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QUANTITATIVE AND QUALITATIVE VARIATION IN THE mRNA FOR CARBOXYLESTERASES IN INSECTICIDE-SUSCEPTIBLE AND RESISTANT *MYZUS PERSICAE* (SULZ)

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Abstract—RNA was extracted from two insecticide-resistant clones and one susceptible clone of *M. persicae*. The resistant clones each produced large amounts of one of two closely related carboxylesterases, the enzymes responsible for cross-resistance to a wide range of insecticides. After purification by affinity chromatography on oligo(dT) cellulose, the mRNA was translated into protein in a rabbit reticulocyte lysate system with [L-^{35}S]methionine. The resultant radiolabelled esterases were immunoprecipitated from the products with IgG prepared from an antiserum to one form of the enzyme, but cross-reacting with both. The bound enzyme was extracted by affinity chromatography on protein *A* sepharose, and characterized alongside the total radiolabelled proteins by SDS electrophoresis and fluorography. The translation products of the two resistant clones each contained large amounts of an immunoprecipitable protein. However, no such protein was detected in the translation products of the mRNA from susceptible aphids showing that resistant aphids produce much more of the mRNA encoding the enzymes responsible for resistance. It was also shown that the enzymes from the two resistant clones had primary structures differing from each other by 1 kDa. In addition, the nascent forms of both enzymes differed from their native forms by 8 kDa and glycosylation was shown to be responsible for this post-translational modification. The likely genetic basis of the changes in mRNA is discussed and related to the karyotype of the resistant clones.

Key Word Index: *Myzus persicae*, mRNA, carboxylesterase, insecticide resistance, immunoassay, glycoprotein, protein synthesis, affinity chromatography

INTRODUCTION

Resistance to organophosphorus and carbamate insecticides by the peach–potato aphid *Myzus persicae* has been reported in many parts of the world (Georghiou, 1981). In the U.K. it was first detected in glasshouses and later in field populations (Needham and Devonshire, 1975); resistant individuals now predominate in both environments but with much higher resistance levels in glasshouses. A correlation between resistance and the activity of carboxylesterases in aphid homogenates was demonstrated by Needham and Sawicki (1971) and subsequently this increased activity was shown to arise predominantly from changes in only one (E4) of the several carboxylesterases in *M. persicae* (Beranek, 1974; Devonshire, 1975). Clonal populations of resistant aphids each have a characteristic E4 activity which is non-inducible and generally stable, although in some of the more-resistant variants there may be a spontaneous decrease in the production of enzyme, with a concomitant loss of resistance (Sawicki *et al.*, 1980). Such variants invariably have an A1, 3 chromosome translocation (Blackman *et al.*, 1978) thought to be involved in the resistance mechanism.

Detailed biochemical studies of the esterase showed that the catalytic centre activity is the same whether purified from susceptible or resistant aphids, demonstrating that the difference in activity between strains is due to different amounts of the same enzyme, rather than to the production of a more efficient enzyme in resistant aphids (Devonshire, 1977). This large amount of enzyme causes resistance by both hydrolysing and sequestering insecticidal esters (Devonshire and Moores, 1982). In some clones a mutant form of the enzyme replaces E4; it causes resistance by the same mechanisms as E4 (Devonshire *et al.*, 1983), but is characterized by its slightly higher (1.5-fold) catalytic centre activity for some insecticides and a molecular weight of 66 kDa compared with 65 kDa for E4. It was first recognized in a non-translocated clone from Ferrara, Italy and is referred to as FE4. The molar amounts of esterase in seven aphid clones, progressively more resistant to insecticides, were shown to form a geometric series in which the enzyme content doubled between each successive variant from 0.4 pmol in the S to 25 pmol in the most resistant. It was suggested that the likely regulatory mechanism was gene amplification arising from a succession of duplications of the structural esterase gene (Devonshire and Sawicki, 1979).

We are therefore studying the changes in nucleic acids associated with increased esterase production to establish whether gene amplification, or some other form of gene regulation, is responsible for resistance. We report here both quantitative and qualitative differences in the mRNA encoding E4 or FE4 between susceptible and resistant aphids.

