THE CHEMISTRY OF ANTI-PERNICIOUS ANÆMIA FACTORS

PART X. SOME CHLORINATION PRODUCTS OF VITAMIN B_{12}

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THE introduction of halogen into a metabolite molecule is generally accompanied by changes in biological properties which may be so marked as to render the product an antimetabolite.[1] It was, therefore, of interest to examine the action of chlorine on cyanocobalamin, when it was hoped to obtain a compound antagonistic in action to vitamin B_{12}.[2,3,4]

Addition of chlorine water step by step to cyanocobalamin solution was accompanied by a gradual colour change to deep purple, after which further addition resulted in destruction of the chromophoric system originally present in the product. It was thus possible to use optical methods to determine the point at which maximum conversion into the purple material had occurred. For this purpose a convenient volume of a 0·01 per cent. aqueous solution of cyanocobalamin at *ca.* *p*H 4 was titrated drop by drop with chlorine water of known concentration and the progress of the reaction followed by absorption measurements with an automatic recording spectrophotometer.[5] The results obtained revealed a progressive change in absorption until 3 moles of chlorine had been added, when the purple solution possessed the highly characteristic spectrum shown in Figure 1, curve 4, after which further addition led to reduction in the absorption intensity in the visible and near ultra-violet regions. By stopping the addition at the point represented by curve 4, Figure 1 and extracting the solution with phenol in the usual way, an almost black amorphous product was obtained designated *CP–AB* (≡ chlorinated product *AB*; *vide infra*). Analysis indicated the presence of 2 atoms of chlorine in the molecule.

The absorption spectrum of *CP–AB* shows a general resemblance to that of cyanocobalamin in the visible and near ultra-violet regions of the spectrum (Fig. 2; see also Table I). It follows that the chromophore present in the chlorinated product is similar to that present in vitamin B_{12}.

Cobalt estimations on *CP–AB* reveal a molecular weight which approximates closely to that of the parent vitamin. The existence of co-ordinated cyanide in the molecule is shown by the observation that oxidation with potassium permanganate leads to the liberation of hydrogen cyanide which is readily detected by the copper benzidine test.[6] Reaction with excess of potassium cyanide solution results in the formation of a deep blue "cyano–*CP–AB*," the absorption spectrum of which is shown in Figure 3. This complex, in striking contrast to dicyanocobalamin,[7] remains unchanged at *p*H 4 and only *slowly* reverts at *p*H 2 to material with the absorption spectrum of *CP–AB*. It is thus far more stable than its B_{12} analogue.

