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Effect of Vitamin B_{12} and Folate on Biosynthesis of Methionine from Homocysteine in the Nematode *Caenorhabditis briggsae*[1] (39289)

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The roles of dietary vitamin B_{12} and folic acid in methionine biosynthesis from homocysteine were first observed in higher animals. Jukes, Stokstad, and Broquist (1) noted that the growth of vitamin B_{12}-deficient chicks was increased by either methionine or homocysteine and vitamin B_{12} but not by homocysteine alone. It was also reported that rats were able to utilize homocysteine in lieu of methionine when folic acid (2) or choline (3) was added to the diet. In microorganisms, *Escherichia coli* is known to possess two alternative pathways for methionine biosynthesis: the vitamin B_{12}-dependent pathway (4) and the vitamin B_{12}-independent one (5). In the present study, we show evidence that dietary vitamin B_{12} and folic acid are required for the biosynthesis of methionine from homocysteine in an axenic free-living nematode, *Caenorhabditis briggsae*. Information related to biochemical nutrition in invertebrates is sparse. Therefore, this study has particular relevance to the fields of comparative nutrition and biochemistry.

Materials and methods. Composition of the basal medium. The chemical-defined medium *Caenorhabditis briggsae* maintenance medium (CbMM) (6) was specially prepared by the Grand Island Biological Company (Grand Island, New York) and supplied as a sterile, double-strength solution. Several of those chemical components which are normally present in the standard medium were deleted from the special medium: vitamin B_{12}, folic acid, calcium folinate (5-formyltetrahydrofolic acid), L-methionine, choline dihydrogen citrate, glutathione (reduced), para-aminobenzoic acid, and nucleic acid substituents (adenosine 2′ and 3′-monophosphoric acid, cytidine 2′ and 3′-monophosphoric acid, uridine 2′ and 3′-monophosphoric acid, guanosine 2′ and 3′-PO_4Na_2 and thymine). These components (calcium folinate was omitted) were prepared in groups or in separate solution, sterilized by filtration (0.22-μm pore size, Millipore Filter Corp., Bedford, Mass.), and combined with the medium at the appropriate concentrations. In the present study, five chemical components (vitamin B_{12}, folic acid, methionine, homocysteine, and choline dihydrogen citrate) were used as test variables. All chemicals were obtained from the Sigma Chemical Co. (St. Louis, Mo.) except for L-methionine (Calbiochem., La Jolla, Calif.).

To all the medium was added 50 μg/ml of β-sitosterol (Sigma Chemical Co., St. Louis, Mo.) (7) dissolved in Tween 80, and 25 to 50 μg/ml of cytochrome *c* (Sigma Chemical Co., St. Louis, Mo.) (8). In addition, an undefined supplement of 5 mg/ml of soy peptone (Sheffield Chemical Co., Union, N.J.) or 70 to 350 μg/ml of *E. coli* "cell residue" was added as a source of the unknown proteinaceous growth factor(s). (See following section.)

Preparation of Escherichia coli cell residue. The *E. coli* "cell residue" fraction was prepared from a culture of *E. coli* (ML 30 strain) maintained on agar (1.6%) slants containing a basal-salts and glucose (0.1%) medium (BSG) (7). One-liter cultures containing basal-salt and glucose (0.25%) medium in 2800-ml Fernbach flasks were inoculated with 1 ml of a smaller "starter culture." They were incubated overnight at 37° on a controlled environment incubator-shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 150 to 200 oscillations/min. The suspension of cells was centrifuged at 9000 *g* for 35 min and washed twice with ca. 10 vol of 0.15 *M* KCl. The cells were

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then suspended in a minimum amount of distilled water (ca. 5 ml). This slurry, containing viable *E. coli* cells, was stored at −20°. For use in experiments, 0.1 ml of the slurry was added to 10 ml of distilled water to give a suspension containing approx 1 mg/ml of solids on a dry-weight basis. The cell suspension was autoclaved at 120° and 15 psi for 8 min, centrifuged at 3000 *g* for 10 min, washed twice with distilled water, and resulting cell residue was stored at 4°.

