CYTOLOGICAL OBSERVATIONS ON *BACT. COLI*, *PROTEUS VULGARIS* AND VARIOUS AEROBIC SPORE-FORMING BACTERIA WITH SPECIAL REFERENCE TO THE NUCLEAR STRUCTURES

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(With Plates 5–8, and 3 Figures in the Text)

INTRODUCTION

Discrete Feulgen-positive chromatinic bodies, occurring in regular numbers and going through a regular cycle of division, were first demonstrated convincingly by Stille (1937) and Piekarski (1937) in several well-known species of sporing and non-sporing bacteria. The literature on the subject has already been briefly reviewed in a previous communication (Robinow, 1942) in which the nuclear apparatus in the spores and vegetative cells of *B. mycoides* and various other bacteria was described.

The first part of the present paper deals with the shape and behaviour of the chromatinic bodies of *Bact. coli* and *Proteus vulgaris*, and in particular with the numerical relationships and distribution of these bodies in growing bacteria from young cultures. The technique of fixing, staining and observing the material has been described in some detail because scepticism about the chromatinic bodies in bacteria is usually due to the use of inadequate cytological methods and unsuitable optical equipment.

The latter part of the paper is concerned with observations on the cellular structure of bacteria from young cultures. Since de Bary's original description of *B. megatherium* (1884), it has been known that the rod forms of some of the aerobic spore-bearing bacilli are not single units but are composed of several separate *cells*. The validity of this structural principle for growing bacteria of many species besides *B. megatherium* is not always realized, though it is recognized by some workers (cf. Migula, 1897; Nakanishi, 1901; Knaysi, 1930; and Badian, 1933). My own observations on suitably stained and plasmolysed bacteria of several species provide further evidence in support of the view that rod forms have a composite structure from the earliest growth stages.

MATERIAL

The following organisms were investigated:

Bact. coli: a strain received from the National Institute of Medical Research, Hampstead;

Proteus vulgaris: No. 5821 Nat. Coll. Type Cultures and a strain isolated from human faeces in the Pathology Department, Cambridge;

and for purposes of comparison:

B. mycoides: No. 16 Nat. Coll. Type Cultures;

B. mesentericus: from the collection of Dr W. J. Dowson of the Botany School, Cambridge;

B. megatherium: isolated from the soil of the Laboratory garden and identified by Dr T. Gibson, Edinburgh;

B. cereus: from the Nat. Coll. Type Cultures.

Although observations were made on only one strain of *Bact. coli* and two strains of *Proteus vulgaris*, experience has shown (Piekarski, 1937–40; Neumann, 1941; E. Klieneberger, unpublished observations; and Robinow, unpublished observations) that there is such a close similarity between the chromatinic structures in different species that nothing is to be gained by a comparative study of many strains belonging to the same species.

TECHNIQUE

Nutrient agar (heart) and broth (heart), both from Messrs British Drug Houses Ltd., were used as culture media throughout the investigation.

The chromatinic structures were differentiated from the cytoplasm by treating the osmium-fixed cells with *N*/HCl at 60° C. before staining them with Giemsa solution (Piekarski, 1937; Robinow, 1942). This method gives preparations of uniform clarity and is equally useful for studying nuclear structures in whole mounts of yeasts, moulds and blue-green algae. The effect of the treatment with hydrochloric acid is not to make the chromatinic bodies more stainable as is sometimes supposed, but merely to remove from the cytoplasm something with a strong affinity for Giemsa's stain which obscures the chromatinic structures in the interior of the cell. The chromatinic structures can be demonstrated without hydrolysis (Badian, 1933; Neumann, 1941) but less clearly and regularly.

The details of the procedures adopted were as follows:

A. *Fixation*

(1) *Spore-forming organisms*

Air-dried films of spores made on glass cover-slips from cultures on potato agar, were covered with two or three narrow strips of nutrient agar and incubated in a moist chamber at 37° C. for 2–3 hr. For the staining of nuclear structures the agar strips were removed and the cover-slips quickly transferred to a deep dish, well sealed by a greased glass plate, where 5 ml. of 2% osmium tetroxide wetting three layers of glass balls produced a strong concentration of osmic vapour. After 2–3 min. the films were allowed to dry in air, then treated for 1–2 min. in Schaudinn's sublimate alcohol warmed to 45–50° C., and finally rinsed and stored in 70% alcohol. The secondary fixation with sublimate, recommended

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by Giemsa (1912), is not indispensable, but the contrast between chromatin and cytoplasm in the stained preparation is much sharper and the staining is more uniform throughout the preparation than after osmic vapour fixation alone.

For the demonstration of cytoplasmic cell boundaries (vide infra) E. Klieneberger's (1934, 1942) method of fixation through the agar with Bouin's fluid was used in a simplified form. The cover-slips bearing the agar strips were left for 30–45 min. or longer in a large volume of the fixative after which the agar was removed and the preparation stored in 70 % alcohol.

(2) Proteus *and* Bact. coli

After many disappointments the making of wet smears was completely abandoned in favour of impression preparations or of dry smears from material fixed in osmic vapour while still on the agar. To make an impression preparation, a square was cut from the agar plate, not larger than could easily be handled by the tip of an iridectomy knife and accommodated on a $\frac{7}{8}$ in. sq. cover-slip. This square was exposed to osmic vapour for 2–3 min. and was then placed face downwards on a clean, dry cover-slip. After removal of the agar, the film of fixed bacteria deposited on the cover-slip was dried, treated with warm sublimate alcohol as described above (§ A (1)), and finally rinsed and stored in 70 % alcohol. Klieneberger's method of fixation through the agar with Bouin's fluid was also used (see § A (1)).

Not only are the bacteria in the impression films broader than those in ordinary smears but all of them are dried within a few seconds which prevents them from becoming arranged in clusters; also they stick to the glass surface more evenly and tend to form well-spaced groups with all their elements at the same level of focus. Impression preparations from plate cultures have the further advantage of providing ample material during the first few hours of incubation, the stage of the growth cycle when much the clearest results are obtained but when there is very little to scrape off an agar slant.

To obtain preparations with many cells in the earliest stages of growth a loopful of bacteria from an 18–24 hr. slant culture, incubated at 37° C., was emulsified with 2–3 ml. of tap water, and three or four drops of this milky suspension were spread on a freshly poured and dried nutrient agar plate. The plate was again dried, incubated and impression preparations were made in the usual manner from small pieces of the medium, cut out and fixed at suitable intervals; an '0 hours' sample was taken immediately the inoculum had dried on the plate.

Impression preparations were found profitable only if there were not more than one layer of bacteria on the agar. Accordingly plates spread with a few drops from a very rich suspension were used only to study the earliest stages of development and more dilute inocula forming microscopically well-separated colonies, were used for preparations of the growth stages from 4 hr. onwards. Films from confluent growths on solid media were obtained by first making a suspension of the material in tap water, then spreading some of it on fresh agar, fixing it immediately after drying and making an impression preparation.

Particularly clear and instructive preparations of the chromatinic structures of *Proteus vulgaris* were obtained both from the earliest growth stages after subculture on a fresh nutrient medium and from dry smears of fixed material from the raised edge of a colony on an agar plate towards the end of one of the periods of 'consolidation' which alternate with periods of rapid swarming (Russ-Muenzer, 1935). Impression preparations of fixed, swarming *Proteus* filaments show very regular spacing of the chromatinic structures within the filaments, but cytological detail is not very clear.

B. Staining

Method 1. From 70 % alcohol films were transferred directly to *N*/HCl warmed to 60° C. and 'hydrolysed' for about 10 min. They were then rinsed in tap water and two changes of distilled water and floated on the staining solution made with 2–3 drops of Gurr's 'improved Giemsa-stain R 66' per ml. of phosphate buffer (Balint, 1926), or a mixture of equal parts of tap water and distilled water. Duration of staining differed according to whether the preparations were to be differentiated and dehydrated in the conventional acetone/xylol mixtures (pure A—A 14 pts, X 6 pts—A 6 pts, X 14 pts—a few seconds in each, followed by three changes of xylol, 10 min. in each), or whether they were to be examined undifferentiated, *mounted in water*. In the latter case it sufficed to stain for half an hour at 37° C., whereas several hours were required when the stained preparations were to be dehydrated and mounted in canada balsam. In water the outlines of the bacterial cytoplasm are much more clearly defined than in balsam-mounted preparations, the bacteria appear broader, and in hydrolysed preparations cell boundaries are visible together with the chromatinic structures, whereas most of the boundaries become invisible during the process of dehydration which precedes mounting in balsam. Photomicrographs too are often more satisfactory in their contrasts and richer in detail when taken from water-mounted material, particularly where very small elements are concerned such as the cells of old cultures. Sealed with wax, water-mounted preparations will keep their colour contrasts for several days; finally they may be stained once more, dehydrated and mounted in canada balsam.

Method 2. A quick way to obtain passably well-defined pictures of chromatinic structures is to dip unfixed, air-dried impression preparations for 5 sec. into boiling *N*/5 HCl, rinse and mount in 0·1 % crystal violet in water.

