iron-sulphur bond has been shown by NMR and spectroscopic measurements to be sensitive to pH, temperature, the presence of alcohols, anions and cations, and chemical modification of nearby residues.

The NMR resonances of the methionine ligand have been studied by various authors (e.g. Moore et al., 1982). Characteristic methyl signals are present only when the ferric methionine-iron bond is intact. Large perturbations to chemical shifts are seen due to delocalisation of the unpaired electron of the low-spin ferric iron. Optical studies have concentrated on the charge transfer absorption band in the near infra-red, at 695 nm. This bond is observed only when the ferric iron-sulphur bond is intact (it is a highly sensitive indicator of the integrity of this bond) and is weak ($\epsilon_{695}$ ca. 810 $mM^{-1}cm^{-1}$). The 695 nm bond is polarized perpendicular to the haem plane; and is probably due to charge-transfer of one electron from a non-porphyrin ligand, such as the $-\ddot{\underset{\cdot\cdot}{S}}-$ of methionine, to a d-orbital on the iron (Eaton and Hochstrasser, 1966).

The charge transfer absorption bond is affected by a variety of conditions:

(i) increasing temperature causes a diminution of the bond (no mention is made of a peak maximum shift) but the magnetic susceptibility remains that of a low-spin ferric iron, and the methionine remains bound to the iron (Ångstrom et al., 1982). NMR spectra confirm that the bond is intact between 15 and 60 °C and that there is a continuous change in the iron-sulphur interaction. Kaminsky et al. (1973) obtained biphasic Arrhenius plots in the presence of denaturants. Transition temperatures for a variety of species of cytochrome c, reflecting stability of the haem crevice, have been determined (Osheroff et al., 1980).

(ii) Theorell and Åkesson (1941) first defined five spectroscopic species of ferric cytochrome c within the pH range 0 - 14, which changed reversibly into one another by altering the pH e.g. for horse-heart cytochrome c:

$$\text{TYPE I} \xrightleftharpoons{\text{pK } 0.75} \text{TYPE II} \xrightleftharpoons{\text{pK } 3.8} \text{TYPE III} \xrightleftharpoons{\text{pK } 9.05} \text{TYPE IV} \xrightleftharpoons{\text{pK } 12} \text{TYPE V.}$$

The transition between types III and IV (the 'alkaline isomerisation') occurs with a pK of 8.8 - 9.3 (depending on the cytochrome c species and the ionic strength). The transition is accompanied by loss of the 695 nm absorption band due to replacement of methionine by a strong field ligand which may be lysine 72, lysine 79 or even water (Bosshard, 1981; Takano and Dickerson, 1981a; 1981b), and results in a more open haem crevice.

(iii) Formylation of tryptophan 59 of cytochrome c causes complete loss of the 695 nm absorption band, which is reversed by alkali treatment (Aviram and Schejter, 1971). Modification of tyrosine 67 by mutation also affects the band (Schejter et al., 1970).

(iv) Denaturation of the crevice and a loss of the 695 nm band is effected by alcohols and other denaturants in order of their hydrophobicity (Greenwood and Wilson, 1971); pH was found to have a cooperative effect with alcohols in the disruption of the crevice structure (Greenwood and Wilson, 1971). Alcohols were found to decrease the $pK_a$ of the alkaline isomerisation (Ilan and Shafferman, 1978).

(v) Anions such as phosphate and citrate appear to bind near the haem crevice of cytochrome c, stabilising the closed form of the crevice at low concentrations (Osheroff et al., 1980). Other anions such as chloride and cacodylate do not interact with this site, but bind to cytochrome c with similar affinity ($K_{app}$ is ca. 70 μM (Nicholls, 1974)) but do not stabilise the crevice at low concentrations. A small loss in the 695 nm bond is observed in the presence of chloride ions and the $pK_a$ is decreased by 0.3 at 0.3 M chloride (Greenwood and Wilson, 1971).

The integrity of the iron-methionine bond is vital to the in vivo functioning of cytochrome c. Where the bond is broken (e.g. in the alkaline isomerisation) cytochrome c is unable to accept electrons from the reductase molecule. The alkaline isomerisation is thought to have a regulatory role in vivo (where the $pK_a$ is expected to be lower than its value in aqueous solution) due to the effect of the conformational change on the oxidation-reduction potential (which falls from 260 to 120 mV) (Davis et al., 1974). When the iron - methionine bond is intact, the closed structure of the haem crevice is present; the $pK_a$ for the alkaline isomerisation reflects the stability of the crevice. Osheroff et al. (1980) found a wide variation for $pK_a$ values in various mammals (8.3 to 9.5) which, when correlated with amino acid sequences, suggested the presence of upper and lower haem crevice bonds (see Chapter 5).

### 1.3.2 Microperoxidase-8

#### (a) History and Structure

The first studies on the digestion of cytochrome c with proteolytic enzymes pepsin, trypsin, chromotrypsin and papain, were carried out by Tsou (1949; 1951a; 1951b). Since then, much attention has been directed at the isolation of peptide fragments of varying sizes containing the covalently linked haem c (see Chapter 6).

One such haem-peptide produced by the consecutive digestion of cytochrome c with pepsin and trypsin enzymes is microperoxidase-8 (MP-8), shown in Figure 1.9. The peptide chain comprises residues 14 to 21 of cytochrome c and is linked to a high spin haem c by thioether linkages from cysteines 14 and 17. Analogous to cytochrome c, histidine 18 is the fifth ligand to the haem iron. The sixth haem ligand is most likely water (Aron et al., 1986). Unlike cytochrome c, MP-8 (and other haem-peptides) are capable of peroxidatic activity (e.g. Baldwin et al., 1985b) a property reflected in the name 'microperoxidase'.

A comparison of the reactivity and other properties of a series of haem-peptides with cytochrome c, is expected to clarify the effect of the protein on the active site. However the chemistry of the haem-peptides is not necessarily a simplified version of related haemoproteins (Tuppy and Paleus, 1955). The peptides lack cytochrome c activity in the succinoxidase system, but can act as peroxidases, and are active oxidases of cytochrome c (Babu et al., 1969; Tu et al., 1968). They are autoxidisable and combine with carbon monoxide. In contrast to cytochrome c they contain high spin iron (III) but react with histidine to give low-spin complexes (Ehrenberg

