MUTANTS OF *NEUROSPORA CRASSA* PERMEABLE TO HISTIDINOL

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IN Neurospora, L-histidinol is the immediate precursor of L-histidine, as shown by the isolation of L-histidinol dehydrogenase and the study of its catalytic functions (AMES 1957; CREASER, BENNETT and DRYSDALE 1965). The failure of histidine mutants, with the histidinol dehydrogenase function intact, to grow on histidinol, suggests that histidinol cannot enter the cell. However, CATCHESIDE (1960) and WEBBER, CASE and GILES (quoted in WEBBER 1960) have independently obtained variants of histidine mutants that can grow on histidinol. CATCHESIDE (1960) suggested that the mutation might involve a change in the system (permease) by which histidine is transported.

The transport of histidine is connected with that of several amino acids. Growth of histidine mutants is inhibited by combinations of a neutral (especially aromatic) amino acid with arginine or lysine (HAAS, MITCHELL, AMES and MITCHELL 1952) and this was shown by MATHIESON and CATCHESIDE (1955) to be due to the prevention of histidine uptake.

The present work sought to determine which genes were able to mutate to allow entry to histidinol and, by comparative physiological experiments, to discover the functions of the normal alleles of these genes. Such work should contribute to an understanding of these mutations and the mechanism of inhibition of uptake of histidine.

MATERIALS AND METHODS

The strains used were the wild type Emerson *a* and the mutants 30300 (*arg-3*) arginine-requiring; K458 (*his-3*) histidine-requiring; A239 (*lys-4*) lysine-requiring; 65001 (*nt*) nicotinic-tryptophan-requiring and E15172 (*sfo*) sulfonamide-requiring. Strains permeable to histidinol will be described later.

The minimal media of VOGEL (1964) and WESTERGAARD and MITCHELL (1947), with appropriate supplements, were used for vegetative growth and crosses, respectively. VOGEL's medium plus 0.5% L-sorbose and 0.1% sucrose (SS) with suitable supplements was used for scoring progeny. VOGEL's medium plus 0.5% L-sorbose, 0.0125% D-glucose and 0.025% D-fructose (SGF) with suitable supplements was used for the isolation of mutants and germination of ascospores. The quantities of supplements (mg per 100 ml medium) used routinely for crosses, maintenance and scoring of cultures were as follows: 20 L-arginine HCl, L-citrulline, L-histidine HCl·H_2O, L-lysine HCl; 60, L-histidinol 2HCl; 1, nicotinamide.

Cultures were tested for ability to grow on histidinol by inoculating conidia on solid VOGEL's medium supplemented with histidinol; scoring was usually possible after 3 days of incubation at 25°C. It may be noted that K458 (*his-3*) also grows very poorly on solid medium containing histidinol but much less as compared to strains permeable to histidinol. Tests for *sfo* and *nt* were also done on solid VOGEL's medium, *sfo* being scored after 1–2 days of incubation at 34°C.

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Growth experiments were carried out in 100 ml Erlenmeyer flasks containing 20 ml of liquid VOGEL's medium with 2% sucrose and various L-amino acids and other compounds added. After incubation at 25°C in the dark on a reciprocating shaker, the mycelial mass was harvested from each flask, squeezed free of liquid, dried at 80–85°C for 5 hrs and weighed.

RESULTS

Genetics: Twelve mutants able to grow on histidinol were obtained from K458 following irradiation by ultraviolet light. Three (*BS37*, *BS38* and *BS39*) were obtained by Dr. B. R. SMITH and nine (*HC111* to *HC119*) by me. All of them also grew on histidine but not on minimal medium. The yield curves of *BS38* (Figure 1) showed a threshold requirement for histidinol, while yield on histidine was proportional to concentration.

BS38 was shown to be located in linkage group VII, first by finding weak linkage (37%) to *nt* and then close linkage (9%) to *sfo*. A three point cross (Table 1) showed that the order is *sfo—BS38—nt*. Among the progeny, two recombinant types, + *BS38* + and *sfo* + *nt*, were relatively common and represent crossing over between *nt* and either *BS38* or *sfo* and so do not help to decide the order. The remaining type, *sfo BS38 nt* (2 individuals), results from crossing over between *sfo* and *BS38*. Since *BS38* and *nt* were not recombined in these progeny, *BS38* must lie between *sfo* and *nt*. If the order were otherwise, these two individuals would be double crossovers, normally quite rare.

