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Permanent Alterations of the L-Forms of *Proteus* and *Salmonella* Under Various Conditions

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L-forms obtained from three strains of *Proteus* and from one strain of *Salmonella* have been kept for 15 to 20 years by weekly or monthly transfers on agar plates containing penicillin. The morphology and growth requirements of these strains have changed. They now grow abundantly on the surface of agar and in broth. The cultures consist of large bodies, small granules, and transitional forms. These organisms are more resistant to distortion and stain more deeply than organisms of the usual L-forms. In broth and to a lesser extent on agar, branching filaments develop, on the ends of which both the large, round organisms and small organisms are produced. The filaments are a transitional stage in the development of the cultures. Usual bacillary forms were not present in the culture and did not appear in successive transfers in the absence of penicillin. Bacilli reappeared on exposure of the L cultures to the influence of a spore-bearing bacillus. A similar transformation of L-forms has also been observed developing within a short time in recently isolated A and B type L cultures of one *Proteus* strain during the process of reversion to the bacterial form. The altered cultures are fixed in a stage of transition between the B type L-form and the regular bacteria.

The observations described in this and in the following paper were made by morphological study of the cultures and of the organisms. They are published in this stage because the author has retired and cannot extend the observations and check them by other methods.

The L-forms of *Proteus* have been studied extensively in our laboratory since their isolation in 1946. The information obtained from these studies and from the study of L-forms of other species was recently reviewed (5). An excellent discussion of the properties of L-forms and of the extensive literature relating to them has been provided by Hijmans et al. (8). The typical L-forms correspond to bacteria without the cell wall and surrounded only by the plasma membrane or, in addition to the plasma membrane, with a greatly altered cell wall. The ability of the L-forms to divide and multiply largely depends on the physical conditions of their environment.

The study of L-forms of *Proteus* is of particular interest, since in this species several strains intermediary in their properties between bacteria and typical L-forms have been isolated. These strains maintain their individuality and can be kept in continuous cultivation. The tendency toward variation in the properties of the cell wall is apparent in *Proteus* during the usual growth of the bacteria, as well as in the development of the L-forms (7). In the bacterial cultures of *Proteus*, the cell walls of the spreading filaments are weakened, as compared with those of the small bacilli. Exposed to penicillin, *Proteus* produces two types of L-forms distinct in their properties. One, designated as A type, corresponds to the typical L-forms; the organisms are surrounded only by a plasma membrane. The other (B type) has, in addition to the plasma membrane, a cell wall, the rigidity of which is lost. The conditions of development and the properties of these two types of L forms have been described several times (2, 6).

During the long period of working with L-forms of *Proteus*, development of several additional permanent varieties of L-forms from one strain of *P. mirabilis* (strain 52) has been observed. Two of these are more similar in their properties to regular bacteria than the A or B type L-forms. Their morphology is extremely variable including growth in branching filaments. The derivation of these different L-forms from *Proteus* seems to be well established. Morphological variations similar to those seen in *Proteus* have been observed in other bacteria and it is usually difficult to obtain definite evidence for the genetic identity of the various forms. The example of *Proteus* may help

in the interpretation of similar variations in other bacteria and provide suggestions for their study.

MATERIALS AND METHODS

Strains. Most of the observations on the morphological variations seen in *Proteus* L-forms were made with strain 52 of *P. mirabilis* isolated in 1946 in our laboratory. Similar variations occurred and were studied in the L-forms of two other old laboratory strains of *Proteus;* one, strain 18, was received from R. Tulasne of Strasbourg; another is *Proteus* XK used for serological diagnosis of scrub typhus. Similar alterations occurred in L-forms of one of four more recently isolated strains of *Proteus* maintained in the laboratory for 2 years. The L-forms of 23 freshly isolated *Proteus* strains were studied with the altered L-forms. An A type L-form of a strain of *Salmonella* sp. maintained in our laboratory for many years was altered in a similar way as the *Proteus* L-forms. The strain is designated as *S. typhimurium*.

The reversion to bacteria of the L-forms of several species was greatly enhanced by growth of the L-forms in the proximity of a culture of a large gram-positive sporebearing bacillus designated as *Bacillus* Y. It was used in the recovery of the bacteria from the altered L-forms.

