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Synthesis of M Protein by Group A Hemolytic Streptococci in Completely Synthetic Media During Steady-State Growth[1]

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Strains of type 6 (S 43) and type 14 group A streptococci were grown with M-protein production in the presence of chemically defined synthetic media slightly modified from that previously employed for the growth of a nonproducer of M protein (type 4). The M protein, which is associated with virulence in group A streptococcus, was previously produced in growing cultures only with complex media. The bacterial growth with the biosynthesis of M protein in synthetic medium was obtained by successive adaptation in steady-state culture with decreasing amounts of Todd-Hewitt broth. The synthesis continued for at least 480 generations at *p*H 7.3 and with a generation time of 84 min. Glucose was the limiting nutrilite and the concentration of reducing agents in the medium was critical. The M protein was identified by gel diffusion against type-specific antisera from the Communicable Disease Center and from R. Lancefield. The yield of M protein obtained from organisms grown in the continuous-culture device was comparable to that from standard broth stationary cultures.

In a previous paper, Davies, Karush, and Rudd (5) investigated the amino acid utilization of a type 4-derived group A streptococcus growing under steady-state conditions in a continuous-culture device with a completely synthetic medium. The type 4 streptococcus was a nonproducer of M protein. Since the M protein is the antigen which confers type specificity upon the group A streptococci and is associated with virulence, it was of interest to establish the growth conditions in a chemically defined medium which would allow its synthesis. There have been many attempts to grow group A streptococci that produce M protein in such a medium. The most recent one was reported by Mickelson and Slade (20) and Mickelson (19), who found that, after repeated subculturing in a protein- or peptide-free medium, the streptococci lacked M protein. The same strains, Richards (type 3), N19 (type 19), and S43 (type 6), when grown on a medium that contained reduced ovalbumin, showed no loss of M protein (26). Fox and Krampitz (8) reported that nonproliferating streptococci required peptides for production of M protein.

The continuous-culture technique provides several advantages for the investigation of M-protein synthesis. In batch culture, essential nutrients must be in excess initially and then decrease in concentration, and metabolic products accumulate. In continuous culture, substrate concentration is controlled by the regulation of the rate at which medium is fed into the growth tube. Since utilization of many essential substrates varies with the age of the batch culture, the interpretation of data involving metabolic uptake is more reliable in steady-state culture because the latter is always in the exponential phase of growth. Another advantage of the steady-state system is that intracellular enzymes released by cell autolysis are held to a minimum due to the continuous renewal of the culture.

MATERIALS AND METHODS

Organism. Lyophilized samples of group A streptococcus S43 MV (type 6) were made available through the courtesy of the late Armine T. Wilson (Alfred I. duPont Inst. of the Nemours Foundation, Wilmington, Del.), and S43 FL (type 6) and T14/46/5 (type 14) were obtained through the courtesy of Rebecca C. Lancefield (The Rockefeller Univ.,

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New York). These cultures were grown for 18 hr in Todd-Hewitt (Difco) broth in which the glucose concentration had been increased by 1.75×10^{-2} M. Portions of the culture were quickly frozen by means of a dry ice-ethyl alcohol mixture and stored at −20 C. Ten-ml samples of the thawed culture were introduced into the growth chamber of the chemostat. The group A streptococcus originally described as type 4 strain H 44 was the same as that used earlier (5).

Serological typing was performed by the precipitin test (27) and by gel diffusion (22) with type 6 and 14 antisera obtained both from the Communicable Disease Center, Atlanta, Ga., and from Dr. Lancefield. In addition, unabsorbed antiserum to the chemostat-grown streptococci was prepared in Hare Brown and New Zealand Red rabbits with heat-killed streptococci according to the procedure of Lancefield (15).

M-protein purification. The method of extraction of M protein from the streptococci was the classical hot-acid precipitation procedure of Lancefield and Perlmann (16). After extraction of the crude antigen, further purification was carried out by incubation with crystalline ribonuclease (Sigma) and column chromatography with Sephadex G-25 (Pharmacia) and carboxymethyl-cellulose (CM-52; Reeves Angel) according to the procedure of Fox et al. (9, 10).