Several of the other mutants were tested for allelism to *BS38* by examining crosses for progeny unable to grow on histidinol. Of course, even two allelic

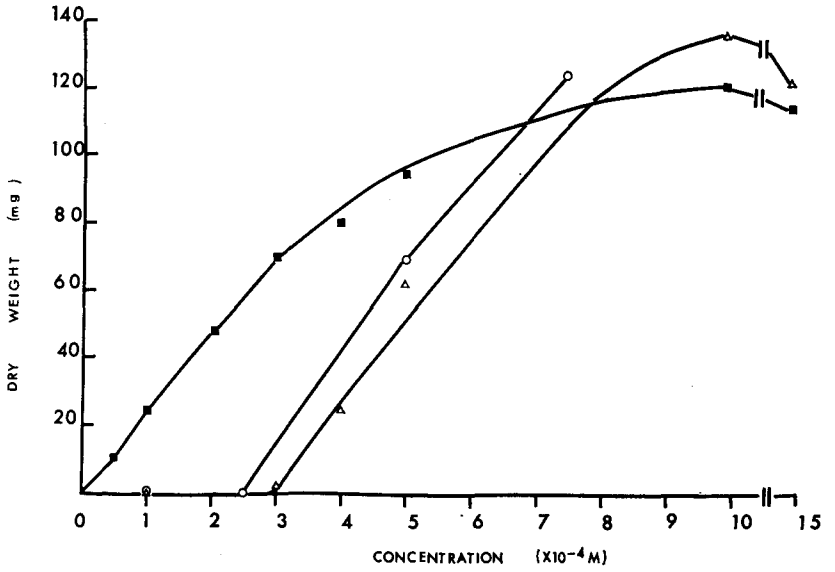

FIGURE 1.—Yield curves of *his-3 hlp-1* (K458 BS38) on histidinol and histidine. Each point is the average of weights of mycelia from two flasks. ■ = histidine (90 hrs). △ = histidinol (90 hrs). ○ = histidinol (104 hrs). Hours given were hrs of incubation.

TABLE 1

Data of three point crosses to place BS38 and HC114 with respect to sfo *and* nt.

All parents carry the K458 gene

	Progeny of crosses + BS38 *nt* × *sfo* + +			+ HC114 + × *sfo* + *nt*
+	*hlp*	*nt*	62	15
sfo	+	+	48	11
+	+	+	0	0
sfo	*hlp*	*nt*	2	0
+	*hlp*	+	19	31
sfo	+	*nt*	25	27
+	+	*nt*	0	4
sfo	*hlp*	+	0	1
			156	89

mutants could recombine, but experience in Neurospora shows that this usually occurs with frequencies less than 10^{-3}. Hence appreciably greater frequencies of recombination suggest non allelism.

Crosses of *HC114* to *BS38* had recombination fractions of 0.25 (63 tested) and 0.08 (79 tested) in two families. A recombination fraction of 0.07 between *sfo* and *HC114* confirmed that *HC114* is also in linkage group VII. The data (Table 1) show that the order is *sfo—HC114—nt*.

It is unlikely that *BS38* and *HC114* are allelic because of the high frequency of recombination between them. The stocks used have been purified, through crossing, from possible contamination with *hlp*$^+$ nuclei. It is also unlikely that the non growers could be pseudowild-type progeny, since complementation between alleles would be unlikely to obliterate completely the mutant's ability to take up histidinol. Also, all 51 single conidium isolates of one histidinol non grower did not grow on histidinol. The purpose of this experiment is to demonstrate that these conidia could not have come from a pseudowild type. The logic is that the pseudowild type would have given rise to a heterokaryon which would not grow on the selective medium, while either of the component homokaryons would grow. Such a heterokaryon would give rise to both heterokaryotic and homokaryotic conidia, and the latter class would grow on the selective medium. Further, all 120 progeny from the control cross *BS38* × *BS38* grew on histidinol indicating that the test for the ability of cultures to grow on histidinol is reliable.

