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INTERRELATIONS OF VITAMIN B_{12} AND FOLIC ACID METABOLISM: FOLIC ACID CLEARANCE STUDIES *

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The studies of many investigators have led to a unified concept of the megaloblastic anemias as a single morphologic entity due to defective nucleoprotein synthesis from various causes. The vast majority of patients with megaloblastic anemia have been found to have deficiency of vitamin B_{12}, of folic acid, or of both vitamins. For this reason, the possible interrelations of these two vitamins have long piqued the curiosity of investigators (1, 2).

Orally administered or injected pteroylglutamic acid (PGA) (folic acid) has been reported to disappear rapidly into the tissues of vitamin B_{12}-deficient patients, as manifested by rapid disappearance of *Streptococcus faecalis* activity from serum and urine (3–6).

The purpose of the present investigation was to determine whether the rapid disappearance of folic acid activity for *S. faecalis* from the serum of subjects with pernicious anemia reflects tissue depletion of folic acid, as believed by prior investigators, or instead indicates inadequate utilization of folic acid due to vitamin B_{12} deficiency. Prior results of part of these studies (7–10) suggest that the latter is the case. Incidental to these observations, the effect of intravenously administered PGA on serum vitamin B_{12} and on erythrocyte folic acid activity was determined.

The studies here presented elaborate on our preliminary reports (7–10), indicating that folic acid activity "piles up" in human serum in the presence of vitamin B_{12} deficiency. The accumulation of this folic acid activity (probably N^5-methyl-tetrahydrofolic acid) provides direct evidence of deranged folic acid metabolism due to vitamin B_{12} deficiency. This folic acid-vitamin B_{12} interrelationship may explain much of the confusion in therapy of pernicious anemia, as well as the fact that the anemias of vitamin B_{12} and folic acid deficiencies are hematologically identical.

MATERIALS AND METHODS

Synthetic pteroylglutamic acid[1] was diluted with saline to a concentration of 1 mg per ml. The concentration was proven by microbiologic assay with both *S. faecalis* and *Lactobacillus casei,* and the solution was stored at 4° C in sterile light-tight bottles.

Procedure. The subjects of the investigation were given 15 μg of PGA per kg of body weight by rapid intravenous injection (5). Blood samples were obtained at zero time (immediately before) and at 3, 8, 15, 30, 60, 120, 240, and 1,440 minutes (24 hours) after the injection. Serum samples were obtained in plain Vacutainers[2] and whole blood samples in heparinized Vacutainers.

Estimations of folic acid activity in serum and erythrocytes. These were carried out by microbiologic assay with *L. casei* and *S. faecalis* as previously reported (8), using both the "standard method" (150 mg per 100 ml ascorbate) and the "aseptic addition method" (1 g per 100 ml ascorbate) (8). The latter method has the advantages of halving the manipulations involved in preparing an assay, and allowing assay of 0.1 ml of serum. (Strict asepsis is not necessary, since *L. casei* grows so rapidly that we have never observed growth of a contaminant.)

In our laboratory, serum *L. casei* values of < 3 mμg per ml are considered indicative of folic acid deficiency; values of 3 to 4.9 mμg per ml are strongly suggestive of folic acid deficiency; values of 5 to 6.9 mμg per ml are diagnostically indeterminate; values of 7 to 15.9 mμg per ml are normal; values of 16 to 24.9 are suggestively elevated and may indicate folic acid ingestion by normal subjects; and values > 25 mμg per ml have never been found in normal subjects unless they were ingesting vitamin tablets containing folic acid.

The folic acid activity of 1 ml of erythrocytes was determined using the same methodology (8) previously applied to serum. "Reticulocyte-rich" and "reticulocyte-

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[1] Folic acid-Folvite, a solution of pteroylglutamic acid, 15 mg per ml, generously provided by Drs. T. H. Jukes and E. L. R. Stokstad of Lederle Laboratories, Pearl River, N. Y.

[2] Becton, Dickinson & Co., Rutherford, N. J.

poor" erythrocytes were prepared as follows. On the eighth day of therapy with 30 μg of vitamin B_{12} daily, 100 ml of blood was obtained from Subject 5 and centrifuged. The buffy coat was discarded and the red cells were thrice washed in 0.9 per cent NaCl, discarding the topmost layer of "residual buffy coat." The middle half of the erythrocyte layer was again centrifuged to yield a "reticulocyte-rich" top layer (26 per cent reticulocytes) and a "reticulocyte-poor" bottom layer (6 per cent reticulocytes); 0.2-ml aliquots of erythrocytes were then assayed by our "standard method" (8).

Serum vitamin B_{12} levels. These were determined using *Euglena gracilis, var. bacillaris,* by the method of Lear, Harris, Castle and Fleming (11), with various trivial modifications. In our laboratory, values < 120 $\mu\mu g$ per ml are low; values of 121 to 200 $\mu\mu g$ per ml are indeterminate; values of 200 to 900 $\mu\mu g$ per ml are normal; and values > 900 $\mu\mu g$ per ml are high.

Erythrocyte vitamin B_{12} levels. The methodology of Spray (12) for extracting vitamin B_{12} from serum was applied to extracting the vitamin from erythrocytes. One ml of erythrocytes, 1 ml of 0.4 N acetate buffer (pH 4.5), 0.4 ml of 0.1 per cent NaCN, and 17.6 ml distilled water were autoclaved together at 118° C for 15 minutes. After cooling and centrifugation, the supernate was assayed as if it were serum (vide supra).

In other studies (13), this extraction procedure was shown to remove approximately 81 per cent of the total vitamin B_{12} radioactivity from 1 g of liver obtained at sacrifice of a baby pig who had been given daily injections of radioactive vitamin B_{12} for almost 2 months. After the final injection and before sacrifice, there was a rest period of 5 days to allow equilibration of the last injections of radioactive vitamin B_{12} with tissue vitamin B_{12}. (The extract contained 81 per cent and the precipitate contained 19 per cent of the total liver radioactivity.) A similar efficiency of extraction is assumed for erythrocytes, although we are not aware of studies using a radioactive marker to demonstrate this probability.

Estimation of formiminoglutamic aciduria. After ingestion of 20 g of L-histidine, urine was collected for 12 hours in a bottle containing 2 ml of concentrated HCl, and the quantity of formiminoglutamic acid (FIGLU) was estimated by a modification (14) of the electrophoretic method of Knowles, Prankerd and Westall (15).

