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ISOLATION OF ^{57}Co-COBALAMIN COENZYMES AT HIGH SPECIFIC ACTIVITY FROM *STREPTOMYCES GRISEUS*

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SUMMARY

The distribution of radio-labelled cobalamins in *Streptomyces griseus* grown in medium containing ^{57}Co-cobalt chloride has been estimated by two-dimensional thin-layer chromatography and bioautography. ^{57}Co-Methylcobalamin (Me[^{57}Co]Cbl) was the major form in the mycelium together with smaller amounts of ^{57}Co-adenosylcobalamin (Ado[^{57}Co]Cbl) and ^{57}Co-hydroxocobalamin (OH[^{57}Co]Cbl). The OH[^{57}Co]Cbl was detected in three forms having, respectively, anionic, cationic and neutral properties.

A simple technique has been developed to isolate and purify Me[^{57}Co]Cbl and Ado[^{57}Co]Cbl from the mycelium using column chromatography on ion-exchange celluloses. Small quantities of each cobalamin coenzyme have been obtained at 90–96% purity and specific activities of 190–230 μCi/μg.

INTRODUCTION

In man and other species the bulk of the cobalamin within the body occurs as 5-deoxyadenosylcobalamin (Ado-Cbl) and methylcobalamin (Me-Cbl) both of which have coenzyme functions. Me-Cbl is the co-factor for N^5-methyltetrahydrofolate-homocysteine methyltransferase (EC 2.1.1.13) responsible for the transmethylation of homocysteine to methionine[1,2]. Ado-Cbl is required as coenzyme with methylmalonyl CoA-mutase (EC 5.4.99.2) for the isomerisation of methylmalonyl-CoA to succinyl-CoA[3,4]. The importance of these reactions is attributable to their involvement in folate and propionate metabolism which in turn influence DNA synthesis and,

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it has been suggested[5], myelin synthesis also. Radioisotopically labelled cobalamins are of considerable value in studying reaction mechanisms in which cobalamins may be involved and when combined with the use of cobalamin markers separated by thin-layer chromatography (TLC) and located by bioautography[6,7] detection of individual cobalamins in amounts down to 10^{-15} g is quite feasible. Cyanocobalamin (CN-Cbl) and hydroxocobalamin (OH-Cbl) have been produced commercially, labelled with ^{57}Co, ^{58}Co or ^{60}Co but CN[^{57}Co]Cbl is the only form of the vitamin available at sufficiently high specific activities for metabolic studies under physiological conditions. Radio-active Me-Cbl and Ado-Cbl may be prepared from labelled CN-Cbl by chemical methods but the specific activities are generally low, which limits their suitability for metabolic studies. Until now radiolabelled cobalamin coenzymes have not to our knowledge been obtained directly from a biosynthetic source. The present communication describes a simple technique using ion-exchange chromatography on small columns of substituted celluloses to isolate from *S. griseus* Me[^{57}Co]Cbl and Ado[^{57}Co]Cbl at high specific activity and purity.

MATERIALS AND METHODS

S. griseus was grown to confluence by subdued light in a medium containing ^{57}Co cobalt chloride (approximately 7 μg/l at 7000 Ci/g). The mycelium was centrifuged down, washed with isotonic saline and stored at −20°. Cobalamins were extracted from the mycelium with hot ethanol (70°). The ethanolic extract was evaporated to dryness and the cobalamins redissolved in 500 μl water[6,8]. Cobalamins in the mycelial extract were separated by two-dimensional chromatography on a thin layer of CM-cellulose–silica gel and identified bioautographically using a cobalamin-responsive strain of *Escherichia coli* and a tetrazolium growth indicator[9]. The extraction and all chromatographic procedures were carried out by red light or in darkness to prevent photolytic changes in the cobalamins.

