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Peptocoagulase: Clotting Factor Produced by Bovine Strains of *Peptococcus indolicus*

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The production of a clotting factor (peptocoagulase) by bovine clinical isolates of *Peptococcus indolicus* and its nature were investigated. Extracellular peptocoagulase was demonstrated in culture filtrates of 93% and cell associated with washed cell suspensions of 100% of the 75 isolates tested. Both citrated and heparinized plasma were clotted. Crude peptocoagulase was nondialyzable, precipitated with $(NH_4)_2SO_4$ at 40% saturation, somewhat resistant to heating at both neutral and acid pH, and chloroform insoluble. Culture filtrate did not contain proteolytic activity with albumin and casein, as substrates and no esterase activity was detected with tosylarginine and benzoylarginine methyl esters as substrates. The clotting reaction required peptocoagulase, prothrombin, and fibrinogen. The activation of prothrombin appeared to involve a stoichiometric reaction with peptocoagulase, possibly by formation of a stable complex.

Several bacterial species have been reported to be able to clot citrated plasmas obtained from different animal species. In some instances this is due to utilization of the citrate in the plasma, which leads to the natural thrombin-catalyzed clotting of fibrinogen, e.g., *Streptococcus faecalis, S. liquefaciens,* and *S. zymogenes* (8) and various gram-negative bacteria (18). In other cases proteolytic activation of prothrombin by a bacterial protease can be the cause, e.g., *Bacteroides melaninogenicus* (4; G. Pulverer, personal communication).

Staphylococcus aureus strains produce coagulase, an extracellular protein that induces clotting of plasmas of different animal species. Staphylococcal coagulase (staphylocoagulase) does not possess proteolytic activity, and its mechanism of action most probably involves activation of prothrombin without proteolysis (33, 34). There has also been a report that a "coagulase" may be produced by *Yersinia pestis* (*Pasteurella pestis*) (7), but its nature and mechanism of action remain to be elucidated. In addition, Wood (30) demonstrated the production of a filterable "coagulase-like" factor by group D streptococci that did not clot heparinized plasma.

Sørensen also demonstrated the production of a "coagulase" by peptococci (22, 23). *Peptococcus indolicus* producing "coagulase" is a new species not included in the 8th edition of *Bergey's Manual of Determinative Bacteriology* (3), but has been placed on the approved list of bacterial names (11). However, the mechanism of clotting by *P. indolicus* coagulase had not been investigated. It was thus possible that the clotting reaction was related to liberation of proteolytic enzymes by these bacteria, to production of a coagulase similar to staphylocoagulase, or to the utilization of citrate in plasma.

In this study data are presented concerning the production, properties, and mechanism of action of *P. indolicus* coagulase (peptocoagulase).

MATERIALS AND METHODS

Strains. Seventy-five strains of anaerobic cocci isolated from heifers or dry cows with summer mastitis were studied (O. Schwan, unpublished data). The strains were identified as *P. indolicus* by the API 20 Anaerobe System (Analytab Products, Inc., New York) (17) and by gas chromatographic analysis of metabolites produced in PRAS peptone-yeast extract-glucose medium (PYG, see below) and were extracted with ethyl ether and methanol-chloroform (9). All strains produced acetic, propionic, and butyric acids. In addition most of the strains could be serotyped with reference typing sera (22) kindly supplied by G. H. Sørensen, State Veterinary Serum Laboratory, Department for Jutland, Århus, Denmark. Additionally, seven reference strains of *P. indolicus* were obtained from G. H. Sørensen (22; personal communication). One of these reference strains, *P. indolicus* PcR 14, was used in the more detailed experiments on the production and characterization of peptocoagulase.

Testing of strains for production of extracellular peptocoagulase. All strains were grown anaerobically at 37°C for 24 h in PYG medium: proteose

peptone, 20.0 g; yeast extract, 5.0 g; NaCl, 2.5 g; D-glucose, 0.5 g; L-cysteine, 0.5 g; distilled water, 1 liter; pH adjusted to 7.2 to 7.4. Inocula comprised 24-h cultures in the same medium. Unless otherwise stated the volume of inoculum was 10% of the volume of medium used for production of peptocoagulase. In some experiments the inoculum was centrifuged at 3,000 × *g* for 20 min at 20°C, the supernatant was discarded, and bacteria were suspended in the same volume of fresh medium. The bacterial cultures were centrifuged at 3,000 × *g* for 20 min at 20°C. The supernatant fluids were then filtered through membrane filters (0.22 μm; Millipore Corp., Bedford, Mass.) and stored at 4°C. Sterile filtrates were assayed for peptocoagulase activity.

