THE FOLIC ACID CONTENT

OF

CERTAIN FRESH VEGETABLES

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INTRODUCTION

The isolation, identification, and synthesis of a new member of the vitamin B-complex constitutes a major event in the science of nutrition, the more so when the substance is shown to play a prominent role in human nutrition. In recent years folic acid has been added to the long list of previously characterized vitamins. Due to the recency of its discovery, information as to its occurence in natural materials is scarce. The recently demonstrated relation of the vitamin to pernicious anemia in humans (32) and in sprue (9) enhances the value of a knowledge of the distribution of this substance in food stuffs.

The history of the discovery of folic acid, in common with most of the other B-complex vitamins, comes from several sources of work. From these individual experiments folic acid has emerged. In 1912 Funk suggested a nutritional origin for sprue. In 1938, Day, Langston, and Darby (10), working with monkeys, showed that on certain diets comprised of nutural foods, an additional factor was necessary. This they called "Vitamin M" and showed that it was found in liver extracts and yeast and that it prevented the development of cytopenia. Also, in 1938, Stokstad and Manning (35) during an experiment on the formulation of a diet for riboflavin assay with chicks, observed that middlings and yeast had a growth-promoting effect which could not be accounted for by their content of the "filtrate factor" or riboflavin. They called this growth factor "Factor U." Snell and Peterson (31), in 1939, found in extracts of yeast and liver a new water-soluble factor for lactic acid bacteria.

Hogan and Parrot, in 1940, (12) produced anemia in chicks on a purified diet. They attributed this anemia to the variability of the commercial

liver extracts which they were using as a source of some of the uncharacterized growth factors. They found that the anemia was of a nutritional nature. The antianemic principle was not identifiable with any vitamin then recognized. Hogan and Parrot designated this substance as vitamin B_6 since it belonged to the B-complex and was essential for the chick. At about the same time Schumacher, Heuser, and Norris (28) found an alcohol precipitate factor which was required by chicks for normal growth and reproduction. The alcohol precipitate factor was composed of two factors found in dried brewer's yeast and might be identical with Stokstad and Koming's "Factor U."

Also in 1940 an unidentified water-soluble factor, "the yeast norite eluate factor," was shown to be necessary for the growth of Lactobacillus casei (13). It was later observed that, when the factor was concentrated from extracts prepared from liver, the potency in promoting the growth of chicks on a purified diet was found to increase at the same time with the potency as measured by L. casei.

Briggs, et al, (5) in 1943, found in working with chicks on a purified ration that factors in liver and other material distinct from folic acid showed differences in the levels of activity of various liver fractions when activity in the chick and in lactic acid bacteria were compared. One factor appeared essential for proper feather development in the chick. A second factor seemed necessary for growth but was not active in producing normal feathers. These two factors were, therefore, tentatively designated as vitamins B_{10} and B_{11}. Koreastesy, et al, (19) believed they had isolated a new growth factor for Streptococcus lactis R. When they used various types of extracts and liver preparations and compared the folic acid and norite eluate factor activity against a standard sample of folic acid, they

found much more activity for S. lactis R. in certain fractions.

The name "folic acid" was applied in 1941 to the factor which was obtained in a concentrate form from spinach (22). This factor was required by Streptococcus lactis R. and also was potent for Lactobacillus casei. Following the concentrate of folic acid from spinach an antianemic factor was isolated in crystalline form from liver, which was thought to be identical to the naturally occurring folic acid and in addition had activity for L. casei and for chicks. The crystalline compound was thin, yellow spear-head-shaped platelets and possessed a characteristic ultra-violet absorption spectrum (27). In 1944 the isolation of another form of the vitamin prepared from a fermentation product obtained from an unidentified bacterium of the genus Corynbacterium was reported (15). By using large scale fermentation, it was possible to prepare this new form in larger amounts than in the form prepared from liver. Degradation studies were carried out with this form, which was termed "fermentation Lactobacillus casei factor."

When the fermentation L. casei factor was heated with NaOH solution in the absence of oxygen, a racemic form of the liver L. casei factor, together with two molecules of a substance containing alpha amino acid nitrogen, was produced.

