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Research Article

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EFFECT OF FOLIC ACID DEFICIENCY ON ABSORPTION OF Co^{60}-LABELED VITAMIN B_{12} BY RATS *

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Deficiency of vitamin B_6 but not of thiamine, riboflavin, or pantothenic acid brings about an impairment of the gastrointestinal absorption of vitamin B_{12}-Co^{60} (1). Will and co-workers (2) recently reported a comprehensive study on the clinical and biochemical effects of vitamin B_{12}, folic acid, and folinic acid in patients with pernicious anemia. The authors presented evidence suggesting that vitamin B_{12} influences folic acid metabolism by releasing folinic acid from its tissue conjugates, by facilitating the reduction of folic acid to folinic acid, or by promoting the formation of the N^{10}-formyl derivative of reduced folic acid. The above findings on the intimacy of the metabolic role of these two vitamins led us to this study on the influence of folic acid deficiency on absorption of Co^{60}-labeled vitamin B_{12} by rats.

EXPERIMENTAL METHODS

Young rats (6 to 8 weeks old), of both sexes of the McCollum strain, were used in this study. They were housed in individual screen-bottom cages in an air-conditioned room and offered food and water *ad lib.* The basal diet (Table I) contained 2 per cent sulfasuxidine and was adequate in all known nutrients except folic acid (diet BDF). In one experiment two groups of animals were used. Group A was given intraperitoneal injections of an aqueous solution of aminopterin (10 μg per 0.25 ml water) three times per week in order to hasten the development of folic acid deficiency. The animals in group B, used as controls, received the supplement of folic acid (30 μg per 0.25 ml water) by intraperitoneal injection every other day. Other experiments differed from this only in that folic acid deficiency was induced solely by dietary deprivation and without the injection of its antagonist. For the purpose of testing the effect of sulfasuxidine on the absorption of vitamin B_{12}, half of the animals in still another experiment were kept on the basal diet. The other half received the same ration, except that the amount of sulfasuxidine in the basal diet was quantitatively replaced by sucrose. In addition, our stock diet supplemented with sulfasuxidine was also employed for the same purpose.

Upon the onset of the deficiency syndrome, both deficient and folic acid-treated animals were placed individually in metabolism cages and provided with the basal diet and water. Control rats received injections of folic acid as usual. After overnight fasting of at least 16 hours, each rat received orally 50 mμg of Co^{60}-labeled vitamin B_{12} with a stomach tube. The administered dose had specific activity of 1 μc per μg of vitamin B_{12}. The urine and feces specimens were collected separately for 4 to 6 days. The animals were then sacrificed and certain tissues were removed. The radioactivity in the various specimens was measured with a gamma scintillation counter. The details for radiometric estimation of urine, feces, and tissue specimens have been previously reported (1).

TABLE I

Composition of the folic acid-deficient basal diet

Ingredients	%
Vitamin-free casein	20.0
L-cystine	0.5
Sucrose	67.5
Salt mixture (3)	4.0
Sulfasuxidine	2.0
Corn oil	6.0
Total	100.0
Vitamin supplements/100 g diet	
	mg
Vitamin A	1,553 IU
Vitamin D	220 USP
α-tocopherol	2.80
2-methylnaphthoquinone	0.26
Thiamine HCl	0.80
Riboflavin	0.80
Pyridoxine HCl	0.60
Ca-pantothenate	1.40
Niacin	5.00
Biotin	0.50
Para-aminobenzinic acid	15.00
Choline chloride	200.00
Inositol	10.00
Vitamin B_{12}	0.002

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TABLE II

Effect of folic acid deficiency induced by aminopterin on excretion and distribution of orally administered Co^{60}-B_{12}

Group	Treatment	Radioactivity				
		Fecal	Urine	Liver	Kidneys	GI tract
		mμg Co^{60}-B_{12}				
A	Folic acid-def.†	22.2 ± 4.01* [5]	2.61 ± 0.93	2.87 ± 0.75	5.03 ± 1.64	3.55 ± 0.58
B	Folic acid-treated	42.6 ± 0.63 [4]	1.92 ± 0.89	1.13 ± 0.11	0.90 ± 0.02	1.58 ± 0.06
t value‡		3.51	0.53	2.31	2.46	3.11

* Standard error of the mean. Figures within brackets indicate number of rats used.
† 10.0 μg aminopterin in 0.25 ml saline, i.p., thrice weekly.
‡ *t* value above 2.4 is considered to be significant at 5% level.

