

# A Stable L-Form of *Haemophilus Pleuropneumoniae*

D. H. Neil, M. M. Garcia and K. A. McKay\*

## SUMMARY

A stable L-form of *Haemophilus pleuropneumoniae* (Shope) was isolated from primary pig kidney cell tissue cultures which had been inoculated 28 days previously with glycine induced spheroplasts of this organism.

*H. pleuropneumoniae* was definitely cytopathic in primary pig kidney cell cultures, producing cell rounding, cytoplasmic vacuolation and nuclear enlargement with peripheral condensation of nuclear DNA. By contrast, the effect of spheroplasts was much less distinct, producing only loss of cytoplasmic granularity coincident with apparent loss of some cytoplasmic RNA, and slight nuclear enlargement.

Both the organism and its L-form were shown to be related by cultural methods, antibiotic sensitivity tests, immunofluorescence and immunodiffusion.

The L-form remained stable after 90 serial passages on agar and 45 in broth, each medium being capable of supporting the growth of both forms of the organism.

## RÉSUMÉ

Nous avons isolé une forme L stable d'*Haemophilus pleuropneumoniae* (Shope) dans une culture de première explantation de rein de porc, ensemencée 28 jours auparavant avec des sphéroplastiques de cette bactérie, induits par la glycine.

L'*Haemophilus pleuropneumoniae* provoque un effet cytopathogène dans ces cellules sans forme d'arrondissement cellulaire, de vacuoles cytoplasmiques, d'hypertrophie nucléaire et de margination de la chromatine. Par contre, l'effet cytopathogène des sphéroplastiques est bien

moins évident. Nous avons observé seulement la disparition des granulations et d'un peu d'ARN cytoplasmiques et une légère hypertrophie nucléaire.

Nous avons démontré une similarité étroite entre la bactérie et sa forme L par les techniques de croissance bactériennes en milieu de culture et par celles des antibiogrammes, de l'immunofluorescence et de l'immunodiffusion. La forme L est restée stable après 90 passages en gélose et 45 passages en bouillon, chaque milieu permettant la croissance des deux formes bactériennes.

## INTRODUCTION

The isolation of the L-forms of bacteria from diseased human tissues has stimulated interest in the possible role of these bacterial variants in chronic disorders and various diseases of known and unknown etiology. Experimental data presented in a recent symposium (4) have stressed the pathogenic potentialities of microbial variants with cell wall defects and their relationship to microbial persistence and latent infection in man. In a review, McKay, Corner and Byrne (10) suggested that similar studies should be extended to veterinary diseases. They emphasized that the prolonged residence of microbial pathogens in animal host tissues without manifestation of clinical signs could pose a real problem in their eradication and control. In the light of reports that some L-forms (5, 6, 8, 9) do not appear to be affected by drug therapy and persist for long periods in the host, it is conceivable that present concepts in animal disease control might have to be modified to a broader perspective.

This report describes the production and characterization of a stable L-form of *Haemophilus pleuropneumoniae*.

\*Animal Pathology Division, Health of Animals Branch, Canada Department of Agriculture, Animal Diseases Research Institute, P.O. Box 1400, Hull, Quebec.

## MATERIALS AND METHODS

## STAINING METHODS

### ORGANISM

The first isolation and study of *H. pleuropneumoniae* from diseased pigs was reported by Shope (12) and Shope, White and Leidy (13). The relationship of *H. pleuropneumoniae* to other *Haemophilus* species was investigated by White, Leidy, Jameson and Shope (15). The strain used in the present study was 38879(S), a capsulated organism with smooth iridescent colonies obtained from Dr. Grace Leidy.<sup>1</sup> Nicotinamide adenine dinucleotide (NAD) but not hemin was found to be essential for growth.

### MEDIA

The capsular organism was grown and maintained in Levinthal agar plates and broth supplemented with 20 µg/ml NAD (Nutritional Biochemical Corporation, Cleveland 28, Ohio). Four per cent defibrinated sheep blood agar plates streaked with a staphylococcus were used to check the purity of the seed cultures and hemolytic activity.

Spheroplasts were produced in a spheroplasting medium made up of 0.01 M sodium phosphate buffered saline (PBS) at pH 7.1, 0.34 M sucrose, magnesium sulphate to give a final concentration of 1.2 µg Mg++ per ml and 3.0% glycine.

Tissue culture monolayers were grown on 7.5 x 2 cm coverslips in Leighton tubes. They were maintained in basal medium (Eagle) in Earles' balanced salt solution plus 5% gamma globulin free swine serum (GIBCO, Grand Island, N.Y. 14072).