Clinical and laboratory criteria for diagnosis (2). Vitamin B_{12} deficiency: hematologic morphologic abnormalities in the peripheral blood (macroovalocytes and hypersegmented polymorphonuclear leukocytes) and bone marrow (megaloblasts and giant metamyelocytes); serum vitamin B_{12} level < 100 $\mu\mu g$ per ml.

Folic acid deficiency: same morphologic criteria as for vitamin B_{12} deficiency; serum folic acid activity for *L. casei* < 3 $m\mu g$ per ml (except in the presence of concomi-

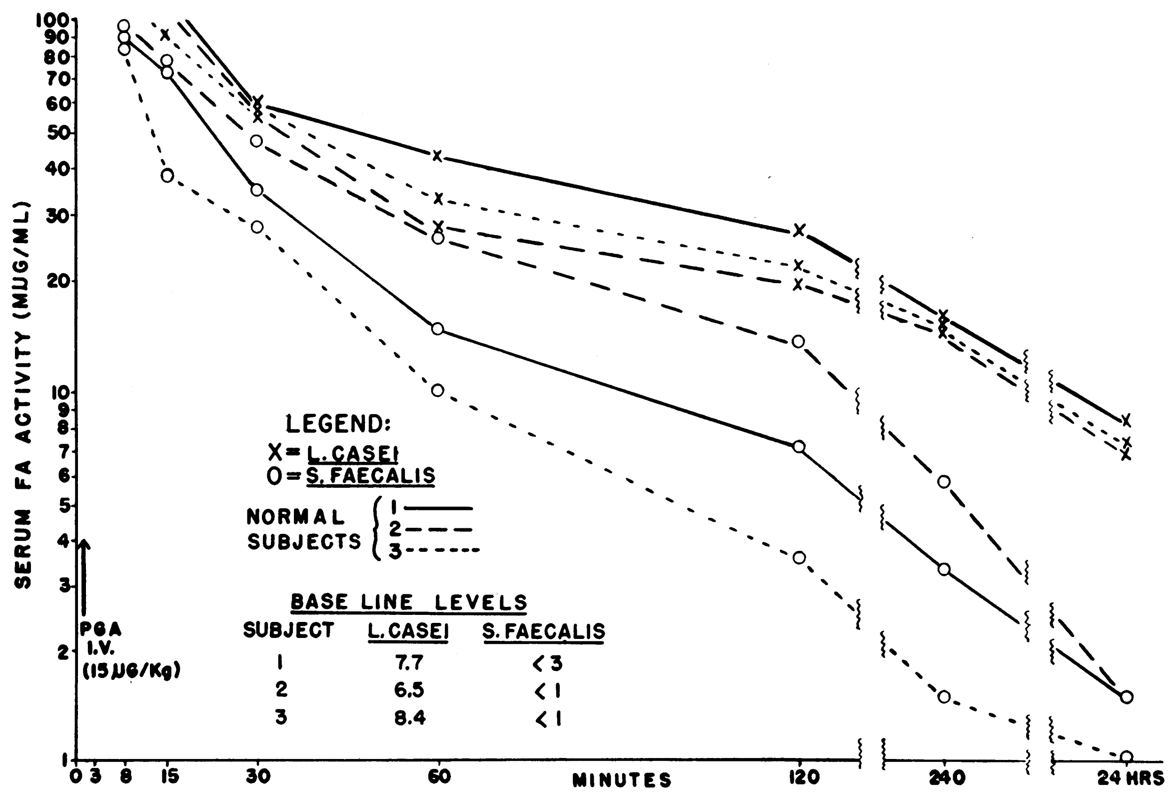

FIG. 1. FOLIC ACID CLEARANCE IN NORMAL SUBJECTS.

TABLE I

*Pteroylglutamic acid disappearance studies**

					Serum folic acid activity ($m\mu g/ml$)																	
					S. faecalis									*L. casei*								
Subjects	PGA dose	Hct	FIGLU	Serum B_{12}	0 min	3 min	8 min	15 min	30 min	1 hr	2 hrs	4 hrs	24 hrs	0 min	3 min	8 min	15 min	30 min	1 hr	2 hrs	4 hrs	24 hrs
	mg		*mg/ 12 hrs*	*μμg/ml*																		
Normal																						
1	1.02	40.0		277	<3	>100	90	74	35	15	7.2	3.4	<3	7.7	>300	>300	>100	60	43	28	16	8.6
2	1.19	40.0		560	<1	>100	98	77	47	26	14	5.8	<3	6.5	>300	>100	>100	57	27	20	15	7.0
3	0.99	41.0		472	<1	>100	85	38	28	10.2	3.6	1.5	<1	8.4	>100	>100	92	57	33	22	15	7.2
Megaloblastic anemia due to vitamin B_{12} deficiency																						
4	0.82	13.0	939	28	1.8	80		4.3	3.0	2.5	3	3.1	3.2	8.5	92		25	19	16	17	16	18
4a	0.82	33.2	15	59	<1	87	19	4.1	<1	<1.1	<3	<1	3	7.2	97	50	29	20.5	19.5	19	24	12.5
5	1.33	13.8	86	18	<1	58	9.9	3.2	1.3	<1	<1	<1	1.3	35	>300	240	165	94	104	62	47	33
5a	1.33	33		692	3.7	>300	240	60	18	6.7	5		3.8	7	>300	212	58	33	26	23		8.4
6	1.0	23.0		73	<3	82	14	4.6	<3.4	<3	<3			16	>220	60	59	38	39	41		
6a	1.0	35.5			<1	155	55	16	3.8	<1	<1			7.4	>300	>240	64	31	25	28		
Megaloblastic anemia due to folic acid deficiency																						
7	0.436	25		371	2.4	36	15.5	6	1.5	1.5	2.4		2.7	1.3	40	16.5	8.8	2.9	3.2	2.3		3.4
8	0.61	30	207	>3,014	<3	74	19	5.4	<3	<3	<3	<3	<3	2.3	88	32	9.6	4.7	4.1	3.7	3.9	3.9
9	0.75	24.2	21	>1,352	<1.8	71	22	5.1	<1.8	1.0	1.8	1.8	1.8	1.5	90	28	7.7	2.9	2.8	3.4	3.3	1.5
Vitamin B_{12} deficiency without overt anemia																						
10	1.125	45		68	<1	140	78	44	18.5	7.6	1.6	<1	<1	21	250	120	96	80	61	55	30	12
Megaloblastic anemia due to deficiencies of both vitamin B_{12} and folic acid																						
11	0.612	25	2,166	56	1.9	91	20	2.7	1.6	1.1	1.2	1.5	1.8	1.3	160	21	4.3	2	3.3	4.3	3.5	2.8
12	0.75	14	177	59	<1	9.4	1.1	<1	<1	<1	<1	<1	<1	4	29	14	7.8	7	7.4	7.9	7.2	6.9

* PGA = pteroylglutamic acid; FIGLU = formiminoglutamic acid.

tant vitamin B_{12} deficiency, which may raise the serum folic acid activity above 3 mμg per ml).

