Isolation and Properties of Crystalline Cobamide Coenzymes Containing Benzimidazole or 5,6-Dimethylbenzimidazole*

H. A. Barker, R. D. Smyth, H. Weissbach,† J. I. Toohey, J. N. Ladd,‡ and B. E. Volcani§

From the Department of Biochemistry, University of California, Berkeley, California

(Received for publication, September 11, 1959)

The isolation of three cobamide coenzymes has been briefly reported (1, 2). Methods used in the assay and purification of the adenylcobamide coenzyme have been described in some detail (3). This paper describes methods used in the isolation of the benzimidazolylcobamide and 5,6-dimethylbenzimidazolylcobamide coenzymes, both of which have been obtained as crystalline products. Some properties of the coenzymes are also reported.

EXPERIMENTAL

Materials and Methods

Bacteria—Clostridium tetanomorphum strain H1 and Propionibacterium shermanii ATCC 9614 were used as sources of the coenzymes. C. tetanomorphum was grown in a glutamate-glucose-yeast extract medium (3), modified by the inclusion of $10^{-4}$ M benzimidazole or its 5,6-dimethyl derivative as required. P. shermanii was grown under anaerobic conditions in a medium containing glucose, cornsteep liquor, cobaltous nitrate, and buffer. To avoid excessive acidification of the medium during fermentation, the culture was stirred continuously and was neutralized at least twice daily to pH 7.6 with 2 N NaOH. Cultures were incubated for 4 to 6 days at 30° before harvesting the cells with a Sharples centrifuge. The cell paste was frozen as soon as it was removed from the centrifuge and was stored at $-10^\circ$ until used.

Chemical and Physical Methods—Most of the methods used in this investigation have already been reported (3). Additional methods are described or referred to in appropriate sections of the text.

Coenzyme Activity Assay—The activity of cobamide coenzyme preparations was estimated by the spectrophotometric assay, based on the catalytic function of the coenzyme in the enzymatic conversion of glutamate to mesaconate (3). Activity measurements can be used to determine coenzyme concentration by direct comparison with the activity of a sample of purified coenzyme of known concentration. Without such a standard only relative coenzyme concentration can be determined. The molar activities of the BC and DBC coenzymes are approximately 5 and 0.1 times as great, respectively, as that of the AC coenzyme (see Table VI). These relative molar activities are applicable only when activity comparisons are made with data obtained from the linear portion of the rate-coenzyme concentration curves.

Estimation of Coenzyme Concentration—The concentration of purified coenzyme preparations, containing only a single colored component, was estimated by absorbancy measurements at 519 mμ or 261 mμ for the BC coenzyme and at 522 mμ or 260 mμ for the DBC coenzyme in 0.01 M potassium phosphate buffer, pH 6.7 to 6.8. The corresponding molar extinction coefficients are given in Table V.

Isolation of Coenzymes

The BC and DBC coenzymes were isolated by small modifications of the methods used to obtain the AC coenzyme (3). The former coenzymes are slightly less basic than the AC coenzyme and therefore are eluted from a Dowex 50, pH 3 column somewhat more rapidly by a sodium acetate buffer between pH 6 and 7. Also, the BC and DBC coenzymes, unlike the AC coenzyme, crystallize readily from water or water-acetone mixtures. All operations were carried out in the dark or in very dim light. The temperature of solutions was kept at 0-4°, except that phenol extractions were done at room temperature, 15-25°.

BC Coenzyme—As starting material, 3.86 kg of frozen cells of C. tetanomorphum, grown in the presence of benzimidazole, were used.

The first four steps (ethanol extraction, treatment with Dowex 50-Na+, treatment with Dowex 2-OH−, and phenol extraction and displacement back into water) were done as previously described (3). The resulting aqueous solution contained 59 μmoles of coenzyme in 70 ml. The solution was acidified to pH 3.2 with 7 ml of 1 N HCl and was then diluted to 400 ml to reduce the salt concentration.

The acidified coenzyme solution was passed into a 1 cm diameter × 80 cm high column of Dowex 50, pH 3, 2% cross linked, 200 to 400 mesh, prepared as previously described (3). After collection of the “pass through” solution (Fractions 1 to 37), the column was eluted first with 0.05 M sodium acetate, pH 5.2

The abbreviations used are: BC, benzimidazolylcobamide; DBC, 5,6-dimethylbenzimidazolylcobamide; AC, adenylcobamide.
FIG. 1. Isolation of BC coenzyme on a Dowex 50W column

(Fractions 38 to 134), and then with 0.03 m sodium acetate, pH 6.2 (Fractions 135 to 240). The volume of each fraction was about 16 ml. The coenzyme eluted in a well isolated and rather symmetrical 260 m\(\mu\) absorbancy peak located between Fractions 175 and 195 (Fig. 1). The peak fractions were reddish orange in color.

