Relation of Vitamin B_{12} and One-carbon Metabolism to Hydrocephalus in the Rat [1,2,3]

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ABSTRACT The purpose of this investigation was to examine the relationship of a number of factors involved in 1-carbon metabolism to the induction of hydrocephalus in the rat. Animals raised with a diet deficient in vitamin B_{12} gave birth to young with congenital hydrocephalus. The addition of an intestinal antibiotic, neomycin, to the dams' diets or the parenteral administration of vitamin B_{12} antagonists did not markedly increase the incidence of congenital abnormalities. The incidence of congenital hydrocephalus was markedly increased by the inclusion in the diet of X-methyl folic acid, a folic acid antagonist. A higher incidence was obtained, however, when females were fed a diet deficient in vitamin B_{12} and choline. Methionine and sarcosine in equivalent methyl groups did not offer the same protective effect as did choline. Abnormalities were similar in newborn animals from vitamin B_{12}-deficient dams raised with the folic acid antagonist diets or with the choline-deficient diets. Lesions noted in offspring from these animals included: 1) stunted embryonic growth, 2) hydrocephalus, 3) hydro-ureter and hydronephrosis, 4) umbilical hernia, 5) spina bifida and cranioschisis, 6) harelip, and 7) skeletal defects.

While studying the nutritional requirements for reproduction and lactation in the rat, Richardson and Hogan (1) observed spontaneous cases of hydrocephalus in newborn animals. Approximately 2% of the offspring born to dams raised with a purified ration were hydrocephalic. The original diet consisted of casein and all of the vitamins available at that time, but it was later shown by O'Dell et al. (2) that supplementation of the diet with folic acid would largely prevent the occurrence of the congenital hydrocephalus. These investigators reported that X-methyl folic acid, an antimetabolite, added to soybean oil meal diets during successive gestations resulted in an increased incidence of hydrocephalic offspring compared with animals fed a casein diet (3). Vitamin B_{12} supplementation was later shown to prevent completely the occurrence of congenital abnormalities in newborn rats whose dams had received the vegetable protein diet (4). The X-methyl folic acid was considered to inhibit vitamin synthesis by intestinal microorganisms.

Newberne and O'Dell (5) were able to produce congenital hydrocephalus in rats by a maternal vitamin B_{12} deficiency; the diets did not contain any antagonist and were supplemented with 5 mg of folic acid and 1 g of choline chloride/kg of diet. Whereas the incidence of hydrocephalus in the folic acid antagonist diets of Hogan et al. (3) was 20%, a 10% incidence was noted when the ration was deficient only in vitamin B_{12} (5). Spina bifida, anophthalmia, harelip, cleft palate and edema were noted with the antagonist diet, but none of these lesions was observed in the newborn animals raised with the vitamin B_{12}-deficient ration without the antagonist.

Because of the differences reported by Hogan et al. (3) and Newberne and O'Dell (5), an attempt has been made to study further, congenital abnormalities in vitamin B_{12}-deficient neonatal rats by determining which accessory dietary factors might influence the incidence or severity of lesions.

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GENERAL METHODS

Weanling albino rats of the Caesarean Derived (CD) Sprague-Dawley strain were obtained from the Charles River Breeding Farm. The animals were housed in air conditioned animal quarters; food and water were supplied ad libitum. Three female animals were housed together in large, raised, screen-wire cages and fed either a vitamin B_{12}-deficient diet or a vitamin B_{12}-supplemented diet during the growth period.[5] The 30% protein diet shown in table 1 was supplemented with 0.3% choline chloride and 0.1% DL-methionine during the growth period; vitamin B_{12} was added to the control diet at the rate of 50 μg/kg of diet. At 13 weeks of age, the rats were placed on the various experimental rations. Females were fed the experimental ration for 4 weeks prior to breeding. Male animals fed a commercial ration were housed separately during their growth period and then placed with females, one male per cage. Females were allowed to litter in their cages, and the offspring were collected as soon as possible after delivery. Hydrocephalus was determined by incising formalin-fixed specimens.

