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# Agar Medium for Use in Susceptibility Testing of Bacteria from Human Periodontal Pockets

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An agar medium (medium V) was formulated to determine the minimal inhibitory concentrations (MICs) of antimicrobial agents for bacteria encountered in human periodontal pockets. The medium contained (per liter) Trypticase, 15 g; yeast extract, 5 g; sodium chloride, 5 g; glucose, 2 g, sodium pyruvate, 2 g; sodium formate, 1 g; sodium fumarate, 1.5 g; sodium succinate, 0.1 g; Tween 80, 0.25 ml; agar, 15 g; hemin, 5 mg; and menadione, 0.5 mg. The growth of 50 oral strains was compared on this and six other media which included: Wilkins-Chalgren agar, Schaedler agar, Brucella agar, Trypticase-soy blood agar, and Schaedler and Brucella agars supplemented with whole blood. Growth, for most strains, was greatest on medium V. Medium V was also compared with Wilkins-Chalgren agar, using the same oral strains, to determine the MICs of the following antibiotics: penicillin, tetracycline, chloramphenicol, clindamycin, and erythromycin. The MICs of these antibiotics were essentially the same on both media when growth was quantitatively similar.

Periodontal diseases are a group of inflammatory diseases having different clinical manifestations and different microbiotas that affect supporting tissues of the teeth. Periodontal pockets harbor as many as 100 to 150 species of bacteria, with 5 to 10 distinct groups predominant in any site. Variation is observed in predominant organisms isolated from different forms of the disease, from patient to patient with similar clinical symptoms, and from site to site within patients. The isolated organisms are dominated by anaerobic, facultative, and capnophilic forms with widely different nutritional requirements.

Antimicrobial therapy is effective in the treatment of periodontal disease either alone (5, 6; A. Haffajee, C. Walker, J. M. Goodson, and S. S. Socransky, J. Dent. Res. **57A**:266, abstr. no. 767, 1978) or in conjunction with mechanical or surgical debridement (5; R. Genco, S. Singh, G. Krygier, and M. Levine, J. Dent. Res. **57A**:266, abstr. no. 768, 1978; H. Reynolds, P. Mashimo, J. Slots, N. Sedransk, and R. Genco, J. Dent. Res. **57A**:267, abstr. no. 769, 1978). Unfortunately, there have been few controlled studies to determine which antibiotics are most effective. Susceptibility testing will help provide a rational approach in the choice of drugs to be used. Investigators have determined the in vitro effect of antimicrobial agents on some of the bacteria encountered (S. Osterberg and B. L. Williams, J. Dent. Res. **57A**:318, abstr. no. 976, 1978; G. Sundquist, Ph.D. thesis, University of Umea, Umea, Sweden, 1976). However, interpretation of these results is difficult because of differences in methodology. Therefore, a medium was developed that can be used for in vitro susceptibility testing of the majority of periodontal isolates. Such a medium had to meet the following criteria: (i) provide adequate growth of organisms most frequently isolated from periodontal lesions, (ii) utilize ingredients readily available, (iii) give minimal inhibitory concentrations (MICs) similar to those obtained with other media in use, and (iv) not require the addition of blood because of the variability different sources might introduce.

## MATERIALS AND METHODS

**Bacterial strains.** Fifty characterized strains representing 24 species or groups of bacteria frequently isolated from human periodontal pockets were used as test organisms. A strain of *Bacteroides fragilis* (VPI 8708AP) with known MICs and growth characteristics was used as a reference strain in each trial.

**Cultivation.** All strains, except *B. asaccharolyticus* and *B. melaninogenicus*, were maintained on Trypticase-soy (T-soy) blood agar (BBL Microbiology Systems) and were transferred weekly. Strains of *B. asaccharolyticus* and *B. melaninogenicus* were maintained on T-soy blood agar supplemented with 5 μg of hemin per ml (equine III, Sigma Chemical Co.) and 0.5 μg of menadione per ml (Sigma) and transferred weekly.

