either for the partial formation of an alkene during ß-elimination, or the partial formation of a planar free radical during homolytic fission (Rüchardt, 1970). The conformational strain for a five-membered ring would be decreased, and for a six-membered ring increased (Rüchardt, 1970), due to the formation of an sp^2 centre.

Homolytic fission under a nitrogen atmosphere is reversible, and is accelerated by oxygen, alcohols and thiols in the following order:

no added reagent < alcohols << thiols << oxygen (Pratt, 1972). Acceleration in the presence of oxygen may be used as evidence that homolytic fission is occuring. ß-Elimination reactions, though potentially reversible, are not in practice, as the cobalt hydride product irreversibly decomposes to give B_{12r} with the liberation of hydrogen (Grate and Schrauzer, 1979), the process being accelerated by buffer anions (Das et al., 1968). The reaction is pH-independent and is not accelerated by oxygen. For some secondary alkylcobalamins, however, decomposition at elevated temperatures was found to be accelerated by oxygen e.g. cyclohexylcobalamin (Chemaly and Pratt, 1980c). This suggests that some homolytic fission can occur in parallel with ß-elimination.

The decomposition reaction of isobutylcobalamin is not accelerated by oxygen; the ß-elimination is rapid (21% decomposition in one hour at 50 °C), and, being more facile, serves as the predominant pathway. Cyclobutylcobalamin, on the other hand, was found to have an enhanced reaction rate in air at 30 °C, pH 1 (Chemaly and Pratt, 1980c); and it was suggested that the ß-hydrogen atom is less conveniently placed for ß-elimination than in isobutylcobalamin, so that steric factors in the transition state might ultimately favour homolytic fission at elevated temperatures (Chemaly and Pratt, 1980c). Evidence for the simultaneous occurrence of two mechanisms of decomposition in cyclohexylcobalamin was adduced by the nature of the organic products: cyclohexane (homolytic fission product) and cyclohexene (ß-elimination product) were detected in the gas phase (Schrauzer et al., 1970).

4.3 The rôle of steric strain in Co-C bond cleavage

The X-ray structure of adenosylcobalamin shows that the Co-C bond

is surrounded at 6.5 Å by two CH_2-groups (C_{26} and C_{37}), and by two methyl groups (C_{46} and C_{54}), forming a square nearly 2 Å above the cobalt atom (Lenhert, 1968). Steric interactions between a bulky upper ligand and the corrin ring are expected. A further source of interaction, in the case of adenosylcobalamin, would be between the oxygen atom of the ribose ring and methylene groups (C_{26} and C_{37}) of acetamide side chains, and methyl groups C_{46} and C_{54} (Lenhert, 1968).

The steric repulsion is expected to be complemented by non-bonded electronic repulsion due to electron pairs in C-C and C-H ligand bonds, and non-bonded cobalt electron pairs (t_{2g} subshells), as well as lone pairs of nitrogen atoms coordinated to the cobalt (Pratt and Craig, 1973). The adenosylcobalamin, where the Co-$\hat{C}_\alpha$-C_β bond angle is increased to 125° from the usual tetrahedral value of 109.5°, probably employs this distortion to diminish the effects of steric and non-bonded electronic repulsion (Lenhert, 1968). Until recently the only organometallic B_{12} compound for which X-ray structural information was available was coenzyme B_{12} (Lenhert and Hodgkin, 1961). In 1985 the structure of a second B_{12} coenzyme, methylcobalamin, was determined by X-ray and NMR methods (Rossi et al., 1985). The Co-C bond length is 1.99 Å in methylcobalamin, as compared to the more sterically hindered adenosylcobalamin, where the Co-C bond length is 2.05 Å. No X-ray structures exist for ethyl and cyclobutylcobalamins, but the Co-C bond length is expected to exceed that of methylcobalamin, and a distortion in the Co-$\hat{C}_\alpha$-C_β bond angle is likely.

In both homolytic fission and β-elimination mechanisms, the lability of the Co-C bond may be ascribed to the distortion of the bond angles around C_α, and the consequent lengthening and weakening of the Co-C bond. Increasing steric compression around the coordinated carbon has been s[illegible]stituting various alkyl ligands (Grate and Schrauzer, [illegible]emaly and Pratt, 1980a; 1980b; 1980c; Schrauzer and Gr[illegible], 1981). Increasing steric compression in organocobalamins in the series methyl < ethyl < isopropyl (i.e. increasing substitution of C_α decreases the Co-$\hat{C}_\alpha$-C_β bond angle), ethyl ~ n-propyl < isobutyl < neopentyl (i.e. increasing substitution on C_β increases the Co-$\hat{C}_\alpha$-C_β bond angle) and cyclopropyl < cyclobutyl < cyclopentyl < cyclohexyl (decreased

Co-$\hat{C}_\alpha$-C_β bond angle), gave rise to parallel changes in the spectra, equilibrium constants and lability of the Co-C bond in organocobalamins. The five-coordinate alkyl corrinoid complexes showed increasing stability relative to the six-coordinate complexes and decreased half-lives for Co-C bond fission in the same order, with increasing steric compression. The absorption band at ca. 460 nm in six-coordinate cobalt(III) complexes was shifted to ca. 440 nm in the spectra of five coordinate forms (with increased steric compression).

It was found that homolytic fission could be induced to the same extent by increasing or decreasing the Co-$\hat{C}_\alpha$-C_β bond angle. Finally, organocobalamins show increasing ease of decomposition with increasing steric compression regardless of the mechanism operative, that is, homolytic fission and/or β-elimination: the alkyl ligands can be arranged in virtually a single order: methyl < cyclopropyl < ethyl ∿ cyclobutyl < isobutyl < neopentyl ∿ cyclohexyl < cyclopentyl ∿ isopropyl (Chemaly, 1980).

Grate and Schrauzer (1981) have shown that homolysis of an alkylcobinamide is negligible relative to that of the corresponding base-on alkylcobalamin ($K_{base\text{-}off} \sim 10^{-3} K_{base\text{-}on}$). This result has been given support by Finke and Hay (1984). In the present work the rates of homolysis of methyl, ethyl and cyclobutylcobalamin were compared with those of the corresponding cobinamides.

4.4 Photolysis of organocobalamins

The first step in the photolysis of organocobalamins is homolytic fission (Pratt and Whitear, 1971; Endicott and Ferraudi, 1977). Flash photolysis has indicated that B_{12r} and methyl or adenosyl radicals are products in the decomposition of methylcobalamin and adenosylcobalamin (Endicott and Ferraudi, 1977; Endicott and Netzel, 1979). Ethyl and adenosyl radicals have also been observed in spin trapping experiments (Joblin et al., 1975). The photolysis results of methyl, ethyl, cyclobutyl and adenosylcobalamins are reviewed here for the insight they lend into the corresponding thermolysis decomposition reactions.

4.4.1 Methylcobalamin

A slow, reversible photolysis occurs under nitrogen giving B_{12r} (Pratt, 1964; Yamada et al., 1966), methane (where the methyl

radical abstracts a solvent or corrin ring hydrogen atom) and ethane (where dimerisation of the methyl radical occurs) (Dolphin et al., 1964). The free radical/B_{12r} recombination occurs very rapidly ($K \sim 1.5 \times 10^9$ mol^{-1} S^{-1}) (Endicott and Ferraudi, 1977) which accounts for the slow rate. Oxygen accelerates photolysis giving B_{12a} (Pratt, 1964) and formaldehyde (the methyl radical is converted to methyl peroxy radical in the presence of oxygen, giving formaldehyde) (Dolphin et al., 1964a; Pratt, 1972). Acceleration of photolysis by the removal of the methyl radical is effected by various reagents in the following order of increasing efficiency: no added reagent < alcohols << thiols << oxygen (Pratt, 1972).

4.4.2 Adenosylcobalamin

The rate of photolysis of adenosylcobalamin is similar under both nitrogen and oxygen (Pratt, 1964); rapid cyclization of the adenosyl free radical to give 8,5' - cyclic adenosine occurs (Hogenkamp, 1963). The rate-determining step is Co-C bond breaking rather than the subsequent reaction of the free radical (Pratt, 1972); recent work supports this conclusion (Finke and Hay, 1984). The reaction is not reversible due to the cyclization of the radical. The effect of alcohols has been studied: isopropanol serves to scavenge the adenosyl radical, producing the strongly reducing α-hydroxypropyl radical which reduces B_{12r} to B_{12s} and is itself oxidised to acetone (Endicott and Netzel, 1979); ethylene glycol (Finke and Hay, 1984) provides a hydrogen radical to terminate the adenosyl radical.

4.4.3 Ethyl and cyclobutylcobalamin

Irreversible photolysis of ethyl and cyclobutylcobalamins is similar to that of methylcobalamin. Under nitrogen the products are mostly alkenes (Dolphin et al., 1964b). Oxygen and alcohols accelerate the reaction giving B_{12a} (Dolphin et al., 1964a; 1964b) and aliphatic aldehydes (Dolphin et al., 1964a). B_{12s} is a product of photolysis under nitrogen (Yamada et al., 1966) as well as B_{12r} (in contrast to methylcobalamin). B_{12s} formation is due either to β-elimination or reduction of B_{12r} by the alkyl radical (Yamada et al., 1966).

