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*ISOLATION AND PROPERTIES OF B_{12} COENZYMES CONTAINING BENZIMIDAZOLE OR DIMETHYLBENZIMIDAZOLE**

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Cell-free extracts of *Clostridium tetanomorphum* convert glutamate via β-methylaspartate to mesaconate. The step from glutamate to β-methylaspartate has been shown[1] to require a coenzyme that has been isolated from *C. tetanomorphum* and identified as a derivative of pseudovitamin B_{12}. This coenzyme, which we designate adenine-B_{12} coenzyme, contains two moles of adenine, one of which is attached to ribose as in pseudovitamin B_{12}, whereas the other is probably attached to a double bond in the corphyrin ring system in such a way as to greatly modify the spectrum.

We now wish to report the isolation of two additional forms of the B_{12} coenzyme.

Several bacteria, such as *E. coli*[2] and *Propionibacterium* species,[3] are known to form different cobalamins when grown in the presence of different heterocyclic bases. We have found that *C. tetanomorphum* possesses the ability to utilize benzimidazole and 5,6-dimethylbenzimidazole[4] in a similar manner in the formation of B_{12} coenzymes. By growing this bacterium in the presence of one of these compounds, the formation of the adenine-B_{12} coenzyme is suppressed, and the corresponding benzimidazole coenzyme is produced in comparable amount. In this way we have prepared, and subsequently isolated by ion exchange methods, micromolar amounts of a benzimidazole-B_{12} coenzyme and a 5,6-dimethylbenzimidazole-B_{12} coenzyme.

The spectrum of a highly purified sample of the benzimidazole-B_{12} coenzyme is compared with that of the adenine-B_{12} coenzyme in Figure 1. The spectrum of the 5,6-dimethylbenzimidazole-B_{12} coenzyme is almost identical with that of the benzimidazole coenzyme and therefore is not shown separately. Both of these coenzymes have absorption maxima at 261, 375, and 519 mμ; the corresponding molar extinction coefficients in neutral solution are 35.5, 9.90, and 7.55×10^6 cm^2/mole, respectively. The most conspicuous difference between the spectra of the benzimidazole- and adenine-B_{12} coenzymes is in the region above 400 mμ. The adenine coenzyme has a broad peak with a maximum at 458 mμ and a relatively slight absorption above 500 mμ, and it is orange in color. The benzimidazole coenzymes have a broad absorption peak with a maximum at about 519 mμ and

they are red. A significant feature of the spectra of the benzimidazole coenzymes, which they share with the adenine coenzyme, is the absence of the high absorption peak in the 360 mμ region, which is characteristic of other cobalamins. The relatively high absorption of the benzimidazole coenzyme in the 260 mμ region, as compared with vitamin B_{12}, is attributable to the presence of an additional mole of adenine in the molecule (see below).

Benzimidazole was identified as a product of acid hydrolysis (6 *N* HCl for 18 hr at 150°) of the benzimidazole-B_{12} coenzyme. The hydrolyzate was made alkaline and the liberated base was extracted with chloroform. Benzimidazole was identified and estimated quantitatively by its characteristic absorption spectrum and by its fluorescence peak at 360 mμ when activated by light at 272 mμ in 0.1 *N* acetic acid; it was further characterized by paper chromatography using 0.1 *N* acetic acid as a solvent (R_f = 0.72). The quantity of benzimidazole re-

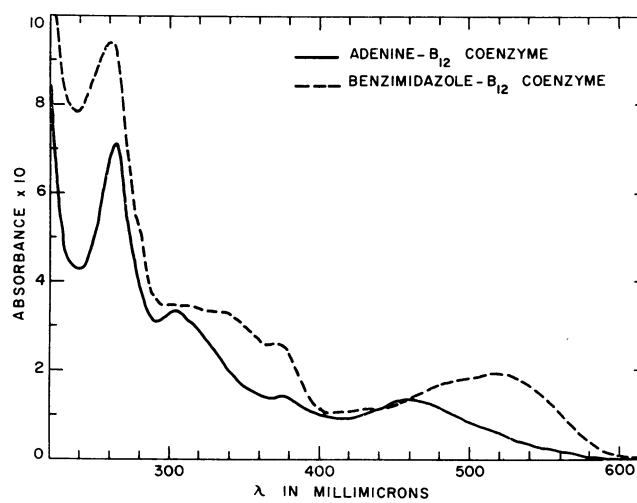

FIG. 1.—Absorption spectra of 1.51 × 10^{-5} M adenine-B_{12} coenzyme and 2.64 × 10^{-5} M benzimidazole-B_{12} coenzyme in 0.02 M sodium acetate buffer pH 6.7.

covered from the hydrolyzate corresponded to 0.80 mole per mole of corphinamide. 5,6-Dimethylbenzimidazole was identified as a product of acid hydrolysis of the corresponding coenzyme by similar methods.

