CHEMICAL AND CYTOLOGICAL STUDIES ON THE FORMATION OF LIPID INCLUSIONS IN B.CEREUS AND OTHER BACTERIA

by

DONALD H. WILLIAMSON, B.Sc.

Thesis submitted to the University of Edinburgh
for the degree of Doctor of Philosophy.

October, 1956.

- CONTENTS -

INTRODUCTION

Part I. General considerations of reserve storage.........................p.1

Part II. A Survey of the literature on bacterial lipid inclusions...p.16

1). Occurrence; staining methods...........................p.16

2). Their chemical nature...p.20

a) Cytochemical evidence...p.21

b) Analysis of cells containing different amounts of lipid inclusions...................p.31

c) Evidence obtained by isolation of the inclusions...p.35

3). Chemical and physical properties of the poly β-hydroxybutyrates.(PHB)...........................p.38

4). Occurrence of poly β-hydroxybutyrates.(PHB)...................p.43

5). Factors influencing the production of lipid inclusions and of PHB...p.46

6). The biological role of bacterial lipid inclusions...p.53

a) The reserve store theory...p.53

b) The "fatty degeneration" theory...........................p.57

MATERIALS AND METHODS

1). Organisms...p.59

2). Composition of solid media...p.63

3). Inoculation, incubation and harvesting...........................p.69

4). Staining methods...p.75

5). Microscopic estimations of inclusion bodies...................p.77

6). Photography...p.79

7). Chloroform extraction of poly β-hydroxybutyrate(PHB)........p.79

8). Measurement of pH...p.80

9). Turbidimetric measurements...p.81

10). Measurement of dry weight...p.81

11). Estimation of total nitrogen...p.81

12)...Estimation of polysaccharide...p.82

13). Phosphorus determination...p.83

14). Ash determination...p.84

SPECIAL METHODS

1). Preparation of the alkaline hypochlorite reagent.............p.85

2). The use of alkaline hypochlorite for quantitative estimation of the lipid inclusions of the Bacillus genus...p.86

3). Preparation of the calibration curve used for estimation of the lipid inclusions.......................................p.88

4). The use of alkaline hypochlorite for large-scale isolation of the lipid inclusionsfor analysis................p.91

5). The isolation and preparation of the lipid inclusions for electron microscopy...........................p.93

EXPERIMENTS AND RESULTS

Part I. Microscopic appearance, cytochemical properties and occurrence of lipid inclusions in the experimental organisms...p.94

1). Lipid stains...p.96

2). Volutin stains...p.103

3). Microscopic appearance of the unstained inclusions...........p.105

4). Simple stains...p.111

5). Thegram stain...p.111

6). Ashby's spore stain...p.112

7). The acid-fast spore stain...p.112

8). Protein stains...p.114

9). The Feulgen reaction..p.115

10). Iodine...p.116

Table 15...p.151

Part II. Observations on the liberation of lipid inclusions by means of hypochlorite...p.118

1). Continuous microscopic observation...............................p.118

2). Turbidimetric observation..,,....................................p.127

3). Experiments relating to preparation of the calibration curve...p.143

Part III. Microscopic appearance and micro-chemical properties of the inclusions isolated by hypochlorite....p.150

Table 15....p.151

Part IV. Isolation of the lipid inclusions by methods other than with hypochlorite, and microchemical observations of the inclusions thus isolated....p.156

1). Isolation of the inclusions with acid....p.156

2). Isolation by mechanical disruption of the cells....p.157

3). Isolation by means of lysozyme....p.159

4). Microscopic staining and solubility characters of mechanically isolated inclusions....p.162

Table 15....p.151

Part V. Chemical examination of the inclusions isolated by the hypochlorite method....p.168

1). Fractionation of the inclusions into an ether-soluble and an ether-insoluble fraction....p.168

2). Analysis of the ether-soluble fraction....p.172

3). Analysis of the ether-insoluble fraction(PHB)....p.174

Part VI. Parallel estimations of poly β-hydroxybutyrate by the hypochlorite and chloroform extraction methods....p.186

Part VII. Electron microscopic appearance of isolated lipid inclusions....p.191

1). The inclusions isolated by hypochlorite....p.191

2). The inclusions isolated by mechanical disruption....p.205

3). The inclusions isolated by lysozyme....p.208

Part VIII. The influence of cultural conditions on the formation of poly β-hydroxybutyrate(PHB) by B.cereus.(strain AC.1)....p.214

1). Purpose of experiments....p.214

2). The choice of methods and their validity....p.215

A note on some of the terms employed....p.226

3). Experimental results....p.228

a) The glucose limited "control" medium....p.228

b) The influence of glucose concentration....p.242

Part VIII(contd.).

c) The influence of concn. of nitrogen source.................. p.250
d) The influence of concn. of K^+ source........................ p.256
e) The influence of concn. of S source........................ p.261
f) The influence of concn. of Phosphate........................ p.265
g) The influence of anaerobiosis................................ p.271

DISCUSSION.................................. p.274

SUMMARY.................................... p.291

REFERENCES.................................. p.299

ACKNOWLEDGEMENTS........................... p.305

INTRODUCTION.

PART I.

GENERAL CONSIDERATIONS OF RESERVE STORAGE

The interest attaching to the process of biological evolution is hardly surpassed by its importance to life as a whole, and it is therefore not surprising that, despite the limited opportunities it presents for experimental work, evolution has been the subject of considerable study. Much of the detail of the process is obscure or controversial, but the overriding importance to it of competition between organisms - in particular competition for food materials - cannot be denied. Oparin (1938) even suggests that competition between colloidal coazervates for organic substances may have played an important part in the gradual transformation of dead matter into the first recognisably "living" bodies.

Be that as it may, competition for food is undoubtedly of prime importance in present-day biological economy, and it is therefore not surprising that most plants and animals are equipped with means of storing any nutrients available in excess of immediate requirements, for use when external food resources fail. The stored materials may either form localised deposits or be dispersed throughout the organism. They are generally high molecular weight compounds, specially suited for storage, which are synthesised by the organism from the available nutrients, and are not simply accumulations of particular substances already present

in the food consumed. A striking general feature of the more prominent reserve materials is that they are all potentially rich sources of energy such as lipids and polysaccharides, and rarely contain nitrogen. Their function would therefore appear to be mainly that of acting as reserves of energy, rather than supplying the "building blocks" for growth. This general feature is not altogether surprising in view of the probable purpose of the reserves, which is presumably that of helping the organism to survive periods of nutritional hardship. The most generally adopted response to starvation (particularly in the case of micro-organisms) is the purely passive one of maintaining minimal metabolism for as long as possible, with the aim of surviving long enough to take advantage of any favourable change in the environment, and obviously the chances of ultimate survival will be greatly enhanced by the possession of reserve nutrients. Little is known of the way in which the resting (i.e. non-growing) cell maintains its life under starvation conditions. It seems likely that the living organism never stops respiring. For instance, Holter and Zeuthen (1948) found that the rate of respiration of the starving amoeba, _Chaos chaos_, remained constant relative to the "reduced weight" (weight in water) right up to death (indicated by disintegration and autolysis). It seems probable, therefore, that a continual small supply of energy is necessary to prevent death. One might regard an organism as comprising a set of complex reversible

reactions which are maintained in an equilibrium favouring the organism's integrity and viability, by a supply of energy. If the energy source fails, the equilibrium is reversed, and death (in the case of micro-organisms probably mediated by autolysis) ensues. Thus, although an energy source is unlikely to be the only requirement for the maintenance of viability, it is certainly of prime importance.

Other possible responses to imminent starvation are the formation of a dormant form such as a cyst or spore, and migration into a better environment. The metabolic rate of the dormant form is negligible, but energy will certainly be needed for its formation, and in the absence of an environmental source, it must come from the organisms endogenous reserves. Moreover, the cyst or spore may require endogenous reserves of energy for subsequent germination, although according to Knaysi (1945a) this is not the case with the spores of B. cereus.

That energy is needed for migration is self evident, and the ultimate chance of survival is probably related to the distance the organism can travel using its energy reserves.

Thus it is apparent that all modes of survival during starvation are largely dependent on the possession of energy reserves, and it is not therefore surprising that energy rich substances are quantitatively so predominant. It should be pointed out, however, that nitrogenous compounds may be stored as frequently as energy rich reserves, and

their apparent scarcity may be simply a reflection of the relatively small amounts required by the cell; for a given amount of growth or metabolic transformation much more carbon and energy source is required, weight for weight, than nitrogen source, and the carbon and energy source is therefore always more obvious and more easily detectable. The purely technical difficulties of detecting a nitrogenous reserve may also play a part; the only known nitrogenous substances which might act as reserves are protein and nucleic acids, and present analytical techniques would make it difficult to detect small storage amounts of these compounds among the essential structural and functional proteins and nucleic acids of the cell.

In this connection there is the added difficulty that there is probably no hard and fast dividing line between materials that function as "expendable" nutrient reserves, and components of the organism that are "essential" to its existence. Under the stress of prolonged starvation, it is conceivable that an organism might, to a limited extent, utilise some of its "essential" constituents in order to maintain its viability. A rather specialised example of this phenomenon was demonstrated by Spiegelman and Dunn (1948). These authors found that when yeasts were adapted to the fermentation of galactose in the absence of an external nitrogen source, there was some loss of glucozymase activity. This loss could be prevented by the addition to the medium of a nitrogen source, and it was deduced that some of the glucozymase protein was being broken down and

utilised for the synthesis of the galactozymase. Since failure to adapt would have resulted in starvation, one can regard this phenomenon as a specialised instance of a response to adverse nutritional conditions, a response which entailed the partial breakdown of an "essential" or "functional" (as opposed to reserve) cell constituent. These findings suggest the possibility that other components of the cell, normally regarded as "essential", might be utilised similarly.

Variable Components of Bacteria.

In general, the distinction between storage materials and essential functional or structural components of the cell is quite clear, the main distinguishing feature being the wide quantitative variability of reserve materials. They increase in amount in times of good nutrition and decrease during starvation. Usually the fluctuations are large enough to be easily detected, and often the materials are deposited in the form of discrete, morphologically distinct bodies. It must be pointed out that the quantitative variability of these materials is not conclusive proof that they function as nutrient reserves. This point will be returned to later. For the present, one might note the chief materials found in bacteria that do vary widely in amount. They are:

1) Materials that occur as discrete cellular structures.

   (a) Polysaccharides.

   These are found as inclusion bodies or granules stainable with iodine, or as capsules.

(b) Lipids.

Many bacteria form discrete intracellular inclusion bodies of lipid. The literature on these inclusions forms the second half of this introduction.

(c) Volutin.

Granules of this enigmatic material, which occurs in widely varying amounts in many micro-organisms, have been the subject of considerable study ever since their discovery. Evidence has recently been accumulating that they consist largely of polymerised meta-phosphate, and the possibility arises that volutin functions as a reserve of energy.

(d) Sulphur.

Certain of the Thiorodaceae obtain their energy by the oxidation of H_2S. If the supply of sulphide is good, the oxidation is only partial, and the resultant sulphur accumulates in the form of intra-cellular inclusions. When the supply of H_2S is exhausted, this sulphur is further oxidised to H_2SO_4.

2) Materials dispersed throughout the cell.

Under this heading one finds mainly lipids and polysaccharides which are usually in complex combination with protein, and do not generally form discrete, microscopically demonstrable deposits. The amount of these substances varies widely with cultural conditions, and it is not unlikely that, to some extent, they can act as reserve nutrients. Not all of the "dispersed" poly-saccharide and lipid of the cell can have this function,

however. Part of it acts as an essential structural or functional component of the cell, and every organism has a certain minimal content of polysaccharide and lipid, which cannot be materially reduced by starvation. Under appropriate conditions, however, the amounts of these materials are greatly increased, and it is this increased portion that is potentially available for reserve use. This concept was clearly stated by Belin (1926). Working with mould fats, he formulated the terms 'élèment constant' for that fraction of the fat which is an essential protoplasmic constituent, and 'élèment variable' for any surplus to this. The 'élèment constant' was considered not to be reducible below a certain minimum value, while the 'élèment variable' was subject to great quantitative variation, and was not truly a part of the protoplasm. Thus he found that the lipid content of <u>Aspergillus niger</u>, which on suitable media rose to considerable proportions, never fell below about 1.4% of the dry weight of the organism, even on prolonged starvation. Similarly, Prill <u>et al</u> (1935) found that the fat content of <u>Aspergillus fischeri</u> dropped, on starvation, from 23% of the dry weight, to 11%, thereafter remaining constant.

Belin's concept can undoubtedly be applied to other cell constituents, and a knowledge of the 'élèment constant' would seem to be a necessary preliminary to any study of a widely variable cell constituent. It can presumably be obtained by growing the organism on

a medium such that growth is limited by deficiency of the material's essential precursors.

Criteria of Reserve Storage.

The quantitative fluctuations shown by the above-mentioned substances, in response to cultural conditions, have frequently been put forward as evidence that they function as reserve nutrients. It must be emphasised that proof that a material acts in this way can only come from a clear cut demonstration that it can be utilised by the cell for the synthesis of essential protoplasmic components, or as a source of energy. In the absence of such proof the materials might have a completely different significance.

For instance, it is not beyond the bounds of possibility that the lipids and polysaccharides that are formed by cells growing on sugar rich media, represent an attempt to render harmless, end-products of metabolism which might otherwise accumulate too rapidly and prove toxic. This could only be a temporary expedient, since, in the case of intracellular materials, unlimited accumulation would eventually burst the cell. Nevertheless, such a function would be strongly indicated if it could be shown that the resulting complexes were, later, simply broken down to soluble units without production of utilisable energy, and thus excreted by the cell. Admittedly, it is unlikely that this would be the case, but nevertheless

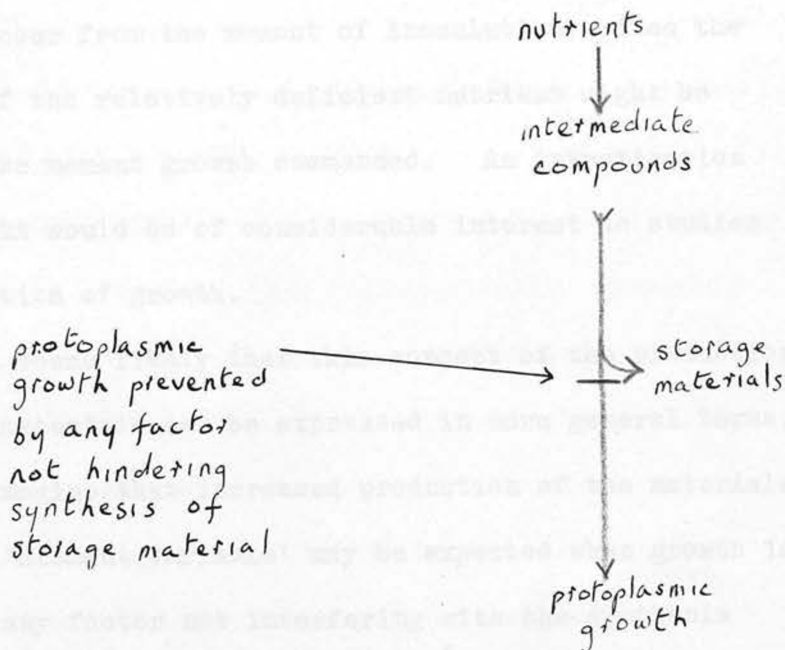
the possible existence of non-reserve functions such as this for the materials comprising Belin's 'élément variable' should not be discounted.

It is clear that the nature of the breakdown of the material is the critical test of its function. If the material is oxidised, it is reasonable to suppose that the energy produced is utilisable, though absolute proof of this would be difficult to obtain. It would be equally difficult to prove that a stored material could be used for growth. The reason for this is twofold: firstly, the total amount of material that a unicellular organism can carry is limited. In general, one finds that not more than about 50% of the dry weight of a bacterium can consist of inert carbon storage materials, the limiting factor being, most probably, cell size. Secondly, the proportion of a carbon containing material that is assimilated, i.e. converted into protoplasmic constituents during growth, is small, since the bulk of the material is used to provide energy and is dissipated as CO_2 and H_2O. In most cases it is probably true to say that not more than about 30% of an exogenous carbon source is assimilated during growth, even when growth is limited by deficiency of the carbon source, and there is no reason to suppose that the assimilation of carbon reserves would be any more efficient. Thus, even maximum storage could only allow a small, fractional increase in growth, and the difficulty of demonstrating this increase perhaps accounts partly for the lack of data on the subject.

Although final proof of the function of a stored material may be elusive, it is relatively easy to demonstrate the material's quantitative variability. In recent years, evidence has been accumulating that the basic mechanism underlying the production of a reserve material (and probably also, the production of all variable cell components) is similar to that stated by Duguid (1948) for extracellular polysaccharide. Duguid found that when the growth of Aero. aerogenes was limited by deficiency of phosphorus or nitrogen, the size of the polysaccharide capsule was greatly increased, and he suggested that any nutritional deficiency which independently limits growth may promote the synthesis of polysaccharide by preserving the carbon and energy source. Similar observations have been made with respect to capsule production by K. pneumoniae (Hoogerheide 1939), polysaccharide production in Chromobacterium prodigiosum and Aero. aerogenes (Bunting et al., 1949; Duguid and Wilkinson 1953) and volutin production by the latter organism (Smith, Wilkinson and Duguid, 1954).

This concept, which is illustrated simply in the accompanying diagram, is not surprising.

(P.T.O)



In effect, cells whose growth has been halted by deficiency of a nutrient can be regarded as comparable to the cells of a washed suspension, except of course, that the cells in a fully grown culture are in a much more complex environment than those of a laboratory washed suspension. Nevertheless they are similar in the sense that further growth is prevented by deficiency of a nutrient, and yet they are still capable of carrying out a variety of metabolic reactions. Chief among these would be the synthesis of whatever storage products could be formed from the remaining nutrients.

It should be pointed out that this concept does not involve any assumption of the time course of the production of the material. One might expect the fastest synthesis of storage materials to occur after the cells had stopped growing, but this does not mean that increased production

might not occur from the moment of inoculation, since the influence of the relatively deficient nutrient might be felt from the moment growth commenced. An investigation of this point would be of considerable interest to studies of the kinetics of growth.

It seems likely that this concept of the production of reserve materials can be expressed in more general terms; one would imagine that increased production of the materials of Belin's 'élément variable' may be expected when growth is limited by any factor not interfering with the synthesis of these materials, provided the necessary precursors and a source of energy are present. In contrast, one would expect no production of a reserve material in cells whose growth had been halted by deficiency of a precursor of the material in question; one assumes that under these 'precursor limited' conditions, any of the 'élément variable' that might be formed would be used up for the further growth permitted by the other nutrients present. If this reasoning is correct, a study of the effect of various nutrient deficiencies on the production of a storage material should provide a convenient way of determining its essential nutrient precursors.

It would seem almost self-evident that, in order for a metabolic product to be of value to the cell as a nutrient reserve, it should remain accessible - i.e. in the cell (or at its surface). The views of Foster (1947) on storage in moulds are of interest in this connection.

Moulds grown on carbohydrate-rich media produce a variety of products which do not accumulate in cultures on carbohydrate poor media. Foster considers that all these products are the result of what he terms "metabolic shunts". They comprise, on the one hand, water-soluble substances such as organic acids, alcohols etc., which may diffuse across the cell membrane, and on the other hand, non-diffusible complexes such as lipid and polysaccharide which accumulate in the mycelium. There is, Foster maintains, no fundamental difference between these two groups of "overflow" product, especially since, as he points out, either group may be used for growth under appropriate conditions, and therefore there is no valid reason for differentiating between them. If, he argues, one regards lipids and polysaccharides as reserve stores, then one must also class the diffusible acids and alcohols as such, a conclusion that he thinks untenable.

In the present author's opinion, Foster has failed to stress sufficiently two important facts.

Firstly, if a shunt product diffuses across the cell membrane, it will, under natural conditions almost certainly be lost to the cell, either by diffusing away from it, or by serving as food supply to an adjacent competitor, and thus cannot function (except under the artificial conditions of a laboratory culture) as a reserve store; a non-diffusible shunt product, on the other hand, by remaining in the cell, is still available for later use, and

Foster's statement that "a water-soluble diffusible compound is by ordinary concepts just as available to the cell as insoluble fat in a vacuole", has little real meaning.

Moreover there is a second striking difference between the two classes of shunt products. Whereas the diffusible organic acids, alcohols, etc., are relatively simple short chain compounds which are formed as intermediate or end products in the breakdown of carbohydrates, lipids and polysaccharides are complex products of anabolism which in most cases are synthesised (probably from the intermediary metabolites) at the expense of considerable energy. *A priori* it seems unlikely that this energy would be thus used unless the resulting synthesis had some survival value to the cell.

- - - - - - - -

The above discussion has outlined some of the problems to be answered in this field of biology. That our knowledge of the nature and functions of the materials considered is incomplete, is a statement that hardly needs reiteration. Of particular interest are the complexes laid down as discrete intracellular structures, since, apart from their probable significance to the general pattern of microbial storage, they have been a source of considerable confusion to bacteriologists, having frequently been the subject of erroneous descriptions of "nuclei",

"gonidia", "chromidia" and the like. While the careful use of modern cytological techniques should enable much of this confusion to be avoided, there is a pressing need for more detailed information concerning the chemical nature of these bodies and the conditions influencing their formation and breakdown. In the long run, such knowledge can only be gained by the use of precise chemical methods of analysis and estimation, and the chief reason for the present lack of knowledge is probably the inadequacy of the methods at present available.

The work recorded in this thesis represents an attempt to remedy this situation in the case of the lipid inclusions of the Bacillus genus. As a first step in this direction, a rapid quantitative method has been developed for their estimation, a method which has also provided some analytical data. Evidence for the value of the method has been gained by its application to a study of the effect of some nutritional deficiencies on the production of the inclusions by B. cereus. A survey of the literature on bacterial lipid inclusions forms the second part of this introduction.

PART II.

A SURVEY OF THE LITERATURE ON BACTERIAL LIPID GRANULES

1. Occurrence: staining methods.

The existence in bacteria of highly refractile intracellular bodies was noted by some of the earliest microbiologists, but it was not until the close of the 19th Century that some of these bodies were identifiable as lipid, for it was not until this period that specific staining methods became available.

Daddi's introduction of Sudan III as a lipid stain (1896) was followed by the discovery of a variety of staining techniques. Among the more important of these was Meyer's use of Sudan III, alkannin, and dimethylamidoazobenzol (1899), and the combined use of naphthol with dimethylparaphenylene diamine (1901a). Dietrich and Liebermeister (1902) described the use of naphthol blue, and their method was modified by Meyer (1903) and others. The year 1909 saw Eisenberg's discovery that many aniline dyes could be treated with precipitating agents to yield selective lipid stains. The principal methods embodied the use of Nile blue sulphate treated with alkali, or basic fuchsin precipitated with various reagents such as iodine, picric acid or alkaline α-naphthol, and the best results were obtained when the cells

were suspended in the precipitating agent, and the suspension then added to the dyestuff.

In the course of the development of these methods, lipid granules were described in many bacteria, including such biologically diverse species as B.anthracis (Grimme 1904), Spirillum giganteum (Ellis 1902-3), and the Timothy Grass bacillus (Grimme 1902b.).

There followed a long period during which little attention was paid to the lipid granules. This may have been partly attributable to the cumbersome nature of the available staining methods. No further advances in staining techniques had been made, and all the early methods employed wet mounts, lacked counterstains, and for the most part did not colour the granules very intensely. Furthermore they afforded no information about the granules other than the fact that they contained "fatty" materials.

Hartman (1940) made the next important technical advance by his introduction of Sudan black B as a bacterial lipid stain, and his method was modified by Burdon, Stokes and Kimbrough (1941). In 1946, Burdon improved this technique still further, and the simple method he described, employing as it does a heat fixed smear, and giving intense coloration of the granules against the counterstained cytoplasm, has virtually superseded all others.

Among the immediate advances made by Burdon was

the demonstration of lipid granules in several species (e.g. Coryne. diphtheriae) previously thought (Lewis 1941) devoid of them. Whatever its cause, this shortcoming of the earlier observations may have been responsible for some of the confusion that lipid granules have caused in the past. Lewis (1941) stated that many of the "gonidia", "nuclei", "chromidia", "spores", "prespores", and even "intracellular parasites" of some earlier workers were in fact lipid granules. Rhizobium and Azotobacter have been particularly concerned in this respect. Many workers have ascribed to these organisms a complex "life-cycle" based partly on the belief that the unstained lipid granules, or the compressed stained cytoplasm between them, were in the fact living bodies, to be released from the cell and form the next stage of the "cycle". (Lewis, 1941).

It seems certain, however, that in most cases, the granules described by Burdon are, whatever their function, lifeless, as they are very irregular in shape, size, and distribution, and their presence is greatly influenced by environmental factors.

Nevertheless, caution should be exercised in interpreting the structures revealed by Sudan black. Any structural entity, or even a diffuse area of the cytoplasm, may take up the stain if its lipid content is high enough. Burdon himself stated that in some cells, a blueish-grey tint (unlike

the dark blue-black colour shown by the inclusions) was imparted to relatively large ill-defined areas of the cytoplasm, and in some bacteria, and most fungi, "a thin irregular peripheral line .... presumably the cytoplasmic membrane" was seen.

Urging the value of the new stain for taxonomic purposes, Burdon surveyed a wide range of yeasts, moulds and bacteria. He concluded that lipid granules occur in "all fungi, and in the great majority of bacteria, whether these are aerobic or anaerobic, saprophytic or parasitic, pathogenic or non-pathogenic".

Table I is a list of the organisms he investigated, grouped according to their lipid forming habits. A list of organisms not studied by Burdon is appended.

TABLE 1.

Group I. Sudan black stainable inclusions regularly present in conspicuous amounts in nearly all the mature cells.		
Gram + ve.	C. xerosis.	Fungi.
Actino. bovis.	Gaffky tetragena.	Aspergillus spp.
Actinomyces species (saprophytic).	Myco. "leprae".	Blastomyces dermatitidis.
	Myco. phlei.	Candida albicans.
B. alvei.	Myco. tuberculosis (avian).	Coccidiodes immitis.
B. anthracis.	Myco. tuberculosis (cold-blooded).	Cryptococcus neoformans.
B. cereus.		Epidermophyton floccosum.
B. circulans.	Myco. spp. (saprophytic).	Histoplasma capsulatum.
B. megatherium.	Sarcina lutea.	Homodendrum pedrosoi.
B. mycoides.	Staph. citreus.	Microsporum gypseum.
Cl. botulinum.	Strept. faecalis.	Mucor spp.
Cl. hystolyticum.	Gram - ve.	Penicillium notatum.
Cl. perfringens.	Acetobacter aceti.	Penicillium spp.
Cl. septicum.	Alkaligenes faecalis.	Phialophora verucosa.
Cl. sporogenes.	Azotobacter beijerinckii.	Rhizopus spp.
Cl. tetani.	Azotobacter chroococcum.	Saccharomyces cerevisiae.
C. diphtheriae.	Chromobacterium violaceum.	Sporotrichum schencki.
C. pseudodiphtheriticum.	Rhizobium leguminosarum.	Trichophyton mentagrophytes.
	Spirillum rubrum.	Rhinosporidium seeberi (in tissue sections).
Group II. Sudan black stainable inclusions usually present, but sometimes absent in cultures on common media.		
Gram + ve.	Myco. tuberculosis (bovine).	Gram - ve.
B. mesentericus.	Myco. tuberculosis (human).	B. brevis.
B. subtilis (Ford).	Staph. albus.	Neisseria catarrhalis.
B. subtilis (Marburg).	Staph. aureus.	Neisseria gonorrhoeae.
Diplococcus pneumoniae.	Strept. pyogenes.	Neisseria Meningitidis.
Lactobacillus acidophilus.	Strept. salivarius.	Neisseria pharyngis.
Group III. Sudan black stainable inclusions usually absent, or present in traces only in a few cells.		
Gram - ve.	K. mutabile.	Ps. fluorescens.
Aero. aerogenes.	K. pneumoniae.	Salmonella enteritidis.
Brucella abortus.	Listerella monocytogenes.	Salmonella schottmülleri.
Brucella melitensis.	P. avi.	Salmonella spp.
Brucella suis.	P. equiseptica.	Serratia marcescens.
Salmonella typhosa.	P. pestis.	Sh. dysenteriae.
Esch. coli.	P. tularensis.	Sh. flexneri.
Esch. communior.	Pr. vulgaris (OX19).	Sh. sonnei.
H. influenzae.	Proteus spp.	Shigella spp.
H. pertussis.	Ps. aeruginosa.	Vibrio cholerae.

Group IV. Organisms containing fatty inclusion bodies, not listed by Burdon.			
Gram - ve.		Spirillum virginianum.	(Lewis 1940).
Azotobacter indicum.	(Starkey & De 1989).	Spirillum volutans.	(Lewis 1940).
Rhizobium japonicum.	(Lewis 1938).	Spirochaeta plicatilis.	(Dyar 1947).
Rhiz. lupini.	(Lewis 1938).	Malleomyces mallei.	(Worley & Young 194
Rhiz. meliloti.	(Lewis 1938).	Fungi.	
Rhiz. trifoli.	(Lewis 1938).	Endomyces vernalis.	(Lindner & Unger, 1919).
Spirillum giganteum.	(Ellis 1902-3).		
Spirillum serpens.	(Lewis 1940).	Hansenula anomala.	(Knaysi 1946c).
Spirillum tenue.	(Lewis 1940).	Nectaromyces reukaufii.	(Rippel, 1943).
Spirillum undula.	(Lewis 1940).	Torulopsis lipofera.	(Kleinzeller, 1944)

2. The Chemical Nature of Bacterial Lipid Inclusions.

Broadly speaking, there are three ways in which information about the chemical organisation of a microscopically visible structure may be obtained.

The first approach is cytochemical, and entails (a) the use of stains specific for particular substances or chemical radicals, and (b) microscopic determination of the effect of solvents or enzymes on the structure concerned. These methods are hampered by lack of knowledge of the specificity of the reagents, by inadequate use of controls, and by the small size of bacteria.

Secondly, there is a technique which deserves wider use than it has enjoyed in the past, and which is applicable to any microscopically demonstrable cell structure which varies widely in size or amount. Cultures containing different amounts of the structure are examined microscopically, and approximate estimates made of the amount of the structure present. Any cell constituent that is found (by macrochemical analysis of the same cultures) to vary in amount in correspondence with the microscopic estimations, is very likely to be a constituent of the structure concerned. Such findings do not constitute a direct proof, but may give a clear indication of the main components of the structure. There is no guarantee, however, that all the components will be revealed.

The third approach is the isolation of the structure, in a more or less entire state, after chemical, enzymic or mechanical degradation of the cell. Analyses of material obtained in this way should yield direct evidence for the nature of the major components of the structure, and may provide the nearest approach to a complete analysis. The chief pitfalls to be avoided are damage to the structure during isolation, and contamination of the isolate with other cell materials.

All three approaches have been used in attempts to elucidate the chemical nature of the lipid inclusions, and the evidence is conveniently discussed under these three headings.

(a) The Cytochemical Evidence.

(i) Staining Methods.

Comprehensive studies of the staining properties of bacterial lipid granules are few, and there are practically no reports of the application to bacteria of established cytochemical techniques for lipids. Probably the main reason for this is the known difficulty and lack of precision inherent in these methods (Gomori, 1952a). However, in a recent review, Cain (1950), has emphasised that, used with care, certain histochemical methods for lipid can yield valuable results, and it is somewhat disappointing that bacteriologists have not paid more attention to them.

Lipid staining methods fall into two categories; (1) those based on the solubility of certain dyestuffs (the "oil-soluble dyes", e.g. Sudan black) in aggregates of lipid, and (2) those dependent on chemical interaction between the lipid and the dye, e.g. the 'acid-haematin' test for phospholipids.

(1) <u>The oil-soluble dyes.</u>

The most important members of this group are the Sudan series of dyestuffs (Sudan III and IV, Sudan black, etc.), Oil red O, and Blue B.Z.L. The bacteriological methods developed by Meyer, Dietrich and Liebermeister, and Eisenberg, which have been referred to already, are also thought to belong in this category.

Their use depends on the fact that the partition coefficient of the dye between the carrier solvent (usually 70% ethanol) and the lipid, is enormously in favour of the lipid. Owing to the similarity in physical properties of all lipids, no distinction between individual lipids is possible with this group of dyes. However, their specificity for lipid is absolute, no other substances being stained (Cain, 1950), and the proper use of Sudan black (nowadays preferred to any of the others) will clearly distinguish lipid from other substances.

Cain's remarks about lipids in the solid state are of considerable interest. Cain stated that a negative reaction with the oil-soluble dyes does not rule out the presence of lipids entirely, since lipids that are solid at the temperature employed in staining are not coloured by these reagents. Now the findings of Lemoigne and his colleagues, which are discussed in detail later, provide evidence that the lipid inclusions of the Bacillus group are comprised largely of a polymer of β-hydroxybutyric acid, which is solid at temperatures below 157°C. If the inclusions consisted only of this polymer, they would not be expected to stain with Sudan black. The fact that they do so stain, as shown by Burdon (1946), suggests that they contain, in addition, other lipids which are sudanophilic. As an alternative, it is not beyond the bounds of possibility that the polymer is in solution, for instances in a complex of fatty acids, in which it is known to be soluble. Such a mixture would almost certainly be sudanophilic.

(2) Stains specific for some classes of lipid.

Nile blue is perhaps the best known of this group. It was introduced in 1908 by Lorraine Smith for distinguishing neutral lipids (triglycerides) from fatty acids. Later workers disputed its value, but Cain (1947) devised a technique for its use which he claimed

to give completely reliable results. A red coloration obtained by his method indicates neutral lipids (esters and/or hydrocarbons). A blue colour signifies the presence of acidic lipids (fatty acids, phospholipines and perhaps some others), though it does not rule out the possible existence of neutral lipids as well. The only recorded report of its use in bacteriology, is that of Delamater (1956). This author stated that the lipid inclusions of B. megaterium were stained blue, thus indicating their content of acidic lipids.

Davis and Mudd (1955) applied Bakers test for phospholipids (Baker 1946, 1947) to Coryne. diphtheriae. They failed to obtain the specific blue coloration necessary for a positive demonstration. Mudd et al (1951) reported similar results with B. megaterium.

Osmium tetroxide is a reagent which, when used under certain specified conditions, is of some value for the detection of reducing lipids, e.g. oleic acid (Cain, 1950). If, however, the conditions used are not stated, or do not conform to those laid down by Cain, no valid conclusions can be drawn. For this reason, the observation by Meyer (1912a) that the lipid inclusions of "B. tumescens"* were not blackened has little value.

* This organism, which was used extensively by Meyer, and which he regarded as a typical representative of the Bacillus genus (Meyer, 1912a), was probably a strain of B. megaterium (Breed et al. 1948). The latter name will be used throughout this discussion.

The observations of Guilliermond (1932) that the lipid inclusions of B. cereus and B. mycoides were not blackened by this reagent, and of King and Beams (1942) that the inclusions of Spirillum volutans were blackened, are also of little significance.

(3) Non-lipid stains.

In contrast to the paucity of strictly specific cytochemical observations, there is a considerable body of somewhat confused and often contradictory evidence concerning the results of applying non-lipid stains. All authors are agreed that lipid granules of various species are not stained with ordinary aniline dyes, or by Gram's method (Meyer, 1899; Grimme, 1902c; Lewis, 1934, 1940). Grimme pointed out that in gram stained bacilli, the granules may be completely masked by the heavy cytoplasmic staining, but where they can be seen, they are evidently completely unstained.

Tests for volutin gave negative results with the lipid granules of B. mycoides (Lewis, 1934), Spirocheta plicatilis (Dyar, 1947), and Spirillum spp. (Lewis, 1940), but Knaysi (1929) found that certain metachromatic granules of Myco. tuberculosis took up fat stains "to a great extent", and Davis and Mudd (1955) demonstrated lipid in the metachromatic bodies of Coryne. diphtheriae. These findings emphasise the caution necessary in applying

results obtained with one species, to another, unrelated one, and also suggest that lipid may sometimes form a mixed deposit with volutin and perhaps other substances.

Lewis (1934) stated that Ziehl-Neelsen's method did not stain the lipid inclusions of B. mycoides. Meyer (1912a), on the other hand, found that the inclusions of B. megaterium, when stained with carbol fuchsin, resisted decolorisation with 5% H_2SO_4, and Bunge (1895) reported similar findings with B. megaterium and B. anthracis.

That the lipid inclusions of the Bacillus genus and Azotobacter are free of glycogen is evident from the observations of Meyer (1899, 1912a), Grimme (1902a), Stapp (1924) and Lewis (1934, 1937). These authors used a solution of iodine in potassium iodide as their test for glycogen, which, according to Grimme (1902c) is coloured a "red-brown" by this reagent. The value of this solution as a cytochemical reagent is somewhat lessened, however, by the fact that the lipid inclusions are also stained, the resultant colour being described as "dark yellow" by Grimme, and "brown" by Meyer.

Knaysi (1945b, 1946a) studied the behaviour of the lipid inclusions and cytoplasmic membrane of B. cereus during growth. He found the granules gave a positive "Sharp" test* (protein) and Feulgen reaction, as well as

* Details of this test were published by Knaysi (1942). It consists of heating a smear with a solution of a reducing sugar for about a day at 100°C. Proteins are said to be specifically stained brown. The test gave a positive reaction with albumen, but no other proof of its validity was reported.

staining with Sudan black. These reactions were also given by the cytoplasmic membrane. Further evidence of a relationship between the inclusions and the membrane was provided by the observation that, towards the end of the growth phase, the cytoplasmic membrane developed protruberances which, under aerobic conditions, were released into the cytoplasm to form typical lipid granules. The author concluded that the lipid granules consisted of lipoprotein, and suggested that the positive Feulgen reaction might be due to the presence of a Feulgen-positive lipid. Feulgen-positive lipids ("Plasmal") are well known to histochemists (Gomori, 1952b) but so far they have not been reported to occur in bacteria; Plasmal was not microscopically visible in several bacteria, including B. megaterium, studied by Dondero et al (1954). Knaysi's observations have not so far been confirmed, though this may be because the staining is very feeble, and easily missed.

A completely unique type of experiment that is most conveniently recorded here, was that of King and Beams (1942). These workers subjected Spirillum volutans to ultracentrifugation. This treatment caused the cells to float horizontally, and they were oriented in a fixed position relative to their longitudinal axis. This behaviour was due to a redistribution

of the lipid granules, which had been forced into the "troughs", thus obliging the organism to float in the horizontal position. It was deduced that the lipid granules were denser than the cytoplasm, an unexpected result, since the granules were thought to be fatty in nature, in which case they would most probably have been less dense than the cytoplasm. Osmic acid was used as a fat stain, and it was found that only a few of the granules were stainable with this reagent. The authors concluded that the granules, which had been shown by Lewis (1940) to be stainable with Sudan black, were not essentially lipid in nature, but that they sometimes became coated with lipid material. Knaysi (1951a) thought it more likely that the granules were lipoprotein.

(ii) <u>Microscopic Solubility Tests.</u>

While most lipids are equally soluble in all lipid solvents, some distinction between lipids can be made on the grounds of differing solubilities in certain solvents. For instance, pure phospholipids are insoluble in acetone, and sphingomyelin is insoluble in ether. Tests of the solubility of lipid inclusions in a variety of solvents, therefore, should yield some information as to their chemical nature. Unfortunately, such solubility tests are fraught with difficulties. Thus, as Cain (1950) pointed out, a lipid

which is insoluble in a given solvent may be partly dissolved in the presence of another, easily soluble lipid. This factor is most important in view of the mixed composition of most natural lipids. Moreover, the accessibility of the inclusion to the solvent is dependent on the nature of the tissue, and the method of fixation employed, as well as on the nature of the solvent, and even if it is known that the solvent has free access to the inclusions, a coating or other barrier of insoluble lipids may render it ineffective. Some bacteriologists have determined the effect of a solvent simply by mounting the cells in it, and noting any change in the appearance of the inclusions. This is not a good method, since it is not always easy to distinguish between a full inclusion and the empty vacuole that would be left by removal of its contents. As Knaysi (1951a) has pointed out, if the solvent has a high refractive index, the inclusions may appear "optically empty" even though no material has been removed.

In view of these difficulties, it would seem advisable to control all microscopic tests of solubility by examining the extracted cells in water mounts, by a control extraction with pyridine (found by Baker (1946) to be the most efficient solvent for the complete removal of all lipids), and by the use of Sudan black. In this connection, however, the investigator should take note of Cain's warning, "the enormous importance of Sudan black must not blot out from

memory its inability to demonstrate solid lipids".

The observations of Meyer (1899, 1901a, 1912a) are a good example of the caution with which solubility tests should be carried out. This author subjected B. megaterium both in suspension and in dried smears, to the action of various solvents and almost always checked the results by examining the cells in water mounts, and by using lipid stains. In this way, he was able to show, with a fair degree of certainty, that glacial acetic acid removed the inclusions completely, leaving empty vacuoles. Chloral hydrate was equally efficient, but alcohol had no effect even after 24 hours treatment, and the only change induced by chloroform was that the inclusions became clumped together in irregular masses. Meyer considered that the last-named solvents probably had difficulty in penetrating the cell membrane. Potassium hydroxide, on the other hand, easily penetrated the cell, and if a 1% solution was allowed to act on a suspension of the cells for 24 hours at 28°C, the inclusions were apparently completely saponified, the resulting soap diffusing out of the cell.

The work of later authors has mostly been insufficiently detailed; as a result, many of the observations are contradictory, and do not lead to any useful conclusions concerning the nature of the inclusions. For this reason, they will not be discussed in detail, but are summarised in Table 2.

TABLE 2.

SOLVENT	METHOD	ORGANISM	RESULT	AUTHOR
Ether	not stated	B. cereus	insoluble	Dyar 1948
	not stated	"sporing aerobes"	insoluble	Imsenecki 1945
	not stated	Azotobacter spp.	insoluble	Bisset 1953
Ether/alcohol	Dry smear fixed in solvent, stained with Sudan III or Indophenol blue	B. cereus B. mycoides	loss of Sudan staining property only	Guilliermond 1932
Alcohol	not stated	Azotobacter spp.	insoluble	Bisset 1953
	not stated	"sporing aerobes"	insoluble	Imsenecki 1945
(hot)	not stated, but effect judged by Sudan black stain	B. cereus	no loss of staining props.	Dyar 1948
	heat fixed smear, effect judged by Sudan black stain	B. cereus	slight loss of staining props.	Burdon 1946
	Dry smear	B. megaterium	insoluble	Meyer 1912a
Chloroform	not stated	"sporing aerobes"	insoluble	Imsenecki 1945
	dry smear	B. megaterium	insoluble	Meyer 1912a
	heat fixed smear, effect judged by Sudan black stain	B. cereus	slowly soluble	Burdon 1946
Glacial acetic acid	wet mount	B. megaterium	soluble	Meyer 1912a
	heat fixed smear judged by Sudan black stain	B. cereus	slightly soluble	Burdon 1946
	not stated	B. cereus	insoluble	Knaysi 1951a
	not stated	"sporing aerobes"	soluble	Imsenecki 1945
Acetone	heat fixed smear, effect judged by Sudan black stain	B. cereus	slowly soluble	Burdon 1946
	not stated	B. cereus	insoluble	Knaysi 1951a
	not stated	"sporing aerobes"	insoluble	Imsenecki 1945
Xylol	not stated	Azotobacter	soluble	Bisset 1953
Carbon tetra-chloride	heat fixed smear, effect judged by Sudan black stain	B. cereus	slightly soluble	Burdon 1946

Another cytochemical test recorded by Meyer (1901b) was his use of "Eau de Javelle" to distinguish between lipid inclusions and spores. When cells of B. megaterium were mounted in this reagent (which is, in fact, alkaline solution of sodium hypochlorite), the cytoplasm would be seen to fade away and the lipid inclusions and spores were soon free inside the cell membrane. After a while, the spores became swollen, and lysed, leaving only their membranes. Finally, the cell membrane disintegrated leaving only the lipid inclusions and the spore membranes. Meyer suggested that the inclusions were so resistant to this reagent that it might be found of value for their large scale extraction, and this thesis presents evidence for the correctness of this view. Meyer also found (1912a) that the inclusions could be liberated from the cytoplasm by the action of 5% H_2SO_4 at 90°C for 5 hours.

(b) Chemical Analysis of Cells Containing Different Amounts of Lipid Inclusions.

Some 33 years ago, Lemoigne, working at the Pasteur Institute, commenced a series of studies of bacterial autolysis that led to the discovery of an apparently unique biological compound, which, after a lapse of nearly 20 years, was shown to constitute a major fraction of the lipid inclusions of certain soil bacteria.

Lemoigne's first publication in this field (1923) concerned the autolysis of "Bacillus M" - a strain of B. megaterium. He found that during the course of several

days autolysis at 30°C, this organism produced a great deal of acid. This acid production was independent of the presence of oxygen, was not accompanied by the evolution of gas, and 60% - 75% of the resultant acidity was due to β -hydroxybutyric acid (1925b). This hydroxy-acid was shown to be present, in relatively small amounts, even in fully viable, non-autolysed cells, and the source of it during autolysis was an intracellular compound that could not be removed by simple washing (1924).

In a search for this compound, two main substances, each having the empirical formula $(C_4H_6O_2)n$, were discovered (1924a, 1925a, 1927b). Both were polymers of β-hydroxybutyric acid. The first, melting at 120°C, formed only a small proportion of the dry weight of the cells, but the second (melting point 157°C) was present in amounts that easily accounted for the acid produced during autolysis.

More recently, Lemoigne and Roukhelman (1940a, b) recommenced the study of this polymer. With the aid of a simplified extraction procedure, they followed its formation and disappearance in B. megaterium grown on solid medium. They found that the polymer was produced in abundance during the first two days of growth, and the cells at this stage were packed with lipid inclusions. Between the second and third days, there was a rapid breakdown of the polymer coincident with the appearance of spores. Unfortunately, the authors did not make detailed observations of the amount of lipid inclusions, or of their fate during

sporulation, and they were apparently unaware of any possible relationship between the inclusions and the polymer.

This relationship was first clearly stated by Lemoigne, Delaporte and Croson (1943, 1944). These authors found that, in the *Bacillus* genus, the polymer was only present in species bearing visible lipid inclusions. It was present in all strains examined of *B. cereus* (49 strains), *B. megaterium* (30 strains), *B. mycoides* (6 strains), *B. anthracis* (1 strain), and *B. polymyxa* (1 strain). It was absent from 17 strains of *B. subtilis* and 57 strains of *B. mesentericus* - both species that do not normally form visible lipid inclusions. These results clearly indicate a close relationship between the inclusions and the polymer. The evidence for this relationship would have been greatly strengthened, however, by analyses of cultures containing different amounts of the polymer, coupled with approximate microscopic estimations of the amount of lipid inclusion material present. Moreover, these findings do not, in themselves, constitute direct proof of the nature of the inclusions. Such evidence will, however, be provided in this thesis, and for the purposes of subsequent discussion, it will be assumed that the inclusions are largely composed of this polymer.

The properties and biological occurrence of these polymers (three closely related ones have been found) will be the subject of a later section of this discussion (p. 38 *et seq.*). It is convenient, however, to record here

Lemoigne's observations of the breakdown of the polymer during autolysis, since it was this breakdown that led him to its discovery.

Lemoigne (1926b) measured the amounts of the two main polymer fractions during autolysis at 30°C of B. megaterium. The amount of free β-hydroxybutyric acid formed was also determined, and the total "β-hydroxybutyric substances" computed, in terms of the free acid. The results are set out in Table 3.

It is clear that the total of these substances remained effectively constant, while the amount of the first fraction (that melting at 157°C), decreased, thus giving rise to increased amounts of the fraction melting at 120°C, and of the free acid.

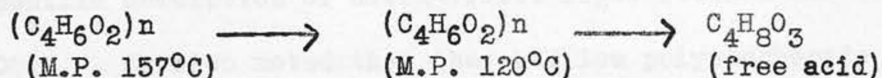
TABLE 3

(Data of Lemoigne 1926b)

	Initial	4 days	9 days	19 days
Acidity (cc. normal acid)	21.91	121.1	136.6	145.0
Free acid	1.6g	10.6g	11.5g	13.2g
Polymer (M.P. 120°C)	1.0g	3.9g	5.7g	5.8g
Polymer (M.P. 157°C)	17.2g	6.9g	4.4g	4.2g
Total compounds	19.5g	19.6g	19.6g	20.9g

The depolymerisation of poly β-hydroxybutyrate during the autolysis of B. megaterium.

Thus, Lemoigne was able to deduce that the autolytic process was as follows:



Lemoigne refers to the polymers as "lipides β-hydroxbutyriques". The present author intends to use the term "poly β-hydroxybutyrate", abbreviated for convenience to "PHB". Unless otherwise indicated, this term and its abbreviation can be taken to refer to the most important member of the group, which is the polymer melting at 157°C.

(c) <u>The Evidence Obtained by Isolation of the Inclusions.</u>

The only work of this type is that reported by Weibull (1953a, b). In the course of experiments on the effect of lysozyme on <u>B. megaterium</u>, Weibull discovered that controlled treatment with this enzyme in the presence of sucrose or other stabilising agents led to the dissolution of the cell wall, the resulting protoplast being fairly stable. On diluting a suspension of protoplasts with phosphate buffer, however, the protoplasts lysed, all that remained being the so called "ghost fraction". The "ghosts" were thought probably to be the cytoplasmic membranes; the lipid granules were still present inside them, and could be separated from the ghosts by centrifugation at 590G for 15 minutes. Weibull referred only briefly to the granules, stating that they could be stained with Sudan

black, were almost completely soluble in warm chloroform or warm alkali, and that the alkaline solution showed no specific absorption of ultra-violet light between 250 and 300mμ. He also noted that they had low polysaccharide and nitrogen contents, the figure for the latter being 0.25% (Weibull, 1956).

All these properties are consistent with the presence of the polyβ-hydroxybutyrate, and Weibull considered his findings to be confirmation of Lemoigne's results. It should, however, be noted that this confirmation would have been greatly strengthened by a more detailed characterisation of the inclusion material. The degree of solubility in ether (PHB is insoluble) would have been of great value.

- - - - - - -

In summary, it is clear that Lemoigne's discovery of polyβ-hydroxybutyrate has provided the most important contribution to our knowledge of the chemical nature of bacterial lipid inclusions. The existence of other materials is likely, however; in particular, the probable presence of Sudan staining components can be deduced, and the work of Knaysi suggests the possibility that protein and perhaps other substances may be found. It should be pointed out that the great bulk of the experimental work recorded in the literature has been concerned with the inclusions of the large-celled <u>Bacilli</u> and <u>Azotobacter</u>.

With the exception of certain suggestive observations that will be discussed in a later section, there is little evidence that the lipid inclusions of other groups are either similar in composition, or radically different.

3. Chemical and Physical Properties of the Polyβ-hydroxybutyrates. $(C_4H_6O_2)n$.

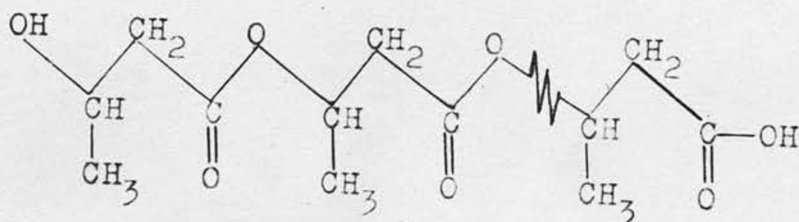
In all, three different polymers have been described by Lemoigne and his colleagues.

Fraction 1: Melting point (nominal) 157°C.

This is the most important of the three, and may occur in amounts as great as 50% of the dry weight of the cells (Lemoigne, 1947). It is insoluble in water, paraffins, ether, acetone and lower alcohols, and soluble in chloroform, glacial acetic acid and other fatty acids, higher alcohols, chlorine-containing solvents, and solvents with a cyclic or heterocyclic nucleus, (Képès and Péaud-Lenoël, 1952). On cooling, solutions in many of these solvents yield gels (Képès and Péaud-Lenoël 1952). On evaporation of a chloroform solution, however, the polymer separates as a film on the bottom of the vessel, described by Lemoigne (1927b) as "transparent if it is thin, white and opaque if it is thicker, but in all cases coherent, and easily and spontaneously detachable from the surface of the vessel".

Képès and Péaud-Lenoël (1952) considered the polymer to be an unbranched polyester of β-hydroxybutyric acid, since they were able to demonstrate free terminal (-OH) and (-COOH) groups. The structure appeared to be:

(P.T.O)



These authors produced evidence that the polymer extracted by Lemoigne's method (which involves an initial treatment of the cells with strong acid) is, despite its sharp melting point, a mixture of homologous polymers having slightly different chain lengths. This range of polymers, having melting points between 136°C and 176°C, and molecular weights (determined by estimation of the terminal groups) lying in the range 500 to 10,000, could be separated by fractional precipitation of a chloroform solution with ether.

The extraction of crushed cells with a mixture of chloroform and dioxane, on the other hand, yielded a homogenous polymer having a melting point of 179°C. The authors considered that Lemoigne's product was a somewhat degraded form of the polymer as it exists in vivo.

Lemoigne realised that his extraction technique might degrade the polymer (Lemoigne, 1947) but he had found the acid treatment necessary to ensure reproducible, quantitative extraction (Lemoigne, 1927b).

Warm alkali dissolves PHB fairly readily, and

strong alkali, by depolymerisation and hydrolysis, converts it to a mixture of β-hydroxybutyric acid (CH_3-CHOH-CH_2-COOH) and its dehydration product α-crotonic acid (CH_3-CH=CH-COOH) (Lemoigne, 1927b). This crotonisation can be largely overcome by the method of hydrolysis used by Péaud-Lenoël (1949), which involves 10 hours hydrolysis at 100°C with 10% sodium carbonate.

α-crotonic acid is also formed when PHB is heated. If the operation is conducted in a small glass tube, the acid sublimes, and small crystals with a characteristic appearance and odour, condense on the cooler parts of the tube (Lemoigne, 1927b).

Fraction 2: Melting point (nominal) 120°C.

This fraction is quantitatively less important than the previous one, and is generally present in amounts of the order of 10% of the dry weight of the cells or less (Lemoigne, 1947). In addition to having a lower melting point than the previous fraction, it is soluble in boiling alcohol (Lemoigne, 1927b) and boiling acetone (Lemoigne, 1947). It can be crystallised out of cold alcohol, and has a molecular weight probably equal to approximately 500 (Lemoigne, 1927b). In all other respects it closely resembles the first fraction, and is undoubtedly a polymer of β-hydroxybutyric acid.

Fraction 3: Melting point 115°C - 116°C.

This polymer was found as a very minor constituent of B. megaterium, constituting less than 0.1% of the dry weight of the cells (Lemoigne, Milhaud & Croson, 1949). It

had the same empirical formula as the other polymers and was soluble in ether as well as in boiling alcohol. Hydrolysis with 50% H_2SO_4 yielded α-crotonic acid, and although too little was available for complete characterisation, this compound was cogently another polymer of β-hydroxybutyric acid, probably representing a late stage in depolymerisation of the other polymers.

It will be noticed that the melting points of the fractions listed above have been quoted as "nominal". This seems advisable in view of the findings of Képès and Péaud-Lenoël (noted above) of the apparent inhomogeneity of the fraction melting at 157°C extracted by Lemoigne's technique. The possibility arises that the 120°C fraction also is inhomogeneous, although in both cases the sharpness of the melting points does not support this view. It would seem that the homogeneity of the fractions should be investigated further, since the findings would have an important bearing on the question of the natural mode of breakdown of the polymer in vivo. If the complete homogeneity of the fractions were proved, one would have to postulate a stepwise mode of breakdown of the polymer, such that the primary fraction is split, in a single step, into two or more identical parts, which would constitute the second fraction, and which would in turn be broken down in a single step to the third fraction, which would then yield the free acid. Such a scheme is represented diagrammatically in Fig. 1.

If, on the other hand, each fraction was found to be a mixture of polymers of different chain lengths, the course of depolymerisation would be more likely to be of the type shown diagrammatically in Fig. 2.

Figure 1.

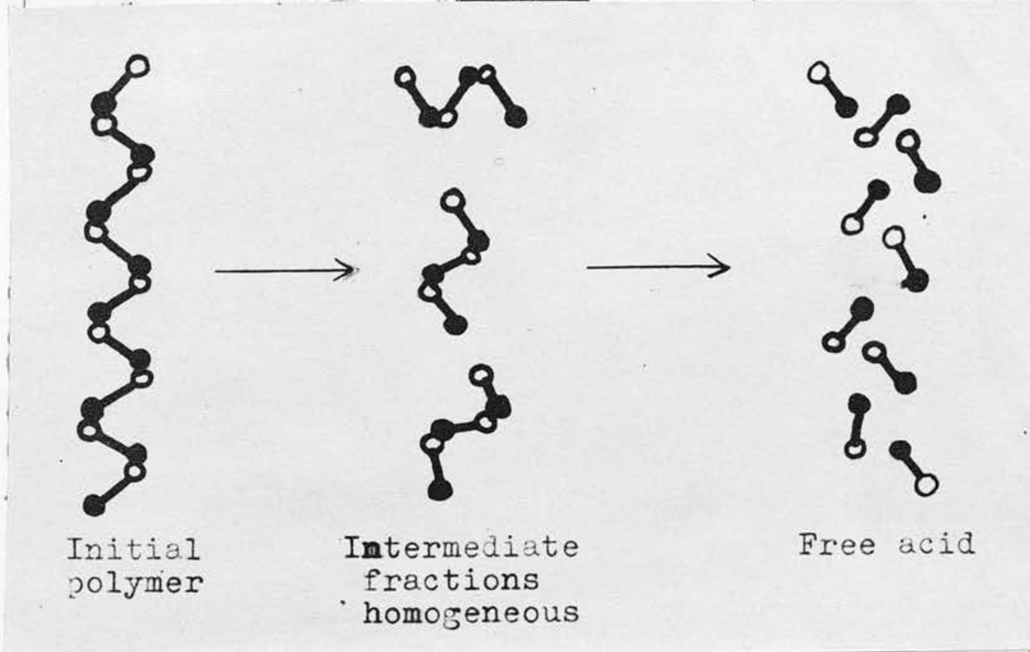
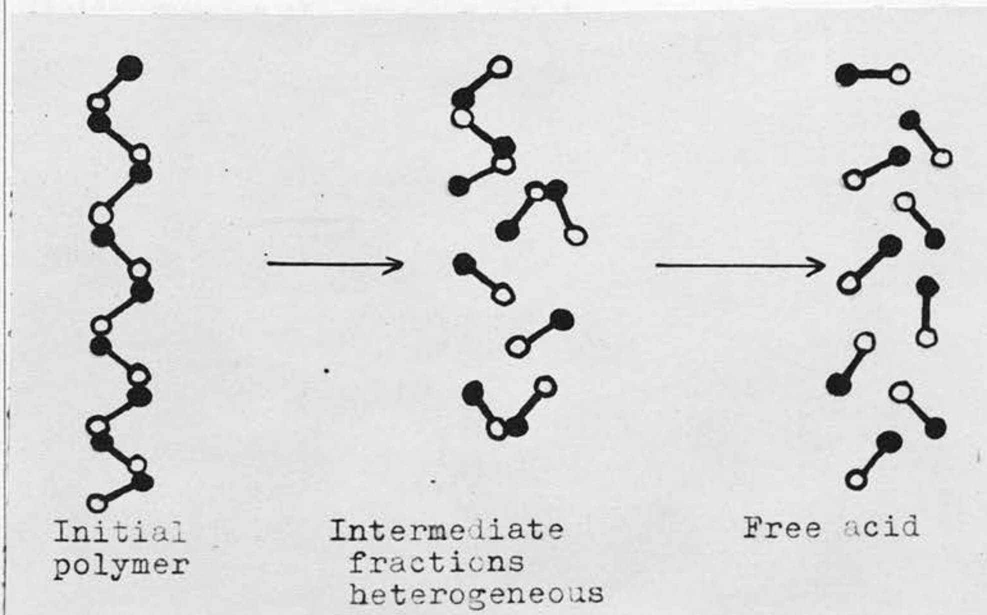


Figure 2.



4. Occurrence of Poly β-hydroxybutyrates.

As far as this author is aware, PHB has not been reported to occur naturally, other than in a few bacteria. Its presence in the Bacillus group has already been noted. In addition, it has been found in Azotobacter chroococcum (Lemoigne & Girard 1943, 1944), a species known to form visible lipid inclusions (Burdon, 1946), and in Micrococcus halodenitrificans (Smithies et al., 1955). In the latter organism it constituted only 2% - 3% of the dry weight of the cells, and in cells disrupted by sonic vibrations, it sedimented with the cell wall fraction. It was not stated if this organism formed lipid inclusions.

Although these reports are the only instances where PHB has been recognised, there are certain observations in the earlier literature which, when allowance is made for faulty techniques and lack of knowledge of the properties of lipids, suggest the possible existence of the polymer in other organisms.

The first of these organisms is Rhizobium, which is known to form prominent lipid inclusions (Lewis, 1938). Möller (1892) found that the inclusions of this organism were insoluble in cold dilute KOH, cold or boiling concentrated NH_4OH, hot ethanol or amyl alcohol, ether, benzene or carbon disulphide. They were soluble in chloroform, acetone, glacial acetic acid, clove oil, and less readily in benzol. Möller concluded that the results were indicative of a fatty or waxy substance corresponding most nearly to cholesterol, but not identical with it. With the exception of the solubility in acetone, these properties are very like those of

PHB. Hopkins and Peterson (1930) extracted Rhizobium meliloti with ether and chloroform. The ether extract constituted about 0.6% - 1.2% of the dry weight of the cells, while 10.2% - 21.7% of the cells' dry weight was found in the chloroform extract. The material in the latter extract had a melting point of 172oC - 173°C, contained only a trace of sterol, and saturated mono-hydroxy acids having an average molecular weight of 117 were liberated from it on saponification.

Secondly, there is the report of Goris (1920) on the chemical composition of the tubercle bacillus. Goris found that about 1% of the dry weight of this organism was composed of a substance which he named "hyalinol". It was insoluble in ether, but soluble in chloroform, and when pure, formed a transparent glassy pellicle rather like a collodion membrane. Its melting point was about 175°C.

The observation of King and Beams (1942) that the lipid inclusions of Spirillum volutans were denser than the cytoplasm has already been noted. Evidence will be presented in this thesis that PHB is a dense material, and it seems likely that the inclusions of this organism may be similar to those of the Bacillus group and contain a large proportion of PHB.

The above observations at least suggest groups of organisms which might profitably be examined for PHB, and in particular, the report of Smithies et al. (1955) of the presence of the polymer in a Micrococcus suggests that it may be much more widely distributed than is at present realised.

