

STUDIES ON THE METABOLISM OF

BACTERIAL LIPID GRANULES.

By

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Part of the work embodied in this Thesis formed the subject of a paper read before the Royal Physical Society of Edinburgh on 24th February, 1958, and is subsequently to be published, viz:-

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INTRODUCTION.

1. Prefatory Remarks.

The fundamental unity of biochemistry has become increasingly evident as discovery after discovery lends its weight to this concept. Many examples of the similarity between widely divergent forms of life could be cited in this connection. Let it suffice, however, to call to mind the omnipresence of carbon compounds and more specifically proteins and nucleic acids which apparently are constituents of all forms of life known on this planet. Not only do these proteins and nucleic acids have similar properties but the component amino acids of the proteins and nitrogenous bases of the nucleic acids are strictly limited in number.

It has been concluded from this underlying integrity that one specific biopoietic event must have been responsible for the biological world as we now know it. By this assumption it is supposed that the event in question took place at an indeterminate time in the past history of the Earth possibly in the order of 2000 million years ago. Contemporary forms of life are thought to have arisen by evolutionary processes resulting in (apart from secondary degeneration) increasing complexity and specialisation.

This point of view has recently been criticised by Pirie (1957) who sees in the unity of biochemistry a condition forced upon primeval dissimilar forms, arising from several biopoietic events, by the terrestrial environment. Thus the most successful biochemical

system would be selected. When viewed from this perspective the occurrence of "peculiar" elements in biological processes takes on a more plausible complexion. That is, that these unusual substances can be explained by the assumption that they represent legacies from a more ancient order where many more elements were concerned in biological functions. Examples which might be taken are a blood pigment of ascidians which contains vanadium (Henze, 1911) and the skeletal function of mineral substances, in place of proteins as in more complex forms, in many coelenterates. Pirie goes on to suggest that one of the most fruitful sources for discovery of these esoteric processes, which might reveal something of the archetypal form, could be the unspecialised saprophytic bacteria. Not the autotrophs, since they must have journeyed far on the road of specialisation, and not parasitic heterotrophs, as they depend upon relatively highly developed complex forms of life for their existence.

Such a suggestion is highly speculative. It is true that there is perhaps a greater diversity of microbial constituents in comparison to those of other life forms and it seems clear that they possess highways and byways of metabolism which occur solely in micro-organisms. Though these facts may allude to such a period of diversity as is postulated by Pirie, other evidence must be considered. For example, in view of their relatively short generation time and haploid structure in addition to their probable early geological

appearance, micro-organisms must have had many times the number of opportunities of most other forms of life to evolve different metabolic pathways.

In some species of the genera Bacillus and Azotobacter there occurs, by the polymerisation of β -hydroxybutyric acid, a compound which appears as a major constituent of certain granules within their cytoplasm. This polymer, which has been called poly- β -hydroxybutyrate (PHB), is apparently of solely microbial occurrence and is perhaps the result of a primeval metabolic pathway of the aforementioned type. It must be borne in mind, however, that its constituent monomer and the substances which probably serve as intermediates in its synthesis and degradation are materials which have a very widespread occurrence in biological systems. One's opinion may therefore incline to the other hypothesis that its existence merely represents a specialisation of metabolic activity developed by the organisms. It would be extremely difficult to decide which view, if either, is correct, and the matter must remain one of conjecture.

This thesis represents an attempt to solve the problem of the functional significance of this β -hydroxybutyric acid polymer and to elucidate to some extent the biochemical processes concerned in its synthesis and degradation.

2. Microbial Lipids.

(a) General Considerations.

In an attempt to place PHB in perspective biochemically, a short review of lipid materials occurring in microbes is proposed. In this context adherence to the definition of the term "lipid" given by Kenneth (1949) will be made; viz. heterogeneous compounds soluble in fats and their solvents, including fats, waxes, chromolipids, sterols, glycolipides, phospholipides. This assignation of lipids to a vaguely delineated class of compounds provides a definition broad enough to encompass the diverse materials of this nature found in micro-organisms. The view may be taken that the inclusion of PHB within the meaning of "lipid" is an unwarranted stretching of the term but for the present purposes it has been assumed that it is sufficiently elastic to bear this stress. This opinion would seem to receive homologation from the works of authorities such as Bloor (1925, 1943) and Deuel (1951).

In recent years little work has been carried out on the qualitative or quantitative nature of microbial lipids, since it has become clear that other fields of study hold out more hope of usefully advancing knowledge. Most available references tend, therefore, to be out of date especially concerning methodology. The contemporary emphasis is clearly on discovering the function of

cell components and their metabolic interactions rather than being content simply with enumerating the substances themselves. A further difficulty, which is reflected in the paucity of information, is the nature of lipids as a class of compounds. They are awkward substances to work with.

(b) Free and Bound Lipids.

Microbial lipids may be divided into two artificial groups, free lipids, which are removed from the cells simply by treating with solvents, and bound lipids which are only so released after preliminary hydrolysis. In the first group are represented free fatty acids, neutral fats and waxes and phospholipids (Knaysi, 1951a) which may now be described briefly.

Palmitic, stearic and oleic acids and less frequently lauric, myristic, tetracosanic, cerotic, linoleic, butyric and caproic acids are among the free fatty acids encountered. Derivatives of these such as hydroxy-stearic acid and combinations like palmito-stearic acid have also been described. Of solely microbial occurrence are tuberculo-stearic, phthoic and diphtheric acids the first two of which occur in the tubercle bacillus (Anderson and Chargaff, 1929) and the last in the diphtheria bacillus (Chargaff, 1933).

The neutral fat of bacteria is apparently largely represented by esters of higher fatty acids with carbohydrate. Normally esters of fatty acids with glycerol are present only in small quantities or absent

altogether (Knaysi, 1951a). Thus bacterial lipid diverges from that usually encountered in animals where the largest part is triglyceride. These observations are supported by the work of Lemoigne, Milhaud and Croson (1949) who found no evidence of glycerol or sterol in their analyses of Bacillus megaterium. On the other hand Crowder and Anderson (1934) found that 12.5% of the neutral fat of Lactobacillus acidophilus was represented as glycerol and 2.5% as cholesterol. Clearly one cannot generalise on the constitution of microbial neutral fat.

Waxes comprise a large class of diverse substances into which are placed, somewhat arbitrarily, compounds occurring in various micro-organisms particularly the mycobacteria. There is a more comprehensive literature on the "waxes" of this genus because of the pathogenicity of some of its members for man and economically important animals. Many studies have been undertaken from a strictly utilitarian viewpoint, an attempt often being made to correlate the occurrence of certain substances in the organisms with their virulence. Bloch (1950) extracted a fatty material from tubercle bacilli which was highly toxic for mice and Asselineau and Lederer (1951) separated two mycolic acids by chromatography on alumina. These mycolic acids were for the most part combined with lipopolysaccharides of high molecular weight, the percentage content of which is apparently roughly correlated with virulence. Recently, infra-red

spectrophotometry has been employed in the recognition of certain waxes of mycobacterial species (Smith, Randall, Gastombide-Odier and Koevoet, 1957). Pound (1958) reports that a lipid from the tubercle bacillus, probably a phosphatide, causes an augmentation of antibody formation to ovalbumin and horse serum albumin if inoculated into guinea pigs with these antigens. A non-specific adjuvant action of lipid materials is not an uncommon observation however.

Phospholipids occurring in microbes are characterised by their low phosphorus and variable nitrogen content (Knaysi, 1951a). Amongst the few organisms which have been investigated in this connection is Lactobacillus acidophilus the phosphatide of which is composed of 55% fatty acids and 20% polysaccharide the remaining 25% being a mixture of glycerophosphoric acid and choline (Crowder and Anderson, 1934).

Bound lipids, it will be recalled, are only removed by solvents after preliminary hydrolysis, usually with hot hydrochloric acid. These lipids normally enter into the constitution of highly complex macromolecules which occur in the cell wall of many Gram-negative bacteria and are discussed below. Smedley-Maclean (1922) and Starkey (1946) found that only after preliminary hydrolysis was the greater part of yeast lipid removed by treatment with fat solvents. The latter author affirms that most of this lipid is bound in situ with sterol, protein or carbohydrate.

As regards quantitative estimates of microbial lipid few dogmatic assertions are possible and Knaysi (1951a) aphorises the position by stating that since all fat solvents do not remove fat from the organisms equally well and since the content of fat is related intimately to the environment, no accurate range of lipid can be given. Some approximate figures are quoted by this author and are reproduced here with a view to providing some indication of the range of lipid contents encountered in different organisms. The subject of microbial lipid granules, as distinct from the lipids themselves, merits separate treatment and will be considered later. In those organisms which do not form lipid inclusions the fat content is usually below 10%, for example Corynebacterium diphtheriae 5% Phytomonas tumefaciens 7% and Lactobacillus acidophilus 7%.

(c) Cytological Localisation of Lipids.

The localisation of lipids in cell structure has received comparatively little attention as an integrated study and it will be profitable to comment on the cytological entities with which these substances are associated.

Cell Wall. Reference has already been made to the occurrence of lipids in complex macromolecules which are present in the cell wall of many Gram-negative bacteria. Such complexes were extracted from the Shiga dysentery bacillus by Morgan (1937) and shown to be composed of

phospholipid, polypeptide and polysaccharide components. The complete substance was responsible for the antigenic behaviour of the organism and it was noted that the phospholipid component could be removed without affecting the antigenicity of the residue. Further analyses (Morgan and Partridge, 1940) showed that of the total complex 9 - 12% was accounted for by the phospholipid but its chemical characterisation was not undertaken nor was its nitrogenous component identified. Studies of this antigenic material were extended by Davies, Morgan and Mosimann (1954) but again no chemical analysis of the phospholipid was carried out. Working with several different Gram-negative organisms Westphal and Lüderitz (1954) extracted a lipopolysaccharide, located in their cell walls, which could be split into 60 - 75% phosphorylated polysaccharide and 25 - 40% lipid. Subsequently seven long-chain fatty acids were identified in hydrolysates of the lipid components derived from the cell walls of six strains of Escherichia coli and five species of Salmonella (Nowotny, Lüderitz and Westphal, 1958). The same series of fatty acids was found in the lipopolysaccharides from each of these organisms. A similar polysaccharide-lipid complex, probably located in the cell wall, has been investigated by Ikawa, Koepfli, Mudd and Niemann (1952, 1953a,b). The lipid constituent was analysed and found to contain the fatty acids lauric, myristic, β -hydroxy-myristic and palmitic and the nitrogenous components glucosamine, ethanolamine,

necrosamine and probably aspartic acid. Phosphoric acid was also present.

The cell walls of bacteria have also been studied by Salton (1952), who showed that 2.3% of the dry matter of the cell wall of Streptococcus faecalis could be extracted with an acidified alcohol/ether solvent. The material removed was a brown greasy solid but was not further characterised. In a more extensive survey (Salton, 1953) it was shown that lipid amounting to 7.2 - 8.6% of the dry weight could be extracted by ether from the dry cell walls of Escherichia coli but only 0.3% from the Gram-positive organism Bacillus subtilis. After hydrolysing with 6N HCl these quantities could be raised to 20.8 - 22.7% lipid from Escherichia coli and 2.5 - 2.7% lipid from Bacillus subtilis. No further examination of the lipid was carried out.

It will be appreciated from the above that information on the chemical character of lipids from bacterial cell walls is rather scanty. The empirical nature of our knowledge of these substances is epitomised by Salton (1956) who, after observing that the lipid components of bacterial cell walls are defined only by the methods of extraction, makes the telling comment that, "There is no information about the chemical nature of the cell-wall lipids". Apart from the data given above, this statement summarises the position.

Cytoplasmic Membrane. According to Knaysi (1951b) the cytoplasmic membrane, which occurs immediately beneath

the cell wall, always gives a positive test for protein and for lipid; he does not specify these tests, however. In certain Bacillus species the lipid content is said to be low in the early stages of active growth but reaches a high concentration as the culture approaches maturity. This is all rather meaningless unless exact details of the growth conditions, particularly with regard to available nutrients, are provided. They are not. It is stated that under aerobic conditions small particles of lipid material are eliminated into the cytoplasm prior to sporulation. Anaerobically, when sporulation does not take place, the lipid material continues to accumulate in the membrane. In view of the fact that treatment with fat solvents does not remove all the lipid from the membrane and because of the constant presence of protein, Knaysi (1946a) affirms that it is composed of lipoprotein perhaps associated with other complex radicles. Whatever the appearance of the original prints, the reproductions of photographs presented in support of these findings are unconvincing.

Although in many circumstances the test for protein was not stated, it would seem that most of the above observations were based on the Sharp test the procedure for which was given by Knaysi (1942). It consisted of placing a heat-fixed smear of the organism under test in a 5% glucose solution at 100° for 24 hr. when proteins were reported to be coloured brown. The test gave a positive result with albumin and "milk proteins" but no other proof of its authenticity was

provided. Since this is a most empirical procedure, results obtained by its use are open to some question without confirmatory evidence.

Mitchell and Moyle (1951) have suggested that a layer of phospho-lipid material may exist beneath the cell wall in Micrococcus pyogenes. This substance was assayed gravimetrically but its chemical composition was not fully investigated.

Other Cell Lipids. There is little information on the non-granular cytoplasmic lipids nor those which may occur as components of nuclear material. Knaysi, Hillier and Fabricant (1950) have stated, on the basis of cytochemical tests, that the bodies, which they supposed were nuclei of the avian strain of Mycobacterium tuberculosis, contained lipid substances. No characterisation of the chemical nature of these lipids was undertaken.

3. Microbial Lipid Granules.

In reviewing the subject of microbial lipids in the preceding Section it was felt that a discussion of lipid granules was best held over for separate treatment, by virtue of its greater relevance.

Lipid granules occur in micro-organisms as discrete intracytoplasmic structures which stain with fat soluble dyes. It seems that not all inclusions which stain with fat soluble dyes are to be regarded as lipid granules, however, since Knaysi (1951d) points out that the sulphur particles of Thiobacillus thio-oxidans are coloured with such reagents. It would appear, therefore, that it is justifiable to regard inclusions as consisting of lipid material only when such inclusions have been shown directly to possess fatty substances. Technically this is seldom a practicable proposition if the cytochemical criterion of ability to take up fat stains is denied. The latter more conventional standard will therefore be recognised in this Section, while bearing in mind its limitations.

These granules have been observed from the early days of bacteriology and more recently have been investigated by Lewis (1934, 1937, 1938) and by Knaysi (1945, 1946b, 1948). Burdon (1946) lists organisms which possess granules demonstrable by staining dry heat-fixed smears with Sudan black B. They were shown to occur in a wide variety of bacteria, yeasts and fungi. In bacteria they are obvious in the genera Acetobacter

Alcaligenes, Azotobacter, Bacillus, Clostridium,
Corynebacterium, Mycobacterium, Rhizobium and Spirillum.
Normally they do not occur in the Enterobacteriaceae,
Parvobacteriaceae or Pseudomonadaceae.

Many of these structures may be, and probably are, of a widely dissimilar character especially in view of the compendious nature of the definitive criterion employed in their recognition. In certain genera, however, it seems likely that they are composed at least partially, of a polymer of β -hydroxybutyric acid and these particular structures will now be considered.

The lipid inclusions of the genus Bacillus, with which the subject matter of this thesis is concerned, are found normally only in the larger-celled species. They are obvious structures often occupying a large part of the cytoplasmic volume, and were observed by the nineteenth century bacteriologists, though initially they could not be stained directly with the usual basic aniline dyes. It was shown by Eisenberg (1909), however, that if certain aniline dyes were treated with precipitating agents fat soluble colouring materials could be produced. Eisenberg noted that the granules of the Bacillus group could be stained with these fat soluble dyes, and concluded that they were composed of lipoidal substances encased in a membrane of protein. Lewis (1934) supported the opinion of Eisenberg that the inclusions were composed of a fatty material on the basis of extensive cytochemical investigations. Many studies of the granules have been made by Knaysi who has put

forward the idea (Knaysi, 1945) that they arise from the cytoplasmic membrane and have a similar chemical structure to it, which he asserts is lipoprotein. This contention is founded on the observation that cytochemical tests for protein (the previously discussed Sharp test) and fat are positive. More recently (Knaysi, 1951c) he has again categorically stated that the lipid of the granules in Bacillus species is undoubtedly combined with protein and probably other complex radicals. However, in another text in the same year (Knaysi, 1951e) he reports, without giving references, that a polymer consisting of β -hydroxybutyric acid units has been extracted from members of the genera Azotobacter and Bacillus. This reference to such a polymer by Knaysi seems to be the first to appear in the English language scientific literature and forms a link with a largely neglected series of researches carried out by Lemoigne and his collaborators at the Pasteur Institute.

In his first publication, Lemoigne (1923) reported that aqueous suspensions of an aerobic spore forming bacillus, similar to Bacillus megaterium, accumulated quantities of β -hydroxybutyric acid. He then went on to make quantitative estimations of this acid (Lemoigne, 1925) and later (Lemoigne, 1926, 1927) he showed that substances having the empirical formula $(C_4H_6O_2)_n$ could be obtained from the bacilli. Boiling alcohol extracted such material which melted at 120° and after acid hydrolysis chloroform extracted a substance of similar empirical composition which had a melting

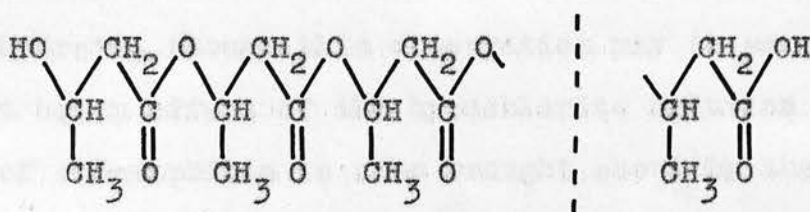
point of 157°. Lemoigne put forward the suggestion that the origin of the β -hydroxybutyric acid in the medium was from these polymers or from a similar, probably homogenous, polymer of higher melting point and molecular weight. The converse process of polymer synthesis was also suggested, the view being taken that the polymer arose from metabolic dehydration and condensation of β -hydroxybutyric acid. The structure suggested was that of a polylactide. The actual form or location of the material in situ was not discovered in this early work but the author stated that in his opinion it was probably bound in some complex substance.