Media. The basal media, except where specified, contained the normal amount of NaCl (0.5%) and consisted of either Trypticase Soy Agar or Broth (BBL) or Brucella agar (Albimi). In most experiments, 10% horse serum or 5% horse blood was added to the media. The concentrations of agar, nutrients, and sodium chloride were varied in several experiments. Gelatin added to nutrient broth in concentrations of 10 and 30%, respectively, was also used. The exact composition of the media was not decisive in the results obtained.

Microsocopic observation. Stained-agar preparations were used for the initial examination of the cultures (4, 10). With this method, the growth and morphology of the organisms, both on the surface and within the media, can be observed with little distortion of the organisms. For bacteria and the altered L-forms, the cell membranes of which are more resistant than of most other L-forms, the procedure described to make permanent preparations (4) can be simplified by omitting fixation with formaldehyde vapors. The staining can also be shortened to 20 to 30 min. Klieneberger's agar fixation method (9) was also very useful for the study of altered L-forms. This method could also be simplified by using a mixture of equal amounts of orthophosphoric acid and 37% formaldehyde for fixation, instead of Bouin's solution. The agar slices can be lifted after 20 to 30 min of fixation; the imprints are then dried at room temperature and stained. The making of permanent preparations with either method required only a few minutes and could be done during the examination of the cultures. Without permanent preparations, it would be difficult to compare the various morphological forms in cultures obtained at different times and from different bacterial species. The technique of the stained-agar preparation is simple, but experience with the technique and familiarity with artifacts which may be present in the preparations are necessary for their interpretation. Phase-contrast microscopy has been used to study the multiplication of different morphological elements but has been successful in only a few cases. It does not replace the stained preparations. The great advantage of stained-agar preparations for the study of L-forms and other pleomorphic organisms is that they can be used with any type of solid media and also with blood-agar plates and coagulated serum. Diffusion of stain from the cover slips does not alter the morphology of these delicate organisms; and it is helpful because the color distinguishes the living, intact organisms from autolyzed, disintegrating organisms. Observation of the development of the cultures with phase microscopy needs careful controls. Placing a cover slip on the media changes the physical properties of the surface of agar and in some cases alters the development of the cultures.

The cultures were maintained for many years by four different persons; however, during their intensive study, all cultures were transplanted and studied personally by the author. He also prepared and stained all microscopic preparations. This was necessary to avoid errors and to obtain the information needed for the step-by-step conduct of the experiments. Since it is not known whether the various morphological forms in the cultures have significant differences in properties or in potentialities of development, it seems reasonable to use the simplest terms for their description, such as large bodies (5 μm or larger), small round forms (between 1 and 5 μm), and small granules (less than 1μm). The expression "altered strain" means a strain which has undergone significant and permanent changes in properties compared with cultures of A and B type L-forms.

RESULTS

The strains, the conditions under which alteration of L-forms were observed, and the most important properties of the altered L-forms are indicated in Table 1.

The first permanent variation of the L-form of *Proteus* 52 was observed in 1952 (3). It developed from B type colonies growing from bacteria inoculated into melted agar and solidified as slants in test tubes. The slants were partially covered with nutrient broth. From two strains of *Proteus* (52 and XK), a thin film, similar to young growth of tubercle bacilli, developed on the surface of the broth. These organisms differed in several respects from the A and B type L-forms. The growth consisted of very small granules (between 0.1 to 0.5 μm) and relatively few larger forms, but no large bodies were seen in them. They were well preserved in smears and on electromicroscopic grids. Bacteria did not reappear in cultures transplanted without penicillin. However, one spreading bacillus colony developed after 1.5 years in a sealed petri dish inoculated with the culture obtained from strain XK. The

TABLE 1. *Permanent alterations observed in the L-forms*

Conditions under which alterations developed	L strains	Properties of altered strains
Cultivation of L-forms for 15 to 20 years on agar plates containing penicillin	*Proteus* 52—A type *Proteus* XK—type does not conform to types A or B *Proteus 18*—type unknown *Salmonella* sp.—A type	Abundant growth in broth and on surface of agar—cultures consisted of large bodies, small granules, and transitional forms. Transient development of branching filaments. No development of regular bacterial forms in absence of penicillin. Organisms more resistant and more deeply stained than in the A and B types.
Cultivation of L-forms for 2 years on agar plates containing penicillin	Four *Proteus* sp. strains freshly isolated A type	Properties of one strain similar to those of strains cultivated for longer periods. Three strains similar in many respects to the B type. Reproduced the usual bacteria in the absence of penicillin.
Alterations of L-forms observed within a short time under various conditions.	*Proteus* 52 and XK variants observed in 1952 (3)	Growth on surface of broth. Cultures consisted of small and larger granules. Bacteria did not reappear in the absence of penicillin. Bacteria reappeared in strain XK after 1.5 years in a sealed plate.
	Proteus 52—mucoid growth in broth.	Large bodies, mostly disintegrated.
	Proteus 52—B type L-forms (N20) *Proteus* 52—A type L-forms (AJ)	Properties similar to those of strains cultivated for long periods. Bacteria reappeared in N20 for 1 month after incubation without penicillin. Later, no reappearance of bacteria. In AJ, bacteria continued to reappear during the period of observation.

