PART III

IN VIVO STUDIES
A survey of the early literature on aberrant bacteria has shown that what are now considered to be L forms or cell wall defective variants were well recognised entities long before Klieneberger drew attention to the presence of these microbial variants in the cultures of Streptobacillus moniliformis. The review of the more recent interdisciplinary investigations carried out on L forms and other wall defective microbial variants has revealed that there is still a considerable lack of information on their biological and pathogenic properties.

Investigations carried out on ten strains of Salm. gallinarum have shown penicillin, glycine and a number of other substances used in the present work are capable of inducing L transformation in this bacterial species. Considerable variations occurred between the strains in their ability to respond to L transformation. Isolates from more recent clinical material were found to undergo L transformation more readily than the standard laboratory strains. Likewise, it was easier to induce L transformation in the smooth than the rough strains of Salm. gallinarum. The ability of field isolates to undergo spontaneous L transformation without the aid of any known incitant is believed to be a new finding that has not been recorded in the Salmonella group of organisms.

A number of cultural and environmental factors that aid in the transformation and propagation of these L forms were investigated and discussed. All ten strains of Salm. gallinarum produced only an unstable L growth on a solid hypertonic medium. A hitherto unrecorded finding is that in the two laboratory strains which, though unable to produce stable L growth, were able to do so in a liquid medium; furthermore the elimination of the serum requirement for stabilisation in a liquid medium is hoped will provide the basis for the future immunological and biochemical characterisation of the L forms of Salm. gallinarum.
Biochemical investigations on some of the L forms and revertants arising from these altered variants showed that they resembled in general their parental forms denoting absence of mutational changes.

Filtration studies have revealed that not all elements in L cultures were filterable.

Investigations on the ultrastructure of L forms of Salm. gallinarum show that the inducing agents used in the current studies bring about varying degrees of cell wall damage resulting in the production of cell wall defective variants not only varying in their sizes but also in the amount of cell wall layers retained.

The pathogenic properties exhibited by the unstable L forms of Salm. gallinarum were shown to be due to their reversion to the vegetative form in vivo and the death of the experimental host was ascribed to the endotoxic properties of the organism.
PART III

PATHOGENICITY OF CELL WALL DEFECTIVE VARIANTS AND BACILLARY FORMS OF SALMONELLA GALLINARUM IN CHICKEN EMBRYOS

GENERAL INTRODUCTION

Since the first isolation of L forms of bacteria by Klieneberger in 1935, their role in the pathogenesis of disease conditions in man and animals has not been clearly defined. Over the past 20 years in particular many publications have appeared centred on the question of the pathogenicity of the L phases of bacteria but the findings reported are far from clear-cut and many problems remain unresolved. These investigations can be broadly categorised into 4 groups viz.: a. Proof of non-patnogenicity of L forms of bacteria. b. Demonstration of pathogenicity of L forms in experimental animal models or in natural hosts. c. Isolation/demonstration of L forms in pathological conditions in which classical bacterial forms could not be found; the possibility of L forms being present to account for the recrudescence of infection despite aggressive antibiotic therapy and their frequent appearance in carriers or disseminators of infection with no clinical evidence of active disease. d. The demonstration of pathogenicity of L forms due to
their reversion to classical forms *in vivo*.

The proof of non-pathogenicity of L forms was first shown by Klieneberger (1938) who found that mice could not be infected with L cultures of *Streptobacillus moniliformis*. Three years later Heilman (1941a & b) arrived at a similar conclusion based on the results of inoculating \(L_1\) cultures into mice and embryonated chicken eggs. Dienes and his group of workers also mentioned the non-pathogenicity of L forms derived from a number of bacterial species without providing much experimental data (Dienes, 1942, 1950; Dienes et al., 1950a; Weinberger et al., 1950). The L forms of *Vibrio cholerae* have been reported to be non-pathogenic (Minck & Minck, 1951). Silberstein (1953) inoculated mice intraperitoneally with stable L forms of *Salm. typhimurium* and found it to lack virulence in contrast to the rapid fatalities produced by the parent vegetative forms. Freundt (1956), in his studies on the L forms of *Streptobacillus moniliformis*, showed that stable L forms of this organism possessed little or no pathogenicity in mice. Minck and Lavillaureix (1956) reported that L transformation of a bacterium entails in almost every case (with the exception of *Vibrio L forms*) the total disappearance of the initial ability of the organism to cause disease, and they ascribe this lack of pathogenicity to the rapid destruction of L forms by substances that were detrimental to their survival
The L phase of *Clostridium perfringens* has been reported to be non-virulent for the guinea-pig (Kawatomari, 1958). Lipnicki (1958) reported that mice inoculated intraperitoneally and subcutaneously with L forms of *Salm. typhosa* showed no evidence of infection. Kagan and Koptelova (1963) seemed to have investigated in detail the pathogenic properties of 7 stable L forms of *Salm. typhosa* and of these only 2 retained their original virulence while the remaining five did not prove fatal to experimental animals and on this basis they concluded that in the process of forming stable L forms, these variants lost their virulence, though not their viability. The L phase of a virulent Group A streptococcus injected into mice did not prove to be pathogenic although they persisted *in vivo* for long periods (Schmitt-Slomska, Sacquet & Caravano, 1967). L forms of *Listeria monocytogenes* lacked pathogenicity when inoculated into mice by various routes (Brem & Eveland, 1968b). Intravenous inoculation of stable L forms derived from some bacterial species have failed to initiate renal infection in experimental animals (Young & Dalquist, 1967; Watt, 1970; Watanakunakorn & Bakie, 1974). Similarly in experimental endocarditis produced in animals, stable L forms of *Staphylococcus aureus* failed to colonise the heart valve lesion (Linnemann, Watanakunakorn & Bakie, 1973).

Among the earliest to ascribe pathogenicity to L
forms was Minck (1950, 1951) who demonstrated experimentally the lethality of L forms of *Vibrio cholerae* for mice. Tulasne and Lavilleareix (1954, 1955) injected into mice by various routes suspensions of L forms of a *Vibrio* spp. which in one to six days killed mice from whose tissues L forms could be re-isolated. They showed that the pathogenicity of the L forms was due to a thermostable toxic factor showing the properties of endotoxin. Following these reports, Scheibel and Assandri (1959) found that stable L forms of *Clostridium tetani* were highly toxigenic and when injected into mice intramuscularly produced tetanic syndromes, indicating that the capacity to produce exotoxins was retained by the L phase variants. Glycine induced L forms of *Cl.tetani* also appeared to be toxigenic (Rubio-Muertos & Gonzalez-Vazquez, 1960). Kagan, Shchegolev & Prozorovsky (1964) found that L forms of *Salm. typhimurium* when injected intravenously or intraperitoneally into rabbits seldom killed the experimental models but were dermatotoxic when given subcutaneously. Intra-articular injections of L phase variants of streptococci caused arthritis similar to that following multiple injections of streptolysin S produced by the parent organisms (Lack, 1967).

Apart from their ability to kill their hosts by their toxigenic properties, L forms derived from various
bacterial species are also known to initiate infection in susceptible experimental animals and their natural hosts. Minck (1955) reported that stable L forms of Proteus inoculated into the allantoic and yolk sacs of embryonated chicken eggs resulted in the death of the embryos. In the experiments of Kagan and Koptelova (1963) of the seven stable L forms of Salm. typhosa tested for their pathogenic properties two proved to have retained their virulence for mice which clearly indicated that some of the stable L phase variants of Salm. typhosa were potential pathogens.

Perhaps the first report of L forms being able to cause disease in a natural host is that of McKay, Abelseth and Vandreumel (1966) who reported that protoplasts of Haemophilus parainfluenzae produced enzootic pneumonia in pigs. The histopathological lesions produced resembled more the viral and PPLG type of pneumonia rather than the bronchopneumonic type of lesions produced by the vegetative forms of H. parainfluenzae. G.Y. Kagan (1968) showed that with a series of injections of L forms of a streptococcus given intravascularly or into the paratonsillar tissue of monkeys produced cardiac anginas and in some instances myocardial organic lesions. The same author also demonstrated that suboccipital injections of L forms from a β-haemolytic streptococcus, Staphylococcus aureus and Salm. typhosa produced meningitis in rabbits,
taking a more protracted course and producing a lower mortality than that caused by the corresponding vegetative forms. Bohnhoff and Page (1968) showed that intraperitoneal injections of large doses of stable L forms of Neisseria meningitidis killed mice. Experimental pyelonephritis in rats could be produced by inoculation of E. coli L forms or by the direct inoculation of these variants into the renal medulla (Higuchi, 1969). Urease producing L forms of Proteus have been shown to produce bladder stones in rats (Braude, 1970). Merline, Golden & Mattman (1971) produced myocarditis and endocarditis in mice with cell wall deficient variants of Streptococcus viridans; recent studies of Iesmantantaite, Ptasekas, Astrauskaite, Astrauskas and Rimkunas (1975) appear to confirm that L forms of streptococci can cause experimental cardiopathies. There have also been recent reports that L forms of Strep. faecalis injected intramurally into the bowel of rabbits produced granulomatous lesions in the intestines (Orr, Tamarind, Cook, Fincham, Hawley, Quilliam & Irwing, 1974).

The credit of the first isolation of L forms of bacteria from diseased tissues must also go to Klieneberger (1938) who isolated these variants from pneumonic lesions of rats. Dienes and Smith (1944) subsequently isolated L forms of a Bacteroides spp. from the pus accumulated in the peritoneal cavity of
of a patient with a history of salpingitis. The next report of the isolation of L forms from clinical materials was the demonstration of L forms of *Streptobacillus moniliformis* from the blood of two patients with rat-bite fever (Dolman, Kerr, Chang & Shearer, 1951). At about the same time *Haemophilus pertussis* (*Bordetella pertussis*) was isolated from the lung lesions of mice (Wittler, 1952).

Following these early reports a spate of publications has appeared associating cell wall defective variants with numerous clinical conditions in man and animals. L forms of streptococci have been associated with endocarditis, rheumatic fevers, septicaemias of unknown aetiology, meningitis, Whipple's disease and renal conditions (Nativelle & Deparis, 1960; Tunstall & Mattman, 1961; Klodnitskaya, 1962; Kagan & Mikhailova, 1963; Guze & Kalmanson, 1964; Mattman & Mattman, 1965; Kagan, Koptelova & Pokrovsky, 1965; Gutman, Turek, Wedgwood & Petersdorf, 1965; Charache, Bayless, Shelly & Hendrix, 1966; Neu & Goldreyer, 1968; Turck, Gutman, Wedgwood & Petersdorf, 1968; Charache, 1968). Recently L forms of *Strep. agalactiae* were isolated from an outbreak of mastitis in a herd of cattle (Wilson, Little & Roberts, 1970). L forms of staphylococcus have been found associated with meningitis, endocarditis, cystic fibrosis of the pancreas, acute and chronic osteomyelitis, recurrent boils and abscesses and other
chronic staphylococcal infections. (Godzeski, Brier, Griffith & Black, 1965; Charache & Kaslick, 1965; Kagan et al., 1965; Rosner, 1966; Godzeski, 1968a; Godzeski, Brier & Glenn, 1968; Charache, 1968; Mattman, 1968; Bruns & Brown, 1970). Lipnicki (1958) and Shchegolev & Starshinova (1964) have demonstrated L forms of *Salmonella typhi* in convalescent patients and carriers in typhoid fever. The persistence of L forms of *Corynebacterium* spp. in endocarditis has been reported (Wittler, Malizia, Kramer, Tuckett, Pritchard & Baker, 1960; Zierdt & Wertlake, 1969). Akton and Akton (1960) reported the isolation of L forms of *Corynebacterium pyogenes* during treatment of bovine mastitis with penicillin. L forms of *Haemophilus* spp. have been closely linked with respiratory infections and occasionally in meningeal diseases (Kagan et al., 1965; Lapinski & Flakas, 1967; Mattman & Karris, 1969). L forms of *E. coli*, *Proteus* spp., *Klebsiella* spp., *Aerobacter* spp. and *Pseudomonas* spp. have been implicated in urological disease, cystic fibrosis and miscellaneous clinical conditions (Voureka, 1951a & b) Gutman et al., Coleman & Little, 1967; Charache, 1968; Kalmanson and Guze, 1968; Braude, Sieminski & Lee, 1968; Bruns & Brown, 1970). Pease (1967, 1969 & 1970) isolated L forms of *Listeria* from the synovial fluid and blood of arthritic subjects and further established this
relationship by demonstrating their presence in the erythrocytes by phase and electron microscopy. Charache (1970) isolated L forms of Listeria monocytogenes from the cerebrospinal fluid from a patient with a chronic intracranial inflammatory lesion. L forms of Brucella spp. have been isolated from clinical materials (Nelson & Pickett, 1951; Carrère & Roux, 1953b). Using phase microscopy as well as immunofluorescence identification techniques, L forms of Mycobacterium tuberculosis have been demonstrated in sputum and cerebrospinal fluid of patients with pulmonary tuberculosis or tubercular meningitis (Mattman et al., 1960; Garvin & Mattman, 1967; Mattman, 1970). The first infection of the erythrocytes of human beings with cell wall deficient forms of Clostridium sordelli has been reported by Mattman, Dowell and Neblett (1971). Cell wall defective variants of Candida spp. have been associated with endocarditis and mycohaemia (Rosner, 1966; Sweizkowski, Mattman, Truant & Wilner, 1970). L forms of Herella have been associated with a number of miscellaneous disease entities (Charache, 1968).