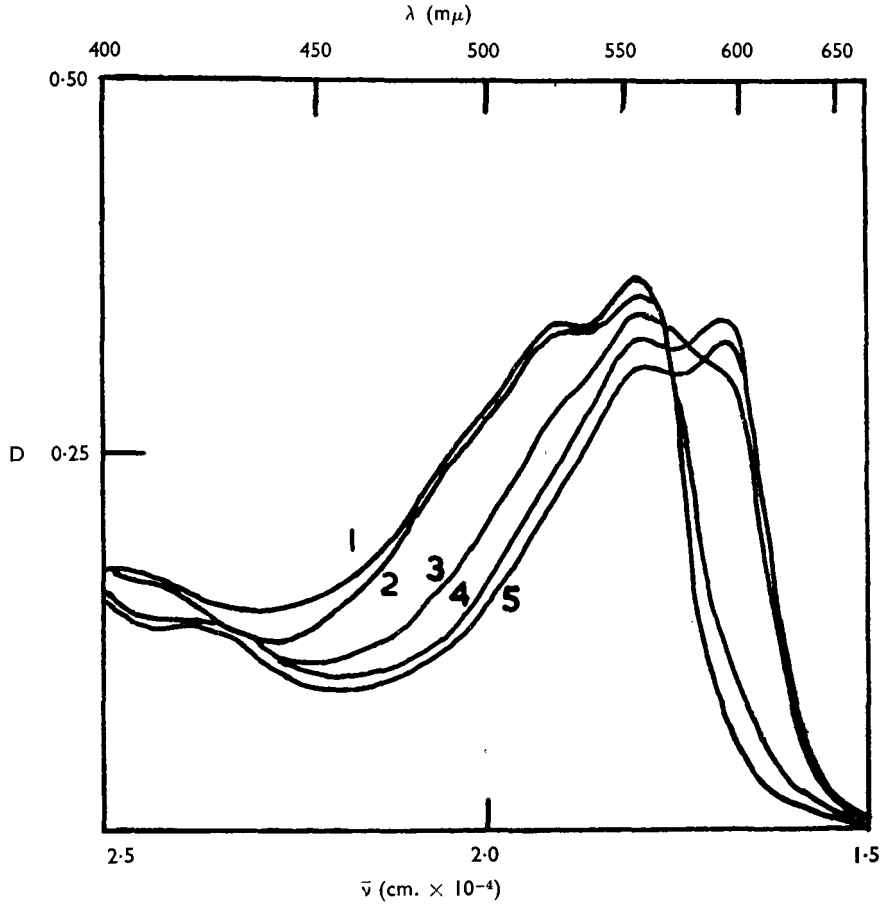

FIG. 1. Visible absorption spectra in 0·001N acetic acid of 1, Vitamin B_{12}; 2, 3, 4 and 5, products obtained after 24 hours from treating vitamin B_{12} with 1, 2, 3 and 4 mol. equivalents of chloramine-T, respectively.

TABLE I

Compound	*p*H	$\lambda_{max.}$ (mμ) in aqueous solution									
CP–AB	2	278	286	—	(315)	(250)	363	(405)	(510)	555	—
"	6	282	289	—	(315)	(345)	365	(415)	(525)	555	585
"	10	279	287	—	—	(355)	366	(420)	—	(560)	580
"cyano-*CP–AB*"	10	281	289	(305)	(315)	(355)	370	(425)	(525)	575	615
CP–A	6	*	*	*	*	—	366	—	—	(560)	588
CP–B	6	279	288	—	—	(350)	366	417	—	(555)	588
"cyano-*CP–A*"	10	*	*	*	(320)	(355)	372	—	(540)	(585)	615
"cyano-*CP–B*"	10	279	288	—	(320)	(355)	372	—	(550)	(583)	619
"red fragment" from *CP–AB*	2	—	286	—	—	(340)	353	408	(500)	526	552
" " " "	10	—	289	—	—	(330)	358	—	—	550	578
"cyano-complex" from "red fragment".	10	—	290	(300)	(315)	(355)	370	(425)	(525)	571	610

* Accurate data not available.
Values in parentheses refer to inflections, which are given to ± 5 mμ.

