

tance between the ruthenium ions increases, the difference in reduction potential of the two rutheniums decreases as does the degree of delocalization of the mixed valence state. At the same time, the energy of the IT band increases with concomitant decrease in molar absorptivity.

## ACKNOWLEDGEMENTS

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PS5.47 — TH

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## SYNTHESIS OF METHYLCOBALAMIN FROM THE GLUTATHIONE-COBALAMIN COMPLEX

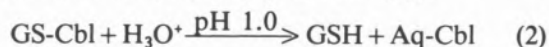
Mechanistic details on the conversion of vitamin B12 (cyanocobalamin, CN-Cbl) to its coenzyme forms adenosylcobalamin (Ado-Cbl) and methylcobalamin (Me-Cbl) by mammalian cells are largely unknown. Some information is available on Ado-Cbl formation by prokaryotes (reviewed in [1]), but the origin of Me-Cbl, the principal circulating Cbl in man, is obscure. Twenty years ago WAGNER and BERNHAUER proposed that the glutathione-cobalamin complex GS-Cbl might serve as a precursor for Cbl coenzymes based on its reactivity with alkylating agents [2]. We have recently obtained evidence that GS-Cbl is a naturally occurring intracellular Cbl in murine L1210 cells [3]. This paper concerns the reactivity of synthetic GS-Cbl in model systems for Me-Cbl formation.

GS-Cbl was prepared by reacting a 10-fold molar excess of glutathione (GSH) with hydroxocobalamin (HO-Cbl) in 0.10 M sodium acetate pH 4.5 (Reaction (1)). This complex was purified from excess



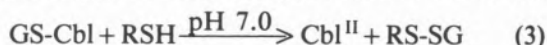
GSH by gel filtration on a 2.5 × 50 cm P2 polyacrylamide (Bio-Rad) column, or for smaller levels (<1 μmole) of GS-Cbl, on Sep-Pak C<sub>18</sub> cartridges (Waters/Millipore). The isolated complex had principal absorbance bands at 534, 428, 372,

and 289 nm (pH 7.0) and was relatively stable if stored anaerobically in the dark at pH 4.5. At lower pH (<2) the complex rapidly hydrolyzed to aquocobalamin (Aq-Cbl) (Reaction (2)).



At elevated temperature (80°C), GS-Cbl underwent thermolytic decomposition to Aq-Cbl. Since intracellular Cbls are usually extracted at elevated temperatures, this may explain why GS-Cbl has not been observed in the past.

In the presence of other thiols, GS-Cbl is very unstable and decomposes to what appears to be cob(II)alamin (Cbl<sup>II</sup>) spectrophotometrically (Reaction (3)). If Reaction (3) is carried out in the



presence of methyl iodide (MeI), the final spectrum appears to be a complex mixture of products (Fig. 1). When analyzed by HPLC, the major Cbl

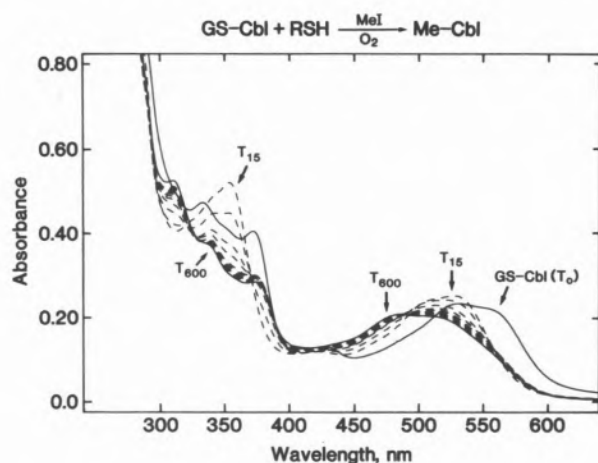


Fig. 1

Formation of Me-Cbl from GS-Cbl in the presence of MeI. The main cuvette compartment contained 2.5 ml of 25  $\mu\text{M}$  GS-Cbl in 0.1 M NaHCO<sub>3</sub> (pH 9.0). The reaction was initiated by adding MeI and 2-mercaptoethanol to final concentrations of 25 mM and 50 mM, respectively. Spectra were recorded every 15 sec on a Hewlett-Packard 8450A diode array UV-visible spectrophotometer

product was identified as Me-Cbl (Fig. 2). Further proof of its formation was obtained by photolyzing the reaction mixture prior to HPLC. After photolysis the Me-Cbl peak disappeared and the Aq-Cbl peak increased proportionately (Fig. 2). Me-Cbl can also be formed in this model system

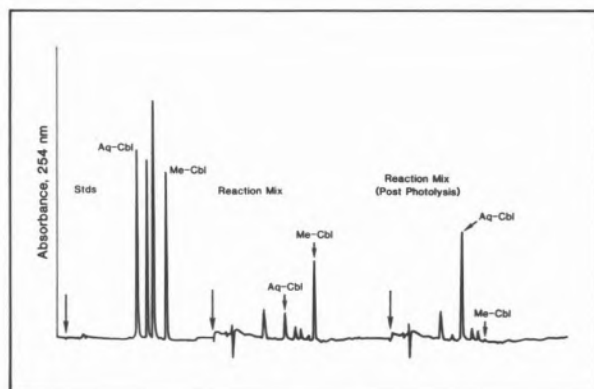
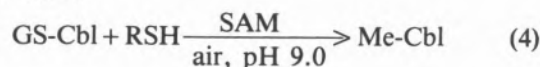


Fig. 2

Identification of Me-Cbl in Reaction Mixtures by HPLC. The reaction mixture from Fig. 1 was passed through an activated Sep-Pak C<sub>18</sub> cartridge. Corrinoids were then eluted with 50% acetonitrile and concentrated to dryness. The residue was dissolved in 100  $\mu\text{l}$  of water, clarified by centrifugation and analyzed (20  $\mu\text{l}$  aliquots) by gradient HPLC according to JACOBSEN et al. [3]. A portion of the reaction mixture was exposed to light from a tungsten-filament bulb (300 W) for 30 min prior to HPLC

in the presence of the physiological methyl donor S-adenosylmethionine (SAM) (Reaction (4)). These studies demonstrate that intracellular GS-Cbl could serve



as a substrate for Me-Cbl biosynthesis in mammalian cells.

