THE CHEMISTRY OF ANTI-PERNICIOUS ANÆMIA FACTORS

PART VI. THE MODE OF COMBINATION OF *COMPONENT* α IN VITAMIN B_{12}

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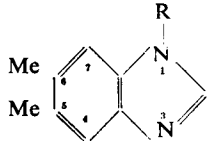
IN the course of studies on the acidolysis of vitamin B_{12}, Beaven *et al.* (Part III[1]) established the formation of three closely related hydrolytic products which they termed *components* α, β and γ. Comparison of the absorption spectra of these compounds with those of known heterocycles led to the identification of *component* γ with 5:6-dimethylbenziminazole (III), a conclusion also reached by Brink and Folkers[2], and of *components* α and β as 1-substituted 5:6-dimethylbenziminazoles. In addition, the view was expressed that the two latter compounds might well prove to be sugar derivatives of 5:6-dimethylbenziminazole.

Subsequent work outlined in Part V[3], and the independent studies of Brink, Holly, Shunk, Peel, Cahill and Folkers[4], and of Buchanan, Johnson, Mills and Todd[5], have now established the main structural features of the *component* α and β molecules. Thus the constitution of a 5:6-dimethylbenziminazole-1-α_a-D-ribofuranoside (II)* has been assigned to the latter compound[4,5] and the formulation confirmed by direct synthesis[4]. The structure of *component* α, however, is based on less secure experimental evidence. The compound is undoubtedly the 2′ or 3′ phosphoryl derivative of (II)[3,5], the 3′-formulation (I) being, in our view, the preferred structure.

Acidolysis of B_{12} thus leads to initial formation[1,3] of *component* α, which then undergoes stepwise hydrolysis into *component* β, and finally into *component* γ.

Structure of Components α, β and γ



Component α (1, 3, 5) : R = —CH(OH)(OPO$_3$H$_2$)—CH(H)—CH(H)—CH$_2$OH (I)

Component β (1, 3, 4) : R = —CH(OH)—CH(OH)—CH(H)—CH$_2$OH (II)

Component γ (1, 2, 3) : R = H (III)

*See footnote on page 954

By using the extinction coefficient of 5:6-dimethylbenziminazole (III) as a model for reckoning molar extinctions, Beaven *et al.*[1] concluded that one molecule of vitamin B_{12} gives rise to approximately one molecule of 5:6-dimethylbenziminazole (calc. as *components* α, β and γ) on acidolysis. In addition, the recognition of two bands at λ = 2885Å and 2785Å in the B_{12} absorption spectrum as characteristic of the benziminazole chromophore led them to the further postulate that a benziminazole nucleus exists preformed in the B_{12} molecule.

We now find that the synthetic 5:6-dimethylbenziminazole glycosides (IV) described in Parts IV[6] and V[3] of this series possess absorption spectra indistinguishable from each other and from those of *components* α and β. The absorption spectrum of such a 5:6-dimethylbenziminazole glycoside, together with the curve for vitamin B_{12}, is shown in Figure 1. It is now clear that the absorptivity of B_{12} in the region of absorption of the benziminazole is not sufficiently high to accommodate two equivalents of the latter. We may thus conclude, with confidence, that vitamin B_{12} contains but one preformed benziminazole nucleus in its molecule. This nucleus, in our view, consists of the intact *component* α, structure (I), as only by this assumption does it appear possible to explain the facility with which this fragment is released on acidolysis[3].

The Anomaly Present in the B_{12} Absorption Spectrum

Inspection of the curves shown in Figure 1 reveals an anomaly. Thus whereas the benziminazole curve (2) is characterised by a well-marked

