Nutrition and Disease: Folic Acid Deficiency in the Mouse

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IT HAS BEEN generally accepted that the growing mouse fed a purified diet low in folic acid would not become deficient in folic acid unless a vitamin antagonist, low casein diet, stress agent, or intestinal germicidal agent were given.

For example, Franklin, Stokstad, and Jukes,[1] in 1947, fed mice diets low in folic acid without obtaining deficiency signs unless a folic acid antagonist was given. In their experiments even the addition of sulfasuxidine to the diet did not result in a deficiency. In 1948 a folic acid deficiency was produced in mice by Weir, Heinle, and Welch[2] by the addition of a crude folic acid antagonist to the diet. These authors also observed changes in the blood components. Woolley[3,4] reported that mice grew normally on a purified diet containing the then known B vitamins (not including folic acid) and inositol.

In 1944, Cerecedo and Vinson[5] reported that they were able to rear three strains of mice through several generations on highly purified diets. Their diets contained casein, salts, fiber, fat, sucrose, and all vitamins except folic acid. Growth without folic acid was actually superior to that of control mice kept on stock diets. However, reproduction and lactation were inferior in the mice fed the purified diet unless a concentrate of folic acid was supplied. Later, in 1947, Cerecedo and Mirone[6] demonstrated that the addition of crystalline folic acid to the diet had a similar effect.

Fatterpaker and co-workers[7] produced a folic acid deficiency in mice on diets low in casein and containing 0.3 per cent of iodinated casein. In 1957 Schneider, Lee, and Olitsky,[8] reported that mice grew normally when fed diets low in folic acid (and biotin and vitamin B_{12}), although they did find that the addition of these three vitamins, alone or together, restored the susceptibility of the mouse to acute disseminated encephalomyelitis. A large number of studies have been reported in the cancer journals on various effects of folic acid antagonists in the mouse. No attempt is made here to review such studies.

Saucier and Demers[9] reported that mice fed diets low in casein and choline grew poorly and had anemia and hypercholesterolemia unless folic acid or vitamin B_{12} were given.

Nielsen and Black[10] in 1944 published what appears to be the only previous report of the production of a folic acid deficiency in mice fed normal purified diets (with 20 per cent of casein). Mice 7 to 9 g in weight were used in experiments lasting eight weeks. Differences of 6 to 7 g were noted in this time. Unfortunately, pure folic acid was not available to prove that the results could be attributed to folic acid (a crude liver preparation was used).

This paper presents full details of our nutritional procedures and results, and reviews our recent studies[11-13] in which we have found means of routinely producing an "uncomplicated" folic acid deficiency in the mouse without the use of an antagonist or sulfa drug. In addition, these studies have provided a new tool for the study of certain relationships between nutrition and disease as will be described.

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TABLE I

Composition of Control Diets with Folic Acid

Constituent	Diet M4 g/kg	Diet C2 g/kg	Diet M9 g/kg
Glucose (cerelose)	538	615	610
Casein (vitamin-free)	200	200	200
Gelatin	—	80	80
DL-methionine	—	3	8
Corn oil	20	40	40
Hydrogenated vegetable oil (Crisco)	180	—	—
Salts A[14]	60	60	60
Water-soluble vitamins[a]	2	2	2
Fat-soluble vitamins[b]	mg/kg	mg/kg	mg/kg
Vitamin A acetate	6	3	3
Vitamin D_3	0.04	0.02	0.02
α-Tocopherol acetate	20	10	10
2-Methyl-1,4-naphthoquinone	2	1	1
TOTAL	1000 g	1000 g	1000 g

[a] The water-soluble vitamins were added in the form of a premix of glucose as follows (mg/kg of diet): thiamine HCl 8, riboflavin 8, calcium pantothenate 20, nicotinic acid 100, pyridoxine HCl 8, D-biotin 0.3, vitamin B_{12} 0.02, and pteroylglutamic acid (folic acid) 3.0 when indicated. Crystalline choline chloride was also added at a level of 2 g/kg of diet. The glucose in the premix was included in the total amount of glucose shown in the table.

[b] The fat-soluble vitamins were added from a stock solution in corn oil (in such amounts that 1 per cent of corn oil supplied the amounts indicated). Vitamins A, D, and K were kept in one stock solution and vitamin E in another. The stock solutions were kept refrigerated.

