MEDICAL GRAND ROUNDS

Parkland Memorial Hospital
February 11, 1965

Current Status of PPLO and Protoplasts

[redacted]

The patient is a 54-year-old [redacted] man who 10 days prior to admission developed cough, fever, headache and myalgias. Cough was initially dry but became productive of thick yellow sputum on the 3rd day. On the 5th day of his illness, he consulted his family physician, who diagnosed bronchopneumonia and instituted therapy with penicillin 600,000 units daily. This program was continued for 5 days without any detectable change in symptoms and because of the persisting illness, the patient was hospitalized on [redacted]64.

Physical examination on admission revealed temperature 101°, blood pressure 160/90, pulse 80, respirations 18. There was no cyanosis. The chest examination revealed scattered expiratory wheezes throughout and inspiratory rales in both posterior bases, most marked on the left. The remainder of the physical examination was normal.

Admission x-ray revealed a patchy bronchopneumonia in the left lower lobe. Admission laboratory work revealed a total white count of 12,500 with a left shift plus an occasional nucleated red blood cell. A reticulocyte count obtained on the basis of the nucleated red cells was 12%. Serum bilirubin was 2.45 mg.% with 1.8 indirect. The remainder of the liver battery including transaminase was normal. Urine revealed neither bile nor urobilinogen. Cold agglutinins obtained on admission were positive 1:1248. Sputum culture grew Neisseria species and Streptococcus viridans. Indirect Coombs was 1+. Bone marrow revealed erythroid hyperplasia.

The patient was treated 72 hours with Combiotic (penicillin and streptomycin) without any detectable change in symptoms. On the 4th day hemoglobin fell to 10 gm.% and the patient was begun on chloramphenicol and steroids, the latter ostensibly directed at his hemolytic state. Defervescence occurred in 36 hours on this program and his subsequent course was one of improvement. Chloro and steroids were discontinued after 7 days' therapy. During his 3-week hospital stay, x-ray very gradually cleared. On discharge his hemoglobin was 12.4 gm.%, reticulocyte count 3%, white count 7,000 with normal differential and the cold agglutinin titer was 1:2048. Presumptive diagnosis was Eaton agent pneumonia.

[redacted]

The patient is a 22-year-old [redacted] male admitted on [redacted]/64 with history of his present illness beginning some 36 hours earlier with severe headache, fever and vomiting with subsequent rapid progression to stupor and deep coma.

Physical examination revealed temperature 104°, blood pressure 110/70, pulse 110, respirations 30. He was unresponsive to various stimuli and had marked nuchal rigidity but no papilledema or lateralizing reflexes. Examination of the ENT and chest were normal, as was the remainder of the physical examination.

Admission laboratory work revealed a hemoglobin of 15 gm.% and a white count of 30,000 with left shift. Lumbar puncture revealed opening pressure of 500 mm.H_2O, cloudy fluid with 3500 white cells, of which 98% were polys, Pandy of 100 mg.%, and glucose of 60 mg.% with concomitant blood sugar of 185 mg.%. Gram-positive diplococci were visualized on smear and pneumococci were subsequently grown from culture.

Therapy was instituted with penicillin 30 million units per day and Solu-Cortef in large doses. On this program he became afebrile on the 5th day but response was disappointing in other respects in that his sensorium deepened and intermittent convulsions appeared. Repeat lumbar puncture during the first 48 hours revealed persisting high pressure, pleocytosis in the range of 6- to 11,000/mm^3, and sugars of 26 to 30 mg.% with blood sugars of 100 mg.%. The lumbar puncture done on the 4th hospital day was the first one showing improvement in that the pressure was normal (180 mm.H_2O), cells were reduced to 341/mm^3 and glucose was normal. After the first week the course was one of very gradual improvement with return of sensorium to functioning status, and by the time of discharge on the 18th day he was thought by most observers to be back to pre-illness status. He had received steroids for 5 days and penicillin for 14 days.

It is of note that spinal fluids obtained on hospital days 2, 3, 4 and 9 were sterile for bacteria but yielded growth interpreted as L forms. His discharge lumbar puncture on 6/8 was negative for both bacteria and L forms.

Because of a retrospective history of repeated infections over a 4-year period and the finding of a gamma globulin of 1.1 gm.% during this admission, his globulin status was evaluated and it was established that he had a form of hypogammaglobulinemia characterized by very low levels of 7S gamma with relatively normal quantities of 19S.

The patient was discharged on no medications on [redacted]/64 and two days later noted a mild frontal headache. On [redacted] he had a rigor with mild increase in his headache. On the following day his headache was quite severe and he had a measured temperature of 103.4°. On readmission he was found to have a temperature of 102°, pulse of 116, respirations 28, bbod pressure 120/90. ENT and chest examinations were normal. Sensorium was clear and there was no nuchal rigidity. There was no papilledema. Reflexes were normal except for absent right knee jerk with a 2+ on the left. The spleen was palpable 2 fb below the left costal margin and several small posterior cervical and axillary nodes were noted.

