Structure of Biologically Produced Malathion Monoacid

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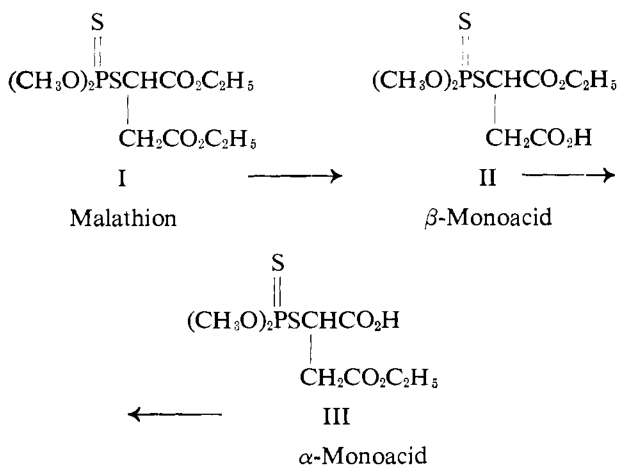
Two structures are feasible for malathion monoacid. Using NMR and infrared spectroscopy, the structure of the monoacid produced by carboxylesterase hydrolysis of malathion and the monoacid isolated in rat urine was identified as *O,O*-dimethyl-*S*-(1-carboxy-2-carbethoxy)ethyl phosphorodithioate (III). The NMR spectra demonstrated that only the α-monoacid was produced biologically.

Malathion, *O,O*-dimethyl-*S*-(1,2-dicarbethoxy)ethyl phosphorodithioate (I), a selective insecticide, is metabolized in mammals to a number of hydrolytic products, the major metabolite being a monoacid (Knaak and O'Brien, 1960; Krueger and O'Brien, 1959; O'Brien, 1960). March *et al.* (1956) suggested that this metabolite resulted from hydrolysis of one of the carbethoxy groups of the succinate portion of the malathion molecule. This suggestion was confirmed by Cook and Yip (1958), who found that the action of a rat liver acetone powder incubated with malathion produced a monoacid. Subsequent studies by Main and Braid (1962) showed that a partially purified rat liver carboxylesterase hydrolyzed only one of the carbethoxy groups of malathion and did not attack the remaining ester function.

The previous investigations left unanswered the question of which of the two carbethoxy groups of malathion underwent hydrolysis. Although Krueger and O'Brien (1959) suggested that the structure of the metabolite is II, no evidence was offered to support this assignment, and structure III is equally feasible.



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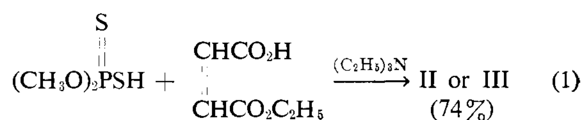
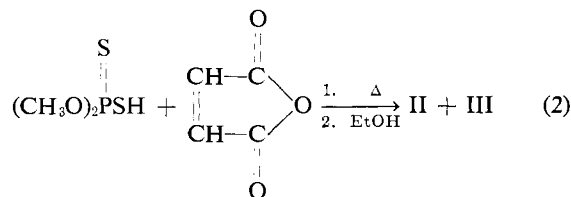
Since the hydrolysis of malathion by carboxylesterase accounts in part for the selective toxicity exhibited by the insecticide, an understanding of the enzymatic action should aid in the design of other insecticides which would utilize this selective detoxication mechanism. This study was undertaken to determine the structure of the carboxylesterase-produced monoacid of malathion and that of the monoacid excreted in the urine.

METHODS AND MATERIALS

Synthesis of Malathion Monoacids. In presence of 3 drops of triethylamine, 0.12 mole of *O,O*-dimethyl phosphorodithioic acid and 0.2 mole of ethyl hydrogen maleate were heated for 4 hours (Hoegberg, 1953). At the end of the reaction, the mixture was dissolved in 300 ml. of distilled water at pH 7, and extracted with an equal volume of chloroform. The aqueous layer was adjusted to pH 1 and extracted again with chloroform. The chloroform was removed after drying over magnesium sulfate. A single malathion monoacid was obtained with a yield of 74% (Equation 1).



