Response to and Assay of Vitamin B_{12} by a Mutant of *Escherichia coli*.* (20382)

K. R. JOHANSSON.

From the Department of Bacteriology and Immunology, University of Minnesota, Minneapolis.

The difficulties encountered in the assay of vit. B_{12} by strains of *Lactobacillus lactis* and *L. leichmannii* are well known(1-7). Since Davis and Mingioli(8) suggested that certain B_{12}-requiring mutants of *Escherichia coli* might be employed for the assay of this factor, several workers(9-11) developed an agar plate-disc assay of moderate sensitivity for B_{12} with one of these cultures. Others(12,13) have utilized *E. coli* 113-3 (strain designation of Davis and Mingioli)(8) for the assay of vit. B_{12} by measuring the response turbidimetrically.

It is the purpose of this report, an elaboration of a preliminary note(14), to describe a turbidimetric assay for B_{12} in which *E. coli* 113-3 is the test organism, and to present data concerning the influence of several factors upon the growth stimulus exerted by vit. B_{12} and methionine† upon this mutant.

Materials and methods. The mineral salts-citrate-glucose medium of Davis and Mingioli (8), modified by the addition of 0.05% sodium chloride, was adopted as the basal medium. The procedure was carried out in chemically-clean Pyrex test tubes (16 x 150 mm) in the conventional manner. Sequential quantities, 5 ml or less, of the standard solutions (from 2 to 70 $\mu\mu$g Merck's crystalline B_{12}‡ per ml, final concentration) or of a material being assayed, were added to a duplicate series of tubes, the volume adjusted to 5 ml with distilled water and 5 ml double strength basal medium added. The tubes were covered with aluminum caps, autoclaved at 120°C for 5 minutes and rapidly cooled. The inoculum consisted of one drop (0.05-0.07 ml) per tube of a 1:50 dilution of saline-washed cells of *E. coli* 113-3, previously grown for 18 hours at 30°C in the basal medium containing 15 μg L-methionine per ml. The stock culture was maintained by monthly transfer on slants of Difco's nutrient agar. Following inoculation, the tubes were incubated without agitation at 37°C for 16 hours, cooled and the amount of growth measured in a Klett-Summerson photoelectric colorimeter (blue filter). The readings of at least 4 tubes containing different amounts of test material were used in estimating the potency of samples assayed. Tissues were usually digested under toluene with trypsin at a pH of 8.0 and a temperature of 37°C for 24 hours prior to the assay. The response of *E. coli* 113-3 to methionine or

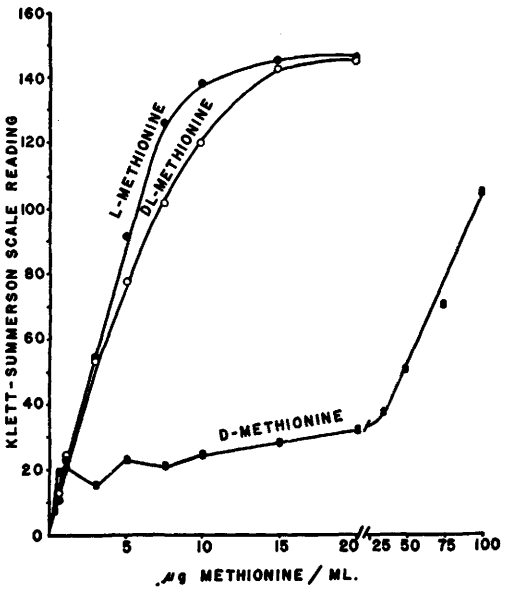

FIG. 1. Response of *Escherichia coli* 113-3 to methionine. Static incubation at 37°C in small tubes (16 × 150 mm) for 18 hr.

* Supported in part by research grant from the National Microbiological Institute of the National Institutes of Health, Public Health Service.

† This strain of *E. coli* responds to either methionine or vit. B_{12} in a medium otherwise free of organic growth factors. No other substance, except possibly cytochrome c(15), will replace its demand for these factors.

‡ Kindly furnished by Dr. Karl Folkers, Merck and Co., Rahway, N. J.

