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Simplified Complete Medium for the Growth of *Neisseria gonorrhoeae*

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A complete medium for the growth of *Neisseria gonorrhoeae* has been developed, using the same basic ingredients contained in commercial media but with fewer supplements. Based on a comparison of plating efficiencies, this medium (designated TTU Complete Medium) supported the growth of laboratory strains of *N. gonorrhoeae* equal to that obtained on commercial GC agar base medium supplemented with IsoVitaleX. It was also equivalent to the commercial medium in supporting the growth of 67 clinical isolates and 6 auxotypes of *N. gonorrhoeae* when tested by the streak plate method. Based on turbidity measurements and viable plate counts, the liquid equivalent of TTU Complete Medium supported maximal growth yields of *N. gonorrhoeae*. The toxicity of different brands of agar appeared to be a major factor in preventing the growth of *N. gonorrhoeae* strains on solid media. The addition of starch neutralized the toxicity of some types of agar but not of others.

Most media designed for the growth of *Neisseria gonorrhoeae* have extremely complex compositions (1, 3, 5, 6, 8, 10). These media are often supplemented with blood, hemoglobin, or serum and also various mixtures of amino acids, vitamins, metabolic intermediates, and nitrogenous bases. The actual role played by many of these supplements in the growth and nutrition of the organism is not known.

An inexpensive and easy-to-prepare medium of relatively simple composition that supports maximal growth of most strains of *N. gonorrhoeae* would be useful for nutritional, genetic, and physiological experiments. This study describes the development of such a simplified complete medium for the routine propagation of *N. gonorrhoeae*. Also, an attempt was made to quantitatively distinguish between the absence of growth caused by agar toxicity and that caused by the absence of specific nutrients required for growth.

MATERIALS AND METHODS

Bacterial strains. *N. gonorrhoeae* laboratory strains 9, 62, and 2686 and 37 clinical isolates from different geographical locations in the United States were obtained from D. S. Kellogg, Jr. (Center for Disease Control, Atlanta, Ga.).

Six auxotypes of *N. gonorrhoeae* described by B. Wesley Catlin (2) were obtained from the American Type Culture Collection, Rockville, Md. An additional 30 clinical isolates of *N. gonorrhoeae* were obtained from the City-County Health Department, Lubbock, Tex.

All strains were reisolated by three successive single-colony selections on GC agar base medium (Baltimore Biological Laboratories [BBL], Cockeysville, Md.) containing IsoVitaleX (10 ml/liter) and V-C-N (BBL) (10 ml/liter) and confirmed as *N. gonorrhoeae* by Gram stain, positive oxidase test, and fermentation of glucose. Strains were stored at −70°C in a buffered proteose peptone no. 3 (Difco) broth containing 20% (vol/vol) glycerol.

Media. GC agar base medium (BBL) containing IsoVitaleX (10 ml/liter) was used as a prototype medium in our initial studies.

The composition of TTU Complete Medium is given in Table 1. The liquid counterpart of TTU Complete Medium was of the same composition as the solid medium except for the addition of 0.05% $NaHCO_3$ and the omission of soluble starch and agar.

The diluent used in all experiments was buffered saline gelatin (BSG) containing: NaCl, 0.85%; KH_2PO_4, 0.03%, Na_2HPO_4, 0.06%; and gelatin, 100 μg/ml (4).

Amino acids, vitamins, and purine and pyrimidine bases were purchased from Calbiochem, Los Angeles, Calif. Regent-grade inorganic chemicals were from Mallinckrodt Chemical Works, St. Louis, Mo.

For some experiments V-C-N inhibitor (BBL) was added (10 ml/liter). The resulting medium contained 3.0 μg of vancomyin, 7.5 μg of colistin, and 12.5 U of nystatin per ml, respectively.

Purified agar, regular agar, Noble agar, and soluble starch were from Difco Laboratories, Detroit, Mich. Regular agar was obtained from BBL. Ionagar no. 2 (Oxoid) was from Flow Laboratories, Rockville, Md. Agarose was from Bio-Rad Laboratories, Richmond, Calif.

