THE CHEMISTRY OF ANTI-PERNICIOUS ANÆMIA FACTORS

PART XII. THE 1-AMINOPROPAN-2-OL COMPONENT OF CYANOCOBALAMIN

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ACIDOLYSIS of cyanocobalamin, followed by examination of the hydrolysates by unidimensional paper-strip chromatography, revealed the presence of a "ninhydrin-reacting substance" chromatographically indistinguishable from 2-amino-1-propanol (Ellis, Petrow and Snook[1,2,3]). The result thus obtained was later confirmed by Chargaff, Levine, Green and Kream[4] who, in addition, drew attention to the identity in chromatographic behaviour between the ninhydrin-reacting substance and the isomeric 1-aminopropan-2-ol. Though making no definite claim as to the nature of the former component the American authors nevertheless attempted its quantitative assay, obtaining a value of 2 moles/mol. of cyanocobalamin (calculated as aminopropanol). Its identity with 1-aminopropan-2-ol was subsequently established by Wolf, Jones, Valiant and Folkers[5] and by Cooley, Ellis and Petrow.[6]

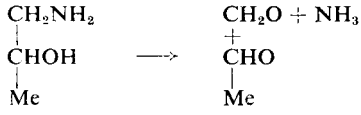
The appearance of 1-α-D-ribofuranosyl-5:6-dimethylbenziminazole phosphate alongside the 1-aminopropanol on mild hydrolysis of cyanocobalamin led Buchanan, Johnson, Mills and Todd[7] to suggest that the vitamin may be ". . . a trisubstituted derivative of phosphoric acid, the base and the red cobalt-containing fragment being attached to the glycosyl benziminazole phosphate through the two free acidic groups of the latter either by ester or amide linkages." Combination of 1-aminopropan-2-ol in this manner, however, was inconsistent with evidence submitted by Cooley, Ellis, Mamalis, Petrow and Sturgeon in Part V.[8] Beaven, Holiday, Johnson, Ellis and Petrow[9] were therefore led to propose an alternative formulation in which the 1-α-D-ribofuranosyl-5:6-dimethylbenziminazole phosphate residue was directly linked to the rest of the structure through a single molecule of aminopropanol. Such a formulation (XI), though having the obvious advantage of structural conformity with other natural products containing aminoalcohol residues, was nevertheless difficult to reconcile with the observation of Chargaff *et al.*[4] that two molecules of aminopropanol are obtained by hydrolysis of vitamin B_{12}. We therefore determined afresh the propanolamine content of hydrolysates of cyanocobalamin. In addition, we examined the preparation of propanolamine phosphate as we wished to study the effect of this compound and of the free base on the yield of vitamin B_{12} obtained by fermentation procedures.

ESTIMATION OF PROPANOLAMINE IN CYANOCOBALAMIN

Initial attempts to determine propanolamine in cyanocobalamin hydrolysates involved separation of the aminoalcohol present in suitable aliquots by use of paper chromatography, followed by its elution and colorimetric

estimation employing the Moore and Stein[10] stannous chloride-ninhydrin reagent. The results obtained, however, were too variable to be of value. Similar difficulties using this reagent in conjunction with paper chromatography have since been reported by other workers,[11,12,13,14] who likewise observed marked interference in the estimation by trace impurities present in the filter papers employed. We therefore turned our attention to the more direct method of assay in which the chromatograms are sprayed with ninhydrin in *n*-butanol to develop the colour *in situ*, after which the coloured zones are eluted and compared absorptiometrically with controls. The values obtained, however, showed marked disagreement with those previously recorded by Chargaff *et al.*[4] Thus a series of 5 experiments gave an average propanolamine content of 5·24 per cent. (req. 5·56 per cent.), from which it follows that only *one* molecule of base is present in the cyanocobalamin molecule.*

The above finding was confirmed by employing an adaptation of the Conway microdiffusion technique[15] which additionally allowed estimation of the ammonium salts also present in hydrolysates of vitamin B_{12} (Ellis, Petrow and Snook[3]). To this end the hydrolysate, placed in the outer chamber of the Conway diffusion unit, was treated with saturated potassium carbonate solution thus decomposing co-present ammonium salts with liberation of ammonia which, in turn, was determined by absorption in 0·01M sodium dihydrogen phosphate contained in the centre chamber, followed by back titration with 0·01M hydrochloric acid. The aminopropanol present in the hydrolysate was then determined by oxidation with periodate as indicated below:



followed by estimation of the ammonia formed in the manner previously described. As before, the results clearly indicated the presence of only *one* aminopropanol residue in the cyanocobalamin structure.