Reference has already been made to the virtual absence of PHB from species such as B. subtilis which do not form appreciable quantities of lipid inclusions. It appears that, in fact, very small amounts of the polymer may be present. Thus, 24.0g. of dried B. subtilis cells yielded about 7.2 mg. of PHB, i.e. about 0.029% of the dry weight (Lemoigne, 1947). It would appear that this material was dispersed throughout the cells, presumably in the form of sub-microscopic granules. Its presence in such small amount in no way invalidates the close relationship between the presence of PHB and the presence of lipid inclusions, especially in view of the fact that B. subtilis does sometimes form the latter (Burdon, 1946).

5. Factors Influencing the Production of the Lipid Inclusions, and of PHB.

It is clear that the lipid inclusions are not essential to the immediate life of the cell, since, in most species, they are formed in widely varying amounts depending on the age of the culture and the nature of the culture medium (Burdon, 1946; Grelet, 1952; Meyer, 1912a). The factors governing these variations are not so well understood, however. It is unfortunate that no systematic investigation has been made, the few observations that do exist being incidental to studies of other aspects of the behaviour of the cell. Nevertheless, one may draw some limited conclusions from a study of the literature.

It has often been observed that media rich in sugar favour the growth of cells bearing large amounts of (total) lipids (Larson & Larson, 1922; Dawson, 1919; Cramer, 1893; Lyons, 1897). While the same general relationship appears to hold good for the lipid inclusions as such, it seems that the presence of sugar is not essential, since Burdon (1946) found the formation of lipid inclusions to be "independent of the presence of glucose, glycerol or other fermentable carbohydrate". This author also noted that special media may be necessary for the formation of the inclusions by some organisms. Thus, when B. subtilis was grown on plain or glucose nutrient agar, only traces of Sudan stainable lipid were formed, but appreciable quantities appeared when the same species was grown on potato slants or glucose starch agar.

Knaysi (1951b) found that, under good aerobic conditions,

the inclusions of the Bacillus group were found in about the same number whether or not a fermentable sugar was present, but he also observed that in the presence of the sugar, the volume of the inclusions was greatly increased. Imsenecki (1945) stated that the lipid inclusions of various spore bearers "increased" on carbohydrate rich media. Thus it seems clear that the presence of sugar or other carbohydrate increases the amount of lipid inclusion material, though it is not essential to its formation.

Aeration, on the other hand, is a crucial factor, at least as far as the Bacilli are concerned. Knaysi (1951b) stated that these organisms do not form lipid inclusions anaerobically. The inclusions occur regularly, however, in certain strict anaerobes (Burdon, 1946; Knaysi, 1951b). The reason for this apparent anomaly is not clear. Perhaps the chemical composition of the inclusions of the two groups is different.

In the course of a study of the sporulation of B. megaterium, Grelet (1952) described the microscopic appearance of cells grown on different media. He noted that when growth was limited by deficiency of phosphorus, sulphur or potassium, the lipid inclusions appeared in large numbers, whereas limitation of growth by deficiency of manganese had no such effect. These findings suggest that manganese is specifically required for the formation of the inclusions, but the process appears to be relatively independent of the presence of potassium, sulphur and phosphorus.

Of considerable importance is the fact that a nutritional deficiency may, theoretically, exert its influence on the production of a storage material in one of two ways. It may produce its effect by limiting the total growth attainable on the medium, or it may act by reducing the rate of growth independently of the rate of production of the storage material, thereby causing a temporary relative increase in the amount of the latter. A given deficiency, may of course act in both these ways, but in any case, the two effects are easily distinguished, since the "rate limiting effect" is only operative during the phase of active growth, and is accompanied by a reduced rate of growth, and the "yield limiting effect" applies to the final level of production in the stationary phase cells. All observations of the production of a storage material should therefore be related to the stage of the growth cycle reached by the culture at the time of observation. Unfortunately, few authors seem to have been aware of these considerations, and most observations have not been accompanied by any indication of the cultural phase at which they were made. There is some indication that "rate limiting effects" may apply to the production of the lipid inclusions, since Burdon (1946) stated that any factor that retarded cell division tended to give rise to an increased production of Sudan black stainable material. More precise data will be presented in the course of the cultural studies reported in this thesis.

With regard to the formation of PHB, Lemoigne (1927a) found that on media containing casein as sole carbon source, B. megaterium grew feebly and contained little PHB. Good growth, on the other hand, with considerable production of PHB, resulted when the cells were cultured in the presence of glucose, sucrose or maltose (Lemoigne, 1947).

The data of Lemoigne et al. (1945) are of considerable interest, since they illustrate two of the fallacies which must be guarded against in any study of the production of storage materials.

The authors grew B. megaterium in liquid and in solid synthetic media containing different amounts of glucose. Growth was estimated (over a period of a few days) by measurements of the total dry weight, and parallel measurements were made of the PHB contents of the cells. Some representative results are shown in Table 4.

TABLE 4.

(Data of Lemoigne et al. 1945)

Glucose content of medium (per 100 ml)	Age of Culture (hours)	Total dry weight (per 100 ml)	Total PHB (per 100 ml)	PHB as % of dry weight	"Corrected dry weight of cells" (see text)
6.30g. liquid medium.	71	1.08g	631mg.	58	0.449g.
3.03g. liquid medium.	56	1.03g	556mg.	54	0.474g.
3.0g. solid medium.	72	0.96g.	252mg.	25.9	0.708g.

The authors made no statement concerning the effect of the concentration of carbon source on the amount of PHB formed by the cells. The unwary observer might, however, be led to the conclusion that the concentration of carbon source had no effect, since doubling the amount of glucose in the liquid medium did not appreciably affect the cell's content of PHB. However, it is apparent that both the liquid media contained more glucose than was necessary for the maximum growth supported by the other nutrients present, since the growth obtained on the second medium was no greater than that obtained on the first. Moreover, it is likely that the amount of PHB stored by these cells (on both the liquid media) was near the maximum possible, since 60% of the dry weight is the highest figure so far reported for any organism (Lemoigne, 1947). Certainly one would expect limitation of intracellular space to set an upper limit to the amount of PHB that could be stored, and the amounts present in both these cultures must have been approaching this limit. If this were so, no increase in the supply of glucose could have led to an increased PHB content, and one is therefore not justified in concluding that the concentration of glucose (at lower levels) would have no effect on the amount of PHB stored.

The second interesting feature of these results concerns the difference between the cultures on the liquid and solid media. It will be seen that the cell content of PHB (in terms of % dry weight) on the 3% glucose liquid medium is

just over twice that in the solid medium, although the total dry weight of cells is about the same in each case. The authors attributed this difference in PHB levels to a difference in the rate of diffusion of oxygen and nutrients in the two cultures. They claimed that in the liquid medium (which was aerated by agitation) the cells were in continual contact with oxygen, and with the homogeneous solution of glucose and other nutrients. On the solid medium, on the other hand, diffusion of nutrients to the layer of growing cells was slow, and only the cells on the upper surface were in contact with air. This would very probably account for the faster rate of growth which was reported in the liquid medium, but the present author suggests that the difference in PHB levels can be accounted for in other terms.

The first point to notice is that in experiments of this type, where one wishes to distinguish between growth (i.e. formation of protoplasm) and storage, the total dry weight of the cells is not a very satisfactory estimate of growth, since, as was pointed out by Belin (1926), it includes the weight of any storage materials as well. Thus, an increase in the total dry weight may be due to an increased synthesis of protoplasm, or to increased storage, or to both. A much more satisfactory estimate of growth is obtained by a measurement of total protein, which, in bacteria, is most easily achieved by determining the total (non-dialysable) nitrogen. The data of Lemoigne _et al_. do not include any measurements of protein, but nevertheless it is possible to calculate a better estimate of growth than is provided by

their dry weight figures. This is done by subtracting the figure for total PHB from the corresponding figure for total dry weight. The figure obtained in this way includes the total protein plus, of course, any stored polysaccharides, and lipids other than PHB. It is therefore not a perfect estimate of total protein, but it is undoubtedly better than the simple measurement of dry weight used by the authors.

The corrected figures obtained in this way are shown in column 6 of the table. It is clear that the true 'growth' (as distinct from storage) on the solid medium, was much higher than that on its liquid counterpart. This means that on the solid medium, there was relatively much less glucose available for conversion into PHB than in the liquid medium, since much more glucose had been used for the synthesis of protoplasm. This is probably the real reason for the lower PHB level attained on the solid medium. At any rate, this suggestion seems more satisfactory than the rather vague proposals of Lemoigne and his colleagues.

For the reasons outlined above, most of the data referring to cell content of PHB that are recorded in this thesis are related to the total nitrogen of the cultures, and not to the total dry weight.

\- - - - - -

In summary, it would seem that aerobic conditions are essential to the production of lipid inclusions by the <u>Bacilli</u>, and that a supply of sugar or other carbohydrate is stimulatory, though not essential.

6. The Biological Role of Bacterial Lipid Inclusions.

(a) The Reserve Store Theory.

Several authors have reported, on the basis of microscopic observations, that the lipid inclusions of the Bacillus group are formed in abundance on carbon rich media, and disappear during starvation or sporulation (Preisz, 1904; Meyer, 1899; Lewis, 1934; Imsenecki, 1945). These observations have given rise to the commonly accepted theory that they function as "reserve materials". In general, the meaning of this term is fairly clear, and the fact that it has so frequently been used without precise explanation of its meaning, is probably mainly a reflection of the difficulty of compounding a definition that is completely comprehensive and unambiguous. It may also, however, be due to recognition of the fact that microscopic observations of the behaviour of the inclusions, of the type noted above, do not by themselves provide an adequate basis for the characterisation of a reserve material. This point has already been discussed; it is only necessary here to emphasise that the function of a suspected reserve storage material can only be proven if it can be clearly shown that the material in question can be used, under the appropriate conditions, for the synthesis of essential cell constituents, or as a source of energy. While the conditions influencing the formation and disappearance of the lipid inclusions are highly suggestive, they do not constitute such a proof, and the role of the inclusions must remain in doubt.

A special development of the reserve store theory is the commonly made suggestion that the inclusions of the large-celled species of Bacillus act as reserves for the process of spore formation. This theory was initially developed on the basis of microscopic ovservations of the inclusions during sporulation. As will be seen, the cytological evidence is not very satisfactory, but recent studies of the behaviour of PHB in the sporulating cell have provided more convincing support for this view.

It is generally agreed that during, or sometimes just prior to the appearance of the spore, there is a reduction in the size and/or number of the lipid inclusions (Bayne-Jones and Petrilli, 1933; Lewis, 1934; Knaysi, 1946b; Burdon, 1946; Chapman, 1956). This breakdown is well substantiated. It is not due to absorption of the intact inclusions by the spore, since they have been seen to strike the intangible boundary of the zone of spore formation without entering it, (Bayne-Jones and Petrilli, 1933; Knaysi, 1951c), and there is no evidence that the spore contains stainable lipid. Moreover, Lemoigne and his colleagues (Lemoigne & Roukhelman, 1940b; Lemoigne, Milhaud & Croson, 1949; Lemoigne, 1947; Lemoigne, Delaporte & Croson, 1944; Lemoigne & Roukhelman, 1940a) have repeatedly observed a fall in PHB during sporulation, and Tinelli (1955a) has shown that there is no PHB in the spore. There is, therefore, little doubt that the inclusions are broken down to some extent while the spore is being formed. However, the observation that sporulation can occur in cells devoid of inclusions (Knaysi, 1951d), and that some inclusions

persist well after the spore is completed (Chapman, 1956), even, in some cases, surviving the sporangium, (Knaysi, 1946b) casts doubt on the significance of the breakdown. Cytological studies will not settle this point, since no microscopic observations can distinguish between a useless degradative breakdown, and an energy yielding oxidation. Biochemical studies are more hopeful, and an encouraging start has been made by Tinelli (1955b,c). She harvested cultures of B. megaterium towards the end of the growth phase (before sporulation had commenced), washed the cells, and suspended them in a medium lacking only a carbon source. Provided this medium was well aerated sporulation started in 6 to 7 hours and was complete (all the cells having sporulated) in 15 to 18 hours. By chemical examination of the vegetative cells, the medium, (both before and after the experiment), and the spores, and by measurement of the CO_2/O_2 exchange, she was able to show that all the PHB of the cells and most of the polysaccharide was completely oxidised to CO_2 and H_2O. About 20% of the cell protein was also oxidised with the liberation of ammonia. The energy requirements of sporulation are not known, but it is highly probable that some, at least, of the necessary energy was supplied by the oxidation of PHB, and the role of the lipid inclusions under these conditions is fairly certain.

Tinelli also observed that if the suspension of cells in the carbon free medium was inadequately aerated, sporulation was only partial, and some of the cells lysed, with liberation

of lipid inclusions into the medium. Partial sporulation also occurred in ordinary ageing cultures, an observation that may account for the reports, mentioned above, of the persistence of the inclusions after sporulation. It also seems likely that the energy needed for the sporulation of the inclusion free cells observed by Knaysi (1951d) could have been obtained either from the oxidation of other reserves, (e.g. polysaccharide), or from the oxidation of an exogenous energy source.

Taking into account both the chemical and the cytological observations, it would seem that the role of the inclusions in sporulation can be summarised as follows:- They do not appear to be essential to the process, and there is no evidence that any of the inclusion material finds its way into the spore, but the energy liberated by the partial or complete oxidation of the inclusions that occurs during sporulation may be utilised for the formation of the spore.

It is of considerable interest that Lemoigne and Girard (1944) stated that PHB in Azotobacter was broken down during the process of "encystment". The nature of this process is not well understood at present. It was described by Pochon et al (1948), and appears to consist of the formation of a "cyst" by the development of a tough double wall around the single cell. Bisset (1955) has described what appears to be the same process, and he considered that the "cyst" had many spore like properties, thus strengthening the relationship which he claimed to exist between Azotobacter and the

Bacillaceae. In the present author's opinion, the formation of the "cyst" and its significance is not sufficiently well understood to warrant its comparison with the endospore, and further research is needed to establish the role of PHB in its formation.

(b) The "Fatty Degeneration" Theory.

Lewis (1941) stated that lipid inclusions, while in some cases acting as reserves of food, might also sometimes be the result of degenerative processes analogous to "fatty degeneration" of higher organisms. He cited the findings of Almon (1933) and of Muller and Stapp (1926) that the granulated bacteroids of the root nodule were non-viable, as support for this theory.

Knaysi (1945b) proposed a similar hypothesis. He found that the lipid inclusions of B. cereus resulted from the migration into the cytoplasm of "protruberances" which appeared on the inner surface of the "cytoplasmic membrane" towards the end of the growth phase. At the time of sporulation, the cells usually contained 3 - 5 of these bodies. Knaysi suggested that they were the result of a breaking up of the cytoplasmic membrane, which represented an abortive attempt at cell division.

This suggestion received some support from the observations of Burdon (1946). From the limited observations he made, the latter author considered it likely that the lipid inclusions originated at the cell periphery, presumably in some relationship to the cytoplasmic membrane, which itself was stainable with Sudan black in some bacteria. Burdon shared

Knaysi's opinion that the usual conception of the inclusions as reserve materials was inadequate, since he noted that in ageing cultures on ordinary media, the bulk of the stainable lipid appeared to remain unchanged, while the cells containing it disintegrated. He also noted the appearance of "ghost" forms - cells packed with masses of lipid, but containing little or no stainable cytoplasm - in old cultures of many bacteria. These degenerate forms were seen in several species that did not store lipid during their actively growing stage, as well as in strains which regularly formed lipid inclusions. Murray and Truant (1954) found, similarly, that granules of lipid, glycogen and volutin could be seen in old cultures of Moraxella, but not in the young, fully viable cells.

These observations certainly point to the possible appearance of lipid inclusions as the result of degenerate metabolic processes, but more precise information is required before this view can be accepted as proven fact. In particular, one would like detailed information as to (a) the viability of the inclusion bearing cells, and (b) the nutritional value of the medium employed, particularly in the late stages of culture, when the inclusions appeared, since their production in a medium in which precursors had been exhausted would be of much greater significance than their appearance in a medium in which the precursors were still available. In the latter case, their production might represent merely a belated form of storage, rather than the result of degenerate metabolism.

- - - - oOo - - - -

MATERIALS AND METHODS.

1. Organisms.

(a) B. cereus.

(i) Strain BC.

This strain is an old stock culture of this department, and was originally obtained from the National Collection of Type Cultures. It is a typical weakly gram-positive chain forming bacillus, about 1.5μX 5μ, bearing large, oval, sub-terminal spores which do not swell the sporangia. Abundant lipid inclusions are formed on most media, and volutin granules are sometimes present, but there is no capsule. The organism is weakly motile. Slight growth occurs anaerobically, but the organism is essentially aerobic, and grows well between 20°C and 37°C, with the optimum at about 35°C. The colonies, which on nutrient agar are often as much as 7mm in diameter, are slightly convex, non-pigmented, opaque and butyrous, and have a rough surface and an irregular outline. After about 24 hours aerobic growth at 37°C, however, abundant sporulation occurs, and the surface of the colony becomes slightly smoother. Glucose is fermented with acid production, but no gas is formed. The organism produces acetyl methyl carbinol, and liquefies gelatin rapidly. In liquid media it requires several amino-acids, notably valine and leucine, but no other growth factors. Some biochemical characteristics are shown in Table 5. The organism was maintained on nutrient agar slopes in closed screw-capped bottles, at room temperature, and there was no loss of viability even after a year of storage.

(ii) Strain AC.1.

This organism was obtained by ultra-violet irradiation of an agar plate heavily inoculated with strain BC, the dose being adjusted so that nearly all the cells were killed. A single colony of the mutant AC.1 was detected after plating out several of the colonies derived from the cells surviving the irradiation. It differs from the parent strain mainly in being completely asporogenous under all cultural conditions tested so far. It is apparently non-motile, and forms longer chains than the parent. The colonies are very similar, but since sporulation does not occur, the older colonies remain fully rough in appearance, a character which facilitated the original detection of the mutant. This strain gives the impression of growing better, and of being more active biochemically than the parent strain, which it otherwise closely resembles (see Table 5). The viability of strain AC.1 is much less than that of the parent, however, few viable cells being detectable on an agar plate culture after two weeks' storage at room temperature. In order to avoid frequent subculturing, a more suitable means of storage was looked for. It was found that if the organism was inoculated as a gelatin stab, and the culture (in a closed screw-capped bottle) kept in the dark at room temperature, viable cells were easily isolated by plating a loopful of the liquefied gelatin, even after as much as six months. Sporogenous back mutants were never detected.

TABLE 5

Some Properties of Strains
AC.1 and BC of B. cereus

Character	Strain	
	BC	AC.1
Motility	+	-
Voges Proskauer	+	+
Catalase production	+	+
Gelatin liquefaction	rapid +	rapid +
Liquefaction of coagulated serum	+ (complete in 4 days).	+ (partial in 4 days).
Haemolysis (blood agar)	Weak β type.	Strong β type.
Amylase production	-	+
Caseinase production	-	+
Fermentation of:		
glucose	$\pm_1$	$\pm_1$
lactose	-	-
dulcitol	-	-
sucrose	$\pm_1$	$\pm_1$
mannitol	-	-
maltose	$\pm_1$	$\pm_1$
glycerol	$\pm_3$	$\pm_5$
salicin	$\pm_4$	$\pm_3$
arabinose	-	-
rhamnose	-	-
xylose	-	-
raffinose	-	-
inulin	-	-

Key: $\pm$ = acid produced, but no gas. Subscript indicates number of days required.
+ = possession of character.
- = absence of character.

(b) B. megaterium.

(i) Strain KM (Northrop 1951).

A culture of this organism, which is sensitive to lysozyme, was obtained through the courtesy of Dr. K. McQuillen.

(ii) Strain NCTC 7581.

The National Collection of Type Cultures supplied this strain; it is not sensitive to lysozyme.

(c) B. subtilis.

A typical culture of this organism was kindly provided by Dr. T. Gibson.

2. Composition and Preparation of Solid Synthetic Media.

Petri dishes and bottles used in the preparation of media were washed with hot detergent solution and rinsed many times in tap water and several changes of distilled water before use. Pipettes were cleansed in potassium dichromate/ sulphuric acid cleaning fluid, and thoroughly rinsed in tap and distilled water before drying and sterilisation. Pipettes and Petri dishes (4" diameter, with an aluminium lid) were sterilised by heating for 1 hour at 160°C in the hot air oven.

All pure chemicals were A.R. standard. The casein hydrolysate used for some experiments was "Bacto casamino acids".

(a) "Glucose Limited Control" Medium.

This solid synthetic medium was the starting point for all the experimental studies of the effect of nutritional variation on growth. Its composition was such that the total growth (in terms of total nitrogen) of strain AC.1 was limited only by exhaustion of the carbon source (0.3% glucose), and the resulting stationary phase cells contained minimal amounts of lipids, volutin and polysaccharide. All the other nutrients were present in amounts determined by experiment to be in considerable excess of the requirements for this complete utilisation of the carbon source.

Plates of the medium were prepared by adding small volumes of concentrated solutions of glucose, ammonium chloride, pooled amino-acids, and phosphate buffer (all solutions having been separately sterilised by autoclaving for 5 minutes at 10

pounds pressure) to 80 ml. amounts of a molten salts/washed-agar base contained in 100ml. "medical flat" bottles. The additional nutrients made the volume up to approximately 100ml., and plates, each containing 50ml. of medium, were poured immediately.

(i) Salts/agar base.

The base was prepared as follows: a weighed quantity of Japanese agar fibre was placed in an appropriately graduated screw-capped bottle (usually 1 or 2 litres capacity) and washed with several changes of distilled water, over a period of 24 hours. After the last washing, appropriate amounts of concentrated solutions of individual salts were added and the volume made up with distilled water to a graduation at 4/5 of the final volume required (e.g. 800ml. in the case of the 1 litre bottles). The base was then melted and sterilised by $1\frac{1}{2}$ hours steaming or by autoclaving for 15 minutes at 15 pounds pressure. Before use, the level was adjusted, if necessary, with sterile distilled water, and the base dispensed in 80ml. amounts in sterile "medical flat" bottles graduated at 50ml, 80ml, and 100 ml.

Each 80ml of the base contained:

washed Jap. agar......	1.5g
KCl..................	0.02g
Na_2SO_4................	0.01g
$MgCl_2$.................	0.002g
$CaCl_2$.................	0.0001g
$MnCl_2$.................	0.00006g
$FeCl_3$.................	0.00002g

(ii) Phosphate buffer.

The medium was buffered by the presence of a mixture of Na_2HPO_4 and NaH_2PO_4. The final concentration of phosphate in the medium, reckoned as the combined weights of the two anhydrous salts was 1.0% (w/v). This was attained by the addition of 20ml of a 5% (w/v) sterile mixed solution of the salts to 80ml of the molten agar base. A final concentration in the medium of 8.7 g/L Na_2HPO_4 and 1.3 g/L NaH_2PO_4 gave an initial pH of 7.2 - 7.4, and had ample buffering capacity for most purposes.

(iii) Amino-acids.

B. cereus is known to require amino-acids for growth (Knight and Proom, 1950). For the purposes of most of the present experiments it was felt that the use of complex mixtures of amino-acids such as casein hydrolysate or peptone was undesirable, since the composition of these mixtures is not known accurately, and their use would almost certainly have entailed an unknown contamination with mineral elements. Trial experiments in liquid media showed that both strain AC.1 and strain BC had an absolute requirement for valine and leucine. Their requirements for other amino-acids were not investigated fully, but it was found that good growth occurred in the presence of a mixture of the above two acids and nine others, and while the removal of any one of these nine appeared to reduce the level of growth somewhat, it was not significantly increased by the substitution of casein hydrolysate for the mixture. It is not suggested that the

mixture employed represented the minimal amino-acid requirements of these organisms. Made up as it was of measured amounts of fairly pure amino-acids, it represented a compromise between the minimal nutritional demands of the organisms and the unknown complexity of casein hydrolysate.

The pure amino-acids were all as supplied commercially, and were not subjected to any further purification. 100ml. amounts of the mixture shown in the accompanying table (Table 6) were made up in distilled water, and sterilised as indicated previously. 0.5ml. of this solution was added aseptically to 100ml. of the complete molten medium, just prior to pouring plates. The final concentration of amino-acids in the medium is shown in the table.

TABLE 6.

Concentration of amino-acids in stock solution (w/v)		Concentration in complete medium (w/v)
dl-valine	0.2%	0.001%
l-leucine	0.2%	0.001%
l-alanine	0.2%	0.001%
l-cysteine HCl	0.05%	0.00025%
l-glutamic acid	0.05%	0.00025%
l-glycine	0.05%	0.00025%
dl-threonine	0.05%	0.00025%
dl-histidine di-HCl	0.05%	0.00025%
l-proline	0.05%	0.00025%
l-lysine HCl	0.05%	0.00025%
dl-isoleucine	0.05%	0.00025%
Total	1.0%	0.005%

(iv) Ammonium chloride.

One ml of a sterile concentrated solution in distilled water containing 15% (w/v) NH_4Cl, was added to 100ml. of the molten medium just before pouring, to give a final concentration in the medium of 0.15% (w/v). The stock solution was acidified with 2 - 3 drops concentrated HCl to prevent loss of ammonia during sterilisation.

(v) Glucose.

The concentrated stock solution of glucose was acidified in the same way as the ammonium chloride solution, but in this case to prevent caramelisation during autoclaving. Its concentration was 30% (w/v), and one ml of this solution was added to 100ml. of the molten medium to give a final concentration in the medium of 0.3% (w/v).

The composition of the "glucose limited" control medium was as follows:

glucose	0.3%	(w/v)
NH_4Cl	0.15%	(")
Total amino-acids (see p. for details)	0.005%	(")
Na_2HPO_4) buffer, pH 7.2 - 7.4	(0.87%	(")
NaH_2PO_4)	(0.13%	(")
KCl	0.02%	(")
Na_2SO_4	0.01%	(")
$MgCl_2$	0.002%	(")
$CaCl_2$	0.0001%	(")
$MnCl_2$	0.00006%	(")
$FeCl_3$	0.00002%	(")

It should be pointed out that the preparation of the medium involved a slight error, in that the actual volume of a nominal 100ml. of medium was, in fact 102.5ml., and similar slight errors applied to all the other media employed. This error was not regarded significant however, in view of the natural variation of the cultures and the relative insensitivity of the analytical methods used.

(b) Media Employed for Nutritional Experiments.

These were prepared exactly as above, but the amounts of the nutrient under investigation was varied as required. In the case of media made deficient in potassium, sulphate, or phosphate ions, the distilled water used to wash the glassware and to make up the solutions was previously purified by passage through successive columns of "Amberlite" resins IR-4B(OH) and IR-120(H).

Phosphate deficient media were buffered with TRIS (tris(hydroxymethyl)aminomethane). Its use will be detailed in the description of the experiments involved.

(c) Media Employed for the Large Scale Cultivation of Cells for Macro-chemical Analysis.

These solid media were used in quantities of the order of 10 litres. They were prepared in the same way as above, but were poured directly into large enamel trays (described below) and were not dispensed into 100ml. bottles. The purity of the amino-acids was not considered critical for these experiments, and in view of the large quantities involved, it was found convenient to replace the pure amino-acids with 0.005% (w/v) casein hydrolysate.

(d) Nutrient Agar.

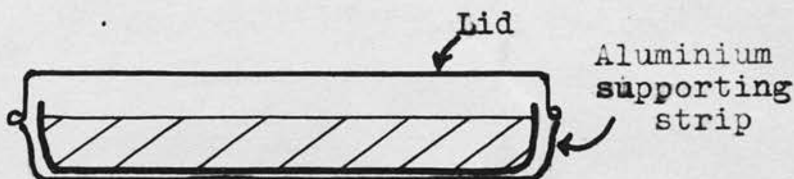
This medium, which was used for some routine purposes, was supplied by the Preparation Room of the Department. It has the following approximate composition: 1.0% (w/v) Peptone (Melville and Hunter), 1.0% (w/v) Lab-Lemco meat extract (Oxoid), 0.5% (w/v) NaCl, 2.0% (w/v) Japanese agar fibre, made up in tap water. For some purposes, additional glucose was incorporated.

3. Inoculation, Incubation, and Harvesting.

(a) Experiments on Nutrition and Growth.

The inocula for these experiments were standardised, as far as possible, in the following way:

A loopful of the stock gelatin culture of the organism (usually strain AC.1) was plated out on nutrient agar. After incubation overnight at 37°C, part of a single colony was thoroughly emulsified in sterile normal saline, the suspension being made just visibly turbid to the naked eye. This density corresponded approximately to a drum reading of 0.01 on the "Spekker" absorptiometer. 0.3ml of this suspension was placed on a plate of the "glucose limited" medium, and spread evenly over the surface with a sterile glass rod. The plate was then placed on a small strip of aluminium (1½" x 4½") having upturned ends which supported the lid about 2 mm. above the rim of the dish, to allow free aeration (see sketch).



This device was used for all cultures.

After incubation for 16 hours at 37°C, a little of the confluent growth was emulsified in a sterile suspending fluid which was used to inoculate and incubate a second plate of the same medium as before. The suspending fluid in this case consisted of the control medium, minus agar, and without

glucose or the nutrient whose effect was under investigation, and was used in order to minimise the possibility of osmotic damage to the cells. A little of the 16 hour growth on this second plate was used similarly for the inoculation of the experimental plates. This procedure was adopted in order to ensure that the colony selected was typical of the strain, any gross abberation being detected from the appearance and amount of the growth and by microscopic examination of a sample. It also gave the cells some opportunity to adapt themselves to the synthetic medium. The 16 hours-old cells were roughly in the middle of the period of active growth, and were chosen as it was found that cells from older cultures did not always grow as well. Particular attention was paid to the emulsification of the inocula, as it was found that if this was not thorough, the growth on the plates was liable not to be perfectly confluent, a feature of importance to the attainment of uniform samples. As far as possible the plates were kept in a strictly horizontal position during the initial stages of incubation, to prevent the inoculum drifting to one side of the plate and causing heavy growth there. Plates bearing non-uniform on non-confluent growth were discarded. The plates used for these experiments were not dried before use, as the free access of air allowed by the raised lid otherwise tended to result in the drying up of the agar during lengthy periods of incubation.

The aim of the cultural experiments was construction of the complete growth curve of the organism coupled with observation of the formation and breakdown of the lipid

inclusions and (in most cases) volutin and intracellular polysaccharide, throughout the period of culture. For this purpose, a number of plates, each containing 50ml of a given medium, were placed in the incubator at the start of the experiment, one or more plates being removed at each sampling time & the total growth per plate and the other chemical determinations carried out. Usually about 12 such samples were taken during the course of any one culture, and in this way curves representing the fluctuations in the culture on a single plate were built up. In the case of the early samples, and in cultures where the growth was low, several plates were required for each sample, in order to provide enough material for all the determinations. Thus at 12 hours as many as 12 plates were required on some occasions. This entailed the use of a large number of plates for any one experiment, usually at least 40 being incubated at the same time. In order to prevent cooling of the incubator due to the rapid influx of this quantity of plates at the start of the experiment, it was found necessary to warm all the plates to 37°C beforehand, the inoculation and transference to the incubator being carried out as rapidly as possible. Usually the first of a batch of plates was placed in the incubator 15 minutes before the nominal start of the experiment and the last about 15 minutes after the start, and it was found that the incubator temperature did not drop more than 3 degrees, and rapidly recovered. As far as possible, there was only one layer of plates per shelf, and the temperature of each shelf was checked from time to time.

In view of the relatively large amount of material needed for each sample, it was not possible to carry out complete estimations on the 6 hour samples, as at least 24 plates would have been needed for these samples alone. For this reason, a single plate was used for the 6 hour sample, and observations were restricted to measurements of turbidity, pH, and staining observations, as described below.

Most experiments were started at 6.0 p.m. It was not convenient to start the plates required for the 6, 12, 36, and 56 hour samples at this time, and the necessary plates for these samples were incubated from 10.00 a.m. This practice was thought justifiable, as it was found that there were not usually any major discrepancies between samples incubated in the two batches.

As soon as a sample plate was taken out of the incubator, heat fixed smears were made, to be stained for lipid and volutin. The quantitative harvesting of the culture was then accomplished with the aid of a blunt spatula to remove the bulk of the growth, followed by thorough rinsing with saline to remove all the residual cells, all the growth being collected in a small volume of saline. The resulting suspension (termed the "initial suspension") was thoroughly shaken to ensure homogeneity, and made up to a measured volume (usually 50 or 100ml) and the turbidity estimated by means of the "Spekker" absorptiometer (see below). The turbidity (in "Spekker" units) was recorded in terms of an initial suspension volume of 50ml.

The initial suspension was then passed through a

coarse sintered glass filter to remove any small pieces of agar, and the cells were washed three times by centrifugation in normal saline. Finally they were made up to a volume of not less than 15ml, and as far as possible, to an accurately measured turbidity equivalent to a reading of 2.5 - 3.0 units on the "Spekker" absorptiometer (see below). This suspension (termed the "working suspension") had a convenient density for the chemical estimations, and was stored in a stoppered flask in the refrigerator. As far as possible, all the determinations were carried out within the next two or three days, but the large number of samples handled sometimes necessitated storage of the suspensions for up to one week. No serious discrepancies appeared as a result of this, however.

The techniques employed for the determination of total nitrogen, lipid inclusions and intracellular polysaccharide are described below. It is only necessary here to point out that the estimations made enabled the total amounts of these substances to be calculated at all stages of the culture cycle, as well as the ratio of lipid inclusions and polysaccharide to the total nitrogen. Volutin was only estimated by examination of stained smears, and thus only an arbitrary and approximate estimate of the volutin per cell was obtained.

(b) <u>Large Scale Cultures for Macrochemical Analysis.</u>

Solid media were used in preference to liquid, as better growth was obtained, and the knowledge gained from the cultural experiments facilitated the control of the lipid contents of the cells.

The media were poured into white enamel trays, 12" x 16" x 1", each tray holding up to $1\frac{1}{2}$ litres. For sterilisation the trays were wrapped in "Kraft" paper and steamed for $1\frac{1}{2}$ hours. As soon as the first tray had been poured, two strips of hardwood, previously wiped with 70% ethanol, were laid across the edges of the tray, the next tray placed on top of these strips and filled with medium at once. This process was repeated until there was a pile of some half a dozen trays, the top one being protected with a large aluminium lid, previously sterilised at 160°C for 1 hour in the hot air oven. The trays were left in this position to solidify, and were then immediately inoculated. This was carried out by placing 10ml of a heavy saline suspension of cells on the surface of the medium and spreading it evenly with a sterile glass rod. The assembled pile of trays was then placed in the incubator. Where large numbers of trays were used, they were distributed evenly throughout the incubator to minimise the possibility of large localised temperature variations, the uppermost tray in each pile being protected by an aluminium lid. The incubator door was opened at intervals during the culture to ensure adequate aeration.

Microscopic checks of the purity of the cultures were always made prior to harvesting, but the only contamination encountered was a rare growth of B. subtilis, an aerial contaminant which was always apparent on naked eye examination of the plate.

To harvest the cultures, the growth was carefully scraped off the surface with a clean glass slide and washed into saline. After passage through a coarse sintered glass filter

to remove small pieces of agar, the cells were washed several times by centrifugation in normal saline, and analyses carried out at once. One advantage of the solid media was that harvesting was a very rapid process.

4. Staining Methods.

(a) Lipids.

The dry smear Sudan black method of Burdon (1946) was employed with the modification that basic fuchsin was used as the counterstain instead of safranin. Lipid inclusions are stained deep blue-black against the pink stained cytoplasm.

In some cases wet mounts were employed, the cells being mounted in a saturated solution of Sudan black B in either ethylene glycol (Hartman, 1940) or 50% diacetin (Leach, 1938).

(b) Volutin.

Albert's method, as modified by Laybourne (1924), was used routinely; the inclusions appear jet black against the green stained cytoplasm. For some purposes, Lindegren's wet mount method (Lindegren, 1949) was employed, the inclusions being stained red against the blue cytoplasm.

(c) Spores.

The modified Ziehl-Neelsen stain (Mackie and MacCartney, 1953) was at first employed to demonstrate spores, but it was soon found that slight uncontrollable variations

in the time of heating and of decolorisation sometimes resulted in volutin and lipid inclusions being stained acid-fast. Experiments with a wide range of Bacillus species showed that the malachite green method of Ashby (1938) was far superior, as only spores were stained, and there was no confusion with lipid or volutin inclusions. Using this technique, the spores are stained green and the cytoplasm pink. In the few experiments involving spore counts, the number of cells that had spored was estimated by examination of malachite green smears.

(d) Sudan black/Tannic Acid/Methyl Violet Stain.

This staining technique was devised to facilitate photography of stained preparations of cells bearing lipid inclusions. The technique is as follows:

1) Burdon's Sudan black B solution is applied to a heat fixed smear for 10-12 minutes, the excess stain is drained off, and the film blotted dry.
2) Precipitated stain is rinsed off the slide with benzol, and the slide air-dried.
3) 5.0% aqueous tannic acid is applied for 30 seconds, washed off at once in tap water, and the slide blotted dry.
4) 0.01% Methyl violet is applied for 10 - 15 seconds, washed off with tap water, and the slide blotted dry.

The lipid inclusions are stained a deep blue-black, and the cell wall a deep violet, providing a clear outline to the cells and good contrast with the background.

5. Microscopic Estimations of Inclusion Bodies.

(a) Lipid Inclusions.

Sudan black stained inclusions in heat fixed smears were estimated in terms of an arbitrary scale of + signs ranging from ± for cells containing "trace" amounts, i.e. few small inclusions, usually peripherally, through +, ++, +++, for increasing amounts, to ++++ for cells packed with large lipid inclusions. Films were made so that there were about 100 - 200 cells per field, using the oil-immersion objective. Many fields were examined, and in each field the percentages of each type of cell (i.e. ±, +, ++, etc.) were recorded, and these percentages multiplied by appropriate factors. Thus, the percentage of ± cells was multiplied by $\frac{1}{2}$, of + cells by 1, of ++ cells by 2, of +++ cells by 3, and of ++++ cells by 4. The resulting figures were added, and the totals assigned to a + value according to the following scale:

0 - 50	±	"trace"
50 -100	+	"little"
100 -200	++	"moderate"
200 -300	+++	"much"
300 -400	++++	"maximum"

Individual cells devoid of all Sudan-stainable inclusions were rare, and cultures even more so.

The fields examined were chosen at random, and care was taken to see that the staining of the smear was uniform, and as intense as possible. If the lipid inclusions were not stained an intense blue-black, one was liable to get

the impression that the cells contained less inclusion material than in fact they did.

(b) Volutin Inclusions.

These were estimated in Albert stained smears in a similar manner, but the estimation was less laborious, since the volutin inclusions showed a much greater uniformity of distribution among the cells of a given sample than did the lipid inclusions, and the differential count was not necessary. The volutin granules appeared at one or both poles of the cell (rarely anywhere else), and varied in size from hardly resolvable specks to large bodies the full width of the cell, sometimes appearing to bulge the cytoplasm of the shrunken heat fixed cells. The arbitrary + scale was as follows:

Most cells containing:-

-	No volutin.
±	One very small granule.
+	Two small granules or one moderate one.
++	Two moderate granules.
+++	Two large granules.

6. Photography.

The Watson "Bactil" microscope with a 1/12" "Fluorite" phase contrast oil immersion objective and a high intensity lamp were used throughout.

Phase contrast photomicrographs were taken on Ilford "Selochrome" plates at an exposure of approximately 20 seconds.

For photographs of stained preparations, the phase contrast annulus was omitted, and Ilford "Rapid Process Panchromatic" plates were used, with an exposure of approximately 1 second.

The initial magnifications were determined with the aid of a stage micrometer.

7. Chloroform extraction of polyβ-hydroxybutyrate (PHB).

The method adopted was slightly modified from that of Lemoigne and Roukhelman (1940b), and was as follows:

The washed cells were centrifuged and suspended in 20%HCl (w/v) to a concentration of approximately 4.0g (dry) cells per 100ml. The suspension was boiled for three minutes, cooled and centrifuged. The deposit was washed thoroughly by centrifugation in distilled water, followed by several washes with absolute alcohol until the supernatant was colourless. Many washes with ether followed, and finally the residue was transferred to a flask of chloroform and boiled under reflux for about half an hour. At the end of this time the mixture was cooled and filtered through a

small filter paper on a Buchner funnel. The filter paper plus residue was transferred to the flask and again extracted with chloroform. The process was repeated three times and the filtrates combined. After concentration by boiling off some of the solvent, the chloroform solution was transferred to a small tared flask, and the solvent removed by distillation <u>in vacuo</u>. Final traces of chloroform were removed <u>in vacuo</u> over a trap of wax chips, and the weight of the residual PHB determined.

8. <u>Measurement of pH of Cultures.</u>

The pH of the cultures on solid media were measured immediately after harvesting the cells. A block of the medium, about $1cm^3$ was cut from the plate with a clean scalpel, and the resulting hole filled with distilled water. After standing for about 30 minutes, the pH of the water was determined colorimetrically with a capillator. The initial pH of the medium was at first measured on a spare plate of the uninoculated medium, but it was found that it was always the same as that of the 6 hour sample, and this practice was discontinued.

9. Turbidimetric Measurements.

All measurements of turbidity of suspensions of bacteria and of lipid inclusions were made with the "Spekker" photoelectric absorptiometer, using cells of 10ml capacity.

Concentrated suspensions were diluted to a density lying on the linear part of the curve relating turbidity to concentration, and the actual turbidity of the test suspension calculated as the product of the turbidity of the diluted suspension and the dilution factor.

10. Measurement of the Dry Weight of Cell Suspensions.

The dry weight of a suspension of cells in distilled water was determined by centrifuging a known volume, transferring the cells quantitatively to a tared watch-glass and drying to constant weight in the oven at 120°C.

11. Estimation of Total Nitrogen.

All nitrogen estimations were made in duplicate by the micro-Kjeldahl method, and each batch of tests included a pair of reagent blanks. Measured volumes of a suspension of cells thoroughly washed in distilled water or normal saline were digested for 2 hours with concentrated H_2SO_4 in the presence of a $CuSO_4/K_2SO_4$/Selenium catalyst on a "Simmerstat" controlled electric heater. Each sample contained approximately 100μg N. The digested mixture was then made alkaline with NaOH, and the ammonia steam-distilled.

A Markham still was used for the early determinations; this was later replaced by a modification of the apparatus of Scandrett (1953) which reduced the time and labour involved considerably, and gave slightly increased accuracy. The distilled ammonia was trapped in boric acid, (1% w/v), Nesslerised, made up to volume in a 50ml graduate flask, and the colour read against a reagent blank in the "Spekker" using an Ilford Spectrum Violet (No. 601) filter. The nitrogen content of the suspension was then determined from a calibration curve.

12. Estimation of Polysaccharide.

For the purposes of the present experiments, a detailed analysis of the polysaccharides of the experimental organism was not necessary, but an approximate estimate of the quantitative fluctuations of the polysaccharide at different stages of culture was obtained by the use of the anthrone method of Fairbairn (1953). It should be pointed out that the anthrone method probably did not measure all the polysaccharide of the organism. Duguid and Wilkinson (1953) found that only about 65% of the total polysaccharide of Aero. aerogenes was measured by this method, and there is no reason to suppose that any greater proportion of the polysaccharide of strain AC.1 was revealed. Nevertheless, the method provided a rapid means of following changes in the amount of polysaccharide in the cells, and enabled a large number of samples to be handled in a relatively short time.

A measured volume of a suspension of cells in normal saline or distilled water was placed in a 10ml pyrex boiling tube, and the volume made up to 2ml with distilled water. 10ml of the anthrone reagent (1% anthrone in 72% H_2SO_4) was slowly added, the tube being kept cool by shaking in a bath of cold water. All determinations were made in duplicate, and every batch of tests included a pair of reagent blanks, and a pair of standards each containing 100μg glucose. The tubes were assembled in a rack which was immersed in a boiling water bath for exactly 8 minutes, a time found by Dudman (1954) to give the highest colour intensity. The tubes were then rapidly cooled in a bath of cold water, and the colours read in the "Spekker" against a reagent blank, using the Ilford Spectrum Orange (No. 607) filter. The amounts of polysaccharide in the test suspensions were calculated (in terms of μg glucose equivalent per ml) from the colours of the standard tubes.

13. Phosphorus Determinations.

The total and inorganic phosphorus contents of samples of PHB and lipid inclusion bodies were measured by the method of Fiske and Subbarow, the samples of dry material being weighed directly into boiling tubes. All determinations were made in duplicate, and each batch of tests included a pair of reagent blanks. The colours developed were measured in the "Spekker" against a reagent blank, using the Ilford Spectrum Red (No. 608) filter, and the phosphorus content of the samples determined with the aid of a previously prepared calibration curve.

14. Ash Determinations.

The ash contents of samples of PHB and of lipid inclusions, were determined by incineration in a platinum crucible. Samples of about 100mg were generally employed, and the weight of the residue after heating to constant weight determined with a micro-balance. Care was taken to avoid over-rapid heating, and the sample was not allowed to catch fire.

SPECIAL METHODS.

The methods detailed here are those relevant to the estimation and isolation of the lipid inclusion bodies, and are recorded here in their final form for ease of reference. The experimental background to their development is described in the "Experiments and Results" section.

1. Preparation of the Alkaline Hypochlorite Reagent.

This reagent was prepared as follows: 200g of fresh bleaching powder was thoroughly triturated with a little distilled water and transferred to a large conical flask. More water was added, to bring the total volume of water up to 1 litre. 300g of Na_2CO_3 were dissolved in a second litre of boiling water, and this solution added to the flask with stirring. After standing for 2 - 3 hours (with shaking at intervals) the mixture was filtered through a Buchner funnel and the residue discarded. The reaction of the greenish-yellow filtrate (initially it was generally about pH 12) was adjusted to pH 9.8 with concentrated HCl, about 120 ml being required. In the course of this adjustment a flocculent precipitate appeared, and this was removed by filtration, after placing the solution in a water bath at 37°C for a few minutes. The resultant crystal clear solution, which contained about 2% (w/v) available chlorine, was stored in a well stoppered bottle in the refrigerator, where it would last several months.

2. The Use of Alkaline Hypochlorite for Quantitative Estimation of the Lipid Inclusions of the Bacillus genus.

Only the practical details of the technique will be recorded here; proof of the validity of the method and the significance of the results obtained by its use, form the major part of the experiments described in the "Experiments and Results" section.

A washed suspension of the test organism in normal saline was prepared, and its turbidity adjusted to a suitable value, using the "Spekker" absorptiometer. For strain AC.1 of B. cereus, a concentration of about 1.0mg (dry weight) per ml., corresponding to a turbidity equivalent to about 2.5 "Spekker" units was satisfactory for most purposes. For accurate measurement of low amounts of inclusions, however, it was sometimes found desirable to double this concentration. Suitable concentrations for other organisms should be determined by experiment. 10ml. of this suspension was placed in a graduated centrifuge tube, and, after centrifugation, the supernatant wholly removed and discarded. The latter operation was conveniently performed with a Pasteur pipette attached to a water pump. Care was taken to see that a minimum of cells was removed, even if this meant that two or three drops of the supernatant fluid remained. The standard alkaline hypochlorite solution, (previously warmed to 37°C and filtered), was added to the mark, and the tube gently agitated to disperse the cells. The tube was then placed in a water bath at 37°C for 90 minutes and at the end of this period the turbidity was determined with the "Spekker".

As will be shown later, this treatment has the effect of completely dissolving the cells, with the exception of the lipid inclusions, and the final turbidity is therefore a measure of the concentration of the latter in the initial suspension. The actual dry weight of the inclusions was determined from the final turbidity by the use of a previously prepared calibration curve (see below). Provided the concentration of cells in the initial suspension was known, the cell content of lipid inclusions could be calculated, and expressed in any desired units. In most of the experiments to be described, "total nitrogen" was used as a measure of cell growth and lipid inclusion contents were recorded as mg lipid inclusions/mg total nitrogen; for certain purposes, the lipid inclusion content was expressed as a percentage of the dry weight of the cells. Chemical evidence will be provided that the lipid inclusions of strain AC.1 as isolated in this way, contained essentially about 89% of their dry weight as polymerised β-hydroxybutyric acid (PHB), the remaining 11% being ether soluble lipids, and this composition was approximately constant. It was therefore possible to convert the estimation of "lipid inclusions" obtained from the calibration curve into an estimate of the PHB content of the suspension, and this was done by multiplying the figure for "lipid inclusions" by 0.89. As will be shown, the error involved in this approximation was relatively small, and the estimation of PHB was probably accurate to within about ± 5%. If extreme accuracy was desired, the actual PHB content of a sample of the isolated lipid inclusions could be determined,

but it would probably be more convenient to use the rather cumbersome method of Lemoigne and Roukhelman (1940b) for the extraction of PHB. It should be emphasised that the value of the present technique is that it provides a simple rapid means of making a large number of reasonably accurate estimations, rather than a limited number of extremely precise determinations. 30 or 40 individual estimations can quite conveniently be handled by a single worker in a day. Using the method of Lemoigne and Roukhelman, on the other hand, the operator would have difficulty in making more than 2 or 3 estimations in the same time.

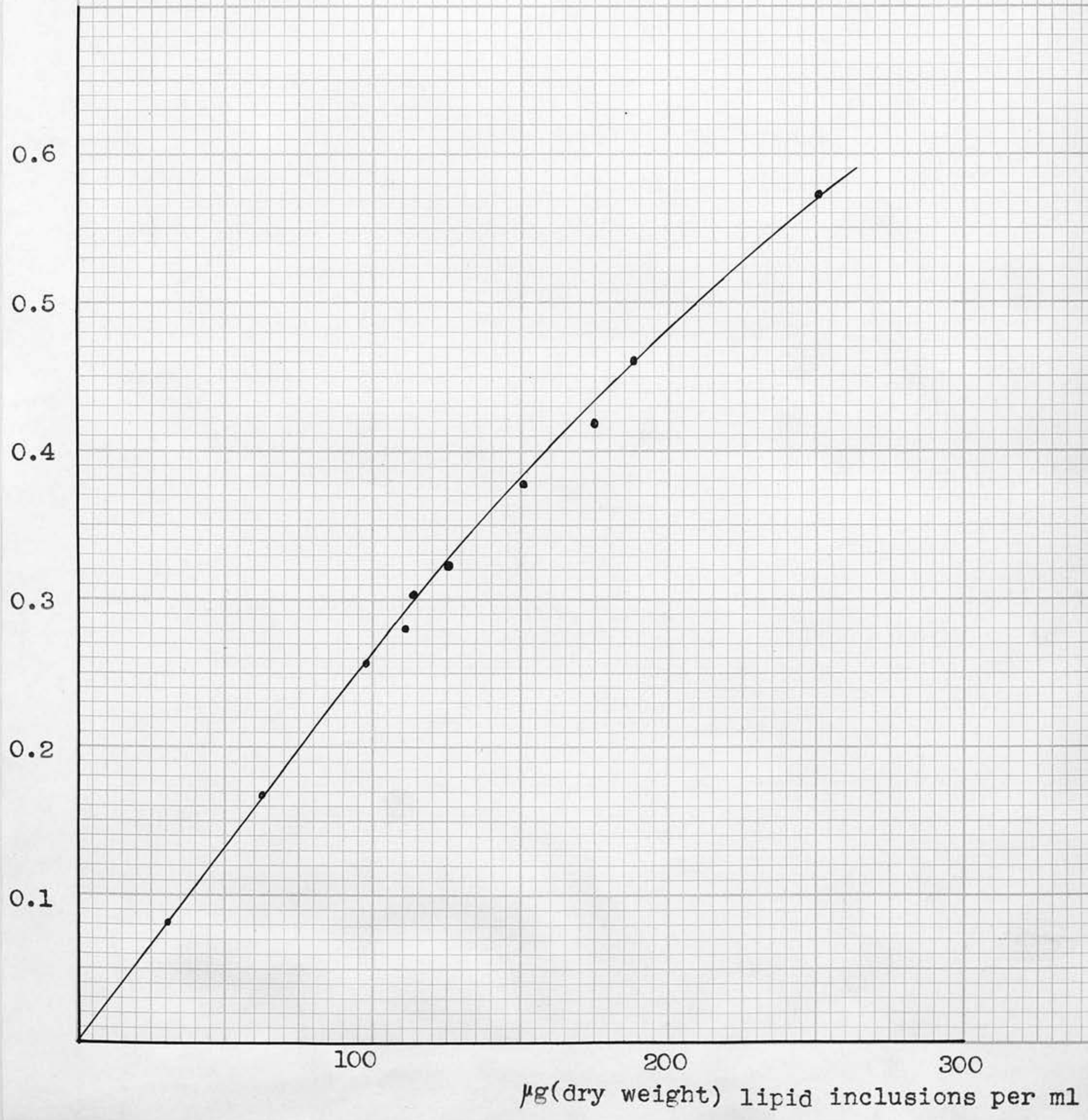
Another advantage of the hypochlorite method is that only about 10 mg. of (dry) cells are required for each estimation, an amount comparable with the quantities required for other conventional microchemical techniques, and which is certainly much more convenient than the several hundreds of milligrams required for the chloroform extraction technique. It will be noted that the hypochlorite measurements were made on single samples. The use of duplicate determinations was dropped after a long series of experiments showed that the tubes of a pair never differed by more than 2% of their mean.

3. <u>Preparation of the Calibration Curve Used for the Estimation of the Lipid Inclusions.</u>

The method adopted for the preparation of the calibration curve was as follows:

A suspension of lipid inclusion rich cells of strain AC.1 of <u>B. cereus</u> (grown on 1% glucose synthetic

medium), containing about 8.0mg. (dry weight) cells per ml. was centrifuged and the deposit resuspended in an equal volume of the standard alkaline hypochlorite at 37°C, for 90 minutes. A portion of the resulting suspension of lipid inclusions was centrifuged at high speed and passed through a fine sintered glass filter to remove all the suspended material, thus providing a quantity of "used" hypochlorite solution. A suitable series of dilutions of the remainder of the suspension was made in this "used" hypochlorite, and the turbidities measured. (When the turbidity of the suspension was above 0.4 units, it was determined by dilution of a portion with "used" hypochlorite, and the actual turbidity calculated as the product of the turbidity of the dilute suspension and the factor of dilution). Measured volumes of each dilution were centrifuged at about 8000 r.p.m. ("M.S.E." high speed centrifuge; glass tubes) for about 20 minutes. The supernatants were carefully removed with a Pasteur pipette, and the deposits resuspended as far as possible in distilled water. The deposited inclusions of strain AC.1 were particularly prone to clump together, and complete resuspension was difficult. Nevertheless, with care, it was possible to get most of the pellet of inclusions back into suspension, and the process of washing by centrifugation was repeated at least six times. It was found that the last traces of the hypochlorite reagent were very difficult to remove by simple washing in water; a slight smell of chlorine still lingered even after many washings. It was therefore the practice to add one or two drops of N/10 sodium thiosulphate


GRAPH 1.
Calibration curve for estimation of lipid inclusions by the hypochlorite method
Turbidity of Suspension
0.6
0.5
0.4
0.3
0.2
0.1
100
200
300
µg(dry weight) lipid inclusions per ml

to the water of the third wash. This completely removed all traces of the smell of chlorine, and, being itself washed away by the subsequent centrifugations, in no way affected the accuracy of the weighings. After the final washing, the deposited inclusions were carefully transferred to small weighed watch-glasses, and dried to constant weight in the oven at 60°C. (Experiment showed that there was no advantage to be gained by freeze-drying the inclusions; there was no apparent weight loss in the oven.). The weights measured were in the order of a milligram or less, and the micro-balance was accordingly employed. Three separate sets of determinations were made, on different occasions, and the results combined in the final curve (Table 7 and Graph 1). The lipid inclusion contents of the three cultures used varied from 10.0% of dry weight of the cells to 25%.

TABLE 7.

The Relationship of the Turbidity of a Suspension of Lipid Inclusions in Hypochlorite to the Dry Weight of Lipid Inclusions per ml.

Turbidity	μg lipid inclusions (dry weight) per ml.
0.082	32
0.168	63
0.257	99
0.280	112
0.302	118
0.322	126
0.378	151
0.418	175
0.461	188
0.572	251

It may be thought that this method of preparing the calibration curve was rather circuitous. There were, however, good reasons for using it, and these are fully discussed in the second part of the "Experiments and Results" section, on p. 143.

4. The Use of Alkaline Hypochlorite for Large-Scale Isolation of the Lipid Inclusions for Analysis.

A washed suspension of the cells was centrifuged and the deposited cells suspended in the standard alkaline hypochlorite reagent at 37°C, the density of the suspension being not more than about 8.0mg (dry weight) per ml. The lysis was continued for 2 hours, and the suspension mechanically stirred. At the end of this time microscopic examination showed that the milky white suspension consisted only of the lipid inclusions. If desired, the inclusions could then be carefully centrifuged and washed by successive centrifugations in distilled water. This process, however, lead to a certain amount of irreversible clumping, which was especially marked if the density of the suspension was high; indeed, if a concentrated suspension was centrifuged at high speed, the inclusions sometimes clumped into tough "buttons" which were virtually impossible to break up. This clumping, which was more marked with strain AC.1 of B. cereus than with B. megaterium (NCTC 7581), could be avoided by dilution of the suspension and slow centrifugation, but this made the separation of the inclusions rather laborious, and an alternative method was developed, as follows:

The suspension was diluted with an equal volume of

distilled water, sealed in lengths of "Visking" dialysis tubing and dialysed for 48 hours against either running tap water, or many changes of distilled water. If tap water was used, a precipitate of inorganic phosphates was formed inside the tubing, and had to be removed at a later stage. Nevertheless, it was sometimes more convenient to use tap water than to make the frequent changes of distilled water. After the dialysis, the suspension was made slightly alkaline with a little 0.2N NaOH, and the inclusions could now be concentrated by centrifugation at about 8000 r.p.m. for a few minutes, without risk of undue clumping. They were resuspended in 0.2N NaOH, and a few drops of N $Na_2S_2O_3$ added to remove all traces of free chlorine, (as indicated by a negative test with starch/ iodide paper). The dialysis was then repeated for a further 24 hours against several changes of distilled water. At this stage, if the initial dialysis had been made against tap water, the precipitated phosphate had to be removed, and this was accomplished by successive centrifugation in 0.2N HCl, until a test on the washings for inorganic phosphate (Fiske and Subbarow) was negative. Finally, the acid was removed by several centrifugations in distilled water and the inclusions were then dried in vacuo over P_2O_5 , when they formed a clean white flaky powder. Chemical analysis of inclusions prepared in this way will be reported later; they showed that the preparation was free of any gross contamination, and microscopical observation indicated that the inclusions were not unduly clumped.

5. The Isolation and Preparation of Lipid Inclusions for Electron Microscopy.

The purification of the suspended inclusions followed essentially the same course as described above, but since only small volumes were needed for electron microscopy, the whole process could be conveniently carried out in dialysis tubing, and the intermediate stage of concentration by centrifugation was omitted. This resulted in a preparation that was virtually free of clumping, at least as judged with the phase contrast microscope. It transpired, however, that some clumping did occur by the time the inclusions were dried on the electron microscope grid, but it was not of a very serious nature. Fixation of the inclusions was accomplished by adding osmic acid to a concentration of about 1%. The osmic acid was removed by dialysis against distilled water, and the inclusions transferred to formvar membranes mounted on copper grids. Some of the earlier preparations were allowed to dry at room temperature, but later specimens were freeze-dried, as it was thought that freeze-drying to some extent prevented the inclusions from collapsing.

EXPERIMENTS AND RESULTS.

PART I.

MICROSCOPIC APPEARANCE, CYTOCHEMICAL PROPERTIES, AND OCCURRENCE OF LIPID INCLUSIONS IN THE EXPERIMENTAL ORGANISMS

The observations recorded here enabled the in situ lipid inclusions of the observed organisms (B. cereus, strains AC.1 and BC, and B. megaterium, (NCTC 7581) to be positively identified and distinguished from other cell inclusions. A wide range of staining methods was employed, some being more cytochemically specific than others, and with few exceptions, the results obtained agreed closely with the generally accepted observations of Meyer (1912a), Burdon (1946) and others.

It will be seen that in many cases, negative results were recorded. These require care in their interpretation, and should not be taken as proof of the absence of a given component from the inclusions. There are several possible reasons why a particular substrate may not be stained, or if stained, may not be made visible. Chief among these causes of pseudo-negative results are:

(a) The substrate may be inaccessible to the dye by virtue of a coating or barrier of impermeable material. In the case of the lipid inclusions, this difficulty is very likely to be encountered when attempting to stain non-lipid components with aqueous dyes, since the substrate may be surrounded by or dispersed through a water-impermeable lipid.

(b) The distribution of the substrate in the

inclusion, coupled with its intrinsic power to bind the dye, may be such that the substrate appears to the observer to be unstained even when it has absorbed a maximal amount of dye. In the case of spherical bodies such as lipid inclusions, a component constituting ten per cent of the volume of the inclusion would, if distributed as a shell or membrane around it, only have a thickness of less than one twentieth of the diameter of the inclusion, and a shell of this thickness might well appear unstained unless the absolute intensity of staining was very high. If, on the other hand, the same amount of material was present as a central core, its diameter would be almost half that of the inclusion, and it would be far more likely to be visible when stained.

(c) A third possible cause of a pseudo-negative result applies to dyes that stain cortical components of the inclusions to the same intensity as the surrounding cytoplasm. In these cases, the peripheral staining of the inclusion body may be masked by the stained cytoplasm.

Thus, it is clear that negative results may not be significant. The usefulness of positive results, on the other hand, is only limited by the known degree of specificity of the stain employed.

Unless otherwise indicated, stained preparations were of heat-fixed dry smears. In most cases they were examined both directly (i.e. under immersion oil) as well as mounted in water, as it was thought that examination in both ways would increase the possibility of detecting

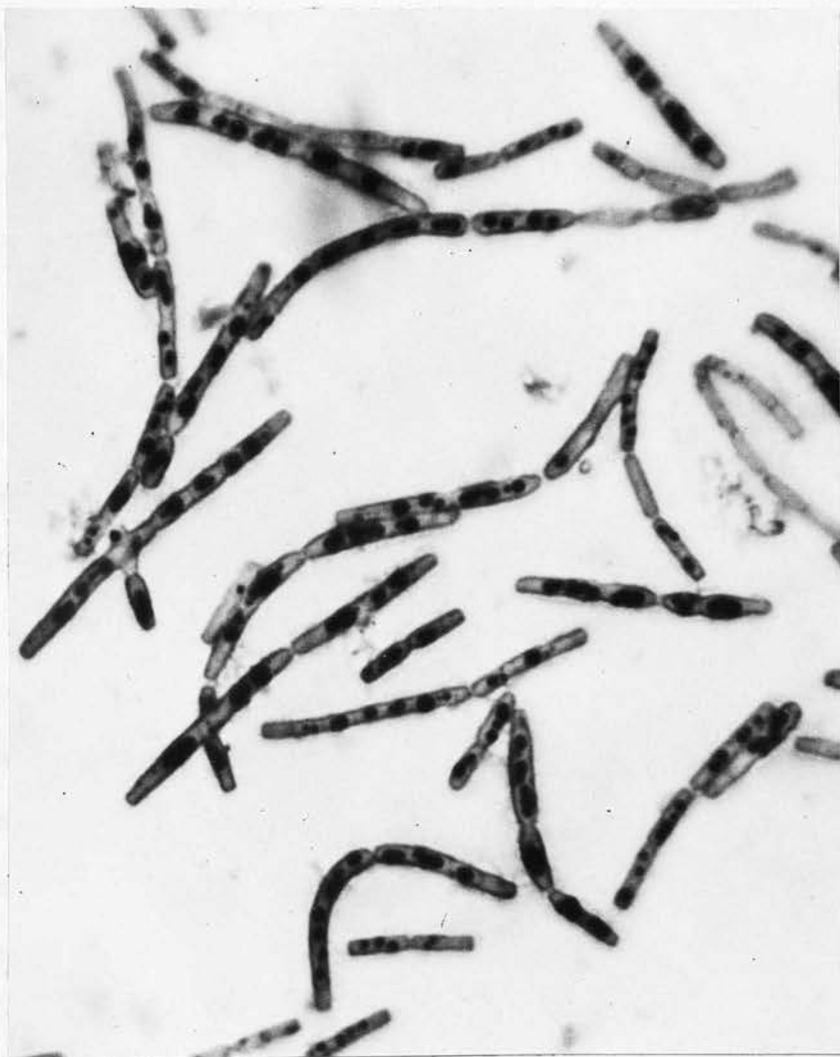
weak staining.