Testing of strains for cell-associated peptocoagulase activity. The *P. indolicus* strains were grown for 24 h in 10-ml volumes of PYG medium at 37°C. Bacterial pellets were obtained by centrifugation at 3,000 × *g* for 20 min at 20°C. The pellets were resuspended in 10 ml of 0.15 M NaCl, and the suspensions were centrifuged to obtain firm bacterial pellets. The washed cells were taken up in 0.1-ml volumes of 0.15 M NaCl with the aid of a vibrator-mixer, and 0.5-ml volumes of citrated rabbit plasma, which had been diluted three times in 0.15 M NaCl, were added. The mixtures were incubated at 37°C, and the tubes were observed hourly for clotting reactions.

Crude peptocoagulase preparation. A crude preparation of peptocoagulase was prepared as follows. *P. indolicus* PcR 14 was incubated in PYG medium for 18 h. The culture was centrifuged at 3,000 × *g* for 20 min, and the supernatant fluid was concentrated about 20 times overnight at 4°C by dialyzing against polyethylene glycol (PEG 20,000) and then dialyzed against 0.05 M sodium phosphate buffer (pH 6.8) overnight at 4°C.

Assay of peptocoagulase activity. Doubling dilutions of 0.1-ml volumes of sterile culture filtrates were performed in sterile 0.15 M NaCl. Portions (0.5 ml) of fresh citrated rabbit plasma diluted three times with sterile 0.15 M NaCl were added to these dilutions. Titrations were incubated at 37°C for 24 h. Clotting was frequently observed earlier. The different batches of rabbit plasma used did not appear to vary significantly with respect to clotability. The titer obtained with a standard crude peptocoagulase preparation from strain PcR 14 varied by no more than one tube up or down from the initial value determined after concentration and dialysis, over a 3-month period. One peptocoagulase unit (U) was defined as the amount of peptocoagulase in the highest dilution of the filtrate producing clotting of plasma under the above conditions. The titer of peptocoagulase was defined as the reciprocal of the highest dilution producing clotting.

Bacterial growth curves. Growth of *P. indolicus* was measured spectrophotometrically as absorbance at 600 nm in 10-mm cuvettes.

Other assays. Proteolytic activity with casein or albumin as substrates was assayed by the method of Kunitz (13) with the modifications of Takahashi and Ohsaka (27). Trichloracetic acid-soluble protein was determined by using Folin-Ciocalteau reagent (14). The abilities of peptocoagulase to hydrolyze *p*-tosyl-L-arginine methyl ester (TAME) and α-*N*-benzoyl-L-arginine methyl ester (BAME) were measured by the method of Hummel (10).

Testing of properties of crude peptocoagulase. The following tests were performed. (i) The ability to dialyze was measured by continuous dialysis of a sample against 0.05 M sodium phosphate buffer (pH 6.8) at 4°C for 5 days with buffer changes every 12 h. (ii) Solubility in chloroform (32) was measured as follows: a sample was shaken with an equal volume of chloroform for 30 min, then the mixture was kept at room temperature for 30 min and centrifuged at 3,000 × *g* for 10 min. The water and chloroform phases were separated. Chloroform was evaporated, and the residue was suspended in the initial volume of 0.05 M sodium phosphate buffer, pH 6.8. (iii) Sensitivity to heating was measured as follows: the pH of samples was adjusted to 4.0 with 0.1 M acetic acid or to 10.0 with 0.1 M sodium hydroxide. Samples were incubated in a water bath at 60, 80, or 100°C. To test for peptocoagulase activity, samples were first dialyzed overnight against 0.05 M sodium phosphate buffer, pH 6.8.

Prothrombin activation. The ability of a crude peptocoagulase preparation to activate bovine prothrombin was tested. Bovine prothrombin was obtained by the method of Malhotra and Carter (16). Prothrombin was assayed by the two-stage method of Ware and Seegers (28) and expressed in NIH units. One NIH unit of prothrombin (thrombin) is the amount that produces 0.37 μmol of *N*-terminal glycine in 20 min at pH 9.0, 1 = 0.2 at 25°C with fibrinogen as substrate (0.3% wt/vol) (15).