bacilli were agglutinated by the specific serum. Similar cultures were obtained in the spring of 1952 in repeated experiments with *Proteus* strains 52 and XK, and no similar cultures were obtained from 14 other strains isolated at that time. During the following 15 years, several trials to reproduce similar cultures with strains 52 and XK and with freshly isolated strains were unsuccessful. The development of this variant L-form, which grew abundantly and had no exacting nutritional requirements, apparently depended on unknown factors which were present in two strains and in the media for a while, but which were not present in later experiments.

During the attempt to reproduce the surface growth in broth by using the technique outlined above, another variation of the B type L culture was obtained. Large mucoid masses developed in broth without general turbidity. These masses could be serially transplanted in broth. The growth consisted of granules and of large bodies, most of which were autolyzed.

The next alteration of the L cultures of *Proteus* was observed in L cultures of three *Proteus* strains and one *Salmonella* strain maintained for 15 to 20 years by weekly or monthly transfers on agar plates containing penicillin. In more recent years, the medium has also contained 3% NaCl. The plates were incubated at 30 C for a few days and then kept in the cold room until subcultures were made. One of the cultures was originally an A type L culture of *Proteus* 52. Another L culture was a descendant of *Proteus* XK and corresponded neither to the A nor to the B type. The third was the L-form of *Proteus* strain 18. The fourth was an A type L culture of a *Salmonella* sp.

In contrast to the original L-forms, the altered old cultures grew abundantly on the surface of agar and in broth. On agar, penetration into the medium was rare and slight. The cultures grew with a moderate concentration of penicillin (400 units/ml) as well as without it. High concentrations of penicillin noticeably decreased growth, but even 10,000 units/ml did not prevent it. Bacterial forms did not reappear from these altered L-forms on consecutive transplants without penicillin nor in transplants on 30% gelatin. However, bacilli reappeared when the cultures were exposed to the influence of *Bacillus* Y. The bacilli were not motile, and no spreaders developed. Exposed to penicillin, they produced altered L-forms, different from the A and B types. The properties and morphology of the altered L-forms obtained from *Proteus* and from *Salmonella* were similar. Those obtained from *Proteus* fermented urea and had the characteristic odor of *Proteus* cultures; the strain obtained from *Salmonella* did not ferment urea.

Type A L cultures of four other strains of *Proteus* kept in subculture for 2 years on agar plates containing penicillin were transferred to broth and to soft horse serum agar, both with and without penicillin. One strain produced altered L-forms similar to those described above. The three other cultures transplanted without penicillin reproduced the usual bacterial growth. The bacilli were not motile immediately after they reappeared; however, after 72 hr of incubation, spreading forms developed and covered the plates. With penicillin they produced B type L cultures. Thus, these L cultures, originally of the A type, presented the usual properties of the B type; the slow development of spreaders, however, indicates some similarity to the L-forms maintained in successive transplants for longer periods.

Alteration of the L cultures was observed in both A and B type L cultures of *Proteus* 52 within a relatively short period of time. The parent bacterial strain which has been maintained for more than 20 years on agar continues to produce spreaders, and, exposed to penicillin, produces A and B type L colonies as it did immediately after its isolation (2). The B type colonies have the typical appearance, and bacteria reappear in them immediately after the elimination of penicillin, but they are changed in one respect. Originally, B type colonies either did not grow in transfer on the surface of agar or they produced a few A type colonies with an occasional B type. They now grow well in successive transfers on the surface of agar, although they remain of the B type in other respects.