**Broth media.** The broth media used for growth in inocula were as follows: Todd-Hewitt broth (Difco) supplemented with 0.1% sodium formate, 0.15% sodium fumarate, and 0.1% $KNO_3$ (TH-FF); mycoplasma broth base (BBL) supplemented with 0.3% glucose

(MBB); TYB broth consisting of 1.0% trypticase-peptone (BBL), 0.5% yeast extract (Difco), 0.3% beef extract (Difco), 0.5% NaCl, and 0.2% glucose; and CS4 broth, which consisted of 1.7% trypticase-peptone (BBL), 0.3% yeast extract (Difco), 0.5% NaCl, 0.12% $K_2HPO_4$, 0.05% sodium thioglycolate, 0.1% Tween 80, and 0.2% glucose. All broth media were supplemented with 5 μg of hemin per ml and 0.5 μg of menadione per ml. All were prepared prereduced (3).

**Inocula.** Inocula were prepared by transferring three to five colonies from a T-soy blood agar plate to a broth medium flushed with an $O_2$-free gas consisting of 80% $N_2$, 10% $H_2$, and 10% $CO_2$. *Selenomonas, Streptococcus, Fusobacterium nucleatum,* oral *Campylobacter,* and *B. fragilis* strains were inoculated into TYB broth. *Capnocytophaga, B. asaccharolyticus, B. melaninogenicus, B. ureolyticus, Eikenella corrodens,* anaerobic *Vibrio,* and the "corroding" *Bacteroides* group (14) were inoculated into TH-FF broth. CS4 broth was used for *Actinomyces,* and MBB broth was used for *Actinobacillus* and anaerobic streptococci. Broth cultures were incubated at 37°C for 24 to 48 h in an atmosphere of 80% $N_2$, 10% $H_2$, and 10% $CO_2$ until turbidity was approximately equal to a no. 1 McFarland standard (1). Bacterial strains which did not grow to sufficient turbidity in broth media were grown for 2 to 3 days on T-soy blood agar, scraped from the agar surface, and suspended in prereduced one-fourth strength Ringer solution (1).

**Antimicrobial agents.** Antibiotics tested were: clindamycin-HCl (Upjohn Co., Kalamazoo, Mich.); erythromycin, tetracycline-HCl, and benzylpenicillin (Sigma Chemical Co., St. Louis, Mo.); and chloramphenicol-HCl (Parke-Davis, Detroit, Mich.).

All antibiotics, except erythromycin, were dissolved in distilled water. Erythromycin was dissolved in 95% ethanol and then brought to volume with nine parts distilled water. The antibiotics were filter sterilized, and then twofold dilutions were made in sterile distilled water.

**Agar media.** Brucella agar (BBL) and Schaedler agar (BBL) were supplemented with hemin (5 μg/ml) and menadione (0.5 μg/ml). Menadione was added aseptically after the media were autoclaved and had cooled to 50 to 55°C. Brucella blood agar and Schaedler blood agar, in addition to hemin and menadione, were supplemented with 5% (vol/vol) defibrinated whole sheep blood (BBL). T-soy blood agar plates (BBL) were purchased commercially. Wilkins-Chalgren agar (WCA) was prepared as described (15). The composition of medium V is given in Table 1. The pH of medium V was adjusted to 7.0 to 7.2 before autoclaving, and, after 48 h of anaerobic incubation, the pH of uninoculated medium was 6.4 to 6.7.

Antibiotic plates were prepared by pipetting 0.5 ml of the desired antibiotic concentration into a plastic petri dish (100 by 15 mm) and adding 25 ml of base. The plates were swirled gently and the medium was allowed to cool. The agar plates were allowed to dry overnight at room temperature and used the following day.

Immediately before use, the medium was dried for 30 to 60 min at 37°C to facilitate rapid drying of the inocula. The bacteria were inoculated aerobically onto the agar surface with a Steers replicator (13). As soon as the inocula were dry, the agar plates were placed in an anaerobic chamber containing an atmosphere of 80% $N_2$, 10% $CO_2$, and 10% $H_2$ which was constantly circulated through a palladium catalyst. The cultures were exposed to air for less than 30 min before anaerobic incubation at 37°C for 48 h.