Coenzyme activities of alternate peak fractions were determined and their apparent specific activities were calculated. The results (Table I) show that the fractions containing relatively high concentrations of cobamide had fairly constant apparent specific activities, indicating that they were relatively homogeneous with respect to light-absorbing components.

Fractions 178 through 193, containing an estimated 53 \(\mu\)moles of BC coenzyme in 284 ml, were combined and the coenzyme was extracted into phenol and displaced back into water by the addition of ether-acetone (3). The resulting solution, containing 48 \(\mu\)moles of coenzyme in a volume of 8 ml, was placed in a vacuum desiccator over concentrated sulfuric acid and left at 4\(^\circ\). After 2 days the volume had been reduced to about 4 ml and the walls and bottom of the container were found to be incrusted with small rectangular or diamond-shaped crystals which were either red or yellow in color depending upon the angle of view (Fig. 2A). The solution was left under reduced pressure for another day during which time it concentrated to approximately 2.5 ml and additional crystalline material separated. The mother liquor was removed and the crystals were vacuum dried and weighed. The crystals were transferred to a vacuum desiccator and allowed to stand for several days and the crystal habit and color changed.

FIG. 2. Crystals of the BC and DBC coenzymes. A shows the BC coenzyme obtained by slow evaporation of an aqueous solution at 4\(^\circ\). The crystals were washed with acetone and ether and photographed in the dry state. Magnification 340 X. B shows the DBC coenzyme crystallized from 87.5% acetone at 4\(^\circ\). The crystals were photographed in the mother liquor. Magnification 63 X.
washed with 1 ml of 90% acetone, 1 ml of 100% acetone, and finally with several milliliters of ethyl ether. The solid material was placed in a vacuum desiccator for a few minutes to remove ether. The resulting product, which contained 11.8% moisture removably by prolonged drying under reduced pressure at 4°C over P₂O₅, weighed 68.7 mg and was estimated to contain 38.2 μmol of coenzyme. An additional 3 to 4 μmol of crystalline coenzyme were later recovered from the mother liquor of the first crop of crystals.

Table II gives data on the volume of solution and amounts of coenzyme at each step in the isolation.

### Table II

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume of solution (ml)</th>
<th>Quantity of coenzyme (μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ethanol extraction</td>
<td>1,200</td>
<td>68.5</td>
</tr>
<tr>
<td>2. Dowex 50-Na⁺ treatment</td>
<td>1,800</td>
<td>72.6</td>
</tr>
<tr>
<td>3. Dowex 2-OH⁻ treatment</td>
<td>2,200</td>
<td>76.8</td>
</tr>
<tr>
<td>4. Phenol extraction</td>
<td>70</td>
<td>59.4</td>
</tr>
<tr>
<td>5. Peak from Dowex 50, pH 3 column</td>
<td>284</td>
<td>53.0</td>
</tr>
<tr>
<td>6. Phenol extraction</td>
<td>8</td>
<td>48.4</td>
</tr>
<tr>
<td>7. Crystallization</td>
<td></td>
<td>41</td>
</tr>
</tbody>
</table>

* Estimated by the spectrophotometric coenzyme assay by comparison with a purified sample of benzimidazolylcobamide coenzyme previously isolated. The apparent increase in the amount of coenzyme in Steps 2 and 3 is attributable to errors in the coenzyme assay caused by interfering substances in the cruder fractions.

After the appearance of several minor absorbancy peaks, containing yellow, orange, or red components but no coenzyme activity, the red coenzyme was eluted by the pH 6.4 buffer in a prominent absorbancy peak between Fractions 336 and 380 (Fig. 3). At least two red compounds, probably hydroxocobalamin and factor B, remained on the column after elution of the coenzyme.

Determinations of visible and ultraviolet absorption spectra and coenzyme activities of peak fractions indicated the major component was DBC coenzyme, slightly contaminated in the end fractions by small amounts of other cobamide coenzymes. Fractions 341 to 370, containing 145 μmol of coenzyme in 840 ml, were combined and extracted three times with, successively, 120, 50, and 40 ml of 92% aqueous phenol. After washing the combined phenol phase twice with 30 ml of water, the 158 ml of aqueous phenol containing the coenzyme were diluted with 3 volumes of ethyl ether and 1 volume of acetone and extracted twice with 20 ml and twice with 10 ml of water. The combined aqueous phase, containing the coenzyme, was washed twice with 20 ml of ether to remove phenol and was then aerated with N₂ to remove dissolved ether. The final solution was intensely red and contained 358 μmol of coenzyme, estimated by its absorbancy at 522 μm, in 60 ml.

The first crystals of DBC coenzyme were obtained by adding 2.1 ml of acetone to 0.3 ml of the above solution, scratching the walls of the tube, and leaving the solution at 3°C. After 3 days small clusters of crystals were found on the walls of the tube. Crystallization was also induced in another sample by allowing an aqueous solution of the coenzyme to concentrate slowly under reduced pressure at 3°C for several days.