Both of the benzimidazole-B_{12} coenzymes differ from the corresponding vitamins by containing adenine. The adenine can be removed by vigorous acid hydrolysis, by treatment with cyanide ion or by exposure to light. Adenine has been isolated from an acid hydrolyzate (1 *N* HCl, 1 hr, 100°) by paper or column chromatography and identified by its R_f, its characteristic absorption peak at 260 mμ in neutral solution and at 267 mμ in alkali. Quantitative determinations showed the presence of approximately one mole of adenine per mole of corphinamide.

All three of the B_{12} coenzymes are active in the enzymatic assay system. The benzimidazole-B_{12} coenzyme is much the most active, whereas the 5,6-dimethylbenzimidazole-B_{12} coenzyme is the least active. The relative molar activities of the benzimidazole-, adenine-, and dimethylbenzimidazole-B_{12} coenzymes are

approximately 100, 17, and 1.6, respectively. The absolute activity of the benzimidazole-B_{12} coenzyme under our usual assay conditions is approximately 2,000 units per μmole, the activity unit corresponding to a change of one absorbance unit per minute at 240 mμ.[1] Therefore it is possible to assay with some accuracy a sample containing approximately 10^{-5} μmole (1.5×10^{-2} μg) of benzimidazole-B_{12} coenzyme, 10^{-4} μmole of adenine-B_{12} coenzyme or 10^{-3} μmole of 5,6-dimethylbenzimidazole-B_{12} coenzyme.

The benzimidazole-B_{12} coenzyme, like the adenine-B_{12} coenzyme,[1] is readily inactivated by exposure to visible light or cyanide ion. On exposure to light, the absorption maximum at 375 mμ disappears, the single peak at 519 mμ is changed to a double peak with maxima at 498 mμ and 522 mμ, and a large new peak appears at 350 mμ (Fig. 2). The spectrum of the light-inactivated benzimidazole-B_{12}

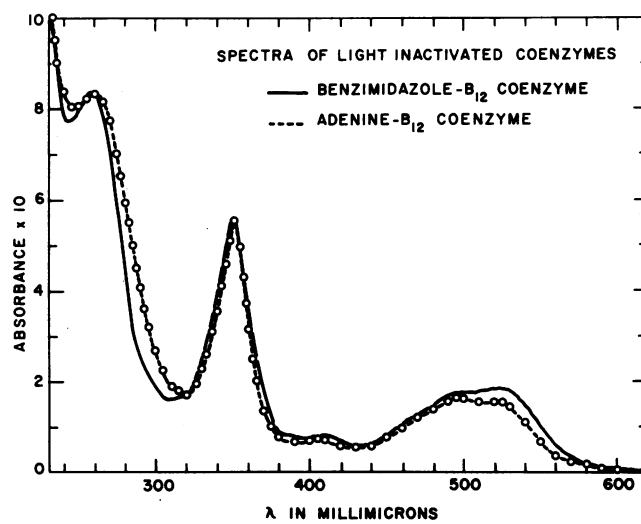

FIG. 2.—Absorption spectra of light-inactivated B_{12} coenzymes. Solutions of both coenzymes were exposed to a 100 watt tungsten lamp for 25 minutes at a distance of 15 cm at 0°C. The benzimidazole-B_{12} coenzyme solution was 2.64×10^{-5} M. To facilitate comparison of the two spectra, the absorbance of the light-inactivated adenine-B_{12} coenzyme was arbitrarily assigned the same value as that of the light-inactivated benzimidazole-B_{12} at 351 mμ.

coenzyme is very similar to that of the light-inactivated adenine-B_{12} coenzyme. On treatment with 0.1 *M* cyanide ion, the color of the benzimidazole-B_{12} coenzyme changes from red to purple, and the new spectrum is typical of dicyanocobamides except for the larger absorption in the 260 mμ region (Fig. 3). The spectrum of the dimethylbenzimidazole-B_{12} coenzyme is modified in the same ways by exposure to light or cyanide. With all of the B_{12} coenzymes, treatment with cyanide causes rapid liberation of one mole of adenine and formation of the corresponding B_{12} vitamin.