TABLE 1. *Composition of medium V*

| Component | Source | Amt/liter |
|---|---|---|
| Trypticase peptone | BBL | 15 g |
| Yeast extract | Difco | 5 g |
| Glucose | Fisher | 2 g |
| Sodium pyruvate | Sigma | 2 g |
| Sodium chloride | Sigma | 5 g |
| Sodium formate | Sigma | 1 g |
| Sodium fumarate | Sigma | 1.5 g |
| Tween 80 | Fisher | 0.25 ml |
| Sodium succinate | Fisher | 0.1 g |
| Hemin | Sigma | 0.005 g |
| Menadione | Sigma | 0.0005 g |
| Agar | Difco | 15 g |

Agar plates without antibiotics were used to determine the ability of the media to support growth of the organisms. The MIC was determined as the least antibiotic concentration that yielded no visible growth.

## RESULTS

**Growth on media free of antibiotics.** Medium V supported sufficient growth of most organisms commonly isolated from human periodontal pockets to permit antibiotic susceptibility testing.

Medium V was compared to T-soy blood agar, a medium often used for the isolation and maintenance of oral bacteria, and to five media that have been used previously in susceptibility testing. The organisms tested and the results are presented in Table 2.

Medium V was similar to T-soy blood agar in ability to grow different organisms and the quantity of growth obtained. Growth was significantly better on medium V for oral *Campylobacter,* anaerobic *Vibrio,* "corroding" *Bacteroides,* and *B. ureolyticus. B. asaccharolyticus* was the only group that did not grow as well on medium V as on T-soy blood agar or WCA. However, the growth of these organisms on medium V was sufficiently heavy to permit susceptibility testing.

Medium V and WCA gave comparable results for 17 of the 24 groups tested. In six of the seven instances where differences in growth occurred, the growth was better on medium V. With three of these groups, *Actinobacillus actinomycetemcomitans,* oral *Campylobacter,* and anaerobic *Vibrio,* growth was very good on medium V, but was either very light or absent on WCA.

Medium V was clearly superior to both Schaedler and Brucella agars, which failed to allow

TABLE 2. *Comparison of the growth of periodontal isolates on medium V and six other media*

| Organisms | No. of strains | Growth of periodontal isolates[a] on: | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Medium V | WCA | Brucella | Brucella blood | Schaedler | Schaedler blood | T-soy blood |
| *Actinobacillus actinomycetemcomitans* | 5 | 3+ | ± | ± | + | NG | + | 3+ |
| *Actinomyces israelii* | 2 | 4+ | 4+ | + | 4+ | + | 2+ | 4+ |
| *Actinomyces naeslundii* | 1 | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ |
| *Actinomyces odontolyticus* | 1 | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ |
| *Actinomyces viscosus* | 2 | 4+ | 4+ | + | 4+ | 2+ | 2+ | 4+ |
| Anaerobic *Vibrio* | 8 | 3+ | NG | NG | NG | NG | ± | 2+ |
| *Bacteroides asaccharolyticus* | 2 | 3+ | 4+ | + | 2+ | NG | + | 4+ |
| *Bacteroides fragilis* | 1 | 4+ | 4+ | 4+ | 4+ | 3+ | 4+ | 4+ |
| *Bacteroides melaninogenicus subsp. intermedius* | 2 | 4+ | 4+ | 3+ | 4+ | NG | 2+ | 4+ |
| *Bacteroides melaninogenicus subsp. melaninogenicus* | 1 | 4+ | 4+ | 3+ | 4+ | + | 2+ | 4+ |
| *Bacteroides ureolyticus* | 1 | 4+ | 3+ | 3+ | 3+ | NG | + | 2+ |
| *Capnocytophaga gingivalis* | 1 | 4+ | 4+ | 2+ | 3+ | + | 2+ | 4+ |
| *Capnocytophaga ochracea* | 2 | 4+ | 4+ | ± | 3+ | + | 2+ | 4+ |
| *Capnocytophaga sputigena* | 1 | 4+ | 4+ | 2+ | 3+ | NG | 2+ | 4+ |
| "Corroding" *Bacteroides* | 3 | 4+ | 3+ | 4+ | 4+ | 2+ | 3+ | 3+ |
| *Eikenella corrodens* | 3 | 4+ | 3+ | 2+ | 2+ | NG | 2+ | 4+ |
| *Fusobacterium nucleatum* | 4 | 4+ | 4+ | 3+ | 4+ | + | 2+ | 4+ |
| Oral *Campylobacter* | 3 | 4+ | ± | ± | + | + | + | 2+ |
| *Peptostreptococci* | 3 | 4+ | 4+ | 4+ | 4+ | 3+ | 4+ | 4+ |
| *Selenomonas sputigena* | 2 | 4+ | 4+ | 3+ | 3+ | | 3+ | 4+ |
| *Streptococci* | 3 | 4+ | 4+ | 4+ | 4+ | 3+ | 4+ | 4+ |

