THE CHEMISTRY OF ANTI-PERNICIOUS ANÆMIA FACTORS

PART XI. THE PREPARATION AND PROPERTIES OF SOME COBALT PORPHYRINS

BY (MISS) R. J. McCONNEL, B. G. OVERELL, V. PETROW
and B. STURGEON

From the Research Laboratories, The British Drug Houses Ltd., London, N.1

IN 1948, at the start of our studies on the chemistry of vitamin B_{12}, we[1] were led to interpret the close association of this compound with hæmopoiesis as possibly indicating its structural similarity to the porphyrins. Further indications in this direction were provided (i) by the absorption spectrum of the material, which showed a superficial resemblance to that of certain metalloporphyrins, and (ii) by the existence of cobalt in the molecule, a metal related to iron and likewise capable of reversible oxidation-reduction reactions. We therefore turned our attention, during 1949, to the preparation of certain cobalt porphyrins and cobalt azaporphyrins. Work on the latter group seemed specially pertinent to the enquiry as Lemberg[2] had previously shown that replacement of a bridge methine group in hæmin by nitrogen was accompanied by increased stability of the complex towards concentrated sulphuric acid. Cobaltiprotoporphyrin, its dimethyl ester, cobaltihæmatoporphyrin, cobaltiazamesoporphyrin, and cobaltipheophytins were prepared and studied during this phase of the work, which terminated in the early months of 1950. The results obtained are reported in *Section A*.

The second phase of the investigation derived from the observations of Beaven, Holiday, Johnson, Ellis and Petrow[3] that the benziminazole nucleus present in the B_{12} molecule was co-ordinately linked to cobalt as indicated in (I). The structural feature so presented, however, had

(where Py = pyridine)

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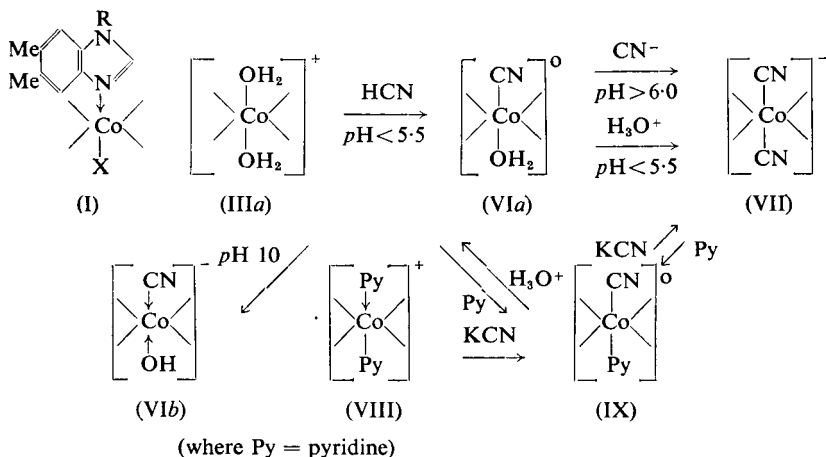
no direct analogy to known types. It was therefore deemed necessary to seek additional evidence in its support. For this purpose experiments were undertaken on the co-ordination of benziminazole derivatives with certain cobalt complexes (Parts VIII[4] and IX[5]) and with the cobalt porphyrins. The latter group, in particular, seemed specially suitable, as we[3] envisaged the cobalt-containing fragment of the B_{12} molecule in terms of a planar structure[6] spatially akin to the porphin ring.

Parallel with these developments, Brink, Kuehl and Folkers[7] presented evidence to show that a cyano-group was bound directly to the central cobalt atom in vitamin B_{12}. We therefore extended our co-ordination studies to include the cyano-cobaltiporphyrins and the corresponding benziminazolo-cyano types (cf. I; X = CN). The data so obtained is collected in *Section B*.

The study of systems involving metalloporphyrins is both complicated and difficult as many of the reactions undergone by these compounds are reversed with extreme facility. The common methods of organic chemistry are thus of limited value in this field. We have, therefore, been forced to rely almost exclusively on spectrophotometric methods in studying the co-ordination phenomena undergone by the cobalt porphyrins. Such methods, however, have obvious limitations in this instance. The conclusions reached herein must consequently be regarded, at least in part, as tentative.

Spectra were measured with a Hartridge reversion spectroscope and with a Unicam spectrophotometer. The latter instrument was kindly placed at our disposal by Dr. R. E. Stuckey and Mr. P. Stross, B.Sc. (Analytical Department, The British Drug Houses Ltd.), whose help we most gratefully acknowledge.

SECTION A. THE PREPARATION AND SPECTRAL CHARACTERISTICS OF SOME COBALT PORPHYRINS

Cobaltihæmatoporphyrin was obtained some 50 years ago by Laidlaw.[8] Since then other compounds of this type have been prepared by treating the appropriate porphyrin with a cobalt salt. Hæmin has naturally formed the starting point for many of these investigations as its conversion into the metal-free porphyrin is readily effected by reductive or hydrolytic methods which can be so chosen as to effect simultaneous reduction or hydroxylation of the vinyl side-chains.

Cobaltiprotoporphyrin chloride (II; R = H), obtained as indicated above, was characterised by its absorption spectrum, which is shown in curve 2, Figure 2 (cf. Holden[9]). The metalloporphyrin so formed was almost insoluble in the usual solvents and thus not entirely suitable for co-ordination studies with heterocyclic ligands. We therefore turned our attention to the methyl ester of (II; R = H) which, in addition to greater solubility, offered the added advantage that salt formation between the propionic acid side-chains and a co-ordinating base would no longer be possible (cf. Hamsik[10]).

The required compound was readily obtained by treating protoporphyrin dimethyl ester with cobalt*ous* acetate in acetic acid solution.

Its constitution as cobalt*ic* protoporphyrin dimethyl ester (II; R = Me) followed from its absorption spectrum (curve 1, Fig. 1) which showed the two bands typical of cobaltic compounds of this type. The material, however, could not be obtained crystalline, whilst analytical data, though consistent for different batches, failed to distinguish between a structure possessing a hydroxide anion and one possessing an acetate anion associated with 2 moles of acetic acid of crystallisation. Treatment with hydrochloric acid led to the formation of cobaltiprotoporphyrin-dimethyl ester chloride, a compound soluble in organic solvents and thus suitable for co-ordination work. Its absorption spectrum in methanol (containing 1 per cent. of chloroform) revealed two bands at 561 and 530 mμ, respectively.

