Activities of various cobalamins for *Euglena gracilis* with reference to vitamin B₁₂ assay with *Euglena*

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SYNOPSIS Coenzyme B₁₂ and methylcobalamin in water are less active in promoting growth of *Euglena gracilis* Z strain than the same concentrations of cyanocobalamin and hydroxocobalamin which are equally active. When bound to human serum or human liver homogenate, however, the activities of these four cobalamins do not differ significantly with one exception. The results suggest that the *Euglena* assay using cyanocobalamin standards is not satisfactory for quantitation of coenzyme B₁₂ and methylcobalamin in water but acceptable when coenzyme B₁₂ and methylcobalamin are bound to serum or liver. Sulphitocobalamin in water is as active as cyanocobalamin and hydroxocobalamin but nitritocobalamin is less active. Factor B, the monocarboxylic acids of cyanocobalamin and hydroxocobalamin, and the dicarboxylic acid of cyanocobalamin in water were inactive.

The measurement of the 'vitamin P₁₂' concentration in tissues by microbiological assay using *Euglena gracilis* is a widely used procedure. *Euglena gracilis* variant *bacillaris* was used by Hutner, Provasoli, Stokstad, Hoffman, Belt, Franklin, and Jukes (1949) and by Ross (1950 and 1952) who described techniques applicable to clinical material and the Z strain was used by Hutner, Bach, and Ross (1956). Previous studies of the growth-promoting effects of cobalamins and other substances for *Euglena gracilis*, summarized by Smith (1965), preceded the discovery of the roles of methylcobalamin and coenzyme B₁₂ in human metabolism (Toohey and Barker, 1961; Lindstrand and Ståhlberg, 1963; Ståhlberg, Radner, and Nordén, 1967), and as the activity of these cobalamins and certain analogues for *Euglena gracilis* Z strain had not been studied, it seemed desirable to do so.

Materials and Methods

In all studies the assay methodology was essentially that of Hutner *et al* (1956) using *Euglena gracilis* Z strain and commercially available medium (Difco Laboratories Inc.). The incubation period was five days at a bath temperature of 29°C and illumination by two 'warm white' 30 watt fluorescent tubes. Samples were assayed in triplicate and the optical density of the cultures was measured in a Unicam SP 300 photometer using an Ilford 204 filter and cells with an optical path of 2.5 mm.

Received for publication 18 June 1970.

Solutions of cyanocobalamin, hydroxocobalamin, coenzyme B₁₂, methylcobalamin, sulphitocobalamin, nitritocobalamin, factor B, and the 'red acids'—the monocarboxylic acid of cyanocobalamin, the monocarboxylic acid of hydroxocobalamin, and the dicarboxylic acid of cyanocobalamin—were made available in known concentration by Dr L. Mervyn of Glaxo Ltd. Various batches of cyanocobalamin, hydroxocobalamin, methylcobalamin, and coenzyme B₁₂ were used, the concentrations being confirmed by absorption spectrometry with a Unicam SP 800 spectrophotometer. Unless otherwise stated, solutions were stored at +4°C and all manipulations except those immediately preceding microbiological assay were carried out in a dim red light.

Initially all samples were assayed on at least three occasions in final concentrations of 1.25, 2.5, 5, 10, 15, 25, and 50 µg/ml and the values compared to those obtained with cyanocobalamin at the same final concentrations. In view of the results further studies were undertaken as follows: (1) the effect of increasing concentrations of factor B and the 'red acids'; (2) repeated assays of aqueous solutions of coenzyme B₁₂ and methylcobalamin in concentrations of 750 µg/ml and 375 µg/ml; and (3) repeated assays of cyanocobalamin, hydroxocobalamin, methylcobalamin, and coenzyme B₁₂ in aqueous solution and bound to pools of serum and human liver homogenate. For the last-named study materials were prepared in a dim red light at 17°C by adding 4,000 µg cobalamin in 1 ml