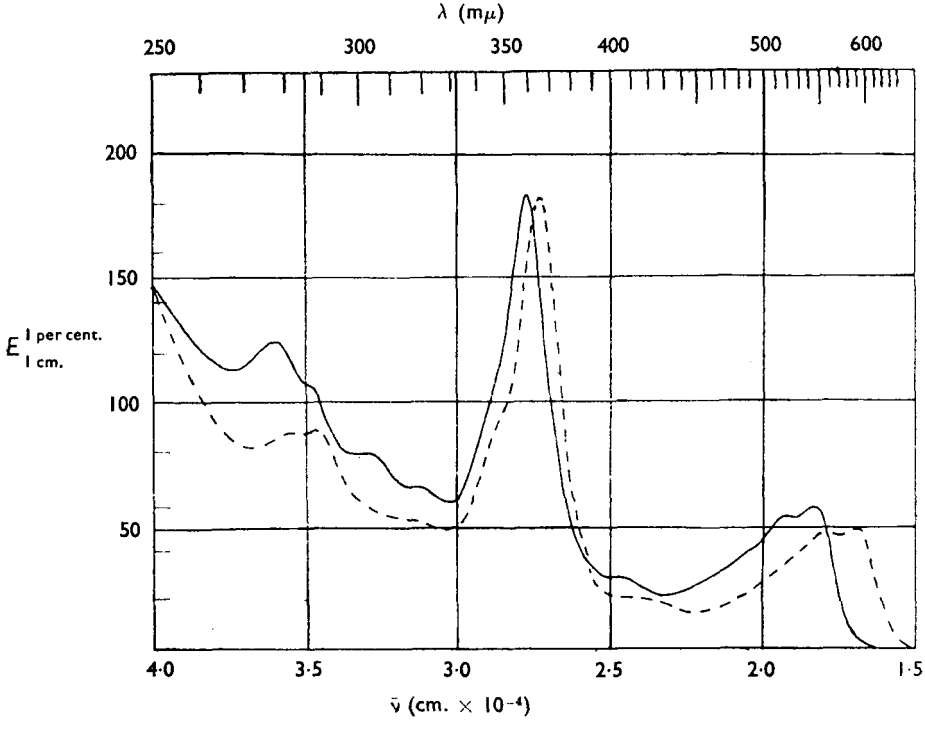

FIG. 2. Vitamin B_{12} (——), and *CP–AB* (— —) in water at *p*H 6.

A further point of difference between *CP–AB* and cyanocobalamin lies in the nature of the cobalt-containing fragments formed on mild hydrolysis with 5N hydrochloric acid at room temperature. Vitamin B_{12}, on such treatment, gives a complex mixture of products from which a single coloured species has not, so far, been isolated. By limiting the time of contact with the acid, however, material is formed consisting essentially of related cobalt-containing fragments of similar absorption spectra. This product, conveniently referred to as "red fragment," resembles the parent vitamin in its behaviour towards cyanide when a purple "cyano-complex" is obtained.[8] *CP–AB*, in contrast, gives reddish-purple material on mild acid hydrolysis, characterised by formation of a blue "cyano-complex" (Fig. 4). It follows that conversion of cyanocobalamin into *CP–AB* is accompanied by changes in the structure of the cobalt-containing fragment present in the vitamin. The benziminazole part of the molecule, in contrast, remains unchanged as hydrolysis leads to the formation of the substituted benziminazole and ultimately of 5: 6-dimethyl-benziminazole itself.[9]

Paper chromatography of *CP–AB* employing butanol (4 vols.)-acetic acid (1 vol.)-water (5 vols.) as irrigation solvent, led to its resolution into two coloured components, hereafter termed chlorinated product A (*CP–A*) ($R_F = 0{\cdot}3$) and chlorinated product B (*CP–B*) ($R_F = 0{\cdot}47$),

respectively. The latter was nearly always present in major amount. With one and the same batch of material, however, the relative proportions of the two components varied from one chromatogram to another. Thus on rare occasions approximately equal quantities of *CP–A* and *CP–B* would be obtained, whilst on others *CP–A* could not be detected by visual inspection of the paper. The position was further complicated by the observation that rechromatography of *CP–B*, following elution, led to its resolution into two spots identified with