The malathion monoacids (mixture of II and III) were prepared using the method of March *et al.* (1956) with some modifications. Equal molar amounts of *O,O*-dimethyl phosphorodithioic acid and maleic anhydride were heated at 85° C. for 1 hour. To this solution an equimolar amount of absolute ethanol was added and the reaction mixture heated for an additional hour (Equation 2). The isomers were separated as described below.



Partial Purification of Malathion Monoacids. To 15 grams of the monoacid mixture of II and III, 75 ml. of benzene were added. This solution was extracted with 20 fractions of 5% potassium bicarbonate solution (5 ml. each), totaling 1 molar equivalent based on the expected half ester. Each extraction was acidified with concentrated hydrochloric acid and extracted twice with chloroform. After evaporation of chloroform, the fractions were combined and dissolved in 10 ml. of benzene per gram of combined weight (124 ml. were added). This benzene solution was extracted four times with 12.4 ml. of water, five times with 12.4 ml. of 2.5% potassium bicarbonate solution, and four times with 12.4 ml. of 5% potassium bicarbonate solution. Each of the three fractions was acidified with concentrated hydrochloric acid and extracted twice with chloroform. The organic layer was dried over anhydrous sodium sulfate and evaporated. One isolated fraction of a monoacid of malathion was obtained in the 2.5% aqueous potassium bicarbonate and water extraction (yield, 4.2 grams). Attempts at crystallization of this monoacid fraction were unsuccessful. On acidification of the 5% aqueous potassium bicarbonate extraction (yield, 3.7 grams), a second monoacid fraction of malathion was obtained. It was crystallized at −70° C. from methylene chloride–hexane mixture and melted at 51–52° C.

Liver Carboxylesterase Preparation. The carboxylesterase was partially purified from a rat liver acetone powder by the method of Hassan and Dauterman (1968). The enzyme was obtained with a 77-fold purification based on the specific activity with 21% recovery. The specific activity of the final enzyme preparation was 97.3 units per mg. of protein. The unit is defined as the initial rate of hydrolysis of 1 μmole of *p*-nitrophenyl butyrate per minute at pH 6.3, 27° C.

Isolation of Enzymatically Produced Malathion Monoacid. The substrate solution for the carboxylesterase was prepared by dissolving 1.5×10^{-3} mole of malathion in 10 ml. of methanol and then diluting to 30 ml. with distilled water.

A 10-ml. enzyme solution containing 80 units and 30 ml. of substrate solution were incubated in the reaction cell of a Radiometer pH Stat at 25° C., pH 7.6. The pH was kept constant with 0.02*M* sodium hydroxide.

After 6 hours of incubation, the reaction mixture was extracted with chloroform at neutral pH to remove the unhydrolyzed malathion. The aqueous phase was adjusted to pH 1 with concentrated hydrochloric acid and extracted again with chloroform. The chloroform was removed under vacuum, and 120 mg. of a pale yellow oil were obtained.

Isolation of Monoacid from Rat Urine. Malathion at a dose of 1500 mg. per kg. was administered orally, diluted in corn oil, to four 200- to 300-gram male Duplin white rats. The animals were held for 24 hours in metabolism cages, which allowed separation of urine and feces (Comar,

