THE B. MYCOIDES N HOST-VIRUS SYSTEM

III. Release of Phage from the Lysogenic Strain*

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The investigation to be described in this paper is concerned with the release of phage from the lysogenic strain of *B. mycoides* N.

Lwoff and his coworkers (1950 *a*) approached the problem of determining how phage is liberated in the lysogenic *B. megatherium*, strain 899, by micromanipulation studies. They found that bacteria in broth microcolonies can undergo many cell divisions without releasing phage. When free phage did appear in the medium, it almost invariably followed lysis of a bacillus. Two kinds of lysis took place: slow and instantaneous. In a few cases in which free phage cropped up in the absence of any observed lysis, Lwoff contended that a bacterium had lysed so rapidly that it had gone undetected. He calculated that 1 per 300 to 1200 log phase cells lysed out, thereby releasing phage.

The mechanisms postulated by the Lwoff group (1950 *a*, *b*, 1951) center about two types of bacteria existing in a lysogenic culture, namely, "*lysogènes potentiels*" and "*producteurs*." The first are bacteria which are capable of multiplying; each cell contains a specific particle (the probacteriophage) possessing genetic continuity. This particle exists in a state of suspended animation; *i.e.*, in an immature, latent, inactive, non-duplicating, gene-like state. As long as the phage remains immobilized and only perpetuates itself at the time of cell division, the "*lysogènes potentiels*" (which will henceforth be referred to as "perpetuators") retain the capacity to divide. These cells release no free phage into the medium. Because of unknown disturbances in metabolism, the prophage starts to develop in 0.08 to 0.33 per cent of the perpetuators. They then are "producers." (Every cell in the lysogenic culture can be "induced" to become a producer by exposing the suspension to ultraviolet, x-rays, and certain reducing substances such as ascorbic acid.) Once phage multiplication begins—once a cell is transformed into a producer—that cell is doomed to lyse (thereby releasing phage). The situation is now precisely as if one had a sensitive cell exposed to phage for the first time. To Lwoff, phage synthesis and its liberation are incompatible with the existence of a viable, intact cell.

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Lwoff's theories on the release of phage in a lysogenic culture find support in the work of Welshimer (1951) on the same strain of *B. megatherium* and of Bertani (1951) on a lysogenic *E. coli*. Welshimer determined the phage output and colony count of germinates of a highly diluted suspension of spores distributed to a number of tubes so that 40 to 88 cells were present in each tube. After 2.5 hours' incubation at 30°C., only a few tubes showed plaques in excess of the number of cells present. He concluded that but a few bacilli in any given suspension were liberating phage.

Bertani (1951) employed essentially the same technique with a log phase suspension of the *Li* strain of *E. coli*. He used more highly diluted samples than Welshimer. His data, confirming the observations of Lwoff, indicate that a constant proportion of *E. coli* cells (about 1 in 10,000) liberate phage at each generation.

EXPERIMENTAL RESULTS

By the centrifuged-sediment capillary tube method (Krueger, 1930), it was shown that the growth rate in broth of the indicator strain of *B. mycoides* N was considerably faster than that of the lysogenic strain during a 23 hour interval of observation (Baer and Krueger, 1952). For periods of time up to 4 hours —occasionally longer—a difference in growth rates between the two strains could also be shown turbidometrically. The important feature in obtaining a culture suitable for Klett readings was to "homogenize" a 16 to 24 hour old broth culture of the lysogenic strain in a Waring blendor to break up the large clumps in which the cells grew. This gave a well dispersed suspension whose density could be estimated photometrically. The suspension was then diluted with broth or centrifuged and resuspended in broth, giving a culture in the lag phase of the growth curve. At this time the suspension was uniform and remained so for an hour or two—sometimes even for 4 hours—before gradually aggregating into small clumps, and later into larger and larger clumps. As long as the cells stayed evenly scattered after blending, the Klett readings were a reliable estimate of bacterial numbers. Even when the cells started grouping into small clusters which stayed more or less evenly distributed throughout the medium, the expected magnitude of experimental error probably was not more than 10 per cent. As time went on (after 4 hours), larger cell aggregates formed and sank rapidly to the bottom of the Klett tube; accordingly turbidometric readings were abandoned.