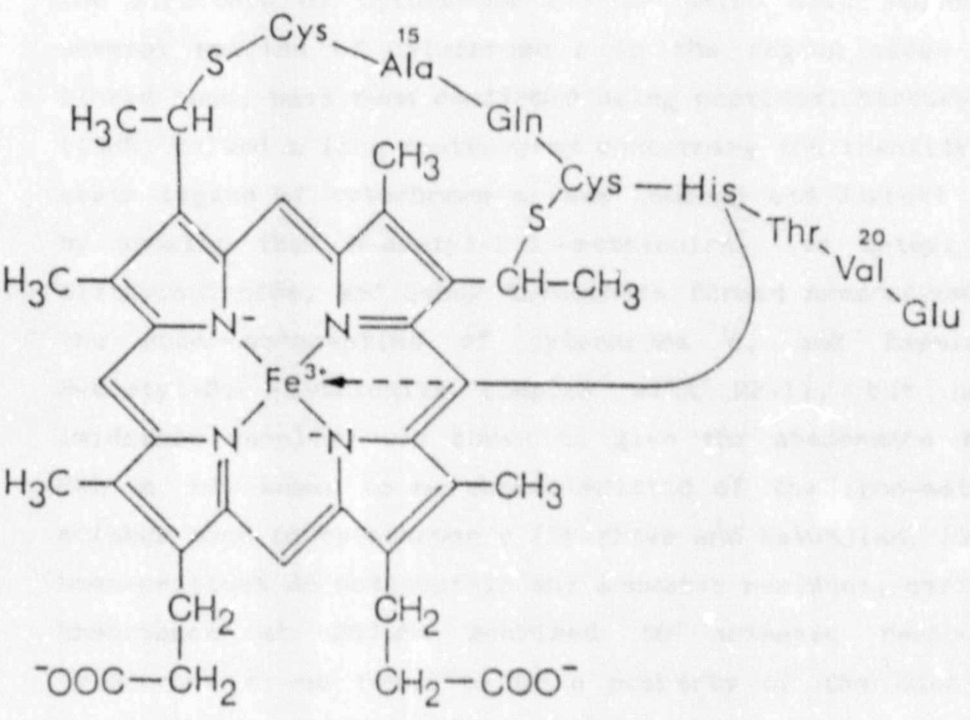

Fig 1.9 The structure of microperoxidase-8.

and Theorell, 1955b). In comparison to a redox potential of 260 ± 20 mV for cytochrome c, the redox potential of MP-8 is ca. -207 mV, and that of the undecapeptide, microperoxidase-11 (MP-11), is ca. -195 mV (Wilgus et al., 1978; Harbury and Loach, 1959).

Haem-peptide complexes have provided valuable insight into the structure of cytochrome c. The amino acid sequences of several species of cytochrome c in the region close to the linked haem, have been confirmed using peptides. Harbury et al. (1965) solved a long controversy concerning the identity of the sixth ligand of cytochrome c (see Lemberg and Barrett (1973)) by showing that N-acetyl-D,L-methionine, its methyl ester, diethylsulphide, and other thioethers formed haemochromes with the haem-octapeptide of cytochrome c, and haemin. The N-acetyl-D,L-methionine complex with MP-11, but not the imidazole complex, was shown to give the absorbance band at 695 nm, now known to be characteristic of the iron-methionine sulphur bond in cytochrome c (Shechter and Saludjian, 1967). As haem-peptides do not contain any aromatic residues, part of the absorbance at 280 nm ascribed to aromatic residues in cytochrome c was found to be a property of the haem itself (Urry, 1967a; 1967b). In addition, part of the absorbance between 240 and 250 nm, ascribed previously to the α-helix, was due to haem (Urry, 1967a; 1967b); thus the α-helix percentage in cytochrome c was shown to have been overestimated (Urry, 1967a). The extent of haem exposure to solvent in cytochrome c (small) was assessed by comparison to the chromotryptic hexadecapeptide (Stellwagen, 1967).

Aside from the structural clarification afforded to cytochrome c by haem-peptides, they have considerable potential as models for the haemoproteins.

(b) Models for haemoproteins

The complexity and size of haemoproteins and enzymes argues for the development of good model systems, of well-defined structure and state, to lend insight into their function and properties in solution. Until recently, models for haemoproteins have comprised haemin (e.g. Adams et al., 1979;

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Baldwin et al., 1985b) and synthetic analogues of varying complexity (e.g. Budd et al., 1979; Spiro and Burke, 1976).

MP-8 has some advantages over these models in aqueous or aqueous-methanol solution. The haem-peptide is water soluble over almost the entire pH range; it may be studied as a monomer in aqueous solution below 2 x $10^{-7}$ M and in 20% methanol:water (v/v) below 2 x $10^{-6}$ M (Aron et al., 1986). The coordination of histidine 18 of the peptide chain as fifth axial ligand, and of an easily removed ligand (probably water in aqueous solution (Aron et al., 1986)) as sixth ligand, facilitates the modelling of mono-imidazole haemoproteins (e.g. myoglobin, horse-radish peroxidase and cytochrome c).

In contrast, haemin dissolves only in alkaline solution where it exists as a dimer (Shack and Clark, 1947). Monomeric haemin in detergent micelles (Simplicio and Schwenzer, 1973; Hambright and Chock, 1975) and a monomeric haemin-caffeine adduct (Campbell, 1980) have been reported; but clearly an artificiality is introduced in these systems, which would affect ligand binding and other reactions (Simplicio, 1972). Both haemin and synthetic models present difficulties in the modelling of mono-imidazole haemoproteins, as the low-spin bis-imidazole adduct is greatly favoured over the mono-imidazole product (Walker et al., 1976). However, complex synthetic work has resulted in mono-imidazole porphyrin models by means of steric interaction (e.g. the picket fence porphyrin of Jameson et al. (1978)).

MP-8 has been used as a model for the cytochromes (e.g. Yang and Sauer, 1982; Kimura et al., 1981; Harbury and Loach, 1960a; 1960b), the activation of hydrogen peroxide by the peroxidases (Baldwin et al., 1985b) and anion binding in haemoproteins (e.g. Blumenthal and Kassner 1979; 1980). The reactions of cytochrome P-450 have up till now been modelled using haemin (e.g. Adams et al., 1979; Adams and Adams, 1986) and synthetic thiolate-based iron porphyrin complexes (see Coon and White (1979)). Although model studies and EXAFS evidence (Cramer et al., 1978) strongly suggest that the fifth ligand to haem is sulphur, the identity of the sixth ligand remains controversial: evidence for water (Philson et al., 1979) and

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## 1.4 The aims of this dissertation

The aim of this work was to investigate the rôle of conformational change in the protein's control of the active site in haemoproteins and enzymes, by studying:

(i) cobalt corrinoid cofactors for the $B_{12}$-dependent isomerase reactions,
(ii) the reactivity of the iron-methionine bond in cytochrome c, and
(iii) the usefulness of MP-8 as a model for cytochrome c.

This was done by:

1. using methyl, ethyl and cyclobutylcobalamins and their corresponding cobinamides (prepared in this study) and adenosylcobalamin, in kinetic studies, both to support a proposed mechanism for isomerase enzymes (Pratt, 1984), and to estimate the half-life of decomposition for the isomerase cofactor, adenosylcobalamin, at room temperature, and hence the labilisation energy necessary for the protein to activate the metal complex (Chapters 3 and 4);
2. confirming the literature $pK_a$ value for the alkaline isomerisation of cytochrome c, and investigating the reactivity of the haem iron-methionine bond in the region of this $pK_a$ through binding studies of the haem iron with various drugs and anions (Chapter 5);
3. developing an improved procedure for the preparation and purification of MP-8, and a rapid test of purity (Chapter 6);
4. using prepared MP-8 in its monomeric form in aqueous-methanol solution to study the binding of D,L-methionine and N-acetyl-D,L-methionine to ferric MP-8, and to bind drugs for direct comparison with cytochrome c (Chapter 7).

