of adrenodemedullated rats whether they had thyroid glands or not, yet it is only the thyroidectomized-adrenodemedullated rats that succumb to reserpine. Indeed, Stern and Maickel(11) failed to show any effect of reserpine on plasma free fatty acids of adrenodemedullated rats housed at room temperature. Thus, temperature and thyroid status both play an important role in the response to reserpine. An explanation for the effect of thyroidectomy in the adrenodemedullated rat may come from the observation in several laboratories that thyroidectomy significantly reduces the lipolytic effect of epinephrine on adipose tissue(12,13). The fact that adrenodemedullated rats with intact thyroid glands survive pretreatment with reserpine while thyroidectomized-adrenodemedullated rats died may reflect the relatively severe impairment in the latter of the capacity to mobilize free fatty acids. The similarity of response of adrenodemedullated rats with and without thyroid glands to reserpine at 30° may well reflect the fact that as the temperature rises, thyroid function is diminished (14). At 30° the adrenodemedullated rats may be behaving like functionally thyroidectomized rats.

Summary. Reserpine increased hepatic glycogen in normal, hypothyroid, and adrenodemedullated rats but not in adrenalectomized or hypophysectomized rats. The elevation in plasma free fatty acids in fasted rats was impaired in reserpinized rats without adrenal medullas.

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Development and Growth of L Forms of Bacteria and PPLO on Membrane Filters.* (30772)

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Dr. J. R. Ward of Salt Lake City and Dr. C. W. Molander of Los Angeles called to our attention that the L forms of bacteria grow well on Millipore® membrane filters placed on appropriate solid media. Molander *et al* found that L cultures of staphylococcus grow through filters with pore sizes given by the manufacturer as 0.1 μ and 0.05 μ(1). We confirmed these observations and also observed that L forms develop from the bacteria on the filters. Thus far the development of L type growth from bacteria has been observed only on media solidified with agar, and not, with rare exceptions, on solid media of other type or in liquid media. The study of the growth in membrane filters

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promises to give some information on the role which the gel structure of the medium plays in the development and growth of L forms.

Materials and methods. We examined 3 brands of membrane filters: Millipore, Gelman and Schleicher and Schuell.† They behaved similarly in most respects. The results with Millipore filters were most clear cut, and these will be presented with more detail.

The filters are opaque and the development of growth in them cannot be seen without staining. Good staining can be obtained by first fixing the filters bearing the cultures on the surface of the medium with formalin vapors for 24 hours or longer. Without removing them from the agar, the filters are then covered with a few drops of a solution of 0.2% Azur II in water containing 10% maltose and about 0.1% tartaric acid. Good staining can also be obtained, but not as consistently, with an aqueous solution of safranin. After 10-15 minutes, while still on the agar, the filters are washed with a few drops of tap water, which is absorbed into the agar. The filters are then removed and placed on coverslips, culture side down, slightly pressed under 2 layers of filter paper and allowed to dry. When completely dry, they are cleared by xylol and mounted with Canada balsam.

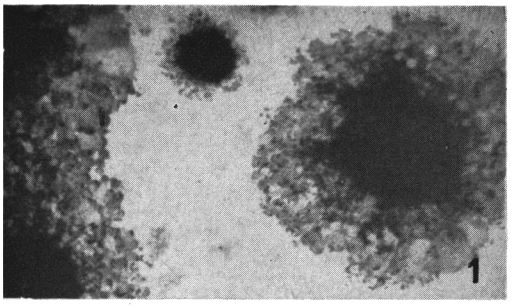
To be sure that the growth on the surface is not disturbed by the liquid stain, we also used a staining procedure, briefly indicated previously(2), in which the filters are gently pressed on a stained coverslip and allowed to dry before mounting.

We examined Millipore filters with pore sizes given by the manufacturer as 0.8 μ, 0.45 μ, 0.3 μ, 0.22 μ, 0.1 μ and 0.05 μ, respectively. They were tested with L cultures obtained from Proteus, Salmonella, Gram positive cocci, diphtheroids and from a Gram positive spore-bearing bacillus. We also tested several strains of PPLO, 2 from the human genitals and one from an epizootic of goats. Growth of L forms from bacteria in the presence of penicillin was tested with Proteus, Salmonella, *H. influenzae,* and the Gram positive spore-bearer. Pieces of filters 2-3 cm square were placed on the surface of appropriate solid media. They were inoculated with bacterial cultures using a loop. The L forms and PPLO were inoculated by pushing small blocks cut out from the agar cultures over the surface of the filters.

