Effects of intracameral Na$_2$EDTA and EGTA on aqueous outflow routes in the monkey eye

Anders Bill, Elke Lütjen-Drecoll, and Björn Svedbergh

Intracameral perfusion with 4 to 6 mM Na$_2$EDTA or 4 mM EGTA for 40 to 80 min caused a very large increase in gross outflow facility. This effect was partly reversible when followed by perfusion with mock aqueous humor. Eyes perfused with Na$_2$EDTA were studied morphologically. In the trabecular meshwork the cells separated due to a splitting of the cell junctions. A distention of the cribiform meshwork, a wash-out of extracellular material, and a disintegration of the denuded trabecular cores were also noticed. The inner wall of Schlemm’s canal protruded in a “balloonlike” manner into the lumen of the canal and showed frank ruptures, especially after prolonged perfusion times. The conventional outflow pathways beyond Schlemm’s canal showed no abnormalities. In the uveoscleral outflow routes the anterior and middle parts of the ciliary muscle demonstrated very wide intermuscular clefts and many degenerated muscle fibers. The posterior third of the muscle was normal. So were the ciliary epithelium, the choroid, and the retina. The pupillary sphincter also showed degeneration. The corneal endothelial cells separated, starting at the apical junctional complex.

Key words: aqueous humor, facility of outflow, Na$_2$EDTA, EGTA, cornea, morphology

It is well known from animal experiments that perfusion of the anterior chamber with isotonic solutions of NaCl tends to reduce the resistance to outflow in the routes draining aqueous humor. The mock aqueous humor described by Bárány, which is buffered and contains glucose and calcium and magnesium ions, reduces the tendency to spontaneous reduction in outflow resistance. But in prolonged experiments even refined solutions tend to cause a considerable rise in facility compared to perfusion with aqueous humor. The calcium ions are of importance for cell adhesion and the permeability in many systems. It seemed likely then that the marked spontaneous change in resistance seen with calcium-free perfusates was at least partly due to a lowering of the calcium concentration in the outflow routes. And it seemed possible that a short-lasting complete elimination of calcium ions from the anterior chamber fluid might be a procedure of interest in the treatment of glaucoma.

This communication reports on experiments in which the anterior chamber was perfused with calcium-free fluid containing...
Table I. Effect of Na₂EDTA perfusion of the anterior chamber on outflow facility

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Facility of outflow (µl/min/mm Hg)</th>
<th>Fluid on control side</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treated eye</td>
<td>Control eye</td>
</tr>
<tr>
<td></td>
<td>Start</td>
<td>End</td>
</tr>
<tr>
<td>1</td>
<td>0.19</td>
<td>3.54</td>
</tr>
<tr>
<td>2</td>
<td>0.22</td>
<td>4.35</td>
</tr>
<tr>
<td>3</td>
<td>0.51</td>
<td>3.26</td>
</tr>
<tr>
<td>5⁺</td>
<td>0.36</td>
<td>2.31</td>
</tr>
<tr>
<td>6</td>
<td>0.43</td>
<td>2.72</td>
</tr>
<tr>
<td>7*</td>
<td>0.57</td>
<td>2.99</td>
</tr>
<tr>
<td>8</td>
<td>n.d.</td>
<td>4.48</td>
</tr>
<tr>
<td>9†</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

* 6 mM Na₂EDTA, otherwise 4 mM.
† Investigated morphologically.

Na₂EDTA or (ethyleneglycol-bis-β-aminoethyl ether)-N,N',tetraacetic acid (EGTA). These chelating agents were used to bind Ca ions entering the anterior chamber with the aqueous humor or by diffusion from the tissues. Previous experiments by Warner and Chu⁴ had demonstrated that EDTA injections into the anterior chamber in cats causes a marked and reversible increase in outflow facility.

The results of the present study indicated marked effects on the outflow resistance in monkeys. These effects were analyzed morphologically. Effects on the cornea, briefly described elsewhere,⁵ are also reported.