TABLE 1. *Comparison of the composition of GC agar base supplemented with IsoVitaleX and TTU Complete Medium*

Components	GC agar base	TTU Complete Medium
Basal (g/liter)		
Polypeptone peptone	15	15
K_2HPO_4	4	4
KH_2PO_4	1	1
NaCl	5	5
Corn starch	1	1[a]
Agar	10	10[b]
Supplements (μg/ml)		
Glucose	1,000 (0.1%)	5,000 (0.5%)
Thiamine (B_1)	0.03	2
Ferric nitrate	0.2	5
Cysteine	259	55
Guanine	0.3	5[c]
Adenine	10	10[d]
Glutamine	100	100
Cystine	11	–[e]
Vitamin B_{12}	0.1	–
Coenzyme I	2.5	–
Cocarboxylase	1	–
p-Aminobenzoic acid	0.1	–

[a] Soluble starch used in place of corn starch.
[b] Purified agar (Difco) used in place of regular agar.
[c] Hypoxanthine used in place of guanine.
[d] Uracil used in place of adenine.
[e] –, Not added.

Selection of supplements for TTU Complete Medium. With the development of certain chemically defined media (3, 8), the nutritional requirements of *N. gonorrhoeae* are beginning to be elucidated. These recent nutritional studies indicate that IsoVitaleX-supplemented GC agar base medium might contain components not essential for the growth of most strains of *N. gonorrhoeae*. Table 1 compares the compositions of commercial GC agar base medium supplemented with IsoVitaleX and the TTU Complete Medium that was developed. The individual components are given in either grams per liter or micrograms per milliliter, final concentration. The same basal ingredients used in GC agar base were also used in TTU Complete Medium: polypeptone peptone (BBL), 15 g/liter; K_2HPO_4, 4 g/liter; KH_2PO_4, 1 g/liter; and NaCl, 5 g/liter. Soluble starch (Difco) was substituted for corn starch at a concentration of 0.1%. There was no apparent difference in the abilities of these two types of starch to neutralize the toxic components of agar. Purified agar (Difco) was used in place of regular agar at a concentration of 1%.

With regard to supplements, we wanted only the minimal number required by most strains for growth. Glucose was used as a major energy source, with its concentration increased to 0.5%. Morse and Bartenstein (11) showed that this concentration supported maximal growth of *N. gonorrhoeae* in liquid medium.

Catlin (3) found that about 1% of the 250 *N. gonorrhoeae* strains she studied required thiamine (B_1) for growth. Thiamine (B_1) was, therefore, added for the detection of those strains that require it for growth. The concentration of thiamine (B_1) (0.03 μg/ml) in IsoVitaleX-supplemented media is ~100-fold lower than that customarily used for thiamine (B_1)-requiring strains, so its concentration was increased to 2 μg/ml.

Kellogg (7) showed that ferric ions stimulated the growth of *N. gonorrhoeae*, so ferric nitrate was added as a supplement in a final concentration of 5 μg/ml.

All strains of *N. gonorrhoeae* examined by Catlin (3) and by La Scolea and Young (8) had an absolute requirement for the amino acid cysteine. In the present study the concentration of cysteine was reduced from 259 μg/ml (concentration in IsoVitaleX-supplemented media) to 55 μg/ml. This lower concentration supports the growth of cysteine-requiring mutants of *Escherichia coli*, and there is no evidence that *N. gonorrhoeae* should require higher levels.

Catlin (3) found that about 20% of the strains she studied had a requirement for hypoxanthine or for both hypoxanthine and uracil. Based on these findings, hypoxanthine and uracil were substituted for the guanine and adenine used in IsoVitaleX.

Since glutamine was included in most media designed for the growth of *N. gonorrhoeae*, it was added as a supplement. It now appears that glutamine is not required for the growth of most *N. gonorrhoeae* strains.

Our medium was not supplemented with cystine, vitamin B_{12}, coenzyme I, cocarboxylase, or *p*-aminobenzoic acid.

Quantitative and qualitative plating experiments. For each experiment, strains were thawed and streaked on GC agar base medium supplemented with IsoVitaleX or to TTU Complete Medium and incubated in a candle jar (~2.5% ambient CO_2) at 35°C. After incubation for 24 h, suspensions were made in warm BSG, vortexed for 15 to 20 s to break up clumps, and adjusted to an absorbance reading of 1 on a Bausch and Lomb Spectronic 20 spectrophotometer at a wavelength of 550 nm. The suspension was then diluted in warm BSG, and 0.05-ml portions of each dilution were plated in duplicate. The inoculum was spread to dryness using a glass spreader, and the plates were incubated in candle jars for 48 h at 35°C.

