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High-Resolution Neutron Study of Vitamin B_{12} Coenzyme at 15 K: Structure Analysis and Comparison with the Structure at 279 K

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Abstract

$C_{72}H_{100}N_{18}O_{17}PCo.nH_2O$, where $n = 17$–18, $M_r =$ 1597 dalton (assuming that 19 coenzyme H atoms have been exchanged for D atoms), $P2_12_12_1$, $a =$ 27.550 (7), $b = 21.568$ (5), $c = 15.343$ (3) Å, $V =$ 9117 (4) Å^3, $Z = 4$, $D_x = 1.38$ (1) Mg m^{-3}; Cu(220) monochromator, $\lambda_1 = 1.5446$ (5) Å, 5505 unique reflections to nominal resolution 0.98 Å, $\mu =$ 2.20 cm^{-1}; Ge(115) monochromator, $\lambda_2 =$ 1.3169 (3) Å, 2361 unique reflections from nominal resolution 1.02 to 0.9 Å, $\mu = 1.6$ cm^{-1}; $T = 15$ K, final $R = 0.085$ for 7378 reflections. A high-resolution and low-temperature structural study of the vitamin B_{12} coenzyme has been undertaken using neutrons. Details of the data collection and refinement of the low-temperature structure are described, and a comparison of the coenzyme molecule and solvent structures at 15 and 279 K made. A shrinkage of around 2% is observed in the volume of the unit cell, and the orientation of the coenzyme molecule is rotated within the unit cell by approximately 2°, about an axis almost parallel to c and close to the Co atom. Incomplete or reverse exchange between D and H atoms led to some difficulties assigning certain exchangeable H positions in the coenzyme. 56 solvent atoms have been identified from neutron difference Fourier maps.

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1. Introduction

It is well accepted that the role of the solvent in the various activities associated with proteins (for example, protein folding, enzymatic catalysis, transport across cell membranes) and other biological molecules is of importance. A full understanding of the role and the interactions of solvent water with biomolecules, at the molecular level, depends to some extent on the availability of a range of accurate experimental data. Single-crystal diffraction data enable the determination of positional parameters, although because of the motions of molecules and atoms and the timescale of data collection, it is recognized that these are at best spatial and time-averaged positions. Despite this, high-resolution neutron studies enable the identification of atoms not easily visible with X-rays (H and D) and allow positional parameters to be determined accurately.

Vitamin B_{12} coenzyme is an ideal model compound for the study of water around groups of biological importance. It is of intermediate size and contains a corrin ring, several amide groups, a 5′-deoxyadenosyl moiety, a phosphate group and a ribose attached to a 5,6-dimethylbenzimidazole side chain. Crystals large enough for single-crystal neutron diffraction (minimum 5 mm^3) can be grown. 17–18 water molecules per coenzyme molecule were estimated to be present from density measurements in the original X-ray structure determination (Lenhert, 1968) and 17 water molecules were identified by the high-resolution (0.95 Å) neutron study carried out at 279 K by Savage, Lindley, Finney & Timmins (1987). The latter study proposed several water networks within the vitamin B_{12} coenzyme crystal and identified the importance of non-bonding O···H and H···H interactions within water networks (Savage, 1986; Savage & Finney, 1986).

This paper describes the details of the collection and analysis of the neutron diffraction data at 15 K and the subsequent refinement of the coenzyme molecule to this data. Preliminary results on the identification of water networks are also described. The solvent structure falls into two categories: (*a*) an ordered region where all peaks in difference maps have been identified and the solvent atoms fully refined, and (*b*) a more disordered region where a majority of peaks have been assigned and which exhibits a complex network of interconnected O—H···O hydrogen bonds; refinement of this region is still in progress, and will be reported elsewhere.

2. Data collection and processing

Vitamin B_{12} coenzyme (Glaxo) was dissolved in D_2O (10 mg ml^{-1}) and volumes of between 0.6 and 1.0 ml of this solution were placed in 1 ml melting point tubes which were filled with acetone. The tubes were covered with dialysis tubing, placed in an acetone bath and stored in the dark to prevent light contact with the coenzyme as this leads to the breaking of the labile C—Co bond.

The density of the crystals was determined to be 1.38 (1) Mg m^{-3} at 294 K from measurements carried out by flotation in an acetone/bromoethanol mixture.

A 5 mm^3 crystal was selected and mounted in a quartz capillary tube on the D19 diffractometer equipped with an Eulerian cradle sample holder and a position sensitive detector (p.s.d.) with a 4 by 64° aperture at the high flux reactor of the Institut Laue–Langevin, Grenoble, France (Thomas *et al.*, 1983). The crystal was cooled in steps of 0.1°/6 s to 200 K and then in steps of 0.1°/3 s to 15 K using a helium cryostat mounted on the χ circle of the diffractometer. A wavelength of 1.5446 Å was selected using a Cu(220) monochromator. The data collection was carried out by moving the detector in steps of 2° (γ) and for each detector position the crystal was rotated to yield a volume corresponding to slightly more than an eighth of reciprocal space; a full complement of reflections up to $\gamma = 102°$ was collected. The unit cell was determined from 2685 reflections for detector angles between $\gamma = 22$ and 100°. The data reduction was carried out using strong reflections to form a library of peak shapes. These were then employed to obtain an optimal estimate of the intensity of the weak reflections by using an algorithm that minimizes the relative error $\sigma(I)/I$ (Wilkinson, Khamis, Stansfield & McIntyre, 1988), where I is the integrated Bragg intensity and $\sigma(I)$ is the standard deviation based on counting statistics. The absorption is mainly due to the incoherent scattering of the hydrogens, which is assumed to be proportional to the wavelength in the region of interest; for $\lambda \simeq 1$ Å, the cross section was taken to be 40 b (40×10^{-28} m^2) which then gives $\mu = 2.20$ cm^{-1}.

The intensities of six standard reflections were measured once a day over the measurement period of five weeks in order to monitor variations in the experimental conditions; the overall variation was just under 9% over the five weeks and appropriate corrections were made to the data. The $\lambda/2$ contamination of the data was determined by comparing the reflections that were expected to be systematically absent with the values of their first-order harmonics [*e.g.* 003 ($\lambda/2$) and 006 (λ)], and other general reflections with their second-order harmonics: $I_0(hkl)(\lambda/2)/I_0(hkl)(\lambda)$. The $\lambda/2$ component was estimated to be 1.6%, and no corrections were made.

15 399 reflections were collected at this wavelength of which 5505 were unique with $R_{int} = 0.069$; 88% had $I > 3\sigma(I)$. $0 \le h \le 27$, $0 \le k \le 21$, $0 \le l \le 15$ (data set 1).

When the mechanical limits of the apparatus were reached, the wavelength was changed to 1.3169 Å by selecting a Ge(115) monochromator, and a full complement of unique reflections collected from λ = 82 to 94°. 6722 reflections were collected of which 2361 were unique with $R_{int} = 0.157$; of these 63% had $I > 3\sigma(I)$. $28 \le h \le 30$, $22 \le k \le 24$, $16 \le l \le 17$ (data set 2).

The completeness of the data from 12.0 to 0.9 Å resolution is shown in Table 1.

Table 1. *Completeness of the data from* 12.0 *to* 0.9 Å *resolution*

Resolution shell (Å)	2.50	1.79	1.46	1.27	1.14	1.04	0.96	0.90
Theoretical number of unique reflections in shell	390	618	768	885	1007	1094	1182	1261
Measured number of reflections in shell	377	614	767	883	1006	1092	1179	1260
Percentage observed	96.7	99.4	99.9	99.8	99.9	99.8	99.7	99.9

3. Structure refinement

The atomic positions of the higher temperature B_{12} coenzyme structure determined by Savage, Lindley, Finney & Timmins (1987) were used as the starting model for refinement; no waters were initially included. Because of its size, 209 atoms, it is possible to refine the model for vitamin B_{12} coenzyme by considering it either as a small macromolecule or as a large small molecule. The crystallographic calculations were based on $|Fhkl|$ of all unique reflections measured, and the neutron scattering lengths used were C 0.665, O 0.581, N 0.921, H −0.374, D 0.667, P 0.510, Co 0.280×10^{-12} cm (Koester & Rauch, 1981).

