Comparative Study of Vitamin B_{12} Assay in Urine. (22357)

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In a study of cyanocobalamin (vit. B_{12}) excretion in the urine of patients, following an intramuscular (I.M.) dose of 50 μg of crystalline B_{12}, striking variations in the apparent content of B_{12} were observed(1,2). These variations stemmed from differences in the specificity of the assay organisms. It is known that some of the microorganisms serving for the microbiological assay of B_{12} respond to congeners of the vitamin and also deoxyribonucleosides(3,4). Specificity, better than that of bacteria, is shown by *Euglena gracilis* and, above all, by *Ochromonas malhamensis* (5,6). Here we compare the response of *Lactobacillus leichmannii, E. gracilis* and *O. malhamensis* to the B_{12} family of vitamins as they occur in urine.

Methods. The maintenance media for the 3 organisms have been given(7-9). The assay technics used have been outlined in previous publications(10). *L. leichmannii* grew in 24 hours, the protozoans within 4-6 days. The inoculum for the bacillus was prepared by 1:10 dilution with basal medium of a 24 hour culture. The inoculum for *E. gracilis* was prepared by 1:50 dilution of a 4-6 day culture in the basal medium. The *Z* strain rather than the *bacillaris* strain of *Euglena* was employed, as it attained higher densities in a shorter period and had the same sensitivity and specificity(11,12). *O. malhamensis* inoculation was directly from a 4-6 day culture in the maintenance medium without significant carry-over of B_{12} activity. The assay media for the organisms were given(6, 8,13). Urines from control patients, who had not received I.M. B_{12}, were diluted 10 X with buffer at pH 4.5,† and autoclaved for 30 minutes to convert bound cobalamin into its microbiologically active form. This procedure allows estimation of total B_{12}(8). All urines were diluted, as necessary, for the measurements to fall on the straight-line portion of the standard curve. Urines from control patients were further diluted 1:2 - 1:100 with assay medium (final dilution of the urine 1:20 - 1:100). Urines from patients, who received 50 μg of B_{12} I.M., were diluted 50 X, autoclaved, and subsequently diluted again 1:2 - 1:100 (final dilution 1:100-1:5000). *L. leichmannii* was incubated at 37° for 24 hours; the protozoans were kept at room temperature (not exceeding 30°C) for 4-6 days under constant illumination provided by six 40 W. warm white fluorescent tubes at an average distance of 0.8 m. Growth was measured as optical density units with a Welsh Densichron equipped with a red-sensitive probe.

Results. Recovery of added vit. B_{12}: To test the validity of the assay methods, recovery experiments of B_{12} in urine were done with 4 concentrations of B_{12} (Table I). The 2 protozoans yielded satisfactory recoveries from urine. In recovery experiments with sera and cerebrospinal fluids, we noticed that *O. malhamensis* may be slightly stimulated by other materials in cerebrospinal fluid and serum when compared to *Euglena*. These stimulants did not affect the results in urine.

B_{12} in Urine. Table II shows results with urine collected during the first 8 hours after intramuscular injection of 50 μg of vit. B_{12} in 23 individuals. In all except 2 urines (Nos. 1 and 5) values obtained with *O. malhamensis* were lower than for that with *Euglena*. The values with *L. leichmannii* were higher than that with *Euglena* except in 2 cases (Nos. 18

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† One-half gram of trans-aconitic acid/100 ml of distilled water, to which triethanolamine had been added to reach pH 4.5. One hundred mg of Na metabisulfite had also been added to 100 ml of buffer for stabilization of the analogs of B_{12} e.g. hydroxycobalamin(14,15).

TABLE I. Recovery of B_{12} Added to Normal Undiluted Urine.

Conc. (μg/ml)	*L. leichmannii*	*E. gracilis*	*O. malhamensis*
5	10.8	3.6	3.7
10	16.5	8.4	9.5
15	24.1	14.2	14.2
20	31.0	18.2	18.2

and 19). In no instance were values with *L. leichmannii* lower than with *O. malhamensis*.

Discussion. Ford(6) has evaluated the response of the 3 microorganisms to pseudovitamin B_{12} and to other B_{12}-like factors. This evaluation is used here as the basis for calculation of true B_{12} values as compared with values for pseudo-B_{12}'s. Ford and Hutner also have discussed the positive response of *L. leichmannii* to deoxyribosides(4). The main difference of practical interest between *Euglena* and *Ochromonas* is the high response of the former to pseudo-vitamin B_{12}. With *L. leichmannii* the response to pseudo-B_{12} is half that of *Euglena*. We assume that the response to *O. malhamensis* (OM) gives the true value for vit. B_{12}. Therefore: $B_{12} = OM$. The extra response of *Euglena* (E) as compared with *Ochromonas* is assumed to reflect the pseudo-B_{12}'s (Ps_{12}) in the sample. Pseudo-B_{12}'s may be estimated by the formula $Ps_{12} = \frac{E - OM}{8}$.

