Contribution of Interstitial Diffusion in Drug Absorption from Perfused Rabbit Muscle: Effect of Hyaluronidase on Absorption

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[^{3}H]Water and [^{14}C]inulin were injected into perfused rabbit muscle with or without hyaluronidase (300 units/ml) and their absorption into venous effluent from muscle was determined. Hyaluronidase accelerated the absorption of both compounds but the enhancement of [^{14}C]inulin was much larger than that for [^{3}H]water. The pharmacokinetic analysis of venous appearance curves based on a physiological diffusion model elucidated that interstitial diffusion of [^{14}C]inulin was remarkably increased by hyaluronidase treatment, suggesting the existence of steric hindrance for it by the polysaccharide network under normal conditions. Enhancement of [^{3}H]water diffusion was also detected although enhancement ratio was about one-half of that of [^{14}C]inulin. Mean time necessary for each process was calculated using the statistical moment concepts. The results suggested predominant contribution of the interstitial diffusion process and secondary and little contribution of local perfusion flow and permeation process across the capillary wall, respectively, in total absorption of [^{14}C]inulin. Effect of hyaluronidase on transcapillary movement of [^{14}C]inulin was studied using an *in vitro* diffusion experiment with cultured endothelial cell monolayer and no enhancing effect was shown on [^{14}C]inulin transport across the cell monolayer. The contribution of the local perfusion flow, on the other hand, was shown to be almost equivalent to that of the diffusion process in the total absorption of [^{3}H]water.

Keywords intramuscular injection; absorption; statistical moment; physiological diffusion model; hyaluronidase; interstitial diffusion; rabbit hind leg perfusion

Drug absorption after intramuscular (i.m.) injection is thought to be regulated by many physiological variables such as interstitial diffusivity, capillary permeability and local blood flow. However, the quantitative contribution of each process in the total absorption is not well understood.

We developed a new experimental method using a local perfusion system, in which the absorption behavior of injected drugs could be directly estimated from their appearance in the venous effluent.[1] Physiological variables among water soluble drugs of various molecular sizes could be estimated by the pharmacokinetic analysis of venous appearance curves based on a physiological diffusion model.[2] The results suggested that diffusion of [^{14}C]inulin in the interstitial space was depressed by the steric interaction with the interstitial polysaccharide network.[2]

In this study, [^{3}H]water and [^{14}C]inulin were injected with hyaluronidase (300 units/ml), which destroys the structure of a polysaccharide network in the interstitial space, and the effect of hyaluronidase treatment was analyzed using a physiological diffusion model. In addition, the effect of hyaluronidase on the transcapillary movement process was evaluated with the use of the cultured endothelial cell monolayer system. From the results, quantitative contributions of physiological variables in total absorption are elucidated based on statistical moment concepts.

Experimental

Animals Male rabbits (1.8—2.1 kg) with free access to a commercial diet and water were used.

Chemicals [^{3}H]Water and [methoxy-^{14}C]inulin were purchased from New England Nuclear, U.S.A. Bovine serum albumin (BSA, fraction V) and all other chemicals of reagent grade were obtained commercially from Nacalai Tesque, Japan. Bovine testis hyaluronidase (510 NF units/mg) was purchased from Sigma Co., U.S.A. and used without further purification.

Procedure of Absorption Experiment from Perfused Rabbit Muscle An absorption experiment was performed as described previously.[1] The right legs of anesthetized rabbits were perfused at 1.7 ml/min. The perfusion medium was Tyrode's solution with BSA (5% w/v) oxygenated with 95% O_2–5% CO_2 to pH 7.4 at 37 °C. After the animal was given a lethal i.v. injection of pentobarbital, a solution (100 μl) containing radiolabeled compounds with hyaluronidase (300 units/ml) was injected over 40 s into the center of *musclus gastrocnemius* at a depth of 8 mm. This injection solution was prepared with phosphate buffer solution (pH 7.4) and solid hyaluronidase was freshly dissolved each experimental day. Twenty seconds after injection sampling began and the venous effluent was collected for 120 min. The injection point was covered with surgical adhesive to prevent fluid leakage. The radioactivities of ^{3}H and ^{14}C in the supernatant were measured with a liquid scintillation counter (LSC-900, Aloka Co., Japan) after centrifugation of the outflow effluent (3000 rpm × 5 min).

