TABLE II. Agglutinin Absorption Tests with Bacillary and L Immune Sera (Proteus Strain 3)

Rabbit antisera	Bacillary antigen 1:20	1:40	1:80	1:160	1:320	L antigen 1:20	1:40	1:80	1:160	1:320
				Agglutination Before Absorption.						
3 Bac	+	2+	3+	3+	2+*	+	2+	3+	3+	2+
3 L	3+	3+	3+	2+	2+†	3+	3+	3+	2+	0
				Agglutination after absorption with bacilli						
3 Bac	0	±	±	0	0	+	±	±	0	0
3 L	0	0	0	0	0	0	0	0	0	0
				Agglutination after absorption with L						
3 Bac	2+	3+	2+	2+	2+	±	±	±	0	0
3 L	±	0	0	0	0	0	0	0	0	0

* The titer of this serum was 1:5120.
† " " " " " " 1:2560.

bits. The L forms of strain 3 which gave a marked common reaction with the bacilli seem to lack certain antigens which are present in the bacilli. The agglutinin absorption experiment conducted with Proteus strain 3 is recorded in Table II.

Summary. The serological properties of 3 Proteus strains and the L forms isolated from them have been studied. The L antigens were agglutinated both by the bacillary and L immune rabbit sera in a relatively low titre and agglutination occurred in large loose flocci. Clear filtrates of L emulsions gave a good precipitation. The reactions of 2 L type cultures were strain specific. A marked common reaction was present between the bacillary and L forms of 2 strains both in the production of antibodies and in the *in vitro* tests. There was only a slight overlapping in the reactions of the L forms and of the bacilli in the third strain. The similarity of the serological reactions of the bacillary and L forms of 2 Proteus strains is further evidence for the genetical identity of the L forms and the bacilli. The nature of the serological dissimilarity between the bacillary and L forms of one strain, apparent both in *in vitro* tests and in antibody production, can not be interpreted at present.

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Isolation of L Type Cultures from Clostridia.*† (18214)

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Under certain conditions many Gram negative bacilli grow in very small soft pleomorphic forms which are usually designated as pleuropneumonia-like or L forms. The development of similar forms has been observed in the cultures of a few large aerobic Gram positive bacilli probably belonging to the genus Bacillus(1). The isolation of L forms from 2 strains of Clostridium is the subject of this paper. The most efficient method for the isolation of L forms is exposure of the bacilli to penicillin on agar plates. Many Gram negative bacilli produce L type colonies abundantly if they are inoculated on soft horse serum agar plates containing either 400 or 1600 units of penicillin

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1. Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 30.

per ml of the medium. L forms were isolated from Gram positive aerobic bacilli also on similar plates originally free of penicillin but on which 1000 units of penicillin was deposited in a small trough cut out of the agar. Several Clostridia strains (*Cl. welchii, tetani, botulinum* and *sporogenes*) were tested with both these techniques. These were old laboratory strains from the culture collection of the Bacteriology Department at Harvard Medical School. The plates were incubated anaerobically with Fortner's biological method.

None of the strains showed any growth or changed its morphology on the penicillin plates. On penicillin-free plates growth of the bacilli was inhibited in a large area around the penicillin trough. Except in the area immediately surrounding the trough the bacilli started to multiply, but the multiplication stopped and many bacilli swelled to large bodies or disintegrated into small granules when they were reached by the diffusing penicillin. Outside the area of inhibition normal bacterial growth developed and, in the case of *Cl. tetani,* covered the plates as a spreader. No further growth developed in the area of inhibition on the plates inoculated with *Cl. welchii, botulinum* or *sporogenes.* On the plates inoculated with *Cl. tetani,* L type colonies started to grow from the large bodies after 24 hours' incubation and within a few days developed to macroscopically visible size. These colonies grew without difficulty in subcultures on similar media either with or without penicillin.