The qualitative growth response was determined as follows. Suspensions of each strain were prepared as described for quantitative platings. Plates were divided into four sectors, and each strain was streaked within a sector using a calibrated loop (0.001 ml) to obtain isolated colonies in the more dilute areas. The total number of viable bacteria delivered by the calibrated loop was approximately 2×10^6. Plates were incubated in candle jars for 48 h at 35°C.

Growth studies using liquid TTU Complete Medium. For growth studies using liquid medium, suspensions of *N. gonorrhoeae* were made in warm BSG, vortexed, and adjusted to an absorbance reading of 1 (based on plating experiments, this reading corresponds approximately to 2×10^9 viable cells/ml). A 0.1-ml sample of the suspension was aseptically pipetted into a 250-ml Nephelo culture flask (Bellco Glass Inc., Vineland, N.J.) containing 20 ml of prewarmed liquid TTU Complete Medium with

0.05% $NaHCO_3$ (the addition of 0.05% $NaHCO_3$ to the liquid medium was found to reduce the lag phase from about 3 to 1 h). At zero time, absorbance readings were made and a sample was taken for the determination of viable count. The viable count at zero time for most experiments was approximately 10^7 cells/ml. The culture flasks were incubated at 35°C in a gyratory water-bath shaker at 200 rpm (New Brunswick Scientific, model G67). At specific time intervals, absorbance readings were recorded, and 0.5-ml samples were aseptically transferred to sterile tubes and vortexed for 15 to 20 s to disperse clumps. The sample was then diluted in prewarmed BSG, and appropriate dilutions were plated in duplicate on the medium being tested. All plates were incubated at 35°C for 48 h in candle jars.

RESULTS

Plating efficiency of *N. gonorrhoeae* strains on TTU Complete Medium and on GC agar base medium supplemented with IsoVitaleX. To compare these two media in their ability to support the growth of *N. gonorrhoeae*, quantitative plating experiments were performed using the three laboratory strains 9, 62, and 2686 (see Materials and Methods for details). Table 2 compares the mean number of colony-forming units (CFU) obtained from eight separate plating experiments. No significant difference was noted in the plating efficiencies of strains 62 and 2686 on the two media. The mean count for strain 9 was slightly lower on the TTU Complete Medium than on the GC agar base medium supplemented with IsoVitaleX. In addition to the three laboratory strains, 67 clinical isolates and 6 auxotypes of *N. gonorrhoeae* were qualitatively tested for growth on both types of media (see Materials and Methods). All strains grew as well on the TTU Complete Medium as they did on the GC agar base medium supplemented with IsoVitaleX.

TABLE 2. *Comparison of mean number of CFU obtained by plating N. gonorrhoeae strains on two types of complete media*

Strain	Mean CFU[a]	
	GC agar base + IsoVitaleX	TTU Complete Medium + supplements
9	9.5396 ± 0.13	9.4102 ± 0.29
62	9.5853 ± 0.22	9.5585 ± 0.21
2686	9.5793 ± 0.18	9.5348 ± 0.11

[a] Mean number CFU are expressed as logarithms to the base 10 ± 2 standard deviations. These mean numbers were determined from counts obtained from eight separate plating experiments. Comparisons of mean numbers using an unpaired Student's *t* test showed no statistically significant difference in the EOP of strains 62 and 2686 on the two media. The EOP of strain 9 was slightly lower on the TTU Complete Medium than on GC agar base medium supplemented with IsoVitaleX.

When the three laboratory strains were plated on TTU Complete Medium containing V-C-N inhibitor (BBL), the efficiency of plating (EOP) was equal to that obtained on control media without V-C-N.

Growth of *N. gonorrhoeae* in liquid TTU Complete Medium. Figure 1 shows a typical growth curve for *N. gonorrhoeae* strain 2686 based on turbidity measurements obtained from two separate experiments. After inoculation of TTU Complete Liquid Medium with approximately 2×10^7 viable cells of *N. gonorrhoeae*, the turbidity of the culture (based on an increase in percent absorbance readings at 550 nm) steadily increased from 1 to 8 h and then leveled off into an apparent stationary phase of growth.

