Purification of the Vitamin B₁₂-Active Material from the Haemoflagellate, *Crithidia fasciculata*

By F. SANDERS AND G. R. SEAMAN

Carter Physiology Laboratory, University of Texas Medical Branch, Galveston, Texas, U.S.A.

(Received 4 December 1958)

Extracts of the haemoflagellate, *Crithidia fasciculata*, replace vitamin B₁₂ for the growth of *Escherichia coli* 113-3, for the soil bacterium referred to as 'Lochhead 38' and for *Euglena gracilis* (Seaman & Sanders, 1957). The substance differs, however, from members of the B₁₂ series of vitamins in that its activity is completely destroyed by exposure for 24 hr. at room temperature to pH 3.5 or 10.7 (Seaman & Sanders, 1957). The B₁₂ vitamins are stable at pH 3.5 and at pH 10.7 undergo only slight degradation, with the release of unidentified fragments which replace cyanocobalamin in the growth of *E. coli* 113-3; alkaline destruction of the *Crithidia* material does not release such fragments. In spite of these differences of stability in acid and alkali, the biological activity of the material from *Crithidia* suggests that it is a functional member of the B₁₂ series of vitamins.

The present report describes a procedure for the partial purification of the vitamin B₁₂-like material from *Crithidia* and describes some of its biological properties. The active material will be referred to as vitamin B₁₂(Cr). It is not suggested that this be adopted as a term for a newly described member of the vitamin B₁₂ series, but rather that the term is used merely for convenience of reference.

EXPERIMENTAL

Materials used and assay procedures

Crithidia fasciculata. Stock cultures were grown in tubes containing the yeast hydrolysate-liver concentrate-haemin medium described by Cowperthwaite, Weber, Packer & Hutner (1953). Cultures were grown at 25°, and transfers were made at monthly intervals. Large-scale cultures were grown for 4-5 days at 25° in stainless-steel drums (capacity 200 l.) containing 50 l. of the Cowperthwaite 'final' synthetic medium (Cowperthwaite *et al.* 1953) to which sterile glucose and vitamins were added after autoclaving. Each drum was inoculated with 1 l. of a 4- to 5-day-old culture which had been grown in the same synthetic medium. Aeration was provided by drawing air through the drums by means of a vacuum line.

To obtain information about the physiological importance of vitamin B₁₂(Cr), the effect of the purified vitamin on the growth of *Crithidia* was studied. Assays were carried out in 25 ml. nephelometer flasks containing 4 ml. of the 'final' synthetic medium of Cowperthwaite *et al.* (1953); sorbitol (1.5%, w/v) was used as energy source. The flasks were

covered with aluminium caps. Both flasks and caps were obtained from the Bellco Glass Co., Vineland, New Jersey, U.S.A. Flasks were inoculated with one drop of a 1:20 dilution of the stock culture, the diluent being the synthetic medium. The cultures were incubated at 25° and growth was periodically measured in a Klett-Summerson photometer equipped with a green filter (Klett-Summerson no. 65).

Vitamin B₁₂ assays. These were carried out by the filter-paper disk method with *E. coli* 113-3, details of which have been described (Sanders & Seaman, 1959). Purified samples of vitamin B₁₂(Cr) were assayed with the chrysomonad, *Ochromonas malhamensis*, which is specific in its requirement for cyanocobalamin (Ford & Hutner, 1955). Stock cultures of this organism were maintained on the basal medium described by Hutner, Bach & Ross (1956), supplemented with 0.1 µmg. of cyanocobalamin/ml. Assays were carried out in 25 ml. nephelometer flasks containing 5 ml. of basal medium and covered with aluminium caps. One drop of a 1:20 dilution of the stock culture was used as inoculum, the diluent being unsupplemented basal medium. Flasks were illuminated with a 40 w fluorescent lamp (Ken-Rad, F40, T-12, type CW) at a distance of 20 cm. and incubated for 5 days at 25°. Growth was measured in a Klett-Summerson photometer with a green filter (Klett-Summerson no. 65).

