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# Anaerobic Specimen Transport Device

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A device is described and evaluated for the anaerobic transport of clinical specimens. The device limits the amount of oxygen entering with the sample to a maximum of 2%, which is rapidly removed by reacting with hydrogen in the presence of a palladium catalyst. The viability on swabs of 12 species of anaerobes, four strains of facultative anaerobes and a strain of *Pseudomonas aeruginosa*, was maintained during the length of the tests (24 or 48 h). The results demonstrated that this device protected even the more oxygen-sensitive clinical anaerobes from death due to oxygen exposure. This device can be used for swabs as well as for anaerobic collection and liquid and solid specimens.

The quality of anaerobic bacteriology practiced in clinical laboratories has greatly improved during the past few years. However, these improvements must be coupled with adequate anaerobic transport of specimens to obtain the full benefit of these improvements. Many investigators have stated that poor transport methods cause the loss of many anaerobic bacteria prior to delivery of the sample to the clinical laboratory, thus making isolation impossible (2).

This paper describes the development and evaluation of a new system for protecting clinical samples from oxygen.

## MATERIALS AND METHODS

**Bacterial cultures.** All anaerobic bacteria were from the culture collection of the Virginia Polytechnic Institute (V.P.I.) Anaerobe Laboratory and were identified by methods given in the *Anaerobe Laboratory Manual* (6). The aerobic and facultative organisms were from the culture collection of the Biology Department of Virginia Polytechnic Institute. The V.P.I. culture collection numbers for the anaerobic bacteria used were as follows: *Bacteroides fragilis* subsp. *fragilis* 2556-1, *Bacteroides fragilis* subsp. *thetaiotaomicron* 5482, *Bacteroides melaninogenicus* subsp. *asaccharolyticus* 4198 (ATCC 25260), *Fusobacterium nucleatum* 8748, *Fusobacterium necrophorum* 6054-A, *Peptococcus magnus* 8532, *Peptostreptococcus anaerobius* 5750, *Streptococcus intermedius* 3372, *Eubacterium lentum* 1947-1B, *Clostridium ramosum* 8546, *Clostridium innocuum* 8593, *Clostridium perfringens* type A 5201.

**Inocula.** All cultures were maintained in prereduced chopped meat (CM) broth (6). One drop from a Pasteur pipette of an overnight culture in CM broth was added to 10 ml of prereduced brain heart infusion (BHI) broth (6); serial 10-fold dilutions were made in BHI broth, and all tubes were incubated overnight at 37 C. The next day the tube with an optical density nearest to 0.2, but not at maximum turbidity, was used as the inoculum. Optical density was measured at 650 nm in 18-mm diameter test tubes with a Bausch and Lomb Spectronic 20. This tube was placed in an anaerobic chamber with an atmosphere of 10% $H_2$, 5% $CO_2$, and 85% $H_2$, and 0.2-ml aliquots were placed in the wells of microtiter plates. Cotton swabs (Sani-Swab, A. H. Thomas Co.) were then placed in each well to absorb the inoculum. One set of swabs was placed in humid, rubber-stoppered tubes and stayed inside the chamber as anaerobic controls. Two sets of swabs were taken out of the glove box. One set was immediately inserted in the transport devices made in our laboratory and described in this paper. The other set was placed into aerobic, humid, rubber-stoppered tubes. The humid tubes were prepared by placing 0.1 ml of water into each tube, which was then closed with a rubber stopper and autoclaved in a press (6). After 2, 4, 24, and, in some cases, 48 h, one swab of each type was taken into the chamber, and viable plate counts were made from each swab. For aerobic and facultative organisms the above procedure was modified by the use of aerobic BHI medium, and viable plate counts were done aerobically.

**Viable plate counts.** For anaerobic plate counts, the swabs were taken into the anaerobic chamber and placed in tubes containing 10 ml of anaerobic dilution fluid (6). This tube was vigorously agitated on a Vortex-type mixer for approximately 10 s, and serial 10-fold dilutions were made. Duplicate 0.1-ml amounts of the dilution were spread on plates of appropriate media. The plates were incubated for 48 h at 37 C in an incubator inside the anaerobic chamber. The coefficient of variation of the plate count results was within ±20%.