L-forms were isolated and maintained on Frey's mycoplasma base medium (Albimi, Flushing 54, New York) supplemented with 10% gamma globulin free horse serum (GIBCO), 7% yeast extract and 20 µg/ml NAD. This medium was used either as a broth or solidified with 2% proteose peptone agar, henceforth called mycoplasma broth and mycoplasma agar respectively.

The May Grunwald-Giemsa (MGG) technique was used to stain tissue monolayers after fixation for ten minutes in methanol. Acridine orange (AO) was used to stain tissue culture cells for DNA and RNA distribution (2). Spheroplasts were stained with phosphomolybdate (1) for the presence of cell wall.

Colonies of *H. pleuropneumoniae* and the L-form on agar were stained by Dienes stain (Hyland Laboratories, Los Angeles, Calif. U.S.A.). For permanent preparations agar blocks were fixed with Bouin's fixative overnight, washed, and stained with Giemsa (Gradwohl Laboratories, St. Louis, Mo., U.S.A.). The Giemsa stain was prepared by mixing one part concentrated Giemsa to 200 parts distilled water.

### PREPARATION OF FLUORESCENT ANTIBODY

A hyperimmune serum against *H. pleuropneumoniae* was prepared by inoculating ten week-old specific pathogen free pigs at four sites subcutaneously with 1 ml of a *H. pleuropneumoniae* antigen made by suspending an 18 h plate culture in a mixture of 0.5% formaldehyde in PBS at pH 7.1 plus an equal quantity of 4% sodium alginate containing 0.67% calcium gluconate (Colab Products (Canada) Ltd., Weston, Ontario). Three further intravenous injections with 2 ml each of the formalized PBS cell suspension (Brown's tube no. 4) were made at seven day intervals. The pigs were bled five weeks following the initial inoculation, their sera pooled, the globulin precipitated by adding an equal volume of saturated ammonium sulphate and dialyzed against PBS (pH 7.1). Dialysis was continued until all of the ammonium sulphate was removed. The resulting dialyzed globulin was dissolved in 0.85% saline, adjusted to 10 mg dry wt per ml, and conjugated with fluorescein isothiocyanate in carbonate/bicarbonate buffered saline (pH9.0) for 18 hours at 4°C. The conjugate was adjusted to pH 7.1 by dialysis against PBS (pH 7.1) and was freed from unreacted fluorescein material by filtration through a G-25 fine Sephadex column. Specificity of the fluorescent immune globulin was tested against various bacteria such as streptococci, staphylococci, pasteurellae, and *H. pleuropneumoniae* (S) and (R) strains.

<sup>1</sup>Address: College of Physicians and Surgeons, Columbia University, N.Y.

The evaluated conjugate then was used to examine tissue culture and for comparing the *H. pleuropneumoniae* rods with the L-forms in smears from broth cultures or from colonies on agar. Impressions of the L-form colonies were made by twice passing a no. 1 coverslip (22 mm square) through a bunsen burner flame and rapidly applying it to the surface of an agar block.

#### MICROSCOPY

All microscopic examinations were made with a Reichert Zetopan microscope provided with Binolux illumination. The morphology of cells grown in liquid media was studied using anoptical contrast. Tissue culture preparations stained by AO and FAT were examined by ultraviolet illumination (UV) with a BG 12 exciter filter, and GG9 plus OG1 barrier filter.

#### SPHEROPLAST PRODUCTION

An overnight broth culture of strain 38879(S) was centrifuged, washed with PBS and inoculated into tubes of spheroplasting medium, the concentration of the bacteria being adjusted to about  $2 \times 10^9$  total cells per ml. Inoculated tubes were agitated in a 37°C water bath for 72 hours. Twice daily samples were removed for examination microscopically and for inoculation of the various broths and solid media. Spheroplasts produced after 60 hours were harvested by centrifugation and divided into three fractions. One was re-suspended in distilled water, another in PBS and the third used to inoculate tissue culture. The spheroplasts suspended in distilled water and PBS were kept at room temperature and examined microscopically over a period of three days.

#### TISSUE CULTURE TECHNIQUE

Primary cultures of pig kidney cells (PKC) were obtained from newborn animals in a herd from which *Mycoplasma* species, known to occur in pigs, are absent. The tissue cultures were divided into three groups. The first was inoculated with the *Haemophilus* rods, the second with the spheroplasts and the third with sterile tissue culture medium to serve as uninoculated controls. The infected tissue culture medium was changed every five days and a

portion was inoculated to the various artificial media. The infected and uninfected control monolayers were observed periodically and representative coverslips were removed, washed in Hank's balanced salt solution and subjected to the AO, FA and MGG techniques.