Cobaltihæmatoporphyrin (III; $\equiv$ IIIa) was prepared as described by Hill.[11] The absorption spectrum of the compound revealed two bands at 559 and 525 mμ over the pH range 2 to 7. Increase in alkalinity to pH 11 was accompanied by a spectral shift to longer wavelengths with bands at 565 and 531 mμ.

Cobaltiazamesoporphyrin (V). Initial experiments were directed to the preparation of cobaltiazaprotoporphyrin. Aerial oxidation of pyridine protohæmochrome in the presence of hydrazine led to the formation of verdohæmochrome (IV) (Lemberg[12]), transformed by ammonium hydroxide into monoazahæmin, which was isolated as the dihydrate.[12] Conversion of this compound into the metal-free porphyrin, however, proved unexpectedly difficult. Attempts to repeat the method quoted by Fischer and Muller[13] employing hydrazine hydrate in glacial acetic acid, led only to partial removal of the metal atom. By raising the temperature of the reaction to 80° C., however, an iron-free product was ultimately obtained but in such low yield that we were forced to discontinue the proposed reaction sequence.

Attention was then turned to the preparation of the corresponding monoazamesoporphyrin. Fischer and Pützer[14] had previously effected the conversion of hæmin into mesoporphyrin-IX by heating with a palladous oxide catalyst in formic acid solution. Monoazahæmin, in contrast, proved unstable to this reagent, being rapidly decomposed on boiling for periods of 15 minutes or longer. By limiting the time of heating to 10 minutes, however, and working in very dilute solution, it proved possible to effect the desired reaction in moderate yield to give monoazamesoporphyrin, identified by its absorption spectrum.[12] The intermediate formation of the corresponding protoporphyrin, which can be detected in similar reductions of hæmin,[15] was not observed in the present instance.

Reaction of monoazamesoporphyrin with cobaltous acetate in acetic acid solution saturated with sodium chloride, led to the formation of a metalloporphyrin which separated from chloroform-ether in small, purple-red, matted needles. The constitution of cobaltimonoazamesoporphyrin (V) has been assigned to this compound in preference to its alternative formulation as the corresponding cobalto-base. It is true the material showed certain spectral characteristics not hitherto recorded in the

cobalti series of compounds. Thus, the β-band was evident in alkaline solution as a small inflection at *ca.* 535 mμ (curve 1, Fig. 3). Again, the acid-alkali shift of the main absorption band (from 568 mμ at *p*H 10·5 to 610 mμ at *p*H 2·0) was greater than for (II) and (III). Nevertheless, its formulation as the cobalti-compound is rendered likely by the observation that reduction with sodium hydrosulphite in alkaline pyridine solution leads to the formation of a reduced form which now shows the typical absorption characteristics of a cobalto-porphyrin with a single (diffuse) absorption band at about 558 mμ (cf. Taylor[15]).

In addition to exerting a marked effect on the absorption spectrum of the compound, the methine-replacing nitrogen atom of (V) increased somewhat the stability of the metalloporphyrin towards elimination of the cobalt atom. Thus removal could not be effected with concentrated sulphuric acid, reduction with palladous oxide/formic acid being required for regeneration of the azaporphyrin. *Inter alia*, we observed that reduction to the cobalto-form could not be effected by sodium hydrosulphite in alkaline solution except in the presence of pyridine (see above).

Attempts to oxidise cobaltiprotoporphyrin (II) to the cobalt analogue of verdohæmochrome (IV) and thus simplify the preparation of cobalt azaporphyrins proved unsuccessful, the starting material being recovered unchanged.

Cobalt pheophytins. Studies on the cobalt derivatives of the plant porphyrins have not hitherto been described in the literature. We now find that by treating the mixture of pheophytins *a* and *b* from *Urtica dioica* with cobaltous acetate, a lustrous green material is obtained which is apparently composed of the corresponding cobalt(ic) pheophytins *a* and *b* as the absorption spectrum of the product shows double peaks in the regions 660 to 670 mμ and 410 to 430 mμ, respectively.

Regeneration of the pheophytin mixture from the cobalt derivatives could not be accomplished. The material was unaffected by acidolysis under conditions preserving the porphyrin framework, whilst methods employing reductive fission led always to hydrogenation of the ring system.

SECTION B. CO-ORDINATION STUDIES

Work on the co-ordination of benziminazoles with cobalt porphyrins was initiated prior to studies employing cyanide as the ligand (cf. p. 180). Discussion of the results obtained, however, is more conveniently effected by reversing the order of presentation.

The action of cyanide on the cobalt porphyrins. Taylor[15] has reported the spectral changes produced by adding cyanide to cobalti- and cobalto-mesoporphyrin, whilst Holden[9] has recorded the spectrum of a cyano-protoporphyrin. Little else is described in the literature. The association of cyanide with hæm, in contrast, has been the subject of much study.[16] It seems reasonably certain that step by step association occurs to give monocyanide and dicyanide ferroporphyrin according to the conditions employed. At high *p*H values the two steps of association are quite

distinct, the monocyanide being formed almost to completion before production of the dicyanide becomes evident.

Conversion of hæm to monocyanide ferroporphyrin is accompanied by marked changes in absorption with production of a hæmochrome type of spectrum (intensity α-band > β-band). Further addition of cyanide to the metalloporphyrin fails to alter the general pattern of absorption, but leads instead to reversal in the relative intensities of the α- and β-bands, together with a general bathochromic shift.[17] Evidence regarding other cyanide-porphyrin adducts, however, is somewhat fragmentary in character. Conclusions based solely on spectral data must consequently be accepted with some reserve.

Reaction of cobalti-protoporphyrin dimethyl ester (II; R = Me) in methanol (+1 per cent. of chloroform) with methanolic potassium cyanide led to the formation of a reddish-yellow adduct ("b"-adduct) stable at pH > 8 and characterised by a di-cyanide type of absorption spectrum with a broad β-band at 555 mμ overshadowing in intensity a weak α-band at 588 mμ (curve 3, Fig. 1). Addition of acetic acid to the solution produced a colour change to red with changes in the absorption spectrum which now resembled the monocyanide type with α- and β-bands of approximately equal intensity at 567 and 535 mμ, respectively (curve 2, Fig. 1) ("a"-adduct). Addition of alkali caused reversion of the spectrum to that of the "b"-adduct.

