The University of Glasgow

# Methods and Applications of Bacterial Cytology

A critical examination by cytological techniques of the nuclear structures in bacteria

by

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VOLUME I

# C O N T E N T S



For the convenience of the reader, all photographs and diagrams (referred to as figures throughout this thesis) have been bound separately in volume II.

# P R E F A C E

# PREFACE

The question whether bacteria possess an organised nucleus has been one of the most controversial points of bacterial cytology. Although there is still a certain amount of scepticism in the minds of some bacteriologists who consider that the structures described as bacterial nuclei are merely artefacts of the staining technique, many now agree that there is some form of nucleus containing hereditable material in the bacterial cell. As one trained in the methods and outlook of plant and animal cytology, I have a natural interest in bacterial cytology. Applying the standards learned in plant and animal studies to observations on bacteria, I find myself in agreement with the conclusion that the bacterial nucleus is a reality.

A review of the literature made it appear to me that many bacterial cytologists had made only an isolated study of the bacterial nuclear material. Their work was isolated in the sense that they very often examined the nuclear material alone, by a single method, and not in correlation with the rest of the cell structures and cell metabolism. It also became clear to me that there were two schools of thought about the state and mode of division of nuclear material in bacteria.

My interest in bacterial nuclei was first aroused during my final honours year as an undergraduate, when I made a study of how to demonstrate the bacterial nucleus by the osmic acid-hydrochloric acid-Giemsa technique. I required a good deal of time before bringing this technique to an adequate level; having accomplished this, however, I thought that the method, especially if correlated with other methods — such as phase-contrast and electron microscopy — would form a good basis for further inquiry into the methods and applications of bacterial cytology — particularly with reference to the status of the bacterial nucleus.

I thought also that a more realistic approach to the subject could and should be made by thinking of the nucleus as a vital part of the cell, instead of regarding it as an isolated, unconnected stainable piece of material that sometimes formed rather beautiful patterns — patterns which have been and still are interpreted by some bacterial cytologists as mitotic and meiotic figures. Because of such interpretations, I thought it very important to study whether changes in the size and shape of the nucleus could be brought about by changes in the nutritional environment of growing cultures of bacteria.

In this thesis I have also attempted to show that

cytology, especially nuclear cytology, could usefully become a recognised tool of the bacteriologist in solving problems in other fields of research, and that bacterial cytology is more than a fanciful mind-exercise in which imaginative diagrams and drawings play too great a part.

# REVIEW OF LITERATURE

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The published literature on the subject of bacterial cytology is extensive and often controversial. An adequate review of the whole of it is beyond the scope of this thesis even if it was desirable. Therefore only those papers connected with the work carried out for this thesis are reviewed, with special emphasis on those concerned with: (i) the state and mode of division of the bacterial nucleus; (ii) the methods used for its demonstration; and (iii) the manner in which nutrition can alter both its demonstration and structure.

Going back to what may be termed the historical literature of bacteriology, it is perhaps significant that Leeuwenhoek, the first person ever to see living individual bacteria, studied them only for their interesting movement and morphology. Leeuwenhoek, indeed, could be regarded as the first bacterial cytologist even if not the first bacteriologist. Leeuwenhoek's discoveries were followed by a very inactive period from the point of view of bacterial cytology, when chemists, pathologists, and doctors were interested in bacteria only because of their industrial importance or their power to produce disease. Two historic exceptions were Ferdinand Cohn, who observed

granules in bacterial cells and demonstrated their endospores, and Robert Koch, who studied the morphology and spore-production of the anthrax bacillus.

The advances made in dye synthesis, the application of these dyes to the study of the bacterial cell, and improvements in the light microscope were largely responsible for the great number of papers that appeared from about the end of the 19th century on bacterial cytology and on the question of a nucleus in bacteria.

In 1924, a further impetus was given by the introduction of the Feulgen technique (see Knaysi 1951,p.83). This technique (discussed on p. 52 of this thesis) was claimed to be able to demonstrate the nucleic acid (now known to be desoxyribonucleic acid) found in the nuclei of higher plants and animals and thought to be present as the nuclear material of bacteria. Papers of importance in this era were those of Stille (1937) and Piekarski (1937).

Independent discoveries were made by these two workers on the intracellular structures of bacteria. They both observed discrete Feulgen-positive bodies in the bacteria they studied, and saw that division of these bodies preceded division of the cell.

Piekarski named these bodies "nucleoids" and considered that Gram-negative non-sporing bacteria at one stage contained two of these bodies — the "binucleate" stage — and at another and older stage contained only one — the "mononucleate" stage. Piekarski, although he used the Feulgen technique almost exclusively, mentioned a modification of it in which Giemsa's stain was used after fixation and hydrolysis.

A good although short review of the literature on the cytology of bacteria was given by Knaysi (1938). A more thorough review was made by Lewis (1941) who quoted 202 references and dealt with cell inclusions, (including fat-bodies which are reviewed later in this thesis p. 98 ) reproductive structures, cell division, the cell membrane, flagella, and the bacterial nucleus, subjects which, as Lewis said, "have long been the source of much controversy" and about which "many conflicting reports have been published". Lewis's aim in this review was to "present both sides of controversial matters as impartially as possible and to draw such conclusions as the evidence appears to warrant". I think that he accomplished his task successfully. The section of Lewis's review dealing with the bacterial nucleus is the most important with regard to the work of this thesis.

The subject of the bacterial nucleus has long been of great interest to cytologists, and at the time when Lewis wrote his review he was of the opinion that "no wholly satisfactory answer" had as yet been found. Theoretically, however, there was nothing against and everything in favour of the supposition that it should be reasonable to expect to find a true nucleus or a functional equivalent in bacteria.

The extensive literature on the bacterial nucleus, however, is often contradictory and highly controversial — many conflicting reports and theories have arisen from a study of even one organism, Bacillus anthracis. It is certain, however, that other structures have been mistaken for bacterial nuclei — for example, cell inclusions, immature spores, and other cytoplasmic structures. Often the organisms studied were not bacteria nor were the methods used always suitable for cytological study. Some of the investigators were not adequately trained and lacked previous cytological experience. For these reasons Lewis considered that much of the older uncritical work was of little value, and he did not review it.

From the extensive literature Lewis found it convenient to divide the theories concerning the bacterial nucleus into eight groups. Each of these groups was treated in turn in the review, and the names and views of the

principal supporters of the theory were given as well as the conflicting opinions of other workers. Lewis's eight groups of theories (the headings being in his own words) were as follows:-

(1) The bacteria do not possess a nucleus or its equivalent

The title here is almost self-explanatory. The workers supporting this theory could find no trace in the organisms they studied or by the methods with which they studied them of any structures that could be claimed to represent a bacterial nucleus, and they concluded, therefore, that there was no nucleus in the bacterial cell.

(2) The cell is differentiated into a chromatin-containing central body and peripheral cytoplasm

Supporters of this theory believed that the bacterial cell had a central body similar to that found in the members of the Cyanophyceae (Blue-green algae). This body was thought to consist of a mass of chromatin, not surrounded by a membrane. The exact nature of the central body in the Cyanophyceae has long been a subject of controversy among phycologists, and recent developments (Cassol and Hutchinson (1954) see p.51 of this thesis for a review of this paper) show that at least some of the algae of this group possess at some stage of their development nuclear structures very like those seen by other workers in bacteria (Robinow 1945).

(3) The bacterial body is a nucleus devoid of cytoplasm; a naked nucleus or nuclear cell

This theory arose mainly from the observation that certain bacteria had a similar staining reaction to that of nuclear material in other cells; it was therefore presumed that they were wholly composed of nuclear material and devoid of cytoplasm. Various evolutionary ideas were put forth as theoretical grounds for the acceptance or rejection of this theory.

(4) The nucleus consists of several chromatin bodies, a chromidial system, scattered throughout the cytoplasm

Supporters of this theory maintained that there were several chromatin bodies scattered throughout the cytoplasm, some believing that under certain conditions some or all of these bodies could aggregate to form a larger structure. Many of their observations, however, appear to have been made on cells where fat bodies had formed, and the chromidia were possibly compressed cytoplasm.

(5) The form of the nucleus is not constant throughout the growth cycle; it may occur as a discrete spherical body, an elongated chromatin thread, or scattered chromidia, depending on the stage of development: a polymorphic nucleus

This theory was devised to include the variable forms that had been observed when different bacteria were studied. In

my opinion this variability in form may have been due to the stage of development of the organism, not from the point of view of nuclear organisation but in regard to the development of food-storage granules that cause a re-arrangement of the nuclear material.

(6) The nuclear substance consists of fine particles of chromatin dispersed uniformly in the cytoplasm but is not distinguishable as morphological units: a diffuse nucleus (This theory is given by Lewis as no.6 in his list of theories but dealt with as no.7 in his review)

The term "diffuse nucleus" was meant to denote that nuclear material was present in the bacterial cell but was so finely divided and dispersed that it became undifferentiated from the cytoplasm. Nuclear stains (such as the Feulgen reaction) would therefore give a diffuse general reaction and this was observed with various organisms. Several of the supporters of this theory believed that under certain conditions the diffuse nuclear material could, like the visible chromidial bodies, aggregate to form large visible structures.

(7) The protoplast contains one or more true vesicular nuclei (This theory is given by Lewis as no.7 in his list but dealt with as no.6 in his review)

This theory, that bacteria possess true nuclei, was

supported by many "most able" investigators, but there is still doubt about the real nature of the so-called true nuclei described. In all work on bacterial nuclei there is need to pay strict attention to rigid criteria such as: showing that the organism studied belongs to the bacteria and not to the yeasts or lower fungi; that a definite, well differentiated body is a consistent feature of each cell; that genetic continuity of the body is maintained in nuclear and cell division; and that the body is not a cell inclusion, a vacuole, a spore primordium, a cytoplasmic body or an artefact. Such criteria have not always been applied to published observations, and many invalid claims have been made because of this. Elsewhere in this thesis (p.5) I have reviewed the work of Stille and Piekarski which supports this theory.

(8) <u>The nucleus is a naked invisible gene string or a chromatin-encrusted gene string analogous to a single chromosome</u>

This theory is based on the knowledge of the cytology and genetics of higher organisms, in which the gene is the fundamental particulate living unit without which life is impossible. A nucleus reduced to its lowest essentials might take the form of a gene string, either naked or

encrusted with chromatin. Such a simple form of nucleus might take the form of a small granule or rod-like body rather than a definite vesicle with a confining membrane. This theory brings in the haploid and diploid state of the cell.

The task of reaching a satisfactory conclusion about the state of the bacterial nucleus from a study of these theories and the evidence upon which they are based is a difficult one.

Lewis believed that claims for a naked nucleus, a central body, a polymorphic nucleus, and a true vesicular nucleus were based on faulty evidence and must therefore be rejected. This statement by Lewis is rather dogmatic and I do not consider that it deals adequately with the theory of a true bacterial nucleus. It does not do justice, for example, to the manner in which workers like Stille and Piekarski demonstrated nucleus-like structures in some bacteria. Lewis was also of the opinion that much of the confusion was due to the failure of many workers to distinguish between volutin and chromatin, and I agree with this. Both these substances contain a great deal of nucleic acids and consequently react to nuclear stains in a similar manner. Tests for volutin have long been known, but most investigators have tended to regard without further investigation any structures or substances that are stained

deeply either as nuclei or chromidia. In Lewis's opinion the presence of stainable granules in a bacterial cell is not significant if specific tests for volutin have not been made. This criticism he considers is sufficient to disprove claims concerning the nucleus of Spirillum volutans, Azotobacter chroococcum, Bacillus bütschlii and many other species.

There has also been much confusion about stainable material in fat-depositing species. When cells of these species were fixed and stained, the protoplasm, compressed and distorted by fat granules, appeared as deeply stained compact masses, zig-zags, or spiral threads that have been described as nuclei. When both volutin and fat was deposited the picture was even more complicated. Lewis had the conviction that many observers have failed to recognise the nature of the stainable structures, and because of this have projected a subjective element into the problem. Even with more recent literature, reviewed later, I still find that this is true. Lewis did not draw definite conclusions about the theory that the nucleus might consist of minute particles of chromatin dispersed uniformly throughout the cytoplasm; on this question there were conflicting reports, based on results from the Feulgen technique. He mentions, however, the possibility that although the bacterial cell did not contain a demonstrable nucleus, it might yet contain

an invisible nucleus essentially the same as the gene strings in the higher organisms; but not encrusted with chromatin. It remains a question whether knowledge of the hereditary mechanism of bacteria can be enhanced by genetical technique. Lewis's review stated no final conclusions regarding the nature of the bacterial nucleus. I think that this lends value to the review, because, although Lewis was himself a bacterial cytologist, his review of the literature was conducted without the bias of a preconceived idea. In a field where impartial, objective assessments are none too frequently met, it seems important that a critical and informed observer — as Lewis was — did not judge that the evidence presented was sufficient to prove or disprove the proposition that bacteria have nuclei.

Knaysi (1951a) in his book "Elements of Bacterial Cytology" wrote an excellent section on the bacterial nucleus, which expanded his earlier review (Knaysi 1938) on this subject and included a discussion of later work. Instead of considering that three different morphological pictures deserved consideration in the light of published work (as he did in the 1938 review) Knaysi (1951a) believed that five fundamental hypotheses deserved consideration: (1) the bacterial cell has no nucleus; (2) the bacterial cell is a naked nucleus; (3) the bacterial cell contains

nuclear material in the diffuse state; (4) the bacterial cell has a central body; (5) the bacterial cell has one or more nuclei.

Knaysi (1951a) also pointed out that there are two main reasons, apart from the small size of the bacterial cell, why the problem of the bacterial nucleus has required so long to resolve. The first is the masking of the nucleus by the nucleic acids and nucleoproteins of the cytoplasm and the second is the inability of the biologist to define criteria by which a nucleus may be recognised. A third reason which I think should be mentioned is that emphasised by Hale (1953)— namely, that the common method of studying bacteria in dried heat-fixed preparations, although very convenient for routine examination, is not at all suitable for cytological study.

Many other papers on the bacterial nucleus have been published since the review article by Lewis in 1941. Many different suggestions have been put forward on the nature of the bacterial nucleus, and to the uninitiated there seems to be only a great volume of controversial literature which is more bewildering than intelligible. Better informed reading, however, discloses two main schools of thought, as I shall now attempt to establish.

To the first school belong those workers who believe

that the bacterial nucleus is a simple structure that divides in a simple way -- not by a process of mitosis or meiosis. The main supporters of this first school are, in my opinion, Robinow and Deleporte. The second school comprises those workers who regard the bacterial nucleus as a more complicated structure that divides by a process of mitosis. Important supporters of this view are DeLamater and his co-workers; also Beutner, and Dergersen. Also in this latter school I have included those workers who are of the opinion that the bacterial nucleus may divide by a process of meiosis. The two schools of thought are now discussed in turn.

The First School

One of the major contributions to bacterial cytology and one that aroused considerable interest was the study of the internal structure of bacteria by Robinow (1942). Apart from the results, I think that Robinow's paper aroused interest because it was one of the first to be published in English and in an easily accessible and well-read publication — namely, the Proceedings of the Royal Society — and because it contained excellent photographs in which the actual structures described by the author could be clearly seen. Robinow used Piekarski's modification of Giemsa's staining method, in which the bacteria were treated with normal hydrochloric acid at 60°C for

10 minutes and then stained by Giemsa's stain. Robinow also used the Feulgen technique in his study, but he found, when identical preparations were stained by each method and compared, that there was a close correlation between the appearance of the two preparations but that the structures stained by the Giemsa stain were easier to observe and to photograph. The organisms studied by Robinow were Bacillus mycoides, Bacillus megatherium, Bacillus subtilis, Bacillus mesentericus, two unnamed Bacillus species, Proteus vulgaris, Sarcina lutea, Sarcina aurantiaca, and two strains of yeasts. From his results Robinow drew the following conclusions. The nuclear material in aerobic spore-forming bacilli (Bacillus genus) is in the shape of dumbell structures, generally two in a vegetative cell, which are Feulgen-positive and show a strong affinity for basic dyes. Resting spores of these organisms each contain one of these dumbell-shaped bodies which is attached to a rod of protoplasm that is non-chromogenic. In a few spores a dividing dumbell structure could be seen. At the beginning of germination the dumbell-shaped body enters the protoplasmic rod and cannot be demonstrated until a later stage of germination when it is seen to have divided to form two closely contiguous dumbell bodies. In dividing cells,

division of these dumbell-shaped bodies precedes the division of the cell. Cells in the normal vegetative state have generally two of these closely contiguous bodies. Similar dumbell-shaped bodies were also demonstrated in P.vulgaris and the two strains of Sarcina studied. Robinow concluded that these dumbell-shaped bodies are comparable to the chromosomes of plant and animal cells.

A second paper by this author (Robinow 1944) described in some detail the method for the demonstration of the nuclear structure of bacteria for as he rightly pointed out "scepticism about the chromatinic bodies in bacteria is usually due to the use of inadequate cytological methods and unsuitable optical equipment". He now studied Escherichia coli along with P.vulgaris, B. mycoides, B.cereus, B.mesentericus and B.megatherium — organisms which he had previously investigated — for comparative purposes. The organisms were studied as impression preparations, fixed in osmic-acid vapour, treated by normal hydrochloric acid at 60°C for 10 minutes, and stained with Giemsa stain. The preparations were generally examined first as wet mounts.

Dumbell-shaped bodies similar to those observed in aerobic Gram-positive spore-forming bacilli were seen in young broth cultures of E. coli and P. vulgaris.

These bodies were dumbell-shaped and usually one cell of these organisms contained one body, or one or two pairs of these bodies representing division forms. Cells of older cultures also contained one or more chromatinic bodies, usually centrally placed granules or dumbells extending right across the cell, although there were some atypical forms.

In this paper Robinow (1944) also made an investigation of the composite structure of organisms from young cultures. When fixed through agar with Bouin's mixture and stained briefly, bacteria from young actively-growing cultures of E. coli, P. vulgaris, B. mesentericus, and B. megatherium were seen to have a banded appearance indicating that each bacterium truly consisted of two, three, or four separate cells. Moreover, studies of the effect of plasmolysis on B. megatherium brought direct proof of the composite nature of the individual bacillus as the cytoplasm in each cell could be seen shrinking away from the cell wall. In the Addendum to "The Bacterial Cell" by Dubos (1945) Robinow reviewed his technique and reviewed and expanded his previous results. He also devoted a section each to the nuclear structures of germinating and resting spores. In this former section he observed that at the beginning of germination of the spores of

B. mycoides, when the nuclear structures became indistinct, the affinity of the cytoplasm for the blue component of the Giemsa stain was increased and gradually the whole spore stained blue all over and no internal differentiation could be seen. This change in the staining affinities of the bacterial spore during germination had been previously reported by Howie and Cruickshank (1940).

In brief, from his investigations he suggested that the chromatinic material in resting spores was either a "biconcave disc" or a circular body corresponding to a nucleus rather than a chromosome. The nuclear structure could be seen at the periphery of the spore, often causing a bulging-out of the spore coat. In a later paper Robinow (1951) considered that this bulging was probably due to treatment with hydrochloric acid. In the work described in this paper (1951) he used cold nitric acid and thought that it gave a truer picture of the internal structure of the spore, but he still considered that the position of the nucleus was eccentric, lying as a crescent-shaped structure on the outside of the cytoplasm. These descriptions of the appearances of the spore nucleus were criticised by Bisset and Hale (1951) and Bisset (1952) who suggested that as the nuclear material in germinating spores was always found

in the centre, it was likely that resting spores also had their nuclear material in the centre. They considered that the peripheral situation of the nuclear material in the resting spore was due to acid hydrolysis, during which a rise in pressure forced the nuclear material into an eccentric position, which was therefore an artefact of the technique.

Realising the value of those workers' criticisms Robinow (1953) made further investigations into this phenomenon. From ultra-thin sections which were made from non-hydrolyzed spores, and treated in various ways, the internal structure of the spore was revealed. Inside the spore coat, which may be a single or double layer, was a core of dense, basophilic protoplasm, with Feulgen-positive, ribonuclease-resistent chromatinic elements dispersed within the periphery of the core. Sections of spores treated with hydrochloric acid were then examined and the eccentrically placed bulging nuclei were seen. Robinow concluded from the results that the suppositions of Bisset and Hale were correct and that his original theory was wrong.

From these results and those of Fitz-James (1953), who cracked open whole spores with minute glass beads, Robinow (1953 and 1953a) drew a diagrammatic representation of what he considers is the internal structure of

the resting spore based chiefly on the observations of B. megatherium.

The spore coat of this organism has two layers, which may be composed of layers of still finer membranes. Then there is a cortical layer and a dense central core. The chromatinic elements, which have a low density in electron micrographs, are embedded in the surface of the dense core.

Robinow has never at any time described mitotic or meiotic figures or suggested that they may exist. He has been of the opinion that the nuclear bodies of bacteria divide in a simple way. In a personal talk which I had with him in 1954 he confirmed this opinion, saying that he thought the bacterial nucleus divided in a simple amitotic manner.

Delaporte, another important worker in the field of bacterial cytology, made observations over a period of years; these also have now been published in one paper (Delaporte 1950). The value of Delaporte's work has not been fully appreciated by many other investigators. Her research on various types of bacteria included: (i) the demonstration of nuclear material (by Feulgen technique, ferric and other haematoxylins, and Giemsa technique); (ii) the use of stains for metachromatic, lipoid, and glycogen granules; (iii) studies

of living unstained organisms; and (iv) vital staining of organisms.

This sensible and versatile approach to bacterial cytology has much to recommend it, since bacteria do not consist merely of a nucleus in a sac of cytoplasm. Many are known to contain, besides other by-products of metabolism, one or other or all of the types of granules mentioned above, and the presence of these in the bacterial cell can alter the shape of the nucleus.

From her studies Deleporte postulated that the nuclear material, which has never been demonstrated to have an exterior membrane, might be very easily deformed by nearby elements and displaced or penetrated by globules. The shape of the bacterial nucleus or the "nuclear element", she maintained, could depend on the shape of the cell and on the age and conditions of the culture. The nuclear shapes that would be visualised as probable on purely theoretical grounds would be:- for a round organism, a central round body; for an ovoid organism, a central ovoid body; for a short rod, an ovoid body or very short rod; in long rods, an axial thread. But because in the majority of bacteria reserve substances and granules will be present, these shapes will be displaced, deformed, constricted, or fragmented although there may be an invisible link of

the fragments.

In Deleporte's picture division of the nuclear element takes place, without any special internal structure being visible, by a simple stretching and separation into two parts. A granule lengthens, takes on a dumbell shape, and divides into two granules that separate from each other. An axial thread separates in the middle. A small rod divides in the centre and the parts move away from each other in the direction of the long axis of the cell. During a stage of active multiplication the nuclear element may divide more rapidly than transverse membranes can be formed and in these cells four or more nuclear elements can be seen. The spore of the Bacillus genus develops from a fragment of nuclear material which becomes surrounded by homogeneous cytoplasm to form a pre-spore. This pre-spore becomes ovoid in shape, with the nuclear element in the form of a rod, which on growth of the spore migrates to the periphery of the cytoplasm after taking a ring shape. The spore cytoplasm alters its staining affinities and the spore becomes enclosed in a refractile membrane. During spore formation, the nuclear material and cytoplasm that have not been included in the spore progressively disappear, and the spore is eventually liberated. On

germination the spore swells, the nuclear element becomes central, enlarges, and divides. One, two, or three divisions may occur before transverse septa are laid down.

These two workers, Robinow and Delaporte, have never described mitotic figures; and I interpret their writings as showing that they are of the opinion that the bacterial nucleus is normally a simple structure dividing in a simple way. These essentials are definite enough to be regarded as representing one school of thought on the state of the nuclear material of bacteria.

Various ideas on the bacterial nucleus regarded as a simple or fairly simple structure have been put forth by Murray (1953), Hoffman (1951), Clark and Webb (1953), and Fitz-James (1954). After studying the problem of the different effect of various fixatives on the demonstration of bacterial nuclei Murray (1953) gave a schematic drawing of his concept of the nuclear structure as observed in young cultures of B.cereus. He considered that there is a matrix in which the chromatin is embedded, usually irregularly and at the periphery of the matrix. The chromatin surrounds a central core which may contain ribonucleic acid. The whole composite structure may be surrounded by a nuclear membrane.

The idea of a hollow spherical-type nucleus with a central area that may contain ribonucleic acid is supported to a certain extent by Robinow (Personal communication 1954) who thinks that at some stage in the endospore this is probably the shape of the nucleus.

A study of Fusobacterium polymorphum, a lactobacillus, and Clostridium septicum was made by Hoffman (1951) with the HCl-Giemsa technique, the Feulgen technique, treatment with ribonuclease, and a recently developed technique whereby desoxyribonucleic (desoxypentose) acid is demonstrated by methyl-green and ribonucleic acid (pentose) by pyronin. From his results Hoffman concluded that, apart from that present in the cytoplasm, ribonucleic acid was present in the "intracellular" structures as a cortex, with the desoxyribonucleic acid as a medulla. But, as he pointed out, the fact that these structures contain ribonucleic acid does not mean that they are not nuclei, for this acid has been found in plant and animal chromosomes also, but he considers that it is highly likely that the nuclear organisation may be different in bacteria from that in plant and animal cells.

In a study of Gaffkya tetragena, Micrococcus aureus, and Nocardia corallina by Clark and Webb (1953) vesicular nuclei were demonstrated by the crystal violet

nuclear stain described by Chance (1952) and further considered on page 59 of this thesis. There was little correlation between this type of nucleus and the stage of development of the cultures. Thus, the authors conclude that these nuclear forms do not indicate the stage of development of the organisms and may be staining artefacts; alternatively, they suggest, these forms may represent the true morphological form of the bacterial nucleus.

A very recent paper on the bacterial nucleus is that of Fitz-James (1954). He studied germinating spores of B.cereus and Bacillus megatherium. The nuclear structures were demonstrated by Azure A or thionin after fixation and hydrolysis. The content of desoxyribonucleic acid was also estimated. He observed a continuous increase of this substance in the germinating spores during separation of the chromatinic material; this suggested to Fitz-James that the process was not one of typical mitosis. He proposed an alternative scheme based on triads of granules. In this scheme each granule represents a distinct genetic structure. The triad duplicates to give six granules, which separate to give two triads, each of which goes to a daughter cell. Basically this is a process of amitotic division.

The views of Bisset are individualistic. Yet, in my assessment they usefully come between the views of those who consider the bacterial nucleus simple and those who consider it extremely complex. Bisset's views up to 1950 on the bacterial nucleus are well expressed in his chapter on the subject in his book "The Cytology and Life History of Bacteria". He considers that in resting cells (i.e. cells whose metabolic activity is low) the bacterial nucleus is a spherical or vesicular structure lying centrally in the cell but often staining eccentrically. In this form it is found in spores and the resting stages of most bacteria. In active stages of the development of certain cocci and mycobacteria a similar type of nuclear structure can be seen. In young cultures of most bacteria, the nucleus — "the vegetative nucleus" — is in the form of paired chromosomes or chromosome complexes. These chromosomes are short rods lying transversely to the long axis of the cell. When the cell divides, these rod-like structures split longitudinally. Bisset postulated a "complex, possibly sexual, method of division in which nuclear fusion is followed by elongation of the bacterium as a filament, redistribution of the nucleus and finally fragmentation to produce a new generation of bacilli".

Bisset does not think that the distribution of nuclear material at the division of the bacterial cell is similar to that in plant and animal cells. Both members of the pair of rod-like chromosomes are thought to be of equal value, and one is transmitted to each daughter cell. If more than one pair is present, half of the nuclear complement passes to each daughter cell, each chromosome dividing to become a pair in the next generation.

There is usually one or two pairs of chromosomes in cells from young smooth cultures. As the culture ages, the nucleus — "the secondary nucleus" — is either a centrally placed single or double structure, but it does not stain clearly and its exact structure is difficult to determine. Or it may appear as a central chromatinic rod. Bisset considers that a resting nucleus may arise from either of these nuclear phases by an autogamous or sexual process. Bisset firmly believes that bacteria have a process of autogamous or sexual fusion accompanying the formation of the resting nuclei.

Many of Bisset's own observations, I think, have led to rather hasty conclusions charged with a considerable amount of imagination. Much of his published work is of value, however, especially that in which he has criticised the conclusions of other investigators. Bisset's sharp

criticisms have made general readers more critical of cytological papers, especially those concerned with the bacterial nucleus, and Bisset's strictures have also caused various workers either to re-examine their conclusions or to produce more convincing evidence so that their opinions might be as hotly defended as they were attacked.

## The Second School

Numerous workers have described a process of mitosis or have observed mitotic figures in bacteria. Of these, I think the papers of the following workers are important: Barnard, Beutner, Bergersen, and DeLamater and co-workers. The views of these workers, whom I consider to represent a second school of thought on the bacterial nucleus, will now be examined.

An early article by Barnard (1930) is of extreme interest in that it is probably one of the first serious attempts made to study the internal structure of living bacteria. The photographs illustrating this paper are of extremely good quality, far surpassing many of those in some recent papers on the bacterial nucleus.

Various organisms were grown on a thin layer of agar on a glass slide. This was illuminated by an annular beam of ultra-violet light and the image formed

was photographed with a suitable quartz objective. B. megatherium, B.mycoides, B.bronchisepticus, Micrococcus pyogenes var. aureus, Bact. coli and Bact. prodigiosum were photographed after 24 hours' incubation at 37° C. The pictures of B.megatherium show an extraordinary amount of intracellular detail. The spore can be clearly seen as a highly refractile structure. From his observations Barnard suggested that the bacterial cell possesses a nucleus which undergoes mitotic division. He also compared photographs of B. megatherium taken as described above with photographs taken with an ordinary apochromatic objective. The image taken by ultra-violet light through such an objective shows only bodies that fluoresce, which nuclei are thought to do. Although this method was in an early stage of development, fluorescent bodies could be seen, one or two per cell, and they corresponded to a certain extent to the bodies seen in the other photographs.

Beutner (1953) made a cytological study of B. megatherium. In an attempt to make his conclusions less subjective than those so commonly produced in this work, he devised a more objective approach and he also carried out growth-curve studies. Demonstration of the nuclear material was mainly accomplished by a perchloric

acid-azure A technique but a modified Feulgen technique was also used. He found that during the lag phase, the type of nucleus that predominated was a single central aggregate of chromatin, which he called a "resting form". During the logarithmic phase of growth many mitotic nuclei were seen, whereas at the end of this phase, cells with an axial type of nuclear structure were predominant. Such cells were interpreted as being one of a number of evolution forms that appear at this stage of the growth cycle.

From rather poor indefinite photographs Beutner drew diagrams of what he claims are "mitotic" nuclei. In my opinion there are two main objections to this interpretation. Firstly, I do not think that the quality of his preparations or photographs is such that he can draw these conclusions. Secondly, from a study of the diagrams where prophase, metaphase, anophase, and telophase stages of mitosis are claimed to be represented, the organisms appear rather longer than those normally expected in the logarithmic phase of growth where active division is taking place. My own views are that the techniques employed for the demonstration of nuclear material did not demonstrate cross-septa or developing transverse cell-walls; consequently the nuclear bodies of some 3 or 4 cells, which in fact show simple constriction

and separation, are regarded by Beutner as the chromosomes of one cell undergoing mitotic segregation. This point is also mentioned in this thesis under the section on lysozyme (p. 86).

A probable growth cycle in Bacillus megatherium is drawn up by Bergersen (1953). Bergersen observed the organisms under the phase-contrast microscope, demonstrated nuclear material by the osmic acid-hydrochloric acid-Giemsa technique, and used Nadi reagent to determine the presence of cytochrome oxidase and Janus green B to confirm oxidation-reduction activity of granules appearing in phase-contrast preparations. Most of his discussion is spent on the growing points which have some of the properties of mitochondria of the cells of higher plants, in that they are centres of oxidation-reduction processes which involve cytochrome oxidase. These bodies, Bergersen claims, appear at the site of cell division before it takes place. The rest of the discussion merely says :- "The behaviour of the nuclei during the life cycle of B. megatèrium (sic) does not differ from that observed with many other species of Bacillus". (No specific references are given here either from his own work or to that of others on which he bases this assumption). He continues:- "There is a fusion of chromatinic material before vegetative

division and a probable sexual stage involving morphologically distinct gamete-like cells". This is illustrated by a diagram showing the various forms.

I do not agree with the interpretation that Bergersen has made of the forms he observed in his stained preparations, illustrated as those are by photographs which are few and not of exceptional quality. The structures which he suggests are the beginning and end of his postulated life-cycle appear to me to be very like stained developing endospores. He has no evidence that the long threads of nuclear material which he observed after 3 hours' incubation are "fusion nuclei". He states dogmatically that in some microscopic fields cells were found which were fusing and apparently transfixing nuclear material; that he does not show photographs of this process leads one to grave doubts about his interpretation of the structures he has seen.

This paper has something to recommend it — namely, the idea of studying the "growing points" of the cells; but the section on the nuclear material and its development is so unsatisfactory that it could only create a great deal of prejudice against the discipline of bacterial cytology and confer upon bacterial cytologists the reputation for making wild statements without sufficient proof to back them up. Happily this is not true of all workers in this field.

DeLamater and his co-workers, Hunter and Mudd, are the main supporters of the theory that the bacterial nucleus undergoes, at certain stages, a mitotic division resembling that seen in cells of higher plants. They have claimed to have seen centrioles, mitotic spindles, and arrangements of chromosomes in prophase, metaphase, anaphase, and telophase. Numerous papers have been published on this subject. Recent examples are; DeLamater (1951 and 1951a); DeLamater and Mudd (1951); DeLamater, Mudd and Hunter (1952); and DeLamater and Woodburn (1952). From the work described in these and other papers and from other later developments, DeLamater (1953) has given the current views of his group on the structure and mode of division of the bacterial nucleus.

Most of DeLamater's conclusions were based on a study of B. megatherium during normal growth and during growth in the presence of various antibiotics, although observations of Salmonella typhosa grown in the presence of colchicine are also referred to. One of the methods used for the demonstration of bacterial nuclear material was a modified Feulgen technique in which an azure A-$SO_2$ complex or a thionin-$SO_2$ complex was substituted for Schiff's reagent (leuco-basic fuchsin). This technique was applied to other preparations of plant and animal

cells, and chromosomes were demonstrated. DeLamater concluded from this that since these methods stain the chromosomes of plant and animal cells, what they stain in bacteria must also be chromosomes. To me this reasoning is not correct. These methods may indeed be staining the same type of material in all the cells, but just because at certain stages this material is in the form of chromosomes in plant and animal cells it does not follow that it takes up the same form in bacterial cells. This means that much of the work of DeLamater and his colleagues is under suspicion if it is based on this assumption.

In brief DeLamater claims that from his preparations of bacteria undergoing normal growth there is definite evidence for the existence in the bacterial cell of a nucleus possessing a nuclear membrane; and that this nucleus can at various stages be seen to be composed of chromosomes that go through the process of mitotic division complete with centriole and spindle fibres. He also claims that these facts are confirmed by studying the organisms when they are grown in the presence of various antibiotics.

Numerous photographs are produced by DeLamater to show the structures which he has described. In many

of these there are patterns of nuclear material that could be interpreted, by a keen enough supporter of such a view, as stages of mitotic segregation of chromosomes; but on the other hand there are possible alternative explanations of the observed configurations. One of the important things in bacterial cytology is to study an organism by as many different methods as possible and not to base conclusions on the results of only one or two similar methods.

Criticism of DeLamater's interpretations has come from various workers, among them Bisset (1953) who pointed out, quite fairly as I think, that the structures which DeLamater and his colleagues regarded as centrioles and spindles are more likely to be the result of the dehydration by freezing alcohol on the demonstration of developing or developed cross-septa and cell walls (see p. 58 of this thesis). Bisset also criticised the photographic material published in various papers (though he gives the authors of these papers he does not give the exact references to them) by DeLamater and his co-workers on the ground that all but two plates presented as illustrating metaphase nuclei in B. megatherium were from the same photomicrograph of the same cell reproduced at different magnifications and in one case inverted. This criticism cannot, however, be levelled at DeLamater's

(1953) paper on the subject since it is well illustrated with numerous different photographs.

In a previously mentioned paper (DeLamater 1951a) DeLamater claimed to have seen conjugation in B.megatherium by"fusion tubes". Bacilli of smaller diameter found among chains of larger organisms were interpreted as fusion tubes. Bisset (1953) pointed out that such smaller bacilli are often septate, as seen in electron micrographs and in stained films, and in most cases are attached only at one end to a larger bacillus. In Bisset's (1953) opinion, therefore, they "appear incapable of performing" the function of a fusion tube. The structures which were claimed by DeLamater(1951a) to be fusion tubes were further investigated by DeLamater and Hunter (1953) and as a result of their investigation this claim was renewed. But further evidence supporting the view of Bisset (1953) that these structures were not fusion tubes was given by Bergersen (1954), who observed filamentous forms in B.megatherium. These filaments were narrow and were found especially when the organisms were grown on blood-containing media. They appeared to arise from large basal cells and contained pairs of nuclear bodies throughout their length; they were generally non-septate, although a septum was sometimes seen dividing off the terminal part. The results of differential staining suggested that the chemical structure of the cell

wall of these filaments was different to that of the cell wall of the other normal forms found in the culture and that it lacked cellulose-like material, which might account for the flexibility of these filaments. Bergersen (1954) considers that these filamentous forms of B.megatherium are produced by a factor in human serum, and that they are the same as those described by DeLamater and Hunter (1953) as fusion tubes. In living slide-cultures he did not, however, find any evidence of true fusion of filaments and bacilli although the preparations were observed for long periods, and he considers that the fusion tubes described by DeLamater and Hunter (1953) were in fact the beginning of filament formation and not the result of fusion.

DeLamater and Woodburn (1952) also claimed to have observed a typical mitotic process in Micrococcus cryophilus, which they regarded as being unicellular, characterised by centrioles, spindles, elongation and contraction of chromosomes, doubling of chromosomes, and cell-plate formation. The technique which DeLamater used for that particular work was that described later in this thesis (p.57) — a method in which thionine was used as the stain and dehydration was accomplished by freezing alcohol. From studying

DeLamater's (1952) photographs, which are not of exceptionally good quality, I am surprised that his original preparations showed to his eyes the detail revealed in the diagrams drawn from these photographs and presented in his descriptions.

Bisset (1954) has strongly criticised these interpretations of DeLamater and Woodburn (1952) saying that this micrococcus is not unicellular but composed of two or four separate cells, each containing one or two nuclear bodies. These facts he demonstrates quite convincingly by various techniques. It is highly probable that the presence of these cross cell-walls led DeLamater and Woodburn to the wrong interpretation of the structure and nuclear apparatus of this organism, especially as the technique that they used did not completely demonstrate cross-walls or septa; in fact what they interpreted as centrioles and spindles were most probably no more than cross cell-walls and septa incompletely demonstrated.

The multicellularity of micrococci has also been emphasised by Webb and Clark (1954), who used the methods of Chance (1952) for the demonstration of nuclear material (see p. 59 of this thesis) and of Chance (1953) for the demonstration of cell walls (see p. 67 of this thesis) in Micrococcus pyogenes var. aureus. Although they could not see mitotic figures and although the nuclear

structures appeared to divide in an amitotic way, Webb and Clark nevertheless surprisingly affirmed their belief that mitosis and meiosis are probably a part of cell division in bacteria. In the light of their actual observations their attitude is not intelligible to me.

The multicellularity of organisms of the Bacillus genus was emphasised and convincingly demonstrated by Robinow (1944 and 1945) and the complexity of the bacterial cell of members of the Bacillus genus ( as well as of the cocci) was also stressed by Bisset and Hale (1953) when they studied these organisms by the phosphomolybdic-acid-methyl green cell-wall stain. The cross cell-walls of these organisms were well demonstrated, revealing that the supposition of other workers — DeLamater, for example — that these organisms were unicellular was very far from the truth. This also added to existing doubts about DeLamater et al's interpretations of the figures which they claimed to represent mitosis.

The fact that these organisms cannot be considered to be unicellular raises many problems, such as a consideration of the work described when these organisms were thought to be so and re-examination of the structures observed then, as they may now prove to be developing

cross-walls and cross-septa. Another problem raised is the manner in which these cross-walls are formed after there has been a division of the cell contents.

There are divergent views on the manner of the division of the bacterial cytoplasm. Chance (1953a) studied cultures of Gaffkya tetragena stained by his crystal-violet nuclear stain (Chance 1952) and observed a structure very similar to a cell plate, which formed in the nuclear staining material and extended out to the walls dividing the cell into two. Since this first paper was published Chance (1953b) has observed cell plates in fifteen other species including Gram-positive and Gram-negative cocci and Gram-positive and Gram-negative rods.

During the study of Gaffkya tetragena Chance (1953a) found that in many organisms the cell-plate configuration appeared similar to a metaphase picture observed in higher plants, but that subsequent behaviour of the nucleus did not simulate that of higher plants. In fact, some of the cells appeared to show amitotic division as the daughter nuclei were unequal in size. Chance (1953a) correctly insists that an accurate description cannot be given of the behaviour of the nuclear material since its size is near the limits of microscopic

resolution.

Mitosis-like figures were also seen by Clark and Webb (1954) in other bacteria and most frequently in "Corynebacterium pseudodiphtheriticum". Using the modified technique of Chance (1952) they stained preparations of this organism for nuclear material and photographed the preparations. They then treated these preparations by a modification of the tannic acid-crystal violet method (Webb 1954) to demonstrate cross-septa and the same organisms were again photographed. They found that many nuclear configurations that could readily have been interpreted as various mitotic figures were in fact nuclear bodies in separate cells of a "multicellular" organism. What could appear to be chromosomes at an anaphase was probably only two nuclear masses separated by a mature cross septum!

Clark and Webb (1954) do not claim that these results signify that bacteria cannot have classical mitotic figures; but they rightly argue that nuclear study alone is not sufficient to establish their existence, and that just because some cells have been shown to contain a nuclear mass which resembles a mitotic figure there is little justification to claim a mitotic cycle on this basis.

This, I think, is an important paper with sound logical thinking behind it. It would have been easy to

rush into print as some authors do claiming to have shown the mitotic cycle in yet another organism, but these workers refrained from doing this. They used a second method to study these configurations and concluded that, although there might be a form of mitosis in bacteria, the configurations seen were probably not part of such a mitotic cycle.

The results of the work of Clark and Webb (1954) and of Chance (1953a and 1953b) help, I think, to clear up the question of the mitotic configurations observed in bacteria, and stress how important it is to realise that cross-septa and cross-walls if ignored or inadequately demonstrated will lead to very misleading and wrong interpretations of the structure and mode of division of the bacterial nucleus.

Not only have mitotic figures been described, but those characteristic of meiosis have also been claimed by some workers — but not many — to be present in growing bacterial cells. An early paper on this subject was that of Allen et al.(1939); a paper published by Flewett (1948) is also of interest.

Figures suggestive of meiosis in an organism isolated from grass and stained by a vital-staining method (Stoughton (1929),p. 49 of this thesis) were described by Allen et al.(1939). These figures were seen prior to

spore formation, during germination of some spores when sown on to fresh medium, and in liberated spores in an old medium in the "resting" condition. To a certain extent these figures resemble those seen during meiosis in higher plants. But the low magnification, the staining method, the fact that some photographs of bodies claimed to represent asci in old cultures are probably yeasts or fungi, and a close study of the figures lead to the conclusion that it is highly unlikely that the organism studied showed meiosis. Some of the figures described are more like the appearance of two nuclei with a cell-plate between them as demonstrated by Clark and Webb (1954). Flewett (1948) made a study of the nuclear changes in 35 strains of B.anthracis and their relation to variants, by the osmic acid-hydrochloric acid-Giemsa technique. In the early stages of growth the bacilli were seen by Flewett to contain dumbell-shaped bodies dividing at right angles to the cell. Four of these chromatinic bodies were seen in some cells, the number varying with the strain. These bodies were often seen fused together and this fusion was followed by a breakup of the mass into four bodies — one of which was incorporated in the cell and the other three disintegrated. Some cells did not show fusion before one of the bodies was incorporated

into the cell, but the other three disintegrated in the same way. Rough-colony strains showed a tendency to form smooth-colony variants more readily if they had a high incidence of fusion. Strains showing a low incidence of fusion were stable in this respect.

The incidence of variants of other types such as coccal and ghost-medusa colonies was not related to the incidence of nuclear fusion in the strain.

Appearances were seen by Flewett that were "reminiscent of the appearance of the chiasmata in the diakenesis stage in the meiosis of higher plants and animals", but he considered that this was possibly due to the presence of the developing vacuoles and had no other significence. In some of the photographs of his preparations vacuoles may be seen that correspond in position to fat globules stained by sudan black,

DeLamater (1953) considered that evidence for meiotic division in bacteria was scanty, but "some interesting stages have been observed which are suggestive of a meiotic process".

If there is a process of meiosis there must be a fusion of nuclear material. Not many workers have described fusion of nuclear material so an observation of Knaysi (1952) is of value here.

While studying Mycobacterium thamopheos by phase-

contrast microscopy Knaysi (1952) observed polar bodies that sometimes fused together. These bodies gave a positive, though often weak, Feulgen reaction. He did not, however, make any reference to the possibility of this fusion being connected with meiosis. In my opinion the fusion could equally well be explained by the coalescence of two lipoid granules and lipoid granules can give a positive Feulgen reaction, a point which is discussed by Dondero et al (1954).

Many of the studies of the bacterial nucleus have been carried out on members of the Bacillus genus. Bacteria of this group produce endospores, which, as I think, has greatly complicated the issue; but a short review of the literature on the cytology of the bacterial endospore should be included here.

In his review of the literature on the cytology of bacteria Lewis (1941) devoted a small part to endospores. He mentioned several conflicting reports on the formation of endospores. There appear to be two main theories: the first, that the spore is formed by the fusion of numerous sporogenic granules; the second --and more widely accepted -- that it is formed from a clear polar spore primordium set off from the rest of the cell by a membrane and that it results from the condensation of material inside this primordium.

Numerous papers have been published about the bacterial endospore since Lewis's (1941) review, either on the spore alone or on the cytology of the spore and the vegetative cell.

Besides shorter works there are two excellent comprehensive articles on the bacterial endospore. The first of these is by Knaysi (1948), who reviewed the literature on the cytology of spore-formation, the resting period, and the germination of the endospore. Knaysi (1948) discussed his own conclusions in the light of this review and his own observations. The other comprehensive text is really a "symposium on the biology of bacterial endospores", to which 10 workers contributed. In the symposium Knaysi (1952a) discussed the cytology of sporulation. Briefly his conclusions are as follows. A potential spore-producing cell may contain one or several nuclei. Dense material is deposited around one terminal nucleus, which is of moderate size and appears to initiate the formation of the forespore. Presporal inclusions and other nuclei which may be present in the fertile part of the sporangium are moved or pushed into the sterile part. The nucleus continues to remain in a central position in the forespore; it may, however, be seen to consist of a pair of chromosome-like bodies which divide to form two pairs. A highly refringent coat is formed within the boundary of the forespore, the remaining peripheral layer becomes the spore coat. Knaysi (1952a) does not think that the endospore has any sexual rôle in the life cycle of the organism.

Allen et al. (1937) examined the chromatin arrangements in spore-forming organisms. They studied six organisms

including B.subtilis and B.megatherium, and used the vital-staining method of Nakanishi modified by Stoughton (1929), in which the stain is Ziehl's carbol-fuchsin diluted with an equal amount of water. In young cultures, many cells showed a sharp division into two sections—one with little staining affinity and the other deeply staining. In older cultures such two-section cells divide to give rise to one deeply stained cell and one poorly stained cell. In three of the organisms studied further observations, which are not described, led the authors to the conclusion that each cell differed in its subsequent cytological development and though each produced a spore, the structures extraneous to the spore were different. (The nature of these structures was likewise not stated). Allen et al. considered that there may be two alternative methods of spore-formation and that this might be the cause of different descriptions by different workers of the same organism. Although the spores were refractile for some time after formation and release from the cells, they might later take up the stain revealing a deeply stained granular structure, which showed re-arrangement over a considerable period of time. This mixture of observation and hypothesis suggested to Allen et al. that in some instances the spore might have a function other than that of a resistant resting stage—namely, that it might provide an opportunity for the

re-arrangement of chromatin material.

To me, Allen et al's. paper seems of importance though it lacks some essential descriptions. The division of the cell into two parts, one deeply stained and the other not, could at first be explained by saying that all the cytoplasmic contents had gone to one end of the cell and had been cut off by a cross-septum or cross-wall which was not demonstrated by the method used and that the part of the cell that did not stain was dead. But it would appear that both parts were capable of growth into organisms of normal size, which one would not expect to happen with a dead or dying cell. These two cells gave rise to endospores but, according to Allen et al., by different cytological developments and with different extraneous structures after the spore is formed. Neither these nor the cytological developments are described. It is a point that should prove worthy of further investigation, however. If two different types of cell produce endospores by different methods, it may mean that the spores are different and this leads one to the conclusion that this may be related to the problem of sex and the recombination of characters, which has now been reported by various workers — for example, Lederberg (1951) and Hayes (1953).

Although it is concerned with the nuclear structures of the small Myxophyceae and not of bacteria, a paper by

Cassel and Hutchinson (1954) is of importance in bacterial cytology. Preparations of these Myxophyceae treated by either the osmic acid-hydrochloric acid-Giemsa technique or the Feulgen technique followed by treatment with tannic acid, showed definite nuclear bodies. These bodies might be spherical, netlike, or rodlike, and at the time of cell-division they appeared to divide equally by a constrictive process. One organism, a species of Oscillatoria, is of particular interest in that it resembles very closely a chain of organisms of B. cereus in an active stage of division. (Figs.166-168 for B.cereus and Fig.169 for Oscillatoria).

Cytological studies on Oscillospira guilliermondii by Tuffery (1954) revealed that this species was a large multicellular organism each segment containing a pair of rodlike nuclear bodies. From these studies and others where the demonstration of cell walls and flagella of " a distinctly bacteriological character" were demonstrated (Tuffery 1954a) the author concluded that this organism, though very large, is definitely eubacterial in nature and not a member of the Myxophyceae.

## Methods used in bacterial cytology

The methods and reagents used in bacterial cytology have naturally been many and varied. Demonstration of the nuclear material of bacteria is not easy by any means, and

this has given rise to the great variety and many modifications of method. Theoretically, there are 3 main steps in the demonstration of bacterial nuclear material: the first, the fixation of the organisms; the second, the removal or breaking down of the ribonucleic acid in the cytoplasm; and the third, the staining of the nuclear structures.

When it was brought out in 1924, the Feulgen technique gave a great impetus to study of the question of the bacterial nucleus. This method demonstrates what we now know to be desoxyribonucleic acid. First, the organisms are fixed. Originally fixation was done with sublimative acetic fixative, but others such as alcohol formalin and acetic alcohol are now used. As Hillary (1939) pointed out the technique is carried out in two stages after fixation. The first — mild hydrolysis — breaks the binding between the purine base and the carbohydrate complex of the nucleic acid. This splitting off of the purine forces the liberation of the aldehyde group of the aldo-pentose sugar. The second stage is the chemical reaction between the liberated aldehyde group and the leuco-basic fuchsin to form a violet colour complex.

This technique was and is used with success on plant and animal cells to demonstrate nuclear material. Knaysi (1951), however, has constantly warned investigators using

this technique for bacteria not to presume that it is specific, because other substances with a free aldehyde group would give similar colour reactions. He suggests that it should never be used alone or applied in a rigidly fixed manner.

In Bradfield's (1954) modified Feulgen technique for use with the electron microscope the liberated aldehyde reduces ammoniacal silver hydroxide, thus depositing metallic silver, which can easily be detected by the electron microscope.

In a recent paper on the Feulgen reaction in bacteria Dondero et al. (1954) described a semi-quantitative test whose results suggested that some of the variation in the microscopic Feulgen reaction depended on the age of culture, the nature of the substrate, and the species. These authors also developed quantitative test-tube Feulgen method, in which the Feulgen-stained material was removed from the bacterial cells with 5% perchloric acid. Application of this test showed that lipids did not contribute significantly to the colour of the Schiff reagent. Some workers had previously doubted this. The reliability of this quantitative Feulgen technique was verified by the decrease in Feulgen value when the cells were treated with desoxyribonuclease, and the high correlation between the Feulgen value and the diphenylamine tube test for desoxy-

ribonucleic acid.

In the discussion the authors emphasise that there is no reason why "some lipid could not be associated with desoxyribonucleic acid", i.e. why the nucleus could not contain some lipid.

The Feulgen technique has been largely superseded by the osmic acid-hydrochloric acid-Giemsa technique which was really popularised by Robinow (1942) although Piekarski (1937) originally introduced it as a modification to the Feulgen technique. This technique is not specific but is comparatively quick and easy to do. Its first stages consist of fixing the organisms in osmic acid (osmium tetroxide)vapour and treating them with normal hydrochloric acid at 60° C for about 10 minutes, the actual time being varied according to the state of the cultures. After being washed, the organisms are stained with Giemsa stain. Robinow (1942) used this technique and the Feulgen technique on parallel preparations, making a constant comparison between the two. He found that there was a close correspondence between the structures stained by the Giemsa stain and those stained by the Feulgen technique. The former were optically superior, being easier to see and photograph, and spore nuclei were also well demonstrated by this

method.

