INFLUENCE OF OSMOTIC PRESSURE ON THE MORPHOLOGY OF THE REITER TREPONEME

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ABSTRACT

HARDY, PAUL H., JR. (Johns Hopkins University, Baltimore, Md.) AND E. ELLEN NELL. Influence of osmotic pressure on the morphology of the Reiter treponeme. J. Bacteriol. **82:** 967–978. 1961.—Spherical bodies similar to those that develop spontaneously in cultures of treponemes, and which have been considered by many investigators to represent one stage in a complex life cycle of these organisms, can be produced rapidly with the Reiter treponeme by merely altering the medium in which the organisms are suspended. Osmotic pressure appears to be the major factor responsible for this effect, as shown by the observation that treponemes suspended in NaCl solutions of 0.15 to 0.10 M retain their spirochetal morphology, whereas organisms suspended in more dilute salt solutions rapidly become spherical. Moreover, the concentration of salt appears to influence both the rate and extent of sphere formation. Further evidence that osmotic pressure is primarily involved is demonstrated by the selectivity of the conditions under which spheres form. Treponemes suspended in various 0.3 osmolal solutions either retain their spiral shape or form spheres, depending upon the nature of the solute. Viability studies of suspensions containing predominantly spherical forms, which have developed spontaneously or have been artificially induced, have failed to produce evidence that the resulting growth of treponemes came from the spheres. It is concluded, therefore, that the naturally occurring spheres probably arise as the result of an osmotic imbalance which develops between the cells and their environment, and that the spheres represent degenerative forms rather than an intermediate stage in a life cycle.

Since the earliest descriptions of spirochetes, especially those now classified as *Treponemataceae*, investigators have regularly observed in cultures, and occasionally in infected tissues, the presence of spherical bodies associated with the typically spiral organisms. These round bodies, which may be either free or attached to some portion of a spirochete, vary tremendously in size, with diameters ranging from slightly more than the width of a spirochete to greater than 10μ. Their origin from the spirochetes themselves has been repeatedly demonstrated by cultural methods and it is well recognized that they usually develop as a result of aging of the culture, or growth under adverse environmental conditions. Considerable interest and speculation have been stimulated through the years concerning the nature of these round bodies, but to date their exact relation to the treponeme has still to be satisfactorily demonstrated. Some investigators have maintained that they are degenerative or involution forms of treponemes, but only Rose and Morton (1952) have presented experimental evidence to support such a hypothesis. In contrast to this, many workers have held to the theory that the spheres are cysts, germinating units, or some similar form of reproductive bodies. Numerous observations have been reported in the literature to support such a belief, most of which have been thoroughly reviewed by Ingraham (1932) and by Campbell and Rosahn (1950). However, none, including the more recent papers of Hampp (1946, 1950, 1951), Hampp, Scott, and Wycoff (1948), Gelperin (1949), and DeLamater, Haanes, and Wiggall (1950, 1951*a*, *b*), has presented completely convincing evidence that such is the case.

Although it is now generally accepted that treponemes under in vitro cultivation multiply primarily by transverse division, the possibility of a more complex reproduction still exists. Such a life cycle hypothesis is important in the study of spirochetes, and proof of its presence or absence would be of marked significance to the

proper phylogenetic classification of these organisms. At the present time, spirochetes are classified among the schizomycetes, where they comprise a separate order (Breed, Murray, and Smith, 1957), but their position in this classification scheme is not a firm one and is subject to change upon the advent of additional basic knowledge concerning their structure and growth conditions.

In addition to the biological significance of the life-cycle hypothesis, it is also important from the clinical point of view, for the existence of the causative agent of syphilis in a nonspirochetal form has long been used to explain latency and the infectiousness of tissues devoid of demonstrable treponemes (Levaditi and Vaisman, 1938, 1951; Wile, 1947; Gueft and Rosahn, 1948). Since the presence of spheres in treponeme cultures and infected tissues has represented the main support for the life-cycle theory, it would be of considerable interest to acquire further knowledge as to the nature of these bodies and the conditions responsible for their formation.

In the course of studies undertaken to devise methods of extracting soluble antigenic material from Reiter treponemes, it was observed that some suspensions in weak salt solutions rapidly decreased in turbidity. Microscopic examination revealed that most of the organisms no longer possessed their spiral configuration, but had assumed a spherical shape instead. Moreover, the spheres so produced were morphologically similar to those that develop spontaneously in cultures. Therefore, the conditions responsible for this rapid change were investigated. The results, presented in this paper, indicate that spirochetal spheres arise when osmotic imbalances develop between the organisms and their environment, and that they are, most probably, degenerative forms rather than part of a life cycle.

