Coupled Amidolytic Assay for Factor VII: Its Use With a Clotting Assay to Determine the Activity State of Factor VII

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A coupled amidolytic assay for factor VII (VII) has been developed that when used with a clotting assay for VII enables detection of activated VII. In the assay, VII in a test material determines generation of factor Xe in a mixture of purified factor X, tissue factor, and calcium; factor Xe is measured with a chromogenic substrate. Factor VII activity in the coupled amidolytic assay (VIIe) correlated well with VII activity in a one-stage clotting assay (VII,) in 57 healthy subjects, 5 patients with hereditary VII deficiency, and 11 patients with liver disease. Activation of plasma VII by kaolin, clotting, or cold strikingly increased VII, but not VIIe levels. Thus the ratio VII,/VIIe (VII activity ratio) is a measure of VII activation. In 27 warfarin-treated patients the mean VII activity ratio was significantly decreased, reflecting a greater decline in VII, than in VIIe. This probably stems from partially carboxylated VII being able to act during the 3-min incubation of the amidolytic assay but unable to act rapidly enough to affect the clotting assay. Measurement of VII,/VIIe should enable evaluation of the activity state of VII in thrombotic disorders and in components for transfusion therapy.

Factor VII, tissue factor, and calcium ions interact to yield activity that can initiate blood coagulation through the activation of factor X and IX. Factor VII activity has usually been measured in clotting assays containing tissue factor and a factor VII-deficient substrate. Manipulations in vitro may increase the apparent factor VII activity of plasma in such clotting assays, e.g., contact with a negatively charged surface, storage at 4°C, or allowing plasma to clot. Such manipulations are thought to convert native factor VII into an activated molecule that reacts more readily with tissue factor. One frequently does not know whether the clotting time obtained in a factor VII clotting assay reflects an effect of only native factor VII or of a mixture of native and activated factor VII.

The availability of a chromogenic substrate for activated factor X (factor Xa) enabled us to develop a new assay for factor VII in which its measurement is coupled to the generation of factor Xa (coupled amidolytic factor VII assay). We present herein a description of the assay and its use, together with a clotting assay, to calculate a factor VII activity ratio that allows evaluation of the activity state of factor VII in test materials. Data are also presented on measurement of factor VII activity by the two assays in patients with hereditary factor VII deficiency, patients with liver disease, and patients receiving warfarin.

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MATERIALS AND METHODS

Tissue factor was a saline extract of human brain tissue, centrifuged twice at 12,000 g to remove large particulate material. It clotted recalcified normal plasma in 17 sec. Purified factor X was prepared from normal human plasma by a method involving the following procedures: barium sulfate or barium citrate absorption, DEAE-cellulose chromatography, preparative polyacrylamide gel electrophoresis, and heparin-agarose column chromatography. The technique is described further elsewhere. Tris-buffered saline (TBS) was a solution containing 0.15 M NaCl and 0.05 M Tris, pH 7.5. The following materials were obtained commercially: the chromogenic substrate Bz-Ile-Glu-Gly-Arg-p-nitroanilide (S-2222) (Ortho Diagnostics, Raritan, N.J.); hereditary factor VII deficiency plasma (George King Bio-Medical, Salem, N.H.); kaolin (Mallinkrodt, St. Louis, Mo.). A factor VII reference standard assumed to contain 1 U factor VII activity/ml was prepared by pooling plasma from 12 healthy males and was stored in plastic tubes at -20°C.

Preparation of plasma and serum samples for factor VII assays. Venous blood was drawn with a plastic syringe through a 21-gauge needle from healthy subjects (hospital personnel), from patients with chronic liver disease, and from patients who had been receiving warfarin for over 6 mo. Nine parts blood were mixed with one part buffered citrate anticoagulant in a plastic tube; plasma was prepared by centrifugation twice at 12,000 g for 10 min at 4°C. Fresh plasma was kept on ice in plastic tubes and was tested within 4 hr. Additional plasma samples were frozen immediately by being placed in a -20°C freezer. Stored frozen plasma samples were also available from five patients with severe hereditary factor VII deficiency; one of these was provided through the courtesy of Dr. Christina Beurling-Harbury of Stanford University, Calif.

