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Effect of Diethylaminoethyl Dextran on the Growth of *Mycoplasma* in Agar

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The growth of certain strains of *Mycoplasma* is inhibited by substances present in commercial agar preparations. The addition of diethylaminoethyl (DEAE) dextran (10 mg per 100 ml) to agar media appears to enhance the growth of some strains. Of eight strains initially tested, the presence of DEAE dextran grossly enhanced the growth of three strains. One strain appeared not to be affected, and a clearly enhancing effect was not evident with four strains. Quantitative studies revealed that growth enhancement varied from 10 colony-forming units (CFU) for *M. hominis* type II (strain Campo) to $10^{3.3}$ CFU for *M. pulmonis* (strain 880). The growth-enhancing effect is probably due to the ability of DEAE dextran to bind the sulfated polysaccharide moieties in agar and not to the DEAE dextran, per se.

Several investigators have shown that sulfated polysaccharides normally present in agar can inhibit the growth of certain mutants of poliovirus (12), encephalomyocarditis virus (13), dengue virus (11), herpes simplex virus (10, 14), western equine encephalomyelitis virus (1, 15), and other arboviruses (3, 9). These inhibitory effects were prevented by the addition to agar of certain polycationic substances (1, 9, 13, 15), such as diethylaminoethyl (DEAE) dextran and protamine. The available data indicate that the inhibitor reacts directly with the virus particles, preventing further virus-cell interaction (3, 4). A unique feature of the growth of *Mycoplasma* species on solid media is their characteristic growth into the agar. The present study was designed to investigate the effect of the agar inhibitor on the growth of *Mycoplasma* species in solid medium.

MATERIALS AND METHODS

Mycoplasma strains. *M. gallisepticum* S6 and *M. arthritidis* Jasmin were obtained from American Type Culture Collection. Michael F. Barile provided *M. gallinarum* PG-16, *M. hominis* type II, strain Campo [shown by some investigators (5) to be identical or very closely related to *M. arthritidis*], and *M. pulmonis* PG-34. *M. gallisepticum* A-1 was obtained from Joseph P. O'Malley; *M. pulmonis* 880, from James T. Grace, Jr.; and *M. pneumoniae* FH-Liu, from the Research Reference Reagents of the National Institutes of Allergy and Infectious Diseases.

Stock cultures of *Mycoplasma* were grown in medium A (Table 1) from which agar was omitted. The cultures were harvested at the time of optimal growth, dispensed in 1-ml samples, sealed in glass ampoules, and stored at −70 C. These stock *Mycoplasma* cultures were thawed just prior to use and were considered to be undiluted at this point in the experiments.

Medium. Our initial experiments were performed on several different media, all of which were modifications of a previously described medium (2). Table 1 describes the composition of the media used in these experiments.

DEAE dextran. DEAE dextran was obtained from Pharmacia Laboratories, Uppsala, Sweden, and was used in the agar media at a final concentration of 10 mg per 100 ml. At this concentration, the sulfated agar polysaccharides (SAPS) are effectively bound and their effects are prevented (6).

RESULTS

Experiments were performed to determine what visible effect DEAE dextran had upon the growth of *Mycoplasma* cultures on agar media. One drop each of undiluted culture and of 10^{-2} and 10^{-4} dilutions of stock *Mycoplasma* culture was placed on the surface of the agar. After the drop had dried, the plates were inverted and incubated in an humidified incubator with an atmosphere of 5% CO_2 and 95% air. Plates were examined daily under a compound microscope at 31 × magnification by an observer who was unaware of the composition of the medium.

Figure 1 demonstrates the enhancing effect of DEAE dextran on the growth of *Mycoplasma*. The growth of some strains appeared to be markedly enhanced by DEAE dextran; others were only moderately enhanced. Table 2 gives a listing of the strains used in these experiments, a

visual estimate of their growth on eight preparations of media, and our conclusions as to whether growth was enhanced by the presence of DEAE dextran. The presence of DEAE dextran enhanced the growth of three of the eight *Mycoplasma* strains tested. One strain appeared not to be affected, and clearly enhancing effects were not evident with four strains. In no instance did growth appear to be better in agar not containing DEAE dextran.

Media C and F contained Agarose (Mann Research Laboratories, New York, N.Y.), a preparation of agar which has had most of the sulfated polysaccharides removed. Although Agarose-containing media C and F appeared to support *Mycoplasma* growth better than did regular agar-containing media A, D, and G, the growth-enhancing properties of Agarose did not equal those of regular agar plus DEAE dextran (media B and E). With *M. pulmonis* 880, Agarose possessed no growth-enhancing properties.