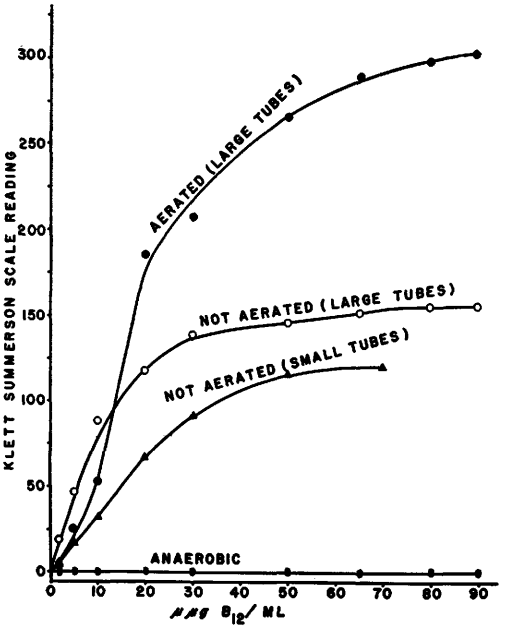

FIG. 2. Effects of degree of aerobiosis upon response of *Escherichia coli* 113-3 to vit. B_{12}. Test tubes used were 25 × 150 mm (large) and 16 × 150 mm (small).

B_{12} could be determined without autoclaving by employing usual aseptic methods with sterile glassware and reagents. For anaerobic incubation, aluminum pressure cookers, equipped with palladium-asbestos catalyzers and contained in an incubator, were filled with the inoculated tubes (25 x 150 mm), evacuated twice by means of a water pump and brought to atmospheric pressure with a 1:9 mixture of carbon dioxide and hydrogen.§

Results. Influence of several factors on response of Escherichia coli 113-3 to vit. B_{12}. It may be seen in Fig. 1 that this bacterium responded to L-methionine at concentrations of from <0.5 to >10.0, and to DL-methionine from <0.5 to 20.0 μg per ml. D-methionine was not appreciably stimulatory in concentrations of less than 25.0 μg per ml, although low concentrations (<1.0 μg per ml) were more stimulatory to growth than either the L or DL isomers.

The response of *E. coli* to vit. B_{12} was shown to vary with the degree of aerobiosis (Fig. 2). This effect, not surprising in view of the ability of B_{12} to stimulate the oxidative action of *E. coli* 113-3(15) and the influence of the redox potential upon the B_{12} requirement of *L. lactis*(3,6,16), has been noted by others (8,13). Thus, continuous shaking of graded levels of vit. B_{12} in large tubes during a 16-hour period resulted in markedly heavier growth than did static cultures in either the large or the small tubes, as did the greater surface area exposed in non-aerated large tubes than in small tubes. Strict anaerobiosis resulted in complete suppression of the growth stimulating property of B_{12} for this mutant. Likewise, L-methionine did not promote growth if the atmosphere were oxygen-free, although the presence of both B_{12} and methionine, at concentrations of greater than 100 $\mu\mu$g and 10 μg per ml, respectively, permitted submaximal growth under anaerobic conditions. The curve in Fig. 2 designated "not aerated (small tubes)" is representative of the usual conditions employed in this study and may be considered to be, therefore, a typical standard curve. Under these "standard" conditions, *E. coli* 113-3 responded linearly to B_{12} at concentrations of from <2.0 to approximately 20.0 $\mu\mu$g per ml while the maximal stimulating level was 75.0 $\mu\mu$g. On occasion, a response could be measured with 0.5 $\mu\mu$g, but 1.0 $\mu\mu$g invariably evoked a growth stimulation.

Cyanide has been shown to react with cobalamins resulting in a heat stable form analogous to cyanocobalamin(17,18), while certain reducing agents have been demonstrated to "protect" the heat labile forms of this vitamin(4,5,13,19,20). Table I shows that concentrations of potassium cyanide greater than 1.0 μg and of sodium thioglycolate or ascorbic acid greater than 10.0 and 5.0 μg per ml, respectively, suppressed the B_{12}-induced growth response of *E. coli*. If, however, the tests were made aseptically, cyanide at a level as low as 0.5 μg per ml was inhibitory, while thioglycolate was not inhibitory at a concentration as great as 500 μg per ml; ascorbic acid was just as inhibitory without as with the autoclaving treatment. At higher concentrations, B_{12} overcame the antagonistic action of cyanide and thioglycolate. It may be seen

§ An apparatus designed and built by Dr. D. W. Watson of this department.

