TABLE I. Susceptibility of Human Embryonic Skin-Muscle Cultures to the MEF_1 Strain of Poliomyelitis.

Virus dilution (log)	0	1	2	3	4	5	6	No virus
Results	2/2*	2/2	2/2	3/3	3/3	2/3	1/3	0/3

* Denominator represents number of tubes inoculated with 0.1 ml virus suspension; numerator, the cultures showing degeneration in 3 days.

ducing cytopathogenic effects in a cultured strain of skin-muscle fibroblasts.

The thin clot technic has been successful with all the tissues so far studied, including adult human uterus and testicle, and embryonic skin-muscle, liver, lung and kidney. It appears, however, that for the optimum growth of each cell strain that may be studied, the nutritional requirements, the pH range and other factors will need to be established. Thus, for example, embryonic skin-muscle fibroblasts seem to be more easily maintained in roller tubes than in stationary cultures, whereas testicular and uterine cells may be maintained under either condition. Also, skin fibroblasts appear to maintain undiminished vigor in nutrient fluid containing chick embryo extract, whereas uterine fibroblasts seem to do better in beef embryo extract. On the other hand, it appears that while fibroblasts of testicular origin can be cultured initially in fluid containing chick embryo extract, their rate of growth eventually declines and can be restored to normal by substitution of beef embryo extract.

Summary. A technic is described by which human tissues may be established readily *in vitro* either on glass or in thin plasma clots. Susceptibility to infection with the MEF_1 strain of poliomyelitis is retained after prolonged cultivation.

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L Type Cultures Isolated from Streptococci.*† (20424)

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Pleuropneumonia-like colonies and larger colonies of similar structure corresponding to L type colonies of various bacteria grow usually in cultures made from the human mouth, especially from tooth scrapings, if bacterial growth is inhibited by penicillin. The observations to be described originated from an effort to determine the origin of these colonies and their eventual connection with bacteria. Numerous colonies of various types of bacteria were picked from anaerobic mouth cultures and were tested for the production of L forms. None of the bacteria produced L type colonies similar to those of the oral pleuro-

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pneumonia-like organisms, but larger colonies similar to those seen in the plates directly inoculated with tooth scrapings were produced by an alpha-hemolytic streptococcus (strain 40). Another streptococcus strain with similar properties (Ch 45) was isolated later from the tonsillar exudate of a patient with a sore throat.

Streptococcus *strain 40* grows on horse blood agar plates in small alpha hemolytic colonies consisting of pairs and short chains. Broth cultures consist of long chains. The coccus is not bile-soluble and is not heat-resistant. It is moderately sensitive to penicillin. Eight consecutive transfers of isolated colonies did not change the properties of the culture, including the production of L forms. The peculiarity of the strain becomes apparent on ascitic agar plates. The colonies develop to uneven size and consist of pleomorphic chains in which some of the cocci swell to large round bodies of 5 μ or more. No L type colonies develop on this medium. A similar pleomorphism develops on horse serum agar if growth is inhibited by penicillin, but here some of the large bodies grow into L type colonies.