# CHAPTER 2 - MATERIALS AND METHODS

## 2.1 **Materials**

### 2.1.1 Cobalt corrinoids

(a) Cobalamins and cobinamides

Samples of vitamin $B_{12}$ and $B_{12a}$ were supplied by Mr. A.P. Domleo of Glaxo-Allenbury (Pty) Limited. Adenosylcobalamin was purchased from Sigma. The organocobalamins and organocobinamides used in this work were prepared as described in Chapter 3.

(b) Alkyl halides

Methyl iodide (Merck, 99%), ethyl bromide (Hopkin and Williams, GPR) and cyclobutyl bromide (Fluka, purum, 98%) were used as received.

(c) Buffers

Phosphate buffers pH 6, 7 and 8 with ionic strength: $\mu = 0.1$, were prepared according to Long (1971), using sodium dihydrogen phosphate (Merck) and disodium hydrogen phosphate (Merck).

(d) Other reagents

The materials used in the preparative work were: ceric nitrate (Schuchardt), phenol (BDH, AnalaR), potassium cyanide (BDH), sodium hydroxide (Sigma), 1,1,1-trichloroethane (BDH, AnalaR), diethylether (Hopkin and Williams), perchloric acid (Merck), sec-butanol (BDH, AnalaR), sodium borohydride (Merck, GR), DE52 and CM32 cellulose (Whatman) and glacial acetic acid (Merck, GR).

### 2.1.2 Cytochrome c studies

(a) Cytochrome c

Horse-heart cytochrome c (Sigma, Type VII) was used without further purification.

(b) Buffers

Tris-hydrochloric acid buffers in the range pH 8.6 to 9.5 with ionic strength: $\mu = 0.1$, were prepared according to Long (1971) using tris (BDH, AnalaR) and hydrochloric acid (Merck).

(c) Other reagents

The following reagents were used without further purification: caffeine (Merck), tryptophol (Sigma), sodium barbitone (BDH), sodium perchlorate (Merck, GR), ephedrine (S.A. Druggists Ltd), salicylic acid (Heyden Chemical Corporation) and benzoic acid (BDH, AnalaR). Chlorpromazine was donated by Maybaker (SA) (Pty) Limited.

### 2.1.3 Microperoxidase-8 studies

(a) Porphyrins

Microperoxidase-8, microperoxidase-11 and horse-heart cytochrome c (Type VII) were obtained from Sigma.

(b) Buffers

In the preparative work 0.1 M ammonium bicarbonate (BDH, AnalaR), pH 8.0, was used. For equilibrium studies, phosphate buffers in the range pH 6.0 - 7.4, with ionic strength: $\mu = 0.1$ were prepared according to Long (1971), using potassium dihydrogen phosphate (Merck) and dipotassium hydrogen phosphate (Merck).

(c) Other reagents

Materials used in the preparative work were hydrochloric acid (Merck), Biogel P6 (200-400 mesh) (Biorad), Sephadex G-50 Superfine (Pharmacia), sec-butanol (BDH, AnalaR), potassium cyanide (BDH), bovine trypsin (Sigma, Type III) and twice recrystallized porcine pepsin (Sigma). Materials used in the equilibrium studies were methanol (Merck, GR), phosphoric acid (Merck, GR) D,L-methionine (Hopkin and Williams), N-acetyl-D,L-methionine (Sigma) and sodium dithionite (Hopkin and Williams); tryptophol, caffeine, sodium barbital, sodium perchlorate and chlorpromazine, were used as described above.

In all cases, the water used was twice distilled in a Büchi Fontavapor 285 still and further purified by a Millipore-Q system (18 MΩcm).

## 2.2 **Methods**

The theoretical principles underlying the application of various methods are elucidated in this section. The context in which they are used in this study is made apparent in later chapters. These methods have for the most part been used previously, but have been adapted to suit the requirements of this work.

### 2.2.1 Phenol-chloroform extraction

Cobalt corrinoids are endowed with a large number of amide side chains which can participate in hydrogen bonding with phenol. Corrinoids are therefore soluble in mixtures of phenol and chloroform and 'phenol-chloroform' extraction can be used to purify them (Friedrich et al., 1954b). In this work, the non-carcinogenic solvent 1,1,1-trichloroethane was substituted for chloroform. The purification process serves to remove both inorganic and organic impurities. An aqueous solution of the corrinoid is extracted with portions of phenol/1,1,1-trichloroethane in the ratio 1:2 (weight:volume). The corrinoid dissolves in the phenol/1,1,1-trichloroethane phase, while inorganic impurities remain in the aqueous phase. The phase containing the corrinoid is then washed twice with water and the corrinoid displaced back into water by the addition of two volumes of ether. Organic impurities remain in the organic phase. To remove traces of phenol and 1,1,1-trichloroethane, the aqueous extract is washed twice with ether.

### 2.2.2 Column chromatography

(a) Cobalt corrinoid studies

In the purification of organocorrinoids, two ion-exchange celluloses were utilised to separate mixtures of organocobalamins or organocobinamides, on the basis of charge.

Carboxymethylcellulose (CM) contains negatively charged $R-O-CH_2COO^-$ (R = cellulose) groups and acts as a weakly acidic cation exchanger. Diethylaminoethylcellulose (DE) is a weakly basic anion exchanger containing positively charged groups: $R-O-CH_2CH_2-\overset{+}{N}HEt_2$. The grades of ion-exchangers used were microgranular forms CM32 and DE32 (Whatman). For part of the work, DE52 and CM52 (Whatman) preswollen microgranular celluloses were employed.

Precycling of DE32 and CM32 and the methods of equilibration and degassing of ion-exchange celluloses, outlined in the Whatman information leaflet, were followed. The activation of CM and DE ion-exchange celluloses is necessary as they are supplied in the hydrogen and free base forms, respectively. Once activated, the ion exchange cellulose was dispersed in water to yield a slurry of consistency 30 $m\ell g^{-1}$ of dry cellulose. The slurry was stirred and allowed to settle in a measuring cylinder, in an environment free from draughts, for thirty minutes. The 'fines', which slow the flow rate of the column, were removed by decanting the supernatant solution. Water was then added to the cellulose (20% of the wet settled volume of the cellulose). Columns were packed by carefully pouring the stirred, degassed slurry down a glass rod into a vertical, glass column, plugged at its base with glass wool. The effluent was allowed to run to waste. When all the slurry had been added, a reservoir was attached to the top of the column and water allowed to run through the column, under the influence of gravity, until the bed height was constant. The dimensions of the columns used varied with the scale of the preparation. For a large scale preparation, a 2.5 x 30 cm column was used for DE cellulose and approximately a quarter of this length of column for CM cellulose. Concentrated solutions were applied to the top of the column using a Pasteur pipette, taking care not to disturb the surface of the column.

Separation of corrinoids is very efficient but the flow rate of eluant is slow. The separation depends both on the overall charge of the molecules and specific charges at local sites:

(1) $B_{12a}$, which on elution by water has a positive charge, is retained by CM cellulose but passes through DE cellulose;

(2) Organocobalamins, uncharged when water is the eluant, are unretarded by DE cellulose, but selectively retained by CM cellulose. These organocobalamins are eluted at a rate qualitatively dependent on their $pK_a$: those with high $pK_a$'s are eluted more slowly (Firth, 1967).

(3) Organocobinamides, aquocyanocobinamide and diaquocobinamide (overall charge +1) all pass through DE cellulose on elution with water, but are retained to varying degrees on CM cellulose. Dicyanocobinamide is retained by DE cellulose.