Test Sample		Mean Values and Standard Deviation of Eight Assays ($\mu\text{g/ml}$ from cyanocobalamin standards)
Water (9 ml) + water (1 ml)		2.5
Water (9 ml) + cyanocobalamin	(1 ml - 4,000 μg)	458 (62.5)
Water (9 ml) + hydroxocobalamin	(1 ml - 4,000 μg)	392 (65.5)
Water (9 ml) + methylcobalamin	(1 ml - 4,000 μg)	293 (66.2)
Water (9 ml) + coenzyme B ₁₂	(1 ml - 4,000 μg)	313 (47.4)
Pool serum (9 ml)		
Pool serum + water (1 ml)		357 (64.0)
Pool serum + cyanocobalamin	(1 ml - 4,000 μg)	527 (89.3)
Pool serum + hydroxocobalamin	(1 ml - 4,000 μg)	538 (108.6)
Pool serum + methylcobalamin	(1 ml - 4,000 μg)	507 (111.4)
Pool serum + coenzyme B ₁₂	(1 ml - 4,000 μg)	504 (92.9)
Pool liver homogenate (9 ml)		
Pool liver + water (1 ml)		397 (39.3)
Pool liver + cyanocobalamin	(1 ml - 4,000 μg)	842 (219.2)
Pool liver + hydroxocobalamin	(1 ml - 4,000 μg)	720 (78.6)
Pool liver + methylcobalamin	(1 ml - 4,000 μg)	776 (80.0)
Pool liver + coenzyme B ₁₂	(1 ml - 4,000 μg)	710 (95.9)

Table I Constitution of test samples and mean values and standard deviation from eight assays¹¹Separate aliquot of each test sample and appropriate cyanocobalamin standards were assayed on each occasion.

water to 9 ml pooled tissue, and after mixing for 15 min, dispensing in aliquots of 1.25 ml in plastic tubes; dilutions of the same batch of cyanocobalamin used in the preparation of test samples were prepared for use as standard at the same time. Batches of standards, blanks, and test samples were then incubated at 17°C for 12 hours, exposed to daylight for 20 minutes, and then stored at -20°C in the dark until assayed. Each batch was assayed once and then discarded. Assays were performed at intervals of one week. The test samples were assayed at dilutions of 1:20 and the activity was compared to that of the cyanocobalamin standards which, after final dilution at 1:20, had values of 2.5, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, and 60 $\mu\text{g/ml}$. The serum pool was prepared from blood obtained from eight healthy subjects and was stored in glassware at -20°C. The liver homogenate pool was prepared from equal volumes of liver homogenates (1 g per 1,000 ml), the tissues being obtained at necropsy on eight patients who had died of cardiac or cerebrovascular disease and had not received antibiotics.

Results

The activities of aqueous solutions of hydroxocobalamin and sulphitocobalamin were always the same as those of similar concentrations of cyanocobalamin in the range 1.25 to 50 $\mu\text{g/ml}$ as judged by the optical densities of the cultures. Methylcobalamin, coenzyme B₁₂, and nitritocobalamin were always less active than corresponding concentrations of cyanocobalamin in the range 1.25 to 50 $\mu\text{g/ml}$

and their activities were about 65% that of cyanocobalamin.

Factor B and the red acids were inactive at low concentrations. At a concentration of 10,000 $\mu\text{g/ml}$ factor B had an activity equal to cyanocobalamin 10 $\mu\text{g/ml}$ and the monocarboxylic acid of cyanocobalamin at 100,000 $\mu\text{g/ml}$ was as active as cyanocobalamin 50 $\mu\text{g/ml}$. The monocarboxylic acid of hydroxocobalamin and the dicarboxylic acid of cyanocobalamin were inactive at concentrations up to and including 200,000 $\mu\text{g/ml}$.

An aqueous solution of coenzyme B₁₂ 750 $\mu\text{g/ml}$ assayed on six occasions gave a mean value of 483 $\mu\text{g/ml}$ (standard deviation 81.4) as expressed from the cyanocobalamin standards and a solution of 375 $\mu\text{g/ml}$ a value of 256 $\mu\text{g/ml}$ (standard deviation 65.0) the relative activities being 64% and 68%. Similarly, six assays of methylcobalamin 750 $\mu\text{g/ml}$ gave a mean value of 544 $\mu\text{g/ml}$ (standard deviation 123) an activity of 72% and methylcobalamin 375 $\mu\text{g/ml}$ a mean value of 232 $\mu\text{g/ml}$ (standard deviation 96) an activity of 62%.

