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Chemical Modification of the Bovine Parotid Hypocalcemic Protein

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The hypocalcemic protein purified from bovine parotid gland was modified with chemical reagents or by digestion with carboxypeptidase A, the hypocalcemic activities of the treated samples were assayed, and the effect of treatments was examined statistically. Cleavage of the tryptophanyl and tyrosyl residues in the sample was oxidatively done with N-bromosuccinimide, and tyrosyl residues were acetylated with N-acetylimidazole. In both treatments, the hypocalcemic activities of the treated samples were somewhat low compared to the controls but the effect of the treatments was not significant. However, both treatments in the presence of urea became effective. Oxidation of methionyl residues of the sample with hydrogen peroxide resulted in the retention of the activity, and the effect of oxidation in the presence of urea was not significant. In the modification of free amino groups, the treatment was ineffective when 51.6% of lysyl free amino groups was blocked with trinitrobenzenesulfonic acid, but the effect appeared when free amino groups were eliminated with nitrous acid. After the reduction of disulfide bonds of the sample with 2-mercaptoethanol, the resulting SH residues were modified with 5,5′-dithiobis(2-nitrobenzoic acid), and the histidyl residues of the sample were acylated with ethoxyformic anhydride, by which effect of these treatments became significant. The sample was digested with carboxypeptidase A at 25° for 4 hr failed to show effect of the treatment. From these results, tryptophanyl, tyrosyl, free amino, disulfide, and histidyl residues may play a role in appearance of the hypocalcemic activity.

Keywords——bovine parotid gland; hypocalcemic protein; parotin; chemical modification

We previously reported that average molecular weight of the hypocalcemic protein isolated from bovine parotid gland was 46500±1500 by means of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, sedimentation equilibrium, and viscometry in a solution of 6 M guanidine-HCl.[2] This protein had the isoelectric point of pH 5.3, and presumably bore a rigid structure containing 54% of α-helix from its circular dichroism spectrum.[3] This protein also had Lys-Leu- as an amino-terminal sequence and -Thr-Val-Leu as a carboxyl-terminal, and was confirmed to be a single polypeptide chain since both dissociation and association were not found by SDS-polyacrylamide gel electrophoresis.[4] Meanwhile, besides having the hypocalcemic action, this protein produced increase in the lymphocyte/polymorph ratio as determined by the method of Hand, *et al.*[5] and antibody-producing cells as determined by the method of Jerne and Nordin,[6] in neonatal mice.[7] We also found previously that the hypocalcemic activity of the samples decreased or disappeared through the modification of phenyl radicals with iodine and of free amino groups with ketone, nitrous acid, or formaldehyde, and by the reduction of disulfide bonds with thioglycollic acid by

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2) A. Mizutani, T. Kitamura, N. Yamada, H. Inaba, Y. Naito, and T. Mizutani, *Chem. Pharm. Bull.* (Tokyo), **22**, 1955 (1974).
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using the fraction precipitated at 7—15% concentration of $(NH_4)_2SO_4$.[8] The hypocalcemic activity disappeared in the sample denatured with urea but appeared in the sample solution from which urea was removed by dialysis.[8]

In the present study, we used the purified hypocalcemic protein and examined treatments with different reagents, and compared with the previous results obtained by using ammonium sulfate fractionated sample.[8] We also studied the effect of modification of other amono acid residues on the hypocalcemic activity of the sample.

Material and Methods

Materials

The hypocalcemic protein, as previously reported,[2] was purified by chromatography on DEAE-cellulose, gel chromatography on Sepharose 6B, preparative polyacrylamide gel electrophoresis, and second gel chromatography on Sepharose 6B, which resulted in a single substance by analytical disc electrophoresis. N-Bromosuccinimide (NBS) and urea were a product of Wako Pure Chem. Co., Tokyo, N-acetylimidazol was from Nakarai Chem. Co., Kyoto, trinitrobenzenesulfonic acid (TNBS) and ethoxyformic anhydride were from Tokyo Kasei Co., and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and carboxypeptidase A were from Boehringer Mannheim Co., West Germany.