Examination of this topic seems to have been in abeyance from 1927 until 1940 when Lemoigne and Roukhelman published a simplified extraction procedure for the isolation of this polymerised material which shall henceforth be referred to as poly- β -hydroxybutyrate (PHB). Quantitative estimations of PHB by Lemoigne and Roukhelman (1940) yielded a figure of up to one quarter of the dry weight of the bacilli and subsequently it became clear from the work of Lemoigne, Delaporte and Croson (1944) that there was a correlation between the amount of PHB which could be extracted and the fatty inclusion material exhibited microscopically. This observation was strong circumstantial evidence for regarding PHB as a major constituent of the granules especially in view of the high proportion of the cell mass accounted for by this substance. For example Lemoigne, Grelet, Croson and

Le Tries (1945) were able, by growing cells in liquid media, to raise the PHB content to 57% of the bacterial dry weight.

Further efforts were made by the Lemoigne group to arrive at a more definitive chemical characterisation of the various poly- β -hydroxybutyrates which could be extracted from Bacillus megaterium. Cryoscopic observations using phenol as solvent gave a figure of six β -hydroxybutyric acid residues in the PHB melting at 120° (Lemoigne, 1947) and it was reported that the product melting at 157° had a molecular weight considerably in excess of this. The view was again expressed that the PHB contained by the cells was of a higher molecular weight than the products extracted by solvents after hydrolysis. Confirmatory evidence for this hypothesis was presented by Képès and Péaud-Lenoël (1952) who extracted another PHB from dry, disintegrated bacilli with a chloroform/dioxane mixture. This substance, which had a melting point of 179°, could not be fractionated by differential precipitation techniques and seemed homogeneous. On the other hand it was possible to prepare a series of poly- β -hydroxybutyrates with melting points ranging from 136° to 176° after acid hydrolysis of the initial material. The solubility properties of the poly- β -hydroxybutyrates were described as :- insoluble in water, ether, acetone, lower alcohols and paraffins but soluble in fatty acids, alcohols with more than three carbon atoms, chlorine containing

solvents and solvents with a cyclic or heterocyclic nucleus. The presence of an acid group in these polymers was verified by potentiometric observations and of an alcohol group by acetochromic oxidation. On the basis of these observations (Képès and Péaud-Lenoël, 1952) the following structure was suggested for the poly- β -hydroxybutyrates:-



Significant points are, the polylactide structure, one terminal secondary alcohol group and one terminal carboxyl group.

More direct confirmation that the lipid inclusions of Bacillus megaterium are composed largely of PHB has come from the work of Weibull (1953a, b). He submitted the protoplasmic contents of the cell to differential centrifugation after dissolution of the cell wall with lysozyme. Analyses of the granules isolated in this manner showed the presence of only small amounts of protein or carbohydrate and almost complete solution in warm alkali or chloroform. Williamson and Wilkinson (1958), who noted loss of sudanophilia of the free granules subsequent to centrifugation or other mechanical shock, were able to confirm directly that they were composed, certainly largely, of PHB by treating whole cells with an alkaline solution of sodium

hypochlorite which solubilises cell material without dissolving the granules. This process can be followed easily by phase contrast microscopy. The inclusions may then be recovered by high speed centrifugation and submitted to chemical analysis. Typical results showed the presence of some 11% ether soluble material, perhaps partly triglyceride and 89% of PHB, soluble in chloroform. There was no evidence for protein or carbohydrate, though this observation may to some extent be an effect of the hypochlorite solution since loss of sudanophilia is also brought about by the reagent.

The tendency of the granules to lose their sudanophilic properties emphasises the caution which must be employed in the interpretation of cytochemical data obtained from staining procedures. Thus Spiegelman, Aronson and Fitz-James (1958) concluded that since certain granules isolated from Bacillus megaterium did not stain with Sudan black B they were not to be regarded as lipid material and they employed this observation to substantiate their case for regarding these structures as nuclear bodies. Since the granules were obtained by centrifugation of lysed, lipase-treated cells they would not be expected to stain with Sudan black B if they were composed of PHB. The criterion of staining with the fat soluble dyes is valueless in these circumstances and giving undue credence to it could lead to misinterpretation of the true nature of the material studied. This is especially so in the case of nuclear

material since Fairman (1956a,b) has shown that lipid granules are often closely associated with, and cause deformation of, nuclear substance.

In the nitrogen-fixing organism Azotobacter inclusions having similar cytochemical properties occur. As in the case of the granules of Bacillus there has been much disagreement over their significance and constitution, and in general the same diverse descriptions were applied to both. These various theories reached their apogee in the suggestion of Zeigenspeck (1930) that the inclusions were invading parasites. Lemoigne and Girard (1943) have observed that important quantities of PHB are formed by Azotobacter and it seems likely that the granules are composed largely of this material. Quantitative estimations showed that it constituted up to 20% of the dry weight of the organisms. Confirmatory results were obtained by Lemoigne, Girard and Jacobelli (1951).

Bacteria which occur in the root nodules of the Leguminosae exhibit inclusions which display similar staining characteristics. No references have been found in which it is stated that these granules contain PHB but Hopkins and Peterson (1930) record some data which suggests that this may be the case. Working with the organism Rhizobium meliloti, which occurs in the root nodules of alfalfa, they showed that ether extraction of the dried powdered cells removed from 0.6% to 1.2% of the dry weight. The extracted material contained neither phosphatide nor sterol. Further treatment was carried

out with chloroform which removed some 10% to 20% of the dry weight. The chloroform soluble substance was a brittle material melting at 173° and yielding on saponification a soluble product. When this product was analysed it gave 66% acetic acid and 34% non-volatile acids. Titrimetric assays gave the average molecular weight of the latter as 117, and iodine number and acetylation studies indicated saturated monohydroxy-acids. Reasons for believing that the granules have a similar composition to those of Bacillus species are firstly, the proportion of ether soluble material to chloroform soluble material is roughly similar at 6%. Secondly, the amount of material extracted by chloroform as a function of the dry weight is of a similar order and the melting point of 173° falls within the range of 136° to 176° recorded for PHB from Bacillus megaterium by Képès and Péaud-Lenoël (1952). Lastly, the solubilisation of the substance by saponification and the occurrence of saturated mono-hydroxy acids in the products. It is known that alkaline hydrolysis converts PHB partially to β -hydroxybutyric acid which has a molecular weight of 104 comparing approximately with the reported figure of 117. Confirmation that the substance does occur in these organisms could be easily obtained, but in interpreting the above data it must be recalled that the analyses refer to extracts of whole cells, without preliminary hydrolysis, and not granules isolated by the hypochlorite method nor extracts derived by the Lemoigne procedure.

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These observations on the nature of the lipid granules of Azotobacter and Rhizobium may lend some support to Bisset's hypothesis (Bisset, 1955, Hale and Bisset, 1956, Bisset et al, 1957) that there is a relationship between these two genera and the Bacillaceae.

Investigations of the fatty inclusions of Spirillum volutans by King and Beams (1942) have shown that they possess a specific gravity higher than the surrounding cytoplasm. This was done by submitting the organisms to ultracentrifugation and observing that the granules accumulated centrifugally. In view of the fact that the granules stained with the usual fat soluble dyes and working on the assumption that the sudanophilic material was lighter than water they concluded that the granules must be composed of a reserve food material coated with fat. It is known that the specific gravity of PHB is considerably higher than unity (1.24) and presumably higher than the cytoplasm. It seems at least possible, therefore, that its presence may afford an explanation of the behaviour of the inclusions of Spirillum volutans. This suggestion is reinforced by the possible analogy of the sudanophilic envelope which seems to occur surrounding the PHB of Bacillus species.

4. The Metabolism and Significance of the Lipid Granules of Bacillus Species.

In his initial observations on the poly- β -hydroxybutyrates extracted from Bacillus species Lemoigne (1927) suggested that they arose from a substance of similar empirical composition and higher molecular weight which occurred in the intact organisms and subserved a reserve function. He put forward the idea that the poly- β -hydroxybutyrates melting at 157° and 120° were stages in a degradation process which led ultimately to the release of β -hydroxybutyric acid into the medium. In later work (Lemoigne and Roukheldman, 1940) assays were made of the levels of PHB after various periods of incubation of a Bacillus culture in wort liquor. The figures obtained showed that the level fell from 22.7% of the dry weight after one day's incubation at 30° to 6.2% after 15 days' incubation. Commenting upon the results these observers stated that PHB was formed rapidly prior to sporulation and disappeared speedily at the time of spore formation. They also noted that whereas the level of PHB fell, the dry mass of the organism continued to rise, and formed the opinion from these results that PHB was not a waste product but a utilisable reserve. After a survey of the value of PHB as a taxonomic criterion, Lemoigne, Delaporte and Croson (1944) went on to discuss the significance of PHB in the metabolism of the organisms. It was stated that β -hydroxybutyric acid was formed at the expense of carbohydrates and that this

acid was then dehydrated and polymerised to PHB. The organisms hydrolysed and depolymerised this material at sporulation. It was concluded that the lipid metabolism of those species forming large quantities of PHB was almost wholly reduced to the formation and degradation of this reserve material. In three further publications (Lemoigne, 1946, 1947, Lemoigne, Milhaud and Croson, 1949) the same general conclusions were re-iterated. Heitzmann (1943a,b) was able to demonstrate the reduction of acetoacetate to β -hydroxybutyrate by Bacillus "M" in the presence of glucose, which was concomitantly oxidised to acetate and lactate. Following up this work Lemoigne, Péaud-Lenoël and Croson (1949) attempted to discover if washed suspensions of Bacillus megaterium could oxidise sodium β -hydroxybutyrate. Equivocal results were obtained with cells washed at room temperature and it was found necessary to wash in chilled saline, after which oxidation of the β -hydroxybutyrate could be demonstrated. From the fact that neither CO₂ nor acetate was formed it was concluded that the product of the oxidation was acetoacetic acid. It was stated that with non-proliferating cells oxidation stopped at this stage. In an attempt to discover something of the biosynthetic pathway of PHB formation Lemoigne, Grelet and Croson (1950) tried various substances as substrates with washed suspensions of organisms. These experiments were uniformly negative, however, and it was necessary to study the formation of PHB during growth. This is not

as satisfactory as observations made on washed suspensions since processes connected with growth must inevitably interfere with PHB metabolism. Such important side effects as rate of growth, the pH value of the culture medium and the availability of essential nutrients were not controlled and the figures provided by these experiments have little quantitative value. It was shown, however, that PHB was formed during growth on the ammonium salts of lactic, pyruvic and β -hydroxybutyric acids but was not produced from ammonium acetate or acetoacetate unless glutamate was also present. These workers concluded that, although the technique employed did not permit any definite views on the intermediates concerned in PHB formation, it was possible that the formative sequence between glucose and PHB was:-

Pyruvate \rightarrow acetate \rightarrow acetoacetate \rightarrow β -hydroxybutyrate.

Other growth experiments (Lemoigne, Péaud-Lenoël and Croson, 1950) confirmed that β -hydroxybutyrate could be utilised alone and that acetoacetate required the addition of glutamate, aspartate or malate. In their conclusions from these experiments the authors stated that it was uncertain whether PHB was a waste or a storage product thus altering the previous views of the Lemoigne group on the significance of this material. The work of Tinelli (1955a,b,c), however, brought about a return to the initial hypothesis of a reserve function for PHB. She found that when cultures of Bacillus megaterium were

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allowed to spore in the absence of a utilisable external carbon and energy source, the significant amount of PHB which was lost in the sporulation was completely oxidised to CO₂ and water. It was shown further that strains of this organism which had lost the faculty of sporulation, underwent an autolysis during which the PHB which they contained was in part released into the surrounding medium, but was not fully oxidised. It was concluded, therefore, that PHB formed a useful, utilisable energy reserve, the metabolism of which was intimately connected with sporulation.

The views of workers, other than those at the Pasteur Institute, on the functional significance of the lipid granules occurring in the Bacillus group may be summarised briefly.

Koch and many workers of his period believed that the granules gradually grew into mature spores and other contemporary workers were of the opinion that spores were formed from the material of the granules after preliminary fusion. Generally it was assumed that the granules were more or less directly concerned in spore formation. Other workers held the view that the granules represented stages in the life cycle of the organisms.

Lewis (1934, 1942) swept away these early ideas on the function of the granules by extensive cytochemical tests and careful observation. He maintained that the granules were composed of a "lifeless", fat-like material with no direct role in

spore formation. The observation that the granules may diminish in number during the ripening of the spore was recorded, however, and the author comments that perhaps the granular material is utilised by the metabolism of the spore or mother cell.

On the other hand Knaysi (1945, 1951c) has adhered to the hypothesis that the granules are lipo-protein entities, formed in the cytoplasmic membrane, which have no role whatever in spore formation. The view is put forward that these granules are not utilisable by the cell and may be the result of abortive attempts at cell division. Photographs reproduced in the second reference (Knaysi, 1951c) do not contribute to (nor do they refute) the author's contention that the granules of Bacillus cereus are not utilised. Further the evidence presented in support of this hypothesis is of little value. It is based on the microscopic observation that the granules do not diminish in size or number when the organisms commence growing after transfer to fresh medium. It seems unlikely that a reserve food material (if the granules were such) would be utilised in the presence of an abundance of external nutrients in fresh medium.

The antithesis of the storage function hypothesis was put by Sparrow (1933) who suggested that, since many Bacillus cultures high in fat had a lesser mass than low fat control cultures, the production of fatty material might be the result of katabolic not anabolic processes.

After extensive experiments on the effect of various nutrient deficiencies on growth and PHB production in an asporogenous strain of Bacillus cereus, Williamson (1956) did not feel able to state whether the functional significance of PHB was primarily that of a carbon and energy reserve. He held the opinion that PHB was formed in large quantities when growth was arrested by the deficiency of a nutrient or nutrients not directly concerned in PHB synthesis.

From the foregoing review it can be appreciated how fragmentary and controversial is the present state of knowledge regarding the primary functional importance of the lipid granules of Bacillus species. The reasons for this unsatisfactory position are several, but in the author's opinion stem prepotently from the dearth of accurate quantitative metabolic studies in which the chemical environment is carefully controlled.

The limitations of growth experiments are many since the environment is constantly altering in an unpredictable fashion. Further, the changes which occur are often difficult to follow accurately. The use of washed suspension techniques is therefore to be preferred since greater control is possible. By suspending washed organisms in phosphate buffer without a source of nitrogen little growth takes place, and if the experimental period is then kept as short as possible, the environment can be more easily controlled. Despite the fruitless attempts of previous authors to study PHB formation in washed suspensions, this has been

successfully carried out in the present study. From the results obtained in these experiments a strong case is made for regarding PHB as a reserve of carbon and energy. In the use of cell-free bacterial extracts and also of isotopically labelled substrates data have been derived which provide information on the biochemical pathways concerned in the formation and degradation of PHB.

MATERIALS AND METHODS

1. Organisms

Two species of the genus *Escherichia* were employed in this research, *E. coli* strain AT (Wollman and Tinkler, 1958) and *E. coli* strain K12 (Wollman, 1954). Strain AT was an sporogenous mutant of a laboratory stock strain of *E. coli*. It was obtained by submitting the latter to ultraviolet irradiation and selecting rough colonial forms which developed. Old colonies of the parent strain became smooth subsequent to their profuse sporulation and the sporogenous mutant colonies could be readily distinguished from the parent.

EXPERIMENTAL

Other details of the two organisms are specified in Table I. All results refer to the K12 strain unless otherwise stated. This organism has two main advantages: firstly, like AT it is avirulent thus obviating the interference of spore formation or the metabolism of H₂S and secondly, it is amenable to techniques which facilitated the preparation of well-kept cultures for major studies.

MATERIALS AND METHODS.

1. Organisms.

Two species of the genus Bacillus were employed in this research, B. cereus strain AC (Williamson and Wilkinson, 1958) and B. megaterium strain KM (Northrop, 1951). Strain AC was an asporogenous mutant of a laboratory stock strain of B. cereus. It was obtained by submitting the latter to ultra-violet irradiation and selecting rough colonial forms which developed. Old colonies of the parent strain became smooth subsequent to their producing spores and the asporogenous mutant colonies could thus be easily recognised. Other details of the two organisms are appended in Table 1.

All results refer to the KM strain unless otherwise stated. This organism has two main advantages. Firstly, like AC it is asporogenous thus obviating the interference of spore formation on the metabolism of PHB and secondly, it is sensitive to lysozyme which facilitates the preparation of cell-free extracts for enzyme studies.

Table 1.

