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MICROBIAL DEGRADATION OF CORRINOIDS

I. VITAMIN B_{12}

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ABSTRACT

SCOTT, W. M. (Wayne State University, Detroit, Mich.), R. C. BURGUS, J. B. HUFHAM, AND J. J. PFIFFNER. Microbial degradation of corrinoids. I. Vitamin B_{12}. J. Bacteriol. **88**:581-585. 1964.—Microorganisms isolated from a variety of soil, sewage, and mud samples, and stock cultures, were examined for the ability to degrade vitamin B_{12}. More than 200 isolates which attack the vitamin were examined, and they all demonstrated reversible fading of the red vitamin. The color was restored by aeration. Very few microorganisms were able to degrade the vitamin to permanently colorless products, although many were able to degrade it partially, to produce new pigments. Some of these pigments appeared similar, if not identical, although they were produced by different bacteria. Radiotracer and electrophoretic mobility data are presented to show that the transformation products are derived from the vitamin. All the degradative microorganisms isolated were bacteria, and the most active was *Pseudomonas rubescens*.

Numerous studies have been carried out on transformations of vitamin B_{12} (Brown and Reynolds, 1963; Stokstad, 1962), but no information is available on the metabolic degradation of the corrinoid nucleus. Corrinoids occur in the animal body only in trace amounts (Wokes and Smith, 1962); therefore, studies were undertaken on microbial degradation in the hope of obtaining degradation products in sufficient quantities for characterization and structural studies, as well as for use as reference compounds in metabolic studies in animals and man.

Sewage, faunal excreta, and the bodies of dead creatures should enrich the soil and waters with the vitamin, and it seemed likely that organisms capable of degrading the vitamin could be isolated from such sources. While our work was in progress, Helgeland, Jonsen, and Leland (1961) reported the production of two yellow pigments from vitamin B_{12} by a strain of *Aerobacter aerogenes* isolated from river mud. The present report is concerned with our search for microorganisms which have the ability to degrade vitamin B_{12}.

MATERIALS AND METHODS

Culture isolation. Nutrient broth (Difco) containing 1.0% glucose, and minimal salts medium (Davis and Mingioli, 1950), were employed. Vitamin B_{12} (Merck, Sharp and Dohme & Co., Inc., Rahway, N.J., or Squibb Institute for Medical Research, New Brunswick, N.J.) was either autoclaved separately or filter-sterilized. The vitamin was added to 5 to 10 ml of medium in test tubes (16 by 150 mm) to a final concentration of 5 to 10 mg per 100 ml of medium. This concentration was sufficient to give an observable red color.

The media were inoculated with soil, water, or sewage samples and were incubated at 30 C. The disappearance of the red color was used as presumptive evidence that the vitamin had been attacked. After several serial transfers, the cultures were streaked on agar plates and isolated colonies were picked.

In addition, a chemostat was constructed and was employed as a vitamin B_{12}-enrichment chamber. The growth chamber was inoculated with a soil or sewage sample and was continuously flushed with minimal salts medium containing vitamin B_{12} and a limiting concentration of glucose as the carbon sources. The effluent was sampled intermittently and new cultures were isolated and tested.

Cultures from the American Type Culture Collection and many natural isolates were tested for the ability to degrade vitamin B_{12}. The most active cultures were examined for degradation products.

Radiotracers. Co^{57}-labeled vitamin B_{12} was obtained from Abbott Laboratories, North Chicago,

Ill. C^{14}-labeled vitamin B_{12} was prepared by biosynthesis from δ-aminolevulinic acid-4-C^{14} (Miller and Rosenblum, 1960). *Propionibacterium freudenreichii* (ATCC 6207) was employed, and the synthesis was carried out by the method of Speedie and Hull (1960).

Extraction procedure. The test organism was allowed to grow in the presence of Co^{57}- or C^{14}-labeled vitamin until no observable red color remained. The cells were removed by centrifugation, the corrinoids were extracted from ammonium sulfate-saturated broth with two volumes of benzyl alcohol, and the emulsion was separated by centrifugation. Three volumes of diethyl ether and less than one-tenth volume of 0.001 M potassium cyanide were shaken with the alcoholic phase. The corrinoids passed into the aqueous phase. The water was evaporated under reduced pressure, and the residue was extracted with anhydrous methanol to separate the corrinoids from salt.

Paper electrophoresis. Electrophoresis of corrinoids was carried out on strips (1 by 35 cm) of Whatman no. 3 MM paper with free horizontal suspension of the paper. The best separations were obtained with the use of 1 N acetic acid containing 0.001 M potassium cyanide to prevent the formation of hydroxocorrinoids. Most separations took 3 hr at 500 v.

Assay of radioactivity. The electrophoresis strips were dried and taped to strips (2 by 25 cm) of Kodak No-Screen X-ray film, and the film was developed after 5 to 10 days of exposure. As little as 50 counts per min of Co^{57} or 15 counts per min of C^{14} activity could be detected as a spot on the film after 5 days of exposure. For semiquantitative measurements of the relative activities of spots on electrophoresis strips, the strips were cut into 1-cm^2 pieces, and the pieces were placed in planchets for counting. Radioactivity was determined by use of a thin-window, gas-flow, proportional counter (750 PF, Baird-Atomic, Cambridge, Mass.).

