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Transitional Forms of *Corynebacterium acnes* in Disease

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A clear-cut triad of sequential *Corynebacterium acnes* transitional forms from disease has been discovered. This entity includes three major forms which are capable of stabilization in culture, the spherical, the intermediate, and the definitive *C. acnes*. During conversion or reversion among the three forms, a variety of forms with mixed characteristics was observed. The spherical form was gram-negative and osmotically fragile, but it possessed a vestigial cell wall and mesosomes which excluded it from the L forms. In lieu of the L-form designation, the term "transitional" was adopted for all forms leading up to the definitive *C. acnes*. Culture of the spherical form was successful only on *Mycoplasma*-type media. The intermediate form was gram-negative, had mixed spherical and filamentous morphology, and bore a striking resemblance to *Streptobacillus moniliformis*. Like the spherical form, it was nutritionally exacting. The definitive form of *C. acnes* was preceded by gram-positive transitional forms of *C. acnes* morphology. It lacked, however, the carbohydrases and proteinases of *C. acnes* and susceptibility to *C. acnes* bacteriophages. Reversion was often blocked at this stage. A series of blood cultures from a patient with endocarditis was studied. Postmortem stain sections of the heart-valve lesion included intracellular masses of gram-negative spherical organisms. Indirect fluorescent antibody staining of these masses was strongly positive with antiserum to the spherical form and weakly positive with antiserum to the intermediate form.

Infections caused by *Corynebacterium acnes*, the only anaerobic species of *Corynebacterium* (9), have been studied extensively by Prévot and others (5-7). These infections, both localized and disseminated, have included septicemia, endocarditis, deep abscesses of muscle and organs, pustular abscesses of the skin including acne, purulent sloughing abscesses of the skin, pneumonia, lymphangitis, and meningitis.

A solid case has been built for the establishment of *C. acnes* as a disease agent. However, *C. acnes* is not yet widely regarded as an agent of pathogenic significance. As Reid (6) has pointed out, even multiple isolations of *C. acnes* from blood cultures of a feverish patient may be termed contamination by a "diphtheroid" (6).

Wittler et al. (8), after thorough study of a 4-yr-old girl with long-term endocarditis, reported alternating infection with an "L-phase variant" during penicillin administration and a definitive *Corynebacterium* when no antibiotic was given. This *Corynebacterium* was not speciated, because its reactions were unlike those of any described species. Reversion to the parent form was incomplete.

This report is concerned with transitional forms of *C. acnes*, thus far observed only in infection and in normally sterile tissues or fluid, such as blood, marrow, or lymph nodes.

MATERIALS AND METHODS

Clinical evaluation. Case histories were examined of 93 patients from whom *C. acnes* or its transitional forms were recovered. Criteria for selection were (i) isolation from more than one specimen, (ii) isolation from body fluids or tissues that were normally sterile, and (iii) presence of disease.

Endocarditis study. A 65-yr-old man with subacute bacterial endocarditis failed to improve with penicillin therapy. Diagnosis was supported by three, consecutive, daily blood cultures that were positive for transitional forms of a bacterium, later identified to be *C. acnes*. Blood samples were distributed in 50-ml amounts of four media: Trypticase Soy Broth (BBL; with 0.1% agar) with and without 0.5% mucin, Thioglycollate Medium (Difco), and Ac Medium (Difco). Subcultures were made from the primary culture bottles to Tryptose Agar (Difco) with 5% rabbit blood (TRBA);

Barile, Yaguchi, Eveland (BYE) medium (1) composed of brain heart infusion agar with 15% human blood, 1% 0.1 M arginine solution, and 1% yeast extract; and AC Medium, a semifluid medium in tubes, plus 20% human serum, selected for intensive work. Gross and microscopic autopsy findings supported the diagnosis.

Antigens and antisera. Each of the three forms was grown on BYE agar under appropriate conditions for 9 days at 37 C; the cells were harvested in saline, and the turbidity was adjusted to the no. 8 McFarland tube. The suspensions were heated at 56 C for 15 min, and Merthiolate was added in a 1:10,000 concentration. These suspensions were used both for serological reactions and antiserum production. Antisera were prepared in rabbits with 50% (v/v) of Freund's complete adjuvant mixed with the suspension. Initial intramuscular injections of 0.5 ml were followed by 1-ml injections at 4 days, 2 ml at 8 days, and 4 ml at 18 days. Two weeks later, blood was obtained from the ear veins to test the serum antibody levels; this was followed by exsanguination. Antisera were inactivated at 56 C for 30 min.

