Isolation of Coenzyme B₁₂ from Liver*

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The identity of the major cobamide in liver has not been satisfactorily established. Cyanocobalamin (vitamin B₁₂, 5,6-dimethylbenzimidazolylcobamide cyanide), which has been obtained in crystalline form with liver as a source material (1, 2), is certainly a very minor component of the cobalamins in liver. Analyses of liver concentrates, both by a paper chromatographic-bioautographic method (3) and by a chemical method (4), have shown that cyanocobalamin is usually not present in detectable amounts and seldom constitutes as much as 10% of the total cobalamin. The preparation of cyanocobalamin from liver probably was made possible by the presence of cyanide in the charcoal used in the isolation process. Examination of a liver extract by the chromatographic-bioautographic method (3) indicated that hydroxocobalamin (vitamin B₁₂₅, 5,6-dimethylbenzimidazolylcobamide hydroxide) was the most abundant cobalamin. This compound was also obtained in pure form from liver (1, 5). However, the recent discovery of the cobamide coenzymes in bacteria (6, 7) and the demonstration that they constitute a large part of the total cobalamides in these organisms (8, 9) raised the possibility that coenzyme B₁₂₅ (5,6-dimethylbenzimidazolylcobamide coenzyme, DMBC coenzyme, DBC coenzyme) may be a constituent of liver. Since coenzyme B₁₂₅ like the adenylcobamide coenzyme (10), is relatively unstable and is readily converted to hydroxocobalamin or cyanocobalamin by treatment with light or cyanide, respectively, the inadvertent decomposition of the coenzyme, if present in liver extracts, could account for previous observations on the occurrence of the two cobalamin vitamins.

The present paper reports some observations on the occurrence and abundance of a cobamide coenzyme, which has been identified as coenzyme B₁₂₅₇ in livers of rabbit, chicken, sheep, and man. A brief report of the occurrence of coenzyme B₁₂₅₇ in rabbit liver has already appeared (7).

**EXPERIMENTAL PROEDURE**

**Biological Material**—Chicken and rabbit livers were obtained from laboratory animals. Sheep (lamb) liver was obtained from the slaughter house. The human liver was from a man, aged 72, who died suddenly of a coronary occlusion. The livers were generally stored at −10°C until used.

**Physical Methods**—Absorbancy measurements were made with a Beckman model DU spectrophotometer equipped with a photomultiplier with silica cells and a 1-ml volume and 1-cm light path. Absorption spectra were recorded with a Cary model 14 spectrophotometer. Fluorescence properties were determined with an Aminco-Bowman spectrophotofluorometer equipped with a photomultiplier-microphotometer and a Moseley X-Y recorder. Turbidity of bioassay cultures was measured with a Klett-Summerson colorimeter with a green filter. Radioactivity of Co²⁶ was measured with a Geiger-Müller counter. The sample, dissolved in 1.0 ml of water, was placed in a metal planchet 2.4 cm in diameter.

**Bioassay Method**—The total cobamides and cobinamides that support the growth of Escherichia coli 113-3 were determined by the tube assay with Burkhoder's medium (11) as modified by Ford et al. (12).

