JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1983, p. 1167–1173
0095-1137/83/111167-07$02.00/0

Vol. 18, No. 5

Utility of Egg Yolk Medium for Cultivation of *Mycoplasma pneumoniae*

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Received 4 April 1983/Accepted 8 August 1983

Chicken egg yolk broth medium containing 10% (vol/vol) phosphate-buffered saline extract of egg yolk instead of horse serum showed better growth-promoting activity for *Mycoplasma pneumoniae* than did Chanock medium and was effective in promoting the growth of small numbers of *M. pneumoniae*. Moreover, the phosphate-buffered saline extract of egg yolk proved superior to horse serum in the following respects: (i) it was consistent in growth-promoting activity for *M. pneumoniae* among different batches; (ii) it could be preserved at 4°C for at least 2 years; (iii) it is inexpensive and easy to obtain; and (iv) it contains large amounts of lipoproteins, cholesterol, and phospholipids.

Mycoplasmas require cholesterol for growth. Among the many mammalian sera, horse serum (HS) is widely used as an excellent cholesterol source in the cultivation of mycoplasmas, but the growth-promoting ability of HS for mycoplasmas often differs considerably among different lots of serum. For diagnosis of *Mycoplasma pneumoniae* infection, the quality of HS has a direct effect on the isolation of *M. pneumoniae*, and thus it is important to obtain HS of good quality. In addition, when large quantities of mycoplasmal antigen are to be prepared, the use of HS becomes costly.

Many investigators have tried to find other materials to replace HS in the growth medium for mycoplasmas (8, 10, 11, 24, 26). Among the many substitutes tried, chicken egg yolks have been found by Hayatsu (8), Jensen et al. (10), and Tamura et al. (24) to be one of the most suitable. They have used egg yolks extracted with organic solvents or with distilled water for cultivation of mycoplasmas.

Considering the instability of lipoproteins in egg yolks, which are the natural cholesterol source for the growth of mycoplasmas in vitro (16), it may be undesirable to denature the lipoproteins with organic solvents. Although native lipoproteins are not always necessary to sustain the growth of mycoplasmas (4, 25), it is difficult to dissolve the lipid components separated from the lipoproteins with organic solvents in the medium, and these components are not as effective in promoting the growth of mycoplasmas as is HS (8, 10). Although the growth-promoting ability of the water extract of egg yolks is comparable to that of HS (8, 24), the extract is unstable and increases in turbidity during long periods of storage.

In the present study, we investigated the efficacy of the supernatant of a chicken egg yolk suspension in phosphate-buffered saline (referred to as EY) for cultivation of *M. pneumoniae* by comparing EY-containing medium with the standard medium reported by Chanock et al. (2), which contains HS. We found that EY was stable over a long period and that its growth-promoting ability was superior to that of HS.

MATERIALS AND METHODS

Mycoplasmas. The FH and Mac strains of *M. pneumoniae* have been maintained for many years in our laboratory. Organisms grown in seed culture medium were stored at −20°C until use.

Preparation of EY. Fresh unfertilized chicken eggs (White Leghorn) were disinfected in 0.1% (wt/vol) Hibitane digluconate solutions (Sumitomo Chemical Industries, Ltd., Osaka) for 10 min and air dried. The egg yolks were suspended in sterile phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS(−)] at a concentration of 20% (wt/vol). After being stirred thoroughly, the suspension was centrifuged at 15,000 × *g* for 40 min. Penicillin G was added to the supernatant fluid to a concentration of 500 IU/ml. This supernatant fluid is referred to as EY. After passing tests for absence of mycoplasmas and for growth-promoting activity for *M. pneumoniae*, EY was stored at 4°C in glass vessels with rubber stoppers.

Yeast extract. Yeast extract was prepared by heating a 25% (wt/vol) aqueous suspension of Nitten Dry Yeast (Nippon Beet Sugar Manufacturing Co., Ltd., Tokyo) in a boiling water bath for 30 min; it was stored at −20°C until use (14).

Media. Basal broth medium consisted of 2.1% (wt/vol) PPLO broth (lot 671893, Difco Laboratories, Detroit, Mich.), 1% (wt/vol) glucose, and 0.002% (wt/vol) phenol red. Basal agar medium consisted of 2.1% (wt/vol) PPLO broth and 1.2% (wt/vol) Wako special agar B (Wako Pure Chemical Industries, Ltd., Osaka).