Character or Biochemical Reaction.	Organism.	
	KM.	AC.
Acid production from glucose.	±1	±1
Sucrose.	±1	±1
Mannitol.	±1½	-
Arabinose.	±2	-
Xylose.	±2	-
Glycerol.	±2	±2
Lactose.	±1½	-
Salicin.	-	±2
Other Characters:		
Motility.	+	-
Gram reaction.	±	±
Spore formation.	-	-
Anaerobiosis.	-	+
Nitrate reduction.	+	-
Caseinase production.	+weak	+strong
Gelatin liquefaction.	+slow	+rapid
Amylase formation	+	+
Catalase production.	+	+
Lecithinase reaction.	-	+
Indole production.	-	-
Methyl red reaction.	-	-
Acetylmethylcarbinol formation.	-	+

Table 1 (contd.)

Character or Biochemical Reaction.	Organism.	
	KM.	AC.
Citrate utilisation	+	(with [†] added <u>aa</u>)
H ₂ S production	-	-
Coagulated serum liquefaction.	+slow	+rapid.
Growth in nutrient broth.	precipitate formed: no pellicle: clear supernatant.	medium turbidity: no pellicle.
Growth on tyrosine agar.	no black pigment.	no black pigment.
Growth on glucose/nitrate agar.	scanty.	scanty.
Growth on potato.	heavy, glistening, bright orange pigment.	heavy, smooth creamy colour.
Growth on blood agar.	diffuse late haemolysis.	discrete β haemolysis. 1 day.
Growth in litmus milk.	coagulation and indicator reduction 10.	alkalinisation and peptonisation 5.

Key:

- no reaction.

$\frac{1}{2}$ acid formation.

$\alpha\alpha$ amino acids.

+ possession of character.

+ variable reaction.

Subscripts denote the number of days incubation at 30° required for the manifestation of a decisive reaction.

2. Media and Culture Methods.

Strain AC was initially grown on a solid medium consisting of a salts/agar base with the addition of casein hydrolysate, phosphate buffer, ammonium chloride and glucose. The exact composition is given in Table 2.

Table 2.

Constituent	g./l. of medium.
KCl.	0.2
Na ₂ SO ₄ .	0.1
MgCl ₂ .6H ₂ O.	0.43
CaCl ₂ .	0.001
MnCl ₂ .4H ₂ O.	0.001
FeCl ₃ .6H ₂ O.	0.0003
NaH ₂ PO ₄ .2H ₂ O.	1.7
Na ₂ HPO ₄	8.72
"Casamino acids", (Difco).	0.05
Agar, (Jap fibre).	15.0
NH ₄ Cl.	1.5
Glucose.	3.0 or 10.0

All chemicals were of analytical reagent grade and the agar fibre was well washed with distilled water prior to use. Sterilisation was effected by steaming 1½ hr. and the initial pH value of the sterile medium was 7.2 - 7.4. The NH₄Cl and glucose were added separately

with aseptic precautions. For cells rich in PHB 10.0 g./l. glucose was used and for PHB-poor cells 3.0 g./l.

For all experiments using the solid medium the method of culture was the same. One litre aliquots of the sterile medium were poured into enamel trays (12" x 16" x 1", previously autoclaved) and after cooling were inoculated with 10 ml. of a dense saline suspension of an overnight culture of the organisms. The inoculum was distributed over the surface of the agar with a sterile glass rod and the trays were incubated at 37°. The incubation period was 16 - 18 hr. for PHB-poor cells and 40 - 42 hr. for PHB-rich cells. Harvesting was effected by scraping the growth from the surface of the agar with a clean glass slide and resuspending the organisms in physiological (0.85% w/v) saline. The suspension was passed through surgical gauze to remove particles of agar and washing was carried out by duplicate centrifugation and resuspension in saline.

The advantages of growth in liquid media were the greater chemical control possible in the absence of the agar, which could contain traces of many interfering substances, the avoidance of diffusion artefacts due to nutrients and metabolites, the enhanced aeration possible and the facility and convenience of producing quantities of cells in an easily prepared pabulum.

Details of the derivation of the media employed are given in the Results Section of this work, the composition and methods of use are set out below.

For all experiments employing organisms grown in liquid media the salts/casein hydrolysate base shown in Table 3 was employed.

Table 3.

Constituent.	g./l. of medium.
Na_2HPO_4 .	6.0
KH_2PO_4 .	3.0
NaCl .	3.0.
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$.	0.1
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$.	0.01
Na_2SO_4 .	0.1
NH_4Cl .	1.0
"Casamino Acids," (Difco).	0.1

In order to avoid the time consuming practice of repeatedly weighing quantities of salts and casein hydrolysate it was found convenient to make them up as a dry mixture. This was effected by carefully powdering the dry, weighed constituents and, after thorough mixing, storing in a tightly closed bottle. The ^{solid} desiccating effect of such additives as anhydrous disodium hydrogen phosphate obviated the chance of difficulties arising from dampness and caking of the mixture. It was necessary only to add 13.3 g. of this mixture to each litre of distilled water to give the properly balanced salts medium.

Glucose, sterilised separately, was added aseptically after sterilisation of the salts medium by steaming $1\frac{1}{2}$ hr. A final glucose concentration of 0.3%(w/v) for PHB-poor, and 2%(w/v) for PHB-rich cells was used. To give a balanced medium for ordinary growth purposes, for example to produce cells for the enzyme studies, a 0.5%(w/v) final concentration of glucose was employed.

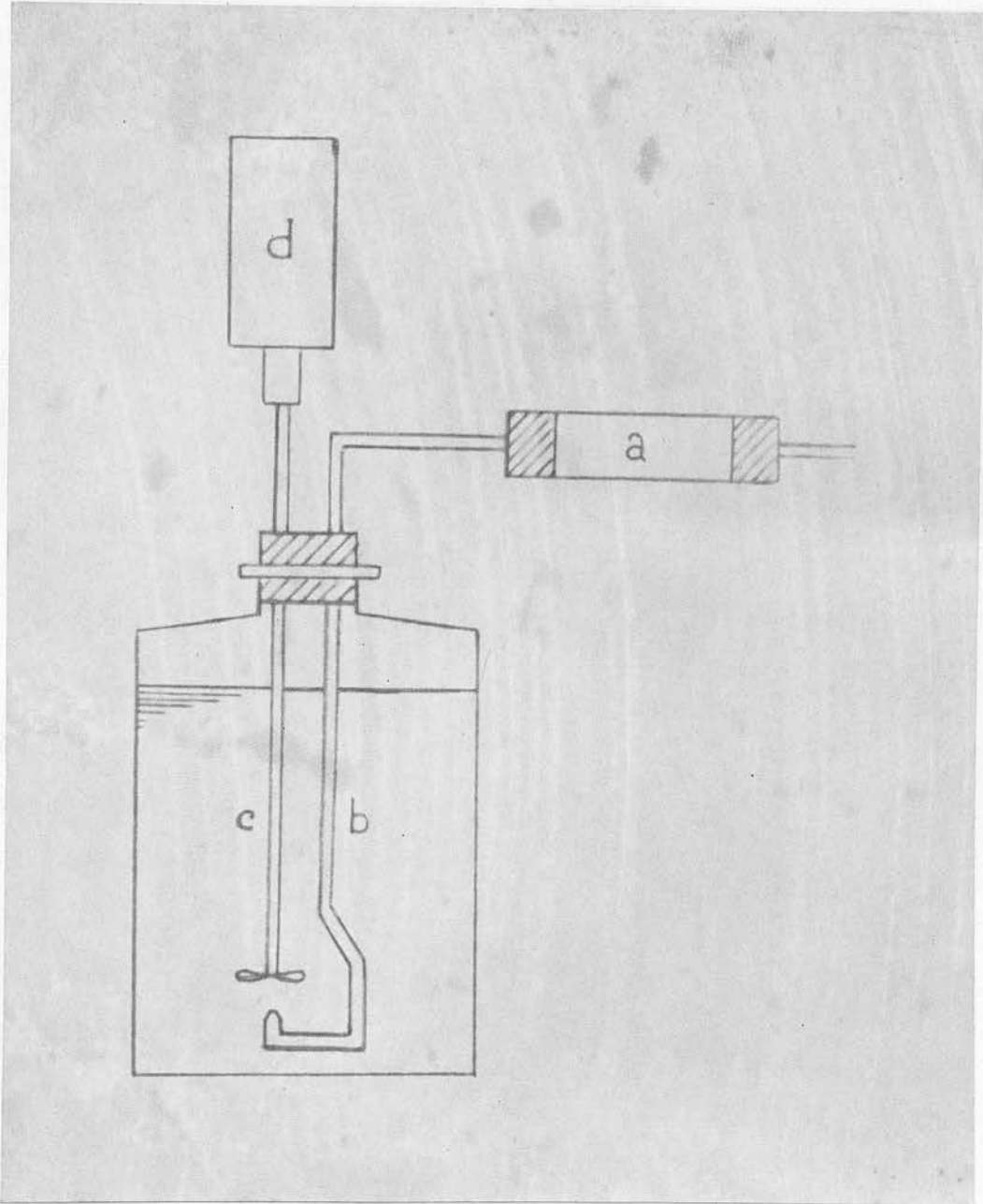
Two methods of culture were used, one for quantities of medium up to 500 ml. giving some 600 mg. dry weight of cells in the balanced medium, and another for quantities of medium up to 10 l. yielding 12 g. dry weight of cells.

In the first method 500 ml. or less of distilled water were added to the requisite quantity of the salts mixture placed in the foot of a glass culture tube (20" x 2"). A sintered glass gas distributing tube was then placed inside and the culture tube closed with a wad of cotton wool. After sterilisation and addition of glucose the tube was inoculated with 5 ml. of an overnight culture of the organism (AC or KM) in the balanced medium. A small electric vibrator air pump (designed for use with aquaria) was then connected to the gas distributing tube and the whole culture tube assembly placed in a water bath at 30°. The air from the pump was filtered by passing through a tube of sterile cotton wool. The period of incubation (to yield cells toward the end of the

log phase) was 18 hr. for 0.3% and 0.5% glucose cultures and 22 hr. for 2% glucose cultures. Harvesting was effected by centrifugation and the cells were washed twice with normal saline.

The method developed for bulk culture was similar in the initial preparation and inoculation of the medium but a large glass culture vessel of 10 l. capacity was employed. Fig. 1 shows the general layout of the aeration and circulatory system. Air is supplied from a similar pump as in the small culture tube technique and, after passing through the filter (a), is bubbled through the medium by means of a glass delivery tube (b). The air entering the medium impinges on the stainless steel paddle (c) which is driven at high speed by the electric motor (d). This has the dual effect of breaking up the air stream into very small bubbles and bringing about efficient circulation of the medium. The final effect is one of excellent aeration and homogenisation of the medium in the culture vessel and provides B. megaterium, an obligatory aerobe, with suitable conditions for growth. The incubation period was comparable with that given above, the temperature being maintained by immersion of the culture vessel in a heated water bath. The stainless steel paddle, glass delivery tube and filter were sterilised separately from the medium by autoclaving at 15 lb. pressure for 15 min. Harvesting of the organisms was effected by passing the culture through a "Sharples super centrifuge". Washing was performed with two changes of normal saline.

Fig. 1.



5. Spectrophotometric Measurements.

In all procedures requiring the use of spectrophotometric techniques a Unicam S.P. 500 spectrophotometer was employed with cuvettes of 1 cm. light path and 4 ml. capacity.

6. Measurement of pH.

The hydrogen ion concentration was estimated by the use of a "Pye" glass electrode, direct-reading pH meter. Routine spot-checks were made of the initial and final pH of growth media and of washed suspensions before and after the experimental period. In all cases the buffering capacity of the medium or suspending fluid was found to be adequate, *never falling below pH 7.*

7. Estimations of Bacterial Dry Weight.

Routinely, these were performed by estimations of the turbidity of cell suspensions and converting to dry weight figures by means of a calibration curve relating turbidity to dry weight. In view of the effect of the size and number of included lipid particles on the turbidity of the cells it was felt advisable to construct two calibration curves one for PHB-poor, and one for PHB-rich cells. The method was identical in both cases. A suspension of organisms containing some 0.5 mg./ml. dry weight was prepared in distilled water after washing the cells thrice in

distilled water. Three 10 ml. aliquots of this suspension were then dried at 105° in a hot air oven and brought to constant weight in vacuo over P_2O_5 . The average mass of these three samples was found to be 4.470 mg. for PHB-rich and 5.160 mg. for PHB-poor cells. The remaining suspension was used to make suitable dilutions for turbidity measurements and the graph shown in Fig. 2 was compiled from the results. The PHB content of the organisms in relation to their dry weight was found to be 23.7% for the PHB-rich, and 3.3% for the PHB-poor cells. It will be seen from the two curves in Fig. 2 that there is only a small difference in the turbidity/dry weight relationship between the two groups of cells and the curves were therefore regarded as satisfactory for routine dry weight estimations. The PHB-poor curve was used for cell suspensions with a PHB content up to about 13% of their dry weight and the PHB-rich curve thereafter.

8. Assay of Poly- β -hydroxybutyrate (PHB).

The turbidimetric method of Williamson and Wilkinson (1958) was used routinely for the estimation of the level of PHB in whole cells. Since the data of these workers referred solely to the AC strain of B. cereus it was necessary to construct another calibration graph relating turbidity to the amount of PHB for B. megaterium strain KM. This curve is given in Fig. 3 and represents the mass of the granules as

FIG. 2.

READING.

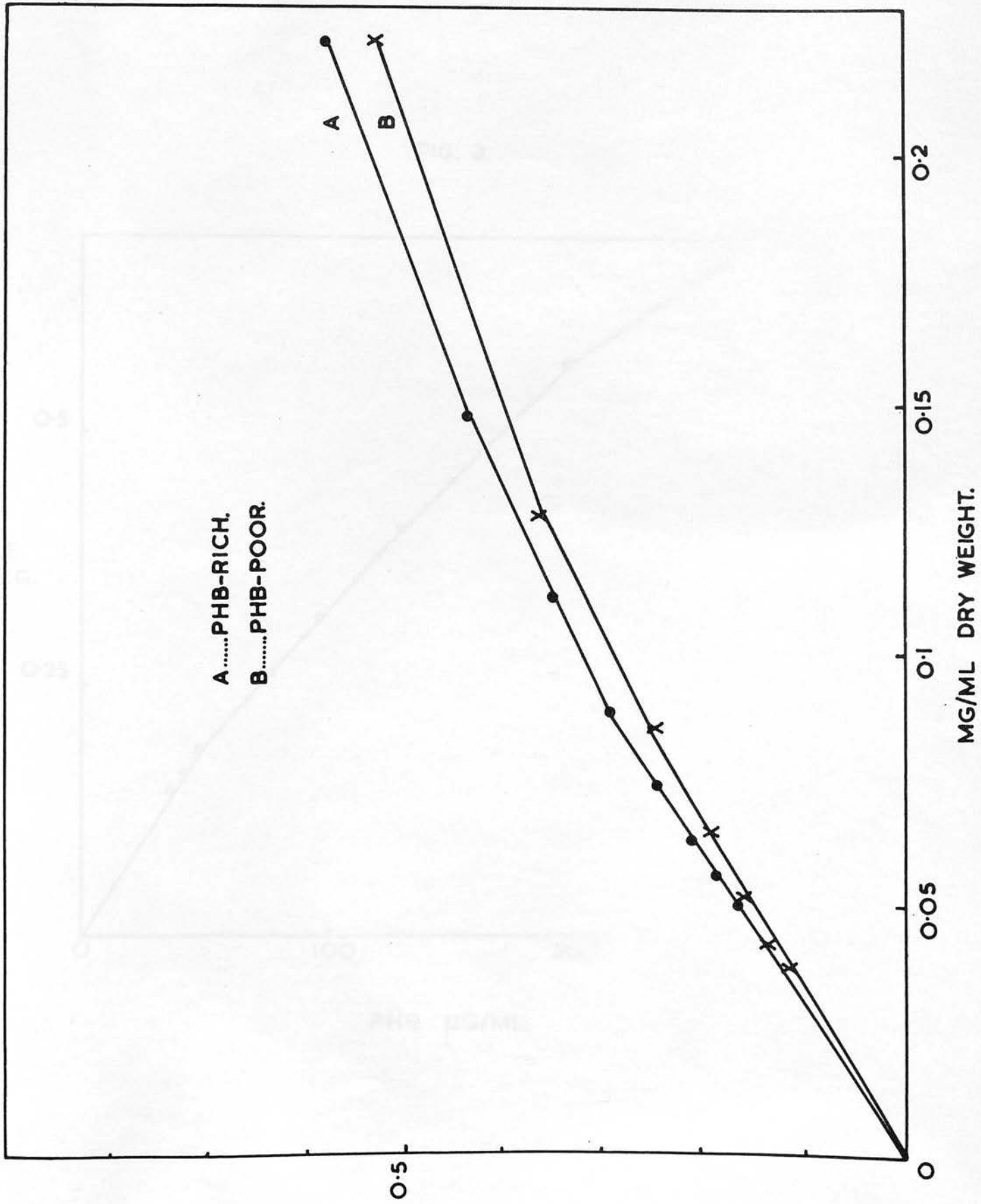
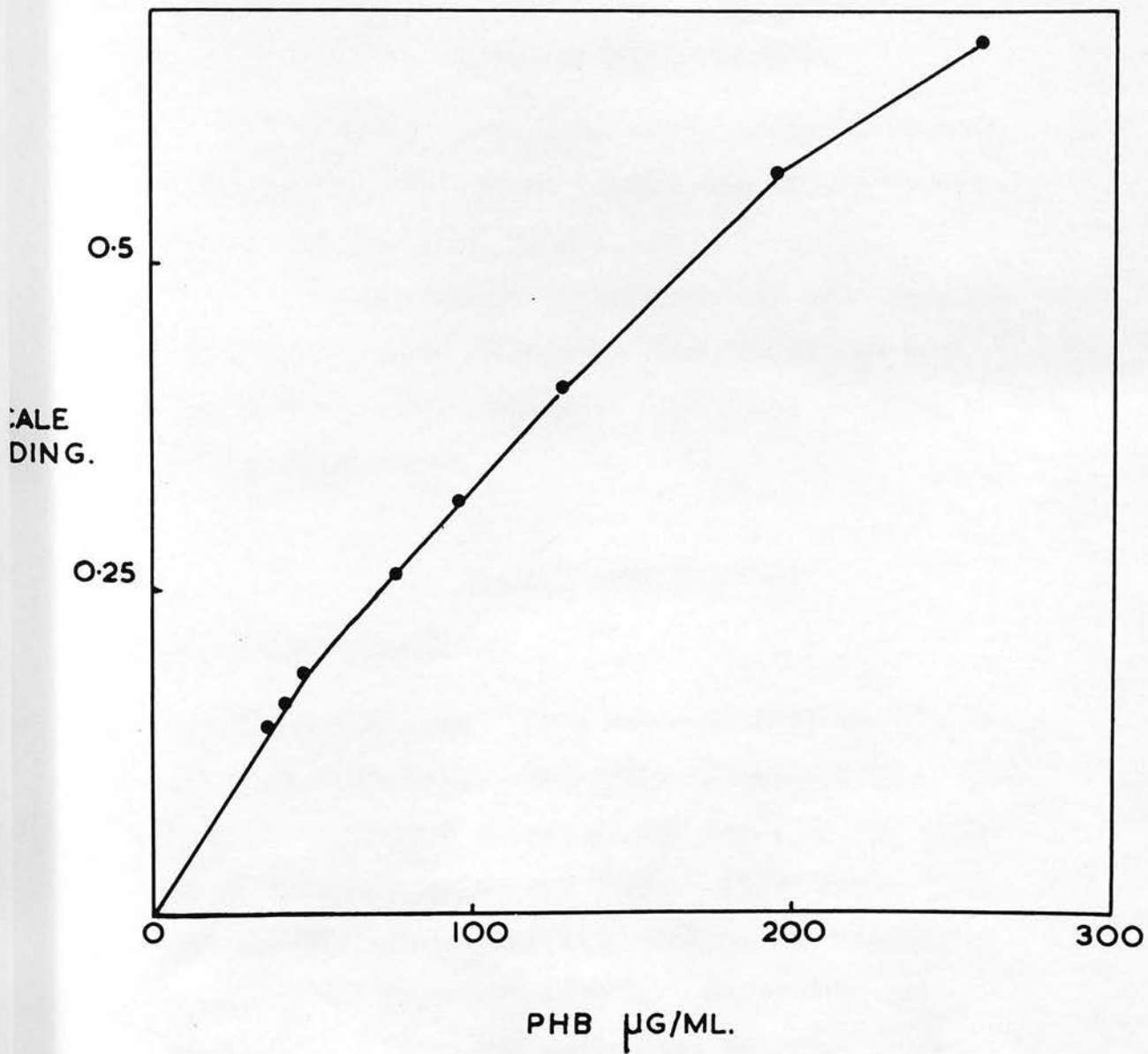