Materials and methods

Cynomolgus monkeys (Macaca fascicularis) of both sexes and weighing 2.2 to 2.6 kg were employed. The animals were anesthetized with sodium methohexitol (Brietal Sodium; Lilly), 50 to 100 mg given intramuscularly. Anesthesia was maintained by a slow intravenous infusion of sodium pentobarbital. The animal was placed prone and kept warm with a heating pad. The arterial blood pressure was measured in a tail artery. The anterior chamber of each eye was connected to an infusion apparatus through a needle and polyethylene tubing. Another needle connected it to a continuously weighed reservoir, and a third needle to a pressure transducer. A needle gun was used to cannulate the eyes. The needles have been described elsewhere.⁶ At the start of an experiment the reservoir and tubing connecting it to the anterior chamber were filled with mock aqueous humor, which contained CaCl₂ (0.170 gm/L) and MgCl₂ (0.064 gm/L). The syringe of the infusion apparatus contained modified mock aqueous humor as will be described under results. In one experiment, push-pull–coupled 5 ml syringes were used to perfuse the eyes at their spontaneous pressures. The rate of perfusion in this experiment was 20 µl/min.

Facility measurements. Gross outflow facility was determined in two different ways. One way was to observe the inflow from the reservoir into the anterior chamber at two different reservoir heights. The difference in inflow rate was divided by the difference in pressure. With this procedure there was no inflow from the infusion apparatus. The other way was to have a continuous infusion, 20 µl/min, from the infusion apparatus into the eye and flow from the eye into the reservoir which was varied by changing the level of the reservoir. The flow into the reservoir was less than the inflow from the infusion apparatus; that is, there was a net inflow from the external circuits into the anterior chamber. Gross outflow facility was calculated by dividing the change in net inflow from the external system caused by a rise in reservoir level by the change in pressure. In one experiment without facility determination, the intraocular pressure (IOP) was maintained at its spontaneous level throughout the experiment. In all the other experiments the IOP was kept between the spontaneous values and 10 mm Hg above those values.

Morphology. Three eyes perfused with Na₂EDTA (Titriplex III; Merck, Darmstadt, West Germany), and their controls were investigated.
morphologically. At the end of the physiological part of the experiment the animal was killed by an overdose of pentobarbital sodium, and the eyes were immediately perfused with the fixative with the use of push-pull–coupled precision syringes connected to the tubings previously used for infusion and facility determinations. In monkey 4 the perfusion fixative was 4% glutaraldehyde in 0.1M Sørensen’s phosphate buffer, pH 7.4; in monkeys 5 and 9 the fixative was a mixture of 2% glutaraldehyde and 1% formaldehyde in the same buffer. The eyes were dissected according to earlier described principles. The eye pressure was maintained at 5 to 8 mm Hg during fixation.

For scanning electron microscopy (SEM) the preparative procedures have been described earlier, briefly consisting of postfixation in osmium, critical-point drying with carbon dioxide from acetone, gold-sputtering, and examining in a JEOL SM-U3, including counting and measuring of the pores of the inner wall of Schlemm’s canal.

For transmission electron microscopy (TEM) the specimens were processed as earlier, briefly summarized as postfixation in osmium, embedding in Epon 812, ultrasectioning, double-staining, and examining in a Siemens Elmscope 1A and a JEOL 100-B. Semithin sections were also examined by light microscopy (LM).

Results

Experiments with EDTA. Eight experiments were performed in which the anterior chamber was perfused with mock aqueous humor1 less Ca++ and Mg++ and with 4 or 6 mM Na2 EDTA added. Table I shows that during the infusion of Na2 EDTA there was a marked rise in gross outflow facility at 4 mM as well as at 6 mM. Some 20 min after the start of the perfusion the outflow facility started to increase and was near maximal at 40 to 50 min. In two experiments, monkeys 6 and 7, the infusion of EDTA was stopped after 60 to 80 min, and mock aqueous humor was perfused instead for 90 min. There was then a reduction in facility to 0.64 and 0.79 μl/min/mm Hg, levels similar to the starting values.

