113. Thermodynamic *trans*-Effects of the Nucleotide Base in the B_{12} Coenzymes

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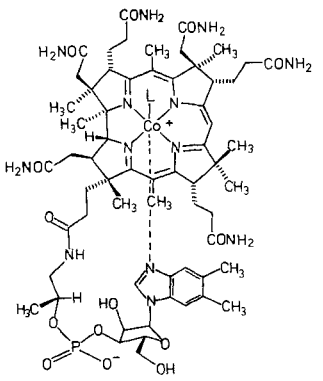
The thermodynamic effects of the nucleotide coordination on the Co–C bond strengths in the B_{12} coenzymes were analyzed. Methyl group transfer reactions from methylcob(III)inamides to cob(II)inamides and cob(I)-inamides in neutral aqueous solution were used in equilibration experiments to determine the effect of the intramolecular coordination of the nucleotide function on the Co–C bond dissociation energies of methylcob(III)-alamin (**4**). In the equilibrium between **4**, cob(I)inamide (**11**), cob(I)alamin (**10**) and methylcob(III)inamide **6** *(Scheme 2)*, **4** and **11** were found to predominate (**4** + **11** ⇄ **10** + **6**, equilibrium constant $K_{I/III} \approx 0.004$), while the equilibrium between **4**, cob(II)inamide **9**, cob(II)alamin (**5**), and **6** *(Scheme 1)* proved to be well balanced (**4** + **9** ⇄ **5** + **6**, equilibrium constant $K_{II/III} = 0.60$). These equilibrium values indicate the nucleotide coordination to stabilize the Co–C bond in **4** both against homolysis (slight effect) and against nucleophilic heterolysis (considerable effect). They reflect a stabilization of the complete corrins **4** and **5** by the nucleotide coordination, which is also indicated for **4** and **5** by their (nucleotide) basicity. The latter information, where available for other organocobalamins, allows the analysis of the thermodynamic nucleotide *trans* effect there as well: *e.g.* in coenzyme B_{12} (**1**), the nucleotide coordination is found this way to weaken the Co–C bond towards homolysis by *ca.* 0.7 kcal/mol.

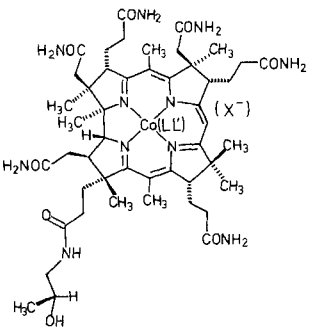
Introduction. – Of the remarkable structural features of the vitamin-B_{12} derivatives [1], the organometallic bond, originally discovered by X-ray analysis [2] in the 'coenzyme B_{12}' (**1**) [3], has been most closely associated[1]) with their biological roles [4]. In particular, the ability of **1** to cleave its weak Co–C bond[2]) homolytically is considered the most relevant reactivity for its coenzymic activity [7] since the 5'-deoxyadenosyl radical produced thereby reversibly appears to induce the complex coenzyme-B_{12}-catalyzed enzymatic reactions [8]. The (comparatively) high rates of these enzymatic processes [9] are attributed to a drastic acceleration of the Co–C homolysis in the protein-bound **1** [10]. In this respect, the intramolecular coordination of the nucleotide function can yield a significant contribution to the weakening of the Co–C bond in organocobalamins, as is shown by the 1400-fold higher rate (at r.t.) of Co–C bond homolysis in neopentylcob(III)alamin (**2**) than that in the nucleotide-free neopentylcob(III)inamide (**3**) [10].

Apparently, less spectacular roles than those of **1**, but nevertheless (similarly) of fundamental biological importance, are assigned to methylcob(III)alamin (**4**) and related methylcorrinoids, the second organometallic B_{12}-coenzyme forms [11]. Their biological functions in CH_3-group transfer and activation [11] presumably depend on the ease of the

[1]) Besides their roles as organometallic catalysts, B_{12} derivatives have recently been proposed to function as electron-transfer agents also in methanogenic bacteria [5].

[2]) The Co–C bond homolysis energy of **1** has been determined in two laboratories [6] by kinetic methods to amount to *ca.* 30 kcal/mol (for aqueous solutions of **1**).





1 Co(L) = 5′-deoxyadenosyl-Co(III)

2 Co(L) = neopentyl-Co(III)

4 Co(L) = methyl-Co(III)

5 Co(L) = Co(II)

7 Co(L) = CN-Co(III)

10 Co(L) = Co(I)$^{-}$ (base not coordinating)

13 Co(L) = H_2O-Co(III)$^{+}$

3 Co(L) = neopentyl-Co(III)$^{+}$, L′ = H_2O

6 Co(L) = methyl-Co(III)$^{+}$, L′ = H_2O, X = OAc

8 Co(L,L′) = $(CN)_2$-Co(III)

9 Co(L) = Co(II)$^{+}$, L′ = H_2O, X = OAc

11 Co(L,L′) = Co(I)

12 Co(L) = HO-Co(III)$^{+}$, L′ = H_2O

corrin-bound Co-ion to methylate and to demethylate by nucleophilic (two-electron) displacement reactions as well as, possibly, by homolytic organometallic processes [12]. The energetics of these processes as well as their stereochemical course [13] might be controlled also by the ability of the nucleotide function to coordinate intramolecularly.

