CHEMICAL STUDIES ON VITAMIN B₁₂ AND ITS RELATED COMPOUNDS

III. ON THE BIOLOGICAL ACTIVITY OF HYDROXOCOBALAMIN FOR OCHROMONAS MALHAMENSIS

TADAKATSU KATO AND SHOICHI SHIMIZU

Department of Industrial Chemistry, Faculty of Engineering, Kyoto University, Sakyo, Kyoto

(Received March 15, 1963)

Hydroxocobalamin (OH-B₁₂) was isolated by Pierce et al. (1) in a crystalline form from the culture of Streptomycetes aureofaciens and was designated as vitamin B₁₂b, which was soon ascertained to be the same as B₁₂a: obtainable by catalytic reduction of cyanocobalamin (CN-B₁₂) using platinum as a catalyst (3, 4). It was also found that OH-B₁₂ was formed by irradiation of CN-B₁₂ in an acidic solution and that it was converted to CN-B₁₂ by treatment with cyanide in a neutral medium (5). Thus OH-B₁₂ is the compound most closely related to CN-B₁₂. Though the effect of OH-B₁₂ on animals and bacteria requiring B₁₂ has been studied by many investigators with comparison with that of CN-B₁₂ (4, 6—10), the results showed great variation, i.e., 20—100 % (4, 6—10).

It is now well known that OH-B₁₂ shows the same effect on pernicious anemia as CN-B₁₂ and no remarkable difference is seen between both the compounds in the response for Lactobacillus leichmannii, Lactobacillus lactis Dorner and Euglena gracilis. But the activity of OH-B₁₂ on Ochromonas malhamensis, an organism most specific to CN-B₁₂, has not been studied in detail.

Kamikubo (11) noticed that vitamin B₁₂ in beef liver gave a very low value, when extracted and determined without addition of cyanide in the Ochromonas method and assumed it to be due to the existence of OH-B₁₂ in the organ without any definite conclusion whether it is due to the lability of OH-B₁₂ or to the low activity of OH-B₁₂ for the organism.

The authors (2) have investigated the non-cyano type B₁₂ in natural sources and found that the Ochromonas values varied markedly when determined with the use of Ford’s medium (13), and presumed it to be due to the lability of non-cyano type B₁₂. In the assay of B₁₂ in natural sources using Ochromonas, addition of cyanide is recommended by Analytical Committee (14). In this method non-cyano type B₁₂ is determined after complete conversion to the cyano type. Therefore, the activity of OH-B₁₂ per se on the growth of Ochromonas can not be obtained.

The present study was undertaken in order to obtain the exact information...
about the activity of OH-B₁₂, a representative of non-cyano type B₁₂, for the growth of *Ochromonas*.

**EXPERIMENTAL**

**Materials and Methods**

1. **Preparation and Physicochemical Properties of OH-B₁₂**

   *Preparation of OH-B₁₂* —— According to Kaczka et al. (2), crystals of OH-B₁₂ was obtained after catalytic reduction of CN-B₁₂ with Adams’ platinum catalyst under normal temperature and pressure. The sample obtained after photolysis of CN-B₁₂ in aqueous solution acidified with acetic acid was used as a reference compound.

   *Identification by Paper Electrophoresis* —— OH-B₁₂ is known to show a basic character in an acidic solution, migrating toward the cathode in paper electrophoresis, whereas CN-B₁₂ is neutral, hardly moving from the starting point. At first, OH-B₁₂ was identified using this test. The condition of paper electrophoresis and the result are shown in Fig. 1. Concentrated solution of OH-B₁₂ crystal (No. 1) showed two spots, a dark red one, greatly migrating toward the cathode, and a very faint red one, hardly migrating from the start. The latter spot seemed to be due to the CN-B₁₂ contaminated in OH-B₁₂ crystal since its mobility agrees with that of standard CN-B₁₂ (No. 3). The former is in coincidence with the photolytic product of CN-B₁₂ (No. 4), possibly due to the main component OH-B₁₂. Further, since the solution of OH-B₁₂ in KCN solution, pH 6.5, (No. 2) showed a red spot in the same position as CN-B₁₂, it was ascertained that OH-B₁₂ had been converted to CN-B₁₂ by cyanide.