Undoubtedly some of the pathogenic properties attached to L forms could also be due to the in vivo reversion to the vegetative state. The first mention of pathogenicity due to reversion was by Heilman (1941a) who reported that when reversion occurred in
L cultures they proved to be highly virulent. Silberstein (1953) noted that mice inoculated with unstable L forms of Proteus succumbed to bacillary infection with the same organism. Earlier Wittler (1952) noticed that L forms of Haemophilus pertussis (Bordetella pertussis) reverted in the tissue of the mice. The classical experiments of Freundt (1956) lend further support to this concept of in vivo reversion. In his experiments mice inoculated intraperitoneally with L forms of Streptobacillus moniliformis died though in smaller numbers than those inoculated with the bacillary forms. Furthermore the course of infection in mice injected with L cultures was more protracted, deaths occurring during a much longer period than in those infected with the bacillary forms, but nevertheless the symptoms and lesions produced were similar in both groups. He attributed this delay in mortality between the two groups to the period taken for the L phase to revert in vivo and this assumption was clearly supported by his experiments in which he used penicillin to suppress the in vivo reversion of the L forms. Carrere, Roux and Mandin (1955a & b) injected L forms of Salm. typhimurium, B. melatsensis and Vibrio cholerae into the chorio-allantoic membrane of five-ten day old embryonated chicken eggs and they reported that after four-five days only the bacillary forms could be
re-isolated, indicating reversion had already occurred. Hannoun, Vigouroux, Levaditi and Nazimoff (1957) used embryonated eggs to study the pathogenicity of an altered variant (granular form) of a streptococcus. They inoculated these forms into the yolk sac and after lapse of time lesions appeared in the embryo from which the normal streptococcal forms were recovered. L forms of *Staphylococcus, Proteus, Salmonella* and *E. coli* reverted in chick embryos unless the embryonated eggs were treated with the appropriate antibiotics (Brier, Ellis & Godzeski, 1962; Godzeski, Brier & Farran, 1967; Godzeski, 1968b). Wittler *et al.*, (1960) who conducted a long term study of a patient with recurrent episodes of a sub-acute bacterial endocarditis caused by a *Corynebacterium* suggested that the bacillary forms of the organism were associated with the active phases of the disease whereas the antibiotic resistant transitional forms were associated with the latent stages of infection. In the experiments of Alderman and Freedman (1963) the experimental pyelonephritis produced by the protoplasts of *E. coli* was evidently caused by the *in vivo* reversion of these altered forms to the vegetative phases. Stable L forms of *Proteus* reverted to the bacillary phase in the renal tissue of rats (Gutman *et al.*, 1965). Winterbauer *et al.*, (1967) also demonstrated that L forms which proved to be stable *in vitro* rapidly reverted *in vivo*
when injected into the renal medulla creating an acute pyelonephritis indistinguishable from that caused by the parent organism. Ponig, Dominique and Schlegel (1972) obtained similar results when they gave intravenous injections of stable L forms of \textit{E. coli} in oxamide treated rats. They concluded that infection did not occur unless reversion to the parent form took place. The udder of cows experimentally infected with stable L forms of \textit{Staphylococcus} reverted \textit{in vivo} to produce only the parent form (Little & Bosbery, 1973).

In the present study a series of experiments was carried out to (a) evaluate the pathogenic properties of cell wall defective variants and the bacillary phases of \textit{Salm. gallinarum} strain 892/71 in 11-day-old chicken embryos (b) investigate the nature of the injury sustained by the embryos that had succumbed to infection and also to determine whether embryos that had survived infection would hatch out, chickens which would subsequently remain as "carriers" or "disseminators" of the bacillary phases of the organisms (c) study the effects of penicillin in causing L transformation and (d) compare the effects of endotoxin of L and bacillary phases of \textit{Salm. gallinarum} for chicken embryos.
MATERIALS AND METHODS (USED FOR IN-VIVO STUDIES)

MEDIA

The following additional media were used in the study pertaining to the pathogenicity of bacillary phases and their cell wall defective variants in 11-day-old embryonated chicken eggs.

Tryptone water (TW)

Tryptone (Difco) .................. 10g
Sodium Chloride (Analar) ........ 5g
Glass Distilled water ............. 1L

The above medium was made alkaline with 5 ml of 1ON NaOH and steamed at 100°C for 10 minutes. The medium was filtered through Whatman filter paper No. 2 and the pH adjusted to 7.6 with 1N HCl. It was then sterilised at 15 lb/sq inch for 15 minutes.

Brilliant green agar (BGA)

Bacto Brilliant green agar (Difco) 58g
Glass distilled water ............ 1,000ml

The suspension was autoclaved at 15 lb/sq inch for 15 minutes and then plated out aseptically into sterile petri dishes.

Salmonella enrichment broth (SEB)

This was prepared according to the method of Rappaport and Konforti (1958).
Selenite broth*

This media was prepared according to the manufacturers' instructions.

STAINING METHODS

The following staining methods were used in addition to those mentioned for in vitro studies.

May-Grunwald/Giemsa staining method (MGG)

Giemsa stain

Giemsa powdered stain [BDH] ............ 3.8g
Methyl alcohol (Analar) ............ 250ml
Glycerine (Analar) ............ 250ml

The powdered Giemsa stain was dissolved in the methyl alcohol in a stoppered flask and the stain was allowed to dissolve at 37°C with continuous stirring for 24 hours using a magnetic stirrer. Glycerine was then added and the stain was filtered prior to use.

Phosphate buffer solution

Potassium dihydrogen phosphate ....... 0.7g (Analar)
Disodium hydrogen phosphate ........... 1.0g (Analar)
Glass distilled water ............... 1L

The pH of the buffer was adjusted to 7.2.

* Oxoid Ltd., U.K.
Staining procedure

1. The film was fixed with May-Grunwald stain for 3 mins. in a coplin jar.
2. It was then transferred to another coplin jar containing 8 per cent. Giemsa stain made up in the phosphate buffer and allowed to stain for 30-45 mins.
3. The film was washed with buffer and allowed to dry.

Fixation and staining tissues for histopathological studies.

Tissues from embryos and chickens were fixed in 10 per cent. buffered formol saline. Sections of organs and tissues were stained by haematoxylin and eosin (H & E), Gram's and Giemsa stains.

EXPERIMENTAL ANIMALS

Eggs

Grade C (weighing approximately 1.6oz) white shelled eggs from a *Salmo pullorum*-free commercial flock were used. This commercial flock was also reportedly free of *Mycoplasma gallisepticum* infection. The eggs were received at the laboratory as 5 day incubated fertile eggs. The eggs, on arrival from the farm, were wiped clean with absorbent cotton wool, moistened with 50 per cent. methylated spirit and then incubated at 37.5°C.

Incubation of eggs.

Eggs were incubated in an egg incubator at 37.5°C until the time of hatching.
Candling of eggs.

Eggs were candled on arrival and all the dead eggs were discarded. Eggs were also candled prior to inoculation on the 11th day of incubation and those with dead embryos were discarded. The embryo was judged to be dead if there was absence of movement and collapse of the large vessels. After inoculation eggs were candled daily up to the time of hatching.

Intra-allantoic inoculation (1/A).

All inocula were introduced into the eggs by the intra-allantoic route. The technique of carrying out this procedure was as follows:-

The eggs were candled and with a pencil a mark was made on the lowest point of the air space on the side showing the least number of blood vessels. With a drilling machine a 2-3 mm single linear line was drilled over the marked area of the shell in such a manner so as not to expose the egg membrane. The inoculum was drawn up in a 1.0ml capacity syringe (calibrated to deliver 0.1ml volumes) fitted with a 1½-2" long needle (size 25BWG). The injection was done by inserting the needle vertically downwards to a depth of 1-1½". Prior to the introduction of the inoculum the egg shell over the area of the airspace was sterilised by wiping the area with Tincture iodine mitis B.P. Following the introduction of the inoculum the drilled area was sealed with melted paraffin wax. Unless otherwise mentioned eggs were
inoculated with 0.1 ml of the appropriate dilutions of cultures.

**In vivo introduction of penicillin.**

Penicillin was introduced into the eggs in 0.1 ml volumes by the l/A route as described earlier with the difference that the drilled area was not sealed with melted paraffin wax but was covered with a removable adhesive cellophane paper which was replaced daily when more of the penicillin was required to be inoculated into the eggs.

**Measurement of volume of allantoic fluid in 11-day embryonated eggs.**

Twenty grade C embryonated eggs from the same commercial source were used to measure the volume of allantoic fluid in 11-day-old embryonated eggs. The amount of allantoic fluid ranged from 4.8ml to 6.6ml giving an average of 5.5ml per egg.

**Chickens.**

Day-old White Leghorn chickens were periodically obtained from a pullorum-free commercial farm to serve as additional controls in some of the experiments.

**CULTURES**

**Induction of L, heteromorphic L and revertant L colonies for pathogenicity experiments.**

The induction of L, heteromorphic L and revertant L colonies were obtained by seeding AJA medium with a
concentrated TW culture of *Salmonella gallinarum* strain 892/71 which had been incubated at 37°C for 20 hours. The TW cultures were concentrated by centrifuging the liquid cultures and resuspending the pelleted organisms in TW to one tenth the original volume.

To produce the various L, heteromorphic L, and revertant L colonies the concentration of the inducing agents (glycine and penicillin) were varied and the serum was either omitted or incorporated to give a 10 per cent. concentration. The period of incubation was also varied to produce revertant L colonies. All AJA plates were incubated at 30°C.

Harvesting of growth on AJA medium.

Growth on AJA medium was harvested with AJB. In the case of heteromorphic and revertant L colonies, 10ml of AJB was used to harvest out the growth from 6-8 plates, whereas for L growth 5-6ml of AJB was used for harvesting 10 AJA plates.

Purity of inocula for egg inoculation.

Prior to harvesting the growth (L, heteromorphic L and revertant L colonies) from AJA medium, all plates containing such growth were visually examined and Gram’s smears made from each of the individual plates. This procedure was helpful in detecting yeast, fungi and Gram positive organisms. Growth from all AJA plates not contaminated by any of these organisms was harvested
and pooled and used as inoculum for injection of eggs. To further test the purity of the inoculum, 1.0 ml of the harvested and pooled growth was inoculated into AJB and incubated at 37°C for 18-20 hours. From this incubated AJB culture subcultures were carried out on several blood and McConkey agar plates which were incubated at 37°C for aerobically and anaerobically. The growth from these plates was checked for its identity and if any contaminations were found all the inoculated eggs were discarded.

L cell count.

Total L cell counts were carried out in a haemocytometer. To obtain viable L cell counts the harvested growth was diluted from $10^{-1}$ to $10^{-8}$ in AJB. 0.1ml of the appropriate dilutions were added to 9.9ml of molten AJA medium containing approximately 100 units/ml of penicillin and 10 per cent. serum. The penicillin and serum were added to the molten AJA medium at a temperature of 45°C-50°C, thoroughly mixed and then poured into petri dishes. The inoculated plates were incubated at 30°C for period of up to 7 days and counts performed. The counts obtained on these plates were referred to as L colony forming units (L.c.f.u.) and these colonies were L colonies when examined microscopically. Counts conducted on a similar AJA medium containing no penicillin were referred to as reverting colony forming units.
(R.c.f.u.) as these contained large numbers of bacillary elements.

**Bacillary cultures for egg inoculation**

All bacillary cultures for egg inoculation were obtained by inoculating TW with *Salmonella gallinarum* strain 892/71 and incubating the culture at 37°C for 20 hours.

**SEROLOGICAL TESTS**

**Rapid whole blood test for detection of *Salmonella pullorum-gallinarum* agglutinins**

This test was carried out by placing 0.04ml of stained Pullorum antigen* with the standard dropper, provided by the manufacturers, on a white glazed tile. 0.02ml of blood from the wing vein was collected with a nichrome wire loop (provided in the pullorum antigen kit) and mixed with the stained antigen and the plate was gently rocked for 1-2 minutes to note for flocculation. Reactions occurring later than 2 minutes after adding the blood were ignored.

**DETECTION OF L FORMS FROM ORGANS OF EMBRYOS AND CHICKENS BY FILTRATION TECHNIQUES.**

Embryos that failed to hatch, and chickens that died following hatching and those that were killed during the course of these investigations were checked for the presence of L forms in their tissues and organs. The

*Wellcome Reagent, U.K.*
method adopted for detecting L forms was as follows: -

The liver, lungs, kidneys, spleen, heart and occasionally the intestines of embryos or young chicks and ovaries of adult birds were triturated with a pestle and sterile sand in a mortar with 10-20 ml of AJB. The fluid part of the mixture was decanted and prefiltered through a Whatmans filter paper Grade 2. The filtrate was drawn up into a 10 ml polypropylene syringe and then filtered through a pre-sterilised Millipore unit containing a 13 mm diameter Millipore filter membrane of 45 µm A.P.D. The filtrate was plated out on serum AJA plates which were incubated at 30°C and examined periodically up to 14 days. The filter units were dismantled after filtration procedures and the filter membranes were visibly checked for leaks, cracks and other physical defects.

PREPARATION OF CRUDE ENDOTOXIN

For the preparation of crude endotoxin from the bacillary phases, the growth from 20 nutrient agar slants were harvested in 20 ml of TW. The harvested growth was washed and centrifuged at 3,000g thrice using normal saline. After the third washing the pelleted organisms were suspended in sterile glass distilled water to one fifth the original volume and then frozen to -20°C for 2 hours, after which it was thawed. This procedure of freezing and thawing was repeated 10 times. The cultures were then steamed at 100°C for 1 hour and
freezing and thawing procedure repeated 4 more times. The opacity of the solution was adjusted to give a reading of 3 x Tube 10 (Brown's opacity Tube). The inoculum was cultured on blood and MacConkey agar to check for viable organisms prior to inoculating the eggs.