Method 3. To stain the cell boundaries, Bouin-fixed films were floated on Giemsa stain of the same strength as that used for nuclear structures, for not more than 3 min., rinsed and taken through 70, 96 and 100 % alcohol (5–10 sec. in each) to xylol and canada balsam. When examining such preparations an orange light filter was often used.

Method 4. The Feulgen reaction was carried out according to the prescriptions given for ordinary cytological work, except in staining *B. megatherium* for which the optimal conditions of hydrolysis were found to be 10–15 min. at 40 instead of 60° C.

Method 5. To demonstrate the cell wall a plasmolysis-like effect was produced in *B. megatherium* by dipping air-dried, unfixed vegetative cells grown from spores for 2–2½ hr. under agar strips (see § A (1)) into boiling 2·5–8·0 % NaOH for 15–30 sec. The films were then rinsed in water and mounted in a 0·5 % watery solution

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of crystal violet. The shrunk cytoplasm stains deep blue to purple and the cell wall a light pink. The sodium hydroxide solution was prepared by dissolving one pellet of the reagent in 10–30 ml. of water.

Optical methods

In all staining operations much 'trial and error' can be avoided by using a *water immersion lens* before the preparations are mounted. Economy in the use of fixing and staining reagents can be achieved by employing cover-slips instead of slides. The cover-slips should be mounted on slides of a thickness not greater than that tolerated by oil-immersed achromatic substage condensers of high numerical aperture. The light source should be minute, at the correct distance from the microscope and fitted with a field stop. For work on the bench the Koehler principle of illumination has been found convenient and has been used throughout. The immersion objectives should be of a quality permitting the use of a 15 times eyepiece, since the magnification of about a 1000 times, commonly employed in bacteriological routine, is not high enough for a detailed study of the chromatinic structures.

RESULTS

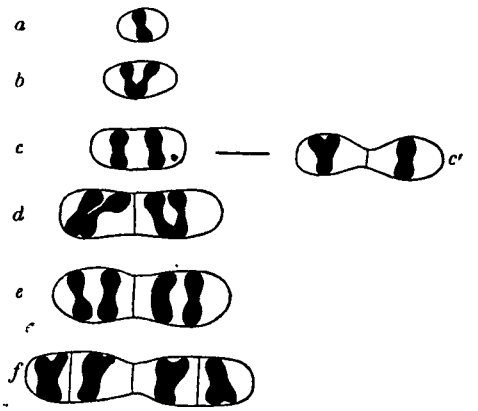
(1) *The chromatinic structures of the cells of young cultures of* Bact. coli *and* Proteus vulgaris

Impression preparations made from agar plates during the first few hours of growth show masses of transparent bacteria containing brilliantly stained, sharply defined and regularly spaced chromatinic structures.

The 18–24 hr. slant cultures from which the plate cultures are seeded consist chiefly of three different types of bacteria. Small coccoid elements, short plump bacilli and small slender rods. This diversity of form persists during the first few hours of growth on the plate (Pl. 5, figs. 1, 2).

The division processes of the chromatinic structures during the development of the coccoid forms (type 1) of *Bact. coli* into the familiar rod-shaped bacteria follow the simple pattern previously outlined for *B. mycoides* (Robinow, 1942) (Text-fig. 1). The first visible changes in the chromatinic structures of coccoid elements from 18 to 24 hr. slant cultures awakening on a fresh nutrient medium are: increase in size, in the depth of staining and in the intensity of the Feulgen reaction. Next the chromatinic structure divides into two closely contiguous dumbbells. The daughter dumbbells increase in width, their contours become asymmetrical and often before completing their separation, they in turn proceed to split longitudinally (Pl. 5, figs. 1, 2). A small round granule or strands of some faintly coloured or of some chromatinic material often persist for some time between separating dumbbell bodies (Pl. 5, figs. 1 *e*, *f*, 2 *b*, *c*, 9 *a*). After the first division of the original chromatinic structure, the cell may divide directly by constriction into two separate ovoid daughter cells. Usually, however, division of the young bacterium does not take place until after the second or third division of the chromatinic bodies. The early development of coccoid forms of *Proteus vulgaris*, whether from an 18 hr. agar slant or from the first belt of confluent growth proximal from the zone of swarming in an agar plate culture, is essentially the same as that of *Bact. coli* (Pl. 5, figs. 10–12). In the two strains of *Proteus* used in this study awakening of the cells was, however, much more protracted than in *Bact. coli*, and consequently a higher proportion of cells with single chromatinic bodies were found in young subcultures.

If the early growth stages of *Bact. coli* and *Proteus* are compared with those of *B. mycoides*, there is found to be a close similarity (compare Pl. 5, figs. 1, 2 with Pl. 7, figs. 25–27). The smallest elements of *Bact. coli* and *Proteus* resemble the first vegetative generation of



Text-fig. 1. Diagram of successive division stages of the chromatinic bodies from the beginning of the lag phase, after transfer to a fresh nutrient medium, to the first division of the growing bacterium. The diagram is based mainly on preparations of *Bact. coli* but applies equally well to the early development of *Proteus vulgaris*. *c*–*c'* and *c*–*f* are alternative modes of development, *c*–*f* being that most commonly followed.

Compare:

Stage *a*, *b*	...	with smallest elements in	... Pl. 5, figs. 1, 2, 11
Stage *c*	...	with	... (*c*) in Pl. 5, figs. 2, 12
Stage *d*	...	with	... Pl. 5, fig. 4
Stage *e*	...	with	... Pl. 5, figs. 5–7
Stage *f*	...	with	... dividing bacterium (*c*) in right-hand bottom corner of Pl. 6, fig. 20, and (*d*) Pl. 7, fig. 24

Compare stage *c'* with constricted bacterium (*c*) near the top in Pl. 5, fig. 11.

Cell boundaries in (*f*) inferred from Bouin-Giemsa preparations.

B. mycoides in their ovoid shape and in the double structure and variable position in the cell of the chromatinic body. In all three species the mode of division of the chromatinic body is essentially the same and the products of one division frequently split precociously for the next division before separation is complete *B. mycoides* (as well as *B. megatherium*) differs from *Proteus* and *Bact. coli*, however, in so far as division of the bacterium does not occur until after the third division of the chromatinic bodies (stage of four double chromatinic structures), whereas in the two non-sporing organisms,

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as stated above, it may already occur after the first division of the chromatinic body.

When plump bacillary forms (type 2) are reactivated by transference to a fresh nutrient medium the two, three or four chromatinic structures which each contains first stretch and enlarge, and then divide into dumbbell bodies which become symmetrically distributed along the bacillus. In this way the same type of bacterium with two or four resting or dividing chromatinic bodies is produced, as that derived from the coccoid forms.

When the slender rod forms (type 3) are about to resume their growth activities, their chromatinic structures are at first arranged in a long, almost homogeneous core (Pl. 5, figs. 1, 2, and fig. 9, top corners). During the first 2 hr. after subculture, the chromatinic core, which is presumably a column of tightly packed chromatinic elements, breaks up and forms groups of regularly spaced dumbbell bodies between which cytoplasmic boundaries then develop. Eventually large rod forms result which have the same structure and nuclear complement and follow the same development as the rods directly derived from the coccoid elements. For example, in Pl. 5, fig. 1, the very large bacterium, above (*a*), has probably developed from a rod with three chromatin masses like that at the extreme right edge of the same figure; this would explain the unusual asymmetrical distribution of the chromatinic bodies in the large bacterium.

The redistribution of the chromatinic matter and its differentiation into dumbbell bodies in the slender rod forms of reactivated cultures of *Bact. coli* and *Proteus vulgaris* is essentially the same process as that previously described in the vegetative cells from old cultures of *B. mycoides* after transference to fresh nutrient medium (Robinow, 1942).

During the second hour of incubation the bacteria, which are now predominantly rod-shaped (Pl. 6, figs. 20, 21) begin to react differently to Giemsa's stain. The chromatinic bodies in the early stages are stout and very deeply stained (cf. Pl. 5, figs. 1, 2 and 7) and the cytoplasm assumes a reddish colour; in the older bacilli the dumbbell bodies become slender and in between divisions stain comparatively faintly, while the cytoplasm, though it may show a reddish tinge while still in the staining solution, becomes light blue in the acetone-xylol mixtures by which it is now very easily decolorized.

At all stages of growth division of the chromatinic bodies may result either in two single dumbbells which separate completely before beginning a fresh process of division (Pl. 5, figs. 5–7), or the daughter bodies may start to divide before they have yet completely separated, as described above for the first division of the chromatinic structure. From the second hour onwards, the greater transparency of the cytoplasm renders the differences between faintly stained, slender, resting dumbbells and deeply stained, stout, dividing dumbbells much more distinct than in the earliest growth stages when the cytoplasm is more heavily stained.