Calculation of Moment Parameters for Venous Appearance Curves after i.m. Injection Venous appearance curves of injected compounds were analyzed based on statistical moment theory to evaluate overall absorption. The fraction absorbed (F_a), the mean arrival time to venous sampling point ($\bar{t}_a$) and variance of arrival time (σ_a^2) were defined as follows[1]:

$$F_a = \int_0^\infty J\mathrm{d}t \tag{1}$$

$$\bar{t}_a = \int_0^\infty tJ\mathrm{d}t/F_a \tag{2}$$

$$\sigma_a^2 = \int_0^\infty (t-\bar{t}_a)^2 J\mathrm{d}t/F_a \tag{3}$$

where t is the sampling time and J is the absorption rate of each compound normalized by the injected dose, *i.e.*, as a percentage of the dose per minute. Moment parameters were calculated by a numerical integration using a linear trapezoidal formula and extrapolation to infinite time based on a monoexponential equation. The $\bar{t}_a$ and σ_a^2 values presented here were not corrected for the lag time of the catheter or its variation. The results were statistically analyzed by Student's t-test. Differences with a p value of less than 0.05 were considered significant.

Analysis of Venous Appearance Curves Based on a Physiological Diffusion Model The effect of hyaluronidase on absorption of [^{3}H]water and [^{14}C]inulin was analyzed using the physiological diffusion model shown in Fig. 1.[2] The Laplace transform of the equations for [^{3}H]water and [^{14}C]inulin absorption rates was derived as follows[2]:

$$\bar{J}_a\,([^3\mathrm{H}]\text{water}) = \frac{k_q r_p X_0}{aq(s) + sV_d(r_i aq(s))^{1/2}/da} \tag{4}$$

$$\bar{J}_a\,([^{14}\mathrm{C}]\text{inulin}) = \frac{k_m k_q da X_0}{sV_d((s+k_p+k_q)bq(s))^{1/2} + dabq(s)} \tag{5}$$

where s is the Laplace variables with respect to time and r_p and r_i are the volumes of the vascular and interstitial spaces, respectively, as fractions