In one experiment, monkey 8, albumin labeled with 131I was present in the Na2 EDTA–containing infusion fluid, and the rate of inflow from the external system into the eye was compared with the rate of appearance of labeled anterior chamber fluid in the general circulation at a time when the facility of the Na2 EDTA-perfused eye was 4.5 μl/min/mm Hg as compared to 0.72 on the control side. This experiment demonstrated that most of the radioactive fluid leaving the eye appeared in the general circulation. At a net inflow from the external system of 12.7 μl of radioactive fluid per minute, the rate of increase in plasma radioactivity corresponded to an inflow of 12.3 μl of the perfusate per minute into the general circulation. It was assumed in these experiments that the blood equivalent albumin space used to determine the amount of radioactivity in the general circulation was 7.2% of the body weight.

Dilatation of the pupil occurred usually within 20 min in the Na2 EDTA–treated eyes, and in some experiments there was a slow development of cloudiness of the cornea. Dilatation and cloudiness remained for 1 to 2 weeks in three eyes and then subsided. The arterial blood pressure was not affected during the experiment.

The fluid perfused on the control side was mock aqueous humor in some experiments and mock aqueous less Ca++ or Ca++ and Mg++ in others. A tendency toward increasing facility was observed irrespective of the composition of the fluid. The physiological results of monkeys 4 and 5 are of special interest because these eyes were processed for electron microscopy. In the third monkey investigated morphologically—monkey 9—the IOP was allowed to change spontaneously during the perfusion period of 75 min. Dur-
Fig. 1. Schlemm’s canal and trabecular meshwork after 80 min perfusion with mock aqueous humor alone. The appearance is basically normal. Invaginations (arrowhead) of the inner wall endothelium are common. However, in some places the trabecular cells (arrow) tend to take up a more angular orientation in relation to Schlemm’s canal (SC), suggesting a localized distention of the meshwork. (TEM; monkey 5.)

During this time the spontaneous pressure decreased from 7 to about 5 mm Hg both in the Na$_2$EDTA eye and the control eye. No facility determinations were made in this experiment in order to avoid any artificial increase of IOP.

Experiments with EGTA. Seven experiments were performed with outflow facility determination followed by 80 to 90 min of perfusion of 4 mM EGTA (Sigma Chemical Co., St. Louis, Mo.) in Ca$^{++}$-free mock aqueous humor followed by about 80 min perfusion with complete mock aqueous humor. Table II shows that EGTA caused a marked increase in gross outflow facility and that the effect was partly reversible. Dilatation of the pupil and in some experiments' cloudiness of the cornea developed as in the experiments with Na$_2$EDTA. The arterial blood pressure was not affected.

Morphological findings

Schlemm’s canal and trabecular meshwork. In the control eyes the appearance was normal except for a few places where the cribriform meshwork was distended, as indicated by a more angular orientation of the meshwork cells in relation to Schlemm’s canal (Fig. 1). In Na$_2$EDTA eye 4 and 9, large “balloons” protruded into the lumen of Schlemm’s canal. The wall of such a balloon consisted of both the inner wall endothelium and the attached first subendothelial cell layer. The latter had apparently lost all contact with the underlying cribriform meshwork. The balloons often distended to touch the outer wall of Schlemm’s canal, and ruptures were seen allowing extracellular material and cell debris to pass out (Fig. 2). Such ruptures were, however, rarely observed and then mostly with platelet aggregates in close vicinity. The tight junctions between the inner wall endothelial cells were mostly intact. Invaginations (“giant vacuoles”) were less frequent than in the control eyes. A quantitative SEM study of the inner wall in monkey 4 showed that the number of pores and the estimated outflow conductivity across...
Fig. 2. Schlemm's canal (SC) and trabecular meshwork after 40 min perfusion with 6 mM Na₂EDTA. The inner wall endothelium and the first subendothelial cell layer are detached from the underlying cribriform meshwork and balloon into the lumen of Schlemm's canal. At one place, the balloon (asterisk) shows a large rupture (arrow), allowing swollen collagen fibers and cell debris to pass out. (TEM; monkey 4.)

The inner wall endothelium were no different from those of a normal monkey group, kept at an IOP of 12 mm Hg. Table III. In animal 9, kept at the spontaneous IOP which was 5 to 7 mm Hg throughout the 80 min experiment, the number of pores was much lower. The low pressure probably reflected a low aqueous humor production and poor outflow via the canal of Schlemm. Previous studies indicate that the number of pores is increased with increasing flow into the canal of Schlemm. The relative pore size–frequency distribution of the control eyes 4 and 9 were normal (Fig. 3). The Na₂EDTA eyes 4 and 9 showed a significant deviation from normal (chi-square distribution test, \( p < 0.01 \)) in having a
smaller proportion of the smallest pores and a greater proportion of the larger ones (Fig. 3).