Similar changes were observed in the action of cyanide on cobalti-hæmatoporphyrin (III). In this case, however, the solubility of the porphyrin in aqueous media allowed a more precise study of the systems involved. Addition of potassium cyanide to an alkaline solution of cobaltihæmatoporphyrin (at pH 10·0) led to the formation of the "b"-adduct, characterised by a broad absorption band of β-type at 537 to 560 mμ, together with an α-band of lower intensity at 582 mμ. When
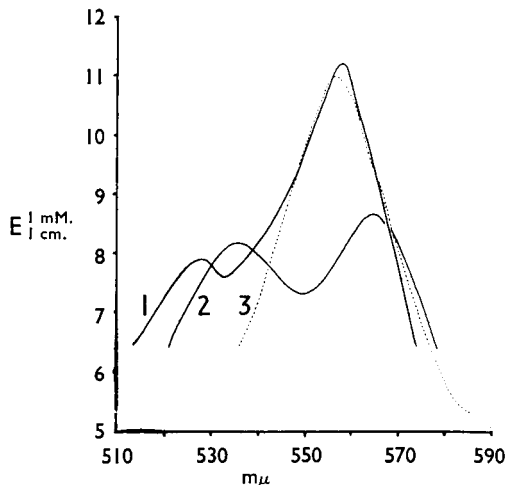

FIG. 1. Stock solution: 0·25 mM Cobaltiprotoporphyrin dimethyl ester in methanol containing 5 per cent. of chloroform.

Curve 1. Cobaltiprotoporphyrin dimethyl ester; 2 ml. of stock solution diluted with 8 ml. of methanol.

Curve 2. Cyanocobaltiprotoporphyrin dimethyl ester; 2 ml. of stock solution, 2 ml. of acetic acid and 6 ml. of 100 mM methanolic potassium cyanide.

Curve 3. Dicyanocobaltiprotoporphyrin dimethyl ester; 2 ml. of stock solution and 8 ml. of 100 mM methanolic potassium cyanide solution.

this solution was titrated spectrophotometrically with acetic acid a spectral change was observed at *ca.* pH 6·0 which, when complete, had altered the character of the absorption spectrum to that of an "a"-adduct with α- and β-bands of approximately equal intensity at 563 and 530 mμ, respectively.

Before concluding from this evidence that (II; R = Me) and (III) were able to form monocyanide and dicyanide adducts, it seemed necessary
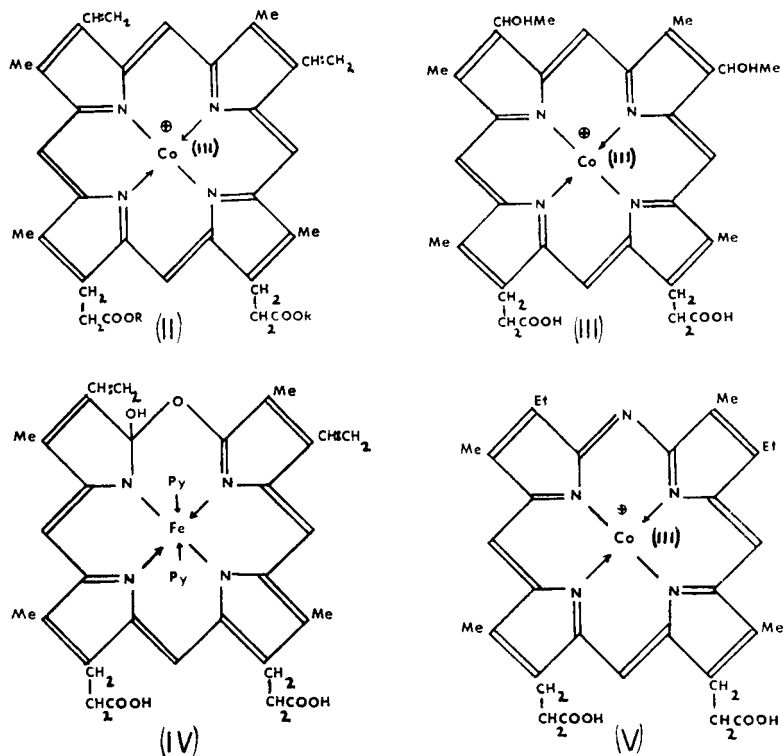
to eliminate the possibility that the observed spectral changes were simply manifestations of an acid-alkali shift. For this purpose we undertook a semi-quantitative study of the action of cyanide on (III ≡ IIIa).

A series of solutions of cobaltihæmatoporphyrin (1 mM) in aqueous acetic acid was prepared to which quantities of cyanide varying from 0·5 to 10 molar proportions were added. Time was allowed for equilibration, after which the solutions were examined spectroscopically. The following results were obtained: (i) Solutions containing one or more moles of cyanide gave spectra identical with that of the "a"-adduct. (ii) The solution containing 0·5 mole of cyanide gave a blurred spectrum intermediate in character between that of the "a"-adduct and the unchanged metalloporphyrin. One mole of cyanide is therefore necessary and sufficient to convert (III) into the "a"-cyanide adduct which is

accordingly formulated as monocyanide cobaltihæmatoporphyrin (partial formula VI*a*).

The solutions were then basified to *p*H 10·0 and the spectra again determined. The following changes were observed. (i) The α- and β-bands in the monocyanide now occupied positions at 570 and 537 m*μ*, respectively. The extent and direction of this movement recalls that previously recorded for the acid-alkali shift of cobaltihæmato-porphyrin (see p. 181) and leads to the conclusion that the compound is now present in the hydroxycyanide form (VI*b*). (ii) The absorption spectra of solutions containing more than one mole of cyanide showed a gradual change towards that of the "b"-adduct with increasing cyanide concentration. A considerable excess of cyanide was required, however, to effect complete conversion into this adduct for which the dicyanide structure (VII) is clearly the preferred formulation.

Similar results were obtained employing the "a"- and "b"-adducts from cobaltiprotoporphyrin dimethyl ester (II; R = Me), which are accordingly formulated as the monocyanide and dicyanide, respectively. The conclusions thus reached on spectroscopic grounds were further confirmed, in this instance, by isolation of the monocyanide in the solid state. Good analytical figures could not be obtained, however, as the adduct proved somewhat unstable, undergoing facile dissociation into its component parts. Thus its spectrum in chloroform showed marked evidence of dissociation with blurring of bands when the solution was warmed, with recombination on cooling. 2·5 moles of potassium cyanide sufficed to effect complete conversion of the free metalloporphyrin into the dicyanide.