The macroscopic appearance and morphology of the L type cultures of *Cl. tetani* are similar to those of the third Gram positive aerobic bacillus previously described(1) and of the L type cultures of Bacteroides(2). The photographs illustrating these two could be used to illustrate the L type cultures of *Cl. tetani* as well. These, like the L type cultures isolated from the aerobic Gram positive bacilli, grew better on media without animal serum. No growth was obtained in broth, and the bacillary form of tetanus was not recovered from the L type cultures. No tetanus developed in several mice injected with the L type cultures.

It is possible that most of the Clostridia studied gave negative results because they were old stock strains. It has already been noted that stock strains of Gram negative bacilli also produce L forms less readily. A slight L type growth starting from the large bodies was observed in another stock strain of *Cl. welchii* examined in a similar manner, and L type colonies were isolated without difficulty from an unidentified Clostridium recently cultivated in this laboratory. The observations made with this strain are of interest because the L forms developed under markedly different conditions from those observed with *Cl. tetani.*

This Clostridium strain (2124) was obtained from an apparently normal lung at post mortem examination. No sign of gas bacillus infection was present. The bacillus was strictly anaerobic; it coagulated milk and produced gas abundantly in milk and in meat tubes in 24 hours. Central spores developed in moderate numbers. Immediately after isolation the colonies on agar plates were large, mucoid and with even contours. After several transfers a spreading growth started from the colonies if the plates were incubated for several days. Injection of 0.1 ml of broth culture into guinea pigs produced extensive liquefaction of the subcutaneous tissue and death in 24 hours. These characteristics identify the strain as a Clostridium; further identification is not important, because the phenomena described are more characteristic of the individual strain than of the species and they could be observed only for a short period following isolation of the strain. Clostridium strain 2124 was studied first with the same technique as described with the other Clostridia. The bacilli swelled to large bodies in the neighborhood of the penicillin trough, but no L type growth developed. The culture was slightly pleomorphic on blood agar plates and a few large bodies developed without exposing the cultures to penicillin. In a 2-day-old culture with abundant growth a few colonies were spread again over the previously uninoculated areas of the plate. Growth was

2. Dienes, L. and Smith, W. E., *J. Bact.,* 1944, v48, 125.

inhibited in a wide area around the primary culture. The bacilli inoculated in that area swelled to large bodies and many L type colonies started to develop from them. They grew without difficulty in subcultures either in the presence or absence of penicillin. In this strain the growth of L type colonies was not induced by penicillin, but by exposure of the bacilli to their own metabolic products. Accumulation of metabolic products is probably responsible for the spontaneous development of L type colonies in Gram negative bacilli. This was especially apparent in a strain of Bacteroides in the cultures of which accumulation of metabolic products and the presence of penicillin produced a similar pleomorphism and the development of L forms(2). It is possible that L type cultures could be obtained from our strain 2124 by penicillin also, if the conditions under which the bacilli exposed to it were sufficiently varied.

As previously mentioned, the development of L type cultures in strain 2124 was observed only for a short period of time following isolation. Later the pleomorphism of the strain disappeared and the spreading growth made impossible the use of the technique which had given positive results initially. The L forms isolated from this strain were similar in appearance and morphology to the L forms of *Cl. tetani*. They grew well on nutrient agar without animal serum; they did not grow in broth and the bacilli were not regained from them. The L type colonies of both Clostridia strains studied continued to grow for several months if the medium was appropriate and if the colonies were well-spaced. The L type colonies of tetanus grew sometimes to a size of 6 mm spreading in star-like streamers under the surface of the agar. Some L type colonies of strain 2124 developed to a size of 10 mm or more during a period of 2 months. The colonies spread slowly both on and beneath the surface of the medium. The spreading edge of these large colonies consisted of small forms similar to those present in young colonies. The center of the colonies was autolyzed and only a few large bodies remained intact and could be stained in them.

The genus Clostridium has been added to the groups of bacteria in which L type cultures have been observed. Such cultures have been observed in several families of Gram negative bacilli(3), in the Neisserriae and in aerobic Gram positive spore-bearing bacilli. It is possible that the ability to grow in such forms is a general property of bacteria. The appearance and morphology of the L forms and the conditions under which they develop in the cultures are similar in all cases. There seems to be a marked difference in growth requirements between the L forms isolated from Gram negative and those from Gram positive bacilli. The former can be maintained in continuous growth only in media containing fresh animal protein, whereas the L forms of Gram positive bacilli grow better if fresh protein is absent.