MATERIALS AND METHODS

Aphids

Susceptible (S) aphids, clone US1L, and two resistant clones, 794 (R) (translocated; from a glasshouse population equivalent to G6) and Ferrara (F) (normal karyotype) were used. The origins and rearing conditions are described elsewhere (Sawicki *et al.*, 1980; Devonshire *et al.*, 1983).

Radiochemicals

[L-^{35}S]Methionine (Code SJ. 204; sp. act. >800 Ci mmol^{-1}), [^{14}C]formaldehyde (Code CFA.343; sp. act. 10–20 mCi mmol^{-1}) and [^{3}H]DFP (Code TRK.207; sp. act. >2 Ci mmol^{-1}) were purchased from Amersham International plc.

Electrophoresis

Proteins were denatured and characterized by SDS electrophoresis as described by Devonshire and Moores (1983) except that the running gel contained 0.03% dithiothreitol (Sigma). Proteins were located either by staining with Coomassie brilliant blue R or, in the case of glycoproteins, with thymol-H_2SO_4 (Gander, 1984). Radiolabelled proteins were visualized fluorographically at -75°C, using either Amplify® (Amersham) or sodium salicylate (Skinner and Griswold, 1983), and preflashed Kodak *X*-omat S X-ray film.

Radiolabelling of protein standards

Proteins of standard molecular weight (Sigma MW-SDS-70) were radiolabelled by reductive alkylation with [^{14}C]formaldehyde and sodium cyanoborohydride (Dottavio-Martin and Ravel, 1978). Purified E4 and FE4 were radiolabelled by irreversible phosphorylation of their catalytic centres with [^{3}H]DFP (Devonshire and Moores, 1982).

Sugar-affinity chromatography

Purified E4 and FE4, either as uninhibited enzymes or after labelling with [^{3}H]DFP, were chromatographed on Con *A*-sepharose in 20 mM Tris, pH 7.5, containing 0.5 M NaCl, as described in "Affinity chromatography: Principles and Methods" (Pharmacia), using columns of 50 μl packed volume in a disposable pipette tip.

Antiserum

E4 was purified as described previously (Devonshire and Moores, 1982), and 2 mg protein emulsified in Freund's complete adjuvant and injected intramuscularly into a rabbit; this was followed, 4 months later, by a second injection of 2 mg E4 in Freund's incomplete adjuvant. Blood samples were collected at 2 week intervals, the serum separated by centrifugation and immunoglobulin *G* (IgG) purified from the serum by affinity chromatography on Protein *A*–Sepharose CL-4B (according to "Affinity chromatography: Principles and Methods", Pharmacia). The IgG, at a protein concentration of *ca* 1.25 mg ml^{-1}, was stored in phosphate buffered saline (150 mM NaCl, 20 mM phosphate buffer pH 7.0) at -20°C. This IgG solution had a titre of 1:64 and gave a single precipitation line in the Ouchterlony double diffusion test (Weir, 1967) against either E4 or FE4 (20 μg ml^{-1}) when using 6 mm wells at 8 mm centres in 1% agar. We have shown by other techniques that the IgG reacts only with E4 and FE4 in aphid homogenates (Devonshire and Moores, 1984; Devonshire *et al.*, 1986).

Immunoassay of E4 and FE4 in aphid homogenates

Aphids (15) from each of clones S, R or F were homogenized in Triton/Tris buffer (750 μl; 50 mM Tris pH 7.7, 50 mM NaCl, 2% Triton) and IgG solution (5 μl, as above) added. After 16 hr at 4°C the homogenates were passed through protein *A*–Sepharose columns (20 μl packed volume prewashed with 0.5% bovine serum albumin to reduce non-specific binding) to trap the IgG/esterase complex and the columns then washed with Triton/Tris buffer (*ca* 2 ml) followed by 10 mM Tris pH 7.5 (*ca* 2 ml). The protein *A*–Sepharose was then removed from the columns and the bound IgG/esterase dissociated by heating to 60°C for 20 min with 20 μl SDS denaturing mix (Devonshire and Moores, 1983). The released immunoprecipitated proteins were then characterized by SDS electrophoresis.