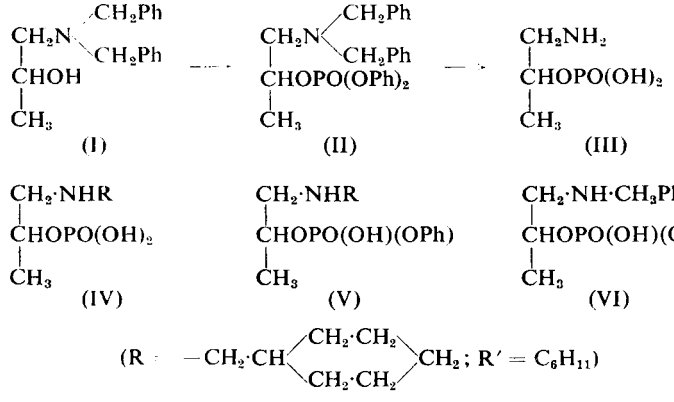
Preparation of 1-Aminopropan-2-ol Phosphate

The preparation of ethanolamine *O*-phosphate has previously been recorded by Cherbuliez and Weniger[16] who employed "polyphosphoric acid," obtained by dehydrating syrupy phosphoric acid in a current of air at 250° C., as phosphorylating agent. Extension of this reaction to 1-aminopropan-2-ol resulted in ready formation of a compound, $C_3H_{10}O_4NP$, which seemed to be the required 1-aminopropan-2-ol *O*-phosphate (III) on the basis of its colour reaction with ninhydrin. It was, nevertheless, felt desirable to seek further evidence on this point by attempting the preparation of (III) by an unambiguous route.

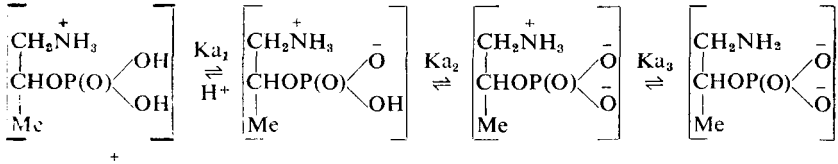
For this purpose l-dibenzylaminopropan-2-ol of established structure

* Dr. J. J. Pfiffner kindly informed the senior author during informal discussions at the 2nd International Congress of Biochemistry that he had independently determined the propanolamine content of cyanocobalamin and likewise obtained a result corresponding to one molecule per molecule of the vitamin.

(I)[17] was phosphorylated with diphenylchlorophosphonate in pyridine solution to give 1-dibenzylaminopropan-2-ol-*O*-diphenylphosphate (II), which was isolated as the hydrochloride. Catalytic hydrogenation of this compound with Adam's catalyst in ethanolic solution resulted in removal of the protective benzyl- and phenyl-groups with formation of authentic 1-aminopropanan-2-ol *O*-phosphate (III), accompanied by smaller quantities of two by-products separately identified as 1-hexahydrobenzylaminopropan-2-ol *O*-phosphate (IV) (*vide infra*) and as a hexahydroderivative of 1-benzylaminopropan-2-ol *O*-phenyl phosphate (V or VI). The 1-aminopropan-2-ol *O*-phosphate (III) thus obtained, moreover, proved identical in every way with the product formed by direct phosphorylation of the base. The constitution of the latter compound as the *O*-phosphate (III) may therefore be regarded as established.

