DETERMINATION OF AVAILABLE CARBOHYDRATES IN PLANT AND ANIMAL FOODS

by

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ABSTRACT The principal objectives of this study were to present a choice of several simple and convenient chemical methods whereby the nutritionist may determine the available carbohydrate, either in a single analysis of the sample, or in separate assays of the soluble sugars and starch; to suggest improved methods for calculating the total available hexose (AHex) or carbohydrate (ACho), and their calorie equivalents, from the raw titration or spectrophotometric data; to relate the ACho to the crude carbohydrate, nitrogen-free extract (NFE), and the digestibility coefficient.

Five procedures were described, with the following principal steps: method A, digestion with 1.0 N HCl during 3.0 hours at 95° or 4.5 hours at 90°; method B, digestion with 0.1 N HCl during 60 minutes at 95° or 90 minutes at 90°, followed by enzymatic hydrolysis; method B1, digestion with water during 60 minutes at 95° or 90°, enzymatic hydrolysis; method C, extraction of soluble sugars with 20% ethanol-10% ether (method C_{20}) or with 40% isopropanol (method C_{40}), removal of the alcohol, enzymatic hydrolysis; method D, extraction of soluble sugars by alcohols as in methods C, washing of residue with 20% ethanol (method D_{20}) or with 40% isopropanol (method D_{40}), gelatinization of starch during 60 minutes at 95° or 90°, enzymatic hydrolysis. All hydrolysates were clarified by $Zn(OH)_2$, and reducing sugars (Rs) were determined by reduction of ferricyanide in Na_2CO_3 during 30 minutes at 80°.

The procedures are adapted for determinations at all altitudes up to 2,700 m, at which the temperature of the vigorously boiling water bath is about 90°. At higher altitudes the hydrolysis of native and retrograded starches, particularly the latter, by 1.0 and 0.1 N HCl is very greatly delayed, making the determination unfeasible.

Enzymatic hydrolysis is carried out at pH 4.7 during 6 hours at 50° with Rhozyme-S, a standardized, sugar-free, commercially available preparation from *Aspergillus oryzae*. Criteria for the choice of enzyme were summarized. Standardization of the preparation in terms of maximal Rs yield was described. Rhozyme-S quantitatively, or almost so, hydrolyzed native starches, glycogen, sucrose, maltose, cellobiose and lactose. The activity toward other saccharides was discussed. Retrograded starches gave low Rs yields with Rhozyme-S (method B1); preliminary hydrolysis with 0.1 N HCl (method B) increased the enzymatic yield of Rs, which paralleled Rs yields by hydrolysis with 1.0 N HCl (method A), but near quantitative yield was never attained from retrograded starches. Native pentosans in plant samples are apparently firmly bound within the plant structures and are not hydrolyzed by Rhozyme-S until liberated by digestion at 90° with acids or buffers at pH 3 or less. Thus, method B1 apparently did not yield Rs from native pentosans in plant samples, whereas the average by method B was about 64%; the respective average degrees of hydrolysis of isolated hemicelluloses were 51 and 77% by methods B1 and B.

Methods D_{20} and D_{40} represent a complete revision of old and current procedures. The lengthy preliminary extraction of fat by ether and several concentrations of ethanol is eliminated. The degree of precipitation, or extraction, of starch and its oligosaccharides depends upon the kind of alcohol used, the concentration of alcohol, the presence of

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electrolytes which accelerates precipitation, the presence of a flocculating agent which completes the precipitation, the temperature, and the duration of extraction or precipitation. Precautions were given for preventing retrogradation during the analysis. Method D_{20} determines only the hydrolyzable starch of high molecular weight; even very slight depolymerization reduces the yield, and the filtrates may give a deep blue color with I-KI. Therefore, method D_{20} should not be applied to prepared foods except as a sensitive indicator of dextrination during processing. Commercial dextrins yield only a small amount of hydrolyzable precipitate in 40% isopropanol. However, method D_{40} assays most of the oligosaccharides of extensively dextrinized cereals, such as puffed rice and puffed wheat; the filtrates contain only traces of oligosaccharides giving blue or violet color with I-KI. Methods D_{20} and D_{40} gave identical results in unheated plant materials and foods. In all samples, the soluble sugars by methods C_{20} and C_{40} were reciprocally related to the starch by methods D_{20} and D_{40}, and both sets of determinations were related to the results by method B1, as follows: $B1 = C_{20} + D_{20} = C_{40} + D_{40}$.

Methods were given for calculating AHex, expressed as glucose, and ACho representing the sum of available carbohydrates, consisting of starch, sucrose, etc., that may have been present in the original, unhydrolyzed sample; AHex = Rs × conversion factor; and ACho = Rs × conversion factor. From these calculations, the caloric equivalent may be estimated, thus: kcal of AHex = AHex × 3.743; kcal of ACho = ACho × kcal conversion factor. Thus the available energy was determined with greater ease and accuracy from the AHex or ACho than by the conventional method for estimating metabolizing energy of crude Cho which requires (a) assays of moisture, crude protein, crude fat, and crude ash; (b) use of assumed factor 6.25 × N for calculating crude protein; (c) bomb calorimetry; (d) use of approximate factors 5.65 and 9.4 for calculating heats of combustion of protein and fat; and (e) determination of human digestibility. The calculations were shown to apply whether Rs was determined with 0.1, 0.04, 0.004, or 0.001 M FeCy. The accuracy of calculations was demonstrated by analyzing known mixtures of carbohydrates. Tables of conversion factors for representative human foods were given. General factors were suggested for calculating AHex and ACho of whole human diets.

The methods were applied to 4 animal feeds and roughages; 3 samples consisting of corn cobs, oat hulls and green whole oats; 33 natural and prepared human foods; 2 series of 11 and 17 samples each of composites of whole human diets; and a series of 4 composites of U. S. Army C-Ration. In 7 breakfast cereals and mixes and 4 whole grains, containing 0.6 to 3.0% crude fiber (CF) and 0.2 to 5.9% pentosans (P), total carbohydrates (TCho) = CF + P + ACho by method B1. TCho = crude Cho within 96.5 to 102.0%, average 99.5%. The calculated TCho gives a better proximation of the total carbohydrate content in animal and human foods than crude Cho. Metabolizable crude Cho = crude Cho − (3 × CF) is suggested as a better approximation of the metabolizable Cho than NFE in human foods. The effect of heat processing on the carbohydrates of cereal grains was discussed.

In the analysis of single human foods, the apparent recovery of ACho by method B, 100 × [ACho/crude Cho], approximated the upper limits of human digestibility of crude Cho of similar foods; the recovery by method B1 generally approximated the lower limits of digestibility. In the 3 series of analyses of whole human diets, the average recoveries of ACho by method B were 94.2 ± 3.1 (SD), 98.0 ± 6.7, and 94.2% of the crude Cho; the average metabolizable energy per gram of crude Cho in the 3 series was 3.805 ± 0.136, 3.928 ± 0.268, and 3.825 kcal. Use of 3.85, rather than 4.0, kcal/g crude Cho is recommended for calculating carbohydrate energy in whole human diets.

INTRODUCTION

The problem of determining the carbohydrates of foods and plant and animal tissues that are available for metabolism in the animal has engaged the nutritionist's attention for more than a century, during which 3 general methods have been developed: first, indirect estimation by difference, using the Weende chemical methods; second, biological determination of digestibility coefficient, in which the Weende chemical methods are used to analyze the feces and ingested food; and third, direct chemical determination of carbohydrates, either single or by categories.

The first 2 procedures were suggested by Henneberg (1, 2) and Henneberg and Stohmann (3, 4) who applied them in nutritional balance experiments with ruminants at the Versuchs-Station Weende-Göttingen (5) during 1859–1861. In a continuation of this work, Weiske (6) determined the digestibility of crude fiber (CF) by humans. The authors set up a system of classification of the principal nutrients; they introduced refinements in the determination of moisture, ash, urea, and total nitrogen, and they recommended the factor 6.25 for calculating protein, N $\times$ 6.25; they described the present-day method of determining CF; they used anhydrous ether for extracting fat; and they noted precautions for collecting and preserving samples in animal feeding experiments. The crude carbohydrate (Cho) and the nitrogen-free extract (NFE) were calculated as follows:

(1) % Crude Cho = 100 − [% crude protein + % crude fat + % H_2O + crude ash],

(2) % NFE; presumably digestible or metabolizable Cho } = % crude Cho − % CF.

Henneberg and Stohmann recognized the proximate character of the analyses by designating the nutrients "Rohprotein," "Rohasche," and "Rohfazer"; they realized that the accuracy of the calculations was affected by the cumulative errors of the several chemical methods—4 in the calculation of crude Cho, and 5 in the case of NFE; and their results showed that only 39 to 67% of the NFE was digested or not recovered.

Atwater (7, 8), Krauch (9), König (10), Tollens (11), Browne (12), Maynard (13–15) and others severely criticized the procedures and expressed the need for direct chemical methods for total and metabolizable carbohydrate. In later years, Henneberg, quoted by Tollens (11), had stated that "the present method of food analysis is in urgent need of improvement. . . ." Maynard noted that "no group of nutrients that makes up 40% of the total (nutrients in animal feeds) should be determined by difference." In human foods, carbohydrates may make up an even greater percentage of the total nutrients. However, the proximate Weende methods still provide all of the data for crude Cho and CF in the standard tables of composition of human foods, from which the presumably metabolizable carbohydrate, the NFE, is further calculated by difference; they still provide the data for the presently accepted values for the digestibility coefficients of human foods.

The adherence to tradition has been aided no doubt by adoption of the Weende methods as "official" methods (16); and it has perhaps contributed to the lack of interest in the third category of methods for determining the available metabolizable carbohydrate which, in the human dietary, consists principally of glucose and fructose and the readily hydrolyzable carbohydrates, starch or glycogen, sucrose, lactose, and maltose. Cellulose and the hemicelluloses are not usually classified as metabolizable by humans. Yet 20 to 80% (6, 17–25) of the crude fiber and pentosans[2] in the diet is not recovered in the

[2] For the purpose of approximating the hemicellulose content of human diets, the ratio of CF to pentosan may be considered to be approximately 1:1, although it may differ greatly in individual foods. Thus, the relative quantity of pentosan (CF = 1) in the fodders and whole grains in the present work varied from 0.4 to 3.0. The data of Watt and Merrill (26) and McCance and Lawrence (27) indicate the following relative quantities of pentosans (CF = 1): in 21 vegetables, the range is 0.3 to 1.3, average 0.85; in 19 fruits, the average is 1.36. Fruit juices contain only traces of pentosans (27).

[3] In unpublished experiments with human subjects at the U. S. Army Medical Research and Nutrition Laboratory, in cooperation with one of us (N.F.W.), a similar loss of recovery was observed when crystalline cellulose was given in the diet over an extended period of time. The number of fecal cellulolytic bacteria increased greatly during the experiment.

feces[3] and, therefore, this unrecovered fraction is included not only in the calculation of the digestibility quotient but also of the energy value of the food (14, 15, 25).

The chemical methods differ considerably with respect to details of technique, depending upon the choice of properties upon which the individual methods are based. For example, Maercker (16, 28) gelatinizes and hydrolyzes starch in 2 successive treatments, in each of which the sample is boiled and then incubated with malt extract, after which the sample is further hydrolyzed by HCl to reducing sugars; Mannich and Lenz (29) and Hopkins (16, 30) extract and slightly hydrolyze the starch with hot, concentrated acidified $CaCl_2$ solution and then determine the optical rotation of the soluble starch. The other metabolizable carbohydrates may be determined individually or by groups, based upon similar combinations of several of their salient properties. The sum of all of these assays, expressed as reducing sugar (Rs) or glucose, has been designated "available carbohydate" (27, 31–35).

Some authors (31–33, 36–40) hydrolyze the whole sample, making only one analysis:

(3) Available Cho = Rs from mono-, di-, polysaccharides, etc., after enzymatic hydrolysis of the whole sample.

The available carbohydrate is determined more specifically by 2 assays:

(4) Available Cho = [Rs from soluble sugars] + [Rs from starch].

A large series of such determinations on native and prepared human foods is summarized by McCance and Lawrence (27) and McCance and Widdowson (35).

The complete hydrolysis of starch and soluble sugars to Rs has been obtained by several methods as follows: by acid hydrolysis (16, 41–45); with malt extract, followed by acid hydrolysis (16, 28, 43, 44, 46, 47); with Taka-Diastase, followed by acid hydrolysis (31–33, 36, 38, 39, 43); with malt extract alone (37) or with Taka-Diastase alone (48–52). Hydrolysis by acid, which was introduced by Siegert in 1865 (41) and is still used in some of the present "official" methods (16, 42), destroys about 3 to 4% of glucose and at least 50% of fructose (43, 48); therefore acid hydrolysis cannot be used when the sample contains labile sugars. Because of this loss, starch should be calculated approximately as 0.93 $\times$ Rs (35, 37, 38, 42, 44, 45, 47, 53–55), instead of 0.90 $\times$ Rs (16).

The use of malt extract, which was introduced by Maercker in 1885 and is still used today in this country in many "official" enzymatic methods (16), has many disadvantages: the extract must be prepared fresh; it contains considerable reducing sugar, varying in each extract and requiring large blank corrections (43); the content of amylases and other enzymes is variable, depending upon the conditions of preparation of the malt grain and the extract; and the hydrolysis of starch is apparently incomplete, requiring additional digestion or boiling with HCl which destroys about 3 to 4% of the Rs. Taka-Diastase, on the other hand, is a commercially available, stable, readily soluble, powdered preparation with high amylase activity. It converts starch quantitatively into glucose (31, 40, 51, 52, 56), provided the following conditions are observed as pointed out by Horwitt et al. (40): (i) presence of excess of enzyme, and (ii) low concentration of substrate and the resulting Rs. Taka-Diastase has considerable activity towards "wood starch" or isolated hemicelluloses (56, 57); yet, in the determination of starch in animal feeds and fodders which are rich in hemicelluloses, Fraps (43) obtained slightly lower results with Taka-Diastase in 8 of 11 samples than with malt extract which is presumed to have minor action on hemicelluloses in the method (16) used.

In the present paper, we shall present 5 procedures, employing either acid hydrolysis or enzymatic hydrolysis, or a combination of the two. In all methods, Rs is determined by ferricyanide (FeCy) reduction (58) after clarification of the digest by $Zn(OH)_2$ (59). The principal purpose is (i) to present a choice of simple and convenient methods whereby the nutritionist may determine the available Cho in a single assay by methods A, B, or B1 (as in equation 3), or in separate assays of the soluble sugars and starch by methods C and D (as in equation 4); (ii) to suggest

improved methods for calculating the available Cho and its calorie equivalent from the raw titration or spectrophotometric data; (iii) to relate the available Cho to the crude Cho, the NFE, and the digestibility coefficient. The salient and novel features of each method will be noted and commented upon briefly.

Method A gives the highest Rs yields from plant materials of any of the 5 methods; it indicates the maximal available Cho, provided the sample contains no fructose and other labile sugars. The whole sample is hydrolyzed by 1.0 N HCl (55) in a constant-temperature water bath. Thus, reproducible conditions are maintained—unlike the Sachsse method (16, 42), in which the digestion mixture, consisting of approximately 10 volumes of water and approximately one volume of 1.125 sp gr HCl (approximately 7.8 M), yielding approximately 0.7 N HCl, is boiled under a reflux condenser or is heated in a boiling water bath. Our hydrolysis with 1.0 N HCl is carried out during 3 hours at 95°, or 4.5 hours at 90°. Thus, the method may be used at higher altitudes up to 2,700 m. At 90°, the yield of Rs from starch is approximately the same as that given by method B, and there is no apparent marked loss of Rs as in the Sachsse method at 100°. Although the method is not recommended for general use in food analysis, it has been used in the present work for the following principal reasons: (i) to demonstrate that 1.0 N HCl-hydrolyzability differs greatly among starch preparations and that this parallels enzymatic hydrolyzability; (ii) to show that this parallel relation is not found with dextrans, celluloses, hemicelluloses, and pectins, some preparations yielding more Rs with the enzyme than with HCl, others yielding more Rs with HCl; and (iii) to obtain data on the maximum yield of Rs in the analysis of plant samples.

Method B yields somewhat less Rs from plant materials than method A; it determines the maximal available Cho under mild conditions of hydrolysis as follows without apparent loss of fructose and other labile sugar.

(i) The sample is digested with 0.1 N HCl at 90 or 95° until the starch-iodine reaction becomes almost negative, after which the HCl is neutralized. Fat does not apparently interfere in the analysis; therefore, the sample need not be extracted with fat solvents prior to digestion, as has been considered necessary in previous methods. The digestion converts the starch gel within the cell into smaller, more readily diffusible particles; it hydrolyzes sucrose; and at the end of digestion it yields an approximately neutral, almost sterile digest. (ii) The saccharides are hydrolyzed at pH 4.7, during 6 hours at 50°, by a single digestion with a commercially available, standardized enzyme preparation of low Rs content; thus, without large blank correction, it is possible to add sufficient enzyme to convert starch, sucrose, lactose and maltose completely into Rs.

Method B1 yields less Rs from plant materials than either methods A or B; the yield agrees closely with *the sum* of the Rs which is obtained by the separate assays of the hydrolyzable soluble carbohydrates and starch by methods C and D. The distinctive features of method B1 are: (i) digestion of the sample with water at 90° or 95°, which gelatinizes and mobilizes starch from the plant structures but apparently does not liberate the pentosans; and (ii) enzymatic hydrolysis and determination of Rs as in method B.