Immunoassay of E4 synthesized by isolated aphid embryos

Strings of embryos were dissected from apterous virginoparae in a solution of amino acids (5 mM each of the common protein–amino acids in 120 mM Hepes buffer pH 7.6, but lacking methionine) and then 10 μCi [L-^{35}S]methionine was added to the intact embryos from 20 aphids in 20 μl solution. After 3 hr at room temperature, the incubations were centrifuged at 3000 ***g*** and the supernatant removed. The embryos were washed three times in non-radioactive amino-acid solution (at 4°C) and then crushed in 200 μl Triton/Tris buffer, to which 2 μl IgG solution was added. After 1 hr at room temperature the IgG-bound radiolabelled E4, synthesized by the embryos, was separated by protein A–Sepharose, denatured and located by fluorography after SDS electrophoresis.

Extraction of poly(A)$^+$ RNA from aphids

Sterile solutions (autoclaved 15 min at 15 p.s.i.) and sterile glassware (heated to 200°C overnight) were used for the following techniques.

Method 1 (phenol). Aphids (1–5 g) were frozen in liquid nitrogen and homogenized (10% w/v) in extraction buffer (200 mM Tris pH 8.8 containing 100 mM NaCl, 10 mM EDTA, 2 mM adenosine 2′ and 3′-mono-phosphate, 0.5% SDS and 0.05% heparin) in a loose fitting Dounce homogenizer. The homogenate was centrifuged at 3000 ***g*** at room temperature, the supernatant collected and thoroughly mixed with 3 vol of redistilled phenol (equilibrated with the extraction buffer) and then extracted with chloroform–isoamyl alcohol (24:1, v/v) according to the method of Brock and Roberts (1983). Poly(A)$^+$ RNA (mRNA) was isolated by affinity chromatography on oligo(dT) cellulose (Bantle *et al.*, 1976), precipitated by addition of 0.05 vol 4 M ammonium acetate and 2.5 vol of ethanol, and redissolved in water (20–100 μl). The yield was routinely *ca.* 100 μg poly(A)$^+$ RNA g^{-1} aphid, based on $A^{1\%}_{260} = 250$.

Method 2 (guanidine hydrochloride). Aphids (1–5 g) were homogenized as above (10% w/v) in 20 mM sodium acetate buffer pH 5.0, containing 7 M guanidine HCl, 1 mM dithiothreitol and 10 mM EDTA, and the RNA extracted as described by Kahn *et al.* (1981). Poly(A)$^+$ RNA was again isolated using oligo(dT) cellulose. This method yielded *ca* 20 μg poly(A)$^+$ RNA g^{-1} aphid.

In vitro *translation and assessment of products*

Poly(A)$^+$ RNA, extracted from S, R or F aphids, was translated into protein in a rabbit reticulocyte lysate system (Amersham Code N.90), according to the manufacturer's directions, with the addition of 10 U human placental ribonuclease inhibitor (Amersham Code N.120). Each translation (total volume 15 μl) contained 20 μCi [L-^{35}S]methionine and 0.5–1 μg RNA; the total proteins synthesized (1 μl from 15 μl), and E4 or FE4 immunoprecipitated, as described above, from the remainder (14 μl) of each translation, were characterized by SDS electrophoresis and fluorography.

RESULTS AND DISCUSSION

Aphid homogenates

E4 purified from R aphids had an apparent molecular weight on SDS gels of 65 kDa compared