Pernicious anemia: anemia due to vitamin B_{12} deficiency caused by idiopathic lack of adequate intrinsic factor secretion.

RESULTS

Normal subjects. In three normal subjects, folic acid activity for both *L. casei* and *S. faecalis* remained elevated for at least 4 hours after intravenous injection of PGA, returning to baseline levels some time between 4 and 24 hours after the intravenous dose (Figure 1 and Table I, Subjects 1–3).

Megaloblastic anemia due to folic acid deficiency. In three such subjects, within 30 minutes after the intravenous injection of PGA, an acute rise in serum folic acid activity occurred, *S. faecalis* activity had fallen below 2 mμg per ml, and *L. casei* activity had fallen to below 5 mμg per ml (Figure 2 and Table I, Subjects 7–9).

Megaloblastic anemia due to vitamin B_{12} deficiency. In three such subjects, serum folic acid activity for *S. faecalis* fell to 3 or less mμg per ml of serum within 30 minutes after the acute rise produced by the intravenous PGA injection (Figure 3). However, serum folic acid activity for *L. casei* remained elevated for at least 4 hours after intravenous PGA injection (Figure 3 and Table I, Subjects 4–6).

After the folic acid clearance study, Subject 4, who has been reported elsewhere in connection with his high FIGLU excretion (16), was treated with 5 μg of vitamin B_{12} daily for 7 days, inducing a 51.9 per cent reticulocytosis and hematologic improvement. He was then allowed to relapse and, 2 months after the first clearance study (Table I, Subject 4), a second one (Table 1, Subject 4a) was performed; the results were similar.

Immediately after the intramuscular administration of 30 μg of vitamin B_{12} daily for 18 days, Subject 5 cleared folic acid activity for both organisms at a rate which was relatively normal (Table I, Subject 5a) compared with these clearances prior to therapy (Table I, Subject 5).

Immediately after the administration of 1 mg of vitamin B_{12} intramuscularly daily for 8 days, Sub-

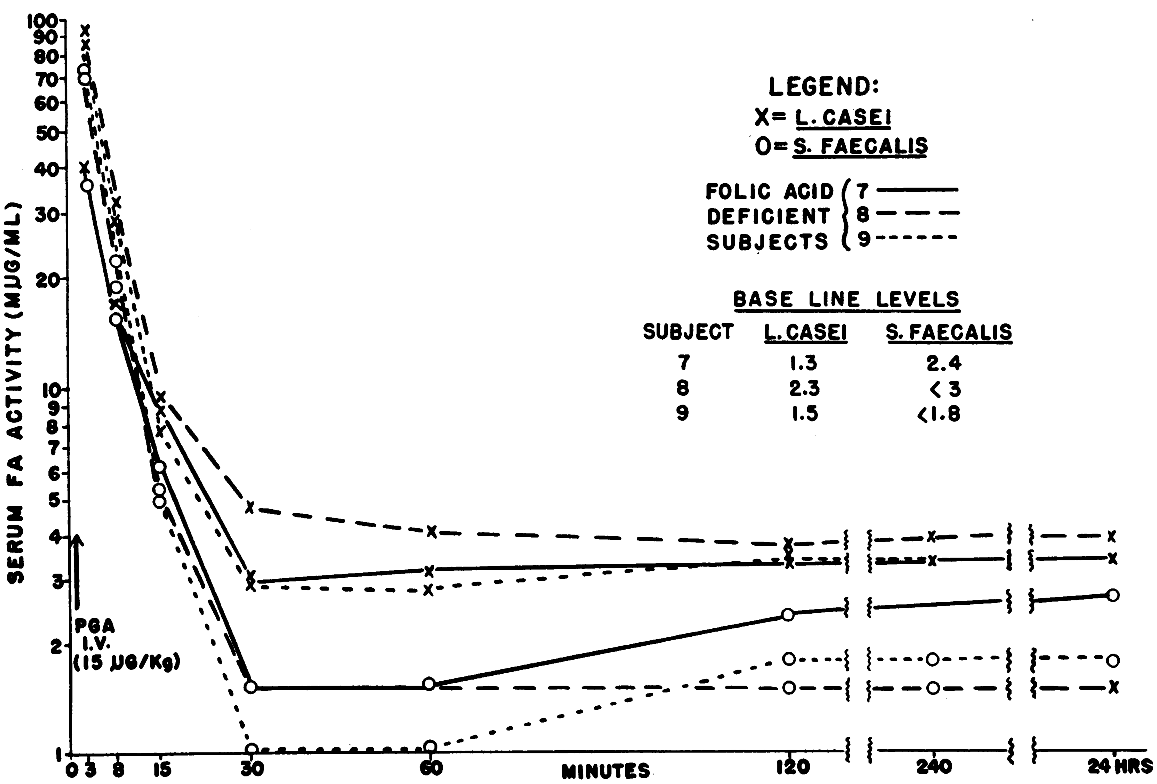

FIG. 2. FOLIC ACID CLEARANCE IN FOLIC ACID-DEFICIENT SUBJECTS.