Uniform results were not obtained. Probably one reason is that the culture tended to clump more on some days than on others. Moreover, the "growth rate" itself (*i.e.*, the number of "producers" present in the population) probably varied from day to day as a consequence of shifts in bacterial metabolism due to uncontrolled variations in the external environment tending to shift the host-virus equilibrium.

Lwoff and his coworkers (1950 *b*) have also experienced difficulty in getting reproducible density readings with the lysogenic strain 899 of *B. megatherium* toward the end of the phase of negative growth acceleration. Often they ob-

served a drop in density of the suspension, accompanied by a release of phage. At other times, there was no diminution in turbidity, but a great deal of phage was liberated. This has greatly troubled Lwoff (1950 *b*) who has steadfastly insisted that cell lysis must precede escape of phage into the medium. He suggested that phage is released from cells during the process of diluting in order to plate for plaque count—not in the undiluted suspension. Experiments disproving this concept are reported recently by Price (1952) for *Staphylococcus muscae* and by Northrop (1952) for *B. megatherium*. He also contended that the work of Price (1948), in which he showed that the maximum virus titer in a sensitive, phage-infected *Staphylococcus muscae* suspension was reached about 20 minutes before lysis, can also be explained in this way.

Despite the error introduced as a result of progressive clumping with time, the turbidity of the lysogenic suspension either remained approximately constant or went down in eight out of nine experiments during the first 4 to 8 hours after resuspension in fresh broth, whereas the density of the indicator suspension increased considerably after a 0.5 to 1.0 hour lag period. In those trials in which the Klett reading is essentially unchanged for 4 hours or longer, one may postulate that the lysogenic cells require a longer period of adjustment to fresh broth than indicator cells before beginning to multiply, but is equally reasonable to suppose that—instead of a static initial stationary phase—there actually exists a dynamic equilibrium in which growth of the "perpetuators" more or less balances lysis of the "producers."

In two experiments, well washed suspensions were assayed for their phage titer at intervals (in one experiment every 0.5 hour, in another every 0.1 hour) for 4 and 3 hours respectively. In the first experiment, the preparation on which photometric determinations were made was plated for plaque count; in the second experiment, assays of phage content were conducted on a 10^{-5} dilution of a suspension giving visible turbidity. In both runs, there was a fairly steady, continuous release of phage over the entire interval during which plating occurred. In the first experiment, a 70-fold increase in phage, in the second, an eight-fold increase in phage was recorded. The Klett readings at the end of each run were the same as at the beginning. While the regular increases in free phage might have been caused by constant, random lysis of cells in the culture, one could not rule out the possibility that phage was being "secreted" into the medium from all the cells—cells which possessed an abnormally long lag phase.

The following experiment appears to eiminate the "secretion" hypothesis. A well washed suspension of lysogenic organisms was diluted with broth and distributed to 60 small tubes in 1.5 ml. aliquots. A 0.1 ml. sample from each tube was directly mixed with indicator bacteria and hot agar and plated for plaque count before and after a 2.0 hour incubation period at 30°C. The average number of filaments in each tube (Table I) was 39; approximately twice that number of cells were present; (microscopically the suspension consisted of single cells and diplobacilli). The total initial concentration of filaments in the 60 tubes ranged from less than 14 to 98. At the end

TABLE I

Phage Output in Dilute Suspensions of Lag Phase Lysogenic Cells of B. mycoides

Plaque count per 0.1 ml.* (1.4 ml. is the entire volume of each of 60 tubes during 2.0 hrs.' incubation at 30°C.)					
Tube No.	0 hrs.	2 hrs.	Tube No.	0 hrs.	2 hrs.
1	2	2	10	1	5
2	0	15	11	0	0
3	0	6	12	4	7
4	1	1	13	1	1
5	0	0	14	1	26
6	2	6	15	0	7
7	2	17	16	2	0
8	0	9	17	4	14
9	2	3	18	4	11
19	0	3	36	1	4
20	5	3	37	3	28
21	5	36	38	3	16
22	7	9	39	13	18
23	1	26	40	0	21
24	6	40	41	2	32
25	7	9	42	1	32
26	1	0	43	1	1
27	2	14	44	2	6
28	7	24	45	0	43
29	2	34	46	2	10
30	3	4	47	0	30
31	1	3	48	0	22
32	0	17	49	1	0
33	0	2	50	1	16
34	1	2	51	4	0
35	2	1	52	1	3
53	3	8			
54	6	26			
55	2	5			
56	1	4			
57	1	16			
58	1	20			
59	41	23			
60	2	25			