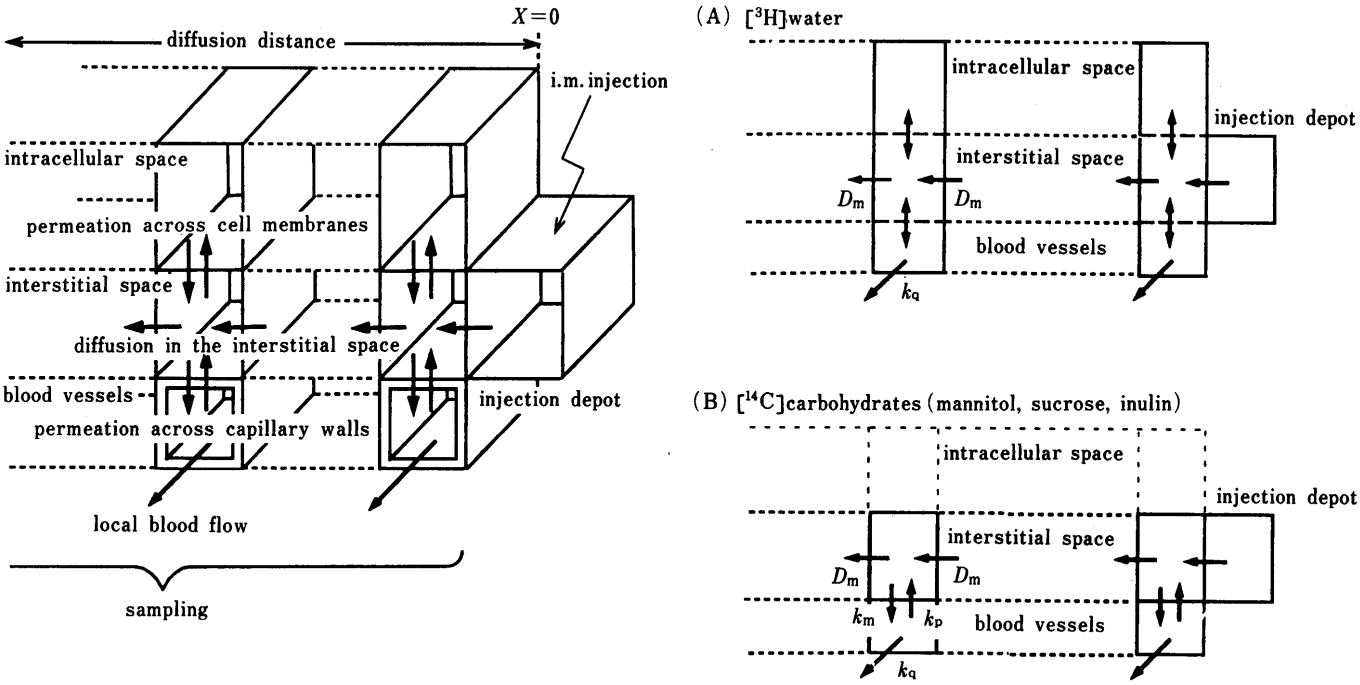

Fig. 1. A Physiological Diffusion Model Used for Analysis of the Venous Appearance Curve of [^{3}H]Water (A) and [^{14}C]Inulin (B)

D_m: Apparent interstitial diffusion coefficient, k_m: permeation rate constant from interstitial side, k_p: permeation rate constant from vascular side, k_q: perfusion rate constant.

of total water space.[2] k_q, k_p and k_m are the perfusion rate constant (local perfusion rate per unit vascular volume), permeation rate constant at the capillary wall from the vascular and interstitial sides, respectively. da (diffusion parameter), $aq(s)$, and $bq(s)$ are defined, respectively, as follows:

$$da = D_m^{1/2} A \tag{6}$$

$$aq(s) = s + r_p k_q \tag{7}$$

$$bq(s) = s^2 + (k_m + k_p + k_q)s + k_m k_q \tag{8}$$

where D_m and A are the apparent diffusion coefficient in the interstitial space and apparent diffusion area, respectively. The r_p and r_i values were previously estimated to be 0.0486 and 0.2454, respectively.[2] k_q value was also previously estimated to be 1.02 (1/min) and local perfusion rate (Q_p) was calculated to be 3.77 (ml/min/100 g muscle) from k_p and the volume of vascular space (V_p: 3.69 ml/100 g muscle).[2] Since [^{14}C]inulin permeates the capillary wall by passive diffusion, the following relation is valid and vascular permeation clearance (CL_p) is derived from k_p and V_p as follows:

$$k_p = (r_i/r_p)k_m \tag{9}$$

$$CL_p = k_p V_p \tag{10}$$

Eqs. 4 and 5 in which the estimated values had been substituted for r_p, r_i and k_q, were fitted to the mean venous appearance curves of [^{3}H]water and [^{14}C]inulin, respectively, to obtain the da and k_m values. Curve-fitting was performed with MULTI (FILT), a nonlinear regression program combined with a fast inverse Laplace transform (FILT) algorithm.[3] This computer program is written in FORTRAN77 and developed on the main frame computer M-382 of Kyoto University Data Processing Center.

Calculation of First Moment for Each Absorption Process Mean arrival time ($\bar{t}_a$) was also calculated with the values of parameters obtained from model analysis.[2] The quantitative contribution of each physiological variable in overall absorption is discussed using this parameter. The $\bar{t}_a$ values for injected [^{3}H]water and [^{14}C]inulin were calculated, respectively, as follows:

$$\bar{t}_a\ ([^3\mathrm{H}]\text{water}) = \frac{1}{r_p k_q} + \left(\frac{r_i}{r_p k_q}\right)^{1/2} \frac{V_d}{da} \tag{11}$$

$$\bar{t}_a\ ([^{14}\mathrm{C}]\text{inulin}) = \frac{1}{k_q} + \frac{1}{k_m} + \frac{k_p}{k_m k_q} + \frac{V_d}{da}\left(\frac{k_p}{k_m k_q} + \frac{1}{k_m}\right)^{1/2} \tag{12}$$