Admission laboratory work revealed a hemoglobin of 12.7 gm.%, white count of 6,600 with left shift, and normal urinalysis. Lumbar puncture revealed opening pressure of 300, 6 mononuclear cells, protein 24 mg.% and glucose 50 with concomitant blood glucose of 100 mg.%. All smears and cultures for bacteria were negative. He was observed 72 hours without change in medical status or spinal fluid findings and on the basis of a presumptive diagnosis of recurrent bacterial meningitis and/or brain abscess, high-dose penicillin therapy was re-instituted and continued for 14 days. He became afebrile on the 4th day of therapy and his subsequent course was symptom-free. Arteriograms done in evaluation of possible brain abscess were negative. An EEG showed only non-specific findings.

It is of interest that spinal fluids obtained on the 2nd, 4th and 24th hospital days revealed L form growth similar to that observed during the previous admission.

The first of these cultures was obtained before institution of antibiotic therapy. Bone marrow obtained on the 5th hospital day was similarly positive. Attempts to revert these organisms to pneumococci by alterations in media have been unsuccessful.

The relationship of the observation of these forms to the initial prolonged course and to possible relapse of disease is speculative at best.

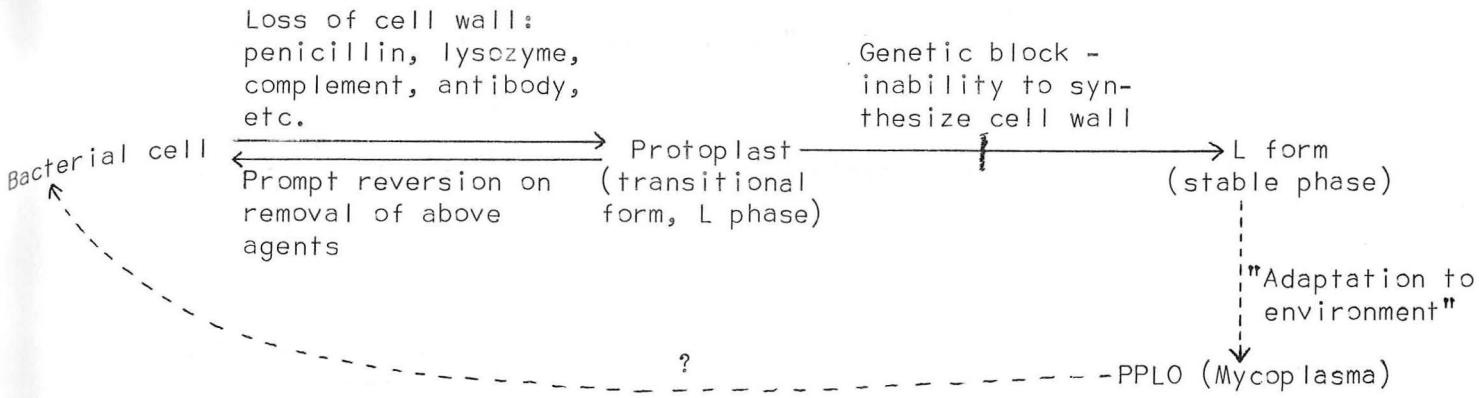
PROPOSED NOMENCLATURE OF PPLO

Order: Mycoplasmatales
Family: Mycoplasmataceae
Genus: Mycoplasma
Species: Decided arbitrarily according to source or founder

COMPARISON OF PPLO (MYCOPLASMA) AND BACTERIAL L FORMS

	PPLO	L Forms
Colony size	0.1-0.3 mm.	0.5-1.0 mm.
Organism size	125 mμ	200-300 mμ
Structure	Homogeneous	Pleomorphic
Resistance to penicillin	+	+
Resistance to thallium acetate	+	+
Osmotic fragility	+	+
Sterol requirements	+ (pathogenic strains only)	0
Sensitivity to anti-microbials altering protein metabolism	+	+
Revert to bacterial form	0	±
Occur free in nature	+	-
Associated with disease	+	-

