LISTERIA MONOCYTOGENES:

A REVIEW AND COLONIAL DISSOCIATION

By

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A THESIS

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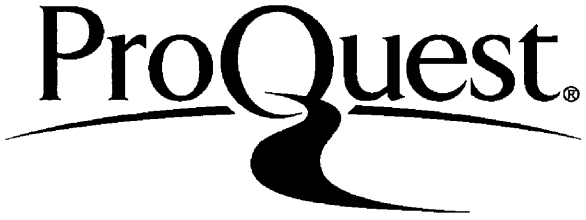

Affectionately dedicated

to

Nell

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The thesis perhaps is the most concrete evidence of the earning of a graduate degree. It is something one can pick up and hold and even occasionally scan its pages. But it is a cold thing of hard facts and no emotion. Only the author and the few closely associated with him see among the cold facts the high pinacles of elation and the deep, wide valleys of disappointment, and the even deeper canyons of despair. Many times there seemed no escape up the steep and rocky canyon wall. In the silence and loneliness of discouragement one heard only the echoing admonition of Solomon --- "of making many books there is no end and much study is a weariness of the flesh" --- or the cry of Job --- "would that my adversary had written a book". But fortunately there was also a louder, more compelling cry - not an echo - a command --- "have dominion over the earth and subdue it". With an ideal no wall is too steep, no rock too sharp and one can always reach the plateau. But the plateau is a hot desert and surely no stopping place. The goal is the high mountain top on the horizon still far away. There is more climbing, more slipping, more sliding, another bruise; but another view of the mountain top, and this time a view perhaps obscured by thick storm clouds of apprehension. But it is closer --- one more stream to ford, one more valley to cross and then finally - the top. And far below stretches all the disappointments, all the hopes, all the accomplishments, all blended in a hazy film of distance. But even the mountain top is cold and windy and one can never linger long, but must go back to plateaus and valleys and canyons. There are more mountains to climb and one must climb many if he is to have dominion over the earth and subdue it. All of this the author sees between the cold facts on the printed page.

To the many who helped me to reach the first mountain top I want to say "thanks". One couldn't quickly forget the many kind words of encouragement of Dr. H. J. Stafseth, major professor of the guidance committee. His soft spoken voice always conveyed sincerity and was not easily ignored or forgotten. Dr. Frank Thorp, Jr. because of his more intimate association with the problem was in a better position to aid, and his broad knowledge and past accomplishments were a continual source of inspiration. Perhaps the intense compelling influence of other years has dimmed a bit, but its memory still compels those who were fortunate enough to experience it. Dr. C. A. Hoppert and Dr. C. F. Huffman by their accomplishments in their chosen fields provided a continual source of inspiration which was keenly felt in personal contacts. There are many others who deserve thanks: the technicians, Miss Sylvia Laine and Miss JoAnn Shriver who helped with the many blood counts and fermentation studies; Mrs. Alubowicz, reference librarian, for her assistance in securing the many inter-library loans; Dr. R. A. Runnells, head of the Department of Animal Pathology; all my colleagues in the department and the Michigan Agricultural Experiment Station who granted a leave of absence in order that the work could be accomplished in a shorter time. I will always be grateful to Nell for the many hours she spent typing the manuscript. And it would be unjust not to mention the three dandy boys who never audibly protested the many hours they lost with Dad. One never can forget the tiny, spontaneous voice of the little boy with hands folded at the dinner table - bless the food --- and help daddy with his thesis ---.

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Introduction

Almost three decades have passed since the first description of the bacterium known as Listeria monocytogenes. The passing of twenty eight years has added little to our knowledge of either the bacterium or to the host of diseases with which it has been associated. To many it is thought to be of little or no economic importance. It is often relegated to the class of laboratory curiosities, a bacterium that can produce a high circulating monocytosis in the peripheral blood or a marked purulent conjunctivitis in a susceptible laboratory animal. But to those who have studied it closely it is a vicious killer, and the few who survive its attack are usually left with permanent physical or mental defects. The constantly mounting evidence suggest that this bacterium is actually widely distributed in nature and that all warm blooded animals and man are susceptible hosts; indeed, all may be carriers. Still unknown are the factors which dictate that in certain species infection results in encephalitis, while in others infection is manifested by septicemia, or abortion, conjunctivitis or some other disorder.

Several factors contribute to the lack of knowledge of the genus Listeria. These are an unawareness of the bacterium which often results in its being discarded as a contaminating diphtheroid; the difficulty of isolating it from certain animal tissues with the consequence that the culture is reported as negative; the misconception that infection with L. monocytogenes is rare; the inability to reproduce in the laboratory some of the diseases with which it has been associated and the failure to determine its natural reservoir.

It was felt that a through review of the world's literature dealing with infections with L. monocytogenes might reveal trends or clues that would aid in the solution of the many unsolved problems surrounding the epidemiology and pathogenesis of the many forms of listeriosis. It was also felt that a study of the colonial dissociation of L. monocytogenes might lead to the recognition of additional colonial forms; forms that conceivably might be found in nature or in inapparent infections, and thus aid in the search to establish both the natural reservoir and the mode of transmission from host to host. These were the goals --- the results unfold on the following pages.

PART I

REVIEW

REVIEW

Listeriosis is one of the most recently described and least understood of all the bacterial infections of man, his domestic animals, and the wild life which share his cities and farms. Hulphers, a Swedish worker, appears to be the first to record an encounter with the causative agent, Listeria monocytogenes. In 1911 he isolated a bacterium from the necrotic liver of a rabbit, and his description of the organism, published in Svensk Veterinartidsskrift, corresponds very closely to that of L. monocytogenes. No doubt the small epidemic of meningitis reported by Atkinson in 1917 also was due to this bacterium. The case reported by Dumont and Cotoni in 1921 was confirmed by Cotoni in 1942 as due to L. monocytogenes. These reports indicate that infections with members of this genus were known prior to its first description in 1926 by Murray, Webb and Swann. They isolated the bacterium from the liver of rabbits and guinea pigs and named it Bacterium monocytogenes. The following year Pirie (1927) reported isolation from the liver of the African jumping mouse (Tatera lobengulae) of a bacterium which he named Listerella hepatolytica. The first recognized association of this bacterium with infection in man was in 1929 when Nyfeld in Denmark isolated it from the blood of patients with infectious mononucleosis.

Matthews (1928) reported an outbreak of encephalitis of unknown etiology in cattle which very likely was listeriosis. But the first reported isolation of L. monocytogenes from a domestic animal was by Gill (1931) in New Zealand. He isolated the microbe from the brain of sheep and established the relation of this microorganism to the etiology of

"circling disease", a name still often applied erroneously to listeriosis. Pletnova and Stiksove (1950) claim its isolation from pigs in the Soviet Union as early as 1924.

In recent years infections with this bacterium have become so wide spread that today it is considered to be the eleventh in economic importance among the diseases of domestic animals. Its proper place in human medicine has not yet been determined, but with an increased awareness of the disease, and the mounting number of case reports, it may eventually be of far greater significance than previously suspected. The role of wild life in the spread of the infection is still to be determined, but judging from the numerous species from which it has been isolated, it would appear that it may have an important part. If man is to protect himself, and also his domestic animals and wild life from this infection which very often kills or leaves its victims physical or mental cripples, it is imperative that a complete study be made to determine effective methods of diagnosis, mode of transmission, methods of treatment and a system of control.

Until 1940 there was considerable confusion in the nomenclature of this bacterium due to the interchange of the generic names Listerella and Listeria. Pirie (1927) chose Listerella as the generic name in honor of Lord Lister, the well known pioneer in the field of bacteriology. However, this name had already been applied to a group of slime molds (Mycetozoaire) by Jahn (1906). Bacterium as applied by Murray et al. (1927) was undesirable because the microbe did not possess the characteristics of this genus. In 1936 Mesnil proposed that in all duplication of generic names, only the one first applied should be considered valid.

This resolution was adapted by the committee on nomenclature of the Third International Congress for microbiology, New York, 1939, (Prevot, 1944). This invalidated the generic name proposed by Pirie and in 1940 he proposed the name Listeria, which was adopted in the sixth edition of Bergey's Manual of Determinative Bacteriology (1948), and is now the official generic name. Wilson and Miles (1948) in the third edition of Topley and Wilson's Principles of Bacteriology and Immunity, employed the generic name Erysipelothrix. This was based on the work of Barber (1939) who showed that there were striking similarities between Listeria and Erysipelothrix. It is hoped that these authors will conform to the newer nomenclature in their next edition. The species name monocytogenes is based on the fact that large numbers of monocytes are often found in the peripheral blood of both naturally and artificially infected animals. Other synonyms in addition to those already mentioned are: Bacterium hepatis (Hulphers, 1911); Listerella monocytogenes hominis (Nyfeldt, 1932) Corynebacterium parvulum (Schülz et al., 1934) Cornybacterium infantisepticum (Potel, 1950) and Listeria infantiseptica (Potel, 1952).

Listeria monocytogenes is a small gram positive, non-spore forming diphtheroid-like rod. It is very easily confused with members of the genus Corynebacterium, and no doubt often has been discarded as a "contaminating diphtheroid". Several reports in the literature bear this out (Atkinson, 1917; Tesdal, 1934; Marcellus et al., 1936; Kennedy, 1947; Potel, 1950). It is extremely resistant to heat (Ozgen, 1951; Zink et al., 1951) and dessication, but is killed by most of the common disinfectants (Kaboyashi, 1951). It is easily cultivated on the common bacterial media. Difco* tryptose agar affords an excellent substrate for growth

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*Difco Laboratories, Inc., Detroit, Mich.

and preservation of cultures. It is aerobic to microaerophilic and when grown at room temperature exhibits a peculiar tumbling motility which usually is absent when grown at 37° C. On blood agar it is beta hemolytic and is not known to produce either endo- or exotoxins. Intravenous inoculation of rabbits, guinea pigs or mice stimulates the formation of a high percentage of circulating monocytes. Most strains produce a characteristic conjunctivitis and keratitis when instilled into the conjunctival sac of the rabbit. The monocytosis and conjunctivitis often are used for quick identification of suspect-cultures.

The various forms of listeriosis are widely distributed and have been reported from all continents. The disease is most commonly found in the temperate zone and has not been reported from the tropics. In the United States it has been reported in all but a few southern states. In Canada it has been reported as far north as the Hudson Bay country. Sheep, cows, and goats appear to be the most susceptible species. Man, chickens, rodents, pigs, horses and dogs follow in that order.

The epidemiology of listeriosis in both animals and man is obscure, and the natural reservoir of L. monocytogenes has never been determined. However, there are strong indications that rodents, swine or fowls may play a part in transmission. The role of insects and parasites often has been suggested, but supporting evidence is lacking. It also may be that this bacterium is widely distributed in nature, perhaps in the form of a soil bacterium or as a saprophyte and becomes a pathogen only when the host is subjected to certain physical or physiological stress. Listeriosis in ruminants is most prevalent between November and April and seldom occurs during the summer. The only exception to this is the

report of Khalimbekov (1952) who found that in Azerbaijan the disease is most prevalent during the hot summer months. Records kept at the Michigan Agricultural Experiment Station indicate that the number of outbreaks increases two to four days following sudden drops in temperature or heavy snow falls, suggesting that climate may play a part. Usually outbreaks fall off rapidly when animals have access to green pasture. The feeding of ensilage was suggested by Olafson (1940) as a predisposing factor in susceptibility. However, in an investigation of this, Olson et al. (1953) could draw no definite conclusions. The possibility of a virus as a "trigger" mechanism has been suggested but not confirmed (Olson et al., 1951; Seeliger and Leineweber, 1952; Geurden and Devor, 1954).

The mode of entry in the host in all forms of the disease is obscure. Enteritis often is found in encephalitic listeriosis, but it is unlikely that food or water can be incriminated. With the exception of mice, it is almost impossible to infect artificially an animal by the oral route. The often present rhinitis supports the possibility of entry through the upper respiratory tract. Pallaske (1940) on two occasions successfully isolated L. monocytogenes from the nasal exudate of ewes with listeriosis. The conjunctivitis often found in listeriosis suggests that the eye may play a part in transmission (Van Driest, 1948; Slooff, 1948; Beute et al., 1948; Gray et al., 1951). Graham et al. (1943) and Gray et al. (1948) each reported one instance where the encephalitic form of the disease developed in a pig and rabbit respectively, following ocular instillation. Asahi and Hosoda (1952) successfully produced symptoms and lesions of listeric encephalitis in both goats and rabbits following ocular instil-

lation of culture. Also postulated is entry through skin breaks and survival in the host until stress creates an environment favorable for the disease (Seeliger et al., 1952).

Eveleth et al. (1953) found that when healthy sheep are imported from infected flocks into non infected flocks the disease often appears in the new flock although it may be several months before symptoms are observed. Gray et al. (1951) reported that cows from the same southwestern feeding ranges developed the disease at about the same time when introduced into northern feed lots, even though the lots were widely separated from each other, but subject to the same climatic conditions. These findings strongly suggest that some animals may be non clinical carriers, and one should use caution when introducing new animals into a flock or herd. Further support for this is the fact that many domestic animals display high agglutinating titers for the bacterium (Belin, 1946; Graham et al., 1943; Hirato et al., 1954; Potel, 1954).

It is relatively easy to isolate L. monocytogenes from the liver or other organs in the septicemic or abortive form of the disease, provided a fairly large inoculum is used, as it has been found that the number of organisms present may be small. For an unexplained reason, it is difficult to isolate the bacterium from the blood of both naturally and artificially infected animals. This tissue should not be used for isolation attempts if other organs are available.

In attempting to isolate L. monocytogenes from the encephalitic form it is essential that cultures are prepared from the medulla oblongata. Spencer et al. (1944), Olson et al. (1953a), and Hirato et al. (1954)

found the number of organisms in this portion of the brain to be far greater than in any other area. The method of isolation found to be most successful is a modification of a technique described by Biester and Schwarte (1939) and consists in aseptically macerating a fairly large portion of the medulla in a sterile mortar or Waring blendor to which has been added 10 to 15 ml sterile distilled water or broth. Approximately 0.2 ml of the resulting suspension is plated on a tryptose agar plate and incubated 18-24 hr at either 37° C or at room temperature. With this technique it is relatively easy to isolate the microorganism from sheep or goats, but often cultures prepared in this manner from the bovine remain sterile. In this event the prepared medulla suspension should be refrigerated for several days or weeks and replated. With the employment of this technique, Gray et al. (1951) were able to demonstrate the bacterium in large numbers in all instances where the disease was suspected and where tissue sections revealed lesions characteristic of listeriosis. These findings have been confirmed by a number of investigators (Bein, 1951; Van den Schaaf et al., 1951; Zink et al., 1951; Seeliger and Linzenmeier, 1953; Geunden and Devor, 1954). The number of colonies which develop in initial cultures from the bovine is always small compared to those of the initial isolation from the ovine or caprine. Gray et al. (1948) suggested that this phenomenon may be associated with a bacteriostatic factor in the bovine brain which may account for the greater resistance to the disease displayed by cows. Tissue sections reveal the bacteria to be incarcerated in the focal lesions and often intracellularly in the macrophages and glial cells. This makes it essential that the medulla be ground.

Pallaske (1940), Belin (1943) and Olson et al. (1953) reported

the successful isolation of L. monocytogenes from the liver and spleen of sheep with encephalitic listeriosis. The number of colonies which developed was always small. No similar findings are reported for cattle. Except in man, antemortem isolation is unreported.

Olson et al. (1953b) compared the efficacy of various isolation techniques in attempts to isolate L. monocytogenes from the brain and other organs of both naturally and artificially infected sheep. These techniques included duplicate cultures from each organ, inoculation of mice with suspensions of suspected tissue, storage of ground tissue in glycerine, centrifugation and selective media. The best results were obtained by grinding duplicate samples of fresh tissue. With this technique in another study Olson et al. (1953a) successfully isolated L. monocytogenes from the brain of 94% of the sheep brains cultured. Unfortunately they did not include in their study the refrigeration technique described in the preceding paragraphs which gave 100% isolations from sheep brains which showed perivascular cuffing and focal necrosis characteristic of listeric encephalitis. (Gray et al., 1951). In respect to the potassium tellurite medium described by Gray et al. (1950), Olson et al. (1953) found inhibition of many individual strains. This finding has been confirmed in unpublished studies and limits to a marked degree its use in isolation attempts.

The inoculation of rabbits or Guinea pigs with tissue suspensions or body fluids suspected of harboring L. monocytogenes has been employed with success in the Soviet Union. With this technique Gudkowa and Sacharoff (1946) and Bilibin (1949) demonstrated the presence of L. monocytogenes in the blood of patients with an infectious mononucleosis-like syndrome and who showed rather high agglutinating anti-

body titers against the specific bacterium. In view of the existing difficulty in successfully isolating L. monocytogenes from blood, there is a need for wider application of this technique in both human and veterinary medicine.

At present encephalitis in ruminants is the form of listeriosis most frequently recognized and is of greatest economic importance. It generally occurs during the period from late November to the first of May, and is most prevalent during February and March. It may affect animals of all ages although there are indications that the young are more susceptible than older animals. Encephalitis without septicemia is unknown before the rumen is functional. It may affect both sexes but there is evidence that pregnant ewes are infected more frequently than non-pregnant. Beef cattle appear to be more susceptible than dairy cattle. The higher incidence in beef cattle may be explained by the fact that generally they are exposed to a more vigorous winter environment.

In sheep and goats the disease is extremely acute and death may occur in four to 48 hr after appearance of symptoms. Some survive several days. Recovery is rare in sheep and goats that show definite symptoms of infection. Mortality may range from three to 30% or more. Recently attention has been focused on a more chronic non-fatal form of encephalitis in sheep. This usually occurs simultaneously with the more typical form. Many of these animals are said to recover, but until a better antemortem diagnosis is developed it can not be stated definitely whether these sheep have had listeriosis or not. If they are actually infected with L. monocytogenes, there is the possibility that they

may act as carriers for long periods of time. Further research is needed to confirm or deny this hypothesis. For the present it appears that such recovered animals should be eliminated from the flock as quickly as possible.

In cattle the disease is more chronic in nature and most cows survive from four to 14 days. Pounden et al. (1947) reported one of the few acute outbreaks in cattle in which deaths were sudden and a high percentage of the herd was affected. Usually no more than eight to 10% of a herd is affected. Spontaneous recovery may often be observed in cattle (Biester and Schwarte, 1941; Jensen and Mackey, 1949), but many of these animals, and also those which respond to therapy, show evidence of permanent brain damage. These animals are unsightly and unable to compete with the rest of the herd. This presents no particular problem in either management or public health in beef cattle unless they continue as carriers, which is not yet established, but does have esthetic disadvantages in dairy herds.

The symptoms in sheep, goats and cattle are similar and differ only in severity. At the onset the infected animal usually separates itself from the rest of the herd. It appears depressed, confused and indifferent to surroundings. Then follows incoordination and torticollis. Often local paralysis of the facial and throat muscles interferes with swallowing and results in marked salivation and drooping of one or both ears. Intermittent twitching of these muscles is often observed, but frank convulsions are rare. There is often strabismus, conjunctivitis and the animal may appear blind. A marked nasal discharge, anorexia and a temperature of as much as 108° F or more are common. In the early

stages the animal tends to crowd into a corner or lean against stationary objects as if unable to stand unsupported. If the animal walks it often moves in a circle, and if it circles, it is always in the same direction. Only a small percentage of the animals with listeric encephalitis show this symptom. In the terminal stages the animal falls and can not get up unassisted. When it is down there are generally involuntary and aimless running motions. The head, neck, and fore legs usually are more tense than the posterior part of the body. Visciousness is not seen except occasionally in cattle. It is unlikely that all these symptoms would appear in any one animal. Many cows show only an inability to swallow due to paralysis of facial and throat muscles.

Antemortem diagnosis of listeriosis is virtually impossible as there is no satisfactory diagnostic test. Listeriosis can be confirmed only by isolation and identification of L. monocytogenes. Some progress is being made toward developing a serological test, especially in human medicine, in Germany (Seeliger, 1953). The blood picture usually shows no variation from normal and the peripheral monocytosis often found in man, rodents, and artificial infections seldom is seen in infected ruminants.

In man L. monocytogenes has been isolated on numerous occasions by means of spinal puncture. This technique applied to sheep and cattle has thus far resulted in failure.

It has often been demonstrated that clinical symptoms alone are not satisfactory as a diagnostic criterion. Subsequent deaths following similar symptoms in the same herd after positive diagnosis may be presumed to be listeriosis. However, even this often proves unreliable

particularly in sheep flocks where listeriosis, enterotoxemia and ketosis may occur simultaneously and be distinguished only at necropsy. In listeriosis there are no distinguishing lesions such as the fatty liver in ketosis and the characteristic hemorrhages of enterotoxemia. Acetone may be found in urine of animals with listeriosis as in ketosis.

Listeriosis in cattle may often be confused with rabies, poisoning, acute gastroenteritis, ketosis, Aujesky's disease, or viral encephalitis.

The encephalitic form of listeriosis occasionally may occur in swine. It has been reported by Biester and Schwarte (1940), Eveleth et al. (1953a) and in an atypical form by Helmboldt et al. (1951). In most instances when pigs show symptoms suggestive of listeriosis cultures prepared from the brain remain sterile even after long periods of refrigeration. The lesions found in the medulla oblongata of these pigs differ from the usual ones characterizing listeriosis, and more closely resemble those of one of the viral infections. It is very possible that these symptoms and lesions indicate a neural manifestation of hog cholera.

Usually there are no detectable gross lesions in animals that die with encephalitic listeriosis. Rarely slight clouding of the meninges or pin-point greyish white foci may be observed. There may be slight congestion of the brain and a few sheep brains show marked congestion. Usually there is an increase in the amount of cerebrospinal fluid. Lesions in other viscera are rare but occasionally fatty liver, duodenitis or pulmonary edema are seen. Pallaske (1940) reported marked focal hepatic necrosis in an adult sheep. These lesions have not been reported for cattle.

It has been well established that the microscopic lesions are confined primarily to the pons, medulla and anterior spinal cord. Both the white and grey matter may be involved. It is felt that the primary lesions develop in the brain substance and that the meningeal lesions result from extension. The predominant lesions are marked perivascular cuffing with varying degrees of focal necrosis. The perivascular cuffs consist mainly of mononuclear cells in all species of ruminants. However, Gray and Moore (1953) reported marked differences between sheep and cows in the cellular constituents of the focal lesions. In sheep and goats the foci contain a preponderance of polymorphonuclear leukocytes and in some instances may appear purulent. Both Olafson (1940) and Pallaske (1940) call attention to the purulent nature of the lesions in sheep. In some instances the normal parenchyma may be almost completely disintegrated. There is edema and hemorrhage, and neurons in the affected areas show various stages of degeneration. Neuronaphagia also may be present. The blood vessels are congested, frequently contain thrombi and show degenerative changes particularly of the endothelial lining.

In cattle the perivascular cuffs are much smaller and the focal lesions usually are limited to edema and small focal collections of microglial cells and lymphocytes. Rarely are lesions as extensive as reported for sheep. This reemphasizes the more chronic nature of the disease in cattle.

L. monocytogenes is never found in the perivascular cuffs but quite readily may be demonstrated in the focal lesions in sections stained by either the Goodpasture or Gram-Weigert method. The bacteria

occur most frequently near the periphery of the focal lesion and may be either extra- or intracellular. In cattle they tend to occur singly or in small clumps, while in sheep they may form dense plaques.

The known reported outbreaks of listeric encephalitis in sheep, goats and cattle are shown in tables 1, 2, 3, pages 28 to 31.

Stockton et al. (1954) reviewed the subject of abortion due to L. monocytogenes. In addition to the outbreaks cited by these authors the following reports should be included. Poppensiek (1944) isolated a gram positive rod which differed only slightly in certain biochemical reactions from L. monocytogenes. Jansen (1945) reported abortion in the rabbit. Levy et al. (1952) described two outbreaks of abortion in cattle, and van Ulsen (1952) reported the isolation of L. monocytogenes from three aborted bovine fetuses. The known reported cases of abortion in all species is shown in table 4, page

This form may be more prevalent than is generally suspected, and presents a broad field for further research. Olson (1945) and Ferguson (1951) suggested that this bacterium may be a normal inhabitant of the female genital tract, but this has not been confirmed. Gray and McWade (1954) in one instance were able to isolate this agent from the cervix of a so-called repeat-breeder cow showing symptoms of nymphomania. Repeated attempts to reisolate the bacterium from this cow resulted in failure but it does establish L. monocytogenes as a transient inhabitant of the female bovine genital tract. Furthermore Levi et al. (1945) encountered lesions in the uterus and spleen but not in the central nervous system of one heifer slaughtered two and a half months after abortion and L. monocytogenes was recovered from the ovary on the side where the

pregnancy had occurred; thus indicating that the bacterium may survive for relatively long periods of time in the bovine reproductive tract.

In cattle abortion usually occurs between the fourth and seventh month of gestation and the bacterium can be isolated from the liver, blood, fetal membranes and most commonly, from the abomasal contents of the fetus.

Gray et al. (1954) reported a condition in cattle which appears to be similar to the outbreak of human abortion reported by Potel (1953), and others (table 4, page 33). They observed instances in the same herd where the calves were born at or near term but lived only a few days if born alive. The living calves always were weak and some showed a marked bloody diarrhea. L. monocytogenes was isolated in pure culture from the liver of eight calves and microscopic focal hepatic lesions were demonstrated in two calves not cultured. At necropsy the most conspicuous alteration was marked hemorrhagic gastritis and enteritis. Some calves showed a few pinpoint necrotic foci in the liver. A similar condition was reported in one calf by Harbour (1941). At no time was L. monocytogenes isolated from the dam, but intrauterine infection was strongly suggested. A similar condition in lambs has been reported by Jepsen (1942) and Gray et al. (1949). The role of L. monocytogenes in the early death of calves and lambs needs further investigation. It may be that this bacterium plays a far more important part in disorders of the very young than previously suspected.

In sheep abortion occurs at or near term and isolations can be made from the same organs as in the bovine. As in the bovine, there are no distinguishing lesions to suggest the etiology. Abortion and

encephalitis are not known to occur simultaneously in the same flock or herd. With the present paucity of information it can not be stated whether L. monocytogenes plays a primary or secondary role in these abortions.