For the preparation of crude endotoxins of L forms (both glycine and penicillin induced) a similar procedure was adopted except that the growths from 10-12 AJA plates were harvested with 5-6 ml of AJB. The cells were pelleted by centrifugation and the AJB was removed and replaced with the same volume of sterile 7.5 per cent. NaCl solution. They were washed once in this salt solution and the pelleted organisms resuspended in 3 ml of sterile glass distilled water. The rest of the procedures were similar to those described for the preparation of crude endotoxin from the bacillary forms. The opacity of the crude endotoxin preparation from L forms was adjusted to Brown's opacity tube $10^3$. 
Experiments to determine the pathogenicity and ELD_{50} of the bacillary phases of *Salmonella gallinarum* strain 892/71.

(a) In the preliminary series of experiments to carry out these pathogenicity tests a total of 44 eleven-day-old embryonated chicken eggs were used.

A 20-hour TW culture of *Salmonella gallinarum* strain 892/71 incubated at 37°C was diluted in TW from $10^{-1}$ to $10^{-7}$ and a plate count was conducted on $10^{-5}$, $10^{-6}$ and $10^{-7}$ dilutions to determine the viable cell count. Three treatment groups of 12 eggs per group were inoculated with undiluted culture, $10^{-4}$ and $10^{-7}$ dilutions respectively. A control group of 8 eggs was inoculated with 0.1 ml of TW.

Twenty-four hours following inoculation 9 of the 12 eggs that received the undiluted TW culture (2x$10^3$ organisms/ml) and 6 of the 12 eggs inoculated with the $10^{-4}$ dilution of TW culture were found with dead embryos. Forty-eight hours after inoculation, 3 of the remaining eggs in the group that received the undiluted culture, 4 from the group that received the $10^{-4}$ dilution of the culture and 2 eggs from the group that received the $10^{-7}$ dilution were dead. Seventy-two hours after infecting the eggs the remaining 2 eggs from the group that received the $10^{-4}$ dilution of the culture and 9 eggs inoculated with the $10^{-7}$ dilution
were found dead. Only 1 egg from the group that received 10^{-7} dilution remained alive until the time of hatching.

Cultures of the allantoic fluid from the dead eggs on blood, MacConkey and AJA agar yielded pure growths of \textit{Salm. gallinarum}. Gram's smears made from the blood agar plate showed mostly tiny Gram negative rods and coccobacillary forms, many of them occurring in short chains. Smears of the same growth stained by MGG method showed similar elements. The growth on the blood agar plates was also stained by Dienes' technique. In none of the blood agar plate cultures could L elements be demonstrated. However in AJA plates inoculated with the infected allantoic fluid a confluent growth was present. Preparations of these colonies stained by Dienes' technique showed it to be comprised of large numbers of L elements. The L elements consisted mostly of tiny and small spherical elements. Medium sized spherical elements occurred in lesser numbers. In many of the Dienes' stained preparations dark blue staining granules were present both extracellularly and within the cells. L elements constituted almost 50 per cent. of the total cellular elements. The bacillary forms present were also very pleomorphic and consisted of long tapering rods, swollen medium sized rods and ovoid bodies. There were also few short serpentine and filamentous forms.

Gram's smears of the allantoic fluid showed the
bacillary forms to be pleomorphic. Many of the bacilli occurred in long chains. Most of the bacilli also appeared swollen. Many of these Gram's smears also showed yeast-like bodies and some transitional filamentous forms. Occasionally a few tiny and small spherical elements could be detected and these elements were always Gram negatively stained. Direct smears of the infected allantoic fluid stained by the MGG technique showed similar elements, but compared with the Gram stained smears there were more tiny and coccobacillary forms many of which occurred in long chains. These MGG, stained smears always contained more tiny and small spherical elements than the Gram's smears.

**TABLE 18(a)**

Mortality of chick embryos following inoculation of Salm. gallinarum strain 892/71

<table>
<thead>
<tr>
<th>Time after inoculation (Hrs)</th>
<th>Dilution of cultures used for inoculation</th>
<th>No. dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>$10^{-5}$</td>
<td>1</td>
</tr>
<tr>
<td>48</td>
<td>$10^{-7}$</td>
<td>1</td>
</tr>
<tr>
<td>72</td>
<td>$10^{-5}$</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>$10^{-6}$</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>$10^{-7}$</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>$10^{-8}$</td>
<td>1</td>
</tr>
</tbody>
</table>
TABLE 18(b)
Total mortality of chick embryos inoculated with Salm. gallinarum strain 892/71

<table>
<thead>
<tr>
<th>Challenge dose</th>
<th>No. died</th>
<th>No. used</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 ml of dilutions used</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>6/6</td>
<td></td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>6/6</td>
<td></td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>6/6</td>
<td></td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>1/6</td>
<td></td>
</tr>
<tr>
<td>$10^{-9}$</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>$10^{-10}$</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>0/6</td>
<td></td>
</tr>
</tbody>
</table>

ELD50 (estimated by Karber's method). 0.1 ml of $10^{-7.6}$ dilution of 20 hour TW culture of Salm. gallinarum strain 892/71.

The allantoic fluid from the control group yielded no growth on AJA, blood agar or MacConkey medium. Smears of the allantoic fluid from the control egg stained both by Gram's and MGG methods failed to reveal any organisms.

(b) In this experiment 42 eleven-day-old embryonated
chicken eggs were used to determine the ELD50 of the bacillary phase of *Salmonella gallinarum* strain 892/71. A 20 hour TW culture of the organisms was titrated from $10^{-1}$ to $10^{-10}$. A plate count was conducted on $10^{-5}$, $10^{-6}$ and $10^{-7}$ dilutions to determine the viable cell count. The undiluted TW culture was calculated to contain $2.5 \times 10^8$ viable bacteria per ml. Eggs were inoculated with 0.1 ml of $10^{-5}$, $10^{-6}$, $10^{-7}$, $10^{-8}$, $10^{-9}$ and $10^{-10}$ dilutions of the titrated TW culture, and the controls were inoculated with 0.1 ml of TW by the same route. Six eggs were used for each dilution and the control group also consisted of 6 eggs. The pattern of egg mortality following infection of the eggs and the final mortality figures are given in Table 18 a & b.

The allantoic fluid from all dead eggs cultured on MacConkey, blood agar and AJA medium produced a pure growth of *Salmonella gallinarum*. The microscopic appearance of the smears made from the cultures obtained on these media were similar to that described in the preceding experiment. Likewise smears of the allantoic fluid stained by both Gram's and MGG techniques showed that these were the same as in the earlier experiment.

The allantoic fluid from 2 eggs of the control group cultured on AJA, blood agar and MacConkey failed to produce any growth. Smears of the allantoic fluid stained by Gram's and MGG methods also failed to reveal
any organisms.

Incubation of all embryos that had not died was continued until the time of hatching. Two eggs from the control group and one from the $10^{-8}$ dilution group failed to hatch. These were cultured on MacConkey, AJA and blood agar but no significant organisms were isolated from them.

The chickens that hatched out from the eggs that had received the bacterial inoculum were killed between the 3rd and 7th days after hatching. Pieces of the liver, spleen and intestines were triturated and the pooled organs were inoculated into Selenite broth and SEB and incubated at $37^\circ$C for 24 hours. Subcultures were made from the SEB and Selenite broth medium on to MacConkey and BGA medium but from none of the chickens was *Salmonella gallinarum* isolated. Filtrates of the triturated organs failed to produce any growth on AJA.

2. **Experiment to evaluate the pathogenicity of bacillary and cell wall defective variants of *Salmonella gallinarum* strain 892/71 produced in liquid and solid medium in 11-day-old embryonated chicken eggs.**

The 30 eggs used were divided into 5 treatment groups of 6 eggs per group.

**Group A** - (L forms induced in liquid medium)

This group of 6 eggs were inoculated with L
forms of strain 892/71 induced in AJB. The method of producing L forms in this strain was the same as described for strain 9S (see page 359) A good growth was obtained after 6 days of incubation at 30°C. The eggs were inoculated with 0.1 ml of undiluted AJB L cultures. The inoculum was examined under a phase microscope and it showed mostly spherical elements of various sizes. Although there were no rod forms, a few transitional elements were present. Gram's and MGG smears showed similar morphological elements. All the various forms also were Gram negative.

Group B - (mixed AJB cultures containing cell wall defective variants, transitional and bacillary elements)

The 6 eggs in this group were inoculated with a mixed growth of L forms, transitional elements and bacillary phases of strain 892/71 produced in AJB. The mixed growth was obtained by subculturing L colonies into AJB containing 10 per cent. horse serum but no penicillin, in the same way as for strain 9S (see page 361). The eggs were inoculated with a 6-day incubated mixed AJB growth. Phase microscopy examination of the inoculum
showed it to consist of a variety of elements namely spherical L elements, transitional and bacillary forms which in Gram's smears were all shown to be Gram negative. MGG smears of the inoculum showed similar morphological elements.

Group C - (heteromorphic L growth produced on AJA medium)

Six eggs in this group were inoculated with heteromorphic L colonies produced on AJA medium. Heteromorphic L growth was produced on AJA plates containing 50 units/ml penicillin but no serum and incubated for 6 days. The inoculum consisted of 0.1 ml of the harvested growth which in wet preparations examined under phase microscopy showed large numbers of spherical elements of various sizes, transitional and bacillary forms. All these elements stained Gram negative.

Group D - (bacillary cultures)

The six eggs in this group received a $10^{-4}$ dilution of a 20-hour incubated TW culture of strain 892/71. The undiluted culture contained an estimated $2.9 \times 10^8$ viable bacteria per ml. This group served as the positive control.
Group E - (controls)

The six eggs in this group served as a negative control and were inoculated with 0.1 ml of AJB.

The results of this experiment are shown in Table 19. (See page 510)

The allantoic fluid from all dead eggs gave a pure growth of *Salm. gallinarum* on blood agar and MacConkey plates. On AJA plates a bacillary type of growth was present, but when these were stained (both by Gram's and Dienes' methods) and examined microscopically it consisted of both L and bacillary elements. Smears of the allantoic fluid from the infected dead eggs stained by Gram's method showed predominantly short rods, coccobacillary forms and a few reddish staining tiny spherical bodies. Many of the bacillary forms were swollen and appeared to occur in chains. Corresponding smears of the allantoic fluid stained by the MGG method showed similar elements but by this method a higher proportion of the tiny spherical bodies were seen than in the Gram's smears.

3. Experiments to evaluate the pathogenicity of bacillary, heteromorphic L, L cultures and L cultured with added penicillin.

Thirty eggs were used in this experiment and
were divided into 5 treatment groups each of 6 eggs.

Group A - (bacillary cultures)

This group of six eggs was inoculated with a $10^{-4}$ dilution of a 20 hour TW culture of the bacillary forms. The undiluted culture gave a viable cell count of $2.9 \times 10^8$ organisms/ml. The inoculum was examined microscopically prior to inoculation and was seen to consist of Gram negative short and medium sized rods, coccobacillary forms and occasional long rods.

Group B - (heteromorphic L cultures)

This group of six eggs was inoculated with heteromorphic L colonies which had been produced on AJA plates containing 100 units/ml penicillin but no serum incubated for 4 days. The eggs were inoculated with 0.1 ml of the undiluted harvested growth. The undiluted harvested heteromorphic culture gave a count of $3 \times 10^7$ reverting colony forming units (R.c.f.u.) and $1 \times 10^3$ L colony forming units (L.c.f.u.) per ml. Wet preparations of the inoculum examined by phase microscopy showed numerous spherical elements of varying sizes, few to moderate numbers of transitional elements, few coccobacilli and some rod shaped
bacilli. Stained smears (both by Gram's and MGG methods) of the inoculum showed no normal bacillary forms but some transitional rod forms were present. As in the wet preparations, the stained smears showed the L elements (particularly the small and medium sized spherical bodies) to be the largest single type of cell present.

Group C - (glycine with penicillin induced L growth)

The eggs in this group were inoculated with L colonies produced on AJA medium containing 10 per cent. serum, 1.5 per cent. glycine and 100 units/ml penicillin which had been incubated at 30°C for 4 days. The eggs were inoculated with the undiluted harvested growth the estimated counts of which were $3.1 \times 10^7$ R.c.f.u./ml and $1 \times 10^3$ L.c.f.u./ml. Both stained and unstained preparations of the inoculum showed no bacillary forms but transitional forms were present.

Group D - (L cultures with penicillin)

The eggs were inoculated with the same inoculum as in Group C except that 500 units/ml penicillin was added to the inoculum. The inoculum with the added penicillin was incubated for 1 hour at 30°C prior to inoculating the
eggs. After the addition of penicillin the undiluted culture gave counts of $6.3 \times 10^3$ R.c.f.u./ml and $1 \times 10^3$ L.c.f.u./ml. The purpose of adding the penicillin to the inoculum was to prevent the transformation of the transitional elements into bacillary forms. When the inoculum was examined before inoculating the eggs there were large numbers of L elements, but there were also moderate numbers of transitional filamentous forms although no bacillary elements.

**Group E - (controls)**

The eggs in this group were inoculated with 0.1 ml of AJB.

The pattern of mortality of the eggs following infection with L, heteromorphic L and bacillary cultures are shown in Table 20.*

As shown in Table 20 it will be seen that all eggs inoculated with L, heteromorphic L, bacillary and L cultures with added penicillin succumbed to infection within 72 hours of infecting the eggs. In the eggs that received the bacillary cultures, the infected allantoic fluid yielded pure growths of *Salmonella gallinarum* on blood agar and MacConkey's medium. Smears of the growth obtained on the blood agar

*See page 511
showed mainly Gram negative tiny and short rods, coccobacillary forms and occasional long rods and short filaments. The blood agar cultures stained by Dienes' method showed no L elements. On AJA medium a confluent bacillary type of growth was present. The growth on AJA medium stained by Dienes' method showed it to consist of approximately 20 per cent. of L elements, 20 per cent. transitional filamentous forms and the rest bacilli. The allantoic fluid from the dead eggs stained by Gram's method showed numerous Gram negative swollen rods and a few tiny reddish filamentous forms were also encountered. MGG stained smears showed similar morphological elements but there were more of the tiny spherical bodies than in the corresponding Gram's smears of the infected allantoic fluid.