TABLE I. Effect of Cyanide and Reducing Agents on Response of *E. coli* 113-3 to Vit. B_{12}.

B_{12}	KCN (μg/ml)				Na thioglycolate (μg/ml)				Ascorbic acid (μg/ml)				
(μμg/ml)	1	5	10	25	10	100	250	500	1	5	100	250	Control
5	16*	14	9	1	15	12	9	2	15	16	12	2	17
10	33	28	19	2	31	23	13	3	32	31	21	2	32
30	90	83	52	5	91	55	20	2	89	87	60	5	92
60	119	113	96	6	117	97	29	4	115	114	91	7	117

* All tubes autoclaved at 120°C 5 min. Growth expressed as scale reading of Klett-Summerson photoelectric colorimeter (avg of several trials). Zero settings made with uninoculated medium.

TABLE II. Effect of Autoclaving on Response of *E. coli* 113-3 to Vit. B_{12} and B_{12a}.

B_{12} or B_{12a}	B_{12}		B_{12a}	
(μμg/ml)	Autocl.*	Aseptic	Autocl.*	Aseptic
5	17	15	9	12
10	34	32	16	23
30	92	90	52	68
60	116	113	92	104
100	120	119	105	115

* See footnote, Table I.

TABLE III. Demonstration, with *E. coli* 113-3, of Action of Cyanide and Reducing Agents upon Vit. B_{12a}.

B_{12a} (μμg/ml)	KCN (1 μg/ml)	Na thioglycolate (5 μg/ml)	Ascorbic acid (5 μg/ml)	Control
5	17*	14	7	8
10	31	28	14	15
30	89	79	41	54
60	109	99	81	89
100	119	116	103	104

* See footnote, Table I.

(Table II) that vit. B_{12a},‖ unlike B_{12}, lost activity (approximately 30%) upon autoclaving, unless low concentrations of cyanide and thioglycolate (Table III) were present. Moreover, cyanide caused conversion of B_{12a} to a cyanocobalamin nearly equivalent to vit. B_{12}, in terms of activity for the test organism. At the concentrations used, ascorbic acid was of no value in "protecting" vit. B_{12a} from heat inactivation. Vit. B_{12a}, when tested aseptically, was approximately 70% as active as B_{12} for this mutant (Table II); this is a favorable comparison with the 75% figure reported by Chiao and Peterson(13).

Assay of vit. B_{12}. Only those materials, *e.g.*, soy bean meal, which contain inappreciable quantities of B_{12} and are relatively rich in methionine were difficult, if not impossible, to assay. The calculated potency of B_{12} in certain materials was found to be influenced by the presence of cyanide or thioglycolate in the basal medium, presumably as a consequence of the presence of heat labile cobalamins. The results of a study with such a substance, a dried streptomyces fermentation concentrate,¶ are assembled in Table IV. Note that, while the standard deviation for a single test was relatively low, a noticeable discrepancy between trials was encountered. The latter may be a reflection, to a large extent, of variations between the one-gram aliquots weighed for each determination. As expected, cyanide (1.0 μg per ml) and, to a lesser extent, thioglycolate (5.0 μg per ml) raised the calculated potency of B_{12} over that estimated in the unsupplemented basal medium. If the concentrate was assayed without autoclaving, the assay values were similar to those obtained by autoclaving in the presence of cyanide. Results with the latter methods were comparable to those obtained with *L. leichmannii* 4797 (method of Capps *et al.*(21), modified by the addition to the basal medium of 0.1% sodium thioglycolate, but not corrected for a response to desoxyribosides). The assay consistently accounted for all (98 to

‖ Crystalline vit. B_{12a} furnished through generosity of Drs. G. E. Boxer and D. Hendlin, Merck and Co., Rahway, N. J. Although it had initially, $<5.0\%$ of other forms of this vitamin, it was found after several months by Dr. Boxer to contain approximately 18% cyanocobalamin. Unless contamination with cyanide occurred, this transformation is difficult to explain.

¶ Kindly furnished by Dr. A. M. Hanson, Grain Processing Corp., Muscatine, Ia.

