METABOLISM OF COBALAMINS BY A SOIL MICROORGANISM

F. SANDERS AND GERALD R. SEAMAN

Carter Physiology Laboratory and Department of Microbiology, University of Texas Medical Branch, Galveston, Texas

Received for publication August 13, 1959

The occurrence in soil of a large group of bacteria which require vitamin B₁₂ has been reported by Lochhead and Thexton (1952). Thirty cultures obtained from 534 isolates showed dependence upon vitamin B₁₂ for growth. Lochhead and Burton (1954) grouped these 30 cultures into 10 types, based on morphological and physiological characteristics. A study of the nature of the vitamin B₁₂ requirements of 19 of these cultures, representing all of the 10 types, has been made by Ford and Hutner (1957). Goldberg et al. (1957) reported on the nutrition of one of these organisms, culture no. 38, a member of the type III group provisionally classified by Lochhead and Burton (1954) as belonging to the genus Arthobacter, and indicated that the organism is specific in its growth requirement for 5,6-dimethylbenzimidazole-cyano-cobalamin. However, in the course of investigations of the biological properties of several B₁₂ vitamins it was observed (Seaman and Sanders, 1957) that the cobalamin requirement of this organism, commonly termed "Lochhead 38" could be replaced by factor B, the compound formed from 5,6-dimethylbenzimidazole-cyano-cobalamin by removal of the nucleotide portion of the molecule. This unanticipated finding prompted a reexamination of the vitamin B₁₂ requirement of Lochhead 38 and an investigation of the metabolism of the cobalamins which support growth.

MATERIALS AND METHODS

Bacterial strains. The culture of Lochhead 38 used was obtained from Dr. S. H. Hutner and was maintained on slants consisting of the chemically defined basal medium described by Goldberg et al. (1957), supplemented with 20 μg per ml of 5,6-dimethylbenzimidazole-cyano-cobalamin, 0.1 per cent trypticase, and 1.5 per cent agar. Microscopic examinations of the culture revealed nonmotile, irregularly shaped rods, most of which were gram-positive. Physiologically also the organisms corresponded to the type III grouping of Lochhead and Burton (1954) in that they neither hydrolyzed starch nor coagulated milk. In agreement with the findings of Goldberg et al. (1957), the cobalamin requirement was not spared by methionine, purines, pyrimidines, yeast extract, nor by acid or alkaline hydrolyzates of ribonucleic acid or deoxyribonucleic acid.

The cultures of Escherichia coli strain 113-3 used were obtained from Drs. D. D. Davis and J. E. Ford, and were maintained on slants of nutrient agar (Difco).

Cobalamin preparations. Factor B was obtained from Dr. S. H. Hutner, 2-methyladenine-cyano-cobalamin from Dr. K. Bernhauer, and 5,6-dimethylbenzimidazole-cyano-cobalamin from a commercial source (Nutritional Biochemical Corporation). The preparations were chromatographically pure when examined in a standard solvent system of the organic phase of a mixture containing sec-butanol-water-acetic acid-4 per cent KCN (100:15:50:0.25).

Factor C was prepared by directed biosynthesis using E. coli according to the procedure of Ford et al. (1955). The organisms were grown in the defined medium described by Burkholder (1951), modified by including 2 μg per ml of NaCN. Fifty-ml amounts of a 5-fold concentrated stock medium were diluted to 240 ml with distilled...
water and dispensed into 1-L Roux culture flasks. After autoclaving, the flasks were cooled to 30 C and 10 ml of a 25 per cent dextrose solution, 0.4 ml of factor B solution containing 10 μg per ml, and 0.25 ml of inoculum were added aseptically. The glucose and factor B solutions were sterilized separately by autoclaving. The inoculum was prepared by washing cells from stock slant cultures with sterile 0.9 per cent NaCl and adjusting the concentration to an optical density of 0.7 to 0.8 measured at a wave length of 560 mμ. After 18 hr incubation at 30 C, cells were harvested by centrifugation at 6000 × G for 15 min at 4 C, washed twice in 250 ml of saline, and once in 10 ml of distilled water. The washed cells were resuspended in 3 ml of 0.033 N hydrochloric acid. Then, 0.2 ml of 1 per cent NaCN solution was added and the suspension was hydrolyzed for 30 to 40 min in a vigorously boiling water bath. The mixture was cooled and adjusted to pH 5 with 1 N potassium hydroxide. After centrifugation at 6000 × G, the supernatant fluid was removed for chromatography and for microbiological assay.