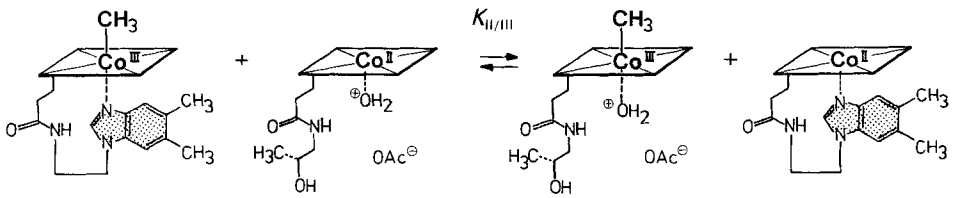
To date, knowledge on the thermodynamic effects of the coordination of the nucleotide function on the Co–C bond strengths in organocob(III)alamins (such as **1**, **2** and **4**) is still lacking, but it would be accessible experimentally by comparison of the cob(III)alamins with the corresponding nucleotide-free organocob(III)inamides. We have recently reported on the thermal CH_3-group transfer between methylcob(III)yrinates and cob(II)yrinates (in one example) and pointed out there, that alkyl-transfer equilibria give access to information on relative Co–C bond homolysis energies in the equilibrating cobalt corrinates [14].

In this report, CH_3-group equilibria between cobalamin and cobinamide derivatives are now used to determine the thermodynamic *trans* effect of the nucleotide on the Co–CH_3 bond in methylcob(III)alamin (**4**). Analysis of the (nucleotide) basicity in the complete vitamin-B_{12} derivatives **4** and cob(II)alamin (**5**), which characterizes the strenght of the nucleotide coordination in these cobalamin derivatives (compared to that of the solvent H_2O), allows the independent determination of the nucleotide effect on the Co–C bond strength of **4**, consistent with the value obtained from the CH_3-transfer

equilibrium. Based on this correlation and on the available data on protonation equilibria in other organocob(III)alamins, the corresponding analysis of the thermodynamic *trans* effect of the nucleotide on their Co–C bond strengths is possible and is also derived, *e.g.* for coenzyme B_{12} (**1**).

Results. – *Equilibration Experiments with Methylcob(III)inamides and Cob(II)inamides.* Storage of a solution of cob(II)alamin (**5**; 'B_{12r}') [14] and methylcob(III)inamide acetate **6** in 0.02M phosphate buffer (pH 7) at r.t. and with careful exclusion of light and air[3]) led to extensive equilibration of the Co-bound CH_3-group within 1 h (see *Scheme 1*, analysis by UV/VIS and HPLC). The equilibration was oxidatively quenched after 65 h by addition of the mixture to air-saturated 1% HCN in CH_3OH. Removal of the solvents at r.t. and in the dark furnished a sample, whose ^{1}H-NMR spectrum (see *Fig. 1*) and HPLC trace[3]) indicated the presence of **4**, **6**, vitamin B_{12} (**7**; from **5** after oxidative

Scheme 1[a])



[a]) Simplified representation of the α-D-ribofuranose 3-phosphate residue.

[b]) On oxidative workup of the equilibrium mixture, **9** was transformed to **8** and **5** to **7**.

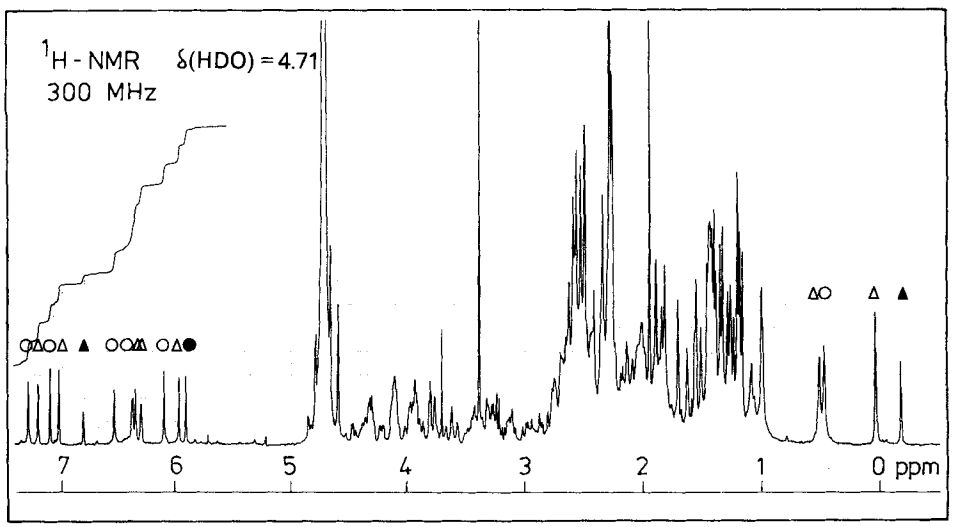


Fig. 1. *300-MHz ^{1}H-NMR analysis of the CH_3-group equilibration, starting with cob(II)alamin* (**5**) *and methylcob(III)inamide* **6**. △ signifies selected signals of **4**, ▲ those of **6**, ○ those of **7**, and ● those of **8**.

[3]) See *Exper. Part.*

workup), and *Coα, Coβ*-dicyanocob(III)inamide (**8**; from **9** after oxidative workup) in a ratio **4**/**6**/**7**/**8** = 1:0.47:1.15:1.05.