In Na₂EDTA eye 5, which was infused twice as long as eye 4, ruptures of the inner wall endothelium were extremely frequent, and ballooning was no longer readily observed. Invaginations were virtually absent. At some places the endothelial cells rounded up, presumably due to loss of cell contact to each other and the first subendothelial layer. Pore estimation by SEM was impossible because of the manyfold ruptures with associated obscuring platelet aggregates. Beneath undamaged parts of the inner wall there was often an accumulation of cell debris, homogeneous material, elastic material, curly collagen, and basement membrane-like material (Figs. 4A and 4B).

In the uveal and the corneoscleral meshwork of all Na₂EDTA eyes a loss of cell contact was a general finding, both between different sheets as well as between individual cells on the cores. The cells tended to round up. Clusters of curly collagen were sometimes observed outside the zone of the basement membrane on denuded parts of the trabecular cores. In the denuded parts of the cores, the content of collagen fibrils, curly collagen, and basement membrane-like material was often rarefied, whereas the elastic material was unaffected.

In the cribriform meshwork the cells appeared normal in configuration but had lost the contact to each other. A distention of the meshwork and a loss of the extracellular material were also observed (Fig. 2).

The changes in the meshwork were less pronounced in Na₂EDTA eye 4, showing normal internal cell structures. In Na₂EDTA eyes 5 and 9 the cells showed internal vesiculation and tentacle-like cytoplasmatic protrusions. Such changes were also noticed in the inner wall endothelium.

The outer wall of Schlemm’s canal appeared normal by TEM. Clusters of platelets could occasionally be observed by SEM. The collector channels were patent and normal by LM and TEM.

Corneal endothelium. The control eyes had a normal appearance, even the eyes perfused with mock aqueous humor without Ca⁺⁺ and Mg⁺⁺.

In the Na₂EDTA eyes most cells rounded up, giving the endothelium a conspicuous cobblestone appearance (Figs. 5 and 6, A). The interdigitating apical junctions split up, the gap being overbridged by slender cytoplasmatic processes, border bars (Fig. 6, B).

In the corneal periphery—zones III and IV—the rounding up of the cells was often more pronounced (Fig. 5). The plasma membrane then showed a very rough surface (Fig. 6, C). The cells kept contact with stretched basal-lateral junctions (Fig. 6, C). At places, however, the cells were completely detached from each other or Descemet’s membrane (Fig. 5). The solitary central cilium had a normal appearance on the cells not showing a severe rounding up (Fig. 6, C). The cilium could not be detected on severely affected cells. Intracellular vesiculation was commonly observed in Na₂EDTA eyes 5 and 9. The morphological changes were uniform in the different quadrants, indicating a thorough mixing of Na₂EDTA in the anterior chamber.

Ciliary body, iris, choroid, and retina. Structural changes were observed in the
Fig. 4A. Schlemm's canal and cribriform meshwork after 80 min perfusion with 4 mM Na₂EDTA. The inner wall endothelium shows a large rupture (R). An accumulation of homogeneous material (arrows), curly collagen (C), and elastic material (E) are observed under the intact part of the inner wall endothelium. Note the many small, intracellular empty vesicles. (TEM; monkey 5.)

Table III. Quantitative SEM data on the pores of the inner wall of Schlemm's canal

<table>
<thead>
<tr>
<th>Animal</th>
<th>Eye</th>
<th>Pores per mm²</th>
<th>Estimated outflow conductivity (µl/min/mm Hg)</th>
<th>Area* examined (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Exp.</td>
<td>1260</td>
<td>2.59</td>
<td>146,500</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>1170</td>
<td>1.75</td>
<td>154,000</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td>102,500</td>
</tr>
<tr>
<td>9</td>
<td>Exp.</td>
<td>430</td>
<td>1.54</td>
<td>70,000</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>500</td>
<td>2.09</td>
<td></td>
</tr>
<tr>
<td>Normal material</td>
<td>18401</td>
<td>2.22</td>
<td></td>
<td>3,216,000</td>
</tr>
</tbody>
</table>

*Projected into a flat surface.
†Data from 12 eyes kept at an IOP of 12 mm Hg.
‡Arithmetic mean; 95% tolerance limits in parentheses.