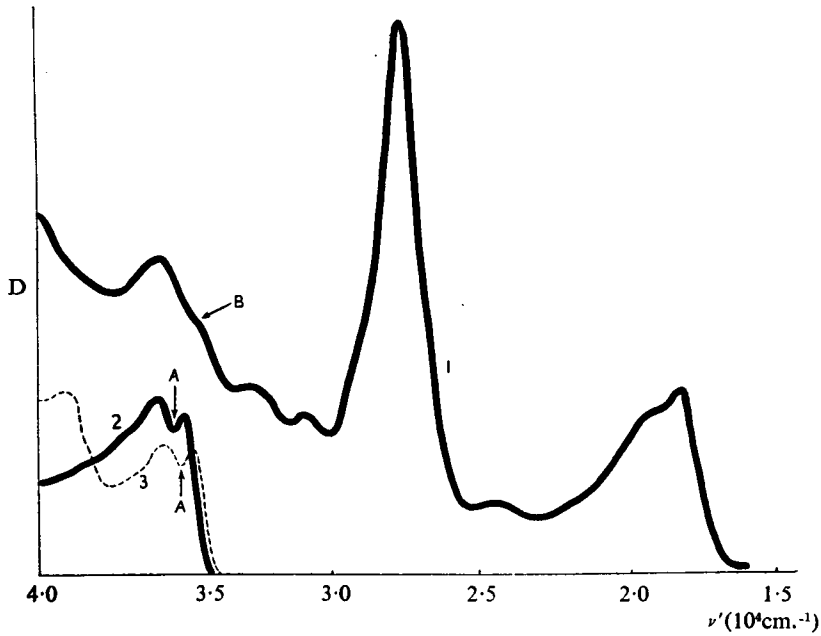


FIG. 1. Absorption spectra of 1. Vitamin B_{12}, 2. 5:6-dimethylbenziminazole-1-glycoside (*p*H 2), 3. 5:6-dimethylbenziminazole-1-glycoside (*p*H 12).

narrow band and "notch" (marked A), the curve for vitamin B_{12} shows only an inflection (marked B) at $\lambda = 2885$Å. Since the absorption of a mixture of two chromophore-bearing compounds is additive provided there is no interaction between them, the non-appearance of the "notch" in the absorption curve of vitamin B_{12} must be interpreted in one of two ways: (i) the absorption spectrum of the rest of the B_{12} molecule, which we shall term the "coloured fragment," masks the benziminazole band; or (ii) the contribution of the benziminazole chromophore to the B_{12} spectrum is altered by some combination or linkage of *component* α within the B_{12} molecule.

The first interpretation is unlikely. It can be shown graphically that for this situation to obtain the absorption curve of the "coloured fragment" must possess a small band which "fills up"—for want of a better term—the small minimum (A; curve 2) present in the benziminazole spectrum. Interpretation (ii), however, requires closer study.

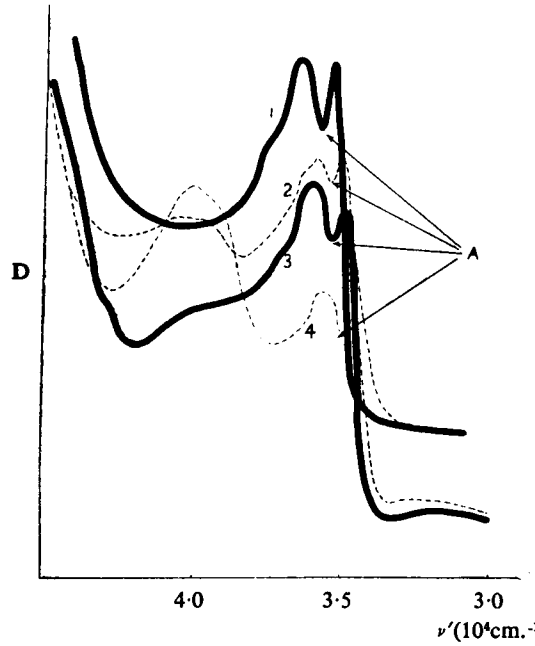
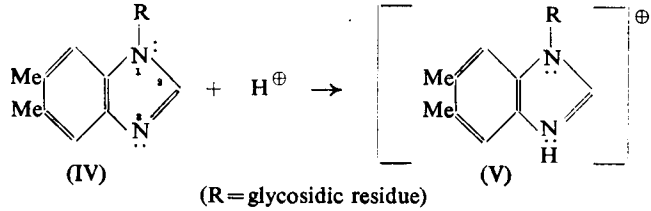


FIG. 2. Absorption curves of 1. *Component* γ (*p*H 2), 2. *Component* γ (*p*H 12), 3. *Components* α and β (*p*H 2), 4. *Components* α and β (*p*H 12).

