THE CHEMISTRY OF ANTI-PERNICIOUS ANAEMIA FACTORS

PART III. 5:6-DISUBSTITUTED BENZIMINAZOLES AS PRODUCTS OF ACID HYDROLYSIS OF VITAMIN B_{12}

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ELLIS, Petrow and Snook have shown[1] that hydrolysis of vitamin B_{12} with 20 per cent. (w/v) hydrochloric acid at 100°C. leads to the formation of a coloured cobalt-containing complex which may be quantitatively extracted from the aqueous phase with *n*-butyl alcohol. Examination of the aqueous phase showed that, in addition to phosphate[2] and a "ninhydrin-reacting" substance[1,3], material showing selective absorption in the ultra-violet with bands and inflections at 2850, 2768, 2690, 2585, and 2500 Å ("the 285-component") was also present[3].

The presence of two main absorption band systems (2850, 2768, 2690 Å) and (2585, 2500 Å) of almost equal intensity in "the 285-component" pointed to the existence of a dicyclic chromophore of unsaturated or aromatic character. The absorption spectra of aromatic compounds of this type differ from that of "the 285-component" from which it was concluded that a heterocyclic chromophore was present in the latter material. The marked fine structure of the absorption spectrum, moreover, indicated a heterocyclic compound probably containing nitrogen. On examining the absorption spectra of a number of heterocyclic compounds containing one or more nitrogen atoms in the molecule, it soon became clear that ring systems containing two fused six-membered rings could be excluded from further consideration as the absorption invariably extended to too long a wavelength. The study of compounds containing a six-membered ring fused to a five-membered ring, on the other hand, revealed the significant fact that only benziminazole (III; R=R′=H) and indazole gave spectra resembling that of "the 285-component," benziminazole approximating the more closely of the two. The characteristic long wavelength fine structure band of benziminazole corresponding to the "285"-band of the B_{12} material, however, was at much too short a wavelength, namely, $\lambda = 2730$ Å in acid solution.

While these experiments were in progress, concurrent work briefly referred to in Part II[3] revealed the complex character of "the 285-component." By employing *n*-butyl alcohol-acetic acid as the irrigation solvent for the chromatograms in place of *iso*butyric acid which had been used for the earlier work, resolution of "the 285-component" was achieved. The chromatograms, when examined under a low-pressure mercury resonance lamp fitted with a Corning 9863 glass filter[4], now showed three violet fluorescent zones in place of the single fluorescent area formerly obtained and ascribed to "the 285-component." A typical chromatogram is represented in Figure 1 (left hand side).

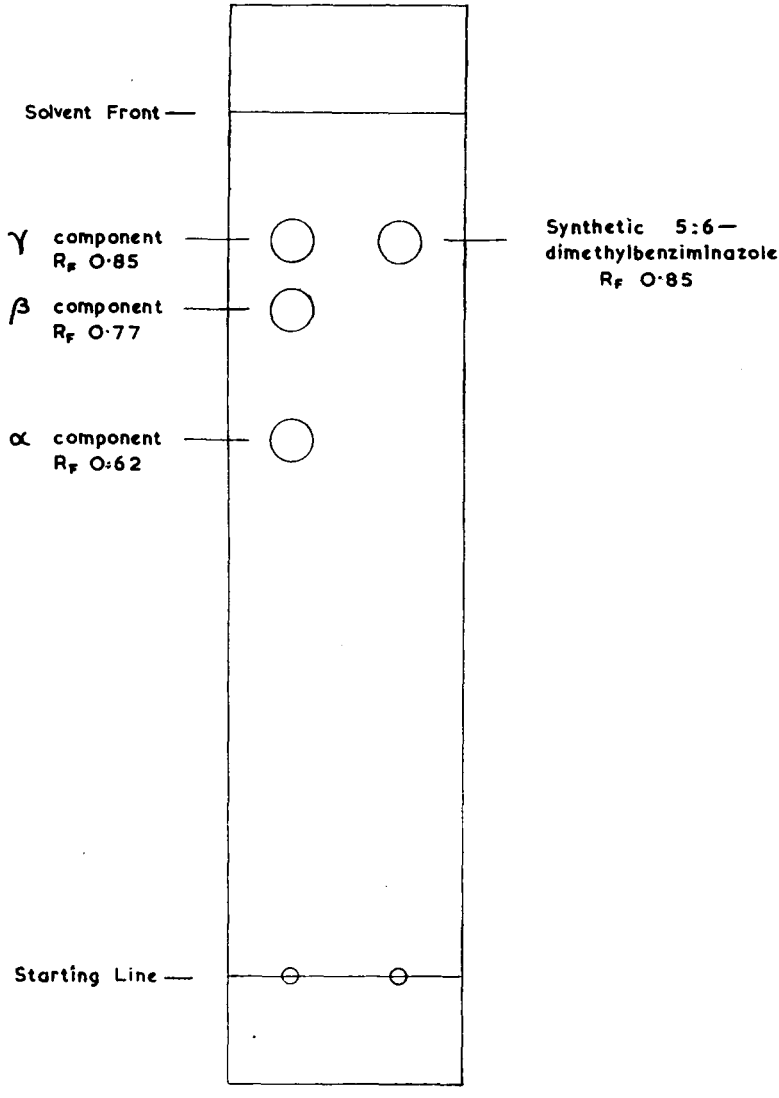


FIG. 1.—Product from vitamin B_{12} hydrolysis. Paper chromatogram irrigated with *n*-butyl alcohol-acetic acid.