For convenience, the cytochemical results, which are fully discussed below, have been recorded in table 15 (p.151).

(1) Lipid stains.

(a) Sudan black.

The method of Burdon (1946) which stains lipid an intense opaque blue-black against the pink-stained cytoplasm, was used routinely. It revealed the presence of roughly spherical lipid inclusions in all the "large-celled" species of Bacillus examined, though the size and number of the inclusions varied widely according to cultural conditions, and also from cell to cell of a given culture. In general, on glucose rich media, the inclusions of B.megaterium (NCTC 7581), the asporogenous B.cereus (strain AC.1), and the sporogenous B.cereus (strain BC), were large (i.e. diameters of the order of 1.0μ), and there were often as many as 2-3 inclusions per cell. In some cases the inclusions appeared to have coalesced, giving rise to irregularly shaped masses which occasionally appeared to almost fill the cell. The appearance of inclusion rich cells of strain AC.1 is illustrated in photograph 1, p. 97. (It should be noted that in order to obtain an easily photographed cell outline, the fuchsin of Burdon's method was replaced by tannic acid/methyl violet, (see Methods), and the latter dye was solely responsible for the heavily stained cell outlines. The staining of the lipid inclusions, on the



Photograph 1. B.cereus (AC.1). 28hrs., 1% glycerol nutrient agar, 37°C. Sudan black/tannic acid/methyl violet. Note the variation in size of the lipid inclusions and their distributions throughout the length of the cell, not usually at the poles. Their shape is generally spherical, but in some cases they appear to have coalesced into irregularly shaped masses. The cell wall, and in some cases, cell septa, (stained by the methyl violet) can be clearly seen. The microscopic estimation of lipid inclusions for this culture was "+++".

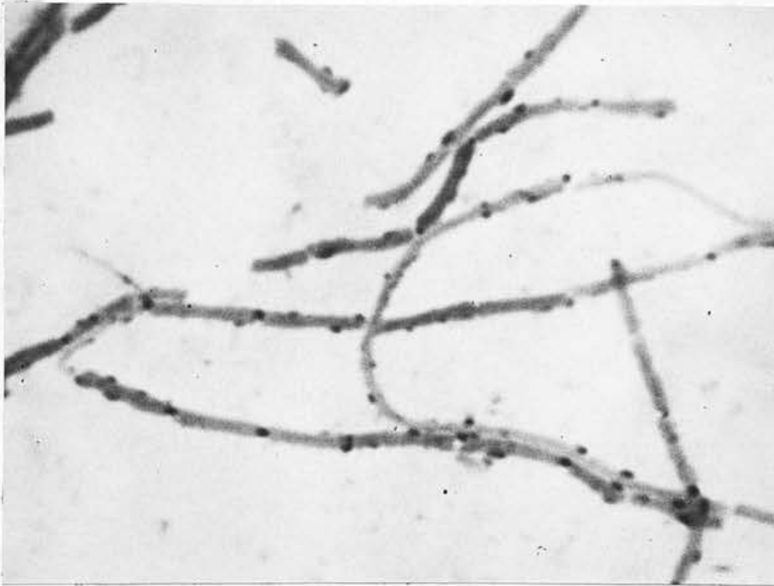
Photograph 1 at a final magnification of X 3000.

other hand, was due entirely to the Sudan black). In contrast to the "large-celled" Bacilli, it was found that B. subtilis never formed more than a trace of lipid inclusion material, even on glucose rich media.

It will be seen that the inclusions of strain AC.1 were essentially central in position in the cell, and showed no tendency to predominate either at the poles of the cell, or at its periphery. This was quite general for cultures of 12 hours or later, but in very young cultures (i.e. 6 hours), the inclusions (which were small) were clearly distributed at the periphery of the cell, and in these heat fixed preparations, sometimes even appeared as "buttons" external to the shrunken cytoplasm (see photograph 2, p. 99). This appearance was probably a shrinkage artefact, but the peripheral occurrence of the inclusions of young cultures, and their apparent relationship to the cytoplasmic membrane was noted by Burdon (1946), and his observations are thus confirmed. Whether the peripheral inclusions later migrate into the cytoplasm, or whether the inclusions of older cultures are formed there, de novo, is not known.

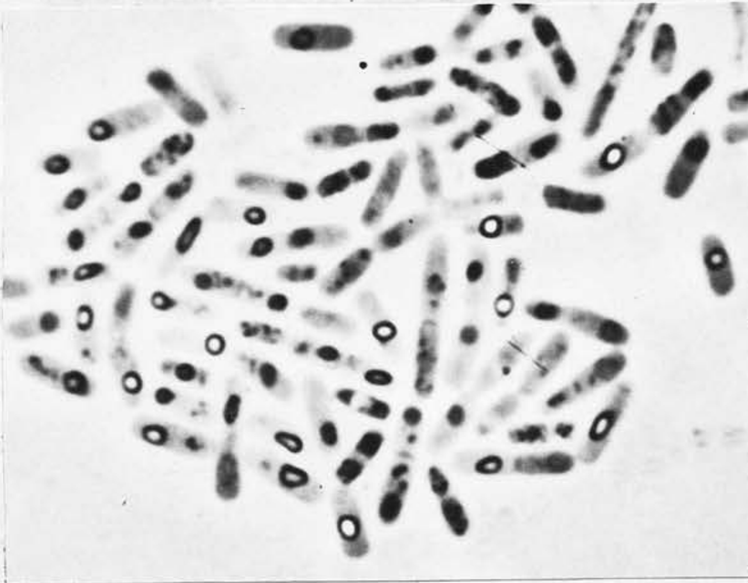
Use was sometimes made of wet mounts of cells in saturated solutions of Sudan black in either 50% diacetin (Leach 1938) or in undiluted ethylene glycol (Burdon 1946). Both these reagents stained the inclusions an intense opaque blue-black, though the ethylene glycol solution acted rather more rapidly.

Observations made on parallel preparations stained



Photograph 2. B.cereus (AC.1). 6hrs., 0.1% peptone agar, 37°C. Sudan black, counterstained methyl violet. Note the peripheral distribution of the small lipid inclusions in this young culture. In some cases, they appear as "buttons" external to the cytoplasm of the shrunken cells.

Photograph 2 at a final magnification of X 2600.



Photograph 3. B.cereus (var mycoides). 30hrs., nutrient agar at 37°C. Unstained living cells by phase contrast. Note the spores, which appear a refractile blue colour, with a thick black (optical) periphery. Prespores and small lipid inclusions are also evident.

Photograph 3 at a final magnification of X 2600.

by Albert's method for volutin and by Ashby's method for spores (see below) showed that spores and volutin granules were never stained by Sudan black, and conversely, bodies that stained with Sudan black never gave positive results with these two methods.

The cytochemical conclusions to be drawn from the use of Sudan black are limited. Cain (1950) has pointed out that while Sudan black stains only lipids, it will stain all liquid or semi-solid (i.e. "greasy") lipids equally. It shows no chemical specificities, since the staining depends entirely on the solubility of the dye in the lipids. However, Cain emphasised a not too widely realised point, namely that the oil-soluble dyes, of which Sudan black is an example, will not stain lipids that are in a solid state at the temperature of staining. This fact is of particular importance in connection with the present studies, since it will later be shown that the major component of the inclusions was found to be a solid. For the present, it is sufficient to record that the use of Sudan black indicated that the inclusions contained some lipids in a liquid or semi-solid state at room temperature. Unfortunately the use of Sudan black gave no indication of the distribution of this material within the inclusions, since the latter were stained quite opaquely, and the existence or otherwise of a more densely stained periphery was not demonstrable.

(b) <u>Sudan III</u>.

This stain (in the form of a saturated solution

in 70% ethanol, and employed as a wet mount) coloured the inclusions uniformly a very pale transparent orange, thus confirming their content of a lipid or semi-solid lipid. The cytoplasm of the cells was completely unstained. If the stainable material was distributed only as a peripheral shell around the inclusions the use of a transparent stain such as this should reveal the existence of the shell. The uniform staining might therefore be taken to indicate that such a shell was not present, but the staining was extremely feeble, and it would be unwise to place too much reliance on the results obtained with such a weak stain.

(c) Cain's Nile blue staining method.

The value of Nile blue, first introduced by Lorraine Smith (1908) to distinguish between neutral and acidic lipids, has been disputed by several authors, but the recent work of Cain (1947) seems to have clarified the matter, and provided a theoretically satisfactory basis for its use. Cain's method was developed for staining sections fixed in formol-calcium; for present purposes, formalin fixed smears were used instead, and this was the only modification of the technique. Duplicate smears (of each of strains AC.1, BC & NCTC 7581) were prepared; they were immersed in a 1% solution of Nile blue at 60°C for 5 minutes, then washed quickly in water at the same temperature, and differentiated in 1% acetic acid (still at 60°C) for 30 seconds. One section was drained and blotted dry. The other, in accordance

with Cain's instructions, was immediately restained in a 0.02% solution of Nile blue at 60°C, washed and differentiated as before. This somewhat complicated staining procedure requires some explanation. The use of the high temperature was adopted by Cain in order to melt, and guarantee the staining of lipids which might be solid, and therefore not stainable, at lower temperatures. The need for the duplicate smears is rather too complex to be discussed here, and the reader is referred to the original paper for details. Suffice it to say that the use of the two solutions may enable the observer to detect neutral lipids (stained red) when present as a mixture with acidic lipids (stained an intense blue). It should be pointed out that valid conclusions can only be drawn from Cain's technique if it is known that the structure observed is lipid, since the free base of Nile blue is a simple basic stain, and will therefore stain non-lipid structures weakly blue. Moreover, some acidic lipids may not be coloured blue, so that a structure staining only red, - indicative of neutral lipids - may also contain acidic lipids.

It was found that the lipid inclusions of the three organisms were stained red (in both smears), and this result was therefore interpreted as indicating the presence of neutral lipids, while not ruling out the simultaneous existence of acidic lipids. This result was contrary to that of DeLamater (1956), who reported the inclusions of B.megaterium to be stained blue,

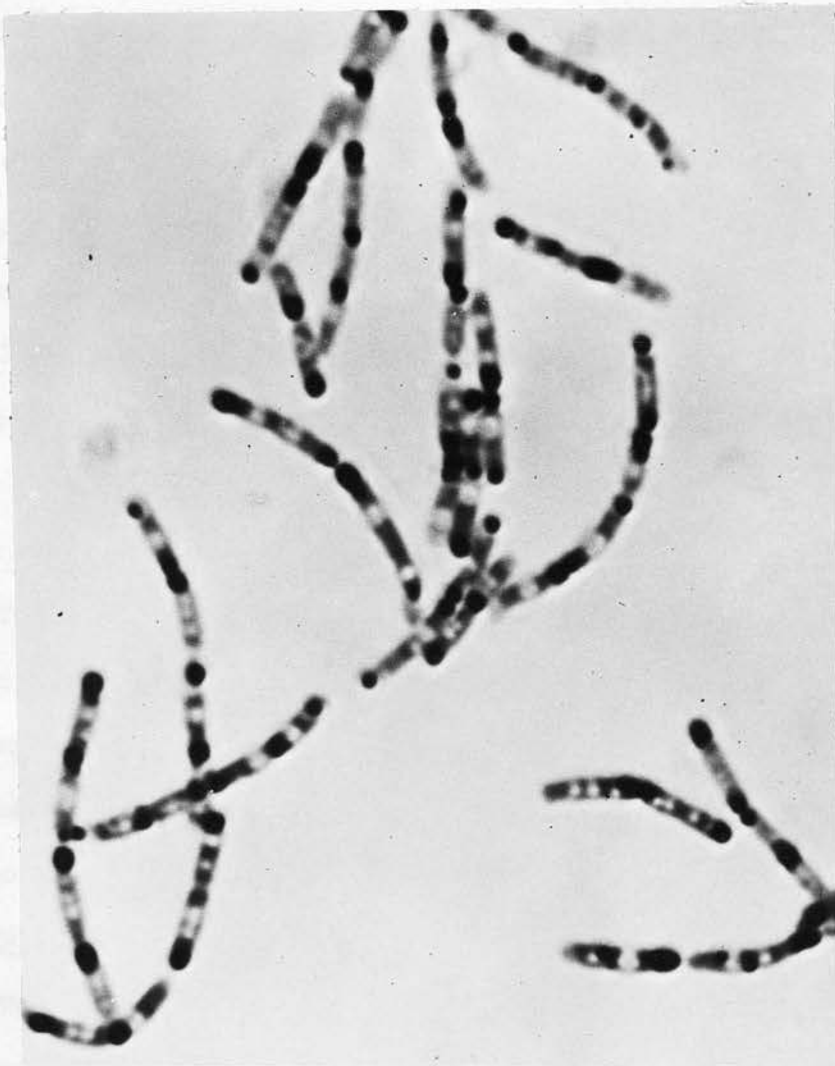
indicating the presence of acidic lipids. The cause of this discrepancy is not understood. It may be due to an intrinsic difference between strains, or perhaps to a difference in the cultural conditions employed.

In summary, the use of the above stains showed that the lipid inclusions of these organisms contained some lipids in a liquid or semi-solid state at room temperature, and that this lipid was of a "neutral" nature.

(2) Volutin stains.

(a) Albert's stain.

This stain revealed the presence of spherical granules of volutin in most cultures of both strains of B.cereus. The asporogenous strain AC.1 tended to form more volutin than its parent strain BC, though in both strains, the amounts present, while varying considerably with cultural conditions (see later), were generally small. In contrast to the lipid inclusions, the volutin granules (usually either one or two per cell) were almost always situated at the poles of the cell, and varied in size from hardly discernable specks to large spherical bodies having a diameter of the order of 1.0μ, sometimes bulging the shrunken cytoplasm of the heat fixed preparations. The appearance of cells of strain AC.1 grown on 1.0% glucose synthetic medium and containing maximal amounts of volutin, is shown in photograph,4p. 104. The volutin granules showed a much greater uniformity of size distribution through the cells of a given culture than did the lipid inclusions, which in the photograph appear as clear unstained



Photograph 4. B.cereus (AC.1). 40hrs., 1% glucose synthetic medium, 37°C. Alberts stain. Note the large black stained volutin granules, predominantly at the poles of the cells. They show a much greater uniformity of size than do the lipid inclusions, which appear as clear unstained "vacuoles".
Photograph 4 at a final magnification of X 3000.

"vacuoles". The identity of these vacuoles with the lipid inclusions was shown by staining parallel smears with Sudan black, and the independence of the lipid and volutin inclusions was strikingly demonstrated by smears simultaneously double stained for lipid and volutin. For this purpose, a heat fixed smear was taken through Burdon's Sudan black method, but without the final application of the counterstain. Instead, the film was carried directly through Albert's procedure. The lipid inclusions were stained brown, volutin black, and the cytoplasm green. It was found impossible to obtain a clear photograph of this preparation, as the colours did not permit good photographic contrast, but the drawing on p. 105a shows the appearance of the cells. It was quite clear that the lipid and volutin inclusions were independent bodies.

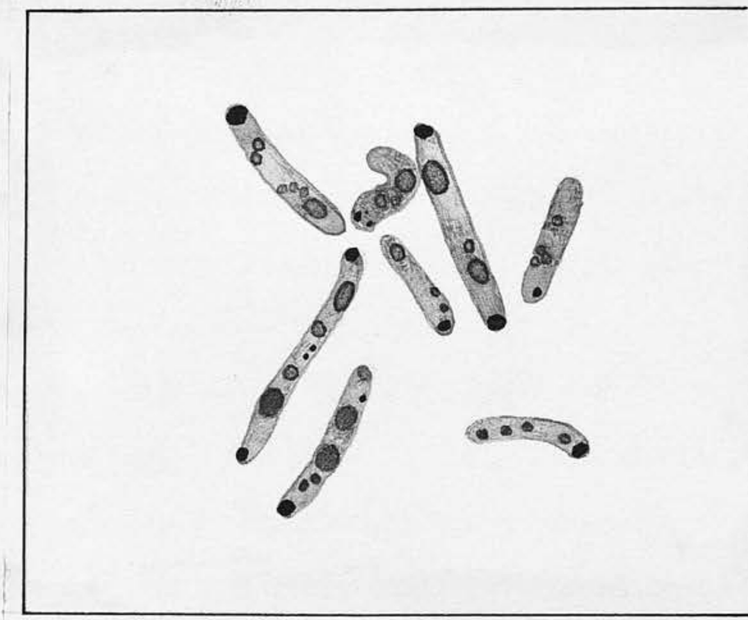
(b) <u>Lindegren's method.</u>

When cells containing volutin were mounted in Lindegren's solution (Lindegren 1949), the volutin inclusions were stained reddish, the cytoplasm blue, and the lipid inclusions appeared unstained.

(3) <u>Microscopic appearance of lipid and volutin inclusions as revealed in unstained preparations.</u>

(a) <u>With the ordinary optical microscope.</u>

With the condenser racked down, the larger lipid inclusions were clearly visible as spherical refractile bodies, but volutin granules were never apparent. The lipid inclusions were fairly readily distinguished from spores by virtue of their spherical as opposed to oval shape,



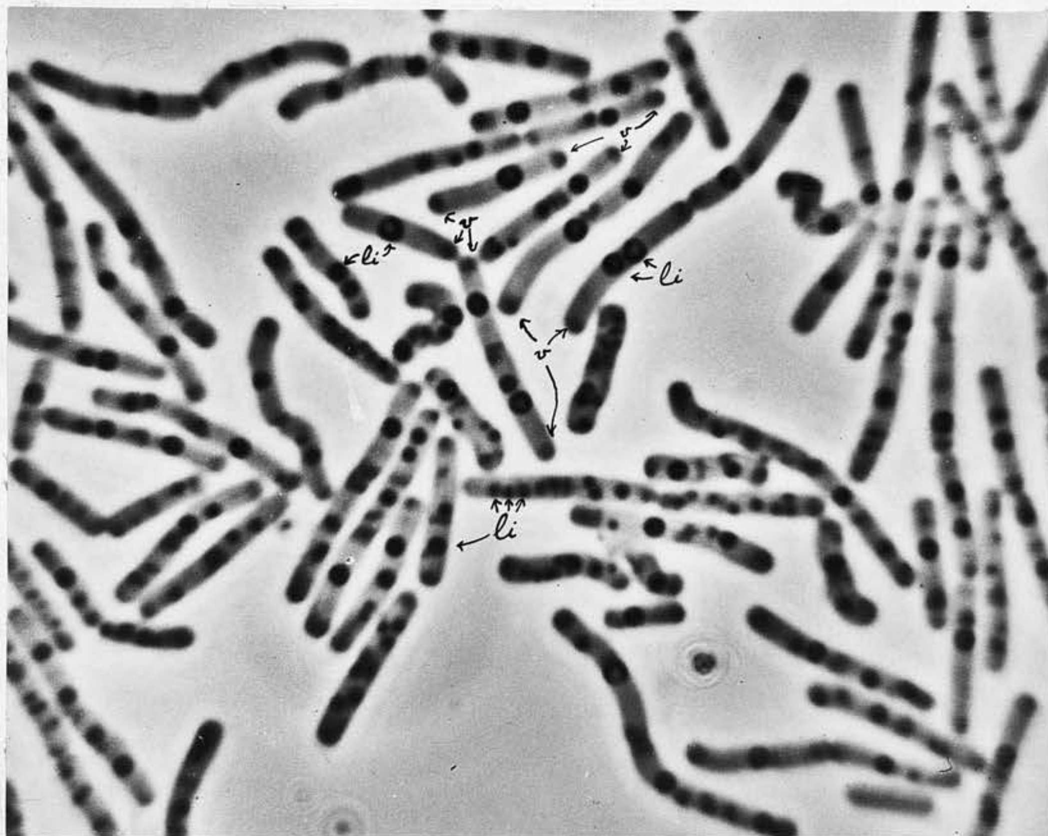
B. cereus (AC.1) double-stained with Sudan black and Albert's stain as described in text (p.105). Volutin granules (at the poles of the cells) are stained black. Lipid inclusions are stained brown, and the cytoplasm green. The two types of inclusion are quite distinct, and separate from one another.

generally smaller size, and greater number per cell.

(b) With the phase contrast microscope.

The appearance of cells of strain AC.1 grown on 1.0% glucose mycoides synthetic medium, and containing large numbers of lipid inclusions and maximal amounts of volutin, is illustrated in photograph 5 , p.107.

It will be seen that the lipid inclusions vary greatly in size and number per cell, and are distributed throughout the length of the cell. The most striking features of the lipid inclusions are their regular spherical shape and high refractility. The smaller bodies appeared a dark brown or black (the cytoplasm being a pale brown), while the largest lipid inclusions were a "glassy" refractile blue colour with a heavy black periphery, thought to be an optical artefact. In contrast, the volutin granules (which can be seen at the poles of some of the cells) were much less refractile and had a less distinct outline. The identity of the lipid inclusions was established as follows: an unfixed dry smear was mounted in water and a suitable field located. The coverslip was removed, the film air dried and remounted in alcoholic Sudan black. Examination of the same field (the phase effect having been removed by withdrawal of the annular diaphragm) showed that all the bodies tentatively identified as lipid were stained, while the volutin granules and other parts of the cell remained unstained. Similar experiments using Lindegren's solution, established the identity of the volutin granules.



Photograph 5. B. cereus (AC.1). 40hrs., 1% glucose synthetic medium, 37°C. Unstained living cells by phase contrast. The lipid inclusions (li) show great variation in size, the larger ones appearing a refractile blue colour, and having a thick black (optical) periphery. The smaller lipid inclusions are black or brown, and stand out clearly against the paler cytoplasm. They are distributed throughout the length of the cells, but generally not at the poles. The volutin granules, on the other hand (v) are very much less refractile, have a less distinct outline, and occur almost invariably at the poles of the cells. The identity of the two types of inclusions was determined by staining parallel smears as indicated in the text. The amount of lipid in this culture is equivalent to a microscopic (Sudan black) estimation of "+++".

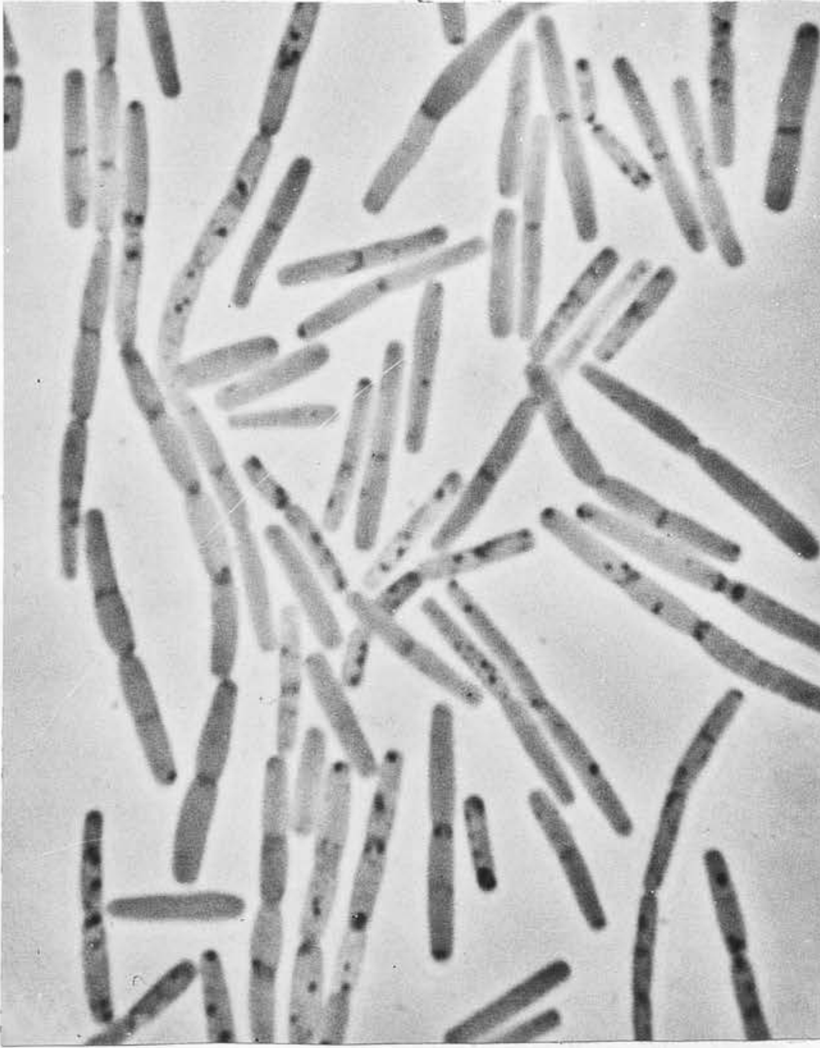
Photograph 5 at a final magnification of X 3000.

The appearance of spores of B. cereus (var mycoides) is shown in photograph 3 , p. 99. The spores are easily recognised by their large size and generally oval shape. Spores were never formed by strain AC.1 under any of the wide range of cultural conditions employed in the growth experiments (reported later), so there is no question that the larger spherical bodies in photograph 5 might be spores. In any case, these inclusions were never stained by Ashby's method for spores, and spores, on the other hand, were never stained by Sudan black.

The general pattern of occurrence of lipid and volutin inclusions in the three test organisms was confirmed by phase contrast observations. The cells in photograph 5 should be compared with those of the same strain grown on the glucose limited control medium, shown in photograph 6, 109. It will be seen that the cells of the latter preparation are practically devoid of inclusions. These findings agree absolutely with the results obtained with Sudan black, since the Sudan black estimations of lipid inclusions in the two cultures were, for photograph 5 , "+ + +", and for photograph 6 , " ± ".

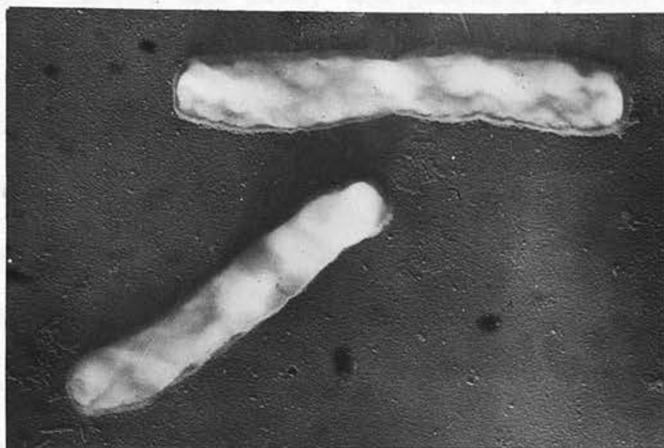
(c) Electron microscopy.

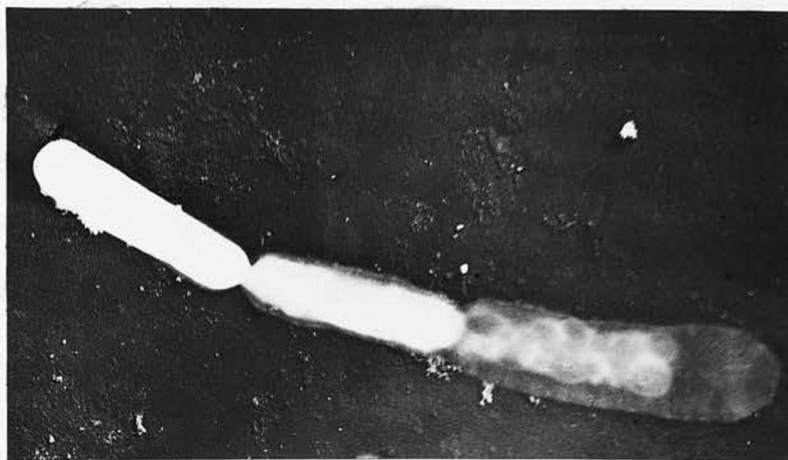
The electron microscopic appearance of lipid rich cells of strain AC.1 is shown in the electron micrograph of a shadowed preparation on p. 110. It will be seen that there was no possibility of detecting the lipid inclusions in such preparations.



Photograph 6. B.cereus (AC.1). 35hrs., glucose limited "control" medium (0.3% glucose), 37°C. Unstained living cells by phase contrast. Note the almost complete absence of inclusions. Cultures of this type give a microscopic estimation of lipid of "1".

Photograph 6 at a final magnification of X 3000.





<u>Photographs 7 and 8.</u> <u>B.cereus</u> (AC.1). 40hrs., 1% glucose synthetic medium, 37°C. Shadowed electron micrographs. Note that the lipid inclusions are not discernable except in the partly autolysed cell in photograph 8.

Photographs 7 and 8 at a final magnification of X10000.

The second electron micrograph on p.110 shows three cells, two of which appear to be in different stages of autolysis. In the end cell, the globular bodies are thought to be lipid inclusions, which have aggregated in the centre of the cells and have been made visible by virtue of the autolytic destruction of the cytoplasm. However, it was clear that in normal cells, the electron opacity of the lipid inclusions was not sufficiently different from that of the other cell contents to render the inclusions visible in electron micrographs.

(4) <u>Simple stains.</u>

When cells of both strains of <u>B.cereus</u>, and of <u>B.megaterium</u> were stained with simple aniline dyes such as methylene blue and basic fuchsin, the lipid inclusions appeared as clear unstained "vacuoles" in the stained cytoplasm. Volutin granules were apparently stained no more intensely than the cytoplasm, since they were never made visible. It is assumed that any acidic protein materials would be stained by these dyes, and the results therefore gave no positive indication of the presence of such materials in the lipid inclusions. Nevertheless, bearing in mind the previously mentioned difficulties inherent in this type of observation, one should not interpret this result as indicating the definite absence of such materials from the inclusions.

(5) <u>The gram stain.</u>

In gram stained cells of any of the three test organisms, the lipid inclusions were sometimes completely

masked by the heavy cortical gram positive staining of the bacilli, but in some cases, the inclusions could be clearly seen, and were apparently completely unstained by this method.

(6) Ashby's malachite green spore stain.

This method, which was described by Ashby (1938), consists essentially in gently heating an ordinary heat fixed smear of cells with a 5.0% solution of malachite green; spores and cytoplasm are stained at this stage, but the application of basic fuchsin decolourises the cytoplasm and stains it pink, leaving the spores stained an intense green. The present author tested the method on a wide range of aerobic and anaerobic sporeformers, including the three Bacilli already mentioned, and found that spores were invariably stained green, and lipid inclusions, if present, (identified by parallel preparations stained with Sudan black) were never stained at all. The method is therefore to be recommended for the demonstration of spores, as distinct from lipid inclusions.

The physico-chemical basis of this staining method is not known, but examination of the cells after staining and before decolourisation, showed that the malachite green did not penetrate the inclusions at all.

(7) The acid fast method of staining spores.

Some variability was found in the response of the inclusions to this method, which was that recommended by Mackie and McCartney (1953). Provided care was taken that the slide was not heated above the temperature at

which steam began to rise, the inclusions were found to remain unstained, while the spores were coloured a deep pink. It was soon found, however, that a slight increase in temperature during the period of staining resulted in the inclusions being stained a pale pink. Only a very slight deviation was required to obtain this result, and the inclusions were frequently stained inadvertantly. For this reason the use of the acid fast stain for spores is not recommended, the method of Ashby being preferred.

It was noted that when the inclusions had deliberately been stained by slightly overheating the slide, they appeared to have lost their regular shape to some extent, and irregular blobs of pink stained material appeared outside the cells. The inclusions and these external blobs of material could not be decolourised even with 20% sulphuric acid. It was concluded that the inclusion material was to some extent soluble in the hot carbol fuchsin, and some of it was being dissolved out of the cells and deposited as amorphous masses when the slide was allowed to cool. This solubility probably also allowed the stain to penetrate the inclusions. They were apparently impermeable to the sulphuric acid, however, and thus exhibited an acid fast character. The correctness of these conclusions was indicated by the chemical and physical properties of the inclusion material which will be reported later.

(8) Protein stains.

(a) The "Sharp" test.

Details of this test were published by Knaysi (1942). It consists of the immersion of a heat fixed smear in a 5.0% (w/v) glucose solution at 100°C for 24 hours. The smear is then rinsed with water, blotted dry, and examined with the oil-immersion objective. Knaysi claimed that proteins were specifically stained brown, and he found the lipid inclusions of B. cereus to give a positive reaction (Knaysi 1945-b), which led him to the conclusion that they consisted of lipoprotein.

When applied to the three organisms used in this study, there was no indication of any staining of the lipid inclusions, which appeared as unstained "vacuoles" in the pale brown-stained cytoplasm. The feeble colouration of the cytoplasm suggested that even if the inclusions did contain appreciable amounts of protein, the staining would be too weak to be visible, and the negative results were not therefore taken to indicate the definite absence of protein.

(b) The mercuric-bromophenol blue protein stain (Mazia, Brewer & Alfert (1953).

This staining method was considered to be much better substantiated than the "Sharp" test, and its' authors claimed it to be highly specific for protein, giving an intense colouration.

The method described by Mazia et al. was developed for the demonstration of protein in tissue sections. It

consists essentially of applying a mixed solution of mercuric chloride and bromophenol blue to the section, and differentiating with dilute acetic acid. Proteins are stained blue, and are not decolourised by the acid. The present author used formalin fixed smears, but the technique was otherwise the same.

Application of this stain to the usual three organisms gave negative results, although the cytoplasm of the cells was weakly stained. The interpretation of these results was similar to that of the negative "Sharp" test, and the possibility of a peripheral shell of protein around the inclusion being marked by the cytoplasmic staining, should not be overlooked.

(9) <u>The Feulgen reaction.</u>

Knaysi (1945b) reported that the lipid inclusions and cytoplasmic membrane of <u>B.cereus</u> gave a positive Feulgen reaction, and it was thought desirable to confirm this finding. The procedure used was that of Robinow (1943-4) and was applied to cells of <u>B.cereus</u> (strains AC.1 and BC). In neither case was there any indication of staining of the inclusions. It was thought possible that a Feulgen positive material of the "plasmal" type, which would only be stainable after pretreatment with mercuric chloride might be present. Accordingly formalin fixed smears of the two organisms were carried through the technique of Gomori (1952b). Negative results were obtained in this case also, and it was therefore concluded that, subject to the considerations outlined previously,

there was probably no Feulgen positive material present in the inclusions.

(10) Iodine.

Wet mounts of cells of strains AC.1 and BC of B.cereus and of B.megaterium (NCTC 7581) were prepared in a solution of iodine in potassium iodide (Albert's No. 2 solution diluted 1:1 with water was found suitable). The lipid inclusions were found to be stained a golden brown colour while the cytoplasm was a much paler yellow. All attempts to stain the inclusions in dry smears failed, unless the smear was mounted in the staining solution. There was certainly no indication of the "red-brown" characteristic of glycogen, and these results were therefore in agreement with those of Meyer (1912a). The chemical and physical basis of the colouration with iodine is not understood but it seemed likely that there was a slight superficial adsorption of the reagent, which was lost immediately the slide was rinsed with water.

In summary, the observations recorded above provided means of identifying the lipid inclusions, and established their distinctive character beyond any doubt. There was no question of the lipid material being formed in either the spores or the volutin granules or vice versa. The cytochemical results, which are recorded in table 15, p.151, indicated only that the inclusions contained some lipids in a liquid or semi-solid state at room temperature, and that part, at least, of this lipid was of a "neutral" nature. Some indication was obtained that the inclusion

material was slightly soluble in hot phenolic solution, and the inclusions appeared to be impermeable (at room temperature) to 20% sulphuric acid. There was no positive indication of the presence in the inclusions of proteins, Feulgen positive materials, metachromatic materials, or iodophilic polysaccharides, but the absence of these materials could not be categorically proved.

PART II.

OBSERVATIONS ON THE LIBERATION OF LIPID INCLUSIONS BY MEANS OF ALKALINE SODIUM HYPOCHLORITE

Preliminary experiments showed that when bacterial cells were suspended in an alkaline solution of sodium hypochlorite, there was a rapid partial loss of turbidity which was visible to the naked eye, and microscopic observation indicated that this loss of turbidity was due to lysis of the cells. The experiments reported below, however, showed that lipid inclusion bodies (if present) were resistant to the reagent, and the final turbidity of a suspension of cells treated with the hypochlorite reagent was due almost entirely to the liberated inclusions, which could therefore be estimated turbidimetrically.

The action of hypochlorite was first investigated by microscopic examination of cells undergoing lysis:

1. Continuous microscopic observation of the course of lysis with alkaline hypochlorite.

(a) At 37°C.

It will have been noted (see methods) that the routine turbidimetric estimation of the lipid inclusions using hypochlorite was carried out at 37°C, and the pH of the reagent was about 9.8.

For reasons not fully understood, the lysis of cells mounted in hypochlorite between slide and coverslip was much slower than in suspension in the water bath. It seems likely that this was due to reduced rates of

diffusion and circulation in the relatively thick suspension, of dissolved cell materials and the reagent itself, which would lead to reduced contact between the latter and the cells. Whatever its' cause, this phenomenon made the use of the standard reagent for microscopic observation rather difficult, since the time taken for completion of the process was greatly increased and even after 4 hours in the incubator at 37°C, the lysis was not always completed. It was found, however, that if the alkalinity of the hypochlorite reagent was increased, the whole lytic process took only a few minutes, even at room temperature, a finding which greatly facilitated the observations. Description of the lysis will therefore be mainly limited to the observations made with the more highly alkaline reagent at room temperature. It should be stressed, however, that several experiments with the standard reagent (at 37°C, the slide being kept in the incubator in between observations) showed that the course of lysis was substantially the same in this reagent, the only differences being the speed with which it took place, and the fact that spore membranes tended to persist.

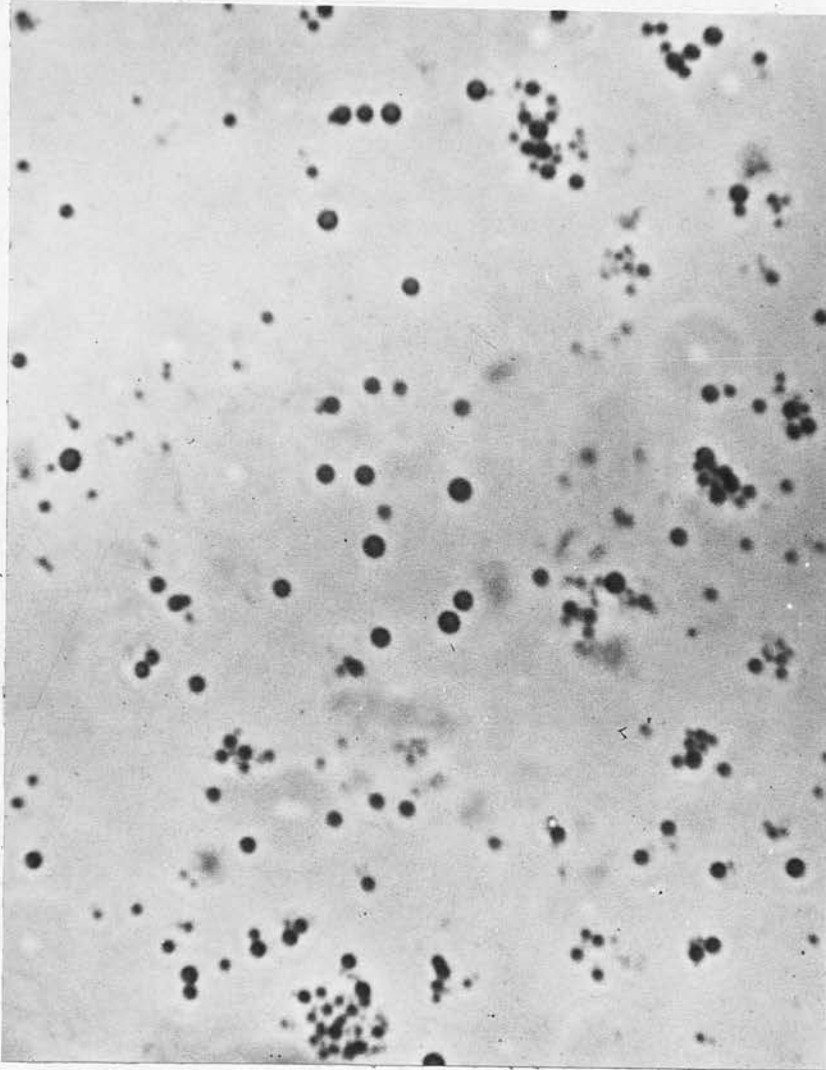
(b) <u>At room temperature.</u>

The strongly alkaline reagent used for these microscopic experiments consisted of a mixture of two volumes of the standard hypochlorite solution with one of 40% NaOH. A loopful of the culture was emulsified in a little of the mixture, a wet film mounted at once and examined (under the phase contrast microscope) without delay,

since the lysis was very rapid.

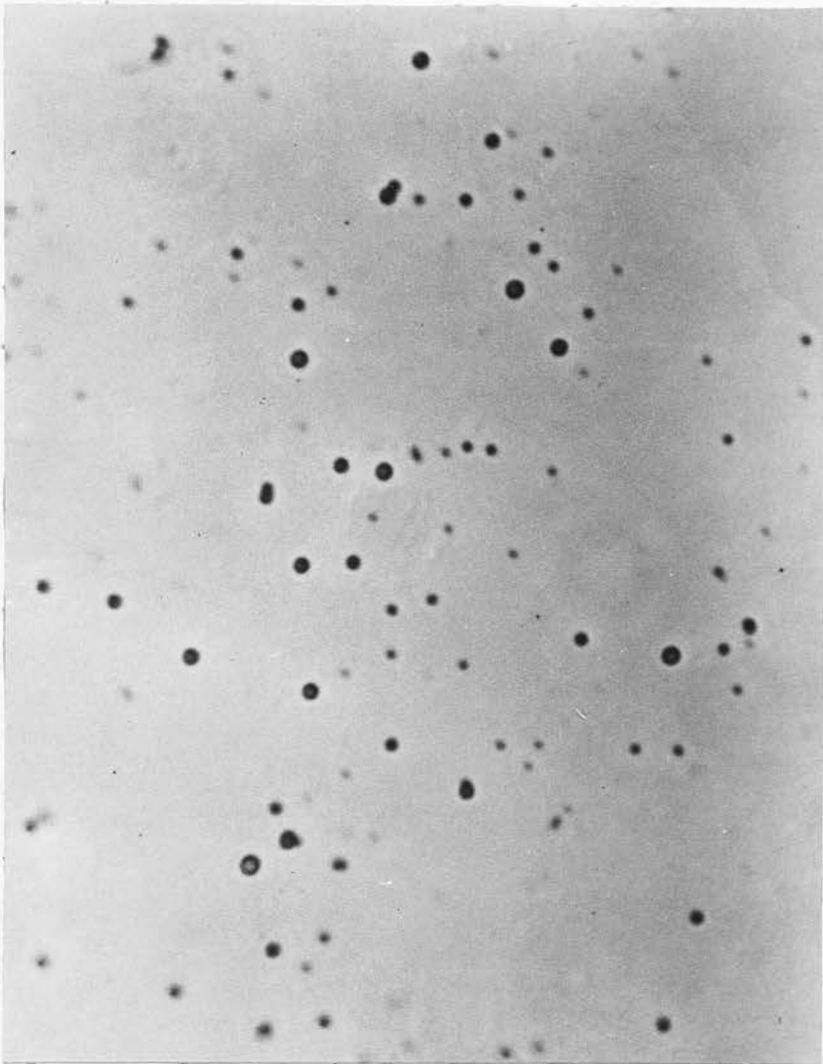
The appearance of cells of strain AC.1 at the start of such an experiment was shown in photograph 5 (p.107). Within about 2 minutes from the start of the experiment, the cytoplasm was seen to start fading away, until, a few minutes later, the outline of the cells was hardly discernable. Apparently the cell membrane was not entirely dissolved at this stage, since the inclusions, though now exhibiting brownian movement, were restricted in their movements and held together by the invisible remnants of the membrane. Eventually (within about 6 minutes of the start of the experiment) the last of the membrane was dissolved, and the inclusions were liberated into the surrounding liquid. The newly liberated granules tended to remain attached to each other, and pairs or trios, linked by some invisible material, were common. In a short while, however, most of these groups were broken up, and eventually practically all the inclusions were completely isolated. The appearance of the isolated inclusions is illustrated in photographs 9 and 10 (p.121/2). They were found to persist indefinitely, and there was no apparent change in their shape, size or refractility, during the lytic process.

Striking direct evidence that the bodies observed microscopically to be resistant to the action of hypochlorite were in fact the lipid inclusions, was provided by the following experiment: a portion of a lipid inclusion rich culture of strain AC.1 was suspended in 70%



Photograph 9. Lipid inclusions of B. cereus (AC.1) isolated with hypochlorite. Unstained, phase contrast. The lipid inclusions normally display marked Brownian movement. In order to take this photograph, a drop of saline suspension was mounted between the coverslip and a thin layer of agar spread on the slide. Comparison of this photograph with photograph 5, shows that the inclusions appear unchanged after treatment of the cells with hypochlorite.

Photograph 9 at a final magnification of X 3000.



Photograph 10. Lipid inclusions of B. cereus (AC. 1) isolated with hypochlorite. Unstained, phase contrast. In order to immobilise the inclusions, a dry smear was made and mounted in water.
Photograph 10 at a final magnification of X 3000.

ethanol for a few minutes. After centrifugation, the cells were resuspended in Burdon's alcoholic Sudan black solution (Burdon 1946), and allowed to stand for about an hour. The cells were then centrifuged and microscopic examination showed that the lipid inclusions were stained a fairly dark blue colour. A little of the pellet of stained cells was emulsified in the strongly alkaline solution of hypochlorite, a wet film made and examined at once with the optical microscope. Cells that had been stained in this way were found to lyse somewhat more slowly than living unstained cells, but the inclusions surprisingly did not lose their colour; within about 15 minutes, most of the cells were lysed, and the Sudan black stained (and therefore cogently lipid) inclusion bodies liberated.

At this point the action of the more highly alkaline reagent was found to differ slightly from that of the standard reagent at 37°C. It was found that for reasons not understood, the standard reagent decolourised the stained inclusions quite rapidly. Provided, however, the preparation was examined without delay, individual stained inclusions could be located, and even though they became decolourised, their persistence during the lysis of the rest of the cell was easily established.

The effect of hypochlorite on spores was investigated by following the lysis of a partly spored culture of <u>B.cereus</u> (strain BC) with the phase contrast microscope. The phase contrast appearance of spores was illustrated in photograph 3, p.99. They were generally easy to distinguish from the larger lipid inclusions on direct inspection, but in any case behaviour under the action of hypochlorite was characteristic. Initially, the lysis

of the cells followed the course outlined above, but the spores, unlysed, were liberated from the cells at the same time as the lipid inclusions. About five minutes later, however, individual spores could be seen to suddenly change from their glassy blue refractile appearance to an opaque brown, the change occurring in a fraction of a second. One could see a 'wave' of brown opacity passing rapidly from one end of the spore to the other. Thereafter the spore became slightly swollen, and slowly faded away until all that could be seen was the faint ghost of the swollen spore membrane. Eventually, this also disappeared, and about 15 minutes after the start of the experiment, only the lipid inclusions were left. The spores of B.megaterium and B.subtilis were lysed in an exactly similar fashion, and these experiments therefore indicated that the presence of spores would not affect the level of the final turbidity of a suspension treated with hypochlorite. Confirmation of this finding was provided by the turbidimetric observations to be reported in the next section.

It was stated above that phase contrast observation of the lytic process showed that only the lipid inclusions were resistant to hypochlorite. This was only true when the cells contained no volutin, for careful observation of cells of strain AC.1 bearing maximal amounts of volutin showed that the volutin granules (recognised by their polar position, low refractility and indistinct outline) tended to remain undissolved. The observation of volutin was

hampered by the presence of the lipid inclusions, which were so much more prominent than the volutin granules, and tended to obscure them. However, it was quite clear that the volutin granules were liberated from the cells with the lipid inclusions, and it was noted that they tended to stick to any lipid inclusions with which they chance to come in contact, a property which added to the difficulties of observing them.

The persistence of volutin granules was much more easily observed in volutin rich cultures of Aero. aerogenes (strain A3(o)), since this organism, when grown on the medium recommended by Duguid et al. (1954) for the production of volutin through the development of an acid reaction, formed no lipid inclusions, but abundant volutin (+++), and was therefore ideal for observation of the fate of volutin granules. The cells were mounted in the strongly alkaline hypochlorite reagent and examined under the phase contrast microscope as before. The granules appeared as large spherical bodies slightly darker than the cytoplasm, but generally not very distinct. There were usually three or four granules per cell. As the hypochlorite started to act (in a few minutes) the cytoplasm started to fade away, and the inclusions became much more prominent. Eventually they were liberated from the disintegrating cells, but showed a great tendency to remain clumped together, and it was only after several minutes that most of these clumps were broken up.

The identity of the bodies thus liberated was

further indicated by the fact that if lightly heat fixed smears of volutin rich cells of either B.cereus (AC.1) or Aero.aerogenes (A3(o)) were stained by Albert's method and mounted in hypochlorite, the jet black volutin granules remained coloured and could be seen to be liberated into the medium, while the green stained cytoplasm was rapidly decolourised and completely dissolved.

The fact that the volutin granules remained undissolved by hypochlorite was of considerable interest, since it suggested the possibility that they might be isolated from the cells by the use of the reagent. It was also thought that the presence of volutin granules in the cell and their persistence when the cell was lysed might introduce an error into the hypochlorite estimation of the lipid inclusions. It will be shown, however, that in B.cereus at any rate, the estimation of the lipid inclusions was not materially affected by the presence of volutin, largely because of the low refractility of the latter inclusions.

The conclusion drawn from the above experiments was that only the lipid and volutin inclusions resisted the action of hypochlorite, and the experiments in the following section were made in order to determine the value of the reagent for the estimation of the lipid inclusions, and to design a suitable technique for its use.

2. Turbidimetric observations of the lytic process.

Washed suspensions of cells of B.cereus (strains AC.1 and BC), B.megaterium (strain NCTC 7581), B.subtilis, and Aero.aerogenes (strain A3(o), Duguid and Wilkinson 1953) were employed. Measured volumes were centrifuged, the supernatants removed, and the cells suspended in the same volume of the appropiate reagent (previously warmed to the desired temperature) in conical flasks. The temperature was maintained by agitating the flasks in a thermostatically controlled water bath. 10ml. samples were withdrawn at intervals and their turbidities determined with the "Spekker" as rapidly as possible. About 15 seconds was required to remove each sample and record its' turbidity, and the times recorded in the tables and graphs were taken as the moments at which the drum was read. Lysis proceeded too rapidly for the initial turbidity of the cells in the hypochlorite reagent to be determined, and the "0 time" readings recorded in the tables are the turbidities of the saline suspensions of cells prior to centrifugation.

(a) pH.

The effect of the pH of the reagent on the lysis was studied by comparing the curves obtained when strain AC.1 of B.cereus (grown on 2.0% glucose agar, and containing no spores, but lipid inclusions estimated microscopically as +++) was suspended in the standard hypochlorite reagent adjusted to pH's 8.4, 9.8, and 11.6 with varying amounts of concentrated HCl. The results are shown in table 8 and

GRAPH 2: The effect of pH on the lysis of B.cereus(AC.1) by hypochlorite at 37°C.

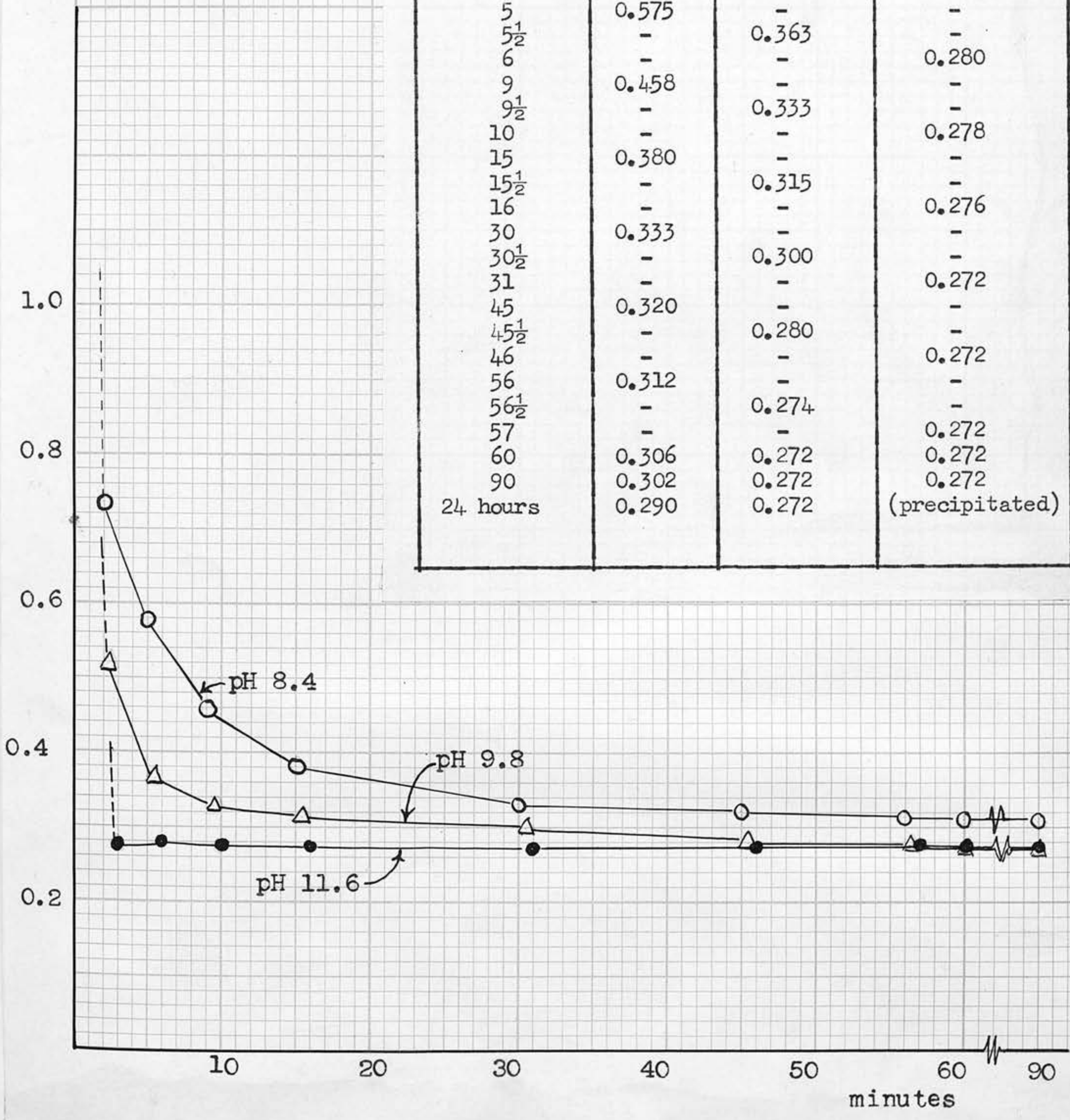


TABLE 8.

Time (mins)	Turbidity at pH:		
	8.4	9.8	11.6
0	1.935	1.935	1.935
2	0.735	-	-
2½	-	0.515	-
3	-	-	0.275
5	0.575	-	-
5½	-	0.363	-
6	-	-	0.280
9	0.458	-	-
9½	-	0.333	-
10	-	-	0.278
15	0.380	-	-
15½	-	0.315	-
16	-	-	0.276
30	0.333	-	-
30½	-	0.300	-
31	-	-	0.272
45	0.320	-	-
45½	-	0.280	-
46	-	-	0.272
56	0.312	-	-
56½	-	0.274	-
57	-	-	0.272
60	0.306	0.272	0.272
90	0.302	0.272	0.272
24 hours	0.290	0.272	(precipitated)

graph 2. In all cases lysis of the cells commenced immediately, rapidly at first, but gradually slowing up until the turbidity reached a constant final level. It is clear that although the rate of lysis was greatly dependent on the pH of the reagent, the rate being higher in the reagents of higher pH, the final level was essentially independent of the pH. The pH of the reagent to be used for purposes of routine estimation was chosen as pH 9.8, though in practice it was found that slight deviations from this value, within a range of 9.5 - 10.0, had no appreciable effect on the final level of turbidity. This range of pH for the final reagent was chosen since it was apparent that a lower value enormously increased the time taken for the attainment of the final steady turbidity, and if the pH was higher than about 11.0, the residual material was eventually agglutinated and thrown out of suspension in large floccules. In the reagent of pH 9.8, however, the final steady turbidity was reached in not more than 90 minutes, and the supension appeared to be stable for a long period. In the experiment recorded, the turbidity remained unchanged over a period of 24 hours, and suspensions in this reagent have been maintained for several days without any appreciable loss of turbidity.

(b) Temperature.

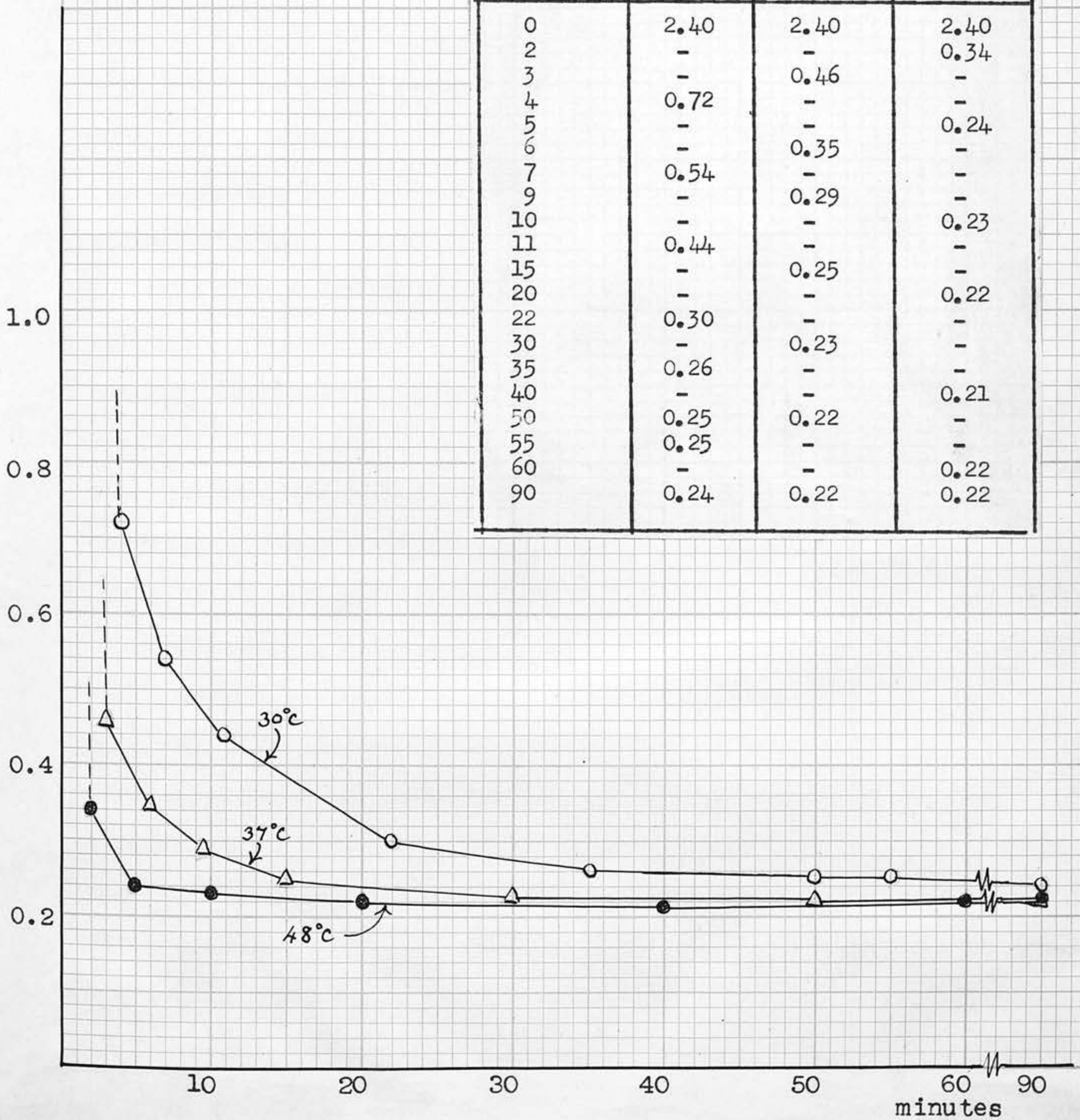
The effect of temperature is shown by the results obtained with strain AC.1 of B.cereus shown in table 9 and graph 3. The rate of lysis was evidently greater at

GRAPH 3.

The effect of temperature on the lysis of B.cereus (AC.1) with alkaline hypochlorite; standard reagent, pH 9.8 for 90 minutes

TABLE 9.

Time (mins)	Turbidity at:		
	30°C	37°C	48°C
0	2.40	2.40	2.40
2	-	-	0.34
3	-	0.46	-
4	0.72	-	-
5	-	-	0.24
6	-	0.35	-
7	0.54	-	-
9	-	0.29	-
10	-	-	0.23
11	0.44	-	-
15	-	0.25	-
20	-	-	0.22
22	0.30	-	-
30	-	0.23	-
35	0.26	-	-
40	-	-	0.21
50	0.25	0.22	-
55	0.25	-	-
60	-	-	0.22
90	0.24	0.22	0.22



higher temperatures, but as before, the final turbidity was independent of temperature. 37°C was chosen as the standard temperature to be used routinely.

(c) Duration of lysis.