(4) Hydrolysis products of organocobinamides, usually possessing zero overall charge, are retained by DE cellulose, presumably due to specific interactions with the carboxylate groups (Thorpe, 1967).

### (b) Porphyrin studies

#### (i) The preparation of microperoxidase-8 (MP-8)

Gel permeation chromatography comprised three essential steps in the preparation of MP-8 from cytochrome c (by peptic and tryptic digestion) and its subsequent purification. Two different gels were utilised: Biogel P6 (200-400 mesh), a gel consisting of polyacrylonitrile beads with exclusion limit $M_r \sim 6\ 000$, was used in the preparative steps; Sephadex G-50 Superfine, a bead formed cross-linked dextran gel fractionating in the range $M_r \sim 1\ 500 - 30\ 000$ with extremely high resolution, in the purification step. Molecules are eluted through these gels in order of decreasing molecular size. Molecules larger than the largest pores in the swollen gel beads (i.e. above the exclusion limit) are not able to enter the gel and are eluted first; the passage of smaller molecules through the gel is retarded to varying degrees, depending on their size and shape.

Both gel-types were swollen for one hour on a boiling water bath, prior to packing: Biogel P6 in ammonium bicarbonate buffer; Sephadex G-50 in deionized water.

After standing for one hour, the 'fines' and excess eluant were removed from the gel mixture to form a fairly thick slurry, which was degassed under vacuum.

The column sizes employed were 4 x 100 cm for the Biogel separation, and 1.5 x 30 cm for the Sephadex column. The vertically mounted column was filled to 15% of its total volume with buffer, and the slurry poured carefully into the column down a glass rod. When all the slurry had been added and buffer used to fill the column completely, the top extension was fastened to the column and the flow begun.

A constant flow was maintained using the Pharmacia Peristaltic Pump P-1, a one-channel laboratory pump, with a range of flow rates from 0.6 to 500 $m\ell cm^{-2}hr^{-1}$. The flow rate used for packing the column was also used to elute the product. It was calculated using Darcy's Law where the resin beads are assumed to be rigid spheres:

$$U = k\Delta p\ell^{-1},$$

where U is the linear flow rate ($m\ell\ cm^{-2}hr^{-1}$);

$\Delta p$, the pressure drop over the gel bed, expressed in cm water;

$\ell$, the bed height in cm; and

k, a constant of proportionality dependent on the properties of the bed material and eluant.

Employing the assumption that the viscosity of the eluant is 1 cp, the expression is simplified to: $U = k_o\Delta p\ell^{-1}$, where $k_o$ is the specific permeability. For Sephadex G-50 Superfine, $k_o$ is 13.5; for Biogel P6, $k_o$ is about 25 (Sephadex pamphlet, 1972): the flow rates employed were 4 $m\ell cm^{-2}hr^{-1}$ and 20 $m\ell cm^{-2}hr^{-1}$, respectively. The peristalic pump was calibrated against each column and gel to obtain the correct setting for the appropriate flow rate. Two to three column volumes of eluant were passed through the column to stabilise and equilibrate the gel bed.

Great care was required in sample application, for a sharp zone of sample is necessary where proteins of similar molecular mass are to be separated. The eluant above the gel bed was allowed to drain away and the sample carefully layered on top of the bed. After the sample had drained into the bed, the gel surface and column wall in contact with the sample were washed with a small amount of eluant.

A completely uncharged substance can be satisfactorily eluted with distilled water. As MP-8 carries a positive charge, an eluant with a small ionic strength would be expected to give improved results. As the final product of both preparative and purification steps in this study was to be lyophilised, a volatile buffer was chosen in each case to avoid lengthy lyophilisation times and desalting procedures. For Biogel P6, ammonium bicarbonate buffer, pH 8, was used; in the case of Sephadex G-50 Superfine, the sample was dissolved in ammonia and eluted with deionized water.

Fractions were collected using an ISCO Model 1850 fraction collector.

Details of the separation can be found in Chapter 6.

(ii) The purification of cytochrome c

Cytochrome c was purified by eluting a sample dissolved in deionized water through a Sephadex G-50 Superfine column (1.5 x 30 cm), equilibrated with 0.5 M ammonium bicarbonate buffer, at a flow rate of 4 m$\ell$cm$^{-2}$hr$^{-1}$.

### 2.2.3 Thin layer chromatography (TLC)

(a) Cobalt corrinoid studies

TLC was used to identify products and assess the purity of prepared products. Corrinoid are intensely coloured and can be detected visually. Where yellow spots are present e.g. organocorrinoids, not easily detectable on white plates, photolysis yields red $B_{12a}$ or diaquocobinamide. TLC is sensitive to changes in the organoligand of organocorrinoids, making it a useful technique for identifying particular organocorrinoids. The technique is very sensitive: impurities of the order of 1 per cent of the total sample can be detected.

Cellulose coated TLC plates of 0.5 mm layer thickness (Merck, cellulose precoated, 20 x 20 cm) were used in solvent systems based on *sec*-butanol/water. A glass cutter was used to give fragments 5 x 20 cm. The four solvent systems employed were:

| | | |
|---|---|---|
| I | *sec*-butanol/water | [95:40] |
| II | *sec*-butanol/water/glacial acetic acid | [100:50:1] |
| III | *sec*-butanol/water/0.88 M ammonia | [95:40:6.75] |
| IV | *sec*-butanol/water/0.88 M ammonia/1M KCN | [250:100:0.4:0.3] |

Concentrated aqueous solutions of the corrinoid sample were applied to the TLC plate using a capillary glass tube drawn to a point, and the spots dried using an electric drier.

The plates were developed for four hours in glass tanks (approximately 25 x 25 x 10 cm in dimension) lined with filter paper and containing about 150 mℓ of the appropriate solvent. The sealed tanks were allowed to stand for at least one hour prior to the chromatography to saturate the vapour in the tank with solvent vapour.

$R_f$ values (distance travelled by the spot divided by distance travelled by the solvent front) for TLC in cellulose are sensitive to small changes in experimental conditions such as temperature, solvent composition, cellulose thickness and development time. The $R_f$ value of cyanocobalamin is used as a standard in the TLC of cobalt corrinoids (Firth et al., 1968): $B_{12}$ is spotted next to, or on the same position as the corrinoid spot. It would appear that the ability of the organoligand to form strong hydrogen bonds is a determining factor for the $R_{B12}$ value ($R_f$ value of corrinoid divided by the $R_f$ value of cyanocobalamin): the stronger the hydrogen bonding potential, the higher the resultant $R_{B12}$ value (Chemaly, 1980). The results are quoted as $R_{B12}$ values which are more

Cellulose coated TLC plates of 0.5 mm layer thickness (Merck, cellulose precoated, 20 x 20 cm) were used in solvent systems based on sec-butanol/water. A glass cutter was used to give fragments 5 x 20 cm. The four solvent systems employed were:

| | | |
|---|---|---|
| I | sec-butanol/water | [95:40] |
| II | sec-butanol/water/glacial acetic acid | [100:50:1] |
| III | sec-butanol/water/0.88 M ammonia | [95:40:6.75] |
| IV | sec-butanol/water/0.88 M ammonia/1M KCN | [250:100:0.4:0.3] |

Concentrated aqueous solutions of the corrinoid sample were applied to the TLC plate using a capillary glass tube drawn to a point, and the spots dried using an electric drier.