We already know[1] that the "notch" in question (marked A) is resolved in the spectra of *components* α, β and γ (Figure 2). In addition, it is quite certain that the resolution of the "notch" in the *component* α spectrum will not be impaired by possible linkage of the phosphoryl residue to the rest of the B_{12} molecule (*vide infra*). Consideration of the ways in which the chromophoric character of *component* α can be affected sufficiently to abolish the resolution of the "notch" without interfering with the wavelength position of the main band and its subsidiary features leads, by a process of elimination, to the conclusion that the nitrogen atom in position 3 of the benziminazole nucleus in *component* α (I), is involved in some form of combination. Evidence supporting this view is recorded in the following section.

Observations Regarding the Dissociation Constants of Vitamin B_{12} and of Certain Benziminazoles

The benziminazole nucleus contains two basic nitrogen atoms and can thus, theoretically, accept a maximum of two protons. Detailed electrometric studies by Davies, Mamalis, Petrow and Sturgeon[7] show that benziminazole, its alkyl derivatives, and its alkylated-l-glycosides, have pK_a values in the region of 5. This pK_a, subsequently referred to as pK_{a_1}, corresponds to the

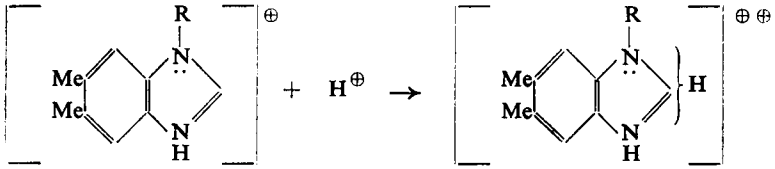


(IV) (V)

(R = glycosidic residue)

acceptance of one proton by the benziminazole (IV) to give the benziminazolinium ion (V) ($pK_{a_1} \doteq 5{\cdot}0$). The electron pair available on the nitrogen atom at position 3, subsequently referred to as the N^3 nitrogen, is utilised for this purpose, addition of proton being accompanied by a marked shift in the position of the absorption bands as shown in Figure I, curves 2 and 3.

By spectrometric titration we now find that the foregoing benziminazole derivatives (IV) give evidence for a second dissociation constant in the region of *p*H 0·0. Thus when the concentration of hydrochloric acid is raised to normal, a new spectral change occurs which consists of a shift to longer wavelengths. The effect is still more marked in 5 N and 10 N hydrochloric acids.



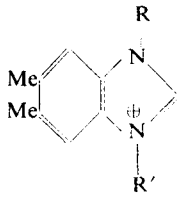
(V) (VI)

This second pK_a, subsequently referred to as pK_{a_2}, is clearly dependent upon the benziminazolinium ion (V) accepting a proton to give (VI), which will obviously exist only in a very high hydrogen ion concentration.

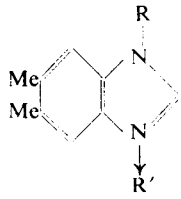
Bearing these facts in mind, examination of the absorption spectra in Figure 1 leads to the following conclusions: (i) The synthetic 5:6-dimethylbenziminazole-1-glycoside (IV) shows, as expected, a pronounced wavelength shift in passing from *p*H 12 (curve 3) to *p*H 2 (curve 2). This spectral shift corresponds to the existence of a pK_{a_1} of *ca.* 5·0 (i.e., IV→V). (ii) The inflection at $\lambda = 2885$Å in the vitamin B_{12} spectrum, for which the benziminazole chromophore present in (I) is responsible, fails to show a wavelength shift in passing from *p*H 12 to *p*H 2. On increasing the acid concentration to 0·1 N, the B_{12} spectrum undergoes a

definite but reversible shift to shorter wavelengths. Further increase in acid concentration leads to spectral changes which are partly irreversible.

Consideration of these facts leads to the conclusion that the "*component* α combination" existing in the B_{12} molecule differs from the 5:6-dimethylbenziminazole-1-glycoside (IV) by the absence of a pK_{a_1}, as defined on p. 947, but resembles (V) by giving the evidence of a pK_{a_2} at a *p*H of *ca.* 0·0. This fact must surely mean that N^3 in the "*component* α combination" must lack the free electron pair responsible for the pK_{a_1}. If this is indeed the case, the conclusion that the N^3 electron pair is involved in some form of linkage within the B_{12} molecule appears to be inescapable. Theoretically, two types of linkage are indicated:



(VII)



(VIII)

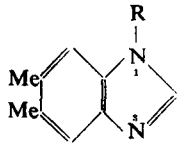
(i) the N^3 electron pair may be shared with a grouping R′ in the form of a covalent bond giving a compound of the quaternary salt type (VII), or (ii) the N^3 electron pair may be donated to a cationic grouping R′ in the form of a coordinate link as indicated in (VIII).