Results. All L forms and PPLO tested produced well-developed colonies on filters with pore sizes of 0.22 μ and higher, and none on 0.1 μ and 0.05 μ filters. After long incubation, from one to several weeks, growth developed in the medium under the 0.1 μ filters with PPLO and with the L forms of staphylococcus, streptococcus, diphtheroids and the Gram positive bacillus. The explanation for growth through the filter into the agar without visible growth on the surface is that a slight growth which progresses only in patches and soon degenerates may not be visible in the filters.

The L colonies on the filters have the typical appearance and structure of those developing on agar (Fig. 1 and 10). The smallest colonies are embedded in the filters (Fig. 3 and 11), and the large colonies on the surface have dense centers grown into the filter and a light periphery, usually consisting of large bodies. The size of well-distanced colonies is larger than on agar. The growth of the colony starts, as on agar, by enlargement of the transferred organisms and penetration of growth into the filter (Fig. 2, 6 and 7). Multiplication at the early stage of development of the colony is not apparent on the surface. The extension into the filter is different from that observed on agar. Growth extends into the agar, insofar as is visible with the light microscope, as distinct small granules or as short elongated filaments. In the filter, the extension is apparently in the form of connected irregular branching masses or filaments (Fig. 8). The thickness and irregularity of these filaments is influenced markedly by the pore size of the filters. In filters of 0.8 μ, the extension appears like irregular masses; in 0.22 μ filters, the extension is more like a delicate branching filament (Fig. 7 and 8). The shape of the extending organisms is not determined exclusively by the shape of the available free