Sectional elution of the different regions with dilute hydrochloric acid and spectroscopic examination of the eluates (Figure 2) showed that the compounds responsible for the three fluorescent zones had absorption characteristics similar to one another and to "the 285-component" from which they had clearly been derived. For convenience they have been designated *components* α, β, and γ. *Components* α and β, it should be added, have indistinguishable absorption characteristics which differ but slightly from those of *component* γ.

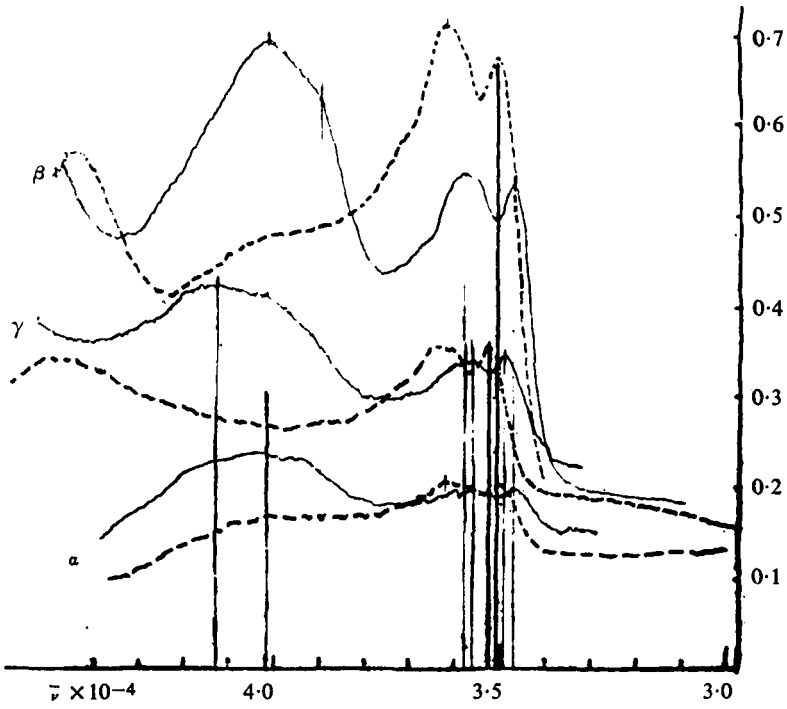
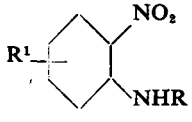


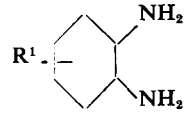
FIG. 2.—Absorption curves of eluates of fluorescent spots from a paper chromatogram of a vitamin B_{12} hydrolysate: continuous line in 0·01N sodium hydroxide, broken line in 0·01N hydrochloric acid.

The absorption spectra of *components* α, β, and γ bore a general resemblance to that of benziminazole (see Figure 3), but the characteristic long wavelength fine structure band of the latter compound still lay at a much shorter wavelength. The general form of the absorption spectrum of such a chromophore is usually little affected by substitution when the substituent groups are not conjugated to form a new or additional chromophore. The band systems are, however, shifted to a greater or lesser extent by substitution, usually in the direction of longer wavelengths.

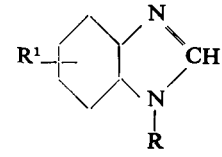
These observations led us to conclude that *components* α, β, and γ were all substituted benziminazoles, and with the object of putting this theory to the test 22 alkylated benziminazoles were synthesised to serve as model compounds for spectroscopic measurements.



I.



II.



III.