It has been shown that L. monocytogenes can attack a wide range of avian species. Besides being isolated from the chicken (table 5, page 35) it has been isolated from the canary (Bigland, 1950; van der Schaaf and de Jong, 1951), duck and goose (Zeller, 1949) wild wood grouse (Lilleengen, 1942), and blue eagle (Schulze, 1950). In fowls the disease occurs as a septicemia and the most conspicuous lesions are massive areas of myocardial degeneration, pericarditis and an increased amount of pericardial fluid. Other lesions may be focal hepatic necrosis, generalized edema, splenomegalia, peritonitis and enteritis. In acute cases the necrotic lesions are less marked and one only finds the lesions usually associated with a septicemia. The bacterium is easily cultivated from the abdominal organs.

As in other species, the young fowl appears to be more susceptible than the older bird. Outbreaks are usually sporadic and mortality in the individual flock may vary within wide limits. There are no specific symptoms in chickens with this disease. Adult birds usually die suddenly while in the young there may be a slow wasting before death. There is disagreement as to whether there is a circulating monocytosis before death.

It has been suggested that L. monocytogenes often is a secondary invader. Infections have been associated with salmonellosis, lymphomatosis, and Newcastle disease (Paterson, 1937; Bolin and Turn, 1951). It has

been isolated from the spleen of apparently normal chickens (Felsenfeld, 1951). Geurden and Devos (1952) reported, but without strong support, that latent infection may be more frequent than suspected and that these latent infections may play an important part in epidemiology. They also showed that sparrows and pigeons could be infected artificially and thus may play a part in transmission. Pigeons previously had been reported to be refractory to artificial infection with L. monocytogenes (Verge and Goret, 1941; Lesbouyries, 1943). These authors suggest the egg as a possible source of infection. They isolated L. monocytogenes from extensive necrotic lesions in the oviduct of one hen. Unfortunately, no eggs were available for culture, but egg infection does present an interesting epidemiological implication. They feel that contact plays only a very small part, if any, in the transmission of the disease in poultry.

The presence of latent carriers in a chicken flock presents a definite public health hazard. Felsenfeld (1951) reported an outbreak of listeric conjunctivitis in workers in a poultry processing plant. He isolated L. monocytogenes from the spleen of five apparently healthy birds which were being processed at the plant. It was found that these birds originated from a poultry farm where Graham et al. (1943) had diagnosed listeriosis some years before. This perhaps is the only instance where a definite mode of transmission could be established, and also the only report that gives definite information that L. monocytogenes may persist on the same premise for long periods of time.

Listeric septicemia is found most commonly in the monogastric animals. It has been reported in pigs (DeBlieck and Jansen, 1942; Kerlin and Graham, 1945), rats (Machiavello, 1942), groundmoles (Levy, 1948), raccoons

(Gifford and Jungherr, 1947), Lemming (Plummer and Byrne, 1950; Barrales, 1953), chinchilla (Kennedy, 1947; McKay et al., 1949; Shalkop, 1950; Smith, 1953), foxes (Cromwell et al., 1939; Wramby, 1945; Jansen and Peperkamp, 1947), domestic and wild rabbits (Hulphers, 1911; Murray et al., 1926; Paterson, 1940; Henricson, 1943; Lesbouyries, 1943; Jansen and van der Hurk, 1945; Gudkowa and Sacharoff, 1946; Schoop, 1946; Vallee, 1952), guinea pigs (Murray et al., 1926; Viera and Castelo, 1944, Gudkowa and Sacharoff, 1946; Roine et al., 1953), and horses (Grini, 1943; Krage, 1944; Svenkerud, 1948). The principal lesion is focal hepatic necrosis. In most instances a circulating monocytosis can be demonstrated in the peripheral blood a short time before death. With the exception of the report of Plummer and Byrne (1950), no information is available dealing with alterations in the central nervous system in these cases. They failed to find microscopic lesions in sections prepared from the medulla of one lemming. This form also has been found in young lambs (Jepsen, 1942; Gray et al., 1949) and calves (Harbour, 1941; Gray et al., 1954) before the rumen is functional.

Gudkowa and Sacharoff (1946) indicated that rodents constitute a reservoir for the infection. They claim that L. monocytogenes is widely distributed in mice, rats and rabbits, and that they were able to isolate the bacterium from the masal mucous membrane of 0.5% of all healthy rabbits. However, this has not been confirmed and both Olafson (1940) and Graham et al. (1943) failed to isolate L. monocytogenes from any of a large number of rats caught on farms where outbreaks of ovine listeriosis were in progress.

There is no satisfactory explanation as to why localized encephalitis

does not occur in these animals. Gudkowa and Sacharoff (1946) postulate a difference in adaptation in the strains which produce encephalitis and those which produce either septicemia or infectious mononucleosis and that the particular disease produced is a manifestation of the specific adaptation. They report that passage through the brain of laboratory animals increased the virulence of the strain for brain tissue. Such strains may produce encephalitis following intramuscular or intravenous inoculation but soon lose their affinity for brain tissue after repeated inoculation by these two methods. They found that strains passed through brain tissue lose their ability to ferment sucrose and lactose and the fermentation of these substances may be an index of affinity for neural tissue. It is well established that there is wide strain difference in ability to ferment sucrose and lactose but further research is necessary to confirm the relationship expressed by them.

Also unexplained is the occurrence of large numbers of monocytes in the circulating blood of infected monogastric animals and the apparent absence of this cell in the blood of ruminants. The development of a monocytosis following intravenous inoculation in the common laboratory animals is such a specific reaction that it has been described in the species name of the bacterium; "monocytogenes", generator of monocytes. However, Olson et al. (1950) failed to find an increase in the number of monocytes in the blood of sheep following intravenous exposure. They found that the polymorphonuclear leukocyte was the predominant cell under these conditions. Also most investigators have failed to find monocytes in the blood of ruminants affected with encephalitic listeriosis.

The monocyte producing factor (MPF) of L. monocytogenes was found

by Stanley (1949) and confirmed by Girard and Murray (1951) to be a non-antigenic lipid fraction of the bacterial cell. This MPF was found to be liberated chiefly in the liver and Seitz filtered saline extracts of necrotic liver produced monocytosis in rabbits. Similar extracts prepared from other infected organs or normal liver failed to elicit response. Resistance in the ruminant liver to this response may explain in part at lest, the failure of L. monocytogenes to localize in the liver of adult ruminants. Failure to localize in this organ may result in localization in the brain. However this speculation requires further investigation as very little has been done in this field to date.

Septicemia in the adult ruminant has been reported but is rare (Pallaske, 1940; Belin, 1943; Olson et al., 1953a; Hirato et al., 1954).

The various medicaments for the treatment of listeriosis were reviewed by Gray and Moore (1953) and Linzenmeier and Seeliger (1954) reported extensive in vitro studies of the effect of the various sulfonamides and antiobiotics against L. monocytogenes. The various antibiotics and sulfonamides differ greatly in their effectiveness on individual strains of L. monocytogenes. Penicillin was shown to be highly variable, and may be most effective when combined with one of the sulfonamides. Gray et al. (1949a) found that streptomycin in low concentration inhibited the growth of L. monocytogenes, but the microorganism very quickly developed a resistance to it. It is possible to produce a streptomycin-dependent strains of this bacterium. In vivo studies showed this antibiotic to be ineffective. Aureomycin and terramycin were found to be highly effective in both in vitro and in vivo studies (Zink et al., 1951; Norman et al., 1951).

The report of Gray and Moore (1953) is one of the few devoted to trials of an antibiotic under field conditions. These authors treated with aureomycin 13 sheep and 25 cows suspected of being affected with listeriosis. In the dosage used (1.25 gm I.V. per day for four days for sheep and 2.50 gm I.V. per day for four days for cows) the antibiotic was ineffective for sheep but did have some therapeutic value for cows. Only one sheep survived the four day treatment period. Many of the cows which recovered following treatment later showed symptoms suggestive of permanent brain damage. In several instances owners disposed of the animals soon after treatment. Some animals appeared to recover but suddenly relapsed and died. It was possible to demonstrate microscopic lesions in the brains of several animals sent to slaughter approximately one year after treatment. In sheep, brain damage appeared to be too extensive for the antibiotic to be effective. In cattle where alterations in the brain are less severe, treatment may be effective if initiated early. The owner should be made aware of the fact that symptoms may persist if the animal survives. This is no particular problem in beef cattle but presents numerous difficulties in a dairy herd.

The failure of therapy once symptoms are evident, emphasizes the need for a prophylactic agent if livestock are to be protected against this disease. Graham et al. (1940) found that both living and formalin killed bacterins had no effect on the course of infection in sheep. The results of Olson et al. (1951) who used both bacterins and whole blood from a recovered sheep were not very encouraging. However, Eveleth et al. (1952) have employed a commercially made vaccine for the past several years and report some benefit from its use. The use of bacterins for cattle is unreported. However, further research is necessary in this area.

The high mortality of listeriosis in man makes it imperative that all suspected material be handled with caution. This is especially true during necropsy of aborted fetuses and septicemic cases where the number of organisms is apt to be very high. In the encephalitic form the bacteria are generally confined to the brain and present little danger unless the brain is removed. Pallaske (1940), Belin (1943) Olson et al. (1953) Hirato et al. (1954), found the bacterium in the liver and spleen of a few adult sheep. This has never been reported in the cow. However, meat from animals showing evidence of active infection should not be used for human consumption. On the basis of observations in laboratory animals and field observations, Gray and Moore (1953) suggested that recovered animals are safe for food at least three weeks after abatement of symptoms. Under laboratory conditions L. monocytogenes seldom has been found to persist in an exposed animal for more than two weeks, and usually not more than five or six days. However, occasionally L. monocytogenes can be isolated from a localized lesion long after initial exposure (Levy et al., 1952; Thompson, 1954). If this also occurs under field conditions, it may account for the presence of carrier animals.

In man listeriosis usually occurs as meningitis (table 6, page 36). The incidence very likely is much higher than published reports indicate. There are no distinctive symptoms in the meningeal form and it easily can be confused with meningitis of other etiology. However, attempts to isolate L. monocytogenes from the spinal fluid have been quite successful. This presents a definite aid in diagnosis which is lacking in ruminants. The mortality due to this form of listeriosis is about 70% and either physical or mental defects are common sequel to infection. Less serious is the conjuntival form. This is usually a local infection

followed by complete recovery (Felsenfeld, 1944; Bilibin, 1949; Pletneva and Stiksove, 1950; Shmeleva, 1953). Pletneva and Stiksove (1950)call attention to the similarity of this form of listeriosis and the ocular form of tularemia, both in the lesion which develops and the fact that both appear to be carried by animals. A low grade septicemic form characterized by chills, fever, rash and swelling of the lymph nodes has been reported from the Soviet Union by Bilibin (1949) and Pletneva and Stiksove (1950).

The relation of L. monocytogenes to infectious mononucleosis has been reviewed by Benazet (1943), Stanley (1949) and Girard and Murray (1951). The results of these investigations indicate that the relation, if any, of this bacterium to the disease is obscure. Further work is necessary also in this area.

The role of L. monocytogenes in human abortion has been touched on in an earlier section and is expanded in the work of Potel (1953), Erdmann (1953), Stark (1953) and many others. The significant findings of these reports are that in a vast majority of the cases, the women were from rural areas and included raw cow or goat milk in the diet. This strongly suggests the mouth as the avenue of entrance in these instances. On one occasion Potel (1953) isolated L. monocytogenes from an atypical bovine mastitis. A woman drinking raw milk from this cow aborted twins and L. monocytogenes was isolated from the liver of each. Bacteriological examination of several hundred other milk samples failed to yield significant growth. Wramby (1944) also reported the isolation of this bacterium from a case of bovine mastitis. Mastitis due to this organism presents a serious public health problem. The resistance studies of Ozgen (1951) and Zink (1951) indicate that L. monocytogenes can survive

high temperatures for long periods of time, and Ozgen (1951) indicated that the bacterium can survive pasteurization. This factor should be considered in case of any abortion or early infant death found to be due to this bacterium.

Listeriosis does not appear to be a serious problem in canine populations. Graham et al. (1943) were unable to infect this species artificially. Cox (1945) reported the isolation of L. monocytogenes from the medulla oblongata of four dogs which showed symptoms suggestive of rabies. However, all tests for this disease were negative. All these occurred within a period of several months. In the one case reported by Chapman (1947) a differential leukocyte count made three days before death showed 25% monocytes. Necropsy revealed meningitis and a few colonies of L. monocytogenes were obtained from the meninges, pons and lateral ventricle. These appear to be the only reported instances of listeriosis in the dog. Nevertheless, there is reason to suspect that the incidence might prove to be much higher if the brains of all dogs showing nervous symptoms were submitted for culture of this bacterium.

The horse also appears to be relatively refractory to infections with this microorganism. Belin (1946) reported the isolation of L. monocytogenes from the brain of three horses on the same farm within a period of four weeks. The symptoms and lesions at necropsy were similar to those found in other forms of meningitis. Zeller (1949) mentions without elaboration two stains of L. monocytogenes isolated from horses with borna disease. Tabuchi et al. (1952) described what appeared to be encephalitic or meningeal listeriosis in an adult horse. No cultures were prepared, but tissue sections of the medulla revealed perivascular cuff-

ing, focal necrosis and large numbers of gram positive bacteria. Septicemia in young foals was discussed earlier under septicemia in monogastric animals. The reported association of L. monocytogenes with periodic ophthalmia (Jones, 1940) has never been confirmed.

L. monocytogenes also has been incriminated in several other conditions which further emphasize the ubiquitous nature of the bacterium. Felsenfeld (1948) recovered the microbe from the blood of a patient with a low-grade fever and moderate enlargement of the cervical lymph nodes following Cesarean section. Morris and Norman (1950) isolated it from the liver of apparently normal ferrets. Slabospits'kil (1938) isolated the microorganism from pox-like lesions in pigs. On two occasions it has been isolated from the liver of pigs that obviously died from other causes. Rhoades and Sutherland (1948) reported it in connection with an outbreak of hog cholera and Bolin and Eveleth (1951) made isolations from the liver of pigs which died of lye poisoning. Instances such as these strongly suggest that swine may play an important role as carriers. This may be speculation but does present intriguing implications in the epizoology of listeriosis.

The accumulated case reports give little suggestion to the actual mode of transmission under natural conditions. The fact that in most outbreaks, especially in animal populations, more than one individual was involved establishes the contagious nature of the disease. This was clearly brought out in the report of Eveleth (1952) who found that when apparently normal sheep from infected sheep flocks were introduced into new flocks, the disease often appeared in the new flock. In this manner 12 flocks became infected over a nine year period by introduction of sheep from a single infected flock. Furthermore, in a study of 44 sheep

flocks where listeriosis had occurred, Eveleth (1952a) found that in 30 of the flocks the disease appeared after introduction of animals from infected flocks. In the remaining 14 cases sheep from flocks of unknown history had been introduced during the year preceding the outbreak. Poppinsiek (1944) reported abortion in a ewe that was thought to be due to L. monocytogenes and that subsequently a cow grazing on the pasture where the abortion occurred died from listeric encephalitis. Gray et al. (1951) reported an instance where a sheep and a cow on the same pasture both died from listeric encephalitis. The patient of Berry (1950) who expired with listeric meningo-encephalitis possessed a pet chipmunk which became sick and died just shortly before the patient became ill. Most of the patients reported by Gudkowa and Sacharoff (1946), Bilibin (1949), Pletneva and Stiksove (1950) and Shmeleva (1953) had contact with either rats, mice, cat, dogs or other animals, but the contribution of these animals to infection could not be determined. The report of Odegaard et al. (1952) deserves special mention. The patient reported by them had cleaned a sheep barn in which manure had accumulated during the winter. A short time later when the patient suffered exposure as a result of a storm he became ill and died of listeric meningitis. A short time later one of the sheep in the late patient's sheep flock died of listeric encephalitis. When the remaining sheep were sent to slaughter, L. monocytogenes could not be isolated from several sheep brains cultured. This case not only implies contagion but also that exposure to adverse weather conditions may be a predisposing factor in infection.

In none of the above cases could the role of carrier animals be definitely established, but the circumstances demand that further work

should be undertaken in an attempt to establish the part that carrier animals play in the spread of infection. Until this is determined it will be difficult to control the disease in both human and animal populations.

The tables which appear on the following pages are intended only as references to known outbreaks of the disease. Except for the reports in man no attempt was made to list the number of individuals involved as such an attempt would be inaccurate at best and serves no particular purpose. The date indicated refers only to date of publication and not to the date outbreaks occurred as many reports covered several years observation. The primary purpose of the tables is to show the wide distribution of L. monocytogenes and to present a readily available source of bibliographic material. They do not include reports in the less commonly infected species which have been cited in the text. Neither included are review articles nor those dealing with the characteristics of the bacterium as such. These will be included in a later study.

Table 1

Isolation of L. monocytogenes from sheep

Author	Year	Country
Gill	1931	New Zealand
Doyle	1932	USA
Jungherr	1937	USA
Graham et al.*	1938-1942	USA
Morin	1938	USA
Biester & Schwarte	1939,1944	USA
Dobberstein**	1940	Germany
Olafson	1940	USA
Pallaske	1940,1941,1943	Germany
Cross	1941	USA
Henderson	1941	USA
Hoffman	1941,1942	USA
Jensen & Gay	1941	USA
Muth & Morrill	1942	USA
Belin & Lagriffoul	1943,1944	France
Grini	1943	Norway
Pomeroy et al.	1943	USA
Pothmann	1944	Germany
Spencer et al.	1944	USA
van Ulsen	1944	Netherlands

*Summarized by Graham et al. (1953).

**Found typical lesions but made no isolation attempts.

Table 1 (cont.)

Isolation of L. monocytogenes from sheep

Author	Year	Country
Gifford & Jungherr	1947	USA
Gray et al.	1947,1951,1951a	USA
Pounden & Edgington	1947	USA
Ryff & Lee	1948	USA
Jensen & Mackey	1949	USA
Stoenner et al.	1949	USA
Eieland & Finborud	1950	Norway
Naerland	1950	Norway
Pletneva & Stiksove	1950	USSR
Pouska	1950	Czechoslovakia
Viswanathan & Ayyar	1950	India
Kaboyashi	1951	Japan
Bain	1952	Canada
Eveleth	1952,1954	USA
Janos & Gyula	1952	Hungary
Khalimbekov	1952	Azerbaijan
Moll	1952	USA
Odegaard et al.	1952	Norway
Hirato et al.*	1954	Japan
McGrath	1954	USA

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*Cites four references in Japanese.
Tajima, M, Y. Fujimoto and M. Ishiguro. 1951. J. Vet. Med. 56: 75.
Sugawa, A and K. Miyairi. 1951. J. Jap. Vet. Med. Assoc. 4: 80.
Hyogo, Y. 1951. Jap. J. Vet. Sci. 13: 362.
Tabuchi, E, Y. Akiyama and T. Hosoda. 1952. Exp. Rep. Govt. Exp. Stat. Animal Hyg., No. 25, 83.

Total reports - 44.

Table 2

Isolation of L. monocytogenes from goats

Author	Year	Country
King	1940	USA
Olafson	1940	USA
Gifford & Eveleth	1942	USA
Jansen & van den Hurk	1943	Netherlands
Kaplan & Lager	1945	USA
Gifford & Jungherr	1947	USA
Naerland	1950	Norway
Asahi et al.	1951	Japan
Hyago & Kato	1951	Japan
Kaboyashi	1951	Japan
Gray et al.	1952	USA
Khalimbekov	1952	Azerbaijan

Total reports - 12.

Table 3

Isolation of L. monocytogenes from cows

Author	Year	Country
Matthews*	1928	USA
Jones & Little	1934	USA
Graham et al.**	1938-1942	USA
Olafson	1940	USA
Biester & Schwarte	1941	USA
Schwarte & Biester	1942	USA
Pomeroy et al.	1943	USA
Poppensiek	1944	USA
Wramby	1944	Sweden
Hatch	1946	USA
Cole	1946	USA
Belin***	1946	France
Boucher	1946	USA
Pounden et al.	1947	USA
Thorp et al.	1947	USA
Gray et al.	1948-1951	USA
Fish & Schroder	1949	Canada
Jensen & Mackey	1949	USA
Sellers et al.	1949	USA
Zeller	1949	Germany

*Found typical lesions but made no isolation attempts.

**Summarized by Graham et al. (1943).

***Symptoms only.

Table 3 (cont.)

Isolation of L. monocytogenes from cows

Author	Year	Country
Bain	1950	Canada
Chivers	1950,1951	USA
Ward	1950	USA
Boyer et al.	1951	USA
Zink et al.	1951	USA
van der Schaaf et al.	1951	Netherlands
Anderson	1952	USA
van der Schaaf & de Jong	1952	Netherlands
Geurden & Devos	1954	Belgium
McGrath	1954	USA

Total reports - 30.

Table 4

Isolation of L. monocytogenes from abortions

Author	Year	Country
Ovine		
Paterson	1940	England
Poppensiek	1944	USA
Olson	1945	Sweden
Charles	1950	Australia
Eveleth et al.	1953	USA
Total reports - 5.		
Bovine		
Graham et al.	1939	USA
Evans & Sawyer	1942	USA
Wramby	1942	Sweden
Olson	1945	Sweden
Ferguson et al.	1951	USA
Levi et al.	1952	Israel
van Ulsen	1952	Netherlands
Stockton et al.	1954	USA
Gray et al.*	1954	USA

*Some born prematurally, others at term but born dead.

Total reports - 9.

Table 4 (cont.)

Isolation of L. monocytogenes from abortions

Author	Year	Country	Cases reported
Human			
Alex and Potel	1953	DDR	?
Erdmann & Potel	1953	DDR	10
Hagemann & Simon	1953	DDR	14
Linzenmeier et al.	1953	Germany	1
Linzenmeier	1953	Germany	2
Stark	1953	DDR	4
Potel*	1953/1954	DDR**	40
			71 as minimum (almost all are fatal)

*Summary of numerous previous reports. Calls bacterium Listeria infantiseptica.

**German Democratic Republic.

Table 5

Isolation of L. monocytogenes from chickens

Author	Year	Country
Seastone	1935	USA
Paterson	1937,1939	England
Belin*	1941	France
Cole	1941	USA
Hurt et al.	1941	USA
Pallaske	1941	Germany
Huffman & Lenarz	1942	USA
Pothmann	1944	Germany
Wramby	1944	Sweden
Peperkamp & Jansen	1947	Netherlands
Sparapani	1947	Argentina
Bigland	1950	Canada
Pletneva & Stiksove	1950	USSR
Bolin & Turn	1951	USA
Chadkowski & Czarnowski	1951	Poland
Geurden & Devos	1952	Belgium
van Ulsen	1952	Netherlands
Thompson	1954	USA

*Found typical lesions but made no isolation attempts.

Total reports - 18.

Table 6

Isolation of L. monocytogenes from man

Author	Year	Country	Disease	Age of patient	Number	Prognosis
Atkinson*	1917	Australia	Meningitis	C	5	4 fatal-1 recovered
Dick*	1920	USA	Meningitis	A	1	fatal
Dumont & Cotoni[1]	1921	France	Meningitis	A	1	fatal
Baldridge et al.*	1926	USA	Infectious Mononucleosis	-	5	recovered
Kessel & Romanoff*	1930	USA	Meningitis	A	1	recovered
Nyfeldt	1932	Denmark	Infectious Mononucleosis	A	3	recovered
Anton*	1934	Austria	Conjunctivitis	A	1	recovered
Schultz et al.[2]	1934	USA	Meningitis	A	1	recovered
Tesdale*	1934	Norway	Meningitis	-	1	fatal

*Diphtheroid recovered, but identification not confirmed.
[1]Identification confirmed by Paterson (1940) and Cotoni (1942).
[2]Identification confirmed by Webb and Barber (1937).

A - Adult
C - Child
I - Infant

Table 6 (cont.)

Isolation of L. monocytogenes from man

Author	Year	Country	Disease	Age of patient	Number	Prognosis
Gibson[1]	1935	Scotland	Meningitis	A	1	fatal
Burn	1936	USA	Meningitis	3I-1A	4	fatal
Carey	1936	USA	Meningitis	C	1	recovered
Marcellus et al.[2]	1936	USA	Meningitis	A	1	recovered
Poston et al.	1937	USA	Meningitis	C	1	fatal
Cislaghi*	1938	Italy	Meningitis	-	-	-
Porzecarski & Baygorria	1938	Uruguay	Meningitis	A	1	recovered
Schmidt & Nyfeldt	1938	Denmark	Infectious Mononucleosis	A	4	recovered
Thatcher	1938	England	Meningitis	C	1	fatal
Pons & Julianelle	1939	USA	Infectious Mononucleosis	C	1	recovered
Porter & Hale	1939	USA	Meningitis	C	1	recovered
Savino	1939	Argentina	Meningitis	A	1	recovered
Wright & MacGregor	1939	Scotland	Meningitis	C	1	fatal

*Diptheroid recovered, but identification not confirmed.
[1]Identification confirmed by Webb and Barber (1937).
[2]Identification confirmed by Schultz et al. (1934).

A - Adult
C - Child
I - Infant

Table 6 (cont.)

Isolation of L. monocytogenes from man

Author	Year	Country	Disease	Age of patient	Number	Prognosis
Fischer	1941	Uruguay	Meningitis	-	-	-
Kapsenberg	1941	Netherlands	Meningitis	3C-1A	4	2C fatal 3 recovered
Harvier et al.	1942	France	Meningitis	A	1	recovered
Webb	1943	England	Infectious Mononucleosis	A	1	recovered
Gudkowa & Sacharoff*	1946	USSR	Meningitis	-	11	9 fatal-2 recovered
			"Mononuclear Angina"	-	3	-
Handelman et al.	1946	USA	Meningitis	C	1	recovered
Martin et al.	1947	France	Meningitis	A	1	fatal
Beute et al.	1948	Netherlands	Meningitis	I	1	recovered
Felsenfeld	1948	Puerto Rico	Low grade infection	A	1	recovered
Sedallian et al.	1948	France	Meningitis	I&C	2	I fatal-C recovered
			Pleuritis	A	1	recovered
Sloof	1948	Netherlands	Meningitis	I	1	fatal
Stanley	1948	Australia	Meningitis	A	1	fatal

*L. monocytogenes not isolated in all instances. Diagnosis made on basis of symptoms and agglutination titers.

A - Adult
C- Child
I - Infant

Table 6 (cont.)

