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CHARACTERIZATION OF MITOCHONDRIAL NEUTRAL PROTEASE ACTIVITY AND THE RESPONSE OF LYSOSOMAL ENZYMES TO CLOFIBRATE FEEDING IN RAT LIVER

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Abstract—The phosphate-inhibitable neutral protease activity of the heavy mitochondrial fraction of rat liver is of lysosomal origin. The activity is essentially due to the thiol proteinases of the lysosomes. Digitonin treatment of the mitochondrial fraction results in the release of about 85 per cent of the neutral protease activity and the residual activity has an alkaline pH optimum and is not inhibited by phosphate. Clofibrate feeding at 0.5 per cent level in the diet results in enhanced levels of lysosomal enzymes. The increase is however restricted to the lysosome-rich fraction such that the activities associated with the heavy mitochondrial fraction show a significant decrease. It is suggested that clofibrate inhibits engulfment of mitochondria by lysosomes and this results in enhanced mitochondrial protein content.

Studies on neutral protease activity of cellular organelles are gaining importance in view of their possible role in organelle turnover [1, 2].

Ethyl-α-*p*-chlorophenoxyisobutyrate (CPIB, clofibrate) treatment induces a significant increase in the mitochondrial protein content of rat liver [3, 4]. There is also evidence to suggest that CPIB treatment elevates the number of microbodies [5] and lysosomes [6], but evidence for the increase in lysosomes is considered inadequate [7].

Gear *et al.* [7] have reported that CPIB depresses the phosphate inhibitable neutral protease activity of the mitochondrial fraction assayed at pH 7.4 and suggested that this could possibly lead to decreased turnover of freshly synthesized mitochondrial proteins. They argued that the CPIB-sensitive neutral protease activity is truly mitochondrial and not of lysosomal origin. However, Rubio and Grisolia [8], on the basis of digitonin extractability of the neutral protease activity from mitochondria, have concluded that lysosomes are responsible for this activity. Again, Subramanian *et al.* [9] have detected neutral protease activity in digitonin-washed mitochondria and have reported that the enzyme activity is not depressed under conditions of CPIB treatment. The activity was assayed at the reported pH optimum of 6.0 in phosphate buffer.

The present study seeks to clarify some of the controversial results presented above. First of all, attempts have been made to examine the question of whether the neutral protease activity of the mitochondrial fraction is of lysosomal or mitochondrial origin. Secondly, the effect of CPIB on the neutral protease level has been studied. Finally, in view of the findings that hormones which affect cellular protein degradation bring about a redistribution of lysosomal enzymes between the heavy and the light mitochondrial fractions [10], the question whether CPIB influences this picture has also been examined.

MATERIALS AND METHODS

Materials. Digitonin (Sigma) was recrystalized from ethanol before use. Bovine hemoglobin (type II, Sigma) was denatured with acid according to Barrett [11]. Pepstatin and leupeptin were purchased from Peptide Institute, Osaka, Japan. Ethyl-α-*p*-chlorophenoxyisobutyrate (clofibrate, CPIB) was a gift from DR. C. K. R. Kurup. α-*N*-Benzoyl-DL-arginine-β-naphthylamide HCl (BANA) and glucose-6-phosphate (sodium salt) were purchased from Sigma. Triton WR 1339 was a kind gift from Serva Fine Chemicals, Heidelberg, Germany.

Treatment of animals. Male albino rats (180–200 g) of the Indian Institute of Science strain were maintained on a 20 per cent casein diet and CPIB, when fed, was mixed with the diet at 0.5 per cent (w/w) level.

Preparation of mitochondria. Mitochondria from the livers of rats were prepared by two different methods. In the first method described by Ragab *et al.* [12], a 15 per cent (w/v) homogenate of the liver was prepared in 0.25 M sucrose containing 1 mM EDTA (adjusted to pH 7.0). The homogenate was spun at 1020 *g* for 10 min and the supernatant was spun at 3300 *g* for 10 min to yield the heavy mitochondrial pellet. This pellet was washed by resuspension and centrifugation five times to get rid of the fluffy layer as well as the lower dark brown lysosome contamination. The second method was that of Lowenstein *et al.* [13]. A 15 per cent (w/v) homogenate of rat liver in 0.25 M sucrose–1 mM Tris (pH 7.5) was spun at 3200 *g* for 1 min and the superanant at 17,000 *g* for 2 min to yield the mitochondrial pellet. This pellet was suspended in 0.25 M sucrose–1 mM Tris (pH 7.5) to a volume of 1 ml per g original tissue and treated with an equal volume of 0.25 M sucrose–1 mM Tris (pH 7.5) containing digitonin (1.75 mg/ml) for 2 min, to disrupt the contaminating lysosomes. After dilution and subsequent washings by centrifuga-

tion at 19,000 *g* for 4.5–5.5 min, the pellet was suspended in 0.25 M sucrose.