Slide agglutination with undiluted serum. The bacterial suspensions used are described above. Two drops of suspension and one drop of undiluted antiserum were placed together in a 1-in square marked on a 1- by 3-in glass slide. The slides were rotated by hand for 2 min and the results were read.

Polystyrene latex particle flocculation. Flocculation tests were performed, by use of antigens and antisera previously described, according to the method of Florman and Scoma (4). The polystyrene particles (Hyland Laboratories, Los Angeles, Calif.) were 0.81 μm in diameter.

Indirect hemagglutination. Sheep red-blood-cell agglutination tests were performed with the same antigens and antisera, in a technique modified from that of Caille and Toucas (2), using whole-cell suspensions of the growth forms instead of extracts. The results indicated antigenic relationships between all of the forms, but not as strongly as did those of the latex flocculation method.

RESULTS

Pathological findings. A 4- by 8-mm polypoid vegetation was present on the noncoronary leaflet of the aortic valve. Microscopically, the lesion consisted of fibrinous material, inflammatory cells, large colonies of microorganisms, and scattered macrophages. In fixed sections stained by the Brown-Brenn modification of the Gram stain, the colonies were composed of small, gram-negative, spherical organisms. In some of the macrophages, small gram-negative forms were present; these occurred diffusely and in small clusters within the cytoplasm.

Bacteriological findings. The earliest growth observed in blood cultures was spherical. Most of the organisms were lysed by Gram stain, except for dense areas of the smear where deep-red spheres of widely varying size remained. Staining with Giemsa avoided extensive lysis and revealed masses of spherical organisms with extreme size range. There was a tendency to failure of decolorization in Gram stain, leaving some cells gram-positive.

Indirect, fluorescent antibody reactions. Sections (3 to 4 μm in thickness) of the polypoid lesion from the aortic valve were rapidly deparaffinized, washed three times with phosphate-buffered saline (*p*H 7.4), and air dried. Rabbit antisera to the cultured spherical form and the definitive form were applied to separate sections for 30 min. Antihuman rabbit sera (Comb's sera) were applied to other sections of the lesion. The sections were washed three times, and then fluorescein-conjugated antirabbit sheep serum mixed with rhodamine bovine albumin (1:4 dilution) was applied to the sections for 30 min. The sections were washed, and cover slips were applied over 25% buffered, nonfluorescent glycerin. The sections were examined with ultraviolet light by means of a Zeiss Photomicroscope with dark-field condenser, the Zeiss no. 53 and 44 barrier filters, and exciter filter BG 12.

Aerobic and anaerobic incubation on the three media elicited startling changes in the organisms. The definitive *C. acnes* developed in AC Medium on the first subculture of culture no. 318. Its appearance required 3 months for no. 298 and 1 yr for no. 308. Subcultures were made of the spherical forms on BYE agar every 3 to 4 days and of the definitive *C. acnes* in AC Medium at 2-week intervals.

The first subculture of cultures no. 298 and 308 to BYE agar incubated anaerobically developed faint growth of the spherical organism and of another organism, a pleomorphic gram-negative rod with filaments that included swelling. Coccoid forms were also present. This organism was subsequently termed the intermediate form. The more salient cultural characteristics of the two major transitional and the definitive forms from the three blood cultures are listed in Table 1.

Spherical form. In its stable state, the spherical form (Fig. 1) was strictly anaerobic. It required an enriched medium such as BYE for growth, although sparse growth was obtained on TRBA for one or two transfers. Colonies on BYE were 1 mm in diameter, smooth, moist, entire, transparent, and nonhemolytic. There was no growth into the agar. Colonies on a transparent medium (i.e., heart infusion agar with 20% horse serum) are shown in Fig. 2. Chaining of the spheres was common but was usually not seen in smears, as the mere act of suspending disrupted the chains.

An attempt was made to measure osmotic fragility of the spheres. Distilled-water treatment

TABLE 1. *Transitional forms and culture conditions favoring each form*

Blood culture no.	Spherical[a]	Intermediate[b]	Corynebacterium[c]
298	Anaerobic BYE	Aerobic tryptose rabbit blood (5%) agar best; also aerobic BYE	AC Medium derived from spherical form; slow reversion (3 months)
308	Anaerobic BYE	Aerobic tryptose rabbit blood (5%) agar; also aerobic BYE	AC Medium reversion occurred from spherical form after 1 yr in culture
318	Anaerobic	Aerobic tryptose rabbit blood (5%) agar best; also aerobic BYE	AC Medium; *Corynebacterium* appeared on first subcultures

[a] May be obtained in stable form.
[b] Usually mixed spherical and intermediate forms; difficult to maintain, converting readily to spherical forms.
[c] Stable, but have been observed to convert to spherical forms on long-term culture in serum-free medium.