A four point cross, *his-3* + + *HC114 nt* × *his-3 sfo BS38* + + gave 8 progeny, out of 261 tested, which would not grow on histidinol instead of histidine. None of these eight required threonine but two required nicotinamide. Hence six of the *hlp*$^+$ recombinants were *his-3* + + + +, while the other two were *his-3* + + + *nt*.

The data presented are consistent with *BS38* and *HC114* being non allelic and being in linkage group VII distributed between *sfo* and *nt* in the order *sfo–BS38–nt*. Thus, possibly two loci are concerned with the ability to grow on histidinol. That defined by *BS38* is designated as *hlp-1* and that defined by *HC114 hlp-2*.

Physiology: It was assumed that the *hlp* mutants have altered permeases, per-

mitting the entry of histidinol. It seemed likely that a normal permease had been altered and that, therefore, an alteration in the uptake of histidine and perhaps other amino acids may have occurred simultaneously.

The method of study adopted was based on the principle that a histidine mutant could grow only if histidine entered the cell. Hence, if conditions prevented growth in the presence of histidine in the medium, the cause was likely to be prevention of uptake. Under the same conditions, the growth of wild type would not be inhibited. If growth inhibition also occurred with wild type, it would indicate that these substances were toxic to Neurospora.

Table 2 shows the effect of various amino acids, singly and in combination with arginine or lysine, on the growth on histidine of *his-3 hlp-1*$^+$ and *his-3 hlp-1*.

The inhibition of the *hlp-1*$^+$ strain was similar to that reported by HAAS *et al.* (1952) and MATHIESON and CATCHESIDE (1955). There is one major difference between the inhibition of *hlp-1*$^+$ and *hlp-1* strains. While inhibition of the *hlp-1*$^+$ required the combination of a neutral amino acid with arginine or lysine, the *hlp-1* strain was inhibited by neutral amino acids alone. The addition of arginine or lysine resulted in a small increase over the considerable inhibition caused by neutral amino acids alone. It may be noted that alanine alone at 12×10^{-4}M prevented the growth of the *hlp-1*$^+$ strain. The growth of wild type was not affected by any of the combinations of amino acids found inhibitory to the *his-3* mutant.

If the basic amino acid permease were the sole transport system for basic amino acids, and the *hlp-1* mutation resulted in their exclusion by the modified permease, arginine and lysine mutants carrying the *hlp-1* allele would be unable to grow on arginine and lysine respectively. One *arg-3 hlp-1* and two *lys-4 hlp-1* double mutants have been bred. The *hlp-1* constitution in these double mutants has been determined by crossing them to *his-3 hlp-1*$^+$ strains and examining progeny for their ability to grow on histidinol. Citrulline was used as supplement in the isolation of the *arg-3 hlp-1* stock, in case it proved to be inviable on arginine.

It was found that the *arg-3 hlp-1* and *lys-4 hlp-1* strains grew as well as *arg-3 hlp-1*$^+$ and *lys-4 hlp-1*$^+$ on arginine and lysine, respectively. The *hlp-1* mutation caused an increased sensitivity of *arg* and *lys* mutants to inhibition by lysine and arginine, respectively (Table 3). Histidine stimulated the growth of *arg-3 hlp-1*$^+$ and *arg-3 hlp-1* mutants. Histidinol had little effect on the growth of *arg-3 hlp-1*$^+$ and *arg-3 hlp-1* except that at 1×10^{-2}M histidinol, growth on arginine (1×10^{-4}M) was inhibited slightly in *hlp-1*. This high concentration of histidinol did not inhibit the wild type.

hlp-2 differs from *hlp-1* in several properties. The growth of *his-3 hlp-2* on histidine (1×10^{-4}M) plus phenylalanine, tryptophan, tyrosine, leucine, arginine or lysine (6×10^{-4}M) is not inhibited but rather stimulated to about 150%. However, there was a 60% inhibition by 6×10^{-4}M methionine while phenylalanine with arginine (both at 6×10^{-4}M) completely inhibited growth on histidine.

Very high concentrations of amino acids revealed a difference between the growth of *his-3 hlp-2*$^+$ and of *his-3 hlp-2* on histidine (Table 4). Methionine, isoleucine, valine and asparagine inhibited the growth of *hlp-2* but have no effect on *hlp-2*$^+$.

