effectiveness of penicillin, but without necessary precautions its use leads to error and will confuse the study of P.P.L.O.

One source of error is that in the area of inhibition, the colonies may remain very small, and the organisms may swell up into large round bodies. They can not be distinguished in an impression preparation from colonies of P.P.L.O. It is probable that the colonies which Salaman identified as mixed colonies of Gonococci and P.P.L.O. were such altered Gonococcus colonies. The author has never seen P.P.L.O. and Gonococcus together in cultures from male patients.

This source of error can be eliminated by using stained agar preparations in which penicillin does not interfere with the identification of P.P.L.O.

The second source of error is the fact that P.P.L.O. can develop from bacteria under the influence of penicillin. It has been shown in another paper that this occurs in pure culture of *H. influenzae*.[7] From a series of 14 throat cultures which were recently studied, P.P.L.O. developed in 10 in the vicinity of the penicillin cups. The distribution of the colonies indicated that they were produced by the penicillin. They were situated in the area of inhibition and were absent in the area not exposed to penicillin. Colonies of P.P.L.O. never develop in throat cultures without the use of penicillin, while they grow very well together with bacteria in cultures from the genito-urinary tract.

According to these observations, penicillin can be used only to screen the specimens for the presence of P.P.L.O. In order to be sure that they are present in the specimens and are not produced from bacteria, their colonies must be seen also in cultures not treated with penicillin. The P.P.L. strains isolated from human patients form a heterologous group, and it is important to distinguish those which are connected with bacteria from those which, like the animal pathogens, do not show such connection.

Summary. Penicillin is of considerable help in isolating pleuropneumonia-like organisms from specimens contaminated with bacteria. Penicillin alters the colonies of certain bacteria in such a manner that in impression preparations they become indistinguishable from pleuropneumonia-like organisms. Furthermore penicillin may induce the growth of pleuropneumonia-like organisms from bacteria. To prove that these organisms are present in the specimens, the characteristic colonies must be apparent in the cultures without the use of penicillin.

[7] Dienes, L., PROC. SOC. EXP. BIOL. AND MED., in press.

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Isolation of Pleuropneumonia-like Organisms from *H. influenzae* with the Aid of Penicillin.*

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It has been previously noted[1] that certain strains of *H. influenzae* show a pleomorphism similar to that observed in *Streptobacillus moniliformis*[1] and bacteroides.[2] The bacilli swell up into large round bodies which either disintegrate into bacteria of usual appear-

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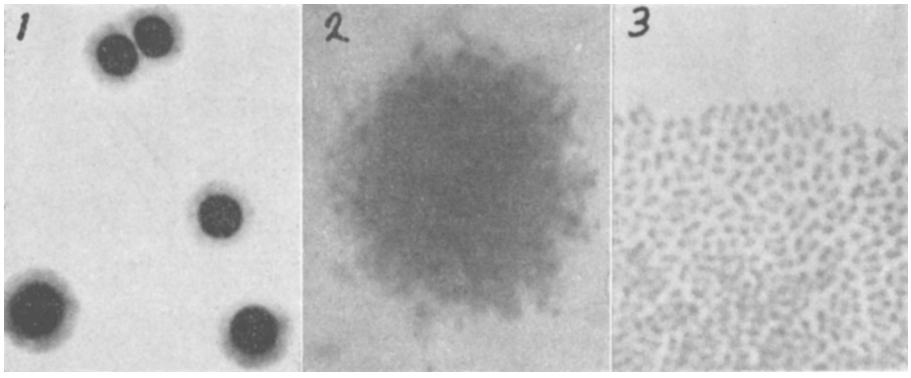
[1] Dienes, L., *J. Bacteriology*, 1942, **44**, 37.

[2] Dienes, L., and Smith, W. E., *J. Bacteriology*, 1944, **48**, 125.



Photograph 1 shows well-developed colonies of the P.P.L.O. isolated from *H. influenzae* (× 90).

Photograph 2 shows a medium-sized colony with high magnification (× 3000). The colony is embedded in the agar and the shape of the individual organisms is not clearly defined.

Photograph 3 shows the edge of the colony of the parent *H. influenzae* strain (× 3000). The organisms are larger than in Photograph 2 and have a sharp contour.

Photographs 1 and 2 were made from stained agar preparations; No. 3 was made from an impression preparation after agar fixation.

ance or develop into tiny colonies resembling the organisms of bovine pleuropneumonia. The pleuropneumonia-like (P.P.L.) growth of *H. influenzae* remained very small, and it could not be determined whether or not they were able to grow indefinitely since they were always overgrown by regular bacterial forms.

