

# A Palatable Diet for Producing Experimental Folate Deficiency in Man

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FOR many years, it has been believed that folate deficiency on a nutritional basis could not be produced in man because of folate synthesis by the bacterial flora of the intestine.<sup>1</sup> Indeed, no hematologic abnormalities were noted in human volunteer subjects after subsisting on a synthetic diet<sup>2</sup> of vitamin free casein, Crisco,<sup>®</sup> dextrimaltose, a mineral mixture, cod liver oil concentrate and a supplementary vitamin mixture containing no folic acid for eighteen months. This diet contained 14 to 17  $\mu\text{g}$ . of folate activity per day as measured by S. lactis R.<sup>1</sup>

In 1961 an abstract<sup>3</sup> appeared which stated: "A study has been made of four patients fed on an artificial liquid diet<sup>†</sup> which appears to provide a complete replacement of all known dietary requirements except folic acid. Estimation has shown that its folic acid content is less than 0.5  $\mu\text{g}$ ./kg. (Fleming, A., personal communication). On such a diet, individuals showed an abnormal excretion of FIGlu after 4-5 weeks. A further patient fed on the same diet with the addition of foods containing 2-5

$\mu\text{g}$ . of folic acid daily<sup>‡</sup> did not excrete excess FIGlu after histidine (30 g) loading even after a period of 3 months. Thus, it would appear that the minimum folic acid requirements in man are of the order of 5  $\mu\text{g}$ . per day, which would suggest that the total body stores do not normally exceed 0.2 mg. After treatment with folic acid, the FIGlu excretion returns to normal, but in contrast to the rapid effect of folic acid on the megaloblastic bone marrow, a matter of hours, there is a delay of several days before FIGlu excretion returns to normal."

The same workers subsequently reported<sup>4</sup> that at the time the abnormal FIGlu excretion was present, there were no hematologic or bone marrow changes and that after ten months one patient of the original group further studied was still not anemic. This failure to observe hematologic abnormality is different from the experience with the diet to be presented here.

It was recently reported<sup>5</sup> that megaloblastic anemia had been produced in a healthy thirty-five year old male physician who had adhered to the diet to be described for four and a half months; this diet contains approximately 5  $\mu\text{g}$ . of folate per day. It consists primarily of food thrice-boiled in large quantities of water to extract water-soluble vitamins, which are replaced, except for folate, by supplementation. In the study, hematologic morphologic abnormalities *preceded* an increase in the urinary excretion of FIGlu.

<sup>‡</sup> Drs. Knowles and Pranker<sup>6</sup> have recently informed us that their assays and referral tables did not use ascorbate to protect folate activity, and thus their figures are approximately tenfold too low. This correction would bring their results more in conformity with our own.

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<sup>†</sup> This artificial diet was Complian, Glaxo. Using L. casei assay, without conjugase treatment, Waters and Mollin (personal communication) found this diet to contain approximately 50  $\mu\text{g}$ . of folate activity per kg. Confirming that finding, we found 58  $\mu\text{g}$ . folate activity per kg. Incidentally, Glaxo's Complian is now made with added folic acid.

TABLE I  
Folate-Deficient Diet Menu

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*Breakfast*  
Black coffee *ad libitum* with sugar *ad libitum*

*Lunch and Dinner*  
Sliced white meat of chicken or sliced frankfurters, plus rice or diced potato with margarine or  
Maine sardines in soybean oil or  
Casserole (100 gm. beef hamburger, 100 gm. potato or rice, 50 gm. onions, 33 gm. egg white)

*Ad libitum*  
Seasonings  
Salt, pepper, monosodium glutamate, Worcestershire sauce, lemon extract

Beverages  
Cola, ginger ale, Seven-up, coffee (instant), whiskey

Desserts  
Flavored gelatin, corn starch pudding, thrice-boiled apple-sauce

Sweets  
Marshmallows, and a limitless variety of hard and soft candies consisting of the following ingredients: sugar, corn syrup, gelatin, corn starch, shortening, artificial flavor, U. S. certified color

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Typical Three-Day Menu

Day 1

*Breakfast*  
Coffee, sugar (no cream)

*Lunch*  
5 frankfurters (total of 200 gm.)  
Rice, 150 gm.  
Oleomargarine, 20 gm.  
Corn starch pudding with rice  
Ginger ale, 360 ml.

