Production, Purification and Chemical Properties of an Antistaphylococcal Agent Produced by *Staphylococcus epidermidis*

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An antibacterial agent produced by *Staphylococcus epidermidis* strain 5 was isolated from culture supernatants; its spectrum of activity is restricted to staphylococci and micrococci. The bacteriocin-like substance was purified to homogeneity by column chromatography on Servachrome XAD-2, CM-Sephadex C-25 and Sephadex G-50. Chemical analysis showed it to be a peptide with a molecular weight of about 6000 and an isoelectric point of about 10.5. Purified material was rapidly inactivated by trypsin, chymotrypsin and pronase and it was relatively heat-stable. After acidic hydrolysis 10 amino acid residues and two unidentified ninhydrin-positive substances were detected. Production of the bacteriocin-like substance was not inducible with mitomycin C.

INTRODUCTION

Staphylococci produce several types of antibacterial substances which differ with respect to their chemical nature and mode of action. Apart from lytic enzymes like lysostaphin and lysozyme, there exist two main groups – bacteriocins (staphylococcins) and low molecular weight antibiotic-like substances.

In contrast to the colicins and pyocins, only a few staphylococcins have been purified and studied in detail: these include staphylococcin 414 (Gagliano & Hinsdill, 1970), staphylococcin 462 (Hale & Hinsdill, 1973, 1975), staphylococcin A (Lachowicz & Walczak, 1968) and bacteriocin Bac R₁ (Rogolsky & Wiley, 1977). However, most work has been done on staphylococcin 1580, which is produced by a *Staphylococcus epidermidis* strain (e.g. Jetten *et al.*, 1972; Jetten & Vogels, 1972 *a, b*; Weerkamp *et al.*, 1977, 1978), and on staphylococcin C55, which is produced by a *Staphylococcus aureus* strain (e.g. Dajani *et al.*, 1970; Dajani & Wannamaker, 1973 *a, b*). Staphylococcins are usually high molecular weight molecules (100 000–500 000) that can be dissociated into smaller subunits under suitable conditions. They have a wide spectrum of activity, though only Gram-positive bacteria are susceptible. This property is shared by bacteriocins of other Gram-positive organisms (Tagg *et al.*, 1976) and by low molecular weight antibiotic-like substances produced by staphylococci, e.g. epidermidins (Hsu & Wiseman, 1971, 1972) and micrococci (Pulverer & Jeljaszewicz, 1976). The epidermidins are peptides consisting of 11 amino acids with molecular weights of 1200–1400 and the micrococci are thiazole-like components with molecular weights in the same range.

In the course of screening coagulase-negative strains of staphylococci and micrococci, we isolated a highly inhibitory strain, *S. epidermidis* strain 5, active only against staphylococci and micrococci. This paper describes the characterization of the inhibitory substance, designated Pep 5, produced by this strain.

METHODS

Organisms and culture conditions. *Staphylococcus epidermidis* strain 5, the indicator *S. cohnii* strain 22, and all other strains used for screening antagonistic activities and recording the spectrum of inhibition were isolated from current routine material in the diagnostic laboratories of the Institute for Medical Microbiology and Immunology of the University of Bonn. *Staphylococcus epidermidis* strain 14490, used as an indicator in some experiments, was obtained from Dr K. H. Schleifer, Munich. Stock cultures were maintained either on Tryptone Soya Agar (TSA; Oxoid) slants in screw-capped bottles at 4 °C or were lyophilized. Currently used strains were subcultured weekly on TSA or blood agar plates.

Detection of antagonistic activities. Screening for antagonistic strains was performed by means of a deferred antagonism test. Producer strains were inoculated as a spot on TSA. After 18–24 h incubation at 36 °C, cells were killed with a few drops of chloroform. Indicator strains were cultured overnight in Tryptone Soya Broth (TSB; Oxoid). Portions (2 ml) of liquefied 0.7% TSA were inoculated with indicator strains to yield 2×10^7 colony-forming units (c.f.u.) ml⁻¹, and then layered on the agar plates containing killed producer strains. The diameters of the inhibition zones were measured after incubation for 18–24 h.

