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I. VARIANTS AND THE USE OF SODIUM AZIDE IN THEIR PROPAGATION†

Microbiologists with diverse interests are currently investigating, by varied approaches, the L variants of bacteria. From the cytologist's standpoint, L variants are remarkable in that they lack a cell wall; thus, in their L-stage even rod-shaped bacteria tend to become spherical. Also, the major force which regulates size is removed by this lack of a wall, and particles varying in size from 0.1 μ to 25 μ may exist in the same L colony.[7] The smaller particles may spontaneously pass through a cellophane membrane, thus providing a method for separating the L from the bacterial form when such a mixture exists.[18, 23]

Many studies of L-type variation concern their physiology. The ability to synthesize cell wall is usually reversible, and most variants, when removed from the agent which forced the L-type growth, will again develop walls and thus resume the bacterial state.

Growth requirements of L variants differ markedly from those of the parent strain. Up to the present it was believed that high protein, usually supplied by from 10 per cent to 30 per cent serum, was necessary in the culture media. Now, to the contrary, recent reports[21, 22] emphasize that serum is not required in synthetic medium and that serum serves to detoxify[19, 21] certain substances in natural media rather than to supply essential nutrient. At least in the species studied in this respect it appears that L variants are fastidious, not so much in their demands as in their tendency to be inhibited by certain factors in complex media. The medium of Medill and O'Kane,[21] which contains only vitamin-free casamino acids, salts, lactic acid, and glucose, has supported growth of L forms of four species of *Proteus*[21, 18] and of *Salmonella typhimurium*.[20] L variants of *E. coli* have been cultivated on a similar medium.[13]

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A highly important recent finding shows that for growth, L variants often require hypertonic conditions as provided by a high salt content[24] or 10 per cent sucrose,[13, 14] and that even different strains within a species may vary in their sensitivity to osmotic conditions.[1]

The oxygen requirements of an L form may be the reverse of those needed by the parent culture from which it is derived. The L variants of Salmonella species[8] and of *E. coli*[24] prefer anaerobic conditions and will not grow aerobically in most media.

L variation may play a role in infectious disease, since the L variant of a bacterium may be produced by antibodies[4] or by antibiotics[8] and in most instances is antibiotic resistant. Although L variants tend to be non-pathogenic,[6, 10] the reversing of a pathogen to and from the L stage may be one causative factor in the relapse of infections. *In vivo* production of L variants has been observed by Grasset and Bonifas.[11]

Immunological examinations of L type cultures indicate that the L variant contains many of the same antigens as does the bacterial stage, but not the complete antigen complex.[5, 20] As would be expected, the H antigen is lacking and some of the O antigens also are absent.[27]

The size of a colony of the L form varies with the bacterial species, but it is always much smaller than in the bacterial stage. The L colonies of *Proteus mirabilis* may measure 0.5 mm. in 48 hours and thus be macroscopically visible; for most species studied, the L colony is microscopic.

Presumably because of the lack of a cell wall, the L colony does not stain well with aniline dyes. The dyes employed in staining erythrocytes or protozoa are used for revealing L variants. Dienes' stain,[2] containing both azure II and methylene blue, is commonly used.

A link between protoplasts and L variants has also been sought. Protoplasts are cells stripped of wall substance by a lytic agent, commonly by lysozyme. Protoplasts continue growing for about six hours only, and during this time they may undergo a single multiplication. The dumbbell forms observed represent dividing cells. The majority of cells, not dividing, are nearly identical in diameter, neatly rounded, and several times larger than the bacterial cells from which they originate. Excellent photographs of protoplasts have been published.[25] The cell membrane and possible remnant traces of the cell wall stain intensely with vital stains. Several rod-shaped internal structures are also easily stained. Recent studies indicate that protoplasts may exchange genetic material with each other and with bacterial rods.[14] Protoplasts may still serve as sites for the attachment of certain phages[34] and when cells are infected with phage before protoplast forma-

tion, phage synthesis will continue despite changes involved by protoplast production.[10]

Evidence which indicates that a protoplast is an arrested stage in the transition to the L form is accumulating rapidly. Whereas L variants require agar, protoplasts are usually produced in a liquid medium; and when produced by the action of penicillin in liquid they may progress to form L colonies if transferred to agar-containing medium.[13] Protoplasts may, however, be found in agar medium if conditions are unfavorable for continuing L colony development. On medium buffered at pH 8.0, slightly more alkaline than suitable for L type growth, protoplasts of *Proteus rettgeri* may appear in the presence of penicillin in gradient concentrations.[20] Also with penicillin as the lytic agent, abundant protoplasts have been observed when *Salmonella typhimurium*[20] was cultured on medium containing agar in concentrations excessive for L type growth.