When two plump dumbbell bodies of symmetrical build are closely contiguous it is impossible to see whether their long axes are parallel or at right angles to the long axis of the cell. (For an illustration of this difficulty, as well as its solution, in the case of a large spore-forming bacterium see Pl. 6, figs. 15 and 16.) The latter arrangement is the usual one; Neumann (1941) describes cases, however, in which one or two dumbbell bodies lie near the surface of the bacterium and parallel with the *long* axis of the cell. As a rare occurrence I have confirmed this observation in my own cultures.

As the culture gets older the bacteria become shorter and narrower and their chromatinic structures increasingly difficult to resolve (Pl. 6, figs. 20, 21); at the edge of isolated colonies, however, bacteria with well-differentiated chromatinic structures can be demonstrated until the growth of the colony ceases.

At the edge of colonies of *Proteus vulgaris* growing on a suitably moist solid medium, periods of intense reproductive activity alternate with the well-known phenomenon of *swarming*. The chromatinic structures in the long filaments of the swarming zone are very regularly spaced (Pl. 6, fig. 14) but the individual elements are less clearly resolved than in the short rods (Pl. 6, fig. 13) which predominate in the marginal zone of a *Proteus* colony between the periods of swarming. The long filaments may divide by breaking into pieces of equal length (Pl. 6, fig. 14 *b*), but usually fragments of variable length break off from one end. The cause of the periodic changes in the periphery of *Proteus* plate cultures remains obscure (cf. Russ-Muenzer, 1935).

(2) *The composite structure of rod-shaped bacteria*

The mono- and multinucleate elements described above are really single and multiple forms of a basic building unit possessing a single chromatinic body (Pl. 5, fig. 10 *a*). The simplest form of this unit is represented by the coccoid elements found in old cultures and in the marginal zone of *Proteus* colonies between the periods of swarming and the composite form by the rod-shaped bacteria which predominate in young cultures. Although the rod-shaped bacteria appear homogeneous in ordinary preparations, their composite structure can be clearly demonstrated in the following ways:

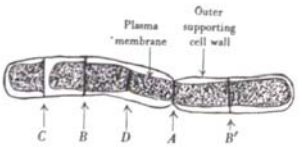
(*a*) *Staining (Bouin fixation)*. When bacteria are fixed through the agar with Bouin's fluid (see p. 414) and stained for not more than a few minutes with Giemsa solution, the nuclear bodies and the cell wall are not revealed but most of the rod-shaped elements show a regular pattern of dark transverse lines and their tips also are deeply stained (Pl. 8, figs. 28, 29; Pl. 7, fig. 24). The lines are absent in the smallest elements though the poles of the cell may be deeply stained (Pl. 8, fig. 28 *a*).

The relative position of the dark lines and the chromatinic bodies can be determined by comparing bacteria treated by the Bouin-Giemsa method with elements of equal length in preparations made by the OsO_4-HCl-Giemsa technique (cf. Pl. 6, figs. 17, 18). This comparison shows that the dark lines always occupy the space *between* the chromatinic structures, which explains why the smallest elements with only one chromatinic body lack transverse lines.

Young bacilli in Bouin-Giemsa preparations have the following appearance (Pl. 8, figs. 28, 29). Usually a very broad and dark double band runs across the middle of the rod with a fine line symmetrically placed on either side of it so that the rod is subdivided into four equal sections. In long bacteria there may be several thick double lines evenly distributed on either side of the broad central band, with a fine line midway between each pair of thicker ones.

If the bacteria are studied in the order of gradually increasing length, it is easy to see how the pattern of lines in the long forms is derived from the simple arrangement in the short rods by the transformation of fine lines into broad ones and by the appearance of new fine lines midway between those which are beginning to thicken. The contour of the cytoplasm, which is deeply stained, has many irregularities similar to those observed by Knaysi (1930) in cells of *B. subtilis*: 'The ectoplasm shows thickenings and appendages of various sizes and shapes on its internal surface.'

When the bacterium divides, the plane of division always coincides with the broad central band. The constriction occurs in the middle of this double band which at this stage has the appearance of a plump *x* (Pl. 7, fig. 24 *c*, *d*, *e*). This suggests that the cytoplasm in the deeply stained region is concerned with the formation of the new transverse cell wall (cf. bacillus (*a*), Pl. 8, fig. 30, and fig. 1 of Knaysi, 1930). Since the dark lines are visible long before the bacterium has begun to show signs of constriction it must be assumed that transverse divisions, either cytoplasmic or of the same nature as the outer cell wall, are laid down long before the bacterium has begun to 'divide' in the accepted sense of the term.



Text-fig. 2. Semi-diagrammatic drawing of a plasmolysed, almost completely divided specimen of *B. megatherium* from a preparation made as described under Method 5, p. 414. Dimensions: $12{\cdot}4 \times 1{\cdot}5\,\mu$. Letters indicate transverse cell walls (*septa*) in order of decreasing age; *D* points to a cytoplasmic membrane which resulted from the division of the protoplast *AB*. A transverse septum, continuous with the outer cell wall laid down in the plane of this boundary, would have completed the spatial separation of the two sister protoplasts. Compare with Pl. 8, figs. 31–34.

(*b*) *Plasmolysis.* In studying the chambered structure of bacteria it would, however, be of advantage to see the cell wall clearly stained together with the cytoplasm. Knaysi (1930) obtained this result in *B. subtilis* by suspending the cells in a drop of 25 % watery solution of NaCl containing one per mille of crystal violet. Using a different method suggested by the experiences of Ruhland & Hoffmann (1925) with *Beggiatoa mirabilis* and staining with either crystal violet or Giemsa (see Method 5, p. 414) I have obtained preparations of the much larger cells of *B. megatherium* and also of *B. cereus* in which cell wall and cytoplasm were differentially stained and the cytoplasm had shrunk from the cell wall which, as Knaysi (1930) has already pointed out, is a great advantage in studying the mode of division of the bacilli. Unfortunately, I was unable to produce this retraction of the cytoplasm at all regularly in *Bact. coli* and *Proteus*, so that the observations made by this method refer almost exclusively to *B. megatherium* and *B. cereus*.

Although permanent mounts can be made, freshly stained preparations mounted in water give the sharpest and most brilliant pictures. In such material the cell walls are pink and the cytoplasm a dense mauve or purple. The rods consist of two, three or four separate portions divided from each other by transverse septa giving the same staining reaction as the cell wall. Owing to the shrinking of the cytoplasm the protoplasts are retracted from both the outer cell wall and the transverse septa, so that the septa are very clearly seen (Text-fig. 2; Pl. 8, figs. 30–34). The youngest cells are nearly cubical; they grow and elongate until their length is about three times their width when a new transverse septum is formed and the growth process repeated. Heavy staining of plasmolysed* films, which is necessary to demonstrate the cell wall, tends to obscure the cytoplasmic boundaries which are the precursors of the transverse septa. Light staining of plasmolysed films, as well as the OsO_4-HCl-Giemsa and the Bouin-Giemsa technique show that oblong photoplasts, even though they may have retracted as a whole, actually consist of two more or less completely divided cells. Thus had the bacillus at the top of Pl. 8, fig. 30 been adequately plasmolysed and more heavily stained it would probably have presented the same appearance as the right-hand bacillus in Pl. 8, fig. 33. Text-fig. 2 gives an example, at (*D*), of a protoplast that is still only divided by a cytoplasmic membrane, while neighbouring cells in the same bacterium are already separated from this protoplast and from each other by transverse partitions which are continuous with the outer cell wall.

These results confirm de Bary's original description (1884) of the multiple structure of the large *Megatherium* rods. They also show that the process of annular furrowing by which whole bacteria are seen to divide is really the splitting of a preformed transverse partition (Text-fig. 2 A; Pl. 8, fig. 33). In the description of bacteria the term 'cell division' should be restricted to the formation of the cytoplasmic membranes—precursors of transverse partitions—which develop in the protoplasts after the separation of recently divided chromatinic structures.

Although it was not possible to make good plasmolysed preparations of *Bact. coli* and *Proteus* with the NaOH method, the number of separate cells in the plasmolysed bacteria of *B. megatherium*, i.e. two, three or four, corresponds with the number of sections into which the transverse lines seen in Bouin-Giemsa preparations usually divide the rods in young cultures of *Bact. coli* and *Proteus* (cf. Pl. 7, fig. 24, and Pl. 8, figs. 30–34).

(*c*) *Chilling.* If the average bacterium in a young culture consists of two to four cells as the foregoing results indicate, it was thought that a mildly damaging treatment might not affect all the cells of the same rod equally. To test this, young cultures of *Bact. coli* were chilled in the refrigerator at 2–4° C. for periods ranging from 20 hr. to several days. The expected result was obtained, and among many hundreds of normal or severely damaged bacteria, a few regularly presented the appearance shown in Pl. 8, figs. 35–37.

* In this paper the term 'plasmolysed' is used, for the sake of convenience, in a purely morphological sense and in disregard of the physico-chemical differences between the (irreversible) method of fixation with NaOH and the reversible response of plant cells to which the term normally refers.

