THE TRANSFORMATION OF TYPHOID BACILLI INTO L FORMS UNDER VARIOUS CONDITIONS[1, 2]

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Received for publication March 6, 1950

That L type cultures can be isolated from *Salmonella typhosa* and many other bacterial species on agar plates containing high concentrations of penicillin was reported in previous papers (Dienes, 1948*a*,*b*, 1949). Transformation into L forms was observed under various other conditions. It often occurred as a result of spontaneous autolysis of cultures (Dienes, 1942). In *Proteus*, refrigeration of the cultures, antagonism between strains, and exposure to tap water resulted in the development of these forms (Dienes, 1949). The study of the conditions inducing L transformation has extended over many years. The behavior of various bacilli and cocci was studied in the presence of bacteriostatic agents and after exposure to injuries that bacteria encounter in their natural life. Since the studies in *Proteus* were reported, most significant observations have been made in *S. typhosa*. In this species L forms developed upon exposure of the bacilli to various types of chemical injuries, to antibody and complement, and to bacteriophage. Observations relating to the conditions in which typhoid bacilli were transformed into L forms are presented in this paper. In the following paper the characteristics of *Salmonella* L type cultures, including *S. typhosa*, will be described.

The behavior of six freshly isolated typhoid strains was studied on penicillin plates. All behaved similarly and produced abundant L type colonies, which grew without difficulty in subcultures. It was previously mentioned (Dienes, 1948*a*) that an old laboratory strain produced L type colonies poorly. The medium and techniques most advantageous for the production of *Proteus* L forms also were most favorable for typhoid; however, more uniform results were obtained by using anaerobic incubation in the typhoid experiments. Anaerobic incubation is also necessary for the maintenance of typhoid L strains in subcultures. The conditions essential for the growth of typhoid L type cultures in the presence of penicillin are the following: the agar must be soft (the usual nutrient agar should be diluted with 50 to 100 per cent broth); the medium must contain 10 to 20 per cent animal serum; and, as in the case of *Proteus*, horse serum obtained from defibrinated blood is superior to human serum or ascitic fluid. L type colonies were rarely obtained with rabbit serum and never with mouse

[1] The expenses of this investigation have been defrayed in part by a grant from the Commonwealth Fund.

[2] This is Publication No. 110 of the Robert W. Lovett Memorial Foundation for the Study of Crippling Diseases.

serum. High concentrations of penicillin (100 to 5,000 units per ml of the media) yielded more uniform results.

Previously various antibiotics in addition to penicillin and many bacteriostatic chemicals were studied to determine whether these induced the growth of L type colonies (Dienes, 1949). Large bodies were produced from bacteria by several chemicals, but only one, carboxylmethoxylamine, produced L type colonies. The effects of this chemical and of glycine, acriflavine, crystal violet, aureomycin, and chloramphenicol were studied with *S. typhosa* and a strain of *Salmonella typhimurium*.

Carboxylmethoxylamine, 0.1 per cent concentration, in soft horse serum agar plates inhibited all growth. With concentrations of 0.05, 0.025, and 0.012 per cent the bacilli swelled into large bodies and many L type colonies developed. With 0.05 per cent these remained very small and soon disintegrated. They grew to larger size with the lower concentrations, but a few bacillary colonies also developed. The margin between the growth-inhibiting concentrations for the bacilli and the L type colonies is narrow. The strain of *S. typhimurium* behaved in a similar way.

Inhibition of certain bacteria and swelling into large bodies in the presence of glycine were observed by Gordon and Gordon (1943). Concentrations of 1 per cent glycine in soft horse serum agar allowed good growth of typhoid bacilli, and only a few of the bacilli swelled into large bodies. With 2 per cent, all bacilli became swollen and many L type colonies developed together with a few large bacillary colonies. With 4 per cent, only numerous small L type colonies developed. The effects of higher concentrations were not studied. The L type colonies grew well in subculture in the presence of glycine. The L1 isolated from *Streptobacillus moniliformis* also grew abundantly in the presence of 1 per cent glycine. Its growth was inhibited with 2 per cent and absent with 4 per cent. Glycine induced a transformation in typhoid bacilli similar to that induced by penicillin, but only in a narrow range of concentration.