Bioassay

Hypocalcemic activity was assayed by the method described previously.[2] The sample dissolved in physiological saline was injected into the rabbit aural vein and mean of percentage decrease in serum calcium level (hypocalcemic rate) after injection was calculated to that before the injection. The *t*-test was used for the evaluation of difference in the hypocalcemic rate in the test group and the control group injected with physiological saline, and significant values at the 5% level of significance were taken as effective. The amount of serum calcium was determined by the atomic absorption spectrophotometer.

Examination for the Validity of Treatments with Various Agents

Validity of the treatment was examined by the *t*-test by comparison of the hypocalcemic activity of the treated sample with that of the control sample, which was treated in the same way but without the reagents. When the difference between the hypocalcemic rate of the test group and that of the control group was significant at the 5% level of significance, the treatment was taken as effective.

1. Cleavage of Tryptophanyl and Tyrosyl Residues——Oxidative cleavage of tryptophanyl and tyrosyl residues with NBS was done by the method of Spande and Witkop.[9,10]

a) A solution of 5 mg of the sample dissolved in 2 ml of phosphate buffer (pH 7.0) or acetate buffer (pH 4.0) was mixed with 0.3 ml of 0.01 M NBS, and the resulting mixture was stirred at room temperature for 30 min.

b) The sample (5 mg) dissolved in 2 ml of acetate buffer (pH 4.0) containing 6 M urea was treated as in a). The control was treated in the same manner but without NBS. In both experiments *a*) and b) the reaction mixture was applied to a column (1×30 cm) of Sephadex G-10 and the effluents were lyophilized for the assay after dialysis.

In the following experiments, the reaction mixture was subjected to gel filtration for removing the reagent and was lyophilized for the assay.

c) Oxidized tryptophanyl residues were determined by the method of Spande, *et al.*[9,10] from the decrease in absorbance at 280 nm of the reaction mixture which was prepared by adding 10 μl portions of 0.01 M NBS solution to the solution of the sample (4.0 mg/ml) dissolved in phosphate buffer (pH 7.0) or acetate buffer (pH 4.0). The number of oxidized tryptophanyl residues per mol of protein (n) was calculated from the following expression.

$$n = \frac{\Delta OD \times 1.31 \times \text{mol. wt.} \times v}{w \times 5500}$$

where ΔOD is the corrected optical density decrease at 280 nm, 1.31, the empirical factor, mol. wt., the molecular weight of the protein used, v, the initial volume (ml) of titrated solution, w, the weight (mg) of protein titrated, and 5500, the molar extinction coefficient of tryptophanyl residue at 280 nm.

2. Acetylation of Tyrosyl Residues——Free tyrosyl residues were acetylated with N-acetylimidazole by the method of Riordan, *et al.*[11]

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a) The sample (10 mg) was dissolved in 2.5 ml of 0.05 M sodium borate buffer (pH 7.5) containing 60-fold mol of N-acetylimidazole, and the solution was allowed to stand for 1 hr at room temperature.

b) The sample dissolved in 0.05 M sodium borate buffer (pH 7.5) containing 6 M urea was treated as in *a*). The control was treated in the same manner but without N-acetylimidazole. In both experiments a) and b), the reaction mixture was loaded on a column (1 × 30 cm) of Sephadex G-25, and the effluents were lyophilized after dialysis. The number of tyrosyl residues acetylated was estimated from the decrease in absorbance at 278 nm.

3. **Oxidation of Methionyl Residues**——Methionyl residues were oxidized according to the method of Schachter and Dixon.[12]

a) The sample was dissolved in 0.5 mM ethylenediamine tetraacetic acid (EDTA) solution to a protein concentration of 4 mg/ml. The solution was adjusted to pH 3.2 with 0.001 M $HClO_4$, and 30% H_2O_2 was added up to 0.38 M. To the mixture which was incubated at 30° for 70 min, 5 μl portions of 1% catalase solution (pH 5.5, 0.005 M phosphate buffer) was added until bubbling ceased, and the reaction mixture was lyophilized for the assay after gel filtration.

b) The sample dissolved (4 mg/ml) in 0.5 mM EDTA solution (pH 3.2) containing 6 M urea was treated as in a), and the sample solution without H_2O_2 was treated in the same way, as a control.