Activity of peptocoagulase-activated prothrombin was assayed in a system containing 0.1 ml of the mixture of prothrombin and peptocoagulase and 0.4 ml of 0.3% bovine fibrinogen solution in Palitsch buffer (0.006 M disodium tetraborate(borax), 0.176 M boric acid, and 0.044 M NaCl, pH 7.6). Clotting time of this mixture at 37°C was inversely proportional to the activity of activated prothrombin. Bovine fibrinogen was produced by the method of Kekwick et al. (12) and assayed by the method of Blombäck (2).

Chemicals. Tosylarginine and benzoylarginine methyl esters, bovine serum albumin, and casein were obtained from Sigma Chemical Co., St Louis, Mo.; citrated rabbit plasma was obtained from Statens Bakteriologiska Laboratorium, Stockholm; proteose peptone and yeast extract were obtained from Difco Laboratories, Detroit, Mich. All other chemicals were of analytical grade or the best grade commercially available. Heparin (1,000 IU/ml) was purchased from Vitrum, Stockholm.

RESULTS

Extracellular peptocoagulase production by *P. indolicus* strains. All but five of the bovine strains produced a filterable factor clotting citrated rabbit plasma. The titer of peptocoagulase produced varied considerably from strain to strain between 10 to 320 U per ml of culture filtrate after 24 h of cultivation. The limit of detection of the assay used was 10 U/ml of filtrate, i.e., 1 U/0.1 ml of undiluted culture filtrate in the assays. Some strains producing

low titers of peptocoagulase were peptocoagulase negative on retesting. Production studies with two strains, PcR 14 and 199, using four different batches of PYG medium revealed wide ranges in peptocoagulase titer (<40 to 320 U per ml). Strains were rechecked with a batch of medium that gave maximum yields with strain PcR 14, but five strains were still negative for peptocoagulase production within the limits for the assay used. Peptocoagulase activity was the same in unfiltered and sterile, filtered supernatant fluids.

The abilities of culture filtrates to clot heparinized (10 IU/ml) rabbit plasma were also tested. All citrated plasma-positive filtrates also produced clotting of heparinized plasma. The five strains that failed to produce a factor clotting citrated plasma were negative in heparinized plasma tests. Again the peptocoagulase titer depended on the strain and was affected by the batch of medium. Culture filtrates from the reference *P. indolicus* strains of Sørensen all produced clotting reactions with citrated and heparinized rabbit plasma.

Cell-associated peptocoagulase. The possible association of peptocoagulase activity with washed peptococcal cells was also investigated. Washed bacterial suspensions of all 75 strains produced positive clotting reactions within 3 h. Thus, the five strains which were negative for free peptocoagulase possessed a cell-bound clotting factor. One of the reference *P. indolicus* strains from Sørensen was negative even after prolonged incubation and thus did not appear to possess a cell-associated form of peptocoagulase. Tests were positive with both citrated and heparinized rabbit plasma.

Growth and peptocoagulase production. Growth and peptocoagulase activity in the course of cultivation of *P. indolicus* PcR 14 are presented in Fig. 1. When a 10% by volume inoculum was used, the culture reached stationary phase after 10 h of incubation. After prolonged incubation the absorbance of the culture decreased slightly. The highest titer of peptocoagulase activity was obtained after 12 h, remained at this level during the next 20 h, and then decreased. pH values during the 3-day incubation period varied slightly from 6.2 to 6.7.

The relationship between growth phase and peptocoagulase production was studied. Different inocula were used, namely 1, 3, and 10% by volume. Inocula were centrifuged, and bacteria were suspended in fresh medium to avoid the introduction of peptocoagulase with the inoculum (Fig. 2). Regardless of inoculum size peptocoagulase production started with initiation of the logarithmic phase of growth, reaching the highest titer in stationary phase. Identical results were obtained with washed cells and unwashed preculture as inocula.