Before discussing these alternative formulations, it is perhaps pertinent to restate once more the spectroscopic features which distinguish the "*component* α combination" existing in B_{12} from those of a simple 5:6-dimethylbenziminazole-1-glycoside (IV). Briefly, these differences involve: (i) The absence of a spectral shift in passing from *p*H 12 to *p*H 2 (Figure 1, compare curve 1 with curves 2 and 3), i.e., the absence of a pK_{a_1} and (ii) The absence of the "notch" (Figure I; marked **A** in curves 2 and 3) characteristic of (IV) but evident in B_{12} as an inflection (Figure 1; marked B).

Returning now to a consideration of (VII) and (VIII), substances possessing either structure may confidently be expected to lack a pK_{a_1} and thus satisfy the first of the conditions formulated above. The effect of the N^3-R′ linkages on the resolution of the benziminazole "notch," however, cannot be predicted at this stage, but must be determined experimentally by spectroscopic study of model compounds.

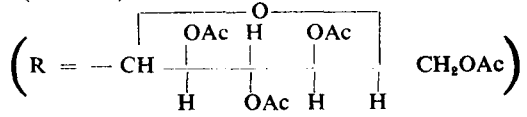
The Effect of Quaternation on the Absorption Spectra of 1-Substituted 5:6-Dimethylbenziminazoles

The absorption spectra of: 1:5:6-trimethylbenziminazole (IX) and of 5:6-dimethylbenziminazole-1-β-tetraacetyl-D-glucopyranoside[6] (X) are given in Figure 3. Both compounds possess pK_{a_1} dissociation constants

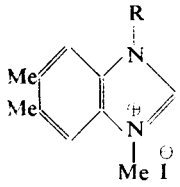


(IX) (R = Me)

(X)



and thus show pronounced spectral shifts in passing from *p*H 12 (curves 2 and 4, respectively) to *p*H 2 (curves 1 and 3, respectively). In addition, both compounds show a well-resolved "notch" (marked A) in the region of λ = 2885Å. The "notch," it will be noted, is more pronounced



(XI) (R = Me) I

(XII) (R = —CH(OH)—CH(H)—O—CH(OH)—CH(H)—CH(OH)—CH(H)—CH_2)

in the case of (IX) (curves 1 and 2), a difference no doubt due to the contrasting inductive effects of the 1-methyl (+I) and 1-glycosido (−I) substituents on the benziminazole chromophore.

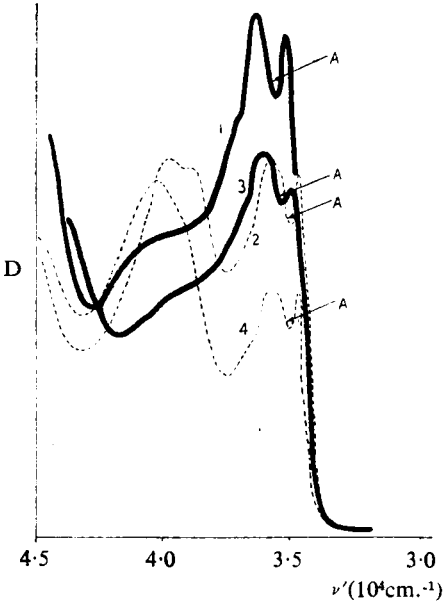


FIG. 3. Absorption curves of 1. 1:5:6-trimethylbenziminazole (*p*H 2), 2. 1:5:6-trimethylbenziminazole (*p*H 12), 3. 5:6-dimethylbenziminazole - 1 - β - tetra-acetyl-D-glucopyranoside (*p*H 2), 4. 5:6-dimethylbenziminazole - 1 - β - tetra-acetyl-D-glucopyranoside (*p*H 12).