MATERIALS AND METHODS

The Reiter treponeme used in these studies was originally obtained from G. D'Alessandro of Palermo, Italy. It is reputed to be a strain of *Treponema pallidum*, one of a number of treponeme strains isolated from syphilitic lesions and adapted to in vitro cultivation by H. Reiter about 1923 (Reiter, 1960). Although this organism is now avirulent, Mulzer and Nothhaus (1928) reported as proof that it was a strain of *T. pallidum* the production of typical syphilitic-orchitis in rabbits after approximately 200 subcultures in vitro.

Cultivation was carried out at 35 C in USP alternate thioglycollate broth fortified with 10% normal calf serum. Diffuse growth was obtained by continuous agitation, either in screw-cap test tubes containing a small glass bead and rotated on a tissue culture drum modified to turn 7 rev/min, or in stoppered Florence flasks suspended above a magnetic mixer. In initial attempts to use the latter method of mixing, it was found that heat generated by the mixer, especially when run at slow speeds, was sufficient to raise the temperature of the culture above that of the incubator. This difficulty was overcome by positioning the culture flask so as to leave an air space of approximately ½ in. between it and the top of the mixer, and by regulating the mixer speed with an external transformer instead of the enclosed rheostat control.

For most experiments, 3- (or occasionally 4-) day cultures were employed, which were just beyond the logarithmic phase of growth. In experiments where it was of interest to investigate conditions in the stationary phase, 7- to 14-day cultures were used.

Direct counts of treponemes were performed under darkfield microscopy, either in a Petroff-Hausser counting chamber, or by the method of Turner and Hollander (1957), which utilizes a volume of 0.005 ml under a 22 mm^2 coverslip.

Osmotic pressures of solutions were determined by multiplying the molal concentration of solute by the number of its dissociated ions. Although this method is obviously less accurate than determinations obtained by means of colligative properties, it was considered satisfactory for the purposes of the present study.

RESULTS

Initial investigations revealed that Reiter treponemes suspended in physiological NaCl solution retained their spiral shape, whereas organisms suspended in distilled water rapidly became spherical. The spheres so formed resembled closely those that develop naturally in aging cultures of this organism, as can be seen in Fig. 1 to 4. The protective effect of salt upon the retention of spirochete morphology was studied more carefully with aliquots of a culture in which the sedimented organisms were resuspended in

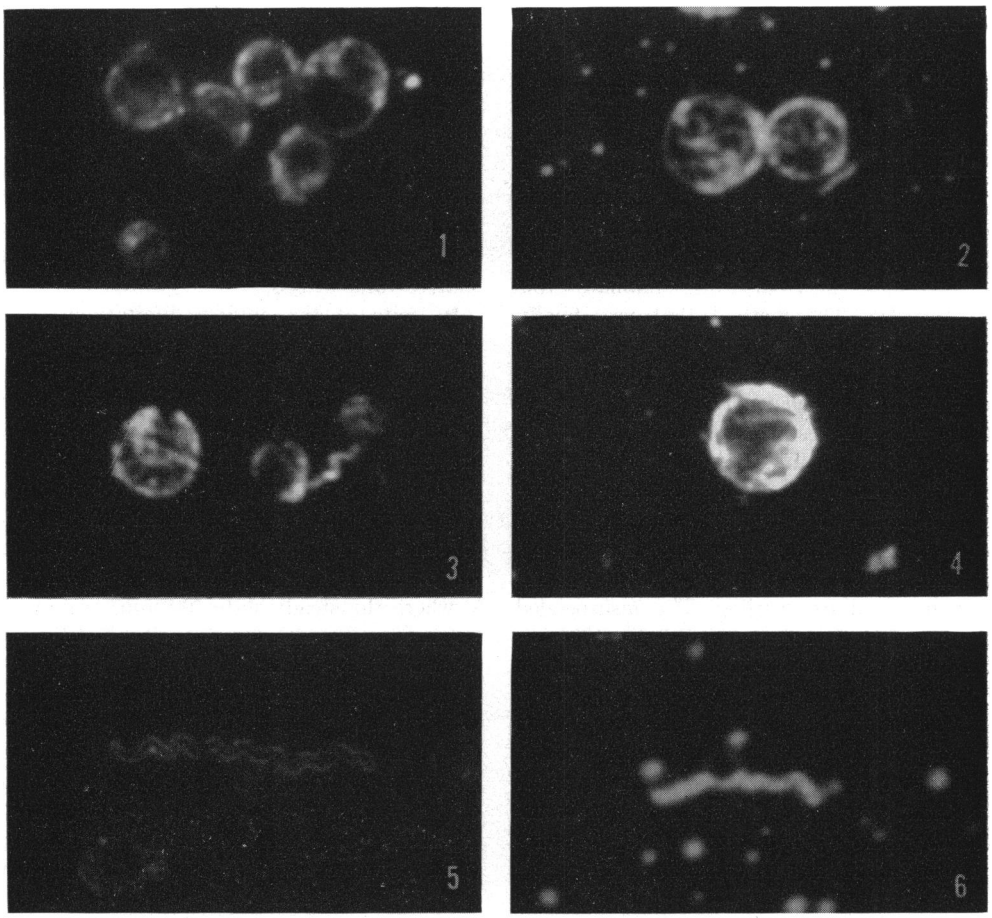

FIG. 1-2. *Darkfield photomicrographs of spheres produced from young Reiter treponemes by suspension in 0.01* M *phosphate buffer, pH 7.6 (1980* ×).