Medium. The medium used for the growth of type 6 streptococcus was slightly modified from that which was previously employed for the growth of type 4 streptococcus (5). The modifications were the presence of 4×10^{-4} M L-proline, a decrease in the concentration of thioglycolic acid to 9.3×10^{-3} M, and a 1.5-fold increase in the concentration of glucose to 3.75×10^{-2} M. The medium used for type 14 was the same as that used for type 6 with the presence of 1×10^{-3} M glutamine (Gallard Schlesinger Chemical Mfg. Corp., Carle Place, N.Y.). The medium was sterilized by pressure filtration through a 0.22-μ Millipore filter (Millipore Filter Corp., New Bedford, Mass.) on a stainless-steel pressure filter holder.

During the autoclaving of the Millipore pressure-filter holder and pad, it was found advisable to have an unbleached muslin ruff with one end tied around the neck of the bottle and the other end tied around the rubber stopper of the filter. In this way, the filter could be tilted during autoclaving, permitting an equal pressure of steam on both sides of the filter. Upon the release of the steam pressure, the filter could be placed in the upright position on the bottle without contamination.

Continuous-culture apparatus. The apparatus used was the continuous-culture device described earlier (13) and modified as described in a previous publication (5). The emergent suspension of bacteria was collected in a bottle immersed in an ethyl alcohol bath at 4 C. The cooling prevented further bacterial growth as evidenced by no additional lactate accumulation or turbidity increase. The volume in the growth tube was kept constant at 133 ml, and medium was supplied to the growth tube at the rate of 66.5 ml/hr. The *p*H was maintained at 7.3 ± 0.08 by a *p*Hstat which neutralized with 1.0 N NaOH the lactic acid produced by the culture. Figure 1 is a record of the maintenance of this steady-state *p*H.

Each of the amino acids in the control medium was present at a concentration approximately twice that required for maximal turbidity with the type 4 organism, for which type the glucose concentration was 2.5×10^{-2} M. Frequent measurements of turbidity of 5-ml samples of the effluent of the growth tube were made using a Klett colorimeter with a 540 filter. Under conditions of glucose limitation, the bacterial density was found by dry-weight measurements of washed cells to be 0.288 mg/ml of culture for a turbidity reading of 100 Klett units. Unless otherwise indicated, turbidities shown are steady-state values which remained constant for at least 24 hr.

RESULTS

Adaptation of type 6 group A streptococci to synthetic medium. The approach employed for the establishment of a steady state was to initiate continuous culture with Todd-Hewitt medium and then to replace it with defined medium containing supplemental and decreasing quantities of

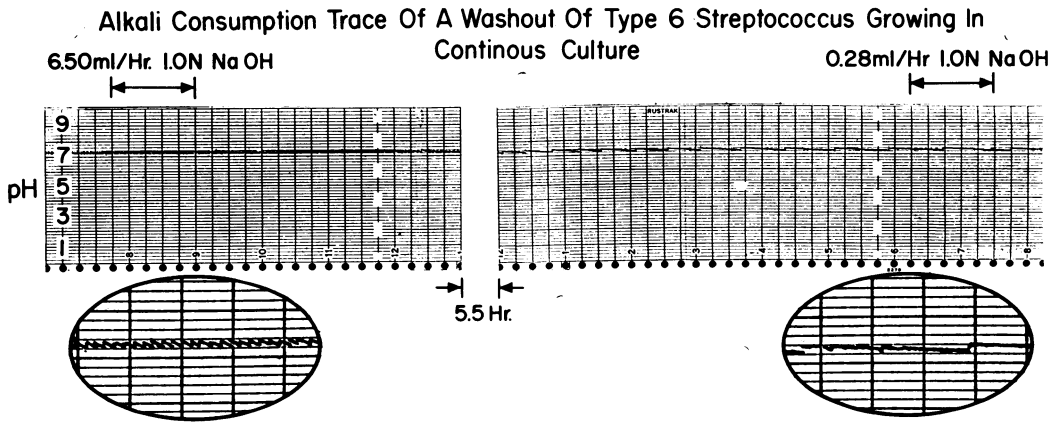

FIG. 1. *Comparison of the fluctuations of pH during steady state and transient state.*