The seed culture medium consisted of 87% (vol/vol) basal broth medium and 13% (vol/vol) serum fraction for mycoplasma (Kitasato Institute, Tokyo). The agar medium for colony counting consisted of 77% (vol/vol) basal agar medium, 13% (vol/vol) serum fraction for mycoplasma, and 10% (vol/vol) yeast extract.

The broth media to be tested for their growth-promoting activity for *M. pneumoniae* were as follows: HS medium consisted of 80% (vol/vol) basal broth medium and 20% (vol/vol) HS (lot 29211115; Flow Laboratories, Rockville, Md.). Chanock medium consisted of 70% (vol/vol) basal broth medium, 20% (vol/vol) HS, and 10% (vol/vol) yeast extract (2). EY medium consisted of 90% (vol/vol) basal broth medium and 10% (vol/vol) EY. EYY medium consisted of 80% (vol/vol) basal broth medium, 10% (vol/vol) EY, and 10% (vol/vol) yeast extract. A single lot of each ingredient was used for all the test media.

Separation of lipoprotein fractions in EY. The method used was essentially that of Havel et al. (7). Lipoproteins in EY were separated sequentially into four fractions of increasing density (d): fraction 1 (Fr. 1), d was <1.019; Fr. 2, d was 1.019 to 1.063; Fr.3, d was 1.063 to 1.12; and Fr. 4, d was 1.12 to 1.21. The density of the background salt solution was adjusted to 1.019 by the addition of solid NaCl. The EY solution was ultracentrifuged at 34,500 rpm for 20 h in a Beckman SW 41 Ti rotor and cellulose nitrate tubes (14 by 89 mm) without caps. The top layer (Fr.1) was reemulsified in PBS(−), the solvent density of the infranatant was adjusted to 1.063 with NaCl, and ultracentrifugation was repeated. The top layer (Fr. 2) was reemulsified in PBS (−), and Fr.3 was obtained in the same manner. Fr.4 was obtained by the addition of solid NaBr in place of NaCl. All fractions were dialyzed for 24 h against 1,000 volumes of PBS(−) containing penicillin G at 500,000 IU/liter. After dialysis, each fraction was sterilized by filtration through a 0.45-μm filter (Millipore Corp., Bedford, Mass.).

Assessment of mycoplasmal growth on agar media. The surfaces of agar plates to be tested for their growth-promoting activity were spotted with 10 0.01-ml portions of each of serial dilutions of a mycoplasmal suspension. In 10 spots in which an appropriate dilution was inoculated, the number of colonies was determined and expressed as the log CFU per milliliter of the original suspension.

The diameters of 12 colonies randomly selected in one spot which contained about 30 to 70 colonies were microscopically measured and expressed as the division of the micrometer. One division of the micrometer equaled 35 μm.

Assessment of mycoplasmal growth in the test broth media. After inoculation of 1 ml of an appropriate dilution of seed culture into 200 ml of each of the test broth media, the media were dispensed in 15-ml quantities into test tubes with cotton plugs and incubated at 37°C. Every day or two, one tube was taken from each group of the test media, four 0.01-ml portions of serial dilutions of the culture in the tube were inoculated onto the agar plates for colony counting, and the pH of the medium in the tube was sometimes measured. The concentration of the viable organisms in the broth culture was expressed as the log CFU per milliliter.

Chemical analysis. Protein and total lipids were determined by the method of Gornall et al. (6) and the method of Pande et al. (15), respectively. Total cholesterol and free cholesterol were determined by enzymatic methods with the commercial kits Cholesterol C-Test (Wako) and Free Cholesterol C-Test (Wako), respectively. Phospholipids were determined by an enzymatic method with the commercial kit Phospholipids B-Test (Wako).

RESULTS

Transparency of EY medium. Many granules and lipid lumps were contained in the egg yolk-PBS(−) suspensions before centrifugation. When such a suspension was added to the basal agar medium, it was impossible to distinguish the mycoplasmal colonies from the lipid lumps dispersed in the agar medium. However, these lipids and granules were easily removed from the suspension by centrifugation.

The supernatant fluid obtained at a centrifugal force of 5,000 × *g* for 40 min contained very few lipid lumps, and no lipid lumps were found in the supernatant fluid obtained at 7,500 × *g* or higher. Moreover, with increases in centrifugal force, clearer supernatant fluids were obtained. Accordingly, the conditions for centrifugation were fixed at 15,000 × *g* for 40 min. EY agar and EY broth media supplemented with 10% (vol/vol) EY were sufficiently transparent.