FIG. 3-4. *Darkfield photomicrographs of spheres that developed spontaneously in an aged culture of treponemes. Figure 3 also shows partial retention of spiral configuration by a treponeme; this has been interpreted by others as emergence of a young organism from cyst (1980* ×).

FIG. 5. *Darkfield photomicrograph of a doubly refractile treponeme, suggestive of longitudinal separation as the first step in sphere formation. This organism was in same preparation as Fig. 1 (1980* ×).

FIG. 6. *Darkfield photomicrograph of treponeme showing localized herniation produced under same conditions as for spheres in Figs. 1-2 (1980* ×).

NaCl solutions of graduated concentrations, ranging from 0.015 to 0.15 M. Microscopic examination revealed spheres in all suspensions where the NaCl concentration was less than 0.1 M, with the proportion of treponemes converted to spheres increasing as the salt concentration decreased. Further investigation demonstrated that the amount of salt in the suspending medium also influenced the rate of sphere formation. This, too, increased somewhat as the salt concentration was lowered. This latter observation made it apparent that microscopic examination was unsatisfactory as a means of following the reaction quantitatively because of the time required for the preparation and examination of slides. Therefore, the drop in optical density (OD) that accompanied sphere formation was investigated as a method to quantitate the reaction, but, before reproducible results could be obtained, it was necessary to control several other factors that were found to influence the reaction. Aeration, as occurred in the process of washing

treponemes free of culture medium or in the repeated transfer of suspensions to and from cuvettes, was found to induce sphere formation, even with organisms suspended in 0.15 M NaCl; this necessitated the elimination of such manipulations. The suspension of treponemes, even in a concentrated form, in physiological salt solutions prior to resuspension in other solutions altered their reaction to the second environment, and it was, therefore, necessary to eliminate this procedure also. The method that was finally employed is described below.

Samples (5 ml) of a broth culture of treponemes were transferred by means of volumetric pipettes to 15 by 125 mm test tubes and centrifuged at 1000 × *g* for 30 min. The supernatants were carefully decanted from the sedimented organisms, and the lips of the tubes were wiped free of residual medium. The cells were immediately resuspended in exactly 8 ml of test solution and evenly dispersed by twirling. The resuspended cells were then transferred to 13 by 100 mm round cuvettes, which were closed with rubber stoppers that permitted only a small air space to remain at the top of the tubes. The suspensions were kept undisturbed thereafter except when readings were made over a prolonged period of time; then they were occasionally gently inverted to insure even dispersion. OD measurements were made in a Bausch and Lomb Spectronic 20 colorimeter at a wave length of 540 mμ, and the actual readings obtained were converted to relative OD readings to overcome the slight daily variations that occurred as the result of small differences in the total number of organisms present from one culture to another. In every experiment a suspension of treponemes in 0.15 M NaCl, pH 7, was included as a reference, and the OD reading of this was arbitrarily given the relative value of 100.

In spite of the obvious limitations of this procedure, it gave satisfactory results, as can be seen in Fig. 7 and 8, where spectrophotometric readings are recorded that substantiate the microscopic observations noted above. Figure 7 shows that the rate of sphere formation, as determined by the time required for the development of a steady OD reading, is a function of the salt concentration of the suspension; this is also true for the extent of sphere formation shown in Fig. 8, where the steady-state (90 min) readings for treponeme suspensions in varying NaCl concentrations are recorded.

Effect of pH. In studies on the influence of various carbohydrates upon the growth of treponemes, Akatsu (1917) found that *T. microdentium* underwent early degenerative changes in the form of spherical bodies attached laterally to the organisms, and concomitantly produced acid when grown in the presence of glucose, but not in