Serum was prepared by allowing 2 ml freshly drawn blood to clot at 37°C in a 12 × 75 mm glass tube. After incubation at 37°C for 2.5 hr the serum was separated by centrifugation two times at 12,000 g for 10 min at 4°C.

The coupled amidolytic assay for factor VII is carried out in two steps. In the first step, a test material containing factor VII is incubated with tissue thromboplastin, calcium, and purified factor X. Conditions are standardized so that the amount of factor Xa generated depends only upon the factor VII concentration of the test material. The formation of factor Xa is stopped after 3 min by the addition of EDTA and by cooling. In the second step, the factor Xa activity is measured by adding a subsample of the incubation mixture to an aliquot of S-2222 and determining the initial rate of cleavage of this chromogenic substrate.

The details of the method are as follows: Test material (0.1 ml), 0.05 ml tissue factor, and 0.05 ml factor X reagent (1.8 U/ml initial concentration) are warmed together in a plastic tube for 1 min at 37°C, following which 0.025 ml 50 mM CaCl2 is added. Exactly 3 min later, 0.025 ml 0.3 M Na2EDTA is added and the tube is placed in ice. A 0.1-ml subsample is transferred to a cuvette containing 0.1 ml S-2222 (1 mg/ml) and 0.6 ml TBS at 37°C. The rate of increase of absorbance at 405 nm (Abs/min) is measured using a Gilford 250 spectrophotometer attached to a Gilford 6050 recorder. Results are converted to factor VII activity in U/ml from a dilution curve made with 1:200 to 1:80 dilutions in TBS of the factor VII reference standard. Unknown samples were routinely tested in a 1:100 dilution in TBS. If very low levels were found, samples were tested again at a 1:50 dilution; if high levels were found, samples were tested again at a 1:200 dilution. A new dilution curve was made each time the assay was used. A representative dilution curve is shown in Fig. 1.

The clotting assay for factor VII activity was carried out by incubating 0.05 ml hereditary factor VII deficiency plasma, 0.1 ml tissue factor, and 0.05 ml adsorbed ox plasma in a glass tube for 3 min at 37°C. Then 0.05 ml test material and 0.1 ml 35 mM CaCl2 were added and the clotting time noted. A 1:10 dilution in TBS was used for test samples with an expected normal or low factor VII activity; 1:20 and 1:100 dilutions in TBS were used for test samples with an expected elevated factor VII activity. Clotting times were converted to factor VII activity in U/ml from a dilution curve prepared with 1:10 to 1:80 dilutions of the factor VII reference standard.

RESULTS

Standardization of the Coupled Amidolytic Assay

Factor X reagent. An ideal factor X reagent for the coupled amidolytic assay should contain neither factor Xa activity nor factor VII. Various prepara-
DILUTION OF FACTOR VII REFERENCE STANDARD. U/mL

Fig. 1. Representative dilution curve for coupled amidolytic assay, ΔAbs/min versus 1:80 to 1:400 dilutions of factor VII reference standard. ΔAbs/min of blank incubation mixture (TBS instead of dilution of reference standard) was 0.016, subtracted from value for each dilution of factor VII reference standard to give values shown.

Dilutions of our factor X reagent were free of factor Xa activity, i.e., no amidolytic activity could be measured in incubation mixtures of factor X reagent (final concentration 0.4 U/mL) and calcium when TBS was substituted for tissue factor and factor VII. However, all of our preparations of factor X reagent apparently contained traces of contaminating factor VII; when factor X reagent was incubated with tissue factor and calcium without added factor VII, a small amount of amidolytic activity invariably formed (ΔAbs/min 0.014-0.029). It was therefore necessary in calculating assay results to subtract the ΔAbs/min of an incubation mixture containing a TBS blank from the ΔAbs/min of incubation mixtures containing test materials (e.g., see legend to Fig. 1).

Figure 2 is a plot of the ΔAbs/min obtained when different concentrations of factor X reagent were incubated for 3 min with full-strength tissue factor, calcium, and a 1:100 dilution of the factor VII reference standard. One can see that a prohibitively high final concentration of factor X, exceeding 1.5 U/mL, would be required to standardize the assay on the plateau of the curve, i.e., to eliminate differences in assay results due to variations in the factor X concentration of incubation mixtures. Therefore as a compromise we chose a final concentration of factor X reagent of 0.4 U/mL, a value on the bend of the curve of Fig. 2. At this concentration small variations in the final concentration of factor X in the incubation mixture due to technical error had little apparent influence upon assay results (see data on reproducibility below).