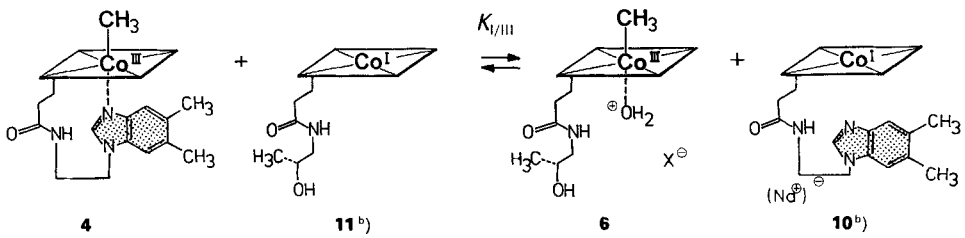
The reverse experiment where methylcob(III)alamin (**4**) and cob(II)inamide acetate (**9**)[3]) analogously were equilibrated, worked up, and analyzed yielded the oxidized products of equilibration in a ratio of **4**/**6**/**7**/**8** = 1:0.65:0.65:0.6. From both (and two analogous) equilibration experiments and after their oxidative quenching, a ratio of products ([**6**]·[**7**])/([**4**]·[**8**]) = 0.60 ± 0.15 was obtained. This value reflects the ratio of the concentrations in the equilibrating mixture (before oxidation), based on the control experiments described below. Thus, for the equilibration of **4**, **5**, **6**, and **9** in neutral aqueous solution, an equilibrium constant $K_{II/III}$ = 0.60 ± 0.15 is indicated. In the control experiments, *ca.* 30 sec after mixing, the mixtures **5**/**6** as well as **4**/**9** were oxidatively quenched by addition to 1% HCN in CH_3OH. Analysis by HPLC indicated only minor (< 5%) formation of the respective products of CH_3-group transfer.

Complementary pairs of equilibration experiments, similarly carried out in the temperature range 5–60° and analyzed by HPLC[3]), showed the equilibrium distribution to change only little with temperature ($K_{II/III}(5°) = 0.40 \pm 0.1$; $K_{II/III}(20°) = 0.56 \pm 0.15$; $K_{II/III}(50°) = 0.73 \pm 0.15$; $K_{II/III}(60°) = 0.92 \pm 0.2$), corresponding to ΔH_o = 2.5 ± 0.5 kcal/mol and ΔS_o = 7.1 ± 1 e.u.

To obtain qualitative information on the CH_3-transfer rates at r.t., the equilibration of O_2-free, buffered, neutral aqueous solutions was followed by UV/VIS during storage at r.t. and under exclusion of light. Solutions that were (originally) 1.3 mM in **5** and 0.93 mM in **6** or 1.3 mM in **4** and 1.0 mM in **9**, respectively, equilibrated with half-times of *ca.* 9 min, based on observed changes at 655 and 525 nm[4]).

Equilibration Experiments with Methylcob(III)inamides and Cob(I)inamides. To a solution of cob(I)alamin (**10**; obtained by electrochemical reduction of cob(II)alamin (**5**)[3])), methyl cob(III)inamide acetate **6**[3]) was added under inert atmosphere and with protection from light. After 3 min, the mixture was oxidatively quenched by addition to *ca.* 1 ml of air-saturated 1% HCN in CH_3OH under protection from light. Workup and analysis as before (by ^{1}H-NMR and HPLC) indicated extensive methylation of the cobalamin and demethylation of the cobinamide (see *Scheme 2* and *Fig. 2*), with a ratio **4**/**6**/**7**/**8** = 1:0.02 (±0.01):0.2:1.05.

Scheme 2[a])



4 **11**[b]) **6** **10**[b])

[a]) Simplified representation of the α-D-ribofuranose 3-phosphate residue.

[b]) On oxidative workup of the equilibrium mixture, **11** was transformed to **8** and **10** to **7**.

[4]) In the earlier experiments [14] using **4** and the relatively lipophilic (heptamethyl cob(II)yrinate) perchlorate (in CH_3OH/aq. phosphate buffer 2:1), an equilibration half-time of *ca.* 3 days was estimated, under (otherwise) comparable experimental conditions.

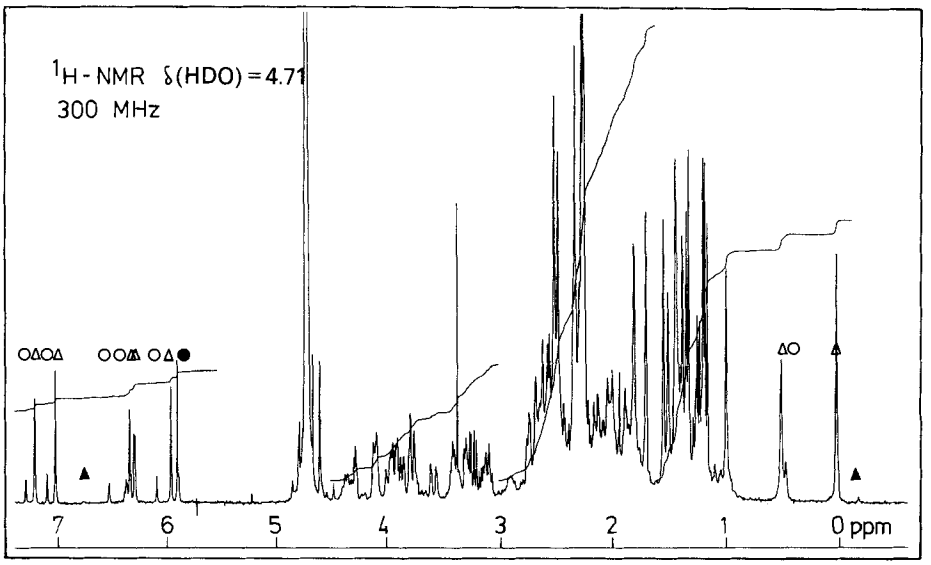


Fig. 2. *300-MHz ^{1}H-NMR analysis of the CH_3-group equilibration, starting with cob(I)alamin* (**10**) *and methylcob(III)inamide* **6**. △ signifies selected signals of **4**, ▲ those of **6**, ○ those of **7**, and ● those of **8**.

