Nuclear changes in *Bacillus anthracis* and their Relation to Variants

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SUMMARY: The nuclear changes leading to spore formation in *Bacillus anthracis* were investigated in 35 strains. Fusion of four chromatinic bodies into one mass and its subsequent break up into four bodies was observed. One of these became incorporated in the spore; the other three disintegrated. Some cells showed no fusion before spore formation; in these one chromatinic body was incorporated in the spore and the other three disintegrated. The percentage of cells in which fusion occurred varied with the strain. Rough colony strains showing a high incidence of fusion tended readily to throw smooth colony variants. Strains showing a low incidence of fusion were stable in this respect. The incidence of variants of other types bore no relation to the incidence of nuclear fusion in the strain.

Nuclear structures in the spore-forming aerobic bacilli were described by Nakanishi (1901), Badian (1933), Stille (1937), Piekarski (1938), and in greater detail by Robinow (1942), who studied them particularly in spore germination and in the early logarithmic phase of growth. Badian described the fusion of the nuclear elements into one mass and its subsequent breaking up into four parts before spore formation; he recognized that one body entered the spore while the remaining three disintegrated. Klieneberger-Nobel (1945) observed fusion of chromatinic structures both in aerobic and anaerobic spore-forming organisms, and also in a non-spore-forming organism, *Sphaerotilus natans*. In later papers (1947*a, b*) she described the changes occurring in myxobacteria and actinomyces, and showed that fusion of chromatinic structures occurred also among them. It was suggested both by Badian and Klieneberger-Nobel that this fusion might represent an autogamic mechanism.

MATERIALS AND METHODS

The following 35 strains of *Bacillus anthracis* were examined.

A. National Collection of Type Cultures: nos. 109 (Paddington VI); 1328; 1712 (Pasteur's Second Vaccine); 1607; 2620 (Hankow Hide); 4991, 5130, 5249, 7200.

B. Strains isolated from a recent shipment of sheep hides, many of which were contaminated, causing a human infection: strains A, C1, CC2, H2, H3, H5, H7, H8, F1, H3 F2, mouse F, mouse 5.

C. Strains isolated from the lesions of human and bovine infections: Hillsborough, Newtownards, Carrickfergus, R.V.H., Tinsdale, McLean 1A, McLean 2, Lindsay, Davis.

D. Strains maintained in this laboratory: A/stock, S/stock, Dean, 2160, 2161.

The media used were Lemco broth, pH 7.6, and Lemco agar (2% agar), pH 7.6.

The staining methods described by Robinow (1944) were used to demonstrate the nuclear structures. For the study of 'spore germination and of young cultures up to 4 hr. old, the agar was cut into strips and mounted on cover-slips, with the organisms outwards, and fixed in the vapour of 2 % osmic tetroxide for 3–5 min. A second cover-slip was then placed on the agar surface, and the two cover-slips with the agar between them immersed in 30 % ethanol for 2–3 hr. The second cover-slip, bearing most of the organisms upon it was then removed and placed for a few minutes in Schaudinn's fixative, then in N-HCl at 56° for 7 min., washed, and stained with Giemsa's stain (0.15–2 ml. phosphate buffer, pH 6.2) for 10–15 min. For the details of this technique, I am indebted to Dr C. F. Robinow (personal communication).

For older cultures, a portion of growth was emulsified in a small drop of water, allowed to dry, and immediately fixed in osmic tetroxide vapour, hydrolyzed and stained as above. All preparations were made on cover-slips. It was found that a more transparent stain was produced by diluting the Giemsa stain with a mixture of 2 vol. horse serum and 1 vol. M/200 phosphate buffer, pH 6.2.

Fat inclusions were stained by the method of Burdon, Stokes & Kimbrough (1942).

All preparations were examined and photographed mounted in water or in the staining solution.

Optical equipment. The optical equipment consisted of a 2 mm. Leitz apochromatic objective, N.A. 1.32, with compensating eyepieces $\times 12$ for photography and $\times 20$ for visual work, and a Watson 'Holoscopic' oil immersion condenser, N.A. 1.3. Critical illumination was used throughout. A green filter was used both for photography and for visual work. It was impossible to resolve some of the structures without oil immersion of the condenser.