The results for eight assays of cyanocobalamin, hydroxocobalamin, methylcobalamin, and coenzyme B₁₂ in aqueous solution and bound to pooled tissues are summarized in Table I. Analysis of the results by the Wilcoxon test (2 tail) showed significant differences in the results from aqueous solutions of cyanocobalamin and coenzyme B₁₂ ($P < 0.01$), cyanocobalamin and methylcobalamin ($P < 0.01$), hydroxocobalamin and coenzyme B₁₂ ($P < 0.01$), hydroxocobalamin and methylcobalamin ($P < 0.01$), and in the results from serum-bound hydroxocobalamin and serum-bound coenzyme B₁₂ ($P < 0.02$).

Discussion

The most interesting results are those relating to the 'physiological cobalamins'—coenzyme B₁₂, methylcobalamin, hydroxocobalamin, and cyanocobalamin. Photolysis of coenzyme B₁₂ and methylcobalamin leads to the formation of hydroxocobalamin which was found by Robbins, Hervey, and Stebbins (1950) to be as active as cyanocobalamin for *Euglena gracilis* var. *bacillaris* and confirmed for the Z strain in this study. The results of assays of several batches of coenzyme B₁₂ and methylcobalamin of known, that is spectrophotometric concentration, were therefore unexpected. Further enquiries were indicated because these results raised the suspicion that values obtained by *Euglena* assay of tissues were underestimates. Because of the considerable interassay variation (see Ross, Hutner, and Bach, 1957; Anderson, 1964) repetitive assays were necessary. The results formalized statistically the previous finding that coenzyme B₁₂ and methylcobalamin in water have an activity which is significantly inferior to similar concentrations of cyanocobalamin and hydroxocobalamin. In addition it is clear that this inferiority is abolished when the cobalamins are bound to serum or liver homogenate, the sole exception being serum-bound coenzyme and serum-bound hydroxocobalamin. Any explanation of the results must take into account the fact that hydroxocobalamin is a product of photolysis of coenzyme B₁₂ and methylcobalamin, and this suggests that other products of photolysis act as inhibitors to the growth of *Euglena* unless tissues are also present. Treatment of coenzyme B₁₂ in water with potassium cyanide, before and after photolysis, by prolonged photolysis in aerobic conditions, and by autoclaving at 15 lb for up to 30 minutes did not affect the activity, and attempts at isolating an inhibitor from solutions of photolysed coenzyme B₁₂ were unsuccessful.

From the practical aspect it is clear that quantitation of solutions of coenzyme B₁₂ and methylcobalamin in water by *Euglena* assay using cyanocobalamin or hydroxocobalamin standards will result in a significant underestimate of the true concentration. The absence of significant differences, with one exception, between cobalamins bound to serum or liver homogenate does not necessarily mean, however, that quantitation of serum or liver-bound coenzyme B₁₂ and methylcobalamin can be achieved with cyanocobalamin or hydroxocobalamin standards. Taking the mean values for cyanocobalamin or hydroxocobalamin, pool serum, and pool liver homogenate (Table I) the recovery rates for cyanocobalamin and hydroxocobalamin added to liver are 98% and 91% respectively

which give rise to confidence. With pool serum, however, the recovery rates for cyanocobalamin and hydroxocobalamin are 65% and 72%. Such low rates give rise to doubts about the quantitation of serum-bound vitamin B₁₂ but may, however, simply be manifestations of the inhibitory effect of serum on *Euglena* growth (Anderson, 1964).

The use of the conventional terms for concentration of cobalamins, that is mass of solute in volume of solution, was convenient in this study. On occasion this convention may result in misleading results and it is relevant to point out that calculation of the results in terms of molarity does not affect the conclusions.

There is no evidence that nitritocobalamin, factor B, and the three 'red acids' tested play a part in vitamin B₁₂ metabolism in man and the results are of little practical interest. We ascribe the trivial activities of the red acids and factor B as due to traces of cyanocobalamin and the results for factor B accord with those found by Coates and Kon (1957) using *Euglena gracilis* var. *bacillaris*. It seems inherently more likely that the lack of B activity of factor B and the 'red acids' tested is due to considerable differences in structure than to the presence of inhibitors.

We are grateful to Dr L. Mervyn, to Dr E. H. Hastings, and to Dr W. S. T. Thomson, for assistance. We acknowledge with thanks grants from the Secretary of State for Scotland and the Scottish Hospital Endowment Research Trust.

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