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Alterations in Circulating Folate Induced by B_{12} Deficiency.* (29617)

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Folate metabolism is abnormal in frank vit. B_{12} deficiency(1-5). This report deals with the fate of ingested synthetic pteroylglutamic acid (PGA) during a B_{12} deficiency as compared with a folate deficiency. The investigation was undertaken to learn more about circulating folates in these deficiency states.

There has been much conjecture about the nature of the circulating folates in B_{12} deficiency. To identify them, serum was chromatographed and activities charted by bioautography with 3 folate-requiring bacteria whose known differential responses permit a closer identification of folates.

Materials and methods. Lactobacillus casei (ATCC 7469), *Pediococcus cerevisiae* (ATCC 8081), and *Streptococcus faecalis* (ATCC 8043) have different responses to folates(6, 7) (Fig. 1). Assays with *S. faecalis* and *P. cerevisiae* were carried out as described for *L. casei*(8); *L. casei* basal medium was used for *S. faecalis*. *Ochromonas malhamensis* served to assay serum B_{12}(8).

An oral loading test with 5 mg of PGA was carried out as before(8,9); 15 subjects with a normal serum folate and B_{12}; 6 with normal folate and low B_{12}, and 6 with low folate and normal B_{12} were used for this study. Folate and B_{12} deficiencies were due to nutritional defects except for one case of pernicious anemia.

Serum, prepared for assay with ascorbate and buffer(8), was concentrated 50 times; non-PGA and 2-hour PGA loaded serum was used. One-tenth ml of the concentrate was spotted and dried on Whatman no. 1 paper (4 × 30 cm). Saturated Na_2HPO_4 was used as a solvent system for the 5 hour ascending chromatography; afterward the paper was dried at 55°C. Following chromatography, bioautography with the 3 organisms was employed to identify folates. A mixture of 300 ml of basal medium together with 6 g of agar was autoclaved and cooled to 45°C; 225 mg of tetrazolium red was added aseptically. Tetrazolium red becomes bright red on reduction and helps define growth spots. The bioautography medium, inoculated with 1 ml of organism grown for 18 hours in

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	PGA → Reduced → THF				
	Mono-glutamate	Poly-glutamate	Mono-glutamate	Poly-glutamate	N^5-methyl THF
L. casei	+	+	+	+	+
S. faecalis	+	—	+	—	—
P. cerevisiae	—	—	+	+	—

FIG. 1. Differential growth responses of 3 folate-requiring bacteria.

TABLE I. Range of Serum Folate Activity (mμg/ml) After Oral PGA.

Subjects with (No.)		Hours 0	2	4	6	8	B_{12} (μμg/ml)
Normal PGA and B_{12} (15)	*L. casei*	7.2–12.6	81–375	65–422	37–173	26–132	136– 308
	S. faecalis	0	17– 30	15– 30	12– 28	8– 25	
Deficient PGA, normal B_{12} (6)	*L. casei*	1.9– 3.4	112–120	65–200	37– 69	26– 38	130– 310
	S. faecalis	0	17– 30	15– 24	12– 20	11– 15	
Normal PGA, deficient B_{12} (6)	*L. casei*	6.1–11.4	90–193	130–207	89–160	63–180	10– 76
	S. faecalis	0	56–184	60–200	55–110	50–130	
Normal PGA, deficient B_{12} after B_{12} treatment (6)	*L. casei*	6.6–16	81–120	65–200	69–173	26– 50	4900–50000
	S. faecalis	0	20– 25	20– 30	20– 27	11– 30	

maintenance medium, was allowed to solidify in sterile glass trays (20 × 36 cm). A developed dried paper chromatogram was placed on the moist agar. The trays were incubated at 37°C for 18-24 hours. Red growth zones were conspicuous; these zones were outlined on the dried paper and the R^f calculated. Only PGA and N^5-formyl tetrahydrofolic acid (N^5-formyl THF) were available for use as standards; 1 mμg each of PGA and N^5-formyl THF were spotted. Comparisons of unknown folate spots were made to R^f's obtained by others using a similar system(5).

