Derivatization of Cysteine and Cystine for Fluorescence Amino Acid Analysis with the o-Phthaldialdehyde/2-Mercaptoethanol Reagent*

(Received for publication, November 21, 1978, and in revised form, January 30, 1979)

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Previous reports (Drescher, D. G., and Lee, K. S. (1978) Anal. Biochem. 84, 559-569; Lee, K. S., and Drescher, D. G. (1978) Int. J. Biochem. 9, 457-467) have shown that high performance liquid chromatographic analysis of amino acids with the o-phthaldialdehyde/2-mercaptoethanol reagent (OPA/2-ME) is one of the most sensitive procedures currently available for micro amino acid analysis. In the present paper, methods are presented for the modification of cysteine and cystine in proteins for micro amino acid analysis using OPA/2-ME. Cysteine and cystine, which both show low fluorescence with OPA/2-ME, are converted to cysteic acid with performic acid directly, or to S-3-sulfopropylcysteine with 1-propane sultone after reduction of cysteine with tri-n-butylphosphine. Cysteic acid and S-3-sulfopropylcysteine form highly fluorescent adducts with OPA/2-ME. The formation of S-3-sulfopropylcysteine in proteins and the subsequent hydrolysis of the proteins with methanesulfonic acid are particularly useful for complete amino acid analysis at the picomole level using a single sample.

Since Roth (1) first reported that amino acids form highly fluorescent adducts in basic aqueous solutions with o-phthaldialdehyde and 2-mercaptoethanol, amino acid analysis using this fluorometric reagent has gained acceptance as an alternative to amino acid analysis using ninhydrin (2–6). The reaction of OPA and 2-ME with amino acids is shown in Fig. 1. The OPA reagent, used in conjunction with high performance liquid chromatography, is at least 10 to 100 times more sensitive than ninhydrin for the detection of most amino acids (6). Although OPA/2-ME does not form fluorescent adducts with proline and forms only weakly fluorescent adducts with lysine, proline is accurately detected after oxidation with performic acid directly, or to S-3-sulfopropylcysteine after addition of intermediates fluorescence after adding the detergent Brij to the reaction mixture (2, 11). Cysteine and cystine both form adducts of low fluorescence with the OPA reagent, but no

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§ The abbreviations used are: OPA, o-phthaldialdehyde; 2-ME, 2-mercaptoethanol; MSA, 4N-methanesulfonic acid containing 0.2% tryptamine; FS, 3-hydroxypropanesulfonic acid γ-sultone (1,3-propane sultone); TBP, tri-n-butylphosphine.

The reason for the low fluorescence of OPA/2-ME adducts of cysteine and cystine is not known. It is possible that disulfhydryl groups methods have yet been detailed for derivatizing these amino acids to yield high fluorescence adducts specifically for OPA/2-ME analysis. In a previous study (6), we determined the relative fluorescence of OPA/2-ME adducts of six common cysteine derivatives and showed that the adducts of cysteic acid and S-3-sulfopropylcysteine have 30- to 40-fold greater fluorescence than the "half-cystine" adduct. In the present study, we therefore, compare the suitability for fluorescence amino acid analysis of sulfopropylation (12) and performate oxidation (13, 14), the reactions which form S-3-sulfopropylcysteine and cysteic acid, respectively. We have chosen egg white lysozyme (containing 4 cystines) and human carbonic anhydrase C (containing 1 cysteine) for these studies. In doing so, we establish a general, highly sensitive method for complete micro amino acid analysis of proteins using OPA/2-ME.