After several trials with different methods of crystallization, the following procedure was found to be convenient and effective.

The aqueous solution of the coenzyme was concentrated, if necessary, under reduced pressure at 4°C over sulfuric acid until it was 0.005 to 0.01 M. Any crystals formed by excess concentration were dissolved in a minimum volume of water. To 20 ml of the concentrated aqueous coenzyme solution in a glass-stoppered flask were added 100 ml (5 volumes) of acetone. After standing at 4°C for 2 to 3 hours the solution was centrifuged for 10 minutes at 10,000 × g in stainless steel tubes to remove the crystalline coenzyme. An additional 3 to 4 μmol of crystalline coenzyme were later recovered from the mother liquor of the first crop of crystals.
any amorphous precipitate that was present. The clear supernatant solution was seeded with a microscopic amount of crystalline DBC coenzyme and was allowed to crystallize overnight at 4°C. Dark red crystals formed on the walls and bottom of the container (Fig. 2B). Usually one-third to one-half of the coenzyme crystallized in this time. Another 40 ml (2 volumes) of acetone were added and the solution was left 4 or 5 days to crystallize. The course of crystallization was followed by measuring the absorbancy of the solution at 522 nm. The absorbancy gradually fell to 0.25, corresponding to a concentration of \(3 \times 10^{-4} \text{ M}\). With an initial coenzyme concentration of 0.005 M, this corresponded to the crystallization of about 94% of the total cobamide. The supernatant solution was decanted and saved for recovery of a second crop of crystals. The crystals were washed twice with a few milliliters of cold 90% acetone, twice with 10% acetone, and twice with ethyl ether. During the acetone and ether wash most of the crystals separated from the glass and after removal of residual ether with a gentle stream of air, they were easily transferred to a weighing bottle. The coenzyme can be readily recrystallized by the same method.

Table III summarizes the data on the isolation of the DBC coenzyme.

With the use of the above crystallization method, 554 mg of crystalline DBC coenzyme were obtained in the first crop and an additional 24 mg were obtained in a second crop, once recrystallized. Since the crystals contained 8.2% of moisture (see below) and since the molecular weight of the dry coenzyme was estimated to be approximately 1660, the total yield of crystalline coenzyme corresponded to 320 \(\mu\text{moles}\), or 80 \(\mu\text{moles}\) per kg of starting cell paste.

Properties of Coenzymes

Crystal Form and Color—The BC coenzyme crystallizes readily from water as small (\(<0.2 \text{ mm long}\)) six-sided prisms with four rectangular and two diamond-shaped faces or as very small needles with blunt ends (Fig. 2A). The larger crystals are formed on the glass walls of the container near the surface of the liquid. Even with slow evaporation of an aqueous solution, much of the solid that forms is microcrystalline. Addition of 85% acetone to an aqueous solution of the coenzyme also gives the greatest mobility, with both coenzymes. When 0.02 \(\mu\text{mole}\) of DBC coenzyme is used, the material moves as a single reddish-orange and ultraviolet-absorbing spot toward the cathode at a rate of approximately 12.8 cm per hour in a potential gradient of 40 volts per cm. Under the same conditions the BC coenzyme moves a little faster, 13.8 cm per hour. No evidence of a second component could be observed with either coenzyme under any of the conditions tested.

Paper chromatography of the BC and DBC coenzymes on Whatman No. 1 paper with acid, neutral, and alkaline solvents. The acidic solvent, consisting of 0.5 M acetic acid, gives the greatest mobility, with both coenzymes. When 0.02 \(\mu\text{mole}\) of DBC coenzyme is used, the material moves as a single reddish-orange and ultraviolet-absorbing spot toward the cathode at a rate of approximately 12.8 cm per hour in a potential gradient of 40 volts per cm. Under the same conditions the BC coenzyme moves a little faster, 13.8 cm per hour. No evidence of a second component could be observed with either coenzyme under any of the conditions tested.

Paper chromatography of the BC and DBC coenzymes on Whatman No. 1 filter paper by the descending method, with a sec-butanol-glacial acetic acid-water (100:3:50 by volume) solvent for 20 hours at room temperature, showed the presence of only one visibly colored or ultraviolet-absorbing spot in each preparation. The \(R_f\) values were 0.16 and 0.22, respectively, for the BC and DBC coenzymes. By comparison on the same chromatogram, the \(R_f\) values of cyanocobalamin and the AC coenzyme were 0.34 and 0.09, respectively.

The solubility test for purity consisted of adding successive amounts of crystalline DBC coenzyme to water until the total quantity exceeded the solubility of the coenzyme by about 60%. After each addition, the solution was shaken until equilibrium was reached and the absorbancy of the solution at 260 nm was determined. Fig. 4 shows that no significant increase in absorbancy occurred after the solution became saturated with coenzyme. This indicates that the crystalline DBC coenzyme contains less than 2% of ultraviolet light-absorbing impurities.

