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LACK OF CORRELATION BETWEEN CYCLIC AMP SYNTHESIS AND FREE FATTY ACID RELEASE IN BROWN FAT OF COLD-ADAPTED RATS

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Abstract—Correlation between cyclic AMP and glycerol and free fatty acid release was studied in brown fat of normal rats and of rats exposed to cold for different periods, varying from 20 days to 6 months. Comparison with white epididymal fat was always made at the same time. Cyclic AMP synthesis and final triglyceride hydrolysis, are strictly related in brown fat of normal rats, both under basal conditions and under stimulation (in the presence of noradrenaline and/or theophylline). Such a correlation was maintained in brown fat of rats exposed to 4° for 6 months, but only under basal conditions. In contrast, when brown fat of cold-acclimated rats was incubated in the presence of noradrenaline and theophylline, cyclic AMP accumulation was still increased, but this increase was not associated with a stimulation of lipolysis. An analogous dissociation was found in epidymal fat incubated under anaerobic conditions. Results obtained in the presence of propranolol and phenoxybenzamine indicate that in brown, as well as in white fat, the interaction of catecholamines with the beta receptor is associated with the activation of adenylate cyclase. The alpha effect seems to be associated with the inhibition of adenylate cyclase and reduced cyclic AMP accumulation.

THE HEAT production is generally regarded as the prominent role of interscapular brown adipose tissue in cold-adapted homeothermic animals.[1–6] The increase in heat production during cold-adaptation is sustained by a non-shivering thermogenesis related to an increased metabolic rate of the tissue. A greater oxygen uptake[4,6] is accompanied by an increased fatty acid oxidation.[1,7,8]

In cold-acclimated rats[9] the basal free fatty acid and glycerol release was increased, but the hormone stimulation of lipolysis was no longer evident.

Other interesting adaptive changes of brown adipose tissue in cold-exposed rats are represented by changes in some biochemical parameters of energy metabolism.[10]

We have investigated the relationship between lipolysis and energy metabolism in interscapular brown fat, especially that between cyclic AMP level and free fatty acid (FFA) and glycerol release in brown fat of normal rats and of rats exposed to 4° for different periods, varying from 20 days to 6 months. Comparison with white epididymal fat was made. Experiments were carried out in the presence and absence of two lipolytic agents, noradrenaline and theophylline.

The results clearly indicate that in brown fat of normal rats hormone-stimulated lipolysis was accompanied and probably mediated by an increase of cyclic AMP

level, as in epididymal white fat. In contrast to the white fat, cyclic AMP accumulation in brown fat of cold-acclimated rats was not associated with a stimulation of lipolysis.

MATERIALS AND METHODS

Wistar rats (130 ± 20 g) were exposed at 4° in a cold room for different periods, varying from 20 days to 6 months. During these periods the animals were maintained on a standard laboratory diet with water *ad lib.* up to the time of sacrifice. After light ether anaesthesia, epididymal (white) and interscapular (brown) fat pads were excised from the same animals (4–6 rats) and immediately prepared for incubation. Interscapular brown fat was sliced and pooled, whereas samples of epididymal fat were simply randomized. Determination of lipolysis and assays for cyclic AMP were carried out in parallel in white and brown adipose tissue of normal and acclimated rats.

The control animals were housed at normal room temperature. The control values reported in each experiment for brown and white fat, were obtained from animals of the same age and maintained on the same diet.

Treatment of adipose tissue, assays of lipolysis and determination of cyclic AMP levels, were described previously.[11–15] Free fatty acids were titrated into the tissue and in the incubation medium according to Dole[16] and glycerol according to Korn.[17] For the assay of cyclic AMP, fat pads were removed from different animals (normal and acclimated rats) and pooled to maximum random distribution, were weighed (200 ± 5 mg) and placed in 1·90 ml of Krebs–Ringer bicarbonate buffer pH 7·2 containing bovine albumin 2·5 per cent. After a preliminary incubation in a metabolic shaker at 37° for 30 min, noradrenaline and/or theophylline were added and the samples incubated for a further 11 min at 37° under air in a shaking apparatus. The reaction was stopped by addition of 0·15 ml of ice-cold 50% TCA.