Tissue factor reagent. The effect of diluting the tissue factor reagent was studied in incubation mixtures containing factor X reagent at a final concentration of 0.4 U/mL, calcium, and a 1:100 dilution of the factor VII reference standard. Although a dilution of more than 1:4 was required to decrease markedly
the amount of factor Xa generated, it was decided to standardize the assay using full-strength tissue factor reagent.

**Incubation time.** The effect of variation of the incubation time of the assay on the amount of factor Xa generated is shown in Fig. 3. A linear increase in factor Xa activity was found with increasing incubation time when the test material was either a 1:100 or 1:1000 dilution of the factor VII reference standard. This linear increase in generation of factor Xa persisted for at least 1 hr in the incubation mixture containing a 1:1000 dilution of the reference standard (final factor VII concentration in the incubation mixture 0.00044 U/ml). These data established the need for an exact incubation time; the assay was standardized to use an exact 3-min incubation time.

**Storage of plasma samples.** Fresh and frozen plasma samples from eight normal subjects were assayed for factor VII activity in the coupled amidolytic assay and in the clotting assay. No significant change in activity was observed in either assay between fresh samples and samples stored at -20°C for 1, 3, and 10 wk.

**Reproducibility of the assay.** A plasma sample from a healthy subject was tested ten times in a single day in the coupled amidolytic assay. A mean value of 1.10 ± 0.09 (SD) U/ml was obtained with a coefficient of variation of 0.078. The SE of duplicate determinations from 100 consecutive different plasma samples

![Fig. 2. Effect of variation of factor X concentration in incubation mixture upon ΔAbs/min in coupled amidolytic assay. Incubation mixtures contained following final concentrations of reagents: tissue factor 22%; CaCl2 5.5 mM; factor VII reference standard 0.0044 U/ml; variable concentrations of factor X reagent, as shown on abscissa. Incubation time 3 min.](image)

![Fig. 3. Effect of variation of incubation time upon generation of factor Xa in coupled amidolytic assay. Final concentrations of reagents of incubation mixture: factor X, 0.4 U/ml; tissue factor, 22%; CaCl2 5.5 mM; factor VII as shown.](image)
was calculated from the equation

$$SE = \pm \sqrt{\frac{(\text{difference between duplicates})^2}{2 \times \text{number of pairs}}}.$$ 

This yielded a value of 0.09 U/ml for both the coupled amidolytic and the clotting assays.

**Plasma Factor VII Activity in the Coupled Amidolytic and Clotting Assays**

**Healthy subjects.** The mean values for plasma factor VII of 57 healthy subjects (29 females, 28 males) were as follows: for the coupled amidolytic assay, 1.13 ± 0.25 (SD) U/ml; for the clotting assay, 0.99 ± 0.24 U/ml. The difference between these means was significant ($t = 2.978$, $p < 0.01$). The individual values obtained in the coupled amidolytic assay are plotted against the individual values obtained in the clotting assay in Fig. 4. A correlation coefficient of $r = 0.800$ ($p < 0.01$) was found. Note that the value for the intercept was 0.30 U/ml, a value that differs significantly from zero ($p < 0.01$).

A factor VII activity ratio was derived by dividing the value for factor VII activity measured in the clotting assay by the value for factor VII activity measured in the coupled amidolytic assay ($\text{VII}_c / \text{VII}_\text{am}$). Mean values for this ratio were as follows: for the 29 females, 0.85 ± 0.14; for the 28 males, 0.92 ± 0.11. The difference between the means was not significant ($t = 1.914$, $p > 0.05$).

**Hereditary factor VII deficiency.** Stored plasma samples from five patients with severe factor VII deficiency yielded the following corresponding values: in the coupled amidolytic assay, 0.069, 0.006, 0.001, 0.001, and 0.001 U/ml; in the clotting assay, 0.072, 0.007, 0.007, 0.006, and 0.005 U/ml.