Inspection of any of the graphs showing the time course of the lysis indicated that the final steady turbidity was invariably reached in about 1 hour from the start. It was decided, as a precautionary measure, to adopt 90 minutes as the standard duration for the purposes of quantitative estimation. It was thought that this time would prevent any errors due to possible slight variations between different batches of the hypochlorite reagent. For the large scale isolation of the inclusions for analysis, however, the lysis was continued for 2 hours, since a much heavier cell suspension was employed for this purpose, and it was thought advisable to use the longer time to guarantee complete dissolution of the cells.

(d) Concentration of cells.

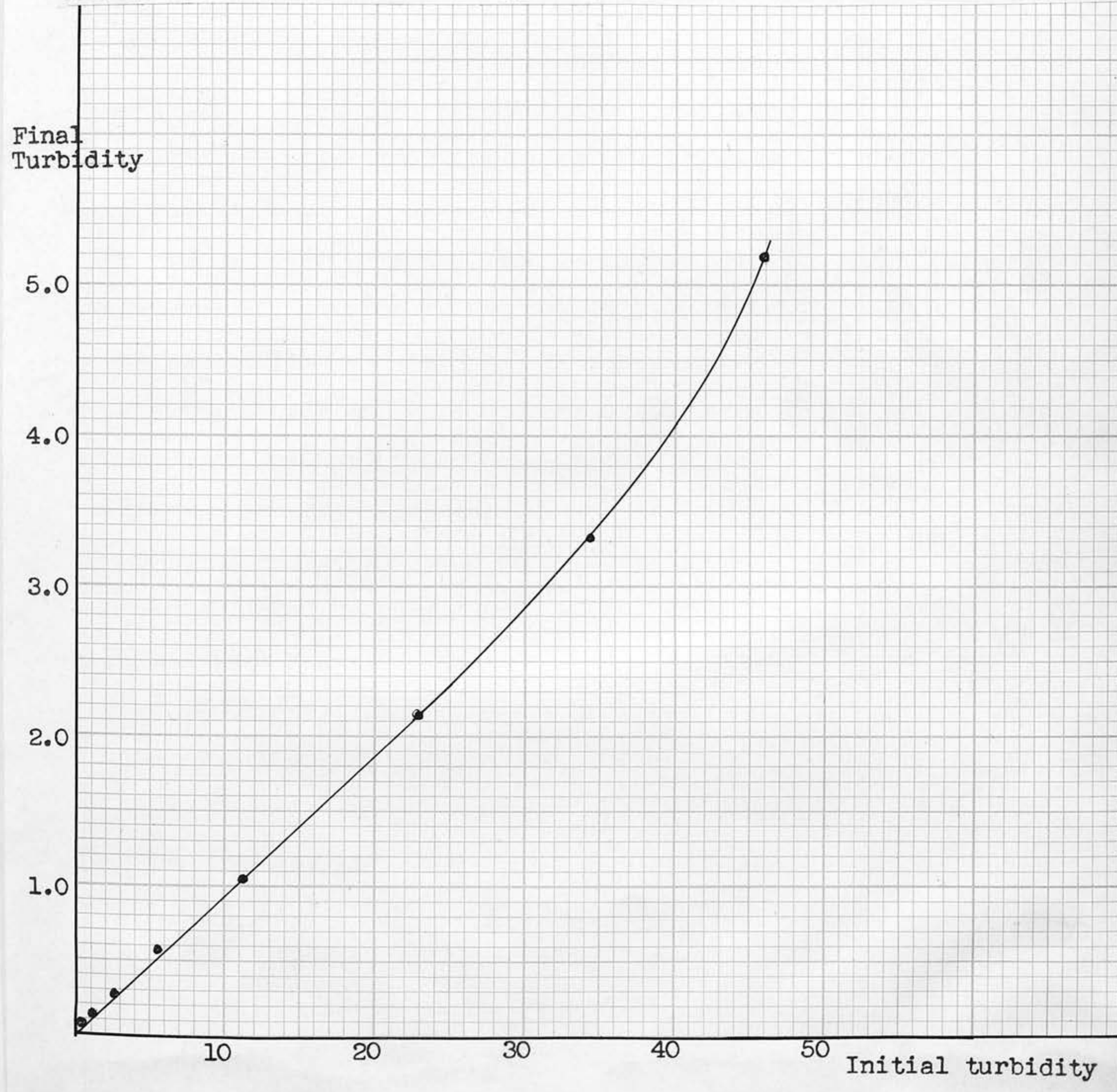
Cells of strain AC.1 (grown on glucose agar and containing no spores but lipid inclusions estimated microscopically as +++) were suspended in various concentrations in saline. Aliquots of each suspension were centrifuged, and the deposits suspended in equal volumes of the standard reagent (pH 9.8) for 90 minutes at 37°C. The final turbidities were read and then plotted against the initial concentrations of the cell suspensions estimated as their turbidities. (table 10 and graph 4). It is clear that over a limited range of cell concentrations

GRAPH 4.

Relationship between initial turbidity of a suspension of a sample of B.cereus (AC.1) and the final turbidity attained on hypochlorite treatment: lipid inclusions "+++". Hypochlorite treatment as described in text.

TABLE 10.

Initial Turbidity	0.515	1.150	2.875	5.750	11.500	23.000	34.500	46.000
Final Turbidity	0.058	0.116	0.262	0.560	1.025	2.120	3.320	5.175



the final turbidity was directly proportional to the initial concentration of cells. When the initial concentration was increased to more than about 25 Spekker units, however, the final turbidity increased disproportionately, presumably because there was not sufficient hypochlorite present to complete the digestion.

For the purposes of routine estimation of the lipid inclusions of strain AC.1, a concentration of cells of approximately 1.0mg. (dry weight) per ml. was employed, the equivalent Spekker reading being about 2.5. Not only was this concentration on the linear part of the curve relating the initial to the final turbidity, but it was also suitable for routine estimations of nitrogen and polysaccharide made on the same samples in the course of later experiments. In large scale isolation of the inclusions for analysis, however, the total volume of reagent handled was kept low by using initial cell concentrations of the order of 8·0mg. (dry weight) per ml., the equivalent turbidity being about 20 Spekker units, which is still on the linear part of the curve relating the initial and final turbidities.

(e) The effect of cell inclusions on the final turbidity.

(i) Lipid inclusions.

A series of experiments were made to determine the relationship between the cell content of lipid inclusions, estimated microscopically, and the ratio of the final steady turbidity obtained with hypochlorite treatment, to the initial concentration of cells in the test suspension, the latter being

TABLE 11.

RELATIONSHIP OF "TURBIDITY RATIO" TO MICROSCOPICALLY ESTIMATED LIPID INCLUSION CONTENT OF B.CEREUS (AC.1)

The results recorded here clearly indicated that the ratio of turbidities was proportional, in a statistical sense, to the lipid inclusion content of the cells as estimated by microscopic examination of Sudan black stained smears. These observations, coupled with the other results in this section, provided the preliminary experimental basis for the use of hypochlorite in estimating lipid inclusions.

Ratios of final turbidity obtained on treatment with hypochlorite to initial turbidity for B.cereus strain AC.1, in 73 cultures having microscopically estimated lipid inclusion contents of :

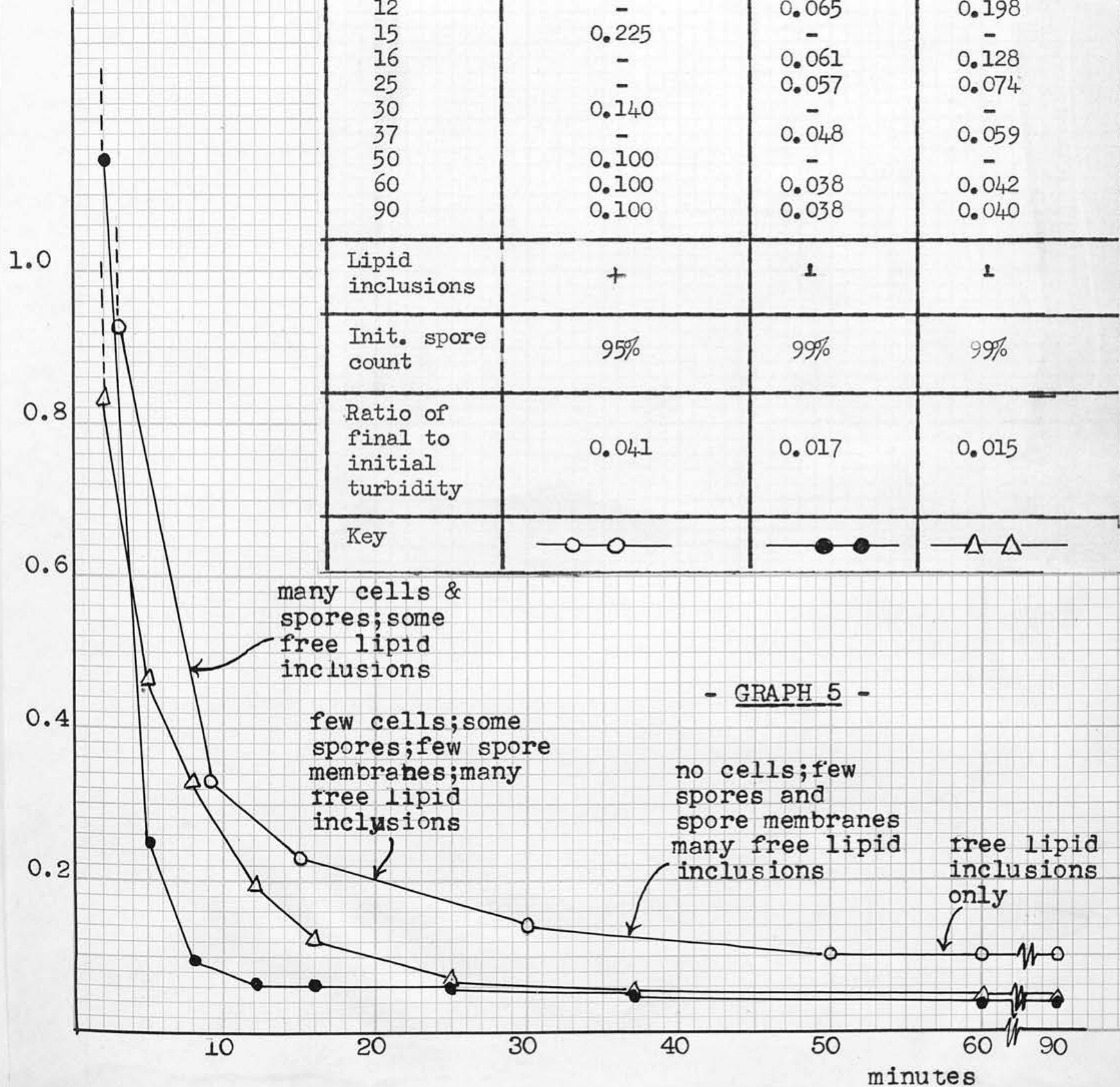
± (17 cultures)	+ (17 cultures)	++ (17 cultures)	+++ (14 cultures)	++++ (8 cultures)
0.024	0.034	0.041	0.148	0.248
0.034	0.035	0.052	0.158	0.267
0.041	0.044	0.042	0.114	0.277
0.029	0.025	0.040	0.130	0.264
0.026	0.035	0.034	0.200	0.253
0.022	0.026	0.048	0.185	0.287
0.017	0.040	0.033	0.185	0.262
0.017	0.029	0.082	0.145	0.236
0.023	0.027	0.041	0.133	
0.028	0.027	0.055	0.130	
0.028	0.034	0.097	0.150	
0.019	0.045	0.071	0.183	
0.018	0.032	0.049	0.192	
0.019	0.031	0.067	0.160	
0.020	0.042	0.064		
0.022	0.027	0.036		
0.019	0.041	0.068		
average 0.024	0.034	0.052	0.158	0.262

estimated by the turbidity of the initial suspension. It was soon found that the ratio of the turbidities was proportional to the lipid inclusion content of the cells, which finding indicated beyond any doubt that the hypochlorite method could be used to obtain an estimate of the lipid inclusions. The results of a large number of separate determinations of the "turbidity ratios" of different samples are shown in table 11, where the figures have been placed into groups according to the microscopic estimations of lipid inclusion content. It will be seen that the figures in each column display a considerable scatter. This is only to be expected, since the microscopic estimation of lipid inclusions is very insensitive and is subject to a considerable subjective error. Proof of the reliability of the hypochlorite estimations will be given later when it will be shown that in fact they yield precise estimates of the major component of the inclusions, PHB. For the present, it is only necessary to note that the figures clearly showed that, in a statistical sense, the ratio of the turbidities varied in parallel with the microscopic estimations of lipid inclusion content. These observations, strengthened by the knowledge that virtually the only hypochlorite resistant materials in the cells were the lipid inclusions, provided the preliminary experimental basis for the use of the method.

Lysis of spore-bearing cultures of B.cereus(BC), B.megaterium(NCTC 7581) and B.subtilis, with standard hypochlorite at 37°C.

TABLE 12.

Time (mins)	Turbidities of:		
	B.megaterium	B.cereus	B.subtilis
0	2.460	2.300	2.660
2	-	1.146	0.832
3	0.925	-	-
5	-	0.252	0.466
8	-	0.097	0.335
9	0.330	-	-
12	-	0.065	0.198
15	0.225	-	-
16	-	0.061	0.128
25	-	0.057	0.074
30	0.140	-	-
37	-	0.048	0.059
50	0.100	-	-
60	0.100	0.038	0.042
90	0.100	0.038	0.040
Lipid inclusions	+	±	±
Init. spore count	95%	99%	99%
Ratio of final to initial turbidity	0.041	0.017	0.015
Key	—○—○—	—●—●—	—△—△—



- GRAPH 5 -

It was still necessary, however, to determine what effect (if any) the presence of spores and volutin might have on the final level of turbidity of the suspension treated with hypochlorite. The following experiments were made with this aim in view.

(ii) Spores.

As has already been recorded, phase contrast examination of cells undergoing lysis at room temperature with the strongly alkaline solution of hypochlorite showed that spores were completely dissolved, and it was not expected that the presence of spores would affect the final level of turbidity, an expectation that was, in fact confirmed.

Heavily spored suspensions of B.subtilis, B.cereus (strain BC) and B.megaterium (NCTC 7581) were treated with the standard hypochlorite reagent, and the lytic process followed turbidimetrically as above. In addition, samples were taken at intervals and examined with the phase contrast microscope, and the presence of cells, spores, spore membranes and lipid inclusions recorded. The results are shown in table 12 and graph 5. It is clear that the presence of spores had no appreciable effect on the final level of turbidity or on the ratio of the final turbidity to the initial turbidity of the cell suspension. Thus, the ratios obtained with the B.cereus and

B.subtilis cultures, (each having spored heavily and bearing only trace (±) amounts of lipid inclusions), were of the same order as those obtained with spore free cells of strain AC.1 of B.cereus containing trace amounts of lipid (table 11). Also, the heavily spored culture of B.megaterium containing "+" lipid inclusions gave a ratio comparable to that obtained with "+" cells of the asporogenous strain AC.1 (table 11). It should be pointed out that the absolute values of the ratio of turbidities for a given cell content of lipid inclusions may vary according to the strain employed, and direct comparison between different strains may not be strictly justifiable unless turbidity characteristics of the strains are known. Moreover, the turbidity ratio of a culture that has wholly or partly spored has little real significance when compared with that of a culture that has not spored, since the initial turbidity of a partly spored suspension is dependent on the percentage of cells spored, and on the degree of breakdown of the sporangia, and variations in these factors may affect the mass/turbidity ratio of such a suspension in an unpredictable fashion. Nevertheless, the close similarity of the figures obtained in this instance suggests that no serious error was involved in the assumption that the presence of spores did not affect the final level of turbidity. The correctness of this assumption

was also indicated by the microscopic observations, which showed that the final suspension was devoid of spores or spore membranes.

(iii) Volutin.

As previously reported, microscopic observations indicated that volutin granules were apparently not entirely dissolved by the hypochlorite reagent. Experiment showed, however, that the resistance of these inclusions was of little consequence to the estimation and isolation of lipid inclusions.

Bacilli were not suitable organisms for the purpose of testing the effect of volutin on the final turbidity, as they invariably contained lipid inclusions, which complicated the interpretation of the results. For this reason Aero. aerogenes (strain A3(o)) was employed. It was grown as previously indicated, and the cells contained no lipid inclusions, but large amounts of volutin (+++). The microscopically observed refractility of these inclusions was relatively low, however, compared with that of the lipid inclusions of the Bacilli, and this low refractility meant that the final turbidity of a suspension of volutin rich cells was relatively low compared with that of a suspension of lipid rich cells. This is shown in table 13, (p.140) which includes for comparison the figures (from table 11) obtained with strain AC.1 of B. cereus bearing various amounts of lipid inclusions. It will be

TABLE 13.

EFFECT OF VOLUTIN ON THE RATIO OF FINAL TURBIDITY (AFTER HYPOCHLORITE TREATMENT) TO INITIAL TURBIDITY OF CELL SUSPENSION

ORGANISM	VOLUTIN CONTENT	LIPID INCLUSION CONTENT	RATIO OF TURBIDITIES
Aero. aerogenes	+++	-	0.037
	-	-	0.015
B. cereus (AC.1)	+±	++++	0.262x
	+± *	+++	0.158x
	±*	+	0.034x
	-*	±	0.024x

*average estimate of the samples used for calculation of the corresponding ratio.

x data from table 11.

seen that the ratio given by the Aero. aerogenes cells containing "++++" (i.e. maximal) amounts of volutin inclusions was substantially no greater than that found for strain AC.1 containing only "+" lipid inclusions, and was less than a quarter of the ratio given by the B. cereus cells bearing "+++" amounts of lipid inclusions. It was, moreover, only about a seventh of the ratio given by B. cereus with "++++" lipid inclusions.

Attention should be drawn to the fact that comparison between different strains may not be entirely legitimate. Nevertheless the assumption that the volutin granules of B. cereus would normally contribute little to the value of the final turbidity, was found, in practice, to be fully justified, and supporting chemical evidence will be presented later. The error involved would be greatest in cells containing small amounts of lipid inclusions and many volutin granules. In fact, in strain AC.1, cells of this type practically never occurred, and a "++" value for volutin was rarely exceeded.

It was also found that the presence of volutin granules did not affect the accuracy of the calibration curve* used for estimation of lipid inclusions. Experiments showed that if the residue obtained by the lysis of volutin rich cells of Aero. aerogenes was centrifuged, and resuspended in distilled water, this volutin residue was completely dissolved. Since the preparation of the calibration curve entailed washing the deposited residue with water, any volutin that might be present would be dissolved and would not affect the accuracy of the curve. This finding also indicated that when the lipid inclusions were isolated on a macro-chemical scale, the dialysis technique used to purify them would result in the complete dissolution and loss during centrifugation, of any volutin that might be present. Micro-chemical and macro-chemical observations confirming this supposition will be presented later.

The microscopic and turbidimetric experiments described above provided the basis for the validity of the method of estimating lipid inclusions by treatment of the cells with hypochlorite. They indicated that the final turbidity of a suspension of cells treated with hypochlorite was proportional (for a given concentration of cells) only to the cell content of lipid inclusions,

* see page 143.

as indicated by microscopic examination of Sudan black stained smears. It will be shown later that the final turbidity was in fact found to be directly proportional to the cell content of polymerised β-hydroxybutyric acid which, it will be shown, was the main component of the lipid inclusions. As a first step in this direction, it was necessary to determine the relationship between the turbidity of the final suspension of hypochlorite treated cells and the concentration of lipid inclusions in the suspension. The calibration curve constructed with the data thus obtained, could then be used for the conversion of the turbidity of the final suspension into its lipid inclusion content.

3. Preparation of the calibration curve relating the turbidity of the final suspension obtained on treatment of a cell suspension with hypochlorite, to its content of lipid inclusions.

The method used to prepare the calibration curve

consisted essentially of the preparation of a series of dilutions of a concentrated suspension of freshly isolated inclusions. These inclusions were still suspended in the hypochlorite that had been used to isolate them, and had not been centrifuged or purified in any way. The suspending fluid used to prepare the dilutions was so-called "used" hypochlorite, which had been prepared by centrifuging and filtering a portion of the above suspension so as to free it of all suspended inclusions, thus providing a clear fluid. The turbidities of these dilutions prepared in this fluid were then measured, aliquots centrifuged, the deposited inclusions washed in distilled water by centrifugation, dried, and weighed. Full details of the technique, together with the results obtained, and the curve itself, are recorded in "Materials and Methods" (p. 88). It will have been noted that in moderately dilute suspensions of lipid inclusions, the turbidity was directly proportional to the dry weight of lipid inclusions per ml., but that as the concentration of inclusions was increased, the proportionality was not accurately maintained. This is a normal feature of any mass/turbidity curve, and it meant that concentrated suspensions of inclusions should be diluted so that their turbidities lay on the linear part of the curve, and the actual mass per ml. of the concentrated suspensions calculated accordingly.

It might be thought that the calibration curve could have been constructed by simply suspending known weights of washed and purified inclusions in distilled

water, and measuring the resultant turbidities. Unfortunately, this was not the case. Initially a curve constructed along these lines was in fact used for some preliminary experiments. One test of its reliability was the determination of the actual dry weight of suspended material in some routine test samples and comparison of this figure with that obtained from the curve. It was found that the real weight of suspended material was only 75% of that estimated from the curve. Investigation of the reasons for this discrepancy brought to light two important factors, both of which are of fundamental significance to any turbidimetric determinations of mass.

Firstly, it had been overlooked that the turbidity of a particulate suspension is dependent, among other things, on the difference between the refractive indices of the particles and of the suspending fluid. It is common knowledge that if the refractive indices are the same, a suspension appears completely transparent, and the greater the difference between the two refractive indices, the greater the ratio of turbidity to mass. It was therefore important to ensure that the refractive index of the suspending fluid used in constructing the calibration curve was the same as that of the routine test samples, and the employment of "used" hypochlorite was the easiest way of satisfying this condition. Secondly, the particles used for the preparation of the calibration curve must be dispersed to the same degree as those of the routine test samples, since if they are appreciably clumped together, the effective particle size may be greatly increased,

a factor which may lead to a reduction of the turbidity/ mass ratio. Now, it has been mentioned (see Methods, p. 91) that these inclusions were particularly liable to clump together when centrifuged, and it was virtually impossible to obtain 100% dispersion of the inclusions once they had been so treated. Even the best suspensions prepared from such centrifuged inclusions were found, microscopically, to consist largely of aggregates of perhaps 30 or 40 inclusions. The freshly isolated inclusions, on the other hand, were completely dispersed and free of such clumps. It was for this reason that freshly isolated inclusions had to be used for the construction of the calibration curve.

The practical importance of these two factors was shown by the following experiments:

0.5ml. of a heavy suspension of freshly isolated inclusions prepared by the treatment of a sample of cells of strain AC.1 with hypochlorite, was added to 9 ml. of each of (a) distilled water, (b) fresh hypochlorite, and (c) "used" hypochlorite. Being fresh and uncentrifuged there was no clumping in any of these suspensions, yet their turbidities were markedly different (see table 14). This difference could only have been due to the different refractive indices of the three fluids, since in all other ways, the suspensions were identical.

A fourth suspension (d) was prepared, in distilled water, from a sample of inclusions washed and purified by centrifugation, and its turbidity adjusted to that of suspension (a) above. The dry weight of material in one ml. of this suspension (d) was determined and is recorded

in table 14. It will be seen that it contained 1.6 times the amount of material in suspension (a). Microscopic examination of the two suspensions showed that the inclusions in suspension (d) were markedly clumped (even though this was not apparent to the naked eye) - while those of suspension (a) were completely dispersed. Since the only difference between these suspensions lay in the degree of clumping, it was concluded that this was the factor responsible for the difference in their turbidity/mass ratios.

TABLE 14.

		Turbidity	Mass/ml.	Turb/mass
Fresh (uncentrifuged) inclusions, suspended in:	(a) distilled water	0.360	0.124mg.	2.90
	(b) Fresh hypochlorite	0.288	0.124mg.	2.32
	(c) "Used" hypochlorite	0.302	0.124mg.	2.43
Washed, centrifuged inclusions, suspended in:	(d) distilled water	0.360	0.198mg.	1.82

In the erroneously constructed calibration curve, both these factors - clumping and refractive index - were operative, since in preparing it, washed (i.e. centrifuged), inclusions had been suspended in distilled water. Using the data of the above experiments, it will be seen that the true turbidity/mass ratio of suspension (c) (which was

equivalent to a routine test sample) was 2.43. In suspension (d), on the other hand, (which was equivalent to the suspensions used in preparing the erroneous calibration curve), the ratio was only three quarters of this, i.e. 1.82. Therefore, the apparent mass per ml. of suspension (c), determined from a calibration curve prepared from suspension (d), would be 1.33 times the true mass. In other words the true mass would be only 75% of the indicated mass, which, it will be remembered, was exactly the error which indicated the need for the above experiments, and led to the development of the correct method of preparing the calibration curve.

In summary, the results of the above experiments showed that when cells of strain AC.1 of B. cereus were treated with hypochlorite, the entire cell with the exception of the lipid and volutin inclusions, was dissolved. Volutin inclusions, however, were never present in very large amounts, and their refractility was so low, compared with that of the lipid inclusions, that they contributed very little to the final turbidity of a suspension of cells treated with hypochlorite. This final turbidity was in fact shown to be proportional to the lipid inclusion content of the cells as estimated by microscopic examination of Sudan black stained smears. A calibration curve was constructed, relating the turbidity of a suspension of lipid inclusions to its dry weight content. Using this curve, it was now possible to determine the dry weight of

lipid inclusions in a suspension of cells simply by treating the cells with the standard alkaline hypochlorite reagent under the appropiate conditions, and determining the final turbidity. As will be shown later, the figure thus obtained for "lipid inclusions" could in fact be converted into one for polymerised β-hydroxybutyric acid, which it will be shown, comprised the major fraction of the inclusions isolated in this way.

PART III.

MICROSCOPIC APPEARANCE AND MICROCHEMICAL CHARACTERS OF THE LIPID INCLUSIONS AFTER ISOLATION WITH HYPOCHLORITE

The observations described here were made with the aim of detecting by microchemical and staining tests any alteration of the inclusions that might have been caused by the hypochlorite reagent, and the results should be compared with those obtained with the exactly similar tests applied to the inclusions *in situ*, as recorded in Part I. For easy comparison, the results of both series of tests have been recorded in table 15, p. 151.

The inclusions used were those of strain AC.1 of *B. cereus* and had been isolated on the macro scale with hypochlorite, and purified by dialysis as described in "Methods". Unless otherwise indicated, they were examined as unfixed dry smears.

As has already been noted, phase contrast observation showed that no apparent change in the shape, size, or refractility of the inclusions took place during the course of lysis with hypochlorite. This is illustrated by comparison of the photographs on p.121/2, which show the microscopic appearance of the isolated inclusions, with the phase contrast photograph of whole cells shown on p.107.

From table 15, it will be seen that most of the results obtained with the isolated inclusions were the same as those obtained with the inclusions *in situ*. Thus the application to the inclusions of simple aniline dyes, the "Sharp" and mercuric-bromophenol blue tests for protein,

Staining method	Lipid Inclusions in situ	Lipid Inclusions isolated with hypochlorite	Lipid inclusions freshly isolated be mechanical disruption of the cells, and not purified in any way.
Simple aniline dyes	no staining; no "acidic" proteins	same as for in situ inclusions	same as for in situ inclusions
"Sharp" test (protein)	no staining; no proteins	same as for in situ inclusions	-
Mercuric-bromophenol blue test (protein)	no staining; no proteins	same as for in situ inclusions	same as for in situ inclusions
Alberts stain	no staining; no volutin or metachromatic material present	same as for in situ inclusions	same as for in situ inclusions
Lindegren's method	no staining; no volutin or metachromatic material present	same as for in situ inclusions	-
Ashby's spore stain	no staining; no spore material present	same as for in situ inclusions	same as for in situ inclusions
Acid fast spore stain	no staining unless slide overheated during staining, when inclusions stained pink, lost regular shape, and irregular blobs of pink material appeared on slide. Colour fast to 20% H_2SO_4. Indicates solubility of inclusion material in hot phenolic solution, and impermeability to cold 20% H_2SO_4.	same as for in situ inclusions	-
Feulgen reaction	no staining; no Feulgen + ve material	same as for in situ inclusions	-
"Plasmal" test	no staining; no "Plasmal" present	same as for in situ inclusions	-
Iodine/potassium iodide	inclusions stained golden brown in wet mounts only; no glycogen, starch or "iogen" present, but reversible adsorption of iodine	same as for in situ inclusions	same as for in situ inclusions
Nile blue	red staining; neutral lipids present but "acidic" lipids not excluded	blue staining; "acidic" lipids present but neutral lipids not excluded	same as for in situ inclusions
Sudan III	pale orange staining; sudanophilic (i.e. liquid or semi-solid)	no staining; sudanophilic lipids absent	same as for in situ inclusions
Sudan black	intense opaque blue-black staining; sudanophilic (i.e. liquid or semi-solid) lipids present	no staining; sudanophilic lipids absent	same as for in situ inclusions

TABLE 15. The microchemical and staining properties of the lipid inclusions of B.cereus (AC.1) in situ, and after isolation by hypochlorite, and by mechanical disruption of the cells.

N.B. Where negative results were obtained, the conclusions are only regarded as tentative; for discussion see p.94.

the Feulgen and Plasmal tests, and Ashby's method for spores, all gave negative results. Tests for volutin were also negative; this result confirmed the fact that volutin was removed during the process of washing the inclusions, since the cells used to prepare the inclusions used for this series of tests had contained some ("+ 1") volutin granules, as well as the lipid inclusions. Positive results were obtained with wet mounts in iodine/potassium iodide, the inclusions being stained a pale golden brown, exactly comparable to the colour obtained with the in situ inclusions. The isolated inclusions also reacted with the acid fast spore stain in exactly the same way as their counterparts in situ, since a little over-heating during the staining period resulted in their losing their regular shape, and being stained a pale pink. In all these cases, therefore, the hypochlorite isolated inclusions reacted in exactly the same way as the inclusions in situ, and the conclusions drawn were the same.

The only important differences between the inclusions before and after isolation with hypochlorite lay in their response to lipid stains, and the results obtained with these stains were of considerable significance to the question of the chemical constitution of the in situ inclusions.

Very surprisingly, it was found that the hypochlorite isolated inclusions could not be stained by Burdon's Sudan black method, in contrast to the inclusions in situ, which were stained an intense opaque blue-black.

It was at first thought that this lack of staining might be due to readier removal of the stain from the naked, unprotected inclusions by the benzol used to rinse the slide, and accordingly, other methods of staining were adopted. The inclusions were mounted in the ethylene glycol and diacetin solutions of Sudan black. Both these solvents are reputed to be almost incapable of dissolving lipids, so the likelihood of removing a sudanophilic component of the inclusions with the solvent used to carry the dye was reduced to a minimum. It was found that these reagents also failed to stain the isolated inclusions to anything like the intensity they displayed before isolation. They did in fact, show a slight blue tinge, but it was negligible compared with the intense opaque blue-black shown by the in situ inclusions, and it was therefore quite clear that the hypochlorite isolated inclusions should be described as being non-sudanophilic.

Turning to the results obtained with Sudan III, it will be seen that here also, the isolated inclusions failed to be stained, a result which confirmed the finding with Sudan black.

In the case of Nile blue, it was found that the isolated inclusions were still stained, but whereas the inclusions in situ were stained red, the hypochlorite isolated inclusion bodies were stained a pale blue.

It was also found that this staining could be abolished entirely by extracting the isolated inclusions with ether, and it was therefore deduced that the blue

staining was due to the presence of an ether-soluble "acidic" lipid, a finding which was later confirmed by chemical analysis.

The results obtained with these lipid stains clearly indicated that the hypochlorite had altered the lipid staining properties of the inclusions, a finding of considerable importance. It must be emphasised that the non-sudanophilic nature of the hypochlorite-isolated inclusions should not be taken as evidence of the non-identity of the inclusions in situ with the bodies isolated by hypochlorite. The microscopic and turbidimetric observations of the lytic process provided clear evidence of their identity, and the chemical analyses to be reported in Part V strengthened this identification. These showed that the main reason why the hypochlorite-isolated inclusions were not Sudanophilic was the fact that they consisted mainly of poly β hydroxybutyrate which, being a solid, was not stainable with oil-soluble dyes. About 11% of the isolated inclusions however, consisted of the ether soluble "acidic" lipid indicated above by Nile blue. This material was semi-solid, and therefore stainable by Sudan black, but for reasons that will be fully discussed later, it was apparently incapable of rendering the hypochlorite-isolated inclusions Sudanophilic. For the present, it is important to realise that the alteration in staining properties brought about by hypochlorite could have been due either to removal from the inclusions in situ of another sudanophilic lipid component or to physico-chemical alteration of this

component making it no longer stainable, or perhaps to a combination of both these factors. Unfortunately, the staining results were of little help in deciding between these alternatives, and this whole question is more conveniently discussed later. The outstanding result of these experiments, however, was quite clear, namely that the inclusions, in situ, contained a strongly sudanophilic lipid in addition to a non-sudanophilic component, and that the sudanophilic lipid was either dissolved by hypochlorite or in some way rendered by it incapable of dissolving oil-soluble dyes.

PART IV.

ISOLATION OF LIPID INCLUSIONS BY METHODS OTHER THAN WITH HYPOCHLORITE, AND MICRO-CHEMICAL OBSERVATIONS OF THE INCLUSIONS THUS ISOLATED

The observation that hypochlorite destroyed or altered the sudanophilic component of the lipid inclusions indicated the need for the development of other, more gentle methods of isolation, in order that the nature of this component and its amount might be determined. Three potentially suitable methods were investigated. It will be seen that although two of the methods were capable of yielding fully sudanophilic inclusions, neither of them was suitable for the large-scale isolation necessary for macro-chemical analysis. It was, however, possible to apply some micro-chemical tests to the isolated inclusions, and some interesting features of the sudanophilic component of the _in situ_ inclusions were revealed.

1. Isolation of the inclusions with acid.

Meyer (1912a) observed that the lipid inclusions of _B.megaterium_ could be isolated by treatment of the cells with 5% H_2SO_4 at 90°C for 5 hours, and it was thought this might provide a means of isolating the inclusions in a fully sudanophilic state. Cells of strain AC.1 of _B.cereus_ were used, and phase contrast examination of a suspension of cells that had been treated in this way confirmed Meyer's observation. It was found that practically all the cell material had been destroyed with the

exception of the lipid inclusions, which were, however, still endowed with small adherent shreds of cytoplasmic material. The acid suspension was carefully neutralised with NaOH and dialysed against distilled water for 24 hours. A dry smear was then stained with Sudan black, when it was found that the inclusions were completely non-sudanophilic. It was clear, therefore, that the sudanophilic component of the inclusions was labile to hot acid, and further experiments with this method were not carried out.

2. Isolation of the inclusions by mechanical disruption of the cells.

The "Mickle" vibrator was employed for this purpose. Cells of strain AC.1 were employed; they were grown on 2% glucose agar at 37°C, and contained many large lipid inclusions. A suspension in saline was shaken on the "Mickle" vibrator with grade twelve ballotini beads, and the concentrations and relative proportions of cells and ballotini were adjusted so that about 90% of the cells were disrupted in as short a time as possible. Usually the period of vibration was about 15 minutes. Phase contrast examination of the suspension at the end of this period showed it to consist essentially of (a) some intact cells, (b) many free lipid inclusions, (c) fragments of cell wall, and (d) a certain amount of fine debris, much of it apparently granular. A drop of this suspension was dried as a smear on a slide, and

mounted in the diacetin solution of Sudan black. It was found that practically all the lipid inclusions at this stage were stained an intense blue-black, i.e. they were still sudanophilic. Moreover, it was found that the alcoholic solution of Sudan black was equally effective, which indicated that the sudanophilic component of the inclusions was not soluble in 70% ethanol.*

The next step was to separate the lipid inclusions from the remaining cells and cell debris by differential centrifugation. This proved a lengthy and laborious process, and was not very successful, the main difficulty being to free the inclusions of fragments of cell walls, which appeared to sediment in saline at about the same rate. After many careful centrifugations, however, a fairly homogenous suspension was obtained; by phase contrast examination it was free of the smaller debris, but still contained a proportion of cell walls. It is doubtful whether this suspension was pure enough to be of much use for analytical purposes, but in any case, on staining a smear with Sudan black, it was found, most surprisingly, that the inclusions were now no longer sudanophilic. Further attempts at purification were therefore abandoned.

Experiment showed that this loss of sudanophilia apparently was caused by mechanical damage to the inclusions;

* It was found convenient to use the phase contrast microscope for this series of observations; the condenser could be lowered slightly, out of phase, and the stained bodies noted; on raising the condenser, the phase effect was brought back into play, and any unstained structures became clearly visible.

if a little of the freshly disrupted suspension (not purified in any way) was left to stand for several days at room temperature, the inclusions remained fully sudanophilic, thereby indicating that enzymic breakdown and simple chemical processes such as hydrolysis or oxidation were not involved. If, on the other hand, the newly disrupted suspension was shaken in the "Mickle" for about 30 minutes (without ballotini) about half the inclusions lost their sudanophilia. Even more surprisingly, two centrifugations at 3000 r.p.m. were sufficient to destroy the sudanophilia of practically all the inclusions, and the addition of formalin to a concentration of 1.0% offered no protection.

These findings suggest that the sudanophilic material lies exposed on the surface of the inclusion body and is in a liquid state, since it is difficult otherwise to account for the ease with which it can be removed. A more detailed discussion of the observations will be provided below.

Repeated attempts to obtain a pure suspension of these mechanically isolated inclusions in a sudanophilic state were unsuccessful, being every time frustrated by the loss of sudanophilia. Attention was therefore directed to another potentially useful method, the use of lysozyme.

3. The isolation of the lipid inclusions by means of lysozyme.

It was thought that the use of this enzyme should

provide a very good possibility of isolating the lipid inclusions in a fully sudanophilic state. Weibull (1953a,b) showed that when cells of B.megaterium (strain KM) were suspended in a concentrated sucrose solution and treated with lysozyme, the cell wall was completely dissolved and the naked protoplast liberated. Transfer of a suspension of protoplasts to either phosphate buffer or normal saline resulted in the immediate lysis of the protoplasts, only the lipid inclusions (some free) and the so-called "ghost" membranes (thought to be the cytoplasmic membranes and in some cases still enveloping lipid inclusions) remaining. The lipid inclusions could be separated from the "ghosts" by differential centrifugation.

The present author grew strain KM on 1.0% glucose synthetic medium for about 20 hours at 30°C; staining with Sudan black showed that most of the cells contained large lipid inclusions. The technique used in treating the cells with lysozyme was that of Weibull(1953a). The washed cells were suspended in a 7.5% (w/v) solution of sucrose in 0.15M phosphate buffer (pH 7.0) containing approximately 0.25 mg/ml of pure crystalline lysozyme (Armour's). The concentration of cells was adjusted to approximately 2.0 mg. (dry weight) per ml.

Phase contrast examination showed that at room temperature the liberation of protoplasts commenced after a few minutes in this solution, and, exactly as described by Weibull, the process was complete in about 30 minutes. The protoplasts were deposited by centrifugation and

resuspended in an equal volume of 0.15M phosphate buffer at pH 7.0. This caused their immediate lysis; phase contrast examination showed that only the "ghosts" and the lipid inclusions remained intact. Examination of a Sudan black stained smear of the suspension at this stage showed that the free lipid inclusions were still fully sudanophilic.

The next step was to separate the inclusions from the "ghosts" by differential centrifugation. Weibull claimed this was easily done by centrifuging at 590G for 15 minutes, when the bulk of the inclusions would be deposited as a separate white layer on the bottom of the tube. The present author found that although most of the inclusions were deposited by this amount of centrifugation, they were far from pure, and were mixed with considerable quantities of "ghosts" and fragments of "ghosts". An added complication was the presence of large quantities of a slimy substance, possibly a polysaccharide resulting from the growth on sugar rich medium. Repeated attempts to obtain a completely pure suspension of the inclusions by differential centrifugation failed, and phase contrast examination showed that the deposited inclusions always contained a fairly high proportion of contaminating material.

This was not the most important difficulty, however, for staining with Sudan black showed that during the course of the differential centrifugations, the lipid inclusions had completely lost their sudanophilia. Since the purification of the inclusions necessitated repeated

centrifugation, there was no hope of preparing the inclusions in a pure and fully sudanophilic state, and the use of lysozyme for their large-scale isolation was therefore reluctantly abandoned.

The experiments were not completely fruitless, however. It had been established that the sudanophilic component of the inclusions was sensitive to 5% H_2SO_4 at 90°C and to mechanical disturbance, as well as being labile to hypochlorite. Moreover it was possible to apply to the crude suspensions of still sudanophilic inclusions some staining and microscopic solubility tests, which will now be discussed.

4. Microscopic staining and solubility characters of the lipid inclusions as isolated by mechanical disruption.

For these purposes, cultures of B.cereus (AC.1) were employed. They were grown on 1.0% glucose synthetic medium and contained lipid inclusions microscopically estimated as "+++". They were disrupted in the "Mickle" vibrator as previously indicated, and care was taken to ensure that the period of vibration was a minimum, and that the isolated inclusions were still fully sudanophilic. Unless otherwise indicated, dry smears of this crude suspension of inclusions were employed and the details of the methods used were those recorded in Part I (p. 94 et seq). It should be emphasised that using the phase contrast microscope as previously described, there was no difficulty in distinguishing the lipid inclusions from the other cell debris, and observing their staining

reactions. After staining, the preparations were examined both directly (i.e. under immersion oil) and as wet mounts, since this increased the chances of detecting weak staining.

For convenience, the staining results have been recorded in table 15, (p. 151). It will be seen that results obtained with the methods applied (i.e. Sudan black, Sudan III, Nile blue, Alberts stain, Ashby's spore stain, simple aniline dyes, the mercuric-bromophenol blue test for protein, and iodine/potassium iodide) were exactly the same as those obtained with the inclusions _in situ_. Thus no new information was gained about the chemical composition of the inclusions, and those isolated mechanically appeared to be identical with those _in situ_.

Microscopic tests of the solubility of the sudanophilic component of the mechanically isolated inclusions were carried out by immersing coverslip smears of the crude suspension of inclusions in various solvents (at room temperature) for a period of 48 hours. The smears were then mounted in alcoholic Sudan black, and the degree of intensity of staining of the free inclusions compared with that of a control smear that had not been treated with any solvent. The results were recorded in terms of an arbitrary scale of "+" signs, as shown in the accompanying table (table 16).

TABLE 16.

Solvent	Result	Solvent	Result
None (control)	++++	Ether	+++
Water	++++	Xylol	+
Alcohol	++++	Benzol	-
Carbon tetra-chloride	++++	Acetone	-
N.NaOH	++++	Glacial acetic acid	-
N.HCl	++++	Pyridine	-
		Chloroform	-*

The effect of various solvents on the sudan black staining properties of crude (i.e. full sudanophilic) lipid inclusions isolated by mechanical disruption of B.cereus (AC.1).

KEY : ++++ = opaque blue-black staining as intense as that of the control.

+++, ++, and + = progressive reductions in the intensity of the staining.

- = no staining at all.

* in the case of chloroform, the entire inclusion body was dissolved.

Now, for reasons which were outlined in the introduction to this thesis, the conclusions that can be drawn from microscopic solubility tests are limited. Nevertheless,

in this case two striking results stand out. Firstly, ether was practically incapable of removing the sudanophilic material, and secondly, all the effective solvents were of a more or less polar nature.

These results suggested that the material was not simply a mixture of triglycerides, since these are soluble in ether. The results with Nile blue indicated that the material was "neutral", however, and it would appear to have a somewhat polar character since only slightly polar solvents were capable of dissolving it. It was of interest, however, that alcohol was so incapable of dissolving it while acetone was so efficient. It was found in fact that acetone removed the bulk of the sudanophilic material in as short a period as ten minutes. Examination of a smear treated with acetone for about 5 minutes, revealed an interssting feature. The intensity of Sudan staining of this smear was greatly reduced by this brief treatment with acetone, and it was now apparent that the Sudan black was concentrated in a peripheral shell around the outside of the inclusion body. This observation was interpreted as an indication of a peripheral distribution of the sudanophilic material; acetone had apparently dissolved away so much of it that it was now transparent in the centre, while the vertically viewed thickness of the material at the edges was sufficiently great to be rendered visible when stained. It is difficult to see how any other distribution of the sudanophilic material within the inclusion body could have led to this result.

The idea of a peripheral shell or membrane of sudanophilic material receives some support from the observations of the "mechanical fragility" of the material. In considering this problem, one has to account for the virtually complete loss of staining properties during such apparently mild operations as shaking (in water) on the "Mickle" vibrator without Ballotini beads, and centrifugation at 3000r.p.m. It is unlikely that simple chemical processes such as hydrolysis or atmospheric oxidation could be responsible, since the crude suspension of inclusions could be left standing undisturbed at room temperature for several days without any loss of sudanophilia. Moreover, no loss of staining properties was incurred when such suspensions were allowed to sediment under normal gravity for several weeks in the refrigerator.

It is tentatively suggested that the sudanophilic material is an ether-insoluble liquid lipid lying on the surface of the inclusion body. If this were the case, it is conceivable that on centrifugation the material might be stripped from the surface of the inclusion body, since it would be subjected to the following stresses: firstly, the "downwards" movement of the inclusion through the water would create friction acting "upwards", and the exposed material on the surface would bear the full brunt of this. Secondly, being a lipid, the material would almost certainly be less dense than water, and would therefore be subject to a centrifugal force acting effectively in the same direction, i.e. "upwards". Both

these stresses would be opposed by the centrifugal force which, acting on the dense core of poly β-hydroxybutyrate (see Part V), causes the inclusion to sediment. The shearing action of these opposed forces might well be sufficient to tear a liquid from the surface of the body. The liquid state of the material is further indicated by its intensely sudanophilic properties, and the suggestion that it lies on the surface of the inclusion is supported by the peripheral staining observed after a short treatment with acetone.

Although necessarily extremely tentative, this theory offers a rational explanation of the observed facts. Further discussion of the problems of the structure of the inclusions will be provided later.

PART V.

CHEMICAL EXAMINATION OF THE INCLUSIONS ISOLATED BY THE HYPOCHLORITE METHOD

Large scale cultures of strain AC.1 of B.cereus were incubated at 37°C on the solid synthetic medium containing 1.0% glucose. They were harvested after about 40 hours, and lipid inclusions isolated with hypochlorite and purified in the manner indicated in "Materials and Methods" (p.91). At the time of harvesting, the cells contained lipid inclusions estimated microscopically as about "++++", and some volutin (about "++"). The purified inclusions had the appearance of a fine white powder.

Samples of the purified inclusions were examined for total nitrogen, total phosphorus, polysaccharide (glucose equivalent, by the anthrone method) and ash. The results are shown in table 17p.170. It will be seen that the amounts present were low and variable. It is thought that their presence was probably the result of contamination of the inclusions with other cell material and, to some extent, with dust particles unavoidably acquired during the lengthy isolation process. The absence of appreciable amounts of phosphorus provided further evidence that volutin (polymetaphosphate) did not survive the purification.

1. Fractionation of the isolated material into an ether-soluble, and an ether-insoluble fraction.

Preliminary experiments showed that the inclusion material could be split into two parts, one soluble in ether

and chloroform, the other soluble in chloroform but not in ether.

The ether-soluble fraction was estimated first, by extracting weighed amounts of the inclusions with dry (distilled over sodium) methylated ether in weighed "Quickfit" stoppered tubes. The inclusions were thoroughly shaken up in the ether and allowed to stand for about 24 hours at room temperature, with shaking at frequent intervals. They were then centrifuged, the bulk of the supernatant quantitatively removed with a pasteur pipette and transferred to a small weighed conical flask. More ether was added to the tube and the process repeated. In all six such extractions were made, all the supernatants being combined in the same flask. Experiment showed that no more ether soluble material could be removed by further extraction. The ether was removed from the combined supernatants by directing a current of air onto the surface of the solution, the flask being held in a water bath at 37°C. Traces of moisture were removed by drying _in vacuo_ over P_2O_5. The flask and contents were then weighed and the amount of ether-soluble material calculated. The analysis of this material will be described below.

The ether-insoluble residue in the "Quickfit" tube was freed of traces of ether by placing it in an evacuated dessicator over a trap of wax chips, and it was finally dried _in vacuo_ over P_2O_5. The melting point of this material (determined in a conventional apparatus) was found to be fairly sharp, lying between 166°C and 169°C, and it was therefore assumed to be a pure substance. It

TABLE 17.

Isolated lipid inclusions of:	B.cereus (AC.1)	B.cereus (AC.1)	B.cereus (AC.1)	B.megaterium (NCTC 7581)
N(% dry weight)	0.14	0.11	0.05	0.39
Polysaccharide (glucose equiv. % dry weight)	0.37	0.27	0.1	trace
Total P (% dry weight)	0.10	0.07	0.08	0.03
Ash (% dry weight)	0.75	0.13	0.18	0.83
Ether soluble lipid (% dry weight)	9.60	10.8	10.9	8.2
PHB* (% dry weight)	89.1	88.6	88.8	90.6

* PHB: polymerised β-hydroxybutyric acid. (insoluble in ether).

Analysis of the lipid inclusions of B.cereus (strain AC.1 ; 3 samples), and B.megaterium (NCTC 7581), isolated by treatment with hypochlorite.

dissolved completely in warm chloroform. There were a few flecks of dust, which were removed by filtration, and the material was reprecipitated by the addition of ether. Some difficulty was experienced in washing the material by centrifugation in ether, as it tended to float in this solvent; after the initial precipitation, therefore, the precipitated material was washed by centrifugation in acetone. Attempts to remove the acetone by placing the material in an evacuated dessicator resulted in the formation of a hard mass of material which was difficult to break up. A rather better technique was developed; the acetone suspension was placed in a water bath at 37°C, and most of the solvent blown off with a current of air. Before it was all removed, a little water was added, and the flask thoroughly shaken up. This resulted in a coarsely flocculent suspension, and when the flask was placed in an evacuated dessicator containing P_2O_5 and some wax chips, the suspension froze, and when dry, yielded a fine white powder. The analysis of this ether-insoluble powder will be described below.

It was found that the ratio of ether-soluble and ether-insoluble components of the inclusions was essentially constant and was independent of the amount of lipid inclusion material in the cells. The results of analyses of the isolated inclusions of six different cultures, having lipid inclusion contents ranging from 10.7% to 42.6% of the dry weight of the cells, are shown in table 18 , p.172.

TABLE 18.

Lipid inclusion content of cultures (by routine hypochlorite estimation) (% dry weight)	Ether-soluble content of inclusions isolated with hypochlorite	Ether-insoluble content of inclusions isolated with hypochlorite
42.6%	10.8%	89.2%
31.6%	9.6%	90.4%
24.7%	11.3%	88.7%
19.6%	10.9%	89.1%
15.4%	10.9%	89.1%
10.7%	10.2%	89.8%

It will be seen that the ether-insoluble material comprised between 88.7% and 90.4% of the hypochlorite isolated inclusions. It will be shown below that this material was in fact a pure polymer of β-hydroxybutyric acid, and its constancy in amount in the hypochlorite isolated inclusions had a special significance for the hypochlorite method of estimation, which will be referred to later.

2. Analysis of the ether-soluble fraction of the inclusions.

The ether-soluble material had the appearance and consistency of fat. It was a pale yellowish brown greasy substance which was semi-solid at room temperature, but melted on warming and was fairly fluid at 37°C.

The "acrolein test" for glycerol (Fiegl 1947) was positive, and triglycerides were therefore indicated. Estimations of the total nitrogen and phosphorus contents were made by transferring directly weighed amounts into the appropiate vessels. 0.14% of the dry weight was found to

be nitrogen, and 0.07% phosphorus. These results showed that the material was not essentially a phospholipid, the N and P contents being only great enough to account for a phospholipid content of about 10%. The similarity of the figures for N and P with those obtained with the entire inclusions suggested that contaminating dust particles were responsible for the N and P present. The lipid was of an acidic nature, having an acid number of 60. Its saponification number was 218. The chemical nature of the material was not further investigated, but the results suggested that it was a mixture of triglycerides and free acids. The observation of its acidic nature was in agreement with the blue staining obtained when the hypochlorite-isolated inclusions were stained with Nile blue.

In order to clarify the results obtained on applying Sudan black to the hypochlorite isolated inclusions, an attempt was made to determine the Sudan staining properties of this "fatty" component. Small droplets were mounted in the diacetin solution of Sudan black and examined microscopically. For comparison, small pieces of tristearin (solid at room temperature) and droplets of triolein (liquid) were examined in the same way. It was found that small droplets of the "fatty" material under investigation, i.e. droplets having diameters of the order of 2μ - 3μ, were stained an intense opaque blue-black throughout. In the case of larger pieces, the stain could be seen to have penetrated to a depth of about 5μ, but did not seem capable of getting any deeper. Droplets of the liquid triolein, on the other hand, were stained an intense opaque blue-black

throughout, no matter what the size of the droplet; in contrast, small pieces of tristearin remained completely unstained. These findings confirmed the statement of Cain (1950) that only lipids in a liquid or semi-solid state would be stainable with oil-soluble dyes. It would also appear that semi-solid lipids are only stained to a limited depth. This depth was, however, great enough in the case of the "fatty" component of the inclusions to ensure that small droplets of the pure material would be stained intensely with Sudan black. This finding immediately posed the question as to why the hypochlorite isolated inclusions should be non-sudanophilic in view of their content of this ether-soluble Sudan stainable lipid. The answer to this problem may be found in the distribution of the material within the inclusion or in its inaccessibility to staining solutions, or perhaps in a combination of these factors. Full discussion will be provided later.

3. Analysis of the ether-insoluble fraction of the isolated inclusions.

The purity of this fraction of the inclusions was indicated by its sharp melting point (166°C - 169°C), and by the low figures obtained for N, P, ash and polysaccharide (see table 20 p.181). The amounts present are almost certainly due to contamination. The material comprising this fraction was in fact found to be a polymer of β-hydroxybutyric acid (PHB), and it will simplify description of its properties if this finding is anticipated, and the material referred to simply as "the polymer". The chief evidence for this assertion lay in the following:

(a) Appearance.

If a chloroform solution of the polymer was allowed to evaporate in a water bath, the solid was deposited as a membrane on the bottom of the vessel, a membrane which was easily, and to some extent spontaneously, detachable from the glass. In the thicker parts of the membrane, deep cracks were often formed; where the membrane was thin, it was usually intact, translucent with a slightly shiny surface, and very flexible. It rather resembled thin plastic sheeting, but had no great strength and was easily torn. In these respects it closely resembled the polymerised β-hydroxybutyric acid described by Lemoigne and his colleagues.

(b) Solubility properties.

The solubility of the polymer was tested (qualitatively) in several organic solvents and other reagents. The results are set out in table 19. It is evident that the solubilities of the material corresponded closely with those of the PHB described by Képès and Péaud-Lenoël (1952) as "insoluble in water, paraffins, ether, acetone, and lower alcohols; soluble in fatty acids, alcohols longer than 3C, chlorine containing solvents, and solvents with a cyclic or heterocyclic nucleus". The more important points are the insolubility in water, ether, alcohol, and mineral acids, and the solubility in chloroform, glacial acetic acid, phenol, and alkalies. The application of heat speeded the dissolution in all cases, and was essential in some. The fact that the material is soluble in hot 5% phenol, and insoluble in 20% H_2SO_4 explains why

TABLE 19.

Solvent	Result
Water	Insoluble
Acetone	
Alcohol	
Ether	
Carbon tetrachloride	
Benzol	
HCl (dilute)	
H_2SO_4 (20%)	
Standard alkaline hypochlorite reagent at 37°C	
Formamide (hot)	Soluble, reprecipitates on cooling
sec-Undecyl alcohol (hot)	Soluble, reprecipitates on cooling
Benzene (hot)	Soluble, reprecipitates on cooling
Na_2CO_3 (hot; Molar) *	Slowly soluble
Chloroform	Soluble
Glacial acetic acid	
Pyridine	
Salicylaldehyde	
Octyl alcohol	
Phenol (5% aqueous)	
NaOH (5% or more)*	
Formic acid (96%; hot)	
Triolein	
Tripalmitin (molten)	
Butter fat (molten)	

* Depolymerisation may occur.

The solubility of PHB isolated from strain AC.1 of B.cereus in various solvents.

the inclusions were stainable by the Ziehl-Neelsen method. The insolubility of the polymer in the standard alkaline hypochlorite reagent provides further justification for the use of this reagent in the isolation of the inclusions. The solubility of the polymer in various heated fats is an observation of some importance to the question of the structure of the inclusions in situ, and will be referred to again later.

(c) The effect of heat.

On heating in an ignition tube, the polymer melted and then gave rise to dense white fumes, to the accompaniment of charring. These fumes had an acrid smell, and condensed on the cooler parts of the tube to form needle shaped crystals which were freely soluble in water, and had a melting point of 71°C - 72°C. This behaviour was exactly the same as that described by Lemoigne (1926c) in the case of PHB from B.megaterium, and the crystals were presumably of α-crotonic acid.

(d) Alkaline hydrolysis.

As shown in table 19, the polymer was insoluble in mineral acids, but dissolved fairly readily in hot concentrated alkalie. About 15 minutes in boiling 10% NaOH or KOH was sufficient to dissolve it, and if the resulting solution was cooled and neutralised with HCl, the polymer was completely reprecipitated. If the heating was continued for some time, progressively smaller amounts of the polymer could be recovered on neutralisation, until, after several hours heating, no precipitate resulted on the addition of HCl, indicating that the polymer had

been completely broken down into water-soluble units. The saponification value of the polymer was found to be 640.

(e) Elementary analysis.

An elementary analysis of the polymer (kindly carried out by Dr. Minnis of the Biochemistry Department of this University) gave the following results:

C: 55.84%

H: 6.99%

O: 37.17% (by difference)

Thus the empirical formula was $(C_4H_6O_2)n$. Together with the findings described above, this result provided the final proof that this ether-insoluble constituent of the hypochlorite isolated inclusions was a polymer of β-hydroxybutyric acid (PHB), similar to that isolated by Lemoigne and his colleagues from B.megaterium.

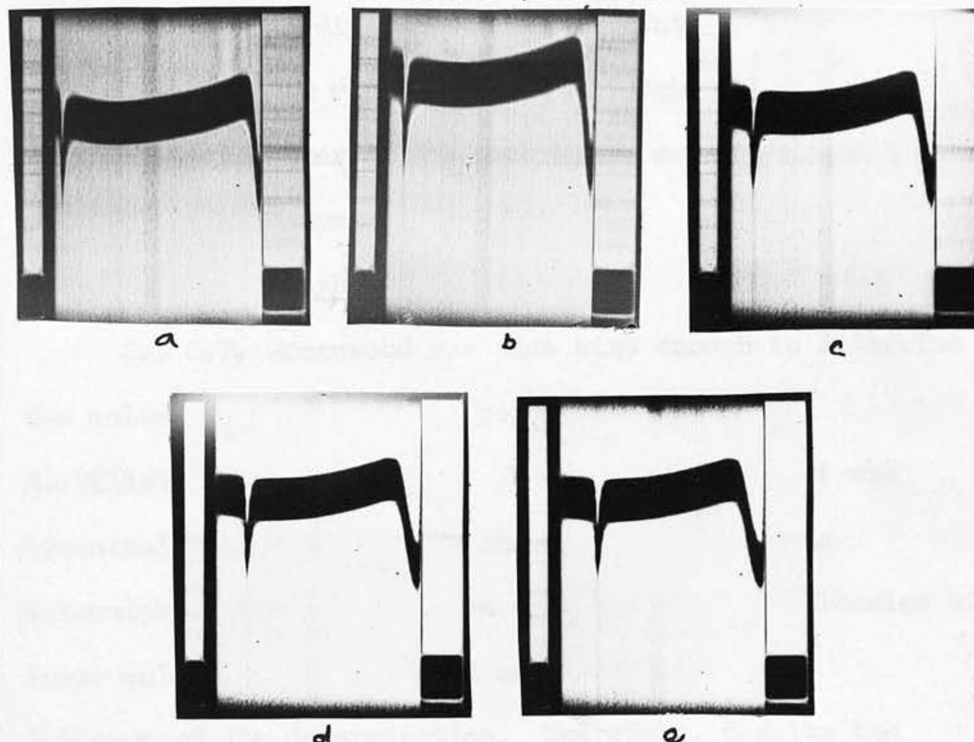
Further characters of the polymer were then investigated, as follows:

(f) Density.

The density of the polymer was determined by suspending small pieces of it in sucrose solutions of measured densities. The pieces of polymer chosen were free of fissures, and great care was taken to see that no air bubbles remained attached to them. They were found to rise in solutions of density 1.25 g/cc or greater; they sank in solutions of density 1.23 g/cc or less. Thus the density of the polymer was found to lie between 1.23 g/cc and 1.25 g/cc.

(g) Ultracentrifugation.

Through the courtesy of Dr. C.T. Greenwood of the Chemistry Department of this University, a chloroform solution of the polymer was subjected to ultracentrifugation on a "Spinco" analytical ultracentrifuge. The sedimentation diagrams are shown below. Three general



Sedimentation diagrams of PHB from strain AC.1 of B. cereus.

"Spinco" analytical ultracentrifuge.

Speed: 60,000 r.p.m.

Solvent: chloroform.

Concentration of PHB: c.1%.

Photographs made at: (a) 4 mins., (b) 11 mins., (c)15 mins., (d) 21 mins., and (e) 36 mins., after reaching full speed.

points are of interest; firstly, the peak representing the sedimenting polymer is inverted. This is taken to indicate that, for reasons not understood, the refractive index of chloroform was greater than that of the solution of polymer. Secondly, the peak travelled towards the meniscus, a consequence of its having a lower density than chloroform. Thirdly, and most important for present purposes, the peak remained sharp throughout the period of centrifugation, thereby indicating the molecular homogeneity of the polymer.

(h) <u>Molecular weight determination.</u>

Dr. C.T. Greenwood was also kind enough to determine the molecular weight of the polymer by isothermal distillation in chloroform. For this purpose, it was essential that the material should be a homogenous molecular species, since even small amounts of molecules of lower molecular weight would seriously affect the accuracy of the determination. Therefore, despite the indication from ultracentrifugation that the polymer was homogenous, a sample was subjected to fractional precipitation out of a chloroform solution with ether. It was however, found impossible to obtain fractions of different melting points in this way, and this finding was taken as further evidence of the molecular homogeneity of the polymer.

Dr. Greenwood reported the molecular weight of the polymer to be of the order of 5000. In the light of the elementary analysis reported above, this polymer would appear to have a chain length of about 60 β-hydroxybutyrate

TABLE 20.