**Preparation of Cobalt²⁶-labeled Coenzyme B₁₂—Propionibacterium shermanii ATCC 9614 was grown in 10 ml of a medium containing 1 g of Difeo yeast extract and 1 g of sodium lactate per 100 ml of distilled water. After incubation at 30°C for 48 hours, 5 ml of the culture were used to inoculate a medium containing 1 g of Difeo yeast extract, 1 g of glucose, 0.5 g of CaCO₃, and 50 ml of distilled water in a 100-ml flask. After incubation at 30°C for 48 hours, this culture was used to inoculate a medium containing 15 g of glucose, 12.5 g of Difeo yeast extract, 10 g of CaCO₃, 20 ml of 1 M potassium phosphate buffer pH 7.0, 1.0 ml of 1 M MgSO₄, 1.0 ml of 0.1 M FeSO₄, 0.5 ml of 10⁻⁴ M CoCl₂, 5.0 ml of 0.04% phenol red solution, 400 ml of distilled water, and approximately 1 mc of carrier-free CoCl₂, contained in a 1-liter flask. The glucose was autoclaved separately. After all additions had been made, sterile 20% Na₂CO₃ was added aseptically until the phenol red indicator was orange (pH 7.4). The culture was incubated under semianaerobic conditions at 30°C for 8 days. The pH was readjusted to approximately 7.4 twice a day by addition of 20% sodium carbonate solution; a total of 162 ml were added during the 8 days of incubation. On the third, fifth, and seventh days, acid production stopped because the glucose supply had been exhausted. On each of these days, 50 ml of a sterile 30% glucose solution were added to the culture. After incubation at 30°C for 48 hours, this culture was used to inoculate a medium containing 15 g of glucose, 12.5 g of Difeo yeast extract, 10 g of CaCO₃, 20 ml of 1 M potassium phosphate buffer pH 7.0, 1.0 ml of 1 M MgSO₄, 1.0 ml of 0.1 M FeSO₄, 0.5 ml of 10⁻⁴ M CoCl₂, 5.0 ml of 0.04% phenol red solution, 400 ml of distilled water, and approximately 1 mc of carrier-free CoCl₂, contained in a 1-liter flask. The glucose was autoclaved separately. After all additions had been made, sterile 20% Na₂CO₃ was added aseptically until the phenol red indicator was orange (pH 7.4). The culture was incubated under semianaerobic conditions at 30°C for 8 days. The pH was readjusted to approximately 7.4 twice a day by addition of 20% sodium carbonate solution; a total of 162 ml were added during the 8 days of incubation. On the third, fifth, and seventh days, acid production stopped because the glucose supply had been exhausted. On each of these days, 50 ml of a sterile 30% glucose solution were added to the culture. On the fourth day, an additional 0.5 μmole of CoCl₂ was added.

The cells were collected by centrifugation on the eighth day. The Co²⁶-labeled coenzyme was isolated by the method previously described (9), except that the final product was not crystallized. The yield was 0.2 μmole of coenzyme B₁₂ containing approximately 0.2 mc of Co²⁶. Behavior of the product in paper chromatography, paper ionophoresis, and ion exchange chromatography on a Dowex 50 column indicated that it did not contain significant amounts of Co²⁶-labeled impurities.

**PURIFICATION OF COENZYME FROM LIVER**

The purification procedure is a modification of that used to isolate cobamide coenzymes from bacteria (9). Modification was
required because of the relatively small amount of cobamide and the large quantity of lipids in liver. Co\textsuperscript{60}-labeled coenzyme B\textsubscript{12} was added at an early stage of the purification to serve as an indicator of the location of the coenzyme.

All operations, unless otherwise specified, were carried out at room temperature in very dim light.

**Acetone powder**—Fresh or frozen liver (1 Kg) was cut into small chunks. Batches (500 g) were homogenized with 1 liter of acetone at room temperature in a large Waring Blender for 5 minutes at high speed. The suspension was filtered through Whatman No. 1 paper on a large Buchner funnel. The solid residue was homogenized with 1 liter of ethyl ether for 3 minutes in the blender. The suspension was again filtered with suction and washed with 200 ml of ether on the funnel. The solid residues from the two batches were combined, dried in a stream of air at room temperature, and weighed.

**Ethanol Extraction**—The dry powder, weighing approximately 300 g, was homogenized with 1 liter of hot 80\% (volume per volume) ethanol in a blender for 2 minutes. The suspension was transferred with washing to a 2 liter beaker and heated near the boiling point for 20 minutes. A tracer amount of Co\textsuperscript{60}-labeled coenzyme B\textsubscript{12} (6 \times 10\textsuperscript{-4} mole, 10\textsuperscript{6} c.p.m.) was added at this point. The suspension was cooled in ice and filtered through Whatman No. 1 paper with suction. The solid residue was homogenized once more with 1 liter of 80\% ethanol, heated for 15 minutes, cooled, and filtered. The combined ethanol filtrate was concentrated to 250 ml in a rotary flush evaporator at 40° to remove ethanol. The remaining aqueous solution contained a milky suspension of phospholipids.

**Ether Extraction**—The aqueous solution was extracted three times with equal volumes of ether in a separatory funnel to remove lipids, and the ether phase was discarded. The aqueous phase, after removal of dissolved ether by evaporation in a vacuum to 100 ml, was clear and orange in color.