Tissue and medium were rapidly transferred to Potter homogenizers with Teflon pestles and carefully homogenized for 3 min. Cyclic AMP (100 pmoles) was added to a control sample to determine its recovery. The samples were then neutralized by the addition of 0·4 ml 1 M Tris and then centrifuged for 20 min at 12,000 *g*.

Cyclic AMP was purified from the neutralized supernatants by the $BaSO_4$ method of Krishna, Weiss and Brodie.[18] To the extracts 1·5 ml each of 5% $ZnSO_4$ and 2·6% Ba $(OH)_2$ were added.

As described previously,[12–15] after the removal of the $BaSO_4$ precipitated by centrifugation, cyclic AMP was further purified by ion exchange chromatography according to Kuo and Greengard.[19,20]

The nucleotide levels were measured by the method of Kuo and Greengard,[19,20] based upon the ability of a protein kinase from bovine heart to catalyze the transfer of ^{32}P to histone from $\gamma^{32}P$-ATP in a reaction dependent on the presence of cyclic AMP. Protein kinase was prepared according to Kuo and Greengard[21] and Kuo *et al.*[22]

The amounts of synthetic cyclic AMP used in each experiment to obtain the standard curve were from 0·5 to 12 pmoles. Under our experimental conditions, the slope of the curve relating the activity of the protein kinase to the concentration of cyclic AMP was constant between 0·5 and 10 pmoles and the apparent K_m value for cyclic AMP was $1{\cdot}2 \times 10^{-8}$ M. Overall recovery of synthetic cyclic AMP added to the con-

trol samples was between 60 and 75 per cent. The data have been corrected in each experiment for the recovery.

Cyclic AMP, free fatty acid and glycerol levels, were respectively expressed as pmoles, μ-equivalents and μmoles/g of fresh tissue.

Materials. DEAE-cellulose (medium mesh, 0·85 m-equiv./g), histone (Type II), cyclic AMP and bovine serum albumin (Fraction V) were purchased from Sigma.

γ^{32}P-ATP was obtained from Radiochemical Centre, Amersham. AG 50W-X8 resin (200–400 mesh) purchased as analytical grade from Bio Rad Laboratories in hydrogen form, was washed repeatedly in distilled water to remove the fine particles and kept as a stock at 4° at 50% (v/v) suspension in distilled water. Noradrenaline bitartrate monohydrate was from Recordati, theophylline from C. Erba, sodium fluoride from Merck and monoiodoacetic acid from British Drug Houses. $N^6C_2^1$-dibutyryl cyclic 3',5'-AMP was a generous gift of Dr. M. Carissimi (Maggioni, Milan, Italy). Propranolol 1-(isopropylamino)-3-(1-naphthyl oxy)-2-propanol hydrochloride was purchased from Imperial Chemical Industries (Wilmslow, England) and phenoxybenzamine hydrochloride, from Smith Kline and French (Philadelphia, U.S.A.).

RESULTS

Effect of cold-exposure on spontaneous cyclic AMP production and on basal FFA and glycerol release in brown and white adipose tissue from the same animals

Spontaneous FFA and glycerol release. Acclimated rats used in these experiments were exposed to 4° for 3 months. The spontaneous FFA and glycerol release in normal rats was higher in brown than in white fat. After cold acclimation, the FFA mobilization was doubled in brown fat, whilst it remained unchanged in epididymal fat (Table 1).

TABLE 1. SPONTANEOUS LIPOLYSIS IN BROWN AND WHITE FAT OF NORMAL AND COLD-ACCLIMATIZED RATS

Rats	FFA (μ-equiv.g^{-1} 150 min^{-1}) (medium + tissue)		Glycerol in the medium (μM g^{-1} 150 min^{-1})	
	Brown fat	White fat	Brown fat	White fat
Normal	20·1 ± 3·1	8·6 ± 1·6	11·2 ± 1·6	4·9 ± 0·9
Acclimated	45·4 ± 10·2*	9·1 ± 1·0‡	16·1 ± 1·0†	5·3 ± 0·8‡

Rats were acclimatized at 4° for 3 months. Samples of interscapular (brown) and epididymal (white) fat (100 ± 5 mg) were incubated in 2 ml of Ca^{2+}-normal Krebs–Ringer bicarbonate buffer pH 7·2 containing 2·5% bovine albumin at 37° for 150 min in a metabolic shaker. Each value represents the mean ± S.E. of five assays from two experiments.