* 0.1 ml. sample was taken from each tube at 0 and 2 hours and plated directly for plaque count by mixing with indicator cells and agar.

of 2 hours, 29 of the 60 tubes showed a rise of more than 5 plaques per 0.1 ml. over the plaque count per 0.1 ml. in that particular tube at 0 time. (21 of the 29 tubes showed an increase of more than 15 plaques per 0.1 ml.) The Klett reading (Table I) of the undiluted suspension remained constant.

If we assume (1) that the same (relative) bacterial concentration existed at 2.0 hours in the diluted as in the concentrated suspension (*i.e.*, that there were still about 80 cells present per tube after 2.0 hours) and (2) that at least one bacterium lysed in each of the 29 tubes which showed a rise in titer, then the increase in plaque count in about half the tubes would indicate that approximately 0.6 per cent of the cells in the lysogenic population were "producers." This does not take into account the possibility that some tubes contained more than one lysing bacterium or that a turnover in cell numbers occurred during the 2 hour period. The incubation period should have been shortened to 0.5 or 1.0 hour to insure production of phage from only one generation of bacteria.

Incidentally, manometric studies with resting cell suspensions of the lysogenic and parent indicator strains of *B. mycoides* N showed no differences in the rates of oxidation of 2 sugars, 11 amino acids, and 1 dicarboxylic acid.

In the hope that a more precise determination of the number of "producers" present in a population could be obtained, the following procedure was used: A washed lysogenic supension was highly diluted and 0.5 ml. aliquots were distributed to each of a series of small tubes (Tables II and III). This time the entire contents of half the tubes were plated for plaque count at 0 time; the remaining tubes were shaken for 1.5 hours (Table II) or 2.0 hours (Table III) at 30°C. Again the whole volume of each tube was used for assay. In the experiment described in Table II each tube contained an average of 15 filaments (mostly single cells—the initial filament to cell ratio was 1.3). 20 out of the 21 tubes plated at 1.5 hours showed an increase in plaque count at the onset of the experiment. In this experiment, however, the turbidity of the undiluted suspension (and of the direct count also) fell instead of remaining constant (as in Table I). There is little reason to doubt that phage was released from those cells—a rather large percentage of the total population—which lysed out; at the least, 1 in 15 cells was a "producer."

Table III tells much the same story. In one series of 44 tubes, only 5 cells were present per tube at the onset of incubation. After 2.0 hours, 17 out of 22 tubes contained more phage than the highest plaque count obtained from any of 22 tubes plated at 0 time. Possibly 14 per cent (1 in 7) of the bacteria (originally present?) which lysed out produced phage.

The variation in Klett readings and in the proportion of tubes showing increases in different runs suggests that a variable rather than a constant proportion of cells liberated phage under the experimental conditions described in the preceding studies, possibly indicating that the equilibrium in this system between host and parasite is a precarious delicately balanced affair which is easily upset by undefined environmental factors; *e.g.*, slight changes in the concentration of inorganic ions in the medium, in temperature, or in manipulation of the culture in preparation for the experiment.

Table IV shows that no phage was released from lysogenic cells when sus-

TABLE II

Release of Phage from Lysogenic Bacteria, as Studied by Method of Plating Entire Contents of Tubes Containing 3 to 48 Filaments per Sample

Plaque count at 0.0 hours for undiluted suspension
(Regular assay procedure)
10^{-6} dilution: 311 plaques
Titer = 3.1×10^8 plaques per ml.

