the dial so the pointer is at 0 before each weighing.

Weigh separately the ingredients of foods such as sandwiches. For example, when making a cheese sandwich for serving have the pointer at 0, add the bread and record its weight. Reset the dial so the pointer is at 0, butter the bread and note the weight of the butter. Again reset so the pointer is at 0, add the cheese and write down its weight. Proceed in a similar manner where several foods are eaten together such as cereal served with fruit, sugar, milk or cream, baked apple with cream, pudding with a sauce, coffee with cream and sugar, or tea with sugar and lemon. Some salads are made of finely divided ingredients which are well mixed with the salad dressing so that a representative sample can be obtained. Where large pieces of foods are used, however, it will be more accurate to weigh each item as well as the salad dressing separately following the plan outlined for the cheese sandwich.

*Eggs,* poached, hard-cooked, soft-cooked, or fried. Weigh after cooking.

*Toast.* Weigh bread before toasting.

*Milk.* Mix well before weighing.

*Candy.* Weigh and record number of candy bars or pieces eaten.

*Water, tea, and coffee.* (Instructions given to the subject will depend upon the measurements to be made by the laboratory. For example, if calcium analyses are to be made, the laboratory may provide a bottle of distilled water or a supply of tap water of known composition to be used for beverages. In some cases, the subject may be directed to weigh or measure all beverages consumed and to save aliquots of them. Sometimes all that is desired is a statement of the number of glasses of water and of the number of cups of tea or coffee ingested. Obviously beverages made from fruit juices or milk or containing chocolate or cocoa should be treated like other foods.)

## 3. Weighing samples of foods for the laboratory

**The laboratory may train the subject to do the weighing of the food aliquot to be used for analysis, or the laboratory may prefer that representative samples of each food eaten be sent to the laboratory where an aliquot will be weighed by a skillful worker. Regardless of the method used, it is of the utmost importance that the aliquot represent accurately** the food intake of the subject.

### a. Preparation of food aliquot for the laboratory

#### (1) Aliquot weighed by the subject

The laboratory needs samples of food equal to _____* of the amount of these eaten. Enough extra food should be purchased and prepared so that the amounts eaten are not reduced in order to provide the laboratory samples. The quantities weighed for the aliquot should be precisely _____ of the amount eaten and should be placed in the food sample containers designated by the laboratory. Weigh the laboratory sample at the same time that the serving of food is weighed. Check the food record carefully and be sure that a sample of everything eaten has been saved.

#### (2) Aliquot weighed by the laboratory

The laboratory needs samples of food equal to _____* of the amount of those eaten. Enough extra food should be purchased and prepared so that the amounts eaten are not reduced in order to provide the laboratory samples. The quantities saved do not need to be precisely _____ of the amount eaten, but they should not be less than these amounts. Weigh the laboratory sample at the same time that the serving of food is weighed, except as indicated under b, below. Check the food record carefully and be sure that a sample of everything eaten has been saved. Place each type of food in a separate container provided by the laboratory and cover tightly. Label each container as to content.

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* The ratio of the weight of the sample to the weight of the food actually eaten must be the same for all the foods in the day's meals and is usually 1/5 or 1/4, but this will vary according to the number and type of analyses to be done. A table showing how many grams in 1/5 or 1/4 of a range of serving sizes, for example, from 10 to 300 gm, may be prepared and given to the subject so he will not have to make arithmetical calculations.

b. Suggestions for weighing the samples of some of the foods to be sent to the laboratory

*Eggs.* If eggs are served in the same form more than once during the day, i.e., poached, hard-cooked, soft-cooked, or fried, add the total amount eaten and save _____ of the total. For omelets, souffles, scrambled eggs, and other dishes where eggs are combined with other ingredients, an amount equal to _____ of that eaten should be saved.

*Bread.* If the same kind of bread has been used more than once during the day, add the total amount used and save a sample equal to _____ of the total. If any of the bread is toasted, the untoasted weight should be included in the total.

*Milk.* In the same way add the total amount of milk used as a beverage and save _____ of the total.

*Butter.* Add the total amount of butter used as a spread or placed on foods and save _____ of the total.

*Candy.* Save one whole candy bar, or if pieces of candy are eaten, save _____ of the total. Place in a container separate from the other foods.

*Soft drinks and beer.* Save one bottle or can. Keep separate from the other foods.

*Other beverages.* Save an amount equal to _____ of that eaten. Keep separate from the other foods.

