THE MEASUREMENT OF LYSOZYME ACTIVITY AND THE ULTRA-VIOLET INACTIVATION OF LYSOZYME

by

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A relatively simple and rapid method for the measurement of lysozyme activity, capable of good accuracy and reproducibility, is described and applied to the study of the ultra-violet inactivation of this enzyme.

The method of activity measurement is based on the continuous reading of the change in turbidity of a bacterial suspension, at neutral or alkaline pH, in a spectrophotometer; and is dependent for its accuracy principally on good temperature control.

In view of the known variation of quantum yield, for the ultra-violet inactivation of enzymes, with molecular weight[1], it appeared to be of value to compare the behaviour of two enzymes of similar molecular weights, such as lysozyme and ribonuclease, the molecular weights of which are, respectively, 14,700 and 15,000. The studies on ribonuclease are under way and will be reported in a later communication.

Additional interest in the study of quantum yields for low-molecular weight enzymes is furnished by the finding of MANDL *et al.*[2] that the quantum yield for photolysis of peptide bonds in solution is of the order of 10^{-3}, which corresponds with the inactivation yields obtained for high molecular weight enzymes, but is a little more difficult to reconcile with the quantum yields of the order of 10^{-2} reported for several enzymes of low molecular weights[1].

EXPERIMENTAL

Preparation of lysozyme

The enzyme was prepared from egg-white according to the method of ALDERTON AND FEVOLD[3] and recrystallized three or four times as the isoelectric or carbonate form. Contrary to the experience of these and later authors[4] it was found that, in the course of three separate preparations, initial crystallization occurred spontaneously, although it took somewhat longer to start than when seeding was used. In one preparation the enzyme first settled out as an amorphous precipitate which, after about 36 hours, was transformed into crystals.

Contrary to information given in the literature, the enzyme was found to be quite stable even in alkaline solution. In fact, exposure to pH 12 in NaOH medium appeared to have no deleterious effect on the activity, at least over a period of many hours. Dialysis at room temperature for several days resulted in no drop in specific activity. Curiously, however, the enzyme passes quite readily through cellophane membranes; in one experiment an enzyme solution containing 1.2 mg/ml dropped to 0.4 mg/ml following three days dialysis. This phenomenon has also been observed by FRAENKEL-CONRAT[5] who ascribes it to the basic nature of the enzyme.

Method of Irradiation

The light source used was a Thermal Syndicates Ltd., low-pressure mercury resonance lamp, shaped in the form of a U-tube. About 95% of the emission of this lamp is concentrated at 2537 A.

The enzyme was irradiated in the 5 mm quarts cells of the Beckman spectrophotometer. The

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cell was supported in a metal jig about 5 cm from the lamp with its face parallel to one of the arms of the U. Although the radiation emitted by the lamp below 2537 A is rated as negligible, an acetic acid filter was used in front of the irradiated cell. The light intensity incident on the irradiated solution was measured actinometrically[6]. Determinations made at different times gave results agreeing to within 5% of an intensity of $1.25 \cdot 10^{17}$ quanta/cm²/min.

Absorption Spectrum of Lysozyme

Fig. 1 shows the absorption spectrum of isoelectric lysozyme in $M/15$ phosphate buffer, pH 7.1. For lysozyme carbonate, the concentration of which was adjusted to give the same optical density at 2820 A, the curve coincides with that for the isoelectric enzyme above 2820 A. Below 2820 A the carbonate curve is about 2% below that of the isoelectric enzyme. The ratio of the optical density at the maximum, 2800 A, to the minimum, 2500 A, for isoelectric lysozyme is 2.16; for the carbonate it is 2.21. KERTESZ[7] has published an absorption spectrum for lysozyme for which this ratio is 2.36, while another publication[8] shows a similar curve with the ratio equal to 1.96. It has been the author's experience that this ratio is of some value in the comparison of protein spectra of different authors[9] and that it may be linked to differences in composition of different preparations.