The liver L. casei factor, a bright yellow substance only very slightly soluble in water, was isolated from liver by adsorption and elution, esterification and extraction of the methyl ester, and fractional precipitation of the ester from water and methanol. The fermentation factor and the liver factor differed in the number of glutamic acid residues. The structure for the liver compound showed only one glutamic acid, while the fermentation factor appeared to contain three glutamic acid residues. Both factors yielded p-amino-benzoic acid and the same pteridines upon degrada-

tion. The liver L. casei factor was named pteroylglutamic acid (1). The sodium salt is fairly soluble, and sunlight has destructive action on the solution. Pteroylglutamic acid is destroyed fairly rapidly by heating with dilute mineral acids (18).

A large portion of the pteroylglutamic acid content of foods has been found to be present in the conjugated form, which, although utilizable by chicks and rats, gives no response in the microbiological assay using Streptococcus faecalis and may not be available to certain other species or to humans with pernicious anemia (8). Since pteroylglutamic acid exists in food to a considerable extent in the form of microbiologically inactive conjugate, specific enzymes are required for the liberation of the free vitamin (2). The conjugated forms contain more than one glutamic acid residue. A triglutamic acid derivative has been isolated from a fermentation of Corynbacterium (15) and pteroylheptaglutamic acid has been found to be identical with vitamin B_c conjugate (1).

In 1942 Cheldelin and Williams (7) published a study of the vitamin B content of foods. At that time crystalline vitamin B_c was not available so the results were based on a standard of assumed potency. They reported large losses of folic acid due to cooking. The results of analysis by Ives, et al, (16) of several canned foods indicated that much of the folic acid present in vegetables may be lost in canning. Using taka-diastase digestion they found the average folic acid content of spinach, when assayed with L. casei, to be 7.4 mcg. per 100 gm. Canned green asparagus, green beans, carrots, yellow corn, peas, and tomatoes gave values less than 10 mcg. per 100 gm. when assayed with either this organism or S. faecalis. About 65 per cent of the vitamin present was found in the solid portion. Olson, Burris, and Elvehjem (26) reported that since the leafy vegetables of deep

green color were all high in folic acid, the folic acid content of vegetables may be correlated with the chlorophyll content of the plant. Vegetables of light green color were shown to be low in folic acid. During these studies on the effect of different storage conditions on the folic acid content of vegetables, it was found that in all cases an appreciable loss occurred when the vegetables were stored at room temperature; that storage at normal refrigerator temperature was quite effective in maintaining the folic acid content; and that storage in ice prevented losses for periods of two weeks or more.

The folic acid content of meat was studied by Schweigert, et al, (29). They found liver to be an excellent source of the vitamin; kidney, good; beef and veal muscle, fair; and pork and lamb muscle, poor. They also found that only from 5 to 40 per cent of the vitamin was retained after frying and steaming pork loin or ham and veal chops. They found the per cent retention of mutton shoulder to be: 12 after roasting, from 8 to 27 after roasting, braising, or stewing, as measured by S. faecalis, and from 10 to 40 as measured by L. casei.

MATERIALS AND METHODS

The turbidimetric method of assay using Streptococcus faecalis was used in this study. The organism, Streptococcus faecalis (S. lactis R), was obtained from the American Type Culture Collection, No. 8043. The stock cultures were carried as stabs in yeast extract - glucose agar (1% glucose, 1% yeast extract, 1.5% agar). Stock cultures were transferred every two weeks. After transfer, the cultures were incubated at 30° C. for 14 to 30 hours and then stored in the refrigerator (25).

Crystalline pteroylglutamic acid served as a standard. The standard was made up in about 0.05 N NaOH at a concentration of 1 mg./ml. and placed in a dark bottle to protect it from light and refrigerated. The standard was diluted to 0.1 micrograms per ml. Assay levels of the diluted standard used were 0.0025, 0.005, 0.0075, 0.01, 0.015, 0.02 micrograms. Figure 1 presents a typical standard curve for the assay.