The control experiment in which cob(I)inamide (**11**; obtained by electrochemical reduction of cob(II)inamide acetate **9**[3])) was treated with (([^{13}C]methyl)cob(III)alamin ([^{13}C-**4**][3]) for 30 min under inert atmosphere and with protection from light likewise yielded a mixture of cobinamides after workup that was analyzed to contain **4**, **6**, **7** and **8** in a ratio of 1:0.05 (±0.02):0.1:1.1. The high-field signals of the ^{1}H-NMR spectrum (*d*'s with J = 138 and *ca.* 140 Hz) showed the Co-bound CH_3-groups of ^{13}C-**4** and ^{13}C-**6** to contain 96 ± 2% and 75 ± 30% of ^{13}C, respectively, and, therefore, to be derived from that of the ^{13}C-labelled CH_3-group (98% ^{13}C) of the starting ^{13}C-**4**.

From both experiments, a ratio of products ([**6**]·[**7**])/([**4**]·[**8**]) ≈ 0.004 ± 0.003 was estimated, taken as the ratio of concentrations in the equilibrating mixtures before the oxidative quenching (*i.e.* $K_{I/III} \approx 0.004$).

Discussion. – Thermal CH_3 group transfer reactions were found to occur rapidly between methylcob(III)inamides and cob(II)inamides as well as cob(I)inamides in aqueous solution. These findings on one hand extend earlier experiments involving simple B_{12}-model compounds on CH_3-transfer from CH_3–Co(III) to Co(II) complexes, in particular by the groups of *Endicott* [15] and of *Johnson* [16]. These CH_3-transfer reactions were found to occur without formation of free CH_3 radicals and were classified as 'methyl bridged electron transfer reactions' [15]. Secondly, they extend also earlier studies on the CH_3-transfer [16] between CH_3–Co(III) and Co(I) forms of the cobaloxime-B_{12} models [17], as well as between vitamin-B_{12} analogs [18] which have been noted to occur rapidly [16] [18] in aqueous solution.

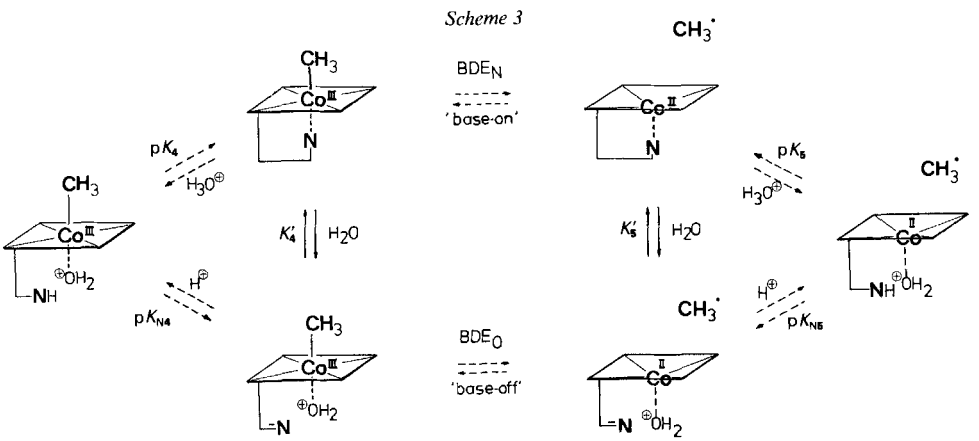
Similar to these [15–18] and to our earlier experiments [14], the CH_3 transfer from methylcob(III)inamides to cob(II)inamides and cob(I)inamides in aqueous solution was

found here to proceed rapidly[5]) and apparently without free CH_3 species (radicals or cations). Its rate presumably depends crucially on the accessible metal-metal distance. Preliminary computer-assisted studies, intended to model an activated complex for the CH_3 transfer and based on the 3-dimensional structures of **4** [19] and of (heptamethyl cob(II)yrinate) perchlorate [20], indicate intermolecular interactions of the peripheral CH_3 groups and acetic-acid side chains to build up substantially upon coaxial β-sided approach of the two corrin moieties at a metal-metal distance of less than *ca.* 5.8 to 6 Å[6]).

Alkyl group transfer equilibria between corrinoid Co complexes can be used to gain information on the strengths of the Co–C bonds involved [14]. In particular, from the equilibrium experiments reported here with cobalamins and cobinamides, the effect of the intramolecular nucleotide coordination on the Co–C bond dissociation energies can be determined. With an equilibrium constant $K_{II/III} = 0.60 \pm 0.15$ from the equilibration experiments in aqueous solution between CH_3–Co(III) and Co(II) forms of cobalamins and cobinamides[7]) (see *Scheme 1*), the Co–C bond in methylcob(III)alamin (**4**) is shown to be slightly more stable with respect to homolysis than that of the nucleotide-free methyl cob(III)inamide **6**. The intramolecular nucleotide coordination barely affects and does not destabilize the Co–C bond of **4** towards homolysis in aqueous solution.

The situation is different, when the heterolytic modes of Co–C bond dissociation are considered: The equilibration experiments between CH_3–Co(III) and Co(I) forms of cobalamins and cobinamides (see *Scheme 2*) yielded an equilibration constant $K_{I/III} \approx 0.004 \pm 0.003$. The Co-bound CH_3 group of methylcob(III)inamide **6** is transfered to cob(I)alamin (**10**) with formation of **4** and cob(I)inamide (**11**), indicative of a considerable stabilization of the Co–C bond in **4** due to the nucleotide coordination. This

Scheme 3



[5]) The rapid CH_3 transfers from methylcob(III)- to cob(II)- and to cob(I)inamides observed here suggest the consideration of the easily accessible and persistent radicaloid cob(II)inamides in biological CH_3-transfer reactions as alternative to the more reduced Co(I) forms.