Method C determines the Rs which is derived from the carbohydrates that are soluble in alcohol solutions in the presence of electrolytes and a flocculating agent; method D determines Rs derived from the insoluble fraction. The distinctive features of method C are: (i) extraction of fatty substances by absolute ethanol or isopropanol; (ii) extraction of carbohydrates by exactly 20% ethanol or 40% isopropanol by volume, in the presence of 1% NaCl and Celite; (iii) removal of alcohol by boiling; and (iv) enzymatic hydrolysis and determination of Rs as in methods B and B1.

The essential steps in method D are: (i) and (ii) as in method C; (iii) filtration and washing of the precipitate on the filter paper with 20% ethanol or 40% isopropanol in 1% NaCl; (iv) quantitative transfer of the precipitate to the original vessel by means of water; (v) gelation of starch by

heating in a water bath at 90° or 95°; and (vi) enzymatic hydrolysis and determination of Rs as in methods B, B1, and C.

Gelatinized starch is precipitated and recovered almost quantitatively under the conditions of methods C and D; therefore, the methods should be useful in the analysis of prepared foods and diet composites. However, slight hydrolysis or dextrination reduces the yield of starch and reciprocally increases the soluble sugars; therefore, methods C and D should be useful for indicating slight changes in the metabolizable carbohydrates during the home- and factory-preparation of foods.

From this brief description of methods, it is evident that we are presenting 2 schemes for enzymatically assaying available Cho; (i) method B, which employs acid hydrolysis and enzymatic hydrolysis and determines some other readily hydrolyzable carbohydrates besides soluble sugars and starch, and (ii) methods B1 and C and D which determine principally the soluble sugars and starch.

Which of these 2 schemes most nearly approximates the metabolizable carbohydrate for man? Unfortunately, this question cannot be answered at present with any degree of certainty because of the crudeness of the Weende biological method for determining metabolizable carbohydrate, which is affected not only by the accuracy of methods for collecting the samples of food and feces and analyzing them for crude carbohydrate, but also by the variable and considerable bacterial digestion and metabolism in the human gut. No biological method has been devised for assessing the contribution of bacterial processes; none has been described for accurately estimating the carbohydrate (or its bacterial metabolic products) which has been actually absorbed and is available for metabolism. In the absence of such methodology, the Henneberg digestibility coefficient[4] of the crude carbohydrate (by difference) has continued to be the accepted measure of availability, and this is based upon the assumption that

(5) Crude Cho of food not recovered in feces = digested Cho = absorbed Cho = metabolizable Cho.

In this paper, we will show that the "available Cho" as determined by method B approximates the upper range of values of the metabolizable Cho as determined by the biological method; the results by method B1 and methods C + D approximate the lower range.

METHODS

Reagents

Hydrochloric acid solutions, exactly 0.7 N and exactly 2 N.

Sodium hydroxide solutions, exactly 0.7 N and approximately 0.5 N.

Sulfuric acid solution, approximately 0.5 N.

4 M Acetate buffer. Dissolve 330 g anhydrous sodium acetate in 1,000 ml water. Add exactly 240 ml (or 251 g) glacial acetic acid; cool and dilute to 2,000 ml.

0.4 M Acetate buffer, pH 4.7 to 4.8 when diluted to 0.04 M (determined by glass electrode).

Enzyme preparation: Rhozyme-S concentrate, Factor 4 (manufacturer's activity factor), with low reducing sugar content.[5] Protect from moisture and strong light, and store in the refrigerator. Before use each day, prepare a 2% solution in water. If the activity factor is greater than 4, or less than 4, then dissolve proportionately less, or more, of the enzyme.

Zinc sulfate solution, 10% solution of $ZnSO_4 \cdot 7H_2O$.

Absolute ethanol.

Ethanol-ether mixture. Add 1,000 ml absolute ethanol to 500 ml absolute ether.

20% Ethanol-1% NaCl solution. Dilute 200 ml absolute ethanol and 10 g NaCl with water to 1,000 ml at about 20°.

Isopropanol, 99 to 100%. USP or reagent grade.

40% Isopropanol-1% NaCl solution. Dissolve 10 g NaCl in water; add 400 ml isopropanol on the basis of 100% purity; dilute to 1,000 ml at about 20°.

[4] Merrill and Watt (25) have reviewed the literature up to 1955 pertaining to the basis and derivation of digestibility and available energy of foods. Their table 23 lists the results of 902 determinations of apparent digestibility of the crude Cho (by difference) by man.

[5] The preparation was obtained through the courtesy of Dr. C. V. Smythe. It may be purchased as Rhozyme-S, High Potency Concentrate, low reducing sugar content, from the Rohm and Haas Company, Special Products Division, Philadelphia.

Celite analytical filter-aid.[6]

Talcum.

2-Octanol (capryl alcohol), ketone-free, BP 178-180°.[7]

Phenolphthalien indicator, 1% solution in 70% ethanol.

Reagents for determining reducing sugars, as described previously (58): 0.04 M ferricyanide (FeCy) in 4% anhydrous Na_2CO_3; 20% KI solution; $ZnSO_4$-acetic acid reagent; starch indicator; 0.01 N thiosulfate solution; and glucose standard solution.

Apparatus

Weighing funnels, glass, 15 × 45 mm.[8]

J-rods. Glass rods, 3-mm diameter and 300-mm long, shaped at one end to fit the round bottom of 25-mm diameter test tubes.

Test tubes, 29 × 200 mm, calibrated at 35- and 50-ml capacity, used for acid and enzymatic digestions of samples and for determination of reducing sugars; 32 × 200 mm test tubes, calibrated at 50- and 75-ml capacity, used in the determination of starch.

Test tube racks (58, 59).

Kohlrausch flasks, 100-ml capacity, fitted with rubber stoppers.

Water baths, electrically heated, thermostatically controlled within ± 1°; fitted with electric stirrer, 35.5 × 61 cm overall dimensions.

Cooling bath, with running water, preferably at 20 to 25°.

Method A, hydrolysis of whole sample by 1.0 N HCl

Do not use this method for analysis of samples which contain sucrose, fructose, or hemicellulose. It may be used for analysis of starch, or other hydrolyzable carbohydrates which yield equally stable monosaccharides, whenever HCl hydrolysis is recommended in "official" methods (16).

To a 250- to 1,000-mg sample in a 29 × 200 mm test tube, weighed and transferred as in method B, add 25 ml water and 25 ml of exactly 2.0 N HCl. Insert a J-rod; mix by rotating the rod. Incubate 3 hours in the water bath at 95°, or 4.5 hours in the bath at 90°. Mix the contents at intervals; add water as needed to maintain the 50-ml initial volume. Cool; quantitatively transfer the contents to a 250-ml volumetric flask. Carry out the $Zn(OH)_2$ precipitation and Rs determination as in method B. Prepare several reagent blanks.

Method B, enzymatic hydrolysis of whole sample, preceded by hydrolysis with 0.1 N HCl

Transfer a 250- to 1,000 mg sample to a 29 × 200 mm test tube. For samples of less than 500 mg use a weighing funnel, allowing the funnel and sample to slide to the bottom of the tube. The sample should contain not more than 400 mg available Cho. If the sample is acid, add a previously determined volume of 0.1 N NaOH sufficient to bring the reaction to pH 4.5, using an appropriate indicator. Add 10 to 15 ml water; insert a J-rod and thoroughly mix and suspend the sample by rotating the rod. Add exactly 5 ml 0.7 N HCl; adjust the volume to 35 ml, plus 1.5 ml to allow for the volume of the weighing funnel. Incubate 60 minutes at 95°, or 90 minutes at 90°[9], or for a period of time at which starch gives only a faintly violet color with I-KI reagent; mix the contents at intervals. Add water as needed to maintain the acidity at approximately 0.1 N. Prepare several reagent blanks.

Cool; neutralize by adding exactly 5 ml 0.7 N NaOH; mix. Add 5 ml 0.4 M acetate buffer and 5 ml 2% enzyme solution; mix again. Incubate 6 hours at 50°, mixing the contents at intervals of 15 minutes or so; cool.

Quantitatively transfer the contents to a 250-ml volumetric flask. Add 10 ml $ZnSO_4$ solution,[10] 2 to 3 drops phenolphthalein indicator, and 1 to 2 drops octanol. Then,

[6] Johns-Manville, New York.

[7] Distillation Products Industries, Rochester, New York.

[8] Similar to no. 30287, Van Waters and Rogers, Inc., Denver, Colorado, or no. 12803, New York Laboratory Supply, Inc., New York.

[9] This period of incubation is recommended at the altitude of Boulder and Denver, 1,600 m, at which water boils at about 95°. At the altitude of Chicago, or at sea level, 25 to 30 minutes incubation in the boiling bath (99-100°) should give comparable hydrolysis of starch.

[10] $Hg(OH)_2$ precipitation is recommended for use with samples that contain small quantities of sugars in the presence of very large quantities of amino acids and other interfering substances (60).

while rotating the flask, rapidly add 0.5 N NaOH until precipitation of $Zn(OH)_2$ begins; thereafter carefully add the alkali until the contents are faintly pink. Wash down the sides of the flask and add 0.5 N H_2SO_4 drop-by-drop until the solution is colorless; dilute to the mark; let stand 10 minutes or so, mixing frequently; filter. Store the sample solution in the refrigerator. Analyze within one week, preferably within 48 hours. Prepare blanks that have been carried through the same procedure.

The determination of Rs by FeCy reduction is preferred. Transfer exactly 2, 3, 4, or 5 ml of sample and blank solutions to the bottom of 29 × 200 mm test tubes, covered with small beakers or, preferably, with large glass bulbs. Add 3, 2, 1, or zero ml water from a pipette to bring the volume to exactly 5 ml; add exactly 5 ml 0.04 M FeCy reagent; mix immediately by gently rotating the tube; incubate exactly 30 minutes in a water bath at 80°. Rapidly cool in a bath of running water, preferably at 20 to 25°. Prepare blanks with 5 ml water in each set of analyses. The greatest accuracy is obtained with a volume of sample solution which contains 3 to 3.5 mg glucose, resulting in approximately 50% reduction of FeCy. Care and accuracy in measurement of the reagent is important since any errors are increased many-fold in the determination of residual FeCy.

Remove the cover; add 1 ml KI solution and 5 ml $ZnSO_4$-acetic acid reagent, mixing by gentle rotation after each addition. To prevent loss of iodine, cover the tubes immediately after adding the $ZnSO_4$-acetic acid reagent, and keep the tubes covered until ready for titration. Let stand *at least* 20 minutes,[11] during which time the contents are mixed twice again. Titrate with 0.01 N thiosulfate until almost colorless, adding the first few milliliters around the sides of the tube to prevent loss of iodine vapors. Now add about 0.5 ml starch indicator; wash down the walls with a fine stream of water, and continue the titration drop-by-drop until the color is pure white.

Alternate spectrophotometric procedure. Since phenolphthalein interferes slightly in the spectrophotometric determination of residual FeCy, carry out the $Zn(OH)_2$ precipitation without the indicator. To the enzymatic digest in the 250-ml volumetric flask, add 10 ml $ZnSO_4$ solution and sufficient 0.5 N NaOH to bring the reaction to about pH 7.5 (red to phenol red, and colorless to phenolphthalein indicator). Determine the volume of 0.5 N NaOH to be added by titrating blanks which contain 5 ml 0.4 M acetate buffer and 10 ml $ZnSO_4$ solution. If all reagents are accurately measured, 12.4 ml of exactly 0.5 N NaOH are required. Dilute to the mark; mix; filter.

Carry out the FeCy reduction as already described. Dilute the FeCy reaction mixture to 250 ml; determine transmittance at 418-mμ wave length, using 1-cm square Corex cuvettes. Prepare color blanks if needed.

This alternate procedure is not recommended for general use because of the uncertainties in the correction for colored substances that may be present in filtrates of food samples or colored plant materials.

Number of Rs determinations; standardization. Make duplicate Rs assays; repeat; calculate the average of the 4 assays. Standardize the procedure, using 2, 2.5, 3.0, 3.5, 4 mg glucose in 5 ml standard solution containing 40, 50, 60, 70, 80 mg/100 ml.

Method B1, enzymatic hydrolysis of whole sample, preceded by digestion with water at 90 or 95°

To the sample in a 29 × 200 mm test tube as in method B, add water to the 35-ml mark; mix thoroughly to assure complete suspension. Heat 60 minutes in the boiling water bath, mixing frequently with the J-rod. If the sample shows any tendency to "explode" or foam over, add 1 to 2 drops octanol; digest at 90°. Cool and, without delay, carry out the enzymatic hydrolysis. Storage in the refrigerator overnight or longer may significantly decrease the yield of Rs from the gelatinized starch. Add acetate buffer and enzyme, and proceed further as in method B. Prepare several reagent blanks.

[11] The importance of allowing sufficient time for completion of the reaction of residual FeCy with iodide ion was not fully appreciated in previous work. Apparently, the reaction proceeds slowly in the presence of sugar-oxidation products and other substances in sample extracts, unlike the almost immediately complete reaction in a water blank. The concentration of KI is important. Return of the blue starch-I-KI color after completion of the thiosulfate titration indicates that insufficient KI has been added.

Method C_{20}, determination of soluble carbohydrates, using 20% ethanol

Transfer a 500- to 5,000-mg sample, containing not more than 800 mg soluble sugars, to a dry 100-ml volumetric flask or Kohlrausch flask. Add 1 g NaCl; add 20 ml absolute ethanol from a pipette; let stand about 10 minutes, mixing frequently by rotating the flask to allow extraction of fatty substances. Add sufficient water to bring the volume to about 90 ml (about 22% ethanol). Keep 60 minutes in a water bath at about 20°, mixing frequently by rotation. Add water to the mark; mix; again adjust the volume to the mark; mix. Add about 200 mg Celite; continue the extraction 30 minutes (thus a total of 90 minutes) at about 20°, mixing frequently. Filter through a 15-cm diameter Whatman no. 54 filter paper. Cover the funnel to prevent evaporation of alcohol. Prepare several reagent blanks.

Transfer 50 ml of filtrate (at 20°) to a 250-ml volumetric flask. Indicate the volume in the flask by a grease-pencil line or scratch mark. Add a small amount of talcum, 1 to 2 drops octanol, and 50 to 60 ml water. Remove ethanol by boiling on an electric hot-plate in a well-ventilated hood; continue boiling until the volume is 40 to 45 ml; cool. Watch the flask constantly during boiling, adding 1 to 2 drops octanol whenever foaming is noted.

Add 5 ml 0.4 M acetate buffer and 5 ml enzyme preparation; incubate 6 hours in a water bath at 50°. If the contents of the flask contain less than 50 mg carbohydrates, then 3 hours incubation will suffice. Carry out the $Zn(OH)_2$ precipitation and Rs determination as in method B.

Method C_{40}, determination of soluble carbohydrates, using 40% isopropanol

Proceed as in method C_{20}; but, from a pipette, add 40 ml isopropanol (on the basis of 100% purity) instead of ethanol to the sample in a dry 100-ml volumetric flask or Kohlrausch flask.

Method D_{20}, determination of starch, using 20% ethanol

To a 250- to 1,000-mg sample in a 32 × 200 mm test tube, containing not more than 400 mg starch, weighed and transferred as in method B, add 500 mg NaCl; add 15 ml ethanol-ether mixture. Insert a J-rod; suspend the sample and mix thoroughly by rotating the rod; let stand about 10 minutes, mixing frequently. Add water to the 50-ml mark; let stand 60 minutes in a water bath at 20°, mixing frequently to assure solution of the soluble carbohydrates. Add 200 mg Celite and continue the extraction 30 minutes (thus a total of 90 minutes) at 20°, mixing frequently. Similarly, prepare blanks.

Pour the contents onto a 15-cm diameter Whatman no. 54 filter paper,[12] letting the tube drain well. Allow the liquid to drain completely from the filter paper; then transfer the remaining solids in the tube as completely as possible to the filter, using 20% ethanol-1% NaCl solution which has been cooled to 15 to 20°. Save the tube and J-rod. Wash the paper and contents with small volumes of the cooled alcohol-NaCl solution, allowing the contents to drain after each washing. Use about 150 ml of the cooled alcohol-NaCl solution for transfer and washings. Keep the funnel covered throughout.

With cool distilled water, transfer the residue from the filter paper to the original test tube. The quantitative removal from the filter paper requires care and previous practice. To remove the last trace of sample, rub the entire surface several times with a rubber policeman, which is followed each time by washing with fine jets of water. Finally, wash the entire surface and the funnel. The volume of water in the test tube should not exceed 75 ml. At this point, the analysis may be delayed 24 to 48 hours without significant loss of digestibility. Keep the tube in the refrigerator.

Add 1 to 2 drops octanol; heat 60 minutes in a water bath at 95° or higher temperature, mixing frequently, especially during

[12] Filtration on a Gooch crucible is not recommended because the rate of filtration of most samples is extremely slow through the asbestos mat. Filtration through Whatman no. 54 filter paper is preferred. Thus, precipitates from gelatinized or degraded starches filter rapidly, yet the paper appears to retain fragmented or very small starch grains. The precipitates and washed residues do not stick to the hardened smooth surface and, therefore, they are readily removed. The paper has a high wet strength, which allows repeated rubbing and washings of the surface to remove the last traces of precipitate without pulping or shedding fibers into the washings.

the first 10 minutes. Particles of samples which contain much starch, such as ground grains, wheat bran, and breakfast foods may "explode" and the contents may suddenly foam over. Digest at 90° if the sample exhibits any tendency to foam over; this is the lowest recommended temperature. Cool the tube, and *without delay carry out the enzymatic hydrolysis.* Storage in the refrigerator overnight or longer may significantly decrease the yield of Rs from the gelatinized starch.

Add 5 ml 0.4 M acetate buffer and 5 ml enzyme solution; incubate 6 hours in a water bath at 50°. Then carry out the $Zn(OH)_2$ precipitation and Rs determination as in method B.