**Preparation of composite food samples in the laboratory**

The number of analyses required for balance experiments can be reduced by preparing composites of several foods having similar physical and chemical properties. For nitrogen and mineral measurements, the composites may be refrigerated, frozen, or converted into digests. Special manipulation of samples is required where food energy values or amino acid contents are to be determined, or where labile compounds such as thiamine, riboflavin, or ascorbic acid are to be measured.

If the food composite has not been weighed by the subject, the laboratory will need to make a composite of the food aliquots sent by the subject. The type of composite made will depend on the analyses to be made, but a common method is to divide the food samples into liquid, fatty, and solid composites in the following manner:

1. Liquid foods

Select a balance which has a sensitivity of 0.1 gm and weigh accurately a proportional aliquot (the amount will depend upon the number and kind of analyses to be made) of all milk, beverages containing milk, fruit and vegetable juices, ice cream, sherbets, ices, soft custard, and soups consumed in one day. Usually water, tea, or coffee are not included with the liquid foods but are analyzed separately. If these are included, the aliquots may be concentrated to a convenient volume by evaporation. Alcoholic beverages and soft drinks also may be handled more easily by separate analysis. Keep a record of the weight of each food added.

2. Fatty foods

Weigh accurately a proportional aliquot of all butter, margarine, cream, mayonnaise, and French dressing. Record weights. Most of the mineral and nitrogenous compounds measured in metabolism studies are not associated with fat, which is difficult to handle in a digest. Such constituents as are present may be separated by treating the fatty foods as follows: heat aliquots of the fatty foods with 50 to 100 ml of water until the fat is melted; cool and remove the layer of fat by letting it solidify; add the liquid quantitatively to the liquid food composite. If solid particles appear in this composite separate them by strain-

* While the subject will not weigh the amount of food that actually will be saved by the laboratory, it is often wise to ask him to weigh a set aliquot. This will ensure that enough food is saved by the subject so the laboratory will have the desired amount. Usually he is asked to save from $\frac{1}{4}$ to $\frac{1}{2}$ of the amount of food eaten, but this will vary according to the number and type of analyses to be performed. Each laboratory should fill in the blank spaces to fit its needs.

ing and add them to the solid food composite. Obviously the above procedure to remove the fat would not be carried out if food energy is to be determined.

3. Solid foods

Include all foods not covered in *1* and *2* above. Record weights. Check the samples weighed against the food record for the day. Many laboratories may prefer to combine the above composites into one. If proportional aliquots of the food eaten have been weighed, the composite will be representative of the subject's intake. The composites may be stored in a jar or a flask until ready for analysis. If the composite is to be kept at refrigerator temperature more than one day, an acid preservative such as HCl or $H_2SO_4$ should be added if the analyses planned permit.

**Preparation of special composites for thiamine, riboflavin, or ascorbic acid analysis or for amino acid composition determination**

1. Thiamine and riboflavin analysis

Solid and liquid foods are kept in a 2-liter wide-mouth Erlenmeyer flask which contains 500 ml of 1 N $H_2SO_4$. Stopper with a cork covered with aluminum foil or cellophane. Be sure that the food is covered with acid at all times. The volume of acid used is usually adequate for a 3-day period if the flask is carefully swirled after the food is added to it. Keep the flask in a cool place and protect the material from light by placing the flask in a #10 paper bag or by using a low-actinic glass flask. Collect aliquots of fatty foods in a beaker. No preservative is necessary. Protect from light.

THIAMINE is determined by the method of Hennessy (1941); RIBOFLAVIN is determined by the method of the Association of Vitamin Chemists (1947) or Conner and Straub (1941).

2. Ascorbic acid analysis

REFERENCE: Loeffler and Ponting (1942)

Two composites are usually made, a milk, ice cream, and cream composite; and a fruit and vegetable composite. These foods contribute the major share of the vitamin to the normal diet. If the subject is consuming a diet very low in ascorbic acid content, however, the contribution of the vitamin by other foods is important. Even foods which contribute small amounts of ascorbic acid should be analyzed. A common aliquot used is 1/5 of the total intake.

a. Milk composite
Collect aliquots in a brown-glass jar, with a screw top, containing 50 ml of freshly made 5% metaphosphoric acid. Refrigerate the composite until it is complete. The composite should be analyzed daily, if possible.

b. Fruit and vegetable composite
Include all fresh and processed fruits and vegetables. Collect aliquots in a brown-glass jar with a screw top, containing 150 ml of freshly made 5% metaphosphoric acid. Refrigerate the composite until it is complete. The composite should be analyzed daily, if possible.