Isolation of heavy and light mitochondrial fractions. These fractions from the liver were isolated with a view to study the relative distribution of lysosomal enzymes and were isolated by slight modification of the procedure described by De Martino and Goldberg [10]. A 15 per cent (w/v) homogenate of rat liver in 0.25 M sucrose–1 mM EDTA (adjusted to pH 7.0) was spun at 600 *g* for 10 min and the supernatant was spun at 3300 *g* for 10 min to yield the heavy mitochondrial pellet. The pellet was washed three times and the combined supernatants were spun at 16,300 *g* for 20 min to yield the relatively lysosome-rich, light mitochondrial pellet. This pellet was also washed three times by resuspension and centrifugation before being suspended in 0.25 M sucrose.

Preparation of 'tritosomes'. Pure lysosomes were isolated in the form of 'tritosomes' after injecting Triton WR 1339 intraperitoneally to rats, essentially according to Trouet [14].

Assay of enzymes. Cathepsin D activity was assayed by measuring the release of tyrosine from acid-denatured hemoglobin [15]. The assay conditions were similar to those employed by Liao Huang and Tappel [16]. The enzyme fraction was incubated with 5 mg of acid-denatured hemoglobin and 0.1 M acetate buffer, pH 4.0 in presence of 0.1 per cent (w/v) Triton X–100 in a total volume of 1 ml at 40°. After 30 min the reaction was terminated by the addition of 1 ml of 10 per cent trichloroacetic acid. The mixture was kept in cold for 30 min and then filtered. Aliquots of the filtrate were analyzed for tyrosine-equivalents using Folin–Ciocalteau reagent [17]. The enzyme activity is expressed as μg tyrosine released/mg protein/min using a tyrosine standard curve.

Cathepsin B was assayed using the synthetic substrate ← α- ←*N*-benzoyl-DL-arginine β-naphthylamide (BANA) and measuring the liberated β-naphthylamine after coupling it to fast garnet GBC according to Barrett [18]. To 0.5 ml of enzyme sample, 1.5 ml of EDTA-phosphate buffer (0.1 M in phosphate, pH6.0) containing cysteine (0.324 mg/ml) was added. After preincubation for 5 min at 40°, the reaction was started by the addition of 50 μl of the substrate in DMSO (40 mg/ml). The incubation was carried out at 40° for 10 min at the end of which 2 ml of the coupling reagent was added. After 10 min the colour developed was read at 520 nm. The specific enzyme activity is expressed as nmoles of β-naphthylamine released per mg protein per 10 min using a β-naphthylamine standard curve.

Neutral protease assay was similar to that of Gear *et al.* [7]. Mitochondrial suspension in 4 mM Tris buffer, pH 7.4 was incubated in a total volume of 1 ml containing around 5 mg of protein, at 37° for 1 hr. Triton X-100 was not included in the assays. The reaction was stopped by adding 1 ml of 10 per cent TCA. After keeping in ice for 30 min the mixture was centrifuged and aliquots of the supernatant were used for estimation of amino acids using ninhydrin reagent [19]. The specific enzyme activity is expressed as μmoles of glutamic acid equivalents released/mg protein/hr using glutamic acid as the standard.

Acid phosphatase was assayed using *p*-nitrophenyl phosphate (0.008 M) as described before [20]. The incubation mixture in a total volume of 2 ml contained: 0.2 ml of *p*-nitrophenyl phosphate (0.008 M), 1 ml of 0.05 M acetate buffer, pH 5.0 and 0.1 ml (1 mg) of Triton X-100. Incubation was carried our at 37° for 15 min. Reaction was stopped by adding 3 ml of 0.2 M glycine–NaOH buffer, pH 10.0. The yellow colour of *p*-nitrophenol released was measured in a Klett–Summerson colorimeter using 42 filter. The specific enzyme activity is expressed as μmoles of *p*-nitrophenol released/mg protein/min.

