Studies in the Biochemistry of Micro-organisms

86. THE MOLECULAR CONSTITUTION OF MYCOPHENOLIC ACID, A METABOLIC PRODUCT OF *PENICILLIUM BREVI-COMPACTUM* DIERCKX. PART 3. FURTHER OBSERVATIONS ON THE STRUCTURAL FORMULA FOR MYCOPHENOLIC ACID

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Alsberg & Black (1913) first isolated and named mycophenolic acid, $C_{17}H_{20}O_6$, from culture filtrates of *Penicillium stoloniferum* Thom. The acid was next reported by Clutterbuck, Oxford, Raistrick & Smith (1932) as a metabolic product of twelve species or strains in the *P. brevi-compactum* series, a series to which *P. stoloniferum* belongs. Clutterbuck & Raistrick (1933), as the result of an extensive chemical examination of mycophenolic acid, advanced a tentative partial structural formula for it which was modified and amplified by Birkinshaw, Bracken, Morgan & Raistrick (1948). These workers suggested structures (I) or (II) as most clearly illustrating the known properties and chemical behaviour of mycophenolic acid, and preferred (I) to (II). Further work on this subject which supports (I) is reported in the present communication.

Clutterbuck & Raistrick (1933) obtained, by vigorous oxidation of mycophenolic acid monomethylether with alkaline potassium permanganate, a tetracarboxylic acid which was analysed as its anhydride. From this analysis the empirical formula $C_{12}H_6O_8$ was deduced for, and structure (III) was assigned to, the oxidation product.

In a recent communication on cyclopolic and cyclopaldic acids, which are metabolic products of strains of *P. cyclopium* Westling, Birkinshaw, Raistrick, Ross & Stickings (1952) showed that when methyl cyclopolate monomethyl ether is

vigorously oxidized with hot alkaline potassium permanganate there is formed a tetracarboxylic acid, analysed as its anhydride which was shown conclusively to be the anhydride of 4:6-dimethoxybenzene-1:2:3:5-tetracarboxylic acid of structure (III). Hence Clutterbuck & Raistrick's anhydride, m.p. 220° (decomp.), and the anhydride of Birkinshaw *et al.* (1952), m.p. $225.5-228^\circ$ (decomp.), should be one and the same substance, but proved to be different since the melting point of a mixture of Clutterbuck & Raistrick's original sample with the anhydride of Birkinshaw *et al.* was depressed to $202-206^\circ$ (decomp.).

We have now repeated the permanganate oxidation of mycophenolic acid monomethyl ether substantially as described by Clutterbuck & Raistrick (1933), and have obtained their oxidation product in good yield. After exhaustive crystallization we found it to melt at $223-225^\circ$ (decomp.) and not to depress the melting point, 220° (decomp.), of Clutterbuck & Raistrick's original sample, although it did depress the melting point of the anhydride of 4:6-dimethoxybenzene-1:2:3:5-tetracarboxylic acid (III), m.p. $225.5-228^\circ$ (decomp.), to $203-208^\circ$ (decomp.).

Since we regard as incontrovertible the evidence for the permanganate oxidation product of methyl cyclopolate monomethyl ether being assigned structure (III), it is clear that another constitution must be sought for the permanganate oxidation product of mycophenolic acid monomethyl ether.

A review of the analytical results obtained for the old sample (A) of the mycophenolic acid monomethyl ether oxidation product quoted from Clutterbuck & Raistrick's (1933) paper and of fresh determinations on the new sample (B) (see Table 1) reveals the following facts. (i) The analyses of

Table 1. Analyses of oxidation products of mycophenolic acid monomethyl ether

	C (%)	H (%)	OCH ₃ (%)	Titration equivalent
(A) Clutterbuck & Raistrick's original sample	48.1	2.7	20.9	80.9
	48.1	2.6	20.9	79.5
(B) Present sample	48.1	2.7	19.1	81
(C) Hydrogen peroxide oxidation product of (B)	48.5	2.7	20.7	75
Calc. for C ₁₃ H ₈ O ₉	48.7	2.7	20.95	74*
Calc. for C ₁₃ H ₈ O ₁₀	48.2	2.5	19.1	81*

* Titrating as a tetracarboxylic acid.