[a] Symbols: NG, no growth on medium; ±, 1 to 5 small colonies or faint haze; +, 5 to 20 colonies or very light confluent growth; 2+, light confluent growth; 3+, medium to heavy confluent growth; 4+, very heavy confluent growth.

growth of a number of the organisms tested and allowed many others to grow only lightly. The addition of blood increased the growth on Brucella agar but did not significantly improve growth on Schaedler agar.

**Susceptibilities.** Since WCA is the medium recommended for susceptibility testing of clinical anaerobic bacteria (15), the same series of organisms was tested for susceptibility to five antibiotics using medium V and WCA as bases. The results are given in Table 3. For strains that grew equally well on both media the MIC was the same or very similar on each medium. In most cases, variation in the MICs between the two media was within an acceptable variation range of two dilution factors. The major exception to this was the "corroding" *Bacteroides* group for which the MICs were consistently higher on medium V than on WCA.

## DISCUSSION

Medium V was derived to provide adequate growth for susceptibility testing of the majority of bacteria isolated from human periodontal pockets. Organisms encountered in such lesions vary widely in atmospheric and nutritional requirements. In primary isolation and routine maintenance, nutritional requirements are often satisfied by the addition of whole blood to the agar medium. In susceptibility testing, however, the addition of blood is undesirable because of the variation it can introduce. Blood varies from supplier to supplier as well as from the same supplier at different times. Blood also deteriorates with age and is easily contaminated. In addition, certain antibiotics may bind to blood proteins, and the degree of binding can vary with the source of blood.

TABLE 3. *Comparative MICs with medium V and WCA of five antibiotics for periodontal isolates*