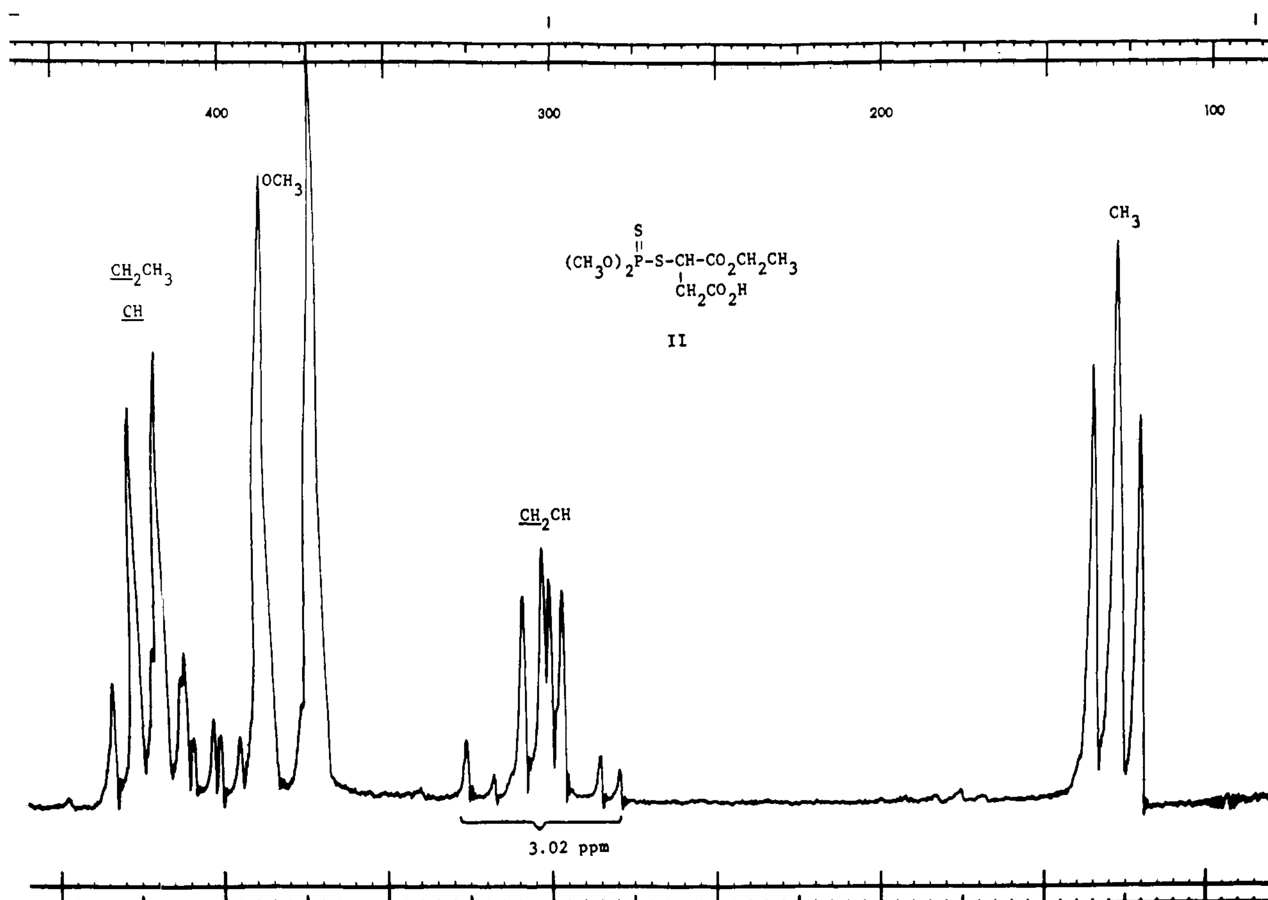


Figure 1. NMR spectrum of crystalline monoacid II
Chemical shifts in p.p.m. downfield from tetramethylsilane internal standard

1955). The urine was extracted with an equal volume of chloroform, after which the urine was acidified to pH 1 and re-extracted three times with $CHCl_3$. The chloroform was dried over $MgSO_4$ and removed under vacuum, and the monoacid isolated.

Chromatography. All of the monoacids were purified by paper or thin-layer chromatography before being analyzed by infrared or nuclear magnetic resonance spectroscopy. Paper chromatography utilized Whatman No. 1 paper and the solvent system acetonitrile-water-ammonia (85:14:1). Thin-layer chromatography was carried out using 0.5-mm. silica gel G plates with chloroform–petroleum ether (9 to 1) and benzene–methanol (7 to 3) to purify the monoacids further. These techniques allowed removal of impurities, but in no case could the isomers be separated from each other chromatographically.

Spectroscopic Analysis. The infrared spectra of the various monoacids were obtained as 10% solutions in chloroform with a Perkin-Elmer Model 237 B infrared spectrophotometer.