samples (A) and (B) are in good agreement except for the methoxyl estimations on sample (A) on which much reliance was originally placed. (ii) With the exception of the methoxyl estimations on (A) all the other estimations are in better agreement with an empirical formula C₁₃H₈O₁₀ than with C₁₃H₈O₉, as originally assigned. This is consistent with the presence of an additional CO group and can be readily explained only on the assumption that a CO.COOH group is present as a substituent of the benzene nucleus in place of one of the carboxyl groups originally postulated. Confirmation of this hypothesis was obtained by further degradation with hydrogen peroxide at 0° of the sodium salt of the permanganate oxidation product of mycophenolic acid monomethyl ether, which then afforded in good yield the anhydride of a dimethoxybenzene tetracarboxylic acid (C) structure (III), C₁₃H₈O₉, identical with the permanganate oxidation product obtained from methyl cyclopolate monomethyl ether.

Structure (IV), i.e. the anhydride of 3:4:5-tricarboxy-2:6-dimethoxyphenylglyoxylic acid, C₁₃H₈O₁₀, for the permanganate oxidation product of mycophenolic acid monomethyl ether has been

established as follows. The permanganate oxidation product was refluxed in a stream of nitrogen with hydriodic acid and red phosphorus when it was demethylated and 1.97 molecular equivalents of carbon dioxide were evolved after 4 hr. heating. The resulting decarboxylation product was isolated as a cream solid which was remethylated with dimethyl sulphate and anhydrous potassium carbonate in dry acetone giving, after alkaline hydrolysis of the methylation product, 4-carboxy-2:6-dimethoxyphenylacetic acid (structure V) as colourless needles, m.p. 286–288°, not depressing the melting point of an authentic synthetic specimen, m.p. 284–285°. This identification was confirmed by

converting the decarboxylation product (V) into its dimethyl ester which was obtained as colourless jagged needles, m.p. 107.5–109° not depressed on admixture with an authentic synthetic specimen of the dimethyl ester of 4-carboxy-2:6-dimethoxyphenylacetic acid, m.p. 106.5–108°.

This experimental evidence establishes two important points. (a) The arguments advanced by Birkinshaw *et al.* (1948) for the constitution of mycophenolic acid (I or II) were based in part on structure (III) for its permanganate oxidation product, from which it was concluded that mycophenolic acid is a hexasubstituted benzene derivative. This conclusion has now been doubly proved with the establishment of structure (IV) for the permanganate oxidation product and structure (III) for the further degradation product obtained from it with hydrogen peroxide. (b) The CO.COOH side chain in (IV) must arise by oxidation of one of the substituents in the benzene ring in mycophenolic acid. It must therefore arise from the HOOC.CH₂.CH₂.C(CH₃)=CH.CH₂ side chain, since this is the only substituent containing more than one carbon atom. Hence it follows that this side chain must lie between the hydroxyl and methoxyl groups in mycophenolic acid. Structure (II) for mycophenolic acid must therefore now be abandoned and structure (I), which was that preferred by Birkinshaw *et al.* (1948), becomes almost certain, except for: (i) a slight doubt as to which of the two nuclear hydroxyl groups in normycophenolic acid is methylated in mycophenolic acid itself; (ii) a slight doubt as to the relative positions in (I) of the CO and CH₂ groups in the phthalide ring

i.e. as to whether the potential CH₂OH group is in the *ortho* position to —CH₃ or *meta* to it.

In an attempt to resolve the doubt expressed in (ii) the aldehyde, C₁₃H₁₄O₆, obtained by ozonolysis of mycophenolic acid monomethyl ether (Birkinshaw *et al.* 1948), was oxidized with alkaline silver nitrate to the corresponding carboxylic acid, C₁₃H₁₄O₆. Since the —CH₂.CHO group in the aldehyde C₁₃H₁₄O₆ and hence also the —CH₂.COOH group in the acid C₁₃H₁₄O₆ arises from the long side

chain in mycophenolic acid monomethyl ether, it now follows that there are only two possible structures, i.e. (VI) or (VII), for the acid $C_{13}H_{14}O_6$, of which (VI) is preferred. It was hoped to confirm structure (VI) by decarboxylating the acid by