Melting point	166°C - 169°C	
N(% dry weight)	<0.05	
Polysaccharide (glucose equiv. % dry weight)	<0.06	
Total P (% dry weight)	trace	
Ash(% dry weight)	0.11	
C(%)	55.84	empirical formula; $(C_4H_6O_2)n$
H(%)	6.99	
O(%) (by difference)	37.17	
Approx. molecular weight *	5000	
Saponification value	640	

Analysis of pure PHB of B. cereus (strain AC.1) isolated by hypochlorite.

* by isothermal distillation in chloroform.

residues, i.e. a formula of $(C_4H_6O_2)_{60}$. Képès and Péaud-Lenoël (1952) found the chain length of the PHB isolated from B.megaterium to lie between 6 and 110 residues, but were unable to obtain a more precise figure.

The above results showed that the inclusions isolated by the hypochlorite method had an essentially constant composition, containing about 11% of an ether-soluble "fatty" material, and 89% of poly β-hydroxybutyrate, similar to that obtained by Lemoigne and his colleagues from B.megaterium. This therefore, was the first direct and comprehensive proof of the existence of this type of material in the lipid inclusions of the Bacillus. The only known difference between the polymer of strain AC.1 of B.cereus and that obtained by Lemoigne from B.megaterium lay in their melting points. Lemoigne's polymer melted at 157°C; Képès and Péaud-Lenoël, on the other hand, isolated (from the strain of B.megaterium used by Lemoigne), a polymer having a melting point of 179°C, which they considered to be more homogenous than Lemoigne's product. It remains to be seen whether the melting point of the polymer isolated from B.cereus by the present author, (166°C - 169°C), is in fact the true melting point of this polymer as it occurs in vivo, but the apparent homogeneity of the pure substance suggests that hypochlorite did not degrade it in any way.

The minor non-lipid components of the isolated inclusions are thought to be the result of contamination, particularly since they varied so much in amount. It

was especially evident that the nitrogen and phosphorus were due to contamination, since the amounts found in the "fatty" component were virtually the same as those present in the unfractionated inclusions. The pure PHB, on the other hand, which had been filtered to remove small particles of dust, was virtually free of nitrogen and phosphorus. It seems highly probable that the contaminating dust particles were responsible for the N and P values of the isolated inclusions.

It must be emphasised, however, that these results should not be taken as indicating the absence of significant amounts of non-lipid substances from the inclusions _in situ_. The difficulty arises of deciding what "significant" amounts would be. One might expect the _in situ_ inclusions to incorporate part, at least, of the enzyme system responsible for their formation, and one might therefore expect to find small amounts of protein. The amounts present, however, would in all probability be too small to be detected with any certainty. Moreover, one would not expect the hypochlorite reagent, which is a powerful oxidising agent, to leave the inclusions completely unscathed. Indeed the cytochemical observation of the loss of sudanophilia suffered by the inclusions during isolation was clear evidence that they were in fact damaged by the reagent. While on this point, it should be mentioned that attempts to stain small pieces of the pure polymer, extracted either by hypochlorite or by chloroform, in any of the Sudan black staining solutions, failed to colour them at all. This finding was therefore in complete agreement with the

statement of Cain (1950) concerning the non-sudanophilic nature of solid lipids, and it afforded a partial explanation of the non-sudanophilic nature of the hypochlorite isolated inclusions.

The origin and significance of the "fatty" component of the hypochlorite isolated inclusions is not clear. Its constancy in amount suggested that it was an integral part of the in situ inclusions, but it might quite plausibly represent lipids from other parts of the cell, which during the treatment with hypochlorite, were caused to adhere to, or be adsorbed by the inclusions. This point will be more fully discussed in connection with the problem of the structure of the inclusions.

The finding of the constancy of the composition of the hypochlorite isolated inclusions was of very great significance with regard to the hypochlorite quantitative method of estimation. It indicated that the routine hypochlorite method of estimating "lipid inclusions" might be extendedto measure poly β-hydroxybutyrate. Knowing the amount of PHB in the isolated inclusions, it was only necessary to multiply the figure obtained from the calibration curve for "lipid inclusions" by the appropriate factor, to convert the figure into an estimate of PHB. In the case of the lipid inclusions of strain AC.1, the average PHB content of the isolated inclusions was found to be 89.6%. This figure, however, ignored the small amounts of contaminating N, P, ash and polysaccharide, and it was thought sufficiently accurate to

use a factor of 0.89 for the conversion of "lipid inclusion" figures into estimates of PHB.

It was however, still not known whether or not the hypochlorite method estimated all the PHB of the cell, and the experiments of Part VI were carried out with this aim in view.

PART VI.

PARALLEL ESTIMATIONS OF POLY β-HYDROXYBUTYRATE (PHB) BY THE HYPOCHLORITE METHOD, AND BY CHLOROFORM EXTRACTION.

The experiments recorded in Part V showed that the lipid inclusions of B.cereus contained poly β-hydroxybutyrate, and that this substance formed a constant fraction (in the case of strain AC.1, 89%) of the inclusions as isolated by alkaline hypochlorite. This observation indicated that the hypochlorite method of estimating lipid inclusions could be conveniently adapted to the measurement of PHB, since it was only necessary to multiply the figure obtained for "lipid inclusions" by the appropiate factor (i.e. in the case of strain AC.1, 0.89) to obtain a fairly accurate estimate of PHB. These observations, however, gave no direct proof that the hypochlorite method would estimate all the PHB in the cell, and the experiments described here were undertaken in order to obtain such a proof. They consisted essentially of parallel estimations of PHB on the same culture samples, by two methods, the hypochlorite method, and Lemoigne's chloroform extraction technique (Lemoigne and Roukhelman 1940b). As will become apparent, the results not only provided the necessary proof that the hypochlorite method estimated all the PHB of the cell, but they were also a direct demonstration of the reliability of the method.

Large-scale cultures of strain AC.1 of B.cereus were incubated and harvested as described in "Methods". Usually

about 6 or 8 trays containing 9-12 litres of medium were employed, and they yielded 8-10 grams (dry weight) of cells. An exact knowledge of the PHB contents of the cultures was obtained by extracting the bulk of the cells with chloroform, after the technique of Lemoigne and Roukhelman (1940b). The routine hypochlorite method was used to estimate the "lipid inclusion" content of the same batch of cells; about 10mg. (dry weight) were required for this purpose. The results of the latter determination were converted into figures for the PHB content of the cells by multiplying them by 0.89, and the estimates of PHB obtained in this way were compared with the results obtained by chloroform extraction.

In all, five such experiments were carried out, four of them being with cultures grown on 1% or 3% glucose synthetic media, and containing from 9.4% to 36.6% of their dry weight as PHB. The variation in PHB contents of these four cultures was due mainly to harvesting them at different times, although they were all harvested between 30 and 50 hours after inoculation. The fifth culture was grown for 35 hours on the 0.3% glucose "control" medium, and the cells contained minimal amounts of PHB. The results are recorded in table 21, p.188.

It will be seen that in cultures 1 to 4, the results obtained by the two methods were in extremely good agreement. It was clear that over this range of cell contents of PHB, the hypochlorite method estimated all the PHB of the cells, and could be relied on to give accurate results. In the case of the fifth culture, however,

TABLE 21.

Expt No.	Lipid inclusion content of cells by routine hypochlorite method; per cent dry weight.	PHB content of cells by routine hypochlorite method; per cent dry weight.**	PHB content of cells by chloroform extraction; per cent dry weight.
1	42.6%	37.9%	36.6%
2	24.7%	22.0%	20.2%
3	19.6%	17.4%	17.5%
4	10.7%	9.5%	9.4%
5	1.8%	1.6%	0.2%

** i.e. "lipid inclusion" content multiplied by 0.89.

Comparison of results obtained in estimating poly β-hydroxybutyrate (PHB) contents of different cultures of B. cereus (AC.1) by the hypochlorite method, and by chloroform extraction.

there was a considerable discrepancy between the results obtained by the two methods. The hypochlorite method gave a result nearly ten times as great as that given by chloroform extraction. There was unfortunately too little material available to carry out a complete analysis, and the reason for this discrepancy was not clear. It was apparent however, that the hypochlorite method was not to be relied on to measure accurately the PHB in these cells containing very low amounts. This is not a serious limitation on the method, however, for the results obtained with the other cultures showed clearly that it gave accurate results for cells containing moderate or high amounts of the polymer. It is important to emphasise that the hypochlorite method offers two great advantages as compared with chloroform extraction. These are the small amounts of cells required (about 10mg. dry weight) and the ease and rapidity with which the determinations can be carried out. A loss of accuracy in measuring very low cell contents of polymer does not detract from practical value of the method for most purposes and the method is particularly well suited to the estimation of the polymer in a large number of small samples. The experiments on growth reported in the last part of this thesis gave a practical demonstration of its usefulness. In the course of those experiments, 30 or 40 estimations were frequently made in a single day; using chloroform extraction, on the other hand, it would have been difficult to make more than 2 or 3 estimations in the same time, and the size of the culture samples required would have made the

experiments impossible.

The above experiments enable one to assess the probable limits of accuracy of the hypochlorite method in measuring moderate cell contents of PHB. The percentage errors obtained with the hypochlorite method in cultures 1 to 4, expressed as the deviations about the averages of the results given by the two methods, were respectively, + 1.6%, + 3.3%, -0.3%, and -0.5%. It appears therefore, that except in cultures containing very low amounts of PHB, the hypochlorite method of measurement can be expected to be accurate to within ± 5%. This accuracy compares well with that of other commonly employed methods such as the estimation of total cell protein by measurements of nitrogen, and the estimation of total cell polysaccharide by the anthrone method.

PART VII.

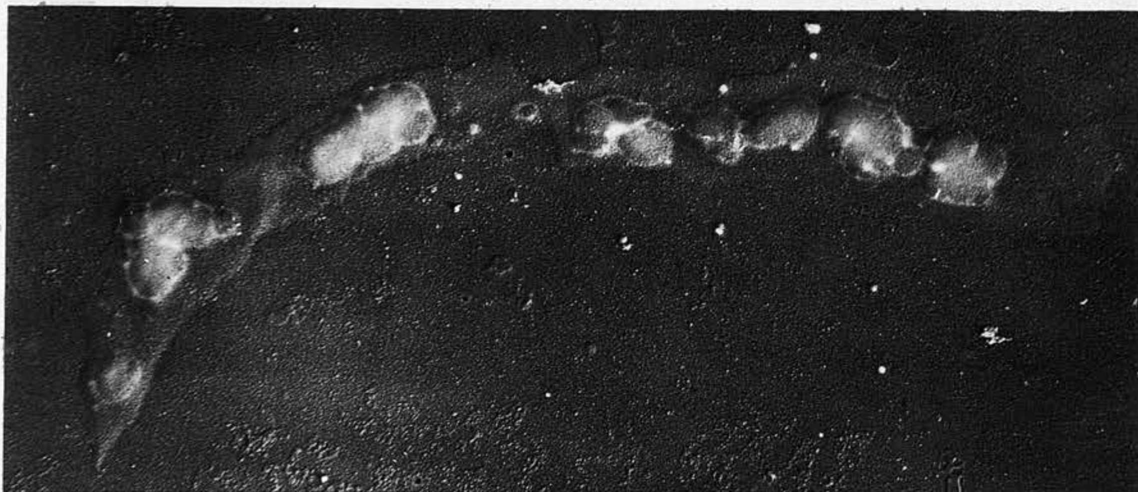
THE ELECTRON MICROSCOPE APPEARANCE OF THE ISOLATED LIPID INCLUSIONS.

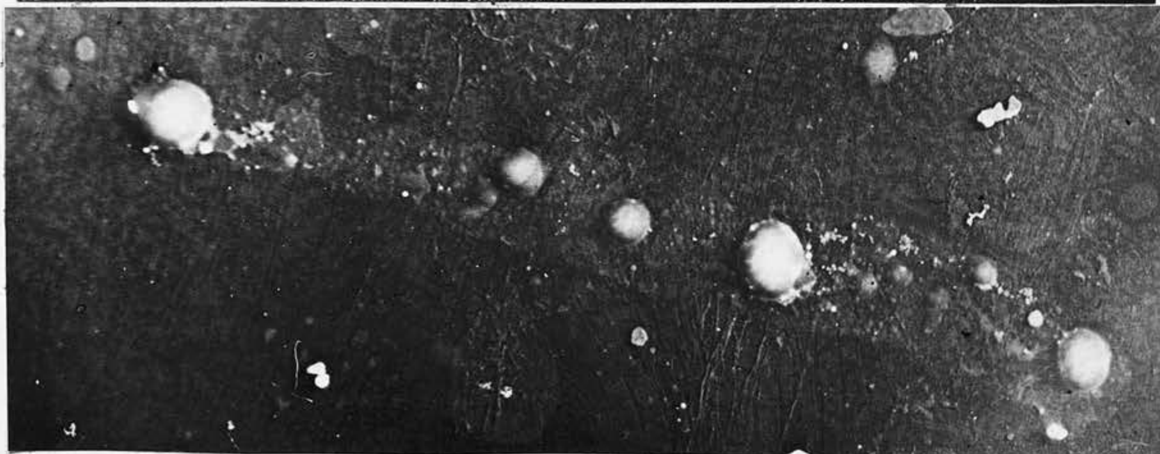
(1). The inclusions isolated by hypochlorite.

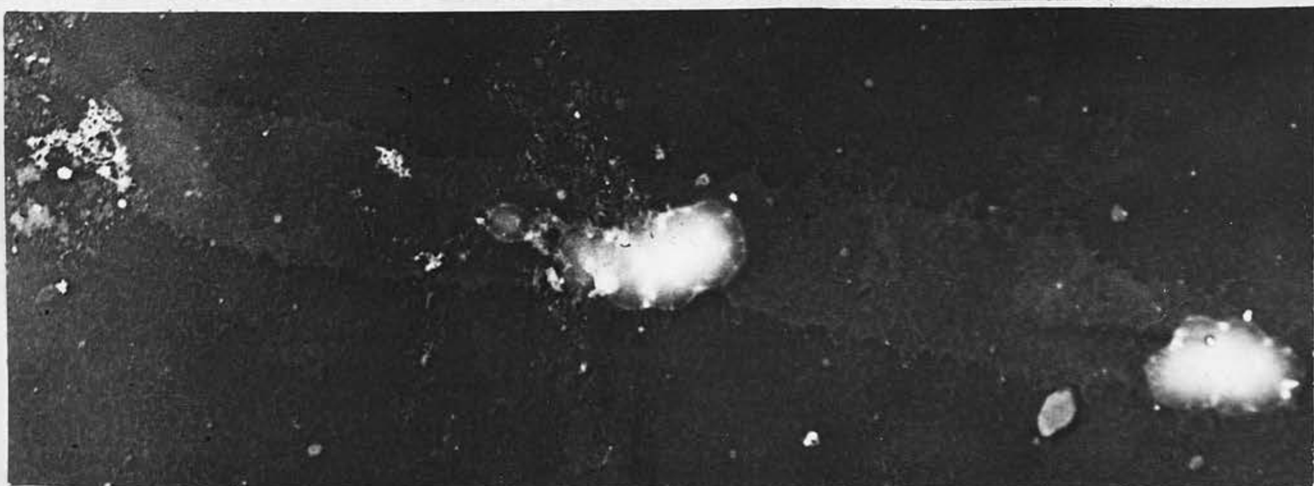
The method used to prepare the hypochlorite isolated inclusions for electron microscopy has already been described. All the preparations were shadowed with gold/palladium at 15°. Collodion membranes were at first employed, but most of the preparations were made on formvar membranes, which were found to be stronger than collodion ones.

It has already been noted that the lipid inclusions *in situ* were not visible in shadowed electron micrographs except in cells that had undergone a certain amount of autolysis (see photographs 7 & 8, p.110). As shown in photographs 11, 12 and 13 (p.192), they also became visible in cells that had undergone a short treatment with hypochlorite. In photograph 12, the globular nature of the individual inclusions is apparent; in the other two examples, the inclusions appear to have clumped together into fairly irregular masses. In all cases, it will be noted that the inclusions appear to have a fairly smooth surface.

Photograph 14, (p.193), which gives a good idea of the homogeneity and purity of the completely isolated inclusion preparations, (in this case of strain AC.1), reveals several interesting features. Firstly, it will be noted that several small clumps are visible. These clumps were numerous in many of the hypochlorite preparations, but as far as possible they were avoided in photographing specimens.

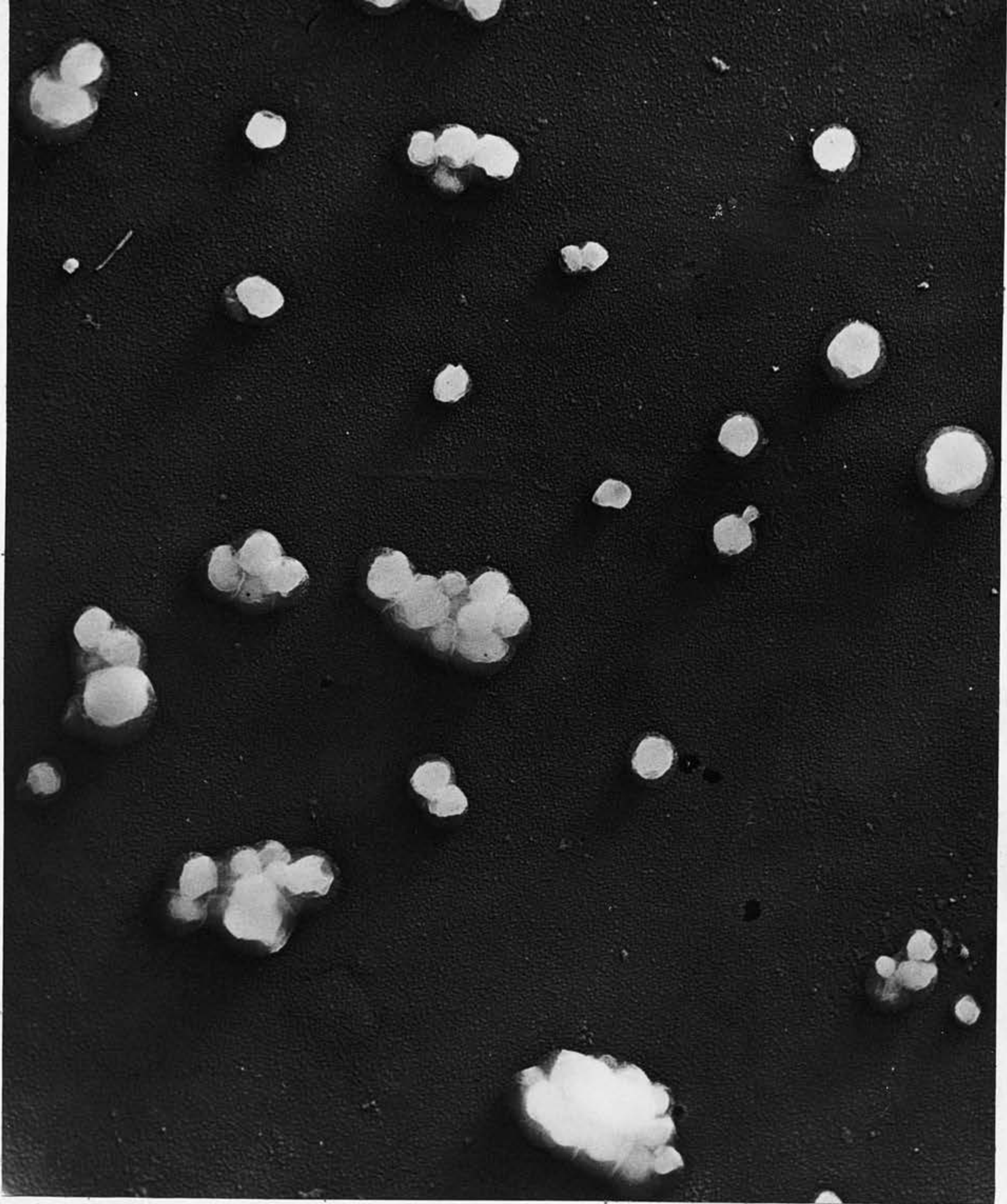






Photographs 11, 12 and 13. B.megaterium (NCTC 7581). 24hrs., nutrient agar, 37°C. Treated with standard hypochlorite reagent for 1 hr. at room temperature. Shadowed electron micrographs. These cells are incompletely lysed with hypochlorite. The partial dissolution of the non-lipid parts of the cell has made the lipid inclusions clearly visible. In 11 and 13, the inclusions appear to have clumped together into irregular masses. Photograph 12 shows clearly the regular spherical shape of the individual inclusions. Note the smooth surface of the inclusions in these preparations.

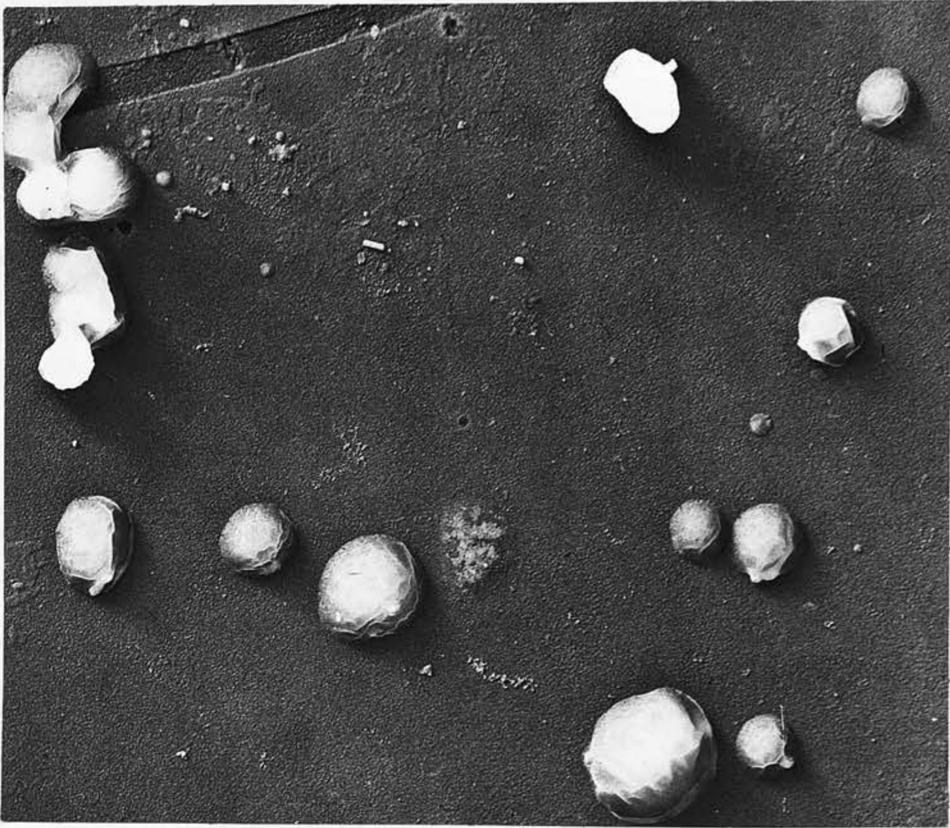
Photographs 11, 12 and 13 at a final magnification of X 10000.



Photograph 14. Lipid inclusions of B. cereus (AC.1) isolated with hypochlorite. Culture grown for 40hrs. at 37°C on 1% glucose synthetic medium. Shadowed electron micrograph. This preparation clearly shows the purity and homogeneity of the hypochlorite preparations. Note the size range of the inclusions, and their tendency to aggregate into clumps, despite the fact that the preparation was not centrifuged at any stage of its preparation. All the inclusions display a fairly electron opaque central core, with a more electron transparent peripheral zone. In some cases, the wrinkled nature of the inner zone can be seen. The length of the shadows of this preparation (the shadowing angle was 15°) suggests that the inclusions in this preparation had not collapsed too greatly; this was rather unusual.

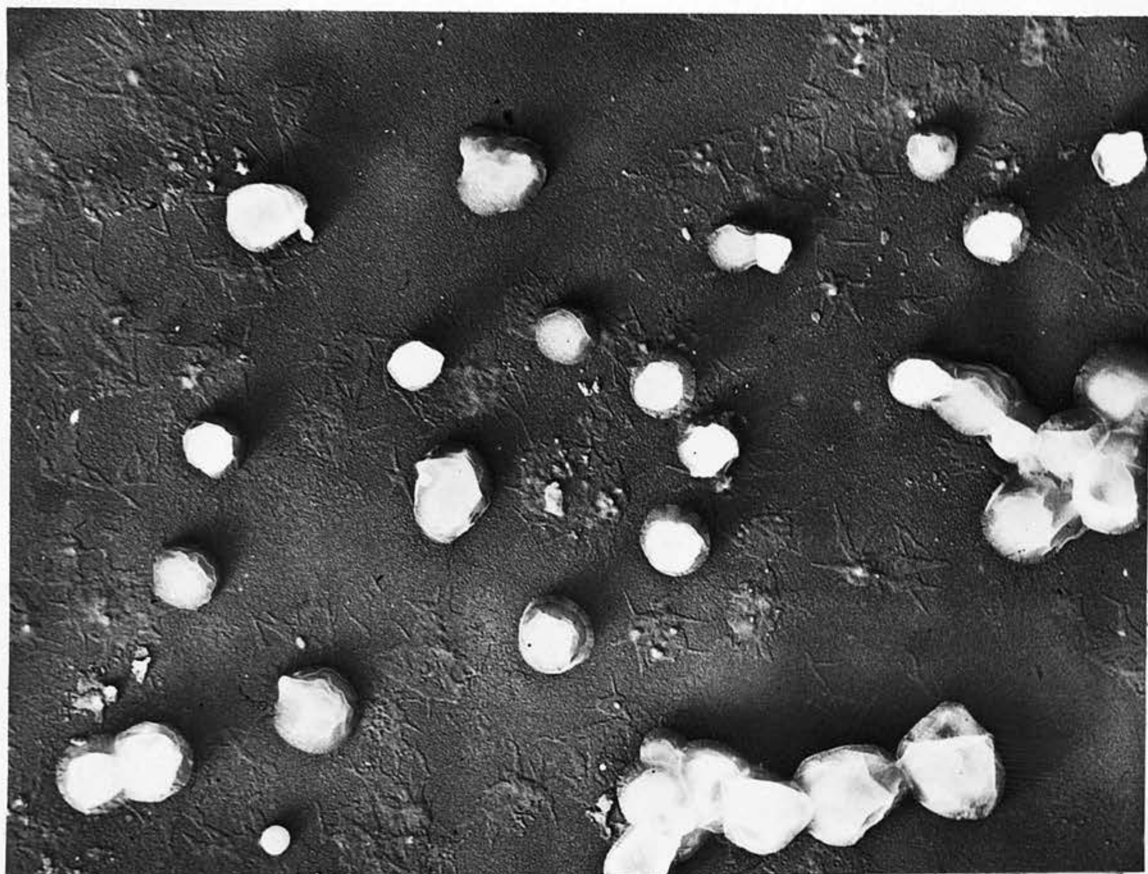
Photograph 14 at a final magnification of X 15000.

Secondly it will be seen that the individual inclusions appear to be differentiated into two parts - an outer, relatively electron-transparent zone, and a central, more electron-scattering core. This photograph shows little extra detail of the structure of the inclusions, although in some instances, the slightly wrinkled nature of the surface of the inner core is apparent. This wrinkled appearance is much more clearly seen in the photographs of the inclusions of <u>B.megaterium</u> (strain KM) on pages 195, 196 and 197. (Photographs 15, 16 and 17). In photograph 15, the wrinkled surface of the inclusions is very clear, and in this case, the outer transparent zone is not apparent. That the absence of the outer zone is not due to an intrinsic difference between strains KM and AC.1, is clear from inspection of photograph 16, where, in some cases, the outer zone is clearly seen. This degree of variability in appearance was a common feature of the inclusions of all the strains examined, and it may be due partly to the way in which individual inclusions happened to dry on the membrane. It will be noted that in most cases, the inclusions appeared to have flattened somewhat on the membranes. At the shadowing angle of 15°, the length of the shadows should be roughly three times the vertical height of the bodies. In most cases, the shadows are less than the apparent diameter of the inclusions, so it is evident that a considerable degree of flattening or collapsing had occurred, and it must have occurred before shadowing. It was found that the amount of flattening appeared to be a matter of chance and varied among different preparations, as well as among the individual inclusions of the same preparation. Thus, in photograph 18,



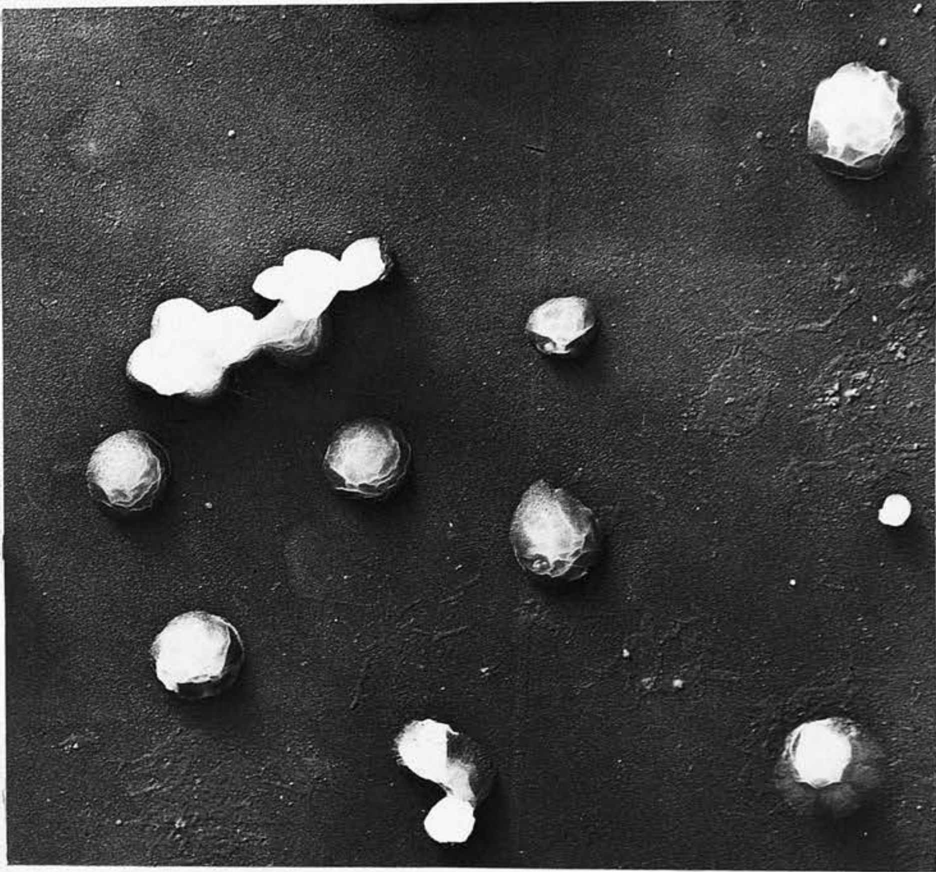
Photograph 15. Lipid inclusions of B. megaterium (KM) isolated with hypochlorite. Shadowed electron micrograph. The cells were grown on 1% glucose synthetic medium for 24hrs. at 30°C. Note that in most cases the inclusions are not very electron opaque. The outer transparent zone seen in photograph 14 is not apparent here, but the "wrinkled" nature of the inclusions is very clear. The short shadows (the shadowing angle was 15°) shows that the inclusions have collapsed considerably, indicating that they are not solid bodies.

Photograph 15 at a final magnification of X 15000.



Photograph 16. Lipid inclusions of B.megaterium (KM) isolated with hypochlorite. Shadowed electron micrograph. The cells were grown on 1% glucose synthetic medium for 24hrs. at 30°C. The transparent peripheral zone is apparent in some cases, and the wrinkled membranous nature of the inclusions can also be seen. In one or two instances, the outer zone appears to be cracked or torn.

Photograph 16 at a final magnification of X 15000.



Photograph 17. Lipid inclusions of B.megaterium (KM) isolated with hypochlorite. Shadowed electron micrograph. The cells were grown on 1% glucose synthetic medium for 24hrs. at 30°C. The wrinkled nature is clear, and in one case, (lower right-hand corner) a broad transparent outer zone showing marked "cracking" can be seen. The apparent differences in electron opacity between some of the inclusions is thought to be due to the way in which they dried on the formvar membrane.

Photograph 17 at a final magnification of X 15000.

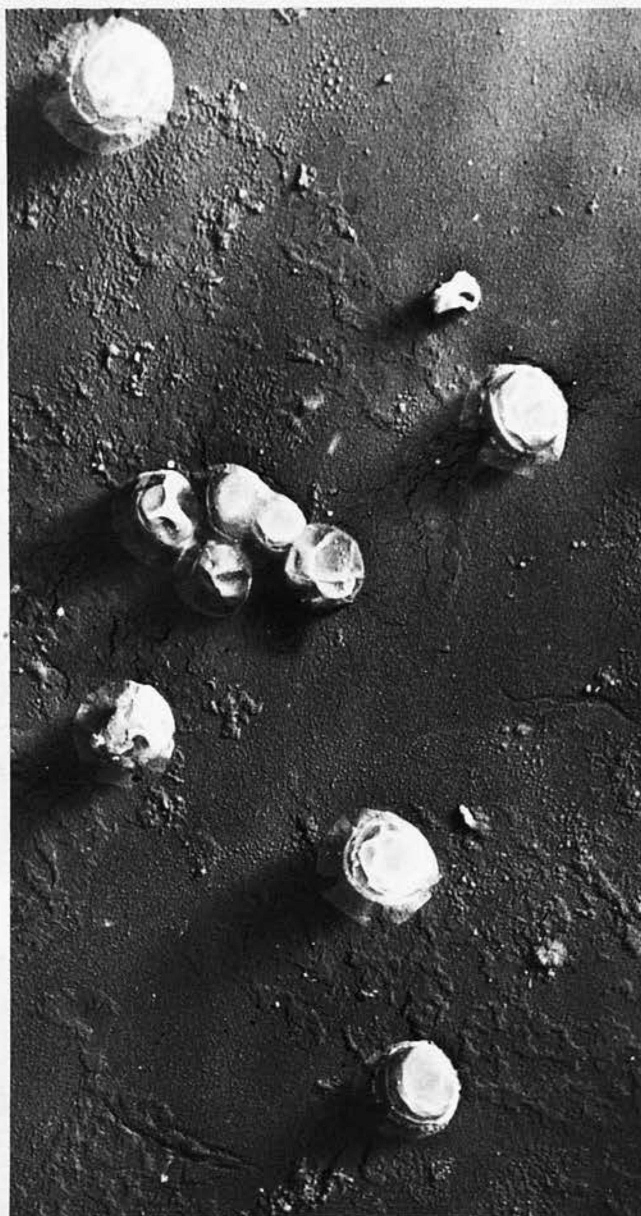
Photograph 18. Lipid inclusions of B.megaterium (NCTC 7581) isolated with hypochlorite. Shadowed electron micrograph. The cells were grown on nutrient agar for 24hrs. at 37°C. These inclusions appear not to have collapsed too greatly on drying, and are fairly electron opaque. The wrinkled outer surface can be seen.

Photograph 18 at a final magnification of X 15000.

of the inclusions of B.megaterium (NCTC 7581), the length of the shadows suggests that little flattening had occurred and, probably as a result, the inclusions appeared very electron-opaque.

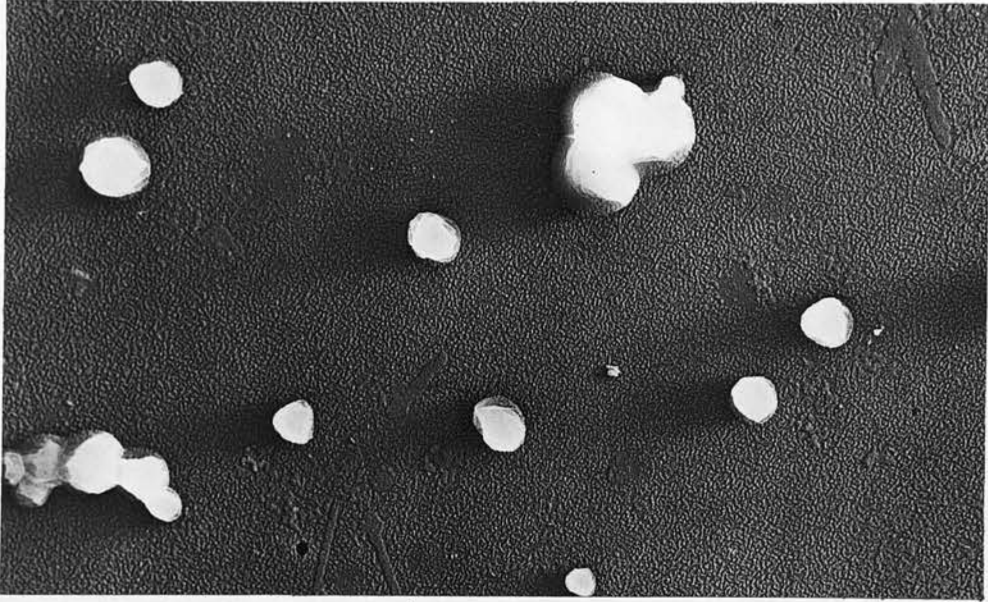
Turning back to photograph 17, the inclusion body in the lower right hand corner is a good illustration of an appearance that can be also seen in some of the inclusions of photograph 16, and is strikingly evident in photograph 19. (p.200). The outer transparent zone of the inclusions appears to have been torn, or to have cracked, presumably on drying. It was at first thought that this outer zone might be composed of the "fatty" component shown to be present by chemical analysis. Experiment showed that this was not the case, however. Reference to photographs 20 and 21, (p.201) which are of inclusions which had been exhaustively extracted with ether prior to their preparation for the electron microscope, shows that ether extraction made no apparent difference to the appearance of the inclusions; the outer transparent zone is easily visible, and some of the inclusions show a slight degree of "wrinkling". Thus the outer transparent zone does not appear to be composed of "fatty" material, and must therefore be of poly β-hydroxybutyrate (PHB). It would appear that this outer "zone" is in fact a shell of PHB. It is thought that in some cases, this shell became fairly strongly stuck to the formvar membrane. As the inclusion body dried, a certain amount of shrinkage of the inner mass of material took place, resulting in tearing of the outer shell.

This shrinkage and collapse of the inclusions probably also accounts for the wrinkled appearance to be seen in so



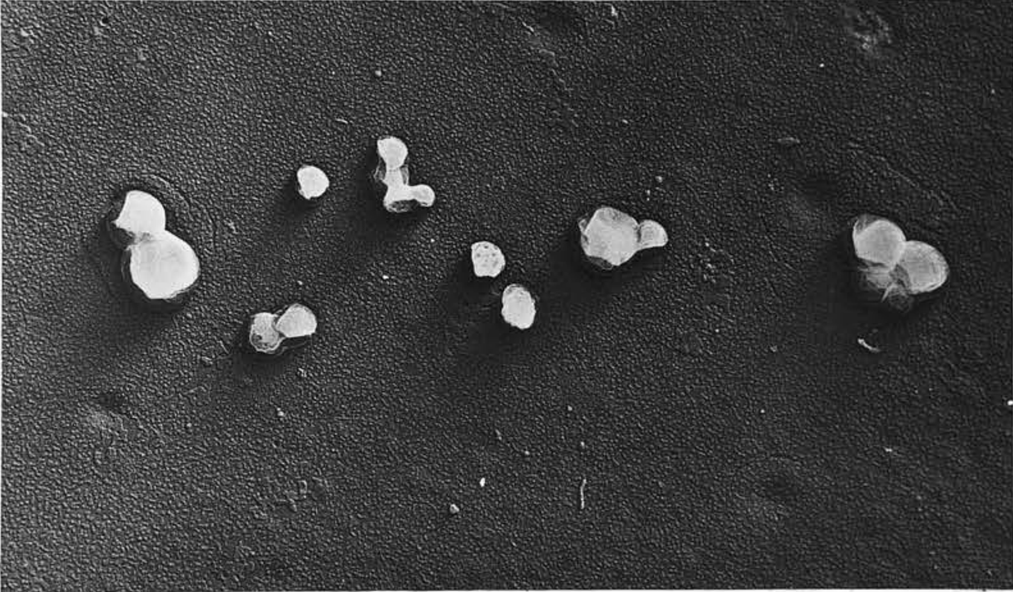
Photograph 19. Lipid inclusions of B. cereus (AC.1) isolated with hypochlorite. Shadowed electron micrograph. The cells were grown on 1% glucose synthetic medium for 40hrs. at 37°C. The wrinkled and partly collapsed nature of the inclusions is apparent. "Cracking" of the outer transparent zone is also evident.

Photograph 19 at a final magnification of X 15000.



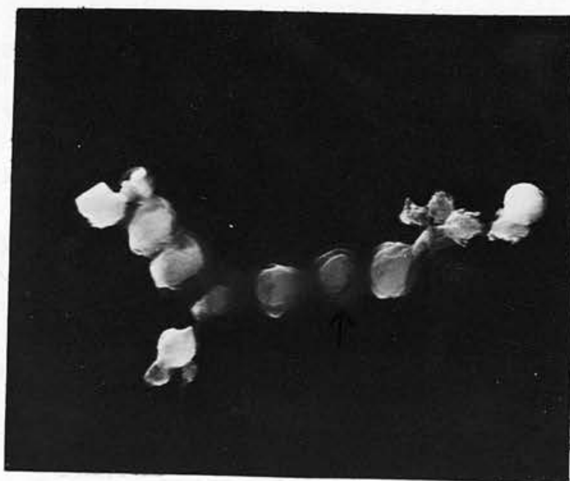
Photographs 20 and 21. Lipid inclusions of B. cereus (AC.1) isolated with hypochlorite, and extracted with ether. Shadowed electron micrographs. The cells were grown on 1% glucose synthetic medium for 40hrs., at 37°C. These inclusions should be compared with those that had not been extracted with ether. It will be seen that there is no significant difference between them. The wrinkled nature and transparent peripheral zone is evident. It is clear, therefore, that the ether-soluble constituent of the inclusions was not visible in the non-ether extracted inclusions.

Photographs 20 and 21 at a final magnification of X 15000.

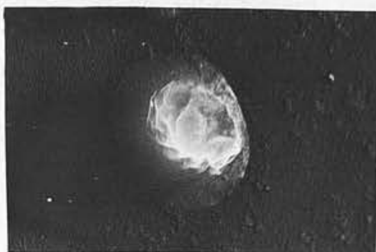


Photograph 22. Lipid inclusions of B.cereus (AC.1) isolated with hypochlorite. Shadowed electron micrograph. The cells were grown on 1% glucose synthetic medium for 40hrs. at 37°C. Note the many wrinkles and folds of the inner mass of the inclusions.

Photograph 22 at a final magnification of X 15000.



23



24

Photographs 23 and 24. Lipid inclusions of B.megaterium (NCTC 7581) isolated with hypochlorite. Shadowed electron micrographs. The cells were grown on 2% glucose agar for 24hrs. at 37°C. Both these photographs have been specially printed to show the detail of the inner part of the inclusions. The greatly wrinkled nature of the inner structure is particularly clear in 24. In photograph 23, the arrow indicates an inclusion which gives the impression of being comprised of concentric shells of material.

Photograph 23 at a final magnification of X 25000.
Photograph 24 at a final magnification of X 19000.

many of the preparations. While in many cases (e.g. photographs 15 and 17) the wrinkling is fairly clearly on the surface of the inclusion, it does not always seem to be confined to the surface, and in photographs 22, 23, and 24, it would appear that the inner mass of material has many folds and wrinkles.

Any interpretation of the structure of the inclusions from electron micrographs such as these is necessarily highly subjective, and at best, can only be put forward tentatively. It must first be emphasised that in the photographs so far discussed, one is dealing essentially with the PHB fraction of the inclusions. The microchemical observations described earlier showed that hypochlorite destroyed the sudanophilic component of the in situ inclusions, and the remaining "fatty" component of the hypochlorite isolated inclusions was apparently not visible in the electron micrographs. Thus, in interpreting the structure of the hypochlorite isolated inclusions as seen in these electron micrographs, one is really only interpreting the structure of the PHB core of the inclusions. It is perhaps rather surprising that the PHB fraction had any "structure" at all. One might have expected it to be a solid ball of polymer. That it is not, is obvious from the apparent shrinkage and collapse of the inclusions. It is also apparent that this collapse and shrinkage occurred before shadowing, since the shadows of so many of the inclusions are so short. This would appear to rule out the possibility that the shrinkage and collapse of the inclusions was an artefact caused by the electron beam. It must be assumed that some material was lost from the centre

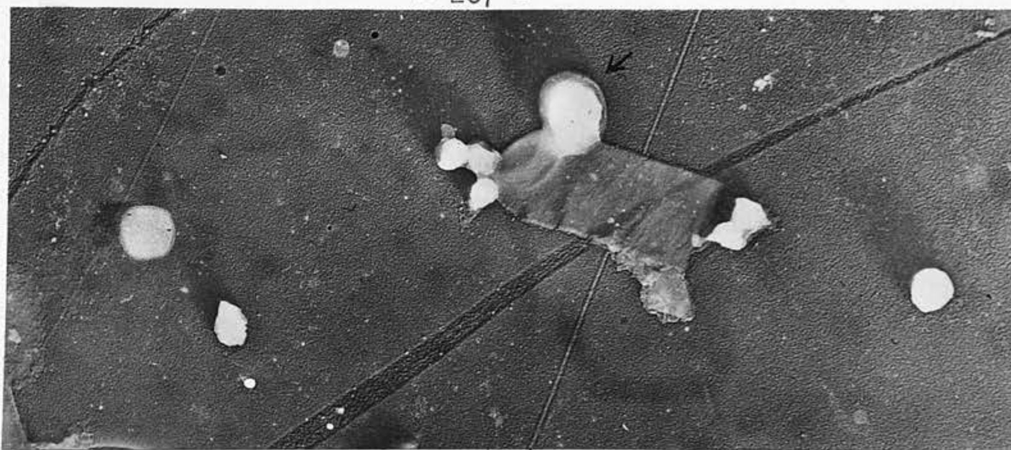
of the inclusion bodies on drying. Though loss of water would seem the most obvious cause of the shrinkage, it is just possible that a fatty material of low melting point might have evaporated under the influence of the high vacuum on freeze-drying. It has been shown that the hypochlorite isolated inclusions contained about 11% of a "fatty" material, so it is possible that it was the loss of this substance that caused the collapse of the inclusions. It does, however, seem more likely that loss of water was responsible. It is conceivable that during the isolation of the inclusions, a fatty or other component was dissolved by the hypochlorite and replaced with water during the subsequent purification. The loss of this water on freeze-drying would certainly account for the shrunken collapsed appearance of the PHB fraction of the inclusions.

As to the structure of this PHB fraction, it would appear that there is at least one peripheral shell of the polymer. Beyond this, the interpretation is difficult, but it is the author's opinion, and it is stressed that it is only put forward as a very tentative suggestion, that the rest of the polymer may take the form of a series of concentric shells. It is at least clear that the polymer is not in a granular form inside the inclusion body, and its membranous folded appearance does give some support for the above suggestion. Electron microscopic studies of ultra-thin sections might solve this problem.

(2). <u>The inclusions isolated by mechanical disruption of the cells.</u>

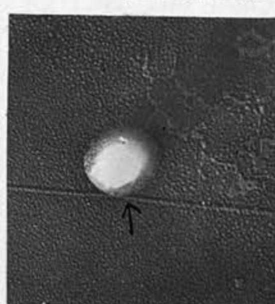
Photographs 25 to 30 (p. 207) show a selection of

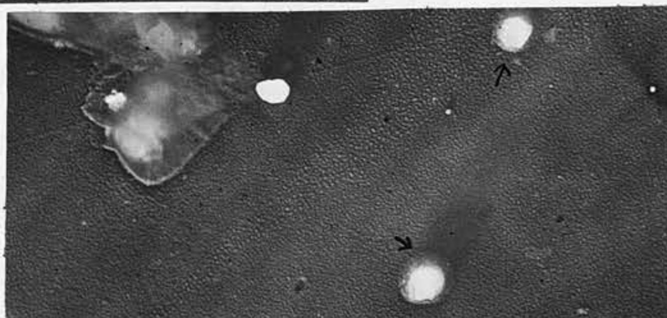
inclusions in preparations of cells that had been freshly disrupted by shaking in the "Mickle" vibrator with Ballotini beads. The freshly disrupted cell suspensions had been centrifuged once, and Sudan black stained smears indicated that about 90% of the isolated inclusions were fully sudanophilic. The deposited material had then been fixed for 30 minutes in 1% osmic acid, and dialysed againsted distilled water for 24 hrs before being freeze-dried on the membranes. It was hoped that in this way, electron micrographs of fully sudanophilic inclusions would be obtained. It will be seen that many of the inclusions appear very electron opaque, and not to have collapsed or shrunk on drying. By comparison with the earlier photographs it would appear that little material was lost on drying these mechanically isolated inclusions. Moreover, their surface is of quite a different texture from that of the hypochlorite isolated inclusions. It appears slightly "fuzzy" and irregular, and the wrinkled nature of the hypochlorite bodies is not apparent. It is possible that these mechanically isolated inclusions were still coated with the sudanophilic lipid material whose existence was indicated in Part IV. But it is also possible that the granules were still embedded in remnants of cytoplasmic material. It will be seen that some of the inclusions (indicated by arrows) corresponded much more closely in appearance with the hypochlorite isolated ones, the transparent outer zone being clearly visible. It is suggested that the opaque bodies may represent inclusion bodies that were still in a fully sudanophilic state, and that the less opaque bodies were inclusions from which the

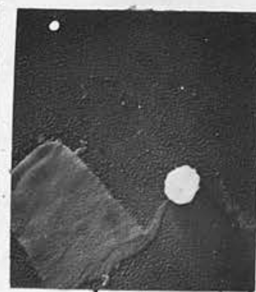

25

26

27


28


29


30

Photographs 25 to 30. The lipid inclusions of B.cereus (AC.1) after isolation by mechanical disruption of the cells, which had been grown on 3% glucose synthetic medium for 40hrs. at 37°C. Shadowed electron micrographs. As explained in the text, the preparations had been subjected to minimal handling, and it was shown that prior to fixation they were mostly in a fully sudanophilic state. Fragments of cell walls and other debris can be seen. Most of the inclusions appear to be coated with an electron-scattering material, and they do not seem to have collapsed too greatly. In photographs 25, 28 and 29, the arrows indicate inclusions that look very similar to those isolated with hypochlorite. The implications of these observations are discussed in the text.

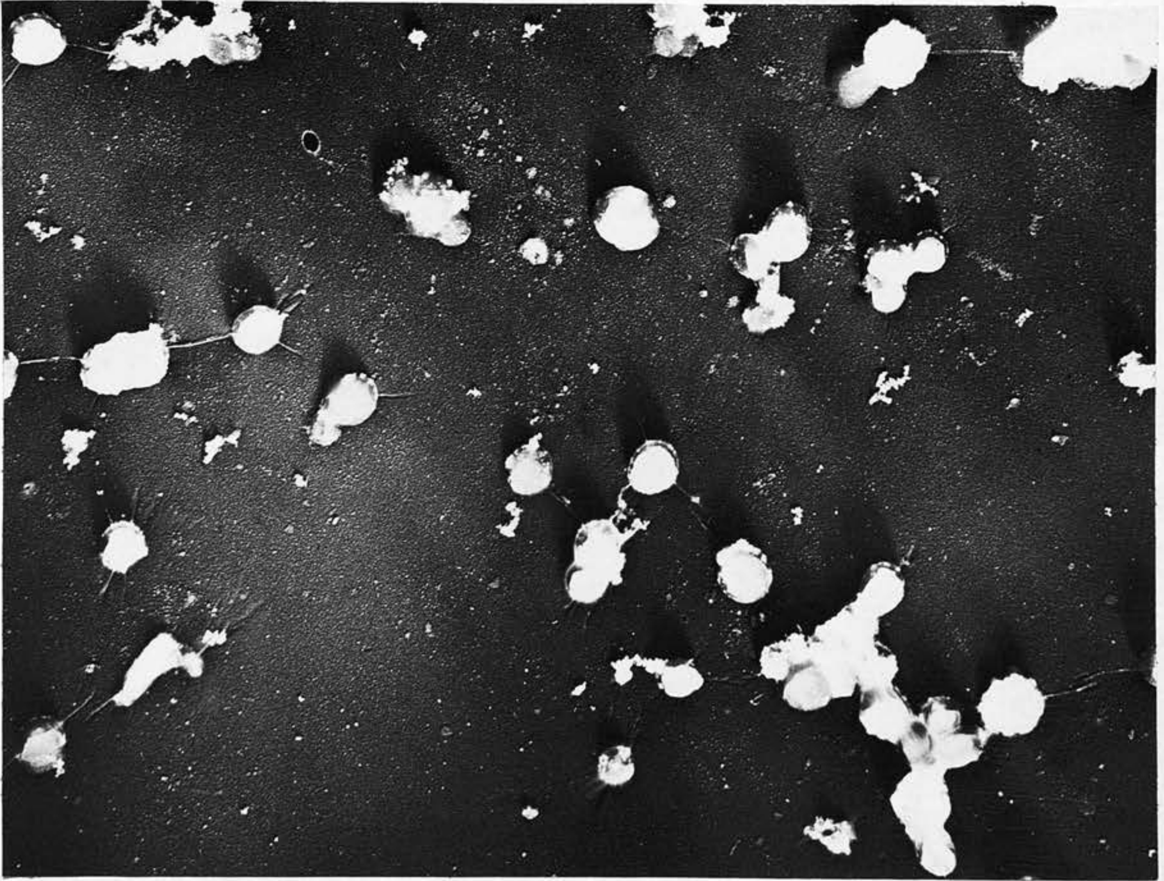
All photographs at a final magnification of X 10000.

outer layer of electron-scattering material had been stripped, revealing the core of PHB.

(3). <u>The inclusions isolated by lysozyme treatment of the cells.</u>

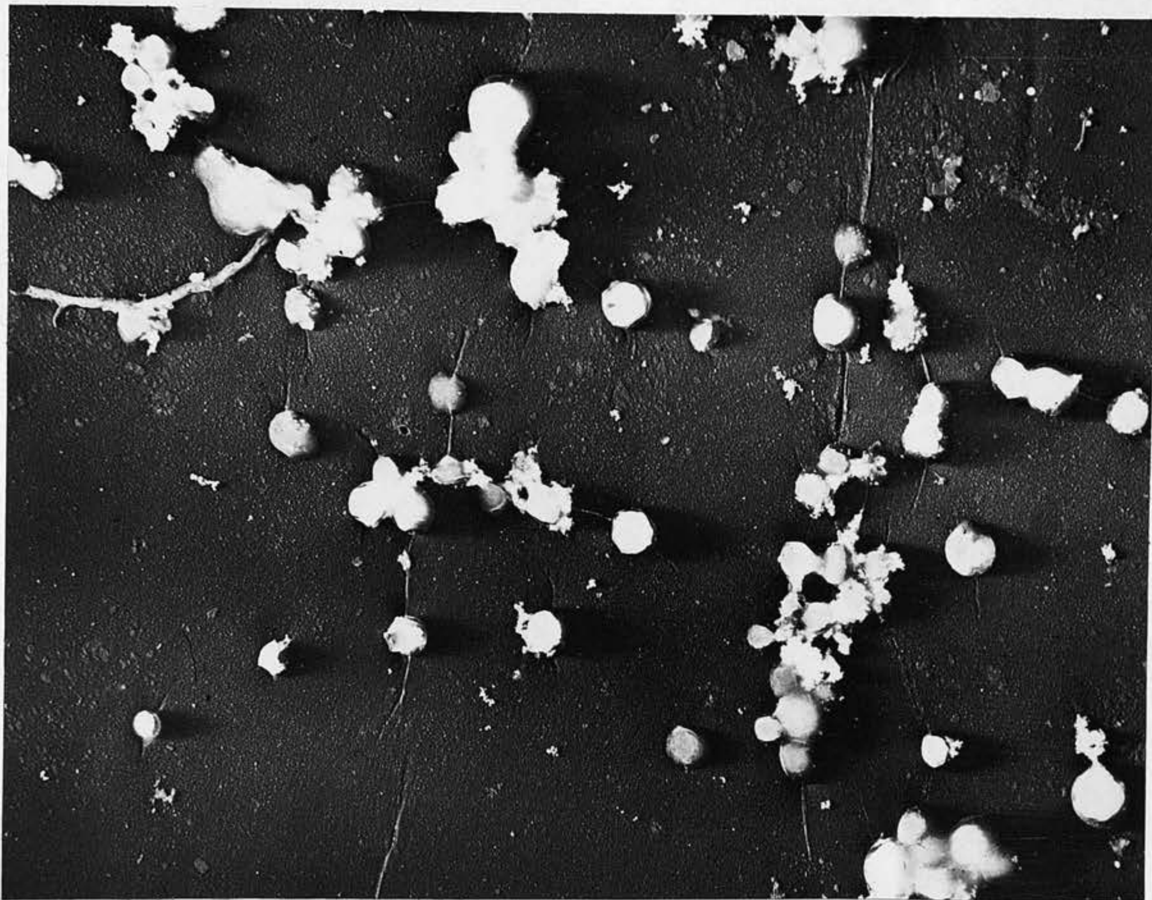
Photographs 31 to 37 are of the residual material obtained on lysis of cells of strain KM of <u>B.megaterium</u> with lysozyme in the manner indicated on p. 159. Samples of the mixed suspension of "ghost" membranes and lipid inclusions obtained on transferring the protoplasts to 0.15M phosphate buffer were removed and the suspended material deposited by a single gentle centrifugation. Sudan black staining showed that at this stage the inclusions were still fully sudanophilic. Fixation of the material was accomplished by suspending it in 1% osmic acid for half an hour, after which the osmic acid was removed by dialysis against distilled water for 24 hours. Droplets of the suspension were then freeze-dried on the formvar membranes and shadowed with Au/Pd at 15°. This unpurified suspension was used in the hope that the inclusions would have retained their full degree of sudanophilia. There was naturally a considerable amount of fine debris still admixed with the inclusions, and there was a fair proportion of "ghost" membranes (see photographs 36 and 37). The latter were in fact much more common than the photographs would suggest, but as far as possible they were avoided in photographing specimens.

Photographs 31 and 32 show the general appearance of the inclusions. On the whole they appear fairly opaque, and the wrinkled appearance seen in the photographs of the



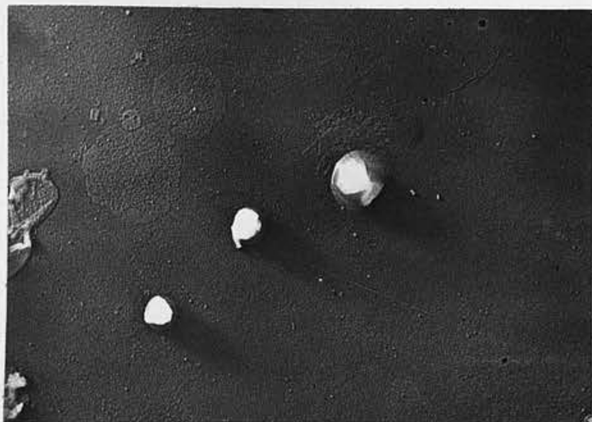
Photograph 31. The lipid inclusions of B.megaterium (KM) prepared by lysozyme treatment of the cells which had been grown on 1% glucose synthetic medium for 24hrs. at 30°C. Shadowed electron micrograph. As explained in the text, the preparations had been subjected to a minimum of handling, and it was shown that prior to fixation, the inclusions were mostly in a fully sudanophilic state. The inclusions, which are admixed with a certain amount of fine debris, appear fairly electron-opaque, but the short shadows indicate that in some cases they have collapsed, and many display the outer transparent zone seen in the hypochlorite-isolated inclusions.

Photograph 31 at a final magnification of X 10000.

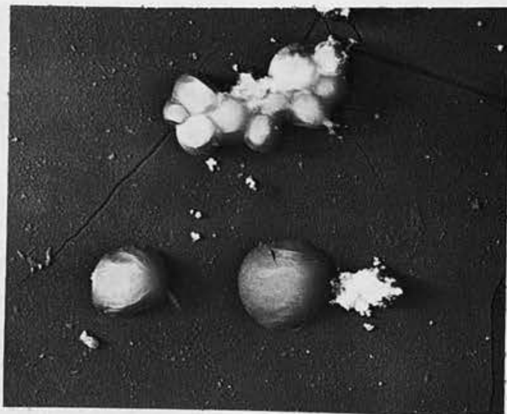


Photograph 32. The lipid inclusions of B. megaterium (KM) prepared by lysozyme treatment of the cells, which had been grown on 1% glucose synthetic medium for 24hrs. at 30°C.

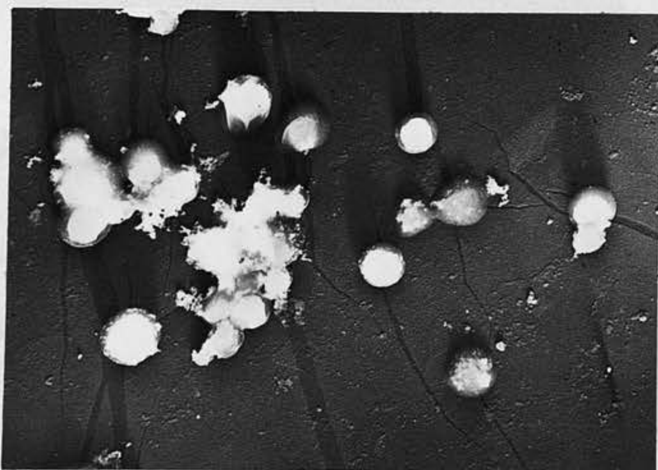
Photograph 32 at a final magnification of X 10000.



33



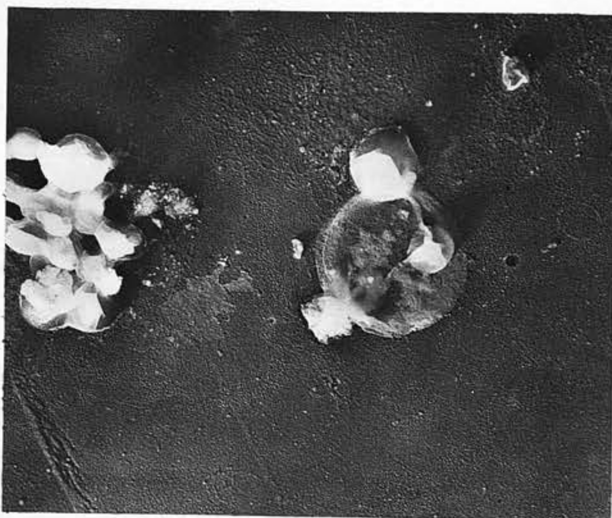
34



35

Photographs 33, 34, and 35. The lipid inclusions of B.megaterium (KM) prepared by lysozyme treatment of the cells, which had been grown on 1% glucose synthetic medium for 24hrs. at 30°C. Shadowed electron micrographs. The three bodies in 33, the clump near the top of 34, and one or two of the inclusions in 35, appear very similar to the inclusions as isolated by hypochlorite. The transparent outer zone and the "wrinkles" seen in the latter inclusions can also be seen here. The implications of this observation are discussed in the text.