Nuclear magnetic resonance spectroscopy was utilized for verification of structure of the monoacids of malathion. The NMR spectra were determined using a Varian Associates HA-100 high resolution spectrometer. The samples were run in deuterochloroform at room temperature with tetramethylsilane as the internal reference.

RESULTS AND DISCUSSION

Both NMR and infrared spectroscopy established that the carboxylesterase-produced monoacid and the monoacid isolated from rat urine were identical. The single isomer obtained from the ethyl hydrogen maleate reaction (Equation 1) was identical to the crystalline material isolated from the maleic anhydride reaction (Equation 2). The carboxylesterase-produced monoacid (and that found in rat urine) was identical to the noncrystalline isomer isolated from the maleic anhydride adduct based on the NMR and infrared spectra. With the biologically produced monoacid and noncrystalline isomer an extra peak at 1340 cm.$^{-1}$ was present, which was absent from the crystalline monoacid and the monoacid from the ethyl hydrogen maleate reaction.

Attempts to separate the mixture of monoacids from maleic anhydride adduct using both paper and thin-layer chromatography and a variety of solvent systems and absorbents were unsuccessful. However, other impurities could be removed chromatographically and the isomers were partially separated by the extraction procedure. One isomer was obtained free of the second by crystallization from methylene chloride–*n*-hexane at −70° C. The other isomer remained as an oil and was approximately 80% pure, based on the NMR spectra.

Assignment of structures II and III to the synthetic malathion monoacids was based primarily on NMR spec-