Preservation of EY. Attempts to preserve EY by freezing or lyophilization were completely unsuccessful, owing to the denaturation of lipoproteins. When EY was stored frozen for a long time, insoluble debris formed as a result of dissociation of lipoproteins, and it was difficult to distinguish colonies from this insoluble debris in EY agar medium. However, EY stored at 4°C for 2 years showed good growth-promoting activity, equal to that of freshly prepared EY, and was free from precipitates (data not shown).

Growth of *M. pneumoniae* on agar media. Various amounts of EY or HS were added to the basal agar medium with or without 10% (vol/vol) yeast extract. After inoculation with the *M. pneumoniae* strains FH and Mac, the plates were incubated aerobically for 12 days, and the number and diameters of colonies were determined (Fig. 1). There were no distinct differences in the log CFU on the plates supplemented with 5% (vol/vol) or more EY or HS. The colony diameters for strain FH were markedly increased by the addition of yeast extract to the medium containing HS, but the colony diameters for neither strain were increased by the addition of yeast extract to the medium containing EY.

Growth of *M. pneumoniae* in broth media. The growth curves of *M. pneumoniae* in each of the test broth media are presented in Fig. 2. A close correlation was observed between the changes in pH of each medium and the CFU in the same medium. The *M. pneumoniae* strains showed

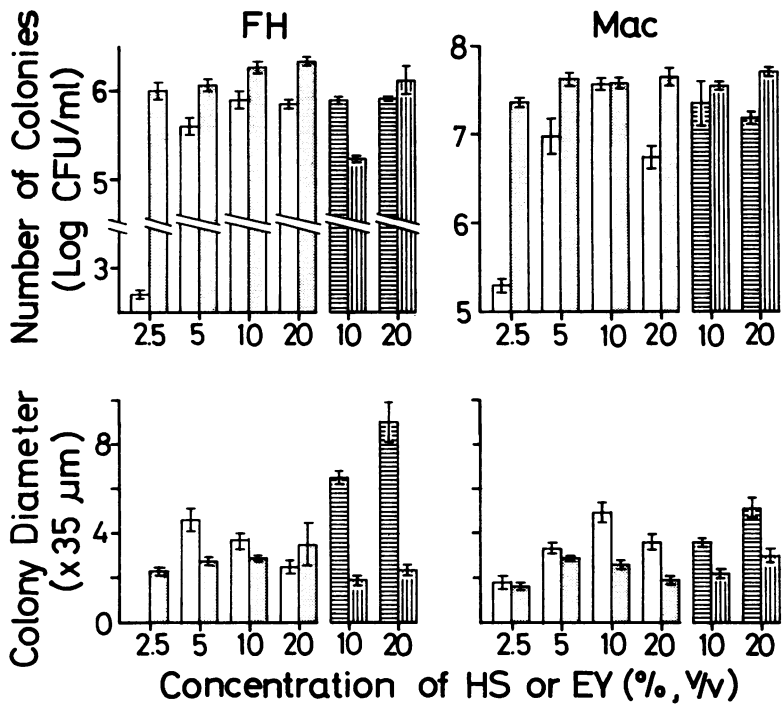

FIG. 1. Growth of *M. pneumoniae* strain FH and Mac on agar media containing various concentrations of HS or EY. Basal agar medium was supplemented with HS (□), HS and 10% (vol/vol) yeast extract (▤), EY (▦), and EY and 10% (vol/vol) yeast extract (▥). The number of colonies formed in a spot on agar plates is expressed as the log CFU recovered per milliliter of a mycoplasmal suspension inoculated on the agar plates, and each column on the upper graphs represents a mean of the values enumerated in 10 spots. Each column on the lower graphs represents the mean diameter of 12 colonies in a spot. Bars indicate 95% confidence intervals of the means.

the most rapid growth in EY medium. In Chanock medium and HS medium, the CFU on the first day after inoculation decreased sharply below the number of CFU in the inoculum. Consequently, the time until maximum growth in these media was longer than that in EY medium. In EYY medium, strain FH showed good growth, whereas the growth of strain Mac was poor.

Relationship between inoculum size and the growth of *M. pneumoniae*. Growth of the FH and Mac strains in the four test broth media was determined by the pH changes in the medium (Fig. 3). At each inoculum size, both strains showed a more rapid growth in EY medium than in any other medium, as shown by an earlier pH change. EY medium was especially effective in promoting the growth of small numbers of *M. pneumoniae* organisms. In addition, EY medium always showed higher growth-promoting activity for both strains than did EYY medium.

When the chanock medium and HS medium were inoculated with concentrations of organisms of about 40 to 60 CFU/ml or lower, no changes in pH could be observed during the 2-week incubation period, but in EY medium, both FH and Mac strains grew even when inoculated at 4 and 6 CFU/ml, respectively.