EXPERIMENTAL PROCEDURES

Unless otherwise indicated, C57 black male mice weighing 8 to 10 g were used in all the dietary studies. They were obtained from the National Institutes of Health colony. The mice were kept in stainless steel "hanging" cages with wire screen bottoms. Each group consisted of six mice housed in one cage. The mice were fed three times a week or more often if needed. The feed cups were constructed in such a manner that feed wastage and feed contamination were kept at a minimum. Experiments were conducted for a six-week period unless otherwise indicated. Weighings were made once each week.

The composition of the diets used in these experiments is given in Table I. No folic acid antagonists or sulfa drugs were used in these diets unless otherwise indicated. All diets were kept refrigerated until used, thus making it possible for sufficient diet to be mixed at one time to last through an entire six-week experiment.

The method of administration of the lymphocytic choriomeningitis virus has been previously described.[12]

RESULTS AND DISCUSSION

In our early studies we found that folic acid deficiency was not produced in mice by removing folic acid from our standard laboratory mouse diet, M4. This is comparable to the experience of others. However, in our studies with chicks we had been routinely very successful in obtaining a folic acid deficiency by diet alone (diet C2). For this reason we fed mice the chick diet which was low in folic acid (diet C2 minus folic acid); rather unexpectedly this resulted in a retarded growth rate in a total of three experiments unless folic acid was present. The major difference in these two diets (see Table I) were in the levels of fat and fat-soluble vitamins and the presence of added gelatin and methionine in the chick diet (diet C2). It was soon determined that the presence of the gelatin and the methionine accounted for the folic acid deficiency in the mouse produced by feeding the chick diet, C2. A level of 0.8 per cent of methionine appeared to be best for the production of a folic acid deficiency in the mouse, and this level was used in all succeeding experiments.

Further preliminary studies to find the amino acid(s) in gelatin responsible for producing the deficiency showed that by merely adding 1.7 per cent of glycine (the amount supplied by 8 per cent of gelatin) to the mouse diet (M4) a good folic acid deficiency could be produced in the presence of methionine. So as not to cause too much of an amino acid imbalance, gelatin was used in all of the following experiments instead of pure glycine. The level of fat and fat-soluble vitamins in the diet was not a significant factor in the production of a folic acid deficiency. Therefore, in formulating the mouse diet (M9) which was used in all succeeding studies, these nutrients were maintained at the same level as in diet C2. In fact, the mouse diet M9 is the same as the chick diet, C2, except for the change in the level of methionine.

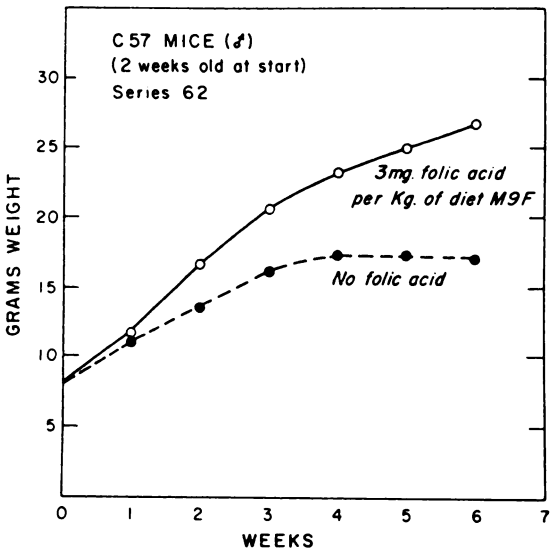

Fig. 1. Growth curves of folic acid deficient mice on diet M9 as compared with growth of control mice. There was no mortality in this series.

When C57 male mice were fed diet M9 minus pteroylglutamic acid they grew at a rate about equal to that of control mice fed pteroylglutamic acid for one or two weeks and then, as in a typical experiment shown in Figure 1, grew at a slower rate. At about four weeks of age a growth plateau was reached in nearly all experiments. In 17 of 20 different experiments of six C57 male mice per group, deficient mice weighed an average of 15 g at the end of six weeks, whereas control mice receiving 3 mg of pteroylglutamic acid per kg grew normally, had no mortality, and weighed an average of 25 g. (Female C57 mice behaved similarly in two six-week experiments.) Mortality within individual deficient groups varied—usually all mice survived but in a few instances most mice in a group of six were dead by six weeks. Contrary to these results, in 3 of 20 experiments, deficient mice weighed as much as the control mice at six weeks of age. The significance of this is discussed later.