FIG. 3.



extracted by the hypochlorite procedure less 5.5% which is the average proportion of ether soluble lipid material present. In this organism the amount of ether soluble material occurring in the granules isolated by the hypochlorite method is more variable than in AC.

The average % deviation between the turbidities of duplicate samples was 1.05, and the standard deviation from this average 0.93.

9. Manometric Procedure.

Standard techniques were adopted in studies involving the use of the Warburg Manometer (Umbreit, Burris and Stauffer, 1949).

Carbon dioxide was measured by the direct method and estimations of anaerobic acid production were carried out in bicarbonate buffer pH 7.01 under an atmosphere of 5% CO₂ in nitrogen.

10. Chemical Methods.

(1) Assay Techniques.

(a) Total Nitrogen. This was estimated by the micro-Kjeldahl procedure. Digestion of the samples, which normally contained about 100 µg. total N, was effected on an electrically heated rack. Steam distillation was carried out using a modified form of the apparatus described by Scandrett (1953). In view of the importance of accurate estimation of total (non-dialysable) nitrogen all unknowns were assayed in triplicate.

(b) Polysaccharide Determinations. These were performed in duplicate according to the anthrone method of Dreywood (1946) as modified by Fairbairn (1953) using glucose as the reference sugar. The method is only useful in giving an approximate indication of the content of polysaccharide, some two-thirds of the actual amount normally being measured. In the few circumstances in which these estimations were employed in this work they were found to be satisfactory.

(c) Total Phosphorus. This was estimated in duplicate by the method of Fiske and Subbarow (1925).

(d) β -hydroxybutyrate, Acetoacetate and Acetone. The method of Thin and Robertson (1952) was employed with the modification suggested by Bahner (1952) in measuring the colour developed spectrophotometrically at 490 m μ . Estimations were carried out in duplicate, and refluxing with the oxidising agent was performed in stoppered "Quickfit" tubes (6" x 1"). Diffusion of acetone into the colour reagent was effected in Conway vessels by incubation at 37 $^{\circ}$ for 3 hr. The method was rigorously tested using mixtures of authentic samples of β -hydroxybutyrate, acetoacetate and acetone.

(e) Acetaldehyde. This substance was assayed by the bisulphite binding method of Friedemann and Graeser (1933) as modified by Elliott, Benoy and Baker (1935).

(ii) Preparative Methods and Special Compounds.

(a) Sodium 2-hydroxy-1-propane sulphonate. This was synthesised by the method of Stewart and Cordts (1952).

(b) β -mercaptobutyric acid. This was prepared by Dr. D. Leaver to whom appreciation is gratefully extended. The sodium salt of the free acid was formed by adding stoichiometric amounts of N NaOH at room temperature.

(c) Pantoyl-tauryl-anisidide. The American Cyanamid Company of New York are thanked for a gift of this material.

11. Chromatographic Procedures.

(i) Fatty Acids.

Aliphatic straight-chain fatty acids with from two to six carbon atoms were separated by the method of Duncan and Porteous (1953). The ammonium salts of authentic samples of the acids were used as references.

(ii) Keto-Acids.

These were converted to their 2 : 4 dinitrophenyl-hydrazone derivatives which were submitted to paper partition chromatography by the method of Cavallini and Frontali (1954).

Both chromatographic techniques were used qualitatively only.

12. Isotopic Assay.

All counting was performed using "Panax" type D 554 counting equipment employing a thin window Geiger-Müller tube. Specimens of barium carbonate were plated on nickel planchets.

Chemical degradative procedures are given along with the Experimental Results.

The methods of preparation and plating of the barium carbonate were as follows. The carbon dioxide produced in the requisite chemical degradative method (see pps. 116, 117) was trapped in N sodium hydroxide solution and precipitated as barium carbonate by the addition of saturated barium chloride solution. The yield of carbon dioxide was ascertained by titrating the excess sodium hydroxide with 0.1N HCl using phenolphthalein as indicator. The barium carbonate was washed thrice with 95% (v/v) ethanol by centrifugation and resuspension and c. 0.5% (w/w) polyvinyl alcohol added as a spreading agent. Enough 95% ethanol was added to form a thin slurry and this was dropped on to tared planchets and dried under heated wire gauze. The planchets were held in vacuo over phosphorus pentoxide overnight and were weighed before counting was carried out.

The background radiation was deducted in each case and corrections for self absorption were made, the counts being extrapolated to those of infinitely thin samples.

RESULTS.

1. The Criterion of Total Nitrogen Content.

Throughout this work the basic reference of cell populations and metabolic activity has been taken as the total non-dialysable nitrogen level. This has many advantages over the total mass of dry matter. It will be recalled that the nitrogen content normally bears a direct relation to the amount of protein and this in turn to the potential enzymic activity. The use of this criterion also avoids the interference of materials such as PHB which may form a significant proportion of the dry weight without in any way altering the amount of functional protoplasm. Misinterpretations due to the confusion of structural with metabolically active material are also largely obviated, since the former normally contain relatively small amounts of nitrogen, though the polypeptide component of the capsule in B. megaterium is a notable exception.

It will be seen, therefore, that the ratio of PHB/total nitrogen gives a more useful implication of the balance between the level of PHB and metabolic potentiality than any comparison depending upon the mass of dry matter.

2. Growth of Organisms.

(a) Use of Salts Medium.

As indicated in the Materials and Methods Section the organisms were grown in the initial stages of this research on solid media (B. cereus strain AC, only was grown on solid medium). In view of the disadvantages of this method, culture in the liquid medium (hereafter termed medium "A") described by McQuillen (1955) was attempted. The composition of this medium is given in Table 4.

Table 4.

Constituent.	g./l. of medium.
NH ₄ Cl.	2.0
Na ₂ HPO ₄ .	6.0
KH ₂ PO ₄ .	3.0
NaCl.	3.0
Mg ⁺⁺ (as MgCl ₂).	0.010
S (as Na ₂ SO ₄).	0.025
glucose.	5.0

McQuillen was able to promote the growth of B. megaterium (strain KM) in this medium "A" which yielded about 1 mg./ml. dry weight of cells when growth was complete. Confirmation of this ease of culture could not be obtained in the present study despite the

organism having been "trained" to grow in medium "A" by several subcultures as suggested by McQuillen. The results of a typical growth experiment on Medium "A" are given in Fig. 4.

When the glucose concentration was raised from 0.5% to 1.0% the final nitrogen level obtained was not increased suggesting that some factor other than the carbon and energy source must be limiting growth. Further, since there was 2 mg./ml. NH_4Cl present representing 0.523 mg./ml. nitrogen it seemed most unlikely that scarcity of this substance could be the limiting factor.

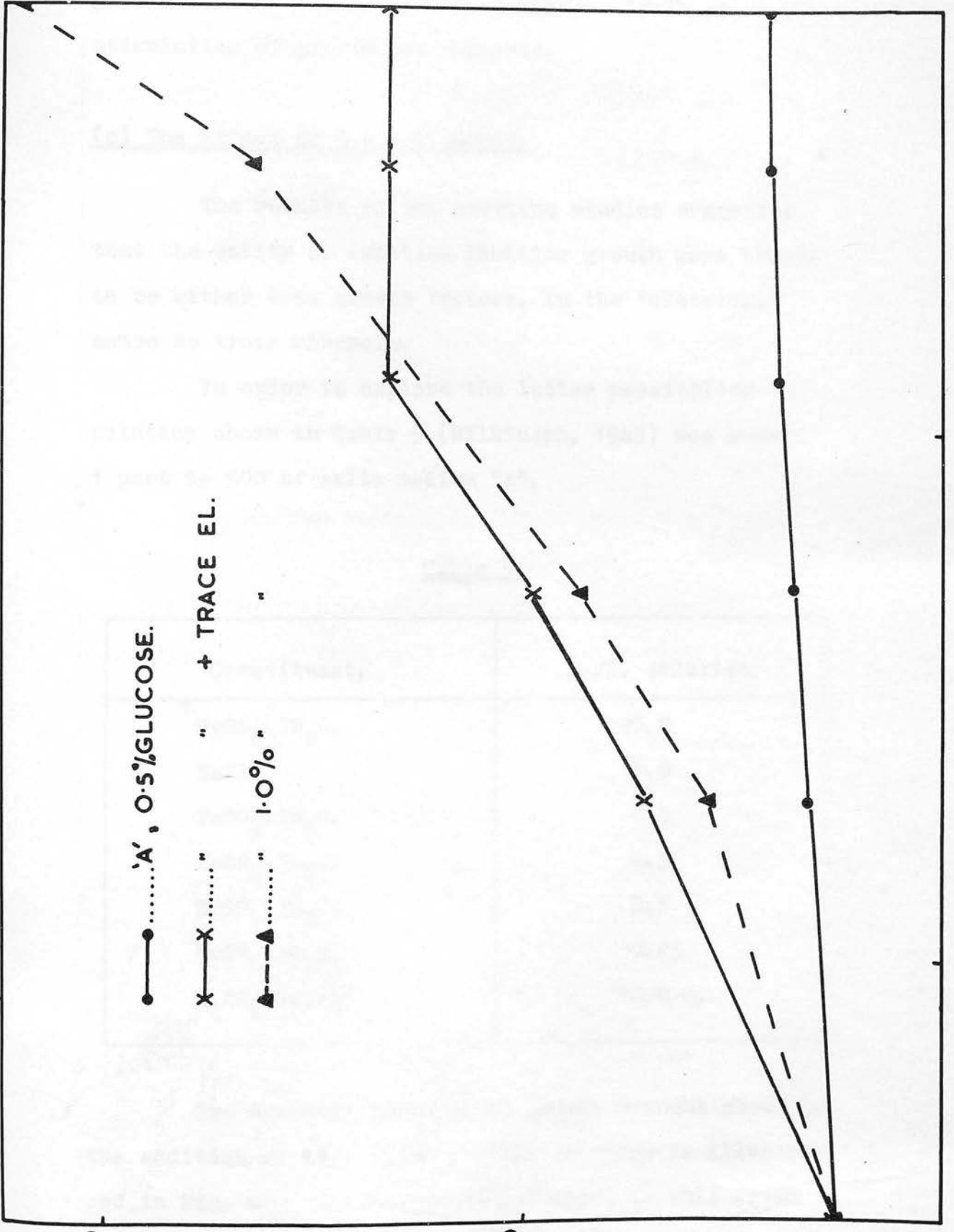
(b) The Effect of Glutamic Acid and Casein Hydrolysate.

Since growth was very poor on Medium "A" it was decided that perhaps some "sparkling" type stimulus was required. The amino acid glutamic acid is normally actively utilised by many organisms and is rapidly and easily converted to the tricarboxylic acid cycle intermediate α -keto glutaric acid. This therefore seemed a likely growth enhancing additive. No discernable effect was observed, however, upon the addition of 0.002% glutamic acid to medium "A".

In view of the fact that many organisms of the cereus/megaterium group require amino acids in amounts similar to growth factors it was decided to supply these in Medium "A". Casein hydrolysate (Casamino acids", Difco) was therefore admixed with the culture fluid to

FIG. 4.

µG N/ML.



●.....'A', 0.5% GLUCOSE.

X....." " + TRACE EL.

▲....." " 1.0%

15

20

TIME HR



give a final concentration of 0.005%. Again no stimulation of growth was observed.

(c) The Effect of Trace Elements.

The results of the previous studies suggested that the entity or entities limiting growth were likely to be either true growth factors, in the "classical" sense or trace minerals.

In order to explore the latter possibility the solution shown in Table 5 (Wilkinson, 1949) was added, 1 part to 500 of salts medium "A".

Table 5.

Constituent.	g./l. solution.
MgSO ₄ ·7H ₂ O.	20.0
NaCl.	1.0
FeSO ₄ ·7H ₂ O.	0.5
ZnSO ₄ ·7H ₂ O.	0.5
MnSO ₄ ·3H ₂ O.	0.5
CuSO ₄ ·5H ₂ O.	0.05
H ₂ SO ₄ (0.1N)	10.0 ml.

The dramatic increase in growth brought about by the addition of this trace element solution is illustrated in Fig. 4. Another point indicated in this graph is that the medium is not limited by the nitrogen source (0.2% NH₄Cl) up to 24 hr. when the glucose

concentration is raised to 1%.

(d) Identification of Growth Stimulating Mineral.

Since medium "A" already possessed magnesium, sodium and sulphur it seemed likely that the factor was iron, zinc, manganese or copper or a combination of two or more of these. A further possibility was that some impurity occurring in the salts making up the trace elements solution was the sought after growth stimulant. Cobalt and molybdenum were considered as the most likely substances from this point of view.

Apart from indicating that even very low concentrations of copper were inhibitory, preliminary studies using flat screw-capped bottles of 100 ml. capacity yielded irregular results when each of the trace minerals was added separately to aliquots of salts medium "A". The following procedure was therefore adopted; 1 l. screw-capped bottles were boiled with dichromate cleaning fluid and washed thoroughly, first with distilled and then with de-ionised water. They were then dried and sterilised at 160° for 1 hr., the caps being separately autoclaved. Into each of 6 bottles was placed 20 ml. of sterile medium "A" containing respectively 0.0001% concentrations of:-

1. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$
2. $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$
3. $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$
4. All constituents of 1, 2 and 3.

5. Constituents of 1, 2 and 3 + 0.005%
"Casamino acids".

6. No additions.

The bottles were inoculated with 0.1 ml. of an overnight culture of the organisms in medium "A" which had been washed twice with saline and made up to its original volume. Incubation was at 30° and aeration was effected by rotation of the culture vessels, one cycle being completed every two seconds. The amount of growth after 22 hr. incubation is given in Table 6.

Table 6.

Culture Vessel No. (see text)	Mineral added	µg. N/ml.
1	Fe	39
2	Zn	36
3	Mn	110
4	Fe, Zn, Mn	130
5	Fe, Zn, Mn, Amino Acids	120
6	None	32

The most important observation is the stimulation of growth brought about by 0.0001% $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$. Similar experiments undertaken to study the effect of cobalt and molybdenum showed that neither of these elements could replace manganese as an essential trace mineral. The results of several studies of a similar nature confirmed that the growth stimulation brought about by the addition of the trace element solution could be duplicated simply

by adding manganese. It was found that the inclusion of 0.01% Casamino Acids in the medium yielded more reproducible growth results and this along with 0.001% $MnCl_2 \cdot 4H_2O$ was subsequently added to medium "A". This modified salts/casein hydrolysate mixture is subsequently referred to as medium "B".