Compounding the complete medium. A preliminary mixture (basal medium) was prepared for each experiment by combining all the fixed components (CbMM + deleted components except the variable components + β-sitosterol + cytochrome *c* + *E. coli* cell residue or soy peptone). This mixture was, in turn, combined with the variable components (vitamin B_{12}, folic acid, choline, methionine, or homocysteine) at the appropriate concentrations. The amounts of vitamin B_{12} and folic acid in the basal medium were determined by Euglena assay (9) and *L. casei* assay (10), respectively.

Bioassay procedures. The *C. briggsae* stock culture was maintained in 4% soy peptone + 1% yeast extract + 10% heated liver extract (SP-YE-HLE) stock medium as described by Rothstein and Cook (11).

Individual larval assay. As reported previously (12), three newly hatched larvae were inoculated into 10 × 75-mm tubes containing 0.25 ml of medium and incubated at 20°. After approx 20 to 30 days, the nematode population was estimated manually by the method of Tomlinson and Rothstein (13). This involved diluting a 0.1-ml sample such that no more than 100 worms were on the counting slide. Counting was made with the aid of a dissecting microscope at 15× magnification.

Mass culture assay. The procedure was adapted from that of Tomlinson and Rothstein (13) as described by Pinnock, Hieb, and Stokstad (14). Culture tubes (18 × 150 mm) containing 5 ml of medium were inoculated with 40 to 1000 worms/ml and incubated at 20° on a tissue culture-type rotator at 1 rpm. The population size was estimated after approx 20 to 30 days by the counting procedure described above for the larval cultures.

Results. The standard *C. briggsae* maintenance medium (CbMM) contains 3750 ng/ml of vitamin B_{12} and 7500 ng/ml of folic acid (6). However, medium supplemented with *E. coli* cell residue and containing 100 ng/ml of folic acid supported nearly maximum population growth, and was later proved to be an optimal level (15). Media supplemented with *E. coli* cell residue and containing 12, 20, and 60 ng/ml of folic acid were folic acid-deficient, while media containing 1500 to 7500 ng/ml were folic acid-supraoptimal.

Table I shows the results of replacing methionine with homocysteine on the population growth. In media containing supraoptimal levels of folic acid (7500 ng/ml) and vitamin B_{12} (3750 ng/ml), the deletion of methionine caused an 80% reduction in population. When methionine was replaced by D,L-homocysteine (assuming only L-form is active), the medium was equally as effective in supporting the population growth as that containing methionine. This indicated that *C. briggsae* can utilize homocysteine in lieu of methionine when supraoptimal levels of vitamin B_{12} and folic acid were provided.

Table II shows the results of varying concentrations of vitamin B_{12} in media containing homocysteine in place of methionine with optimal concentration of folic acid (100 ng/ml). When vitamin B_{12} was omitted, moderate population growth occurred in the

TABLE I. EFFECT OF METHIONINE DEFICIENCY AND ITS SUBSTITUTION WITH HOMOCYSTEINE ON POPULATION (INDIVIDUAL LARVAL ASSAY).

Basal medium[a] + variables		Population on 19th day (number of worms/0.25 ml of assay medium)[b]
Amino acid	Folic acid (7500 ng/ml)	
Methionine[c]	+	410
Methionine	−	0[d]
−	+	70
−	−	0[d]
Homocysteine[e]	+	420
Homocysteine	−	0[d]

[a] The basal medium consisted of CbMM, 25 μg/ml of cytochrome *c*, 50 μg/ml of β-sitosterol, and 70 μg/ml of *E. coli* cell residue. The basal medium furnished 3750 ng/ml of vitamin B_{12} (9) and 3 ng/ml of folic acid (10).

[b] Average of four tubes.

[c] 2.60 m*M* or 389 μg/ml of L-methionine.

[d] Contains the original three larvae.