The monolayers inoculated with the complete *H. pleuropneumoniae* cells and with the spheroplasts were inspected for changes in bacterial morphology seven and 28 days respectively after inoculation. This was done by breaking up the tissue cells through repeated freezing of the Leighton tubes in alcohol ice mixture and thawing at 37°C. The resulting residue was mixed with the tissue culture medium and 0.2 ml removed for inoculation to blood agar plates streaked with staphylococcus, Levinthal agar and broth, and mycoplasma broth and agar. Growth after 72 hours in any of the media was subcultured to another set of these media.

#### TESTS FOR THE COMPARISON OF THE ROD AND L-FORM

The L-form isolated from tissue culture inoculated with spheroplasts was compared with the whole organism employing the following characteristics: a) hemolysis, b) specific staining with fluorescent antibody, c) immunodiffusion, d) antibiotic and sulfonamide sensitivity, e) NAD requirement, and f) cytopathic effect. The latter was determined by transferring blocks of agar supporting a 72 hour growth of the L-form to tubes containing mycoplasma broth. After 72 hours of incubation these cultures were inoculated onto PKC monolayers which were then maintained for three weeks. At this time re-isolation was attempted. For the immunodiffusion comparisons hyperimmune sera against the rods and the L-forms isolated from tissue culture were prepared. The antigens used for the production of appropriate antisera in rabbits (New Zealand Whites) were formalized, dense suspensions (Brown's tube no. 10) of each form of the organism in PBS. The animals were inoculated intravenously at three-day intervals over a period of three weeks. The immunodiffusion tests were conducted at both 37°C and room temperature (about 25°C) in 1% Noble agar (Difco) with 0.01% Merthiolate, the wells being 2.0 cm in diameter and set 4.0 mm apart. For each test, antigens were

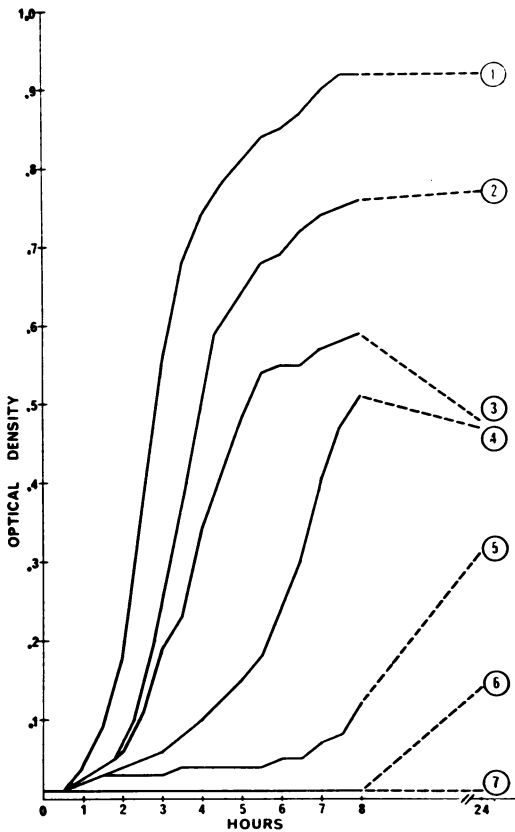


Fig. 1. Optical density (OD) at 520  $m\mu$  of *Haemophilus pleuropneumoniae* in Levinthal broth (LB) 1; LB plus 0.34 M sucrose and 12  $\mu\text{g}$  per ml  $\text{Mg}^{++}$  (LBSM) 2; LBSM plus the following concentrations of glycine expressed as percentages: 0.24 3; 0.26 4; 0.28 5; 0.40 6; and 0.42 7; incubated at 37°C. The solid black lines represent the period of frequent monitoring and the dotted line represents the unmonitored overnight growth, correlating the OD at 8 and 24 hours.

freshly prepared and consisted of dense suspensions (Brown's tube no. 10) of living organisms in PBS. The antibiotic sensitivity was assessed using discs impregnated with oxytetracycline, neomycin, dihydrostreptomycin, chloramphenicol, furandantin, triple sulphonamide, and penicillin placed on freshly inoculated Levinthal and mycoplasma agar plates.

## RESULTS

When spheroplasting medium was inoculated with the rods in capsulated form, no increased growth could be detected. The morphological transition to the less refractile spheroplast form began within six hours after inoculation and was apparently completed after 24 hours. However, cells

kept up to 30 hours in the spheroplasting medium were still capable of reversion and produced colonies of the capsulated rod forms on Levinthal agar. With cells kept more than 30 hours in spheroplasting medium, no growth was detectable when they were inoculated onto Levinthal agar. Such "non-reverting" spheroplasts remained intact in PBS or distilled water. Subsequent staining with phosphomolybdate indicated that most "non-reverting" spheroplasts had cell wall material covering all or part of their surfaces. A few cells, apparently without any cell wall material, characteristically appeared in small clumps in films. The significance of this observation is not understood.