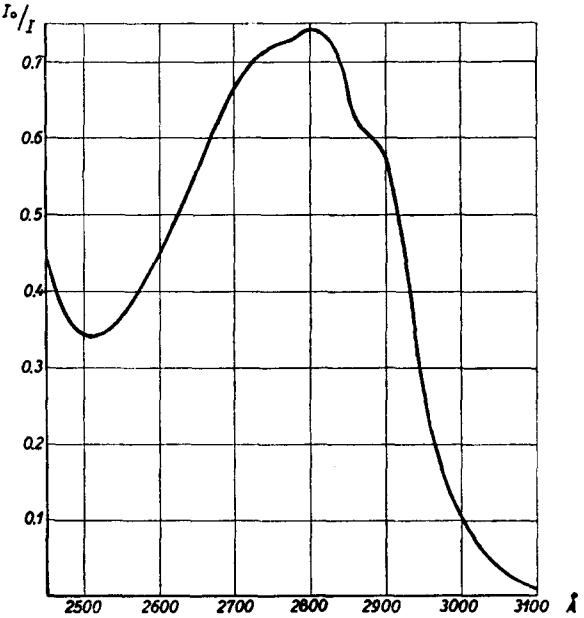

Fig. 1. Absorption curve of isoelectric lysozyme, 0.337 mg/ml in $M/15$ phosphate buffer pH 7.1

Enzyme concentrations were measured spectrophotometrically by relating the height of the absorption maximum at 2800 A to the nitrogen content measured by the Kjeldahl method. For the isoelectric preparation, the nitrogen content of which is 18.6%[10], the concentration is related to the optical density at 2800 A by the relation

Conc. (mg/ml) = 0.473 · (optical density)

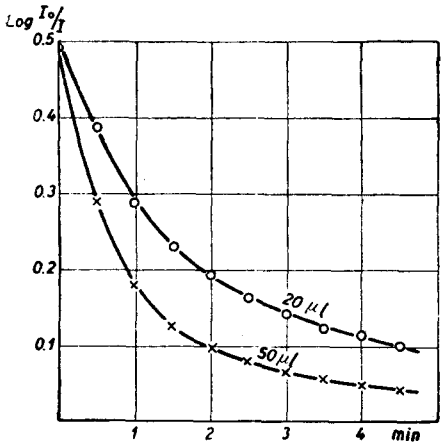

Fig. 2. Decrease with time of optical density of suspensions of *Micrococcus Lysodeicticus*, following addition of 20 µl and 50 µl of a 0.33 mg/ml solution of lysozyme carbonate to a 3 ml suspension of bacteria at pH 7.1 in $M/15$ phosphate buffer

Measurement of enzyme activity

Micrococcus lysodeicticus, a strain of which was kindly made available by Dr J. WIAME, was grown on agar slants for 48 hours, using the medium recommended by MEYER AND HAHNEL[11]. The bacteria were washed off the agar with $M/15$ phosphate buffer pH 7.1, filtered through glass wool and diluted to give an optical density of from 0.5 to 0.75 in the 10-mm corex cells of the Beckman spectrophotometer at 4500 A, measured against a blank cell containing distilled water. A volume of 3 ml was used for activity measurements.

A volume of from 20 to 50 µl of enzyme solution was then quickly added to the cell, using a constriction pipette, and the contents rapidly mixed by mouth with a glass syringe. Optical densities were then read at 30 second intervals. Fig. 2 shows the course of the reaction for 4½ minutes for two different enzyme concentrations.

In a given experiment a suspension of enzyme crystals was centrifuged down at 10,000 r.p.m. in the Multispeed Head of the International Centrifuge, the supernatant poured off, and the pellet of crystals dissolved in the appropriate buffer medium to desired concentration for irradiation.

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Aliquots were removed and diluted to different concentrations, which were then used to make up a calibration curve of effective enzyme concentration *vs.* decrease in optical density of the bacterial suspension after 1 minute. Fig. 3a shows a typical series of such reactions and Fig. 3b the corresponding calibration curve. The enzyme sample was then irradiated and aliquots removed for activity measurements at given time intervals.

A new calibration curve was always prepared along with each series of experiments, thus eliminating possible variations in the different preparations of bacteria and the optical density of the suspension used. It was easily verified that the use of bacterial suspensions of different optical densities, along with their corresponding calibration curves, gave results in good agreement with each other.

