552. *Chemistry of the Vitamin B_{12} Group. Part I. Acid Hydrolysis Studies. Isolation of a Phosphorus-containing Degradation Product.*

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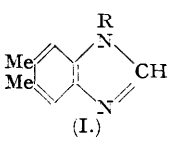
The acid hydrolysis of vitamin B_{12} has been examined in detail. The experimental conditions for the formation of 5 : 6-dimethylbenziminazole and two *N*-substituted 5 : 6-dimethylbenziminazoles have been ascertained, as well as for the preparation of the basic hydrolysis product giving a positive ninhydrin reaction. Similar degradations have been carried out with another member of the vitamin B_{12} group, vitamin B_{12c}, which differs from vitamin B_{12} only in the portion of the molecule represented by the red cobalt-containing hydrolysis product. The location of the phosphorus atom in vitamins B_{12} and B_{12c} has been determined by the isolation, in the form of its barium salt, of a *N*-substituted 5 : 6-dimethylbenziminazole bearing a phosphorylated side-chain. Removal of the phosphoryl group yields a glycoside, evidently identical with 5 : 6-dimethyl-1-α-D-ribofuranosylbenziminazole (Brink, Holly, Shunk, Peel, Cahill, and Folkers, *J. Amer. Chem. Soc.*, 1950, **72**, 1866). The two *N*-substituted benziminazoles obtained on hydrolysis of the vitamin are thus inter-related in a manner exactly analogous to the pyrimidine and purine nucleotides and nucleosides. A new method of synthesis of benziminazole *N*-glycosides is described.

A crystalline anti-pernicious anæmia factor was isolated from liver independently by Rickes, Brink, Koniuszy, Wood, and Folkers (*Science*, 1948, **107**, 396; **108**, 134) and by Lester Smith and his colleagues (*Nature*, 1948, **161**, 638; **162**, 144; *Proc. Roy. Soc.*, 1949, *B*, **136**, 592). Later isolations from the same source were reported by Ellis, Petrow, and Snook (*J. Pharm. Pharmacol.*, 1949, **1**, 60) and by Pierce, Page, Stokstad, and Jukes (*J. Amer. Chem. Soc.*, 1949, **71**, 2952). Lester Smith *et al.* used as a bioassay in their isolation procedure the clinical effect on patients suffering from pernicious anæmia. Rickes *et al.*, on the other hand, employed a microbiological

assay based on the effect of the factor on the growth of certain organisms, notably *Lactobacillus lactis* Dorner; no doubt for this reason they described the isolated anti-pernicious anæmia factor as vitamin B_{12}, a name which has been generally adopted. The discovery that vitamin B_{12} could also be isolated from the culture fluid of *Streptomyces griseus* (Rickes *et al.*, *Science*, 1948, **108**, 634) has led to a somewhat greater availability of the vitamin, which is now being increasingly used in clinical practice. Vitamin B_{12} is closely related to the previously described animal protein factor of Jukes *et al.* (*J. Biol. Chem.*, 1949, **180**, 647, and earlier papers).

Since the latter part of 1948 we have carried out a series of investigations on the material isolated from liver and kindly supplied by Dr. Lester Smith of Glaxo Laboratories Ltd., and by Dr. T. H. Jukes of Lederle Laboratories Inc. The very small amounts of material available, particularly in the early stages, considerably hampered chemical investigation and we devoted most of our attention to the acid hydrolysis of the vitamin, a reaction which has been the subject of most of the work published so far by other investigators. Vitamin B_{12}, which forms dark red needles, owing their colour, in part at least, to the molecule containing co-ordinately bound cobalt, was ascribed a formula $C_{61-64}H_{86-92}O_{13}N_{14}PCo$ by Brink, Wolf, Kaczka, Rickes, Koniuszy, Wood, and Folkers (*J. Amer. Chem. Soc.*, 1949, **71**, 1854). Our material, dried to constant weight *in vacuo* at room temperature, gave analytical values corresponding approximately to a formula $C_{63}H_{97}O_{20}N_{14}PCo$, which has a higher content of hydrogen and oxygen than that of the American workers, but since their sample was dried at 100° the difference is probably caused merely by a greater degree of hydration in our analytical sample. The identity of the two preparations of the vitamin dried under the same conditions has been established by direct comparison (Fantes, Page, Parker, and Lester Smith, *Proc. Roy. Soc.*, *loc. cit.*). In early experiments on the acid hydrolysis of slightly impure specimens of vitamin B_{12}, we observed the presence of various common amino-acids in the hydrolysate but quickly found that these acids derived from the impurities and were not formed when the pure vitamin was hydrolysed. When pure vitamin B_{12} was heated with 6N-hydrochloric acid at 100° for 48 hours there were produced (1) phosphoric acid, (2) ammonium chloride, (3) a colourless compound showing no selective absorption in the ultra-violet region but giving a purple colour with ninhydrin, (4) two colourless compounds with characteristic ultra-violet absorption spectra but giving no reaction with ninhydrin, and (5) a deep-red non-crystalline material containing co-ordinately bound cobalt. These results agree in the main with those of other workers. The substance (2) giving the ninhydrin reaction has been obtained also by Ellis, Petrow, and Snook (*loc. cit.*); although it was at first thought by them (*ibid.*, 1949, **1**, 735, 950) to be 2-amino-1-propanol on the basis of its behaviour on paper chromatograms, they later withdrew their claim (*ibid.*, 1950, **2**, 128) when they failed to obtain alanine from it on permanganate oxidation. Using the technique of paper chromatography and applying the method of Crumpler and Dent (*Nature*, 1949, **164**, 441) involving the formation of copper complexes, we have shown that the base (2) is not an α-amino-acid and, since treatment of it with nitrous acid and then 4N-hydrochloric acid yields a product which no longer reacts with ninhydrin, we conclude that it contains a primary rather than a secondary amino-group. The similarity between the behaviour of substance (2) and certain alkanolamines has also been confirmed by us, but identification by paper chromatography alone seems dangerous, especially when compounds such as 2-amino-1-propanol and 1-amino-2-propanol have virtually the same R_F value in various solvents. We are in full agreement with Ellis, Petrow, and Snook that the base (2) is an authentic degradation product of vitamin B_{12} and that it is produced in substantial quantity under the conditions of hydrolysis from which Brink *et al.* (*loc. cit.*) apparently failed to detect it.*

Brink and Folkers (*J. Amer. Chem. Soc.*, 1949, **71**, 2951) isolated from acid hydrolysates of vitamin B_{12} a crystalline degradation product which they identified as 5 : 6-dimethylbenziminazole (I; R = H); on this basis vitamin B_{12} was ascribed a structure (I; R = $C_{53-54}H_{77-81}O_{13}N_{12}PCo$).



(I.)

