THE DEVELOPMENT OF TOOTH GERMS *IN VITRO*

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INTRODUCTION

Much experimental work has been done recently on the growth of isolated embryonic tissues *in vitro* with a view to studying the capacity of these tissues for self-differentiation. Although deprived of a vascular and nervous system and connections with neighbouring structures many tissues develop in a comparatively normal way during cultivation. Bone, for example, differentiates readily *in vitro* (Fell, 1932), and since bone and dentine are very closely related it seemed possible that dentine might develop under the same conditions. If this were so a new field of dental research would be opened, as dental tissue cultures would provide ideal material for analysing many of the physiological conditions of tooth development. The experiments recorded in the present communication were therefore undertaken in order to find whether embryonic dental tissues would differentiate normally when isolated and cultivated *in vitro*.

Probably the first experiments on the explantation of teeth on record were those of Cadmus, who, it may be remembered, explanted in the soil dragons' teeth which grew into armed men (Stesichorus, 6 B.C.). Apart from these legendary experiments of Cadmus which had such singular results, very little work has been done on the self-differentiating capacity of dental tissues outside the mouth.

Legros and Magitot (1874) removed tooth germs from dogs 21–28 hours after birth and grafted them subcutaneously into guinea-pigs or adult dogs. The grafts were portions of maxilla including tooth germs, isolated enamel organ, complete tooth germs, pulp and the isolated dentine cap. All tooth germs grafted into guinea-pigs were lost by suppuration or resorption. Of the grafts in dogs, seven out of the twenty-six complete tooth germs formed enamel and dentine, and in the isolated pulp grafts, two out of sixteen formed dentine. A few of the grafts showed no growth at all and the majority of them were lost by resorption or suppuration. As these experiments were done in the days before asepsis was practised the results obtained were highly commendable.

Further work on the transplantation of tooth germs was done by Huggins, McCarroll and Dahlberg (1934). They grafted the unerupted canine teeth of dogs between the ages of 3–6 weeks on to the connective tissue of the abdominal wall or thigh of the same dogs. They removed the calcified cap of dentine and enamel and explanted the enamel epithelium and the dentine papilla separately.

The transplants of the isolated epithelial layer formed no new enamel, but the isolated dentine papilla had produced dentine 14 days after transplantation. They also grafted the enamel epithelium and dentine papilla together in various ways. Both enamel and dentine differentiated in this series, enamel being deposited only on dentine, but the dentine being formed independently of the enamel. In other experiments the centre of the pulp was completely freed of its odontoblasts and transplanted; no dentine was formed even after 29 days, although the odontoblast layer which was removed from the pulp and transplanted separately formed small islands of dentine during the same period. The results of Huggins, McCarroll and Dahlberg thus showed that the soft tissues of tooth germs were able to differentiate readily when transplanted into abnormal surroundings outside the mouth.

As far as the present writer is aware no previous work has been done on the growth of teeth *in vitro*.

MATERIAL AND METHODS

The tooth germs of 18–21-day rat embryos were cultivated. Molars and incisors which had not yet begun to form dentine were explanted whole; in those incisors where a cap of dentine had formed on the tip, this cap together with the enamel epithelium was removed and the dentine papilla itself was explanted. In performing this last operation some of the odontoblasts remained adherent to the cap of dentine whilst some were left behind on the tip of the dentine papilla. In some of the explants a little of the enamel epithelium also remained attached. Owing to the small size of the dentine papilla it was not possible to remove the ameloblasts completely without irreparable damage to the tissue.

As explantation had to be done with the strictest aseptic precautions and the removal of teeth from post-foetal rats under such conditions is almost impossible, the necessary material had to be obtained from embryos.

The tooth germs for cultivation were dissected out with a cataract knife and a needle from both upper and lower jaws and washed in saline. In order to determine the stage of development of each tooth, one tooth was removed for explantation, whilst the corresponding tooth from the opposite side of the jaw was fixed, either *in situ* in a fragment of jaw or after removal, and sectioned to serve as a control.