Peshkoff (1945) introduced the use of light-green as a counterstain for preparations fixed in alcohol/chloroform/acetic acid fixative and stained with Giemsa's stain. He claimed that this led to a better demonstration of the nuclear material.

DeLamater (1948) studied the action of 5 distinct nuclear stains and staining procedures which utilised basic fuchsin as the dye on a Feulgen-weak fungus Blastomyces dermatitidis and other fungi. He found that aqueous basic fuchsin was an excellent stain but it faded very quickly. Treating the cells with formaldehyde produced a more permanent staining effect. Treatment with formaldehyde should be done after hydrolysis but before staining.

This technique as well as the HCl-Giemsa technique described by Robinow (1942) gave rise to a modified technique suggested by Smith (1950). This technique consists of fixation with osmium tetroxide vapour for 1-3 minutes, hydrolysis in normal hydrochloric acid at 60°C for varying periods depending on the state of the organisms, mordanting in 1% formaldehyde for 2-4 minutes and staining in 0.3% aqueous basic fuchsin for 15-30 seconds. The organisms which Smith treated by this method were E.coli, Proteus, Staphylococcus aureus and B.megatherium. From the results the author concluded that this technique "possesses advantages in terms of sharpness of definition of nuclear chromatin, of keeping qualities, of simplicity and rapidity of preparation".

No attempt was made in Smith's (1950) paper to interpret a nuclear cycle in the organisms studied, nor to establish any genetical significance to the chromatinic bodies demonstrated even though Smith was convinced that they represented the nuclear apparatus of the cell.

During the discussion of his modified technique for the demonstration of bacterial chromatin Smith concluded that it would give a truer definition of chromatin distribution than the same preparations treated by the hydrochloric acid-Giemsa and other techniques. He considered that Giemsa stain, which is a rather complicated mixture of various thiazin-eosinates, is less selective in its staining reaction than the comparatively simple basic fuchsin in pure form (pararosaniline). Comparison of preparations treated by these two stains after appropriate treatment gave him the impression that structures stained with Giemsa stain were more often in the form of unresolved pairs and that those stained by basic fuchsin were more delicate in structure.

From his observations on B.megatherium, E.coli, Proteus and Staph.aureus which were made with his modified basic-fuchsin method for the demonstration of bacterial chromatin, Smith stated that "of the four species the chromatinic structure of B.megatherium appears to be the most complicated and hardest to interpret". The chromatinic material of this organism was not similar in distribution to that seen with

E.coli and Proteus. It was more often in granular masses or strands. The individual chromatinic bodies ranged from dumbell-shaped structures to definite rodlets. The problem of over-fixation was not considered for this organism so it could not be said if the arrangement of these chromatinic structures were due to this. As Smith pointed out Robinow (1942) found this organism "difficult to differentiate satisfactorily" with the Giemsa technique and found that the Feulgen technique gave the best results.

A new staining procedure introduced by DeLamater (1951) was based primarily on the observation by two other workers Atwood and Orinstein (1949) that azure A or thionine in the presence of $SO_2$ stained chromosomes intensely and apparently selectively. Preparations of bacteria after hydrolysis in N.HCl at 60°C for a specific time were treated by either of these stains (thionyl chloride was used as a source of $SO_2$ in each case) for a minimum of 2 hours. After being stained the preparations were immersed in absolute alcohol chilled to about -50°Cfor at least 12 hours. This is a process of dehydration introduced by Blank et al.(1951) which is claimed to help in the making of undistorted permanent preparations. The results seen by DeLamater when this new technique was carried out on preparations of B.megatherium were considered by him to be further proof for the existence of a mitotic cycle in bacteria.

Bisset (1953a) criticised this method of DeLamater. He pointed out that although DeLamater claimed that the method of dehydration preserved the material intact, and that the staining technique was specific for nuclear structures, no controls were submitted to support these contentions. Bisset studied similar preparations of B.megatherium and found that dehydration in freezing alcohol caused considerable shrinkage and distortion of the contents of the cells. He also showed that thionin did not stain the cell-walls of the organisms, but stained the cross-septa. He considered that the "spindles" and "centrioles" seen by DeLamater were derived from cross-septa and cross-walls, shrunken by dehydration, and that this technique obscured the true multicellular structure of B.megatherium. This in turn would lead to wrong interpretation of the nuclear arrangements. Bisset considered that the descriptions of mitotic figures in B.megatherium by DeLamater were invalid because of these reasons.

Another method is the orcein staining method which is regarded to be "cytogenetically specific" for "nuclear" structures in the cells of higher plants and animals, and has been suggested by Scaletti et al. (1952) as a suitable method for the demonstration of bacterial nuclear material. Twenty-four-hour cultures of B.cereus, B.megatherium, E.coli and Azotobacter chroococcum were examined. Discrete

deeply-staining bodies were observed in all cultures except A.chroococcum and these structures were similar to those observed in the same organisms by other workers with other methods.

Still another method for the demonstration of nuclear material was suggested by Chance (1952), in which aqueous solutions of crystal violet, mercuric chloride, and nigrosin were used. Studies of Gaffkya tetragena were made by this method and the author claimed that the staining procedure was dependable for demonstrating the nucleus of this organism at any age.

The evolution of a direct method of staining the two types of nucleoprotein was claimed by Hartman and Payne (1954). Their staining procedure was adapted from that described by Jacobson and Webb (1952) for other cells; by it the ribonucleoprotein is stained blue and the desoxyribonucleoprotein is stained red. Application of this procedure to cultures of E.coli in the log phase of growth revealed that the cytoplasm was largely composed of a homogeneous distribution of ribonucleoprotein which resembles the dense material seen under phase-contrast, but desoxyribonucleoprotein was confined to the nucleus and occasional small perinuclear granules.

The important problem of the fixation of the bacteria has given rise to numerous papers. A study of the nuclear

structures of E.coli fixed in osmic tetroxide (osmic acid) and chromic acid and observed under the phase-contrast microscope or stained by the Feulgen technique was made by Guha et al.(1954). They showed that, when this organism was examined by phase-contrast microscopy after fixation with osmic tetroxide, a regular pattern of dark and lighter zones was observed. This pattern was reversed when the organisms were fixed in chromic acid. When the bacteria fixed in chromic acid were treated by the Feulgen technique, longitudinal thread-bodies were demonstrated but in bacteria fixed by osmic tetroxide the Feulgen-positive bodies were round in shape.

Murray (1953) devoted a paper to this subject (i.e. to the problem of fixation for studies of bacterial nuclei) at the International Congress of Microbiology at Rome. Cells of a two-hour culture of B.cereus were fixed with osmium tetroxide or methanol with 10% formaldehyde solution, with or without 1% picric acid (MFP or MF). After fixation by one of these two reagents the cells were subjected to one of the following five procedures: (1) direct staining with thionin; (2) hydrolysis with N.HCl (at 60°C for 10 minutes) and staining with Giemsa; (3) treatment with ribonuclease and staining with Giemsa; (4) treatment with ribonuclease, hydrolysis with N.HCl, and staining with Giemsa; and (5) Feulgen technique. Unfixed, unstained

organisms were also examined by phase-contrast.

There was a marked difference between the cells fixed by the two different reagents and treated by procedures 1 and 2. There was a certain amount of correlation between cells fixed by the two reagents and treated by procedures 4 and 5 and very good correlation between those fixed by the two reagents and treated by procedure 3.

From these results Murray concluded that the different arrangement of nuclear structures might be due to the "differing reactivity of cell constituents after fixation with reagents that seem adequate in other aspects". He agrees that the strictures of Dalaporte (1950) on the importance of the comparison of different combinations of fixatives and nuclear stains are well founded.

Murray also drew up a provisional hypothesis from these results on the nuclear structure of B.cereus in the early stages of the growth cycle. This is discussed on p.25 of this thesis.

With regard to the fixation of bacteria with osmium tetroxide vapour a paper by Smith (1950) is of interest. Fixation of cultures of E.coli and Proteus showed that the chromatinic structures ( as demonstrated by basic fuchsin after hydrolysis) tended to become condensed and rounded and that there was a certain amount of shrinkage of cells

if the preparations were fixed for 3 minutes instead of for 1 minute. There is real danger, therefore, in prolonging the time of fixation and in using too great a strength of osmic acid, e.g. if it is new. The facts reported in these papers make it important that, in any study of the bacterial nucleus, the exact details of reagents and methods should be given.

The appearances and configurations seen when bacteria are stained with cytological preparations need very careful interpretation. This interpretation needs to be as objective as possible. Objectivity has not always been attained and I think that the descriptions in some papers are very imaginative. Bisset (1952) emphasises the dangers of these interpretations, rightly insisting that all forms of fixation and dehydration are liable to cause distortion, and the important but often ignored fact that many types of bacteria are multicellular. Yet, even with these facts in his mind, Bisset's own interpretations of the true state of the bacterial cell and its nuclear structure from fixed stained preparations are rather imaginative, nearly all the configurations being interpreted to fit in to his own particular theory.

The treatment of the bacterial cell by hydrochloric acid at 60°C has been criticised because it is a harsh treatment and is quite likely to give rise to artefacts.

Alternative methods have been suggested for the removal of the cytoplasmic ribonucleic acid which masks the staining of the nuclei. Two important contributions are those of Tulanse and Vendrely (1947) and Cassel (1950).

Tulanse and Vendrely treated various species of organisms with ribonuclease (pancreatic) and found that nuclear bodies could be well demonstrated. Care had to be taken with the choice of fixative; osmium tetroxide could not be used since the osmium interferes with the action of this engyme. Instead, Chaubard's mixture (sat. aqu. mercuric chloride 2 pts./abs.alcohol 1 pt.) was used. After treatment with ribonuclease at 60° C, the organisms were stained with Giemsa solution. The following results were obtained. With cultures of Bact. coli single central nuclei could be seen in some cells, while in others there were regularly spaced groups and chains of nuclei. Organisms of B.anthracis showed very clear pictures of nuclei, generally round but in some cases constricted. In the culture of C.diphtheriae there was a "bewildering" variety of nuclear structures, even though it was known that the particular strain examined had no metachromatic granules. The authors promised that they would try to find a correlation of these figures with the growth cycle of the organism. Gonococci showed well defined nuclei. Pyogenic staphylococci were very resistant

to ribonuclease and also to treatment with hydrochloric acid, although it was known that these organisms contained as large amounts of ribo- and desoxy-ribonucleic acids as other organisms. The authors postulated that in these staphylococci there must exist some special complex between the ribonucleic acid and the protein of the cytoplasm that could not be attacked.

The best pictures of nuclei were obtained with cells from the end of the lag phase or the beginning of the logarithmic phase. Here the nuclei were very large and difficult to demonstrate without pre-treatment as there was a high ribonucleic acid content which gave an intense basophilic reaction. In older cultures the cells were far less basophilic and the nuclei were often seen without pre-treatment. This was also observed by Poshkoff (1945) who studied 24 hour-old cultures of the Enterobacteriaceae by his previously mentioned Giemsa-light green method ( p. 55 of this thesis) where no hydrolysis or similar treatment was given. A possible explanation of the above results is given by Tulasne and Vendrely. It may be that many bacteria contain an active ribonuclease, but produce only a little desoxyribonuclease; in old cultures, where many of the cells are dead, the ribonuclease breaks down the ribonucleic acid but leaves the nuclei untouched. This may not be so with all bacteria as there is variation in

the amount of ribonuclease produced. Bact. coli produces very large amounts of this enzyme, which is very active and has been used as a substitute for the one taken from the pancreas. On the other hand, B.anthracis and staphylococci produce much less of this enzyme.

Cassel (1950) investigated the action of perchloric acid on the demonstration of nuclear bodies in B.cereus. Preparations of organisms grown at 37°C for 3 hours were first fixed either by Schaudin's fixative for 20 minutes or by osmium tetroxide vapours for 1½ minutes. They were immersed in a bath of perchloric acid at 4°C, removed at varying intervals of time, washed with water, and stained with 0·1% aqueous basic fuchsin. The results showed that after treatment with perchloric acid for about 3½ hours there was the first suggestion of chromatinic bodies; a definite image was seen after 7 hours. After 18 hours the cytoplasm still stained rather heavily, but after 31 hours the cytoplasm stained poorly and the chromatinic bodies were well demonstrated. Results were the same for both fixatives. Comparisons were also made with preparations of organisms treated by normal hydrochloric acid at 60°C for 7 minutes after osmium fixation. A comparison was also made of treatment with perchloric acid and hydrochloric acid at 70°C after osmium fixation. With perchloric

acid chromatinic bodies were well demonstrated after 15-30 seconds' treatment. But after 1½ minutes they became faint and after 7 minutes they disappeared completely. Treatment with hydrochloric acid revealed obscure chromatinic bodies after 30 seconds, but after 2 minutes there was a clear picture of them which remained even after 7 minutes.

From these results Cassel (1950) concluded that the perchloric acid is of value in bacterial cytology, for it may help to resolve the controversy about artefacts due to heating. This acid treatment at 4°C would appear to remove or denaturate the ribonucleic acid in the cytoplasm, thus allowing the chromatinic bodies to be deomstrated, but at higher temperatures the desoxyribonucleic acid of these structures is also affected.

As already pointed out the multicellularity of some bacteria has been ignored and many confusing descriptions have resulted. The staining of the bacterial cell-wall and especially the cross-walls is, therefore, of importance in bacterial cytology. Various staining methods have been used. One of these methods consists of treating the cell with tannic acid and was brought into general use by Robinow (1945) although treatment with tannic acid had been suggested earlier by Gutstein (1924 and 1924a). This treatment with tannic acid mordants the cell-wall, increasing

its affinity for a dilute solution of aqueous crystal violet. The preparations are temporary water mounts.

Hale (1953a) describes a phosphomolybdic acid-methyl-green cell-wall stain, which has proved of value and is especially applicable to some bacteria that do not stain by the last method. The preparations here are also temporary water mounts.

A method suggested by Chance (1953) involves the use of a basic dye, such as crystal violet or new fuchsin and the decolourisation of the cell contents by use of an acid dye such as congo red. This method has proved successful with most bacteria especially such forms as were difficult to stain by the above two methods i.e. Nocardia and Streptomyces, but it has the disadvantage that the cross-septa are only very faintly stained.

Webb (1954) developed a technique that combines both the tannic acid-crystal violet and fuchsin-congo red methods. Tannic acid is used as a mordant, congo red as a selective decolourising agent and a 0·5-1·0% solution of crystal violet can be used to stain the cell-walls and cross-septa leaving the cell cytoplasm uncoloured. The cell-walls and especially the cross-septa are very sharply defined and this method has the advantage of yielding permanent dry mounts.

The importance of demonstrating the cell-wall as

well as the chromatinic structures of bacteria was realised by Cassel (1951) who suggested the following treatment. Osmic acid fixation, followed by hydrolysis with hydrochloric acid, staining with 0·1% basic fuchsin, treatment with 10% tannic acid for 5 minutes and re-staining with basic fuchsin. In this way the cross-walls as well as the nuclei were demonstrated. Organisms from young cultures of members of the Bacillus genus showed many cross-walls usually each unit having one or two nuclear structures. When E.coli and Micrococcus pyogenes var. aureus were studied in this manner, little success was obtained in the demonstration of the cross-walls.

The various staining methods described have usually been examined by the ordinary light microscope, which has the disadvantage that fixed, dead, stained organisms are being observed at a magnification which is not high, yet cannot be increased. Other methods of microscopy, however, have been used in the study of the internal structure of the bacterial cell. These methods are phase-contrast microscopy and electron microscopy. They have been used alone or in conjunction with staining methods. Some investigators have used the three methods for the examination of similar organisms and this has proved of

value as will be revealed later.

Phase-contrast microscopy — although it is now overshadowed by its more colourful sister, interference microscopy — has proved of value in the study of the internal structure of the bacterial cell. It has the advantage of showing internal differentiation of living unstained and unfixed bacterial cells. This method is also of value when it is used in conjunction with other methods, such as examination of fixed stained preparations by ordinary light microscopy, electron microscopy, or by both methods.

An examination of the morphology of Corynebacterium diphtheriae by phase-contrast microscopy was carried out by Hewitt (1951). The organisms were generally fairly transparent, sometimes unicellular, but often divided up into segments by well-defined cross-septa. In gravis and mitis strains it was noticed that the cross-septa were few in number and the segments long, but in intermedius strains there were many cross-septa and often the organisms resembled a chain of fused cocci. As in fixed stained preparations of killed bacteria, swollen club-shaped cells were seen in the living organisms and occasionally branched forms were encountered.

A study of a culture of B.serous grown on "broth agar plate" (the composition of the agar is not given, nor

the time and temperature of incubation) by phase-contrast microscopy was made by Hewitt (1951a). He found that there was a great variety of forms, some cells were almost uniformly opaque, some had opaque and clear patches, while others were nearly transparent except for opaque circular inclusion granules. These granules varied in the number per cell, generally 3 to 6, and in their arrangement. While mentioning the postulate that there might be nuclear structures with a unit of three chromosomes to each cell (a similar idea to that of Fitz-James (1954) p. 27 of this thesis) and that their re-arrangements might resemble mitosis, Hewitt pointed out the danger of such reconstruction since there was no supporting evidence that such granules had any nuclear significance. When the cells were stained by the osmic acid-hydrochloric acid-Giemsa technique and examined by the phase-contrast microscope these granules appeared dark and opaque and unstained. Under the light microscope they were unstained and had a light appearance. Hewitt concluded that "the granules are not nuclear in nature but probably fat-storage depots".

Hewitt (1951a) also found phase-contrast microscopy valuable in the study of the effect of cultural conditions on bacterial cytology (see p. 94 of this thesis).

Clifton and Ehrhard (1952) made a study of actively dividing cells of B. anthracis (variant R563) by dark-phase

contrast. They present evidence that the nuclear structures of these cells are spherical nuclear structures and that possibly the nucleus assumes a resting stage between divisions.

Phase-contrast microscopy was also used by Tomcsik and Guex-Holzer (1954) to show the reaction between the cell-wall and homologous antibody in a bacillus related to B.anthracis. This reaction was very clearly demonstrated when the organisms were treated with lysozyme or trypsin. The specific antibody was shown to react with the transverse septa and polar bodies of the capsule rendering them also visible by phase-contrast.

Phase-contrast microscopy has also been used in conjunction with electron microscopy and staining methods. Experiments on those lines by Bringmann (1952) and Eisenstarke et al.(1950) are reviewed on p. 78 and p. 76 of this thesis respectively.

The advantage of the electron microscopy is the increased magnification that can be obtained. Bacterial cytologists at first thought that this increase would enable them to determine the finer details of intracellular structures seen by the light microscope and suitable staining methods, and would also reveal new structures. While the electron microscope has proved of value in the study of flagella and the morphological structure of various

spirochaetes, apart from general applications in the field of virology, it has so far not proved of great value to the cytologist interested in the nuclear material and other intracellular structures of bacteria. The main trouble lies in the fact that most bacteria during normal growth are opaque to the electron beam. Special treatment is usually required to reveal any internal structure.

McFarlane (1949) points out that the electron microscope has not settled many problems since many organisms that show bodies when stained by the Feulgen or Giemsa technique are too opaque to be studied with electrons. He adds, however, that it may be that in some of these stained organisms what is taken to be the nucleus is in fact the shrunken body of the organism itself, especially since the cytoplasm of most bacteria is diffusely basophilic. From the results of my own observations by phase-contrast microscopy of comparable preparations to those stained or examined under the light microscope I have formed the opinion that this is not so.

Robinow and Cosslett (1948) studied the internal structure of various bacteria by the electron microscope. The bacteria examined were either naturally transparent to the electron beams at 50-90 kv (kilovolts) or they were

specially treated — for example, by brief exposure to glacial acetic acid, to vapours of ether or of ether plus 5-10% acetic acid, or to alcohol followed by treatment with osmic acid or formalin. Structures which these authors took to be nuclei by reason of their "characteristic shape and arrangement" were of lower density than the surrounding cytoplasm.

Many other attempts by a variety of methods have been made to render bacterial cytoplasm transparent to the electron beam — for example, by growth on poorly nutrient media; treatment with glass beads; use of higher voltage; treatment with osmic acid and other reagents. Successful results were obtained by Knaysi and Baker (1947), McClung (1950), and Bringmann (1953) with different organisms when they were grown on various media poor in nutrients. The internal structures of the organisms were revealed by this method (These three papers are reviewed on p. 90 and p. 92 of this thesis under the heading "Nutrition").

Dawson and Stern (1954) studying the cell-wall structure of Staph. aureus and Strep. faecalis during division, found that these organisms were opaque to the electron beam. Shaking with glass beads, however, removed the cytoplasmic contents and the organisms became transparent to the electron beam. So treated, they consisted only of an empty "sac",

in which cross-septa could be observed. The study of these septa was the main purpose of the investigation. No mention was made of any discrete bodies that might have been nuclei in the liberated cytoplasmic contents.

Increase in the voltage has helped to reveal internal structures in organisms that were opaque to the electron beam at lower voltages. Knaysi and Mudd (1943) showed that one or more granules were demonstrated in cells of Staph.flavocyaneus at 200 kv which were not visible at 60 kv. These bodies often appeared constricted or in pairs. Cells from young cultures, however, were mostly homogeneous at the higher voltage. A strain of Neisseria meningitidis also showed granules at 200 kv and not at 60 kv. Cells of strains of Neisseria gonorrhoeae, Staph.aureus and Strep.pyogenes were opaque to the electron beam at both voltages.

In very young actively growing cultures of Brucella abortus and Pasteurella pestis the cells appeared uniform or showed one or two transparent areas, which Knaysi and Mudd (1943) regarded as vacuoles or reserve material. In older cultures one or two dense bodies with sharp outlines were seen. These observations were all at the higher voltage. The authors are of the opinion that "different bacteria may contain nuclear material in different states and that the nuclear material may change with the development

of the cell".

It would also appear from a paper by Mudd and Smith (1950) which is more fully reviewed on p. 77 of this thesis, that osmic acid helped to demonstrate nuclear material in organisms examined by electron microscopy.

Knaysi (1951a) put his finger on the problems of electron microscopy when he said "The obstacles in the way of demonstrating the bacterial nucleus with the electron microscope are identical with those which for many years hindered its demonstration by the light microscope". In both cases the nucleus has to be unmasked and then recognised. This statement could apply equally well to phase-contrast microscopy.

Studies of the nuclear material by varying methods helped a great deal towards the solving of these problems. An attempt in this direction was the introduction by Bradfield (1954) of a modified Feulgen technique (described on p. 53 of this thesis) for use with electron microscopy. In this way the position of the nuclear material could be determined. In his examination of Staph. aureus and a paracolon bacillus by this technique, Bradfield found that the nuclear material was central in both organisms and, although the limits were not well

defined, generally spherical in shape.

Other attempts to solve this problem have been made by studying bacteria by electron microscopy, phase-contrast microscopy, and HCl-Giemsa technique. For example, a study was made by Eisensterke et al. (1950) of a pleomorphic strain of Azotobacter by these three techniques. They found that in young cultures of this organism deeply staining intracellular bodies were demonstrated by the staining technique. In organisms from cultures less than 24 hours' old there were definite bands of this material across the cells. There was some resemblance between these stained structures and those seen by phase-contrast microscopy (where they appeared I think as dark bands; this is not definitely stated, but it appears to be so from the photographs) and electron microscopy (where they appeared as light bands). In older cultures these staining structures gradually disappeared from the cell although some cells show a peripheral staining area.

The authors consider that these stained intracellular granules are not fat bodies, volutin, gonidia, or reproductive organs but probably represent nuclear bodies similar to these described in other bacteria — though why the disappearance of this material in older cultures should be used as proof of this idea I do not know.

A method for making comparable specimens for examination

by light and electron microscopy has been devised by Murray and Wyckoff (1953). After examination of impression preparations by the light microscope, the organisms can be stripped off by a layer of collodion, which can be mounted on a grid for examination by the electron microscope.

Another method has been described by Mudd and Smith (1950) whereby parallel preparations of organisms may be made for comparative study by the electron microscope and the light microscope. By this method the action of the various reagents used in cytological procedures was studied. The specific method was that described by Smith (1950). The organisms investigated were Erberthella typhosa, E.coli and Proteus. It was found that osmic acid vapour was an excellent fixative in that it preserved the existing pattern of the nuclear sites and cytoplasm and yet increased the contrast between the two, both in photographs taken by light and electron microscopy (where the nuclear sites appeared light against a dark cytoplasmic background). Treatment with hydrochloric acid, however, was found to reverse completely the pattern of density in the electron micrographs. The nuclear sites were dark against a light cytoplasmic background. This increase in density was probably due to a coagulation of nuclear material. The nuclear structures were discrete bodies corresponding to

the chromatinic structures described by Robinow. This acid treatment permits the demonstration of stained nuclear bodies by the light microscope. Treatment with 1% formaldehyde for 3 minutes followed by washing in distilled water did not change the results just described. When the preparations were stained with 0.3% basic fuchsin for 15-30 seconds stained nuclear bodies were well demonstrated by the light microscope but by the electron microscope the pattern of nuclear sites and cytoplasm was obscured by the stain.

From the results the authors suggested that "bacterial nuclei have a lower density (mass per volume) of solid matter to effect electron and photons and to fix osmium than the surrounding cytoplasm", and that they have characteristic shapes which stain with chromatin dyes and do not merge with the cytoplasm. They express as they say "in the idiom of the cytologist" that "bacteria possess vesicular nuclei containing chromatin". This paper is of value in showing how different pictures of the bacterial nucleus may be produced by differing methods of treatment and examination.

A comparative study of the nuclear material in 30 strains of E.coli and two strains of paracolon organisms by staining methods, phase-contrast microscopy, and electron microscopy was made by Bringmann (1952). He expected like many other workers to be able to see finer details of the

nuclear structures of Bact. coli by electron microscopy but preliminary examination of young cultures showed no internal differentiation at all. Attempts to obtain differentiation with hydrochloric acid and perchloric acid were not successful. A method was evolved, however, where the organisms were grown on a poorly nutrient crushed wheat-extract agar. Organisms grown in this way, when examined by the electron microscope, were found to contain dense polar bodies with lighter cytoplasm in between. Preparations of similar organisms were examined by phase-contrast microscopy embedded in a viscous solution of high polymer polyvinyl alcohol and were found to contain dark polar granules in lighter cytoplasm. This was the picture seen when similar preparations were treated by the HCl-Giemsa method—the nuclear staining material also being present as polar granules. This work is an example of the demonstration of nuclear bodies by three different methods when the ribonucleic acid content of the cytoplasm had been removed or broken down. It was also noted that, although actively growing, organisms from a glucose-peptone yeast-extract agar did not show differentiation under the electron microscope at first, treatment with osmic acid demonstrated structures which possibly were nuclear structures.

An excellent review of technical methods by Hale (1953) deals admirably with the precise details to be observed in carrying out most of the techniques at present in general use in bacterial cytology.

As already pointed out, bacteria grown in normal media have generally been found to be opaque to the electron beam and little internal structure has been revealed by the electron microscope. Other techniques have helped to render the bacteria less opaque, but bacterial cytologists have mostly concluded that the electron microscope was not being used to its fullest degree in the study of the internal structure of the bacterial cell.

Then in 1953 a rather startling new technique came into bacterial cytology. This was the cutting of ultra-thin sections of bacteria for examination by the electron microscope. Although the technique was new, its value had been realised as early as 1927 when Churchman (1927) (see also p. 88 of this thesis) studying the loss of the Gram reaction in B.anthracis under certain conditions put forth a hypothesis about a Gram-positive cortex and Gram-negative medulla to explain the results but ended by saying "positive proof of the correctness of this explanation must await the evidence furnished by cross-sections of bacteria".

Chapman and Hillier (1953) cut sections of a rough

variant of B.cereus, less than 0·1μ thick. The organisms grown in heart brain infusion medium for 7 hours were fixed with osmic acid, dehydrated in alcohol and finally embedded in a mixture of n-butyl methacrylate monomer, ethyl methacrylate monomer, and "luperco CDB" ( composition of this was not given). Polymerisation of this mixture was usually complete after 24 hours' incubation at 47°C. Both longitudinal and cross sections are shown in this paper and the amount of internal structure that they reveal is quite amazing. A particularly fine micrograph of an ultra-thin longitudinal section of one organism, magnification X 60,000, is claimed by the authors to show: the cell-wall; peripheral bodies; the beginning of centripetally growing transverse cell-wall in the middle of the bacterium; a completed transverse cell-wall before thickening; low density fibrous components of nuclear apparatus; a dense body in this apparatus which may be an inclusion of cytoplasmic material; small dense particles which appear to be the main constituent of the cytoplasm; and unidentified cytoplasmic inclusions. This list is given to show the large amount of internal structure that is revealed. Other micrographs of longitudinal and slightly oblique sections show: distribution of peripheral bodies (which may be mitochondria); formation of super-

nummerary transverse cell-walls. Micrographs of transverse and oblique sections show the distribution of the peripheral bodies, and the position of the nuclear material.

From a study of these sections the authors have drawn up a possible plan of the division of these cells. Without giving a description of this procedure I should like to mention a very few significant points that emerged in the discussion. The first was that the nuclear material seemed to consist of low density spherical bodies, the second and more important one, that "at present no structures have been observed which could be interpreted as being the mitotic figures described by DeLemater" and various other workers. The nuclear structures bore some resemblance to those described by Delaporte although she was studying older cultures. The authors point out that the sections shown represent only one tenth of the total volume of cells and that consequently no definite statements regarding the existence of mitotic figures can be made. It would seem to me, however, that one tenth represents a good proportion of the cells in a culture, and that it would include a very good cross-section of the types of nuclear structures present in them. The bacteria are from a young culture and therefore would be in an active state of division. If mitotic figures are formed I should

have expected them to be formed at this stage.

The last point is that no nuclear membrane could be detected in any of the cells.

Ultra-thin sections of E.coli were made by Birch-Andersen, Maaløe and Sjöstrand (1953) using a technique adapted from that used for sectioning of tissues. The treatment of the bacteria was similar in principle to that mentioned in the previous paper. The sections were cut of bacteria in the logarithmic phase of growth and at the resting phase of growth and were not more than about 200 A.U. (0·02μ) thick. Examination of these sections by the electron microscope revealed that the bulk of the material inside the organisms consisted of a sponge-like structure of uniform pattern surrounded by a membrane, which was often loose, attached to the cell only at irregular intervals (this was probably the result of previous treatment).

In the central part of the bacteria, different types of structures were present depending on what stage of growth the organisms had reached. In the logarithmic phase, one or more large low density vacuoles were present containing irregularly folding threads so tightly wound that they appeared as dense bodies in an empty vacuole. There was no indication of a membrane surrounding these

vacuoles. In the resting phase, the vacuoles become narrow winding ducts each containing a single thread.

The authors point out that it is difficult to draw conclusions since the cells were fixed in osmic acid and some of the structures observed may be due to that, and the cells were also treated with the other materials. Also since study of successive sections had not been made, it could not be said if E.coli exhibits "true mitotic patterns" or not.

By indirect methods the authors concluded that the threads observed in the ultra-thin section were composed in part of desoxyribonucleic acid.

The significant points that emerge from this paper are again:- the inability to demonstrate mitotic figures or a nuclear membrane, and the high density of the nuclear material.

Electron micrographs of ultra-thin sections of Staph.aureus and a paracolon bacillus of about 0·035μ in thickness, used to illustrate a previously mentioned paper by Bradfield (1954) are not of the excellence of those described in the two previous papers. They are of importance, however, in that the nuclear material was taken to be the region of low density and that no nuclear membrane could be observed, and there was no strong proof that mitotic figures ever occurred. Of interest were the sections of an older

culture of the paracolon bacillus, which showed a very irregular distribution of the nuclear material, which appeared to ramify throughout the cytoplasm.

Thus in studies of a Gram-positive spore forming bacillus, a Gram-negative non-spore forming bacillus and a Gram-positive coccus, mitotic figures were not observed, nor was there any evidence for the existence of a nuclear membrane. In two of these studies the nuclear material appeared as low density material, but in the other the nuclear material was a dense contracted mass of tightly wound threads in a clear vacuole-like structure.

Ultra-thin sections of bacterial spores have been cut and examined by Robinow (1953). The main body of the results is reviewed elsewhere ( p.21 of this thesis) but the sections of about 0·3 to 0·4μ show the internal structure of the spore clearly. One striking fact was that the coat of B.cereus spores has only one layer, but two layers can be seen in the coat of B.megathorium spores.

A technique recently described by Alexander and Jackson (1954) that may have an application in bacterial cytology (see p.268of this thesis) is that where soil organisms were studied undisturbed in their natural relationships to one another. Soil samples were impregnated with a resin mixture, and thin sections of this were then cut which could be stained to show the micro-organisms present.

There is no reason why this technique should not be adapted so that the nuclear structure of these organisms could be demonstrated.

Lysozyme

Treatment of bacteria with lysozyme has also been of value in bacterial cytology. The action of lysozyme on a strain of B.megatherium has been shown by Welshimer (1953) to disrupt the chains of organisms as a result of lysis of the walls of adjoining cells. It also brings about the breakdown of the capsular material. It has been shown that lysozyme depolymerizes and hydrolyses a mucopolysaccharide consisting of an acetyl-aminopoly-saccharide, and the conclusion drawn is that the cell-wall and capsule of this organism consist at least in part of an acetyl-aminopolysaccharide.

The ability to break up chains of bacilli into individual cells has its advantages in the study of the bacterial cell and especially of nuclear structures. I think that wrong interpretations have often been made because the method of demonstrating the nuclear material has not also defined the actual limits of the bacterial cell. I think in particular of a paper by Beutner (1953) (see p.31 of this thesis for review and criticism) in which he claimed to demonstrate mitotic figures in B.megatherium.

Lysozyme has further applications in bacterial cytology.

Weibull (1953) found that when cells of B.megatherium were treated with lysozyme in a dilute phosphate buffer the resulting lysis left only two microscopically detectable elements: granules, which were probably of lipid(lipoid) material, and spherical empty "ghosts". If sucrose solutions were used instead of phosphate buffer only the cell-wall of the organisms was depolymerized, the rest of the cell remaining complete as the protoplast. These protoplasts could by lysed by dilution of the solute, leaving ghosts and granules as already described. It is important to note that flagella were still attached to the protoplasts, which confirms their protoplasmic origin.

Further studies of this subject by Weibull (1953a) led him to the belief that the ghosts might represent the cytoplasmic membrane. The cytochrome system of B.megatherium was deposited with the ghost fraction in differential centrifugation. Weibull (1953a) did not detect discrete nuclear material, but he thought that by use of the proper medium isolation of nuclear bodies might eventually be possible.

I think that the use of enzymes such as ribonuclease and lysozyme can play a great part in the unravelling of the complicated structure that is the bacterial cell, provided that they are used in conjunction with other methods.

Nutrition.

The nutritional requirements of bacteria, especially pathogenic bacteria, have long been a subject of intensive study. Little use, however, has been made of the knowledge gained or the methods used in their possible relation to bacterial cytology. One of the major contributions to this aspect was that of Knaysi and Baker (1947) who studied cultures of B.mycoides by the electron microscope; but before reviewing this paper I consider that the efforts of some earlier workers deserve mention although they were not all concerned with the effect of nutrition on the internal structure of the bacterial cell but rather with the effect of nutrition on the reaction to Gram's staining method.

Churchman (1927) studied the effect of small amounts of aqueous gentian violet, acriflavine, or acriviolet on young cultures of B.anthracis. He found that the antiseptic dyes, apart from reducing the diameter of the cells, caused them to lose their Gram-positivity. He also noted similar changes in other aerobic spore-formers but non-sporing organisms were generally not affected. Churchman suggested that a Gram-positive corter might be peeled off and a Gram-negative medulla exposed, but he noted that cross sections of bacteria would need to be made to confirm this — a forecast of some prescience in the light of recent developments. The most important of the earlier papers is that

of Stearn and Stearn (1929), who showed that when Bacillus cereus var. Frankland was grown in physiological salt solution (no formula is given for this solution but I would expect it to consist of 0.85% sodium chloride in distilled water) for 48 hours at 37°C the cells showed variation both in their reaction to Gram's method and in their size. There were full-sized Gram-positive and Gram-negative organisms as well as vacuolated Gram-negative and small Gram-negative cells. Twenty-four-hour cultures of B.subtilis and a colon organism were used as controls for the Gram reaction. When the 48-hour saline culture of B.cereus was sub-cultured on nutrient agar slants, 24 and 48 hours' growth on these gave mainly Gram-negative organisms. When these organisms were grown in physiological salt solution for 7 weeks at 37°C and smears made at various intervals, it was found that the reaction to Gram's method was negative, the organisms staining only faintly by a counterstain of basic fuchsin or of safranin. A 48-hour nutrient-agar sub-culture from the above was composed , to about 40% of Gram-negative organisms, the rest being stippled with Gram-positive granules ("partially starved cells"). Gram-positivity was not lost when a culture of B.cereus in the salt solution was left at ice-box temperature.

Stearn and Stearn (1929) concluded from these results that the loss of Gram-positivity was due to the use of

acidic food reserves such as nucleins and nucleoproteins by the organisms in the absence of a nutritive environment. At ice-box temperature they suggested that the organisms were probably dormant and were not undergoing any significant change. Stearn and Stearn were not in agreement with Churchman's hypothesis of a Gram-positive cortex and Gram-negative medulla, but thought that in view of the stippled appearance of the "partly starved" cells the material responsible for the retention of the Gram stain was more or less uniformly distributed throughout the cytoplasm.

An earlier paper by Enderlein (1925) stated that the nuclear body was observable only when cells held little food reserve. In his view, this reserve material, which consists mainly of ultra-microscopic granules (trophocania), represents the actual chromatinic material of the cell and stains strongly with basic dyes because of the high content of nucleic acid and nucleoprotein. When bacteria were starved by keeping them in distilled water, the last remnants of food reserve clung tenaciously about the nuclear bodies. Alcohol extraction could be used as an alternative method to remove this reserve material from the cells.

Knaysi and Baker (1947) found that when B.mycoides was grown in the usual laboratory medium before examination, the organisms were generally opaque to the electron beam

and little of the internal structure could be seen. When the organisms were grown in a nitrogen-deficient medium, however, bodies could be seen inside the cells. The medium used consisted of 0·2% glucose plus 0·23% of an equimolar solution of dipotassium hydrogen phosphate ($K_2HPO_4$) and potassium dihydrogen phosphate ($KH_2PO_4$); sodium acetate could be substituted for the latter solution. The organisms were grown on a nutrient-agar slope and washed off with sterile distilled water; a heavy inoculum was then placed in the deficient medium. It was claimed that many of the endospores germinated in this medium using up their supply of ribonucleic acid and that the germ cells and subsequent generations of vegetative cells were transparent to the electron beam and showed two types of opaque bodies.

The first type of body was relatively large and very opaque, and it showed evidence of division, although it did not appear to divide by simple constriction. It was totally enclosed in the forespore and is thought by the authors to be the nucleus of the cell.

The second type of body was small and very thin, consisting of beaded threads and granules in the cytoplasmic membrane. It did not appear to take part in the formation of the endospore, but there was evidence that it was endowed with synthetic powers and was possibly involved in the formation of cross-plates at the time of cell division.

From this paper it seems probable that the ribonucleic acid of the bacterial cell renders it opaque to the electron beam and that if the supply of this substance is exhausted in metabolism, as when the organisms were grown without a source of nitrogen, the internal differentiation of the cell was thereby revealed, nuclei and other bodies becoming visible.

Other papers concerned with the demonstration of intracellular structure by growth on poor or deficient media are those of McClung (1950) and Bringmann (1953).

McClung, while studying the morphology of various members of the Nocardia genus, found that when Nocardia.ruber was grown on nitrogen-free medium the cytoplasm became more transparent to the electron beam (at 50 kv.). This increased transparency made the study of the internal structure easier and two types of bodies were seen, spherical opaque bodies at the poles and a larger elongated body generally in the centre with a diffuse outline.

Bringmann (whose paper is more fully reviewed on p. 78 of this thesis) grew E.coli on poorly nutrient wheat-extract agar and found that organisms from this medium showed polar bodies by electron microscopy, phase-contrast microscopy, and the HCl-Giemsa method.

A very valuable contribution to the study of the effect of nutrition on the internal structure of the bacterial cell was made by Duguid (1948). When studying

Bacterium (=Aerobacter)aerogenes grown in a synthetic medium with carbohydrate and peptone present, or a combination of phosphate and ammonium sulphate in adequate amounts to replace the peptone, he found that the morphology of the bacteria was influenced by the nutrient balance and the pH of the culture medium. On media containing little peptone and a large quantity of carbohydrate and in media that were deficient in either phosphate or ammonium sulphate in relation to their carbohydrate content, cells of an unusual type were formed. These cells had very large capsules, and when stained by 1% aqueous methyl violet, deeply stained bodies in weakly stained cytoplasm were seen. When bacteria of this type were sub-cultured on nutrient agar the cells grew and multiplied rapidly and in a few hours they stained uniformly.

The deeply stained intracellular bodies were thought by Duguid to be nuclear bodies identical with those described by Robinow and other workers as being present in many bacteria. He based this claim on the following reasoning:- the bodies have a high affinity for basic dyes; their position and their constant presence in the cell is characteristic of nuclei; they are not volutin granules since they do not give a metochromatic staining reaction; they can be demonstrated "vitally" stained in unfixed wet

films; on sub-culture to normal medium the cells reproduce rapidly and normally therefore they do not represent the shrunken protoplasm of degenerate or plasmolysed cells. Duguid's reasoning is perfectly sound, but the results would have been of more value if the observed bodies had been demonstrated by alternative methods — by the Feulgen technique or after treatment with ribonuclease, for example.

From the results Duguid suggested that a deficiency of either phosphate or nitrogeneous nutrient prevented the accumulation of ribonucleic acid in the cytoplasm and this meant that the desoxyribonucleic acid of the nucleus could be demonstrated by basic dyes.

Phase-contrast microscopy has been used by Hewitt (1951 and 1951a) to study the effect of cultural conditions on bacterial cytology. This method has the advantage that the organisms are observed in their living unfixed state, which eliminates the possibility of artefact production inherent in staining methods and electron microscopy.

Hewitt's (1951a) studies on Pasteurella pseudotuberculosis were of interest. When grown on agar plates, the organism consisted of small oval almost transparent cells. When grown in broth the organisms were longer, containing refractile material at the poles, and they remained together in long festoons. Hewitt suggested that

the differentiation of refractile material in broth cultures could account for the bipolar staining of the organisms.

An unnamed Gram-positive bacillus also showed a variation in morphology when grown on agar and in broth. In broth, long filaments were formed and the organisms contained refractile bodies which, according to Hewitt, (1951a) resembled chromatinic structures; on agar the growth consisted of separate organisms. As already mentioned the morphology of Corynebacterium diphtheriae was also studied by this method by Hewitt (1951); (see p. 69 of this thesis). He noted that when the gravis strain of this organism was grown in a magnesium-deficient broth, long filaments were formed with cross-septa. When the same strain was grown in the presence of 0.1% magnesium sulphate, it formed small short cells, possessing more highly refractile areas at the poles but without cross-septa.

It was known that the diphtheria bacillus could reduce tellurite and that on selenite medium colonies of this organism became scarlet red due to the production of selenium. From his studies of this bacillus grown on tellurite and selenite media, Hewitt (1951) concluded that the elementary tellurium and selenium formed might be deposited locally in the cell, possibly in the region of nuclear structures.

Hewitt (1951a) also made a study of the cells of

B.cereus grown in the presence of selenite. Filamentous forms with cross-septa were seen containing refractile patches which the author claimed might represent nuclear structures. Similar studies on Corynebacterium fascians grown in the presence of selenium revealed dark refractile patches resembling nuclear structures. Hewitt (1951a) considered it possible that the nuclear material of these three organisms might have an affinity for tellurite and selenite or the nuclear material might constitute regions of intensive metabolic activity where preferential reduction of the elements could take place. Webb's (1949) conclusion that magnesium ions were necessary for the fission of bacteria during multiplication was also emphasised by Hewitt (1951a) with reference to cultures of B.cereus grown in magnesium-deficient medium. In such a medium the organisms form long filaments with cross-septa and segments of varying refractive index, as compared with the normal form which shows individual organisms containing dark granules.

Knight and Proom (1950) studied the nutritional requirements of 296 strains of the genus Bacillus. B.megatherium, they found, could grow with ammonia as a nitrogen source and in the absence of growth factors, whereas B.cereus grew in the absence of added growth factors

but required mixtures of amino acids instead of ammonia. These points are expanded in the section of this thesis marked "Discussion".

One other important paper in connection with nutrition and the Bacillus genus is that of Foster and Perry (1954), who studied the intracellular changes in B.mycoides during sporluation and concluded that this sporulation involved a synthesis de novo of at least some spore matter. They thought that the synthetic process probably used intracellular material formed from the degredation of existing components of the cell, and regarded spore formation as "endotrophic" — a term intended to convey that spore formation is independent of exogeneous material, using only the constituents within the cell at the time of sporluation.

Clearly, changes in the nutritional environment and the conditions of culture may have a marked effect on the internal structure of the bacterial cell. It seems to me that cytologists have not yet made enough use of this method of exploring matters which may influence in readily ascertainable fashion both the formation and the conformation of structures suspected of being nuclei.

Inclusion bodies

In the review article by Lewis (1941) there is a very

interesting section on bacterial inclusion bodies. In my opinion, the most important of these bodies in regard to the bacterial nucleus are those composed of fat. Lewis points out that fat may be seen in some bacteria as highly refractile, spherical, oval or elongated bodies, which resemble endospores and were often mistaken for such by early investigators. These bodies may vary in shape but they are generally spherical. They may also vary in size often reaching a diameter equal to that of the cell, or even larger, in which case the cell becomes bulged. Fat-bodies are not stained by the usual methods used in bacterial cytology, and in stained material they appear as clear areas which have been mistaken for vacuoles as well as for endospores. Stained cells containing them have been described as being interrupted, speckled, granulated, barred, beaded, vacuolated, and alveolar. The biological significance of these bodies in bacteria appears to be the same as in other cells. In some cells they probably function as reserve food; in others they may denote fatty degeneration.

In some of his earlier investigations Lewis (1934) found that young cells of B.mycoides stained evenly with aniline dyes. Older cells had an uneven staining appearance due to numerous highly refractile non-stainable granules resembling oil globules. He made suitable tests for fat,

volutin, and glycogen, and concluded from the results that these granules consisted of a "non-living substance of a fat-like nature which serves as a food reserve".

Lewis (1934) also made a study of spore formation in this organism grown on agar, and observed the fate of these granules during the process. As far as it was possible to determine, these granules played no direct part in the early stages of spore formation; they generally diminished in number during the ripening of the spore and were generally absent in old sporangia. Two explanations were possible: the first, that the fat substance might be broken down to contribute material for the development of the spore; the second, that it might be used by the mother cell for its own metabolic processes. Lewis concluded that it was not impossible for the granules to serve the two functions, but thought that the second was the less probable.

In old broth cultures the organisms contained numerous granules but few or no spores. Other workers had suggested that under these conditions, which were not suitable for spore formation, the granules could be gonidia; but Lewis (1934) did not think that they had any reproductive functions. Lewis (1941) said that the conditions necessary for deposition of fat-bodies in the form of visible droplets were not fully understood, for many species

that did not show these droplets had a large amount of extractable fat.

There are also some very interesting points about fat-bodies (or lipid granules) in a paper by Delaporte (1950). These lipid granules may be seen as refractile globules in living bacteria and are generally formed by polymerization of β-hydroxybutyric acid. Delaporte points out the importance of these granules in connection with the arrangement of the nuclear material. In cells where these granules are present "the nuclear substance is generally divided into several granules located in the interstices between" the globules. She considers that the nuclear substance of bacteria is very easily deformed by nearby elements and displaced and penetrated by globules. Whenever the edge of a nuclear mass presents a concave surface, it can usually be seen that a lipid granule or some other granule is present there, for example in the "horse-shoe" structures. Delaporte emphasises that it is likely that other shapes are due to the presence of adjoining elements and are not determinate structures but altered passive ones. This, I think, is a very important point. It must also be remembered that because of their high surface tension lipoid granules will be spherical and can more easily disturb other material than be deformed by it.

When studying the results of a modified technique (see p. 55 of this thesis) on the demonstration of chromatinic structures in B.megatherium, Smith (1950) noticed structures which he described as vacuoles in organisms grown on enriched tryptone glucose yeast-extract agar at 37°C for 2½-3 hours. These vacuoles consisted of clear areas, generally circular and seen in nearly every "cellular unit". They were associated with chromatin, sometimes as a single granule, or several granules, and at other times as "strands of chromatin streaming away from opposite poles". In my opinion, these vacuoles were most probably composed of lipoid material. This point is enlarged in the section of this thesis marked "Discussion".

Volutin or metachromatic granules have also aroused the interest of the bacterial cytologist mainly because they are intensely basophilic and have often been mistaken for nuclei in the bacterial cell. A paper by Winkler (1953) given to the International Congress of Microbiology at Rome summarises the present idea about these granules. After a review of the literature he came to the conclusion that these granules, which are intensely basophilic and dense compared with the cytoplasm, are not an essential part of the bacterial cell, as nuclei are, but that they may have an important function in the aerobic metabolism of some organisms. They have a high content of meta-

phosphates of heterogeneous composition and correspondingly heterogeneous activity.

A more recently observed type of inclusion was described by Hannay (1953) in B.cereus var. thuringiensds, an insect pathogen. During a study of spore formation in this organism Hannay noted that the spores were invariably accompanied by a diamond-shaped crystalline structure, which varied in size but not in shape. These crystals were observed also in other spore-forming insect pathogens (variants of B.cereus)but not in strains of B.cereus from other sources. As Hannay explains, it is hard not to speculate about a possible connection between the crystals and the pathogencity of the organisms. It may be that they are some form of crystalline inclusion body composed of virus of phage, or the crystals may be formed as a genetical characteristic that is connected with the formation of toxic substances.

The purpose of including this paper in the review is to emphasise how complex the internal structure of the bacterial cell may be and that there are many types of inclusions.

From this review of only a few of the many papers on the subject of inclusion bodies in bacteria I hope to have made it clear how important it is to realise that inclusion bodies are often present in the bacterial cell.

These bodies may have a quite marked effect on the structure and distribution of the nuclear material in the cell and its subsequent interpretation. For a composite and true picture of the bacterial nucleus, nuclear stains are not enough, other methods of examination and staining must be carried out to show the presence and nature of these inclusions and effect on the nucleus.

Bacteriophage

On first consideration there did not appear to me to be any relationship between bacteriophage and the bacterial nucleus. On further thought, however, I became aware that what affected the bacterial cell and its metabolism was bound to affect the bacterial nucleus. That is why I consider the following papers to be important.

The changes during the action of specific bacteriophages on B.cereus and E.coli were found by Delaporte (1950) to be essentially the same in both organisms until the liberation of the phage. Within the cell, the nuclear elements fuse; then there is such an increase in the nuclear substance that it fills practically all of the bacterial cell; thereafter the whole cell enlarges. When the phage is liberated from B.cereus the chromatinic substance disappears little by little from the periphery of the cell, whereas in

E.coli the cell swells rapidly into a sphere and bursts.

A very interesting paper by Murray and Whitfield (1953) showed that infection of E.coli with the bacteriophage T5 and related bacteriophages caused a loss of nuclear staining material during the first half of the latent period. During the second half of this period, however, new nuclear material was synthesised and took the form of a fine granular core, with some of the phages a spherical nuclear mass was also formed. Murray and Whitfield suggested that this arrangement of the newly-formed material may be a hereditary phage-characteristic and may be of use in classification. But even if this is proved not to be so the paper is valuable in that it shows that the nuclear material is affected by and connected with the bacteriophages.

Observations by Luria and Human (1950) on the cytological changes in E.coli when attacked by bacteriophage revealed that different phages gave specific changes. Phages of the T-even group, when active, caused a disruption of the chromatinic bodies followed by a swelling of the cells and a large increase of granular chromatin. When the phage was inactivated by ultra-violet light, the chromatin gradually disappeared. Other phages caused the accummulation of the chromatin even when inactivated. The authors raised

the question whether the granular chromatin could be phage nucleoprotein.

The status of bacterial nuclei is therefore open. Chromatinic material there undoubtedly is. Sometimes it is arranged in patterns that are reminiscent of those observed in dividing plant or animal cells. One school of thought has striven to establish the reality of bacterial nuclei by the closeness of the analogies between such appearances in bacteria and those in plant and animal cells in which their structure and function is not in doubt.