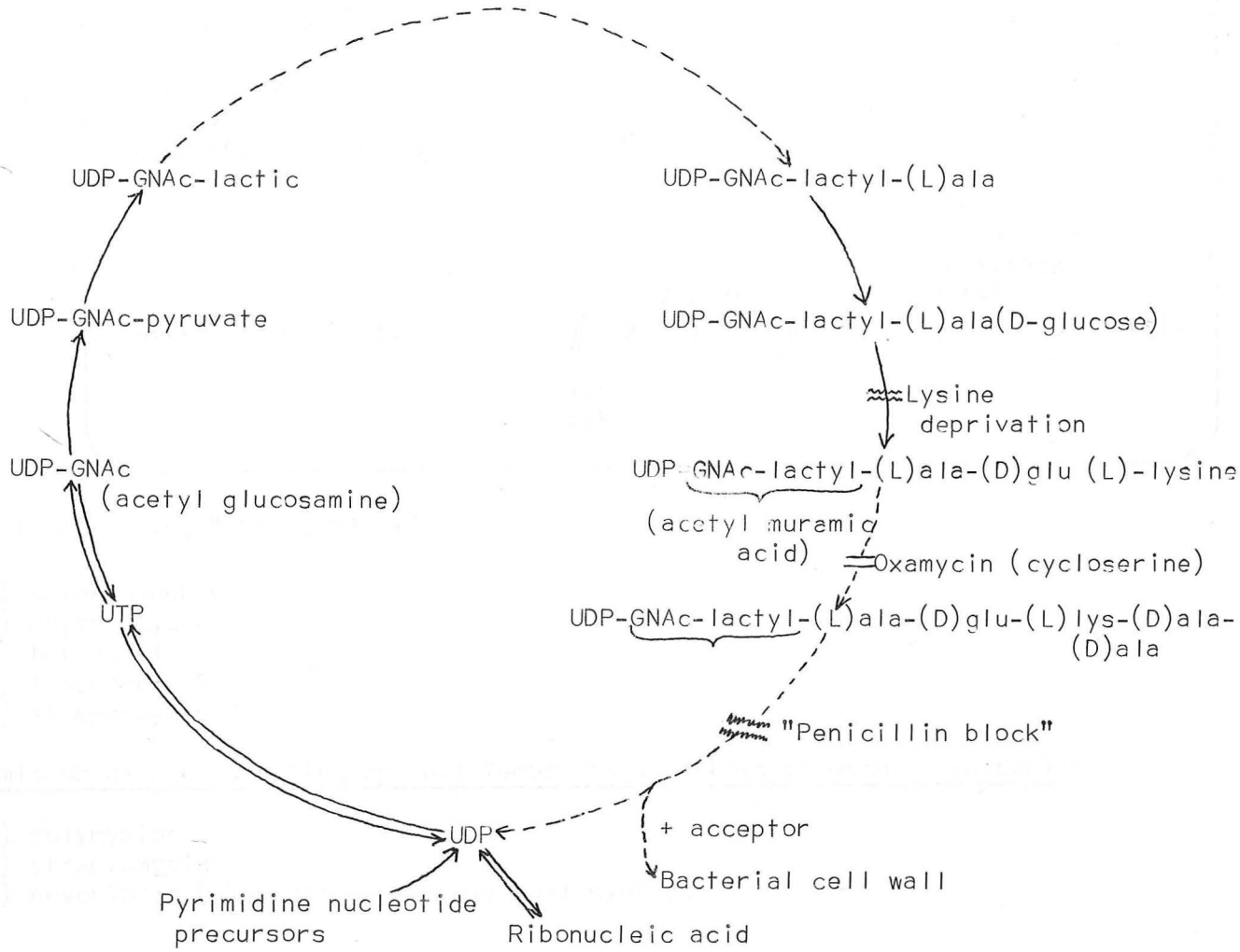
POSSIBLE RELATIONSHIP OF PROTOPLASTS TO PPLO (MYCOPLASMA)



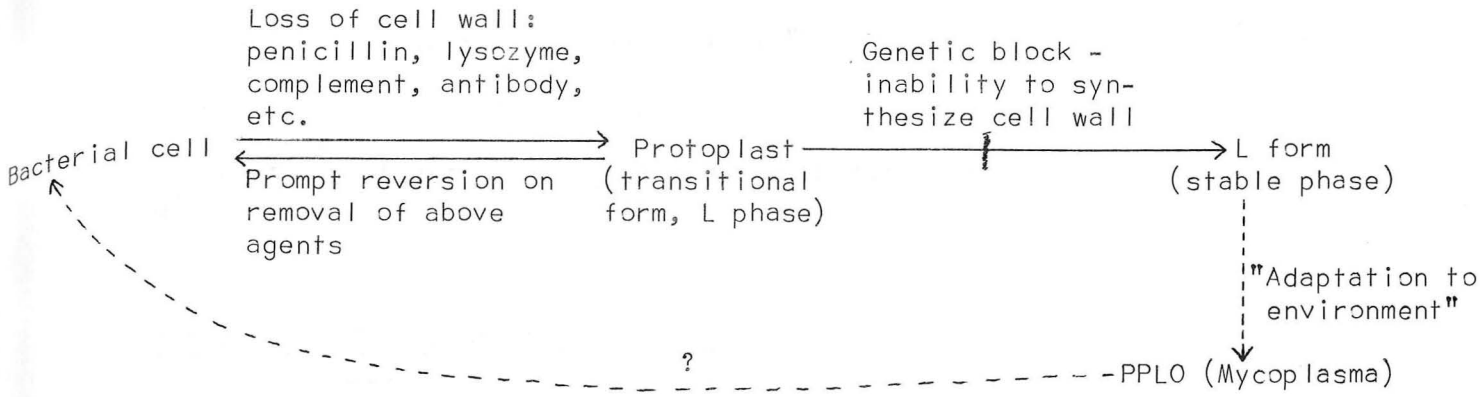
RELATION OF ANTIMICROBIAL AGENTS TO BACTERIA, L FORMS AND MYCOPLASMA

There are 3 general modes of action recognized: 1) interference with cell wall synthesis, 2) disruption of protein synthesis and 3) disruption of integrity of protoplasmic membranes.