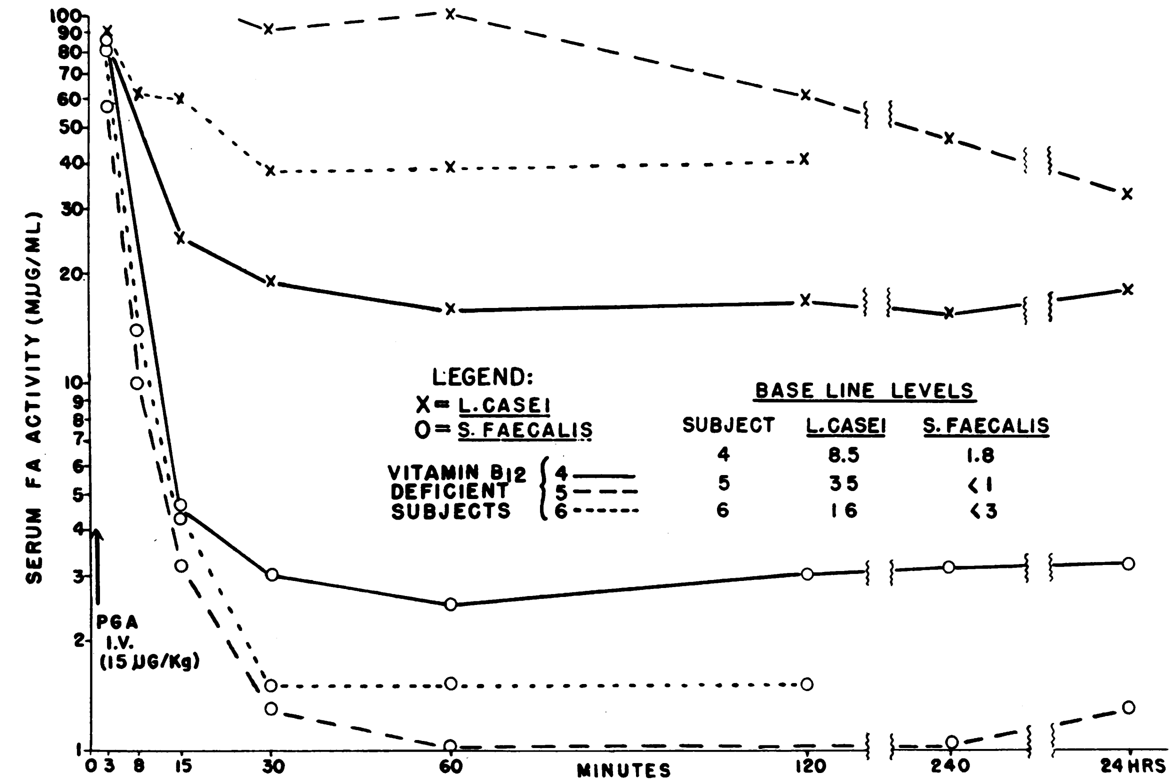

FIG. 3. FOLIC ACID CLEARANCE IN VITAMIN B_{12}-DEFICIENT SUBJECTS.

ject 6 [3] cleared folic acid activity for *S. faecalis* somewhat more rapidly than did normal subjects (Table I, Subject 6a), and cleared folic acid activity for *L. casei* at a rate (Table I, Subject 6a) similar to that prior to therapy (Table I, Subject 6).

Vitamin B_{12} deficiency without overt anemia. An 80 year old white male was studied for 9 months, after routine evaluation prior to cholecystectomy led to discovery of macroovalocytes and hypersegmentation of polymorphonuclear leukocytes in his peripheral blood. His serum vitamin B_{12} level was low but no therapy was given, since it was desired to determine how long it would take for a fall in hematocrit. The disappearance of *S. faecalis* activity from his serum after intravenous PGA was normal, but the disappearance of *L. casei* activity appeared suggestively prolonged (Table I, Subject 10; note 2- and 4-hour levels).

[3] This patient of the Peter Bent Brigham Hospital was made available for study by Drs. Alan Keitt and Stanley Yachnin.

Megaloblastic anemia due to deficiencies of both vitamin B_{12} and folic acid. Two such patients were studied. Subject 11 had idiopathic steatorrhea (nontropical sprue); Subject 12 had pernicious anemia with associated folic acid deficiency (presumably due to protracted anorexia). Subject 11, who had more marked folic acid deficiency, rapidly cleared folic acid activity for both microorganisms from his blood stream. Subject 12, with less marked folic acid deficiency, cleared

TABLE II

Serum folic acid activity for L. casei of 100 consecutive subjects with vitamin B_{12} deficiency characterized by megaloblastic anemia and a serum vitamin B_{12} level < 100 $\mu\mu g$ per ml

No. of subjects	"Folic acid"	Interpretation
	mµg/ml	
9	0 to 2.9	FAD*
24	3 to 4.9	Strongly suggestive of FAD
7	5 to 6.9	Diagnostically indeterminate
34	7 to 15.9	Normal
9	16 to 24.9	Diagnostically indeterminate
17	25 to 83 (Mean: 39)	High

* FAD = folic acid deficiency.

TABLE III

Serial serum vitamin B_{12} levels after intravenous pteroylglutamic acid

Subjects	PGA dose	Hct	Serum "folic acid"	Serum vitamin B_{12} levels ($\mu\mu g/ml$)								
				0 min	3 min	8 min	15 min	30 min	1 hr	2 hrs	4 hrs	24 hrs
Controls	*mg*		*mμg*									
3	0.99	41	8.4	472	835	464	845	581	464	731	632	560
Megaloblastic anemia due to vitamin B_{12} deficiency												
4	0.82	13	8.5	28	30		28	25	26	31	27	29
4a*	0.82	33.2	7.2	51			44			62		57
5	1.33	13.8	35	18	22	22	30	28	19	22	24	21
5a†	1.33	33	7	440	357	416	485	365	307	419		325
6	1.0	23	16	16	22	60	59	38	39	41		
Megaloblastic anemia due to folic acid deficiency												
7	0.436	25	1.3	371	301	321		292	359	304		
8	0.61	30	2.3	>3,200	>3,200		>3,200		>3,200			>3,200
Megaloblastic anemia due to deficiencies of both vitamin B_{12} and folic acid												
11	0.612	25	1.3	56	71		54	51			54	51
12	0.75	14	4	59	57	56	49	51	42	55	51	52

* Subject 4 in a second relapse (see text).
† Subject 5 after therapy with vitamin B_{12}.

TABLE IV

Erythrocyte folic acid activity after intravenous injection of pteroylglutamic acid

Subjects (controls)	PGA dose	Hct	*S. faecalis* ($m\mu g/ml$)									*L. casei* ($m\mu g/ml$)								
			0 min	3 min	8 min	15 min	30 min	1 hr	2 hrs	4 hrs	24 hrs	0 min	3 min	8 min	15 min	30 min	1 hr	2 hrs	4 hrs	24 hrs
	mg																			
2	1.19	40	7.6	8.6	8.6	9	7.8	8.4	7.8	8.6	7.4	44	24	26	30	24	22	28	22	16.4
3	0.99	41	5.6	8.2	8.0	7	7.2		8.2	7.2	16	33	43	39	38	27		23	25	14

S. faecalis activity as rapidly as did subjects with severe folic acid deficiency. Her initial clearance of *L. casei* activity was rapid but then appeared to "plateau" at a level approximately 3 mμg per ml above baseline (Table I).