Greater moderation, better sense, and a harder discipline seem to be accepted by those who think that an essentially experimental and more fundamentally bacteriological approach is required before reaching conclusions about the probable nature and significance of the differentially-stained material. The work to be described in the rest of this thesis is intended to show some possibilities of an approach of this kind.

# MATERIALS AND METHODS

# M A T E R I A L S  A N D  M E T H O D S

## MATERIALS AND METHODS

In the work now to be described, investigations of various kinds were carried out with ten different species of micro-organism grown in ordinary and special media. Besides common staining methods, specialised techniques were employed to demonstrate particular structures or aspects of bacterial morphology. The details of organisms, media, and methods of examination are given in this section.

### ORGANISMS

The organisms studied are listed below with the sources from which they were obtained. With the exception of the Rhizobium and Clostridium species, all the organisms were maintained in meat-extract agar or broth.

| Organism No. | Species | Source |
|---|---|---|
| 1 | Bacillus anthracis (Avirulent strain 34F2) | *N.C.T.C. No.8234 |
| 2 | Bacillus cereus | N.C.T.C. No.8035 |
| 3. | Bacillus megatherium | N.C.T.C. No.7581 |
| 4. | Anthracoid bacillus | Isolated in the laboratory as a contaminant on an agar plate. |

* N.C.T.C. = National Collection of Type Cultures, Colindale, London.

| Organism No. | Species | Source |
|---|---|---|
| 5 | Micrococcus lysodeikticus | N.C.T.C. No.2665 |
| 6 | Aerobacter cloacae | Laboratory culture |
| 7 | Rhizobium species | **Botany Department, |
| | i) clover strain | University of |
| | ii) lucerne strain | Glasgow |
| | iii) field bean strain | |
| 8 | Clostridium putrificum | N.C.T.C. No.4718 |
| 9 | Clostridium sphenoides | N.C.T.C. No.507 |
| 10 | Clostridium tetanomorphum | N.C.T.C. No.2909 |

MEDIA

The following media were used:-

1) Meat-extract agar and broth

For the purpose of maintaining the aerobic organisms to be studied these media were prepared as described on page 144 of the 1948 (8th) edition of Mackie and McCartney's "Handbook of Practical Bacteriology"

2) Robertson's medium

For the purpose of maintaining the anaerobic organisms to be studied this media was prepared as described on page 185 of the 1948 (8th) edition of Mackie and McCartney's "Handbook of Practical Bacteriology".

3) Citrate medium (Ministry of Health,1939)

| | |
|---|---|
| Sodium chloride | 5.0 gm. |
| Magnesium sulphate | 0.2 gm. |

** I am grateful to Dr.G.B. Bond for the cultures of the three strains of Rhizobium.

| | |
|---|---|
| Ammonium dihydrogen phosphate($NH_4H_2PO_4$) | 1.0 gm. |
| Dipotassium hydrogen phosphate(anhydrous) | 1.0 gm. |
| Distilled water | 1000 ml. |

To the above solution (pH 6.8) 2 gm. of citric acid are added and the reaction is brought back to pH 6.8 by adding normal sodium hydroxide solution.

This medium was used in studies of Aerobacter cloacae.

4) Basal medium (modified from that of Meiklejohn,1950)

a) As a fluid

| | |
|---|---|
| Dipotassium hydrogen phosphate($K_2HPO_4$) | 1 gm. |
| Sodium chloride | 2 gm. |
| Magnesium sulphate | 0.5 gm. |
| Ferric chloride | trace |
| Calcium carbonate | 10 gm. |
| Tap water | 1000 ml. |

b) As a solid

2.0% of washed agar is added to the fluid.

This medium and the solutions given below were used in various nutritional studies.

10% soluidons of the following compounds were made in distilled water:-

Ammonium sulphate

Asparagine

Peptone

Sodium nitrate

Urea

Glucose

These solutions were added in appropriate amounts to basal medium, in either its fluid or solid form (see above), to give a 1% concentration.

For brevity the fluid medium with basal nutrients, chemically defined, is hereafter referred to as "basal broth" and the solid medium as "basal agar".

5) Yeast-extract mannitol agar (Fred et al. (1932))

| | | |
|---|---|---|
| Mannitol | 10 | gm. |
| Dipotassium hydrogen phosphate ($K_2HPO_4$) | 0.5 | gm. |
| Magnesium sulphate | 0.2 | gm. |
| Sodium chloride | 0.1 | gm. |
| Calcium carbonate | 3.0 | gm. |
| Yeast water | 100 | ml. |
| Glass-distilled water | 900 | ml. |
| Agar | 15 | gm. |

Yeast water

10 gm. of baker's yeast are added to 100 ml. of distilled water. After shaking, the mixture is allowed to stand at room temperature for 1-2 hours and thereafter is autoclaved for 40 minutes at 15 lb. pressure. The supernatant clear fluid — yeast water — is pipetted off after the mixture has been allowed to settle in the refrigerator for five days.

This medium was used for the maintenance of Rhizobium species;

6) Yeast extract-tryptone broth (Welshimer, 1953)

| | |
|---|---|
| Yeast extract (Difco) | 20 gm. |
| Tryptone | 2 gm. |
| Distilled water | 1000 ml. |

The pH is adjusted to 7.2, and the broth tubed in 10 ml. amounts, accurately measured.

This medium was used for the investigation of the action of lysozyme on cultures of Bacillus megatherium.

## METHODS OF EXAMINATION

The methods employed to demonstrate the different structures of the bacterial cell depend upon the microscope.

In the present work, three types of microscope were used: the ordinary light microscope, the phase-contrast microscope, and the electron microscope. The methods appropriate to each will be described separately and an account will be given of some problems of method alone which had to be overcome before certain techniques could be brought into use.

## ORDINARY LIGHT MICROSCOPY

### Impression preparations.

The great majority of the films of organisms studied were made as impression preparations. The value of this type of preparation is that it allows examination of the bacteria in their true relationship with one another, i.e. they remain in the positions in which they have grown. Generally, in impression preparations, the organisms are all in the same plane; this makes the preparation easier to examine and to photograph than a smear preparation. A particularly important reason for using impression preparations is that they require little material and that enough is available on a spread plate during the first few hours of incubation although there is very little visible growth at that time.

To obtain impression preparations the following method was carried out: about three drops of an overnight meat-extract broth culture were placed on the surface of a meat-extract agar plate and spread very thoroughly over the whole surface by means of a glass spreader in order to ensure an even film of growth on incubation. The spread plates were incubated at a suitable temperature for the required period of time. Then, half-inch squares were cut from the agar with a scalpel previously sterilised by flaming it with methylated spirit and allowing it to cool.

The squares of agar were lifted on the tip of the scalpel and carefully inverted on to clean, grease-free No.1 coverslips, the film of bacteria coming in contact with the coverslips. (The coverslips were of the large size, 7/8" square; they were stored for at least a week in methylated spirit, and before use they were dried with a piece of clean silk material.) After a few seconds the agar was carefully removed on the tip of the scalpel leaving films of bacteria on the coverslips as impression preparations. While making impression preparations of the organisms and throughout the whole of the staining procedure, it was extremely difficult to prevent the organisms from becoming dry at one stage or another. For this reason, the preparation was allowed to dry at this stage so as to know exactly when drying happened and to ensure that it was at a uniform time throughout. The agar squares were discarded into an empty petri dish for disposal. With care, a single agar plate will provide many preparations, either all at the same time, or at successive periods of incubation. The plate should not become contaminated, but as more agar is removed it becomes more liable to drying. When the bacterial growth becomes heavy, impression preparations made from it are very thick. This is not desirable, because with many layers of organisms it is difficult to see the internal structure of individual organisms and still more

difficult to photograph them. Also, thick films tend to become detached from the coverslip during treatment with hydrochloric acid. To overcome this, after the organisms had been fixed a sterile scalpel was used to scrape away some of the excess bacteria. This left on the coverslip patches where there was a very thin film of organisms.

Impression preparations were made on coverslips, instead of on glass slides, because the use of coverslips saves space for storage and reagents for staining. Because coverslips are very fragile and liable to break easily, two identical impression preparations were always made. They were given exactly the same treatment and unless otherwise stated in the various experiments they showed the same staining reaction. In certain cases, impression preparations were made on slides e.g. when the organisms were to be stained to demonstrate spores by methods requiring the use of hot carbol fuchsin. From fluid cultures, smear preparations were made on coverslips directly from the medium or after concentrating the organisms by centrifugation and washing them in distilled water. These preparations were treated in exactly the same way as were the impression preparations. When the effect of nutrition on the nuclear structures of various organisms was being studied, slight modifications in the cultures used for the inoculation of the agar were made. These modifications are described in

the introduction to the various experiments.

The staining methods used were as follows:-

Osmic acid-hydrochloric acid-Giemsa method

(modified from that suggested by Robinow 1944)

The organisms on the impression preparations were fixed in osmic acid (osmium tetroxide) vapour. To obtain an atmosphere of osmic acid vapour, I used an airtight glass jar containing two or three layers of glass beads. To this a quantity of 2% osmic acid (as supplied by G.T. Gurr Ltd.) was added so that the first layer of beads was covered. The osmic acid vapourizes readily, producing an atmosphere of osmic acid vapour inside the jar. The osmic acid in the jar must be renewed as required to replace that lost as vapour when the jar is opened. The film-bearing coverslips were placed on the top layer of beads, with the film of organisms upper-most, and left for 2-3 minutes — or for 5 minutes if the spores predominated in the culture. They were then placed in small staining racks to carry out the rest of the method. This meant that all the preparations were treated in exactly the same way, and could therefore be compared with each other as regards staining reaction and demonstration of nuclear material.

After fixation of the films the coverslips in the racks were placed in 70% (v/v) ethyl alcohol where they could be stored successfully for at least a month. Normally

they were not left in the alcohol for more than a day. When these coverslip-film preparations were due to be stained, the excess alcohol was drained away, and they were placed in normal hydrochloric acid at 60°C for 10 minutes, or if spores predominated in the culture, for 7 minutes. Then they were removed, washed in running tap water for several seconds, passed through two changes of Sórensen's phosphate buffer mixture (pH 6.9), and finally put into Giemsa's staining solution. This solution was made by adding 2.5 ml. of Gurr's Improved Giemsa Stain R 66 to 40 ml. of Sórensen's phosphate buffer mixture at pH 6.9. Preparations were left in the stain for ½ hour at 37°C if they were to be mounted temporarily; for permanent mounting they were left for 1 to 1½ hours.

Temporary mounting

After the organisms had been stained the coverslip-films were rinsed well in the phosphate buffer mixture and mounted in fresh buffer. Excess buffer was removed and the top of the coverslip dried with blotting paper. The edges of the coverslip were sealed with paraffin wax by means of a heated drawing pen. The preparations were then examined under the 2 mm. oil-immersion objective. Preparations sealed in this way could be kept for over one week without any radical changes taking place.

Permanent mounting

After removal from the staining solution the

preparations were rinsed in the phosphate-buffer mixture and taken through acetone alone for a few seconds followed by acetone/xylol (A/X) mixtures, also for a few seconds, in the following proportions, A14/X6, A6/X6, A6/X14. Next they were immersed in 3 changes of xylol, 10 minutes in each. Finally the preparations were mounted in Gurr's Neutral Mounting Medium, which was found to be the most suitable. They were left for about a week to set firmly before being examined under the 2 mm. oil-immersion objective.

To obtain good results by this method care was necessary about two matters in particular: (1) in ensuring that the pH of the phosphate buffer remained at 6.9; and (2) that the preparations were well washed after treatment with hydrochloric acid. In good preparations the chromatinic material, which stains reddish purple, can be clearly seen and photographed, being well differentiated from the very pale pink-mauve colour of the cytoplasm. When photographed, the chromatinic bodies stand out black against the pale grey of the cytoplasm, as seen in Fig. 9.

Quick differential method (Robinow 1944).

This useful method quickly provides reasonably well defined pictures of the chromatinic material. The point of using it is to indicate whether the particular culture has reached the desired stage of development at which preparations by more elaborate methods are worth making. Air-dried, unfixed impression preparations were placed in boiling

N/5 hydrochloric acid for about 5 seconds, rinsed in distilled water and mounted in 0.05% (w/v) aqueous crystal violet, and sealed with paraffin wax. The chromatinic bodies stain purple and the cytoplasm a light mauve. When photographed these bodies appear black against a pale grey cytoplasmic background as shown in Fig.67.

Tannic acid-crystal violet method

(Robinow 1945, Bisset 1952a)

This method demonstrates extremely well the cell-walls and the cross-septa of bacteria. The tannic acid has a mordanting effect on the cell-walls and septa, increasing their affinity for stains. It also seems to alter the protein of the cytoplasm in such a way that it is hardly stained by the very dilute solution of crystal violet used, and so does not obscure the cross-septa.

Impression preparations were made and placed in a watch glass containing 10% (w/v) tannic acid solution. The tannic acid is made up as a 20% (w/v) solution, which can be stored successfully after sterilization by steaming for 20 minutes on three successive days as suggested by Bisset (1952a). Dilution with an equal volume of distilled water gives the 10% solution as required. How long the preparations were left in the tannic acid depended on the organisms studies, but generally good results were obtained

after half-an-hour. After removal from the tannic acid the preparations were washed with tap water and mounted in 0.01% (w/v) or weaker solution of aqueous crystal violet and sealed with paraffin wax.

The cell-walls and cross-septa of the organisms stain pale purple and the cytoplasm a very pale mauve. When photographed, the cell-walls and septa appear as a black line, against the pale grey of the cytoplasm as seen in Figs. 162-163.

Lipoid staining method

Impression preparations were made on slides and stained for 15 minutes in one of the following stains, which were filtered before use.

(a) sudan black — a saturated solution in a mixture (v/v) composed of equal parts of 70% (v/v) ethyl alcohol and acetone.

(b) sudan IV — a saturated solution in a mixture (v/v) composed of equal parts of 70% (v/v) ethyl alcohol and acetone.

After 15 minutes the stain was washed off with 70% (v/v) ethyl alcohol. The lipoid material was well demonstrated as dark granules in a light coloured cell (Figs. 57 and 61 ).

Phase-contrast Microscopy

This method of examining bacteria is a very important

one, in that the organisms are in a living, unstained and unfixed state. The internal differentiation observed is due to the differences in refractive index of the constituents of bacterial protoplasm (see Fig.133). When interpreting photographs of organisms taken under phase-contrast, it is necessary to realise, therefore, that the differences in colour and density are due to these differences in refractive index and not to differences in affinity for stains.

The phase-contrast microscope used was that designed by W. Watson & Sons Ltd. with the annular type of diaphragm and phase plate. Preparations to be examined were made on glass slides, covered with No.1 coverslips, and drained free of all excess moisture with blotting paper. Care had to be taken to ensure that there was only a very thin film between the slide and the coverslip — too thick a film, or one containing a large number of organisms, made it very difficult to superimpose the ring of light on to the phase plate. The edges of the coverslips were sealed with paraffin wax, but the preparations were never kept long before they were examined and photographed, since the organisms were living and still undergoing internal changes.

Electron Microscopy

Electron microscopy, with the advantage of great

resolving power, has been of value in examining the shapes and external structures of bacteria, such as flagella. It has so far proved to be of limited use in studying the internal structure of the bacterial cell, because most bacteria in their normal state are opaque to the electron beam. Under certain abnormal conditions of growth, and by treatment with various reagents, I have observed the internal structures of some bacteria. The technique of cutting ultra-thin sections of bacteria has now opened up a vast new field for examining the internal structure of bacteria with the electron microscope. I have taken some preliminary steps towards establishing this technique but have not yet reached the stage of cutting sections.

The type of microscope used was Phillips Metalix 60-100 kv, (25 A lens). Copper mesh grids covered with a thin film of "formvar" acted as mounts for the organisms. To cast a formvar film, I worked out the following procedure after trial of various methods. A drop of liquid formvar was spread very thinly over a clean dry microscope slide and allowed to dry. The film was then cut into pieces of about one cm. square with a sharp scalpel. One end of the slide was gently lowered into a bath of clean, dust-free water and the squares of film floated from the

slide on to the surface of the water. With a large wire loop these squares of formvar were picked up, gently placed on the copper grids, and allowed to dry.

A suspension of the organisms to be examined was made in sterile distilled water, either from an agar plate or slope, or from a centrifuged broth culture. The organisms were washed three or four times in distilled water to remove as much extraneous material as possible. If they were to be fixed, 0.25 ml. of 2% osmic acid (osmium tetroxide) was added to 10 ml. of suspension and left for five minutes, rewashed twice, and resuspended in sterile distilled water. A drop of the organisms, either fixed or unfixed, was then placed on the copper grids and the water allowed to evaporate. The preparations were not shadow-cast.

During the casting and drying of the formvar films, and the drying of the organisms on the grids, great care had to be taken to ensure that they were kept as free as possible from dust-particles and extraneous micro-organisms. Because of this, all the above operations were carried out in an inoculating cabinet sterilized by an ultra-violet lamp. The organisms were then examined by the electron microscope and photographs were taken of suitable fields. It was planned to take electron micrographs of cultures of two organisms, Aerobacter cloacae and Bacillus megatherium,

grown under various conditions and for different periods of time. Certain preliminary experiments had to be carried out before this could be done.

The first was to see if the two organisms would grow in the presence of the copper mesh grids. The grids were sterilized by boiling, and one was added to each of three tubes of meat-extract broth. One of these 3 tubes remained uninoculated to act as a control. The other two tubes, along with a tube of meat-extract broth without a grid to act as a growth control, were inoculated with a culture of A.cloacae. This was repeated for B.megatherium, and all four tubes were incubated at 37 °C for 48 hours. Growth was observed in all the inoculated tubes, showing that the presence of copper grids did not inhibit the growth of either organism. No growth took place in the uninoculated control, but it was observed as expected in the growth control.

The second investigation was to find some way to sterilize the copper grids without damaging the formvar film which they supported. Boiling was found to be unsuitable as it disrupted this film. Experiments were then carried out with an ultra-violet ray tube emitting radiations at 2357 A.U., enclosed in a sterilizing cabinet. An old, sporing culture of B.megatherium and copper grids with formvar films were used in these experiments. Five

of the grids were exposed to atmospheric contamination, and five other grids were deliberately contaminated with a drop of spore suspension, which was allowed to dry. One grid of each type was placed on meat-extract agar plates before exposure to ultra-violet radiation, to act as growth controls. The other grids were exposed to the ultra-violet radiation for periods of 1 hour 5 minutes, 2 hours 30 minutes, 3 hours 25 minutes, and 4 hours 45 minutes. After exposure the grids were placed on meat-extract agar plates, and all the plates were incubated at 37°C for 48 hours. The results showed that ultra-violet radiation for about one hour was sufficient to sterilize grids exposed only to atmospheric contamination, but that about 4½ hours' radiation was necessary to kill all the spores of B.megatherium.

The final problem was to determine if the copper grids could float on the surface of liquid culture medium, and if organisms would grow successfully on the formvar film, so that they could be examined in their natural growth patterns under the electron microscope. This was done very simply by placing copper grids on the surface of meat-extract broth in a petri dish, already placed in the 37°C incubator. The broth was then inoculated with a culture of A.cloacae and incubated for 3 days. This was repeated for B.megatherium. The grids were then

carefully removed, dried in air, stained with carbol fuchsin, and examined under the ordinary microscope. Numerous organisms were present on the grids and it was concluded that this method was quite satisfactory.

Photomicrography

Photography is an essential technique of the bacterial cytologist. Though proficiency in this work comes only with hard practice and bitter experience, its importance cannot be over-estimated. Photographs permanently record a true picture of the intracellular structures and are necessary for the interpretation of such structures. Throughout the present work photographic recordings were made whenever possible. Diagrams were also made from the photographs — and this may require a word of explanation. Diagrams are of value in that they give a simpler picture of the internal arrangement of the bacterial cell, making interpretation of the various structures easier. For this reason resort to diagrams alone is not satisfactory to illustrate a text on bacterial cytology. The ideal is to combine diagrams, which make interpretation simpler, with the original photographs which can always be consulted to check that simplification has not led to falsification.

Some of the diagrams illustrating work done for

the earlier part of this thesis were made directly from preparations examined under the microscope; this was done as a necessity, however, and only because there was no camera available at the time to take photographs.

For ease of reproduction all the diagrams were photographed. This enabled several prints of one diagram to be made from one negative, saving a great deal of time and arduous eyestrain in copying faithfully the original diagrams.

Nearly all the photographs illustrating this thesis were taken by a "Laboratory" photomicrographic camera (W.Watson & Sons Ltd.). This horizontal type of camera was adapted so that it could take photographs of organisms under phase-contrast, as well as those examined by ordinary light microscopy. Some of the earlier photographs, however, were taken with a Cooke microcamera adapter, which was attached to the tube of an ordinary microscope. Ilford Rapid Process Panchromatic quarter plates were used and developed with I.D.2 developer. The photographs were printed on Ilford Bromide paper, either the hard or extra-hard grade, depending on the density of the negative; I.D.20 developer was used.

35 mm. film was used to take photographs under the electron microscope, and these were developed for me in the Chemistry Department. Prints were made from these

negatives either on normal or hard-grade bromide paper using I.D.20 developer.

When diagrams were copied, Ilford Selochrome or Rapid Process Panchromatic quarter plates were used and developed with I.D.2 developer or Kodak Contrasty developer. Prints were made on hard-grade Ilford bromide paper with I.D.20 developer.

# EXPERIMENTS AND RESULTS

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To describe all the experiments conducted during the present investigation would make the thesis tediously and unnecessarily long. Many experiments were essentially of a developmental and exploratory character. These assisted in evolving new and in modifying old methods. Other experiments were either confirmatory or were negative; for not every idea yielded a fruitful result! The experiments described here include all those which influenced the development of my current thoughts about bacterial nuclei as well as certain others which, although they may have appeared to yield no important result, were of significance either in persuading me to abandon or postpone a particular line of inquiry or in guiding me to an approach which proved useful.

Two points should be emphasised:-

1. In all the experiments, unless it is stated otherwise, the written descriptions were made from microscopic examination of the actual preparations — not from the photographs or diagrams; these are used only to illustrate and corroborate the written account.

2. A cautious attitude would be to avoid the use of the words "nucleus" and "nuclear structure". The shortest of the possible substitutes would be the expression "chromatinic material", which begs no questions. Nevertheless, I have

used the word nucleus and its appropriate adjective throughout because: (1) this is the convention in other papers on bacterial cytology; (2) I myself understand that "nucleus" really means "nucleus-like in staining properties but not necessarily in all other respects"; (3) the frequent repetition of a cautious or burdensome phrase would become more tedious to the reader than the presumption implied in using the word nucleus with its limited meaning fully understood as defined here.

Experiment 1 A study of the nuclear material of three strains of Rhizobium, as revealed by the HCl-Giemsa technique and phase-contrast microscopy.

The experiment with three strains of Rhizobium had three aims. The first was to determine the position of the nuclear material. The second was to find out whether the appearance of the nuclear material in a stained preparation corresponded, or did not correspond, with any appearance revealed by the phase-contrast microscope. The third was to determine if there was any relationship between the internal structure or nuclear structure of these organisms and the effectiveness of nitrogen-fixation in the strain.

The three strains, chosen because they grew well on yeast extract-mannitol agar, were (a) clover, (b) lucerne, (c) field-bean. The lucerne and field-bean strains were effective nitrogen-fixers, the clover strain was not. Spread plates were made in the way described in the methods, the plates being inoculated from a three-week-old agar culture. Impression preparations were made after three weeks' incubation at 25° C. These preparations were treated by the HCl-Giemsa technique. Other preparations made of living organisms at the same stage of growth were examined under the phase-contrast microscope. At the time this experiment was carried out, there was no

camera available so diagrams were made of the most striking organisms. The results were as follows:-

HCl-Giemsa technique (All strains examined after 3 weeks at 25° C).

(a) Clover strain

The organisms at this stage of growth were large bacilli. The distribution of the nuclear material, which was clearly demonstrated, varied considerably from one organism to another. The majority of the organisms showed bipolar granules linked by a thick band of nuclear material (Figs. 1 and 2). In some organisms there were definite and separate bipolar granules (Fig.3) whereas in others the nuclear material was in the form of three large separate granules (Fig.4). Clear unstained areas resembling vacuoles, or possibly spores, and associated with large masses of nuclear material were present in some organisms (Figs. 5 and 6). One interesting organism noted had clear unstained separate bipolar granules, and the rest of the cell contained nuclear-staining material (Fig. 7). Some of the organisms were of the cocco-bacillary type and contained a large amount of nuclear material (Fig.8).

(b) Lucerne strain

The organisms were small cocco-bacilli, and took up the stain evenly; there was no internal differentiation.

(c) Field-bean strain

In this organism, which was a fairly large bacillus, there was no definite arrangement of the nuclear material; this material was present, however, in the form of small granules scattered throughout the cytoplasm.

Phase-contrast microscopy

(a) Clover strain

Some organisms had a single terminal granule; others had bipolar granules; and a few showed banding. The majority appeared to have numerous smaller granules distributed throughout the cytoplasm.

(b) Lucerne strain

This organism was actively motile, which made it difficult to see the internal structure.

(c) Field-bean strain

Some organisms showed a certain amount of banding, but in the majority the cytoplasm was granular.

With the HCl-Giemsa technique, the nuclear apparatus of clover strain was well demonstrated, whereas the other strains either showed no differentiation (lucerne) or granulation (field-bean). The discrepancies in these results were probably due to the time of treatment in hydrochloric acid, 10 minutes being suitable for the clover strain, but not for the other strains. To overcome this,

each strain would have to be treated separately and preparations treated for various periods to find the best "hydrolysis" time.

There appeared to be a certain amount of correlation between the picture seen when the organisms were stained to show nuclear material, and when the organisms were examined under the phase-contrast microscope. Although with the clover strain no granular appearance was seen by the staining method it was seen under the phase-contrast microscope. With the field-bean strain, banding was seen under the phase-contrast and not by the staining method. A comparison could not be made with the lucerne strain.

As the morphology of these three strains of organisms differed so considerably each from the other, it was impossible to find any relationship between the nuclear structure and the effectiveness of nitrogen-fixation. To examine this further it would be better to use similar strains that differed only in their ability to fix nitrogen, and not strains symbiotic with different hosts as with the three strains examined. Because of the difficulty of growing some other strains and of the comparatively slow growth even of those that grew well, it was decided not to continue a cytological study of this particular group for the present.

Experiment 2 A study of the nuclear material of A.cloacae as revealed by the HCl-Giemsa technique and the Quick differential method.

This experiment was set up to examine the nuclear structures of A.cloacae in a young culture, where active division was taking place, and in an older culture, where active division was known to have ceased. From previous studies which I had made on the nuclear structure of Escherichia coli at similar stages of growth, I had recorded full descriptions. E.coli is closely related to A.cloacae, and in the experiment I hoped to make a brief comparison of the nuclear pattern of the two organisms.

A.cloacae was grown on a meat-extract agar plate, inoculated with an overnight broth culture. Impression preparations were made from the spread plate after 2 hours' and after 3 days' incubation at 37° C and were treated by the HCl-Giemsa technique. Some of the two-hour-old preparations were treated by the Quick differential method. Photographs were taken, and some diagrams were made.

Results

The nuclear material of the organisms was well demonstrated by the HCl-Giemsa technique.

The two-hour-old culture gave a very clear picture of some organisms containing spherical bodies, but the majority contained dumbell-shaped bodies,(Fig.9a) and

double dumbell-shaped bodies (Fig.9b). One interesting feature was that a few organisms possessed a core of nuclear material (Fig.10). In most of these there were two bacilli not completely separated and the core of one organism appeared to be attached to the core of the other (Fig.11).

The majority of organisms in the three-day-old culture showed well defined spherical nuclei. There were very few dumbell-shaped bodies.

Impression preparations of the two-hour-old culture treated by the Quick differential method gave the following results. No dumbell shapes could be seen at all, instead there were darkly stained spherical structures.

The discrepancy between these structures and those demonstrated by the HCl-Giemsa method could be due to the fact that the latter is a very delicate method, whereas the Quick differential method may deposit a lot of stain on the structures making it difficult to resolve the dumbell shapes, which appear instead as spheres. Alternatively, the discrepancy may be due to the two differing methods giving rise to different patterns of nuclear material.

The nuclear organization of A.cloacae would appear to be simple. No mitotic figures were ever observed, and the nuclear core was demonstrated in only a few organisms, which may have been dead or may have been pleomorphic forms.

The general picture of the nuclear structures in the cultures of A.cloacae studied was very similar to that which I had previously demonstrated in E.coli. Unfortunately I had no photographs or diagrams of the structures seen in E.coli, but from the account which I had written on them at the time there appeared to be an appreciable similarity between the two organisms. This point is enlarged in the section of this thesis marked "Discussion".

Experiment 3 An investigation of the internal structure of A.cloacae when grown in various media and examined by the electron microscope, the phase-contrast microscope, and the HCl-Giemsa technique.

The question of artefacts in connection with the bacterial nucleus has already been emphasized in the preface. It was thought that if similar structures could be demonstrated in A.cloacae by three different methods of examination this would help to repudiate the suggestion that the nuclear structures observed in bacteria were artefacts. The first method used was that of electron microscopy; the second, the HCl-Giemsa technique for the demonstration of nuclear material; and the third, phase-contrast microscopy for the examination of living organisms. In describing this experiment it is difficult to isolate the results from the experimental details, since one result led to the setting up of another part of the experiment. The results have been drawn up into a table (no.I,p.142).

A preparation of A.cloacae was made for examination by the electron microscope. The organisms were grown in meat-extract broth for three days at 37°C. The organisms were spun down, washed four times in sterile distilled water, and finally resuspended in it. Drops of the suspension were placed on the formvar films supported by copper grids and allowed to dry. Examination of these preparations under the electron microscope revealed that

the organisms were so heavily fringed with protein and other contaminating substances that they were very difficult to study and that no photographs could usefully be taken. No internal differentiation could be seen in the few organisms that were not so heavily fringed.

In the review of the literature on nutrition of bacteria, I pointed out that growth of the organisms in a simple medium led to a clearing of the cytoplasm and a subsequent uncovering of the internal structure, especially the nuclear structures. Because of this, steps were taken to grow A.cloacae in as simple a medium as possible. I also hoped that this would eliminate the heavy fringing with protein. The organism grew well in citrate medium ( p.107); accordingly, to a flask containing 10 ml. of this medium, a small amount of washed organisms was added. After three days' incubation at 37°C the organisms were spun down and washed three times with sterile distilled water. Two smear preparations of the organisms were then made on coverslips and treated by HCl-Giemsa technique. The rest of the organisms were resuspended in 10 ml. of sterile distilled water. To this suspension 0.2 ml. of 2% osmic acid was added and left for 5 minutes to fix the organisms. A further 10 ml. of sterile distilled water was added to decrease the effect of the osmic acid while the organisms were being spun down. The organisms were

then washed with and finally resuspended in sterile distilled water. Small drops of this suspension were placed on the formvar film on the copper grids and allowed to dry. When examined under the electron microscope, the organisms were found to be practically free from contaminating proteins and other substances, and photographs were taken.

The majority of organisms were opaque to the electron beam but a certain amount of internal structure, mainly in the form of granules both large and small, could be seen in some of them (Fig.12). One organism in particular showed two definite small granules and two less definite larger granules (Fig.13). Another large organism was seen with a very granular internal structure (Fig.14). This may have been a contaminant or a "giant" form of A.cloacae.

When treated by the HCl-Giemsa technique the majority of organisms showed well stained bipolar bodies with very palely stained cytoplasm between them (Fig.15).

There did not appear to be any striking correlation between the structures observed under the electron microscope and those shown by the staining method. The real fault lay in the fact that the majority of the organisms still tended to be opaque to the electron beam and consequently very little of their internal structure

was revealed.

It was then decided to use a very deficient medium for growth in the hope that definite starvation of the organisms would lead to the use of some of the ribonucleic acid of the cytoplasm, thus helping to uncover the nuclear material. Distilled water to which a few drops of ammonia had been added was inoculated with organisms which had been grown in meat-extract broth for 2 days at 37° C, spun down, and washed twice in sterile distilled water. After leaving this culture at room temperature for three weeks, slight growth took place, as seen by increase in opacity. The organisms were spun down, washed twice in distilled water, and resuspended in the same. One half of the suspension was treated with osmic acid as previously described in this experiment and the other half was left untreated. Treated and untreated organisms were examined under the electron microscope. Wet preparations of both types of organisms were also examined under the phase-contrast microscope.

Under the electron microscope, the preparations were not very clean or clear, but individual organisms could be picked out, showing dense bipolar bodies with very clear cytoplasm in between them (Fig.16). In one particular organism (Fig.17) these bodies were not as regular in shape as those observed previously (i.e.Fig.16)

and resembled the configurations observed at late anaphase or early telophase of mitosis in plant cells, as shown in Fig.171. These bodies could be seen in the preparation not treated with osmic acid, but they were sharper and denser in the treated preparation.

When examined under the phase-contrast microscope, both the fixed and unfixed, living organisms showed dark bipolar granules. There appeared to be no difference at all between the two preparations. At the time of the experiment no photographs could be taken of the organisms under the phase-contrast microscope.

At least two significant points emerged from this experiment. Firstly, A.cloacae grown in media adequate for its growth requirements tended to be opaque to the electron beam and little of the internal structure could be revealed by this instrument. Only by growing the organism in a deficient medium could details of its internal structure be revealed. It was striking that these structures were dense bipolar granules, which were also demonstrated by the phase-contrast microscope in living organisms.

The second point was that treatment with osmic acid helped to demonstrate these bipolar granules under the electron microscope by increasing their density and yet made no difference when the organisms were examined

by the phase-contrast microscope. This meant that when osmic acid was used as a fixative it did not alter the shape or size of the internal structures.

The bipolar bodies observed under the electron microscope and the phase-contrast microscope were also demonstrated in organisms grown in citrate broth by the HCl-Giemsa technique. This meant that these bodies were probably not produced as a result of autolytic processes in the cell.

Similar dense bipolar bodies have also been observed by Bringmann (p. 78 of this thesis) in cultures of E.coli grown on poorly nutrient medium and demonstrated by the electron microscope, the phase-contrast microscope, and the HCl-Giemsa technique.

The results of this experiment (summarised in table I) show that the application to the same culture of these three different methods for the study of bacterial cytology is to be recommended since there was good correlation between the structures revealed by them. This increases confidence in each of the methods and reduces the opportunity for critics to cry: "Artefacts!"

Table I

Experiment 3. To show the structures revealed in A.cloacae grown in various media and examined by different methods

| Medium and treatment | Electron microscope | Phase-contrast microscope | HCl-Giemsa technique |
|---|---|---|---|
| Meat-extract broth | Heavily fringed and opaque | ... | ... |
| Citrate broth | Majority opaque some showing granules | ... | Deeply stained bipolar bodies |
| Distilled water plus ammonia:- | | | |
| (a) No treatment | Dense bipolar bodies | Dense bipolar bodies | ... |
| (b) Osmic-acid treatment | Bipolar bodies sharper and denser than above | Bipolar bodies no different from above | ... |

... = Not examined

Experiment 4 An investigation of the changes in the demonstrability of nuclear material and the stainability of the cytoplasm of A.cloacae brought about by differences in the nutritional environment and revealed by the HCl-Giemsa technique.

It is known that changes of nutrition affect the metabolism of the bacterial cell and that these changes in metabolism bring about changes in the internal structure of the cell. This experiment was devised to determine whether various nitrogen sources with glucose would make any difference to the demonstration of nuclear material of A.cloacae. Basal broth and basal agar,(p.108) solutions of glucose and various nitrogenous substances — ammonium sulphate, asparagine, peptone, sodium nitrate, and urea — were prepared. Agar plates were poured having the following composition (summarized in Table II,p.152 ).

| | | | | |
|---|---|---|---|---|
| Plate | 1 | Basal | agar | alone |
| " | 2 | " | " | plus glucose |
| " | 3 | " | " | plus glucose and ammonium sulphate |
| " | 4 | " | " | plus glucose and asparagine |
| " | 5 | " | " | plus glucose and peptone |
| " | 6 | " | " | plus glucose and sodium nitrate |
| " | 7 | " | " | plus glucose and urea |

Plate 8 Basal agar plus ammonium sulphate
" 9 " " plus asparagine
" 10 " " plus peptone
" 11 " " plus sodium nitrate
" 12 " " plus urea
" 13 " " plus all the above nitrogenous compounds

A meat-extract agar plate was used as a control, for the demonstration of the nuclear structures (see experiment 2)

0.1 ml. of a 5-hour meat-extract broth culture was spread over the surface of each of the 14 plates, which were incubated at 37° C. Two impression preparations were made from each plate after 2 hours' incubation and treated by the HCl-Giemsa technique. This was repeated after 20 hours' incubation.

Unless stated otherwise the staining reactions of the two identical impression preparations were the same. In cases where there was a difference this is pointed out, but for the purpose of compiling table II the better staining reaction was taken. The staining reaction of the organisms as well as the demonstration of nuclear material was observed and recorded.

Results

(a) 2-hour cultures

The preparations made from growth on meat-

extract agar demonstrated the nuclear bodies very well and this was taken as a standard, the other preparations being compared with it.

A.cloacae grew quite well on basal agar alone, and nuclear bodies could be seen. The addition of 1% glucose did not change the demonstration of nuclear bodies, although in one of the preparations the staining reaction was poor.

Ammonium sulphate and glucose together appeared to inhibit the demonstration of nuclear bodies and the organisms stained only poorly. Yet with ammonium sulphate in the absence of glucose the organisms stained well and nuclear material could be seen.

Asparagine and glucose together did not appear to inhibit either staining reaction or demonstration of nuclear material, although one preparation stained much better than the other. Asparagine alone, however, resulted in very poor staining of the organisms and no nuclear bodies could be seen.

When peptone was present in the medium with glucose, the staining reaction was poor and nuclear bodies were not demonstrated. A similar result was obtained with peptone alone. The presence of sodium nitrate alone and with glucose gave similar results to those obtained with peptone.

Urea when present with glucose resulted in poor staining reaction, but nuclear bodies could be seen, though not very well. One preparation did not show nuclear bodies. When glucose was absent the reaction was poor and nuclear bodies were not demonstrated.

In the absence of glucose the presence of all the nitrogenous substances together in the medium resulted in poor staining reaction of the organisms and nuclear bodies were not seen.

(b) 20-hour cultures

The preparations made from growth on meat-extract agar stained well and nuclear bodies could be clearly seen; this was taken as standard for the other preparations. The staining reaction of organisms grown on basal agar alone was comparable with the results for the 2-hour cultures. The staining reaction was quite good and nuclear bodies could be demonstrated, although only in certain parts of the film; the rest of the film stained only poorly. The addition of glucose to the basal medium resulted in good staining of the organism and demonstration of the nuclear material.

Ammonium sulphate with glucose again appeared to inhibit the demonstration of nuclear bodies but when the glucose was left out the organisms and nuclear bodies stained very well.

When asparagine was present with glucose the staining reaction and demonstration of nuclear material was good —although in one preparation it was poor. Asparagine without glucose also gave good results.

The presence of peptone and glucose gave a poor staining reaction and poor demonstration of nuclear material. When glucose was left out, the organisms stained well and nuclear bodies could be seen in patches of the preparation — the rest showing only poor reactions.

Sodium nitrate plus glucose resulted in poor staining reaction and demonstration of nuclear bodies; yet when glucose was left out staining reaction and demonstration of nuclear bodies was good.

Similar results were obtained with urea and glucose, and urea alone.

When all the nitrogenous substances were present, staining reaction and nuclear demonstration were good.

The poor staining with glucose and peptone was unexpected. It was thought that these substances would encourage the demonstration of nuclear material.

When, as occasionally, the two identical impression preparations did not show the same results it was assumed that there was a fault in the staining technique; but this might be a wrong conclusion.

With two-hour cultures it would appear that in

general when glucose was absent the staining reaction and the demonstration of nuclear material was poor. When it was present, the results were variable depending on the source of nitrogen.

The observations with twenty-hour cultures showed that the presence of glucose appeared in some way to result in poor staining of the organisms and poor demonstration of the nuclear material. In most cases absence of glucose gave good reactions.

These observations were primarily concerned with the staining reactions of the organisms and the demonstration of the nuclear material; and not with the shape or position of the nuclear bodies. It appeared that stainability of the organsisms as a whole and of the nuclear material was affected by the presence or absence of glucose (possibly a pH effect) and by the nature of the nitrogen source.

Since there were certain discrepancies between similar preparations, the experiment was repeated in exactly the same manner to see if some or all of the results were reproducible.

Results of repeated experiment 4 (Expt.4a)

(a) 2-hour cultures

All the preparations showed stainable nuclear material. Those made from growth on basal agar with

glucose and asparagine, with glucose and peptone, with glucose and urea, with ammonium sulphate alone, with asparagine alone, with peptone alone, and with all the nitrogen sources, stained less clearly than those made from growth on the other agar plates; but the difference was not striking. Results from the control plate were good.

(b) 20-hour cultures

All the preparations showed stainable nuclear material. Those made from growth on basal agar with glucose and asparagine, with glucose and sodium nitrate, with glucose and urea, with peptone, with sodium nitrate, and with all the nitrogen sources, stained less clearly than those taken from growth on the other plates but the difference was not striking. Results from the control plate were good.

The results of this repeated experiment showed great variation from the results of the previous attempt so I decided to repeat the experiment yet again using only the nitrogenous substances that showed the greatest differences from the control and examining only the two-hour cultures.

Results of second repetition of experiment 4 (Expt.4b)

2-hour culture

The organisms showing a poor staining reaction

and poor demonstration of nuclear material were those grown on basal agar with glucose and ammonium sulphate, basal agar with asparagine, and basal agar with urea. Good staining and demonstration of nuclear material was seen in organisms grown on basal agar alone, basal agar with glucose and peptone, basal agar with glucose and urea, and basal agar with peptone alone. Very good demonstration of nuclear bodies was seen with organisms grown on basal agar and on the control as was expected. From the results of this experiment and its two repetitions, which are summarised in Table II, it would appear that differences in nutrition, such as the presence or absence of glucose and the nature of the nitrogen source, may affect the demonstration of nuclear material. But the results were not constant in the three attempts. Some results of the first attempt showed striking effects, but these were not borne out by the second and third attempts although there was better correlation between their results.

The discrepancies noted in this experiment may have been due to faults in technique, especially in the first attempt; but by the time that the third attempt was made the technique had reached a level of proficiency which made for ready and easy reproducibility of results with ordinary media and a variety of organisms. The

probability is that nutrition affects the nuclear structure of A.cloacae, but to establish this conclusively, and still more to identify the most important nutritional influences, many known variables — such as pH, for example — and some unknowns would have to be ascertained, checked, and stabilised. Such a task might well be worth undertaking, but it would certainly be of some magnitude. At this stage to embark upon it seemed to be to accept too great a diversion for too uncertain a reward. In view of many other possibilities open to investigation I resolved merely to note the results of experiment 4 for the present and to pass to other matters.

# TABLE II.

Experiment 4 (twice repeated.) To show the effect of nutrition on the nuclear staining reaction in Aerobacter cloacae

| Plate Number | Substances added (+) or not added (-) to the basal medium. | | | | | | Nuclear staining reaction after 2 hours | | | Nuclear staining reaction after 20 hours | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Glucose | Ammonium Sulphate | Asparagine | Peptone | Sodium Nitrate | Urea | Expt.4 | Expt.4a | Expt.4b | Expt.4 | Expt.4a | Expt.4b |
| 1 | - | - | - | - | - | - | +++ | ++++ | ++++ | +++ | ++++ | ... |
| 2 | + | - | - | - | - | - | +++ | ++++ | ... | ++++ | ++++ | ... |
| 3 | + | + | - | - | - | - | + | ++++ | + | + | ++++ | ... |
| 4 | + | - | + | - | - | - | +++ | +++ | ... | ++++ | +++ | ... |
| 5 | + | - | - | + | - | - | + | +++ | +++ | + | ++++ | ... |
| 6 | + | - | - | - | + | - | + | ++++ | ... | + | +++ | ... |
| 7 | + | - | - | - | - | + | + | +++ | +++ | + | +++ | ... |
| 8 | - | + | - | - | - | - | + | +++ | + | ++++ | ++++ | ... |
| 9 | - | - | + | - | - | - | + | +++ | ... | ++++ | ++++ | ... |
| 10 | - | - | - | + | - | - | + | +++ | +++ | +++ | +++ | ... |
| 11 | - | - | - | - | + | - | + | ++++ | ... | ++++ | +++ | ... |
| 12 | - | - | - | - | - | + | + | ++++ | ++ | ++++ | ++++ | ... |
| 13 | - | + | + | + | + | + | + | +++ | ... | ++++ | +++ | ... |
| 14 | NUTRIENT AGAR | | | | | | ++++ | ++++ | ++++ | ++++ | ++++ | ... |

Staining reaction very poor = +
" " poor = ++
" " quite good = +++
" " good = ++++
Not attempted = ...

Experiment 5 An investigation into the changes in the nuclear material of B.cereus brought about by difference in the nutritional environment of the organisms.

This experiment was set up to study the changes of nuclear material in a Gram-positive sporing organism — in contrast to the Gram-negative non-sporing organism A.cloacae studied in experiments 2-4.

The nutritional plates were the same as those used for experiment 4. 0.1 ml. of a washed distilled-water suspension of organisms grown in meat-extract broth at 30°C for eighteen hours was spread over each of the nutrition plates. Impression preparations were made after 2 hours' and 24 hours' incubation at 30°C and treated by the HCl-Giemsa technique. The type of growth was observed after 24 hours' incubation. The results summarised in Table III (p.161) were as follows:-

(a) 2 hours' incubation at 30°C.

(1) Basal agar alone

Many short coccal forms were present, some forming short chains. Demonstration of nuclear material was poor, but some internal differentiation was seen with the aid of a green filter.

(2) Basal agar plus glucose

As for (1)

(3) Basal agar plus glucose and ammonium sulphate

The organisms were irregularly shaped and internal differentiation was poor.

(4) Basal agar plus glucose and asparagine

Many small poorly-stained organisms were present. Some showed nuclear granules.

(5) Basal agar plus glucose and peptone

Organisms were generally large, and appeared singly, in pairs, and in short chains. The nuclear material and cross-septa were well demonstrated.

(6) Basal agar plus glucose and sodium nitrate

Small organisms that stained deeply were present, showing very little internal structure.

(7) Basal agar plus glucose and urea

Generally large organisms were present, showing good nuclear differentiation. A few of the organisms present had a small bright area, usually central, surrounded by well-stained nuclear material. Septa were not demonstrated.

(8) Basal agar plus ammonium sulphate

As for (3)

(9) Basal agar plus asparagine

As for (4)

(10) Basal agar plus peptone

Generally large organisms in short chains were

present. Nuclear material and cross-septa were well demonstrated as for (5).

(11) Basal agar plus sodium nitrate

Organisms differed in shape and size but all were poorly stained.

(12) Basal agar plus urea

Small poorly-stained organisms were present.

(13) Basal agar plus all nitrogen sources

Square, deeply-stained organisms showing little nuclear differentiation but well marked septa were present.

(14) Meat-extract agar

Organisms were present in chains of medium length; thoyshowed well-demonstrated nuclei, some spherical in shape,(Fig.166) other dumbell-shaped (Fig.167). Cross-walls were also well deomstrated (Fig.168).

(b) 24 hours' incubation at 30 °C.

(1) Basal agar alone

Large deeply-stained organisms were present, many having rounded or pointed ends. Some had a vague darkly stained central area and bright polar areas, but definite structures were not outlined.

(2) Basal agar plus glucose

As for (1)

(3) Basal agar plus glucose and ammonium sulphate

Large well stained organisms were present, generally

with a dark central area and bright polar regions; but some showed good nuclear differentiation.

(4) Basal agar plus glucose and asparagine

The size of the organisms varied in this preparation. Many had pointed ends. Some showed nuclear structures; others showed only a dark central area.

(5) Basal agar plus glucose and peptone

As for (1) except that some organisms showed definite nuclear structures.

(6) Basal agar plus glucose and sodium nitrate

As for (3)

(7) Basal agar plus glucose and urea

Large organisms appeared singly, in pairs, and in short chains. Nuclear material and cross-septa were well demonstrated (Fig.18). One notable structure consisted of a very bright clear central area surrounded by a band of well stained nuclear material. In some organisms this band was complete (Fig. 19), in others it was horse-shoe like (Fig.20), and in some it was uneven and appeared to be composed of granules (Fig.21). On examination of the photographs taken of this preparation, there were two interesting organisms. The first contained nuclear material in the shape of a spiral (Fig.164). A similar spiral structure was also observed in B.megatherium (Fig. 128). The second was a long bacillus containing a large

amount of nuclear material in which there were numerous clear round areas (Fig.165).

(8) Basal agar plus ammonium sulphate

As for (1)

(9) Basal agar plus asparagine

Large organisms in long chains were present with darkly staining central areas. Many showed a long core of nuclear material (Fig.22) that looked similar to the type of structure seen during the diakinesis stage of meiosis in plant cells (Fig.172). For convenience of reference I called these configurations observed in B.cereus "cross-over" structures.

(10) Basal agar plus peptone

As for (5)

(11) Basal agar plus sodium nitrate

As for (6)

(12) Basal agar plus urea

Large deeply staining organisms were present with little differentiation apart from a few granules.

(13) Basal agar plus all nitrogen sources

As for (5)

(14) Meat-extract agar

Organisms were large, deeply stained and in long chains. In some, nuclear differentiation was good; in others, a vague dark central area with light polar regions

could be seen.

The results showed that differences in nitrogenous substances and in the presence or absence of glucose have a very marked effect on the nuclear material of B.cereus. The staining reaction after 2 hours' growth was generally poor except where peptone was present, as in plates 5, 10 and 13, or where there was glucose and sodium nitrate (no.6) or glucose and urea (no.7). After 24 hours' growth, the staining reaction was good or quite good in all the preparations. A good staining reaction was not necessarily associated with abundant growth. Only on plates nos. 5, 10, 13 and 14 was there abundant growth, the other plates showed only a thin film of organisms.

There was a fair range in the size and shape of the organisms, depending on the composition of the agar they were grown upon. There were differences also in the staining of the cytoplasm, the demonstrability of the nuclear material, and the arrangement of this material.

The predominant structures seen when B.cereus was grown on basal agar plus glucose and urea were those composed of a clear bright central region surrounded by a ring of deeply stained nuclear material (Fig.19). For convenience of description and identification I shall name these structures "X structures" and refer to them by this name throughout the thesis. When these structures

were first observed, I, and others who saw them, thought that the clear central area might be a growing endospore which had begun to develop within the nucleus. Because of this I decided to study the development and growth of these structures and if possible to determine their relationship with the bacterial nucleus and the developing endospore. I was aware of the possibility that the clear areas might be lipoid granules and this also was investigated.

Organisms grown on basal agar and asparagine also showed interesting nuclear structures. These "cross-over" structures resembled the figures seen at the diakinesis stage of meiosis. They were also studied further in experiment 14 (p.208).

I first observed X structures in B.cereus grown on basal agar plus urea after 24 hours' incubation at 30°C. The 30°C incubator was kept in a classroom used for lectures and practical classes. In my next experiment I planned to make a thorough investigation of the development of these new and interesting X structures; accordingly I first made observations to discover if X structures were formed at 37°C., appropriate incubators being more conveniently available for half-hourly preparations to be made. A plate with basal agar and urea (as before) was poured and inoculated and incubated at 37°C. After 18, 22, and

24 hours' incubation I made impression preparations and treated them by the HCl-Giemsa technique.

X structures were seen in all the preparations. Although they were small in the 18-hour preparation, they were large and easily demonstrated in the 22 and 24 hours' preparations. This meant that the next experiment could be set up and incubated at 37° C.

# TABLE III

Experiment 5. To show the effect of nutrition on the nuclear staining reaction in Bacillus cereus

| Plate Number | Substances added (+) or not added (-) to the basal medium. | | | | | | Nuclear staining reaction after 2 hours at 30°C. | Nuclear staining reaction after 24 hours at 30°C. | Type of growth after 24 hours at 30°C. |
|---|---|---|---|---|---|---|---|---|---|
| | Glucose | Ammonium Sulphate | Asparagine | Peptone | Sodium Nitrate | Urea | | | |
| 1 | - | - | - | - | - | - | ++ | +++ | Thin film |
| 2 | + | - | - | - | - | - | ++ | ++++ | " " |
| 3 | + | + | - | - | - | - | + | ++++ | " " |
| 4 | + | - | + | - | - | - | + | ++++ | " " |
| 5 | + | - | - | + | - | - | ++++ | ++++ | Abundant growth |
| 6 | + | - | - | - | + | - | ++++ | ++++ | Thin film |
| 7 | + | - | - | - | - | + | +++ | ++++ | " " |
| 8 | - | + | - | - | - | - | + | ++++ | " " |
| 9 | - | - | + | - | - | - | + | ++++ | " " |
| 10 | - | - | - | + | - | - | ++++ | ++++ | Abundant growth |
| 11 | - | - | - | - | + | - | + | ++++ | Thin film |
| 12 | - | - | - | - | - | + | + | +++ | " " |
| 13 | - | + | + | + | + | + | ++++ | ++++ | Abundant growth |
| 14 | NUTRIENT AGAR | | | | | | ++++ | ++++ | " |

Staining reaction very poor = +
" " poor = ++
" " quite good = +++
" " good = ++++

Experiment 6 A study of the development of the X structures of B.cereus grown on basal agar plus glucose and urea.

