Kinetics of the Firefly Luciferase Catalyzed Reactions†

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ABSTRACT: Evidence is presented which indicates that at least two distinct rate-limiting steps occur after rapid mixing of ATP, luciferin, and firefly luciferase before maximum light emission occurs. There is an initial lag of 25 msec at 25° before any light is emitted and this is followed by a slow rise of light emission which requires approximately 0.3 sec to reach the maximum rate. From thermodynamic and other data we conclude that there must be several sequential and large conformational changes in the luciferase prior to light emission. These conformational changes must occur after the formation of the enzyme–luciferyl adenylate complex and prior to the addition of oxygen to this intermediate.

Enzymatic catalysis of light emission offers a unique tool for investigating the mechanism of enzyme action. Since every catalytic event results in the emission of a photon it is possible to continuously monitor the rate of product formation. This is conveniently measured by a photocell without having to remove samples or stop reactions as is usually done when following enzymatic activity.

An interesting property of several bioluminescent reactions is the relatively long time it takes to reach maximum rate after the substrate and enzyme are mixed. In addition there is a very slow or no turnover of the enzymes (M. Cormier, personal communication). Chance *et al.* (1940) have studied the kinetics of light emission of intact bacteria as well as crude extracts of the enzyme and substrate. They concluded that a minimum of four reactions was necessary to explain the kinetics. Hastings and Gibson (1963) have analyzed the rapid kinetics of the purified bacterial luciferase and find that luminescence rises to a maximum intensity in about 2 sec and declines with a $t_{1/2}$ of about 10 sec. Under these conditions the luciferase only reacts once with $FMNH_2$ since the excess $FMNH_2$ is rapidly autoxidized. However, this enzyme does turnover if additional $FMNH_2$ is added.

Bellisario *et al.* (1972) have examined the luciferase from *Diplocardia longa* and they concluded that this enzyme does not turn over probably due to the fact that H_2O_2, one of the substrates, inactivates the enzyme. In all of these systems there is a significant lag time between mixing of all components and the appearance of maximal light intensity, usually of the order of a fraction of a second. This implies a slow rate limiting step or steps in the reaction sequence prior to light emission.

McElroy and Hastings (1956) studied the flash pattern of the intact firefly. The normal flash has a $t_{1/2}$ rise time of about 50 msec and a duration of about 0.2 sec. McElroy and Seliger (1961) have suggested that a typical light flash catalyzed by firefly luciferase *in vitro* (Figure 1) was composed of three parts: first, the slow initial rise was attributed to mixing time; second, the initial decrease was thought to be due to product inhibition or the formation of E·L-AMP;[1] and third, the slower decrease in rate represented some turnover of the enzyme.

Recent studies indicate the formation of E·L-AMP is not a factor in the decrease of light intensity after the initial flash. The evidence indicates that the product of the light reaction, which has been identified (Suzuki and Goto, 1973), is the true inhibitor of luciferase. Furthermore, in the presence of high concentrations of ATP and LH_2 some of the product is removed allowing the enzyme to turn over. This leads to a slow light production after the initial peak rate.

We shall present evidence in this paper which indicates there are at least two distinct rate limiting steps that occur after mixing ATP, luciferin, and luciferase before maximum light emission occurs. There is a lag after mixing of about 25 msec at 25° before any light is emitted and then following this phase the rate of light emission slowly rises to the maximum. Neither mixing time nor product inhibition plays a role in these rate limiting steps. This slow response of product formation has been observed with other enzymes and has been defined as hysteresis (Frieden, 1970).

For purposes of discussion the reactions catalyzed by firefly luciferase are shown below. The evidence for these reactions is discussed in detail in a recent review (McElroy and DeLuca, 1973).

$$E + LH_2 + ATP \overset{Mg^{2+}}{\rightleftharpoons} E\cdot LH_2\text{-}AMP + PP_i \quad (1)$$

$$E\cdot LH_2\text{-}AMP\ O_2 \longrightarrow [P^*\text{-}E\cdot AMP] + CO_2 \quad (2)$$

$$\downarrow$$

$$E\text{-}P + h\nu + AMP$$

$$E + L + ATP \overset{Mg^{2+}}{\rightleftharpoons} E\cdot L\text{-}AMP + PP_i \quad (3)$$