*Dinner*  
Chicken, 100 gm.  
Rice, 150 gm.  
Oleomargarine, 20 gm.  
Gelatin dessert

Day 2

*Breakfast*  
Coffee, sugar (no cream)

*Lunch*  
Chicken, 100 gm.  
Rice, 150 gm.  
Oleomargarine, 20 gm.  
Applesauce, 100 gm.  
Cola beverage, 360 ml.

*Dinner*  
Hamburger, 100 gm. }  
Rice, 100 gm. } Mixed together  
Oleomargarine, 20 gm. } and heated in  
Onion, 50 gm. } a casserole  
1 egg white  
Corn starch pudding with rice

Day 3

*Breakfast*  
Coffee, sugar (no cream)

*Lunch*  
Sardines, 1 can (120 gm.)  
Rice, 150 gm.  
Oleomargarine, 20 gm.  
Gelatin dessert  
Ginger ale, 360 ml.

*Dinner*  
Chicken, 200 gm.  
Rice, 150 gm.  
Oleomargarine, 20 gm.  
Corn starch pudding  
Cola beverage, 360 ml.

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In addition to producing megaloblastic anemia due to pure nutritional folate deficiency, this diet has been used in a study<sup>6</sup> which indicates the minimal daily adult folate require-

ment to be in the range of 50  $\mu\text{g}$ . It has the advantage of reasonable palatability and thus of acceptance by patients. A synthetic diet used previously<sup>7</sup> had the disadvantages that most patients found it unpalatable (the majority refused to tolerate it for more than a few days) and in most of those who continued to take it diarrhea developed within two to fourteen days. Diarrhea is not a side-effect of the present diet.

MATERIALS AND METHODS

Table I presents illustrative diet menus.

Each 100 gm. aliquot of meat or vegetable was cooked for ten minutes in 2 L. of boiling water. The water was then discarded and the procedure was repeated twice in order to extract the residual water-soluble vitamins. Prior to cooking, chicken was sliced thin, frankfurters were sliced lengthwise into quarters, potatoes were diced and onions were chopped. When potassium depletion developed,<sup>5</sup> in the first subject given this diet, the fact that water extraction removes potassium<sup>8</sup> was appreciated, and in subsequent studies subjects received a daily supplement of 2 gm. of potassium chloride. A salt mix probably should have been used, but it was not. At the end of four and a half months on this diet, the serum magnesium level\* was still normal (2.3 mEq. per L.).

The folate activity of the components of this diet was determined both before and after treatment with chick pancreas conjugase<sup>†</sup> by microbiologic assay with *Lactobacillus casei* ATCC No. 7469. Assay was also performed with *Streptococcus faecalis* ATCC No. 8043.

Solid samples were prepared for assay as follows: 3 gm. of sample were homogenized in a Waring blender for one minute with 27 ml. of 0.05 M sodium phosphate buffer (pH 6.1) in which 350 mg. per cent ascorbic acid had been freshly dissolved. Half of this material was set aside in a 40 ml. centrifuge tube for assays without and the other half for assays with added chick pancreas conjugase. Difco desiccated chick pancreas was dissolved in water (3 mg. per ml.), the solution centrifuged for five minutes and the precipitate discarded. One milliliter of the supernate was added to the approximately 14 ml. of sample. After gentle shaking, the mixture, layered with 1 ml. of toluene, was placed in an incubator at

\* Kindly determined by Dr. W. E. C. Wacker of the Peter Bent Brigham Hospital.

† Bacto chicken pancreas, purchased from Difco Laboratories, Detroit, Michigan.

TABLE II  
Folate Activity\* for *L. casei* and *S. faecalis* of Constituents of Folate-Deficient Diet Menu