This experiment was an attempt to see if any correlation could be found between the nucleus, the endospore, and the X structures which I observed on B.cereus when the organism was grown on basal agar plus urea and glucose. The development of these structures was studied during a period of 4 days.

Two plates of basal agar plus glucose and urea were spread in the manner described for experiment 5. They were both incubated at 37° C. Impression preparations were made from the first plate after various periods of incubation. The first preparation was made after 18 hours and then at half-hourly intervals till 24½ hours. Then two impression preparations were taken from plate II which was then kept in the refrigerator overnight (16½ hours), being re-incubated the next morning. Preparations were again taken at half-hourly intervals (apart from preparation no.20, which was made after three-quarters of an hour) until 33½ hours. The plate was kept in the refrigerator overnight (16½ hours) and further preparations were made after re-incubation for 34¼, 35¼, and 36½ hours. The plate was then removed from the incubator and kept at

room temperature for the rest of the experiment. Impression preparations were taken after 42½, 66½, and 81 hours. In all 37 impression preparations were made. They were then treated by the HCl-Giemsa technique. Photographs were not taken, but diagrams were made.

Two plates were poured to provide enough agar for making the impression preparations. Both plates were incubated together, but the first plate was removed every half hour to make preparations, while the second plate remained in the incubator. Any slight discrepancies at the change over of plates can be explained by this fact. The second plate was used when all the agar with growth on it in the first plate had been used. It was kept in the refrigerator overnight and it was presumed that growth did not take place during this period. After 31 impression preparations had been taken at half-hourly intervals, I decided to make only a few more after longer periods of incubation, partly because of the time factor and partly because there was not much agar with growth on it left in the second plate. The plate was left at room temperature to prevent drying of the agar and to encourage spore formation. After 37 preparations had been made the agar left was found to have a fungal contaminant on it,

so it was discarded.

Results of experiment 6

When the impression preparations were examined I regularly observed certain forms. To prevent repetition these are described below and are given names by which they are referred to throughout the experiment.

(1) X structures. These forms were the most important. They consisted of a clear, unstained, central area which was usually spherical, though sometimes slightly oval, surrounded by deeply stained nuclear material. In shape, the surrounding material was like a circular band (Figs. 23 and 24), or a horse-shoe, (Fig.25) or a double-dumbell (Fig.26); or it showed variations of these three shapes. The clear areas ranged in size from very small (Fig.23) to very large (Fig.24). They were also inconstant in their position in the surrounding nuclear material and were sometimes eccentrically placed (Fig.27). The nuclear material was the only nuclear material in some of the cells; if so, the rest of the cytoplasm was evenly stained. Other cells, however, were larger and contained other nuclear material in different patterns and sizes.

(2) Spore with eccentric nucleus. The spore cytoplasm was stained evenly, but the nucleus was eccentrically

placed, often bulging out of the side of the spore (Fig.28).

(3) Blue structures. These were light-blue, refractile structures that had no apparent connection with any nuclear material (Fig.29). They were seen lying in some of the cells, often slightly crosswise(Fig.30). They may have been a stage in spore formation and were similar to those described by Robinow (see p.20 of this thesis) in germinating cultures of B.mycoides.

(4) Y structures. These structures, which filled practically the whole of the bacterial cell, consisted of a central (Fig.31) or paracentral (Fig.32) deeply staining granule surrounded by a palely staining oval or round area, which was enclosed in a mass of well-stained nuclear material. The results were as follows:

1. 18 hours

Well stained large bacilli were present singly, in pairs, and in short chains.

On first examination it appeared that only a few X structures were present, but a closer and more detailed examination revealed a considerable number. This misapprehension arose because the central clear space was small and quite hard to see. In other organisms, instead of a clear continuous band of nuclear material there were

a number of granules. There were also other nuclear structures in the same cells as contained X structures. These nuclear forms showed a variety of shapes: very few of the "resting" type of nuclei were seen; there were more of the actively dividing granular type, some of these showing 4 polar granules.

It was noticed also that the ends of some organisms were "cut off". These lacked nuclear elements, and had not very much cytoplasm. They were usually separated from adjacent cells by well defined septa.

2. 18½ hours As for (1)

3. 19 hours As for (1) except that the organisms showed a less granular type of nucleus. More X structures were present and these were mainly in cells by themselves, without other nuclear structures present.

4. 19½ hours As for (3)

5. 20 hours As for (3) except that the clear central areas in the X structures were bigger than those seen in (1.)

6. 20½ hours As for (5)

7. 21 hours As for (5)

8. 21½ hours As for (5) except that central areas in X structures were bigger than those seen in(5.)

9. 22 hours This was a satisfactory preparation very

like the first that showed X structures (experiment 5, Fig.18). The clear edges of the cells were well defined and most of the cytoplasm was an evenly stained pink. X structures were clear and numerous and central areas were transparent, large, and well defined. As yet there were no signs of spore-formation.

10. 22½ hours As for (9)

11. 23 hours As for (9) except that for the first time blue structures were seen. None of these were found in no. 10 when it was re-examined more closely.

12. 23½ hours As for (11)

13. 24 hours As for (11) except that there were more X structures and the central areas were large.

14. 24½ hours As for(13)

15. 25 hours (Change of plate). Spores were present in this preparation. There were a few free ones with eccentric nuclei. Blue structures were also present as well as some Y structures. Preparation 14 was re-examined; the main difference between it and no.15 was that although there were no spores or Y structures in no.14, there were more X structures. The change of plate may have been responsible for these changes. Impression preparations were made on slides after 24½ and 25 hours incubation and stained by Fleming's spore method.

14a. 24½ hours (Fleming's spore method). Some spores appeared single and normal, but there were others with smaller dots or other shaped structures inside them. In some cells there was a densely stained spore with some less dense structure next to it. A considerable number of cells showed two spores without dividing septa between them. There was great variety in the density of stain taken up.

15a. 25 hours (Fleming's spore method). Most organisms had spores inside them but most of these spores were eccentrically placed in the cell. A few spores were normal (i.e. staining uniformly) but others were bright red outside and clear inside (Fig.33). A number of spores of this type had next to them a bright red structure without a hole inside (Fig.34). Free spores were notably square in shape.

16. 25½ hours As for (15)

17. 26 hours (Taken after the plate was kept in the refrigerator overnight). A considerable difference was noted here. There were many more free spores and also more spores within the vegetative cells. There was also a considerable reduction in the number of X structures and those present were smaller. Some spores within vegetative cells had eccentric nuclei. Y structures were also present.

18. 26½ hours As for (17) except that some of the X structures were clearer and better defined.

19. 27 hours As for (18) except that there were fewer free spores.

20. 27½ hours As for (19) except that there were more blue structures but fewer X structures.

21. 28¼ hours There were free spores tending to form clusters. Some of the smaller clusters were composed entirely of free spores. Vegetative cells were absent. There were also fewer blue structures and fewer spores with eccentric nuclei inside the vegetative cells. X structures were still present; although not so numerous, they were bigger and better defined than in earlier films.

22. 28¾ hours Many more spores were free in and around vegetative cells. Some X structures were present although these were smaller than in 21. There were a number of Y structures, but hardly any blue structures.

23. 29¼ hours Free spores were still present but they did not seem to be so numerous as in (22). Blue structures were present; X structures were larger and better defined.

24. 29¾ hours As for (23) except that there were more free spores and Y structures.

25. 30¼ hours As for (24)

26. 30¾ hours As for (24) except that X structures were

larger.

27. 31½ hours As for (26) except that there were considerably more free spores, blue structures, and Y structures and fewer X structures

28. 31¾ hours As for (27) except that X structures were more prominent and there were a number of well-defined Y structures.

29. 32¼ hours As for (28) except that there were fewer X structures and that those present were smaller and less well-defined. There were more X structures.

30. 32¾ hours As for (29) but there was an increase in the number of smaller X structures.

31. 33¼ hours As for (30) but there was an increase in the size and number of X structures. Some Y structures were present.

32. 34¼ hours (Taken after plate kept overnight in the refrigerator). Some X structures were present — quite large and distinct — but few blue structures. Numerous empty cells were present with pale cell walls. Spores were present, both in the free state and inside vegetative cells. Nuclei in other vegetative cells were most distinctive, not like any of the above forms. They were very large and solid and not granular at all (Fig.35).

There were numerous organisms with the ends of their

cells cut off and apparently without cytoplasm in the end structures (Fig.36).

33. 35½ hours As for (32) but there were more empty end structures.

34. 36½ hours As for (32) but there were fewer spores.

35. 47½ hours (Taken after the plate was kept in the refrigerator overnight). There was a good deal of debris, presumably derived from dead cells. The organisms present had stained, but were completely different in their nuclear structures from any of the organisms examined in the previous impression preparations. There was an occasional X structure but not many. The predominant forms were those with nuclear cores of various shapes (Fig.37).

36. 66½ hours As for(35) except that more spores were present and a considerable number of rather large organisms showing even, reddish-purple staining throughout. Very few X structures were seen, and blue structures were not seen. There were many cells with their ends cut off by septa ( as in 32 except that the rest of the cell stained evenly).

37. 81 hours As for (36) except that the outlines of the organisms were less distinct and there were not quite so many spores.

Summary of experiment 6

As explained in detail in the preceding pages,

when B.cereus was grown on basal agar plus glucose and urea, four prominent types of structure were seen. The first was the X structure; the second was the endospore with the eccentrically placed nuclei; the third was the blue structure, which was probably some stage in endospore formation; and the fourth was the Y structure, which may be connected with the X structure.

These four structures were studied over a period of 4 days and the following points emerged. After 18 hours' incubation at 37°C, X structures were visible but they were small and not easily recognised. They appeared to grow in size until about 22 hours, when the clear central area was very large. At this time also, blue structures were observed for the first time. After 25 hours' incubation spores were seen and there appeared to be a decrease in the number of X structures. It was perhaps significant that blue structures were observed three hours before the spores — an observation which helped to substantiate the theory that these structures might be a stage in spore formation.

The presence of spores and spore-staining material was demonstrated by Armstrong's method after 24½ hours' incubation when spores had not already been demonstrated by the HCl-Giemsa technique. Spores were also observed

by Armstrong's method after 25 hours' incubation as well as by the HCL-Giemsa technique.

After 25 hours' incubation there appeared to be a decrease in the number of X structures and an increase in the number of spores and blue structures. This was followed by a decrease in the number of blue structures and an increase in spore formation.

The number of small X structures increased after 33 hours' incubation, but after this time it was hard to follow the development of the four structures, as other forms were developing. Y structures were seen after 25 hours and were present until about 34 hours.

After 47 hours' incubation the whole picture of the preparation changed. The predominant forms, apart from spores, were cells with dense solid nuclear material, or nuclear cores. There was also a considerable number of large evenly staining organisms, some with empty cell-ends.

My general impression was that there appeared to be some connection between X structures and spores, between spores and blue structures, and between X and Y structures.

The idea that there might be a connection between the X structures and the endospore has already been mentioned.

This connection could be imagined as a strong and definite one, the picture being that the endospore was actually developed inside the nucleus and that the clear areas seen inside the X structures were in fact developing endospores. The connection could also be imagined as a less definite but equally important one, in which the clear central areas were composed of lipoid material and this material was used either as material for inclusion in the developed endospore, or by the spore mother-cell as a source of energy for the production of new materials for the endospore.

The first of the two possible connections envisaged would mean that the nuclear material should always be found at the site where the endospore was to be formed, i.e. in the centre of the cell for centrally-formed spores and at the end for terminally-formed spores. If in a study of terminal spore-bearing organisms, the nucleus was constantly found at the end of the cell this would give strong support to the hypothesis. This point was investigated in the next experiment (no.7). Consultation of the sixth edition of Bergey's "Manual of Determinative Bacteriology" did not reveal the names of any easily available members of the Bacillus genus that consistently formed terminal endospores, so three terminal spore-bearing members of the genus Clostridium were studied

instead. (Experiment 7).

The hypothesis that the clear areas might be composed of lipoid material was also investigated in experiments 8, 9, and 10.

Experiment 7 An investigation to determine if there was any constant relationship between the nucleus and the endospores of three terminal spore-bearing organisms.

The three organisms studied in this experiment were Cl. putrificum, Cl. sphenoides and Cl. tetanomorphum. They were chosen because it was known that their endospores developed in a terminal position. A study was made to determine if the nuclei of these organisms were consistently in a terminal position, and if they bore any relationship to the developing spore. After these organisms had been grown on meat-extract agar for three days at 37°C under anaerobic conditions impression preparations were made and treated by the HCl-Giemsa technique. The results were as follows.

Clostridium tetanomorphum

Small slender organisms with very large spherical spores were present. The spores stained well and generally consisted of two definite areas. On the outside there was a fairly thick, bright red-purple area; inside this there was a less bright area, lighter and bluer in colour (Fig.38). Some of the spores had slight points on them (Fig.39). With such a large spore it was very difficult to see any connection between it, and the vegetative organism.

Nuclear material was present in most cells, consisting of a small spherical nucleus or nuclei, singly (Fig.40), in pairs (Fig.41), or in groups of three (Figs. 42 and 43), mainly central or subterminal; an occasional one was terminal (Fig.44).

Clostridium putrificum

The organisms were slender and spores were not formed under the conditions described. Most of the bacilli stained evenly and densely. Some showed nuclei but none was terminal.

Clostridium sphenoides

The organisms were small and slender and quite a number formed spores. The free spores, and some of those still inside the vegetative cells, stained blue. Some spores showed a general pink area with a blue dot in the centre, the cytoplasm of the vegetative cell staining red (Fig.45). Some organisms lacking spores showed a nucleus or nuclei at the tip of the cell. One peculiar organism worth mentioning had a faint blue area of developing spore with a red dot on either side of it (Fig.46).

From these results it would appear that there was no connection between the nucleus and the endospore of the three Clostridia studied. Generally the nuclei were in or near the centre of the cell, whereas the spores

were all formed terminally. No structures similar to the X structures of B.cereus were seen in any of the preparations, but it must be remembered that it was only under abnormal nutritional conditions that these peculiar structures were seen.

The Clostridia were not ideal organisms from a cytological point of view, because it is not convenient to make impression preparations every hour or so to watch the development of certain structures when this means resetting the anaerobic jar each time. Because of this and because it might require a considerable amount of time and trial to study the nutritional factors to discover if it were possible to produce X structures or similar structures, the investigations were not continued any further. The distribution of nuclei observed in the organisms studied did not, moreover, support the idea that X structures were endospores developing in close relation to nuclear material.

Experiment 8 An examination of the X structures of B.cereus when the organisms were treated by the HCl-Giemsa technique and methods to demonstrate lipoid material.

This experiment was set up to examine further the nature of the clear areas in the centre of the X structures of B.cereus. Fat stains were used either on untreated preparations, which were then treated by the HCl-Giemsa technique, or on preparations previously stained by this technique. This was to determine if the clear areas of X structures could be shown to be composed of lipoid material surrounded by a ring of nuclear material. A preparation for the purpose of comparison was stained by Armstrong's method to demonstrate spores.

Impression preparations were made from a 24-hour culture grown at 37°C on basal agar plus glucose and urea (inoculated as in experiment 5) and treated in the following manner:-

Preparation 1 stained by the HCl-Giemsa technique

Preparation 2 stained by sudan black for 15 minutes

Preparation 3 stained by sudan IV for 15 minutes

Preparation 4 stained by the HCl-Giemsa technique and then treated with sudan black for 15 minutes.

Preparation 5 stained by the HCl-Giemsa technique and

then treated with sudan IV for 15 minutes.

Preparation 6 stained by sudan black for 15 minutes and then treated by the HCl-Giemsa technique.

Preparation 7 stained by sudan IV for 15 minutes and then treated by the HCl-Giemsa technique.

Preparation 8 This preparation was made on a glass slide and stained by Armstrong's method for the demonstration of spores.

Preparations 1, 2 and 3 acted as controls to show the effect of each of the stains applied singly.

The effect of the treatment by osmic-acid fixation and by hydrolysis in N. hydrochloric acid at 60° C for 10 minutes was also considered in this experiment. Preparations similar to those above were treated in the following manner:-

Preparation 9 treated with osmic acid vapour for three minutes and stained with sudan black for 15 minutes.

Preparation 10 as for 9 but stained with sudan IV.

Preparation 11 treated with osmic acid vapour for three minutes, immersed in N.HCl at 60° C for 10 minutes, and stained with sudan black for 15 minutes.

Preparation 12 as for 11 but stained with sudan IV.

Results of experiment 8

Preparation 1 (HCl-Giemsa technique)

Organisms on this preparation showed a tendency

to form long chains, which had not been noticed before in other 24-hour cultures on this agar. Nuclear structures and septa were well demonstrated.

However, there were not quite so many X structures as I had expected in a preparation of this sort. Those present were small, but could be seen quite clearly.

Preparation 2 (sudan black)

Although the stain was filtered, there was a considerable amount of debris in this preparation. Numerous lipoid granules were present but they did not stain particularly well.

Preparation 3 (sudan IV)

As for preparation 2 except that there was not so much debris present.

Preparation 4 (HCl-Giemsa technique/sudan black)

The Giemsa stain was completely removed when the preparation was treated with sudan black. Lipoid granules were well demonstrated and appeared to be surrounded by a ring of clearer material, which was where the nuclear material was expected to be.

Preparation 5 (HCl-Giemsa technique/sudan IV)

As for preparation 4 except that the lipoid granules were not so definite.

Preparation 6 (sudan black/HCl-Giemsa technique)

The lipoid stain was completely removed by the HCl-Giemsa technique, and the preparation did not stain well by the latter method; nuclear differentiation was not good.

Preparation 7 (sudan IV/HCl-Giemsa technique)

As for preparation 6.

Preparation 8 (Armstrong's method for spores)

There were very few mature spores inside the vegetative cells and none free. From the majority of cells stainable material was absent, but some cells showed small stained granules which were possibly forespores.

These results were disappointing in that it was not possible to superimpose either a fat stain on a nuclear stain or vice versa.

By a rough study of the position and relative numbers of the fat granules and the X structures it appeared, however, that there was good correlation between the two. This aspect of the investigation was enlarged in Experiment 10.

There was no correlation between the preparation stained to demonstrate spores and that stained to show X structures.

The results from the preparations treated to

show the effect of pre-treatment with osmic acid or hydrochloric acid or a combination of the two on the demonstration of fat granules were as follows:-

Preparation 9 (osmic acid/sudan black)

The lipoid granules stained clearly and well, although a considerable amount of debris was present.

Preparation 10 (osmic acid/sudan IV)

Lipoid granules were well demonstrated. A certain amount of debris was present.

Preparation 11 (osmic acid/HCl/sudan black)

Lipoid granules were present; they were not so well stained as in preparation 9 but better than in preparation 2. Debris was present.

Preparation 12 (osmic acid/HCl/sudan IV)

Lipoid granules stained well though they appeared to be smaller in size than those demonstrated in preparation 10.

When the preparations were treated with osmic acid vapour for 3 minutes and stained by sudan black, the lipoid granules were very well demonstrated. If these preparations were treated by osmic acid and hydrochloric acid (at 60°C for 10 minutes) and then stained, the granules were not so well demonstrated. The results were better, however, than preparations that had no previous

treatment.

When sudan IV was used as the stain, pre-treatment with osmic acid gave good results, but when hydrochloric acid was used as well, the lipoid granules appeared to be smaller, though they stained well.

It would seem that pre-treatment with osmic acid helps to demonstrate fat granules when stained by sudan black and sudan IV.

The plate of basal agar plus glucose and urea that was inoculated with B.cereus in this experiment was kept, and an impression preparation was made after 66 hours' incubation. It was not examined until about 5 days after being stained, but the results were very interesting. The organisms were present in long chains and chains of medium length. Nuclear material was well demonstrated, but it showed a variety of patterns both in shape and distribution.

Some cells were packed with densely-stained nuclear material (Fig.47), but in others there was none of this material or only a small deeply-stained granule (Fig.49); others had a large amount of lightly staining nuclear material inside them, often with one or two clear round granules in this material (Fig.48).

Numerous structures showing active movement

had been seen in another preparation (p.210) of a 23-hour culture of B.cereus grown on basal agar plus asparagine and treated by the HCl-Giemsa technique. These structures, which for convenience I named "dancing bodies", were seen also in this preparation. There were not many of these bodies, but those present were in very active movement from one side of the cell to the other and up and down. One was photographed (Fig.50). It was quite large and densely stained, and the contents of the cell were colourless.

This appearance of dancing bodies in an occasional preparation may not be of major importance, but I think that it is of interest. Various possibilities about their exact nature have been considered. It may be that dancing bodies appear in organisms that were dead before they were fixed and in which the cytoplasm has undergone lysis, leaving only the possibly more resistant nuclear material as a dancing body inside the cell. The dancing movement might be Brownian in character, the molecules which seem to bombard the nuclear structure coming from the mounting buffer solution that had penetrated into the dead cell. Alternatively, the dancing bodies might be an aggregated mass of cytoplasm containing nuclear material that had shrunk because of the treatment

given and had taken up the stain deeply. All this is naturally speculative and not perhaps of major interest, but it seemed worth noting as a phenomenon that might at some stage deserve further consideration than it has received here.

Experiment 9 A comparison of the position of the X structures and lipoid granules of B.cereus.

At the outset of this experiment photographs taken of preparations from the last experiment (no.8) were critically re-examined. Diagrams were made to show the position and size of the clear central areas of X structures and of other clear areas that were associated with nuclear material but not necessarily surrounded by it. Diagrams were also made of the position and size of the lipoid granules and the associated lighter material.

A wet preparation was also made from the same culture of B.cereus grown on basal agar plus glucose and urea after 24 hours incubation (i.e. at the same time the other preparations were made). This preparation of living organisms was examined and photographed under the phase-contrast microscope.

The photgraphs particularly re-examined were those taken of:-

(a) preparation 1 — treated by HCl-Giemsa technique.

(b) preparation 9 — treated with osmic acid and stained by sudan black.

(c) preparation 10 — treated with osmic acid and stained by sudan IV.

(d) the wet preparation examined by phase-contrast microscopy.

In the preparation treated by the HCl-Giemsa technique, nuclear material was well demonstrated. Cross-walls could be seen, the organisms being in chains of about 9-12 members. X structures were present, although they were not quite so distinct as Fig.19. Some of the X structures were composed of clear areas of different sizes completely surrounded by a band of nuclear material (Fig.51), with others this band was not complete (Fig.52) or appeared to be composed of granules (Fig.53) which were often opposite each other (Fig.54). In many of the organisms there were one or more clear areas associated with nuclear material but not necessarily surrounded by it (Figs. 55 and 56). These areas differed in size from large to small and in the number per cell.

There was no essential difference between the preparations stained with sudan black or sudan IV, so for the purpose of description the two are treated as one preparation stained to show lipoid granules. These staining methods do not delimit separate organisms, but from the appearances observed in the previous experiment their approximate size could be judged. The lipoid granules differed in shape and size and position in the cells. Some were large (Fig.57); other were small (Fig.58). The small granules were often in a group (Fig.59). Nearly all the granules, large and small, were associated

with lighter material, which either completely surrounded them (Fig.60) or only partly surrounded them (Fig.61). This lighter material may have been of nuclear character.

The dark granules observed in the preparation examined by phase-contrast microscopy (Fig.62) corresponded in shape, size, and distribution to the lipoid granules seen in the above preparations. They were also associated with a lighter material which may have been nuclear material (see experiments 12 and 13).

From the general appearances of the photographs it appeared that the number of clear areas in the HCl-Giemsa-treated preparation, whether they were part of X structures or merely associated with nuclear material, corresponded to the number of lipoid granules in the other photographs. For this reason it seemed that a count both of clear areas and of lipoid granules would prove of value, and such a count is described in experiment 10. It also seemed that the dark granules seen by phase-contrast microscopy were lipoid granules. A count of these granules was not possible as the preparation had dried up by the time that the other preparations were examined and the structures counted.

The interpretation of these results was that the clear areas in the centre of the X structures were almost

certainly lipoid material, and that this material was not necessarily surrounded by nuclear material but often associated with it. In fact I agree wholeheartedly with Deleporte (1950) who considered that peculiar arrangements of nuclear structures could be due to the presence of these lipoid granules, which could readily displace nuclear material from its normal position and break it into smaller fragments. To emphasise this point I have made drawings from the photographs that were taken of (a) organisms stained to show nuclear material, (b) organisms stained to show lipoid granules. Figures 63 and 64 show the way in which I think that nuclear material may be distorted and pushed out of place by lipoid granules (Figs.63 and 64 drawn from Figs. 55 and 56 respectively). Figures 65 and 66 show the position of the lipoid granules in relation to the lighter areas, which I consider to be composed of nuclear material (Fig.65 includes a drawing of Fig.58; and Fig.66 includes drawings from Figs. 59 and 60).

Experiment 10 An attempt to show by statistical methods a relationship between X structures and lipoid granules of B.cereus.

In this experiment, preparations from experiment 8 were examined and the X structures and lipoid granules were counted in an attempt to find a statistical relationship between the two. The preparation treated by the HCl-Giemsa technique (preparation 1, experiment 8) and that treated by osmic acid and stained by sudon black (preparation 9, experiment 8) were the two examined.

Sixteen fields were examined in each preparation. In each field one chain of organisms was chosen and the X structures or lipoid granules were counted along with the organisms in the chain.

The results were as follows:- (see p.192)

(a) X structures

| Field No. | No. of X structures per chain | No. of organisms per chain |
|---|---|---|
| 1 | 4 | 7 |
| 2 | 5 | 10 |
| 3 | 6 | 13 |
| 4 | 3 | 14 |
| 5 | 3 | 7 |
| 6 | 2 | 5 |
| 7 | 2 | 5 |
| 8 | 4 | 5 |
| 9 | 2 | 4 |
| 10 | 3 | 9 |
| 11 | 9 | 13 |
| 12 | 3 | 10 |
| 13 | 2 | 10 |
| 14 | 1 | 5 |
| 15 | 1 | 10 |
| 16 | 4 | 4 |
| Totals | 54 | 131 |

i.e. Number of X structures to 100 organisms = 41·2

(b) Lipoid granules

| Field no. | No. of lipoid granules per chain | No. of organisms per chain |
|---|---|---|
| 1 | 3 | 4 |
| 2 | 2 | 5 |
| 3 | 4 | 12 |
| 4 | 6 | 15 |
| 5 | 1 | 4 |
| 6 | 2 | 5 |
| 7 | 7 | 14 |
| 8 | 6 | 14 |
| 9 | 4 | 7 |
| 10 | 4 | 8 |
| 11 | 7 | 16 |
| 12 | 1 | 4 |
| 13 | 3 | 10 |
| 14 | 4 | 9 |
| 15 | 8 | 17 |
| 16 | 3 | 8 |
| Totals | 65 | 152 |

i.e. Number of lipoid granules to 100 organisms = 42·7

From these results it appeared that there was a strong correlation between the ratio of X structures

and of lipoid granules to organisms. This led to the conclusion that there was good evidence in favour of the hypothesis that the clear central area of the X structure was composed of lipoid material.

Experiment 11 An investigation to determine if B.cereus would form X structures when grown in basal broth plus glucose and urea.

It was known that when B.cereus was grown on basal agar plus glucose and urea X structures could be demonstrated inside the organisms when they were treated by the HCl-Giemsa technique. It was thought that organisms producing these structures would be very interesting material to examine as ultra-thin sections under the electron microscope. Preparations for electron microscopy, if made from agar, were often found to be rather difficult to see clearly because of contamination with agar and other substances. Preparations made from broth cultures, when well washed, were easier to examine. Consequently this experiment was set up to determine if B.cereus would produce X structures when grown in basal broth plus glucose and urea.

0·1 ml. of washed organisms from an 18-hour meat-extract broth culture was added to a tube of 10 ml. basal broth plus 1% glucose and 1% urea. The same inoculum was spread over a basal agar plate of similar composition. Impression preparations from the plate and smear preparations from the broth were made after 5, 8, 23, 26, 31, 47, and 50 hours' incubation at 30 °C.

These preparations were treated by the HCl-Giemsa technique, and the results from the broth and the agar are given together so that a true comparison may be made.

Results

After 5 hours' incubation

(a) agar - a few organisms were present, mostly as singles but sometimes in pairs. The organisms stained a dense, even reddish colour. Nuclear structures were not visible.

(b) broth - organisms could not be detected in the preparation.

After 8 hours' incubation

(a) agar - organisms were present singly, in pairs, and in short chains. Cell walls and dividing septa were well defined. Small distinct nuclei were present, mainly round in shape. In a few cells very small X structures could be seen.

(b) broth - as for broth after 5 hours' incubation.

After 23 hours' incubation

(a) agar - organisms were observed singly, in pairs, and in short chains. Nuclear material was well demonstrated; X structures were present. Other nuclear material was very granular and definite forms were not observed.

(b) broth - the few organisms present stained poorly and did not show internal structures.

After 26 hours' incubation

(a) agar - organisms were present singly, in pairs, and in short chains. Some of the organisms were clearly divided into two by well defined septa. X structures were numerous and quite large, although the surrounding band of nuclear material was rather thin. Other nuclear material was present; generally it was diffuse and granular, and there were not many definite structures.

(b) broth - as for broth after 23 hours' incubation.

After 31 hours' incubation

(a) agar - this preparation was similar to that made after 26 hours' incubation, except that more chains of organisms were present. Blue structures were quite numerous, but the number of X structures decreased. Free spores were not present.

(b) broth - organisms could not be seen in the preparation.

After 47 hours' incubation

(a) agar - numerous spores were present, generally still inside the vegetative cells, although there were a few free spores. The spores inside the cells had nuclei and peripheral red staining areas which were generally restricted to part of the circumference of the spores. A few large

X structures were present. Other nuclear forms were mainly of the granular type.

(b) broth - no organisms could be seen in the preparation.

After 50 hours' incubation

(a) agar - many free spores with eccentric nuclei were present, though some were still inside the vegetative cells. Nuclear material of the vegetative cells still present was granular and diffuse, without definite structures. The outlines of these cells were generally hazy and ill defined and a lot of debris was present, suggestive of the beginning of autolysis.

(b) broth - no organisms could be seen in the preparation.

From the results of the experiment it appeared that B.cereus did not multiply in basal broth containing glucose and urea, although it produced growth on agar of similar composition. It may have been that the inoculum used was not large enough or that the tube of broth should have been aerated. It was decided, however, that it would be better to use the growth from basal agar for the preparation of ultra-thin sections. This could probably be made suitable if care was taken in removing the organisms from the agar and if the cells were well enough washed.

Experiment 12 A comparison of the demonstration of X structures when preparations of B.cereus were examined by three different methods: the HCl-Giemsa technique, the Quick differential method, and phase-contrast microscopy.

The purpose of this experiment was to see if, with organisms grown on basal agar plus urea and glucose, there was any correlation between the demonstration of X structures when the organisms were treated by the HCl-Giemsa technique and when they were examined by phase-contrast microscopy. The organisms were also treated by the Quick differential method to see whether this method gave a similar picture to that obtained when the organisms were treated by the HCl-Giemsa technique. If this were so, the method could be used as a preliminary to determine if X structures were present in the preparation.

Organisms from a 3-day meat-extract agar slope were spread over the surface of a basal agar plate containing 1% glucose and 1% urea. After 26 hours' incubation, impression preparations were made and treated by the HCl-Giemsa technique and the Quick differential method. Wet preparations were also made and examined under the phase-contrast microscope. Photographs were taken of

all preparations.

Results of experiment 12

(a) Quick differential method

Quite large organisms were present, most of them in singles, in pairs, or in short chains. Internal differentiation was good, some cells containing large deeply-staining nuclear bodies (Fig.67). Numerous spores in various stages of development were present inside the vegetative cells, and there were a few free spores. Certain cells had X structures inside them (Fig.68) although these were not numerous. In some cells X structures appeared by themselves, in other cells granules were also present: generally two and rarely three. The majority of the X structures were small, although a few were very big. In most cells without X structures there was a single round definite granule, although a few had two granules. Some cells had cap-like material which stained well (Fig.69).

(b) HCl-Giemsa technique

This preparation was similar to that stained by the Quick differential method but the nuclear differentiation was clearer. X structures were better defined and there appeared to be more of them (Figs. 70 and 71). Other nuclear structures (Fig.72) were not quite so round and

regular as they appeared in the Quick differential method. Numerous cells showed peripheral nuclear-staining material. Blue structures were also present as well as free spores and spores inside vegetative cells, both types of spore showing eccentric nuclei. Some cells showed deeply-staining cap-like material (Fig.73).

(c) Phase-contrast microscopy

Free spores, and spores inside vegetative cells were very clearly demonstrated. Granules and septa could also be seen. Some structures consisting of a dark central round area surrounded by a lighter clear area were present (Fig.74). Some cells showed caps of darker material (Fig.75).

From a careful study of the photographs it could be seen that there was good correlation between preparations treated by the Quick differential method and the HCl-Giemsa technique, although the latter gave the clearer picture. This meant, however, that the rapid method could usefully be applied to see if cultures were at a stage when they were worth staining by the more troublesome and time-consuming HCl-Giemsa technique. It was important to note that X structures were demonstrated by the Quick differential method.

On first examination, there did not appear to be

much correspondence between the structures demonstrated by the phase-contrast microscope, and those demonstrated by the two staining methods. But closer examination revealed that cap-like material was demonstrated by the phase-contrast microscope as well as by the other two methods. It did appear, however, that the phase-contrast microscope did not demonstrate X structures, but further examination revealed that many of the dark areas were surrounded with lighter material. This appearance is exactly the opposite in photographic appearance of X structures in stained preparations. Phase-contrast microscopy picks out differences in refractive index, not density or ability to stain with nuclear dyes, so that it was quite possible that these structures were in fact X structures, this method only serving to give a different view of them.

Experiment 13 A comparison of the structures observed in B.cereus when grown on various media and demonstrated by the HCl-Giemsa technique and phase-contrast microscopy.

As already mentioned, the criticism is often made that the appearance of nuclear structures in bacteria may be the result of artefact production. This experiment was set up to determine how much correlation there was between the appearance of organisms grown on different media when they were examined after treatment by the HCl-Giemsa technique (dead fixed bacteria) and by the phase-contrast microscope (unfixed living bacteria). Such an approach, it seemed, might help to clarify the status of material under the question: nucleus or artefact? Impression preparations were made from organisms grown on meat-extract agar, basal agar plus glucose and urea, and basal agar plus asparagine — all incubated for 24 hours at 37°C. These preparations were treated by the HCl-Giemsa technique. Wet preparations from the same plates were made and examined under the phase-contrast microscope. Photographs were taken of each preparation.

Results of experiment 13

Meat-extract agar

(a) HCl-Giemsa technique

The organisms were present in chains of medium length

(Fig.76). Nuclear material was well demonstrated, but there was variety in its shape and distribution. Spores were still present inside the vegetative cells, the majority taking up the stain well (Fig.77).

(b) Phase-contrast microscopy

The organisms were present in chains of medium length (Fig.78). Spores were well demonstrated (Fig.79) as were cross-walls (Fig.80). Some internal differentiation could be seen in some cells but this was not at all clear or definite.

Basal agar plus glucose and urea

(a) HCl-Giemsa technique

The organisms were present in chains of medium length (Fig.81). Nuclear material was well demonstrated but showed variety in shape and distribution. Numerous X structures were present (Fig.82) but in many, the band of nuclear material was uneven in shape (Fig.83). In some organisms there were one or more small clear areas which were not surrounded by nuclear material but were often associated with it (Fig.84).

Spores could not be seen.

(b) Phase-contrast microscopy

The organisms were present in chains of medium length (Fig.85). Spores were absent. Dark granules in the

centre of a lighter area could be seen (Fig.86). In some cells one or more of these granules was associated with lighter material but not always surrounded by it (Fig.87). From the results of experiment 9 these granules would appear to be lipoid granules and the lighter material may be nuclear material.

Basal agar plus asparagine

(a) HCl-Giemsa technique

Long chains of organisms were present (Fig.88); nuclear material was well demonstrated, but of varied shape and distribution. Some cells contained spherical nuclear structures (Fig.89), whereas others contained a central core of nuclear material (Fig.90). Clear unstained areas were also present in some organisms (Fig.91). Cross-walls were also well demonstrated (Fig.92).

(b) Phase-contrast microscopy

Long chains of organisms were present (Fig.93). Cross-walls were well demonstrated (Fig.94) and numerous spores were present in various stages of development. Many were very refractile showing on the photographs as white structures with a dark edge (Fig.95); some were very dark (Fig.96) and others were intermediate between the two stages (Fig.97). Little internal structure was visible in the other cells.

From these results it would appear that there was a good deal of correlation between the demonstration of spores and cross-walls by the two methods, but that phase-contrast microscopy gave the clearer picture of these structures. With regard to lipoid material in organisms grown on basal agar plus urea and glucose, there was a correlation between the clear areas in stained preparations of the X structures and the dark granules shown by phase-contrast microscopy. There was not, however, any correlation between stained and phase-contrast preparations in the demonstration of the nuclear material, because in most of the preparations examined by phase-contrast microscopy little internal structure was visible, apart from the dark granules.

Phase-contrast microscopy, although excellent for the demonstration of cross-walls and cross-septa, is not good for the demonstration of nuclear material. Lipoid granules show in phase-contrast as dense bodies that could readily be mistaken for nuclear structures unless alternative methods of examination were carried out. In resorting to phase-contrast microscopy, therefore, to answer the question — artefact or nucleus? — I had to reach the conclusion that the answer to this particular question was beyond the method. I learned, however, that the method had uses in defining certain non-nuclear structures.

The right lesson to be learned is that bacterial cytologists bound to any single method of examination are likely to err — and to err handsomely and often. Replication of methods of examination is a most useful discipline and its regular application to bacterial cytology would do much to reduce the number and length of published papers and to increase their value to the reader.

Experiment 14 A study of the nuclear structures formed when B.cereus was grown on basal agar plus asparagine.

From experiment 5 it was known that, on basal agar plus 1% asparagine and after about 24 hours' incubation at 30° C, B.cereus grew in long chains of organisms with definite and characteristic nuclear structures. This experiment was set up to watch the development of these structures. Details of method were exactly the same as in experiment 5. Impression preparations were made after 23, 26, and 28 hours' incubation at 30° C and treated by the HCl-Giemsa technique. The results follow:

After 23 hours' incubation

Generally the organisms were present in long chains (Fig.99) although in patches of the preparation the chains were not very long, and there were some single and double organisms (Fig.98). Separating cell-walls were well defined (Fig.100). The nuclear material was definite and well stained. Some forms were very interesting (Figs. 101 and 102) in that they resembled the structures seen at the diakinesis stage of meiotic division of plant and animal cells. I had previously named these forms "cross-over" structures. A few organisms showed clear bright areas, similar to those found in the centre of the X structures (Fig.103).

After 26 hours' incubation

Spore development had begun and numerous spores with well stained nuclei and other peripheral nuclear material could be seen (Fig. 104). There were still long chains of organisms, although some of the chains had broken up into small fragments (Fig. 105). Some organisms still showed the cross-over type of nuclear arrangement (Fig. 106). Clear bright areas are also present in some of these organisms (Fig. 107).

After 28 hours' incubation

Many spores were present, mostly with well stained nuclei (Fig.108). A few vegetative cells were left, but the outline of these were hazy and the internal structure irregular (Fig. 109).

Thus, when asparagine alone was incorporated into the basal agar, the organisms generally formed long chains and many of them showed a characteristic type of nuclear arrangement. The nuclear structures observed were very different from those seen when urea and glucose were present in the agar, but in a few organisms there were clear areas which were probably lipoid granules.

Spore development did not appear to have started up to 23 hours' incubation, but 3 hours later numerous spores were present and many of the long chains

of organisms were broken up. After 28 hours' incubation the preparation was composed almost entirely of spores. This may mean that spore formation took place in the relatively short time of 5 hours (i.e. between the 23rd and 28th hour of incubation).

I do not think that these nuclear structures, which are so similar to the cross-over structures seen in the meiotic divisions of plants and animals, are proof that mitotic or meiotic division is carried out in the bacterial cell. In my films such appearances were isolated structures, meiotic forms previous to this stage were not seen, not were later forms. They are more likely to be due to the passive alteration of the nuclear material by inclusion bodies and other metabolic by-products resulting from the altered nutrition of the organism.

The impression preparation of 23 hours' growth was kept for over two weeks and re-examined. On re-examination, numerous peculiar structures were observed which were similar in shape to the nuclear structures and had stained deeply in a similar manner. These structures, which were constantly moving about inside cells in which there was little or no cytoplasmic material, were called "dancing bodies"; their possible significance has already been discussed on p.185 of this thesis.

Experiment 15 A study of the internal structure of B.megatherium as revealed by various methods.

The various methods of this experiment were carried out over a period of time, but are grouped together to give as comprehensive a picture as possible of the internal structure of a Gram-positive spore-bearing bacillus: B.megatherium.

The methods used were:

(a) HCl-Giemsa technique.

(b) Tannic acid-crystal violet method.

(c) Quick differential method.

(d) Electron microscopy.

(e) Phase-contrast microscopy.

Each method is treated separately.

(a) HCl-Giemsa technique

Impression preparations were made of a 2-hour meat-extract agar culture of B.megatherium and treated by the HCl-Giemsa technique. Photographs were taken of the various nuclear arrangements observed and in some cases diagrams were drawn from the photographs to try to simplify the arrangements of the nuclear structures. The diagrams have the same figure numbers as the photographs of the organisms they are drawn from, but they are indicated by a letter "a" after the number.

The nuclear material of many of the organisms

was spherical. In some organisms there was only one nuclear structure per cell (Fig.110); in others, two per cell (Fig.111). Generally the organisms appeared to be grouped in pairs with two nuclei in each cell, sometimes with a dividing septum between each nucleus (Fig. 112). Many of the nuclei were in various stages of division. Constriction of the nuclei into two daughter nuclei had started in some organisms (Figs.113 and 114). In others, where division was not complete, the daughter nuclei were still joined (Figs. 115 and 116). A gap could clearly be seen between the daughter nuclei in some organisms (Figs. 117 and 118). The appearance of nuclear-staining material was also noted on or near the dividing septa (Figs. 119 and 119a). One organism was of particular interest; it was divided into two by a septum, each part having two nuclei. Three of these nuclei were spherical, the other was of a boomerang shape (Figs. 120 and 120a). This type of shape was also seen in another organism where both nuclei were boomerang-shaped (Figs. 121 and 121a). Another structure of interest consisted of a double dumbell of nuclear material with a clear central area (Figs. 122 and 122a). It was very similar to a structure observed in B.cereus (Fig. 26).

Accepting the general impression conveyed by those figures it would seem that the nuclear structure of

this organism, which was in an early stage of active growth and division, was relatively simple. It appeared that the nuclei were normally spherical but that during growth they constricted to form two daughter nuclei. These daughter nuclei might themselves divide before a cross-cell-wall had been formed between them. Some of their peculiar shapes could be explained by the fact that constriction of the daughter nuclei has begun before they themselves had become completely divided. Comparing the nuclear structures of this organism with those of A.cloacae at a similar stage of growth, there did not appear to be any significant difference between the nuclear structures of young cultures of B.megatherium and A.cloacae, apart from the fact that vertical dumbell shapes were seen in the latter organism (Fig. 9).

(b) Tannic acid-crystal violet method

Impression preparations of a 2-hour meat-extract agar culture were treated by this method. The majority of the organisms were only single cells and had no cross-septa, but their cell walls were well demonstrated (Fig. 123). At first the cytoplasm of these organisms was stained an even mauve colour, but when the preparation was examined and photographed some hours later, many of them could be seen to have a central granule (Fig.124).

A chain of three very long organisms was also present, but they may have been contaminants (Fig. 125).

From these results it would seem, in this 2-hour culture, that the cells were in an active state of nuclear division but that cross-septa could not be demonstrated. Many organisms showed small central granules which might be the beginning of development of cross-septa or might be composed of nuclear material, though this method was not designed to show this material.

(c) Quick differential method

Smear preparations from a 24-hour meat-extract agar were treated by this method. The results were disappointing in that little internal structure could be seen. The vegetative organisms stained densely and evenly (Fig.126); the endospores were taken to be the round unstained areas (Fig. 127).

(d) Electron microscopy

The organisms were grown in meat-extract broth for three days at 37° C and then washed carefully four times in distilled water. On examination by the electron microscope, most of the organisms were found to be very heavily fringed with proteins and other contaminating substances, but a few relatively clean bacilli were photographed.

The first bacillus photographed was a long organism

with a very striking internal structure. Running down the centre was a core of dense material and in the central portion of this core about 5 turns of a spiral could be seen (Fig.128). The rest of the organism consisted of rather pale cytoplasm.

The small group of bacilli next photographed was not very well separated but it would seem that in one bacillus at least there was a similar core of dense material, and in all bacilli the cytoplasm appeared very pale. (Fig. 129).

The majority of the organisms, however, were opaque to the electron beam and little internal structure could be seen. The next step, therefore, was to try to grow the organism in as simple a medium as possible to eliminate the protein fringes and perhaps help to reveal more of the internal structure. Citrate medium was tried. B.megatherium did not grow in it, but growth was obtained when a few drops of 10% glucose solution were added. After incubation at 37°C for 7 days the organisms were spun down, washed, and examined by the electron microscope.

The results were not at all what had been expected. There was a considerable amount of debris and many curiously shaped organisms. There were also numerous small regularly shaped particles and it was thought that these might be bacteriophage particles which had attacked the culture of B.megatherium. The curiously shaped organisms and structures

were probably bacterial cells in a state of lysis. A few photographs were taken and these are shown for the sake of interest. (Figs.130,131 and 132).

(e) Phase-contrast microscopy

I had made numerous unsuccessful attempts to take photographs of living organisms under the phase-contrast microscope with a vertical camera. As soon as a horizontal camera was used a very good photograph of B.megatherium was obtained. The organisms examined were from a 6-day culture on meat-extract agar (2 days at 37°C; 4 days at room temperature). The endospores were very well demonstrated, appearing as bright structures with a dark edge to them. (Fig.133). Cross-septa could also be seen (Fig.134), and numerous granules were present (Fig.135).

The results of this experiment were disappointing to the extent that the methods used did not demonstrate similar structures. But it must be remembered that the organisms were from cultures of different ages so that many of the appearances should not be expected to be similar.

The young cultures studied by the HCl-Giemsa technique add proof to the gradually hardening view about the simple nature of the bacterial nucleus and its amitotic mode of division.

The spiral structure observed by the electron microscope is of interest in that a similar structure has

been observed in B.cereus (Fig.164) and it is similar to the nuclear cores seen in A.cloacae and also in B.cereus and B.megatherium. It is an attractive hypothesis that the bacterial nuclear material may at times assume the spiral shape, but I do not think that there is enough proof to support it. It is more likely that the shape is due to the material being distorted by inclusion bodies. Apart from showing this one structure very clearly the electron microscope was not of great help in this particular study. The best conditions for its use are worthy of further investigation.

Phase-contrast microscopy was excellent in the demonstration of spores and septa. The nature of the internal granulation is rather harder to interpret and may have been due to lipoid material.

Experiment 16 An investigation into the action of lysozyme on the morphology of B.megatherium.

Much confusion has arisen in the interpretation of what may be nuclear structures of bacteria because the method of examination used did not demonstrate cross-septa as well as nuclear material (see paper by Beutner (1953) reviewed on p.31 of this thesis). To overcome this difficulty, two methods — one to demonstrate nuclear material and the other to demonstrate cross-septa and the approximate length of individual organisms — may be carried out on two similar preparations of the same material. This, although it avoids error, is not ideal, so I sought a new approach to the problem. It was known that lysozyme can break down chains of B.megatherium into individual cells (Welshimer (1953) reviewed on p.86 of this thesis). If the individual cells thus produced could be treated by the HCl-Giemsa technique to show nuclear material, a much better picture of the arrangement and division of nuclear structures would be obtained. With this in mind the following experiment was set up.

A preparation of egg-white, with chloroform added as a preservative, was used as a source of lysozyme. 1 gm. of this preparation was added to 5 ml. of meat-extract broth and thoroughly shaken. To this 0.1 ml. of a 24-hour

meat-extract broth culture of B.megatherium was added and the tube incubated at 37°C for 7 days. A control was set up without egg-white. Smears were made after 1 hour, 24 hours, 2 days, and 7 days, stained by dilute carbol fuchsin, and examined under the microscope for the breaking up of the chains. The organisms remained in chains throughout the whole 7 days. After 4 days, however, a general raggedness was seen in the organisms treated with egg-white which was not apparent in the control; after 7 days a considerable amount of debris appeared in the treated culture.

From these results it was decided to modify the experiment in three respects:-

(a) to use a known solution of lysozyme, such as Armour & Co's egg-white lysozyme, so that concentrations would be known;

(b) to grow the organisms in basal broth plus 1% peptone, in which they form very long chains, and in which normal growth has a fluffy appearance visible to the naked eye; any change could possibly be used as a rough indicator for the break-up of the chains;

(c) to use a culture of Micrococcus lysodeikticus as a control for the action of lysozyme.

With these modifications the experiment was set

up again. 0.5 gm. of the crystalline lysozyme was dissolved in 5 ml. of $\frac{M}{15}$ Sørensen's phosphate buffer mixture pH 6.6 and added to tubes of 10 ml. amounts of basal broth plus 1% peptone to give final lysozyme concentrations of 1µg., 2µg., 4µg., and 6µg. per ml. of broth. A loopful of a washed saline suspension of a five-hour basal broth (as above) culture was added to each tube and to a tube of broth without lysozyme. A control was set up with a meat-extract broth culture of M. lysodeikticus and a final lysozyme concentration of 0.4µg. per ml. of broth. The tubes were incubated at 37° C, and smears were taken after 1 hour, 24 hours, 2 days, 4 days and 7 days, stained by dilute carbol fuchsin, and examined under the microscope. No break-up of the chains was observed. The culture of M. lysodeiktious showed no lysis either to the naked eye or when examined under the microscope. It was concluded from these results that the solution of lysozyme, which had been made up and kept in the ice-box, might have deteriorated or that its action might be being inhibited by the salts in the basal broth.

A further modification of the experiment was then set up. A freshly prepared solution of lysozyme was used. The organisms were grown in yeast water-tryptone broth (YWT broth, of Welshimer (1953)) for 24 hours at 37° C.

Then a final concentration of 15% formaldehyde was added to the culture. This was to inhibit the action of the autolytic enzymes and to ensure that any change that might occur was due to the lysozyme system. The organisms were then spun down, washed three times with saline, and ultimately suspended in $\frac{M}{15}$ phosphate buffer mixture pH 6.6. To half of this suspension and to a control broth culture of M.lysodeikticus, lysozyme solution was added to give a final concentration of 4µg. per ml. The other half of the suspension was left untreated to act as a control. Both suspensions and the control of micrococci were incubated at 37°C and examined microscopically after 1 hour, 2 hours, 24 hours, and 2 days. Lysis of the micrococci in the control tube did not take place even after 2 days' incubation but it was thought that this culture of M.lysodeikticus was composed primarily of a non-lysogenic variant ( and this was later found to be so) as I was fairly confident of the activity of the lysozyme. Nor were the chains of organisms observed to break up in either suspension.

It was therefore presumed from these results that although the culture of micrococci did not show lysis, it was highly likely the strain of B.megatherium studied was unfortunately resistant to the action of lysozyme.

While this experiment was being carried out,

I also stained the nuclear material in B.megatherium grown in the various media and treated with formaldehyde. Nuclear bodies could be demonstrated well when the organisms were grown in basal broth plus 1% peptone for 5 hours at 37°C and in yeast-water tryptone broth for 24 hours at 37°C (Fig.136). After treatment with formaldehyde nuclear structures could be seen, but they were not definite or clear cut.

Although the results of this experiment were not successful, I am still convinced that the possible usefulness of this approach to bacterial cytology warrants a renewal of the attempt to break chains of organisms into individual cells. The use of lysozyme for this purpose may not be the best or only approach, but this trial of it seems worth noting if only to record the need for this kind of analysis of bacterial structure and a few of the inherent difficulties.

Experiment 17 An investigation of the changes in the internal structure of B.megatherium grown in different nutritional environments.

This experiment was exactly similar to experiment 5 except that B.megatherium was studied to see if X structures or any comparable characteristic structures were formed when it was grown in differing nutritional environments. Impression preparations were made after 2 hours' and 24 hours' incubation at 37°C and treated by the HCl-Giemsa technique. The amount of growth on the plates was also noted after 24 hours' incubation. (Summarized in table IV on p.235 of this thesis).