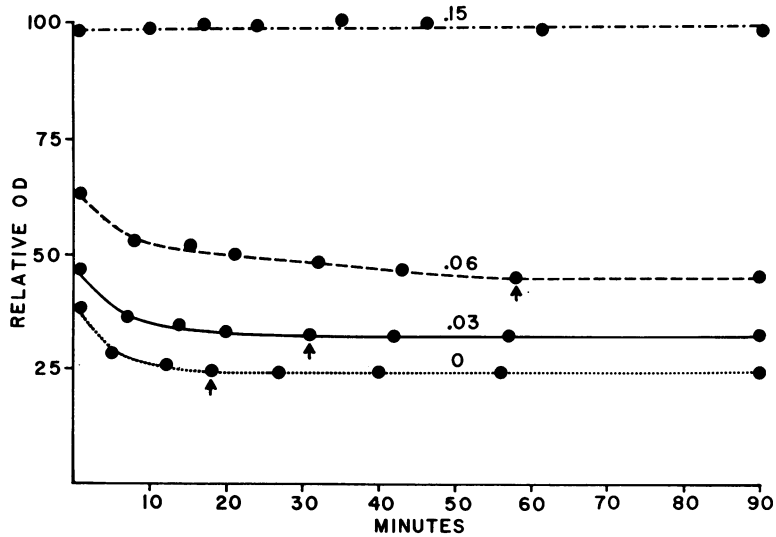

FIG. 7. *Rate of sphere formation, as measured by decrease in OD, of treponemes suspended in NaCl solutions of indicated molarity. Arrows indicate times at which steady OD readings were obtained.*

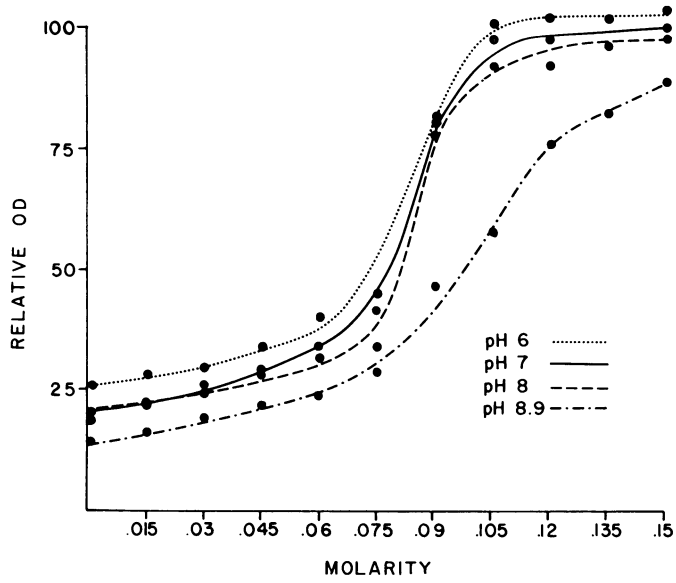

FIG. 8. *Extent of sphere formation of treponemes in NaCl solutions of different molarities and pH. OD readings were made after 90 min incubation at room temperature.*

its absence. Rose and Morton (1952) also noted an association between spontaneous sphere formation and pH drop in the culture medium. It was of interest, therefore, to determine whether the pH of the suspending fluid exerted an influence upon the artificial formation of spheres. This was done by resuspending treponemes in graded salt solutions as before, but buffered at different pH levels. For pH 6, 7, and 8, phosphate buffers at a final concentration of 0.01 M were used, and for pH 8.9, borate buffer of the same strength was employed. The results, which are also recorded in Fig. 8, show that at pH 6, 7, and 8 the suspensions reacted in essentially the same manner, whereas at pH 8.9 spheres were formed at much higher salt concentrations. However, in other experiments where a universal buffer (Veronal-acetate) was employed at all pH levels, the suspensions at pH 9 reacted like those at lower pH's. From this, it was concluded that pH, in the ranges studied, had little effect upon sphere formation, and that the results recorded in Fig. 8 for pH 8.9 were probably due to some effect of borate upon the organisms, rather than pH.

Effect of temperature. Cultures accidentally subjected to excessive heat during incubation were observed to consist almost entirely of spherical forms and this led to an investigation of the effect of temperature upon sphere formation. It was found that treponemes exposed to temperatures of 40 C and above were rapidly converted to spheres, not only in physiological salt solutions, but also in the broth medium used for cultivation. Moreover, as shown in Fig. 9, the rate at which spheres formed was directly related to the temperature to which the organisms were exposed. Although the temperatures studied here were lethal for the treponemes, and therefore higher than used for cultivation of these organisms, the results suggest that incubation of cultures at temperatures above the optimal for growth may make the organisms more prone to spontaneous sphere formation.

Relation of osmotic pressure to sphere formation. When the behavior of treponemes in solutions of salts other than NaCl was investigated, the concentrations at which spheres began to form followed more closely the osmolality than the molarity of the different solutions. This indication that the osmotic pressure of the suspending medium might be a major factor in the formation of spheres suggested that these organisms must be unusually susceptible to environmental changes; although many bacteria may swell somewhat when placed in water or weak salt solutions, they do not usually undergo profound morphological changes. Such hypersusceptibility to osmotic changes would imply that the external surface of

treponemes must lack the rigidity usually associated with a bacterial cell wall and possess, instead, the physical properties of a cell membrane. Treponemes should, therefore, respond to environmental changes in a manner somewhat similar to that of protoplasts; these, as shown by Mitchell and Moyle (1956*a*,*b*, 1959) have selective permeability characteristics that make their response to osmotic pressure changes dependent not only on the concentration of solute in the suspending medium, but also on the nature of the solute or solutes, since those compounds that readily cross the cell membrane exert no pressure effect.