**Passage through Dowex 2-OH**—The solution was passed through a 2.1-cm diameter \times 18-cm high column of Dowex 2-OH, 50 to 100 mesh, 8\% cross-linked, at a flow rate of 1 ml per minute, and the column was then sucked free of liquid.

**Phenol Extraction**—The effluent solution was distributed among four or five conical, glass-stoppered 40-ml centrifuge tubes. The solution was saturated with phenol and then was extracted five times with 1-ml volumes of 85\% (weight per weight) phenol. The tubes were centrifuged to break the emulsion after each shaking. An equal volume of ether was added to the combined p\textsuperscript{b}-enol phase, and the mixture was extracted five times with 1-ml volumes of water. The combined aqueous phase was washed four times with equal volumes of ether to remove phenol, and the ether phase was discarded. Dissolved ether was removed by bubbling nitrogen gas through the aqueous solution.

**Passage through Dowex 50-Na\textsuperscript{+}**—The solution was adjusted to pH 7.0 and was passed through a 6-mm diameter \times 6-cm high column of Dowex 50 in the sodium form, 200 to 400 mesh, 2\% cross-linked, at a rate of 1 ml per minute.

**Fractionation on Dowex 50, pH 3**—The solution was then adjusted to pH 3.0 with 1 n hydrochloric acid and was placed on a 6-mm diameter \times 6-cm high column of Dowex 50-W, 200 to 400 mesh, 2\% cross-linked, which had been previously adjusted to pH 3 (8). The column was washed with water and eluted with sodium acetate buffers as indicated in Fig. 1. The column was operated at room temperature under air pressure of 1 to 2 pounds per sq in at a flow rate of 2 ml per 5 minutes. The 2-ml fractions were collected manually, and the elution pattern was followed by absorbancy and radioactivity measurements with 1-ml aliquots. The fractions containing the radioactivity peak were combined, and the coenzyme was concentrated by extracting through phenol and back into water. The spectrum of the resulting solution was recorded, and coenzyme activity in the glutamate isomerase system was compared with that of crystalline coenzyme B\textsubscript{12} (8).

**Method of Isolation and Identification of Dimethylbenzimidazole**—A sample containing approximately 0.09 \mu mole of coenzyme from human liver was made 0.1 n with potassium cyanide and left at room temperature for 2 hours. The pH was adjusted to 3.0 with hydrochloric acid, and the solution was passed through a 6-mm diameter \times 2-cm high column of Dowex 50, pH 3.0. The absorption spectrum of the solution was identical with that of vitamin B\textsubscript{12}. The solution was made 6 n with hydrochloric acid, sealed in a vacuum in a glass tube, and heated at 150° for 20 hours. After removal of excess hydrochloric acid by evaporation to dryness over sodium hydroxide in a vacuum at 37°, the residue was taken up in 2.0 ml of 0.1 n hydrochloric acid and extracted twice with 2-ml volumes of chloroform. The chloroform extracts were discarded. The water phase was made 0.1 n with respect to sodium hydroxide by addition of 0.15 ml of 2.5 n NaOH and was extracted six times with 1-ml volumes of chloroform. The chloroform was evaporated in a stream of nitrogen and the residue was taken up in 1.0 ml of 0.1 n hydrochloric acid.

The fluorescence spectra of a suitable aliquot of the product were determined in 0.1 n acetic acid. The absorption spectra were determined in both 0.1 n HCl and 0.1 n NaOH. The quantity of 5,6-dimethylbenzimidazole recovered was calculated from the absorbancy at 223 m\textmu of the solution in 0.1 n HCl and the molar absorbancy index of 8100 cm\textsuperscript{2} per mole.

**RESULTS**

A typical elution pattern of a liver coenzyme preparation from a Dowex 50, pH 3 column is shown in Fig. 1. Most of the red color of this human liver preparation and most of the Co\textsuperscript{60}, added as Co\textsuperscript{60}-labeled coenzyme B\textsubscript{12}, were eluted together in the position characteristic of the cobamide coenzymes containing a benzimidazole derivative. A small amount of Co\textsuperscript{60} appeared also in the solution passed through the column. This represents a neutral or acidic decomposition product of the labeled coenzyme B\textsubscript{12}. No red color was detected in this region, indicating that no substantial amount of cyanocobalamin was present in the preparation. A small amount would have been concealed by the...
yellow impurity eluting in the same position. Any hydroxocobalamin in the preparation would have remained on the column after the coenzyme was eluted. No effort was made to recover this compound.