Extraction and purification of vitamin B₁₂(Cr)

Preliminary extraction. Haemoflagellates were collected by passage through a Sharples supercentrifuge at 4°. The organisms were washed by centrifuging twice with 0.1M-phosphate buffer, pH 8.5, once with 0.9% NaCl and once with water. They were then suspended in 0.03N-HCl to give a concentration of approx. 80 mg. of solids/ml. After the addition of 0.5 mg. of NaCN/ml., hydrolysis was carried out in a boiling-water bath for 45 min. After cooling to 4° the pH was adjusted to 4.5-5.0 with 2N-KOH and the insoluble material was removed by centrifuging.

Purification of extract. Ethanol (95%, v/v; 5 vol.) at 4° was added slowly to the extract with stirring, and the mixture was allowed to stand at 4° for 4 hr. The precipitate was removed by centrifuging and the ethanolic extract was evaporated to near dryness *in vacuo*. The residue was dissolved in a minimum volume of 3 mN-HCl and was extracted into 5 vol. of water-saturated phenol. The phenol-rich layer was extracted three times with equal volumes of water and the protein-free aqueous solutions were combined and evaporated to dryness *in vacuo*. The residue was dissolved in the minimum volume of 0.05M-phosphate buffer, pH 6.0. The redissolved extract was taken up in a small amount of ether-washed Celite 535 (Johns-Manville Co. Ltd.) and was placed on top of a 3 in. x 30 in. chromatographic column packed with Celite 535 (Swim & Krampitz, 1953). Several

Table 1. Summary of purification of vitamin B₁₂(Cr)

Vitamin activity was assayed by the filter-paper-disk method with *E. coli* 113-3.

Fraction	Total dry wt. of solids (mg.)	Total volume (ml.)	Total vitamin B ₁₂ activity (μmg.)	Activity per mg. dry wt. of solids (μmg.)
Cell extract	184800	2400	720	0.004
Phosphate extract	3720	620	625	0.17
Celite eluate	11	25	250	22.7

Fig. 1. Elution of vitamin B₁₂(Cr) from chromatographic column packed with Celite 535. The solvent system was the organic phase of a mixture of butan-2-ol-0.05 M-phosphate buffer (pH 6.0)-4% (w/v) KCN (100:50:0.25, by vol.). Vitamin B₁₂ activity was assayed by the filter-paper-disk method with *E. coli* 113-3.

litres of the organic phase of butan-2-ol-0.05 M-phosphate buffer (pH 6.0)-4% (w/v) KCN (100:50:0.25, by vol.) were passed through the column under a pressure sufficient to deliver 2 l. of eluate/hr., and collections were made of successive 1 l. volumes. Fig. 1 shows that the maximum vitamin B₁₂(Cr) activity was eluted between 8 and 12 l. These five fractions of tawny-coloured solution were combined and evaporated *in vacuo* to a volume of 25 ml. The concentrate was a reddish brown colour. The degree of purification achieved by the procedure is shown in Table 1.

RESULTS

Activity of vitamin B₁₂(Cr) for Ochromonas. Fig. 2 shows that purified vitamin B₁₂(Cr) replaces cyanocobalamin for the growth of *Ochromonas*; however, it is only one-hundredth as active, on the basis of cyanocobalamin equivalents for growth of *E. coli* 113-3. When added in the presence of cyano-

Fig. 2. Growth response of *Ochromonas malhamensis* to vitamin B₁₂(Cr) and to combinations of vitamin B₁₂(Cr) with cyanocobalamin. The amounts of added cyanocobalamin and vitamin B₁₂(Cr) were determined by assay with *E. coli* 113-3. ○, No added cyanocobalamin; ●, 0.01 μmg. of cyanocobalamin; ◐, 0.03 μmg. of cyanocobalamin; ◑, 0.1 μmg. of cyanocobalamin.

cobalamin, vitamin B₁₂(Cr) augments the growth response. As illustrated in the figure, augmentation is maximal at low levels of cyanocobalamin. As the concentration of cyanocobalamin is increased the stimulation by vitamin B₁₂(Cr) decreases, until at levels of cyanocobalamin which support half-maximal to maximal growth, addition of vitamin B₁₂(Cr) is without appreciable effect.