TABLE 1

Composition of basal diets

	1	2
	% of diet	
Sucrose	55	65
Soybean protein [1]	30	20
Salts [2]	5	5
Cottonseed oil [3]	10	10
Vitamins [4]	*mg/kg of diet*	
Thiamine·HCl	16	16
Pyridoxine·HCl	16	16
Riboflavin	16	16
Ca pantothenate	40	40
Nicotinamide	12	12
Folic acid	5	5
Inositol	250	250
dl-α-Tocopheryl acetate [5]	112	112
	IU/kg of diet	
Vitamin A	22,800	22,800
Vitamin D	2,280	2,280

[1] Alpha Protein, Central Soya Company, Chicago (1.05% methionine).
[2] Hegsted et al. J. Biol. Chem., *138:* 459, 1941.
[3] Wesson oil, Wesson Sales Company, Fullerton, California.
[4] Vitamin B_{12} (50 μg/kg of diet) was added to the control diets.
[5] Hoffmann-LaRoche, Inc., Nutley, New Jersey.

Three separate experimental trials were conducted. The first trial was performed to determine the effects of feeding an intestinal antibiotic or vitamin B_{12} antagonists (monobasic acids of vitamin B_{12}) (6, 7). In the second trial the effect of a choline deficiency and the effect of feeding the folic acid antagonist, X-methyl folic acid, were determined. The third trial examined effects of lowering dietary protein levels, of substituting various methyl donors for choline, of parenteral administration of vitamin B_{12} antagonists and of feeding X-methyl folic acid in a choline-deficient diet.

METHODS AND RESULTS

Experimental trial 1

During the growth period, changes in body weight were measured weekly. Averages for the groups fed the vitamin B_{12}-deficient growth diet (diet C, table 2) and the control diet (diet D) are presented in figure 1 as a growth rate curve. When a 30% soybean protein diet contained 0.1% DL-methionine and 0.3% choline chloride, the growth rate of vitamin B_{12}-deficient rats was found to be similar to that of control animals. After the animals reached maturity the vitamin B_{12}-deficient group was divided into 3 subgroups and either maintained with the growth diet (diet C), given a diet containing neomycin (diet B), or fed a ration containing vitamin B_{12} antagonists (diet A). The adult vitamin B_{12}-deficient animals gave birth to hydrocephalic young; the control animals did not. Inclusion of an intestinal antibiotic, neomycin, in the diet did not greatly alter the incidence of hydrocephalus, whereas supplementation with monobasic acids of vitamin B_{12} caused infertility of that group. One apparently normal litter from the vitamin B_{12} antagonist group was obtained after the males in the cages were exchanged with those maintained with a commercial ration. The incidence of hydrocephalus is shown in table 2.

[5] Vitamin B_{12} antagonists were generously supplied by Dr. E. Lester Smith, Glaxo Research Laboratories, Ltd., Greenford, Middlesex, England. The X-methyl folic acid was kindly donated by Dr. E. H. Dearborn of Lederle Laboratories, Pearl River, New York. The soybean protein, Alpha Protein, was supplied through the courtesy of Central Soya Company, Chicago.

TABLE 2
Incidence of hydrocephalus (trial 1)

Diet (Ingredients added to basal diet 1, see table 1)		No. females fed diet	No. litters born	Avg no. animals/ litter	Newborns found dead	Avg wt newborn animals	Litters with hydrocephalic animals	Hydrocephalic animals
	mg/kg diet				%	*g*	%	%
Diet A		6	1	8.0	—	—	0	0
DL-methionine	3000							
choline chloride	1000							
monobasic acids of vitamin B_{12}	10							
Diet B		6	15	6.8	6.7	5.2	13.3	2.0
DL-methionine	3000							
choline chloride	1000							
neomycin sulfate	125							
Diet C		60	149	6.6	5.1	5.3	9.8	1.3
DL-methionine	3000							
choline chloride	1000							
Diet D		30	86	6.7	5.7	5.7	0	0
DL-methionine	3000							
choline chloride	1000							
vitamin B_{12}	0.05							