Antimicrobials and Cell Wall Synthesis



POSSIBLE RELATIONSHIP OF PROTOPLASTS TO PPLO (MYCOPLASMA)



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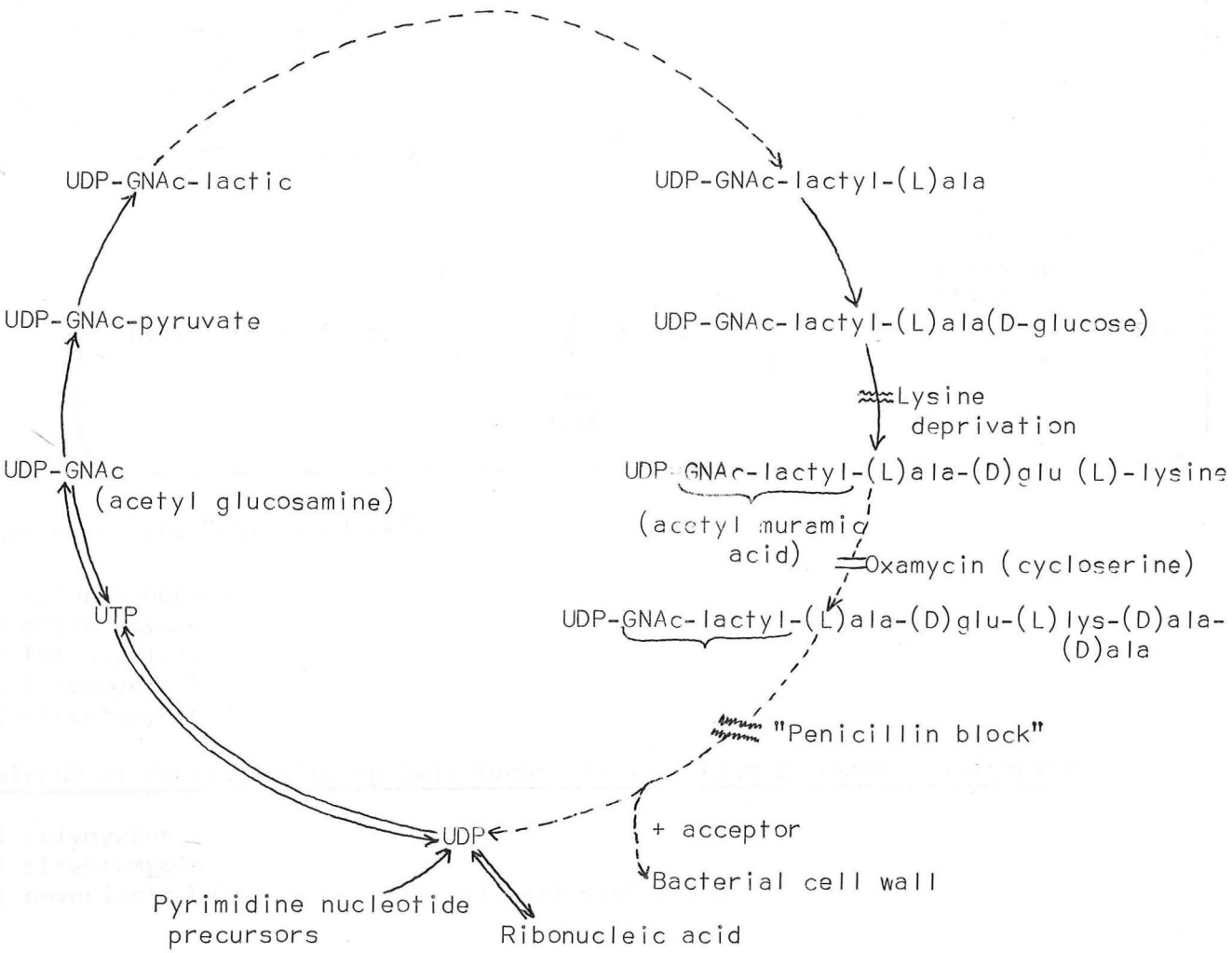
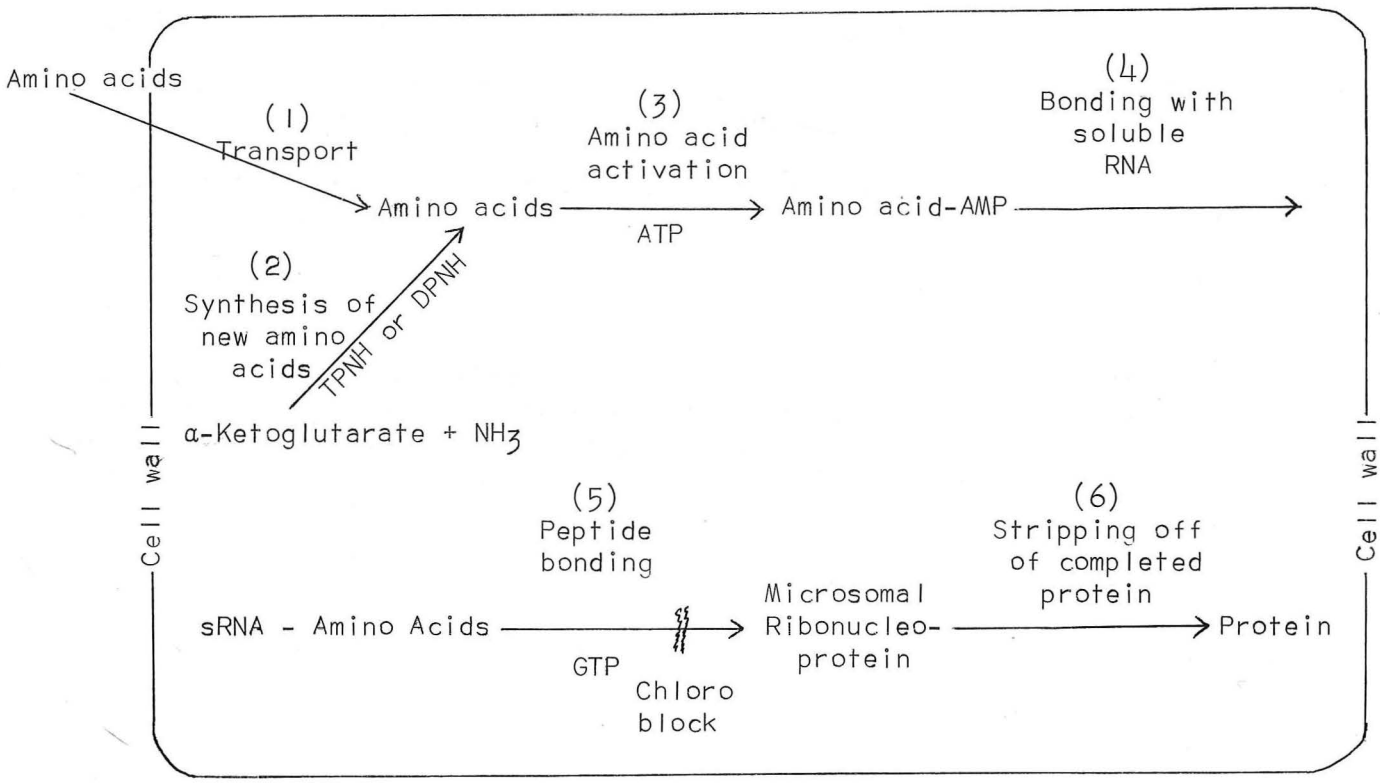
Antimicrobials and Cell Wall Synthesis