3. Amino acid composition determinations

REFERENCE: Futrell *et al.* (1952)

The foods which are pooled in the composites will depend upon the type of hydrolysis employed and the amino acids determined.

a. Procedure I, employed by one laboratory, separates the foods into three categories, liquid, fatty, and solid, and adds the water-soluble fraction of the fatty-food composite to the liquid-food composite as described previously. Aliquots amounting to

1/5 of each day's solid food intake are collected in an Erlenmeyer flask containing 500 ml of 20% HCl. The liquid food is collected in an Erlenmeyer flask containing 200 ml of HCl. An aliquot of water ingested is evaporated to 100 ml and added to the composite. The water-soluble fraction of the fatty-food composite is added.

b. Procedure II, used by another laboratory, separates the foods in the following manner: cereals, consisting of bread, cake, and dry breakfast cereals; food mixture, consisting of fruits, vegetables, and prepared dishes that do not belong in the other categories; meats; eggs; cheese; and milk. All aliquots are stored in a refrigerator with no acid preservative until the end of the collection period.

**Preservation for analysis**

1. Preparation of brown digest for certain mineral analyses and for nitrogen analysis

REFERENCE: Stearns (1929)

A brown (acid) digest of food samples is a convenient way of preserving samples for certain mineral analyses and for nitrogen analysis. If made properly and sealed tightly it will keep for an indefinite period at room temperature and allow the laboratory worker to perform the analyses at his convenience. Brown digests may be made with either HCl or $H_2SO_4$ acting upon the 3 types of food composites (liquid, fatty, and solid) described previously, upon a composite of the entire diet, or upon any fraction thereof depending upon the analyses to be made. The digest may be heat-treated by the use of an electric hot plate, autoclave, steam bath, or water bath, depending upon the facilities available. The length of heat-treatment may vary from one hour to a week depending upon the type of heat used. A long period of heat-treatment results in a greater digestion of the food particles and a finer suspension of the material, thus aiding in the handling and sampling of the digest. There is no danger of loss of nitrogen and most minerals unless the material is charred. The digests should be made and stored in Pyrex or other hard-glass containers, since the acid may release calcium from soft glass and thus inadvertently add calcium to the food digest.

a. Heat-treatment by autoclave, hot plate, or steam bath

(1) Transfer the food composite quantitatively to an electric blender. Do not fill the blender more than 2/3 full. Add water if the composite is too thick to permit thorough blending. Blend thoroughly; approximately 15 minutes for solid foods.

(2) If only a part of the sample is to be digested, the blended composite may be weighed or made up to a known volume.

(3) Transfer the total composite or a known volume or weight of the composite to an Erlenmeyer flask and add acid in the following approximate amounts:

100-150 ml HCl or $H_2SO_4$ : 1 liter of blended solid food
100 ml HCl or $H_2SO_4$ : 1 liter of liquid food

(4) The flask may be plugged with cotton and the contents autoclaved at 15 pounds pressure for 1 to 2 hours, or the flask may be heated on a steam bath or on a hot plate set at low heat until the mixture is uniform in consistency, brown in color, and evaporated to an amount that can be made up to the desired volume. If a steam bath or hot plate is used, contents of the flask must be mixed by stirring or occasional shaking.

(5) With the aid of a pestle or other device*, pass the solid food digest through a wire mesh sieve (approximately 20 mesh; copper or brass if iron is to be determined) placed in a funnel supported by a ring clamped to a ringstand. Insert

* A paddle made of a tough but flexible plastic material attached to a 1/4" shaft, which is inserted in the chuck of a hand drill, enables the operator to force the digest rapidly through the sieve.

the stem of the funnel into a weighed volumetric flask. Add water rinsings from the Erlenmeyer flask, sieve, pestle, and funnel to the volumetric flask before making up to volume with water. Weigh the flask plus its contents; sampling and subsequent calculations can then be made on the basis of weight. (If calculations are to be made on the basis of volume, the weighings may be omitted. Because of the nature of the digest, one may experience some difficulty in sampling on the basis of volume.) Mix contents of the flask by inverting and rotating 50 times. Save duplicate samples. Invert and rotate the flask 25 times before filling the second bottle.

b. Heat-treatment by water bath

Carry out step (*1*) above.

(2) Sampling blended composite

(a) If the volume is to be the basis for calculations transfer the blended composite quantitatively to a volumetric flask, add acid as in step **(3)** above, and make to volume with water. Stopper and mix 50 times before filling the first jar and 25 times before filling the second jar.

(b) If weight rather than volume is to be the basis for calculations, add the acid to the composite in the blender and weigh. Add water to bring the weight to a multiple of 10 gm. Blend thoroughly. Pour the contents of the blender into an Erlenmeyer flask and swirl 30 times

(3) Fill 2 chemically clean quart jars (with glass tops, metal rings and rubber rings) ¾ full of the composite. Adjust the lids and process one jar in a boiling water bath for one hour. If it is not needed discard the unprocessed duplicate which served as a safeguard against loss by breakage in processing.