Transport Experiment with an Endothelial Cell Monolayer An *in vitro* transport experiment was performed as reported previously.[4] Endothelial cells isolated from bovine aorta were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (Gibco Laboratories, U.S.A.) at 37 °C in a 95% air–5% CO_2 humidified atmosphere. Endothelial cells from 2 to 10 passages were subcultured with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in Hank's balanced salt solution (HBSS) and seeded on fibronectin coated (10 μg/cm^2) microporous polycarbonate membranes (3.0 μm pore size, 6.5 mm diameter) fixed to cylinders (Transwells, Coaster, U.S.A.). The endothelial cells were seeded at 7.0×10^3 cells/cm^2 and culture medium was changed every other day until the confluent cell monolayer was formed (7 d after seeding). Transport experiments of [^{14}C]inulin were performed at 37 °C for 2 h. Hyaluronidase was added to the abluminal side at the concentration of 300 units/ml. At each sampling period, 10 μl of sample was removed from the abluminal side and an equal volume of HBSS buffer was immediately added to it. The fibronectin coated polycarbonate membrane without cell monolayer was used for measurement of the membrane permeability. ^{14}C-Radioactivity in each sample was measured in a liquid scintillation counter (LSC-900, Aloka Co., Japan).

Calculation of the Permeability Coefficient across the Cell Monolayer If transendothelial movement of [^{14}C]inulin occurs by diffusion, its concentration in abluminal compartment (C_a) is written as follows[4]:

$$C_a = X_0 \times V_a/(V_1 + V_a) \times [1 - \exp\{-PA(1/V_a + 1/V_1)t\}] \tag{13}$$

where V_a and V_1 are the volume of abluminal compartment (0.7 ml) and luminal compartment (0.13 ml), respectively; t is time (min), P is the permeability coefficient, A is the surface area of membrane (0.33 cm^2), and X_0 is the applied dose. Eq. 13 was fitted to the penetration profiles using a nonlinear least-squares computer program MULTI and the P values were obtained. The permeability coefficient of the cell monolayer alone (P_c) was corrected from the following relationship:

$$1/P_c = 1/P_{m+c} - 1/P_m \tag{14}$$

where P_m and P_{m+c} are the permeability coefficient of polycarbonate membrane alone and polycarbonate membrane plus cell monolayer, respectively.

Results and Discussion

Figure 2 shows the venous appearance curves of [^{3}H]water and [^{14}C]inulin after i.m. injection with or without hyaluronidase (300 units/ml). Hyaluronidase enhanced the [^{14}C]inulin absorption from muscle and caused a sharp peak in the venous appearance curve. Hyalu-

ronidase also increased the maximum absorption rate of [^{3}H]water. However, the extent of enhancement was much larger in [^{14}C]inulin than in [^{3}H]water and mean arrival time value ($\bar{t}_a$) for ^{14}C-inulin became about one-third of control condition while that for [^{3}H]water was not diminished as much (Table I). This molecular size dependent enhancement of drug absorption by hyaluronidase is in good agreement with the results observed in previous studies.[5] Fraction absorbed (F_a) of [^{3}H]water and [^{14}C]-inulin was not significantly influenced by the addition of hyaluronidase and both compounds were also completely absorbed from muscle (Table I).

The basic structure of the interstitial space is considered to be a polysaccharide network composed of hyaluronate and proteoglycans dispersed in the collagen and elastic fibers.[6] The molecular size-dependent effect of hyaluronidase has been explained as being caused by the steric hindrance due to the interaction between the test molecules and interstitial network formation, although no direct evidence was shown.[7] In a previous paper, we analyzed absorption processes of water soluble drugs of various molecular sizes using a physiological diffusion model.[2] It was found that interstitial diffusion of large molecules such as [^{14}C]inulin was somewhat restricted due to steric hindrance by interstitial polysaccharide network formation. On the other hand, diffusion of small molecules such as [^{3}H]water and [^{14}C]sucrose was not greatly much depressed by the steric interaction.

The pharmacokinetic parameters obtained by computer fitting of Eqs. 4 and 5 to the venous appearance curves are summarized in Table II. Hyaluronidase treatment extremely increased the *da* value of [^{14}C]inulin; however, the enhancing extent (*da* value ratio) for [^{3}H]water was a good deal less than that for [^{14}C]inulin. These results clearly demonstrate that hyaluronidase accelerates the diffusivity of [^{14}C]inulin by destroying the polysaccharide matrix which restricts its interstitial diffusion and enhances its absorption from muscle.