* $P < 0·005$.

† $P < 0·02$.

‡ $P > 0·80$.

Basal cyclic AMP levels. In the absence of drugs, that is, under basal conditions, the cyclic AMP levels in brown adipose tissue were progressively increased by prolonging the cold exposition time. After 6 months of cold exposure, the level was about 70–90 times higher than that in normal rats (Table 2).

In contrast, in white adipose tissue of the same animals the basal level of cyclic AMP decreased with time (Table 2). Moreover, from the comparison of data from

TABLE 2. EFFECT OF COLD-EXPOSURE ON CYCLIC AMP LEVELS IN BROWN AND WHITE FAT

Cold-exposure time (days)	pmoles Cyclic AMP/g fresh tissue (medium + tissue) Brown fat	White fat
0	12·83 ± 9·07	226·86 ± 37·62
20	61·38 ± 11·30*	145·21 ± 4·69*
90	209·58 ± 19·13†	83·97 ± 27·15†
180	908·21 ± 25·70†	51·44 ± 8·57†

Rats were acclimatized at 4° for 20, 90 or 180 days. Samples of interscapular (brown) and epididymal (white) fat (200 ± 5 mg) were incubated in 2 ml of Ca^{2+}-normal Krebs–Ringer bicarbonate buffer containing 2·5% bovine albumin at 37° for 41 min in a metabolic shaker. At the end of the incubation, tissue and medium were treated as indicated in the methods. Cyclic AMP was then extracted, purified and titrated by the use of bovine heart protein kinase (12 μg). The data are the means ± S.E. of eight determinations from two experiments.

* $P < 0·05$.

† $P < 0·01$ with respect to zero time.

brown and white fat it is interesting to note that in normal rats, the cyclic AMP concentration was much higher in white than in brown fat (Table 2).

Effect of cold-exposure on cyclic AMP production and on FFA release induced by noradrenaline and by theophylline in brown and white adipose tissue from the same animals

Spontaneous FFA release in brown and white fat in the presence of 10^{-5} M *noradrenaline.* Rats were maintained at 4° for 90 days. Noradrenaline was normally active in white adipose tissue both of normal and cold acclimated rats. In contrast, in brown fat of cold adapted animals, the stimulatory effect of noradrenaline disappeared in parallel with the increased basal lipolysis (Table 3). In brown fat of normal rats, the stimulatory effect of noradrenaline is still present, even if quantitatively lower in comparison with that induced in white fat (Table 3).

Cyclic AMP levels in brown and white fat in the presence of noradrenaline. Rats used for these assays were exposed to 4° for 20 or 90 days. As shown in Table 4 noradrenaline by itself clearly increased the cyclic AMP synthesis both in brown and in white

TABLE 3. EFFECT OF NORADRENALINE ON FFA RELEASE IN BROWN AND WHITE FAT OF NORMAL AND COLD-ACCLIMATIZED RATS

Cold-exposure time (days)	Drugs in the medium	FFA μ-equiv. g^{-1} 150 min^{-1} (medium + tissue) Brown fat	White fat
0	—	20·10 ± 3·39	8·64 ± 1·64
	NE 10^{-5} M	40·24 ± 2·34*	50·94 ± 3·24*
90	—	45·43 ± 10·16	9·10 ± 9·00
	NE 10^{-5} M	54·93 ± 8·40†	53·64 ± 1·51*

Rats were acclimatized at 4° for 3 months. Samples of interscapular (brown) and of epididymal (white) fat (100 ± 5 mg) were preincubated in 2 ml of Ca^{2+}-normal Krebs–Ringer bicarbonate buffer pH 7·2 containing 2·5% bovine albumin at 37° for 30 min in a metabolic shaker. At that time where indicated 10^{-5} M noradrenaline (NE) was added and the incubation of both treated and untreated tissue was continued for a further 120 min.

Each value represents the mean ± S.E. of five to ten assays.

* $P < 0·005$.

† N.s.