† Samples of membrane filters were kindly supplied by the manufacturers.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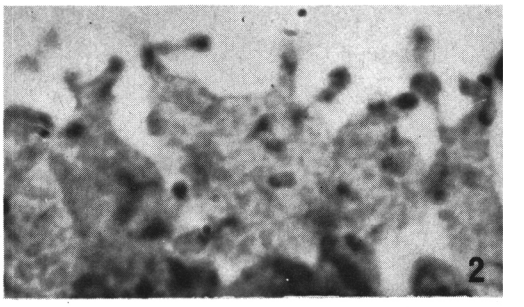
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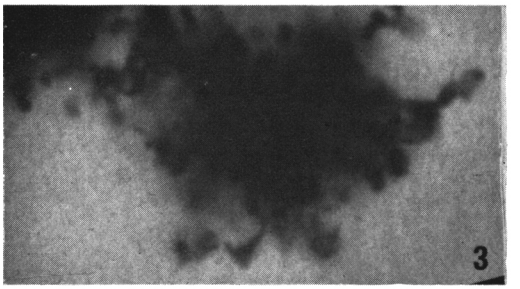
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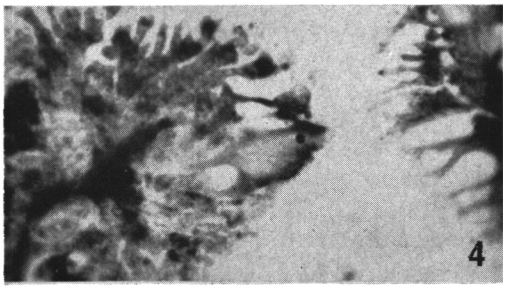
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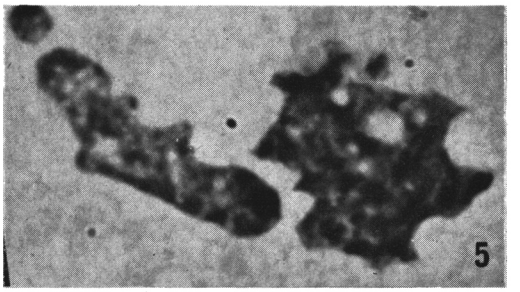
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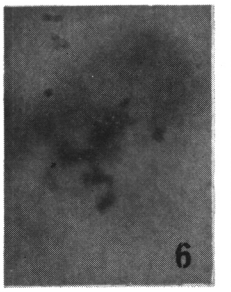
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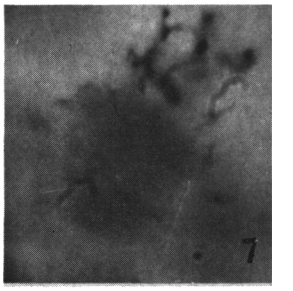
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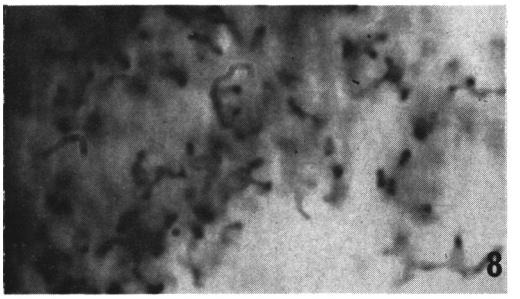
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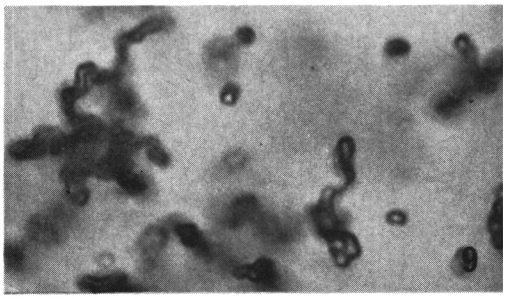
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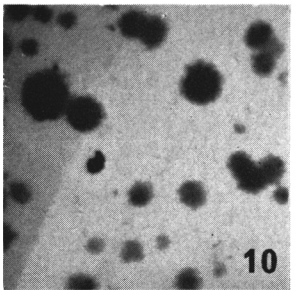
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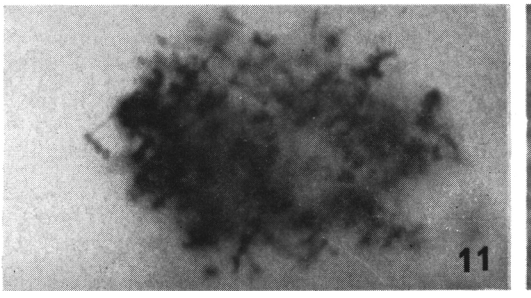
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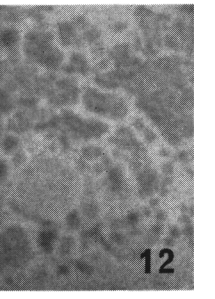
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space inside the filter. On filters of similar pore sizes, the different organisms may grow in coarser or more delicate elements.

We did not succeed in determining the size and shape of the spaces in the filters into which the organisms extend by injecting into the filters under pressure a solution of gelatin strongly stained with crystal violet. The extending edge consisted of a maze of faintly stained very narrow passages, surrounding areas which did not stain. More information was obtained by observing the shape taken by entrapped air during the clearing of the filters with Canada balsam. Both water and xylol are quickly imbibed into the filter. Entrapped air is under pressure and, even in viscous solutions of the balsam, is expelled in bubbles. The last traces of air are quickly absorbed from the passages of the membrane. It is apparent in the photographs that the spaces filled by the air and by the extending organisms are similar. They are elongated interconnected passages of fairly large dimension even in filters of pore size as small as 0.22 μ (Fig. 9). In contrast to the spaces penetrated by the crystal violet solution, these passages filled with air and the similar structures produced by the growth of the organisms do not seem to be preformed in the filter, but are produced by the pressure exerted by air or by growing organisms.