We have kept folic acid deficient mice on the deficient diet for longer periods of time than six weeks with varying results. Sometimes there was a high death loss in several weeks and in some instances the entire group of six mice lived for many months. Such results indicated to us early in the experiments that the deficient animals live in quite a delicate balance with respect to synthesis of folic acid by intestinal microorganisms or by some other mechanism. It was determined rather early in our studies that it was important to clean the mouse cages and feeding equipment whenever they became contaminated with fecal matter; coprophagy appeared to be a factor in causing the variability between experiments. Variability was kept at a minimum when this was done and greater differences were obtained with and without folic acid.

TABLE II

Effect of Age of Mouse on Folic-Acid Deficiency

Age of mouse 1 *wk*	Weight at start *g*	Diet	Weight after 6 wk on experiment *g*
2	7.4	M9 minus folic acid	12.6 ± 1.0
2	7.4	M9 plus 3 mg folic acid/kg	22.5 ± 0.3
3	12.8	M9 minus folic acid	22.3 ± 0.3
3	12.8	M9 plus 3 mg folic acid/kg	22.8 ± 0.6

Factors in Production of Folic Acid Deficiency

Effect of Age of Mouse: It was found early in these studies that the age and size of the mouse at the time of starting the experiment were important in determining the results obtained. Table II gives the results of two experiments on six mice each. Although a deficiency of folic acid has been produced in our laboratory in older mice over 12 g in weight, the deficiency is more consistently obtained with smaller mice. We now routinely use mice 8 to 10 g in weight.

Effect of Strain of Mouse (Genetics): To find whether or not the genetics of the mouse were responsible for our unexpected results, several strains of mice produced in the National Institutes of Health colony were fed the folic acid deficient diet (M9 minus folic acid) and compared with control mice. The results are shown in Table III. It is clear that all strains of mice tested became folic acid deficient as evidenced by reduced growth rates and increased mortality rates. It is assumed, therefore, that differences in strain of mice used in the various laboratories are not responsible for the differences in experimental results.

TABLE III

Effect of Genetics on Folic Acid Deficiency in the Mouse[a]

Strain	Supplement to diet M9[b]	Weight after 6 wk ± S.E. g	Mortality/ no. mice
C57B1	None	17.1 ± 1.0	3/12
	Folic acid	24.5 ± 0.5	0/12
NIH general-purpose (Swiss)	None	20.6 ± 1.2	4/12
	Folic acid	22.6 ± 0.8	0/12
CFW	None	20.2 ± 1.8	5/12
	Folic acid	28.3 ± 0.6	1/12
DBA	None	12.0 ± 1.5	10/12
	Folic acid	23.9 ± 0.4	1/12
A/LN	None	14.9 ± 1.0	2/12
	Folic acid	23.0 ± 0.5	0/12
CDBA	None	20.0 ± 1.0	0/12
	Folic acid	26.0 ± 0.4	0/12

[a] All groups averaged approximately 8 g at start of experiment and 12 male mice were used in all instances (average of two experiments).

[b] Control mice were all fed 3 mg of pteroylglutamic acid (folic acid) per kg of diet.

Effect of Various Substances on Growth of Deficient Mice: Various compounds have been tested for their effect on growth of deficient mice. A considerable number of substances have had no effect on growth when added to the diet of deficient mice in various experiments (details not given to conserve space). These include: Aureomycin® (50 mg/kg of diet), cellulose (15 per cent), Crisco (up to 20 per cent), ascorbic acid (0.5 per cent), cystine (1 per cent, proline (1 per cent), isoleucine (1 per cent), tryptophan (0.2 per cent), phenylalanine (0.5 per cent), histidine (0.5 per cent), various mixtures of these amino acids, and 0.1 per cent each of several purines and pyrimidines (adenine, guanine, thymine, orotic acid, uridylic acid, and cytidine).

