Forms of Vitamin B$_{12}$ Compounds Containing SulfitoB$_{12}$ in Corbiculas

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Summary Forms of vitamin B$_{12}$ (B$_{12}$) compounds in young and aged corbiculas were examined by bioautography using B$_{12}$-requiring microorganisms combined with cellulose acetate membrane electrophoresis or high-performance liquid chromatography (HPLC). Both corbiculas (per 100 g) contained ca. 30 µg of cobalamin, a complete type of B$_{12}$. Five known B$_{12}$ compounds, adenosylB$_{12}$ (AdoB$_{12}$), methylB$_{12}$, hydroxoB$_{12}$, sulfitoB$_{12}$ and cyanoB$_{12}$, were identified by bioautography. Young corbicula contained more B$_{12}$ compounds, assumed as methylB$_{12}$, AdoB$_{12}$ and sulfitoB$_{12}$, as compared to aged corbicula. All of the B$_{12}$ compounds detected around the location corresponding to those of methylB$_{12}$, AdoB$_{12}$ and sulfitoB$_{12}$ in the bioautography were converted into compounds that behaved like hydroxoB$_{12}$ after photolysis. Young corbicula was found to contain an unidentified B$_{12}$ compound using bioautography combined with HPLC. A large portion of the B$_{12}$ compound that moved like hydroxoB$_{12}$ during cellulose acetate membrane electrophoresis using 0.5 N acetic acid might be identical with the unidentified B$_{12}$ compound detected in the HPLC-bioautography.

Key Words vitamin B$_{12}$, forms of vitamin B$_{12}$, sulfitoB$_{12}$, corbicula

Vitamin B$_{12}$ (B$_{12}$), discovered as an antipernicious anemia factor, is now noted in connection with diseases such as arteriosclerosis (1) and senile dementia of the Alzheimer type (2) through the CI metabolism affecting plasma homocysteine concentration. Furthermore, it has been reported that B$_{12}$ acts as an immunomodulator for cellular immunity (3), which is also very intriguing. However, there are still many problems remaining regarding the physiological roles of B$_{12}$. Bioautography to identify the forms of B$_{12}$ compounds is a useful method for obtaining a clue to B$_{12}$ roles, by which it would be possible to estimate B$_{12}$-dependent enzymatic reactions functioning in the cells.

Farquharson and Adams (4) reported on the forms of B$_{12}$ in several animal foods. By using a thin-layer chromatography-bioautography, they identified five forms of B$_{12}$ (cobalamin): AdoB$_{12}$ (adenosylcobalammin), methylB$_{12}$, hydroxoB$_{12}$, sulfitoB$_{12}$ and cyanoB$_{12}$. Methyl-, sulfito- and cyanoB$_{12}$ were detected only in a few foods and in small quantities, although methylB$_{12}$ was detected in significant quantities in egg yolk and cheese. It is noteworthy that sulfitoB$_{12}$ was found mainly in canned foods and considered to be an artifact (5).

Among food materials, the content of B$_{12}$ is known to be highest in livers and shellfishes such as corbiculas, clams and oysters. However, there are few reports on the forms of B$_{12}$ compounds in shellfishes. The identification of B$_{12}$ forms in shellfishes will be useful not only from a nutritional point of view, but also from the viewpoint of exploring the physiological roles of B$_{12}$. In the present paper, we thus examined the forms of B$_{12}$ in corbicula by bioautography using B$_{12}$-requiring microorganisms, combined with cellulose acetate membrane electrophoresis or HPLC.

Materials and Methods

Materials. Corbiculas, Japanese freshwater clams called "shijimi," were obtained from Lake Shinji in Shimane and stored at −80°C until use. They were separated into two groups: young corbiculas of nearly 2 to 3 y old, whose shell size was ca. 1 cm; and aged corbiculas of nearly 5 to 6 y old, whose shell size was ca. 2.5 cm. Ado-, cyano- and hydroxoB$_{12}$ were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). MethylB$_{12}$ and sulfitoB$_{12}$ were prepared according to procedures previously described (6).

Extraction and determination of B$_{12}$ compounds. Twenty grams of the stripped corbiculas were suspended in 30 mL of distilled water, and the cells were disrupted by a Trio blender (Trio Science Co. Ltd., Japan).

For determination of the total B$_{12}$ content, 1 mL of the homogenate was mixed with 9 mL of 0.1 M acetic buffer (pH 4.5) and 0.5 mL of 0.1% KCN solution. B$_{12}$ compounds were extracted by boiling for 20 min. After adjusting the volume to 50 mL, the B$_{12}$ extract was centrifuged at 12,000×g for 20 min at 4°C. The super-

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natant was used for B12 determination with Escherichia coli 215 (6-8) or Lactobacillus delbrueckii subsp. lactis ATCC 7830 (6). For exclusion of methionine from samples for B12 determination, a Sep-Pak C18 cartridge (Waters, Division of Millipore, Milford, Mass., USA) was used after prewashing with 5 mL of 70% ethanol and then 30 mL of distilled water. Five milliliters of the sample for B12 determination were applied on the cartridge. B12 compounds were eluted with 10 mL of 50% ethanol after washing the cartridge with 10 mL of distilled water. The residue obtained by evaporating the eluate to dryness was dissolved in 5 mL of distilled water. This was used as a Sep-Pak-treated sample for B12 determination.