heating it with copper chromite and quinoline and thus obtaining 5:7-dimethoxy-4:6-dimethylphthalide, which has been isolated as a degradation product of cyclopolic acid and has been synthesized (Birkinshaw *et al.* 1952). Unfortunately, although carbon dioxide was evolved, no crystalline decarboxylation product could be isolated. However, indirect support for structure (VI) was obtained by heating the acid with hydriodic acid and red phosphorus. One molecule of carbon dioxide was evolved and a product, $C_{10}H_{10}O_3$, m.p. 184–185°, was isolated, which gives no colour with ferric chloride and is insoluble in aqueous sodium bicarbonate though readily soluble in aqueous sodium carbonate. We believe it to be one of the two possible lactones derived from 2:6-dihydroxy-3:4-dimethylphenylacetic acid (VIII).

The loss of a molecule of carbon dioxide under these experimental conditions is much more readily explained from structure (VI) than from (VII). It is known that the $-\text{CH}_2\text{COOH}$ side chain is stable to boiling hydriodic acid (cf. the stability of V). Hence, it is probable that the molecule of carbon dioxide which is evolved arises from the potential carboxyl group in the phthalide ring, the CH_2OH group being reduced to CH_3 . On the basis of (VI) this COOH group would be situated both ortho to one and para to the other of the two hydroxyl groups formed by demethylation of the parent acid. It is our experience that a carboxyl group so placed is readily removed on heating with hydriodic acid and red phosphorus, whereas a COOH group meta to both the hydroxyl groups, such as is necessitated by (VII), would not be readily eliminated under these experimental conditions.

It is hoped to synthesize (VIII) and thus decide between (VI) and (VII) as the structure of the acid $C_{13}H_{14}O_6$, and so finally settle the constitution of

normycophenolic acid. The only outstanding point with regard to the constitution of mycophenolic acid itself would then be the relative positions of its hydroxyl and methoxyl groups.

Support for structure (I) for mycophenolic acid is afforded by the work of Barer, Cole & Thompson (1949) who examined the infrared spectra, with the aid of a reflecting microscope, of a minute crystal of 'bialin' obtained from cultures of *P. bialowiezense* Zaleski (a synonym of *P. brevi-compactum* Dierckx) and compared it with a similar small crystal of mycophenolic acid obtained from *P. brevi-compactum* Dierckx. The two compounds were shown to be identical. In addition, however, their results suggested that the 5'-carboxy-3'-methylpent-2'-enyl grouping was *ortho* to the OH and OCH_3 groups in mycophenolic acid as required in structure (I).

The tree *Chlorophora excelsa* is the source of iroko, one of the principal commercial hardwoods of tropical Africa. Iroko, during recent years and because of the shortage of teak, has been extensively used for the construction of laboratory benches. King & Grundon (1949, 1950) have isolated from iroko, by ether extraction, a new polyphenolic body which they have named chlorophorin and which they have shown to have structure (IX). Comparison of this structure with that of mycophenolic acid (X), written with the phthalide ring opened, shows a noteworthy resemblance between the structures of these two naturally occurring compounds and illustrates the essential unity, from the point of view of comparative biochemistry, in some of the metabolic processes of microscopic fungi at one extreme of the plant kingdom and those of a forest tree at the other extreme.

(IX) Chlorophorin

(X) Mycophenolic acid (hydrated)

EXPERIMENTAL

Oxidation of mycophenolic acid monomethyl ether with hot alkaline KMnO_4 . Isolation of (IV)

The method used was substantially that described by Clutterbuck & Raistrick (1933). A solution of mycophenolic acid monomethyl ether (5.0 g.) in aqueous KOH (2%; 100 ml.) was refluxed, and finely ground KMnO_4 (30 g.) was