NITROGEN content of the digest is determined by the method of A.O.A.C. (1950), Meeker and Wagner (1933), Scales and Harrison (1920), or Triebold (1946). CALCIUM is determined by the method of McCrudden (1911); PHOSPHORUS by the method of Fiske and Subbarow (1925).

2. Freezing of food composite for determination of the vitamins of the B complex

**Refrigerate the food samples until the aliquot is weighed. Add a solution of one part of HCl to one part of water to the aliquot to make a slurry. Blend and weigh the slurry, and place a portion in a sample bottle or other suitable container. Freeze the material until needed.**

**The NIACIN, RIBOFLAVIN, and THIAMINE contents of the food are determined by the methods of the Association of Vitamin Chemists (1947); VITAMIN $B_6$ by the method of Atkins *et al.* (1943).**

3. Treatment of food composite for ascorbic acid analysis

a. Milk composite

Tranfer the milk composite preserved with 5% metaphosphoric acid to a 200-ml volumetric flask. Rinse the collection jar with 5% metaphosphoric acid and add the rinsing to the flask. Make to volume with 5% metaphosphoric acid. Filter the solution through Whatman ♯12 filter paper.

b. Fruit and vegetable composite

Transfer the composite preserved with 5% metaphosphoric acid to a blender. A few drops of caprylic alcohol may be added to prevent foaming. Blend for 2 minutes. Transfer the blended material quantitatively to a 500-ml volumetric flask using 5% metaphosphoric acid to rinse. Make to volume with 5% metaphosphoric acid. Filter the solution through Whatman ♯12 filter paper.

The 1-ASCORBIC ACID content of the filtrates is determined by the method of Loeffler and Ponting (1942).

4. Treatment of food composite for energy value determination

Material for measuring food energy should include all solid foods, all liquids containing carbohydrate, fat, protein, or alcohol, and all fats. HCl or $H_2SO_4$ is not used as a preservative, instead aliquots are kept in a freezer. One tenth of the total intake is a common aliquot used. Dishes in which to dry the blended material should be shallow enough to allow the material to dry in a thin layer. Evaporating dishes, 15 cm in diameter, or pyrex casserole dishes, 5½ inches in diameter, are recommended. A glass stirring rod 20 cm long is placed in each dish. The weight of the dish plus the rod is determined.

a. Thaw the food composite by placing the container in a pan of slightly warm water. Place before an electric fan. If the composite is in a wide-mouthed dish, thaw slightly so that it can be slipped out of the dish. Transfer the composite quantitatively to an electric blender. Rinse container with a small amount of hot water. Blend material.

b. Sample the blended material using either a known weight or a known volume of material.

(1) If calculations are to be made by weight and the amount of aliquot is small, quantitatively transfer the blended material into a dish. Rinse blender with hot water and add rinsings to the dish. If the amount of blended material is too large for one dish, weigh the total amount of material and put a weighed portion into one or more dishes.

(2) If calculations are to be made by volume, transfer the blended material quantitatively to a 1- or 2-liter volumetric flask. Rinse the blender with water and transfer rinsings to the volumetric flask. Use warm water for final rinsings. Make up to volume with water. Mix blended material by inverting and rotating the flask 50 times. Fill three 250-ml volumetric flasks, inverting and rotating the large volumetric flask 25 times before filling the second and third small flasks. Quantitatively transfer the contents of each 250-ml flask to a dish.

c. Dry the composites by one of the following methods depending upon the facilities available.

(1) Place the dishes in a drying oven equipped with a fan, and set at 40°-60°. Stir samples frequently to avoid uneven drying and layering of fat. Leave for approximately 4 days.

(2) Place the dishes under 250-watt infra-red heat lamps, keeping the distance from the bulb to the food between 8 to 12 inches. At 1- to 1½-hour intervals stir the food with the glass stirring rod to prevent the formation of a crust and to facilitate drying. When the material is no longer gummy, transfer the dishes and rods to a drying oven or vacuum oven set at 40°-60°. The vacuum oven should be provided with a water trap.

d. Transfer the dishes with the dried material and the rods to a desiccator. Cool and weigh.

e. Scrape the dried product from the dish and grind with a pestle until all of the material is finely divided, uniform, and well mixed. A ball-mill may be used to grind the sample, provided the fat content of the sample is not so high that it will form a gum around the pebbles. The use of a small-mesh (20 mesh) sieve is helpful to insure uniformity. All of the material in the dish must be ground because fat has a tendency to layer during the drying process. Store material in a tightly stoppered bottle in a refrigerator or freezer. Make certain the sample does not pick up moisture during storage.

f. Prepare the ground sample for ignition by one of the following methods:

(1) Prepare pellets using a pelleting press (Parr Instrument Co.). Press the pellet against a clean piece of filter paper. If fat is visible on the paper, discard the pellet and prepare a second, using less pressure. Weigh pellet.