Reaction 1 is the activation of luciferin in the presence of ATP, Mg^{2+}, and luciferase to form enzyme-bound luciferyl adenylate ($E\cdot LH_2$-AMP). This enzyme-bound luciferyl adenylate then reacts with oxygen and several intermediate steps must occur prior to the formation of the excited complex (P*–E–AMP) which then emits light resulting in E–P, the enzyme–product complex in the ground state. We indicate the chromophore in the excited state is still bound to the enzyme since we know that the structure of the luciferase affects the color of light emitted (Seliger and McElroy, 1964). Even though AMP must be removed from the luciferin before decarboxylation occurs it must remain bound to the enzyme through the light emitting step since modified AMP, such as

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[1] Abbreviations used are: LH_2, luciferin; L, dehydroluciferin; LH_2-AMP, luciferyl adenylate; L-AMP, dehydroluciferyl adenylate; E·L-AMP, enzyme-bound dehydroluciferyl adenylate.

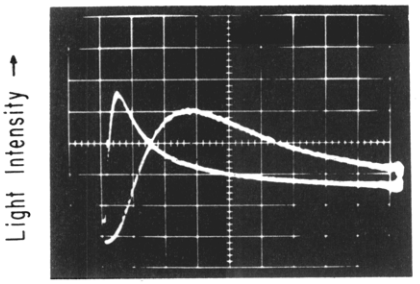

FIGURE 1: Time course of light emission upon rapid mixing of equal volumes of ATP with luciferin, luciferase, and $MgCl_2$. Time scale is 0.5 and 0.1 sec/division. Substrate concentrations are: ATP, 1.3×10^{-3} M; $MgCl_2$, 3.6×10^{-3} M; LH_2, 8×10^{-5} M; luciferase, 2×10^{-7} M.

Σ-AMP or *i*-AMP, also affect the color of the light (DeLuca *et al.*, 1973).

Reaction 3 is the activation of the competitive inhibitor dehydroluciferin (L) to form enzyme-bound dehydroluciferyl adenylate (E·L-AMP). This complex cannot react with oxygen to give light and the reaction is freely reversible.

LH_2-AMP can be chemically synthesized and when this is added to luciferase in the presence of oxygen light emission occurs without any other requirements (Rhodes and McElroy, 1958).

Materials and Methods

Four times recrystallized firefly luciferase was prepared as described by Green and McElroy (1956). The enzyme was stored as a concentrated solution, 6–10 mg of protein/ml, in 10% ammonium sulfate. This stock solution was diluted into cold buffer just prior to use. The protein concentration was determined by reading the optical density at 278 mμ where 1 mg of luciferase/ml has an optical density of 0.75.

Crystalline luciferin and dehydroluciferin were synthesized according to the method of Seto *et al.* (1963) by Dr. Lemuel Bowie. The concentrations of L and LH_2 were determined by measuring optical densities at their respective absorption maxima at neutral pH using the following extinction coefficients: L, 353 mμ (24,000); LH_2, 327 mμ (18,000) (Morton *et al.*, 1969).

LH_2-AMP was synthesized as described by Morton *et al.* (1969). The product was dissolved in 0.01 M NaOAc–0.04 M NaCl (pH 4.5) and used without further purification. Due to the extreme lability at neutral pH, the compound was diluted into pH 7.0 buffer just immediately prior to use.

Stopped Flow Experiments. These experiments were done using an Aminco-Morrow stopped flow instrument with a 1P28 phototube and equipped with a Tectronix R-564B storage oscilloscope. The dead time of the instrument is 5 msec. Bioluminescent reactions were followed without the light source activated and the figures presented are photographs of the oscilloscope traces.

For fluorescent measurements in the stopped flow instrument the phototube was mounted for observation at right angle to the activating xenon light source. Dehydroluciferin fluorescence was measured by activating at 390 mμ and a Corning filter 3-72 was mounted in front of the phototube to filter out light below about 480 mμ. In some experiments the voltage on the phototube was increased from the normal 400 to 800 V to give additional sensitivity.

