Crystalline Methylcobalamin from a Microorganism

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The isolation of methylcobalamin from a microorganism and liver has been briefly reported.[1] The work has been extended and it has been possible to obtain methylcobalamin in crystalline form. This report deals with the purification and characterisation of methylcobalamin from a microorganism.

Material and methods. The microorganism used was a patented organism, kindly supplied by Glaxo Research Ltd., Greenford, Middlesex, and can only be referred to as a microorganism. The organism was a good source of cobamide coenzyme analogues.

Methylcobalamin was synthesized as described by Lester Smith *et al.*[2]

Partition chromatography on Sephadex G25 in a mixed aqueous-organic solvent system. Dry Sephadex G25 (medium) was soaked in the solvent system to be used for partition chromatography, butanol-isopropanol-water (10:7:10 by volume) for several hours (decanted if necessary) and packed into a glass column.

The ionic exchange resins were repeatedly treated with alternating 0.5 M NaOH and 0.5 M HCl and washed with water in between. The resins were finally converted to the desired form with the appropriate solution and thoroughly rinsed with water until they were neutral.

Amberlite CG50/II was used in the sodium form; after sodium hydroxide treatment it was washed with 0.5 M NaCl before rinsing with water.

Absorbancy measurements for ultraviolet and visible light were carried out in a Unicam SP500 spectrophotometer. Samples (approximately 1 mg) for infrared measurements were pressed into tablets with sodium chloride and read in a Perkin-Elmer Model 21.

All operations were carried out in the dark or in dim light and all vessels were covered with black cloth or metal foil.

The microorganism was grown, harvested and extracted by Glaxo. Extraction of the coenzyme analogues was done immediately after the cells had been separated from the broth by suspending them in ethanol and heating to 80°C, keeping the solution at this temperature for 30 min.

The cell residue was separated and the clear solution (approximately 30 l) was concentrated by evaporation under reduced pressure to about 4 l when no ethanol remained. The B_{12} analogues were extracted with phenol and displaced from phenol back into water by addition of acetone and ether. Sometimes it was necessary to spin the mixtures in a centrifuge in order to break the emulsions.

Ether was removed from the aqueous solution and this was filtered through a thin layer of Kieselguhr in a Büchner funnel, then adjusted to pH 5.0 and fed onto a column, Dowex $50-Na^+$ (25×3.5), which was then washed thoroughly with water. The aqueous eluate was then fed onto a column containing Amberlite CG50/II$-Na^+$ (15×2) and the column was thoroughly washed with 0.01 M sodium acetate.

The cobamide analogue was adsorbed on Amberlite and then eluted with 1000 ml of 1 M sodium acetate and extracted with phenol in a separating funnel. The combined phenol solution was washed carefully with additional water in order to get rid of remaining salt. The coenzyme analogue was displaced back to water by addition of acetone and ether. The aqueous solution was washed twice with ether and finally concentrated to dryness by evaporation. The residue was dissolved in a minimal volume of solvent for partition chromatography (butanol-isopropanol-water, 10:7-10) and placed on top of a Sephadex G 25 (18×3) column. The chromatogram was developed with the same solvent mixture and the fractions collected in tubes (10 ml/tube). The first red zone was collected in tubes Nos. 7, 8, and 9. These were combined, an equal amount of ether was added, and the aqueous phase was separated in a separating funnel. The remaining ether was removed from the aqueous solution by evaporation. The water solution was then passed through Permutit (strongly basic) (5×1) and the aqueous eluate was lyophilized to dryness. The powder was dissolved in 0.2 ml of water, 1.8 ml of acetone was added and the solution was left overnight in a sealed tube. The crystals formed overnight were washed with a small amount of acetone and then with ether. The yield was 1.5 mg.

The 5′-deoxyadenosylcobamide coenzyme was recovered from the Sephadex G25 column, and was further purified and crystallized. 60 mg of crystalline coenzyme was obtained.

Results. It is known that preparations of mixed cobamides can crystallize and

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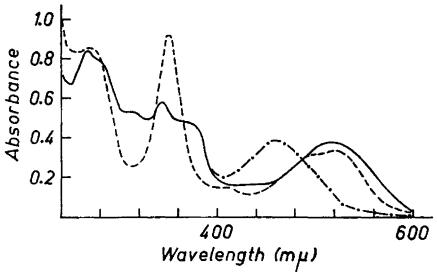

Fig. 1. Absorption spectra of methylcobalamin, from microorganism, in water (——); the same solution after acidification (-•-•-); then light treatment (- - -).

accordingly it is not a proof of purity if a crystalline product is obtained. In order to get further evidence that the isolated cobamide was homogeneous and to establish its identity, electrophoresis at different pH, chromatography, and spectroscopic investigation have been done.

Paper electrophoresis at pH 7.0 and 3.0 has been done and in both cases the cobamide preparation and synthetic methylcobalamin behaved exactly the same, *i.e.* both were neutral at the two pH's. No other visible or ultraviolet absorbing impurities could be detected.

Chromatography on Whatman No. 1 by the descending method in two different solvent systems gave identical R_F values for the cobamide preparation and methylcobalamin. The different solvents were *sec*-butanol:glacial acetic acid:water (100:3:50 by volume) and butanol:isopropanol:water (10:7:10 by volume). The R_F values were 0.45 in *sec*-butanol:acetic acid:water and 0.5 in butanol:isopropanol:water. In comparison, vitamin B_{12} had an R_F value of 0.3—0.35 in these solvents. No other visible or ultraviolet spots were observed.

Absorption spectra from synthetic and natural methylcobalamin were identical. After addition of a drop of 1 M acetic acid both absorption spectra changed in the visible region (Fig. 1). The cuvettes with the acidified solutions were left in the light and new absorption spectra were obtained. Both gave spectra similar to hydroxocobalamin in the ultraviolet and the visible region. However, a minor difference was found in infrared spectra (see discussion).

Discussion. The limited amounts of methylcobalamin available in this microorganism made it very difficult to estimate with accuracy the recovery of the substance and accordingly no attempt was made either to figure out the content of the crude extract or to estimate the losses during isolation procedure. However, the poor yield of methylcobalamin is not due to heavy losses during the isolation procedure, rather that methylcobalamin does not exist in substantial (though significant) amounts in this particular source.

No attempt to carry out an elementary analysis was made because of the small amounts available and also because Johnson *et al.*[3] could not get consistent analytical results of synthetic methylcobalamin. However, there can be little doubt about the identity of the isolated compound. The ultraviolet spectrum clearly bears a close resemblance to that of methylcobalamin and there is no indication whatsoever of any purine moiety or other group attached to the cobamide. The change of colour in weak acid and the change of spectrum after light treatment as well as electrophoresis and chromatography gave further proof that it is methylcobalamin.

A slight difference in infrared spectra could be detected. Synthetic methylcobalamin gives a peak at 850 cm^{-1}, which is more pronounced than at 865 cm^{-1}. This was reversed with methylcobalamin from the microorganism. However, hydroxocobalamin shows a distinct peak at 865 cm^{-1} and it was presumed that some methylcobalamin had been converted to hydroxocobalamin which could explain the discrepancy. Reextraction of the sodium chloride disc and chromatography of the extracted cobamide confirmed the suspicion that some hydroxocobalamin had been formed although there was no sign of it before it was pressed into the disc.

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2. Lester Smith, E., Mervyn, L., Johnson, A. W. and Shaw, N. *Nature* **194** (1962) 1175.
3. Johnson, A. W., Merwyn, L., Shaw, N. and Lester Smith, E. *J. Chem. Soc.* **1963** 4146.

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