Results. Table I shows the results of PGA-loading. Because *P. cerevisiae* values were not affected during this study, they are not listed; total reduced folate ranged from 0.6-3.0 mμg/ml.

Subjects with normal circulating B_{12} and folate activities showed a normal PGA absorption pattern as judged by *L. casei* activity(9); *S. faecalis* activity was not significantly altered during the study; the values never exceeded 30 mμg/ml. Patients with deficient circulating folates and normal B_{12} showed normal *L. casei* and *S. faecalis* activity in the serum after a PGA load. In contrast, patients with normal folate levels and low serum B_{12} had elevated *S. faecalis* activity. Treatment with 100 μg of cyanocobalamin (vit. B_{12}) intramuscularly, twice a day for 10 days, brought *S. faecalis* activity down to normal without affecting the *L. casei* pattern. Identical patterns were noted in 2 subjects with a combined B_{12} and folate deficiency after B_{12} treatment.

Table II shows results of the bioautography study. After PGA loading, B_{12}-deficient serum showed an intense growth zone *L. casei* and *S. faecalis* at R^f 0.16; PGA has the same R^f. After B_{12} treatment the R^f values resembled the normal pattern. The R^f of 0.80 with *L. casei* corresponds closely to N^5-methyl THF(5). *P. cerevisiae* had no growth zones in serum; only the N^5-formyl THF standard (R^f 0.52) permitted growth.

Discussion. When the B_{12} deficient subjects were loaded with PGA, serum *S. faecalis*-active folate increased 3-6 times more than normal (Table I). B_{12} treatment lowered the *S. faecalis* activity to normal, suggesting that B_{12} favored the metabolism of the *S. faecalis*-active folate. It is possible that tetrahydrofolate (THF) accumulated and was oxidized to PGA and thus may

TABLE II. Bioautography of Serum Folates Before and After PGA Loading.*

Serum	*L. casei* R^f	*S. faecalis* R^f
(1) Normal	.80 (+)	—
(2) Normal + PGA load	.16 (±), .80 (++)	.16 (±)
(3) B_{12} deficient	.80 (+)	—
(4) 3 + PGA load	.16 (+++), .80 (+)	.16 (+++)
(5) 3 After B_{12} treatment	.80 (+)	—
(6) 5 + PGA load	.16 (±), .80 (+)	.16 (±)
(7) PGA standard	.16 (+++)	.16 (+++)
(8) N^5-formyl THF standard	.52 (+++)	.52 (+++)

* — = no growth; (±) = trace-, (+) = slight-, (++) = moderate-, and (+++) = heavy growth intensity.

account for the high *S. faecalis* activity. Since the assay system is not anaerobic, labile THF produced would not be assayable(10, 11), hence no detectable rise in *P. cerevisiae* activity in serum was noted. THF accumulation could explain the increased formiminoglutamic acid (FIGLU) excretion seen in patients with B_{12} deficiency(1,3,4). If, as surmized(4), formimino transferase is B_{12} dependent, then enzyme activity would be decreased during a B_{12} deficiency; THF would accumulate, and FIGLU excretion would increase. When B_{12} is introduced, THF would be converted to 5-formimino THF by formimino transferase, the formimino group coming from FIGLU; FIGLU excretion would then decrease. B_{12}-deficient patients treated with PGA alone still excrete increased amounts of FIGLU(4,12), further emphasizing dependence of formimino transferase on B_{12}. During a B_{12} deficiency, PGA treatment would not affect formimino transferase and FIGLU excretion would still persist.