A possible effect of acriflavine on bacilli was suggested by its production of small colony variants in yeast (Ephrussi *et al.*, 1949). In parallel experiments the influence of acriflavine and crystal violet was studied. Both dyes allowed good growth of *S. typhosa* in the highest concentration tested (0.02 per cent). Acriflavine markedly changed the morphology of the bacilli. These grew as long filaments, and large bodies were produced in moderate numbers. L type colonies did not develop. Crystal violet only occasionally produced a few long filaments and large bodies. The L type cultures of *S. typhosa*, *S. typhimurium*, and two pleuropneumonialike strains isolated from patients were inhibited by both dyes in the lowest concentrations tested (acriflavine, 0.002 per cent, and crystal violet, 0.0005 per cent). The bacillary forms were more resistant to these dyes than the L forms.

Attempts to produce typhoid L type colonies with streptomycin were unsuccessful, as in previous experiments with other bacteria. Bacillary growth was markedly inhibited by streptomycin in concentrations of 4 μg per ml. Long filaments and large bodies were produced, but no L type colonies developed.

The growth of typhoid L type cultures was completely inhibited by this concentration. It is of interest that growth of the bacillary and L forms of *S. typhosa* and *S. typhimurium* and of pleuropneumonialike organisms isolated from humans and rats was almost completely inhibited by 10 μg per ml; a few colonies, however, developed on plates with the highest concentration tested (45 μg per ml).

Aureomycin in concentrations of 10 μg per ml allowed a slight growth of *S. typhosa*. The bacilli grew as long filaments, which then swelled into large bodies. A few tiny L type colonies started to grow but later disintegrated. No growth developed with higher concentrations. The L type cultures of *S. typhosa*, *S. typhimurium*, and strains of pleuropneumonialike organisms grew sparsely on plates containing 10 μg per ml and not at all with the higher concentrations. Chloramphenicol markedly inhibited the growth of typhoid bacilli in concentrations of 0.004 μg per ml, but a few small colonies also developed with concentrations of 0.016 μg per ml. Long filaments and large bodies developed on these plates, and a few small L forms started to grow from the large bodies. The L forms of *S. typhosa* and *S. typhimurium* were slightly more sensitive than the bacilli; their growth was markedly inhibited by 0.002 μg per ml, and was absent with 0.016 μg per ml. The L1 isolated from *Streptobacillus moniliformis* was completely inhibited by 0.016 μg per ml, and the multiplication of the pleuropneumonialike strains was barely recognizable with this concentration of chloramphenicol.

Refrigeration and exposure of cultures to distilled water, which induces pleomorphism in the spreading filaments of *Proteus*, exerted no effect on *S. typhosa* cultures. It was not possible to produce L forms by heating the cultures. The few typhoid bacilli surviving heating of broth cultures to 56 C for 30 and 60 minutes did not become pleomorphic and no L type colonies were produced. Occasionally a few long filaments and large bodies were observed in colonies developing from heated bacilli.

The effects of penicillin apparently are nonspecific. The swelling of bacilli and the development of tiny L type colonies were induced also by other antibiotics (aureomycin and chloramphenicol). L type colonies developed abundantly under the influence of carboxylmethoxylamine and glycine. It is probable that a systematic study of a large number of bacteriostatic agents would disclose others with similar effects. The swelling of bacteria into large bodies occurs more often than the development of L forms. Further development into L forms depends in part at least upon whether the L forms are more or less sensitive than the bacteria to the unfavorable influences to which they are exposed. The toxicity of penicillin is characteristically low for the L forms whose growth is not inhibited even by the presence of 10,000 units per ml. The margin between the inhibiting dose for L forms and bacteria is small with carboxylmethoxylamine and glycine, and it is scarcely discernible with aureomycin and chloramphenicol.