Isolation of L. monocytogenes from man

Author	Year	Country	Disease	Age of patient	Number	Prognosis
van Driest	1948	Netherlands	Meningitis	I	1	recovered
Bilibin**	1949	USSR	"Mononuclear Angina" Conjunctivitis	-	12	-
Schamesow*	1949	USSR	"Mononuclear Angina" Conjunctivitis	A	18	some recovered
Berry	1950	USA	Meningitis	A	1	fatal
Pletneva & Stiksove*	1950	USSR	"Mononuclear Angina" Conjunctivitis	8C-1A	9	recovery implied
Bergstrom	1951	USA	Meningitis	I	1	fatal
Felsenfeld	1951	USA	Conjunctivitis	A	2	recovered
Bennett et al.	1952	USA	Meningitis	C	1	recovered
Hein	1952	Germany	Meningitis	A	1	fatal

*L. monocytogenes not isolated in all instances. Diagnosis made on basis of symptoms and agglutination titers.

**L. monocytogenes isolated from 3 cases.

A - Adult
C - Child
I - Infant

Table 6 (cont.)

Isolation of L. monocytogenes from man

Author	Year	Country	Disease	Age of patient	Number	Prognosis
Line & Cherry	1952	USA	Meningitis	I	2	fatal
Line & Appleton	1952	USA	Meningitis	I	1	recovered
Odegaard et al.	1952	Norway	Meningitis	A	1	fatal
Seeliger & Leineweber	1952	Germany	Meningitis	A	1	recovered
Seeliger et al.	1952	Germany	Septicemia	A	1	fatal
Tompkins	1952	USA	Meningitis	C	1	recovered
Winkler	1952	USA	Meningitis	A	1	fatal
Binder et al.	1953	USA	Meningitis	A	1	recovered
Schulze	1953	USA	Meningitis	A	1	recovered
Shmeleva*	1953	USSR	Conjunctivitis	3C-3A	6	recovered
Simon	1953	Germany	Meningitis	-	4	fatal
Wenkebach	1953	Argentina	Gonorrhea**	A	5	recovered
Gray***	1954	USA	Meningitis	4A-3C	7	3C recovered-4 fatal

*Did not mention isolation attempts.
**Isolated L. monocytogenes from urethral exudate.
***Reports have not been published but cultures submitted for identification and found to be L. monocytogenes.

A - Adult
C - Child
I - Infant

Total reports - 56.

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PART II

COLONIAL DISSOCIATION

COLONIAL DISSOCIATION

INTRODUCTION

From the literature review in Part I, it appears that L. monocytogenes is extremely ubiquitous and suggests that this microorganism is actually widely distributed in nature, perhaps in the form of a soil bacterium or as a saprophyte, and that only under specific conditions does it occur as a pathogen. It is possible that failure to isolate this organism from sources other than the animal body reflects a failure to recognize it in a non-pathogenic form. Accordingly an investigation was carried out to determine how many different colonial forms would develop under normal laboratory conditions.

The occurrence of wide variation in colonial morphology between virulent and non virulent strains within the same bacterial species was recognized early in the history of bacteriology. It was suggested as early as 1877 by Nageli. Hadley (1927) published an extensive review of the subject. It also formed the basis for the more recent review of Braun (1947). Huddelson et al. (1952) recognized at least 71 variations within the three species of the genus Brucella. That fewer variations are reported for other genera may be only a reflection of less intensive study.

In spite of the rather extensive bibliography available for L. monocytogenes, there is a paucity of information relating to its colonial morphology. Webb and Barber (1937) appear to have been the first to publish on this subject. They reported the existence of at least two colonial forms designated rough and smooth. Barber (1939) in a comparative

study of L. monocytogenes and Erysipelothrix rhusiopathiae described smooth, intermediate and rough colonial forms in each species. Paterson (1940) in a study of the pathogenicity of L. monocytogenes for the developing chicken embryo found that three "rough" strains were non pathogenic while "smooth" strains produced death in 48 hr. Grini (1943) reported that an "avirulent" strain failed to kill rabbits when inoculated intravenously but produced a marked monocytosis in the circulating blood. No mention was made of colony morphology. Sohir et al. (1948) isolated a "non pathogenic" strain of L. monocytogenes from cooked beef, but did not mention colonial characteristics. Several other French authors, Verge and Goret, 1941; Belin, 1946; Harvier et al., 1943; Sedallian et al., 1948; called attention to the presence of "rough" forms developing in "smooth" cultures of this bacterium. Schoop (1951) observed "rough" colonies which consisted of filamentous cells in one culture of L. monocytogenes. Seeliger and Linzenmeier (1952) and Hirato et al. (1954) described two different colonial types designated "smooth" and "rough", but did not elaborate on the two types. In general, all investigators who reported the presence of rough forms called attention to the filamentous nature of the cells of rough type colonies and their striking resemblance to rhusiopathiae.

As far as can be determined none of these investigators employed oblique lighting as described by Henry (1933), an invaluable technique for studies of this type. Hunter et al. (1950) observed 19 colonial variations by this technique in a culture of L. monocytogenes exposed to beta particles from radio phosphorus. They found that only a few of the variant cultures differed essentially from the parent strain in fermentative ability, biochemical reactions, or antigenic structure. In general the

variants showed greatly decreased pathogenicity for embryonating chicken eggs and white mice. No mention was made of the ability of the variants to produce a circulating monocytosis in the infected mice. It was found, however, that large intraperitoneal inocula of the least virulent strains failed to protect white mice when six weeks later they were exposed to a lethal inoculation of the parent strain.

MATERIALS AND METHODS

Twenty cultures of L. monocytogenes were employed in this study. These included strains isolated from sheep, cows, goats and man. They were streaked on dry Difco* tryptose agar plates in such a manner that both confluent and well isolated colonies developed. Approximately 20 ml of agar were poured into sterile Petri dishes, allowed to harden and incubated at 37° C for 24 hr. They were incubated an additional 24 hr at room temperature. The surface of the agar, then, was free of condensation and ready for use. A small platinum loop (1.0 mm diameter) was used to inoculate the plates. This loop was sufficiently flexible to prevent marring the agar surface. The culture to be examined was streaked heavily along a quadrant of the plate, the loop then was flamed, allowed to cool and lightly touched in the previously inoculated area and the remainder of the plate inoculated with a light rapid series of widely spaced streaks. The colony distribution of an ideally inoculated plate is shown in fig. 1. The plates were then incubated at 37° C for 24 hr and examined with a binocular scanning microscope equipped with a 1.5 objective and 10 X oculars. Oblique lighting was used at all times. Several of the colonial types were virtually indistinguishable from each other without this technique. The relation of the microscope to the light source is shown in fig. 2.

*Difco Laboratories, Inc., Detroit, Michigan.

Fig. 1. An ideally inoculated agar plate showing both confluent and well isolated colonies. S culture at 3 days.

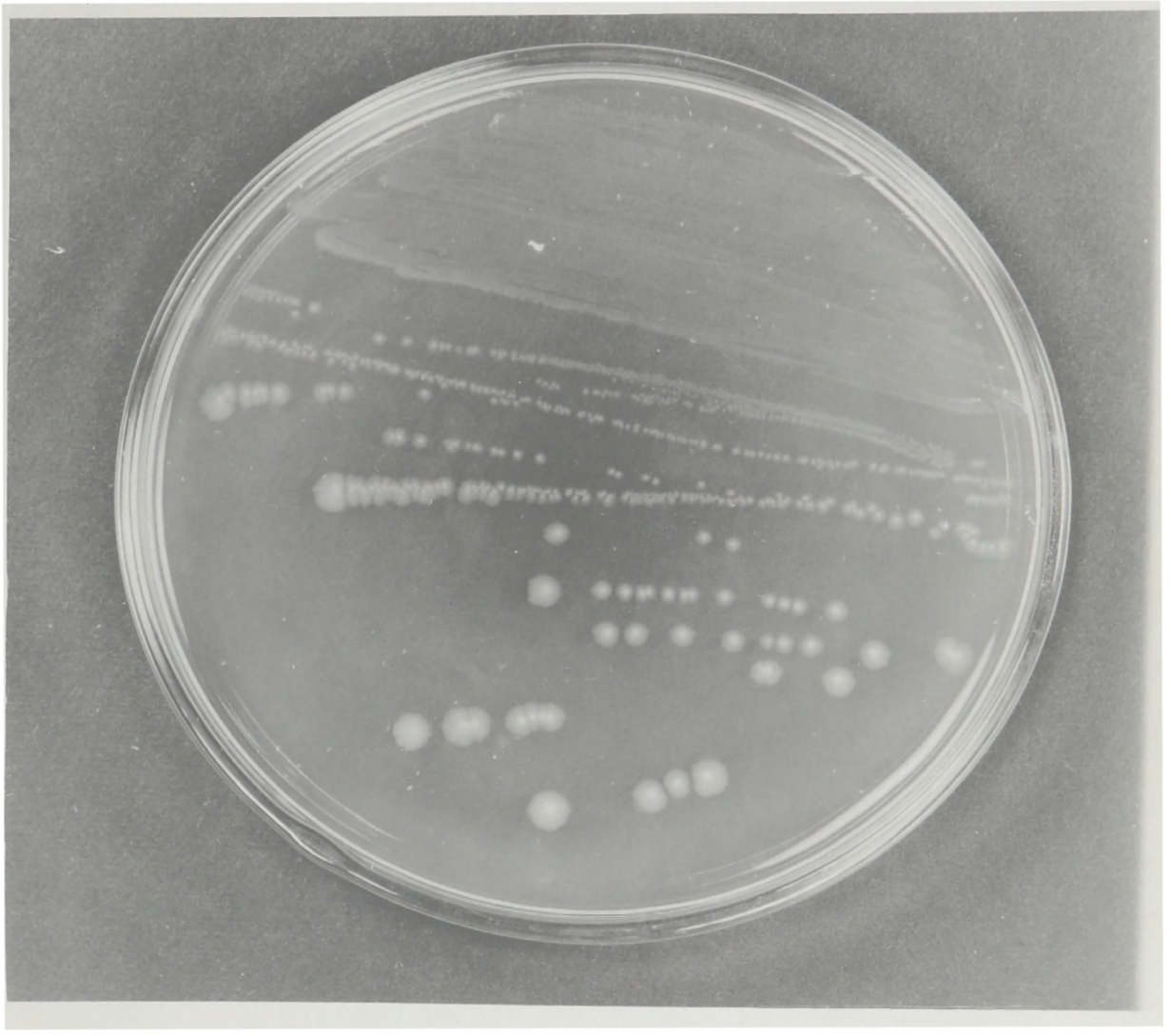
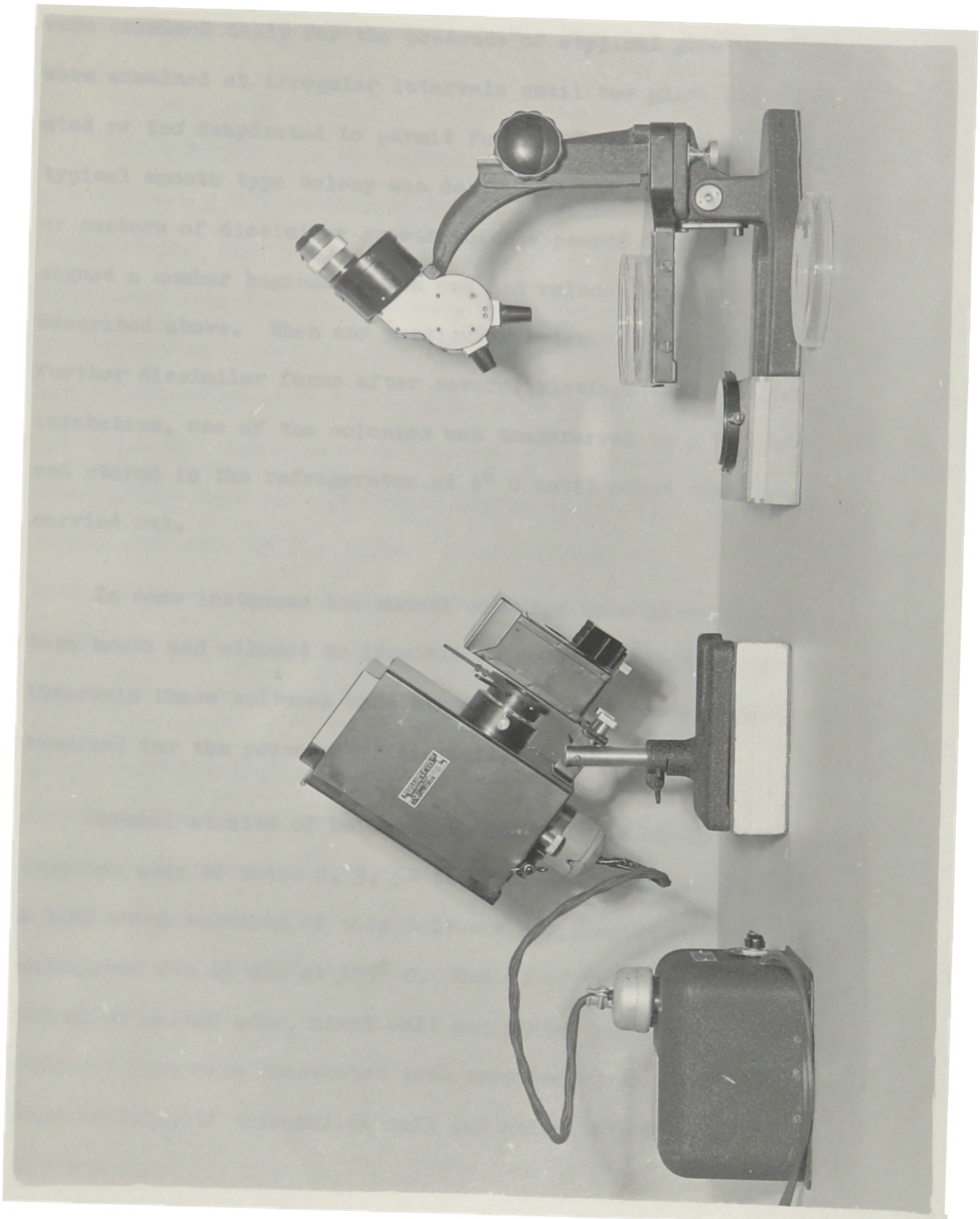


Fig. 2. Arrangement of light source, mirror and binocular scanning microscope. The lamp was tilted downward at an angle of approximately 40° at a distance of 12.5 cm from the flat surfaced microscope substage mirror placed on a microscope slide box set in front of the microscope. The green glass microscope stage was replaced by a photographic plate from which the emulsion had been removed. All cultures were examined with the Petri dish open.



After the initial incubation at 37° C, the plates were allowed to stand at room temperature until discarded. During the first week they were examined daily for the presence of atypical growth. Thereafter they were examined at irregular intervals until the plate was either contaminated or too dehydrated to permit further colonial development. The typical smooth type colony was designated "S", and any secondary growth or sectors of dissimilar growth which appeared in the colonies was assigned a number beginning with one and reinoculated and incubated as described above. When any particular colonial form failed to yield further dissimilar forms after several platings and long periods of incubation, one of the colonies was transferred to a tryptose agar slant and stored in the refrigerator at 4° C until other tests were to be carried out.

In some instances the smooth colonies were inoculated into tryptose broth and allowed to incubate as described above. At appropriate intervals these cultures were streaked on agar plates, incubated and examined for the presence of dissimilar forms.

Several strains of both smooth and rough cultures were plated on tryptose agar to which 2, 3, 5- triphenyltetrazolium chloride was added. A 1.0% stock solution of this salt was prepared in distilled water and autoclaved for 15 min at 115° C. One ml of this solution was added to 100 ml of melted agar, mixed well and plates prepared as described above. Cultures also were inoculated into tryptose broth containing the same concentration of tetrazolium salt and plated after varying periods of incubation.

Smears of colonies representative of each of the various types were

stained with either the Hucker modification of the gram stain or with Wright's stain. Consistency of the colonies was determined by stroking or pushing them with a small loop. This was done under the scanning microscope.

The colonies were photographed by placing over the stage of the microscope a 5 X 7 Eastman Clinical Camera equipped with a 42 mm microtessar lens. The light source was a Bausch and Lomb 6 volt ribbon filament lamp. The rheostat was set at the number 3 position. The color photos were made on 2 1/4 X 3 1/4 sheet type B Ektachrome. Exposure was 1 1/2 seconds with the lens diaphram open. Development was in Eastman Kodak Ektachrome processing chemicals supplied in kit form. The color prints were made by Drewry Photocolor Corp.* The black and white photos were made on 5 X 7 sheet Isopan exposed for 1/2 second with the lens diaphram open. They were developed five minutes in Eastman Kodak DK-11 and fixed in Eastman Kodak rapid fix for 3 min. The magnification was the same for all photos and was approximately 12 times.

The biochemical and fermentation reactions of a number of non-smooth cultures were compared with the reactions of the parent smooth culture. Fermentation reactions were carried out on 20 fermentable substances as indicated in results. These compounds were added to a basal medium containing bactotryptose, beef extract, sodium chloride and distilled water. Brom cresol purple was used as indicator. All media were sterilized by autoclaving at 15 pounds pressure for 15 min with the exception of dextrin and maltose which were filtered through a fritted glass filter. All reactions were observed for 14 days.

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*Chicago, Ill.

Biochemical reactions were carried out using media and methods described in "The Manual of Methods for the Pure Culture Study of Bacteria".

Pathogenicity studies in rabbits were carried out with several cultures from non-smooth colonies. Each culture to be employed was transferred to tryptose agar slants and incubated at 37° C for approximately 18 hr. The resulting growth was washed off with sterile distilled water and a distilled water suspension was prepared which corresponded in density to the number one tube of the McFarland nephelometer. Generally a 0.5 ml portion of this suspension was inoculated intravenously. In a few instances larger or smaller injections were given. Dosage was based on unpublished observations which revealed that this approximated a minimum lethal dose. Differential leukocyte counts were taken immediately preceding inoculation and at 24 hr intervals for the next several days. In some instances body temperatures also were recorded.

To determine the ability of some of the non-smooth cultures to produce a conjunctivitis in the rabbit eye, dense distilled water suspensions were prepared from cultures grown at 37°C for approximately 18 hr. With the aid of a capillary pipette a few drops of this suspension were instilled into the conjunctival sac of the left eye. The right eye served as control.

Cultures from four different colonial types were used to determine pathogenicity in rabbits by the oral route. These were type S, R-7, R-11 and R-14. Each type was grown on tryptose agar in a flat sided antigen bottle for 24 hr at 37° C. The growth was washed off with tap water and this constituted the only source of fluid intake for the rabbits

for a period of two months. The resulting suspension corresponded roughly to the number 10 tube of the McFarland nephelometer when compared in a photolometer. For a period of two weeks the mouth of one rabbit in each group was deeply lacerated every third day just before the culture was given. Two rabbits in the group receiving type S and one rabbit in each of the other groups were fed only ensilage during the two month period. The remaining rabbits received Kellogg rabbit pellets. At intervals the feces were cultured for the presence of the colonial type being fed. The fecal pellets were macerated in a Waring blendor together with approximately 15 ml of tryptose broth containing 0.15% potassium tellurite. The resulting suspension was incubated overnight at 37° C and then plated on tryptose agar. The plates were incubated at 37° C for 18 hr and examined under the scanning microscope for the presence of the appropriate colonial type.

Agglutination reactions were carried out using antisera from rabbits which survived the intravenous inoculations. Antigens were prepared by growing the various cultures to be studied on tryptose agar slants. Incubation took place at 37° C for approximately 18 hr. The resulting growth was washed off and suspended in 0.85% saline and standardized with 0.85% saline to a density corresponding to the number two tube of the McFarland nephelometer. The cultures were killed by incubation for one hr in a water bath at 80° C. Dilutions were prepared as outlined in "The Manual of Methods for the Pure Culture Study of Bacteria". After addition of the antigen the tubes were incubated for two hr in a water bath at 37° C and stored in the refrigerator overnight. After removal from the refrigerator the tubes were allowed to remain at room temperature for 30 min after which the results were read.

As some non-smooth cultures produced high agglutinating titers against smooth cultures, attempts were made to vaccinate rabbits with these non-smooth cultures. The cultures used were prepared as described for intravenous inoculation. These were inoculated subcutaneously in doses ranging from 1.0 ml to 5.0 ml. As earlier unpublished work revealed that a localized necrotic lesion developed at the site of subcutaneous inoculation, 15 TR (turbidity-reducing) units of Wydase* were added to some of the suspensions before inoculation. After two months the rabbits were challenged with a lethal dose of virulent smooth culture.

RESULTS

Examination of the agar plates with the scanning microscope revealed the presence of at least 19 different colonial forms arising either directly from the parent smooth culture or from one of its non-smooth colonies. Differences were detected by variations in texture, consistency, color, opacity, or morphology of the resulting growth. It often was difficult to distinguish two colonial forms when they grew widely separated from each other on the agar plate, but it was relatively easy to detect the more subtle differences in either color or texture when they were adjacent to each other. Fig. 14 and 25. The density and character of the population had a marked effect on the appearance of many of the colonial types. Dense mixed populations produced marked changes in colony size and color, and the true description of the colony could be made only if it could be grown in pure culture. Many of the colonies were never produced in pure form in spite of repeated platings, but always gave rise to a mixture of colonial forms.

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*Wyeth Inc., Philadelphia, Pennsylvania, brand of hyaluronidase.

It should be pointed out that the terms "smooth" and "rough" as applied to bacterial colonies do not always carry the same connotation. As this study was devoted primarily to a recognition of colonial types the two terms were employed rather loosely. Studies on agglutinability in acriflavine (Braun and Bonestell, 1947) and stability in salt solution as criteria of smooth, rough, or mucoid types were not carried out. The following terms were applied to characterize the various colonial types:

Smooth (S) - The small circular type colony with entire margin, finely textured surface and found thus far only in animal tissue or body fluids. Highly pathogenic for rabbits.

Intermediate (I) - The large flat spreading type daughter colony arising from smooth type colonies and which always gives rise to a mixture of smooth and non-smooth colonies, but never to a pure culture. Pathogenic for rabbits.

Rough (R) - The relatively large type colony with erose to lobate edge, coarsely textured surface. Most frequently found as sectors or daughter colonies developing from the intermediate type. Occasionally found directly as sectors or daughters in smooth type colonies. May or may not be obtained in pure culture. Non pathogenic for rabbits. In spite of entire margin and smooth surface, type 11 was included here on the basis of development.

Non-smooth (N-S) - Any form other than that described under smooth.

Color often played an important part in distinguishing one colonial form from another. As color interpretation is often largely subjective it is difficult to assign definite color values to an object unless it is

compared to some standard such as the "Munsell Book of Color, 1942", a technique which Huddelson (1952)employed. In this study no such comparison was made. However, the most important colonial types are reproduced by color prints. Unfortunately, due to the inherent difficulties of color printing, the colors are not too exact and in some, most of the topographical features are lost. However, it is hoped that they will convey to a small degree some of the more subtle features of the various colony types which can be fully appreciated only when viewed directly through the microscope.

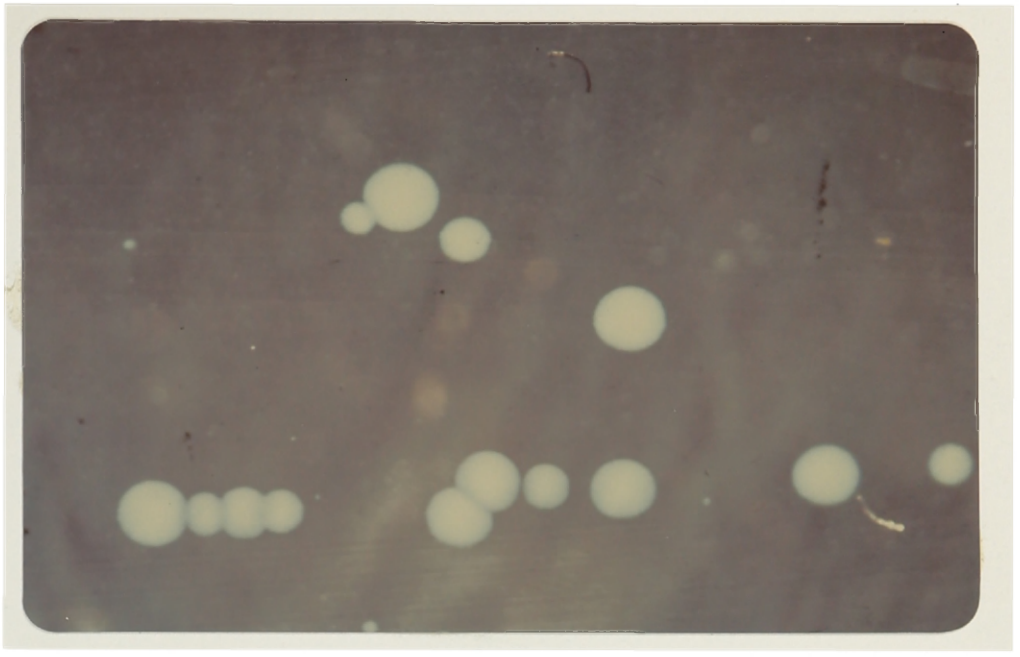
The origin and description of the principal colony types is given opposite the photographs which appear on the following pages.

Fig. 3. Type S.

Found in all isolations from animal or human tissue or body fluids.

Agar colonies at 24 hr are puntiform, translucent, slightly raised with a finely textured surface and entire margin, blue-green in color. Watery in consistency. After five to ten days well isolated colonies reach a diameter of two to five mm, are more blue in color, slightly umbonate and rubbery in consistency. May show evidence of daughter colonies or sector formation. Cells: short rods 1.0 to 1.5 X 0.5 microns. Some coccoid, curved, Y and T forms. Palisade arrangement common. Few long forms up to 7.0 microns in length. Tumbling motility. Highly pathogenic.

Fig. 4. Ten day old S culture on agar containing 2, 3, 5-triphenyltetrazolium chloride, showing four distinct growth zones; deep purple center, red, pink and pale green at periphery. It has not been determined if each zone gives rise to a characteristic culture.