Results of experiment 17

2-hour culture

(1) Basal agar alone

The organisms stained well, but definite nuclear shapes could not be seen in any of them. Granules were present in some and in others the ends of the organisms had stained deeply. Some spores were present.

(2) Basal agar plus glucose

As for (1)

(3) Basal agar plus glucose and ammonium sulphate

As for (1)

(4) Basal agar plus glucose and asparagine

Organisms appeared rather larger and less densely stained

than those seen in the first preparation. In some organisms, a single small granule was present; in others there were several, some central and others peripheral. A few spores were present.

(5) Basal agar plus glucose and peptone

Some long chains of organisms were seen, though these were not numerous. Some organisms had small granules and in others the cross-septa had stained red and could be clearly seen. Spores were present.

(6) Basal agar plus glucose and sodium nitrate

As for (1), except that the organisms stained less densely.

(7) Basal agar plus glucose and urea

As for (1), but some long chains of organisms were present.

(8) Basal agar plus ammonium sulphate

As for (1)

(9) Basal agar plus asparagine

As for (1)

(10) Basal agar plus peptone

Organisms were present in long chains, but they showed little internal differentiation apart from a few granules and deeply stained cross-septa. A few spores were present.

(11) Basal agar plus sodium nitrate

As for (10), but more spores were present.

(12) Basal agar plus urea

Most of the organisms were in small chains. Granules were present in some, and in others there was a thin core of nuclear material; the majority, however, showed no internal differentiation. Numerous spores were present.

(13) Basal agar plus all nitrogenous compounds

As for (10)

(14) Meat-extract agar

Organisms were present in chains of moderate length. The nuclear structures were well demonstrated, generally consisting of simple type of dividing nucleus as described in experiment 15 and shown in Figs. 110 and 113.

24-hour culture

(1) Basal agar alone

Organisms were generally single or in short chains. The cytoplasm stained poorly and evenly, although some organisms could be seen to possess nuclei. Some of the chains of organisms showed structures consisting of a dense central purple dot in a pale pink area, surrounded by an area of reddish-staining material (Fig.137). Spores, both free and within the vegetative cells, showed eccentrically placed nuclei.

(2) Basal agar plus glucose

Organisms were present singly, in pairs, and in long

and short chains. Nuclear material in some of them was well demonstrated, but the appearances ranged from single spherical structures to complicated spiral forms.

(3) Basal agar plus glucose and ammonium sulphate

A fair amount of debris was present in this preparation but not many organisms, and these showed little internal structure.

(4) Basal agar plus glucose and asparagine

The organisms were mainly in long chains. The nuclear material of many of them was well demonstrated. Spherical nuclei as well as nuclear cores were present. One organism (Fig.138) showed what could be interpreted as a late anaphase or early telophase of mitotic division. A few free spores with eccentric nuclei were also present.

(5) Basal agar plus glucose and peptone

Very long chains of organisms as well as shorter ones were seen. Some organisms had nuclear material, but the majority stained homogeneously. A lot of debris was present.

(6) Basal agar plus glucose and sodium nitrate

A considerable amount of debris was present, but there were very few organisms in the preparation. These organisms showed a lack of internal differentiation, — apart from some with spores inside them.

(7) Basal agar plus glucose and urea

As for (6)

(8) Basal agar plus ammonium sulphate

As for (6)

(9) Basal agar plus asparagine

Organisms were present in long and short chains. Some organisms showed red peripheral granules, others red septa, others dense spherical nuclear bodies. In the long chains some of the organisms showed very complex nuclear structures.

(10) Basal agar plus peptone

Organisms were present in long chains, some showing nuclear material but the majority showing no internal differentiation apart from some with small peripheral granules and others with septa.

(11) Basal agar plus sodium nitrate

Organisms were present in chains of different lengths. Some showed a granular type of nuclear material and there were nuclear cores in others. There were numerous free spores and spores with eccentric nuclei still inside the vegetative cell. Some of the free spores were very poorly stained with red rims and caps and others appeared to have two thin nuclei.

(12) Basal agar plus urea

As for (11) except that some of the organisms in the long chains were very large and had long nuclear cores. Various atrophied forms with complex contents were also

present.

(13) Basal agar plus all nitrogenous substances

The organisms had not taken the stain well, but nuclear material was well demonstrated and complicated in structure. Two organisms showed very good spiral structures (Fig.139). No spores were present.

(14) Meat-extract agar

Organisms of different sizes were present in chains and their nuclear material was well demonstrated; there were also a few developing spores. Some organisms had nuclear cores, others spherical nuclei, and others red peripheral granules.

The results were disappointing in that any characteristic structure was not observed in B.megatherium: there was nothing comparable in interest to the X structure of B.cereus, and B.megatherium did not itself form X structures. The apperance of the organisms on the various nutritionally-manipulated agar plates was different from that seen with organisms grown on meat-extract agar and also from cultures of B.cereus on the same range of media. Since the two organisms have different nutritional requirements, the differences in morphology need not be surprising.

However, on certain of the plates, the appearance of growth was so slight that it may be that what was being examined after 2 hours' incubation and even after 24 hours' incubation was in fact the organisms from the 18-hour broth

culture. Because of this the experiment was repeated but basal broth was used instead of basal agar. The amount of growth that took place was noted. Unfortunately this had to be estimated by naked-eye appearances since the amount of insoluble salts in the basal broth made it impossible to use the nephelometer. Smear preparations were made only after only 2 hours' incubation, although the amount of growth was estimated after 2 and 24 hours' incubation.

Results

(1) Basal medium alone

Organisms could not be found in the preparation.

(2) Basal medium plus glucose

As for (1)

(3) Basal medium plus glucose and ammoniumsulphate

As for (1)

(4) Basal medium plus glucose and asparagine

As for (1)

(5) Basal medium plus glucose and peptone

Long chains of organisms were seen with evenly stained cytoplasm but nuclei were not demonstrated. Cross-septa were visible although very lightly stained.

(6) Basal medium plus glucose and sodium nitrate

As for (1)

(7) Basal medium plus glucose and urea

As for (1)

(8) Basal medium plus ammonium sulphate

As for (1)

(9) Basal medium plus asparagine

As for (1)

(10) Basal medium plus peptone

Very long chains of organisms were seen which were divided by well defined thin lightly stained bands. Some chains showed what appeared to be true branching. This interesting phenomenon was repeatable and deserves further attention. Nuclei were well demonstrated and consisted of central cores, peripheral granules and spherical forms (Fig. 140). Some chains showed very clear fairly thick bands at the dividing septa (Fig.141). On naked-eye examination, the growth was fluffy.

(11) Basal medium plus sodium nitrate

As for (1)

(12) Basal medium plus urea

As for (1)

(13) Basal medium plus all nitrogenous compounds

Long chains of organisms were formed and many of the chains showed branching. There were some individual separate organisms in parts of the preparation. There did not appear to be much nuclear material in the cells. That

which was present was masked to a certain extent by the intense staining of the cytoplasm.

(14) Nutrient broth

As for (13) except that the chains or organisms were longer. The results of growth in basal broth are summarized in table IV (p.235 of this thesis).

As previously noted (pp.223 - 225), after two hours' growth on agar, the staining of nuclear material was poor in many of the preparations (viz.nos. 1, 2, 3, 4, 6, 7, 8, 9, 11 and 12). The general appearance of most of these preparations was similar and led to the belief that little or no growth had taken place and it was organisms from the inoculum — an 18-hour meat-extract broth culture — that were being examined. When basal broth was used instead of basal agar, it was confirmed that a poor nuclear staining reaction on agar corresponded with absence of organisms in the preparations made from broth and with lack of visible growth in the broth after two hours' incubation.

Preparations 5, 10, 13 and 14 made from agar after 2 hours showed good staining reactions, although the nuclear material itself was not well demonstrated in preparations 5, 10, and 13.

In broth of similar composition after 2 hours' incubation, growth was observed in the corresponding 4 tubes ( 5, 10, 13 and 14) and organisms were demonstrated in smear

preparations made from these broth cultures. The organisms were generally in long chains and stained well, but in preparations 5, 13 and 14 the nuclear material was not so well demonstrated as it was in preparation 10.

It was noted that there were more long chains of organisms in preparation 10 than in 5 and that there was more growth in tube 10 than in tube 5, apart from the difference in nuclear demonstration described above. These facts are probably explained by the presence of glucose in tube 5, which would cause a fall in pH when it was used as a metabolite. Presumably the acid produced would be responsible for the observed differences between organisms grown in the presence and absence of glucose.

The quality of the nuclear staining reaction after 24 hours' growth on agar depended on the preparation studied. In preparations 3, 6, 7, and 8, internal structure was not visible in the few organisms that were present. Nuclear material was seen in some of the organisms in preparations 1, 2, 11 and 12. Growth on agar after this period was either poor or non-existent in these preparations, and there was no visible growth in broth apart from tube 3, in which there was moderate growth although a pellicle was not formed.

The most notable changes took place in preparations 4 and 9, in which the organisms were present mainly in long chains and in which the nuclear material was well demonstrated and showed a range from spherical shapes to the core type of structure.

These results corresponded with growth in fluid medium after 24 hours' incubation. Growth with pellicle formation took place in tubes 4 and 9. Growth on agar was poor for preparation 4 and moderate for preparation 9.

The interesting facts that emerge from these results are firstly that long chains of organisms were present in agar preparations 4 ( basal agar plus glucose and asparagine) and 9 ( basal agar plus asparagine) and that nuclear material was well demonstrated in these also. These appearances corresponded to growth with pellicle formation in basal broth, showing that the organisms were able to adapt themselves to use asparagine as a source of nitrogen and that glucose did not appear to be necessary because tube 9 gave as good results as tube 4. Even though growth on agar was poor or moderate, the observed change in the nuclear demonstration was associated both with growth on agar and good growth in the broth. This point I think is significant.

The second point of note is that preparation 3 from agar (basal medium plus glucose and ammonium-sulphate) did not show nuclear material or growth on agar but showed growth in broth although without pellicle formation. In this

preparation there was no change in the nuclear demonstration.

After 24 hours' incubation on agar many organisms were present on preparations 5, 10, 13 and 14. The nuclear material was well demonstrated in preparations 13 and 14 but not so well in preparations 5 and 10. Growth on agar was good for 10, 13 and 14, but only poor for 5, which again may be due to differences in pH. Growth in broth was good in all cases, with pellicle formation.

The conclusions that can be drawn from this experiment are that B.megatherium differs from B.cereus in its nutritional requirements and its metabolic activities. A study of nuclear structure may prove of value along with studies of the nutrition and growth of this and other organisms. The observed facts cannot yet be explained in terms of the effect of the presence or absence of a single chemical — perhaps they never will — but they again show clearly that the demonstrability of nuclear material in bacteria may be profoundly influenced by nutritional environment. The field is one which should be cultivated.

# T A B L E IV

## To show the amount and type of growth of Bacillus megatherium on basal agar medium and in basal broth medium

| Plate Number | Substances added (+) or not added (-) to the basal medium | | | | | | Growth on agar after | Growth in broth after | |
|---|---|---|---|---|---|---|---|---|---|
| | Glucose | Ammonium Sulphate | Asparagine | Peptone | Sodium Nitrate | Urea | 24 hours | 2 hours | 24 hours |
| 1 | - | - | - | - | - | - | + | - | - |
| 2 | + | - | - | - | - | - | + | - | - |
| 3 | + | + | - | - | - | - | - | - | ++ |
| 4 | + | - | + | - | - | - | + | - | ++P |
| 5 | + | - | - | + | - | - | + | + | +++P |
| 6 | + | - | - | - | + | - | - | - | - |
| 7 | + | - | - | - | - | + | - | - | - |
| 8 | - | + | - | - | - | - | - | - | - |
| 9 | - | - | + | - | - | - | ++ | - | ++P |
| 10 | - | - | - | + | - | - | +++ | ++ | +++P |
| 11 | - | - | - | - | + | - | + | - | - |
| 12 | - | - | - | - | - | + | + | - | - |
| 13 | - | + | + | + | + | + | +++ | ++ | +++P |
| 14 | Meat extract agar or broth | | | | | | +++ | +++ | +++P |

Good growth = +++
Moderate growth = ++
Poor growth = +
No apparent growth = -
Pellicle formed = P

Experiment 18 A study of the internal structure by the HCl-Giemsa technique of an avirulent strain of B.anthracis grown on two different types of media.

This experiment was devised primarily to see if X structures or similar characteristic structures could be observed in B.anthracis grown on basal agar plus glucose and urea. Organisms were grown on meat-extract agar for the sake of comparison and also to see the nuclear structure of this organism on a normal laboratory medium. Although an avirulent strain of B.anthracis was used thoughout this experiment, very careful sterile precautions were taken during the making and staining of impression preparations and in the disposal of cultures in case a virulent mutant should appear.

0.1 ml. of washed organisms from an 18-hour meat-extract broth culture was spread over the following plates; meat-extract agar, basal agar plus 1% glucose and 1% urea. Impression preparations were taken after 2, 5, 8 and 24 hours' incubation at 37° C and treated by the HCl-Giemsa technique.

Results of experiment 18

Meat-extract agar

(a) 2 hours' growth

The organisms were present in long chains.

Nuclear structures could be seen although they were not very clear. In general they were either spherical granules or dumbell-shaped, lying along the horizontal axis of the cell. Lightly-stained bands were observed between the cells of the chains. Figure 142 is a general photograph of the preparation.

(b) 5 hours' growth

The organisms were still present in chains. The nuclear structures were easier to see but had the same form as in the previous preparations: either spherical (Fig.143) or dumbell-shaped (Fig.144). Cross-walls were also well demonstrated (Fig.145).

(c) 8 hours' growth

The organisms were present in long chains. The nuclear structures were well demonstrated and were either spherical (Fig.146) or dumbell-shaped (Fig.147) as in the two last preparations. Cross-walls were well demonstrated (Fig.148).

(d) 24 hours' growth

The long chains of organisms were completely broken up and the preparation consisted almost entirely of spores, the majority free (Fig.149) but some still inside the vegetative mother cell (Fig.150). An occasional organism without a spore but with a nuclear core could be seen (Fig.151).

On meat-extract agar the nuclear material of B.anthracis which was best demonstrated after 8 hours' growth, consisted of spherical structures, usually one but sometimes two per cell. The nuclei were always small or dumbell-shaped bodies lying along the horizontal axis of the cell. The only conclusions that can be drawn are that the nucleus of this bacterium is normally spherical in shape and that there is only one nucleus per cell. This nucleus divides by constriction to form daughter nuclei which are small to begin with but grow in size until they are as big as the parent. One of these daughter nuclei may divide before a cell-wall has been laid down between them, as is seen in Fig.147. When grown on meat-extract agar, long chains of organisms are formed, but spore formation takes place with this strain after only 24 hours' incubation.

Basal agar plus glucose and urea

(a) 2 hours' growth

Long chains of organisms were present. Nuclear material was demonstrated but appeared to have a diffuse granular appearance and distribution (Fig.152); occasional large nuclear structures could be seen (Fig.153).

(b) 5 hours' growth

Long chains of organisms were present. The nuclear material in some chains appeared to be well

demonstrated, either as large spherical granules (Fig. 154) or as nuclear cores (Fig.155), but in other chains it was still in a diffuse granular form (Fig.156).

(c) 8 hours' growth

Short chains of organisms were present showing good nuclear differentiation. In the majority of organisms, the nuclear structures were dumbell-shaped (Fig.157), but in others a clear area could be seen in the centre of those dumbell shapes (Fig.158).

One organism had a large clear area with associated nuclear material that looked very like an X structure (Fig.159). Numerous free spores were present (Fig.160).

From these results it would appear that the nuclear structure on this medium is rather diffuse and granular or in the form of nuclear cores in the early stages of growth. After 8 hours' growth dumbell-shapes are seen, the nuclear material appearing simpler although clear areas resembling lipoid granules are also observed. A preparation was not made from the 24 hours' growth.

No typical X structures were observed in any of these preparations, but clear areas resembling lipoid granules were seen after 8 hours' incubation on basal agar plus urea and glucose. Neither were any other structures observed that could possibly be taken as being characteristic of B.anthracis.

On meat-extract agar the nuclear material appeared to be simple in form and division. At first young cultures on basal agar plus glucose and urea showed diffuse granular nuclear material but after eight hours the material was aggregated in dumbell-shapes or spherical structures. This early diffuse granular appearance may mark a period of adaptation to new cultural conditions.

Experiment 19 A study of an anthracoid bacillus by the tannic acid-crystal violet method.

An unknown anthracoid bacillus which produced spores very readily was examined. A 3-day growth on meat-extract agar at 30° C was composed to nearly 95% of spores. When a further plate of meat-extract agar was inoculated from the above culture and incubated for two hours at 30° C, germinating spores could be very clearly seen. Impression preparations were made and treated by the Tannic acid-crystal violet method. The empty spore case (Fig.161), the cell-walls (Fig.162), and cross-septa (Fig.163) of the developing organisms were clearly demonstrated by this method. Germination of the spore was equatorial and the remains of the spore case could be seen still clinging to some organisms, and there were many free spore cases.

This experiment is included to show the usefulness of this method of examining bacteria and to show by figures how clearly the individual cell may be delimited.

# D I S C U S S I O N

# D I S C U S S I O N

Of all the experiments that were carried out, those dealing with the effects of nutrition on the nuclear structure of B.cereus proved the most fruitful and interesting. (Experiments nos. 5 and 6). When this organism was grown on basal agar plus urea and glucose many of the bacilli contained peculiar structures which have not been previously described. The structures, which for the sake of convenience I have named "X structures", were composed of a clear circular area surrounded by a band of deeply staining nuclear material. As I have already mentioned, my first thought about these structures was that they were probably endospores developing inside the bacterial nucleus. My second thought was that the clear areas might merely be composed of lipoid material. The considerable number of these structures and their many different sizes seemed to support the first view. A further study of the development of these X structures revealed that they were in some way connected with the endospore. As the bacterial culture aged, the X structures, which had at first been small, grew in size until they reached a maximum; then they decreased in number. At the same time, mature

endospores, both free and still inside the vegetative cell, were seen for the first time. There appeared to be a definite relationship between these X structures and the bacterial endospore.

In an attempt to determine more exactly the nature of this relationship, terminal spore-bearing organisms of the Clostridium genus were studied. The results were not encouraging to the first view. X structures were not demonstrated, nor did there appear to be any relationship between the position of the nucleus and the site of formation of the endospore. These results tended to support the second view --namely, that these clear areas were not developing endospores but were more likely to be lipoid granules.

Application of appropriate staining techniques for lipoid and counts of X structures and lipoid granules confirmed that the numerical relationship was so close as to leave no doubt that the clear areas of X structures were indeed composed of lipoid material.

What probably happened was as follows. The organisms cultured under abnormal nutritional conditions formed large amounts of lipoid material as a result of their altered metabolism. This material aggregated together in the form of granules which grew in size as the culture aged. The

granules were generally formed centrally or paracentrally, the position normally taken up by the nucleus of the cell. These granules being lipoid and thus of high surface tension penetrated their way through the rather easily deformed nuclei to give these typical X structures. This argument is strengthened by the observation that the surrounding nuclear material was not always in a regular band, but was often horse-shoe shaped or composed of granules, and that other clear areas were observed which were not necessarily associated with nuclear material. The nuclear material and the lipoid granules probably have no connection with each other apart from the passive de-formation of the nucleus by the granules.

The possibility of passive deformation of bacterial nuclei is held by Delaporte (1950). It is mentioned also by Flewett (1948) who considered that appearances in B.anthracis "resembling chiasmata in the diakenesis stage of meiosis in higher plants" were possibly due to the development of vacuoles probably of lipoid material and that these appearances had no other significance. Neither of these authors had such clear evidence in support of their ideas as is now provided by an analysis of the composition of the X structures of B.cereus.

The results observed when B.cereus was grown on basal

agar plus asparagine (Experiment no. 14) must also be mentioned. On this medium I saw similar structures to those described by Flewett; for convenience I called them "cross-over" structures in my account of the experiment. I considered that this nuclear arrangement was probably due to the altered metabolism of the organisms and was not due to meiotic division. The fact that small clear areas very similar in appearance to lipoid granules were also observed in some organisms strengthened the view that altered nutrition produced lipoid inclusions and that these in turn caused the nuclear material to become arranged in this way.

Proving that there was no absolute connection between the nucleus and the lipoid material did not mean, however, that there was no connection between this lipoid material and the development of the bacterial endospore. I believe that this material is either included in the spore as a reserve storage material, or that it is used by the spore mother cell as a metabolite in the formation of the spore. These theories, which are not inherently improbable, — they could both be true — would account for the disappearance of lipoid material as spore-formation progressed. The work of Foster and Perry (1954) helps to confirm these views.

The alternative hypothesis is that the lipoid material is a definite part of the nucleus. Murray (1953), for example, considers that the bacterial nucleus is spherical with the nuclear material disposed at the periphery of the sphere. It may be that the middle of the sphere contains lipoid material among other substances. Dondero et al. (1954) think it not unlikely that lipoid may be associated with nuclear material.

The effect of nutrition on the nuclear structure of B.megatherium (Experiment no. 17) and A.cloacae (Experiment no. 4) was also studied. The first results with A.cloacae were very encouraging, giving rise to the speculation that the presence or absence of glucose in the medium was important in the demonstration of nuclear material. This was considered not improbable since it was known that the staining of nuclear material was influenced by changes in pH. These first results could not, however, be repeated under the conditions of subsequent experiments. In such work variables are undoubtedly numerous and still too poorly understood to be strictly standardized. This experiment showed, however, that changes in nutrition could affect the demonstrability of the nuclear material of A.cloacae and led up to more regularly repeatable observations with other organisms.

The experiment with B.megatherium under a variety of nutritional conditions was also of interest in that it showed how different this organism was from B.cereus. None of the structures (e.g. X structures) observed in B.cereus was demonstrated in B.megatherium. Differences could be expected since Knight and Proom (1950) showed that the two organisms have different nutritional requirements. Experiment 17 was of interest in that it showed that changes in the demonstrability of nuclei were associated with growth in broth and on agar. I think that this difference may be a very useful one and might be exploited with other organisms -- not inculture only but also in their natural environment.

Altering the nutrition has a definite effect, therefore, not only on the growth of certain representative organisms but also on their nuclear structure. I think that this has been shown in the work described in this thesis; and I believe that this method of tackling the problems of bacterial cytology has only to be systematically applied to yield other results which may well be as interesting as those recorded here about B.cereus in particular. Naturally it is only one of many possible approaches but so far it has been surprisingly little investigated.

One important fact has emerged from my own observations

and from those of other investigators on members of the Bacillus genus (B.cereus, B.megatherium, B.anthracis). In the early stages of growth, the structure of the nucleus is simple, usually spherical, and it divides in a simple amitotic way. It is only as the culture ages and by-products of metabolism accumulate, and endospore formation begins, that the nucleus appears to be a complicated structure.

As endospore formation does not begin in different species under the same conditions or even in the same species under different conditions at the same time, and as the metabolic processes of these species are not exactly similar, it is small wonder that there are few similarities in older cultures in the appearances of the nuclear structure even of organisms fairly closely related. This point, I think, has not been sufficiently emphasised.

Lipoid granules were observed by myself in cultures of B.cereus grown on basal agar plus glucose and urea, and were best demonstrated after about 24 hours' growth at 37°C. I also observed bright granules in B.anthracis grown on similar medium after 8 hours' growth at 37°C, which I took to be composed of lipoid material. Other workers have observed granules of the same appearance in other organisms under different conditions of growth. They were described

by Smith (1950) as "vacuoles" in B.megatherium grown for 2½--3 hours at 37°C on tryptone glucose yeast-extract agar — a very rich medium (Fig.170). They were also described in B.anthracis by Flowett (1948), who considered that they might be fat granules, when the organism was grown for 5 hours on "Lemco" agar at 37°C.

These observations are significant in that they show under what different conditions visible granules probably composed of lipoid material may be formed in the same organism and in different organisms. Knowing that these granules may cause a dis-arrangement of the nuclear material it is not surprising that little correlation has been found between the appearances of the nuclei of different members of the Bacillus genus.

When more is known about the bacterial nucleus, probably from studying another family, the facts about nuclear conformation may actually prove to be of use as a further means for classification of the Bacillus genus.

As already mentioned, I had studied the nuclear structure of Escherichia coli as an undergraduate. When I began the present series of experiments I decided to take my undergraduate observations as the starting-point. Unfortunately, or perhaps fortunately as things turned out, I worked with a culture labelled E.coli which proved to be Aerobacter cloacae. This wrong labelling was discovered when the

culture was found to be able to grow in citrate medium. But from this error one feature clearly emerged. In all the observations that I carried out on A.cloacae when I thought that it was E.coli, I was not struck by any differences in the appearance of the nuclear structure of this organism from what I expected of E.coli -- not even when I compared my own with the observations of other workers who studied E.coli or when I went back to my own earlier records of nuclear appearances in E.coli. This point, I think, is significant as showing that in these two organisms the nuclear structure -- which is simple and divides simply -- is basically similar.

The similarity is further emphasised by my own demonstration of dense bipolar bodies in A.cloacae by phase-contrast and electron microscopy, which are comparable with those demonstrated by Bringmann (1952) in E.coli by the same two methods and by the HCl-Giemsa technique.

From the review of the literature and from my own observations, it can be seen that different staining methods demonstrate the nuclear material in different ways. In the experiment with A.cloacae a different picture of the shape of the nucleus was given by the HCl-Giemsa method and the Quick differential method, the latter giving a spherical type

of structure, the former a dumbell shape. Differences in the shape of the nucleus can also be brought about by differences even in the time taken for osmic acid fixation as shown by Smith (1950). Differences in fixatives also give different nuclear appearances as shown by Murray (1953) and Guha et al. (1954).

Because of this and the effects of nutrition now demonstrated both on the demonstrability and the shape of the bacterial nucleus, any serious cytological study should clearly indicate the following: the strain of bacteria studied; the exact composition of the medium used; the full particulars of the inoculum; the exact conditions of growth; the exact details of the technique used to demonstrate nuclear material including the fixative used, the method for the removal of the cytoplasmic ribonucleic acid, and the stain employed. It is only in this way that adequate comparison of results can be made, and only by such comparisons that an idea of the structure of the bacterial nucleus can be built up.

The importance of examining bacteria by more than one technique has been stressed, I hope, by my own work. The need for such an approach may certainly be inferred from the results of other investigators, whether they show themselves aware of it or not. The HCl-Giemsa technique and the phase-contrast microscopy are very important in the study of the bacterial

nucleus. The HCl-Giemsa technique demonstrates the nuclear material in fixed dead specimens, but does not demonstrate lipoid or other inclusion granules. The phase-contrast microscope reveals the internal structure of living, untreated bacteria, and granules composed of lipoid material can be demonstrated by it.

When using different methods it is important not only to realise their usefulness, but also to keep in mind the special interpretation that is necessary. A probable first reaction when organisms of the Bacillus genus are examined under the phase-contrast microscope is to presume that the dark granules observed are nuclei. This, I think, is unlikely to be correct. The granules are far more likely to be lipoid granules, the nuclear material not being well demonstrated by this microscope. This became clear to me only because of my interest in the relation of lipoid to nuclear material and because I examined for both by a variety of methods. It is differences in refractive index — not in density nor in staining affinity — that the phase-contrast microscope reveals. This microscope is of great value, however, for the rapid and unequivocal demonstration of endospores, cross-walls and cross-septa.

The electron microscope has proved of limited use in the examination of whole bacteria. The variety of

observations recorded by various workers on the appearance of the nuclear material has depended, I think, on the preliminary treatment given to the organisms. In general and from my own observations the nuclear material of organisms like A.cloacae and E.coli would appear to be composed of dense material. This view is borne out by the appearance of the sections of E.coli cut by Birch-Andersen et al.(1953), which showed that the nuclear material formed dense, tightly wound threads. On the other hand the nuclear material of the members of the Bacillus genus was shown in sections by Chapman and Hillier (1953) to be of a low density, appearing light-coloured in the micrographs. These appearances depend, however, on the treatment given to the organisms especially on the fixative used. It is interesting to note this difference between these genera, and to speculate whether the genera containing Gram-negative organisms may be regarded as being simpler than the Bacillus genus, because the nuclear structure of these organisms appears to be less complicated in pattern and possibly of simpler composition.

The technique of cutting ultra-thin sections of bacteria is a very important one and I think that it will prove of great value in the elucidation of the true nature and function of the bacterial nucleus.

It is important for those who care about bacterial cytology not only to use more than one technique for the cytological study of bacteria, but also to increase the ratio of observed fact to ingenious speculation in the interpretation of the structures observed.

During the past 50 years many of the cytological studies of bacteria, especially those connected with the problem of the nucleus, have been made on members of the Bacillus genus.

These organisms are doubtless chosen because they are aerobic; easy to cultivate in the ordinary laboratory; do not require any special media for growth; are large bacilli; are easily photographed, and are almost all non-pathogenic. As a result of these studies various life cycles have been drawn up, (e.g. Bergersen's (1953) on B.megatherium). Many drawings and diagrams have been published claiming to show mitotic (e.g. DeLamater 1953) and even meiotic (e.g. Allen et al.1939) figures resembling those seen in the cells of higher plants and animals.

I consider, however, that the Bacillus genus has three main disadvantages which make it unsuitable for a study of the bacterial nucleus. First, these organisms are spore-formers. The formation, cytology, nuclear structure and exact function of the bacterial endospore

have not yet been fully elucidated. Consequently the formation of the spore greatly complicates the investigation of the nuclear structures of the vegetative cells. Since the spore does not begin development at the same time in different members of the Bacillus genus, or even in the same species under different conditions, it becomes rather difficult to determine if the nuclear arrangements observed are concerned with the vegetative cell or if they are due to the development of the spore.

In my opinion the second is probably the most important disadvantage of the Bacillus genus for cytological work, and is the one least recognised by a great many investigators. This is the formation of numerous inclusion granules during metebolic processes. Inclusion bodies composed of lipoid seem to be common in this group. Lipoid granules, because of their high surface tension, are generally spherical and it is difficult to alter their shape. Because of this they usually cause the alteration or rearrangement in shape and distribution of the other cytoplasmic contents of the bacterial cell, especially the nuclear material. In my opinion, many of the figures claimed to represent mitosis or meiosis are probably the result of this rearrangement — or, strictly speaking, disarrangement of the nuclear material by lipoid inclusion

bodies. Further complications arise when comparative studies of members of the Bacillus genus are made, because the conditions for the formation of visible lipoid granules differ from organism to organism even in the same media and times and temperatures of incubation.

The third disadvantage is that members of the Bacillus genus tend to form chains of organisms, and that the organisms themselves are often multicellular. Thus it is hard to determine the exact boundaries of one cell or "cellular unit". This leads to difficulty in interpretation of nuclear structures and nuclear division, for many of the techniques employed to demonstrate nuclear material in bacteria do not demonstrate the cross cell-walls and cross-septa which are often delicate. The failure of a particular technique to do this has again and again led to the description of mitotic division in what appears to be a large organism. More accurate examination by appropriate methods would probably reveal that the "large organism" was composed of several cells. If the cross-walls were demonstrated, each cell would then appear to have a simple nucleus dividing in a simple way.

Because of the multicellularity of many bacteria, especially in the Bacillus group, which is stressed many

times by both Robinow and Bisset, I consider that the enzyme lysozyme can play an important part in the study of the bacterial nucleus. This enzyme is thought to break down by depolymerization a mucopolysaccharide in the cell-wall and capsule of B. megatherium and probably of other similar organisms, releasing the individual cells of a chain of organisms. This appeared to me to offer a more satisfactory way than any other of demonstrating the nuclear material and its behaviour in individual cells. The usual procedure is to rely upon a technique to demonstrate nuclear material which will also show up cross-walls, or to use a combination of methods to demonstrate nuclei and cross-walls.

The less treatment the organisms are subjected to, the less is the risk of distortion. It would seem useful, therefore, first to separate the individual cells by an enzyme such as lysozyme and then to demonstrate their nuclear structure. In this way there would be no doubt at all about the mode of nuclear division. Although the experiment that I carried out on this problem was not successful, I remain convinced of the usefulness of this approach and intend to reopen it.

One of the advantages which the members of the Bacillus genus have over non-sporing organisms in connection with bacterial cytology is simply that they

produce spores. As Knaysi (1948) pointed out, the spore "constitutes the only reproducible cyclostage in bacteria". The full meaning of this rather simple statement may at first seem rather obscure, but its value was pointed out to me by Robinow (personal communication). The state of the nuclear material in the bacterial endospore during its resting stage and at the beginning of germination is — for any one genus or species — the stage that is the most likely to be the same for all members no matter how and under what conditions the spore was formed. The spore therefore forms ideal material for the beginning of an investigation of the nucleus. It has the added advantage that it is comparatively easy to obtain a culture composed entirely of endospores, so that when they germinate a great many of the vegetative organisms would be at the same stage of growth. The disadvantage of using a five-hour or more especially an eighteen-hour culture of organisms for investigation is that the organisms are in all different stages of growth and nuclear division. This makes it hard to relate the many different pictures of the nucleus to any particular stage of development.

Various other types of microorganism have been studied besides the Bacillus genus. These include the Gram-positive cocci and Gram-negative bacilli especially

those of the Enterobacteriaceae. Members of this group are smaller than those of the Bacillus genus and neither form endospores nor produce inclusion bodies in anything like the same profusion as the members of the Bacillus genus; moreover they are generally not multicellular. Because of these three reasons I think that they form excellent material for the study of the bacterial nucleus. The conclusions of an extensive study would probably help to elucidate the nuclear structure of other genera, including the Bacillus genus. To my mind it is significant that arrangements thought to resemble mitosis have not been observed in the Gram-negative bacilli. In young cultures of A.cloacae the nuclear structures were simple and they were simple also in older cultures; conclusions quite unlike those usually reached with members of the Bacillus genus. This was also observed by other workers for other members of the Enterobacteriaceae — for example Bringmann (1952).

Such conclusions are of interest as they can lead to hypothesis on the evolutionary status of the Enterobacteriaceae and the Bacillaceae. Are the Enterobacteriaceae simple organisms from which the family of the Bacillaceae has evolved? Endospore production is regarded by many workers as denoting a higher form of

bacteria. Is this supposition strengthened by the observation that the metabolism of spore-formers appears to be more complex and that the beginning of cell-differentiation is marked by the formation of definite storage granules? I think that it is!

Again, the Gram-positive cocci are smaller than the members of the Bacillus genus, but although mitotic figures have been reported in the cocci I think that they have been satisfactorily explained by the presence of cross-septa which were not demonstrated clearly enough by the technique employed to reveal the nuclear structures. Apart from their small size, these cocci are often multi-cellular and are difficult to examine under the electron microscope; this makes them not an ideal group for a basic cytological study.

It will be seen quite clearly from the review of the literature and from my own observations that although many nuclear structures have been described, they were found in many different organisms studied by many different methods. This is an important point and too much stress cannot be laid upon the necessity for all bacterial cytologists to state exactly the type of organism studied, the cultural conditions, and the method of examination. Variation even in fixation with osmic acid has been shown by Smith (1950) to cause differences in structure. It

should also be stressed that bacteria should be examined by more than one method to obtain a true picture of the internal structure of the cell.

Apart from these important points, the views of the various investigators resolve themselves into two groups. Those of one group believe that there is a mitotic and perhaps even meiotic division in the bacterial nucleus. Some have claimed to have seen these figures, others only accept the assumption that they should be there. Those of the other group believe that bacteria have a simple nucleus with a simple form of division. But even in this group there are differences about the exact nature and structure of this simple nucleus.

That the bacterial cell has a type of nucleus I have never doubted, but from my own research and with a knowledge of the controversial literature already published, I have quite naturally formed my own opinions about the state and the behaviour of the nuclear material found in bacteria. My own views are now summarised in what follows.

The bacterial nucleus is essentially a simple structure which either consists of or contains hereditable factors, either as genes comparable with those of higher plants or as modified types of genes, characteristic of bacteria and other lower forms of life. These hereditable factors, which are linked, are present in many sets, so

that if some of the nuclear material is damaged there will still be a linked set of factors left to be passed on to the next generation. Or, as in spore formation, only part of the nuclear material is included in the endospore, the other part presumably being released into the surrounding environment. In passing, would it be, perhaps, too incredible to suggest that there might be a connection with this discarded nuclear material and bacteriophage! This is discussed later (p.265 of this thesis).

The division of this nuclear material, when the cell is dividing to form two daughter cells, is also simple. The material simply constricts, generally in the centre, to form two daughter nuclei which are equal in size, one daughter nucleus passing into each of the daughter cells. The division of the nuclear material, however, may not be even, and one daughter nucleus may be much larger than the other. But as the sets of linked hereditable factors are duplicated many times, this does not bring about any differences in the characters of the daughter cells. In other words, the division of the nucleus is amitotic. I do not think that the nuclear material is normally aggregated into chromosomes, or that there is a process of mitosis or meiosis in the bacterial cell. As I have already pointed out the figures that have been interpreted

as mitosis and meiosis are more likely to be the result of distortion or aggregation of the nuclear material due to the staining techniques used or of inclusion bodies causing the disarrangement and de-formation of the nuclear material. The nuclear material is, I think, very easily damaged and split up by inclusion bodies of higher resistance, such as lipoid granules. This leads to the idea that the nuclear material is not surrounded by a nuclear membrane, and so far no real proof of the existence of this membrane has been found. The nuclear material is probably located in the centre of the bacterial cell, possibly as a spherical structure but without a definite binding membrane. Or it may be that the nuclear material is contained in a vacuole surrounded by a delicate vacuolar membrane. There is some evidence from the ultra-thin sections of Birch-Andersen et al. (1953) that this may be so. If it were true, it would help to explain many results and observations. For instance, why the nuclear material is so easily broken up, why a definite nuclear membrane has not been successfully demonstrated, and why so many different nuclear configurations have been seen. Some at least of the many different descriptions are probably due to the way in which the material was fixed treated and stained.

During normal growth of the bacterial cell, the

nuclear material, what ever its exact physical state may be, divides in a normal amitotic way, and it is only when inclusion bodies or spores are formed in certain organisms that it becomes distorted.

The question now arises whether this aggregation of nuclear material can be called a nucleus. Should another term be devised for it or could it be called a simple nucleus? I think that it could, for there are no definite criteria by which a nucleus may be recognised morphologically. It is more by their reactions to certain reagents and their behaviour that nuclei are recognised.

That bacteria should be regarded as simple organisms is generally recognised; one would expect therefore that the organisation of their nuclear material would also be simple. As organisms become more complicated, with differentiation of parts for such various processes as reproduction, nutrition, and so forth, it would also be expected that the nuclear material should become more complicated.

That I consider the bacterial cell to have a simple nucleus does not mean that I do not think it is not possible to have an occasional fusion of nuclear material; for wherever there is hereditable material, I believe that re-organisation of it can take place. If this were not

so there would be no evolution. But I do not consider that there are male and female bacteria; and I think that the introduction of the term "sex" into discussions of bacteriology was a grave mistake. But this rather thorny subject is one that I prefer not to handle until further investigations are carried out, especially on the endospores of the genera Bacillus and Clostridium.

To summarise my views on the bacterial nucleus I should say that it is normally a simple spherical structure, not bounded by a definite nuclear membrane, but containing numerous sets of linked hereditable characters and dividing in a simple amitotic fashion. It is only due to distortion by staining techniques and disarrangement by inclusion bodies that the nuclear material sometimes appears to be complicated, occasionally acquiring even a distribution similar to the figures seen during mitosis in the cells of higher plants and animals.

That bacteriophage has a definite effect on the nuclear material of bacteria cannot now be doubted. Luria and Human (1950) have suggested that "granular chromatin" may possibly be phage nucleoprotein. This line of thinking is of importance. It had often struck me how remarkable it was that during lysis of the bacterial cell nuclear bodies were never observed. These suppositions about bacteriophage may help to account for this and I think that

this hypothesis is worth considering and investigating further.

Up to now, little use has been made of the demonstration of the state of the bacterial nucleus in other fields of bacterial research. The cytological study of the bacterial cell and of the bacterial nucleus has so far been purely academic. This phase has indeed yielded plenty of interest but it is tending to become static, repetitive, and merely argumentative with some exponents. I believe that the use of cytological methods as a tool in other branches of bacteriology might do much both for cytology itself and for bacteriology in general.

To my knowledge there are at least four important applications of bacterial cytological techniques. The first has already been mentioned in connection with the experiments on nutrition, but it is enlarged here. Under certain special conditions of cultivation and treatment, characteristic reproducible X structures were observed in B.cereus. If equally characteristic structures could be demonstrated in pathogenic organisms of importance in bacteriological warfare such as B.anthracis by similar conditions and techniques, this would greatly aid in making or confirming the identification of these organisms. This earliest knowledge of the type of organism used by an

enemy would be of immense help in devising or putting into practice measures to combat the spread of the disease. Work by Pearce and Powell (1951) on a nutrient medium containing lysozyme and haematin which permits the free growth of B.anthracis while suppressing more than 95% of other Bacillus spp. in soil is of interest here. It may prove possible to link up these findings with cytological techniques for the quick identification of B.anthracis.

I sincerely hope that bacteriological warfare will never become a reality, but I think that if there is the threat of its possibility, as much preparation as possible for dealing with it should be made now and the potential contribution of cytology should not be ignored.

The second application is the study of mixed populations of bacteria from natural habitats, such as soil, silage, and the intestinal tract of animal and man. Bacteriologists are interested not only in the type of organisms from these habitats but also in the state of division and growth of these organisms. The questions arise in particular circumstances: Are these organisms in a resting state and not likely to be concerned at this period with metabolic or other processes? Are these organisms actively dividing and growing and consequently more likely to be the cause of some particular effect that

is being produced? Those questions are of special importance in a consideration of the varied effects following a disturbance of the intestinal tract of man.

One condition in particular, in which I think that it is important to know what type of organism or organisms are actively growing is in the investigation of the cause of diarrhoea after partial gastrectomy. According to Howie et al.(1953), this condition may have some connection with abundant growths of Cl.welchii in the gastric remnant. The state of the bacterial nucleus, whether recognisably in the resting or actively dividing state, would possibly act as a very good indicator of the state of the bacterial cells of a particular species.

There are and there no doubt will be many difficulties in the demonstration of the bacterial nucleus by cytological techniques applied to mixed bacterial populations in natural habitats. I do not consider this idea impossible — nor does Dr. K.A.Bisset with whom I discussed this possible application.

A new technique devised by Alexander and Jackson (1954) for cutting thin sections of soil samples in which the microorganisms are preserved in their natural relationships to one another will doubtless prove of value in this application of cytological techniques.

The third important application is concerned with

the nutrition of bacteria. As my experiments have shown, differences in nutrition of an organism lead to differences in nuclear structure and arrangement. This may be due to one of two factors: (a) altered metabolism of the cell leads to a definite intrinsic change in the nuclear structure; or (b) the change in metabolism leads either to the production of different by-products, or to differences in the amounts of normal by-products, and these differences may lead to the passive de-forming of nuclear material. It may be possible that both these factors operate together.

These facts are of interest, but is it possible to turn them round? That is, can we use the appearance of the nuclear material to detect and determine changes caused by nutritional differences in the same species, and to study differences in response between different species to similar nutritional environments. It has already been pointed out that visible lipoid granules are produced by different organisms under different conditions of growth. Facts like these make me consider that this application would be of value not only in studying different metabolic processes of members of the same genus, but also in the elucidation of natural relationships within the group, which would also help in the classification of bacteria.

The fourth application is really an offshoot of

the above. There is great interest in the reaction of bacteria to antibiotics. Properly understood, this is a study of responses to change in the nutritional environment of the organism. For example, what cytological changes are produced in staphylococci during the action of penicillin upon these organisms? And are the effects the same or different with a range of doses? The answers to these questions may be both interesting and important for Saz and Eagle (1953) have shown that within a very limited range of optimum dosage penicillin may kill both sensitive and resistant staphylococci — that is, staphylococci unaffected by relatively large doses of penicillin. Is it possible to correlate particular cytological effects with response — or lack of it — to particular doses of penicillin and with resistance or susceptibility on the part of the organisms?

By including among their inquiries problems of such a kind as those discussed here bacterial cytologists might find themselves advancing in directions hitherto unthought of — which might well be a good thing not only for their fascinating speciality but for themselves.

# C O N C L U S I O N S

# C O N C L U S I O N S

This thesis embodies a critical examination of the question whether bacterial cells have "nuclei". The evidence shows that the bacterial cell undoubtedly possesses chromatinic material aggregated to form a type of nucleus. The chromatinic material divides in a simple amitotic way. By more than one method of examination the chromatinic material and its mode of division has been clearly demonstrated and photographed in organisms from a young actively growing culture. The demonstration of this material by more than one method of examination, including the study of living untreated organisms, is important as a means of refuting the suggestion that bacterial nuclei are only staining artefacts.

At later stages of growth, changes in the metabolism of the cell and the constitution of the cytoplasm lead to formation of inclusion bodies. These bodies are not necessarily stained by methods for demonstrating nuclear material, but their formation may lead to an alteration in the previously simple shape of the nuclear material, often splitting it up into numerous separate fragments. The pattern that these fragments take up depends on the size of the inclusion bodies and the way in which they have

developed. At times this rearrangement of nuclear material resembles the mitotic and even meiotic figures seen during the various stages of division of plant and animal cells. Among bacteria, however, these patterns do not appear regularly or consistently. Such patterns, however striking, should therefore be disregarded as evidence for the possession by the bacterial cell of a complex nucleus with numerous chromosomes. Attempts to interpret the appearances in this light have greatly confused the literature on bacterial cytology; their weakness is often apparent from the irregularity with which the striking pattern is observed in a particular culture of bacteria.

In work of this kind members of the Bacillus genus have been most fully studied. The illustrations of this thesis confirm that these organisms form such inclusion bodies and show consequent changes in the pattern of their nuclear material. The work also shows how morphological changes due to endospore formation may complicate interpretation. Various attempts to gain some control over the production of particular nuclear patterns led to the demonstration that when bacilli were grown under abnormal nutritional conditions the formation of inclusion bodies was intensified. In particular, when B.cereus was grown on basal agar with glucose and urea, very large lipoid granules were formed.

These granules appear to push their way through the nuclear material, which is generally central, and give it the appearance of a ring type of nuclear structure.

Close study of the experimental material strongly suggests that there may be some connection between the formation and growth of these lipoid granules, the position and shape of the nuclear material, and the formation of the endospore. This observation, which does something to correlate an interesting morphological appearance with controllable influences in the environment, encourages the idea that an experimental approach to bacterial cytology may be capable of development. This could usefully lead to more objective assessments than have hitherto been either usual or possible.

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The University of Glasgow

# Methods and Applications of Bacterial Cytology

A critical examination by cytological techniques of the nuclear structures in bacteria

by

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University Assistant in Bacteriology

Thesis submitted for the Degree of Ph.D. in the Faculty of Science

Volume II

Department of Science Bacteriology May 1955

Volume II

C O N T E N T S



NOTE

In some of the photographs within more than one significant appearance is shown. In such cases, for convenience of reference between text and figures, each significant appearance is given a figure number to itself.

Experiment 1. Rhizobium (Clover Strain) Figs. 1 - 4

FIG. 1

FIG. 2

FIG. 3

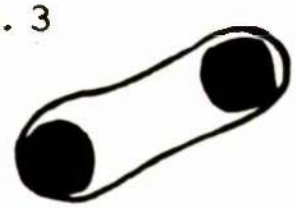


FIG. 4

**Figs. 1 & 2** Organisms showing linked nuclear bipolar granules

**Fig. 3** Organism showing separate nuclear bipolar granules

**Fig. 4** Organism showing three large separate nuclear granules

Organisms grown on yeast-extract-mannitol agar at 25°C for 3 weeks.

Stained by the HCl-Giemsa technique. (Drawn by hand)

x 10,00(approx.)

Experiment 1. Rhizobium (Clover strain) Figs. 5 - 8

FIG. 5

FIG. 6

FIG. 7

FIG. 8

**Figs. 5 & 6** Organisms showing unstained areas associated with a large mass of nuclear material

**Fig. 7** Organism showing unstained bipolar granules in nuclear material

**Fig. 8** Cocco-bacillary type of organism showing distribution of nuclear material

**Organisms grown on yeast-extract-mannitol agar at 25°C for 3 weeks.**

**Stained by the HCl-Giemsa technique.** **(Drawn by hand)**

x 10,000(approx.)

Experiment 2. Aerobacter cloacae Fig. 9

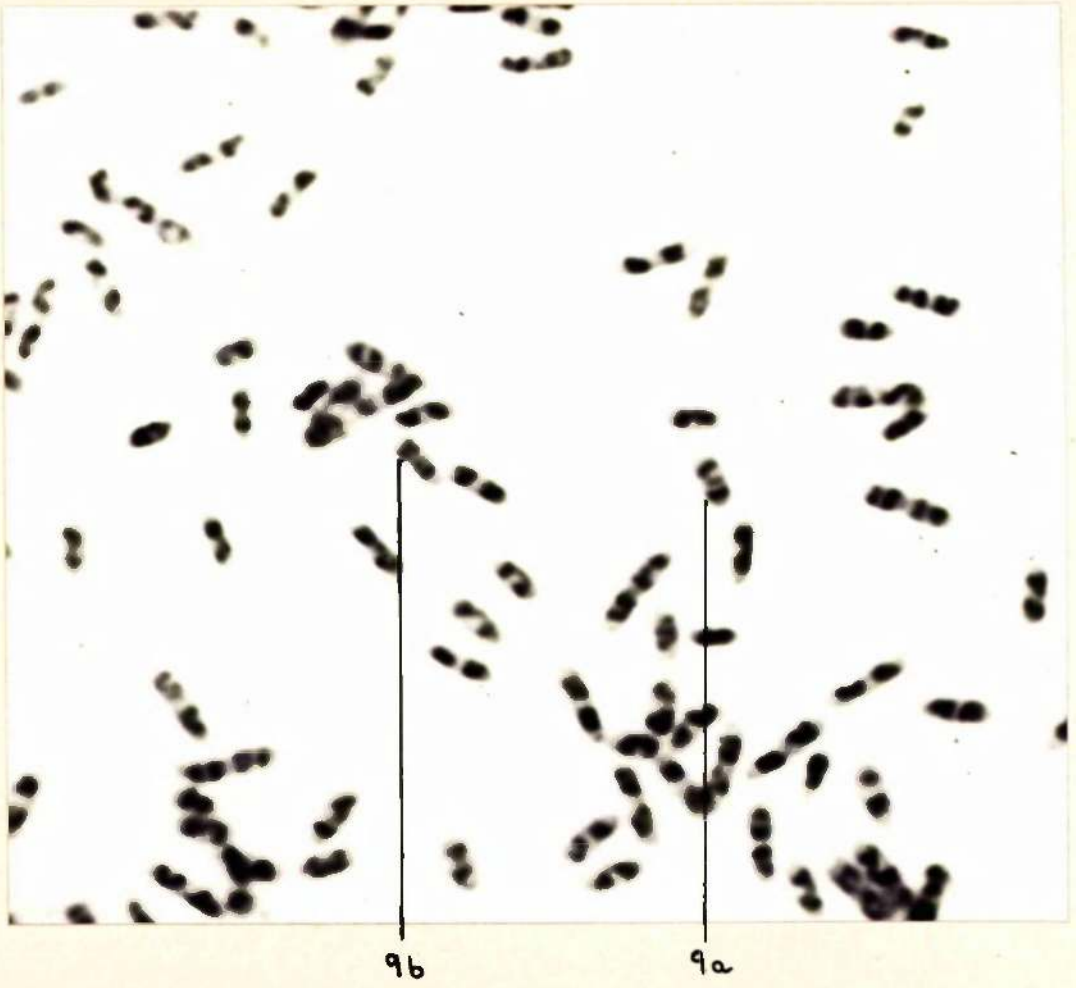



Fig. 9 Photograph showing organisms with dumbell-(a) and double-dumbell-(b) shaped nuclear structures

Organisms grown on meat-extract agar at 37°C for 2 hours.

Stained by the HCl-Giemsa technique.

x 3000

Experiment 2. Aerobacter cloacae Figs. 10 - 11

FIG. 10

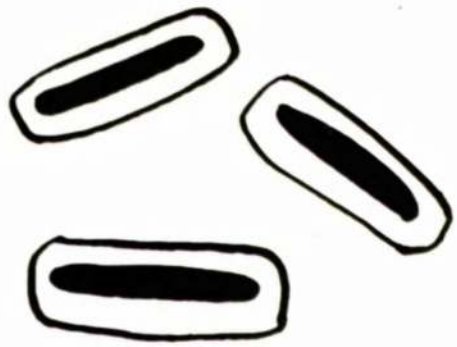


FIG. 11

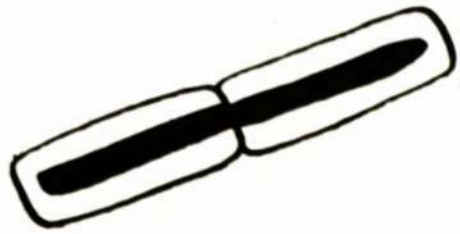


Fig. 10 Organisms showing core of nuclear material

Fig. 11 Organisms showing attachment of nuclear cores

Organisms grown on meat-extract agar at 37°C for 2 hours.