Item	Folate Activity (µg./kg.)		
	L. casei Pretreatment		Strep. faecalis
	None	Conjugase†	
Chicken, white meat, sliced, thrice-boiled	7.5	8.6	7.5
Frankfurters, Colonial brand, sliced lengthwise into quarters	7.5	17.8	8.3
Chopped beef (hamburger), thrice-boiled	17.5	27.5	11.6
Sardines, Maine, Port Clyde brand, in soybean oil	10.7	22.5	6.2
Lipomul-oral brand corn oil high calorie supplement	3.6	4.1	...
Rice, thrice-boiled	2.0	2.0	2.0
Potato, Maine, diced, thrice-boiled	8.7	10.3	5.1
Onion, chopped, thrice-boiled	9.2	27.5	2.1
Egg white, once-boiled	7.8	14.0	7.0
<i>Seasonings</i>			
Margarine, Blue Bonnet brand	6.5	19.5	6.2
Lard, Hormel brand	1.9	2.0	...
Salt, Morton brand	0	0	0
Sugar, granulated, Jack Frost brand	0	0	0
Monosodium glutamate, Accent brand	0	0	0
Worcestershire sauce, French brand	9.0	16.0	6.0
Lemon extract, Baker brand	0	0	0
<i>Beverages</i>			
Coffee, instant, Maxwell House brand	0	0	0
Coca Cola	<1	<1	<1
Pepsi Cola	<1	<1	<1
Seven-up	<1	<1	...
Ginger ale, Canada Dry brand	<1	<1	<1
Ginger ale, Cott brand	<1	<1	<1
<i>Whiskey</i>			
Blanchard 777 brand	<1	<1	<1
Hiram Walker Imperial brand	<0‡	<0	<0
White Horse brand (scotch)	<0	<0	<0
Cutty Sark brand (scotch)	<0	<0	<0
Old Grand Dad brand	<0	<0	<0
<i>Desserts</i>			
Flavored gelatin powder, Jell-O brand, assorted artificial flavors	2-7.4	3-8.6	1-5.8
Corn starch, Argo brand	2.7	8.4	1.9
Applesauce, thrice-boiled	6.0	8.0	5.0
<i>Candies</i>			
<i>Hard candies</i>			
Tavener brand fruit drops	<1	<1	...
Lifesaver brand fruit drops	<1	<1	...
<i>Soft candies</i>			
Orange slices, Mel-O-Sweet brand	1.9	1.9	...
Spearment leaves, Mel-O-Sweet brand	1.9	1.9	...
Jelly centers, Richardson brand	<1	<1	...
Marshmallows, Princess brand	1.9	1.8	1.1

\* Three separate batches of each item assayed for *L. casei*. Results averaged.

† Chicken pancreas.

‡ The designation <0 means bug growth inhibited. This was probably not a property of the alcohol in the whiskey, since autoclaving prior to assay vaporized the alcohol away.

TABLE III  
Folate Activity for *L. casei* of Various Foods

Item	Folate Activity (µg./kg.) <i>L. casei</i>
Alaska king crab, Pride of the Ocean brand	9.3
Anchovies, Jose Antonio Ritta brand, in olive oil	>100
Applesauce, Stop & Shop brand, grade A	8.1
Arrowroot	4.1
Beer, Dawson brand	136.0
Butterfly cocoons, fried, Castle Hill brand	>100
Carrots, diced, thrice-boiled	23.0
Charcoal seasoning, McCormack's brand	27.0
Clams, fresh	>150
Cognac, Remy Martin brand	<0
Complan brand liquid diet (powder)	58.0
Corn bread	>100
Corn syrup, Karo brand	<0
Crackers, Saltine brand	>100
Cream of Mushroom soup, Campbell brand	22.7
Dietene brand liquid diet (powder)	>150
Egg yolk	>100
Flour, wheat	>100
<i>Folic acid-deficient diet, Nutritional Biochemical Corp.</i>	
Regular	5.1
Chick	14.8
Grape juice, Westfield brand	9.5
Hamburger, Waring-blended and twice-boiled	2.1
Herring, Vita brand	51.0
Ice cream, vanilla	46.0
Ketchup, Monarch brand	>100
Lobster, fresh, once-boiled, caught off uninhabited area	1.7
Lobster, fresh, once-boiled, caught close to inhabited area	119.0
Lobster, canned, United Maritime Fisherman brand	195.0
Lobster, South African rock, Albatross brand	39.0
Milk, powder, non-fat dry	>100
U. S. Dept. of Agriculture brand	>100
Carnation brand	>100
Meat, pressed, prepared, Mor brand	51.0
<i>Mushrooms</i>	
Oxford Royal brand	>100
Oxford Royal brand, thrice-boiled	42.0
Mustard, French brand	39.0
Olive oil	3.9
Oysters, fresh	>100
Paprika, powdered	>100
Peanut butter, Finast brand	>100
Potato, instant, mashed	>100
Minute brand	>100
French brand	>100
Betty Crocker brand	>100
Rice, Minute brand	137.0
Sardines,* Portugal, Nice brand, in olive oil	66.0
Sardines, Norway, Sea Fair brand, in sardine oil	128.0
Sardines,* Maine, Mooseabec brand, in salad oil	10.8
Sardines,* Underwood brand, in soy bean oil	39.0
Sardines,* Underwood brand, in mustard sauce	14.4
Sardines,* Beach Cliff brand, in soy bean oil	28.5
Sauerkraut, Silver Floss brand	>100
Broth, George Washington brand	27.0
Shrimp, fresh cooked	>100
Tuna, Geisha brand, packed in water	72.0
Vinegar, Heinz, distilled white	1.1
<i>Wines</i>	
Home-made Italian red	12.0
Concord grape, Mogen David brand	3.0
<i>Muscatel</i>	
Blanchard brand	10.1
Guild brand	12.9
Sherry, Blanchard brand	<1

\* Maine "sardines" are herring.