TABLE 4. EFFECT OF NORADRENALINE ON CYCLIC AMP LEVELS IN BROWN AND WHITE FAT OF NORMAL AND COLD-ACCLIMATIZED RATS

Cold-exposure time (days)	Drugs in the medium	Cyclic AMP pmoles/g fresh tissue (medium + tissue)	
		Brown fat	White fat
0	—	12·83 ± 9·07	468·50 ± 127·64
	NE 10^{-5} M	229·47 ± 26·94*	1779·41 ± 124·47*
		(× 18)	(× 4)
20	—	61·38 ± 11·30	155·35 ± 54·14
	NE 10^{-5} M	46·11 ± 12·01†	708·38 ± 55·15*
			(× 5)
90	—	209·58 ± 19·13	51·44 ± 8·57
	NE 10^{-5} M	146·94 ± 11·72	252·55 ± 13·63*
			(× 5)

Rats were acclimatized at 4° for 20 or 90 days. Samples of interscapular (brown) and of epididymal (white) fat (200 ± 5 mg) were preincubated in 2 ml of Ca^{2+}-normal Krebs–Ringer bicarbonate buffer containing 2·5% bovine albumin at 37° for 30 min in a metabolic shaker. At that time where indicated 10^{-5} M noradrenaline (NE) was added and the incubation continued for a further 11 min. The experimental conditions for extracting, purifying and titrating cyclic AMP were as described in Table 2.

The data are the means ± S.E. of eight determinations from two experiments.

* $P < 0.01$.

† N.s.

fat of normal rats. However, after 3 months of cold-exposure the effect was maintained only in white adipose tissue. Instead, in brown fat, in parallel to the increase in basal level (Table 2), there was a lack of stimulation by noradrenaline (Table 4).

TABLE 5. EFFECT OF THEOPHYLLINE ON THE LEVELS OF CYCLIC AMP IN BROWN AND WHITE FAT OF COLD-ACCLIMATIZED RATS

Cold-exposure time (days)	Theophylline in the medium	Cyclic AMP pmoles/g fresh tissue (medium + tissue)	
		Brown fat	White fat
0	—	12·83 ± 9·07	694·42 ± 62·97
	3×10^{-3} M	542·21 ± 18·00*	1145·55 ± 118·70*
		(× 42)	(× 1·6)
20	—	61·38 ± 11·30	97·11 ± 14·52
	3×10^{-3} M	67·41 ± 12·31†	198·20 ± 18·01*
			(× 2·0)
90	—	209·58 ± 19·13	51·44 ± 8·57
	3×10^{-3} M	198·66 ± 17·51†	149·54 ± 3·46*
			(× 2·9)
180	—	908·21 ± 25·70	54·90 ± 5·08
	3×10^{-3} M	1030·67 ± 30·17†	175·18 ± 5·95*
			(× 3·2)

Rats acclimatized at 4° for 20, 90 or 180 days. Samples of interscapular (brown) and of epididymal (white) fat (200 ± 5 mg) were incubated in 2 ml of Ca^{2+}-normal Krebs–Ringer bicarbonate buffer containing 2·5% bovine albumin, for 30 min at 37° in a metabolic shaker. At that time where indicated 0·003 M theophylline was added and all the samples further incubated for 11 min. The experimental conditions for extracting, purifying and titrating cyclic AMP were as described in Table 2. The data are the means ± S.E. of eight determinations from two experiments.

* $P < 0.01$.

† N.s.

TABLE 6. EFFECT OF NORADRENALINE PLUS THEOPHYLLINE ON CYCLIC AMP LEVELS IN BROWN AND WHITE FAT OF NORMAL AND COLD-ACCLIMATIZED RATS

Cold-exposure time (days)	Theophylline in the medium	Cyclic AMP pmoles/g fresh tissue (medium + tissue)	
		Brown fat	White fat
0	—	12·85 ± 9·07	220·07 ± 32·26
	3×10^{-3} M	3794·20 ± 176·01*	26066·24 ± 1487·50*
	+ NE 10^{-5} M	(× 295)	(× 118)
90	—	209·58 ± 19·13	83·97 ± 27·15
	3×10^{-3} M	1965·14 ± 92·77*	22170·01 ± 847·25*
	+ NE 10^{-5} M	(× 9)	(× 264)
180	—	908·21 ± 25·70	51·44 ± 8·57
	3×10^{-3} M	1505·95 ± 52·12*	15284·34 ± 1046·68*
	+ NE 10^{-5} M	(× 1·7)	(× 297)

Rats were acclimatized at 4° for 90 or 180 days. Experimental conditions are described in Table 4. The data are the means ± S.E. of eight determinations from two experiments.
NE = noradrenaline.
* $P < 0.001$.