The allantoic fluid of the dead eggs in Group B also yielded pure growth of *Salm. gallinarum* on blood and MacConkey medium. On AJA plates a confluent growth was obtained which when stained by Dienes' method showed very large numbers of L elements of varying sizes and transitional forms. Very few normal bacilli were present. The smears of the allantoic fluid stained
by both Gram's and MGG methods showed large numbers of long and medium sized rods and many short filaments. There were also a few tiny spherical bodies. All elements were stained Gram negative.

The eggs that died in Group C and D produced a pure growth of *Salm. gallinarum* on blood agar and MacConkey medium. Gram's smear of the growth showed large numbers of long rods and fewer short and medium sized rods. There were also some tiny spherical bodies and coccobacilli. All elements stained Gram negatively. The blood agar cultures of the allantoic fluid of the dead eggs in Group D showed in addition few to moderate numbers of transitional filamentous forms on Gram's smears. The findings in the stained preparations of the growth on AJA plates were similar to those described for Group C.

None of the eggs died in Group E but two eggs were killed two days after inoculation and cultures and smears made from the allantoic fluid. No growth of any bacterial colonies was obtained on blood agar and MacConkey medium incubated at 37°C both aerobically and anaerobically nor were any organisms seen in smears from either egg stained by Gram's and MGG methods.

4. Experiments to evaluate the pathogenicity of bacillary cultures with and without added penicillin, heteromorphic L colonies and L colonies produced on different induction media.
Fifty-three eggs were used and were divided into eight treatment groups as follows:

Group A - (heteromorphic L colonies)

The six eggs in this group were inoculated with a $10^{-3}$ dilution of a 4-day growth of heteromorphic L colonies produced on AJA plates containing 100 units/ml penicillin but no serum. The inoculum was estimated to contain $7.5 \times 10^4$ R.c.f.u./ml and $1 \times 10^2$ L.c.f.u./ml. The microscopical findings of the stained smears showed the same type of elements as described previously for the inoculum of heteromorphic colonies in experiment 3 (see page 479).

Group B - (L colonies induced with 150 units/ml penicillin)

Seven eggs in this group were inoculated with a $10^{-3}$ dilution of L growth harvested from 10 plates of AJA medium containing 150 units/ml penicillin incubated for 4 days. This harvested growth contained $1 \times 10^5$ R.c.f.u./ml and $1 \times 10^2$ L.c.f.u./ml. Wet preparations of the inoculum examined under the phase microscope showed mostly spherical elements of varying sizes, transitional forms and some elements resembling bacillary rods. Stained smears of the inoculum did not reveal any normal bacillary forms.
Group C - (L colonies induced with 200 units/ml penicillin)

Seven eggs were inoculated with L colonies produced on a medium containing higher amounts of penicillin than in the previous group. The L forms were induced in exactly the same manner as in Group B except that the inducing medium contained 200 units/ml penicillin and 10 per cent. serum. The eggs were inoculated with a $10^{-3}$ dilution of the harvested growth which gave counts of $4 \times 10^5$ R.c.f.u./ml. No L.c.f.u were formed in this instance. The microscopic appearance of both wet and stained smears of the inoculum was similar to that described in Group B except that even under phase examination no bacillary forms could be seen.

Group D - (L cultures produced on 400 units/ml penicillin)

Seven eggs were inoculated with L colonies produced on AJA medium containing 400 units/ml penicillin and 10 per cent. serum. The induction of the L colonies was similar to that described in Groups B and C. The eggs were inoculated with a $10^{-3}$ dilution of the harvested growth. One ml of the undiluted harvested growth, gave approximately $1 \times 10^5$ R.c.f.u. and $1 \times 10^3$ L.c.f.u. The inoculum examined microscopically (both wet and stained) showed the same picture as the inoculum in Group B.
Group E - (L cultures produced with penicillin and glycine)

The seven eggs in this group were inoculated with L cultures induced by the combined action of penicillin and glycine. The L forms were induced in a manner similar to that in Groups B, C and D except that the inducing medium contained 100 units/ml penicillin, 1.5 per cent. glycine and 10 per cent. serum. Wet and stained preparations showed no bacillary forms but transitional forms were present. L elements were present in large numbers. Eggs were inoculated with a $10^{-3}$ dilution of the harvested growth which gave counts of $3 \times 10^5$ R.c.f.u./ml and $2 \times 10^4$ L.c.f.u./ml.

Group F - (bacillary cultures with added penicillin)

Seven eggs in this group were inoculated with bacillary cultures with penicillin added to the inoculum. To a 20 hour $37^\circ C$ incubated TW culture of strain 892/71 penicillin was added to give a final strength of 1,000 units/ml. The culture with the added penicillin was incubated for 1 hour at $30^\circ C$ and a $10^{-3}$ dilution of the culture used to inoculate the eggs. The undiluted culture gave a viable count of $3 \times 10^8$ of bacilli/ml. (prior to the addition of penicillin). Both wet and stained preparations of the inoculum after the addition of penicillin showed numerous coccobacilli
short rods and many disrupted bacilli but no spherical elements were detected.

Group G - (bacillary cultures)

This group served as the positive control for this experiment and was inoculated with the same culture as in Group F but before adding the penicillin. The undiluted inoculum gave a viable bacillary count of $3 \times 10^8$ bacilli per ml.

Group H - (controls)

The six eggs in this group were inoculated with 0.1 ml of AJB.

The results of the egg inoculation and pattern of mortality are shown in Table 21. (page 512)

Allantoic fluid from dead eggs which succumbed to infection within 5 days of inoculating the eggs in all groups yielded pure growths of *Salmonella gallinarum* in both blood agar and MacConkey's agar medium. The allantoic fluid from the infected eggs from all groups cultured on AJA medium produced a confluent growth consisting microscopically of varying numbers of L elements, transitional and bacillary forms. Generally there were more L elements in the growth produced by the allantoic fluid of eggs inoculated with L forms. The allantoic fluid from dead eggs of Group F (i.e. eggs infected with bacillary cultures with added penicillin) on AJA medium produced also a confluent
growth but microscopically it contained mostly tiny spherical elements and coccobacillary forms. Large, medium and small spherical elements and transitional filamentous forms were rarely encountered; however small numbers of slender rods were present. The smears of the allantoic fluid from dead eggs in Group A showed elements microscopically similar to those in the allantoic fluid of eggs in the previous experiments infected with heteromorphic L cultures (see page 478) Stained smears (both Gram's and MGG methods) of the allantoic fluid from Groups B, C, D & E showed pleomorphic bacillary forms, a few short filaments and some tiny spherical elements. All of them stained Gram negative. There were no filamentous forms in the allantoic smears of the dead eggs from Group F. The stained smears of the allantoic fluid from dead eggs of Group G showed mostly medium sized and short rods with small numbers of coccobacilli.

One egg each from Groups A, B, E and H failed to hatch. No growth of Salmonella gallinarum was obtained in AJA, blood agar or MacConkey medium from any of the dead embryos, in Groups A and B. Two eggs, one each from Groups E and H, were overgrown with a fungus.

Of the 53 eggs inoculated, 13 hatched out live chickens, 5 of them being from the negative control group. Two chickens (one each from Groups A and C) died within 48 hours of hatching. Cultures of the
liver, spleen, heart, lungs and kidneys on blood agar and MacConkey medium did not yield any growth of Salmonella gallinarum colonies; the filtrate of the triturated pooled organs on AJA medium failed to yield any L colonies.

Faecal excretions from all the remaining 11 chickens from Groups B, D and H were cultured initially into Selenite broth and SEB medium at 37°C for 24 hours and then subcultures were made from these media to MacConkey agar plates to detect the presence of Salm. gallinarum colonies. Examination of the faeces of the chickens was done when the birds were 5 days old and for the next 5 days and also when the birds were 40, 45 and 60 days old. No Salmonella organisms were detected in the faeces by these cultural procedures. All 11 birds were tested serologically for Salm. pullorum-gallinarum agglutinins by the rapid whole blood test when the birds were 26, 40, 45 and 60 days old and all were found to be negative. Three birds of similar age were injected intramuscularly with 0.5 ml of a 10⁻² dilution of a 20 hours 37°C incubated TW culture of Salm. gallinarum strain 892/71 at the age of 14 days. The undiluted inoculum gave a viable count of 2.25 x 10⁸ organisms per ml. These artificially infected birds were kept as positive controls to check for detecting of Salm. pullorum-gallinarum agglutinins by the rapid whole blood test.
Of the 3 birds artificially infected only 2 showed detectable agglutinins 2 weeks after infection, but only one bird remained consistently positive when tested at 40, 45 and 60 days of age. The other bird, which was serologically positive at 28 days old, gave a negative result at 40 days old but, when retested 5 days later, it was again positive, becoming negative when last tested at 60 days old. Faecal cultures from all these birds were carried out on 8 separate occasions but only once was *Salm. gallinarum* isolated from the faeces of the bird that was consistently serologically positive.

All birds (including the 3 artificially infected) were killed when they were 2½ months old but from none of the organs (liver, spleen, kidney, lungs, heart and intestines) could *Salm. gallinarum* be isolated. The filtrate from the triturated pooled organs (liver, spleen, kidney, lungs and heart) plated out on AJA medium did not yield any L colonies.

5. **Experiments to determine the pathogenicity of bacillary L and reverting L colonies with and without added penicillin (in vivo).**

The purpose of this experiment was to determine whether the pathogenicity of the bacillary and cell wall defective variants would be significantly reduced if daily doses of penicillin were introduced *in vivo*
after infecting the eggs and whether this procedure would
(a) convert the bacillary forms into cell wall
defective variants in vivo,
(b) inhibit the in vivo reversion of L elements into
the bacillary phases and
(c) retransform, in vivo, the transitional and bacillary
elements in the reverting L cultures back into L
forms.

Forty-two eggs were used and were distributed
into 9 treatment groups. From the mean volume of
allantoic fluid determined previously it was estimated
that the introduction of 100 units penicillin into the
eggs would give an approximate concentration of 18-20
units penicillin per ml in the allantoic fluid.

Group A - (bacillary cultures)

The five eggs in this group were inoculated
with a $10^{-5}$ dilution 20 hour 37°C incubated TW
cultures of strain 892/71. The undiluted inoculum
gave a viable count of $2.5 \times 10^8$ bacilli per ml.

Group B - (bacillary cultures with penicillin)

This group of 5 eggs were also given the same
quantity and type of inoculum as in Group A.
All the inoculated eggs in this group received 100
units of penicillin immediately after the eggs
had been inoculated. One hundred units of
penicillin were given on each of the next two
days in eggs with surviving embryos.

Group C - (reverting L colonies)

This group contained 6 eggs which were
inoculated with reverting L growth. The L
colonies were induced on a serum AJA medium
containing 150 units/ml penicillin continuously
incubated at 30°C for 12 days. This prolonged
incubation had the effect of inducing reversion.
The growth on the 12th day of incubation AJA
plates stained by Dienes' method showed a mixture
of long slender rods, short filaments, large
numbers of L elements, a few transitional and
irregularly shaped forms. The growth from these
plates was harvested with AJB and the eggs were
inoculated with a $10^{-3}$ dilution of the harvested
growth. The undiluted inoculum gave a R.c.f.u.
count of $1.6 \times 10^5$/ml and a total L count of
$1.8 \times 10^5$/ml.

Group D - (reverting L cultures with penicillin)

Five eggs were used in this group and were given
the same quantity and type of inoculum as in
Group C. The eggs in this group were given
100 units of penicillin soon after infection.
In the only surviving egg 100 units of
penicillin was given on the first day post
inoculation.

Group E - (L colonies)

Six eggs in this group were infected with L colonies produced in the same way as in Group C except that the L colonies were produced on serum AJA medium containing 1.5 per cent. glycine and 100 units/ml penicillin. The 12 day incubated growth stained by Dienes' method showed mostly L elements and many irregularly shaped bodies but there were no normal bacilli. Smears of the growth stained by Gram's method also showed no bacillary forms. Eggs were inoculated with 0.1 ml of a $10^{-3}$ dilution of the harvested L growth. The undiluted inoculum gave a total L count of $1.25 \times 10^5$ cells/ml and $8 \times 10^7$ R.c.f.u.

Group F - (L colonies with penicillin)

Five eggs in this group were inoculated with the same amount and type of inoculum as in treatment Group E. Soon after the eggs were inoculated, 100 units of penicillin were introduced into these eggs. Eggs that survived were given 100 units of penicillin daily for two additional days.

Group G - (L colonies with penicillin)

Six eggs in this group received L colonies
produced on serum AJA medium containing glycine. The L colonies were produced in the same way as in Group C, except that the inducing medium contained 3 per cent. glycine. The growth on these 12 day incubated plates stained by Dienes' method showed it to consist mostly of small and tiny spherical elements; the large spherical elements constituted less than 20 per cent of the growth. Smears of the growth stained by Gram'd method also showed large numbers of well stained Gram negative small and tiny spherical elements. Occasional medium sized and large spherical elements were present but there were no normal bacillary forms. The eggs were inoculated with 0.1 ml of a $10^{-3}$ dilution of the harvested growth. The undiluted harvested growth gave a total L cell count of $1.6 \times 10^5$ cells/ml and $2 \times 10^8$ R.c.f.u./ml. They also received 100 units of penicillin soon after infection and daily for 2 days in eggs that survived infection.

Group H - (control)

This group was made up of 4 eggs inoculated with 0.1 ml of AJB.

The results of egg mortality and pattern of embryo deaths are presented in Table 22.* Of the 42 eggs inoculated 4 hatched out live chickens.

* See page 513
2 of which were from the negative control group. One egg from the control group failed to hatch but no growth of any organisms was obtained from the embryo. The remaining embryo of the negative control group died on the 4th day after inoculation and was found to be contaminated by a fungal growth.