The following modification of the basal medium of Tepley and Elvehjem (36) was used:

Sodium citrate	50 gm.
K_2HPO_4	5 gm.
Casein (acid hydrolyzed)	10 gm.
Glucose	10 gm.
Cystine	400 mg.
Tryptophane	400 mg.
DL-alanine	400 mg.
Adenine	20 mg.
Guanine	20 mg.
Uracil	20 mg.
Xanthine	20 mg.
Thiamine	400 mcg.
Riboflavin	400 mcg.
Nicotinic Acid	1200 mcg.
Pyridoxine	2400 mcg.
Ca. Pantothenate	800 mcg.
Biotin	0.8 mcg.
p-Aminobenzoic Acid	20 mcg.
Salt Solution B.	10 ml.
Distilled water to	1000 cc.

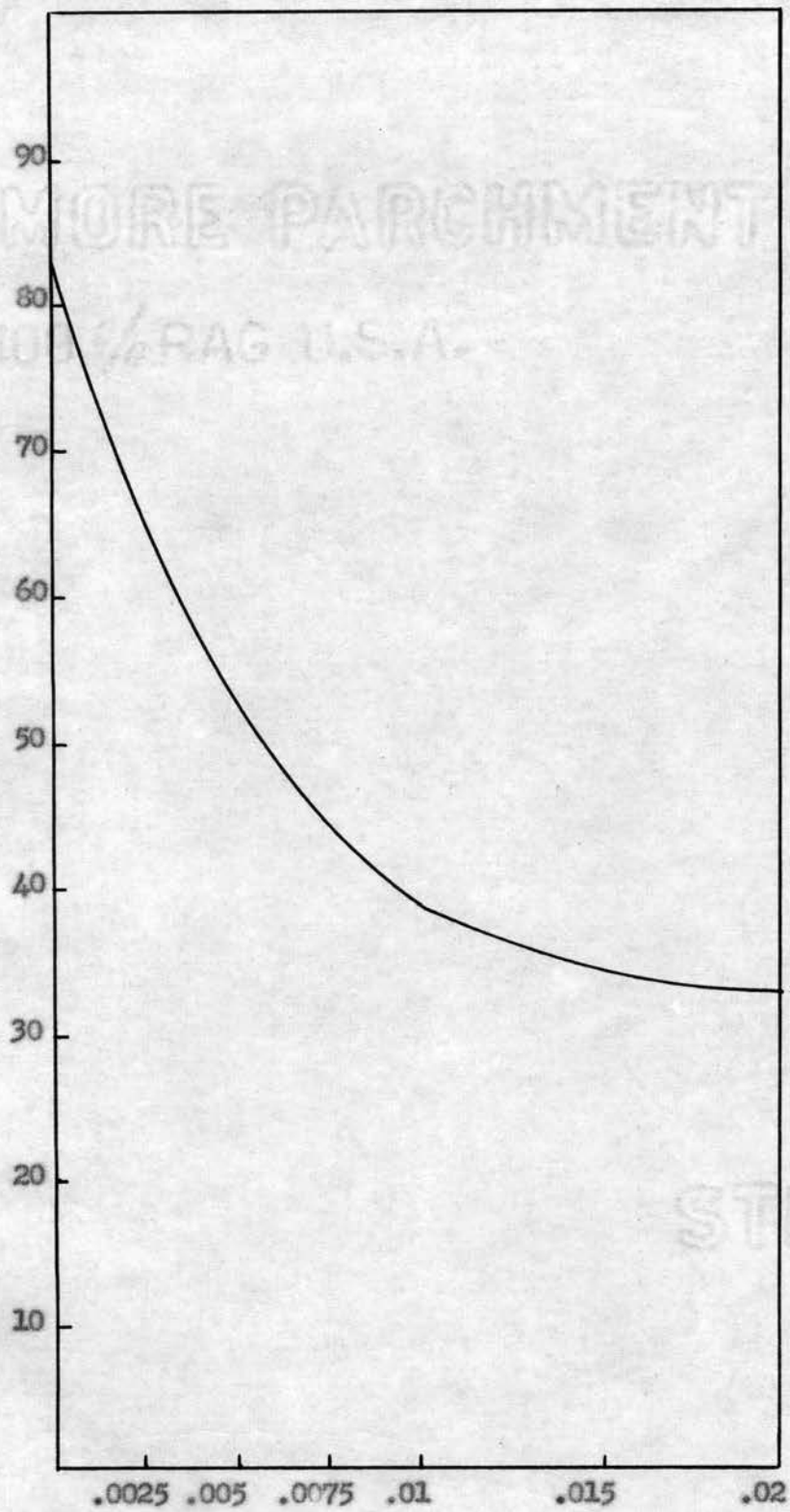

Figure I

Standard Curve for Folic Acid

The media was adjusted to pH 6.8, using bromthymol blue.

