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MECHANISM BY WHICH WARFARIN BINDS TO HUMAN SERUM ALBUMIN

STOPPED-FLOW KINETIC EXPERIMENTS WITH TWO LARGE FRAGMENTS OF ALBUMIN

OCTAAF J. M. BOS,* MARCEL J. E. FISCHER, JAAP WILTING and LAMBERT H. M. JANSSEN
Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Utrecht, Catharijnesingel 60, 3511 GH Utrecht, The Netherlands

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Abstract—In order to obtain information about the kinetics of the process by which warfarin binds to human serum albumin at a molecular level, we performed stopped-flow kinetic experiments on albumin and on a large peptic fragment (residues 1–387) and a large tryptic fragment (residues 198–585) of albumin. From these experiments it can be concluded that the first interaction between warfarin and the proteins is almost certainly diffusion-controlled and is dependent on the net charges of the reactants. The next step in the binding process involves the formation of an activated warfarin–protein complex, whereafter the final complex is formed.

The warfarin–albumin complex forms more slowly than the warfarin–fragment complexes, because the formation is sterically hindered by the albumin structure. We think it very unlikely that albumin has an oblate ellipsoid structure; it is much more likely to have a U-shaped structure, where the domains make contact with each other. If this hypothesis is correct, then this indicates that the domains do not act independently of each other. The formation of the activated warfarin–albumin complex is further influenced by the conformational state of the albumin molecule, i.e. the N–B transition. The possible role of this N–B transition in albumin-mediated transport of drugs through cellular membranes is discussed.

Albumin,† being the most abundant blood protein, functions as a transport and depot protein in the circulation. The molecule is composed of three domains, each containing three loops. The domains appear to function more or less independently of each other [1, 2]. Because many drugs bind with high affinity to albumin ($K > 10^5\ M^{-1}$), binding can be an important determinant in the pharmacokinetic behaviour of these drugs [1–5]. Unfortunately, the binding constant itself provides little information about the rate of formation and dissociation of the drug–protein complex. Therefore, it is useful to study the kinetics of the binding of drugs to albumin. Moreover, knowledge of these kinetics can give us deeper insight into the mechanism of the drug–protein interaction.

The kinetics of the binding of warfarin to albumin has been studied in previous papers [6, 7]. The binding process could be described in terms of two steps: a diffusion-controlled fast step and a slow step in which the final warfarin–albumin complex is formed. The association rate of the complex, like the binding constant itself, was found to be dependent on the so-called neutral-to-base or N–B transition which in albumin occurs around physiological pH [6, 8, 9].

Warfarin is a representative drug which binds to one of the best characterized drug binding sites on the albumin molecule, namely the so-called drug binding site I [10, 11]. Site I is located in domain two of the albumin structure and actually consists of partly overlapping binding sites for drugs such as warfarin and azapropazone. It is very probable that Trp-214 participates in the binding of warfarin [1–3, 8, 12].

Many questions about the molecular mechanism of the warfarin–albumin interaction remain unanswered. Better insight into this mechanism may give us a better understanding of the possible role of albumin in the protein-mediated uptake of drugs through cellular membranes [13–15]. Therefore, we have investigated the kinetics of the binding of warfarin to fragments of albumin by means of stopped-flow spectrophotometry. Because these fragments are composed of intact domains [16, 17], the stopped-flow experiments may also reveal whether or not the domains actually act independently of each other.

MATERIALS AND METHODS

Preparation of the albumin and the fragment solutions. Albumin was isolated from human plasma according to the method described by Kremer [18]. The albumin used for all experiments was from one batch. The peptic fragment (the P46 fragment) and the tryptic fragment (the T45 fragment) were obtained as described previously [8, 16, 17]. The P46 fragment represents residues 1–387 and therefore comprises domains one and two of the albumin structure. The T45 fragment corresponds to residues 198–585 or domains two and three of the albumin structure. All protein solutions were deionized before use

* To whom all correspondence should be addressed.

† Abbreviations: albumin is human serum albumin unless otherwise stated; T45, the 45 kDa tryptic fragment; P46, the 46 kDa peptic fragment; I, the ionic strength of a solution.

as described recently [8].

