COBALAMIN CATALYZED OXIDATION OF SULFHYDRYL GROUPS

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Vitamin B_{12} has been shown to function as a coenzyme in a number of enzymatic oxidation-reduction reactions (Brownstein and Abeles,1960), (Eggerer et al,1960). Peel (1962) has recently reported that cobalamins greatly accelerate the auto-oxidation of mercaptoethanol. While studying the role of vitamin B_{12} in the methylation of homocysteine, we noticed that both homocysteine and DPNH were rapidly oxidized in the presence of aquocobalamin. This communication describes the non-enzymatic oxidation of mercaptans by vitamin B_{12} derivatives, mediating the oxidation of reduced diphosphopyridine nucleotide(DPNH), and the formation of a mercaptan-cobalamin complex.

Oxidation of mercaptans was followed by measuring oxygen uptake, disappearance of sulfhydryl groups, or reduction of triphenyl tetrazolium chloride (TTC). Calibration curves of reduced TTC were prepared by reduction of TTC with dithionite, or with excess mercaptan in presence of cobalamin. Sulfhydryl groups were determined by the nitroprusside method of Grunert and Phillips (1951). Aquocobalamin was prepared from cyanocobalamin by photolysis at pH 4. DPN^+ was identified with the aid of the alcohol dehydrogenase system (ADH) according to the method of Racker (1950).

Aquocobalamin or factor B catalyzed the oxidation of homocysteine, cysteine, glutathione, 2-mercaptoethanol, and 2,3-dimercaptopropanol at approximately equal rates. In the absence of any cobalamin the rate of auto-oxidation was very low (Table 1). The oxidation rate was found to be directly

Table 1

Oxidation of homocysteine by vitamin B_{12} derivatives

Cobalamin added	Oxygen consumed (ul./5 min.)						
	Cobalamin concentration (uM)						
	0.0	0.4	2	10	50	100	200
Factor B		28	48	57			
Aquocobalamin			8	19	34		
Cyanocobalamin				3	4	12	17
None	1						

Warburg flasks contained: homocysteine, 30 umoles; phosphate buffer pH 8, 50 umoles; total volume, 2 ml. Incubated in air, at 37°C.

proportional to the concentration of both catalyst (oxidant) and substrate (reductant). There was essentially no oxidation of homocysteine at pH 4; the rate increased with increasing pH up to 8.5. A similar pH dependence was observed with TTC, indicating that the anion RS^- is the oxidizable form of the mercaptan. Factor B was the most active of the vitamin B_{12} derivatives tested. The maximal oxidation rate of homocysteine (1.5×10^{-2}M, at pH 8, in air) was obtained with 1×10^{-5}M factor B or with 2×10^{-4}M aquocobalamin. Dimethyl benzimidazole cobamide coenzyme (DBCC) was almost as active as aquocobalamin, while cyanocobalamin was considerably less active(Table 1). Cobalt and iron ions (Co^{2+}, Co^{3+}, Fe^{2+}, Fe^{3+}) were inactive at concentrations up to 5×10^{-4}M.

KCN (2×10^{-4}M) completely inhibited the uptake of oxygen and the reduction of TTC in the presence of 1×10^{-4}M aquocobalamin. Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (2×10^{-4}M) lowered the rate of oxygen uptake, suggesting that flavins may act anaerobically as electron acceptors from cobalamins. Direct measurements of changes in the absorbancy of flavins at 450 mu under anaerobic conditions confirmed this assumption.

DPNH was not oxidized by cobalamins. However, addition of a mercaptan caused a rapid oxidation of DPNH in air. DPNH significantly decreased the oxidation rate of the mercaptan, thus suggesting that free radicals of the

substrate were formed as a result of the cobalamin activity. DPNH was quantitatively oxidized to DPN^+ as shown by the instantaneous increase in absorbancy to the original level upon addition of the ADH system (fig.1).