RESULTS

The changes found in spore germination confirm the results of Robinow (1942). The spores of *B. anthracis*, however, take 60–75 min. to germinate, a much longer time than that taken by the spores of his strain of *B. mycoides*.

In the earlier stage of logarithmic growth the rods contain dumb-bell-shaped bodies which divide at right angles to the long axis of the cell (Pl. 1, figs. 1, 2; Fig. 1A). When they separate they leave between them a single thread of chromatin which later ruptures. In the rough colony variants the chromatinic bodies are widely separated. In the smooth colony variants they are closer together, and the intercellular septa occur at an earlier stage than in the rough phase.

In the later logarithmic growth stage, after about 5–5½ hr. growth, 'vacuoles' appear in between the dividing chromatinic bodies; and now, instead of a single chromatin thread joining the separating bodies, two threads can be seen (Pl. 1, fig. 3; Fig. 1B), which are separated by the 'vacuole'. The nature of this 'vacuole' is not certain. Vacuoles may often be seen by dark-ground illumination after about 5 hr. growth in a position corresponding to that of the

spaces seen in the Giemsa-stained preparation. They often contain a small granule in violent Brownian movement, showing that the contents are fluid. In many strains fat globules can be stained by Sudan Black in 5 hr. cultures corresponding in position to the vacuoles seen in dark ground and in the Giemsa-stained preparations.

From $5\frac{1}{2}$ to 10 hr. of growth (the time varying from one strain to another, but fairly constant for the same strain on the same medium) four (occasionally eight) chromatinic bodies become joined end to end and then fuse into a single chromatin rod in the long axis of the cell. At this stage the vacuoles disappear (Pl. 1, fig. 4; Fig. 1C-E). Fusion occurs only in a certain percentage of the cells; with some strains, in less than 1 %, with others, in over 95 %. Just before this stage, each cell contains four chromatinic bodies, surrounded by a cell membrane; a few cells contain eight. The cells which do not show this change remain as before, with their chromatin structures in pairs separated by vacuoles. As a rule, the axial chromatin rods remain unchanged for at least 45 min.: in one strain (N.C.T.C. 1328) they persisted for nearly 8 hr. Vacuoles then appear in the rods (Pl. 1, figs. 5-7; Fig. 1F, G) which break up to form four chromatinic bodies in two pairs, the members of each pair separated by a vacuole. Around any one of the four chromatinic bodies an eosinophil substance is laid down, usually around the terminal body (Pl. 2, fig. 8; Fig. 1G; cf. the diagrams of Badian and Klieneberger-Nobel). A limiting membrane around this eosinophilic area could not be seen. This eosinophil substance increases in size and becomes the spore body. Inside it the chromatinic body becomes orientated into the long axis of the cell, and then becomes spherical in shape. It increases in size, until the whole spore body becomes so densely basophilic that no internal structure can be distinguished (Pl. 2, figs. 9-13; Fig. 1H-J). The appearance is similar to that seen in one stage of the germinating spore. The basophilic material then clears and the chromatinic body appears lying on the surface of the spore body. Meanwhile, the three remaining chromatinic bodies disintegrate, and eventually the rest of the cell disappears leaving the free spore (Pl. 2, fig. 14; Fig. 1K, L). The cells in which fusion of nuclear substance does not take place go on to spore formation in the same way.

Attempts were made to induce fusion of nuclei in strains in which this rarely happened. Following a suggestion of Dr Klieneberger-Nobel, cultures at various stages of growth of the strain 'Carrickfergus' were examined after 3 hr. at 0°. Clumping of the chromatinic bodies occurred in the early stage of logarithmic growth (Pl. 2, fig. 15; Fig. 1M), but at the stage immediately before spore formation, when fusion might have been expected, no fusion occurred, possibly because of the vacuoles between them.

When this strain was grown on Lemco agar containing 1/2000 (w/v) CaCl_2 , as recommended by Bordet & Renaux (1930) for production of asporogenous variants, about 15 % of fusion cells occurred, instead of the usual 1 %. Similarly, the strain Hankow Hide, normally producing about 40 % fusion cells, produced over 90 % when grown on this medium.