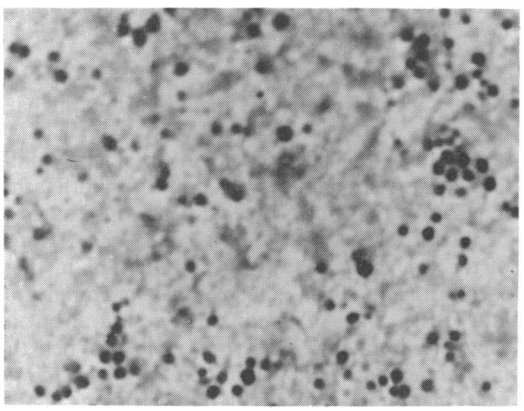

FIG. 1. *Spherical form organisms. Giemsa stain.* × *1,650.*

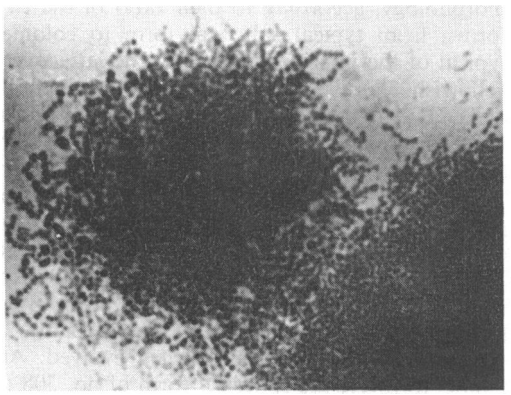

FIG. 2. *Spherical form of C. acnes transitional forms. Dienes stain.* × *800.*

of a suspension of the spheres (turbidity of no 3. McFarland tube) for 24 hr resulted in about 75% lysis, as judged by phase microscopy, but the membranes retained their shape. The difference in refractivity apparently reflected leakage. Increase in light transmission of this lysed material at 530 nm was only 21% more than a suspension in buffered saline after incubation for 24 hr at 4 C.

Spherical form. Subcultures of this form proved culturally stable if a serum- or blood-containing medium was used. Attempts to cause reversions to either the intermediate or the *Corynebacterium* form were usually successful, but they varied in time required. To achieve the intermediate form, subcultures were made on TRBA and incubated aerobically at 37 C. The *Corynebacterium* reversion was made by heavy inoculation of serum-free media; this occurred on agar plates as well as in liquid media.

Electron microscopy of the spherical form disclosed it to have cell wall material as a continuous layer, but it appeared thin and atypical. This finding satisfied the perplexing question of partial resistance of the spherical form to lysis by distilled water. The presence of mesosomes was established.

Intermediate form. Aerobic plating of the spherical form to TRBA favored reversion to this highly pleomorphic form (Fig. 3) which closely resembled *Streptobacillus moniliformis* in morphology. The similarity ended with morphology, as it did in the series of Wittler et al. (8). The Gram-stain reaction, although usually negative, tended to be variable. The microscopic morphology was striking, with areas of intense staining of a filament next to nonstained areas, tapering coiled filaments that included large bulbous bodies, spheres, and rod forms.

This form would not grow on the usual laboratory media for biochemical testing and did not react with *S. moniliformis* antisera. It was un-

stable, usually converting to the spherical form but occasionally reverting to the *Corynebacterium* form. It required cultural manipulation, using TRBA and Tryptose sheep blood agar alternately, to maintain its typical morphology. Plating from broth cultures of spherical forms to aerobic BYE or TRBA was conducive to growth of the intermediate form.

Spherical cells were present with the intermediate form, but they did not grow aerobically as separate colonies on surface media. The proportion of spherical to intermediate form organisms in the individual colonies varied from almost pure spherical to pure filamentous intermediate form. Although morphologically similar, the spherical form accompanying the intermediate form and the stable pure form were very unlike metabolically. The colonies themselves varied in morphology, according to their ratio of the two forms, from typical spherical form to colonies typical of the intermediate form. The latter were yellowish, 3 mm in diameter, with green hemolysis on TRBA, and they possessed radial striations, serrated edges, peaked centers, and rough surfaces.