Stability—The dry crystalline coenzymes are moderately stable. No changes in spectrum or activity have been detected in samples stored several months at \(-10^\circ\) or several days at room temperature. Solutions of the coenzymes appear to be most
Progressively larger amounts of crystalline DBC coenzyme were added to 1 ml of water at 24° ± 0.5°. After stirring the solution for 15 minutes and centrifuging down any remaining solid material, aliquots of 5 μl were diluted to 1.00 ml in 0.01 M KPO₄ buffer, pH 6.8, and the concentration of the original solution calculated from the absorbancy of the diluted sample at 260 nm.

stable at pH 6 to 7. Neither coenzyme is appreciably inactivated by heating for 20 minutes at 100° in a 0.01 M sodium acetate buffer, pH 6.0, or by storage in dilute neutral solution for several months at −10°. As previously noted, the BC and DBC coenzymes are more stable under acid conditions than the AC coenzyme (2). Nevertheless, heating in 0.07 N HCl at 85° causes slow inactivation; in one experiment the BC coenzyme lost 8% of its activity during 5 minutes under these conditions.

The great instability of solutions of the BC and DBC coenzymes under exposure to light or cyanide ion has already been reported (2). Either treatment causes a loss of activity and a dramatic change in the adsorption spectrum. The rates of decomposition increase with light intensity or cyanide ion concentration. These effects will be discussed more fully in a subsequent paper.

Solubility—The coenzymes show a solubility pattern similar to that of cyanocobalamin. They are rather soluble in water,
ethanol, and phenol, but insoluble in acetone, ether, dichlorehxyl-
ene, dioxane, and other relatively nonpolar solvents.

Data on the solubility of the coenzymes in water and acetone-
water solutions are given in Table IV. Noteworthy is the rather
striking difference between the solubility of the two coenzymes
in water at room temperature. The relative ease with which
the BC coenzyme can be crystallized from water is a reflection
of its lower solubility in this solvent.

Absorption Spectra—Fig. 5 shows absorption spectra of solu-
tions of crystalline BC and DBC coenzymes. The compounds
have very similar spectra. The most distinctive difference be-
tween the two spectra is the position of the rather inconspicu-
ous inflection on the side of the main absorbancy peak. With the
DBC coenzyme it is located at 288 mp whereas with the
BC coenzyme it is located at 288 mp. There are also some
other small differences between the two spectra. The BC co-
enzyme has maxima at 261 and 519 mp whereas the corre-
ponding maxima of the DBC coenzyme are at 260 and 522 mp.
The BC coenzyme also has an inconspicuous maximum at about
305 mp which is lacking in the latter compound. Both of these
coenzymes differ from the AC coenzyme by their much redder
color in neutral solution, by the position of the broad maximum
in the visible region close to 520 mp rather than at 458 mp,
and by other features.

Molar Extinction Coefficients—Treatment of any one of the
cobamide coenzymes with 0.1 M KCN for 60 minutes at 25° causes
the formation of a product with an absorption spectrum above
350 mp characteristic of the dicyanocobamides (1, 2). By mak-
ing the assumption (see below) that the molar extinction coeffi-
cient of the 367 mp peak of the product formed in this way from
a cobamide coenzyme is the same as that of cyanocobalamin
in the presence of 0.1 M KCN, namely 30.4 X 10⁻⁴ cm² per mole,
the molar concentration of a coenzyme solution can be calculated.
Knowing the concentration, the molar extinction coefficients of
the coenzyme at various wave lengths can be estimated from its
absorption spectrum in the absence of cyanide.

Table V gives the molar extinction coefficients of the BC,
DBC, and AC coenzymes in neutral solution and in 0.1 M KCN,
estimated by the above method. A comparison of the extinction
coefficients of the three coenzymes in the presence of cy-
nide at 350, 367, 540, and 579 mp, demonstrates that their spectra
are very similar if not identical in this region. Cyanocobalamin
in the presence of 0.1 M KCN also has the same spectrum above
350 mp. The virtual identity of the spectra of all these com-
pounds appears to be a consequence of the fact that excess cy-
nide ion displaces the purine or benzimidazole moiety from the
cobaltato-coordinat form its dicyano derivative. This structure is
evidently entirely responsible for the spectrum above 350 mp.
The nucleotide bases absorb light of shorter wave lengths and
only influence the spectrum above 350 mp when they are able to
form a coordinate linkage with the cobalt. Under these cir-
cumstances, the assumption that the molar extinction coeffi-
cients of the cobamide coenzymes are the same as that of cyanocoba-
lanin at 367 mp in 0.1 M KCN appears to be justified. Further
evidence for the approximate correctness of this assumption is
provided by analytical data on the phosphate and cobalt contents
of the coenzymes (see below).