The preparation of the various constituents of the medium was as follows:

Vitamin free, Acid-hydrolyzed Casein. 50 gm. of General Biochemical vitamin free casein were mixed with 500 ml. HCl (280 ml. to 500 ml.) and refluxed for 48 hours. The hydrolysate was concentrated to a thick syrup, redissolved in water, and reconstituted four times. It was then taken up to 500 ml. with water, brought to pH 3.0 (bromphenol blue) with NH_4OH, and 5 gm. Norite A was added. The mixture was stirred for 30 min. and filtered. Then 10 gm. Darco G 60 was added, and the mixture was stirred for 30 min. and then filtered. The filtrate was neutralized with NH_4OH (bromthymol blue). The filtrate was autoclaved at 15 lbs. for 20 min., preserved with toluene, and refrigerated.

L-cystine, L-tryptophane, DL-alanine: 2 gm. of each were dissolved in a small amount of conc. HCl with heat and made up to 1000 cc. (2 mg./ml.), covered with toluene and refrigerated.

Adenine, guanine, uracil: 1 gm. of each were dissolved in a small amount of conc. HCl with heat and made up to 1000 cc. (1 mg./ml.), covered with toluene, and refrigerated.

Xanthine: 200 mg. of xanthine was dissolved in a small amount of conc. HCl and made up to 200 cc (1 mg./ml.), covered with toluene, and refrigerated.

Salt Solution B.: $MgSO_4.7H_2O$, 10 gm.; NaCl, 0.5 gm.; $FeSO_4.7H_2O$, 0.5 gm.; $MnSO_4.4H_2O$, 0.5 gm.; water to make 250 ml. The salts precipitated from this solution when it stood in air. The salts needed to be renewed only when a uniform suspension could no longer be obtained by shaking.

Vitamins.

Thiamine: a stock solution containing 100 mcg. of thiamine chloride per ml. was prepared, dissolved in a buffer solution, and stored in the refrigerator.

Pantothenic Acid: a stock solution containing 100 mcg. of thiamine chloride per ml. was prepared, dissolved in a buffer solution, and stored in the refrigerator.

Para-Aminobenzoic Acid: a solution of para-aminobenzoic acid containing 100 mcg. per ml. was prepared, dissolved in water, and stored in the refrigerator.

Riboflavin: a stock solution of riboflavin containing 100 mcg. per ml. was prepared in 0.02 N acetic acid and refrigerated. Unnecessary exposure to light was avoided, and a fresh solution was prepared at frequent intervals.

Nicotinic Acid: a stock solution of nicotinic acid containing 100 mcg. per ml. was dissolved in water and refrigerated.

Biotin: a stock solution of biotin was prepared from commercial concentrates that are low in other vitamins. The solution, containing 0.1 mcg. per ml. of biotin was preserved under benzene and refrigerated.

Pyridoxine: a stock solution containing 100 mcg. per ml. of pyridoxine hydrochloride dissolved in water was prepared and stored in the refrigerator.

Medium for Inoculum: folic acid free media, diluted to the concentration used in the final test (5 ml. media, 5 ml. water) was prepared to which was added 0.01 mcg. of folic acid. 10 ml. was placed in each inoculum tube.

Preparation of Inoculum: a transfer was made from the stab to the inoculum media and incubated for 20 to 24 hours. The cells were centrifuged

out and resuspended in 0.9% saline three times, in order to eliminate any folic acid that might cling to the cells.

Part of the vitamin present in natural foods is in a form that is ineffective as a growth stimulant for the organisms. Bird, et al., (3) found that desiccated hog kidney was a practical source of the enzyme and that it contained a blank low enough for most purposes. Fresh hog kidney was blended and mixed with three parts of distilled water. This was blended and then centrifuged, and the centrifugate was filtered through a thick pad of diatomaceous earth (John Manville "Super Cel"). The clarified extract was dispensed in 5 cc. amounts in test tubes and frozen. To a 1 gm. sample to be tested 4 cc. of hog kidney enzyme plus 10 cc. of 0.1% Na acetate buffer was added.