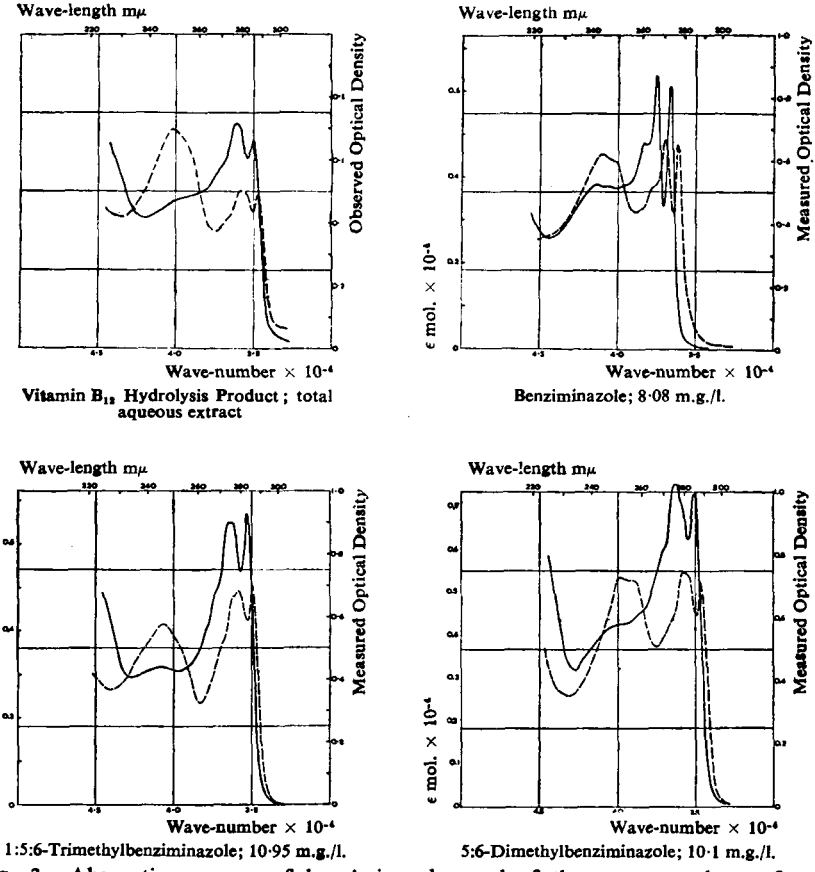


FIG. 3.—Absorption curves of benziminazoles and of the aqueous phase after *n*-butyl alcohol extraction of the vitamin B_{12} hydrolysate: continuous line in 0·01N hydrochloric acid, broken line in 0·01N sodium hydroxide.

The general procedure of Phillips[5] was employed for their preparation whereby the *o*-diamine (II) was heated with formic or acetic acid in the presence of 4N hydrochloric acid. The required *o*-diamines (II) were prepared by catalytic reduction of the corresponding *o*-nitroanilines (I) employing a palladium-charcoal catalyst. N-Methyl-*o*-nitroanilines (I; R = Me) required for the preparation of the 1-methyl substituted benziminazoles (III; R = Me) were obtained by methylation of the corresponding *o*-nitroanilines (I; R = H) using the method described by Usherwood and Whiteley[6]. The preparation and properties of the following benziminazoles have not previously been reported in the literature:

1:6-, 1:7-, 2:4-, 4:5-, 5:6-*Dimethylbenziminazole.*

1:2:7-, 1:4:5-, 1:5:6-, 2:4:5-, 2:5:6-*Trimethylbenziminazole.*

1:2:4:5-, 1:2:5:6-*Tetramethylbenziminazole.*

1- and 2-monomethyl and 1:2-dimethylbenziminazole, the first benziminazoles to be synthesised and examined, showed spectra resembling that of the parent ring system, but with some alteration in

form and small shifts in wavelength which did not approach the required values of $\lambda = 2850$ for *components* α and β, and $\lambda = 2832$ for *component* γ *(vide infra).* A greater shift in the fine structure bands was observed with the Bz-alkylated derivatives, particularly with the 5-methyl-, 6-methyl-, 1:5-dimethyl-, and 1:6-dimethyl analogues. We therefore concentrated our synthetic efforts on the preparation of 5:6-dimethyl- and 1:5:6-trimethylbenziminazoles and, with these compounds at our disposal, were able to confirm our speculations and obtain evidence for the identity of *components* α and β with 1- substituted 5;6-dimethylbenziminazoles and of *component* γ with 5:6-dimethylbenziminazole.

The positions of the fine-structure bands observed with these two sets of compounds in both acid and alkaline solution are recorded in Table I, and the agreements between the sets of figures is indeed seen to be remarkably good.

TABLE I

POSITION OF FINE-STRUCTURE BANDS (Å) OF HYDROLYSIS COMPONENTS OF VITAMIN B_{12} AND OF THE CORRESPONDING METHYLATED BENZIMINAZOLES

	0·01N Hydrochloric Acid							
	a_1	a_2	a_3	a_4	a_5	b_1	b_2	
α	2850	2787	2753	2683	—	—	—	
β	2851	2787	2751	2689	2657	—	—	
1.5.6 T.M.B.	2850	2787	2751	2690	2652	2597	2470	
γ	2833	2770	2731	2673	2639	2580	—	
5.6 D.M.B.	2832	2770	2730	2672	2635	2580	2440	
	0·01N Sodium Hydroxide							
	a_1	a_2	a_3	a_4	a_5	b_1	b_2	b_3
α	2880	2812	2782	—	—	—	—	—
β	2880	2816	2782	2720	2679	2558	2492	2418
1.5 .6 T.M.B.	2880	2820	2784	2723	2685	2582	2498	—
γ	2861	2800	2760	2702	2660	2535	—	—
5.6 D.M.B.	2862	2802	2760	2705	2655	2535	2460	2380

T.M.B. = 1 : 5 : 6-Trimethylbenziminazole D.M.B. = 5 : 6-Dimethylbenziminazole

Spectroscopic examination of the remaining alkylated benziminazoles provided further data supporting these conclusions. The difference between *components* α and β, it should be added, probably lies in the nature of the substituent groups in position 1.