The allantoic fluid from all eggs which died from Group A to G yielded pure growths of *Salm. gallinarum* on blood agar and MacConkey medium. On AJA medium inoculated with the infected allantoic fluid a confluent growth was usually produced which, when stained (Dienes' method) and examined microscopically, was seen to be composed of L, transitional and bacillary forms. The differences in the proportion of the various cellular elements were generally small in that usually the allantoic fluid from dead eggs infected with L forms or those in which penicillin was introduced produced proportionately more L elements on AJA medium. L elements were not demonstrated from blood agar colonies stained by Dienes' method but in a few blood agar cultures of the allantoic fluid from eggs that received penicillin, there were some swollen long transitional filamentous forms.

Smears of the allantoic fluid from dead eggs from Group A to H stained both by Gram's and MGG methods
showed great variation. In Group A eggs the allantoic fluid smears showed pleomorphic Gram negative bacillary forms most of which were swollen with coccobacilli and short rods occurring in chains. Occasional transitional filamentous forms and tiny spherical elements were also encountered. Stained smears of the allantoic fluid from dead eggs of Groups B, C, D, F and G, on the other hand, showed mostly Gram negative long rods, short filaments, transitional filamentous forms, and a few tiny, small, medium and large spherical elements. Smears of the allantoic fluid from dead eggs from Groups E did not show any large spherical elements or filamentous transitional forms.

The chickens from Group G and H which survived infection were tested for *Salmonella pullorum-gallinarum* agglutinins by the rapid whole blood test when the birds were 11, 33, 40 and 45 days old. None of them reacted serologically. The droppings from the birds were cultured on 6 different occasions but were negative for any *Salmonellae*. All 4 birds were killed when they were 50 days old and the liver, spleen, lungs, heart, kidney and intestines were cultured on blood agar and MacConkey medium, but no *Salmonella* organisms could be isolated from any of these birds. All these organs except for the intestines were triturated with AJB and the filtrates subcultured on AJA medium. No L colonies formed on AJA medium.
6. Experiments to study the effect of high doses of penicillin \textit{in vivo} on 11-day-old embryonated eggs infected with bacillary and L forms of \textit{Salm. gallinarum} strain 892/71.

Fifty-four eggs were used to compare the pathogenicity of L forms and bacillary phases of strain 892/71 in eggs receiving high doses of penicillin \textit{in vivo} and in those not receiving penicillin. The eggs were distributed into 8 treatment groups. The introduction of 10,000 units of penicillin into the egg was estimated to produce a penicillin concentration of 1800-2000 units/ml of allantoic fluid.

Group A - (glycine induced L forms)

Seven eggs in this group were inoculated with L colonies produced on serum AJA medium containing 3 per cent. glycine. The eggs were inoculated with undiluted harvested growth from 4 day incubated growth. The growth on these plates stained by Dienes’ method consisted mostly of medium sized and small spherical elements. The large and tiny spherical elements were present in lesser numbers. The undiluted harvested growth gave a total L count $2.5 \times 10^6$ cell/ml and a viable count of $5 \times 10^4$ L.c.f.u./ml and $1 \times 10^8$ R.c.f.u./ml.
Group B - (glycine induced L forms with daily introduction of penicillin)

The seven eggs in this group were given the same amount of inoculum as in Group A and in addition each egg was given 10,000 units of penicillin soon after infection and daily for the next 5 days.

Group C - (penicillin induced L forms)

The seven eggs in this group were inoculated with the undiluted harvested growth of L colonies induced in 4 days on serum AJA medium containing 1,000 units/ml penicillin. The inoculum stained by Dienes' method showed mostly large and medium sized spherical elements and lesser numbers of the smaller L elements. Some of the larger L elements contained rod-like structure (see plate 52). In MGG stained smears of the inoculum in addition to the spherical L elements blue staining granules, a few fine short filaments and some 'V' shaped forms were also seen (see plate 53) but these did not appear to resemble the bacillary forms of strain 892/71 as they stained bluish in Gram's smears (see plate 54).

The undiluted harvested growth gave a total L count of $2 \times 10^6$ L cells/ml, and a viable count of $7 \times 10^7$ R.c.f.u. and $3 \times 10^4$ L.c.f.u./ml.
Group D - (penicillin induced L forms with daily introduction of penicillin)

Seven eggs were given the same inoculum as in Group C but penicillin (10,000 units) was introduced soon after infection and daily for 5 days.

Group E - (bacillary cultures)

Seven eggs were inoculated with a $10^{-5}$ dilution of a 20 hour, $37^\circ C$ incubated TW culture of strain 892/71. The undiluted culture gave a viable bacterial count of $2 \times 10^8$ cells/ml.

Group F - (bacillary cultures with daily introduction of penicillin)

Seven eggs were given the same culture as in Group E with daily introduction of 10,000 units of penicillin.

Group G - (AJB with penicillin with daily introduction of penicillin)

The six eggs were given 0.1 ml AJB followed by 10,000 units penicillin daily for 5 days.

Group H - (TW with penicillin, daily introduction of penicillin)

Six eggs were inoculated with 0.1 ml of TW, followed by 10,000 units penicillin daily for 5 days.
The results of these experiments are shown in Table 23* from which it can be seen that penicillin in high doses prevented the embryo from dying of a *Salmonella gallinarum* infection. Only one embryo (from Group D) of those receiving daily doses of penicillin died within 24 hours as a result of *Salmonella gallinarum* infection.

The allantoic fluid cultured on blood agar and MacConkey medium produced only 4 colonies on the blood agar plates and none on the MacConkey plates. Smears of the colonies from the blood agar plates stained by Gram's method showed it to consist of large numbers of grossly swollen Gram negative filaments and a few long rods. The allantoic fluid from this egg subcultured on AJA medium produced a more luxuriant growth which when stained by Dienes' method showed large numbers of L elements of various sizes, swollen and distorted filamentous forms and long bacilli. The colonies on the blood agar plates were further subcultured and their identity was confirmed both serologically and biochemically as *Salmonella gallinarum*.

Smears of the allantoic fluid stained both by Gram's and MGG method showed a few extremely long filaments.

All infected eggs that did not receive penicillin succumbed to infection within 72 hours of inoculation and *Salm. gallinarum* was consistently obtained in pure

*See page 514.*
culture from the infected allantoic fluid on blood agar, MacConkey and AJA media.

Three eggs, one each from the groups that were receiving penicillin daily, were killed on the third day after infection. Smears of the allantoic fluid showed no bacillary or L elements. Cultures of the allantoic fluid on blood agar, MacConkey and AJA media produced no growth.

Several of eggs that received penicillin daily failed to hatch, including one from the control groups which was given only AJB and penicillin. Cultures of the organs from any of these embryos on blood agar and MacConkey media incubated both aerobically and anaerobically failed to produce any growth. Likewise cultures of the organs on AJA medium failed to produce any growth.

Two birds, one each from the negative control groups G and H, died when they were 7 and 14 days old respectively, but no organisms were isolated from their tissues.

Faecal cultures and serological testing of all the birds were carried out when they were 7, 14, 21, and 28 days old. Faecal cultures were negative in all instances. The birds were also negative to the rapid whole blood test for *Salmonella pullorum-gallinarum* agglutinins.

All the birds were killed at 30 days of age and the liver, spleen, kidney and lungs were cultured on
blood agar and MacConkey medium. No salmonellae were isolated. The filtrate from the triturred organs also failed to produce any growth on AJA medium.

7. Experiment to determine the ELD\textsubscript{50} of penicillin-induced L forms of Salm. gallinarum strain 892/71.

Sixty two eggs were used for this experiment. L forms were induced on serum AJA medium containing 1,000 units/ml penicillin, the 4th day incubated growth being harvested in AJB and diluted also in AJB from $10^{-1}$ to $10^{-8}$. The inoculum stained both by Gram's and MGG methods showed no normal bacillary forms (see plates 53 & 54) nor did the Dienes' stained colony preparation from these 4 day incubated AJA plates (see plate 52).

The undiluted inoculum gave a viable L count of $3 \times 10^3$ L.c.f.u./ml and $1.2 \times 10^8$ R.c.f.u./ml. Four eggs inoculated with 100 ELD\textsubscript{50} doses of the bacillary forms of strain 892/71 in TW culture served as positive controls and another four eggs, each inoculated with 0.1 ml of AJB, served as negative controls. Six eggs were also inoculated with 0.1 ml of undiluted harvested L growth.

The results of the egg inoculation are shown in Table 24*. Four eggs, one each from the $10^{-6}$, $10^{-7}$, $10^{-8}$ dilutions and from the negative control group failed to hatch. The organs of these embryos were cultured but

*See page 515
no salmonellae could be isolated. However, *Proteus* spp. was isolated from the organs of the embryo of the egg that failed to hatch in the $10^{-6}$ dilution group. In the group inoculated with $10^{-7}$ dilution of the L growth one egg died on the first day post inoculation but no growth of any organisms was obtained from the allantoic fluid cultured on the routine isolation media. Smears of the allantoic fluid stained both by Gram's and MGG methods also failed to show any organisms. All other eggs that died yielded *Salm. gallinarum* on culture. In calculating the ELD$_{50}$ of the L forms, eggs from which no *Salmonella gallinarum* was isolated were disregarded. The ELD$_{50}$ of these penicillin-induced L forms was calculated to be 0.1 ml of a $10^{-6}$ dilution of a 4 day incubated L growth which was approximately 12 viable L forms.

8. *Experiment to determine the ELD$_{50}$ of glycine-induced L forms of Salm. gallinarum strain 892/71.*

The L forms were produced on serum AJA medium containing 3 per cent. glycine. The 4 day growth was harvested and diluted from $10^{-1}$ to $10^{-8}$ using AJB as the diluent. The inoculum was stained both by Gram's and MGG methods to check for the absence of filamentous transitional or bacillary forms. The undiluted harvested growth gave a viable count of $1.1 \times 10^3$ L.c.f.u./ml and $1.5 \times 10^8$ R.c.f.u. per ml. The eggs were inoculated with all dilutions of the L growth, six eggs being used for each dilution. The positive control group of six eggs
was inoculated with 0.1 ml of a $10^{-5.6}$ dilution TW culture of the bacillary forms (100 ELD$_{50}$ doses). The negative control group also containing six eggs was inoculated with AJB. The results are shown in Table 25. (see page 516)

All eggs inoculated with 100 ELD$_{50}$ doses of the bacillary forms and $10^{-5}$ dilution of L forms succumbed to infection. Of the six eggs inoculated with $10^{-6}$ dilution of the L growth five died. The allantoic fluid from all these eggs yielded pure growths of *Salm. gallinarum*.

The ELD$_{50}$ of the glycine induced L forms was calculated to be 0.1 ml of a $10^{-6.3}$ dilution of a 4 day incubated L growth which was approximately 10 viable L forms.

One egg each from the negative control and $10^{-3}$ dilution groups failed to hatch. The organs of the embryos from these two eggs did not yield any growth of *Salmonella* colonies.

As in the preceding experiment in calculating the ELD$_{50}$ of these L forms, eggs from which no *Salm. gallinarum* were isolated were disregarded.

9. **Experiment to determine the effects of crude endotoxins of L forms and bacillary phases of *Salm. gallinarum* strain 892/71 on 11 day old embryonated chicken eggs.**

Thirty-nine eggs were used in this experiment
and were distributed into 8 treatment groups.

Six eggs each in Group A, B and C were inoculated with 0.2 ml of crude endotoxins prepared from glycine-induced L forms, penicillin induced L forms and bacillary phases respectively of strain 892/71.

Five eggs each in Groups D and E were inoculated with 0.1 ml of undiluted harvested L growths (4 day incubated) produced respectively on 3 per cent. glycine serum AHA medium with 1,000 units/ml penicillin. The five eggs in Group F were inoculated with 0.1 ml of a $10^{-5}$ dilution of 20 hour 37°C incubated TW bacillary culture of strain 892/71. Groups D, E and F served as positive controls. The negative control groups G and H had 3 eggs per group and were inoculated with 0.2 ml of sterile glass distilled water (GDW) and AJB respectively. The pattern of mortality and result of the egg inoculation are shown in Table 26. (See page 517)

All eggs infected with the bacillary and L forms of strain 892/71 succumbed to salmonella infection. *Salmonella gallinarum* was isolated in pure culture from the allantoic fluid of these infected eggs.

In group C, inoculated with crude endotoxin preparation of the bacillary forms only one egg died within 24 hours of inoculation but no organisms were isolated from the culture of the allantoic fluid of the dead egg. In Groups A and B inoculated with crude endotoxins of the
two L forms, 3 eggs (i.e. 2 from Group B and one from Group A) died but no organisms were isolated from the allantoic fluid of the dead eggs. No organisms were isolated from organs of embryos that failed to hatch.

Pathological changes in embryos infected with bacillary, cell wall defective variants, and crude endotoxins of Salmonella gallinarum strain 892/71.

Gross Pathology.

All embryos infected with bacillary and cell wall defective variants of strain 892/71 and that succumbed to infection showed almost identical gross pathological lesions. The vessels of the chorioallantoic membrane were intensely congested and often showed haemorrhagic patches. The membranes appeared oedematous and thickened. The affected embryos appeared almost scarlet red. The liver was yellowish and enlarged and was friable. These lesions were less marked in embryos dying within the first 24 hours after inoculation.

Similarly in embryos inoculated with crude endotoxin preparations of the bacillary and L forms of strain 892/71 gross pathological lesions were minimal. The chorioallantoic membrane showed a slight to moderate generalised congestion. The embryos were only slightly reddened.
In the control groups the embryos were pale pink and did not show congestion of the vessels of the chorioallantoic membrane.

**Histopathological changes.**

There were no appreciable differences in the histopathological lesions of embryos infected with bacillary and wall defective variants. In all dead embryos infected with bacillary phases and L forms of strain 892/71 there was a marked bacteraemia but this was absent in embryos that died as a result of inoculation with crude endotoxins. The following organs were examined.