added continuously in 1–2 g. portions. All the KMnO_4 was utilized. The precipitated MnO_2 was separated by filtration and washed thoroughly with hot water. The pale-yellow filtrate and washings were adjusted to pH 1–2 with HCl , and CaCl_2 (5 g.) was added. The precipitated calcium oxalate (0.06 g.) was separated by filtration and discarded. The filtrate was evaporated *in vacuo* at 50–55° to 40 ml. Conc. HCl (2 ml.) was added and the solution was extracted continuously with ether for 30 hr. Removal of the solvent gave a yellow gum (3.95 g.) which could not be obtained crystalline. It was therefore re-oxidized. It was dissolved in 2N- KOH (50 ml.) and refluxed. KMnO_4 (7.5 g.) was added in 0.3 g. portions until a permanent pink colour remained after heating for 20 min. The excess KMnO_4 was destroyed with Na_2SO_3 , and oxalic acid was removed as calcium oxalate (1.40 g.) as described above. The acidified filtrate from the calcium oxalate was continuously extracted with ether for 10 hr. Removal of the solvent gave a pale-yellow gum which, on heating *in vacuo* at 70° for 1.5 hr., gradually solidified, wt. 2.05 g., m.p. 187–190° (decomp.). Repeated crystallization of this product from ethyl acetate-light petroleum (b.p. 60–80°) finally gave colourless microrods, m.p. 223–225° (decomp.), which did not depress the m.p. 220° (decomp.) of the original specimen of this oxidation product prepared by Clutterbuck & Raistrick (1933). On the other hand, on admixture with the anhydride of 4:6-dimethoxybenzene-1:2:3:5-tetracarboxylic acid (III), m.p. 225.5–228° (decomp.), obtained by KMnO_4 oxidation of the monomethyl ether of methyl cyclopentate (Birkinshaw *et al.* 1952), the mixture melted at 203–208° (decomp.). (Found, on sample dried to constant weight at 100° *in vacuo*: C, 48.1; H, 2.7; OCH_3 , 19.1; titration equivalent to phenolphthalein in water, 81. $\text{C}_{13}\text{H}_8\text{O}_{10}$ requires C, 48.2; H, 2.5; 2OCH_3 , 19.1%; equivalent, titrating as a tetrabasic acid (di-acid mono-anhydride), 81.)

Brady's reagent (2.5 ml.) was added to a solution of the acid (10 mg.) in water (1 drop). On standing overnight a little yellow amorphous precipitate (0.5 mg.), which could not be obtained crystalline, was collected. It gave a deep-red colour, typical of a mono-2:4-dinitrophenylhydrazones, when it was dissolved in ethanolic NaOH (Neuberg's reaction).

On addition of a few drops of conc. H_2SO_4 to a suspension of the acid in benzene containing a trace of thiophen the H_2SO_4 layer rapidly became intense brown-red in colour, changing on standing overnight to an intense greenish-blue colour which became a deep pure blue on dilution with water. Control experiments with H_2SO_4 + benzene + thiophen and also with H_2SO_4 + the acid alone, gave only a moderate orange-red colour. Under the same conditions phenylglyoxylic acid gives a deep-red colour changing to reddish violet on standing (Claisen, 1879), and the 2:4-dihydroxyphenylglyoxylic acids give characteristic colours (Hunsberger & Amstutz, 1948).

Degradation of the acid $\text{C}_{13}\text{H}_8\text{O}_{10}$ with H_2O_2 .

Isolation of (III)

A solution of the above acid $\text{C}_{13}\text{H}_8\text{O}_{10}$ (0.25 g.) in water (2 ml.) was neutralized with N- NaOH using phenolphthalein as an external indicator. The solution was cooled to 0° and H_2O_2 (90–100 'vol.'; 0.14 ml.) was added. After standing overnight at room temperature the colourless solution was acidified with 2N- HCl (2 ml.), saturated with NaCl and extracted with ethyl acetate (4 × 10 ml.). On removal of the solvent *in vacuo* at 50° from the dried (Na_2SO_4) extract, there

remained a colourless solid (0.237 g.), m.p. 225–226.5° (decomp.), which, on crystallization from ethyl acetate-light petroleum (b.p. 60–80°) gave colourless micropisms, m.p. 225.5–227° (decomp.) not depressed on admixture with the anhydride of 4:6-dimethoxybenzene-1:2:3:5-tetracarboxylic acid, m.p. 225.5–228° (decomp.), obtained by KMnO_4 oxidation of the monomethyl ether of methyl cyclopentate (Birkinshaw *et al.* 1952). On the other hand, on admixture with the starting acid $\text{C}_{13}\text{H}_8\text{O}_{10}$, m.p. 223–225° (decomp.) the mixture melted at 204.5–209° (decomp.). (Found, on sample dried to constant weight at 110° *in vacuo*: C, 48.5; H, 2.7; OCH_3 , 20.7; titration equivalent to phenolphthalein in water, 75. $\text{C}_{13}\text{H}_8\text{O}_8$ requires C, 48.7; H, 2.7; 2OCH_3 , 21.0%; equivalent, titrating as a tetrabasic acid (di-acid mono-anhydride), 74.)