Relationship between protein synthesis and appearance of extracellular peptocoagulase. Addition of chloramphenicol at the beginning of the logarithmic phase of growth before peptocoagulase production started caused marked inhibition of bacterial growth, and no extracellular peptocoagulase appeared during the next 12 h (Fig. 3). When the antibiotic was added in the middle of the logarithmic phase, the results were similar, i.e., bacterial growth was inhibited and there was no increase in peptocoagulase titer. The concentration of chloramphenicol used was 4 μg/ml, which was slightly higher than the minimal inhibitory concentration for strain PcR 14 (3.12 μg/ml).

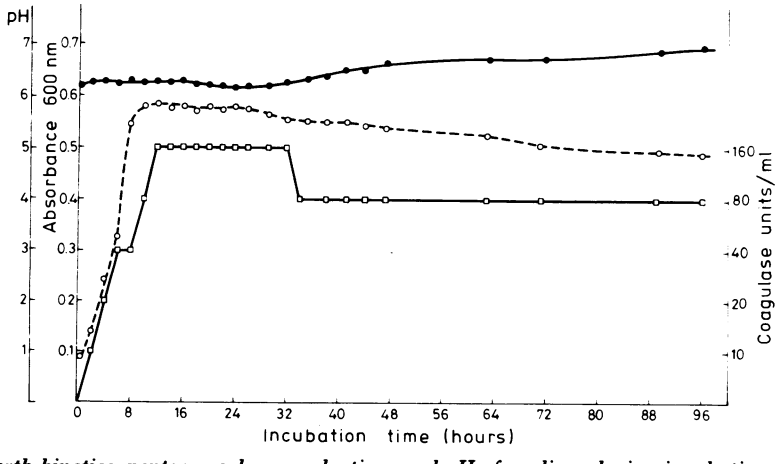

FIG. 1. *Growth kinetics, peptocoagulase production, and pH of medium during incubation of P. indolicus PcR 14 for 4 days at 37°C. pH (●), absorbance at 600 nm (○), peptocoagulase activity (□).*

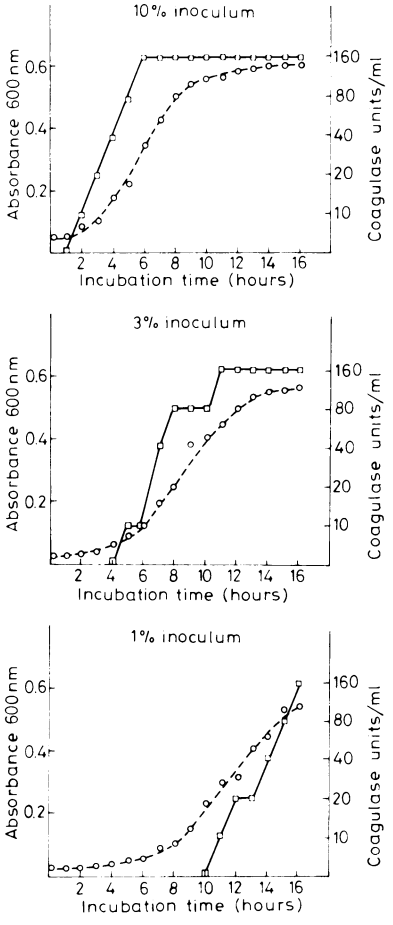

FIG. 2. *Influence of the inoculum size on the production and growth of peptocoagulase by P. indolicus PcR 14 at 37°C. Absorbance at 600 nm (○); peptocoagulase activity (□).*

Properties of *P. indolicus* coagulase. The properties of crude peptocoagulase are summarized as follows: (i) It was nondialyzable; peptocoagulase activity did not decrease during 5 days. (ii) Peptocoagulase activity did not change after shaking in the presence of chloroform; all activity remained in the water phase. (iii) When ammonium sulfate was added to crude peptocoagulase, the clotting factor could be precipitated at about 40% saturation at 4°C. (iv) Peptocoagulase had neither proteolytic nor esterase activity when tested with albumin, casein, and synthetic esters of arginine as substrates.

The sensitivity of crude peptocoagulase to heating with varying pH is shown in Fig. 4. These data indicate that crude peptocoagulase was to some extent resistant to heating, especially at neutral and acidic pH. During incubation at acidic and alkaline pH at 100°C, its activity decreased rapidly.