Taka-diastase was first used by Cheldelin and Williams (6) to release folic acid from tissue. Luckey, Briggs, et al., (21) also used this enzyme preparation. They used 20 mg. of taka-diastase to a 1 gm. sample plus 10 ml. of 0.1% Na acetate buffer.

The vegetables used in this study were obtained from either the college foods units or the horticultural plots of the college and were of uniformally high quality. Those vegetables from the college food units had been secured from produce houses in cities located nearby and shipped here. The vegetables were washed and cut into pieces suitable for cooking. Only the edible portions of all the vegetables were taken for analysis, and only fresh, succulent tissues were selected. All the vegetables were cooked by steaming in an autoclave that came up to about 4 lbs. pressure, thus simulating, under laboratory conditions, institutional cooking methods. After cooking, the vegetables were reweighed.

A 25 gm. portion of representative sample of the raw and the cooked vegetables respectively were homogenized with water, blended in a Waring

Blendor for 3 min., and made to 250 ml. with distilled water. A 10 ml. (1 gm.) aliquot was then taken for incubation with either taka-diastase or hog kidney enzyme. Also, a 10 ml. (1 gm.) aliquot for autolysis was taken. A few drops of toluene were added to each and the samples were incubated at 37° for 24 hours.

After incubation the samples were neutralized to a pH 6.5 with NaOH or HCl and then autoclaved for 20 min. at 15 lbs. pressure. They were then filtered and made to 100 ml. with distilled water. Aliquots of three levels in not fewer than duplicate were used so that an average could be obtained and drift could be detected. The aliquots were placed in test tubes, and distilled water added to make 5 ml., and then 5 ml. of media was added. The tubes were autoclaved for 15 min. at 15 lbs. pressure. After cooling to room temperature, 1 drop of the inoculum was added to each tube. The assay was incubated in a water bath at 30° for 18 hours.

The growth response was determined for 18 hours of incubation by measuring the turbidity with the Evelyn photo-electric colorimeter, using the 660 millimicron filter. A standard curve was made by plotting galvanometer readings of the folic acid concentration against the folic acid content of the sample at the various levels of addition was determined. The figure reported is an average of values obtained at the three levels of addition.

The values obtained by taka-diastase and hog kidney preparation digestion are presented in Tables I to III. Comparative values when no enzymatic procedure was employed are given in Table IV.

RESULTS AND DISCUSSION

The green leafy vegetables (beet tops, mustard greens, spinach, and swiss chard) had a relatively high folic acid content, as shown in Table I. With the exception of zucchini squash and golden bantam corn, which were quite high, the lighter colored green leafy and yellow vegetables gave intermediate values as shown in Table II.

The results in Table III show that root vegetables had only a fair amount of folic acid; that beets and new potatoes had higher values. For the other vegetables in general, the values are lower. Because eggplant contained such small quantities, it was not measurable; and acorn squash also contained a very small amount.

A red and a white variety of old and new potatoes were sampled. Old potatoes that had been stored for approximately seven months were compared with recently harvested summer potatoes. For 100 gm. of new white potatoes there were 87 mcg. of folic acid, as compared with 4 mcg. in old white potatoes when hog kidney enzyme was used. 100 gm. of red new potato contained 52 mcg., as compared with 16 mcg. in an equal amount of old red potato. This decrease of folic acid during storage of potatoes is in accordance with the observations of Olson, Elvehjem, and Burris (26), who found severe losses in vegetables held at room temperatures for various lengths of time. This loss may be of serious magnitude and requires further investigation.