**Patients receiving warfarin.** In patients receiving warfarin, significantly higher values were found for factor VII activity in the coupled amidolytic assay than in the clotting assay. Mean values for 27 patients were as follows: in the coupled amidolytic assay, 0.49 ± 0.24 (SD) U/ml; in the clotting assay, 0.25 ± 0.15 U/ml. The factor VII activity ratios for healthy subjects and for patients

![Fig. 4. Factor VII activity as measured in coupled amidolytic assay (\text{VII}_am) versus factor VII activity as measured in clotting assay (\text{VII}_c) for 57 healthy subjects. Equation of regression line $y = 0.30 + 0.834x$.](image-url)
receiving warfarin are plotted in Fig. 5. Clearly, patients receiving warfarin have a lower factor VII activity ratio (mean ratio 0.5) than healthy subjects.

Patients with chronic liver disease. Mean values for factor VII activity in 11 ambulatory patients with chronic parenchymal liver disease were as follows: in the coupled amidolytic assay, 0.84 ± 0.4 (SD) U/ml; in the clotting assay, 0.66 ± 0.28 U/ml. The difference between these mean values was not significant. Moreover, in contrast to the patients receiving warfarin, the mean factor VII activity ratio of patients with chronic liver disease (mean ratio 0.82) did not differ significantly from the mean factor VII activity ratio of healthy subjects (see Fig. 5).

Normal plasma stored at 4°C. Storage at 4°C increases factor VII activity in plasma from women taking oral contraceptives and in certain healthy subjects who are not taking such drugs. Plasma samples from 12 males and 3 females (one of whom was taking oral contraceptives) were stored for 24 hr at 4°C to compare the effect of such storage upon the plasma factor VII activity measured by the coupled amidolytic and clotting assays. Four subjects were identified as “cold activators,” i.e., the factor VII activity in their plasma increased strikingly when measured in the clotting assay (mean initial value 1.05 U/ml; mean value after cold storage 7.16 U/ml). Eleven subjects were identified as “non-cold activators,” i.e., the factor VII activity of their plasma failed to increase after storage in the cold. However, in the coupled amidolytic assay plasma from neither group changed significantly in factor VII activity. Consequently, the factor VII activity ratio of the “cold activators” rose strikingly after storage in the cold, whereas the factor VII activity ratio of the “non-cold activators” failed to change significantly (see Table 1).

When the standard 3-min incubation time of the coupled amidolytic assay was shortened to an incubation time of seconds, a difference could be demonstrated...
between plasma stored at 4°C from “cold activators” and from “non–cold activators.” Factor Xa activity formed within 15 sec after adding CaCl₂ when the former plasma was used as the test material. In contrast, factor Xa activity did not form until 37 sec after adding CaCl₂ when the later plasma was used as the test material (Fig. 6). The difference in reactivity of the two types of plasma lessened progressively as the incubation time was increased. This experiment was carried out four times using plasma from two “cold activators” and yielded similar results.

**Normal plasma exposed to kaolin.** Fresh plasma from ten healthy subjects who were “non–cold activators” was exposed to kaolin powder to induce contact activation of the plasma. Kaolin suspension (20 mg/ml) was added (0.05 ml) to 0.5 ml fresh citrated plasma, the mixture was incubated at 4°C for 4.5 hr with occasional shaking, and the kaolin was removed by centrifugation. The plasma was then tested immediately for factor VII activity in both the clotting and the
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coupled amidolytic assays. A subsample was stored at 4°C for a further 20-hr period and tested again for factor VII activity in both assays. The samples tested immediately showed a five to tenfold increase in factor VII activity in the clotting assay but no increase in factor VII activity in the coupled amidolytic assay (see Table 1). As a consequence, the mean factor VII activity ratio increased about eightfold. Further storage at 4°C did not change the factor VII activity of the samples.

Decay in vitro of factor VII activity in plasma from two "cold activators." Plasma from two "cold activators" was kept for several days at 4°C in plastic tubes. Kaolin was added to one of the samples in the amount described above. Subsamples were removed at different times and assayed for factor VII activity in the coupled amidolytic and clotting assays (Fig. 7). Factor VII activity as measured in the coupled amidolytic assay progressively declined to about 30% of the initial activity in 5–7 days. In contrast, factor VII activity as measured in the clotting assay increased markedly during the first 48 hr and then began to decline. However, after 5 days of storage in the cold, large amounts of activated factor VII were still present.