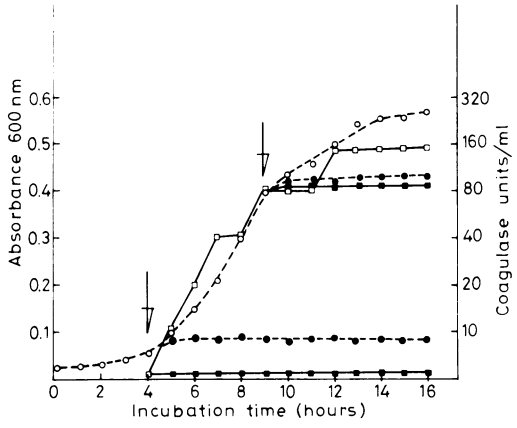

FIG. 3. *Effect of addition of chloramphenicol on the growth and production of peptocoagulase by P. indolicus PcR 14. The arrows indicate addition of antibiotic after 4 or 9 h of incubation of the culture at 37°C. Inoculum, 3% by volume of washed bacteria. Absorbance (○, ●) and peptocoagulase activity (□, ■) in the absence (open symbols) and presence (closed symbols) of 4 μg of chloramphenicol per ml.*

Peptocoagulase activation of prothrombin. To elucidate the mechanism of peptocoagulase action, two types of experiments were performed. In the first, varying concentrations of prothrombin were incubated in the presence of the same amount of peptocoagulase; in the second, prothrombin was incubated with varying concentrations of peptocoagulase.

Crude peptocoagulase was preincubated for 15 or 150 min with varying amounts of prothrombin. The lowest concentration of prothrombin tested was sufficient on activation to clot all the fibrinogen present in the test mixture. The results presented in Table 1 indicate that (i) clotting time was independent of the amount of activated prothrombin formed in this experimental system, and (ii) prolonged preincubation neither decreased nor increased the amount of activated prothrombin formed.

The results presented in Table 2 demonstrate that when prothrombin was in excess, clotting time, which is inversely proportional to the amount of activated prothrombin, was related only to the concentration of peptocoagulase. The preincubation period had no influence on the amount of activated prothrombin formed.

DISCUSSION

To date, only a few papers on *P. indolicus* have been published. Recently, Sørensen reported the isolation of *P. indolicus* from bovine summer mastitis (22–25). Usually these strains were isolated together with *Corynebacterium*

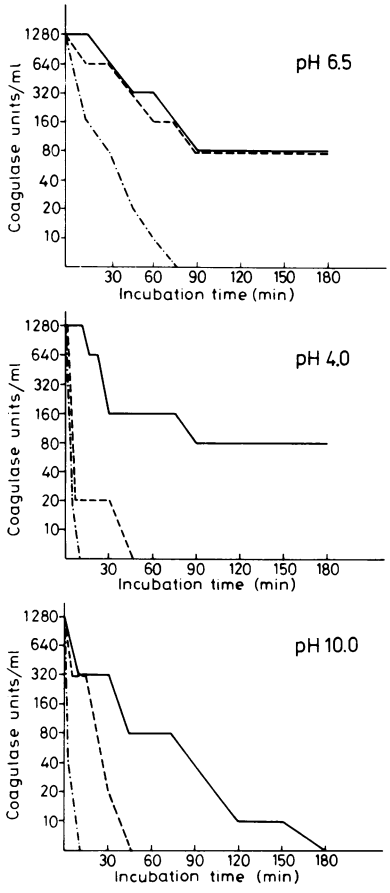

FIG. 4. *Effect of heating at neutral, acidic, and alkaline pH on the activity of P. indolicus peptocoagulase activity. Temperatures: 60°C (——), 80°C (---), and 100°C (-·-).*

TABLE 1. *Effect of prothrombin concentration on the formation of activated prothrombin in the presence of peptocoagulase*[a]

Prothrombin (NIH units)	Clotting time (s)	
	15 min of preincubation	150 min of preincubation
63	160	155
31.5	159	154
15.75	156	152
7.88	159	150
3.94	156	150
1.97	157	149
0.98	153	148
0.49	154	152

[a] 256 U of peptocoagulase were added to each assay.

pyogenes. The pathogenesis of this infection remains obscure. It can be postulated that peptocoagulase produced by *P. indolicus* might be one of the pathogenicity factors. Moreover, to