It has thus been shown that protoplasts may progress to L colonies, and that one of the best inducers of L variation, penicillin, is also capable of producing protoplasts from many species: *P. rettgeri, P. mirabilis,*[13] *E. coli,*[13,14] *Salmonella typhimurium, Salmonella enteriditis.*[23]

The relationship of L variants to the pleuropneumonia-like (P.P.L.O.) forms is not clear. Are the agents of bovine pleuropneumonia and the other P.P.L.O. strains merely L variants which have lost the ability to return to the bacterial stage? Freundt, who has investigated the characteristics of over 200 isolates of P.P.L.O.,[9] has compared such organisms with L variants and concluded that their morphological differences preclude any basic similarity.[8] On the other hand, Kandler and Kandler[12] have reported that with the methods they employed the mode of reproduction (the pinching off of extruding buds) was identical for both types of organisms. An antigenic relationship between certain P.P.L.O. strains and diphtheroids has also been found.[26]

The research here reported concerns a method of stabilizing L growth, permitting its study *in vitro* without its reversion to the bacterial stage. The stabilizing agent, sodium azide, selectively inhibits Gram-negative rods, and this study is concerned only with the Enterobacteriaceae. However, the principle involved, combining a labile substance which induces L variation with a second stable agent which prevents growth of the bacterial stage, should prove useful in maintaining L growth from many types of bacteria.

When L variants are produced by antibiotic action they tend rapidly to revert to the bacterial form as the antibiotic degrades. Even when pure cultures of L variants are obtained by filtration through cellophane mem-

branes, many species soon revert to the rod form, often before complete development of L colonies.

The culture of L forms of certain Enterobacteriaceae in the presence of azide was studied to determine whether sodium azide could prevent reversion of L colonies to the bacillary type. It was believed that a synergistic action might be exerted by azide and penicillin and that therefore less penicillin would be required to produce variation.

MATERIALS AND METHODS

The bacteria studied included three strains of *Proteus mirabilis,* and one each of *Proteus vulgaris, Proteus rettgeri, Proteus morgani, Salmonella typhimurium,* and *Shigella alkalescens.*

Bacteria for inocula were grown in nutrient broth for preliminary tests and on nutrient agar for all later work.

Sodium azide was tested in concentrations from 0.0004 M to 0.004 M.

Tryptose Blood Agar Base (Difco) and P.P.L.O. agar (Difco) were used as basal media. These were enriched with either P.P.L.O. Serum Fraction (Difco), 20 per cent human serum or 20 per cent horse serum. The sera were inactivated at 56° C. before use.

Initial tests employed slide cultures, in which 1.0 ml. of the enriched agar was used to coat a glass slide. After the cultures had been evenly streaked over the surface, a strip of filter paper saturated with bicillin* was placed at one end. This end of the culture was then elevated 2 mm. to facilitate diffusion of the antibiotic. The work specifically described in this paper employed 22 mm. by 50 mm. cover slips instead of slides, which increased resolution of the stained colonies.

After the *modus operandi* for L growth on sodium azide medium had been established, it was found expedient to make cultures on plates, inoculating four or more sectors successively with $\log_{10}$ dilutions of the culture. The organisms were streaked over the surface of the medium and bicillin in a filter paper disc was placed in the center of the plate to diffuse into each sector.

L colonies and bacillary growth were studied with oil immersion objective after staining with Dienes' method. Slide cultures permitted examination and photography of colonies *in situ.* For photography with high magnification a 1 per cent alcoholic solution of basic fuchsin was a superior stain not absorbed by agar.

RESULTS

With concentrations of sodium azide less than 0.003 M, swarming of *Proteus* rods occurred, and thus Azide Blood Agar Base (Difco) which contains this concentration of azide was chosen for the investigation of factors necessary for L growth on azide medium.

When diluted broth cultures were employed, as are commonly used in L variant work with *Proteus,* no L variants were obtained. Such a negative

* Combined dibenzylethylenediamine dipenicillin G, procaine penicillin G, and potassium penicillin G. Wyeth Laboratories, Inc., Philadelphia, Pennsylvania.

series is shown in Table 1. On the azide medium the bacillary growth did not invade the inhibition area, but no L forms appeared. Studies with such moderate inocula were done with all six strains of *Proteus,* none giving L variants on the azide medium.

Large numbers of organisms, taken from solid medium, did give L variants in the presence of sodium azide, as demonstrated by Table 2.