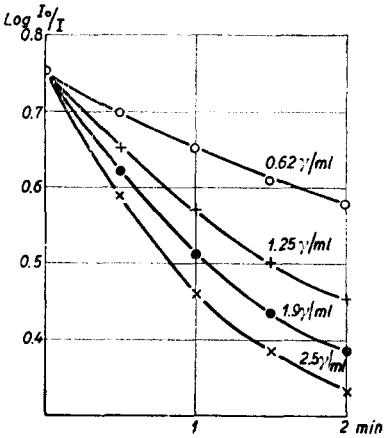

Fig. 3a. Rate of lysis of *Micrococcus Lysodeicticus* as a function of lysozyme concentration, in *M*/15 phosphate buffer, pH 7.1 at 20° C

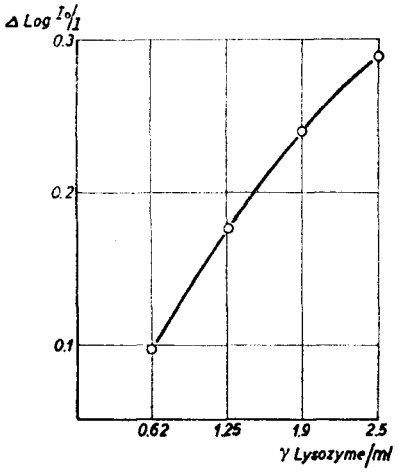

Fig. 3b. Calibration curve of lysozyme concentration *vs.* decrease in optical density, produced during first minute of reaction, of suspension of bacteria of initial optical density 0.75 at 4500 A, in *M*/15 phosphate buffer, pH 7.1 at 20° C

It is apparent that the above method must be modified if it is desired to study the reaction in acid media[12]. For this purpose it is preferable to reduce the enzyme concentration, in ordents to reduce the velocity of the reaction, and then to add 50 or 100 μl of 5 *N* NaOH after a time interval suitably selected. In these circumstances it is, of course, not possible to follow the reaction continuously.

The principal problem to be overcome is that of temperature control. No kinetic studies have as yet been made*. However a few rough observations indicated a temperature coefficient (Q_{10}) greater than 3, so that a difference of temperature of 0.2° C would correspond to an error in the measured activity of 3% or more. To resolve this problem the cell compartment of the spectrophotometer was regulated to room temperature by means of a thermostatic bath which circulated water through the auxiliary blocks supplied with the instrument, and a sheet of thick asbestos was placed between the hydrogen lamp and the cell compartment to reduce conduction of heat from the lamp to the latter. In addition the reaction cell was placed in the compartment several moments before the start of the reaction. The use of these precautions made possible the duplication of control activities to 2 or 3%, which is practically the limiting accuracy dictated by the necessity of taking a density reading and a stop-watch reading simultaneously.

RESULTS

Fig. 4 shows two typical inactivation curves, plotted as first order reactions, $\ln \frac{C_0}{C}$ being plotted *vs.* time of irradiation, where C_0 represents the initial concentration of

* Fig. 3b shows, however, that the rate of reaction is nearly proportional to enzyme concentration over a four-fold range of the latter, while from Figs 2 and 3a it is readily shown that the reaction is first-order for at least 60% of its course.

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active enzyme and C the concentration at time t. Curve a is that for 0.25 mg/ml enzyme in $M/15$ phosphate buffer pH 7.1. Curve b, for the same enzyme concentration in 0.01 N NaOH is of particular interest; it appears at first sight as though the rate of inactivation is faster in this case. In reality, however, it is due to the increased absorption of the enzyme at this p_H, about 12[17]. The last row in Table I shows that the resulting quantum yield is unaltered.