Holiday, Petrow, *et al.* (*J. Pharm. Pharmacol.*, 1949, **1**, 734, 957) identified the same compound (I; R = H) in acid hydrolysates by its characteristic absorption spectrum. They obtained evidence for the presence in their hydrolysates of two further unidentified materials which seemed on spectroscopic evidence to be *N*-substituted 5 : 6-dimethylbenziminazoles. These two unidentified

* Since this paper was written, the base has been identified as D$_5$-1-amino-2-propanol (Wolf, Jones, Valiant, and Folkers, *J. Amer. Chem. Soc.*, 1950, **72**, 2820). Furthermore, Chargaff, Levine, Green, and Kream (*Experientia*, 1950, **6**, 229) have shown that the ratio, aminopropanol produced on hydrolysis : phosphorus in vitamin B_{12}, is two to one.

substances we believe to be identical with the two compounds which we obtained as fraction (4) in our hydrolysis experiments described above. Under the conditions of hydrolysis specified by Holiday and Petrow (*loc. cit.*) or in the experiments mentioned above, we have never obtained any of the simple benziminazole derivative (I; R = H); we have, however, obtained it by hydrolysis under more vigorous conditions (16 hours at 150° with 6N-hydrochloric acid).

The supplies of vitamin B_{12} isolated from liver and available to us were too limited to permit of adequate degradative studies involving isolation of the colourless hydrolysis products as distinct from their study by such techniques as paper chromatography, but the recognition that there exist several closely related " vitamins B_{12} " has recently made possible an extension of our investigations and we have devoted our main attention to a new member of the vitamin B_{12} group, for which the name vitamin B_{12c} is proposed. The existence of more than one red clinically active factor in liver extracts was first recognised by Lester Smith and his colleagues (*loc. cit.*), and the crystallisation of a second active compound from this source has been reported from the Lederle Laboratories (Pierce, Page, Stokstad, and Jukes, *loc. cit.*). Biologically, this new compound was indistinguishable from the original vitamin B_{12} in its effect on the growth of *L. leichmanii* 313 (Hoffmann, Stokstad, Hutchings, Dornbush, and Jukes, *J. Biol. Chem.*, 1949, **181**, 635) and in the chick assay (Stokstad, Jukes, Pierce, Page, and Franklin, *ibid.*, 1949, **180**, 647), and it was named by its discoverers vitamin B_{12b}. A similar product was also obtained from cultures of *S. aureofaciens* used for the production of aureomycin (Stokstad *et al.*, *loc. cit.*; *Fed. Proc.*, 1950, **9**, 235) and from *S. griseus* cultures (Fricke, Lanius, de Rose, Lapidus, and Frost, *ibid.*, p. 173). The name vitamin B_{12b} was selected because B_{12a} had been earlier applied to a product which had been obtained by Kaczka, Wolf, and Folkers (*J. Amer. Chem. Soc.*, 1949, **71**, 1514) by catalytic hydrogenation of vitamin B_{12} followed by aerial oxidation and was reported to have only some 20% of the activity of the original vitamin when assayed using *L. leichmanii* 313. Whether vitamin B_{12a} does in fact represent merely an impure preparation of vitamin B_{12b}, as has been claimed by Pierce, Stokstad, Jukes, *et al.* (*J. Amer. Chem. Soc.*, 1950, **72**, 1042, 2615), has not yet been finally established.

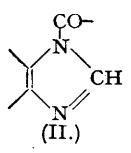
Vitamin B_{12c} is quite distinct from any of the factors so far discussed. It has been supplied to us by Dr. Lester Smith of Glaxo Laboratories Ltd., who has produced it from culture broths of *S. griseus*. Full details of its isolation and its relationship to other members of the B_{12} group will be published elsewhere, but its activity in promoting the growth of *L. lactis* Dorner ATCC 8000 (Cuthbertson *et al.*, *Biochem. J.*, 1949, **44**, v) and *L. leichmannii* 313 (Lees and Emery, *ibid.*, **45**, ii) is similar to that of vitamin B_{12} and it is also highly active clinically in the treatment of pernicious anæmia. Vitamin B_{12c} is the " unnamed factor " mentioned in papers by Lester Smith and Ungley, and by Mollin and Dacie at a meeting of the Royal Society of Medicine on February 14th, 1950 (*Lancet*, 1950, i, 353; *Proc. Roy. Soc. Med.*, in the press) and at times referred to as vitamin B_{12x}. Crystallised in the same way as vitamin B_{12} itself, vitamin B_{12c} forms dark red needles and gives analytical values approximating to a formula $C_{63}H_{97}O_{22}N_{14}PCo$, *i.e.*, it appears to be more highly oxygenated than vitamin B_{12} which it otherwise closely resembles.

We are at present engaged on a detailed investigation into the structure of vitamin B_{12c}, and some of our results concerning its behaviour on acid hydrolysis are now presented. It should be emphasised that, although our results refer strictly to vitamin B_{12c}, careful comparisons with vitamin B_{12}, treated under identical conditions, have revealed no differences in behaviour in so far as the colourless products of hydrolysis are concerned; the difference between the two vitamins seems to concern that part of the molecule which is represented by the red cobalt-containing fission product. Acid hydrolysis of vitamin B_{12c} yields, in addition to phosphoric acid, ammonia, the base giving a positive ninhydrin reaction, and a red amorphous product, three substances all of which appear to be benziminazole derivatives (cf. Holiday and Petrow, *loc. cit.*). Of these three, one is 5 : 6-dimethylbenziminazole and is produced only under drastic conditions of hydrolysis (150° for 5—16 hours with 6N-hydrochloric acid), while the other two are the more complex substances which, on the evidence of Holiday and Petrow (*loc. cit.*), have been regarded as *N*-substituted 5 : 6-dimethylbenziminazoles; we will refer to the more and the less complex of these compounds as the α- and β-benziminazole respectively, as has been done by Holiday and Petrow.

The α-benziminazole is the sole colourless product showing strong ultra-violet absorption when vitamin B_{12c} is hydrolysed under relatively mild conditions, *e.g.*, with N-hydrochloric acid at 100° for 1—20 hours. We have isolated this substance in the form of its barium salt and shown it to be a substituted phosphoric acid. Further hydrolysis of the α-benziminazole, or hydrolysis of the original vitamin B_{12c} under more vigorous conditions (100° for 48 hours with

6N-hydrochloric acid), gives the phosphorus-free β-benziminazole accompanied by some of the unchanged α-compound, and still more drastic hydrolysis yields the unsubstituted 5 : 6-dimethylbenziminazole. These reactions suggest that the α-benziminazole contains a phosphorylated side-chain attached to one of the nitrogen atoms in the benziminazole nucleus and that the β-benziminazole contains the same side-chain without the phosphoryl group; this is in accordance with the behaviour of the compounds on paper chromatography and with their absorption spectra. Our efforts have therefore been directed towards determining the nature and mode of attachment of this side chain and the position of the phosphoryl group.

Analysis of the barium salt of the α-benziminazole indicates a formula $C_{14}H_{17}O_7N_2PBa,H_2O$ or $C_{15}H_{19}O_8N_2PBa,H_2O$ from which it may be surmised that the side-chain grouping of five or six carbon atoms bears several hydroxy-groups as well as a phosphoryl residue. If this side-chain is assumed to be attached to nitrogen, the simplest types of linkage which might be involved are represented by the partial structures (II), (III), (IV), and (V). Of these, (II) and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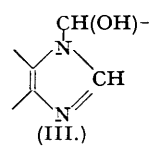
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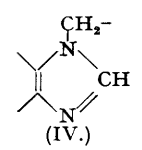
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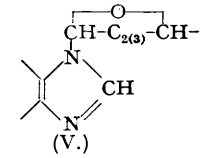
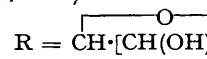

(V.)