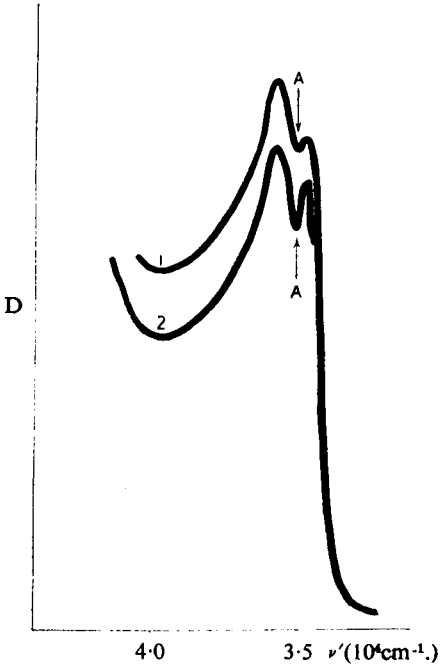


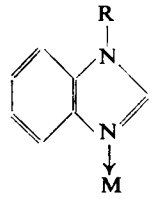
FIG. 4. 1. 5:6-dimethylbenziminazole-1-β-D-arabopyranoside-3-methiodide (*p*H 2 and 12), 2. 1:5:6-trimethylbenziminazole-3-methiodide (*p*H 2 and 12).

Quaternation of the N^3 atom leads, as expected, to the abolition of the pK_{a_1}. This point is evident from the absorption spectra of 1:5:6-trimethylbenziminazole 3-methiodide (X) (Figure 4; curve 2) and of 5:6-dimethylbenziminazole-1-β-D-arabopyranoside 3-methiodide (XII) (Figure 4; curve 1) which, respectively, give identical absorption spectra in both alkaline and acid solution. Quaternation fails, however, to abolish the well-resolved "notch" (marked A) which is clearly

present in the absorption spectra of both these compounds. The spectroscopic features of 1-substituted 5:6-dimethylbenziminazole-3-quaternary salts are thus incompatible with the second of the conditions formulated on p. 948. The stability of such compounds as (XI) and (XII) towards hydrochloric acid, too, stands in marked contrast to the facility with which release of *component* α occurs under the influence of acid. 1-Substituted 5:6-dimethylbenziminazole-3-quaternary salts may therefore be excluded from further consideration.

The Effect of Coordination by the N^3 Atom on the Absorption Spectra of 1-Substituted Benziminazoles

Considerable difficulty was experienced in preparing model compounds of type (XIII)



(M = Metal) **(XIII)**

for spectroscopic study. Although complex formation between the base and such compounds as cobalt chloride was achieved in many cases[8], the resulting products proved, in general, to be compounds of low stability which dissociated into their component parts during solution prior to spectroscopic study. Discussion of these results is accordingly postponed. Complexes formed employing platinic chloride[8], however, proved sufficiently stable for our purpose and, moreover, contained the structural feature (VIII).

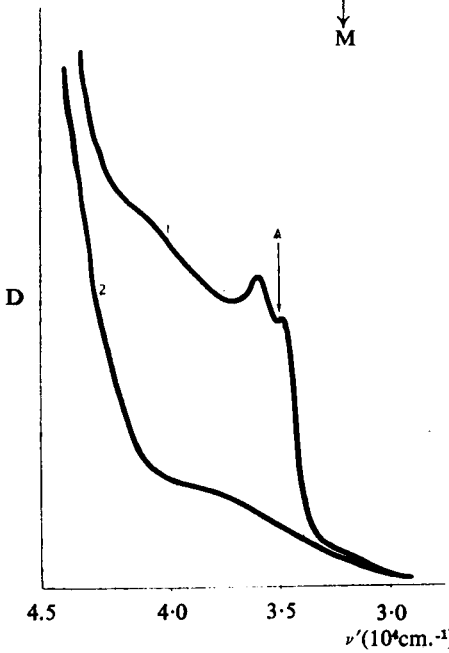


FIG. 5. 1. Platinum complex of 5:6-dimethylbenziminazole - 1 - arabopyranoside, 2. $[Pt(NH_2)_2Cl_4]$. Curve 2 is given to indicate the absorption characteristics of a platinum complex which does not contain another specific chromophore.

The absorption spectrum of the 5:6-dimethylbenziminazole-1 - α - L - arabopyranoside/platinic chloride complex is shown in Figure 5. Examination of the curve reveals: (i) the absence of an acid/alkali spectral shift, i.e., the absence of a pK_{a1}, as defined on p. 947 and (ii) a marked decrease in resolution of the long wave fine structure maximum at $\lambda = 2860$ Å (marked A).