The use of 3% glycine in the spheroplasting medium was based on preliminary experiments on the minimum inhibitory concentration (MIC) of this substance for *H. pleuropneumoniae* 38879(S). The initial level was 0.42% (Fig. 1), but serial passage through increasing glycine concentrations was directly correlated with increasing tolerance (Fig. 2). Thus the glycine level

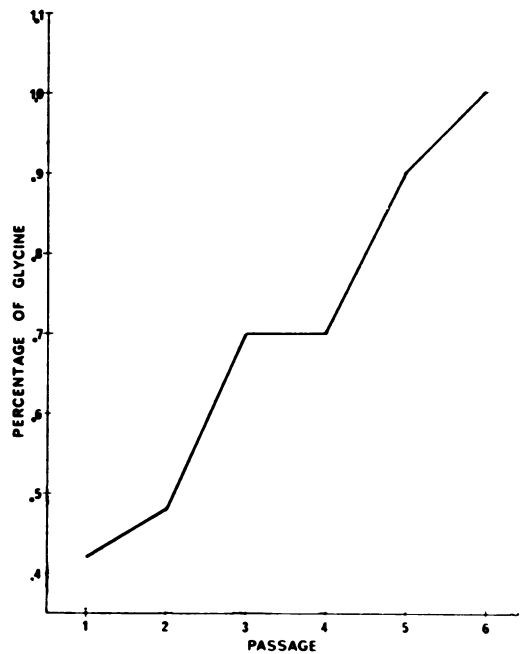


Fig. 2. The highest concentration of glycine permitting detectable growth of *Haemophilus pleuropneumoniae* in Levinthal broth with 0.34 M sucrose and 12  $\mu\text{g}$  per ml  $\text{Mg}^{++}$  added was used to inoculate another range of glycine media (0.2 to 1%). This process was repeated to the sixth passage. The figure demonstrates an increased tolerance from 0.42% initially to 1% at the sixth culture.



Fig. 3. Uninoculated primary pig kidney cell monolayer maintained in basal medium (Eagle) in Earles' balanced salt solution plus 5% gamma globulin free pig serum after ten days. (May Grunwald-Giemsa stain. X 1000).

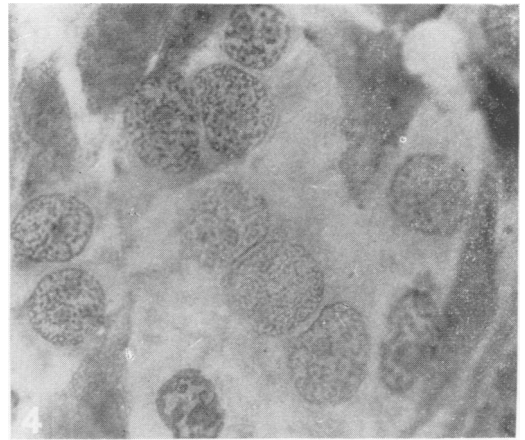


Fig. 4. Primary pig kidney cell monolayer 72 hours after infection with *Haemophilus pleuropneumoniae* 38879S. Note similar cytoplasmic changes to those in Fig. 5. Some nuclei have become enlarged, and contain blobs and fine strands of DNA. (May Grunwald-Giemsa stain. X 1000).

employed for spheroplast production was well over the MIC obtained after the sixth passage. Dienes and Zamecnick (3) obtained satisfactory L-forms with 3% glycine, in plate media.

#### ASPECTS OF TISSUE CULTURE

Observations of the viable tissue cultures at 400 magnifications indicated that the uninoculated controls were healthy and adherent to the glass for the first two weeks of the experiment. Fig. 3 shows the uninoculated tissue culture cells with granular cytoplasm, and rich in RNA as stained by MGG and confirmed by AO. The cell nuclei contained one or two discrete nucleoli. Between the 14th and 23th day there was a progressive loss of the original cells and apparent replacement with fibroblast-like cells. The *H. pleuropneumoniae* rods produced a distinct cytopathic effect on the tissue cells. Fig. 4 illustrates this effect at 72 hours with vacuolation of the cytoplasm and nuclear enlargement. Blobs of condensed DNA appeared peripherally in these nuclei as stained by AO. This nuclear enlargement was not preceded by pyknotic changes. Cell cultures inoculated with spheroplasts (Fig. 5) showed a slight cellular enlargement with darkened non-granular cytoplasm. Staining by AO suggested that the loss of granularity of the cytoplasm was to some extent associated with the loss of the orange fluorescing cytoplasmic RNA. The nuclei appeared to be more refringent than those of the control cells and were

slightly larger; each nucleus contained from one to six nucleolus-like bodies. The specificity of these changes is not known.