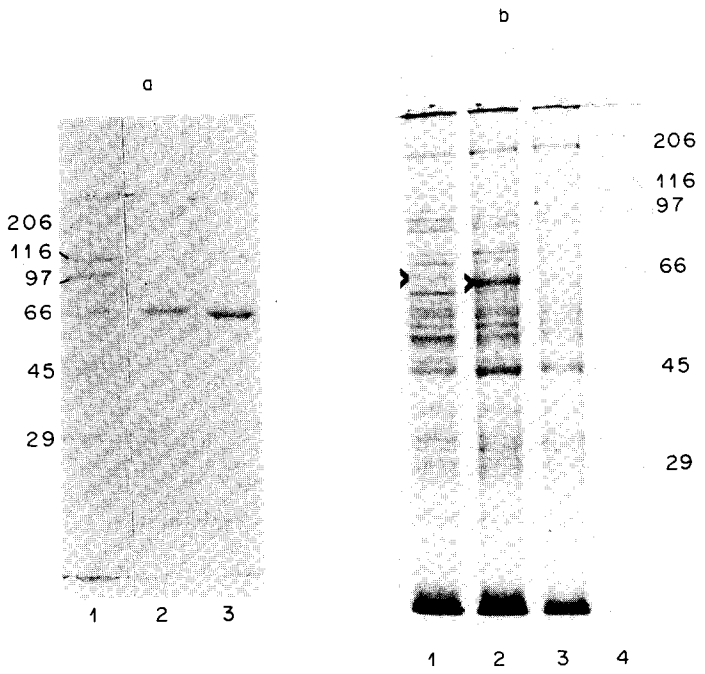

Fig. 1. Polyacrylamide gels stained with Coomassie blue. (a) Lane 1. Molecular weight standard proteins, myosin (206 kDa), β-galactosidase (116 kDa), phosphorylase *B* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa). Lane 2. FE4. Lane 3. E4. (b) Lane 1. Homogenate of F aphids. Lane 2. Homogenate of R aphids. Lane 3. Homogenate of S aphids. Lane 4. Molecular weight standard proteins. Arrows indicate positions of FE4 and E4 in F and R aphid homogenates. Lanes 1, 2 and 3 each contain homogenate from 8 aphids (approx. 2.5–3.0 mg tissue).

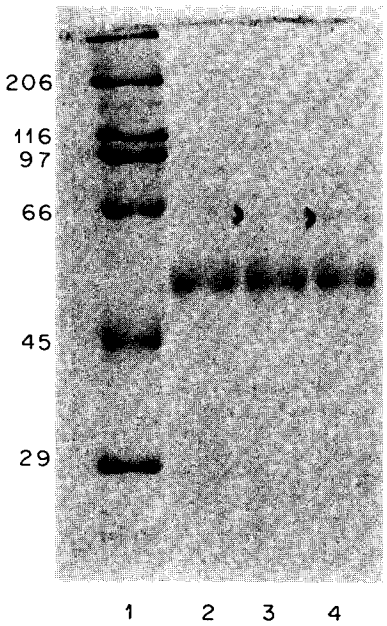

Fig. 2. Polyacrylamide gel stained with Coomassie blue. Molecular weight standard proteins as Fig. 1 (lane 1). Immunoprecipitated proteins from homogenates of S (lane 2), F (lane 3) and R (lane 4) aphids (10 of each, approx. 3 mg). Arrows indicate positions of FE4 and E4 in lanes 3 and 4, respectively. The dark band of *ca* 52 kDa was present in control experiments without aphid homogenate.

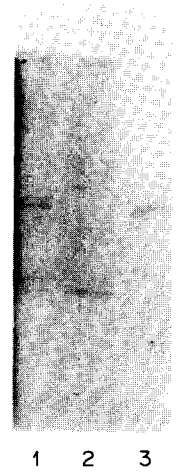

Fig. 3. Fluorograph of polyacrylamide gel with immunoprecipitated proteins from R aphid embryos (lane 1) and S aphid embryos (lane 2) after incubation with [^{35}S]methionine. Lane 3 is [^{3}H]E4.