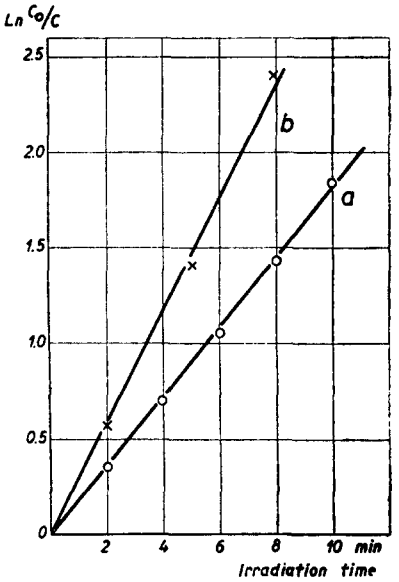

Fig. 4. Ultra-violet inactivation of lysozyme plotted as a first order reaction (a) 0.25 mg/ml in $M/15$ phosphate pH 7.1 (b) 0.25 mg/ml in 0.01 N NaOH

Table I lists the relevant data, including the inactivation rate constants, k_1, and the calculated quantum yields, ø, under various conditions of irradiation. The irradiation cell was in all cases a 5-mm Beckman cell, and the intensity of light incident on the solution was $I = 1.25 \cdot 10^{17}$ quanta/cm²/min.

The quantum yield

$$ø = \frac{\text{number of molecules inactivated}}{\text{number of quanta absorbed}}$$

is calculated from the formula $ø = \frac{C_o k_a}{I_1}$, which takes into account the decrease in absorption of light by active molecules during the course of the reaction[13,14]. C_o is expressed in molecules/cm² of light path, using 14,700 as the molecular weight of lysozyme[10]; k_1 in min^{-1}; while $I_a = I \cdot$(% light absorption of non-irradiated solution).

In several instances the values given represent the mean of several measurements. For example, the value for p_H 7.1 at a concentration of 0.25 mg/ml is the mean of three sets of determinations conducted at different times, each utilizing a new calibration curve for enzyme activity.

TABLE I

INACTIVATION RATE CONSTANTS, k_1, AND QUANTUM YIELDS, ø, FOR ULTRA-VIOLET INACTIVATION OF LYSOZYME

(For further details see text under heading "Results")

Preparation	Concentration (mg/ml)	pH	Buffer	Absorption (%)	k_1 (min^{-1})	$ø = \frac{C_o k_1}{I_a}$
Isoelectric	0.25	3.6	$M/10$ acetate	25.8	0.147	0.023
Isoelectric	0.25	4.6	$M/15$ phosphate	25.8	0.157	0.025
Isoelectric	0.25	7.1	$M/15$ phosphate	25.8	0.160	0.025_5
Isoelectric	0.25	7.1	$M/15$ phosphate + $3 \cdot 10^{-3}$ M cysteine	25.8	0.155	0.024_6
Isoelectric	0.25	7.1	$M/15$ phosphate + $8 \cdot 10^{-3}$ M arginine	25.8	0.140	0.022
Isoelectric	0.125	7.1	$M/15$ phosphate	14.0	0.150	0.022
Isoelectric	0.50	7.1	$M/15$ phosphate	43.8	0.090	0.017
Carbonate	0.235	7.1	$M/15$ phosphate	25.8	0.155	0.023_3
Isoelectric	0.25	9.0	$M/15$ phosphate	26.5	0.157	0.024_3
Isoelectric	0.25	12.0	00.1 N NaOH	47.0	0.293	0.025_3

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In view of the previous finding of a pronounced protecting effect of cysteine against ultra-violet inactivation of triosephosphate dehydrogenase[14], an -SH enzyme, an attempt was made to see whether a similar behaviour manifests itself in the case of lysozyme, which contains -SS-groups. Cysteine, however, had no influence on the activity or the rate of inactivation of the enzyme, as can be seen from Table I. Arginine, which was tried in view of the high content of guanidyl groups in lysozyme, did show a slight protecting effect.

Fig. 5 shows the change in absorption resulting from irradiation of a solution of lysozyme carbonate at a concentration of 0.235 mg/ml in $M/15$ phosphate buffer at pH 7.1.Measure ments on the absorption spectrum were made directly on the solution in the 5-mm cell. Note how slowly the maximum rises initially. During the first 6 minutes' irradiation, corresponding to an inactivation of about 55%, the maximum shows no change at all. Following the 15-minute irradiation period, the absorption curve continued to rise, in the absence of the irradiation source, at a rate too fast to record the entire spectrum. About 17 minutes later a faint turbidity was visible which continued to increase with time. At pH 12 in 0.01 N NaOH, the rate of increase in enzyme absorption with irradiation time paralleled the above results.