Reaction conditions for stopped flow measurements were as follows. All reactions were carried out in 0.025 M glycylglycine buffer (pH 7.8) at 25° unless specified otherwise. For maximal bioluminescent activity the final concentrations of reactants were: luciferin, 7.5×10^{-5} M; ATP, 1.33×10^{-3} M; $MgCl_2$, 3.3×10^{-3} M; and luciferase, $2–5 \times 10^{-6}$ M. Since the order of mixing did not affect the kinetics most experiments were done with luciferin, luciferase, and $MgCl_2$ in one syringe and ATP in the other syringe. Experiments in which the substrate concentrations were varied are described under Results. For the LH_2-AMP experiments the LH_2-AMP was dissolved in 0.025 M glycylglycine (pH 7.0) in one syringe and enzyme and buffer were in the other syringe.

Experimental conditions for following the formation of E·L-AMP were the same as for bioluminescence except dehydroluciferin, L, was used in place of luciferin and the decrease in fluorescence was observed with time after mixing.

Anaerobic Experiments. ATP, Mg^{2+}, LH_2, and buffer were mixed and placed in the side arm of a flask. Luciferase and buffer were placed in the bottom of the flask. The flask was fitted with a rubber stopper which had an inlet glass tube and an outlet with a three-way stopcock and a syringe for removal of the sample. N_2 (99% pure) was bubbled using a sintered glass filter through a flask containing 0.025 M glycylglycine buffer and 0.01 M dithionite, then through a second flask containing buffer and a leuco Methylene Blue indicator, and then into the flask containing the enzyme and substrates. The system was allowed to equilibrate with the N_2 for 1.5 hr with gentle shaking. The substrates were then mixed with the enzyme and a low level of light emission was observed initially which ceased within 2 min. While the N_2 flow continued the enzyme–substrate mixture was removed into a syringe and immediately placed into the stopped-flow apparatus. The other syringe contained air equilibrated buffer. Bubbling O_2 through the buffer did not change either the kinetics or light intensity.

Once the anaerobic mixture was in the syringe all experiments were carried out within 10 min. The final concentration of substrates and enzyme was the same as indicated previously.

Temperature Experiments. The conditions for these experiments are the same as indicated above except all solutions were preequilibrated at the specified temperature and the cell compartment of the stopped flow was maintained at the appropriate temperature by means of a circulating water bath. Once the solutions were placed in the syringes all experimental data were obtained within 2 min.

Results and Discussion

Effect of the Order of Addition of Substrates. Figure 1 shows a typical light emitting reaction catalyzed by firefly luciferase upon the rapid mixing of ATP with enzyme, Mg^{2+}, and excess luciferin. In this case all of the substrates are in excess and the enzyme is the limiting component in the system. ATP was contained in one syringe while a mixture of luciferase, LH_2, and Mg^{2+} was present in the other. Since the dead time of the instrument is 5 msec it is apparent that the rise to maximal intensity of luminescence is not due to mixing time. It takes 0.25–0.3 sec to reach the peak intensity. The time delay for the appearance of luminescence and the time to reach peak intensity remain the same regardless of the order of addition of substrates. This is in agreement with the studies of Denburg *et al.* (1969) in which they concluded that substrate addition was of the random type.

In addition there is a lag of approximately 25 msec before any significant light emission is observed. This lag becomes much more pronounced at lower temperatures. If the enzyme concentration is varied over a 100-fold range there is no change

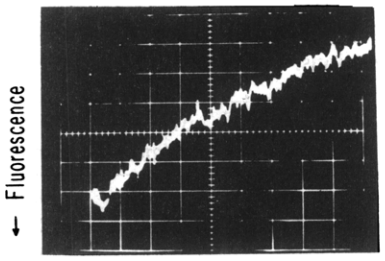

FIGURE 2: Time course of formation of E·L-AMP followed by decrease in fluorescence after mixing equal volumes of L with luciferase, ATP, and $MgCl_2$: L, 8×10^{-5} M; luciferase, 1×10^{-6} M; ATP, 1.3×10^{-3} M; $MgCl_2$, 2.6×10^{-3} M. Time is 20 msec/division. The upper line represents the final equilibrium fluorescence.