Chromatography columns (8.0 × 0.7 cm I.D.) were filled either with diethylaminoethylcellulose (Whatman DE-32) or carboxymethylcellulose (Whatman CM-32). A visible loading of cobalamin markers (approximately 100 μg each of Me-Cbl, Ado-Cbl, OH-Cbl and CN-Cbl in 200 μl water) was applied to a DE-32 column and eluted with water. Fifteen 0.5-ml fractions were collected at a flow-rate of 0.5 ml/min and the optical density of each was measured at 361 nm in a Unicam SP 500 spectrophotometer. A further aliquot of the visible cobalamin mixture was chromatographed on a CM-32 column, eluting first with water, 80 fractions (0.25 ml each) being collected, then with 0.01 *N* HCl, and a further 40 (0.25 ml) fractions collected. Peaks were identified from the optical density of the fractions at 361 nm. Since the mycelium available contained only 10–20 ng total cobalamin the above procedure was repeated with a smaller loading of cobalamin markers (10 ng of each in 200 μl water) to see whether this affected the separation. Peaks were located from the total cobalamin concentration of each fraction estimated by radioisotopic assay[10].

Mycelial extract (200 μl) was then applied to a DE-32 column and the cobalamins eluted with water, as above. Fractions were counted in an auto-γ-spectrometer and those containing radioactivity were pooled and evaporated to dryness. The deposited film was redissolved in water (200 μl), the cobalamins were separated on a CM-32 column as described above and the peaks identified from the radioactivity of

the fractions. Each cobalamin peak was then concentrated by rotary evaporation, checked for purity by thin-layer chromato-bioautography, and the radioactivity in each zone estimated. To minimise the degradation of Ado-Cbl which occurred during storage at low pH, the pooled fractions in which Ado-Cbl eluted were adjusted to pH 6.0 without delay, using 0.1 *N* NaOH. Nevertheless, since the purity of Ado-Cbl obtained at this stage was low (*ca.* 50%), the following steps were introduced to improve this. Ado-Cbl-containing fractions were evaporated to dryness and redissolved in 0.05 *M* sodium metabisulphite (200 μl). After standing at room temperature for 15 min the mixture was applied to a fresh DE-32 column. Fractions (0.5 ml) were collected as above and the radioactivity in each was estimated. A single peak was obtained containing Ado-Cbl at high purity. The specific activities of Me-Cbl and Ado-Cbl were calculated from the total cobalamin estimated microbiologically using *Euglena gracilis* and the radioactivity compared with that of a standard CN[^{57}Co]Cbl sample of known specific activity.

RESULTS

Estimation of cobalamins in S. griseus

Thin-layer chromato-bioautography showed that the *S. griseus* mycelium contained Me-Cbl, Ado-Cbl and OH-Cbl (Fig. 1), the cobalamins normally present in human tissues. In all five batches of mycelium, Me-Cbl was the major form, accounting for between 39 and 49% of the total cobalamin (Table I). The remainder comprised Ado-Cbl and OH-Cbl in almost equal proportions. Estimates of the radioactivity in each growth-zone showed that 44–58% was associated with Me-Cbl, 23–31% with Ado-Cbl and 10–20% with OH-Cbl (Table I). A small proportion of the radioactivity on the chromatogram (5%) was detected close to the origin. Later investigation showed

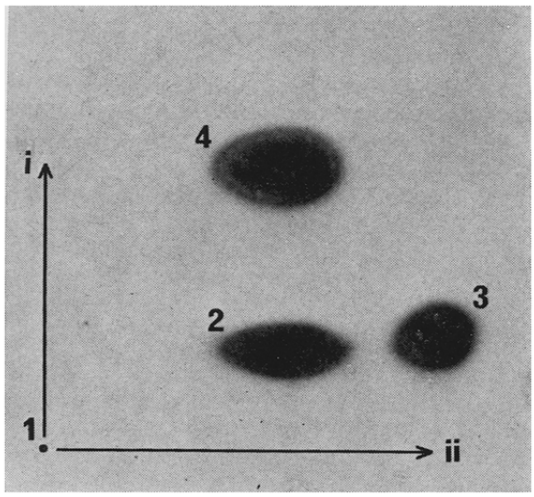

Fig. 1. Bioautogram of cobalamins from *S. griseus* separated by two-dimensional chromatography in: (i) *sec.*-butanol–water–0.880 ammonia (72:25:2); (ii) water saturated with benzyl alcohol. Zones: 1 = origin; 2 = Ado-Cbl; 3 = OH-Cbl; 4 = Me-Cbl. Zone 1 corresponds also to the cobalamin eluting as OH-Cbl$_2$ from the CM cellulose column. Zone 3 corresponds to that eluting as OH-Cbl$_1$.