Refinement to 1.0 Å using data set 1 was carried out using the macromolecular refinement program *RESTRAIN* (Driessen *et al.*, 1989). The positional parameters and isotropic temperature factors were refined and difference neutron maps obtained. The refined models and the difference maps were displayed on an Evans and Sutherland PS 390 using the *FRODO* graphics package (Jones, 1978). 42 solvent atoms were incorporated into the refinement model to give a final $R = 0.103$.

Some difficulties were experienced in scaling data set 2 to data set 1 presumably due to the relatively small number of overlapping reflections (different scaling and merging procedures do not affect positional parameters or their e.s.d.'s, though they affect the atomic temperature factors slightly). The scaling was carried out using the *SCALEIT* program from the Science and Engineering Research Council program suite for crystallography (SERC Daresbury Laboratory, 1986). The two sets of F_{obs} were scaled to the F_{calc} values from the model to 1.0 Å. Preliminary scaling and merging gave $R = 0.121$ for 7378 unique reflections. The model and this combined data set were refined using *SHELX* (Sheldrick, 1976) in blocked least-squares refinement mode. The weighting scheme was chosen to give a constancy in the value of $\sum w(F_{obs} - F_{calc})^2$ when analyzed in terms of increasing $|F_{obs}|$ and $\sin\theta/\lambda$, $w = 0.09/(\sigma^2 + 0.004F_{obs}^2)$. At this stage 14 more solvent atoms were assigned. Positional and isotropic temperature-factor refinement gave an R of 0.095 for all data, which reduced to 0.085 for anisotropic temperature-factor refinement for all atoms excluding exchangeable H/D's and solvent atoms.

4. Results and discussion

Fig. 1 indicates the atom labelling for the vitamin B_{12} coenzyme molecule. Fractional atomic coordinates and equivalent isotropic thermal parameters are listed in Table 2.*

The structures at 15 and 279 K are very similar, but there is evidence for some incomplete or back-exchange between deuteriums and hydrogens in the lower temperature structure. This could be due to the length of time the crystals and the mother liquor were exposed to air during the transfer from the melting point tubes to the quartz capillary tubes. The transfer was completed under safe-light conditions in a darkroom and took several minutes. This back exchange has given rise to difficulties in assigning sites to three out of the 19 exchangeable hydrogen/deuteriums where partial exchange seems to have taken place. On the other hand it has led to an unambiguous identification of solvent sites, as all water molecules are now H_2O rather than D_2O and the hydrogens therefore show up as negative peaks in the neutron-density maps.

Differences between the structures at 15 and 279 K can be categorized as follows:

(i) *Unit-cell differences.* Table 3 indicates that the unit-cell parameters at 15 K differ significantly from those at 279 K. There is an overall shortening of the unit-cell dimensions leading to a shrinkage of ~ 2% in unit-cell volume.

(ii) *Precision of the data.* The lowering of the temperature to 15 K does not seem to result in an improvement in the precision of the model for those atoms which are close to the corrin ring centre because these are tightly bound with no static and very little dynamic disorder, and leads only to a

* A list of structure factors has been deposited with the British Library Document Supply Centre as Supplementary Publication No. SUP 55813 (29 pp.). Copies may be obtained through The Technical Editor, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England.

lowering of the temperature factors. Table 4 gives a few examples of these effects. The cobalt-atom precision is also not improved, for, in addition to the tight binding, it has a relatively small scattering contribution. For those atoms further away from the cobalt atom, the lower temperature gives rise to slightly more precise data; this possibly reflects the fact that the dynamic disorder is now greatly reduced.

(iii) *Comparison of the coordinates.* The study carried out by Savage, Lindley, Finney & Timmins (1987) showed that at 279 K there is evidence that the N40 atom on group *b* (see Fig. 1) is disordered with an alternative site (N640) with occupancy 0.26. At 15 K there is no evidence of such disorder. A statistical analysis of the coordinate differences (excluding the solvent molecules) between the structures at 279 and 15 K was carried out using a fitting program (Maclachlan, 1979) (see Table 5). The 15 K model can be seen to have rotated by $\sim 2^\circ$ about an axis which is almost parallel to the *c* axis of the unit cell, and passing close to the Co atom (Figs. 2*a* and 2*b*). Inspection of r.m.s. coordinate differences between the two structures as a function of the coenzyme groups shows that the benzimidazole (*h*) has moved less than the other groups possibly reflecting that it is bound to the corrin ring *via* a side chain on group *d* and directly to the centre of the ring system *via* the tightly held Co atom. Groups *a*–*d* have flexible side chains which contribute to the slightly larger r.m.s. (Fig. 3).

(iv) *Bond lengths.* The bond lengths calculated from the coordinates for the structure at 15 K are given in Figs. 4 and 5 along with those for the structure at 279 K (Savage, Lindley, Finney & Timmins, 1987). There seems to be no correlation between temperature and bond lengths, *i.e.* a shortening of bond lengths with a fall in temperature.

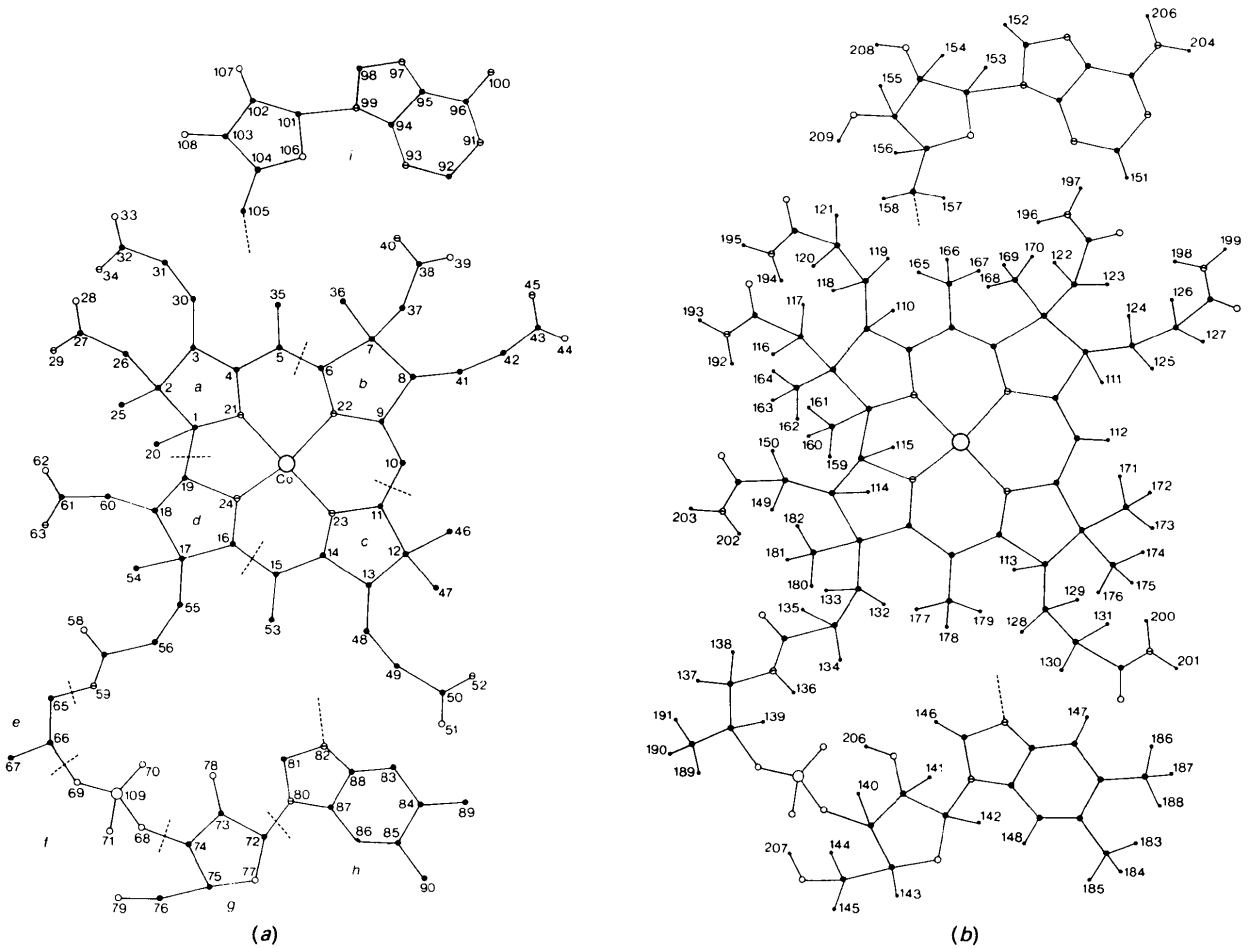

Fig. 1. (*a*) Atom types and numbering in the vitamin B_{12} coenzyme molecule for non-hydrogen atoms: filled circles, C; open circles (small), O; open circles with horizontal lines, N; open circles (large), Co and P. Groups: *a*, *b*, *c*, *d*, pyrrole rings including side chains; *e*, propionic acid; *f*, phosphate; *g*, ribose; *h*, benzimidazole; *i*, adenosyl. (*b*) Atom numbering for hydrogen atoms in the coenzyme molecule.