The plates were developed for four hours in glass tanks (approximately 25 x 25 x 10 cm in dimension) lined with filter paper and containing about 150 mℓ of the appropriate solvent. The sealed tanks were allowed to stand for at least one hour prior to the chromatography to saturate the vapour in the tank with solvent vapour.

$R_f$ values (distance travelled by the spot divided by distance travelled by the solvent front) for TLC in cellulose are sensitive to small changes in experimental conditions such as temperature, solvent composition, cellulose thickness and development time. The $R_f$ value of cyanocobalamin is used as a standard in the TLC of cobalt corrinoids (Firth et al., 1968): $B_{12}$ is spotted next to, or on the same position as the corrinoid spot. It would appear that the ability of the organoligand to form strong hydrogen bonds is a determining factor for the $R_{B12}$ value ($R_f$ value of corrinoid divided by the $R_f$ value of cyanocobalamin): the stronger the hydrogen bonding potential, the higher the resultant $R_{B12}$ value (Chemaly, 1980). The results are quoted as $R_{B12}$ values which are more reproducible than $R_f$ values.

A list of $R_{B12}$ values for some cobalt corrinoids is given in Chapter 3.

### (b) Porphyrin studies

A new TLC solvent system was developed to detect the presence of impurities in MP-8 formed by the digestion of horse-heart cytochrome c. Silica gel plates were used with the following solvent:

88 mℓ water/88 mℓ *sec*-butano[illegible]0.2 mℓ 0.38 M ammonia/0.114 g KCN. The plate was developed for 1.5 hours.

Further details can be found in Chapter 6.

### 2.2.4 Rotary evaporation and freeze-drying

A Büchi Rotavapo RE rotary evaporator connected to an oil vacuum pump was used to remove water (at about 40 °C) during the preparation of aquocyanocobinamide.

The freeze-drying apparatus used was from the Virtris company. The solution to be lyophilised (freeze-dried) was placed in a round-bottomed flask and frozen in stages in liquid nitrogen such that the entire surface of the flask was coated in a homogeneous layer. This serves to expose the maximum surface for sublimation under vacuum, and to reduce considerably the time for lyophilisation. The flasks were insulated, and protected from light in the case of light-sensitive cobalt corrinoids. Non-crystalline, very dry solids were obtained.

### 2.2.5 Photolysis

A tungsten lamp of 200 W was used to photolyse samples placed at a distance of 20 cm, and immersed in cool water. Photolysis was used to determine the end point of slow thermal reactions of organocobalamins and organocobinamides, in some cases (see Chapter 4).

### 2.2.6 The measurement of pH and $pK_a$ values

The pH of solutions was measured using a Metrohm Herisau E532 digital pH-meter. Metrohm series EA 6.0203 combination glass electrodes were calibrated against standard buffers (BDH). The reference electrolyte was 3 M potassium chloride. The pH reading of solutions in spectrophotometer cells was facilitated by the use of a Metrohm glass microelectrode (series EA 6.0203).

The $pK_a$ determination of the alkaline isomerization of cytochrome c is described in Chapter 5. A thermostatted glass cell (25.0 ± 0.2 °C) containing a Radiometer combined electrode (GK 2402 B) was used. Aliquots of sodium hydroxide solution were added to the cell with the aid of a micro-burette. The pH was read off a Radiometer pHM84 digital pH-meter.

### 2.2.7 Ultraviolet-visible spectrophotometry

Ultraviolet-visible absorption spectra were recorded in the range 750 - 280 nm using two spectrophotometers: the Cary 219 UV-Vis and the Cary 2300 UV-Vis-NIR. The stability of the Cary 2300 made it particularly suitable for work in low absorbance ranges, sometimes necessitated in this study. The spectrophotometers were calibrated using Holmium glass as a standard. Spectra were recorded using quartz cells of 1 cm and 10 cm pathlengths. A thermostatted cell holder was employed to maintain the temperature of the sample cell at 25.0 ± 0.2 °C.

### 2.2.8 Determination of the concentration of solutions

#### (a) Corrinoids

Organocorrinoids were converted to the dicyanide form (dicyanocobalamin or dicyanocobinamide) by photolysis after the addition of a small crystal of potassium cyanide. Extinction coefficients were determined with reference to that of dicyanocobalamin ($\epsilon = 3.04 \times 10^4$ for the $\gamma$-band at 368 nm) (Hill et al., 1964): the same value is used for dicyanocobinamide, positions and relative intensities for peaks above 300 nm being the same in the two compounds (Thorp, 1967).

#### (b) Cytochrome c

The spectrum of horse-heart cytochrome c containing 0.456 per cent iron, in the reduced and oxidised forms, and quoting millimolar extinction coefficients from 600 to 200 nm, has been published by Margoliash and Frohwirt (1959).The concentration of cytochrome c solutions were determined using extinction coefficients at 550 nm ($0.92 \times 10^4$ $cm^{-1}$ $M^{-1}$) and 528 nm ($1.12 \times 10^4$ $cm^{-1}$ $M^{-1}$) for the oxidised species. An alternate value for the extinction coefficient at 550 nm (oxidised species) has been given as $0.85 \times 10^4$ $cm^{-1}$ $M^{-1}$ (Margalit and Schejter, 1970).

The determination of the value of the extinction coefficient at 695 nm for oxidised cytochrome c, is described in Chapter 5.

### (c) Microperoxidase-8

#### (i) Pyridine-haemochrome method

Haemochromes are complexes formed by the coordination of two molecules of base to a ferroporphyrin. Pyridine-haemochromes have sharp and characteristic α-bands which can be used for identification, and the determination of concentration. Pyridine-haemochrome spectra are measured in aqueous alkaline solution after reaction with sodium dithionite. A final concentration of 0.075 M NaOH and 2.1 M pyridine has been recommended; the pyridine to haem ratio should be at least 150 000:1 (Paul et al., 1953).

The extinction coefficients for the haem c pyridine-haemochrome are $29.1 \times 10^3$ $M^{-1}$ $cm^{-1}$ (551 nm) and $18.6 \times 10^3$ $M^{-1}$ $cm^{-1}$ (522 nm) (Falk, 1964).

The method used for concentration determination was as follows: to 1.5 mℓ 0.5 M NaOH and 0.5 mℓ pyridine, 100 uℓ of a haem-containing solution (the absorbance of which was between 0.3 and 0.9 in the wavelength region 450 - 650 nm) was added. The haem was reduced as soon as possible by the addition of small quantities of solid sodium dithionite. The wavelength region 400 - 600 nm was rapidly scanned to monitor the increase of the 550 nm band; excess dithionite causes a decrease of the band due to decomposition of haem. The ratio of the intensity of the 550 nm band to the minimum between the α and β bands is expected to be about

3.5; variation between repeated readings was found to be less than one per cent.

(ii) Iron determination

Iron analysis of MP-8 is expected to yield values 3.51 to 3.59 per cent depending on the formula assumed (Harbury and Loach, 1960a).