Todd-Hewitt broth. Table 1 shows the steady-state turbidities of type 6 streptococci using this stepwise decrease of Todd-Hewitt broth, which is normally present at a concentration of 30 g/liter. Section A, representing 7 days of continuous culture, shows the result of an attempt at rapid adaptation. Each medium listed was fed into the growth tube for a minimum of 24 hr. Based on the experience described below with type 4 streptococci in a synthetic medium, the thioglycolate of the synthetic medium was used at a concentration of 9.3×10^{-3} M, and the temperature in the growth tube was maintained at 36 C. After 6 days of growth of the organism in the steady state, the Todd-Hewitt broth, which had been present at a concentration of 15 g/liter of synthetic medium, was entirely removed from the medium and a gradual decline of turbidity ensued. Figure 1 shows the effect of this withdrawal of Todd-Hewitt from the medium over a 10-hr period in terms of the fluctuation of *p*H resulting from the automatic addition of 1.0 N NaOH.

A successful adaptation was achieved with smaller stepwise decreases in Todd-Hewitt as seen in section B of Table 1, representing 12 days of continuous culture. Following the decrease of the Todd-Hewitt concentration from one-eighth to one-sixteenth of the normal level, there was a decrease of more than 50% in the steady-state turbidity. Adaptation occurred in steps, each taking approximately 24 hr to proceed to the next turbidity level. Subsequent to adaptation to the synthetic medium, the turbidity levels reached and remained at 325 units. The process of adaptation to a synthetic medium was repeated five times with type 6 streptococci. The apparatus was maintained in continuous operation with the type 6 organism for over 480 generations, and at that time the streptococci were still producing M protein. They appeared normal on phase-contrast examination, were typically β-hemolytic on human red blood plates, and grew normally when transferred to Todd-Hewitt broth.

The M protein obtained from the streptococci was trypsin-sensitive, and upon digestion could not be identified by a precipitin test (27) and gel diffusion (22) against type 6 antisera from the Communicable Disease Center and from Dr. Lancefield. The Ouchterlony pattern shown in Fig. 2 demonstrates the presence and type-specificity of the M protein in the culture. In the continuous-culture device on synthetic medium, 7 g (wet weight) of bacteria per liter was obtained, compared to 2 g (wet weight) of bacteria per liter

TABLE 1. *Adaptation of type 6 group A streptococcus to a chemically defined medium*

Medium	Day	Steady-state turbidity[a]
A- Todd-Hewitt 30.0 g/liter	1	170
Todd-Hewitt 30.0 g/liter + glucose 3.2 g/liter	2, 3	317
Todd-Hewitt 30.0 g/liter + glucose 7.0 g/liter	4	360
Defined + Todd-Hewitt 15.0 g/liter	5, 6	395
Defined	7	—[b]
B- Defined + Todd-Hewitt 7.50 g/liter	1	338
Defined + Todd-Hewitt 3.75 g/liter	2, 3	317
Defined + Todd-Hewitt 1.88 g/liter	4, 5, 6	134, 160, 300[c]
Defined + Todd-Hewitt 0.94 g/liter	7, 8	230
Defined (first 2 days)	9, 10	280
Defined	11, etc...	325

[a] Turbidity readings are in Klett units at 540 mμ; 100 Klett units is equivalent to 0.288 mg (dry weight) of cells per ml.

[b] No steady state.

[c] These are three values on 3 successive days which remained fairly constant for at least 8 hr.

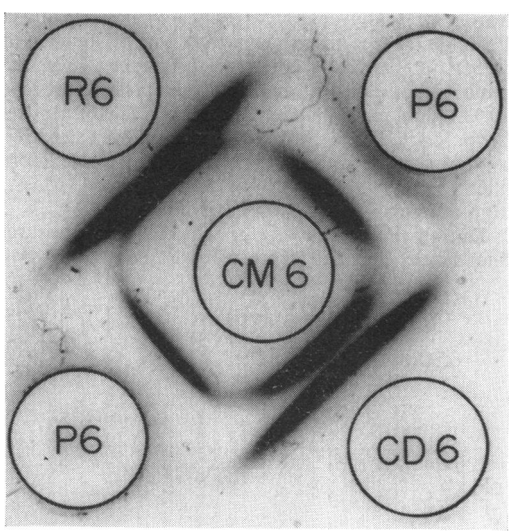

FIG. 2. *Gel-diffusion analysis of M protein from type 6 streptococcus grown in the steady state in synthetic medium. M protein in center well (CM6); R6: type 6 specific antiserum from Rockefeller University; CD6: type 6 specific antiserum from Communicable Disease Center; P6: polyvalent unabsorbed antiserum against type 6 streptococcus grown in the steady state on synthetic medium.*

from an 18-hr standard Todd-Hewitt broth in stationary culture. The yield of crude M protein (hot-acid extract) ranged from 5 to 6 mg/g of cells (wet weight) for both types of culture. In addition, the intensity of the specific precipitin reaction was approximately the same for both preparations.