Protein and lipid content of EY and HS. Table 1 shows the protein, phospholipid, and cholesterol content of EY and HS. EY was characterized by high quantities of free cholesterol and phospholipids, which were about eight times higher than those in HS. In contrast, the amounts of protein and esterified cholesterol in EY were about one-fourth and one-half of those in HS, respectively.

Effect of each lipoproteins fraction of EY on the growth of *M. pneumoniae*. Figure 4 shows the distribution of protein, total lipids, and total cholesterol among the four lipoprotein fractions in EY. Low-density lipoprotein fractions, Fr. 1 and Fr. 2, constituted most of the lipoproteins present in EY.

To investigate whether there are differences in the growth-promoting activity for *M. pneumoniae* strain Mac among the lipoprotein fractions, each fraction was added to the basal agar medium to give the cholesterol concentration or the volume equivalent to that of EY added at a concentration of 2% (vol/vol), which was found by a preliminary experiment to be the minimum

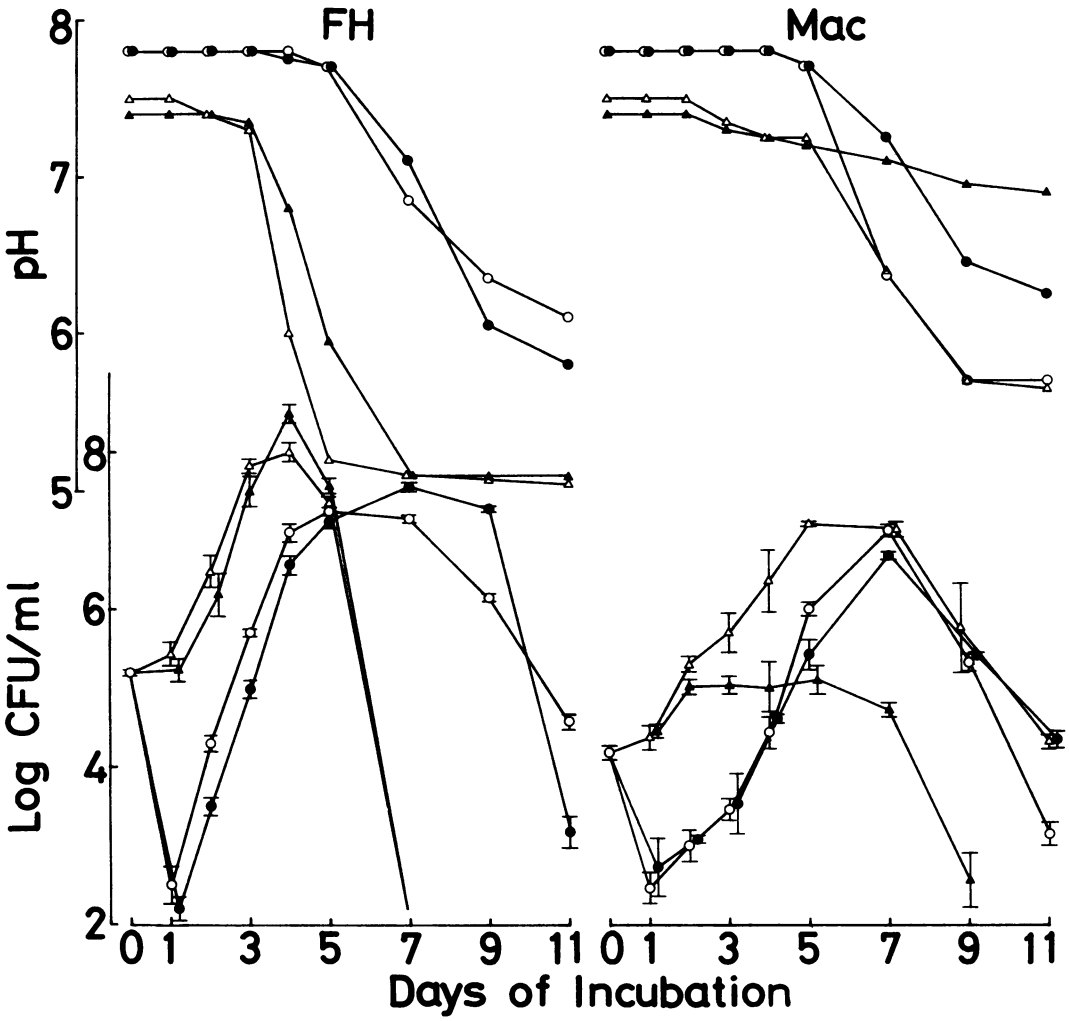

FIG. 2. Growth curves of *M. pneumoniae* strains and pH changes in the four test broth media. The mean value of log CFU per milliliter measured in four spots is represented by each point, and the 95% confidence interval of the mean is indicated by each bar. Symbols: ○, HS medium; ●, Chanock medium; △, EY medium; ▲, EYY medium.

concentration required to yield good growth of *M. pneumoniae* (data not shown).