4. **Modification of Free Amino Groups**——a) Blocking of Lysyl Free Amino Groups: The lysyl free amino groups were blocked with TNBS according to the method of Habeeb.[13] To a mixture of 2 ml of aqueous sample solution (0.6—0.7 mg/ml) and 1 ml of phosphate buffer (pH 7.5) or 4% $NaHCO_3$ solution (pH 8.5), 1 ml of 0.1% TNBS was added. The resulting mixture was incubated at 40° for 2 hr, and subsequently subjected to dialysis and lyophilization for the assay after gel filtration. For measurement of the absorbance of the blocked lysyl amino groups, 1 ml of 10% SDS and 0.5 ml of 1 N HCl were added to the reaction mixture (3 ml) which was treated in the same way as above. The amount of blocked amino groups was estimated from increase in the absorbance of the solution at 344 nm using $\Delta\varepsilon_{344}=1.09\times10^4\ M^{-1}\ cm^{-1}$.[14] For the blank, 2 ml of water was used in place of the sample solution.

b) Elimination of Free Amino Groups with Nitrous Acid:——The sample (4 mg) dissolved in 0.4 ml of 1 M acetate buffer (pH 4.0) was mixed with 0.12 ml of 4 M $NaNO_2$, stood at room temperature for 30 min, the solution was adjusted to pH 7.0 with 4 N NaOH, and lyophilized for the assay after dialysis.[15] Another portion of the sample (4 mg) was treated in the same way as above, besides incubation with $NaNO_2$ for 60 min.

5. **Modification of Disulfide Bonds**——To a solution of 5 mg of the sample dissolved in phosphate buffer (pH 8.0), 2-mercaptoethanol was added to give 1% concentration, the mixture was allowed to react at room temperature for 30 min, subsequently was mixed with 0.2 ml of 3.96% DTNB in phosphate buffer ($\mu=0.1$, pH 7.0), and the mixture was incubated for 10 min.[16] The reaction mixture was lyophilized for the assay after gel filtration.

6. **Acylation of Histidyl Residues**——Histidyl residues were acylated with ethoxyformic anhydride as performed by Melchior and Fahrney.[17] To the sample dissolved (0.8 mg/ml) in acetate buffer (0.1 M, pH 4.0), ethoxyformic anhydride was added to give 15 mM solution, and absorbance of the solution at 240 nm was measured after a few seconds of agitation. The amount of acylated histidyl residues in the protein was estimated from increase in absorbance of the solution using $\Delta\varepsilon_{240}=3.2\times10^3\ M^{-1}\ cm^{-1}$, and the solution was lyophilized for the assay after gel filtration.

7. **Digestion of C-terminal Area of the Protein with Carboxypeptidase A**——The sample was digested with carboxypeptidase A at 25° for 4 hr according to the method previously reported,[4] followed by lyophilization for the assay after gel filtration.

Results and Discussion

Dose-Response Curve

Figure 1 shows the hypocalcemic responses produced in rabbits injected intravenously with purified sample in various doses. The hypocalcemic rate was insignificant in a dose of 0.01 mg/kg compared to that of the control injected with physiological saline, and the effective doses were found to be 0.03 mg/kg or more.

The results of various treatments and assays of the treated samples and controls on hypocalcemic activity are listed in Table I.

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1. Cleavage of Tryptophanyl and Tyrosyl Residues——a) Although NBS reacts with SH group as well as with tryptophanyl and tyrosyl residues, there was no necessity for considering the reaction of NBS with SH group because this group was absent in the purified sample.[18] In the treatment of tryptophanyl and tyrosyl residues with NBS at pH 7.0, tryptophanyl residue was not modified because changes in the absorbance at 280 nm could hardly be observed. From this fact, most of tryptophanyl residues appear to be buried in the protein molecule at nearly around pH 7.0. The relation of the absorbance at 280 nm to the volume of 0.01 M NBS solution added at pH 4.0 were plotted and shown in Fig. 2. The minimum absorbance on this curve shows terminal point of the reaction and absorbance decreasing from the initial to the terminal point was 0.472 by the addition of total 180 μl of 0.01 M NBS. The