The culture methods employed were the hanging-drop and watch-glass techniques. In the hanging-drop method the explants were placed in a clot of medium composed of one drop of fowl plasma and one drop of embryo extract made with Pannett and Compton's saline from 10–11-day chick embryos. The cultures were grown on $1\frac{1}{4}$ in. square cover-slips inverted over a hollow-ground slide sealed with paraffin wax and incubated at 37° C. The explants were transferred to fresh medium every 72 hours. In the watch-glass method four drops of fowl plasma and four drops of embryo extract were mixed in a watch-glass which was enclosed in a Petri dish surrounded by cotton-wool saturated

in sterile water. A large hole was cut in the cotton-wool to allow trans-illumination of the explants for microscopic observation. The tissue which was grown on the surface of the clot was removed from the old medium every 72 hours, washed in saline and transferred to a fresh clot.

The explants were fixed at different periods of growth in Zenker's solution containing 3 per cent. glacial acetic acid for 30 min., then washed, dehydrated, cleared in cedar-wood oil and embedded in paraffin wax. Serial sections were cut and were stained with iron haematoxylin and chromotrope.

THE STRUCTURE OF THE TOOTH GERMS AT THE TIME OF EXPLANTATION

As mentioned above the stage of development at the moment of explantation was ascertained from the series of controls obtained by fixing and sectioning one of each pair of teeth and cultivating the corresponding tooth.

Whole tooth germs

The whole tooth germs at the time of explantation consisted of the dentine papilla, which was a mass of stellate connective tissue cells, surrounded by the enamel organ. In some of the explants the peripheral cells of the dentine papilla were in an early stage of differentiation into odontoblasts; they were rounded in form with large nuclei and a small amount of cytoplasm. In other explants the formation of odontoblasts had not yet begun. In the molars the shape of the cusps was already determined by the enamel organ dipping into the dentine papilla.

The dentine papilla

The dentine papilla of the incisors (Plate I, fig. 1), which was removed from the teeth of 19–21-day embryos, consisted of connective tissue cells with some odontoblasts at the periphery, mostly near the tip. As mentioned above a number of odontoblasts were torn away in the process of removing the dentine cap. The connective tissue cells towards the base had not yet begun to differentiate into odontoblasts. In some of the explants a small part of the internal enamel epithelium was present in the basal region, owing to the difficulty of removing it without serious injury to the papilla itself.

GROWTH AND DIFFERENTIATION OF THE EXPLANTS

Observations on the living explants

The explants did not enlarge during cultivation but usually contracted slightly, particularly those of the dentine papilla. In the hanging-drop cultures the original odontoblasts and ameloblasts were clearly seen as bands of columnar cells. During cultivation odontoblasts and dentine continued to differentiate. The newly formed tissues were not seen quite so clearly but were usually marked by a translucent area. In some of the explants of the dentine papilla the tip, with its peripheral layer of odontoblasts, contracted and degenerated

by the third day. This was due to the fact that this portion of the papilla was rather narrow, and in removing the dentine cap it was severely injured and unable to recover *in vitro*. In the watch-glass cultures the original ameloblasts and odontoblasts could be distinguished as light bands. Considerable growth in the cusps of the molars was observed during the period of cultivation, and in the incisors, in some of the cultures, some distortion was noticed. For detailed observation of living tissue the watch-glass method is not very suitable.

Histological differentiation

Histological examination of the explants at different stages of cultivation showed normal differentiation of the cells of the dentine papilla to form odontoblasts and dentine, although a few explants degenerated at about the third or fourth day. The formation of the dentine proceeded much more slowly *in vitro* than *in vivo*. An explant after 20 days *in vitro* (Plate I, fig. 2) showed no more dentine than a normal tooth germ 4 days after the formation of odontoblasts.

Dentine papilla

In the absence of any epithelium, the odontoblast layer invaginated into the pulp and deposited irregular bands of dentine (Plate I, fig. 3). By the third day, wherever a fragment of enamel epithelium existed, a new odontoblast layer had been formed adjacent to it. The enamel epithelium continued to grow and showed numerous mitoses. Its extension was always followed by the development of an underlying layer of odontoblasts (Plate II, fig. 4).