The method of iron determination used was derived from Paul (1948). A sample containing 5 to 70 ug iron was boiled in a Pyrex test tube over a free gas flame with 1.0 mℓ sulphuric acid and 3 drops of hydrogen peroxide. The colour disappeared and then reappeared (2-5 minutes). Boiling was continued, and a drop of hydrogen peroxide added every minute until the solution remained colourless. Boiling was continued for a further 10 minutes. The solution was cooled in ice-water, diluted with a few drops of water, and neutralised with ammonia solution. A yellow colour was achieved by the addition of 0.30 mℓ 20 per cent sulphosalicylic acid and ammonia solution. The solution was transferred to a 15 mℓ volumetric flask and made to the mark with water. The absorbance at 424 nm was measured: 1.00 μg iron per mℓ in a 1 cm cell gives an optical density of 0.103. One determination yielded the value 3.5 per cent.

### 2.2.9 Crystallization

Two methods were used in unsuccessful attempts to crystallize MP-8. The HPLC analysis of MP-8 prepared in this study [see Chapter 6, Section 6] indicated that the MP-8 was impure. The methods used are recorded here as they were deemed particularly suitable for MP-8: it is to be hoped that they would be successful using purer samples.

(a) The 'hot-box technique', using the temperature gradient of solubility, was attempted (Blundell and Johnson, 1976). A solution of MP-8/cyanide adduct, buffered at low ionic strength, was brought close to the isoelectric point of MP-8 (pI ∿ 5.4 (Gerber et al., 1975)). At the first sign of turbidity, the protein solution was heated to about 35 °C, and resulted in a clear solution. A test tube sealed with parafilm containing the heated MP-8 solution was placed in a thermos flask containing water at 35 °C. This flask was sealed and enclosed in a polystyrene container, packed tightly with pieces of polystyrene.

(b) A special cellulose, EDTA treated dialysis tubing (Cole-Palmer, Spectra/Por 7) with a molecular weight cut off point of 1000 was used. To about 1 mg MP-8 was added 1 mℓ acetate buffer, pH 5.2, containing a slight excess of potassium cyanide. Portions of a known mass of ammonium sulphate were added until cloudiness was apparent. Acetate buffer was added drop by drop until the turbidity disappeared. The solution was contained within a piece of dialysis tubing, clamped with plastic tubing enclosures on either end. The tubing was suspended in acetate buffer with the same concentration of potassium cyanide, and containing ammonium sulphate at the concentration that first resulted in turbidity in the sample.

### 2.2.10 Numerical methods

Linear regression analyses were carried out using the SAS package on an IBM 3083 computer.

# CHAPTER 3 - THE PREPARATION AND PURIFICATION OF ORGANOCORRINOIDS

## 3.1 Features of preparation, purification and identification

The thermal decomposition reactions of organocorrinoids, described in Chapter 4, required the synthesis of three organocobalamins and three organocobinamides. This chapter describes the general features of the preparation, purification and identification of organocorrinoids, and methods followed in the preparation of methyl, ethyl and cyclobutylcobalamins and the equivalent cobinamides.

### 3.1.1 Preparation

The preparation of organocorrinoids occurs in two stages: a cobalt(III) corrinoid such as $B_{12}$, $B_{12a}$, diaquocobinamide or aquocyanocobinamide is reduced by sodium borohydride, at room temperature, in neutral or alkaline solution, under nitrogen, to the corresponding Co(I) derivative, via the cobalt(II) species. It is then reacted with an appropriate alkylating agent to yield the product. The rate determining step is the conversion of cobalt(II) to cobalt(I), and may be catalysed by the addition of transition metal ions (Schrauzer, 1968; Chemaly, 1980). Cobalt(I) corrinoids are strong nucleophiles and participate in rapid addition and substitution reactions with a large variety of electrophiles. Commonly used alkylating agents include alkyl halides, alkenes (with electron withdrawing substituents), alkynes, tosylates and epoxides.

In this work, all preparations of organocobalamins and organocorrinoids were carried out starting with $B_{12a}$ or aquocyanocobinamide and alkylating with the appropriate alkylhalide. All preparative work was carried out under subdued red light in a dark room, as organocorrinoids are light sensitive.

### 3.1.2 Purification

The purification of organocorrinoids requires the removal of excess inorganic (e.g. reducing agent and metal salts) and organic (e.g. alkyl halides) reagents, which was accomplished in this work by phenol-chloroform extraction [Section 2.2.1]. Separation of the desired organocorrinoid from the corrinoids present (e.g. unreacted starting material and corrinoids with hydrolysed side chains) was achieved using cellulose ion-exchange chromatography [Section 2.2.2]. The purity of the corrinoid was estimated using cellulose TLC [Section 2.2.3].

### 3.1.3 Identication

The identity of prepared organocorrinoids was confirmed by comparison of electronic spectra to published spectra, and of $R_{B12}$ values to published values (Chemaly, 1980).

The presence of the cobalt-carbon bond was confirmed by the decomposition of organocorrinoids in light and air to yield aquocorrinoids (Pratt, 1972, p.266), in light, air and cyanide to yield cyanocorrinoids (Pratt, 1972, p.266), and, in the case of organocobalamins, the pH-dependent equilibrium between the red 'base-on' form and the yellow 'base-off' form (Pratt, 1972, p.185) was monitored.

## 3.2 **The preparation of organocobalamins**

Methyl, ethyl and cyclobutylcobalamins were prepared according to the method given below (Pratt, 1972, p.222):

### The preparation of ethylcobalamin

$B_{12a}$ (0.5 g, $3.7 \times 10^{-4}$ moles) in 25 mℓ of deionized water was reduced under nitrogen using sodium borohydride (0.6 g, 0.016 moles), giving the greyish-green $B_{12s}$. The flow of nitrogen was terminated and ethyl bromide (0.3 mℓ, $3.7 \times 10^{-3}$ moles) was added. The solution was allowed to stand for an hour. After this time, a red colour, indicative of the formation of ethylcobalamin, was present. Phenol-chloroform extraction [Section 2.2.1] was used to remove unreacted alkylbromide and inorganic ions. The solution was chromatographed on a short (2 x 5 cm) column of CM32 ion-exchange cellulose [Section 2.2.2] using deionized water as the eluant. A small amount of unreacted $B_{12a}$ was retained at the top of the column and the ethylcobalamin was slowly eluted from the column. The eluate was concentrated using the phenol-chloroform procedure and then lyophilised to yield a fluffy red solid. The yield was 70 to 80 per cent.

The spectra of prepared cobalamins corresponded closely with published spectra (Chemaly, 1980). The characteristics of absorption spectra (650 to 300 nm) observed in this study are shown in Table 3.1 for all organocorrinoid complexes studied. The absorption spectrum of 'base-on' ethyl cobalamin may be seen in Chapter 4 (Figure 4.5).

TLC studies showed the cobalamin preparations to be ca. 95 per cent pure; $R_{B12}$ values compared reasonably well with the published values (Chemaly, 1980). A list of $R_{B12}$ values for organocobinamides and organocobalamins observed in this study, are given in Table 3.2. Expected reactions in the presence of light and cyanide were observed and the presence of the pH-dependent equilibrium confirmed. Organocobalamins were stored in the dark.