Stained by the HCl-Giemsa technique. (Drawn by hand)

x 15,000(approx.)

Experiment 3. Aerobacter cloacae Figs. 12 - 13

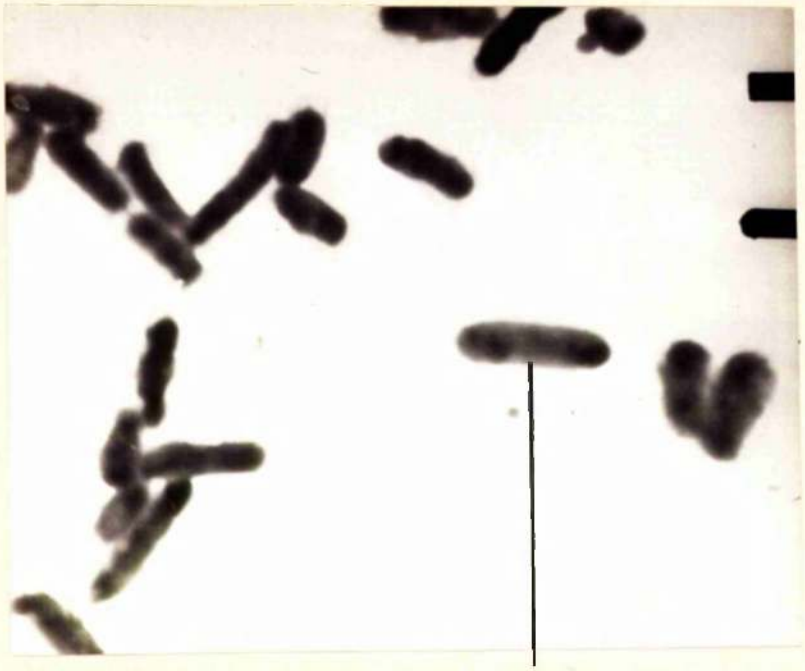



Fig. 12 Photograph to show majority of organisms opaque to the electron beam, but a few showing granular internal appearance

Fig. 13 Organism showing two definite small granules and two less definite larger granules

Organisms grown in citrate broth, at 37°C for 3 days, washed with and suspended in sterile distilled water, treated with osmic acid.

Examined by the electron microscope.

x 8000

Experiment 3. Aerobacter cloacae Fig. 14

**Fig. 14 Large organism showing very granular internal structure**

**Organisms grown in citrate broth, at 37°C for 3 days, washed with and suspended in sterile distilled water, treated with osmic acid.**

**Examined by the electron microscope.**

x 8000

Experiment 3. Aerobacter cloacae Fig. 15

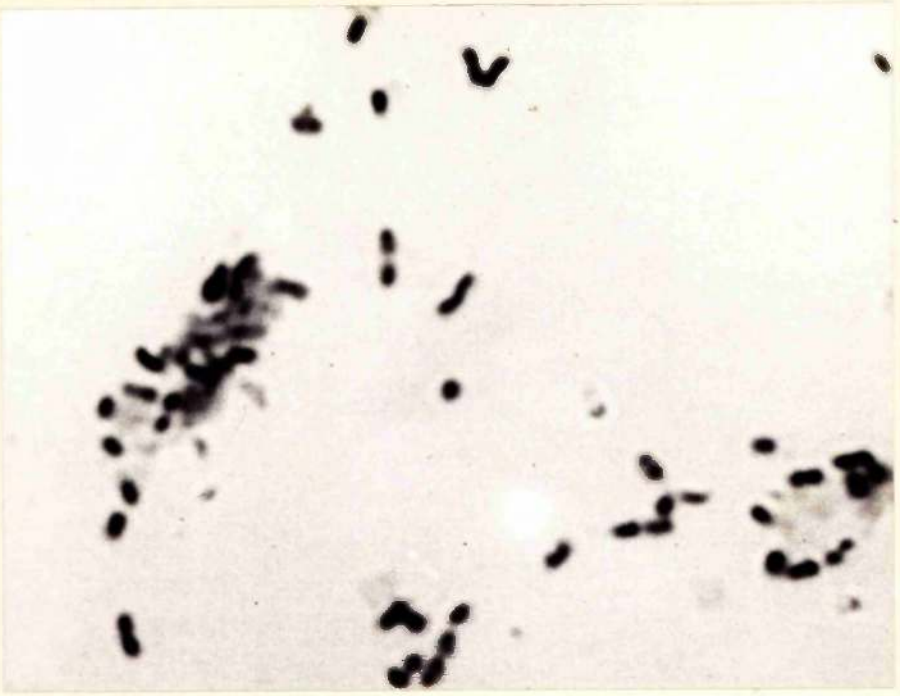


**Fig. 15 Photograph to show organisms with bipolar bodies**

**Organisms grown in citrate broth at 37°C for 3 days.**

**Stained by the HCl-Giemsa technique**

x 1500

Experiment 3. Aerobacter cloacae Fig. 16

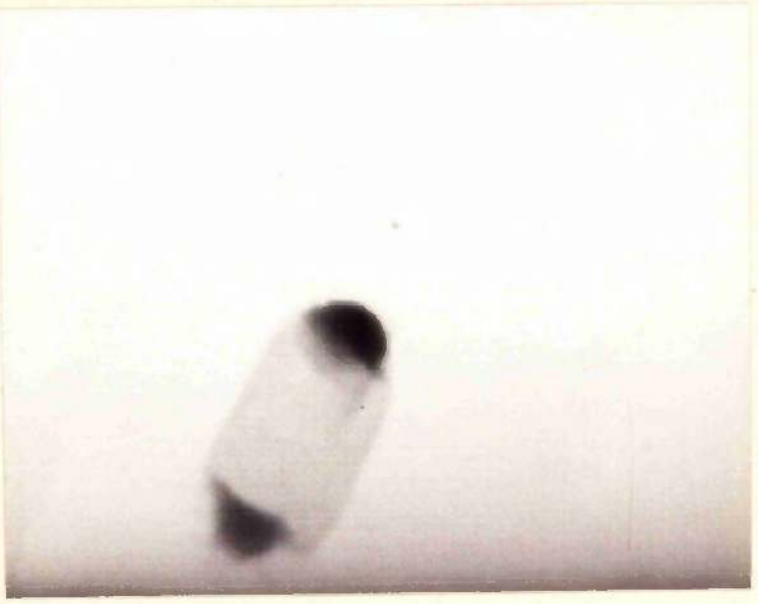


**Fig. 16 Organism with dense bipolar bodies**

**Organisms grown in sterile distilled water plus a little ammonia, at room temperature for 3 weeks, washed with and suspended in distilled water, treated with osmic acid.**

**Examined by the electron microscope.**

x 15,000

Experiment 3. Aerobacter cloacae Fig. 17

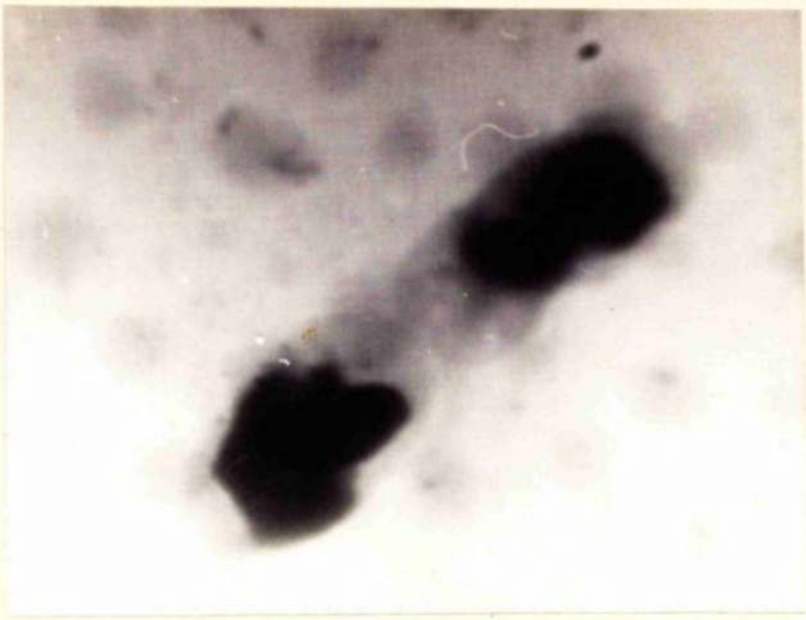


**Fig. 17 Organism with dense bipolar bodies**

**Organism grown in sterile distilled water plus a little ammonia, at room temperature for 3 weeks, washed with an suspended in distilled water, treated with osmic acid.**

**Examined by the electron microscope.**

x 15,000

Experiment 5. Bacillus cereus Figs. 18 - 21

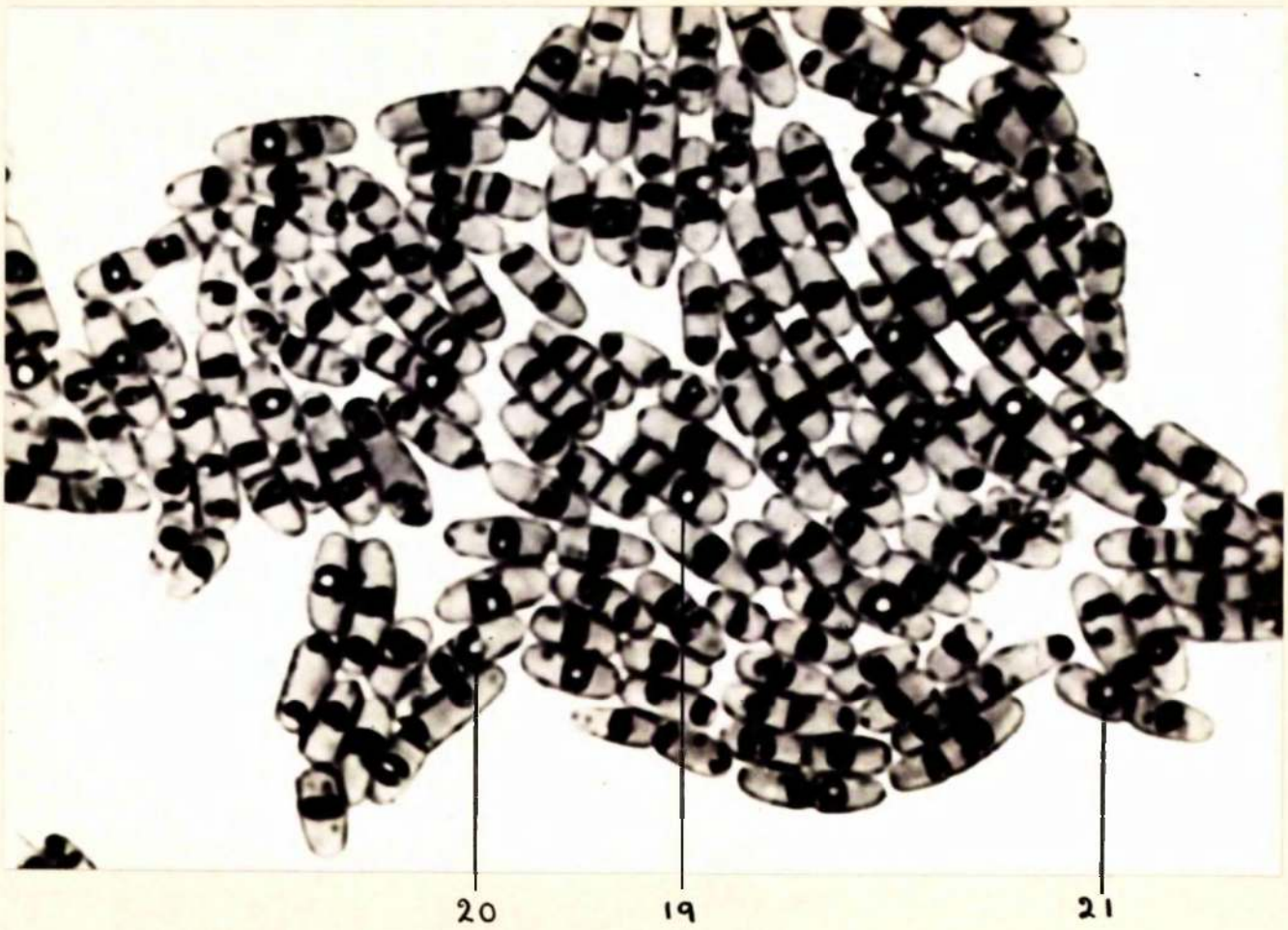



Fig. 18 Photograph to show organisms with well-demonstrated nuclear material and cross-walls

Fig. 19 Organism showing bright central area surrounded by band of nuclear material

Fig. 20 Organism showing broken band of nuclear material

Fig. 21 Organism showing nuclear band composed of granules

Organisms grown on basal agar plus 1% glucose and 1% urea at 30°C for 24 hours.

Stained by HCl-Giemsa technique.

x 4500

Experiment 5. Bacillus cereus Fig. 22

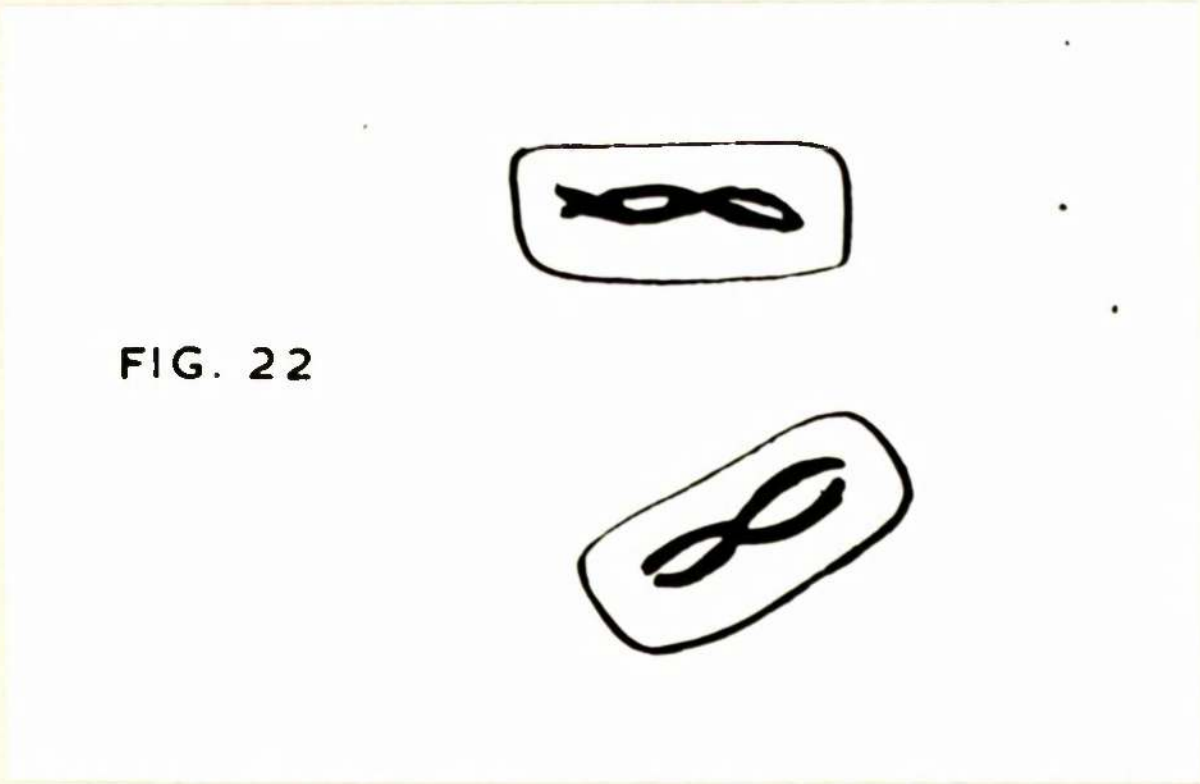



**Fig. 22 Organisms showing "cross-over" type of structure**

**Organisms grown on basal agar plus 1% asparagine at 30°C for 24 hours.**

**Stained by HCl-Giemsa technique. (Drawn by hand)**

x 15,000(approx.)

Experiment 6. Bacillus cereus Figs. 23 - 27

FIG. 23

FIG. 24

FIG. 25

FIG. 26

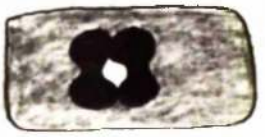


FIG. 27

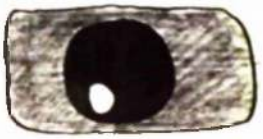


**Fig. 23** X structure with unbroken circular band of nuclear material with small central area

**Fig. 24** X structure with unbroken circular band of nuclear material with large central area

**Fig. 25** X structure with horse-shoe (broken) band of nuclear material

**Fig. 26** X structure with nuclear material in the shape of a double-dumbell

**Fig. 27** X structure with eccentrically placed clear area

**Organisms grown on basal agar plus 1% asparagine and 1% glucose at 37°C for 24 hours.**

**Stained by HCl-Giemsa technique.** **(Drawn by hand)**

x 13,000(approx.)

Experiment 6. Bacillus cereus Figs. 28 - 32

FIG. 28

FIG. 31

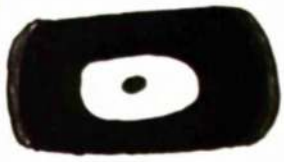


FIG. 29

FIG. 32

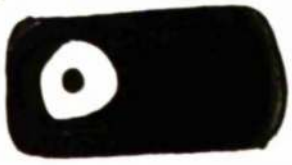


FIG. 30

**Fig. 28** Spore with eccentrically placed nucleus

**Fig. 29** Organism containing a "blue" structure

**Fig. 30** Organism containing a "blue" structure lying crosswise to the cell

**Fig. 31** Organism containing a Y structure with deep staining central granule

**Fig. 32** Organism containing a Y structure with the deep staining granule in a paracentral position

Organisms grown on basal agar plus 1% asparagine and 1% glucose at 37°C for 24 hours.

Stained by HCl-Giemsa technique. (Drawn by hand)

xI3,000(approx.)

Experiment 6. Bacillus cereus Figs. 33 - 37

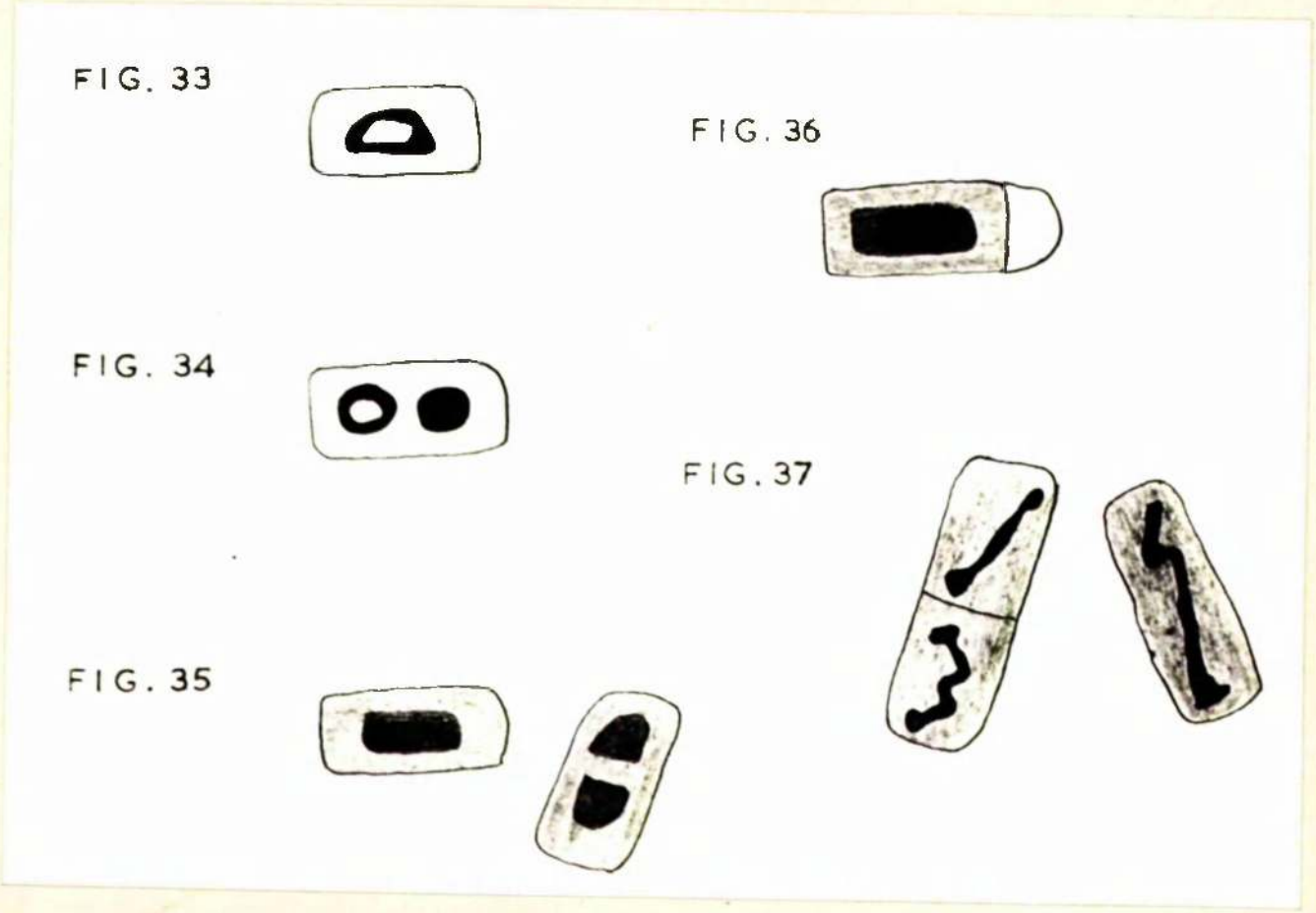



**Fig. 33** Organism containing spore with stained outside, but clear unstained central area

**Fig. 34** Organism containing normally stained spore and also the type of spore seen in Fig.33

**Fig. 35** Organisms showing large solid nuclear structures

**Fig. 36** Organism showing large solid nuclear structure, with the end of the cell cut off and having no cytoplasmic contents

**Fig. 37** Organisms showing nuclear cores of various shapes

Organisms grown on basal agar plus 1% glucose and 1% urea at 30°C,
Figs. 33 & 34 for 25 hours, stained by Fleming's spore method
Figs. 35 & 36 for 34½ hours, stained by HCl-Giemsa technique
Fig. 37 for 47½ hours, stained by HCl-Giemsa technique. (Drawn by hand)

x 10,000(approx.)

Experiment 7. Clostridium tetanomorphum Figs. 38 - 44

FIG. 38

FIG. 39

FIG. 40

FIG. 41

FIG. 42

FIG. 43

FIG. 44

**Fig. 38** Large spore showing two definite areas

**Fig. 39** Large spore showing slight point

**Figs. 40 - 44** Slender organisms containing a nucleus or nuclei in varying numbers and in varying positions

Organisms grown anaerobically on meat-extract agar at 37°C for 3 days.

Stained by HCl-Giemsa technique. (Drawn by hand)

x 10,000(approx.)

Experiment 7. Clostridium sphenoides Figs. 45 - 46

FIG. 45

FIG. 46

**Fig. 45** Spore showing two definite areas

**Fig. 46** Developing spore with red dots on either side of it

**Organisms grown anaerobically on meat-extract agar at 37°C for 3 days.**

**Stained by HCl-Giemsa technique.** **(Drawn by hand)**

x 10,000(approx.)

Experiment 8. Bacillus cereus Figs. 47 - 49

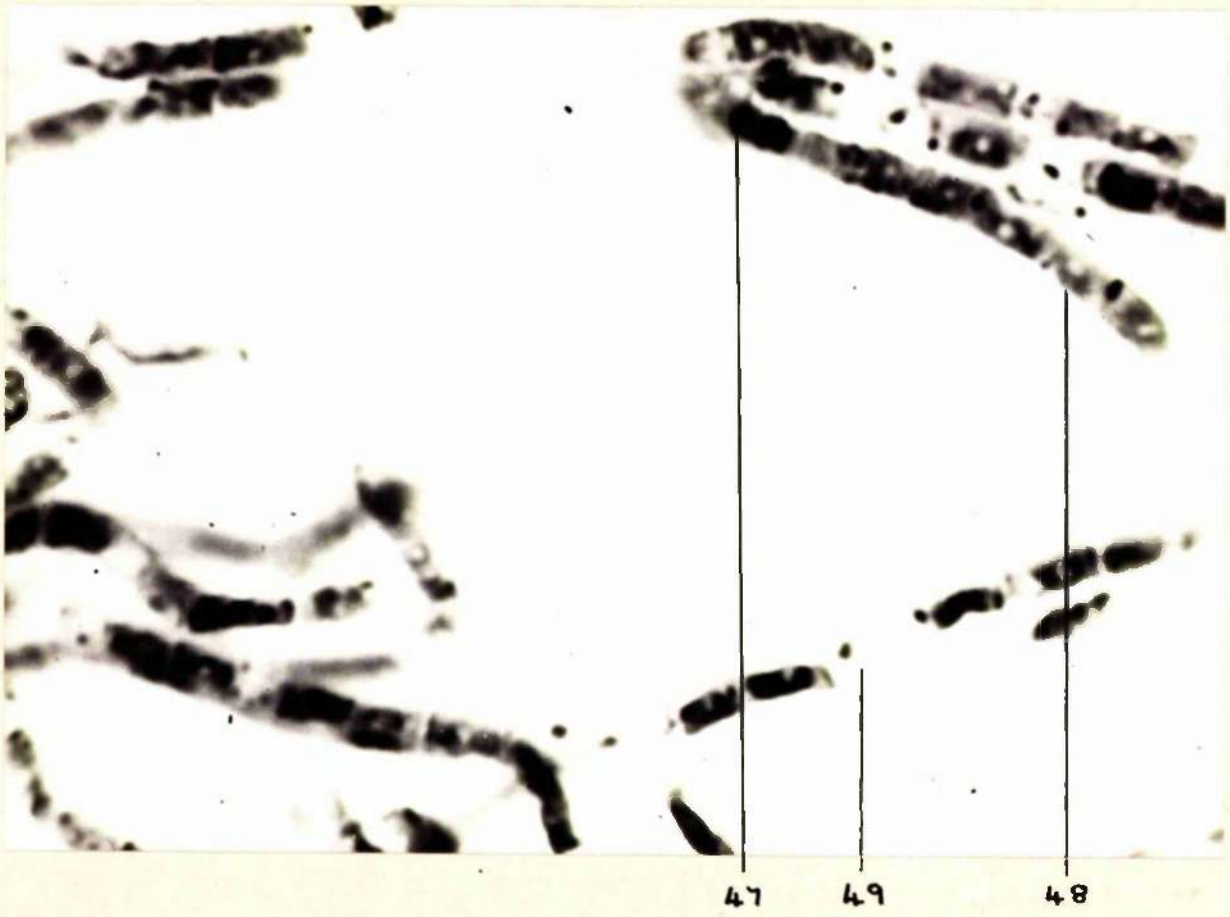



**Fig. 47** Organisms with densely packed nuclear material

**Fig. 48** Organism containing a mass of lightly-stained nuclear material with a clear area in it

**Fig. 49** Organism with only a small deeply-stained granule

Organisms grown on basal agar plus 1% glucose and 1% urea at 37°C for 66 hours.

Stained by the HCl-Giemsa technique.

x 4000

Experiment 8. Bacillus cereus Fig. 50


**Fig. 50 "Dancing body"**

**Organisms grown on basal agar plus 1% glucose and 1% urea at 37°C for 66 hours.**

**Stained by the HCl-Giemsa technique.**

x 3500

Experiment 9. Bacillus cereus Figs. 51 - 56

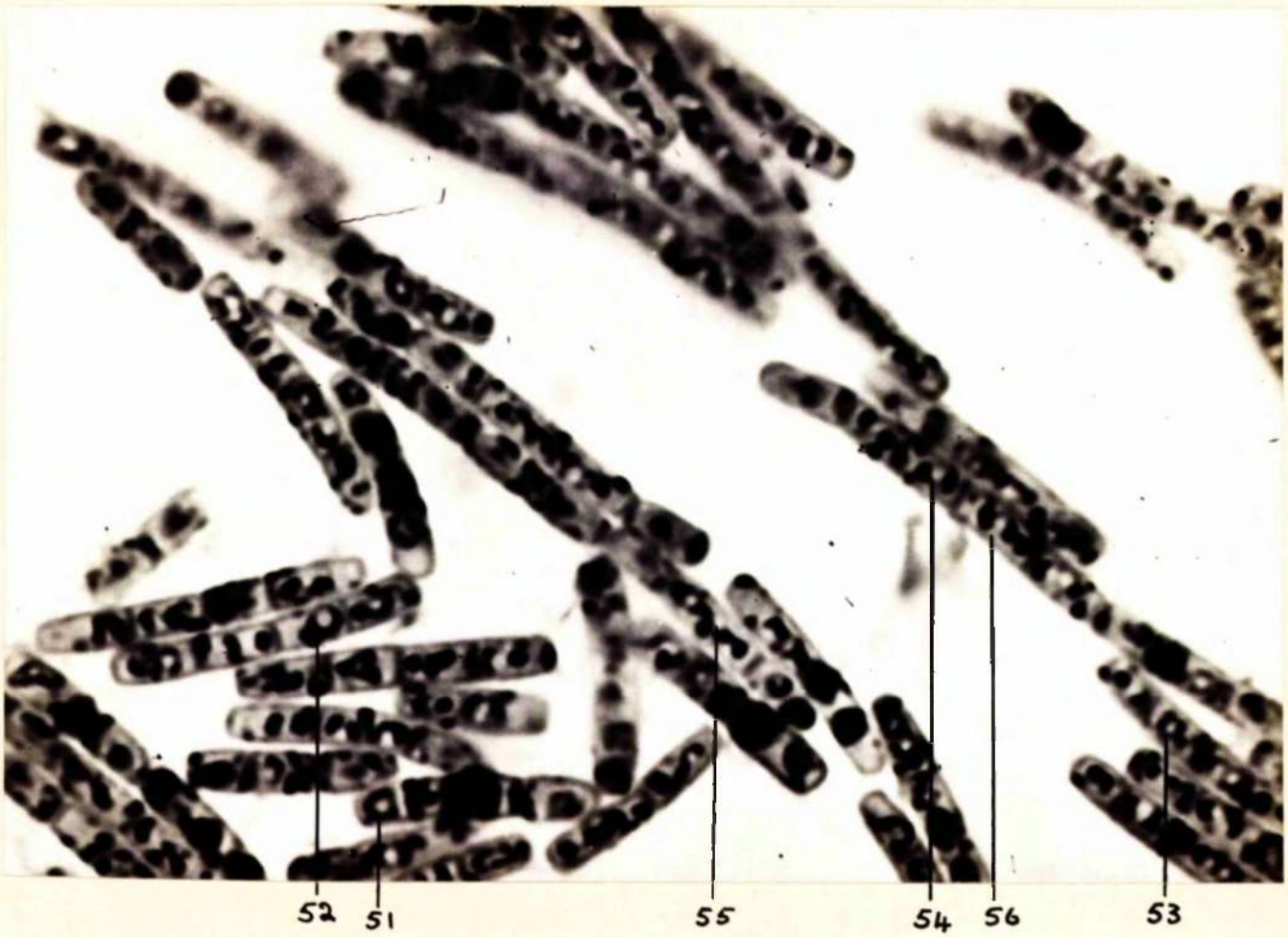



Fig. 51 X structures with complete band of nuclear material

Fig. 52 X structures with incomplete band of nuclear material

Fig. 53 X structures with granules of nuclear material

Fig. 54 X structures with granules of nuclear material opposite one another

Figs. 55 & 56 Organisms where clear unstained areas are associated with nuclear material

Organisms grown on basal agar plus 1% glucose and 1% urea at 37°C for 24 hours.

Stained by HCl-Giemsa technique.

x 4500

Experiment 9. Bacillus cereus Figs. 57 - 60

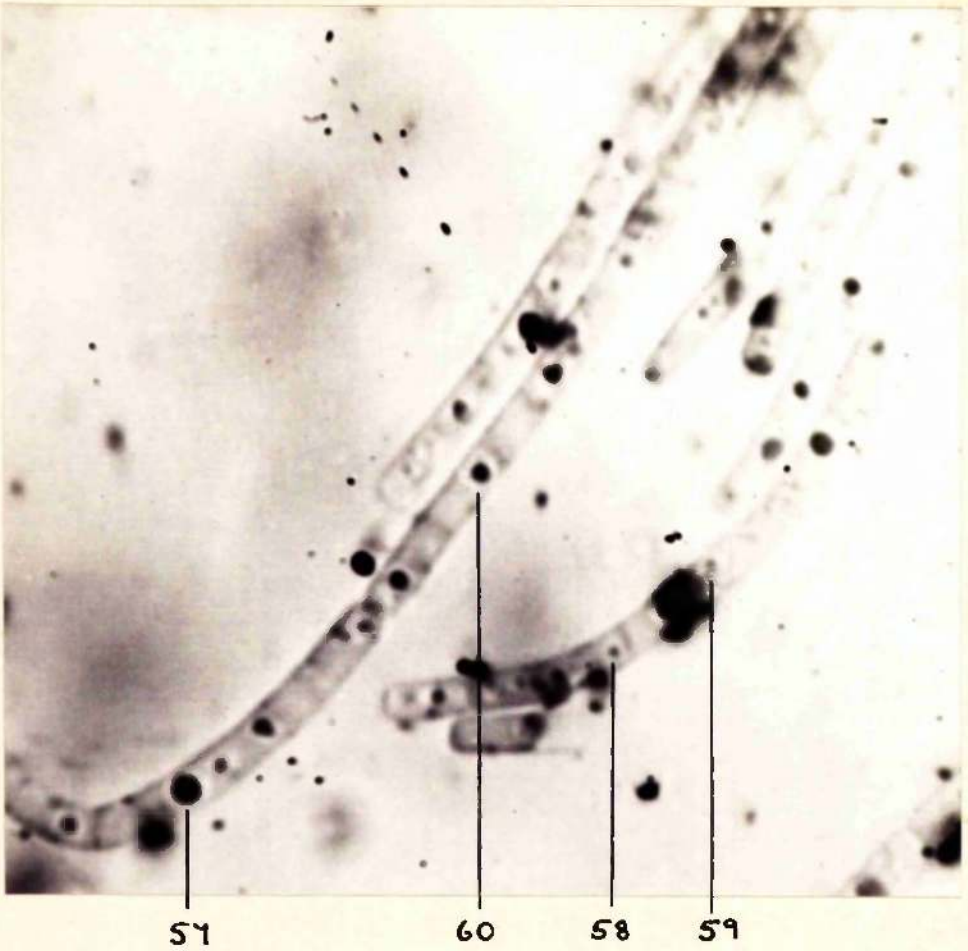



Fig. 57 Very large lipoid granules

Fig. 58 Small lipoid granule

Fig. 59 Group of small lipoid granules

Fig. 60 Lipoid granule completely surrounded by lighter area

Organisms grown on basal agar plus 1% glucose and 1% urea at 37°C for 24 hours.

Stained by Sudan black for the demonstration of lipoid granules.

x 4500

Experiment 9. Bacillus cereus Fig. 61

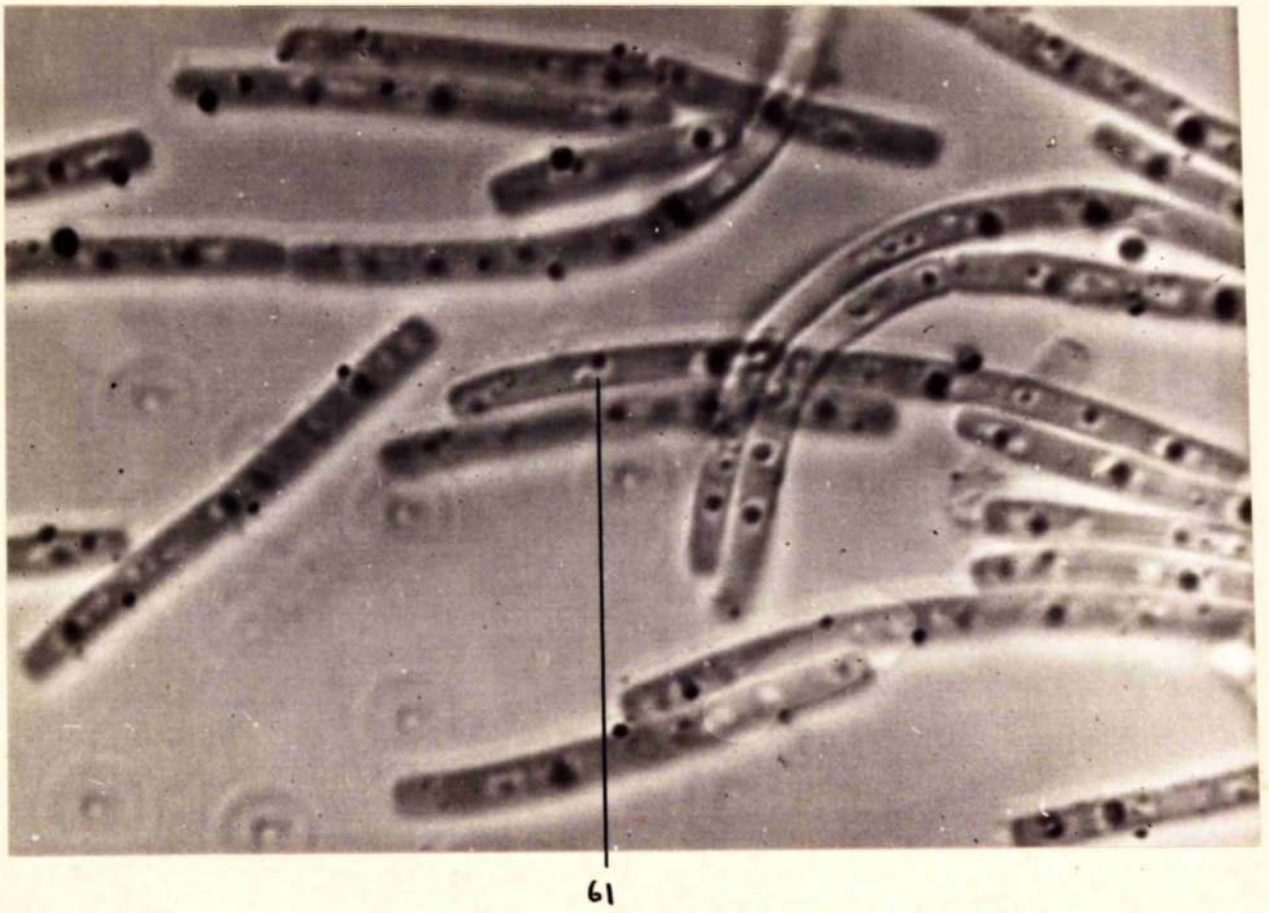



**Fig. 61 Lipoid granule not completely surrounded by lighter area**

**Organisms grown on basal agar plus 1% glucose and 1% urea at 37°C for 24 hours.**

**Stained by Sudan IV for the demonstration of lipoid granules.**

x 4500

Experiment 9. Bacillus cereus Fig. 62

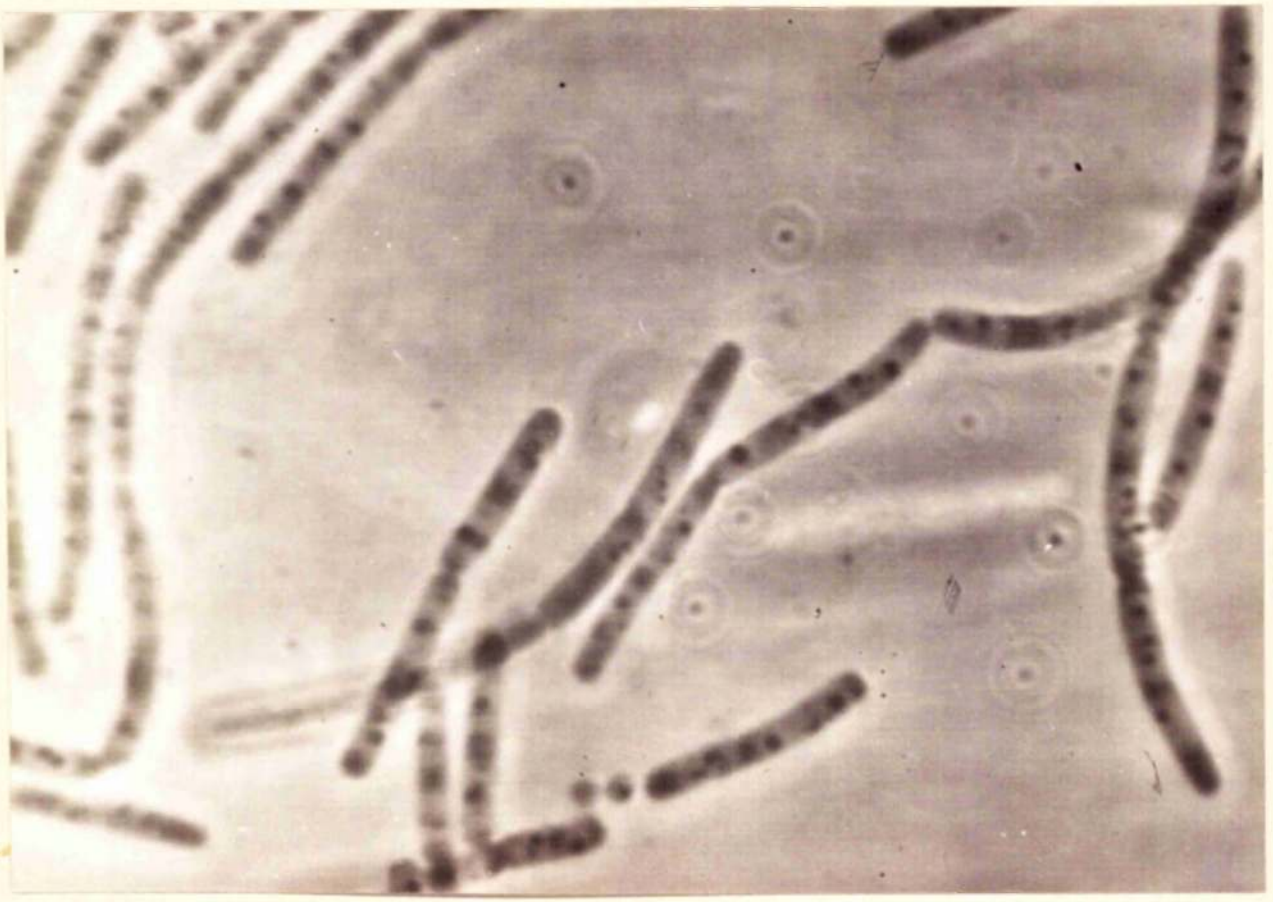


**Fig. 62 Organisms with dark granules associated with lighter material**

**Organisms grown on basal agar plus 1% glucose and 1% urea at 37°C for 24 hours.**

**Examined by the phase-contrast microscope.**

x 4500

Experiment 9. Bacillus cereus Figs. 63 - 66

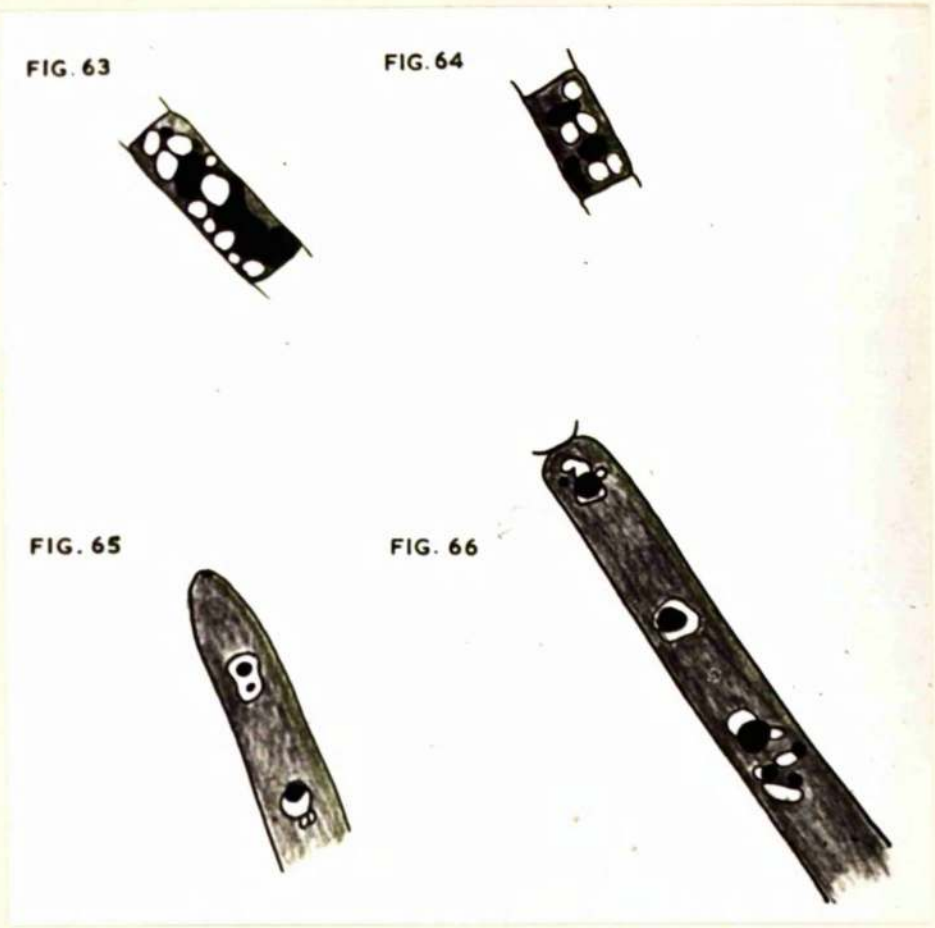



Fig. 63 Drawing of Fig.55 to show the way in which the nuclear material is distorted by the lipoid granules

Fig. 64 Drawing of Fig.56 to show the same as above

Fig. 65 Drawing to show position of lipoid granules in relation to the clear areas (this includes a drawing of Fig.58)

Fig. 66 Drawing to show the same as above (this includes drawings of Figs.59 & 60)

Organisms grown on basal agar plus 1% glucose and 1% urea at 37°C for 24 hours.

Stained by the HCl-Giemsa technique. (Drawn by hand)

x 10,000(approx.)

Experiment 12. Bacillus cereus Figs. 67 - 69

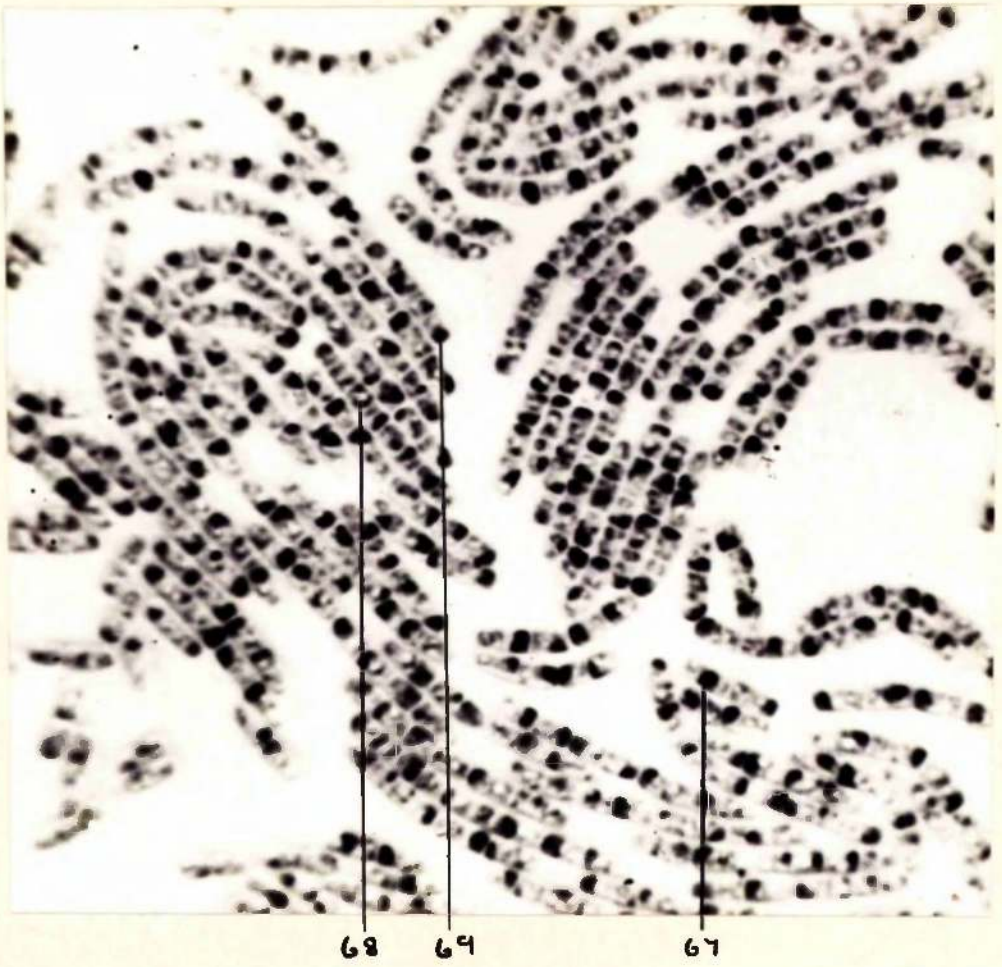



**Fig. 67** Organism containing a deeply stained nuclear body

**Fig. 68** Organism containing an X structure

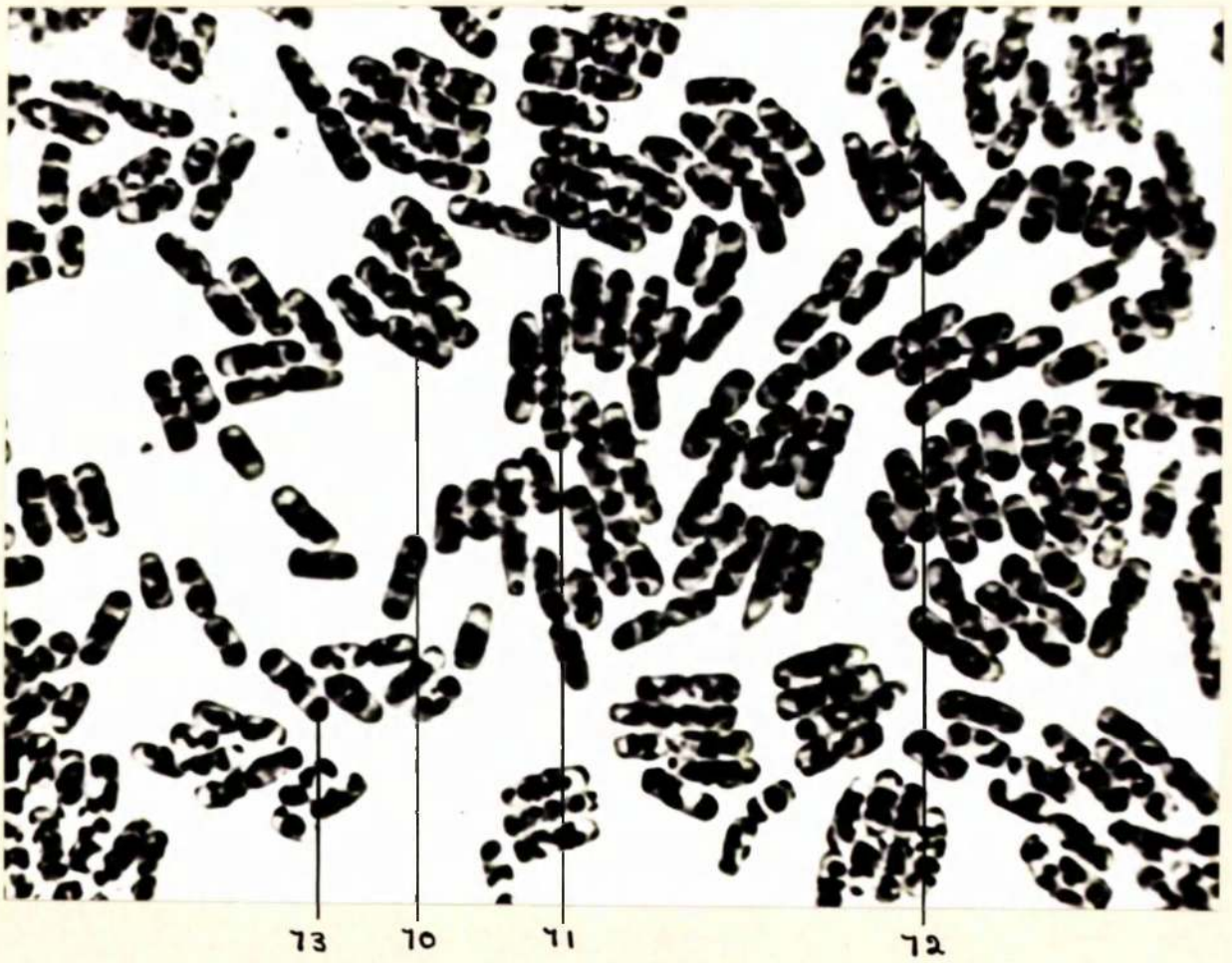
**Fig. 69** Organism with well stained cap-like material

Organisms grown on basal agar plus 1% glucose and 1% urea at 37°C for 26 hours.