RESULTS

Reversible fading. Reversible fading of the red color of vitamin B_{12} occurred in over 200 cultures which attacked the vitamin. If the tube was closed tightly after growth occurred, the red color faded, leaving a medium that varied from brown to pale yellow. When air was present to allow growth, the red color often faded in the butt of the tube, but remained near the surface, where more oxygen was dissolved in the medium. Cultures which grew in a pellicle often faded the color near the surface. The time required for visual observation of the fading depended on the organism and the cultural conditions, but usually the fading occurred after 1 or 2 days.

If the tube was aerated soon after the red color had disappeared, the color was partially, if not completely, restored. The major recolorized product was red and behaved electrophoretically like vitamin B_{12}. A crystalline red product isolated from one recolorized culture was identical to vitamin B_{12} in activity towards *Lactobacillus leichmannii*, ultraviolet and visible spectrum, and electrophoretic mobility in 1 N acetic acid buffer.

Effect of light. The effect of light on recolorization was investigated. Three tubes containing 5 ml of nutrient broth plus 1.0% glucose and 0.25 mg of vitamin B_{12} per tube were inoculated with *Pseudomonas rubescens* (Pivnick, 1955; ATCC no. 12099). Two tubes were incubated in the dark at 30 C. When the control tube had faded, examination of one of the tubes incubated in the dark showed that it, too, had faded. Hence, light is not essential to the fading reaction. The second tube incubated in the dark was aerated in the dark, and on examination the red color had returned. Thus, the oxidation of the reduced product did not require light. The results were the same when the experiment was repeated with the use of culture RS2A1 in minimal salts medium.

Permanent fading. After prolonged incubation (5 days to several weeks), some of the cultures which initially exhibited reversible fading failed to recolorize to a comparable extent upon aeration. Many crude, mixed cultures from lake-bottom mud, sewage effluent or sludge, and various soils enriched with animal feces faded the red color irreversibly in 1 day. Isolates from these sources and stock cultures were much slower in their action than were the crude cultures. No organism able to use vitamin B_{12} as a sole carbon source was isolated from cultures enriched with the vitamin. All the isolates that attacked vitamin B_{12} required an energy source such as glucose. None of the isolates was able to fade the color of the vitamin under conditions of vigorous aeration.

Cultures that did not exhibit degradative activity were *Bacillus stearothermophilus*, *Clostridium haemolyticium*, *C. thermosaccharolyticum*,

Propionibacterium freudenreichii, *Pseudomonas fluorescens*, and *P. riboflavina*. None of the isolated molds was active, and several streptomycetes showed either very little activity or no activity. The cultures which showed the most activity were *P. rubescens* (ATCC 12099); Chemo-11 (soil isolate), D-37, and J-30 (air contaminants); and RS2A1 (sewage isolate).

Radioactive degradation products. Figure 1 shows the location of radioactive spots on electrophoresis strips spotted with extracts from several cultures. Table 1 shows the cultural conditions and percentage of recovered radioactivity found in the electrophoresis fractions. The results with either C^{14} or Co^{57} label demonstrate that the pigment fractions were derived from added vitamin B_{12} and were not synthesized de novo. With *P. rubescens*, comparable results were obtained when either label was used.

In our searches for degradation products, the extracted corrinoids were the most extensively studied, but other fractions of the fermentation broth were checked for radioactivity in a few cases. Among the cultures listed in Table 1, none of the whole-cell fractions or diethyl ether extracts of broth supernatant contained appreciable Co^{57} activity. After saturation of the supernatant fluid with ammonium sulfate and extraction with benzyl alcohol, the aqueous phases usually showed little radioactivity

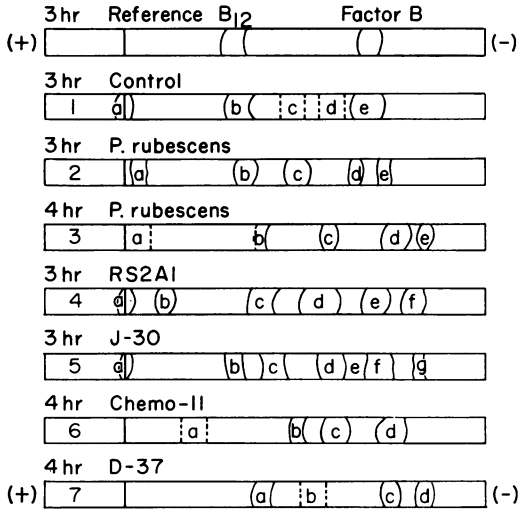

FIG. 1. *Electrophoresis of corrinoids in acetic acid-KCN buffer at 500 v. Time of each run is shown above strip. See Table 1.*