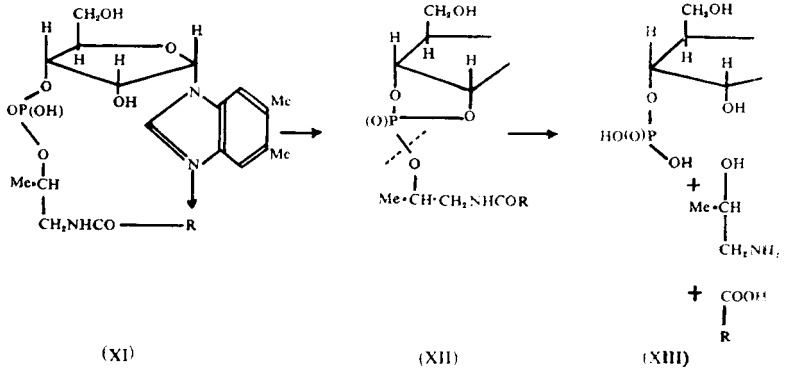


The conclusion thus reached on chemical grounds proved to be in complete accord with the behaviour of the phosphorylated product on potentiometric titration. Thus whereas a propanolamine *N*-phosphate should function as an *N*-substituted amide of phosphoric acid, a propanolamine *O*-phosphate of structure (III) should exist in aqueous solution as the zwitterion (VIII), and behave on ionisation as an ampholyte, (BH_2), of low basicity and possessing two weakly acidic hydrogen atoms. In strongly acid solution, moreover, it should pass into the tribasic acid (BH_3^+) (VII).



Its dissociation should therefore follow the sequence (VII) > (X), and examination of Figure 1 shows that this is indeed the case. Thus the titration curve shows evidence for the conversion of (VIII) into both

(VII) and (IX). In addition, the sigmoid shape assumed by the curve between points A and B is highly typical of ampholytes. Independent proof is thus furnished of the formulation of the phosphorylation product as 1-aminopropan-2-ol *O*-phosphate (III).



(where R is the cobalt-containing macrofragment present in cyanocobalamin.)

Attempts to hydrolyse (III) under the reaction conditions which liberate aminopropanol from cyanocobalamin proved unsuccessful, the starting material being recovered unchanged. Such a result was not unexpected, as simple monoesters of phosphoric acid are known to be stable to hydrolysis.[18] Disubstituted phosphoric esters of the type represented by (XI), in contrast, behave in a different manner,[19] undergoing facile cleavage as indicated in (XIII), presumably by way of the cyclic intermediate (XII).[20] The stability of (III) is thus fully compatible with (XI) and with the observation that mild acidolysis of the latter leads to the release of 1α-D-ribofuranosyl-5:6-dimethylbenziminazole phosphate (cf. XIII), followed subsequently by liberation of aminopropanol.

Experimental

Microanalyses are by Drs. Weiler and Strauss, Oxford. M.pts. are uncorrected.

Determination of 1-*Aminopropan*-2-*ol in Cyanocobalamin*

1. *Paper chromatographic method.* 3·0 mg. of cyanocobalamin (dried at 70° C.) were hydrolysed for 6 hours with 2·0 ml. of 20 per cent. hydrochloric acid in a sealed tube at 100° C. The product, after dilution to 4 ml. with distilled water, was extracted 3 times with 1 ml. quantities of freshly redistilled *n*-butanol. The combined intensely-coloured extracts were then back-extracted with 3 × 0·5 ml. quantities of water, and the aqueous extracts combined with the almost colourless butanol-extracted hydrolysate. Evaporation to dryness gave a faintly pink residue which was dissolved completely in exactly 250 μl. of 0·05N acetic acid. 6 μl. aliquots of this solution were spotted on to a series of filter paper strips (Whatman No. 1) alongside 3, 6 and 12 μl. aliquots of an accurately

standardised solution of 1-aminopropan-2-ol containing 0·5 μg. of base per μl. The strips were irrigated for 18 hours with 70 per cent. *iso*butyric acid, dried at room temperature, sprayed with a 0·1 per cent. solution of ninhydrin in *n*-butanol, and then heated at 100° C. for 10 minutes. Each developed spot was cut out and the coloured material eluted with a mixture consisting of equal volumes of acetone and water. The eluates were made up to 2 ml. and the colour intensities compared employing a "Spekker" photoelectric absorptiometer and Ilford filter (606).

5 separate estimations were performed giving the percentage of 1-aminopropan-2-ol in cyanocobalamin as 5·39, 5·56, 4·96, 5·21 and 5·09 per cent. (average 5·24 per cent.).

During preliminary experiments designed to establish the method of assay it was found that (*a*) 1-aminopropan-2-ol alone is stable under the conditions used for hydrolysis of the cyanocobalamin, and (*b*) the foregoing extraction and chromatographic procedures involve virtually no loss of the base.