Ciliary muscle and the pupillary sphincter in all Na₂EDTA eyes and to minor extent in control eye 4, infused with mock aqueous humor without Ca²⁺ and Mg²⁺.

In the ciliary muscle the intermuscular clefts were very wide (Fig. 7, A and B). Many muscle fibers demonstrated a breakdown of the myofilament organization and a degeneration of the mitochondria. The cell nucleus became electron-optically more translucent (Fig. 8). The nerve endings were normal. These changes were more pronounced in the anterior part of the muscle, whereas the posterior third of the innermost portion of
Fig. 4B. Endothelial cells in the meshwork are losing contact. Upper arrow shows intact desmosome; lower arrow indicates dense region probably representing remains of desmosome. (TEM, monkey 5.)

the muscle appeared unaffected. In the treated eyes the overall configuration of the muscle was similar to that of the control eyes. Similar muscle fiber changes were also observed in the pupillary sphincter (Fig. 7, C and D). The pupillary dilator was unaffected.

The ciliary epithelium, the iris stroma and epithelium, the vessels of the anterior uvea, the choroid, and the retina had a normal appearance.

**Discussion**

Both Na₂EDTA and EGTA caused a large decrease in aqueous outflow resistance when perfused intracamerally into the normal cynomolgus eye. This effect was probably caused by binding of Ca²⁺, EGTA being a rather specific Ca²⁺-chelator with much less affinity for Mg²⁺ than Na₂EDTA. One cannot, however, rule out the possibility of unspecific effects of the chelating agents or a binding of cations other than Ca²⁺.

About three fourths of the total resistance to aqueous humor outflow is normally located between the anterior chamber and Schlemm’s canal. Since the conventional outflow route...
Fig. 6. Corneal endothelium after 40 min perfusion with 6 mM Na₂EDTA. A, Survey of the chamber angle. Note the increasing bulging of the cells towards the periphery of the corneal endothelium (E). I, Iris; TM, trabecular meshwork; SC, Schlemm's canal, widened by retroreflection of the iris; M, ciliary muscle. B, These endothelial cells show the initial splitting of the apical junction but are still anchored to each other by slender cytoplasmic processes, "border bars" (arrow). C, One cell (top) has rounded up profoundly, showing a rough plasma membrane and stretched but still preserved basal-lateral cell junctions (arrow). Another cell (bottom left) has a normal flat surface with a solitary central cilium (C). (SEM; monkey 4.)

distal to Schlemm's canal had a normal appearance in the present experiments, one must conclude that the characteristic morphological changes observed between the anterior chamber and Schlemm's canal—the damaged trabecular cores, distention of the cribiform meshwork with loss of the extracellular material, ballooning and ruptures of the inner wall—most probably resulted in the large decrease in outflow resistance. Part of the effect in monkeys 4 and 5 might be due to high perfusion rates in the chamber angle. But
Fig. 7. Ciliary muscle and pupillary sphincter after 80 min perfusion. A, Normal appearance of ciliary muscle in the control eye with slightly broader intercellular clefts than usual (arrows). SP, Scleral spur. B, In the Na₂EDTA eye the muscle fibers are severely damaged, with loss of cytoplasmatic organization (arrowheads). Note the very wide intermuscular clefts (arrows). SP, Scleral spur. C, Normal appearance of the pupillary sphincter (P) in control eye. ST, Iris stroma. D, Similar damage (Na₂EDTA eye) of the pupillary sphincter (P) as in the ciliary muscle. The pigment epithelium has been artifactually torn in this preparation. ST, Iris stroma. (LM; monkey 5.)
monkey 9, perfused at its spontaneous IOP, showed very much the same changes as 4 and 5, indicating that overperfusion was not the main reason for the changes in structure observed.