Baseline serum folic acid activity for L. casei of patients with untreated vitamin B_{12} deficiency. Of 100 consecutive such subjects, none of whom were ingesting vitamin tablets, 17 had initial serum folic acid activity for *L. casei* of 25 mμg per ml or more (Table II). Minimal criteria for characterizing these patients as having vitamin B_{12} deficiency were the presence of a megaloblastic anemia and of a serum vitamin B_{12} level < 100 μμg per ml.

Serial serum vitamin B_{12} levels after intravenous PGA administration. These showed no significant pattern of increase or decrease (Table III).

Erythrocyte folic acid activity for L. casei. No significant measurable increase in erythrocyte folic acid activity followed the standard intravenous injection of PGA (Table IV), suggesting that the mature erythrocyte is relatively impermeable to folic acid.

Folic acid activity (and vitamin B_{12} activity) was much higher in "reticulocyte-rich" than in "reticulocyte-poor" erythrocytes obtained during vitamin B_{12} therapy for pernicious anemia (Table V), suggesting the relative permeability to folic acid and vitamin B_{12} of young erythrocytes. [It has previously been observed (17) that there is an increased concentration of radioactive vitamin B_{12} in the stroma protein of erythrocytes during active blood regeneration in anemia in dogs.] *L. casei* folic acid activity of leukocytes appears to be higher than that of erythrocytes (18).

Effect of specific therapy with vitamin B_{12} on serum folic acid activity for L. casei. Table VI

TABLE V

Concentration of vitamin activity in reticulocytes

Subject 5 (day 8 of vitamin B_{12} therapy)	"Folic acid"	Vitamin B_{12}
	mμg/ml	*μμg/ml*
Serum	5.8	891
"Reticulocyte-rich" erythrocytes (26% reticulocytes)	273	2,004
"Reticulocyte-poor" erythrocytes (6% reticulocytes)	29	621

TABLE VI

Effect of vitamin B_{12} therapy on serum folic acid activity for L. Casei

Subject	Before therapy		B_{12} therapy (i.m.)	Reticulocyte peak		Serum FAA* during hematologic response							
						Day†							
	Hct	Serum B_{12}			Day†	0	1	2	3	4	5	6–10	11–51
		μμg/ml	μg/day	%		mμg/ml		mμg/ml		mμg/ml		mμg/ml	mμg/ml
13	15	23	1 × 9‡	18	6	7		6.8	7.8		8.4	13.3[6]§; 10.1[7]	
14	18.5	12.5	1 × 11	12.2	9	8.6	7.2	7.3	5.8	6.4	15.3	8.2[7]; 9[8]; 9.3[9]; 11.5[10]	12.5[13]
15‖	29	50	1 × 12	6.8	6	28.3						22.7[8]	12.6[21]
16	16.7	26	1 × 26	24.9	6	27	¶						21[13]; 15.3[28]
4	13	28	5 × 7	51.9	6	8.5			3.9			1[7]; 4.1[8]	5[11]; 8.9[26]
21	26	26	10 × 10	16.5	7	8.1	8		5.3	7.3	8.3	8.9[7]; 7.2[8]; 6.5[10]	
17	25	20	30 × 7	15.1	7	12	5						3.6[12]
5	13.8	18	30 × 20	40.7	5	35						5[6]; 5.8[7]	7[19]; 11[22]
22	20	24	50 × 6	9.5	7	9.4	4.2	3.4	4.5	3.7			
20**	28	25	1,000 × 1			25			8				
23	24	33	1,000 × 5	15.5	5	9.8	3.6						
6	23	73	1,000 × 7	19	6	16	5		3.9			7.4[9]	7.4[13]
18	17	38	1,000 × 14	27.8	4	14.5							5[11]; 10.9[51]
19	13.2	42	1,000 × 23	21.2	6	10.4					5.1		

* FAA is folic acid activity for *L. casei*.
† Under day 0 appear the values before therapy; under days 1, 2, 3, etc., are those on days after therapy was begun.
‡ Second number indicates the number of days therapy was given.
§ Numbers in brackets indicate the day the value was determined.
‖ Diagnosis: B_{12} deficiency due to total gastrectomy. In all other subjects, the diagnosis was pernicious anemia (except in Subject 20).
¶ Values during first 24 hours: 26 (5 min); 24 (15 min); 22.3 (30 min); 24.5 (1 hr); 25 (2 hrs); 28.5 (3 hrs).
** Diagnosis: B_{12} deficiency due to regional enteritis with resection of part of the ileum (patient of Dr. Matthew Block, University of Colorado Medical Center).

shows that serum folic acid activity falls slowly during specific therapy with daily doses of 1 μg of vitamin B_{12}, but may fall relatively sharply with larger doses (5 to 1,000 μg).

DISCUSSION

As previously reported by Chanarin, Mollin and Anderson (5), we found that the clearance of folic acid activity for *S. faecalis* from the serum, after injection of 15 μg of PGA per kg of body weight, was rapid in anemic subjects with folic acid deficiency and also in anemic subjects with severe vitamin B_{12} deficiency. This was also noted in a vitamin B_{12}-deficient subject (Subject 6a) after 8 days of administration of 1 mg of vitamin B_{12} intramuscularly daily, when his hematocrit was 35.5, as well as in a patient with pernicious anemia (Subject 4a) in early hematologic relapse, with a hematocrit of 33.2 per cent. Thus, rapid clearance of *S. faecalis* activity may also occur in subjects with moderate vitamin B_{12} deficiency who are only slightly to moderately anemic. However, the clearance of *S. faecalis* activity from the serum of a patient (Subject 5a) with vitamin B_{12} deficiency, after 18 days of therapy with 30 μg of vitamin B_{12} intramuscularly daily, when the hematocrit was 33 per cent, was essentially normal.

Microbiologic assay with *L. casei* also revealed rapid disappearance of folic acid activity from the serum after intravenous injection of PGA in patients with folic acid deficiency. However, in patients with vitamin B_{12} deficiency, serum *L. casei* activity did *not* disappear as fast. In fact, a "plateau phenomenon" may be present, manifested by a tendency for serum *L. casei* activity to remain elevated well above baseline at a fairly constant level for at least 0.5 to 2 hours after the intravenous injection of PGA.