Stained by the Quick-differential method.

x 2500

Experiment 12. Bacillus cereus Figs. 70 - 73




**Figs. 70 & 71** Organisms with well-defined X structures

**Fig. 72** Organism with irregular nuclear structure

**Fig. 73** Organism with well-stained cap-like material

**Organisms grown on basal agar plus 1% glucose and 1% urea at 37°C for 26 hours.**

**Stained by HCl-Giemsa technique.**

x 3000

Experiment 12. Bacillus cereus Figs. 74 - 75

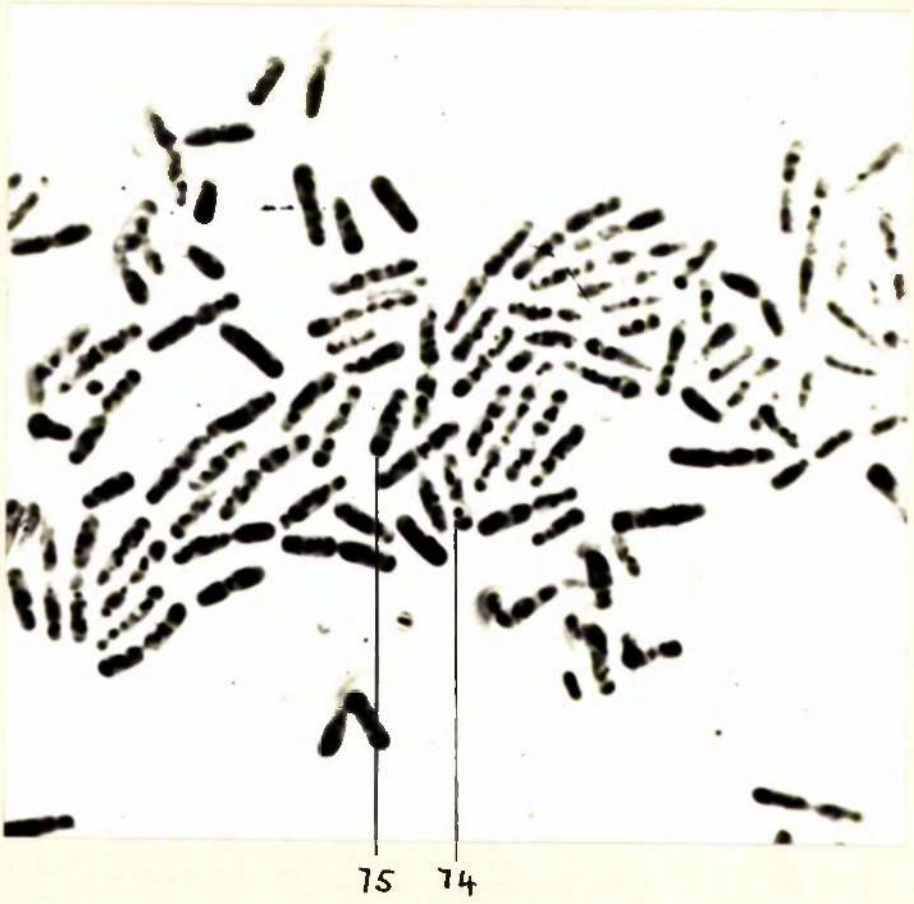



Fig. 74 Organism containing structure composed of dark central round area, surrounded by lighter clear area

Fig. 75 Organism with darker cap-like material

Organisms grown on basal agar plus 1% glucose and 1% urea at 37°C for 26 hours.

Examined by the phase-contrast microscope.

x 2000

Experiment 13. Bacillus cereus Figs. 76 - 77


**Fig. 76** Photograph to show general appearance of organisms and nuclear material

**Fig. 77** Well-stained spore still inside the vegetative cell

Organisms grown on meat-extract agar at 37°C for 24 hours.

Stained by HCl-Giemsa technique.

x 4000

Experiment 13. Bacillus cereus Figs. 78 - 80

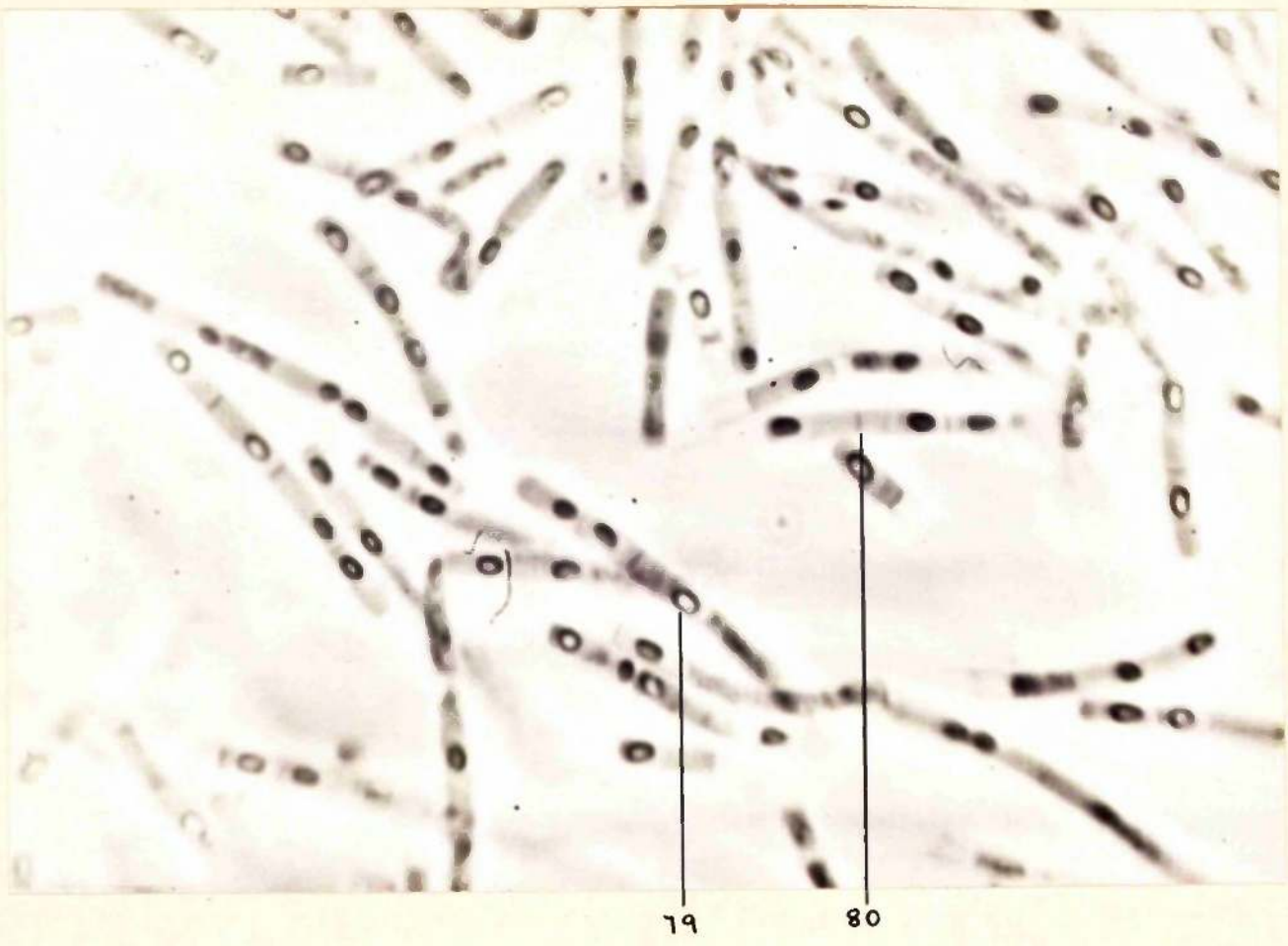



**Fig. 78** Photograph to show general appearance of organisms

**Fig. 79** Well-demonstrated spore

**Fig. 80** Well-demonstrated cross-walls

Organisms grown on meat-extract agar at 37°C for 24 hours.

Examined by the phase-contrast microscope.

x 3000

Experiment 13. Bacillus cereus Figs. 81 - 84

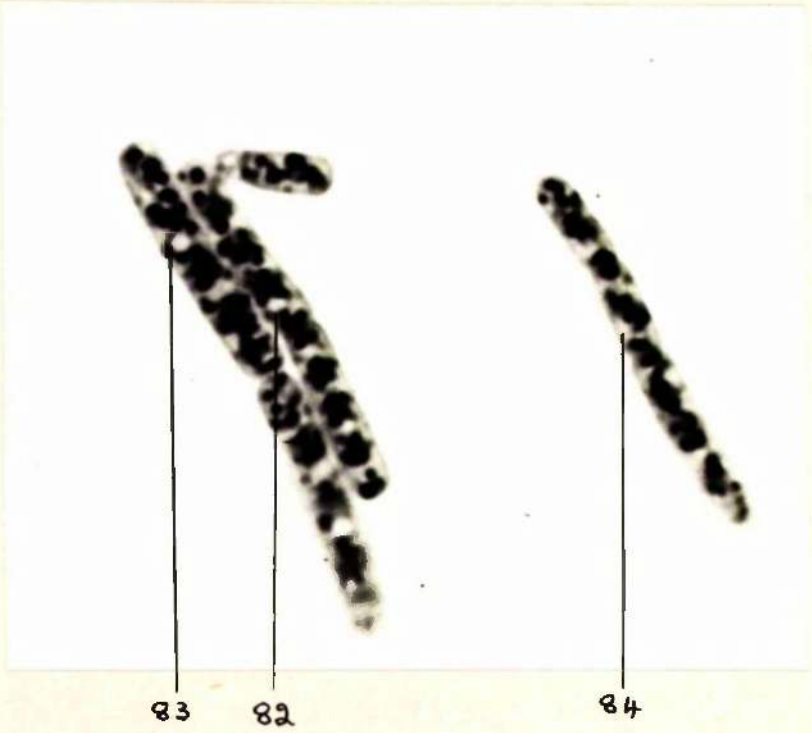



**Fig. 81** Photograph to show general appearance of organisms

**Fig. 82** Organism containing an X structure

**Fig. 83** Band of nuclear material of X structure uneven in shape

**Fig. 84** Clear areas associated with nuclear material

**Organisms grown on basal agar plus 1% glucose and 1% urea at 37°C for 24 hours.**

**Stained by the HCl-Giemsa technique.**

x 4500

Experiment 13. Bacillus cereus Figs.85 - 87

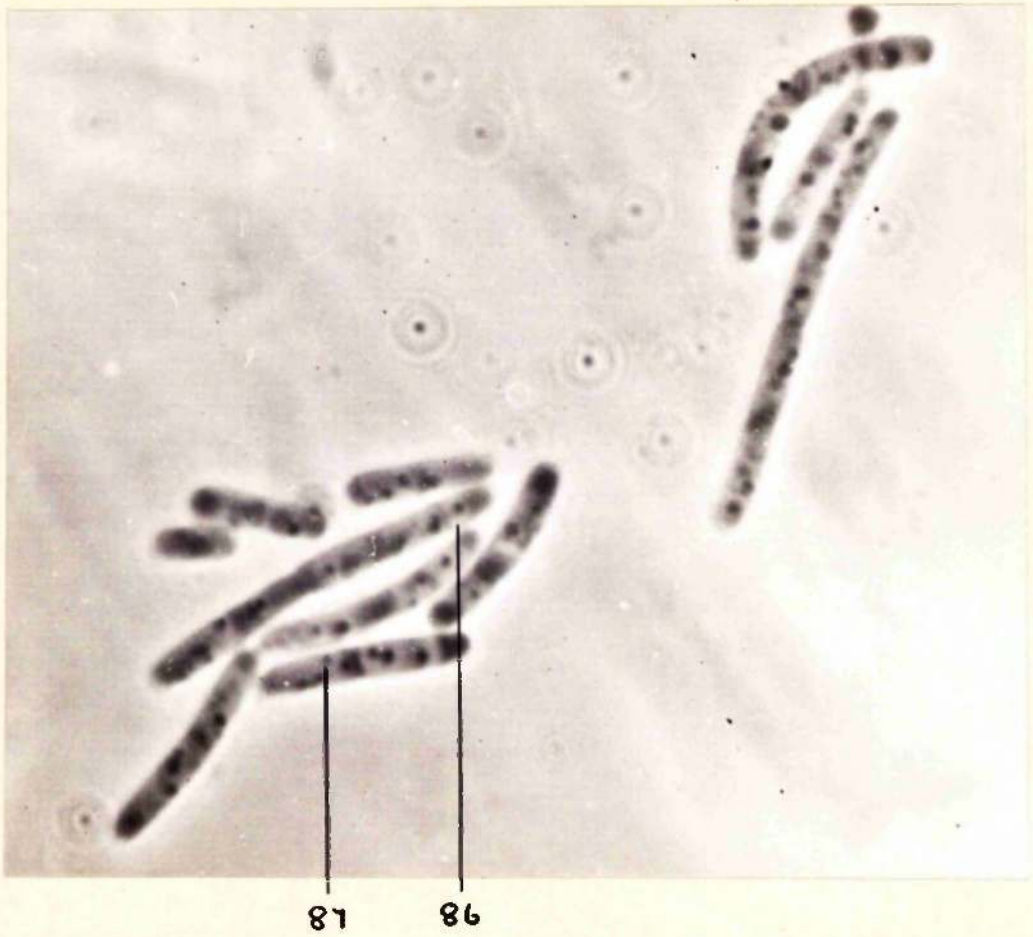



Fig. 85 Photograph to show general appearance of organisms

Fig. 86 Dark granule surrounded by "nuclear" material

Fig. 87 Dark granule associated with "nuclear" material

Organisms grown on basal agar plus 1% glucose and 1% urea at 37°C for 24 hours.

Examined by the phase-contrast microscope.

x 3000

Experiment 13. Bacillus cereus Figs. 88 - 92

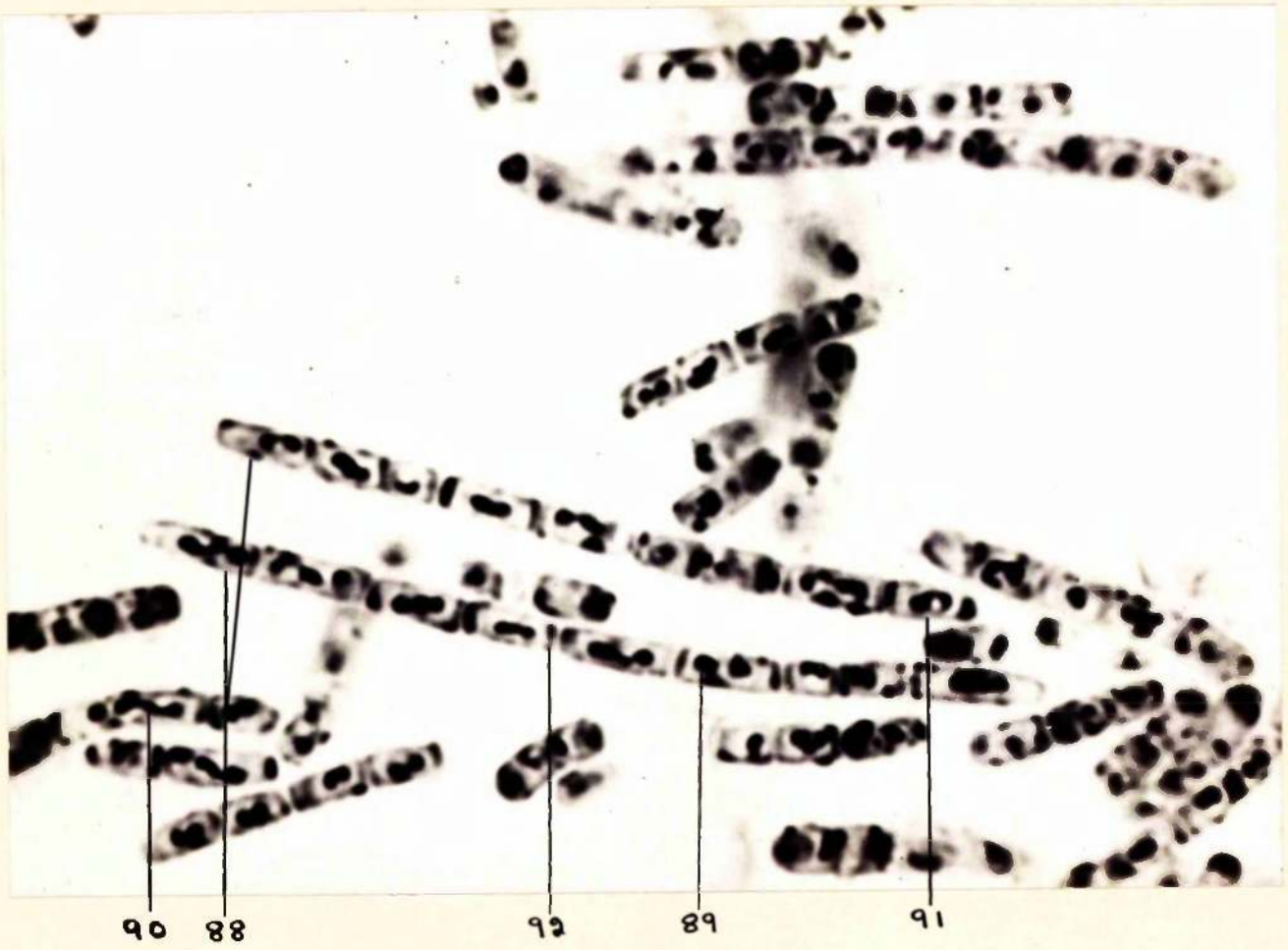



Fig. 88 Long chains of organisms

Fig. 89 Spherical nuclear structures

Fig. 90 Central core of nuclear material

Fig. 91 Clear unstained area

Fig. 92 Well demonstrated cross-walls

Organisms grown on basal agar plus 1% asparagine at 37°C for 24 hours.

Stained by the HCl-Giemsa technique.

x 4000

Experiment 13. Bacillus cereus Figs. 93 - 97

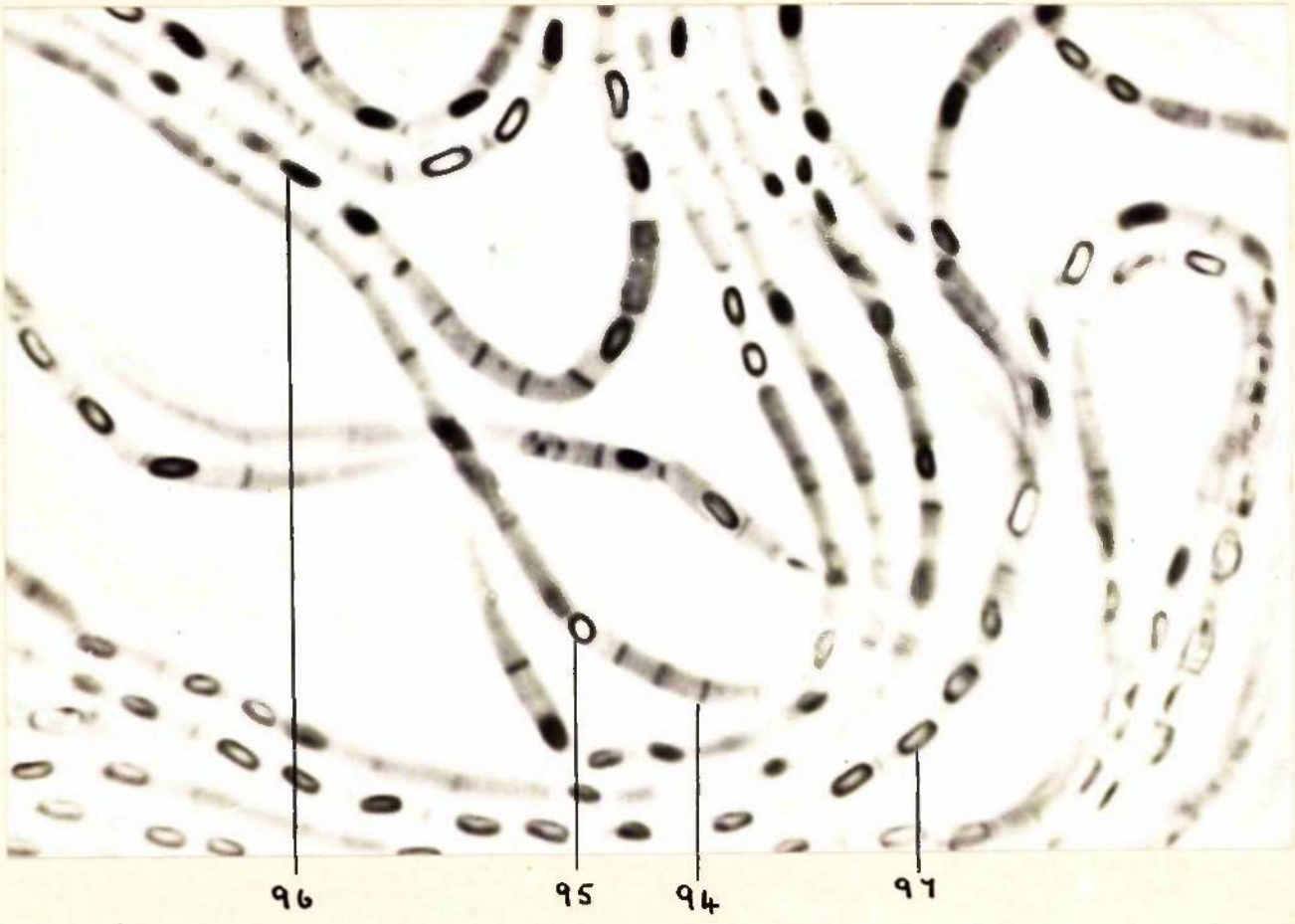



**Fig. 93** Photograph to show general appearance of organisms

**Fig. 94** Well demonstrated cross-walls

**Fig. 95** Very refractile spore

**Fig. 96** Dark spore

**Fig. 97** Intermediary type of spore

Organisms grown on basal agar plus 1% glucose and 1% urea at 37°C for 24 hours.

Examined by the phase-contrast microscope.

x 3000

Experiment 14. Bacillus cereus Fig. 98

Fig. 98 Photograph to show general appearance of organisms

Organisms grown on basal agar plus 1% asparagine at 30°C for 23 hours.

Stained by the HCl-Giemsa technique.

x 4000

Experiment 14. Bacillus cereus Figs. 99 - 103

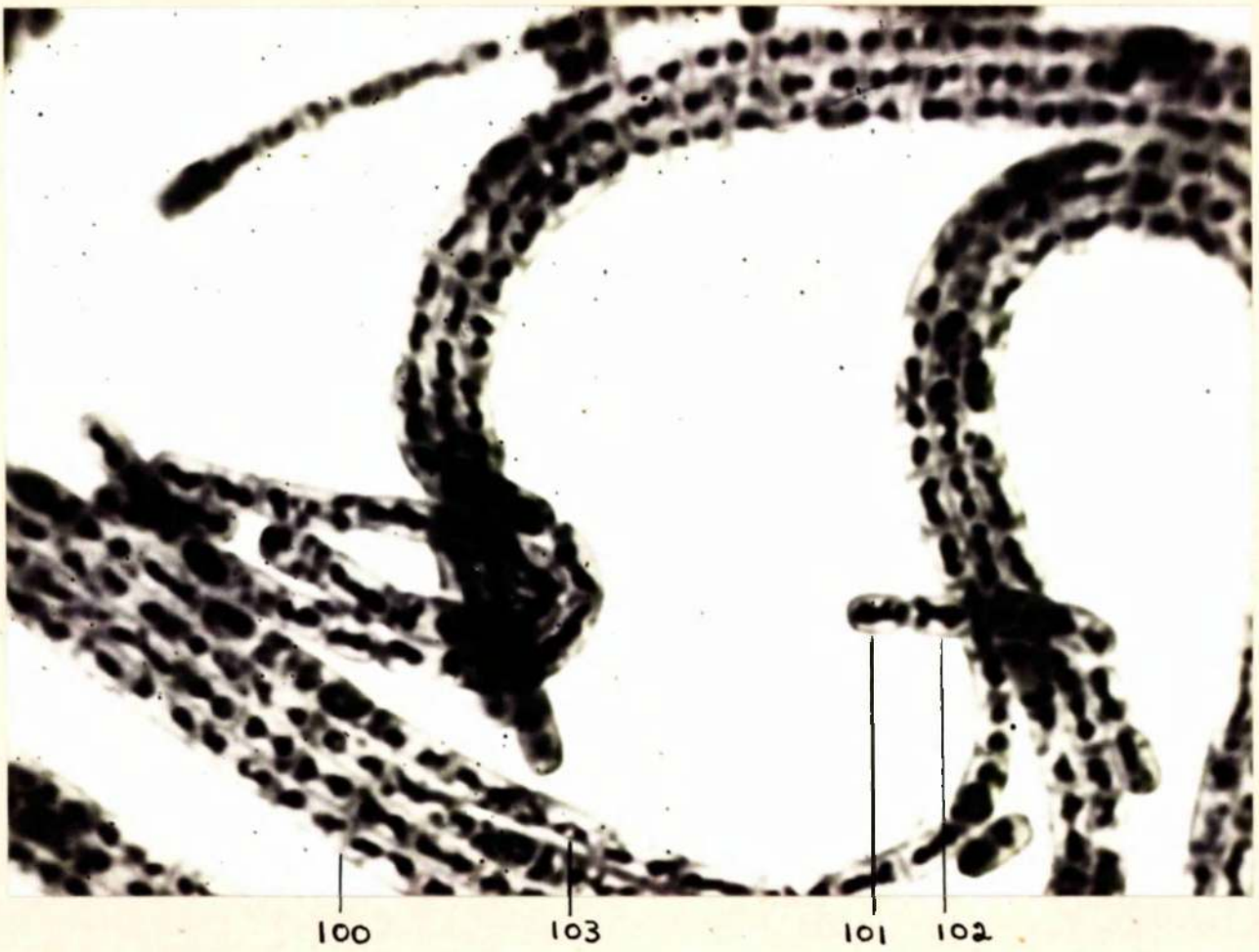



Fig. 99 Photograph to show long chains of organisms

Fig. 100 Well-defined separating cell walls

Figs. 101 & 102 Nuclear structures resembling "cross-over" forms

Fig. 103 Bright clear area

Organisms grown on basal agar plus 1% asparagine at 30°C for 23 hours.

Stained by the HCl-Giemsa technique.

x 4000

Experiment 14. Bacillus cereus Figs. 104 - 107

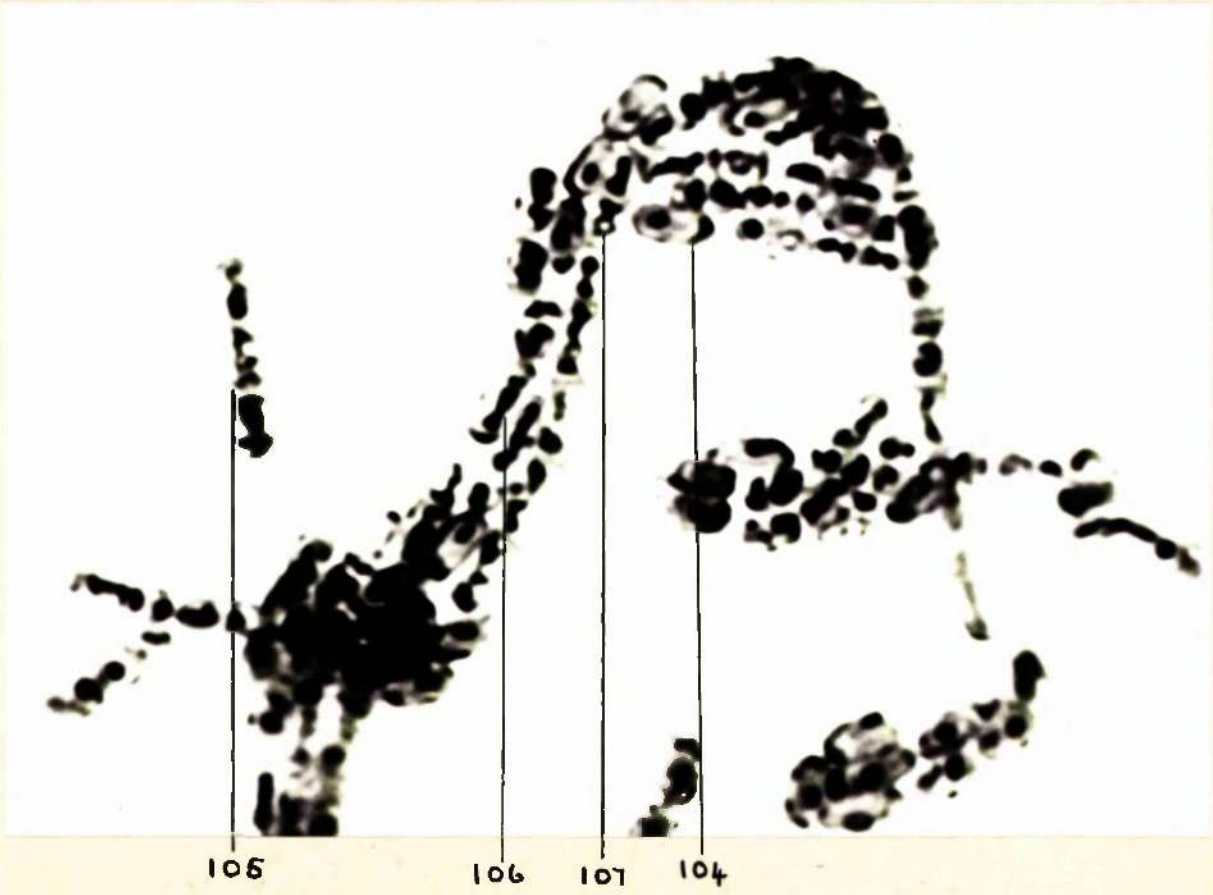



Fig. 104 Spore with well-stained nucleus and other peripheral nuclear staining material

Fig. 105 Fragments of the long chains

Fig. 106 Nuclear structures resembling "cross-over" forms

Fig. 107 Bright clear area

Organisms grown on basal agar plus 1% asparagine at 30°C for 26 hours.

Stained by the HCl-Giemsa technique.

x 4000

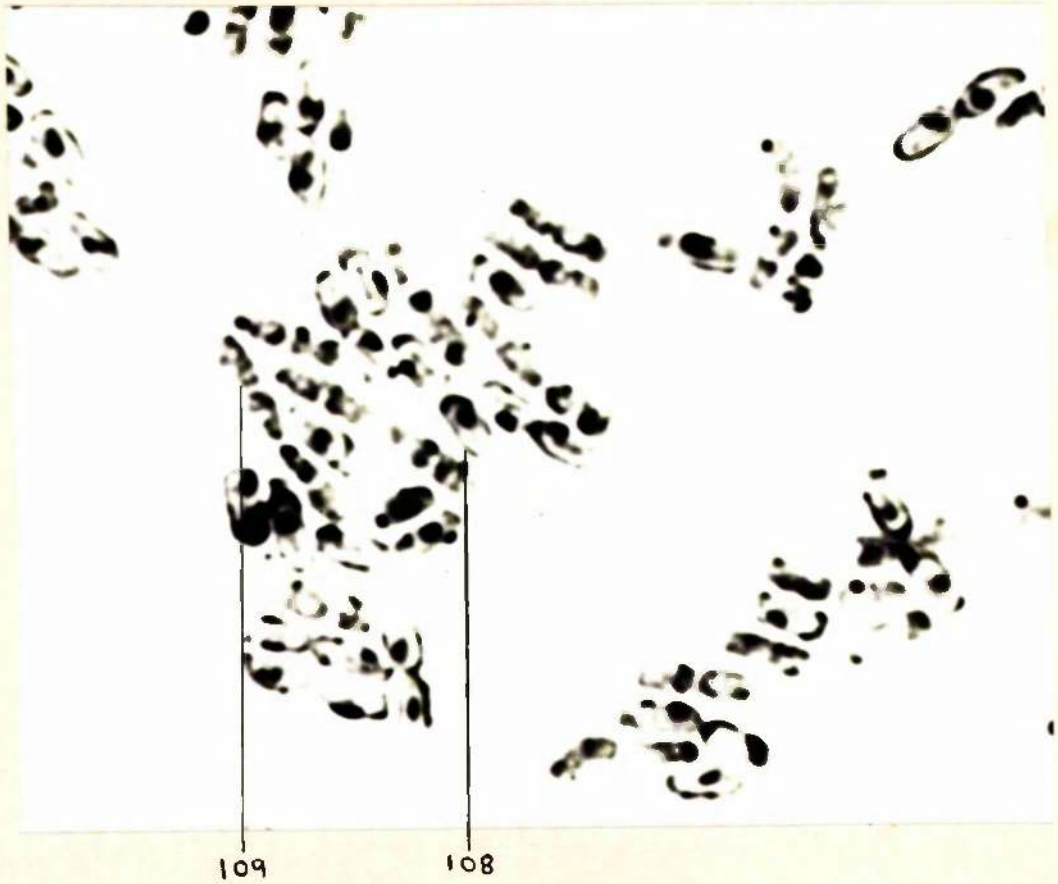



**Fig. 108** Spore with well-stained nucleus

**Fig. 109** Vegetative cells with hazy outline and irregular nuclear structure

Organisms grown on basal agar plus 1% asparagine at 30°C for 28 hours.

Stained by the HCl-Giemsa technique.

x 4000

Experiment 15. Bacillus megatherium Figs.110 - 112

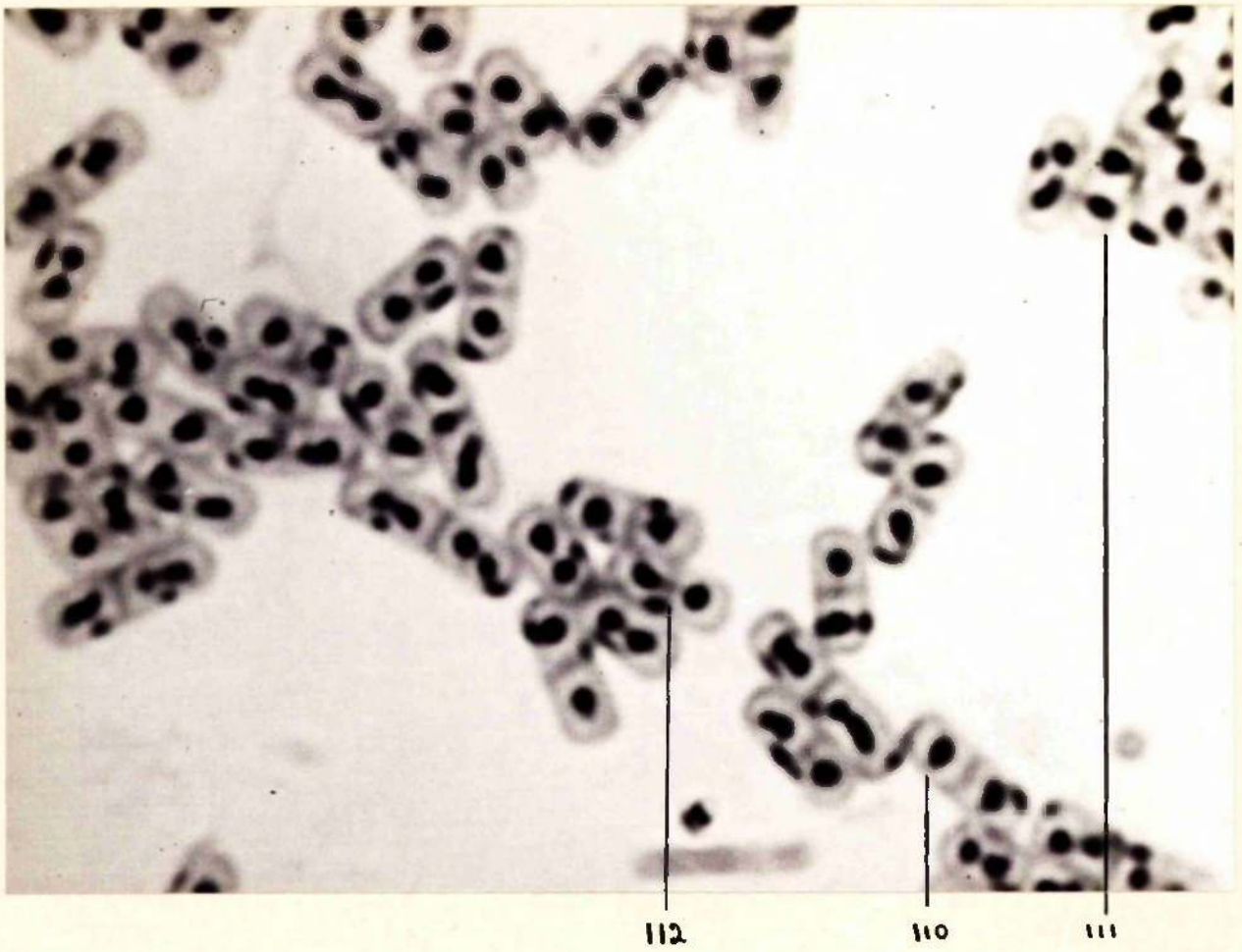



Fig. 110 Organism with one nucleus

Fig. 111 Organism with two nuclei

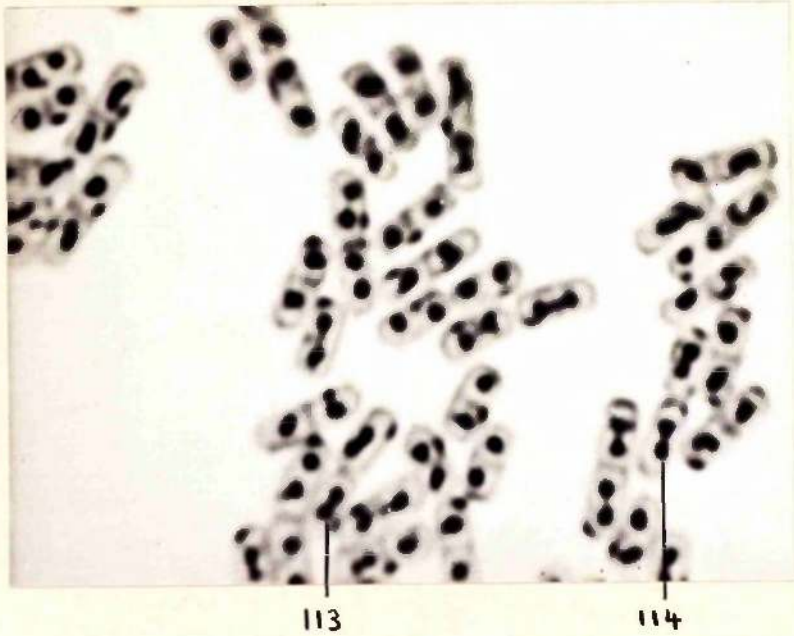
Fig. 112 Organism with two nuclei separated by a dividing septum

Organisms grown on meat-extract agar at 37°C for 2 hours.

Stained by the HCl-Giemsa technique.

x 6000

Experiment 15. Bacillus megatherium Figs. 113 - 114

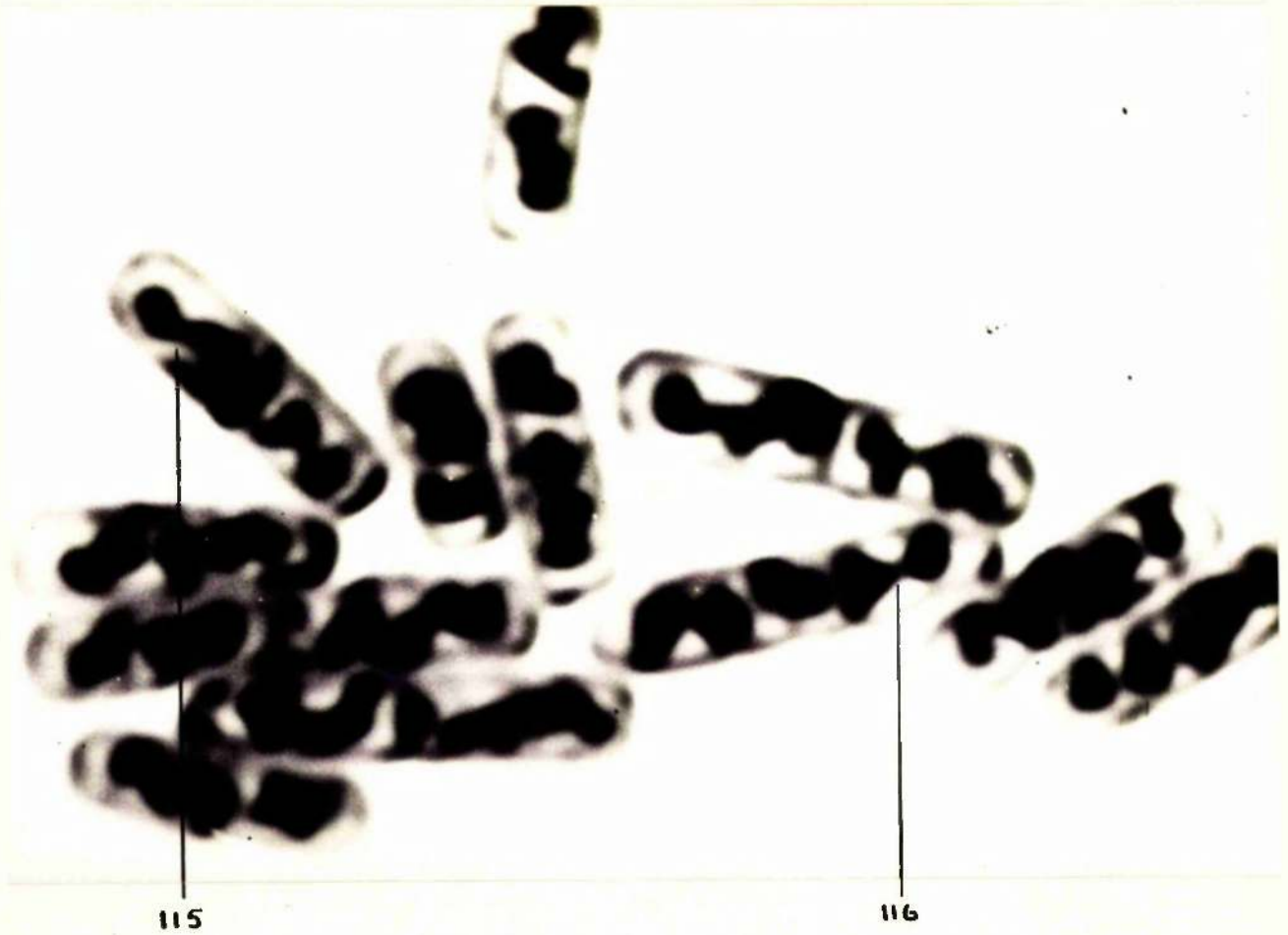



**Figs. 113 & 114 Organism where nucleus is constricting into two daughter nuclei**

**Organisms grown on meat-extract agar at 37°C for 2 hours.**

**Stained by the HCl-Giemsa technique.**

x 3500



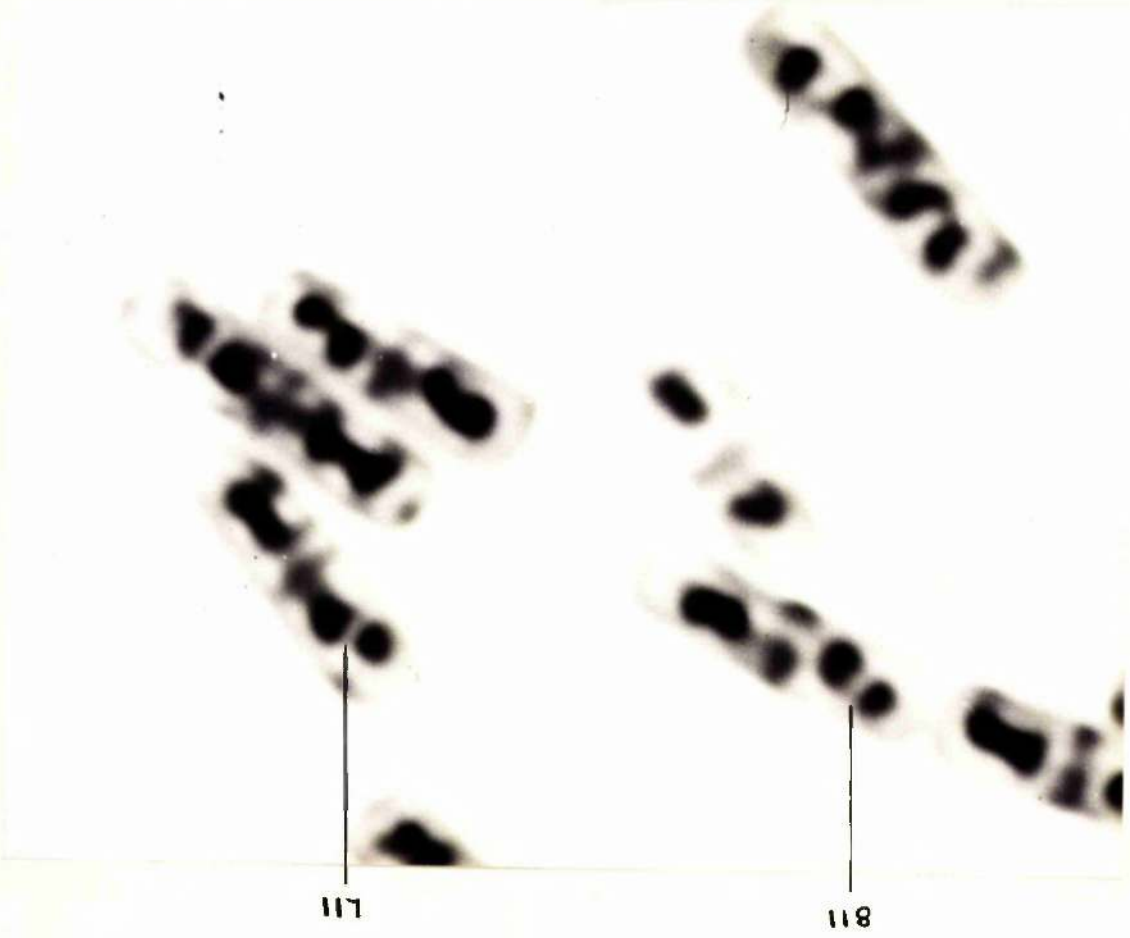

**Figs. 115 & 116 Organism where daughter nuclei are still joined**

**Organisms grown on meat-extract agar at 37°C for 2 hours.**

**Stained by the HCl-Giemsa technique.**

x I2000

Experiment 15. Bacillus megatherium Figs.117 - 118




**Figs. 117 & 118 Organisms where there is a very clear area between daughter nuclei**

**Organisms grown on meat-extract agar at 37°C for 2 hours.**

**Stained by the HCl-Giemsa technique.**

x 10,000

Experiment 15. Bacillus megatherium Fig. 119

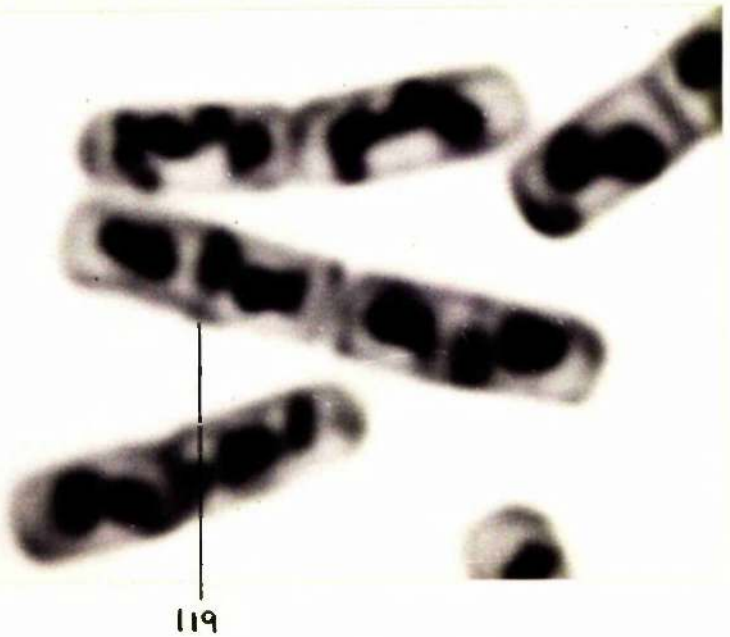



**Fig. 119** Organism showing nuclear-staining material on or near the dividing septum

Organisms grown on meat-extract agar at 37°C for 2 hours.

Stained by the HCl-Giemsa technique.

x 10,000

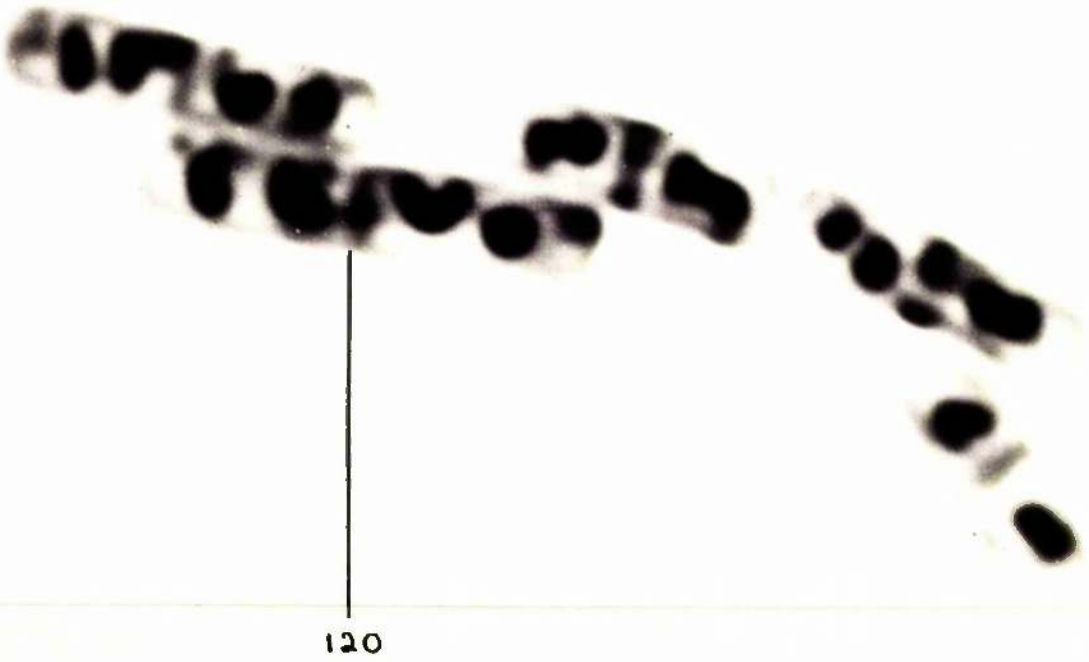



**Fig. 120** Organism with three spherical nuclei and one boomerang-shaped nucleus

Organisms grown on meat extract agar at 37°C for 2 hours.

Stained by HCl-Giemsa technique.

x 10,000

Experiment 15. Bacillus megatherium Fig. 121

**Fig. 121** Organism with two boomerang-shaped nuclei

Organisms grown on meat-extract agar at 37°C for 2 hours.

Stained by the HCl-Giemsa technique.

x 10,000

Experiment 15. Bacillus megatherium Fig.122

**Fig. 122** Organism with double-dumbell type of nucleus with clear area in the centre

Organisms grown on meat-extract agar at 37°C for 2 hours.

Stained by the HCl-Giemsa technique.

x 8000

Experiment 15. Bacillus megatherium Figs. 119a - 122a

FIG. 119a

FIG. 120a

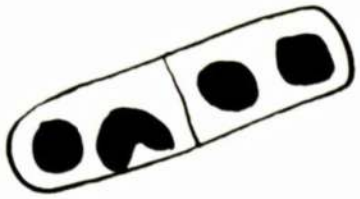


FIG. 121a

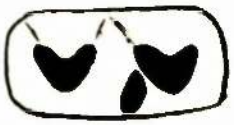
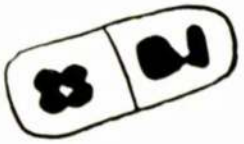


FIG. 122a



**Figs. 119a - 122a Diagrams of the organisms described in Figs. 119 - 122**

**(Drawn by hand)**

x 10,000(approx.)