[e] 5.20 m*M* or 800 μg/ml of D,L-homocysteine thiolactone HCl.

original culture ("0" culture) and then decreased to almost zero population by the second subculture. This process of subculturing is necessary for the mass culture assay in order to deplete the vitamin B_{12} stores of the organism and to induce a complete vitamin B_{12} deficiency. Based on the results of the second subculture, it can be concluded that 0.04 ng/ml of vitamin B_{12} (the actual total concentration at the 0.15 ng-ml of vitamin B_{12} level) was required to give a detectable response and 6 ng/ml (the actual total concentration at the 6.1 ng/ml level) to give a maximum response. It should be noted at this point that the low levels of added vitamin B_{12} (i.e., 0.04 ng/ml) are greatly influenced by the additional 0.11 ng/ml of vitamin B_{12} which was furnished by the components in the basal medium. Soy peptone, a plant protein, contains a negligible amount of vitamin B_{12} (1 pg/mg dry weight) (9) and a low level of methionine (8.2 μg/mg dry weight) (16). It was used as the proteinaceous supplement for developing vitamin B_{12} deficiency studies in the mass culture assay (Tables II and V). However, soy peptone was not used for developing a severe folic-acid deficiency (Tables III and IV) because it contains 5 ng/mg dry weight folic acid (10).

TABLE II. DEVELOPMENT OF VITAMIN B_{12} DEFICIENCY IN MEDIUM CONTAINING HOMOCYSTEINE IN PLACE OF METHIONINE AND OPTIMAL CONCENTRATION OF FOLIC ACID (MASS CULTURE ASSAY).

Basal medium[a] + vitamin B_{12} (ng/ml)	Population in continuous cultures (number of worms/ml of medium)		
	"0" Culture[b] (19th day)	"1" Subculture[c] (19th day)	"2" Subculture[d] (26th day)
3750.00	135,000	38,000	37,000
6.00	80,000	34,000	40,000
1.20	105,000	–	–
0.24	110,000	7,500	250
0.04	50,000	3,300	190
0.01	40,000	2,800	100
0.00	43,000	3,200	40

[a] The basal medium consisted of CbMM, 800 μg/ml of D,L-homocysteine thiolactone HCl, 50 μg/ml of cytochrome *c*, 50 μg/ml of β-sitosterol, and 5 mg/ml of soy peptone. The basal medium furnished 0.11 ng/ml of vitamin B_{12} (9) and 125 ng/ml of folic acid (10).

[b] The initial population was 2500 worms/ml which was inoculated from a stock culture (SP-YE-HLE).

[c] The initial population was 300–350 worms/ml which was inoculated from the "0" culture containing no vitamin B_{12}.

[d] The initial population was 40–60 worms/ml which was inoculated from the "1" subculture containing no vitamin B_{12}.

Table III ("0" culture) presents results of an experiment to determine the role of choline in a medium containing homocysteine. It can be seen that omission of choline (88.5 μg/ml) from the chemically defined medium, either in the presence or absence of vitamin B_{12}, had no effect on population size. This was true whether the medium contained low (20 ng/ml) or supraoptimal (2000 ng/ml) levels of folic acid. Thus, under the experimental conditions, choline did not appear to decrease the requirement for

TABLE III. EFFECT OF VITAMIN B_{12}, FOLIC ACID, AND CHOLINE ON POPULATION GROWTH IN MEDIA CONTAINING HOMOCYSTEINE (MASS CULTURE ASSAY).

Basal medium[a] + variables		Population in two continuous cultures (number of worms/ml of medium)			
		"0" Culture[b] (33rd day)		"1" Subculture[c] (34th day)	
Vitamin B_{12} (3750 ng/ml)	Choline (88.5 μg/ml)	Folic acid (20 ng/ml)	Folic acid (2000 ng/ml)	Folic acid (20 ng/ml)	Folic acid (2000 ng/ml)
+	+	3,100	9000	—[e]	—[e]
+	−	—[d]	8300	270	16,000
−	+	430	3600	—[e]	—[e]
−	−	330	3200	0	13

[a] The basal medium consisted of CbMM, 800 μg/ml D,L-homocysteine thiolactone HCl, 50 μg/ml of β-sitosterol, and 70 μg/ml of *E. coli* cell residue ("0" culture), 350 μg/ml of *E. coli* cell residue ("1" culture). The basal medium furnished 0.056–0.057 ng/ml of vitamin B_{12} (9) and 3–7 ng/ml of folic acid (10).