MATERIALS AND METHODS

Materials—Reagents of the highest purity available were used for amino acid analyses (5–9). All other chemicals were of reagent grade or better. Purified carbonic anhydrase C from human erythrocytes was obtained from Worthington. Egg white lysozyme (3 times crystallized, dialyzed, lyophilized), OPA, and cysteic acid were from Sigma, and 2-ME was from Calbiochem. Amino acid standards, 4N-methanesulfonic acid containing 0.2% tryptamine, constant boiling HCl (Sequanal grade), S-3-sulfopropylcysteine, Brij-35 (30% solution), and thioglycol (25% solution) were from Pierce Chemical Co., Rockford, IL. Hydrogen peroxide (30%) was from Fisher, and tri-n-butylphosphine and 1-propane sultone were obtained from Aldrich Chemical Co. Inc., Milwaukee, WI. Ignition tubes (Corning 9860, 14 × 100 mm) were from Corning Glass Works, Corning, NY. The tubes were prewashed in a solution of concentrated H2SO4 and HNO3 (9:1) before use.

Performic Acid Oxidation—The method of performic oxidation described by Hirs (13, 14) was modified in this study for use with microgram amounts of protein. One hundred μl of a 10 μM solution of egg white lysozyme or human carbonic anhydrase C (1 nmol of each protein) was placed in an ignition tube, and the solution was frozen on dry ice and lyophilized. Performic acid was prepared by adding 0.1 ml of 30% hydrogen peroxide to 1.9 ml of 88% formic acid. The solution was allowed to stand in a closed container at 25°C for 2 h. Then the mixture was cooled to 0°C and used immediately. One hundred μl of performic acid was added to the ignition tube containing the protein, and the mixture was allowed to stand at 0°C for 2.5 h. The reaction was terminated by adding 2 ml of cold deionized water, and the solution was frozen on dry ice and lyophilized.

Sulfopropylation—Either carbonic anhydrase or lysozyme was treated with TBP and PS. TBP reduces cysteine to cysteine (15–17), and PS alkylates cysteine to S-3-sulfopropylcysteine (12), and these reactions can be performed simultaneously. The method of Ruegg and Rudinger (12) was modified for microanalysis as follows. One nmoi of protein that had been lyophilized in an ignition tube was dissolved in 0.1 ml of aqueous 0.5 M NaHCO3 mixed with n-propanol (1:1, v/v). Ten μl of 50 mM TBP in 100% n-propanol and 6 μl of 240 which are present in pure cysteine or in cysteine formed from cystine in the presence of 2-ME compete intramolecularly with 2-ME for position 1 in the isoindole (Fig. 1, and S. S. Simons, Jr., personal communication).
mm PS in 60% n-propanol (both solutions prepared immediately before use) were added, and the reaction mixture was kept under N₂ at 25°C for 2 h. The reaction was terminated by adding 50 μl of constant boiling HCl, and the mixture was diluted with 0.5 ml of deionized water, frozen, and lyophilized. Lyophilization, instead of rotary evaporation as reported by Rüegg and Rudinger (12), was performed because the small amounts of protein were apparently more prone to rotary evaporation, evidenced by amino acid analyses of decreased accuracy (data not shown). Because PS is a suspected carcinogen (18,19) the following precautions were taken. Three Pyrex vapor traps (Corning 7229, 28 X 200 mm) cooled with dry ice in acetone were connected in series between the lyophilization flask and the lyophilized sample. The sample tube, insulated with a piece of Styrofoam, was placed inside the flask. One ml of 100% NaOH was placed inside each trap. Thus the PS was condensed and hydrolyzed to noncancerogenic products.

Preparation of Samples for Amino Acid Analysis—One nmol protein samples were hydrolyzed with 100 μl of MSA at 115°C for 24 h according to the method of Simpson et al. (20) modified by Lee and Drescher (6). After hydrolysis, the samples were partially neutralized with 100 μl of 3.5 N NaOH. Some samples were hydrolyzed with 100 μl of constant boiling HCl at 110°C for 22 h (12) instead of with MSA, and these samples were partially neutralized with 100 μl of 5.25 N NaOH (21). When necessary, neutralized samples were appropriately diluted with the first buffer used for amino acid analysis. Analytical performance was carried out with a Hitachi high-performance liquid chromatography system (American Instrument Co., Silver Spring, MD) using OPA/2-ME (6, 6).