Agents shown to effect "penicillin block:"

1) penicillin and synthetic analogues
2) bacitracin
3) novobiocin (also ↓ integrity of protoplasmic membrane)
4) vancomycin
5) cephalosporins

Antimicrobial Agents and Protein Synthesis



Agents effecting "chloro block":

1) chloramphenicol
2) erythromycin
3) tetracycline
4) lincomycin ?
5) streptomycin ?

Antimicrobial Agents Acting on Cell Membranes With Loss of Osmotic Integrity

1) polymyxins
2) streptomycin
3) novobiocin (also decreases cell wall synthesis)

Mycoplasma, protoplasts and L forms are resistant to those drugs acting on cell membrane synthesis and susceptible to those altering protein metabolism or protoplasmic membrane integrity.

CURRENT STATUS OF MYCOPLASMA, PROTOPLASTS AND L FORMS

-Summary Statements-

1. While mycoplasma and L forms may have common bacterial origins, there is insufficient evidence to justify this postulate at present.

2. The role of Mycoplasma pneumoniae in an important respiratory disease, Eaton agent pneumonia, is firmly established.

3. Evidence that T strain mycoplasma is an important agent in a form of venereal disease outweighs objections to that view.

4. There is insufficient evidence to incriminate mycoplasma in Reiter's and "autoimmune" states.

5. Protoplasts have not been shown to have inherent pathogenicity. Their principal threat comes from their ability to exist in the face of host defense mechanisms and/or antimicrobial agents and to opportunistically revert to the parent pathogen. The importance of this feature is at present speculative.

General Characteristics of PPLO (Mycoplasma), Protoplasts and L Forms

1. Klienenberger-Nobel, E. PPLO: Mycoplasmatacae. Academic Press, London and New York, 1962.
   A review

2. Klienenberger-Nobel, E. L forms of bacteria; Chapter 7 in The Bacteria: A Treatise on Structure and Function; vol. i: Structure, p. 361. Academic Press, New York, 1960.
   A review

3. Edward, D. G., and Freundt, E. A. The classification and nomenclature of organisms in the PPLO group. J. Gen. Microbiol. 14:197, 1956.

4. Klienenberger-Nobel, E. A study of organisms of the PPLO group by electron microscopy. J. Gen. Microbiol. 12:95, 1955.

5. Dienes, L. Controversial aspects of the morphology of PPLO. Ann. N. Y. Acad. Sci. 79:356, 1960.

6. Hayflick, L., and Stinebring, W. R. Intracellular growth of PPLO in tissue culture and in ovo. Ann. N. Y. Acad. Sci. 79:433, 1960.