Table 2. *Fractional atomic coordinates* (× 10^4) *and equivalent isotropic thermal parameters* (Å^2 × 10^3) *and occupancy factors*

$U_{eq} = (1/3)\sum_i \sum_j U_{ij} a_i^* a_j^* \mathbf{a}_i . \mathbf{a}_j .$

	x	y	z	U_{eq}	Occ.		x	y	z	U_{eq}	Occ.
Co64	488 (5)	1877 (7)	199 (10)	11 (3)	1.00	H173	1258 (7)	1939 (9)	2664 (13)	52 (4)	1.00
C1	174 (2)	2673 (3)	−1222 (4)	10 (1)	1.00	H174	813 (5)	321 (7)	3147 (9)	28 (3)	1.00
C2	379 (2)	2824 (3)	−2155 (4)	9 (1)	1.00	H175	192 (6)	485 (7)	3140 (10)	35 (3)	1.00
C3	502 (2)	2168 (3)	−2507 (4)	10 (1)	1.00	H176	596 (5)	758 (7)	3994 (10)	31 (3)	1.00
C4	626 (2)	1822 (3)	−1677 (4)	12 (1)	1.00	H113	411 (6)	2109 (8)	3501 (11)	38 (4)	1.00
N21	432 (2)	2080 (2)	−994 (3)	8 (1)	1.00	H128	−299 (5)	1280 (6)	2582 (9)	24 (3)	1.00
C20	−379 (2)	2558 (3)	−1204 (4)	13 (1)	1.00	H129	−479 (6)	2026 (7)	2851 (10)	34 (3)	1.00
C25	23 (2)	3189 (3)	−2741 (4)	14 (1)	1.00	H130	−473 (6)	1846 (8)	4379 (11)	40 (4)	1.00
C26	867 (2)	3174 (3)	−2073 (4)	12 (1)	1.00	H131	−27 (7)	1285 (9)	4317 (13)	52 (4)	1.00
C27	1151 (2)	3218 (3)	−2927 (4)	15 (1)	1.00	H200	−1266 (7)	440 (9)	3634 (13)	37 (4)	0.87
O28	1018 (3)	3556 (3)	−3531 (5)	12 (1)	1.00	H201	−1054 (7)	1030 (9)	3046 (13)	36 (4)	0.85
N29	1546 (2)	2858 (2)	−2998 (3)	13 (1)	1.00	C53	91 (3)	3179 (4)	2710 (5)	25 (2)	1.00
C30	112 (2)	1791 (3)	−3039 (4)	14 (1)	1.00	H177	142 (7)	2937 (10)	3296 (14)	57 (5)	1.00
C31	86 (2)	1949 (3)	−4016 (4)	14 (1)	1.00	H178	321 (6)	3618 (8)	2717 (11)	38 (4)	1.00
C32	579 (2)	1890 (3)	−4455 (4)	10 (1)	1.00	H179	−273 (6)	3326 (8)	2742 (11)	37 (3)	1.00
O33	784 (3)	1374 (3)	−4485 (5)	13 (1)	1.00	C15	212 (2)	2779 (3)	1920 (5)	19 (1)	1.00
N34	767 (2)	2398 (2)	−4809 (3)	16 (3)	1.00	C16	203 (2)	3037 (3)	1052 (4)	13 (1)	1.00
H159	−487 (5)	2171 (7)	−1621 (9)	27 (3)	1.00	C17	108 (2)	3726 (3)	806 (4)	10 (1)	1.00
H160	−505 (5)	2459 (6)	−562 (8)	21 (3)	1.00	C18	−6 (2)	3657 (3)	−189 (4)	7 (1)	1.00
H161	−562 (5)	2981 (7)	−1459 (10)	33 (3)	1.00	C19	312 (2)	3105 (3)	−442 (4)	11 (1)	1.00
H162	191 (5)	3306 (7)	−3369 (10)	30 (3)	1.00	N24	299 (2)	2710 (2)	353 (3)	9 (1)	1.00
H163	−329 (5)	2930 (7)	−2834 (9)	27 (3)	1.00	C54	587 (2)	4091 (3)	967 (4)	17 (1)	1.00
H164	−84 (5)	3624 (7)	−2424 (9)	27 (3)	1.00	C55	−302 (2)	4065 (3)	1278 (4)	9 (1)	1.00
H116	1103 (4)	2936 (6)	−1567 (8)	19 (3)	1.00	C56	−802 (2)	3727 (3)	1311 (4)	17 (1)	1.00
H117	822 (6)	3652 (7)	−1848 (10)	34 (3)	1.00	C57	−1132 (2)	4074 (3)	1956 (4)	13 (1)	1.00
H110	827 (4)	2180 (5)	−2918 (7)	12 (2)	1.00	O58	−1002 (3)	4158 (4)	2714 (5)	18 (2)	1.00
H118	−259 (5)	1828 (6)	−2748 (9)	22 (3)	1.00	N59	−1537 (2)	4295 (2)	1625 (3)	13 (1)	1.00
H119	206 (5)	1286 (7)	−2964 (9)	28 (3)	1.00	C60	83 (2)	4236 (3)	−752 (4)	13 (1)	1.00
H120	−170 (5)	1660 (7)	−4326 (9)	27 (3)	1.00	C61	−328 (2)	4712 (3)	−711 (4)	14 (1)	1.00
H121	−36 (5)	2439 (7)	−4092 (10)	30 (3)	1.00	O62	−756 (3)	4552 (4)	−800 (5)	20 (2)	1.00
D194	1111 (5)	2359 (6)	−5055 (9)	47 (3)	0.72	N63	−189 (2)	5296 (2)	−613 (3)	16 (1)	1.00
D195	590 (4)	2806 (5)	−4791 (8)	34 (2)	0.68	H180	545 (5)	4577 (6)	846 (9)	24 (3)	1.00
C35	1177 (2)	1066 (3)	−2481 (4)	15 (1)	1.00	H181	888 (6)	3939 (8)	569 (10)	36 (4)	1.00
H165	1281 (5)	1492 (7)	−2827 (10)	30 (3)	1.00	H182	697 (6)	4025 (8)	1661 (10)	36 (4)	1.00
H166	978 (6)	782 (7)	−2926 (10)	35 (3)	1.00	H132	−368 (5)	4515 (6)	941 (9)	25 (3)	1.00
H167	1506 (5)	848 (6)	−2294 (9)	25 (3)	1.00	H133	195 (5)	4177 (7)	1941 (10)	28 (3)	1.00
C5	899 (2)	1249 (3)	−1659 (4)	13 (1)	1.00	H134	−742 (5)	3251 (7)	1551 (10)	32 (3)	1.00
C6	911 (2)	889 (3)	−906 (4)	9 (1)	1.00	H135	−953 (5)	3680 (7)	663 (10)	31 (3)	1.00
C7	1178 (2)	264 (3)	−812 (4)	12 (1)	1.00	D136	−1631 (4)	4185 (6)	1018 (8)	43 (3)	0.74
C8	922 (2)	15 (3)	26 (4)	14 (1)	1.00	H114	−392 (5)	3523 (6)	245 (9)	25 (3)	1.00