## 3.3 **The preparation of organocobinamides**

The preparation of organocobinamides from $B_{12}$ proceeds in two phases; first, the cleavage of the 5,6-dimethylbenzimidazole nucleotide, and secondly, the alkylation of the nucleotide free product. In this study, the nucleotide was removed from $B_{12}$ using cerous hydroxide. The products of the cerous hydroxide hydrolysis of $B_{12}$ are aquocyanocobinamide, 5-6-dimethylbenzimidazole nucleoside and phosphate (Friedrich and Bernhauer, 1954a). The aquocyanocobinamide was then reduced to the cobalt(I) corrinoid and alkylated to yield the desired organocobinamide.

### 3.3.1 Aquocyanocobinamide

The preparative procedure followed was than of Thorp (1967), which was based on the procedure developed by Friedrich and Bernhauer (1954a).

Aquocyanocobinamide was prepared using $B_{12}$ (0.6 g $3.6 \times 10^{-4}$ moles), cerous nitrate (50 mℓ of 0.33 M Ce $(NO_3)_3$ $6H_2O$), sodium hydroxide (15 mℓ 2 M NaOH) and potassium cyanide (0.5 g KCN in 7.5 mℓ water).

The preparation proceeded in five stages:

1. The reagents above were added to 100 mℓ of water and heated on a boiling water bath for thirty minutes, with frequent shaking.

The mixture was cooled in ice, adjusted to pH 8.5 by addition of 0.88 M ammonia, and left in a refridgerator overnight to allow the cerous hydroxide to coagulate.

2. The solution was filtered with a sinter-glass funnel and freed of organic and inorganic impurities by phenol-chloroform extraction [Section 2.2.1]. The product at this stage was mainly in the form of the dicyanide. To obtain the aquocyano-form, the solution was adjusted to pH 3 with dilute perchloric acid. Care had to be exercised not to decrease the pH below this value, to minimise hydrolysis of the amide side chains (Thorp, 1967). The solution was protected from the light to avoid photolysis to diaquocobinamide.

3. The removal of hydrogen cyanide from this acidic solution was facilitated by passing nitrogen through the solution; the flow was stopped when the spectrum of a neutralised (red) sample indicated that only the aquocyano-species was present. The solution was neutralised with sodium hydroxide, phenol extracted, and concentrated by rotary evaporation to a small volume. Figure 3.1 gives the spectrum of aquocyanocobinamide in water and of $B_{12}$.

4. For purification, the concentrated aqueous solution was rendered faintly alkaline (pH 8) with sodium hydroxide and chromatographed on columns of DE and CM cellulose, connected in series, with deionized water as the eluant.

   The main band, aquocyanocobinamide, passed through the DE column, but was retained at the top of the CM column. Products of further hydrolysis, and traces of dicyanocobinamide were retained by the DE column, rendering the column faintly purple. Unchanged cyanocobalamin passed through both columns. Diaquocobinamide also passed through both columns as a broad slow moving band. The extent to which diaquocobinamide was present was proportional to the degree of exposure of the solution to light after acidification. The two columns were disconnected as soon as the aquocyanocobinamide was clear of the DE cellulose, and elution

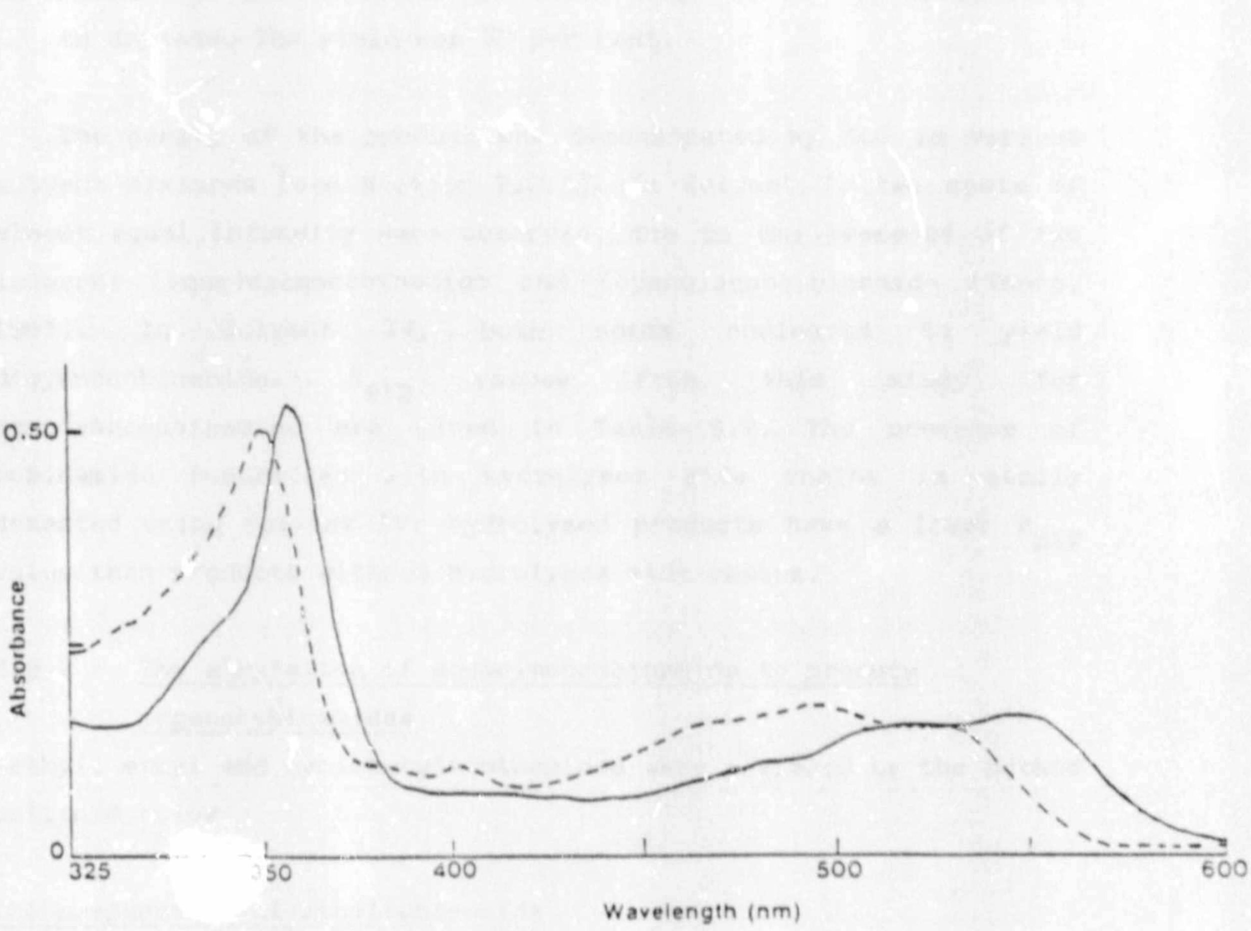


Fig. 3.1 Absorption spectra of $B_{12}$ ( —— ) and aquocyanocobinamide ( --- ) in water (adapted from Chemaly (1980), p. 96).

continued on the CM column. The CM column was eluted until the diquocobinamide band was well separated from the aquocyanocobinamide. The column was then dissected and the product removed from the ion-exchange resin with 0.05 M NaOH (Betterton, 1982). The resultant solution was phenol extracted, reduced to small volume by rotary evaporation and lyophilised to dryness. The yield was 70 per cent.