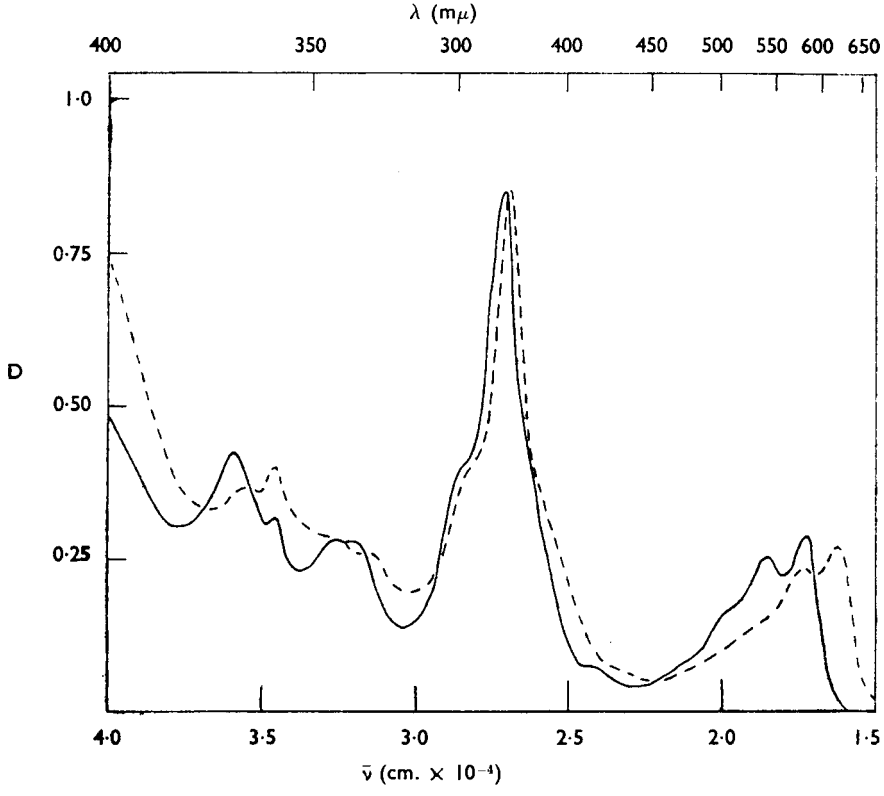

FIG. 3. Dicyanocobalamin (——), and "cyano–*CP–AB*," (– – –) *p*H *ca.* 10 in water (excess of potassium cyanide present).

CP–B and *CP–A*, respectively. The latter material, in contrast, behaved as a single species. It follows that conversion of *CP–B* into *CP–A* occurs on the paper chromatogram.

A similar phenomenon has previously been recorded by Woodruff and Foster[10] who observed the formation of aquocobalamin phosphate[11] from cyanocobalamin on buffered-paper chromatography. As this change is likewise producted by irradiation,[12] the effect of the latter process on *CP–B* was examined when it was hoped that *CP–A* might be obtained. Exposure of *CP–B* in aqueous solution to ultra-violet light, however, was accompanied by a hypsochromic shift of the absorption band at

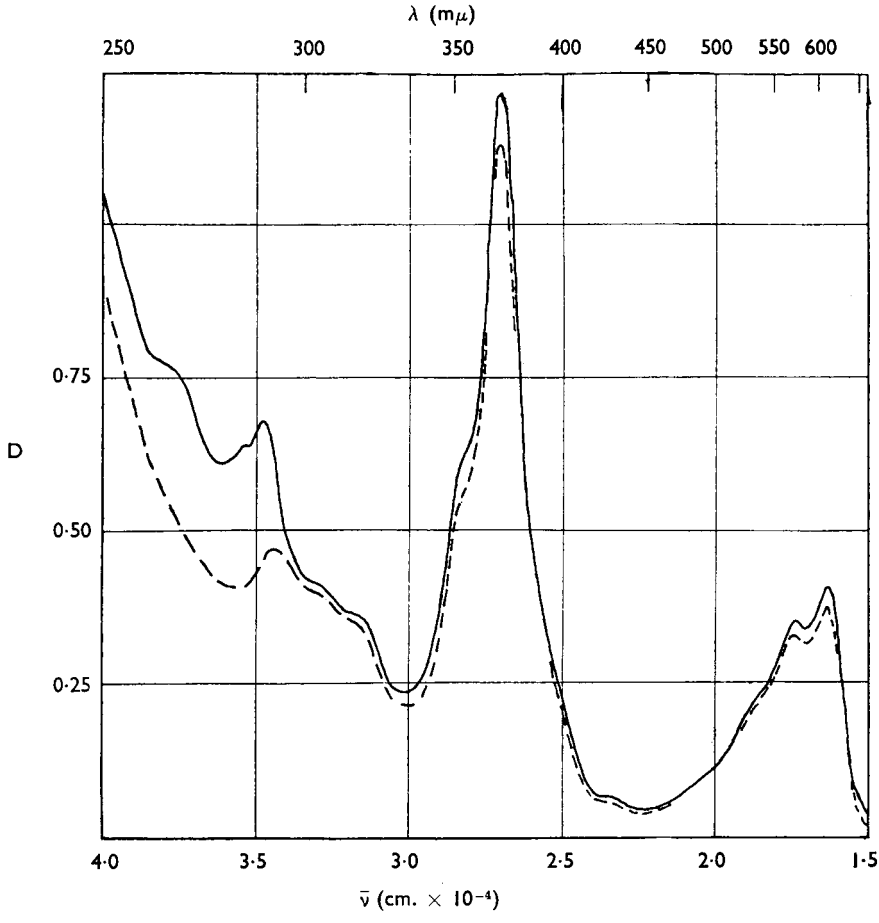

FIG. 4. "Cyano-*CP–AB*" (——), and "cyano-complex" obtained from "red fragment" derived from *CP–AB* (– – –); *p*H *ca.* 10 in water (excess of potassium cyanide present).