In preparations made by the Bouin-Giemsa technique but more heavily stained than for the demonstration of cell boundaries, one-half of such a differentially affected rod is seen to have partly disintegrated and stains faintly pink, while the other half has the deep blue colour characteristic of bacteria which were alive and growing at the time of fixation. The half damaged rods show no sign of a medial constriction but have the usual smooth contour of resting bacteria; yet under the conditions of the experiment each behaves as if consisting of two physiologically independent units. Presumably this independence is not absolute and the fact that most of the affected rods disintegrated as a whole implies some kind of plasmatic connexion between the two cells of which each is composed.

(3) *The relationship of the transverse septa to the arrangement of the chromatinic bodies.* As stated above, if bacteria stained to show the cytoplasmic boundaries are compared with bacteria of equal length stained for the chromatinic bodies, it is found that the chromatinic bodies lie between the boundaries. This alternation of the two structures can also be demonstrated in the same bacteria by a slight modification of the osmium-HCl-Giemsa method.

In ordinary hydrolysed preparations of *B. megatherium* and *B. mycoides* stained with Giemsa's solution, red lines, corresponding in position with the transverse partitions made visible by the sodium hydroxide treatment are often visible in the differentiated, dehydrated films mounted in canada balsam (Pl. 7, fig. 25), but such lines are rare in similar preparations of *Bact. coli* and *B. mesentericus*. If, however, films of these organisms are mounted in water immediately after staining and without differentiation the lines are clearly seen bisecting the rod-shaped elements and intervening between recently divided dumbbell bodies (Pl. 6, figs. 15, 16, 17). They are more distinct in *B. mesentericus* than in *Bact. coli* in which they are usually too thin and faintly stained for good photography (Pl. 5, fig. 9).

Normally cytoplasmic cell boundaries divide both short rods and long winding filaments into a series of cells, each with its own single or multiple, i.e. dividing, chromatinic structure.

If for some reason growth occurs without division of the cytoplasm there is no orderly arrangement of the chromatinic bodies. Long filaments are formed in which no cell boundaries can be demonstrated and in which the distribution of numerous chromatinic bodies is chaotic. Such filaments (Text-fig. 3) arise spontaneously in cultures of *Bact. coli* and *Proteus*, where they were also observed by F. Neumann (1941), especially if derived from old stock and they can be produced experimentally (Robinow and Lea, unpublished observations) by certain doses of ionizing radiations which do not interfere with cytoplasmic growth (Lea *et al.* 1937).

(4) *The chromatinic structures of the cells of old cultures.* Even in old cultures kept from 18 hr. to 4 days at 37° C., or for 2 days to several months at room temperature, cells with well-developed chromatinic structures are never completely absent, a fact already noted by Neumann (1941).

As stated above, such cultures contain three main types of bacteria: small coccoid forms, short stout rods and small slender rods. The first predominate in old cultures of *Proteus vulgaris*, while all three are represented in 18–24 hr. slant cultures of *Bact. coli*. When stained for chromatinic matter by the OsO_4-HCl-Giemsa method, old cultures present a variegated appearance. A few colourless and structureless elements are obviously dead remains (cf. cell above (*e*) in Pl. 5, fig. 2), but most bacteria of all three types contain one or more chromatinic bodies (lower half of Pl. 7, fig. 2). The chromatinic structures are either deeply stained and centrally placed not touching the periphery, or less densely stained dumbbell forms extending right across the cell (Pl. 7 fig. 22 *b*). The minute coccoid forms are best studied in preparations mounted in water in which it is possible to see the delicate layer of cytoplasm surrounding the relatively large chromatinic structures; after dehydration and decolorization this marginal cytoplasm is often invisible.

Neumann (1941) has reported a diffuse Feulgen reaction in all the cells of old cultures, I have found examples of both diffuse and localized positive reactions in cells from 18 to 24 hr. slant cultures, but these elements are so small that interpretation is very difficult and I have been unable to reach any definite conclusions about the results of the Feulgen test at this stage.

Preparations of old cultures made by the Bouin Giemsa method for the demonstration of cell boundaries present a remarkable contrast to similar preparations of young growing cultures. Instead of the bright, pure blue coloration and conspicuous banded or chambered appearance characteristic of the young bacteria (Pl. 7 fig. 24), the great majority of the old bacilli of all three types stain a faint mauve or dull light blue and show little or no sign of the differential staining which indicates division processes (Pl. 7, fig. 23).

These differences in staining reaction reflect the well-known change seen in living bacteria after sub culture, when the pale transparent cytoplasm of the old cells acquires a brightly refractile appearance during the so-called 'lag-phase' (which comprises all but the last developmental stages shown in Text-fig. 1).

Quantitative observations on five slant cultures of *Bact. coli* incubated for 24–18 hr. at 37° C. were made from films prepared by the Bouin-Giemsa method for the demonstration of cell boundaries; 500–600 bacteria from each culture were examined. In these five cultures active bacteria with deeply stained and clearly subdivided cytoplasm constituted only 4·7, 4·8, 5·5, 8·0 and 12·0 % respectively of the total count. The remaining bacteria were faintly and uniformly stained and the cell boundaries were either absent or indistinct. On the other hand, osmium-HCl-Giemsa preparations from two of the same cultures showed that 65–67 % of the bacteria contained more than one chromatinic body. Whether all the bacilli in which cell boundaries are indistinguishable are really single cells, or whether the intercellular boundaries are sometimes present but at this stage are unstainable by the methods used, is not certain.

In spite of the diffuse Feulgen reaction and apparent absence of cell boundaries in most bacteria in old (18–24 hr.) cultures, the great majority of these organisms are fully viable. Neumann's view that there is an 'early degeneration of the cells in well-grown cultures is untenable when the changes induced in the chroma

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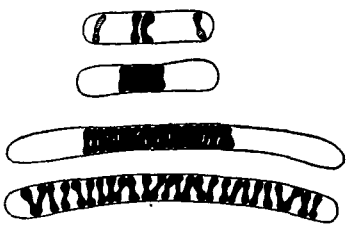
tinic structures by 45–90 min. incubation (i.e. during the lag phase) on a fresh nutrient medium are studied. The results of such observations show that all but a small proportion of the bacteria undergo the growth changes described in the preceding sections.

(5) *Atypical forms.* To complete this account of the chromatinic structures, three abnormal types of cells must be mentioned which occur in cultures of *Bact. coli*, *Proteus vulgaris* and many other species (Text-fig. 3). Of these, the most interesting are bacteria of normal length which apparently are not divided into two cells; they have a faintly stained dumbbell body at each end and a more deeply stained pair of these bodies near the middle.

The second atypical form may be short or several times the normal length and contains a variable number of chromatinic dumbbells compacted into a central deeply staining mass. The third and fourth forms are long filaments with many discrete but irregularly spaced chromatinic bodies. Though always rare, the last two types are more common in young cultures derived from very old (e.g. 4 weeks) material, than in subcultures from slants incubated for only 18 hr. Both have been accurately described and illustrated by Neumann, whose inability to determine their function I unfortunately share.

Speculation about the significance of all these enigmatic cells such as their connexion with autogamic processes, is bound to be unprofitable until it is possible to isolate them from normal living material and follow their development separately.



Text-fig. 3. Diagram of certain peculiar types of cells, small numbers of which are regularly found in cultures of *Proteus vulgaris* and *Bact. coli*. Tentatively arranged in order of what may be their true genetical relationship. At present any such connexion between the different types is purely hypothetical.

DISCUSSION

The chromatinic structures described in this communication obviously correspond to those which J. Badian, in a series of papers since 1930, has reported in the cells of myxobacteria, actinomycetes and aerobic spore-forming bacteria and which were also described in the spores and vegetative forms of *B. mycoides* and *B. mesentericus* by Robinow (1942). There is good reason to believe that such structures occur very widely among bacteria.

The chromatinic dumbbell bodies are also identical with the 'nucleoids' of Piekarski (1937–40). The difference between Piekarski's and my own description of the nuclear structures is twofold and is due to differences in technique. According to Piekarski the nucleoids are round bodies and each cell has two, one near each end. My observations make it clear that (1) the nucleoids in Piekarski's preparations are optically unresolved dumbbell bodies, or, more often, configurations of these and (2) that the 'cell' or 'Primaerform' of his terminology is actually a two-cell rod with a resting or dividing dumbbell body in the centre of each of its component cells. In other words: the binucleate form which Piekarski puts at the beginning of his sequence of developmental stages is really composite and derived from small, uninucleate coccoid or rod-shaped elements (Piekarski's 'Secundaerform') through growth and cell division. This process is evident during the lag-phase and is less common during the first few hours of a culture's active growth, the stage to which Piekarski paid particular attention.