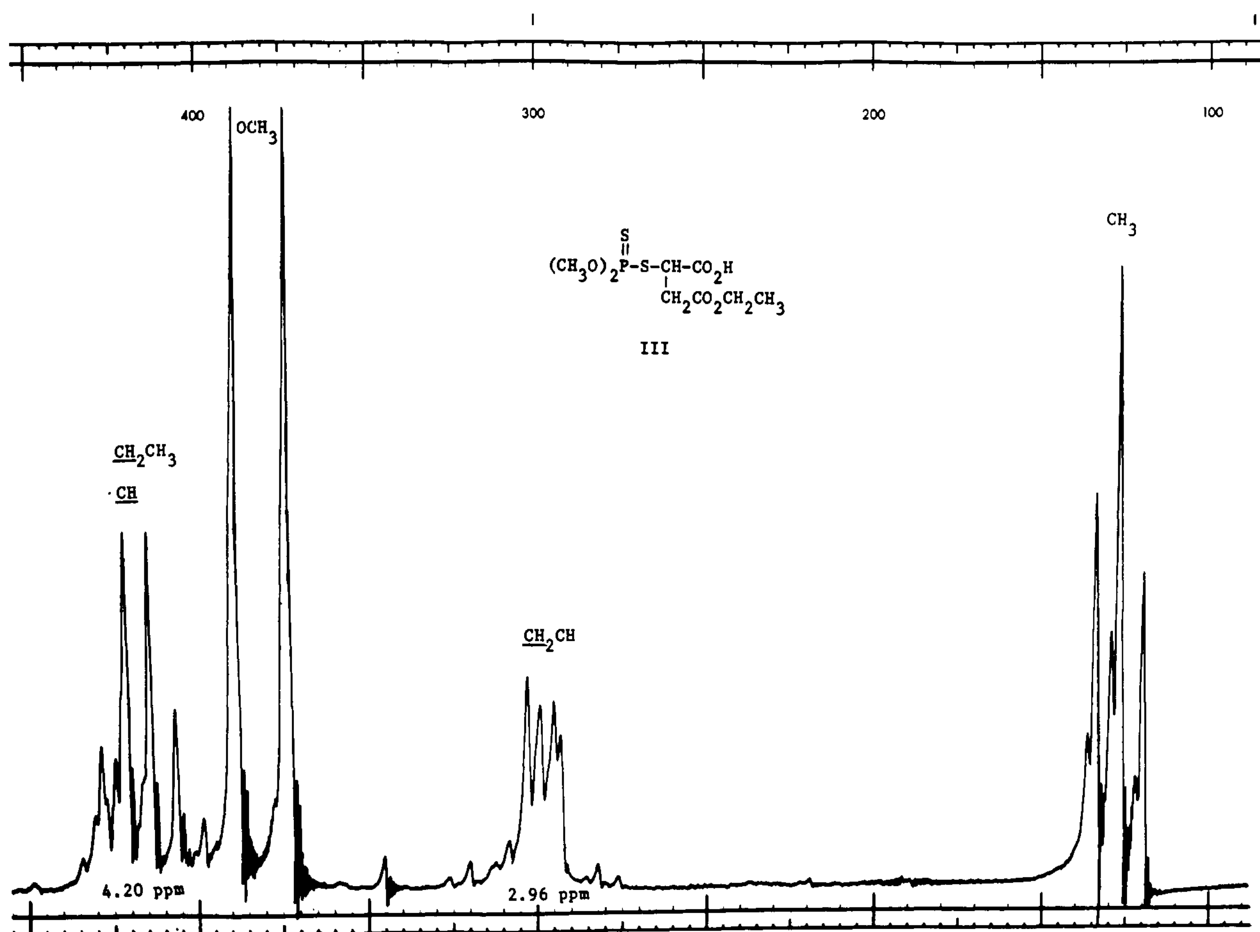


Figure 2. NMR spectrum of synthetic monoacid III
Chemical shifts in p.p.m. downfield from tetramethylsilane internal standard

tral analysis. Only minor absorption differences are detected in the spectra shown in Figures 1 and 2. In both spectra the pattern at ca. 3.0 p.p.m. is the *AB* portion of an *ABX* system composed of protons of the succinate chain. This system includes the nonequivalent methylene protons and the methine proton. The pattern for the methine absorption (the *X* proton of the *ABX* system) is complicated by the fact that it is additionally coupled to phosphorus and is at least partially obscured by the methylene absorption of the ethyl ester (a quartet at ca. 4.0 p.p.m.). The chemical shifts for the *AB* patterns are easily measured (2.96 p.p.m. in Figure 1 and 3.02 p.p.m. in Figure 2). However, frequency-sweep experiments using double and triple resonance techniques were employed to simplify the spectra and to locate the center of the methine absorption precisely.

It was hoped that the difference in chemical shifts between methylene and methine protons could be used to distinguish between isomers II and III. Such a distinction makes use of the fact that the absorption of a methine or methylene group adjacent to a carboxylic acid function is slightly more deshielded (shifted downfield) than the same group adjacent to an ester (Bible, 1965; Jackman, 1959). This being the case, in isomer II one would expect to find the methylene absorption at a lower field position than in isomer III. Likewise, the methine absorption in II should