366 mμ, the final spectrum being quite different from that of *CP–A*. Formation of the latter compound by loss of CN^- from *CP–B* is thus unlikely. Irradiation of *CP–A* gave similar results.

Separation of *CP–AB* into the same two components was also achieved by a 21 transfer counter-current distribution of the material, employing a two-phase system given by a mixture of carbon tetrachloride (8 pts. vol.)-water (8 pts. vol.)-phenol (6·7 pts. wt.) in which the distribution of *CP–A* is wholly in favour of the upper phase and in which *CP–B* has a distribution coefficient of *ca.* unity. The visible spectra of the isolated compounds, neither of which could be obtained crystalline, is shown in Figure 5.

As halogenation by means of chlorine water presumably involves reaction between cyanocobalamin and the solvated chlorine cation, the use of chloramine-T as a source of "positive" chlorine ions was next examined.

Treatment of vitamin B_{12} in aqueous acetic solution with this reagent resulted in spectral changes similar in character to those previously observed employing chlorine water. Quantitative studies revealed that reaction was virtually complete when 3 moles of chloramine-T had been added to each molecule of cyanocobalamin and that the material so formed was relatively stable to further contact with the reagent (see Fig. 1). The behaviour of chloramine-T thus stands in marked contrast to that of chlorine water, where addition of quantities in excess of 3 moles results in marked degradation of the *CP–AB* produced.