2. *Microdiffusion method.* The Conway microdiffusion method[15] for the estimation of ammonia was used in conjunction with the titration technique described by Hawes and Skavinski.[21]

Ca. 3 mg. of cyanocobalamin were used for each estimation, the compound being hydrolysed as under (1) above. The hydrolysate containing both ammonium salts and propanolamine was transferred to the outer chamber of a Conway microdiffusion cell and treated in experiments i, ii and iii with 1 ml. of saturated potassium carbonate solution to liberate the ammonia. The latter was absorbed in 1 ml. of 0·01M sodium dihydrogen phosphate solution contained in the centre chamber and estimated by back titration with 0·01N hydrochloric acid delivered from an "Agla" micrometer syringe, a methyl red-bromocresol green mixture being used as indicator. After completion of the determination, the propanolamine present in the hydrolysate was oxidised to ammonia by addition of 0·5 ml. of 0·2M periodic acid and the ammonia formed estimated as before.

"Total ammonia" only was determined in experiments (iv) and (v). This was done by treating a hydrolysate with the potassium carbonate solution, followed immediately by the periodate solution, and then estimating total ammonia evolved in one operation. A typical experiment gave a (back-titration) reading of 1·10 ml. of 0·01N hydrochloric acid.

The following results were obtained:—

Experiment	Nitrogen as ammonia (per cent.)	Nitrogen as aminopropanol (per cent.)	Total nitrogen (per cent.)
(i)	5·92	0·76	6·68
(ii)	5·71	0·83	6·54
(iii)	5·58	1·07	6·65
(iv)			6·53
(v)			7·22

(Required for 1 mol. of aminopropanol, N = 1·04 per cent.)

1-*Aminopropan*-2-*ol* O-*phosphate* (III). Polyphosphoric acid (18 g.) was thoroughly mixed with 1-aminopropan-2-ol (7·5 g.) and after the exothermic reaction had subsided, the mixture was heated on the steam bath for 30 minutes. The melt was dissolved in water (300 ml.) and adjusted to *p*H 9·0 by addition of concentrated barium hydroxide solution. The barium salts were separated and washed with water and the combined filtrates rendered acid to *p*H 4·5 with sulphuric acid. The barium sulphate was removed by filtration and the filtrate evaporated to dryness. The residue was recrystallised from water and ethanol from which 1-*aminopropan*-2-*ol* O-*phosphate* (5 g.) separated in fine white needles, m.pt. 240 to 241° C. Found: C, 23·5; H, 6·5; N, 9·0. $C_3H_{10}O_4NP$ requires C, 23·2; H, 6·3; N, 9·0 per cent.

1-*Dibenzylaminopropan*-2-*ol*. Dibenzylamine (39·4 g.) was added in portions to a mixture of propylene oxide (11·6 g.) and ethanol (25 ml.) and the mixture heated under reflux for 6 hours. After removal of solvent, the residue was fractionated under reduced pressure to give 1-dibenzylaminopropan-2-ol as a colourless oil (30 g.), b.pt. 130° C. 0·15 mm. The hydrochloride separated from acetone-ether in white needles, m.pt. 184° C. (Kerwin *et al.*[17] give m.pt. 181 to 183° C.).

1-*Dibenzylaminopropan*-2-*ol diphenylphosphate hydrochloride* (II) (with Mr. P. Mamalis). A solution of dibenzylamino*iso*propanol (5·1 g.) in dry pyridine (15 ml.) was chilled in ice and treated with diphenylchlorophosphonate (5·4 g.) in pyridine (10 ml.). After standing at room temperature overnight, the solution was poured into water and the heavy oil washed by decantation with water. The product was taken into ether and the solution washed with dilute acetic acid, sodium bicarbonate solution and water. After drying over magnesium sulphate, a little acetone was added, and a stream of dry hydrogen chloride bubbled in, whereupon the *hydrochloride* of 1-dibenzylaminopropan-2-ol *O*-diphenylphosphate (7·4 g.) separated in rosettes of soft white needles, m.pt. 141° C. Found: N, 2·5; Cl, 6·7; P, 6·2. $C_{29}H_{30}O_4NP,HCl$ requires N, 2·7; Cl, 6·8; P, 6·1 per cent.