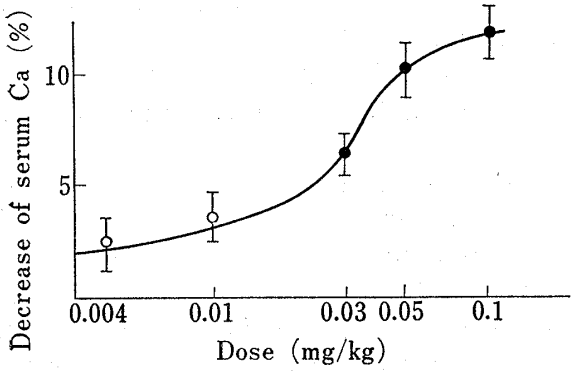

Fig. 1. Dose Response Curve

●, significantly different from control ($p<0.01$).
○, $p>0.05$.

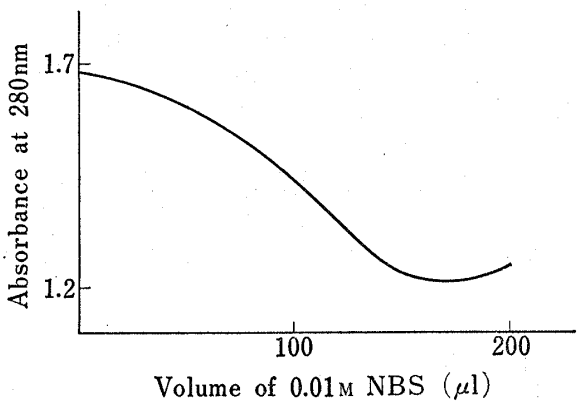

Fig. 2. Titration Profile of the Sample by Addition of NBS in Acetate Buffer (pH 4.0)

TABLE I. The Hypocalcemic Activities of the Samples Treated

Attacked amino acid	Experimental number	Reagent and condition		Mol modified/mol protein (% modified)	Dose (mg/kg)	Hypocalcemic rate (%) Mean±S.E. ($n=6$) Treated	Control	The effect of treatment
Tryptophan (Tyrosine)	1-a	NBS	pH 7.0	0 (0)	0.03	6.43±0.56[a]	6.58±0.91[a]	
			pH 4.0	2.7(52.9)	0.03	4.33±1.01	6.58±0.91[a]	
					0.05	7.25±1.09[a]	10.04±2.04[a]	
	1-b		pH 4.0, urea	4.3(84.3)	0.05	3.59±1.41	8.67±1.19[a]	effective[b]
Tyrosine	2-a	N-Acetyl-imidazole	pH 7.5	2.2(26.2)	0.03	4.58±0.56	6.58±0.91[a]	
					0.05	7.37±1.36[b]	10.40±1.23[a]	
	2-b		pH 7.5, urea	7.9(94.0)	0.05	2.17±2.44	9.14±1.52[a]	effective[b]
Methionine	3-a	H_2O_2	pH 3.2		0.03	6.18±1.22[b]	6.58±0.91[a]	
	3-b		pH 3.2, urea		0.05	7.56±0.96[a]	8.40±0.39[a]	
Lysyl free NH_2	4-a	TNBS	pH 7.5	6.3(24.6)	0.03	6.53±0.97[a]	6.58±0.91[a]	
			pH 8.5	13.2(51.6)	0.03	6.30±0.58[a]		
Free NH_2	4-b	$NaNO_2$	pH 4.0, 30 min		0.05	5.02±0.99	10.40±1.23[a]	effective[a]
			pH 4.0, 60 min		0.05	2.87±0.79		
Disulfide	5	DTNB	pH 8.0		0.03	3.71±1.00	6.58±0.91[a]	
					0.05	5.07±1.25	10.40±1.23[a]	effective[b]
Histidine	6	Ethoxyformic anhydride (pH 4.0)		3.8(97.4)	0.03	2.26±0.95	6.58±0.91[a]	effective[a]
					0.1	4.03±2.01	12.00±1.20[a]	
C-terminal amino acid	7	Carboxypeptidase A pH 8.5			0.03	6.56±0.62[a]	6.58±0.91[a]	

a) 1% level of significance.
b) 5% level of significance.