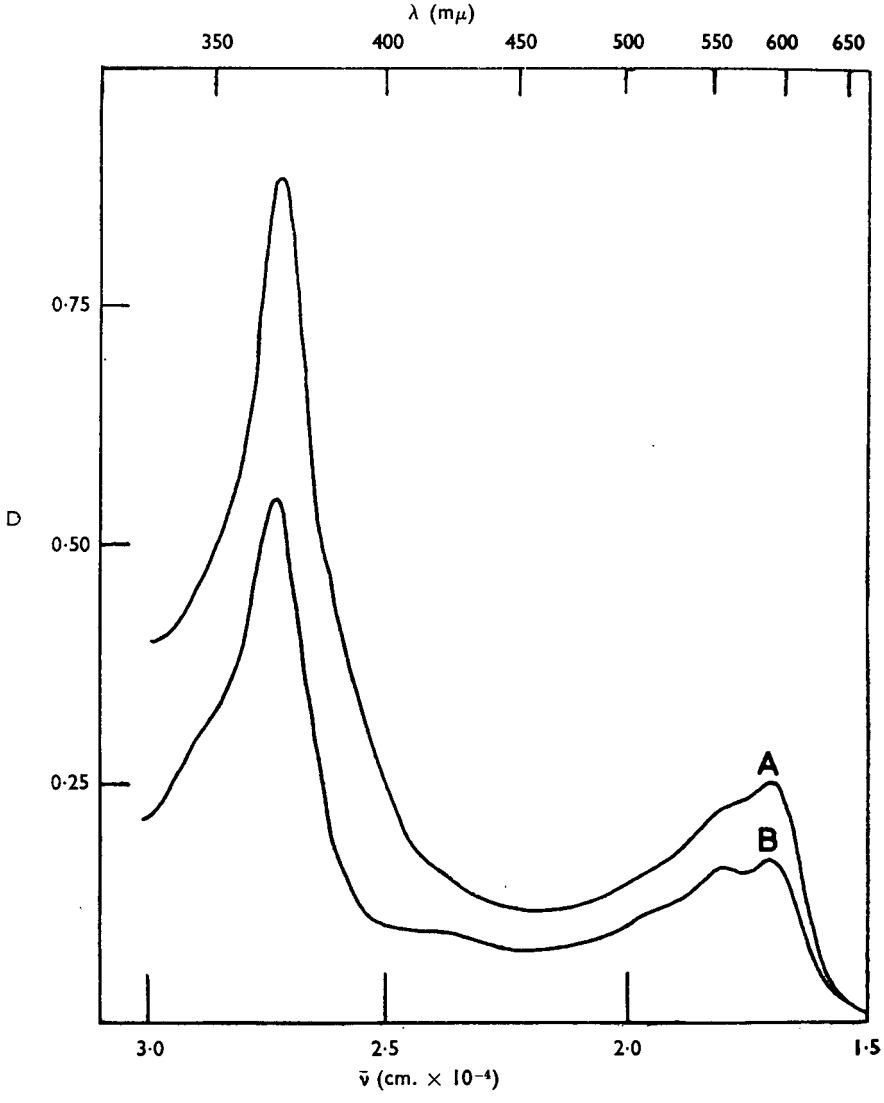

FIG. 5. Visible absorption spectra, in water *p*H *ca.* 6, of *CP–A* and *CP–B*.

The product obtained in this way formed an almost black amorphous solid which could not be induced to crystallise. Paper chromatography employing butanol-acetic acid as irrigation solvent, led to its resolution into three coloured components, two of which were identical with *CP–A* and *CP–B*, respectively. The third component ($R_F = 0{\cdot}65$), designated *CP–C*, was present in quantity comparable to that of *CP–B*, from which it could not be distinguished spectroscopically. Rechromatography of this material was accompanied by its complete conversion into *CP–B*, together with smaller quantities of *CP–A*. Its instability in solution was further demonstrated by the observation that on a series of chromatograms prepared daily for 10 days from aliquots of a freshly dispensed solution of the solid chlorination product (2 mg./100 μl.), the intensity of the coloured zone of $R_F = 0{\cdot}65$ decreased regularly until by the tenth day all trace of *CP–C* had disappeared.

Biological study of *CP–AB* by Dr. S. W. F. Underhill (Physiological Research Laboratories, the B.D.H. Ltd.) has shown that the material cannot replace cyanocobalamin in supporting the growth of *E. coli*. Furthermore, it does not seem to function as an antagonist to this metabolite. Its animal protein factor activity has been kindly assayed by Dr. T. H. Jukes (Lederle Laboratories) who reports that it has no detectable potency.

Inter alia, we have observed that addition of bromine water to an aqueous solution of vitamin B_{12} results in a colour change leading in the course of *ca.* 15 minutes to the formation of a purple product with an absorption spectrum very similar to that of *CP–AB*. Further action of bromine water results in progressive destruction of the material. The same compound is likewise formed in glacial acetic acid, in which solution it appears to be less susceptible to the action of excess of halogen. On adjusting an aqueous solution of the purple material to *p*H 9 and adding cyanide, a blue solution is obtained the absorption spectrum of which is undistinguishable from that of "cyano–*CP–AB*." Attempts to demonstrate an analogous reaction between cyanobocobalamin and iodine proved unsuccessful.

Experimental

Absorption measurements. Absorption spectra were determined with a twin-beam automatic recording spectrophotometer designed and built by Holiday and Sutton.[5] The spectra obtained are linear in wave number ($\bar{\nu}$) and optical density (D), as shown in Figures 1 to 5 which are direct tracings of the records after correction for instrumental zero error.

Paper chromatography. Whatman No. 1 filter paper was used for the chromatograms. These were irrigated overnight at room temperature by the descending technique, employing the upper phase given by a mixture of *n*-butanol (4 vols.)-acetic acid (1 vol.)-water (5 vols.). The materials under examination were applied to the paper strips as visible purple spots (50 to 70 μg. solids). Chromatogram segments were eluted by the method of Dent.[13]