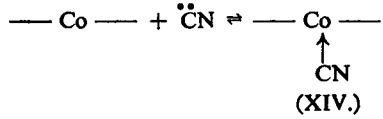
The spectroscopic characteristics of compounds of type (XIII) (and hence VIII) thus fully satisfy the two conditions formulated on p. 948. We may therefore conclude, with reasonable certainty, that the N^3 electron pair in *component* α is deployed in some form of co-ordinate link in the combination existing in B_{12}. Evidence regarding the nature of this linkage is supplied by studies on the action of cyanide on vitamin B_{12}.

The Effect of Cyanide on Vitamin B_{12}

When potassium cyanide or hydrocyanic acid is added in excess to a solution of vitamin B_{12}, the colour changes from red to deep violet with a correspondingly marked change in the absorption spectrum both in the visible and ultraviolet regions (*vide infra.*). The B_{12} cyanide complex so formed is stable only in solution in the presence of excess of cyanide, reverting to a red compound on reducing the *p*H to below 7, or, when hydrogen cyanide is employed, on simply allowing the solution to evaporate. The identity with vitamin B_{12} of the material so recovered is established by its absorption spectrum, its general crystallographic appearance, its behaviour on paper chromatograms[9], and its microbiological activity as measured with *Lactobacillus lactis* Dorner A.T.C.C. 10697. For this latter determination, we are indebted to Dr. S. W. F. Underhill and Miss F. E. Larkin. We may therefore conclude that: (i) cyanide adds reversibly to the B_{12} molecule, (ii) addition of cyanide occurs at an electrophilic centre in the B_{12} molecule, and (iii) addition is not accompanied by profound structural changes in the molecule.

Evidence concerning the point of attachment of cyanide is furnished by the observation that the coloured cobalt-containing fragment obtained by Ellis, Petrow and Snook[10] by acidolysis of B_{12} likewise adds cyanide under similar experimental conditions. The cobalt-containing part of the B_{12} molecule must therefore be the seat of the electrophilic centre involved in "B_{12}-cyanide complex" formation. This centre is, in our view, the central cobalt atom, the cyanophilic properties of which are well established and require no further comment (cf. Ephraim, *Inorganic Chemistry*, 3rd English Edition, 1939, p. 302).

The co-ordination of cyanide ion with the central cobalt atom, as shown diagrammatically in formula (XIV),



(XIV.)

is accompanied by profound changes in the B_{12} absorption spectrum as shown in Figure 6 (*vide supra*). Comparison of curves 1 and 2 (Figure 6) reveals the important fact that the benziminazole inflection (marked B) at $\lambda = 2885$Å present in the B_{12} curve (curve 1) has been replaced in the "B_{12}-cyanide complex" by a well-resolved notch (marked A) (curve 2). In fact, the *component* α contribution to the absorption spectrum of the "B_{12}-cyanide complex" may be considered as "normal," and the corresponding anomaly previously observed in the B_{12} spectrum (cf.

Figure 1) as absent. The co-ordination of cyanide ion with cobalt is thus able to influence directly the contribution made by the benziminazole chromophore to the B_{12} spectrum. Some form of linkage between the cobalt atom or chromophore and the benziminazole chromophore is thus established.