Effect of vitamin B₁₂(Cr) on growth of Crithidia. Fig. 3A shows that addition of vitamin B₁₂(Cr) to cultures of *Crithidia* shortened the lag period and increased the total growth. Although the shortening of the lag phase of growth is not proportional to the amount of vitamin B₁₂(Cr) added, Fig. 3B

Fig. 3. Effect of vitamin B₁₂(Cr) on growth of *Crithidia fasciculata*. The amounts of vitamin B₁₂(Cr) added were determined as vitamin B₁₂ equivalents by assay with *E. coli* 113-3 by the filter-paper-disk method. (A) Time course of growth response. ○, No added vitamin; ●, 0.1 μmg. of vitamin B₁₂(Cr); ◐, 0.3 μmg. of vitamin B₁₂(Cr); ◑, 1.0 μmg. of vitamin B₁₂(Cr). (B) Effect of vitamin B₁₂(Cr) on total growth (12 days' incubation) achieved by *Crithidia*. Data were taken from (A).

shows that the growth finally attained is dependent upon the concentration of added vitamin. Addition of either cyanocobalamin or factor B (the compound formed by removal of the nucleotide portion of cyanocobalamin) had no effect on the growth response.

It appears that vitamin B₁₂(Cr) is a growth factor for *Crithidia* and that the growing organisms are capable of synthesizing it in limited amounts. The growth of this organism is not stimulated by cyanocobalamin or by factor B. The vitamin is apparently also not identical with bipterin (2-amino-4-hydroxy-6-(1:2-dihydroxypropyl)-pteridine), the pteridine which under certain conditions is required for the growth of *Crithidia* (Patterson, Broquist, Albrecht, von Saltza & Stokstad, 1955) since in media containing large amounts of folic acid, as does the medium used in the experiments shown in Fig. 3, the pteridine requirement is abolished.

SUMMARY

1. The vitamin B₁₂ activity [vitamin B₁₂(Cr)] from the haemoflagellate, *Crithidia fasciculata*, has

been purified 5500-fold with a recovery of 34% of the initial activity present in cell extracts.

2. Vitamin B₁₂(Cr) is approximately one-hundredth as active as cyanocobalamin for the growth of *Ochromonas malhamensis*.

3. The growth of *Crithidia* is stimulated by vitamin B₁₂(Cr); the degree of stimulation is dependent upon the concentration of vitamin added.

This investigation was aided by a grant (E-159) from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service, and from the Elsa U. Pardee Foundation.

REFERENCES

- Cowperthwaite, J., Weber, M. M., Packer, K. & Hutner, S. H. (1953). *Ann. N.Y. Acad. Sci.* **56**, 972.
- Ford, J. E. & Hutner, S. H. (1955). *Vitam. & Horm.* **13**, 101.
- Hutner, S. H., Bach, M. K. & Ross, G. I. H. (1956). *J. Protozool.* **3**, 101.
- Patterson, E. L., Broquist, H. P., Albrecht, A. M., Saltza, M. H. von & Stokstad, E. L. R. (1955). *J. Amer. chem. Soc.* **77**, 3167.
- Sanders, F. & Seaman, G. R. (1959). *J. Bact.* (in the Press).
- Seaman, G. R. & Sanders, F. (1957). *Science*, **125**, 398.
- Swim, H. E. & Krampitz, L. O. (1953). *J. Bact.* **67**, 418.