18) T. Mizutani, P-F. Kuo, and A. Mizutani, *Chem. Pharm. Bull.* (Tokyo), **25**, 2821 (1977).

number of oxidized tryptophanyl residues was estimated to be 2.7 mol/mol protein from the equation of Spande, *et al.*[9,10] using 0.472 for the decreased absorbance and 48000 for the molecular weight of the sample protein from SDS-polyacrylamide gel electrophoresis.

The hypocalcemic rate of the sample treated at pH 7.0 was 6.43±0.56% in a dose of 0.03 mg/kg and the activity was retained (1-a in Table I). Effect of the treatment was insignificant against intact sample. The sample treated at pH 4.0 gave the hypocalcemic rate of 4.33 ±1.01% in a dose of 0.03 mg/kg and the value was not significant, and administration of the same sample in a dose of 0.05 mg/kg resulted in a rate of 7.25±1.09% (1-a in Table I). This value was somewhat low compared to the rate (10.04±2.04%) of the control which was treated at pH 4.0 but without NBS. However, the activity remained, and effect of the treatment was not shown.

b) In the sample treated at pH 4.0 in the presence of 6 M urea, the oxidized residues were estimated to be 4.3 mol/mol of the protein, which corresponded to 84.3% of total tryptophanyl residues. Its hypocalcemic rate was 3.59±1.41% and the activity disappeared (1-b in Table I). Effect of the treatment was also found against the control, 8.67±1.19%, at the 5% level of significacnce.

2. **Acetylation of Tyrosyl Residues**——a) Figure 3 shows the absorbance curves of the intact sample and the sample treated at pH 7.5 with N-acetylimidazole. The acetylated tyrosyl residues were calculated to be 2.2 mol/mol of the protein from decrease in the absorbance at 278 nm and 26.2% of tyrosyl residues in the protein was acetylated. The hypocalcemic rate of the acetylated sample was 4.58±0.56% in a dose of 0.03 mg/kg, and it was not significant (2-a in Table I). The dosage of 0.05 mg/kg of the sample gave the hypocalcemic rate of 7.37±1.36% and the activity was retained though it somewhat decreased, and the treatment was not effective.

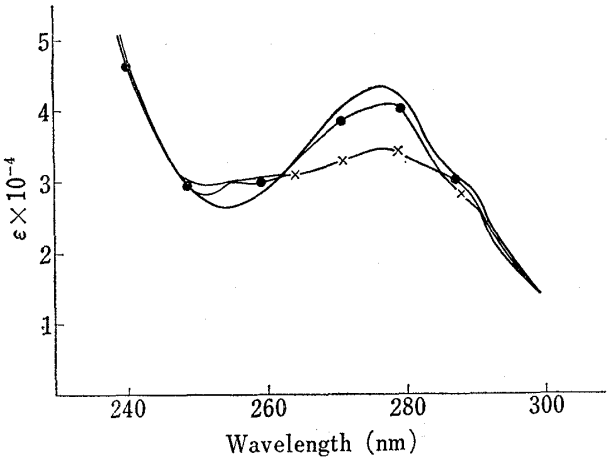

Fig. 3. Acetylation of Tyrosyl Residues in Protein with N-Acetylimidazole

—— intact sample; —●— sample treated in sodium borate buffer; —×— sample treated in sodium borate buffer containing 6M urea.

b) In the sample treated with N-acetylimidazole at pH 7.5 in the presence of 6 M urea, the acetylated tyrosyl residues were 7.9 mol/mol of the protein, which corresponded to 94.0% of total tyrosyl residues. The hypocalcemic rate of this sample was 2.17±2.44% in a dose of 0.05 mg/kg and the activity disappeared as shown in Table I 2-a. Effect of the treatment was significant against the control, 9.14±1.52%.

3. **Oxidation of Methionyl Residues**——a) The sample in which methionyl residues were oxidized with H_2O_2, gave the hypocalcemic rate of 6.18±1.22% in a dose of 0.03 mg/kg and the treatment was not effective (3-a in Table I).

b) The hypocalcemic rate of the sample oxidized with H_2O_2 in the presence of 6 M urea was 7.56±0.96% in a dose of 0.05 mg/kg and the effect of the treatment was also not significant against the control, 8.40±0.39% (3-b in Table I).