The material. It was also necessary to discover how to grow at will, cells rich or poor in PAB. The development of the media with sodium and the sulfur isotopes have been described. The present section concerns the examination of the effect of the concentrations of glucose as carbon and energy source and amount of chloride as nitrogen source.

The results of the growth experiments are given in Fig. 5. Medium "B" was employed with varied concentrations of the carbon and energy and nitrogen source.

In Fig. 5(a) where the medium contained 0.1% glucose and 0.1% sodium chloride, the growth of the organism was limited by the quantity of the carbon and energy source. The rate of growth is poor as indicated by the total nitrogen level which reached a maximum of only 33 $\mu g./ml.$ The decrease which occurs after about 16 hr. indicates the onset of autolysis. The highest content of PAB is 9 $\mu g./ml.$ reflects poor synthesis of this material. The rate of degradation roughly parallels the rate of exponential nitrogen.

The next graph, Fig. 5(b) depicts the levels of nitrogen and PAB in cells grown in a balanced medium

3. The Influence of Cultural Conditions on PHB Formation.

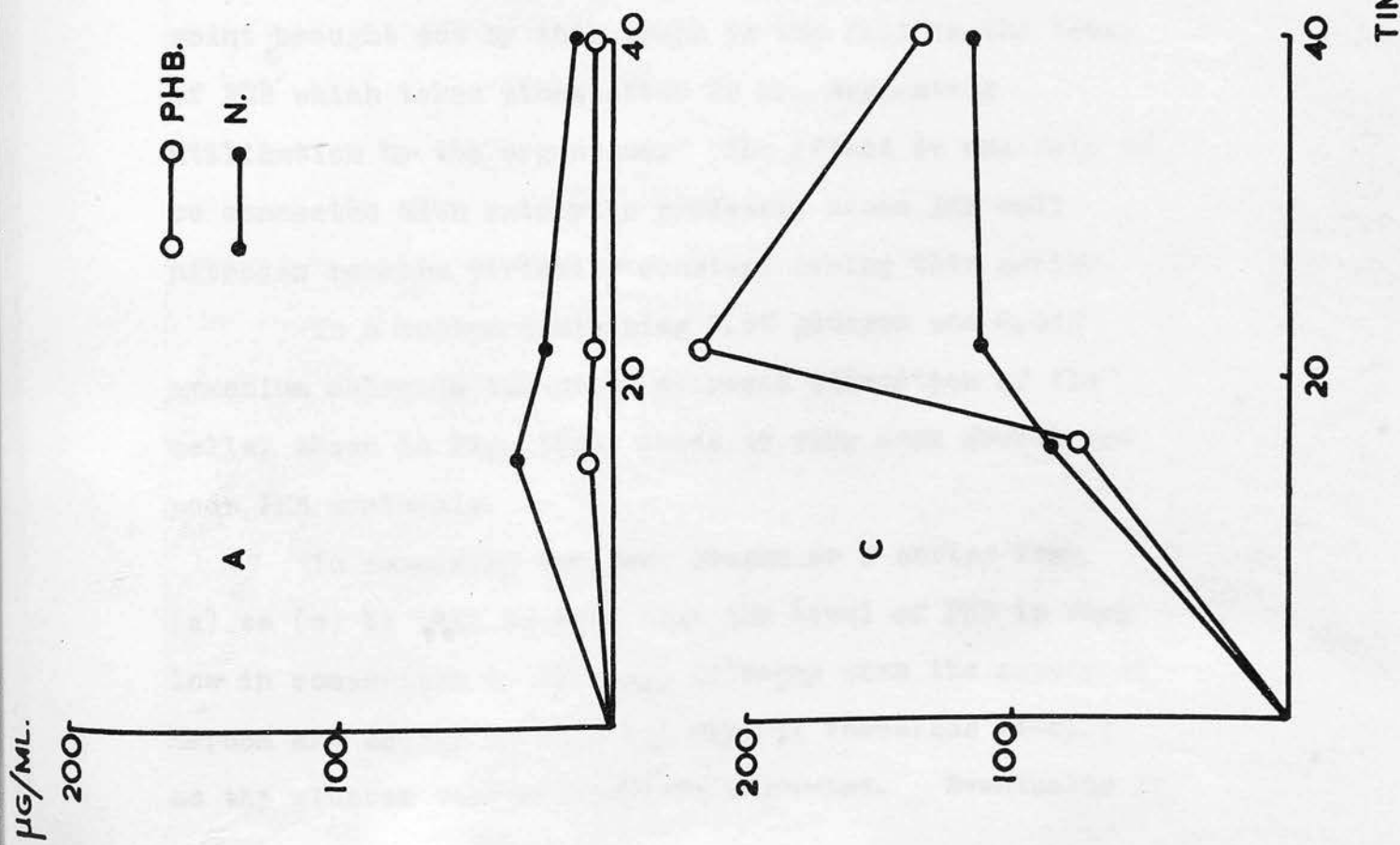
It was thought that some indication of the function of PHB might be gleaned from a study of the cultural conditions under which the organisms synthesised the material. It was also necessary to discover how to grow at will, cells rich or poor in PHB. The development of the basic salts medium and the culture technique have been described. The present section concerns the examination of the effect of the concentrations of glucose, as carbon and energy source and ammonium chloride as nitrogen source.

The results of the growth experiments are given in Fig. 5. Medium "B" was employed with varied concentrations of the carbon and energy and nitrogen sources.

In Fig. 5(a) where the medium contained 0.1% glucose and 0.1% ammonium chloride, the growth of the organisms was limited by inadequacy of the carbon and energy source. The total amount of growth is poor as indicated by the total nitrogen level which reaches a maximum of only 33 $\mu\text{g./ml.}$ The decrease which ensues after about 16 hr. indicates the onset of autolysis. The highest content of PHB at 9 $\mu\text{g./ml.}$ reflects poor synthesis of this material. Its rate of degradation roughly parallels the loss of non-dialysable nitrogen.

The next graph, Fig. 5(b), depicts the levels of nitrogen and PHB in cells grown in a balanced medium

FIG. 5.



containing 0.5% glucose and 0.1% ammonium chloride. The total extent of growth, which attains a level of 140 $\mu\text{g. N/ml.}$, represents a good yield of cells from the culture fluid. The production of PHB is what might be regarded as average, and it is instructive to note that the extent of its formation follows the total nitrogen level.

Where the growth of the organisms is arrested due to exhaustion of the nitrogen source, Fig. 5(c), the formation of PHB continues rapidly after 16 hr. incubation. In the experiment illustrated, where the medium contains 0.5% glucose and 0.05% ammonium chloride, PHB forms a large part of the bacterial mass and even higher levels are possible by further increasing the relative superfluity of the carbon and energy source. An important point brought out by this graph is the fall in the level of PHB which takes place after 22 hr. suggesting utilisation by the organisms. The effect is unlikely to be connected with autolytic processes since the cell nitrogen remains virtually constant during this period.

In a medium containing 0.5% glucose and 0.01% ammonium chloride the gross nitrogen starvation of the cells, shown in Fig. 5(d), leads to very weak growth and poor PHB synthesis.

In examining the four graphs as a series from (a) to (d) it will be seen that the level of PHB is very low in comparison to the cell nitrogen when the supply of carbon and energy is poor but that it increases steeply as the glucose concentration is augmented. Eventually it

increases beyond the cell nitrogen level and even where growth is extremely poor in Fig. 5(d) the presence of a carbon and energy excess leads to the establishment of a high PHB/nitrogen ratio.

Microscopical observations on the amount of cytoplasmic sudanophilic material confirmed the general pattern of these results.

Further experiments of a similar nature showed that a suitable medium for the growth of cells poor in PHB, and which yielded a practicable quantity of organisms contained 0.3% glucose and 0.1% ammonium chloride. It was also possible to show that very high levels of PHB could be obtained by culturing the organisms in the presence of 2% glucose and 0.1% ammonium chloride. The balanced medium containing 0.5% glucose and 0.1% ammonium chloride has already been described.

The employment of the above concentrations of glucose and ammonium chloride had the advantage of holding the concentration of the latter constant and producing the type of cells required simply by altering the amount of the carbon and energy source.

4. Analysis of Granules Isolated by the Hypochlorite Method.

(a) Comparison between "Standard" and Commercial Hypochlorite Solutions.

The method of preparation of "Standard" alkaline hypochlorite solution is described by Williamson and Wilkinson (1958) and is repeated here, briefly, for the sake of convenience.

Reagents.

Fresh bleaching powder	200 gm.
Sodium carbonate (anhydrous)	300 gm.
Distilled water	2000 ml.

The sodium carbonate is dissolved in 1500 ml. of water and 500 ml. aqueous suspension of the bleaching powder added. After standing 2 - 3 hr., with occasional shaking, the mixture is filtered and the pH adjusted to 9.8 with concentrated HCl. Incubation at 37° causes the formation of a precipitate which is removed by further filtration.

The commercial hypochlorite solution was obtained from British Drug Houses, Limited, and was adjusted to pH 9.8 with concentrated HCl.

It was foreseen that work on the metabolism of PHB would involve large numbers of hypochlorite estimations, and the use of a commercial solution was investigated in order to obviate, if possible, the frequent preparation of the "Standard" reagent.

Duplicate samples of cells were treated in parallel with "Standard" and BDH" hypochlorite solutions.

Table 7 shows how the turbidity of granule suspensions is affected by the concentration of the commercial solution.

Table 7.

Hypochlorite Reagent.	Turbidity ("Spekker" Scale Readings of Duplicate Estimations).	
"Standard"	0.250	0.248
BDH 100%	0.141	0.145
" 50%	0.226	0.226
" 33%	0.260	0.262
" 25%	0.270	0.281

Thus the concentration of "BDH" hypochlorite solution which gives an equivalent turbidity when used to treat the cells lies between 33% and 50%.

In an attempt to rationalise the potency of these solutions, which did not depend upon pH, estimations of the amount of available chlorine were carried out. Suitable dilutions of the two reagents were titrated with alkaline As_2O_3 solution using a starch/iodide indicator. The average results of several assays gave the available chlorine content of the "Standard" reagent as 0.91%(w/v) and 8.85% for the "BDH" preparation.

Since the amount of available chlorine is only about a tenth as great in the "Standard" solution as compared with the commercial hypochlorite it is obvious

that factors other than this must account for the difference in turbidity noted. As the effect of pH was excluded these factors may have been differences in refractive index, osmotic pressure or ionic concentration. Experience showed that the "Standard" reagent gave more reproducible results and, in view of its ancillary nature, the further investigation of the differences between the two solutions did not seem warranted.

(b) Granules Derived from Hypochlorite Treatment of Whole and Lysed Cells.

Attempts to assay PHB by the hypochlorite method after dissolution of the cell wall by the action of lysozyme were shown to yield consistently low results as compared with estimations using whole cells. A series of experiments were therefore initiated to investigate this discrepancy.

A 2 l. culture grown to produce cells rich in PHB was washed and the organisms resuspended in 200 ml. phosphate buffer, the suspension containing 2.69 mg./ml. total N. To half of this preparation was added 0.3 mg./ml. lysozyme and the mixture incubated at 37° for 30 min. after which time lysis was complete by microscopic observation. Both 100 ml. aliquots were then treated with 500 ml. of "Standard" hypochlorite reagent and incubated at 37° for 90 min. A check on the turbidities at this stage showed a "Spekker" scale reading of 0.307 x 10 for whole and 0.257 x 10 for lysed cells representing a granule mass from the calibration curve of

0.97 and 0.89 mg./ml. respectively. These figures were in accord with several observations of the discrepancy between the granule turbidities of whole and lysed cells.

The granules from both suspensions were recovered by centrifugation at 65,000 g. for 10 min. in a "Spinco" preparative ultracentrifuge. After washing thrice with distilled water they were dried at 95° in a hot air oven and brought to constant weight in vacuo over P₂O₅. The granules from the whole cells weighed 0.5805 gm. and from lysed cells 0.5379 gm. These figures when reduced to mg./ml. by dividing by the original volume of each suspension after addition of hypochlorite (600 ml.) gave 0.970 mg. for whole and 0.895 mg. for lysed cell preparations in excellent agreement with the turbidity measurements.

From each batch of dried granules two samples containing about 100 mg. were accurately weighed. These were exhaustively extracted with boiling ether and the combined extracts weighed after removal of the ether, firstly by evaporation and then in vacuo over wax chips. The average of the duplicate determinations yielded values of 6.6% and 3.3% for the ether soluble material from whole and lysed cell granules respectively. This difference in the contents of ether soluble substances does not explain completely the variance in weight between the two lots of granules. After subtracting the mass of the ether-extracted material the granules weigh 0.906 mg./ml. for whole cells and 0.865 mg./ml. for lysed cells leaving 0.041 mg./ml., a significant quantity,

to be accounted for. The substance remaining after ether treatment of the granules is completely soluble in chloroform and has been shown to consist solely of PHB (Williamson, 1956).

To investigate any further difference between the two sets of granules total nitrogen and total phosphorus determinations were carried out in triplicate. The average results of these measurements showed that granules from whole cells contained 0.254% nitrogen and 0.140% phosphorus and from lysed cells 0.127% nitrogen and 0.082% phosphorus. Thus the proportion of nitrogen found exactly paralleled the difference in the ether soluble lipid content and so, passably, did the percentages of phosphorus.

Examinations of the supernatant solution from the formation of protoplasts by the action of lysozyme (see p. 105) showed that it contained insignificant amounts of ether or chloroform soluble material, without preliminary hydrolysis.

These results on the effects of hypochlorite on cells of Bacillus species show that the PHB assay method based on this reaction is only reliable in certain conditions. In particular it is suitable only for intact cells under carefully controlled conditions of pH and available chlorine content. When the cell wall is destroyed with lysozyme a lower yield of PHB, and an even lesser yield of ether soluble material, is obtained. This suggests that at least part of the ether soluble fraction, which is associated with the granules isolated

by hypochlorite, may be derived from other cell components probably located in the cell wall. Other experiments have suggested that greatly increased yields of ether soluble material and smaller amounts of PHB are obtained as the available chlorine content or alkalinity of the reagent is raised. This might be explained by the greater hydrolysis of bound cell wall or cytoplasmic lipids with the consequent release of their ether soluble components. The quantities of phosphorus and nitrogen found, which have been shown to parallel the amount of ether soluble fraction, further suggest that this material might be derived by hydrolysis of complex phosphorus and nitrogen containing lipids by the action of the hypochlorite. A further possibility causing an increased ether soluble fraction is suggested by the fall in the PHB yield as this is probably accounted for by partial hydrolysis which enhances its solubility in ether. It has been observed that strong hypochlorite gives rise to fractions (probably of low molecular weight) which are only precipitated from chloroform by ether when the latter is in great excess. These observations of differing solubility in chloroform and ether are confirmed by the work of Képès and Péaud-Lenoël (1952) who prepared a series of β -hydroxybutyric acid polymers by differential precipitation by ether from chloroform after preliminary acid hydrolysis. Further work, directed at analysing the ether soluble lipid fraction obtained from whole and lysed cells with different concentrations of hypochlorite, should prove fruitful

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in resolving the problem of the site of origin of this material.

4. Influence of ...

(a) The influence of ...

The results described in this report were obtained in three runs (A, B, C) and in two experiments exploring the effect of ...

A phosphate buffer was used throughout the experiment. A 0.05 M phosphate buffer was used in all runs. The pH of the buffer was maintained at 7.0. In one run air was filtered through a ... in the other runs air was not filtered. The temperature was kept constant at 25°C. The composition of the gas mixture was kept constant by frequent replenishment. The results of the experiments are given in Fig. 6 which shows that the ... both measurements fall ... according to ... than especially when it was ... was that in water the loss was ... most of the degradation having occurred ... in nitrogen the level ... experimentally period of 1 hr.

Comparable results were obtained when ...

5. Washed Suspension Experiments.

A. Breakdown of PHB.

(a) The Influence of Aerobic and Anaerobic Conditions.