4.5 Adenosylcobalamin-dependent isomerase reactions

The essential first step in the reaction of adenosylcobalamin-dependent isomerase enzymes involves the reversible homolytic cleavage of the Co-C bond of the B_{12} coenzyme (Dolphin, 1982) to yield B_{12r} and the adenosyl free radical. Recent evidence points to this homolysis as the key (and only) rôle played by the adenosylcobalamin cofactor (Finke and Hay, 1984). The next step in the reaction is the abstraction of a hydrogen atom from the substrate by the radical to give 5'-deoxyadenosine and a substrate free radical. The substrate free radical, either directly or via a carbocation or organocobalt complex, rearranges to give a product free radical which abstracts a hydrogen atom from 5'-deoxyadenosine, so producing the product and an adenosyl free radical; adenosyl cobalamin is then regenerated. The overall reaction scheme is presented in Figure 4.1.

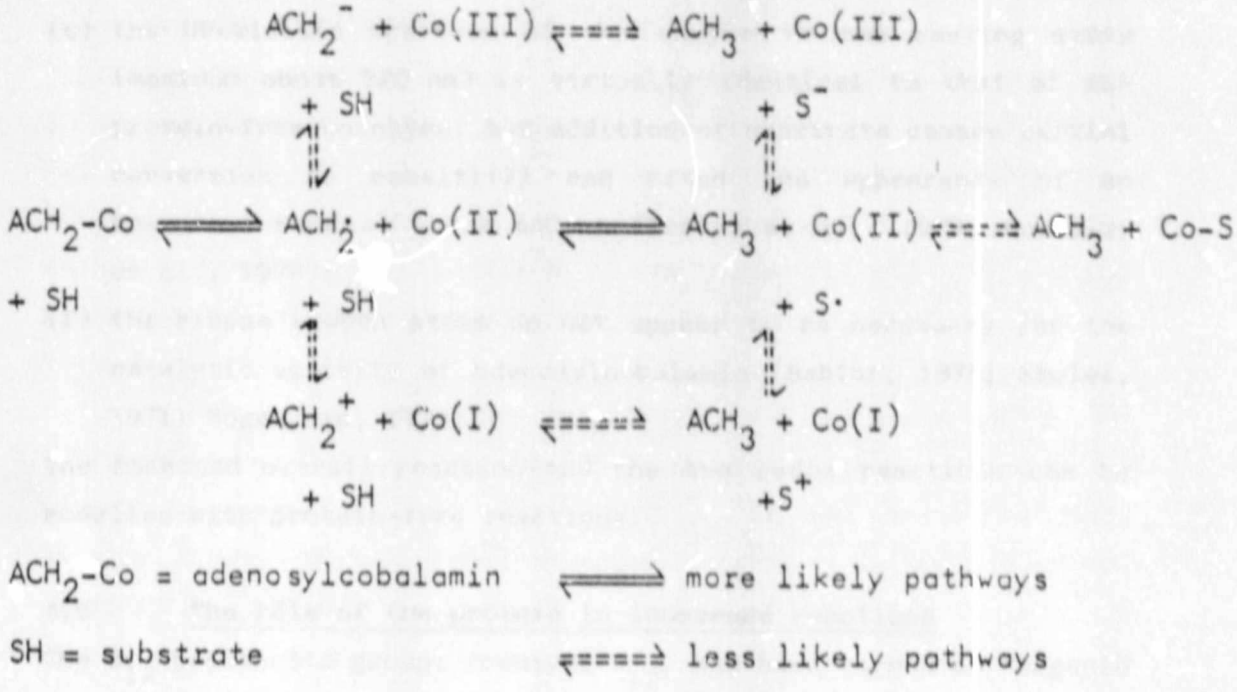

Fig. 4.1 Scheme of possible reaction mechanisms for the isomerase enzymes (source: Chemaly (1980)).

Experimental evidence in support of this scheme is as follows:

(a) the migrating hydrogen atom for all enzymes studied equilibrates with both hydrogen atoms on the coordinated (C_α) atom of ACH_2 but not with the solvent (Babior, 1975; Abeles, 1979);

(b) there is evidence for the formation of 5'-deoxyadenosine as an intermediate for several enzymes (Babior, 1975; Abeles and Dolphin, 1976; Abeles, 1979);

(c) unusual ESR (electron spin resonance) spectra, obtained by freezing the enzyme during the steady state, indicate the presence of cobalt(II) with overlap between the atomic orbitals of the cobalt atom and the carbon atom of the substrate radical, at a distance of ≥ 10 Å, for three enzymes (Valinsky, 1974; Babior, et al., 1974; Schepler et al., 1975; Buettner and Coffman, 1977; Boas et al., 1978);

(d) B_{12r} and organic free radicals have been detected using UV-visible and ESR spectroscopy (Babior, 1975; Cockle et al., 1972).

(e) the UV-visible spectrum of the enzyme in the resting state (maximum about 520 nm) is virtually identical to that of the protein-free coenzyme; but addition of substrate causes partial conversion to cobalt(II) and often the appearance of an anomalous band at about 440 nm (Toraya et al., 1979; Hollaway et al., 1979);

(f) the ribose oxygen atoms do not appear to be necessary for the catalytic activity of adenosylcobalamin (Babior, 1975; Abeles, 1971; Hogenkamp, 1979).

The combined overall reaction and the two redox reactions can be modelled with protein-free reactions.

4.6 The rôle of the protein in isomerase reactions

The B_{12} prosthetic group, coenzyme B_{12}, has been termed an "organic radical carrier" (Halpern, 1983); the readily cleaved Co-C bond is a prerequisite for the formation of a protein-bound substrate radical, which undergoes rearrangement reactions often not favoured by the substrate radical in the absence of the protein. Analogous to haemoglobin, where the protein modulates the ability of the metal centre to bind dioxygen, the protein in B_{12}-dependent enzymes

influences the binding of the substrate radical via its ability to labilise the Co-C bond.

The Co-C bond in the protein-free coenzyme under physiological conditions in the dark is very stable: less than 5 per cent decomposition was observed after adenosylcobalamin was held for five hours in the dark at 94 °C in water under argon (Hogenkamp et al., 1975). The principal rôle of the protein appears to be to labilise this bond (by a factor of $\geqslant 10^{11}$ [see Section 4.11]). The manner in which this is accomplished is still a matter of controversy. Some of the evidence has implicated repulsive interactions between the bulky adenosyl ligand and the corrin macrocycle in Co-C bond cleavage (Chemaly and Pratt, 1980a; 1980b; 1980c; Grate and Schrauzer, 1979; Toraya et al., 1979, Hollaway et al., 1978; Dolphin, 1982). Pratt (1984) suggests that the protein uses steric distortion of the Co-$\hat{C}_{\alpha}$-C_{β} bond angle, caused by a substrate-induced conformational change, to promote Co-C bond cleavage. The lability of the Co-C bond is increased dramatically by increased steric compression: the change in half lives ($t_{½}$) for decomposition of alkylcobalamins at 25 °C increases as the alkyl ligand is changed from methyl ($t_{½} \geqslant 1$ year) through ethyl ($t_{½} \sim 6$ months) to isopropyl ($t_{½} \sim 3$ minutes), neopentyl ($t_{½} = 30$ minutes) and cyclohexyl ($t_{½} = 44$ minutes) (Grate and Schrauzer, 1979). The adenosyl ligand, on the basis of spectra and equilibria, would be expected to occupy a position between methyl and ethyl. The predicted $t_{½}$ at room temperature would thus be larger than 6 months.

At the time that this work was carried out, no value of $t_{½}$ for the homolytic fission of the Co-C bond in adenosylcobalamin, neutral solution, at room temperature, had yet been reported. However, Finke and Hay (1984) reported a first-order rate constant of $1.12 \times 10^{-4} s^{-1}$ for the homolytic fission of adenosylcobalamin at 110 °C, in ethylene glycol. The anaerobic thermolysis of adenosylcobalamin was followed by optical spectroscopy in the temperature range 90 - 120 °C in ethylene glycol using TEMPO (nitroxide 2,2,6,6-tetramethyl- piperidinyl-1-oxy) as a trap for 5'-deoxyadenosyl radical. Co-C bond energies were estimated in two studies (Halpern et al., 1984; Finke and Hay, 1984). Finke and Hay (1984) postulated a lowering by at least 14.7 K cal mol^{-1} of the

barrier for Co-C bond homolysis for a rate acceleration of at least 10^{10} by the adenosylcobalamin-dependent enzyme.

In this study the ratio of rates of decomposition of adenosylcobalamin and ethylcobalamin at 96 °C, together with the rate of decomposition of ethylcobalamin at room temperature, were used to calculate a value of $t_{1/2}$ for the room temperature decomposition of adenosylcobalamin. Hence an estimate of the labilising effect of the protein could be calculated and compared to the Finke and Hay (1984) value. The effect on $t_{1/2}$ values of decomposition of the heterocyclic base was assessed using ethyl and methylcobinamides. Finally, the decomposition of cyclobutylcobalamin, expected to decompose at elevated temperatures by both β-elimination and homolysis mechanisms, was examined.

The scheme in Figure 4.2 illustrates the complexity of reactions initiated by homolytic fission, both thermal and photochemical (R is methyl, ethyl or adenosyl). Recent evidence shows that overall β-elimination also proceeds via initial homolytic fission to give the caged pair (Baldwin et al., 1985a) and by detection of the unstable hydride (Chemaly and Pratt, 1984).