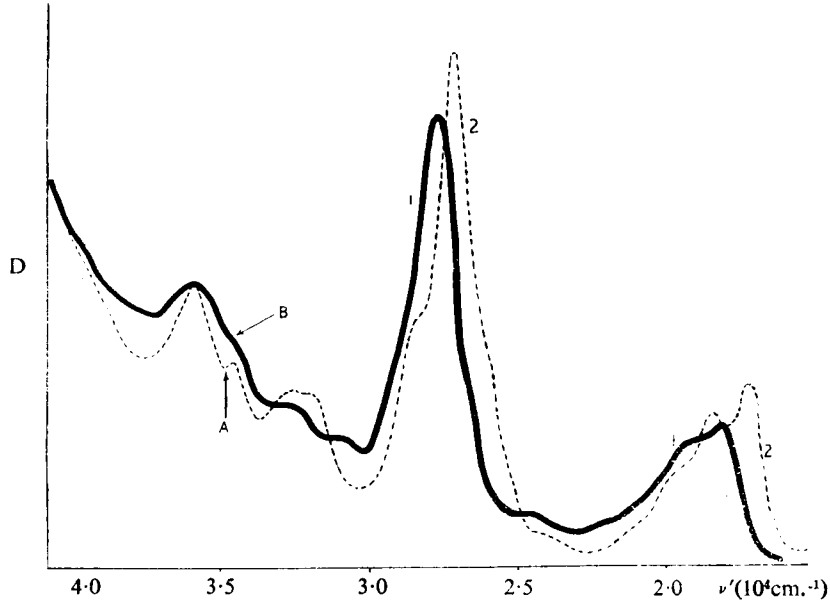


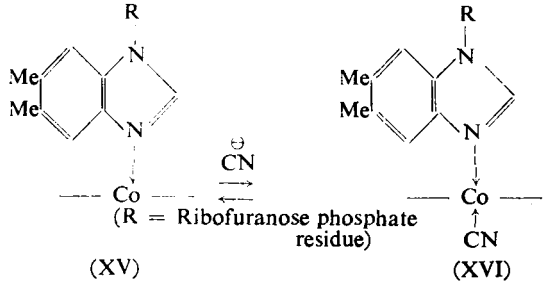
FIG. 6. 1. Vitamin B_{12}, 2. Vitamin B_{12}-cyanide complex.

The benziminazole chromophore (I), for reasons outlined above, is considered as bound through its N^3 atom by means of a co-ordinate linkage as shown in (VIII). The N^3 atom of the latter compound must therefore be involved in co-ordinate linkage with either the cobalt atom or chromophore. An unequivocal decision between these two alternative sites of combination is, unfortunately, impossible at the present time, owing to an almost complete absence of information regarding the detailed structure of the cobalt chromophore. The probable existence of a structural analogy between B_{12} and the hæmatin group of compounds, however, to which Ellis, Petrow and Snook[10] have already drawn attention, points to the hypothesis that N^3 in the benziminazole glycoside (I) is co-ordinately linked to cobalt as shown in (XV).

It is perhaps relevant to add that we visualise the cobalt-containing fragment as a planar structure somewhat akin spatially to a porphyrin, with *component* α lying perpendicularly to this plane.

The resolution of the benziminazole "notch" (Figure 6) in the "B_{12}-cyanide complex" spectrum is readily explained on this basis by assuming that combination of cyanide ion with cobalt to give (XVI) leads to an increase in the electronegativity of the cobalt atom and a corresponding decrease in the electronic contribution of the N^3 benziminazole nitrogen

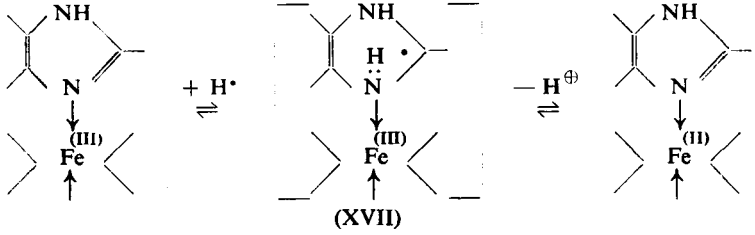
to the cobalt electron cloud. Decrease of this electronic contribution will, conversely, diminish the changes produced by the N^3 – Co linkage in the benziminazole chromophore spectrum and lead to an absorption curve more closely resembling that of an unattached 5:6-dimethylbenziminazole-1-glycoside (IV), as is indeed found to be the case. Thus the co-ordination hypothesis fulfils all the spectroscopic desiderata established during the course of this investigation. Its unequivocal proof, however, must await a closer knowledge of the internal architecture of the B_{12} molecule.



A precedent for a similar co-ordination is furnished by hæmoglobin and by cytochrome c in which a histidine residue is co-ordinated with the porphyrin iron atom. The analogy with hæm compounds is further strengthened by the recent observation of Wallman, Cunningham and Calvin[11], and of Gruen and Menassé[12], who have shown that the cobalt atom in vitamin B_{12} is trivalent and possesses octahedral symmetry. The effect of cyanide on the spectrum of B_{12} is likewise reminiscent of the effect of cyanide on hæm compounds described by J. Keilin[13], and of cyanide on cobalt porphyrins described by Taylor[14].