RESULTS

Table I shows the amino acid compositions of (A) egg white lysozyme and (B) human carbonic anhydrase C, determined for four different treatments (columns 1 to 4) namely: 1) no treatment of the protein, followed by hydrolysis of the protein in MSA; 2) performic acid oxidation and hydrolysis in MSA; 3) reduction with TBP, sulfohprolylation with PS, and hydrolysis in HCl; and 4) reduction with TBP, sulfohprolylation with PS, and hydrolysis in MSA. The actual amino acid compositions, determined from sequence data (22, 23), are shown in column 5. For the untreated proteins (column 1), cysteine and cystine were difficult to detect because of the known acid lability of these amino acids (24) and the low fluorescence of the cystine-OPA/2-ME adduct (5, 6), and, therefore, were not determined. However, for proteins treated with performic acid (column 2), cysteine and cystine were accurately determined because of the relatively high fluorescence of the cysteic acid-OPA/2-ME adduct (6). The methionine sulfone resulting from performic acid oxidation of methionine (13, 14) was also determined using OPA/2-ME. However, with performic acid oxidation, tryptophan was completely lost and tyrosine was partially destroyed. When the proteins were treated with TBP and PS and hydrolyzed in HCl according to the modified method of Rüegg and Rudinger (12) (column 3, and “Materials and Methods”), the recoveries of all of the amino acids were good, except that tryptophan

3 Incomplete recovery of tyrosine has been shown to be due to halides such as chloride (13, 14, 25). Halide impurities in the reagents are probably important at the low concentrations of protein employed in the present study (M. Doscher, personal communication).

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### Table I

#### A. Lysozyme

<table>
<thead>
<tr>
<th>Protein</th>
<th>1. Untreated (MSA)</th>
<th>2. Treated with performic acid (MSA)</th>
<th>3. Treated with TBP and PS (HCl)</th>
<th>4. Treated with TBP and PS (HCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>20.4 ± 0.3</td>
<td>22.0 ± 0.4</td>
<td>21.1 ± 0.3</td>
<td>21.5 ± 0.3</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.5 ± 0.2</td>
<td>6.7 ± 0.1</td>
<td>6.6 ± 0.1</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>Serine</td>
<td>8.9 ± 0.1</td>
<td>9.2 ± 0.3</td>
<td>9.1 ± 0.2</td>
<td>9.4 ± 0.3</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>5.2 ± 0.1</td>
<td>5.5 ± 0.1</td>
<td>5.4 ± 0.1</td>
<td>5.5 ± 0.3</td>
</tr>
<tr>
<td>Alanine</td>
<td>12.3 ± 0.2</td>
<td>12.2 ± 0.1</td>
<td>11.7 ± 0.3</td>
<td>12.5 ± 0.2</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.4 ± 0.1</td>
<td>8.4 ± 0.1</td>
<td>8.4 ± 0.1</td>
<td>8.4 ± 0.1</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.3 ± 0.1</td>
<td>1.8 ± 0.2</td>
<td>2.9 ± 0.3</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.0 ± 0.1</td>
<td>3.1 ± 0.2</td>
<td>3.0 ± 0.1</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.4 ± 0.4</td>
<td>1.8 ± 0.2</td>
<td>1.6 ± 0.3</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>8.5 ± 0.1</td>
<td>N.D.</td>
<td>8.5 ± 0.1</td>
<td>5.0 ± 0.2</td>
</tr>
<tr>
<td>Lysine</td>
<td>6.5 ± 0.1</td>
<td>6.1 ± 0.2</td>
<td>6.2 ± 0.3</td>
<td>5.9 ± 0.3</td>
</tr>
<tr>
<td>Arginine</td>
<td>11.3 ± 0.2</td>
<td>10.8 ± 0.3</td>
<td>11.0 ± 0.3</td>
<td>10.6 ± 0.3</td>
</tr>
</tbody>
</table>