In subjects with vitamin B_{12} deficiency, the combination of rapid clearance of *S. faecalis* activity and slow clearance of *L. casei* activity suggests that in such subjects PGA (which is available to both *S. faecalis* and to *L. casei*) is rapidly converted, perhaps in the liver, to a form only available to *L. casei*. This *L. casei*-active form then appears to "pile up" in the serum, suggesting that vitamin B_{12} is required for its utilization.

During the course of therapy with daily doses of 1 μg vitamin B_{12}, changes in serum folic acid activity for *L. casei* appear to occur very slowly, as may changes in serum iron when folic acid deficiency is treated with 50 μg of pteroylglutamic acid daily (19). When larger daily doses (5 to 1,000 μg) of vitamin B_{12} are used, serum folic acid activity for *L. casei* appears to fall much more sharply, and may reach levels below normal before rising again into the normal range. This drop in serum folic acid activity for *L. casei* may have a meaning similar to the drop in serum iron (2, 20) which occurs during vigorous specific therapy of megaloblastic anemias.

Of the original ten patients with pernicious anemia in whom serum folic acid activity for *L. casei* was measured, one had a value of 43 mμg per ml (21). This value was described in the original report as "high, but of unknown significance." In the present report, we are able to throw some light on the significance of that finding. In the present study, review of 100 consecutive patients with vitamin B_{12} deficiency revealed that 17 had initial serum folic acid activity for *L. casei* of 25 mμg per ml or more, and nine had values of 16 to 24.9 mμg per ml (Table II), despite frequent protracted anorexia, which would be expected to lower such activity. Waters and Mollin (22) have also observed increased serum folic acid activity for *L. casei* in untreated Addisonian pernicious anemia. The majority of our 100 patients had pernicious anemia. Those with serum *L. casei* folic acid activity < 7 mμg per ml frequently had debilitating complications, which may have led to associated anorexia with inadequate ingestion of folic acid, such as chronic genitourinary tract infection, alcoholism, or marked neurologic disability due to past cerebrovascular accident. One patient also had lupus erythematosus. Serum folic acid activity < 7 mμg per ml was also frequent among the patients with vitamin B_{12} deficiency who did not have pernicious anemia. These were mainly patients with gastrointestinal dysfunction due to structural or functional small bowel damage, which may result in malabsorption for folic acid, and included patients with partial small intestine resection, idiopathic steatorrhea, total or subtotal gastrectomy with subsequent malabsorption, and carcinoma with abdominal metastases. Although in presumably normal subjects values of 7 to 24 mμg per ml have been observed, values above 16 mμg per ml are infrequent. These findings, like the PGA clearance studies, suggest a tendency of the *L. casei*-active form of folic acid activity to accumulate in the serum of subjects with vitamin B_{12} deficiency, as does the finding of normal serum *L. casei* activity despite moderately protracted anorexia in many other patients with pernicious anemia (10).

In view of the tendency of *L. casei*-active folic acid activity to accumulate in the serum of subjects with vitamin B_{12} deficiency, it is possible that a low normal value for such activity may be present in the serum of a vitamin B_{12}-deficient subject with folic acid stores inadequate to sustain normal hematopoiesis, just as a normal serum iron level may be present in patients with untreated megaloblastic anemia who do not have iron stores adequate to sustain normal hematopoiesis (2).

Recent studies (23) suggest that most of the *L. casei* activity in human serum is due to a material similar or identical to N^5-methyl-tetrahydrofolic acid (N^5-methyl THFA), the folic acid coenzyme active as an intermediate in methionine biosynthesis (24–29), which requires vitamin B_{12} in order to act (25, 26, 30, 31). Table VII summarizes present knowledge concerning the folic acid activity for microorganisms of various folic acid analogues.

Earlier clinical investigation of patients with vitamin B_{12} deficiency has also provided evidence suggesting that vitamin B_{12} is required for normal folic acid metabolism: *1*) While normally the liver folic acid stores appear to be mainly folinic acid-like material, in vitamin B_{12} deficiency states the stores had appeared to be mainly folic acid (32, 33). However, more recent studies indicate that the bulk of normal liver stores may be N^5-methyl THFA which is only active for *L. casei* (23, 28, 34, 35). It is evident that much of the data in the literature will have to be re-evaluated in the light of this recent work. In severely vitamin B_{12}-deficient sheep, grazing on land deficient in cobalt, liver folic and folinic acid activity for *L. casei* and *L. citrovorum,* respectively, plummet to very low levels (36). *2*) After an oral test dose of PGA, less folinic acid appears in the urine of pernicious anemia patients than in the urine of normal subjects (37). In vitamin B_{12}-deficient subjects previously treated with folic acid, the injection of 1 mg of vitamin B_{12} doubles the urinary folic acid activity excreted (38). *3*) Whole blood folic acid activity for *S. faecalis* appears to be low in one-half of patients with pernicious anemia (39). *4*) Large doses of folic acid will almost invariably induce at least temporary or partial hematologic remission in vitamin B_{12}-deficient subjects (40). Conversely, large quantities of vitamin B_{12} will induce partial hematologic remission in subjects with folic acid deficiency (41). *5*) Formiminoglutamic acid (FIGLU), an intermediate in the catabolism of histidine, found in the urine (sometimes only after an oral dose of histidine) in folic acid deficiency (42, 43), also appears in the urine of some vitamin B_{12}-deficient subjects, sometimes in very large quantities (2, 14, 16, 21, 40, 44, 45), and is generally present in large quantities in the urine of vitamin B_{12}-deficient rats (46) and chicks (47).

TABLE VII

Folic acid activity for microorganisms of various folic acid analogues

	Leuconostoc citrovorum	*S. faecalis*	*L. casei*
Reduced pteroylmonoglutamates (except N^5-methyl)	+	+	+
Pteroylglutamic acid Pteroyldiglutamates*	−	+	+
N^5-methyl folate-H_2 N^5-methyl folate-H_4 Pteroyltriglutamates*	−	−	+

* *S. faecalis* does not grow well on some diglutamates; *L. citrovorum* may grow on some reduced di- and triglutamates (51, 52, 63).

Figure 4 presents, in abbreviated diagrammatic form, a hypothetical explanation [4] for the "piling up" of *L. casei* activity in serum and of FIGLU in urine in vitamin B_{12} deficiency. In this system, vitamin B_{12} acts as coenzyme and folic acid as substrate. If one considers the two agents to interact in this relationship, one has a facile explanation for the fact that a relatively small increase (to 400 μg) (48) above the approximate minimal daily requirement (50 μg) (19) for folic acid may produce hematologic response in pernicious anemia, whereas a much larger increase (to 100 to 500 μg) (41) above the approximate minimal daily requirement (0.1 μg) (49) for vitamin B_{12} appears necessary to produce a hematologic response in folic acid deficiency. Figure 4 may also explain the apparent decrease in FIGLU excretion by folic acid-deficient subjects when treated with 500 μg of vitamin B_{12} daily (41).