When cholesterol was supplied at that minimal amount or more to the basal medium by the EY lipoprotein fractions, there were no significant differences in the growth of *M. pneumoniae* on the agar media containing the different fractions (Fig. 5).

DISCUSSION

M. pneumoniae showed better growth in EY broth medium than in Chanock broth medium. EY medium was especially effective in promoting the growth of small numbers of *M. pneumoniae*. In addition, the growth-promoting activity of EY for *M. pneumoniae* was consistent among different EY batches (data not shown). One of the reasons for the good growth-promoting activity of EY medium for *M. pneumoniae* may be the absence of toxic substances in EY. Generally, *M. pneumoniae* is sensitive to HS, and its growth is often markedly affected by HS. As shown in Fig. 2, in Chanock medium, the CFU after incubation for 1 day decreased from that of the inoculum, which may be due to the presence of toxic substances in HS (9, 23). In our experience, the degree of inhibitory effect of HS on the growth of *M. pneumoniae* differed considerably among different lots of HS, and the inhibitory effect was decreased to a great extent by heating the serum at 55°C for 30 min. Therefore, the toxic substance in HS may be proteinaceous.

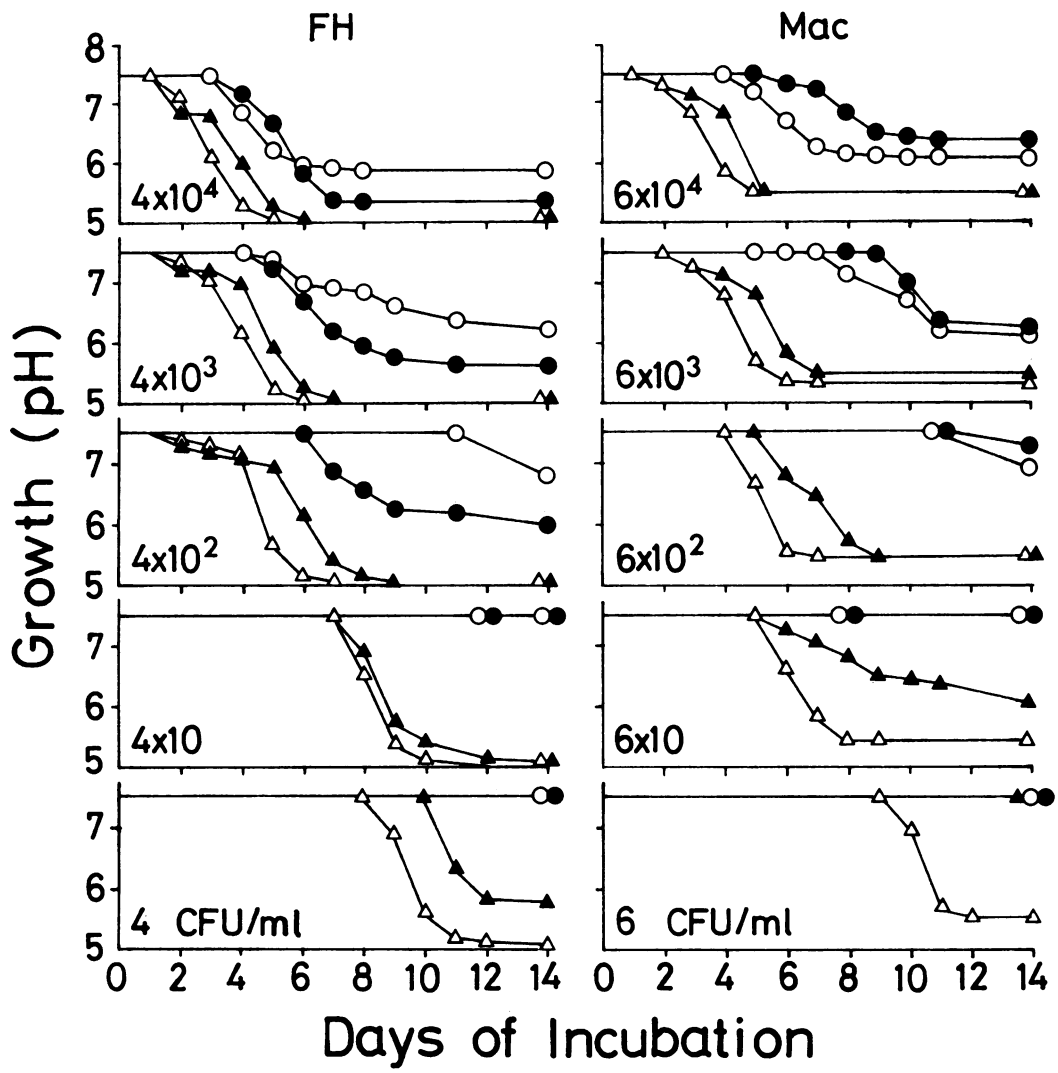

FIG. 3. The relationship between the inoculum concentration and the growth of *M. pneumoniae* strains in the four test broth media. Inoculum concentrations are indicated on each graph. Symbols: ○, HS medium; ●, Chanock medium; △, EY medium; ▲, EYY medium.

Another possible explanation for the good growth-promoting activity of EY medium for *M. pneumoniae* is the high content of lipoproteins rich in cholesterol and phospholipids. It is well known that serum lipoproteins are the natural cholesterol source for animal mycoplasmas in vitro and in vivo and that the growing cells of mycoplasmal species take up significant quantities of exogenous cholesterol and phospholipids into their plasma membrane from the medium (5, 16, 17).

HS is much richer in esterified cholesterol than in free cholesterol; in contrast, EY contained much more of the latter, about eight times that of HS. Razin and co-workers (16, 17, 21) reported that mycoplasmal organisms incorporate more free cholesterol than esterified cholesterol into their plasma membranes during growth in a serum-containing medium. Therefore, a high content of phospholipids and free cholesterol in EY may be favorable for the growth of *M. pneumoniae*.

Most of the lipoproteins in mammalian sera belong to the high-density lipoprotein class (18, 26, 27), whereas those in EY belong to the low-density lipoprotein class. Smith et al. (22) reported that alpha-1 lipoprotein (high-density class) in bovine serum is responsible for the growth of *Mycoplasma* spp., and Sayed et al. (20) also reported that alpha lipoprotein in equine serum had growth-promoting activity for *Ureaplasma urealyticum*. However, in the case