Method D_{40}, determination of starch using 40% isopropanol

Proceed as in method D_{20}; but, to the sample in the test tube, add 20 ml isopropanol (on the basis of 100% purity) from a pipette instead of 15 ml ethanol-ether mixture. Use cooled 40% isopropanol-1% NaCl solution, instead of 20% ethanol-1% NaCl, for transfer of the sample from the test tube to the filter paper and for washing the residue on the paper.

This method is preferred over method D_{20} in the analysis of starches and heat-processed foods in which the starch grains may have been disrupted and the starch may have been gelatinized and perhaps depolymerized to a considerable extent.

Calculations

Reducing sugar, uncorrected Rs by methods A, B, C, D. Let Rs = reducing sugar expressed as glucose; T = ml difference between 0.01 N thiosulfate titration of blank and sample solution; a = mg glucose equivalent per ml thiosulfate, i.e., mg standard glucose/T; V = ml final volume of digest which is clarified with $Zn(OH)_2$ (250 ml in the present work); v = ml aliquot of $Zn(OH)_2$ filtrate taken for analysis; and W = mg weight of sample. Then,

$$(6) \qquad \% \text{ Rs} = \text{aT}\left[\frac{100\text{V}}{\text{v W}}\right];$$

or

$$(7) \qquad \% \text{ Rs} = \text{bA}\left[\frac{100\text{ VX}}{\text{v W}}\right],$$

in which A = difference between absorbances obtained with blank and sample solutions; b = mg glucose equivalent per unit absorbance and dilution, i.e., mg standard glucose/*AX*; X = dilution factor, i.e., volume to which FeCy reagent has been diluted/ml FeCy reagent used. In the recommended procedure, X = 50.

Available hexose; Rs corrected for recovery from starch and reduction of FeCy by galactose. The further calculations are based upon the following assumptions: (i) that starch (or glycogen), lactose, sucrose, and maltose are the principal hydrolyzable sugars in foods, and that glucose and fructose are the principal preformed monosaccharides; (ii) that lactose, sucrose, and maltose are hydrolyzed completely, but that starch yields an average of 97.5% calculated Rs; (iii) that glucose, fructose, and galactose are the principal reducing sugars in the enzymatic digest; (iv) that galactose is derived solely from lactose; and (v) that the reductions given by galactose and digest of lactose are, respectively, 78% and 88% as great as an equal weight of glucose (58).

Let AHex = available hexose, consisting of glucose, fructose and galactose, which are derived from the preformed monosaccharides and the hydrolyzable carbohydrates. Let St, Lac, Mal, Suc, Gal, Glc and Fru = the respective fractions of the total carbohydrate mixture in the food, consisting of starch, lactose, maltose, sucrose, galactose, glucose, and fructose (or invert sugar). These respective fractions may be calculated readily from recipes, standard tables of food composition, and other published data, as discussed in considerable detail in a later section. Generally,

$$(8) \quad \% \text{ AHex} = \% \text{ Rs}\,[1 + 0.1364\ \text{Lac} + 0.2821\ \text{Gal} + 0.0256\ \text{St}].$$

In dairy products, without added sugars, Lac approaches 1.00 and equation 8 then becomes

$$(9) \quad \% \text{ AHex of dairy products} = 1.1364 \times \% \text{ Rs}.$$

In grains, fodders, and other plant materials, which contain only small quantities of soluble sugars, St approaches 1.00 and equation 8 then becomes

$$(10) \quad \% \text{ AHex of plant materials} = 1.0256 \times \% \text{ Rs}.$$

The energy equivalent of AHex of 100-g sample is simply calculated:

(11) AHex in 100-g sample, kcal = 3.743 × % AHex.

Examples of calculations are given in column 14, table 1.

Comparison with Weende methods; available carbohydrates in original unhydrolyzed sample. If it is desired to express Rs in terms of the whole mixture of available carbohydrates, consisting of starch, sucrose, etc., that may have been present in the sample *before* hydrolysis, then the procedure is as follows. Let ACho = sum of all available mono-, di-, and polysaccharides. As before, let St, Lac, Suc, Mal, Glc and Fru = the respective *fractions* of starch, lactose, sucrose, etc., of the available saccharides. Then,

(12) % ACho = % Rs [1.080 Lac + 0.950 (Suc + Mal) + 1.000 (Glc + Fru) + 0.923 St],

or

(13) % ACho = % Rs × K_1.

K_1 is the conversion factor for the mixture of reducing sugars expressed as glucose, which is derived by enzymatic hydrolysis of the sample. K_1 varies greatly in foods: from 0.923 in cereal grains to 1.080 in dairy products, and from 0.924 to 0.973 in recipe foods and composites of daily menus, depending upon the relative quantity of milk and dairy products, fruits and fruit juices (in which the sugars are largely glucose and fructose), and sucrose and starch-containing foods.

The energy equivalent of the available carbohydrate mixture of 100-g sample is

(14) ACho in 100-g sample, kcal = % ACho [3.945 (Lac + Suc + Mal) + 3.743 (Glc + Fru) + 4.150 St],

or

(15) ACho in 100-g sample, kcal = % ACho × K_2.

K_2 is the energy equivalent per g ACho. It varies greatly: from 3.743 to 4.149 in native and recipe foods, and from about 3.99 to 4.05 in human mixed diets. Examples of calculation of the energy equivalent of carbohydrate mixtures are given in table 1.

TABLE 1

Analysis of carbohydrate mixtures by method B

Sample no.	Carbohydrate mixture: Fraction of anhydrous sugars/ 100 g ash-free dry matter					Calculated total carbohydrate; ash-free dry matter			Results of analysis					
									Reducing sugar (Rs) (eq. 6)	Available carbohydrate (ACho)			Available hexose (AHex)	
	Lactose	Sucrose	Maltose	Glucose	Starch[1]	Wt[2]	Energy equivalent (eq. 15)	Calculated total hexose[3]		Wt (eq. 13)	Energy equivalent (eq. 15)	Wt (eq. 8)	Energy equivalent (eq. 11)
	%	%	%	%	%	%	*kcal/g*	%	*% Rs*	*% ACho*	*kcal/g*	*% AHex*	*kcal/g*
1	49.07	25.86	14.73	10.34	none	96.63	3.792	101.19	94.82	96.62	3.791	101.17	3.787
2	4.98	26.23	none	15.74	53.05	95.27	3.832	102.45	100.64	95.61	3.845	102.69	3.844
3	19.82	26.11	none	15.66	38.41	95.71	3.821	102.11	98.64	96.00	3.832	102.28	3.828
4	20.09	10.58	25.11	5.29	38.93	94.43	3.790	101.29	97.96	94.85	3.807	101.62	3.804

[1] Argo Cornstarch (Corn Products Company, New York) sample 6, table 4.
[2] Ash-free dry matter, calculated on the basis of ash and moisture of the individual sugars. Compare the results of column 7 with those of column 11.
[3] Total hexose = dry matter − [(Lac + Suc + Mal)/0.95) + (Glc + Fru) + (St/0.90)]. Lac, Suc, etc., represent the *fraction* of lactose, sucrose, etc., in the carbohydrate mixture. Compare the results of column 9 with those of column 13.

Comparison with Weende methods; digestibility coefficient. Assume that crude Cho = TCho + M + N, in which TCho = total carbohydrate, or the sum of soluble sugars, starch, CF and pentosans; M = metabolizable noncarbohydrate substances such as organic acids and sugar alcohols; and N = nonmetabolizable, noncarbohydrate substance, such as lignins and the resins derived from browning and other decompositions of carbohydrates. Then the apparent recovery of Rs after enzymatic (or acid) hydrolysis, expressed as ACho, is

(16) % Apparent recovery of ACho =

$$\frac{\text{ACho}}{\text{TCho} + \text{M} + \text{N}} \times 100.$$

The true recovery cannot be calculated from the crude Cho because of the indeterminable quantities of *M* and *N;* the true recovery is always greater than the apparent recovery by equation 16. In dairy products and in cereal grains or meals, the true recovery approaches or equals the apparent recovery.

Assume, further, that the crude Cho not recovered in the feces = absorbed Cho + M + B, in which B = the fraction of crude Cho which has been metabolized by intestinal bacteria, mainly in the lower bowel, without absorption of significant quantities of metabolizable products. Then

(17) % Apparent digestibility =

$$\frac{\text{absorbed Cho} + \text{M} + \text{B}}{\text{TCho} + \text{M} + \text{N}} \times 100.$$

If we assume that, as an approximation, ACho = crude metabolizable Cho, then

(18)
$$\frac{\text{ACho}}{\text{TCho} + \text{M} + \text{N}} \times 100 =$$
$$\frac{\text{absorbed Cho} + \text{M} + \text{B}}{\text{TCho} + \text{M} + \text{N}} \times 100.$$

Therefore,

(19) % Apparent recovery of ACho = % apparent digestibility of crude Cho.

However, by inspection of equation 18, it is evident that, generally, the apparent recovery may be expected to be lower than the apparent digestibility. With starch and cereal grains, the apparent recovery may be expected to approach or equal the apparent digestibility.

The apparent digestibility of crude Cho by humans may be approximated without using the cumbersome biological method. Assume the following digestibilities: 99% of soluble carbohydrates and starch determined by method B1 or by methods C and D; 30% of CF and pentosans. Then

(20) % Apparent digestibility of crude Cho =

$$\frac{0.99[\text{Cho by method B1}] + 0.30\,[\text{CF} + \text{pentosans}]}{\text{crude Cho}} \times 100.$$

Application to mixture of carbohydrates. Application of equations 6, 8, 11, 13 and 15, to mixtures of carbohydrates is shown in table 1.

Application of calculations when 0.100 to 0.001 M *FeCy reagents are used instead of 0.04* M *FeCy.* Depending upon the homogeneity of the sample, the methods may be altered considerably, without affecting the calculations by equations 8-15, using larger or smaller samples and enzyme than recommended and carrying out the sugar oxidations with 0.100 to 0.001 M FeCy reagents. The calculations in equations 8-15 are based upon the ratio of the reducing capacities of the principal hexoses in the enzymatic digest which, by iodometric titration of the 0.02 M FeCy reaction mixtures, was approximately 1.00 glucose: 1.00 fructose: 0.78 galactose. The results in table 2, which were obtained by spectrophotometric determination of residual FeCy, show that the ratio of the relative reducing capacities of the 3 sugars remained unchanged over the entire range of FeCy concentrations.

This ratio also was maintained within wide ranges of the total volume of reaction mixture, from 4 to 20 ml. Although the relative reducing capacities of the sugars thus remained constant, *the reduction per mole of each sugar* decreased in a quantitatively parallel manner for each sugar as the FeCy concentration was decreased in the order given, from 0.05 to 0.0005 M in the reaction mixture: 112.0, 100.0 (with 0.04 M reagent, or 0.02 M in the reaction mixture), 85.2, and 81.4%. The analysis of highly dilute sugar solutions, using 0.001

TABLE 2

Reducing capacities of hexoses at 0.05 to 0.0005 molar FeCy concentrations in the reaction mixture[1]

Hexose standard solution	Composition of ferricyanide reagent			
	0.100 M in 12.5% Na_2CO_3	0.040 M in 5.0% Na_2CO_3	0.004 M in 2.0% Na_2CO_3	0.001 M in 2.0% Na_2CO_3
	Relative %	*Relative %*	*Relative %*	*Relative %*
Glucose[2]	100.0	100.0	100.0	100.0
Fructose	98.6	99.7	100.1	100.4
Galactose	78.7	80.2	80.5	80.2

[1] In the respective order of determinations in columns 2-5, the technique was as follows: hexose standards contained 1.5, 0.6, 0.06, 0.015 mg hexose/ml; ml standard + ml FeCy reagent in 25 × 200 ml test tubes were 2 + 2, 5 + 5, 5 + 5, 10 + 10; all tubes were heated 30 minutes at 80°; after cooling, the reaction mixtures were diluted to 500, 500, 50, 25 ml (20 + 5 ml water); all spectrophotometric readings were made at 418 mμ wave length.

[2] In the order, from 0.05 M to 0.0005 M FeCy in the test tube, the relative reduction given by the glucose standard was 112.0, 100.0 (authors' recommended reagent), 85.2, 81.4%.

M FeCy reagent for example, is not recommended. The determination not only loses sensitivity as the Rs concentration is decreased, but also is subject to indeterminate losses of Rs from the highly dilute solution during precipitation, filtration and subsequent handling before analysis.

EXPERIMENTAL

Materials and preparation of samples

Sugars. Glucose and sucrose were obtained from the U. S. Bureau of Standards. The other sugars were obtained from commercial sources.

Polysaccharides. Starches and other polysaccharides were obtained from many sources. Starch samples 6A, 7A, 8A, 13A were prepared from samples 6, 7, 8, 13, as follows: The material from the original package was soaked overnight with cold water and then well-washed with cold water; it was extracted 10 hours with CH_3OH in a Soxhlet extractor, filtered, well-washed with CH_3OH and, finally, air-dried one week at room temperature. Hemicellulose samples 23-26 were prepared by the method of Wise et al. (61).

Plant materials, cereal grains. Samples 1-20 were prepared and analyzed by AOAC (16) methods under the direction of Dr. Richard W. Carroll and Dr. F. N. Peters, (Quaker Oats Company, Research Laboratories, Barrington, Illinois). Each of the 5 pairs of samples 11-20 consisted of a heat-processed cereal grain product and the identical grain or mix before processing.

The ingredients of the wheat flakes mix were 90.0% wheat, 5.0% sucrose, 2.5% malt syrup, 2.5% salt. From this formulation, the calculated fractions of metabolizable carbohydrates were approximately 0.897 St, 0.096 Suc + Mal, and 0.007 Glc; % ACho by equation 12 = % Rs × 0.926.

The ingredients of the corn flakes mix were 91.0% yellow grits, 5.5% sucrose, 1.5% malt syrup, 2.5% NaCl. From this formulation, the calculated fractions of metabolizable carbohydrates were approximately 0.913 St, 0.083 Suc + Mal, 0.004 Glc; % ACho by equation 12 = % Rs × 0.925.

Bulger wafer, no. 22 ["Wafer, Survival, All Purpose (Civil Defense), Bulger Type," procured by QM Corps, Department of the Army], was prepared and analyzed by AOAC (16) methods by Richard S. Harding at the U. S. Army Medical Research and Nutrition Laboratory (USAMRNL), Denver, Colorado. According to specifications, the components were: 79.1% partially debranned, water-steeped, autoclaved, whole bulgur (sic) wheat (10% final moisture); 10.0% hydrogenated shortening; 10.0% dry powdered malt or malt cereal syrup (calculated 3% moisture); and 0.9% added salt. The baked product had 3.68% moisture, 9.10% protein (N × 5.70), 9.90% fat, 2.24% ash, 2.68% crude fiber; it was crisp and had a marked brown color similar to zwieback. On the basis of formulation, ACho = Rs × 0.928.

Samples 1-20 were carefully chosen by Dr. Carroll and Dr. Peters to represent the great differences in composition that are found among plant materials. The ranges of composition were 0.6 to 38.5% crude fiber, 0.7 to 34.5% pentosans, 1.2 to 81.9% starch, 0.8 to 19.3% soluble sugars, 1.8 to 21.1% protein. The samples represent animal feeds (nos. 1–3, 6), human foods (nos. 8, 9, 11–22), and materials that are used in the manufacture of furfural (nos. 4, 5). All samples were ground to pass completely through a 20-mesh sieve.

Human foods; diet composites. Four or more large samples, nos. 23-42, were obtained from different sources. Solid foods were rapidly mixed and ground several times in a meat grinder until it was felt that homogeneity had been attained. Fruits and canned foods were mixed in a large Waring Blendor as below.

Composites of weighed portions of all of the items of daily diets and military rations, including milk, cocoa, fruit juices and other liquid juices were placed in one or more weighed, cold Waring Blendor containers of 4-liter mixing capacity. The containers were kept in the refrigerator and, at the end of each day, the contents were homogenized and mixed. The containers were weighed again. The diet composites were prepared in the course of experiments at the USAMRNL[13] in cooperation with one of us (N.F.W.). The ration samples were prepared at the USAMRNL during the course of ration trials (62, 63) in cooperation with one of us (T.E.F.). All analyses by AOAC methods (16) were made by Richard S. Harding.

Portions of all food composites were placed in 250-ml sample bottles and were either analyzed immediately or were quickly frozen and stored in the deep-freeze until time for analysis.

Method A, hydrolysis by 1.0 N HCl

The reasons for using method A in the present work, and some of the disadvantages and precautions against its use, were noted in the introduction and the section on methods. The data in tables 3, 4, 5 illustrate these points and others, which will be summarized briefly.

(i) As expected, great differences of stability were noted among the sugars. The loss of fructose was variable: from about 60 to 80% of reducing capacity to FeCy and from 70 to 90% to cupric-tartrate reagent. FeCy apparently oxidized some of the decomposition products more readily than the cupric-tartrate complex. The low yields from inulin and sucrose were caused by decomposition of the fructose moiety (tables 3, 4).

In contrast with the ketoses, the aldoses and uronic acids were considerably more stable. On the basis of experience, the following losses have been noted: 1 to 3% of glucose, mannose, and galactose; about 5% of the pentoses and about 10% of glucuronic acid.

(ii) Starches (table 4) yielded the maximal Rs within 2 to 3 hours at 95°, within 3.5 to 4.5 hours at 90°, and within 7 to 8 hours at 85°. Low Rs-yielding starches

[13] Obtained through the courtesy of Lt. Colonel John E. Canham, M.C.