Each time turbidity measurements were made, a small sample was withdrawn for the determination of viable count. Figure 2 shows the growth curve for *N. gonorrhoeae* strain 2686 based on viable counts obtained from two separate experiments. The kinetics of growth based on viable counts are generally similar to those obtained from turbidity measurements. After 1-h lag phase, exponential growth began and continued for the next 7 h. (Viable counts were also determined at 30-min intervals dur-

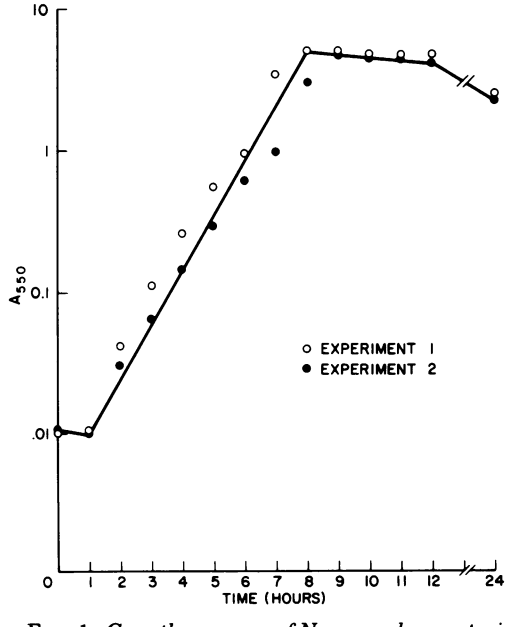

FIG. 1. *Growth response of N. gonorrhoeae strain 2686 in liquid TTU Complete Medium based on turbidity measurements obtained from two separate experiments.*

ing the exponential phase of growth, and the generation time for strain 2686 was calculated to be 30.5 min.) Maximal growth was achieved after 8 h of incubation, with viable counts reaching levels of ~5 × 10^9 CFU/ml. At this point, a steady (exponential) decrease in viability was noted, which continued for the next 16 h until the population reached levels of ~2 × 10^7 CFU/ml. Forty-eight hours after initial inoculation (data not shown in Fig. 2), the viable count was down to ~10^4 CFU/ml. These results are similar to those reported by Morse and Bartenstein using a proteose peptone no. 3 medium supplemented with IsoVitaleX (11). These authors noted a similar sharp decrease in viability after growth of *N. gonorrhoeae* in liquid medium. They further showed that *N. gonorrhoeae* underwent spontaneous lysis when the glucose content of the medium became exhausted. Microscopic examination of our cultures during the period of decreasing viability showed extensive cell lysis.

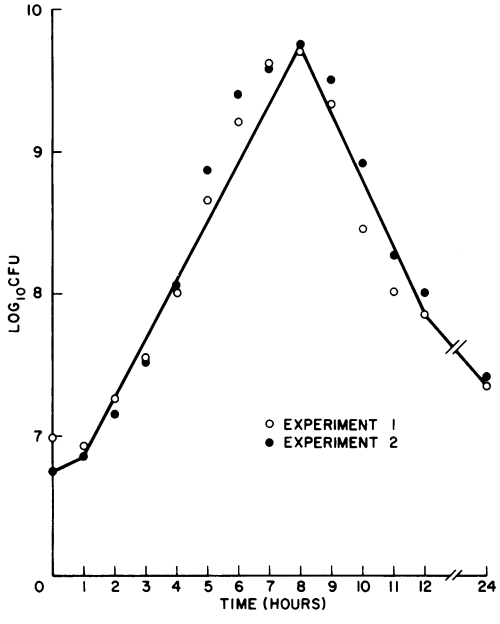

FIG. 2. *Growth response of N. gonorrhoeae strain 2686 in liquid TTU Complete Medium based on viable plate counts obtained from two separate experiments.*