Cytochrome oxidase was assayed by the method of Wharton and Tzagoloff [21]. The rate of oxidation of reduced cytochrome *c* was recorded in a Cary 14 spectrophotometer at 550 nm, starting 15 sec after the addition of an appropriate amount of the mitochondrial or homogenate suspension in 10 mM phosphate buffer containing 1 per cent cholate to the cuvette containing 40 μM reduced cytochrome c and 10 mM phosphate buffer, pH 7.0. 0.01 ml of potassium ferricyanide (0.5 M) was added to the reference cuvette. The specific enzyme activity is expressed as μmoles of ferrocytochrome *c* oxidized/mg protein/min.

Glucose-6-phosphatase was assayed by the method of Aronson and Touster [22]. The incubation mixture in a total volume of 0.5 ml consisted of 0.05 ml of enzyme and 0.45 ml of the substrate solution prepared by mixing 0.1 M glucose-6-phosphate (sodium salt), pH 6.5, 35 mM histidine, pH 6.5, 10 mM EDTA, pH 7.0 and water in a ratio of 2:5:1:1 respectively. Incubation was carried out at 37° for 15 min and the reaction terminated by the addition of 1 ml of 10 per cent TCA. After centrifuging the tubes in cold, 1 ml of the supernatant was assayed for phosphate. The enzyme activity is expressed as μmoles of phosphate released/min.

Protein was estimated by the method of Lowry *et al.* [17].

RESULTS AND DISCUSSION

In the earlier studies mentioned, neutral protease activity has been assayed in mitochondrial fractions isolated with [8, 9] and without [7] digitonin treatment. Therefore, in the present study mitochondrial fractions have been isolated by two different methods, one involving differential centrifugation and repeated washing [12] and the other involving the additional use of digitonin [13]. The lysosomal contamination index of the two preparations has been assessed on the basis of the assay of acid phosphatase and cathepsin D as lysosomal enzyme markers and cytochrome oxidase as the mitochondrial enzyme marker. The results presented in Table 1 indicate that the mitochondrial fraction prepared by the procedure of Ragab *et al.* [12] shows about 8–10 per cent lysosomal contamination, whereas that prepared by the method of Lowenstein *et al.* [13] shows a contamination index of 1–3 per cent. The two mitochondrial fractions show less than 2 per cent contamination with the endoplasmic reticulum. The lysosome-rich mitochondrial fraction shows about 10 per cent endoplasmic reticulum contamination based on the recoveries of cathepsin D and glucose-6-phosphatase in the fraction.

Next, attempts have been made to characterize the neutral protease activities of the two mitochondrial preparations. The incubation conditions mentioned in

Table. 1. Assessment of the lysosomal contamination of the mitochondrial preparations

Preparation	Protein content (mg/g liver)	Cathepsin D	Acid phosphatase	Cytochrome *c* oxidase	Glucose-6-phosphatase
		(Units/g liver)			
Homogenate	200.1	458.9	25.2	20.3	16.10
Mitochondrial preparation I	8.01	21.4	1.16	10.05	0.15
Mitochondrial preparation II	6.03	4.34	0.22	7.15	0.04
Lysosome-rich fraction	20.40	182.3	16.51	3.32	0.80

Mitochondrial preparation I is obtained by the procedure of Ragab *et al.* [12] without the use of digitonin. Preparation II is obtained by the procedure of Lowenstein *et al.* [13] with the use of digitonin treatment. The enzyme activity units are: Cathepsin D—μg of tyrosine released/min/g liver; acid phosphatase—μmol of *p*-nitrophenol released/min/g liver; cytochrome *c* oxidase—μmol of cytochrome *c* oxidised/min/g liver; glucose-6-phosphatase—μmol *Pi* liberated/min/g liver.