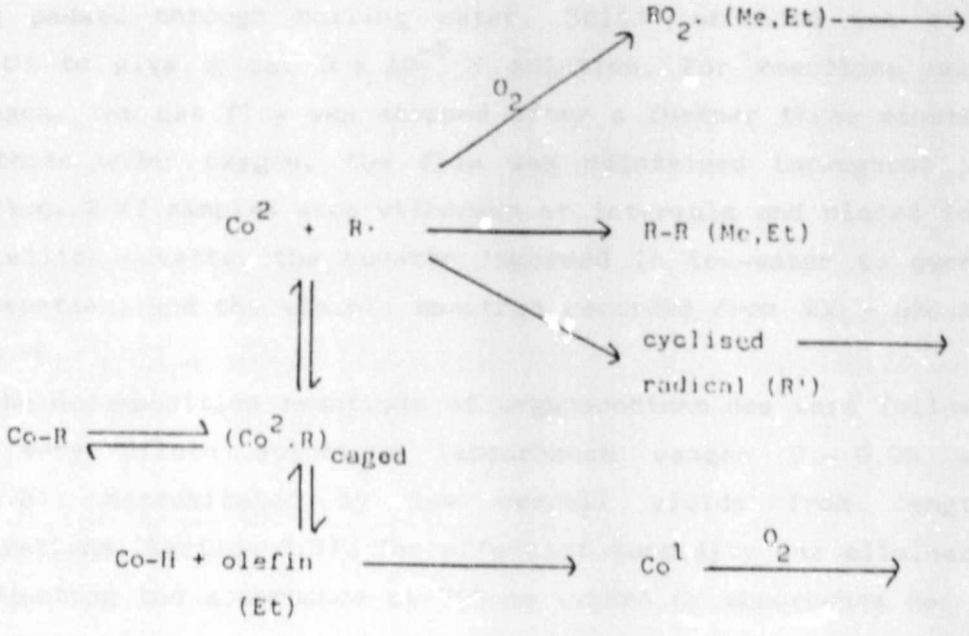

Fig. 4.2 Scheme of reactions initiated by homolytic fission of the Co-C bond in organocorrinoids; R may be methyl, ethyl or adenosyl, and Co-R denotes five- and/or six-coordinate species.

The rates of thermolysis of methyl, ethyl, adenosyl and cyclobutylcobalamins in aqueous solution at 96 °C in this study were compared in the presence and absence of oxygen (to ensure the maximum rate of Co-C bond fission), and in the case of adenosylcobalamin, at varying pH (to confirm that the observed reaction is pH-independent homolytic fission, not heterolysis as occurs at high and low pH (Pratt, 1982)).

4.7 Experimental procedure

Adenosylcobalamin was purchased from Sigma and used without further purification. Methyl, ethyl and cyclobutylcobalamins and the corresponding cobinamide complexes, were prepared as outlined in Chapter 3. Phosphate buffers at pH 6, 7 and 8, with ionic strength μ = 0.1, were prepared according to Long (1971).

200 mℓ of buffer solution was placed in a 500 mℓ three-necked round-bottomed flask, fitted with a water-cooled reflux condensor and a gas inlet. The solution was refluxed gently (ca. 96 °C, boiling point at alt. 1760 m) and purged with a stream of fine bubbles of nitrogen or oxygen. To decrease evaporation of the hot solution, the purging gas was first saturated with water vapour by being passed through boiling water. Solid corrinoid was added (t = 0) to give a ca. 3×10^{-5} M solution. For reactions under nitrogen, the gas flow was stopped after a further three minutes; for those under oxygen, the flow was maintained throughout the reaction. 2 mℓ samples were withdrawn at intervals and placed in a 1 cm silica cuvette, the cuvette immersed in ice-water to quench the reaction, and the visible spectrum recorded from 300 - 650 nm, at 25 °C.

The decomposition reactions of organocobinamides were followed with very dilute solutions (absorbance ranges 0 - 0.05 and 0 - 0.2), necessitated by low overall yields from lengthy preparations [Section 3.3]. The effect of turbidity was eliminated by adjusting the absorbance at 750 nm (where no absorbance due to the corrinoid is expected) to zero, before running the spectrum of organocobinamides. A plentiful supply of the corresponding organocobalamins allowed the use of 0 - 1.0 and 0 - 2.0 absorbance ranges. Alkyl corrinoids are very light sensitive (Pratt, 1972); appropriate precautions were taken at all stages: the

round-bottomed flasks were insulated and covered with aluminium foil and the cuvette sealed from light.

The reaction products were identified from their main absorption bands (Pratt, 1972): cobalt(III) (aquocobalamin) 350 nm; cobalt(II) 473 and 311 nm (slowly oxidised by oxygen at room temperature); stable yellow corrinoid (SYC) in which the corrin ring has been broken (Pratt, 1972; Gossauer et al., 1977) ca. 460 nm (stable under oxygen). The spectral features of the organocobalamins and organocobinamides studied were discussed in Chapter 3.

For the faster reactions of ethylcobalamin and cyclobutylcobalamin, A_∞ values were determined by following the reactions to completion. For adenosylcobalamin the endpoint was calculated from the ratio (1.85) of A_{522} for ethylcobalamin and cobalt(II), determined by photolysis of [illegible]cobalamin under nitrogen. Values for the half-life of decomposition ($t_{½}$) were determined from plots of $\ell og|A_\infty - A|$ against time [see derivation in Appendix (a)]. The SAS package on an IBM 3083 computer was employed for linear regression analyses.

4.8 Results

Preliminary experiments established that adenosylcobalamin decomposes at similar rates in the pH range 6 - 8 (i.e. by pH-independent homolytic fission) but more rapidly at pH 5 (presumably due to acid catalysed heterolytic fission). Ethylcobalamin and cyclobutylcobalamin also decompose at similar rates pH 6 - 8, but faster under oxygen; the observed changes were easier to analyse at the lower pH value. Most experiments were therefore carried out at pH 6.

4.8.1 Adenosylcobalamin

Decomposition in a nitrogen atmosphere produced cobalt(II) with good isosbestic points at ca. 335, 390, 490, and 585 nm (see Figure 4.3); the changes in spectrum were similar to those reported for photolysis under nitrogen (Brady and Barker, 1961). The reaction (analysed from the decrease in A_{522}) followed first-order kinetics (see Figure 4.4a) with $t_{½}$ = 180 and 190 minutes (duplicate experiments) at pH 6 and 170 minutes at pH 7, i.e. the rate is

round-bottomed flasks were insulated and covered with aluminium foil and the cuvette sealed from light.

The reaction products were identified from their main absorption bands (Pratt, 1972): cobalt(III) (aquocobalamin) 350 nm; cobalt(II) 473 and 311 nm (slowly oxidised by oxygen at room temperature); stable yellow corrinoid (SYC) in which the corrin ring has been broken (Pratt, 1972; Gossauer et al., 1977) ca. 460 nm (stable under oxygen). The spectral features of the organocobalamins and organocobinamides studied were discussed in Chapter 3.

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4.8.1 Adenosylcobalamin

Decomposition in a nitrogen atmosphere produced cobalt(II) with good isosbestic points at ca. 335, 390, 530, and 585 nm (see Figure 4.3); the changes in spectrum were similar to those reported for photolysis under nitrogen (Brady and Barker, 1961). The reaction (analysed from the decrease in A_{522}) followed first-order kinetics (see Figure 4.4a) with $t_{½}$ = 180 and 190 minutes (duplicate experiments) at pH 6 and 170 minutes at pH 7, i.e. the rate is

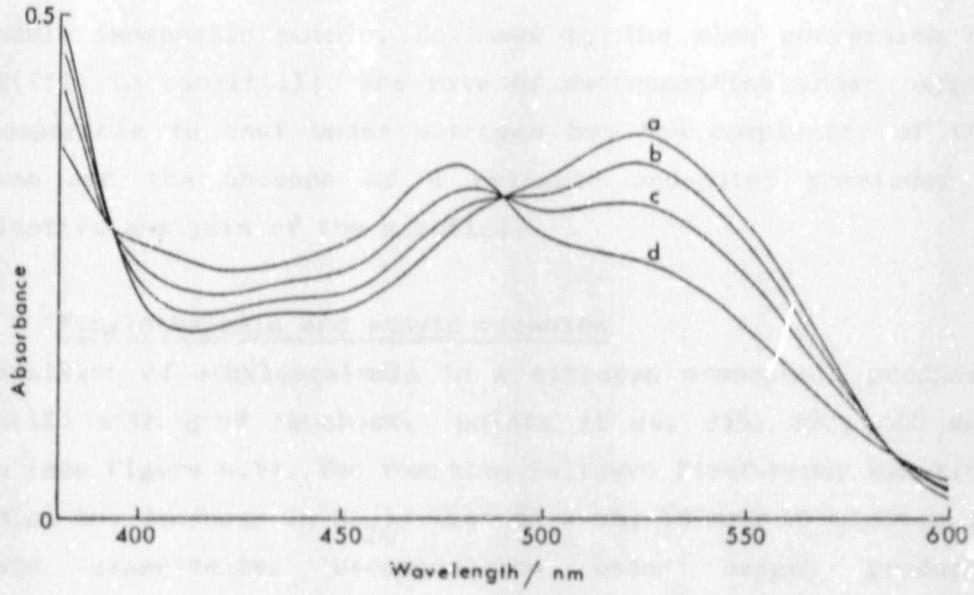

Fig. 4.3 Spectral changes observed during the decomposition of adenosylcobalamin at 96 °C under nitrogen: A = 2 minutes, B = 66 minutes, C = 145 minutes and D = 345 minutes.