All unspecified chemicals used were of analytical grade (J. T. Baker, Deventer, The Netherlands; Janssen Chimica, Beerse, Belgium; Sigma Chemical Company, St Louis, U.S.A.; or Merck, Darmstadt, F.R.G.).

Stopped-flow experiments. The stopped-flow experiments were performed at 25° on a Durrum stopped-flow apparatus (model D 110). Equal volumes of two solutions (100 μl each) were rapidly mixed (mixing time 3–4 msec) in a cell with an optical pathway of 2 cm and a total volume of 60 μl. One solution contained albumin or one of the fragments in a phosphate or borate buffer at a given pH (I = 0.1). The second solution contained warfarin or diazepam in the same buffer at the same pH. The concentrations of the proteins after mixing varied between 1.0×10^{-6} and 3.0×10^{-4} M, depending on the experiment. In all cases, the warfarin-to-protein ratio (r) was 0.1. As soon as the warfarin–protein complexes had formed, the changes in fluorescence intensity were monitored as described previously [7]. Excitation was induced at 330 nm and the emitted light was measured at 380 nm. A cut-off filter prevented light with a wavelength below 360 nm from entering the detector. The changes in fluorescence intensity during the reactions were recorded and stored in a transient recorder (Biomation, model 805). Next, the stored signals were registered, processed and analysed on an Apple II Europlus computer. The observed rate constants (k_{obs}) are calculated from the traces stored in the computer, using a non-linear least-squares computer program.

Treatment of the kinetic data. The kinetic data were treated with the following equation:

$$k_{obs} = k_2 \frac{K_1 [P]}{K_1 [P] + 1} + k_{-2}. \qquad (1)$$

This equation is based on the two-step reaction mechanism postulated by Wilting *et al.* for the binding of warfarin to albumin (D, drug; P, protein; $K_1 = k_1/k_{-1}$) [7]:

$$D + P \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [D \approx P]^* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} DP \quad \text{(Scheme 1)}$$

I II

In this scheme a fast diffusion-controlled step I is followed by a slow step II in which the stable drug–protein complex is formed. The first step is very fast, the association and dissociation rates having half-lives in the microsecond range [7]. In other words the half-lives are within the mixing time of the stopped-flow apparatus. The last step is rate limiting and can be monitored by the stopped-flow instrument. Because we measure at the low drug-to-protein ratio of 0.1, both the reaction kinetics of step II and the reaction kinetics of step I can be described by a (pseudo) first-order process. Since k_{obs} is found to be dependent on the protein concentration, it was possible to calculate the equilibrium constant (K_{eq}) of the binding of warfarin to albumin for all pH values between 6 and 9 [7, 19].

Because it is difficult to obtain enough of the two fragments to perform the protein dependent kinetic experiments (often more than 50 mg being required per experiment), we performed our experiments only at pH 7.6.

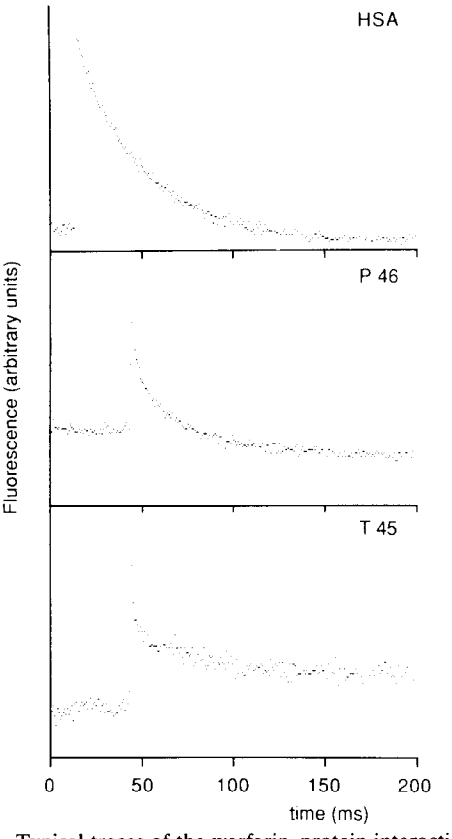


Fig. 1. Typical traces of the warfarin–protein interactions. Shown are the changes in fluorescence intensity vs time: (A) albumin, $k_{obs} = 16\ sec^{-1}$; (B) the P46 fragment, $k_{obs} = 68\ sec^{-1}$; (C) the T45 fragment, $k_{obs} = 97\ sec^{-1}$. Conditions: $[P] = 10^{-4}$ M, $r = 0.1$ pH = 7.6 (phosphate buffer, $I = 0.1$); temperature = 25°.