In the presence of Todd-Hewitt broth (15 g/liter) in synthetic medium, a single colony of the type 6 streptococcus from a blood plate was sufficient for a successful seeding of the growth tube, and, in 24 hr, a steady-state turbidity of over 300 units was established. When only synthetic medium was used in the growth tube, 10 ml of inoculum (at a turbidity of 300 units) of organisms that had been maintained in the frozen state in synthetic medium was necessary for growth to occur within 24 hr. When the frozen organisms had been thawed and frozen two or more times, no growth was observed in the synthetic medium up to 72 hr. These bacteria could, however, be grown in synthetic medium to which Todd-Hewitt broth (15 g/liter) had been added. After 18 hr in this medium, the streptococci could be readapted over a period of 5 days to the synthetic medium. Postgate and Hunter (24) reported that recovery of bacterial populations damaged by freezing and thawing usually showed more viable bacteria when plated on a rich medium than on a poor one. The recovery of the streptococcal cells in Todd-Hewitt broth may be due to an additional requirement for the resynthesis of essential proteins denatured by freezing.

Growth of type 14 streptococci. Type 14 streptococci were also adapted to growth in continuous culture in synthetic medium by gradual withdrawal of Todd-Hewitt broth. However, the synthetic medium used for type 6 was able to support the steady-state growth of type 14 streptococci for only 24 hr, after which time the turbidity fell rapidly. The addition of 2×10^{-3} M glutamine prevented this decrease in turbidity, whereas 5×10^{-4} M glutamine was inadequate. Although McIlwain (17, 18) demonstrated the importance of glutamine in the nutrition of the streptococci, neither type 4 nor type 6 required glutamine for growth. The M protein from type 14 streptococci was also identified by double diffusion in gel with type-specific antiserum (Fig. 3) and did not cross-react with the type 6 antiserum, as is evident by the absence of a precipitin band.

Inverse temperature effect and its relationship to reducing agents. A difference was noted between the optimal temperature for the growth of type 4 streptococcus when grown in Todd-Hewitt broth and when grown in synthetic medium under certain conditions. Increasing the temperature from 32 to 37 C, which is the optimal temperature for

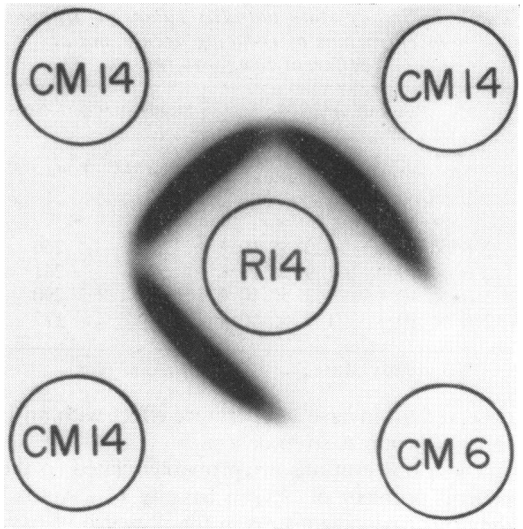

FIG. 3. *Gel-diffusion analysis. Type 14 specific, absorbed antiserum from Rockefeller University in the center well (R14). Outer wells contain M protein from streptococci grown in the steady state in synthetic media. CM14: M protein from type 14 streptococcus derived from three cultures harvested 5 days apart; CM6: M protein from type 6 streptococcus.*