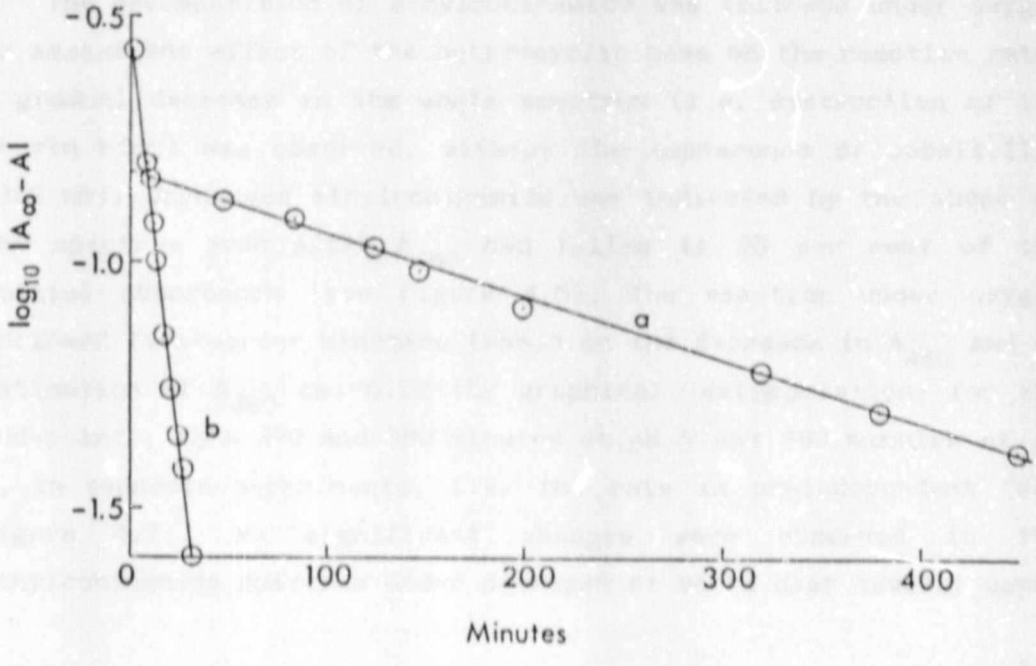

Fig 4.4 First-order kinetic plots of the decomposition at 96 °C of a) adenosylcobalalamin under nitrogen and b) ethylcobalamin under oxygen.

pH-independent. Decomposition under oxygen is more complex; the spectra indicated the formation of cobalt(III) with some SYC with reasonable isosbestic points, followed by the slow conversion of cobalt(III) to cobalt(II). The rate of decomposition under oxygen was comparable to that under nitrogen but the complexity of the reaction and the absence of a reliable end-point precluded a quantitative analysis of the kinetics.

4.8.2 Ethylcobalamin and ethylcobinamide

Decomposition of ethylcobalamin in a nitrogen atmosphere produced cobalt(II) with good isosbestic points at ca. 335, 390, 485 and 585 nm (see Figure 4.5). The reaction followed first-order kinetics (based on the decrease in A_{510}) with $t_{½}$ = 15, 15 and 16 minutes in separate experiments. Decomposition under oxygen produced cobalt(III) and a little SYC with isosbestic points at about 335, 364, 455, 480 and 600 nm, followed by a much slower conversion of cobalt(III) to cobalt(II). The reaction followed first-order kinetics (based on the increase in A_{350}) for over three half-lives (see Figure 4.4b) with $t_{½}$ = 9, 9.5 and 9.5 minutes in separate experiments, i.e. significantly shorter than under nitrogen.

The decomposition of ethylcobinamide was followed under oxygen to assess the effect of the heterocyclic base on the reaction rate. A gradual decrease in the whole spectrum (i.e. destruction of the corrin ring) was observed, without the appearance of cobalt(III) (350 nm). Unchanged ethylcobinamide was indicated by the shape of the spectrum even after A_{460} had fallen to 20 per cent of the initial absorbance (see Figure 4.6). The reaction under oxygen followed first-order kinetics (based on the decrease in A_{460} and an estimation of A_{460} ca. 0.02 (by graphical extrapolation) for the end-point); $t_{½}$ = 370 and 380 minutes at pH 6 and 390 minutes at pH 7, in separate experiments, i.e. the rate is pH-independent (see Figure 4.7). No significant changes were observed in the ethylcobinamide spectrum under nitrogen at 96 °C over several days.

4.8.3 Methylcobalamin and methylcobinamide

No significant changes were observed for methylcobalamin under nitrogen over six days. The presence of oxygen, as observed with ethylcobinamide, caused a gradual decrease in the whole spectrum

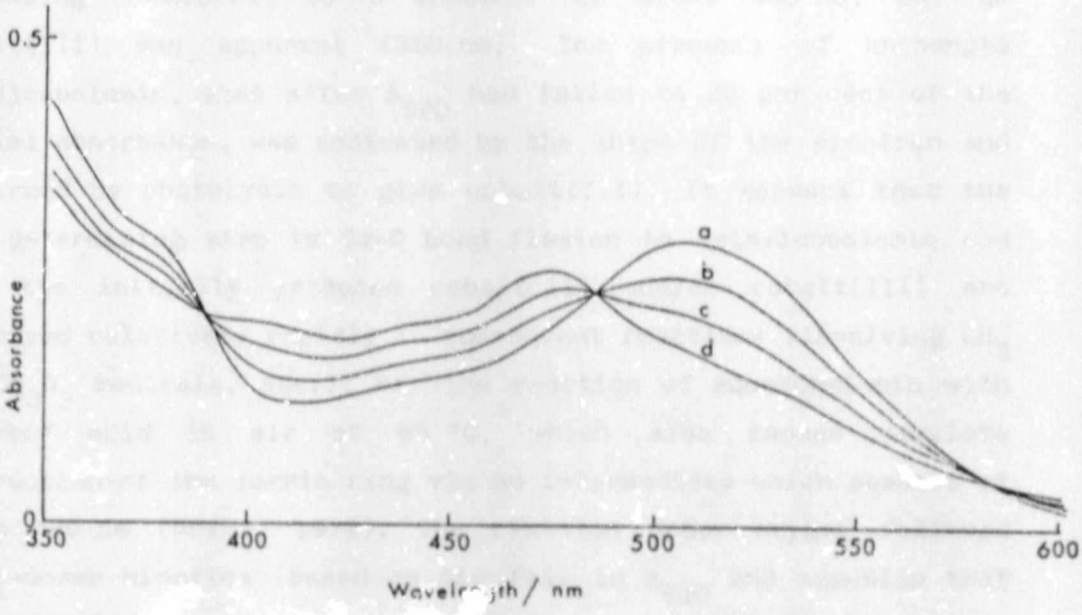

Fig. 4.5 Spectral changes observed during the decomposition of ethylcobalamin at 96 °C under nitrogen: A = 1 minutes, B = 7 minutes, C = 18 minutes and D = 37 minutes.

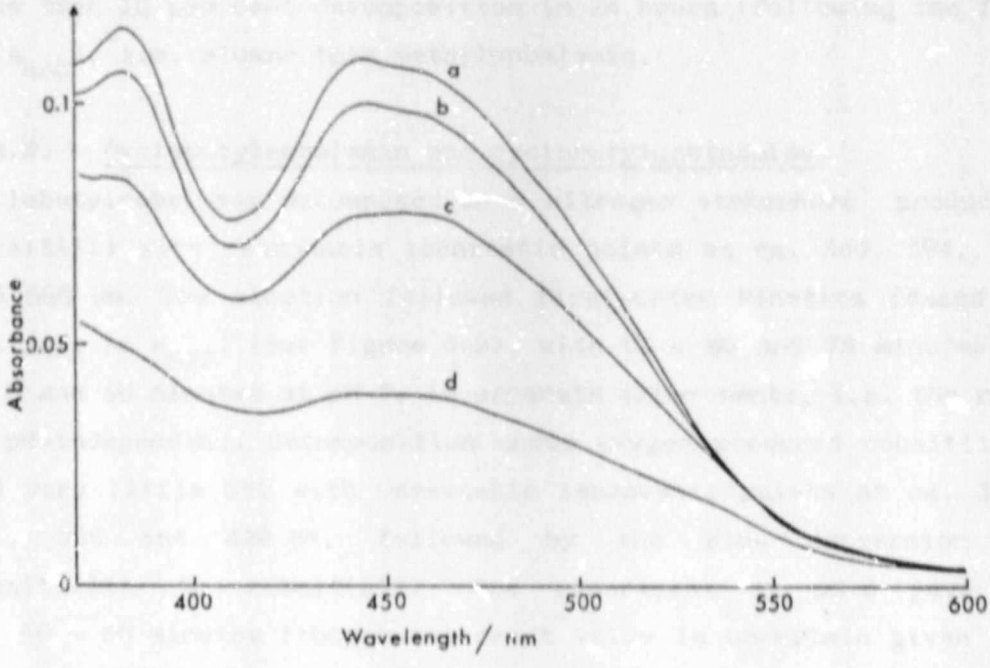

Fig. 4.6 Spectral changes observed during the decomposition of ethylcobinamide at 96 °C, pH 7, under oxygen: A = 3 minutes, B = 46 minutes, C = 264 minutes and D = 1598 minutes.