The FA technique alone was able to demonstrate the presence of the spheroplasts in tissue culture. Comparison of the results from anoptral contrast examination (Fig. 6) with that under UV illumination (Fig. 7) shows the intense fluorescence of the spheroplasts in the cytoplasm of tissue culture cells. Examination of control tissue culture cells by FAT indicated the absence of non-specific staining of tissue artifacts.

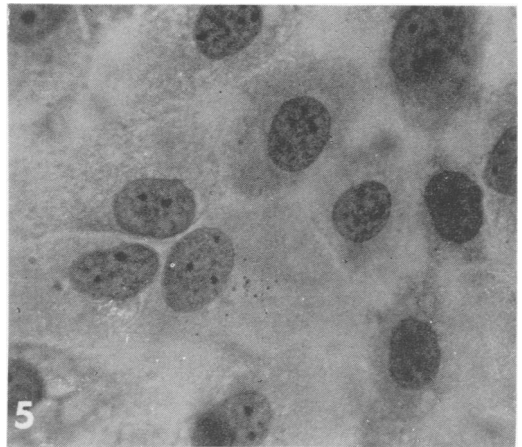
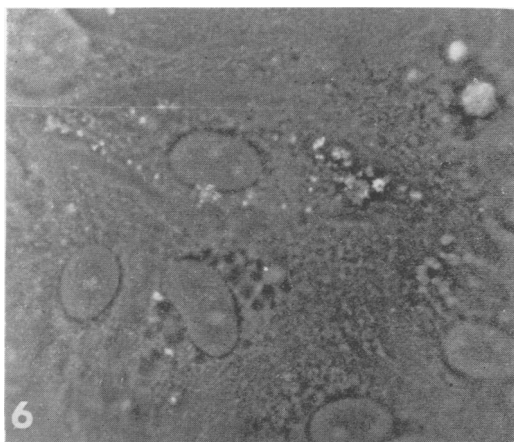


Fig. 5. Tissue monolayer from same experiment as that in Fig. 3 having been infected for six days with *Haemophilus pleuropneumoniae* spheroplasts (X 1000). Note the clouddiness and loss of granularity of the cytoplasm, compared with the coarse granularity of the negative control in Fig. 3. Note slight nuclear enlargement and increased number of nucleoli compared with Fig. 3. (May Grunwald-Giemsa stain).



**Figs. 6 and 7.** These are both exactly the same field on a tissue cell monolayer five days after infection with *Haemophilus pleuropneumoniae* spheroplasts, subjected to

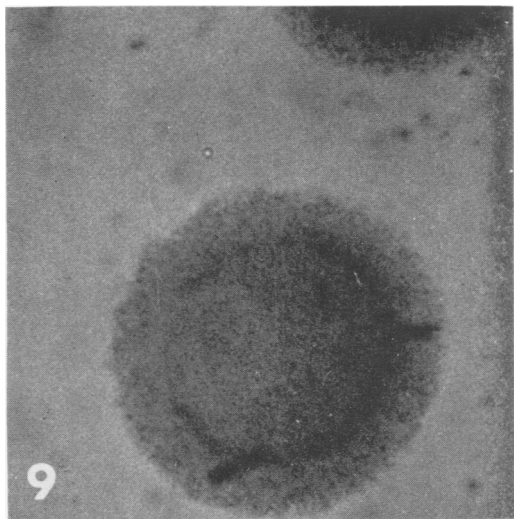
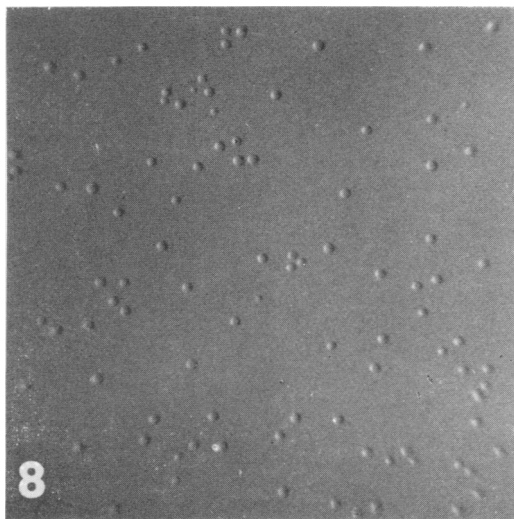
the fluorescent antibody technique. Fig. 6 demonstrates what can be seen by anoptral contrast and Fig. 7 by ultraviolet-light through the same condenser.