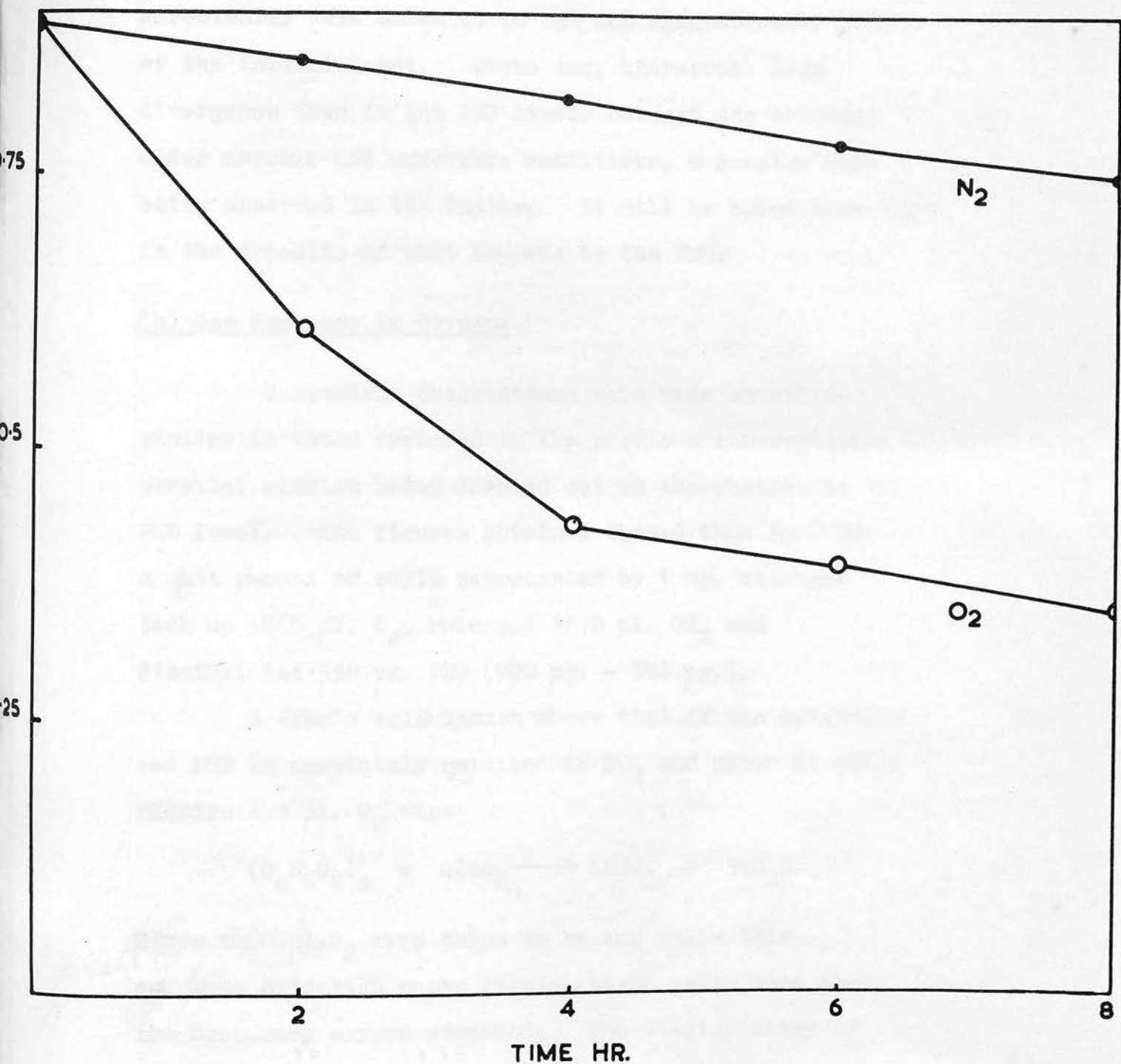
The results described in this sub-section (a) and in three which follow (b), (c) and (h) were derived from experiments employing the AC strain of B. cereus grown on solid media.

A phosphate buffer cell suspension, containing 0.089 mg./ml. total N and rich in PHB, was divided into two 50 ml. aliquots which were placed in separate 250 ml. flasks. In one the air was flushed out with nitrogen, in the other with oxygen. The vessels were shaken at 30° for 8 hr., the composition of the gas phase being kept constant by frequent replenishment. Traces of oxygen in the nitrogen were absorbed by passing over heated copper. Samples were removed initially and at 2, 4, 6 and 8 hr. and assayed for PHB content. The results are given in Fig. 6 which shows that the PHB level in both suspensions fell. Anaerobically this loss, amounting to 17% of the original level, was much less than aerobically where it was 61%. A further difference was that in oxygen the loss was most rapid initially, most of the degradation having occurred by 4 hr., whereas in nitrogen the level steadily declined throughout the experimental period of 8 hr.

Comparable results were obtained using the KM strain of B. megaterium grown on liquid media.

PHB/N.

FIG. 6.

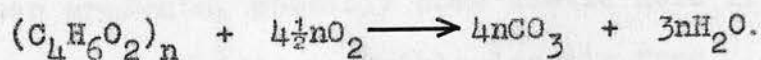


In a similar experiment, in which measurements of the anthrone value for polysaccharide were made, it could be shown that this material was also partially lost. Aerobically this amounted to 13% and anaerobically 30% of the initial level. There was, therefore, less divergence than in the PHB levels between the decrease under aerobic and anaerobic conditions, a greater fall being observed in the latter. It will be noted that this is the opposite of what happens to the PHB.

(b) Gas Exchange in Oxygen.

Manometric observations were made on cells similar to those employed in the previous sub-section, parallel studies being carried out on the changes in the PHB level. The figures obtained showed that in 8 hr. a unit amount of cells represented by 1 mg. nitrogen took up 1460 μ l. O_2 , released 1270 μ l. CO_2 and dissimilated 550 μ g. PHB (900 μ g. - 350 μ g.).

A simple calculation shows that if the metabolised PHB is completely oxidised to CO_2 and water it would require 642 μ l. O_2 viz:-



Since 1460 μ l. O_2 were taken up by the cells this complete oxidation seems likely, there being more than the necessary oxygen absorbed. The dissimilation of other compounds, particularly polysaccharide, presumably accounts for the remainder of the oxygen uptake. Other experiments were carried out which confirmed the general

pattern of these results.

(c) Anaerobic Acid Production.

Again, as in sections (a) and (b), PHB-rich cells (0.70 mg.N/ml.) were employed in these measurements which were conducted by manometric methods in bicarbonate buffer with 5% CO₂ in nitrogen as the gas phase. Side by side with the measurement of acid production the decrease in the PHB level was followed by assays of this material.

When calculated to 1 mg. cell nitrogen the total amount of acid formed in 4 hr. was 4.67 μ .mole. The amount of PHB lost was 128 μ g./mg.N and if this was simply hydrolysed to β -hydroxybutyric acid according to the equation:-



then the amount degraded would result in the formation of 1.49 μ .mole acid. Thus more acid is formed than can be explained by a simple hydrolysis of the dissimilated PHB. Attention must be drawn to the probable formation of other products, possibly some acetic acid from PHB but presumably acidic materials largely from polysaccharide substances. As shown in (d) below a maximum of about 10% of the PHB lost could be converted to acetic acid. The results suggest that the oxygen uptake aerobically and the acid production anaerobically cannot be explained wholly in terms of PHB and polysaccharide dissimilation, but the point was not

further investigated. A partial explanation may be afforded by the low value given by the anthrone method for polysaccharide estimation, and by the dissimilation of cell lipids.

(d) The formation of β -hydroxybutyric and Acetoacetic Acids from PHB.

Three aliquots of PHB-rich suspension in phosphate buffer containing 0.59 mg./ml. cell nitrogen were shaken at 30° for 3½ hr. in nitrogen, hydrogen and air respectively. Initial and final assays of PHB, β -hydroxybutyrate, acetoacetate and acetone were made. No acetone was present in any of the samples. The other results are given in Table 8.

Table 8.

Sample.	PHB * (μ mole/ml.)	PHB lost,* (μ mole/ml.)	β -hydroxybutyrate. (μ mole/ml.)	Acetoacetate. (μ mole/ml.)
Initial	28.8	-	-	-
N ₂ 3½ hr.	27.0	1.86	1.08	0.58
H ₂ "	27.0	1.86	1.11	0.50
Air "	22.3	6.75	0.03	0.44

* Calculated as C₄H₆O₂ residues.

The same amount of PHB is lost in nitrogen as in hydrogen and 89% of the quantity dissimilated in the former and 87% of that in the latter can be accounted for in terms of β -hydroxybutyrate and acetoacetate. In air only 7% of the amount lost can be explained in this way.

In nitrogen and hydrogen the discrepancy between the observed fall in PHB and the formation of β -hydroxybutyrate and acetoacetate might be represented, wholly or partly, by inaccuracies inherent in the experimental assay methods.

The much more extensive dissimilation of PHB under aerobic conditions was again displayed, in this case using the KM strain, and the low levels of β -hydroxybutyrate and acetoacetate found suggest that these products were further oxidised.

(e) Chromatographic Analysis of Breakdown Products.

Cells with a high content of PHB were suspended in phosphate buffer giving a concentration of 0.74 mg./ml. total nitrogen. Two 65 ml. aliquots of this suspension were shaken at 30° for 4 hr. one in nitrogen the other in air. Initial and final PHB assays showed that the level had fallen by 60 μ g./ml. (1580 μ g. - 1520 μ g.) anaerobically and 220 μ g./ml. (1580 μ g. - 1360 μ g.) aerobically. The cells were centrifuged off and two 25 ml. portions taken from each supernatant.

One 25 ml. sample from each was chromatographed after the formation and extraction of the 2 : 4 dinitrophenylhydrazone derivatives of any reactive compounds present. The chromatograms showed the presence in both supernatants of acetoacetic acid. By treating β -hydroxybutyric and acetic acids in the same manner reference spots were obtained which in the case of β -hydroxybutyric acid corresponded with a spot in the

unknowns. This was very weak, however, in the aerobic supernatant sample and the presence of a spot corresponding to acetic acid was doubtful. Neither pyruvic acid, acetone nor spots relating to any other compounds were found.

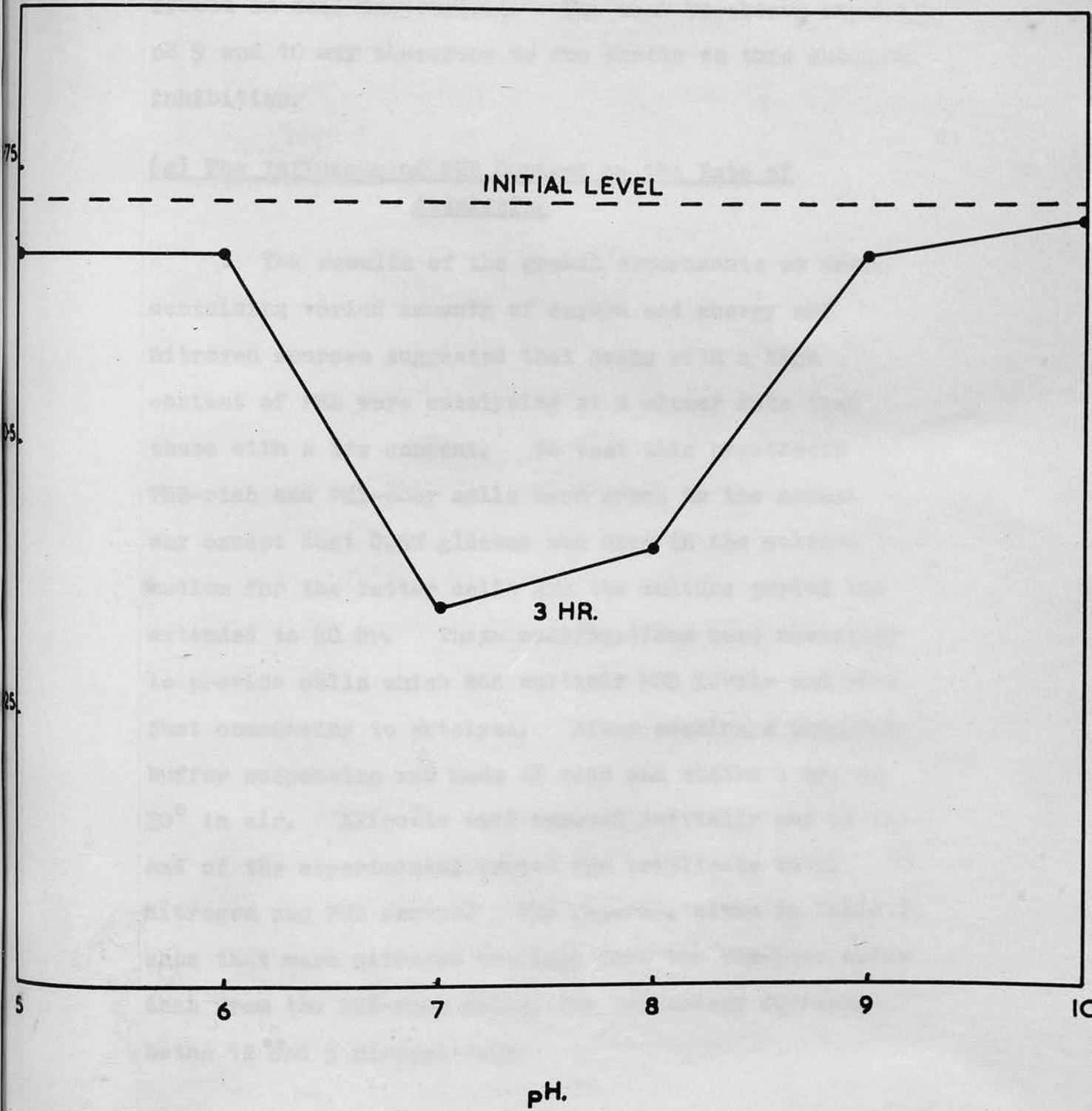
The other 25 ml. portions were de-ionised by the addition of 10%(w/v) Amberlite IR - 120 (H) resin and then submitted to ascending chromatography for the presence of C₂ - C₆ straight chain fatty acids. The resulting chromatograms showed that despite excellent resolution of the reference acids there were no spots corresponding to C₃ - C₆ fatty acids present in either unknown sample. Whether acetic acid was present or not was uncertain as by the method employed its flow rate was similar to that of β -hydroxybutyric acid which was there in relatively large quantities.

(f) The Effect of the Hydrogen Ion Concentration.

Aliquots of 20 ml. of a suspension of cells in normal saline containing 0.043 mg.N./ml. were added to 20 ml. quantities of 0.2 M. buffers of pH 5, 6, 7, 8, 9 and 10. The buffers of pH 5 and 6 were potassium acid phthalate/sodium hydroxide, of 7 and 8 potassium dihydrogen phosphate/sodium hydroxide and of 9 and 10 boric acid/sodium hydroxide (with added potassium chloride). Samples were taken initially and after shaking for 3 hr. at 30° in air and assayed for PHB content. The results, shown in Fig. 7, reveal that degradation of PHB is greatest around pH 7, little

HB/N.

FIG. 7.



breakdown occurring at pH 5, 6, 9 or 10. Experiments to be described in the section dealing with PHB synthesis have shown that borate exerts an inhibitory effect on cell suspensions. The weak breakdown shown at pH 9 and 10 may therefore be due partly to this chemical inhibition.

(g) The Influence of PHB Content on the Rate of Autolysis.

The results of the growth experiments on media containing varied amounts of carbon and energy and nitrogen sources suggested that cells with a high content of PHB were autolysing at a slower rate than those with a low content. To test this hypothesis PHB-rich and PHB-poor cells were grown in the normal way except that 0.5% glucose was used in the culture medium for the latter cells and the culture period was extended to 40 hr. These modifications were necessary to provide cells which had suitable PHB levels and were just commencing to autolyse. After washing, a phosphate buffer suspension was made of each and shaken 4 hr. at 30° in air. Aliquots were removed initially and at the end of the experimental period for triplicate total nitrogen and PHB assays. The figures, given in Table 9, show that more nitrogen was lost from the PHB-poor cells than from the PHB-rich cells, the percentage decrease being 12 and 5 respectively.

Table 9.

Type of Cells	PHB $\mu\text{g./ml.}$		Total N $\mu\text{g./ml.}$	
	Initial	Final	Initial	Final
PHB-rich	78	63	160	152
PHB-poor	42	37	152	134

This experiment and others of a similar nature lend support to the contention that high levels of PHB slow down the rate of autolytic processes.

It was thought that in view of its effect on autolysis, a high level of PHB might lead to assimilation of an extraneous source of nitrogen in the absence of an external carbon and energy source. Repeated attempts to demonstrate this were unsuccessful, however, using a wide variety of PHB levels and concentrations of external nitrogen source.

(h) The Effect on Endogenous Respiration of the PHB Level.

In view of the effect of the PHB content on the rate of autolysis it seemed likely that other general criteria of metabolic behaviour, such as the endogenous respiration rate should be similarly influenced. To determine whether this was so, a phosphate buffer suspension of washed PHB-rich cells containing 0.076 mg.N/ml. was prepared. From this initial samples were removed for PHB, polysaccharide and total nitrogen assays. Manometric observations in oxygen were carried

out in duplicate for 8 hr. at 30°. Parallel studies were made of the PHB and polysaccharide levels at 2 hr. intervals throughout the course of the experiment. The cells for these latter measurements were contained in a 2 l. oxygen-filled flask which was shaken in the same bath as the manometers. The initial volume of the suspension in this vessel was 250 ml. The results are given in Table 10 and Fig. 8.

Table 10.