RESULTS

The kinetics of the binding of warfarin to albumin, to the P46 fragment and to the T45 fragment

The kinetics of the binding of warfarin to its high-affinity binding site on albumin can be followed by measuring the change in fluorescence intensity vs time [6, 7]. Because the P46 fragment contains the intact primary warfarin binding site and the T45 fragment contains at least the main part of this binding site [8], it was possible to follow the kinetics of the binding of warfarin to the fragments as well. Figure 1 gives some typical traces of the reaction between warfarin and the proteins; the traces were obtained directly from the data stored in the Biomation. In order to make comparisons, the same time axis was chosen for all three proteins.

Figure 2 shows the observed rate constant, k_{obs}, as a function of the protein concentration at pH 7.6. In all cases k_{obs} increases with the protein concentration until a plateau is reached. This indicates that all three proteins have a comparable warfarin binding mechanism. For the binding of warfarin to albumin, the reaction mechanism of Scheme 1 was postulated

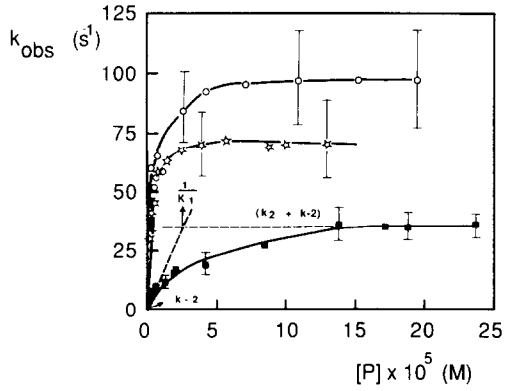


Fig. 2. k_{obs} vs $[P]$ for albumin (■—■), the P46 fragment (☆—☆) and the T45 fragment (○—○). For further conditions see Fig. 1. Assuming a two-step reaction model [6, 7], k_2, k_{-2}, K_1 and K_{eq} can be calculated: the intersection on the ordinate axis gives k_{-2}; the plateau level for the higher $[P]$ levels corresponds to $k_2 + k_{-2}$; K_1 can be derived from the point of intersection of the two straight lines; $K_{eq} = K \times k_2/k_{-2}$. The values represent the mean of four to seven experiments. The vertical bars correspond to the SD.

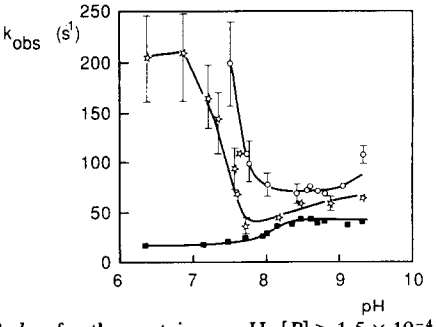


Fig. 3. k_{obs} for the proteins vs pH. $[P] > 1.5 \times 10^{-4}$ M for albumin (■—■), $[P] > 6 \times 10^{-5}$ M for the P46 fragment (☆—☆) and $[P] > 8 \times 10^{-5}$ M for the T45 fragment (○—○). For further conditions see Fig. 1.

[7]. Therefore, for the fragments too, the kinetics can in all probability be described by such a reaction mechanism. The equilibrium constants (K_{eq}) can be calculated from the kinetic constants derived from the protein dependent measurements [7, 19]. Table 1 lists these values, together with the values of k_2, k_{-2}, K_1 and K_{eq}, obtained from equilibrium dialysis experiments [8]. From this table, it can be seen that in all cases the K_{eq} values derived from the kinetic experiments are of the same order of magnitude as those obtained from the equilibrium dialysis experiments. For albumin, this is in agreement with the situation described before [6, 7].