[6]) A relevant part of the observed rate retardation for the CH_3 transfer from **4** to the heptamethyl cob(II)yrinate[4]) compared to the transfer to the cob(II)inamide **9**, could be due to the intermolecular interactions of hydrophilic and lipophilic peripheral substituents in the former transfer.

[7]) The equilibrium value for the analogous equilibration between cobalamins and heptamethyl cobyrinates was determined as $K_e = 0.63 \pm 0.15$ [14].

'inverse' *trans* effect observed here contrasts with *Hogenkamp*'s finding [21] on the equilibrium involving **4**, aquocob(III)inamide **12**, aquocob(III)alamin (**13**), and **6** where **13** and **6** are strongly favoured in aqueous solution.

These findings on the CH_3-transfer equilibria in aqueous solution between cobalamins and cobinamides, used to determine the thermodynamic *trans* effect of the nucleotide in **4** on the three modes of cleavage of the Co–C bond of **4**, are consistent with the information on the strength of the nucleotide coordination, extractable from protonation equilibria in **4** ($pK_4 = 2.7$ [10a]), in **5** ($pK_5 \approx 2.9$ [22]), in **10** ($pK_{10} \approx pK_N = 5.65$ [23]), and **13** ($pK_{13} = -2.4$ [24]). With the approximation that the basicity of the 'noncoordinating nucleotide' in various 'base-off' B_{12} forms is practically invariant and equal to that in the isolated nucleotide portion (*e.g.* $pK_{N4} \approx pK_{N5} \approx pK_N = 5.65$, see *Scheme 3*), the pK_a values are a measure of the stabilization of complete corrinoids by the nucleotide coordination, *i.e.* of the equilibria K_4' and K_5'. Based on this, the conclusion can be drawn from the difference of 0.2 between pK_4 and pK_5 that **4** gains more stabilization by *ca.* 0.3 kcal/mol than **5**[8]) upon coordination of the nucleotide (resulting in a stabilization of the Co–C bond in **4**, which would correspond to a $K_{II/III} = 0.61$). Similarly, the formation of the nucleotide-Co bond which accompanies the methylation of **10**[8]) yielding **4** can be analyzed [14] to drive the CH_3 abstraction from **6** by **10**, (the difference of *ca.* 2.9 of pK_4 and pK_{10} corresponds to a stabilization of *ca.* 4.2 kcal/mol in **4**, *i.e.* to an equilibrium constant $K_{I/III} \approx 0.0013$), in qualitative agreement with the experimental result. Also the CH_3 transport in the reverse sense, from **4** onto diaquocob(III)inamide **12** to give **6** and aquocob(III)alamin (**13**) [21] can be rationalized [14] by an increased strength of the

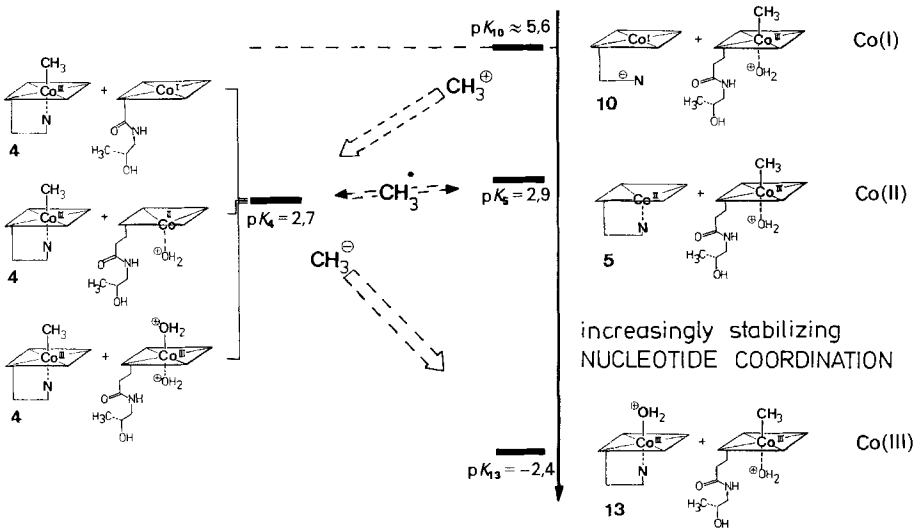


Fig. 3. *The CH_3-transfer equilibria between cobalamins and cobinamides.* Effect of the nucleotide coordination on the Co–C bond strengths in methylcob(III)alamin (**4**).

[8]) Co(II) corrins such as **5** and **9** presumably contain a 5-coordinate Co(II) center [20] [22] [25a, b], while the Co(I) center in **10** and **11** presumably is 4-coordinate, in analogy to the situation in the Co(I) form of *Scheffold*'s B_{12} model [25c] [26].

nucleotide coordination in **13** compared to **4** (the difference of *ca.* 5.1 between pK_4 and pK_{13} corresponds to an additional stabilization of *ca.* 7 kcal/mol in **13**, see *Fig. 3*).