1-*Aminopropan*-2-*ol* O-*phosphate* (III). 1-Dibenzylaminopropan-2-ol diphenylphosphate hydrochloride (15 g.) in absolute ethanol (80 ml.) was shaken with hydrogen in the presence of Adam's platinum oxide catalyst (250 mg.) until uptake ceased. Water (50 ml.) was added to dissolve the material which had separated, and the solution filtered and evaporated to dryness. The residue was dried by thrice evaporating with ethanol, and the thick residual oil was dissolved in absolute ethanol (50 ml.). On standing, 1-aminopropan-2-ol *O*-phosphate separated, and after recrystallisation from water and ethanol formed fine needles, m.pt. 237° to 238° C., not depressed in admixture with the compound obtained by direct phosphorylation. Found: C, 23·7; H, 6·8; N, 9·0; P, 19·4. $C_3H_{10}O_4NP$ requires C, 23·2; H, 6·5; N, 9·0; P, 20·0 per cent.

The ethanolic mother liquors on standing deposited 1-*hexahydrobenzyl aminopropan*-2-*ol* O-*phosphate* (IV) which, when recrystallised from water and ethanol, separated in needles, m.pt. 241° to 242° C. Found: C,

47·6; H, 8·7; N, 5·3; P, 12·1. $C_{10}H_{22}O_4NP$ requires C, 47·8; H, 8·8; N, 5·6; P, 12·3 per cent.

The original ethanolic mother liquors were then evaporated to dryness and the residue stirred with a little water, when crystallisation occurred. Purification from water afforded 1-*benzylaminopropan-2-ol* cyclo*hexyl-phosphate* (VI) or its *isomer* (V) in silver needles, m.pt. 204° to 205° C. Found: C, 58·0; H, 8·1; N, 4·2; P, 9·5. $C_{16}H_{26}O_4NP$ requires C, 58·7; H, 8·0; N, 4·3; P, 9·5 per cent.

Potentiometric titrations. The apparatus used for this investigation has been described in Part VIII.[22] In titrations where the *p*H exceeded 10, a "High-Alki" glass electrode was employed as indicator. The electrode was standardised over the range *p*H 4·01 to 9·18 and the sensitivity found to remain constant up to *p*H 12 in solutions of low ionic strength.

For the determination of $p\mathrm{Ka}_2$, approximately 0·01M solutions of 1-aminopropan-2-ol *O*-phosphate were prepared in $2{\cdot}5 \times 10^{-3}$N hydrochloric acid and titrated in 25 ml. portions with 0·1N sodium hydroxide solution. For titrations leading to the determination of $p\mathrm{Ka}_1$ and $p\mathrm{Ka}_3$ a solution of similar strength was prepared in water, and 0·4N hydrochloric acid or sodium hydroxide solution added respectively.

In attempting to calculate $p\mathrm{Ka}_1$ from Figure 1 the relationship

$$p\mathrm{Ka}_1 = p\mathrm{H} + \log_{10}\frac{a - [H^+]}{(c - a) + [H^+]} \quad \ldots\ldots\ldots\ldots \text{(i)}$$

was employed, where a is the concentration of the chloride ion.

Approximate values of $[H^+]$ were calculated from the *p*H readings employing the simplified Debye-Huckel expression

$$-\log_{10}\int_i = A\, z_i\, {}^2\sqrt{I} \quad \ldots\ldots\ldots\ldots \text{(ii)}$$

where A approximates to 0·509 at 25° C. for aqueous solutions, z_i is the valence of the ion i (BH_3^+ in this instance), and I is the ionic strength of the solution. In all cases where the ionic strength was sufficiently small for (ii) to be valid, it was found that $[Cl^-] \simeq [H^+]$, i.e. that coordination of H^+ with the zwitterionic species (VIII) had not occurred. It may be presumed that such association with formation of (VII) would nevertheless take place under conditions of high acidity when (ii) would no longer be valid. The value of $p\mathrm{Ka}_1$ will therefore be not greater than 2.