In order to investigate the pH dependence of the kinetic processes of the binding of warfarin to the proteins, we measured k_{obs} as a function of the pH at protein concentrations where k_{obs} is independent of the protein concentration, i.e. in the range where k_{obs} corresponds to $k_2 + k_{-2}$ (Fig. 2). We assume that the fragments behave like albumin [6], namely that the plateau observed at the higher protein concentrations at pH 7.6 is also present at the other pH values. The results of these experiments are given in Fig. 3. It can be seen that at the lower pH values, the observed rate constants of the binding of warfarin to the fragments are very high. It is also obvious that for albumin, k_{obs} increases as the pH is raised from 6 to 9, whereas for the fragments the reverse is the case. For the T45 fragment, k_{obs} cannot be measured at pH values below 7.4, because the reaction time falls within the mixing time of the apparatus.

DISCUSSION

Figure 2 reveals that for all three proteins the kinetics can be described by the reaction mechanism of Scheme 1. This is further supported by the agreement between the values of K_{eq}, based on the kinetic parameters given in Table 1, and the values of K_{eq}, based on the equilibrium dialysis experiments. From Table 1 it can also be seen that in all cases k_2 is larger than k_{-2} (although for the T45 fragment this is not significant). The value of k_2 for the three proteins is of the same order of magnitude and corresponds to half-lives of about 10–21 ms. In contrast, the value of k_{-2} for the fragments is larger than for albumin, the T45 fragment having the largest k_{-2} value. The half-lives for these dissociation rates were found to be approximately 170 ms, 39 ms and 15 ms for albumin, the P46 fragment and the T45 fragment respectively. Figure 3 shows that this is not the only difference between albumin and the fragments. The decrease in the rate constants of the binding of warfarin to the fragments, as the pH is raised from 6 to 9, points to the possibility that electrostatic interactions are involved in these binding processes. This is what we might expect: warfarin, having a pK_a of about 5.0, is negatively charged over the pH range under investigation. Since the net proton charge of the proteins decreases from −5 to −24 for albumin, from −3 to −16 for the T45 fragment and from −4 to −22 for the P46 fragment in the pH range 6 to 9 [20], one expects an increase in the electrostatic

Table 1. Kinetic parameters for the interaction of warfarin with albumin and the fragments

	k_{-2} (sec^{-1})	k_2 (sec^{-1})	$K_1 \times 10^{-5}$ (M^{-1})	$K_{eq} \times 10^{-5}$ (M^{-1})	$K_{eq} \times 10^{-5}$ (M^{-1})*
HSA	4 ± 1	33 ± 4	0.36 ± 0.1	3.0 ± 0.4	3.0 ± 0.1
P46	18 ± 8	50 ± 20	1.0 ± 0.1	2.8 ± 1.0	2.0 ± 0.1
T45	45 ± 10	55 ± 22	0.33 ± 0.1	0.4 ± 0.2	0.72 ± 0.04

The values represent the mean ± SD of four to seven experiments.
* Derived from equilibrium dialysis experiments [8].

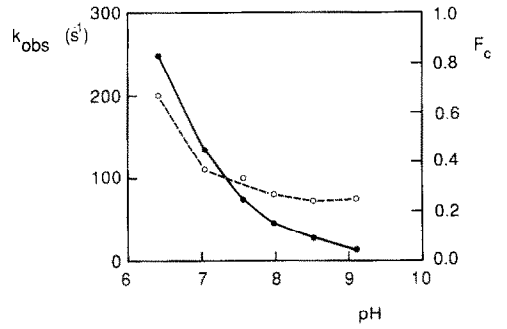


Fig. 4. k_{obs} for the T45 fragment ($[P] > 8 \times 10^{-5}$ M) vs pH (○—○), together with the calculated Debye factor f_c (●—●) for the T45 fragment vs pH. The factor f_c is calculated without correcting for the double layer effect that is due to the presence of salts ($I = 0.1$). This will result in a slope less steep than the one presented, but this does not alter the conclusion drawn.