| Organisms | No. of strains | MIC of: | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Penicillin G | | Tetracycline | | Clindamycin | | Erythromycin | | Chloramphenicol | |
| | | V[a] | WCA[a] | V | WCA | V | WCA | V | WCA | V | WCA |
| *Actinobacillus actinomycetemcomitans* | 5 | 1.0–4.0 | —[b] | 1.0–2.0 | — | 2.0 | — | 1.0–32.0 | — | 0.25–1.0 | — |
| *Actinomyces israelii* | 2 | ≤0.25 | ≤0.25 | 0.5–1.0 | 0.5 | 0.06 | ≤0.06 | ≤0.06 | ≤0.06 | ≤0.125 | ≤0.125 |
| *Actinomyces odontolyticus* | 1 | ≤0.25 | ≤0.25 | 0.25 | 0.5 | ≤0.06 | ≤0.06 | 0.125 | ≤0.06 | ≤0.125 | ≤0.125 |
| *Actinomyces naeslundii* | 1 | ≤0.25 | ≤0.25 | 1.0 | 0.5 | 0.5 | 0.5 | 0.5 | ≤0.06 | ≤0.125 | ≤0.125 |
| *Actinomyces viscosus* | 2 | ≤0.25 | ≤0.25 | 0.5 | 0.25 | ≤0.06 | ≤0.06 | ≤0.06 | ≤0.06 | ≤0.125 | ≤0.125 |
| Anaerobic *Vibrio* | 8 | ≤0.25 | — | ≤0.06–2.0 | — | 0.25 | — | 0.5–1.0 | — | 0.25 | — |
| *Bacteroides asaccharolyticus* | 2 | ≤0.25 | ≤0.25 | 0.5 | 0.25 | ≤0.06 | ≤0.06 | 0.5 | 1.0 | ≤0.125 | ≤0.125 |
| *Bacteroides fragilis* | 1 | >128 | >128 | 32.0 | 32.0 | 0.25 | 0.5 | 8.0 | 32.0 | >64.0 | >64.0 |
| *Bacteroides melaninogenicus subsp. intermedius* | 2 | ≤0.25 | ≤0.25 | 0.5 | 0.25 | ≤0.06 | ≤0.06 | 1.0 | 1.0 | ≤0.125 | ≤0.125 |
| *Bacteroides melaninogenicus subsp. melaninogenicus* | 1 | 0.5 | 0.5 | 0.5 | 0.5 | ≤0.06 | ≤0.06 | 0.25 | 0.5 | 0.25 | 0.25 |
| *Bacteroides ureolyticus* | 1 | 0.25 | 0.25 | 0.125 | 0.125 | 0.25 | 0.06 | 0.125 | 1.0 | 0.125 | 0.125 |
| *Capnocytophaga gingivalis* | 1 | 0.5 | ≤0.25 | 1.0 | 1.0 | ≤0.06 | ≤0.06 | 1.0 | 2.0 | 0.5 | 0.5 |
| *Capnocytophaga ochracea* | 2 | ≤0.25 | ≤0.25 | 1.0 | 1.0 | ≤0.06 | ≤0.06 | 1.0 | 1.0 | 0.25 | 0.25 |
| *Capnocytophaga sputigena* | 1 | ≤0.25 | ≤0.25 | 1.0 | 1.0 | ≤0.06 | ≤0.06 | 2.0 | 4.0 | 0.25 | 0.25 |
| "Corroding" *Bacteroides* | 3 | 2.0–8.0 | 0.5–2.0 | 0.5–2.0 | 0.25–1.0 | 1.0 | 0.25–0.5 | 1.0–4.0 | 0.5–1.0 | 2.0–8.0 | 0.5–4.0 |
| *Eikenella corrodens* | 3 | 0.25–0.5 | 0.25 | 0.5 | 0.5 | 8.0 | 4.0–32.0 | 2.0 | 0.5 | 0.5 | 0.25 |
| *Fusobacterium nucleatum* | 4 | ≤0.25 | ≤0.25 | 2.0 | 0.5 | ≤0.06 | ≤0.06 | 32.0 | 16.0 | <0.125 | ≤0.125 |
| Oral *Campylobacter* | 3 | 0.25 | — | 1.0 | — | 1.0–2.0 | — | 8.0 | — | 0.25–2.0 | — |
| *Peptostreptococci* | 3 | ≤0.25 | ≤0.25 | 0.5 | 1.0 | 0.25 | ≤0.06 | 16.0 | 32.0 | 0.5 | 0.125 |
| *Selenomonas sputigena* | 2 | ≤0.25 | ≤0.25 | 0.5 | 0.25 | ≤0.06 | ≤0.06 | 4.0 | 2.0 | ≤0.125 | ≤0.125 |
| *Streptococci* | 3 | ≤0.25 | ≤0.25 | 1.0 | 0.25 | 0.125 | ≤0.06 | 0.125 | ≤0.06 | ≤0.125 | ≤0.125 |

[a] Medium V or WCA used as base.

[b] MIC not determined due to inability of medium to support growth of the organisms.