(III) seemed most unlikely in view of the known instability, towards acids, of *N*-acylbenziminazoles (cf. Oddo and Ingraffia, *Gazzetta*, 1932, **62**, 1092) and of *N*-(1-hydroxyalkyl)benziminzoles, the latter being in effect substituted aldehyde-ammonias. Structures of type (IV) seemed at first attractive since they would include 5 : 6-dimethyl-1-D-ribitylbenziminazole, a compound bearing a relationship to riboflavin which might be biogenetically significant. Comparison on paper chromatograms showed, however, that the phosphorus-free β-benziminazole is not identical with 5 : 6-dimethyl-1-D-ribitylbenziminazole. The α-benziminazole is unaffected by periodic acid and so contains no free α-glycol grouping; the β-benziminazole, on the other hand, is oxidised by periodic acid, from which it may be deduced that the α-compound contains the grouping $CH(OH)\cdot CH\cdot O\cdot PO_3H_2$. In this laboratory, a method for the detection of α-glycols on paper chromatograms, based on oxidation with periodic acid followed by a colour reaction with Schiff's reagent (Buchanan, Dekker, and Long, forthcoming publication), has recently been developed. Application of this method to the phosphate-free β-benziminazole showed that its behaviour was exactly analogous to that of the pyrimidine and purine ribonucleosides, the periodate-oxidation product being similar in its colour reaction on paper to that of the dialdehyde formed from the natural nucleosides. In this respect, it differed markedly from both 1-(2 : 3-dihydroxypropyl)-5 : 6-dimethylbenziminazole and 5 : 6-dimethyl-1-D-ribitylbenziminazole, with which the method fails, owing, apparently, to further reaction of the side-chain on periodate oxidation. This anomalous behaviour with periodate has also been observed in the case of riboflavin itself by Dr. H. S. Forrest in this laboratory (private communication); the course of the reaction in this and similar cases is not yet clear and will be investigated as opportunity permits. It appears from these experiments that a linkage of type (IV) with a side-chain of a type analogous to that in riboflavin cannot be present in the benziminazole derivatives obtained from vitamin B_{12c}.

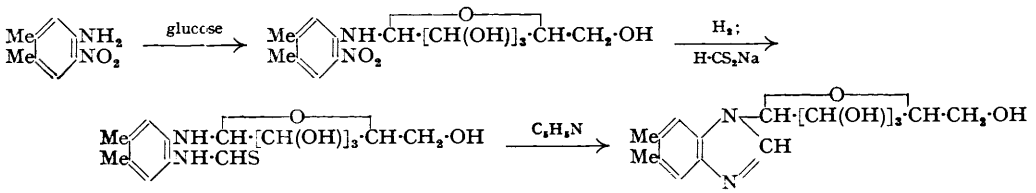
The linkage represented by structure (V) is, of course, glycosidic and we were at first inclined to reject this as a possibility in view of the comparative ease with which *N*-glycosides undergo fission with acids. If the α-benziminazole were a phosphorylated *N*-pentoside or *N*-hexoside of 5 : 6-dimethylbenziminazole its resistance to acids (it appears to be virtually unaffected by 15 hours' heating at 100° with 6N-hydrochloric acid) would seem to be at variance with commonly accepted ideas, the more so as 9-glycosylpurines are easily hydrolysed by dilute acids. Nevertheless, the failure of the α-benziminazole to react with periodate and the reaction of the β-compound to give a product very similar to those obtained from the purine and pyrimidine nucleosides would be most readily explained on the basis of a glycosidic structure in which the α- and β-compounds stand to one another in the same relation as a purine nucleotide to its phosphorus-free nucleoside. It was, therefore, decided to prepare model benziminazole glycosides in order to compare their behaviour towards acid with that of the vitamin degradation products. Both 1-D-glucosyl-5 : 6-dimethylbenziminazole (I; R = $C_6H_{10}O_5$) and 1-D-glucosylbenziminazole (the latter kindly provided by Dr. I. Goodman of the University of Colorado) were unaffected by 20 hours' heating at 100° with 6N-hydrochloric acid and failed to give a Molisch reaction under normal conditions. In these and other respects, the synthetic compounds

closely resembled the β-benziminazole and it seemed almost certain that that they had the same type of structure, *i.e.*, that the α-compound was a benziminazole nucleotide and the β-compound the corresponding nucleoside.

Since this work was carried out, Brink, Holly, Shunk, Peel, Cahill, and Folkers (*J. Amer. Chem. Soc.*, 1950, **72**, 1866) have announced the isolation of 5 : 6-dimethyl-1-D-α-ribofuranosyl-benziminazole (I; R = ) from the hydrolytic degradation of vitamin B_{12} and confirmed the structure by direct comparison with a synthetic specimen. In their paper, Brink *et al.* have suggested that the name ribazole be given to the vitamin B_{12} degradation product 5 : 6-dimethyl-1-α-D-ribofuranosylbenziminazole. We feel that there is little to be gained by the use of such a term; the systematic name of the compound is not unduly complicated. Moreover, the name ribazole is open to strong objection on a variety of grounds; it certainly does not indicate that the compound is a glycoside and the use of a trivial name for a substance which contains two alkyl substituents and a carbohydrate residue (any or all of which might be varied) on the basic nucleus is much to be deplored.

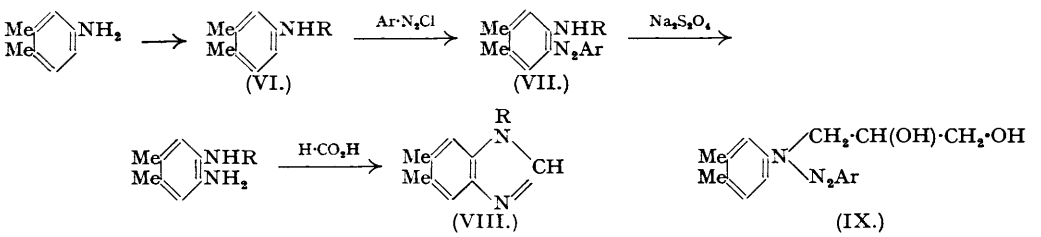
5 : 6-Dimethyl-1-α-D-ribofuranosylbenziminazole is doubtless identical with our β-benziminazole and, if we accept the structure assigned by the Merck group, then other observations can be made on the structure of our phosphorylated benziminazole and of vitamins B_{12} and B_{12c}. The benziminazole nucleotide must have the empirical formula $C_{14}H_{19}O_7N_2P$ and, as it contains no α-glycol grouping, the phosphoryl group must be located at $C_{(2)}$ or $C_{(3)}$ in the ribose residue; its isolation thus determines the position of the phosphorus atom in vitamins B_{12} and B_{12c} (within these limits).