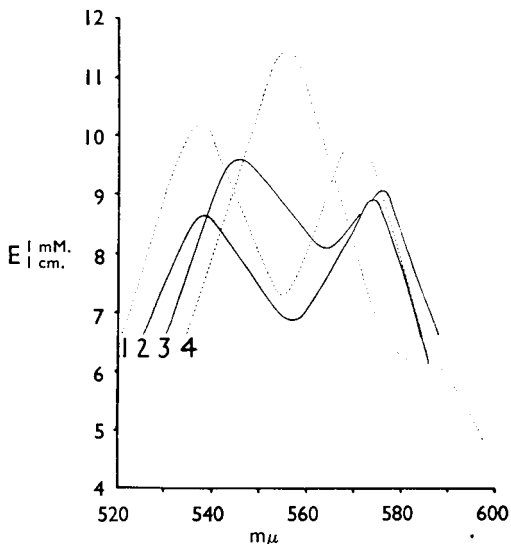The action of cyanide upon cobaltiprotoporphyrin (II; R = H) in

FIG. 2. Stock solution: 0·3 mM cobaltiprotoporphyrin chloride in 0·16 N sodium hydroxide solution.

Curve 1. Dipyridinocobaltiprotoporphyrin; 2 ml. of stock solution, 2 ml. of pyridine and 6 ml. of water.
Curve 2. Cobaltiprotoporphyrin chloride; 2 ml. of stock solution diluted to 10 ml. with water.
Curve 3. Pyridine cyanocobaltiprotoporphyrin; 2 ml. of stock solution, 2 ml. of pyridine, ·0·5 ml. of 50 mM aqueous potassium cyanide solution and 5·5 ml. of water.
Curve 4. Dicyanocobaltiprotoporphyrin; 2 ml. of stock solution and 8 ml. of 50 mM potassium cyanide solution.

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alkaline solution led to the formation of the adduct described by Holden[9] with bands at *ca.* 589 and 554 mμ (curve 4, Fig. 2). This spectrum is clearly of the "b"-type from which it seems likely that (II) exists under these experimental conditions as the dicyanide. Attempts to convert the latter into a monocyanide by acidification proved unsuccessful, precipitation occurring at *p*H 7·0. The cyanide complex of cobaltimesoporphyrin, described by Taylor,[15] likewise shows an absorption spectrum of the "b"-type. A dicyanide structure is consequently indicated. The absorption spectrum of cobaltiazamesoporphyrin (V) in alkaline cyanide solution (curve 2, Fig. 3) gives evidence for adduct formation, but fails to indicate the molar proportion of ligand involved in the combination.
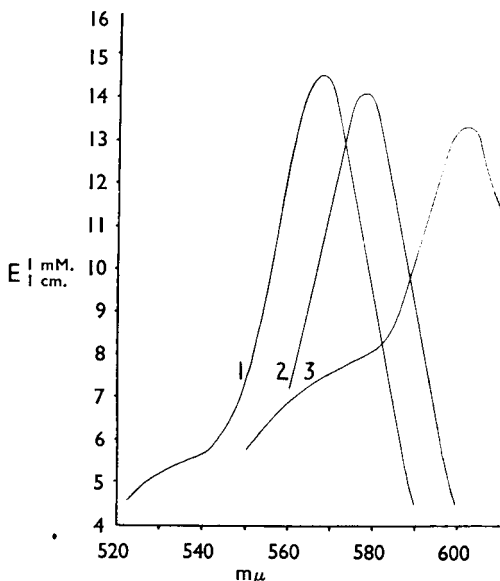

FIG. 3. Stock solution: 0·3 mM Cobaltiazamesoporphyrin in 0·08 N sodium hydroxide solution.

Curve 1. Cobaltiazamesoporphyrin (alkaline); 1 ml. of stock solution diluted to 5 ml. with water.
Curve 2. Cobaltiazamesoporphyrin — cyanide adduct; 1 ml. of stock solution, 1 ml. of 100 mM potassium cyanide and 3 ml. of water.
Curve 3. Cobaltiazamesoporphyrin (acid); 1 ml. of stock solution diluted to 5 ml. with 0·1 N hydrochloric acid.

The co-ordination of benziminazoles with metalloporphyrins. The co-ordination of simple nitrogenous ligands with ferroporphyrins to form hæmochromes (VIII) is readily detected spectrophotometrically. Thus reduction of hæmatin in aqueous pyridine solution gives rise to pyridine protohæmochrome which is characterised by a very sharp α-band at 558 mμ, accompanied by a β-band of less intensity at 525 mμ.[18] The position regarding dicyclic ligands, in contrast, is less satisfactory. Thus quinoline apparently fails to form a hæmochrome owing to steric hindrance by the aryl ring[19] (see also the supporting publication by Cowgill and Clark[19] which appeared after completion of the present investigation), whilst 1-methylbenziminazole is stated by Keilin[20] to form a hæmochrome, although the experimental evidence permitting this conclusion is not reported in full.

We now find that by reducing alkaline hæmatin in the presence of 1-methylbenziminazole, material giving a typical hæmochrome-type spectrum with bands at 557·5 and 532·5 mμ is readily formed (curve 1, Fig. 4; the extinction is not necessarily maximal as the ligand was present

in a concentration relatively low for measurements of this type). Coupled with the observation that similar spectra are likewise obtained from the series of benziminazoles given in Table I, it seems reasonable to infer that formation of benziminazole hæmochromes does, in fact, take place under these experimental conditions.

TABLE I

BENZIMINAZOLE HÆMOCHROMES

Benziminazole	Hæm concentration $M \times 10^{-5}$	Ligand concentration $M \times 10^{-2}$	Ratio of ligand concentration to hæm concentration	pH	α-Band		β-Band		pKa of the ligand, in ethanol (50 per cent.)
					Position mμ	$E_{1\,cm.}^{1\,mM.}$	Position mμ	$E_{1\,cm.}^{1\,mM.}$	
1-Ethyl-	2·45	1·00	414	12·1	560·0	26·2	527·5	11·1	4·88
1-n-Propyl-	2·52	1·03	407	11·6	562·5	24·5	530·0	10·7	4·83
1-isoPropyl-	2·43	1·02	422	12·2	562·5	34·6	530·0	11·0	4·97
1-Allyl-	2·52	1·00	398	12·0	562·5	25·7	530·0	11·0	4·58
1:5-Dimethyl-	2·49	1·01	407	12·3	565·0	25·9	530·0	10·8	5·22
5-Chloro-1-methyl-	2·52	1·0	398	11·6	567·5	26·9	537·5	11·5	3·88
5-Bromo-1-methyl-	2·52	0·025	9·9	11·5	567·5	21·3	532·5	10·0	—