The data of Tables 3 and 4 clearly indicate that noradrenaline looses its stimulatory power both on lipolysis and on cyclic AMP synthesis in brown fat of cold-exposed rats.

Cyclic AMP levels in brown and white fat in the presence of 3×10^{-3} M *theophylline.* Theophylline by itself has the same effect as noradrenaline on cyclic AMP synthesis in brown and white fat of normal and cold adapted rats (Table 5).

TABLE 7. EFFECT OF BETA (PROPRANOLOL) AND ALPHA (PHENOXYBENZAMINE) ANTIADRENERGIC DRUGS ON CYCLIC AMP LEVELS IN BROWN FAT OF NORMAL AND COLD-ACCLIMATIZED RATS

Drugs in the medium	pmoles Cyclic AMP/g fresh tissue (medium + tissue)	
	Normal rats	Cold-acclimatized rats
—	12·83 ± 9·07	209·58 ± 19·13
Propranolol 10^{-4} M	111·28 ± 6·31*	122·15 ± 4·15†
Phenoxybenzamine 10^{-3} M	441·83 ± 16·37*	490·43 ± 8·94*
Noradrenaline 10^{-5} M	3794·20 ± 176·01*	1695·06 ± 92·77*
+ theophylline 3×10^{-3} M		
Noradrenaline 10^{-5} M	1128·88 ± 77·93†	399·45 ± 6·00*
+ theophylline 3×10^{-3} M	(−70%)	(−80%)
+ propranolol 10^{-4} M		
Noradrenaline 10^{-5} M	4908·75 ± 192·30‡	1849·52 ± 136·21
+ theophylline 3×10^{-3} M	(+29%)	(no increase)
+ phenoxybenzamine 10^{-3} M		

Rats were acclimatized at 4° for 3 months. Samples of interscapular (brown) fat (200 ± 5 mg) were incubated in 2 ml of Ca^{2+}-normal Krebs–Ringer bicarbonate buffer containing 2·5% bovine albumin and, where indicated, the antiadrenergic drugs for 30 min at 37°. Noradrenaline and theophylline were then added and all the assays further incubated for 11 min. The experimental conditions for extracting, purifying and titrating cyclic AMP, were as described in Table 4. The data are the means ± S.E. of eight determinations from two experiments.
* $P < 0.001$.
‡ $P < 0.005$.
† $P < 0.05$.
P values were determined vs respective control.

Cyclic AMP levels in brown and white fat in the presence of 10^{-5} M *noradrenaline plus* 3×10^{-3} M *theophylline.* When noradrenaline was added together with theophylline (to prevent cyclic AMP hydrolysis by phosphodiesterase) a significant potentiation of cyclic AMP accumulation due to noradrenaline, was observed in brown fat of normal rats. As reported in Table 6, the stimulatory effect induced by the two adipokinetic agents was still maintained in brown fat of animals exposed to cold for 3 and for 6 months (from 908·21 to 1505·95 pmoles of cAMP/g of fresh tissue), but to a lower degree than in brown fat of normal rats.

Comparison with epididymal adipose tissue (Table 6) clearly emphasizes that also after 6 months of cold-exposure, noradrenaline plus theophylline stimulated the cyclic AMP accumulation to enormous levels (15,000 pmoles of cAMP/g of fresh tissue) in white tissue. These levels were markedly higher than those found in brown tissue both of normal and of cold-acclimated rats under analogous experimental conditions. Moreover, it is interesting to note that the stimulatory effect exerted by the two lipolytic agents on cyclic AMP accumulation in brown fat of cold-adapted rats, is quantitatively correspondent to that found in white epididymal adipose tissue by incubating the fat under anaerobic conditions.[23]

Effect of phenoxybenzamine and propranolol on cyclic AMP level in brown fat of normal and of cold-exposed rats

Taking into consideration the high noradrenaline content of brown tissue[24–26] we studied the effect of alpha (phenoxybenzamine) and beta (propranolol) antiadrenergic drugs on cyclic AMP synthesis in the presence and absence of noradrenaline plus theophylline.