The growth of these *L colonies* is markedly influenced by the composition of the medium. It is helped by dextrose, starch, gelatin, and low concentrations of glycine. Animal serum is not necessary, but horse serum makes the growth more abundant. Ascitic fluid and rabbit serum are inhibitory. Anaerobic cultivation is more favorable than aerobic. The most favorable medium found thus far is hard nutrient agar containing 1% gelatin, 2% soluble starch, 0.25% glycine and 10% horse serum. To induce transformation of the cocci into L forms, 200 to 1,000 units of penicillin are deposited at one site on the inoculated plate in a small trough cut into the agar. The plates are incubated anaerobically using Fortner's biological method. Microscopical L type colonies become visible after 24 hours. They require 3 to 5 days for full development. The culture is transferred by a piece of agar cut out with colonies and smeared over a fresh plate. After a few transfers the culture grows abundantly both with and without horse serum. It grows as well without as with high concentrations of penicillin. Growth has never been observed in liquid media. These L type cultures remained stable when transferred every few days on horse serum agar even if penicillin was absent. However, when the cultures were serially transferred on dextrose agar without horse serum and without penicillin, and especially when the plates were kept for several weeks, the cocci reappeared in the cultures. In one case the L forms were cultivated for more than two months in the presence of high concentrations of penicillin (about 1000 units per ml). After a few transfers without penicillin, the cocci reappeared. As in the case of other L forms, it is probable that the cocci were reproduced from the L forms. The cocci could not survive through many transfers on penicillin plates. They were sensitive to penicillin when they were recovered from the L forms. They were never seen in microscopic preparations made from the L cultures and did not grow when the L cultures were transferred to broth. The coccus recovered from the L forms was similar in every respect to the original culture and produced L colonies when exposed to penicillin. This is rare in streptococci and indicates that the recovered strain was identical with the original and was not a contamination. The L type cultures were similar in appearance and morphology to the L forms of other bacteria (1). They were most similar in appearance to the L type cultures of *Salmonella*. The L forms were derived from the cocci in the same way as in the case of other bacteria. The cocci grew to flat, large bodies, sometimes of very large size (10 to 20 μ) and small granules started to grow from these into the medium. Photograph 2 of Fig. 1 shows large bodies developed from the cocci before the growth of granules. The small L colony which developed from a large body shown in photograph 3 of Fig. 1 extends in many directions as strands of small granules into the medium. Photographs 4 and 5 show the appearance of the colonies in stained and unstained preparations. The L forms of streptococcus, like L forms of gram-positive bacilli, are gram-negative. Their morphology is illustrated in photographs 6 through 8 of Fig. 1 and in the electron micrographs in Fig. 2. Electron micrographs made of L type cultures of *Proteus*(2) show, in addition to the elements visible with

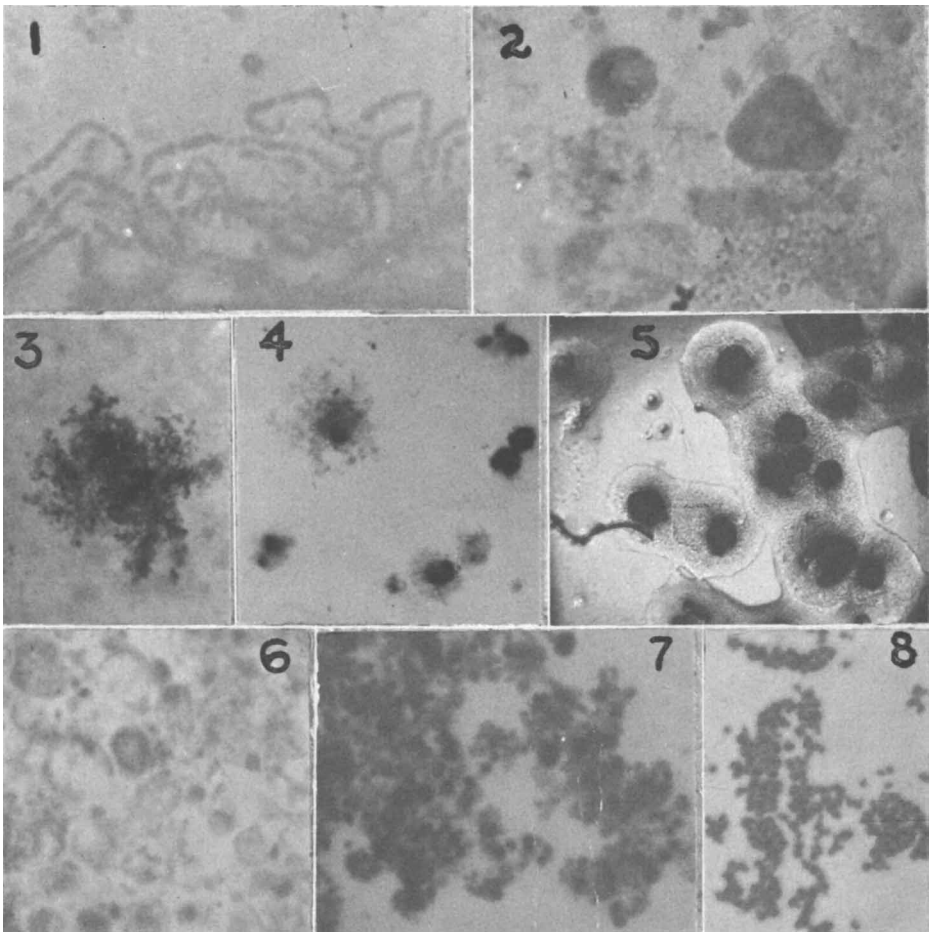

FIG. 1.