All the methylated benziminazoles so far examined show characteristic spectra which are readily distinguished from each other. Each compound shows 5 or 6 bands in acid solution which are shifted in characteristic manner in alkaline solution. There are, therefore, 10 or 12 bands (12 in the case of 5:6-dimethylbenziminazole) for comparison.

As the band positions of the unknown benziminazoles agreed within the error of placing with those of the synthetic compounds, we are confident that the position of the substituents in the two sets of compounds is the same. It cannot be inferred from the identity of the spectra that the substituent groups in the vitamin B_{12} products are methyl groups. It is certain, however, that they are small unreactive groups, otherwise distortion of the spectrum to a recognisable degree could be expected. It is hardly relevant to the present communication to report the detailed spectroscopic data for all the methylated benziminazoles synthesised and examined in the course of this investigation, but it is hoped to publish this part of the work elsewhere at a later date.

Spectroscopic identification of a compound for the structure of which there is, *a priori*, no chemical evidence, is not to be undertaken lightly. Since the method is quite empirical, one is entirely dependent on the specificity of the absorption spectra for the degree of certainty with which the positive statement of identity with a model compound may be made. The spectra are so characteristic in this series of compounds, however, that they offer more strongly presumptive evidence of identity than is usually the case. In addition, preliminary experiments on the behaviour of *component* γ and 5:6-dimethylbenziminazole on paper chromatograms support the view that the two compounds are, indeed, identical.

By using the extinction coefficient of 5:6-dimethylbenziminazole as a model for reckoning molar extinctions—an assumption justified by our observation that all the benziminazoles so far examined possess very similar molar extinction coefficients—it has been possible to determine that one molecule of vitamin B_{12} gives rise to approximately one molecule of 5:6-dimethylbenziminazole (calc. as *components* $\alpha+\beta+\gamma$) on acid hydrolysis. This result accords with our preliminary analytical studies on the products of hydrolysis of vitamin B_{12}, and leads to the conclusion that *components* α, β and γ represent different stages of degradation of a common precursor.

Re-examination of the absorption spectrum of vitamin B_{12} (cf. Ellis, Petrow and Snook[1]) by the moving plate method[7] shows that two bands characteristic of benziminazoles are apparent at $\lambda=2895$ and 2785 Å, and a trace of the third and fourth bands at $\lambda=2630$ and 2500 Å (see Table II). Recognition of a chromophore with a complex band system such as a benziminazole is difficult, however, as the absorption due to the rest of the molecule obscures and distorts to a great extent the absorption in the benziminazole region of the spectrum. The spectrograms reproduced in Figure 4 show, however, that the moving plate method picks out the characteristic maxima and inflections. The bands are at wavelengths considerably longer than those of *components* α, β, and γ, and, in addition, the acid-alkali shift is found to be negligible (Table II, 1 and 2). These differences might be due to the nature of the group through which the benziminazole is united to the rest of the

molecule, or to intramolecular forces polarising the benziminazole chromophore. An example of the latter effect is to be found in the shift to longer wavelengths of the spectrum of the aromatic amino-acids in native proteins[8], and of purines and pyrimidines in nucleic acid[9]. In both cases mild hydrolysis releases the respective fragment from the effects of these polarising forces and the spectrum reverts to that of the free chromophore.