The finding that methionine decreases FIGLU

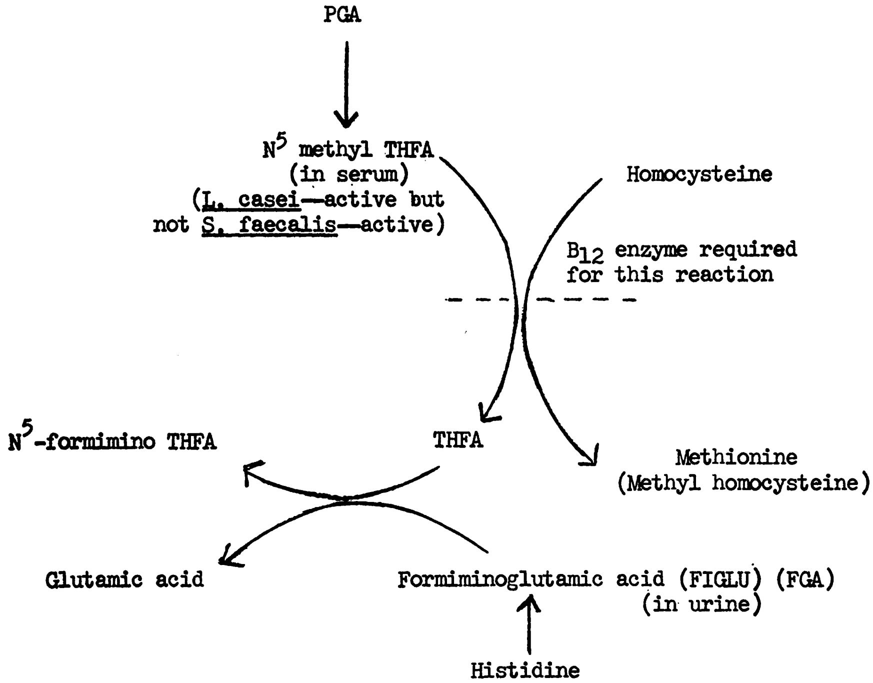

FIG. 4. HYPOTHETICAL EXPLANATION IN ABBREVIATED DIAGRAMMATIC FORM OF THE "PILING UP" OF *L. casei* ACTIVITY IN SERUM AND OF FIGLU IN URINE IN VITAMIN B_{12} DEFICIENCY. THFA is tetrahydrofolic acid. The dashed line represents the reaction blocked by lack of vitamin B_{12}.

excretion in vitamin B_{12}-deficient rats (46) and chicks (47) has been discussed in terms of its possible biochemical meaning by Noronha and Silverman (50). They noted that methionine administration to the vitamin B_{12}-deficient rat eliminated FIGLU excretion and changed the folate pattern of the liver from predominantly N^5-methyl THFA to N^5- and N^{10}-formyl THFA. They concluded that methionine provides an acceptor, directly or indirectly, for the methyl group of N^5-methyl THFA, thus releasing THFA for the metabolism of carbon 2 of histidine (the formimino carbon), which then appeared as the N^{10}-formyl group of N^{10}-formyl THFA. It is also possible, however, that providing methionine may spare the entire pathway involving N^5-methyl THFA, allowing greater production of THFA via other (unblocked) pathways. This could occur if, by negative feedback, there was suppression of the activity or synthesis of an enzyme required for the production of the metabolically blocked N^5-methyl THFA.

As Figure 4 indicates, the increase in both serum *L. casei* activity and urine FIGLU may be

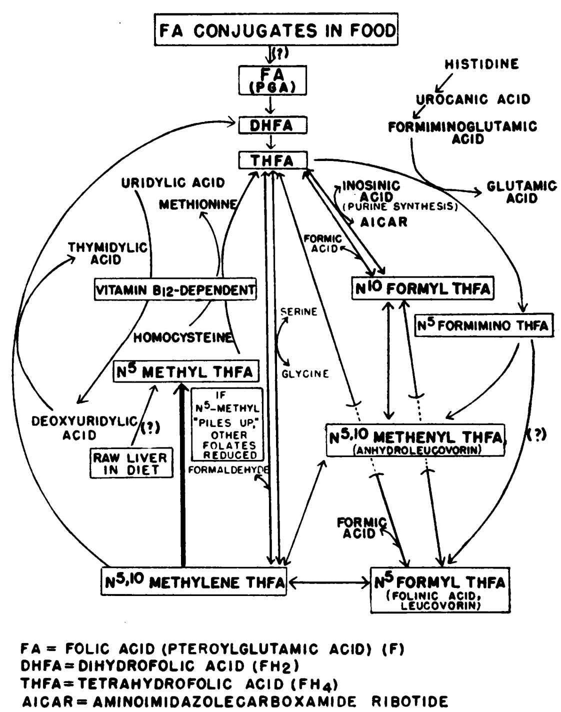

FIG. 5. INTERRELATIONS OF FOLATE COENZYMES, WITH REACTIONS DEPENDENT ON VITAMIN B_{12} INDICATED.