(2) Place approximately one gm of ground sample in a weighed gelatin capsule standardized for calorific purposes (Parr Instrument Co.). Weigh capsule plus sample.

(3) Ignite a weighed amount of the dry, pulverized material.

TOTAL ENERGY VALUE of the food is determined by the method given in the brochure of the Parr Instrument Company (1948).

5. Treatment of food composite for determination of amino acid composition

a. Blend composites from Procedure I, p. 12, and make to a known volume with water. Invert and rotate the flasks 50 times before placing duplicate samples of the solid-food composite and of the liquid composite in sample bottles.

HYDROLYSATES of the above composites are prepared by the methods of Kuiken *et al.* (1947) and Schweigert *et al.* (1949). The methods of the above authors are also used to determine AMINO ACID composition.

b. Blend and freeze composites from Procedure II, p. 12. Thaw the composite when ready to hydrolyze. Weigh 10 gm of the milk sample and 20 gm of the food mixture. Dry the cereal mixture at 60° in a vacuum oven, then grind to a powder in a mortar. Weigh 2 gm of this powder. Dry the meat, eggs, or cheese at 60° in a vacuum oven and extract the dried material for 12 hours in a Goldfisch apparatus. Use approximately 0.2 gm for hydrolysis. Hydrolyze the samples for 8 hours in an autoclave at 15 pounds pressure in 4 N HCl. For determination of tryptophan, hydrolyze the samples in 4 N NaOH for 15 hours. After hydrolysis, adjust the samples to pH 6.8, dilute to 100 ml, and filter. Further dilute the samples for assay as needed.

The AMINO ACID composition of the hydrolysates is determined by the method of Futrell *et al.* (1952), using the media of Steele *et al.* (1949).

## COLLECTION, SAMPLING, AND PRESERVATION OF FECES

REFERENCE: Peters and Van Slyke (1932)

### Equipment and supplies provided for the subject by the laboratory

Gelatin capsules, No. 0, tightly packed with carmine (approximately 3 grains)

Containers in which to collect feces (coffee cans, Seal-rite Thermerex cartons, one pt. or one qt., low form, or paper cartons, paraffin-lined, are satisfactory). To line paper cartons with paraffin, pour hot paraffin, m.p.55°-60°, into the carton and rotate rapidly until the inside has been covered completely. Coat the inside of the lid but not the flange. Cartons may be discarded after use or washed with warm water and a detergent if reused. (If the paraffin lining cracks, the carton should be discarded or relined.)

Containers in which to carry cartons (galvanized iron lunch pails with lids, painted coffee cans with lids, baskets, or flight bags are satisfactory).

Platform or length of strong paper to fit under toilet seat and hold paper carton, or a commode

### Written instructions given to the subject

One-half hour before breakfast on the first day of the experimental period, take the carmine capsule supplied by the laboratory. On the morning following the last day of the balance period take a carmine capsule one-half hour before breakfast. Carmine is a harmless dye which is used to color feces.

Collect all feces in the cartons supplied by the laboratory, using one carton per day or one carton per bowel movement as instructed by the laboratory. To hold the carton under the toilet seat, a platform or a length of paper may be placed between the toilet seat and the china bowl. Place the carton on top of the paper. This should allow normal posture for the bowel movement. Discard toilet paper unless instructed to do otherwise. Indicate the hour and day when each sample is collected. Collect all feces from the time of the first meal of the experiment until the carmine appears after the end of the experiment.

Place cartons in the containers provided and keep in a cool place until collected by the laboratory.

**Separation of the feces belonging to the experimental period**

Whenever possible the procedures outlined for working with feces should be carried on in a well-ventilated hood.

Well-formed feces are essential in making good separations. The use of mineral oil and other cathartics by the subject is to be avoided because very soft or loose feces make it difficult to decide whether the uncolored or the dyed portion of the feces left the bowel first. With very dehydrated and compact feces, the dye remains in the intestine for a relatively long time and may fade to a point where only a different shade of brown indicates the location of the portion originally colored red. If in doubt, the next collection should be watched for evidence of carmine. All separations should be completed within one week from the date of collection to avoid fading of the carmine.