Corynebacterium form. This form (Fig. 4) appeared with the spherical and intermediate forms on the first subculture of blood culture no. 318 to TRBA. Transition from the spherical and intermediate forms of blood culture no. 298 was very slow. Almost 3 months had elapsed before a typical anaerobic *Corynebacterium* evolved. Attempts to revert the spherical form of no. 308 to *Corynebacterium* were unsuccessful for over a year, but thereafter reversion was accomplished repeatedly in one transfer by heavily inoculating serum-free media. Large inocula may have provided a greater chance of including mutant bacteria.

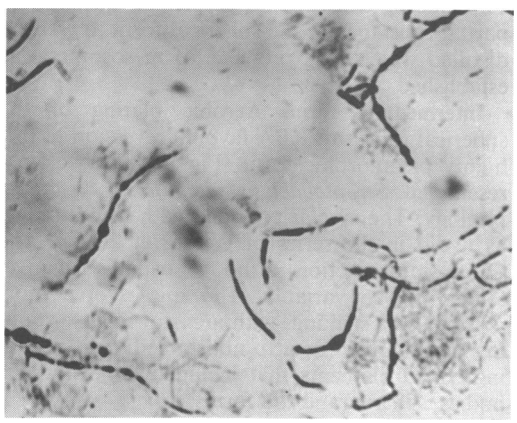

FIG. 3. *Intermediate or "Streptobacillus-like" form of the C. acnes transitional forms. Gram stain.* × *1,000.*

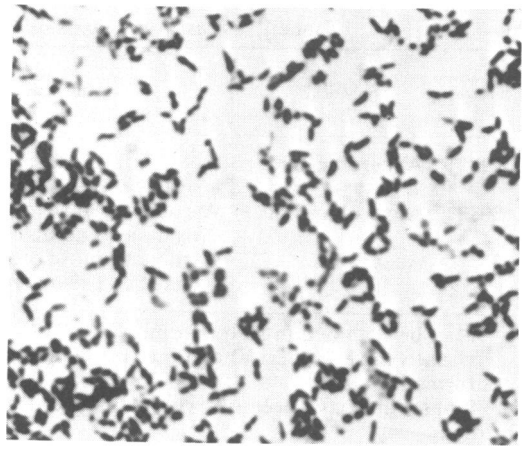

FIG. 4. *Corynebacterium form of the C. acnes transitional forms. Gram stain.* × *1,250.*

Action of the bacterial form on sugars, milk, and gelatin sometimes was not seen until after incubation for 2 or 3 weeks. This represented a period of reversion to the typical *C. acnes*. Some of the *Corynebacterium* reversion strains in their early transitional stage formed gas in Thioglycollate (BBL) and AC Medium plus serum, but as they stabilized this property was lost. When the metabolic characteristics of the *Corynebacterium* were acquired, the ability to ferment a few carbohydrates occurred first, followed by the ability to reduce nitrate, liquefy gelatin, form indol, and coagulate and digest litmus milk. The *Corynebacterium*, even after appearance as a recognizable form, continued to evolve in colonial and microscopic morphology. Final stabilization required weeks of continuous culture in AC Medium. This medium, used without serum, effected stabilization to the definitive *Corynebacterium*. Salient biochemical reactions were as follows. Positive reactions occurred for indol, methyl red, nitrate, gelatin, and catalase. Litmus milk was coagulated and digested. For sugar fermentations, a semisolid base without serum was used with the following composition: beef extract, 3 g; Proteose Peptone no. 3 (Difco), 3 g; gelatin, 80 g; K_2HPO_4, 0.3 g; NaCl, 2.0 g; agar, 2.0 g; phenol red, 0.025 g; and H_2O, 1 liter. The final *p*H was 7.4 to 7.6. In this medium, fermentation without gas was obtained in dextrose, lactose, sucrose, adonitol, inositol, maltose, mannose, trehalose, levulose, galactose, and starch. Subculture of the *Corynebacterium* forms was usually made in AC Medium. Occasionally,

the organisms deteriorated in AC Medium. This deterioration was usually associated with large numbers of gram-negative spherical forms, but these could not be successfully subcultured.

Slide agglutination. Table 2 depicts cross reactions between the antisera and antigen of the spherical and intermediate forms. Neither of these cross-reacted with antiserum to the *Corynebacterium*. Antisera diluted 1:10 and 1:100 gave similar reactions to the undiluted antisera when tested with the three forms.