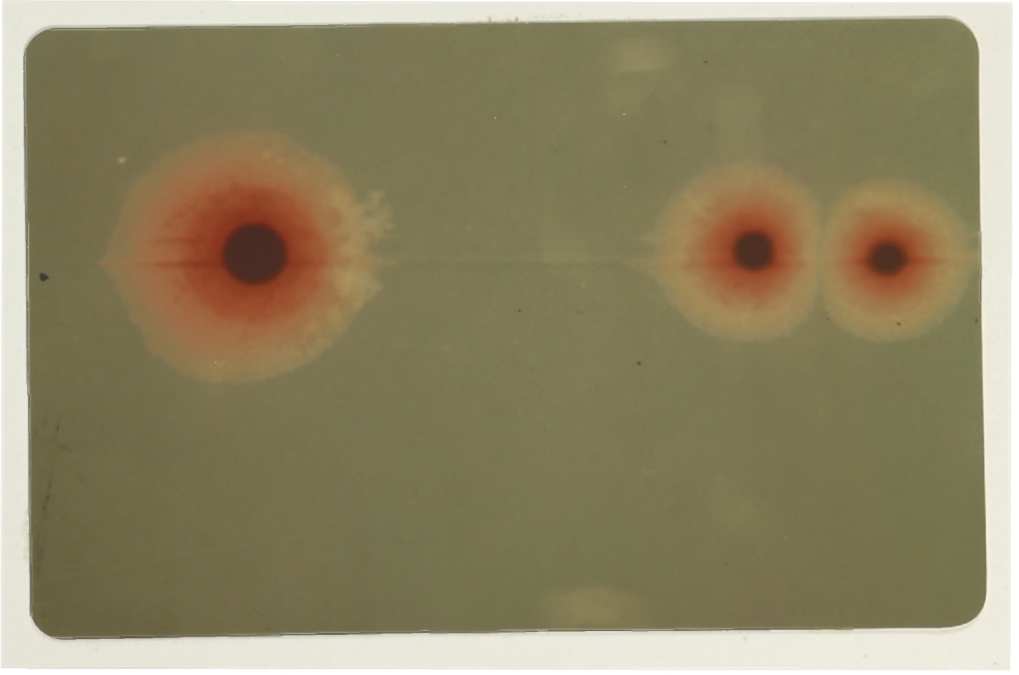


Fig. 5. Types I-1, I-2, R-4 and R-5.

First appear after about five days incubation of S type stock cultures. Rarely develop in freshly isolated S type cultures. Large spreading intermediate type, flat coarse surface, translucent, filamentous margin, stringy in consistency.

I-1. Are pale to bright green depending on age.

I-2. Are pale to bright blue. Plating gives rise to colony types S, R-4, and R-5 in different proportion. I-2 gives more S than does I-1.

R-4. Arise from platings of I-1 or I-2. Agar colonies at 24 hr are small irregular, slightly opaque, raised to umbonate, coarsely grooved surface, undulate to lobate margin, blue, watery. Cells: Similar to type S but more long forms. Tumbling motility. Pathogenic.

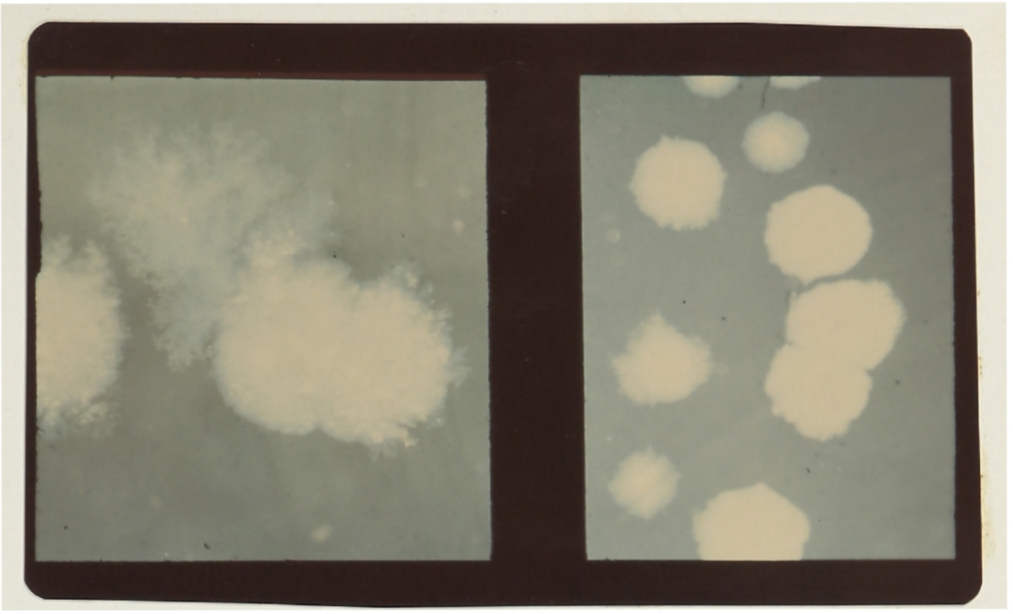
R-5. Arise from platings of I-1 or I-2. Agar colonies at 24 hr are larger, irregular to filamentous, opaque, slightly raised, finely textured to deeply grooved surface, lobate to filamentous margin, cream to pale yellow, watery. Cells: Similar to R-4 but more long forms. May show few filaments. Tumbling motility. Pathogenic.

These types cannot be maintained pure. Plating gives rise to type S colonies which very quickly develop type I-1, I-2 and R-3 daughter colonies; type R-4 and R-5 colonies; occasionally type R-3, R-7 and R-11 colonies. R-5 dissociates more rapidly than R-4, and type R-3 daughter colonies can be seen as early as 24 hr.

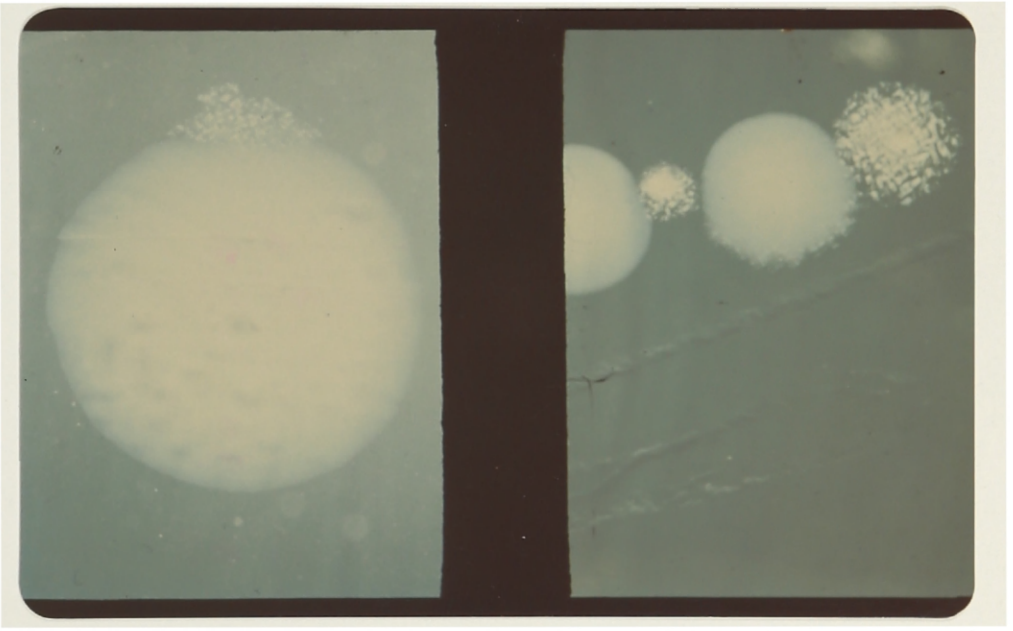
Fig. 6. Type R-3.

Arise as daughter colonies from five to ten-day old type S colonies, occasionally from platings of R-4 and R-5. Develop rapidly in some freshly isolated S-type cultures.

Agar colonies at 24 hr are filamentous to irregular, slightly raised with opaque center, coarsely ridged surface, erose to filamentous margin, green color. After five to ten days colonies reach a diameter of two to five mm or more. Little change in color. Become rubbery in consistency. Cells: Mostly rather long slender filaments and long rods three to seven microns in length, few short forms. Non motile. May be pathogenic.



At left, ten day old S type colony from plating non pure R-3 colony showing large and small I-1 and I-2 daughters. At right, small blue, umbonate R-4 and larger flat cream to yellow R-5 colonies. 24 hr culture from I-1 plating. Small R-3 daughters can be seen on the R-5 colonies. Also see fig. 19 and 20.

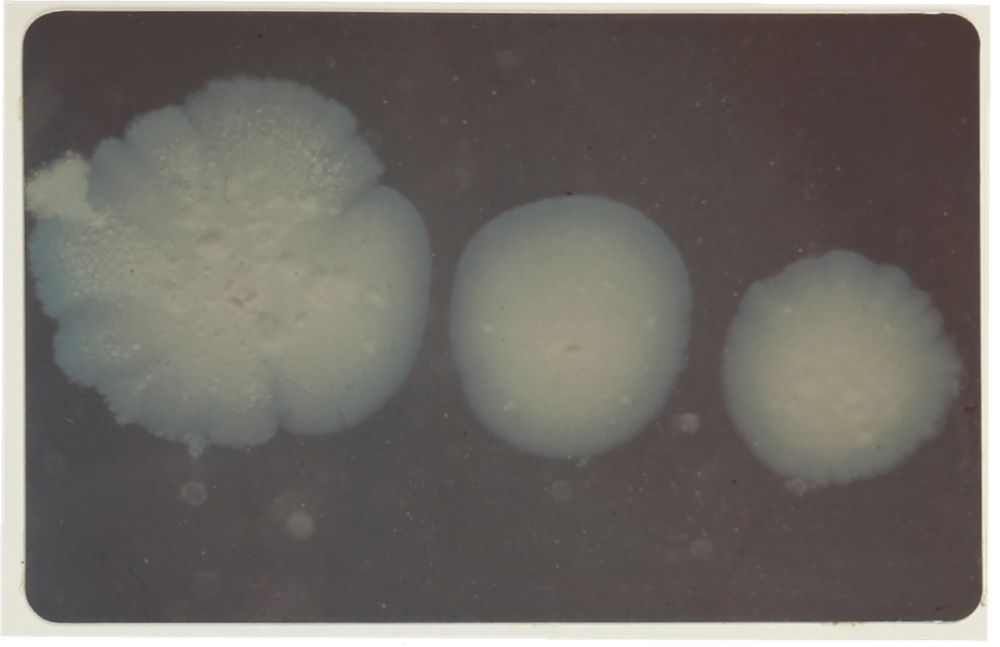


At left, five day old S type colony with type R-3 daughter. At right, 48 hr culture resulting from plating of R-3 daughter showing mixture of S and R-3 grows more slowly than type S. Can be produced pure after repeated platings.

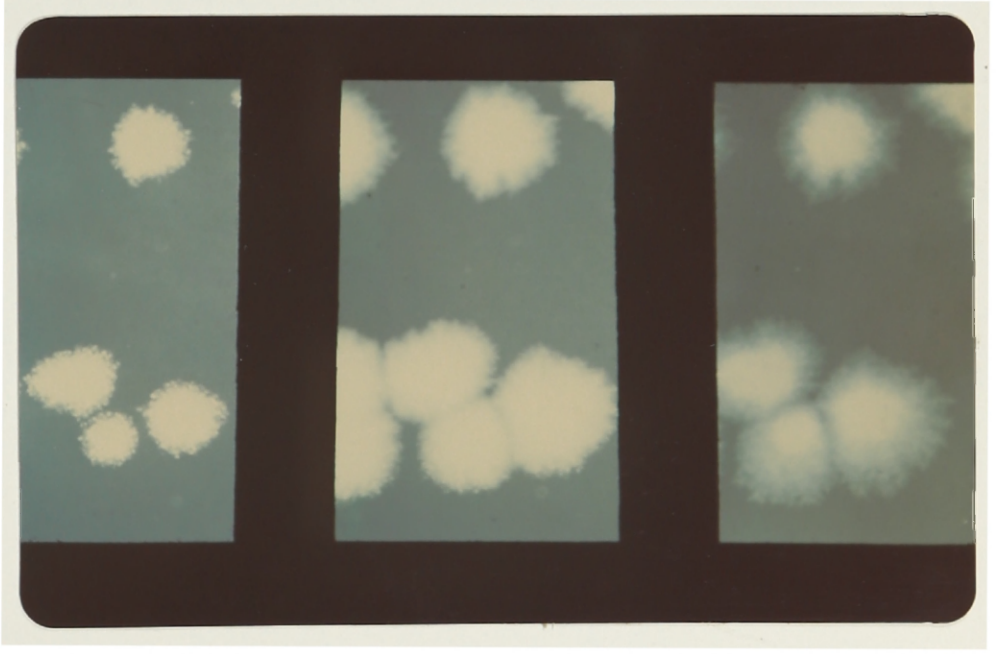
Fig. 7 and 8. Type R-7.

Arise as sectors in type R-3, R-4 or R-5 type colonies or from I-1, I-2, or R-3 type daughters developing on S colonies. Occasionally arise directly from S type colonies which result from plating of I-1, I-2, or R-3 type colonies. Seldom develops directly from S colonies isolated from animal tissue. Not pathogenic.

Agar colonies at 24 hr are filamentous to irregular, translucent, slightly raised deeply ridged surface with erose to filamentous margin, pale pink in color, watery in consistency. After two to four days the colony becomes more flattened with a highly filamentous margin and changes to a pale green color. After about four days pink to gold filaments, designated type R-13, develop from the margin, which when plated give rise to type R-11 and R-14 colonies, occasionally to R-3. Cells: usually short plump rods 2 to 4 X 1 microns, often in pairs or short chains, no palisading or filaments, non motile.



At left, type S colony from plating of R-3 colony after seven days incubation. Large sectors of I-1, I-2, and R-3. Conspicuous sector of R-7. S colony at center.

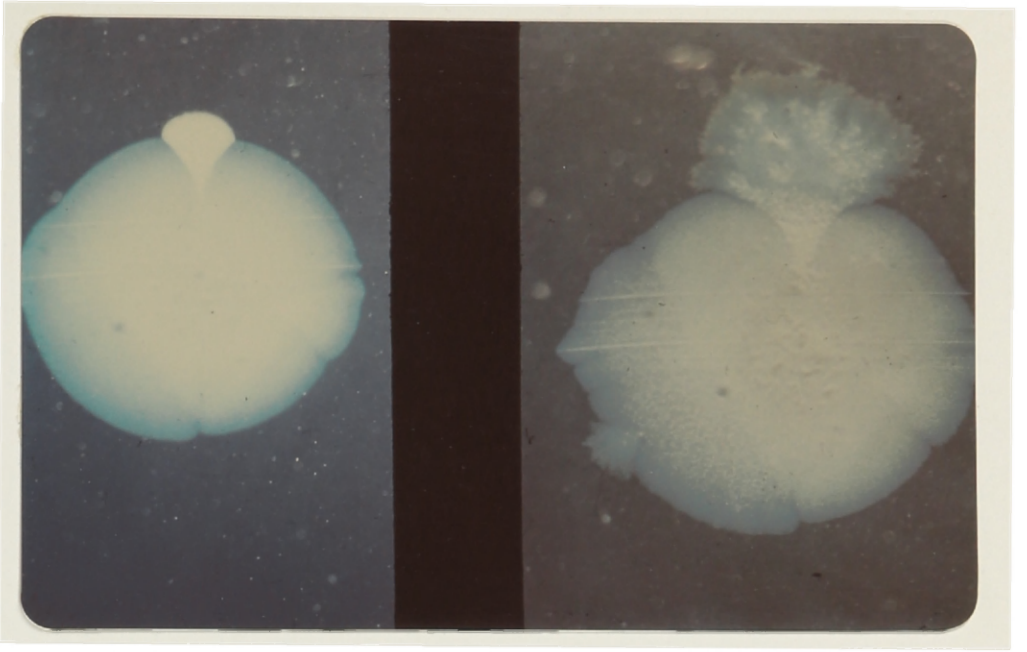


From left to right, pure culture of R-7 at 24, 48 and 96 hr incubation showing marked change in size, contour and color. Same colonies in each section.

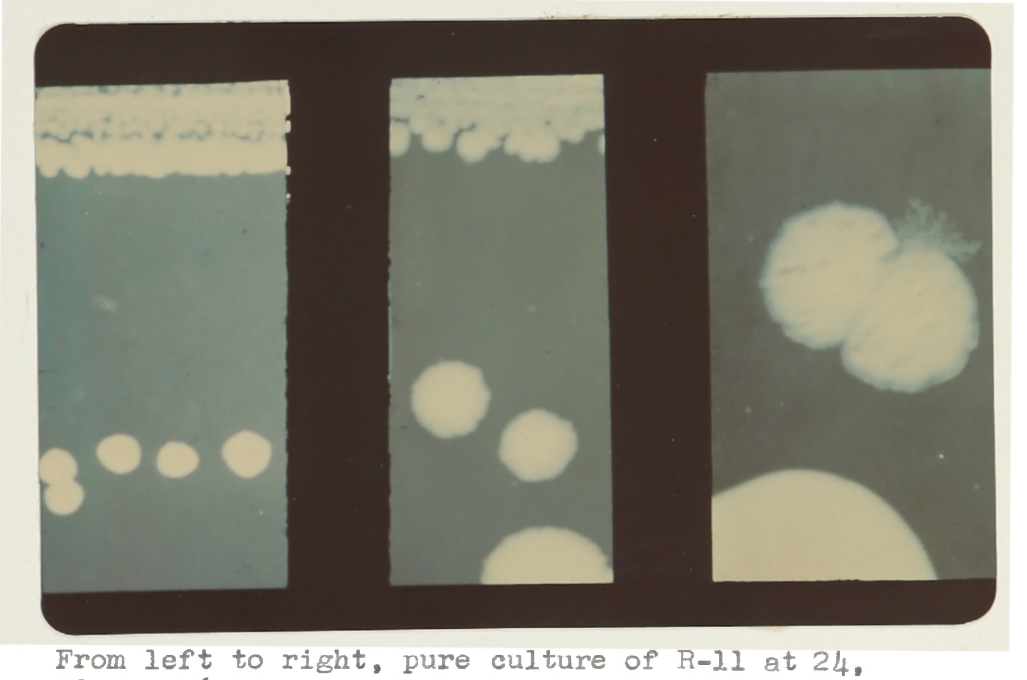
Fig. 9 and 10. Type R-11.

Arise as filaments from four to ten-day old R-7 colonies. Occasionally from similar filaments developing an old type R-3 colonies. Rarely develops directly as a sector in type S colonies. Not pathogenic.

Agar colonies at 24 hr are circular, opaque, flat to convex with smooth surface and entire margin, pale to bright pink in color, watery to stringy in consistency. After about four to six days the colonies are 2-4 mm in diameter and appear very flat with entire to slightly irregular margin. May or may not develop secondary filaments. Colonies which develop no secondary filaments retain the pink color, but when filaments are produced the colony becomes a pale green with slightly cream center. This may represent two distinct colony types, but has not been confirmed. Filaments when plated give rise to type R-14 and R-15 colonies. Cells: Short plump rods 2 to 4 X 1 microns in pairs, chains, or palisades, many curved, rarely short filaments, non motile.



At left, seven day old S type colony with conspicuous R-11 sector at top. Actually this is rarely seen. At right, same colony at 11 days. R-11 sector now blue-green with faint gold R-13 at margin. I-2 at lower left.

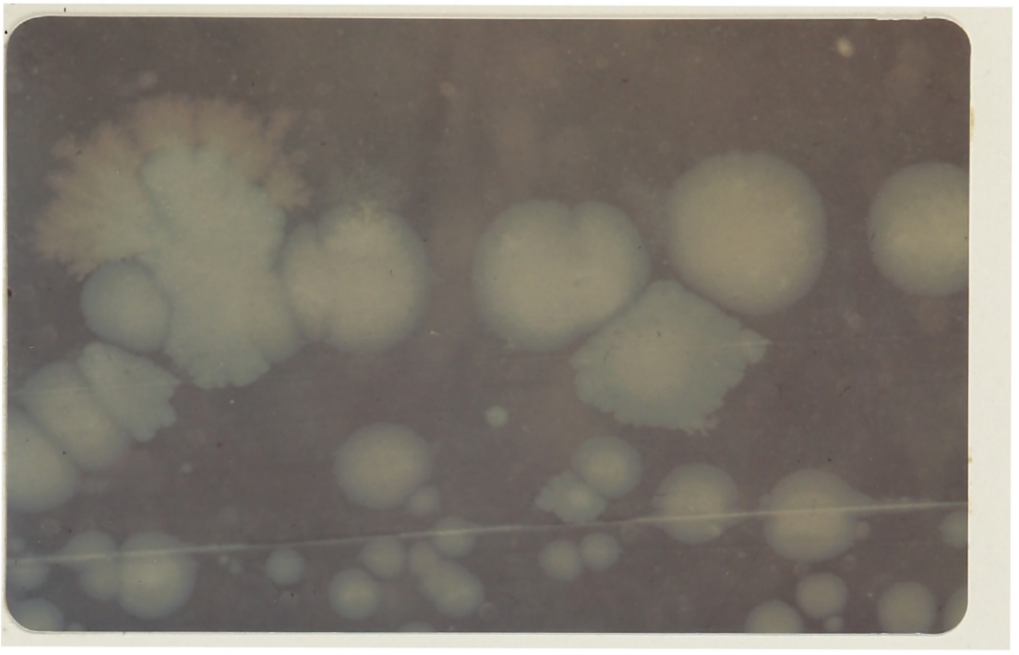


From left to right, pure culture of R-11 at 24, 48 and 96 hr incubation showing marked change in size but similarity of margin and color. At right, secondary filaments with R-13 at margin. Same colonies in each section. Contaminant at bottom.

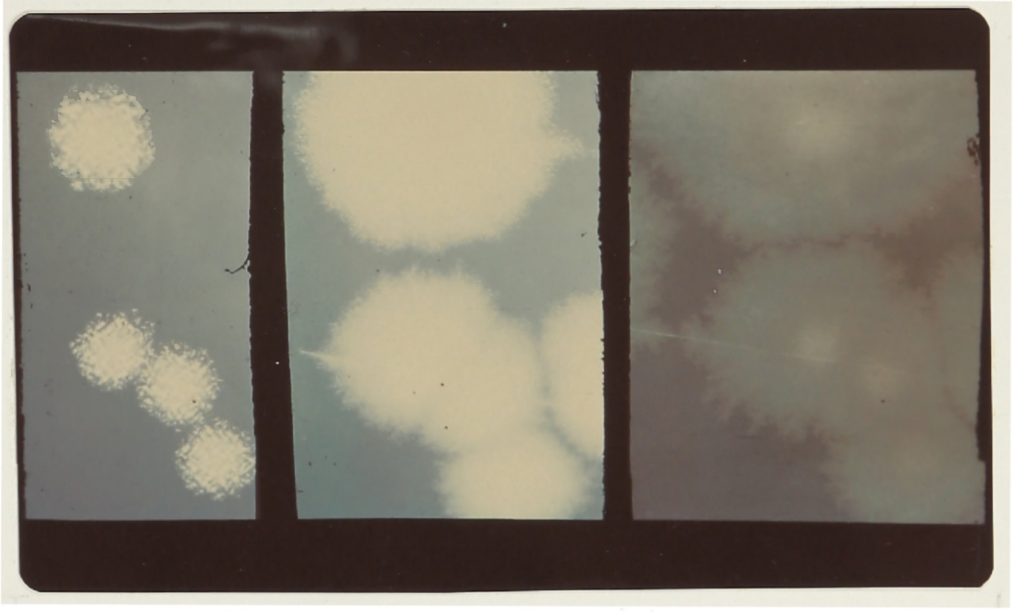
Fig. 11 and 12. Type R-14.

Arise as filamentous daughters from five to ten-day old type R-7 or R-11 colonies. Never develops directly from type S colonies. Tendency to be unstable, but can be obtained in pure form. Non pathogenic.

Agar colonies at 24 hr are filamentous to irregular, translucent to opaque, slightly raised, with deeply ridged and irregular surface, erose to filamentous margin, bright pink, watery. After 48 hr the colonies are flat, filamentous, spreading and become pale green in color. After several days may reach 10 to 15 mm diameter. Filaments when plated give rise to type R-15 colony. Cells: Long plump rods 3 to 7 X 1 microns in chains or long heavy filaments, many curved, no slender or S type cells. Non motile.



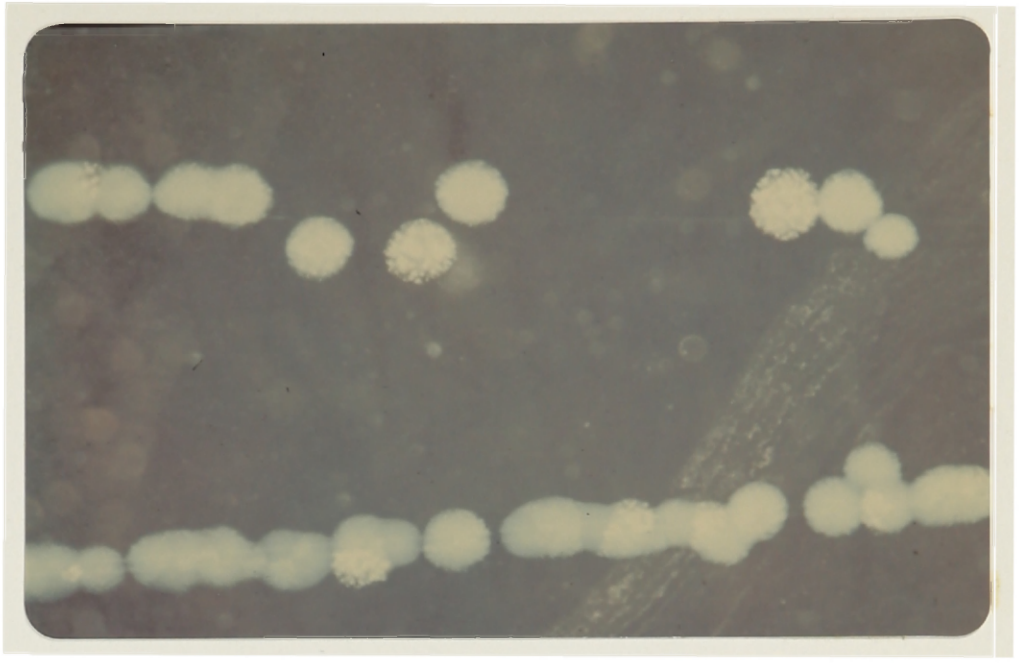
Mixture of 5 day old S and R-7 colonies from plating of I-2. At upper left pink R-13 which gives rise to R-14 and R-15 colonies. At bottom a few S colonies with blue halos.