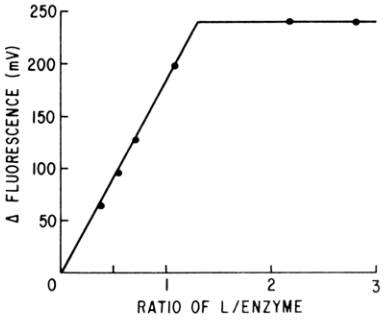

FIGURE 3: Fluorescent titration of luciferase with dehydroluciferin: luciferase, 2.2×10^{-5} M; ATP, 1.3×10^{-3} M; $MgCl_2$, 2.6×10^{-3} M; increasing amounts of L were added as indicated.

in the time of onset of bioluminescence. However, the peak intensity varies linearly with enzyme concentration when the substrates are in excess. If the enzyme is in excess of the substrates, the 25-msec lag is still present and the time to reach peak intensity remains at 0.3 sec.

Effect of Varying the ATP Concentration. The effect of varying the ATP concentration (1×10^{-3} to 1×10^{-6} M) on the peak intensity and the lag time indicates that at lower concentrations the peak height is proportional to ATP. However, the time required to reach 50% peak height is constant and independent of the peak intensity. This demonstrates that the onset of luminescence is controlled by something other than the binding of ATP to the enzyme and that this process follows first-order kinetics.

The time required to reach 50% inhibition due to product formation is not constant but is related to the peak height. The lower the peak height (less product formation) the longer it takes to reach 50% inhibition.

If one plots the log of the *initial* decay rate against time (Figure 5) this extrapolated rate indicates it takes 0.8 sec to reach 50% inhibition while the observed time required to reach 50% of initial peak intensity is 2 sec. If one assumes the peak intensity is proportional to (E–P*) and if every molecule of enzyme is inhibited by product, then the decay of luminescence should follow first-order kinetics with a constant $t_{1/2}$. Since this is not observed there must be some turnover of the enzyme. This is substantiated by observations of Rhodes and McElroy (1958) and McElroy and Seliger (1961) that enzyme–substrate mixtures may continue to emit light at a low level for hours.

Studies on the Formation of E·L-AMP. By using the competitive inhibitor dehydroluciferin it is possible to follow the activation reaction in the absence of light emission (eq 3). This is done by measuring the decrease in fluorescence of L which occurs upon the formation of E·L-AMP (Rhodes and McElroy, 1958). A typical experiment is shown in Figure 2. The fluorescence decrease is followed as a function of time to the final level at which all of the enzyme has been converted to the form of E·L-AMP and no further reaction can occur. It is important to note that there is no lag before the fluorescence begins to decrease. This is also true at lower temperatures.

By varying the concentration of L with a constant amount of enzyme it is possible to obtain the stoichiometry of L-AMP formation to enzyme. Such a titration is shown in Figure 3 in which it can be seen that 1.3 mol of L-AMP are formed per mol of luciferase. Many other experiments have shown the number is 1.0 ± 0.3. This is in agreement with previous experiments (Denburg *et al.*, 1969). At all of the concentrations of L studied (7×10^{-5} to 7×10^{-6} M) the first 60% of the reaction is first order and independent of the concentration of L. After about 60% of the reaction is completed linearity falls off.

Studies of LH_2-AMP Reaction. It seemed possible that the slow step or steps involved in bioluminescence were the actual synthesis of the acyl–anhydride bond (reaction 1). We therefore synthesized LH_2-AMP and studied the appearance of luminescence when this compound was mixed with luciferase in the stopped flow instrument. Figure 4 shows the results of this experiment. As can be seen the time to reach the peak is still 0.3 sec. Furthermore, the upper curve, which is 20 msec/division, still has a similar lag to that observed with the ATP light flash. It appears that the synthesis of the LH_2-AMP is not a limiting factor in the rise time or the lag. It is interesting to note that when the log of the decay is plotted *vs.* time, Figure 5, the reaction with LH_2-AMP remains logarithmic much longer than that observed when ATP is the substrate. Rhodes and McElroy (1958) demonstrated that the enzyme did not turn over with LH_2-AMP and found that only 2 quanta/mol of enzyme are produced and further addition of LH_2-AMP did not give any additional light, suggesting the enzyme is completely inhibited at this stage. The molecular weights of the active species for the activation of L and for the emission of light with LH_2-AMP are discussed in detail in a recent review, McElroy and DeLuca (1973). The discrepancy between the formation of one L-AMP/100,000 mol wt and the oxidation of 2 mol of LH_2-AMP/100,000 mol wt is unresolved.