To determine whether the formation of spheres was, in fact, the result of osmotic pressure changes, the behavior of treponemes suspended in equal osmolal concentrations of a number of different compounds was investigated. A solute concentration of 0.3 osmolality was employed, as this was well above the threshold level of sphere formation with NaCl. Representative results are shown in Fig. 10, where it can be seen that the reaction of the treponemes varied greatly in different solutions. Organisms suspended in NaCl, or in lactose, maintained a steady OD and retained their spiral shape, as found by microscopic examination, throughout the period of observation. On the other hand, treponemes suspended in other solutions were converted to spheres, but at different rates depending upon the solute. These varied from almost instantaneous conversion in glycerol to very slow conversion over a period of several hours in sucrose. The results obtained with all compounds tested are recorded in Table 1, which shows that spheres were not formed in any of the salt or disaccharide solutions, except sucrose. They were, however, formed in all of the other sugar and in the two amino acid solutions tested, with the rate of formation in the sugars being inversely related to their molecular size.

These findings demonstrated the selective permeability of the treponeme surface and confirmed the impression that sphere formation was the result of an osmotic imbalance between the internal and external environment of the organism.

Bacterial cells usually possess a greater internal osmotic pressure than their environment, and the phenomenon of plasmoptysis, as sphere formation appears to be, occurs only when the pressure difference, i.e., turgor pressure, is abnormally great. Since turgor pressure varies not only with environmental changes but also with the physiological state of the organisms, and is greatest during the logarithmic phase of growth (Knaysi, 1951), the possibility arose that the young treponemes used for these studies might possess a greater internal osmotic pressure than older cells and were, therefore, more susceptible to environmental changes. This was investigated by de-

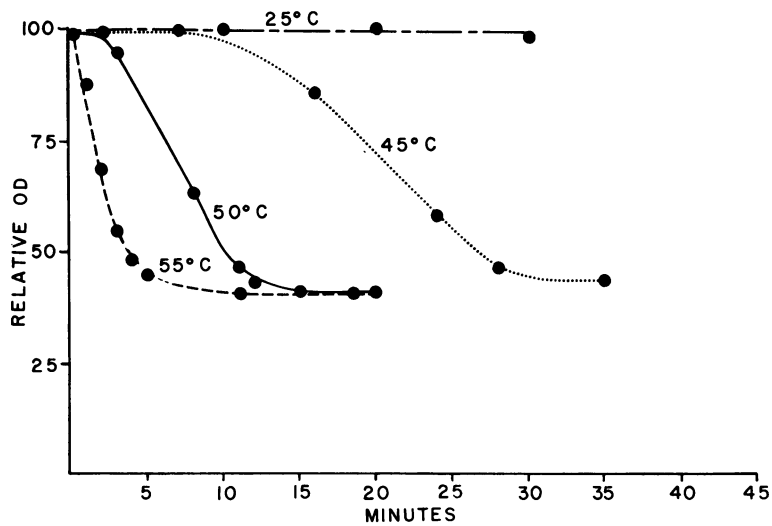

FIG. 9. *Rate of sphere formation of treponemes suspended in 0.15* M *NaCl and incubated at various temperatures.*

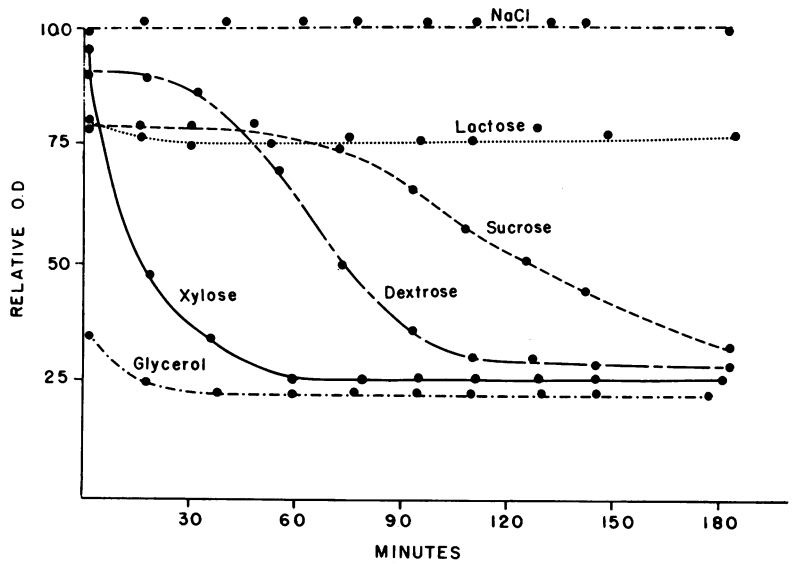

FIG. 10. *Rate and extent of sphere formation of treponemes suspended in various 0.3 osmolal solutions. Variations in initial OD readings of suspensions in lactose, sucrose, dextrose, and xylose are due to differences in refractive indices as compared with NaCl.*

TABLE 1. *Ability of 0.3 osmolal solutions to form spheres from Reiter treponemes*

Spheres not formed*	Spheres formed—50% time†	
NaCl	Sucrose	102
KCl	Mannose	77
NH_4Cl	Fructose	75
NaCNS	Dextrose	65
$MgCl_2$	Galactose	65
Na_2SO_4	Arabinose	22
$(NH_4)_2SO_4$	Xylose	12
Na citrate	Glycerol	<1
Na acetate	Glycine	<1
Lactose	Alanine	<1
Maltose		
Cellobiose		

* Observed for 180 min.