For bioautography, B12 compounds were extracted from 20 mL of the above-prepared homogenate by adding 80 mL of ethanol and then heating at 90°C for 20 min. The extract was centrifuged at 12,000×g for 20 min at 4°C. The supernatant was evaporated to dryness and the residue was dissolved in 20 mL of distilled water. After centrifugation of the solution thus obtained, 5 mL of the supernatant was treated with a Sep-Pak C18 cartridge as described above. This solution was used as a sample for cellulose acetate membrane electrophoresis or HPLC (6-8).

**Results and Discussion**

**Vitamin B12 contents of corbiculas.** The contents of B12 compounds in young and aged corbiculas are shown in Table 1. B12 contents in corbiculas were fairly close to the B12 levels of beef or pork livers (50-60 μg/100 g), which are known to have the largest content of B12 among foods. This level of B12 is assumed to be very high, since only a very few foods contain over 10 μg/100 g. B12 contents (ca. 30 μg/100 g) determined using L. delbrueckii 7830 were lower than that (ca. 60 μg/100 g) determined by E. coli 215 assay. This might be because the samples contained a sufficient amount of methionine or some incomplete B12 like cobinamide, which promotes the growth of E. coli 215. This assumption seems to be supported by the fact that the B12 contents of the Sep-Pak C18-treated samples determined by the E. coli assay were of a similar level to B12 contents determined by the L. delbrueckii assay, but further study is necessary to obtain conclusive evidence.

**Cellulose acetate membrane electrophoresis-bioautography.** The forms of B12 compounds in corbiculas were examined by cellulose acetate membrane electrophoresis. As shown in Fig. 1, which is the typical result, forms of B12 compounds corresponding to all five authentic samples were detected in the corbiculas samples upon cellulose acetate membrane electrophoresis (0.5 N acetic acid, 600 V, 2 h). However, young corbiculas contained more methylB12, AdoB12, hydroxob12, sulfitob12 and cyanoB12 as compared to aged corbiculas (Fig. 1A). In aged corbiculas, the content of hydroxob12 was higher (Fig. 1B). These facts suggest that the metabolisms of young corbiculas seem to be more active in the coenzymic forms of B12. On the other hand, cyanoB12
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Fig. 2. Cellulose acetate membrane electrophoresis bioautography of vitamin B12 compounds extracted from young corbicula and photo-decomposed using a tungsten lamp. The other conditions and procedures were the same as described in the legend of Fig. 1.

Fig. 3. Cellulose acetate membrane electrophoresis bioautography of vitamin B12 compounds extracted from young corbicula. Electrophoresis was performed in acetate buffer (pH 3.5) at 600 V for 2 h. The other conditions and procedures were the same as described in the legend of Fig. 1.

was also detected in both young and aged corbiculas. The reason for this is not clear, but cyanoB12 might have been formed by a metabolism of cyanide in corbiculas or incorporated into them by the food chain.

When the samples from young corbiculas were exposed to light, light-sensitive B12 compounds were decomposed and only hydroxo- and cyanoB12 were detected as shown in Fig. 2. It could be assumed that methylB12, AdoB12 and sulfitoB12 were converted into hydroxoB12 by the photolysis. Similar results were obtained with the sample of aged corbiculas.

The bioautography with cellulose acetate membrane electrophoresis at pH 3.5 showed that the amount of hydroxoB12 in the young corbiculas was very small (Fig. 3). From these results, a considerable amount of B12 detected in the vicinity of hydroxoB12 upon electrophoresis in 0.5 N acetic acid (Fig. 1) may be different from hydroxoB12 itself.

**HPLC-bioautography.** Bioautography combined with HPLC also demonstrated the presence of five forms of B12 compounds (Fig. 4). The B12 positions active in the microbiological assay were delayed by one fraction, relative to the positions of the authentic B12 detected in UV absorption of 260 nm. This was caused by the existence of a small distance between the detector microcell and the effluent exit connected to the microcell by a tube. By this bioautography, a considerable amount of an unidentified B12 compound was detected in addition to a small amount of hydroxoB12. At present, we do not know whether this unidentified B12 might be artificially formed with hydroxoB12, or actually present in corbiculas. The unidentified B12 might be identical to the B12 compound detected in the vicinity of hydroxoB12 during cellulose acetate membrane electrophoresis in 0.5 N acetic acid (Fig. 1).

Furthermore, the presence of sulfitoB12 in corbiculas, in particular young ones, was confirmed by the HPLC-bioautography. Although Farquharson and Adams (4, 5) reported that sulfitoB12 found in canned foods would be an artifact, we previously found a considerable amount of sulfitoB12 in raw eggs (9), and presently in corbiculas as well as oysters (data not shown). From these results, there is a possibility that sulfitoB12 may not be an artifact and might have some physiological role, which remains to be revealed.

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