Action of HI and red P on the acid $\text{C}_{13}\text{H}_8\text{O}_{10}$. Isolation and identification of 4-carboxy-2:6-dimethoxyphenylacetic acid (V)

A mixture of the acid $\text{C}_{13}\text{H}_8\text{O}_{10}$ (0.307 g.), HI (d, 1.7; 5 ml.) and red P (0.4 g.) was refluxed in a stream of CO_2 -free N_2 . The effluent gases were scrubbed first through a bubbler containing red P and water and then through bubblers containing measured amounts of aqueous 0.2N- $\text{Ba}(\text{OH})_2$ which were titrated with 0.1N- HCl at intervals. CO_2 was evolved as follows: after 0.5 hr., 1.49 mol. equiv.; 0.75 hr., 1.89 mol. equiv.; 2.5 hr., 1.95 mol. equiv.; 4 hr., 1.97 mol. equiv. The reaction was terminated after a further 6 hr. to ensure complete reduction when P was separated by filtration and washed with water. The filtrate and washings were extracted with ethyl acetate (4 × 10 ml.). The combined orange-brown extracts were washed with water, aqueous $\text{Na}_2\text{S}_2\text{O}_3$, and again with water. Removal of the solvent *in vacuo* from the dried (Na_2SO_4) extract gave a cream solid (0.113 g.) which was methylated by refluxing for 1.5 hr. with dimethyl sulphate (0.5 ml.), dry acetone (10 ml.) and anhydrous K_2CO_3 (0.25 g.). The solvent was removed and the residue was hydrolysed for 0.75 hr. at 100° with aqueous N- NaOH (10 ml.). Acidification with HCl gave a cream solid which was extracted with ethyl acetate (4 × 15 ml.). Removal of the solvent *in vacuo* gave small gummy needles (95 mg.) which, on washing with a little cold ether gave colourless needles (50 mg.), free from gum, m.p. 278–282°. This product was purified by sublimation in a high vacuum followed by two crystallizations from aqueous methanol giving 4-carboxy-2:6-dimethoxyphenylacetic acid as colourless needles, m.p. 286–288°, the dark melt rapidly resetting on cooling and remelting at 285–286.5°. On admixture with an authentic synthetic specimen, m.p. 284–285°, remelting at 282–283°, kindly supplied by Prof. F. E. King of Nottingham University, the mixture melted at 285–285.5° resetting and remelting at 283–285°. (Found: C, 55.3; H, 5.1; OCH_3 , 26.1. Calc. for $\text{C}_{11}\text{H}_{12}\text{O}_6$: C, 55.0; H, 5.0; 2OCH_3 , 25.8%.)

The above acid, $\text{C}_{11}\text{H}_{12}\text{O}_6$ (24 mg.), was refluxed for 1.5 hr. with dimethyl sulphate (0.05 ml.), acetone (5 ml.) and K_2CO_3 (50 mg.). The solvent was removed and the residue was shaken with water giving a colourless product (23 mg.), m.p. 105.5–107.5°. Crystallization from aqueous methanol gave the dimethyl ester of 4-carboxy-2:6-dimethoxyphenylacetic acid as small jagged needles, m.p. 107.5–109°, resetting and remelting at 107.5–108.5°. A synthetic specimen of this ester was prepared from the authentic synthetic acid by treatment with ethereal diazomethane and crystallization of the ester from aqueous methanol giving colourless jagged

needles, m.p. 106.5–108°. A mixture of the two specimens melted at 107–108°. King & Grundon (1950) give the melting points of the free acid as 282–284° and of the ester as 108–109°. (Found: C, 58.2; H, 6.0; OCH_3 , 46.3. Calc. for $\text{C}_{13}\text{H}_{14}\text{O}_6$: C, 58.2; H, 6.0; 4OCH_3 , 46.3 %.)

Oxidation of the aldehyde $\text{C}_{13}\text{H}_{14}\text{O}_5$ with Ag_2O .