Supporting this interpretation, B_{12}-deficient, PGA-loaded serum showed a very intense growth zone for *L. casei* and *S. faecalis* at the R^f of PGA, and practically no growth zone for *S. faecalis* after B_{12} treatment, pinpointing PGA as the folate species responsible for the high *S. faecalis* activity seen in B_{12} deficient subjects; correspondingly, N^5-methyl THF does not seem to be the main folate accumulating during a B_{12} deficiency as has been claimed(13).

Many laboratories use variations of the microbiological assays employed here(3,5,14), therefore more significance should be attached to the differentials permitted by the assays used conjointly here, rather than to the absolute values obtained with any one organism as compared with the values obtained with the same organisms in other laboratories. We regard the laboratory-laboratory variations in technique as minor and unlikely to alter the present conclusion.

Summary. Clinical B_{12} deficiency is further characterized by accumulation of *S. faecalis*-active folyl monoglutamates after 5 mg of oral PGA. B_{12} treatment lowers *S. faecalis* activity to normal implying that B_{12} decreased folyl monoglutamate activity; neither normal nor folate-depleted subjects showed this effect. Our findings suggest that B_{12} deficiency interferes with formimino transferase, and that principally PGA, not N^5-methyl THF, accumulates during B_{12} deficiency.

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Isolation of *Leptospira grippotyphosa* from a Cow Following an Abortion. (29618)

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Serologic evidence of *Leptospira grippotyphosa* has been found in cattle in the United States over a number of years but without bacteriologic confirmation. Some cattle with positive sera have shown signs suggestive of *Leptospira pomona* infection, manifested by abortions, weak calves, and reduced milk flow, while other individuals have not exhibited any signs of disease(5,14).

Various serologic studies(4,11,13,14,17,19, 21,24) have indicated the presence of *L. grippotyphosa* antibodies in swine and wild animals as well as cattle. Deer sera obtained in Illinois between 1958 and 1960 had a reactor rate of 6% for *L. grippotyphosa* antibodies(8).

Aseptic meningitis in a man caused by *L. grippotyphosa* was reported in Illinois in 1953(2). The clinical diagnosis was confirmed by serologic studies. A year earlier Spain and Howard(23) reported a case of hepatitis in man caused by *L. grippotyphosa,* also serologically confirmed. A similar case was reported in 1954(12).

Several isolations of *L. grippotyphosa* have been made from wild animals in the United States(13,17,21). Clark *et al*(5) isolated *L. grippotyphosa* from voles on a Pennsylvania farm where several cattle developed antibodies following abortions.

Michin and Azinow(18) were the first to describe outbreaks of bovine leptospirosis due to *L. grippotyphosa* which occurred in the U.S.S.R. The disease varies from severe forms causing death of adult cattle to subclinical forms detectable only serologically(9).

Materials and methods. Abortions involving 2 cows of a dairy herd followed by development of *L. grippotyphosa* antibodies prompted collection of urine 7 days after the abortions. The animals at that time had agglutination-lysis titers of 1:100 and 1:1000. No signs other than abortion were observed in the cows.

Guinea pigs weighing 200 g and young weaned hamsters were intraperitoneally inoculated with ½ ml each of undiluted bovine urine. Gerbils(22) and white mice were used later for further determination of host susceptibility.

Rectal temperatures of the guinea pigs were recorded daily and their blood was taken, cultured either at the time of the first temperature rise or in the absence of a temperature rise at 7 days. Blood samples were obtained by heart puncture from the gerbils, white mice and hamsters 4 to 7 days after inoculation and cultured. Serum samples were obtained from all surviving animals approximately 3 weeks after inoculation for detection of antibodies. The agglutination-lysis test was used to detect *L. pomona, L. grippotyphosa, L. sejroe,* and *L. canicola* antibodies.

Portions of liver, spleen and kidney tissues, obtained from the experimental animals at necropsy, were ground in mortars and cultured. The media used in this study were Fletcher's semisolid and Stuart's liquid media enriched with 5, 10 and 15% rabbit serum and experimental media containing bovine albumin and oleic acid(7) obtained from the National Animal Disease Laboratory. Por-