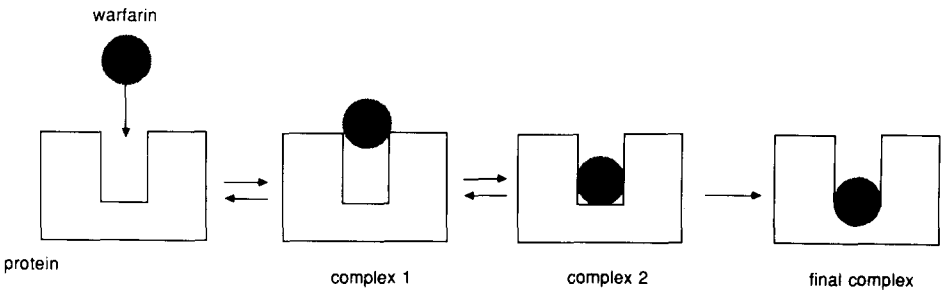
repulsion between warfarin and the proteins. The possible involvement of electrostatic interaction in the binding of warfarin to the fragments is illustrated in Fig. 4. This figure gives the pH dependence of k_{obs} of the T45 fragment together with the pH dependence of the Debye factor f_c of the T45 fragment. The Debye factor describes the effect of the electrostatic interaction on the rate of the reaction [6, 7]. Because f_c and k_{obs} run parallel, this is an indication that k_{obs} of the binding of warfarin to the T45 fragment is at least partly dependent on the net charge of the reactants. The same reasoning holds for the P46 fragment. In other words, in this case too the reaction may depend on the net charges of the reactants, resulting in a k_{obs} that runs parallel with f_c (data not shown). It should be mentioned that, with respect to the warfarin binding site, one should take care in comparing the T45 fragment with albumin. This is because the T45 fragment, in contrast to the P46 fragment, does not contain the intact warfarin binding site [8]. However, because the process by which warfarin binds to the P46 fragment is just as sensitive to the net charge of the molecules as is the process by which warfarin binds to the T45 fragment, it looks as if the electrostatic interaction, i.e. the electrostatic repulsion, may indeed be an important parameter in the warfarin binding process. By contrast, in the binding of warfarin to albumin, k_{obs} does not seem to be dependent on the net charges of the reactants. As mentioned above, such a dependence is expected. Because this is not visualized in the binding of warfarin to albumin, it is clear that other factors must be important determinants in the rate limiting step of this warfarin binding process.

If we compare albumin and the P46 fragment, both of which have comparable affinities for warfarin and have the intact primary structure of the warfarin binding site [8], we can conclude that it is very probably the absence of domain three in the P46 fragment which leads to the different values for the kinetic parameters of the binding of warfarin to albumin and to the P46 fragment. This means that for albumin, the warfarin binding process must be dependent on the three-dimensional structure of the molecule. If albumin has an oblate ellipsoid structure, as is commonly supposed [1, 2], we might expect the kinetic parameters of the warfarin binding process to be the same for the P46 fragment and for albumin. Because we find large differences in these parameters, this indicates that the albumin molecule cannot be completely ellipsoid. In a previous paper [20], we already discussed the possibility that the albumin molecule might be U-shaped, similar to the α-fetoprotein structure. Such a molecule, where all three domains make contact with each other, could explain several of the findings reported in the literature, e.g. the nearly equal distance between Trp-214 and Tyr-411 and Tyr-411 and Cys-34 [2, 21, 22].

Because the molecules of the albumin and the P46 fragment have different structures, we can conclude that the differences in the observed rate constants of the binding of warfarin to albumin on the one hand and of the P46 fragment on the other hand are in all probability due to different kinetic pathways. It is very likely that in our case it is particularly the activation entropy (ΔS^*) for the formation of an activated intermediate complex which is affected by the different reaction pathways: sterical hindrance in the process by which warfarin binds to albumin leads to a more negative ΔS^* value than for the binding of warfarin to the P46 fragment, and therefore to a larger value for ΔG^*. This would lead to a smaller value for the observed rate constant of the warfarin binding to albumin ($k = \text{constant} \times e^{-\Delta G^*/RT}$ [23]), which was indeed what we found. This means that the reaction mechanism for the binding of warfarin to the P46 fragment can be described by two steps, as is illustrated in Scheme 1, whereas for the warfarin binding to albumin the situation is more complex: in this case, an equilibrium probably develops between a complex (compex 1), formed when warfarin and albumin first came in contact with each other, and a second intermediate complex (complex 2) leading to the final complex formation. The equilibrium between complex 1 and 2 is illustrated in Scheme 2. This representation is based on the assumption that the albumin molecule is U-shaped and is supported by the literature [24, 25]: the site I binding area is formed after the first contact between warfarin and albumin, in a hydrophobic region far away from the polar exterior. The negative charge of the warfarin molecule is delocalized over several amino acid residues.