TABLE 3

Available carbohydrate by hydrolysis with 1.0 N HCl in the boiling water bath, ± 95° at Boulder, Colorado, 1,615-m altitude

Duration of digestion	Sucrose	Starch, soluble Merck[1]	Corn cobs, sample 4	Corn meal, yellow, sample 9
hours	*% hydrolyzed*	*% hydrolyzed*	*% of maximum*	*% of maximum*
None	5.4	1.9	6.9	3.2
0.5	93.9	74.0	—	—
1	85.6	94.5	89.6	96.1
2	74.9	99.8	96.6	100.0
3	69.7	99.3	100.0	99.9
4	64.3	99.2	99.7	99.1
5	62.7	99.1	—	—
6	58.8	98.3	100.0	99.2

[1] Merck and Company, Inc., Rahway, New Jersey.

TABLE 4

Saccharolytic activity of Rhozyme-S[1] and 0.1 and 1.0 N HCl on polysaccharides; yield of reducing sugar, calculated as starch[2]

Polysaccharide[3]	Dry matter, ash-free	Dry matter hydrolyzed				
		0.1 N HCl, 1 hr at ± 95°	Enzyme: Method B1, enzyme alone, 6 hr at 50°; pH 4.7	Enzyme: Method B with 0.10 N HCl, followed by enzyme, 6 hr at 50°; pH 4.7	1.0 N HCl: Method A, 3 hr at ± 95°	1.0 N HCl: Method A, 4.5 hr at 90°
	%	%	%	%	%	%
Starches and starch products						
1 Potato, N.F. ref. standard, 1954	86.0	29.6	—	98.6	—	—
2 Potato, N.F. ref. standard, 1962	84.5	26.4	99.7	99.4	99.1[4]	99.7[4]
3 Soluble, Lintner	90.3	25.5	98.3	97.3	99.3	—
4 Soluble, USP	94.3	26.1	98.8	98.2	97.1	98.8
5 Soluble, A.R.	92.4	24.9	83.7	86.8	92.1	92.8
6 Corn	91.9	27.5	98.8	97.6	97.5	98.7
6A Corn, extracted	93.5	26.9	97.8	97.0	96.2	98.4
7 Potato	90.7	28.7	99.4	98.1	99.0	100.0
7A Potato, extracted	93.5	27.8	97.3	96.3	96.5	98.5
8 Arrowroot	87.6	26.6	98.4	99.5	100.3	99.8
8A Arrowroot, extracted	92.9	26.5	97.6	98.5	99.7	98.4
9 Amylopectin	90.4	26.1	95.6	94.4	96.7	96.4
10 Ramelin G (amylopectin)	91.3	25.6	98.9	98.5	99.8	98.4
11 Amylose	89.8	22.8	61.8	76.9	82.6	75.5
12 Superlose (amylose)	90.2	25.0	64.7	77.5	85.3	75.9
13 Linit laundry starch	91.0	26.2	98.3	97.4	99.3	98.1
13A Linit laundry starch, extracted	93.2	24.2	98.7	98.1	100.0	98.5
Other polysaccharides						
14 Glycogen, oyster	90.8	21.5	95.5	95.6	98.1	100.6
15 Inulin, starch-free, C.P.	97.0	89.4	47.6	88.9	(63.8)	—
16 Dextran, mol wt 15-20,000	97.2	9.7	17.3	27.8	85.8	—
17 Dextran, mol wt 200-275,000	94.1	4.1	2.8	14.6	94.3	—
18 Cellulose, crystalline (Avicel)	97.0	none	0.9	0.7	2.0	—
19 Cellulose, carboxymethyl, low viscosity	87.7	0.8	10.0	13.3	55.8	—
20 Cellulose, carboxymethyl, high viscosity	89.3	0.5	9.7	11.7	49.6	—
21 Cellulose, oxycellulose, 12% COOH	99.7	3.0	23.7	24.9	21.7	—
22 Cellulose, oxycellulose, 18% COOH	99.7	5.1	14.3	14.6	17.4	—
23 Hemicellulose, wheat bran no. 1	93.0	46.0	39.9	72.4	88.8	—
24 Hemicellulose, wheat bran no. 2	88.0	44.5	65.3	79.1	91.2	—
25 Hemicellulose, oat hulls	96.0	40.7	51.0	70.6	84.4	—
26 Hemicellulose, corn cobs	89.1	39.8	49.1	84.4	87.3	—
27 Pectin, N.F., 9.5% methoxy	89.4	2.8	40.7	41.7	10.3	—
28 Pectin, N.F., 4.2% methoxy	89.4	2.7	31.4	32.4	10.5	—
29 Pectin, pectic acid	88.6	1.9	45.4	43.8	8.9	—

[1] Rhozyme-S, High Potency Concentrate, low reducing sugar content, obtained from the Rohm and Haas Company, Special Products Division, Philadelphia.

[2] 250 mg sample and 100 mg Rhozyme-S, factor 4.37, were used. All digests (columns 3-7) were precipitated with $Zn(OH)_2$; % starch = % Rs × 90% DM.

[3] The sources of samples were as follows: 1, 2, Committee on National Formulary, National Pharmaceutical Association, Washington, D. C.; 3, Merck and Company, Inc., Rahway, New Jersey; 4, Fisher Scientific Company, Fair Lawn, New Jersey; 5, Mallinckrodt Chemical Works, St. Louis; 6, 9, 11, 13, Corn Products Company, New York; 7, B. Manischewitz Company, Newark, New Jersey; 8, Spice Islands Company, South San Francisco; 10, 12, Stein, Hall and Company, New York; 14, Distillation Products Industries, Rochester, New York; 15, Mann Research Laboratories, Inc., New York; 16, 17, Sigma Chemical Company, St. Louis; 18, Dr. O. A. Battista, American Viscose Corporation, Marcus Hook, Pennsylvania; 19, 20, Hercules Powder Company, Wilmington, Delaware; 21, 22, Eastman Chemical Products, Inc., Kingsport, Tennessee; 23-26, prepared by method of Wise et al. (61); 27-29, Dr. Glenn H. Joseph, Research Department, Sunkist Growers, Inc., Corona, California.

[4] The rate of hydrolysis was very slow at 85°, attaining 98.0% at the sixth and seventh hours in the water bath.

TABLE 5

Analysis of plant samples by AOAC (16) methods and authors' methods A, B, C and D; effect of acidity during preliminary digestion at 95° on results obtained by modifications of method B and effect of heat processing on cereal grains and derived cereal products[1]

	20-Mesh air-dried samples	AOAC methods[2]			Methods $C_{20} + D_{20}$; soluble sugars and starch	500-mg sample, methods B and B1-B5, with preliminary digestion with water only, or buffer, or acids						Method A, maximal hydrolysis by 1.0 N HCl	Crude fiber + pentosans + method B1	
		Crude Cho by difference	Crude fiber	Pentosans		Method B1, water only	Method B2, acetate buffer pH 4.7	Method B3, acetic acid pH 3.0	Method B4, lactic acid pH 2.5	Method B5, 0.05 N HCl, pH 1.4	Method B, 0.10 N HCl, pH 1.1		Sum = TCho	M + N = crude Cho − TCho
		%	%	%	% *ACho*	% *ACho*	% *ACho*	% *ACho*	% *ACho*	% *ACho*	% *ACho*	% *ACho*	% *TCho*	% *M + N*
	Effect of acidity during preliminary digestion; methods B and B1-B5; low starch, high CF, high pentosan content													
1	Cereal grass	61.0	21.7	12.7	11.8	12.9	12.6	13.4	14.4	17.6	21.4	23.2	47.3	13.7
2	Alfalfa meal	59.5	19.3	9.3	12.3	12.0	12.2	12.4	13.4	14.3	17.5	20.2	40.6	18.9
3	Timothy hay	76.0	38.5	17.5	6.4	6.6	6.8	8.0	9.2	11.6	15.5	22.8	62.6	13.4
4	Corn cobs	89.9	35.5	34.5	2.6	2.9	2.9	3.0	3.9	16.3	23.9	35.9	72.9	17.0
5	Oat hulls	84.3	33.1	33.0	3.8	3.5	3.7	3.9	4.9	12.5	23.1	36.1	69.6	14.7
	Effect of acidity during preliminary digestion; methods B and B1-B5; high starch, high and low CF, high and low pentosan content													
6	Wheat bran	64.7	9.5	18.1	30.1	32.2	32.9	34.7	36.2	44.3	47.6	47.4	59.8	4.9
7	Oats, green, whole grain	70.8	10.8	10.9	44.8	45.4	45.5	45.8	45.9	47.2	51.5	59.4	67.1	3.7
8	Oats, rolled (Quaker)[3]	68.3	1.3	2.3	64.6	66.1	66.2	66.1	66.4	67.7	68.2	66.1	69.7	−1.4
9	Corn meal, whole, yellow	77.0	2.1	4.0	66.8	68.2	67.8	67.8	68.4	70.7	72.4	73.2	74.3	2.7
	Effect of heat-processing; odd-numbered samples were not heated; even-numbered samples were heat-processed[4]													
11	Rice for puffed rice	83.6	0.7	0.3	82.6	83.3					83.2	84.2	84.3	−0.7
12	Puffed rice	83.9	0.7	0.2	82.6	83.8					82.9	83.5	84.7	−0.8
13	Wheat for puffed wheat	75.8	2.3	5.5	65.1	67.2					71.3	69.8	75.0	0.8
14	Puffed wheat	76.0	2.5	4.8	69.7	68.0					70.6	70.5	75.3	0.7
15	Wheat for rolled wheat	77.7	3.0	5.9	66.2	67.5					73.3	73.5	76.4	1.3
16	Rolled wheat (Pettijohn's)[5]	77.4	2.6	5.3	67.2	68.1					72.7	73.7	76.0	1.4
17	Wheat flakes mix	73.6	1.8	4.8	68.7	67.7					70.4	67.7	74.3	−0.7
18	Wheat flakes	74.4	1.3	4.6	68.1	67.0					71.3	68.4	72.9	1.5
19	Corn flakes mix	79.7	0.7	0.9	78.1	78.3					79.0	78.4	79.9	−0.2
20	Corn flakes	80.0[6]	0.6	0.7	74.4	74.0					76.0	76.2	75.3	4.7[6]
22	Bulger wheat wafer[7]	75.1	2.7								71.2			

[1] Cho = carbohydrate; TCho = total carbohydrates; and CF = crude fiber.

[2] In samples 6, 13-18, and 22, protein = N × 5.70. Pentosans were determined by John Wayne Leitner in our laboratory.

[3] Quaker Oats, Quaker Oats Company, Chicago.

[4] All results of samples 11-20 were calculated to 10% moisture content. All results of samples 1-9 were calculated to the initial moisture content, 7.0, 6.6, 6.7, 6.4, 7.4, 9.6, 8.9, 7.0, 8.5%, respectively.

[5] Pettijohn's, Quaker Oats Company, Chicago.

[6] These results are obviously too high, due to errors in the assays of protein, fat, moisture or ash upon which the calculation of crude Cho is based. The 5 series of analyses of sample 20 by methods B, B1, A, C + D gave 2.2 to 4.3 less ACho than sample 19.

[7] Bulger wafer, no. 22 ("Wafer, Survival, All Purpose (Civil Defense), Bulger Type") procured by QM Corps, Department of the Army.

(samples 5, 11, 12) required longer digestion than high Rs-yielding starches. The longer period at each temperature was chosen in order to obtain the maximum in all samples.

(iii) Plant samples (table 3) yielded maximal Rs within about the same time as the starches (table 4).

(iv) Starches exhibited large differences in Rs yields, due perhaps, first, to the great differences in the relative content of amyloses and amylopectins among starches and, therefore, to the relative number of α-1,4- and α-1,6-linkages and, second, to the degree of retrogradation undergone by the starch during preparation from the native source. As expected, potato and arrowroot starches gave the greatest calculated yields of Rs, 99%. Soluble starches 4 and 5 gave only 97.1 and 92.1%; amylopectin sample 9, 96.7%; amylose samples 11 and 12, only 82.6 and 85.3%. Relatively mild extraction procedures, used in removing possibly fatty and water-soluble substances, resulted in distinctly lower yields of Rs in three of the four samples 6, 7, 8 and 13, table 4.

(v) At 90°, several of the low Rs-yielding starches gave less Rs than at 95°. The results agreed more closely with those obtained by method B than those obtained at 95°. The temperature of digestion with 1.0 N HCl is very important in the analysis of low Rs-yielding starches.

(vi) Generally, the acid-hydrolyzability of the isolated starches, whether at 95° or 90°, paralleled the enzymatic digestibility; thus the differences among samples 1-13A, given by digestion with enzyme alone, or by method B, were indeed real. However, such parallelism may not necessarily be expected among other polysaccharides. Thus the hydrolysis of dextrans by 1.0 N HCl vs. enzyme alone was 85.8 and 94.3% vs. 17.3 and 2.8%; the reverse was shown by pectins, 8.9 to 10.5 vs. 31.4 to 45.4%.

(vii) The isolated hemicelluloses (table 4) were apparently completely hydrolyzed by 1.0 N HCl. Samples 23-26 yielded 84.4 to 91.2% ACho, or 87.2% weighted average calculated as starch, Rs $\times$ 0.90/DM. The hypothetical yields of ACho from araban and xylan are 85.5 and 94.2%, allowing for 5% losses during hydrolysis and allowing for the differences in relative reducing capacities of the pentoses (58). Assume that the average hypothetical yield of ACho is approximately 90%; then the degree of hydrolysis of samples 23-26 was 94 to 101%, or 97% average.

(viii) The native hemicelluloses within the plant structures of samples 1-7 (table 5) were also apparently completely hydrolyzed by the acid, as indicated by the following rough methods of calculation. Assume that

(21) ACho by hydrolysis = [soluble Cho + ($c \times$ starch)] + [$d \times$ pentosans],

or

(22) ACho by hydrolysis = [ACho by method B1] + [$d \times$ pentosans]

where c and d represent the respective fractions of starch and pentosans that were hydrolyzed.

Assume complete hydrolysis of starch, or $c = 1.00$ from the hydrolysis of samples 1-7. The values for d in equation 21 then are: 0.90, 0.85, 0.94, 0.96, 0.98, 0.96, 1.34; average, 0.99, or 99% hydrolysis of pentosans. The values for d in equation 22 are: 0.81, 0.88, 0.93, 0.96, 0.99, 0.84, 1.28; average, 0.96, or 96% hydrolysis.

Method B, enzymatic hydrolysis, preceded by hydrolysis with 0.1 N HCl

In this section we shall discuss the 2 basic operations of method B which most profoundly affect the yield of Rs, namely, (i) the preliminary digestion with 0.1 N HCl or weaker acid, and (ii) the choice and use of the enzyme. The discussion applies also to methods C and D.

Rate of hydrolysis of granular and gelatinized N.F. reference starch by 0.05 and 0.1 N HCl at 95, 90 and 85°. Two-hundred milligrams of starch were placed into each tube of a series of 11 tubes. The starch was hydrolyzed under the conditions shown in table 6. Colors given by I-KI reagent were recorded (footnotes 3–5). Additional data are given in table 4, representing the effect of hydrolysis during 1 hour at 95°.

The data in tables 4 and 6, allow the following conclusions. (i) Previous gelatinization of starch did not materially change the rate of hydrolysis as compared with that of the granular starch suspension, un-

TABLE 6

Rate of hydrolysis of N.F. reference starch to reducing sugars by 0.05 N and 0.10 N HCl at 95°, 90°, and 85°, calculated to percentage hydrolysis of starch or dry matter[1]

Duration of time of heating	Starch gelatinized before adding HCl	Starch not gelatinized before adding HCl				
	Hydrolysis in 0.05 N HCl	Hydrolysis in 0.10 N HCl				
	Hydrolysis at 95°				Hydrolysis at 90°	Hydrolysis at 85°
min	%	%	%	%[2]	%	%
None	0.8	0	0	0.6	0.5	0.4
5	2.2	1.3	1.5	1.7	.5	.1
10	1.2	3.7	3.2	4.1	1.5	.8
15	3.4	6.4[3]	6.3	6.7[3]	2.4	1.0
20	4.9	9.0	8.9[3]	9.5	4.3	2.3
30	7.6[3]	13.5	13.8	13.9	5.7	3.6
40	9.2	18.6	17.7	18.8	8.5[3]	5.7
50	11.0	22.3	21.6[4]	22.7[4]	10.3	6.4[3]
60	13.6	23.5[4]	25.9	23.6	12.5	7.7
90	20.3[4]	38.0[5]	36.4[5]	39.0[5]	17.7[4]	10.7
120	25.0	46.4	46.5	46.5	23.0	14.3

[1] % Starch or DM hydrolyzed = % Rs × 0.90/0.845. The starch contained 84.5% ash-free DM.
[2] The acid digests in this series were clarified with $Zn(OH)_2$ as in method B.
[3] First definite change from starch-blue to purple or violet color on adding iodine reagent.
[4] Orange or brown, with possibly faint trace of violet, on adding iodine reagent.
[5] Virtually no color as compared with blank plus iodine reagent.

der the prescribed conditions of method B. (ii) Clarification with $Zn(OH)_2$, as in method B, did not significantly affect the recovery of Rs. (iii) At 90°, the rate of hydrolysis in 0.10 N HCl was only *one-half* as fast as at 95°, requiring about 120 minutes to attain the same yield of Rs as at 60 minutes at 95°; at 85°, the rate was reduced to only *one-fourth* of that at 95°. (iv) In 0.05 N HCl at 95° (col. 2), the rate was approximately the same as in 0.10 N HCl at 90° (col. 6), or about *one-half* as great as in 0.10 N HCl at 95° (cols. 3-5). Because the rate of hydrolysis is thus greatly affected by acidity and temperature, it is essential that a fair degree of accuracy be observed in standardizing and measuring the 0.7 N HCl, in adjusting the volume to 35 ml, in maintaining the temperature, and in measuring time. (v) The qualitative changes of color with dilute I-KI reagent in each of the series roughly paralleled the rate of hydrolysis. At the first change of the deep blue starch-iodine color to blue-violet (footnote 3), the degree of hydrolysis in all of the series varied from 6.4 to 8.9%, average 7.4%. At attainment of an orange or brown color, with possibly a trace of violet (footnote 4), the degree of hydrolysis in five of the six series varied from 17.7 to 26.5%, average 21.8%. Such variability was expected, not only because of the subjective element in judging colors, but also because of the great variability of the starch-iodine reaction which is affected by concentration of iodine and KI, by temperature and by many other factors. Despite its limitations, the starch-iodine reaction is useful in standardization of method B by means of a reference starch, so that the same degree of preliminary hydrolysis may be obtained in all laboratories.