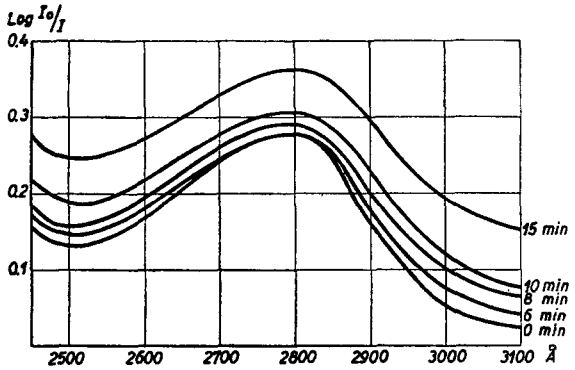

Fig. 5. Change in absorption spectrum of lysozyme carbonate, 0.235 mg/ml in $M/15$ phosphate pH 7.1, as a function of time of irradiation

These results show no apparent correlation with destruction of enzyme activity. The phenomenon is believed to be due to photo-oxidation[15]. A few qualitative measurements were made of the tryptophan content of the irradiated and non-irradiated samples of enzyme, using BRICE's modification of the glyoxylic acid method[16]; it was in fact found that the tryptophan content decreased, and that the response for tryptophan was practically nil following 15 minutes irradiation, which corresponds to nearly complete inactivation.

Studies on other enzymes and proteins have shown that irradiation in some cases results in splitting of the molecules into smaller fragments[15]. This phenomenon does not, however, appear to be general. An attempt was made to see whether splitting off of any amino acids or peptides resulted from irradiation, using paper chromatography. Ascending chromatograms were run in two different solvents, water-saturated butanol and water-saturated phenol. About 100 γ protein was deposited on each spot, and a number of amino acids run alongside as controls. Colour development was by means of ninhydrin, and 0.5 γ of tyrosine and tryptophan were easily detected. Although enzyme samples were used which had been irradiated for periods beyond that corresponding to complete inactivation, no sign of any free amino acid or peptide could be detected.

DISCUSSION

Excluding the result obtained for an enzyme concentration of 0.5 mg/ml, the

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average value for the quantum yield is 0.024 ± 0.01. What is most striking about this value is its apparent independence with respect to pH, a behaviour markedly different from that observed for other enzymes; and rendered even more obvious by the fact that at pH 12, where the absorption spectrum of the enzyme is markedly altered[17], the inactivation rate increases correspondingly to give the same ø.

A measured value of ø exists for ribonuclease, molecular weight 15,000, based on unpublished observations[15]. The figure is 0.026 at a pH of 4.1. Considering the marked differences in amino acid composition of the two enzymes[10, 18], the concordance between the yields for two enzymes of the same molecular weight is what one would expect on the basis of MIRSKY AND PAULING's theory of inactivation as being due to rupture of a peptide bond[19]*.

That rupture of peptide linkages does take place on irradiation was long ago established by MITCHELL AND RIDEAL[20]. That this is the only mechanism operative cannot, however, be said to be established. The experiments of MITCHELL AND RIDEAL involved the use of surface films, which by no means corresponds to the conditions existing in solution. MCLEAN AND GIESE[21] have recently attempted to place in evidence the amino and carboxyl groups which would be expected to appear following the irradiation of egg albumin, on the assumption of the rupture of peptide bonds. No significant change was noted following irradiation. It is, of course, not unlikely that the effect sought for was obscured by secondary reactions, such as those illustrated above by the photo-oxidation of tryptophan and the continuing increase of absorption, leading finally to coagulation, even following removal of the irradiating source. As a matter of fact ROBERTS[22] has shown, in the case of serum albumin, that secondary reactions involve carboxyl as well as other groups.

The decrease of ø with increasing molecular weight would certainly have to be explained by any general hypothesis of the inactivation process. The existence of an intramolecular Franck-Rabinowitch effect, becoming increasingly important with increasing molecular weight, may offer such an explanation, were it not for the low quantum yields found by MANDL *et al.*[2] for the rupture of peptide bonds (see introductory paragraphs above).