Assuming the molar extinction coefficients given in Table V to
be correct, the molecular weight of a coenzyme can be determined
by measuring the absorbancy of a solution containing a known
weight of the dry crystalline compound. Samples of the DBC
coenzyme were dried for 2 hours in a vacuum over silica gel at
56.5, 78.5, and 100°. The percentage weight loss was the same
within experimental error at all three temperatures, the average
being 8.2%. Solutions of the dried samples showed the same
spectrum and the same specific activity in the coenzyme assay
as the undried coenzyme. The molecular weight calculated from
absorbancy measurements at six wave lengths on the three
coenzyme samples dried at different temperatures was found to
be 1660 ± 20.

The molecular weight of the BC coenzyme was estimated by
the same method, with a sample of crystalline coenzyme dried
to constant weight during 6 weeks in a vacuum over P₂O₅ at 4°,
to be 1,610 ± 20.

Elementary Analysis—Cobalt was identified in both the BC
and DBC coenzymes by means of an x-ray fluorescence spectrom-
eter (3). Quantitative cobalt estimations by the nitrosocresol
method (9), after wet digestion with a mixture of nitric, perchlo-
rnic, and sulfuric acids (10), gave values of 0.93 and 0.95 atom
of cobalt per mole of BC and DBC coenzyme, respectively,
estimated spectrophotometrically.

Total phosphate determinations by the method of Fiske and
SubbaRow (11) after digestion with nitric and sulfuric acids and

<table>
<thead>
<tr>
<th>Table V</th>
<th>Millimolar extinction coefficients of cobamide coenzymes in presence and absence of cyanide</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ</td>
<td>BC coenzyme</td>
</tr>
<tr>
<td>λ</td>
<td>0.01 M KPO</td>
</tr>
<tr>
<td>260</td>
<td>25.2</td>
</tr>
<tr>
<td>240</td>
<td>21.6</td>
</tr>
<tr>
<td>230</td>
<td>20.0</td>
</tr>
<tr>
<td>220</td>
<td>18.1</td>
</tr>
<tr>
<td>210</td>
<td>16.2</td>
</tr>
<tr>
<td>200</td>
<td>14.6</td>
</tr>
<tr>
<td>190</td>
<td>13.0</td>
</tr>
<tr>
<td>180</td>
<td>11.5</td>
</tr>
<tr>
<td>170</td>
<td>10.2</td>
</tr>
<tr>
<td>160</td>
<td>9.0</td>
</tr>
<tr>
<td>150</td>
<td>8.0</td>
</tr>
<tr>
<td>140</td>
<td>7.5</td>
</tr>
<tr>
<td>130</td>
<td>7.0</td>
</tr>
<tr>
<td>120</td>
<td>6.5</td>
</tr>
<tr>
<td>110</td>
<td>6.0</td>
</tr>
<tr>
<td>100</td>
<td>5.5</td>
</tr>
<tr>
<td>90</td>
<td>5.0</td>
</tr>
<tr>
<td>80</td>
<td>4.5</td>
</tr>
<tr>
<td>70</td>
<td>4.0</td>
</tr>
<tr>
<td>60</td>
<td>3.5</td>
</tr>
<tr>
<td>50</td>
<td>3.0</td>
</tr>
<tr>
<td>40</td>
<td>2.5</td>
</tr>
<tr>
<td>30</td>
<td>2.0</td>
</tr>
<tr>
<td>20</td>
<td>1.5</td>
</tr>
<tr>
<td>10</td>
<td>1.0</td>
</tr>
<tr>
<td>0.1</td>
<td>0.5</td>
</tr>
</tbody>
</table>
hydrogen peroxide showed the presence of 0.07 and 0.08 atom of phosphorus per mole of BC and DBC coenzyme, respectively, estimated spectrophotometrically.

No sulfur could be detected in 1.1 μmoles of DBC coenzyme by the nitroprusside test (6) after sodium fusion. Under identical conditions 0.3 μmole of thiamine gave a strong positive test for sulfur.

Elementary analysis by a commercial laboratory of a sample of crystalline DBC coenzyme dried in a vacuum for 2 hours at 58° gave the following results: C 53.3%, H 6.9%, N 14.9%. On the basis of the above analytical data and the assumption that the DBC coenzyme contains 18 atoms of nitrogen, its empirical formula can be calculated to be approximately C₇₀H₁₁₀O₃₃N₃₃PCo. This formula gives a molecular weight of 1701 which is 2.6% higher than the molecular weight of 1,660 calculated from dry weight determinations and the molar extinction coefficients given in Table V. If we assume the lower value is more nearly correct, we must reduce the number of oxygen atoms in the empirical formula by one or two and possibly also reduce the number of C and H atoms. On this basis the formula C₇₀H₁₁₀O₃₃N₃₃PCo appears to cover the range of possibilities. The corresponding minimal and maximal molecular weights are 1636 and 1685, respectively. No C, H, and N analyses have been done on the BC coenzyme.