Hydrolysis of sugars, starches, and other polysaccharides by 0.10 N HCl, 60 minutes at 95°. Two-hundred-and-fifty milligrams of polysaccharides (table 4) and sugars (table 7) were placed into test tubes. The oxycelluloses, hemicelluloses and pectins were suspended in 20 ml 0.05 NaOH and then kept 24 hours in the refrigerator, stirring frequently; the dextrans and carboxymethyl celluloses were suspended in water and then kept 24 hours in the cold; the resulting jells were adjusted to pH 4.7 by adding previously determined volumes of dilute HCl or NaOH. Hydrolysis was then made as in method B.

(i) The 0.10 N HCl variably hydrolyzed many classes of polysaccharides besides starch that are found in plant materials. It completely hydrolyzed inulin. The degree of hydrolysis of other polysaccharides, in decreasing order of completeness, was as follows: hemicelluloses, 39.8 to 46.0%, average 41.9%; starch and glycogen, 21.5 to 29.6%, average 26.0%; dextrans, about 4 to 10%; carboxycelluloses (oxycelluloses) and pectins, 2 to 5%; carboxymethylcelluloses, less than 1%; cellulose, none. The data emphasize the marked difference between α-1,4- and α-1,6-glucans in hydrolyzability by dilute acids and amylases, as exemplified by starch and dextran. Although β-1,4-glucans, cellulose for example, are not readily hydrolyzed by dilute acid, solubilization of the molecule by oxidizing carbon-6 to carboxyl, or by adding carboxymethyl groups, increased the hydrolyzability.

(ii) The soluble carbohydrates (table 7) were hydrolyzed to varying degrees of completeness, in the following decreasing order of approximate percentage hydrolysis: sucrose, 99%; raffinose, melizitose, and turanose, 78 to 90%; melibiose, about 50%; maltose, lactose, cellobiose, and trehalose, none to 10%. The loss of reducing capacity of fructose varied from none to 1%; 0.10 N HCl is the maximal acidity that can be used without significant loss of fructose.

(iii) The digestion with 0.10 N HCl contributed materially toward a greater yield of Rs from polysaccharides and soluble sugars in method B over that given in method B1 (by the enzyme alone), provided the Rs resulting from the hydrolysis by 0.10 N HCl was greater than about 5%. The only exception to this was dextran no. 17. The contribution of the preliminary hydrolysis was particularly evident in the case of inulin, dextran no. 16, hemicelluloses, raffinose, melizitose, turanose and melibiose; it was not so pronounced among the α-1,4-glucans, glycogen and the starches, because the hydrolysis with the enzyme alone was generally greater than 95%. However, soluble starch no. 5 and amylose nos. 11 and 12 yielded only 83.7, 61.8 and 64.7% calculated Rs by method B1, but gave 86.8, 76.9 and 77.5% by method B.

Hydrolysis of native polysaccharides by 0.10 N HCl, 60 minutes at 95°. Two

TABLE 7

Saccharolytic activity of Rhozyme-S and 0.1 N HCl on mono, di-, and tri-saccharides[1]

Sugar	Constituent sugars	Reduction given by constituent sugars, expressed as glucose	0.1 N HCl, 1 hour in boiling water bath at ± 95°	Enzymatic hydrolysis	
				Method B1, enzyme alone, 6 hr at 50°; pH 4.7	Method B, with 0.10 N HCl, and followed by enzyme at pH 4.7
		% glucose	*% glucose*	*% glucose*	*% glucose*
		Nonreducing sugars			
Raffinose 1	Gal-Glc-Fru	92.4	79.7	78.3	87.4
Raffinose 2	Gal-Glc-Fru	92.4	79.6	80.5	88.7
Melizitose	Glc-Glc-Fru	99.8	90.4	14.9	92.1
Sucrose	Glc-Fru	99.7	98.4	100.7	100.5
Turanose	Glc-Fru	99.7	77.9	75.8	79.7
Trehalose[2]	Glc-Glc	100.0	9.7	1.2	6.1
		Reducing sugars			
Maltose	Glc-Glc	100.0	81.6[3]	99.2	98.7
Cellobiose	Glc-Glc	100.0	84.3[3]	100.1	100.4
Lactose	Gal-Glc	88.9	82.3[3]	87.5	87.4
Melibiose	Gal-Glc	88.9	75.0[3]	76.6	81.8
Glucose	Glc	100.0	100.6	–	100.6
Fructose	Fru	99.5			
Galactose	Gal	77.8			
Glucuronolactone		94.4	90.1	93.2	90.2

[1] Method as in table 4.
[2] This sugar is a 1,1-glucopyranosyl-pyranoside.
[3] The results indicate little or no hydrolysis by 0.1 N HCl.

groups of samples were hydrolyzed by 0.10 N HCl and then clarified by $Zn(OH)_2$ as in method B, omitting the enzymatic hydrolysis. Group 1 consisted of samples 4 and 5 (table 5) containing 34.5 and 33.0% pentosans, 35.5 and 33.1% crude fiber, only 1.4 and 0.8% soluble sugars and 1.2 and 3.0% starch. Group 2 consisted of samples 8, 9, 11, 12 and 20, containing 62.7 to 81.9% starch and only 0.7 to 12.7% soluble sugars and 0.2 to 4.0% pentosans. The reducing sugars after the hydrolysis, calculated as ACho by equation 12, in the order of the respective samples, were: of group 1, 10.0, 11.2%; of group 2, 16.9, 20.2, 21.1, 29.1, 22.0%.

Assume that $c = 0.26$ in equation 21, which was the average degree of hydrolysis of isolated starches and glycogen by 0.10 N HCl (table 4); then the respective degrees of hydrolysis, d, of native pentosans of the samples of group 1, were 0.241 and 0.291 average 0.266. Thus 24 and 29% hydrolysis was obtained as compared with 39.8 and 40.7% of the corresponding isolated hemicelluloses (which perhaps do not represent all of the hemicelluloses that may be present within the cell structures).

The respective degree of hydrolysis, c, of native starches in the samples of group 2 were as follows by equation 21, when $d = 0.27$ as above: 0.230, 0.262, 0.248, 0.246 and 0.175; average, 0.232.

From these calculated data, it appears (i) that the preliminary digestion with 0.10 N HCl in method B hydrolyzed the native starches in samples 8, 9 and 11 to about the same extent as isolated granular starches; (ii) that the native pentosans also were hydrolyzed, but only about two-thirds as much as the isolated product from the same sample; and (iii) that the available Cho determined by method B includes a portion of the pentosans which are mobilized by the preliminary hydrolysis.

Effect of acidity during preliminary digestion on the overall yield of Rs × 0.923 by method B when applied to plant samples. Methods B1, B2, B3, B4, B5. Duplicate determinations of ACho in 500-mg portions of samples 1-9 (table 5) were made by modifications of method B, in which the 60-minute preliminary digestion was carried out at acidities varying from neutral to 0.05 N HCl acidity. Methods B and B1 were described earlier. The modifications were as follows:

Method B2, with acetate buffer: Add 5 ml 0.4 M acetate buffer and water to the 35-ml mark; mix thoroughly; then proceed as in method B, omitting the additions of 0.70 N HCl and NaOH and the later addition of 0.4 M acetate buffer.

Method B3, with 0.10 N acetic acid: Proceed as in method B, but use 5 ml 0.70 N acetic acid instead of 0.70 N HCl; add 5 ml 0.364 N NaOH which partially neutralizes the acid to pH 4.7. Do not add 0.4 M acetate buffer, since the digest already contains sufficient acetate.

Method B4, with 0.10 N lactic acid: Proceed as in method B, but use 5 ml 0.70 N lactic acid instead of 0.70 N HCl; add 9 ml 0.35 N NaOH or 5 ml 0.63 N NaOH, instead of 0.70 N NaOH, to partially neutralize the acid to pH 4.7. Because of the low buffering capacity of lactate, add 5 ml 0.4 M acetate buffer.

Method B5, with 0.05 N HCl: Proceed as in method B, using 5 ml 0.35 N HCl; neutralize with 0.35 N NaOH.

The following conclusions are suggested by the data: (i) The results by method B1, digesting with water alone, approximated the sum of the soluble sugars and starch by methods C and D; the analysis by method B1 represented the soluble sugars and starch perhaps more accurately than the sum of the separate assays, besides being simpler and involving less work. In the analysis of samples 1-20, the differences between the results by method B1 and the results by methods $C_{20} + D_{20}$ ranged from −1.7 to 2.1, average 0.46. The differences were not related to the starch or pentosan content and were small as compared with the hydrolyzable Cho, including the pentosans. They represented (a) either systematic analytical errors; or (b) oligosaccharides which may have been dissolved in the washings in method D; or (c) losses in recovery of starch from the filter paper in method D; or (d) they were the resultant of all of these factors.

(ii) In samples 8-19, TCho, the sum of soluble sugars and starch by method B1,

and crude fiber and pentosans, approached within —1.4 to 2.7, average 0.42, of the value of the crude Cho by difference (last column, table 5). When crude Cho = 100%, then TCho was 96.5 to 102.0, average 99.5%, of the crude Cho by difference.

This agreement among the 11 samples was unexpected, in view of the proximate character of the methods used, especially those of protein, fat, CF, and pentosan. The small differences were not related to the CF plus pentosan content, which (letting crude Cho = 100%) varied from 1.1 to 11.4% of the crude Cho. Not all samples of grains gave such good chance agreement as samples 8-19; in sample 20, the crude Cho by difference was obviously too high (see footnote 6, table 5), and the TCho was only 94.1% of the crude Cho = 100%.

(iii) On the whole, the sum of the soluble sugars, starch, CF, and pentosans represented the total carbohydrate, TCho, more accurately than the crude Cho by difference. TCho may be determined most simply by 2 assays, namely, as the sum (a) of soluble sugars and starch by method B1, and (b) holocellulose (61, 64, 65) which includes cellulose and hemicelluloses. Generally, the relation between these carbohydrates and the crude Cho by difference is expressed by equation 23.

(23) Crude Cho by difference = [soluble Cho + starch] + [CF + pentosan] + [M + N].

As in equation 16, M + N includes many groups of noncarbohydrate substances, and considerable quantities may be present in plant materials as in samples 1-5 (last column, table 5). The magnitude of M and N may be seen for example, in the data of Ely and Moore (65) which have been calculated to 7% moisture content. In 6 samples of hay prepared from mature clover, grasses, and legumes, the following noncarbohydrate constitutents of the NFE were observed: lignin, 9.0 to 12.7, average, 10.6%; plant pigments, etc., 2.0 to 4.5, average, 3.4%. The latter probably represents minimal values. In the samples of Ely and Moore, the sum of the averages of the 2 categories of noncarbohydrate substances was 14.0%; in our hay samples 1-3, M + N by difference was 13.7, 18.9, and 13.4%.

(iv) The results by methods B1-B5 indicate that the native pentosans of the plant samples were firmly bound within the cell structures and that they were not extracted until a considerable H-ion concentration had been attained. Methods B1, B2, and B3 gave closely agreeing results; however, in most samples, method B4 yielded significantly more ACho than method B1 or B2. Therefore, pH 3 in method B3 probably represents the limiting acidity for determining available carbohydrates without mobilizing pentosans.

(v) At increasingly greater acidities than 0.1 N acetic or lactic acids, the bondings of pentosans within the cells were increasingly broken until, in method B, a considerable liberation of pentosans appeared to have been attained in the preliminary hydrolysis with 0.10 N HCl.

For example, assume complete hydrolysis of starch in method B. Then in samples 1-7, d was 0.52 to 0.97, average 0.66 by equation 21; 0.51 to 0.85, average 0.63 by equation 22. Thus, the average degree of hydrolysis of the native pentosans in these samples was about 64% as compared with 77% of the isolated hemicelluloses (table 4).

Choice of enzyme. The following criteria were applied for selecting the enzyme preparation. (i) It should be a stable, finely powdered soluble enzyme mixture of high potency which is standardized by the manufacturer, particularly with respect to amylase activity; (ii) it should give a low Rs blank value; (iii) it should contain all of the enzymes for hydrolyzing starch, lactose, maltose, sucrose and other metabolizable carbohydrates in the human diet.

Ideally, the preparation should be compounded to contain a balanced mixture of saccharolytic enzymes which will give the same degree of hydrolysis into reducing sugars in vitro as would be obtained in vivo, on the average, during digestion and passage of the sample or food through the intestinal tract of the species to which the analytical data are to be applied. Since the presently accepted Weende biological method includes 20 to 80% digestibility of the small quantities of cellulose and hemicelluloses in the human diet, and since this is presumed to represent a portion of the

metabolizable carbohydrate, then the preparation should contain a modicum of cellulases and hemicellulases.

Among several commercially available preparations, Rhozyme-S most nearly conformed to these criteria. The Rs content of various enzyme preparations was: Rhozyme-S, 8.5 to 9.8%; Taka-Diastase,[14] 86.5 to 72.0%; Rhozyme-35, Cellulase,[15] 10.1%; Takamine cellulase[16] prepared from *Aspergillus niger*, 29.9%; Hemicellulase "Fungal,"[17] 64.9%; pancreatin[18] about 5%.

Taka-Diastase gave approximately the same Rs from plant samples as Rhozyme-S, but the results fluctuated greatly because of the large amount of Taka-Diastase (about 500 mg of enzyme, containing 360-430 mg Rs) required to give as complete hydrolysis as 100 mg Rhozyme-S (containing only about 10 mg Rs). Perhaps Taka-Diastase would have been equally useful as Rhozyme-S if it had been prepared and standardized without lactose diluent. Both Rhozyme-S and Taka-Diastase are derived from cultures of *Aspergillus oryzae*.

Pancreatin, 250 mg, did not fully hydrolyze the starch of plant samples. The 2 cellulase preparations contained amylases, but did not hydrolyze crystalline cellulose under the conditions of method B. All preparations were tested at the optimal temperature and pH recommended by the manufacturer.

Quantity of enzyme preparation to be used in analysis. The preparation was standardized by determining the minimal quantity necessary to attain the maximal yield, or near maximal, of Rs when starch or plant samples containing 100 to 300 mg total carbohydrate were analyzed by method B. In the examples shown in table 8, 200 mg starch, 250 mg cornmeal, and 500 mg timothy hay were analyzed using 3 to 100 mg enzyme. The respective samples contained approximately 169, 175, and 70 mg maximal ACho, determined by method A.

Starch attained maximal hydrolysis with about 35 mg enzyme, factor 2.95, and with about 25 mg enzyme, factor 4.37. These quantities are, roughly, inversely proportional to the manufacturer's factor which is based upon maltose-hydrolytic activity. The hydrolysis of cornmeal and timothy hay attained a plateau with about 20 mg enzyme, factor 4.37, and the yield of Rs continued to increase very slightly with additional quantities of enzyme. This slowly rising plateau is characteristic of plant samples, indicating that the hydrolytic products of pentosans or other polysaccharides besides starch, which have been mobilized by the 0.10 N HCl digestion, are being further hydrolyzed by the enzyme preparation.

[14] Parke Davis and Company, Detroit.
[15] Rohm and Haas Company, Philadelphia.
[16] Miles Chemical Company, Clifton, New Jersey.
[17] Mann Research Laboratories, Inc., New York.
[18] See footnote 14.

TABLE 8

Standardization of enzyme; yield of reducing sugar (Rs) by method B, calculated as % starch or available carbohydrate (ACho)[1]

Wt of Rhozyme-S used	200 mg N.F. reference standard		250 mg yellow corn meal	500 mg timothy hay
	Rhozyme-S, factor 2.95	Rhozyme-S, factor 4.37		
mg.	*% starch*	*% starch*	*% ACho*	*% ACho*
3	84.0	90.2	67.0	12.3
5	90.1	94.2	68.8	12.8
10	94.2	96.2	70.6	13.0
15	95.8	97.7	—	—
20	97.3	97.6	72.2	14.1
25	98.1	98.9	—	—
30	—	—	72.2	14.3
35	99.7	98.8	—	—
50	100.0	98.9	73.8	14.7
100	—	—	73.2	15.6

[1] % starch or DM = % Rs × 0.90/0.845; % ACho = % Rs × 0.923.

Saccharolytic enzymes of Rhozyme-S. The enzymatic activity (tables 4, 7) of 100 mg Rhozyme-S alone toward 250 mg substrate, at pH 4.7, 6 hours at 50°, is summarized in 5 categories according to the percentage hydrolysis to Rs as follows: (i) *quantitative or nearly quantitative hydrolysis,* 95 to 100% of starch, glycogen, maltose, lactose, sucrose, and cellobiose; (ii) *high activity,* 75 to 95% of raffinose, turanose and melibiose; (iii) *intermediate activity,* 30 to 75% of inulin, hemicellulose and pectin; (iv) *low activity,* 5 to 30% of dextran, oxycellulose, carboxymethyl cellulose and melizitose; (v) *very low or no activity,* zero to 5% of crystalline cellulose (Avicel) and trehalose. Thus, Rhozyme-S had considerable hemicellulase and cellulase activity; but the cellulase activity was evident only with soluble cellulose derivatives, such as samples 19-22, which were allowed to become hydrated before the enzyme was added. The hemicellulase activity was not apparent in plant samples unless the hemicelluloses were mobilized from the plant structures and solubilized to some extent, as was pointed out previously.