Neumann's (1941) observations on *Bact. coli* and *Proteus* agree with those of Piekarski. Neumann observed chromatinic dumbbell bodies but thought they were stages in the division, hour-glass fashion, of a single chromatinic granule identical with Piekarski's nucleoids. Neumann was not aware of the multiple cell structure of rod-shaped and filamentous bacteria, but apart from these and other, less important, differences my observations on *Proteus vulgaris* and *Bact. coli* fully confirm Neumann's detailed description of the distribution of nuclear material in the various growth forms of these organisms.

There is some diversity of opinion about the mode of division of the chromatinic bodies in different groups of bacteria. This problem is important in connexion with the possible function of these bodies in the transmission of hereditary characters.

In the rod-shaped bacteria, my own observations indicate that the dumbbell bodies divide like chromosomes by splitting longitudinally. The compact nuclei which Beebe (1941) describes in the vegetative cells of *Myxococcus xanthus* n.sp. and which he believes divide amitotically by constriction, are more probably composed of closely contiguous dumbbell bodies. The appearance of a compact nucleus dividing by constriction is easily simulated when, as described in the present communication, the chromatinic dumbbell bodies divide again before they have completely separated after the previous division, and especially if coloured strands persist between two pairs of closely contiguous bodies as they move towards opposite poles (cf. Pl. 5, figs. 1 *e*, 9 *a*; Pl. 7, fig. 27 *a*). This interpretation of Beebe's results is the more likely since he himself describes the nuclei of cells migrating to the fruiting centres as breaking up into 'four irregularly shaped bodies or chromosomes'. An alternation of amitotic and mitotic division such as this author envisages seems hardly likely.

Direct division of a round nuclear body by elongation and constriction has recently been described by Knyasi (1942) in a new species of yellow coccus. I have previously expressed the view (Robinow, 1942) that the Feulgen positive granules which I noted in most of the cells of two sarcinae were the ends of dumbbell bodies which divided by longitudinal splitting. I now find that my photographs are more satisfactorily explained by Knyasi's assumption that the granules divide directly, the smaller cells containing one and the larger cells two.

On the other hand, Miss E. Klieneberger (unpublished observations) has recently demonstrated unmistakable dumbbell bodies and their V-shaped division stages in large streptococci from a human case of broncho-pneumonia, and I have seen the same appearances in a large, colourless unidentified coccus which was encountered as a contaminant on an old agar plate. One or two Feulgen-positive bodies, similar to those which I have previously described in two sarcinae are also present in the cells of *Staphylococcus aureus* and of *Streptococcus pyogenes* (unpublished observations). I cannot, therefore, agree with the conclusions drawn by Knaysi & Mudd (1943) from electron micrographs, that these organisms lack discrete nuclear structures and that the organization of nuclear material varies widely among different species of bacteria.

Their small size renders cocci rather difficult objects for the study of the chromatinic bodies and even in the more readily investigated rod-shaped bacteria, resolution of the chromatinic structures into chromosome-like bodies is not always possible (cf. *B. mesentericus*, Pl. 6, figs. 17, 19).

It seems plausible that the chromatinic bodies of bacteria are homologous with the chromosomes of more highly differentiated organisms. The positive Feulgen reaction of these bodies, their special affinities for nuclear dyes, their regular cycle of multiplication co-ordinated with cytoplasmic division and their simple and constant numerical relationships strongly suggest that they are true nuclear structures. Moreover, their mode of division appears well suited for the transmission of hereditary factors in linear array such as occurs in chromosome division, though whether they actually perform this function is of course not yet known.

Also, direct proof that the chromatinic bodies contain nucleic acid is still lacking; to obtain this it will be necessary to determine whether they maximally absorb the same wave-lengths of ultra-violet light as the nucleic acids, an undertaking rendered difficult by the small size of the bacteria. Piekarski's claims (1938) to have obtained a selective absorption of ultra-violet light require confirmation.

Further evidence for the nuclear character of the dumbbell bodies in bacteria is afforded, however, by a comparison of these bodies with the nuclear apparatus of yeasts. Badian (1937) has studied several species of yeasts and has shown that they possess two chromosomes which in form and size closely resemble the dumbbell bodies of bacteria. I have been able to confirm these observations for the vegetative stages of *Saccharomyces cerevisiae* and *Schizosaccharomyces Pombe*. My results will be described fully elsewhere but in the present connexion it is interesting to note that in yeasts delicate strands extend between the two pairs of chromosomes at anaphase and between daughter nuclei, which exactly resemble the strands so often seen between separating chromatinic structures in bacteria.

Statistical evidence that the bacterial cell contains an organ of higher radiosensitivity than the rest of the cytoplasm has been provided by D. E. Lea and his collaborators in a series of radiological investigations (1937, 1940, 1941). This organ cannot be regenerated by the cell once it has been destroyed by irradiation and is probably identical with the nuclear material. It is hoped to supplement these observations with morphologica data on the effect of ionizing radiations on the chroma tinic structures.

The chambered appearance that Giemsa's stain lend to bacteria which have been fixed through the agar wit Bouin's fluid, can also be produced by iron alum haema toxylin in the case of the aerobic spore-formers. Usin this stain, Guilliermond (1908) has described such boun daries ('cloisons transversales') in *B. mycoides*. Th pattern of alternating dark and fine transverse marking is also reminiscent of the chambered structure of larg spirochaetes as described by Gross (1911) and Dobe (1912) (cp. Pl. 8, figs. 28, 29 with figs. 143 and 144 o Dobell's memoir).

The existence of a bacterial cell wall as a structur differing physico-chemically from the cytoplasm ha been recognized since the work of A. Fischer (1895 and his contemporaries, but the clear conception of th cell wall, derived from plasmolysing experiments, whic prevailed forty years ago has become somewhat obscure by the difficulty of demonstrating it satisfactorily i most of the bacteria which interest the medical bacteric logist.

Knyasi (1930) has described the morphological rela tionship of the plasma membrane and the outer sup porting cell wall in plasmolysed bacteria. My ow results with the Bouin-Giemsa and NaOH-crystal viole method fully confirm his findings with regard to thi relationship, but differ from them in showing that th aerobic spore-formers have a multiple cell structur from the *earliest* growth stages onwards.

NOTE

In the present account observations on the cell wa in *B. megatherium* have been used to supplement th description of the chambered cytoplasm in rod form and filaments of *Bact. coli* and *B. mesentericus*, i.e. i two species in which the demonstration of the oute supporting cell wall which surrounds the cytoplasr had not proved successful. Since this article first wen to press I have learnt distinctly to stain the cell wa in all three organisms by mordanting Bouin-fixe preparations with 5 % tannic acid and staining fo 10 sec. (!) with 0·02 % crystal violet in water. Th new preparations further emphasise the distinctio between cytoplasmic cell boundaries and transvers cell walls (septa) drawn in the present article and hav allowed the formation of the transverse septa to b studied in detail. These observations will be publishe elsewhere.

SUMMARY

1. The basic observations of Piekarski (1937–40) an F. Neumann (1941) on Feulgen-positive, chromatini structures, going through a regular cycle of division, i the cells of *Bact. coli* and *Proteus vulgaris* are confirme and the view that these structures are nuclear in natur is accepted.

2. The chromatinic structures in bacteria from ol cultures, although usually distinguishable from th cytoplasm, are too small to be resolved accurately After transfer to a fresh nutrient medium the chroma tinic structures increase in size and give rise to shor often dumbbell shaped, rods (chromosomes) whic

multiply by splitting lengthwise in a plane more or less parallel with the short axes of the bacterium.

3. The chromatinic structures of *Bact. coli* and of *Proteus vulgaris* are essentially the same as those previously described in myxobacteria and actinomycetes (Badian, 1930, 1933, 1936) and in various well-known aerobic spore-forming bacteria (Badian, 1933, 1935; Robinow, 1942).

4. A single cell of *Bact. coli* or *Proteus vulgaris* contains one chromatinic body or one or two pairs of these, representing primary and secondary division products.

5. Few bacteria from young cultures are single cells. When fixed through the agar with Bouin's mixture and stained briefly with Giemsa's solution, bacteria from young growing cultures of *Bact. coli*, *Proteus vulgaris*, *B. mesentericus* and *B. megatherium* assume a banded appearance indicating that each bacterium consists of two, three or four separate cells.

6. A plasmolysing treatment has been applied to *B. megatherium* which provides direct proof of the composite structure of this bacillus by inducing its component protoplasts to shrink away independently from the outer supporting cell wall.

I wish sincerely to thank Dr H. B. Fell for the hospitality offered to me at the Strangeways Laboratory, for her sustained interest in this investigation and for her help in preparing the manuscript for the press. I have also pleasure in admitting my indebtedness to Prof. E. G. Pringsheim for much helpful criticism and advice in matters relating to the bacterial cell wall.

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EXPLANATION OF PLATES 5–8

All figures on Plates 5 and 6, with the exception of fig. 18, are of preparations fixed with osmium vapour, treated with *N*/1 HCl at 60° C. and stained with Giemsa's solution. Unless otherwise described, preparations were photographed mounted in water. A scale of magnification is provided on every plate and is the same for all figures.