TABLE 1. Protein, lipid, and cholesterol contents of HS and EY

Component	Quantity (mg/dl)		
	HS[a]	EY[a]	EY/HS[b]
Protein	8,393 (7,598–8,827)[a]	2,017 (1,606–2,427)	0.24[b]
Total lipids	325 (264–385)	2,247 (2,135–2,358)	6.91
Phospholipids	116 (103–128)	1,018 (927–1,108)	8.77
Total cholesterol	110 (97–122)	186 (157–214)	1.69
Free cholesterol	16 (11–20)	131 (126–135)	8.18

[a] The quantities expressed are the means of triplicate to quintuplicate determinations on one sample; numbers in parentheses indicate 95% confidence limits of the mean.

[b] These values are the ratio of the amount of each component in EY to that in HS.

of EY, the growth-promoting activity for *M. pneumoniae* was independent of the class of lipoprotein.

Surprisingly, when yeast extract was added to EY medium, the growth of *M. pneumoniae* was decreased. We obtained similar results in an experiment with 35 clinical isolates (manuscript in preparation). Therefore, in cultivating *M. pneumoniae* strains, it is not necessary to add yeast extract to EY medium. Considering the troublesome preparation of yeast extract, this is an advantage of EY medium. Yeast extract is an important constituent of the media for growth of some mycoplasma strains (3, 12), but little is known about the nature of growth-promoting factors in yeast extract (19). We showed that colony size of *M. pneumoniae* became larger by the addition of yeast extract to agar medium containing HS, but in liquid media containing HS, the effect of yeast extract on the growth of *M. pneumoniae* was not clear. It is well known that the quantitative relationship between the inoculum concentrations of *M. pneumoniae* and the number of colonies formed on the agar media without addition of yeast extract is poor. These results suggested that yeast extract plays an important role in agar media rather than in liquid media for growth of *M. pneumoniae*.

In harvesting the organisms by centrifugation, it is necessary to give sufficient attention to pH changes in EY medium: the growth of *M. pneumoniae* in EY medium caused a rapid drop in pH, and a large amount of deposit resulting from EY was coprecipitated with the organisms collected from the culture at pH values below 5.5. When the culture was centrifuged before the death phase, we were able to collect the organisms with minimum contamination with these deposits. On the other hand, the early and rapid decrease in the pH value makes EY medium a favorable medium for the metabolic inhibition test, because a clear-cut endpoint was obtained early (manuscript in preparation).