The purity of the product was demonstrated by TLC in various solvent mixtures [see Section 2.2.3]. In Solvent I, two spots of almost equal intensity were observed, due to the presence of two isomers: (aquo)cyanocobinamide and (cyano)aquocobinamide (Thorp, 1967). In Solvent IV, both spots coalesced to yield dicyanocobinamide. $R_{B12}$ values from this study for aquocyanocobinamide are given in Table 3.2. The presence of cobinamide impurities with hydrolysed side chains is easily detected using Solvent IV: hydrolysed products have a lower $R_{B12}$ value than products without hydrolysed side chains.

### 3.3.2 The alkylation of aquocyanocobinamide to produce organocobinamides

Methyl, ethyl and cyclobutylcobinamides were prepared by the method outlined below:

The preparation of ethylcobinamide

Aquocyanocobinamide (0.05 g, $3 \times 10^{-5}$ moles) in 25 mℓ deionized water was reduced under nitrogen to the cobalt(I) corrinoid. The solution turned purple due to the simultaneous formation of dicyanocobinamide. Ethyl bromide (30 μℓ, $3.7 \times 10^{-4}$ moles) was added to this solution and the nitrogen flow terminated. After one hour, the yellow organocobinamide was completely formed. The reaction mixture was freed of organic and inorganic impurities by phenol-chloroform extraction. The concentrated aqueous solution was lyophilised to yield a yellow solid.

The organocobinamides prepared by the above method had spectra similar to published spectra (Chemaly, 1980) (see Table 3.1). The cobalt-carbon bond was shown to be present in all cases. Yields varied between 20 per cent and 40 per cent. The absorption spectrum of ethylcobinamide may be seen in Chapter 4 (Figure 4.6).

### 3.3.3 The purity of prepared organocobinamides

It has been noted that organocobinamides are much less stable in the solid state than are the corresponding organocobalamins (Chemaly, 1980). For instance, methylcobalamin is stable for ten years without change, while methylcobinamide over a period of weeks to months may show two further organocorrinoid TLC spots, in equal proportion to the original spot. In some cases more than one spot on TLC is apparent immediately after preparation and purification (Chemaly, 1980). As the UV-visible spectra indicate that only organocorrinoid is present, these extra spots can be ascribed to organocorrinoids with hydrolysed amide side chains, rather than resulting from changes in the organoligand or corrin ring. It has been suggested that hydrolysis of side chains occurs as freeze dried corrinoids pick up water from the atmosphere, the hydrolysis being catalysed by the cobalt atom of the organocobinamide complexes (Chemaly, 1980; Kimura et al., 1970).

**TABLE 3.1:** UV-visible absorption spectra for organocorrinoids in this work.

| Corrinoid | Maxima (wavelength in nm)* | | |
|---|---|---|---|
| | α/β region | | γ region |
| adenosylcobalamin | 522 (0.84) | | 374 (1.1) |
| methylcobalamin | 518 (0.87) | | 372 (1.1) |
| ethylcobalamin | 506 (0.91) | | 375 (0.97) |
| cyclobutylcobalamin | 510 (0.87) | | 375 (0.91) |
| methylcobinamide | 460 | | 374 |
| ethylcobinamide | 455 | 440 | 380 |
| cyclobutylcobinamide | | 438 | 378 |

* Extinction coefficients for organocobalamins are included ($10^{-4}$ ε).

In the case of the organocobinamide complexes prepared for the thermal decomposition studies discussed in the next chapter, the purification step using ion-exchange columns was omitted. TLC results after lyophylisation showed no sign of the parent aquocyanocobinamide complex, but did have three yellow spots at the $R_{B12}$ values expected for the organocobinamide and two products of hydrolysis (Chemaly, 1980). Since the half-life (t½) values for the thermal decomposition reactions would not be expected to be significantly affected by hydrolysis of the side chains, it was considered to be justified to use these samples.

**TABLE 3.2**: $R_{B12}$ values of some corrinoid complexes in Solvent I

| Corrinoid | $R_{B12}$ | |
|---|---|---|
| methylcobalamin | 1.4 | |
| ethylcobalamin | 1.5 | |
| cyclobutylcobalamin | 1.6 | |
| aquocyanocobinamide | 0.34 | 0.96 |
| diaquocobinamide | 0.06 | |
| methylcobinamide | 1.4 | |
| ethylcobinamide | 1.5 | |
| cyclobutylcobinamide | 1.6 | |

## 3.4 **Summary**

The organocobalamins, methyl, ethyl, and cyclobutylcobalamin, were prepared by reduction of $B_{12a}$ with $NaBH_4$ under a nitrogen atmosphere and alkylation of the resulting cobalt (I) corrinoid. The corresponding cobinamide complexes were prepared from aquocyanocobinamide in the same manner. Purification was by phenol-chloroform extraction and ion-exchange chromatography.

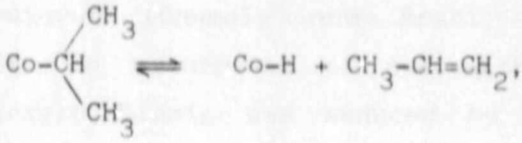
# CHAPTER 4 - HOMOLYTIC THERMOLYSIS OF ETHYL, METHYL AND CYCLOBUTYLCOBALAMINS AND CORRESPONDING COBINAMIDES, AND ADENOSYLCOBALAMIN

## 4.1 Introduction

The rôle of cobalt corrinoids as cofactors in the enzymatic isomerase reactions was discussed in Chapter 1. This chapter examines a proposed mechanism (Chemaly and Pratt, 1980a; 1980b; 1980c) for the labilisation of the cobalt-carbon bond (Co-C) towards homolytic fission, emphasizing the rôle of conformational change in the protein. The decomposition reactions of various organocobalamins and organocobinamides, in addition to the cofactor, adenosylcobalamin, were examined at elevated temperature in aqueous solution. The order of reaction rates confirms earlier studies at lower temperatures (Chemaly and Pratt, 1980b) and lends insight into the decomposition mechanism operative in some secondary alkylcobalamins at elevated temperatures. The final order supports the proposed mechanism of sterically induced Co-C bond homolytic fission. Finally, the results are used to calculate an approximate value for the decomposition rate of the cofactor at room temperature, and thus an estimate of the labilising effect of the protein in the isomerase enzyme.

## 4.2 β-Elimination and homolytic fission

There are two possible mechanisms by which alkylcobalamins can decompose. If there is a hydrogen atom on the β-carbon, β-elimination will be operative, i.e.



$$\text{Co-CH}(\text{CH}_3)_2 \rightleftharpoons \text{Co-H} + \text{CH}_3\text{-CH=CH}_2,$$

giving rise to a cobalt hydride and an alkene. Homolytic fission is the mechanism observed on thermolysis of alkylcobalamins with no hydrogen in the β-carbon position, such as methylcobalamin, i.e.

$$\text{Co-CH}_3 \rightleftharpoons \text{Co(II)} + \text{CH}_3^{\cdot},$$

giving rise to $B_{12r}$ and an alkyl radical. In both decomposition pathways, the transition state is expected to have sp$^2$ character,

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