Experiments were performed to obtain more quantitative measurement of the enhancing effect of DEAE dextran. Serial 10-fold dilutions of *Mycoplasma* stock cultures were made in a complete liquid medium similar in composition to that used in growing the initial stock cultures. A measured portion (0.1 ml) of each dilution was delivered on the surface of two agar plates. The first plate contained medium A (without DEAE dextran), and the latter plate contained medium B (with DEAE dextran).

Colonies were counted by careful and systematic observation at 31 × magnification, starting on the 2nd day postinoculation and continuing every other day thereafter until the last reading on day 10 to 12. The last reading was always performed on plates which had been flooded with 1 to 2 ml of a 1:100 dilution of Dienes' stain (8). The *Mycoplasma* colonies which were readily stained could be clearly observed. This method allowed confirmation of any questionable colony by direct observation at a higher magnification. Although slightly more laborious, we have found this method of titration on solid medium followed by direct counting of stained colonies to be most reliable, reproducible, and accurate as a *Mycoplasma* assay system.

Table 3 lists the strains of *Mycoplasma*, the titers of colony-forming units (CFU), and the differences in titers observed on media with and without DEAE dextran. Differences in titer of CFU varied from 10 for *M. hominis* type II, strain Campo to $10^{3.3}$ for *M. pulmonis* 880. Although there was no difference in titer of the S6 strain of *M. gallisepticum* on the two media, the individual colonies appeared larger on the DEAE dextran medium (Fig. 2D). In no experiment was the titer higher in the medium not containing DEAE dextran.

DISCUSSION

A unique feature of the growth of *Mycoplasma* species on solid media is their characteristic growth into the interstices of the agar. This intimate association could make these delicate or-

TABLE 1. *Composition of media*

Components	Quantity	Medium							
		A	B	C	D	E	F	G	H
PPLO broth[a] + 1% dextrose	70 ml	+[b]	+	+	−	−	−	−	−
PPLO broth + 1% dextrose and 0.002% phenol red	70 ml	−	−	−	+	+	+	−	−
Purified agar[a]	1.2 g	+	+	−	+	+	−	−	−
Agarose	1.0 g	−	−	+	−	−	+	−	−
DEAE dextran	10 mg	−	+	−	−	+	−	−	+
PPLO agar[a]	70 ml	−	−	−	−	−	−	+	+
Yeast extract[c]	10 ml	+	+	+	+	+	+	+	+
Horse serum[d]	20 ml	+	+	+	+	+	+	+	+
Penicillin G	500 units per ml	+	+	+	+	+	+	+	+

[a] Difco Laboratories, Detroit, Mich.
[b] Presence (+) or absence (−) of a particular component in the medium.
[c] Fresh yeast extract, 25% (2).
[d] Agamma horse serum, Hyland Laboratories, Los Angeles, Calif.