#### ISOLATION OF THE STABLE L-FORM

The stable L-form was recoverable from frozen and thawed tissue culture cells inoculated with the spheroplasts 28 days previously. Isolation had to be made by first inoculating the disintegrated tissue culture cells into mycoplasma broth. From this medium colonies could be obtained by inoculation of mycoplasma agar whereas direct transfer of disintegrated tissue culture material to mycoplasma agar was uniformly

unsuccessful. Similarly, when the L-form isolated by the process described was re-inoculated into PKC monolayers it could be recovered only by following this same procedure each time.

Microcolonies which developed after 72 hours on mycoplasma agar plates were discernible under the dissecting microscope at a 25X magnification (Fig. 8). These colonies stained well with Dienes special stain (Fig. 9), and Giemsa stain. Good colony fixation with Bouin's fixative for the Giemsa staining technique proved un-



**Fig. 8.** Colonies of *Haemophilus pleuropneumoniae* L-form on an enriched mycoplasma medium containing yeast extract, NAD and gamma globulin free horse serum. Taken at X 25.

**Fig. 9.** A colony from the mycoplasma agar plate in Fig. 8, at X 1000. Stained by Dienes' special stain.

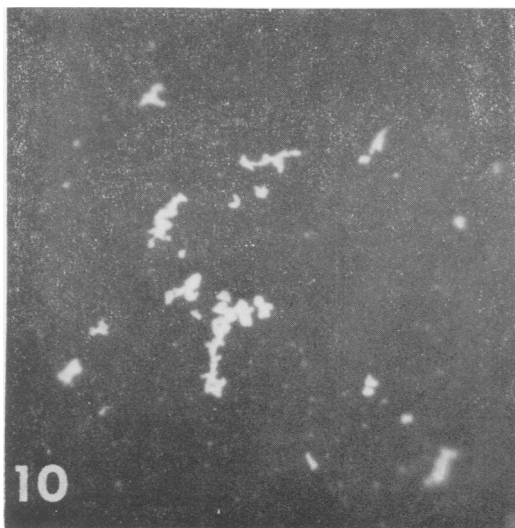


Fig. 10. *Haemophilus pleuropneumoniae* L-form grown in mycoplasma broth and subjected to the fluorescent antibody technique using fluorescein isothiocyanate conjugated *H. pleuropneumoniae* immune globulin. X 1000.

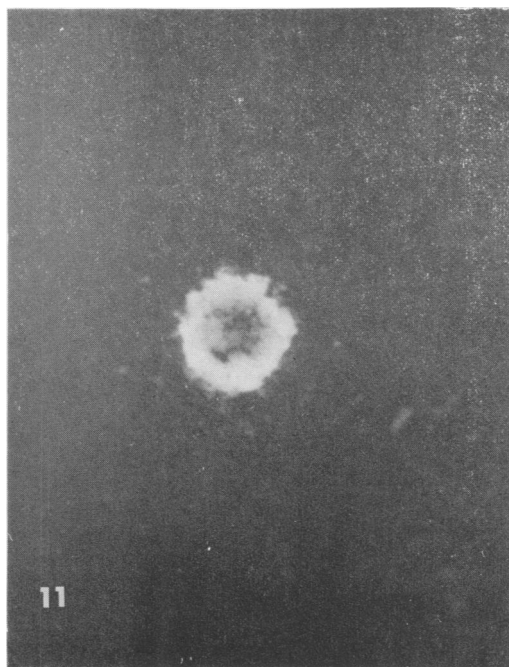


Fig. 11. Impressions from the mycoplasma agar plate seen in Fig. 8 subjected to the fluorescent antibody technique using fluorescein isothiocyanate conjugated *H. pleuropneumoniae* immune globulin. Ultra-violet illumination X 400.



Fig. 12. Comparison of *H. pleuropneumoniae* and the L-form by immunodiffusion in 1% Noble agar plates (X 1.75). The well at the right of the photograph contained a fresh dense suspension of the L-form; the well at the left contained a fresh dense suspension of the entire organism. The top well was filled with antiserum against the L-form, and the bottom well contained antiserum for the haemophilus, both sera being of rabbit origin. Note the common confluent line produced by both antigens, against the L-form antiserum, with no apparent spurs indicating antigenic similarity, and the same with both antigens against the antiserum for the entire organism, plus the extra complex of lines between the haemophilus antigen and its antiserum. The complex represents antigens peculiar to the entire bacterium, as indicated by the spur it produces, and is thought to represent the wall antigens.

usually difficult. Micromanipulation of the colonies indicated their centres to be embedded in the agar surface, and the component units of the colony firmly aggregated. Removal of the coverslip after fixation often resulted in damage to the colony.