1. Edge of a small colony of streptococcus strain 40 on horse serum agar stained with methylene blue on the surface of the medium. × 2000.

2. Cocci and large bodies on an area exposed to penicillin after 24 hours' incubation. The majority of the large bodies are faintly stained and contain small granules. Stained on the surface of the medium. × 2000.

3. A young L-type colony spreading in the medium. Dried stained agar preparation with moderate magnification. × 900.

4. L-type colonies on starch-glycine horse serum plates, inoculated with strain 40 and exposed to penicillin. The colonies are of variable size and several have peripheral growth. Stained as in No. 1 and 2. × 100.

5. L-type colonies as in No. 4, developed to larger size but some remaining very small. Unstained. × 100.

6. Surface growth of L-type colonies suspended in water and stained with crystal violet. × 2000.

7 and 8. Material and staining similar to that in No. 6, but preparations dried. In the area shown in No. 8, the large bodies all disintegrated into small granules. × 2000.

the light microscope, granules 0.3 to 0.1 μ in diameter. Micrographs made from the L culture of streptococcus (Fig. 2) show similar forms. In some parts of the preparation, the size of the smallest granules is between 0.2 and 0.5 μ (Micrograph 3) but at certain places much smaller ones (0.1 to 0.2 μ) are also present. That granules of the size of 0.3 to 0.5 μ may be viable is indicated by the extension of the young colonies into the agar by such forms. The smallest granules (0.1 to 0.2 μ) are produced in the large bodies

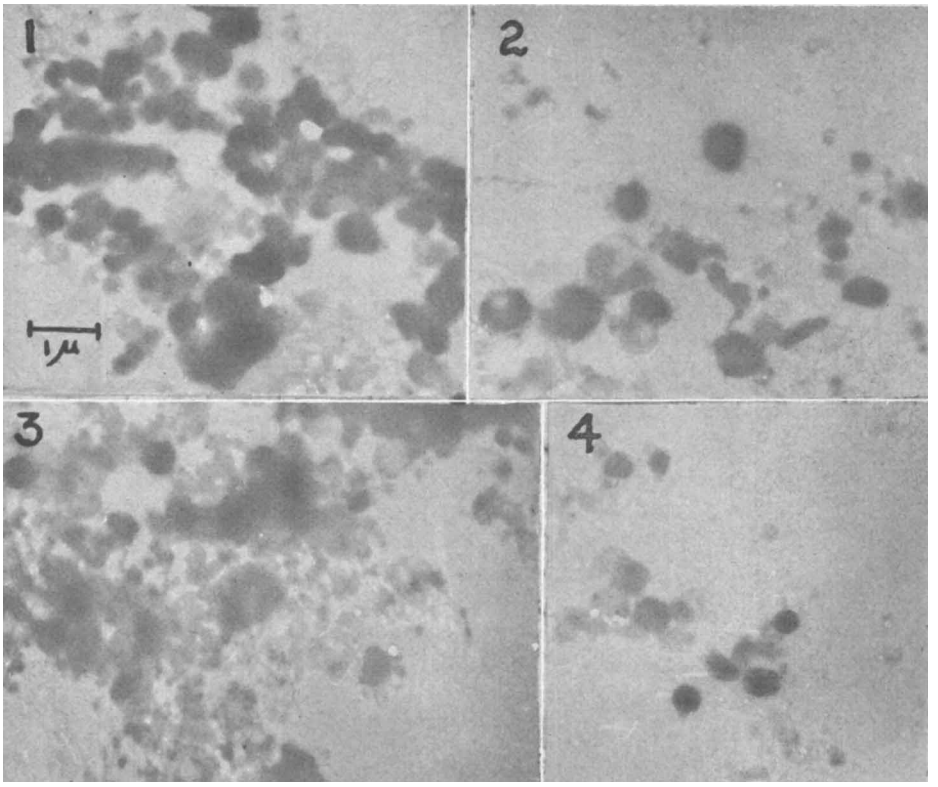

FIG. 2. Electron micrographs made from impressions from agar cultures. × 9000. Most organisms are between 0.5-1.0 μ, but many are considerably smaller. In micrograph 3, the small granules were probably produced by disintegration of larger forms. In micrograph 2, the density is uneven in several of the larger organisms.

and are freed by their disintegration. Nothing definite can be said at present about their nature.