RESULTS

The results of the urinary and fecal excretion of radioactivity and the radiovitamin uptake by various organs after oral administration of Co^{60}-B_{12} to folic acid-deficient rats induced by the injection of its antagonist and by the controls are summarized in Table II. Data indicate that the amount of radioactivity in the fecal matter of aminopterin-treated rats is approximately one-half that of the folic acid-treated rats. Likewise, the radioactivity in the target organs of deficient animals was greater than that of treated ones by twofold in liver, fivefold in kidneys, and twofold in the gastrointestinal (GI) tract. These differences are statistically significant at a 5 per cent level with 7 degrees of freedom. The difference in the urinary excretion, however, did not reach the 5 per cent level. These findings, taken as a whole, may be interpreted as an increase of absorption of Co^{60}-B_{12} by aminopterin treatment.

In order to substantiate that the effect of aminopterin on vitamin B_{12} absorption is due to folic acid deficiency and not to its pharmacological properties, this vitamin deficiency was produced by feeding 8-week old male rats BDF alone for 10 weeks. These animals (A) as well as our folic acid-treated ones (control B), were then given the oral test for the study of vitamin B_{12} absorption. The results are shown in Table III. In general, deprivation of dietary folic acid for 10 weeks resulted in a slightly, but significantly smaller magnitude of weight gain (36 ± 2.8 for group A vs 49 ± 4.1 g for group B) and a marked reduction of leukocyte counts (2,166 ± 89 vs 9,925 ± 285 per mm^3), for the deficient and treated groups, respectively. Data given in Table III showed that these folic acid-deficient animals absorbed more Co^{60}-B_{12} than did the treated ones; that is, the concentration of radioactivity in fecal matter of deficient rats is less than that of the control animals by 50 per cent. On the other hand, the urinary excretion of Co^{60}-B_{12} was increased approximately fivefold in deficient rats

TABLE III

Effect of folic acid deficiency on excretion and distribution of orally administered Co^{60}-B_{12}

Group	Treatment	Radioactivity					Serum B_{12}
		Feces	Urine	Liver	Kidneys	GI tract	
		mμg Co^{60}-B_{12}					*μμg/ml*
A	Folic acid-def.	16.8 ± 3.25* [5]	3.40 ± 0.50	7.18 ± 0.73	4.53 ± 0.27	9.13 ± 1.05	421 ± 33.5
B	Folic acid-treated	38.4 ± 1.58 [5]	0.67 ± 0.23	0.99 ± 0.25	1.47 ± 0.69	1.54 ± 0.56	624 ± 40.8
t value		3.43	4.96	8.68	4.36	6.18	3.80

* See footnotes to Table II.

as compared with the controls. It should be pointed out that the serum B_{12} level of the deficient animals (see Table III) is lower than that of the treated ones, in spite of the greater absorption and the abundance of B_{12} in the diet. This observation might be due to a reduction of food intake which usually accompanies nutritional deficiency or a faster turnover of vitamin B_{12}.

The following experiments, therefore, were conducted to determine whether dietary intake of vitamin B_{12} affects the apparent absorption of vitamin B_{12}. In the first study, 10 adult male rats with an average body weight of 200 ± 10 g (group A) from our stock colony were fed a basal casein diet containing 10 μg added vitamin B_{12} per 100 g diet, and another group of 10 comparable rats was fed a basal soybean diet (group B) essentially devoid of vitamin B_{12}, both *ad lib.* Two months later, these animals were given an oral absorption test of radioactive vitamin B_{12} (50 mμg per rat). It was found that these two groups of animals excreted in the feces 18.8 ± 0.6 and 18.0 ± 0.8 per cent of the total dose, respectively. Four days after feeding, their organs were removed for radioactivity measurement as well as for the determination of vitamin B_{12} content in the plasma. It was found that, in spite of the marked difference in the B_{12} plasma levels (626 ± 48 for group A and 296 ± 14 $\mu\mu$g per ml for group B) the amounts of radioactive B_{12} in the tissues of the two groups of animals were essentially the same.