Pierce[3] has observed that penicillin facilitates the isolation of L_1 from *Streptobacillus m.* by the elimination of the regular bacterial forms. After some experimentation with penicillin a procedure was found by which pleuropneumonia-like organisms (P.P.L.O.) can be isolated from cultures of *H. influenzae* and grown in pure culture.

H. influenzae is planted heavily on blood agar plates. One or several small troughs are made on the agar without cutting through it, and in each is placed a drop of solution containing 2000 units of penicillin per ml. After absorption of the penicillin solution the plates are made anaerobic by growing *B. prodigiosus* together with the culture and sealing the petri dish with paraffin. The plates are incubated at 30°C and 36°C and are opened after 1, 2, 3, and 4 days. Bacterial growth is completely inhibited in an area 2 to 3 cm in diameter around the trough. It is apparent in stained agar preparations that in the area of inhibition the originally deposited bacteria swell up into large round bodies sometimes as large as 15 to 20 micra without any indication of multiplication. In the border area where growth becomes apparent the organisms are swollen in a similar way. Further out they are replaced first by filaments and then by bacilli of the usual appearance. The swollen organisms do not show any sign of development if they are transplanted. However P.P.L. growth begins to develop from a few large bodies in the area in which bacterial growth is inhibited. Often after 48 hours many P.P.L. colonies are visible in stained agar preparations, and after 3-4 days a crop of tiny colonies, visible with the hand lens, grows in the zone of inhibition. These colonies are embedded in the agar and correspond in appearance and morphology to the organisms of the pleuropneumonia group.

The tiny colonies can be transplanted by cutting out a piece of agar containing them and smearing it on blood agar and boiled blood ascitic agar plates. The plates are treated with penicillin as were the original ones and are incubated anaerobically with *B. prodigiosus*. The cultures later can be grown

[3] Pierce, C. H., personal communications.

[4] Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 470.

without penicillin.

The culture so obtained differs from the parent organism in the following characteristics: (1) The organisms in young cultures are much smaller. They do not have a sharp contour like usual bacilli and they are very soft. The slightest injury deforms or destroys them. (2) The colonies do not grow on the surface of the agar but extend below the surface. The well-developed colonies as visible in Fig. 1 have the appearance typical of the pleuropneumonia group. (3) In older colonies the organisms swell into round bodies. (4) The organisms do not show satellite growth and are very resistant to penicillin. In appearance, staining and physical properties these organisms are very similar to the L_1 isolated from *Streptobacillus moniliformis,* to the corresponding organism isolated from bacteroides and to the whole pleuropneumonia group.

Five strains of *H. influenzae* were studied by the technic described. The strain from which the P.P.L.O. were most easily isolated was cultured from the sputum of a case of bronchiectasis treated with penicillin aerosol. The second strain, an *H. influenzae* Type B, from which large colonies of P.P.L.O. developed but which could not be kept in continuous cultivation was isolated from a blood stream infection. Immediately after isolation this culture was pleomorphic and produced tiny P.P.L. colonies. This property had been lost by the time experiments with penicillin were started. The other strains were not pleomorphic. Two, isolated from blood and from the conjunctiva respectively, produced a few fairly large P.P.L. colonies in the area of inhibition. The third strain, isolated from a sputum did not produce P.P.L. colonies. The strains with the exception of the second were studied immediately after isolation. Most freshly isolated strains of *H. influenzae* apparently can be induced to produce P.P.L.O. by exposure to penicillin. There is considerable variability among the strains concerning the ease with which P.P.L.O. are produced and with which they can be kept in cultivation. A similar variability has been observed in the viability of P.P.L.O. isolated from different bacteroides strains.[2] Use of the same technic has thus far not been effective in isolating P.P.L.O. from gonococci, *E. coli* and several strains of streptococci.

The role of penicillin in the growth of P.P.L.O. is unknown. It is possible that it consists mainly in the elimination of regular bacterial forms. The fact that swelling of bacteria induced by penicillin is very similar to the swelling occurring in naturally pleomorphic strains, may indicate that the effect of penicillin is more complex. The growth of P.P.L.O. does not seem to be a degenerative process because a viable organism, closely similar to an important group of pathogens, is produced.

Summary. With the aid of penicillin a strain of pleuropneumonia-like organism has been isolated from a culture of *H. influenzae.* This strain could be kept in continuous cultivation. Colonies of similar organisms were seen in 3 other cultures of *H. influenzae,* but their propagation in pure culture was not successful.