INFLUENCE OF OXYGEN ON THE PHOTOLYSIS OF COENZYME B_{12}*

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Coenzyme B_{12} and its analogues are rather stable in aqueous solution in the dark, but are rapidly decomposed by visible or ultraviolet light with the formation of the corresponding aquocobamides (Weissbach et al., 1959; Barker et al., 1960b; Weissbach et al., 1960). This decomposition is accompanied by a major change in the absorption spectrum which may be the result of an oxidation of the cobalt atom from the divalent to the trivalent form or of an increase in the conjugation in the corrinoid ring system. In either event, the over-all reaction may involve an oxidation of the coenzyme. This possibility was investigated by Pawelkiewicz et al. (1960) using a light-sensitive corrinoid conjugate isolated from Propionibacterium shermanii which has a spectrum similar to that of the adeninylcobamide (AC) coenzyme (Barker et al., 1960a). This compound, designated SB_{12p}, presumably is structurally similar to coenzyme B_{12} except that it lacks a part of the nucleotide side chain and therefore is a derivative of cobinamide (corphinamide). Pawelkiewicz et al. briefly reported that the photolysis of SB_{12p} occurs only in the presence of oxygen, and is prevented by the addition of ascorbate or by the use of a hydrogen atmosphere. They also reported that SB_{12p} can be synthesized by reducing cobinamide with hydrosulfite in the presence of adenine.

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We have attempted to confirm the report of Pawelkiewicz et al. by determining the influence of oxygen on the photolysis of coenzyme B_{12}. We have found that oxygen is not involved in the initial photolytic process, but it intervenes in a subsequent step and determines the nature of the final product. An attempt to synthesize coenzyme B_{12} by the reduction of aquocobalamin in the presence of adenine or adenosine was unsuccessful.

Experiments on the photolysis of coenzyme B_{12} were done with an approximately 28 μM solution of the crystalline coenzyme (Barker et al., 1960b) in water. The solution was placed in a special silica cuvette, 1 cm light path, having a top constructed like a conventional Thunberg tube. Oxygen was removed from the coenzyme solution, when necessary, by bubbling either hydrogen or oxygen-free helium through it for at least 15 minutes. The cuvette was then closed, evacuated until the solution started to boil and refilled to atmospheric pressure with oxygen-free gas; this procedure was repeated four times. The absorption spectrum of the coenzyme solution was determined with a Model 14 Cary spectrophotometer that had a modified cover for the absorption cell compartment designed to accommodate the Thunberg-type cells. All these operations were carried out in very dim light or total darkness. The light absorbed during determination of the spectrum did not cause detectible decomposition of the coenzyme.

The spectrum of an O_2-free solution of coenzyme B_{12} was determined before and after exposure to a 100 watt flood light at a distance of 10 cm (Fig. 1). This exposure caused complete decomposition of the coenzyme as indicated by the degree of spectral change. The absorption spectrum of the photolyzed coenzyme is very different from that of the intact coenzyme and is virtually identical, above 300 mμ, with that of vitamin B_{12r} (Diehl and Murie, 1952; Beaven and Johnson, 1955). Absorbancy maxima are located at 263, 310, 404 and 474 mμ, shoulders at about 286, 347 and 530 mμ, and minima at 242, 294, 387 and 423 mμ. The 263 mμ absorbancy maximum, not present in vitamin B_{12r}, is largely attributable to the adenine moiety of

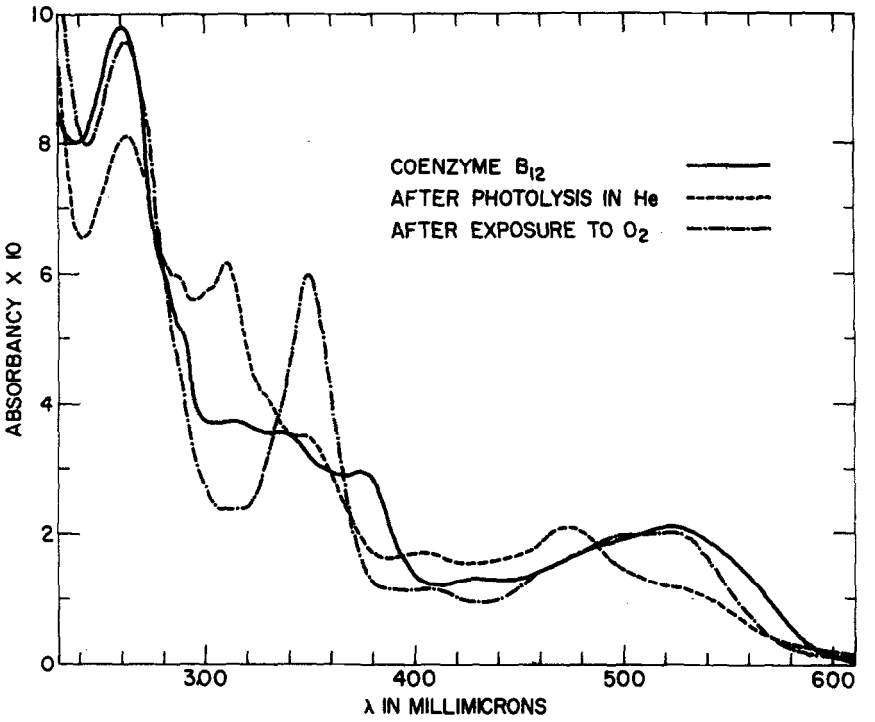

Fig. 1. Spectra of coenzyme B_{12} and the products of its anaerobic photolysis before and after exposure to oxygen. The experimental conditions are described in the text.