The study of the development of L forms under the adverse conditions encountered by bacteria in their natural life is of particular interest. One such situation is exposure of bacilli to immune serum and complement. This induced the development of L forms from typhoid bacilli. The arrangement of the

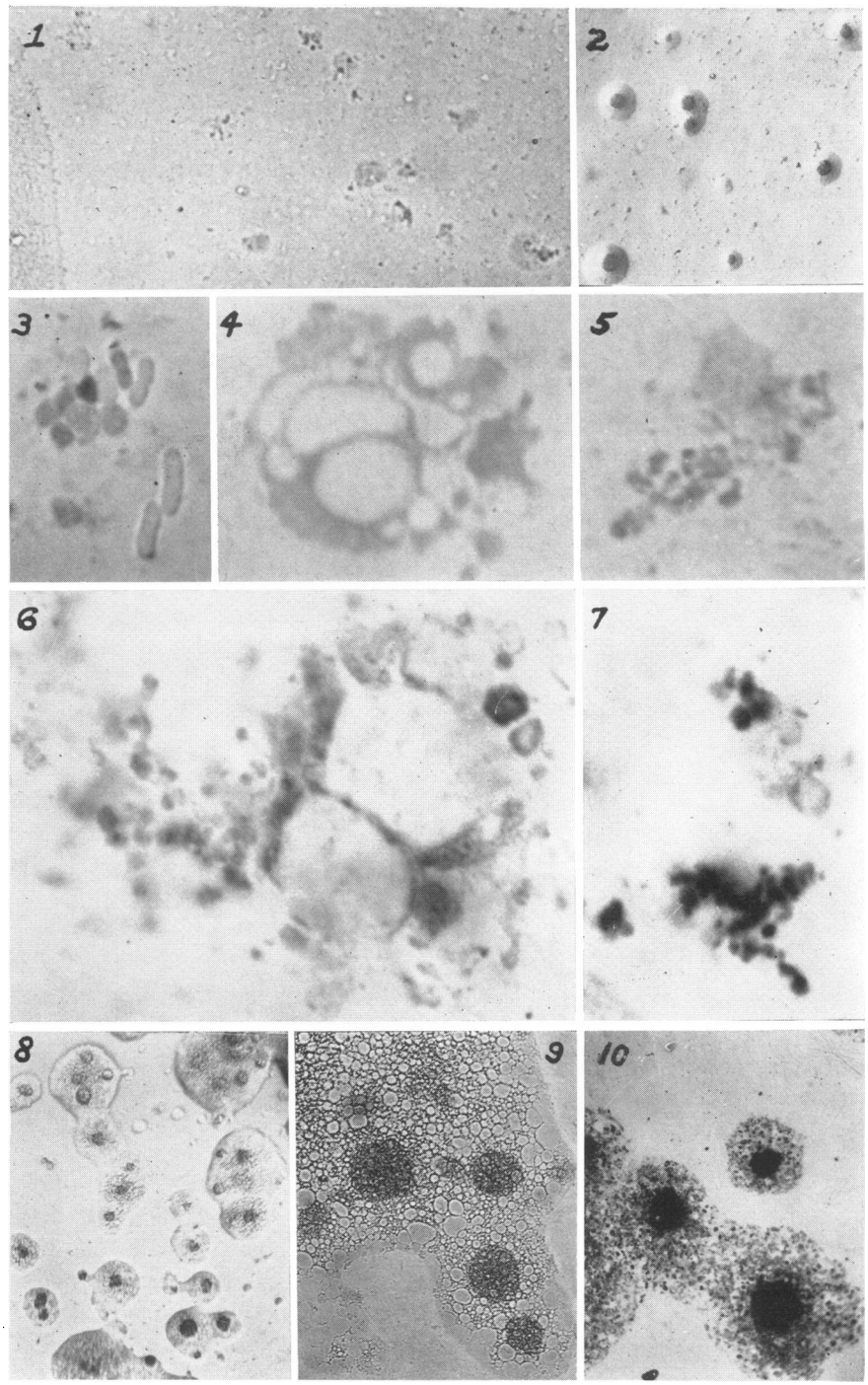

Figure 1. 1. Surface of a soft horse serum agar plate inoculated with typhoid bacilli previously exposed to antibody and complement. The edge of a bacillary colony and 11 tiny L type colonies are visible. Unstained, × 100.