#### B. Carbonic anhydrase

<table>
<thead>
<tr>
<th>Protein</th>
<th>Untreated (MSA)</th>
<th>Treated with performic acid (MSA)</th>
<th>Treated with TBP and PS (HCl)</th>
<th>Treated with TBP and PS (HCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>28.2 ± 0.9</td>
<td>29.5 ± 1.0</td>
<td>28.1 ± 1.0</td>
<td>29.6 ± 0.9</td>
</tr>
<tr>
<td>Threonine</td>
<td>11.5 ± 0.2</td>
<td>11.5 ± 0.1</td>
<td>11.8 ± 0.2</td>
<td>12.7 ± 0.2</td>
</tr>
<tr>
<td>Serine</td>
<td>16.6 ± 0.3</td>
<td>16.4 ± 0.6</td>
<td>17.3 ± 0.3</td>
<td>18.0 ± 0.3</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>24.2 ± 0.1</td>
<td>24.4 ± 0.5</td>
<td>24.5 ± 0.1</td>
<td>25.7 ± 0.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>17.2 ± 1.5</td>
<td>17.8 ± 0.8</td>
<td>17.7 ± 1.2</td>
<td>16.4 ± 1.9</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>22.3 ± 0.3</td>
<td>22.0 ± 0.7</td>
<td>21.7 ± 0.3</td>
<td>22.8 ± 0.3</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>13.9 ± 0.2</td>
<td>13.5 ± 0.4</td>
<td>13.9 ± 0.2</td>
<td>14.0 ± 0.2</td>
</tr>
<tr>
<td>Histidine</td>
<td>16.7 ± 0.2</td>
<td>16.4 ± 0.9</td>
<td>16.4 ± 0.3</td>
<td>15.8 ± 0.7</td>
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<tr>
<td>Tryptophan</td>
<td>1.2 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.0</td>
</tr>
<tr>
<td>Lysine</td>
<td>8.8 ± 1.0</td>
<td>8.4 ± 0.4</td>
<td>8.5 ± 0.1</td>
<td>7.7 ± 0.3</td>
</tr>
<tr>
<td>Arginine</td>
<td>27.2 ± 0.4</td>
<td>27.2 ± 0.9</td>
<td>26.7 ± 0.9</td>
<td>26.7 ± 0.9</td>
</tr>
<tr>
<td>Asparagine</td>
<td>8.4 ± 0.4</td>
<td>8.2 ± 0.3</td>
<td>7.5 ± 0.3</td>
<td>8.6 ± 1.3</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>11.9 ± 0.4</td>
<td>11.0 ± 0.6</td>
<td>11.5 ± 0.3</td>
<td>12.4 ± 0.7</td>
</tr>
<tr>
<td>Histidine</td>
<td>11.1 ± 0.5</td>
<td>13.0 ± 1.0</td>
<td>12.1 ± 0.3</td>
<td>12.5 ± 0.7</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>6.4 ± 0.1</td>
<td>N.D.</td>
<td>6.5 ± 0.2</td>
<td>5.5 ± 0.2</td>
</tr>
<tr>
<td>Lysine</td>
<td>25.3 ± 0.8</td>
<td>23.9 ± 1.0</td>
<td>23.8 ± 0.3</td>
<td>22.9 ± 0.6</td>
</tr>
<tr>
<td>Arginine</td>
<td>7.3 ± 0.1</td>
<td>7.3 ± 0.3</td>
<td>7.3 ± 0.1</td>
<td>7.1 ± 0.1</td>
</tr>
</tbody>
</table>

a Column 1, A and B, shows the analysis of unmodified lysozyme and carbonic anhydrase, respectively, performed as described under “Materials and Methods.” MSA refers to hydrolysis in 4N-methanesulfonic acid containing 0.5% tryptophan. For lysozyme, the number of determinations, N, equals 6; for carbonic anhydrase, N = 3.
b Column 2 shows the analysis of performate-oxidized proteins (see text for details). For lysozyme, N = 4; for carbonic anhydrase, N = 4.
c Column 3 shows the analysis of protein treated with tri-n-butylphosphine (TBP) and 1,3-propane sultone (PS). “HCl” refers to hydrolysis in constant boiling HCl. For lysozyme, N = 8; for carbonic anhydrase, N = 3.
d Column 4 shows the analysis of proteins treated as in column 3, except that hydrolysis was performed with MSA. For lysozyme, N = 5; for carbonic anhydrase, N = 4.
e Column 5 shows the theoretical amino acid compositions for egg white lysozyme (22) and human carbonic anhydrase C.