† Calculated time, in min, for 50% of treponemes to form spheres.

termination of the response to various salt concentrations of treponemes from 7- to 14-day cultures. They were found to react in essentially the same manner as the younger organisms, and the conclusion was drawn that under the conditions of these studies age did not influence susceptibility to sphere formation.

In addition to the above investigations, the reaction of treponemes to hypotonic solutions of a variety of salts was determined, and it was found that spheres developed under essentially the same conditions as in NaCl, with the exception of $MgCl_2$. Treponemes in the presence of this salt, at concentrations as low as 0.01 M, retained their spiral shape. This apparent exception to the formation of spheres by decreased osmotic pressure may possibly be due to a direct action of magnesium ions upon the cell membrane, as will be discussed later.

Mechanism involved in sphere formation. With the recognition that sphere formation was the result of osmotic imbalance, it was of interest to investigate the nature of the reaction. This was done by direct observation of wet preparations under the darkfield microscope. Treponemes in saline solution were observed while distilled water was pulled into the preparation by capillary action, and it was found that although all treponemes in a field were not changed to spheres simultaneously, the conversion of any single one took place instantaneously. The process appeared to consist of a retraction of both ends of the organism, with a ballooning of the central portion. Moreover, the reaction was found to be irreversible; once spheres formed, the organisms could not be made to regain their spirals.

It was also noted that under no condition could the entire population of treponemes be

converted to spheres. There were always a few organisms that retained a gross spirochetal morphology. However, close scrutiny revealed that many of these were altered, some were doubly contoured as though split down the center (Fig. 5), while others possessed one or more small laterally placed bleb-like protusions suggestive of a local herniation (Fig. 6).

Although the formation of spheres did not appear to be a lytic process, this possibility was investigated by examination of the suspending medium, after sphere induction, for the presence of high molecular weight intracellular products. When the spheres were removed by centrifugation, spectrophotometric examination revealed an absorption peak at 260 mμ, indicative of the presence of nucleic acids. However, when the cells were removed by the more gentle procedure of gravity filtration through a 0.8 μ membrane filter, no such absorption peak was found in the filtrate. It was concluded from this that lysis was not an inherent part of sphere formation and that the nucleic acid appearing in the supernatant fluid after centrifugation was probably the result of mechanical rupture; it had been found previously that spheres were very fragile and easily broken.

Determination of the viability of spheres. Since many investigators have attributed to naturally occurring treponemal spheres a role in the life cycle of these organisms, and some have even reported observations interpreted as the emergence of treponemes from these round bodies (DeLamater et al., 1950, 1951*a*,*b*), it was of interest to determine whether the spheres produced by laboratory manipulation could give rise to the growth of treponemes. This could not be accomplished with absolute certainty, for the presence of an occasional treponeme in every suspension of spheres made it impossible to determine definitively that growth upon subculture arose from a spherical and not a spiral organism in the inoculum. Nevertheless, it was felt that a probable answer might be obtained by means of viability counts, since the number of spheres were far greater than the number of spirochetes in a suspension. For this it was necessary to utilize a procedure that would determine the number of viable organisms in broth cultures, because, in our hands, growth of the Reiter treponeme on solid media has not given colonies of sufficient size or discreteness of character to permit counting by the usual plating technique.

The procedure that was employed consisted of the determination of the number of "viable units" (VU) per ml in a dilution end-point fashion. In practice, 10-fold dilutions of a suspension were made, and four replicate 1-ml aliquots of appropriate dilutions were inoculated into tubes of broth medium. From the tubes in which treponemes grew, a 50% end point was calculated by the method of Reed and Muench (1938), and this, in turn, was multiplied by the constant 0.69 to obtain the VU per ml. (The constant 0.69 is the calculated exponent n in the Poisson probability distribution equation, $P(0) = e^{-n}$, where $P(0)$, probable number of tubes showing

TABLE 2. *Comparison of direct microscopic count of Reiter treponemes and spheres with the number of viable units per ml*

Material examined	Microscopic count per ml		Viable units per ml
	Treponemes	Spheres	
4-Day-culture	3.3×10^8	—	2.8×10^8
7-Day-culture	3.3×10^8	—	2.8×10^8
4-Day treponemes in saline	5.1×10^8	—	5.1×10^8
4-Day treponemes in 0.01 M buffer	2.0×10^7	3.2×10^8	3.2×10^5
7-Day treponemes in saline	3.5×10^8	—	1.3×10^9
7-Day treponemes in 0.01 M buffer	2.0×10^7	2.2×10^8	2.7×10^7
9-month culture	—*	6.4×10^7	1.1×10^5
14-month culture	3.0×10^5	1.4×10^7	2.2×10^5

* No treponemes were seen in the counting chamber, but 10^5/ml could have been present without being observed.

no growth, is 0.5 as represented by a 50% end point, and e is the Naperian constant.)