TABLE 1. SODIUM AZIDE MEDIUM WITH MODERATE INOCULUM*

Penicillin	*Control, no sodium azide*	*0.003 M sodium azide in medium*
30,000 units	Large vacuolated colonies 2½ cm. from antibiotic; smaller L colonies to end of slide.	No L colonies; no bacilli.
3,000 units	Large L colonies start 2 cm. from antibiotic, extend to end of slide.	No L colonies; no bacilli.
300 units	As above, except L colonies appear 1½ cm. from antibiotic.	No L colonies; no bacilli.
30 units	L colonies appear 1 cm. from antibiotic. They lack penetrating central growth seen on slides with higher antibiotic concentration.	No L colonies; bacillary growth over entire slide.
3 units	No L colonies. Bacillary growth starts 2 mm. from antibiotic source.	No L colonies; bacillary growth over entire slide.

Inoculum = 10,000 *Proteus mirabilis* per slide.
Eight days' incubation at 37° C.

* Even an excess of antibiotic produces no L variation on sodium azide medium if the inoculum of organisms is moderate.

Typical L colonies, usually of the type with vesicular periphery, were consistently obtained with the three strains of *P. mirabilis* tested.

As soon as it became established that a specific large number of organisms must constitute the inoculum, a change was made to plated medium, in which several inocula could be tested simultaneously in different sectors of the same plate. Table 3 shows the advantage of examining more than one concentration of organisms. The section with 200,000 organisms gave isolated L colonies which were ideal for subculturing; other sections either had confluent L growth, or in the more highly diluted inoculum at times no L growth was formed. Sectored plates were similarly made with *P. morgani, P. rettgeri, P. vulgaris, S. typhimurium,* and *Sh. alkalescens,* revealing in each instance that it was possible to obtain L colonies which would not be

overgrown by swarming bacilli and which would not revert to the bacterial form. Such L colonies may be stored under refrigeration for at least two months without bacillary invasion or reversion of the L phase to the rod stage. Predetermination of ideal inoculum, which varies with strain as well as species, was not required when such sectors were made.

TABLE 2. SODIUM AZIDE MEDIUM WITH HEAVY INOCULUM*

Penicillin	*Control, no sodium azide*	*0.003 M sodium azide in medium*
30,000 units	Results similar to findings with smaller inoculum (see Table 1) except L growth is 1-4 mm. closer to antibiotic.	Large L colonies start 1½ cm. from antibiotic, and extend to end of slide. No bacilli.
3,000 units	Results similar to findings with smaller inoculum (see Table 1) except L growth is 1-4 mm. closer to antibiotic.	Large L colonies start approximately 1 cm. from antibiotic. No bacilli.
300 units	Results similar to findings with smaller inoculum (see Table 1) except L growth is 1-4 mm. closer to antibiotic.	No L colonies. Bacillary growth begins 1 cm. from antibiotic.
30 units	Results similar to findings with smaller inoculum (see Table 1) except L growth is 1-4 mm. closer to antibiotic.	Bacillary colonies only, over entire slide.
3 units	No L colonies. Growth of bacilli over entire slide.	Bacillary colonies only, over entire slide.

Inoculum = 2,400,000 *Proteus mirabilis* per slide.
Eight days' incubation at 37° C.

* L variants are produced on sodium azide medium when the bacterial inoculum is large.

Earlier studies without azide[19] had shown that all of the *Proteus* strains could produce L colonies when P.P.L.O. serum fraction was the added protein. When azide was in the medium the growth requirements became more strict and with the exception of one strain of *P. mirabilis* all of the cultures required horse serum which was definitely superior to human serum.

Increasing the tonicity of the medium by addition of 10 per cent sucrose did not change the antibiotic requirement or the need for a large bacterial inoculum.

For *P. mirabilis* strains two types of L colonies were seen, one vesicular with a dense center (the so-called "fried egg" colony), the other composed of irregular granules. Their appearance was similar with and without azide. For other bacterial species the L stage consisted of sheets of vacuolated growth, enormous vacuolated colonies without differentiated center, or the granular type of colony.

TABLE 3. SHIGELLA ALKALESCENS, VARIED INOCULA ON ANTIBIOTIC DIFFUSION PLATE*

Inoculum	*Growth after 7 days' incubation*
2,000,000 organisms	Sheets of L growth centrally. Small rim bacillary growth peripherally.
200,000 organisms	Isolated L colonies centrally. Bacillary L growth peripherally.
20,000 organisms	No growth, L or bacillary.
2,000 organisms	No growth, L or bacillary.

Medium, Azide Tryptose Agar.
60,000 units penicillin in central disc.
Seven days' incubation at 37° C.

* Varied inocula on same plate give optimum isolation of L colonies.