7. Weibull, C., and Beckman, H. Growth of bacterial L forms and bacterial protoplasts. J. Bacteriol. 79:638, 1960.

Relationship of Protoplasts and L Forms to PPLO (Mycoplasma)

8. Whittler, R. G., et al. Reversion of a PPLO to a corynebacterium during tissue culture passage. J. Gen. Microbiol. 14:763, 1956.

9. Smith, P. S., et al. Conversion of PPLO to bacteria. Proc. Soc. Exp. Biol. & Med. 96:550, 1957.
   A standard PPLO strain ("Campo") propagated 10 years in lab without change. Reverted to corynebacterium when grown in liquid medium without thallium acetate. Could not be reconverted to PPLO.

10. Kelton, W. H., and Gentry, R. F. Derivation of gram-positive cocci from PPLO. Ann. N. Y. Acad. Sci. 79/Art. 10:410, 1960.
    Beginning with 21 standard avian PPLO, 3 human strains and 3 canine strains, by gradually reducing serum content of medium was able to convert all to streptococci.

11. McKay, K. A., and Truscott, R. B. Reversion of avian PPLO to bacteria. Ann. N. Y. Acad. Sci. 79/Art. 10:465, 1960.
    On serial yolk sac passage, Mycoplasma gallinarum, a PPLO responsible for acute coryza in chickens, was reverted to Hemophilus gallinarum, a bacterium responsible for avian chronic bronchitis.

12. Pease, P., and Laughton, N. Observations on corynebacteria and related PPLO. J. Gen. Microbiol. 27:3, 1962.
    Cultured both Mycoplasma hominis and Corynebacterium cervicis from lung of

hyaline membrane disease. By appropriate culture was able to convert each to the other.

13. Pease, P. Evidence that Streptobacillus moniliformis is an intermediate stage between a corynebacterium and its L form or derived PPLO. J. Gen. Microbiol. 29:91, 1962.

14. Dienes, L. Comparative morphology of L forms and PPLO. VIII International Congress for Microbiology, p. 511, 1962.
Points out difficulty in distinguishing PPLO from L forms by present criteria.

Relation of Antimicrobial Agents to Mycoplasma and Protoplasts

15. Perkins, H. R. Chemical structure and biosynthesis of bacterial cell walls. Bact. Rev. 27:18, 1963.

16. Park, J. T., and Stroninger, J. L. Mode of action of penicillin. Science 125:99, 1957.

17. Mandelstam, J. Preparation and properties of cell walls of gram-negative bacteria. Biochem. J. 84:294, 1962.

18. Stroninger, J. L. Mononucleotide acid anhydrides and related compounds as intermediates in metabolic reactions. Physiol. Rev. 40:55, 1960.

19. Mandelstam, J., and Rogers, H. J. The incorporation of amino acids into the cell wall mucopeptide and the effect of antibiotics on the process. Biochem. J. 72:654, 1959.

20. Rogers, H. J., and Mandelstam, J. Inhibition of cell wall mucopeptide formation in E. coli by benzyl penicillin and ampicillin. Biochem. J. 84:299, 1962.

21. Collins, J. F., and Richmond, M. H. A structural similarity between n-acetylmuramic acid and penicillin as a basis for antibiotic action. Nature 195:142, 1962.

22. Brock, T. D., and Brock, M. L. The similarity in mode of action of chloramphenicol and erythromycin. Biochim. et Biophys. Acta 33:274, 1959.

23. Brock, T. D. Chloramphenicol. Bact. Rev. 25:32, 1961.
Good review of this drug

24. Tissieres, A., et al. Amino acid incorporation into proteins by E. coli ribosomes. Proc. Natl. Acad. Sci. 46:1450, 1960.

25. Vasquez, D. Antibiotics which prevent protein synthesis: The uptake of C^{14} chloramphenicol by bacteria. Biochem. & Biophys. Res. Comm. 12/5:409, 1963.

26. Lamborg, M. R., and Zamecnik, P. C. Amino acid incorporation into protein by extracts of E. coli (inhibition by chloramphenicol). Biochim. et Biophys. Acta 42:206, 1960.

27. Rendi, R., and Ochoa, S. Enzymic specificity in activation and transfer of amino acids to ribonucleoprotein particles. Science 133:1367, 1961. Tetracycline action similar to chloramphenicol.

28. Brock, T. D., and Brock, M. L. Effect of novobiocin on permeability of E. coli. Arch. Biochem. & Biophys. 85:176, 1959. Postulate that action is by decreasing new cell wall synthesis.

29. Stroninger, J. L. The optical configuration of the alanine nucleotide residues in cell wall of Staph. aureus. Biochim. et Biophys. Acta 33:280, 1959.

30. Smith, J. L., and Weinberg, E. D. Mechanisms of antibacterial action of bacitracin. J. Gen. Microbiol. 28:559, 1962.

31. Wolfe, A. D. Erythromycin, mode of action. Science 143:1445, 1964.

32. Jordan, D. C. Effect of vancomycin on the synthesis of cell wall mucopeptide of Staph. aureus. Biochem. & Biophys. Res. Comm. 6/3:167, 1961.