The various stages in the development of these new areas of odontoblasts could be followed in cultures of different ages. After 2 days' growth *in vitro*, at the future site of the odontoblast layer adjacent to the ameloblasts, the connective tissue cells had begun to change their appearance, becoming larger, rounded and containing relatively larger nuclei. By the third day these cells had enlarged still further, were very closely packed, and changed from a rounded to a cylindrical form. The oval nuclei lay at the ends of the cells farthest from the site of the future dentine. The dental surface of the cytoplasm was flat but continued into protoplasmic strands, passing into a layer of collagenous substance which had now been deposited in the odontogenic zone. By the fourth day a band of normal tubular dentine had been formed, and the protoplasmic strands were enclosed in the tubules as dentinal fibrils (Plate II, figs. 5, 6).

After the fourth day the formation of dentine progressed very slowly until the fourteenth day, when the odontoblasts began to degenerate. This degeneration of odontoblasts continued, and on the twentieth day the connective tissue cells also began to degenerate rapidly. As described above, in some of the cultures the odontoblasts and the connective tissue cells at the tip degenerated soon after explantation. This necrotic tip was removed, leaving part of the dentine papilla composed of connective tissue cells alone, which remained alive but failed to differentiate. If in these cultures some ameloblasts were

present, new odontoblasts and dentine were formed irrespective of the fact that the tip had been removed.

Whole tooth germs

The histological differentiation in the whole tooth germs was the same as in the cultures of the dentine papilla. The peripheral pulp cells which were in an early stage of differentiation formed odontoblasts and dentine. Those cultures where the peripheral pulp cells at the time of explantation had not yet begun to differentiate did the same eventually. The presence of the enamel organ controlled the position of the newly formed dentine which appeared next to the ameloblast layer. As the enamel organ had already determined the shape of the cusps by dipping into the dentine papilla, regular cusps were formed by the new dentine, except that in some of the explants there was some distortion of the tooth as a whole.

The development of abnormal dentine

In some of the cultures, besides the normal tubular dentine, an abnormal dentine was formed comparable to the "scar tissue" described by Fish (1932). In the early stages of the development of this "scar tissue", some of the odontoblasts became rounded, and instead of forming tubular dentine produced a mass of collagenous fibrillar material which later became much more abundant. The odontoblast layer disappeared, but irregular-shaped cells with large nuclei were found embedded in this "scar tissue" and possibly represented abnormal odontoblasts (Plate II, fig. 7).

DISCUSSION

The results obtained from these experiments confirm the conclusions of Huggins, McCarroll and Dahlberg that isolated dental tissue is self-differentiating. Whereas their evidence was derived from the transplantation of tooth germs into the abdomen, the results of the present study were obtained from the cultivation of tooth germs *in vitro*, which, by eliminating all the vascular and nervous connections, produced a more complete isolation than grafting.

As described above, in the absence of the enamel epithelium, the peripheral layer of odontoblasts produced convoluted bands of dentine, but wherever dentine was formed in contact with the epithelium it was deposited as regular sheets. This result clearly showed that one of the functions of the enamel epithelium is to determine the morphological structure of the tooth, thus confirming the views of earlier investigators (von Brunn, 1887, 1891; Ballowitz, 1892).

Of particular interest is the question of the influence of the enamel epithelium over dentine development. It had previously been held that this epithelium, which even occurs in teeth which do not form enamel, is necessary for the deposition of dentine. Hertwig (1874) described the presence of an epithelial sheath in certain Amphibia, which he believed was essential for tooth develop-

ment. Tomes (1876) discovered the existence of an enamel organ in the armadillo, whose teeth have no enamel, and later found that in all Edentata an enamel organ occurs although enamel is absent. Von Brunn (1887, 1891) carried the investigations further and showed that many Mammals in other groups possess enamel epithelium in the absence of enamel, notably the Rodents, whose incisors have no enamel on the dorsal surface but yet are entirely invested by an enamel organ. He came to the conclusion that the epithelial sheath is responsible for the development of odontoblasts and dentine, and that its primary function is that of determining the form of the future tooth. Ballowitz (1892), in his paper on the enamel organ of the Edentata, confirmed von Brunn's conclusions.