In brown fat of normal rats the basal level of cyclic AMP was increased by 10^{-4} M propranolol (Table 7), whereas the hormone-induced cyclic AMP synthesis was strongly antagonized by the drug (−70 per cent). In the case of cold acclimated rats, (Table 7) both the basal and the hormone-induced cyclic AMP synthesis were significantly inhibited by propranolol (−80 per cent).

Phenoxybenzamine (10^{-3} M) increased the basal cyclic AMP concentration both in the tissue of normal and of cold acclimated rats. In addition this antiadrenergic drug potentiated the stimulatory effect of noradrenaline plus theophylline in brown fat of normal rats and did not modify the hormonal action in brown fat of cold-exposed rats (Table 7).

DISCUSSION

The results described indicate a good relationship between the variations of cyclic AMP levels and FFA release in brown fat of normal rats, both under basal conditions and hormonal stimulation. In this tissue, as in epididymal fat, cyclic AMP synthesis was increased by noradrenaline or by theophylline, and theophylline clearly potentiated the hormonal action on the process (295 fold over the basal value). Thus, in the presence of the two adipokinetic agents cyclic AMP concentration reached the level needed for the increased lipolytic rate (Table 1). In epididymal adipose tissue, cyclic AMP was increased by 10^{-5} M noradrenaline or by 3 mM theophylline alone, to levels sufficient for a maximum stimulation of lipolysis (two- to five-fold over the basal value). In the presence of both noradrenaline and theophylline a supramaximal

accumulation (100- to 400-fold over the basal value) of cyclic AMP, not related to activation of lipolysis was found.[12–15,22,50]

This situation was not altered in white fat of cold-acclimated rats. However, in brown fat after cold exposure, both cyclic AMP levels and spontaneous lipolysis were increased, but, when theophylline was present together with noradrenaline the cyclic AMP synthesis, even if still increased to a certain degree, was not accompanied by a stimulation of lipolysis.

An analogous situation was found by incubating epididymal adipose tissue of rats under anaerobic conditions. That is, a cyclic AMP increase, largely sufficient to induce a maximum stimulation of the lipolytic process, was not followed by a correspondent increase in lipolysis.[23] Thus, in brown fat of acclimated rats, as in white fat incubated in the absence of oxygen, the final triglyceride hydrolysis becomes insensitive to hormonal stimulation and cyclic AMP synthesis is no more the rate-limiting step in the process.

The lack of correlation between cyclic AMP synthesis and the final FFA release, suggests a deficiency of ATP, which is necessary at different steps of the process, before, and particularly after cyclic AMP synthesis.[11,14,15,27–29]

Taking into consideration that in brown fat of cold-acclimated rats, FFA largely accumulate inside the tissue,[9] this high intracellular level of FFA could exert a feedback control of lipolysis directly, or, indirectly by uncoupling the oxidative phosphorylation.[30–37] Modulation of lipolysis in adipose tissue by intracellular fatty acid concentration, and the negative effects of an increased FFA concentration in fat cell are well known.[38,39] Moreover the effects of added fatty acids are similar to those of a typical uncoupler of oxidative phosphorylation, such as 2,4-dinitrophenol.[40] Alternatively, according to Prusiner *et al.*[41] a deficiency of ATP disposal to the lipolytic process could be dependent on the great demand of ATP for re-esterification[42,43] or, mainly, for the oxidation of FFA, a process that in brown fat mitochondria was found only ATP-dependent.[44]

To conclude, in brown adipose tissue of normal rats, the metabolic pathways seem to be similar to those in white fat. However, after cold-acclimation, that is, during maximal physiological activity, during heat-production, the two phases of the lipolytic process (the cyclic AMP synthesis and the final FFA and glycerol release) become separated presumably through the lack of ATP availability before and, mostly, after cyclic AMP synthesis. Finally, experiments carried out in the presence of beta and alpha antiadrenergics, propranolol and phenoxybenzamine, confirm that in brown, as well as in human[47–50] or in rat epididymal white fat,[49] the interaction of catecholamines with the beta receptor is associated with the activation of adenylate cyclase. Instead, the alpha effects seem to be associated with the inhibition of adenylate cyclase and reduce cyclic AMP accumulation as in human white adipocytes.[45,50,51]

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