Representative results are recorded in Table 2. It can be seen that in young untreated cultures, which consisted almost entirely of spirochetes, the number of VU found per ml agreed closely with the number of organisms determined by direct microscopic count. This was taken as evidence that one viable treponeme was sufficient to promote growth, and it represented a confirmation of the findings of Rose and Morton (1952). When viability counts were made of suspensions of spheres, the results were quite different, as also shown in Table 2. In these studies, a control was always included to eliminate the effect of manipulation upon the viability of the organisms; one aliquot of sedimented treponemes was resuspended in dilute buffer to produce sphere formation, whereas the other was resuspended in similarly buffered physiological saline to preserve spirochetal morphology. It can be seen that the number of VU per ml in the sphere suspensions were far less than the number of spheres present. This indicated that few, if any, of the spheres were viable, and since there were always a few spiral organisms in every suspension it appeared that where growth occurred it most probably arose from the spirochetes and not the spheres.

The medium and cultural conditions employed in our laboratory for the propagation of the Reiter treponeme are not conducive to the spontaneous formation of spheres; few, if any, can be found in young cultures. Moreover, only in very aged cultures are the organisms predominantly spherical. For this reason, very few viability studies upon spontaneously formed spheres have been made, but, where they have been done the findings indicate that these are no more viable than the artificially produced spheres. This too, can be seen in Table 2. In every culture studied, the number of VU per ml has always been far less than the number of spheres, and with one exception, has never exceeded the number of spirochetes known to be present. In the exception, no spirochetes were seen microscopically, but the calculated number that could be present without being observed was equivalent to the number of VU found per ml.

With the finding that spheres were almost certainly nonviable, the question arose as to whether viable treponemes were necessary for the formation of spheres. Since it was not possible to use heat-killed organisms, treponemes were rendered nonviable by the use of formalin or Merthiolate, both at a final concentration of 0.1%. Treponemes killed by either chemical formed spheres as readily as living organisms.

DISCUSSION

The recognition of markedly aberrant forms of treponemes came almost simultaneously with the first description of these organisms, but to date, adequate evidence as to the exact nature of these spherical bodies has never been presented. Ample proof that this has not been due to lack of interest can be found in the extensive literature on the subject, much of which has been reviewed by Ingraham (1932) and by Campbell and Rosahn (1950). The existence of these round bodies has, in fact, been the basis of much argument in the past concerning the proper phylogenetic classification of spirochetes. Thus, McDonagh (1913), and other advocates of a protozoan classification, considered them to be gametocytes, whereas those who classified the organisms among the higher fungi, e.g., Meirowsky (1930), considered the round bodies to be the result of budding and, therefore, fruiting bodies.

Although today the spirochetes are generally classified as bacteria, there are still proponents of a complicated life cycle for these organisms, and who consider the round forms to be cysts or germinating bodies (Levaditi, 1941; Mudd, Polevitzky, and Anderson, 1943; Hampp, 1946, 1950, 1951; Gelperin, 1949; DeLamater et al., 1950, 1951*a*,*b*). There is no doubt that the spheres develop from spirochetes, but evidence that they are viable and that the reverse reaction, spheres to treponemes, occurs is for the most part indirect. The only direct studies are those of DeLameter et al. (1951*a*, *b*), who examined daily, by phase microscopy, slide cultures that were incubated for periods as long as 30 days. These investigators described treponemes emerging from round bodies. However, the prolonged period of cultivation and the intermittent nature of the examinations make the interpretation of these observations open to question.

Most proponents of the germination of treponemes have based their arguments primarily on the viability of cultures (Hampp, 1946, 1951) or the infectiousness of tissues (Levaditi and Vaisman, 1938, 1951; Wile, 1947; Gueft and Rosahn, 1948) which contain spherical bodies and are devoid of demonstrable treponemes. With the

exception of Bessemans, Wittebolle, and Baert (1948) who failed, no one has attempted to determine the viability of individually isolated spheres. Most investigators have worked on the theory that the failure to observe treponemes in microscopic preparations is sufficient proof of their absence. The fallacy of such reasoning becomes obvious when one recalls that in the usual preparation for darkfield or phase microscopy (0.005 to 0.01 ml under a 22 mm^2 coverslip) the volume of material under each oil immersion field is not greater than 5×10^{-7} ml, or that the calibrated volume of the Petroff-Hausser bacterial counting chamber is only 2×10^{-5} ml. It would take an exceptionally persistent investigator to find one organism in a preparation that contained as many as 10^3 treponemes per ml. In light of the observations reported in this paper, which agree with those of Rose and Morton (1952) for the Reiter treponeme, and Magnuson, Eagle, and Fleishman (1948) for *T. pallidum*, one organism is sufficient to initiate growth. It is not surprising that many investigators have failed to observe treponemes in infectious material.