All photographs at a final magnification of 10000.



36

37

Photographs 36 and 37. The lipid inclusions of B.megaterium (KM) prepared by lysozyme treatment of the cells, which had been grown on 1% glucose synthetic medium for 24hrs. at 30°C. Shadowed electron micrographs. A "ghost" membrane can be seen in each photograph. Note that, with the exception of the single inclusion body at the lower centre of 37, which appears very opaque and uncollapsed, the inclusions appear very similar to those isolated with hypochlorite. The implications of this observation are discussed in the text.

Photograph 36 at a final magnification of X 15000.
Photograph 37 at a final magnification of X 20000.

hypochlorite-isolated inclusions is not apparent. Nevertheless many of the inclusions have an outer electron-transparent zone, and the short shadows of some clearly indicate that some degree of collapse had occurred. Turning to photographs 33, 34 and 35, there are some clear examples of inclusions which appear very similar to those isolated by hypochlorite, showing the outer transparent zone as well as a slight amount of "wrinkling". This similarity to the hypochlorite-isolated inclusions is even more apparent in photographs 36 and 37, which also show the appearance of the "ghost" membranes.

The interpretation of these photographs is not easy, but it would seem that most of the lysozyme-isolated inclusions contained some electron opaque material in addition to the core of PHB, but that in some cases, this material had been more or less completely removed, presumably during the handling subsequent to the initial centrifugation.

In summary, the electron microscopic appearance of the inclusions as isolated in the three different ways suggest that <u>in situ</u>, they may consist of an outer shell of electron-scattering material (perhaps the sudanophilic lipid whose existence was indicated in Part IV) surrounding a core of poly β-hydroxybutyrate. This core does not appear to be solid, however, and the suggestion is made that it consists of a series of concentric shells of the polymer, perhaps interspersed with more lipid material. Further investigation of this finding is desirable, however.

PART VIII.

THE INFLUENCE OF CULTURAL CONDITIONS ON THE FORMATION OF POLY β-HYDROXYBUTYRATE (PHB) BY B.CEREUS (STRAIN AC.1)

(1) Purpose of experiments.

The aim of these experiments was twofold. Firstly, it was desired to assess the practical usefulness of the hypochlorite method of estimating PHB. Secondly, it was desired to obtain preliminary information about the influence of cultural conditions on the production of the polymer by the asporogenous strain of B.cereus. As was indicated in the Introduction to this Thesis, little is known of either the cultural conditions leading to the formation of PHB, or of its role in the life of the cell. It was pointed out that studies of the formation in culture of a "lifeless" material such as PHB can provide a valuable preliminary indication of the function of the material, as well as indicating some of the essential nutrient precursors required for its synthesis. As was discussed in the Introduction, such studies cannot prove the function of a suspected reserve store material, but they can and do provide a useful pointer to this function. As will be seen, the results obtained in this case did provide support for the suggestion that "lifeless" materials of the type commonly regarded as reserve storage materials, are laid down in increased amounts in cultures whose growth has been limited by any factor not interfering with their synthesis.

In addition to measurements of the formation of PHB at all stages of the cultures, some experiments included measurements of intracellular polysaccharide and of volutin. Though not so comprehensive as the data obtained for PHB formation, these observations brought to light some interesting features of the conditions of formation of volutin and polysaccharide, and in this way, helped to provide a fairly complete picture of the behaviour of the cell.

(2) The choice of methods and their validity.

In planning these experiments, it was realised that it would be essential to determine the growth curves obtained in every culture. As pointed out by Lamanna and Mallette (1953), "the chronological age of a culture is of little biological importance", and observations of the cell contents of PHB at unspecified stages of the culture cycle would have had little real value. The correctness of this viewpoint has found ample support in the experimental results, and will be referred to later. The growth curve was determined by measuring the total non-diffusible nitrogen (effectively the total cell protein) in the culture at all stages of growth. Coupled with determinations of the ratio of PHB to nitrogen in the culture (i.e. effectively the PHB per cell), these figures for total nitrogen enabled the total PHB contents of the cultures to be determined at all stages of growth. The resultant figures obtained for total PHB, in turn facilitated the interpretation of the fluctuations observed in the cell

content of PHB.

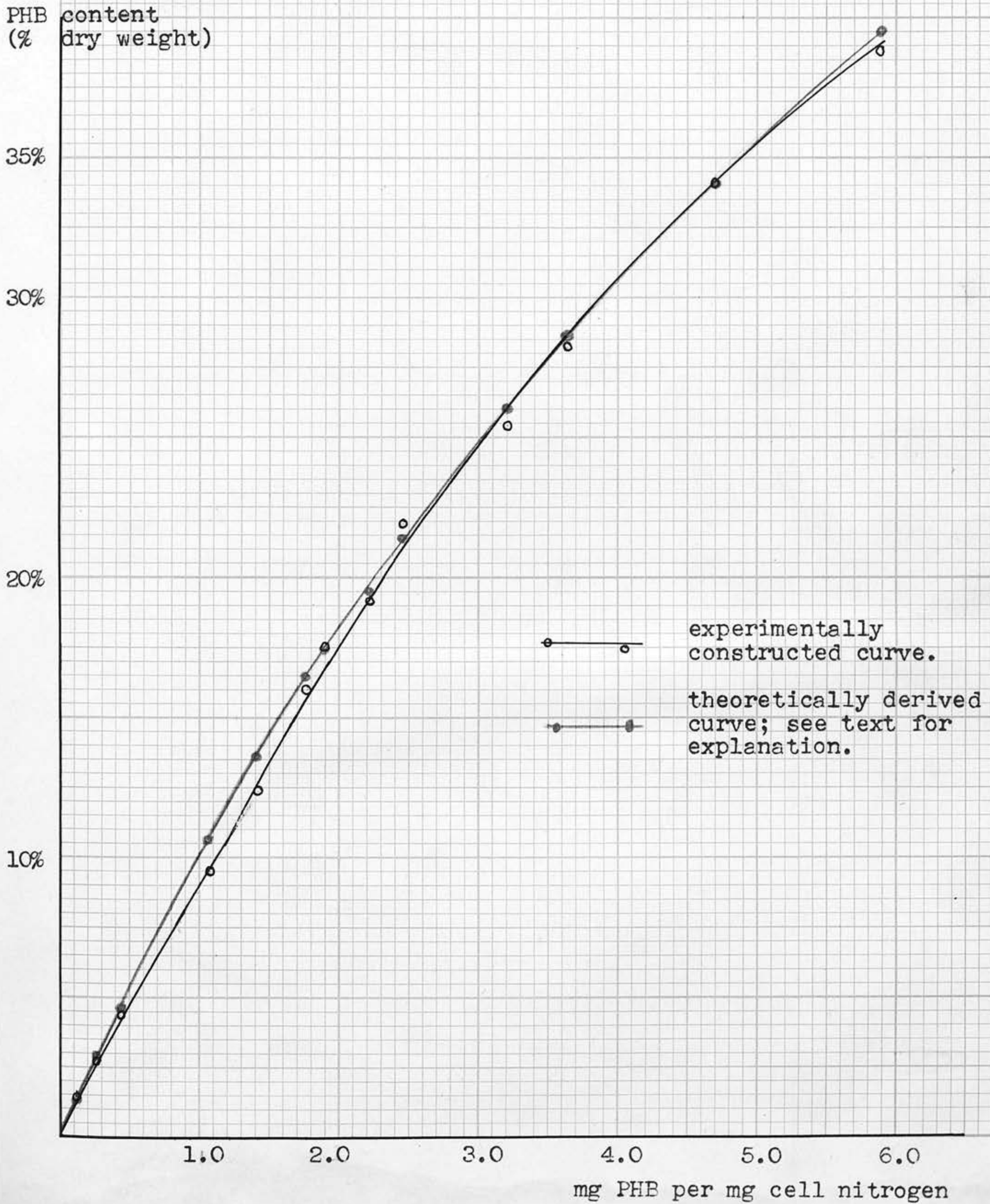
The total nitrogen content of the cells has many advantages over their dry weight as a parameter of growth. These have already been outlined in the Introduction. Chief among them, the use of total nitrogen avoids fallacies due to the assumption that an increase in the total dry weight represents an increase in growth when in fact, it may only be due to increased deposition of one particular "lifeless" material. Moreover, it was simpler in practice to estimate nitrogen than to measure the dry weights of the large numbers of samples involved. Nevertheless, it was felt that the cell content of PHB in terms of its proportion of the dry weight of the cell gives a more meaningful "picture" of the state of the cell than does the ratio of PHB to nitrogen. For this reason, in a few instances, the cell content of PHB as a percentage of the dry weight has been shown in addition to the PHB/N ratio. Unless otherwise indicated, the figures for the dry weight content of PHB in the cells were obtained indirectly, using the curve (marked in black) shown in graph. 6p.217. This curve was constructed with the data obtained from a series of measurements of the PHB, nitrogen and dry weight contents of suspensions of different cultures of strain AC.1 grown on synthetic media, (table 21) and it shows the relationship that exists between the ratio of PHB to nitrogen, and the amount of PHB in the cells as a percentage of their dry weight. It enables one to determine the percentage PHB content of the culture without

TABLE 21.

PHB content of cells as % dry weight (found)	mg. PHB per mg. cell nitrogen	PHB content as % dry weight calculated from $y = Kx/(1 + Kx)$
1.34	0.12	1.3
2.67	0.27	2.9
4.33	0.45	4.7
9.50	1.07	10.6
12.4	1.42	13.6
16.0	1.78	16.4
17.5	1.90	17.3
19.2	2.21	19.5
21.8	2.45	21.3
25.4	3.20	26.0
28.4	3.63	28.6
34.1	4.70	34.0
38.9	5.90	39.5

These data are plotted in GRAPH 6; see text for full explanation.

Relationship between PHB content of cultures of B.cereus(AC.1) expressed as % dry weight and as mg. PHB/mg. cell nitrogen.


GRAPH 6.
Relationship between PHB content of cultures of B.cereus(AC.1) expressed as % dry weight and as mg PHB per mg cell N.
PHB content (% dry weight)
35%
30%
20%
10%
1.0
2.0
3.0
4.0
5.0
6.0
mg PHB per mg cell nitrogen
experimentally constructed curve.
theoretically derived curve; see text for explanation.

actually making any measurement of the dry weight of a cell suspension. The ratio of PHB to nitrogen is all that need be determined; the percentage content of PHB can then be read off the curve.

It is of considerable significance that a curve that closely fits the experimentally determined one, can be constructed from purely theoretical considerations as follows:

Suppose a cell of dry weight m units and containing no PHB, has a nitrogen content of K units per unit dry weight i.e. a total nitrogen content of Km units. Suppose further that when this cell is induced to form p units of PHB, thereby increasing its dry weight to $(m+p)$ units, the total nitrogen present in the cell remains unaltered at Km units.

let y = total PHB/total dry weight

and let x = total PHB/total nitrogen

then $y = p/(m+p)$

and $x = p/Km$

whence $y = Kmx/(m + Kmx)$

i.e. $y = Kx/(1 + Kx)$

Using this equation, and assuming a value for K of 0.11mg. N per mg. dry weight of the cells (a figure which closely corresponds to the actual nitrogen content of cells of strain AC.1 containing practically no PHB), the curve shown in red on graph 6 can be constructed. The close fit of this theoretical curve to the experimentally determined data is evident. The closeness of the fit

is strong evidence for the practical validity of the assumption made above in calculating the equation of the theoretical curve, namely that when a cell forms PHB, the total nitrogen in the cell remains constant. Thus no protein or other nitrogenous material is displaced or synthesised in order to accomodate the PHB. Changes in the nitrogen content of a culture are therefore a reliable indication of growth or cell autolysis, as distinct from changes in the total dry weight, which may merely be due to synthesis or breakdown of PHB.

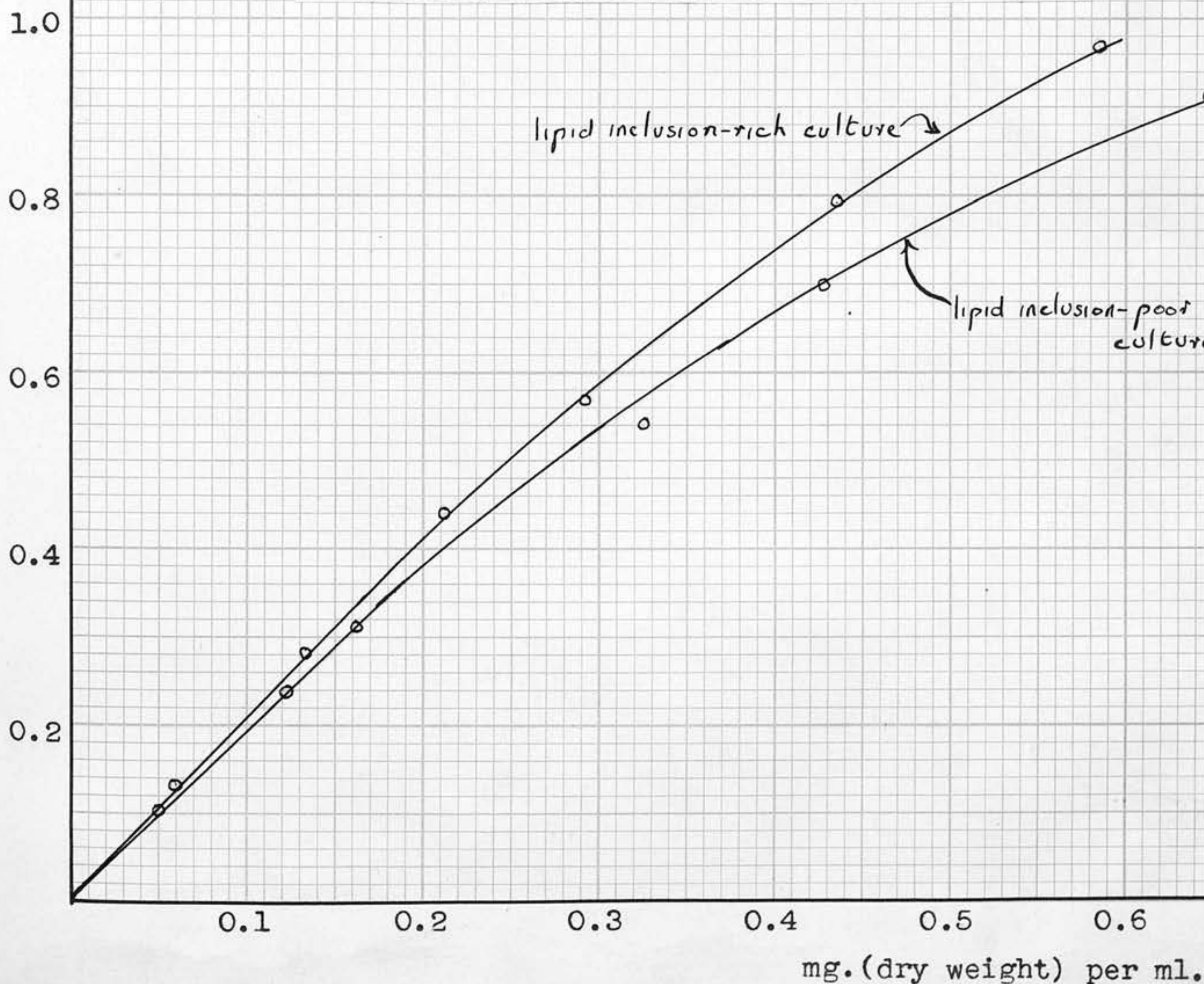
It may be suggested that the dry weight content of the cultures could have conveniently been estimated from their turbidities. Unfortunately this was not so. Graph 7 (table 22) shows the dry weight/turbidity curves for two cultures containing different amounts of PHB. It will be seen that the turbidity of a given dry weight of cells is appreciably increased by the presence of moderate amounts of PHB. Remembering that the lipid inclusions are much more refractile than cytoplasm, this finding is not altogether surprising, and it underlines an important source of error in the use of turbidity as a measure of dry weight in the case of organisms forming refractile inclusions. Graph 8 (table 23) shows that the presence of the lipid inclusions also effects the ratio of turbidity of a suspension to its nitrogen content. It will be seen that the points display a wide scatter; this is perhaps due to yet another source of error inherent in turbidimetric estimates of cell mass, i.e. that due to variation in the

GRAPH 7.

Turbidity/dry weight relationships of two cultures of B.cereus(AC.1) containing different amounts of lipid inclusions.

TABLE 22

cells containing microscopically estimated lipid inclusions as:			
⊥		+++	
Turbidity	μg(dry wt.) per ml.	Turbidity	μg(dry wt.) per ml.
0.10	52	0.13	60
0.24	123	0.28	135
0.31	163	0.44	213
0.54	325	0.57	293
0.70	428	0.79	435
0.91	648	0.97	585



GRAPH 8.

The effect of the presence of PHB (i.e. lipid inclusions) on the ratio of the turbidities of suspensions of B. cereus (AC.1) to their cell nitrogen contents.

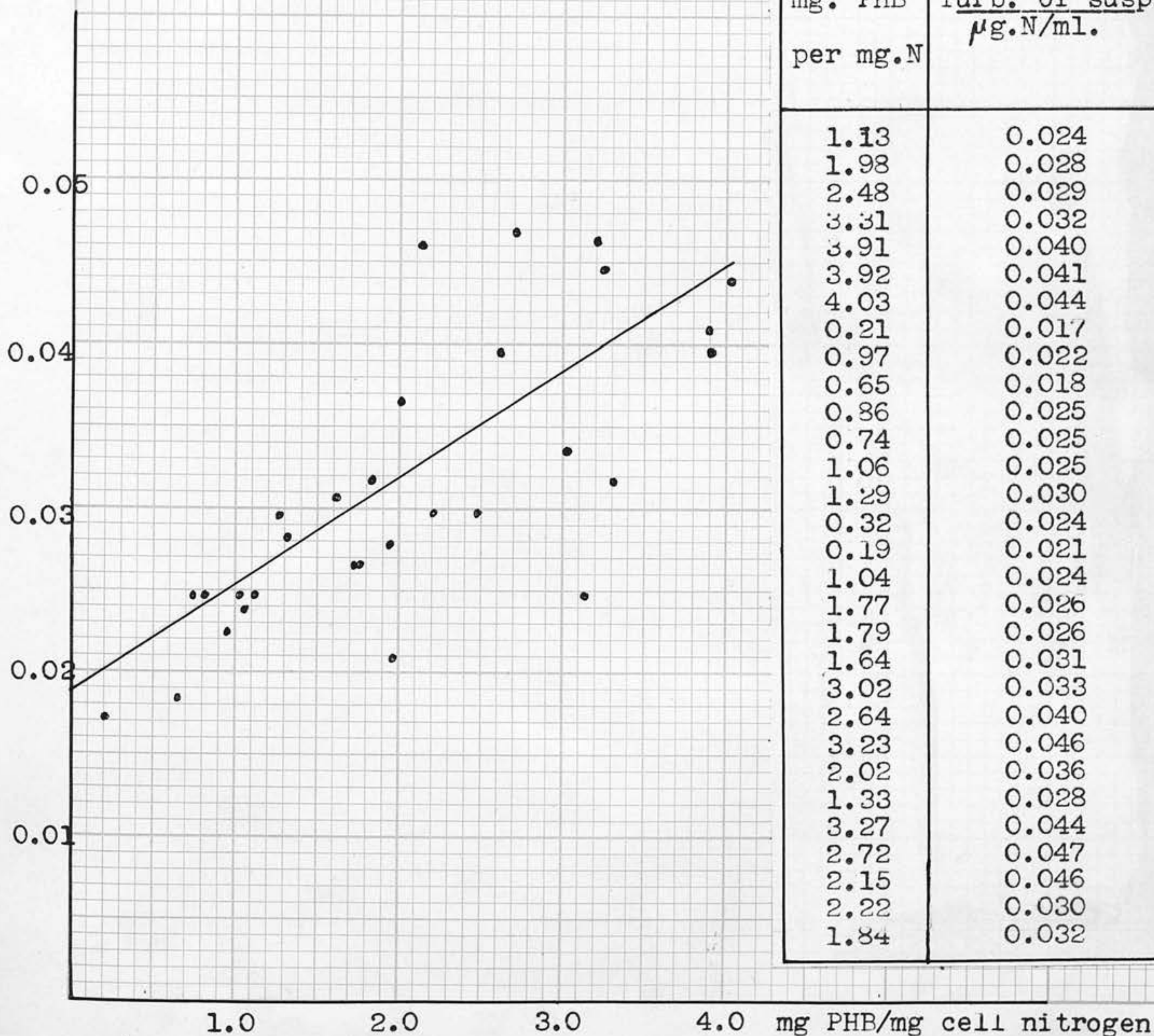


TABLE 23

mg. PHB per mg.N	Turb. of susp. / μg.N/ml.
1.13	0.024
1.98	0.028
2.48	0.029
3.31	0.032
3.91	0.040
3.92	0.041
4.03	0.044
0.21	0.017
0.97	0.022
0.65	0.018
0.86	0.025
0.74	0.025
1.06	0.025
1.29	0.030
0.32	0.024
0.19	0.021
1.04	0.024
1.77	0.026
1.79	0.026
1.64	0.031
3.02	0.033
2.64	0.040
3.23	0.046
2.02	0.036
1.33	0.028
3.27	0.044
2.72	0.047
2.15	0.046
2.22	0.030
1.84	0.032

refractility of the cell caused by variations in cell size and in content of dispersed materials such as polysaccharides and lipids, etc. In this connection it is perhaps significant that the data plotted in Graph 6, which did not involve the measurement of turbidities of cells suspensions, displayed very little scatter.

The line drawn on Graph 8 was constructed from the regression equation calculated after Snedecor (1940), and represents the best estimate of the relationship from the available data. The correlation coefficent (r) was found to be 0.839 which indicated the significance of the relationship at the 0.001 level of probability.

The choice of solid media may require some explanation. There are several valid objections to the use of solid media for experiments of this type. Notably, there is the objection that the use of agar entails an unknown contamination with mineral elements; secondly, the growth on the surface of a solid medium may not be uniform, since the cells at the surface are relatively further from the source of nutrients, yet better aerated than those in contact with the agar. The use of solid media however, was indicated, in this instance, by the following observations. Firstly, it was found that repeated attempts to obtain reproducible growth curves in shallow layers of liquid media failed. Secondly, the total growth was only about half that obtained in comparable solid media. Thirdly, and most important of all, the growth in liquid media was approximately linear; fluctuations in lipid inclusion content were slight, and there was little in-

GRAPH 9.

Growth curve of B.cereus in a shallow layer of a liquid synthetic medium; strain AC.1, 37°C.

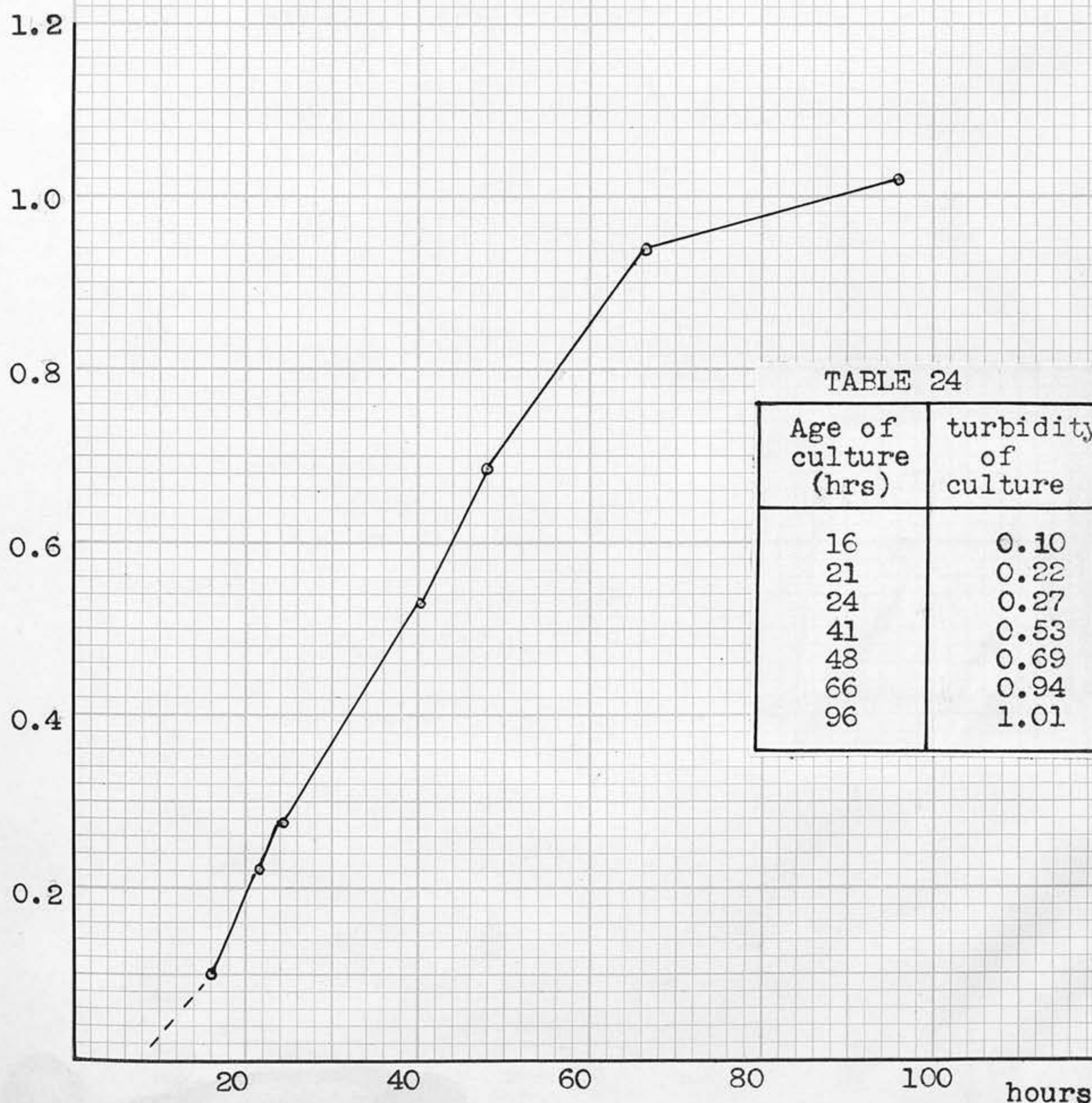


TABLE 24

Age of culture (hrs)	turbidity of culture
16	0.10
21	0.22
24	0.27
41	0.53
48	0.69
66	0.94
96	1.01

dication that an increase in carbon source had any affect on the content of PHB. An example of the type of growth curve obtained is shown in Graph 9 . Its linear nature suggests that a factor such as inadequate aeration was limiting the rate of growth. For practical reasons, the use of cultures aerated by bubbling or by agitation was not possible, and attention was therefore turned to the use of solid media. It was at once found that the growth curves obtained were nearly logarithmic, and there were marked and regular fluctuations in PHB content in response to variations in the supply of carbon source. The causes of this difference between liquid and solid media are irrelevant to the present discussion; suffice it to say that the observed difference in the organism's response far outweighed the objections to solid media, and the choice of media was obvious.

The details of the technique used for this series of experiments have already been given. It might be added that when the effect of variation in amount of a given nutrient was being investigated, it was usual to set up media representing two concentrations of the nutrient at the same time. Usually one of these concentrations was in excess of the amount required by the organism for maximum growth from the available carbon source, and the culture thus served as an effective control for the other set of plates, on which the total growth was limited by deficiency of the nutrient in question.

A certain amount of uncontrollable variation was

observed between identical cultures made on different occasions. In the case of the cultures containing high concentrations of glucose, this variation was sufficiently great to warrant the inclusion of results from more than one experiment. In all other cases, however, only a single set of results has been recorded for each experiment, since the variations between identical cultures made at different times were relatively insignificant. It is stressed, however, that in all cases, at least two experiments were made with each type of medium, and the reported results are a reliable indication of the typical response of the cells.

The interpretation of the graphical results requires some explanation. During the phases when the total nitrogen is changing, i.e. during the phases of active growth and of decline, a change in the ratio of PHB (or polysaccharide) to nitrogen (effectively the amount of the material per cell) may not be due to any active synthesis or breakdown of the material in question. It may simply be the case that the total amount of the material in the culture remains constant, while the nitrogen level changes. Inspection of the curve showing the total level of the material in the culture will reveal whether in fact any active breakdown or synthesis occurs, and for this reason the "total PHB" and "total polysaccharide" curves are of great importance during the growth phase and the phase of decline. During the stationary phase, the curve showing the amount of material per cell (i.e. per unit of nitrogen) is

probably more meaningful, since any change in it clearly represents a synthesis or breakdown of the material concerned and, for reasons which will become apparent later, the amount of material per cell is a better indication of the effect of altering the concentration of a nutrient on the production of the material concerned than is the total amount of material in the culture. It will be seen that in some of the experiments, the level of PHB per cell was not constant throughout the stationary phase, but after rising to a peak declined fairly rapidly. Without making any assumptions about the reason for this decline, it is thought that the peak level of PHB per cell attained during the stationary phase is probably the best general indication of the effect of the nutrient concerned on the formation of PHB. For this reason, comparison of the curves obtained during the stationary phase is important, and it has been simplified by adjusting the positions of the curves of a set so that they all enter the stationary phase at the same point on the time axis of the graph. Reference to one of the graphs will make this point clear.

<u>A Note on some of the terms employed.</u>

For convenience, and in order to avoid laborious repetition, certain recurrent expressions have been abbreviated to simpler forms. Thus when a medium contained more of a given nutrient than was necessary for the maximum growth (in terms of nitrogen) supported by the other nutrients present in the medium (or by the prevailing physical conditions) the medium has been described as "rich"

or "excess", e.g. " a glucose rich medium", or "an excess glucose medium". Similarly, the terms "deficient" or "limited" (e.g. "potassium deficient" or "phosphorus limited") have been used to denote media in which the total growth (in terms of nitrogen) was limited by exhaustion of the nutrient concerned.

(3). Experimental results.

(a) The glucose limited "control" medium

It has already been explained that this medium was designed by experiment to be such that growth was limited by exhaustion of the carbon and energy source (0.3% glucose), the formation of PHB, volutin and polysaccharide being minimal. The evidence for this contention is shown in graph 10 p.230.(table 25), where the total growths obtained on media containing different amounts of glucose have been plotted against the concentrations of glucose in the media. It will be seen that a reduction of the glucose content from 0.3% to 0.1% was accompanied by a proportionate drop in the total growth. An increase in the glucose content on the other hand, from 0.3% to 1%, was accompanied by an increased total growth, but the increase was proportionately lower than the increase in glucose concentration. There was therefore, a surplus of glucose in the 1% medium, and this surplus was available for conversion into PHB and polysaccharide(as will be shown later). It will also be noted that the total growth on the 3% glucose medium was slightly less than that on 1% glucose. The reasons for this will be discussed later. It is clear, however, that the total growth on media containing 0.3% glucose or less was limited by exhaustion of the carbon and energy source, glucose. It will also be shown that, in accordance with expectations, the levels of PHB, volutin and polysaccharide in the stationary phase cells grown on this medium were minimal. The levels obtained, did in fact represent the "élément constant" (Belin 1926) for each of the materials concerned, since, as will be shown,

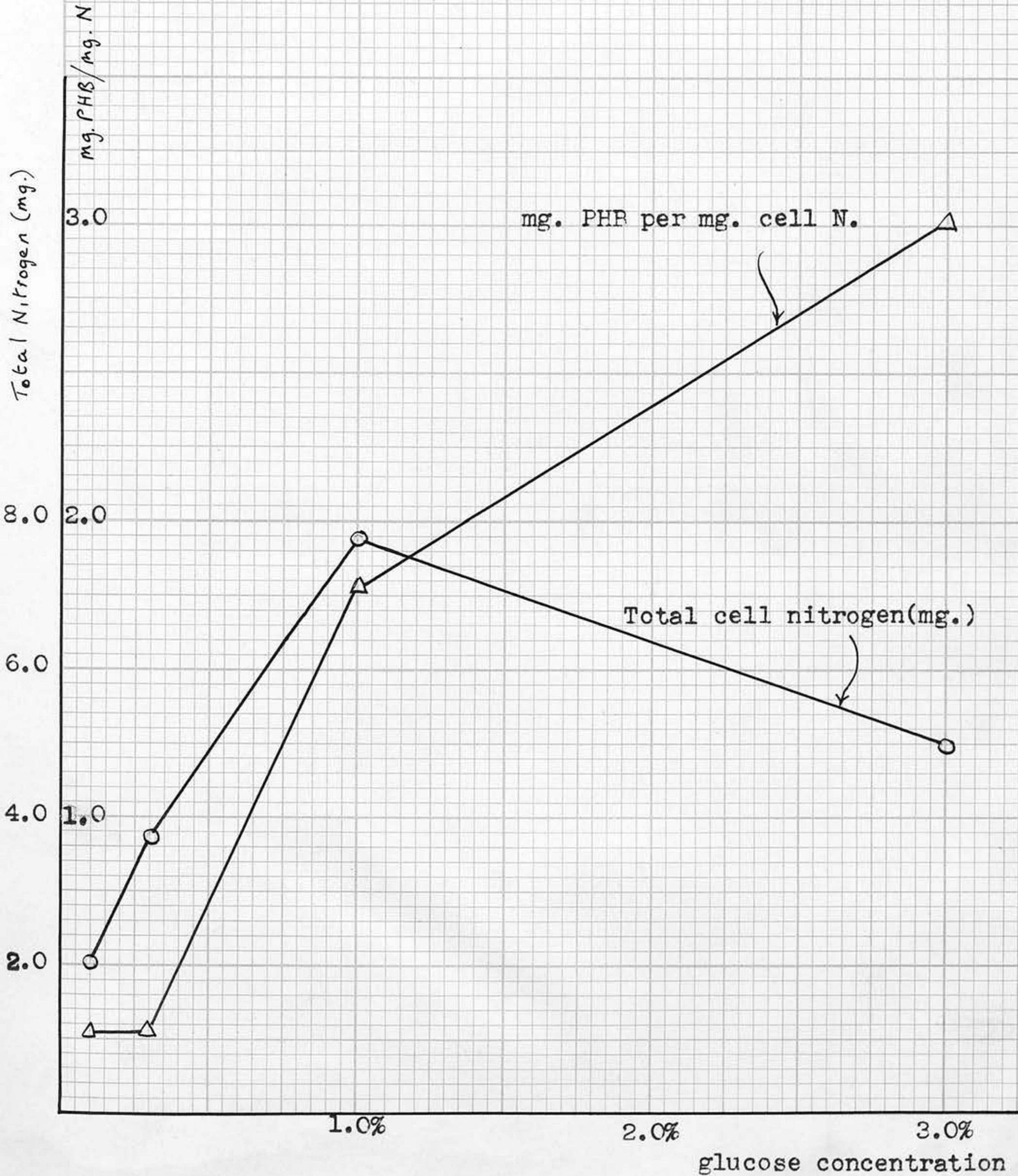
TABLE 25.

Concentration of glucose in medium (W/V).	Total growth (stationary phase average mg nitrogen).	mg PHB per mg cell N.(peak stationary phase value).	mg polysacch-aride per mg cell N(peak stationary phase value).	Volutin (Albert's stain peak stationary phase value).
0.1%	2.0 mg	0.23	0.71	±
0.3%	3.7 mg	0.23	0.94	+
1.0%	7.7 mg	1.79	2.00	++±
3.0%	5.0 mg	3.02	1.87	++±

The influence of glucose concentration on the total growth and stationary phase cell contents of PHB, Volutin and intra-cellular polysaccharide of B.cereus.(AC.1).

GRAPH 10.

The influence of glucose concentration on the total growth and cell content of PHB of B.cereus (AC.1) grown on synthetic media at 37°C



reduction of the glucose concentration from 0.3% to 0.1% did not result in a reduced cell content of the materials.

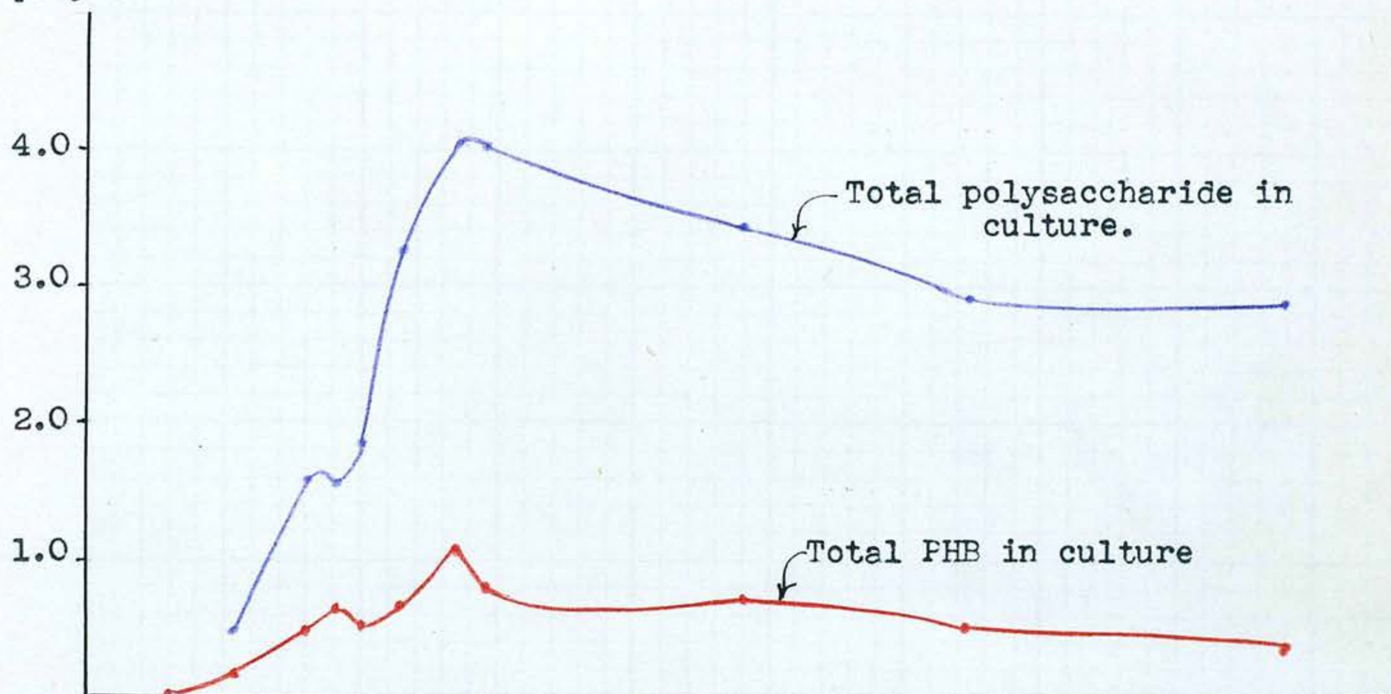
The concentrations in the medium of all the other added nutrients were adjusted to be many times in excess of the amounts needed to support complete utilisation of the glucose for growth.

Examples of the curves obtained on this "control" medium are shown in graphs 11 and 13. (pp.232 and 240). They illustrate several interesting features.

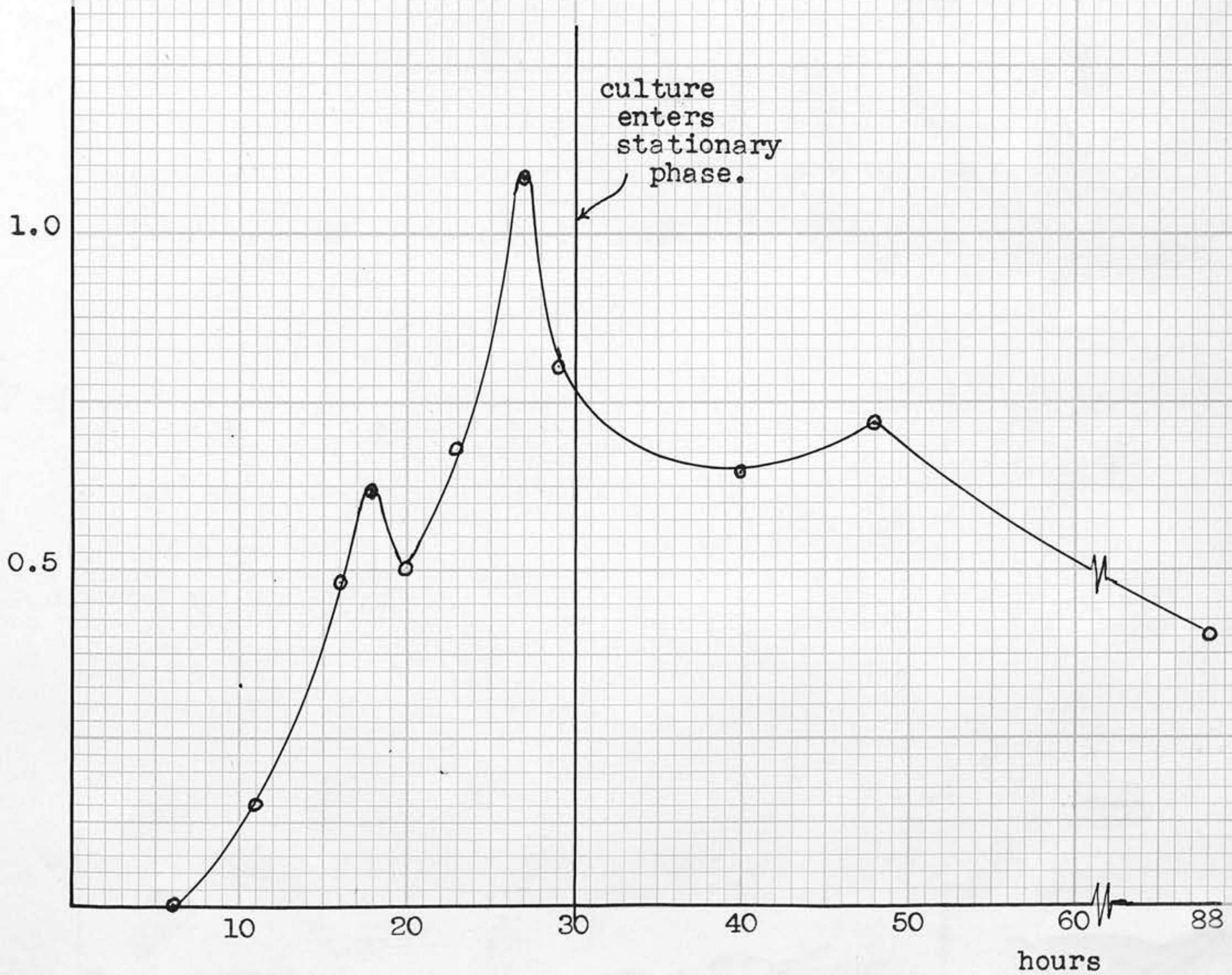
Inspection of the PHB/N curve in graph 11 reveals that although the level of PHB per cell (PHB/N) was low once the stationary phase was reached, moderate amounts of PHB appeared in the cells during the phase of active growth. This result emphasises the importance of relating observations of this nature to the stage of the growth cycle at which the experimental observations are made. The conclusions reached by a single examination of this culture at 12 hours, for instance, would have been quite different from those of a similar observation at, say, 35 hours, and would have been quite misleading since, as will be shown later, similar fluctuations occurred on several media during growth, and appeared to be quite independent of the final level attained during the stationary phase, (see for instance graph 15).

It will be seen that the PHB/N curve in this example displayed the three peaks marked (a), (b), and (c). Though not always apparent, these peaks occurred with sufficient regularity to indicate that they were not simply chance variations. Peak (a) (at about 12 hours) provides a good example of the sometimes misleading nature of the PHB/N

Growth, PHB and polysaccharide production of B.cereus (AC.1) on the glucose-limited "control" medium



GRAPH 12. (data from table 28,p.250b;0.15% NH_4Cl). The production of PHB by B. cereus (AC.1) growing on the glucose limited"control" medium

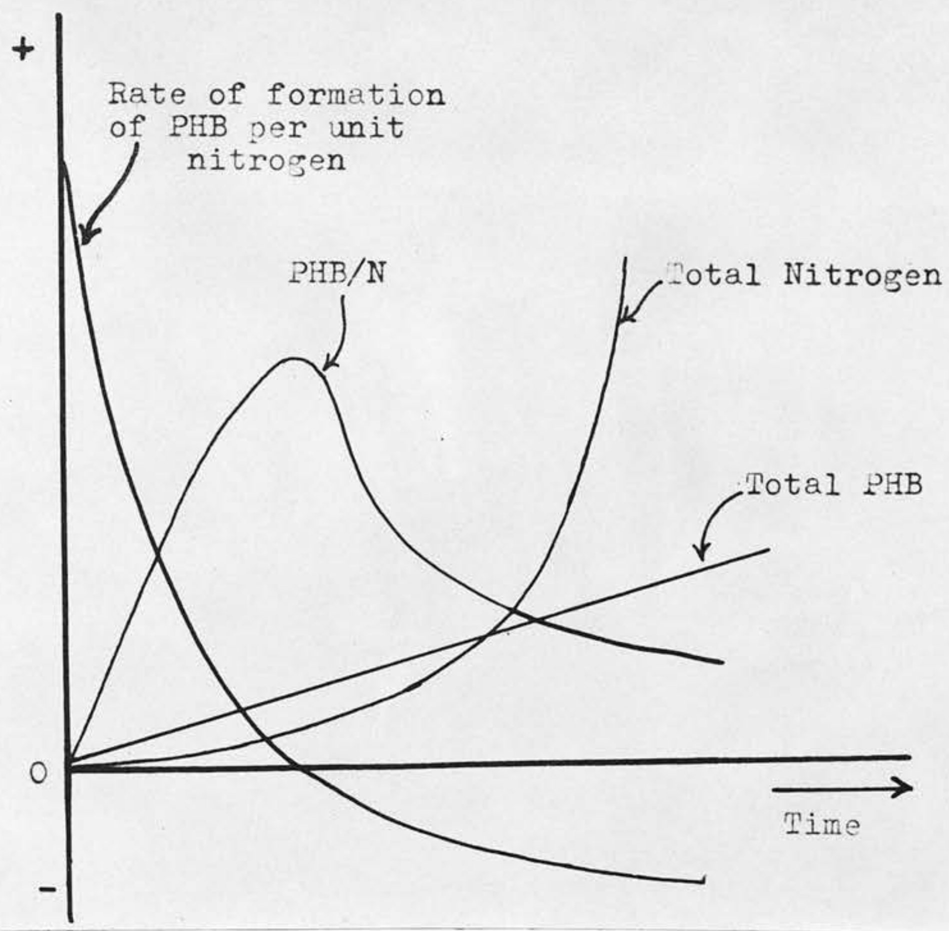


curve, since the turning point does not represent an actual breakdown of PHB in the culture. This is shown by examination of the curve for total PHB, which at this stage is steadily increasing at an approximately linear rate, without any break. It appears that at the start of growth the total rate of production of PHB in the culture was relatively much greater than the absolute growth rate. Accordingly, the PHB per cell began to increase. As the growth rate began to increase (logarithmically) it soon outstripped the rate of total production of PHB and the amount of PHB per cell started to decline. This idea can be expressed more precisely in mathematical terms. In a hypothetical ideal case, where the growth follows a strictly logarithmic course, and assuming the total PHB production to be at a steady rate, it can be shown that the curve for PHB/N has the equation:

$$P_t = \frac{kt + P_o}{n_o (R)^t} \quad \text{.....................} (1)$$

where P_t is the PHB/N at time t, k is the total PHB production per unit time, Po is the total PHB at time 0, n_o the total nitrogen at time 0, and R the factor of increase in total nitrogen per unit time.

The shape of the curve is indicated in the accompanying diagram (p235). It is unfortunately not possible to test the fit of equation (1) to the experimental data, since the latter are not sufficiently numerous, and in particular, n_o and k could not be measured with sufficient accuracy. However, these considerations do emphasise the point that the peak (a) did not represent an actual breakdown of the PHB in the culture, but only a drop in the amount of PHB



The curves diagrammatically represented here are of the total nitrogen, total PHB, PHB per unit nitrogen, and rate of production of PHB per unit nitrogen, in a theoretical culture where the growth(in terms of nitrogen)is perfectly logarithmic and the total production ofPHB in the culture is at a constant rate.

The curves have the following equations:

Total PHB: $P = kt + P_o$ ....(1)

Total N: $N = n_o(R)^t$ ....(2)

PHB/N: $P_t = \frac{kt + P_o}{n_o(R)^t}$ ....(3)

PHB/N/unit time: $r = \frac{k - (kt + P_o)\log_e R}{n_o(R)^t}$ ....(4)

Where P is total PHB at time t, k is total production of PHB per unit time, P_o is total PHB at time 0, N is total nitrogen at time t, n_o is total nitrogen at time 0, R is the factor of
crease of total nitrogen per unit time, P_t is PHB/N at time t, and r is production of PHB per unit nitrogen per unit time. Equation (4) was derived from (3) by differentiation.

It is significant that in such a culture there is no real breakdown of PHB. The apparent drop in PHB/N is due to the dilution of the slowly produced PHB among the more rapidly dividing cells.

per cell resulting from the logarithmic growth rate and the nearly linear production of total PHB.

The significance of a linear total production of PHB in the culture is not understood. Such a constant rate of production would be consistent, however, with a fixed amount of enzyme working at a steady maximal rate. It is conceivable that at this early stage of the culture (and it should be stressed that these considerations only apply to the very young culture) the amount of glucose in the medium would be in excess of the amount of PHB-forming enzyme, which would therefore be kept working at its maximal rate. Provided the total amount of the enzyme in the culture were constant, a linear total production of PHB would result. As the cells multiplied, the enzyme would be "diluted" amongst them, and the rate of production of PHB per cell would decrease from the moment cell division commenced. At the precise moment at which peak (a) occurred, the rate of production of PHB per cell would be zero since the cells would be dividing so rapidly that any PHB formed would be immediately diluted amongst them, without any change in the level of PHB per cell, and immediately after this moment, the rate of production of PHB per cell would become negative,& the level of PHB per cell would diminish, as in fact, it did. The shape of the curve showing the rate of production of PHB per cell is indicated on the diagram. It is of some interest that Duguid and Wilkinson (1953) found a similar relationship to hold for the rate of production per cell of capsular polysaccharide by Aero aerogenes, the rate being maximal at the start of growth,

and gradually declining thereafter.

Returning to the suggestion that the amount of PHB-forming enzyme in the culture is constant from the start of cell division, it will be recalled that the inocula used for these experiments were 16 hour cultures on the "control" medium. If the above suggestion is correct, the amount of the enzyme per cell of the inoculum should be very small, much smaller in fact than the amount of the enzymes per cell at the start of cell-division. One must therefore assume that during the lag phase, a stock of the enzyme was built up, but as soon as growth was initiated, this production of enzyme ceased. Studies with washed suspensions might provide information on this point.

Turning back to graph 11, one finds peak (b) in the PHB/N curve. This peak, unlike the previous one, is apparently due to an actual breakdown of PHB. At first sight there is apparently no corresponding peak in the curve of total PHB, but when a more suitable scale is used (graph 12)*, a slight peak at the time when peak (b) occurs becomes apparent. Despite its small size, this peak in the total PHB curve was always apparent when peak (b) was evident, and it clearly indicated a slight increased production of PHB followed by a breakdown. The cause of this phenomenon is not understood.

The third peak (c) appears to be a natural consequence of the total PHB production being maintained at a steady or slightly increased rate while the rate of growth tails off. Just before growth stops completely, however, the PHB starts to be broken down, perhaps to be utilised for the final stage of active growth. In the case of media such as the

glucose rich ones, however, there is no final breakdown of PHB, but a rapid increased production occurs, and peak (c) is not visible (e.g. graph 15).

As the stationary phase is entered (at about 30 hours) the PHB/N decreases to a fairly steady minimum value. Finally, (about 20 hours later) the phase of decline is reached, and the nitrogen falls away fairly rapidly. In cases where the phase of decline was marked, the culture at this stage was found to have a strong, putrefactive odour. It will be seen that the PHB/N curve remained steady during this phase, and inspection of the total PHB curve shows that a steady breakdown of PHB occurred at the same time. It seems probable that the autolytic process occurring during this stage was equivalent to the autolysis of B.megaterium observed by Lemoigne (1926b).

Inspection of the curves showing the behaviour of the intracellular polysaccharide (graph 11) shows that they are largely similar to those for PHB. The type (a) peak is not so apparent since there is no measurement of polysaccharide before 12 hours. Nevertheless, since the inoculum was a 16 hour culture on the control medium, its polysaccharide content must have been less than that at 12 hours, and the presence of the type (a) peak is inferred. The type (b) peak is less marked in this example and peak (c) hardly apparent. There is a fairly steady decline in the total polysaccharide level during the stationary phase, but it levels off during the phase of decline and in consequence the polysaccharide/N curve rises sharply. This again illustrates

the sometimes misleading nature of the "ratio" curves (i.e. the PHB/N and polysaccharide/N curves) during the phases of growth and decline, since without reference to the curve of total polysaccharide, one might have been persuaded that polysaccharide was being synthesised during the stage of decline.

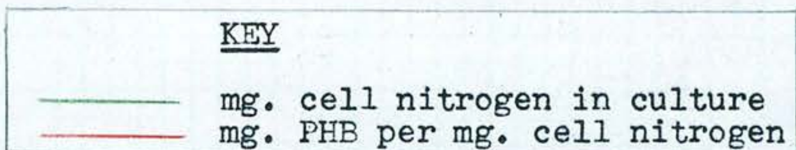
As has been pointed out, volutin was measured only by microscopic examination of stained smears, and the course of volutin production has not been presented graphically but is recorded in the appropriate tables. From table 26, it will be seen that as in the case of PHB, volutin is formed fairly abundantly during the period of active growth on the control medium, but disappears soon after the cells enter the stationary phase, and the level thereafter is low.

Some indication of the normal degree of variability encountered in these cultural experiments is given by the curves on graph 13 (p. 240). The total nitrogen and PHB/N curves obtained with several cultures on the glucose limited control medium are shown (the data for these curves were abstracted from the experiments on nutrient deficiencies reported later, and the figures can be found in the appropriate tables). It will be seen that although there was a certain amount of variability, the curves are basically similar, and their general reproducibility is not doubted.

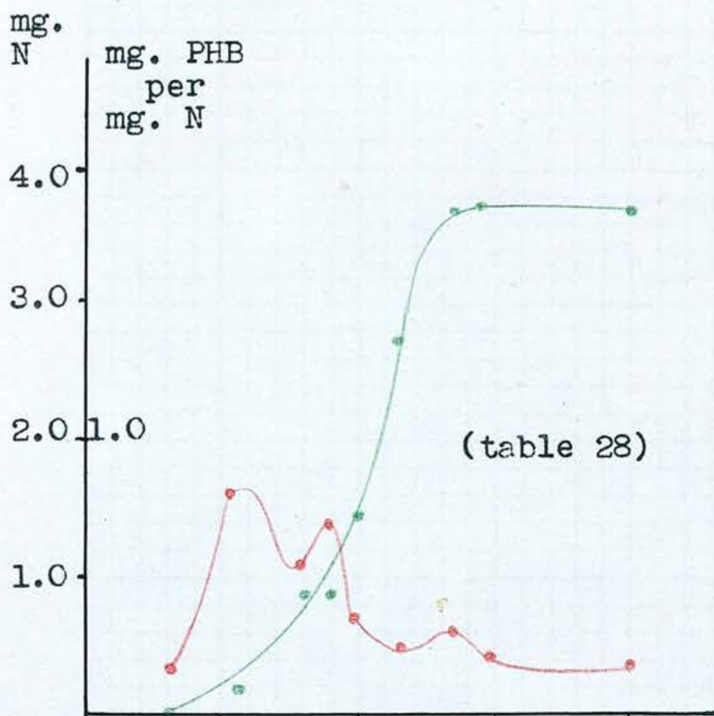
In summary, moderate amounts of PHB, volutin and intracellular polysaccharide appeared in the cells on this glucose limited "control" medium during the phase of active

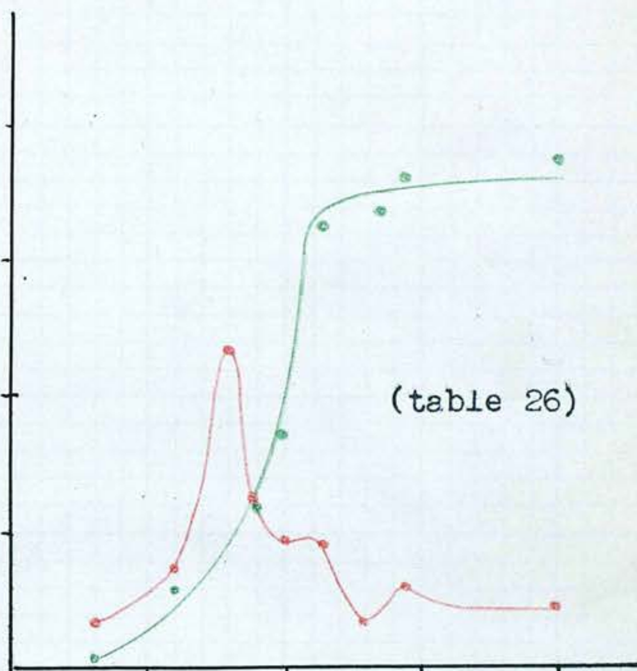
GRAPH 13.

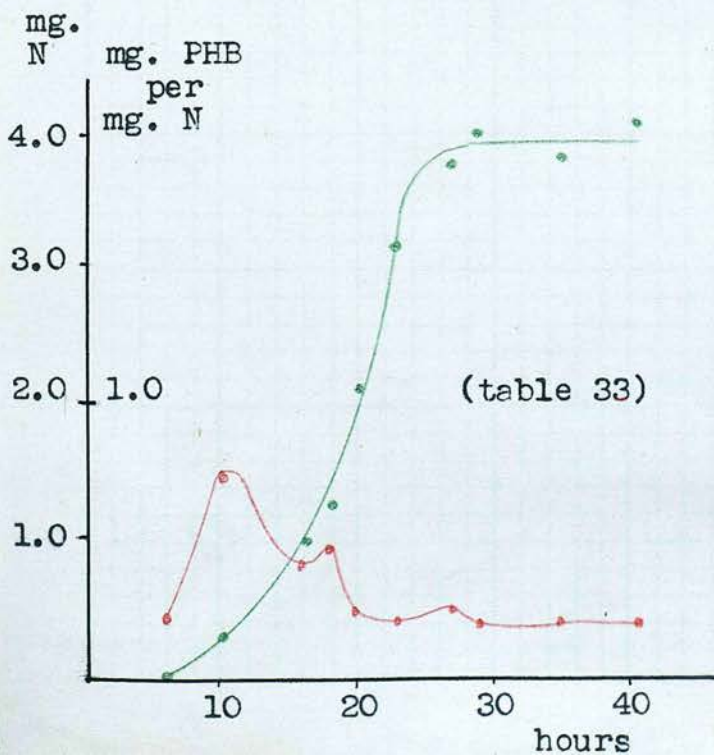
Growth and formation of PHB per unit nitrogen(i.e. per cell) of B.cereus (AC.1) in four different cultures on the glucose-limited "control" medium.

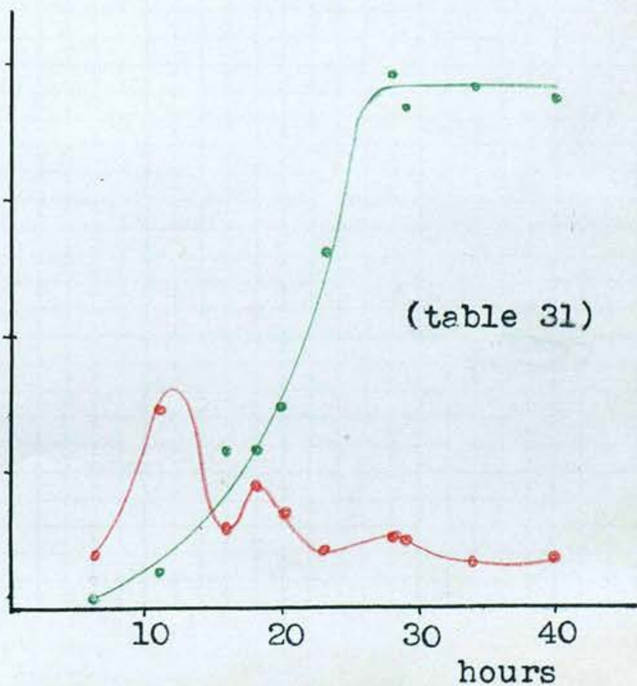


Note: the data for each set of curves can be found in the tables indicated.









growth. As the stationary phase was entered, however, the amounts of these materials in the cells decreased to a steady minimal level which was maintained throughout the stationary phase. Finally, as the phase of decline was entered, there was a drop in the total nitrogen accompanied by an equivalent drop in the total PHB, so that the level of PHB/N remained constant. The level of total polysaccharide remained constant during the phase of decline, however, resulting in an apparent increase in the amount of polysaccharide per cell. Volutin was absent at this stage.

As has been mentioned, the minimal cell contents of PHB, volutin and polysaccharide attained during the stationary phase on this medium did in fact represent the "élement constant" levels of these materials, since it will be shown that they were not reduced by cutting the glucose content of the medium below 0.3%. The values were; for PHB, 0.2 mg per mg N, or approximately 2.0% of the dry weight of the cell;** for polysaccharide, 1.0 mg per mg N, or approximately 10% of the dry weight of the cell; for volutin a "trace".

** bearing in mind the error in estimates of PHB by the hypochlorite method at low levels of PHB, this figure is probably too high, but throughout this series of experiments, this error has been neglected.

(b) The influence of glucose concentration.

The following levels of glucose in the medium were tested: 0.1%, 0.3% (the "control" level), 1.0%, and 3.0%. It has already been shown (p.228, and graph 10) that growth on the first two of these media was limited by exhaustion of the carbon and energy source, glucose. On the 1% and 3% media, on the other hand, the glucose was in excess of the amount required for the maximal growth (in terms of nitrogen) supported by the media, since on both these media, the total growth was less than, and not proportional to the glucose concentrations. The actual growth curves obtained are shown in graph 14, p. 243.

It has already been mentioned that the curves obtained with these media containing different amounts of glucose showed some degree of variability. This has been indicated on graph 15 by the inclusion of the PHB/N curves obtained for the same media on different occasions. It will be seen that during the phase of active growth, the curves for PHB/N on all four concentrations of glucose displayed fluctuations closely similar to those seen on the control medium. At about the middle of the phase of active growth, however, on all the glucose excess media, the levels of PHB/N rose sharply and continued to rise well into the stationary phase. It was at this point on these glucose rich media that the major differences between different cultures on the same media were elicited. In general, one of two courses was followed; either the PHB/N rose to a sharp peak during the stationary phase

TABLE 26. The Influence of glucose concentration on growth and formation of PHB, polysaccharide and volutin by B. cereus (AC.1).