Formation of the acid $\text{C}_{13}\text{H}_{14}\text{O}_6$ (VI)

The aldehyde $\text{C}_{13}\text{H}_{14}\text{O}_5$, obtained by the ozonolysis of mycophenolic acid monomethyl ether (Birkinshaw *et al.* 1948) was treated with AgNO_3 and alkali according to the method of Delépine & Bonnet (1909). A solution of AgNO_3 (605 mg.) in water (15 ml.) was added to a solution of the aldehyde $\text{C}_{13}\text{H}_{14}\text{O}_5$, m.p. 108–111.5° (294 mg.), in warm methanol (6 ml.). To the resultant slightly turbid solution aqueous 0.5N-NaOH (8.4 ml.) was added dropwise with vigorous shaking over 1 hr. Shaking was continued for a further 0.5 hr. The resulting black precipitate was separated by filtration through kieselguhr, and the filtrate was extracted with ether (2×20 ml.) to remove neutral material which was discarded. The extracted aqueous solution was acidified with HCl, saturated with NaCl and re-extracted with ether (3×25 ml.). On removal of the solvent from the dried (Na_2SO_4) ether extract the residual gum rapidly solidified to colourless needles (270 mg.), m.p. 145–148°. Crystallization from ether-light petroleum (b.p. 40–60°) gave colourless needles (183 mg.), m.p. 150–151°, resetting and remelting at the same temperature and not depressed on admixture with the acid $\text{C}_{13}\text{H}_{14}\text{O}_6$, m.p. 151–152°, obtained, in smaller yield, by oxidation of the aldehyde $\text{C}_{13}\text{H}_{14}\text{O}_5$ with alkaline iodine (Birkinshaw *et al.* 1948). The mixed melt reset and remelted at the same temperature.

Decarboxylation of the acid $\text{C}_{13}\text{H}_{14}\text{O}_6$

(a) *With copper chromite and quinoline.* Although CO_2 was evolved when the acid $\text{C}_{13}\text{H}_{14}\text{O}_6$ (100 mg.) was heated in N_2 with copper chromite (100 mg.) and carefully purified quinoline (1 ml.), the decarboxylation product was obtained as a dark-coloured gum which could not be obtained crystalline.

(b) *With HI and red P.* A mixture of the acid $\text{C}_{13}\text{H}_{14}\text{O}_6$ (100 mg.), HI (d, 1.7; 3 ml.) and red P (0.3 g.) was refluxed in a stream of CO_2 -free N_2 . CO_2 , evolved as follows, was estimated as described on p. 633: after 2 hr., 0.18 mol. equiv.; 7 hr., 0.50 mol. equiv.; 12 hr., 0.76 mol. equiv.; 19 hr., 1.00 mol. equiv. The reaction was terminated after 19 hr. Red P was removed by filtration and the filtrate and washings were extracted with ethyl acetate (4×15 ml.). The combined extracts were washed with water and aqueous $\text{Na}_2\text{S}_2\text{O}_3$. Removal of the solvent *in vacuo* from the dried (Na_2SO_4) extract gave a gummy solid (50 mg.) which was sublimed at 110–130° in a high vacuum giving a colourless solid (15 mg.), m.p. 140–160°. This was crystallized from benzene and the resulting colourless needles were resublimed giving the *decarboxylation product* (6 mg.), m.p. 184–185° unchanged on recrystallization from benzene. (Found: C, 67.1; H, 5.4. $\text{C}_{10}\text{H}_{10}\text{O}_3$ requires C, 67.4; H, 5.7 %.) The product is insoluble in cold saturated aqueous NaHCO_3 , but dissolves rapidly in aqueous 2N- Na_2CO_3 . Its aqueous solution gives a yellow colour with FeCl_3 .

SUMMARY

1. Experimental evidence is recorded which establishes the constitution of the permanganate oxidation product of mycophenolic acid monomethyl ether as the anhydride of 3:4:5-tricarboxy-2:6-dimethoxyphenylglyoxylic acid.

2. The sodium salt of this acid, on further oxidation with hydrogen peroxide, gives the anhydride of 4:6-dimethoxybenzene-1:2:3:5-tetracarboxylic acid which was believed by Clutterbuck & Raistrick (1933) to be the structure of the permanganate oxidation product of mycophenolic acid monomethyl ether.

3. This evidence confirms the preferred structure advanced for mycophenolic acid by Birkinshaw *et al.* (1948).

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