2. The same plate as in no. 1 photographed after 3 days' incubation. The L type colonies are larger in size and show the characteristic appearance. Unstained, × 30.

experiments was simple and is apparent in the tables. Lysis was induced with both rabbit immune sera and guinea pig complement and with sera from both typhoid patients and normal subjects. In the experiment described in table 1 decreasing amounts of rabbit antityphoid sera were mixed with fresh guinea pig serum and typhoid bacilli. The final mixture contained a 1:6 dilution of guinea pig serum and about 100,000,000 typhoid bacilli per ml. Immediately after the bacilli were added and after varying periods of incubation in a 37 C water bath, a loopful of the mixture was transferred to soft horse serum agar plates. The plates were incubated anaerobically, using Fortner's technique (Dienes and Smith, 1944). The antibody and complement solutions were each cultivated separately in a similar way. The complement alone did not markedly reduce the growth of typhoid bacilli. The reduction was marked after 15 minutes' exposure of the bacilli to antibody and complement, and, in addition to bacillary colonies, a moderate to a large number of L type colonies developed. In figure

TABLE 1

Production of L type colonies by exposure of typhoid bacilli to immune rabbit serum and complement

ANTITYPHOID RABBIT SERUM, DILUTION	FRESH GUINEA PIG SERUM, DILUTION	TYPHOID BACILLI PER ML (APPROX.)	ONE LOOPFUL PLANTED AFTER 15-MIN INCUBATION	
			No. of bacterial colonies	No. of L colonies
1:20	1:6	100,000,000	63	Moderate
1:40	1:6	100,000,000	20	Moderate
1:80	1:6	100,000,000	3	Abundant
None	1:6	100,000,000	Abundant	None

The tubes were planted again after 45 minutes. The number of bacterial colonies decreased further, and no L colonies developed.

1, no. 1, are shown the edge of a bacillary colony and many L type colonies, which developed after overnight incubation. The L type colonies after 3 days' incubation are shown in no. 2. Subcultures were obtained without difficulty, and the

3. Typhoid bacilli exposed to antibody and complement showing partial swelling into round forms immediately after inoculation on the plate. Wet-stained agar preparation, × 2,000.

4 and 5. Tiny L type colonies from plate illustrated in no. 1. In no. 4 large bodies with multiple vacuoles are visible. Wet-stained agar preparation, × 2,000. In no. 5 the earliest L type growth from a large body is shown. Dry-stained agar preparation, × 2,000.

6 and 7. A more advanced stage in the development of L type colonies. In no. 6 the surface of the colony is shown with two large empty blebs and several large bodies. New growth of small forms is seen on the left. In no. 7 the same field is shown as in no. 6 with the focus set beneath the surface of the agar. The L forms are growing into the agar as strands of darkly stained granules. Wet-stained agar preparation, × 2,000.

8, 9, and 10. Fully developed L type cultures obtained by exposing typhoid bacilli to antibody and complement. No. 8, unstained, low magnification, × 30. No. 9, unstained, × 100. The foamlike appearance of the surface of the colonies is produced by vacuolization of the large bodies. No. 10, same colonies as in no. 9, stained, × 100.

L type cultures have been carried for a period of more than a year on penicillin-free plates. Six similar experiments were made in which two different rabbit antityphoid sera and two freshly isolated strains of typhoid bacilli were used. All results were essentially similar. The antityphoid sera were prepared in the laboratory and contained no preservatives.

The patient's serum (table 2) was obtained in the third week of typhoid fever. It showed a moderately positive Widal reaction (1:160). The blood culture was positive for typhoid bacilli in broth, and negative in agar pour plates. Efforts to isolate L type colonies directly from the blood were unsuccessful. The serum, undiluted and diluted 1:5 and 1:20 with saline, was inoculated with typhoid bacilli so that the mixture contained about 100,000,000 bacilli per ml. The mixtures were incubated and transferred as in the experiments with rabbit serum. Another experiment was performed with the inactivated patient's serum and guinea pig complement. The inactivated patient's serum and complement

TABLE 2

Production of L type colonies by exposure of typhoid bacilli to patient's serum

FRESH PATIENT'S SERUM, DILUTION	TYPHOID BACILLI, PER ML (APPROX.)	ONE LOOPFUL MIXTURE PLANTED AFTER 25-MIN INCUBATION	
		No. of bacterial colonies	No. of L colonies
Undiluted	100,000,000	Moderate	Moderate
1:5	100,000,000	Moderate	None
1:20	100,000,000	Abundant	Few
Undiluted (inactivated)	100,000,000	Abundant	None