**Plate media.** Viable plate counts were made on one of the following media, depending on which gave optimal growth for each organism: BHI agar with 0.5% yeast extract, with and without 5% whole sheep blood; brucella agar with 5% whole or laked sheep blood.

**Resazurin agar indicator.** The indicator consisted of: resazurin, 0.01%; cysteine, 0.05%; $KH_2PO_4$, 0.6%; $K_2HPO_4$, 0.87%; and agar, 0.7%. The

resazurin and phosphate buffer were boiled, the cysteine was then added, and the pH was adjusted to 7.0 with KOH while flushing with $N_2$ gas. This solution was dispensed into large tubes containing the agar, and the tubes were then flushed with nitrogen while rubber stoppers were inserted. These tubes were placed in a press, and sterilized by autoclaving (121 C, 15 min). For use, a tube was melted, and a Pasteur pipette was used to fill the small tubes (25 by 6 mm).

**Specifications of transport device.** The devices used in these studies were made in our laboratory in the following manner. A hole was drilled in a size 4 rubber stopper (Fig. 1, B) to fit glass tubing (9-mm diameter). A 9-cm-long piece of this tubing with one end closed by heating was inserted partway into the bottom of the stopper (Fig. 1, C). Ten palladium catalyst pellets (Fig. 1, G) were dropped into a roll tube (142 by 25 mm; Fig. 1, D; Bellco Glass Inc.), along with a small tube (25 by 6 mm; Fig. 1, F) containing resazurin agar indicator (formula given separately). The large tube was flushed with a gas mixture of 10% $H_2$, 5% $CO_2$, and 85% $N_2$ (Fig. 1, E). The stopper holding the sample tube was inserted into the large roll tube and was tightened into place. Aluminum foil was placed over the stopper; the device was placed in a press (6) and sterilized by autoclaving (121 C, 15 min). A plunger made of solid glass tubing (9 by 30 mm; Fig. 1, A) was sterilized separately.

**Use of device.** A commerical prototype is shown in Fig. 2. In the commercial device the cotton swab is attached to the plunger. The device is used by placing the specimen, which can be a biopsy, liquid, swab, etc., in the central tube, inserting the plastic or glass plunger in the hole in the rubber stopper, and pushing the plunger down. This drops the cen-

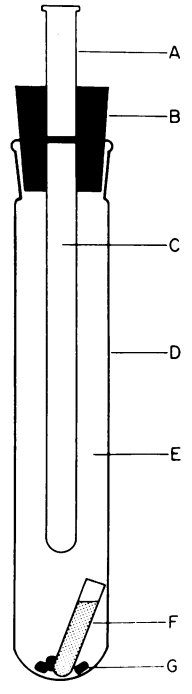


FIG. 1. *Sectional drawing of original device as developed by us. A, Plunger; B, size 4 rubber stopper; C, sample holding tube; D, 142- by 25-mm roll tube; E, atmosphere inside roll tube consisting of: 10% $H_2$, 5% $CO_2$, and 85% $N_2$; F, small tube containing the resazurin indicator; G, palladium-coated asbestos pellet.*