The same general procedure was used for recording the activity spectrum of *S. epidermidis* strain 5: streptococci were cultured in Trypticase Phosphate Broth (Oxoid), pneumococci, anaerobic cocci and *Clostridium* species in Brain Heart Infusion Broth (Oxoid) and *Enterobacteriaceae* in Peptone Broth (Oxoid). For preparation of the semi-solid agar, 0.7% agar was added to these media.

Activity assay. The indicator strains used for assay of Pep 5 were *S. epidermidis* strain 14490 and *S. cohnii* strain 22 which were cultured as described above. Preparations of Pep 5 were diluted 1 in 3 and 1 in 5; twofold dilution series of these dilutions as well as of the undiluted sample were prepared in microtitre plates. This procedure reduced the possible error associated with a single series of doubling dilutions. Drops (10 µl) of each dilution were placed on the indicator plate. The highest dilution yielding a clear zone of inhibition was defined as containing 1 arbitrary unit (A.U.) ml⁻¹. Specific activities were expressed as A.U. mg⁻¹.

Purification. The following procedure was used to prepare about 100 mg of purified Pep 5: 40 l TSB in two 20 l carboys were each inoculated with 100 ml of an overnight culture of *S. epidermidis* strain 5, and incubated at 35 °C. The cultures were magnetically stirred and aerated (1 l air min⁻¹ for each carboy) after the addition of 1 ml silicone anti-foam emulsion H-30 (Serva, Heidelberg). After 20 h growth ($A_{600} = 3$) each culture was centrifuged and the supernatant was applied to a glass column of Servachrome XAD-2 (Serva) (column diam., 9 cm; bed volume, 1 l). The column was washed with 3 bed volumes of 50% (v/v) methanol in 1 mM-K₂HPO₄, pH 7.0 and then Pep 5 was eluted with 2 bed volumes of 70% (v/v) methanol in 1 mM-acetate buffer, adjusted to pH 2.0 with 1 M-HCl. Methanol was evaporated in a Büchi apparatus, precipitated protein was removed by centrifugation, and the clear supernatant was adjusted to pH 5.8 with 200 mM-K₂HPO₄. This solution (about 500 ml) was applied to a column of CM-Sephadex C-25 (Pharmacia) (column diam., 2.7 cm; bed volume, 80 ml) previously equilibrated with 200 mM-KH₂PO₄ buffer, pH 5.8. The gel was then washed extensively with at least 10 bed volumes of equilibration buffer, and Pep 5 was eluted with a linear potassium phosphate gradient (0.2–1.4 M, 500 ml total volume) at pH 5.8. Active fractions were pooled and solid (NH₄)₂SO₄ was added to give 90% saturation. The precipitate was harvested by centrifugation and dissolved in a small volume of 100 mM-citrate/phosphate buffer, pH 4.5 supplemented with 100 mM-KCl, and this solution was applied to a column of Sephadex G-50 (Pharmacia) equilibrated with the same buffer. Active fractions were pooled, desalted on a column of Sephadex G-25 (Pharmacia) and freeze-dried.

Polyacrylamide gel electrophoresis. This was performed as described by Maurer (1968) using 15% gels at pH 4.3: 20–50 µg of the purified substance was applied to the test tubes (0.5 × 8 cm), methylene blue was used as tracking dye and gels were run at a constant current of 4–5 mA per tube. Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) was carried out according to Weber *et al.* (1972). The final concentrations of polyacrylamide and *N,N'*-methylenebisacrylamide (Bio-Rad) were 17% and 0.45%, respectively. To avoid too rapid polymerization, the concentrations of *N,N,N',N'*-tetramethylethylenediamine and ammonium persulphate were lowered to 40% of the amount recommended by Weber *et al.* (1972). Gels were stained in Coomassie blue (1 h for gels without SDS and overnight for SDS gels) and destained in 5% (v/v) methanol/7.5% (v/v) acetic acid for several days. Proteins with the following molecular weights were used as markers: 2512, 6214, 8159, 14404, and 16949 (protein standard mixture no. 1, Merck, 15124).

Chemical analyses. For lipid determination, 5 mg of the freeze-dried substance was extracted with 1 ml CHCl₃/methanol/water (75:25:4, by vol.). The supernatant was concentrated and chromatographed on silica-gel thin-layer plates using the following mobile phases: CHCl₃, CHCl₃/*n*-hexane (1:3, by vol.), and CHCl₃/methanol/water (75:25:4, by vol.). Chromatograms were sprayed with 10% (v/v) sulphuric acid and heated at 110 °C for 15 min.