For the estimation of the percentage of cells in which nuclear fusion took

place, the Lemco agar plate was heavily and evenly inoculated over an area of at least 2 sq.in., so that the cells in preparations made from the centre of this area were as nearly as possible at the same stage of development. In strains showing almost 100 % nuclear fusion, few preparations were necessary; in strains whose cells showed little fusion, very many preparations were made

Fig. 1.

to ensure that fusion was not taking place. The figures in Table 1 are those of the preparations showing the highest incidence of fusion cells found in the strain in question.

All the strains were inoculated into 100 ml. of Lemco broth, pH 7.6, and incubated at 37°. The cultures were thoroughly shaken after 10 and 17 days' growth and plated out to give single colonies. Between 50 and 100 colonies of each strain were examined. The 17-day period was chosen because variation takes place in most strains of *B. anthracis* by this time; and if cultures in a large volume of broth are incubated longer, motile variants sometimes appear. When this happens all or nearly all of the colonies found on plating are smooth colony motile variants; the other forms are overgrown in the flasks. In estimating the incidence of cells showing fusion the difficulty was greatest in strains showing an incidence between 10 and 90 %; some cells are seen in which it is hard to decide whether or not an early or late stage of fusion is taking place. Over this range the percentage error is probably at least ± 10 %. In the table the letter 'R' indicates the rough medusa-head colony variant and 'S' the smooth, usually avirulent, colony variant. 'RS' indicates the intermediate form.

Bacteriophage was present in the strains C1, H2, and F1. The strain C1,

Table 1. *The incidence of nuclear fusion in variants of Bacillus anthracis*

Strain	Incidence of fusion (%)	Type of colonies after 10 days' incubation	Type of colonies after 17 days' incubation
N.C.T.C. 109	70	All R	RS 20 % R 80 %
N.C.T.C. 1328	30	All R	All R
N.C.T.C. 1607	4	All R	All R
N.C.T.C. 1712	95	All R	S 52 % R 48 %
N.C.T.C. 2620	40	All R	A few coccal variants Remainder R
N.C.T.C. 4991	90	All R	S 12 % RS 67 % R 15 %
N.C.T.C. 5180	1	All R	S 1 % RS 1 % Remainder R
N.C.T.C. 5249	95	All R	S 40 % RS 33 % R 27 %
N.C.T.C. 7200	20	All R	All R
A	1	All R	All R
CC2	10	RS 1 % R 99 %	RS 5 % R 95 %
H3	50	S 50 % R 50 %	RS 60 % R 40 %
H5	95	RS 10 % R 90 %	S 30 % R 70 %
H7	80	All R	S 20 % RS 35 % R 45 %
H3 F3	60	S 30 % R 70 %	S 30 % R 30 %
Mouse F	95	RS 1 % R 99 %	RS 30 % R 70 %
Mouse 5	5	All R	All R
Hillsborough	1	All R	All R
Newtownards	10	Ghost colonies (R) 50 % R 50 %	Ghost colonies 70 % R 70 %
Carrickfergus	2	All R	All R
R.V.H.	20	All R	Coccal variants 75 % R 25 %
Tinsdale	5	All R	All R
McClean IA	10	Small smooth 1 % R 99 %	1 coccal variant Remainder R
McClean II	40	All R	S 65 % R 35 %
A/stock	50	All R	Coccal variants only
S/stock	25	All R	All R
Lindsay	75	Coccal variants 65 % R 35 %	S 15 % R 85 %
Dean	70	All RS as original	All S
2160	25	All R	S 10 % R 90 %
2161	60	1 smooth colony Remainder R	S 4 % R 96 %
Davis	95	RS 5 % R 95 %	All R

with an incidence of fusion of 95 % regularly produced about 50 % of smooth colony variants after 24 hr. incubation. The strains H2 (fusion incidence 1 %) and F1 (fusion incidence 5 %) did not throw any smooth colony variants from broth cultures. (Smooth colony variants were obtained on plate culture from the strain F1 in the presence of bacteriophage of high titre which was specific for the rough colonies (N. C. Graham, personal communication).)