37°C. overnight. As a control, a third sample was prepared consisting of 1 ml. chick pancreas conjugase supernate in 14 ml. of phosphate-ascorbate buffer. One milliliter aliquots of each sample were assayed using the "standard method" previously described.<sup>9</sup> Table II presents the results obtained for the folate activity of the ingredients of the diet.

In Table III may be found the L. casei folate activity (without conjugase treatment) of various food-stuffs. This table is presented for its possible value in preparing diets of various "natural" folate content. Experience of the past fifteen years suggests that L. casei folate activity of foods *without* conjugase treatment is a much closer approximation of the food folate available to man than L. casei activity *with* conjugase treatment.\*

#### COMMENTS

Considerable differences will be noted between the data here recorded for folate content of certain foods and the data of others.<sup>10,11</sup> A factor in these differences is the use in the present study of ascorbate to protect labile forms of folate against oxidative destruction.

Using Table II as a guide, a diet can be constructed containing no more than 5 µg. of folate activity per day, with any desired level of total daily caloric intake and protein, carbohydrate and fat.

Girdwood<sup>12</sup> has noted that "Since folic acid is present both in vegetables and meats, it would be difficult to devise a diet to produce deficiency of this substance alone." The water extraction procedure here used has overcome this difficulty. Since the water extraction probably removed all water-soluble vitamins (as well as minerals such as potassium), the diet here described could probably be used to produce deficiency of some other water-soluble vitamin or mineral that was not added as a daily supplement. (Assay of the diet here described reveals no vitamin B<sub>12</sub> activity for *E. gracilis*.)

#### SUMMARY

A palatable diet for producing experimental

\* This point has been reviewed previously. (SPRAY, G. H. The role of pteroylglutamic acid and related compounds in macrocytic anaemia. *Proc. Nutrition Soc.*, 15: 119, 1956.)

folate deficiency in man is described. The folate content of each ingredient of this diet was determined before and after treatment with chick pancreas conjugase by microbiologic assay with *Lactobacillus casei*, and also with *Streptococcus faecalis*.

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#### REFERENCES

1. NAJJAR, V. A. and BARRETT, R. The synthesis of B vitamins by intestinal bacteria. *Vitamins & Hormones*, 3: 23, 1945.
2. NAJJAR, V. A. and HOAK, L. E., JR. The biosynthesis of thiamine in man. *J. A. M. A.*, 123: 683, 1943.
3. KNOWLES, J. P., PRANKERD, T. A. J. and WESTALL, R. G. Folic acid requirements in man. *J. Physiol.*, 157: 24P, 1961.
4. KNOWLES, J. P. and PRANKERD, T. A. J. Megaloblastic anaemias of gastrointestinal origin. *Postgrad. M. J.*, 37: 755, 1961.
5. HERBERT, V. Experimental nutritional folate deficiency in man. *Tr. A. Am. Physicians*, 75: 307, 1962.
6. HERBERT, V., CUNNEEN, N., JASKIEL, L. and KAPFF, C. Minimal daily adult folate requirement. *Arch. Int. Med.*, 110: 649, 1962.
7. ZALUSKY, R. and HERBERT, V. Megaloblastic anemia in scurvy with response to 50 µg. of folic acid daily. *New England J. Med.*, 265: 1033, 1961.
8. COTLOVE, E. Mechanism and extent of distribution of inulin and sucrose in chloride space of tissues. *Am. J. Physiol.*, 176: 396, 1954.
9. HERBERT, V. The assay and nature of folic acid activity in human serum. *J. Clin. Invest.*, 40: 81, 1961.
10. TOEPFER, E. W., ZOOK, E. G., ORR, M. L. and RICHARDSON, L. R. Folic Acid Content of Foods. Microbiological Assay by Standardized Methods and Compilation of Data from the Literature. Agriculture Handbook No. 29. Washington, D. C., 1951. U. S. Dept. of Agriculture.
11. FLYNN, L. M. Report on folic acid. *J. A. Official Agr. Chem.*, 33: 633, 1950.
12. GIRDWOOD, R. H. Folic acid, its analogs and antagonists. *Adv. Clin. Chem.*, 3: 235, 1960.