(i.e. the destruction of the corrin ring), together with the increasing prominence of a shoulder at about 480 nm, but no cobalt(III) was apparent (350 nm). The presence of unchanged methylcobalamin, even after A_{520} had fallen to 20 per cent of the initial absorbance, was indicated by the shape of the spectrum and confirmed by photolysis to give cobalt(III). It appears that the rate-determining step is Co-C bond fission in methylcobalamin and that the initially produced cobalt(II) and/or cobalt(III) are destroyed relatively rapidly in subsequent reactions (involving CH_3 and CH_3O_2 radicals, etc.); cf. the reaction of aquocobalamin with ascorbic acid in air at 65 °C, which also causes complete destruction of the corrin ring via an intermediate which absorbs at about 480 nm (Pratt, 1972). The reaction under oxygen followed first-order kinetics (based on the fall in A_{520} and assuming that A_{520} = 0 for the end-point) for over two half-lives (see Figure 4.8) with $t_{½}$ = 80 hours. Two other, more qualitative, experiments in air also gave $t_{½}$ as ca. 80 hours.

A qualitative experiment following the decomposition of methylcobinamide under oxygen showed a similar pattern of corrin ring destruction to the ethylcobinamide reaction under oxygen, with less than 10 per cent decomposition in 24 hours (following the fall in A_{460}), i.e. slower than methylcobalamin.

4.8.4. Cyclobutylcobalamin and cyclobutylcobinamide

Cyclobutylcobalamin decomposed in a nitrogen atmosphere producing cobalt(II) with reasonable isosbestic points at ca. 340, 394, 490 and 580 nm. The reaction followed first-order kinetics (based on the fall in A_{522}) (see Figure 4.9), with $t_{½}$ = 80 and 75 minutes at pH 6 and 80 minutes at pH 7, in separate experiments, i.e. the rate is pH-independent. Decomposition under oxygen produced cobalt(III) and very little SYC with reasonable isosbestic points at ca. 329, 371, 395 and 428 nm, followed by the slow conversion of cobalt(III) to cobalt(II). One experiment at pH 6 gave $t_{½}$ ca. 50 - 60 minutes (though the exact value is uncertain given the absence of a reliable end-point), i.e. the rate of decomposition is accelerated by oxygen.

A qualitative experiment with cyclobutylcobinamide under oxygen at pH 7 was followed from the decrease in A_{460}; a rough value of $t_{½}$ ca. 200 minutes was obtained.

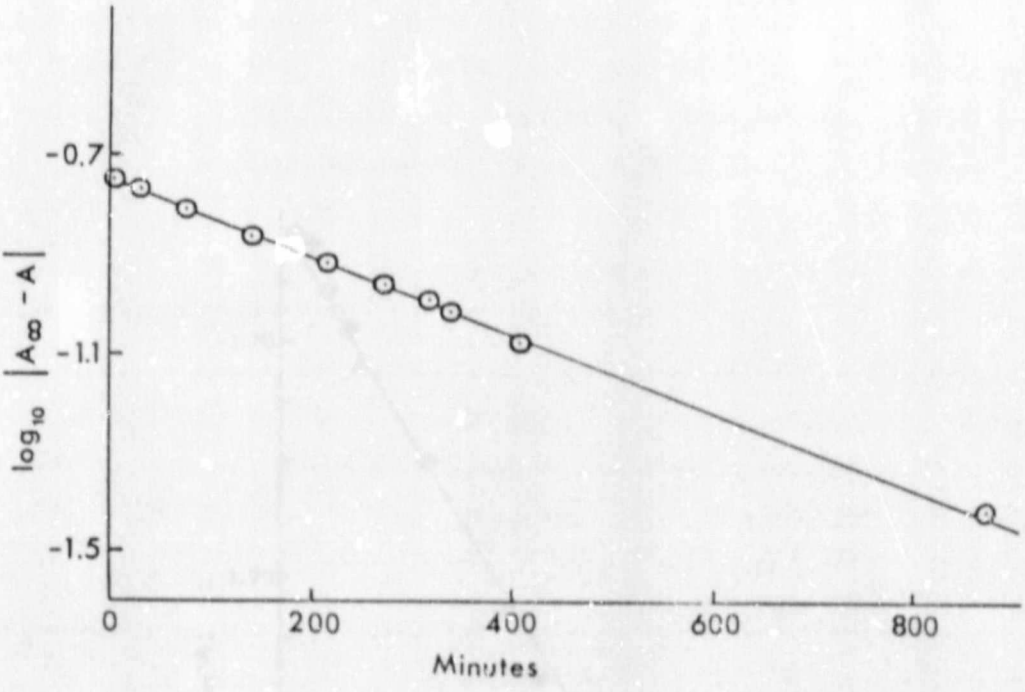

Fig. 4.7 First-order kinetic plot of the decomposition at 96 °C of ethylcobinamide under oxygen.

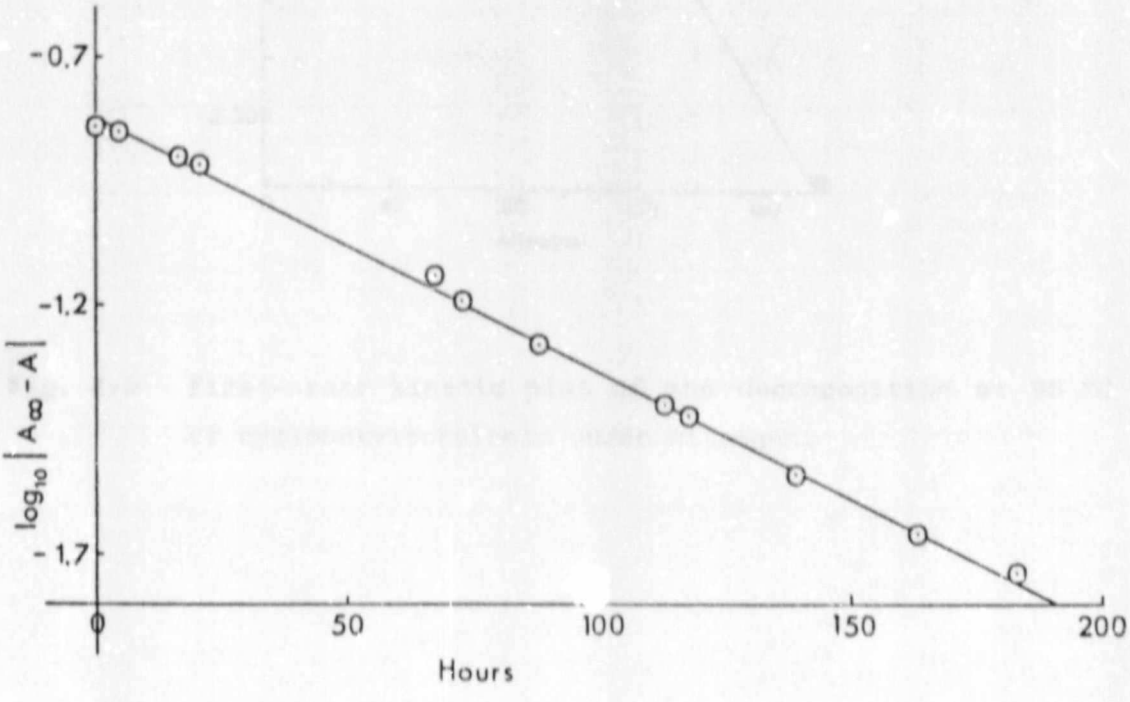

Fig. 4.8 First-order kinetic plot of the decomposition at 96 °C of methylcobalamin under oxygen.

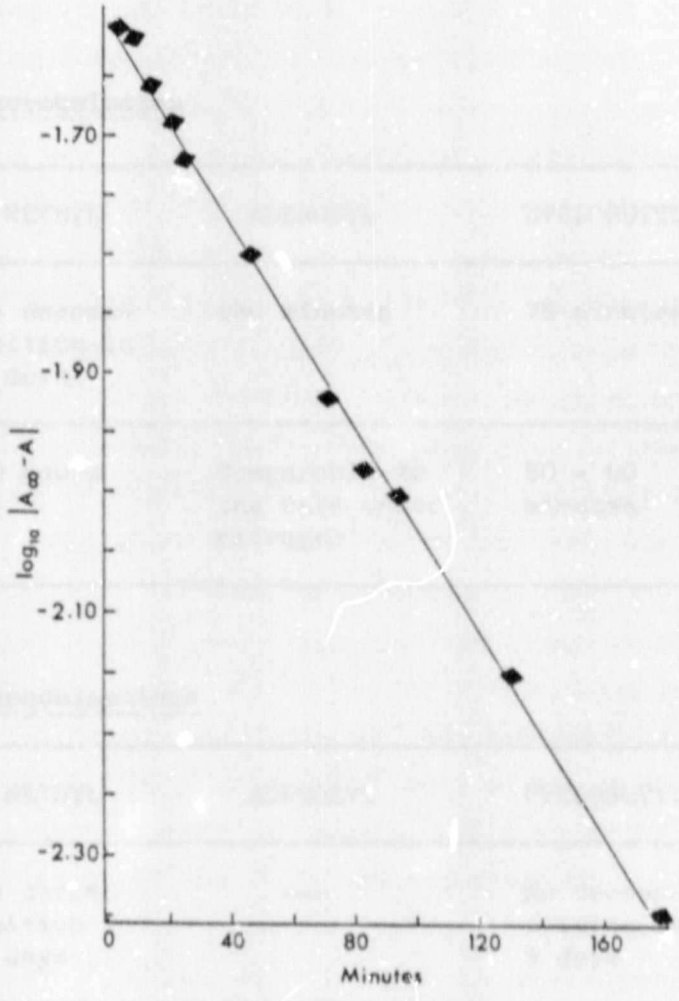

Fig. 4.9 First-order kinetic plot of the decomposition at 96 °C of cyclobutylcobalamin under nitrogen.