TABLE IV

Comparison of Activity of Folic Acid and Para-aminobenzoic Acid (PABA) in Male C57 Mice

A typical experiment

Supplement to diet M9 minus folic acid	No. mice	Mortality at 6 wk	Weight at 6 wk ± S. E. g
None	6	0	16.8 ± 0.7
1 mg PABA/kg	6	0	22.9 ± 0.9
5 mg PABA/kg	6	0	24.3 ± 0.7
50 mg PABA/kg	6	0	24.8 ± 0.4
0.5 mg PGA/kg	6	0	24.8 ± 0.7
1.0 mg PGA/kg	6	0	25.0 ± 0.5
3 mg PGA/kg	6	0	24.8 ± 0.4

Several substances have increased the severity of the deficiency, including additional glycine, gelatin (up to 15 per cent), and sulfasuxidine (0.5 per cent), as would be expected.

On the other hand, several substances have a marked sparing effect on folic acid under these conditions. These include procaine penicillin G, which at a level of 50 mg/kg of diet completely replaced the need for folic acid in three experiments.* Also, para-aminobenzoic acid, which is a constituent of pteroylglutamic and is known to spare folic acid in other animals, had a marked sparing effect on folic acid (see Table IV for results of a typical experiment). The exact folic acid requirement has not been determined under these conditions but it appears to be less than 0.5 mg/kg of diet. Para-aminobenzoic acid was not as active as folic acid, but the exact relationship needs further study. Calcium leucovorin (Lederle) was fully as active as pteroylglutamic acid when compared at low levels in the diet. This was rather unexpected, since in the chick calcium leucovorin is not as active as folic acid when present in the diet.[14]

Observations Related to Folic Acid Deficiency

Excretion of Formiminoglutamic Acid: In a trial with pooled urine from six mice on a deficient diet a total of 11.9 micromols of formiminoglutamic acid was excreted in a 24-hour period, whereas none was excreted in control animals fed 3 mg of pteroylglutamic acid per kg.† This is similar to results which have been obtained with rats fed diets low in folic acid.[15]

Other Observations: There was no change in the black hair color in the deficient mice. Depigmentation might have been expected from studies with folic acid in other animals. Alopecia was observed only rarely and was not apparently correlated with changes in the diet.

* Studies made in cooperation with Dr. Victor H. Haas. It is possible that the procaine portion of the molecule is responsible for this effect because of its relationship to PABA.

† I wish to thank Dr. Milton Silverman and Miss Rita Gardiner for making these assays.

Mice on the deficient diet for extended periods of time showed gross evidence of anemia. Preliminary blood studies on four deficient mice showed a significant drop in hemoglobin as compared with control mice. Complete blood studies were made by Drs. Martin Leibling and Emil Frei and will be reported elsewhere.

Effect of Folic Acid Deficiency

Relationship to Virus Infection: Folic acid deficient mice obtained in these studies have been used in experiments conducted in cooperation with Drs. V. H. Haas and S. E. Stewart. The results of these experiments have been published[12] and are only briefly described here.

It has been shown that the injection of amethopterin, a folic acid antagonist, prevented death of mice given an intercerebral injection of the virus of *lymphocytic choriomeningitis* (LCM) in doses that killed all untreated control mice.[16] The spared mice had prolonged viremia and developed immunity. It was of interest, therefore, to see whether similar results could be obtained in mice made folic acid deficient by dietary means without the use of drugs. It was found that death from LCM injections did not occur in mice fed the low folic acid diet M9 for a six-week period. This is similar to the results with amethopterin. Control mice, fed 3 mg pteroylglutamic acid, were all killed by similar injections. Details of three typical experiments are shown in Table V. Similar results have been obtained in other trials. It is obvious, therefore, that the effect of amethopterin is truly due to its folic acid inhibiting effect and not to any other nonspecific effect it might have.

It is of interest that in mice in which death was prevented by folic acid deficiency or by amethopterin an immunity developed after several weeks.[11,12] These mice could be placed later on a normal stock diet with folic acid and would live indefinitely. Active virus, capable of killing other mice, was obtained from the brains of the spared mice as late as the twenty-ninth day. Thus, a high level of virus production occurs in the spared mice. Apparently, in the absence of excess folic acid in the brain, an immunity develops before the virus has a chance to kill the animal.[12] The exact explanation is not known.