Serum factor VII activity. Factor VII activity in plasma and serum from 21 healthy subjects was measured in the clotting and coupled amidolytic assays (see Table 1). In the clotting assay, the apparent mean factor VII activity of the serum was four times greater than the mean factor VII activity of the plasma. In the coupled amidolytic assay, the mean factor VII activity of plasma and serum did not differ significantly.

Further experiments were carried out to rule out error in the measurement of factor VII activity in the clotting assay due to possible traces of thrombin or

![Fig. 7. Factor VII activity measured in clotting (VIIc) and coupled amidolytic (VIIam) assays in plasma from two "cold activators" standing at 4°C for 5–8 days. Kaolin powder was also added to plasma from one subject (solid line).]
factor Xa in serum separated from the clot 2.5 hr after clotting. Serum samples from ten subjects, each of whose plasma factor VII activity was known not to rise on storage in the cold, was tested for factor VII activity 2.5 hr after clotting and again after storage overnight at 4°C to allow for decay of possible trace thrombin and factor Xa activities. The mean values obtained in the clotting assay for factor VII were as follows: plasma 0.98 U/ml; serum 2.5 hr after clotting 4.34 U/ml; serum 24 hr after clotting 3.52 U/ml.

Serum prepared 2.5 hr after clotting was diluted 1:100 as for assay for factor VII and tested for thrombin by an amidolytic assay with the chromogenic substrate S-2238 (H-D-phenylalanyl-L-pipecolyl-L-arginineparanitroanilide-2HCl). Six such samples were found to contain from 0.0025–0.0080 U/ml of thrombin. When different amounts of thrombin were then added to a 1:100 dilution of normal plasma immediately before testing the diluted plasma for factor VII activity in the clotting assay, shortening of the clotting time due to thrombin was not observed until its concentration in the test plasma exceeded 0.075 U/ml.

Serum prepared 2.5 hr after clotting and diluted 1:100 did not contain enough factor Xa activity to be measurable when the diluted serum was added directly to the chromogenic substrate S-2222. Moreover, in a supplemental experiment two samples of factor X-deficiency plasma were tested for factor VII activity in the clotting assay before and 2.5 hr after recalcification. The following mean values were found: before recalcification 0.71 U/ml; after recalcification 3.62 U/ml. It therefore appears that error in the assay due to either factor Xa or thrombin could not account for the increased factor VII activity of serum in the clotting assay for factor VII.

DISCUSSION

The data presented herein establish that factor VII may be measured reproducibly by a coupled amidolytic technique based upon the ability of factor VII in a test material to catalyze the generation of factor Xa. The assay differs from the one-stage clotting assay for factor VII in several respects. First, and most important, the coupled amidolytic assay measures the total factor VII content of a test material irrespective of the activity state of factor VII; for example, the same level of factor VII is found before and after activation of factor VII in plasma with kaolin (see Table 1). Second, the coupled amidolytic assay is carried out with a 1:100 dilution of the test material, when the expected factor VII level is about 1 U/ml. This enables one to assay minute subsamples of a test material. Finally, since the coupled amidolytic assay measures generation of factor Xa, the assay may be used to measure factor VII accurately in test samples containing activated factors participating after factor Xa in the blood coagulation reactions, namely, thrombin-activated factor V or thrombin itself. Thus the assay has wide potential applicability. The major factor limiting its use is the need to prepare a purified human factor X reagent.

To comprehend why the clotting assay is sensitive to activated factor VII whereas the coupled amidolytic assay is not, one must understand what each assay measures. In the clotting assay, the endpoint reflects the initial rate of increase of thrombin activity in the clotting mixture, a rate that depends upon the initial rate of increase of factor Xa activity. The latter, in turn, is critically dependent upon the initial rate of the interaction between tissue factor, factor VII, and factor X. Activated factor VII, the two-chain molecule,9,10 is thought
to react more rapidly with tissue factor and factor X than does native factor VII and hence will shorten the clotting time of the assay.