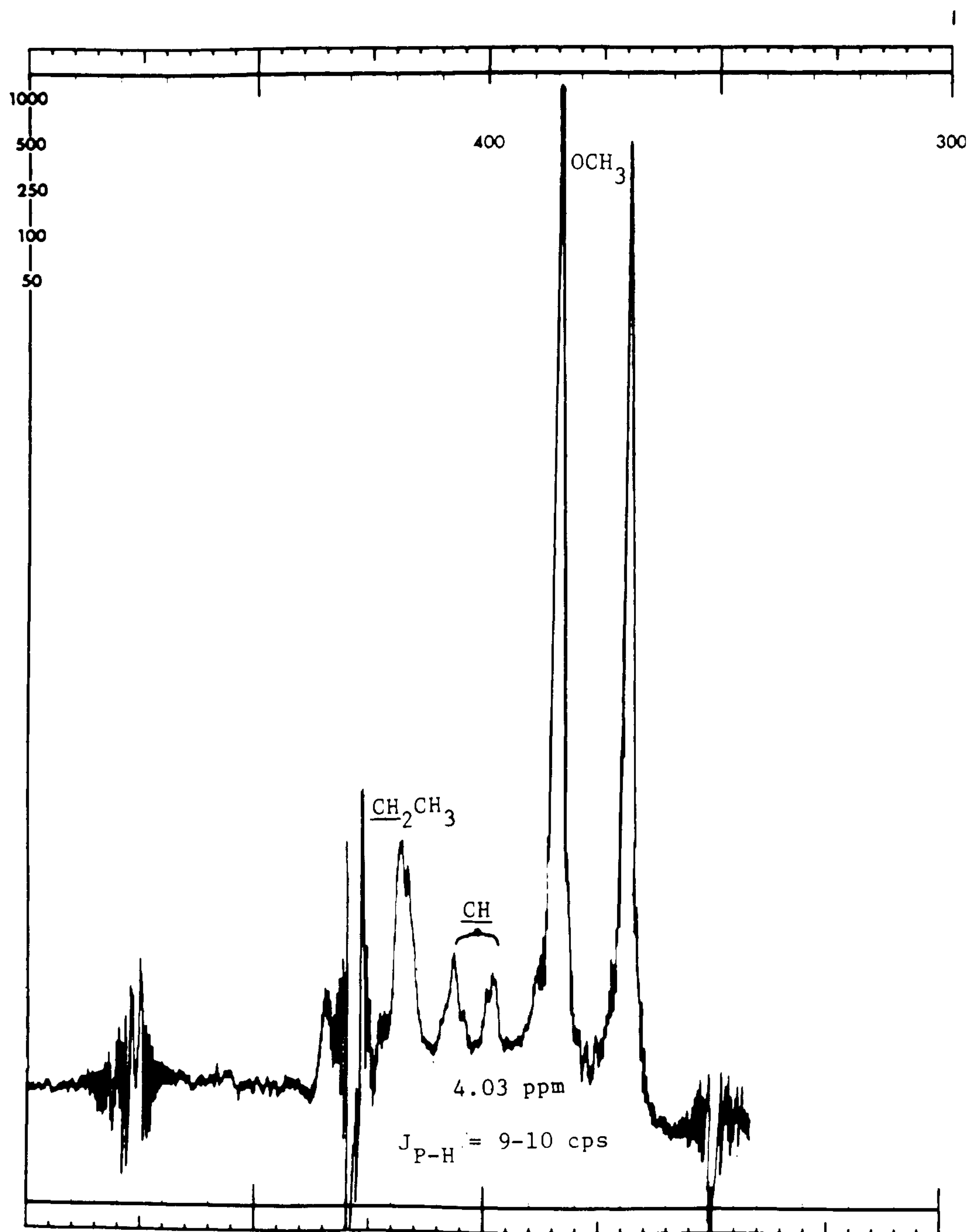


Figure 3. Decoupled portion of NMR spectrum of monoacid II
Result of irradiation of sample at both ethyl CH_3 and CH_2 of succinate chain

be at slightly higher field than in III. This would give a greater difference in chemical shift between the protons of the succinate chain in III than in II.

The result of a triple resonance experiment used to locate the methine absorption is shown in Figure 3. A sample of the crystalline monoacid was doubly irradiated to remove the coupling of *AB* from *ABX* and to collapse the quartet of the ethyl methylene group to a singlet. The methine absorption now appears as a doublet (coupled with phosphorus, J_{p-H} = 9 to 10 c.p.s.) and its chemical shift is easily measured. In the spectrum of the isomer shown, the *AB* absorption is at 3.02 p.p.m. and *X* is at 4.03, a chemical shift difference of 1.01 p.p.m.

The results obtained with the other noncrystalline isomer are not so clearly defined. The methine absorption is apparently very nearly centered under the ethyl quartet at 4.18 p.p.m. and its exact chemical shift was not measured. However, a conservative estimate centers this absorption at 4.20 p.p.m. and the chemical shift difference between the methylene (2.96 p.p.m.) and methine groups is at least 1.24 p.p.m. Since this is a larger difference than that exhibited by the first isomer, the structure of this monoacid is assigned as III. This is the carboxylesterase-produced material. The crystalline monoacid is the other isomer and is assigned structure II.

These assignments are consistent with the acid dissociation constants determined for the two isomers. The crystalline monoacid (II) has a pK of 3.74, while monoacid III has a pK of 3.62. This is comparable to the difference in the pK values between acetic and propionic acid.

The infrared and NMR spectra show that only one isomer of the monoacid is produced in vitro and in vivo, and that the site of hydrolysis is strictly at the α-carbethoxy group of malathion to form structure III or malathion α-monoacid.

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