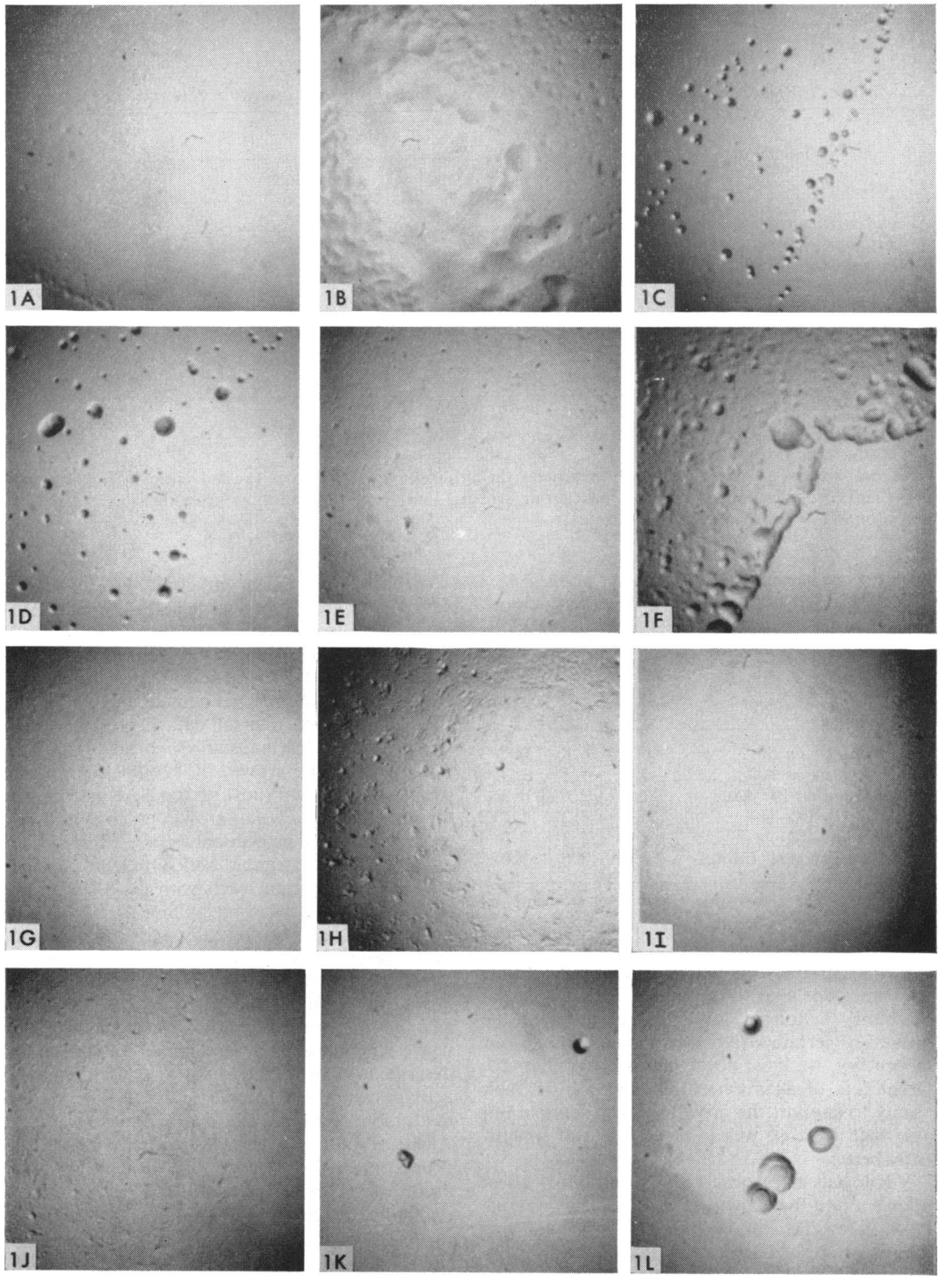

FIG. 1. *Enhancing effect of DEAE dextran upon the growth of Mycoplasma colonies on different agar media. All photographs (taken at 31 × magnification) demonstrate the growth of Mycoplasma colonies on agar inoculated with the undiluted stock culture. Each Mycoplasma strain is shown growing on media without DEAE dextran and on media with DEAE dextran (10 mg per 100 ml). (A and B) M. gallisepticum S6 on medium A, without DEAE dextran (W/O), and on medium B, with DEAE dextran (W), respectively. (C and D) M. pneumoniae FH-Liu on medium D (W/O) and E (W), respectively. (E and F) M. pulmonis PG-34 on medium D (W/O) and E (W), respectively. (G through J) M. pulmonis 880 on medium A (W/O), B (W), D (W/O), and E (W), respectively. (K and L) M. hominis type II, Campo on medium A (W/O) and B (W), respectively. Although the number of colonies was almost the same on both media, the individual colonies appeared much larger on medium B, which contained DEAE dextran.*

TABLE 2. *Visible comparison of growth of Mycoplasma species on several different agar media*

Organism	Media[a]								Growth enhancement by DEAE dextran
	A	B[b]	C[c]	D	E[b]	F[c]	G	H[b]	
M. gallisepticum S6	±[d]	4	2	1	3	3	2	4	yes
M. gallisepticum A-1	4	4	4	2	3	3	4	4	?
M. pneumoniae FH-Liu	2	2	2	1	2	2	2	4	?
M. pulmonis PG-34	2	4	3	1	4	3	1-2	3	yes
M. pulmonis 880	2	4	1	±	3	±	1	2	yes
M. hominis type II, Campo	±S	±L	±	0	±	0	±L	±L	?
M. gallinarum PG 16	4	4	4	4	4	4	4	4	no
M. arthritidis Jasmin	±	±	0	0	±	0	0	0	?