A rabbit antiserum produced with the L strain agglutinated the L strain and the parent streptococcus. It did not agglutinate 3 other alpha-hemolytic streptococci or the L type cultures produced by streptococcus *strain Ch 45*. The L forms were not agglutinated by antisera produced against 2 heterologous coccus strains, or by antisera against oral strains of pleuropneumonia-like organisms. The serological similarity of the L forms and of the coccus is additional evidence for their essential identity.

Streptococcus *strain Ch 45* behaved essentially similarly to *strain 40*. It produced L type colonies under similar conditions, and the cocci reappeared in these in a similar way. However, marked differences were present between the 2 strains. The tendency to produce L forms decreased rapidly in *strain Ch 45* and at the same time the properties of the L cultures changed. The L colonies obtained immediately after isolation of the strains resembled the 3B L type colonies of *Proteus* and *Salmonella* (1). The organisms grew to large bodies not only on the surface but also in the depth of the colonies, and after a few days the cocci reappeared in many of the large bodies. The L colonies isolated somewhat later grew abundantly but reproduced cocci only occasionally in aging cultures. The viability of L type colonies produced by the coccus further decreased, and within a month after isolation the strain lost entirely the tendency to produce L forms and was not made pleomorphic by penicillin.

Many strains of alpha- and beta-hemolytic streptococci were picked from throat and genital cultures and examined for the production of L forms. Thus far only the two strains

described produced such forms. However, on plates inoculated from the mouth, colonies similar to the L type colonies of streptococcus often develop, occasionally in great numbers, in the area inhibited by penicillin. It is probable that they are produced by streptococci. Their appearance and size distinguish them from the oral pleuropneumonia-like organisms, and their growth in the absence of animal serum distinguishes them from the L type colonies sometimes produced in the plates by gram-negative bacilli. An L strain obtained from a mouth culture has been carried in our laboratory. It gives good agglutination with an anti-streptococcus rabbit serum, and on one occasion streptococci have been recovered from it. The connection between this streptococcus strain and the L type culture is at present under study. If the above-mentioned colonies develop from streptococci, they indicate that there are usually a few, and occasionally many, streptococci in the mouth which produce L type colonies. There is some evidence that most of these cocci lose the tendency to produce L forms after they start to multiply on artificial medium. If the penicillin is not put on the plates immediately after inoculation but after the bacteria multiply for a few hours, no L type colonies corresponding to those of streptococci will develop. This explains probably the difficulty in isolating streptococcus strains producing L forms. The variability of the tendency of different strains to produce L forms and the diminution of this tendency on cultivation have been observed in many other species producing L forms.

Discussion. The observation of L forms in streptococci adds a new important group of bacteria to those in which L forms have been observed previously. Their occurrence in streptococci is of special interest because of the claims repeatedly made for the production of filterable forms by these cocci. It is impossible to tell at present whether the observation of the L forms will confirm some of these claims.

The concept of L forms was derived from the observation that bacteria sometimes grow with a changed morphology consisting of small granules and large bodies. In the instances described in this paper, the L forms were grown in pure culture, and their identity with the bacteria is supported by good evidence. Recently Klieneberger-Nobel(3) and some other authors following her(4) designated as L forms granules and pleomorphic bacterial forms without offering evidence that these forms reproduce independently from the bacteria. Such use of the term "L form" can create only confusion because certainly not all pleomorphic forms, and especially not all granules visible in bacterial cultures, are analogous to L forms.

Summary. L cultures were isolated from two strains of alpha-hemolytic streptococci obtained from the human mouth. The appearance of these cultures and their morphology are similar to those of other bacteria, and they originated in a similar way from the parent streptococci. When exposed to certain influences, especially to penicillin, the cocci grew to large bodies and, on appropriate media, L type colonies developed from these. The L forms of streptococcus are not sensitive to penicillin and, like the L forms of gram-positive spore-bearing bacilli, do not require animal serum for growth. The cocci reappeared several times in cultures of the L form. Thus far, only two strains of streptococci could be induced to produce L forms. However, colonies similar in appearance and growth requirements to the L type colonies of streptococcus often develop on plates inoculated from the human mouth, suggesting that streptococci which produce L forms are often present in the mouth.

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