**Liver** - there are acute congestion of the vessels and sinusoids of the liver. In most instances there were massive extravascular haemorrhages. In addition to the vascular changes the liver cells showed acute cellular swelling and fatty changes. Bacilli and transitional forms were present in the lumen of blood vessels (see plate 55).

**Heart** - there was moderate to severe congestion of the vessels. Bacilli were present in the chambers of the heart.

**Lungs** - severe vascular congestion and in some instances extravascular haemorrhages were evident.

**Kidneys** - severe vascular congestion, extravascular
haemorrhages and interstitial oedema were present and bacilli and L elements were seen in the lumen of blood vessels and kidney parenchyma (see plate 56).

Tibiometatarsal joints - there was mild to severe congestion of the periarticular vessels. Bacilli were present in the bone marrow.

Chorioallantoic membrane (CAM) - the changes produced on the CAM were most marked (see plate 57 & 58). There were marked subepithelial oedema and severe necrotic changes. Bacilli were present in large numbers within the lumen of blood vessels as well as extravascularly (see plates 59 & 60) and in some of the embryos infected with L forms, both bacillary and L elements were present (see plate 60).

Karyorrhexis of the epithelial cells was marked. There was vacuolation and proliferation of the lining cells of the CAM. Infiltration of the membrane with heterophils was noticed indicating that a purulent inflammation was setting in. The degenerative changes in the walls of the blood vessels were very marked.

In the eggs that died as a result of receiving crude endotoxin the changes in the organs were limited to blood vessel wall damage. The CAM showed more focalised necrotic
changes (see plate 61) unlike the diffused lesions seen in eggs infected with live organisms.
Table 19

Pathogenicity of bacillary and cell wall defective variants of *Salm. gallinarum* strain 892/71 produced in liquid and solid medium inoculated into 11-day-old chick embryos

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Day(s) after inoculation</th>
<th>No. of embryos dead</th>
<th>No. died</th>
<th>No. used</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>A-L forms from liquid medium</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>B-Mixed AJB cultures</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>C-Heteromorphic L cultures</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>D-Bacillary cultures</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>E-Controls</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 20

Pathogenicity of bacillary, heteromorphic L and L cultures and L cultures with added penicillin of *Salm. gallinarum* strain 892/71 in 11-day-old chicken embryos

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>No. of embryos dead</th>
<th>No. died</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 10</td>
<td></td>
</tr>
<tr>
<td>A- Bacillary culture</td>
<td>1 0 5 0 0</td>
<td>6/6</td>
</tr>
<tr>
<td>B- Heteromorphic L culture</td>
<td>0 5 1 0 0</td>
<td>6/6</td>
</tr>
<tr>
<td>C- L culture</td>
<td>0 2 4 0 0</td>
<td>6/6</td>
</tr>
<tr>
<td>D- L culture + penicillin</td>
<td>0 2 4 0 0</td>
<td>6/6</td>
</tr>
<tr>
<td>E- Control</td>
<td>0 0 0 0 0</td>
<td>*0/6</td>
</tr>
</tbody>
</table>

*2 embryos killed.
Table 21

Pathogenicity in 11-day-old chick embryos of bacillary cultures with and without added penicillin and heteromorphic L growth compared with L growth of Salm.

gallinarum strain 892/71 induced on different induction media

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>No. of embryos dead</th>
<th>No. died No. used</th>
<th>No. failed to hatch</th>
<th>No. hatched</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-Heteromorphic L growth</td>
<td>0 0 4 0 0</td>
<td>4/6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>B-L growth from 150/u-pen.AJA</td>
<td>0 0 3 0 0</td>
<td>3/7</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>C-L growth from 200/u-pen.AJA</td>
<td>0 3 3 0 0</td>
<td>6/7</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>D-L growth from 400/u-pen.AJA</td>
<td>0 1 3 0 0</td>
<td>4/7</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>E-L growth from gly-pen.AJA</td>
<td>0 1 4 0 1</td>
<td>6/7</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>F-Bacillary culture with pen.</td>
<td>0 1 0 6 0</td>
<td>7/7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G-Bacillary culture</td>
<td>1 0 6 0 0</td>
<td>6/6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H-Control</td>
<td>0 0 0 0 0</td>
<td>0/6</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

\[u = \text{units/ml}\] \hspace{1cm} \text{pen. = penicillin} \hspace{1cm} \text{gly = glycine}
**Table 22**

Effect of penicillin given in vivo on the pathogenicity of bacillary, reverting L and L colonies of *Salm. gallinarum* strain 892/71 in 11-day-old chick embryos

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Day(s) after inoculation</th>
<th>No. of embryos dead</th>
<th>No. died</th>
<th>No. hatched</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 2 3 4 5 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-Bacillary culture</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-Bacillary culture + pen.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-Reverting L growth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Reverting L growth + pen.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-L growth (gly-pen. induced)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F-L growth (gly-pen. induced) +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pen. treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-L growth (gly. induced)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*failed to hatch  pen. = penicillin  gly = glycine
Table 23

Effect of high doses of penicillin in vivo on 11-day-old chick embryos infected with bacillary and L forms of Salm. gallinarum strain 892/71

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Day(s) after inoculation</th>
<th>No. of embryos dead</th>
<th>No. died</th>
<th>No. killed</th>
<th>No. failed to hatch</th>
<th>No. hatched</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 2 3 7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-Gly. induced L forms</td>
<td></td>
<td>0 5 2 0</td>
<td>7/7</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>B-Gly. induced L forms + pen. treatment</td>
<td></td>
<td>0 0 0 0</td>
<td>0/7</td>
<td>1</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>C-Pen. induced L forms</td>
<td></td>
<td>0 5 2 0</td>
<td>7/7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D-Pen. induced L forms + pen. treatment</td>
<td></td>
<td>1 0 0 0</td>
<td>1/7</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>E-Bacillary cultures</td>
<td></td>
<td>0 0 0 0</td>
<td>0/7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F-Bacillary cultures + pen. treatment</td>
<td></td>
<td>0 0 0 0</td>
<td>0/7</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>G-AJB + pen. treatment</td>
<td></td>
<td>0 0 0 0</td>
<td>0/6</td>
<td>0</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>H-TW + pen. treatment</td>
<td></td>
<td>0 0 0 0</td>
<td>0/6</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

pen. = penicillin  
Gly = glycine
Table 24

Mortality of 11-day-old chick embryos following inoculation of penicillin induced L forms of Salm. gallinarum strain 892/71

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>No. died</th>
<th>No. used</th>
<th>No. failed to hatch</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-1}$ L forms</td>
<td>6/6</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>$10^{-2}$ L forms</td>
<td>6/6</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>$10^{-3}$ L forms</td>
<td>6/6</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>$10^{-4}$ L forms</td>
<td>6/6</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>$10^{-5}$ L forms</td>
<td>6/6</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>$10^{-6}$ L forms</td>
<td>3/6</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>$10^{-7}$ L forms</td>
<td>1/6</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>$10^{-8}$ L forms</td>
<td>0/6</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>POSITIVE CONTROL</td>
<td>4/4</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>NEGATIVE CONTROL</td>
<td>1/4*</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>UNDILUTED HARVESTED L GROWTH</td>
<td>6/6</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

*Killed

ELD$_{50}$ (estimated by Karber's method) = 0.1ml of $10^{-6}$ dilution.
Table 25
Mortality of 11-day-old chick embryos following inoculation with glycine induced L forms of Salm. gallinarum strain 892/71

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>No. died</th>
<th>No. failed to hatch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. used</td>
<td></td>
</tr>
<tr>
<td>$10^{-1}$ L forms</td>
<td>6/6</td>
<td>0</td>
</tr>
<tr>
<td>$10^{-2}$ L forms</td>
<td>6/6</td>
<td>0</td>
</tr>
<tr>
<td>$10^{-3}$ L forms</td>
<td>6/6</td>
<td>0</td>
</tr>
<tr>
<td>$10^{-4}$ L forms</td>
<td>6/6</td>
<td>0</td>
</tr>
<tr>
<td>$10^{-5}$ L forms</td>
<td>6/6</td>
<td>0</td>
</tr>
<tr>
<td>$10^{-6}$ L forms</td>
<td>5/6</td>
<td>0</td>
</tr>
<tr>
<td>$10^{-7}$ L forms</td>
<td>0/6</td>
<td>0</td>
</tr>
<tr>
<td>$10^{-8}$ L forms</td>
<td>0/6</td>
<td>1</td>
</tr>
<tr>
<td>Positive Control</td>
<td>6/6</td>
<td>0</td>
</tr>
<tr>
<td>Negative Control</td>
<td>0/6</td>
<td>1</td>
</tr>
</tbody>
</table>

$ELD_{50}$ (estimated by Kärber's method) = 0.1ml of $10^{-0.3}$ dilution
Table 26
Effect of crude endotoxin from bacillary and L forms of Salm. gallinarum

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Day(s) after inoculation</th>
<th>No. of embryos dead</th>
<th>No. died</th>
<th>No. failed to hatch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 2 3 10</td>
<td>No. used</td>
<td></td>
</tr>
<tr>
<td>A-Endotoxin (Gly. induced L forms)</td>
<td></td>
<td>1 0 0 0</td>
<td>1/6</td>
<td>3</td>
</tr>
<tr>
<td>B-Endotoxin (pen. induced L forms)</td>
<td></td>
<td>2 0 0 0</td>
<td>2/6</td>
<td>3</td>
</tr>
<tr>
<td>C-Endotoxin (bacillary forms)</td>
<td></td>
<td>1 0 0 0</td>
<td>1/6</td>
<td>3</td>
</tr>
<tr>
<td>D-Gly. induced L forms</td>
<td></td>
<td>3 2 0 0</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td>E-Pen. induced L forms</td>
<td></td>
<td>2 0 3 0</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td>F-Bacillary cultures</td>
<td></td>
<td>1 2 2 0</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td>G-GDW</td>
<td></td>
<td>0 0 0 0</td>
<td>0/3</td>
<td>0</td>
</tr>
<tr>
<td>H-AJB</td>
<td></td>
<td>0 0 0 0</td>
<td>0/3</td>
<td>1</td>
</tr>
</tbody>
</table>

Gly = glycine  
Pen = penicillin  
GDW = glass distilled water
DISCUSSION

The initial experiments described in the present investigations demonstrate that *in ovo* introduction of live *Salm. gallinarum* organisms in embryonated chicken eggs brings about a rapid and high mortality of the affected chick embryos. These findings are in agreement with the observations of Goodpasture and Anderson (1937) who noted that *Salm. typhi* deposited on the chorio-allantoic membrane of developing chick embryos killed them within 72 hours with occasionally survival up to the 4th day after inoculation. Buxton (1954) reported that minute doses of *Salm. pullorum* were fatal to chick embryos and likewise Solomon (1968) stated that less than five live organisms of *Salm. gallinarum* are required to kill chick embryos. The present studies confirm the observations of both these workers in that very small numbers of viable *Salm. gallinarum* organisms were necessary (*ELD*<sub>50</sub> equals approximately 3 organisms) to initiate a rapidly fatal infection thus illustrating the extreme sensitivity of the developing chick embryo to artificial infection with salmonellae and hence its suitability as an experimental animal model to test the pathogenicity of L forms and other cell wall defective variants of bacteria which have been claimed by a number of workers to be non-pathogenic.

The mortality noted in embryos inoculated with
heteromorphic L and L cultures containing transitional elements could be directly attributed to these untransformed or partially transformed elements which had multiplied in ovo to cause the death of the embryo. These cultures were pathogenic irrespective of the method employed in producing them showing that the microscopic composition of the inoculum determined the pathogenicity of the L cultures and not the method used to obtain them.

Carrère, Roux and Mandin (1955a) inoculated embryonated chicken eggs with bacillary cultures of *Salm. typhimurium*, *Brucella melitensis* and *Vibrio cholerae* which had been in contact with penicillin and human plasma and noted that only the bacillary forms of these organisms could be reisolated from the infected embryos although the inoculum contained globular forms and elements undergoing L transformation. It is unlikely that the short period of contact of their cultures with penicillin would have completely transformed all the bacillary forms into L elements (although they did mention that their inoculum contained forms undergoing transition to the L phase) and therefore at best their cultures used for infecting the chick embryos could be considered as heteromorphic L cultures consisting of L elements, transitional forms and very likely a few bacillary forms. It would thus appear that the results obtained in the present studies in embryonated eggs following the inoculation of heteromorphic L cultures and L cultures containing
transitional forms of *Salm. gallinarum* can be equated with the findings of Carrere and co-workers, although the methods used to obtain these altered variants differ.

The pretreatment of *L* cultures containing transitional elements with a brief exposure to penicillin failed to convert all these forms into the *L* phases although it reduced their numbers and ensured the complete removal of bacillary forms that were occasionally present; the inoculation of these pretreated *L* cultures into embryos generally resulted in a fulminating infection indistinguishable from that caused by bacillary cultures showing that the transitional forms had rapidly reverted to the bacillary phases which in turn could have caused the death of the embryos. These findings however, did not exclude the possibility that *L* forms themselves had reverted *in ovo* to the vegetative forms. This was best illustrated when *L* cultures induced on very high concentrations of penicillin or glycine medium containing low bacillary or transitional elements proved equally fatal to developing chick embryos indicating that *in ovo* reversion to the classical forms had taken place with concomitant overt infection and subsequent death of the embryos. The relationship of *in vitro* reversion and causation of disease can be seen in the investigations of a number of workers who had attempted to produce experimental pyelonephritis in laboratory animals using *in vitro* induced *L* forms derived from a number of Gram-
negative bacteria confirmed that the pathological conditions that followed were always associated with the reappearance of the parent vegetative forms (Alderman & Freedman, 1963; Gutman et al., 1965; Winterbrauer et al., 1967; Ponig et al., 1972).