Components—No cyanide could be detected in either coenzyme by the method of Boxer and Rickards (12).

Adenine was identified and estimated semiquantitatively after acid hydrolysis of the BC coenzyme in the following manner. A sample containing 0.11 μmole of the coenzyme was hydrolyzed for 60 minutes in 1 N HCl at 100°. After cooling, the solution was concentrated to dryness and the residue was dissolved in a little 60% (volume per volume) ethanol. The ethanol extract was divided into two portions to one of which was added some adenine as an internal control. Both solutions and separate adenine, adenosine, and adenylic acid standards were chromatographed on Whatman No. 1 paper by the ascending method with a solvent containing 50% (volume per volume) ethanol and 50% aqueous 0.5 M sodium acetate, pH 7.2. When the paper was examined by ultraviolet light, a quenching spot appeared in the hydrolyzed sample at Rₚ 0.63 that did not separate from the added adenine, but readily separated from adenylic acid (Rₚ 0.56) and adenosine (Rₚ 0.70). The quenching spots at Rₚ 0.63 were eluted and their absorption spectra determined. The unknown showed a typical adenine spectrum in neutral solution with an absorbancy maximum at 260 μM which shifted to 267 μM on addition of alkali. From the absorbancy at 260 μM in neutral solution, corrected for a suitable paper blank and for a 60% recovery of an adenine standard, the yield of adenine was estimated to be 1.0 ± 0.2 μmole per μmole of BC coenzyme hydrolyzed.

The presence of adenine in an acid hydrolysate of the DBC coenzyme was established by similar methods. In this case, a colorless compound with an absorbancy maximum at 260 μM was separated from the other products of acid hydrolysis by means of a Dowex 50W, pH 3 column. The compound eluted with 0.03 M sodium acetate, pH 6, in a well defined peak which was only slightly contaminated by a faster moving red compound. The elution rate of the colorless compound was like that of adenine. The hydrolysate product was further identified as adenine by its absorption spectrum in neutral and alkaline solution and by its Rₚ in paper chromatography. The yield of adenine, calculated from the absorbancy at 260 μM of the elution peak fractions, was 0.92 mole per mole of DBC coenzyme.

Benzimidazole was shown to be a component of the BC coenzyme and 5,6-dimethylbenzimidazole of the DBC coenzyme. These compounds were separated from the corresponding coenzymes by acid hydrolysis, alkalization of the hydrolysate, and extraction of the heterocyclic base with chloroform. After removal of the organic solvent, the base was identified by its absorption spectrum, its fluorescence spectrum, and by paper chromatography.

The experimental details of the identification of benzimidazole in the BC coenzyme are as follows:

A sample containing 0.17 μmole of BC coenzyme was dissolved in 6 ml of 6 N HCl and heated in an evacuated and sealed tube for 18 hours at 150°. The solution was transferred to a 10-ml beaker and dried under a stream of warm air. A little water was added and the solution was again evaporated to dryness to remove excess HCl. The residue was dissolved in 1 ml of water, alkalized with 0.01 ml of 10 N KOH, and shaken with 12 ml of chloroform. The organic phase was transferred to a small beaker and the chloroform was removed by a stream of warm air. The residue was dissolved in a little ethanol, and an aliquot was used for ascending paper chromatography. A blue fluorescent spot corresponding to benzimidazole was observed at Rₚ 0.78, with sec-butanol-acetic acid-water, 100:3:50 by volume, as the developing solvent, and at Rₚ 0.72 with 0.1 N acetic acid as a solvent. Another aliquot was evaporated to dryness, dissolved in 0.1 N acetic acid, and its ultraviolet absorption spectrum determined. The spectrum was identical with that of benzimidazole with characteristic absorption maxima at 239, 260, 266, and 273 μM, and a sharp minimum at 270 μM. The fluorescence of the hydrolysate product was compared with that of benzimidazole with an Amino spectrophotofluorometer. Both samples showed a strong fluorescence in 0.1 N acetic acid that was maximally activated by light at 272 μM and was most intense at 365 μM. The fluorescence of both samples was quenched by addition of 0.1 N HCl.

The yield of benzimidazole, determined from the absorbancy at 273 μM and the corresponding molar extinction coefficient of 8100 cm² per mole, was 0.80 mole per mole of coenzyme.

In the quantitative determination of benzimidazole or dimethylbenzimidazole, a standard of the appropriate benzimidazole and a reagent blank were carried through the entire extraction and estimation procedure and used to determine appropriate corrections for the unknown sample. It was also determined that benzimidazoles are not appreciably decomposed under the conditions of acid hydrolysis employed.