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PS5.48 — MO

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**<sup>31</sup>P-NMR STUDIES OF COBALAMINS**

<sup>31</sup>P-NMR observations of aquocobalamin (H<sub>2</sub>OCbl) in H<sub>2</sub>SO<sub>4</sub>/H<sub>2</sub>O mixtures show that both the base-on and base-off species may be observed and quantitated. Correlation of the base-off <sup>31</sup>P-chemical shifts with a generalized acidity function [1] gives values of -1.59 and -0.09 for

the two macroscopic pK<sub>a</sub>'s for phosphodiester deprotonation, virtually identical to the values previously obtained for base-off CNCbl and CH<sub>3</sub>Cbl (Table, [2]). Similar observations of several other base-off cobalamins (Table) show that all the base-off cobalamins have the same phosphodiester macroscopic pK<sub>a</sub>'s (-1.59 ± 0.02 and -0.04 ± 0.02) and the same chemical shifts for the phosphodiester protonated (-339.88 ± 3.80 Hz) and deprotonated (-36.70 ± 2.80) base-off species. However, the base-on species of these cobalamins have chemical shifts that vary (from -50.16 to +9.37 Hz) in a regular way with the apparent free energy of coordination of the dimethylbenzimidazole ligand (ΔG<sub>Co</sub>, Table) so that Δδ<sub>31P</sub> (the difference in chemical shift between the base-on and base-off species) is a linear function of -ΔG<sub>Co</sub> (slope = 7.93 ± 0.40 Hz kcal<sup>-1</sup>mol, intercept = -28.06 ± 1.90 Hz, correlation coefficient r<sup>2</sup> = 0.964). In addition, both the chemical shift of the base-on species and the value of Δδ<sub>31P</sub> are linearly related to the axial Co-N bond length for adenosylcobalamin (AdoCbl), d<sub>Co-N(Bz)</sub> = 2.24 Å [3], CH<sub>3</sub>Cbl, 2.14 Å [4] and CNCbl, 2.06 Å [5].

These results may be interpreted in terms of the

Table  
<sup>31</sup>P-Chemical Shifts and Phosphodiester Macroscopic pK<sub>a</sub>'s of Cobalamins <sup>a)</sup>

Cobalamin	pK <sub>base-off</sub> <sup>b)</sup>	ΔG <sub>Co</sub> <sup>c)</sup>	pG <sub>4</sub>	pG <sub>5</sub>	Base-Off Species		Base-on δ <sub>31P</sub> <sup>d)</sup>	Δδ <sub>31P</sub> <sup>e)</sup>
					Phosphodiester Protonated δ <sub>31P</sub> <sup>d)</sup>	Phosphodiester Deprotonated δ <sub>31P</sub> <sup>d)</sup>		
CH <sub>3</sub> (CH <sub>2</sub> ) <sub>2</sub> Cbl	4.10	- 1.97	-1.58	-0.05	-337.76	-38.10	-50.16	-11.98
AdoCbl	3.67	- 2.57	—	—	—	-36.70	-47.15	-10.44
NC(CH <sub>2</sub> ) <sub>3</sub> Cbl	3.50	- 2.81	-1.60	-0.02	-342.14	-36.63	-43.66	- 7.03
CH <sub>3</sub> Cbl	2.89	- 3.64	-1.62	-0.02	-335.43	-38.47	-35.66	2.81
CF <sub>3</sub> CH <sub>2</sub> Cbl	2.60	- 4.04	-1.56	-0.02	-345.50	-33.51	-32.91	0.60
CF <sub>2</sub> HCbl	2.15	- 4.66	-1.61	-0.03	-342.31	-33.47	-25.58	7.89
NCCH <sub>2</sub> Cbl	1.81	- 5.19	-1.60	-0.02	-342.14	-34.33	-25.91	8.42
CF <sub>3</sub> Cbl	1.44	- 5.62	—	—	—	-36.70	-11.99	24.71
CNCbl	0.10	- 7.44	-1.57	-0.04	-335.81	-37.40	- 1.72	35.68
H <sub>2</sub> OCbl	-2.13	-10.48	-1.59	-0.09	-337.10	-41.64	- 0.37	51.01
Average:			-1.59 ± 0.02	-0.04 ± 0.02	-339.88 ± 3.80	-36.70 ± 2.80		

a) 25 ± 1°C.

b) Apparent pK<sub>a</sub> of the base-on-base-off reaction.

c) Apparent free energy of coordination of the free-base benzimidazole ligand.

d) In Hz, from external 85% H<sub>3</sub>PO<sub>4</sub> (negative shifts upfield from the reference) at 80.988 MHz.

e) Difference in chemical shift (in Hz) between the base-on and base-off species.