The present findings are in agreement with the observations of Brier et al. (1962) who reported that unstable L forms of E. coli, Proteus and Staphylococcus reverted in ovo and caused death of infected embryos. Similar observations have been made by Hannoun et al. (1957) who found that they could recover only normal Streptococcus viridans following injection of embryonated chicken eggs with L phases of the organism. In contrast to these reports and the results obtained in the present investigations, Minck (1955) was able to recover the L forms of Proteus from the yolk sac of embryonated chicken eggs which had been injected with stable variants of this organism. However, he also reported that when the stable L forms were injected into the allantoic sac they not only caused the death of the embryo but they were not recoverable. Since the method used to infect embryos in the current work was via the allantoic sac, it is likely that the in ovo reversion of the L phases of Salm. gallinarum can be attributed in part to the route of infection possibly due to substances present in the allantoic fluid which favoured the reversion of L forms. This possibility could not
be entirely ruled out as shown in the experiments of Minck (1955). Furthermore allantoic fluid has been shown to be inhibitory for the development and propagation of bacteria in their L phase (Godzeski et al., 1967).

Another investigation that is pertinent to the present work is that of Carrère et al. (1955b) who observed that when L forms of Salm. typhimurium, consisting of globular bodies and "dwarf" forms were inoculated into embryonated eggs, reversion was noticed and L forms could not be demonstrated, but when these cultures were filtered and the filtrate containing only the "dwarf" forms was inoculated on to the chorio-allantoic membrane of the eggs, reversion did not occur. Since the L cultures of Salm. gallinarum used in the present studies were not filtered and as the inoculum used for the eggs contained not only the smaller L elements (referred to as the "dwarf" forms by the French workers) but also many of the larger L elements (large round pale-staining bodies, large and medium-sized spherical elements) reversion could then be attributed to the presence of some of these larger L elements; support for this assumption is based on the findings of the current in vitro findings where photographic evidence is presented showing some of the larger L elements containing bacilli and the development of bacilli from some of these large cell wall defective variants.
It thus seems reasonable to conclude that in ovo reversion of *Salm. gallinarum* L phase cultures in which no bacillary or transitional elements were present was also due to the production of bacilli from many of the larger L elements present in these cultures.

Treatment of eggs infected with unstable L forms of bacteria has been reported by Brier *et al.* (1962), as well as Godzeski (1968b) to combat reversion in ovo. The present results with the unstable L forms of *Salm. gallinarum* have confirmed these findings but extremely high doses of penicillin were required to prevent infected embryos from succumbing to a generalised bacterial infection. However, eggs infected with the bacillary cultures and treated in a similar manner survived infection thus suggesting the possibility that the administration of such high doses of penicillin merely killed all bacillary and L elements and survival of the embryo was the result of total elimination of infective agents. This possibility could not be ruled out altogether as no bacillary or L elements could be demonstrated in treated embryos killed during the course of the antibiotic therapy. This is unlikely however, as shown by the fact that one of the embryos infected with L cultures succumbed to infection despite therapy and transitional elements (though extremely few in number) could be demonstrated. The failure to
demonstrate L elements in the allantoic fluid could be due to the fact that the L forms are located intracellularly in the ectodermal cells of the chorio-allantoic membrane as has been suggested by Carrère et al. (1955a & b). There is evidence that L forms of Salm. gallinarum often occur within the cells as shown in plate 60 and therefore the failure to see or cultivate them from the allantoic fluid does not necessarily indicate that L forms are not present. On the basis of these findings it is concluded that there is a distinct possibility that the high daily doses of penicillin administered to eggs infected with bacillary and L form cultures had converted bacillary as well as L elements into intracytoplasmic forms which were probably non-virulent as suggested by Carrère et al. (1955a & b).

Godzeski et al., (1967) reported that glycine induced L cultures were not identical to antibiotic induced L cultures particularly in an in vivo environment without indicating how they differed. The experimental data presented here do not support this statement is as far as the pathogenicity or reversion of these forms are concerned in an in ovo environment; the two types of L cultures of Salm. gallinarum were not significantly different in their pathogenicity for chick embryos as can be seen in the ELD\textsubscript{50} values.

A comparison of the ELD\textsubscript{50} values of the bacillary
and L cultures (both penicillin and glycine induced) showed that considerably larger numbers of L elements were necessary to infect embryos than of bacillary forms. These differences are much greater if one considers that on reversion a single L element of *Salm. gallinarum* can give rise to several bacilli as shown in the present *in vitro* studies and this is further supported by the investigations of Hirokawa (1962) who showed that a single reverting spheroplast of *E. coli* can give rise to several rods with an average of four bacilli per reverting form.

Experiments by previous workers involving the determination in embryonated eggs of ELD$_{50}$ values of both bacillary and L form cultures have not been reported and therefore it is not possible to evaluate the data presented here in relation to investigations of a similar nature. It would appear, however, that revertants arising from L forms are less virulent than the original parental vegetative forms. Kagan and Levashov (1957) also noted that some of the revertants of *Salm. typhi* arising from L phases had a slightly lower virulence than the original vegetative forms. Guze, Harwick and Kalmanson (1976) recently reported that a strain of *Klebsiella* virulent for mice, after being passaged for several times *in vitro* as L forms gradually lost its virulence when made to revert. On the contrary other studies have shown that revertants arising from
L forms possessed virulence comparable to the parental forms (Bohnhoff & Page, 1968; Hughes, Schwarz & Gomolka, 1972).

The effects of crude endotoxin preparations of bacillary and L form cultures of *Salm. gallinarum* on developing chicken embryos showed that the immediate lethal effects of endotoxins are most marked within the first 24 hours after administration of the crude endotoxin preparations with no subsequent deaths occurring up to the time of hatching. These observations are similar to the findings of Smith & Thomas (1956) who noted that deaths in 10 day old embryos due to endotoxin damage seldom occurred more than 24 hours after the administration of these toxins. However, in the current experimental work it was observed that relatively large numbers of embryonated eggs that received crude endotoxin preparations of L and bacillary forms of *Salm. gallinarum* failed to hatch indicating that these crude preparations had in some way produced tissue damage that decreased their ultimate ability to survive. In the experiments of Smith and Thomas their observations were not extended beyond the 48-hour following administration of the endotoxin, but had they extended them up to the time of hatching, it is possible that some affect on the hatchability of the embryos would have been noticed. These workers suggested that vascular damage was the main lethal effect of the administration of
bacterial endotoxins to living 10 day old chick embryos. It is possible therefore that the failure of embryos to hatch could be attributed to vascular damage of a sublethal nature, not severe enough to cause immediate death in most of the embryos, but nevertheless, sufficient to cause decreased hatchability or affect their ultimate survival. The effects of crude endotoxin prepared from L forms of Salm. gallinarum induced by penicillin as well as by glycine in causing embryo mortality, confirm the findings of Tulasne and Lavillaureix (1955) and Dasinger and Suter (1962) who demonstrated the lethal endotoxic properties of L forms of bacteria in experimental animals. Dasinger and Suter (1962), however, reported that endotoxin prepared from L forms of Salm. paratyphi were between 5.5 and 9 times less toxic for mice than similar preparations made from the parental forms; the present studies do not indicate this to be so, as differences in the mortality caused by crude endotoxins from L and bacillary cultures of Salm. gallinarum were not marked. In contrast to these findings Wittler (1968) found the unstable glycine induced L forms of Haemophilus (Bordetella) pertussis were more toxigenic than the parental forms.

The histopathological changes noticed in embryos that had succumbed to infection with both bacillary and L cultures of Salm. gallinarum were similar to the lesions described
by Goodpasture and Anderson (1937), and Smith and Thomas (1956), suggesting that death in these embryos was the result of endotoxin damage to the host tissues. The inability to demonstrate L forms in the tissues and fluids of embryos that survived infection with L cultures and in chickens that had hatched from such infected eggs is difficult to explain. It is possible that L forms were too few in number to allow detection by the conventional methods used here or their presence in such small numbers was not sufficiently antigenic to evoke immune responses to enable them to be detected by the relatively insensitive serological methods employed in the current work. Because of their intracellular location it is unlikely that positive results would have been obtained in filtration experiments.
KEY TO THE SYMBOLS USED IN THE MICROGRAPHS
IF NOT FOUND IN THE LEGENDS ARE EXPLAINED
ON PAGE 605
PLATE 1 - Dwarf colonies of *Salm. gallinarum* strain 9S on NA medium incubated at 37°C for 48 hours. x 90

PLATE 2 - L colony of strain 9S on AJA medium containing no penicillin incubated at 37°C for 7 days. Note the dark central core surrounded by a lighter peripheral zone. x 110
PLATE 3 - Colony similar to that shown in plate 2. x 300

PLATE 4 - The same colony as shown in plate 3 viewed under a phase objective to give a better contrast between the central and peripheral parts of the L colony. x 300
PLATE 5 - The same colony as plate 3 with the condenser of the microscope lowered and the objective in the 'off click' position to give a 3 dimensional effect. x 300
PLATE 6 - Micro-L colonies of strain 9S stained by Dienes' method on AJA medium containing no penicillin incubated at 37°C for 7 days. Note the lobulated appearance x 420
PLATE 7 - A higher magnification of the colony shown in plate 6.

x 1060
PLATE 8 - Heteromorphic L colonies of strain 9S showing the core placed eccentrically grown on AJA medium containing no penicillin inoculated with AJB culture treated with 4000 units/ml penicillin and incubated at 37°C for 5 days  x 110
PLATE 9 - Heteromorphic L colonies of strain 9S showing classical morphology. Produced by inoculating treated AJB cultures (10,000 units/ml penicillin) on to AJA medium containing no penicillin and incubated at 37°C for 5 days. x 110
PLATE 10 - Dienes’stained preparation of heteromorphic L colonies of strain 9S. Note the large numbers of the larger L elements and filamentous transitional forms. x 2000
PLATE 11 - Gram's smear of a 7 day confluent growth of strain 9S on MC medium inoculated with treated AJB cultures (4000 units/ml penicillin) and incubated at 37°C.  x 1600
PLATE 12 - Gram's smear of a 3 day old bacillary culture of strain 9R on NA medium incubated at 37°C. x 1800
PLATE 13 - Dienes' stained preparation of 7 day old reverting heteromorphic L growth of strain 9R on AJA medium pH 8.1, containing 100 units/ml penicillin incubated at 30°C. x 1060
PLATE 14 - Patchy granular L type growth of strain 9S on AJA medium containing 100 units/ml penicillin incubated at 30°C for 24 hours. x 95
PLATE 15 - Dienes' stained preparation of 5 day old heteromorphic L growth of strain 9R on AJA medium containing penicillin in a central well (500 units) incubated at 37°C. x 1060
PLATE 17 - Gram's smear of the same growth shown in plate 15. Note the vast amount of nondescript material. x 1600
PLATE 18 - Heteromorphic L colony of strain 9S showing the classical L colony appearance on AJA medium containing 10 per cent. serum and 100 units/ml penicillin incubated at 30°C. x 95
PLATE 19 - Large globular atypical L colonies of strain 9R growing within the depths of AJA medium containing 20 per cent. serum and 200 units/ml of penicillin incubated anaerobically at 37°C for 4 days. x 95
PLATE 20 - Colonies of strain 9S showing classical L morphology on AJA medium containing 10 per cent. serum and 150 units/ml penicillin incubated at 30°C for 2 days.  x 40
PLATE 21 - Dienes' stained preparation of a 3 day old patchy granular L growth of strain 9S on AJA medium containing 10 per cent. serum and 200 units/ml penicillin incubated at 30°C. x 1600
PLATE 22 - Dienes' stained preparation of 3 day old L growth of strain 98R on AJA medium pH 6.7 containing 100 units/ml penicillin incubated at 30°C x 1060
PLATE 23 - subsurface growth of L colonies of strain 9S after removal of the surface confluent growth incubated 48 hours. x 110
PLATE 24 - Dienes' stained preparation of the subsurface L growth shown in plate 23. Note the distortion of many of the L elements (L). x 1600
PLATE 25 - Gram's smear of a 10 day old L colony of strain 9S on AJA medium containing 10 per cent. serum and 200 units/ml penicillin incubated at 30°C. x 1600
PLATE 26 - Dienes' stained preparation of the centre of the L colony shown in plate 25. Note the deformed L elements (Arrow) x 1600
PLATE 27 - Dienes' stained preparation of a 9 day old bacillary growth of strain 9R on AJA medium containing no penicillin incubated at 30°C x 1600
PLATE 28 - Heteromorphic L colony of strain 9S produced on subculture on AJA medium with penicillin (1000 units) diffused from a central well incubated at 37°C for 5 days. These colonies show a large central core and a relatively narrow periphery. x 90
PLATE 29 - Reverting L colonies of strain 9S on AJA medium containing 10 per cent. serum and 300 units/ml penicillin incubated at 37°C for 10 days. Note the wavy and poorly defined peripheral zone of the colony and the dark central portion.  x 90
PLATE 30 - A drawing of some of the 'T' elements found in 7-10 day old L and heteromorphic L cultures of strain 9R incubated at 30°C on AJA media containing serum and penicillin.
PLATE 31 - Dienes' stained preparation of a 5 day old heteromorphic L growth of strain 9R on AJA medium containing 20 per cent. serum 1,000 units/ml penicillin incubated at 30°C. Note a cluster of the larger L elements (arrow). x 1250
PLATE 32 - The same preparation as in plate 31 showing bipolar staining of the coccobacillary form, condensation of the cytoplasm towards the periphery of the large spherical elements. Note some of the coccobacillary forms occurring in chains. x 3125
PLATE 33 - Gram's smear of the same growth in plate 31 showing only the coccobacillary forms and a few ring-shaped bodies (Rs). Note the absence of the L elements.