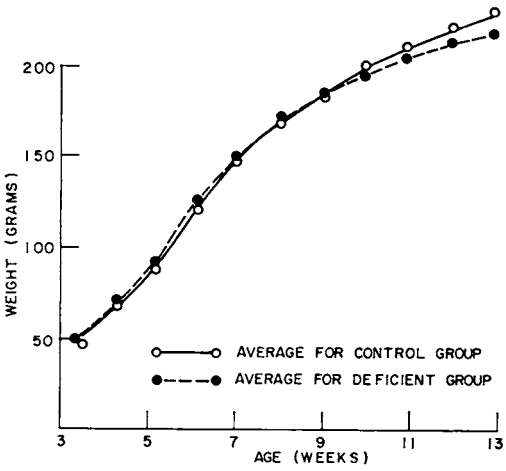

Fig. 1 Growth rate for vitamin B_{12}-deficient and control animals.

Experimental trial 2

The experimental procedure in trial 2 differed from that of trial 1. In trial 2 the mature rats were maintained with the growth diet (diet C, table 3) or with the control diet (diet D) for 2.5 months before being divided into subgroups. During this time, the females of trial 2 gave birth to one and sometimes two litters. All vitamin B_{12}-deficient females that had previously given birth to hydrocephalic young were maintained with a diet deficient in vitamin B_{12} (diet C, subgroup 1-a; table 3). One-third of the deficient animals were given a ration deficient in vitamin B_{12} but containing 10 mg of X-methyl folic acid/kg of diet (diet E, subgroup 1-b; table 3). The animals in the third subgroup were given a vitamin B_{12}-deficient ration which contained no choline (diet F, subgroup 1-c). The control animals were also divided into 3 subgroups (2-a, 2-b, 2-c) and fed diets D, G, or H.

The results (table 3) demonstrated that the addition of X-methyl folic acid to the diet caused a marked increase in the incidence of hydrocephalus (subgroup 1-b); however, a greater incidence of hydrocephalus occurred when the dams' diets were made deficient in choline as well as in vitamin B_{12} (subgroup 1-c). Females maintained with the folic acid antagonist-containing diet or the choline-deficient diets gave birth to offspring which were often small. Small embryos weighing less than 4 g were most common from dams fed the vitamin B_{12}-choline-deficient diets. Umbilical hernia and limb abnormalities were noted in hydrocephalic animals born to dams receiving the folic acid antagonist.

Samples of liver from adult and newborn animals were collected at the time

TABLE 3

Incidence of hydrocephalus (trial 2)

Group	Sub-group	Diet (Ingredients added to basal diet 1, see table 1)		No. litters	Total no. newborn	No. found dead	Avg wt newborn animals	No. animals weighing under 4 g	Litters hydro-cephalic	Newborn animals hydro-cephalic
			mg/kg diet				*g*		%	%
1		Diet C (before group subdivision)		17	161	9	5.6	0	5.8	0.6
		DL-methionine	3000							
		choline chloride	1000							
2		Diet D (before group subdivision)		7	54	1	6.1	0	0	0
		DL-methionine	3000							
		choline chloride	1000							
		vitamin B_{12}	0.05							
1	a	Diet C		16	115	5	5.4	0	18.0	6.0
		DL-methionine	3000							
		choline chloride	1000							
1	b	Diet E		19	101	12	6.1	3	33.3	13.8
		DL-methionine	3000							
		choline chloride	1000							
		X-methyl folic acid	10							
1	c	Diet F		15	72	16	5.8	12	60.0	37.5
		DL-methionine	3000							
2	a	Diet D		3	28	0	6.0	0	0	0
		DL-methionine	3000							
		choline chloride	1000							
		vitamin B_{12}	0.05							
2	b	Diet H		8	62	5	5.6	0	0	0
		DL-methionine	3000							
		choline chloride	1000							
		X-methyl folic acid	10							
		vitamin B_{12}	0.05							
2	c	Diet G		6	36	6	6.4	0	0	0
		DL-methionine	3000							
		vitamin B_{12}	0.05							