Since Huggins, McCarroll and Dahlberg obtained the formation of dentine in the isolated dentine papilla in the absence of the epithelial layer, their results were not in entire agreement with the views of the previous authors mentioned above. They therefore modified von Brunn's statement that "where there is no epithelial sheath, there can be no odontoblast formation and consequently no dentine", and concluded that odontoblasts that have arisen as a result of epithelial influence on connective tissue do not further need for their function the presence of an epithelium.

The results of the present study fully confirm the conclusions of Huggins, McCarroll and Dahlberg. The writer's experiments have shown that odontoblasts differentiated *in vitro* only when ameloblasts were present. Moreover, if the original odontoblast layer degenerated in an explant containing no epithelium, there was no further differentiation of the pulp; the connective tissue cells survived as such, which suggested that they cannot develop into odontoblasts in the absence of the enamel epithelium. It is therefore probable that the enamel epithelium, though not necessarily the whole of the enamel organ, has an organising effect on the dentine papilla inducing odontoblast formation. Once the connective tissue cells have reacted to this influence and have differentiated into odontoblasts, the presence of the enamel epithelium is not necessary for the deposition of dentine.

The "scar tissue" produced in the cultures is interesting, as it is similar to the early stages of "scar tissue" found in the teeth of guinea-pigs suffering from scurvy (Fish and Harris, 1934). The nature and significance of this abnormal dentine is being investigated further.

SUMMARY

1. Whole or partial tooth germs cultivated *in vitro* showed remarkable powers of histological differentiation. The dentine papilla developed normally, forming odontoblasts which deposited normal tubular dentine.

2. The presence of the internal enamel epithelium is essential for odontoblast formation.

3. Dentine formation could take place in the absence of the enamel epithelium provided that odontoblasts were present in the explant.

4. One of the functions of the enamel organ is to determine the gross morphological structure of the tooth.

5. "Scar tissue", similar to the "scar tissue" found in the teeth of animals suffering from scurvy, occurred in some of the cultures.

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EXPLANATION OF PLATES

Plate I

Fig. 1. Longitudinal section of the dentine papilla after removal of the dentine cap, showing the general histological structure (×57).

Fig. 2. Longitudinal section of a 20-day culture of the dentine papilla, showing the convoluted bands of dentine formed in the absence of the enamel epithelium. Also the "scar tissue" formed in the same culture (×110).

Fig. 3. Higher magnification of the convoluted dentine shown in Fig. 2. The original odontoblasts have almost completely degenerated (×460).

Plate II

Fig. 4. Longitudinal section of an 8-day culture of the dentine papilla showing the new layer of odontoblasts and dentine formed adjacent to the ameloblast layer (×360).

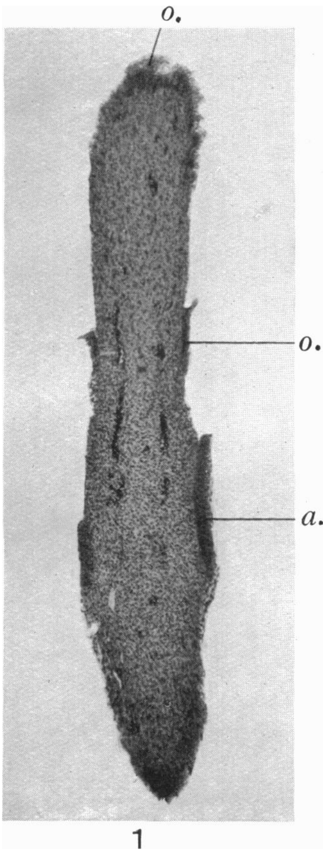
Fig. 5. Transverse section of an 11-day culture of a molar showing the dentinal tubules (×550).

Fig. 6. Longitudinal section of a 12-day culture of an incisor showing the sheet of tubular dentine formed when the enamel epithelium was present (the latter was removed after cultivation) (×450).

Fig. 7. Higher magnification of "scar tissue" shown in Fig. 2, showing its fibrillar structure and the irregular-shaped cells embedded in it (×380).