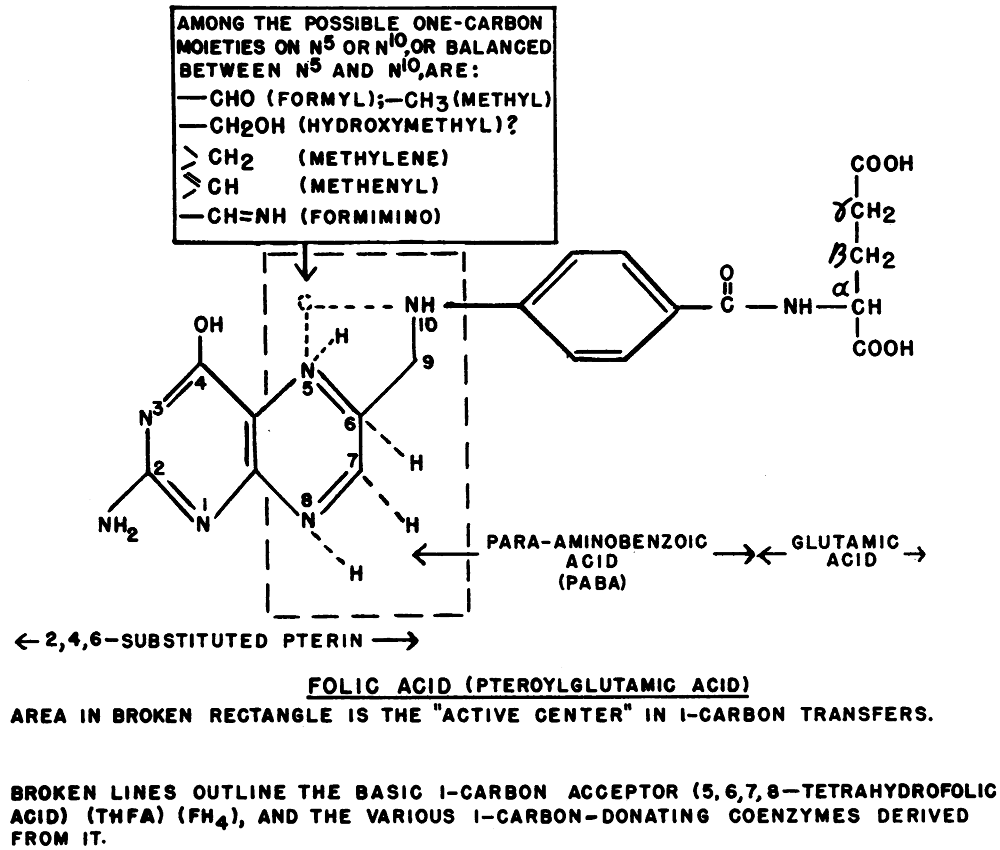

FIG. 6. STRUCTURE OF PTEROYLGLUTAMIC (FOLIC) ACID AND VARIOUS FOLATE COENZYMES.

due to "piling up" of these substrates, whose utilization is blocked by lack of vitamin B_{12}. The "piling up" of N^5-methyl THFA reduces the amount of folic acid available to travel via other metabolic pathways. Thus, this one "metabolic trap" could conceivably produce a generalized slowdown in all 1-carbon transfers. This may explain much of the apparent folic acid deficiency in many patients with vitamin B_{12} deficiency.

Figure 5 presents the interrelations of the folate coenzymes in a more detailed context (23, 25, 26, 29, 51–56), which indicates the possible alternate pathways to THFA, whose variable activity may explain why FIGLU "piles up" in only a third (16, 45) of patients with pernicious anemia. Since N^5-methyl THFA may be both the main circulating (23) and the main storage (liver) (23, 28, 34, 35) folate form in normal man, it is possible that this form of folic acid may play an even larger role in human metabolism than present studies suggest.

Figure 6 depicts the structure of pteroylglutamic (folic) acid, with the various folate coenzymes superimposed thereon. Note the close resemblance of the 5-membered ring of $N^{5\text{-}10}$-methenyl THFA to the hydantoin ring of diphenylhydantoin (Dilantin, Phenytoin). Although prior work using *S. faecalis* led to the belief that "Folic acid tests have not indicated a deficiency, but rather a failure to utilize normal serum levels" (57), we found low folate activity for *L. casei* in the serum of 11 patients who had been receiving anticonvulsant therapy for periods in excess of 6 months (58). One may speculate that the folic acid-responsive megaloblastic anemia which sometimes occurs in such patients (2, 57, 59–62) may be due to weak competitive inhibition by anticonvulsants of the conversion of $N^{5\text{-}10}$-methenyl THFA to N^5-methyl THFA, possibly at the level of absorption of food folates. Competitive inhibition of the 6-membered pyrimidine ring of folic acid, as suggested by Girdwood (62), may explain the megaloblastic anemia infrequently associated with anticonvulsants other than Dilantin.

These studies support the possibility that the megaloblastic anemia which follows vitamin B_{12} deprivation may be partly the result of secondarily deranged folic acid metabolism. This may, in large measure, explain why the hematologic picture is the same in vitamin B_{12} deficiency as it is in folic acid deficiency. Much of this hematologic similarity may also be due to the fact that lack of either folic acid or vitamin B_{12} reduces thymidylate synthesis, as indicated in Figure 5 (51–56).

SUMMARY

In slightly to severely anemic vitamin B_{12}-deficient subjects, after the intravenous injection of 15 μg pteroylglutamic acid (PGA) per kg of body weight, folic acid activity for *S. faecalis* disappears rapidly but activity for *L. casei* disappears slowly from the serum.

Markedly elevated serum folic acid activity for *L. casei* (25 or more mμg per ml) was observed in 17 of 100 consecutive subjects with vitamin B_{12} deficiency.

During specific therapy with daily doses of 5 to 1,000 μg of vitamin B_{12}, serum folic acid activity for *L. casei* may fall sharply and may reach levels below normal before rising again into the normal range. The phenomenon may be due to release of the block in utilization of *L. casei* folic acid activity caused by lack of vitamin B_{12}, with subsequent rapid utilization in hematopoiesis, and may be similar to the fall in serum iron during therapy. Serum folic acid activity for *L. casei* may fall more slowly during specific therapy with smaller (1 μg) daily doses of vitamin B_{12}.

These findings suggest that in the vitamin B_{12}-deficient subject, PGA is rapidly converted to an *L. casei*-active and presumably metabolically useful form (probably N^5-methyl-tetrahydrofolic acid) which then "piles up" in the serum because vitamin B_{12} is required for its normal utilization. This "piled up" folate activity would tend to reduce the amount of folic acid available for other 1-carbon unit transfers. These studies, by providing evidence for the concept that vitamin B_{12} is required for normal folic acid metabolism, support the possibility that the apparent folic acid deficiency in many patients with vitamin B_{12} deficiency may be in large measure due to secondarily deranged folic acid metabolism.

Two minor observations of the present study were:

1. The intravenous injection of 15 μg of PGA per kg of body weight did not appear to affect significantly either the serum vitamin B_{12} level or the folic acid activity of the red cell for *L. casei*. The latter finding suggests that the mature erythrocyte is relatively impermeable to folic acid.

2. Folic acid activity for *L. casei* and vitamin B_{12} activity for *E. gracilis* both may be much higher in reticulocyte-rich than in reticulocyte-poor erythrocytes after vitamin B_{12} therapy. This suggests that the reticulocyte or its precursors, or both are relatively permeable to folic acid and vitamin B_{12}.

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