On 20 November 1965 in a freshly isolated B type L culture of strain 52, after 1 week of incubation on a plate containing penicillin (400 units/ml), regular bacteria started to reappear at a few places at the periphery of confluent growth. A transplant was made from an area away from the bacterial growth to horse serum agar containing 400 units of penicillin/ml. The L culture that developed was different from the B type and resembled closely the cultures of the old altered L-forms. This L culture was designated as N20. Growth was less abundant in the presence of large concentrations of penicillin, but was not inhibited by 10,000 units per ml. During the first month after isolation, when transferred to media without penicillin, bacteria reappeared in the cultures within a few hours. These bacilli grew in thin long filaments and were not motile. Spreading forms were not produced. It is of interest that the L-forms reverted to bacteria en masse. Microscopic examination of the cultures at successive short intervals indicated that both the small and the large organisms grew into bacteria. After a few hours of incubation, before bacteria covered the surface of agar, no organisms originally present were seen in the transplants, only bacteria and a few large bodies. The cultures were maintained on plates containing penicillin and, when tested 1 month or later after isolation, bacteria could not be regained from them either by removal of the penicillin or by exposure to the strain of *Bacillus* Y.

In 1965, another variation of the L-forms of *Proteus* 52 developed within a relatively short period of time. A recently isolated, abundantly growing A type culture of strain 52 was kept at room temperature in a sealed petri dish on soft horse serum agar containing penicillin. After several months the original colonies were autolyzed and did not stain. Secondary growth which stained well and corresponded morphologically to L-forms was seen at the periphery of many of the old autolyzed colonies. The organisms developing in transplant in the presence of penicillin were morphologically similar to the old altered L-forms and to strain N20. This strain was designated as AJ. Transplanted without penicillin, bacteria were reproduced within a few hours in the same way as in N20. During the 6-month period that this culture was studied, it retained the tendency to reproduce bacteria upon withdrawal of penicillin.

Morphology of altered L-forms of Proteus and Salmonella. The morphology and the mode of reproduction of the L-forms altered by long cultivation and of strains N20 and AJ are similar, insofar as can be established, and will be described

together. The cultures consist of organisms varying greatly in size and shape: large organisms of 5 μm or more in diameter, smaller round forms, granules less than 1 μm, and filaments. The large bodies are of two types. One, less often present, is similar to the large bodies seen in A and B type L-forms. They are flat, moderately refractile, and slightly stained. The majority of the large bodies are as refractile and as darkly stained as growing bacteria; they are smaller than those of the A and B type L-forms and more resistant to mechanical distortion (Fig. 10). In young cultures they are often irregular and elongated with protrusions, and many intermediate forms between them and the smaller round forms (Fig. 11) may be seen. The small round forms (1 to 3 μm) in most cases are highly refractile, darkly stained, and they are not easily distorted (Fig. 24, 25). Old colonies often contain masses of unstained small granules. The filamentous forms present the greatest pleomorphism. They are often less refractile than bacteria and stained faintly. They are easily deformed and disrupted and tend to disappear as the colonies become well developed. The filaments can be thick as bacteria but usually they are less thick (Fig. 12, 13) and they also can be very thin (Fig. 14). True branching is present; the filaments do not divide or segment. The filaments and their branches usually end in round knobs of various sizes; some of these may grow to the size of large bodies. The best photographs of these filaments and knobs were obtained from broth cultures of the altered *Salmonella* L-forms (Fig. 16, 17). In some of the young colonies on agar, it is apparent that the small round forms are the knobs of filaments (Fig. 18, 23). Colonies composed of very small granules may also start to grow as thin filaments which later break up into granules (Fig. 19, 20). On a medium containing 2% agar and 10% urine, many small colonies of this type were seen after 24 hr of incubation. This interrelation of filaments with other morphological elements in the cultures suggests that the cultures are the result of the growth of a single strain and are not a mixture of strains with different hereditary properties.

In most instances individual colonies in a well-developed culture consist of a mixture of morphological elements, but sometimes the whole culture may consist preponderantly of one type of organism: regularly or irregularly shaped large bodies, medium-sized forms, or small granules. The filamentous forms were present in broth or shortly after transplant from broth to agar (Fig. 18); they did not form colonies on agar as did the other forms. Under the conditions of cultivation used, no regular bacterial forms were visible at any time in the cultures of these organisms.

The multiplication of round forms and filaments is apparent. The filaments grow from the round bodies and reproduce them. The multiplication of large, round forms by fragmentation, as it is seen in various L-forms (5), is also indicated. The development of young colonies consisting of small granules suggests the multiplication of the granules.