Materials and Methods were arrived at to ensure assay of activity in the linear phase of increase of enzyme activity with respect to protein concentration and time of incubation. The effect of pH on the release of ninhydrin-positive material from the two mitochondrial preparations is depicted in Fig. 1. The mitochondrial fraction prepared without digitonin treatment shows an acidic pH optimum and the activity falls steeply with the increase in pH. Inclusion of phosphate, 50 mM, in the assay medium inhibits the activity significantly. The neutral protease activity detected in this preparation is of a similar magnitude as that reported by Gear *et al.* [7]. Digitonin treatment results in about 85 per cent loss of the neutral protease activity. The pH optimum is around 8.5 and the residual activity is not inhibited by phosphate. On the basis that digitonin treatment employed selectively disrupts the lysosomes [13], it can be concluded that the phosphate inhibitable neutral protease activity of the mitochondrial fraction resides in the contaminating lysosomes.

Additional properties of the phosphate-inhibitable neutral protease activity are presented in Table 2. It can be seen that the activity is significantly inhibited by leupeptin and sulfhydryl reagents but not by pepstatin. The properties of the phosphate-inhibitable neutral protease have also been compared with those of the enzyme

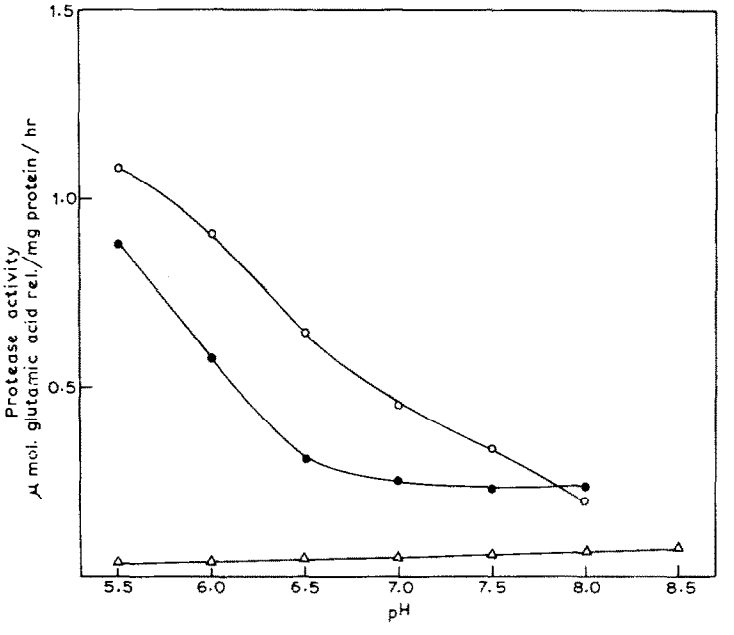

Fig. 1. Effect of pH on the protease activity of the mitochondrial fractions. The enzyme activity was assayed in Tris–maleate buffer to cover the pH range 5.5–8.0. Rest of the incubation conditions are as given in [7] and text. (○——○) protease activity of mitochondrial preparation I; (●——●) protease activity of mitochondrial preparation I in presence of 50 mM phosphate; (△——△) protease activity of mitochondrial preparation II.

Table 2. Properties of the neutral protease activity of the mitochondrial fraction

Additions to the complete incubation mixture	Neutral protease activity
None	100 (0.34 ± 0.03)*
Iodoacetate, 2 mM	29.5
PHMB, 2 mM	17.9
Pepstatin, 0.2 mM	98.7
Leupeptin, 0.2 mM	43.6
Phosphate, 50 mM	42.5

* The activity of complete incubation mixture is taken as 100. The absolute activity is given in parentheses and the units are μmoles of amino acids released/mg protein/hr. The other experimental details are given in text.

Table 3. Properties of the neutral protease activity of the lysosome fraction

Fraction	Additions to the incubation mixture	Neutral protease activity (μmoles of amino acids released/mg protein/hr)
Tritosomes	Nil	Not detectable
Tritosomes	Casein	34.0
Tritosomes	Digitonin-washed mitochondria	7.7
Tritosomes	Hemoglobin	2.8
Tritosomes	Bovine serum albumin	1.2
Tritosomes	Casein + PHMB	10.3
Tritosomes	Casein + leupeptin	10.5
Tritosomes	Casein + phosphate	15.1
Lysosome-rich fraction	Nil	0.19

Tritosomal extract (140 μg protein) was incubated with or without added substrates (5–6 mg protein) at 37° for 1 hr in presence of Tris buffer, 20 mM, pH 7.4. The results represent the average of two experiments. Suitable substrate and zero time blanks were employed.