To ensure good separation of the feces and to take care of any fecal "lag" at the end of the experiment, it is often wise to ask the subject to continue on his diet for one more day. This day is not considered a part of the balance period but is used to avoid drastic diet changes which might alter gastro-intestinal action.

**Preparation of fecal composite in the laboratory**

**The composite should contain all feces from the first appearance of the carmine, up to, but not including, those colored by the second capsule. From the feces collected during the first day, separate and discard that part which preceded the carmine.** During the collection period, the samples may be frozen by placing the cartons containing the feces in a freezer, in which case no preservative is used; or the feces may be rinsed with water from the cartons into a 2-liter wide-mouth Erlenmeyer flask containing enough HCl so the final concentration of acid in the brown digest is approximately 10%. The flask should be kept covered and in a refrigerator until the collection is completed. The procedures chosen for treating the composite will depend upon the laboratory facilities for processing and storage, and upon the analyses to be made.

**Preservation for analysis: brown digests**

See *Brown digest* part of *Food* section.

REFERENCE: Stearns (1929)

1. Heat-treatment by autoclave, hot plate, or steam bath
   a. Frozen composite
      (1) After thawing slightly, ½ to 1 hour, remove feces from the cartons. The following equipment has been found useful: chisel, ice pick, putty knife, hammer, tongs, scissor-type forceps, rubber policeman, glass rod, spatula, paring knife, or spoon. All metal tools should be free from rust.
      (2) Transfer samples to a wide-mouthed 2-liter Erlenmeyer flask. Add enough HCl so that the final concentration of acid in the brown digest is approximately 10%.
      (3) Carry out steps *(4)* and *(5)* under *Brown Digest, a.* of food section.

b. Refrigerated composite
Carry out steps *(4)* and *(5)* under *Brown digest*, *a*, of food section.

2. Heat-treatment by water bath and or autoclave
   a. Transfer the feces to a tared 2-qt. jar (weighed with glass lid, metal ring and rubber ring). Store in a freezer or add 20% HCl (see *b* below) and refrigerate until collection is complete. A 2-qt. jar will accommodate feces for an ordinary 5- to 7-day period. The jar should not be more than ⅔ full including spaces around the feces.
   b. Add 20% HCl to fill the jar ⅔ full. This should practically cover all the feces. Leave at least 3" head space. Seal the jar and release the band ½ turn.
   c. Exhaust air from jar by one of the following procedures:
      (1) Set the jar on a wooden rack in a water bath containing cold water up to the acid line. Slowly and cautiously heat the water to boiling or near boiling. If the contents of the jar foam excessively, open the jar, stir the contents with a glass rod to remove some of the air, and leave rod until the foaming stops. Rinse the rod and the jar rim with water and partially close the jar as before. In from 4 to 8 hours the air is usually well exhausted and the feces and acid are thoroughly mixed.
      (2) Place the jar in a deep sink which has very hot water running into it. Shake jar occasionally. Air exhaustion is complete when the feces are no longer well formed and a sediment appears in the jar.
   d. Finish the heating process by one of the following procedures:
      (1) Tighten the lid and continue processing the jar in a simmering water bath for 8 to 12 hours or until the contents are dark brown and well digested. At the end of the processing period leave the jar in the water bath until the water comes to room temperature, or remove the jar from the hot water and allow to cool on an asbestos mat.
      (2) Tighten the lid and place the jar in an autoclave at 15 pounds pressure for 3 hours.
   e. Record the weight of the jar, complete with contents, glass lid, rubber ring and metal ring.

The NITROGEN CONTENT of the digest is determined by the method of A.O.A.C. (1950), Meeker and Wagner (1933), Scales and Harrison (1920), or Triebold (1946). CALCIUM is determined by the method of McCruddon (1911); PHOSPHORUS by the method of Fiske and Subbarow (1925).

## COLLECTION, SAMPLING, AND PRESERVATION OF MENSES

**Equipment and supplies provided for the subject by the laboratory**

Sanitary pads or tampons
Small-sized waxed or plastic bags
Paper bags, size 16
Paper clips

**Written instruction given to the subject**

Save all sanitary pads or tampons used. Place each one in a separate waxed or plastic bag. Save one unused pad or tampon from each box from which pads or tampons have been used. (If the laboratory buys those supplies in large quantities, reserve three unused pads or tampons from each large box for blank analyses.)

Label each small bag with your name and the date; fold the top and fasten it with a paper clip. Place in the large paper bag and store in a cool place until collected by the laboratory.