Methods C and D, soluble sugars and starch

Fat extraction. Removal of fatty substances is essential for rapid entry of the aqueous solvent into cellular structures. This is necessary, particularly in such samples as prepared foods, diet composites and nuts, in which the particles may be enveloped with fat. This is accomplished very simply by adding a measured volume of absolute ethanol-ether mixture or isopropanol. In the case of fatty samples, the subsequent addition of water to the specified volume may give a fatty emulsion which may pass through the filter paper. Neither the emulsion in the filtrate, nor the fat emulsion (if any) on the filter paper, affect the subsequent enzymatic hydrolysis and Rs assay of the filtrate or of the residue after washing.

A period of 10 minutes is sufficient for the extraction. Long exposure to solvents may bring about retrogradation of starch.

Recommended conditions for precipitation of starch and extraction of soluble sugars. The recovery of starch is apparently readily accomplished in the assay of fresh or air-dried, unheated plant materials, in which the starch is already present in an oriented, nondispersible form in the starch granules. The results by method D in such unheated or ungelatinized samples agree very well whether 10 or 35% ethanol, or 40% isopropanol, is used in the extraction and subsequent washing; 20% ethanol represents a median concentration within the range of 10 to 35% which is being used in presently accepted methods (16, 46, 66).

However, complete precipitation may not be obtained when the starch has been previously gelatinized or solubilized as in home-prepared or factory-processed foods. The samples, with 20 to 40% ethanol or isopropanol, may yield opalescent or turbid supernatants and filtrates. Addition of NaCl immediately flocculates much of the material, but the filtrate still may show some opalescence or turbidity. However, if Celite is added, in addition to the salt, then clear filtrates are obtained. The precipitation proceeds slowly, and for this reason a sufficiently long period of 90 minutes is recommended for extraction and precipitation.

The temperature is important, since it affects the solubility of dispersed starch and its oligosaccharides; 20° is recommended because it represents a uniform, reproducible condition. Furthermore, the glassware is calibrated at 20° and, because alcoholic solutions have a considerable temperature coefficient of expansion, the adjustment to volume and measurement of the aliquot should be made at this temperature.

Recovery of granular starch by method D_{20}. The starch content (Rs $\times$ 0.923) of samples 1-4, table 1, was as follows: by analysis, 0.02, 51.6, 36.8, 36.7%; calculated ash-free DM of starch, none, 50.5, 36.8, 36.8%.

Analysis of a fifth sample, N.F. reference starch, yielded 85.7% starch, 85.6% ash-free DM. The close agreement is undoubtedly accidental.

Use of factor 0.923 for calculating starch. The factor, as used in equation 12 and applied in the 2 sets of analyses immedi-

ately above, is based upon an assumed overall recovery of 97.5% which allows for the following: (i) incomplete enzymatic hydrolysis which in potato, corn, and arrowroot starches (nos. 2, 6-8, table 4) varied from 97.6 to 99.7, average 98.8%; (ii) decreased hydrolyzability due to retrogradation either before analysis or during the analysis; (iii) incomplete mobilization from the cell structures; and (iv) incomplete recovery from the filter paper. Justification for the use of factor 0.923 is evident in the general consistency of the data throughout this work.

Recovery of soluble sugars and starch from gelatinized and acid-hydrolyzed starch solutions. Six-hundred milligrams of N.F. reference potato starch were placed into seven 100-ml volumetric flasks; 25 ml water were added to flasks 1 and 2, and 25 ml 0.10 N HCl to the other five. Flasks 1 and 2 were vigorously agitated while heated, 60 minutes at 90°. Flasks 3-7 were heated during periods from 5 to 30 minutes; while cooling, 25 ml 0.10 N NaOH were added. Soluble sugars were determined by method C_{20}.

In a second series, 300 mg of the starch were placed into 9 test tubes. Tubes 1 and 2 contained 15 ml water, and they were heated 60 minutes at 90°. Tubes 3-9 contained 15 ml 0.10 N HCl, and they were heated 5 to 50 minutes; while cooling, 15 ml 0.10 N NaOH were added. Starch was determined by method D_{20}.

All hydrolyzed solutions at 5 to 50 minutes gave the characteristic blue I-KI color. The degree of hydrolysis of each solution may be judged by the results in column 6, table 6. All results were calculated to starch by equation 12; the recoveries were based upon 85.65% ash-free DM. The data in figure 1 permit the following conclusions.

(i) Methods C_{20} and D_{20} are extremely sensitive to very slight changes in the degree of polymerization (DP) of starch. Thus, at 5 and 10 minutes of hydrolysis by the HCl, 43.2 and 12.2% starch were re-

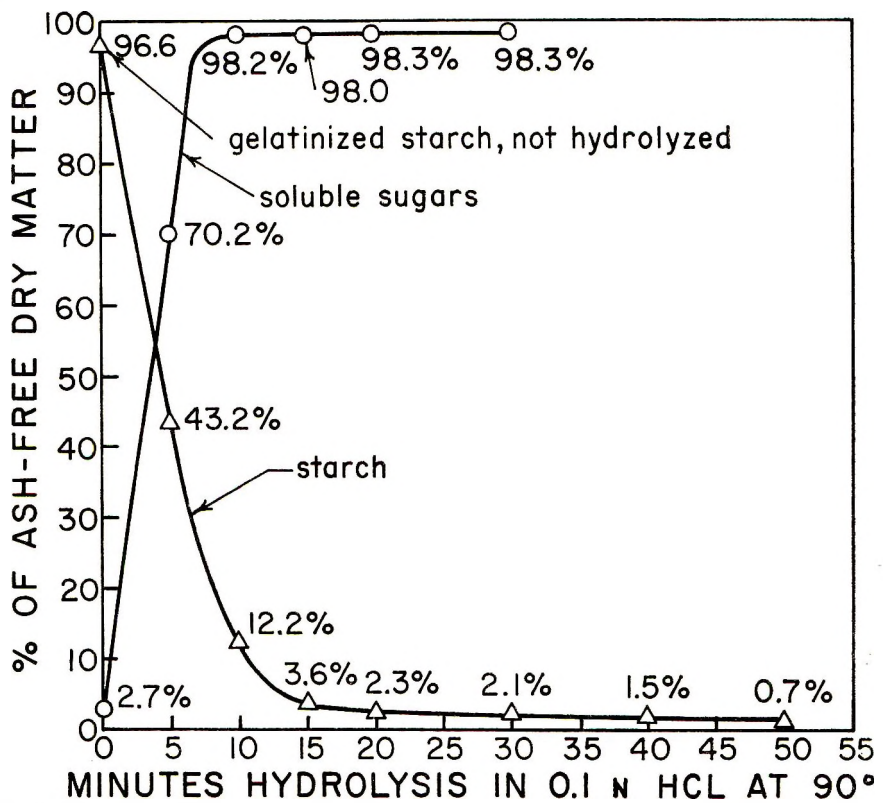

Fig. 1 Recovery of soluble oligosaccharides and starch by methods C_{20} and D_{20} from starch solutions that have been heated zero to 50 minutes in 0.1 N HCl at 90°.

covered, and 70.2 and 98.2% soluble Cho. The rate of hydrolysis during the critical first 10 minutes of heating was apparently greater in the thinner layer of solution in the flasks than in the tubes, resulting in 113.4 and 110.4% recovery of the total Cho; but at 15, 20 and 30 minutes, the totals were 101.6, 100.6, and 100.4%.

(ii) The great sensitivity is shown further by the recovery of 2.7% soluble Cho, and only 96.6% starch, or 99.3% total Cho, from the gelatinized unhydrolyzed starch. The gelatinized solution therefore contained small fractions of soluble polymers which either were present in the granular N.F. reference starch or were produced during the 60 minutes digestion at 90°.

(iii) Method D_{20} thus determines only the least soluble, higher starch polyglucans, probably equal to or approaching the DP of native starch and, therefore, it should not be applied to prepared foods for the assay of starch, except as a sensitive indicator of depolymerization during processing.

Soluble sugars in granular and gelatinized N.F. reference starch. The preparation and analysis of gelatinized starch in this experiment differed as follows from that of the previous experiment (fig. 1): gelatinization of 4,000 mg in 200 ml water in a 500 ml flask during 30 minutes at 90°, instead of 600 mg in 25 ml water in a 100 ml flask during 60 minutes; gentle mixing during heating instead of vigorous agitation; analysis of the white, opaque solution after standing about 16 hours in the refrigerator, instead of immediate analysis of the limpid, opalescent solution; analysis of 400 ml 20% ethanol extract, instead of 5 ml—both with 5 ml FeCy—thus representing the soluble Cho from 64 mg, instead of 6 mg, starch.

The soluble Cho in 20% ethanol (% Rs $\times$ 0.923/0.8565) of the gelatinized starch was 0.64%; and of the granular, ungelatinized starch, 0.02%.

These results suggest (i) that the method of preparation and subsequent handling of the gel before analysis is important. In the limpid, opalescent unhydrolyzed solution of figure 1, the starch molecules were highly dispersed and hydrated and, therefore, were more soluble, 2.7%, than in the white, opaque stored gell, 0.64%, in which the hydrated molecules were probably considerably aggregated through intermolecular hydrogen bonding.[19] (ii) However, in the granular starch only a trace of the alcohol-soluble fraction was dissolved, due undoubtedly to the high degree of intermolecular bonding between the many molecular species, of variable structure and DP, which are co-crystallized and tightly packed within the starch granule.

(iii) These results, and those of figure 1, indicate that the dry, partially degraded fractions of starch in heat-processed breakfast cereals, should be soluble or extractable under the conditions of methods C_{20} and D_{20}, depending upon the degree of depolymerization and disorganization of the crystalline state. This will be evident in the following discussion.

Effect of heat-processing of cereal grains. The following tests were performed on samples 11-20 (tables 5, 9) in addition to those previously described.

Iodine-binding particles: 500 mg were suspended by vigorous shaking with 25 ml cold water; 2 drops of suspension were mixed on a glass slide with 1 drop I-KI solution; the suspension was covered by a cover glass, and the average relative number of amorphous particles and those having the appearance of whole and fractured starch granules were counted in 10 fields.

Degree of polarization of granules: The suspension and the microscopic slide were prepared as before, except that the addition of iodine was omitted. The number of whole starch grains in 10 fields was counted in 3 categories, according to refraction of polarized light as compared with that of the unheated sample, namely, unchanged, markedly altered, and doubtful or no refraction.

Blue starch-iodine color given by the 20% ethanol extract and water extract: The extract of 2,000 mg in 100 ml 20% ethanol was prepared as in method C_{20}; 50 ml of clear extract were freed of alcohol, diluted to 250 ml and then filtered. An aliquot

[19] See the following reviews in the monograph by Radley (67): The swelling and gelatinization of starch, by J. A. Radley; starch retrogradation, by R. L. Whistler; starch and the hydrogen bond, by G. V. Caesar. See also discussions in the monograph by Ott and Spurlin (68), pertaining to hydration and hydrogen bonding in cellulose.

TABLE 9

Soluble sugars and starch of plant samples; effects of crude fiber and pentosan content; effect of heat processing[1]

	Sample[2]	AOAC (16) method; maltose and sucrose calculated as starch[2]	Methods C_{40} and D_{40} with 40% isopropanol			Methods C_{20} and D_{20} with 20% ethanol			Soluble starch; proximate analysis by reaction with I-KI and determination of absorbance at 610 mμ	
			Method C, soluble sugars, Rs × 0.923	Method D, starch, Rs × 0.923	Other soluble sugars; difference, method B1 minus methods C and D	Method C, soluble sugars, Rs × 0.923	Method D, starch, Rs × 0.923	Other soluble sugars; difference, methods B1 minus methods C and D	Water extract, with 1% NaCl and Celite	20% Ethanol extract as in method C
		% ACho	%ACho	% ACho	% ACho	% ACho	% ACho	% ACho	approx %	approx %
	Low starch, high crude fiber, high pentosan content									
1	Cereal grass	6.4	7.9	5.1	−0.1	7.1	4.7	1.1		
2	Alfalfa meal	3.4	6.7	5.8	−0.5	6.5	5.8	−0.3		
3	Timothy hay	4.6	5.2	1.9	−0.5	4.7	1.7	0.2		
4	Corn cobs	0.9	1.5	1.5	−0.1	1.4	1.2	0.3		
5	Oat hulls	0.2	0.8	2.7	0	0.8	3.0	−0.3		
	High starch, high crude fiber, high pentosan content									
6	Wheat bran	4.8	5.1	24.7	2.4	4.1	26.0	2.1		
7	Oats, green, whole grain	0.9	1.8	42.6	1.0	2.1	42.7	0.6		
	High starch, low crude fiber, low pentosan content									
8	Oats, rolled (Quaker)[4]	1.3	1.8	62.8	1.5	1.9	62.7	1.5		
9	Corn meal, whole, yellow	1.8	2.3	64.0	1.9	2.2	64.6	1.4		
	High starch, low crude fiber, low pentosan content; effect of heat processing (even-numbered samples)[5]									
11	Rice for puffed rice	0.2	0.4	81.6	1.3	0.7	81.9	0.7	0.06	0.04
12	Puffed rice	0.2	1.4	80.3	2.1	12.7	69.9	1.2	6.7	2.3
13	Wheat for puffed wheat	2.9	3.1	62.3	1.8	2.5	62.6	2.1	0.04	0.07
14	Puffed wheat	2.1	5.2	60.9	1.9	19.3	50.4	−1.7	15.3	4.0
15	Wheat for rolled wheat	2.3	2.6	62.3	2.6	2.7	63.5	1.3	0.02	0.03
16	Rolled wheat (Pettijohn's)[6]	2.3	2.4	63.8	1.9	2.6	64.6	0.9	0.03	0.03
17	Wheat flakes mix[7]	7.8	8.6	57.4	1.7	15.5	53.2	−1.0	4.5	0.9
18	Wheat flakes[7]	7.1	8.5	57.5	1.0	15.0	53.1	−1.1	4.2	1.1
19	Corn flakes mix[7]	5.6	7.3	68.8	2.2	7.6	70.5	0.2	0.05	0.04
20	Corn flakes[7]	5.5	7.0	65.7	1.3	10.6	63.8	−0.4	2.9	1.0

[1] Rs = reducing sugar; and ACho = available carbohydrate.
[2] Weight of sample analyzed was 2,000 mg by method C and 500 mg by method D.
[3] % starch or ACho = [Mal + Suc] × 0.923/0.950; although 3% lower than the sum of reducing sugars calculated as disaccharides, this calculation permits comparison of the results of column 2 with those of columns 3, 6, 9 and 10.
[4] Quaker Oats Company, Chicago.
[5] The results from samples 11-20 calculated to 10% moisture content.
[6] Quaker Oats Company.
[7] The mix of samples 17 and 18 had 5.0 and 2.5%, respectively, of added sucrose and malt syrup; the mix of samples 19 and 20 had 5.5 and 1.5%, respectively, of the sugars.

of the filtrate and enough water to bring the volume to 24 ml, and 1 ml 0.01 N iodine in 1% KI, were mixed in a 35-mm diameter Beckman test tube cuvette. A reagent blank was prepared, and the absorbance at 610 mμ was determined. The procedure was standardized against solutions which contained 1 to 10 mg calculated ash-free N.F. reference starch per 100 ml. The quantity of "soluble starch" was roughly approximated by assuming that the amylose content of rice, wheat, and cornstarch is the same as in the reference potato starch and that the iodine-binding per unit weight of amylose, and the light absorbance spectrum, are the same in the shorter chains as in the freshly gelatinized native starch. Water extracts at 20° were similarly prepared, adding NaCl and Celite as in method C_{20}.

The results are summarized as follows: (i) In samples 12, 14 and 20, heat processing resulted in an increased number of amorphous iodine-stainable particles and a marked reduction of refraction of polarized light by starch grains, indicating large-scale fragmentation and alteration of molecular orientation. These were the only samples in which the soluble Cho was greatly increased over that of the unprocessed grain or mix. The soluble, iodine-reacting oligosaccharides of puffed rice and puffed wheat were rapidly extractable.

(ii) The increased soluble Cho in the processed samples 12, 14, 20 represented soluble oligosaccharides of greater molecular weight than the simpler sugars which were determined by the AOAC (16) method, since the sugars by the AOAC method were not increased by the heat-processing. The gain of soluble Cho was at the expense of the insoluble native starch. Thus, in samples 12, 14, and 20, the gain of soluble oligosaccharides by method C_{20} vs. loss of starch by method D_{20} was roughly: + 12.0 vs. — 12.0; + 16.8 vs. — 12.2; + 3.0 vs. — 6.7%.

(iii) The extracts of processed samples 12, 14, 20 gave the characteristic deep blue I-KI color of the amylose of native starch and, therefore, the chain-lengths of the saccharides giving the color must have been still quite large. The increased soluble Cho apparently consisted almost entirely of such large molecules. Thus, the *increased soluble Cho,* by method C_{20}, vs. the *soluble starch* in the alcohol extract giving the I-KI reaction, was: 12.0 vs. 2 to 3%; 16.8 vs. 3.9%; 3.0 vs. 1.0%. The solubilization of the starch in water at 20° is strikingly shown by the data in column 9 of table 9.