Polystyrene latex particle flocculation. These tests indicated a relationship between all three forms (Table 3), unlike the slide test which related the spherical and intermediate but not the *Corynebacterium*. Spherical-form antiserum agglutinated the sensitized latex particles at 1:40 dilution when coated with *Corynebacterium* antigen, and at 1:320 with the homologous spherical-form antigen. Intermediate-form antiserum agglutinated at 1:40 dilution the particles sensitized with *Corynebacterium* antigen, at 1:1,280 with the homologous antigen, and at 1:160 with spherical-form antigens. *Corynebacterium* antiserum agglutinated at 1:320 dilution the particles sensitized with homologous antigen, at 1:320 with intermediate-form antigen, and at 1:160 with spherical-form antigen.

Individual fluorescent antibody reactions. Sections of heart-valve lesion exposed to the anti-spherical-form serum contained foci of strong specific fluorescence that corresponded to the colonies of bacteria. The preparations exposed to the intermediate-form antiserum contained similar foci of low-intensity fluorescence. No fluorescence was obtained with antiserum to the *Corynebacterium* form. The control preparations were negative.

TABLE 2. *Slide agglutination cross reactions*[a]

Antiserum	Antigen		
	Spherical	Intermediate	*Corynebacterium*
Spherical	++	+	−
Intermediate	+	++	−
Corynebacterium	−	−	++++

[a] Undiluted antiserum was used; ++++ indicates the strongest flocculation seen; +, the minimal reaction; −, no reaction.

TABLE 3. *Polystyrene latex flocculation cross reactions*

Antiserum	Antigen		
	Spherical	Intermediate	*Corynebacterium*
Spherical	1:320	1:1280	1:40
Intermediate	1:160	1:1280	1:40
Corynebacterium	1:160	1:320	1:320

DISCUSSION

The possibility of a contaminant role for any or all of the organisms in this study was of continuous concern. Painstaking care was exercised throughout to detect contamination on or in media before it was used and while it was in use. Cultures were made in duplicate and, at times, in triplicate. Beyond the controls just discussed, the organisms in the study were well validated by means of the following mechanisms. (i) A study in parallel of organisms from three positive blood cultures taken on three successive days was done. Each transition was repeated numerous times. (ii) Evolvement of the transitional forms occurred in each of the cultures along the same tortuous route. (iii) The L form in situ was demonstrated by fluorescent staining. (iv) *C. acnes* did not spring full-formed into the cultures. Initially, it was corynebacterial in morphology only. Metabolic conversion was slow, and it often stopped short of the typical *C. acnes*. (v) Serologic relationship among the three major forms in the series was demonstrated through latex particle flocculation.

It has been shown that characteristic spherical, intermediate, and *Corynebacterium* forms may be maintained in stable form on preferred media with selected degrees of aerobiosis. These forms are: (i) a spherical form having many L form characteristics; (ii) the intermediate form, an unstable form morphologically similar to *S. moniliformis;* and (iii) a stable definitive form, *C. acnes*. Each form (except the definitive *Corynebacterium*) may be converted or reverted to the others by culture on appropriate media, again under controlled aerobiosis. The mechanisms of conversion and reversion are not well understood. Perhaps inducible enzyme systems are partially responsible.

The spherical form has, in general, been stable under selected conditions. The intermediate form is very difficult to maintain as the purely filamentous form and is usually mixed with a high proportion of spherical forms. However, it was possible to produce the filamentous form, a necessity before antigen preparation. Once stabilized as a *C. acnes*, conversion did not occur in vitro, but, as stated before, not all reversions to the *Corynebacterium* acquired the full complement of *C. acnes* characteristics. Although *C. acnes* was the definitive and stable bacterial form of this culture

series, it was sometimes slow to develop and often did not occur at all.

Bacteriophage 174, specific for *C. acnes*, lysed the cultures in this series that had acquired *C. acnes* characteristics and lysed the great majority of *C. acnes* strains from widely separated sources. It did not lyse *Actinomyces*, other *Corynebacterium* species, *Propionibacterium*, *Lactobacillus*, *Listeria*, or *Streptobacillus* species. Thus, it appears to be specific for *C. acnes*

Wittler (8) studied a *Corynebacterium* and its transitional forms isolated from a 4½-yr-old endocarditis patient and kindly provided us with cultures of this organism. Our studies proved that one of the *Corynebacterium* cultures (SS M5) is culturally and serologically *C. acnes* and is lysed by phage 174. The others, 114d, SS 52-3, and SS 6-4a-3, are not typical *C. acnes*, having stopped short of this form in their in vitro development. Wittler was successful in precipitating reversion to the bacterial form by supplying filtrates of *Staphlococcus epidermidis* culture to the media. Yeast extract was also added to assist reversion. We applied these agents to our techniques and found them to precipitate reversion.