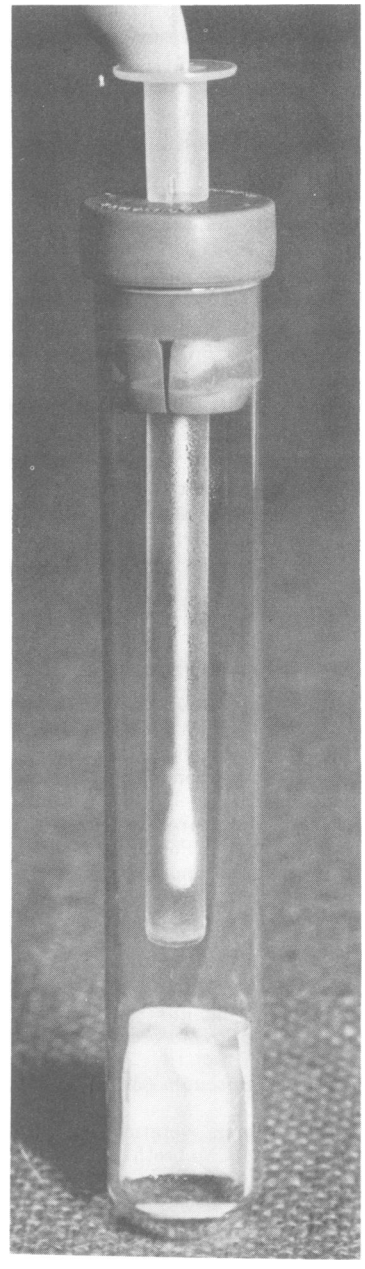

FIG. 2. *Commercial prototype. Cotton swab is fixed to the plunger.*

tral tube with the specimen into the outer tube and simultaneously closes the hole in the rubber stopper (Fig. 3). The air contained in the central tube is immediately diluted from 20% oxygen to less than 2%, with the non-explosive mixture of 10% $H_2$, 85% $N_2$, and 5% $CO_2$ contained in this tube. Reaction of the $H_2$ and $O_2$ immediately commences, catalyzed by the palladium-covered asbestos pellets in the tube. There is approximately a fivefold excess of $H_2$ in the tube over that needed for the complete removal of oxygen.

As a quality control measure to detect defective devices, a resazurin oxidation indicator is included in each tube. The indicator is contained in either an agar-filled tube (original model) or a soaked filter-paper disk (prototype). This indicator should remain colorless until the specimen is inserted. The indicator may turn red (oxidized, Eh > −40 mV) due to the small amount of oxygen introduced with the sample, but it should revert to its colorless state within 30 min. The indicator serves an additional purpose of providing moisture, both for autoclave sterilization and to help prevent deleterious drying of specimens. The atmosphere inside the device is completely saturated with water vapor so moisture cannot evaporate from the sample. However, the swab should be moistened as well as possible with the sample, since the dry swab will absorb water to some extent.

## RESULTS

**Evaluation.** We tested the device for the ability to protect logarithmically growing cells from death due to oxidation. Preliminary experiments showed that resting cells diluted in anaerobic salts solution were relatively resistant to oxygen, in comparison to actively growing organisms. Since bacteria from clinical specimens are assumed to be metabolically active, this seemed a more relevant test than the use of cells in the maximum stationary phase of growth.

For our tests we used one strain of each of 12 species of anaerobic bacteria, including those most frequently isolated in clinical laboratories. For convenience of data presentation, we have grouped these organisms into three groups based on the length of time the cells remained viable on aerobic swabs (detection limit of 100 cells per swab). The "very oxygen sensitive" group was nonviable at the first sampling at 2 h; the "moderately oxygen sensitive" group survived 4 h, but not 24 h; and the "oxygen tolerant" group survived 24 h.

Viable cell counts were made on aerobic swabs, swabs exposed briefly (30 s to 1 min) to air before insertion into the transport device, and control swabs never exposed to air (glove box). With three of the four "very oxygen sensitive" cultures, no significant amount of death occurred prior to the first sample period in the transport device (Fig. 4). With *F. necrophorum* there was a loss in viability from $5 \times 10^6$ cells/ml at 0 time to $10^6$ cells/ml after 2 h in the device, but no further death occurred. *B. melaninogenicus* was the only culture showing any loss in viability after 24 h in the device. Plate count results were variable with the *B. melaninogenicus* culture, however, and it is impossible to know how much of the apparent loss of viability was caused by this variation. With *P.*