ABBREVIATIONS

a.	ameloblasts	*e.c.*	embedded cells
c.d.	convoluted dentine	*f.*	fibrils
d.	dentine	*o.*	odontoblasts
d.o.	degenerate odontoblasts	*s.t.*	"scar tissue"
d.t.	dentine tubules		



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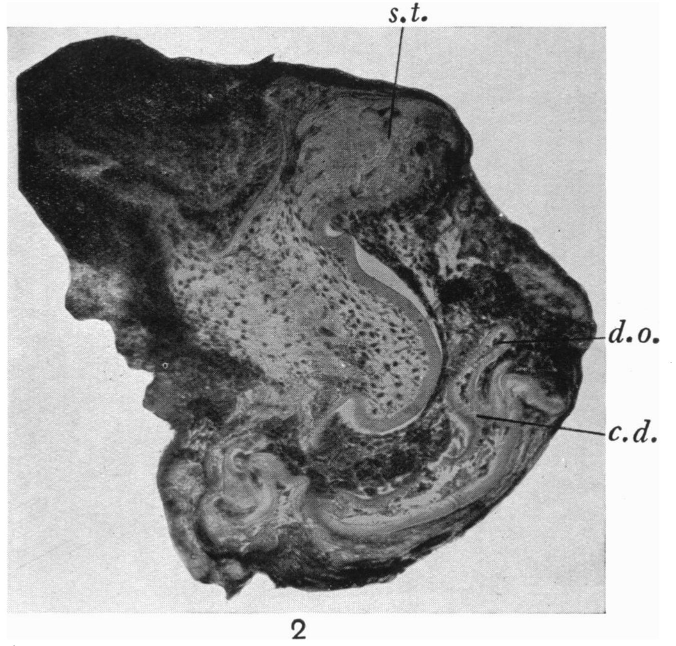

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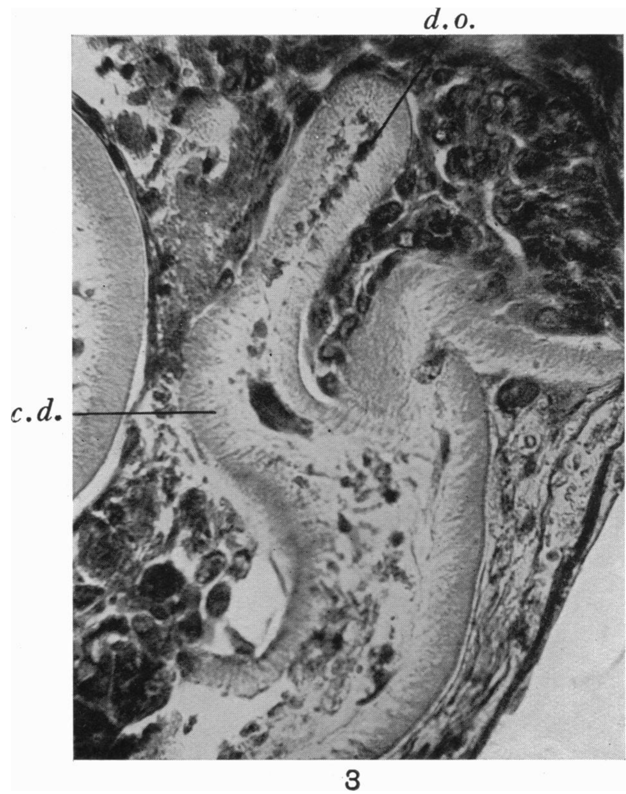

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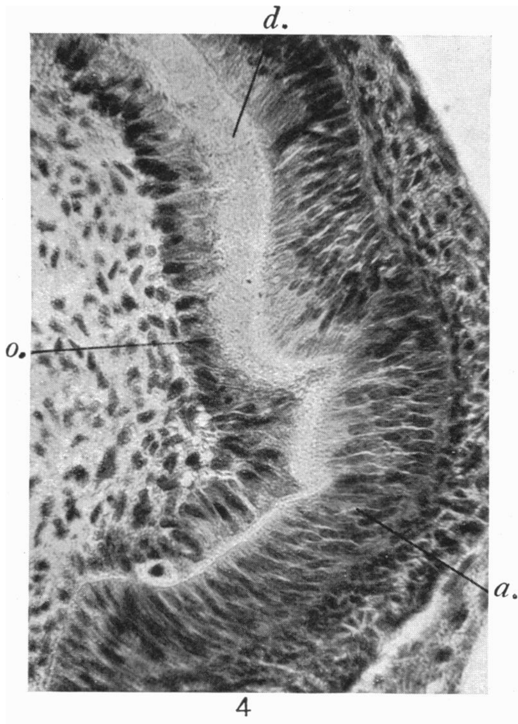