It is unusual that gas should, on occasion, be produced in AC Medium by large inocula of the transitional forms whereas no gas was produced by the *Corynebacterium*. Wittler has obtained similar results (*personal communication*). Protein and carbohydrate enzymes were not demonstrable in the transitional forms. They were characteristic of the stabilized *Corynebacterium*.

Dienes (3) observed transitional forms in *Bacteroides* and *Proteus* strains that possess morphology similar to *S. moniliformis*. There has been no evidence from our study to indicate anything other than a morphological relationship between these intermediate forms and *S. moniliformis*. The basis for occurrence of this gram-negative, bizarre, filamentous–bacillary–spherical phase is unknown. It could be that in every instance of its presence the same uneven condition of presence or lack of cell wall substance was extant. It was not seen in tissue sections, where only spherical forms were seen, and it did not appear until cultures were made.

Intermediate and spherical forms found in this study possessed strong antigenic relationships, as demonstrated by both agglutination (tube and slide) and styrene-particle flocculation. These forms were unrelated to the *Corynebacterium* in tests using the direct agglutination reactions; however, they were strongly related in tests using styrene-particle flocculation. Wittler (8) utilized agglutination and complement-fixation reactions and found no common antigens between the L form and *Corynebacterium*, although she postulated that such antigens must exist.

The failure of penicillin to eliminate the intracellular form in heart tissue of the endocarditis patient was predictable. Wittler (8) reported that penicillin administration induced the L form in the endocarditis patients that she studied. The *Corynebacterium* and its transitional forms reappeared in the blood on cessation of penicillin therapy in her cases. In our case, there was evidence that the *Corynebacterium* form occurred only in vitro, with the spherical form predominant in vivo with or without penicillin therapy.

The incidence of infection via the mechanisms described here is unknown. Three or four verifiable infections are seen each year in the senior author's laboratory service in a 400-bed research hospital. A large number of these transitional form cultures may not be recognized for one or more of the following reasons. (i) The transitional form immediately reverts in culture and is seen only as fully characteristic *C. acnes*. (ii) The transitional form is mixed in vivo with *C. acnes* and is overgrown immediately by the latter. (iii) The transitional form fails to grow even initially on usual media, especially if no anaerobic cultures are included. (iv) The transitional form, like the spherical form does not revert to the intermediate form nor to *C. acnes*, and it either dies out in culture (most common) or is misdiagnosed. (v) The transitional form is reported as a *Streptococcus* because of the presence of gram-variable spheres in stained films. Also, the spherical transitional form of *C. acnes* often occurs in chains in broth. The cells in these chains usually separate during preparation of the film. However, the cells in chains, when seen in films, are strongly flattened on contiguous sides.

The transitional forms isolated from different infections are apt to differ extremely in aerobic and nutritional requirements. The round bodies reported by Wittler, which are probably the same as the spherical form of this study, grew adequately in veal infusion broth plus human serum, whereas the spherical form would not grow in this medium. Thus, it is necessary to treat separate cultural isolations of this dissociation syndrome as individual problems and to accommodate further changes in the physiology of the dissociants by changes in media and growth conditions.

Although the fastidious, fragile spherical form had many characteristics of L forms, examination with the electron microscope showed it to have a complete sphere of cell wall material; this, however, was thin and atypical. Mesosomes were also noted. By rigid definition, L forms should not

have cell wall material, no matter how vestigial, nor should they have mesosomes. Many reports of the L forms of bacteria in disease take no heed of such rigid criteria; indeed, the first description of L forms listed no such criteria.

Numerous names have been employed to indicate the peculiar cellular characteristics of these organisms, such as aberrant, transitional, pleomorphic, dissociation, L, soft, amural, intermediate, variant, spherical, or multiple forms. Each of these names has one or more disadvantages. It is difficult to apply a restrictive epithet to organisms with such extremes of morphology and physiology. The term *transitional forms* indicates the changing character of the group and has been used most extensively in the present work.

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