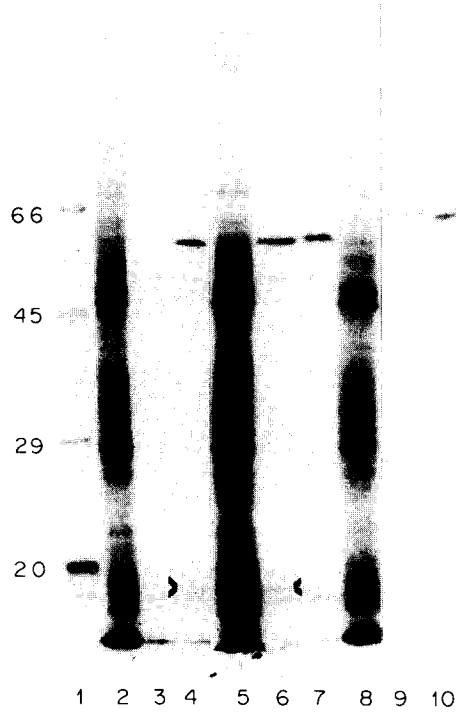

Fig. 4. Fluorograph of polyacrylamide gels. Lane 1. Molecular weight standard proteins, bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and soyabean trypsin inhibitor (20 kDa). Lane 2. Translation products of mRNA from S aphids. Lane 3. Immunoprecipitated proteins from translation products of mRNA from S aphids. Lanes 4 and 6. Immunoprecipitated proteins from translation products of mRNA from R aphids. Lane 5. Translation products of mRNA from R aphids. Lane 7. Immunoprecipitated proteins from translation products of mRNA from F aphids. Lane 8. Translation products of mRNA from F aphids. Lane 9. [^{3}H]FE4. Lane 10. [^{3}H]E4. In addition to the differences in E4 and FE4, arrows indicate the 19 kDa protein immunoprecipitated only from R translation products. Lanes 2, 5 and 8 each contain 1 μl from a 15 μl translation and lanes 3, 4, 6 and 7 contain the immunoprecipitated proteins from the 14 μl remaining.

with 66 kDa for FE4 from F aphids (Fig. 1a). We have shown previously that to avoid breakdown of proteins in SDS homogenates by endogenous proteases, aphids must be boiled before homogenization (Devonshire and Moores, 1983). When samples prepared thus were analysed by SDS electrophoresis, there were both qualitative and quantitative differences between S, R and F clones (Fig. 1b). Protein bands corresponding to E4 and FE4 were clearly present in R and F clones respectively, but not apparent in the S clone.

Preliminary experiments with purified E4 established that 5 μl of IgG solution and a 20 μl protein *A*–Sepharose column had the capacity to immunoprecipitate and bind at least 1 μg E4 or FE4. When homogenates of 15 aphids of the S, R and F clones were examined by this technique, a clear band corresponding to E4 (65 kDa) was detected from the R homogenate and to FE4 (66 kDa) from the F homogenate, whereas neither was detected in the S homogenate (Fig. 2).

Synthesis of E4 in aphid embryo incubations

Incubations of dissected tissues with radiolabelled amino acids have been widely used to study the rate of protein synthesis; for example Sato and Roberts (1983) used this technique to study the synthesis of larval serum proteins by tissues from *Drosophila melanogaster*. Because developing aphid embryos should be actively synthesizing protein, we chose to apply the same technique to this material. During 3 hr incubations at room temperature, embryos from 20 R aphids synthesized enough ^{35}S-labelled E4 to be detected on SDS gels as a clear band after immunoprecipitation and fluorography, whereas corresponding incubations with S embryos did not produce detectable amounts of E4 (Fig. 3). The increased E4 content of R aphids can thus be explained on the basis of a more rapid synthesis of this protein. Some bands due to non-specific binding to the column are also apparent from both homogenates (Fig. 3).

Extraction of poly(A)$^+$ RNA and in vitro *translation*

Poly(A)$^+$ RNA could be extracted from aphids by either of the methods described and successfully translated in the rabbit reticulocyte lysate system. Both methods were investigated because it has been shown that extraction by guanidine–HCl can be an absolute requirement for the isolation of high-molecular weight mRNA (Northemann *et al.*, 1983). In the case of aphids it proved possible to immunoprecipitate E4 or FE4 from translations of poly(A)$^+$ RNA prepared by either method, and the phenol technique then was used routinely because it was quicker and gave a higher yield.

Only slight differences were apparent in the total [^{35}S]proteins produced in translations of S, R or F poly(A)$^+$ RNA (Fig. 4). However, immunoprecipitations of larger amounts (14-fold) of the translations gave a strong band in the R and F but not in the S (Fig. 4) indicating that resistant aphids produce much more mRNA coding for proteins recognised by the E4-specific antiserum. The immunoprecipitated proteins from both R and F each ran faster on SDS gels than the corresponding native E4 and FE4 proteins purified from aphids (Fig. 4). These proteins synthesized *in vitro* from R and F mRNA have apparent molecular weights of 57 and 58 kDa respectively compared with 65 and 66 kDa for E4 and FE4, an 8 kDa difference in each case. This suggests that the *in vitro* proteins are nascent forms of E4 and FE4 and that *in vivo* these are post-translationally modified to give the native forms of the enzymes. Furthermore since the molecular weight difference between E4 and FE4 (1 kDa) is the same in both the nascent and native forms, the proteins must differ in their primary structure and not as the result of different post-translational modification. The immuno-precipitations of translations also showed some minor bands apparently arising from non-specific binding to the protein *A*–Sepharose (despite pre-treatment with bovine serum albumin) since they were common to all 3 clones (Fig. 4). However, a 19 kDa protein, the significance of which is yet to be established was detected only in R translations. It cannot account for the difference between nascent and native forms since it is too large and is absent from translations of F mRNA.