In the second study the urinary excretion test of Schilling was performed with two groups of healthy male Mexican subjects, aged 20 to 30 years. One group (C) consumed a diet consisting primarily of corn and vegetable proteins with a small portion of meat per week and a small daily ration of milk. The other group (D) was derived from the same city and their diet consisted of liberal amounts of animal protein (meat and fish) and milk. The B_{12} serum levels of the two respective groups were 180 ± 19 and 320 ± 38 $\mu\mu$g per ml; these results thus reflect the differences in the B_{12} reserve. When these two groups of subjects were given, orally, 5 μg radioactive vitamin B_{12}, followed by 1 mg unlabeled vitamin B_{12} by injection 2 hours later, the total radioactivity in the 24-hour urine specimens of the two groups was 288 ± 14 and 341 ± 46 mμg, respectively. Again there was no difference in absorption as measured by the urinary excretion test. These results, therefore, demonstrate that the absorption of vitamin B_{12} is essentially independent of the dietary history and vitamin B_{12} status of the tissue.

We have studied further the effect of differences in the amount of vitamin B_{12} intake in the diet on the apparent absorption of radioactive vitamin B_{12}. To this end, a group of male rats was given a daily ration of 5 g casein diet for 20 days. Another group was given the same diet *ad lib.* (approximately 15 g per day). After 20 days an oral test dose of 50 mμg of radioactive B_{12} was given. Four days later the radioactivity in the 24-hour urine, feces, and liver was determined. It was found that the absorption of vitamin B_{12} by these two groups of animals was not statistically different.

Since our basal diet contains 2 per cent sulfasuxidine, one may argue that this drug rather than folic acid deficiency enhances the absorption of vitamin B_{12}. To rule out such a possibility, the effect of sulfasuxidine supplementation to two types of basal diets (namely, BFD and our stock diet) was studied. To this end, 20 female rats, with an average weight of 140 g, were randomly distributed into four groups of five each. Thus, the first two groups were offered BDF with or without sulfasuxidine. The third and fourth groups were placed on our stock diet again, with or without sulfasuxidine. At the end of a 3-week period all the animals were given the oral test for vitamin B_{12} absorption according to the procedure described above. The results shown in Table IV demonstrate that supplementation of the diet with

TABLE IV

Effect of sulfasuxidine on distribution of orally administered Co^{60}-B_{12}

Diet	Supplement	Radioactivity		
		Liver	Kidneys	Intestines
		mμg Co^{60}-B_{12}		
Folic acid-def.	None [5]	3.20 ± 0.33	3.28 ± 0.11	6.42 ± 0.14
	Sulfasuxidine [5]	3.33 ± 0.30	3.14 ± 0.18	4.18 ± 0.25
Stock (4)	None [5]	1.98 ± 0.80	1.42 ± 0.30	3.44 ± 0.90
	Sulfasuxidine [5]	1.97 ± 0.18	2.12 ± 0.24	6.49 ± 1.18

TABLE V

Comparison of the Co^{60}-B_{12} uptake in organs of folic acid-deficient and -treated rats after its injection into intestinal loop *

Treatment	Final weight <	Leukocyte	Radioactivity		
			Intestinal loop	Liver	Kidneys
	g	*mm³*	*mμg Co^{60}-B_{12}*		
Folic acid-def.	149 ± 5.7* [5]	3,200 ± 490	167.9 ± 5.4	11.2 ± 3.4	3.35 ±0.6
Folic acid-treated	238 ± 7.4 [5]	10,800 ± 485	165.1 ± 10.3	14.5 ± 3.5	5.07 ± 4.3
t value	8.9	10.3	0.3	1.1	0.6

* See footnotes to Table II.

2 per cent sulfasuxidine did not influence the uptake of the radioactive vitamin B_{12} in liver, kidneys, or intestinal tract in rats. This was true in both groups kept on folic acid-depleted diet and our stock diet. However, the uptake of Co^{60}-B_{12} by liver of animals on stock diet was lower than that of the animals on the BFD.