TABLE IV. Vit. B_{12} Assays of a Dried Streptomyces Fermentation Concentrate. Comparison between *Escherichia coli* 113-3 under Different Conditions and *Lactobacillus leichmannii* 4797.

	E. coli				
Trial No.	Autoclaved control	Aseptic control	+KCN (1.0 μg/ml)	+Na thioglycolate (5.0 μg/ml)	*L. leichmannii**
1	8.9 ± .32†	12.0 ± .40	12.4 ± .24	11.6 ± .46	—
2	9.7 ± .19	14.9 ± .08	16.0 ± .24	—	13.6 ± .28
3	8.0 ± .16	—	12.6 ± .34	—	13.5 ± .45
4	7.8 ± .24	—	—	10.0 ± .46	11.2 ± .68
Avg	8.60	13.45	13.67	10.80	12.77

* Method of Capps *et al.*(21) modified by addition of 0.1% sodium thioglycolate to basal medium.

† Average of at least 4 levels of test material, ± stand. dev. $\left(\sqrt{\frac{\Sigma x^2}{n-1}}\right)$, expressed as μg/g.

103%) of the B_{12} added in known amounts.

Discussion. It is clear that a number of factors affect the assay of vit. B_{12} with *E. coli* 113-3, *viz.*, 1) the degree of aerobiosis during incubation, 2) the presence of methionine (all 3 isomers) in the test substance, 3) the kinds of cobalamins present, 4) the nature and concentration of reducing agents in the medium, 5) the concentration of cyanide, if present, and 6) the employment of heat. Although aeration during incubation stimulated the response of the culture to B_{12}, the advantage, in terms of an assay, was no greater than with static incubation. The upper limit of response to B_{12} was higher with than without aeration but the sensitivity of *E. coli* 113-3 to small amounts of B_{12} was greater without agitation. Furthermore, agitation caused difficulty in reproducibility. Consequently, aeration, advocated by other workers(12,13), serves to complicate the assay without an improvement in precision or sensitivity. The dual response of *E. coli* 113-3 to B_{12} and methionine causes obvious difficulties. Nevertheless, most materials of animal origin lend themselves to this assay since the half-maximal response of *E. coli* 113-3 to vit. B_{12} and methionine under "standard" conditions was approximately 18 μμg and 2.5 μg per ml, respectively. When a methionine interference is suspected, such suspicion may be confirmed by applying a mild alkaline hydrolysis and assaying directly for methionine. Chiao and Peterson(13) report that if the ratio of methionine to B_{12} on a weight basis is less than 50,000, the former will not influence the specificity of the assay with this mutant. That D-methionine was increasingly stimulatory at high levels while supporting slight growth at lower concentrations is suggestive of a methionine isomerase in these cells.

Unfortunately, the different cobalamins with varying biochemical activities both in bacteria and animals continues to plague any biological assay for vit. B_{12}. While the rat responds equally well to B_{12} and B_{12a}(22), B_{12a} was 0.7 (0.4 with autoclaving treatment) as active toward *E. coli* 113-3 as B_{12}. Chiao and Peterson(13) found vit. B_{12f} to be 0.4 as active for this mutant as B_{12} while the former is inactive for the rat(23). Attempts have been made by numerous investigators to "protect" the heat labile forms of cobalamins by the incorporation of reducing agents in the medium. This study indicates that the nature and concentration of reducing agents are critical. Ascorbic acid was of no value in protecting vit. B_{12a} from partial heat inactivation while sodium thioglycolate was useful in this respect. However, relatively high concentrations of these reducing agents completely destroyed the growth stimulating property of B_{12} and B_{12a}, an observation compatible with earlier reports(24,25). A further complication of reducing agents is the report(26) that thiomalic and thioglycolic acids convert vit. B_{12} to an analogue more active than B_{12} toward *L. leichmannii*. If such a substance was formed during our study, it was no more active—indeed, probably less active—for *E. coli* than vit. B_{12}, *per se*. Apparently, the

optimal concentration of thioglycolate depends on the composition of the basal medium. For example, Burkholder(12) used 0.1 mg sodium thioglycolate per ml in his *E. coli* 113-3 assay medium, an amount shown by our study to suppress this organism's growth to a significant extent. Burkholder's medium was, however, complex, containing more glucose** and 7 amino acids.