Some Implications of the Coordination Hypothesis

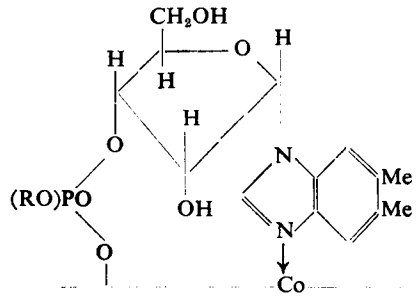
The function of the histidine residue in cytochrome c is to provide a channel for the transmission of electrons to and from the iron atom as indicated in the following scheme[15]:



whereby reversible reduction of the ferric atom is achieved. We are tempted to suggest a similar function to the benziminazole residue in vitamin B_{12} in view of the close and unexpected analogy evident between the two partial structures (XV) and (XVII). Cobalt co-ordination complexes, it is true, exist normally in the more stable cobaltic form. Nevertheless, the possibility that transient reduction of cobaltic to cobaltous

occurs in vitamin B_{12} by electron transmission through the benziminazole nucleus should not be excluded from consideration. It is of interest to note, in this connection, that the oxidation/reduction potential of the $Co^{++} \rightarrow Co^{+++}$ reaction is very much altered by conversion of the simple inorganic cobalt ion into a complex of the covalent octahedral type (see Pauling, *Nature of the Chemical Bond,* 2nd Ed., 1942, p. 96).

A point of difference between the behaviour of the histidine residue in the hæm pigments and the benziminazole residue in vitamin B_{12} is furnished by the action of dilute acids. Hydrolysis occurs in both cases. In B_{12}, however, progressive release of *component* α seems to take place in contrast to an instantaneous cleavage effect observed with hæm compounds. This difference in behaviour would appear to indicate the presence of an additional less readily hydrolysed linkage which, together with the N^3-Co link (cf. XV), binds *component* α to the rest of the B_{12} molecule. Buchanan, Johnson, Mills and Todd[5] have indeed proposed that the phosphoryl grouping in *component* α is joined directly to the cobalt macrofragment and also to the " ninhydrin-reacting fragment "[16] (D-1-amino-2-propanol[17,18]) in tertiary union. Combination with D-1-amino-2-propanol in this fashion is, of course, inconsistent with evidence submitted by Cooley *et al.* in Part V[3]. Nevertheless, we accept the suggestion regarding the attachment of the phosphoryl residue to the cobalt-containing fragment, either directly or through an intermediate molecule, such as D-1-amino-2-propanol, and tentatively propose a two-fold attachment of *component* α as indicated below (XVIII).



(XVIII)

The feasibility of this formulation depends not only upon structural features of which we have no present knowledge, but also upon the stereochemical configuration of the glycosidic centre present in *component* α. Two-fold attachment can only be postulated for one of the anomeric forms of (I). This anomer* must possess the α_a glycosidic configuration established by Butler, Smith and Stacey[19] for the α_a and β_a forms of tetraacetyl-D-galacto-pyranose anilide. The anomeric form of

* In order to avoid ambiguity between the use of α, β γ to designate the hydroyltic products of B_{12}, and the use of α and β to designate the anomeric forms of the benziminazole glycosides, the latter have been termed the α_a and β_a-glycosides. The subscript a implies reference to the anomeric forms of sugars as generally accepted in carbohydrate nomenclature.

component α is not, of course, known with certainty. Nevertheless, it is perhaps more than a coincidence that Brink *et al.*[4] have provisionally assigned it the α_a-configuration on grounds of general usage and practice.

Summary and Conclusions

1. Spectroscopic studies lead to the conclusion that the nitrogen atom in position 3 of *component* α (I) is involved in a linkage of co-ordinate type within the B_{12} molecule.

2. Cyanide ion is shown to add reversibly to the cobalt-containing chromophore present in vitamin B_{12}, such addition being accompanied by a significant change in the anomalous contribution of the benziminazole chromophore of (I) to the ultraviolet absorption spectrum of vitamin B_{12}.

3. It is suggested that the nitrogen atom in position 3 of *component* α (I) is co-ordinately linked to cobalt as shown in (XVIII).

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Note. After Part VI had been submitted for publication, Brink, Kuehl and Folkers (*Science*, 1950, **112**, 354) presented evidence which indicates that a cyano-grouping is bound directly to the central cobalt atom in vitamin B_{12}. This fact in no way invalidates the arguments outlined in Part VI. The purple complex formed by addition of cyanide to vitamin B_{12}, however, which we refer to as the "B_{12}-cyanide complex," will therefore contain two cyano-groups linked directly to cobalt, one of which is readily lost with regeneration of the parent vitamin.

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