Probably most significant is the behavior of the L forms on filters of small pore sizes into which they cannot penetrate. The organisms grow on such filters to bodies of much larger size than on the usual agar media or in broth. The appearance of these large bodies is of two types. With Proteus and *H. influenzae* we have seen thick, more or less irregular forms of 15-30 μ (Fig. 5). They are not uniformly stained and seem to consist of separate parts. On the filters we did not observe the division of these large forms, but division was visible in similar forms developing on hard gelatin. The organisms of the L forms of Gram positive cocci may grow to 50 μ or more. The edges often show multiple protrusions, often very thin, and ending in sharp points (Fig. 4). Detachment of these protrusions and multiplication of the detached parts is not apparent on the filters, whereas it can be seen in appropriate agar cultures. Extension of growth of irregular large bodies into the meshes of the filter is visible in young cultures on filters of large pore size (0.3 μ) (Fig. 2). According to all appearances, the penetration into the filter is similar to the extension on the surface, but inside the filter a continued growth is possible, probably as a result of being completely surrounded by the gel structure.

The growth of PPLO on Millipore filters

The photographs were made from Millipore filters stained with Azur II.

FIG. 1. L form of staphylococcus, large colonies on 0.3 μ Millipore filter. Periphery on the surface of large bodies. × 250.

FIG. 2. Irregular large bodies at the edge of one of the colonies in Fig. 1. They extend with tongue-like protrusions on the surface. Some of these embed themselves into the filter as dark elongated masses. The embedded parts are not in sharp focus. × 2250.

FIG. 3. A small colony of staphylococcus L form extending into the filter. Only small parts of the extension are in sharp focus. ×2000.

FIG. 4. Gigantic growth of large bodies of staphylococcus L forms transplanted from broth culture. The organisms extend on the surface of 0.1 μ pore size Millipore filters with tongue-like, often pointed protrusions. × 900.

FIG. 5. Irregular large bodies of Proteus strain 52 developing on filters of 0.22 μ pore size. Small protrusions begin to embed themselves in the filter from the large body. The large body appears to consist of differentiated parts. × 2250.

FIGS. 6 and 7. Early growth from large bodies into filters of 0.3 and 0.2 μ pore size. Diffuse shadows on photograph indicate the large bodies from which growth started. × 2250.

FIG. 8. Further developed growth of Proteus L forms in the filter under the large bodies. 0.22 μ pore size filter. × 2250.

FIG. 9. Air spaces in the filter just before complete absorption of air. 0.22 μ pore size filter. The air spaces are considerably wider than the extending growth of Proteus. × 2250.

FIG. 10. Colonies of human urethral PPLO strain Campo after 2 days' incubation on filter of 0.22 μ pore size. × 250.

FIG. 11 A tiny PPLO colony from same preparation as Fig. 10, made with × 20 dry lens and enlarged to × 1,000.

FIG. 12. PPLO on 0.1 μ filter after 24 hr incubation. No multiplication occurred, but some of the organisms are considerably enlarged. The staining of the organisms is faint.

is essentially similar to that of the L forms. The organisms do not start to multiply on the surface and the new growth penetrates into the filter. PPLO have a greater tendency than the L forms to grow as small granules in agar and in broth. This is apparent on the filters also, but like the L forms, they too produce branching filaments (Fig. 11). PPLO develop into colonies and grow through filters of similar pore sizes as the L forms. On 0.1 μ and 0.05 μ filters we did not observe multiplication, but the organisms grew to large bodies, although smaller in size than the L forms (Fig. 12).

The way in which L forms develop from bacteria was most clearly visible with *H. influenzae*. Filters of different pore size were inoculated and incubated on horse blood agar plates containing penicillin. The filters were examined after 1, 2, 5, and 16 hours, and after several days of incubation. On all filters the bacteria grew to large bodies in the presence of penicillin. At 5 hours on 0.22 μ filters and on those of larger pore size, fine branching filaments started to grow into the filters. After 18 hours, usually large L type colonies were present. On filters of smaller pore size, the isolated large bodies were similar to those seen in the L forms of Proteus. They remained smaller where the organisms were crowded. The L forms of *H. influenzae* developed more abundantly and to a larger size on the filters than on any medium with which we have experience. We did not observe growth of the L forms of this organism through the filters into the agar.

The development of L forms from Proteus was similar. The filaments penetrating the filter were often thicker than those of *H. influenzae* (Fig. 6 and 7), and in several experiments only a few L colonies developed. The development of L forms from Proteus on agar media is variable also.

A strain of *B. subtilis* produced L forms on filters in the same way, though less abundantly.