The value of including cyanide in the medium when assaying for vit. B_{12} with *L. lactis* and *L. leichmannii* has been established(27, 28). In this work, cyanide appeared to convert B_{12a} to a cyanocobalamin equivalent in activity for *E. coli* 113-3 to that of B_{12}. Inasmuch as this organism is very sensitive to cyanide, the concentration used must be below a level of 5 μg per ml basal medium. The greater inhibition by cyanide of the response of *E. coli* to B_{12} or B_{12a} when tested aseptically than when determined following autoclaving was provoking. Perhaps at higher temperatures cyanide is altered, or a product results from the reaction of cyanide with cobalamins which is inactive for the test organism. Until the significance of the various cobalamins is clarified, it would seem desirable to include cyanide in the basal medium at some level less than 5 μg per ml. However, if the assay is to be made without autoclaving, cyanide should be omitted from the medium. Furthermore, there is good agreement when natural substances, *e.g.* streptomyces concentrate, liver, feces, etc., are assayed simultaneously by autoclaving in the presence of CN^-, and by asepsis without CN^-.

Although this mutant is used by a number of laboratories for the assay of vit. B_{12} by means of the agar plate-disc technic(9,10), or modifications thereof, a few trials in this laboratory found the plate method to have less sensitivity than the more tedious turbidimetric procedures. The assay procedure reported herein compares favorably to the similar one of Chiao and Peterson(13) but avoids, without reduction in precision, both agitation during incubation and pre-treatment with potassium cyanide. The turbidimetric method of Burkholder(12) appears to be less sensitive with no greater a range of linear response to vit. B_{12}. This method of assay for B_{12} has numerous advantages over those requiring a *Lactobacillus* spp., not the least of which is its relative simplicity.

Summary. A mutant organism, *Escherichia coli* 113-3, which responds to vit. B_{12} or methionine in a mineral salts-citrate-glucose medium, was adopted successfully for the assay of vit. B_{12}. The effects of the degree of aerobiosis, and of potassium cyanide and two reducing agents, upon the response of this bacterium to vit. B_{12} and B_{12a} are described.

** To determine whether or not a constituent in the medium augmented destructive action of heat on vit. B_{12a}, it was noted that less destruction occurred if glucose was omitted from the medium. The slight degree of heat inactivation (5 min. at 120°C) of B_{12a} in distilled water was the same as that which occurred in the glucose-free medium.

1. Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, 1948, v175, 475.

2. Shorb, M. S., and Briggs, G. M., *J. Biol. Chem.*, 1948, v176, 1463.

3. Caswell, M. C., Koditschek, L. K., and Hendlin, D., *J. Biol. Chem.*, 1949, v180, 125.

4. Hoffman, C. E., Stokstad, E. L. R., Hutchings, B. L., Dornbush, A. C., and Jukes, T. H., *J. Biol. Chem.*, 1949, v181, 635.

5. Skeggs, H. R., Nepple, H. M., Valentik, K. A., Huff, J. W., and Wright, L. D., *J. Biol. Chem.*, 1950, v184, 211.

6. Cuthbertson, W. F. J., and Lloyd, J. R., *J. Gen. Microbiol.*, 1951, v5, 416.

7. Zygmunt, W. A., *J. Bact.*, 1952, v63, 821.

8. Davis, B. D., and Mingioli, E. S., *J. Bact.*, 1950, v60, 17.

9. Bessell, C. J., Harrison, E., and Lees, K. A., *Chem. Ind.*, 1950, 561.

10. Harrison, E., Lees, K. A., and Wood, F., *Analyst*, 1951, v76, 696.

11. Williams, W. L., Esposito, R. G., and Pierce, J. V., *Fed. Proc.*, 1952, v11, 458.

12. Burkholder, P. R., *Science*, 1951, v114, 459.

13. Chiao, J. S., and Peterson, W. H., *Applied Microbiol.*, 1953, v1, 42.

14. Johansson, K. R., *Bact. Proc.*, 1951, 146.

15. Oginsky, E. L., Smith, P. H., Tonhazy, N. E., Umbreit, W. W., Lichstein, H. C., and Carson, S. F., *J. Bact.*, 1951, v61, 581.