Total sugar determination was carried out by the anthrone method described by Herbert *et al.* (1971) using glucose as a standard.

Protein was determined by the method of Beisenherz *et al.* (1953) using bovine serum albumin as a standard.

For amino acid analysis, Pep 5 was hydrolysed in 6 M-HCl for 18 h at 110 °C and the analysis was carried out chromatographically on silica-gel thin layer plates (Merck, 5721) with *n*-butanol/acetic acid/water (80:20:20, by vol.) in one direction and phenol/water (75:25, by vol.) in the second direction. Amino acids were detected with ninhydrin spray. Quantitative determinations were done in a LKB autoanalyser.

The elementary analysis of Pep 5 was performed by Mikroanalytisches Laboratorium Dr F. Pascher, Bonn.

Basic grade chemicals were obtained from Merck and biochemicals from Boehringer, Mannheim.

RESULTS

Characterization of the strains

Staphylococcal strains selected for further investigations were characterized on the basis of their biochemical reactions according to Kloos *et al.* (1976). Strain 5 was identified as *S. epidermidis* and the indicator strain 22 as sucrose-positive *S. cohnii*. *Staphylococcus epidermidis* strain 5 was resistant to penicillin, ampicillin, mezlocillin and azlocillin, but sensitive to oxacillin, ticarcillin, aminoglycosides, cephalosporins, erythromycin, clindamycin, sisomicin and chloramphenicol, whereas *S. cohnii* strain 22 was sensitive to all antibiotics tested.

Spectrum of activity of S. epidermidis strain 5

The spectrum of activity was recorded as described in Methods and is summarized in Table 1; only staphylococci and micrococci were sensitive to the action of *S. epidermidis* strain 5. To confirm these results, quantitative sensitivity tests against purified Pep 5 were carried out with 11 *S. aureus* strains which proved to be resistant in the deferred antagonism test, 10 streptococcal strains of different serological groups, 5 *Escherichia coli* strains and 1 strain of *Neisseria meningitidis*. By comparison with *S. cohnii* strain 22, which by definition was sensitive at a concentration of 1 A.U. Pep 5 ml⁻¹, the 11 *S. aureus* strains were sensitive in the range 62.5–1000 A.U. ml⁻¹. The other organisms were not inhibited with concentrations up to 40 000 A.U. ml⁻¹, the highest concentration obtainable in aqueous solution.

Production of Pep 5

Staphylococcus epidermidis strain 5 produced Pep 5 under aerobic and anaerobic conditions on agar plates as well as in broth cultures. Comparison of different growth media at several pH values and temperatures showed that the highest yields of Pep 5 were obtained in TSB at pH 7.3, incubated at 35 °C in aerobic culture.

The titre of Pep 5 increased in direct proportion to growth and reached a maximum at the end of the exponential phase (Fig. 1). This is surprising as staphylococci seem to show a low rate of exoprotein secretion during the exponential phase of growth (Coleman *et al.*, 1978; Robinson *et al.*, 1980), whereas in the stationary phase exoprotein production is increased. Among the proteins excreted proteases are usually evident, and it seems likely that they were responsible for the decrease in Pep 5 titre, because the purified substance otherwise proved to be rather stable.

There have been reports of increased yields of bacteriocin following treatment of the producing strain with ultraviolet irradiation or mitomycin C (Tagg *et al.*, 1976). Attempts to increase the yield of Pep 5 from *S. epidermidis* strain 5 in this way (incubation in the presence of 0.1–5 µg mitomycin C ml⁻¹ for 0.5–18 h at 37 °C) were unsuccessful.