From left to right, pure culture of R-14 at 24, 48 and 72 hr showing marked change in size, contour and color. Same colonies in each section.

Fig. 13. Plating of R-10 colony at 24 hr showing mixture of R-3 and R-14 colonies. Difference in color and texture most pronounced in confluent growth and a few S colonies can be seen.

Fig. 14. Plating of R-10 colony at 48 hr showing mixture of R-3, R-7 and R-14 colonies. Colors are not exactly true and the individual colony types are difficult to distinguish on this print.



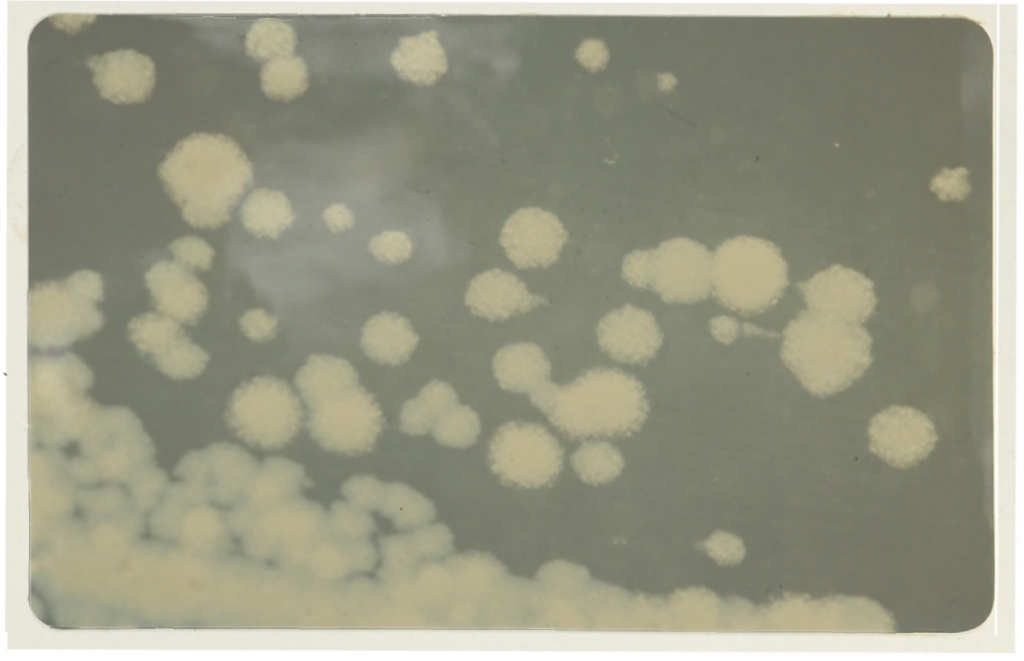
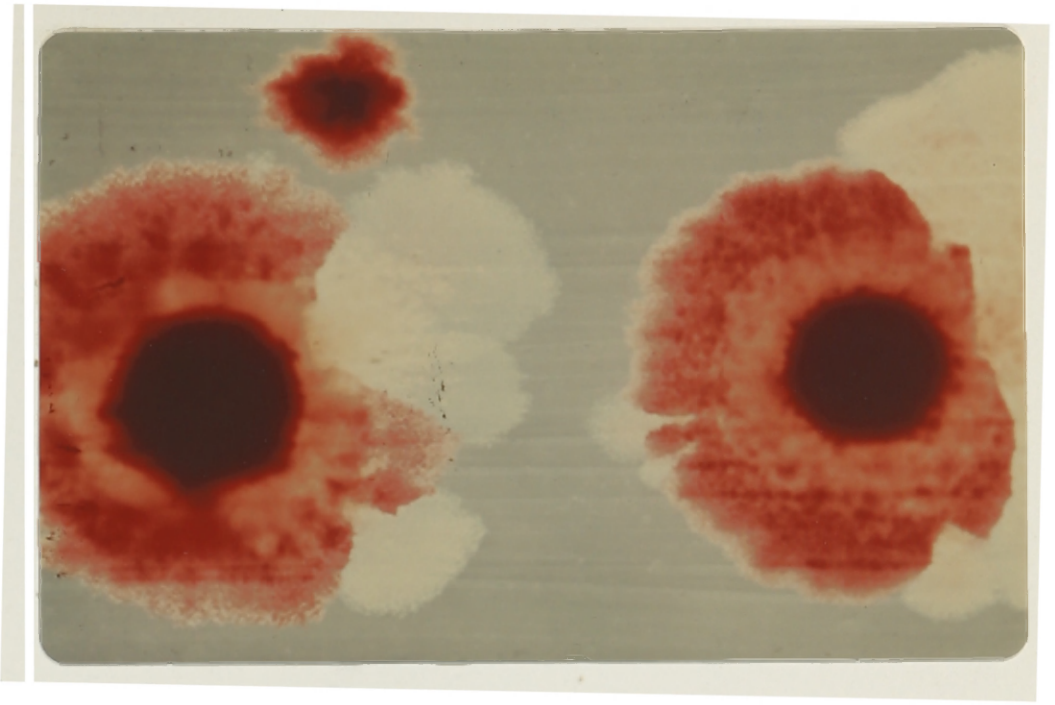


Fig. 15. R-7 colonies at 16 days on agar containing 2, 3, 5-triphenyltetrazolium chloride. Pale pink daughter at far right gives rise to mixture of R-7, R-11, R-14, R-16 and R-17. Pale green sectors at center usually give rise to pure R-3 or mixture of R-3 and R-16. R-16 daughters (not shown are a brighter green, more coarsely textured, and almost transparent. Center of colony gives rise to pure R-7.

Fig. 16. Plating of R-11 at five days on agar containing 2, 3, 5- triphenyltetrazolium chloride, showing mixture of R-7, R-11 and R-14 and difference in ability to reduce the salt. Fine pink growth in background is R-18, a poorly understood variant which appears only in cultures grown on tetrazolium salt. Usually no growth when transferred to plain agar.



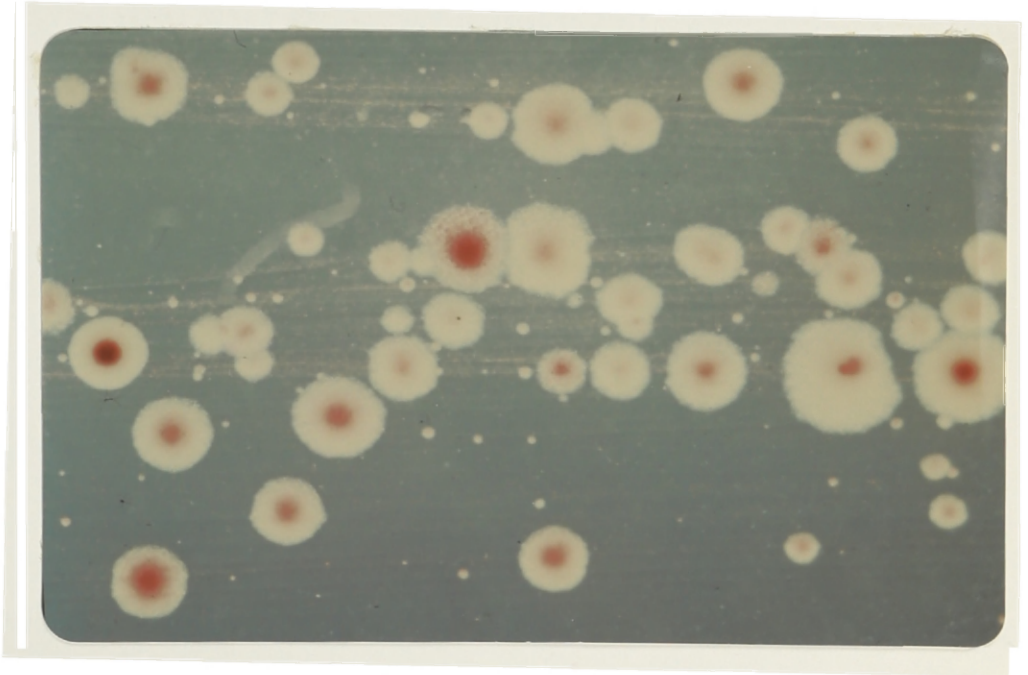
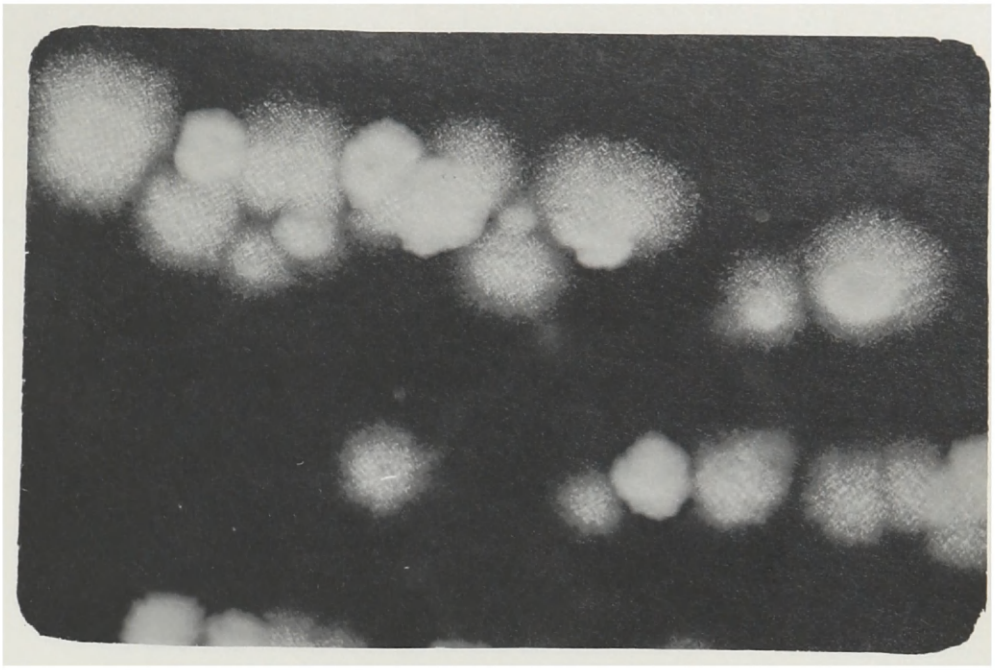


Fig. 17. Plating of I-1 colony at 48 hr showing mixture of R-11 (smooth surface, entire margin) and R-7 (granular surface, filamentous margin).

Fig. 18. Same colonies as in Fig. 17 after five days. R-11 unchanged except for slight increase in size. Still retains pink color. R-7 marked increase in size due to extension of filamentous R-13. Now pale green in color. R-13 pink to gold. Mold and two conspicuous contaminants in field.



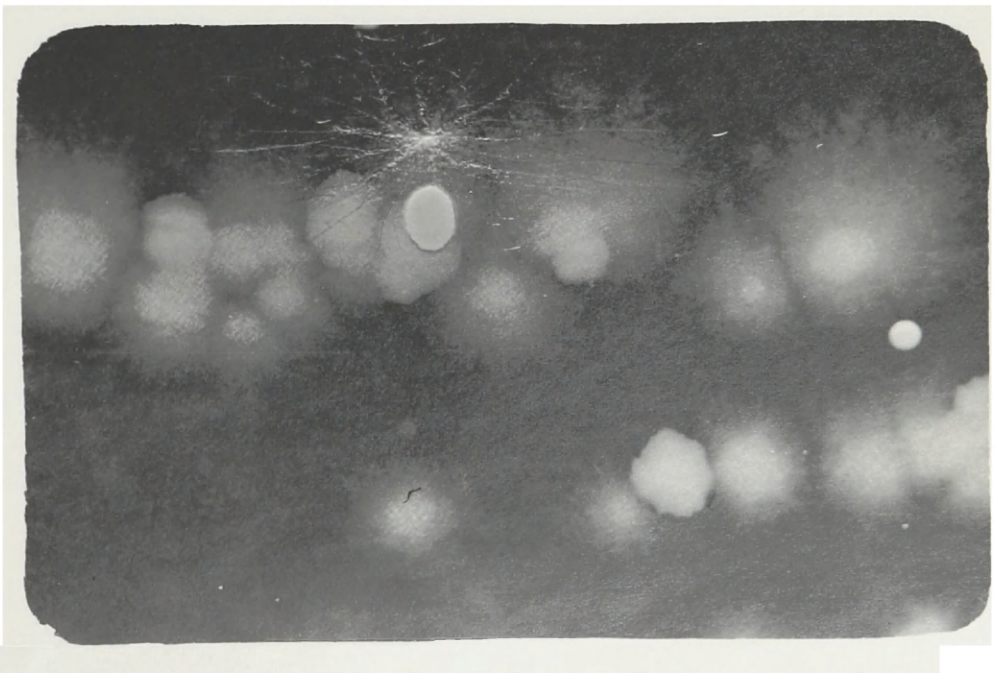


Fig. 19. R-4 and R-5 at three days. Compare with Fig. 5.

Fig. 20. Same colonies as Fig. 19 at seven days showing both I-1 and I-2 daughters.

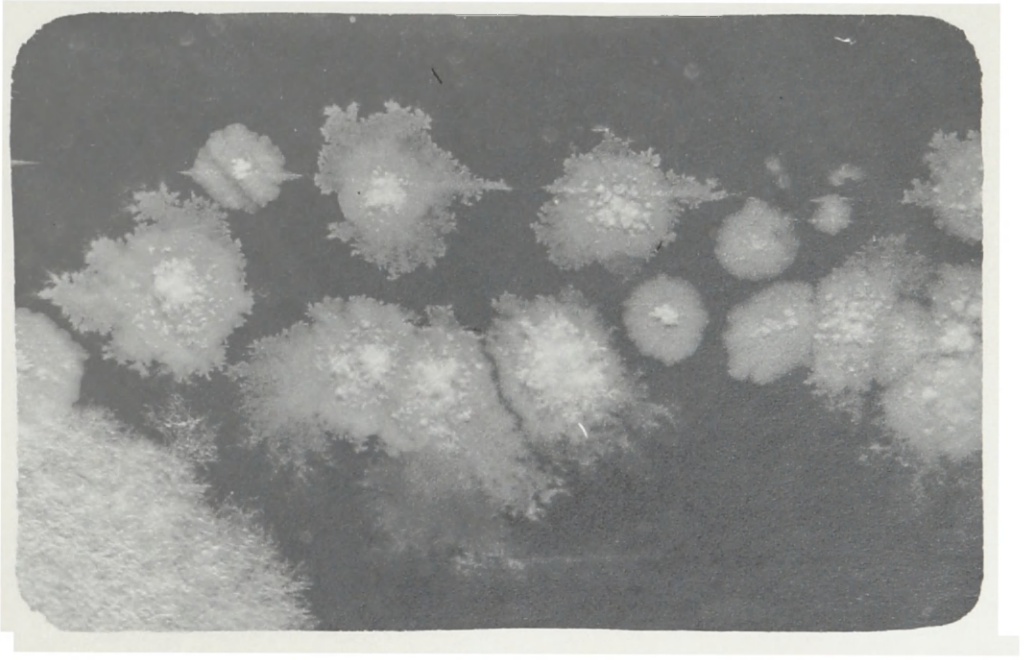
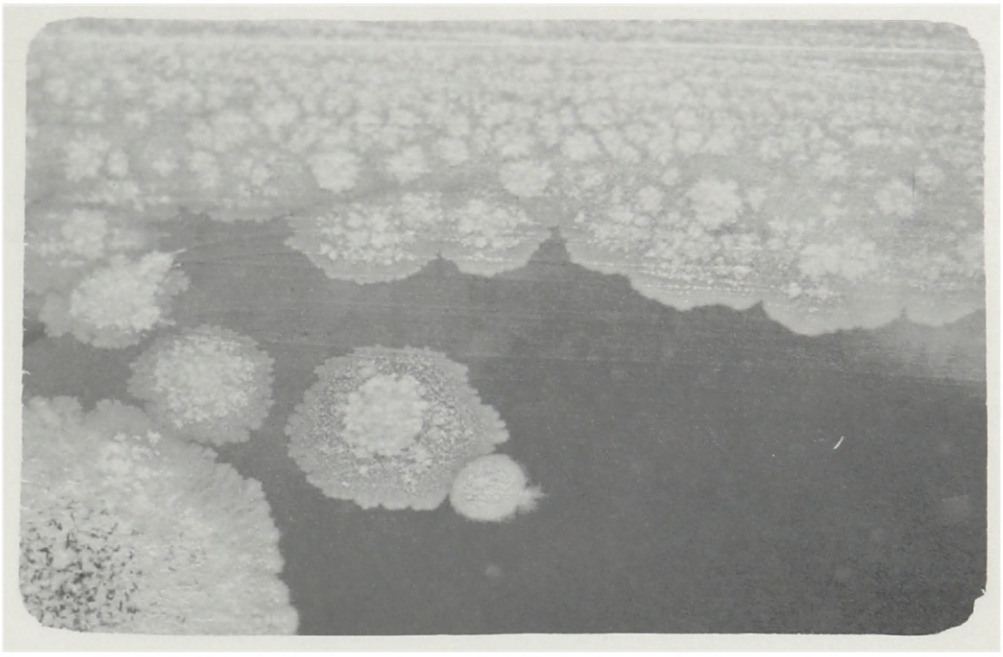


Fig. 21. R-7 and one colony R-11 at 10 days. Filaments coalesced and flattened appearance. Light colored central area marks site of original colony. R-11 has small R-13 daughter.

Fig. 22. Pure R-11 after 20 days showing uniform dirty pink to pale green color and very small filament of R-13.



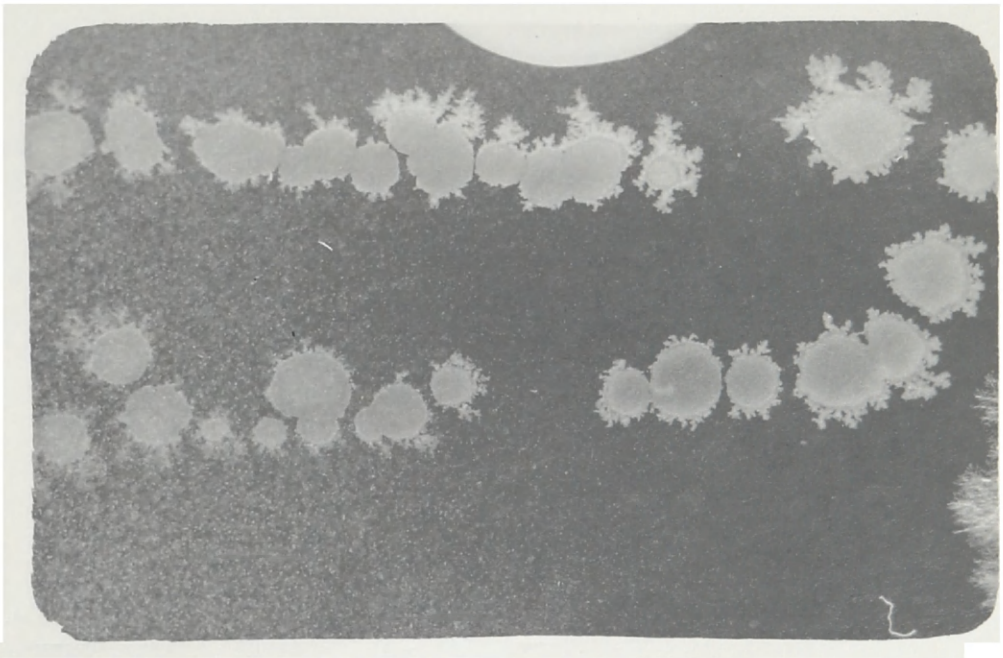
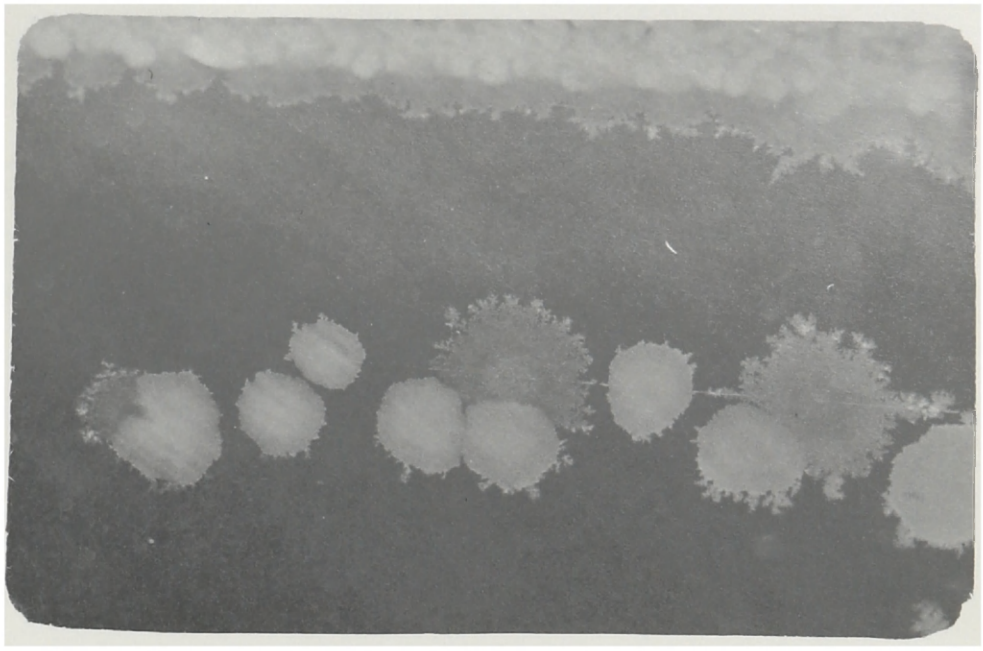


Fig. 23. R-11 from plating R-12 colony at 10 days showing development of large R-13 daughters. R-11 are pink and R-13 daughters are green with gold margin.

Fig. 24. Same area as Fig. 23 at 21 days showing uniform color which is now green. This is a striking characteristic of all R types that regardless of the color of young colonies as they age they become a pale green similar to old S colonies. The only exception is R-11 which retains the pink color when no or only small R-13 daughters develop. See Fig. 18 and 22.



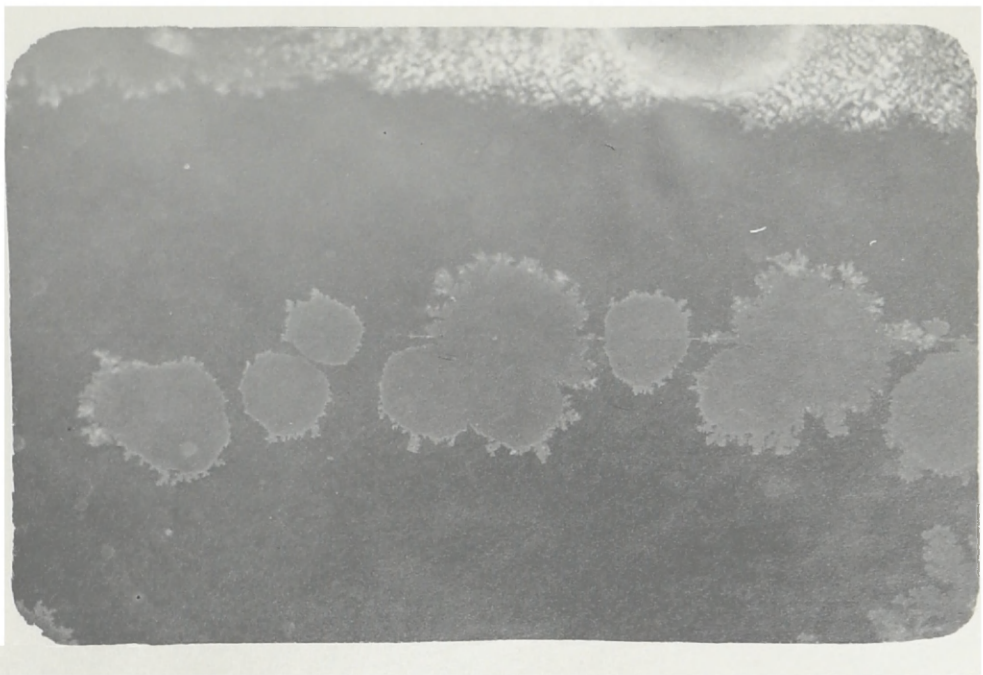
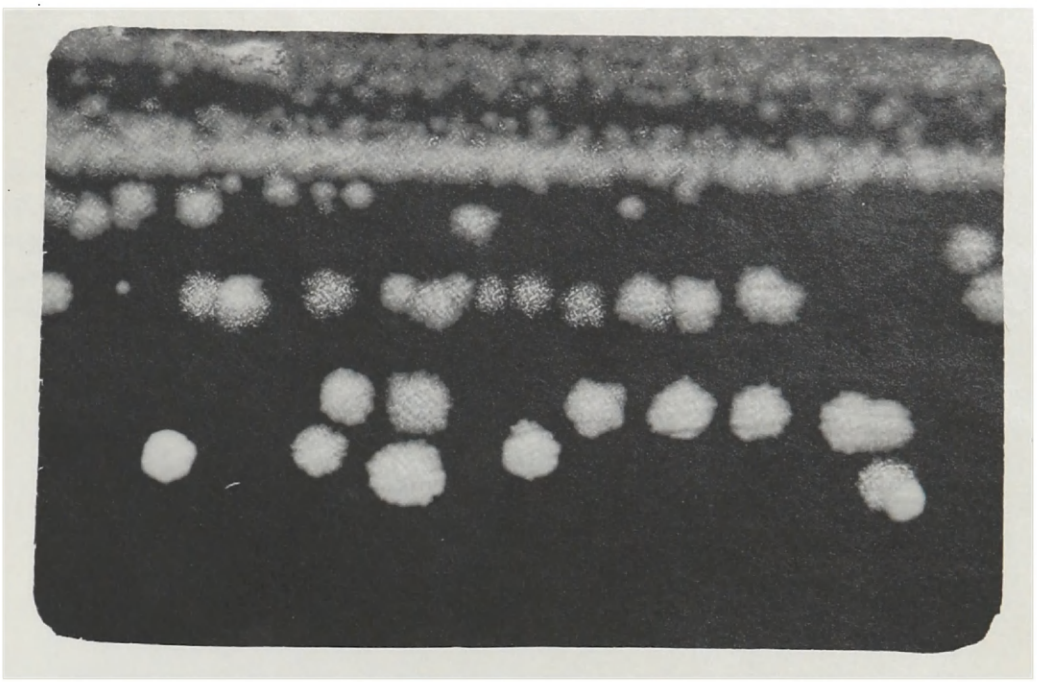


Fig. 25. Plating of R-4 colony showing mixture of R-3, R-7 and R-11 colonies at 24 hrs. R-3 can be distinguished from R-7 (pink) by lighter color (green) and smaller size. R-11 can be distinguished by smooth surface and entire margin. R-3 and R-7 may be difficult to distinguish as isolated colonies.