TABLE 2

Dry weight in mg attained after 90 hrs of growth (with the exception of values marked a, which were harvested after 102 hrs)

Strain / Supplement	*his-3* Minimal	*his-3* + Arg	*his-3* + Lys	*his-3 hlp-1* Minimal	*his-3 hlp-1* + Arg	*his-3 hlp-1* + Lys
None	0 (46)	.....	....	0 (30)	.....	.....
His	28.2±0.5(46)	30.6±0.9(8)	....	24.7±0.5(30)	26.3±0.5(10)	.....
His	32.6a (2)	.....	....	.....	.....	.....
His + Try	32.2±1.2(8)	0 (8)	0 (8)	0.4±0.1(10)	0 (10)	0 (10)
His + Phe	32.0±1.1(4)	0 (4)	0 (2)	0.4±0.2(4)	0 (4)	0 (4)
His + Tyr	32.1±1.5(4)	0.3±0.3(4)	....	0.5 (2)	0 (4)	0 (2)
His + Met	30.2±0.8(8)	0 (8)	0 (8)	0.3±0.1(10)	0 (10)	0 (10)
His + Leu	32.5±1.2(6)	0 (6)	0 (4)	2.5±1.2(4)	0 (4)	0 (4)
His + Ala	30.4±0.5(6)	1.7±1.3(4)	0.7 (2)	0.1 (4)	0.6±0.5(6)	0.5 (2)
His + Ala(12×10^{-4}M)	1.3 (2)	2.8 (2)	....	.....	.....	.....
His + Thr	33.3±0.9(4)	28.4±1.2(4)	2.2 (2)	3.6 (2)	0.1±0.1(4)	0.1 (2)
His + Thr(12×10^{-4}M)	28.6 (2)	2.4 (2)	....	.....	.....	.....
His + Ile	30.6±1.3(4)	30.1±1.5(4)	....	1.4±0.5(4)	0.1 (2)	.....
His + Ile(12×10^{-4}M)	32.4 (2)	0.3 (2)	....	.....	.....	.....
His + Val	33.6 (2)	25.6 (2)	....	0.7±0.2(4)	0 (2)	0 (2)
His + Val(12×10^{-4}M)	29.3 (2)	0.1 (2)	....	.....	.....	.....
His + Gly	30.1±1.7(4)	28.2±1.0(4)	27.6 (2)	10.0±2.0(4)	6.1±3.0(4)	6.9±1.1(4)
His + Gly(12×10^{-4}M)	24.6 (2)	1.6 (2)	....	.....	.....	.....
His + Ser	31.3 (2)	30.7 (2)	....	1.1 (2)	0 (2)	.....
His + Ser(12×10^{-4}M)	32.1 (2)	0 (2)	....	.....	.....	.....
His + Asp(NH_2)	32.0±1.2(4)	29.2±2.1(4)	31.0 (2)	2.7±1.1(4)	0 (2)	0.1 (2)
His + Asp(NH_2)(12×10^{-4}M)	19.4 (2)	30.1 (2)	....	.....	.....	.....
His + Asp(NH_2)(24×10^{-4}M)	24.8a (2)	0a (2)	....	.....	.....	.....
His + Pro	31.7 (2)	29.1 (2)	....	24.6±0.7(4)	19.7 (2)	.....
His + Cys	33.6 (2)	31.3 (2)	....	27.3 (2)	25.1 (2)	.....
His + Cys[b]	33.4±2.5(4)	31.3±1.0(4)	31.4 (2)	25.5 (2)	27.1 (2)	27.4 (2)
His + Glu	31.7 (2)	33.4 (2)	....	25.4 (2)	26.3 (2)	27.1 (2)
His + Asp	26.9±1.4(4)	25.1±1.1(4)	27.5 (2)	24.5±2.3(4)	23.7±2.0(4)	24.5±1.3(4)
His + Lys	29.9±1.2(6)	30.1±1.4(6)	....	26.8±0.9(8)	26.3±0.9(8)	.....