Purification of Pep 5

First attempts to purify the antistaphylococcal substance of *S. epidermidis* strain 5 by means of (NH₄)₂SO₄ precipitation, DEAE-cellulose chromatography, isoelectric focusing and Sephadex gel filtration were unsatisfactory. The main reason for this was the low concentration of Pep 5 in the culture supernatant (1–2 mg l⁻¹) in relation to the high content of peptides which are components of the medium. However, we were able to devise a very effective enrichment procedure (see Methods) on the basis of two characteristic properties of

Table 1. *Spectrum of activity of S. epidermidis strain 5 in the deferred antagonism test*

Organisms	No. of strains tested	No. of strains sensitive
Coagulase-negative staphylococci and micrococci	98	98
<i>Staphylococcus aureus</i>	92	81
Streptococci, serological group A	43	(1)*
serological group B	37	0
serological group C	23	0
serological group D	13	0
serological group G	19	0
<i>Streptococcus pneumoniae</i>	29	0
<i>Peptococcus</i> (4 different species)	8	0
<i>Peptostreptococcus anaerobius</i>	7	0
<i>Corynebacterium</i> (4 different species)	15	0
<i>Clostridium</i> (19 different species)	71	0
<i>Bacillus</i> (7 different species)	12	0
<i>Listeria monocytogenes</i>	2	0
Gram-negative bacteria (<i>Salmonella</i> , <i>Shigella</i> , <i>Enterobacter</i> , <i>Pseudomonas</i> , <i>Proteus</i> , <i>E. coli</i> and <i>N. meningitidis</i>)	27	0

* An atypical small diffuse inhibition zone was observed.

Fig. 1. Pep 5 production during growth of *S. epidermidis* strain 5. Growth (●) was recorded by measuring the A_{600} in suitable diluted samples. Concentrations of Pep 5 were determined as follows: 20 ml culture sample was centrifuged; the clear supernatant was applied to a small Servachrome XAD-2 column (PEGG column, LKB; 1 ml bed volume), washed with 5 ml 50% (v/v) methanol, pH 7.0, and Pep 5 was eluted with 2 ml 70% (v/v) methanol, pH 2.0. Activity assays (○) were performed as described in Methods.

Fig. 2. CM-cellulose chromatography of Pep 5. The 70% methanol fraction eluted from Servachrome XAD-2 was concentrated to 500 ml (see Methods) and applied to a column of CM-Sephadex C-25, previously equilibrated with 200 mM-KH₂PO₄, pH 5.8. After extensive washing with starting buffer, Pep 5 was eluted with a KH₂PO₄ gradient (0.2–1.4 M, pH 5.8). Protein (—) was measured as A_{280} , and Pep 5 activity (●) was assayed as described in Methods.

Fig. 3. Relative position of Pep 5 after SDS-PAGE compared with marker proteins. A: Pep 5 preparation obtained after ion-exchange chromatography (Table 2, step 2), showing inactive (*a*) and active (*b*) fractions. B: Pep 5 preparation obtained after Sephadex G-50 chromatography (Table 2, step 4). C: Marker proteins of the molecular weights indicated (see Methods). Arrows indicate the position of the tracking dye.

Table 2. *Purification of Pep 5*

Purification step	Volume (ml)	Total activity (A.U.)	Protein (mg)	Specific activity (A.U. mg ⁻¹)	Recovery (%)	Purification
Culture supernatant	40000	1 120 000	354 000	3.16	—	—
1. XAD-2, 70 % methanol, pH 2 fraction	1300	1 100 000	5452	201.7	98	64
2. CM-Sephadex column, pooled active fractions	80	1 020 000	156	6538.5	91	2070
3. (NH ₄) ₂ SO ₄ precipitation, 90 % saturation	12	1 010 000	141	7163	90	2267
4. Sephadex G-50 filtration, active fractions	62	980 000	113	8672	88	2744

Pep 5 found later during chemical analysis: (i) the relatively high content of amino acids with a non-polar side-chain, resulting in a high affinity of Pep 5 for hydrophobic Amberlite XAD resins (step 1), and (ii) the high content of the basic amino acids lysine and arginine, resulting in strong binding to cation-exchange resins (step 2). This allowed elution of nearly all contaminating peptides and proteins by extensive washing of the column with 200 mM-KH₂PO₄, pH 5.8 (Fig. 2).

The Pep 5 preparation obtained after this step showed one band in polyacrylamide gel electrophoresis and Sephadex G-50 chromatography in the presence of 10 mM-K₂HPO₄, pH 7. However, when this fraction was subjected to SDS-polyacrylamide gel electrophoresis and Sephadex G-50 chromatography in 100 mM-citrate/phosphate buffer, pH 4.5 supplemented with 100 mM-KCl, it separated into two bands (Fig. 3, A). Antistaphylococcal activity was detected in the fraction with the lower molecular weight, which proved to be the major component. The amount of the inactive higher molecular weight protein differed in several experiments and ranged from 10–40%. The data of a typical enrichment procedure are summarized in Table 2.