The tubes were planted again after 1 hour and 25 minutes' and 4 hours' incubation with essentially similar results.

used alone did not reduce the bacterial growth, and no L type colonies developed in the transplants. The number of viable bacilli was markedly reduced by the undiluted fresh serum and by the 1:5 dilution, and a moderate to a large number of L type colonies grew in the transplants. The number of bacterial colonies was not reduced by the 1:20 dilution of fresh serum, but a few L type colonies grew in the transplants. When guinea pig complement was added to the inactivated serum, the reduction of bacterial growth was marked in all serum dilutions, and in some L type colonies also developed.

In addition to the patient's serum just described, four other sera, obtained from normal female subjects, were studied in a similar way. Two sera exerted a marked and two a slight bactericidal effect. In the transplants made from the latter two and from one with marked bactericidal effect, L type colonies developed among the bacterial colonies. It is not known whether the subjects were previously inoculated against typhoid fever or whether the effects observed were due to specific antibodies or to the normal bactericidal property of fresh human sera. Only one of the four sera showed a slightly positive Widal reaction (1:12).

A strain of *Salmonella typhimurium* was examined in a similar way in the presence of *S. typhimurium* rabbit antiserum. This species is not susceptible to serum lysis. The growth of the bacilli was not inhibited, and L type colonies were not produced. When the strain was exposed to penicillin or to glycine, abundant L type colonies developed.

L type colonies were observed in all experiments done with rabbit immune serum, but their development was variable. In the experiments in which the bactericidal effect was marked, L type colonies developed most abundantly after short exposure to antibody and complement (less than 30 minutes) and were absent after longer exposure. When the bactericidal effect was less marked, many bacterial colonies developed in the transplants, and L type colonies grew together with the bacteria. The bacilli apparently reacted to antibody and complement in the same manner as to penicillin and other bacteriostatic agents. The ability to grow in the usual bacillary form is lost first. The ability to grow in L form persists longer; later this is also destroyed.

The appearance, morphology, and growth requirements of the L type cultures obtained by antibody-complement reactions are similar to those of L type cultures that develop under the influence of penicillin. Both showed a comparably high resistance to penicillin, although the former cultures had not previously been exposed to the antibiotic. The typhoid bacilli were never recovered from these cultures. The L forms produced by penicillin and by antibody and complement react similarly in the agglutination test (Weinberger *et al.*, 1950). In *S. typhosa*, as in *Streptobacillus moniliformis* and *Bacteroides*, the L type cultures are identical regardless of the nature of the influences inducing them.

The circumstances under which L type colonies develop after exposure to antibody and complement leave no doubt as to their origin from the bacilli. The rabbit serum, complement, and human sera planted on soft horse serum plates never grew out L type colonies. The patient's blood was examined with special care since the isolation of L forms from it would be of great interest. The microscopic observation of the development of L type colonies and their serological similarity to the bacilli offer positive evidence for their derivation from the bacilli. The L type colonies develop under the influence of antibody and complement in the same manner as they do after exposure of the bacilli to penicillin. After contact with antibody and complement some of the bacilli become swollen into round forms. These swollen forms are illustrated in figure 1, no. 3. Transferred to soft horse serum plates, these grow to a larger size, and the growth of the L type colony starts from these large bodies. This process is also illustrated in nos. 4, 5, 6, and 7. In the following paper (Weinberger *et al.*, 1950) data will be presented indicating that the sugar fermentation reactions and serological specificity of L type cultures obtained by antibody and complement and by penicillin are similar and correspond to those of *S. typhosa*.