the coenzyme. The spectrum of the photolyzed solution did not change with time, as long as air was excluded. When air was admitted, the absorption spectrum of the solution changed, above 310 mμ, to that characteristic of aquocobalamin (Fig. 1). This change required several minutes; the rate was obviously dependent upon the availability of oxygen. The yield of vitamin B_{12r} resulting from the photolysis of coenzyme B_{12}, estimated from the absorbancy of the solution at 473 mμ and the molar absorbancy index of 7.3×10^6 cm^2/mole[1] (Diehl and Murie, 1952), was 1.05 moles/mole of

[1] By assuming that the photolytic conversion of coenzyme B_{12} to vitamin B_{12r} is strictly quantitative, the molar absorbancy indices of vitamin B_{12r} can be calculated from the spectra of Fig. 1 and the molar absorbancy index of coenzyme B_{12} at 375 mμ to be 22.8, 6.3, 12.9 and 7.7×10^6 cm^2/mole at 311, 405, 345 and 473 mμ, respectively. These values are about 8% higher than those reported by Diehl and Murie (1952).

coenzyme. The yield of aquocobalamin, estimated from the absorbancy at 350 mμ and the molar absorbancy index of 20.4×10^6 cm^2/mole (Friedrich and Bernhauer, 1956), was 1.09 moles/mole of coenzyme. Considering possible errors in the molar extinction coefficients of these compounds, the results indicate that anaerobic photolysis of the coenzyme causes an essentially quantitative formation of vitamin B_{12r}, which can be oxidized quantitatively to aquocobalamin.

When the coenzyme is photolyzed in a solution saturated with oxygen, vitamin B_{12r} does not accumulate in appreciable amounts but is presumably oxidized to aquocobalamin, recognizable by its spectrum. The rate of coenzyme decomposition by light of a given intensity is essentially the same in the presence or absence of oxygen. The rate of decomposition was measured by following the absorbancy increase at 350 mμ in the presence of air and at 312 mμ in the absence of oxygen.

These results demonstrate that the photolytic conversion of coenzyme B_{12} to aquocobalamin involves two readily separable reactions: (a) the photolytic reaction proper which converts the coenzyme quantitatively to vitamin B_{12r} and does not require oxygen, and (b) the oxidation of vitamin B_{12r} to aquocobalamin by O_2. The initial formation of vitamin B_{12r} is consistent with the view that coenzyme B_{12} contains divalent cobalt. The divalent state of the cobalt is strongly indicated by the work of Bernhauer *et al.* (1961) which demonstrates that in solution the coenzyme is paramagnetic. In contrast, cyanocobalamin is diamagnetic and is believed to contain trivalent cobalt (Wallmann *et al.*, 1951).

Coenzyme B_{12} and its analogues are known to be inactivated by light in the presence of oxygen (Weissbach *et al.*, 1960). We have now shown that anaerobic photolysis also destroys coenzyme activity. The experiment was carried out by placing all of the components of the glutamate isomerase coenzyme assay mixture (Barker *et al.*, 1960a), except coenzyme B_{12}, in the main compartment of a Thunberg-type silica cuvette. A suitable aliquot of

a coenzyme solution was placed in the side arm. Both solutions were thoroughly deoxygenated and the tube was filled with helium. The coenzyme was converted to vitamin B_{12r} by photolysis and then mixed with the assay mixture. No activity could be detected, whereas the same coenzyme solution, before photolysis, was fully active.

An attempt to achieve a chemical synthesis of coenzyme B_{12} by the reduction of aquocobalamin in the presence of adenine or adenosine, using the general method reported by Pawelkiewicz et al. (1960), was made in the following manner. Hydrogen gas was bubbled through 3 ml of water in each of three Thunberg tubes for 15 minutes, followed by the introduction of 50 mμmoles of aquocobalamin and 2 mg of platinum oxide per tube. The solution was approximately neutral. The tubes were evacuated and hydrogen gas was introduced at atmospheric pressure. This operation was repeated four times. The tubes were then shaken gently for 5 minutes in the dark, at which time the characteristic spectrum of B_{12r} was obtained. The platinum was removed by centrifugation at slow speed in the dark, and the supernatant solutions were rapidly transferred to Thunberg tube type pyrex cuvettes. The cuvettes were evacuated and refilled with a hydrogen atmosphere. To one tube no further addition was made; to the other tubes, 30 μmoles of adenosine or adenine were added from the side arm. The mixture was incubated for an additional 15 minutes in the dark. No reoxidation of vitamin B_{12r} occurred during these procedures. No spectral evidence of coenzyme formation could be detected either with or without adenine or adenosine. Photolysis caused no change in the spectrum of the reaction mixtures. When air was bubbled through the solutions, the spectrum gradually changed to that of aquocobalamin.

In a separate experiment, using 1.5 μmoles of aquocobalamin and 7.5 μmoles of adenosine in a total volume of 2 ml, we tried to detect the formation of coenzyme B_{12} or other corrinoid conjugate having the same ionic properties by chromatography on a Dowex-50 column. Under the condi-

tions of chromatography used, coenzyme B_{12} and its analogues can be readily separated from aquocobalamin (Barker et al., 1960a). No corrinoid compound was detected in the portion of the elution pattern in which the cobamide coenzymes normally appear. We conclude that no light-sensitive corrinoid conjugate, having a spectrum similar to that of coenzyme B_{12}, was formed under the conditions provided by either of the above experiments. A similar result was obtained when sodium hydrosulfite was used as a reducing agent in place of hydrogen and platinum oxide. We have no explanation for the discrepancy between our results and those of Pawelkiewicz et al. (1960). We have been able to confirm the enzymatic synthesis of cobamide coenzymes from aquocobamides using extracts of P. shermanii as reported by Pawelkiewicz et al. and by Bernhauer et al. (1960).

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