Besides responding to certain pseudo-B_{12}'s, *L. leichmannii* (L) also responds to thymidine and certain other deoxyribosides(3). The quantity of these deoxyribosides (D) may be computed in terms of true vit. B_{12} by the following calculation

$$D = L - OM - 4\frac{E - OM}{8}$$

which leads to $D = L - \frac{OM + E}{2}$.

These formulae are only crude approximations, as they disregard the possible presence of other members of the vit. B_{12} group. If by these calculations interference by deoxyribosides is suspected, it might be desirable to assay for them directly(16). Results in Table II indicate a correlation between pseudo vit. B_{12} and true B_{12}. Deoxyribonucleosides, and other factors, stimulating the growth of *L. leichmannii* appear to interfere in the vit. B_{12} load tests described here; the difference in results between *L. leichmannii* and the others are possibly due to dietary differences of patients, which may be reflected in their urine.

The recoveries of true B_{12}, according to Table I, appear to be 6-11 μg too high with *L. leichmannii*. This excess may perhaps be ascribed to the combination of stimulatory urinary constituents with materials present in the basal medium(17), a reaction, which assumes disturbing proportions, when the growth is dense.

Summary. The response of *Ochromonas malhamensis, Euglena gracilis,* and *Lactobacillus leichmannii* to vit. B_{12} in normal urine and in urine of patients, given an intramuscular dose of 50 μg of vit. B_{12}, was studied. The "true" vit. B_{12} values presumably are given by the *Ochromonas malhamensis* assay. The response of *E. gracilis* and *L. leichmannii* to other members of the B_{12} group and to other metabolites is discussed from the standpoint of how to estimate responses other than *O. malhamensis*-active B_{12}. Calculations are given for the estimation of interfering metabolites.

TABLE II. B_{12} in Urine of Patients within 8 Hours after Intramuscular Injection of 50 μg of B_{12}.

Total output in μg determined by response of		
Ochromonas	*Euglena*	*Lactobacillus*
.6	.5	1.2
1.0	2.4	3.0
1.0	2.9	10.6
1.5	1.7	5.0
1.9	.7	3.8
2.5	4.2	8.3
3.6	5.6	9.9
3.8	7.4	7.4
4.1	6.9	8.5
4.9	10.2	23.4
6.0	14.5	33.0
6.3	11.6	16.8
6.6	13.1	20.0
8.1	10.2	20.4
9.0	9.5	19.0
10.2	16.1	19.5
10.5	22.7	33.1
11.4	38.0	24.7
11.7	17.1	11.5
11.9	15.0	16.1
13.7	23.6	33.0
13.8	24.7	27.6
28.7	30.2	31.3

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Molybdenum Toxicity in the Rat.* (22358)

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The symptoms of molybdenum toxicity have been thoroughly described in several species(1), yet little is known of the underlying defects responsible for these symptoms, except that the resultant anemia can be remedied by copper sulfate supplementation. Weil-Malherbe and Green(2) and Lutwak and Sacks(3) have noted that compounds such as adenosine triphosphate (ATP) and acetyl phosphate are rapidly hydrolyzed in the presence of molybdenum salts. Since molybdate ions *in vitro* catalyze the acid hydrolysis of "high-energy" phosphate esters, the possibility existed that molybdenum might have a similar effect *in vivo*. If this were so, a decrease in the supply of "high-energy" phosphate compounds might then be fundamental to some of the symptoms of molybdenum toxicity. The acetylation of p-aminobenzoic acid (p-ABA) and the conjugation of glycine with benzoic acid have been used extensively in tests for liver function. Both reactions involve ATP, coenzyme A, magnesium ions, and the appropriate enzymes. Lipmann(4) has shown that ATP is involved in the acetylation of sulfonamides by pigeon liver preparations, and Chantrenne(5) has established a requirement for ATP in the enzymatic formation of hippuric acid. If molybdate ions *in vivo* should catalyze the hydrolysis of organic phosphate esters, then the supply of "high-energy" phosphate compounds might be reduced, and presumably, the reduction would be reflected in the ability of the animal to acetylate p-aminobenzoic acid or to conjugate benzoic acid.

The experiments reported here tested this hypothesis. Studies were also conducted on the effect of molybdenum toxicity on the alkaline phosphatase activity of various tissues.

Methods. The basal diet had the following

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