The rate limiting step in the binding of warfarin to albumin therefore seems to be the formation of complex 2, i.e. the step where warfarin passes from the outside of the albumin molecule to its binding area inside the U-shaped structure. This step is very probably absent in the warfarin–P46 fragment complex formation because in the P46 fragment domain three has been cut off, which means that the warfarin encounters less sterical hindrance as it moves towards its binding area. In other words, we can assume that complex 1 is absent in the binding of warfarin to the P46 fragment.

The fact that the T45 fragment has a higher rate constant than the P46 fragment is probably the result of the absence of domain one in the T45 fragment: for the T45 fragment, where the warfarin binding site is located at the edge of the fragment, the binding



Scheme 2.

site is even more accessible than in the case of the P46 fragment.

It is obvious that in the binding of warfarin to albumin, the U-shaped structure is an important factor. As has been described earlier [6], another determinant in the rate limiting step of the binding of warfarin to albumin is the N–B transition, which occurs in the albumin molecule around physiological pH [6, 8, 9]. It is known that the P46 fragment contains an N–B related transition, whereas the T45 fragment does not [8, 20]. Apparently, in the case of the binding of warfarin to albumin, the effect of the N–B transition on the pH dependence of k_{obs} predominates the effect of the electrostatic repulsion on the pH dependence of k_{obs}. On the other hand, for the P46 fragment, the effect of the electrostatic repulsion probably prevails, resulting in a k_{obs} that decreases with the pH. For the T45 fragment, which has no N–B-like conformational change, the pH dependence of k_{obs} can be attributed mainly to the electrostatic interaction between the reactants.

It is of interest to know whether the U-shaped albumin molecule and the N–B transition are involved in the albumin-mediated transport of drugs over e.g. the hepatic membrane. Such albumin-mediated hepatic transport has been described before [13–15]. It has also been found that during this albumin-mediated transport a conformational change in albumin occurs, due to the interaction with the hepatocyte [13]. This conformational change (the N–B transition?) may be facilitated by the lower pH at the membrane surfaces of several tissues [7, 26–28]. The experiments described in this paper reveal that the dissociation rate ($k_{-2} = 4\ sec^{-1}$, corresponding to $t_{1/2} = 0.17$ sec) for the warfarin–albumin complex is fast enough to allow the albumin-mediated transport process to occur. In other words, the half-life of the dissociation is much shorter than the transit time of an albumin molecule in the liver, which is about 10 sec [7, 29]. Moreover, in preliminary experiments we have done recently, we found that a shift in the $N \rightleftharpoons B$ equilibrium in the physiological pH range is also fast ($t_{1/2}$ is approximately 0.4 sec); so this indicates the N–B transition may well play a role in the albumin-mediated membrane process. Studies are in progress to verify this phenomenon.

CONCLUSIONS

The binding of warfarin to albumin can be described by a three-step reaction mechanism: the first contact between warfarin and albumin is very fast and diffusion controlled. The second step is rate limiting and involves the movement of warfarin from the outside of the molecule to the binding site. Finally, the stable drug–protein complex is formed. In the case of the binding of warfarin to the fragments the second step is missing, because one of the three domains present in albumin has been cut off. As a result, warfarin is not sterically hindered from moving to the warfarin binding area. It is likely that in the binding of warfarin to the fragments, the rate constant is dependent on the net charges of the reactants. As has been described in previous articles [6, 7], it is particularly the first step of the reaction mechanism which is dependent on this electrostatic interaction. An important parameter in the binding of warfarin to albumin and perhaps (to a lesser extent) to the P46 fragment is the N–B transition. It is very likely that both the formation of complex 2 (in the case of albumin) and the formation of the final complex are dependent on the conformational state of the proteins. The N–B transition may be involved in the albumin-mediated transport of drugs through cellular membranes.

Another important conclusion is that whereas the binding affinity of a drug to an isolated domain may be the same as to intact albumin, the binding processes can never be exactly the same. In other words, the domains do not act independently of each other.

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