The extracts were streaked on large (18½ by 22½ in) sheets of Whatman no. 4 filter paper and were developed in the ascending direction for 48 hr at 27 C in the standard sec-butanol-water-acetic acid-KCN solvent system. The areas on the filter paper which contained vitamin B₁₂ activity were identified by bioautography as described below. Since factor C moves very slowly in the standard solvent system and the 2 component factors, C₁ and C₃, are poorly separated (R₁ 0.09 and 0.15), the factors were eluted from the paper as a mixture; water was used as eluant.

Small amounts of benzimidazole-cyano-cobalamin were also prepared by bio-synthesis using E. coli. The procedure was as described for the preparation and isolation of factor C, except that in addition, 5 ml of an autoclaved solution of benzimidazole containing 1 mg per ml were added aseptically to the culture vessels before inoculation. The biosynthetic product was extracted from the cells as described above, chromatographed, and located by bioautography as the area at R₁ 0.43. The activity was eluted from the filter paper with water.

Factor B, 2-methyladenine-cyano-cobalamin, and 5,6-dimethylbenzimidazole-cyano-cobalamin were dissolved in 0.01 per cent KCN and the optical density of each was determined at a wave length of 367 mμ in a Beckman model DU spectrophotometer. The concentration of each factor was calculated by assuming that the specific extinction coefficient of each is the same as that of 5,6-dimethylbenzimidazole-cyano-cobalamin (Gregory and Holdsworth, 1953). Following standardization, solutions were diluted with 0.01 per cent KCN to a concentration of 5 μg per ml, and autoclaved. For microbiological testing, the sterile solutions were serially diluted with 30 per cent ethyl alcohol.

The small amounts of factor C and of benzimidazole-cyano-cobalamin isolated did not permit spectrophotometric standardization. Therefore, the concentration of these materials was determined as 5,6-dimethylbenzimidazole-cyano-cobalamin equivalents by filter paper disc assay with E. coli as described below. After standardization, these factors were diluted with 0.01 per cent KCN to a concentration of 5 μg of 5,6-dimethylbenzimidazole-cyano-cobalamin equivalents per ml.

Growing responses of Lochhead 38. The response of Lochhead 38 to the various cobalamins was measured by the filter paper disc method. A solution of 250 ml of the defined basal medium containing 1.5 per cent agar, was sterilized by autoclaving and cooled to 45 C. Inoculum (0.5 ml) prepared by suspending organisms from the stock culture in sterile 0.9 per cent NaCl to an optical density of 0.7 to 0.8 (measured at a wave length of 560 mμ) was then added. The mixture was poured to a depth of 2 to 3 mm on a glass plate upon which was mounted an 8 by 18 by 1 in stainless steel frame fitted with a cover (Esposito and Williams, 1952). Immediately before pouring the agar the apparatus was rinsed with 70 per cent alcohol and was rapidly air-dried; only very rarely was contamination encountered.

Filter paper discs (Schleicher and Schuell, no. 470 E) of 12.7 mm diameter were dipped into the standard alcoholic cobalamin solutions and were air-dried at 60 C. Discs were then placed on the solidified agar and the plates incubated for 72 hr at 30 C. The diameters of zones of growth were measured and the responses to graded cobalamin concentrations were recorded as zone diameters of growth in millimeters.

Cobalamin metabolism of Lochhead 38. The culture was grown in 2-L Fernbach flasks con-
taining 400 ml of defined basal media. Factor B, 2-methyladenine-cyano-cobalamin, factors C, and 5,6-dimethylbenzimidazole-cyano-cobalamin were each added aseptically from sterile stock solutions to individual flasks to give a final concentration of 10 μg per ml. As is usual practice in directed biosynthetic studies (Ford et al.; 1955; Perlman and Barrett, 1958) this concentration is several-fold greater than that required for optimal growth. The medium was inoculated with 0.5 ml of cell suspension and incubated, without shaking, at 30 C for 5 to 6 days. The cells were harvested and extracted for cobalamin content as described above for E. coli, and samples were spotted in 5-μL amounts to sheets of Whatman no. 3MM filter paper. Approximately 0.3 μg each of 2-methyladenine-cyano-cobalamin, factor B, factors C (C₁ and C₂), benzimidazole-cyano-cobalamin, and 5,6-dimethylbenzimidazole-cyano-cobalamin were applied as marker spots. Chromatograms were developed in the descending direction for 24 hr at 27 C in the standard solvent system. The papers were air-dried and the vitamin B₁₂-active materials visualized by bioautography with E. coli.