Reversion of the L forms into the usual bacilli and the serological similarity of the bacilli and the L forms has been observed in several species of Gram negative bacilli (Proteus(4), Salmonella(3), *H. influenzae*, Bacteroides(2) and *Streptobacillus moniliformis*(5)). These observations leave hardly any doubt that the bacilli and the L forms are different growth forms of the same organism. This opinion is now held by all investigators working in this field(6). However, the nature and significance of the L forms is entirely unknown. The availability of these forms in many different groups of bacteria may provide an opportunity to get information in this matter.

Summary. L type cultures have been isolated from a strain of *Clostridium tetani* with the help of penicillin. Similar cultures were obtained from a freshly isolated unidentified Clostridium when the area surrounding a 2-day-old agar culture was reinoculated with the same strain. The metabolic products diffusing from old culture inhibited growth

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4. Dienes, L., *J. Bact.*, 1949, v57, 529.

5. Brown, T. McP., and Nunemaker, T. C., *Bull. Johns Hopkins Hosp.*, 1942, v70, 201.

6. Klieneberger-Nobel, E., *J. Gen. Microbiol.*, 1949, v3, 434.

and transformed the bacilli into L forms. The L type cultures obtained are similar in appearance and morphology to those isolated previously from a Gram positive spore-bearing bacillus. They differ from the L forms of Gram negative bacilli inasmuch as they grow better in the absence rather than the presence of animal serum and have a tendency to form large spreading colonies increasing in size for several months.

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The Storage of Methoxychlor in the Fat of the Rat. (18215)

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(Introduced by A. J. Lehman)

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Methoxychlor, p,p' dimethoxy diphenyl trichloroethane has been shown to have a considerably lower order of toxicity than DDT(1,2). There is some indication, as in the case of certain isomers of benzene hexachloride(3), that a low order of toxicity may be associated with a reduced tendency for storage in the fat. A study of the storage of methoxychlor has therefore been made to see whether a similar correlation exists.

Method. Three groups of 24 weanling 21-day-old rats, equally divided as to sex were placed on diets containing methoxychlor at concentrations of 25, 100 and 500 p.p.m. After 4-, 9-, 13- and 18-week exposures, 2 males and 2 females from each group were sacrificed and abdominal fat analyzed for methoxychlor. Equal numbers of control animals were treated similarly. At the end of 18 weeks, exposure to methoxychlor was terminated and the survivors placed on control diet. Following 2 and 4 weeks on control diet the remaining animals were sacrificed and the tissues also analyzed for methoxychlor. The basic diet was a ground commercial rat biscuit with a fat content of 5%. Analysis for methoxychlor gave negative results. The experimental diets were prepared by mixing appropriate amounts of corn oil solution of methoxychlor with the ground rat biscuit. The methoxychlor used was the p,p' isomer and was of technical grade. The experimental animals were kept in separate cages and food consumption and weight gains recorded weekly. The control animals were housed in groups, and their weights recorded at approximately four-week intervals. Tissues were prepared for analysis by successive extraction of the fat with ethyl ether. Methoxychlor was determined by a method developed in this laboratory(4). This method consists essentially of saponification of the fats by ethanolic KOH, with simultaneous conversion of the methoxychlor to the dehydrochloride. Separation of the dehydrochloride of methoxychlor from the saponified fats is effected by extraction with petroleum ether. The dehydrochloride is then nitrated by the standard Schechter procedure. Upon treatment with sodium methylate in benzene-methanol solution a stable red color is produced having an absorption maximum at 530 mμ. The sensitivity is sufficiently great to quantitate 5 μg of methoxychlor when contained in a maximum of 5 g of rat fat (1 p.p.m.).

Results. The incorporation of methoxychlor at concentrations of 100 and 500 p.p.m. in the diet produced a retardation of growth in both male and female rats. After 14

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