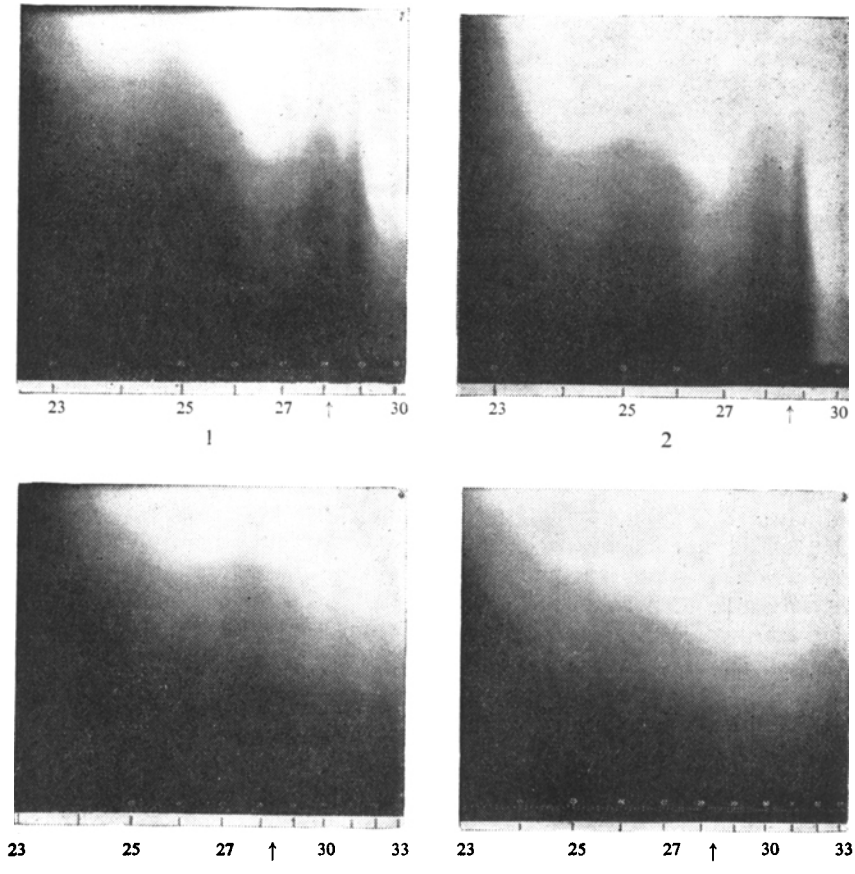


FIG. 4.—Spectrograms of:—(1) Component β; (2) 1:5:6 trimethylbenzinazole; (3) Vitamin B_{12}; (4) Methyl ester of coloured component. All measurements were made in 0·01N sodium hydroxide. The arrows mark the positions of the "285-region" bands.

A similar effect has now been observed in the case of vitamin B_{12} itself. The band positions for solutions in 3N hydrochloric acid are at shorter wavelengths than those in 0·01N hydrochloric acid solutions, and do not revert to the long-wave position on returning to the latter acid concentration. The shift to shorter wavelengths observed in 3N hydrochloric acid solution is thus irreversible. Furthermore, the new

TABLE II

POSITION OF FINE-STRUCTURE BANDS (Å) OF VITAMIN B_{12} UNDER VARYING CONDITIONS

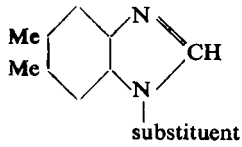
						a_1	a_2+a_3	a_4+a_5	b_1	b_2
1	B_{12} in 0·01N sodium hydroxide	3615	3435	3235	3065	2895	2785		2630	2500
2	B_{12} in 0·01N hydrochloric acid	3610	3435	3235	3065	2895	2785			
3	B_{12} in 3N hydrochloric acid ...	3560	3415	3200	3040	2862	2770			
4	Solution (3) brought back to 0·01N hydrochloric acid ...			3210	3050	2861	2776			
5	Solution (3) brought back to weak alkali	3609	3433	3230	3070	2889	2790		2625	

Note.—Many of the bands are difficult to distinguish. Only those which have been identified with certainty are included.

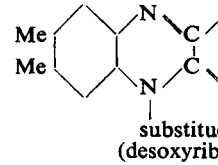
positions occupied by the bands are considerably closer to those of *components* α and β, and these bands now show a comparable acid-alkali shift. As a moving-plate spectrogram of a solution of the " methyl ester " of the cobalt complex also present[1] in hydrolysates of vitamin B_{12} shows, apart from other changes, an almost complete absence of the fine structure bands in the " 285-region " (see Figure 4), it may be concluded that the benziminazole nucleus exists intact in the B_{12} molecule.

The recognition of a 5:6-dimethylbenziminazole nucleus in vitamin B_{12} raises a point of great biogenetic interest. Riboflavine, which likewise contains the 4:5-dimethyl-*o*-phenylenediamine residue, is known to be synthesised by microbial flora in the rumen of the sheep[10]. Tosic and Mitchell[11] have shown that the microbial flora utilise at least part of the cobalt ingested by the ruminant, and have suggested, on the basis of this observation, that pining and other wasting diseases in ruminants may be due to cobalt deficiency of the bacteria, the animal requiring not cobalt, but the bacterial products which have thereby become deficient. Becker, Smith and Loosli[12] have reported that there is no significant response in cobalt-deficient lambs when injected even with comparatively large quantities of vitamin B_{12} (125 μg.) or fed with B_{12} concentrates, although rapid disappearance of the symptoms occurred following cobalt administration by feeding (1 mg. Co per day). They concluded that these preliminary observations do not support the theory that vitamin B_{12} is an important intermediary in cobalt metabolism in lambs. Nevertheless, it seems difficult to avoid drawing the conclusion that the microbial flora of the sheep's rumen utilise cobalt to accomplish some stages in the synthesis of vitamin B_{12} or, more probably, of a closely related derivative. What part, if any, is played by riboflavine in such a process by its action on, or part in, the bacterial metabolism will, of course, only be clarified by further experimental studies.

The formation by acid hydrolysis of vitamin B_{12} of two 1-substituted 5:6-dimethylbenziminazoles (*components* α and β) leads to the conclusion that the latter ring system is attached to the macro-fragment of the B_{12} molecule by a grouping which is relatively stable to acid. The structural similarity between vitamin B_{12} (IV) and riboflavine (V) implict in



IV.



V.

the existence of an N-substituted 4:5-dimethyl-*o*-phenylenediamine residue in both compounds, may well extend to the nature of this substituent. Sugar derivatives of 5:6 dimethylbenziminazole are therefore being synthesised. Their preparation and properties will form the subject of a later communication.

Experimental

M.pts. are uncorrected.

Paper Chromatography of a vitamin B_{12} hydrolysate.