In Fig. 2, the fitting-line of [^{14}C]inulin deviates from experimental data suggesting some limitations in the application of the physiological pharmacokinetic model (Fig. 1) to the present case: in this analysis, interstitial diffusion coefficients of test compounds were assumed to also be constant in the case of hyaluronidase treatment. Actually, however, there should be a gradual fall in enhancement of interstitial diffusivity of drugs with diffusion distance due to the concentration gradient of hyaluronidase. A more complex model considering a declining or uneven hyaluronidase effect gave better fitting curves to venous appearance of both [^{3}H]water and [^{14}C]inulin, suggesting that interstitial diffusion is much enhanced by hyaluronidase at a point closer to the injection site (data not shown). However, this model analysis requires more assumptions

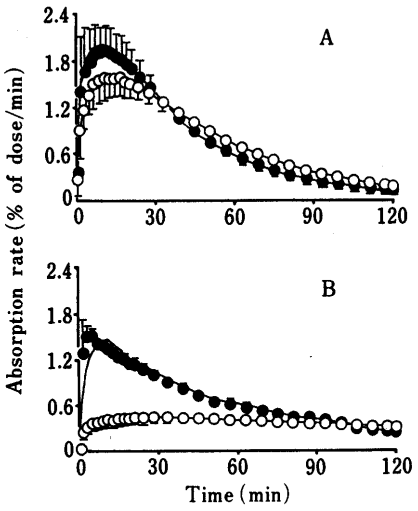

Fig. 2. Mean Venous Appearance Curves of [^{3}H]Water (A) and [^{14}C]Inulin (B) with (●) or without (○) Hyaluronidase (300 Units/ml)

The results are expressed as the means of at least three experiments and the vertical bars indicate standard deviations. The solid lines were calculated using the parameters listed in Table II.

TABLE I. Effect of Hyaluronidase (300 Units/ml) on Absorption of [^{3}H]Water and [^{14}C]Inulin from Rabbit Perfused Muscle[a]

Compound	Treatment	F_a (% of dose)	$\bar{t}_a$ (min)	σ_a^2 (min^2)
[^{3}H]Water	Control[b]	103.5± 4.5	49.2± 5.1	1819± 398
	Hyaluronidase	100.1± 2.6	41.8±10.2	1501± 777
[^{14}C]Inulin	Control[b]	112.0±21.4	215.1±57.7	45270±21263
	Hyaluronidase	94.7±11.3	66.9± 3.1[c]	4685± 531[c]

a) Values are mean±S.D. *b*) From ref. 2. *c*) Significantly different from control at $p<0.01$.

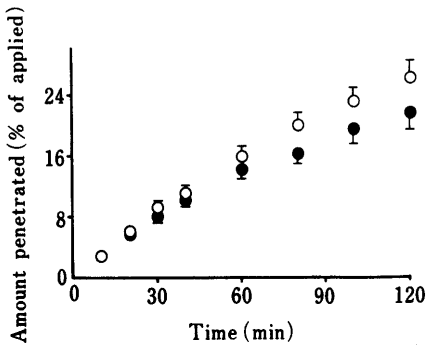

Fig. 3. Transport Profiles of [^{14}C]Inulin across the Endothelial Cell Monolayer with (●) or without (○) Hyaluronidase (300 Units/ml)

The results are expressed as the means of six experiments and the vertical bars indicate standard deviations.

TABLE II. Estimated Pharmacokinetic Parameters for the Absorption of i.m. Injected [^{3}H]Water and [^{14}C]Inulin with or without Hyaluronidase

Compound	Treatment	*da* (ml/min$^{1/2}$)	k_q (min^{-1})	Q_p[a] (ml/min/100 g)	k_m (min^{-1})	k_p (min^{-1})	CL_p[a] (ml/min/100 g)
[^{3}H]Water	Control[b]	0.00804	1.02	3.77	—	—	—
	Hyaluronidase	0.0152	(1.02)	(3.77)	—	—	—
[^{14}C]Inulin	Control[b]	0.00121	(1.02)	(3.77)	1.46	7.37	27.2
	Hyaluronidase	0.00424	(1.02)	(3.77)	1.48	7.48	27.6

a) Values estimated assuming perfused muscle weight was 51.4 g. *b*) From ref. 2.