The distribution of different morphological forms varies in different cultures of a strain and filaments are less prevalent in broth cultures of *Proteus* XK and *Proteus* 18 than in the other altered strains studied.

DISCUSSION

Transplanting cultures from plate to plate over many years offers ample opportunity for contamination. The connection between the altered cultures and the L-forms is supported by two kinds of evidence. One is that the alteration of the cultures occurred only in L-forms of *Proteus* and *Salmonella* and was not seen in cultures of other L-forms or of *Mycoplasma* maintained under similar conditions, nor was it seen in bacterial cultures examined with the same microscopic techniques. The other evidence is that the properties of altered L-forms are transitional between those of L-forms and of the parent bacteria.

All L cultures of *Proteus* and *Salmonella* (four cultures) which were maintained over a period of 15 years were altered. From four A type L cultures of *Proteus* maintained similarly for 2 years, one was altered; the other three strains were altered only slightly in the same direction. Six strains of mycoplasma and the L-forms of the following bacteria were maintained similarly for long periods of time: three strains of beta-hemolytic streptococcus, seven strains of other streptococci, one strain of staphylococcus, two strains of diphtheroids, and one strain of *Erysipelothrix*. No growth similar to the altered L-forms of *Proteus* has been seen in them. *Proteus* and *Salmonella* exposed to penicillin produce A and B type L-forms, whereas in the other species only A type colonies are produced. Electron microscopic studies have shown that the altered L-forms have a cell wall like the B type L-form (Roger M. Cole, *personal communication*).

The transition from the classic A type L-form which lacks the cell wall to the B type, from the B type to the altered L-forms, and from these to the bacteria is apparent. The physical properties of the altered L-forms and the conditions necessary for their growth are similar in many respects to those of the bacteria. This transition to bac-