5,6-Dimethylbenzimidazole was identified as a product of acid hydrolysis of the DBC coenzyme by the same methods. The Rₚ of 5,6-dimethylbenzimidazole and the unknown was found to be 0.52 in sec-butanol-acetic acid-water and 0.60 in 0.1 N acetic acid. The spectra of both samples had absorption maxima at 245, 273, and 283 and a sharp minimum at 250 μM. The fluorescence of both samples was maximally activated by light at 285 μM and was most intense at 350 μM.

The yield of 5,6-dimethylbenzimidazole formed by acid hydrolysis of the DBC coenzyme, calculated from its absorbancy in 0.01 N HCl at 283 μM and the corresponding molar extinction coefficient of 8,100 cm² per mole, was 0.73 mole per mole of coenzyme.

Relative Coenzyme Activities—Activities of the cobamide co-
enzymes in catalyzing the conversion of glutamate to β-methyl-aspartate can be compared either at low or at high coenzyme concentrations. A comparison at low concentrations, where the reaction rate is proportional to concentration, shows that the activity per mole is highest for the BC coenzyme, intermediate for the AC coenzyme, and lowest for the DBC coenzyme (Table VI). This difference in molar activities could be the result of differences either in the affinities (Km values) of the coenzymes for the enzyme system or in their potential maximum activities (Vmax values). Determination of the apparent Km values in this multienzyme system shows that the relative molar activities are largely a reflection of differences in the Km values (Table VI). The product Km x molar activity (Column 4) is almost constant for the three coenzymes, indicating that the molar activities are inversely proportional to the affinities. The maximal activities of the three coenzymes are very similar (Column 5).

**Growth Factor Activity**—Activity was measured in the E. coli B8r-requiring mutant and Ochromonas malhamensis tests as previously described (3). In the E. coli test, both the DBC and BC coenzymes showed 100 ± 20% of the activity of cyanocobalamin, on a molar basis. In the Ochromonas test, the DBC coenzyme showed 90 ± 20% and the BC coenzyme showed 45 ± 10% of the activity of cyanocobalamin on a molar basis. The value obtained with the BC coenzyme is similar to that reported for the benzimidazole analogue of cyanocobalamin (13).

**DISCUSSION**

The methods described for the isolation of the coenzymes are rather effective since they result in an over-all purification from 900-fold with the DBC coenzyme to 8,000-fold with the BC coenzyme, and an over-all yield of crystalline products ranging from 54 to 62% of the extracted coenzymes. It is worth noting that the selected coenzyme peak fractions from the Dowex 50W, pH 3 column contain essentially pure coenzyme, since the specific activity of the coenzyme in these fractions is virtually the same as that of the final crystalline product. Consequently for some of the final purification steps are unnecessary.

The DBC coenzyme content of P. shermanii (130 μmoles per kg of wet cells) is approximately 6.5 times greater than the DBC or BC coenzyme content of C. tetanomorphum (20 μmoles per kg). However, since the molar activity of the BC coenzyme in the C. tetanomorphum assay is about 73 times that of the DBC coenzyme, the total cobamide coenzyme activity per kg is about 11 times greater in C. tetanomorphum cells grown in the presence of benzimidazole than in P. shermanii cells. The latter are grown without addition of 5,6-dimethylbenzimidazole to the medium since the organism synthesizes this compound in adequate amounts.

The once recrystallized DBC coenzyme, carefully protected from light, appears to be at least 98% pure. This estimate is based upon the results of the solubility test for homogeneity and on the failure to detect colored impurities separable by paper ionophoresis or chromatography. The purity of the crystalline BC coenzyme has not been determined as carefully, but the failure to detect color impurities by ionophoresis or chromatography indicates that the coenzyme is at least 95% pure.

The analytical data demonstrate the presence of approximately one mole each of cobalt, phosphorus, and either benzimidazole or 5,6-dimethylbenzimidazole per mole of coenzyme. These results clearly establish a chemical relation of these compounds with cyanocobalamin and its benzimidazole analogue, a relation previously indicated by a study of the absorption spectra of products formed by the action of light or cyanide ion on the coenzymes (2). The main differences in composition so far observed between the BC and DBC coenzymes and the corresponding vitamins are the absence of cyanide and the presence of an adenine moiety in the coenzymes. The AC coenzyme and pseudovitamin B12 differ in the same ways (3). The extra adenine moiety appears to be an essential structural feature of the cobamide coenzymes.