The formation of these hæmochromes seems to depend upon (i) the basicity of the ligand, which must be high enough to permit the formation of a co-ordinated band with the iron atom of the metalloporphyrin, and (ii) the water solubility of the base, which must be sufficient to ensure the formation of the hæmochrome in a concentration which allows its detection spectroscopically. Thus 1:5:6-trimethylbenziminazole (pKa 5·45 in 50 per cent. ethanol[4]) gives only trace quantities of hæmochrome (curve 2, Fig. 4, in which a diffuse α-band at 555 mμ is associated with a slight inflection at 523 mμ). The less basic but somewhat more soluble 1:5-dimethyl-pKa 5·22[4]), 5-chloro-1-methyl- (pKa 3·88[4]) and 5-bromo-1-methyl-
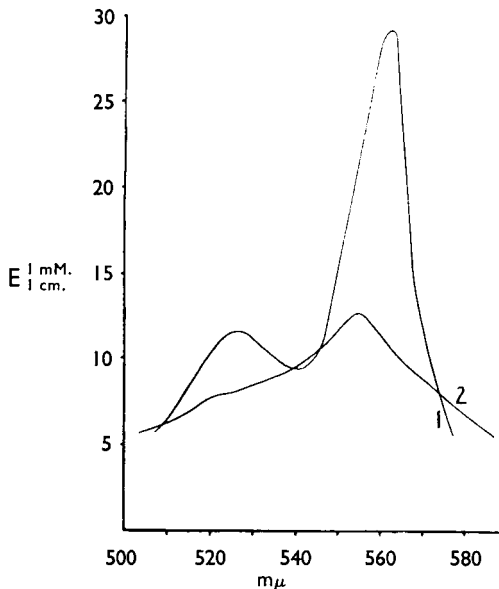

FIG. 4.

Curve 1. Di-(1-methylbenziminazolo)-ferroprotoporphyrin; 0·0025 mM hæm and 10 mM 1-methylbenziminazole in sodium hydroxide at pH 11·7.

Curve 2. Di-(1:5:6-trimethylbenziminazolo)-ferroprotoporphyrin; 0·0025 mM hæm and 7·7 mM 1:5:6-trimethylbenziminazole in sodium hydroxide at pH 12·1.

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benziminazole, in contrast, appear to form hæmochromes in the normal way (cf. Table I).

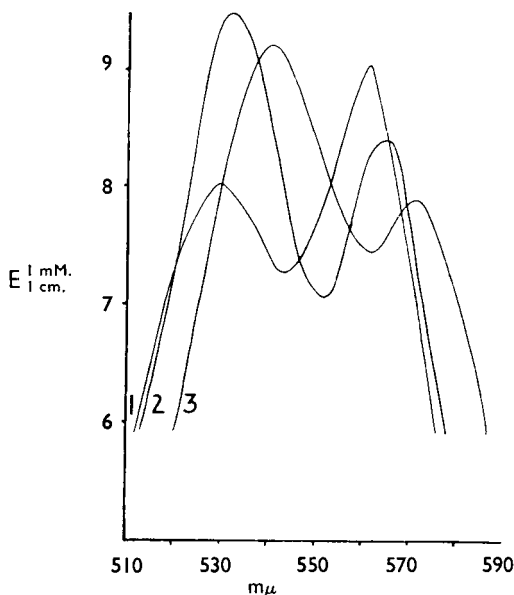Attempts to prepare hæmochromes from glycosyl benziminazoles were not successful. 1-α-D-Arabopyranosyl-[21] and 1-β-D-ribopyranosyl-5 :6-dimethylbenziminazole[22] proved too insoluble for this purpose. 1-β-D-Glucopyranosylbenziminazole,[23] though readily soluble in water, failed to react, a result which may be associated with its low basicity.[4]

Attention was next directed to the co-ordination of benziminazoles with cobalt porphyrins. The spectral changes observed when nitrogenous bases are added to compounds of this type, however, are much less marked than in the hæm series, with consequent difficulty in interpretation. Thus Holden[9] observed slight changes in spectrum on adding pyridine, ammonia, iminazole and globin to alkaline cobaltiprotoporphyrin, but failed to comment on the significance of this observation.

FIG. 5. Stock solution: 0·265 mM Cobaltiproto-porphyrin dimethyl ester chloride in methanol containing 5 per cent. of chloroform.

Curve 1. Cobaltiprotoporphyrin dimethyl ester chloride; 2 ml. of stock solution diluted to 10 ml. with methanol.

Curve 2. Dipyridinocobaltiprotoporphyrin dimethyl ester; 2 ml. of stock solution, 2 ml. of pyridine and 6 ml. of methanol.

Curve 3. Di-(1 : 5 : 6-trimethylbenziminazolo)-cobaltiprotoporphyrin dimethyl ester; 2 ml. of stock solution, 0·1 g. of trimethylbenziminazole and 8 ml. of methanol.

Taylor[15] studied the action of pyridine, picoline and nicotine on cobaltimesoporphyrin and concluded that there was little or no evidence of co-ordination. Our own results, in contrast, provided strong grounds for concluding that cobaltichrome* formation does actually occur.

Thus adding pyridine to methanolic cobaltiprotoporphyrin dimethyl ester chloride (II; R = Me) led, in our hands to (i) a small shift of the absorption spectrum to longer wavelengths and (ii) a typical hæmochrome type of spectrum with a sharp α-band accompanied by a β-band of greater intensity (curve 2, Fig. 5). The same cobaltichrome was formed more readily and at a lower pyridine concentration by using the *acetate* of

* The term "cobaltichrome" is used herein as a generic name for co-ordination compounds derived from cobaltiporphyrins and nitrogenous ligands.

(II; R = Me) as metalloporphyrin. Addition of acetic acid in trace amounts appeared, in fact, to exert a marked catalytic action on reactions of this character.

The use of methanol as solvent for co-ordination studies with (II; R = Me) made it possible to employ 1:5:6-trimethylbenziminazole as ligand (cf. p. 187). On adding this compound to the methanolic solution of the metalloporphyrin a shift of ca. 13 mμ was observed in the positions of the α- and β-bands, the relative intensities of which were now reversed (curve 3, Fig. 5). In this case, moreover, but 4 moles of base were required at a pigment concentration of 10^{-4} M to effect the conversion of (II; R = Me) into the corresponding 1:5:6-trimethylbenziminazole cobaltichrome.

By adding ether to a chloroform-methanolic solution of the latter compound, the cobaltichrome was precipitated as a red amorphous material. The absorption spectrum of this material showed a small reversion towards that of (II; R = Me), which was further increased by reprecipitation. Two further reprecipitations proved sufficient to eliminate practically all the benziminazole present. Attempts to purify the cobaltichrome for combustion analysis were therefore discontinued. Paper chromatography of the crude complex led to the separation of some free benziminazole from the coloured material. Elution of the latter, followed by acidolysis, led to the liberation of a further quantity of the ligand. As cobaltic compounds are nevertheless known to form stable co-ordinated structures with 6 nitrogen atoms, the constitution of di-(1:5:6-trimethylbenziminazolo)-cobaltiprotoporphyrin dimethyl ester is provisionally assigned to this complex.