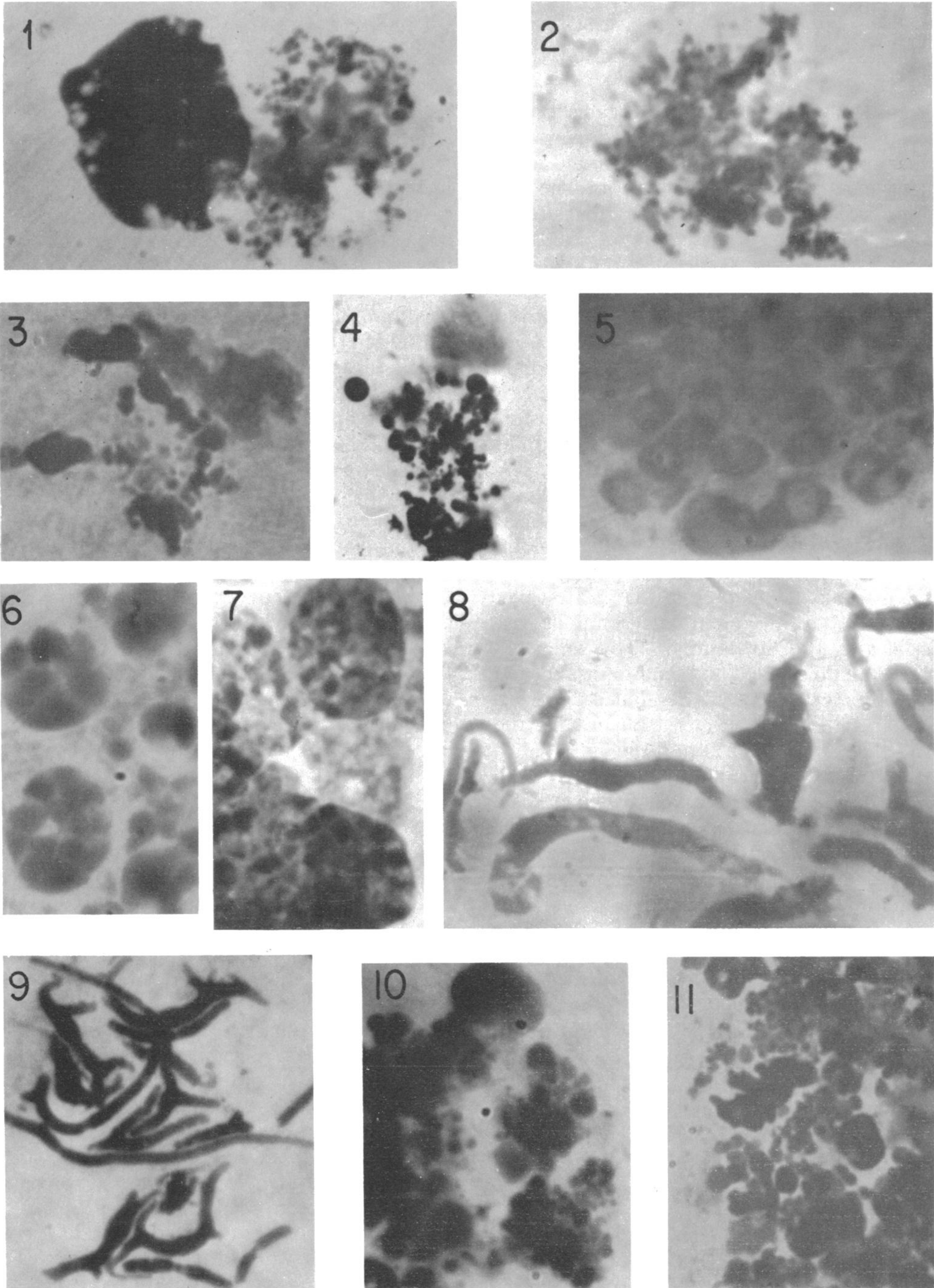

FIG. 1–11. *Photographs were made either from permanent stained-agar preparation or from impressions made by the agar fixation technique. All figures are ×2,250 unless otherwise noted. Figures 1 to 7 show A and B type L-forms for comparison with the altered L-forms (Fig. 10–25). Fig. 1. Large body developed from Proteus bacillus and growth of the small granules in the A type form within the agar from a disintegrating large body. ×3,000. Fig. 2. A type L-form embedded in soft, horse serum agar after 24 hr of incubation. ×3,000. Fig. 3. B type L-form after 24 hr of incubation. Large bodies and granules on the surface of agar. ×3,000. Fig. 4. Similar colony as in Fig. 3. (focus is set beneath the surface.) Darkly stained granules of different sizes. ×3,000. Fig. 5. Large bodies in B type L colony inside the medium after 2 days of incubation. Fig. 6. Large bodies from a B type colony on the surface of agar. The large bodies are disintegrating into smaller round forms. Fig. 7. Large bodies developing from bacteria on the surface of a membrane filter (Millipore) show condensations of their content into small round forms. Fig. 8. Large bodies developed from bacteria on solidified gelatin containing penicillin and transferred to blood-agar plates without penicillin; after 6 hr of incubation, they have increased in size, elongated, and produced branchings. Fig. 9. Culture similar to that in Fig. 8, but the large bodies have developed into thick branching filaments. Fig. 10. Old altered L-form of Proteus 52. One-day-old growth on the surface of agar. Fig. 11. Edge of a 1-day-old colony of N20 on the surface of agar. Small round forms and irregular darkly stained large bodies.*