the streptococci in broth, could decrease the steady-state turbidity values. This inverse temperature effect was related to the reducing agents present in the medium. At 37 C, with control levels of 2.0×10^{-3} M cysteine, 1.9×10^{-2} M thioglycolic acid was inhibitory, showing a 241 turbidity level against one of 290 at a concentration of 9.3×10^{-3} M thioglycolic acid (Table 2). A higher concentration of cysteine, 2.6×10^{-3} M, partially overcame this inhibition, resulting in a steady-state turbidity of 256. Lowering the temperature to 32 C also produced an increased turbidity of 264. The combined effect of increasing cysteine to 2.6×10^{-3} M and lowering the temperature raised the turbidity to 292. At 9.3×10^{-3} M thioglycolic acid, there was a direct temperature effect, as there was also at 1.9×10^{-2} M thioglycolic acid when the cysteine was decreased to 2.6×10^{-4} M. However, in the latter case, the inhibitory effect of the thioglycolic acid that was not compensated by high cysteine was reflected in either lowered turbidity or loss of the steady state. Nevertheless, in the absence of thioglycolic acid, no growth was observed. When glucose was added in high concentrations (13 g/liter) to Todd-Hewitt broth, the inverse temperature effect was again noted, e.g., 200 turbidity units at 37 C and 285 turbidity units at 32 C. At least part of the inhibitory effect of glucose may be due to its action as a reducing agent. Panos and Cohen (23) have

TABLE 2. *Steady-state turbidity values at different concentrations of reducing agents and at different temperatures*

Reducing agents		Steady-state turbidities	
Cysteine	Thioglycolic acid	Temp 32 C	Temp 37 C
M	*M*		
2.6×10^{-3}	1.9×10^{-2}	292	256
2.0×10^{-3}	1.9×10^{-2}	264	241
2.0×10^{-3}	9.3×10^{-3}	280	290
2.6×10^{-4}	1.9×10^{-2}	—[a]	177

[a] No steady state.

observed an inverse temperature effect with an L form of group A streptococci.

These observations are probably related to the general problem of oxygen toxicity (4). Autoxidation of reducing agents in the presence of oxygen has been shown to lead to the formation of hydrogen peroxide and probably of free radicals (1, 2, 3, 14). Gilbert (11) noted that a mixture of cobaltous ions and reduced glutathione, each of which autoxidizes to produce hydrogen peroxide, decomposed hydrogen peroxide much more rapidly than did either component separately. Similarly, in the presence of cysteine, part of the hydrogen peroxide formed by the autoxidation of thioglycolic acid and cysteine could be used to oxidize the thioglycolic acid and cysteine, thus decreasing the amount of hydrogen peroxide available for damage to the streptococci. In the presence of the higher thioglycolic acid concentrations, at the higher temperatures, more hydrogen peroxide and free radicals were probably being evolved, leading to a decrease in turbidity. Thus, increasing the cysteine could compensate for the inhibitory effects of the higher thioglycolic acid concentration. However, at very low cysteine concentrations in the presence of high thioglycolic acid levels at 32 C, there was a loss of the steady-state growth.

Effect of hemin on growth. In view of this apparent role of peroxide, it is probable that in most complex media group A streptococci are protected against peroxides by sulfhydryl groups of proteins or by heme compounds which are present in media containing blood or mammalian tissue infusions. Since heme compounds can act as peroxidases and catalases, we sought evidence for their presence in the chemostat cultures. No cytochromes, hemoglobin, heme-containing peroxidase, or heme-containing catalase could be detected in type 4 streptococci grown in the synthetic medium when examined with Britton Chance's low temperature sensitive spectrophotometer (7), with which it is possible to detect 10^{-10} moles/ml of these compounds (R. W. Estabrook, *personal communication*). Since the addition of some heme compound to the synthetic medium might make it possible to decrease the amount of reducing agents and thus the amount of hydrogen peroxide being formed from them, hemin or hematin was included in the synthetic medium.

The hemin or hematin (solubilized in 0.1 M Na_2HPO_4), at a final concentration of 5.3 mg/liter, was added to the medium before the sterile filtration. Table 3 shows that hemin protected the growth of type 4 streptococcus against the inhibitory effect of 1.9×10^{-2} M thioglycolic acid, especially when the cysteine concentration was low (2.6×10^{-4} M). However, decreasing the thioglycolic acid to 9.3×10^{-3} M in the absence of hemin or hematin resulted in higher turbidities. The addition of hemin to the medium containing 9.3×10^{-3} M thioglycolic acid did not cause an increase in turbidity. Hematin differed from heme in that it was slightly inhibitory. Its presence without thioglycolic acid could not support steady-state growth.