The correlation between the strength of the nucleotide coordination as determined from acid-base equilibria with the strength of the Co–C bond in **4** as determined from the three CH_3-transfer equilibria, is thus established[9]). In **4**, the nucleotide coordination facilitates the transfer of the CH_3 group (formally as 'methyl anion') onto electrophiles such as aquocobinamide **12**, exhibiting a 'normal' *trans* effect [25]. On the other hand, it stabilizes **4** (in comparison to **6**) against the abstraction of the CH_3 group (formally as 'methyl cation') by nucleophiles such as Co(I) corrinates. Thirdly, the nucleotide coordination hardly affects the thermodynamics of the Co–C bond homolysis or of the transfer of the CH_3 group (formally as 'methyl radical') onto radical(oid)s such as Co(II) corrinates. This 'inverse' *trans* effect of the nucleotide in **4** on demethylation to **5** or to **10** correlates with the change of the oxidation state of the Co center from Co(III) to Co(II) or to Co(I)[8]).

On the basis of these correlations, the higher basicity of coenzyme B_{12} (**1**; $pK_1 \approx 3.4$ [10a]) compared to **5** leads to the conclusion that the nucleotide coordination in **1** in aqueous solution weakens the Co–C bond of **1** towards homolysis by an amount of *ca.* 0.7 kcal/mol. Correspondingly, the increased rate of homolysis of some organocobalamins compared to the analoguous organocobinamides [10a] can qualitatively be correlated with the known pK_a values: *e.g.* the mentioned, *ca.* 1400 times faster homolysis of the Co–C bond in neopentylcobalamin (**2**) than in neopentylcobinamide **3** [10] can be traced back largely to a lack of (ground state) stabilization in **2** due to the weak nucleotide coordination ($pK_2 \geq 4.7$ [10]). In the organocobalamins, the degree of the substitution of the Co-bound alkyl group influences the strength of the axial nucleotide coordination and (in this way) the additional weakening of the Co–C bond towards homolysis. An upper limit to this nucleotide-based wakening of the Co–C bond of organocobalamins towards homolysis in aqueous solution should be set by the stabilization of the homolysis product **5** by the nucleotide coordination which amounts to *ca.* 3.9 kcal/mol (corresponding to the difference pK_N–pK_5).

In conclusion, these results point to a *mutual dependence of the Co–C and Co-nucleotide bonds in organocobalamins in aqueous solution in that weak (axial) Co-nucleotide coordination destabilizes the* trans *Co–C bonds.* This is in support of the inferences of experiments on the Co–C bond energies [7][10]) and of X-ray-structural investigations with simple B_{12}-model compounds [27][11]). In this way, the unique intramolecular coordination

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[9]) The axial nucleotide coordination in complete B_{12} derivatives correspondingly also enhances their oxidizability and diminishes their reducibility. Based on the electrochemical half-wave potentials of the redox couples **5**/**10** ($E_{1/2} = -0.85$ V *vs.* SCE) and **9**/**11** ($E_{1/2} = -0.73$ V *vs.* SCE) [22] in aqueous solution, the electron-transfer equilibrium **5** + **11** ⇄ **9** + **10** (equilibrium constant K_e) lies to the left, with $\log K_e = -2.0$. As pointed out to the author by Professor *Scheffold* (Universität Bern, CH-3012 Bern), and since $K_e = K_{I/III}/K_{II/III}$, the equilibrium constants $K_{I/III}$ and $K_{II/III}$ from the CH_3-group transfer equilibria also allow the determination of K_e, with $\log K_e = -2.2 \pm 0.5$. In this way, a second, independent correlation is given as concerns the nucleotide effect in the cobalamins.

[10]) A synchronous variation of the Co–C bond dissociation energies and the basicity of the axial pyridine ligand is observed in organocobaloximes [7].

[11]) A synchronous, mutual dependence of Co–C and Co-(*trans*)ligand bond lengths is observed in organocobaloximes and related 'B_{12} models' [27].

of the nucleotide in **1** and **4** is given a function[12]) presumably of relevance also to the B_{12}-catalyzed enzymatic processes since the protein would be expected to control the strength of the coordination of the nucleotide to the Co center (and, therefore, of the Co–C bond) in enzyme-bound B_{12} coenzymes.

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Experimental Part

1. *General.* Solvents and reagents: Methylcob(III)alamin (**4**), crystalline [30a]; cob(II)alamin (**5**), crystalline, by catalytic reduction [30b]; dicyanocob(III)inamide (**8**), *Sigma*, 95% pure; PtO_2, *Baker-Engelhard*; H_2O, 'nanopure', *Ultrafilter*, Barnstead, USA; *CM*-cellulose, *Serva*, Heidelberg; *XAD-2, puriss., Serva*, Heidelberg; acetone, *puriss. p.a., Fluka*; Bu_4NClO_4, crystalline [30c]; $LiClO_4$, *p.a., Merck*; CH_3OH, *puriss. p.a., Fluka*; AcOH, *puriss. p.a., Fluka*; H_2 gas, *Stickstoff-Wasserstoffwerke*, Luzern; methyl *p*-toluenesulfonate (TsOMe) cryst., *purum*, redistilled, *Fluka*; $^{13}CH_3I$, *Stohler Isotope Chemicals*, 99% ^{13}C; CH_2Cl_2 and acetone, practical grade, redistilled. UV/VIS: *Uvikon 860*, in 0.02M phosphate buffer solution (pH 7). ^{1}H-NMR: 300.14 MHz, *Bruker WM-300*, D_2O (δ(HDO) = 4.71 ppm); sample preparation in the dark room.

2. *Experimental Setup.* The equilibration experiments were carried out with strict protection from light and air (glove box *Mecaplex GB-80*, < 10 ppm of O_2); workup: dark room with minimal exposure to white light. Electrolysis: Two-compartment electrolysis cell [30c], Hg-pool working electrode, Pt-counter electrode, 0.1N-calomel reference electrode (0.1N CE); electrolysis in the glove box; *PAR* model *170*. HPLC (in the dark room): stationary phase, *Rp-18* (5 µm); mobile phase, 0.01M phosphate buffer (pH 7)/CH_3OH 4:6, 0.01M NaCN; λ_{obs} at 500 nm; retention times (relative extinction coefficients at 500 nm): **7**, 6.3 min (1.0); **8**, 7.4 min (1.0); **4**, 8.7 min (1.66); **6**, 18.6 min (major isomer, 1.29) and 14.0 min (minor isomer, *ca.* 1.4).