Various types of evidence that will be presented in a later paper show that hydroxocobalamin can be formed from the DBC coenzyme. If we tentatively accept this as a fact, then the coenzyme must contain the hydroxocobalamin structure with a molecular weight of about 1355, and an adenine moiety with a molecular weight of about 135. If these were the only components, the molecular weight of the coenzyme would be close to 1490. The experimentally determined molecular weight, calculated from the dry weight of the crystalline DBC coenzyme and either its phosphorus content or its estimated molar extinction coefficient, is approximately 1660. This leaves roughly 170 g not accounted for by the known components and indicates that the coenzyme contains an additional compound. This compound cannot contain cobalt or phosphorus since these are already accounted for by the hydroxocobalamin structure. The unknown compound also probably does not contain nitrogen, since the 18 nitrogen atoms present in hydroxocobalamin and adenine satisfactorily account for the elementary analysis and a molecular weight between 1640 and 1700. Consequently it probably contains only carbon, hydrogen, and oxygen. The determined empirical formula of the DBC coenzyme, C57H82N18O19PNC0, in fact has 5 to 8 carbon atoms, 19 to 22 hydrogen atoms, and 5 to 6 oxygen atoms in excess of those contributed by the known components. Some of the hydrogen and oxygen may be present as water of hydration not removed by drying at 100°, but all of

**Table VI**

<table>
<thead>
<tr>
<th>Coenzyme</th>
<th>(a) Km</th>
<th>(b) Micromolar activity</th>
<th>(a) x (b)</th>
<th>Vmax</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC</td>
<td>2.4 x 10^-4</td>
<td>800</td>
<td>1.9</td>
<td>0.24</td>
</tr>
<tr>
<td>AC</td>
<td>1.4 x 10^-4</td>
<td>160</td>
<td>2.2</td>
<td>0.24</td>
</tr>
<tr>
<td>DBC</td>
<td>1.8 x 10^-4</td>
<td>11</td>
<td>2.0</td>
<td>0.17</td>
</tr>
</tbody>
</table>
the carbon and much of the hydrogen and oxygen must be present in the unidentified moiety.

All three of the cobamide coenzymes so far described are equally active in catalyzing the conversion of glutamate to β-methylaspartate, when tested at saturating coenzyme concentrations. However, the coenzymes differ greatly in their affinities for the enzyme system; the BC coenzyme has by far the highest and the DBC coenzyme the lowest affinity. These differences determine the minimum concentration at which the coenzymes can be detected by the enzymatic assay. For example, the BC coenzyme can be estimated at a level of $10^{-5}$ μmole per ml of the assay mixture, whereas approximately $10^{-3}$ μmole of DBC coenzyme is required. The AC coenzyme and several other homologous coenzymes that have been recently isolated are intermediate between these two coenzymes in affinity for the enzyme system.

The identity of the $V_{max}$ values of the AC, BC, and DBC coenzymes clearly indicates that the 6-membered rings of the nucleotide adenine and benzimidazole and their substituent groups are not directly involved in the catalytic activity of the coenzymes.

The activities of the DBC and BC coenzymes as growth factors for the E. coli mutant and for Ochromonas malhamensis are approximately the same as those of the corresponding vitamins. This could mean either that the vitamins are converted quantitatively to the coenzymes by these organisms or vice versa. Further experiments are needed to elucidate this point.

**SUMMARY**

Methods for isolation of benzimidazolylcobamide and 5,6-dimethylbenzimidazolylcobamide coenzymes, active in catalyzing the interconversion of glutamate and β-methylaspartate, have been described. The crystalline benzimidazolylcobamide coenzyme was obtained from Clostridium tetanomorphum in a yield of 10.6 μmoles per kg of moist cells, the crystalline 5,6-dimethylbenzimidazolylcobamide coenzyme from Propionibacterium shermanii in a yield of 80 μmoles per kg. The coenzymes contain approximately one mole each of cobalt, phosphorus, and heterocyclic base, either benzimidazole in the benzimidazolylcobamide coenzyme or 5,6-dimethylbenzimidazole in the 5,6-dimethylbenzimidazolylcobamide coenzyme. In addition, both coenzymes contain one mole of adenine which is not present in the corresponding cobamide vitamins. The coenzymes differ markedly in their affinities for the enzyme system, but have the same maximal activities. Several physical, chemical, and biological properties of the coenzymes are described.

**Acknowledgments**—The authors are greatly indebted to Dr. David Perlman and his associates of the Squibb Institute for Medical Research for providing an ample supply of P. shermanii cells, to Dr. Karl Folkers and Dr. H. B. Woodruff of the Merck, Sharp and Dohme Research Laboratories for a sample of 5,6-dimethylbenzimidazole, to Dr. C. M. Johnson, Department of Soils and Plant Nutrition, University of California, for carrying out cobalt analyses on the coenzymes, to Dr. C. C. Delwiche, Kearney Foundation, University of California, for identifying cobalt by means of the x-ray fluorescence spectrometer, and to Professor A. Pabel, Department of Geology, University of California, for examining crystals of the coenzymes.

**REFERENCES**

Isolation and Properties of Crystalline Cobamide Coenzymes Containing Benzimidazole or 5,6-Dimethylbenzimidazole


Access the most updated version of this article at http://www.jbc.org/content/235/2/480.citation

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/235/2/480.citation.full.html#ref-list-1