[b] The initial population was 300–400 worms/ml which was inoculated from a previous culture containing 0.048 ng/ml of vitamin B_{12}.

[c] The initial population was 10–20 worms/ml which was inoculated from the "0" culture containing no vitamin B_{12}.

[d] Sample lost.

[e] Not tested.

TABLE IV. EFFECT OF VARIOUS CONCENTRATIONS OF FOLIC ACID ON POPULATION GROWTH OF VITAMIN B_{12} DEFFICIENT *C. BRIGGSAE* IN THE MEDIUM CONTAINING HOMOCYSTEINE (INDIVIDUAL LARVAL ASSAY).

Basal medium[a] + variables		Population on 32nd day (number of worms/0.25 ml of assay medium)[b]	Percentage population reduction (Vitamin B_{12} omitted)
Vitamin B_{12} (3750 ng/ml)	Folic acid (ng/ml)		
+	7500	1800	
−	7500	900	50
+	1500	1500	
−	1500	850	43
+	300	1400	
−	300	900	36
+	60	1300	
−	60	240	82
+	12	1000	
−	12	50	95
+	0	0[c]	
−	0	0[c]	–

[a] The basal medium consisted of CbMM, 800 μg/ml of homocysteine thiolactone HCl, 50 μg/ml of cytochrome *c*, 50 μg/ml of β-sitosterol, and 70 μg/ml of *E. coli* cell residue. The basal medium furnished 0.11 ng/ml of vitamin B_{12} (9) and 3 ng/ml of folic acid (10).

[b] Average of four tubes.

[c] Contains the original three larvae.

vitamin B_{12} or folic acid by methylation of homocysteine.

The results for "1" subculture in Table III clearly demonstrated that both vitamin B_{12} and folic acid are required for population growth in *C. briggsae* in the medium containing homocysteine. Choline was omitted, however, from the media since the previous results ("0" culture, Table III) indicated that choline had no effect on the requirement of vitamin B_{12} or folic acid. In the doubly deficient medium (0 ng/ml of vitamin B_{12} and 20 ng/ml of folic acid), no population increase was observed. In the vitamin B_{12}-deficient medium (0 ng/ml of vitamin B_{12} and 2000 ng/ml of folic acid), no population growth was observed. In the folic acid-deficient medium (3750 ng/ml of vitamin B_{12} and 20 ng/ml of folic acid), an increase of approx 14–27 times over the initial population was observed. Furthermore, when the same medium contained supraoptimal vitamin B_{12} (3750 ng/ml) and supraoptimal folic acid (2000 ng/ml), the final population was approx 1000 times over the initial population. Therefore, it is concluded that both vitamin B_{12} and folic acid are required in *C. briggsae* for the biosynthesis of methionine from homocysteine (Tables III and I). The responses of two vitamins are additive.

It was of special importance to select the correct proteinaceous supplements in the medium in order to produce the double vitamin deficiencies. *E. coli* cell residue which contains 95% protein was found low in all three substances: vitamin B_{12} (0.003 ng/mg dry weight) (9), folic acid (1 ng/mg dry weight) (10), and methionine (2–5 μg/mg).[4] It supported a good population growth at as little as 70 μg/ml and was therefore used in developing combined vitamin B_{12} and folic-acid deficiencies in the nematode.

The effect of varying concentration of folic acid on the response to a vitamin B_{12}-deficient medium containing homocysteine is shown in Table IV. At high levels (300 ng/ml and higher) of folic acid, omission of vitamin B_{12} produces a 36 to 50% reduction in population. However, at levels (12 to 60 ng/ml) of folic acid which are below the optimum, omission of vitamin B_{12} produces an 82 to 95% reduction in population, indicating a more severe vitamin B_{12} deficiency at low levels of folic acid. This demonstrates that supraoptimal levels of folic acid can spare the requirement for vitamin B_{12}. The data also indicate that supraoptimal level of vitamin B_{12} spares the requirement for folic acid.