After the development of L type colonies from bacilli surviving serum lysis was observed, another naturally occurring destructive process of bacteria, lysis by bacteriophage, was studied. Phage filtrate effective for our typhoid strains was obtained from Dr. P. R. Edwards. The phage was added to young

broth cultures of two freshly isolated strains in sufficient amounts to produce lysis of almost the whole culture developing in transplants to agar plates. A loopful of the broth culture was transferred to soft horse serum plates immediately after addition of the phage, after 20 minutes' incubation, and after 2 hours' incubation. No change in the morphology of the typhoid bacilli in the broth cultures was visible during this time, and the growth obtained in the successive transfers was similar. The majority of the bacilli became markedly swollen on the plates after 3 hours' incubation. These did not develop into large bodies similar to those produced by penicillin but disintegrated into formless heaps, which later disappeared without a trace. After incubation overnight tiny secondary colonies developed in the lysed areas, between which single bacillary forms were scattered on the surface of the agar. Some of the tiny secondary colonies were markedly pleomorphic and consisted of bacilli growing into long filaments and large bodies. Many of the bacilli scattered on the surface of the agar showed a similar pleomorphism, and L type colonies grew from some of these large bodies. The L type colonies developed to fair size. Isolation from the plate was not attempted. From these observations it appears that the direct action of the phage does not induce the growth of L forms. Some of the bacilli surviving the influence of phage become pleomorphic like those that survive the influence of various other injuries and produce the L type colonies. The morphological changes in the bacteria are similar in all instances in which transformation into L forms was observed.

Whether the phage survives and multiplies in the L forms and whether these forms can be lysed by the phage was not studied. Studies of this type, and those relating to the metabolic and serological properties of L forms, may yield information as to the nature of the differences between the L forms and the bacilli.

DISCUSSION

According to the observations described, L forms are produced from typhoid bacilli under various conditions having in common only that each results in injury to the bacilli. Similar observations were made previously with *Proteus*. Whenever the development of L forms was observed, a great number of the bacteria were destroyed; some of those surviving developed into L forms. A possible exception to this rule was the behavior of colon bacillus strain (12394) previously described (Dienes, 1942).

Certain conditions that induce L type growth—for example, exposure to glycine in high concentration—are entirely artificial and are not encountered by the bacteria in their natural life. It seems doubtful also that typhoid bacilli naturally are exposed to high concentrations of penicillin. On the other hand, lysis by serum and by bacteriophage probably plays an important role in the life of the bacteria. These considerations pose the question of whether transformation into L forms is a useful response in the survival of bacteria. Until more is known of the occurrence and properties of the L forms, this question cannot be answered. There is general agreement among investigators now that the L and bacillus are forms of the same organism. In a recent paper Kleine-

berger-Nobel (1949) abandoned the symbiosis theory and accepted this viewpoint.

The observation that L forms are produced from typhoid bacilli exposed to human sera suggests the possibility that these forms may have a role in the infectious disease process. During many years of experience with these forms, in only one instance has it seemed likely that bacilli are in the L form *in vivo* (Dienes and Smith, 1944). Pleuropneumonialike organisms that are indistinguishable from L forms in appearance and morphology are often recovered from the genitourinary tract and occasionally from other sites (Dienes *et al.*, 1948). Following intensive penicillin treatment, they can also be cultured from the upper respiratory tract and various suppurative processes. Whether or not these organisms are produced *in vivo* by the clinical administration of penicillin has not thus far been determined. The L forms of virulent bacterial strains are not pathogenic for the usual laboratory animals. It is not unlikely that L forms are produced from bacteria *in vivo*, but we have no information as to the significance of this process.

SUMMARY

Freshly isolated strains of *Salmonella typhosa* produce L type colonies abundantly on soft horse serum agar plates containing high concentrations of penicillin. The L type colonies grow well with the highest concentration of penicillin tested, 10,000 units per ml of the media. Carboxylmethoxylamine and glycine induce a similar transformation but only in a narrow range of concentration. The L forms are only slightly more resistant to these agents than the bacteria. L forms start to develop from bacteria exposed to aureomycin and chloramphenicol, but their growth is arrested in the initial stages. The resistance of the L forms and of the bacteria to these antibiotics is about the same. The L forms are more sensitive to acriflavine and crystal violet than are the bacteria. L forms were obtained from typhoid bacilli that survived exposure to specific antibody and complement. Bacilli lysed with bacteriophage did not develop into L forms, but those that survived in lysed cultures were occasionally transformed into these forms. Sublethal injuries of various types induced transformation into L forms, and the development of the latter depends largely upon whether they are more or less susceptible than the bacteria to the particular injury. Certain of the injuries producing the L transformation are encountered by the bacteria in their natural environment suggesting that this phenomenon may occur in the natural life of bacteria.

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