extract prepared from pure lysosomes isolated in the form of 'tritosomes'. The results presented in Table 3 indicate that the 'tritosome' extract has very little neutral protease activity in the absence of added substrate. Casein serves as a good substrate followed by digitonin-washed mitochondria. The enzyme activity is inhibited by phosphate, *p*-hydroxymercuribenzoate and leupeptin. These results clearly establish that the phosphate-inhibitable neutral protease activity of the mitochondrial fraction prepared without digitonin treatment is essentially due to lysosomal thiol proteinases such as cathepsins B [23], H [24] and L [25], which are inhibited by leupeptin.

Next, the effect of CPIB treatment on the levels of lysosomal enzymes and neutral protease activity in the heavy mitochondrial and lysosome-rich fractions was studied. The fractions were isolated basically as described by De Martino and Goldberg [10] and the effect of CPIB on the distribution of lysosomal enzymes between the two fractions was studied. The results presented in Table 4 indicate that CPIB at 0.5 per cent (w/w) level in the diet, when fed for 4 days increases the protein content in the heavy mitochondrial and lysosome-rich fractions. However, after 2 days of feeding there is a statistically significant increase in the lysosome-rich fraction but not in the heavy mitochondrial fraction. The increase in the lysosome-rich fraction under these conditions is not due to any contribution by the endoplasmic reticulum comtamination. In fact, the endoplasmic reticulum contamination index of the lysosome-rich fraction falls from 10 per cent to about 7 per cent in the case of CPIB fed animals. The specific activity as well as the activity per unit weight of liver of acid phosphatase and neutral protease show an increase in the lysosome-rich fraction from CPIB-fed amimals. These activities are significantly decreased in the heavy mitochondrial fraction. It is significant to note that the specific activity of neutral protease of the lysosome-rich fraction in normal animals (0.19) is lower than that of the heavy mitochondrial fraction (0.36). CPIB treatment elevates the activity in the lysosome-rich fraction but depresses the same in the heavy mitochondrial fraction. This observation has been cited by Gear *et al.* [7] to suggest that the neutral protease activity of the heavy mitochondrial fraction as well as its decrease under conditions of CPIB treatment cannot be due to lysosomal contribution. However, the results presented in the present study clearly establish that the neutral protease activity of the

Table 4. Effect of CPIB feeding on acid phosphatase and neutral protease levels in the heavy mitochondrial and lysosome-rich fractions

Treatment	Heavy mitochondrial fraction			Lysosome-rich fraction		
	Protein (mg/g liver)	AP* (Units/g liver)	NP† (Units/g liver)	Protein (mg/g liver)	AP* (Units/g liver)	NP† (Units/g liver)
Normal	10.5 ± 1.1	1.47 ± 0.14	3.78 ± 0.43	21.5 ± 1.31	17.63 ± 1.43	4.08 ± 0.51
CPIB 2 days	11.0 ± 0.9	0.77 ± 0.06	2.53 ± 0.31	26.4 ± 1.42	26.93 ± 2.16	5.07 ± 0.21
CPIB 4 days	15.4 ± 1.0	0.77 ± 0.09	2.77 ± 0.26	31.1 ± 2.50	37.63 ± 3.62	5.27 ± 0.43

*AP—acid phosphatase; †NP—neutral protease.
The enzyme activity units are as expressed in the text and Tables 1–3.
The results represent the mean ± S.D. obtained from four experiments.

Table 5. Effect of CPIB feeding on cathepsin D and cathepsin B levels in post-neclear supernatant, heavy mitochondrial and lysosome-rich fractions

Treatment	Post-nuclear supernatant		Heavy mitochondrial fraction		Lysosome-rich fraction	
	Cathepsin D (Units/g liver)	Cathepsin B (Units/g liver)	Cathepsin D (Units/g liver)	Cathepsin B (Units/g liver)	Cathepsin D (Units/g liver)	Cathepsin B (Units/g liver)
Normal	276.9 ± 15.3	3142 ± 123	30.1 ± 2.1	732.6 ± 25.3	174.1 ± 12.1	3326 ± 125
CPIB 2 days	310.2 ± 12.1	3205 ± 141	26.1 ± 0.7	552.6 ± 15.6	232.5 ± 13.5	3543 ± 134
CPIB 4 days	357.7 ± 25.2	3436 ± 163	29.1 ± 1.2	659.7 ± 25.4	263.1 ± 11.3	4015 ± 223