Anaerobic Experiments. McElroy and Hastings (1956), McElroy and Seliger (1961), and Hopkins (1968) have shown that when buffer containing oxygen is injected into an anaerobic solution of luciferin and luciferase and ATP and Mg^{2+} the onset of luminescence is very rapid. We examined this process using anaerobic enzyme and substrates under anaerobic conditions in one syringe and oxygenated buffer in the other. The results are seen in Figure 6a,b. Clearly the time to reach the peak of luminescence is much faster than any of the previous rates, about 60 msec. More important, the 25-msec lag is absent.

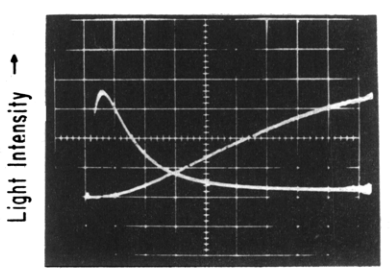

FIGURE 4: Time course of light emission upon rapid mixing of LH_2-AMP with luciferase. Time is 0.5 sec/division and 20 msec/division.

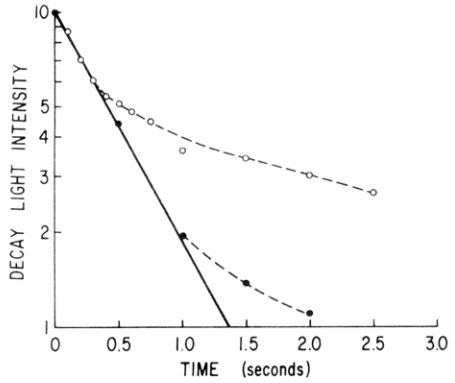

FIGURE 5: Decay of light emission following peak intensity of bioluminescence: (○) decay observed after ATP flash, conditions as in Figure 1; (●) decay rate after LH_2-AMP flash, conditions as in Figure 4. Solid line is extrapolated rate, dashed lines are experimental.

Effect of Temperature. The results observed for light emission starting with either LH_2 and ATP or LH_2-AMP suggest that there may be large steric or conformational factors that must occur before oxidation and subsequent light emission. For this reason we have determined the thermodynamic parameters for the reactions involving the lag and subsequent light emitting step. The data are presented in Table I. The effect of temperature on the lag indicates that the initial transition state involves only enthalpic changes with essentially no contribution to $\Delta F^{\ddagger}$ by the entropy of activation. In contrast, if one studies the appearance of light using $t_{1/2}$ for maximum light to calculate the first-order rate constant one observes large negative entropy of activation. We believe the large $\Delta F^{\ddagger}$ and relatively large and negative $\Delta S^{\ddagger}$ are due to a conformational change in the enzyme after the formation of the E·LH_2-AMP complex.

The effect of temperature on the activation of L indicates that there is a relatively large contribution of the entropy of activation to $\Delta F^{\ddagger}$. However since it appears that the activation reaction is not the limiting step in luminescence this effect should not be observed in the light emitting step.

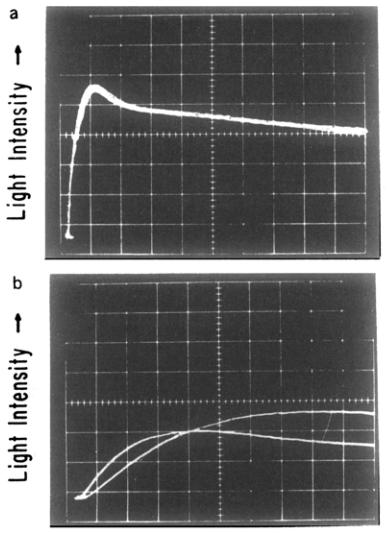

FIGURE 6: (a) Time course of light emission following rapid mixing of aerated buffer with deoxygenated luciferase, luciferin, ATP, and $MgCl_2$; 0.1 sec/division. Experimental conditions are given under Materials and Methods. (b) Conditions same as a except the time is 10 and 20 msec/division.