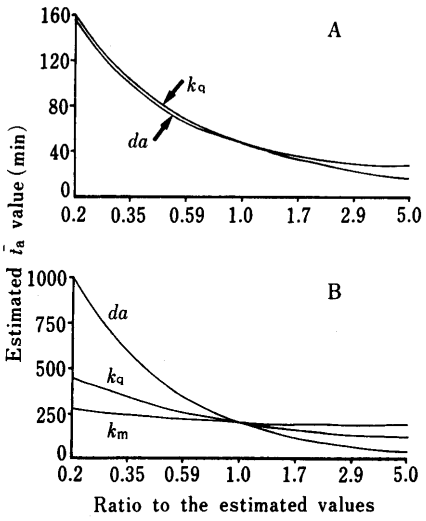

Fig. 4. Simulated Effects of Changes in da, k_m, and k_q Ratio to the Estimated Values under Normal Conditions on Mean Arrival Time ($\bar{t}_a$) for [^{3}H]Water (A) and [^{14}C]Inulin (B)

concerning gradient of the hyaluronidase effect, and increased numbers of unknown parameters would pose difficulties in estimating the effect of hyaluronidase on pharmacokinetic parameters relating to drug absorption. In addition, the time-dependency involved in the hyaluronidase effect remains beyond discussion. In this study, therefore, we applied the model shown in Fig. 1 to the analysis of drug absorption injected with hyaluronidase. Obtained parameters were compared with those of control and the enhancement of the interstitial diffusion was concluded to be the action of the hyaluronidase mechanism.

Concerning other actions of this mechanism, no concrete experimental results have been presented.[7] The increase in capillary permeability has been considered to be a possible mechanism of hyaluronidase.[7] Pharmacokinetic analysis based on a physiological diffusion model demonstrated that hyaluronidase treatment has little influence on capillary permeability and that increase in this permeability is not a mechanism of the enhancement. Figure 3 shows the transport of [^{14}C]inulin across endothelial cell monolayer with or without hyaluronidase (300 units/ml). Permeability coefficients (P_c) of [^{14}C]inulin with or without hyaluronidase were calculated to be 0.117 and 0.084 (cm/h), respectively, and the transport of [^{14}C]inulin across the endothelial cell monolayer was not increased by hyaluronidase treatment in this experimental system. In this monolayer system, however, junctions between endothelial cells are not considered to be as tight as in the *in vivo* system and the effect of hyaluronidase on drug transport across endothelial cells may be underestimated. The increase in lymph flow (convection flow) from the injection site also has been considered to be a possible mechanism of hyaluronidase.[7] However, not only [^{3}H]water but also [^{14}C]inulin was completely recovered in the venous effluent, suggesting that compounds in this size area were absorbed though the blood vessels.

In Fig. 4, the quantitative contributions of physiological variables (interstitial diffusivity, permeability across the capillary wall and local perfusion flow) in the total absorption are simulated based on statistical moment concepts. Pharmacokinetic parameters were altered from one-fifth to five times the values estimated under control condition of the present experiment. For [^{14}C]inulin, the contribution of the diffusion process in the interstitial space was the largest in the three variables and this interstitial diffusion mainly regulated absorption of [^{14}C]inulin from muscle (Fig. 4). Local perfusion rate also influenced the absorption to some extent, but the effect of the permeation process on the total absorption of [^{14}C]inulin was negligible. Hyaluronidase injected with [^{14}C]inulin shortens the time spent for the rate-limiting step of absorption, and accelerates [^{14}C]inulin absorption after i.m. injection. On the other hand, the contribution of local perfusion flow and interstitial diffusion were equivalent in [^{3}H]water absorption, and both interstitial diffusivity and local blood flow are determinant factors of absorption.

In summary, quantitative contributions of physiological variables in the total absorption were studied and it was demonstrated that the absorption from muscle was mainly determined by the diffusion in the interstitial space. As the size of drug molecules becomes larger, the diffusivity is more depressed due to the steric resistivity in the interstitial space, and hyaluronidase increases interstitial diffusion of the drug by destroying the polysaccharide matrix. Coadministration of drugs with hyaluronidase accelerates the rate-limiting step of absorption and enhances drug absorption from muscle.

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