Not determined (N.D.) for untreated proteins; determined as cysteic acid in column 2, and as S-3-sulfpropylcysteine in columns 3 and 4.

* Determined as methionine sulfone after performate oxidation.
human carbonic anhydrase C, even 1 cysteine residue (5, 23).

In the limit, as little as 10 to 20 ng of that S-3-sulfopropylcysteine is easily detected as the OPA/2-TBP and PS and hydrolysis in MSA. In Fig. 2, it can be seen that S-3-sulfopropylcysteine adduct with OPA/Z-ME has about the same fluorescence as the "high fluorescence" adduct (5). However, when MSA instead of HCl is used for hydrolysis (column 4), the sulfopropylation method yields good analyses of tryptophan (about 80%) and excellent analyses of the other amino acids. In addition, only a single hydrolysis sample is required for the determination of this amino acid (6). How- ever, when MSA instead of HCl is used for hydrolysis (column 4), the sulfopropylation method yields good analyses of tryptophan (about 80%) and excellent analyses of the other amino acids. In addition, only a single hydrolysis sample is required.

Inglis and Liu (30), Inglis et al. (31), and Simpson et al. (29) have described methods for obtaining complete ninhydrin amino acid analyses of proteins using single hydrolysates. A central part of these procedures is the formation of S-sulfocysteine from cysteine after treatment of the protein hydrolyzed with dithiothreitol and tetrathionate. However, these methods (20, 30, 31) are not suitable for OPA/2-ME analysis, because S-sulfocysteine yields an OPA/2-ME adduct that is only 2.9 times as fluorescent as the "halfcystine" adduct (6). The modified sulfopropylation method, as discussed above, is well suited for OPA/2-ME analysis because S-3-sulfopropyl- was completely destroyed, presumably because of the HCl used for the hydrolysis (26). The most accurate values, i.e., those closest to the actual amino acid compositions, were obtained after treatment with TBP and PS and hydrolysis in MSA (column 4).

Fig. 2 shows the elution diagrams for OPA/2-ME amino acid analysis of (A) egg white lysozyme and (B) human carbonic anhydrase C after treatment of the proteins with TBP and PS and hydrolysis in MSA. In Fig. 2, it can be seen that S-3-sulfopropylcysteine is easily detected as the OPA/2-ME adduct because of its high fluorescence and its early elution position (8 min). For lysozyme (Fig. 2A), with 8 "halfcystines" and 8 leucines, the S-3-sulfopropylcysteine peak is about the same size as the leucine peak, emphasizing the fact that the S-3-sulfopropylcysteine adduct with OPA/2-ME has about the same fluorescence as the "high fluorescence" adduct, leucine-OPA/2-ME (1, 5, 6). Fig. 2B shows that for human carbonic anhydrase C, even 1 cysteine residue (5, 23) can easily be determined by the method of sulfopropylation combined with OPA/2-ME amino acid analysis.

**DISCUSSION**

High performance liquid chromatographic amino acid analysis using the OPA reagent is probably the most sensitive liquid chromatographic method yet devised for studies of amino acid composition of proteins and peptides. In our studies, only 0.24 μg of hydrolyzed protein per analyzer run was sufficient to detect most of the amino acids with high accuracy (Table 1). In the limit, as little as 10 to 20 ng of material is adequate for compositional studies of proteins and peptides. This is particularly noteworthy, because with fluorescamine and ninhydrin, even under ideal conditions, analyses are 5 to 10 times less sensitive than with OPA/2-ME (2, 6).