TABLE 1. *Distribution of radioactivity in corrinoid extracts*

Strip[a]	Organism and conditions[b]	Spot[a]	Color	Per cent recovered activity[c]
1	Control; C^{14}-vitamin B_{12}; 2,600 counts per min; nutrient broth plus glucose; 13 days	a	Colorless	1
		b	Red[d]	90
		c	Colorless	3
		d	Yellow?	3
		e	Pink[d]	3
2	*Pseudomonas rubescens;* C^{14}-vitamin B_{12}; 2,600 counts per min; nutrient broth plus glucose; 4 days	a	Colorless	12
		b	Red-orange	36
		c	Light yellow	13
		d	Orange	27
		e	Pink	12
3	*P. rubescens;* Co^{57}-vitamin B_{12}; ca. 200 counts per min; nutrient broth plus glucose; 13 days	a	Colorless	4
		b	Pink	28
		c	Light yellow	25
		d	Orange	34
		e	Pink	7
4	RS2A1; Co^{57}-vitamin B_{12}; 4,000 counts per min; minimal salts medium; 25 days	a	Light yellow	1
		b	Light pink	1
		c	Red	65
		d	Yellow	8
		e	Orange	16
		f	Pink	9
5	J-30; Co^{57}-vitamin B_{12}; 4,000 counts per min; minimal salts medium; 25 days	a	Yellow-brown	2
		b	Yellow	3
		c	Red	65
		d	Yellow	10
		e	Orange	10
		f	Orange	8
		g	Pink	1
6	Chemo-11; Co^{57}-vitamin B_{12}; 4,000 counts per min; minimal salts medium; 55 days	a	Colorless	12
		b	Red-orange	62
		c	Light yellow	20
		d	Light yellow	6
7	D-37; Co^{57}-vitamin B_{12}; 4,000 counts per min; nutrient broth plus glucose; 55 days	a	Red	71
		b	Light yellow	8
		c	Light yellow	11
		d	Pink	9

[a] See Fig. 1.

[b] All cultures were incubated at 30 C.

[c] Per cent total radioactivity counted on each strip. In control experiments, there was a large loss of radioactivity due to absorption by the paper. With C^{14} label, about 20% of the added radioactivity was detected on the strip; with Co^{57} label, about 50%.

[d] Electrophoresis was carried out in the presence of unlabeled vitamin B_{12} and factor B as internal reference compounds.

(RS2A1 was not tested). Levels varied from 1 to 4% of the original Co^{57} activity introduced as vitamin B_{12}. The one exception was one sample of Chemo 11, where the aqueous phase contained 36% of the original activity after 55 days of incubation. In agreement with the results with Co^{57} label, *P. rubescens* acting on C^{14}-vitamin B_{12} also showed little radioactivity in noncorrinoid fractions.

DISCUSSION

Although soil and water enriched with vitamin B_{12} yielded microorganisms capable of degrading the vitamin, some of the best degraders found were *P. rubescens*, which was isolated from cutting oil by Pivnick (1955), and some air contaminants encountered in the laboratory. Some of the degraders thus came from rather unlikely sources.

A common feature of more than 200 active cultures studied was their production of a reduced product(s), derived from vitamin B_{12}, which appears yellow and which turns red with aeration. In all cases, the red product seems to be identical to vitamin B_{12}, as judged by color and electrophoretic mobility. In one instance, the isolated crystalline product was proven to be identical with vitamin B_{12}.

Reports of the widespread occurrence of the coenzyme, and the suggestion of Helgeland et al. (1961) that one of their products might be a coenzyme analogue with increased stability to light, forced us to consider the involvement of vitamin B_{12} coenzyme in the reversible fading. The coenzyme is sensitive to light and remains brown after anaerobic exposure to light; the brown product recolorizes to red when air is admitted (Brady and Barker, 1961). Our cultures decolorized vitamin B_{12} anaerobically without exposure to light. Neither of the yellow-brown pigments that were observed was vitamin B_{12} coenzyme because they both showed slower electrophoretic mobility; furthermore, the faded product could be recolorized by air without exposure to light. Therefore, we concluded that we were not studying the coenzyme in our work.

The reversible fading of color should be distinguished from the permanent fading that occurs after more prolonged incubation. The complete degradation of the vitamin to colorless products was not observed in any of the studies in this report. On the other hand, several cultures that can produce yellow, orange, or red pigments from vitamin B_{12} were isolated. Their vitamin B_{12} origin was confirmed by the use of Co^{57}- or C^{14}-labeled vitamin.

P. rubescens, RS2A1, and J-30, yield two radioactive spots of yellow and orange color between vitamin B_{12} and a pink spot with mobility similar to factor B (Fig. 1). It seems likely, though not certain, that these two pigments, as well as factor B, are common degradation products that result from the action of many different microorganisms. Helgeland et al. (1961) previously reported two yellow-brown pigments, referred to as I and II, derived from vitamin B_{12} by *A. aerogenes*. These pigments have properties similar to the pigment fractions formed by our cultures of *P. rubescens*, RS2A1, and J-30. On the other hand, cultures of Chemo-11 and D-37 form pigment fractions, some of which differ from the above, judging by color and electrophoretic mobility. Of the cultures studied, *P. rubescens* attacked vitamin B_{12} most rapidly and gave the largest yields of degradation products.

Microbiological activities and chemical characterization of degradation products will be reported later.

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