All the preparations gave similar elution patterns, although the amounts eluting positions of the impurities varied somewhat.

Table I summarizes the quantitative results on the isolation of cobamide coenzymes from the livers of four animal species. The last line of the table gives the relative molar activity of each preparation in the glutamate isomerase assay.

The properties of the base recovered from the coenzyme derived from human liver, after cyanide treatment and hydrolysis, were identical with those of 5,6 dimethylbenzimidazole. The wave length of maximal fluorescence was 382 mμ and that of maximal fluorescence activation was 285 mμ. The absorbancy maxima were at 274 and 284 mμ in acid and at 281 and 288 mμ in alkaline solution. The dimethylbenzimidazole was recovered in 93% yield based upon the amount of cyanocobamide used for acid hydrolysis. The bases present in the other coenzyme preparations were not examined by chemical methods.

**DISCUSSION**

The isolation procedure was designed to give a coenzyme preparation of sufficient purity to permit characterization of the coenzyme with a reasonable degree of certainty. No serious attempt was made to remove all contaminating materials. As a result, the purity of the various preparations differed considerably.

Comparison of the absorption spectra of the preparations with that of crystalline coenzyme B₁₂ (Fig. 2, Sample 4) indicates that the chicken liver preparation was more heavily contaminated with ultraviolet light absorbing impurities than were the other preparations. The spectrum of the chicken preparation shows an absorbancy maximum at about 520 mμ and gives an indication of a shoulder at about 495 mμ. These features are characteristic of cobamide coenzymes containing a benzimidazole derivative. It should be noted that cyanocobalamin has two absorbancy maxima at 520 and 550 mμ, the latter being the higher. Consequently even the fragment of the spectrum above 460 mμ readily distinguishes the coenzyme from the vitamin.

The lamb and human liver preparations show characteristic spectral properties of the coenzyme in the visible region and down to at least 340 mμ. The spectrum of the human preparation, in addition, shows the inflection at 288 mμ which is specifically indicative of coenzyme B₁₂ (9). The spectrum of the rabbit liver preparation shows all of the above features plus a shoulder at 260 mμ which is indicative of the corresponding absorbancy peak of the pure coenzyme. In summary, the spectra show that the lamb, human, and rabbit preparations, and probably also the chicken preparation, contain a cobamide coenzyme having a benzimidazole derivative; the inflection at about 288 mμ indicates that in the human and rabbit preparations, the base is probably 5,6-dimethylbenzimidazole.

The spectral evidence for coenzyme B₁₂ in the human preparation is confirmed by the isolation in good yield and the characterization of 5,6-dimethylbenzimidazole as a product of acid hydrolysis. Such direct evidence for the specific identity of the coenzyme in preparations from other animals was not obtained. In these cases, specific identification is based largely on the relative molar activities of the preparations in the enzymatic assay (Table I).

The molar activities of all the preparations are the same, within the experimental error of the method, as that of crystalline coenzyme B₁₂. The experimental error in the determinations with lamb, human, and chicken preparations was about ±25%. With the rabbit preparation, the molar activity determination was done on a different occasion, with a less reliable standard; the experimental error could have been ±50%. Despite these uncertainties, the observed relative molar activity values provide strong evidence that all the preparations contained coenzyme B₁₂. The other known cobamide coenzyme analogues containing a benzimidazole derivative have molar activities from 25 to 80 times larger than that of coenzyme B₁₂ (13).