Cobaltichromes were likewise prepared from cobaltihæmatoporphyrin (III) and cobaltiprotoporphyrin (II; R = H) employing pyridine and 1:5:6-trimethylbenziminazole as ligands. Combination, indeed, took place far more readily than in the hæm series. The results obtained are recorded in Table III.

Co-ordination of cobaltipheophytin with 1:5:6-trimethylbenziminazole proved difficult to demonstrate spectroscopically in view of the complex nature of the parent metalloporphyrin spectrum (cf. p. 182). Some evidence for cobaltichrome formation was nevertheless obtained in the following way. The two components were first warmed in ethanolic solution, after which uncombined benziminazole was removed by chromatography. The coloured material so obtained was then submitted to vigorous hydrolysis with hydrochloric acid and the products of acidolysis examined by paper chromatography. A zone corresponding to the benziminazole was readily detected. The formation of a relatively stable complex between the cobalt pheophytin and the benziminazole is thus established. It does not follow, of course, that this complex is necessarily the cobaltichrome. At the same time such a conclusion is not unwarranted.

The preparation of mixed cyanide-base cobaltichromes. We had previously shown (p. 185) that ca. 2 mole. of cyanide are required to convert cobaltiprotoporphyrin dimethyl ester (II; R = Me) into the

dicyanide complex. We now find that in the presence of pyridine very much larger quantities of the anion are required, the amount bearing a direct relation to the concent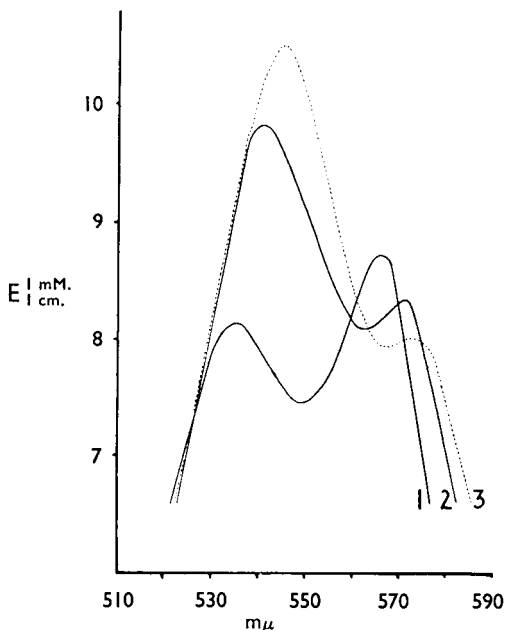ration of pyridine present. Cômpetition between the ligands for co-ordinating positions around the cobalt atom is thus established. The transformation of the pyridine cobaltichrome into the dicyanide complex, is not reversible. By treating the latter with pyridine a new complex is obtained with bands at 570 and 541 mμ (curve 2, Fig. 6) which, though closely similar to the pyridine cobaltichrome, is nevertheless a distinct entity.

This unexpected observation led us to undertake a quantitative study of the action of cyanide upon the pyridine cobaltichrome. For this purpose a series of 0·06 mM methanolic solutions of (II; R = Me) containing pyridine (600 mM) and concentrations of potassium cyanide varying from 2 to 20 mM were allowed to stand for 60 minutes to reach equilibrium and then examined spectroscopically. The results obtained, recorded in Table II,

FIG. 6. Stock solution: 0·265 mM Cobaltiprotoporphyrin dimethyl ester chloride in methanol containing 5 per cent. of chloroform.

Curve 1. Cyanocobaltiprotoporphyrin dimethyl ester; 2 ml. of stock solution, 2 ml. of acetic acid and 6 ml. of 100 mM methanolic potassium cyanide.

Curve 2. Pyridine cyanocobaltiprotoporphyrin dimethyl ester; 2 ml. of stock solution, 0·1 ml. of 100 mM potassium cyanide solution, 4 ml. of pyridine made up to 10 ml. with methanol.

Curve 3. 1 : 5 : 6-Trimethylbenziminazolocyanocobaltiprotoporphyrin dimethyl ester; 2 ml. of stock solution, 1 ml. of 100 mM potassium cyanide solution and 1 g. of 1 : 5 : 6-trimethylbenziminazole made up to 10 ml. with methanol.

show that conversion to the dicyanide takes place in stepwise fashion through intermediate formation of the same complex as that obtained by treating the dicyanide with pyridine. The spectrum of this complex, moreover, reveals no evidence of a mixture, the α- and β-bands at 570 and 541 mμ being sharp and clearly defined (cf. samples 5 and 6, Table III). The same complex was lastly obtained by adding pyridine to a slightly acid solution of cobaltiprotoporphyrin methyl ester monocyanide. It seems evident from these results that formation of the complex depends upon simultaneous association of pyridine and cyanide with the cobalt

porphyrin. The material is therefore assigned the constitution of the mixed pyridine-cyanide cobaltichrome (IX).

The foregoing series of transformations were likewise achieved employing 1:5:6-trimethylbenziminazole as ligand. The spectrum of the resulting mixed cobaltichrome is reproduced in curve 3, Figure 6.

<div align="center">TABLE II</div>

<div align="center">COMPETITION BETWEEN CYANIDE AND PYRIDINE FOR COBALTIPROTOPORPHYRIN
DIMETHYL ESTER</div>

Tube	Cyanide concentration $M \times 10^{-3}$	Spectrum		Absorbing entity
1	0	564	530	Dipyridine cobaltichrome
2	2	570	541	Mixed pyridine cyano-cobaltichrome
3	5	Two vague bands		Intermediate mixtures
4	10	One vague band		
5	20	(588)	555	Dicyanide complex

Cobaltiprotoporphyrin dimethyl ester 0·06 mM and pyridine 600 mM in methanol containing 1 per cent. of chloroform.

Pyridino-cobaltihæmatoporphyrin cyanide was prepared by treating an alkaline solution of the corresponding dicyanide, or a slightly acid solution of the monocyanide, with an excess of pyridine. The latter method was preferred as conversion to the mixed adduct took place instantaneously and at very low pyridine concentrations, whereas alkaline mixtures of the dicyanide with pyridine required long periods for equilibration. Mixed cobaltichromes with 1:5:6-trimethyl-, 1-α-D-arabopyranosyl-5:6-dimethyl- and 1-β-D-glucopyranosylbenziminazole were thus prepared and characterised by their absorption spectra (Table III).