Properties of Pep 5

Stability. Purified Pep 5 preparations appeared to be remarkably stable in aqueous solution. Incubation at 80 °C for 1 h had no effect; boiling for 10 min and for 1 h resulted in 10% and 70% loss of activity, respectively. No significant decrease in activity was found within 3 months when stored at 4 °C. The compound was stable on lyophilization. No loss in

activity was obtained after 24 h incubation at pH values between 2 and 8, but at pH values of 9 and 10 there was a 20% decrease in activity. Rapid inactivation of Pep 5 occurred in the presence of trypsin, chymotrypsin and pronase ($10\ \mu\text{g ml}^{-1}$, treatment for 1 min). No decrease in activity could be detected after incubation for 1 h in the presence of the following enzymes (concentrations in units ml^{-1}): pepsin (porcine stomach mucosa, 250 U), carboxypeptidase A (bovine pancreas, 4 U), α -amylase (*Bacillus subtilis*, 60 U), β -amylase (barley malt, 0.6 U), deoxyribonuclease (bovine pancreas, 200 U), ribonuclease (bovine pancreas, 2 U), lipase (*Aspergillus oryzae*, 18 U), lipase (wheat germ, 9 U), phospholipase A_2 (porcine pancreas, 20 U), phospholipase D (cabbage, 6 U), lysozyme (2200 U), and lysostaphin (27 U). All tests were performed at 25 °C and with optimal assay conditions for each enzyme.

Molecular weight. This was determined by comparing the elution of Pep 5 from a Sephadex G-50 column with the elution profile of reference proteins: ovalbumin (mol. wt 45 000), chymotrypsinogen A (25 000), cytochrome *c* (12 400) and insulin (6000). Values obtained in separate experiments ranged from 5300 to 5900. The average molecular weight of the inactive fraction was 17 800. The molecular weights obtained by comparison of the R_F values of the active and inactive fractions with those of protein markers in SDS-PAGE differed slightly, but fell within the range of the possible error of both methods. They were 6300 to 6800 for the active fraction and about 18 500 for the inactive fraction (Fig. 3).

Isoelectric point. Attempts to determine the isoelectric point of Pep 5 by means of analytical isoelectric focusing on polyacrylamide gel plates (LKB, pH 3–9.5) yielded no exact value; Pep 5 was found to run into the cathode strip. Similar results were obtained in preparative experiments with ampholytes in the pH range 3–10.5; thus, it must be assumed that the isoelectric point is higher than 10.5. This result is in good agreement with the high content of basic amino acids (see below).

Chemical analysis. Purified Pep 5 was analysed with respect to its carbohydrate, lipid and protein contents. No lipids could be detected after chloroform extraction and no carbohydrates were detected by the anthrone method. Protein determination by the biuret method, with bovine serum albumin as a standard, yielded $1.05\ \text{mg protein (mg dry wt)}^{-1}$.

For elementary analysis, Pep 5 was purified further. Freeze-dried material from the last purification step was dissolved in 200 mM-KCl solution and again bound to a cation-exchange resin (CM-Sephadex C-25), equilibrated with 200 mM-KCl solution, pH 6.8. The column was washed extensively with the starting buffer and Pep 5 was eluted with a KCl gradient (200 mM–4 M). The active fractions were concentrated by ultrafiltration and desalted by Sephadex G-25 filtration in twice-distilled water. After lyophilization the substance was dried in high vacuum at room temperature and analysed. The following results were obtained (% w/w. of the dry substance): carbon 49.1%, hydrogen 8.54%, oxygen 17.4%, nitrogen 19.7%, sulphur 3.0%, phosphorus 0.2%; total 97.94%. The missing 2% may be KCl, due to insufficient desalting on Sephadex G-25. The presence of covalently bound phosphorus in Pep 5 is unlikely, as one molecule of phosphorus per molecule of Pep 5 should account for 0.56%, assuming a molecular weight of 6000 for Pep 5. On the same assumption, 3% of sulphur should account for 5–6 molecules of sulphur per molecule of Pep 5. The possibility that the sulphur content may be due to non-covalently bound sulphate can be excluded as the content remained the same after repeated ion-exchange chromatography against KCl. As can be seen from Table 3, no sulphur-containing amino acids were detected in the 6 M-HCl hydrolysate. It must therefore be assumed that the sulphur is incorporated in the two unidentified ninhydrin-positive substances.