**Preparation of composite in the laboratory**

All sanitary pads or tampons used during the menstrual period are collected and preserved in 20% HCl. At the end of the collection period an acid digest is prepared. Aliquots of this are used for chemical analyses.

1. Sanitary pads

Handle pads with tongs made of special alloy if iron analyses are to be made. Transfer the pads to a 2-liter beaker and cover them with 20% HCl. Cover the beaker with cellophane and refrigerate. Place each day's pads in the beaker, adding additional acid as needed. The pads should be covered with acid but the beaker should not be more than one-half full. Since only 6 to 8 pads can be handled in one lot, it may be necessary to use more than one beaker.

2. Tampons

Proceed as above, except use a 1-liter beaker.

**Preservation for analysis: brown digest**

1. Let all pads or tampons stay at least 24 hours in the acid before heating is started.

2. Heat the beaker containing pads or tampons and acid on an electric hot plate at low heat until the digest is uniform in color and consistency and is reduced in volume. Stir occasionally. Cool.

3. Transfer the contents of the beaker quantitatively to a 2-liter volumetric flask, one-liter if tampons are used, and make up to volume with water. If the final volume for one subject exceeds the one volumetric flask, use two and combine the digests in a large Erlenmeyer flask. An alternate procedure is to combine aliquots, such as one-half, from each volumetric flask used.

4. Mix contents of the flask by inverting and rotating 50 times. Preserve duplicate samples in bottles. Before the second bottle is filled invert and rotate the flask 25 times.

5. Blank determination. Place an unused pad or tampon in a 400-ml beaker. Cover with 20% HCl and allow to stand 24 hours. Heat on an electric hot plate at low heat until the digest is uniform in color and consistency. Cool. Transfer the contents of the beaker quantitatively to a 500-ml volumetric flask. Mix and save duplicate samples as described above.

The NITROGEN CONTENT of the digest is determined by the method of A.O.A.C. (1950), Meeker and Wagner (1933), Scales and Harrison (1920), or Triebold (1946). CALCIUM is determined by the method of McCrudden (1911); PHOSPHORUS by the method of Fiske and Subbarow (1925).

## COLLECTION, SAMPLING, AND PRESERVATION OF URINE

REFERENCES: Association of Vitamin Chemists (1947, 1951); Consolazio *et al.* (1951); Peters and Van Slyke (1932)

**Equipment and supplies provided for the subject by the laboratory**

Suitable containers for the urine:

The following containers have been found satisfactory: (1) pint jars with metal rings and enamel-lined or glass lids when each sample collected during the day is to be kept separate and later combined in the laboratory; (2) wide-mouthed 1- to 3-liter bottles; (3) quart-size, paraffin-lined cartons. See section under *Feces* for lining cartons. Containers should have tight-fitting lids or stoppers so no urine is lost by leaking around the lid. Brown bottles or cartons should be used when the urine is to be analyzed for light-sensitive nutrients.

Receiving receptacle to transfer the voided sample to large collection container. If the urine is not voided directly into the collection container, supply the subject with an 8" funnel or a one-liter glass or stainless steel beaker. Water should be supplied by the laboratory for rinsing the funnel or beaker. The subject should be directed to add the rinsings to the urine in the collection container.

**Written instructions given to the subject**

On the first day of the collection period, empty the bladder immediately after the usual rising time. Record the hour. *Discard this sample only on the first day.* All the following collections plus the collection upon rising the day after the study is finished are to be saved. The urine collected upon rising is considered apart of the urine for the preceding day.

When urinating, sit on the toilet seat and hold the receiving receptacle close to the body. If the urine is transferred to a large container, rinse the small container used for collecting with distilled water provided by the laboratory and add the rinsings to the large collection container.

Store the urine in a cool place until collected by the laboratory.

**Preservatives used for 24-hour sample**

The preservative placed in the collection bottle will depend on the combination of analyses to be made and the analytical methods used. A mineral acid should not be used if the mineral added is one for which analysis is to be made. The purpose of the preservative and any effect it may have on the appearance of the urine should be explained to the subject.

1. Nitrogen and mineral analyses
   a. No preservative. Store urine in a cool place. Samples should be brought to the laboratory at least once a day.
   b. Five to 10 ml of toluene. Store urine in a cool place. Samples should be brought to the laboratory at least once a day.
   c. A minimum of 10 ml of HCl per liter of urine.

2. Nitrogen and ascorbic acid analyses. One hundred grams of metaphosphoric acid sticks. Store urine in a cool place. Samples should be brought to the laboratory at least once a day.