16. Koditschek, L. K., Hendlin, D., and Woodruff, H. B., *J. Biol. Chem.*, 1949, v179, 1093.

17. Kaczka, E. A., Wolf, D. E., Kuehl, F. A., Jr., and Folkers, K., *Science*, 1950, v112, 354.

18. Veer, W. L. C., Edelhausen, J. H., Wijmenga,

H. G., and Lens, J., *Biochim. Biophys. Act.*, 1950, v6, 225.

19. Stokstad, E. L. R., Dornbush, A. C., Franklin, A. L., Hoffman, C. E., Hutchings, B. L., and Jukes, T. H., *Fed. Proc.*, 1949, v8, 257.

20. Thompson, H. T., Dietrich, L. S., and Elvehjem, C. A., *J. Biol. Chem.*, 1950, v184, 175.

21. Capps, B. F., Hobbs, N. L., and Fox, S. H., *J. Biol. Chem.*, 1949, v178, 517.

22. Frost, D. V., Fricke, H. H., and Spruth, H. C., *J. Nutrition*, 1953, v49, 107.

23. Lewis, U. J., Tappan, D. V., and Elvehjem, C. A., *J. Biol. Chem.*, 1952, v199, 517.

24. Lang, C. A., and Chow, B. F., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 39.

25. Hendlin, D., and Soars, M. H., *J. Biol. Chem.*, 1951, v188, 603.

26. Broquist, H. P., Stokstad, E. L. R., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 806.

27. Cooperman, J. M., Drucker, R., and Tabenkin, B., *J. Biol. Chem.*, 1951, v191, 135.

28. Soars, M. H., and Hendlin, D., *J. Bact.*, 1951, v62, 15.

Received May 5, 1953. P.S.E.B.M., 1953, v83.

Effect of Injecting Platelet Extract Intravenously.*† (20383)

ERNST EPSTEIN AND ARMAND J. QUICK.

From the Department of Biochemistry, Marquette University School of Medicine, Milwaukee, Wis.

It is now fairly widely accepted that platelets do not contain any significant amount of preformed thromboplastin, but supply a factor which reacts with a plasma constituent, thromboplastinogen, to form active thromboplastin (1). Recent studies indicate that before this reaction can occur, thromboplastinogen must become activated either by contact with a foreign surface such as glass(2) or by the addition of thrombin(3). Since circulating blood probably contains no activated thromboplastinogen, the injection of a platelet extract should not cause intravascular clotting nor have any pronounced effect on the blood. The present investigation was undertaken to test the correctness of this conclusion. As a control, the effect of injecting tissue extract containing active thromboplastin was also studied.

Methods. Platelet extract from rabbit blood was prepared according to the method previously described(2). The procedure consists essentially in obtaining platelets by differential centrifugation, and then causing disintegration by repeated freezing and thawing while suspended in distilled water. This procedure is very effective for disintegrating platelets as revealed by phase contrast microscopy and by the loss of all power to bring about clot retraction. *Rabbit brain extract* was prepared by triturating fresh brain tissue, cleared of blood vessels, with distilled water, then subjecting it to freezing and thawing similar to the procedure used for platelets. The emulsion was centrifuged for 5 minutes at 1000 rpm to remove coarse particles and then was allowed to remain at room temperature for 3 hours to assure inactivation of any adenosine triphosphate(4). Both the platelet extract and the brain extract were adjusted to a sodium chloride concentration of 0.85%. The *fibrinogen concentration* was determined by the method previously described(5) except that instead of employing the Folin Ciocalteu reagent, the fibrin digested in sodium hydroxide was estimated directly by determining the optical density at 280 μ. The *prothrombin time* and prothrombin consumption time were determined by the methods previously described(5). Platelets were counted directly in a hemacytometer using 3.8% sodium citrate solution as diluent. All injections were made into the marginal vein of the rabbit ear and were given at the rate of about 3-5 cc per minute. Blood samples for analysis were taken from the central artery of the ear.

Results. When a potent extract of platelets in an amount equivalent to as high as 210%

* The authors acknowledge the valuable assistance of Dr. Edward Barta of the Department of Pathology who furnished most of the animals used.

† This investigation was supported by a grant from the Division of Research Grants, National Institute of Health, USPHS.