However, the analysis of unaltered cysteine and cystine with OPA/2-ME presents certain problems. Cysteine and cystine yield OPA/2-ME adducts that are much less fluorescent than most of the other amino acid adducts (the "halfcystine" adducts). This is about 40-fold less fluorescent on an equi- molar basis, thus making the determination of cystine and cysteine difficult (1, 6). The problem is compounded by the low abundance of cysteine and cystine in proteins and by the fact that cystine and alanine show peak resolution that is extraordinarily pH dependent. Such characteristics make it important to develop a method for specifically modifying cysteine and cystine in proteins prior to OPA/2-ME amino acid analysis.

In the present paper, we have compared the suitability of four methods for performing complete micro amino acid analyses using OPA/2-ME (Table I). Analysis of unmodified proteins (column 1) yields values that are close to the actual values (column 5), with the exception of cysteine. Performate oxidation (column 2) suffers drawbacks because of the destruction of tryptophan and tyrosine. Sulfopropylation with HCl hydrolysis (column 3) yields good values for all the amino acids except tryptophan, and, therefore, a separate sample is required for the determination of this amino acid (6). However, when MSA instead of HCl is used for hydrolysis (column 4), the sulfopropylation method yields good analyses of tryptophan (about 80%) and excellent analyses of the other amino acids. In addition, only a single hydrolysis sample is required.

In our system (6), cystine and alanine peaks overlap above pH 3.25 and are optimally separated at pH 3.32. This kind of pH dependence occurs in other cation exchange systems as well (27). When protein is not treated with TBP and PS, but is hydrolyzed in MSA (Table 1, column 1), tryptophan is recovered at 91 to 93% yields. This 3 to 9% loss of tryptophan is due to MSA hydrolysis and can be corrected kinetically (20, 28). Protein that is treated with TBP and PS and hydrolyzed in MSA (column 4) gives tryptophan that is recovered at 79 to 83% yields. The difference in tryptophan recoveries between columns 1 and 4, 12 to 14%, is probably due to the TBP/PS reaction itself. Recovery of methionine in Table I appears to be somewhat low, by 10 to 15%, due to the TBP/PS reaction (compare columns 3 and 4 with columns 1 and 2; see also Ref. 12, p. 450). The mechanisms for these losses are unknown, and the losses cannot easily be corrected kinetically. However, for practical purposes, the losses are usually not a problem: methionine is commonly present in low amounts in proteins and can, therefore, be assigned accurate residue numbers despite small errors, and tryptophan is recovered in yields that are as good as, or better than, yields obtained by other hydrolysis techniques (26, 29). It is suggested that constant correction factors of 10% for methionine and 15% for tryptophan be applied when using the TBP/PS/MSA method. In addition, note that the 24-h values for threonine and serine in column 4, Table I, are close to the theoretical values. Values for the hydroxylic amino acids probably need not be corrected kinetically for MSA hydrolytic times of 24 h or less.
cysteine yields an OPA/2-ME adduct that is about 40-fold more fluorescent than the "half-cystine" adduct. In addition, the sulfopropylation method is straightforward; the reduction, alkylation, and MSA hydrolysis are performed in a single hydrolysis tube.

The power of the combined approach of sulfopropylation, MSA hydrolysis, and OPA/2-ME amino acid analysis is well illustrated for human carbonic anhydrase C (Fig. 2B). It is difficult to obtain an accurate determination of 1 cysteine in 259 amino acid residues (5), especially for small amounts of protein. The modified sulfopropylation method reported here yields both an accurate determination of the single cysteine in human carbonic anhydrase C and accurate determinations of all of the other amino acids in the protein (Table I, column 4). It is suggested that the method for micro amino acid analysis using OPA/2-ME presented here and in previous papers (5, 6) has the potential for replacing ninhydrin amino acid analysis as a common biochemical technique.

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Derivatization of cysteine and cystine for fluorescence amino acid analysis with the o-phthalaldehyde/2-mercaptoethanol reagent.

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