C9	770 (2)	611 (3)	471 (4)	12 (1)	1.00	H149	96 (5)	4111 (7)	−1429 (9)	27 (3)	1.00
N22	712 (2)	1069 (2)	−114 (3)	9 (1)	1.00	H150	−442 (5)	4449 (7)	607 (9)	29 (3)	1.00
C36	1144 (2)	−168 (3)	−1584 (4)	16 (1)	1.00	D202	−446 (4)	5651 (6)	−630 (8)	40 (3)	0.74
C37	1705 (2)	449 (3)	−565 (4)	14 (1)	1.00	D203	157 (4)	5402 (5)	−475 (8)	40 (3)	0.76
C38	2068 (2)	−71 (3)	−389 (4)	14 (1)	1.00	H115	692 (5)	3245 (6)	−533 (9)	25 (3)	1.00
O39	1943 (3)	−629 (4)	−346 (5)	17 (2)	1.00	C65	−1874 (2)	4659 (3)	2139 (4)	15 (1)	1.00
N40	2513 (2)	111 (2)	−239 (3)	16 (1)	1.00	C66	−2249 (2)	4277 (3)	2633 (4)	16 (1)	1.00
C41	476 (2)	−384 (3)	202 (4)	14 (1)	1.00	C67	−2603 (3)	4692 (4)	3114 (5)	25 (2)	1.00
C42	138 (2)	−552 (3)	574 (4)	16 (1)	1.00	H137	−2062 (5)	4992 (7)	1705 (9)	28 (3)	1.00
C43	−237 (2)	−982 (3)	194 (4)	16 (1)	1.00	H138	−1662 (5)	4912 (7)	2634 (10)	33 (3)	1.00
O44	−159 (3)	−1544 (4)	91 (5)	20 (2)	1.00	H139	−2068 (5)	3942 (7)	3081 (9)	28 (3)	1.00
N45	−659 (2)	−731 (2)	−40 (3)	17 (1)	1.00	H189	−2870 (6)	4442 (7)	3497 (11)	37 (4)	1.00
H168	797 (5)	−120 (7)	−1952 (10)	29 (3)	1.00	H190	−2404 (6)	4994 (8)	3578 (11)	43 (4)	1.00
H169	1442 (5)	−83 (7)	−2065 (10)	33 (3)	1.00	H191	−2795 (6)	5005 (8)	2650 (11)	38 (4)	1.00
H170	1156 (5)	−669 (7)	−1383 (10)	34 (3)	1.00	O69	−2522 (3)	3913 (3)	1998 (5)	12 (1)	1.00
H122	1698 (5)	765 (7)	−1 (10)	33 (3)	1.00	P109	−2571 (3)	3176 (4)	2115 (5)	8 (2)	1.00
H123	1873 (5)	746 (6)	−1071 (9)	24 (3)	1.00	O70	−2567 (3)	2993 (4)	3061 (5)	17 (2)	1.00
D196	2778 (7)	−184 (10)	43 (14)	32 (5)	0.37	O71	−3001 (3)	2950 (3)	1594 (5)	14 (2)	1.00
D197	2614 (8)	573 (11)	−217 (15)	41 (5)	0.39	O68	−2075 (3)	2949 (3)	1644 (5)	15 (2)	1.00
H111	1161 (4)	−258 (6)	444 (8)	18 (3)	1.00	C74	−1945 (2)	2315 (3)	1703 (4)	12 (1)	1.00
H124	594 (6)	−833 (7)	−487 (10)	35 (3)	1.00	C73	−1751 (2)	2054 (3)	844 (4)	12 (1)	1.00
H125	252 (5)	−147 (6)	−693 (9)	25 (3)	1.00	C72	−1460 (2)	1492 (3)	1204 (4)	13 (1)	1.00
H126	−40 (5)	−148 (7)	854 (9)	27 (3)	1.00	O77	−1344 (3)	1613 (3)	2067 (5)	13 (1)	1.00
H127	349 (5)	−787 (7)	1070 (9)	31 (3)	1.00	C75	−1505 (2)	2233 (3)	2325 (4)	14 (1)	1.00
D198	−880 (5)	−969 (7)	−477 (9)	36 (3)	0.56	C76	−1608 (3)	2265 (4)	3296 (5)	25 (2)	1.00
D199	−732 (5)	−287 (7)	32 (9)	36 (3)	0.61	O78	−1457 (3)	2492 (3)	452 (5)	14 (2)	1.00
C10	733 (2)	666 (3)	1361 (4)	10 (1)	1.00	O79	−2053 (3)	1954 (4)	3536 (6)	28 (2)	1.00
H112	827 (5)	248 (7)	1764 (9)	28 (3)	1.00	H140	−2253 (4)	2027 (6)	1923 (8)	18 (3)	1.00
C11	628 (2)	1210 (3)	1812 (4)	12 (1)	1.00	H141	−2062 (5)	1916 (7)	414 (10)	32 (3)	1.00
C12	663 (2)	1285 (3)	2784 (4)	16 (1)	1.00	H142	−1676 (5)	1073 (7)	1149 (9)	29 (3)	1.00
C13	303 (3)	1828 (3)	2933 (5)	22 (2)	1.00	H143	−1218 (5)	2575 (6)	2138 (9)	26 (3)	1.00
C14	352 (2)	2166 (3)	2059 (4)	15 (1)	1.00	H144	−1608 (6)	2759 (8)	3491 (10)	37 (4)	1.00
N23	496 (2)	1752 (2)	1428 (3)	11 (1)	1.00	H145	−1297 (6)	2056 (8)	3632 (12)	43 (4)	1.00
C46	1184 (3)	1483 (3)	3011 (5)	23 (2)	1.00	H206	−1471 (6)	2423 (8)	−200 (12)	35 (4)	0.94
C47	558 (3)	673 (3)	3280 (5)	20 (1)	1.00	H207	−2295 (7)	2263 (9)	3456 (12)	40 (4)	0.91
C48	−247 (3)	1630 (3)	3060 (5)	20 (1)	1.00	N80	−1016 (2)	1384 (2)	699 (3)	11 (1)	1.00
C49	−350 (3)	1445 (3)	4012 (5)	24 (2)	1.00	C81	−586 (2)	1669 (3)	808 (4)	14 (1)	1.00
C50	−719 (3)	916 (4)	4132 (5)	29 (2)	1.00	N82	−260 (2)	1515 (2)	215 (3)	12 (1)	1.00
O51	−717 (3)	640 (4)	4857 (6)	28 (2)	1.00	C83	−350 (2)	801 (3)	−1105 (4)	13 (1)	1.00
N52	−1025 (2)	788 (3)	3510 (4)	29 (1)	1.00	C84	−682 (2)	443 (3)	−1558 (4)	15 (1)	1.00
H171	1230 (7)	1538 (9)	3695 (12)	47 (4)	1.00	C85	−1176 (2)	376 (3)	−1273 (5)	18 (1)	1.00
H172	1463 (7)	1124 (9)	2806 (12)	48 (4)	1.00	C86	−1325 (2)	686 (3)	−505 (4)	16 (1)	1.00