These results show that the occurrence of smooth colony variants in rough colony cultures is associated with a high incidence of cells showing fusion of the chromatinic bodies and that stability is associated with a low incidence. The only exceptions were the N.C.T.C. strain 5130, where one smooth and one RS colony were produced, and the strain 'Davis', where hardly any variation occurred. The occurrence of coccal and ghost-medusa colonies is not related to the incidence of nuclear fusion.

DISCUSSION

There can now be little doubt that the structures seen in bacteria stained by the osmic acid HCl-Giemsa method represent the nuclear apparatus. They are present in all organisms examined which are large enough to show internal structure under the light microscope (Robinow, 1942, 1944, 1945 and Klieneberger-Nobel, 1945, 1947*a*, *b*). The distribution and appearance of these structures is similar by different staining methods: Nakanishi (1901), using a 'vital' method with methylene-blue; Dobell (1911) and Badian (1933), fixing with osmic acid or formalin, staining with Giemsa, and differentiating with 1 % eosin solution; Stille (1937), Piekarski (1940), and Neumann (1941) using the Feulgen technique; Robinow and Klieneberger-Nobel using the osmic acid HCl-Giemsa method; all saw essentially the same structures.

The Feulgen stain in all other plant and animal tissues is specific for the desoxyribonucleic acid which is found only in nuclei and which is associated with the presence of genes. Vendrely & Lehault (1946) have estimated the dry-weight percentage of ribonucleic and desoxyribonucleic acids and protein in staphylococci, *B. anthracis* and *Escherichia coli*. They found that the amount of desoxyribonucleic acid is in accord with the size of the nuclei described by Robinow, but that the nuclei of Stille and of Piekarski appear too small in their photographs. This may be due to the use of dehydrated and cleared preparations by Stille and Piekarski, whereas Robinow's preparations were photographed mounted in water.

The spore contains one chromatinic body; the coccal variant of *B. anthracis* contains a single nuclear body (Pl. 2, fig. 16; Fig. 1N) and in the phase of growth of *Proteus vulgaris* just before swarming occurs, forms containing one chromatinic body occur (Klieneberger-Nobel, personal communication). The cells of *Esch. coli* and *Proteus vulgaris* (Robinow, 1944) and the cells of *Corynebacterium diphtheriae* in old cultures contain only one nuclear body (Pl. 2, fig. 17; Fig. 1O). It seems, therefore, that the bacterial individual must be considered as having a single nuclear body which may reasonably be considered as a chromosome; and that the rod form of young cultures of species like *Esch.*

coli and *B. anthracis* consists of several individuals still sharing a common cytoplasm and cell membrane. So the fusion cell of *B. anthracis* represents the fusion of four individual organisms which have arisen by two divisions from the same ancestral individual.

Appearances suggesting nuclear fusion are seen in the illustrations of Nakanishi (1901) and Dobell (1911), in the aerobic and anaerobic spore-formers. They have been more fully described by Badian (1933) and Klieneberger-Nobel (1945, 1947 *a, b*) in aerobic and anaerobic spore-forming organisms, myxococci and the aerobic actinomycetes. In a motile Gram-negative non-sporing bacillus Rosca (1937) described cells with X and Y chromosomes or nuclei which underwent fusion to form a diploid cell. His evidence is not convincing. Stapp (1942) described in *Pseudomonas tumefaciens* the formation of aggregates of rod forms like the spokes of a wheel with fusion of cytoplasm and chromatin in the centre, and their subsequent separation. His photographs are quite convincing. Zygo-spore formation has been described by Mellon (1925) in a rough strain of *Esch. coli*, but he did not demonstrate fusion of chromatin. The work of Lederberg & Tatum (1946) and of Tatum & Lederberg (1947) suggests that fusion of cells of one strain of *Esch. coli* may occur with those of another. They think that this does not necessarily occur in all strains, and that it may occur only rarely. It seems, therefore, that some form of fusion mechanism occurs widely among bacteria.