Plaque count		Colony count		Estimation of bacterial numbers
0.0 hr.	1.5 hrs.	0.0 hr.	1.5 hrs.	1. Turbidometric determination Klett reading
Entire contents of each tube (0.5 ml. volume) was plated				

Time	Undiluted suspension	1:10 broth dilution
hrs.		
0.0	181	21.5
1.0	161	20.5
1.5	141	16.0

Plaque count		Colony count		2. Direct count (Petroff-Hausser chamber)
0.0 hr.	1.5 hrs.	0.0 hr.	1.5 hrs.	
Entire contents of each tube (0.5 ml. volume) was plated				
7	67	0	0	
10	73	0	0	
16	33	0	0	
10	166	1?	0	
33	212			
19	158			
13	364			
48	143			
11	81			
9	85			
10	166			
12	90			
8	197			
19	89			
23	161			
7	241			
6	97			
8	92			
8	104			
3	124			
28	226			
122				
Average... 15	141			

Time	Filaments per ml.	Cells per ml.	Ratio of cells to filaments
hrs.			
0.0	3.3×10^8	4.4×10^8	1.3
1.5	8.2×10^7	9.2×10^7	

Actual counts

Small squares (16 small squares in a large square)		Large squares	
Filaments	Cells	Filaments	Cells
15	15	82	82
15	20	67	69
17	24	49	53
15	23	47	57
21	29	77	96
20	23	65	80
11	16	59	69
		72	85

pended in phosphate buffer rather than nutrient broth, although a significant amount of lysis occurred, causing a drop in turbidity and in the plaque count (of both the dilute and concentrated cell suspensions).

Welshimer (1951) reported that actively growing broth cultures of lysogenic strains of *B. megatherium* undergo extensive lysis when placed under anaerobic conditions. In some strains, anaerobic lysis was accompanied by a definite increase of free phage. "Lag phase" suspensions of the lysogenic strain of *B.*

mycoides suffered only limited lysis and produced no phage; rather, there was an actual drop in the number of plaque-forming particles when the oxygen

TABLE III

Phage Liberation from Lysogenic Bacteria, as Studied by Method of Plating Entire Contents of Tubes Containing 0 to 9 Filaments per Sample

Plaque count				Bacterial numbers
(Entire contents of each tube (0.5 ml.) plated)				1. Turbidometric determiuation Klett reading
10^{-7} dilution		10^{-6} dilution		Time / Undiluted / 1μ10 dilution
0 hrs.	2 hrs.	0 hrs.	2 hrs.	*hrs.*
3	83	0	0	0 — 182 — 21
4	82	1	12	2 — 142 — 23
4	43	0	0	2. Direct count (Petroff-Hausser chamber)
4	145	0	9	Time / Filament per ml. / Ratio of cells to filaments
3	178	1	0	*hrs.*
6	270	1	0	0 — 1.2×10^8 — 1.3
3	34	0	0	2 — 4.4×10^7 — 1.5
9	33	1	0	
6	183	1	2	Actual count
9	102	0	0	Time / Large square / Cells / Filaments / No. of clumps in entire ruled area on slide
5	15	1	0	*hrs.*
22	0	1	0	0 — 1 — 136 — 97
5	157	0	0	0 — 2 — 124 — 96 — 33
5	6	0	1	2.0 — 1 — 61 — 41
7	271	0	0	2.0 — 2 — 52 — 32 — 28
2	175	0	6	2.0 — 3 — 52 — 36
3	1	1	0	Plaque count of undiluted suspension at 0 hrs. (Regular assay procedure)
7	14	1	0	Dilution / Plaques per plate / Virus per ml.
2	79	1	0	10^{-6} — 159 — 1.6×10^8
10	24	0	0	10^{-7} — 11 — 1.1×10^8
6	83	0	32	
2	29	1	0	
			0	
			0	
			0	
5	Average per tube..... 89			

supply was cut off for 2.0 to 3.3 hours to about 20 per cent of its original value.

Welshimer (1951) also "observed many times that the number of plaques that arise from a suspension of heated spores or washed vegetative cells of lysogenic strains of *B. megatherium* is always less than the number of colonies

from the same suspension;" nevertheless, subcultures of isolated colonies always contained phage. Similarly Rountree (1949) working with lysogenic staphylococci noted that plaque counts fall below colony counts.