TABLE I: Thermodynamic Characteristics of Luciferase-Catalyzed Reactions.[a]

	$\Delta F^{\ddagger}$ (kcal/mol)	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (eu)
Light emission ($t_{1/2}$(max))	19,700	7,900	−42
Lag time	18,700	18,800	0.4
L activation	15,500	9,900	−22

[a] From the observed rate constants the free energies of activation ($\Delta F^{\ddagger}$) were calculated according to the following equation: rate = $(kT/h)e(-\Delta F^{\ddagger}/RT)$. In the case of the lag, the reciprocal of the lag time was used as the rate at various temperatures. $\Delta F^{\ddagger}$ values were calculated at 25°.

Discussion

These experiments indicate that at least two slow overall and independent steps occur prior to light emission but subsequent to the binding of the substrates and the synthesis of LH_2-AMP. One of these is associated with the 25-msec lag before any light emission is observed. Since there is no observed light emission for almost 25 msec the initial lag phase must involve at least two sequential steps (see Figure 7). The second lag is involved in the rise to maximum light intensity, about 0.3 sec.

Since the lag and the rise time are the same whether the reaction is initiated with LH_2 and ATP-Mg or synthetic LH_2-AMP, these slow steps must occur after the synthesis of LH_2-AMP. Other than conformational change in the protein, the only known chemical change that occurs after the formation of E·LH_2-AMP is the abstraction of a proton by the enzyme to form E·LH^--AMP. The latter complex adds oxygen and a series of reactions occurs that leads to light emission. However, the anaerobic experiment indicates that the two lag phases must occur prior to oxygen addition.

The most likely explanation would seem to reside in the nature of LH_2-AMP binding to the enzyme prior to proton abstraction. We know that LH_2 binds at a very hydrophobic site and there may be considerable induced conformational changes that occur prior to the AMP binding. Proton abstraction may also depend on bringing a specific proton acceptor into the proper steric position with respect to the substrate. It was somewhat surprising, therefore, to observe that the $\Delta S^{\ddagger}$ for the initial lag was essentially zero. However, if several steps are involved in the initial binding of LH_2-AMP and pro-

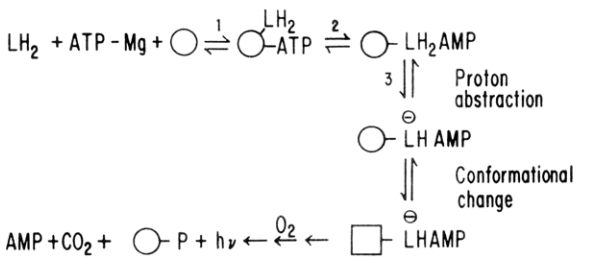

FIGURE 7: Postulated sequence of events occurring during luciferase-catalyzed reactions. In reaction 1, the binding of the substrates and in reaction 2 synthesis of LH_2-AMP occur rapidly relative to later reactions. The enzyme-bound LH_2-AMP after proton abstraction (step 3) undergoes a slow conformational change so that it is in a form to which oxygen adds rapidly. The experiment in which the enzyme was incubated with the substrates in the absence of oxygen would proceed through step 3 and the conformational change.

ton abstraction, there may be a cancelling due to both negative and positive entropies of activation. On the other hand, the large negative entropy of activation (−40 eu) for the slow rise to maximum rate of light emission supports the idea that a large conformational change must occur after proton abstraction. Since neither the lag nor the slow initial rise of light emission was influenced by varying the enzyme concentration, this further supports an intramolecular conformational change as a rate limiting step in the sequence. The fact that a protein conformational change is associated with the formation of E·L-AMP has been reported previously based on tritium–hydrogen exchange rates and optical rotatory dispersion measurements. In the presence of excess L and ATP when the enzyme is fully in the form of E·L-AMP, about 250 backbone hydrogens do not exchange with water over a 7-hr period (DeLuca and Marsh, 1967). This must reflect a large change in the conformation of the enzyme.

There are now many examples in the literature where conformational changes of proteins occur as relatively slow steps following the binding of substrates (Hammes and Schimmel, 1970; Gutfreund, 1971; Frieden, 1970). It would appear that luciferase falls into the classification of a hysteretic enzyme in that it exhibits a slow response to its substrates. What is surprising and apparently unique is the fact that apparently there are two distinct slow steps involving conformation change in the enzyme.

Acknowledgment

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