A mixed cobaltichrome derived from (II; R = H) was obtained by treating its dicyanide with pyridine (curve 3, Fig. 2). The corresponding compound with 1-β-D-arabopyranosyl-5:6-dimethylbenziminazole could not be obtained, however, as the basic ligand proved insufficiently soluble in water (cf. p. 188).

In conclusion, it should be recalled that in Part VI[3] attention was drawn to certain anomalies present in the cyanocobalamin absorption spectrum which were interpreted as indicating partial structure (I) for the vitamin. Some benziminazolo-cobaltic co-ordination complexes were therefore prepared in Part IX[5] in order to show that their absorption spectra fulfil the criteria laid down in the earlier publication[3] as evidence for co-ordination of this type. The results obtained, however, were not fully satisfactory as attempts to prepare similar co-ordination compounds from *glycosyl* benziminazoles had not been successful. It was, of course, possible to interpret this failure as due to an incorrect choice of the cobaltic co-ordinating agents employed. At the same time it might well have been due to the low pKa values of the basic ligands,[4] when structure (I) would have been impossible on theoretical grounds. This eventuality is now finally removed by the successful preparation of the

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mixed cyano-glycosyl-5:6-dimethylbenziminazolo-cobaltichromes des-
cribed in the present communication.

EXPERIMENTAL

Microanalyses were carried out by Drs. Weiler and Strauss, Oxford.

Cobaltiprotoporphyrin chloride. Protoporphyrin dimethyl ester, pre-
pared by the method of Grinstein,[24] was hydrolysed by standing at room
temperature with concentrated hydrochloric acid for 4 to 5 hours. Dilute
sodium hydroxide solution was added, and the precipitate collected by
means of a centrifuge and washed with distilled water. The proto-
porphyrin so obtained was taken up in acetic acid saturated with sodium
chloride and the solution run slowly into a stirred solution of cobaltous
acetate[15] in acetic acid at 30° to 40° C. Crystallisation commenced
almost immediately and after standing for 30 minutes, the product was
removed by centrifugation, washed with acetic acid and water, and dried
in vacuo. Cobaltiprotoporphyrin chloride formed dark red needles.

TABLE III

SUMMARY OF THE SPECTRA OF COBALT PORPHYRINS*

Compound	Solvent	Spectrum	
Cobaltiprotoporphyrin dimethyl ester acetate ..	Methanol containing 1 per cent. of chloroform	559	528
Cobaltiprotoporphyrin dimethyl ester chloride ..	,,	561	527
Cyanocobaltiprotoporphyrin dimethyl ester ..	,,	567	535
Dicyanocobaltiprotoporphyrin dimethyl ester	,,	(588)	555
Dipyridinocobaltiprotoporphyrin dimethyl ester ..	,,	564	530
Di-(1:5:6-trimethylbenziminazolo)-cobaltiproto-porphyrin dimethyl ester	,,	573	538
Di-(1-α-D-arabopyranosyl-5:6-dimethylbenzimin-azolo)-cobaltiprotoporphyrin dimethyl ester ..	,,	572	537
Pyridine cyanocobaltiprotoporphyrin dimethyl ester	,,	570	541
1:5:6-Trimethylbenziminazolocyanocobaltiproto-porphyrin dimethyl ester	,,	571	547
Cobaltiprotoporphyrin chloride	Aqueous solution pH 11 to 12	574	536
Dicyanocobaltiprotoporphyrin	,,	589	554
Dipyridinocobaltiprotoporphyrin	,,	570	535
Di-(1:5:6-trimethylbenziminazolo)-cobaltiproto-porphyrin	,,	582	545
Di-(1-α-D-arabopyranosyl-5:6-dimethylbenzimin-azolo)-cobaltiprotoporphyrin	,,	577	540
Pyridine cyanocobaltiprotoporphyrin	,,	577	543
Cobaltihæmatoporphyrin	Aqueous acetic acid	559	525
Cyanocobaltihæmatoporphyrin	,,	563	530
Dicyanocobaltihæmatoporphyrin	Aqueous solution pH 9·0	582	549
Dipyridinocobaltihæmatoporphyrin	,,	562	530
Di-(1:5:6-trimethylbenziminazolo)-cobaltihæmato-porphyrin	,,	574	536
Di-(1-α-D-arabopyranosyl-5:6-dimethylbenzimin-azolo)-cobaltihæmatoporphyrin	,,	574	536
Di-(1-β-D-ribopyranosyl-5:6-dimethylbenzimin-azolo)-cobaltihæmatoporphyrin	,,	574	536
Pyridinocyanocobaltihæmatoporphyrin	Aqueous acetic acid	568	535
1:5:6-Trimethylbenziminazolocyanocobalti-hæmatoporphyrin	,,	572	539
1-β-D-Glucopyranosylbenziminazolocyanocobalti-hæmatoporphyrin	,,	572	539
1-α-D-Arabopyranosyl-5:6-dimethylbenziminazolo-cyanocobaltihæmatoporphyrin	,,	572	538
Cobaltiazamesoporphyrin	Aqueous solution pH 10·5	568	
	Aqueous solution pH 2	601	
Cobaltiazamesoporphyrin—cyanide compound ..	Dilute alkali	575	
Cobaltiazamesoporphyrin—pyridine compound ..	,,	568	

* Determined with a Hartridge reversion spectroscope.

Found: C, 63·3; H, 5·2; N, 8·3. $C_{34}H_{32}O_4N_4ClCo$ requires C, 62·4; H, 4·9; N, 8·5 per cent.

Cobaltiprotoporphyrin dimethyl ester derivatives (II; R = CH₃). A solution of cobaltous acetate (200 mg.) in acetic acid (50 ml.) was added to protoporphyrin dimethyl ester (200 mg.) in acetic acid (200 ml.). The spectrum of the porphyrin rapidly changed to the two-banded spectrum of the metalloporphyrin. Chloroform was added, and the solution washed free from acetic acid with water.

For the preparation of the *acetate*, the foregoing solution was dried, concentrated to 10 ml., and an equal volume of ether added with stirring and scratching. The product precipitated in the form of small red granules. After standing overnight at 0° C., *cobaltiprotoporphyrin dimethyl ester acetate* (40 mg.) was centrifuged off, washed with chloroform-ether (1:1) and ether, and dried *in vacuo*, forming a bright red, amorphous solid. Found: C, 59·7; H, 5·6; N, 7·8. $C_{36}H_{36}O_4N_4Co^+ \cdot CH_3CO_2^- \cdot 2CH_3CO_2H$ requires C, 61·0; H, 5·7; N, 6·8 per cent.