The presence of glutamic acid is questionable. The high isoelectric point of Pep 5 makes it more likely that the peptide contains glutamine, which is hydrolysed during 6 M-HCl treatment. A quantitative comparison of the amino acid content of the inactive higher molecular weight substance yielded an identical amino acid pattern with two unidentified substances in the same positions on the elution profile as for the Pep 5 hydrolysates.

Table 3. *Amino acid content of Pep 5*

For amino acid analysis, Pep 5 was hydrolysed as described in Methods. Results are shown for a hydrolysate of 2.41 mg Pep 5, corresponding to 400 nmol when a molecular weight of 6000 is assumed. The numbers in parentheses are average values for five different determinations. Peaks A and B were eluted between alanine and valine but were not identical with citrulline or α -aminobutyric acid, which were eluted in the same range.

Amino acid	Amino acid content of hydrolysate (μ mol)	Calculated number of residues per molecule of Pep 5
Alanine	3.61	9.0 (9)
Arginine	1.41	3.5 (4)
Glutamic acid	1.62	4.0 (4)
Glycine	2.62	6.6 (6)
Isoleucine	0.79	2.0 (2)
Leucine	2.06	5.1 (5)
Lysine	4.68	11.7 (12)
Phenylalanine	0.87	2.1 (2)
Proline	0.80	2.0 (2)
Valine	1.08	2.7 (3)
Peak A	ND	—
Peak B	ND	—

ND, Quantitative determination not possible.

The absence of lipid and carbohydrate components, together with its sensitivity only to proteases, indicate that Pep 5 is a pure peptide, although two ninhydrin-positive substances have not yet been identified. A peptide consisting of the amino acids listed in Table 3 would have an elementary composition of 52.8% carbon, 7.7% hydrogen, 18.1% oxygen and 21.3% nitrogen. This differs slightly from the values obtained with Pep 5 but lies within a range which allows for correction after identification of the two unknown substances.

DISCUSSION

The antistaphylococcal agent Pep 5 produced by *Staphylococcus epidermidis* strain 5 is different from other antibacterial substances produced by staphylococci. First, its spectrum of activity is restricted to staphylococci and micrococci. This is unusual, as other antibacterial agents of Gram-positive organisms normally have a wide spectrum of activity against other Gram-positive bacteria. Furthermore, with a molecular weight of 6000 it is significantly larger than the low molecular weight antibiotic-like substances (Hsu & Wiseman, 1972; Pulverer & Jeljaszewicz, 1976), but seems to be too small to be grouped with the bacteriocins (e.g. Jetten & Vogels, 1972*a*). The latter usually occur as aggregates of high molecular weight. They often contain sugar and lipid components besides the essential protein; on SDS treatment the staphylococcins 1580, 414 and 462 dissociate into subunits with molecular weights of 10000–20000. Pep 5 also seems to occur in a higher molecular weight form, which is indicated by the identical amino acid composition of the second substance obtained after gel chromatography at higher ion concentrations. However, it is unlikely that this substance is an aggregate of three molecules of Pep 5, as might be suggested by molecular weight comparison. The fact that it did not dissociate in SDS–PAGE indicates that it is one molecule perhaps consisting of three covalently bound Pep 5 molecules. In addition, it is inactive.

Tagg *et al.* (1973) isolated a group A streptococcus bacteriocin, streptococcin A–FF 22, which has a molecular weight of 8000 and is therefore in the same range as Pep 5. Another antibacterial substance of similar size produced by a different group A streptococcal strain was characterized by Johnson *et al.* (1979). The latter was classified as a bacteriocin-like substance mainly due to its bacteriostatic action. Tagg *et al.* (1976) referred to the lack of exact definitions and the difficulties of dealing with bacteriocins of Gram-positive bacteria.

Following their recommendations, Pep 5 should be classified as a bacteriocin-like substance until further information is obtained about its mode of action and genetic determinants (chromosomal or plasmid).

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