TABLE IV

Release of Phage from Lag Phase Lysogenic Cells in M/15 Phosphate Buffer (pH 6.8)

Plaque count								Estimation of bacterial numbers (Undiluted suspension)		
Undiluted suspension				10^{-6} dilution		10^{-7} dilution				
(Regular assay method)				(0.5 ml. in each tube: entire contents of tube plated)				Time	Klett reading	Direct cell count
0.0 hr.		2.5 hrs.		0.0 hr.	2.5 hrs.	0.0 hr.	2.5 hrs.	*hrs.*		
Dilution	No. of plaques on plates	Dilution	No. of plaques on plates	17	9	2	1	0.0	90	8.2×10^7/ml.
				11	7	4	3	2.5	69	7.4×10^7/ml.
				13	3	3	1	Phage titer (Undiluted suspension)		
10^{-4}	324	10^{-4}	218	9	8	3	1	Time		
10^{-5}	32	10^{-5}	23	8	4	7	5	*hrs.*		
10^{-6}	2	10^{-6}	1	8	5	7	7			
				6	2	5	2	0.0	3.2×10^7 virus/ml.	
				9	2	4	3	2.5	2.3×10^7 virus/ml.	
				9						
Average.....				10.0	5.0	4.4	2.6			
Colony count										
				0	0	0	0	Macroscopic		
								Microscopic examination of culture:		
				0	0	0	0			
				0	0	0	0	Microscopically culture contained diplobacilli single cells predominantly; also a few large clumps were present		
				0	0	0	0	Macroscopically, holding tube against light clumping was detectable		
								However, microscopically and macroscopically at end of 2.5 hrs. culture was no more clumped than at start of experiment		

The data presented in Table V show that precisely the opposite is true of well washed suspensions of the lysogenic strain of *B. mycoides* N. The plaque counts always exceeds the colony count. This cannot be attributed to free phage in the medium; the suspensions are twice washed. Moreover, the plaque counts and direct filament counts of twice washed suspensions are in good

agreement. For example, in Table II, a washed suspension gave a direct filament count of 3.3×10^8 chains per ml., a phage titer of 3.1×10^8 plaques per ml. Likewise, in Table III, the number of chains per ml. was 1.2×10^8; the number of plaques, 1.4×10^8 per ml. On the other hand, there were no colonies —Tables II and IV—demonstrable on plates that yielded an average of 15 and 10 plaques respectively when indicator cells were added. Presumably all the cells originally deposited on the plate—or all their descendants (in the event that they underwent several divisions)—lysed out on the surface of the nutrient agar during the 18 hour incubation period.

Most of the data—the occasional drops in turbidity (Tables II and III), the "inferior" colony counts (Tables II and IV), the fact that phage release in almost every tube (originally containing only a few cells) accompanied a decrease in density—favor the idea that a few or many cells are lysing out when

TABLE V

Differences between the Plaque and Colony Counts per Ml. of Well Washed Lysogenic Suspensions of B. mycoides *N*

Broth cultures (20 to 24 hours old) of the lysogenic strain of *B. mycoides* N were washed at least twice with M/15 phosphate buffer and then resuspended in buffer. The suspensions were homogenized and gauze-filtered after each washing.

Lysogenic cultures (prepared on 3 separate days)	No. of washings	Klett reading (660 mμ filter)	Phage per ml.	Colonies per ml.
A	2	276	2.2×10^8	1.8×10^7
B	2	125	1.4×10^8	1.3×10^7
C	3	191	3.4×10^8	$1.3 \times 10^{7*}$

* The indicator strain (made up to Klett of 190) gave a colony count of 8.3×10^7.

the lysogenic suspension is placed in a fresh medium—the number depending on undefined and evidently uncontrolled variations in environment. But, why should it be necessary to postulate that the "non-lysers" are multiplying? With the exception of two experiments out of fourteen, the readings never go up—always remain constant or go down—for many, many hours past the normal lag phase for the indicator cells. In Table I, if only 1 per cent of the bacteria are "producers," it does not seem reasonable that no increase in turbidity would be detected after 2.0 hours if the rest of the cells were multiplying.[1] Perhaps the "non-producers" are not increasing at all, as a result of stresses imposed by the struggle to suppress development of provirus.

By measuring the growth rates of both strains under conditions in which the lysogenic strain produces no phage, one might be able to decide whether the "perpetuators" in the carrier culture of *B. mycoides* N grow more slowly

[1] One can circumvent this by assuming that a great many cells lyse out, but only a few of the "lysers" produce phage.

than indicator cells. Northrop (1951) has shown that the culture of lysogenic *B. megatherium* 899a grows quite well in an amino acid mixture containing Fe and glucose but in the absence of added Mg^{++} (or in the presence of more than 0.05 M PO_4); however, it liberates no phage. Such a study was not attempted with the lysogenic strain of *B. mycoides* N because both the parent and the virus-harboring strain clump in synthetic media (P. N. Smith, personal communication); at the time these experiments were undertaken, there appeared to be no rapid, accurate way of estimating cell numbers. By use of the Coleman model 7 photonephelometer (Fisher, 1951), this experiment could now be easily and reliably carried out.