x 3125
PLATE 34 - Irregularly shaped wrinkled L colony of strain 9R on AJA medium containing 3 per cent. glycine incubated at 30°C for 9 days  x 40
PLATE 35 - Dienes' stained preparation of 4 day old colonies of strain 9S on AJA medium containing 10 per cent. serum and 3 per cent. glycine incubated at 30°. x 1600
PLATE 36 - Dienes' stained preparations of 14 day old L colonies of strain 9S on AJA medium containing 10 per cent. serum and 3 per cent. glycine incubated at 30°C. Note the presence of intracellular granules in some of the large L elements (arrow) x 1600
PLATE 37 - Dienes' stained preparation of a 48 hour old heteromorphic L growth of strain 9R on AJA medium containing 10 per cent. serum, 1.5 per cent. glycine and 50 units/ml penicillin incubated at 30°C. Transitional filamentous forms appear beaded. x1250
PLATE 38 - Gram's smear of a 4 day old bacillary growth of strain 37/74 on AJA medium containing 10 per cent. serum incubated at 30°C. Note the absence of transitional filamentous forms x 1600
PLATE 39 - L colony of strain 37/74 produced on AJA medium containing 10 per cent. serum and 1.5 per cent. glycine incubated at 30°C for 17 days showing classical L type morphology x 64
PLATE 40 - Dienes' stained preparation of a 4 day old heteromorphous L colony of strain 9R on AJA medium containing 20 per cent serum and 1,000 units/ml penicillin. Fr. fragmentation of the large round pale-staining bodies giving rise to smaller L elements. Note the cluster of rods appearing to arise from one of the larger L elements (arrow) x 1600
PLATE 41 - Gram's smear from a 4 day old confluent L growth of strain 784/71 on AJA medium containing 10 per cent. serum and 1.5 per cent. glycine and incubated at 30°C. Note the absence of transitional filamentous forms and bacilli x 1600
PLATE 42 - Gram's smears of a 4 day old slimy L colony of strain 892/71 on AJA medium containing 10 per cent. serum and 1.5 per cent. glycine incubated at 30°C. Note the absence of transitional and bacillary forms. x 1600
PLATE 43 - Dienes' stained preparation of the same growth as in plate 42 showing transitional forms not seen in Gram's staining.  x 1600
PLATE 44 - Dienes' stained preparation of a 3 day bacillary growth of strain 595/72 on AJA medium containing no penicillin or serum incubated at 30°C. Note the presence of rod-like structures in a large round pale staining body (arrow). x 1600
PLATE 45 - Gram's smear from a 3 day heteromorphic growth on NAJA medium containing 20 per cent. serum with penicillin (10,000 units) diffused from a central well incubated at 30°C. Many of the transitional rod forms stain poorly and occur in clumps (arrow). x 3000
PLATE 46 - Gram's smear from a 3 day old micro-colony of strain 9S produced on M medium containing 100 units/ml penicillin incubated at 30°C x 1600
PLATE 47 - Gram's smear of a 6 day old heteromorphic L growth of strain 9R on AJA medium containing 10 per cent. serum and 1.5 per cent. glycine. Note the bulbous terminal portion of a transitional filamentous form (arrow) x 3125
PLATE 48 - Wet preparation of a 7 day old culture of strain 9S in AJB containing 4,000 units/ml penicillin incubated at 37°C showing spheroplasts (s) x 3125
PLATE 49 - Gram's smear of 15 day old flaky L growth of strain 9S in AJB containing 10 per cent. serum and 1000 units/ml penicillin incubated at 30°C. rz. branching rhizoid bodies. bl. bulbous bodies. y. irregular shaped bodies.         x 1600
PLATE 50 - Gram's smear of a 22 day old 30°C incubated L growth of strain 9S in AJB containing 10 per cent. serum and 1,000 units/ml penicillin showing branched filamentous bodies with knob-like enlargements (fb), small spherical elements and granules x1600
PLATE 51 - 9 day old faint granular growth produced by strain 9R beneath a 0.6 μm Millipore filter membrane placed on AJA medium containing 10 per cent. serum and 200 units/ml penicillin incubated at 30°C x 100
PLATE 52 - Dienes' stained preparation of a 4 day L growth of strain 892/71 on AJA medium containing 10 per cent. serum and 1,000 units/ml penicillin incubated at 30°C. A large round pale staining body containing a rod-like structure (arrow). Note the difference in the staining properties between spherical elements and the large round pale staining bodies. × 1600
PLATE 53 - MGG stained smear of the harvested growth from a 4 day old L culture of strain 892/71 shown in plate 62, and used to inoculate eggs. Note the presence of fine granules, V shaped bodies (arrow) and fine filamentous forms (f) x 1600
PLATE 54 - Gram's smear of the same material as in plate 53. The fine filaments (f) are stained blue.  x 1600
PLATE 55 - Section of liver from 12 day old chicken embryo infected with penicillin-induced L forms of *Salm. gallinarum* strain 892/71. Bacilli and a single transitional filamentous form present in a sinusoid. Giemsa x 1600
PLATE 56 - Section of kidney from a 12 days old chicken embryo infected with glycine-induced L forms of Salm. gallinarum strain 892/71. Giemsa x 1600
PLATE 57 - Section of the chorioallantoic membrane of a 14 day old chicken embryo injected with the bacillary forms of *Salm. gallinarum*. Note the extensive areas of necrosis (arrow), congestion of blood vessels (cv) H & E  x 200
PLATE 58 - Section of the chorioallantoic membrane from a 12 day chicken embryo infected with bacillary cultures of *Salm. gallinarum* strain 892/71. Note the denuding of cells lining the CAM. (arrow); a. loss of endothelial cells lining blood vessels. bc. bacterial colony. vc. vacuolation of cells H & E  x 800
PLATE 59 - Section of chorioallantoic membrane from a 14 day old chicken embryo injected with bacillary forms of Salm. gallinarum strain 892/71.

Giemsa x 2000
PLATE 60 - Section of the chorioallantoic membrane from a 12 day old chicken embryo injected with glycine-induced L forms of Salm. gallinarum strain 892/71. Giemsa \( \times 1600 \)
PLATE 61 - Section of chorioallantoic membrane from a 12 day old chicken embryo inoculated with crude endotoxin of the bacillary form of *Salm. gallinarum*. 

nf. areas of focal necrosis. 
dv. blood vessel damage - loss of lining cells of the blood vessel. 
cv. vascular congestion. H & E x 200
PLATE 62 - Thin section of bacterial cells of *Salm. gallinarum* strain 9S 30°C incubated in AJB (with 10 per cent. horse serum) for 9 days showing the rod shaped bacterial profile. x 20,000
PLATE 63 - Higher magnification (x 80,000) of plate 62 showing the triple electron-dense layered cell wall. The cytoplasmic membrane and part of cell wall have been damaged during preparative procedures.
PLATE 64 - Thin section of bacterial cell of *Salm. gallinarum* strain 9S from a 9 day, incubated (30°C) AJB (with 10 per cent. horse serum) showing cell wall consisting of 4 electron-dense lines. Arrow shows the 4th electron dense line between cytoplasmic membrane and cell wall. x 100,000
PLATE 65 - Thin section of bacillary and transitional filamentous forms of Salm. gallinarum strain 9S in a 9 day incubated (30°C) serum - AJB showing the wrinkled cell wall profile of the bacillary forms. Note cell wall damage at enlarged polar regions of the transitional filamentous form (arrows) x 30,000
PLATE 66 - Thin section of *Salm. gallinarum* strain 9R from 9 day incubated (30°C) on AJA medium, showing bacillary forms with a non-wrinkled thick cell wall and a retracted cytoplasmic membrane.  x 30,000
PLATE 67 - Higher magnification (x 40,000) of plate 66. Arrow points to the gap between cell wall and cytoplasmic membrane.
PLATE 68 - Higher magnification (x 80,000) of plates 66 and 67 the triple layered cell wall.
PLATE 69 - Thin section of *Salm. gallinarum* strain 9R grown on NA at 37°C for 3 days. Note the thick wavy cell wall profile. The cytoplasmic membrane is more closely applied to the cell wall than in plate 68. x 60,000
PLATE 70 - Thin section of L forms in a 22 day 30°C partially stabilised L culture of Salm. gallinarum strain 9S in AJB with penicillin showing L forms (LF) bounded by a cytoplasmic membrane and L forms (LFI) with parts of cell wall layers retained. x 20,000
PLATE 71 - Thin section of 22 day 30°C partially stabilised L cultures of *Salm. gallinarum* strain 9S induced in a 10 per cent. serum AJB medium containing 1000 units/ml penicillin showing cell wall defective variant (CWD). x 30,000
PLATE 72 - Higher magnification of plate 71 (x 80,000) of a cell wall defective variant (CWD) showing the modified cell wall with 2 electron dense lines ($X_1$, $X_2$) and areas of the cell wall with 3 electron dense lines ($X_1$, $X_2$, $X_3$) and the cytoplasmic membrane.
PLATE 73 - Thin section of 4 day 30°C L culture of *Salm. gallinarum* strain 9S grown on AJA medium. The L forms are bounded only by a cytoplasmic membrane. Many vesicular bodies containing ribosomal granular material are present in these L forms. Note the irregular distribution of collections of dense ribosomal material in the L forms. x 15,000
PLATE 74 - Thin section of 14 day 30°C L culture of *Salm. gallinarum* strain 9S on AJA medium. The L forms are bounded only by a cytoplasmic membrane. Vesicular bodies bounded by 'unit' membrane and dense bodies are present in the L forms

x 30,000
PLATE 75 - Thin section of cell wall defective variants (CWD) in a 4 day 30°C L culture of Salm. gallinarum strain 9S on AJA medium. The modified cell wall is shown as an electron dense-light-dense integument. x 40,000
PLATE 76 - Thin section of L and revertant forms in 14 day 30°C L culture of *Salm. gallinarum* strain 9S on AJA medium (3 per cent. glycine and 10 per cent. serum) showing L forms with little internal organisation (LF₁) and L form with well defined ribosomal areas (LF₂) and two L forms with dense and vesicular bodies (LF₃). x 7,500
PLATE 77 - Thin section of 14 day 30°C L culture of *Salm. gallinarum* strain 9S on AJA medium showing a reverting bacillary form. Note the gap between the cell wall and the cytoplasmic membrane (arrowed). The cell wall has been thrown into loose folds.  x 40,000
PLATE 78 - Thin section of a 14 day 30°C L culture of Salm.gallinarum strain 9S on AJA medium containing 3 per cent. glycine and 10 per cent serum showing a segment of the cell wall still attached, with the cytoplasmic membrane alone making up most of the circumference of the cell, most of the ribosomal areas are distributed peripherally. x 20,000
SYMBOLS USED IN MICROGRAPHS

b = Bacilli
cb = Coccobacilli
Cm = Cytoplasmic membrane
CW = Cell wall
D = Distorted large round pale staining bodies
Ds = Distorted large spherical elements
g = granules
L = L elements
Ls = Large spherical elements
Ms = Medium sized spherical elements
N = Nuclear areas
Nd = Nondescript material
R = Ribosomes
RB = Revertants
Rb = Large round pale staining bodies
SDB = Small dense bodies
Sb = Swollen bacilli
Ss = Small spherical elements
Tf = Transitional filamentous forms
Tr = Transitional rod forms
Ts = Tiny spherical elements
VB = Vesicular bodies


Arkwright, J.A. (1931). Variation: pp.320-


Burdon-Sanderson (1871). The origin and distribution of microzymes (bacteria) in water and the circumstances which determine their existence. Q. Jl. microsc. Sci., 11, 323-352.


Charrin (1889) 'La maladie pyocyanique', Paris, 1889. (Cited by Wilson, 1906).


Hirokawa, H. (1962). Biochemical and cytological observations during the reversing process from spheroplasts to rod form cells in *E. coli*. J. Bact. 84, 1161-1168.


van Iterson, W., Ruys, A.C., Botman, M.J. (1964). 


In. I.M. Rivers (ed) Filterable Viruses.
Bailliere, Tindall & Cox, London.

Mycoplasma and bacterial L-form colonies in
relief with an ordinary light microscope by
means of oblique light. Appl. Microbiol., 25,
484-488.

Murray, R.G.E., (1968). Bacterial cell wall anatomy in
relation to the formation of spheroplasts and
Microbial Protoplasts, Spheroplasts and L forms.
The Williams & Wilkins Co., Baltimore.

location of the mucopeptide in sections of the
cell wall of Escherichia coli and other Gram
547-560.

Muschel, L.H. (1968). The formation of spheroplasts by
immune substances against diverse rounded forms.
pp. 19-29. In L.B. Guze (ed) Microbial Protoplasts,
Spheroplasts and L forms. The Williams & Wilkins
Co. Baltimore.

of bacterial protoplasts by serum components.
J. Immun., 82, 38-42.


(cited by Lohnis, 1922).


The properties of L forms isolated from *Salmonella* and the isolation of L forms from *Shigella*. J. Bact., 59, 765-775.


ACKNOWLEDGEMENTS

My thanks are due to Professor A. Buxton and Dr. J.E. Phillips for their help, advice and guidance in connection with the present study. I would also express my thanks to Dr. S. Thuraisingham and Dr. Fadzil bin Hj. Yayaha for making it possible to do part of this work at the Veterinary Research Institute, Ipoh, West Malaysia.

I am deeply indebted to Mr. C.H. Mulholland, his staff, Miss Noor Liala and Miss Tan Lin Jee for their assistance. My thanks are also due to Mr. R.C. James, Mr. R.K. Thomson and Mr. Liew Sin Wah for photography, Miss M.W. Millar for preparation of photocopies, and Miss M. Buchan for her great care in the preparation of the typescript.

I would like to acknowledge the financial support given to me from the Department of Technical Co-operation, Ministry of Overseas Development, U.K.