An attempt has been made to find a relationship between quantum yields and molecular weights for enzyme inactivation[1]. Undoubtedly the availability of more data obtained under well-defined conditions is a prerequisite for further work along this line. In some cases, *e.g.*, triosephosphate dehydrogenase[14], disagreement still exists as to the molecular weight. For a number of enzymes the effect of pH, concentration, etc. has not been sufficiently well studied. And in the only known instance where measurements have been made on the same enzyme by two different observers, the enzyme urease, the results are most discordant[14, 15].

The effect of enzyme concentration has been previously discussed[14]. The decrease in ø noted above for a concentration of 0.5 mg/ml is believed to be significant, and indicative of a secondary effect due to intermolecular forces.

The absence of liberation of free amino acids or peptides is to be contrasted with the finding of MITCHELL AND RIDEAL[20] that insulin monolayers release free tyrosine upon irradiation; and the more recent report of KAPLAN *et al.*[23] to the effect that insulin in solution releases tyrosine upon irradiation. The latter authors point out that the

* Preliminary measurements in this laboratory appear to indicate some anomaly in the ultraviolet inactivation of ribonuclease with a quantum yield somewhat higher than 0.026.

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quantity of tyrosine liberated is insufficient to account for the observed degree of inactivation.

The lack of modification of the absorption spectrum maximum (Fig. 5) during the first 6 mins irradiation, corresponding to over 50% inactivation, apparently indicates that the aromatic amino acids only become involved to an appreciable extent in the later stages of the reaction, as appears to be the case for insulin[23]. In the presence of a photosensitizer such as riboflavine, tryptophan will undergo atmospheric photo-oxidation under the influence of visible or ultra-violet light with a quantum yield equal to 0.038; while lysozyme, under similar conditions is inactivated with a quantum yield equal to 0.006[24]. A comparison of this latter figure with that for direct inactivation, 0.024, tends to substantiate the finding of FINKELSTEIN AND MCLAREN[25] that the quantum yield for chymotrypsin inactivation is the same in an atmosphere of oxygen or nitrogen.

SUMMARY

1. A rapid and reproducible method for measurement of lysozyme activity is described.
2. Using this method, the ultra-violet inactivation of lysozyme has been studied and found to conform to a first order reaction.
3. The measured quantum yield for inactivation at 2537 A is 0.024 and remains unaltered over the pH range 3.6 to 12.0. A small effect of concentration on quantum yield is indicated.
4. Inactivation is accompanied by photo-oxidation and other secondary processes, with no apparent liberation of free amino acids or peptides.
5. The relation of molecular weight to quantum yield, as well as the other findings, are discussed.

RÉSUMÉ

1. Une méthode rapide et reproductible est décrite pour mesurer l'activité du lysozyme.
2. Cette méthode a permis d'étudier l'inactivation ultra-violette du lysozyme, qu'on a constaté être une réaction du premier ordre.
3. Le rendement quantique pour l'inactivation à 2537 A est de 0.024 et n'est pas modifié pour des pH compris entre 3.6 et 12.0; mais il subit une légère modification sous l'influence de la concentration.
4. Le phénomène d'inactivation s'accompagne de réactions secondaires dont la photo-oxydation. On n'a pas remarqué de libération apparente d'acides aminés ou de peptides libres.
5. Le rapport du poids moléculaire au rendement quantique, ainsi que les autres résultats, ont été discutés.

ZUSAMMENFASSUNG

1. Es wird eine schnelle und reproduzierbare Methode der Aktivitäts-Messung des Lysozyms beschrieben.
2. Mit Hilfe dieser Methode wurde die Inaktivierung des Lysozyms durch ultra-violettes Licht studiert; diese ist eine Reaktion erster Ordnung.
3. Der gemessene Quantum-Ausbeute für die Inaktivierung bei 2537 A beträgt 0.024, und bleibt unverändert bei einem pH von 3.6 bis 12.0. Er wird durch die Konzentration schwach beeinflusst.
4. Die Inaktivierung ist von einer Photooxydation und von anderen sekundären Vorgängen begleitet, ohne dass eine Freimachung von Aminosäuren oder Peptiden bemerkbar wäre.
5. Das Verhältnis des Molekulargewichtes zur Quantum-Ausbeute, sowie andere Resultate, wurden erörtert.

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