1·6 mg. of vitamin B_{12} were hydrolysed with 0·5 ml. of 20 per cent. hydrochloric acid for 15 hours at 100°C., the solution diluted to 5 ml. and extracted three times with successive portions (2 ml.) of *n*-butyl alcohol. The combined *n*-butyl alcohol extracts were back extracted several times with 2 per cent. hydrochloric acid, and all the aqueous phases combined. Evaporation of these extracts to dryness gave a crystalline residue. A solution of this material in several microlitres of distilled water was spotted on to a paper strip and the latter irrigated with *n*-butyl alcohol-acetic acid. Examination of the chromatogram in λ=2536 Å radiation revealed the presence of three violet fluorescent spots (*components* α, β, and γ) having R_F values 0·62, 0·77 and 0·85 respectively.

Chromatography of authentic 5:6-dimethylbenziminazole gave rise to a violet fluorescent spot, identical in every respect with *component* γ (see Figure 1).

Spectrographic Methods.

(i) The moving-plate method described in 1937 by one of us[7] has been modified by substituting a new cam which gives a ratio of final to initial rate of motion of the spectrograph plate of 100:1 instead of the original 10:1. This gives greater latitude and sensitivity to the method. The precision of location of fine-structure bands or inflections depends upon their spectral width and resolution. In the case of the sharper long wavelength bands of benziminazoles the error is as little as $\pm$ 1 Å, while for the diffuse short wavelength bands it may increase to $\pm$ 10 Å.

(ii) The spectrophotometric measurements were made with an automatic recording spectrophotometer designed and built by the Telecommunication Research Establishment of the Ministry of Supply in collaboration with Medical Research Council. A full description of this instrument will be published elsewhere. It plots a continuous record of optical density against wave number. Figure 2 shows examples of records taken with this instrument.

(iii) All solutions were examined in both 0·01N hydrochloric acid and sodium hydroxide. Since the change in the absorption spectrum on passing from acid to alkali is different for the different benziminazoles, the " acid " and " alkaline " spectra may be taken as independent properties of the chromophore in question and hence the specificity increased very greatly. In Figure 3 the usual notation is employed.

$$\epsilon_{mol} = \frac{1}{c \times l} \cdot D \quad \ldots\ldots\ldots\ldots (1)$$

where ϵ is the molar extinction coefficient, D is the measured optical density, l the path length and c the concentration in g.-molecules per litre.

Samples of vitamin B_{12} were dried at 70°C. for 1 hour prior to examination.

Spectrophotometry of Paper Chromatogram Eluates.

(i) The sensitivity of spectrophotometry:—The weight of substance (W_s g.) to give a satisfactory optical density (D) is related to the molar extinction coefficient (ϵ_{mol}) and the volume of the photometer cell (V ml.) for a path length of 1 cm.

In most spectrophotometers the volume of solution required to fill the cell adequately is proportional to the path length. We may, therefore, define a specific volume requirement (v_s) of the photometer as the volume required to fill a cell of 1 cm. path length. Introducing W instead of c into the relation (1) we obtain

$$W_s = \frac{v_s}{1000} \frac{DM}{\epsilon_{mol}} \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

where M is the molecular weight.

In our photometer 1·7 ml. is required to fill a cell of 1 cm. path length. If we take an average maximum ϵ for benziminazoles as $0{\cdot}5 \times 10^4$ and a molecular weight of 150, and if we also take as a requirement that the optical density of the solution at the band maxima shall be not less than D=0·8, then it follows that the minimum weight of benziminazole required to fulfil the conditions is given by:

$$W_s = \frac{1{\cdot}7 \times 0{\cdot}8 \times 150}{1000 \times 0{\cdot}5 \times 10^4} = 40\ \mu g.$$

The relation (2) is general for any photometer and any compound, and is useful for rapidly determining whether the spectrophotometric method is sufficiently sensitive for estimating components of a paper chromatogram. W_s may be termed the limiting weight sensitivity of the spectrophotometer.

Where the specific absorption of a compound is high, as in the case of the benziminazoles, complete absorption curves can be derived from quantities of the order of ten micrograms.

(ii) *Identification of the components on the paper chromatogram:* Parallel runs on two spots of hydrolysate were made on the same piece

of paper. After drying the paper in air or in the oven at 95°C., the strips were examined, before cutting the paper, under the mercury resonance arc through Corning 9863 filter which transmits the $\lambda = 2536$ line (Holiday and Johnson[4]). Regions of fluorescence were observed on a typical chromatogram as described above. These were marked off with pencil, the strip cut longitudinally between the two spots, and one half sprayed with the ninhydrin reagent. The flourescent spots were cut out from the unsprayed half, together with control sections of the paper, and each eluted by soaking at least half an hour in 4 ml. 0·01N hydrochloric acid.

It may be well to emphasise that the excitation by the $\lambda = 2536$ Å radiation of the mercury arc is essential for the appearance of the fluorescent spots, which are not seen when the paper is viewed under the same arc, but through Wood's glass.

Figure 2 is a direct reproduction of the record made by the spectrophotometer on the eluates of the three fluorescent spots from such a paper chromatogram. Each pair of curves represents the same eluate in both acid and alkaline solution. In the case of each pair the spectrum shifted to longer wavelength (smaller wavenumber) is that for the alkaline solution.