Although we have never found any mycoplas-

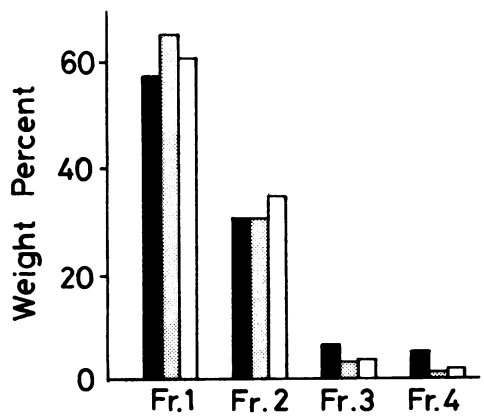

FIG. 4. Distribution of protein, total lipids, and total cholesterol among the different lipoprotein fractions in EY. Fr. 1, d < 1.019; Fr. 2, d = 1.019 to 1.063; Fr. 3, d = 1.063 to 1.12; Fr. 4, d = 1.12 to 1.21. Symbols: ■, protein; ▦, total lipids; and □, total cholesterol. The weight percentage of each component in each fraction was calculated as the ratio of the amount of the component in each fraction to the total amount of the component in the four fractions. Values are the means of triplicate determinations.

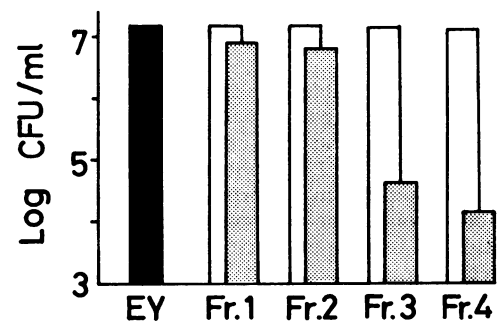

FIG. 5. Effect of each lipoprotein fraction in EY on the growth of *M. pneumoniae* Mac. Each fraction was added to the basal agar medium to give the cholesterol concentration (□) or the volume (▦) equivalent to that of EY added at a concentration of 2% (vol/vol) (■). Growth is expressed as the log CFU per milliliter of undiluted inoculum, calculated from the colony count.

mal contamination in EY preparations obtained only by centrifugation, it is well known that mycoplasmas are transmitted to progeny through eggs laid by infected chickens (1, 13). Therefore, we recommend that EY be sterilized by filtration and tested for mycoplasmal contamination. We can obtain a clear filtrate easily by the same method as that used for the preparation of yeast extract (14).

As antibiotics added to poultry feed contaminate eggs, it is desirable to use eggs from flocks not receiving antibiotic-fortified feed. If this is not possible, EY should be tested for its growth-promoting activity before use, as the growth of mycoplasmas might be affected by antibiotics carried in EY.

The results of the present study show that EY is useful as a substitute for serum in culture media for mycoplasmas. EY medium is useful for cultivation of laboratory strains, for preparation of mycoplasmal antigen free from contamination with HS proteins, and as a metabolic inhibition test medium. Furthermore, EY medium was found to have higher growth-promoting activity than Chanock medium for 35 new clinical isolates (manuscript in preparation). This finding suggests the possible usefulness of EY medium as primary isolation medium. We are currently comparing EY medium and conventional media for direct recovery of *M. pneumoniae* from clinical specimens.

ACKNOWLEDGMENTS

We are grateful to M. Kurokawa for his helpful discussions and to K. Akama for his valuable comments on the manuscript.