Plating efficiency of *N. gonorrhoeae* strains on TTU Complete Medium containing different types of agar. In early experiments, it was noted that starch did not neutralize the toxicity of some lots of purified agar. At this point we decided to quantitatively compare the EOP of *N. gonorrhoeae* strains on media containing different types of commercial agar with and without the addition of soluble starch. Table 3 shows the EOP of *N. gonorrhoeae* strains 9, 62, and 2686 on TTU Complete Medium containing different types of agar with and without starch. The EOP of these strains on TTU Complete Medium with purified agar (control number 617949) plus soluble starch was set at 1. The EOP of all three strains was reduced in the absence of starch. Strain 9 showed a 5- to 10-fold higher EOP than strains 62 and 2686, which suggests that it is somewhat less sensitive to the toxic components of this lot of purified agar

TABLE 3. *EOP of three N. gonorrhoeae strains on TTU Complete Medium using different agars with and without the addition of 0.1% soluble starch*[a]

Type of agar[b]	EOP of strain:		
	9	62	2686
Purified with starch (617949)	1	1	1
Purified without starch (617949)	4.8×10^{-3}	1.7×10^{-3}	6.2×10^{-4}
Purified with starch (598810)	$<4.0 \times 10^{-5}$	$<2.7 \times 10^{-5}$	$<2.7 \times 10^{-5}$
Purified without starch (598810)	$<4.0 \times 10^{-5}$	$<2.7 \times 10^{-5}$	$<2.7 \times 10^{-5}$
Regular (Difco) with starch	4.8×10^{-1}	1.6×10^{-1}	2.9×10^{-1}
Regular (Difco) without starch	3.8×10^{-3}	2.7×10^{-5}	2.7×10^{-5}
Regular (BBL) with starch	1.2×10^{-1}	1.2	0.9
Regular (BBL) without starch	7.2×10^{-3}	4.3×10^{-5}	1.2×10^{-3}
Ionagar (Oxoid) with starch	0.6	0.9	1.0
Ionagar (Oxoid) without starch	3.1×10^{-3}	1.3×10^{-3}	4.9×10^{-4}
Noble (Difco) with starch	$<4.0 \times 10^{-5}$	$<2.7 \times 10^{-5}$	$<2.7 \times 10^{-5}$
Noble (Difco) without starch	$<4.0 \times 10^{-5}$	$<2.7 \times 10^{-5}$	$<2.7 \times 10^{-5}$
Agarose with starch	$<8.0 \times 10^{-9}$	$<5.4 \times 10^{-9}$	$<5.4 \times 10^{-9}$
Agarose without starch	$<8.0 \times 10^{-9}$	$<5.4 \times 10^{-9}$	$<5.4 \times 10^{-9}$

[a] EOP was determined by dividing the number of CFU observed on TTU Complete Medium containing different types of agar with or without starch by the number of CFU observed on control plates containing purified agar (Difco) with soluble starch.

[b] All brands of agar were incorporated into the medium at a concentration of 1%.

than the other two strains.

Some lots of purified agar (e.g., control number 598810) were quite toxic and reduced the EOP of all three strains to almost undetectable levels. The addition of starch did not neutralize the toxicity of these agars (see Table 3).

The three strains showed a slight reduction in EOP (5- to 10-fold) when plated on medium containing regular agar (Difco) plus starch. When plated on the same medium without starch, the EOPs were further lowered 100- to 10,000-fold. Again, strain 9 showed a higher EOP than strains 62 and 2686.

On medium containing regular agar (BBL) plus starch, the EOP of strain 9 was reduced about 10-fold. Strains 62 and 2686 showed EOPs essentially equal to those obtained on the control medium. The EOP of all three strains was reduced if starch was omitted (see Table 3). Strain 62 showed the greatest reduction (approximately 10,000-fold). These data suggest that strain 62 was more sensitive than strains 9 and 2686 to the toxic components in this particular lot of regular agar.

All three strains grew as well on Ionagar (Oxoid) with starch as they did on the control medium. The EOP of all three strains was reduced approximately 1,000-fold on the same medium without starch.

Noble agar was toxic for all three strains whether starch was added or not. The response of the strains on media containing Noble agar was like that observed on media containing bad lots (e.g., control number 598810) of purified agar.

Agarose was the most toxic of the agars tested. Media containing agarose with or without starch completely inhibited the growth of all three strains even on plates spread with undiluted samples (the total number of viable cells of *N. gonorrhoeae* in 0.05 ml of an undiluted suspension is approximately 10^8).

When the results of repeated quantitative plating experiments were compared, counts on media containing regular agars plus starch were somewhat more erratic than those on media containing purified agar plus starch.