[a] Media A through H described in text.
[b] Media containing DEAE dextran.
[c] Media containing Agarose.
[d] Comparison of growth of each strain on the different agar media is graded from 1 to 4, where 4 represents luxurious growth for a particular strain; ± = very few colonies growing; L = large size of colonies; S = small size of colonies.

TABLE 3. *Quantitative determination of the effect of DEAE dextran on growth of Mycoplasma species in agar medium*

Organism	DEAE dextran		Difference in titer
	Without	With	
M. gallisepticum S6	6.8[a]	6.8	0
M. gallisepticum A-1	3.4	5.1	1.7
M. pneumoniae FH-Liu	2.3	2.6	0.3
M. pulmonis PG-34	6.0	7.2	1.2
M. pulmonis 880	2.0	5.3	3.3
M. hominis type II, Campo	2.6	3.6	1.0

[a] Log_{10} colony-forming units per 0.1 ml of inoculum.

ganisms extremely vulnerable to any unfavorable constituent of agar. Lynn et al. (7) observed the inhibitory action of some lots of agar on the growth of certain strains of *Mycoplasma*. In our laboratory, we have observed not only that different lots of agar varied considerably in their ability to support the growth of *Mycoplasma* but also that Agarose was less variable and consistently better.

Virologists have observed the inhibitory effect of agar upon the growth of certain virus mutants (1, 3, 9–15). Takemoto et al. (13) attributed this inhibition to SAPS moieties present in conventional agar preparations and have prevented these inhibitory effects by the addition to agar of certain polycationic substances which would combine with and "neutralize" the SAPS.

The experiments described in this paper show that the growth of certain strains of *Mycoplasma* appears better in agar containing DEAE dextran. This growth enhancement could be due either directly to a growth-promoting property of DEAE dextran per se or indirectly to an effect of DEAE dextran. Our data do not completely exclude the possibility that DEAE dextran per se might have some *Mycoplasma* growth-enhancing properties. We feel, however, that the data suggest that conventional agar has substances, probably SAPS, which inhibit the growth of *Mycoplasma*. Agarose, which has had most of the SAPS removed, manifested *Mycoplasma* growth-enhancing properties. A similar growth-enhancing effect occurs when DEAE dextran is added to conventional agar. DEAE dextran reacts with the SAPS moieties, forming a visible precipitate. The resulting preparation lacks biologically active SAPS, and, in this respect, it is similar to Agarose from which the SAPS has been extracted chemically.

We made no attempt to identify further the agar inhibitory factor. However, in two respects it appears to be quite similar to the substances described in the virology literature: the inhibitory activity can be prevented by DEAE dextran, and the inhibitory effect is reduced in Agarose.

Our results show that the growth-enhancing properties of the single lot of Agarose which we used in these experiments did not equal those of regular agar plus DEAE dextran. Different lots of commercial Agarose vary in the amount of SAPS remaining after chemical extraction. In a preliminary experiment, we demonstrated that the lot of Agarose used in this study contained a small amount of SAPS.

We are unable to explain the differences in the effect of DEAE dextran between the two strains of *M. gallisepticum* and between the two strains