Concentration of histidine 1×10^{-4}M. Concentration of other amino acids 6×10^{-4}M (with exceptions given). The figures in parentheses refer to the number of flasks measured. Abbreviations: His=histidine; Try=tryptophan; Phe=phenylalanine; Tyr=tyrosine; Met=methionine; Leu=leucine; Ala=alanine; Thr=threonine; Ile=isoleucine; Val=valine; Gly=glycine; Ser=serine; Asp(NH_2)=asparagine; Pro=proline; Glu=glutamic acid; Asp=aspartic acid; Lys=lysine; Cys=cystine; Cys[b] means that cysteine was the supplement and it was shown by the nitroprusside test to be converted to cystine after autoclaving.

TABLE 3

Dry weight in mg attained after 90 hrs of growth

Supplement	*arg-3*		*arg-3 hlp-1*	
None	0	(6)*	0	(6)*
Arg(1×10^{-4}M)	12.1±0.4	(6)	14.3±0.4	(6)
Arg(1×10^{-4}M) + Lys(6×10^{-4}M)	23.3±1.9	(4)	2.6±0.2	(4)
Arg(1×10^{-4}M) + Lys(12×10^{-4}M)	0.4	(3)	Trace	(2)
Arg(1×10^{-4}M) + Lys(24×10^{-4}M)	0	(2)	.....	
Arg(1×10^{-4}M) + His(12×10^{-4}M)	28.1	(2)	29.1	(2)
Arg(1×10^{-4}M) + His(30×10^{-4}M)	26.8	(2)	29.0	(2)
Arg(1×10^{-4}M) + His(100×10^{-4}M)	28.9	(2)	28.9	(2)
Arg(1×10^{-4}M) + Histidinol(6×10^{-4}M)	12.2	(2)	15.2	(2)
Arg(1×10^{-4}M) + Histidinol(30×10^{-4}M)	15.9	(2)	15.7	(2)
Arg(1×10^{-4}M) + Histidinol(100×10^{-4}M)	12.6	(2)	9.8	(2)
Supplement	*lys-4*		*lys-4 hlp-1*	
None	0	(2)	0	(2)
Lys(1×10^{-4}M)	10.2±0.3	(6)	10.4±0.6	(6)
Lys(1×10^{-4}M) + Arg(1×10^{-4}M)	11.3	(2)	0.1	(2)
Lys(1×10^{-4}M) + Arg(1.2×10^{-4}M)	7.2	(2)	Trace	(2)
Lys(1×10^{-4}M) + Arg(1.5×10^{-4}M)	0.9	(2)	0	(2)
Lys(1×10^{-4}M) + Arg(3×10^{-4}M)	0	(2)	0	(2)

* The figures in parentheses refer to the number of flasks measured.

DISCUSSION

Evidence presented in this paper supports the theory that histidine normally enters the cell by the aromatic and the basic amino acid permeases. Amino acids from two classes, neutral and basic, are required to produce strong inhibition of *hist* mutants. On the hypothesis of dual permeases for histidine transport, it was predicted and found that serine, threonine, alanine and asparagine, in combination with arginine, were inhibitory to the growth of the *hist* mutant.

Certain compounds, for example, the acidic amino acids, appear to have no affinity for either of the histidine permeases. Their combinations with arginine or with phenylalanine (Ho Coy Choke, unpublished) are not inhibitory to the growth of the *hist* mutant. This interpretation suggests that the inhibition by histidine on the growth of an *amination* mutant (Fincham 1950) is due to an intracellular cause.

The groups of compounds deduced, from the growth inhibition of *hist* mutant, to enter, respectively, through the aromatic and the basic amino acid permeases, agree with those suggested by growth inhibition experiments involving other mutants (Brockman 1964) and by direct competition studies. Neutral amino acids compete with one another for entry (DeBusk and DeBusk 1965; Stadler 1966; Wiley and Matchett 1966 and Kappy and Metzenberg 1967) and basic amino acids also inhibit the uptake of one another (Bauerle and Garner 1964; Roess and DeBusk 1965), but there is little or no competition between the uptake