Cathepsin B activity units are expressed as nmoles of naphthylamine released/10 min/g liver. Cathepsin D activity units are as expressed earlier. The results represent the mean ± S.D. obtained from four experiments.

heavy mitochondrial fraction is essentially due to the lysosomal thiol proteinases. In addition, the results presented in Table 3 indicate that the 'tritosomal' extract has very little neutral proteolytic activity in the absence of added substrate. Digitonin-washed mitochondrial fraction serves as a good substrate for the 'tritosomal' extract. It is thus clear that the neutral protease activity of the lysosome-rich fraction is due to the action of the lysosomal neutral protease on associated mitochondria. The neutral protease specific activity of the lysosome-rich fraction assayed without the addition of Triton X-100 being lower than that of the heavy mitochondrial fraction can be attributed to limitation of engulfed mitochondria in the former fraction.

The idea that CPIB may be altering the pattern of distribution of the enzymes between the heavy mitochondrial and lysosome-rich fractions has been further examined by assaying the levels of cathepsin D and cathepsin B under these conditions. The results presented in Table 5 again indicate that under conditions of CPIB feeding the fraction of the activity residing with the heavy mitochondrial fraction shows a significant decrease. The specific activity pattern obtained is similar to the activity in the fraction expressed per unit weight of liver and hence only the latter data are presented in Tables 4–6. CPIB treatment enhances the total cathepsin D and cathepsin B activities in the post-nuclear supernatant as well as in the lysosome-rich fraction. The higher recovery of cathepsin B in the subcellular fractions compared to the activity present in the post nuclear supernatant can be due to the presence of blood components like α_2 macroglobulin in the latter, which are known to inhibit cathepsin B activity [26].

A clear picture on the effect of CPIB on enzyme distribution is evident from Table 6 where the results are presented as the activity in the heavy mitochondrial fraction expressed as per cent of the activity in the lysosome-rich fraction. This expression overcomes the variation in the enzyme activities of the post-nuclear supernatant caused by the varying contamination by blood components in different samples. CPIB decreases the per cent of activity associated with the heavy mitochondrial fraction even at 2 days of feeding when its effect on the protein content of the fraction is not significant. However, at this time period the protein content of the lysosome-rich fraction, the activity of the lysosome enzymes in this fraction and the post-nuclear supernatant or homogenate have already increased. This shows that CPIB has an early effect on lysosomes. A reasonable explanation is that CPIB inhibits the process of autophagy and thereby restricts the increase in lysosomal enzymes to the lighter lysosome-rich fraction. It is known that when autophagy is promoted lysosomes become heavier and pellet down at lower centrifugal forces [27]. It is interesting to note that insulin brings about a redistribution of lysosomal enzymes similar to CPIB in hepatocytes [28], an effect which is reversed by glucagon [27]. While the effect of CPIB in causing increased mitochondrial and lysosomal content of liver could also involve enhanced synthesis of the organellar proteins, an important effect of the drug appears to be to prevent the process of autophagy of mitochondria. A similar situation may exist for peroxisomes as well, since Jones and Masters [29] have shown that the increase in catalase activity in mouse liver under conditions of CPIB feeding results

Table 6. Effect of CPIB feeding on lysosomal enzyme activity in the heavy mitochondrial fraction expressed as per cent of activity in the lysosome-rich fraction

Treatment	Cathepsin D	Cathepsin B	Acid Phosphatase	Neutral Protease
	(Percent activity in the heavy mitochondrial fraction)*			
Normal	17.3	22.0	8.3	92.6
CPIB 2 days	11.2	15.6	2.8	49.9
CPIB 4 days	11.1	16.4	2.0	52.6

* The data presented in tables 4 and 5 are expressed as:

$$\frac{\text{Activity in the heavy mitochondrial fraction/g liver}}{\text{Activity in the lysosome-rich fraction/g liver}} \times 100.$$

from decreased degradation of the protein. In retrospect this also emphasizes the importance of lysosomes in the normal turnover of organelles.

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