In comparing different varieties of the same vegetable, there was only a slight variation of folic acid content in some vegetable groups, such as white and red cabbage, white and pascal celery, red and white old potatoes, red and white new potatoes. However, a wide variation was found among the varieties of squash. Zucchini squash was very high in folic acid

TABLE I

The Folic Acid Content of Green Leafy Vegetables

Vegetable	Wt. before Cooking gm.	Wt. after Cooking gm.	Cooking Time	Taka-Diastase Mcg./100 gm. Raw	Taka-Diastase Mcg./100 gm. Cooked	Apparent % Decrease or Increase	Hog Kidney Enzyme Mcg./100 gm. Raw	Hog Kidney Enzyme Mcg./100 gm. Cooked	Apparent % Decrease or Increase
Beet Tops	250	237.2	3 min.	17	6	- 65%	190	113	- 40%
Spinach	250	259.2	3 min.	53	13	- 75%	85	71	- 19%
Mustard Greens	229	214	3 min.	23	10	- 57%	62	190	+ 303%
Swiss Chard	250	252.1	3 min.	11	10	- 9%	66	98	+ 49%
Cabbage, White, Cut	249.2	245.3	6 min.	9	7	- 22%	41	48	+ 17%
Cabbage, Red, Cut	250	250.8	15 min.	21	10	- 52%	37	23	- 38%
Lettuce	250	250.4	6 min.	4	3	- 25%	23	24	+ 5%
Celery, Pascal	250	220.5	5 min.	3	0.8	- 73%	29	31	+ 7%

TABLE II

The Folic Acid Content of Light Green and Yellow Vegetables

Vegetable	Wt. before Cooking gm.	Wt. after Cooking gm.	Cooking Time	Taka-Diastase Mcg./100 gm. Raw	Taka-Diastase Mcg./100 gm. Cooked	Apparent % Decrease or Increase	Hog Kidney Enzyme Mcg./100 gm. Raw	Hog Kidney Enzyme Mcg./100 gm. Cooked	Apparent % Decrease of Increase
Okra	255.1	255.8	5 min.	37	4	- 89%	66	70	+ 6%
Corn, Golden, cut from cob	250	256.1	10 min.	67	23	- 66%	70	76	+ 9%
Green Beans	250	239.4	6 min.	21	2	- 95%	52	54	+ 4%
Asparagus	250	248	8 min.	10	3	- 70%	14	26	+ 86%
Lima Beans, Frozen	134	136.4	6 min.	8	26	+ 225%	23	24	+ 4%
Tomatoes	250	227.2	6 min.	0.4	0.3	- 25%	13	19	+ 46%
Carrots	250	226.9	10 min.	8	4	- 50%	24	25	+ 4%
Squash, Yellow Crookneck	250	245.6	10 min.	57	59	+ 3%	31	29	- 6%
Squash, Zucchini	250.2	228.3	10 min.	4	1	- 75%	38	85	+ 123%
Squash, Acorn, Cut	242	236.1	15 min.	6	0	- 100%	6	6	0
Pumpkin, Cut	257	223.7	20 min.	2	0	- 100%	30	34	+ 13%
Sweet Potato	250.6	252.8	15 min.	0	0	0	35	54	+ 54%

TABLE III

The Folic Acid Content of Root and Other Vegetables

Vegetable	Wt. before Cooking gm.	Wt. after Cooking gm.	Cooking Time	Taka-Diastase Mcg./100 gm. Raw	Taka-Diastase Mcg./100 gm. Cooked	Apparent % Decrease or Increase	Hog Kidney Enzyme Mcg./100 gm. Raw	Hog Kidney Enzyme Mcg./100 gm. Cooked	Apparent % Decrease or Increase
Potato, New, White	249.5	249.7	15 min.	17	51	+ 200%	53	87	+ 64%
Potato, New Red, Diced	250	246.3	6 min.	23	15	- 35%	43	52	+ 20%
Potato, Old, White 7 Mo. Storage	250	262.1	6 min.	4	1	- 75%	4	4	0
Potato, Old, Red, 7 Mo. Storage	250	247.6	6 min.	7	3	- 58%	5	16	+ 220%
Beets, Fresh	250	226.2	20 min.	16	5	- 69%	22	29	+ 32%
Cauliflower	250.4	246.4	6 min.	12	7	- 42%	32	44	+ 37%
Eggplant	249.6	255.5	5 min.	00	00	00	00	00	00
Celery, White	250	243	5 min.	4	3	- 25%	24	20	- 17%
Squash, White Summer	246.7	244	10 min.	13	0.1	- 99%	37	27	- 27%

(85 mcg./100 gm.), yellow crookneck squash (59 mcg./100 gm.) and white summer squash (37 mcg./100 gm.) were of an intermediate value, and acorn squash was extremely low (6 mcg./100 gm.) in folic acid.