Certain points of chemical interest have arisen from the syntheses of the *N*-substituted benziminazoles. 1-D-Glucopyranosyl-5 : 6-dimethylbenziminazole was prepared by a method similar to that already used in these laboratories for the synthesis of purine glycosides (Baddiley, Lythgoe, and Todd, *J.*, 1944, 318, and numerous later papers), *viz.*, by the cyclisation of the 2-thioformamidoaniline glucoside :



The product, which was obtained as a sesquihydrate, was also prepared, although in very poor yield, by reaction of α-acetobromoglucose with the silver salt of 5 : 6-dimethylbenziminazole. The scope of the thioformamido-route to benziminazole glycosides is at present under investigation, as are other possible approaches.

The syntheses of 1-(2 : 3-dihydroxypropyl)-5 : 6-dimethylbenziminazole [(VIII); R = CH_2·$CH(OH)$·CH_2·OH] and 5 : 6-dimethyl-1-ribitylbenziminazole {VIII; R = CH_2·$[CH(OH)]_3$·CH_2·OH} were carried out according to the annexed scheme. Under the



conditions employed, the coupling of *N*-(2 : 3-dihydroxypropyl)-*o*-4-xylidine [VI; R = CH_2·$CH(OH)$·CH_2·OH] with 2 : 5-dichlorobenzenediazonium chloride gave a product which appeared to consist mainly of the diazoamino-compound (IX) (cf. Karrer and Meerwein, *Helv. Chim. Acta*, 1936, **19**, 264) which was converted into the isomeric azo-compound [VII; R = CH_2·$CH(OH)$·CH_2·OH] on treatment with warm formic acid; the azo-compound was then converted into the benziminazole [VIII; R = CH_2·$CH(OH)$·CH_2·OH] as indicated. Attempts to prepare the same benziminazole by hydroxylation of 1-allyl-5 : 6-dimethylbenziminazole were unsuccessful.

Experimental.

Vitamin B_{12c}.—The *vitamin* was provided by Dr. Lester Smith of Glaxo Laboratories Ltd. For analysis, a sample was crystallised repeatedly from aqueous acetone, forming dark red needles (Found, in a sample dried at 100° in a high vacuum: C, 50·5; H, 6·5; N, 13·3; P, 2·05; Co, 4·7. $C_{63}H_{97}O_{22}N_{14}PCo$ requires C, 50·7; H, 6·6; N, 13·1; P, 2·1; Co, 4·0%).

The α-Benziminazole.—(1) *Detection.* Vitamin B_{12} (0·5 mg.; Fantes, Page, Parker, and Lester Smith, *loc. cit.*) was heated under reflux with N-hydrochloric acid (2 c.c.) and *n*-butanol (2 c.c.) for 70 minutes. After cooling, the contents of the flask were transferred to a small test-tube, and the aqueous layer separated (absorption spectrum: maxima at 277 and 285 mμ.) and evaporated to dryness. The residue was dissolved in a very small drop of water and introduced on to a paper chromatogram. After neutralisation of the spot with ammonia vapour, the chromatogram was irrigated with the top layer of a *n*-butanol–acetic acid–water mixture (4 : 1 : 5; henceforward described as the butanol–acetic acid–water mixture) for 16 hours and, after it had been dried, the paper was photographed in ultra-violet light (cf. Markham and Smith, *Biochem. J.*, 1949, **45**, 294). The R_F value of the single spot in the track of the hydrolysate was 0·55 and that of a 5 : 6-dimethylbenziminazole standard 0·79.

A repeat experiment with parallel hydrolyses of vitamins B_{12} and B_{12c} gave the following spots when photographed as before: vitamin B_{12} hydrolysate, R_F 0·48; vitamin B_{12c} hydrolysate, R_F 0·47; 5 : 6-dimethylbenziminazole standard, R_F 0·77. This paper was used later to demonstrate the presence of the ninhydrin-reacting base in the hydrolysate (below).

(2) *Isolation of the barium salt.* Vitamin B_{12} (146·6 mg.) was heated under reflux with a mixture of concentrated hydrochloric acid (AnalaR; 7 c.c.), water (63 c.c.), and *n*-butanol (10 c.c.) for 2 hours. The solution was cooled, more *n*-butanol (5 c.c.) added, and the butanol layer removed. The pink aqueous layer was extracted twice with butanol (9 c.c.; 7 c.c.) on the steam-bath for 20 minutes each time and the combined dark red butanol extracts were shaken with 2N-hydrochloric acid (2 × 10 c.c.). After evaporation of the solvent from the combined aqueous layers under reduced pressure, there remained a red crystalline residue (78 mg.).

Meanwhile, a column of paper pulp for chromatography was prepared by packing the dry paper (H. Reeve Angel and Co. Ltd., London; 40 g.) into a column (20 × 2·5 cm.) and then thoroughly wetting the column with water-saturated *n*-butanol (henceforward described as wet butanol). Metallic ions were removed by introducing two successive solutions of 8-hydroxyquinoline (100 mg.) in wet butanol (4 c.c.). The column was then washed with wet butanol (110 c.c. in all) until free from 8-hydroxyquinoline (no colour with ferric chloride).

The red crystalline hydrolysate was dissolved in a mixture of wet butanol (4 c.c.) and butanol (1 c.c.), the latter being necessary to give a homogeneous solution, which was brought on to the column followed the wet butanol washings (2 × 1 c.c.). Wet butanol was used as the eluant, and the ultra-violet absorption spectra of successive fractions of the eluate were measured in a Beckman spectrophotometer. After 45 c.c. of the eluate had been collected, at least four coloured bands were visible on the chromatogram, and after 75 c.c. the spectrum of the eluate showed the presence of a benziminazole (maxima at 277 and 285 mμ.) which persisted until 155 c.c. had been collected. The leading pink band was also eluted (126—140 c.c.) in these fractions which were combined and evaporated to dryness under reduced pressure, leaving a solid pink residue (34·7 mg.). This was taken up in water (1 c.c.), and a small drop of the solution subjected to paper chromatography using wet butanol for irrigation. A spot, R_F 0·14, was observed when the chromatogram was photographed in ultra-violet light, but no purple spots were obtained on spraying the paper with ninhydrin solution and subsequent heating at 100° (cf. Consden and Gordon, *Nature*, 1948, **162**, 180).

Glacial acetic acid (0·05 c.c.) was added to the remainder of the aqueous solution which was boiled, filtered, and combined with aqueous washings (3 c.c.). Evaporation of the solvent under reduced pressure gave a red gum (30·3 mg.) which was taken up in water (3 c.c.), to which a saturated solution of barium hydroxide was then added dropwise (0·8 c.c. in all) until the solution was alkaline to phenolphthalein. The solution was boiled and filtered, and the precipitate washed with water (1 c.c.) followed by ethanol (10 c.c.). The combined filtrates were boiled for 2 minutes and set aside in the refrigerator overnight. The precipitate was separated and dissolved in water (5 c.c.) as far as possible, and any solid material separated again. Ethanol (10 c.c.) was added to the filtrate, and the resulting suspension kept at 0° for 6 hours. The precipitate was spun off (centrifuge) and washed with 75% ethanol (5 c.c.) followed by acetone (5 c.c.), and the faintly pink amorphous solid (19 mg.) dried in a vacuum at room temperature for 16 hours (Found, in a sample dried at 110° at 0·1 mm.: C, 33·1; H, 3·9; N 5·1; P 6·7; $C_{14}H_{17}O_7N_2PBa,H_2O$ requires C, 32·9; H, 3·7; N, 5·5; P, 6·1. $C_{15}H_{19}O_8N_2PBa,H_2O$ requires C, 33·3; H, 3·9; N, 5·2; P, 5·7%). The ultra-violet absorption spectrum in 0·1N-hydrochloric acid showed maxima at 277 and 285 mμ.; $\varepsilon_{max.} = 6730$ and 6360 respectively.