The results are summarised in Table 4.1.

TABLE 4.1: The half-lives of decomposition of some organocorrinoids under nitrogen and oxygen, at pH 6 and 7 (pH-independent), at 96 °C.

A. Organocobalamins

GAS	METHYL	ADENOSYL	CYCLOBUTYL	ETHYL
N_2	No decomposition in 6 days	180 minutes	75 minutes	15 minutes
O_2	80 hours	Comparable to the rate under nitrogen[1]	50 - 60 minutes[2]	9 minutes[3]

B. Organocobinamides

GAS	METHYL	ADENOSYL	CYCLOBUTYL	ETHYL
N_2	No decomposition in 3 days	---	No decomposition in 3 days	No decomposition in 3 days
O_2	Decomposition ca. 10% in 24 hours	---	≈200 minutes	370 minutes

[1] The absence of a reliable end point precluded a quantitative rate analysis.

[2] Chemaly (1980) quotes 10 per cent decomposition at 80 °C under oxygen, pH 6 and 7 in one hour.

[3] Chemaly (1980) gives $t_{½}$ = 30 minutes in under oxygen, pH 6 and 7, at 80 °C.

4.9 Evidence for homolytic thermolysis

The rate of thermolysis for all four organocobalamins studied, and for ethylcobinamide, show pH-independence at 96 °C over the range pH 6-8, suggesting that Co-C bond cleavage is homolytic. Increased rates at pH values below 5 and above 9 for adenosylcobalamin, can be ascribed to the known acid-catalysed heterolysis (Pratt, 1972) and alkaline heterolysis (Schrauzer and Sibert, 1970), repectively. Thermolysis of adenosylcobalamin under oxygen and nitrogen gave comparable results, as expected from photolysis results (due to rapid cyclization of the radical formed (Pratt, 1972)). The accelerating effect of oxygen on the thermolysis of methyl, ethyl and cyclobutylcobalamins parallels its effect on the rates of photolysis (Pratt, 1972; 1982). Thus while thermolysis of ethyl and cyclobutylcobalamins probably occurs by β-elimination at room temperature (Chemaly, 1980), the mechanism is homolytic or to a large extent homolytic, at 96 °C. The kinetics of thermolysis of all four cobalamins studied therefore indicate that homolytic cleavage of the Co-C bond is predominant. The values for rates of thermolysis at 96 °C very likely correspond to the maximum rate; low levels of oxygen are required to attain this maximum rate (e.g. one per cent oxygen for thermolysis of cyclohexylcobinamide at 80 °C) (Baldwin et al., 1985a). Destruction of the corrin ring in methylcobalamin (with increasing prominence of a shoulder at 480 nm) under oxygen at 96 °C shows a similarity to the reaction of aqueous solutions of aquocobalamin (B_{12a}) with ascorbic acid in air at 65 °C (also leading to the complete destruction of the corrin ring via the formation of an intermediate absorbing at 480 nm) (Pratt, 1972).

Grate and Schrauzer (1979; 1981) reported that homolysis of an alkylcobinamide is slower by a factor of ca. 10^3 relative to that of the corresponding alkylcobalamin. The 1979 study examined isopropylcobinamide quantitatively, at room temperature: the corresponding cobalamin is known to decompose predominantly by β-elimination (Chemaly and Pratt, 1980c). The rates of decomposition of organocobinamides in this study are more rapid than would be expected from the Grate and Schrauzer observation: methylcobinamide gives ca. 10% decomposition in 24 hours as compared to ca. 20% decomposition in 24 hours for methylcobalamin;

ethylcobinamide has $t_{½}$ = 380 minutes under oxygen as compared to $t_{½}$ = 9 minutes under oxygen for ethylcobalamin (all at 96 °C). Decomposition appears to be homolytic in both cases (as evidenced by the accelerating effect of oxygen on the reaction rate). The decomposition of cobinamides, however, gave no evidence of cobalt(III) or cobalt(II) peaks: rather the entire corrin ring disappeared, in an analogous fashion to the decomposition of methylcobalamin. Rapid recombination of B_{12r} with the alkyl ligand is likely and destruction of the ring probably occurs via an intermediate radical.

4.10 The order of ligands and estimation of the room temperature half-life for homolytic decomposition of the coenzyme

The half-lives at 96 °C increase (i.e. the rates decrease) with the nature of the ligand in the order ethyl (9 min) < cyclobutyl (75 min) < adenosyl (180 min) < methyl (80 hours) for organocobalamins studied. The order corresponds to that established at room temperature (Chemaly and Pratt, 1980b) except that cyclobutylcobalamin displays a shorter rate of reaction at higher temperature (due to a larger percentage of homolytic fission). It is difficult to estimate the relative steric hindrance offered by ethyl or cyclobutyl ligands: the former serves to decrease the $Co\text{-}\hat{C}_{\alpha}\text{-}C_{\beta}$ bond angle, the latter to increase the angle.

The maintenance of ligand order at higher temperatures suggests that the ratio of rates is probably not strongly temperature dependent. The ratio observed at 96 °C for adenosylcobalamin and ethylcobalamin can thus be combined with the value of $t_{½}$ ca. 6 months reported for ethylcobalamin at room temperature (Grate and Schrauzer, 1979) to give a rough value of $t_{½}$ ca. 10 years for adenosylcobalamin at room temperature. The Co-C bond in the coenzyme is clearly very stable towards homolytic fission in the absence of the protein.

4.11 Estimation of the labilising rôle of the protein

In Section 4.1.4, experimental evidence in support of a reaction scheme (Figure 4.1) was given, in which the first step in adenosylcobalamin-dependent isomerase reactions is reversible

homolytic cleavage of the Co-C bond in the coenzyme e.g.,

$$\text{Co-R} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \text{Co(II)} + \text{R}^{\cdot} \qquad (1)$$

It would appear that the main influence of the protein (in the presence of the substrate) on the active site is to displace this equilibrium to the right. Indeed, in the absence of protein, the equilibrium constant for the reaction has been estimated as $K_{eq} \sim 2 \times 10^{-17}$ M^{-1} (Pratt, 1984). Given that k_r is very high (ca. 2×10^{9} $M^{-1}s^{-1}$ (Endicott and Netzel, 1979)) the effect is likely to be an increase in k_f without affecting k_r, thus increasing $k_f/k_r = K_{eq}$ i.e. by a thermodynamic rather than a kinetic effect (Pratt, 1975).

A value of $t_{½}$ ca. 10 years was estimated in this work for adenosylcobalamin at room temperature. For dioldehydrase at 37 °C, turn-over numbers of up to $370s^{-1}$ (i.e. $t_{½} \sim 2.5$ ms) have been reported (Toraya et al., 1976). Neglecting the difference in temperature, this corresponds to a labilisation of the Co-C bond by a factor of 10^{11} in the presence of substrate. Dioldenydrase is sensitive to oxygen in the presence of substrate; the Co-C bond cleaves (probably via an initial homolytic fission) with $t_{½}$ = 20 min at 30 °C (Wagner et al., 1966; Toraya et al., 1976). This corresponds to a labilisation by ca. 3×10^{5}. As other B_{12}-dependent isomerases appear to be less sensitive to oxygen and have lower turn-over numbers, labilisation due to the apoenzyme alone can be assumed to be $\leq 10^{5}$ at room temperature.

Finke and Hay (1984), using a rate of $\geq$ 100 turnovers/(site/s) for diol dehydratase (Eagar et al., 1975) and thermodynamic data derived from the thermolysis of adenosylcobalamin (90 - 120 °C), suggest that the diol dehydratase enzyme provides at least a 14.7 K calmol^{-1} lowering of the barrier for Co-C bond homolysis, for a rate acceleration of at least 10^{10}.

These results show that the enzymatic reaction requires a Co-C bond labilisation by a factor of $\geq 10^{10}$; this proceeds in two stages: the first, when the coenzyme is bound by the apoenzyme ($\leq 10^{5}$) and the second, when the substrate is added ($\geq 10^{5}$).

The evidence in Section 4.1.2 suggests that the protein

achieves its effect on equilibrium (1) by distorting the Co-$\hat{C}_{\alpha}$-C_{β} bond angle (facilitating Co-C bond fission) by a change in protein conformation at the active site, induced by substrate binding (e.g. Chemaly and Pratt (1980c)). The effect is probably reversed when the substrate has reacted and diffused away (Pratt, 1975). Evidence for a conformational change asscciated with the fission of the Co-C bond has been reported for one enzyme, ethanolamine ammonia lyase (Babior, 1982), based on differing susceptibilities to denaturating agents of the two forms (similar evidence was offered for a conformational change in cytochrome c [Section 1.3.1]).