Preparation of p-*Toluenesulphon*-o-*nitroanilides:*

The following general method was employed: The nitro-amine (0·1 mol.), dissolved in pyridine (15 to 20 ml.), was treated with *p*-toluenesulphonylchloride (0·1 mol.) in portions. The reaction was completed by heating for 2 hours on the steam bath. After addition of dilute hydrochloric acid, the solid was collected, washed with water and purified by crystallisation. The yields were of the order 80 to 95 per cent.

p-*Toluenesulphon*-(2′-*nitro*-4′-*methylanilide*), yellow needles from alcohol, m.pt. 104°C. Found: N, 9·2. $C_{14}H_{14}O_4N_2S$ requires N, 9·2 per cent.

p-*Toluenesulphon*-(2′-*nitro*-6′-*methylanilide*, yellow prisms from alcohol, m.pt. 125°C. Found: N, 8·8. $C_{14}H_{14}O_4N_2S$ requires N, 9·2 per cent.

p-*Toluenesulphon*-(2′-*nitro*-3′:4′-*dimethylanilide*), prismatic needles from alcohol, m.pt. 126° to 127°C. Found: N, 8·9. $C_{15}H_{16}O_4N_2S$ requires N, 8·8 per cent.

p-*Toluenesulphon*-(2′-*nitro*-4′:5′-*dimethylanilide*), yellow blades from alcohol, m.pt. 149° to 150°C. Found: N, 8·9. $C_{15}H_{16}O_4N_2S$ requires N, 8·8 per cent.

Methylation of the p-*Toluenesulphon*-o-*nitroanilides:* The procedure below was adopted: A mixture of the sulphonanilide (0·1 mol.) and 4N sodium hydroxide (26 ml.) was treated under reflux with methyl sulphate (8·0 ml.), the mixture being kept alkaline to phenolphthalein by drop-by-drop addition of 10 N sodium hydroxide solution. A further 8·0 ml. of methyl sulphate was added, and the mixture refluxed for 15 minutes. After cooling, the product was collected, washed with water and purified from alcohol. (Yields, 80 to 95 per cent.)

p-*Toluenesulphon*-(2′-*nitro*-N:4′-*dimethylanilide*), pale yellow prisms, m.pt. 128° C. Found: N, 9·1. $C_{15}H_{16}O_4N_2S$ requires N, 8·8 per cent.

p-*Toluenesulphon*-(2′-*nitro*-N:6′-*dimethylanilide*), silver leaflets, m.pt. 139° to 140°C. Found: N, 8·9. $C_{15}H_{16}O_4N_2S$ requires N, 8·8 per cent.

p-*Toluenesulphon*-(2′-*nitro*-N:3′:4′-*trimethylanilide*), colourless prisms, m.pt. 137°C. Found: N, 8·7. $C_{16}H_{18}O_4N_2S$ requires N, 8·3 per cent.

p-*Toluenesulphon*-(2′-*nitro*-N:4′:5′-*trimethylanilide*), m.pt. 125° to 127°C. Found: N, 8·3. $C_{16}H_{18}O_4N_2S$ requires N, 8·3 per cent.

Hydrolysis of the p-*Toluenesulphon*-N-*methylanilide:* A mixture of the *p*-toluenesulphon-N-methylanilide (0·1 mol.), glacial acetic acid (16 ml.) and concentrated sulphuric acid (37 ml.) was heated on the steam bath for 1 to 2 hours and poured into ice-water. The amine was collected and recrystallised from alcohol. Yields 60 to 75 per cent.

2-*Nitro*-N:3:4-*trimethylaniline hemihydrate,* scarlet prisms, m.pt. 59° to 60°C. Found: N, 14·7. $C_9H_{12}N_2O_2.\frac{1}{2}H_2O$ requires N, 14·8 per cent. Found on material resublimed at 100°C. 0·05 mm.: N, 15·5, $C_9H_{12}N_2O_2$ requires N, 15·6 per cent.

2-*Nitro*-N:4:5-*trimethylaniline* was resublimed at 100°C. 0·05 mm. for analysis, forming orange-red needles, m.pt. 138°C. Found: N, 15·6. $C_9H_{12}O_2N_2$ requires N, 15·6 per cent.

The preparation of Benziminazoles:

The appropriate nitro-amine (0·02 mol.) in ethanolic solution was shaken with hydrogen in the presence of 10 per cent. palladium-charcoal (0·5 g.) until hydrogen uptake was complete. The solution, freed from catalyst, was taken to dryness in an atmosphere of nitrogen and the residue dissolved in 4N hydrochloric acid (20 ml.). The appropriate aliphatic acid (0·1 mol.) was added and the solution refluxed in nitrogen for 40 minutes. The product was then precipitated by addition of dilute ammonia, collected and recrystallised. The yields were 50 to 60 per cent. of the theoretical.

Monomethylbenziminazoles: 1-Methyl-, needles from light petroleum, m.pt. 64°C. (Skraup[13]); 2-methyl-[5], needles from water, m.pt. 176°C.; 4-methyl-, needles from ethyl acetate-light petroleum, m.pt. 140°C. (Gabriel and Thieme[14]); 5-methyl-, needles from ethyl acetate-light petroleum, m.pt. 113°C., b.pt. 169° to 172°C./0·1 mm. (Niementowski[15]).