To compare filters of different makes, we examined filters made by Schleicher and Schuell with pore sizes of 0.2 μ, 0.15 μ, and 0.1 μ and Gelman filters with pore sizes of 0.3 μ, 0.2 μ, and 0.1 μ. Filters of smaller sizes were not available. The development of the cultures on Schleicher and Schuell filters was similar to that observed on Millipore filters of comparable sizes. On the Gelman filters the cultures developed more abundantly than on the other filters, and colonies of typical structure developed on 0.1 μ filters also. Extension into the filters occurred in these also in branching filaments, but these were soon broken to fairly large round granules. The differences between the 3 pore sizes of Gelman filters were not marked. The Gelman filters may prove to be useful for the cultivation and isolation of L forms, but they did not give as much information on the growth and development of L forms as the Millipore filters.

Discussion. Several morphological properties of L forms are apparent in cultures grown on membrane filters. One is that bacteria freed from the regulating influence of the rigid cell wall, under appropriate conditions, continue to increase in bulk and grow in some cases to very large size. The size, like that of bacterial colonies, is probably determined by a balance of the available nutrients and the rate of diffusion of metabolic products from the organisms. The conditions for growth are more favorable in the form of a thin extending sheet, as the large bodies are on the surface of the filters, than in the spherical form assumed by the large bodies in liquid media. The second characteristic apparent is that the size of the large bodies and the division into growing progeny depends on the properties, chiefly the physical properties of the environment. Division of the large bodies into other large bodies has not been observed on the filters, although this is sometimes seen on agar(3), as well as on other solid or liquid media. Penetration of growth into the meshes of the filters permits a large extension of growth and the formation of colonies. The way in which this growth extending in various directions and the analogous growth on agar are produced indicates that the large bodies contain smaller parts which, detached from the large bodies, are capable of independent and continuous growth.

In thin sections studied by electron micro-

scope L forms appear enclosed either by a one-layered cytoplasmic membrane, like PPLO, or by a double-layered membrane, like the Gram negative bacteria. These membranes apparently cannot induce and regulate division. Adherence to a solid surface often permits considerable extension of undivided growth, and under certain conditions makes division possible. Penetration into the filter permits a much more extensive growth than that which develops on the surface. It starts in the filters in undivided branches. Division is better facilitated in agar, and growth inside the agar extends in the form of small granules. Adherence to a gel substitutes in an imperfect way for the functions of a rigid cell wall. In bacteria with full structure, division is self-regulating. In L forms, without the rigid cell wall, division is largely dependent on the influence of environment. This is probably the cause of their often bewildering pleomorphism.

The tendency to undivided growth, the dependence on the properties of the environment for division and morphology, and the ability for continuous growth of small separated parts of large bodies account for most of the routinely observed morphology of the L forms. Observations on the growth of L forms on agar and gelatin media of increasing hardness leads to similar conclusions. However, there are various and at present rare observations which are not explained by these properties of the L forms and by the alteration of the cell wall. For example, the reproduction of viable granules or of bacteria with full bacterial structure inside the large bodies. We have referred previously to these phenomena(4,5).

Summary. We have confirmed that L forms of bacteria and PPLO grow well on membrane filters of about 0.2 μ or larger pore size when placed on the surface of appropriate solid media. The cultures grow through these filters and, according to our observations, also through filters of 0.1 μ on the surface of which no growth is visible. Bacteria exposed to inducing agents on membrane filters continue to grow as L forms, as on agar. With *H. influenzae* this occurs more readily and more abundantly than on agar media. The way in which the L forms develop from bacteria and the structure of the colonies is similar to that observed on agar. The bacteria grow to large bodies on the surface and, from these, growth embeds itself and extends inside the filters. The extension inside the filters consists of irregular connected masses or of irregular branching filaments. The size of these depends partly on the pore size of the filters but also on the individuality of the organisms. Air entrapped at the clearing of the filters with Canada balsam fills spaces similar to but coarser than those occupied by extending L colonies. In agar, the growth of L forms extends as small granules or short thin filaments. Observations on the growth of the L forms of bacteria on membrane filters contribute to our understanding of the nature of these forms. The similar behavior of L forms and PPLO on the filters further emphasizes the morphological similarity of these two groups of organisms.

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