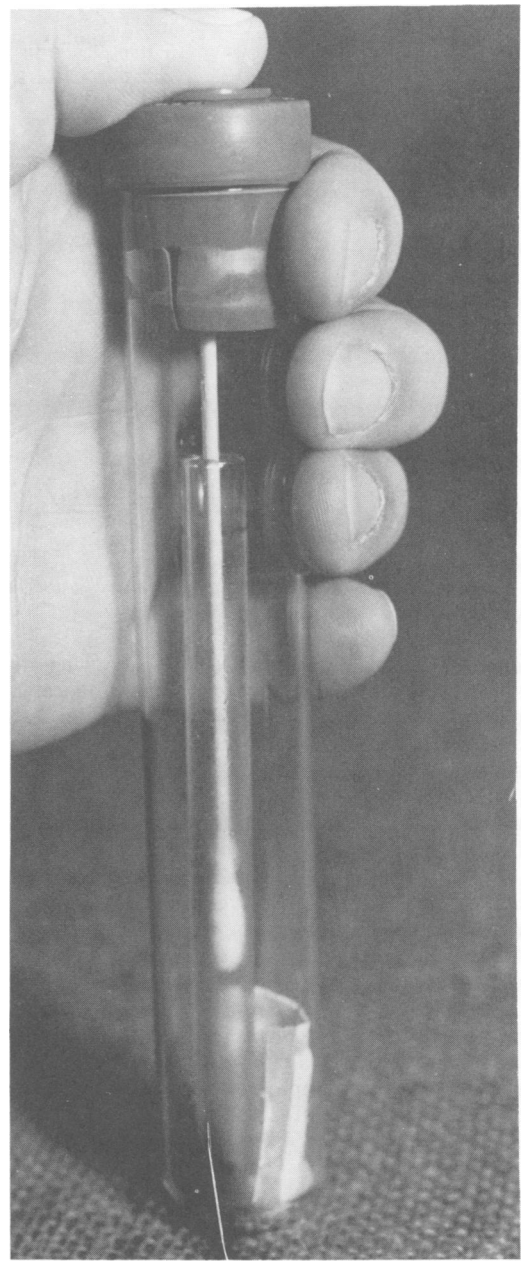

FIG. 3. *Commercial device after introduction of sample.*

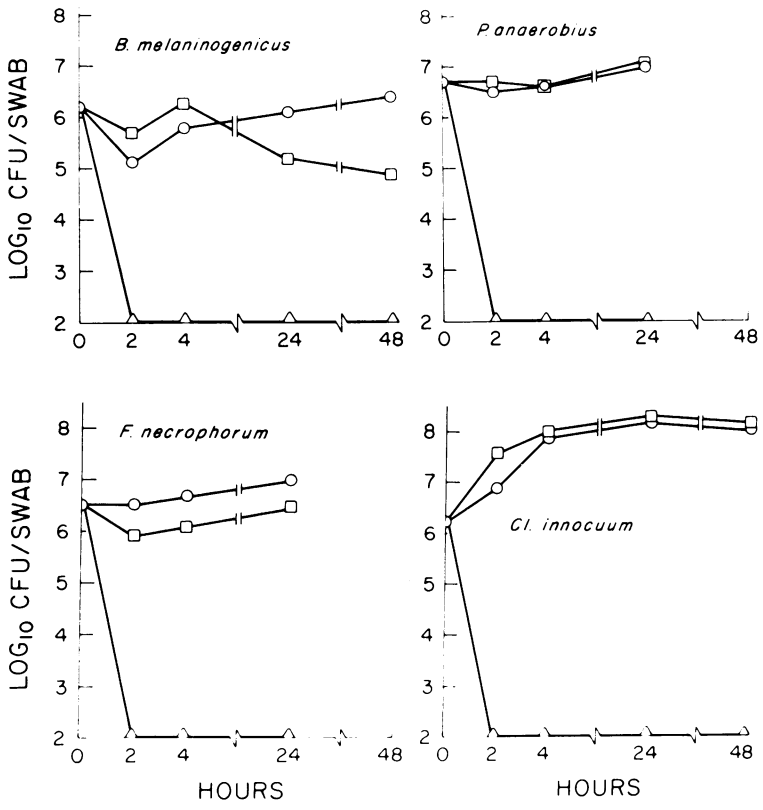


FIG. 4. *Survival of "very oxygen sensitive" anaerobes on swabs. Symbols: △, aerobic control; ○, anaerobic control; □, transport device.*

*anaerobius* and *C. innocuum*, there was no significant difference between the anaerobic control swab and the swab in the transport device. There were also few differences observed with the other eight organisms tested (Figs. 5 and 6). The transport device maintained viability of all of the organisms for the length of the test (24 or 48 h).

To determine whether the transport device could be used for facultative and aerobic bacteria as well as for anaerobes, we performed similar experiments with one strain each of *Escherichia coli, Staphylococcus aureus, Streptococcus pyogenes, Proteus vulgaris,* and *Pseudomonas aeruginosa*. No detrimental effects of the transport device could be detected with these organisms when compared to aerobic and anaerobic controls even after 48 h.