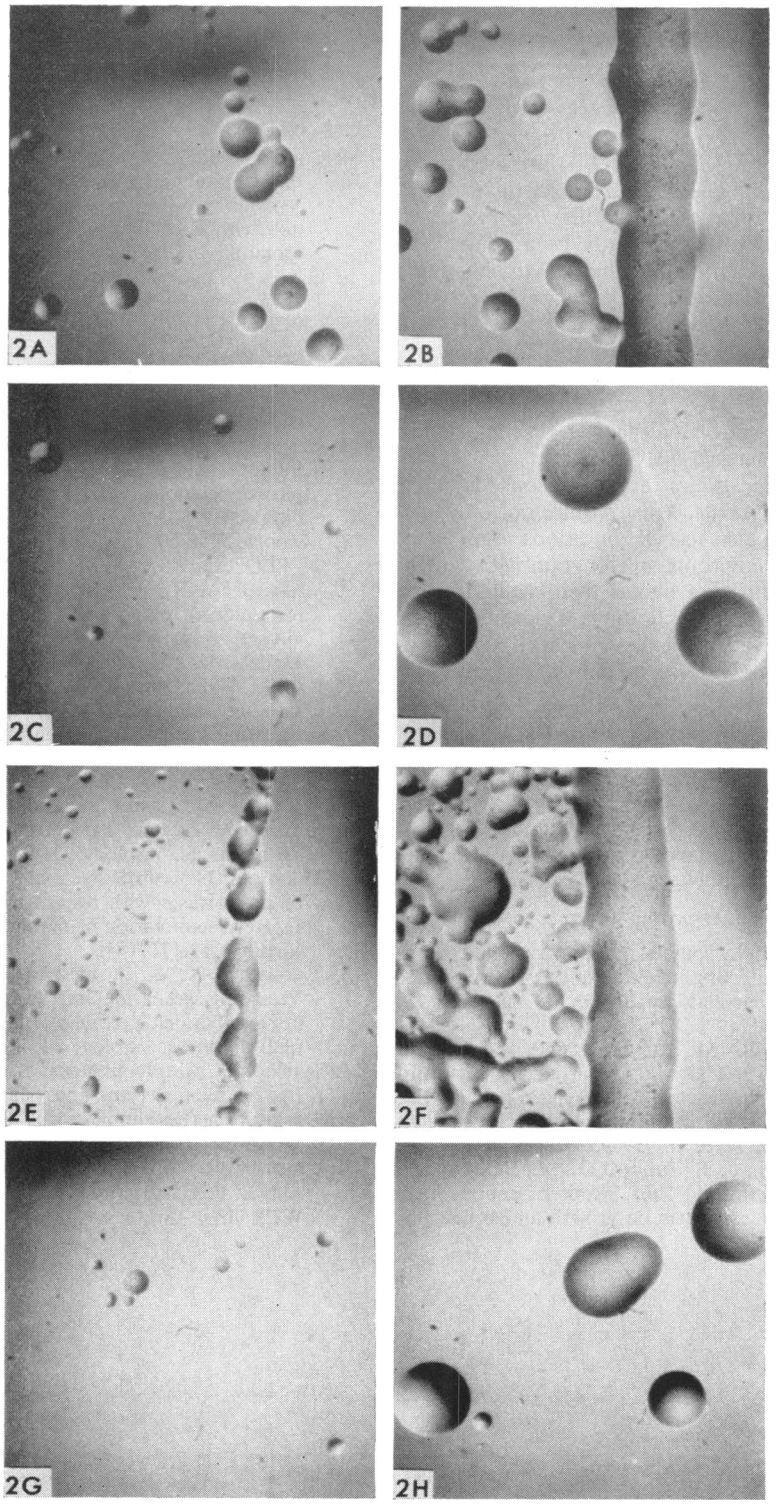

FIG. 2. *Growth of Mycoplasma colonies on agar with and without DEAE dextran. All photographs are taken at 31 × magnification. (A and B) M. gallisepticum S6, 10^{-3} dilution, on medium A, without DEAE dextran (W/O), and on medium B, with DEAE dextran (W), respectively. (C and D) M. gallisepticum S6, 10^{-4} dilution, on medium A and B, W/O and W, respectively. Although the titer is identical with both media, the size of the individual colonies is much larger on medium B with DEAE dextran. (E and F) M. pulmonis PG-34, 10^{-3} dilution, on medium A and B, W/O and W, respectively. (G and H) M. pulmonis PG-34, 10^{-4} dilution, on medium A and B, W/O and W, respectively. The titer is higher and the individual colonies larger on medium B with DEAE dextran.*

of *M. pulmonis* (Table 3). However, the passage history of these strains is interesting. *M. gallisepticum* S6 and *M. pulmonis* PG-34 are laboratory strains which have been cultivated on artificial media for many years. *M. gallisepticum* A-1 and *M. pulmonis* 880 have recently been adapted to grow on artificial media. If the growth-enhancing effect of DEAE dextran is greater with recently isolated strains, then addition of DEAE dextran to agar might enhance isolation of *Mycoplasma* from clinical specimens. However, additional experiments would have to be performed to determine the effect of DEAE dextran on the primary isolation of *Mycoplasma* on agar.

Mycoplasma species are fastidious in their growth requirements. Most require not only special media but also special conditions of moisture and gaseous environment for optimal growth. Their delicate nature makes them vulnerable to toxic substances. The findings reported in this paper may have practical significance in the isolation of *Mycoplasma* from clinical specimens in which the titers of living organisms are likely to be low. Enhancement of growth in these situations might mean the difference between a negative attempt and a positive isolation.

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