Periodate oxidation. Finely divided barium salt (1·428 mg.) was thoroughly mixed with N-sulphuric acid (0·1 c.c.) and water (0·2 c.c.) to precipitate barium sulphate. Sodium metaperiodate (1 c.c.; 0·024M.) was added and the mixture set aside with exclusion of light for 20 hours. Determination of the unchanged metaperiodate iodometrically, after neutralisation of the solution with N-sodium hydroxide (0·1 c.c.), showed that none had reacted with the α-benziminazole. A similar experiment with the barium salt of cytidine 5′-phosphate (1·938 mg.) (Michelson and Todd, *J.*, 1949, 2476) showed that 1·24 mols. of metaperiodate were consumed per mol. of nucleotide.

The β-Benziminazole.—Detection. Vitamin B_{12} (17·7 mg.) was heated at 100° in a sealed tube with 8N-hydrochloric acid for 48 hours and the purple solution so obtained evaporated to dryness. The solid residue was extracted with acetone (3 c.c.) which removed the purple pigment, and the colourless residue was dissolved in wet butanol (3 c.c.) and brought on to a cellulose chromatogram (15·5 × 1·7 cm.; from

17 g. of cellulose and 40 c.c. of wet butanol, heavy metals being removed with 8-hydroxyquinoline as before). Wet butanol was passed through the column, and the ultra-violet absorption spectra of successive 1-c.c. fractions were measured. The benziminazole fraction (from 23 c.c. to 36 c.c. of the eluate) was treated with 8N-hydrochloric acid (0·06 c.c.) and evaporated to dryness in a vacuum, the solid residue was dissolved in water (1 c.c.) and filtered, and the combined filtrate and washings (2 × 0·5 c.c.) were evaporated to dryness. The residue was again taken up in water (0·09 c.c.), and one drop of this solution was subjected to paper chromatography, after neutralisation with ammonia, using the butanol–acetic acid–water mixture as solvent. Two spots were present in the ultra-violet photograph, having R_F values 0·46 and 0·67, the former being due to the α-benziminazole (5 : 6-dimethylbenziminazole standard had R_F 0·77). The R_F value for the α-benziminazole is very sensitive to slight changes in the composition of the butanol–acetic acid–water solvent. In a second chromatogram with wet butanol as solvent, the two spots from the hydrolysate had R_F values 0·12 and 0·78, and 5 : 6-dimethylbenziminazole had R_F 0·89. No ninhydrin-reacting compounds were detected on these chromatograms.

In a similar hydrolysis of vitamin B_{12c} at 100° with 8N-hydrochloric acid for 36 hours, the product was treated exactly as in the previous experiment. The two spots in the track of the hydrolysate had R_F values 0·56 (α-benziminazole) and 0·73, that of 5 : 6-dimethylbenziminazole being 0·79, and the single spot on the same chromatogram from a hydrolysis of vitamin B_{12} with N-hydrochloric acid had R_F 0·55.

The various spots on the above chromatograms were cut out and extracted with water (2 c.c.) for 10 minutes, and the ultra-violet absorption spectra of the solutions determined. The α- and β-benziminazoles showed maxima at 277 and 285 mμ., and unsubstituted 5 : 6-dimethylbenziminazole at 275—276 and 283—284 mμ.

5 : 6-*Dimethylbenziminazole.*—Vitamin B_{12} (1·1 mg.) was hydrolysed with 6N-hydrochloric acid in a sealed tube at 145° for 16 hours. The purple solution, after evaporation to dryness under reduced pressure, was extracted with acetone (1 c.c.), and the colourless residue, in the minimum quantity of water, transferred to a paper chromatogram. Vitamin B_{12c} (1·1 mg.) was treated similarly. The chromatogram was developed with the butanol–acetic acid–water mixture and photographed as before. Each hydrolysate showed a single spot: vitamin B_{12} hydrolysate, R_F 0·75; vitamin B_{12c} hydrolysate, R_F 0·74; 5 : 6-dimethylbenziminazole standard, R_F 0·74.

In another experiment, vitamin B_{12c} (0·9 mg.) was heated with 6N-hydrochloric acid (1 c.c.) at 147° for 5 hours, and the colourless fraction isolated in the same way as previously. Paper chromatography as before showed the presence of a single benziminazole, R_F 0·80 (5 : 6-dimethylbenziminazole standard, R_F 0·78). The benziminazole spot from the vitamin B_{12c} hydrolysis was extracted with N-hydrochloric acid (2 c.c. for 30 minutes), and the absorption spectrum of the extract was shown to exhibit maxima at 275 and 283 mμ.

Examination of the α- and the β-Benziminazole by Paper Chromatography.—The α-benziminazole was obtained from the hydrolysis of vitamin B_{12c} (4·9 mg.) with N-hydrochloric acid (colourless residue dissolved in 0·11 c.c. of water), and a mixture of α- and β-benziminazoles was obtained from the hydrolysis of vitamin B_{12} (17·7 mg.) with 8N-hydrochloric acid, subsequent purification being as described above (colourless residue dissolved in 0·09 c.c. of water)

(1) *Presence of phosphorus in the α-benziminazole.* The mixture of the α- and the β-benziminazole after exposure to ammonia vapour was chromatographed on paper by the ascending technique (Williams and Kirby, *Science*, 1948, **107**, 481), with *n*-butanol as solvent, and photographed in ultra-violet light. The observed R_F values of the two spots, 0·12 and 0·78, corresponded to the α- and the β-compound respectively, and that of a 5 : 6-dimethylbenziminazole standard was 0·89. The paper was then sprayed with the molybdate–perchloric acid reagent of Hanes and Isherwood (*Nature*, 1949, **164**, 1107), heated at 90° for 7 minutes, exposed to water vapour, and finally treated with hydrogen sulphide. A blue spot, indicating the presence of phosphorus, was obtained with R_F 0·12.

(2) *Reaction of the α-benziminazole with sodium metaperiodate.* The α-benziminazole solution (0·015 c.c.) was mixed with an equal volume of sodium metaperiodate solution (0·24M.) on a small watch-glass, and the covered vessel kept in the dark for 20 hours. Water (0·02 c.c.) and glycerol (0·02 c.c.) were added, and the mixture was kept for a further 5 hours. The residue was transferred to a paper chromatogram and developed with the butanol–acetic acid–water mixture. Ultra-violet photography showed two spots, having R_F 0·09 and 0·54 (α-benziminazole standard 0·51), of which the former, after extraction with water, showed no specific absorption in the region 270—300 mμ. characteristic of the benziminazole nucleus. This slow-moving spot was probably caused by the attack of periodate on the filter-paper or by glycerol oxidation products. Examination of the paper for phosphorus showed a single blue spot, having R_F 0·52 (α-benziminazole standard 0·54). The ninhydrin-reactive base present in the original solution (below) was not detectable after the periodate treatment.