## DISCUSSION

Although there is general agreement that anaerobic bacteria should be transported from the patient to the clinical laboratory as well protected from oxygen as possible, the few methods that have been proposed for accomplishing this (4) have not been adequately evaluated. These methods consist of either: rapidly placing the swab or sample into a $CO_2$-filled tube (6), putting it into a transport medium (7), or drawing a liquid sample into a syringe and injecting it into an anaerobic bottle (5, 6). Of these two methods, the latter is more likely to retain strict anaerobiosis and should work well as long as air is not aspirated with the sample during collection. The use of reduced transport media for anaerobes is certainly better than aerobic transport, but the results of available studies on the survival of anaerobic bacteria in such media are not encouraging (J. Yrios, A. Helstad, S. Inhorn, C. Field, and E. Balish, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, M316, p. 119; 3). Barry et al. (3) concluded from their test of Amies transport medium that "the anaerobes survived much better when placed in the reduced environment of the freshly prepared transport medium, but even in such a medium the more sensitive anaerobes survived for only a short period, if at all." Sutter et al.

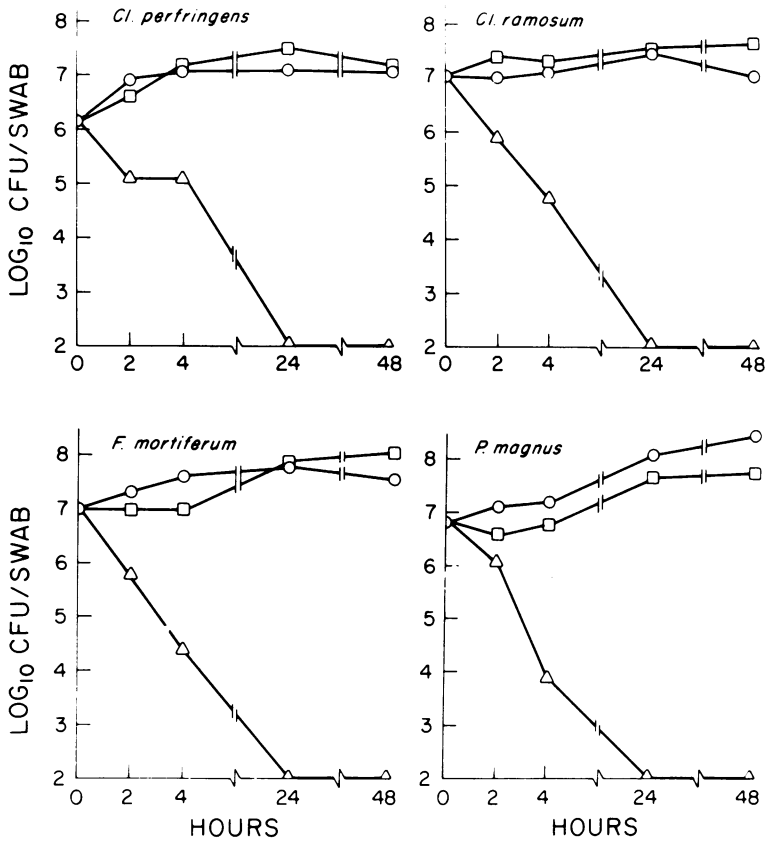


FIG. 5. *Survival of "moderately oxygen sensitive" anaerobes on swabs. Symbols: △, aerobic control; ○, anaerobic control; □, transport device.*

(7) have recently recommended the use of prereduced Cary-Blair transport medium that may prove superior to Amies transport medium, but to our knowledge it has not yet been quantitatively evaluated.

The device described in this paper incorporates the idea of placing the sample as rapidly as possible in an anaerobic tube, but, unlike simply placing the sample into a $CO_2$ filled tube, the amount of air entering with the sample is limited. The maximum amount of oxygen to which the sample is exposed inside the device is 2%, and this is rapidly removed by combination with hydrogen. Theoretically, this is superior even to placing a sample immediately into a GasPak anaerobic jar, since such a jar starts with 20% oxygen and the hydrogen must be generated chemically.