In addition, Dr. Edward A. Adelberg has suggested that since penicillin affects only growing cells, the use of growth-inhibiting concentrations of penicillin might provide a way of deciding whether a true lag phase of the lysogenic strain of *B. mycoides* N existed during the first 4 to 8 hours after suspending the cells in fresh nutrient broth.

DISCUSSION

A perfect infection is one in which "the parasite persists long enough to insure its transfer to a new host and in which the host suffers no significant disability" (Burnet, 1946). The phages (three in all) carried by the *Li* strain of *E. coli* (Bertani, 1951) only exceptionally cause host mortality (one "producer" in 10,000); therefore, this bacterium-bacteriophage system comes close to demonstrating that such a host-virus relationship is not only an ideal concept but actually can be attained. In comparison, the lysogenic culture of *B. mycoides* N might appear at first glance to present a very different situation in which fatal infection ovretakes an unduly large number of the host population. However, one might also conclude that it may actually be an advantage for the phage to kill an appreciable portion—but not all—of the lysogenic bacteria in a phage-infected culture. The result should be the dissemination of a great deal of free phage so that (in nature) the extracellular phage released could reach and invade the cells of any uninfected, sensitive cells of *B. mycoides* present in the environment (mud, lake water, etc.).

Whether the lysogenic strain of *B. mycoides* N could compete successfully with other bacterial species is open to question. The lysogenic culture of the N strain is what might well be called a "laboratory creation," a "domesticated hothouse organism" (Winogradsky, 1937) whose metabolism is so deranged even under favorable environmental conditions that the virus is able to multiply within a considerable proportion of the cells in the lysogenic population. Such a culture might never survive competitively with the fungal and bacterial population of the soil in the utilization of the limited substrates available. But, if the lysogenic bacteria of *B. mycoides* N can persist in such natural mixed cultures *mycoides* phage N may have a better chance of perpetuating

itself than the virus in the *E. coli Li* system in which little free phage is liberated, or in the *Staphylococcus muscae* system in which the phage completely sterilizes a previously unexposed culture (Dr. W. H. Price, personal communication).

Experimental Procedure

Maintenance of Stock Cultures and Preparation of Experimental Cultures.—Blended-filtered 16 to 24 hour old broth cultures of the lysogenic strain of *B. mycoides* N were prepared as described by Baer and Krueger (1952). Such cultures were washed at least twice in M/60 Sorensen's phosphate buffer (pH 6.8) and subjected to additional filtration and blending after each resuspension in fresh buffer, before being resuspended in fresh nutrient broth.

Phage Assay.—The layering method of plaque count described by Gratia (1936) was used. Lag phase gauze-filtered broth cultures of the indicator strain of *B. mycoides* N were used in making plaque counts.

Media.—All experiments were carried out in Difco nutrient broth; plating for plaque count was done on nutrient agar. Serial dilutions in plating were made in nutrient broth.

SUMMARY

Experiments were performed to determine the mechanism of release of phage from the lysogenic strain of *B. mycoides* N. The results suggest that qualitatively the same situation obtains as in the phage-carrying cultures of *B. megatherium* 899 and *E. coli Li; i.e.*, the population consists of two kinds of cells: "*lysogènes potentiels*" and "*producteurs.*" Quantitatively, however, there are more "producers" in a broth culture of the lysogenic *B. mycoides* N, at least during the first 4 to 8 hours after cells have been suspended in fresh medium, suggesting that the interaction between host and parasite is one in which the balance is easily swung in favor of the virus.

These conclusions are based upon the following lines of evidence: (1) the slow "growth rate" of the lysogenic culture, (2) the fact that the colony count falls far below the plaque count or the filament count (which correspond) for a well washed suspension, (3) the increase in phage output in a large number of tubes, each containing a small number of lysogenic cells, after a few hours' incubation in nutrient broth at 30°C.

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