Published UV-visible spectra for three enzymes in the presence of coenzyme and substrate, show near complete conversion to B_{12r}, and the presence of a band at 440 nm, not seen in unstrained or 'normal' B_{12} (Hollaway et al., 1979; Tamao and Blakley, 1973; Torayo et al., 1979; Wagner et al., 1966; Foster et al., 1970). A simulated UV-visible spectrum of a mixture of 80 per cent B_{12r} and 20 per cent cyclohexylcobalamin (a strained species: see Section 4.1.2) has been reported (Chemaly, 1980) and resembles the spectrum of glycerol dehydrase (Foster et al., 1970), with a band at 440 nm.

Pratt (1984) suggests that in the absence of substrate, the protein bound coenzyme is in an unstrained six-coordinate form with the protein in a strained conformation. On binding the substrate, the protein conformation relaxes producing a distortion of the Co-$\hat{C}_{\alpha}$-C_{β} bond angle of the coenzyme. The coenzyme is converted to a strained five-coordinate form displaying the 440 nm band (the base is not coordinated); an equilibrium between the strained five coordinate Co(III) complex and a five-coordinate Co(I) complex and adenosyl radical then results.

4.12 Summary

The thermolytic homolysis of the Co-C bond in adenosylcobalamin, methyl, ethyl and cyclobutylcobalamins and the corresponding cobinamides was examined under nitrogen and oxygen atmospheres at 96 °C in phosphate buffer, pH 6 and 7, and half-lives for decomposition were calculated. The order of ligands in organocobalamins for increasing half-lives (decreased rates of reaction): ethyl < cyclobutyl < adenosyl < methyl, confirms the

order obtained at lower temperatures (Chemaly and Pratt, 1980) and lends support to a proposed mechanism of steric control of the Co-$\hat{C}_{\alpha}$-C_{β} bond angle by the protein, in isomerase reactions (Pratt, 1984). The effect of the coordinated base, 5,6-dimethylbenzimidazole, on the half-lives of decomposition was assessed qualitatively using the cobinamide complexes. The half-life for the homolytic decomposition of the cobalt-carbon bond in the coenzyme adenosylcobalamin, at room temperature, was estimated as ca. 10 years. Hence labilisation due to the protein in isomerase enzymes is by a factor of $\geqslant 10^{10}$, composed of $\leqslant 10^{5}$ when the coenzyme is bound by the apoenzyme and $\geqslant 10^{5}$ when the substrate is added.

CHAPTER 5 - STUDIES ON THE REACTIVITY OF THE IRON-METHIONINE BOND IN CYTOCHROME C

5.1 Introduction

The structure of cytochrome c has been determined by X-ray crystallography in both the ferric and ferrous states (e.g. Dickerson et al., 1971). The salient features of the structure were described in Section 1.3(b) and shown in Figure 1.8 . The prosthetic group, haem c, is contained in a hydrophobic environment, situated sideways in a crevice formed by the folding of the protein about the haem, with one edge exposed at the molecular surface (see Figure 5.1). The upper part of the exposed haem edge is known to be included in the site of the interaction of cytochrome c with cytochrome c reductase and cytochrome c oxidase (e.g. Smith et al., 1977) and is probably the site for electron transfer (Moore et al., 1982).

An oxidation-linked conformational change between the two redox states of the cytochrome c molecule has long been indicated by physico-chemical investigation [Section 1.2(c)] and was confirmed recently by X-ray studies of ferric and ferrous bonito cytochrome c (Takano and Dickerson, 1981a; 1981b). The haem crevice appears to be less open in the reduced molecule, which also has a more stable and rigid conformation than the oxidised species. The porphyrin is flatter in the oxidised molecule and the haem iron is shifted 0.16 Å further out from its crevice than in the reduced molecule.

The identity of the sixth haem ligand was in dispute for decades. Harbury et al. (1965) demonstrated for the first time that thioether bonded sulphur was able to coordinate to haem peptides derived from cytochrome c, to form low spin compounds. Schechter and Saludjian (1967) showed that these compounds exhibited the 695 nm band characteristic of cytochrome c. X-ray crystallographic studies confirmed that the haem iron is coordinated to histidine 18 and the sulphur of methionine 80, in both ferric and ferrous forms, a conclusion which was supported by NMR work (Wütrich, 1969). The extent to which the iron-methionine bond (Fe-S) participates in the conformational change of cytochrome c remains uncertain. NMR

studies indicated a small change in the Fe-S interaction on reduction, and interpreted this as a bond length increase (Moore et al., 1980). But X-ray studies (Takano and Dickerson, 1981a; 1981b) and EXAFS measurements (Labhardt and Yuen, 1979) suggest no oxidation-linked bond length change. This discrepancy may be accounted for if the nature of the interaction detected by NMR is not in fact detectable by X-ray studies. The Fe-S bond is sensitive to a wide range of conditions [Section 1.3 (d)] and it appears that bond stretching occurs which in the extreme leads to rapid bond breaking and remaking (Sutin and Yandell, 1979). Rotation about the Fe-S bond may also occur leading to a poor overlap between the sulphur lone pair and ferric orbitals (Moore et al., 1982).

But aside from the interesting possibility of an oxidation-linked change in the Fe-S interaction, the physiological activity of cytochrome c is dependent upon the integrity of this bond. When the bond is intact the closed haem crevice structure representative of the Type III cytochrome c species, is present. The bond is broken in a reversible alkaline isomerisation of cytochrome c occuring with an apparent pK_a of ca. 9 (dependent upon

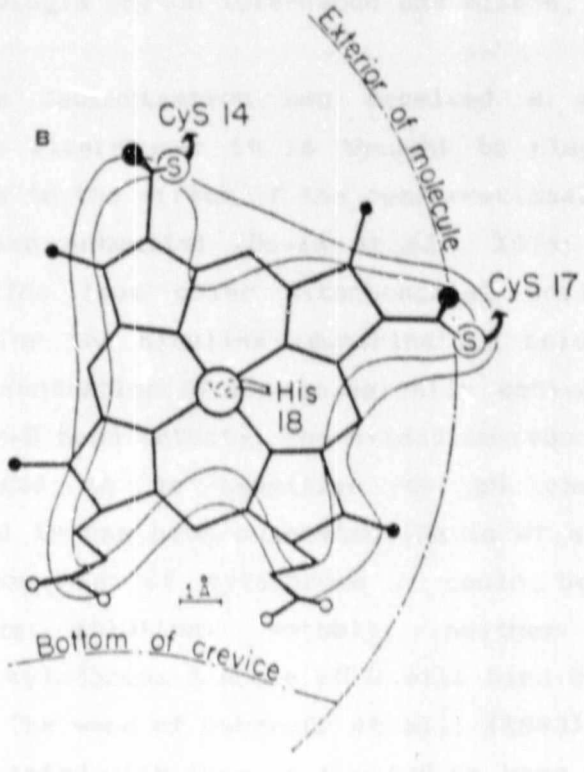

Fig. 5.1 Relationship of haem to the polypeptide chain, to the bottom of its crevice and to the surface of the molecule (source: Lemberg and Barrett (1973)).

the cytochrome c species and ionic strength) and is replaced by a strong field ligand (the identity of which is not certain, but which may be lysine 72, lysine 79 or even water (Bosshard, 1981)), which maintains a low spin complex. The crevice is opened due to the isomerisation, the redox potential is lowered from 260 mV to 120 mV and the haem cannot be reduced by ascorbate or ferrocyanide i.e. it is not physiologically active in the alkaline state (Davis et al., 1974). Both the 695 nm absorption bond and the methyl signal from methionine 80 in NMR (Gupta and Redfield, 1970) disappear on isomerisation [see Section 1.3(d)]. The loss of the near infra-red charge-transfer absorption bond with increasing pH describes a titration curve involving a single proton dissociation. The apparent pK_a of ca. 9 for the titration is due to the ionization of a protein residue with a pK_a' of 11.0 coupled with a conformational equilibrium constant of 125 M^{-1} (pK_c = 2.1). Evidence suggests that rapid deprotonation is followed by a slower conformational change (Davis et al., 1974). Both the temperature and pH-induced conformational changes are reversible and thought to be due to the identical protein chain rearrangement with the ionization of a single proton (Greenwood and Wilson, 1971; Davis et al., 1974).

The alkaline isomerisation has received a great deal of attention in the literature: it is thought to play a regulatory role in vivo, due to the effect of the conformational change on the oxidation-reduction potential (Davis et al., 1974; Greenwood and Palmer, 1965). The less polar mitochondrial environment would reduce the pK_a for the alkaline isomerisation below 9, and thus decrease the concentration of physiologically active cytochrome c (i.e. with the Fe-S bond intact). The oxidation-reduction potential would be expected to be sensitive to pH changes in the mitochondria; and it has been suggested (Davis et al., 1974) that the chemical behaviour of cytochrome c could be modelled in alkaline aqueous solution. Notably, neither mitochondrial cytochrome c nor cytochrome c above pH 9 will bind cyanide (George and Tsou, 1952). The work of Osheroff et al., (1980) suggests that pK_a values associated with loss of the 695 nm bond for cytochrome c from various species of mammal reflect the stability of the haem crevice. The haem crevice in turn plays a crucial role in the control of the cytochrome c - cytochrome c oxidase reaction, via an

open-closed transition. Cytochromes c with similar kinetics on reacting with a particular oxidase tend to display similar pK_a values.