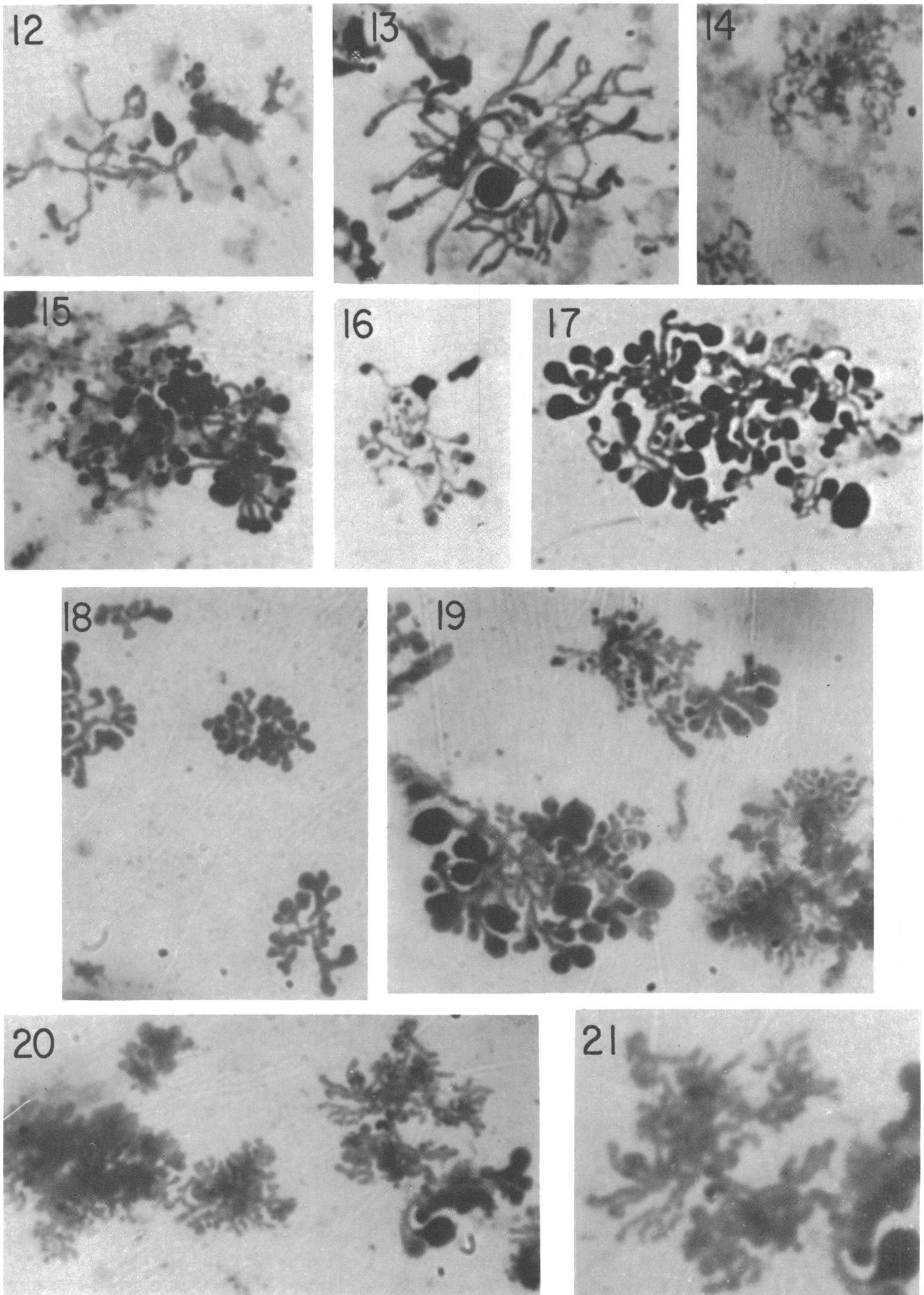

FIG. 12–21. *Photographs made as in Fig. 1–11. All figures are ×2,250 unless otherwise noted. Fig. 12 and 13. Altered L cultures of Proteus strain 52 in broth. Branching filaments with knobs or thickening at the ends. Fig. 14. Two small groups of thin filaments. Culture similar to that shown in Fig. 12 and 13. Fig. 15. Old altered L-form of Proteus. Thick growth of filaments with knobs in broth culture. Fig. 16 and 17. Old altered L cultures of Salmonella sp. in broth. In Fig. 16, very fine branching filaments with knobs at the ends. Fig. 18. Young colonies of old altered Proteus L on agar inoculated from broth. Filaments producing small and larger round forms. Fig. 19. Altered strain N20 transplanted from broth to agar after 1 day of incubation, indicating the early development of the colonies. Filaments with small and large knobs, thick growth radiating from the center, and thin short filaments disintegrating into granules. The medium contained 2% NaCl and 10% urine. Fig. 20. Same culture as in Fig. 19. One day of incubation. Small colonies produced by very thin branching filaments, also colonies of small granules. Fig. 21. Part of Fig. 20, enlarged ×3,500.*