of necropsy. These were analyzed for vitamin B_{12} by the method outlined by the USP (8). Results (table 4) indicated the following: 1) animals raised with a vegetable protein diet deficient in vitamin B_{12} had low levels of vitamin B_{12} when compared with the control animals; 2) the addition of X-methyl folic acid to the diet of vitamin B_{12}-deficient animals did not cause a further reduction in liver levels of the vitamin; this suggested that the folic acid antagonist might exert a systemic action rather than inhibit the intestinal synthesis of vitamin B_{12}; 3) there were no differences in liver levels of vitamin B_{12} between hydrocephalic and nonhydrocephalic littermates; and 4) there were no differences noted in vitamin B_{12} levels between deficient newborn animals from hydrocephalic litters and nonhydrocephalic litters. It should be noted that animal no. 1 from the vitamin B_{12}-deficient adult group gave birth to only one hydrocephalic litter, whereas animal no. 2 gave birth to numerous hydrocephalic litters. The liver levels of vitamin B_{12} were essentially the same in all deficient animals.

Experimental trial 3

In contrast with the 30% protein diets used in trials 1 and 2, the basic ration of trial 3 contained only 20% protein. Three separate control groups were used. The first control group was fed the basic 20% protein diet supplemented with choline and vitamin B_{12} (diet 1, table 5). The second was fed a choline-deficient and vitamin B_{12}-supplemented diet (diet J). The third was fed a vitamin B_{12}-deficient and choline-supplemented diet (diet K). A fourth group was given diet K and, in addition, received intraperitoneally 50 μg of the vitamin B_{12} antagonists (monobasic acids of vitamin B_{12})/week (total of 0.9 mg/animal). Other groups were given rations supplemented with sarcosine hydrochloride, DL-methionine, and DL-homocysteine (diet L); DL-methionine and ethanolamine (diet M); or X-methyl folic acid (diet N). One mole of DL-methionine and 2 moles of sarcosine hydrochloride were considered to have methyl groups equivalent to 1 mole of choline. Three moles of DL-methionine were considered equivalent to 1 mole of choline in methyl groups.

Female animals receiving the vitamin B_{12} antagonists intraperitoneally gave birth to young which were normal in all respects. For this reason, this group of animals was included in table 5, with those receiving the vitamin B_{12}-deficient diet but not receiving the antagonist. No congenital abnormalities were detected grossly in the animals receiving the vitamin B_{12}-deficient ration supplemented with choline. A high incidence of congenital malformations was noted when either sarcosine and methionine or methionine alone was substituted for choline. The addition of X-methyl folic acid to the choline-deficient, vitamin B_{12}-deficient ration caused the adults to lose weight and to become infertile (see table 5).

When the protein content of the basal diet was reduced from 30 to 20%, the females receiving the choline-deficient but methionine - supplemented rations gave birth to offspring which had multiple con-

TABLE 4

Vitamin B_{12} analysis (trial 2)

Group	Litter	Liver vitamin B_{12}
		mμg/g of liver
Adult animals		
Control (D)		143–202
Vitamin B_{12}-deficient (subgroup 1-a)		6–7
Vitamin B_{12}-deficient with X-methyl folic acid added (subgroup 1-b)		9–10
Newborn animals		
Vitamin B_{12}-deficient newborn animals from dam in subgroup 1-a	hydrocephalic	1–2
	nonhydrocephalic	3–4

TABLE 5

Incidence of hydrocephalus (trial 1)