TABLE 4

Dry weight in mg attained after 90 hrs of growth

Supplement	*his-3 hlp-2*		*his-3*	
None	0	(12)	0	(46)
His	28.6±1.0	(12)	28.2±0.5	(46)
His + Ala	0	(2)	0	(2)
His + Citrulline	0	(2)	0	(2)
His + Gly	3.8	(2)	7.3	(2)
His + Ser	4.7	(2)	5.8	(2)
His + Thr	11.7	(2)	14.0	(2)
His + Met	Trace	(2)	27.8	(2)
His + Ile	2.4	(2)	22.0	(2)
His + Val	Trace	(2)	23.7	(2)
His + Asp(NH_2)	8.3	(2)	23.9	(2)
His + Try	31.3	(2)	.....	
His + Phe	35.5	(2)	.....	
His + Tyr	39.9	(2)	.....	
His + Leu	35.1	(2)	.....	
His + Arg	36.0	(2)	.....	
His + Lys	41.0	(2)	.....	
His + Ornithine	27.8	(2)	.....	
His + Asp	37.4	(2)	.....	
His + Glu	37.5	(2)	.....	
His + Cys	37.7	(2)	.....	
His + Cys[a]	35.0	(2)	.....	
His + Pro	33.4	(2)	.....	

Concentration of His 1×10^{-4}M. Concentration of other amino acids 6×10^{-3}M. The figures in parentheses refer to the number of flasks measured. Cys[a] means that cysteine was the supplement and it was shown by the nitroprusside test, to be converted to cystine after autoclaving. For cystine, at 72 hrs, there was about 50% inhibition; however, by 90 hrs, almost complete growth had occurred. The same effect was seen for *his-3 hlp-1*, *his-3 hlp-1*+ and wild type.

of a neutral and a basic amino acid. Thus, there is a clear correspondence between the specificities of permeases determined by growth inhibition and by direct competition studies.

WOODWARD, READ and WOODWARD (1967) and STADLER (1967) have also independently suggested that histidine is transported by both the neutral and basic amino acid permeases.

Since the neutral and basic amino acids have separate transport systems with little overlap between them, it appears that the charge of its side chain is a primary factor that determines the affinity of an amino acid for a particular permease. The case of histidine is unique. The pk^1 value of its imidazole side chain is 6.0 (MEISTER 1965). At the pH of 5.8 for VOGEL's medium and of 5.6 (RYAN, BEADLE and TATUM 1943) for FRIES medium, histidine exists both as a neutral and a basic amino acid with affinities both for the aromatic and basic amino acid permeases.

The observation that the growth of *his-3 hlp-1* on histidine is strongly inhibited by neutral amino acids indicates that the mutation modifies the basic amino acid

permease, gaining affinity for histidinol and losing affinity for histidine. The only major route for entry of histidine in the *hlp-1* mutant is by the aromatic amino acid permease and this can be blocked by neutral amino acids. However, a small amount of growth occurs in the presence of neutral amino acid and this is eliminated by arginine or lysine. Hence, it is probable that histidine and the basic amino acids still have a slight affinity for the modified basic amino acid permease.

The *hlp-1* mutation does not prevent the growth of arginine and lysine mutants on arginine and lysine, respectively, but it does result in an increased sensitivity of arginine and lysine mutants to inhibition by lysine and arginine, respectively. This suggests possibly that although the modified basic amino acid permease may not transport arginine and lysine, they can enter the cells via another permease. This suggestion is consistent with the observation that histidinol does not inhibit the growth of *arg-3 hlp-1*, except at very high concentrations.

The present data are inadequate to suggest which of the permeases has been modified by the *hlp-2* mutation.

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SUMMARY

Strains of *Neurospora crassa* permeable to histidinol result from mutation at the *hlp-1* and *hlp-2* loci in linkage group VII. The growth of an *his-3 hlp-1*$^+$ strain on histidine is inhibited by a combination of a neutral with a basic amino acid but not by either alone. Any neutral amino acid alone strongly inhibits the growth of an *his-3 hlp-1* strain. The *hlp-1* mutation also increases the sensitivity of arginine and lysine mutants to inhibition by lysine and arginine, respectively. It is believed that histidine normally enters by both the neutral (aromatic) and the basic amino acid permeases. In the *hlp-1* strain, it is believed that the latter permease is altered so that it is able to transport histidinol and at the same time becomes defective in the transport of histidine, arginine and lysine. The *hlp-2* mutant differs from *hlp-1*. The growth of *his-3 hlp-2* on histidine is stimulated by aromatic amino acids. Only high concentrations of methionine, isoleucine, valine or asparagine inhibit its growth on histidine. The primary defect of the *hlp-2* mutation is not known.

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