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**Table I**

**Isolation of cobamide coenzyme from liver**

<table>
<thead>
<tr>
<th></th>
<th>Lamb (1000 g)</th>
<th>Human (1000 g)</th>
<th>Chicken (993 g)</th>
<th>Rabbit (230 g)</th>
</tr>
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<tr>
<td>µmole</td>
<td>µmole</td>
<td>µmole</td>
<td>µmole</td>
<td></td>
</tr>
<tr>
<td><strong>Total cobamide recovered</strong></td>
<td>0.27</td>
<td>0.24</td>
<td>0.040</td>
<td>0.44</td>
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<td>After ethanol extraction</td>
<td>0.27</td>
<td>0.20</td>
<td>0.030</td>
<td>0.30</td>
</tr>
<tr>
<td>After ether extraction</td>
<td>0.28</td>
<td>0.19</td>
<td>0.031</td>
<td>0.16</td>
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<tr>
<td>After passage through Dowex</td>
<td>0.22</td>
<td>0.18</td>
<td>0.030</td>
<td>0.16</td>
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<td>0.016</td>
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<tr>
<td>After concentration through phenol</td>
<td>0.128</td>
<td>0.135</td>
<td>0.0174</td>
<td>0.015</td>
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<td>Coenzyme recovered†</td>
<td>0.83</td>
<td>0.71</td>
<td>1.25</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* Determined by the E. coli bioassay and expressed as µmoles of cyanocobalamin.
† Calculated from the absorbancy at 520 mμ and the molar absorbancy index of 8.0 x 10⁴ cm² per mole.
‡ The ratio obtained by dividing the molar activity (ΔA per minute per µmole) of the coenzyme preparation by that of crystalline coenzyme B₁₂. The relative molar activity of coenzyme B₁₂ is 1.00.

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**Fig. 2.** Absorption spectra of cobamide coenzyme preparations from liver in 0.01 M potassium phosphate buffer pH 6.4. The approximate concentrations of the coenzyme solutions, estimated from the absorbances at 522 mμ (9) are as follows: 1, 1.2 x 10⁻⁶ M; 2, 3.4 x 10⁻⁶ M; 3, 2.1 x 10⁻⁶ M; 4, 1.4 x 10⁻⁶ M; 5, 7.5 x 10⁻⁶ M.
The recovery of total cobamide in the final fraction was reasonably high with the three preparations for which the yields could be calculated from bioassay data, varying from 41% for the chicken liver preparation to 73% for the human liver preparation. The coenzyme recovery, based on the absorbancy at 520 nm, was of the same order of magnitude, ranging from 43% to 56% of the initial total cobamide. These yields may be a little high because they would be increased by any impurities absorbing at 520 nm. However, the absence of any large amount of such impurities is indicated, especially for the lamb and human liver preparations, by the absorption spectra (Fig. 2). Also the relative molar activity values, which are based upon coenzyme concentration estimated from the absorbancy at 520 nm, suggest that inactive impurities do not exceed 29% and are generally lower. By making corrections both for possible impurities in the coenzyme preparations, reflected by the deviation of the relative molar activity values from unity, and for the loss of total cobamide between the ethanol extraction and the concentration through phenol steps, we may conservatively conclude that at least 48, 53, and 72% of the total cobamide in the ethanol extracts of lamb, human, and chicken livers, respectively, was in the form of coenzyme B₁₂. Since the coenzyme is less stable than the cobamide vitamins, the true percentage of coenzyme B₁₂ in the cobamides of liver may be considerably higher.

Since coenzyme B₁₂ is a major component of liver cobamides, and since the coenzyme is readily converted to the cyano- and hydroxocobalamin, it is apparent that the latter compounds, isolated from liver, were derived in large part from coenzyme B₁₂. The possibility that cyanoocobalamin, and especially hydroxocobalamin, also occur in liver in smaller amounts is not specifically excluded by our observations.

These experiments do not distinguish between free and combined forms of coenzyme B₁₂ in liver. They simply establish that a large part of the cobamide that can be extracted from liver with boiling ethanol is coenzyme B₁₂.

**SUMMARY**

Cobamide coenzymes have been isolated in moderate purity from sheep, rabbit, chicken, and human livers. The spectra of all the preparations showed characteristics of cobamide coenzymes containing a benzimidazole derivative; the human and rabbit liver preparations showed features specifically characteristic of coenzyme B₁₂ (DBC coenzyme). All four preparations had molar activities close to that of coenzyme B₁₂ in the glutamate isomerase assay. The coenzyme from human liver was shown to yield 1 mole of 5,6-dimethylbenzimidazole on acid hydrolysis. Quantitative data indicate that at least 48 to 72% of the total cobamide in ethanol extracts of these livers was in the form of coenzyme B₁₂. Cyanocobalamin and hydroxocobalamin, which have previously been isolated from liver, appear to be mainly artifacts, produced by chemical decomposition of coenzyme B₁₂.

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