Diet (Ingredients added to basal diet 2, see table 1)		No. females fed diet	No. litters born	Litters with hydrocephalic young	No. newborn animals	Newborn animals hydrocephalic
	mg/kg diet			%		%
Diet I		8	8	0	42	0
choline bitartrate	1814					
vitamin B_{12}	0.05					
Diet J		8	7	0	64	0
vitamin B_{12}	0.05					
Diet K		16	7	0	64	0
choline bitartrate	1814					
Basal diet 2 (vitamin B_{12}- and choline-deficient)		8	3	33	16	25
Diet L		16	7	43	36	22
DL-methionine	1069					
sarcosine·HCl	1312					
DL-homocysteine	1452					
Diet M		16	8	38	65	8
DL-methionine	3207					
ethanolamine	440					
Diet N		16	0	—	—	—
X-methyl folic acid	10					

genital abnormalities. Hydrocephalus was by far the most common abnormality observed; however, on occasion the hydrocephalic animals had other defects as well. These included hydronephrosis, hydro-ureter, harelip, umbilical hernia, spina bifida, cranioschisis, and skeletal defects.

DISCUSSION

Weissbach et al. (9) demonstrated in partially purified fractions from animal liver that the methyl group of methyl B_{12} can be transferred to homocysteine. Buchanan et al. (10) studied the enzyme system in pig liver and provided convincing evidence that the pig liver enzyme is similar to the bacterial vitamin B_{12}-containing enzyme, which transfers a methyl group from 5-methyl-tetrahydrofolic acid to homocysteine to yield methionine and tetrahydrofolic acid. It was suggested by Herbert and Zalusky (11) that in vitamin B_{12}-deficient subjects, pteroylglutamic acid was converted to a metabolically useful form (probably N-5-methyl-tetrahydrofolic acid), which "piles up" in the serum because vitamin B_{12} is required for its normal utilization. The accumulation of 5-methyltetrahydrofolic acid occurs because the reaction catalyzed by the enzyme 5,10-methylene-tetrahydrofolate reductase probably operates irreversibly under physiological circumstances (10). Therefore, in vitamin B_{12} deficiency there appears to be a block in conversion of 5-methyltetrahydrofolic acid to methionine and a failure to regenerate tetrahydrofolate. The failure to regenerate tetrahydrofolate and its derivatives could cause alterations in purine and thymidine methyl synthesis as well as affect the catabolism of histidine.

It has always been assumed that the teratogenic effects of diets deficient in either folic acid or vitamin B_{12} were the consequence of the participation of these 2 vitamins in nucleic acid metabolism. The results of the experiments presented here show that deletion of choline from a vitamin B_{12}-deficient diet greatly increases the incidence of congenital abnormalities in neonatal rats. Choline in the form of phospholipids is important as a structural component of cells, in lipid transport be-

tween tissues, and probably in the metabolism of fatty acids in the liver. Methionine in amounts equivalent to choline in methyl groups would not offer the same protective effects. Thus, a choline-containing compound was probably a critical metabolic intermediate responsible for preventing the congenital abnormalities observed. Vitamin B_{12} could increase the availability of this intermediate so that the demands of pregnancy could be satisfied. It is still possible, however, that a deficiency of choline caused more methyl groups to be utilized for the synthesis of the missing metabolite and that this in turn decreased the methyl groups available for other functions such as purine and pyrimidine synthesis.

The sarcosine diets gave a high incidence of hydrocephalus; however, it was probably not different from that observed with methionine. The data were not inconsistent with the presence of a vitamin B_{12}-containing enzyme in the terminal chain of methionine synthesis from methyltetrahydrofolic acid; however, there were implications that the action of the vitamin may be more involved. This idea is supported by the synergistic action of X-methyl folic acid and vitamin B_{12}. If it is assumed that the X-methyl folic acid is acting as a systemic folic acid antagonist, then it is plausible to postulate that vitamin B_{12} and folic acid gave some independent action.

The feeding of an intestinal antibiotic, the injection of vitamin B_{12} antagonists, and the measurement of liver levels of vitamin B_{12} all indicated that factors other than a deficiency of vitamin B_{12} are concerned with production of congenital hydrocephalus in neonatal rats. The fact that the incidence and severity of lesions are increased when vitamin B_{12}-deficient diets are made deficient in choline or when X-methyl folic acid is added stresses the role of this vitamin in 1-carbon metabolism. Further research must be conducted to determine whether the primary metabolic aberration is in nucleic acid synthesis or is concerned with choline per se.

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