(iv) The soluble sugars of the heat-processed samples had apparently greater chain-lengths than the soluble polysaccharides of commercially available soluble starches and dextrins, as was indicated by the relative insolubility in 40% isopropanol. Solutions of commercial soluble starches and dextrins, plus isopropanol to 40% strength, gave only a slight turbidity, whereas the results of analysis of samples 11-20 indicated a high degree of *insolubility* in 40% isopropanol. Thus, the difference of soluble Cho due to processing, in the respective pairs of samples 11-20, using 40% isopropanol, was: + 1.0; + 2.1; — 0.2; — 0.1; and — 0.3%. The differences in starch content, using 40% isopropanol, were: — 1.3; — 1.4; + 1.5; + 0.1; and — 3.1%.

(v) The soluble Cho of *unheated* plant samples 1-9, 11, 13, 15 (table 9) consisted mostly of sugars of low molecular weight, probably hexoses and disaccharoses which, for convenience, are designated as "maltose" and "sucrose" when determined by the AOAC method (16, 66). The results by method C_{20} differed by 3.1 to — 0.7, average, 0.58, from those by the AOAC method. In 10 of the 12 samples, the differences were not significant.

That the soluble sugars of unheated plant samples consisted largely of sugars of low molecular weight is further indicated by the close agreement of the results from samples 1-9, 11, 13, 15 which were obtained by means of 20% ethanol as compared with 40% isopropanol. The differences between each pair of results ranged from + 1.0 to — 0.3, or to + 0.28 average. In 10 of the 12 samples, these differences were not significant.

It appears, therefore, that the concentration of alcohol is of minor importance in the analysis of unheated native plant materials—in contrast with the major importance of the alcohol concentration in the analysis of mixes and prepared foods.

(vi) The same principle appears to apply also to the assay of starch in *unheated* samples 1-9, 11, 13, 15, in which the differences between the results with 20% ethanol as compared with 40% isopropanol ranged from + 0.4 to — 1.3, or —0.26 average.

(vii) Finally, the data, although few in number, give some indication of the stability of carbohydrates under the particular conditions of processing. (a) The degree of polymerization and the enzymatic digestibility of native starch were apparently unchanged by light cooking and drying (sample 16) and by light toasting (sample 18), as was indicated by precipitation with 20% ethanol and by recovery of Rs.[20] (b) However, starch was depolymerized in 3 samples under more drastic conditions of heating which, in samples 14 and 20, was accompanied by deep browning. Sample 12, puffed rice, browned only slightly, due perhaps to the very low content (only 0.2 and 0.5%) of maltose and sucrose and pentosans in the polished grains. (c) Pentosans, like starch, were probably depolymerized into soluble oligosaccharides under drastic conditions of heating; and in sample 14, which initially contained 5.5% pentosans, they may have contributed 16.8 minus 12.2, or 4.6, of the 16.8% soluble Cho. (d) The soluble oligosaccharides were as stable as the native starch and pentosans, and the recovery of Rs, i.e., the digestibility, was not apparently affected by the processing. (e) Destruction of maltose and glucose, and fructose derived by hydrolysis of the 5.5% added sucrose, probably occurred during the deep browning of sample 20. (f) The degree of browning was not related to the overall loss of carbohydrate, if any, in the 5 cereal preparations.

Application of method B to human foods and diet composites

Calculations: conversion factors. Before applying the method, a table of composition of native human foods was prepared, listing the percentages of moisture, TCho, CF, pentosans, Glc, Fru, Suc, Mal, Lac and St. The principal sources were Watt and Merrill (26), McCance and Lawrence (27), Widdowson and McCance (34), McCance and Widdowson (35), Leach and Winton (69), Winton and Winton (70), Tressler, Joslyn and Marsh (71) and Tressler and Joslyn (72). The conversion factors (table 10) for estimating AHex, ACho, and the kilocalories per gram of ACho were then calculated by equations 8, 12, 14.

A second table was prepared for the recipe foods which were served in the diets of this study and of other foods commonly present in the daily diet, using the data of the first table. The factors (table 11) for estimating AHex, ACho kilocalories per gram were calculated by equations 8, 12, 14 from the fractions of Glc, Fru, Suc, Mal, Lac and St of the total ACho of the food.

The factors for recipe foods may be calculated perhaps more simply if the factor (table 10) and ACho of each native food item, and the total ACho of the recipe, are known. Then

$$(24)\quad K = \Sigma \left[\frac{\text{ACho contributed by food item}}{\text{total ACho of recipe}} \times k \right]$$

in which K = overall factor for calculating AHex, ACho, kilocalories per gram; ACHo = sum of the starch and other available sugars; k = corresponding factor for each native food item. Equation 24 may be used also for calculating K of diet composites.

If the values for ACho of the individual components of the recipe or diet are not available, a fair approximation of K may be obtained by using the data of Watt and Merrill (26). Assume, as in the last column of table 12, that ACho = crude Cho — (3 × CF). The average values for k, as given in tables 10 and 11, may be used if the individual values for k are not available.

These factors are as essential for determining AHex, ACho and their kilocalorie equivalents as are the specific nitrogen conversion factors for estimating protein (1, 3, 73). However, the carbohydrate factors are probably more accurately derived than the protein factors, since they are based upon fairly reliable assays of individual carbohydrates, or groups of carbohydrates,

[20] The enzymatic digestibility of N.F. reference potato starch was decreased only slightly, but significantly, by heating the granular starch 4 hours in an oven at 120° and by autoclaving the freshly gelatinized starch 4 hours at 120°.

TABLE 10

Factors for calculating available hexose (AHex), available carbohydrate (ACho) and the calorie equivalent of ACho in native foods and derived foods in the human diet

Food	Available hexose factor	Available carbohydrates	
		Factor	Kilocalorie factor
			kcal/g
Mature seeds: immature lima beans; flours prepared from beans, corn, peas, rice, rye, wheat	1.0254	0.923	4.149
Potatoes, yams	1.0250	0.924	4.143
Bran	1.0218	0.927	4.119
Sweet corn, sweet peas	1.0174	0.935	4.071
Sweet potatoes	1.0155	0.945	4.024
Nuts	1.0083	0.947	3.986
Sucrose	1.0000	0.950	3.945
Corn syrup, baker's glucose	1.0115	0.956	3.964
Root vegetables: carrots, parsnips, radishes, rutabagas, turnips	1.0050	0.977	3.847
Leaf and stem vegetables: brussels sprouts, cabbage, endive, lettuce, spinach, celery	1.0026	0.981	3.825
Flower vegetables: broccoli, cauliflower	1.0032	0.978	3.844
Fruit vegetables			
Beans: green and yellow snap beans	1.0017	0.989	3.793
Cucumbers, egg plant, melons, squashes	1.0012	0.989	3.793
Tomatoes, tomato juice	1.0007	0.991	3.781
Fruits, frozen, sweetened and candied: blueberries, cherries, blackberries, peaches, pineapples, raspberries, strawberries[1]	1.0000	0.956	3.919
Fruits, fresh and fruit juices			
Apples, pears	1.0002	0.988	3.791
Apricots, peaches	1.0000	0.971	3.860
Bananas	1.0028	0.974	3.857
Berries: blueberries, blackberries, raspberries, strawberries	1.0000	0.994	3.766
Cherries	1.0000	1.000	3.743
Grapes	1.0000	0.997	3.753
Oranges	1.0000	0.976	3.841
Fruits, dried: apricots, dates, figs, peaches, prunes, raisins	1.0000	0.990	3.784
Syrups: cane, maple, sorghum, honey	1.0004	0.983	3.814
Glucose, invert sugar	1.0000	1.000	3.743
Lactose	1.1365	1.080	3.945
Milk, butter, cheese, cream	1.1365	1.080	3.945

[1] Addition of 25 parts sucrose to 75 parts fresh fruit was assumed.

whereas the protein factors are based largely upon approximation of the principal proteins in a small group of foods (73). These carbohydrate factors were used for the calculations in tables 12–14.

Application to composite samples of some native and derived foods in the human diet. Composite samples were prepared as described in the section, Materials and Preparation of Samples, and they were analyzed by method B (table 12). Crude protein (N × specific factor), crude fat, moisture and ash were determined, and from these data the NFE was calculated by difference, using the values for crude fiber given by Watt and Merrill (26). The purpose was to present data from a diverse variety of foods, to show the relation of Rs to AHex and ACho (columns 5–7), to show the relation of these results with the NFE by difference, and to compare our results with published data from similar foods.

(i) Our results for NFE (col. 8) agreed unexpectedly well with the NFE (col. 3) calculated from the data of Watt and Merrill for similar foods. The agreement was particularly close with 15 of the 20 samples. Therefore, the samples may be

TABLE 11

Factors for calculating available hexose (AHex), available carbohydrate (ACho) and calorie equivalent of ACho in some recipe foods in the human diet

Food	Available hexose factor	Available carbohydrate	
		Factor	Kilocalorie factor
			kcal/g
Breakfast cereals			
Unsweetened (puffed rice and wheat)	1.0245	0.924	4.140
Sweetened slightly (corn flakes, shredded wheat)	1.0226	0.926	4.126
Sugar coated	1.0192	0.932	4.089
Breads, bread rolls, and biscuits prepared from graham, rye, and wheat flours			
With water	1.0245	0.924	4.141
With 2-3% NFMS[1]	1.0272	0.924	4.143
With 4% NFMS	1.0275	0.927	4.140
With 6% NFMS (bread, biscuits, English muffins, corn bread)	1.0302	0.931	4.135
With milk, fruits, nuts, sugar (see cakes, etc.)			
Cakes, cookies, muffins, rolls, sweet rolls; without icing[2]			
With milk; medium sweet (corn muffins, cinnamon rolls, hot cross buns, etc.)	1.0232	0.937	4.085
With milk, bran; medium sweet (bran muffins)	1.0261	0.949	4.050
With milk, fruits, nuts; medium sweet (raisin, date-nut and Boston brown breads)	1.0193	0.941	4.045
With milk, (sweet rolls, doughnuts)	1.0179	0.942	4.059
With milk, very sweet (white and chocolate cakes, devil's food, fudge cake)	1.0109	0.945	4.005
With water, eggs; very sweet (white and yellow angel food, sponge cake)	1.0070	0.943	4.000
With water, eggs, butter; very sweet (pound cakes)	1.0118	0.938	4.037
With water, eggs, butter, currants; very sweet English currant cake)	1.0084	0.959	3.940
French toast, griddle cakes, waffles			
With milk; not sweetened (French toast)	1.0369	0.940	4.123
With milk; slightly sweetened (griddle cakes, waffles)	1.0343	0.941	4.112
Pies, with plain crusts[3]			
Double crust, fruit, starch or flour (apple, peach, cherry pies)	1.0112	0.945	4.004
Double crust, mixed fruits (mince pies)	1.0082	0.961	3.929
Single crust, fruit juice, starch or flour, eggs, meringue top (lemon meringue pie)	1.0076	0.943	4.003
Single crust, gelatin, cream (lemon, chocolate chiffon pies)	1.0095	0.947	4.000
Single crust, chocolate, starch or flour, milk, meringue top (chocolate meringue pie)	1.0144	0.952	3.993
Single crust, vegetable fruit, eggs, milk or cream (pumpkin pie)	1.0219	0.958	3.998
Single crust, eggs, milk (custard pie)	1.0279	0.960	4.011
Icings for rolls, sweet rolls, and cakes[2]			
With water, eggs, and/or butter (uncooked and boiled white icings)	1.0006	0.951	3.939
With milk or cream, butter (chocolate, chocolate fudge, caramel)	1.0030	0.953	3.943
Ice cream (chocolate and vanilla)	1.0246	0.973	3.946

[1] Non-fat milk solids, NFMS.
[2] Icing is assumed to be added as follows: 25 parts icing to 75 parts cakes or cookies (26); 15 parts icing to 85 parts muffins, rolls, or sweet rolls.
[3] The data cannot be categorized as readily as those of the other foods of this table because of the use of many types of crusts, of fillings, and of toppings. Therefore, data are given only for the most commonly prepared pies in each of a few categories.

TABLE 12

Analysis of some human foods by method B; comparison of results with published data from similar foods, calculated to the same moisture content[1]

	Composite samples	Watt and Merrill (26); AOAC (16) methods[2]		McCance and Widdowson (35); starch + soluble sugars expressed as glucose[2]	Authors' data				
					Method B, with digestion by 0.1 N HCl			NFE = crude Cho by difference minus CF[2]	Corrected NFE = crude Cho by difference, minus 3 × CF
		Moisture	NFE by difference		Reducing sugar (Eq. 6)	Available hexose (Eq. 8)	Available carbohydrate (Eq. 12)		
		%	%	*% glucose*	*% Rs*	*% AHex*	*% ACho*	%	%
23	Eggs, boiled	73.7	0.9	0	1.60	1.64	1.48	0.6	0.6
24	Eggs, fried	67.7	0.3	0	1.48	1.52	1.37	1.8	1.8
25	Turkey, roasted	57.3	0	0	1.73	1.77	1.60	–	–
26	Beef, lean, raw, round	66.6	0	0	1.12	1.15	1.03	3.8	3.8
27	Milk, whole	87.4	4.9	5.05	4.31	4.90	4.65	5.0	5.0
28	Milk, whole				3.99	4.53	4.31	–	–
29	Oatmeal, cooked	86.5	9.5	10.2	9.67	9.91	8.94	11.5	11.1
30	Bread, 4% non-fat milk solids	35.6	50.3	54.5	51.57	52.99	47.81	51.9	51.5
31	Cookies, ginger snaps	3.1	79.7	74.9	78.57	79.98	74.01	72.4	72.2
32	Noodles, raw, dry	9.8	71.6		72.50	74.34	66.92	69.6	68.8
33	Potatoes, raw	79.8	16.6	17.4	16.82	17.24	15.54	16.9	15.9
34	Catsup	68.6	24.9	21.4	23.40	23.47	22.98	24.7	23.7
35	Beans, lima, raw	67.5	20.3		19.34	19.83	17.85	21.7	18.1
36	Peas, green, raw	78.0	12.4	10.8	10.54	10.72	9.85	14.0	10.0
37	Carrots, raw	88.2	8.7	6.0	6.95	6.98	6.79	8.7	6.7
38	Apricots, raw	85.3	12.2	7.4	12.43	12.43	12.07	13.2	12.0
39	Pears, canned, light syrup	83.8	14.9	13.1	13.35	13.35	13.19	14.6	13.2
40	Peaches, frozen, sweetened	76.5	22.2		20.88	20.88	19.96	22.0	21.2
41	Pineapple, frozen, sweetened	77.1	21.9		20.00	20.00	19.12	21.7	21.1
42	Orange juice, canned	86.5	12.1	10.3	9.76	9.76	9.53	12.2	12.0

[1] NFE = nitrogen-free extract; CF = crude fiber; Rs = reducing sugar; AHex = available hexose; and ACho = available carbohydrate.

[2] The percentages of crude fiber given by Watt and Merrill (26) were used in calculating NFE by difference, column 8. Compare NFE, column 3, with ACho and NFE, columns 7 and 8.

[3] Compare the results for glucose, column 4, with those for AHex, column 6. The results for bread represent the average of McCance and Widdowson's (35) samples 18, 19, 20.

presumed to have been fairly representative of each category.

(ii) The NFE was greater than ACho, with only 2 exceptions (samples 23, 31). However, when the NFE was corrected by further subtraction of CF, as in column 9, then most of the calculated values from the cereals, vegetables and fruits approached those of ACho, as well as could be expected from the crude method of calculating crude Cho. In human foods 6, 8-19 (table 5), the corrected NFE again approximated the ACho, more nearly by method B1 than by method B. Therefore, the relation, corrected NFE = crude Cho $-$ (3 $\times$ CF), approximates the chemically determined ACho and is, undoubtedly, a better measure of the available crude Cho than NFE in human plant foods. The correction, (3 $\times$ CF), allows for the CF, pentosans,[21] and other nonsaccharine substances.

(iii) Depending upon the content of lactose or starch, AHex was greater than Rs: in milk, it was 13.6% greater; in cereal products and potatoes, it was 1.7 to 2.7% greater. Generally, AHex was only slightly greater than Rs in vegetables, and it was equal to Rs in fruits.

(iv) The data obtained by method B indicate that eggs and meats contain significant quantities of carbohydrate. Blood plasma and all animal tissues contain polysaccharides which are associated with proteins; all tissues contain glycogen and glucose, which may disappear rapidly postmortem. This has long been known and verified repeatedly. Yet, the standard tables of food composition (26) give zero per cent crude Cho in all categories of samples of raw beef, pork, lamb, chicken, turkey and fish. McCance and Widdowson (35), using chemical methods, apparently obtained zero per cent carbohydrate in eggs and all varieties and cuts of meats.

Application to diet composites. The diets (table 13) were nutritionally adequate in all respects. They consisted of 22 to 27 natural and recipe foods, and the measured quantities of each food varied greatly among the 28 individual diets. The whole diet per day contained 8 to 77 g

[21] See footnote 2.