#### COMPARISON OF ROD AND L-FORM

The intact rod and the stable L-form hemolyzed sheep red blood cells and required NAD for growth. Groups of the L-form from broth cultures (Fig. 10) and the colonies on agar (Fig. 11) both gave specific fluorescence with *H. pleuropneumoniae* immune globulin conjugate.

In the immunodiffusion test a common line of precipitation occurred with the L-form antibody and both antigens. The *H. pleuropneumoniae* antiserum gave a complex of lines with its own antigen and a single common line with both antigens (Fig. 12).

The bacterium and its L-form were sensitive to streptomycin and neomycin, and were extremely sensitive to oxytetracycline,

chloramphenicol and furandantin. The L-form was completely resistant to triple sulfonamide and penicillin whereas the rod form was sensitive to both.

In contrast to the cytopathic effect produced by the inoculation of *H. pleuropneumoniae* rods to tissue culture cells, no such effect was detected in the cells inoculated with the L-form. Recovery of the L-form introduced to PKC monolayers was only possible by following the original isolation procedure; i.e., by freezing and thawing twice followed by inoculation to mycoplasma broth.

#### STABILITY OF THE L-FORM

At the time of writing, the L-form has been passed serially 90 times on mycoplasma agar and 45 times in Levinthal broth without the occurrence of any tendency of reversion to the original rod form, although both the mycoplasma agar and Levinthal broth were known to support the growth of the parent strain.

### DISCUSSION

The formation of the stable L-form of *H. pleuropneumoniae* appeared to require the passage of the spheroplasts to tissue cells and their subsequent recovery in mycoplasma broth. This observation emphasizes the need for adaptation of the L-form in an environment provided by living cells. Mortimer (11) found that a streptococcal L-form appeared only after infection in mice had existed for some time, suggesting that the production of the L-form might be the result of either some host response like the disruption of streptococcal wall or the provision of conditions protecting the L-form against osmotic lysis. It should be noted that in the present report, preliminary alteration of the bacterial wall was carried out by glycine treatment. However, since no detectable lysis occurred even when the produced non-reverting spheroplasts were suspended in distilled water (up to three days), it would appear that much of the wall remained associated with the spheroplasts prior to inoculation of tissue cultures. This was confirmed by cell wall staining. It is probable that further spheroplast wall disruption might have occur-

red in tissue cells prior to the appearance of the L-form. The requirement for tissue cell adaptation has also been reported by McKay *et al* (9), who prepared *H. parainfluenzae* "protoplasts" from aged colonies. These protoplasts were passaged six times through tissue cultures and were not found to revert to the rod type at any time.

The use of spheroplasts to infect tissue culture was preferred to the cytopathic rod form since spheroplasts produced much less tissue cell alteration than the rods and therefore could be maintained longer in the tissue cultures. Hatten and Sulkin (7) working with their *Brucella* L-forms observed that the stability of the L-forms increased with the duration of the intra-cellular inclusion.

The L-form and the *H. pleuropneumoniae* were linked biochemically and immunologically, as demonstrated by NAD requirements, FA technique and immuno-diffusion. The sensitivity of both types to penicillin and triple sulfonamides appears interesting since both substances are known to interfere with cell wall synthesis. Similarly, glycine is believed to reverse the terminal cross-linking reaction in cell wall synthesis (14).

The hypothetical role of bacterial L-forms in the etiology of recurrent acute infection arising from covert subclinical infection of the host, already under scrutiny in the field of public health (7 and 8), remains largely unexplored in veterinary medicine. The domestic animals present a number of diseases of great economic importance which have been refractory to satisfactory disease control. Investigations of those aspects concerning the prolonged residence of these bacterial variants in relation to the environment provided by animal host tissues could lead to a better understanding of microbial persistence and ultimately, on the ways to control them.

#### ACKNOWLEDGMENTS

The authors wish to express their thanks to Dr. Grace Leidy for supplying the lyophilized cultures of the organisms, and to Mr. R. B. Stewart for technical assistance, especially with the photomicrography.