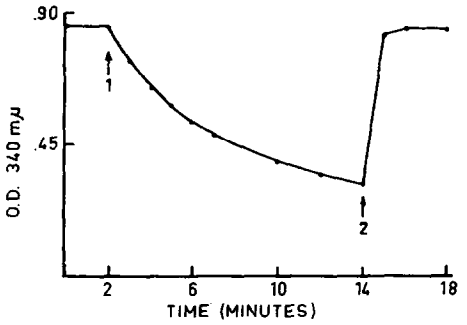

Fig.1. Oxidation of DPNH in the presence of homocysteine and aquocobalamin. DPNH, (0.1 mM); homocysteine, (2 mM); aquocobalamin, (0.02 mM); phosphate buffer pH 8, (5 mM); incubated in air at 25°C. 1. Addition of homocysteine. 2. ADH system added.

Cystine and homocystine were identified chromatographically as the oxidation products of cysteine and homocysteine, respectively. In assays with TTC the molar ratio of thiol added to TTC reduced was found to be 2.2 - 2.8 : 1. Comparison of O_2 uptake and sulfhydryl group oxidation indicated a molar ratio of 4. This ratio was not affected by addition of catalase, suggesting that peroxide was not the final oxidation product.

There was no change in the spectrum of cyanocobalamin or dicyano-factor B upon incubation with an oxidizable mercaptan. However, when aquocobalamin or factor B were incubated with homocysteine or with mercaptoethanol their spectrum changed rapidly; the main absorption band maxima for factor B (at 355, 500 and 530 mu) decreased in intensity and disappeared, while a new band with a maximum at about 475 mu appeared (fig.2). Analogous

spectral changes were observed with aquocobalamin. No reversion to the original spectrum occurred upon aeration, addition of hydrogen peroxide, or cyanide.

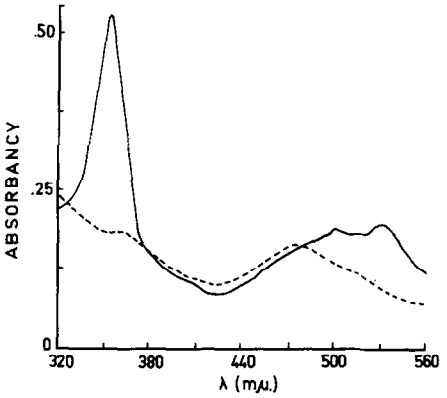

Fig.2. Absorption spectra of factor B and the mercaptan-factor B complex.
(——), factor B (0.02 mM) in phosphate buffer pH 8.
(- - -), after incubation for 30 minutes with mercaptoethanol (2 mM).

Formation of the mercaptan-cobalamin complex (of yellow color) can be followed by measuring the decrease in absorbancy at 355 mu. The spectrum of the complex between 330 - 560 mu resembles that of the pigments isolated by Helgeland (1961), the γ - picoline cobalamin complex described by Pratt and Williams (1961) and the photolyzed cobamide coenzyme (Brady and Barker, 1961). However, in contrast to the cobamide coenzyme, the complex described here is stable towards air and light. The formation of the complex may involve reduction of the cobalt atom followed by coordination to the mercaptide anion RS^-. On the other hand, the lower intensity and the displacement of the absorption bands to shorter wavelengths may indicate a rupture in the conjugated chain of the vitamin.

The basic aquocobalamin or factor B cations, or their reduction products, seem to be the active forms of the catalyst. This is suggested by the relative inactivity of cyanocobalamin and the inhibition by KCN. The

coordinated water molecules in aquocobalamin and factor B are readily displaced by anionic ligands (George et al, 1960). The mercaptide anion RS^- may presumably function as such a ligand forming a mercaptan-cobalamin complex. In presence of cyanide a stable cyano-complex is formed, which does not appreciably dissociate in aqueous solution and is, therefore, relatively inactive.

The aquocobalamin-catalyzed oxidation of DPNH and the reduction of flavins in presence of mercaptans are of interest in view of the recently published observations on the participation of B_{12}, DPNH and FAD in the biosynthesis of methionine from homocysteine and formaldehyde (Hatch et al,1959) (Aronovitch and Grossowicz, 1961). Peel's findings and our own may throw light on the earlier observations of Dubnoff and Barton (1956) on the activation of enzymes by glutathione and vitamin B_{12}, indicating a role of the vitamin in the activation or protection of protein sulfhydryl groups.

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