For these reasons, medium V was formulated so that the addition of blood was not required. The ingredients used in medium V are common and should not vary significantly either among suppliers or from lot to lot. Trypticase peptone is the only ingredient that is not available from different sources. Different peptones, including proteose, Trypticase, gelysate, and phytone, were tested individually and in combination. The best and most uniform results were obtained with Trypticase. Other investigators have also found this peptone to give the best growth for anaerobic bacteria (15, 16). At a 1.5% concentration, Trypticase gave better and more reproducible growth for *Capnocytophaga, Campylobacter, B. melaninogenicus* subsp. *melaninogenicus*, and the "corroding" *Bacteroides* group than did 1.0% Trypticase. Yeast extract was added at 0.5% as a source of vitamins, purines, pyrimidines, and other growth factors. Several organisms grew poorly when the yeast extract concentration was lowered to 0.25%. Glucose was added as an energy source for the saccharolytic organisms. No difficulty was experienced with the 0.2% concentration; however, higher concentrations permitted saccharolytic organisms such as the streptococci to significantly decrease the pH of the surrounding medium. Formate and fumarate have been reported to act as an electron donor and acceptor in the energy metabolism of *B. ureolyticus* and *Vibrio succinogenes* and were necessary to achieve adequate growth in broth with these organisms (10). We found that formate and fumarate greatly enhanced the growth of the anaerobic *Vibrio* group, the "corroding" *Bacteroides* group, *E. corrodens*, and *B. ureolyticus*. Pyruvate was added as an energy source for certain asaccharolytic organisms. This compound enhanced growth of other organisms including certain of the *Capnocytophaga, Peptostreptococcus, Actinobacillus*, and *B. melaninogenicus* strains. The same effect was not achieved when pyruvate was replaced with an equal amount of lactate. Therefore, it would appear that pyruvate may have acted as other than an energy source. Pyruvate has been reported to degrade hydrogen peroxide to form acetate, carbon dioxide, and water (15). This reaction may account for the stimulatory effect of pyruvate in our medium. Sodium chloride was added to make the medium isotonic. Tween 80 is known to stimulate growth of a number of gram-positive organisms. We found Tween 80 at a 0.025% concentration greatly enhanced the growth of *Peptostreptococcus* and *Actinomyces israelii* strains. Higher concentrations were inhibitory for some of the *B. melaninogenicus* and *F. nucleatum* strains. Sodium succinate has been reported to stimulate oral strains of *B. melaninogenicus* (4). Although the deletion of this compound did not have any apparent effect on our strains, we chose to leave it in our medium in case such organisms were later encountered. The requirement for hemin has been documented for a number of the *Bacteroides* species (2, 8). Menadione was added to fulfill the requirements of certain strains of *B. asaccharolyticus* and *B. melaninogenicus* (2).

A number of media have been utilized for the growth and antibiotic susceptibility testing of anaerobic bacteria. Although these media may be admirably suited for growth of organisms isolated from the habitat for which they were developed, there is no reason to presuppose that they would be equally useful for isolates from a different habitat.

Schaedler medium yields excellent growth of many anaerobes (7, 9, 11, 12). In our experiments, however, Schaedler agar without blood gave the poorest growth of all media tested for 16 of the 21 groups. Of these 16 groups, 7 failed to grow and 9 grew below what is considered to be sufficient for susceptibility testing. The addition of blood to this medium enabled us to obtain better growth; but, for many of these organisms, growth was still not adequate for susceptibility testing.

Brucella agar allowed better growth than the Schaedler agar of several groups of organisms, particularly the agar-corroding bacteria. However, there were 10 groups that did not grow well on this medium. When the medium was supplemented with whole blood, growth was improved only for 5 of these 10 groups.

In our experiments, neither Schaedler nor Brucella blood agars were as good as T-soy blood agar. This is contrary to the findings of Murray, who found that quantitative growth was similar on all three (7). Murray also found that, even though growth was similar, overall colony size and rate of growth were best on Schaedler blood agar. The differences in our findings and those of Murray are probably due to the different organisms tested. Murray's results were obtained with anaerobic bacteria from clinical specimens whereas the organisms in this study were from periodontal lesions. The latter bacteria are generally more fastidious and have more varied nutritional requirements.

WCA is the medium recommended for susceptibility testing of anaerobic bacteria from clinical samples. We originally thought to use this medium for susceptibility testing of periodontal isolates. However, certain groups of organisms were encountered which would not grow on the medium. The *Actinobacillus*, oral *Campylobacter*, and anaerobic *Vibrio* strains either failed to grow or else grew too lightly for use. The agar-

corroding organisms, *B. ureolyticus, E. corrodens*, and the "corroding" *Bacteroides* group, grew on WCA but not as well as on medium V. The difference in growth did not affect the MICs obtained for *B. ureolyticus* and *E. corrodens*; however, the MICs for the "corroding" *Bacteroides* group were higher with medium V than with WCA. Presumably, this may be due to better growth obtained on medium V, and the increase in the MICs would reflect the increase in antibiotic required for inhibition.

Based on the experiments reported, medium V is a suitable agar medium for susceptibility testing of bacteria from subgingival plaque samples of periodontally diseased individuals. The growth of organisms from such sites on medium V was as good as, and in some cases better, than on blood-supplemented agar media. The MICs obtained with medium V were reproducible, and the absolute values were comparable to those obtained using WCA.

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