![Fig 1 Paper Electropherograms of OH-B₁₂ Preparation](image)

**TABLE I**

<table>
<thead>
<tr>
<th>OH-B₁₂</th>
<th>OH-B₁₂ value in the literature</th>
<th>OH-B₁₂ + cyanide</th>
<th>CN-B₁₂ value in the literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption maxima (mₜ)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>272</td>
<td>274</td>
<td>278</td>
<td>278</td>
</tr>
<tr>
<td>353</td>
<td>351</td>
<td>361</td>
<td>361</td>
</tr>
<tr>
<td>520</td>
<td>525</td>
<td>548</td>
<td>549</td>
</tr>
<tr>
<td>Extinction ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E₁₀₀/E₅₇₈</td>
<td>1.02</td>
<td>1.23-1.30</td>
<td>—</td>
</tr>
<tr>
<td>E₁₅₁/E₅₇₈</td>
<td>2.67</td>
<td>2.69-2.95</td>
<td>—</td>
</tr>
<tr>
<td>E₃₅₁/E₄₄₄</td>
<td>—</td>
<td>1.40</td>
<td>1.62-1.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.89</td>
<td>2.83-3.45</td>
</tr>
</tbody>
</table>
Identification of OH-B₁₂ by Absorption Spectrum —— The absorption spectra of aqueous solution of OH-B₁₂ alone and that added with cyanide were determined in visible and ultraviolet regions using Shimadzu Spectrophotometer Type QB-50. The wave lengths of the absorption maxima and the extinction ratios in the wave lengths of each absorption maximum were found, as shown in Table I, to be in good accordance with those reported in the literature (6).

2. Microbiological Assay

The microbiological assay of B₁₂ using Ochromonas was conducted by the same procedure as reported previously (12). The composition of the medium was the same as described by Ford, and the medium was sterilized at 120°C for 10 minutes. The cultivation was carried out for 72 hours in test tubes, 16 mm in diameter, containing 5 ml of the medium in an inclined position without shaking. For the comparison, L. leichmannii method was conducted according to Pharmacopeia of USA (XV edition) as described in the foregoing paper (12).

RESULTS

1. Comparison of the Microbiological Activities of OH-B₁₂ and CN-B₁₂

To the aqueous solution of OH-B₁₂ (0.5 µg/ml) equal volume of water or 0.1% KCN solution in phosphate buffer (pH 6.5) was added. The solution was kept at 10°C for 15 hours in the dark and subjected to the assay using L. leichmannii and Ochromonas. The activity of the one to which KCN had been added, was assumed to be 100, supposing the complete conversion into CN-B₁₂, and the activity of the other solution to which water had been added, i.e., OH-B₁₂, is shown in Table II. According to L. leichmannii method, the activity of OH-B₁₂ was about 95 per cent of that of CN-B₁₂, no remarkable difference being observed between both the compounds, whereas according to Ochromonas method, the activity of OH-B₁₂ was only about 20 per cent of that of CN-B₁₂ and the values showed remarkable fluctuations.

<table>
<thead>
<tr>
<th>Bacterium used</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. leichmannii</td>
<td>95.5</td>
<td>95.8</td>
<td>95.2</td>
<td>95.2</td>
<td>95.4</td>
</tr>
<tr>
<td>Ochromonas</td>
<td>20.9</td>
<td>16.7</td>
<td>18.0</td>
<td>18.5</td>
<td>18.5</td>
</tr>
<tr>
<td>Ochromonas</td>
<td>95.4</td>
<td>104.5</td>
<td>119.0</td>
<td>90.3</td>
<td>102.3</td>
</tr>
</tbody>
</table>

CN-B₁₂ = 100

This behavior is practically the same as that observed in the case of the cell preparation previously reported, and possibly in common with non-cyano type B₁₂ compounds.