DISCUSSION

The continuous-culture technique for bacterial growth has important advantages over batch methods for the growth of streptococci. Not only can the substrate concentrations be controlled by regulating the rate at which fresh medium is fed to the culture, but deleterious metabolic end products are maintained at constant and relatively low levels. Although preformed peptides have been implicated in growth and protein production of the streptococci (8, 20, 26), the results with the continuous culture technique show that no peptides are necessary for either growth of streptococci or M protein production. The one possible

TABLE 3. *Effect of heme compounds on growth of type 4 streptococcus in synthetic medium*

Thioglycolic acid	Heme compound (5.3 mg/liter)	Cysteine	Steady-state turbidity
M		*M*	
	Hemin		
1.9×10^{-2}	Yes	2.0×10^{-3}	265
1.9×10^{-2}	No	2.0×10^{-3}	241
1.9×10^{-2}	Yes	2.6×10^{-4}	260
1.9×10^{-2}	No	2.6×10^{-4}	177
9.3×10^{-3}	Yes	2.6×10^{-4}	265
9.3×10^{-3}	No	2.0×10^{-3}	290
	Hematin		
—	Yes	6.0×10^{-4}	—[a]
9.3×10^{-3}	Yes	6.0×10^{-4}	258
9.3×10^{-3}	No	6.0×10^{-4}	282

[a] No steady state.

exception noted was subsequent to repeated freezing and thawing, when complex medium was required during the lag phase. Recovery from freezing may necessitate additional factors for resynthesis of essential structures damaged by the freezing.

Another advantage of growing organisms in continuous culture is that the redox potential can easily be poised at different levels and oxygen poisoning held to a minimum. However, the growth of these organisms without the addition of proteins or peptides necessitates a careful adjustment of reducing agents. Since reducing agents can autoxidize to form peroxides (1, 2, 3, 14), it is necessary to decrease the inhibitory effect of these agents by keeping their concentrations at a minimum and determining what mixtures are the least inhibitory. In batch cultures, there can be no easy poising of the redox system, since reducing agents must be present initially in excess and the concentration of oxidized products continuously increases.

An inverse temperature effect, similar to that shown in streptococci in the presence of relatively high thioglycolic acid concentrations, has been described in *Paramecium* grown in high-oxygen pressures (28). The same mechanism of oxygen toxicity due to hydrogen peroxide formation appears to be operating in the present case. In these terms, the implication is that the increased rate of formation of hydrogen peroxide with increased temperature is not adequately compensated for by the increased rate of synthesis of sensitive components.

It has been noted (25) that increasing concentrations of ascorbic acid inhibited the growth of batch cultures of a group A streptococcus. With respect to a related observation involving the partial protection of *Achromobacter* P6 against oxygen by ascorbate, it was concluded that this effect appeared unrelated to the reducing power of ascorbate, since the protection did not increase with increasing ascorbate (12). When ascorbate in the presence of oxygen is recognized to be both a reducing agent and a hydrogen peroxide-generating agent whose efficiency depends on other compounds present, these apparent inconsistencies become understandable.

A further possible benefit is provided by the fact that the microorganisms in these experiments have been in the growth tube at 36 C for a mean residence time of only 2 hr, determined by the ratio of the culture volume to the flow rate (21). This feature limits the extent to which the streptococcal proteins, in particular the M proteins, are acted upon by the proteinases (6) which are produced by the streptococci, whereas, in stationary systems, the organisms are grown at 37 C for approximately 18 hr and may be subjected to more extensive proteolytic action.

The media commonly used for the growth of group A streptococci contain proteins, peptides, and hemes from mammalian tissue infusions. Since the antigenic specificities of the streptococci are of great interest in relation to the "delayed sequelae" of streptococcal infection, i.e., rheumatic fever and glomerulonephritis, the steady-state growth of these organisms in synthetic media should greatly simplify the comparison of their specificities with those of mammalian tissues.

Thus, the steady-state system permits an investigation of the streptococci and their products under stable defined growth conditions, which include minimal exposure to proteolytic and autolytic activities without contamination from preformed peptides and proteins that may be carried along in some media.

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