For the preparation of the *chloride*, the chloroform solution was washed with a little dilute hydrochloric acid, dried over sodium sulphate and concentrated to small bulk. Cautious addition of ether precipitated the *product* as a dark red solid (45 mg.), which was collected, washed with chloroform-ether (1:1) and dried. Found: C, 62·8; H, 6·4; N, 7·3; Cl, 6·6. $C_{36}H_{36}O_4N_4ClCo$ requires C, 63·3; H, 5·3; N, 8·2; Cl, 5·2 per cent.

Azahæmin was prepared by the method of Lemberg.[12] Found: C, 57·8; H, 5·2. Calculated for $C_{33}H_{31}O_4N_5ClFe \cdot 2H_2O$: C, 57·5; H, 5·1 per cent.

Azamesoporphyrin. A solution of azahæmin (50 mg.) in 90 per cent. formic acid (25 ml.) was gently heated under reflux whilst small portions of palladium oxide[25] catalyst were added. Samples of the reaction mixture were withdrawn every few minutes, diluted with ether, and examined in the reversion spectroscope. Liberation of azamesoporphyrin was complete within 10 to 15 minutes, when the solution was cooled, filtered and poured into ether. The ethereal solution was washed with water and the aqueous extract repeatedly extracted with ether until the extracts were colourless. The combined ether extracts were washed with 2 per cent. hydrochloric acid and then extracted with 10 per cent. hydrochloric acid. The porphyrin was salted out from the hydrochloric acid solution into a large volume of ether by addition of sodium acetate. The ether extract was washed with water. Any interfacial precipitate was redissolved in 10 per cent. hydrochloric acid and the salting out repeated. The combined ethereal solutions were dried and concentrated to small bulk. *Azamesoporphyrin* (7 mg.) crystallised on standing in fine red needles. Found: C, 69·0; H, 6·5; N, 11·3. $C_{33}H_{37}O_4N_5$ requires C, 69·8; H, 6·6; N, 12·3 per cent.

Cobaltiazamesoporphyrin chloride (V). A solution of azameso-porphyrin in acetic acid saturated with sodium chloride was treated with cobaltous acetate in acetic acid at 30° C. Control of the temperature was important as the porphyrin began to decompose above 30° C. After

standing for 1 to 2 hours, chloroform was added, the solution washed with water until neutral, and finally washed with a little dilute hydrochloric acid. The solution was dried over sodium sulphate and concentrated to small bulk. Cautious addition of ether led to the separation of *cobaltiazamesoporphyrin chloride dihydrate* in fine matted needles. Found: C, 57·5; H, 5·2; N, 10·7. $C_{33}H_{35}O_4N_5Cl\cdot Co\cdot 2H_2O$ requires C, 57·0; H, 5·7; N, 10·1 per cent.

Pheophytin a and b. The crude pheophytin, isolated from *Urtica dioica* by the method of Willstätter and Stoll,[26] was recrystallised 4 times from absolute ethanol to give the mixture of pheophytin *a* and *b* as a dark brown wax. Found: C, 75·7; H, 8·2; N, 6·4. Calculated for $C_{55}H_{72}O_5N_4$: C, 75·9; H, 8·5; N, 6·4. Calculated for $C_{55}H_{72}O_6N_4$ C, 74·7: H, 8·1; N, 6·3 per cent.

Cobalt pheophytin a and b. A saturated solution of pheophytin was prepared by shaking the porphyrin with cold acetic acid (50 ml.) followed by filtration from excess solids. 10 per cent. aqueous cobaltous acetate solution was slowly added to it with shaking, until, after filtration, there was no spectroscopic evidence of uncombined pheophytin. Approximately 20 ml. of cobaltous acetate solution was required. The solution was filtered, diluted with water and neutralised with dilute sodium hydroxide. The precipitate was collected, washed with water and dried *in vacuo*. An ethereal extract of the product was stirred and light petroleum added, whereupon *cobalt pheophytin* separated as a lustrous green material. Found: C, 69·9; H, 7·6; N, 6·8; Co (by residue calculated as Co_3O_4), 6·6. $C_{55}H_{72}O_5N_4Co^+\cdot OH$ requires C, 69·9; H, 7·7; N, 5·9; Co, 6·2. $C_{55}H_{70}O_6N_4Co^+\cdot OH$ requires C, 68·9; H, 7·4; N, 5·8; Co, 6·1 per cent.

Hæmin. Hæmin was prepared from fresh ox-blood and was recrystallised twice from pyridine and from chloroform and acetic acid. Found: C, 62·5; H, 5·1; N, 8·6. $C_{34}H_{32}O_4N_4Fe\cdot Cl$ requires C, 62·6; H, 4·9; N, 8·6 per cent.

Benziminazole hæmochromes (see Table 1). The appropriate benziminazole (*ca.* 1 mM, accurately weighed) was dissolved by shaking in distilled water (*ca.* 70 ml.) in a 100-ml. flask. 5 ml. of a 0·025 mM-alkaline hæmatin solution, freshly prepared by dissolving recrystallised hæmin in 0·1 N sodium hydroxide, was added, and followed at once by a solution of sodium hydrosulphite (*ca.* 90 mg.) in water. The clear pink solution was made up to 100 ml. with distilled water, and the spectrum immediately measured with a Unicam spectrophotometer.

SUMMARY AND CONCLUSIONS

1. Cobalti-protoporphyrin, -hæmatoporphyrin, -azamesoporphyrin and pheophytin have been prepared and their absorption spectra determined.

2. The reaction of cyanide with cobaltiporphyrins has been shown to give monocyanide and dicyanide co-ordination compounds, respectively.

3. The co-ordination of certain benziminazoles with cobaltiporphyrins to yield cobaltichromes has been established.

4. Spectroscopic evidence for the existence of mixed benziminazolo-cyanide cobaltichromes has been obtained.

5. The bearing of these results on the structure of cyanocobalamin is discussed.

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Correction.

THE VOLUMETRIC DETERMINATION OF THIOURACIL AND CERTAIN HOMOLOGUES

BY C. F. ABBOTT.

This Journal, 1953, **5**, 53.

On page 55, last paragraph, *beginning*:—Starch does not interfere . . . *should read*:—In the determinations of thiouracil, starch does not interfere. . . .

On page 56, paragraph *beginning*:—The B.P. method in this case appears . . . *should read*:—The B.P. method for methylthiouracil appears. . . .

On page 56, paragraph *beginning*:—Starch does not appear to affect the results . . . *should read*:—Starch does not appear to affect the results of the propylthiouracil determinations. . . .