33. Abraham, E. P., and Newton, G.G.F. Structure and function of some sulfur-containing peptides. In: Amino Acids and Peptides With Antimetabolic Activity (CIBA Symposium), Little, Brown and Co., 1958, p. 205. Show cephalosporin activity against bacteria to be similar to that of bacitracin and penicillin.

34. Hahn, E. E., et al. Effect of streptomycin on the synthesis of proteins and nucleic acids and on cellular multiplication of E. coli. Biochim. & Biophys. Acta 61:741, 1962.

35. Anand, N., and Davis, B. Effect of streptomycin on E. coli: Damage by streptomycin to the cell membrane of E. coli. Nature 185:22, 1960.

36. Kagan, B. M., et al. Antibiotic sensitivity and pathogenicity of L-phase variants of staphylococci. Antimicrobial Agents and Chemotherapy-1963, Braun-Brumfield, Ann Arbor, 1964, p. 517. Found variants insusceptible to antibiotics influencing cell wall synthesis. Those agents affecting protein synthesis were in general more effective L phases than their respective parent strains.

37. Newton, B. A. The properties and mode of action of the polymyxins. Bact. Rev. 20:14, 1956.

38. Leberman, P. R., et al. The susceptibility of PPLO to the in vitro action of antibiotics: aureomycin, chloramphenicol, streptomycin and sodium penicillin G. J. Urology 64:167, 1950.

39. Leberman, P. R. The susceptibility of PPLO to the in vitro action of antibiotics: terramycin and neomycin. J. Urology 68:394, 1952.

Pathogenicity of PPLO (Mycoplasma)

A. "Eaton Agent" Primary Atypical Pneumonia

40. Eaton, M. D., et al. Studies on the etiology of primary atypical pneumonia. I. A filterable agent transmissible to cotton rats, hamsters and chick embryos. J. Exp. Med. 79:649, 1944.

41. Eaton, M. D., et al. Studies on the etiology of primary atypical pneumonia. III. Specific neutralization of the virus by human serum. J. Exp. Med. 82:329, 1945.

42. Liu, C., et al. Studies on primary atypical pneumonia. II. Observations concerning the development and immunologic characteristics in patients. J. Exp. Med. 109:545, 1959.
Using fluorescent technique, antibody appeared during second or third week and persisted for more than one year. Showed fluorescent antibody to be the same as neutralizing antibody and to differ from cold agglutinins.

43. Chanock, R. M., et al. Serologic evidence of infection with Eaton agent in lower respiratory disease in childhood. New Eng. J. Med. 262:648, 1960.
Retrospective study of 152 children showing antibody response to Eaton agent in 10% of non-bacterial pneumonias.

44. Chanock, R. M., et al. Eaton agent pneumonia. JAMA 175:213, 1961.
Parris Island study revealing that 44% of recruits developed antibody to Eaton agent during 12 weeks' training while only 1.5% had clinical disease. Cold agglutinins were present in only 44% of those with pneumonia.

45. Mufsan, M. A., et al. Eaton agent pneumonia—Clinical features. JAMA 178:369, 1961.
Good clinical study. Points out that this disease cannot be distinguished from viral pneumonia without serological studies. Cold agglutinins present in 45% of Eaton agent pneumonia, 17% adenovirus pneumonia, 6% undiagnosed pneumonia.

46. Kingston, J. R., et al. Eaton agent pneumonia. JAMA 176:120, 1961.
A double-blind study clearly showing efficacy of antibiotic therapy in this disease. (D-methylchlortetracycline 0.9 gm. daily x 6 used.)

47. Chanock, R. M., et al. Growth on artificial medium of an agent associated with atypical pneumonia and its identification as a PPLO. Proc. Natl. Acad. Sci. 48:41, 1962.
Unequivocal demonstration that Eaton agent is a PPLO—subsequently termed *Mycoplasma pneumoniae*.

48. Forsyth, B. R., et al. Etiology of primary atypical pneumonia in a military population.
JAMA 191/5:364, 1965.

49. Grayston, J. T., et al. *Mycoplasma pneumoniae* infections: Clinical and epidemiological studies. JAMA 191/5:369, 1965.
Civilian population study showing isolation of *M. pneumoniae* from 20% of

patients with acute febrile respiratory disease with pneumonitis and 7% acute respiratory disease without pneumonitis.

B. Relation of PPLO (Mycoplasma) to Venereal Disease

50. Dienes, L., et al. The role of PPLO in genitourinary and joint diseases. New Eng. J. Med. 238:509, 1948.
Notes high frequency of PPLO from urethrae of males with gonorrhea and "non-gonococcal" urethritis. Records 2 cases of Reiter's with PPLO grown from urethra and joints.

51. Shepard, M. C. Visualization and morphology of PPLO in clinical material. J. Bact. 73:162, 1956.
Demonstrated intracytoplasmic PPLO in cells scraped from urethrae in NGU. Cultures and smears negative after successful therapy.

52. Klienenberger-Nobel, E. Possible significance of PPLO in human genital infection. Brit. J. Venereal Dis. 35:20, 1959.
Reported PPLO in urethral cultures of 48% of 65 patients with NGU and in 30% of 50 patients with gonorrhea. Healthy population yielded 3% positive cultures.