The sparing effect between the two vitamins was further examined. The results (Table V) show that, in the presence of optimal amount of vitamin B_{12} (6 ng/ml), 20 ng/ml of folic acid gave a response which was nearly the same as that at 2000 ng/ml. At a concentration of 1.2 ng/ml of vitamin B_{12}, 2000 ng/ml of folic acid gave a population four times as great as 20 ng/ml of folic acid. Thus, in the medium containing soy peptone, 20 ng/ml of folic acid (the actual total concentration at 45 ng/ml of folic acid) was nearly optimal at high concentrations of vitamin B_{12}, but was not optimal at low vitamin B_{12} concentrations.

Discussion. Our results confirm a previous report that methionine is an essential amino acid for *C. briggsae* (18). We have

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[4] Estimated; approximately one-half of the amount of methionine content (4–10 μg/mg) in *E. coli* cell (17).

TABLE V. EFFECT OF VARYING CONCENTRATIONS OF VITAMIN B_{12} AND FOLIC ACID ON POPULATION GROWTH IN A MEDIUM CONTAINING HOMOCYSTEINE AND IN THE ABSENCE OF CHOLINE (MASS CULTURE ASSAY).

Basal medium[a] + variables	Population on 35th day (number of worms/ml of medium)[b]		
Vitamin B_{12} (ng/ml)	Folic acid (ng/ml)		
	20	500	2000
0	15	10	5
0.24	10	10	35
1.2	50	180	200
6.0	7600	7500	8500

[a] The basal medium consisted of CbMM, 800 μg/ml of homocysteine thiolactone HCl, 50 μg/ml of cytochrome *c*, 50 μg/ml of β-sitosterol, and 5 mg/ml of soy peptone, except choline was omitted for the experiment. The basal medium furnished 0.062 ng/ml of vitamin B_{12} (9) and 25 ng/ml of folic acid (10).

[b] The initial population was ca. 40 worms/ml which was inoculated from a previously twice-depleted culture containing no vitamin B_{12}.

also shown that methionine can be replaced by homocysteine providing supraoptimal levels of vitamin B_{12} and folic acid are present. This indicates that, as in higher animals and microorganisms, this nematode possesses a mechanism for the biosynthesis of methionine from homocysteine.

Further evidence (Table III) suggests that the biosynthesis of methionine from homocysteine is both vitamin B_{12}- and folic acid-dependent, and two vitamins spare each other in the reaction. In the case of the vitamin B_{12}-dependent pathway in *E. coli* (4), homocysteine is converted to methionine with N^5-methyltetrahydrofolic acid as a methyl donor. The enzyme, methyltetrahydrofolate-homocysteine transmethylase, contains a Co-methylcorrinoid as a prosthetic group. Since both vitamins serve as cofactors in one enzymatic reaction, they might spare each other to a certain degree in the same reaction.

In mammals, there are at least two enzyme systems which can methylate homocysteine: one is N^5-methyltetrahydrofolate-homocysteine methyltransferase (vitamin B_{12}-dependent), the other is betaine-homocysteine methyltransferase (vitamin B_{12}-independent). It should be noted that the vitamin B_{12}-independent pathway in mammals is different from the vitamin B_{12}-independent pathway existing in *E. coli.* The former reaction is betaine dependent; the latter reaction is folic acid-dependent ($H_4PteGlu_3$) which is not present in mammals. In mammals, the vitamin B_{12}-dependent pathway has its unique physiological importance in regulation of methionine biosynthesis (19). It was also reported that betaine-homocysteine methyltransferase was found only in rat liver while N^5-methyltetrahydrofolate-homocysteine methyltransferase could be demonstrated in all tissues except small intestinal mucosa. In *C. briggsae*, we found omitting choline (Table III, "0" culture) from the chemically defined medium had no effect on methylation of homocysteine even in the medium containing low levels of folic acid and no vitamin B_{12}. It was possible that the proteinaceous supplements (i.e., *E. coli* cell residue or soy peptone) furnished a sufficient amount of choline. It could also be due to the fact that there was a carryover of choline by the nematode itself. However, an absolute vitamin B_{12} deficiency was developed in *C. briggsae* even when choline was present in the medium (Table II). Assuming choline could be utilized in the same way as betaine in the nematode, this would suggest that the vitamin B_{12}-dependent pathway for methylation of homocysteine is the predominant pathway existing in this organism.