TABLE I

DISTRIBUTION OF ^{57}CO-COBALAMINS IN *STREPTOMYCES GRISEUS* MYCELIUM

No CN-Cbl zone was detected bioautographically in any mycelial sample but in 2 of the 5 batches approximately 5% of the total radioactivity on the chromatogram was recovered in the CN-Cbl position.

Method of estimation	*Total mycelial cobalamin (%)*			
	Me-Cbl	*Ado-Cbl*	*OH-Cbl**	*Zone at origin***
Densitometric scanning of bioautogram	43 ± 1.9***	27 ± 1.1	26 ± 1.5	—
γ-Counting of chromatogram	52 ± 2.7	24 ± 2.5	17 ± 3.0	4.8 ± 1.4

* Corresponds to the peak separated by CM column chromatography as OH-Cbl$_1$.
** Corresponds to the peak separated by CM column chromatography as OH-Cbl$_2$.
*** Mean ± SEM ($n = 5$).

that this was attributable to a cationic form of OH-Cbl. In two of the 5 batches approximately 5% of the radioactivity was recovered in the CN-Cbl position though no growth-zone corresponding to CN-Cbl was detected in any sample.

Column chromatography of cobalamin markers

Visible loadings of Me-Cbl, Ado-Cbl, CN-Cbl and OH-Cbl (100 μg of each) eluted from DE-cellulose as a single peak in fractions 3–10. On CM-cellulose, CN-Cbl eluted in fractions 11–15 and Me-Cbl in fractions 50–72. Ado-Cbl and OH-Cbl remained on the column. These were eluted with 0.01 *N* HCl, OH-Cbl appearing in fractions 9–17, Ado-Cbl in fractions 19–28.

When small amounts (10 ng) of Me-Cbl, Ado-Cbl and CN-Cbl markers were chromatographed on CM-32 cellulose columns R_F values were found to be very similar to those of higher (visible) loadings of these cobalamins, but small amounts (10 ng) of OH-Cbl behaved differently. The majority (80%) appeared as a peak before CN-Cbl during elution with water (OH-Cbl$_1$). The remainder (19%) eluted with 0.01 *N* HCl just ahead of Ado-Cbl (OH-Cbl$_2$). This amphoteric behaviour of OH-Cbl was probably due to the equilibrium between hydroxocobalamin and aquocobalamin, the relative proportions of the two forms depending on the OH-Cbl concentration and the pH of the solution. Aquocobalamin probably predominates at low pH[11]. In our experiments aquocobalamin was represented by the peak eluting with acid from CM-32 and designated OH-Cbl$_2$.

Column chromatography of mycelial cobalamins

When chromatographed on DE-32, cobalamins in mycelial extract eluted from the column as a single peak in fractions 3–10. Approximately 40% of the total radioactivity was retained by the column. Non-cobalamin material in the alcoholic extract accounted for much of this radioactivity but further investigation revealed that it also included an anionic form of OH-Cbl (Fig. 2) which on CM-cellulose eluted as OH-Cbl$_1$ (Fig. 3). None of the other cobalamins were retained by DE-32. After concentrating the peak from the DE-32 column and applying it to a CM-32 column, a series of peaks of radioactivity eluted (Fig. 3) with R_F values very similar to those for small amounts of unlabelled cobalamin markers. The residual radioactivity retained by the