Fig. 26. Pure culture of R-17 at six days. This originated from an R-3 culture exposed to 2, 3, 5- triphenyl-tetrazolium chloride. Similar to the one shown in Fig. 16. This colony is characterized by an almost smooth pink raised center which when young (24-48 hr) is almost identical to R-11 except that it is larger. After several days a coarsely textured halo develops which when plated usually gives rise to R-16. This colony seldom attains a diameter of more than 5 mm. It also may arise from R-16 cultures. It has never been observed in cultures not exposed to the tetrazolium salt. No pathogenicity studies.



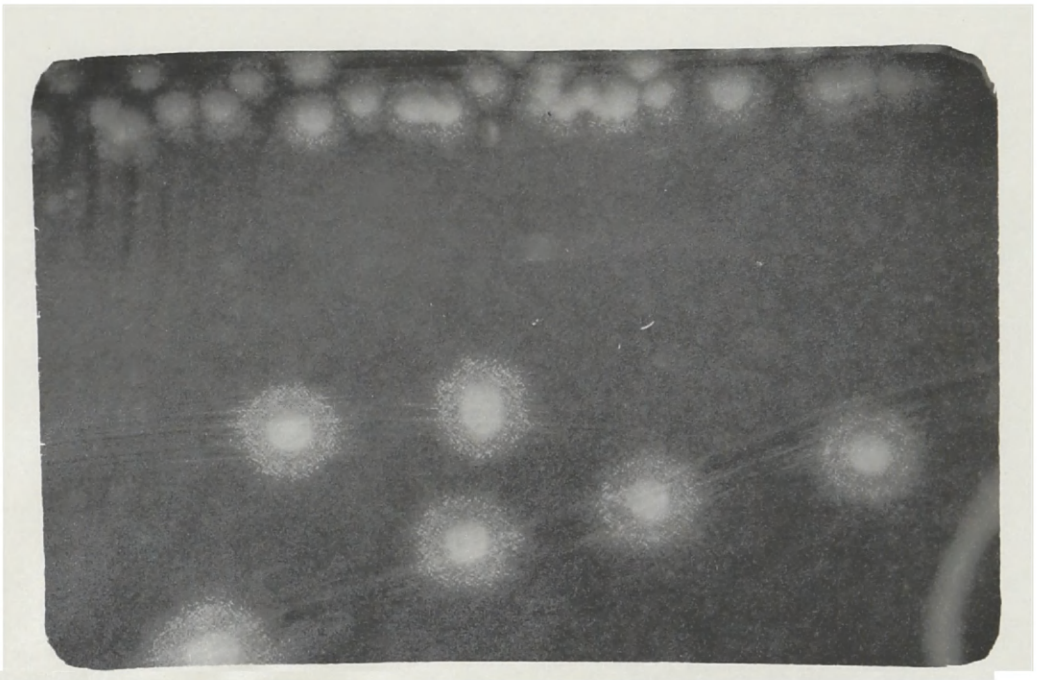


Fig. 27. Pure culture of R-3 at five days. This culture is from plating center of colony shown below. Note irregular shape and filamentous to lobate margin. This colony may reach 20 mm in diameter. Young colony shown in Fig. 6 and 25. Compare with R-16, Fig. 30.

Fig. 28. R-3 colony at 10 days with wide halo of R-16. This usually occurred only in R-3 cultures grown in the presence of 2, 3, 5- triphenyltetrazolium chloride. The halo developed in the first transfer to agar not containing the tetrazolium salt. At times similar appearing halos give rise to R-17 colonies as shown in Fig. 26 or to a mixture of R-16 and R-17.

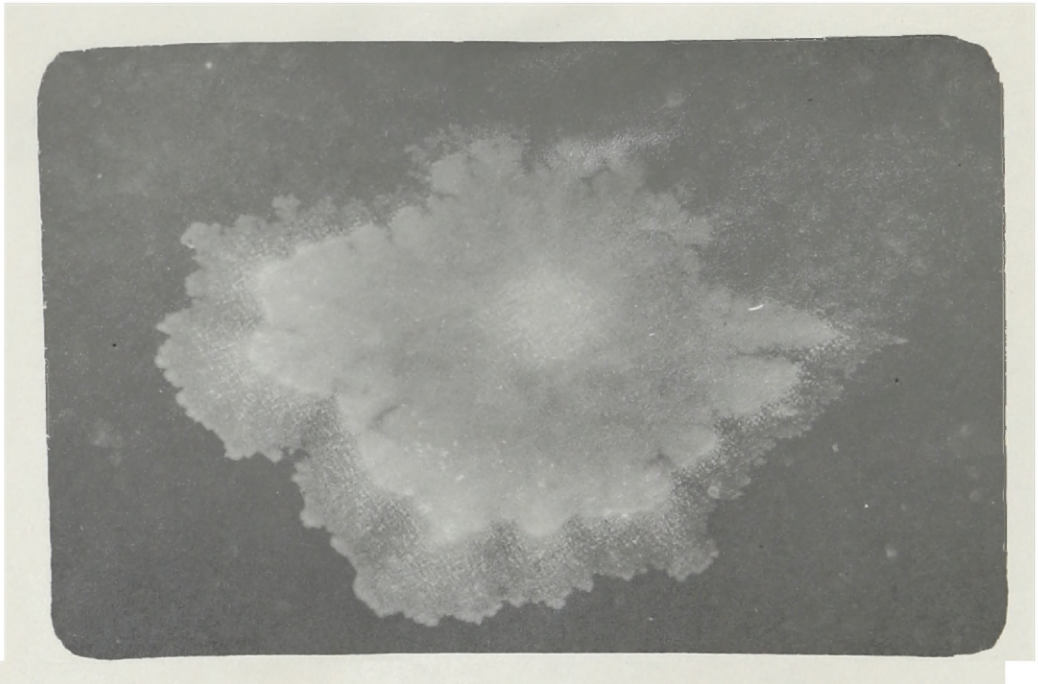
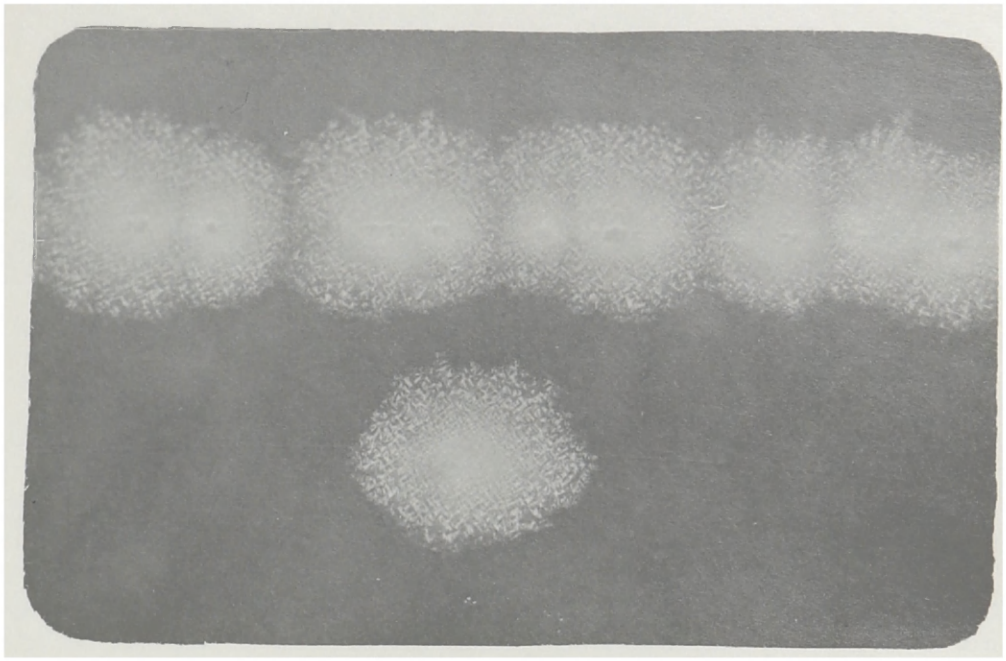


Fig. 29. Pure culture of R-16 at 48 hr. Similar to R-3 in color and texture but much larger in size. See Fig. 6. Usually develops from either S or R cultures exposed to 2, 3, 5- triphenyltetrazolium chloride but may occur without this salt. This culture can be maintained pure. Non pathogenic.

Fig. 30. R-16 at 11 days. Can be distinguished from R-3, Fig. 27, by larger size, raised finely textured center and heavy filamentous to lobate margin.



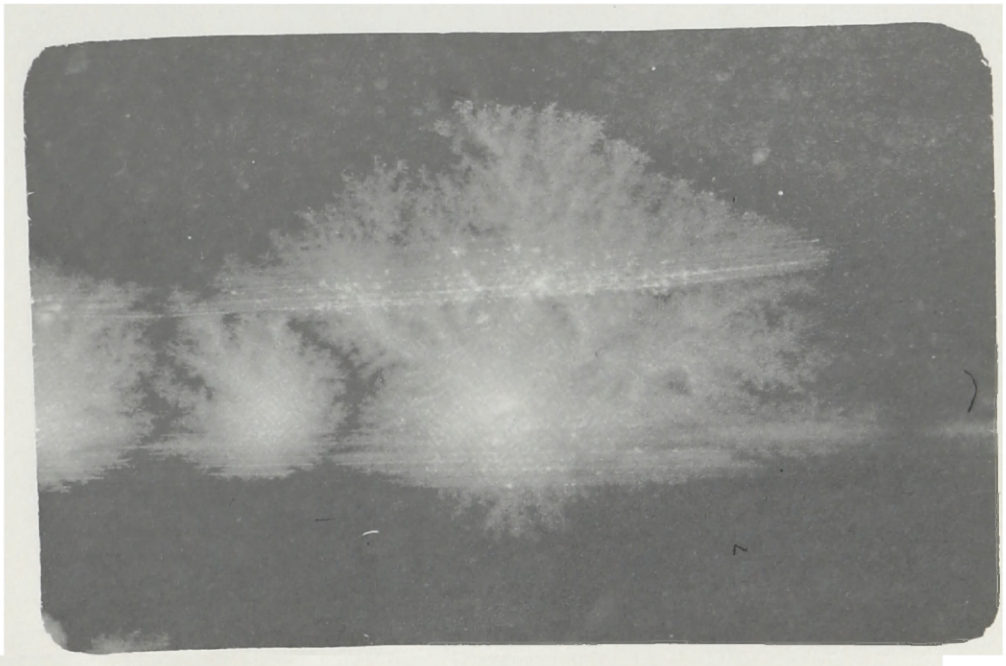


Fig. 31. Pure culture of R-15 at four days. Showing finely textured surface and filamentous margin. This culture is similar to R-14 but bright green in color especially in the center. May reach 20 mm in diameter. Can not be maintained pure. Plating gives rise to R-14, R-15 and R-7. No pathogenicity studies.

Fig. 32. R-15 colony at eight days showing large sector of R-7 at right.

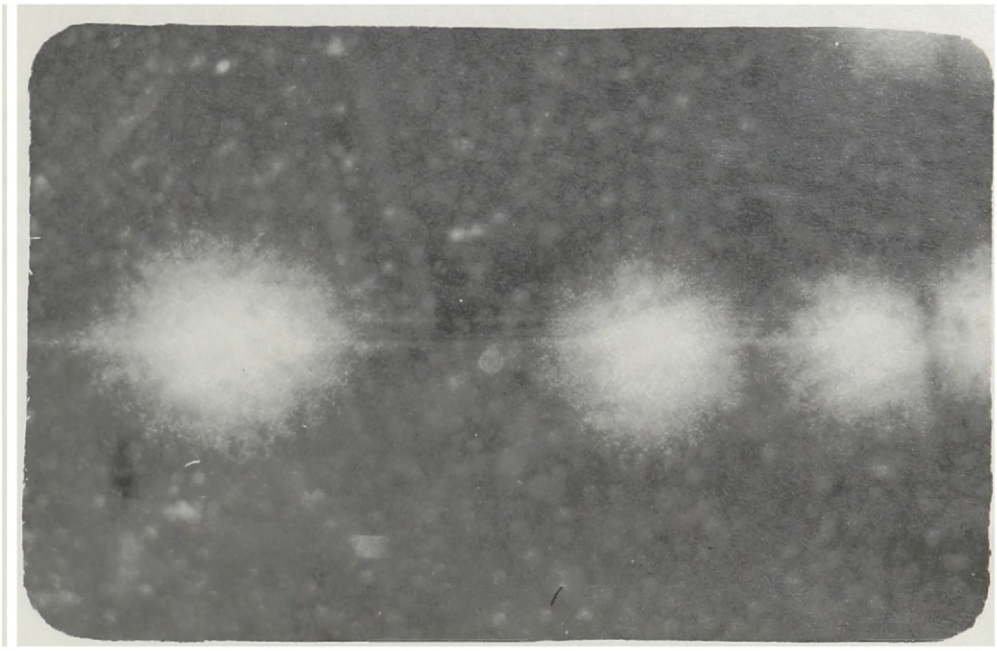
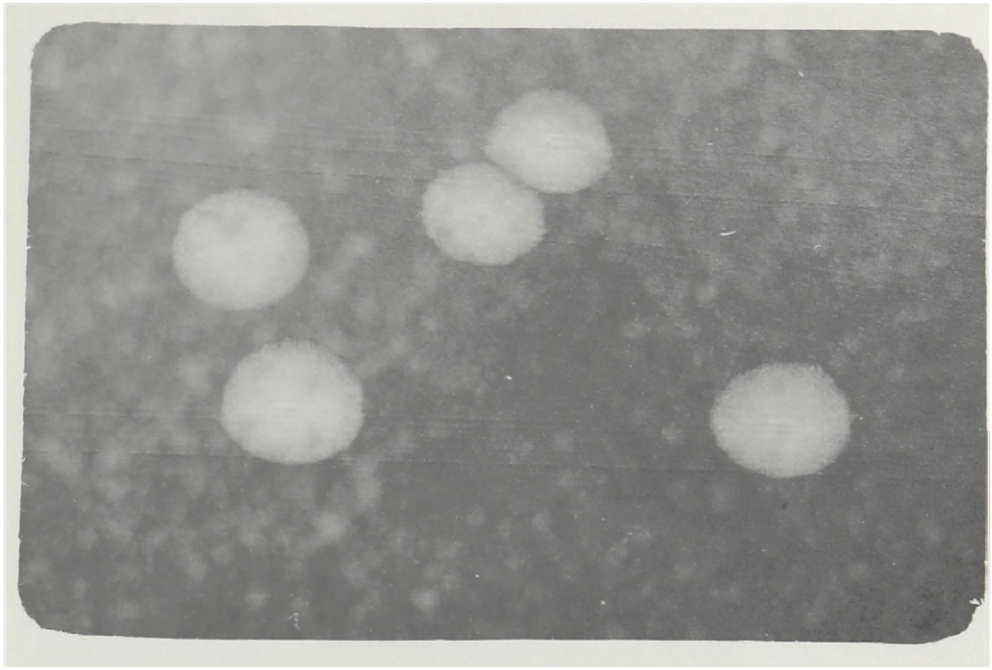


Fig. 33. Possible "reverted" S colony at 48 hr from R-16 colony maintained on tryptose agar containing 2, 3, 5- triphenyltetrazolium chloride. Very similar to S but slightly larger and more coarsely textured at margin. Compare with Fig. 3. No pathogenicity studies.

Fig. 34. Five day old R-6 colonies from plating of I-1 showing filaments and pitted appearance. Found in 10 to 20 day old S cultures. Colonies are large, up to 10 mm diameter. This actually is a highly motile form of S colony and pitted appearance was due to the organisms growing into the agar. These colonies often gave rise to large I-1. I-2 or R-3 daughters which were indistinguishable from the corresponding types from other S colonies. No pathogenicity studies. This is an interesting colony and needs further investigation. Compare with Fig. 6 and 7.



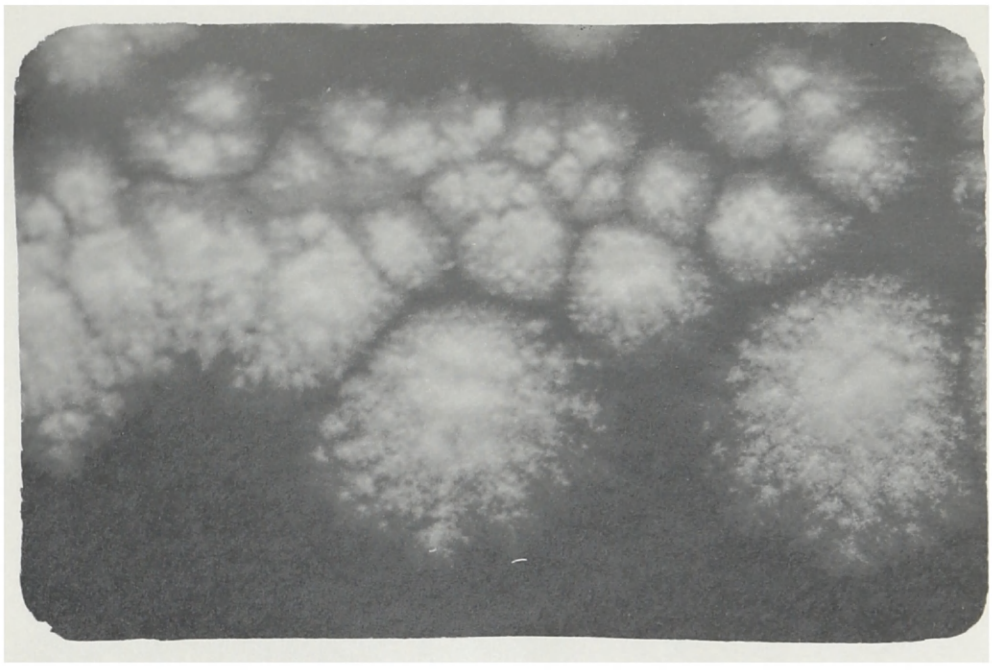
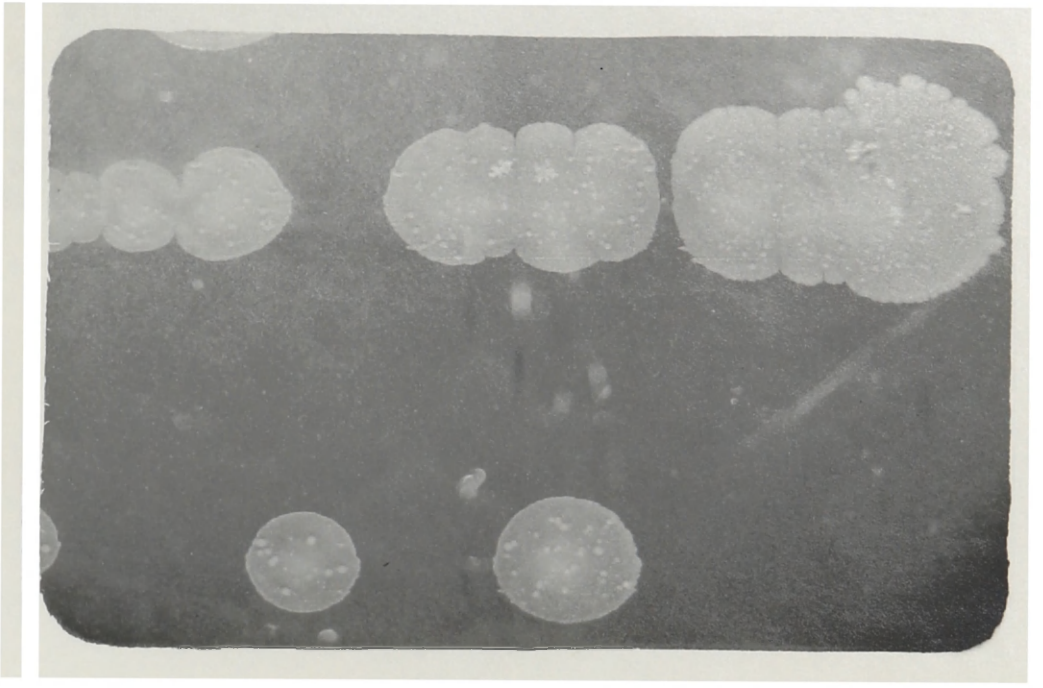


Fig. 35. Fifteen day old S colonies with small R-19 dwarf-like secondary colonies which appear as minute white projections. Careful picking and plating always give rise to what appear to be pure S colonies. No pathogenicity studies.

Fig. 36. Seventy day old S colony with conspicuous R-19 dwarf-like secondary colonies. At right R-3 daughter. Viable cells only at margin of colony and in R-19 colonies.



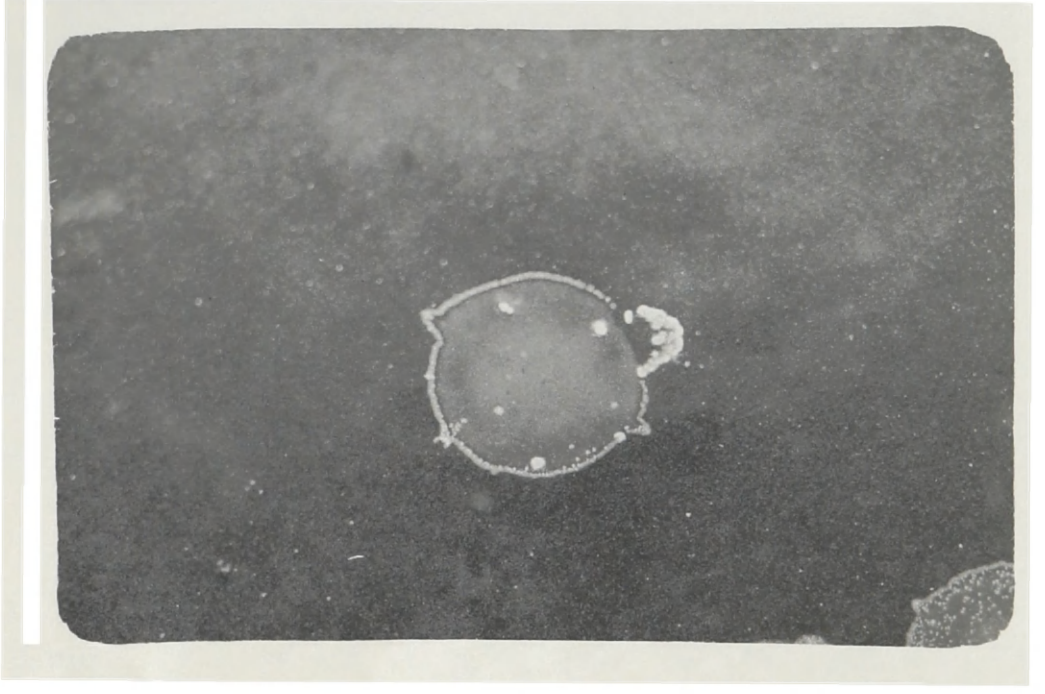
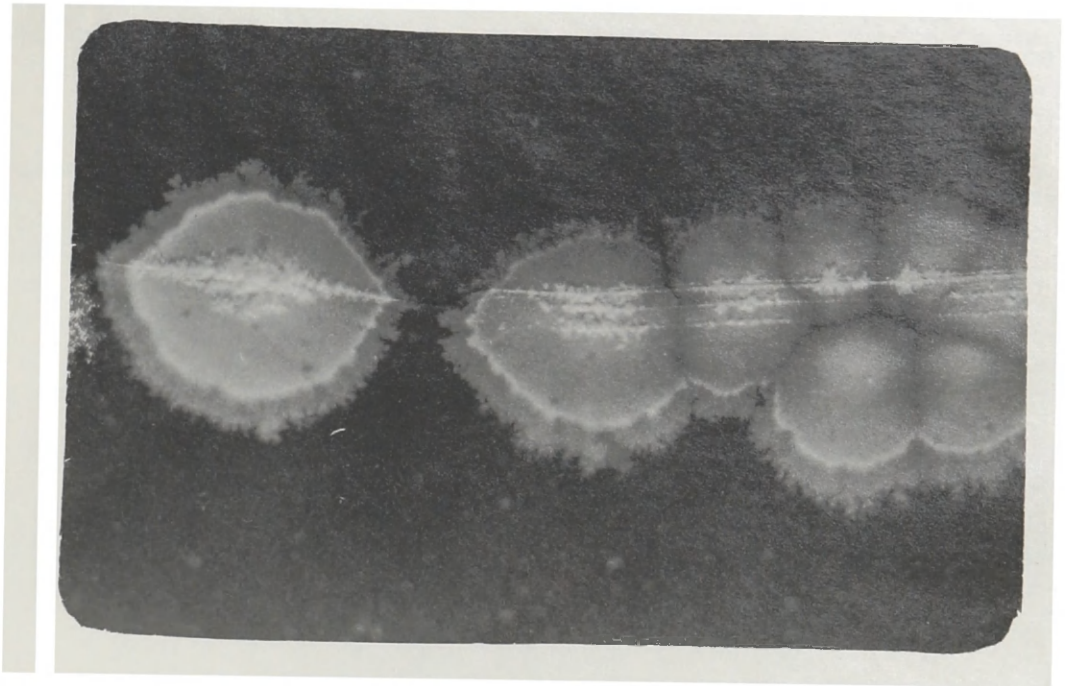


Fig. 37. Six day old S colonies completely surrounded by halo of filamentous growth. This occurred rarely and in only a few cultures. Plating usually gave rise to mixtures of R-3, R-8 and R-16. It has not been determined if cultures which show this halo constitute a distinct group. Also see Fig. 11.