The adenine-B_{12} coenzyme is rapidly inactivated by mild acid hydrolysis (0.07 *N* HCl 10 min, 85°). The two benzimidazole-B_{12} coenzymes, on the contrary, are relatively insensitive to hydrolysis under these conditions, apparently because of

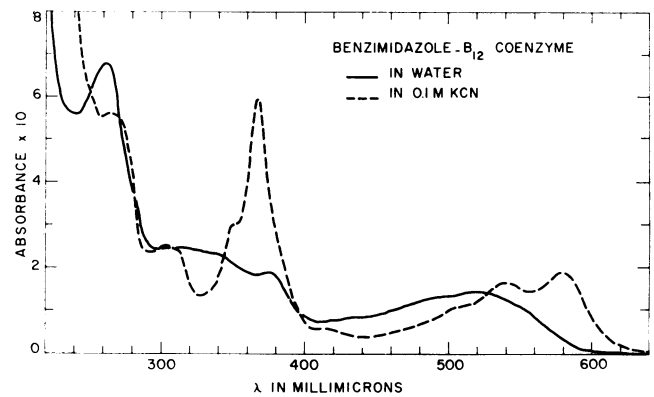

FIG. 3.—Absorption spectra of 1.91×10^{-5} M benzimidazole-B_{12} coenzyme in water and in 0.1 *M* KCN.

the much greater stability of the benzimidazole-ribose linkage as compared to the adenine-ribose linkage. The great difference in sensitivity to mild acid hydrolysis provides a convenient method of distinguishing between purine- and benzimidazole-B_{12} coenzymes in crude preparations. After 5 min hydrolysis under the above conditions, over 90 per cent of the adenine-B_{12} coenzyme is inactivated, whereas only 8 per cent of the activity of the benzimidazole-B_{12} coenzyme is lost.

The occurrence of B_{12} coenzymes in two other biological materials has been observed. A coenzyme with the same spectrum and the same specific activity in the enzymatic assay as the 5,6-dimethylbenzimidazole-B_{12} coenzyme from *C. tetanomorphum* was obtained from rabbit liver and from dried cells of a species of Propionibacterium.[5] With liver, the yield of coenzyme was relatively low, 0.015 μmole per 250 gm of fresh liver, because of losses during extraction and isolation. Since the losses during isolation from liver were at least 80 per cent, the actual coenzyme content per 100 gm of fresh liver must be approximately 0.03 μmole. This corresponds to about 80 per cent of the reported vitamin B_{12} content (0.038 μmole/100 gm) of beef liver.[6] The coenzyme content of the dried propionic acid bacteria was considerably higher, approximately 0.05 μmole per gram. At least 60 per cent of the total cobalamin in this preparation appeared to be in the coenzyme form.

Since the B_{12} coenzymes are present in considerable amount in organisms that have been previously reported to contain B_{12} vitamins, the question arises as to why the coenzyme forms, with their distinctive spectra, have been overlooked. The answer appears to lie in the methods that have been used to extract cobalamins from biological materials. These methods have commonly involved heating for a considerable time in weakly acid solution, addition of cyanide ion, and exposure to light. Each of these treatments accelerates the conversion of the coenzyme forms to the vitamin forms. Even relatively brief exposure to cyanide ion or light can cause a more or less quantitative decomposition of the B_{12} coenzymes.

The isolation of the adenine-, benzimidazole- and 5,6-dimethylbenzimidazole-B_{12} coenzymes from *C. tetanomorphum* and the demonstration that they possess similar spectra and similar sensitivities to light and cyanide ion, establish the existence of a new class of corphinamide derivatives possessing biological activity. The further

identification of the dimethylbenzimidazole-B_{12} coenzyme in propionic acid bacteria and rabbit liver suggests that these compounds may be active in a wide range of organisms. In the two bacteria and in rabbit liver, the only biological materials so far examined, a considerable fraction of the total cobalamin is present in the coenzyme forms. These coenzymes may be the main forms of B_{12} and B_{12}-like compounds that are catalytically active in the enzymatic reactions of living cells.

The discovery of the coenzyme forms of vitamin B_{12} opens new possibilities for the study of enzymatic processes, such as the synthesis of methionine,[7] of protein,[8] and of deoxyribonucleosides[9] in which the vitamin is believed to participate. Also, in the treatment of pernicious anemia, the coenzyme theoretically could be more effective than the vitamin under some circumstances. For example, the coenzyme may be absorbed from the digestive tract more readily than the vitamin in the absence of intrinsic factor.[10] Also, conditions may exist in which, because of a block in the conversion of vitamin to coenzyme, only the latter has therapeutic value. These possibilities still require exploration.

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*ELECTRON MAGNETIC RESONANCE OF $CH(COOH)_2$**

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We have carried out a detailed analysis of the electron magnetic resonance spectra of single crystals of malonic acid that were subjected to 50-kv. X-ray damage at room temperature. A typical spectrum of an aged single crystal is shown in Figure 1. The positions and intensities of the principal signals (1–4 in Fig. 1) depend on the crystal orientation relative to the applied magnetic field (3,400 gauss). All of the observed spectra can be accounted for in terms of the following set of assumptions.