TABLE 13

Analysis of diet composites; relation of crude carbohydrate (Cho) by difference to results obtained by method B, with preliminary digestion by 0.1 N HCl[1]

	Series 1, 1961 (11 diets)	Series 2, 1963 (17 diets)
Moisture, %	62.35-72.08[2]	63.30-74.26
AHex factors	1.018-1.029	1.018-1.040
ACho factors	0.944-0.953	0.947-0.973
Rs, % glucose	16.28-22.59	13.09-19.85
AHex, % hexose	16.75-23.08	13.52-20.48
ACho, % carbohydrates	15.51-21.36	12.65-18.93
Crude Cho by difference, % carbohydrates	15.83-23.01	13.25-19.09
ACho recovery, % crude Cho	87.7-98.0	82.3-105.8
ACho recovery, % crude Cho	94.2$\pm$3.1[3]	98.0$\pm$6.7
Kilocalories/g crude Cho, by calorimetry[4]	3.750$\pm$0.222	3.728$\pm$0.454
Metabolizable kcal/g crude Cho[5]	3.805$\pm$0.136	3.928$\pm$0.268
Kilocalorie factor of ACho by equation 14	4.021-4.046	3.989-4.049
Kilocalorie factor of ACho by equation 14	4.038$\pm$0.008	4.013$\pm$0.016

[1] AHex = available hexose; ACho = available carbohydrate; and Rs = reducing sugar.
[2] Range.
[3] Average $\pm$ SD.
[4] Kilocalories/g crude Cho = $\frac{\text{total kcal} - [5.65 \times \text{crude protein} + 9.40 \times \text{crude fat}]}{\text{crude Cho}}$.
[5] Metabolizable kcal/g crude Cho = $\frac{[\text{ACho} \times \text{kcal factor}]}{\text{crude Cho}}$.

estimated lactose from breads, cakes, milk, other dairy products, etc.; 32 to 169 g sucrose, mainly from sucrose, fruits, fruit juices, salads; 2 to 34 g maltose, mainly from proprietary preparations; 25 to 62 g glucose and invert sugar, mainly from vegetables, salads, fruits, fruit juices; 115 to 262 g starch; 1.7 to 5.6 g crude fiber; 272 to 447 g estimated metabolizable carbohydrates and crude fiber. The composites contained 62 to 74% water, 3.81 to 7.42% protein (N $\times$ 6.25), and 5.17 to 9.93% crude fat; they were liquid and tended to settle.

(i) The maximal variation of Rs among triplicate samples of each of the 28 composites, was less than 0.95% of the mean (= 100%) of each. The standard deviation from the mean (= 100%) in each set of Rs determinations, was 0.56% in series 1 and 0.27% in series 2.

(ii) The range of AHex and ACho factors was small, from 1.018 to 1.040 and from 0.944 to 0.973, representing differences of only 2.2% and 3.1% between the respective minima and maxima. The range was narrower than the range of the factors for recipe foods (table 11). The differences among factors depended largely on the intake of liquid milk and, to a considerably lesser extent, on the intake of starchy foods. The effect of milk is evident also in the examples of recipe foods shown in table 11.

The following factors are suggested for estimating AHex and ACho in nutritionally adequate mixed diets of 2,000 to 3,000 total metabolizable kcal when the exact amounts of the individual foods, except milk, are not known: 1.020 and 0.940, with no liquid milk; 1.025 and 0.950, with 1 to 2 glasses milk; 1.030 and 0.960, with 2 to 4 glasses milk. Generally the factors for complete human diets fall well within 1% of these suggested factors. Such factors may be used whenever the composite is made up of helpings or measures of the foods as served, instead of weighed quantities of each item. When the weight of the individual foods is known, as in controlled dietary experiments, or as was the case in the diets of table 13, then AHex and ACho are estimated more accurately by applying the factors which are obtained from equation 24.

(iii) The recovery of ACho varied greatly among the diets, from 87.7 to 98.0% in series 1 and from 82.3 to 105.8% in series 2. These variations were caused most likely by the cumulative effect of errors or variations in the 4 determinations which are required for estimating the crude Cho by difference.

(iv) The available energy of the carbohydrate was determined with greater ease and accuracy from the AHex or ACho than by the conventional method, since it did not require (a) calorimetry; (b) assays of moisture, protein, fat, and ash; (c) use of approximations of the heats of combustion of crude protein and crude fat (footnote 4, table 13); (d) use of the assumed factor 6.25 $\times$ N for crude protein; and (e) the extremely cumbersome determination of digestibility. The difficulties of calorimetric determinations are particularly great in samples of high moisture content, as in the diets of table 13, which had to be dried in vacuo at low temperature before calorimetry. The results for kilocalories per gram of crude Cho by direct calorimetry were low and, as expected, highly variable. The kilocalories per gram of metabolizable crude Cho also were highly variable (assuming that ACho = metabolizable crude Cho, recognizing that ACho by method B included some of the pentosan in the food); this was caused most likely by errors in determination of crude Cho, since the values for Rs could be duplicated within less than $\pm$ 0.95% *maximal* deviation from the mean in replicates or in repeated analyses.

In both series of diets the mean metabolizable energy of the crude Cho was considerably less than the commonly used factor 4.00 kcal/g for calculating the available energy of crude Cho in whole diets. Factor 3.85 kcal/g crude Cho would apply perhaps more accurately to diets like those of table 13 which contain liberal quantities of milk, fruits and fruit juices.

Application to packaged military rations. Unlike the composites of the 28 greatly varying diets of table 13, the 4 composites of table 14 were prepared from identical items of 6 daily menus of the U. S. Army C-Ration in each of 4 large cartons which

TABLE 14

Analysis of composites of U. S. Army packaged C-Ration; relation of crude carbohydrate (Cho) by difference, with results obtained by method B, with preliminary digestion by 0.1 N HCl; relation of recovery of ACho to digestibility[1]

	Composite of 6 menus of				Avg of all analyses
	Carton 1	Carton 2	Carton 3	Carton 4	
Moisture, loss by lyophilization, %	53.24	53.36	53.37	53.29	53.31
Moisture, 16 hr in vacuo, 60-70°, %	54.28	54.33	54.36	54.18	54.29
Moisture, 24 hr in vacuo, 70°, %	54.72	54.46	54.39	54.37	54.48
Protein, N × 6.25, %	7.60	8.25	8.77	8.83	8.36
Fat by Mojonnier method, %	7.43	7.65	7.25	7.49	7.46
Ash, %	1.33	1.28	1.33	1.73	1.42
Crude Cho by difference, %[2]	28.92	28.36	28.26	27.58	28.28
Rs, %[3]	28.16 27.04	28.40	28.61	28.72	28.33
AHex, Rs × 1.020, %[4]	28.72 27.58	28.97	29.18	29.29	28.90
ACho, Rs × 0.940, %[4]	26.47 25.42	26.70	26.89	27.00	26.63
Kilocalories of AHex/100-g sample, AHex × 3.743	107.50 103.23	108.43	109.22	109.63	108.17
Metabolizable kcal/g crude Cho[5]	3.717 3.569	3.823	3.865	3.975	3.825
ACho recovery, % crude Cho	91.5 87.9	94.1	95.1	97.9	94.2
Digestibility of crude Cho:					
by rats, avg %	— —	—	92.4	91.9	—
by human, avg %	— —	—	—	—	96.5[6]

[1] Rs = reducing sugar; AHex = available hexose; and ACho = available carbohydrate.

[2] In a previous study (74), analysis of composites of 8 cartons, calculated to 54.48% moisture, gave 27.38 to 29.04% maximal range of crude Cho by difference, average 28.25%.

[3] Both sets of the widely disagreeing results of duplicate analyses of sample 1 are given. Unfortunately, the samples were discarded soon after analysis. Both results are therefore given, since the analysis could not be repeated. Duplicate analyses of samples 2, 3, 4 yielded closely agreeing results.

[4] Factors 1.020 and 0.940 were used as recommended in the discussion of the results of table 13. The ration contained no liquid milk, although some of the items contained milk solids. Detailed recipes of all food items were not available.

[5] Metabolizable kcal/g crude Cho $= \frac{\text{AHex} \times 3.743}{\text{crude Cho}}$.

[6] Digestibility by 9 human subjects (62) was as follows: C-Ration, stored 22 months in refrigerator at 5°, 94.6 to 97.2% range, 96.5% average; C-Ration, stored 22 months in warm room at 48°, 94.1 to 97.1% range, 96.1% average; control diet of freshly prepared foods similar to that of C-Ration, 96.9 to 98.5% range, 97.7% average.

had been taken all at one time from the factory assembly line and stored at 5° in the refrigerator until the time for analysis (62). The cans and packages were emptied completely; the average net total weight of all the foods was 1,675 g.

(i) The values for crude Cho shown in table 14 probably did not accurately represent the true carbohydrate content, since the crude Cho depended upon the values for moisture, protein, fat and ash which, in turn, were considerably affected by the methods chosen for the individual determinations. Thus, in the determination of moisture, after the preliminary removal of free water at 70° at atmospheric pressure, at least 24 hours' heating in vacuo at 70° was required to attain relatively constant weight in the fatty, sticky, syrupy residue. The moisture content by 2 methods of heating should be noted. Determination of moisture by lyophilization was carried out at low temperature, followed by continued high vacuum at room temperature during 24 to 36 hours. Crude protein was calculated by using factor 6.25, which is the factor that is customarily applied to whole diets. Factor 6.15 perhaps should have been applied, thus decreasing the crude protein values by about 0.1, since about 40% of the protein was of plant origin, largely of cereals. The Mojonnier method (16) was used to determine crude fat, which was more convenient and gave higher results than the older method of extraction by absolute ether. The crude fat extract was carefully re-extracted with petroleum ether; the extracts were filtered and the

filter paper was well washed with the ether, after which the clear extract was evaporated, dried and weighed. Cumulative small errors in determination, or differences due to the method chosen, especially of moisture, therefore could have considerably affected the values for crude Cho.

(ii) The values for Rs, AHex and ACho *increased* in the order of samples from 1 to 4, whereas the crude Cho *decreased.* The recovery of ACho therefore increased, from about 90% to about 98% in the same order of samples. The divergence of recoveries in these samples of quite uniform composition was as great as in the more watery samples of variable composition of table 13.

(iii) The metabolizable energy per gram of crude Cho varied through the same percentage range as the apparent recovery, since the calculations were based upon [AHex $\times$ 3.743]/crude Cho. Again the results indicate that factor 3.85 kcal/g crude Cho would apply perhaps more accurately to whole mixed diets than the commonly accepted value of 4.0 kcal/g.

Relation between apparent recovery of ACho and human digestibility of crude Cho. The following conclusions are based upon the data of tables 13-15. (i) The apparent recovery of ACho by method B in samples 6 and 8-20 (table 15) approximated the upper limits of human digestibility of similar human foods; the recovery by method B1 generally approximated the lower limits of digestibility.

The result with method B was expected, since the determination included a large part of the pentosans (64% average, as noted previously) which was perhaps greater than in the biological method; but, unlike the biological method, it did not include cellulose. Apparently, the liberation and hydrolysis of the pentosans by 0.1 N HCl and the enzyme preparation compensated for the nondigestibility of cellulose in method B. Thus, apparently, digestible Cho = 0.99 [soluble Cho + starch] + 0.64 pentosans.

However, a similar result may be obtained if an approximately equal average digestibility of 30% of the cellulose and 30% of the pentosans is assumed as in equation 20. This assumption recognizes the fact that a considerable percentage of both cellulose and pentosans disappears in the intestinal tract; the percentage is generally greater than 30% (6, 17–25, 27). For example, in 7 experiments reviewed by Merrill and Watt (table 19 of Ref. 25), 43 to 90%, average 80.1%, of the CF in various types of diets was not recovered. The hemicelluloses appear to have about the same range of digestibility as cellulose, as indicated in animal and human feeding experiments.

The high values for the human digestibility of CF or cellulose are undoubtedly very much overestimated since they depend upon recovery of CF in the feces. The determination is difficult and is subject to several errors: first, a considerable part of the CF may be lost through the relatively coarse filters that are used in the 2 filtrations and washings. Thus, the finely divided, undigested CF of breads and breakfast cereals is probably lost entirely through the filters. The same loss would be encountered with other finely comminuted foods, of which there are many in the human diet. As pointed out by Henneberg (2), and abundantly confirmed since, the determination of CF is greatly affected by the particle size. The fibers of the edible portions of unground foods, such as vegetables and fruits, are more delicate and less stringy than those of the animal forages. Cooking and baking of such foods depolymerizes cellulose to some extent; these processes disrupt cell structures and soften and separate the fibers, even of forages, making them more susceptible to bacterial action which occurs most readily in the regions of amorphous cellulose in the fibers (75). Thus the long cellulose structures may be fragmented into shorter particles, of which some may pass through the coarse filter. It is evident, therefore, that bacterial action, in addition to fineness of grinding, affects particle size and recovery of the residual CF in the feces. The disappearance of CF may be greater in some subjects than in others, depending upon the bacterial flora and the passage-time through the gastrointestinal tract which may vary in healthy subjects from 1 to 5 days.

TABLE 15

Relation between apparent recovery of available carbohydrate (ACho) and human digestibility

	Sample	Apparent recovery of ACho by eq. 16		Human digestibility of crude Cho by difference		
		Method B, with preliminary digestion with 0.1 N HCl	Method B1, with preliminary digestion with water	Data of Merrill and Watt (25) Range	Data of Merrill and Watt (25) Average	Calculated by eq. 20[1]
		%	%	%	%	%
	Low starch, high crude fiber, high pentosan, high lignin content					
1	Cereal grass	35.1	21.1			37.7
2	Alfalfa meal	29.4	20.2			34.3
3	Timothy hay	20.4	8.7			30.5
4	Corn cobs	26.6	3.2			26.2
5	Oat hulls	27.4	4.1			27.3
	High starch, high crude fiber, high pentosan content					
6	Wheat bran	73.6	49.8	28.8-74.7	56	61.9
7	Oats, green, whole grain	72.7	64.1			72.6
	High starch, low crude fiber, low pentosan content					
8	Oats, rolled (Quaker)[2]	99.8	96.8	94.4-99.1	98	97.4
9	Cornmeal, whole grain, yellow	94.0	88.6	92.6-99.7	96	90.1
	High starch, low crude fiber, low pentosan content; effect of heat processing					
11	Rice for puffed rice	99.5	99.6	98.0-99.8	99	99.1
12	Puffed rice	98.8	99.9			99.3
13	Wheat for puffed wheat	94.1	88.6	87.5-98.0	90	90.9
14	Puffed wheat	92.9	89.5			91.6
15	Wheat for rolled wheat	94.3	86.9	87.5-98.0	90	89.4
16	Rolled wheat	93.9	88.0	86.9-95.1	90	90.1
17	Wheat flakes mix	95.6	92.0		[91][3]	93.7
18	Wheat flakes	95.8	90.0			91.6
19	Corn flakes mix	99.1	98.2		[99][3]	97.9
20	Corn flakes	95.0[4]	92.5[4]			92.1[4]
22	Bulger wheat wafer[5]	94.8			[95][3]	

[1] Calculations are based upon the data of table 5.

$$\text{Calculated \% digestibility of crude Cho} = \frac{0.99\,[\text{ACho by method B1}] + 0.30\,[\text{CF} + \text{Pentosans}]}{\text{crude Cho by difference}} \times 100.$$

[2] Quaker Oats Company, Chicago.

[3] Calculations are based upon the formulation (see section on materials and preparation of samples) and the averages given by Merrill and Watt (25).

[4] These data are obviously too low. See footnote 6, table 5.

[5] Bulger wafer, no. 22 ("Wafer, Survival, All Purpose (Civil Defense) Bulger Type"), procured by QM Corps, Department of the Army.

The second source of error results from continued bacterial digestion after collection of the sample. This can be minimized by immediate freezing. But the action continues during unfreezing and mixing of the sample at room temperature and subsequent keeping of the sample at less than freezing temperature until time for analysis. Under these conditions, a considerable portion of cellulose particles may disappear as viewed microscopically (76).

The assumption of equal digestibilities of 30% of cellulose and pentosans is conservative; it permits approximate calculation of the digestible Cho of single foods or diets, without the use of the crude Weende chemical and biological methods. Because of the assumption of equal digesti-

bilities, the calculation is independent of the ratio of CF (or cellulose) to pentosans. The assumed 30% average digestibility of CF and pentosans is slightly less than one-half of the 64% average hydrolyzability of pentosans by method B, mentioned previously. Therefore, the recovery of ACho (col. 2, table 15), should approximate both the biological (col. 4 and 5) and the calculated (col. 6) digestibilities.

The agreement, in samples 6-22 (table 15) between the recovery of ACho by method B and the biological and calculated digestibilities is unexpectedly close when allowance is made for the crudeness of the methods for determining crude Cho, CF, pentosans and biological digestibility. An equally satisfactory agreement was obtained with samples 1-7 which contained larger quantities of CF and pentosans.

The recovery of ACho by method B1 in samples 1-5 was low and probably approximated the lower limits of human digestibility, provided these powdered roughage feeds had been suitably prepared by cooking or baking in order to break down some of the plant structures and to gelatinize and liberate the starch. As noted previously, ACho determined by method B1 is approximately equal to the soluble sugars and starch determined by methods C + D. Thus, the results by method B1 indicate that virtually none of the carbohydrate energy of the crude Cho of such plant materials as samples 3-5 was readily available; however, the results by method B appear to indicate that about one-fifth was potentially available in the green cereal grass and alfalfa, and that wheat bran is a fairly good source of readily available carbohydrate energy if necessity should require its use as a food. The metabolizable portion of the crude Cho in samples 1-5 is probably less than is indicated by method B and perhaps more than is indicated by method B1.

(ii) The recovery of ACho by method B in 28 whole diet composites (table 13) varied from 82.3 to 105.8%, average 96.5%; the recovery in the U. S. Army C-Ration (table 14) varied from 87.9 to 97.9%, average, 94.2%. These values are within the range of digestibility of crude Cho in mixed human diets as determined in human feeding experiments. In a previous experiment (62), the following digestibilities of crude Cho by 9 human subjects were noted: 94.6 to 97.2% range, 96.5% average, in C-Ration which had been stored 22 months at 5°; 96.9 to 98.5% range, 97.7% average, in a control diet of freshly prepared foods similar to that of the C-Ration. The following digestibilities of crude Cho have been calculated from the data given in a review of the literature by Merrill and Watt (25): 93.6 to 96.5% in 4 mixed diets with large amounts of legumes; 93.7 to 97.6% in 3 mixed diets with large amounts of cereals; 97.5 to 98.7% in 3 mixed diets with large amounts of vegetables; 97.5 and 98.0% in "mixed diets"; and 96.4% average digestibility in these 12 diets.

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