Fig. 38. S culture at 48 hr showing a few pale blue colonies which usually give rise to pure cultures of R-7 when plated.



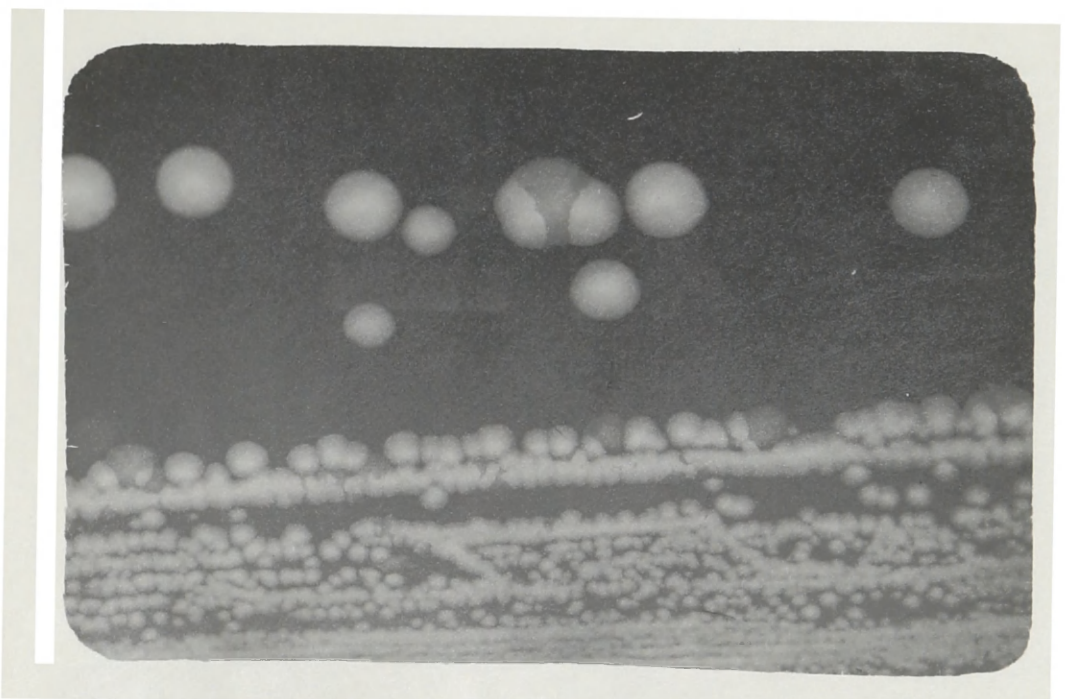
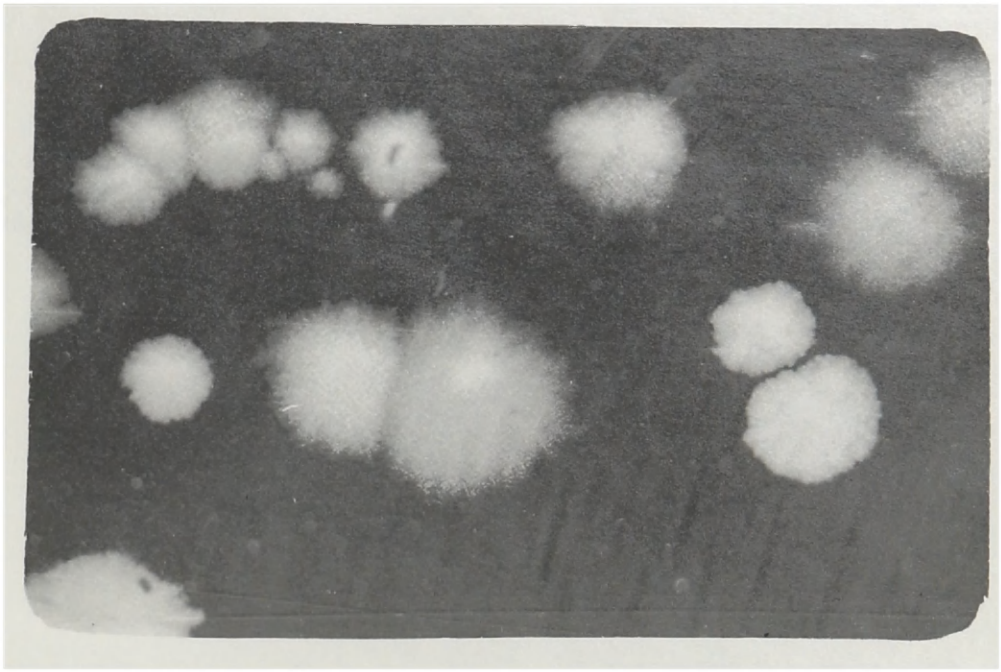


Fig. 39. Mixture of R colonies at 24 hr from broth culture of S colony.

Fig. 40. Mixture of R colonies at eight days from broth culture of S colony.



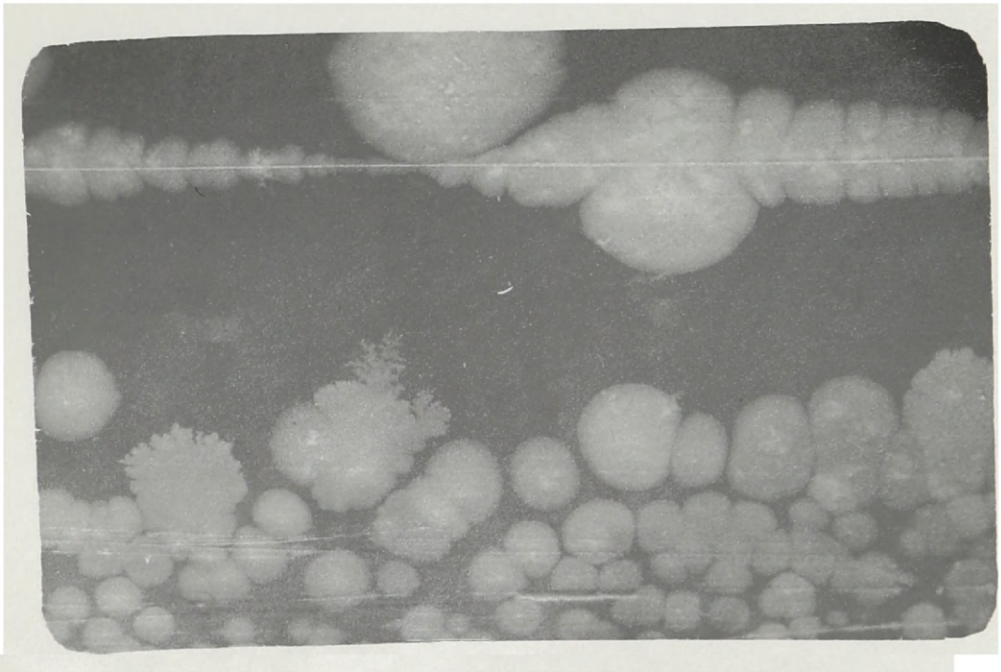
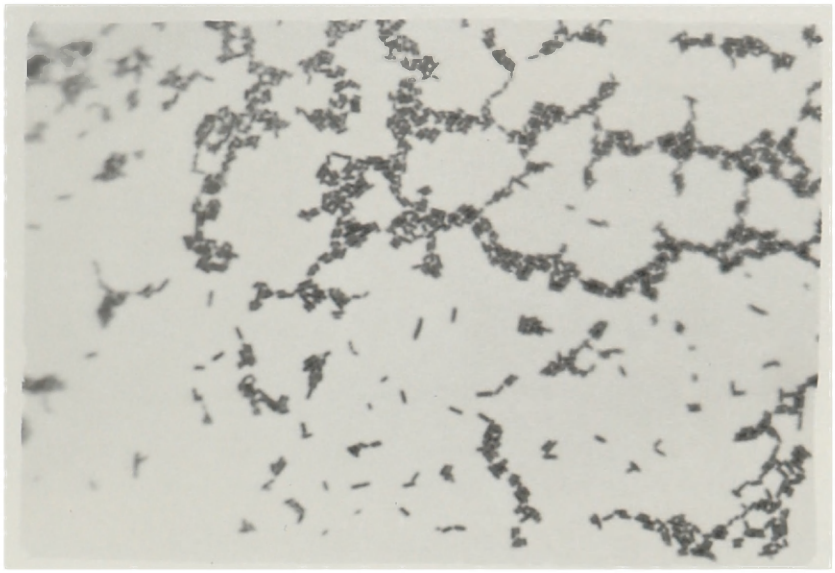
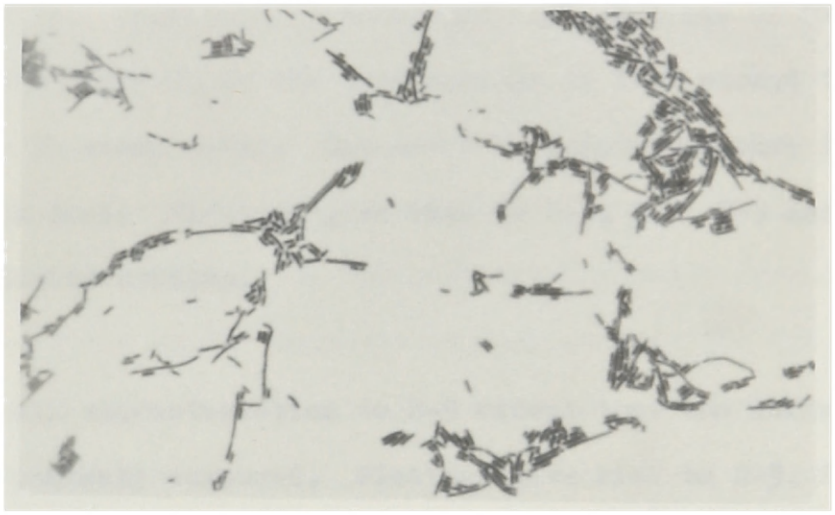


Fig. 41. Cells from an S type colony showing short rods in palisade arrangement, pairs, Y and T forms and a few long cells characteristic of all S cultures of L. monocytogenes. All three fig approx. 1000X, gram stain

Fig. 42. Cells from a non-smooth colony type (R-5) showing mixture of short and long slender rods and a few short slender filaments.

Fig. 43. Cells from a non-smooth colony type (R-14) showing long heavy curved filaments.





Other colony types which developed but which could never be maintained in pure culture are listed below.

Type R-8.

Found most commonly in platings from S cultures maintained for several weeks in broth. Occasionally developed from platings of R-4 and R-5. Agar colonies after 24 hr are very similar to R-7 except that they are gold in color. Plating always gives rise to a mixture of R-3, R-7 and R-11. No pathogenicity studies.

Type R-9.

Found most commonly in platings from S cultures maintained for several weeks in broth. Occasionally developed from platings of R-4 and R-5. Agar colonies after 24 hr are very similar to R-11 except that they are pale yellow to cream color. Can never be maintained pure for more than several days. Platings give rise to R-3, R-7, R-9 and R-11. No pathogenicity studies.

Type R-10.

Similar in all characteristics to R-9 except that the surface of the colony was coarsely textured. Platings give rise to R-3, R-7, R-9, R-10, R-11 and R-14. No pathogenicity studies.

Type R-12.

Similar in all characteristics to R-11 except they have a gold sheen. Platings give rise to R-11, R-14 and R-15. No pathogenicity studies.

S colonies inoculated into tryptose broth and incubated at 37° C for 18 hr and at room temperature for indefinite periods of time, when streaked on agar plates failed to produce colonial forms other than those which developed when the colonies were plated directly on agar plates. Rarely one of the R forms was found as a pure isolated colony.

However the occurrence of the unstable type colony was much more common than in platings from agar cultures.

When 2, 3, 5- triphenyltetrazolium chloride was incorporated in the agar it was possible to distinguish one new colonial from which had not previously occurred. This was type R-17. Fig. 26. In addition the difference in ability of some colonial types to reduce this salt greatly enhanced their detection. Type 16 failed to reduce this salt and the colonies appeared a rather pale green in color while all other types varied from pale pink to a deep red or purple. Fig. 15. After about a week of incubation the S colony was characterized by a dark red central area which approached black. Fig. 4. This intense central area also developed in some R colonies, particularly R-7. Fig. 7.

The degree to which the individual colony type reduced the tetrazolium salt was reflected in the colonies which develop from transfers to plates without the salt. This was evident only in the first transfer. Notable in this respect was type R-15 which took on a much deeper pink than before exposure to the salt. As type R-7 and R-14 did not reduce the salt as readily as did R-15, they could very easily be distinguished from each other, particularly when they were adjacent. This effect undoubtedly was due to a small residual of tetrazolium salt carried over on the first transfer and was an amount sufficient to enhance the color of those colonies which already displayed a tendency toward deep pink.

There were no differences in either fermentative or biochemical characteristics displayed by any of the N-S colonial types when compared with the parent S type. All colonial forms tested produced acid but no gas after 24 hr in glucose, levulose, trehalose and salacin. Acid

production was slow and variable in arabinose, galactose, lactose, maltose, sucrose, dextrin, sorbitol, glycerol, and melezitos. There was no fermentation of xylose, raffinose, inositol, insulin, dulcitol, mannitol or adonitol. There were some slight variations in the rate of fermentation but no significant differences. The variations appeared to be of the same order one would expect to find in any large number of stock cultures of L. monocytogenes exposed to the same fermentable substances.

None of the R types tested reduced nitrates, or produced indole. Gelatin was not liquefied. In general the R types produced either no hemolysin or displayed a much smaller zone than did the parent S type.

By the methods employed, cultures from N-S colony types I-1, I-2, and R-3 were pathogenic for rabbits. However, all types tested produced marked monocytosis and a varying degree of agglutinating antibodies in the peripheral blood. Typical results, following intravenous inoculation, together with agglutinating antibody titer for the homologous type and S type cultures are shown in the protocols which appear on pages 92-96. At no time did any animals which received cultures from R colonies display any marked clinical indication of illness. A few rabbits showed a slight rise in temperature on the second or third post inoculation day. Cultures prepared from the liver, spleen and heart blood of rabbits sacrificed from several days to several weeks after inoculation, failed to yield significant bacterial growth. Rabbits inoculated intravenously with cultures from R colonies very often survived a subsequent lethal dose of S culture.

When cultures from R type colonies were instilled into the conjunctival sac of rabbits, little or no reaction followed. Two rabbits in-

stilled with type R-7 showed marked lacrimation on the second post instillation day, but appeared normal thereafter. Instillation of cultures from type S colonies resulted in a marked conjunctivitis and keratitis in all instances.

Rabbits inoculated with cultures from certain R type colonies, particularly types R-3 and R-7 produced high agglutinating titers against type S cultures. This suggested that these types might be successfully employed in a vaccine that could conveniently be administered by the subcutaneous route. The results indicated that in the amount given, these types did produce fairly high agglutinating titers following subcutaneous inoculation. It was further shown that when 15 TR units of Wydase per ml were added to the bacterial suspension antibody titer was enhanced. The addition of Wydase also prevented the development of the localized necrotic lesion that developed at the site of inoculation when cultures from type S colonies were given subcutaneously. However, in rabbits inoculated with cultures from type R colonies, congestion and small superficial hemorrhages were the principal lesions observed, and necrosis, if present, was limited to small discreet areas which seldom measured more than two to three mm in diameter. These may have resulted from the presence of pathogenic S type cells in the culture.

All rabbits exposed subcutaneously with cultures from either type S or type R colonies developed a marked monocytosis in the circulating blood. The monocytosis developed most quickly and reached the highest value in the rabbits exposed to culture containing Wydase. There was no evidence either <u>in vitro</u> or <u>in vivo</u> that Wydase exerted any unfavorable effect on the cultures.

When these rabbits were exposed five to 10 weeks later to a known lethal dose of culture from S type colonies, death occurred in two to three days. All unvaccinated controls died within the same period. At necropsy all rabbits showed lesions typical of septicemic listeriosis; focal hepatic necrosis and splenomegalia. Type S cultures were recovered in pure culture from the liver, spleen and occasionally from the heart blood of all animals which died. This strikingly demonstrated that the presence of agglutinating antibodies in the blood serum was not a measure of immunity to infection with L. monocytogenes. These results are summarized in table 1 and typical protocols are given on page 97.

Fate of two rabbits inoculated intravenously with 0.3 ml culture 12255-S*

Rabbit 159

Day	Differential leukocyte count P***	L	M	B	E
0**	29	65	0	6	0
1	61	17	19	2	1
2	29	32	38	1	0
3	58	7	35	0	0
4	Dead. Focal hepatic necrosis and splenomegalia. L. monocytogenes isolated from heart blood and liver.				

Rabbit 158

Day	Differential leukocyte count P	L	M	B	E
0	23	72	0	0	0
1	45	52	2	1	0
2	49	12	38	1	0
3	Dead. Splenomegalia. L. monocytogenes isolated from liver.				

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*All cultures standardized to No. 1 tube McFarland nephelometer.

**Inoculation day.

***P - polymorphonuclear leukocyte
L - lymphocyte
M - monocyte
B - basophil
E - eosinophil

Same symbols are employed in subsequent tables.

Typical results following intravenous inoculation of 0.3 ml culture 12255 R-3*

Rabbit 153

Differential leukocyte count

Day	P***	L	M	B	E
0**	38	60	0	2	0
1	63	28	2	6	1
2	60	5	35	0	0
3	47	9	44	0	0
4	40	30	28	2	0
7	45	47	8	0	0

Titer after 100 days

Culture	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
S	++++	++++	±	-	-	-	-	-
R-3	++++	++++	++++	±	-	-	-	-

Challenge at 106 days with 1.0 ml 12255-S I.V.*

Differential leukocyte count

Day	P	L	M	B	E
0	49	42	5	3	1
1	54	42	4	0	0
2	62	17	21	0	0
3	51	13	35	0	1
6	47	25	28	0	0
10	56	31	11	0	2

Survived.

For explanation of symbols see page 92.

Typical results following intravenous inoculation of 0.3 ml culture 12255 R-7*

Rabbit 142

Differential leukocyte count

Day	P***	L	M	B	E
0**	31	66	1	0	2
1	83	15	2	0	0
2	42	18	40	0	0
3	31	23	46	0	0
4	37	26	36	0	1
7	44	52	4	0	0

Titer after 100 days

Culture	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
S	∕	-	-	-	-	-	-	-
R-7	∕∕∕∕	∕∕∕∕	∕∕∕∕	∕∕∕∕	∕∕∕∕	∕∕∕∕	∕∕	-

Challenge at 106 days with 1.0 ml 12255-S I.V.*

Differential leukocyte count

Day	P	L	M	B	E
0	58	32	8	1	1
1	70	30	0	0	0
2	59	38	2	1	0
3	52	12	35	1	0
6	63	17	18	0	2
10	51	27	19	1	2

Survived.

For explanation of symbols see page 92.

Typical results following intravenous inoculation of 0.3 ml culture 12255 R-11*

Rabbit 143

Differential leukocyte count

Day	P***	L	M	B	E
0**	29	63	2	4	2
1	58	41	0	0	1
2	56	6	37	1	0
3	35	20	44	0	1
4	42	17	40	0	1
7		(Poor slide)			

Titer after 100 days

Culture	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
S	++++	+++	±	-	-	-	-	-
R-11	++++	++++	++++	++++	++++	++++	-	-

Challenge at 106 days with 1.0 ml 12255-S I.V.*

Differential leukocyte count

Day	P	L	M	B	E
0	45	47	2	3	3
1	51	46	1	1	1
2	53	24	20	3	0
3	47	27	23	2	1
6	46	20	29	4	1
10	30	59	8	1	2

Survived.

For explanation of symbols see page 92.

Typical results following intravenous inoculation of 0.3 ml culture 12255 R-14*

Rabbit 154

Differential leukocyte count

Day	P***	L	M	B	E
0**	31	68	0	1	0
1	62	22	11	3	2
2	38	17	44	0	1
3	27	36	34	0	3
4	48	27	25	0	0
7	29	63	7	1	0

Titer after 100 days

Culture	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
S	++++	++++	++++	++++	+	±	-	-
R-15	++++	++++	++++	++++	++	±	-	-

Challenge at 106 days with 1.0 ml 12255-S I.V.*

Differential leukocyte count

Day	P	L	M	B	E
0	26	62	0	0	0
1	67	26	2	1	4
2	37	3	60	0	0
3	25	9	66	0	0
6	27	39	31	3	0
10	37	37	22	4	0

Survived.

For explanation of symbols see page 92.

Table 1

Condensation of a vaccination trial

Culture 1007X	Titer S	Titer R	Monocytes % on 6th day	Survival after challenge in days
R-7 with Wydase	1:320	-*	32	2**
R-7 w/o Wydase	-	-	10	2
S with Wydase	1:2560	1:320	43	3
S w/o Wydase	1:640	1:160	18	3
12255				
R-3 with Wydase	1:80	1:40***	27	3****
R-3 w/o Wydase	1:40	-	25	3
R-16 with Wydase	1:320	1:80	19	3
R-16 w/o Wydase	1:320	1:320	18	3

Immunizing dose: 0.1 ml standardized to No. 9 tube McFarland nephelometer.

Challenge dose: 0.5 ml S culture std. to No. 1 tube McFarland nephelometer.

Four rabbits in each group. Percentage of monocytes represents highest value in each group. Titers are average of animals bled.

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*Titer at two weeks.

**Challenge at five weeks.

***Titer at seven weeks.

****Challenge at 10 weeks.

DISCUSSION

The results revealed that at least 20 different colonial forms of L. monocytogenes could be recognized on tryptose agar plates examined by the oblique lighting technique. Of these, six could be maintained in stable form without further dissociation. These were types S, R-3, R-7, R-11, R-14 and R-16. Type S was the typical smooth colonial form characterized by a small circular, blue-green, slightly raised colony with a finely textures surface and entire margin. Fig. 1. It was the type commonly found in initial isolations from infected material. Cultures of this type were highly pathogenic for rabbits.

In general only four stable colony types developed directly from S colonies. These were R-3, R-7, R-11 and R-16. However types R-7, R-11 and R-16 only rarely developed directly from S type cultures. However, they could be demonstrated quite readily in platings from I-1, I-2, R-4 or R-5 cultures. Fig. 5, 19 and 20. R-3 was the most common type and usually appeared as daughter colonies on well isolated S colonies after about five days incubation. Fig. 6. Rarely did it occur in confluent S type cultures.

R-7 usually developed as a daughter but occasionally developed as a sector in S cultures. Fig. 7 and 8. Type R-16 developed only occasionally from S colonies. In S cultures it always appeared as a daughter and platings of young R-16 daughters very often gave rise to S or R-3 types. It often developed as isolated colonies from several of the unstable R forms. Fig. 28, 29 and 30.

R-11 was unique in that it rarely developed in S cultures, but if it did, it always appeared as a very conspicuous smooth surfaced, pink

to gold sector. Fig. 9 and 10. Many of these sectors, if carefully picked, gave rise on first transfer to pure cultures of R-11. Occasionally platings were mixed with types S and R-7, but never with R-3. The young R-11 colony was so different from any of the other types that it first was thought to be contamination. It was the only R type which developed into a round completely smooth surfaced colony. However after several days incubation the colony became more flattened and developed a faint greenish tint. Fig. 10. Some R-11 colonies retained their circular form and entire margin while others developed into a more lobate form. Still others developed long gold colored filaments, R-13. Fig. 21, 22, 23 and 24. Another distinctive characteristic of the R-11 colony was its flattened appearance after several days incubation contrasted to its initial convex form. Colonies which retained their circular form also tended to retain the pink color which made this type easy to identify in old mixed cultures. Fig. 17 and 18.

Type R-14 was the only other stable R colony. Fig. 12. It never developed directly from S cultures, but was found only in platings of the gold colored long filamentous secondary growth or daughters, R-13, which developed on old R-7 and some old R-11 colonies. Fig. 8, 18 and 23. In young cultures R-14 and R-7 colonies were very similar and could very easily be confused. R-14 was a more intense pink than R-7 and some colonies appeared to be almost red. Compare Fig. 8 with Fig. 12. Twenty four hr colonies were approximately 1 to 2 mm or more in diameter whereas R-7 seldom measured more than 1mm at 24 hr. With continued incubation the development of R-14 was much more rapid than that of R-7 and some well isolated colonies reached a diameter of 20 to 30 mm. R-7 seldom attained a diameter of more than 20 mm. Compare Fig. 8 with Fig. 12. One

of the outstanding differences was in the cells of the two colonies. R-7 cells were usually long plump rods in pairs or short chains similar to those shown in Fig. 42, whereas cells of R-14 were usually curved and in long heavy filaments. Fig. 43.

R-16 was unique in that its development appeared to be enhanced in cultures exposed to 2, 3, 5- triphenyltetrazolium chloride. Its presence had been suspected in cultures not exposed to this salt but because of its close resemblance to R-3 it was difficult to establish it as a distinct type. Fig. 6, 27, 28, 29 and 30. However, on agar plates containing the tetrazolium salt it appeared as large, translucent, green daughter colonies while the parent colony, usually I-1, I-2, R-4 or R-5, or one of the unstable N-S colonies, appeared a bright pink to deep red in color. R-3 also failed to reduce the tetrazolium salt but the appearance of the R-3 daughters was quite distinctive from the R-16, being more even textured, more opaque, and did not have the glisten and sparkle of R-16. Fig. 15.

It appeared that the remaining 14 colonial forms consisted of either a highly unstable population which dissociated into two or more of the stable N-S (non smooth) forms, or that they were actually a mixture of several growth forms. Stained smears prepared from the unstable colonial forms revealed a wide variation in cellular morphology. The bacterial cells ranged from small coccoid forms to long filaments with many intermediate forms. No attempt was made to determine the ratio of the several components of the unstable cultures, but the fact that the ratio and the colonial type which resulted from subsequent platings could almost always be predicted suggested that the colonies contained a rather consistent proportion of the individual cell types. Unstable R-types never developed

directly from S colonies on agar plates. However they were found in old broth cultures of S colonies and in platings of N-S colonies.

S colonies seldom developed from platings of either the stable or unstable R cultures. Yet when R cultures maintained for long periods of time in tryptose broth containing 2, 3, 5- triphenyltetrazolium chloride, were streaked on agar plates only S colonies developed. The number of colonies which developed on these plates was always small. This suggested that in the concentration used, the tetrazolium salt exerted a toxic effect on N-S types after exposure of 10 days or more, but to a less degree in the S type.