(3) *Reaction of the mixed α- and β-benziminazoles with sodium metaperiodate.*—The mixed α- and β-benziminazole solution (0·015 c.c.; above) was treated with sodium metaperiodate solution (0·24M.; 0·02 c.c.) as in the previous experiment. Water (0·04 c.c.) and glycerol (0·02 c.c.) were added, and the mixture was kept for a further 24 hours. The resulting solution was subjected to paper chromatography with the butanol–acetic acid–water mixture as solvent, and the dried paper after being photographed in ultra-violet light showed three spots having R_F values, 0·06, 0·50, and 0·94 respectively. The mixed α- and β-benziminazoles, run in parallel for comparison, showed two spots, having R_F values 0·47 and 0·69 respectively. The nature of the slowest-moving spot in the periodate oxidation experiment has been discussed above, where it was shown that this compound does not contain a benziminazole nucleus. The chromatogram was then treated with the phosphate reagent and developed with hydrogen sulphide as described above. The oxidation product gave a single blue spot, of R_F 0·50, and the mixed α- and β-benziminazoles gave a similar blue spot of R_F 0·48 due to the α-compound.

(4) *Use of sodium metaperiodate as a spraying reagent.* A paper chromatogram of the mixed α- and β-benziminazoles was run with the butanol–acetic acid–water mixture as solvent and, after photography in the usual way, the normal two spots were observed, having R_F 0·60 and 0·70 (5 : 6-dimethylbenziminazole standard 0·80). The chromatogram was sprayed with a mixture of equal volumes of 5% sodium metaperiodate solution and 3N-sulphuric acid and kept at room temperature for 30 minutes. It was exposed to sulphur dioxide for 5 minutes in a large beaker, then sprayed with Schiff's reagent (1 g. of rosaniline was dissolved in 50 c.c. of water and the solution just decolorised with sulphur dioxide; the filtered solution was diluted to 1 l.), and again treated with sulphur dioxide to remove the excess of iodine. The paper was kept at room temperature for 24 hours, whereupon a dark blue spot, which intensified with time, appeared at R_F 0·74.

Comparative experiments exactly similar to the above were carried out with a variety of compounds of known structure:

	R_F values of spots observed after ultra-violet photography.	R_F values of dark blue spots obtained after reaction with periodate and Schiff's reagent.
Mixed α- and β-benziminazoles	0·51 (α)	—
	0·68 (β)	0·69
1-(2 : 3-Dihydroxypropyl)-5 : 6-dimethyl-benziminazole	0·71	—
5 : 6-Dimethyl-1-D-ribitylbenziminazole	0·61	—
5 : 6-Dimethylbenziminazole	0·77	—
Mixed α- and β-benziminazoles	0·42	—
	0·63	0·64
Adenosine	0·41	0·39
1-(2 : 3-Dihydroxypropyl)-5 : 6-dimethylbenz-iminazole	0·66	—

The Ninhydrin-reactive Base.—(1) *Detection in the* N-*hydrochloric acid hydrolysate.* The paper chromatogram of the N-hydrochloric acid hydrolysate of vitamins B_{12} and B_{12c} (above), after photography in ultra-violet light to demonstrate the presence of the phosphorylated α-benziminazole, was sprayed with a solution of ninhydrin (0·1%) in *n*-butanol containing 1% of acetic acid. It was then heated at 100° for 5 minutes. The R_F value of the single purple spot in the track of each of the hydrolysates was 0·41.

In a similar chromatogram an alanine standard gave a spot of R_F 0·24; 1-amino-2-propanol and 2-amino-1-propanol after development at 120° for 3 minutes gave spots each of R_F 0·42.

Further chromatograms of the hydrolysate (spots neutralised with ammonia before running of the chromatogram) were carried out as follows: (*a*) In wet butanol with development at 90° for 5 minutes. The base had R_F 0·15. Standards: alanine, 0·06; 1-amino-2-propanol, 0·16; with 2-amino-1-propanol the violet spot appeared only on further heating at 120° for 3 minutes. (*b*) In collidine with development at 120° for 3 minutes: vitamin B_{12c} hydrolysate, 0·33; 1-amino-2-propanol, 0·35; 2-amino-1-propanol, 0·37; alanine, 0·14.

(2) *Partial purification.* Vitamin B_{12} (15·9 mg.) was hydrolysed with 8N-hydrochloric acid (5 c.c.) in a sealed tube at 100° for 48 hours. The contents of the tube were removed and evaporated to dryness in a vacuum, and the coloured material was extracted with acetone. The colourless residue was dissolved in wet butanol (2 c.c.), brought on to a column of powdered cellulose (9·5 × 1·7 cm.; 10 g., with heavy metals previously removed by 8-hydroxyquinoline treatment; wet butanol as solvent), and eluted with wet butanol, and successive 10-c.c. fractions were collected. Determination of the ultra-violet absorption of these fractions showed that the first two contained no absorbing material, and that fractions 3—5 inclusive contained material showing absorption maxima at 276 and 285 mμ. These fractions (3—5) were combined, evaporated to dryness, and used in experiments with the mixed α- and β-benziminazoles.

Examination of fractions 1—11 with ninhydrin reagent on paper showed that faint purple colours were given with all of the fractions but that those from fractions 8, 9, and 10 were the most intense. These were, therefore, combined, 8N-hydrochloric acid (0·06 c.c.) being added and the solution evaporated to dryness. The residue was dissolved in water (2 c.c.), any solid material separated, and the solution evaporated to dryness in a high vacuum at room temperature. The crystalline solid (2·6 mg.), which gave a positive Nessler reaction, was dissolved in water (0·1 c.c.), and a portion of the solution used for paper chromatography using the butanol–acetic acid–water mixture as solvent. Treatment of the paper with ninhydrin solution and subsequent heating (90° for 15 minutes) gave a single purple spot, of R_F 0·36, in the track of the hydrolysate. DL-Valine standard, 0·44; DL-leucine standard, 0·60. When fractions 7 and 11 were treated similarly, both residues gave a positive reaction with Nessler's reagent but no colour with ninhydrin after paper chromatography.

The remainder of the solution of the ninhydrin-reactive substance was used in the following experiments:

(3) *Effect of nitrous fumes.* The solution of the ninhydrin-reactive substance (0·015 c.c.) was diluted with water (0·5 c.c.) and nitrous fumes (sodium nitrite—dilute hydrochloric acid) were passed through the solution for 5 minutes. 6N-Hydrochloric acid (1 c.c.) was added and the mixture boiled for 5 minutes. The solution was then taken to dryness in a vacuum, and the residue dissolved in water (0·04 c.c.) and transferred to a paper chromatogram. After development with mixed butanol–acetic acid–water solvent, the chromatogram was dried and treated with ninhydrin solution in the usual way. No

coloured spot was obtained. The R_F value of purple spot from the untreated solution on the same chromatogram was 0·39; cf. DL-alanine, 0·27; DL-valine, 0·48; DL-leucine, 0·64.