## REFERENCES

1. BISSETT, K. A. and C. M. F. HALE. Complex cellular structure in bacteria. *Expl Cell Res.* 5: 449-454. 1953.
2. CRUICKSHANK, R. (ed.) "Medical microbiology". Section by Swain, R.H.A. 11th ed. p. 633. London: E. and S. Livingstone Ltd., 1965.
3. DIENES, L. and P. C. ZAMECNIK. Transformation of bacteria into L-forms by amino acids. *J. Bact.* 64: 770-771. 1952.
4. GUZE, L. B. (ed.) Microbial protoplasts, spheroplasts and L-forms. Baltimore: The Williams and Wilkins Co. 1968.
5. GUZE, L. B. and G. M. KALMANSON. Persistence of bacteria in "Protoplast" form after apparent cure of pyelonephritis in rats. *Science* 143: 1340-1341. 1964.
6. HATTEN, B. A. and S. E. SULKIN. Intra cellular production of brucella L-forms. II. Induction and survival of *Brucella abortus* L-forms in tissue culture. *J. Bact.* 91: 14-20. 1966.
7. HATTEN, B. A. and S. E. SULKIN. Possible role of brucella L-forms in the pathogenesis of brucellosis. In Guze, L. B. (ed.). (See ref. 4.) pp. 457-471. 1968.
8. LAPINSKI, E. M. and E. D. FLAKAS. Induction of L-forms of *Hemophilus influenzae* in culture and their demonstration in human bronchial secretions. *J. Bact.* 93: 1438-1445. 1967.
9. MCKAY, K. A., M. K. ABELSETH and A. A. VANDREUMEL. Production of enzootic-like pneumonia in pigs with "Protoplasts" of *Hemophilus parainfluenzae*. *Nature* 212: 359-360. 1966.
10. MCKAY, K. A., A. H. CORNER and J. L. BYRNE. Microbial persistence and latent infection. *Can. J. comp. Med.* 31: 256-259. 1967.
11. MORTIMER, Jr., E. A. The Production of L-forms of Group A Streptococci in mice. In Guze, L.B. (ed.). (See ref. 4.) pp. 345-351. 1968.
12. SHOPE, R. E. Porcine contagious pleuropneumonia. I. Experimental transmission, etiology and pathology. *J. exp. Med.* 119: 357-368. 1964.
13. SHOPE, R. E., D. C. WHITE and G. LEIDY. Porcine contagious pleuropneumonia. II. Studies of the pathogenicity of the etiological agent, *Hemophilus pleuropneumoniae*. *J. exp. Med.* 119: 369-375. 1964.
14. STROMINGER, J. L. Enzymatic reactions in bacterial cell wall synthesis sensitive to penicillins and other antibacterial substances. In Guze, L. B. (ed.). (See ref. 4.) pp. 55-61. 1968.
15. WHITE, D. C., G. LEIDY, J. D. JAMESON and R. E. SHOPE. Interrelationship of *Hemophilus pleuropneumoniae* to other species of hemophilus: nutritional, metabolic, transformation and electron microscopy studies. *J. exp. Med.* 120: 1-12. 1964.

## Book Review

LEHRBUCH DER SPEZIELLEN VETERINAR-CHIRURGIE. (*Textbook of Special Veterinary Surgery*) W. Boltz, O. Dietz, H. Schleiter and R. Teuscher. Published by Gustav Fischer Verlag Jena 1968. 996 pages (2 volumes) illustrations throughout the text. Linen binding. Price, DM 88,00. (In German).

This book, written by a team of prominent European teachers in veterinary surgery, appears in two volumes. Volume one deals with afflictions requiring surgical treatment of the head, neck, thorax and abdomen. The second volume deals with sex organs and motility of the limbs including the spine in both large and small animals.

The text is exceedingly well organized and each chapter is comprehensive and presented in considerable detail. Each region of the body and each organ is dealt with separately and in detail. Each chapter describes symptoms and etiology leading to diagnosis and recommends several choices of surgical treatment. Operational techniques for standard surgical procedures are

not given, but presumed to be known by the reader. However, advanced and newer methods such as surgery of the heart and large vessels or the limbs are given in detail including modern aids for diagnosis such as X-rays or nerve block, choice of anaesthesia and post surgical care.

Both volumes are extensively illustrated in black and white as well as color. Numerous X-ray illustrations enhance the clarity of a well written and easily readable text.

The absence of reference to surgery of the eye is a surprise in an otherwise comprehensive work. Also missing are surgical techniques for economic or cosmetic reasons such as dehorning, spaying, trimming of ears, etc. References are not given and would be a definite asset, especially when referring to surgical techniques.

Both volumes will be of invaluable help to practitioners seeking a careful description of the latest possible surgical treatment. It will also be of great help to the advanced veterinary student possessing knowledge of general surgery and basic surgical techniques. *G. Lutte*