Lindegren (1935) postulated on theoretical grounds that a bacterial cell, having life, must possess genes arranged in a string that splits longitudinally in cell division. This linear arrangement of genes is probably represented by the dumb-bell-shaped chromosome. When the four chromosomes fuse, there may be an opportunity for interchange of position of genes on the chromosome. Change in the relative position of genes on the chromosome has been suggested by Darlington (1937) as a mechanism of variation in *Drosophila*, *Chorthippus* and *Tulipa*. There can, of course, be no doubt that change in the gene substance itself is also a mechanism for production of mutants.

The appearance when the fusion cylinder breaks up (Pl. 1, figs. 5-7) is reminiscent of the appearance of the chiasmata in the diakinesis stage in meiosis of higher plants and animals; but it is possible that this appearance is due to the presence of the developing vacuole, and that it has no other significance.

The results suggest the importance of nuclear fusion as an autogamic process for the production of rough colony → smooth colony → motile variants. It is possible that the other variant colony types (coccal, ghost medusa, dwarf medusa, extreme rough) arise by means of gene mutation only; but this has yet to be proven.

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Figs. 8-17

T. H. FLEWETT—NUCLEAR CHANGES IN *BACILLUS ANTHRACIS* AND THEIR RELATION TO VARIANTS. PLATE 2

Figs. 1-7

T. H. FLEWETT—NUCLEAR CHANGES IN *BACILLUS ANTHRACIS* AND THEIR RELATION TO VARIANTS. PLATE 1

EXPLANATION OF PLATES

PLATE 1

All photographs were of cover-slip preparations mounted in water. Magnification $\times 4000$.

- Fig. 1. Strain Hankow Hide, rough colony type, after $3\frac{1}{2}$ hr. incubation, showing widely spaced chromosomes.
- Fig. 2. Strain C1, smooth colony type, after $3\frac{1}{2}$ hr. incubation, showing (a) incomplete division of chromosomes, and (b) divided chromosomes lying close together. The chromosomes are more closely spaced than in Fig. 1. Photograph by Dr C. F. Robinow.
- Fig. 3. Strain Carrickfergus, after $4\frac{1}{2}$ hr. incubation, showing separating chromosomes connected by two threads of chromatin enclosing a vacuole.
- Fig. 4. Strain Hankow Hide, after 7 hr. incubation on agar containing CaCl_2 1 : 2000 (w/v), showing (a) chromosomes joined end to end, and (b) complete fusion.
- Fig. 5. Strain and medium as in Fig. 4; $7\frac{1}{2}$ hr. incubation. Stages in the breaking up of the fusion cylinders: (a) a long form, probably derived from eight chromosomes, and (b) a short form derived from four chromosomes.
- Figs. 6 and 7. Strain and medium as in Fig. 4, showing appearances suggestive of chiasma formation.

PLATE 2

- Fig. 8. Strain Carrickfergus, 8 hr. incubation on ordinary agar, showing an early stage of the spore; the remaining chromatin material is indistinct.
- Fig. 9. Strain C1. A later stage than Fig. 8. The spore is larger than in Fig. 8 and the chromosome is in the long axis.
- Fig. 10. Strain Hankow Hide, $8\frac{1}{2}$ hr. incubation. The chromosome is bent into a horse-shoe shape.
- Figs. 11 and 12. Later stages of Fig. 10 showing expanding rings of chromatin.
- Fig. 13. Completely basophilic spores, similar to those seen in one stage of germination.
- Fig. 14. Strain C1. Resting spores, hydrolyzed at room temperature, by oblique illumination, showing the spore chromosome and the outer spore membrane.
- Fig. 15. Strain Carrickfergus after $3\frac{1}{2}$ hr. incubation at 37° and 1 hr. at 4° showing clumping of nuclear material.
- Fig. 16. Strain Tinsdale. Coccal variants, showing the single chromosome. Dividing forms show two chromosomes. 3 hr. incubation. Photograph by Dr C. F. Robinow.
- Fig. 17. *C. diphtheriae*, 10-day culture on Neill's blood-tellurite medium, showing single chromosome, photographed in light of the 435.8μ band of the mercury arc.

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