Age of culture (hrs).	0.3% glucose. (glucose limited "control" medium).										3.0% glucose (glucose-rich).										
	pH	Turbidity of culture (in 50 ml).	Final turbidity after hypochlorite treatment. Ø	Total PHB (mg)	Total cell nitrogen (mg)	mg PHB per mg cell nitrogen.	Total polysaccharide (mg).	mg polysaccharide per mg cell nitrogen.	Volutin (Albert's stain).	Microscopic estimation of lipid inclusions (Sudan black)	pH	Turbidity of culture (in 50 ml.)	Final turbidity after hypochlorite treatment. Ø	Total PHB (mg)	Total cell nitrogen (mg)	mg PHB per mg cell nitrogen	TOtal polysaccharide (mg)	mg polysaccharide per mg cell nitrogen.	Volutin (Albert's stain)	Microscopic estimation of lipid inclusions (Sudan black)	mg PHB per mg cell nitrogen PHB/N. *
6*	7.3	0.040		0.02	0.09	0.18			±	±	7.3	0.021		0.01	0.05	0.18			±	±	
12	7.3	0.262	0.011	0.22	0.58	0.37	1.25	2.09	±	+±	7.1	0.117	0.007	0.13	0.20	0.64	0.31	1.57	±	+±	
16	7.2	0.345	0.036	0.71	0.60	1.19	1.09	1.90	±	+	7.0	0.525	0.011	0.22	0.55	0.40	1.20	2.21	±	+	
18	7.2	0.481	0.038	0.74	1.20	0.62	1.74	1.45	+	+	7.0										
20	7.2	1.320	0.065	1.26	2.69	0.47	3.70	1.38	+	+	6.8	1.304	0.018	0.35	0.95	0.37	1.90	2.03	±	+	
23	7.2	1.550	0.080	1.48	3.21	0.46	4.46	1.39	+±	+	6.7	1.440	0.020	0.41	1.25	0.33	1.80	1.44	+	++	
26	7.2	1.656	0.031	0.60	3.36	0.18	4.41	1.31	+±	+±	6.7	1.932	0.026	0.62	2.28	0.27			+±	++±	
29	7.2	1.640	0.055	1.08	3.60	0.30	3.56	0.99	+	+	6.6	2.100	0.056	1.22	3.60	0.34	6.81	1.89	++	++±	
35	7.2	1.680							+	±	6.6	2.200	0.158	3.30	4.74	0.70	8.59	1.82	++±	+++	0.93
40	7.1	1.580	0.043	0.85	3.71	0.23	3.50	0.94	±	±	6.2	3.240	0.375	7.70	5.80	1.33	10.4	1.77	++±	+++	1.94
44											5.9	3.232	0.441	9.00	4.45	2.02	8.30	1.87	++±	+++	2.72
48	7.1	1.730	0.041	0.72	3.80	0.19	3.46	0.91	-	±	5.5	3.360	0.720	15.10	3.07	3.02	8.21	1.63	++±	+++	2.92
65	7.1	1.541	0.026	0.50	3.11	0.16	3.53	1.14	-	±											1.70
73											5.5	3.840	0.621	12.8	4.86	2.64	6.90	1.42	++	+++	1.02
95	7.1	0.884	0.021	0.40	1.91	0.21	3.32	1.74	±	±											0.60
115											5.4	3.912	0.660	13.8	4.28	3.23	7.62	1.77	+	+++	

Ø Figures for "final turbidity" have been calculated from experimental readings on actual "working suspensions" (see p.73) to correspond with initial turbidities of the cultures; they are directly proportional to the figures for "total PHB".

Note: The heavy horizontal line denotes the stage at which the stationary phase was entered.

* 6hr. estimate of growth made from turbidities; 6 hr. PHB contents estimated from Sudan black staining.

* Data from culture on same medium made on a different occasion.

TABLE 26. (contd.). The Influence of glucose concentration on growth and formation of PHB, polysaccharide and volutin by B. cereus (AC.1)

	0.1% glucose (glucose-deficient)									
Age of culture (hrs).	pH	Turbidity of culture (in 50 ml).	Final turbidity after hypochlorite treatment. ∅	Total PHB (mg).	Total cell Nitrogen (mg)	mg PHB per mg cell nitrogen.	Total polysaccharide (mg).	mg polysaccharide per mg cell nitrogen.	Volutin (Albert's stain).	Microscopic estimation of lipid inclusion (Sudan black)
6*	7.2	0.043		0.03	0.15	0.18			-	±
12	7.2	0.261	0.010	0.19	0.97	0.20	1.24	1.29	±	+±
16	7.2	0.045	0.018	0.32	1.54	0.21	2.23	1.45	±	++
18	7.2	0.510	0.023	0.41	1.73	0.24	1.51	0.88	+	+
20	7.2	0.539	0.019	0.35	1.96	0.18	1.39	0.71	+±	±
23	7.1	0.603	0.021	0.38	1.99	0.19	1.41	0.71	+	±
26	7.1	0.584	0.024	0.44	2.03	0.22	1.20	0.60	±	±
29	7.1	0.545	0.023	0.41	1.80	0.23	1.25	0.70	±	±
35	7.1	0.493	0.021	0.37	2.00	0.19	1.69	0.85	-	±
40	7.1	0.485	0.023	0.41	1.63	0.25	1.55	0.95	-	+
44	7.1	0.457	0.022	0.40	1.61	0.25	1.75	1.09	-	±
48	7.1	0.445	0.014	0.25	1.21	0.20	1.39	1.15	-	±
65	7.1	0.291	0.009	0.18	0.85	0.21	1.17	1.39	-	±
95									-	±

	1.0% glucose (glucose-rich)										
Age of culture (hrs).	pH	Turbidity of culture (in 50 ml).	Final turbidity after hypochlorite treatment. ∅	Total PHB (mg).	Total cell nitrogen (mg)	mg PHB per mg cell nitrogen.	Total polysaccharide (mg).	mg polysaccharide per mg cell nitrogen.	Volutin (Albert's stain).	Microscopic estimation of lipid inclusion (Sudan black)	mg PHB per mg cell nitrogen. ⁕
6*	7.2	0.041		0.017	0.11	0.15			±	±	
12	7.2	0.317	0.010	0.21	0.93	0.22	1.20	1.28	±	+	0.21
16	7.2	0.454	0.021	0.40	1.33	0.30	2.10	1.58	+	+±	0.30
18	7.2	0.594	0.019	0.36	1.49	0.24	2.20	1.44	+±	+	0.24
20	7.1	0.800	0.025	0.49	2.16	0.23	2.60	1.21	++	+	0.23
23	7.0	1.488	0.048	0.90	4.52	0.20	6.20	1.38	++	+	0.21
26	6.9	1.800	0.056	1.14	5.41	0.21	7.10	1.31	++±	+	0.22
29	6.9										
35	6.8	3.552	0.180	3.40	7.80	0.44	15.6	2.00	++±	++	3.20
40	6.7	3.588	0.710	13.7	7.75	1.77	10.3	1.33	++±	+++	2.72
44	6.8	3.588	0.710	13.6	7.61	1.79	9.8	1.28	++±	+++	1.76
48	6.8					1.64		1.09	++	+++	
65	6.8	3.496			7.25		7.5	1.04	+±	+++	0.59
95	6.6	2.431	0.420	8.02	6.38	1.25	7.7	1.24	+	+++	

∅ Figures for "final turbidity" have been calculated from experimental readings on actual "working suspension" (see p.73) to correspond with initial turbidities of the cultures; they are directly proportional to the figures for "total PHB".

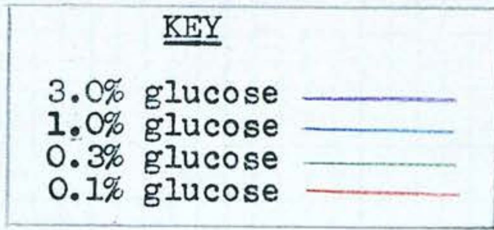
Note: The heavy horizontal line denotes the stage at which the stationary phase was entered.

* 6 hr. estimate of growth made from turbidities; 6 hr. PHB contents estimated from Sudan black staining.

⁕ Data from culture on same medium made on a different occasion.

GRAPH 14.

Growth curves of B.cereus (AC.1) on synthetic media containing different amounts of glucose.



mg. cell nitrogen

cultures enter stationary phase

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0

-25 -15 -5 0 +5 +15 +25 +35 +45 +55 +65

hours

mg. PHB per
mg. cell N

←cultures enter stationary phase

3.0

mg.PHB/mg.N

2.0

1.0

mg.
PHB

14

Total mg. PHB
in culture

12

10

8

GRAPH 15;B.cereus(AC.1)
PHB production on media
containing diff. amounts
of glucose

6

KEY for both sets:

3.0% glucose
1.0% glucose
0.3% glucose
0.1% glucose

(broken lines indicate alternative pathways sometimes found)

4

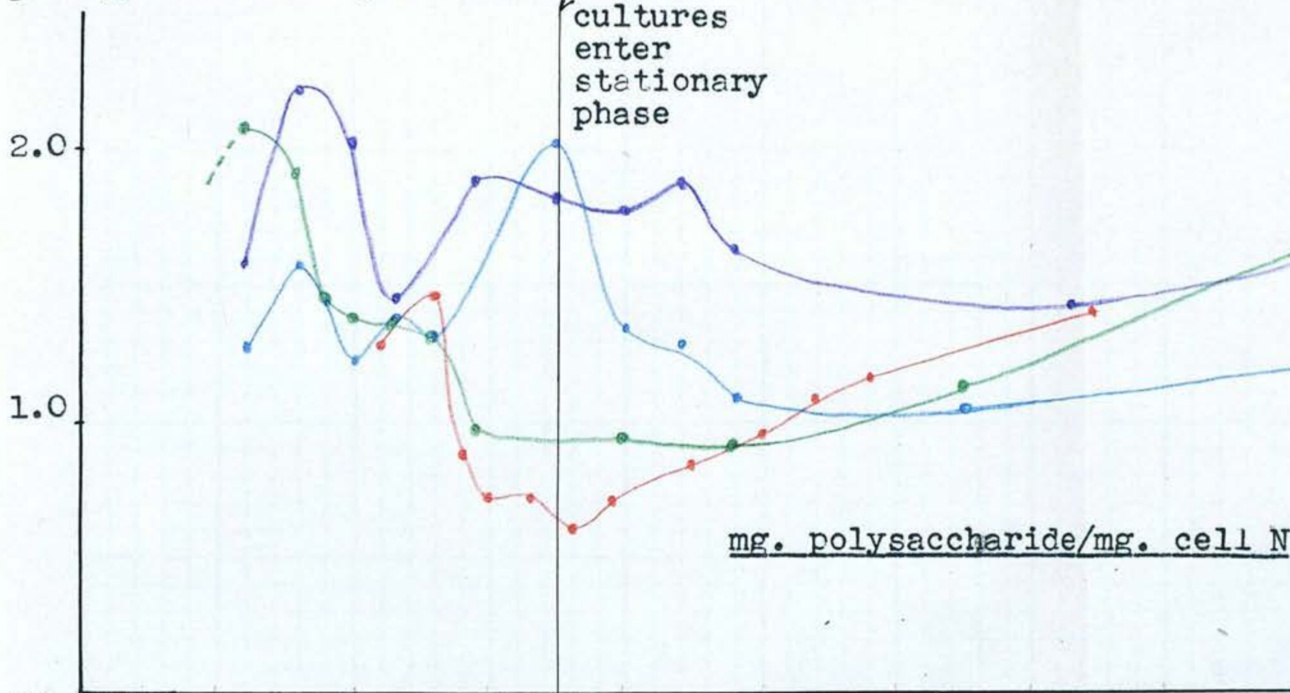
2

-25 -15 -5 0 +5 +15 +25 +35 +45 +55 +65

hours

GRAPH 16

The production of intracellular polysaccharide by B.cereus(AC.1) growing on synthetic media containing different amounts of glucose



mg. poly in | total saccharide in culture

KEY for both sets of curves:

3.0% glucose
1.0% glucose
0.3% glucose
0.1% glucose

14
12
10
8
6
4
2

Total mg. polysaccharide

-25 -15 -5 0 +5 +15 +25 +35 +45 +55 +65

hours

and fell away again almost as rapidly, finally reaching a value not much above that of the control, or after declining slightly from the peak, the PHB/N tended to level off and even to show a slight increase towards the final stages of culture. The cause of this variability is not understood. A clue to it may perhaps be found in the fact that on these glucose rich media, the factor limiting the total growth was not known. Growth was probably limited either by exhaustion of an unknown mineral, perhaps supplied by the agar, or by the accumulation of inhibitory waste products. There is some evidence that the latter factor may have been operative on the 3% glucose medium, since it was seen in graph 10 that the total growth on this medium, though higher than that of the control, was lower than that obtained on the 1% medium. It is not known however, if growth on the 1% medium was also limited by accumulation of toxic products. It is possible that on this medium, growth was halted by exhaustion of a mineral. Whatever the cause of this variability, it was clearly not under experimental control and it is perhaps significant that it was not encountered on any of the other experimental media, on all of which, the factors limiting growth were known.

Despite this variability in response, the main conclusion is clear, namely that an excess of glucose in the medium, over and above that required for the maximal growth supported by the medium (the growth limiting factor being unknown) led to an increased content of PHB in the

cells of the stationary phase. It is important to stress that this result does not indicate that the addition of an "excess" of glucose (in the sense indicated above) to any medium would result in an increased production of PHB by this organism. It was pointed out that the factors limiting growth on the glucose rich media above were not known. Now, it will later be shown that growth limiting deficiencies of certain nutrients (e.g. the nitrogen source, ammonium chloride) did not allow an increased production of PHB, even though, by halting growth before all the carbon source in the medium was exhausted, they created a relative "excess" of glucose. Thus if the addition of glucose to a concentration of 1% to the above medium had resulted in exhaustion of the nitrogen source becoming the growth limiting factor,** there would have been no increased production of PHB. It is therefore only permissible to conclude from the above experiment that an increase in the glucose concentration to 1% or more in this particular medium resulted in growth being limited by an unknown factor, and this factor was one that permitted a greatly increased production of PHB from the resulting "excess" glucose.

The results for PHB/N on the two glucose limited media provide a good illustration of another fallacy that should be avoided in work of this type. It was seen (graph 15) that reduction of the glucose content of the medium from the growth limiting level of 0.3%, to 0.1%,

** evidence will be presented later that nitrogen definitely was not the growth limiting factor on either the 1% or 3% glucose media.

resulted in no drop in the PHB/N level of cells in the stationary phase. This finding was an indication of the validity of Belins' concept of the "élèment constant" (Belin 1926) that was discussed in the Introduction. The importance of the actual existence of an "élèment constant" for PHB, which cannot be reduced below a certain minimum value by reducing the level of glucose below the growth limiting concentration of 0.3%, is that if the effect of concentration of glucose had been judged simply by examination of the stationary phase cells on the two glucose limited media, it would have been concluded that the level of glucose in the medium was without effect on the cell content of PHB, a conclusion that is obviously erroneous in view of the results obtained on the glucose rich media. It cannot be too strongly emphasised that to be meaningful, data of the type recorded here must be related to the level of total growth obtained on the media, and unless the factor limiting total growth is known, the conclusions drawn from such experiments are necessarily limited.

Turning to the curves showing the fluctuations in intracellular polysaccharide (graph 16 p.245), it will be seen that in the glucose rich cultures, the peak levels of polysaccharide/N in the stationary phase were about twice as great as those of the glucose limited media. From the curves of total polysaccharide production it will be seen that practically all this increased production of polysaccharide occurred before the end of the phase of

active growth. It will also be seen that in all four media the total level of polysaccharide remained constant during the phase of decline.

It was concluded that the cell content of intracellular polysaccharide in the stationary phase cells was greatly increased by a concentration of glucose in this medium in excess of that required for the maximum growth supported by the medium. This conclusion was of course subject to the reservations outlined above, due to lack of knowledge of the factor limiting total growth. It was also apparent that the level of polysaccharide/N in the stationary phase cells was not significantly increased by an increase in glucose concentration from 1% to 3%. It is thought therefore, that the total production of polysaccharide was finally limited by some factor. It seems unlikely, at least on the 3% glucose medium, that this limit to the total production of polysaccharide was due to exhaustion of the carbon source, and some other factor was presumably responsible. The nature of this factor was not determined.

The level of volutin in the cells (table 26) was also increased by the presence of excess glucose in the medium. This finding agreed with the observation of Smith _et al_ (1956) of an energy requirement for volutin production.

(c) The effect of concentration of nitrogen.

The following concentrations of ammonium chloride were tested : 0.15% (the control medium), 0.06%, 0.025%, 0.01%. The results are plotted in graphs 17 & 18, pp.251/2 (tables 27 & 28).

From table 27, it will be seen that reduction of the concentration of ammonium chloride from 0.025% to 0.01% was accompanied by a proportionate decrease in the total growth. An increase in the ammonium chloride concentration, on the other hand, from 0.025% to 0.06% resulted in a slightly increased growth, but the increase in total growth was not proportional to the increased concentration of the nutrient, and the levels obtained on both the more concentrated media were roughly the same, and were in fact at the level obtained on the glucose limited "control" media. Thus on the 0.025% and 0.01% media, the total growth was limited by exhaustion of the nitrogen source, ammonium chloride, and on the two more concentrated media, the growth was limited by exhaustion of glucose, and more nitrogen source was available than was required for the total utilisation of the available glucose.

It will be seen that during the period of active growth, the total production of PHB on all concentrations of ammonium chloride followed approximately the course already seen in the control. As a consequence, the usual fluctuations appeared in the PHB/N curve. The most surprising feature of this set of curves is the fact that when the stationary phase was reached, the PHB/N in the N-

TABLE 27.

Concentration of ammonium chloride. (W/V).	Total growth (Stationary phase average mg nitrogen).	mg PHB per mg cell N.(average stationary phase value).	mg polysaccharide per mg cell N.(peak stationary phase value).	Volutin (Albert's stain) peak stationary phase value).
0.01%	1.0 mg	0.25	2.52	++±
0.025%	2.8 mg	0.26	1.71	±
0.06%	3.7 mg	0.15	1.00	-
0.15%	3.8 mg	0.20	1.00	±

The influence of concentration of nitrogen source (NH_4Cl) on total growth and stationary phase cell contents of PHB, intracellular polysaccharide and volutin, of B.cereus(AC.1).

TABLE 28. The Effect of nitrogen deficiency on the growth and production of poly β-hydroxybutyrate(PHB) and intracellular polysaccharide by strain AC.1 of B.cereus.

Age of culture (hrs).	0.15% NH_4Cl. (glucose limited "control")										0.025% NH_4Cl. (N-deficient).									
	pH	Turbidity (in50 ml).	Final turbidity after hypochlorite treatment. Ø	Total PHB (mg)	Total cell N in culture (mg)	mg PHB per mg N (PHB/N)	Polysaccharide (mg) per unit nitrogen(mg)	Total poly-saccharide (mg)	Sudan black estimation of lipid inclusions	Volutin Albert's stain	pH	Turbidity (in 50 ml).	Final turbidity after hypochlorite treatment. Ø	Total PHB (mg)	Total cell N in culture (mg)	mg PHB per mg N (PHB/N)	Polysaccharide (mg) per unit nitrogen(mg)	Total poly-saccharide (mg)	Sudan black estimation of lipid inclusions	Volutin Albert's stain
6*	7.2	0.006		0.003	0.015	0.18			±	±	7.2	0.006		0.003	0.015	0.18			±	±
11	7.2	0.110	0.008	0.15	0.19	0.81	2.49	0.47	++±	±	7.2	0.108	0.005	0.09	0.19	0.48	2.33	0.43	++±	-
16	7.2	0.378	0.025	0.48	0.90	0.54	1.78	1.58	++	±	7.2	0.330	0.016	0.30	0.78	0.39	1.48	1.14	+±	±
18	7.2	0.411	0.033	0.62	0.89	0.70	1.73	1.53	++±	+	7.2	0.348	0.023	0.42	0.74	0.56	1.50	1.10	++	+±
20	7.2	0.608	0.027	0.50	1.43	0.35	1.28	1.82	+	+	7.2	0.441	0.018	0.34	1.08	0.32	1.24	1.34	++	+
23	7.2	1.112	0.037	0.68	2.74	0.25	1.19	3.26	+	+	7.2	0.563	0.023	0.42	1.41	0.30	1.13	1.59	+	+
27	7.2	1.400	0.058	1.08	3.66	0.30	1.10	4.06	+	+	7.2	0.876	0.023	0.41	1.77	0.26	1.33	2.35	+	++
29	7.2	1.468	0.044	0.80	3.73	0.21	1.10	4.10	++	±	7.2	1.182	0.040	0.75	2.85	0.26	1.71	4.87	+	+
40	7.2	1.584	0.035	0.64	3.64	0.18	1.00	3.64	±	±	7.2	1.180	0.041	0.87	2.69	0.30	1.71	4.60	++	±
41																				
48	7.2	1.490	0.039	0.72	3.68	0.20	0.94	3.45	±	-	7.2	1.188	0.034	0.62	2.81	0.22	0.99	2.78	±	±
65	7.2	1.006	0.029	0.53	2.25	0.24	1.30	2.92	±	-	7.2	1.140	0.035	0.64	2.60	0.24	0.82	2.13	±	±
88	7.2	0.776	0.021	0.40	1.69	0.24	1.71	2.88	+	-	7.2	1.016	0.033	0.61	2.33	0.26	0.82	1.92	±	±

Ø Figures for "final turbidity" have been calculated from experimental readings on actual "working suspension" (see p.73) to correspond with initial turbidities of the cultures; they are directly proportional to the figures for "total PHB".

Note: The heavy horizontal line denotes the stage at which the stationary phase was entered.

* 6 hour estimations of total cell N approximated from culture turbidities; 6 hour PHB/N and Total PHB approximated from Sudan Black estimations of lipid inclusions.

TABLE 28(Contd.). The Effect of nitrogen deficiency on the growth and production of poly β-hydroxybutyrate(PHB) and intracellular polysaccharide by strain AC.1 of B.cereus.

Age of culture (hrs).	(glucose limited) 0.06% NH_4Cl										0.01% NH_4Cl (N-deficient).									
	pH	Turbidity (in 50 ml).	Final turbidity after hypo-chlorite treat-ment. ∅	Total PHB (mg)	Total cell N in culture (mg)	mg PHB per mg N. (PHB/N)	Polysaccharide per unit nitrogen	Total poly-saccharide (mg).	Sudan black estimation of lipid inclusions	Volutin Albert's stain	pH	Turbidity (in 50 ml).	Final turbidity after hypo-chlorite treat-ment. ∅	Total PHB (mg)	Total cell N in culture (mg)	mg PHB per mg N. (PHB/N)	Polysaccharide per unit nitrogen	Total poly-saccharide (mg)	Sudan black estimation of lipid inclusions	Volutin Albert's stain
6*	7.3	0.021		0.009	0.052	0.18			±	-	7.3	0.016		0.007	0.04	0.18			±	-
11	7.3	0.134	0.007	0.13	0.22	0.58	2.14	0.48	++	±	7.3	0.106	0.004	0.07	0.22	0.32	1.78	0.39	++±	±
16	7.3	0.470	0.011	0.20	1.17	0.17	1.60	1.87	+±	+	7.3	0.375	0.008	0.14	0.92	0.15	1.82	1.66	+±	+
18	7.3	0.539	0.014	0.24	1.18	0.21	1.83	2.16	+±	+	7.3	0.467	0.010	0.20	1.00	0.20	2.80	2.80	+±	+
20	7.3	0.712	0.018	0.33	1.55	0.22	1.63	2.53	+±	+±	7.3	0.485	0.008	0.15	1.09	0.14	1.98	2.16	±	+
23	7.3	1.044	0.022	0.40	2.35	0.17	1.32	3.10	+	++	7.3	0.534	0.009	0.17	1.02	0.16	2.48	2.53	±	+
27	7.3	1.486	0.033	0.60	3.33	0.18	1.25	4.16	+	+±	7.3	0.538	0.013	0.24	1.00	0.24	2.52	2.51	±	+±
29	7.2	1.584	0.029	0.53	3.74	0.14	1.11	4.15	±	±	7.3	0.561	0.013	0.25	1.05	0.24	2.32	2.45	±	+
40																				
41	7.2	1.574	0.027	0.49	3.73	0.13	1.00	3.73	±	-	7.2	0.426	0.012	0.21	0.95	0.23	1.09	1.03	±	++
48	7.2	1.350	0.026	0.48	3.80	0.13	0.75	2.85	±	-	7.2	0.467	0.015	0.28	1.10	0.25	0.96	1.05	+	++±
65	7.2	0.912	0.019	0.35	2.07	0.17	1.30	2.68	±	-	7.2	0.395	0.011	0.20	0.97	0.21	0.78	0.75	+	+
88	7.2	0.418	0.010	0.18	1.14	0.16	1.30	1.48	+	-	7.2	0.424	0.013	0.24	1.09	0.21	0.71	0.77	+	±

∅ Figures for "final turbidity" have been calculated from experimental readings on actual "working suspension"(see p.73) to correspond with initial turbidities of the cultures; they are directly proportional to the figures for "total PHB".

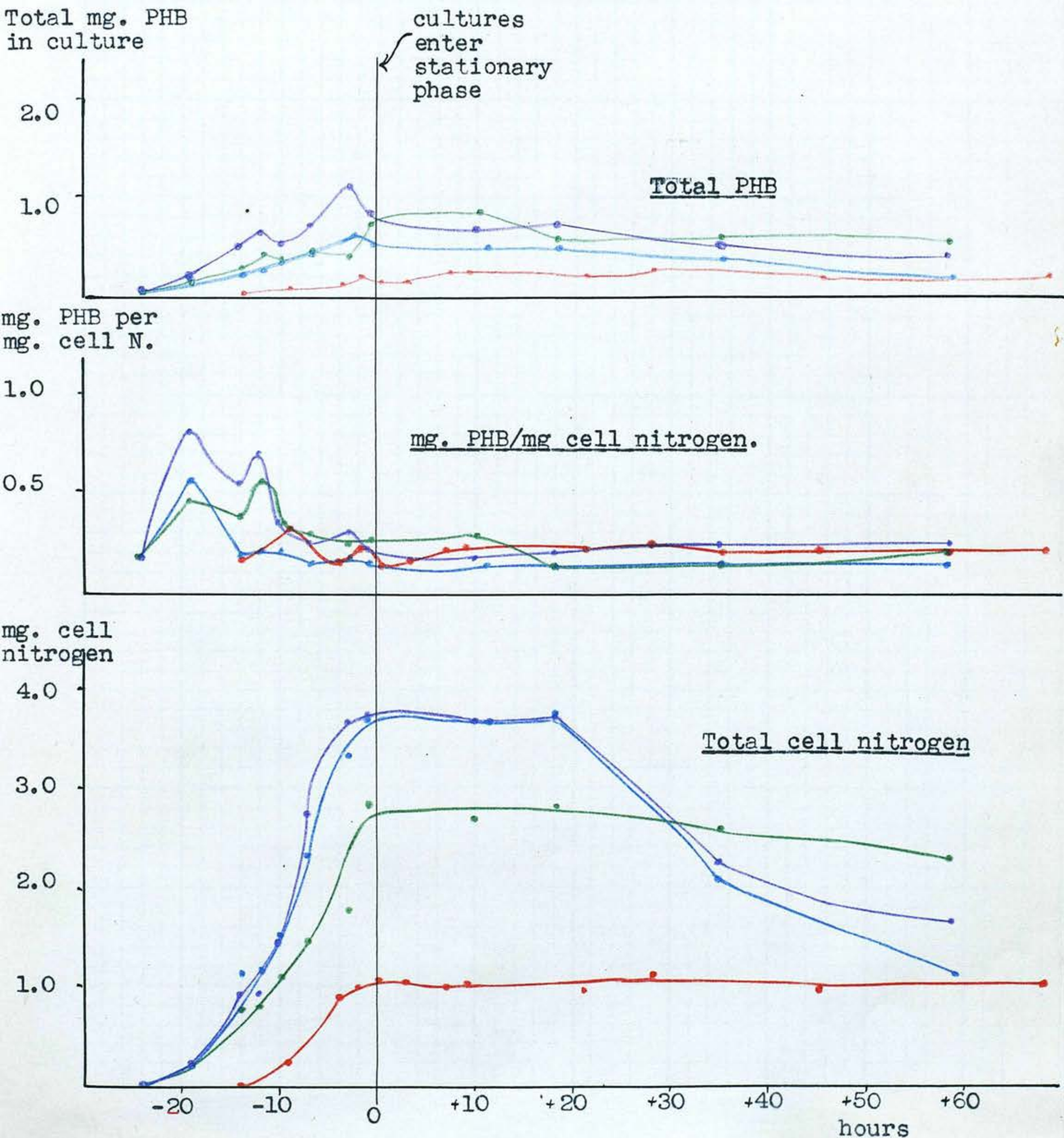
Note: the heavy horizontal line denotes the stage at which the stationary phase was entered.

* 6 hour estimations of total cell N approximated from culture turbidities; 6 hour PHB/N and Total PHB approximated from Sudan Black estimations of lipid inclusions.

GRAPH 17.

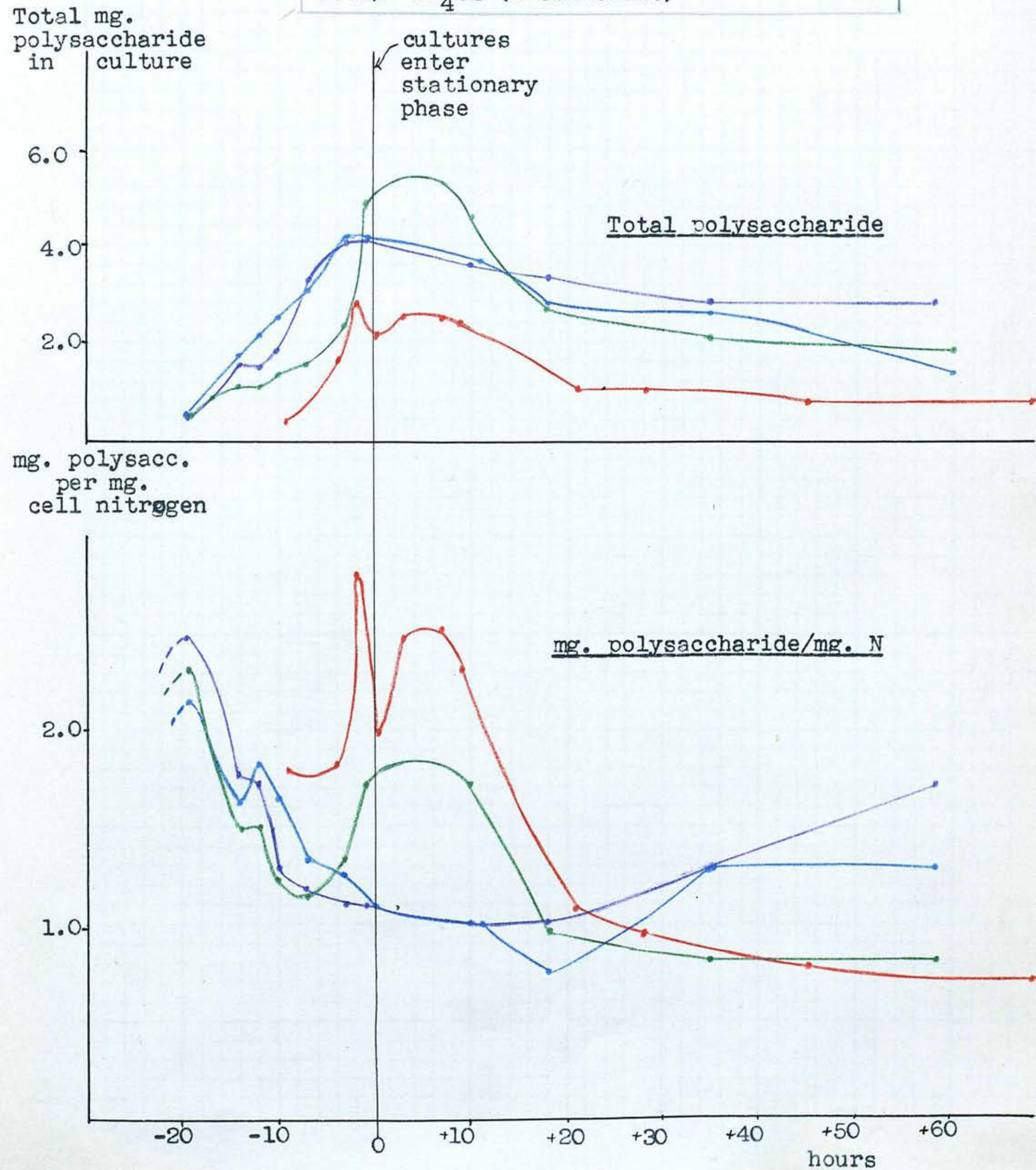
Growth and production of PHB by B.cereus (AC.1) growing on synthetic media containing different amounts of nitrogen source (NH_4Cl).

KEY for all sets of curves:	
0.15% NH_4Cl (glucose limited)	purple line
0.06% NH_4Cl (glucose limited)	blue line
0.025% NH_4Cl (N deficient)	green line
0.01% NH_4Cl (N deficient)	red line



GRAPH 18.

Production of intracellular polysaccharide by B.cereus (AC.1) growing on synthetic media containing different amounts of nitrogen source (NH_4Cl).



limited cultures settled down to the steady minimal level of the controls. This was quite contrary to expectations, for it is a common observation that lifeless carbon materials such as lipids and polysaccharides are laid down in greatly increased amounts when growth is limited by deficiency of nitrogen. One must conclude that nitrogen is necessary for the formation of PHB. Since nitrogen is not a constituent of PHB, it would appear that this requirement may be related to the formation or proper functioning of the enzymes needed for its synthesis. One difficulty to be explained is the reason why the enzyme that was already present during the phase of growth did not continue to yield more PHB once growth stopped. This observation is more profitably discussed later.

With the exception of the 0.01% NH_4Cl culture, the polysaccharide/N curves followed the usual pattern during the phase of growth. The reason for the unusually high peak at the end of the period of growth on the 0.01% NH_4Cl medium is not clear, but it should be remembered that the growth on this medium was very low, and perhaps subject to errors due to the difficulties of handling such small samples. Nevertheless the PHB/N curve of this medium during the stationary phase conformed very closely with that of the other N-limited medium. It will be seen that on both media the polysaccharide/N rose sharply to a peak and almost as rapidly fell away again to the "élèment constant" level, which was maintained through the phase of decline, since no cell autolysis occurred and the total

amount of polysaccharide remained constant. This should be contrasted with the steady low level of polysaccharide/N during the stationary phase, and the rising level during the phase of decline, that occurred on the control media. In none of the media, however, was polysaccharide synthesised or broken down during the phase of decline.

Inspection of the fluctuations in volutin (table 28) reveals that on three of the media, this material was formed in moderate amounts during the growth phase, but disappeared during the stationary phase. On the most highly N-deficient medium, however, the volutin level was maintained during the stationary phase and even increased somewhat during the later stages of culture, finally declining to a low level. This latter finding agrees with the observation of Smith, Wilkinson and Duguid (1954) that volutin was produced by Aero. aerogenes in the later stages of culture on an N-deficient medium.

The finding that limitation of growth by exhaustion of the nitrogen source did not induce the formation of increased cell contents of PHB, despite the relative excess of glucose, underlined the importance of knowledge of the factor limiting growth as discussed in the previous section. The factor limiting growth on the 1% glucose medium described previously could not have been exhaustion of the nitrogen source, since the results of the present experiment showed that had this been the case, there would have been no increased production of PHB. It was thought desirable, however, to obtain direct proof of this

contention. For this purpose, media with the following concentrations of ammonium chloride and glucose were set up: (a) 1% glucose, 0.3% NH_4Cl; (b) 1.0% glucose, 0.15% NH_4Cl; and (c) 1% glucose, 0.03% NH_4Cl. Medium (b) was the normal 1% glucose medium, as used before. Medium (a) differed from it only by having an increased concentration of ammonium chloride, and medium (c) had a greatly decreased concentration of ammonium chloride. These media were inoculated and incubated with strain AC.1 in the usual manner. The results are shown in table 29.

TABLE 29.

Medium	Composition	Total growth (mg N)	Peak PHB/N in stationary phase cells. (mg PHB/mgN)
(a)	1.0% glucose 0.3% NH_4Cl	10.0	1.38
(b)	1.0% glucose 0.15. NH_4Cl	10.1	1.29
(c)	1.0% glucose 0.03% NH_4Cl	3.6	0.18

It will be seen that increasing the ammonium chloride concentration of the 1% glucose medium (b) from 0.15% to 0.3% did not result in an increased growth, or in any significant change in the PHB/N. On reducing the ammonium chloride content of medium (b) to 0.03%, on the other hand, there was a marked reduction in growth, but the reduction was not proportional to the decrease in the NH_4Cl content of the medium. Thus it was clear that

growth on the 1% glucose medium (b) was not limited by exhaustion of the nitrogen source, but this limitation was operative on medium (c), and therefore, the stationary phase cells on the latter medium contained only minimal amounts of PHB, despite the relative excess of glucose that must have been present. This finding further emphasised the importance of knowledge of the factor limiting the total growth, as discussed previously.

(d) The effect of concentration of potassium.

The effect of potassium concentration was studied by the use of media having the following concentrations of added KCl: 0.02% (the glucose limited control medium), 0.006%, 0.002% and 0.00%. It should be pointed out, however, that these concentrations did not accurately represent the true K^+ concentrations in the media, since the level of K^+ contamination from the agar was significantly high. From the amounts of growth supported by the two less concentrated media on which (see below) growth was limited by exhaustion of K^+, it was calculated that the amount of K^+ contributed by the agar was equivalent to circa 0.002% KCl. The true levels of K^+ in the media were therefore taken as equivalent to 0.022%, 0.008%, 0.004% and 0.002% KCl. Table 30 shows the total growths obtained on the four different media. It will be seen that reduction of the KCl concentration from 0.004% to 0.002% resulted in a proportionate decrease in growth. An increase in the KCl concentration, on the other hand, from 0.004% to 0.008% or more, resulted in a slight increase

TABLE 30.

Concentration of KCl(W/V)	Total growth (stationary phase average:mg N).	mg PHB per mg cell N. (stationary phase average).	Volutin(Albert's stain; stationary phase average).
0.002%	1.5mg	3.9	±
0.004%	3.80mg	0.55	±
0.008%	3.85mg	0.15	±
0.022%	3.85mg	0.17	±

The influence of concentration of KCl on the total growth and stationary phase cell contents of PHB and volutin in *B. cereus*. (AC.1).

TABLE 31. The Influence of KCl concentration on growth and formation of PHB, polysaccharide and volutin by B. cereus(A^C.1).

Age of culture (hrs).	0.022% KCl (glucose limited "control")								0.004% KCl (K^+ deficient).								
	pH	Turbidity of culture (in 50 ml).	Final turbidity of culture after hypochlorite treatment. Ø	Total PHB in culture (mg)	Total cell nitrogen in culture (mg).	mg PHB per mg cell N (PHB/N).	Sudan black estimation of lipid inclusions.	Volutin (Albert's stain).	pH	Turbidity of culture (in 50 ml).	Final turbidity of culture after hypochlorite treatment. Ø	total PHB in culture (mg)	Total cell nitrogen in culture (mg)	mg PHB per mg cell N. (PHB/N).	PHB content of cells (% dry weight). ⊠	Sudan black estimation of lipid inclusions.	Volutin (Albert's stain).
6*	7.3	0.020		0.009	0.050	0.18	±	-	7.3	0.002		0.001	0.005	0.18	1.8	±	±
11	7.3	0.126	0.009	0.15	0.210	0.72	+±	±	7.3	0.127	0.009	0.15	0.28	0.53	5.3	++	±
16	7.3	0.474	0.019	0.32	1.15	0.28	+±	±	7.2	0.590	0.039	0.64	1.31	0.49	4.9	++	±
18	7.3	0.532	0.030	0.51	1.17	0.44	++	+	7.2	0.730	0.047	0.80	1.58	0.51	5.1	++	+
20	7.2	0.720	0.028	0.48	1.46	0.33	+±	+	7.2	1.160	0.082	1.46	3.00	0.52	5.2	++	+
23	7.2	1.109	0.030	0.52	2.61	0.20	+±	+	7.2	1.410	0.095	1.66	3.18	0.53	5.3	++	+
28	7.2	1.424	0.057	0.98	3.92	0.25	±	±	7.2	1.620	0.105	1.86	3.50	0.53	5.3	++	+
29	7.2	1.510	0.051	0.88	3.68	0.24	±	±	7.2	1.710	0.113	2.07	3.86	0.45	4.5	++	±
34	7.2	1.603	0.037	0.65	3.83	0.17	±	±	7.2	1.580	0.100	1.76	3.94	0.58	5.8	++	±
40	7.2	1.568	0.042	0.71	3.75	0.19	±	-	7.2	1.690	0.121	2.10	3.64	0.58	5.8	++	-
48	7.2	1.545	0.033	0.59	3.72	0.16	±	-	7.2	1.360	0.093	1.66	3.10	0.53	5.3	++	-
72	7.2	0.924	0.026	0.44	1.93	0.23	+	-	7.2	1.450	0.071	1.27	2.98	0.43	4.3	++	-
90	7.2	0.523	0.013	0.22	1.24	0.18	±	-	7.2	1.050	0.049	0.91	2.45	0.37	3.7	++	-

Ø Figures for "final turbidity" have been calculated from experimental readings on actual "working suspension" (see p. 73) to correspond with initial turbidities of the cultures; they are directly proportional to the figures for "total PHB".

Note: The heavy horizontal line denotes the stage at which the stationary phase was entered.

* 6 hr. estimate of growth made from turbidities; 6 hr. PHB contents estimated from Sudan black staining.

⊠ Estimated from PHB/N and graph 6.

TABLE 31. (Contd.). The influence of KCl concentration on growth and formation of PHB, polysaccharide and volutin by B.cereus(AC.1).

	0.008% KCl (glucose-deficient)									0.002% KCl (K^+-deficient).									
Age of culture. (hrs).	pH	Turbidity of culture (in 50 ml).	Final turbidity after hypochlorite treatment. ∅	Total PHB (mg)	Total Cell nitrogen (mg)	mg PHB per mg cell nitrogen	mg polysaccharide per mg cell nitrogen	Volutin (Albert's stain).	Microscopic estimation of lipid inclusion (Sudan black)	pH	Turbidity of culture (in 50 ml)	Final turbidity after hypochlorite treatment. ∅	Total PHB (mg)	Total cell nitrogen (mg)	mg PHB per mg cell nitrogen	cell content of PHB (% dry weight). ⊠	mg polysaccharide per mg cell nitrogen	Volutin (Albert's stain).	Microscopic estimation of lipid inclusion (Sudan black)
6*	7.3	0.020		0.01	0.050	0.18		±	±	7.3	0.002		0.001	0.005	0.18	1.8		±	±
11	7.3	0.145	0.009	0.17	0.25	0.68	2.08	±	++	7.3	0.132	0.016	0.29	0.28	1.05	10.0		±	++
16	7.3	0.410	0.025	0.46	1.06	0.43	1.54	±	+±	7.3	0.428	0.087	1.53	0.78	1.98	17.6		+	+++
18	7.2	0.551	0.026	0.49	1.24	0.40	1.63	+	+±	7.3	0.773	0.179	3.24	1.31	2.50	21.4		+	++++
20	7.2	0.866	0.028	0.51	1.55	0.33	1.63	+±	+	7.2	0.998	0.265	5.20	1.58	3.31	26.6		+±	++++
23	7.2	1.113	0.035	0.65	2.41	0.27	1.28	+±	+	7.2	1.220	0.338	6.03	1.55	3.91	29.8		+	++++
28	7.2	1.475	0.048	0.86	3.63	0.24	1.21	+±	+	7.2	1.310	0.348	6.29	1.60	3.92	29.8		±	++++
29	7.2	1.650	0.051	0.95	4.05	0.24	1.12	+±	+	7.2	1.252	0.329	5.70	1.42	4.00	30.4	0.87	±	++++
34	7.2	1.575	0.036	0.65	3.85	0.17	0.95	+	±	7.2	1.163	0.334	6.10	1.44	4.20	31.6	0.94	±	++++
40	7.2	1.700	0.029	0.53	3.96	0.13	1.04	±	±	7.2	1.142	0.289	5.15	1.42	3.63	28.3	0.82	±	++++
48	7.2	1.512	0.027	0.48	3.75	0.13	0.85	-	±	7.2	1.121	0.310	6.20	1.57	3.92	29.8		-	++++
72	7.2	1.125	0.028	0.50	2.80	0.18	1.32	-	±	7.2	1.323	0.254	4.40	1.60	2.76	23.3		-	++++
90	7.2	0.650		0.35	2.21	0.16	1.41	-	±	7.2	1.200	0.256	4.50	1.44	3.12	25.5		-	++++

∅ Figures for "final turbidity" have been calculated from experimental readings on actual "working suspension" (see p.73) to correspond with initial turbidities of the cultures; they are directly proportional to the figures for "total PHB".

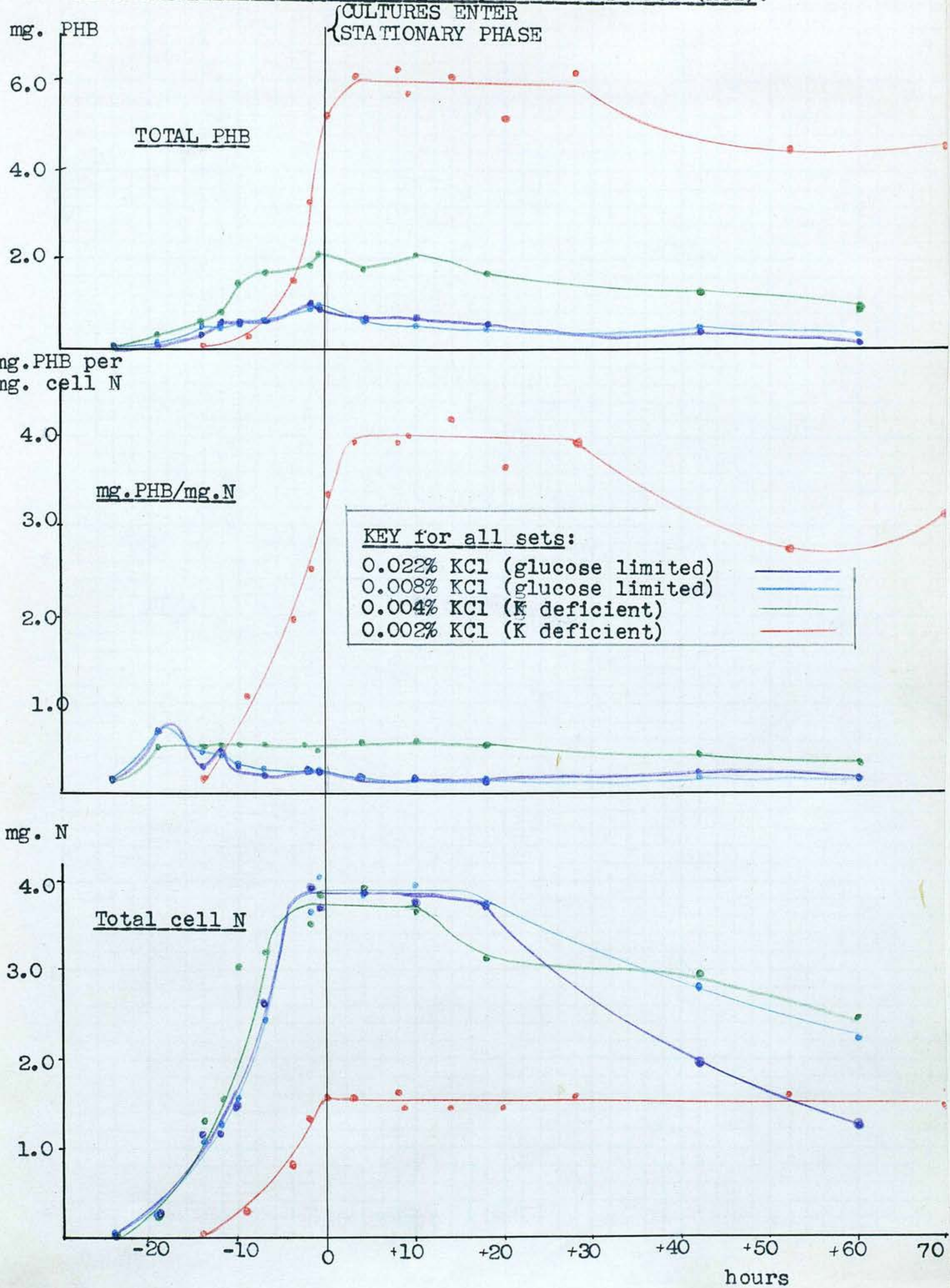
Note: The heavy horizontal line denotes the stage at which the stationary phase was entered.

* 6hr. estimate of growth made from turbidities; 6 hr. PHB contents estimated from Sudan black staining.

⊠ Estimated from PHB/N and graph 6.

GRAPH 19

Growth and production of PHB by B.cereus(AC.1) on synthetic media containing different amounts of potassium source (KCl).



in total growth, but the relative increase was less than, and not proportional to, the increased concentration of KCl. Thus, the 0.004% and 0.002% media were such that the total growth was limited by exhaustion of K^+. On the other two media, there was more potassium than was necessary for the complete utilisation of the available glucose (0.3%), and the growth on these two media was therefore limited by exhaustion of glucose.

Polysaccharide determinations were not made throughout the growth cycle, but a few measurements were made during the stationary phase to indicate the effect of K^+ deficiency on the level of this material during the stationary phase.

It will be seen from graph 19, that the production of PHB on the K^+-deficient media followed a course significantly different from that so far displayed on other media. Whereas, on the controls and on the other media to date, the total PHB curve was at first roughly linear, and the rate of production fairly slow, on the K^+-deficient media, total PHB production started to rise from the moment growth commenced, and the rate of production rose sharply and continued to rise until growth ceased. This resulted in a steady rise in PHB/N without any of the fluctuations apparent on other media. As the stationary phase was reached, the PHB/N levelled off at once and remained stable at this high level for a long period. The rise during the growth phase is clearest on the 0.002% K^+ medium; on the less deficient medium the

final PHB/N level was relatively low, since the limitation of growth was slight, and presumably most of the carbon source was utilised for growth and was not available for PHB production. Nevertheless, the general pattern is apparent on this medium as well. The two do differ somewhat, however, in their final stages. On the more deficient medium, the plateau in the PHB/N curve (which was the highest value obtained in this series of experiments) was interrupted by a breakdown of about a quarter of the total PHB, followed finally by a period when no more PHB disappeared, but there was a slight (and hardly significant) drop in total nitrogen, resulting in a slight rise in the PHB/N curve. On the less K^+- deficient medium, the plateau in the total PHB curve was broken by a steady decline, no drop in total nitrogen being apparent, and therefore a steady drop in PHB/N ensued.

From these results it is concluded firstly, that when growth was limited by exhaustion of K^+, an increased production of PHB occurred, and that K^+ is therefore not "irreversibly consumed" during its formation. (This phrase has been used, and will be used again, as a convenient way of expressing the fact that K^+ was not incorporated into the inclusions or in any other way "inactivated" during the synthesis of PHB; the results could not, however, be interpreted as signifying that K^+ had no role in the formation of PHB. If its role was only a catalytic one, perhaps in connection with an enzyme system, its exhaustion in the medium would not

affect the production of PHB. This point will be more fully discussed later.

Secondly, the deficiency of K^+ affected cultures from the start of growth, and not, as in previous media, and on the controls, only as growth came to an end. Presumably the ratio of potassium to one or more of the other nutrients in the medium must in some way affect the equilibrium between production of PHB on the one hand, and growth, in terms of assimilation of nitrogen, on the other. It is suggested that this result may in fact be an example of the operation of a "rate limiting factor", as was discussed on p.48. Examination of the growth curves obtained on these media suggests that the rate of growth on the most highly K^+-deficient medium was much less than on the controls. Thus the deficiency of potassium would appear to have been limiting the rate of growth as well as the total growth. It is suggested that the effect of the deficiency on the rate of production of PHB was relatively less than its effect on the growth rate, and that in this way the amount of PHB in the culture, (and per cell) was permitted to increase. This suggestion will be fully discussed later.

The few figures for polysaccharide/N during the stationary phase (table 31) suggest that the level of polysaccharide in the cell was not increased when growth was limited by deficiency of K^+, a finding that agreed with the observation by Duguid and Wilkinson (in Wilkinson, Duguid and Edmunds, 1954) that polysaccharide

production by Aero. aerogenes was specifically inhibited by K^+ deficiency. Bernheimer (1953) made similar observations with respect pneumococcal polysaccharides.

Inspection of the results of the volutin estimations (table 31) shows that volutin production was at all times low, though it is perhaps slightly higher during the stationary phase on the 0.002% K^+ medium than on the controls, but the difference is not regarded as significant, and the results are therefore interpreted as indicating that limitation of growth by deficiency of K^+ had an inhibitory effect on the production of volutin. This result is in agreement with the findings of Smith et al (1954) for volutin production in Aero. aerogenes.

(e) The effect of sulphur deficiency

For the purposes of these experiments, the cysteine in the amino-acid mixture was not removed, as this would have upset the balance of the supplied amino-acids, and in any case a good limitation was obtained without having to resort to its removal. Two limiting concentrations of added S were employed, (0.0003%, and 0.001% Na_2SO_4) together with one "control" medium (0.01% Na_2SO_4). From the amounts of growth supported by the two S-deficient media, it was calculated that the "contamination" sulphur in the agar, plus that supplied by the cysteine in the amino-acids, was equivalent to roughly 0.0006% Na_2SO_4, the cysteine being responsible for about a third of this amount. Thus the actual concentrations of sulphur in the medium were taken as equivalent to 0.0009%, 0.0016%,

and (in the control) 0.0106% Na_2SO_4. As in previous experiments, the evidence for the sulphur deficient nature of the two test media was supplied by the total growths obtained on the media, compared with that obtained on the control (table 32). It will be seen that whereas a reduction of the sulphate concentration from 0.0016% to 0.0009% was accompanied by a proportionate drop in the total growth, the increased growth obtained on raising the sulphate concentration from 0.0016% to 0.0106% was slight and not proportional to the increased concentration of the nutrient. Thus, growth on the 0.0009% and 0.0016% sulphate media was limited by exhaustion of the sulphur source, while the medium containing 0.0106% sulphate was the usual glucose limited "control". No measurements were made of polysaccharide, and only a few samples were examined for volutin.

The results are shown in table 33 and graph 20, p.263. From the latter it will be seen that the most striking feature was the rather low levels of PHB/N attained during the stationary phase. Taking the more highly S-deficient culture as an example, it will be seen that there was a peak in the PHB/N curve at about 12 hours, presumably similar to the "type (a) peak" seen in the control. A second peak occurred at about 25 hours and this peak was apparently the result of an increasing total rate of production of PHB in the culture. Just before the stationary phase was entered, however, a breakdown of total PHB commenced, and this breakdown

TABLE 32.

Concentration of Na_2SO_4 (W/V)	Total growth (stationary phase average: mg N).	mg PHB per mg cell N (stationary phase average)	Volutin (Albert's stain: stationary phase average.)
0.0009%	2.2	0.6	++
0.0016%	3.5	0.4	
0.0106%	3.9	0.2	±

The influence of concentration of sulphur source (Na_2SO_4) on the total growth and stationary phase cell contents of PHB and Volutin in B. cereus. (AC.1).

TABLE 33. The effect of concentration of Na_2SO_4 on growth and formation of poly β-hydroxybutyrate(PHB) and volutin by B. cereus(AC.1).

Age of culture (hrs)	0.0106% Na_2SO_4 (glucose limited "control")							0.0016% Na_2SO_4 (sulphate deficient)						0.0009% Na_2SO_4 (sulphate deficient).						
	pH	Turbidity of culture (in 50 ml)	Final turbidity after hypochlorite treatment. ∅	Total PHB (mg)	Total cell N (mg) in culture	mg PHB per mg cell N (PHB/N)	Volutin (Albert's stain)	pH	Turbidity of culture (in 50 ml)	Final turbidity after hypochlorite treatment. ∅	Total PHB (mg)	Total cell N (mg) in culture	mg PHB per mg cell N. (PHB/N)	pH	Turbidity of culture (in 50 ml)	Final turbidity after hypochlorite treatment. ∅	Total PHB (mg)	Total cell N (mg) in culture	mg PHB per mg cell N. (PHB/N)	Volutin (Albert's stain).
6*	7.3	0.010		0.005	0.03	0.18	±	7.3	0.005		0.003	0.015	0.18	7.3	0.014		0.005	0.03	0.18	
10	7.3	0.119	0.007	0.15	0.21	0.72	±	7.3	0.061	0.006	0.11	0.15	0.73	7.3	0.075	0.006	0.10	0.18	0.57	
16	7.3	0.425	0.017	0.38	0.98	0.39	±	7.3	0.284	0.018	0.31	0.75	0.42	7.3	0.357	0.023	0.40	0.76	0.53	
18	7.3	0.488	0.024	0.54	1.22	0.44	+	7.3	0.475	0.023	0.40	1.17	0.35	7.3	0.455	0.031	0.54	1.13	0.48	
20	7.2	0.820	0.020	0.46	2.05	0.22	+±	7.2	0.565	0.028	0.50	1.60	0.31	7.2	0.475	0.033	0.59	1.23	0.48	
23	7.2	1.410	0.025	0.59	3.10	0.19	+	7.2	0.700	0.026	0.47	2.10	0.22	7.1	0.611	0.069	1.34	1.76	0.76	
27	7.2	1.640	0.041	0.91	3.72	0.24	+	7.1	1.020	0.056	1.00	3.52	0.28	7.1	0.675	0.066	1.18	1.73	0.67	++
29	7.2	1.725	0.035	0.76	3.99	0.19	+	7.1	1.100	0.073	1.26	2.80	0.45	7.1	0.765	0.080	1.42	2.07	0.68	++
35	7.2	1.614	0.034	0.72	3.76	0.19	±	7.1	1.400	0.075	1.33	3.75	0.40	7.2	0.780	0.064	1.12	2.02	0.58	++
41	7.2	1.782	0.035	0.75	4.01	0.19	±	7.1	1.370	0.075	1.34	3.49	0.38	7.1	0.785	0.060	1.06	2.08	0.52	++
48	7.2	1.625	0.036	0.78	3.81	0.21	-	7.1	1.425	0.070	1.19	3.45	0.35	7.1	0.775	0.045	0.80	2.43	0.33	
90	7.2	0.995	0.015	0.34	2.31	0.15	-	7.1	1.325	0.051	0.92	3.05	0.30	7.1	0.820	0.040	0.70	2.18	0.32	

∅ Figures for "final turbidity" have been calculated from experimental readings on actual "working suspension"(see p. 73) to correspond with initial turbidities of the cultures; they are directly proportional to the figures for "total PHB".

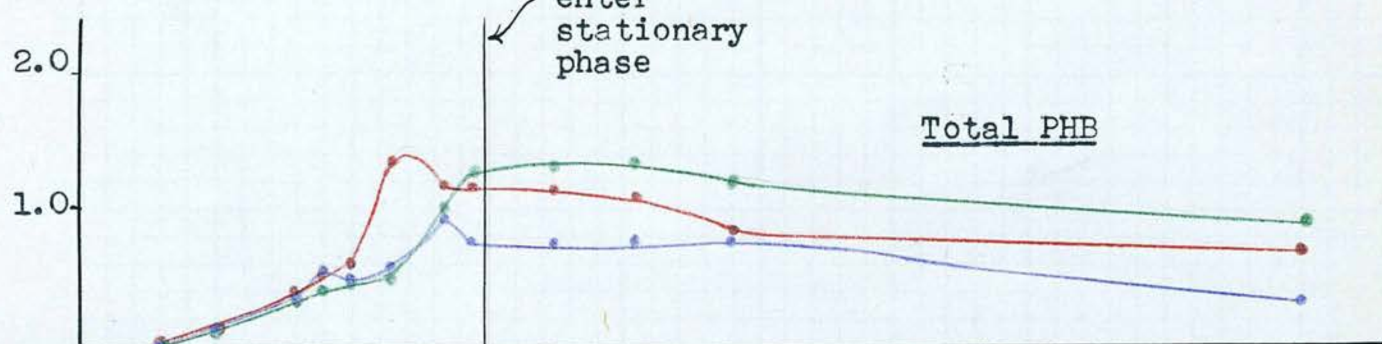
Note: The heavy horizontal line denotes the stage at which the stationary phase was entered.

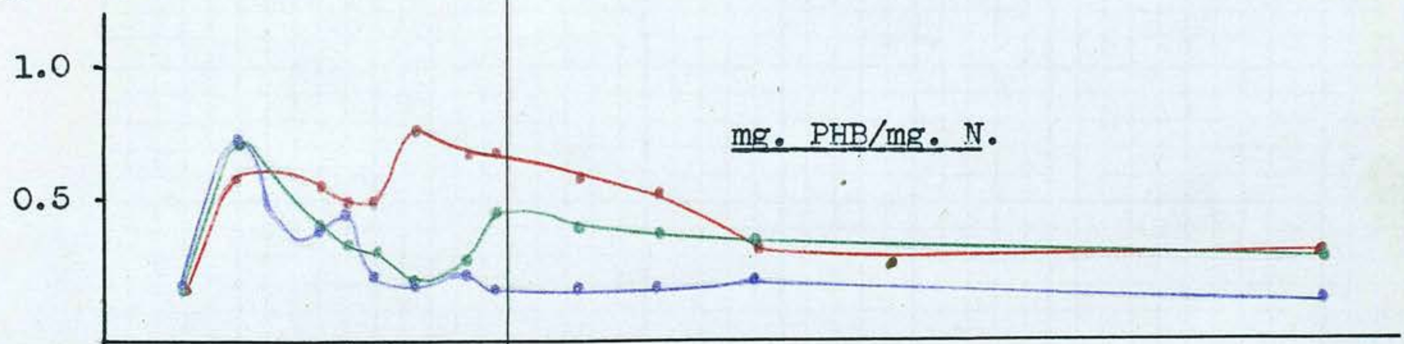
* 6 hr. estimate of growth made from turbidities; 6 hr. PHB contents estimated from Sudan black staining(which was a trace(±) in each case).