TABLE V

Prevention of Death from LCM Injections in Mice Fed Folic Acid Deficient Diets[12]

C57 male mice, 6 weeks on deficient diets

Diet	Experiment	Average weight g	No. mice dead by 14 days/ no. injected	Average day of death
M9 minus folic acid	1	12	0/5	—
	2	22	0/6	—
	3[a]	19	0/6	—
M9 + 3 mg folic acid/kg	2	23	6/6	8.3
	3[a]	21	6/6	8.3

[a] In experiment 3, diet M4 (see Table I) plus 8 per cent of gelatin was used.

It is an interesting observation that the effect of a folic acid deficiency in preventing death was noted in several experiments, with mice which had shown no weight differences compared to control mice. This is a good argument for not using weight differences as an essential criterion of a nutrient deficiency in experimental nutrition studies. In this instance, for example, the effect of a folic acid deficiency in preventing death from LCM virus provides a tool by which a folic acid deficiency can be diagnosed in the mouse when other means, including weight changes, are not available. We have used this tool successfully in other studies (not described here) with folic acid deficient mice.

These results in experiments 2 and 3 (see Table V) also provide good evidence that possible inanition in the deficient mouse was not a contributing factor in the results obtained. The deficient mice in these experiments were eating well throughout the study.

Relationship to Transplantable Lymphocytic Neoplasms: Because of the interesting results with LCM virus it was obvious that similar studies should be made with certain forms of mouse leukemia (lymphocytic neoplasms) known to be sensitive to amethopterin. These studies have been made with Dr. M. Potter of the National Cancer Institute and will be published in detail elsewhere.

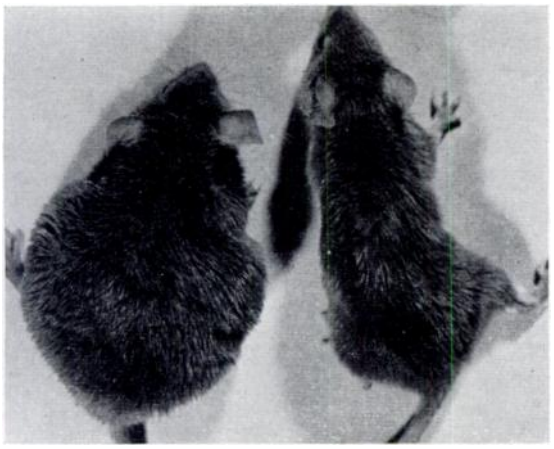

Fig. 2. Typical mice from the studies on lymphocytic neoplasms in folic acid deficiency. Both mice were injected with ascitic tumor cells 14 days before the photograph was taken. The mouse on the left received folic acid and died on day 15. The mouse on the right received the deficient diet and lived 8 days longer.

In brief, ascitic tumor cells of certain transplantable lymphocytic neoplasms (P288, P288AMRII, P388), in varying inocula size, were injected intraperitoneally into groups of hybrid (BALB/c × DBA/2) F1 mice.[17] In mice fed the deficient (M9) rations the onset of ascites and death were significantly delayed in contrast to the control mice, as demonstrated by Figure 2. One unexpected finding was that an amethopterin-resistant tumor responded in the same manner as an amethopterin-sensitive tumor. These details are quite complex and many variables affect the results. Further studies are in progress.

By means of studies such as these it should be possible to use intact animals fed normal diets without drugs to study nutritional factors which affect virus multiplication or growth of neoplasms. It is hoped that additional work will provide solutions to the problems raised here.

SUMMARY

An uncomplicated deficiency of folic acid has been produced by dietary means in young mice of various strains in three to six weeks without the aid of a folic acid antagonist or a sulfa drug. This has been done by keeping mice in cages with screen bottoms (to prevent coprophagy) and feeding them a synthetic diet low in folic acid containing 20 per cent casein, 8 per cent gelatin, and 0.8 per cent methionine, in addition to the other usual ingredients. The deficiency may be overcome by feeding 0.5 mg or more pteroylglutamic acid per kg of diet or by feeding larger amounts of para-aminobenzoic acid or procaine penicillin G.

The folic acid deficient mice have been used to study the relationship of nutrition of infection with the virus of lymphocytic choriomeningitis and to certain transplantable lymphocytic neoplasms. With both types of infection the time of death was significantly delayed in folic acid deficient mice. These studies provide a new tool which may be used to study the relationships between folic acid and disease in normal intact mice not receiving drugs.

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