PLATE 5. *Bacterium coli* and *Proteus vulgaris*

Figs. 1–9. Chromatinic structures in *Bact. coli* from five nutrient agar plate cultures seeded with material from 18 hr./37° C. slant cultures and incubated for 45–90 min. at 37° C.

Fig. 1. *a–h* are successive stages in the development of a coccoid element with a central pair of closely contiguous chromatinic bodies into a typical rod-shaped bacterium with four chromatinic structures. *h* may, of course, also have arisen directly from a plump bacillary form without having gone through the coccoid stage. Note the V-shaped division stage at *d*. The dumbbell body forming the left limb of the V is so much broader than the right one, that it seems plausible to assume that it is preparing for the next division. Compare this stage with Pl. 7, fig. 26. *e* a strand of chromatinic matter is seen connecting recently separated chromatinic structures. Compare with Pl. 7, fig. 27.

Figs. 2, 3. *a–e* successive developmental stages. The two deeply stained dumbbell bodies at c are connected at their base by a faintly stained round body similar to that in *f*, fig. 1. Loss of clear-cut dumbbell shape together with asymmetrical broadening of the chromatinic bodies at *d* indicates impending division. The same applies to the chromatinic structures in the central bacterium in fig. 3.

Fig. 4. Separation of the products of the 2nd division of the chromatinic bodies is almost complete in the right chromatinic structure in the central bacterium. Stage *d* of Text-fig. 1 gives an interpretation of these configurations suggested by their appearance under the microscope at different levels of focus.

Fig. 5 (from a preparation mounted in canada balsam). A typical two-cell bacterium with four chromatinic bodies, three of which are dumbbell-shaped and must be considered as having been in a state of rest at the time of fixation.

In fig. 6 (mounted as in fig. 5) *a* represents a stage comparable to *c* in fig. 2, while *b* is another two-cell bacterium with four dumbbell-shaped chromatinic bodies.

Fig. 7. In the large bacterium below and to the right of the centre the first dumbbell body from the left as well as the fourth are at rest while the greater width and asymmetrical shape of the two middle ones again suggest a process of division (reduplication).

Fig. 8. All four chromatinic structures in the large bacterium are preparing to divide.

Fig. 9. Separation of daughter chromatinic bodies has begun in the two middle ones of the four chromatinic structures in the large bacterium; less densely stained than the chromatinic bodies of fig. 7, they correspond to the stage of fig. 3, i.e. that of impending separation of the products of a recent longitudinal division. A faintly stained cell boundary traverses the gap between the two pairs of chromatinic configurations. The same type of two-cell rod is also found at *e* in fig. 2 and at *h* in fig. 1. At *a* a filamentous structure connects recently separated, complex chromatinic structures. A slender rod with a long core of chromatinic matter is seen in the right-hand top corner; others are visible in figs. 1 and 2. The development of these forms is explained in the text.

Figs. 10–12. From a culture of *Proteus vulgaris* of 1 hr. incubation, prepared from the coccoid elements with poorly differentiated chromatinic structures, that form the first belt of confluent growth inside the zone of swarming filaments at the periphery of an agar plate culture. The preparation was photographed mounted in canada balsam.

Figs. 10, 11. *a*, coccoid cell with a single, diagonally placed chromatinic body. *b*, cell with two chromatinic bodies resulting from recent longitudinal division. *c*, division of this bacterium will give rise to two single cells (stage *c'* of Text-fig. 1), in contrast to the more usual mode of division at the four-cell stage, illustrated by the dividing bacterium above *d* in Pl. 6, fig. 13.

Fig. 12. In the centre are two young rod forms with clearly defined dumbbell bodies. In the one on the right a rounded faintly stained body is visible between the chromatinic dumbbell bodies, similar to *f*, fig. 1 and *c*, fig. 2, Pl. 5.

PLATE 6. *Proteus vulgaris, Bact. coli, B. mesentericus*

All figures except fig. 18 are from preparations mounted in water. Fig. 18 is the same as the top part of fig. 29, Pl. 8 and is from a Bouin-Giemsa preparation mounted in canada balsam.

Figs. 13, 14. From one culture. Fig. 13 shows the short normal bacteria from the raised edge of an agar culture shortly before swarming was due to begin.

Fig. 14. From an impression preparation made after swarming had been in progress for 1 hr. In fig. 13 particularly clear division stages at *a* and *b*; *c* and *d* illustrate further stages in the formation of two-cell bacteria with four chromatinic bodies. In fig. 14 four compact, symmetrically arranged chromatinic configurations are visible in the larger bacterium marked *a*. Many of the chromatinic structures in the long filaments are very broad, which suggests that they may be composed of several pairs of dumbbell bodies. At *b* a large filament is seen to break into two fragments of equal length. A delicate strand of chromatinic matter connects the first two chromatinic structures from the left, in the left daughter bacterium.

Fig. 15. A two-cell rod of *B. mesentericus* containing two pairs of closely contiguous, symmetrically built dumbbell bodies separated by a median cytoplasmic cell boundary. The shadows on top of the bacillus are discarded spore cases.

Fig. 16. An early stage in the separation of the closely contiguous dumbbell bodies. Note the well-defined median cell boundary and compare this photograph with *b* in Pl. 8, fig. 28. The corresponding stage in the development of *Bact. coli* is shown in Pl. 5, fig. 4. Fig. 16 should also be compared with stage *h* of Badian's text-fig. 1 (1933).

Fig. 17. Near the lower edge of the picture a typical composite filament of *B. mesentericus* from the first 2 hr. after spore germination. Note that the middle one of the three septa is particularly deeply stained; it is here that a break will take place like that illustrated in fig. 19. There are also clear-cut median cell boundaries in two other bacteria on this picture.

Fig. 18. Comparison with the previous figure shows that the dark bands in the long bacillus correspond to cell boundaries. No chromatinic structures are shown in fig. 18.

Fig. 19. See legend of fig. 17.

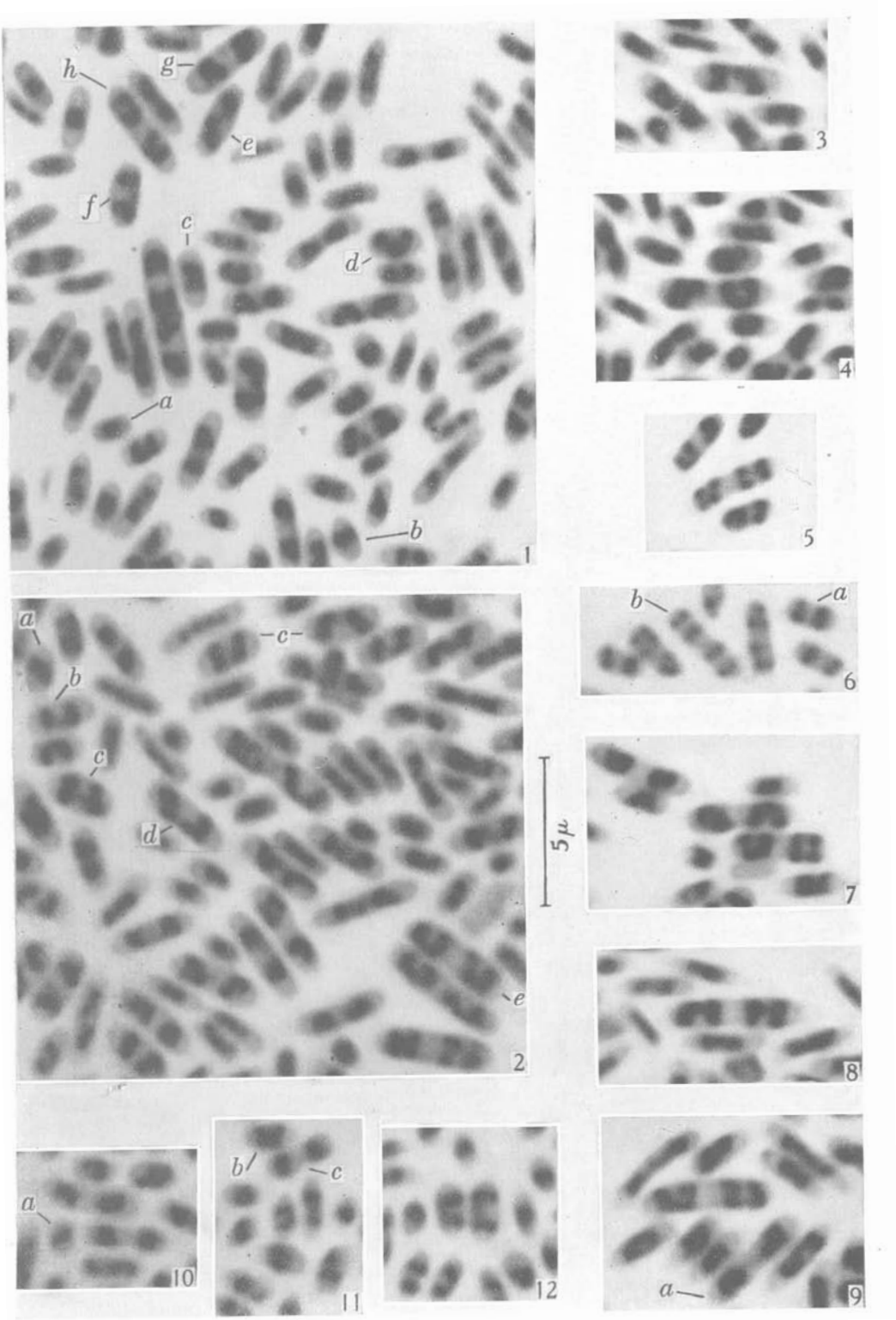
Figs. 20. 21. *Bact. coli*, 4 hr. agar slant at 37° C.; 2 hr. in tap water at room temperature, 1¾ hr. on fresh nutrient agar at 37° C. (Control to a radiation experiment.) The photograph shows slender bacteria typical for growth on crowded plates of more than 2 hr. incubation. At *a*, *b* and elsewhere two-cell rods with four resting or dividing chromatinic bodies are seen. A slightly constricted two-cell rod with a pair of closely approximated chromatinic bodies in each of its cells is visible in the right-hand bottom corner of fig. 20. To the left of this bacterium, at *c*, sister bacteria are seen connected by a slender protoplasmic bridge. Compare these two photographs with Pl. 7, fig. 24, where the distribution of cell boundaries is shown.