Table 2 (*cont.*)

	x	y	z	U_{eq}	Occ.		x	y	z	U_{eq}	Occ.
C87	−975 (2)	1029 (3)	−59 (4)	14 (1)	1.00	H303	3095 (6)	1648 (8)	−310 (11)	40 (4)	1.00
C88	−502 (2)	1111 (3)	−349 (4)	14 (1)	1.00	O212	3485 (3)	−414 (4)	190 (5)	20 (2)	1.00
C89	−520 (3)	93 (4)	−2359 (5)	27 (2)	1.00	H305	3636 (6)	−804 (8)	259 (11)	39 (4)	1.00
C90	−1531 (3)	3 (3)	−1779 (5)	22 (2)	1.00	H304	3701 (6)	−115 (8)	368 (12)	43 (4)	1.00
H146	−513 (6)	1995 (7)	1366 (11)	37 (3)	1.00	O213	3580 (3)	1357 (4)	5822 (5)	15 (2)	1.00
H147	32 (5)	851 (6)	−1349 (8)	20 (3)	1.00	H306	3425 (6)	1084 (8)	5445 (11)	41 (4)	1.00
H148	−1702 (5)	649 (6)	−279 (9)	27 (3)	1.00	H307	3712 (6)	1120 (7)	6336 (10)	35 (3)	1.00
H186	−141 (5)	177 (7)	−2495 (10)	33 (3)	1.00	O214	1792 (4)	4873 (6)	1789 (9)	40 (2)	1.00
H187	−728 (7)	218 (9)	−2943 (12)	47 (4)	1.00	H308	1837 (12)	4560 (17)	7425 (24)	56 (8)	0.60
H188	−568 (7)	−411 (9)	−2285 (13)	52 (4)	1.00	H309	1801 (16)	4801 (21)	2276 (33)	59 (10)	0.50
H183	−1884 (7)	6 (9)	−1509 (12)	50 (4)	1.00	H508	2033 (10)	5180 (12)	1743 (17)	44 (6)	0.70
H184	−1524 (8)	129 (10)	−2492 (14)	60 (4)	1.00	O215	1556 (6)	4593 (9)	3525 (11)	25 (4)	0.50
H185	−1434 (7)	−478 (9)	−1758 (12)	50 (5)	1.00	H310	1533 (9)	4137 (12)	3549 (16)	42 (6)	0.70
N91	1953 (2)	2757 (2)	5097 (3)	18 (1)	1.00	O216	3380 (3)	2605 (4)	1277 (6)	21 (2)	1.00
C92	1738 (3)	3218 (3)	4653 (5)	22 (2)	1.00	H312	3485 (7)	2205 (9)	1474 (13)	50 (4)	1.00
N93	1709 (2)	3290 (2)	3788 (3)	17 (1)	1.00	H313	3495 (7)	2897 (9)	1681 (12)	48 (4)	1.00
C94	1959 (2)	2840 (3)	3357 (4)	16 (1)	1.00	O217	3608 (3)	1307 (4)	1498 (6)	22 (2)	1.00
C95	2199 (3)	2352 (3)	3738 (5)	20 (1)	1.00	H314	3509 (7)	1265 (9)	2111 (14)	55 (5)	1.00
C96	2190 (2)	2303 (3)	4647 (4)	16 (1)	1.00	H315	3865 (6)	1020 (8)	1351 (11)	40 (4)	1.00
N97	2415 (2)	1993 (2)	3104 (3)	20 (1)	1.00	O618	3273 (4)	1407 (5)	3218 (7)	34 (2)	1.00
C98	2292 (3)	2252 (3)	2349 (5)	24 (2)	1.00	H516	3251 (8)	1098 (11)	3610 (15)	60 (5)	1.00
N99	2013 (2)	2776 (2)	2464 (3)	20 (1)	1.00	H517	2965 (8)	1598 (9)	3139 (13)	53 (5)	1.00
N100	2401 (2)	1827 (2)	5098 (3)	20 (1)	1.00	O220	3755 (4)	3332 (5)	2575 (7)	25 (2)	0.85
C101	1757 (2)	3137 (3)	1785 (4)	16 (1)	1.00	H339	3774 (7)	3057 (10)	3010 (14)	38 (4)	0.80
C102	2082 (2)	3305 (3)	993 (5)	19 (1)	1.00	O222	2418 (6)	5633 (9)	4554 (12)	46 (4)	0.70
C103	1940 (2)	2800 (3)	320 (4)	17 (1)	1.00	H251	2442 (13)	5236 (18)	4627 (24)	64 (8)	0.60
C104	1396 (2)	2706 (3)	539 (4)	15 (1)	1.00	O223	700 (4)	7867 (5)	296 (6)	30 (2)	1.00
C105	1206 (2)	2087 (3)	219 (4)	16 (1)	1.00	H326	426 (8)	8059 (9)	205 (13)	54 (5)	1.00
O106	1368 (3)	2755 (4)	1465 (5)	19 (2)	1.00	H327	934 (7)	8123 (10)	490 (13)	54 (5)	1.00
O107	1957 (4)	3889 (4)	692 (6)	31 (2)	1.00	O224	3784 (7)	2452 (9)	3846 (13)	30 (4)	0.50
O108	2020 (3)	2997 (4)	−531 (5)	17 (2)	1.00	H716	3654 (9)	2031 (11)	3621 (16)	40 (5)	0.70
H151	1566 (6)	3561 (7)	5061 (10)	33 (3)	1.00	H717	3975 (10)	2349 (12)	4274 (18)	47 (6)	0.70
H152	2406 (7)	2090 (10)	1710 (14)	56 (5)	1.00	O225	3050 (7)	3310 (10)	4647 (14)	36 (4)	0.50
H204	2401 (6)	1855 (8)	5774 (11)	37 (4)	0.99	H362	3030 (10)	3185 (13)	5191 (20)	41 (6)	0.60
H205	2579 (6)	1492 (8)	4791 (11)	40 (4)	0.96	O422	3688 (8)	5262 (11)	4335 (14)	37 (5)	0.50
H153	1607 (5)	3564 (7)	2096 (10)	30 (3)	1.00	H258	3817 (18)	5018 (24)	4606 (34)	50 (11)	0.40
H154	2484 (6)	3250 (8)	1203 (10)	37 (4)	1.00	O228	2357 (9)	4394 (12)	5056 (17)	30 (5)	0.40
H155	2138 (5)	2368 (6)	456 (9)	26 (3)	1.00	H246	2420 (10)	4376 (13)	5652 (19)	35 (6)	0.60
H156	1194 (5)	3093 (7)	252 (10)	33 (3)	1.00	H247	2548 (16)	4138 (21)	4836 (29)	82 (10)	0.50
H157	1359 (5)	1731 (7)	640 (10)	35 (3)	1.00	O610	2670 (16)	6019 (21)	2912 (31)	24 (9)	0.20
H158	1321 (5)	2018 (7)	−427 (10)	29 (3)	1.00	O431	2762 (11)	5887 (14)	3570 (20)	27 (7)	0.30
H208	2168 (12)	4030 (15)	311 (22)	44 (7)	0.42	H250	2516 (12)	5623 (15)	3996 (23)	36 (7)	0.50
H209	2016 (6)	2643 (8)	−924 (11)	38 (4)	0.93	O229	2900 (16)	3981 (21)	3192 (27)	32 (9)	0.25
						H253	2730 (16)	3770 (21)	3938 (28)	52 (10)	0.40
Solvent positions						H254	2739 (35)	4233 (47)	3119 (60)	63 (22)	0.25
O210	2427 (6)	5861 (9)	1922 (12)	34 (4)	0.60	O428	2424 (14)	5811 (17)	1181 (25)	43 (9)	0.30
H500	2285 (13)	6194 (19)	1887 (23)	45 (8)	0.50	O231	3342 (15)	5967 (19)	3516 (27)	47 (9)	0.30
H501	2662 (11)	5880 (14)	2330 (22)	46 (7)	0.60	O427	3607 (11)	5795 (15)	3909 (21)	32 (7)	0.30
O211	3046 (3)	1271 (4)	−23 (5)	15 (2)	1.00	O426	3587 (12)	4420 (15)	3964 (23)	31 (7)	0.30
H302	3203 (6)	1268 (8)	551 (11)	40 (4)	1.00	H260	3790 (28)	4441 (34)	4349 (52)	46 (17)	0.25
						H261	3263 (17)	4063 (22)	4269 (31)	58 (11)	0.40

Table 3. *A comparison between unit-cell parameters at* 279 *and* 15 K

	a (Å)	b (Å)	c (Å)	V (Å^3)	$\Delta V/V_{279}$ (%)
279 K	27.849 (13)	21.736 (10)	15.368 (7)	9302 (7)	
15 K	27.550 (7)	21.568 (5)	15.343 (3)	9117 (4)	2

Table 4. *Selected coordinates and isotropic temperature factors* U_{eq} (Å^2) *for the high* (*line* 1) *and low* (*line* 2) *temperatures*

Co, N21, C1: inner atoms; C60, C61, O62: side-chain atoms.