3. Nitrogen, thiamine, riboflavin, and mineral analyses. Fifty milliliters of either 2.5 N HCl or $H_2SO_4$.

4. Nitrogen, ascorbic acid, thiamine, riboflavin, and mineral analyses. One hundred milliliters of a sulfuric acid-metaphosphoric acid-8-hydroxyquinoline solution made as follows: fifty ml of 5 N $H_2SO_4$ and 50 ml of 5% metaphosphoric acid solution containing one ml of 8-hydroxyquinoline solution. (Five percent metaphosphoric acid solution is prepared fresh as needed. 8-hydroxyquinoline solution is made by dissolving 1.45 mg of 8-hydroxyquinoline in 100 ml of 95% ethyl alcohol. Keep in the refrigerator; when the solution turns brown it is no longer effective.)

**Preparation of urine composite in the laboratory**

1. Twenty-four hour sample
   a. Combine the urine collected for the day in a graduated cylinder of a suitable size. Rinse containers with water and add rinsings to cylinder.
   b. If no preservative has been added to the collection containers and the analyses to be made permit, add a minimum of 10 ml of HCl per liter of urine. Add water to bring the volume to the nearest 10 ml. Record volume.
   c. Save duplicate samples. Before filling the first bottle, invert and rotate the cylinder 20 times. Invert and rotate 10 times before the second bottle is filled. If HCl or

$H_2SO_4$ is used as a preservative these samples may be kept indefinitely at room temperature.

2. Experimental period composite

a. Mix the 24-hour sample by inverting and rotating the bottle 20 times.

b. Use various combinations of pipettes or use an appropriate size graduated cylinder to obtain an aliquot of each day's sample. Usually composites cover periods varying from 3 to 7 days and an aliquot of 1 5 is taken. Combine the daily aliquots in a stoppered container of suitable size. Record volume.

c. Save duplicate samples. Before filling the first bottle, invert and rotate the cylinder 20 times. Invert and rotate 10 times before the second bottle is filled. If sufficient HCl or $H_2SO_4$ is used as a preservative these samples may be kept indefinitely at room temperature.

The CREATININE CONTENT is determined by the method of Hoffman (1941) or by the method given in the Klett Summerson Clinical Manual (1946); NITROGEN is determined by the method of A.O.A.C. (1950), Meeker and Wagner (1933), Scales and Harrison (1920), or Triebold (1946). CALCIUM is determined by the method of McCrudden (1911); PHOSPHORUS by the method of Fiske and Subbarow (1925). THIAMINE is determined by the method of Association of Vitamin Chemists (1947) or of Mickelsen *et al.* (1945).

**Special precautions for handling of composite**

1. Riboflavin analysis

Protect sample from light by using low-actinic glassware and or working in a darkened room.

RIBOFLAVIN is determined by the method of the Association of Vitamin Chemists (1947)

2. Ascorbic acid analysis

Because ascorbic acid is unstable, analyze the composite as soon as possible.

ASCORBIC ACID is determined by the method of Evelyn *et al.* (1938) or by the method of Roe *et al.* (1943).

3. Niacin, vitamin $B_6$, 4-pyridoxic acid, N'-methyl nicotinamide analysis

Add HCl until the urine is a pH of 4.5 to 5. Store in the freezer. Protect from light.

N'-METHYL NICOTINAMIDE is determined by the method of Huff and Perlzweig (1947); NIACIN by the method of the Association of Vitamin Chemists (1947); 4-PYRIDOXIC ACID by the method of Huff and Perlzweig (1944) as modified by Sarett (1951). VITAMIN $B_6$ is determined by the method of Atkins *et al.* (1943).

**Preservation for analysis**

If the urine composite is not analyzed within a short time and HCl or $H_2SO_4$ has not been added as a preservative, the samples should be stored in a freezer until needed. An acid-urine digest which prevents the formation of a uric acid precipitate and digests such body cells and proteins as may be present in the urine can be made. The digest is satisfacory for nitrogen and mineral analyses by any of the methods given previously.

1. Freezing urine samples

Place sample bottle in freezer. Bottle should not be more than ¾ full to allow for expansion of the liquid during freezing.

2. Acid-urine digest

a. Transfer 1 5 of the urine composite to an Erlenmeyer flask. Add 100 ml HCl.

b. Heat on a steam bath or hot plate at low heat until the solution is dark brown and evaporated to an amount where it can be made up to the desired volume. Cool.

c. Transfer to a volumetric flask. Make to volume with water.
d. Save duplicate samples. Before filling the first bottle, invert and rotate the flask 20 times. Invert and rotate 10 times before the second bottle is filled. These samples may be kept indefinitely at room temperature.

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