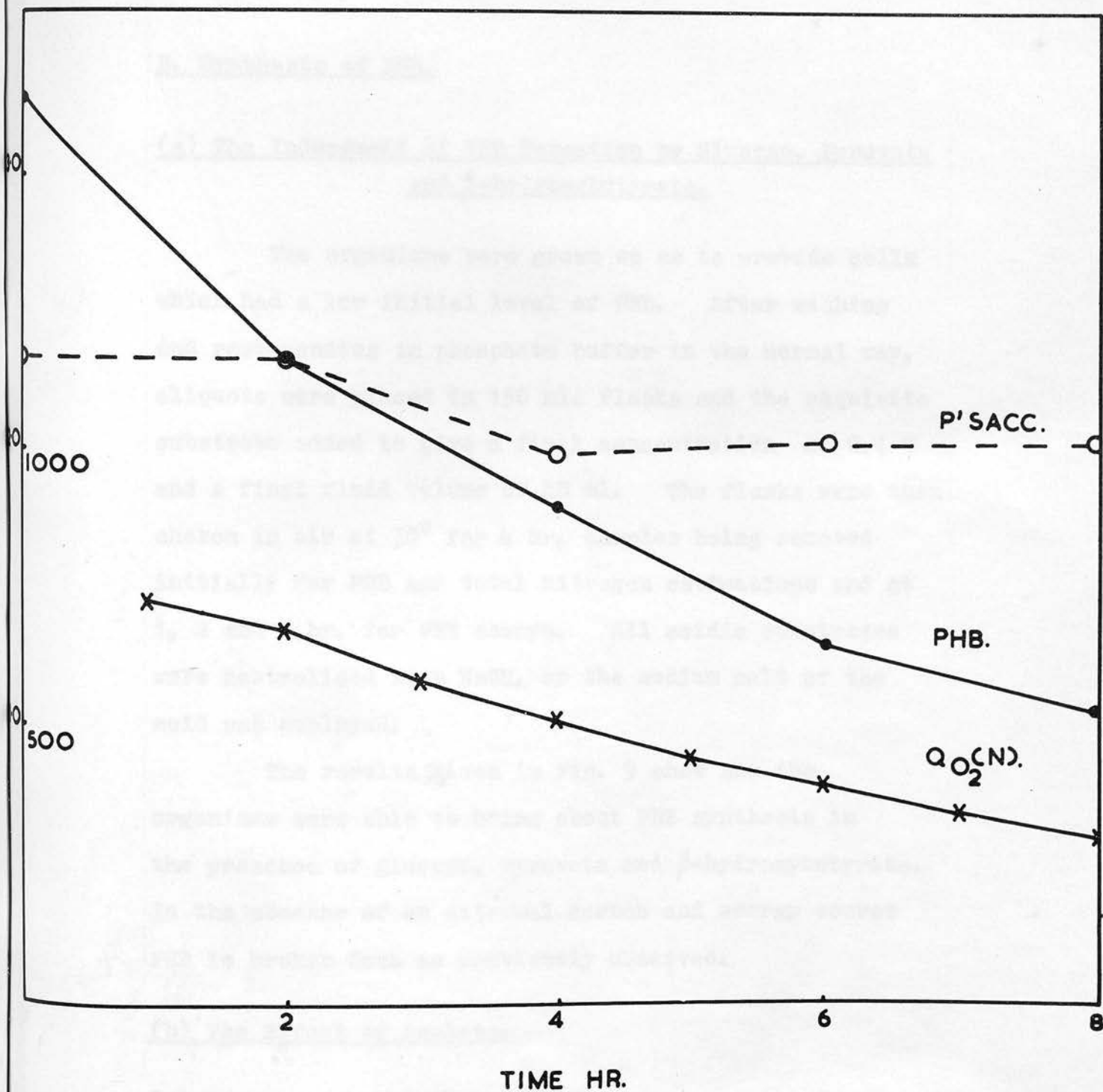
Time (hr)	Q_{O_2} (N).	PHB/N.	Polysaccharide/N.	Q_{O_2} (N)/PHB/N.
0	-	3.27	2.32	-
2	675	2.32	2.33	290
4	510	1.77	1.97	288
6	385	1.28	2.01	292
8	290	1.04	2.01	280

The Q_{O_2} (N) represents the oxygen uptake in μ l. per mg. total cell nitrogen per hr. and in the Table was calculated from the oxygen uptake during the 30 min. before and after the time indicated. The last column which gives the ratio of the Q_{O_2} (N) value to the PHB/N value indicates how closely the endogenous respiration rate is linked to the PHB content. Although too much cannot be read into these figures, it does seem clear that there is a strong connection between the two vectors and that this relationship is little affected, if at all,

PHB OR P'SACC/N.

Q_{O_2} (N).

FIG. 8.



by the amount of polysaccharide present, a deduction which follows from the relative stability of the level of this material throughout the course of the experiment.

B. Synthesis of PHB.

(a) The Inducement of PHB Formation by Glucose, Pyruvate and β -hydroxybutyrate.

The organisms were grown so as to provide cells which had a low initial level of PHB. After washing and resuspending in phosphate buffer in the normal way, aliquots were placed in 150 ml. flasks and the requisite substrate added to give a final concentration of 0.1 M and a final fluid volume of 40 ml. The flasks were then shaken in air at 30° for 4 hr. samples being removed initially for PHB and total nitrogen estimations and at 1, 2 and 4 hr. for PHB assays. All acidic substrates were neutralised with NaOH, or the sodium salt of the acid was employed.

The results given in Fig. 9 show how the organisms were able to bring about PHB synthesis in the presence of glucose, pyruvate and β -hydroxybutyrate. In the absence of an external carbon and energy source PHB is broken down as previously observed.

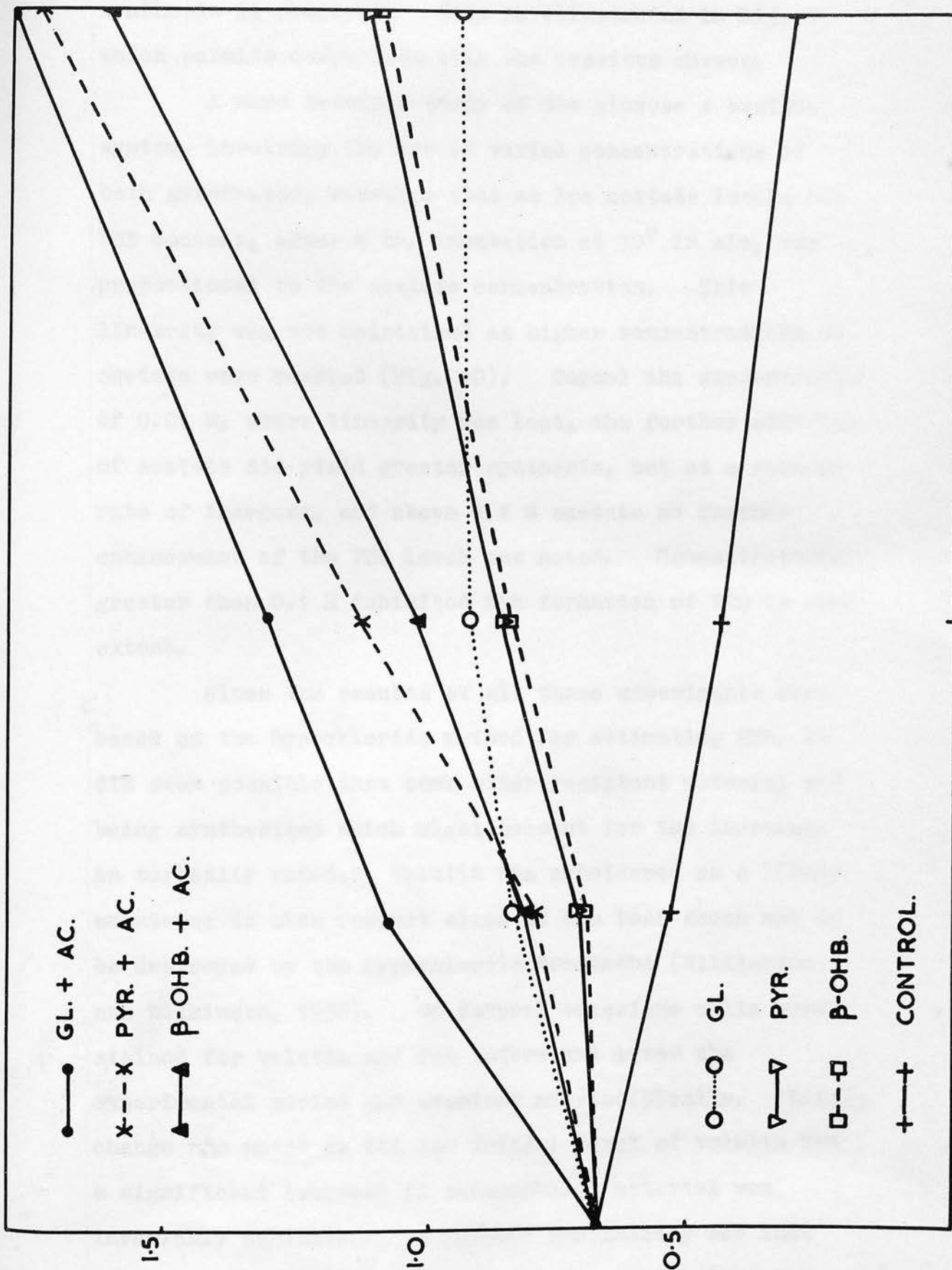
(b) The Effect of Acetate.

(1) Stimulation of PHB Synthesis.

When acetate is added to any of the substrates described in (a) above, a pronounced enhancement of

PHB/N.

FIG. 9.



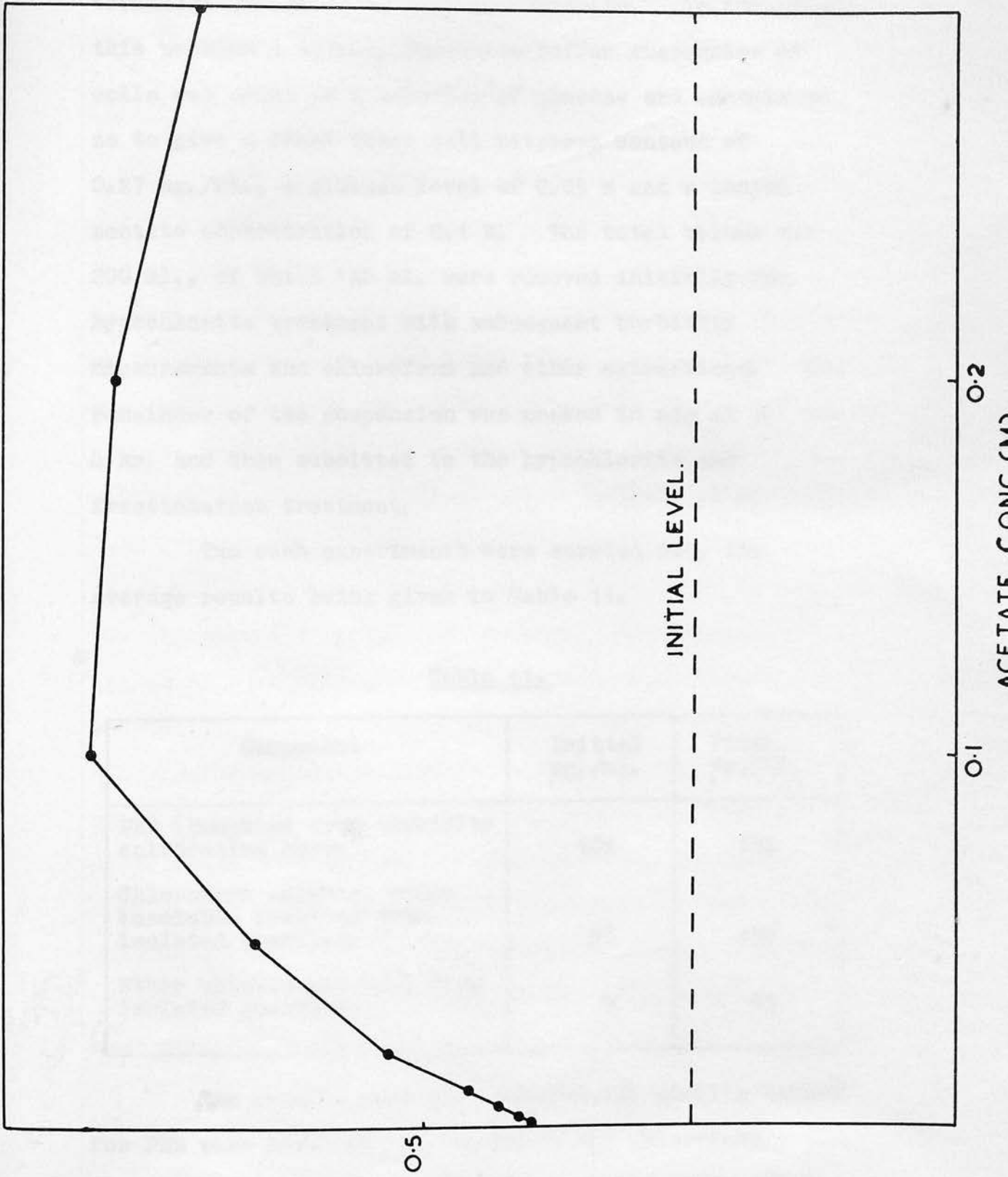
synthesis is observed. This is illustrated in Fig. 9, which permits comparison with the previous curves.

A more detailed study of the glucose + acetate system, involving the use of varied concentrations of both substrates, revealed that at low acetate levels the PHB content, after 4 hr. incubation at 30° in air, was proportional to the acetate concentration. This linearity was not maintained as higher concentrations of acetate were reached (Fig. 10). Beyond the concentration of 0.01 M, where linearity was lost, the further addition of acetate did yield greater synthesis, but at a reduced rate of increase, and above 0.1 M acetate no further enhancement of the PHB level was noted. Concentrations greater than 0.1 M inhibited the formation of PHB to some extent.

Since the results of all these experiments were based on the hypochlorite method for estimating PHB, it did seem possible that some other resistant material was being synthesised which might account for the increases in turbidity noted. Volutin was considered as a likely substance in this respect since it has been shown not to be destroyed by the hypochlorite treatment (Williamson and Wilkinson, 1958). On several occasions cells were stained for volutin and fat before and after the experimental period and examined microscopically. Little change was noted in the low initial level of volutin but a significant increase in sudanophilic material was invariably exhibited. A further possibility was that ether soluble lipid material was being synthesised and

FIG. 10.

PHB/N.



that this was then affecting the turbidity after treatment of the cells with hypochlorite. To investigate this problem a washed, phosphate buffer suspension of cells was added to a solution of glucose and acetate so as to give a final total cell nitrogen content of 0.27 mg./ml., a glucose level of 0.05 M and a sodium acetate concentration of 0.1 M. The total volume was 200 ml., of which 120 ml. were removed initially for hypochlorite treatment with subsequent turbidity measurements and chloroform and ether extractions. The remainder of the suspension was shaken in air at 30° for 4 hr. and then submitted to the hypochlorite and fractionation treatment.

Two such experiments were carried out, the average results being given in Table 11.

Table 11.

Component	Initial µg./ml.	Final µg./ml.
PHB (computed from turbidity calibration curve).	102	138
Chloroform soluble, ether insoluble fraction from isolated granules.	98	139
Ether soluble material from isolated granules.	9	15

The results show that essentially similar values for PHB were given by the turbidity and chloroform extraction procedures. The percentage of ether soluble material in the granules was apparently little affected

in the synthesis of PHB from glucose plus acetate. This places the results of the experiments on PHB synthesis on a firmer footing.

Grown

(ii) Studies on Acetate-Adapted Cells.

Using PHB-poor cells, grown in the normal manner, the presence of acetate alone was insufficient to cause the organisms to form PHB. There was, however, a suppression of breakdown of this material in the presence of acetate concentrations above about 0.01M. The possibility existed that cells previously adapted to acetate might be able to synthesise PHB from this additive alone. Cells were therefore grown in the 0.3% glucose medium with the addition of sodium acetate to give a final concentration of 0.1 M. Grown in this way the organisms presented an unusual morphological appearance, showing long slender cells packed with sudanophilic inclusions and having a PHB/N ratio of 4.53. Experiments with these cells revealed that synthesis of PHB took place in the normal way when glucose and acetate were added to washed suspensions. Results using acetate by itself were variable and it was concluded that these organisms differed little from those unadapted to acetate in their ability to synthesise PHB from this substrate alone.

(iii) Manometric Observations.

These studies were conducted with both normally grown and acetate-adapted cells. The results showed that the organisms tended to vary rather widely in metabolic

activity, reflecting the physiological age of the culture and such vectors as the PHB content which, as has been shown previously, exerts a marked effect on the endogenous respiration rate. It was found to be virtually impossible to grow cells under different conditions (i.e. with or without the addition of acetate) in such a way as to produce organisms of closely similar metabolic potentialities and PHB content. Thus, though the results yielded by any one batch of cells are valid, numerical comparison between different experiments is of little value without recourse to the endogenous respiration rates as a general index of metabolic activity.

Using acetate-adapted cells in the presence of 0.2 M acetate the Q_{O_2} (N) value (corrected for endogenous respiration) was found to be 327 and the corresponding figure for endogenous respiration 330. With cells grown in the usual manner these rates were 217 and 226 respectively. Growth in the presence of acetate did not lead, therefore, to any significant increase in the rate of oxygen uptake by the cells on this substrate.

Since acetate provoked such a sharp increase in the rate and extent of PHB synthesis when added to cell suspensions already containing glucose, it seemed possible that it may have had a synergistic effect with glucose on the oxygen uptake. Cells grown on 0.5% glucose were employed and final concentrations of 0.1 M glucose and 0.2 M acetate were used in the Warburg reaction vessels. An amount of cells represented by

0.625 mg. total nitrogen was placed in each vessel and observations carried out in air at 30°. The $Q_{O_2}(N)$ values which, where substrates were added, were all corrected for endogenous respiration were as follows:- glucose 365, acetate 8, glucose + acetate 310, endogenous 64. Acetate apparently has no stimulatory action on the uptake of oxygen by cells metabolising glucose, if anything a slight inhibition is displayed. The low respiratory activity on acetate reflects the variability between different batches of organisms previously mentioned.

(c) Other Substrates as Inducers of PHB Formation.

Many other substances at a concentration of 0.1 M were tested for their influence on PHB synthesis both with and without the added presence of glucose (0.05 M). These are listed in Table 12 which gives the change in the PHB/N ratio after 2 hr. and 4 hr. incubation in air at 30°.

Table 12.

The Synthesis of poly- β -hydroxybutyrate on a
Variety of Substrates.

Initial poly- β -hydroxybutyrate/total nitrogen
ratio = 0.31.