The toxic effect of 2, 3, 5- triphenyltetrazolium on the R colony types was also evident on agar plates containing this salt. When transfers were attempted from colonies which had incubated for long periods of time, 20 days or more, the inoculated plates remained sterile or only very few colonies developed. Often a few S colonies were observed on these plates, again suggesting that all R colonies contained a few cells capable of producing S colonies. Both S and R colonies on agar plates without tetrazolium remained viable for long periods of time, up to two months or more. Growth failed to occur when transfers were made to fresh plates; only when the agar was desiccated to the point that it was hard and brittle; thus indicating that prolonged exposure to the tetrazolium salt exerted a lethal toxic effect on the R types. This effect was less marked in the S cultures, and growth occurred even after long exposure to the salt.

The variation in color of the mature colonies on agar containing tetrazolium salts indicated that there were differences in the reducing

enzyme systems of the various colony types. The red color which develops in living cells in the presence of tetrazolium salts is thought to depend on the presence of reducing enzymes (dehydrogenases with or without coenzymes I or II) which reduce the colorless compounds to colored formazans.

That all R colonies contained a few S type cells was again suggested by the lesions which developed in rabbits after subcutaneous inoculation of R cultures particularly those containing Wydase. At necropsy of many of these animals very small (1 mm) discrete grey foci of necrosis were observed in an area several cm in diameter surrounding the inoculation site. As large inocula of R cultures administered by various routes had shown no pathogenic effect it was felt that these isolated necrotic foci resulted from S type cells dispersed by the spreading action of Wydase. Rarely a small area of necrosis was found at the inoculation site in animals in which Wydase had not been added to the culture. When pure S cultures were inoculated by this route a large well localized necrotic lesion developed at the site of inoculation. When Wydase was added to S cultures large (5-10 mm) discrete necrotic foci were found in a wide area surrounding the inoculation site. These findings suggested that all R cultures contained a few S cells which in the proper environment could produce lesions.

Apparently the number of S cells in R cultures inoculated intravenously or instilled into the conjunctival sac was too small to produce evidence of infection. It has been found that fairly large inocula of S cultures must be used to produce infection under laboratory conditions, and the number of S cells in the R cultures may have been too small to

incite a clinical response.

Whether the monocytosis which developed in rabbits inoculated intravenously with R cultures resulted from the presence of S cells, or whether the R cultures also contain the monocyte producing factor (MPF) has not been determined. Grini (1943) found that a non pathogenic strain of L. monocytogenes produced a monocytosis when inoculated intravenously into rabbits, but unfortunately gave no description of the colony. Stanley (1949) showed and Girard and Murray (1951) confirmed that the MPF of L. monocytogenes was found in a non-antigenic lipid fraction of the bacterial cell, and was found to be liberated chiefly in the liver of infected animals. The R cultures may apparently also contain this lipid MPF but further work is necessary to confirm this.

None of the rabbits which received dense suspensions of either S or R cultures in the drinking water showed any clinical indication of illness during the two month observation period. The rabbits which received ensilage displayed marked emaciation due to their refusal to accept this ration. Laceration of the mouth made no contribution to susceptibility to infection. Neither did local lesions develop at the laceration sites. Cultures prepared from the feces as described by Gray et al. (1950) gave inconclusive results and it could not be determined whether the cultures added to the drinking water survived passage through the digestive tract or not. Further investigation revealed that the particular strain of culture (12255, isolated from the brain of a calf with listeric encephalitis complicated by vitamin E deficiency) was inhibited by the potassium tellurite in the concentration (0.05%) employed. This confirmed the findings of Olson et al. (1953) who found that certain stains of L. monocytogenes were sensitive to 0.05% potassium tellurite.

With the exception of mice (Julianelle, 1941) most investigators failed to infect artificially either ruminant or monogastric animals by the oral route. Roine et al. (1953) reported death of guinea pigs fed a ration containing aureomycin due to infection with L. monocytogenes. These authors expressed the opinion that aureomycin suppressed other bacterial inhabitants of the intestinal tract and favored the proliferation of L. monocytogenes and that infection was established as a result of this. For the present this must be viewed with reservations as, as indicated in part one of this thesis, L. monocytogenes has been shown to be highly susceptible the antibacterial action of aureomycin.

The results obtained in this study confirmed the inability of either S or R cultures of L. monocytogenes to infect by way of the intestinal tract even when the animals were subjected to emaciation and laceration.

Asahi and Hosoda (1952, 1953) suggested that L. monocytogenes may use the trigeminal nerve as an avenue of entrance in the encephalitic form of the disease. They succeeded in infecting both mice and young goats (2-4 mo old) by scarifying the mucous membrane of the mouth and the cutaneous tissue surrounding the mouth and nose before exposure to L. monocytogenes. Neither Hirato et al. (1954) nor the present study could confirm these findings.

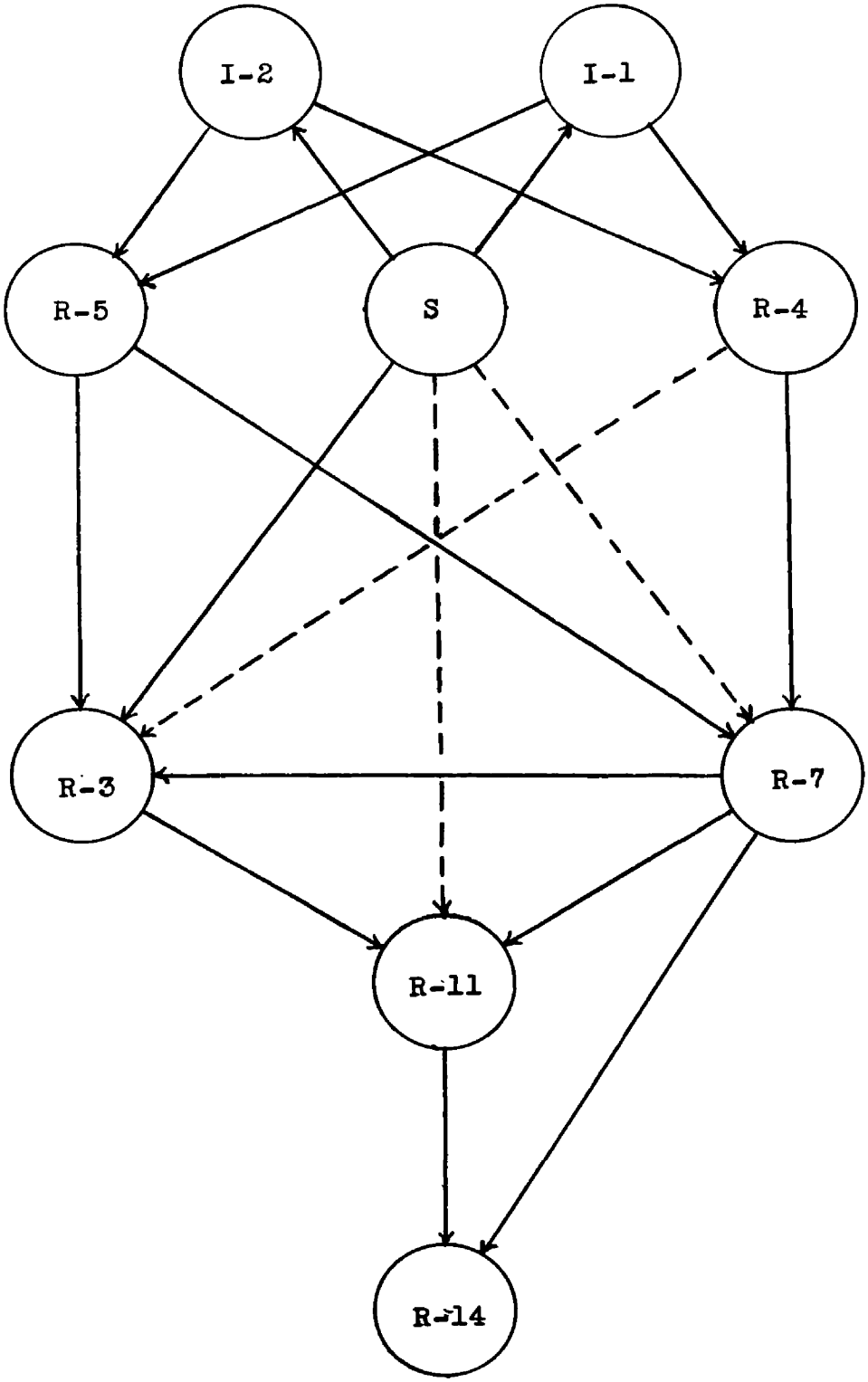
At the present very little is known concerning the nature of the unstable R cultures. Because they could not be maintained in pure form they were not used in pathogenicity studies or tested for biochemical reactions. However it did appear that they were composed of a definite ratio of the various cell types and on this basis can be considered as

distinct colony types; possibly as intermediates because of their rapid dissociation into the other stable R forms. As this study was directed primarily toward the detection and recognition of new colonial forms in the genus Listeria no intensive study has been made to date to further characterize these types. The numerous publications of Huddelson and Braun on dissociation studies in the genus Brucella emphatically emphasize that studies of this nature are not quickly exhausted. It is hoped that this present study is only the beginning of similar studies on the genus Listeria.

From the data now at hand, compiled from several hundred separate platings, a tentative dissociation scheme, shown on the following page, can be derived for the principal stable R types. It is almost certain that future work will show this scheme to be incomplete and many revisions may be necessary. In work of this nature where color values, size and texture of colonies play so important a part in differentiating one colony type from another, it is often difficult to free oneself from subjective prejudices which might lead to misinterpretation of the true picture. In spite of all possible precautions the present scheme may be colored by such misinterpretations and therefore should be considered as only tentative until it can be confirmed or revised by other investigators. It was felt that its inclusion would portray the dissociation pattern in a more graphic manner than an extended description.

In the 20 cultures observed in this study it was noted that several cultures dissociated more rapidly than did others. Certain cultures also tended to produce a predominance of a particular R type while others showed a predominance of another type. Still other cultures displayed almost no tendency toward dissociation whereas some dissociated so rapidly

Fig. 44. Dissociation scheme showing origin of the principal stable R cultures. I-1, I-2, R-4 and R-5 are not stable but are included as most R forms developed from them. R-3 was the only stable R type to commonly develop directly from S cultures. Broken lines indicate less common development. R-14 actually develops from filaments (R-13 on R-7 and some R-11 colonies. R-16 is not shown as it rarely developed in the absence of 2, 3, 5- triphenyltetrazolium chloride.


I-2
I-1
R-5
S
R-4
R-3
R-7
R-11
R-14

that it was difficult to record its direction accurately. The scheme shown on page 106 represents the main trend shown by all the cultures as a group. No doubt certain strains, if taken individually, would present a different picture and certain of the R type colonies would never appear.

At this time the significance of the wide variation in rate of dissociation of the various cultures cqn only be conjectured. Whether cultures isolated from particular disease processes showed a distinctive dissociation pattern has not yet been accurately determined to date. Nevertheless there were strong indications that this might be true. In general it appeared that three strains (1007X, 1192X, 1197X) isolated from the liver of calves which died shortly after birth (Gray et al., 1954) and two strains (455X, 967X) isolated from the spinal fluid of man (both unreported) showed a far more rapid dissociation than did any of the other types. Also these particular strains produced many more R-3, R-7, R-8 and R-9 colonies than did any of the other cultures. Cultures isolated from the brain of sheep and cows with listeriosis showed a rather average consistent pattern and rate of dissociation. However, a few cultures showed almost no dissociation other than the development of small I-1 and I-2 daughter colonies. Cultures freshly isolated from the viscera of artificially infected rabbits (not included in the original 20 cultures) developed a predominance of type R-3 daughters, and I-1 and I-2 types were rarely seen in these cultures. Some of these cultures never gave rise to other than R-3 colonies while others produced a variety of the several types.

Two cultures of Listeria infantiseptica (new species questioned) received from Potel and isolated from aborted infants in the German

Democratic Republic, and a similar culture received from Seeliger, Hygiene Institute, Friedrich-Wilhelms University, Bonn, Germany, showed a wide variety of R forms on initial platings similar to those seen in the cultures isolated from calves. These cultures were received too late for extensive observations to be included in this report. Moreover it was found that these cultures were not agglutinated by rabbit antisera prepared against a strain of L. monocytogenes isolated from a bovine brain. Whether there is a connection between the difference in dissociation and the failure to agglutinate can not be stated at this time, but it does present an interesting subject for further study.

A strain of L. monocytogenes isolated from the intestinal tract of a guinea pig by Roine et al. (1953) in Finland showed a wide variety of R forms on initial platings and is presently under further study. As it was found that all pure R forms were non pathogenic for the rabbit there is a suggestion that these cultures which show a wide variety of R forms may be less pathogenic than those which show only S forms on initial isolation as do the strains isolated from sheep and cows with listeric encephalitis. There is of course the possibility that dissociation of these cultures occurred during transit. However, many of the stock cultures of L. monocytogenes maintained in this laboratory for long periods of time without transfer fail to show the rapid and marked changes as these cultures from less "typical" disease processes. ("Typical" is used here on the questionable assumption that encephalitis of ruminants is the most characteristic and distinctive form of infection due to this bacterium). Sohier et al. (1948) report the isolation of a non pathogenic strain of L. monocytogenes from cooked beef. As non pathogenic strains of this microbe are rather rare, it would prove to be an interesting

strain for further study. Its arrival is anticipated shortly. The five strains isolated by Wenckebach (1953) from the urethral exudate of five men with gonorrhea should also prove interesting. At the present state of our knowledge it seems more than coincidence that strains which dissociate slowly are associated with acute disease processes and the more rapidly dissociating strains are associated with a more chronic type of disease. A striking exception to this generalization is the strain recently isolated by Gray and McWade (1954) from the cervix of a so-called repeat breeder cow which showed no clinical indications of infection. This culture has shown very little tendency toward dissociation. Therefore it appears that many more comparisons will be required before a definite trend can be established.

The role of S and R cultures in the epidemiology of various bacterial diseases has been the subject of numerous investigations. These have been summarized by Hadley (1927) and Braun (1947). The relationship between dissociative changes, accompanied by changes in virulence, to epidemic waves is still a matter of speculation. Braun (1947) cites several instances in which S cultures of various bacterial species were isolated in the acute stages of a disease and that as the disease becomes more chronic, the isolated organisms showed a marked tendency toward R forms. Also cited was an epidemic of Shigella dysentery in which S forms were isolated in the early stages of the epidemic, SR forms as the epidemic, waned and R forms towards the end of the epidemic. Cultures from chronic carriers of typhoid bacilli and Erysipelothrix rhusiopathiae have yielded R forms. It is contended that these changes in the bacterial population are intimately associated with changes in the selectivity of the host environment. Thus normal serum appears to contain factors which suppress

the establishment of R forms and favors the development of S forms. But as antibodies (agglutinins) are produced against the S form, it is suppressed and an environment is established which favors the development of R forms. When the R type has been established in disease carriers, there follows a decrease in S antibodies which may alter the environment by permitting the natural anti R factors to exert their effect on the R form, and a condition favorable to the reestablishment of S forms results.

Fibrinous encapsulations, as found in many bacterial diseases, may also serve to remove the S forms from the total body environment. If in these encapsulations there is an S to R transformation, when the R forms are released into the body, conditions would favor the establishment of S forms. This possibility presents intriguing implications for a disease, such as that produced by L. monocytogenes where the lesions are almost always localized and conceivably could and would be encapsulated if the disease were less acute. Sedallian et al. (1948) reported the isolation of what they considered an R form of L. monocytogenes from a localized lung lesion in a 45 year old man. The authors do not describe the colony but stated that stained smears showed large rods to filaments which were gram negative, motile and required glucose for growth. It failed to kill rabbits and to produce a conjunctivitis. However it did kill mice and guinea pigs which showed a monocytosis and focal hepatic necrosis at necropsy. Helmboldt et al. (1951) reported the isolation of L. monocytogenes from a localized abcess in the brain of a pig which died after showing symptoms of a nervous disorder. Lesions associated with listeric encephalitis were not observed. Unfortunately no mention was made of colonial characteristics of this culture. Old localized fibrotic lesions have often been observed in various viscera, particularly

in the uterus and heart of rabbits that survived intravenous exposure. On culture these lesions are often sterile. In a few instances S-like cultures of L. monocytogenes have been isolated. At no time have any of the ^{R} type cultures identified in this study been isolated.

A notable example of a localized lesion due to L. monocytogenes is the encapsulated necrotic focus which develops at the site of subcutaneous inoculation of cultures from S colonies. This lesion presents interesting possibilities for S to R or R to S studies. At the present time this has been subjected to only a few exploratory observations, and insufficient data have been accumulated to draw positive conclusions. From some of these lesions which develop from S cultures it was possible to reisolate what appeared to be S cultures. However, after 24 hr incubation these colonies, especially those well separated, appeared to be larger and have a more wrinkled surface than did the original S colonies after the same incubation period. Fig. 33. After 48 hr incubation they were indistinguishable from the original S culture. At this time it can not be definitely stated whether these colonies represent the so-called "reverted S" of Hadley (1937), Spicer (1936) and Mellon et al. (1944). "Reverted S" is a term applied to S-like colonies which arise from R cultures and appear to be an R to S reaction but actually is an S to R to S^{1}, S^{1} being the reverted S. The reverted S of Spicer (1936) and Mellon et al. (1944) showed distinct differences from the normal S form. Braun (1946) in in vitro studies with Brucella abortus found only an increased growth rate for reverted S forms, all other characteristics were identical to the normal S. This again presents the question of whether there is true R to S reversion or whether it is only "apparent reversion". Huddelson et al. (1952) failed to find evidence of R to S reversion either in in vitro or in vivo studies with Brucella. Braun

(1946a) claims true reversion is possible providing the environment is such that S forms which develop in R cultures are able to manifest themselves. This is seldom possible under *in vitro* condions but may occur *in vivo*.

In this study there was no evidence that true R to S reversion occurred either *in vitro* or *in vivo*. S forms which developed from R cultures maintained for long periods of time in tryptose broth containing 2, 3, 5-triphenyltetrazolium chloride or from the small localized necrotic lesions that developed in rabbits after subcutaneous inoculation of R cultures to which Wydase had been added, were presumed to be the result of S type cells in the R cultures or reverted S types. Webb and Barber (1937) reported that platings of ^{R} colonies of *L. monocytogenes* resulted each time in approximately equal numbers of both S and R forms, indicating that they may actually have had an I form rather than a true R. Seeliger and Linzenmeier (1953) failed to find evidence of S forms in R cultures even after 18 mo observation. Hirato *et al.* (1954) did not mention R to S reversion. However, from these meagre data it appears that there is no true R to S reversion in cultures of *L. monocytogenes*.

From the photographs of R colonies of *L. monocytogenes* published by Webb and Barber (1937), Barber (1939), Seeliger and Linzenmeier (1953) and Hirato *et al.* (1954), one can only speculate as to which particular colony type they were dealing. None of the photographs appear to have been made with obliquely reflected light. The R colonies described by Webb and Barber (1937) no doubt were type R-4. This would explain the almost equal numbers of S and R forms which developed from successive platings. Other R forms which may have developed perhaps escaped detection due to the fact that oblique lighting was not employed. Only Barber

(1939) mentions the occurrence of intermediate forms. The one pictured appeared to be an R-4 type. The R colonies shown may have been R-3. This was suggested by their irregular margins and long slender filamentous cells. The cells of the intermediate type were a mixture of rods and filaments. In this respect their findings were similar to those reported here.

The colonies shown by Hirato et al. (1954) appear to be R-3. The photomicrograph tends to confirm this. The colonies shown by Seeliger and Linzenmeier (1954) were interesting because they are the only ones to show evidence of sector formation. Other than that it would be difficult to determine with which specific R form they were working as their cultures were grown on blood agar.

Paterson (1940), in studies of the pathogenicity of L. monocytogenes for the embryonating chicken, did not describe the R forms with which he worked. Neither are there suggestions as to which possible R forms the various French workers had encountered. (Verge and Goret, 1941; Belin, 1946; Harvier et al., 1943; Sedallian et al., 1948).

It is remarkable that of the several doctoral theses (Zeller, 1949; Boekels, 1950; Schulz, 1950; Ozgen, 1951; Beinhauer, 1953) presented at the Justus Liebig Veterinary High School, Giessen, Germany, none of the authors mentioned the occurrence of R forms; in spite of extensive studies on cultural characteristics including colonial morphology. Only Zeller (1949) mentions the presence of halos on some colonies after several days incubation. The failure to recognize R forms must have resulted from failure to employ obliquely reflected light.

There is a need for a more effective vaccine against listeric

encephalitis than is presently available. The use of non-virulent variants of several bacterial species as immunizing agents is well established. The most notable type perhaps is the M form of Brucella abortus (Huddelson, 1946). However in the genus Listeria it has been found that agglutinating antibodies are not a measure of protection against infection. Julianelle (1941) on studies in mice was the first to suggest this. Hunter et al. (1950) found that mice immunized with variants of L. monocytogenes produced by exposure to radioactive phosphorus, gave no protection when the mice were challenged with a lethal dose of culture six weeks later. It has been found in unpublished studies that rabbits immunized with S cultures of L. monocytogenes and which showed high agglutinating antibody titers, (1:1280 or more) against S cultures were not always protected from a lethal exposure to the homologous culture. This was again confirmed in the attempts to immunize rabbits with R cultures as reported here. It is obvious that some other means to measure immunity must be devised for this bacterium. Various investigators (Belin, 1946; Graham et al., 1943; Kaboyashi, 1951; Zink et al., ; Potel, 1954; Ehrenhardt, 1954) found that many animal species show high agglutinating titers against L. monocytogenes. Hirato et al. (1954) reported wide fluctuations in agglutinating titers of individual sheep during an outbreak of listeriosis. Until the significance of these titers is determined, they serve no purpose as an indication of either previous exposure or potential resistance.

Hunter et al. (1950) and Seeliger and Linzenmeier (1953) are the only investigators to mention agglutination studies with R cultures. Two of the 19 variants isolated by Hunter et al. (1950) agglutinated spontaneously in saline when agglutination reactions were attempted. No mention was made of autoagglutination in the remaining 17 variants

studied. Seeliger and Linzenmeier (1953) found that R cultures showed a strong tendency toward autoagglutination. Only by the employment of ultrasonics were they able to prepare stable suspensions of R cultures. By this method they were able to demonstrate specific agglutination of R cultures. Autoagglutination did not present a particular problem in this study. Its occurrence was inconsistent and at times invalidated apparent results. Boeckels (1950 suggested that if a 0.2% saline solution were employed for agglutination reactions with L. monocytogenes more consistent results could be obtained than with 0.85% saline. This has not been used with R cultures but may prove useful in the future.

This report established that in the genus Listeria at least 20 distinct colonial forms occur, six of which can be maintained in stable form. Of these 20 forms, one is smooth and the others rough. Whether any of the R forms occur in nature either within or outside the animal body, or occur as common but unrecognized soil organisms or saprophytes remains to be determined. However with the detection and recognition of these 20 types attempts can be initiated to determine the natural reservoir of this microorganism.

SUMMARY

Twenty different colonial forms of Listeria monocytogenes could be detected by means of obliquely reflected light and a binocular scanning microscope. One of these forms was the naturally occurring smooth form found in isolations from infected human or animal tissue. This type was characterized by a small circular, slightly raised, finely textured, translucent, blue-green colony. This gave rise either directly or indirectly to the remaining 19 types designated as rough. They were characterized

by a variety of colonial forms which differed in texture, configuration, or color from the smooth type. Only the smooth form was pathogenic for rabbits when inoculated intravenously or instilled into the eye. Six of the 19 rough forms could be maintained in stable form. There were no indications that rough types reverted to smooth. On the basis of fermentative and biochemical reactions the rough types were indistinguishable from the parent smooth type, but did differ in antigenic structure. Certain rough forms which produced high agglutinating antibody titers against the smooth form failed to protect rabbits against a subsequent lethal dose of smooth culture. It was suggested that antibodies found in the blood of many animals which agglutinated the smooth type culture were neither an indication of exposure nor of immunity. Because of the ubiquitous nature of listeriosis in animals and man, there is a possibility that some of these rough forms may eventually be found in either carrier animals or substances such as soil or water.

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Listeria monocytogenes: A Review and Dissociation Studies

An Abstract

The thesis is divided into two parts.

Part one constitutes a review of the world's literature with emphasis on the various disease processes with which the bacterium *Listeria monocytogenes*, has been associated. Encephalitis in sheep, cows and goats was the most prevalent form. Then followed septicemia with meningitis in man; septicemia with or without meningitis in monogastric animals, especially rodents; abortion in man; abortion in animals and conjunctivitis in man. The accumulated reports failed to reveal clear cut epidemiological trends and the specific mode of transmission was obscure. The blood of many normal animals was found to contain high agglutinating antibody titers for *L. monocytogenes*. In no instance was the bacterium found outside the animal body yet the wide distribution and multiplicity of disease processes with which it has been associated suggests that the bacterium is actually widely distributed in nature, perhaps in an unrecognized form.

Part two is a study devoted to the recognition and isolation of "rough" colonial variants which develop in "smooth" cultures maintained on artificial media under laboratory conditions. By means of obliquely reflected light and a binocular scanning microscope 20 different colonial forms of *L. monocytogenes* could be detected. One of these forms was the naturally occuring smooth form found in isolations from infected human or animal tissue. This was characterized by a small circular, slightly raised, finely textured, translucent, blue-green colony which gave rise either directly or indirectly to the remaining 19 rough types. These were

characterized by a variety of colonial forms which differed in texture, configuration, or color from the smooth type. Only the smooth form was pathogenic for rabbits. Six of the 19 rough forms could be maintained in pure form. Rough types were indistinguishable from the parent smooth type in fermentative and biochemical reactions. Certain rough forms which produced high agglutinating antibody titers against the smooth form failed to protect rabbits against a subsequent lethal dose of smooth culture, suggesting that antibodies found in the blood of many animals which agglutinated smooth type cultures were neither an indication of exposure nor immunity. Because of the ubiquitous nature of listeriosis in animals and man, there is a possibility that some rough forms may eventually be found in either carrier animals or substances such as soil and water.

Approved
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