2. Activity to Ochromonas of OH-B₁₂ Sterilized without Heating

In above experiment OH-B₁₂ was sterilized by heating with the medium without cyanide, and showed an activity corresponding to about 20 per cent of CN-B₁₂ in Ochromonas assay. OH-B₁₂ solution was filtered through a Chamberland filter,
aseptically added to a sterilized medium and the activity was measured. As seen from the result shown in Table II, in which the activity of CN-B₁₂ was assumed to be 100, the activity of OH-B₁₂ agreed quite well with that of CN-B₁₂ within the experimental errors.

### 3. Effect of the Addition of Stabilizing Agents During Heat Sterilization

Since it had been reported that the decomposition of OH-B₁₂ during heat sterilization in the assay using *L. leichmannii* could be prevented by adding ascorbic acid to the medium (8, 9), the effect of the acid was investigated in the assay using *Ochromonas* and the results given in Table III were obtained. As the standard curve with ascorbic acid was somewhat different from that without the acid, the standard curve was made with addition of the acid. With addition of 2 mg/ml ascorbic acid in final concentration, OH-B₁₂ values were almost the same as CN-B₁₂, showing that the decomposition of OH-B₁₂ was completely prevented.

<table>
<thead>
<tr>
<th>Condition for sterilization</th>
<th>2 mg/ml ascorbic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With addition</td>
</tr>
<tr>
<td>Heating at 120° for 10 minutes</td>
<td>90-92</td>
</tr>
<tr>
<td>Filtration through Chamberland filter</td>
<td>86-91</td>
</tr>
</tbody>
</table>

### 4. Activity for *Ochromonas* of OH-B₁₂ Heated with Ascorbic Acid

In order to clarify the cause of the effect of ascorbic acid, 0.25 µg/ml OH-B₁₂ solution, to which ascorbic acid had been added at the concentration of 2 mg/ml, was heated at 120° for 10 minutes and then mixed to the Ford medium. The vitamin was determined with a conventional *Ochromonas* method. OH-B₁₂ showed an activity only about 30 per cent of that of CN-B₁₂, suggesting that the decomposition of OH-B₁₂ was accelerated by ascorbic acid in the absence of the medium components, contrary to the preventing effect of ascorbic acid on OH-B₁₂ in the medium.

**DISCUSSION**

OH-B₁₂ was produced by *B₁₂*-producing bacteria (1, 10) and by photolysis of *B₁₂* coenzyme (15). Furthermore, OH-B₁₂ is assumed to be an important intermediate in the biosynthesis of *B₁₂* coenzyme and *B₁₂* bound with protein. Clinically it is noteworthy that OH-B₁₂ is retained in the body longer than CN-B₁₂.

In the above experiment it has been proved that it is almost equally effective to CN-B₁₂ on the growth of *Ochromonas*, an organism which shows an activity nearest to that for mammals.

OH-B₁₂ is, however, heat-unstable, differing from CN-B₁₂, and its activity is almost completely lost by heat sterilization with the Ford medium. It should therefore be added aseptically to the sterilized medium or sterilized with a stabilizing agent such as ascorbic acid in order to determine the activity of OH-B₁₂ per
se, or it may be determined as CN-B₁₂ after conversion to CN-B₁₂ by addition of cyanide. Therefore when OH-B₁₂ is present together with CN-B₁₂, OH-B₁₂ may be determined by subtracting the value without ascorbic acid from that with the acid. The action of ascorbic acid is the problem of more interest. Ascorbic acid shows a stabilizing effect on OH-B₁₂ when added to the Ford medium, but it accelerates decomposition of OH-B₁₂ when added to OH-B₁₂ solution without the medium ingredients.

SUMMARY

Hydroxocobalamin was found to be as effective as cyanocobalamin on the growth of *Ochromonas* as well as on *Lactobacillus leichmannii*. When it is autoclaved with the Ford medium at 120° for 10 minutes, it is decomposed and the activity was reduced to as low as 20 per cent, and it is not reproducible. In order to determine hydroxocobalamin it is necessary to add it aseptically to the sterilized medium or to autoclave with stabilizing agents such as ascorbic acid.

ACKNOWLEDGEMENT

We are much grateful to Professor emeritus Ryohei Takata for his guidance and advice and to Professor Saburo Fukui for his kind revision of this paper.

REFERENCES