Bioautography. The defined medium of Burkholder (1951), supplemented with 2 μg per ml of NaCN and 1.5 per cent agar, was autoclaved and after cooling to 45 C; glucose and 2,3,5-triphenyl-2H-tetrazolium chloride (Ford and Holdsworth, 1953) prepared separately and sterilized by autoclaving, were added aseptically to final concentrations of 1 per cent and 0.8 mg per ml, respectively. The medium was seeded with approximately 1 ml of E. coli inoculum and was poured upon the steel frame-fitted glass plate.

Figure 1. Growth responses of Lochhead 38 to factor B, 2-methyladenine-cyano-cobalamin, and 5,6-dimethylbenzimidazole-cyano-cobalamin as measured by agar plate assay. Curve O, factor B; curve ●, 2-methyladenine-cyano-cobalamin; curve □, 5,6-dimethylbenzimidazole-cyano-cobalamin. Solid portion of the curves were obtained experimentally, whereas the dotted portion of each curve are extrapolations.
described above. Dried chromatograms were placed on the solidified agar and left there during the 12 to 24 hr incubation at 30 C. Areas of growth, causing reduction of the dye to a red color, indicated regions of vitamin B<sub>12</sub> activities.

RESULTS

Growth response to cobalamins. The vitamin B<sub>12</sub> requirement of Lochhead 38 is satisfied not only by 5,6-dimethylbenzimidazole-cyano-cobalamin, but also by factor B, factors C, and by 2-methyladenine-cyano-cobalamin. However, whereas the zone of growth produced with 5,6-dimethylbenzimidazole-cyano-cobalamin is sharply defined at its circumference, the growth zone produced with factor B is extremely diffuse. Zones of moderate to slight diffuseness are produced when factors C or 2-methyladenine-cyano-cobalamin are the cobalamin source.

Figure 1 illustrates typical agar plate assay curves of Lochhead 38 obtained with various concentrations of 5,6-dimethylbenzimidazole-cyano-cobalamin, 2-methyladenine-cyano-cobalamin, and factor B (the compounds which were standardized by spectrophotometric measurements). Although within the concentration ranges indicated by the solid curves in the figure, the response to each of the cobalamins is linear, the slope for each of the compounds is significantly different. These differences in slope may be due to variations in the rate of migration through agar of the various cobalamins. This variability might be eliminated by the use of liquid tube assays, but as pointed out by Goldberg et al. (1957), the Lochhead 38 organism

\[ \text{factor B} \]

\[ \text{5, 6- dimethyl benzimidazole-cyano-cobalamin} \]

\[ \text{benzimidazole-cyano-cobalamin} \]

\[ \text{2-methyladenine-cyano-cobalamin} \]

\[ \text{factor C<sub>2</sub>} \]

\[ \text{factor C<sub>1</sub>} \]

REFERENCE STANDARDS

<table>
<thead>
<tr>
<th>5, 6-dimethyl benzimidazole</th>
<th>2-methyladenine-cyano-cobalamin</th>
<th>factor B</th>
</tr>
</thead>
</table>

EXTRACTS OF LOCHHEAD 38 GROWN WITH

Figure 2. Representation of bioautographs of paper chromatograms of extracts of Lochhead 38 grown in the presence of various cobalamins.
frequently forms granular, nondispersible flocculates when grown in liquid culture. As a consequence it has not been possible in the present study to obtain reproducible assay curves for any of the cobalamins in liquid culture.

An estimate of relative biological potency of the various compounds tested can be made by extrapolating the observed curves to an ordinate value of 12.7, the diameter of the cobalamin-containing discs applied to the agar plates. At this point, differences in rates of diffusion are at a minimum. As is indicated in the dashed-lined portion of figure 1, 5,6-dimethylbenzimidazole-cyano-cobalamin and 2-methyladenine-cyano-cobalamin are approximately the same in potency, the difference between 0.39 and 0.57 mg of cobalamin at the “0 point” is considered to be within the limits of experimental error. Since the concentration of factor B is well above these values at this point, the factor is approximately one-fourth as effective as is 5,6-dimethylbenzimidazole-cyano-cobalamin for the growth of Lochhead 38.