Summary. (i) Omission of L-methionine from the medium resulted in an 80% population reduction. Substitution of D,L-homocysteine corrected methionine deficiency in *C. briggsae* in the presence of supraoptimal vitamin B_{12} and folic aid.

(ii) An absolute vitamin B_{12} requirement in *C. briggsae* developed in the medium containing homocysteine at the second subculture. Concentration of 6 ng/ml of vitamin B_{12} (at 100 ng/ml of folic acid) was sufficient to support maximum growth of C. briggsae in the medium containing homocysteine.

(iii) It was found that either supraoptimal folic acid (2000 ng/ml) or supraoptimal vitamin B_{12} (3750 ng/ml), with homocysteine, supported very little population growth of *C. briggsae*. However, supraoptimal folic acid and supraoptimal vitamin B_{12} together supported a maximum population growth. Therefore, it was concluded that both vi-

tamin B_{12} and folic acid were required for the biosynthesis of methionine from homocysteine. Studies also showed that the two vitamins spared each other for population growth in the medium containing homocysteine.

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1. Jukes, T. H., Stokstad, E. L. R., and Broquist, H. P., Arch. Biochem. **25,** 453 (1950).
2. Bennett, M. A., Science **110,** 589 (1949).
3. du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., Proc. Amer. Soc. Biol. Chem., J. Biol. Chem. **128,** cviii (1939).
4. Foster, M. A., Dilworth, M. J., and Wood, D. D., Nature (London) **201,** 39 (1964).
5. Guest, J. R., and Woods, D. D., *in* "Vitamin B_{12} and Intrinsic Factor" (H. C. Heinrich, ed.), p. 686. F. Enke, Stuttgart (1962).
6. Buecher, E. J., Hansen, E., and Yarwood, E. A., Proc. Soc. Exp. Biol. Med. **121,** 390 (1966).
7. Hieb, W. F., and Rothstein, M., Science **160,** 778 (1968).
8. Hieb, W. F., Stokstad, E. L. R., and Rothstein, M., Science **168,** 143 (1970).
9. Hutner, S. H., Bach, M. K., and Ross, G. I. H., J. Protozool. **3,** 101 (1956).
10. Tamura, T., Shin, Y. S., Williams, M. A., and Stokstad, E. L. R., Anal. Biochem. **49,** 517 (1972).
11. Rothstein, M., and Cook, E., Comp. Biochem. Physiol. **17,** 683 (1966).
12. Lower, W. R., Hansen, E. L., and Yarwood, E. A., J. Exp. Zool. **161,** 29 (1966).
13. Tomlinson, G., and Rothstein, M., Biochim. Biophys. Acta **63,** 465 (1962).
14. Pinnock, C. B., Hieb, W. F., and Stokstad, E. L. R., Nematologica **21,** 1 (1975).
15. Lu, N. C., Ph.D. thesis, University of California, Berkeley (1973).
16. "Technical Data on Soy Peptone Powder." Sheffield Chemical Co., Norwich, New york (1964).
17. Mondolfo, H., and Hounie, E., Rev. Assoc. Bioquim. Argentina **16,** 416 (1951), *from* Chem. Abs. **46,** 3606 (1952).
18. Van Fleteren, J. R., Nematologic **19,** 93 (1973).
19. Finkelstein, G. D., Kyle, W. E., and Harris, B. J., Arch. Biochem. Biophys. **146,** 84 (1971).

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