The value of $p\mathrm{Ka}_2$ (classical) was obtained from the relation

$$p\mathrm{Ka}_2 = p\mathrm{H} + \log_{10}\left[\frac{c - x}{x}\right] \quad \ldots\ldots\ldots\ldots \text{(iii)}$$

where c is the initial concentration of (III) and x is the alkali concentration, whilst for $p\mathrm{Ka}_3$ the modified equation

$$p\mathrm{Ka}_3 = p\mathrm{H} + \log_{10}\left[\frac{(c - a) + [OH^-]}{a - [OH^-]}\right]$$

was employed. Consistent values were obtained in both the $p\mathrm{Ka}_2$ and $p\mathrm{Ka}_3$ determinations. Thus the value for the $p\mathrm{Ka}_3$ of 1-aminopropan-2-ol

O-phosphate derived from different titrations showed a spread of less than 0·1 *p*Ka units at an ionic strength of approximately 2×10^{-2}M.

The classical values of pKa_2 and pKa_3 for 1-aminopropan-2-ol *O*-phosphate and the pKa_2 for 1-hexahydrobenzylaminopropan-2-ol phosphate are recorded in Table I. The thermodynamic constants were calculated from the classical values by means of activity corrections obtained from

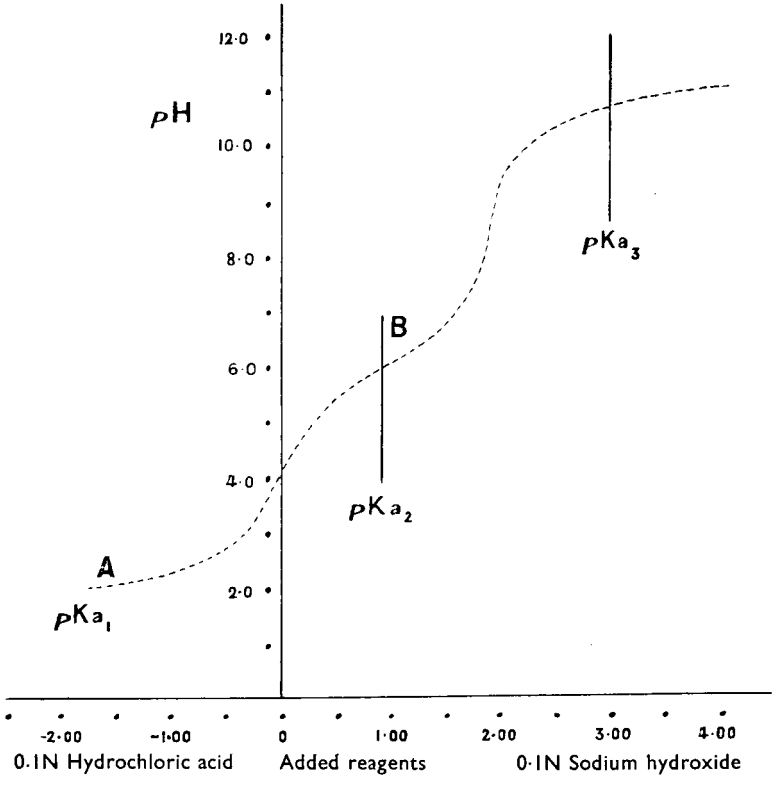


FIG. 1. Titration curve of an aqueous solution of 1-aminopropan-2-ol *O*-phosphate. Concentration = 1·15 g.l.$^{-1}$.

TABLE I

DISSOCIATION CONSTANTS OF PROPANOLAMINE *O*-PHOSPHATES AT 25 ± 1° C.

Substance	Dilution (lit. mol.$^{-1}$)	pKa_2 classical	Activity correction	pKa_2 thermo.	pKa_3 classical	Activity correction	pKa_3 thermo.
1-Aminopropan-2-ol phosphate (by phosphorylation of propanolamine) ..	130 110	5·99	+0·04	6·03	10·76	+0·21	10·97
1-Aminopropan-2-ol phosphate (by the hydrogenation method)	177	5·98	+0·04	6·02			
1-Hexahydrobenzyl aminopropan-2-ol *O*-phosphate ..	230	5·81	+0·03	5·84			

the simplified Debye-Huckel equation. The correction is particularly important in the case of pKa_3 where a divalent ion participates in the equilibrium in a solution of relatively high ionic strength.

Summary and Conclusions

1. Estimation of the 1-aminopropan-2-ol present in hydrolysates of cyanocobalamin shows that only one molar proportion of this amino-alcohol is released on acidolysis.

2. The preparation of 1-aminopropan-2-ol *O*-phosphate is described.

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