PLATE 7. *Bact. coli, B. mycoides*

All figures except figs. 25–27 are from preparations mounted in water.

Fig. 22. Typical sample of the different types of cells in an 18 hr. 37° C. slant culture. *a* are coccoid forms with single dumbbell bodies. Compare the poor definition of the chromatinic structures in this picture with the strong contrasts between cytoplasm and chromatinic structures in Pl. 5, figs. 1, 2.

Fig. 23. Bacteria from an 18 hr. slant culture, spread on agar, fixed with Bouin's fluid and stained briefly with Giemsa's solution. Except for the darkly stained bacterium with median constriction in the left-hand



Figs. 1–12.

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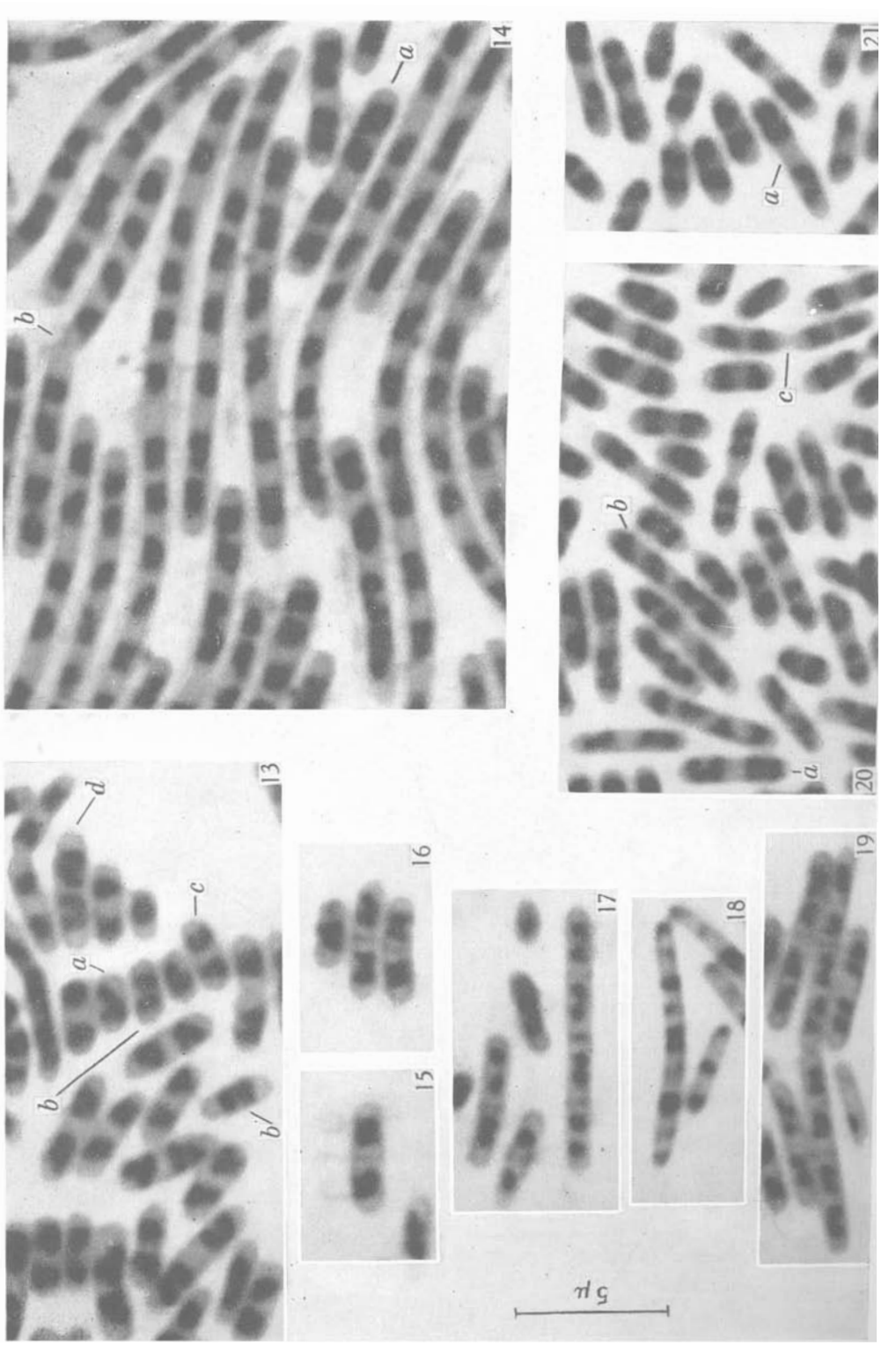

Figs. 13–21.

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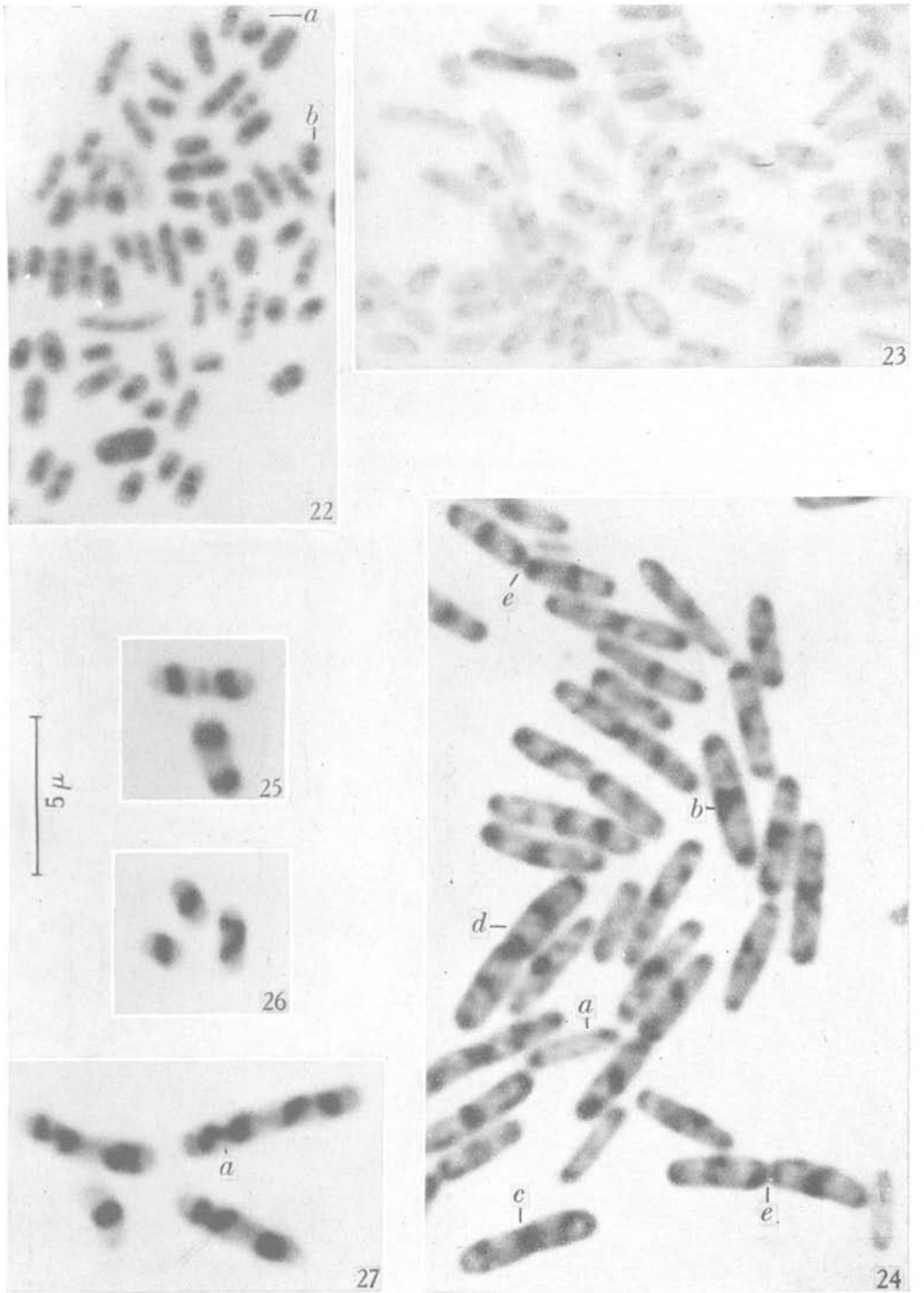

Figs. 22–27.