DISCUSSION

Based on current knowledge of the nutritional requirements of *N. gonorrhoeae*, a complete medium has been developed which is of simpler composition than those presently in use. This medium, designated TTU Complete Medium, can be prepared from scratch at a fraction of the cost of commercial media designed for the growth of *N. gonorrhoeae*. By comparing the plating efficiencies of three well-characterized laboratory strains, it has been shown quantitatively that the TTU Complete Medium is essentially equal to GC agar base medium supplemented with IsoVitaleX in supporting the growth of *N. gonorrhoeae*. There was no significant difference in the EOPs of strains 62 and 2686 on the two media. Strain 9 showed a slight, but statistically significant, reduction in EOP on the TTU Complete Medium (see Table 2). Recent experiments in our laboratory suggest that strain 9 has more complex nutritional requirements than strains 62 and 2686 and may require additional supplements for optimal growth. Qualitatively (as determined by a streak plate method), TTU Complete Medium was also equivalent to GC agar base medium supplemented with IsoVitaleX in supporting the growth of 67 clinical isolates and 6 auxotypes of *N. gonorrhoeae*. Based on the above data, we conclude that probably most strains of *N. gonorrhoeae* will grow on TTU Complete Medium. If additional nutrients are found to be required by some strains of *N. gonorrhoeae*, these could be easily added as supplements.

The growth response of *N. gonorrhoeae* in the liquid equivalent of TTU Complete Medium was similar to that reported by others using different media (8, 11). After a short lag phase, both turbidity measurements and viable counts increased exponentially for 7 h (see Fig. 1 and 2). After reaching a level of $\sim 5 \times 10^9$ CFU/ml, the culture went into what appeared to be a phase of spontaneous lysis, which resulted in an exponential decrease in viable cell numbers. Morse and Bartenstein (11) observed a similar lytic phenomenon after growth of *N. gonorrhoeae* in a liquid medium. These authors showed a correlation between glucose depletion and the onset of lysis and further stated that cultures limited by nitrogen were not susceptible to lysis. To fully understand the physiological mechanisms responsible for the lysis of *N. gonorrhoeae* in liquid medium, further study will be required.

It was observed that different commercial agars varied widely in their toxicity for *N. gonorrhoeae*. By comparing differences in the EOP of the three laboratory strains on TTU Complete Medium containing different types of agar with and without starch, we were able to quantitatively assess the effect various types of agar had on growth (see Table 3). The addition of soluble starch at a concentration of 0.1% neutralized the toxicity of some types of agar, e.g., most lots of purified agar and the three brands of regular agar. Starch did not neutralize the toxicity of other brands of agar, e.g.,

some lots of purified agar, Noble agar, and agarose.

In 1946, Ley and Mueller (9) presented evidence that agar contained fatty acids that inhibited the growth of *N. gonorrhoeae* and further showed that starch neutralized the toxicity. Walstad et al. (12) recently showed that a number of *N. gonorrhoeae* strains both produced and were inhibited by a variety of saturated and unsaturated fatty acids as well as the phospholipid monacyl phosphatidylethanolamine. Both of the above groups noted differences in strain susceptibility to these inhibitory agents. Our results also suggest that some strains of *N. gonorrhoeae* are more sensitive to agar toxicity than other strains (see Table 3). The more toxic brands of agar, which were not neutralized by starch, may contain higher levels and/or more inhibitory types of fatty acids. It is also possible that chemical pollutants other than fatty acids may be responsible for the toxicity of some agars. The isolation and characterization of the toxic components of agar could well be a subject for future study.

When the results of repeated quantitative plating experiments were compared, counts on media containing regular agars were more erratic than those on media containing purified agar. From these results, we conclude that for quantitative platings purified agar is probably superior to regular agar. For most qualitative experiments and for the primary isolation of *N. gonorrhoeae* from clinical specimens, regular agars should be satisfactory if each lot is pretested against known sensitive control strains before routine use.

TTU Complete Medium is a useful general purpose medium to which additional supplements can be added depending upon the particular nutritional requirements of the *N. gonorrhoeae* strains under study. This medium is easy to prepare and less expensive than commercially available media. It is currently of most use to researchers doing genetic, nutritional, and physiological studies which often require rather large batches of media.

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