The pK_a value for the alkaline isomerisation of cytochrome c is affected by various reagents. Denaturants such as dioxane, formamide, ethane 1,2-diol, 1-propanol, methanol and urea cause loss of the 695 nm absorption band and are effective in the order of their hydrophobicity. The hydrophobic haem crevice environment becomes progressively unstable in the presence of these denaturants (Kaminsky et al., 1973; Greenwood and Wilson, 1971). 2.5 M ethanol has been shown to lower the pK_a of horse-heart cytochrome c by 1.1 pH units to pH 7.95 (Ilan and Shafferman, 1978). Denaturants and pH were found to act co-operatively in bleaching the 695 nm band (Grenwood and Wilson, 1971); it is likely that the ionization of a single proton is followed by disruption of the Fe-S bond due to denaturants. Kaminsky et al. (1973) examined the thermal titration of the 695 nm band in the presence of denaturants and attributed the pronounced effect of pH on the half-lives for the temperature transition, to the involvement of protons in the equilibrium reaction.

Anions such as phosphate, citrate, malate and nucleotide triphosphates and diphosphates appear to stabilise the closed form of the haem crevice at low concentrations by binding near the haem crevice (Osheroff et al., 1980; Borden et al., 1978). Anions such as chloride, acetate, borate and cacodylate do not interact with this site, though they are known to bind to cyctochrome c (e.g. K_{app} (chloride) is ca. 50 - 70 μM^{-1} (Nicolls, 1974)). At low concentrations, bromide (Kaminsky et al., 1973) and chloride (Greenwood and Wilson, 1971) appear to decrease the pK_a. At high concentrations (0.5 M) the increase in pK_a has been attributed to a general ionic strength effect rather than specific binding (Osheroff et al., 1980). According to Nicholls (1974) no spectroscopic effects of this binding have been seen. However disruption of the haem crevice by non-haem binding anions does cause a decrease in the 695 nm band (ca. 10 per cent decrease at 1.0 M sodium chloride (Greenwood and Wilson, 1971)). In contrast, iron binding ligands, cyanide, azide and imidazole (e.g. Stellwagen 1966; 1968; Gupta and Redfield, 1970) both abolish the 695 nm band

and show spectroscopic changes in the region of the 530 nm maximum [see Section 5.6]. The effect of the perchlorate anion at pH 7.6 was interpreted by Serre et al. (1981) as favouring the substitution of methionine 80 by the unknown strong field ligand: the pK_a for the transition was lowered to 7.8 in 0.1 M perchlorate. This effect was confirmed by Andersson et al. (1980) and is further discussed in Section 5.5.

The preceding discussion has stressed the importance of the iron-methionine bond in the maintenance of haem crevice stability, and the important biological role of the open-closed transition of the crevice. An understanding of the factors influencing haem crevice stability is a prerequisite for the elucidation of the in vivo functioning of cytochrome c. While the effects of pH, temperature, denaturants and some anions have been well studied, the binding of drugs and other molecules appear to have received scant attention in the literature. In this work, the binding of perchlorate anion to cytochrome c, and donor-acceptor interactions between caffeine and tryptophol, and cytochrome c, in alkaline solution at various pH values, are examined quantitatively. A qualitative study of donor-acceptor interactions between barbital, chlorpromazine, procaine and salicylic acid, and cytochrome c, and the effect of ephedrine, is presented.

5.2 Experimental procedure

Horse-heart cytochrome c (Sigma Type VII) was used without further purification. Tris-hydrochloric acid buffers in the pH range 8.6 - 9.5 were prepared according to Long (1971). Cytochrome c solutions were ca. 1×10^{-4} M and were stored at -20°C. Other reagents used in this chapter are listed in Section 2.1.

Titrations of the 695 nm absorbance band of cytochrome c were conducted by adding weighed quantities of solid ligand to the cytochrome c solution in the cuvette, and mixing thoroughly; visible spectra (750 - 600 nm) were recorded on a Cary 2300 UV-Vis-NIR spectrophotometer. The cell compartment of the spectrophotometer was thermostatted at 25.0 ± 0.2°C. The very low extinction coefficient of the 695 nm absorbance band (ε_{695} ca. 880 $mM^{-1}cm^{-1}$ (Lemberg and Barrett, 1973)) necessitated the use of absorbance range: 0 - 0.1.

The method of Kaminsky et al., (1973) was used to correct for the contribution of background absorbance to the 695 nm band (see Figure 5.2). Whereas A_{695} is the actual absorbance measurement at 695 nm, A_{695} (corr.) represents the absorbance at 695 nm corrected for the contribution of background absorbance by extrapolation of the longer wavelength absorbance bands.

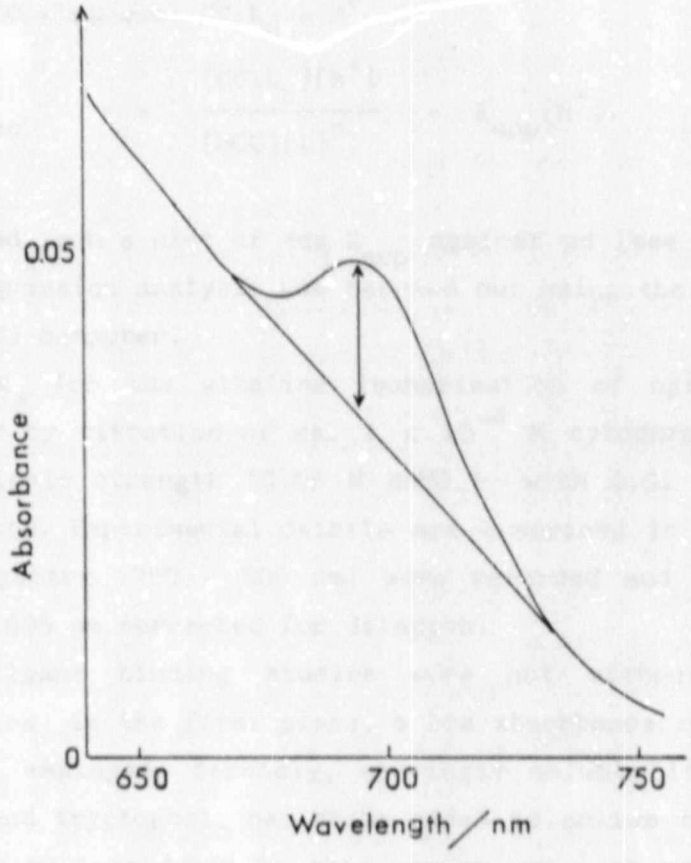

Fig. 5.2 The correction method of Kaminsky et al. (1973) for background absorbance.

The equilibrium constant, K_{app}, for the binding of n ligands, L, to cytochrome c (CC) e.g.,

$$CC + nL \rightleftharpoons CC.L_n$$

where $$K_{app} = \frac{[CC.L_n]}{[CC][L]^n} ,$$

was derived from a plot of $\log|A_o - A|/A$ against $\log[\text{ligand}]$ [see full derivation in Appendix (b)]. The correction method for absorbance employed in this study, obviates the need for an A_∞

value for the titrations (Osheroff et al., 1980).A plot of $1/|A_o-A|$ against $1/[L]$ [see Appendix (b)] for corrected absorbance values, verified that A_∞ approximated to zero. The equilibrium constant, K_{eq}, for the coupled reaction of deprotonation and ligand displacement of methionine:

$$HCC + nL \rightleftharpoons CC.L_n + H^+$$

and $$K_{eq} = \frac{[CC.L_n][H^+]}{[HCC][L]^n} = K_{app}[H^+],$$

was derived from a plot of $\log K_{app}$ against pH [see Appendix (b)]. Linear regression analysis was carried out using the SAS package on an IBM 3083 computer.

The pK_a for the alkaline isomerisation of cytochrome c was determined by titration of ca. 1×10^{-4} M cytochrome c, held at constant ionic strength (0.05 M $NaNO_3$), with 0.01 M NaOH from a microburette. Experimental details are described in Section 2.2.6. Visible spectra (750 - 500 nm) were recorded and the absorbance values at 695 nm corrected for dilution.

The ligand binding studies were not without experimental difficulties. In the first place, a low absorbance range, 0 - 0.1, had to be employed. Secondly, sparingly soluble ligands such as caffeine and tryptophol, had to be added as solids to the cuvette. Any turbidity introduced in this manner was much magnified in the sensitive absorbance range used; the Kaminsky method of absorbance correction thus proved invaluable. Finally, a small error was introduced in the estimation of the lower line (see Figure 5.2) using a flexi-curve. The error is of the order of 0.4×10^{-3} absorbance units, and is represented on the plots by error bars. The error is exaggerated at lower pH values (pH 8.5) where the 695 nm absorbance is at a maximum.

5.3 The use of unpurified commercial cytochrome c without further oxidation

The literature indicates that where commercially prepared cytochrome c is used, it may (Smith and Millett, 1980; Greenwood and Wilson, 1971) or may not (Serre et al., 1981; Kaminsky et al., 1973; Ilan and Shafferman, 1978) have been further purified before