3. *Preparation of* **6**, **9** *and* ^{13}C-**4**. *Aquacob(II)inamide Acetate* (**9**). In the glove box, 55 mg (53 µmol) of dicyanocob(III)inamide (**8**) were dissolved in 5 ml of deoxygenated CH_3OH to which 12 mg of PtO_2 and 40 µl of AcOH were added. The mixture was stirred magnetically under H_2 (*ca.* 1 atm, balloon) for 2.5 h at r.t. (→dark brown; monitoring UV/VIS [22a]), and then the PtO_2 catalyst was filtered off. The solvents were removed, and the residual **9** was precipitated from *ca.* 0.5 ml of H_2O by addition of *ca.* 5 ml of acetone. The precipitate was dried (h.v.) and stored in the glove box.

Co*α*-*Aqua*-Co*β*-*methylcob(III)inamide Acetate* (**6**). To a soln. of 25 mg of **9** in 6 ml of CH_3OH/0.1M Bu_4NClO_4 in the cathode chamber of the electrolysis cell, 50 mg (269 µmol) of TsOMe were added. At a Hg-pool electrode and with magnetic stirring, **9** was reduced at –0.95 V *vs.* 0.1N CE with protection from light (consumption: 2.55 C, *i.e.* = 1.0 F/mol). The mixture was transferred into a dark room, taken up in 50 ml of H_2O and shaken 3 times with 50 ml of CH_2Cl_2 (org. phase discarded). The solvent was evaporated at r.t., the residue taken up in *ca.* 1 ml of H_2O and precipitated by addition of *ca.* 10 ml of acetone. The light-sensitive, orange precipitate was dried (h.v., 16 h, r.t.): 23 mg (*ca.* 92%) of **6**. UV/VIS: see [22c] [31a]. ^{1}H-NMR: –0.15 (*s*, CH_3–Co, major isomer); –0.05 (*s*, CH_3–Co, minor isomer); 1.00, 1.10 (2*s*, 2 CH_3); 1.18 (*d*, *J* = 6, CH_3–C(17^6)); 1.28, 1.36, 1.70 (3*s*, 3 CH_3, minor isomer); 1.44, 1.58, 1.64, 1.85 (4*s*, CH_3); 1.95 (*s*, CH_3CO_2); 2.41, 2.49 (2*s*, CH_3–C(5)/CH_3–C(15)); superimposed by 1.8–2.9 (*m*, in total *ca.* 40 H); 2.99 (*m*, CH(18)); 3.26 (*m*, CH_2(17^5)); 3.50 (*m*, CH(13)?); 3.75 (*dd*, *J* = 8, 4, CH(8)?); 3.94 (*m*, CH(17^6)); 4.09 (*d*, *J* = 8, CH(3)?); 4.47 (*d*, *J* = 10, CH(19)); 6.52 (*s*, CH(10), minor isomer); 6.82 (*s*, CH(10), major isomer); the spectrum indicates an 85:15 mixture of two isomeric forms of **6** [31b].

Upon photolysis in aerated 0.1% HCN/CH_3OH, **6** was cleanly converted to **8**.

((^{13}*C)Methyl)cob(III)alamin* (^{13}C-**4**). A soln. of 3 g (2.2 mmol) of cob(II)alamin (**5**) in 70 ml of a 1:1 mixture of H_2O and CH_3OH/0.02N $LiClO_4$ in the cathode compartment of the electrolysis cell was reduced at the Hg-pool electrode at –0.95 V *vs.* 0.1N CE under inert atmosphere (consumption: 267 C, *i.e.* 1.15 F/mol). Then 300 µl of $^{13}CH_3I$ were added. The now red mixture was protected from light and treated with *ca.* 200 ml of acetone to precipitate the raw ^{13}C-**4** (2.8 g, after drying; contained some aquocob(III)alamin (**13**), by UV/VIS). The raw ^{13}C-**4**

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[12]) Steric distortions, *e.g.* 'steric perturbations involving an enzyme-induced conformational distortion of the corrin ring' [28], have been proposed as the relevant contribution to the weakening of the Co–C bond of the enzyme-bound coenzyme B_{12} [10] [28] [29].

(1 g, *ca.* 0.73 mmol) was taken up in *ca.* 5 ml of H_2O, applied to a column of 10 g of *CM*-cellulose, and washed out with *ca.* 50 ml of H_2O. The solvent was evaporated at r.t. and the residue dissolved in *ca.* 1.5 ml of H_2O and treated with *ca.* 3 ml of acetone. Upon storage overnight, 0.86 g (0.60 mmol) of crystalline ^{13}C-**4** were obtained, uniform by HPLC. ^{1}H-NMR (D_2O): –0.12 (*s*, 0.02 ± 0.01 H, and *d*, *J* = 138.3, *ca.* 0.98 H).