Preparation of CP–AB. A solution of 97 mg. of vitamin B_{12} in 1 l. of

water was brought to *p*H 4 by the addition of a trace of hydrochloric acid, and then treated at intervals of 2 or 3 minutes with 4 quantities, each of 6 ml., of freshly prepared chlorine water containing 0·63 mg. of chlorine per ml. (total quantity of chlorine introduced ≡ 3 mols. per mol. of B_{12}). The colour of the mixture changed rapidly from red to purple during the process, the spectrum in the visible region finally reaching that shown in Figure 2 (curve no. 4). At this stage the coloured product was extracted quantitatively into phenol, from which it was transferred to a small volume of water by the addition of ether. The resulting aqueous solution was extracted several times with ether, evaporated to dryness *in vacuo*, and a solution of the residue in methanol filtered through a column (8 × 2·5 cm.) of neutral alumina. The coloured filtrate was taken to dryness under reduced pressure, and gave 81 mg. of a deep blue-black amorphous solid (*CP–AB*) (Found: Co, 3·75; 4·1. Cl, 5·35; 5·1, 5·82 per cent.), which failed to crystallise from aqueous acetone. It was completely insoluble in non-polar solvents.

Chlorination of vitamin B_{12} employing chloramine-T. Preliminary experiments were carried out in which 4 aliquots (25 ml. each) of a solution of vitamin B_{12} (13·5 mg.) in 0·001N acetic acid (100 ml.) were treated with 1, 2, 3 and 4 mol. equivalents of chloramine-T B.P., respectively. The visible spectra obtained (see Fig. 2) revealed that maximum conversion into the new coloured product occurred when not less than 3 mol. equivalents of the reagent were employed per mol. of B_{12} originally present. For preparative purposes a solution of 27 mg. of vitamin B_{12} in 200 ml. of 0·001N acetic acid was treated with 17 mg. of chloramine-T in 6 ml. of water, and the mixture allowed to stand for 1 hour. Following 2 or 3 extractions of the reaction mixture with ether, the product was isolated with phenol as described above and obtained as an almost black amorphous solid (23 mg.) which failed to crystallise from aqueous acetone.

Summary and Conclusions

1. Addition of 3 mols. of chlorine (water) to cyanocobalamin leads to the formation of a purple product designated *CP–AB*.

2. *CP–AB* contains 2 atoms of chlorine in the molecule.

3. Paper chromatography or counter current distribution leads to the separation of *CP–AB* into two closely related products termed *CP–A* and *CP–B* respectively. The latter undergoes partial conversion into the former component on rechromatography.

4. 3 molar equivalents of chloramine-T react with cyanocobalamin to give a product separated by paper chromatography into *CP–A*, *CP–B* and a third component termed *CP–C*. The latter undergoes change into *CP–B* and thence into *CP–A* on rechromatography.

5. *CP–AB* appears to be biologically inactive.

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REFERENCES

1. Woolley, *A Study of Antimetabolites*; John Wiley and Sons, Inc., p. 219.
2. Holly, Peel, Cahill and Folkers, *J. Amer. chem. Soc.*, 1951, **73**, 332.
3. Antaki and Petrow, *J. chem. Soc.*, 1951, 2873.
4. Woolley, *Abstracts 2nd International Congress of Biochemistry*, p. 486.
5. Holiday and Sutton, *to be published.*
6. Cf. Brink, Kuehl, Jr., and Folkers, *Science*, 1950, **112**, 354.
7. Cooley, Ellis, Petrow, Beaven, Holiday and Johnson, *J. Pharm. Pharmacol.*, 1951, **3**, 271.
8. Beaven, Holiday, Johnson, Ellis and Petrow, *ibid.*, 1950, **2**, 944.
9. Beaven, Holiday, Johnson, Ellis, Mamalis, Petrow and Sturgeon, *ibid.*, 1949, **1**, 957.
10. Woodruff and Foster, *J. biol. Chem.*, 1950, **183**, 569.
11. See Ellis and Petrow, *J. Pharm. Pharmacol.*, 1952, **4**, 152 for nomenclature.
12. Veer, Edelhausen, Wigmenga and Lens, *Biochem. Biophys. Acta*, 1950, **6**, 225.
13. Dent, *Biochem. J.*, 1947, **41**, 240.

Correction.

THE EFFECT OF PENICILLIN AND OF STREPTOMYCIN ON BLOOD COAGULATION IN NORMAL SUBJECTS

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TABLE II

Opposite Week 4, in column 4, insert I.