Experiment 15. Bacillus megatherium Figs.123 - 125

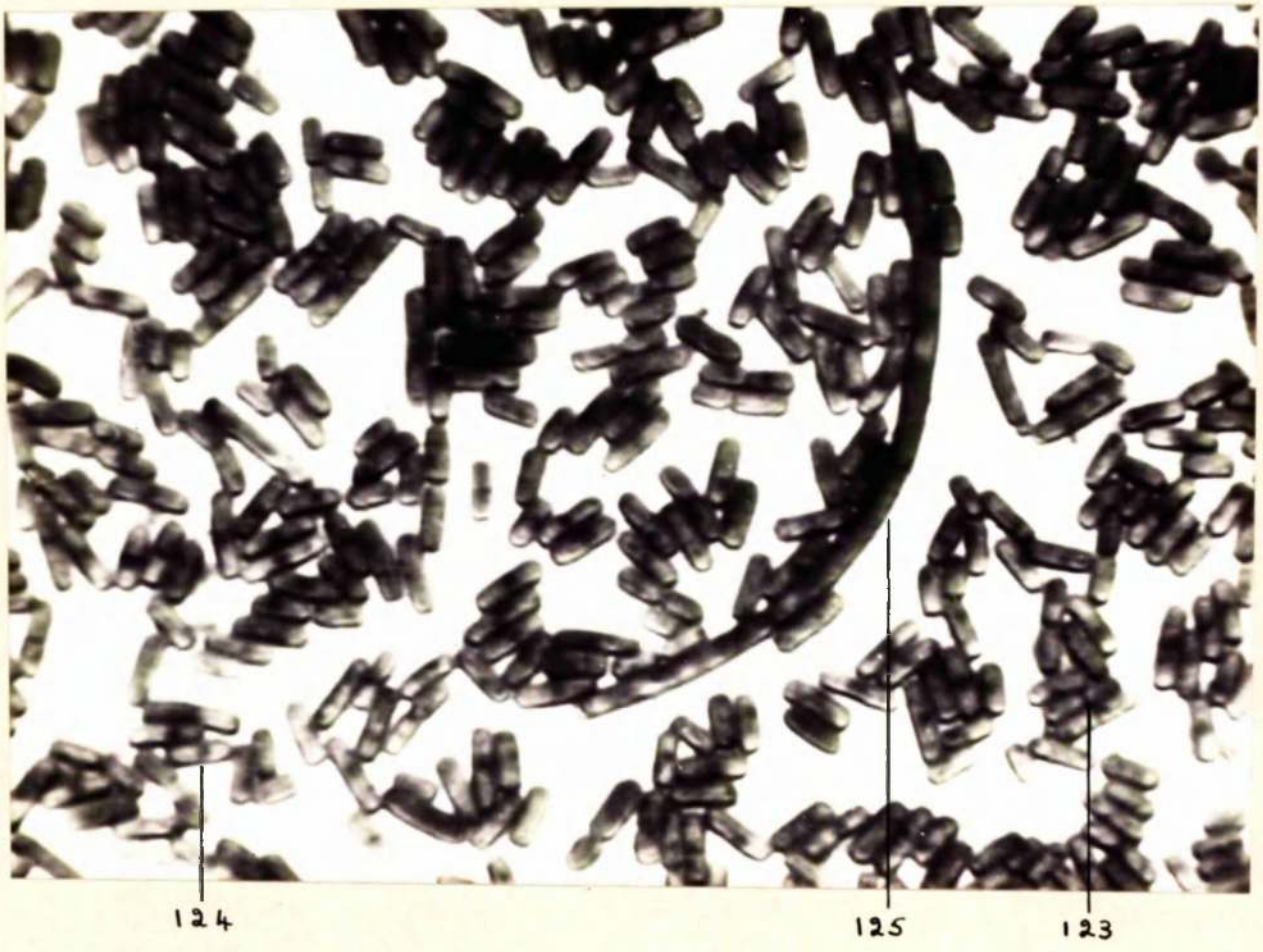



**Fig. 123** Cell-walls well demonstrated

**Fig. 124** Central granule

**Fig. 125** Chain of three long organisms

Organisms grown on meat-extract agar at 37°C for two days.

Stained by the Tannic acid - crystal violet method to demonstrate cell-walls.

x 2500

Experiment 15. Bacillus megatherium Figs.126 - 127

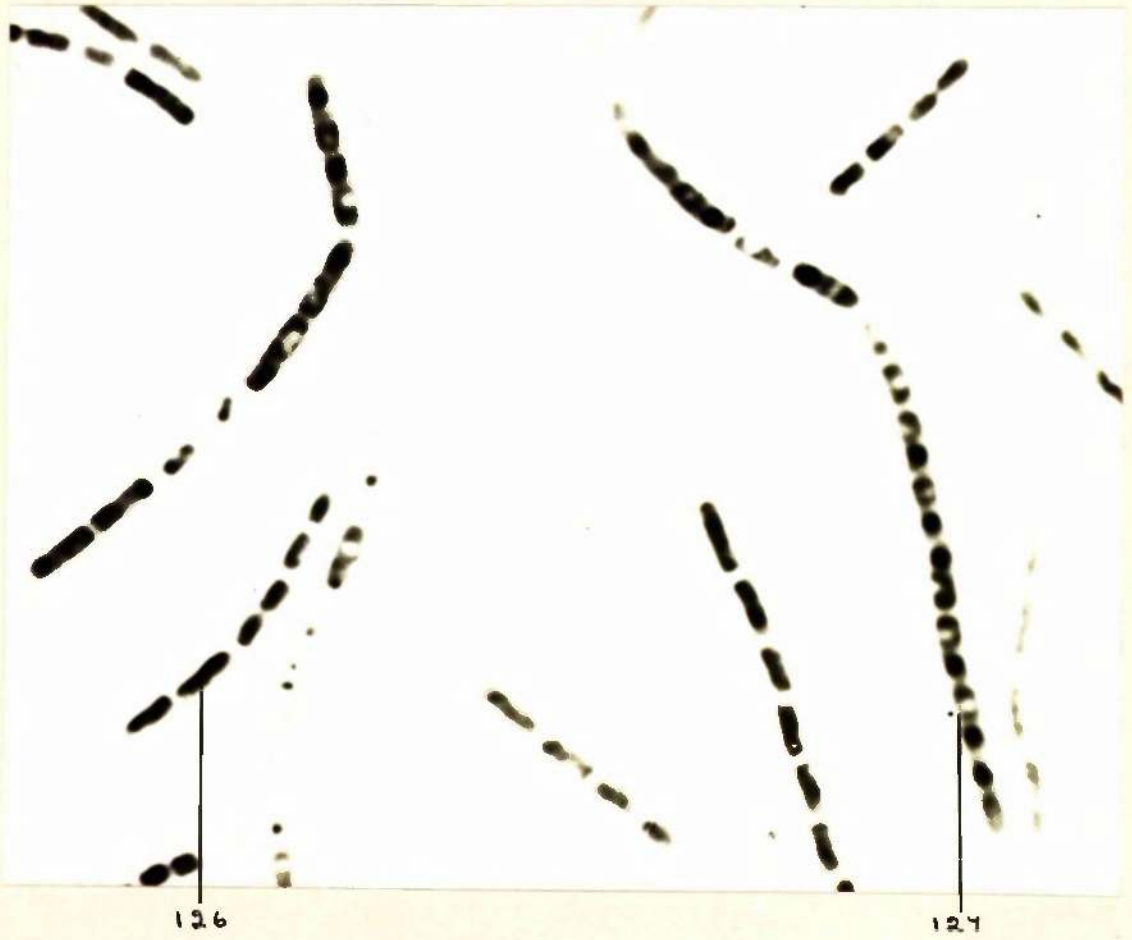



**Fig. 126** Densely stained vegetative organism

**Fig. 127** Round unstained area of endospore

Organisms grown on meat-extract agar at 37°C for 24 hours.

Stained by the Quick-differential method.

x 2500

Experiment 15. Bacillus megatherium Fig. 128

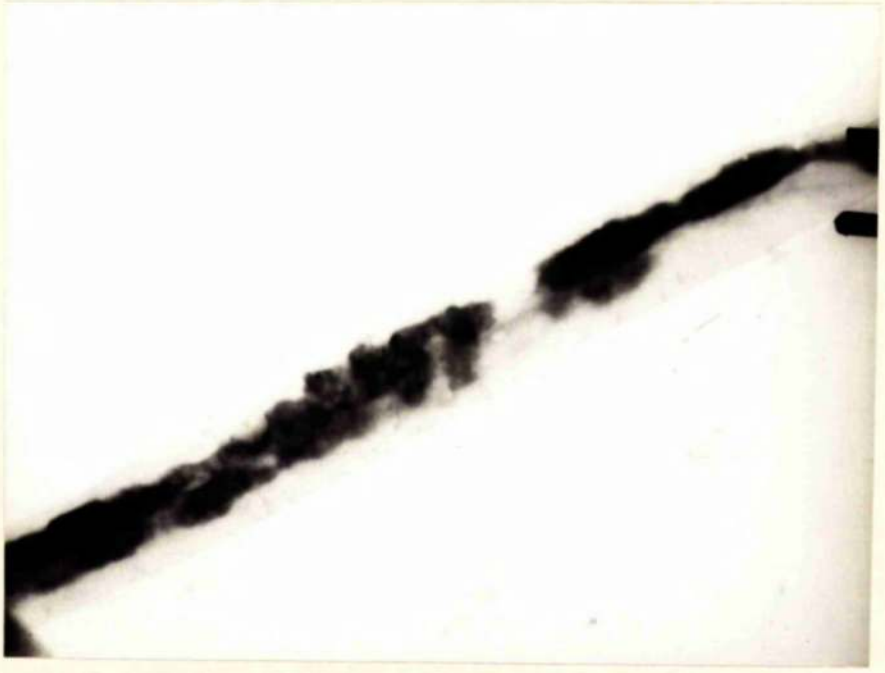


**Fig. 128** Organism with dense spiral structure in the centre of pale cytoplasm

**Organism grown in meat-extract broth at 37°C for 3 days, washed with and resuspended in distilled water.**

**Examined by the electron microscope.**

x 13,000

Experiment 15. Bacillus megatherium Fig. 129

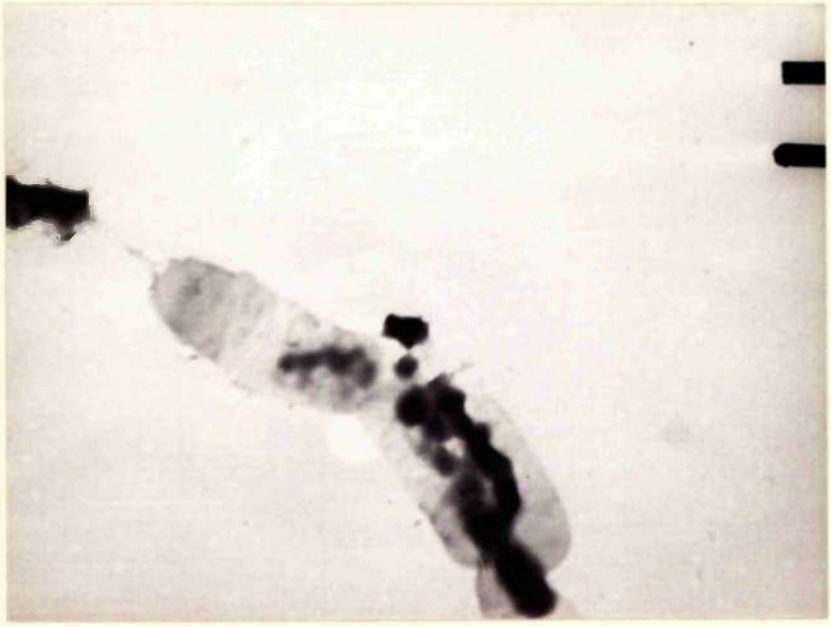


**Fig. 129** Group of organisms, with core of dense material and pale cytoplasm

Organisms grown in meat-extract broth at 37°C for 3 days, washed with and resuspended in distilled water.

Examined by the electron microscope.

x 13,000

Experiment 15. Bacillus megatherium Fig. 130

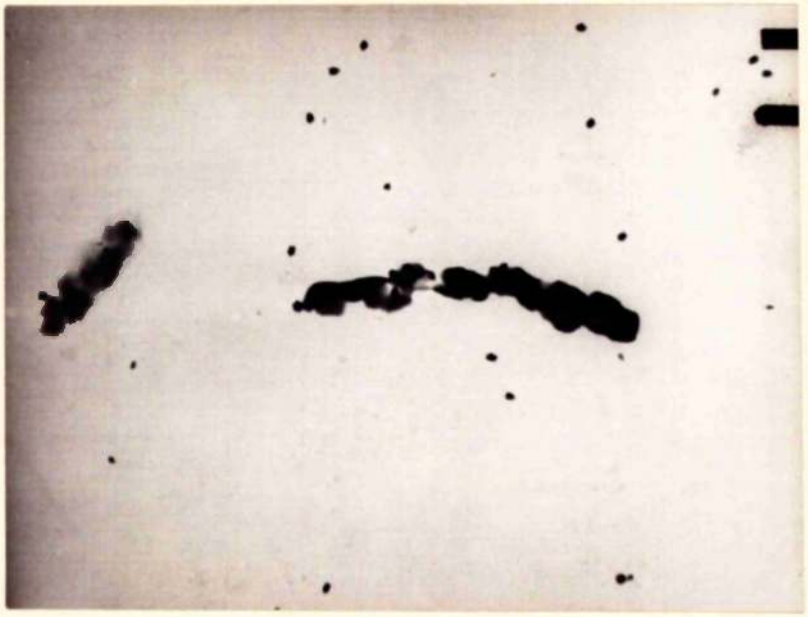


**Fig. 130 Mass of organisms and debris with regular-shaped phage-like particles**

**Organisms grown in citrate medium plus small amount of 10% glucose solution at 37°C for 7 days, washed with and resuspended in distilled water.**

**Examined by the electron microscope.**

x II,000

Experiment 15. Bacillus megatherium Fig. 131

**Fig. 131** Mass of organisms and debris with regular-shaped phage-like particles

Organisms grown in citrate medium plus small amount of 10% glucose solution at 37°C for 7 days, washed with and resuspended in distilled water.

Examined by the electron microscope.

x II,000

Experiment 15. Bacillus megatherium Fig. 132

**Fig. 132** Mass of organisms and debris with regular-shaped phage-like particles

Organisms grown in citrate medium plus small amount of 10% glucose solution at 37° C for 7 days, washed with and resuspended in distilled water.

Examined by the electron microscope.

x II,000

Experiment 15. Bacillus megatherium Figs.133 - 135

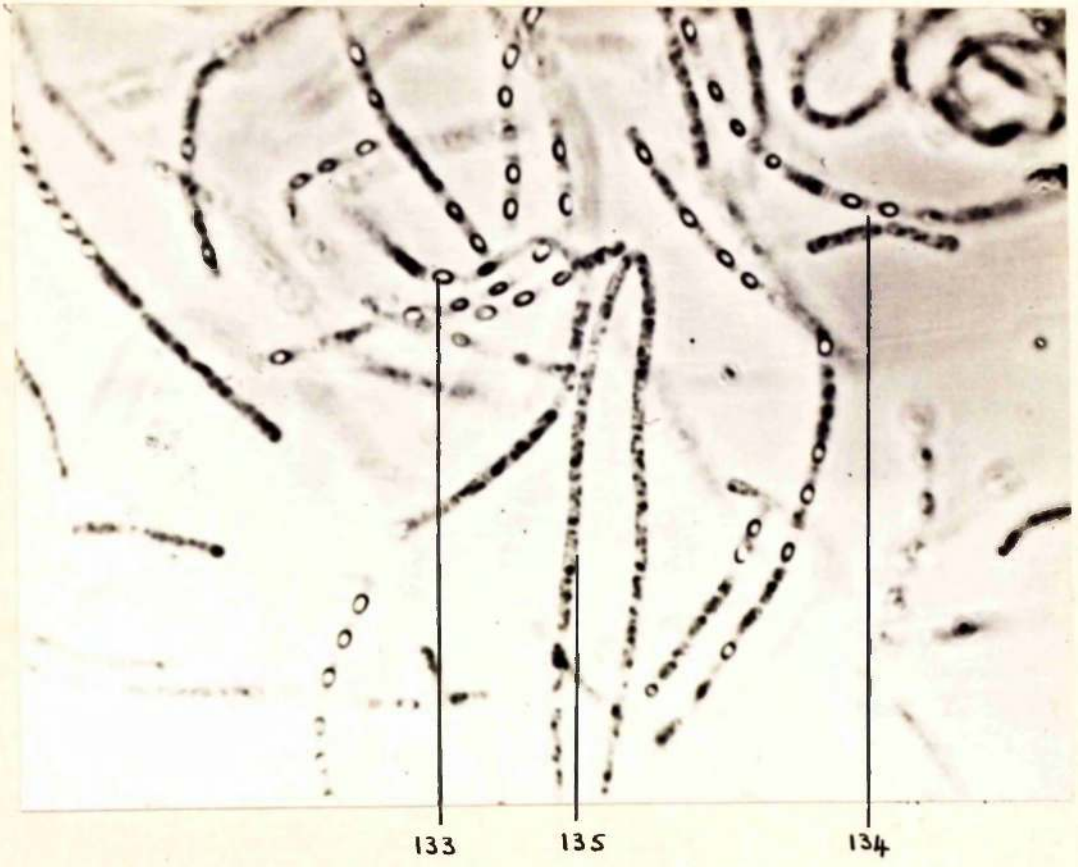



**Fig. 133** Organism with well-demonstrated endospore

**Fig. 134** Organisms with well-demonstrated cross-septa

**Fig. 135** Organism with granular appearance

Organisms grown on meat-extract agar at 37°C for 2 days and at room temperature for 4 days.

Examined by the phase-contrast microscope.

x 1500

Experiment 16. Bacillus megatherium Fig. 136

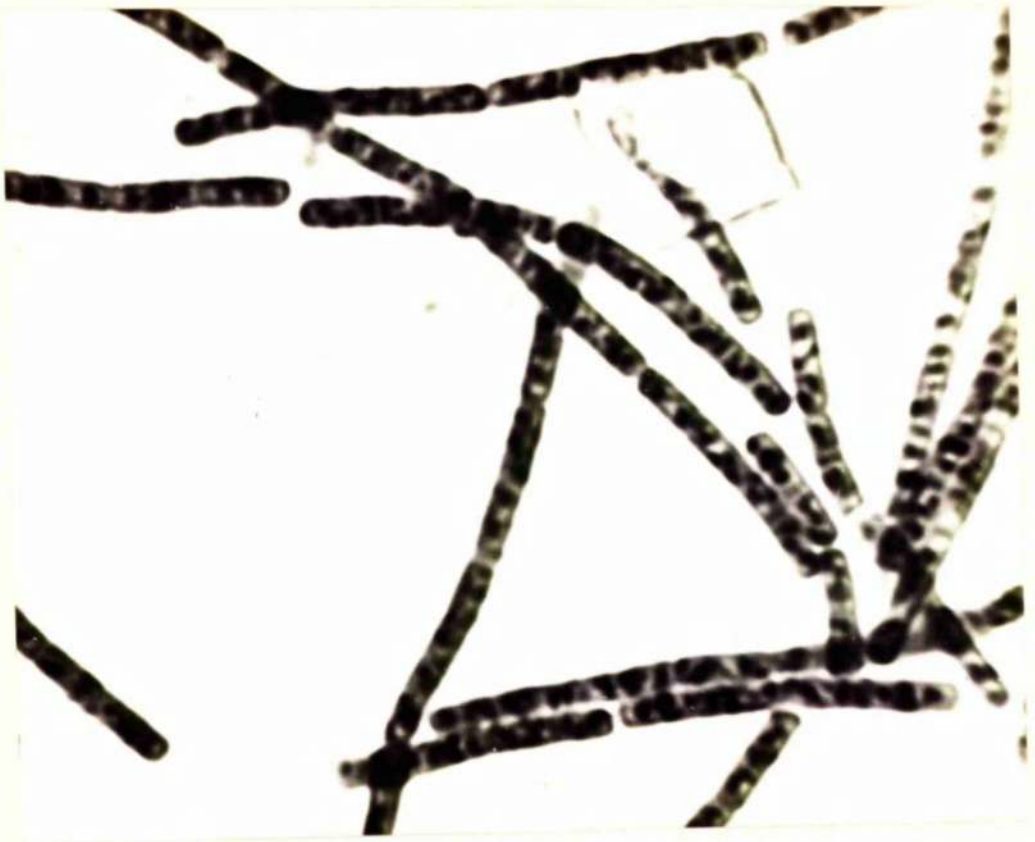


**Fig. 136 Long chains of organisms showing nuclear structures**

**Organisms grown in yeast-water tryptone broth at 37°C for 24 hours.**

**Stained by the HCl-Giemsa technique.**

x 3000

FIG. 137

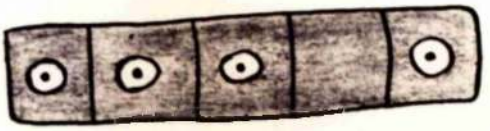


FIG. 138

FIG. 139

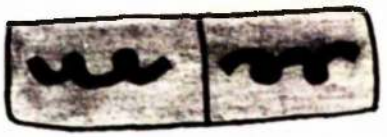


**Fig. 137** Chain of 5 organisms, 4 of which contain structures which consist of a central deeply-staining dot in a pale area surrounded by a reddish-staining area

**Fig. 138** Organism showing structures that could be interpreted as a late anaphase stage of mitotic division

**Fig. 139** Two organisms showing well-demonstrated spiral structures

**Organisms grown on basal agar alone (Fig.137), basal agar plus 1% glucose and 1% asparagine (Fig.138), basal agar plus 1% of all nitrogenous substances (Fig.139) at 37° C for 24 hours.**

**Stained by the HCl-Giemsa technique.**

x 10,000(approx.)

FIG. 140

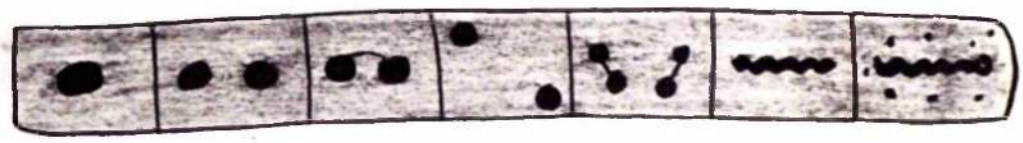


FIG. 141

**Fig. 140** Chain of organisms showing nuclear structures in various stages of division

**Fig. 141** Chain of organisms showing clear bands between dividing septa.

Organisms grown in basal broth plus 1% peptone at 37°C for 2 hours.

Stained by the HCl-Giemsa technique.

x 15,000(approx.)

Experiment 18. Bacillus anthracis Fig. 142

**Fig. 142 Long chains of organisms with nuclear structures present, but not well demonstrated**

**Organisms grown on meat-extract agar at 37°C for 2 hours.**

**Stained by HCl-Giemsa technique.**

x 2000

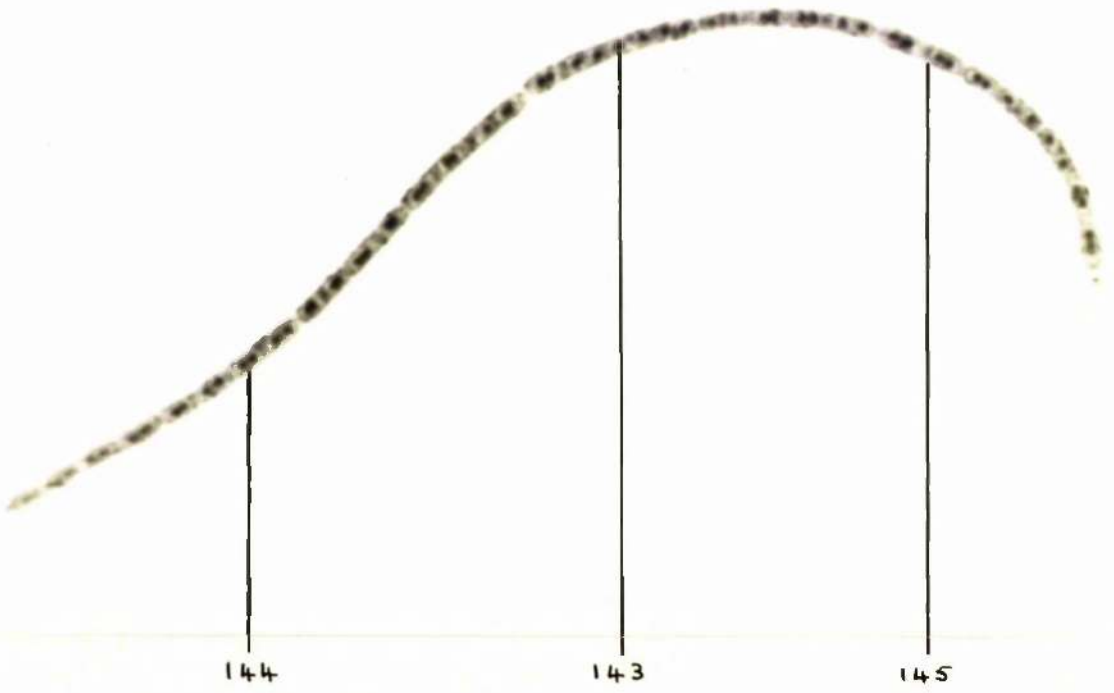



**Fig. 143** Organism with spherical nuclear structures

**Fig. 144** Organism with dumbell-shaped nuclear structures

**Fig. 145** Well-demonstrated cross walls

Organisms grown on meat-extract agar at 37°C for 5 hours.

Stained by HCl-Giemsa technique.

x 2000

Experiment 18. Bacillus anthracis Figs. 146 - 148

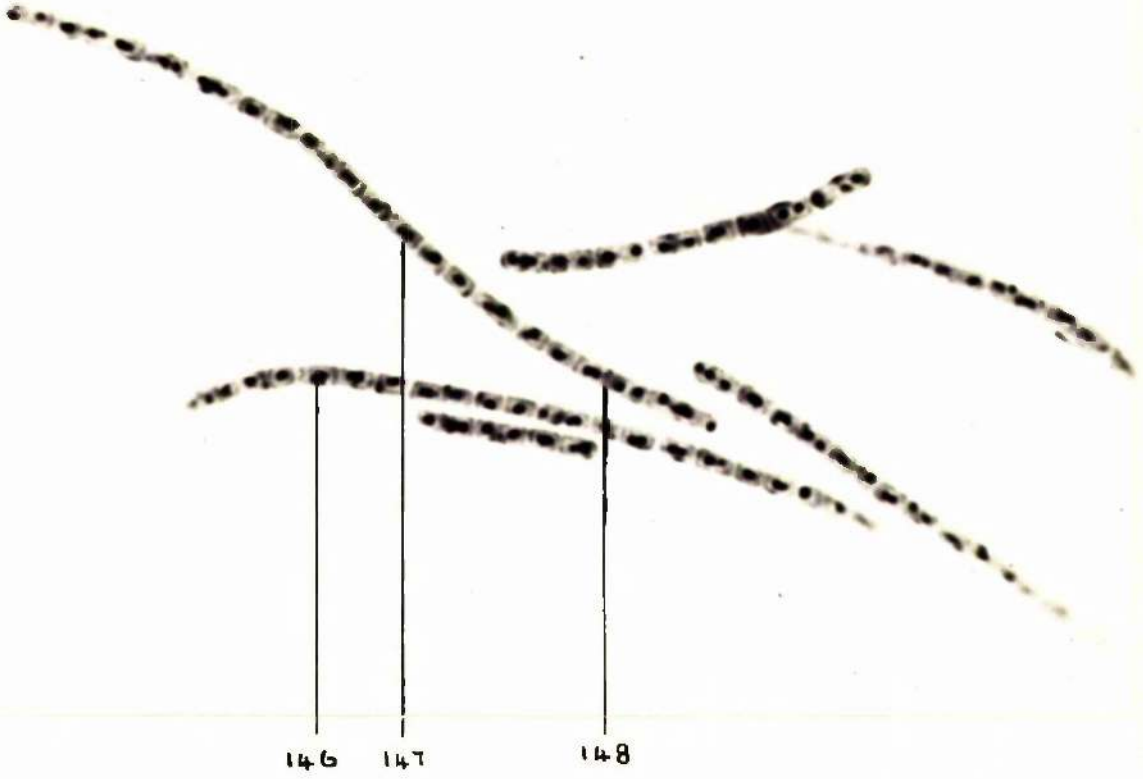



Fig. 146 Organism with spherical nuclear structures

Fig. 147 Organism with dumbell-shaped nuclear structures

Fig. 148 Well-demonstrated cross-walls

Organisms grown on meat-extract agar at 37°C for 8 hours.

Stained by HCl-Giemsa technique.

x 2000

Experiment 18. Bacillus anthracis. Figs. 149 - 151

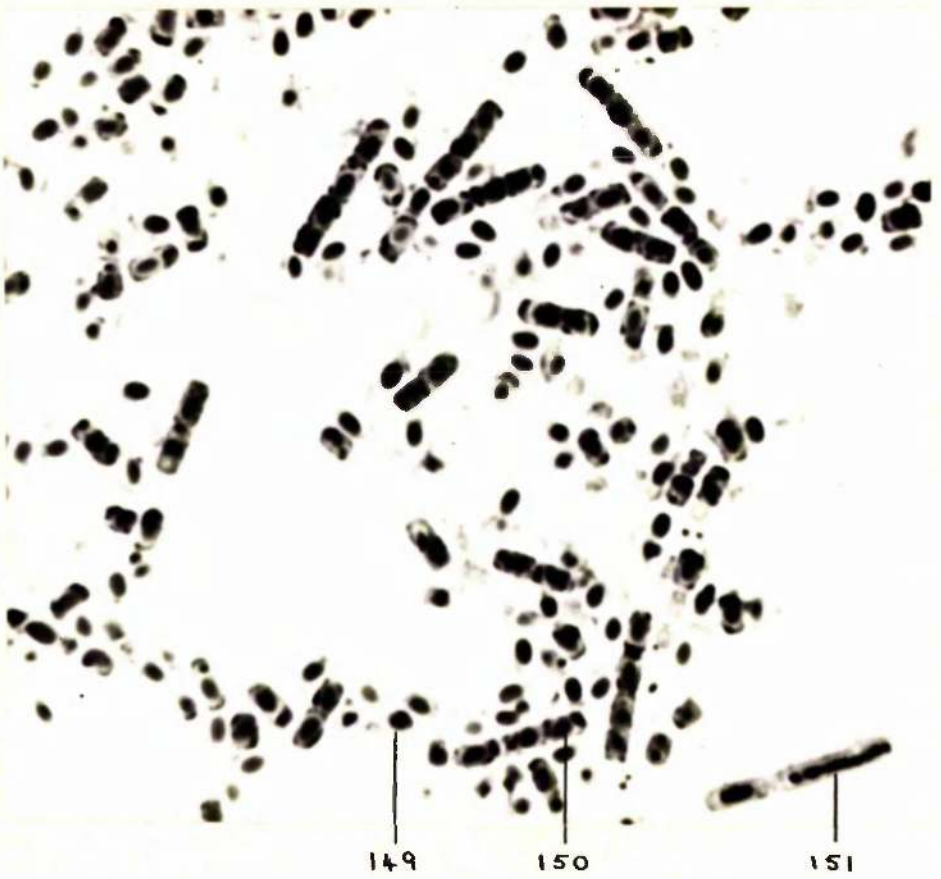



**Fig. 149** Free spores

**Fig. 150** Spore still inside vegetative mother cell

**Fig. 151** Organism with a nuclear core, but no spore

Organisms grown on meat-extract agar at 37°C for 24 hours.

Stained by HCl-Giemsa technique.

x 2000

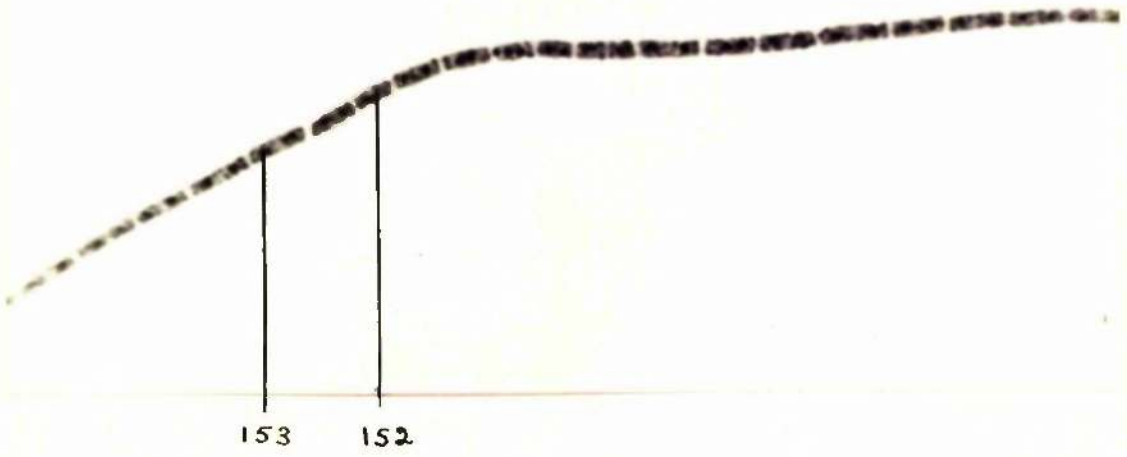



**Fig. 152 Organism with diffuse granular appearance**

**Fig. 153 Organism with large nuclear structure**

**Organisms grown on basal agar plus 1% glucose and 1% urea at 37°C for 2 hours.**

**Stained by the HCl-Giemsa technique.**

x 2000

Experiment 18. Bacillus anthracis Figs. 154 - 156

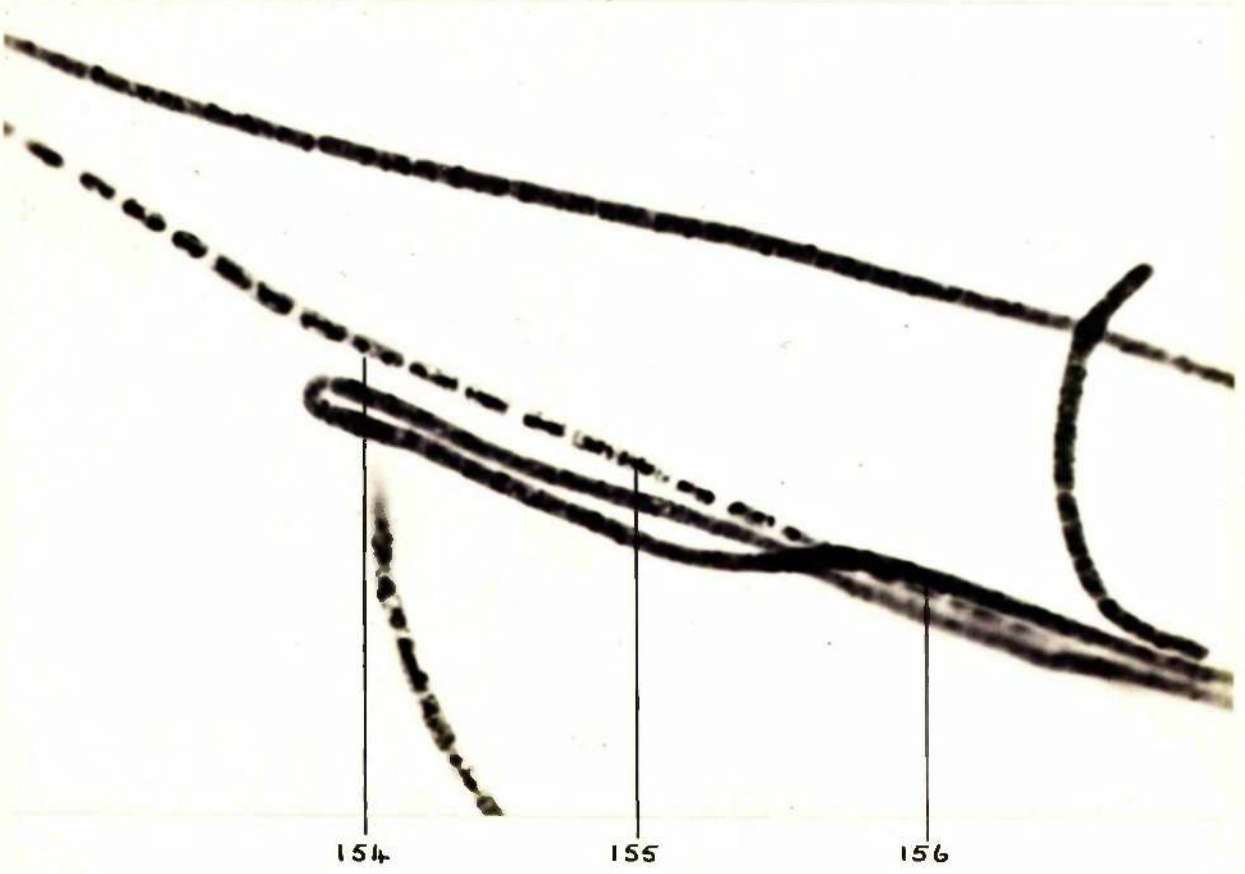



**Fig. 154** Organism with well-demonstrated nuclear structure

**Fig. 155** Organism with well-demonstrated nuclear core

**Fig. 156** Organism with diffuse granular appearance

Organisms grown on basal agar plus 1% glucose and 1% urea at 37°C for 5 hours.

Stained by the HCl-Giemsa technique.

x 2000

Experiment 18. Bacillus anthracis. Figs. 157 - 160


Fig. 157 Organism with dumbell-shaped nuclear structure

Fig. 158 Organism with clear area in the centre of the dumbell-shaped nucleus

Fig. 159 Organism containing a structure very similar to an X structure

Fig. 160 Free spores

Organisms grown on basal agar plus 1% glucose and 1% urea at 37°C for 8 hours.

Stained by the HCl-Giemsa technique.

x 2000

Experiment 19. Anthracoid bacillus Figs. 161 - 163

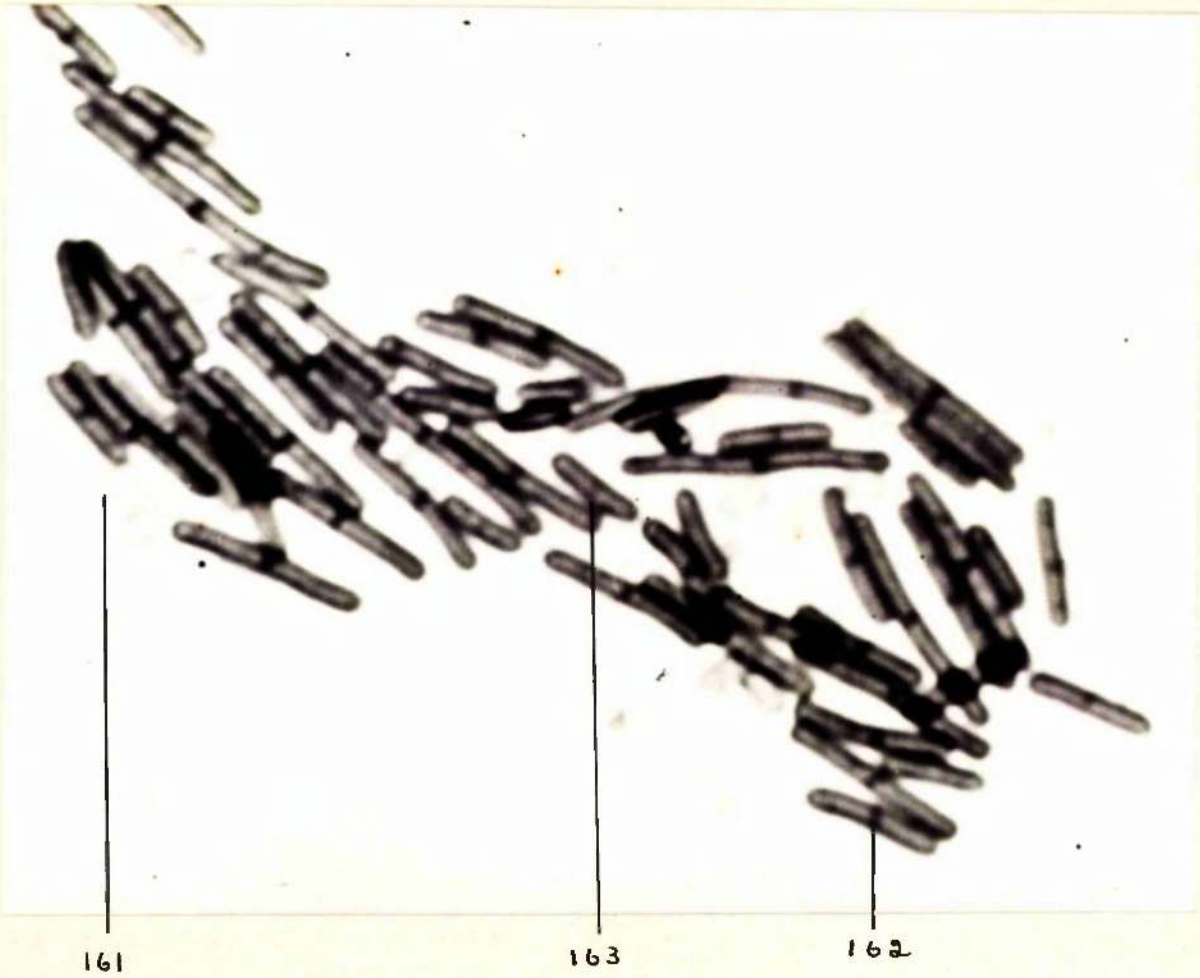



Fig. 161 Empty spore case

Fig. 162 Organism with well-demonstrated cell wall

Fig. 163 Organism with well-demonstrated cross-septum

Organisms grown on meat-extract agar at 30°C for 2 hours.

Stained by the Tannic acid-crystal violet method.

x 2000

Experiment 5. Bacillus cereus Figs. 164 - 165


Fig. 164 Organism with well-demonstrated spiral nuclear structure

Fig. 165 Long organism with a mass of nuclear material, and large clear granules

Organisms grown on basal agar plus 1% glucose and 1% urea at 30°C for 24 hours.

Stained by the HCl-Giemsa technique.

x 8000

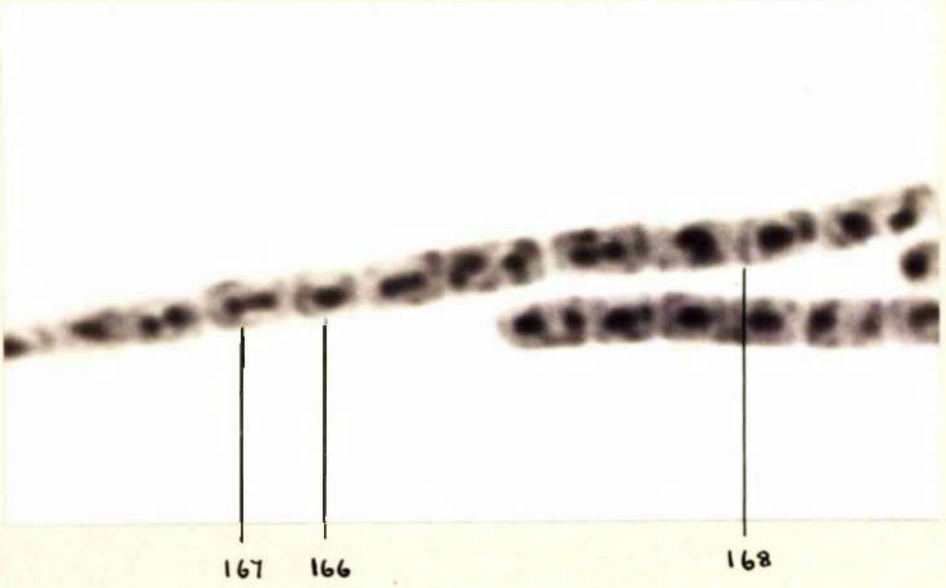



**Fig. 166** Organism with spherical nucleus

**Fig. 167** Organism with dividing nucleus

**Fig. 168** Cross-wall

? cf page 153

Organisms grown on meat-extract agar at 37°C for 2 hours.

Stained by the HCl-Giemsa technique.

x 5000

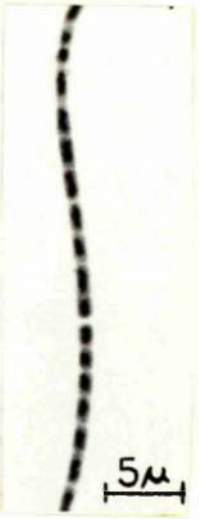



**Fig. 169 Cells of Oscillatoria showing nuclear material**

**Organisms grown in a liquid medium for 1 - 2 weeks.**

**Stained by HCl-Giemsa technique.**

**Photograph from a paper by Cassel & Hutchinson (1954).**

x 2000(approx.)

Fig. 170 Photograph of organisms showing nuclear material and "vacuoles"

Organisms grown on enriched tryptone glucose yeast-extract agar at 37°C for 3 hours.

Stained by Smith's technique.

Copied from a photograph illustrating a paper by Smith (1950).

x 5500(approx.)

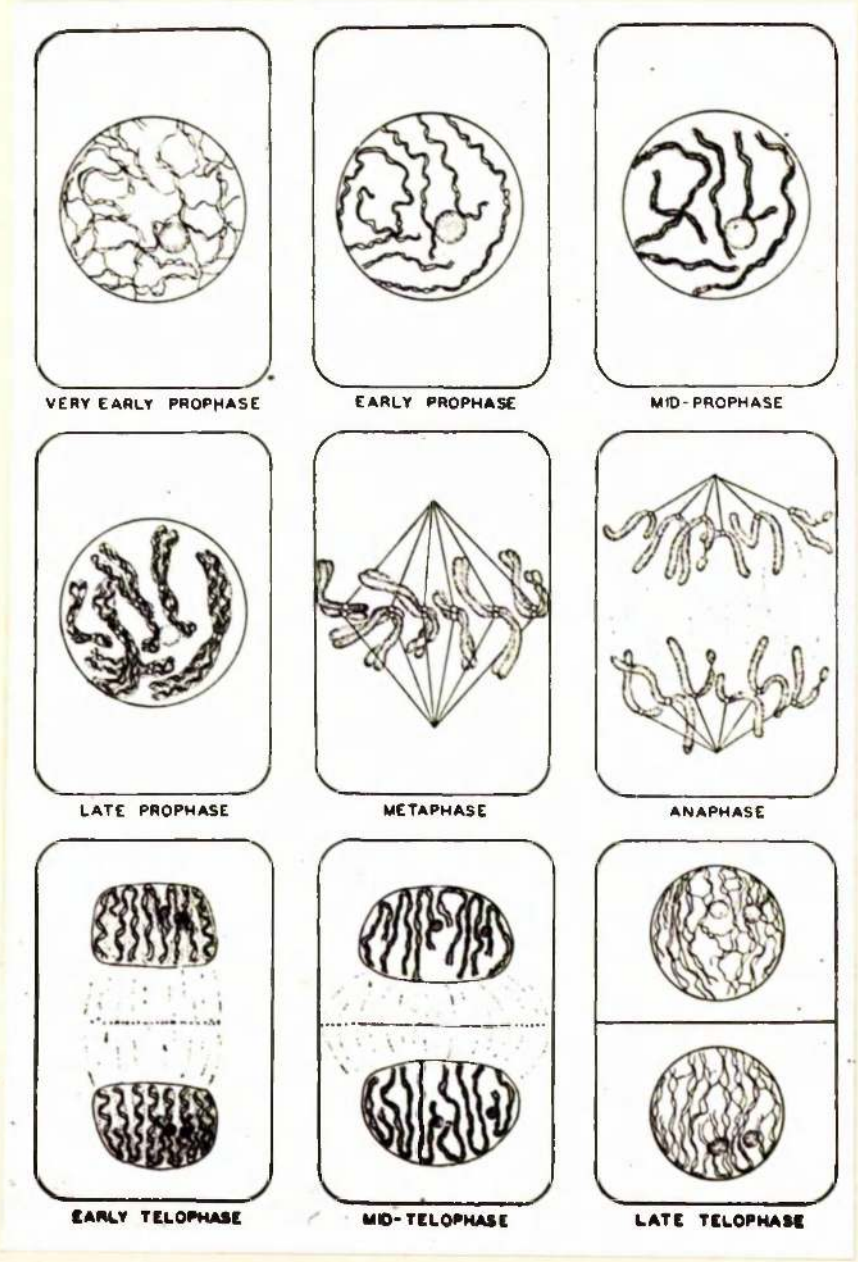



**Fig. 171** **Diagrammatic representation of the stages of mitotic division in plant cells**

Copied from p.60 of "Fundamentals of Cytology" by L. W. Sharp (McGraw-Hill Book Company, Inc. 1943).

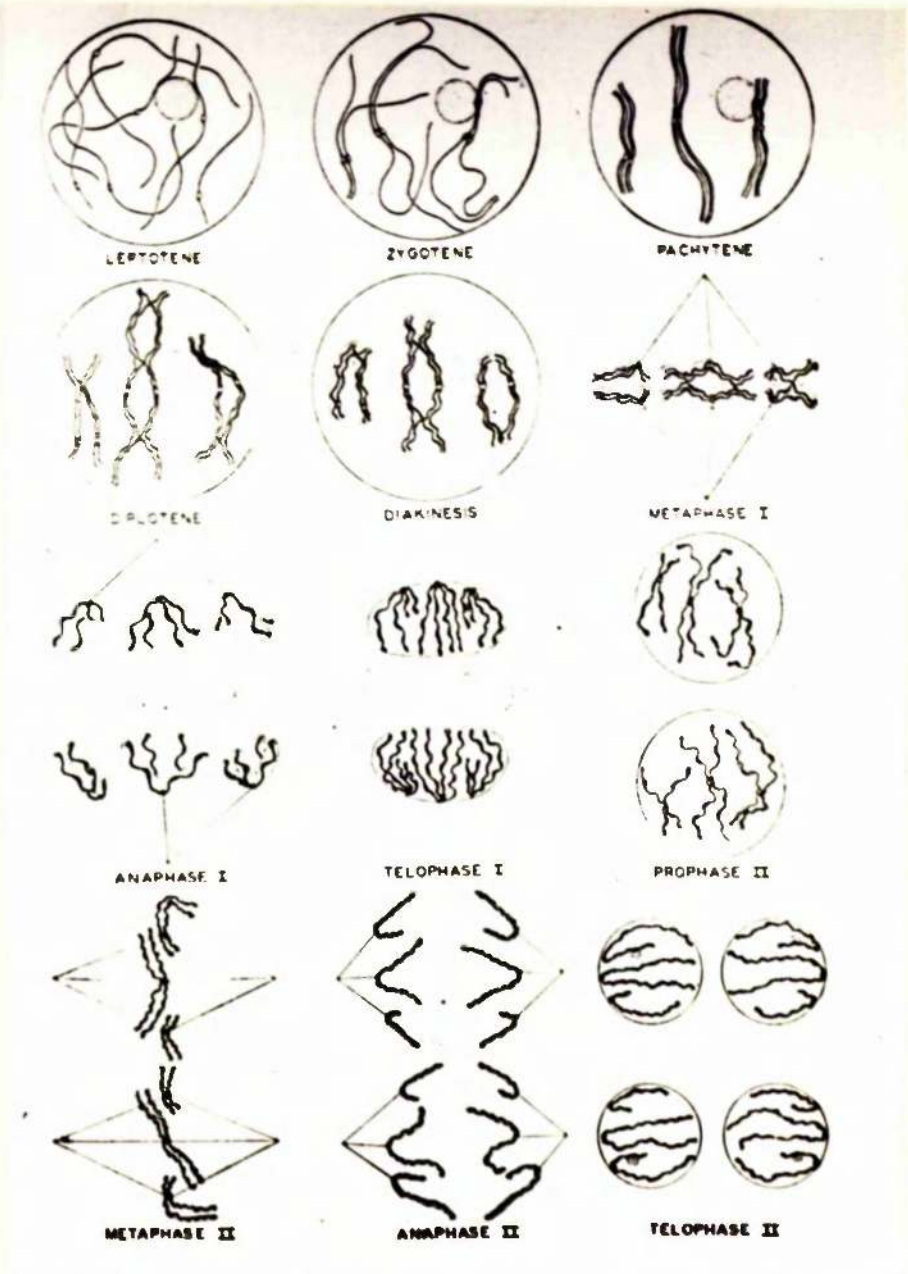



**Fig. 172 Diagrammatic representation of the stages of meiotic division in plant cells**

**Copied from p.107 of "Fundamentals of Cytology" by L. W. Sharp (McGraw-Hill Book Company, Inc. 1943).**