Dimethylbenziminazoles: 1:2-Dimethyl-, needles from ethyl acetate-light petroleum, m.pt. 109° to 110°C. (Fischer[16] gives m.pt. 112°C.); 1:5-dimethyl-, needles from ethyl acetate-light petroleum, m.pt. 94°C. (Fischer[17]); 1:6-dimethyl-, needles from light petroleum, m.pt. 74° to 75°C. Found: C, 73·7; H, 7·0. $C_9H_{10}N_2$ requires C, 73·9, H, 6·9 per cent.) (Fischer and Wreszinski[18] describe this compound as an oil, b.pt. 280°C.); 1:7-*dimethyl-*, prismatic needles from ethyl acetate-light petroleum, m.pt. 68° to 70·5°C. Found: N, 19·0. $C_9H_{10}N_2$ requires N, 19·1 per cent.; 2:4-*dimethyl-*, prisms from ethyl acetate, m.pt. 168° to 169°C. Found: C, 74·0; H, 6·6. $C_9H_{10}N_2$ requires C, 73·9; H, 6·9 per

cent. 2:5-dimethyl-, leaflets from ethyl acetate-light petroleum, m.pt. 202°C. (Green and Day[19]); 4:5-*dimethyl-*, leaflets from aqueous alcohol, m.pt. 196° to 197°C. Found: N, 18·8; $C_9H_{10}N_2$ requires N, 19·1 per cent.; 5:6-*dimethyl-*, needles from ethyl acetate-light petroleum, m.pt. 199° to 200°C. Found: C, 73·5; H, 6·4; $C_9H_{10}N_2$ requires C, 73·9; H, 6·9 per cent.

Trimethyl benziminazoles: 1:2:5-Trimethyl-, plates from ethyl acetate, m.pt. 141°C. (Fischer and Regaud[20]); 1:2:6-trimethyl-, rods from ethyl acetate-light petroleum, m.pt. 119° to 120°C. (Fischer and Rigaud[20] give m.pt. 122°C.); 1:2:7-*trimethyl-*, fine needles from petroleum, m.pt. 146° to 147°C. Found: C, 74·5; H, 7·7; $C_{10}H_{12}N_2$ requires C, 75·0; H, 7·6 per cent.; 1:4:5-*trimethyl-*, white needles from light petroleum, m.pt. 95° to 96°C. Found N, 17·7. $C_{10}H_{12}N_2$ requires N, 17·5 per cent.; 2:4:5-*trimethyl-*, needles from aqueous alcohol, m.pt. 188° to 190°C. Found: N, 171·5. $C_{10}H_{12}N_2$ requires N, 17·5 per cent.; 1:5:6-*trimethyl-*, needles from ethyl acetate-light petroleum, m.pt. 142° to 143°C. Found: N, 17·1. $C_{10}H_{12}N_2$ requires N, 17·5 per cent. 2:5:6-*trimethyl-*, needles from aqueous alcohol, m.pt. 233° to 234°C. Found: C, 75·2; H, 7·1; $C_{10}H_{12}N_2$ requires C, 75·0; H, 7·6 per cent.

Tetramethyl benziminazoles: 1:2:4:5-*Tetramethyl-*, long needles from aqueous alcohol, m.pt. 144° to 145°C. Found: N, 16·1. $C_{11}H_{14}N_2$ requires N, 16·1 per cent.; 1:2:5:6-*tetramethyl-*, pale yellow prisms from ethyl acetate-light petroleum, m.pt. 164°C. Found: N, 16·0; $C_{11}H_{14}N_2$ requires N, 16·1 per cent.

Summary and Conclusions

1. Evidence is presented for the presence of three chemically related substances, *components* α, β, and γ, in acid hydrolysates of vitamin B_{12}.

2. Spectroscopic examination of these components has led to their classification as benziminazole derivatives.

3. Spectroscopic comparison with 22 methylated benziminazoles synthesised to serve as model compounds, has resulted in the identification of *components* α and β as 1:5:6-trisubstituted benziminazoles, and of *component* γ as a 5:6-disubstituted benziminazole.

4. Preliminary paper chromatographic studies point to the identity of *component* γ with 5:6-*dimethylbenziminazole;* from which it is concluded that *components* α and β are both 1-substituted 5:6-dimethylbenziminazoles.

5. Spectroscopic and chemical evidence leads to the conclusion that only one 5:6-dimethylbenziminazole residue is released from vitamin B_{12} on acid hydrolysis and that the benziminazole nucleus exists preformed in the vitamin.

6. It is, therefore, concluded that *components* α, β, and γ represent successive stages of degradation of a common precursor.

7. The bearing of these results on the biogenesis and structure of vitamin B_{12} is briefly discussed.

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Footnote. While preparing this paper for publication we became aware at the meeting of the First International Congress of Biochemistry held at Cambridge in August, 1949, of the findings of Dr. K. Folkers and his colleagues, and at the same session we announced the conclusions we had reached in the work now reported (Holiday and Petrow, *J. Pharm. Pharmacol.*, 1949, **1**, 734).

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