Atom	x	y	z	U_{eq}
Co	0.0495 (6)	0.1841 (7)	0.0212 (11)	0.026 (5)
	0.0488 (5)	0.1877 (7)	0.0199 (10)	0.011 (3)
N21	0.0466 (2)	0.2040 (2)	−0.0992 (3)	0.021 (1)
	0.0432 (2)	0.2080 (2)	−0.0994 (3)	0.008 (1)
C1	0.0288 (2)	0.2632 (2)	−0.1199 (4)	0.018 (2)
	0.0174 (2)	0.2673 (3)	−0.1222 (4)	0.010 (1)
C60	0.0150 (3)	0.4187 (4)	−0.0689 (5)	0.033 (3)
	0.0083 (2)	0.4236 (3)	−0.0752 (4)	0.013 (1)
C61	−0.0243 (4)	0.4627 (4)	−0.0718 (6)	0.040 (5)
	−0.0328 (2)	0.4712 (3)	−0.0711 (4)	0.014 (1)
O62	−0.0671 (6)	0.4461 (7)	−0.0873 (11)	0.076 (5)
	−0.0756 (3)	0.4552 (4)	−0.0800 (5)	0.020 (2)

Table 5. *Coordinate analysis for all atoms excluding solvent, between the structures at* 15 K *and* 279 K

	Before fitting	After fitting
R.m.s. difference (Å)	0.232	0.143
Mean difference (Å)	0.209	0.119
Standard deviation (Å)	0.101	0.080
Angle (°)	1.908	
Translation (Å)	0.057, 0.058	
	0.057	

E.s.d.'s on distances again improve when the dynamic disorder of atoms concerned seems to be substantially reduced (*i.e.* for side-chain atoms) as indicated in Table 6.

The e.s.d. ranges (Å) are as follows:

	Range	Average
Non-H atoms	0.007–0.015	0.009
H atoms	0.013–0.042	0.016

Most of the non-H bond lengths at 15 K are within 3 × 0.009 Å (average bond length e.s.d.) of

those at 279 K. The largest bond length e.s.d.'s at 15 K (0.015 Å) are observed for the four N—Co bonds of the corrin ring (see Fig. 4).

In a similar manner to that found in the high-temperature structure the absence of a bridging C atom between C1 (of corrin group *a*) and C19 (of corrin group *d*) leads to an incomplete delocalization of electrons over the corrin ring nucleus with C=N bonds at C4—N21 [1.30 (1) Å] and C16—N24 [1.31 (1) Å].

Table 6. *Selected bond lengths and e.s.d.'s* (Å), *at* 279 *and* 15 K, *showing little improvement for 'core' distances, but considerable improvement for outer side chains*

	N21—Co	N21—C1	C61—O62	C61—N63
279 K	1.890 (17)	1.482 (9)	1.270 (16)	1.340 (14)
15 K	1.889 (15)	1.503 (7)	1.236 (10)	1.326 (8)

(v) *Thermal parameters.* As expected the atomic temperature factors (U_{eq}) are substantially lower at 15 than at 279 K. A comparison of the temperature factors as a function of atom number for the non-hydrogen/deuterium and non-solvent atoms is shown in Fig. 6(*a*). At 15 K there are no large variations in the thermal parameters, with the average U_{eq} = 0.016 Å^2.

At 279 K the thermal parameters of non-H/D atoms can be seen to increase with distance from the

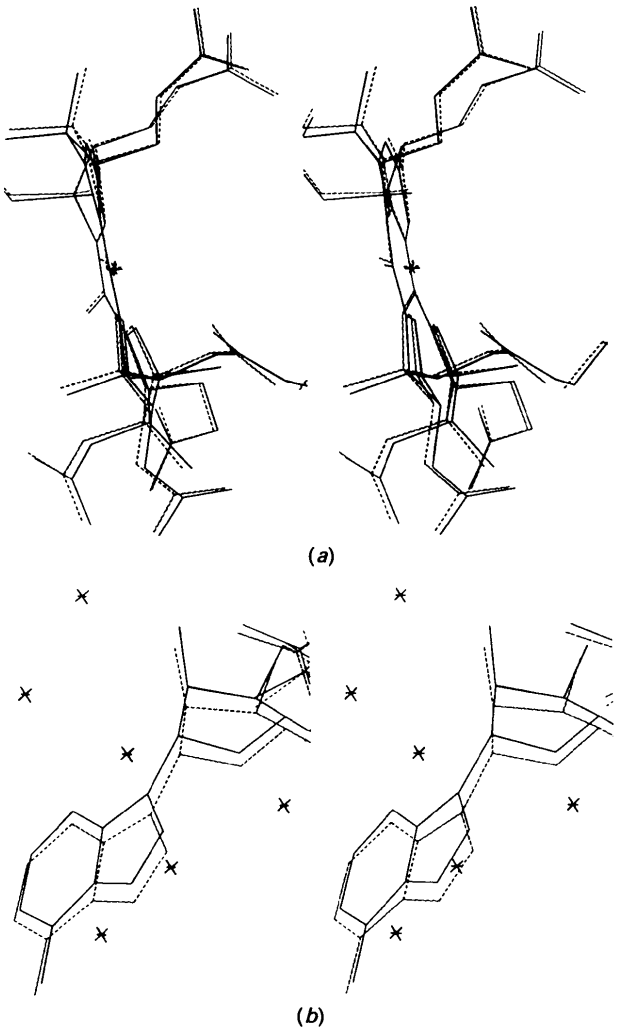

Fig. 2. Structural differences between the vitamin at 279 K (dotted line) and at 15 K (solid line): (*a*) looking at the corrin ring, (*b*) differences in the 5′-deoxyadenosyl group.

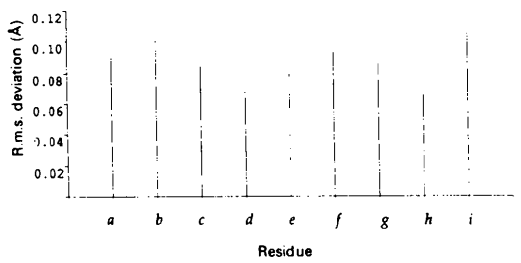

Fig. 3. R.m.s. deviation of the difference in the atomic coordinates between the structures at 279 and 15 K.

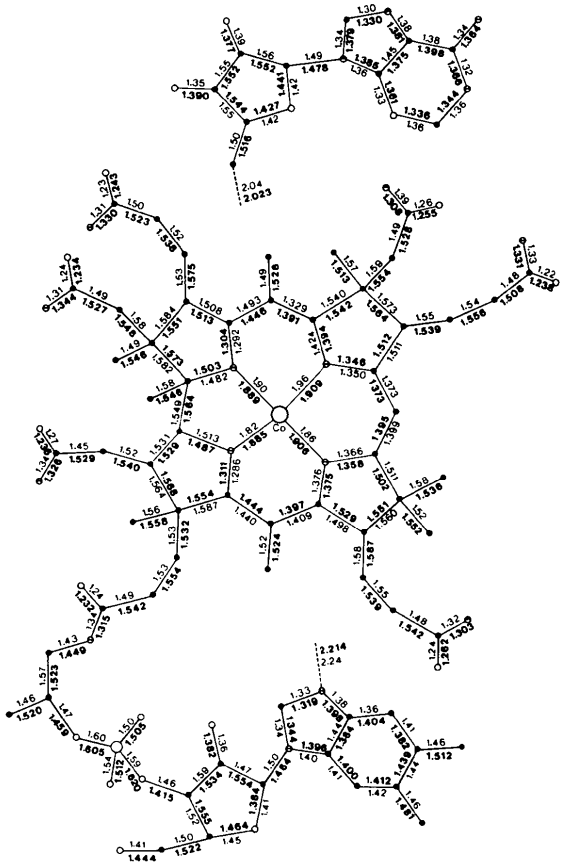

Fig. 4. Interatomic distances (Å) for the coenzyme B_{12} molecule; bold numbers refer to the neutron model at 15 K, the lighter numbers to the 279 K structure.

centre of the corrin ring (Co atom), yet at 15 K there is no evidence of such a trend (Fig. 6*b*).