At concentrations of 1 to 100 mg per ml of 5,6-dimethylbenzimidazole-cyano-cobalamin equivalents, factors C routinely produced zones of growth which were only a fraction the size formed with the standard 5,6-dimethylbenzimidazole-cyano-cobalamin. Since the factors C were not spectrophotometrically standardized, but were equated to 5,6-dimethylbenzimidazole-cyano-cobalamin by plate assay with E. coli, a procedure which introduces additional biological variables, it is not possible to correlate the activity of this factor for the growth of Lochhead 38 with the values obtained with the spectrophotometrically standardized cobalamins.

Metabolism of cobalamins. Figure 2 shows that factors C are present in cells of Lochhead 38 when the cobalamin requirement is supplied by 5,6-dimethylbenzimidazole-cyano-cobalamin, 2-methyladenine-cyano-cobalamin, or factor B. These are the only cobalamins, in addition to the one supplied for growth, detected in cells grown with 5,6-dimethylbenzimidazole-cyano-cobalamin. However, cells grown with either 2-methyladenine-cyano-cobalamin or factor B contain 3 additional chromatographically separable cobalamins, which have mobilities indicating that they are identical with benzimidazole-cyano-cobalamin, 2-methyladenine-cyano-cobalamin, and factor B.

Discussion

The observations on the nature of the growth responses of Lochhead 38 to various cobalamins are in agreement with the findings of Goldberg et al. (1957), who also reported the diffuse response to factor B. However, these authors attribute the diffuse nature of the growth zone to contamination of factor B with 5,6-dimethylbenzimidazole-cyano-cobalamin, and the present investigation indicates that this is not the case. Although as Kon (1955) has pointed out, “chromatographically pure” factor B may contain traces of 5,6-dimethylbenzimidazole-cyano-cobalamin, even if such a trace contaminant were present, it could not account for the magnitude of the growth response observed. Since factor B and 5,6-dimethylbenzimidazole-cyano-cobalamin apparently diffuse through agar at independent rates, it is evident that the growth response obtained with factor B cannot be accounted for as a 5,6-dimethylbenzimidazole-cyano-cobalamin contamination, since as indicated in figure 1, to produce a zone of growth equal in diameter to that obtained with 10 mg of factor B, requires approximately 100 mg of 5,6-dimethylbenzimidazole-cyano-cobalamin; physically impossible amount of contamination.

In addition, with regard to the diffuseness of the zone of growth obtained with factor B, it would be most unusual in a diffusion assay if contamination of an inactive material with traces of an active material created a diffuse zone of growth rather than a smaller zone of response. Rather, such diffuse zones of growth are, in general, usually a reflection of heterogeneity of the culture in response to a growth-limiting or growth-promoting factor. Thus, through the employment of colony selection, it has been possible to derive a substrain of Lochhead 38 which produced a more sharply defined zone of growth, similar to that obtained with 5,6-dimethylbenzimidazole-cyano-cobalamin.

Factor C is the principal product of the metabolism of factor B by E. coli (Ford and Porter, 1953; Brown et al., 1955) and, as shown in the present experiments, it is also formed by Lochhead 38 when grown with either 2-methyladenine-cyano-cobalamin or factor B. The appearance of factor C as the result of growth on 5,6-dimethylbenzimidazole-cyano-cobalamin is apparently unique; the ability of this molecule to stimulate the formation of other cobalamins...
by bacteria has not been previously reported. In addition, the appearance of factor B in cells grown with 2-methyladenine-cyano-cobalamin and of 2-methyladenine-cyano-cobalamin in cells grown with factor B, has not been detected with organisms previously studied.

SUMMARY

The cobalamin requirement for the growth of a soil microorganism previously reported to be specific for 5,6-dimethylbenzimidazole-cyano-cobalamin has been found to be satisfied also by 2-methyladenine-cyano-cobalamin, factor B, and by factor C.

Chromatographic examination of extracts of the soil organism indicates that when grown with 5,6-dimethylbenzimidazole-cyano-cobalamin, factor C is formed. Both factor C and benzimidazole-cyano-cobalamin are recovered from cells when either factor B or 2-methyladenine-cyano-cobalamin is supplied for growth. And in addition, factor B is recovered from cells grown with 2-methyladenine-cyano-cobalamin and this latter compound is also present in cells grown with factor B.

REFERENCES