53. Card, D. H. PPLO of human genital origin. Serologic classification of strains and antibody distribution in man. Brit. J. Ven. Dis. 35:27, 1959.
Found the genital strains isolated in above study to belong to a single serogroup distinct from previously found human strains. 34% of 700 VD Clinic patients had positive PPLO complement fixation while only 2% of 300 blood donors had demonstrable titers.

54. Shepard, M. C. Recovery, propagation and characteristics of T strain PPLO isolated from human cases of nongonococcal urethritis. Ann. N. Y. Acad. Sci. 79: 397, 1960.
Summarizes experience revealing urethral cultures positive for T strain PPLO in 70% of 500 cases of nongonococcal urethritis.

55. Shepard, M. C., et al. Possible role of T strain mycoplasma in nongonococcal urethritis: A sixth venereal disease? JAMA 188/8:729, 1964.
50% of NGU cases were associated with only mycoplasma T and 15% of patients with gonorrhea had concomitant T forms. While penicillin was ineffective in both groups, all subsequently responded to tetracycline.

Possible Relation of Mycoplasma to Reiter's Syndrome and Autoimmune States

56. Jonsson, J. Mycoplasma organisms in synovial fluid from rheumatic joints. Acta Rheum. Scand. 7:287, 1961.
Mycoplasma-like organisms were isolated from synovial fluid in 12/25 non-rheumatoid, "non-infectious" arthriditides. 4 of the cultures had bacterial contaminants. 4 of his patients had Reiter's; only one of these yielded PPLO.

57. Kuzell, W. C., and Markle, E. A. Cultivation of PPLO in Reiter's disease including one incidence of laboratory cross-infection. Ann. N. Y. Acad. Sci.

79:651, 1960.
Reports 5 cases of Reiter's from whom PPLO were obtained from urethral cultures and/or conjunctivae.

58. Bartholomew, L. Isolation of mycoplasma (PPLO) from patients with rheumatoid arthritis, systemic lupus erythematosus and Reiter's syndrome. Arth. & Rheum. 7/3, June 1964 (Abstract).
Isolated mycoplasma in 3 of 4 specimens from Reiter's, 5 of 6 from SLE and 5 of 7 from rheumatoid arthritis. Six specimens from other arthritides were negative.

59. Ford, D. K. The relationship of human genital PPLO to arthritis complicating urethritis. Arth. & Rheum. 3:395, 1960.
Reports inability to grow PPLO from synovial exudates in 12 cases of presumed Reiter's.

Possible Pathogenicity of Protoplasts

60. Brier, G., et al. Survival in vivo (in ovo) of L phase bacteria. Antimicrobial Agents and Chemotherapy-1962, Braun-Brumfield, Ann Arbor, 1963, p. 854.
Organisms remained in L phase while antibiotics were given. Reverted to pathogenic strain and killed embryos when drugs were withdrawn.

61. Muschel, L. H., et al. Formation of bacterial protoplasts by serum complement. J. Immunol. 82:38, 1959.
Demonstrated that in vivo conversion to protoplast is complement-dependent.

62. Carey, W. F., et al. The formation of bacterial protoplasts in vivo. J. Immunol. 84:183, 1960.
Salmonella and other gram-negative pathogens converted to protoplasts when given intraperitoneally to mice. Postulated that protoplast form is parasite defense mechanism affording protection against host factors.

63. Freimer, E. H., et al. Studies of L forms and protoplasts of group A streptococci. I. Isolation, growth and bacteriologic characteristics. J. Exp. Med. 110:853, 1959.
Produced L forms by penicillin or phage lysate. Protoplasts able to produce M protein, streptokinase and DNAse. Were able to revert organisms to original parent strains.

64. Mortimer, E. A. Production of L forms of Group A streptococci in mice. Proc. Central Soc. for Clin. Res. 37:57, 1964.
Demonstrated that group A streptococci transformation can occur in vivo in experimental animals.

65. Kagan, B. M., et al. - Reference #36.
Reported inability to produce staphylococcal L forms in vivo (mice) and inability to produce lesions by subcutaneous injection of those produced in vitro.

66. Braude, A. I., and Siemienski. Protoplast formation in human urine. Trans. Assn. Amer. Phys. 74:234, 1961.

Demonstrated protoplast formation by urinary pathogens exposed to penicillin in vitro or in vivo. Of special interest is the observation that protoplast formation occasionally occurred with tetracycline or chloramphenicol.

67. Kalmanson, G. M., and Guze, L. B. Role of protoplasts in pathogenesis of pyelonephritis. JAMA 190/13:1107, 1964.
Demonstrated protoplasts in renal tissue of rats "cured" of experimental enterococcal pyelonephritis by penicillin. Erythromycin, ineffective against parent strain, was capable of eradicating protoplasts.

68. Andriole, V. T., and Epstein, F. H. Prevention of pyelonephritis by water diuresis: Evidence for the role of medullary hypertonicity in promoting renal infection. J. Clin. Invest. 44/1:73, 1965.