The above data, demonstrating a decrease in fecal excretion and an increase in the radioactivity of the tissue of the folic acid-deficient animals, may be due to an increase in gastrointestinal absorption or perhaps due to the reduction of vitamin B_{12} reserve resulting from the decrease in dietary intake. The latter possibility seems to be unlikely according to the results of yet another type of experiment. In this study, two groups (A and B) of six adult female rats (150 to 200 g) were pair-fed the folic acid-deficient diet (6 g per day per rat) for 6 weeks. Folic acid solution was injected into group A but not group B. The radioactivity in the feces was measured after the oral administration of 50 mμg of radioactive vitamin B_{12} and was found to be 38.2 ± 1.1 (for A) and 18.6 ± 1.7 (for B) mμg. Thus, the same difference was observed in spite of the identical dietary intake. In other experiments, weaning rats were fed a basal diet consisting, in per cent, of vitamin-free casein, 20; salt mixture, 4; fat, 2; and sugar, 74. One group received a supplement of all vitamins except B_6; another group, except B_1; still another group, except folic acid; and a last group, a complete vitamin supplement. Six weeks after feeding, the absorption of vitamin B_{12} by all four groups was measured. It was found again that the folic acid-deficient rats excreted significantly less vitamin B_{12} in feces than did any other group, whereas the B_6-deficient group excreted more than any other group. The groups provided with the complete vitamin supplement excreted the same amount as those with vitamin B_1 deficiencies. The average body weight of all animals on vitamin-deficient diet was approximately the same and was only one-half that of the group on complete vitamin supplement.

To determine whether the increase in absorption of vitamin B_{12} in folic acid-deficient rats is attributable to any alteration of the physical nature of intestinal mucosa, a loop technique described by Okuda (5) was used on five deficient rats and an equal number of controls. After overnight fasting, their abdominal cavities were exposed under light ether anesthesia. An intestinal loop was made by connecting the upper end of the jejunum and the lower end of the ileum with a piece of polyethylene tubing. The openings at the end of the duodenum and cecum were also joined to make a temporary connection in the digestive system. The contents of the intestinal loop were flushed with a warm physiological saline solution in order to clean out any intrinsic factor that might affect the absorption of Co^{60}-B_{12}. A dose of 200 mμg radioactive vitamin B_{12} was injected into the loop of each animal. Precaution was taken to prevent any leakage of radioactive material into the peritoneal cavity during the process of administration. Animals were sacrificed 24 hours after surgery and the radioactivity in the intestinal loop, liver, and kidneys was measured. The results tabulated in Table V show that radioactivity in the intestinal loop of folic acid-deficient rats was essentially the same as that of rats supplemented with folic acid. The concentration of activity in the liver and kidneys

was slightly but insignificantly lower in deficient animals.

DISCUSSION

The above findings demonstrate that rats with syndromes of folic acid deficiency induced by the administration of its antagonist (aminopterin) or by dietary deprivation in the presence of sulfasuxidine absorbed more vitamin B_{12} than did control rats treated with folic acid. One may argue that the alteration in vitamin B_{12} absorption is a result of a decrease in food intake by deficient rats. However, Yeh (6) showed that semistarvation for 10 days did not affect the absorption of vitamin B_{12} by rats. Furthermore, pyridoxine deficiency, which results likewise in a decrease of food intake, brings about a reduction of absorption of orally administered vitamin B_{12} (1). On the other hand, deficiencies of other vitamin B complexes such as thiamine, riboflavin, or niacin which also reduces food intake, have no influence on the absorption of the vitamin by rats. More recently Sauberlich (7) has observed that the rats fed choline retained less of the injected radioactive vitamin B_{12} in their organs than did the choline-deficient animals. All of these observations, taken as a whole, lead us to believe that the alteration of absorption of vitamin B_{12} is due to a specific vitamin deficiency, and, therefore, are in harmony with our hypothesis that deficiency of one vitamin can alter the absorption of another. This observation is particularly interesting, since deficiency of folic acid brings about an increase in efficiency of absorption which did not occur with other deficiencies.

It has been reported that deficiency of folic acid can produce certain damage in intestinal mucosa (8, 9). However, our studies on absorption of vitamin B_{12} (Table V), with the intestinal loop technique, failed to reveal any appreciable difference in radioactivity found in the loop, liver and/or kidneys of folic acid-deficient and -treated animals. Therefore, the epithelial damage induced by folic acid deficiency, if its exists, plays no role in the increased absorption of vitamin B_{12}.

SUMMARY

Folic acid deficiency in rats induced by the administration of antagonist or by dietary deprivation and sulfasuxidine feeding brings about an increase in absorption of orally administered Co^{60}-labeled vitamin B_{12} as measured by the fecal excretion test or by organ uptake method. However, prolonged deficiency of this vitamin results in a lower B_{12} serum level. When Co^{60}-B_{12} is introduced into the intestinal loops of folic acid-deficient or -treated rats, it was absorbed to the same extent. These data demonstrated no absorption defect in the intestine due to folic acid deficiency.

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