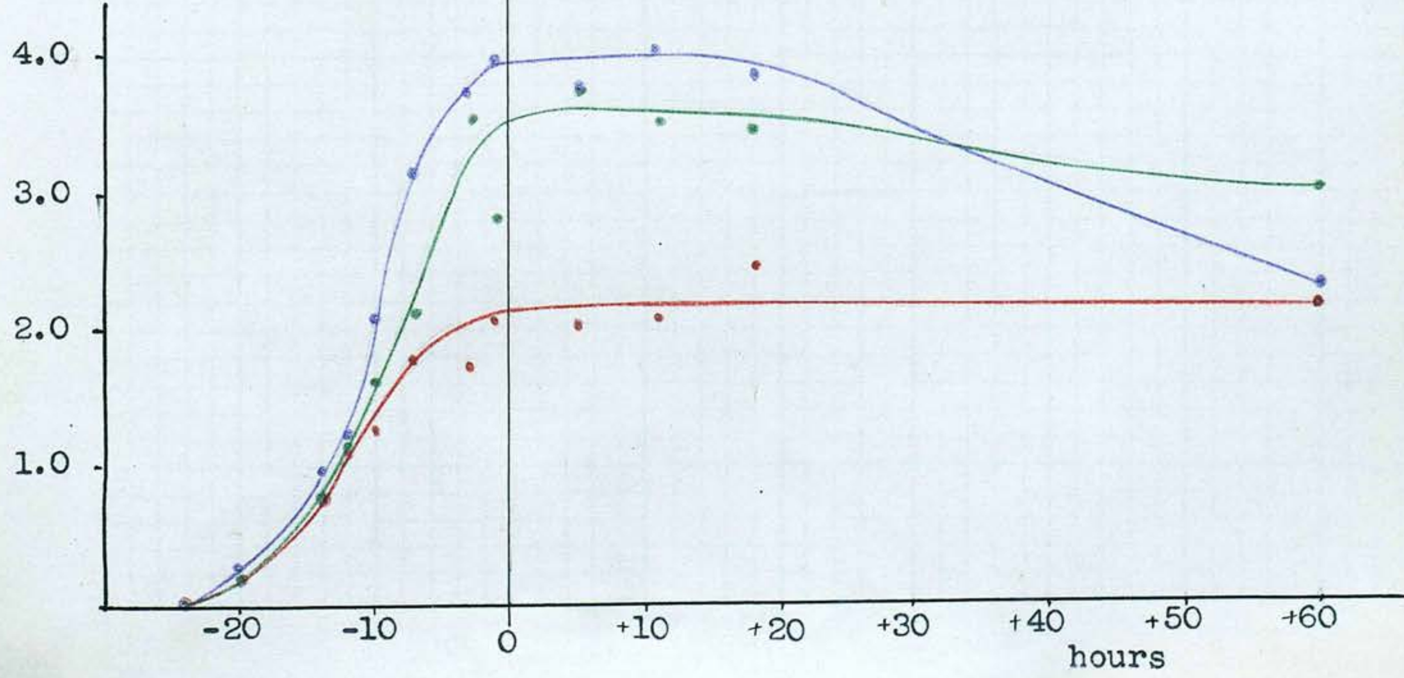
GRAPH 20.

Growth and production of PHB by B.cereus (A.C.1) grown on synthetic media containing different amounts of sulphur source (Na_2SO_4)

KEY for all sets of curves:
0.0106% Na_2SO_4 (glucose limited)
0.0016% Na_2SO_4 (sulphate deficient)
0.0009% Na_2SO_4 (sulphate deficient)







continued right through the stationary phase and resulted finally in a PHB/N level not much above that of the control cells. The pattern displayed by the less S-deficient medium was very similar, but the slightly increased rate of production of PHB during the second half of the phase of active growth was not so marked, and indeed the total PHB curve of this culture was very similar to that of the control during the growth phase. In both media, however, the levels of PHB/N in the stationary phase were low and decreasing. This suggests that sulphur is to some extent required for and "irreversibly consumed" during the synthesis of PHB, since on the more S-deficient medium especially, there must have been a considerable amount of surplus glucose in the medium, but very little PHB was formed. This finding will be discussed in detail later.

It would also appear that when the deficiency of sulphur was slight, the course of total production of PHB during the phase of active growth was very similar to that seen on the control. In the more highly deficient medium, on the other hand, the rate of total production of PHB increased to some extent during the phase of active growth, and the resulting curve of total PHB production was, to a limited extent, similar to that seen in the K^+-limited media. The rate of growth of the more highly deficient culture was apparently slightly less than that of the control, and it would therefore appear that the deficiency of sulphur was limiting the rate of growth as

well as the total crop. Thus it would appear possible that the deficiency of sulphur was displaying a "rate limiting effect" similar to but less marked than that seen on the K^{+}-deficient cultures. This point will be discussed in full later.

The observations of volutin formation (table 33) suggest that sulphur deficiency did not hinder the formation of increased amounts of volutin, a finding in agreement with that of Smith et al (1954) for volutin production in Aero. aerogenes.

(f) The effect of phosphate deficiency.

The buffering of phosphate deficient media has always presented difficulties to bacteriologists, since most buffer systems active at around biological pH's are themselves biologically active. The recent introduction for biochemical work of "Tris" (tris (hydroxymethyl) amino-methane) gave some hope of a solution to this difficulty, however. The author is unaware of any reports of its use as a buffer for bacteriological culture media, and experiments were undertaken to test its buffering capacity and biological activity, if any. A series of pairs of plates of the synthetic control medium were prepared, each plate containing 1.0% Tris, and each pair a different concentration of phosphate buffer, the highest being 0.009% (w/v). The Tris was supplied as a 5% (w/v) solution, adjusted to pH 7.6 with HCl, and sterilised by autoclaving at ten pounds pressure for ten minutes. At a concentration of 1.0% (w/v), the molarity of Tris in

the medium was 0.08M, as compared with a molarity of about 0.06M in the case of 1.0% phosphate buffer. The initial pH of the medium containing 1.0% Tris was 7.4, irrespective of the concentration of phosphate. The plates were inoculated with strain AC.1 of B.cereus and incubated for 35 hours at 37°C, and the turbidities of the cultures measured. The results (table 34) are plotted in graph 21p.267. It will be seen that the final growth was constant at phosphate concentrations between 0.003% and 0.009%, and this level was the same as that obtained on the normal control medium with 1.0% phosphate and no Tris. The final pHs of the media on this part of the curve were 7.2. The cells were morphologically indistinguishable from those of the normal control cultures, and contained traces only of lipid inclusions and volutin. Thus it appeared that the presence of Tris had not affected the cells in any way, and the fact the the level of growth was no greater than that of the control medium indicated that Tris was not being utilised for growth. With lower phosphate concentrations, growth was reduced proportionately, and the lipid inclusion content (indicated on the graph by "+" signs) was increased. The pHs of the media on this part of the curve were virtually unchanged, so it was evident that the Tris had adequate buffering capacity for the present purposes.

Two experimental media were set up, containing 0.0003% and 0.001% phosphate buffer (reckoned as the combined weights of the two anhydrous salts, Na_2HPO_4,

TABLE 34.

Concentration of Phosphate (W/V) *	Turbidity of culture(50ml) at 35 hrs.	Final pH. ∅	Microscopic estimation of lipid inclusions (Sudan black).
0.009%	1.855	7.2	±
0.003%	1.850	7.2	±
0.0009%	1.285	7.2	++
0.0003%	0.610	7.4	++±
0.00009%	0.270	7.4	++±
0.0%	0.134	7.4	++±

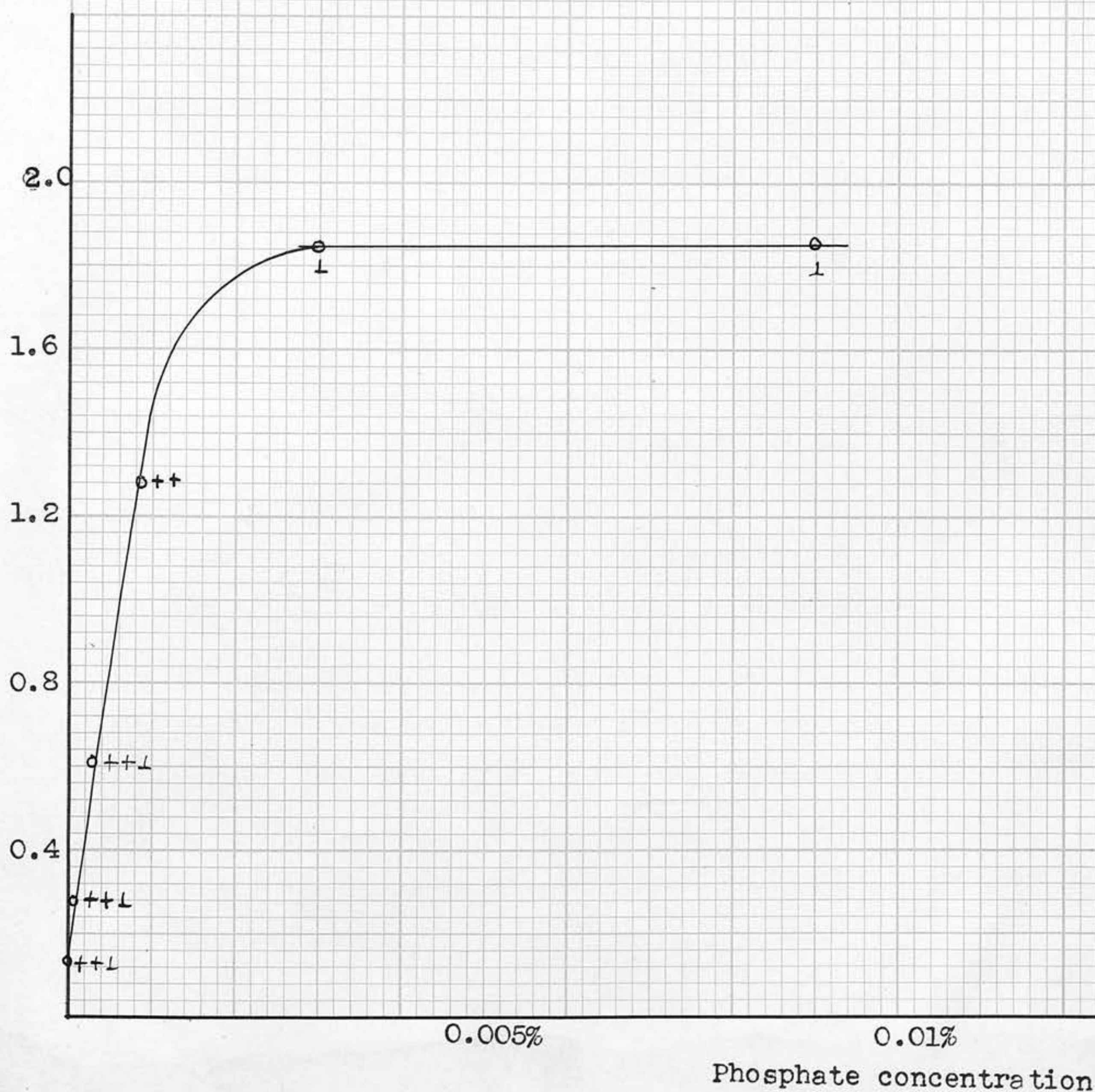
* Medium contains 1% (W/V) tris (hydroxymethyl)aminomethane

∅ Initial pH 7.4

This experiment was made to determine the effect of "Tris" on the growth and pH of B.cereus (AC.1) on the synthetic medium containing various concentrations of phosphate buffer. The results, which are plotted in graph 21 (p.267) also gave a preliminary indication of the effect of phosphate deficiency on production of lipid inclusions. The results(discussed in text) indicated that Tris had no inhibitory or stimulating effect on growth, but had adequate buffering capacity.

GRAPH 21.

The effect of concentration of phosphate on the total growth and lipid inclusion content of B.cereus(AC.1) grown on the "control" medium, buffered with 1.0% TRIS; lipid inclusions estimated microscopically and recorded as "+" signs.



and NaH_2PO_4). The results (tables 35 & 36, graph 22) should be compared with those of the normal glucose limited control (graph 11, p. 232). It will be seen that the growth curves obtained on these P-deficient media were somewhat irregular, but from table 35 it is clear that the peak growths obtained on the two media were proportional to the amounts of phosphate in the medium. On increasing the phosphate content of the medium however, from 0.001 % to the level of the control (1%), there was only a slight increase in growth and the increase was not proportional to the increased phosphate concentration. Thus it was clear that the two media with reduced phosphate concentrations were such that growth was limited by deficiency of phosphorus.

From graph 22, it will be seen that both the cultures tended to grow in two stages, and in the less deficient medium there was no clear stationary phase. The reason for this irregular growth is not understood. It seems unlikely that it is a normal feature of growth on phosphate deficient media, and it may be connected with the presence of Tris in the medium. Whatever its cause, it makes the interpretation of the results a little difficult. Nevertheless, it is clear that the peak levels of PHB/N in both cultures were about ten times the levels seen in the stationary phase cells of the control media. Thus it was clear that when exhaustion of phosphorus was the factor limiting growth, the cells were still capable of converting the excess of carbon source into PHB, and one would therefore assume that phosphorus

TABLE 35.

Concentration of phosphate (W/V) ∅	peak growth level (mg N).	peak level of mg PHB per mg cell N.	Volutin(Albert's stain).
0.0003%	1.22 mg	1.86	-
0.001%	3.21 mg	1.55	-
1.0%*	3.66mg*	0.18*	±*

The influence of phosphate concentration on the peak growth and cell contents of PHB and volutin in B. cereus (AC.1).

∅ media buffered with 1.0% (W/V) tris.

* Data from glucose limited control medium (table 28: "0.15% NH_4Cl "), the figures shown being the average stationary phase values.

TABLE 36. The Effect of deficiency of phosphate on growth and formation of poly β-hydroxybutyrate(PHB) and volutin by B. cereus(AC. 1).

Age of culture	0.001% Phosphate. (Phosphate deficient).								0.0003% Phosphate. (Phosphate deficient).							
	pH	Turbidity of culture (50 ml)	Final turbidity after hypochlorite treatment. ∅	Total PHB (mg)	Total cell N(mg)	mg PHB per mg N (PHB/N).	cell content of PHB (% dry weight) ⊠	Volutin Albert's stain.	pH	Turbidity of culture (50 ml).	Final turbidity after hypochlorite treatment. ∅	Total PHB (mg)	Total cell N(mg)	mg PHB per mg N (PHB/N).	PHB (% dry weight) ⊠	Volutin Albert's stain.
6*	7.2	0.006		0.004	0.02	0.18		-	7.2	0.004		0.002	0.01	0.18		-
11	7.0	0.252	0.009	0.14	0.64	0.22	2.2	-	7.2	0.165	0.008	0.14	0.37	0.36	3.5	-
16	7.0	0.434	0.025	0.45	1.13	0.40	4.0	-	7.2	0.272	0.026	0.45	0.54	0.84	8.0	-
18	7.0	0.568	0.029	0.51	1.50	0.34	3.5	-	7.0	0.298	0.027	0.48	0.66	0.72	7.0	-
20	6.8	0.738	0.049	0.86	1.79	0.48	4.8	-	7.0	0.348	0.035	0.61	0.70	0.87	8.2	-
23	6.8	0.895	0.077	1.34	1.88	0.71	7.0	-	6.8	0.386	0.045	0.78	0.70	1.15	10.5	-
27	6.8	1.125	0.106	1.86	2.00	0.93	8.7	-	6.8	0.392	0.047	0.82	0.55	1.48	13.3	-
29	6.8	1.270	0.138	2.40	2.13	1.13	10.5	-	6.6	0.440	0.058	1.00	0.54	1.86	16.6	-
41	6.6	1.755	0.239	4.18	2.73	1.55	13.8	-	6.6	0.510	0.052	0.90	0.76	1.20	11.0	-
48	6.6	1.905	0.276	4.80	3.21	1.48	13.3	-	6.4	0.705	0.066	1.17	1.04	1.12	10.3	-
72	6.6	0.865	0.091	1.58	1.88	0.84	8.0	-	6.4	0.730	0.041	0.74	1.17	0.63	6.3	-
90	6.6	0.665	0.105	1.82	1.46	1.26	11.3	-	6.4	0.960	0.075	1.31	1.22	1.07	9.8	-

∅ Figures for "final turbidity" have been calculated from experimental readings on actual "working suspension"(see p. 73) to correspond with initial turbidities of the cultures; they are directly proportional to the figures for "total PHB".

* 6 hr. estimate of growth made from turbidities; 6 hr. PHB contents estimated from Sudan black staining(which was a trace(±) in each case).

⊠ Estimated from PHB/N and graph 6.

GRAPH 22.

The effect of phosphate deficiency on growth and production of PHB by B.cereus (AC.1)

KEY for both sets of curves:

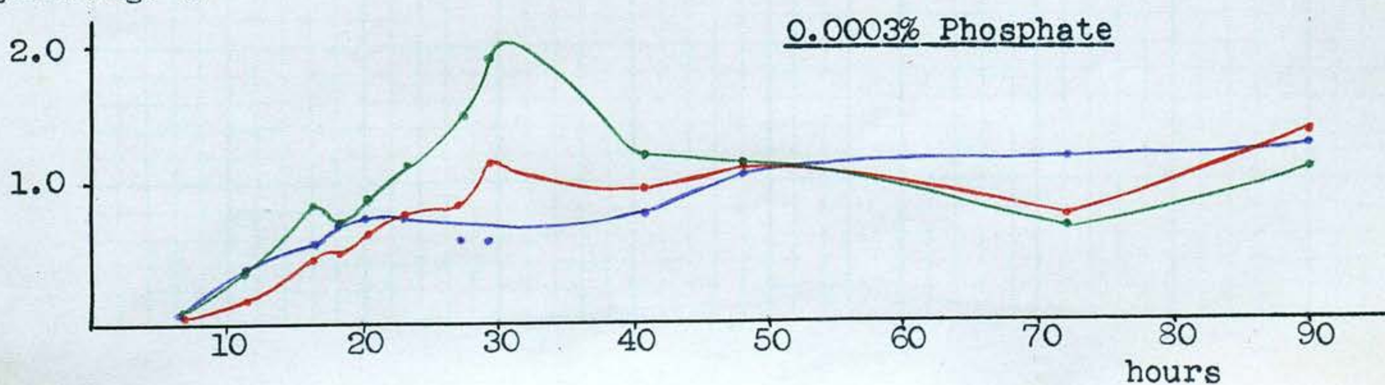
Total nitrogen
Total PHB
mg. PHB per mg. N

Note: as explained in text, both these cultures were such that total growth was limited by exhaustion of phosphate; they should be compared with the culture on the glucose limited "control" medium, graph 11, p. 232

mg. PHB
mg. cell N
mg. PHB/mg. N.

5.0
4.0
3.0
2.0
1.0

0.001% Phosphate



was not "irreversibly consumed" during its synthesis. This finding was in agreement with present knowledge of the role of phosphorus in the transport of energy. The cell presumably always carries with it a certain minimal amount of phosphorus, in both organic and inorganic forms, in order to maintain its basal metabolic functions. Even cells from P-limited cultures will still contain this "élément constant" of phosphorus, and there is no reason therefore why such cells should not synthesise storage materials provided the necessary nutrient precursors and a source of energy are present.

It will be seen, however, that the peak levels of PHB/N on both the phosphate media were about the same. Now the total growth on the more highly P-deficient medium was about a third of that on the other medium, and there must therefore have been much more "excess" glucose available for conversion into PHB on the more P-deficient medium. The peak level of PHB/N was no greater, however, and one must therefore assume that some unknown factor was limiting the total PHB/N level attained. The nature of this factor was not determined; it does not however, seem likely to be connected with intracellular cell "space", since this organism was capable of containing up to 40% of its dry weight as PHB.

Turning to the volutin results, (table 36) it will be seen that no volutin granules appeared at any stage of the cultures. This result provided further confirmation of the observations of a phosphorus require-

ment for the formation of volutin already observed by Elser and Huntoon (1909), Zikes (1922), Duguid et al., (1954), and Smith et al., (1954). It will be seen that the cells were completely free of volutin even during the phase of growth, which indicates that the volutin forming system is sensitive to the relative amount of phosphate in the medium in the same way that PHB formation is sensitive to the relative concentration of K^+, the effect of the deficiency being felt right from the start of growth.

Smith et al (1954) reported that volutin was produced by Aero. aerogenes when cells from a phosphorus limited medium were transferred to a medium containing phosphate. This effect was found to be shown also by B. cereus: some of the cells from a 30 hour culture on the more highly P-deficient medium were transferred as a heavy streak to a plate of nutrient agar, and Albert stained films made at intervals. The results were as follows:

Time	Volutin
0 hrs.	–
0.5hrs.	±
1 hr.	+
2 hrs.	+
3 hrs.	+
5 hrs.	±

(g) The effect of anaerobiosis.

Some indication of the effect of anaerobiosis was obtained by incubating cultures of strain AC.1 on the "control" medium, and on the 1% glucose medium,

TABLE 37.

Factor limiting growth in glucose containing synthetic medium	Maximum stationary phase levels of:		
	PHB per cell	Intra-cellular poly-saccharide per cell	Volutin per cell
glucose (carbon and energy source)	±	±	±
nitrogen source	±	+	++
potassium source	+++	±	±
sulphur source	±		++
phosphorus source	++		-
"unknown" factor in media containing increased added glucose**	++	+	++
oxygen (i.e. in anaerobic culture)	±		

** as explained in the text, the factor limiting growth on the glucose rich media was not determined; it was possibly accumulation of a toxic waste product of growth, or perhaps deficiency of an unidentified mineral. The arbitrary scale of "+" signs has the following approximate meaning: -, none; ±, a trace; +, ++, and +++, increasing amounts.

anaerobically (McKintosh and Fildes' jar), and staining some samples for lipid. There was no difference between the levels of growth obtained on the two media, which were both about a third of that obtained on the aerobically incubated control medium.

Samples were examined at 24, 36, 72, and 96 hours, and the microscopically estimated lipid inclusion contents were, respectively, "-", "±", "-", and "±". Thus it was clear that the formation of the inclusions was essentially dependent on the presence of molecular oxygen, a finding in agreement with that of Knaysi (1951b).

A detailed summary of the results of the above experiments is given in the general Summary to this Thesis (p.291). The table on page 272 however shows, in an approximate manner, the effect of the different cultural conditions investigated on the stationary phase cell contents of PHB, volutin and intracellular polysaccharide.

DISCUSSION.

DISCUSSION

The most striking feature of the literature on the lipid inclusion bodies of bacteria is the lack of real knowledge of the nature and function of these widely distributed and prominent cell structures. The long period of neglect that followed their discovery in the early years of this century suggests that most workers were content with the statement - based on their staining properties only - that the inclusions were "fatty" in nature, and that they functioned as "reserve stores". In fact recent research indicates that the major component of the inclusions is not fat, and there is yet no certain evidence that the inclusions have a "reserve" function. Practically the only significant observation that has been made concerning their nature was the discovery of poly β-hydroxybutyrate (PHB) by Lemoigne, and his observation of a correlation between the presence of this material in the cells and the presence of lipid inclusions. Moreover, until very recently, this observation has been largely neglected, save for a handful of workers at the Pasteur Institute, and progress has been slow.

Perhaps one of the main reasons for the apparent lack of interest in the inclusions is the known difficulties attendant on researches with lipids. Even Lemoigne's chloroform extraction method for the estimation of PHB, which is quite straightforward, suffers from the disadvantages that very large culture samples are

required, and the process is lengthy. In the absence of better methods, progress must necessarily be slow.

The hypochlorite technique described in the present Thesis was developed to remedy this situation; it provides a simple rapid means of making accurate estimations of the lipid inclusions of the members of the Bacillus genus, and can be used to give an accurate estimate of the cell content of PHB.

The microscopic and turbidimetric observations of the effect of alkaline hypochlorite on the cells of the Bacillus species examined, provided overwhelming proof that only the lipid and volutin inclusion bodies are resistant to this reagent. Since the refractility of the volutin granules is relatively low, the final turbidity obtained on treating a sample of cells with hypochlorite is almost directly proportional to the amount of lipid inclusions present. This measurement of turbidity gives a better estimate of the lipid inclusion content of the cells than any method hitherto available, and with the aid of a simple calibration curve it is easily converted into an estimate of the mass of the inclusions. Furthermore, the finding that the hypochlorite-isolated inclusions of strain AC.1 of B.cereus contained a constant proportion of poly β-hydroxybutyrate (PHB), namely 89%, increased the usefulness of the method considerably, by making it possible to derive from the "lipid inclusion" measurement, an accurate estimate of the PHB content of the cells. This method of estimating PHB necessarily depends for its accuracy on the proportion of PHB in the hypochlorite-isolated

inclusions being constant regardless of the cell's content of lipid, the cultural conditions, and other variables. This in fact was proved to be the case with strain AC.1 for cultures containing moderate and large amounts of lipid inclusions; the composition of the hypochlorite-isolated inclusions was found to be constant for a number of different cultures containing from 9% to 40% PHB. Moreover, estimations of the PHB in these cultures made in parallel by the hypochlorite and chloroform extraction methods gave identical results. The only exception was found in the case of the cells containing very small amounts of PHB, the hypochlorite method indicating a PHB content of 1.6%, and chloroform extraction yielding only 0.2% PHB. It is apparent therefore that the hypochlorite method cannot be used to give accurate estimates of PHB in cells containing much less than 9% of the polymer.

The reason for this latter discrepancy was not determined. Possibly the proportion of PHB in the very small inclusions of PHB-poor cultures is less than that of the larger inclusions of PHB-rich cultures. Alternatively the smaller average size of the inclusions of the PHB-poor cultures may increase their turbidity/mass ratio, and thus the estimate of their amount given by the hypochlorite method. As regards the latter possibility, however, it is to be noted that the close correspondence of the results given by the two methods with different cultures containing from 9% to 40% PHB suggests that in these cases, variation in the mean size of the inclusions did not have an appreciable effect on the turbidity/mass ratio of the

inclusions. It is important to emphasise that before the hypochlorite method can be applied to any organism other than strain AC.1 of B.cereus, the composition of the hypochlorite-isolated inclusions of that organism must be determined and its constancy established.

The resistance of volutin granules to hypochlorite digestion means that the presence in the cells of large amounts of volutin may introduce an error into the estimation of lipid inclusions and PHB by the hypochlorite method. This error will be greatest in cells containing large amounts of volutin and small amounts of PHB. In strain AC.1 of B.cereus, cells in this condition were found in only one set of circumstances, namely in the final stages of growth on an extremely nitrogen-deficient medium. These cells contained the maximal amounts of volutin formed by this strain, "++ ±", and their lipid inclusion content, as estimated by microscopic examination of a Sudan black-stained smear was only "+". The hypochlorite method indicated a cell content of 0.25mg. PHB per mg. nitrogen, (table 28: 0.01% NH_4Cl, 48 hour culture) a figure which compares well with the hypochlorite estimate of the PHB content of cells containing "+" lipid inclusions and no volutin, (table 28: 0.15% NH_4Cl, 88 hour culture) which was 0.24mg. PHB per mg. nitrogen. Thus in this case the error incurred by the presence of maximal amounts of volutin was apparently only of the order of 5%. This error would become progressively smaller in cells containing larger amounts of PHB, and for most purposes would probably be negligible.

The close correspondence of the results obtained by parallel determinations of PHB by the hypochlorite method and by chloroform extraction gives some idea of the probable limits of error inherent in the method. With the exception of cultures containing less than 9% PHB, as mentioned above, the apparent error in the hypochlorite method was never greater than about 3%. This accuracy compares well with that of other chemical methods in common use such as the estimation of total cell protein by determination of nitrogen, and estimation of total cell polysaccharide by the anthrone method. Even if the error of the hypochlorite method were as great as ± 10%, the method would still be of considerable value, in view of the small size of the samples required (of the order of 10mg. dry weight of cells) and the speed with which the determinations can be carried out.

The experiments on growth and the production of PHB by B. cereus in culture provided a rigorous test of the practical usefulness of the hypochlorite method. Without it, it would have been impossible to make the very large numbers of determinations of PHB at different times during growth, since the alternative chloroform extraction method would have been too lengthy and would have necessitated prohibitively large culture samples.

The value of hypochlorite is not limited solely to quantitative estimation of PHB. The analysis of the hypochlorite-isolated lipid inclusions provided the first direct and well substantiated proof of the existence of poly β-hydroxybutyrate in these inclusions, and was thus

a direct confirmation of Lemoigne's circumstantial evidence to this effect. It would appear that the PHB from strain AC.1 of B.cereus is not quite identical with the PHB isolated by Lemoigne and his colleagues from B.megaterium; the former had a slightly higher melting point though resembling the latter in all other respects. The two preparations presumably differ only in their chain length. The molecular weight of the PHB from B.cereus, as determined by isothermal distillation in chloroform, was of the order of 5,000; this suggested that the chain length of this polymer was in the neighbourhood of 60 residues of β-hydroxybutyric acid. The PHB from B.megaterium was found by Képès and Péaud-Lenoël (1952) to have a chain length of between 6 and 110 residues. A single run on the ultra-centrifuge suggested that the PHB of B.cereus was a homogenous molecular species.

The examination and analysis of the isolated lipid inclusions revealed some interesting features of the lipid staining properties of these bodies, and although the results do not permit a complete explanation of all the observed phenomena, they do provide a preliminary indication of the complex nature of the chemical composition and structure of the inclusions.

An outstanding, and at first sight surprising observation was that the pure PHB which constitutes nearly 90% of the inclusions which, in situ, are so strongly sudanophilic, was itself entirely devoid of sudanophilic properties. This was so when both thin films and small

pieces of the polymer extracted by either hypochlorite or chloroform, were tested with various solutions of Sudan black, the results being observed both microscopically and with the naked eye. Furthermore, intact isolated lipid inclusions were readily rendered non-sudanophilic by certain procedures (treatment with hypochlorite, vigorous shaking, and centrifugation) which did not remove or destroy the PHB. This observation that PHB is unstainable with Sudan black is nevertheless in agreement with the principle enunciated by Cain (1950), that solid lipids are not stainable with the oil-soluble dyes, PHB being a solid at the temperature of staining. Since the lipid inclusions in situ are intensely sudanophilic, it is concluded that they must contain a liquid or semi-solid lipid in addition to the PHB polymer.

Unfortunately, the nature and amount of this second liquid and sudanophilic component of the inclusions could not be definitely established. This was due to its very labile nature. It was rapidly destroyed (or removed or rendered non-sudanophilic) when the inclusions were isolated by the hypochlorite method and when the sudanophilic inclusions liberated by lysozyme or by mechanical disruption of the cells were deposited by centrifugation. Thus it was not possible to obtain, for measurement and analysis, a concentrated preparation of the inclusions in a fully sudanophilic state.

The observation that the sudanophilic component could be removed by simple centrifugation of the lysozyme-isolated inclusions suggested that this component lay on the surface of the inclusion body. As was previously

described (p. 166), a liquid lipid of low specific gravity might be torn from the surface of the inclusion body by the considerable stresses imposed on such material during centrifugation. Further evidence that the sudanophilic component of the inclusions lay on the surface of the inclusion body was provided by the observation of a peripheral concentration of Sudan black in the stained preparations of isolated inclusions from which part of the sudanophilic material had been removed by a brief treatment with acetone. It was not possible to determine what proportion of the inclusions _in situ_ was taken up by this sudanophilic component. Microscopic observations of individual inclusions during the course of hypochlorite lysis of the cells failed to show any change in their diameter . However, this observation is of little significance, since a considerable proportion of the volume of a spherical inclusion body could be removed from its surface before any microscopically detectable change in its diameter would become apparent. Thus, assuming that a change in diameter of 0.2μ would be barely detectable, a surface film of thickness 0.1μ would have to be removed before any change in the diameter of the inclusion became visible. A surface film of this thickness would constitute about 50% of the volume of an inclusion body of diameter 1.0μ, and correspondingly greater proportions of inclusions of smaller diameters. Thus the lack of any microscopically detectable change in the diameters of the inclusion bodies during isolation with hypochlorite does not rule out the possibility that a liquid sudanophilic substance constituted a considerable fraction of the volume of the inclusions, that it was situated on their surface, and was removed by hypochlorite treatment.

The microchemical tests on the fully sudanophilic inclusions freshly liberated from mechanically disrupted cells revealed that this sudanophilic component was neutral (judged by Nile blue staining) and insoluble in ether, though it was readily dissolved by acetone and some other moderately polar solvents. These properties clearly distinguished this strongly sudanophilic liquid lipid from a third substance, the ether-soluble, acidic, semi-solid "fatty" material, which was found to constitute 11% of the hypochlorite-isolated inclusions.

The significance of this latter component of the hypochlorite-isolated inclusions is not clear. It is not even certain that it was in fact a component of the inclusions prior to their isolation with hypochlorite. It is conceivable that it simply represented lipids from other parts of the cells which were liberated on treatment with hypochlorite and simply adhered to, or were adsorbed by the inclusions. This idea is contradicted however by the finding that the proportion of this material in a number of different preparations of the isolated inclusions was constant, and that Nile blue, (which stained the ether-soluble material but not the PHB), stained all the hypochlorite-isolated inclusion bodies equally intensely. If this ether soluble material was simply adsorbed by the inclusions during their isolation, one would have expected some variation in the amount of the material in the isolated inclusions, and some non-uniformity of staining. It would therefore seem more likely that this ether-soluble "fatty" material was in fact a third component of the inclusions

in situ. There is no evidence to the contrary, and even the red staining observed when the in situ inclusions were stained with Nile blue (indicating the presence of a neutral lipid) does not preclude the simultaneous existence of an acidic lipid. Another barely conceivable possibility is that the ether-soluble, semi-solid, acidic "fatty" substance was not a third component but the end product of the action of hypochlorite on the ether-insoluble, neutral, and strongly sudanophilic liquid lipid postulated to account for the intensely sudanophilic nature of the in situ inclusions. Whatever the origin of the ether-soluble component of the hypochlorite-isolated inclusions, this material, per se, could not account for the intensely sudanophilic nature of the inclusions in situ, since it failed to render the isolated inclusions sudanophilic, although constituting 11% of their mass. In view of this latter finding it was surprising that the ether-soluble lipid, when extracted from the isolated inclusions, was apparently moderately sudanophilic. Microscopic tests showed that small droplets of it appeared as strongly stained by Sudan black, as were small droplets of the strongly sudanophilic lipid, triolein. Assuming that the ether-soluble acidic material in the isolated inclusions was accessible to the stain (and it was certainly accessible to ether, so this seems a reasonable assumption), one can only suppose that the mode of distribution of the material in the intact, isolated inclusions prevented their being visibly stained with Sudan black. If the ether-soluble material was distributed throughout the inclusion body, its dispersal may have

rendered it invisible when stained. Alternatively, it may have been admixed with the polymer in the form of a solid solution, which being a solid, would have been non-sudanophilic. Whatever the explanation of these findings, it is probable that the intensely sudanophilic nature of the inclusions <u>in situ</u> is due to the presence at their surface of a liquid and intensely sudanophilic lipid as suggested above.

The electron microscopic appearance of the hypochlorite isolated inclusions suggested that the PHB core of the inclusions is not a solid ball of material, since it apparently collapsed considerably on drying. This suggests that in the inclusions <u>in situ</u>, the PHB may be admixed with other material which becomes removed by hypochlorite. However, the staining tests on the <u>in situ</u> inclusions failed to reveal any materials other than the sudanophilic component, and chemical analysis of the hypochlorite isolated inclusions showed only the presence of PHB and the ether-soluble acidic "fatty" material. Moreover, it may be mentioned that preliminary observations indicate that the proportion of the total volume of the cell that is occupied by the lipid inclusions is not very much greater than that occupied by the measured content of PHB. This suggests that the inclusions do not contain large amounts of water or materials other than PHB.

These observations enable one to formulate a tentative hypothesis. They suggest that the lipid inclusions of this group of organisms may consist of a central core of PHB admixed with an ether-soluble, semi-solid acidic lipid and

perhaps other substances, the whole being coated with a moderately thin but strongly sudanophilic film of an acetone-soluble and ether-insoluble liquid lipid.

Turning to the results of the growth experiments, there are several interesting features to be discussed. In the first place, support was obtained for the generality of the suggestion made in the Introduction that the production of a lifeless cell material will be increased when the total growth is limited by deficiency of any nutrient which is not an essential precursor or a source of energy for the synthesis of that material.

Evidence supporting this contention was provided by the observation that limitation of the total amount of growth on glucose-containing media by exhaustion of potassium or phosphorus, resulted in there being a greatly increased content of PHB in the stationary phase cells. Presumably growth was halted before all the glucose in the medium was exhausted, and the resulting surplus of glucose was used for this increased PHB production. Another instance of increased production of PHB, presumably through the same mechanism, was observed when a great excess of glucose was added to the balanced synthetic medium. It was not known in this instance what factor was limiting growth (and preventing full utilisation of the glucose for this purpose) except that it was not deficiency of potassium, nitrogen, phosphorus or sulphur, or an unfavourable pH; it may have been the accumulation of some inhibitory waste product of growth, or deficiency

of an unidentified mineral. There is however, little doubt that the increased production of PHB by the cultures on these media containing added glucose was caused by the same type of mechanism that led to increased production of PHB on the potassium and phosphorus deficient media; growth was halted before all the glucose had been used up, and part of the surplus glucose was then used by the cells for the synthesis of PHB.

The results obtained with the cultures whose growth had been limited by deficiency of nitrogen or sulphur, showed, however, that not all growth-limiting deficiencies will lead to increased production of PHB. In these cases, growth was halted before exhaustion of all the glucose in the medium, yet there was no greatly increased production of PHB from the resulting surplus glucose. These findings however, do not disprove the general validity of the principle suggested above, if it is assumed that nitrogen and sulphur are essential to synthesis of the PHB polymer. In contrast, potassium and phosphorus are apparently not essential to its production since greatly increased amounts were formed by the potassium and phosphorus deficient cultures.

The increased production of PHB by the potassium and phosphorus deficient cultures indicates that potassium and phosphorus are not consumed for synthesis of the lipid inclusions. On the other hand, it does not prove that the formation of PHB is entirely independent of these elements. The cells will always contain minimal quantities of them, and if the enzymes responsible for PHB synthesis require them only in catalytic roles or as

activators, the enzymes will function normally and produce the polymer from the excess of carbon and energy source in the medium.

Turning to the finding that PHB production was not greatly increased when growth was halted by exhaustion of nitrogen or sulphur, it is necessary to consider why these elements should be needed for the continued production of PHB from the surplus glucose in the media concerned. In the first place, these elements do not seem to be constituents of the inclusions; at least they are absent from the main component, PHB. A possible explanation is that they are required for the protein moeity of the PHB-forming enzymes. The increasing amount of PHB in the cultures during the phase of active growth showed that the necessary enzymes were present at that stage, and the question arises as to why these enzymes did not continue to produce PHB once growth had stopped. It is suggested that a possible solution to this problem may be that as growth came to a halt on these nitrogen and sulphur deficient media, the cell started to break down any proteins that were not vital to its immediate existence, in order to utilise the sulphur and nitrogen thus acquired for the synthesis of more "essential" proteins. This explanation would certainly be in accord with modern concepts of the "dispensable" nature of certain types of enzyme , as shown by the work of Spiegelman and Dunn (1948), and Virtanen and de Ley (1948). If PHB is simply a carbon and energy storage material, it would seem

a reasonable assumption that the enzymes responsible for its formation would be less "essential" to the cell than many others; they would be among the first to be thus cannibalised and no further PHB could be produced once growth had stopped.

Another interesting feature of these experiments concerned the rate of PHB production in the logarithmic phase of growth. It was found that when the total growth was limited by exhaustion of glucose, and when it was limited by the unknown factor operating on the balanced media to which an excess of glucose had been added, the total rate of production of PHB in the early stages of growth was low and at a roughly constant rate. If the amount of PHB-forming enzyme in the culture had been increasing in parallel with the amount of growth, one would have expected the rate of production of PHB to increase logarithmically. It would appear therefore that the conditions of logarithmic growth on these media did not favour the production of this relatively non-essential enzyme. A different course of events was observed on the media on which growth was halted by exhaustion of potassium. On these media (and to a lesser extent on the highly sulphur-deficient medium), the total rate of production of PHB started to rise from the moment cell division commenced, and the rate of production rose sharply throughout growth. The rate of growth of these cultures was appreciably slower than on the glucose-limited and glucose rich media, the relative deficiency of potassium (or sulphur) limiting the rate of growth as

well as the total crop. It is suggested that the deficiency did not exert a proportionate effect on the rate of synthesis of the PHB-forming enzymes, thus permitting these to increase in amount. Provided they were kept saturated with their substrate (a reasonable assumption in view of the known excess of glucose in the medium) the total rate of production of PHB would increase. Thus, these potassium and sulphur deficient cultures may have been exhibiting a "rate-limiting effect" as discussed in the Introduction (p.48). These suggestions are necessarily tentative. Experiments with washed suspensions of non-proliferating cells might prove fruitful; they should at any rate be capable of demonstrating whether or not the amount of PHB-forming enzyme in the culture does vary in the manner suggested.

The observations of volutin production showed that this material was laid down in greatly increased amounts in cultures whose growth had been limited by exhaustion of nitrogen and sulphur, and by the unknown factor operating on the glucose-rich media. Volutin was not present, however, in more than trace amounts, in stationary phase cells whose growth had been limited by exhaustion of glucose or potassium, and was completely absent from cultures grown on phosphorus deficient media. These findings are in agreement with the observations of Smith _et al_ (1954) on the formation of volutin by _Aero. aerogenes_. Apparently the synthesis of volutin requires sources of energy, phosphorus and potassium.

It is thought that the observed increased

production of volutin was a result of the same type of mechanism that led to the increased production of PHB, i.e. limitation of growth by deficiency of any nutrient that was not an essential precursor or a source of energy for its synthesis.

SUMMARY.

SUMMARY

(1) A comprehensive study was made of the staining and microchemical properties of the intracellular inclusion bodies of B. cereus and certain other Bacillus species. In an asporogenous strain of B. cereus (AC.1), spherical lipid inclusions, which were stained an opaque blue-black with Sudan black, were rarely absent, and were especially large and numerous in cultures on media rich in glucose as carbon and energy source. They were distributed throughout the cells, generally not at the poles. With the phase contrast microscope they appeared very refractile, varying in size from just resolvable specks to bodies as much as 1μ in diameter. The only other kind of inclusion in the asporogenous strain were granules of volutin. Recognised by their metachromatic staining properties, these occurred almost invariably at the poles of the cell, were generally present in only small amounts, and were distinct and separate from the lipid inclusions. With the phase contrast microscope, they appeared much less refractile than the lipid inclusions. Microscopic staining tests failed to show any substances to be present in the lipid inclusions other than neutral, sudanophilic, (and thus presumably liquid) lipids. The observations of Knaysi (1945b, 1946a) of the presence of protein and Feulgen +ve materials were not confirmed.

(2) The observation of Meyer (1901b) that Bacillus cells are soluble in alkaline sodium hypochlorite, was confirmed. Under appropriate conditions this reagent completely

dissolved the cells (and if present, the spores) but not the volutin and lipid inclusion bodies. Proof of the identity of the latter hypochlorite resistant bodies was provided by continuous phase-contrast observation of the lysis of individual cells(both unstained, and when stained for volutin or lipid) by hypochlorite.

(3) Turbidimetric observations showed that the final turbidity of a given mass of cells suspended in hypochlorite almost directly proportional to the cell content of lipid inclusions. The presence of volutin granules had only a small effect on the final turbidity, presumably because of their relatively low refractility.

(4) On the basis of these observations, a simple method was developed for rapid measurement of the lipid inclusion content of small samples of Bacillus cultures. A suspension of the culture is centrifuged and the cells resuspended in the same volume of a standard alkaline hypochlorite solution at 37°C for 90 minutes. With the aid of a calibration curve the final turbidity of the suspension is converted into an estimate of the mass of lipid inclusions present.

(5) A method involving use of hypochlorite was developed for the large-scale isolation of lipid inclusions. Any volutin present in the isolated material is removed by dialysis subsequent to the primary extraction, since volutin was found to be water-soluble. Analysis of the lipid inclusions thus isolated revealed the presence of two lipid components; there were traces only of nitrogen,

phosphorus, polysaccharide and ash.

The lipid components were : (a) an ether-soluble, acidic, semi-solid fat-like material (11% of the mass of the inclusions) and (b) an ether-insoluble solid polymer of β-hydroxybutyric acid (PHB) (89% of the inclusions' mass). The latter was similar to the PHB isolated by Lemoigne and his colleagues from B.megaterium, and supposed by them to be derived from the lipid inclusions; it differed only in having a slightly higher melting point. The first direct proof of the existence of this type of material in the lipid inclusions of the Bacillus genus was thus supplied. It was further shown that the PHB from B.cereus was not stainable with Sudan black; it had a molecular weight of the order of 5,000, a specific gravity of 1.24, and on ultracentrifugation seemed to be a homogenous molecular species. Microscopic tests with the extracted ether-soluble, acidic fat-like component of the inclusions showed that it was moderately sudanophilic, although its presence failed to render the intact hypochlorite-isolated inclusions sudanophilic. The primary source of this ether-soluble material was not finally determined; certain observations however, suggested that it was in fact a component of the inclusions *in situ*, though it may have been chemically altered by the hypochlorite during their isolation.

(6) The proportions of the ether-soluble acidic lipid and PHB in the hypochlorite-isolated inclusions of strain AC.1 were found to be constant (11% and 89% respectively) for a number of different cultures containing from 9% to 40%

PHB. This meant that the hypochlorite method of estimating lipid inclusions could be used to furnish an accurate estimate of the PHB content of the cells, simply by multiplying the figure for lipid inclusions by 0.89.

(7) Estimations of PHB were made in parallel on several different cultures of B.cereus (AC.1) by the hypochlorite method and by Lemoigne's chloroform extraction technique; the two methods gave virtually identical results for cultures containing from 9% to 40% PHB, the hypochlorite method never erring by more than 3% from the mean of the two methods. Thus, in the case of cultures containing moderate or large amounts of PHB the hypochlorite method was found to be just as accurate as chloroform extraction, over which it has the advantage of requiring only a very small amount (about 10mg. dry weight) of cells, and of being much more rapid. In the case of cells containing only traces of PHB, however, the hypochlorite method indicated about ten times the amount of PHB shown to be present by chloroform extraction. This finding showed that the hypochlorite method cannot be used to give accurate estimates of PHB in cells containing much less than 9% of the polymer, though there is no reason to suppose that its estimate of "lipid inclusions" in such cultures is not accurate.

(8) The hypochlorite-isolated inclusions, and those liberated from the cells by lysozyme treatment or by mechanical disruption were subjected to microscopic staining and solubility tests. It was found that the

hypochlorite-isolated inclusions were, like the pure PHB, non-sudanophilic, but Nile blue staining revealed the presence in them of the ether-soluble acidic lipid. The lysozyme and mechanically-isolated inclusions, on the other hand, were apparently identical to the inclusions _in situ_, and were stained an opaque blue-black with Sudan black. The sudanophilic nature of the isolated inclusions was, however, readily destroyed by hypochlorite treatment, by vigorous shaking, by centrifugation or by treatment with acetone. It could not be destroyed, however, by immersing the inclusions in ether at room temperature. It was thus deduced that in addition to the non-sudanophilic, solid PHB, and the ether-soluble acidic fat-like material, the lysozyme and mechanically isolated inclusions(as well as those _in situ_)contained strongly sudanophilic acetone-soluble, ether-insoluble liquid lipid. Its lability to centrifugation unfortunately prevented the large-scale isolation of the fully sudanophilic inclusions, and thus prohibited their complete chemical analysis.

(9) On the basis of these and electron microscopic observations, it is suggested that _in situ_, the lipid inclusions of the _Bacillus_ genus consist of a central core of ether-insoluble, solid, and non-sudanophilic PHB, perhaps admixed with an ether-soluble, acidic, semi-solid (fat-like) material, this core being coated with a moderately thin film of an intensely sudanophilic ether-insoluble, and acetone-soluble, liquid lipid which is responsible for their "fat staining" properties.

(10) Using the hypochlorite method, the formation of PHB by B.cereus (AC.1) was followed through all stages of culture on a variety of different media. The growth of the organism at all stages was measured by estimating cell nitrogen, and in some cases the fluctuations of intracellular polysaccharide were followed by the anthrone method, and of volutin by microscopic examination of Albert stained smears.

(11) The content of PHB was greatly increased in stationary phase cultures on glucose containing media, under three conditions; (a) when the total growth was limited by exhaustion of the potassium source; (b) when it was limited by exhaustion of the phosphorus source; and (c) when a great excess of glucose was added to an otherwise balanced synthetic medium, the factor limiting total growth on this last medium being unknown. In these three cases, growth was halted before the glucose was exhausted, and the increased PHB production is thought to have been due to the resultant surplus of glucose.

(12) The stationary phase cell content of PHB was minimal when the growth was halted by exhaustion of glucose, the nitrogen source or the sulphur source. It is thought that in cultures on the last two media, the exhaustion of nitrogen or sulphur halting growth caused the cells to break down relatively non-essential enzymes including the PHB-forming enzyme, in order to utilise their S and N for the synthesis of more essential proteins; thus, despite the surplus glucose no PHB could be synthesised once growth

had stopped in these cultures.

(13) PHB was produced by the actively growing cells of cultures on all media, regardless of the final cell content attained in the stationary phase. On glucose deficient media, and on the otherwise balanced media containing a great excess of added glucose, the total rate of PHB production in the culture was low and roughly constant during the early stages of growth. On the potassium deficient media on the other hand, the total rate of production of PHB during the early stages of culture increased rapidly. The implications of these findings are discussed.

(14) Lipid inclusions were not formed in more than trace amounts under anaerobic conditions.

(15) The stationary phase cells had increased contents of intracellular polysaccharide when the total growth was halted by exhaustion of the nitrogen source, and when a greatly increased amount of glucose had been added to the otherwise balanced medium. The polysaccharide content was minimal in stationary phase cells whose growth had been halted by deficiency of glucose or potassium.

(16) Volutin was present in greatly increased amounts in stationary phase cultures whose growth had been halted by exhaustion of nitrogen or sulphur, and in the otherwise balanced medium containing an added excess of glucose. Only minimal amounts of volutin were present in stationary phase cultures whose growth had been halted by exhaustion of glucose and potassium, and no volutin appeared at any

stage on phosphorus deficient media. It is concluded that the formation of volutin requires a supply of energy, phosphorus and potassium.

- - - - - - -

REFERENCES.

Almon, L., (1933). Cbl. Bakt., Abt. II, 87, 289.

Ackerman, G.A., (1952). Science, 115, 629.

Ashby, G.K., (1938). Science, 87, 443.

Baker, J.R., (1946). Quart.J.micro. Sci., 87, 441.

Baker, J.R., (1947). Ibid., 88, 463.

Bayne-Jones, S. & Petrilli, A., (1933). J.Bact., 25, 261.

Belin, P., (1926). Bull.soc.chim.biol., 8, 1081.

Bisset, K.A. & Hale, C.M.F., (1953). J.gen.Microbiol., 8, 442.

Bisset, K.A., (1955). J.gen.Microbiol., 13, 442.

Breed, R.S., Murray, E.G.D. & Hitchens, A.P., (1948). Bergey's Manual of Determinative Bacteriology. 6th edition. Bailliere, Tindall & Cox. London.

Bunge. (1895). Fortschritte der Medizin, 13. Cited in Grimme (1902c).

Burdon, K.L., Stokes, J.C. & Kimbrough, C.E., (1941). J.Bact., 43, 717.

Burdon, K.L., (1946). J.Bact., 52, 665.

Cain, A.J., (1950). Biol. Rev., 25, 73.

Cain, A.J., (1947). Quart. J.micr.Sci., 88, 383.

Chapman, G.B., (1956). J.Bact., 71, 348.

Cramer, E., (1893). Arch.f.Hyg., 16, 151.

Daddi, A. (1896). Arch.ital.Biol., 26, 143.

Davis, J.C., Winterscheid, L.C., Hartman, P.E. & Mudd, S., (1953). J.histochem. & cytochem., 1, 123.

Davis, J.C. & Mudd, S., (1955). J.Bact., 69, 413.

Dawson, A.I., (1919). J.Bact., 4, 133.

DeLamater, E.D., (1956). 'Bacterial Chromosomes and their mechanism of division'. 6th Symposium of the Society for General Microbiology, London, 1956, p.219.

Dietrich, A. & Liebermeister, E., (1902). Cbl.Bakt., Abt.I, Orig., 32, 858.

Dondero, N., Adler, H. & Zelle, M., (1954). J.Bact., 68, 483.

Dudman, W.F., (1954). Ph.D. Thesis, Edinburgh.

Duguid, J.P., (1948). J.Path.Bact., 60, 265.

Duguid, J.P. & Wilkinson, J.F., (1953). J.gen.Microbiol., 9, 174.

Duguid, J.P., Smith, I.W. & Wilkinson, J.F., (1954). J.Path.Bact., 67, 289.

Dyar, M.T., (1947). J.Bact., 54, 483.

Dyar, M.T., (1948). J.Bact., 56, 821.

Eisenberg, P., (1909). Cbl.Bakt., Abt.I, Orig., 48, 257.

Ellis, D., (1902-3). Cbl.Bakt., Abt.I, Orig., 33, 1.

Elser, W.J. & Huntoon, F.M., (1909). J.Med.Research, 20, 371.

Fairbairn, N.J., (1953). Chem. and Ind., p. 86.

Fiegl, F., (1947). Qualitative Analysis by Spot Tests. Elsevier Publishing Co., Inc., New York. 3rd edition, p.408.

Foster, J.W., (1947). Bact.Rev., 11, 167.

Gomori, G., (1952a). 'Microscopic Histochemistry': Principles and Practice. Univ. of Chicago Press, Chicago, p.94.

Gomori, G., (1952b). Ibid., pp.104, 111.

Goris, A., (1920). Ann.inst.Pasteur, 34, 497.

Grelet, N., (1952). Ann.inst.Pasteur, 82, 66.

Grimme, A., (1902a). Cbl.Bakt., Abt.I, Orig., 32, 1.

Grimme, A., (1902b). Ibid., 32, 81.

Grimme, A., (1902c). Ibid., 32, 241.

Grimme, A., (1904). Cbl.Bakt., Abt.I, Orig., 36, 352.

Guilliermond, A., (1932). Compt.rend., 194, 2322.

Hartman, T.L., (1940). Stain.Tech., 15, 23.

Holter, H. & Zeuthen, E., (1948). Compt.rend.Lab.Carlsberg, Sér.chim., 26, 227.

Hoogerheide, J.C., (1939). J.Bact., 38, 367.

Hopkins, E.W. & Peterson, W.H., (1930). J.Bact., 19, 9.

Imsenecki, A., (1945). J.Bact., 49, 1.

Képès, A. & Péaud-Lenoël, C., (1952). Compt.rend., 234, 756.

King, R.L. & Beams, H.W., (1942). J.Bact., 44, 597.

Kleinzeller, A., (1944). Biochem.J., 38, 480.

Knaysi, G., (1929). J.Infectious Diseases, 45, 13.

Knaysi, G., (1942). J.Bact., 43, 365.

Knaysi, G., (1945a). J.Bact., 49, 617.

Knaysi, G., (1945b). Science, 102, 424.

Knaysi, G., (1946a). J.Bact., 51, 113.

Knaysi, G., (1946b). Ibid, 51, 187.

Knaysi, G., (1946c). Ibid., 52, 487.

Knaysi, G., (1951a). 'Elements of Bacterial Cytology'. 2nd edition. Comstock Publishing Company, Inc., Ithaca, New York, p.193.

Knaysi, G., (1951b). Ibid., p.194.

Knaysi, G., (1951c). Ibid., p.233.

Knaysi, G., (1951d). Ibid., p.255.

Knight, B.C.J.G. & Proom, H., (1950). J.gen.Microbiol., 4, 508.

Lamanna, C. & Mallette, M.F., (1953). Basic bacteriology; its biological and chemical background. Williams & Wilkins, Co., Baltimore. 1st edition, p.267.

Larson, L.W. & Larson, W.P., (1922). J.Infectious Diseases, 31, 407.

Laybourne, R.L., (1924). J.Am.Med.Assoc., 83, 121.

Leach, E.H., (1938). J.Path.Bact., 47, 635.

Lemoigne, M., (1923). Compt.rend., 176, 1761.

Lemoigne, M., (1924a). Ibid., 178, 1093.

Lemoigne, M., (1924b). Ibid., 178, 253.

Lemoigne, M., (1925a). Compt.rend., 180, 1539.

Lemoigne, M., (1925b). Ann.inst.Pasteur, 39, 144.

Lemoigne, M., (1926a). Compt.rend.soc.biol., 94, 1921.

Lemoigne, M., (1926b). Ibid., 95, 1359.

Lemoigne, M., (1926c). Bull.soc.chim.biol., 8, 770.

Lemoigne, M., (1927a). Ibid., 9, 446.

Lemoigne, M., (1927b). Ann.inst.Pasteur, 41, 148.

Lemoigne, M. & Roukhelman, N., (1940a). Compt.rend., 211, 673.

Lemoigne, M. & Roukhelman, N., (1940b). Ann.Ferment., 5, 527.

Lemoigne, M., Delaporte, B. & Croson, M., (1943). Compt.rend., 216, 819.

Lemoigne, M. & Girard, H., (1943). Compt.rend., 217, 557.

Lemoigne, M., Sanchez, G. & Girard, H., (1943). Ann.inst.Pasteur, 69, 187.

Lemoigne, M., Delaporte, B. & Croson, M., (1944). Ibid., 70, 224.

Lemoigne, M. & Girard, H., (1944). Bull.soc.chim.biol., 26, 443.

Lemoigne, M., Grelet, N., Croson, M. & Le Treis, M., (1945). Bull.soc. chim.biol., 27, 90.

Lemoigne, M., (1946). Helv.Chim.Acta., 29, 1303.

Lemoigne, M., (1947). Congres International des Industries de Fermentation, Gand.

Lemoigne, M., Péaud-Lenoël, C. & Croson, M., (1949a). Compt.rend., 229, 476.

Lemoigne, M., Péaud-Lenoël, C. & Croson, M., (1949b). Ann.inst.Pasteur, 77, 211.

Lemoigne, M. & Péaud-Lenoël, C., (1949). Compt.rend., 228, 1529.

Lemoigne, M., Milhaud, G. & Croson, M., (1949). Bull.soc.chim.biol., 31, 1587.

Lemoigne, M., Grelet, N. & Croson, M., (1950). Bull.soc.chim.biol., 32, 719.

Lemoigne, M., Péaud-Lenoël, C. & Croson, M., (1950). Ann.inst.Pasteur, 78, 705.

Lemoigne, M., Girard, H. & Jacobelli, G., (1951). Ann.inst.Pasteur, 80, 428.

Lewis, I.M., (1934). J.Bact., 28, 133.

Lewis, I.M., (1937). Ibid., 34, 191.

Lewis, I.M., (1938). Ibid., 35, 573.

Lewis, I.M., (1940). Ibid., 40, 271.

Lewis, I.M., (1941). Bact.Rev., 5, 181.

Lindegren, C.C., (1949). The Yeast Cell. Educational Publishers, Inc., St.Louis. 1st edition.

Lindner & Unger, (1919). Woch Braueriei, 36, 188.

Lorraine-Smith, J., (1908). J.Path.Bact., 12, 1.

Lyons, R.E., (1897). Arch.f.Hyg., 28, 30.

Mackie, T.J. & McCartney, J.E., (1953). Handbook of Practical Bacteriology. Livingstone, Edinburgh. 9th edition.

Mazia, D., Brewer, P.A. & Alfert, M., (1953). Biol.Bull., 104-105, 57.

Meyer, A., (1899). Flora, 86, 428.

Meyer, A., (1901a). Ber.d.deutsch.bot.Gesellsch., 19, 428.

Meyer, A., (1901b). Cbl.Bakt., Abt.I, Orig., 29, 809.

Meyer, A., (1903). Cbl.Bakt., Abt.I, Orig., 34, 578.

Meyer, A., (1912a). 'Die Zelle der Bakterien'. G.Fischer, Jena. (pp.225-231).

Meyer, A., (1912b). Ibid., (p.202).

Moller, H., (1892). Ber.d.deutsch.bot.Gesellsch., 10, 242.

Mudd, S., Brodie, A., Winterscheid, L.C., Hartman, P.E., Beutner, E.H. & McLean, R.A., (1951). J.Bact., 62, 729.

Muller, A. & Stapp, C., (1926). Arb.biol.Reichs.Land.u Forst w., Berlin-Dahlem, 14, 455. Cited in Lewis, 1941, 1938.

Murray, R.G.E. & Truant, J.P., (1954). J.Bact., 67, 13.

Northrop, J.H., (1951). J.Gen.Physiol., 34, 715.

Oparin, A.I., (1938). 'The Origin of Life', translated by S.Morgulis. Macmillan, New York.

Péaud-Lenoël, C., (1949). Bull.soc.chim.biol., 31, 1562.

Pochon, J., Tchan, Y.T. & Wang, T.L., (1948). Ann.inst.Pasteur, 74, 182.

Preisz, H., (1904). Cbl.Bakt., Abt.I, Orig., 35, 537.

Prill, E.A., Wenck, P.R. & Peterson, W.H., (1935). Biochem.J., 29, 21.

Rippel, A., (1943). Naturwissenschaften, 31, 248.

Robinow, C.F., (1943-4). J.Hyg., Camb., 43, 413.

Scandrett, F.J., (1953). Analyst, 78, 734.

Smith, I.W., Wilkinson, J.F. & Duguid, J.P., (1954). J.Bact., 68, 450.

Smithies, W.R., Gibbons, N.E. & Bayley, S.T., (1955). Can.J.Microbiol., 1, 605.

Snedecor, G.W., (1940). 'Statistical Methods'. Iowa State College Press, Ames, Iowa. 3rd edition, p.102.

Spiegelman, S. & Dunn, R., (1948). J.Gen.Physiol., 31, 153.

Stapp, C., (1924). Cbl.Bakt., Abt.II, 61, 276.

Starkey, R.L. & De D.K., (1939). Soil Sci, 47, 329.

Tinelli, R., (1955a). Ann.inst.Pasteur, 88, 212.

Tinelli, R., (1955b). Ibid., 88, 364.

Tinelli, R., (1955c). Ibid., 88, 642.

Virtanen, A.I., and de Ley, J., (1948). Arch. Biochem., 16, 169.

Weibull, C., (1953a). J.Bact., 66, 688.

Weibull, C., (1953b). Ibid., 66, 696.

Weibull, C., (1956). 6th Symposium of the Society for General Microbiology, London, 1956, p.118.

Wilkinson, J.F., Duguid, J.P. & Edmunds, P.N., (1954). J.gen.Microbiol., 11, 59.

Worley, G. & Young, G., (1945). J.Bact., 49, 97.

Zikes, H., (1922). Cbl.Bakt., Abt.II, 57, 21.

ACKNOWLEDGEMENTS

The author is deeply indebted to Drs. J.P. Duguid and J.F. Wilkinson for their invaluable guidance, criticism, and inexhaustible patience in the supervision of the work embodied in this Thesis. The author also expresses his thanks to the University of Edinburgh for two successive awards of Post-graduate Studentships for the years 1953 - 1955, and to the Agricultural Research Council for the award of a Research Studentship for 1955 - 1956.

- - - - -