Testing a device for protection of anaerobic bacteria in clinical material is complicated by the difficulty of quantitating and controlling such experiments. With in vitro tests, the effect of oxygen on anaerobes in actual infected tissue and pus cannot be studied, but more adequate controls can be designed and reproducible quantitation is possible. We chose to use in vitro tests for these reasons and because adequate numbers of clinical specimens were not readily available to us. The tests were conducted with logarithmically growing cultures, since this is the most sensitive system we have yet found to detect killing due to oxygen exposure. Swabs were used both because of the criticism that they are the most likely type of sample to oxidize or desiccate, and because they are a very common method of obtaining samples.

The fact that this device adequately protected even the more oxygen-sensitive group of clinical anaerobes, which rapidly died on the aerobic control swabs, indicated that anaerobic conditions were attained rapidly. There was no indication of toxicity of the cotton, and the continued viability of the organsims for 48 h indicated that desiccation did not occur under these conditions. Indeed, the conditions were such in the transport device that most of the organisms

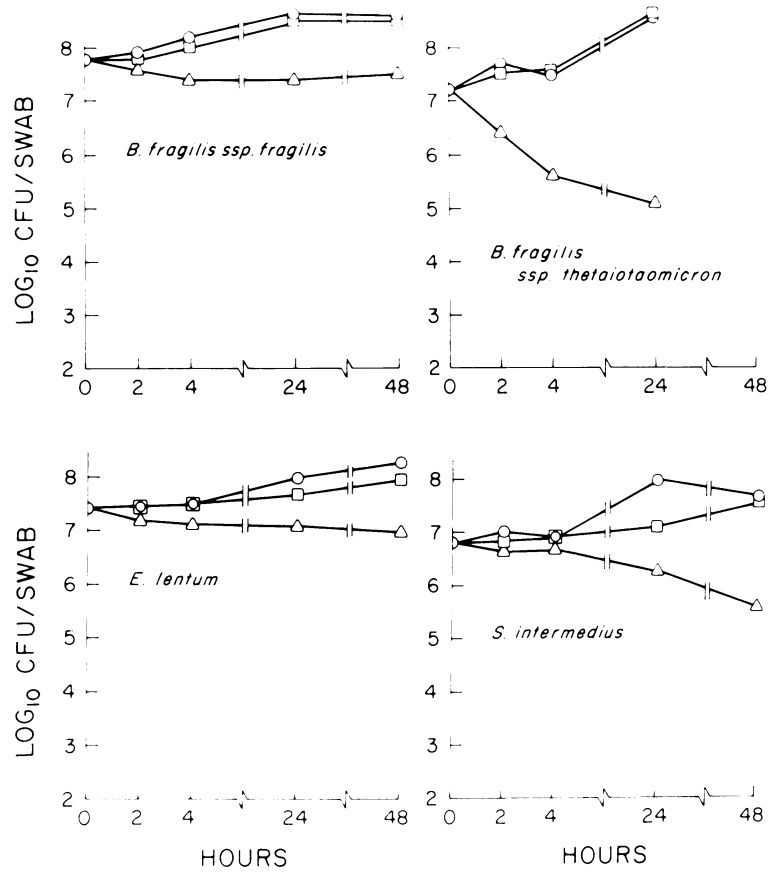


FIG. 6. *Survival of "oxygen tolerant" anaerobes on swabs. Symbols: △, aerobic control; ○, anaerobic control; □, transport device.*

continued to grow at the same rate as those on the control anaerobic swabs never exposed to air.

The tests with aerobic and facultative bacteria demonstrated that anaerobic conditions were not harmful to such organisms. It has been suggested that anaerobic transport approaches the ideal method for almost all bacteria (5), and transport media designed for facultative bacteria contain reducing agents to help promote anaerobiosis. Specimens containing a mixture of anaerobes and facultative organisms would be expected to change in relative proportion of the two organisms, if transported aerobically. This is due, in part, to possible death of the anaerobes, but also to the advantage facultative organisms have in being able to utilize oxygen for growth.

Although we recommend that all specimens be processed as rapidly as possible to recover the maximum number of anaerobic bacteria, we recognize that some samples cannot be adequately processed in many clinical laboratories at night and on weekends. The transport device we have described should be helpful in maintaining the viability of anaerobic bacteria in specimens that are obtained at such times. The transport device should also help prevent the rapid death of the more oxygen-sensitive anaerobes that can occur during the first 2 h of oxygen exposure after sample collection. However, the utility of the device can only be determined by actual clinical experience.

## ACKNOWLEDGMENTS

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