The coupled amidolytic assay measures not the initial rate of generation of factor Xa but the total factor Xa formed during the 3-min incubation period. Despite an initial faster generation of factor Xa in the presence of activated factor VII (Fig. 6), the total amount of factor Xa generated over 3 min is not increased. Two explanations for this observation can be postulated. Conceivably, the initial factor Xa formed in the incubation mixture rapidly activates the native factor VII in the test material, thus compensating over 3 min for initial differences in the activity state of factor VII. Alternatively, tissue factor in the presence of calcium ions could increase factor VII reactivity in a way that compensates over 3 min for initial differences in the activity state of factor VII.

Factor Xa not only activates but also inactivates factor VII, and this inactivation has been postulated to function as a self-damping mechanism for the extrinsic clotting system. If factor Xa inactivated factor VII in the incubation mixture of the coupled amidolytic assay, then the rate of formation of factor Xa should decrease as the incubation period is lengthened. However, factor Xa was found to increase linearly in the incubation mixture of the coupled amidolytic assay even when the incubation period was prolonged to 1 hr (see Fig. 3). Thus at the concentrations of factor VII and factor X used in these experiments inactivation of factor VII by factor Xa was not evident. Whereas these data indicate that inactivation of factor VII by factor Xa does not affect the results of the coupled amidolytic assay, they should not be viewed as evidence against the hypothesis that inactivation by factor Xa dampens factor VII activity at the much higher relative concentrations of factor VII to factor Xa existing during normal hemostasis.

Normal plasma samples yielded a slightly but significantly higher mean value for factor VII activity in the coupled amidolytic assay (1.13 U/ml) than in the clotting assay (0.99 U/ml). This observation is difficult to explain, since the same pooled plasma was used as the reference standard in both assays. Moreover, storage of individual plasma samples for up to 2.5 mo did not result in loss of factor VII activity as measured in either assay. We therefore have to assume that pooling plasma to make the reference standard was associated with the loss of a small amount of factor VII activity as measured in the coupled amidolytic assay but not as measured in the clotting assay.

When in a given sample factor VII activity is measured only by the clotting assay (VIIc), one cannot tell whether the test result represents native factor VII, activated factor VII, or a mixture of both activities. However, when factor VII activity is also measured by the coupled amidolytic assay (VIIam), one can evaluate the activity state of factor VII in the sample by calculating the ratio VIIc/VIIam. High factor VII activity ratios (4–8.5) were found in test materials subjected to manipulations known to activate factor VII (see Table 1).

Interestingly, low factor VII activity ratios (mean ratio 0.5) were found in plasma from patients receiving warfarin. Although both assays yielded reduced factor VII levels, the values obtained in the clotting assay were consistently lower than the values obtained in the coupled amidolytic assay. Warfarin inhibits the formation of gamma-carboxyglutamic acid residues in the vitamin K–dependent clotting proteins. Administration of warfarin has been shown to
give rise to a spectrum of partially carboxylated prothrombin molecules in human plasma. The present finding of higher factor VII activity in the coupled amidolytic assay than in the clotting assay is compatible with the presence of partially carboxylated factor VII molecules in human plasma after warfarin. Such molecules, reacting less effectively with tissue factor than normal factor VII molecules, could fail to react rapidly enough to influence the clotting assay yet still support the generation of factor X during the 3-min incubation period of the coupled amidolytic assay. Presumably, such factor VII molecules with reduced reactivity would also fail to influence the prothrombin time tests used to establish the therapeutic ranges for oral anticoagulant therapy. This possibility raises interesting theoretic questions about the relation between prothrombin time test results and the antithrombotic effectiveness of oral anticoagulants in different clinical settings.

Determination of the factor VII activity ratio has wide potential application in the study of patients with thrombotic disorders, in the characterization of laboratory reagents, and in the monitoring of the activity state of factor VII in blood components for transfusion therapy. This last application seems to us of particular importance. If the increased factor VII activity in plasma from "cold activators" after storage at 4°C persists for several days (Fig. 7) and if 10%–15% of normal men and women are "cold activators," then transfusions of whole blood or plasma stored at 4°C in a blood bank could be associated with the frequent infusion into patients of substantial amounts of factor VII with increased reactivity. A systematic study of this possible hazard of transfusion therapy is planned.

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Coupled amidolytic assay for factor VII: its use with a clotting assay to determine the activity state of factor VII

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