Glycoprotein nature of E4 and FE4

Since E4 is known to be associated with membranes (Devonshire, 1975), and glycosylation is a common form of post-translational modification of such proteins, we examined by affinity chromatography and sugar-specific staining of electrophoresis gels whether the molecular weight difference between native and nascent esterases was attributable to glycosylation.

Both E4 and FE4 bound strongly to Con *A*–Sepharose columns, and a maximum of 25–30% of the bound enzymes could be displaced by eluting the columns with 1 M methyl mannoside or 0.1 M borate, despite using very small columns to restrict the lectin:esterase ratio. Similar results were obtained whether binding was assessed by measuring enzyme activity eluted, or by using ^{3}H-labelled enzyme and measuring both bound and eluted radioactivity. The irreversible binding could not be prevented by loading E4 on to the column in 1 M methyl mannoside, indicating a very high affinity of the enzyme for lectin.

To confirm the glycoprotein nature of E4 suggested by these affinity chromatography studies, homogenates equivalent to 5 aphids of clones S and R [containing approx. 6 and 400 ng E4/aphid respectively (Devonshire and Sawicki, 1979)], and purified E4 (2 μg) were run on non-denaturing electrophoresis gels (Devonshire and Moores, 1982) and stained for sugars with thymol/H_2SO_4. Purified E4 and the R homogenate gave a red band at the expected position for E4, whereas no such band was detected in the S homogenate. Apart from one other clearly-staining band close to the origin and common to both clones, the E4 band in the R homogenate was the only glycoprotein detected.

Glycosylation is therefore involved in the 8 kDa difference between the native esterases, E4 and FE4, and their respective nascent forms produced by *in vitro* translation of mRNA. Although SDS electrophoresis gives anomalous estimates of the molecular weight of glycoproteins, comparable changes in apparent molecular weight were found when a number of glycoproteins were stripped of their

oligosaccharides by digestion with endo-β-*N*-acetylglucosaminidase *H* (Trimble and Maley, 1977). Different numbers of oligosaccharides bound to a protein are recognizable as electrophoretic mobility variants (Trimble and Maley, 1984); such variation in the degree of glycosylation might explain the presence in *M. persicae* of minor esterase bands of similar mobility to E4 in the tissue of the alimentary canal at specific developmental stages, as reported by Takada (1979).

Genetic basis of insecticide resistance

This work has established that insecticide-resistant *M. persicae* produce increased amounts of esterase because they have much more of the corresponding mRNA. This supports the earlier suggestion that gene amplification is the mechanism responsible (Devonshire and Sawicki, 1979), although increased transcription of this particular gene or a more stable mRNA cannot be ruled out. However, the regular increase in "constitutive" E4 content throughout a series of progressively more-resistant clones, is more readily accounted for by changes in the copy number of the structural gene than by the other mechanisms.

The apparent 1 kDa difference between the primary structures of FE4 and E4 proteins might be attributable to a qualitative change in sequence, or to a corresponding loss of amino acids. It might be significant that this difference is associated with the A1,3 translocation in the R clone, thought to be involved with the production of very large amounts of esterase. The molecular basis of this translocation is therefore particularly relevant to insecticide resistance in *M. persicae*, especially since the spontaneous loss of resistance appears to be restricted to translocated clones (Sawicki *et al.*, 1980).

Previous work on similar examples of gene regulation in response to cytotoxic chemicals relied on the study of cells, both prokaryotic and eukaryotic, grown in culture (Schimke, 1982). The present work forms the foundation for further studies to understand the molecular basis of the qualitative and quantitative changes in the genes responsible for the development and spontaneous loss of insecticide resistance in *M. persicae*, an economically important example of the adaptation of an intact higher organism to environmental stress.

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