The belief that spheres are degenerative forms of spirochetes is not new. Akatsu (1917), from his studies on the influence of sugars upon the growth of treponemes, felt that round bodies were the result of degeneration, and even suggested that they were caused by plasmoptysis. Noguchi (1917), in spite of the fact that he held to the germination theory, reported the production of spherical swellings on the sides of treponemes exposed to hypotonic solutions. Warthin and Olsen (1930) believed that the granular forms they observed in aortic lesions of syphilis were not viable. Finally, Rose and Morton (1952), in their extensive study of this problem, reported a number of conditions that gave rise to the formation of spheres, e.g., exposure to Dial soap, saturated salt solution, and osmotic shock (2.0 M glycerin to water). This led them to the conclusion that spheres were the result of degeneration.

In view of the ease with which spheres could be produced in low salt concentrations in the present study, it is surprising that this phenomenon has not been reported previously. Our method of cultivating this organism may offer a possible explanation. As pointed out by Knaysi (1951), certain cultural conditions can markedly influence turgor pressure of cells, which in turn can affect their ability to undergo plasmoptysis. Such factors may also explain a discrepancy between our findings and those of both Gelperin (1949) and Rose and Morton (1952). Gelperin reported that agar-less media promoted the production of spheres, whereas Rose and Morton found just the opposite. In our hands, the presence or absence of agar appeared to have no effect upon the growth of treponemes, and spontaneous development of spheres did not occur readily in either type of medium.

The influence of environment upon cell turgor pressure may also account for a phenomenon observed in the course of these studies which is still under investigation and for which we as yet have no complete explanation. As already mentioned, treponemes suspended in hypotonic magnesium chloride do not form spheres as in other salt solutions, but retain their spiral shape. There is evidence that magnesium ions can alter the response of microorganisms to their environment, possibly by affecting the cell membrane. Both Knaysi (1951) and Weibull (1956) have reported that organisms grown in the presence of 0.2 to 0.5 M magnesium sulfate undergo spontaneous plasmoptysis. Knaysi considered this to be due primarily to increased intracellular osmotic pressure, but Weibull concluded that a direct toxic action was also involved. Evidence that magnesium exerts its action upon the cell membrane comes from the observation that magnesium in low concentration protects from lysis protoplasts formed by penicillin (McQuillen, 1958), and prevents fragmentation of osmotically produced protoplast ghosts (Weibull, 1956). These findings suggest that magnesium ions may in some fashion either increase the tensile strength of the cell membrane or affect its permeability properties, especially to water.

The spheres that are produced artificially by changes in osmotic pressure are morphologically indistinguishable from those that develop spontaneously in cultures. Although suggestive, this observation does not prove that the two are identical. If they are, the question arises as to how conditions of osmotic imbalance develop spontaneously during cultivation of the organisms. Several interrelated conditions must be involved, i.e., concentration of intracellular constituents, permeability of the cell membrane, and composition of the cultural environment.

Although the first two factors cannot be readily separated, evidence that one or both play a role comes from the observations that all treponemes are not equally susceptible to sphere formation under artificial conditions, and that the natural development of spheres in cultures is a slowly progressive one. Proof that environmental changes participate in this phenomenon is more difficult to obtain, for it necessitates demonstration that either the total osmotic pressure of the medium drops markedly, which seems unlikely, or that the effective osmotic pressure decreases. For the latter to occur, the medium would have to change from one composed to a large extent of impenetrable solutes to one composed of metabolic products that freely cross the cell membrane. An attempt to demonstrate such a phenomenon by suspending young treponemes in cell-free broth taken from an aged culture was unsuccessful. However, Rose and Morton (1952) reported that the cultivation of treponemes in media from old cultures resulted in more extensive sphere formation. In view of our failure to confirm this observation, more extensive studies are needed to prove that such a change actually occurs.

The results presented in this paper offer additional, although not conclusive, evidence that spheres are degenerative forms of treponemes and not stages in a complicated life cycle. The observation by DeLamater et al. (1951*a*,*b*) that young treponemes may occasionally emerge from round bodies is not incompatible with this conclusion, for it has been shown with other bacteria that during the course of degeneration there may be a stage when morphological changes take place before complete loss of viability has occurred (Knaysi, 1951; Hughes, 1956).

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