(4) *Chromatography on paper pre-treated with copper carbonate.* The solution of the ninhydrin-reactive substance was chromatographed on paper pre-treated with copper carbonate (Crumpler and Dent, *loc. cit.*), with wet butanol as solvent. The chromatogram was sprayed with ninhydrin and heated at 100° for 10 minutes, the R_F value of the single purple spot so obtained being 0·15. A β-alanine standard gave a spot with R_F 0·03, but no coloured spots were obtained from DL-alanine or DL-valine after similar treatment.

N-D-Monoglucopyranosyl-4 : 5-dimethyl-o-phenylenediamine.—4 : 5-Dimethyl-2-nitroaniline was converted into its *N*-D-glucopyranoside in 25% yield by the method of Kuhn and Ströbele (*Ber.*, 1937, **70**, 773).

The glucoside (1·25 g.) was suspended in absolute ethanol (100 c.c.) and hydrogenated in the presence of Raney nickel (0·5 g.) at atmospheric temperature and pressure, until the theoretical volume of hydrogen had been absorbed (7 hours). The colourless product which had largely separated out from the solution was filtered off, together with the catalyst, and extracted from the nickel with cold water (10 c.c.). The pale yellow aqueous solution was used directly for the preparation of the thioformyl derivative (below). The colourless alcoholic mother-liquors from the hydrogenation were taken to dryness at room temperature and gave a faintly yellow solid (180 mg.), m. p. 105—110° (decomp.). This appeared to be fairly stable in air or in absolute ethanol but when dissolved in water, in which it was very soluble, it rapidly darkened. Crystallisation from 80% ethanol caused large losses but yielded small greyish plates, m. p. 105—115° (decomp.) which gave analytical values for N-D-*monoglucopyranosyl-4 : 5-dimethyl-o-phenylenediamine monohydrate* (Found : C, 53·9; H, 7·7; N, 8·6. $C_{14}H_{22}O_5N_2,H_2O$ requires C, 53·2; H, 7·7; N, 8·9%).

N-D-*Glucopyranosyl-4 : 5-dimethyl-2-thioformamidoaniline.*—Sodium dithioformate (1 g.) was dissolved in the aqueous solution of diamine obtained as above and the solution kept in a loosely stoppered flask at 20—25° for 52 hours. Hydrogen sulphide was evolved and the solution slowly deposited a mass of small crystals. These were separated, washed with water (1 c.c.) and then ethanol (3 c.c.), and dried in air. The yellowish-grey solid (730 mg.) was stable in air but decomposed with evolution of hydrogen sulphide on being heated to 180°. Crystallisation from ethanol gave the *thioformyl* derivative as small faintly yellow needles, m. p. 180° (decomp.) (Found : C, 52·6; H, 6·8. $C_{15}H_{22}O_5N_2S$ requires C, 52·6; H, 6·4%). The product dissolved readily in boiling water, but hydrogen sulphide was evolved and hydrolysis evidently occurred as 5 : 6-dimethylbenziminazole could be isolated from the solution.

1-D-*Glucopyranosyl-5 : 6-dimethylbenziminazole.*—The thioformyl compound (310 mg.) was heated under reflux with anhydrous pyridine (10 c.c.) in an atmosphere of nitrogen for 7 hours, whereafter the evolution of hydrogen sulphide had diminished and the solution had begun to darken. It was cooled and then evaporated to dryness at 30° in a vacuum, and the residual pale brown resin was dissolved in water (1 c.c.). After being kept for 12 hours at room temperature the crystalline product which had formed was separated, washed with cold water (0·5 c.c.), and dried at 100° to a pale grey felted mass (160 mg.), m. p. 165—166°. Recrystallisation from hot water (2·5 c.c.) with charcoal yielded colourless needles (125 mg.), m. p. 166—167° which after air-drying gave analytical figures of 1-D-*glucopyranosyl-5 : 6-dimethylbenziminazole sesquihydrate* (Found : C, 53·6; H, 7·2; N, 8·5; loss at 125° over P_2O_5 *in vacuo*, 8·4. $C_{15}H_{20}O_5N_2,1{\cdot}5H_2O$ requires C, 53·7; H, 6·9; N, 8·4; H_2O, 8·1%). The anhydrous substance was very hygroscopic.

Action of Hydrochloric Acid on 1-D-*Glucopyranosylbenziminazole.*—The glucoside (8·4 mg.) was heated with 6N-hydrochloric acid (2 c.c.) in a sealed tube at 100° for 20 hours. The solvent was removed from the product at room temperature, the gummy residue dissolved in water (0·5 c.c.), and a portion (0·01 c.c.) of the solution used for paper chromatography. A parallel experiment was conducted with the glucoside (8·5 mg.) and N-hydrochloric acid under similar conditions. The chromatogram was developed with wet butanol and finally photographed in ultra-violet light. The various absorbing materials were then extracted from the chromatogram with N-hydrochloric acid (2 c.c. per spot) for 15 minutes and the spectra of the compounds determined :

	R_F value.	Maxima in spectrum (mμ.).
Benziminazole standard	0·84	267, 274
1-D-Glucopyranosylbenziminazole standard	0·49	268, 275
1-D-Glucopyranosylbenziminazole (after 6N-HCl hydrolysis)	0·49	268, 275
1-D-Glucopyranosylbenziminazole (after N-HCl hydrolysis)	0·49	268, 275

Action of Hydrochloric acid on 1-D-*Glucopyranosyl-5 : 6-dimethylbenziminazole.*—The glucoside was hydrolysed with 6N-hydrochloric acid in a sealed tube at 100° for 20 hours and the reaction product treated exactly as described in the previous experiment :

	R_F value.	Maxima in spectrum (mμ.).
5 : 6-Dimethylbenziminazole standard	0·87	274—275; 283
1-D-Glucopyranosyl-5 : 6-dimethylbenziminazole standard	0·60	276—277; 285
1-D-Glucopyranosyl-5 : 6-dimethylbenziminazole (after 6N-HCl hydrolysis)	0·60	276—277; 285

5 : 6-*Dimethyl*-1-D-*ribitylbenziminazole* (with Dr. H. S. FORREST).—4 : 5-Dimethyl-2-(D-ribitylamino)-azobenzene (2 g.; Bergel, Cohen, and Haworth, *J.*, 1945, 165) in ethanol (100 c.c.) was reduced by addition of saturated sodium dithionite solution until all the colour had disappeared. The solution was

then evaporated to dryness and the residue thoroughly extracted with alcohol. The solvent was removed from the extracts, and the residue treated with formic acid (98%; 4 c.c.) and 4N-hydrochloric acid and heated under reflux for 30 minutes. The resulting solution was cooled, filtered, and neutralised with concentrated ammonia solution, and the yellow precipitate so obtained crystallised from very dilute alcohol to give colourless needles (0·5 g.) of 5 : 6-*dimethyl*-1-D-*ribitylbenziminazole*, m. p. 194—196°, raised to 198—200° by recrystallisation from water (Found : C, 59·6; H, 7·1; N, 10·1. $C_{14}H_{20}O_4N_2$ requires C, 60·0; H, 7·1; N, 10·0%). Light absorption : (i) in water : maxima at 251 and 278 mμ.; $\varepsilon_{max.}$ = 5500 and 6040; (ii) in 0·1N-hydrochloric acid : Maxima at 276·5 and 285 mμ.; $\varepsilon_{max.}$ = 7800 and 7450.