LITERATURE CITED

1. **Calnek, B. W., and P. P. Levine.** 1957. Studies on experimental egg transmission of pleuropneumonia-like organisms in chickens. Avian Dis. **1**:208–222.
2. **Chanock, R. M., L. Hayflick, and F. Barile.** 1962. Growth on artificial medium of an agent associated with atypical pneumonia and its identification as a PPLO. Proc. Natl. Acad. Sci. U.S.A. **48**:41–49.
3. **Edward, D. G. ff.** 1947. A selective medium for pleuropneumonia-like organisms. J. Gen. Microbiol. **1**:238–243.
4. **Edward, D. G. ff., and W. A. Fitzgerald.** 1951. Cholesterol in the growth of organisms of the pleuropneumonia group. J. Gen. Microbiol. **5**:576–586.
5. **Efrati, H., S. Rottem, and S. Razin.** 1981. Lipid and protein membrane components associated with cholesterol uptake by mycoplasmas. Biochim. Biophys. Acta **641**:386–394.
6. **Gornall, A. G., C. J. Bardawill, and M. M. David.** 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177**:751–766.
7. **Havel, R. J., H. A. Eder, and J. H. Bragdon.** 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. Clin. Invest. **34**:1345–1353.
8. **Hayatsu, D.** 1978. Acquired immunity to *Mycoplasma pneumoniae* in hamsters. Microbiol. Immunol. **22**:181–195.
9. **Hayatsu, E., M. Yoshioka, K. Kihara, and S. Arima.** 1966. Growth inhibitor of mycoplasma in normal horse serum. Med. and Biol. **73**:141–145. (In Japanese.)
10. **Jensen, K. E., L. B. Senterfit, R. M. Chanock, C. B. Smith, and R. H. Purcell.** 1965. An inactivated *Mycoplasma pneumoniae* vaccine. J. Am. Med. Assoc. **194**:134–138.
11. **Johnson, J. K., and N. L. Somerson.** 1980. Cholesterol as a limiting factor in the growth of *Mycoplasma pneumoniae*. Appl. Environ. Microbiol. **40**:391–399.
12. **Lemcke, R. M.** 1965. Media for the Mycoplasmataceae. Lab. Pract. **15**:712–715.
13. **Lin, M. Y., and S. H. Kleven.** 1982. Egg transmission of two strains of *Mycoplasma gallisepticum* in chickens. Avian Dis. **26**:487–495.
14. **Ogata, M., T. Ohta, A. Obara, and I. Z. Pan.** 1967. Investigation on growth media for mycoplasma: evaluation of infusions, peptones, sera, yeast extracts and other supplements. Jpn. J. Vet. Sci. **29**:259–271.
15. **Pande, S. V., R. P. Khan, and T. A. Venkitasubramanian.** 1963. Microdetermination of lipids and serum total fatty acids. Anal. Biochem. **6**:415–423.
16. **Razin, S., H. Efrati, S. Kutner, and S. Rottem.** 1982. Cholesterol and phospholipid uptake by mycoplasmas. Rev. Infect. Dis. **4**(Suppl.):85–92.
17. **Razin, S., S. Kutner, H. Efrati, and S. Rottem.** 1980. Phospholipid and cholesterol uptake by mycoplasma cells and membranes. Biochim. Biophys. Acta **598**:628–640.
18. **Robie, S. W., C. H. Janson, S. C. Smith, and J. T. O'Connor.** 1975. Equine serum lipids: lipid composition and electrophoretic mobility of equine serum lipoprotein fractions. Am. J. Vet. Res. **36**:1715–1717.
19. **Rylance, H. J., W. Marr, M. G. Malcolm, S. M. Stewart, and B. P. Marmion.** 1979. Growth factors for *Mycoplasma pneumoniae* in yeast and tissue extracts. J. Appl. Bacteriol. **47**:341–345.
20. **Sayed, I. A., and F. W. Sweat.** 1982. High-density lipoprotein that supports *Ureaplasma urealyticum* growth. J. Bacteriol. **151**:629–635.
21. **Slutzky, G. M., S. Razin, I. Kahane, and S. Eisenberg.** 1977. Cholesterol transfer from serum lipoproteins to mycoplasma membranes. Biochemistry **16**:5158–5163.
22. **Smith, P. F., J. G. Lecce, and R. J. Lynn.** 1954. A lipoprotein as a growth factor for certain pleuropneumonialike organisms. J. Bacteriol. **68**:627–633.
23. **Smith, P. F., and H. E. Morton.** 1953. Nature of growth inhibitor in some mammalian sera for pleuropneumonia-like organisms of human origin. Proc. Soc. Exp. Biol. Med. **83**:65–69.
24. **Tamura, H., S. Kuramasu, and Y. Tajima.** 1975. Efficacy of egg yolk medium in cultivation of *Mycoplasma gallisepticum*. Jpn. J. Microbiol. **19**:463–466.
25. **Washburn, L. R., J. H. Hughes, and N. L. Somerson.** 1978. Mycoplasma growth factors in bovine serum fraction. J. Bacteriol. **135**:818–827.
26. **Washburn, L. R., and N. L. Somerson.** 1979. Lipoproteins as substitutes for serum in mycoplasma culture medium. J. Clin. Microbiol. **10**:586–589.
27. **Wendlandt, R. M., and C. L. Davis.** 1973. Characterization of bovine serum lipoproteins. J. Dairy Sci. **56**:337–339.