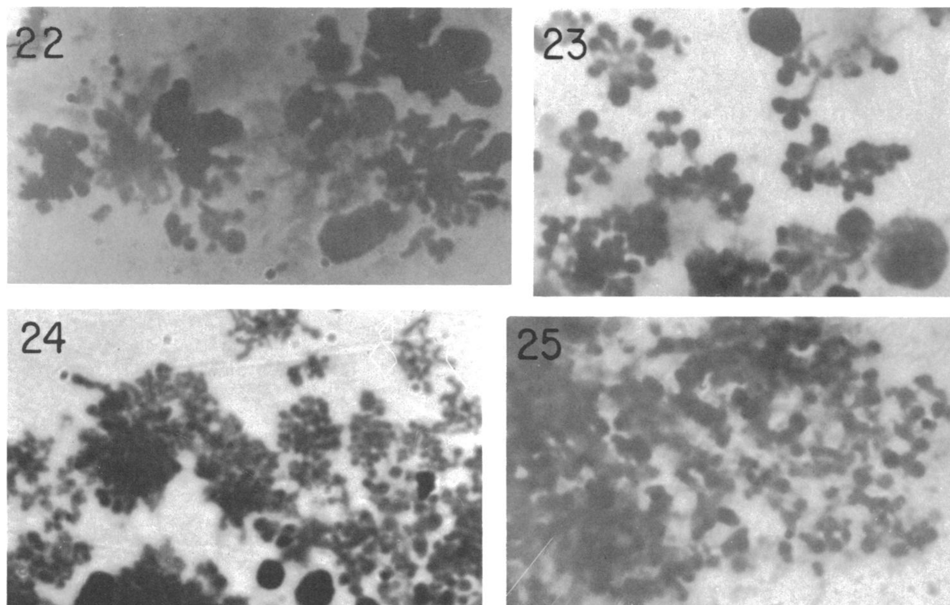

FIG. 22–25. *Photographs made as in Fig. 1–11. All figures are ×2,250. Fig. 22. Large bodies of strain N20 transferred from broth to agar. Fig. 23. Strain N20 transferred from broth to agar. Edge of a colony after 1 day of incubation. Connection of the round forms with the short filaments is apparent. Fig. 24. Strain N20. Growth on agar adjoining a large colony. Multiplication of small granules. Fig. 25. Strain N20. Edge of a colony consisting mostly of medium-sized round forms.*

terial form has been apparent in the cultures of AJ and N20. The altered L-forms developed from freshly isolated A and B type L-forms transplanted to plates containing penicillin. Transferred immediately after isolation to plates without penicillin, almost all organisms continued to grow as bacteria. Their morphology remained similar to that of the altered L-forms only in the presence of penicillin. After longer cultivation their morphology and the ability to multiply like bacteria remained, but the ability to regain full bacterial structure was lost. It is probable that the L-forms were altered by long cultivation in a similar way.

It can hardly be questioned that the altered L-forms were descendants of the typical L-forms of *Proteus* and *Salmonella*. Reconstruction of bacteria from L-forms is evidently a complex process which may be altered in various ways and arrested at various stages; under special conditions, survival and multiplication may be possible at different stages of this process. It is probable that organisms with somewhat different hereditary properties are produced and that some of these may be eliminated in successive transplants. For this miscellaneous group, in which every strain has more or less individual properties, I propose the provisory designation "C type L-forms." All are transitional forms between the classical L-forms and bacteria with full structure. Pleomorphic organisms in some bacterial cultures show similarities to the C type L-forms. One example seen by the author has been the growth in the form of small granules of *Hemophilus hemolyticus* isolated from the human throat.

The filaments observed in the altered L-forms are in many respects different from the usual bacterial filaments. They do not segment into bacilli but produce round forms or small granules. In *Proteus*, branching structures are produced in various conditions under the influence of penicillin. Irregular branching masses and thick branching filaments are often produced when the large bodies produced by penicillin from *Proteus* bacilli return to bacterial form (Fig. 8, 9). Similar thick filaments were produced from L cultures of *Proteus*, partially altered by 2 years of cultivation. On transplant of the L culture of *Proteus* strain 49 on media with penicillin, branching filaments grew out from the large bodies at first. These soon degenerated and disappeared, and only the L-forms developed further. Apparently the conditions created by penicillin which induce growth of branching filaments remain in the altered L-forms. These filaments do not indicate the presence of bacteria with regular structure or of contaminants in the culture.

The study of these altered L-forms needs ex-

tension in various directions. Their connection with the bacteria should be checked by biochemical and genetic methods. Their chemical and serological properties have not been studied, nor have their growth requirements or the influence of different media on their morphology and on their reversion to bacteria. These altered L-forms will probably be useful in the study of the development of the cell wall of bacteria and of its structure. However, not only is it desirable to study the available cultures further, but also the whole process of transformation needs to be investigated. Observation of the development of altered L-forms during a short period of time suggests methods by which such cultures may be obtained.

Considering the variability of L-forms of bacteria, it is inadequate to characterize them either as "stable" or "unstable," especially in those species in which both A and B type L-forms occur. It is necessary to indicate their origin, their morphology, and their characteristic properties.

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