Since taka-diastase did not contain any conjugase activity, incubation of the vegetables with this enzyme did not release any appreciable quantity of folic acid from the conjugated forms. The cooking of vegetables destroyed the major portion of the natural conjugase present in the plant tissues, and therefore, essentially only free folic acid content of the vegetables was measured in the cooked samples incubated with taka-diastase. Relatively high values for free folic acid as compared with the total present as measured by hog kidney hydrolysis was found in lima beans, yellow crookneck squash, potatoes, and corn. This might have been an artifact produced by a failure to completely destroy by cooking the conjugase activity in these particular foods. In order to check further on this point, the amount of folic acid in fresh and cooked vegetables subjected only to autolysis was determined (Table IV). It will be seen from these data that, for example, nearly identical values were obtained when cooked old potatoes were analyzed after autolysis, taka-diastatic digestion, and hog kidney preparation digestion. This suggests that complete destruction of the conjugase occurred. Should such have not been the case, release of folic acid from the conjugate present in the taka-diastase would have been observed. These data again emphasize the need for further research on the changes in folic acid composition of foods during storage. Yellow crookneck squash, head lettuce, tomatoes, and white cabbage had approximately the same amount of folic acid in both raw and cooked form when measured with taka-diastase.

The folic acid content of both raw and cooked vegetables was, as a rule, much higher when the hog kidney enzyme instead of taka-diastase was used.

TABLE IV

A Comparison of The Folic Acid Content As Determined by Taka-Diastase, Hog Kidney Enzyme Preparation, and Autolysis.

Vegetable	Taka-Diastase Mcg./100 gm. Raw	Taka-Diastase Mcg./100 gm. Cooked	Hog Kidney Enzyme Mcg./100 gm. Raw	Hog Kidney Enzyme Mcg./100 gm. Cooked	No Enzyme Mcg./100 gm. Raw	No Enzyme Mcg./100 gm. Cooked
Potato, Red. Old, 7 Mo. Storage	7	3	5	16	6	12
Potato, White, Old, 7 Mo. Storage	4	1	4	4	4	6
Mustard Greens	23	10	62	190	32	8
Lima, Beans, Frozen	8	26	23	24	16	14
Cabbage, Red	21	10	37	23	0	0
Squash, Zucchini	4	1	38	85	9	1
Potato, Sweet	0	0	35	54	4	2
Pumpkin	2	0	30	34	1	0
Egg Plant	0	0	0	0	0	0

The greatest difference was noted with beet tops, mustard greens, swiss chard, sweet potatoes, pumpkin, and zucchini squash. As noted previously, in some vegetables it appears that at least a part of the folic acid may exist in the free form.

Examination of the data clearly indicates that losses of folic acid on cooking are not of serious magnitude. In fact, in many vegetables increases in folic acid content, as measured by S. faecalis following hog kidney preparation digestion, were observed. These increases might be due either to the destruction of a conjugase inhibitor that has been shown to be present in many natural materials (4) or to a release of the conjugated folic acid from protein by the cooking process, thus rendering it more readily attacked on digestion with conjugase. The previously observed apparent loss on cooking was, in all probability, due to the destruction of the natural conjugase present in the vegetable during the cooking process. This fact is substantiated, for example, by the folic acid content of mustard greens (Table IV). Similar results were obtained by autolysis and by taka-diastase digestion in both raw and cooked samples.

SUMMARY

The results of the analysis of certain fresh vegetables for folic acid showed that: the green leafy vegetables had a relatively high folic acid content; the light colored green leafy and yellow vegetables had an intermediate value; the root vegetables had a fair value; and other vegetables were low.

The great loss of folic acid during storage of potatoes at room temperature was serious and needs further investigation on the changes in folic acid composition of foods during storage.

Different varieties of the same vegetable showed only a slight variation of folic acid with the exception of the varieties of squash where a wide variation existed.

The folic acid content of the vegetables was, as a rule, much higher when the hog kidney enzyme instead of taka-diastase was used. In some vegetables it appeared that at least a portion of the folic acid may be in free form.

The loss of folic acid on cooking was not serious. In many vegetables an increase in folic acid content following hog kidney preparation was observed.

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