4. *Equilibration Experiments with Methylcob(III)inamides and Cob(II)inamides.* a) *Cob(II)alamin* (**5**) *and Methylcob(III)inamide Acetate* **6**. In a soln. of 6.0 mg (4.4 μmol) of **5** in 0.5 ml of 0.02M phosphate buffer (pH 7) were dissolved 3.5 mg (3.1 μmol) of **6** with protection from light in the glove box. The soln. was stored at r.t. in a tightly stoppered flask for 65 h prior to removal of the flask from the glove box and rapid addition of its content to *ca.* 0.5 ml of 1% HCN in air-saturated CH_3OH. The mixture was taken into the dark room and evaporated at r.t. The residue was dried (h.v., r.t., 4 h) and analyzed by HPLC (**4**/**6**/**7**/**8** = 1:0.5:1.2:1.1), then taken up in 0.5 ml of D_2O for ^{1}H-NMR analysis (see *Fig. 1;* **4**/**6**/**7**/**8** = 1:0.45:1.1:1.0). The methyl-corrins **4** and **6** and the cyano-corrins **7** and **8** were thus found to be present in a ratio of 1:0.47:1.15:1.05.

b) *Methylcob(III)alamin* (**4**) *and Cob(II)inamide Acetate* **9**. This experiment was carried as described in *a)*, but starting with 5.5 mg (4.2 μmol) of **4** and 3.6 mg (3.4 μmol) of **9** in 0.5 ml of 0.02M phosphate buffer (pH 7). Analysis of the equilibrated and oxidized reaction mixture as before indicated the presence of **4**, **6**, **7**, and **8** (only), in a ratio of 1:0.70:0.70:0.65 (HPLC) and 1:0.6:0.6:0.55 (^{1}H-NMR); average, 1:0.65:0.65:0.60.

c) *Effect of Temperature on the Equilibrium.* Deaerated solns. of 0.5 mg (0.38 μmol) of **5** and 0.31 mg (0.3 μmol) of **6** *(Exper. A)* or of 0.42 mg (0.30 μmol) of **4** and 0.37 mg (0.36 μmol) of **9** *(Exper. B)* in 350 μl of 0.02N aq. phosphate buffer (pH 7) were stored with protection from light at the temp. and for the time indicated in the *Table*. The equilibrated solns. were added to *ca.* 150 μl 1% HCN in aerated CH_3OH and analyzed by HPLC.

Table. *Effect of Temperature on the Equilibrium Obtained from* **5** *and* **6** (Exper. A) *and from* **4** *and* **9** (Exper. B)

Exper.	Temp. [°]	Time [h]	**4**/**6**/**7**/**8**	$K_{II/III}$
A	0	26	1:0.5:0.85:1.0	0.42
B	0	26	1:0.7:0.9:1.7	0.37
A	20	4	1:0.55:1.0:1.05	0.53
B	20	4	1:0.9:0.95:1.45	0.60
A	50	1	1:0.75:1.05:1.05	0.75
B	50	1	1:1.05:1.2:1.8	0.70
A	60	1	1:0.9:1.1:1.1	0.90
B	60	1	1:1.1:1.2:1.4	0.94

5. *Equilibration Experiments with Methylcob(III)inamides and Cob(I)inamides.* a) *Methylcob(III)alamin* (**4**) *and Cob(I)inamide* (**11**). A soln. of 13.8 mg (13 μmol) of **9** in 5 ml of 0.02M phosphate buffer (pH 7) in the cathode compartment of the electrolysis cell was reduced at –0.99 V *vs.* 0.1N CE at a Hg-pool electrode. After 1.40 C (1.08 F/mol) of electricity were consumed, ^{13}C-**4** (17 mg, 12.7 μmol) was added to the now greenish soln. (of **11**) and the mixture stored (with protection from light and in the glove box) for 30 min at r.t.[13]). The mixture then was taken out of the box and added to 1 ml of 1% HCN in aerated CH_3OH (with protection form light). A sample was taken for HPLC analysis (**4**/**6**/**7**/**8** = 1:0.03:0.05:1.1), then the remainder adsorbed to a column of *ca.* 1 g of *XAD-2*, washed with H_2O (5 ml), and eluted completely with H_2O/CH_3OH 1:1 (20 ml). The solvents were evaporated at r.t., and the dried residue was taken up in *ca.* 0.5 ml of D_2O for ^{1}H-NMR analysis (**4**/**6**/**7**/**8** = 1:0.05:0.3:1.1).

b) *Methylcob(III)inamide* **6** *and Cob(I)alamin* (**10**). To a soln. of 8.5 μmol of **10** which was likewise produced by reduction of 11.3 mg (8.5 μmol) of **5** at –1.2 V *vs.* 0.1N CE (1.24 F/mol), 9.3 mg (9.1 μmol) of **6** were added. After 3 min[14]), the mixture was worked up as described above (addition to 1% HCN in aerated CH_3OH, *etc.*), a sample was taken for analysis by HPLC (**4**/**6**/**7**/**8** = 1:0.016:0.16:1.1), and the rest analyzed by ^{1}H-NMR as above (**4**/**6**/**7**/**8** = 1:0.02 (±0.01):0.2:1.0, see *Fig. 2*).

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[13]) Treatment of such a soln. from a parallel experiment with 20 μl of CH_3I in the dark resulted in a mixture of **4**/^{13}C-**4** (60% ^{13}C), **6**/^{13}C-**6** (30% ^{13}C), **7** and **8** in a ratio of 1:1:0.15:0.12 (% ^{13}C refers to ^{13}C-content of Co-bound CH_3, as taken from the high-field signals in the ^{1}H-NMR).

[14]) Treatment of such a soln. from a parallel experiment with 10 μl of $^{13}CH_3I$ in the dark resulted in a mixture of **4**/^{13}C-**4** (38% ^{13}C), **6**/^{13}C-**6** (60% ^{13}C), **7**, and **8** in a ratio of 1:0.53:0.15:0.1 (% ^{13}C: see *Footnote 13*).

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