(vi) *Hydrogen–deuterium exchange.* The expected level of deuteration for the exchangeable hydrogen/deuterium for the coenzyme molecule and the water would be 99–100% as the crystals were grown from 99.8% D_2O. Initial difference Fourier maps had shown that the water was H_2O rather than D_2O, indicating that a substantial amount of incomplete or back-exchange had occurred. In order to determine the extent of H/D exchange in the crystal, the thermal parameters and the occupancies for the atoms were varied in several cycles of refinement. It is possible to determine the proportions of H and D atoms for each site by using:

$$x_H b_H + y_D b_D = \mathrm{occ}_r b_H$$

(or $\mathrm{occ}_r b_D$ depending on whether the associated density is negative or positive), and

$$x_H + y_D = 1.0.$$

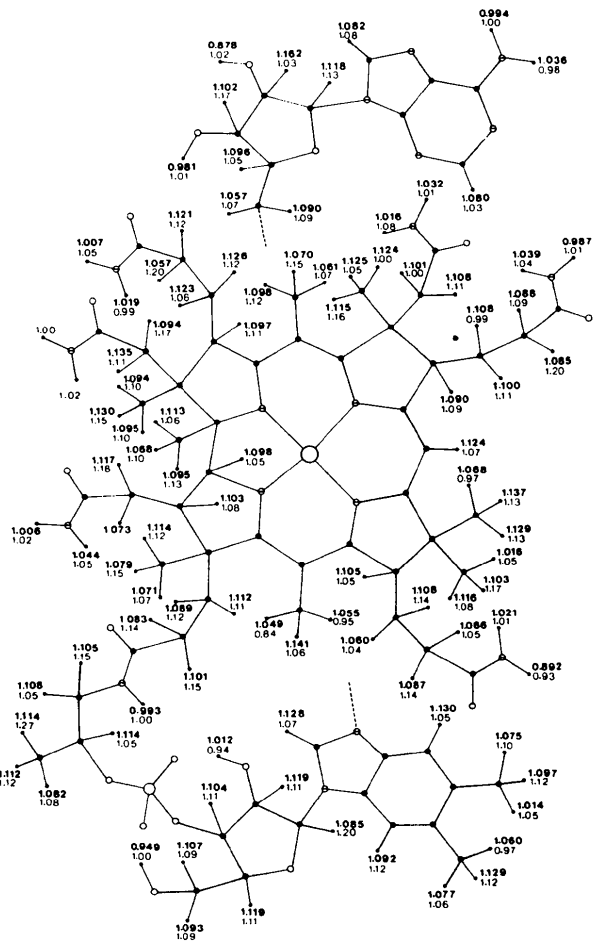

Fig. 5. Interatomic distances (Å) in the vitamin B_{12} coenzyme molecule involving the H and D atoms: bold numbers refer to the neutron model at 15 K, the lighter numbers to the 279 K structure.

Table 7. *Proportion of hydrogen (x_H) and deuterium (y_D) associated with 17 of the 19 exchangeable hydrogens on the vitamin B_{12} molecule*

Occ, is the occupancy from refinement.

Atom No.	Occ,	Density	x_H	y_D
194	0.72	> 0	0.18	0.82
195	0.68	> 0	0.21	0.79
196	0.37	> 0	0.41	0.59
197	0.39	> 0	0.38	0.62
198	0.56	> 0	0.28	0.72
199	0.61	> 0	0.25	0.75
200	0.87	< 0	0.95	0.05
201	0.85	< 0	0.94	0.06
136	0.74	> 0	0.17	0.83
202	0.74	< 0	0.90	0.10
203	0.76	< 0	0.91	0.09
204	0.99	< 0	1.0	0.0
205	0.96	< 0	0.98	0.02
206	0.94	< 0	0.97	0.03
207	0.91	< 0	0.96	0.04
208	0.42	< 0	0.79	0.21
209	0.93	< 0	0.97	0.03

Table 8. *Average positional e.s.d.'s and temperature factors U_{eq} (Å^2) for water molecules*

	O (pocket)	H (pocket)	O (channel)	H (channel)
(*a*) Average positional e.s.d.'s				
279 K	0.0019	0.0023	0.0027	0.0037
15 K	0.0006	0.0010	0.0008	0.0018
(*b*) Average temperature factors				
279 K	0.097	0.130	0.094	0.132
15 K	0.025	0.044	0.037	0.044

Here x_H is the proportion of hydrogen, y_D is the proportion of deuterium, b_H is the hydrogen scattering length, b_D is the deuterium scattering length and occ_r is the occupancy from refinement. [For example when no scattering is observed, *i.e.* $\mathrm{occ}_r = 0$,

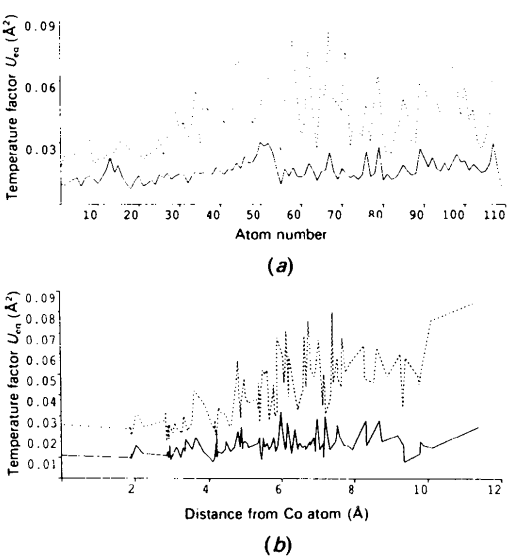

Fig. 6. Individual atomic isotropic temperature factors for non-H/D and non-solvent atoms: (*a*) as a function of atom number (see Fig. 1) and (*b*) as a function of the distance (Å) of the atoms from the cobalt at the centre of the corrin ring (dotted line: structure at 279 K; solid line: structure at 15 K).

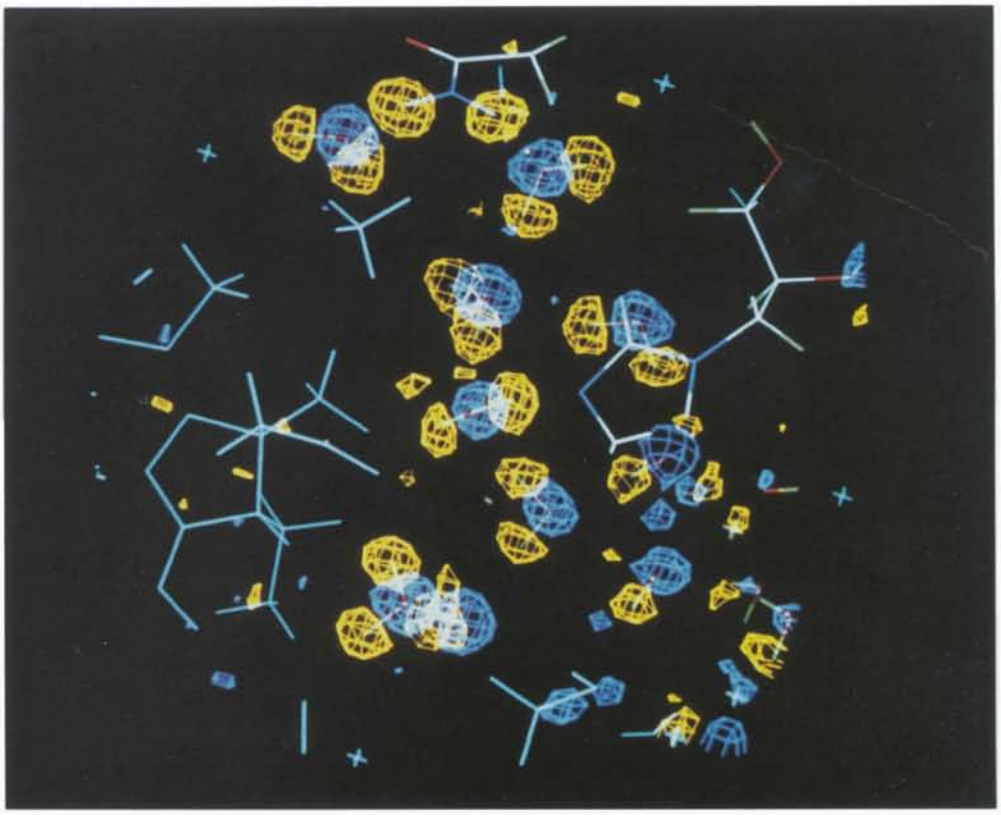

Fig. 7. Difference neutron map ($F_{obs} - F_{calc}$) showing the solvent structure in the ordered pocket region. Deuteriums and hydrogens directly bound to the coenzyme molecule were included in the F_{calc} values and therefore do not appear (contour levels at ± 1.5 r.m.s.).