TABLE 2. *Dependence of the formation of activated prothrombin on the concentration of peptocoagulase*[a]

Coagulase (U)	Clotting time (s)	
	15 min of preincubation	150 min of preincubation
512	94	90
384	120	121
256	161	164
128	285	285

[a] Each assay contained 3.94 NIH units of prothrombin.

date, no other potential virulence factors produced by this species have been described. Extracellular peptocoagulase was produced by 93% of the strains examined in this study, which compares favorably with the data of Sørensen with more extensive material (98%, 274 strains tested) (22). However, peptocoagulase titers may be influenced by as yet undefined differences between batches of PYG medium.

Several bacterial toxins or virulence-associated factors are produced as extracellular and surface-bound forms, e.g., protein A by *S. aureus* (29). According to Sørensen (22) and to the present data, peptocoagulase is produced in cell-associated and extracellular forms. Moreover, in the presence of chloramphenicol (Fig. 3) production of extracellular peptocoagulase and bacterial growth were inhibited in parallel. These data suggest that peptocoagulase is synthesized and released into the medium only during the logarithmic phase of growth (c.f. *Aeromonas hydrophila* hemolysin [31]). By the criteria of Raynaud and co-workers (19, 20), the bound form of peptocoagulase could be intracellular or surface associated. The fact that lysis of peptococci in late stationary phase (the decrease in absorbance in Fig. 1) was not accompanied by an increase in the titer of peptocoagulase would suggest that peptocoagulase is not released from an intracellular pool and thus that cell-associated peptocoagulase is surface bound. However, extracellular activity need not be associated with the same factor as the cell-bound form, e.g., staphylococcal coagulase and clumping factor (26, 33, 34). This question remains to be resolved for peptocoagulase.

Some properties of *P. indolicus* coagulase were also investigated, which suggest that it is protein in nature. Attempts to verify this by using proteolytic inactivation proved problematic, as the common proteases tested also caused clotting of rabbit plasma (data not shown), rendering interpretation of such experiments, with or without addition of inhibitors, difficult and dubious.

The mechanism of peptocoagulase action was partially characterized. The possibility that clotting of plasma was due to citrate utilization was excluded by the following observations: (i) bacteria-free filtrates produced clotting (this would not exclude the possibility of a metabolic enzyme); (ii) *P. indolicus* strains were negative in citrate utilization tests (22); and (iii) clotting of heparinized plasma was as effective as with citrated plasma. Crude peptocoagulase preparations possessed no proteolytic and/or esterase activities, at least on the substrates tested. It is however possible, although unlikely, that it may be a very specific enzyme cleaving some peptide bonds of the prothrombin molecule without liberation of any material, as in the case of activation of chymotrypsinogen by trypsin (6).

Three factors had to be present in the system for a clotting reaction—peptocoagulase, prothrombin, and fibrinogen. The data presented in Tables 1 and 2 indicate that the factor in plasma reacting with peptocoagulase was prothrombin, a fact substantiated by the observation that plasma from dicumarol-treated rabbits showed a very prolonged clotting time with peptocoagulase; this effect was reversed by administration of the antidote vitamin K to the rabbits (L. Świtalski and C. Smyth, unpublished data). The possible involvement of other blood clotting factors in the plasma clotting reaction was not investigated.

The mechanism of activation of prothrombin by peptocoagulase therefore differs essentially from the physiological pathway of its activation, i.e., proteolysis of the prothrombin molecule by a complex of active X-factor, accelerator-globulin, phospholipids, and calcium ions (5). Rather, it resembles, superficially at least, that observed for staphylocoagulase (33, 34). Prothrombin and peptocoagulase appear to interact stoichiometrically in a nonenzymatic manner. Thus, according to our hypothesis, peptocoagulase could be an allosteric effector changing the confirmation of the prothrombin molecule. Activated by peptocoagulase, the prothrombin molecule could possess active and binding centers accessible for fibrinogen. The data (Tables 1 and 2) are suggestive of the formation of a complex between peptocoagulase and prothrombin. However, complexing of peptocoagulase with prothrombin could be a secondary effect after proteolytic activation of prothrombin. Further studies on peptocoagulase-activiation of prothrombin and the potential role of this factor in the pathogenesis of summer mastitis are warranted.

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