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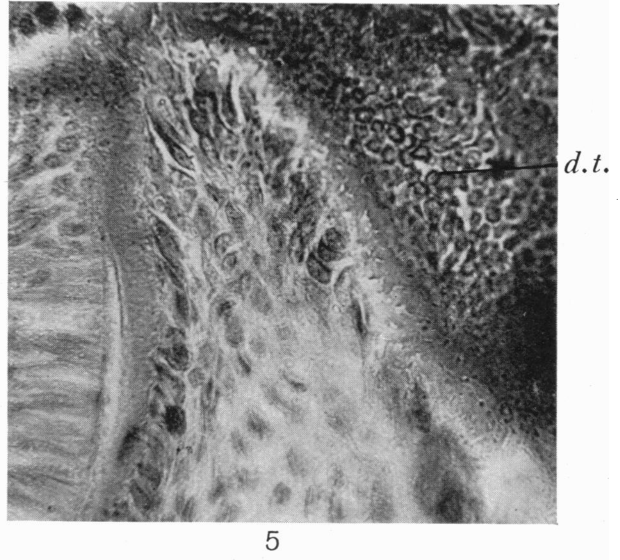

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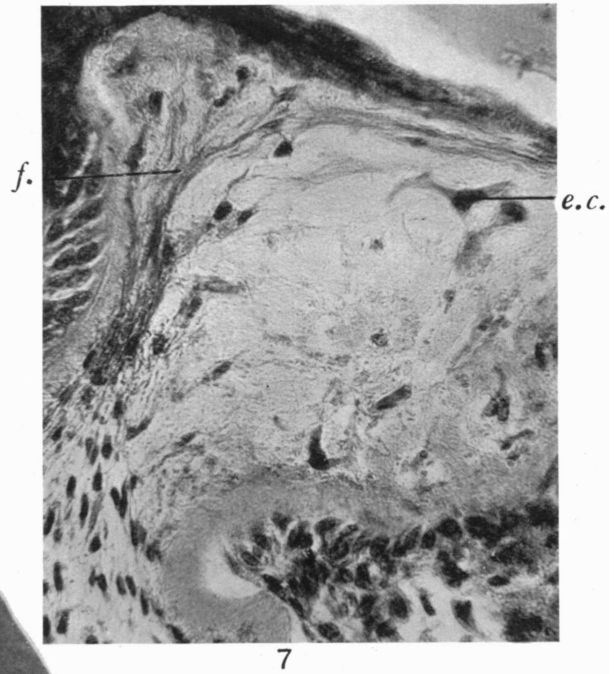

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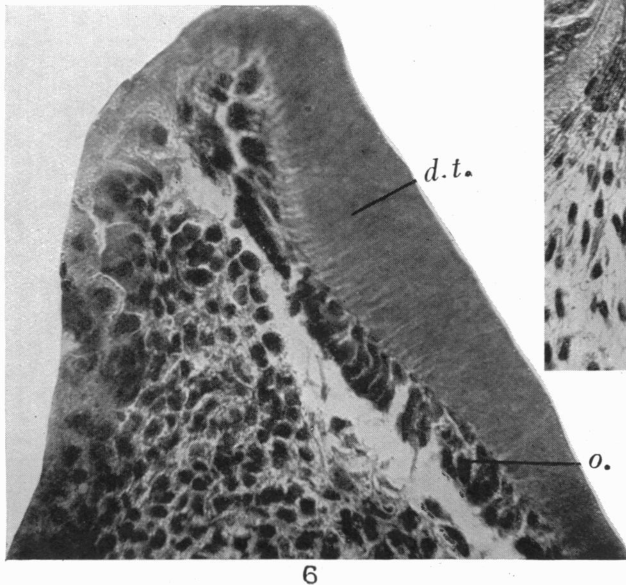

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