4. **Modification of Free Amino Groups**——a) Blocking of Lysyl Free Amino Groups: In the treatment of the sample with TNBS, the blocked lysyl amino groups per mol of the protein were 6.3 at pH 7.5 and 13.2 at pH 8.5, and these corresponded to 24.6 and 51.6% of total lysyl amino groups, respectively, since this protein had 25.6 mol of lysine per mol of the protein. In the former treatment, the hypocalcemic rate was 6.53±0.97% in a dose of

0.03 mg/kg, and in the latter was 6.30±0.58% in the same dose, and both treatments were not effective (4-a in Table I). From these observations, rise in the reactivity of the residues may be due to some changes in the conformation of the protein caused by the shift of pH from neutral medium, and these tendencies of reactivity were analogous to the fact that the reaction of bovine serum albumin or ovalbumin with TNBS is influenced by changes in pH of the solution.[13]

b) Elimination of Free Amino Groups with Nitrous Acid: In treatment of the sample for 30 and 60 min with $NaNO_2$, hypocalcemic rates were 5.02±0.99% and 2.87±0.79%, respectively, in a dose of 0.05 mg/kg and these hypocalcemic activities were undetectable and both treatments were effective at the 1% level of significance (4-b in Table I). Although tyrosyl residue was also modified with $NaNO_2$, NH_2 group mainly influences the hypocalcemic activity since the effect of the treatment was not significant in the sample in which tyrosyl residue was acetylated. These results agree with the fact that the hypocalcemic activity of 7—15% $(NH_4)_2SO_4$ fraction was lost by the modification of its free amino groups with ketene or nitrous acid.[8] The high reactivity of these reagents without the aid of a denaturant, such as urea, may be due more or less to smallness of the molecular size of the reagents.

5. Modification of Disulfide Bonds——The disulfide bonds of the sample, which contained 1.1 mol of cystine, were modified with 2-mercaptoethanol and DTNB, and administration of the treated sample resulted in the hypocalcemic rates of 3.71±1.00 and 5.07±1.25% in doses of 0.03 and 0.05 mg/kg, respectively. The hypocalcemic activity was lost in both cases, and the treatment was not effective in the former dosage and was effective in the latter at the 5% level of significance. This fact was also not contrary to the fact that reduction of the 7—15% $(NH_4)_2SO_4$ fraction with thioglycollic acid led to an inactive product.[8]

6. Acylation of Histidyl Residues——The number of acylated histidyl residues was estimated to be 3.8 mol/mol protein from increase in absorbance, and the value corresponded to 97.4% of total histidyl residues (3.9 mol/mol protein). NH_2 group is also acylated usually with ethoxyformic anhydride, but this group seems to be unmodified since reactivity of NH_2 group is low compared with histidyl residue at pH 4.0. The hypocalcemic rates of the acylated samples were 2.26±0.95% and 4.03±2.01% in doses of 0.03 and 0.1 mg/kg, respectively. The hypocalcemic activity disappeared and the treatment was effective at the 1% level of significance (6 in Table I).

7. Digestion of C-terminal Area of the Protein with Carboxypeptidase A——The hypocalcemic rate of the sample digested with carboxypeptidase A was 6.56±0.62% in a dose of 0.03 mg/kg and the treatment was not effective (7 in Table I). In the previous experiment, this hypocalcemic protein was found to bear -Thr-Val-Leu in carboxyl-terminal area and liberated in turn leucine, valine, and threonine in yields of 0.63, 0.29, and 0.25 mol/mol protein, respectively, by the digestion with carboxypeptidase A at 25° for 4 hr. From this fact, at least leucine residue of the carboxyl-terminus dose not take part in appearance of the hypocalcemic activity.

Conclusion

From the experimental described above, tryptophanyl, tyrosyl, free amino, disulfide, and histidyl residues may play a role in maintaining the hypocalcemic activity through affecting directly the active site of the protein or indirectly keeping its conformation in an active state, and Met residue, whose treatment even under the denaturation with urea was ineffective, and at least leucine residue of the carboxyl-terminus are considered to be not essential for the hypocalcemic activity.

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