DISCUSSION

Culture of the organisms on azide medium without antibiotic revealed that while bizarre thick rods often developed, there was no marked central swelling typical of beginning L variation. Thus it would appear that the toxic effect of azide is not directed even partially toward interruption of cell wall synthesis, as is true with penicillin. Therefore, the lack of synergism of azide and penicillin in relation to L variant formation is not surprising.

The increment in penicillin requirement is probably due to the inactivation of the antibiotic by sodium azide, as found by Marco and Buffi.[17] Azide also inhibits many organisms which otherwise might make the change to L growth, and thus the need for a larger inoculum may be explained.

Additional studies of Salmonella isolates have revealed that many strains require a different basal medium for L development than the ones used in this work.

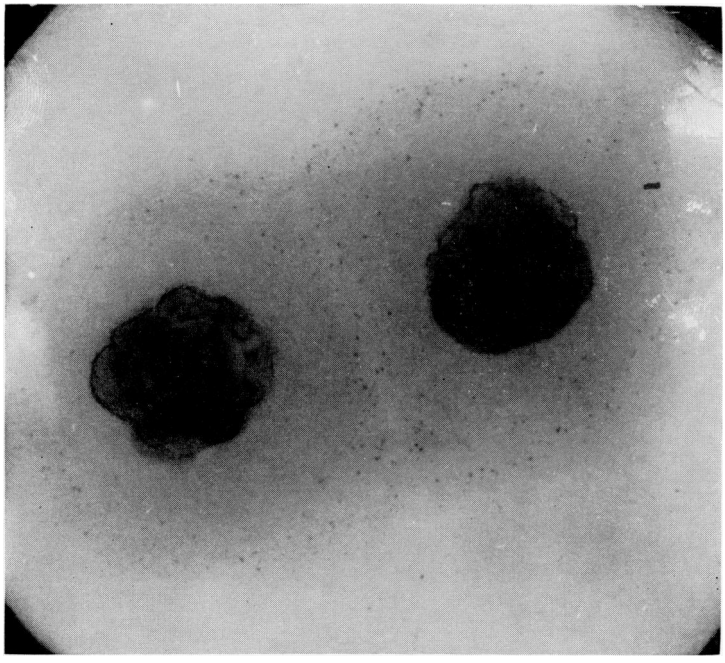

FIG. 1. Typical L colonies of Proteus mirabilis, x110.

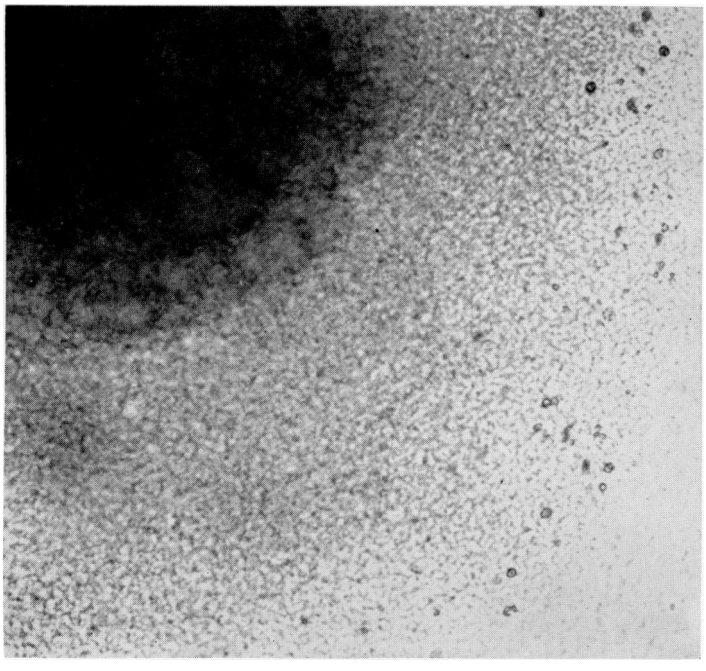

FIG. 2. Edge of one of the colonies shown in No. 1, x1100.

An incidental observation in this laboratory has been that azide medium is useful in isolating P.P.L.O. strains from cervical and urethral specimens, preventing overgrowth by Gram negative rods in the normal flora. Comparative studies will be required before it can be stated that there is no inhibition of P.P.L.O. strains by azide.

SUMMARY

The nature of L variants is reviewed and their relation to protoplasts and the P.P.L.O. forms is discussed.

Experimental work is reported showing that it is possible to prevent reversion of the Enterobacteriaceae L stage to the bacillary form by incorporating sodium azide in the culture medium.

In addition to usefulness in cultivation of L variants, sodium azide medium proved functional for isolation of P.P.L.O. strains from urethral and cervical specimens.

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