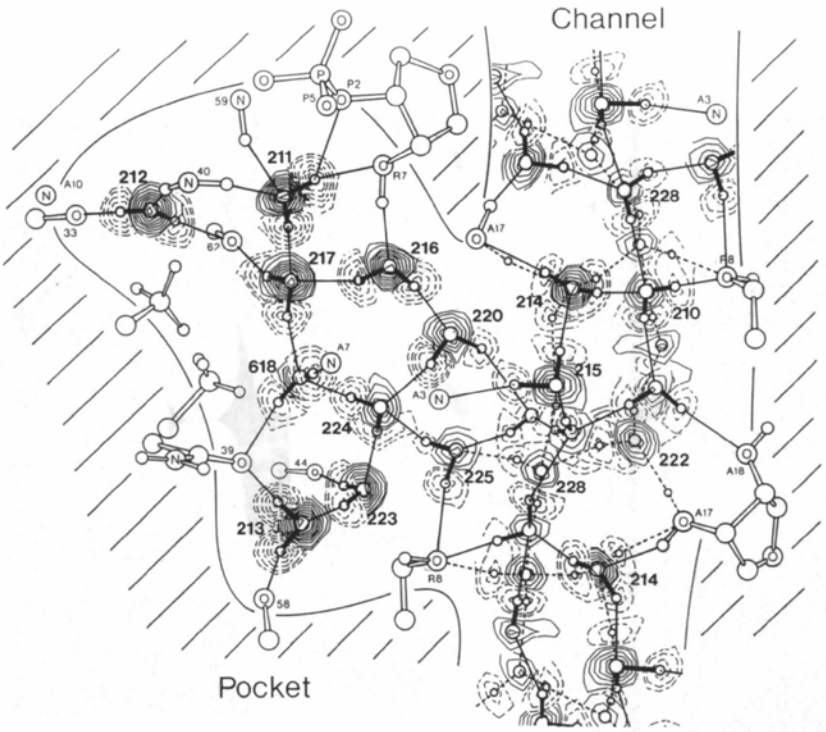

Fig. 8. Water networks in the 15 K crystal of vitamin B_{12} coenzyme. Large open circles: O; small open circles: H. Intervals at 1 r.m.s.; dashed lines are negative contour levels, solid lines positive contour levels. Zero contour level is the first negative contour. Dotted lines between atoms indicate the alternative network.

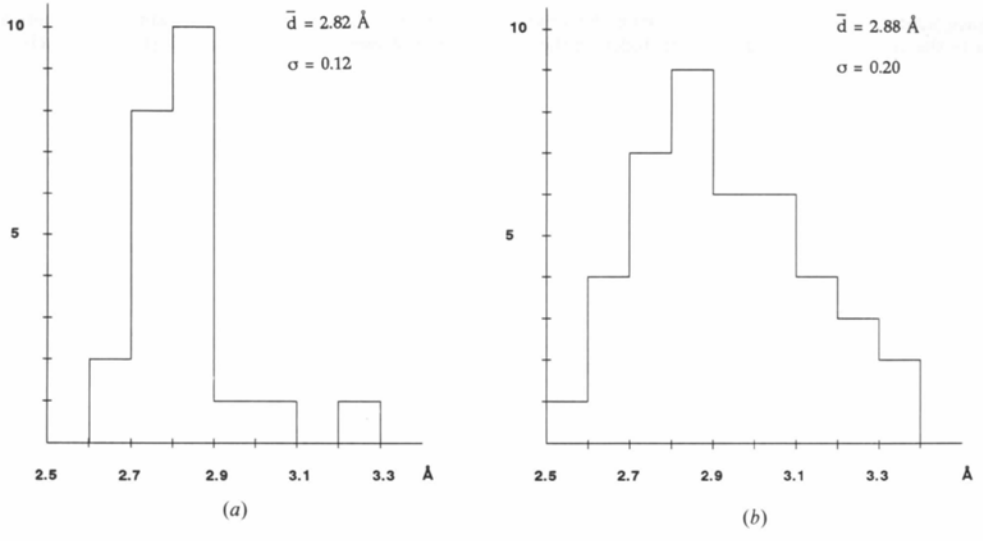

Fig. 9. Distribution of water–water O···O distances (Å) at (*a*) 15 and (*b*) 279 K.

then $x_H b_H = -(1.0 - x_H)b_D$ and $x_H = -b_D/(b_H - b_D)$, $x_H = -0.667/(-0.374 - 0.667) = 0.64$ and $y_D = 0.36$.] The results are outlined in Table 7.

The maximum e.s.d. on occupancies for these atoms is 0.04. At this stage there is no evidence of any peaks associated with H/D's 192 or 193, indicating that the exchange is such that the H and D contributions effectively cancel each other, and $x_H \simeq 0.64$, $y_D \simeq 0.36$.

(vii) *Solvent networks.* In total at this stage 56 solvent atoms (O and H) were identified and distributed over 23 sites. The structure at 279 K has 113 solvent atoms distributed over 47 sites. In the case of the solvent structure, lowering the temperature gives sharper density peaks, thereby improving the precision as the dynamic disorder associated with these molecules at 279 K is substantially reduced at 15 K (see Tables 8*a* and 8*b*). Complete four-, five- and six-membered rings can easily be identified. Figs. 7 and 8 show the water networks in the 15 K structure; these can be subdivided into an 'ordered' region, the pocket, where the solvent sites are clearly defined, with unit occupancies, and a 'disordered' region, the channel, where the sites overlap and have lower occupancies.

The hydrogen-bond lengths in the main water networks at 15 K range from 2.61 to 3.03 Å; they are more tightly centred around 2.8 Å than at 279 K. There is less disorder at 15 than at 279 K, and therefore less sites; this leads to fewer hydrogen bonds. Fig. 9 shows the distribution of hydrogen-bond distances at both temperatures.

5. Concluding remarks

High-resolution single-crystal diffraction studies at low temperature lead, as expected, to very much lower temperature factors and reduced static disorder. The solvent molecules are clearly identifiable with well resolved density peaks; water networks can be readily formulated using neutron diffraction at 1.0 Å resolution; a shrinkage of 2% in the unit-cell volume and a 2° reorientation of the coenzyme molecule were observed. It is interesting to note that a similar study of β-cyclodextrin (156 atoms, with one ethanol and eight water molecules) at low temperature (15 K) by Steiner, Mason & Saenger (1990) shows a similar unit-cell shrinkage (3.5%), together with a 2.4° change in the β angle of the monoclinic unit cell from the values at 295 K. However, to carry out such studies using neutrons, 'large' crystal sizes are needed ($\sim 5\,mm^{-3}$), and for low-temperature studies the crystals must be amenable to a freezing technique. Low-temperature high-resolution neutron studies of protein crystals are more problematic, not only because of the difficulty of growing large enough crystals, but also because the high water content in these crystals often leads to difficulties in freezing. Recently, rapid and flash freezing methods applied to small protein crystals in conjunction with high intensity synchrotron radiation have shown promise, and in the case of the bovine lens protein, γII-crystallin, data have been collected to 1.2 Å at 150 K (Lindley, Glover, Najmudin & Slingsby, 1992); preliminary processing of this data has shown that flexible side chains such as arginine appear well ordered at low temperature, and it will be interesting to see how much more information will result from this study. In the case of the B_{12} study, where both X-ray and neutron data are available, a comparison of scattering-density maps (Savage, 1986) clearly showed that the solvent oxygens are well defined by X-rays and that the neutron data then serves to unravel the patterns of disorder. Whenever solvent structure is of importance for the understanding of the function of the macromolecule, it will be necessary to undertake a neutron study despite the obvious obstacles.

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