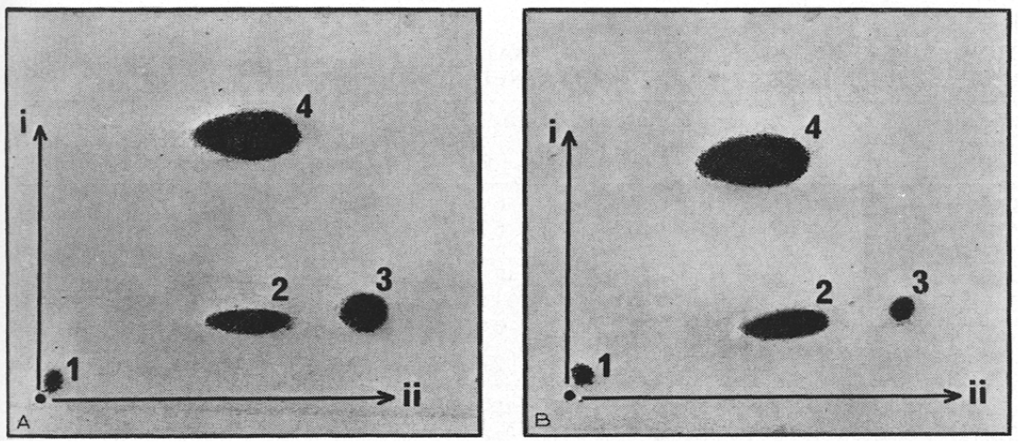

Fig. 2. TLC of cobalamins from mycelial extract (A) before and (B) after elution from a DE-32 cellulose column to remove anions. Solvent systems and key to zones as in Fig. 1.

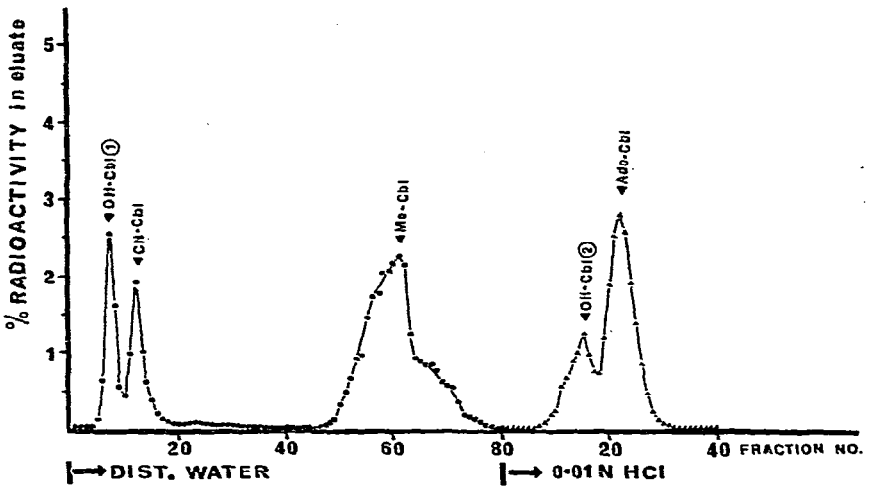

Fig. 3. Column chromatography on CM-32 cellulose of ^{57}Co-cobalamins in the mycelial extract after elution from DE-32 cellulose.

CM-32 column was less than 2% of that applied. Each peak when concentrated and re-chromatographed by two-dimensional TLC showed on bioautography a growth-zone corresponding to the cobalamin eluted from the column. OH-Cbl$_1$ had a high mobility in the second solvent, OH-Cbl$_2$ a low one (Fig. 1). To estimate the radioactive purity of each cobalamin, further two-dimensional thin-layer chromatograms were tun, overspotting with the four unlabelled cobalamin markers (50 pg of each). Most of the radioactivity was detected in the zone corresponding to the respective cobalamin peak (Table II). Values thus obtained for the recovery of OH-Cbl$_1$ and CN-Cbl were 75% and 63% respectively. Of the OH-Cbl$_2$ peak, 62% was recovered at the origin. Purity of Ado-Cbl was lower (49%) since a substantial proportion of OH-

TABLE II

RADIOACTIVE PURITY OF MYCELIAL COBALAMINS ELUTED FROM A CM-32 CELLULOSE COLUMN AND CN[^{57}Co]Cbl OBTAINED COMMERCIALLY

Eluted Peak	*Total radioactivity recovered* (%)*				
	Me-Cbl	*CN-Cbl*	*Ado-Cbl*	*OH-Cbl*	*Zone at origin*
CN-Cbl	2.2	62.8	6.8	25.3	2.9
OH-Cbl$_1$**	1.9	6.8	6.3	74.8	10.2
OH-Cbl$_2$***	3.6	4.8	7.2	22.2	62.2
Ado-Cbl	1.5	4.6	48.5	12.0	33.4
Purified Ado-Cbl§	0	0.7	93.8	3.6	1.9
Me-Cbl	93.7	1.0	0.7	3.2	1.4
Commercial CN-Cbl	0.3	90.2	2.4	4.3	2.8

* Estimated by thin-layer chromato-bioautography and γ-spectrometry.
** Eluted from CM-32 with water in fractions 5–9.
*** Eluted from CM-32 with 0.01 *N* HCl in fractions 9–17.
§ After metabisulphite treatment and rechromatography on DE-32.