In the unconventional outflow routes—the uveoscleral routes—the anterior and middle parts of the ciliary muscle showed degeneration of the muscle fibers and very wide intermuscular clefts. These findings could very well imply a decrease of the resistance in this outflow route. The experiment with perfusion of albumin labeled with $^{125}$I indicated that the major part of the perfusate drained rapidly into the general circulation. The most likely route for this drainage is via the canal of Schlemm, but some albumin may have entered the blood vessels of the uvea.

In most of the control eyes there was a decrease of outflow resistance during the perfusion period. This decrease was probably due to a contraction of the ciliary muscle and/or a "washout" effect. Morphologically we noted a mildly contracted state of the ciliary muscle and occasional localized distentions of the cribiform meshwork with loss of the extracellular material.

The effects of Na$_2$EDTA and EGTA on the outflow resistance were reversible, since a following perfusion period with mock aqueous humor alone brought the outflow facility values toward the starting level. It seems rather unlikely, however, that there could be a restitution of the structure to a normal state within 90 min. A correlation to morphological findings is underway.

At the ultrastructural level, Na$_2$EDTA affected the muscle fibers, the cell junctions, and the internal structure of the cell, probably in that very order. The muscle fibers of the ciliary muscle and the pupillary sphincter appeared to be the structure most sensitive to depletion of calcium and magnesium ions, since degenerative changes were also observed in control eye 4, perfused with mock aqueous humor lacking these ions.

In the Na$_2$EDTA-treated eyes the pupillary dilator and the posterior third of the ciliary muscle were unaffected, possibly because they were not reached by the chelating agent. On their way through the uveoscleral routes, low-molecular-weight substances are lost to the blood, due to diffusion.

In the Na$_2$EDTA eyes the cell junctions
split in the corneal endothelium, the trabecular meshwork, and also in the inner wall endothelium. These effects were clearly observed in Na₂EDTA eye 5, perfused twice as long as Na₂EDTA eye 4. We have so far not used specific techniques to determine which type of junction is most sensitive to Na₂EDTA. Moreover, there are different views on what type of junction is normally situated where. Also best observed in Na₂EDTA eye 5 was the effect of Na₂EDTA on the internal structure of the cell, the cell rounding up, the cytoplasmatic tentacle-like protrusions, the rugged and disintegrated plasma membrane, and the intracellular vesiculation being characteristic features.

Cytochalasin B is an agent recently shown to reduce outflow resistance in monkeys. The prime action of cytochalasin B is probably exerted on the cytoskeleton, whereas the prime action of Na₂EDTA is probably on the cell junctions. Both principal modes of action lead to a distention of the cribriform meshwork with a washout of extracellular material and ruptures of the inner wall endothelium of Schlemm's canal.

In the corneal endothelium, Na₂EDTA caused a splitting of the apical cell junctions and a rounding up of the cells as we have earlier reported. In the periphery the cells might even detach from each other and Descemet's membrane. These findings are similar to those described in the rabbit cornea when perfused with Ca⁺⁺-free medium in vitro. Such morphological changes would be expected to increase the corneal permeability to aqueous humor and thus explain the observed corneal edema. Again it is of interest to compare the effects with those of cytochalasin B. With this agent there were cytoplasmatic blebbing, a loss of cell nuclei, and intercellular dilatation; however, the apical junctions were intact, and there was no separation from the underlying Descemet's membrane.

Clinically Na₂EDTA is mainly used to treat lead poisoning; it has well documented toxic and embryopathic effects. EDTA has also been tried as a therapeutic agent in ocular siderosis, even by intravitreal injection, although this mode of administration has been demonstrated to induce toxic effects on the retina in rabbit experiments. In that study 100 μl were given at a concentration 10-fold that recommended for topical eye application, which is 10 mM solution. Given intracameral in the rabbit eye, a 10 mM solution of Na₂EDTA caused a "diffuse corneal edema and marked hyperemia of the iris and conjunctiva." Since Na₂EDTA does not normally penetrate the corneal epithelium, it has to be administrated directly into the anterior chamber or possibly by iontophoresis in order to exert its effects on the aqueous outflow routes. The obvious question is whether Na₂EDTA might be useful in the treatment of glaucoma as a "pharmacological trabeculectomy" or some sort of outlet cleaner. Before that question can be answered, further studies are required on dose-response relationship, reversibility, local toxicity, and long-term effects.

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