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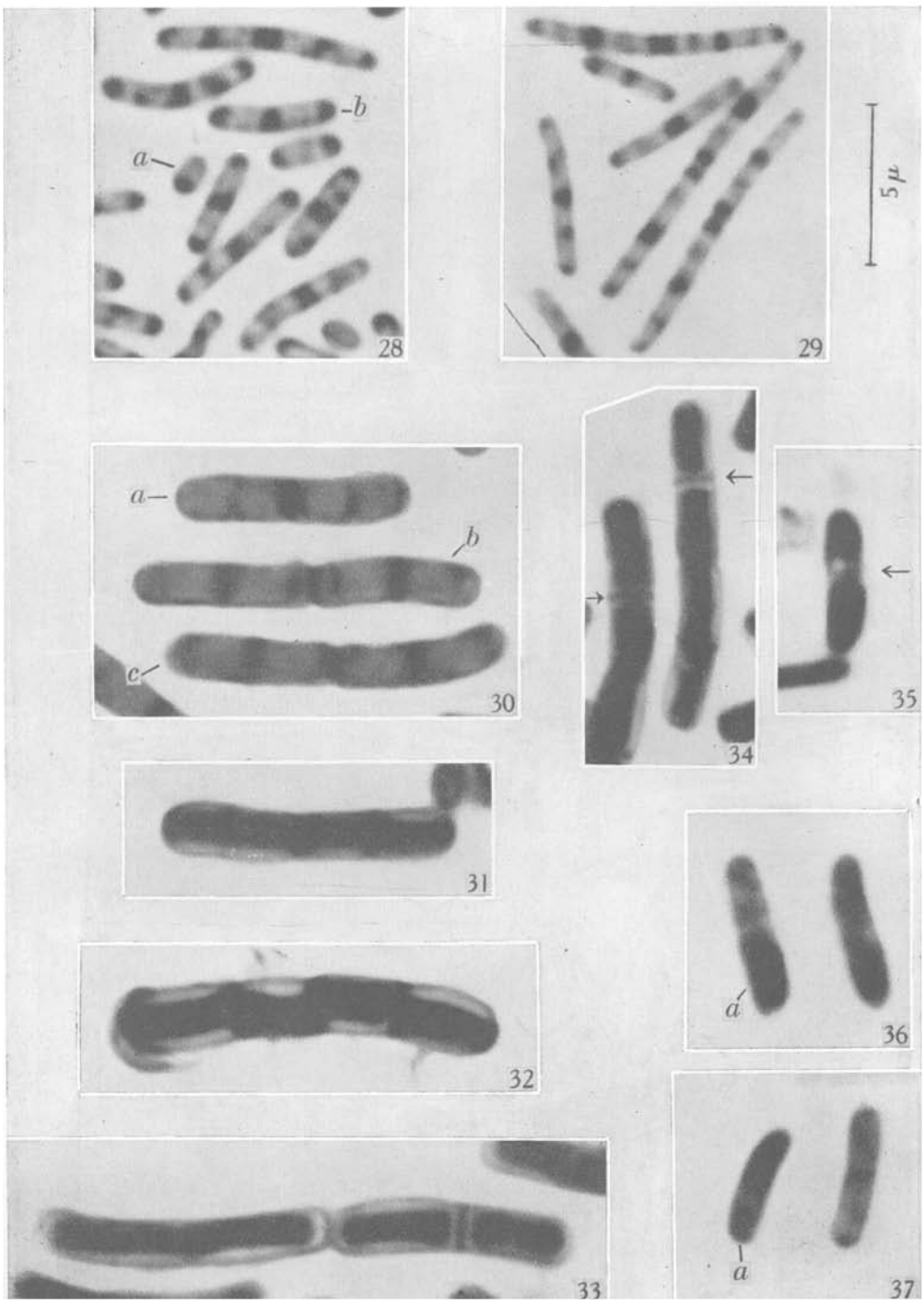

Figs. 28–37.

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top corner the cells in this picture were dormant at the time of fixation.

Fig. 24. The same culture as in fig. 25, incubated for 90 min., fixed and stained in the same way. *a*, a cell of the slender rod type which has not yet begun to develop into a large two-cell bacterium. *b*, a two-cell bacterium. *c*, a slightly constricted four-cell bacterium. *e*, dividing four-cell bacteria.

Figs. 25, 27 are successive stages in the development of *B. mycoides* beginning with spores near the end of the germination period.

Fig. 26 should be compared with the corresponding stages *a*, *b*, *d* in the development of *Bact. coli* in Pl. 5, figs. 1, 2.

Fig. 26. Demonstrates a clear median cell boundary.

The three bacteria in fig. 27 correspond to those in figs. 8 and 9 of *Bact. coli* on Pl. 5. At *a* a strand of chromatinic matter is seen extending between separating chromatinic structures.

PLATE 8

B. mesentericus, figs. 28, 29
B. megatherium, figs. 30–34
Bact. coli, figs. 35–37

No nuclear structures are shown in any of the photographs on this plate. The photographs of *B. megatherium* are from water-mounted preparations, the rest from balsam mounts.

Figs. 28, 29. *B. mesentericus* from spores incubated for 2 hr. and 2¾ hr. respectively. *a* in fig. 28 marks a single-cell stage, all the remaining bacilli in both pictures are composite in nature. Note the dark staining of the tips and of the places where the bacteria will eventually divide by constriction. The 2- and 4-cell bacilli in fig. 28 are all 'resting', the two long diagonally placed filaments in fig. 9 show narrow median constrictions. Each consists of at least four cells. Compare with Pl. 7, fig. 24 of *Bact. coli* and note similarity in structure.

Figs. 30–34. From three different preparations of *B. megatherium*, treated with boiling 1·3 % NaOH as described under method 5. The stain is 0·5 % crystal-violet and the preparations were photographed mounted in water.

Fig. 30. Plasmolysis and differentiation of cytoplasm and cell wall are imperfect but the composite structure of all three bacilli is nevertheless recognizable. *a*, the cytoplasm of the left half is incompletely, that of the right half completely divided by a transverse cytoplasmic partition not yet strengthened by cell-wall material. The specimen might be described as a 3–4 cell bacillus. Had the plasmolysing treatment been more effective, it is probable that the internal configuration would have resembled that of the right half of the large dividing specimen in fig. 33. *b*, note the retraction of deeply stained cytoplasm from the cell wall in the plane of constriction and from the horizontal part of the adjoining region (under side) of the third cell from the left. There has also been a concave retraction of the cytoplasm in cell no. 4 at the right extremity of the bacillus. (Compare this specimen with the large, darkly stained bacterium to the left of the centre in Pl. 7, fig. 26.) There has been some retraction of the cytoplasm from the cell wall at both poles of the third bacillus (*c*).

Fig. 31. The two cells on either side of the future plane of constriction in this bacillus have retracted in two continuous portions from the cell wall where the formation of transverse septa, destined to cut into the cytoplasm, can be seen to have begun. In the right-hand top corner is a discarded spore case.

Fig. 32. All four component cells of this young bacillus have retracted independently from the outer cell wall. The third cell from the left has also retracted from the transverse partition separating it from the fourth cell. The left end of the bacillus is still contained in the spore case.

Figs. 33, 34. All-round retraction of the cytoplasm from the cell wall in the bacillus on the right has provided a clear view of the medium transverse partition. Further examples are seen in the right-hand top corner and near the left margin of fig. 34.

Figs. 35–37. *Bact. coli*, incubated for 3 hr. on an agar plate at 37° C. followed by 20 hr. 2–4° C. Bouin fixation through the agar, Giemsa's stain. Mounted in canada balsam.

Fig. 35. Retraction of the cytoplasm from a median septum. Probably comparable to the condition of the upper half of the right-hand bacillus in the adjoining fig. 34.

Figs. 36, 37. In both figures the bacterium marked *a* is composed of one deeply stained and one lightly stained half. Demarcation of the two is particularly clear in fig. 36. The faint staining is due to disintegration of the cytoplasm in this half of the bacterium. Explanation in the text.

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