Cbl$_2$ (33%) eluted from the CM-32 column with an R_F close to that of Ado-Cbl and the two were incompletely resolved (Fig. 3). After treatment of the pooled fractions containing Ado-Cbl and OH-Cbl$_2$ with sodium metabisulphite and re-chromatographing on a DE-32 column, Ado-Cbl was eluted at high purity (Table II), OH-Cbl$_2$ having been converted to sulphitocobalamin (SO_3-Cbl) and retained by the column.

Me[^{57}Co]Cbl and Ado[^{57}Co]Cbl were thus isolated at radioactivity purities ranging from 90–96%, very similar to those of commercially produced CN[^{57}Co]Cbl (Table II). Specific activities of the final products were estimated on two of the batches of mycelium. Values obtained were 200 and 220 μCi/μg for Me-Cbl, 227 and 191 μCi/μg for Ado-Cbl.

DISCUSSION

Radiolabelled cobalamin coenzymes have not, to our knowledge, previously been obtained directly from a microbial source. Their ready accessibility should be of value in a wide range of studies of cobalamin and folate metabolism. The isolation of OH[^{57}Co]Cbl at high specific activity during this study made it possible for us to investigate in some detail the chromatographic behaviour of this cobalamin which has for many years complicated the separation of cobalamins[6,13,17]. Elution from CM-32 cellulose of two OH-Cbl peaks of widely differing R_F was initially difficult to explain. The first peak (OH-Cbl$_1$) eluted readily from the column with water, was relatively stable, did not react with ammonia or sodium metabisulphite and appeared to be influenced little by changes in pH. The second peak (OH-Cbl$_2$) was more strongly bound to the column and required dilute hydrochloric acid as eluent. Its higher reactivity was demonstrated by the effects of pH, ammonia and sodium metabisulphite, which suggested that this second form of OH-Cbl was capable of behaving as an acid or base under various conditions. Differences were also conspicuous on two-dimensional thin-layer chromatography (2-D TLC). Though both forms remained close to the origin in the first solvent system (*sec.*-butanol–ammonia), in the second

(water saturated with benzyl alcohol), OH-Cbl$_2$ had a low R_F whereas OH-Cbl$_1$ moved almost with the solvent front, its position corresponding to that of OH-Cbl previously reported in animal[15] and human[9,16] tissues, and to SO_3-Cbl reported in a wide range of animal food products[14,17]. 2-D TLC of mycelial extract before and after passing it through DE cellulose indicated that the high R_F OH-Cbl spot consisted of an anionic form retained by the column together with a neutral form which readily eluted (Fig. 3). Taken together these findings suggest that "OH-Cbl" detected in *S. griseus* is present in anionic, cationic and neutral forms.

The finding that Me-Cbl is the major cobalamin in *S. griseus* contrasts with the situation in *E. gracilis* (Carell et al.[18]) and in animal and human cells, where Ado-Cbl predominates[15,16,19]. The difference may be related to the fact that unlike the situation in higher animals, *S. griseus* achieves total synthesis of the cobalamin molecule. Under the culture conditions provided, Me-Cbl synthesis appears to be favoured.

Increases in tissue Me-Cbl have been reported in developing tumours[20], and much higher proportions of Me-Cbl have been found in human foetal tissues than in the respective tissues from children or adults[21]. This finding correlates well with levels of the Me-Cbl-dependent enzyme N^5-methyltetrahydrofolate-homocysteine methyltransferase which are highest in the foetus and decline over the first few months of life[22]. Recent studies in the mould *Rhizobium meliloti* show that methionine synthesis via the cobalamin-dependent methionine synthetase pathway is important for growth of this micro-organism which is stimulated by the addition of inorganic cobalt[23]. It may well be that in *S. griseus* the high proportion of Me-Cbl reflects the importance of the methionine synthetase pathway in this organism also.

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