N-(2 : 3-*Dihydroxypropyl*)-o-4-*xylidine* [VI; R = CH_2·CH(OH)·CH_2·OH].—A solution of potassium hydroxide (3 g.) in absolute ethanol (15 c.c.) was added to *o*-4-xylidine (6 g.) and α-monochlorohydrin (5·5 g.) in ethanol (15 c.c.), and the mixture kept overnight at room temperature. The precipitated salt was separated and washed, and the combined filtrates and washings were evaporated to dryness in a vacuum. Dilute sodium hydroxide solution was added to the residue, a brown oil being precipitated. Benzene (10 c.c.) and light petroleum (20 c.c.; b. p. 60—80°) were added, the mixture was well cooled and emulsified, and crystallisation, induced by scratching, was completed at 0° overnight. The *product* (5·2 g.) had m. p. 97—99°, raised to 100—101° by recrystallisation from water (Found : C, 68·0; H, 8·8. $C_{11}H_{17}O_2N$ requires C, 67·7; H, 8·7%).

N-(2 : 5-*Dichlorobenzeneazo*)-N-(2 : 3-*dihydroxypropyl*)-o-4-*xylidine* (IX).—A clear diazonium solution prepared from 2 : 5-dichloroaniline (3·3 g.) and sodium nitrite (1·7 g.) was added quickly to a stirred solution of *N*-(2 : 3-dihydroxypropyl)-*o*-4-xylidine in acetic acid (100 c.c. of 10%). A gummy material was precipitated from the red solution and after 15 minutes at room temperature, the excess of sodium acetate and the granular orange-red solid were collected, triturated with cold water, washed, and dried (6·2 g.). The *aminoazo*-compound crystallised from dilute alcohol or benzene to give very pale orange felted needles, m. p. 135—136° (decomp. *ca.* 160° with effervescence) (Found : C, 55·1; H, 5·2. $C_{17}H_{19}O_2N_3Cl_2$ requires C, 55·4; H, 5·2%). The product was largely unchanged on treatment with alcoholic sodium dithionite.

1-(2 : 3-*Dihydroxypropyl*)-5 : 6-*dimethylbenziminazole* [VIII; R = CH_2·CH(OH)·CH_2·OH].—The above diazoamino-compound (IX) (5·2 g.) was dissolved in hot ethanol (40 c.c. of 75%), and formic acid (10 c.c. of 85%) added. The red solution was cooled and after 3½ hours the product was precipitated by dilution with water and separated after being kept overnight, forming red needles (3·5 g.), m. p. 130—140°, of the crude amino-azo-compound [VII; R = CH_2·CH(OH)·CH_2·OH). This material was dissolved in boiling alcohol and treated with sodium dithionite solution (8 g. in 30 c.c. of water, added in two portions). Inorganic salts were separated from the pale orange solution, and the alcohol was removed from the filtrate in a vacuum. The separated 2 : 5-dichloroaniline was extracted with benzene–light petroleum (1 : 1), and the aqueous solution taken to dryness in a vacuum. The residue was extracted with hot alcohol (3 × 10 c.c.), and the solvent removed from the combined extracts. The residual solid was then dissolved in formic acid (8 c.c. of 85%) containing concentrated hydrochloric acid (2 c.c.), and the solution boiled for 30 minutes. The formic acid was removed *in vacuo* and the residue made alkaline with dilute sodium hydroxide and cooled in the refrigerator. The solid which separated was washed with benzene–light petroleum and then dissolved in boiling water (20 c.c.), and the solution clarified with charcoal. The solution on cooling deposited almost colourless needles of 1-(2 : 3-*dihydroxypropyl*)-5 : 6-*dimethylbenziminazole monohydrate* (0·5 g.) which were purified by recrystallisation from water, then having m. p. 95—98° with resolidification and re-melting at 151—152° (Found : loss on drying at 100° in a vacuum, 7·1. $C_{12}H_{16}O_2N_2,H_2O$ requires 7·6%) (Found, on anhydrous material : C, 65·8; H, 7·0; N, 12·9. $C_{12}H_{16}O_2N_2$ requires C, 65·5; H, 7·3; N, 12·7%).

1-*Allylbenziminazole*.—(1) *From potassium benziminazole*. Benziminazole (3·5 g.) was added to a solution of potassium hydroxide (1·7 g.) in ethanol (25 c.c.), and freshly distilled allyl bromide (3·6 g.) added to the mixture and shaken vigorously. A rapid exothermic reaction occurred and solid was precipitated after a few seconds. After 2 hours, the potassium bromide was separated and washed with alcohol. The filtrate and washings were evaporated to dryness under reduced pressure, the oily product extracted from the residue with ether, and after removal of the solvent the residual brown viscous oil was distilled and the fraction of b. p. 99—100°/0·1 mm. (3·5 g.) collected as a pale yellow oil. The *picrate* formed needes, m. p. 175·5—177° (decomp.), from ethoxyethanol (Found : C, 49·6; H, 3·2. $C_{16}H_{13}O_7N_5$ requires C, 49·6; H, 3·4%).

(2) *From silver benziminazole*. Benziminazole (2 g.) was dissolved in ethanol (40 c.c.) containing aqueous ammonia (*d* 0·880; 1 c.c.). A slight excess of an aqueous solution of silver nitrate was added and the gelatinous precipitate collected after 2 hours, washed with alcohol, and dried over sulphuric acid in a vacuum. The silver salt (2·2 g., 1 mol.) was ground and suspended in benzene (25 c.c.) and, after a little of the solvent had been distilled off to remove any moisture, allyl bromide (3·6 g., 3 mols.) was added and the mixture heated under reflux for 3 hours. The product was purified as in the previous experiment, to give a pale yellow oil (1·3 g., 83%) which gave a picrate identical with that obtained above.

Attempted hydroxylations of the base with hydrogen peroxide–osmium tetroxide (cf. Mugdan and Young, *J.*, 1949, 2988) or performic acid (cf. Swern *et al.*, *J. Amer. Chem. Soc.*, 1946, **68**, 1504) were unsuccessful.

1-*Allyl*-5 : 6-*dimethylbenziminazole*.—This was prepared from silver 5 : 6-dimethylbenziminazole (0·32 g.) and allyl bromide (0·5 g.) as in the previous experiment. The product was distilled at 0·1 mm. to give a pale yellow oil (0·14 g.) which readily gave a *picrate* as long needles, m. p. 192—193° (decomp.), from ethanol (Found : C, 52·3; H, 3·9. $C_{18}H_{17}O_7N_5$ requires C, 52·1; H, 4·1%).

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