Substrate	Poly- β -hydroxybutyrate content, (PHB/N ratio).			
	2 hr.	4 hr.	+Glucose (0.05 M)	
			2 hr.	4 hr.
None	0.26	0.24	0.36	0.35
Acetaldehyde	0.30	0.31	-	-
Acetaldehyde + acetate	0.30	0.31	-	-
Acetaldehyde + pyruvate	0.29	0.32	-	-
Acetate	0.32	0.30	0.62	0.87
Acetoacetate	0.32	0.31	0.36	0.33
Butyrate	0.28	0.28	0.36	0.37
Caproate	0.28	0.26	0.37	0.37
Citrate	0.30	0.22	0.37	0.36
Crotonate	0.28	0.28	0.36	0.40
Formate	0.28	0.26	0.37	0.36
Fumarate	0.29	0.29	0.38	0.36
Glycerol	0.32	0.28	0.34	0.33
2 - Hydroxy - 1 - propane - sulphonate	0.26	0.24	0.36	0.35
Propionate	0.31	0.28	0.33	0.33
Pyruvate	0.34	0.37	0.58	0.84
n-Valerate	0.31	0.30	0.36	0.36

It will be noted that insignificant synthesis has taken place on any of the substrates not previously employed. Further, acetaldehyde, which seemed a likely two-carbon intermediate on the synthetic pathway, was similarly inactive. It did, in fact, cause complete inhibition of synthesis down to a concentration of 0.005 M.

(d) The Effect of the Addition of a Nitrogen or Magnesium Source on PHB Synthesis.

In all experiments concerning the formation of PHB which follow in this and subsequent sections, standard concentrations of glucose (0.05 M) and acetate (0.1 M) were employed. The cell suspension density was about 0.1 mg.N/ml. and the total fluid volume 40 ml. The flasks (150 ml.) containing the suspension were shaken in air at 30° for an experimental period of 4 hr. Where any variation in these conditions has been made it is given in the relevant Section.

As a source of nitrogen, ammonium chloride was used in concentrations of 0.01 and 0.001 M. Both caused a slight inhibition of synthesis amounting to 19% and 12% respectively. This was interpreted as being due to the diversion of energy and intermediates from glucose and acetate metabolism to processes connected with growth.

The addition of magnesium chloride at a concentration of 0.01 M. led to a 15% inhibition of synthesis thus confirming that a scarcity of the cation magnesium was not a limiting factor in PHB production under the experimental conditions.

(e) The Influence of Certain Inhibitors on PHB Synthesis and Oxygen Uptake.

(i) Fluoroacetate.

When this material was added in suitable concentrations ranging from 0.001 to 0.025 M. it was found that at 0.01 M. and below a slight stimulation of synthesis took place. The degree of this stimulation is shown in Table 13.

Table 13.

Fluoroacetate concentration (M).	% Stimulation of PHB Synthesis.	% Inhibition of Oxygen Uptake.
0.001	13	-
0.005	4	33
0.01	1	35
0.025	-3	34

At 0.025 M. some inhibition of synthesis was displayed.

The effect on the oxygen uptake is one of inhibition as is also shown in Table 13.

These effects are what would be expected if fluoroacetate were blocking the tricarboxylic acid cycle as it is known to do. In this way more intermediates would be available for PHB formation resulting in the observed stimulation of synthesis, and complete oxidation of such compounds would be suppressed

as is suggested by the reduction in the level of oxygen consumption.

(ii) Potassium Cyanide.

At the lowest concentration of this material employed in experimental observations (0.0025 M.) 94% inhibition of PHB synthesis was brought about. Using B. cereus under similar conditions a KCN concentration of 0.005 M. caused 83% inhibition of aerobic synthesis. Mention of KCN as an inhibitor is made again in the section dealing with the effect of the gaseous environment on the synthesis of PHB. In this context it seems likely that its effect is brought about by the inactivation of the cytochrome system with the attendant standstill of oxidative metabolism and subsequent deprivation of energy to the cell. In these circumstances it would be expected that "inessential" (if this teleological epithet may be used) processes such as the synthesis of PHB would be amongst the first to be curtailed.

(iii) 2 : 4 Dinitrophenol.

At concentrations of 1 and 2×10^{-4} M. this substance caused an inhibition of PHB synthesis equivalent to 30% and 57% respectively.

When manometric observations on the oxygen uptake of the cells on glucose were carried out using the same concentrations a stimulation was noted of 8% for 1×10^{-4} M. and 2.5% for 2×10^{-4} M. These results are in accord with the known effects of

2 : 4 dinitrophenol in uncoupling the metabolic mechanisms for the formation of energy rich phosphate bonds. In this manner carbon assimilation is inhibited and oxygen uptake stimulated.

(iv) d-(2 Pantoyl tauryl)-para-Anisidide. (PTA).

Since this compound inhibits the synthesis of co-enzyme A from metabolic precursors (Klein and Lipmann, 1953) it was thought that if this co-enzyme was concerned in PHB formation the addition of PTA should decrease the production of PHB. Studies were therefore made of the effects of added PTA on PHB synthesis and oxygen uptake, the results being shown in Table 14.

Table 14.

PTA Concentration (mg./ml.)	% Inhibition of PHB Synthesis.	% Inhibition of Oxygen Uptake.
0.0625	18	15
0.125	29	15
0.25	35	15
0.5	33	15
1.0	44	-

It can be seen that increasing concentrations of PTA result, by and large, in greater suppression of PHB formation. From the results it would appear that a similar extent of inhibition of oxygen uptake on glucose has been caused by all the concentrations of PTA employed,

despite the greatest being eight times the strength of the weakest. The reason for the absence of a graded response is not known.

(f) Studies using Analogues of β -hydroxybutyric Acid.

The compound sodium 2-hydroxy-1-propane sulphonate ($\text{CH}_3\text{.CHOH.CH}_2\text{.SO}_3\text{.Na}$) was employed as a possible substrate which the cells might utilise for polymerisation. All experiments in this context were negative, however, whether glucose was added or not.

The same compound was examined in a different capacity to determine if it could act as a competitive inhibitor of PHB synthesis. It was found that little inhibition was caused and low concentrations resulted in a slight stimulation of PHB formation. This stimulation amounted to 3% at 0.01 M. and 10% at 0.005 M. levels of the sulphonate. These results were repeated on several occasions and when controlled manometrically no effect on the oxygen uptake of the cells, with glucose as substrate, was displayed.

Another analogue of β -hydroxybutyrate, sodium β -mercaptobutyrate ($\text{CH}_3\text{.CHSH.CH}_2\text{.COONa}$) was tested in concentrations of 0.1 to 0.01 M. to discover if it could substitute for the authentic compound as regards polymerisation by the cell. As in the case of the sulphonate, however, this material was found to be inactive even after the addition of glucose (0.05 M.).

In the case of both these analogues the conclusion that they are inactive in promoting the

formation of a corresponding polymer to PHB rests upon the assumption that any polymer so formed would react in a similar manner to PHB as regards treatment with hypochlorite. It is obvious that this may well be an invalid assumption.

A further complicating factor in the study of these substances (and for that matter all other) as substrates in washed suspension observations is the interference of permeability artefacts. It is almost certain that without specific (or sufficiently non-specific) transport mechanisms these compounds would be excluded from the cell. The properties of the bacilli have not been studied in this respect and their inability to utilise the above substances, or any in Table 12, as substrates for PHB formation may reflect their unavailability to the organism.

(g) The Effect of the Gaseous Environment.

(i) Oxygen.

Following upon an observation that synthesis in air was apparently greater than when oxygen was employed as the gas phase an investigation of the effect of oxygen was carried out.

In pure oxygen the organisms soon ceased to synthesise PHB and commenced to dissimilate their reserves of this material after some 2 hr., even in the presence of glucose and acetate. In air the rate of PHB formation tended to fall off as the incubation period was prolonged.

This oxygen effect could not be reversed by adding reducing agents such as cysteine to cell suspensions.

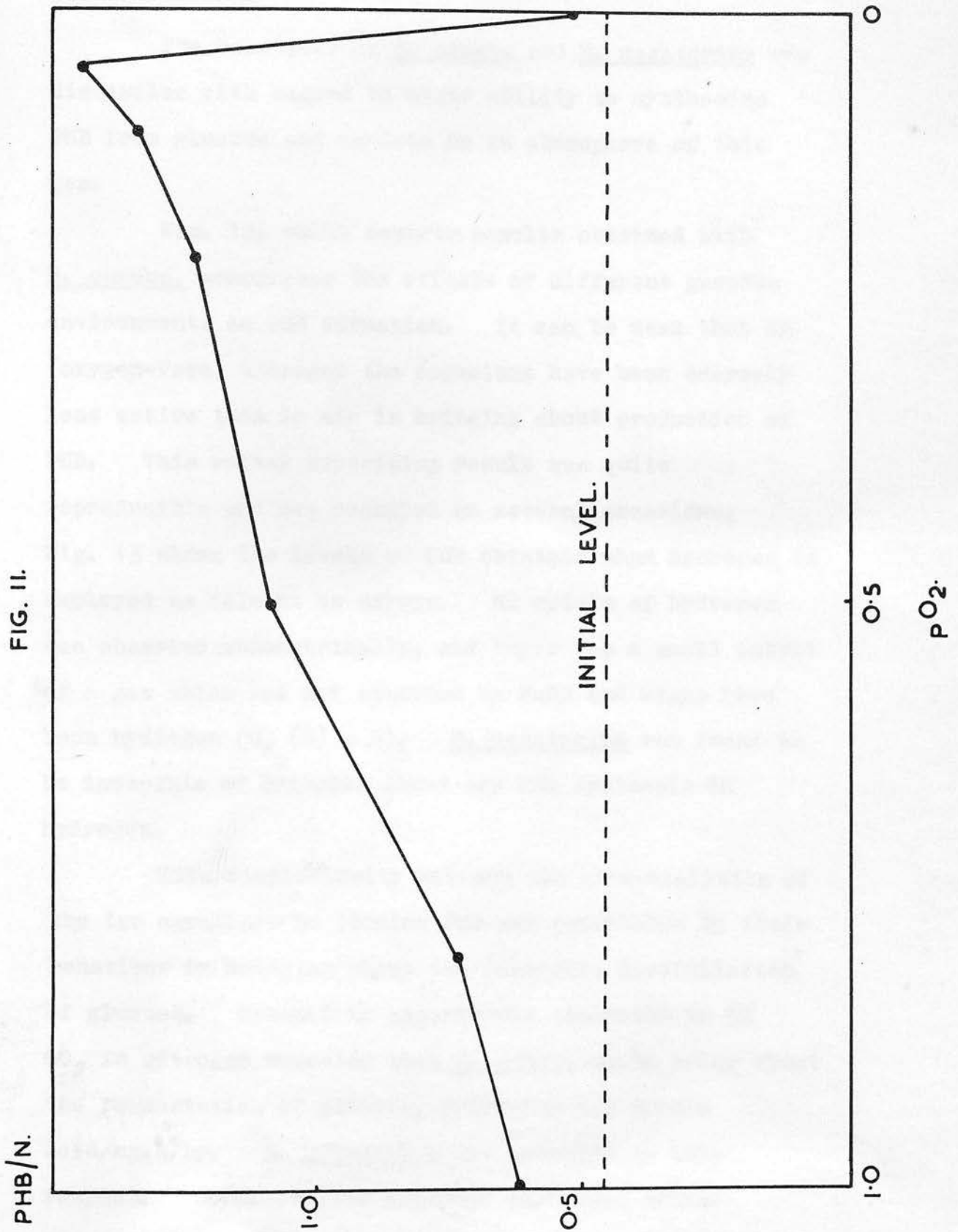
The degree of sensitivity of PHB synthesis to oxygen was dissimilar for different substrate systems. Thus the percentage inhibition brought about by pure oxygen, as compared with air, was 77% for pyruvate + acetate, 44% for glucose + acetate and 11% for β -hydroxybutyrate + acetate.

(ii) Nitrogen.

Using this substance as gas phase (previously passed over heated copper to remove traces of oxygen) no synthesis took place in any of the substrate systems which gave positive results in air. This was true of B. cereus as well as B. megaterium.

Since no synthesis took place in nitrogen it provided an ideal diluent in studying the effect of various partial pressures of oxygen on PHB formation in glucose and acetate. Fig. 11 shows how the content of PHB after 4 hr. incubation at 30° was inversely related to the oxygen concentration down to a level of 5%, after which it fell to about the initial value (in pure nitrogen). It will also be noted that the rate of increase of PHB formation, as a function of the attenuation of oxygen, became accelerated as the lower oxygen concentrations were approached, there being a greater increase between a p_{O_2} of 0.10 and 0.05 than between p_{O_2} 0.21 and 0.10.

FIG. II.



(iii) Hydrogen.

The behaviour of B. cereus and B. megaterium was dissimilar with regard to their ability to synthesise PHB from glucose and acetate in an atmosphere of this gas.

Fig. 12, which depicts results obtained with B. cereus, summarises the effects of different gaseous environments on PHB formation. It can be seen that in (oxygen-free) hydrogen the organisms have been scarcely less active than in air in bringing about production of PHB. This rather surprising result was quite reproducible and was repeated on several occasions.

Fig. 13 shows the levels of PHB obtained when hydrogen is employed as diluent to oxygen. No uptake of hydrogen was observed manometrically, and there was a small output of a gas which was not absorbed by NaOH and might have been hydrogen ($Q_{H_2}(N) = 4$). B. megaterium was found to be incapable of bringing about any PHB synthesis in hydrogen.

This dissimilarity between the potentialities of the two organisms in forming PHB was paralleled by their behaviour in bringing about the anaerobic dissimilation of glucose. Manometric experiments conducted in 5% CO_2 in nitrogen revealed that B. cereus could bring about the fermentation of glucose, producing 4.4 μ .mole acid/mg.N/hr. B. megaterium was inactive in this respect. Corroborative evidence that this latter organism was an obligate aerobe was derived from the appearance of stab cultures and from the absence of

FIG. 12.

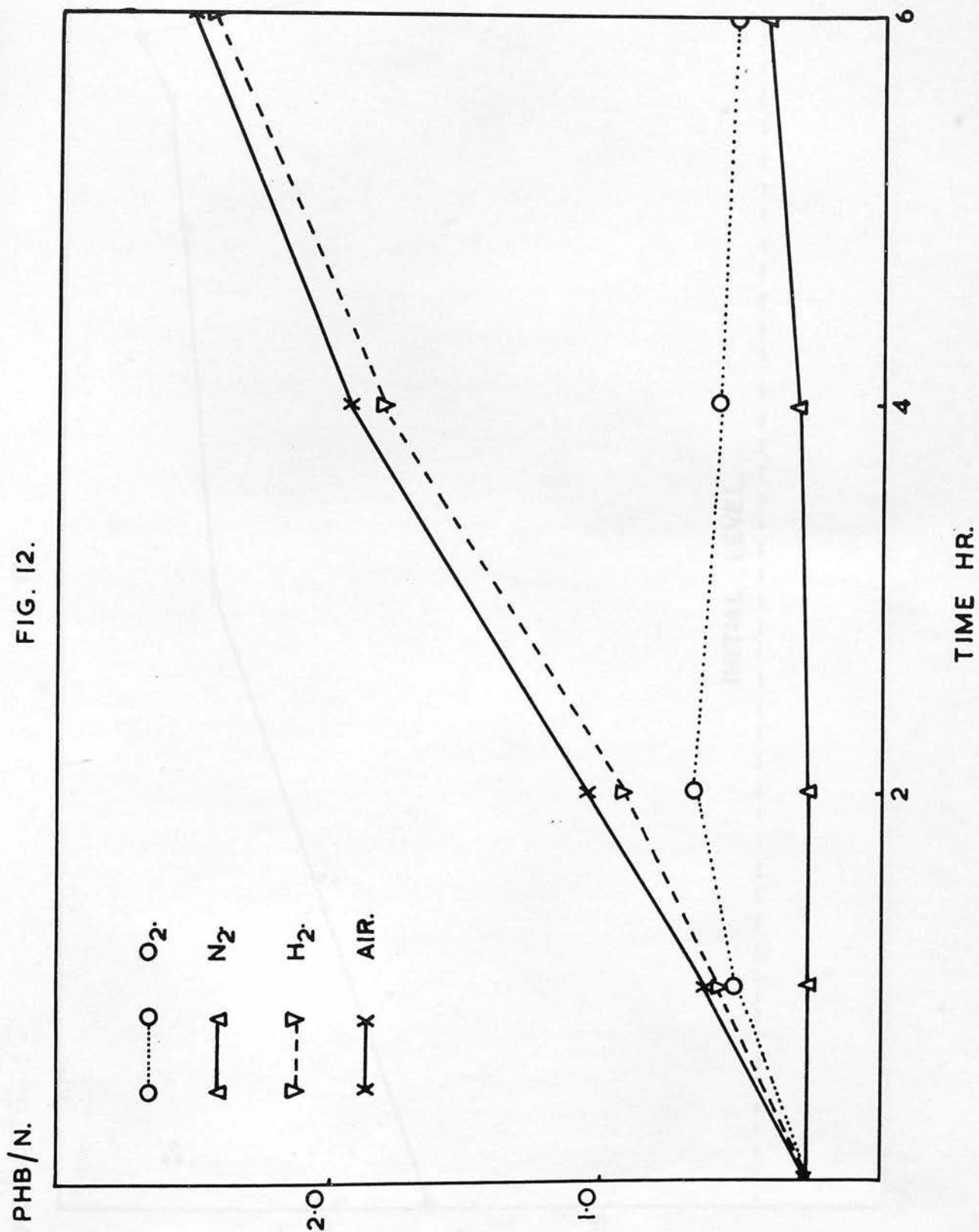
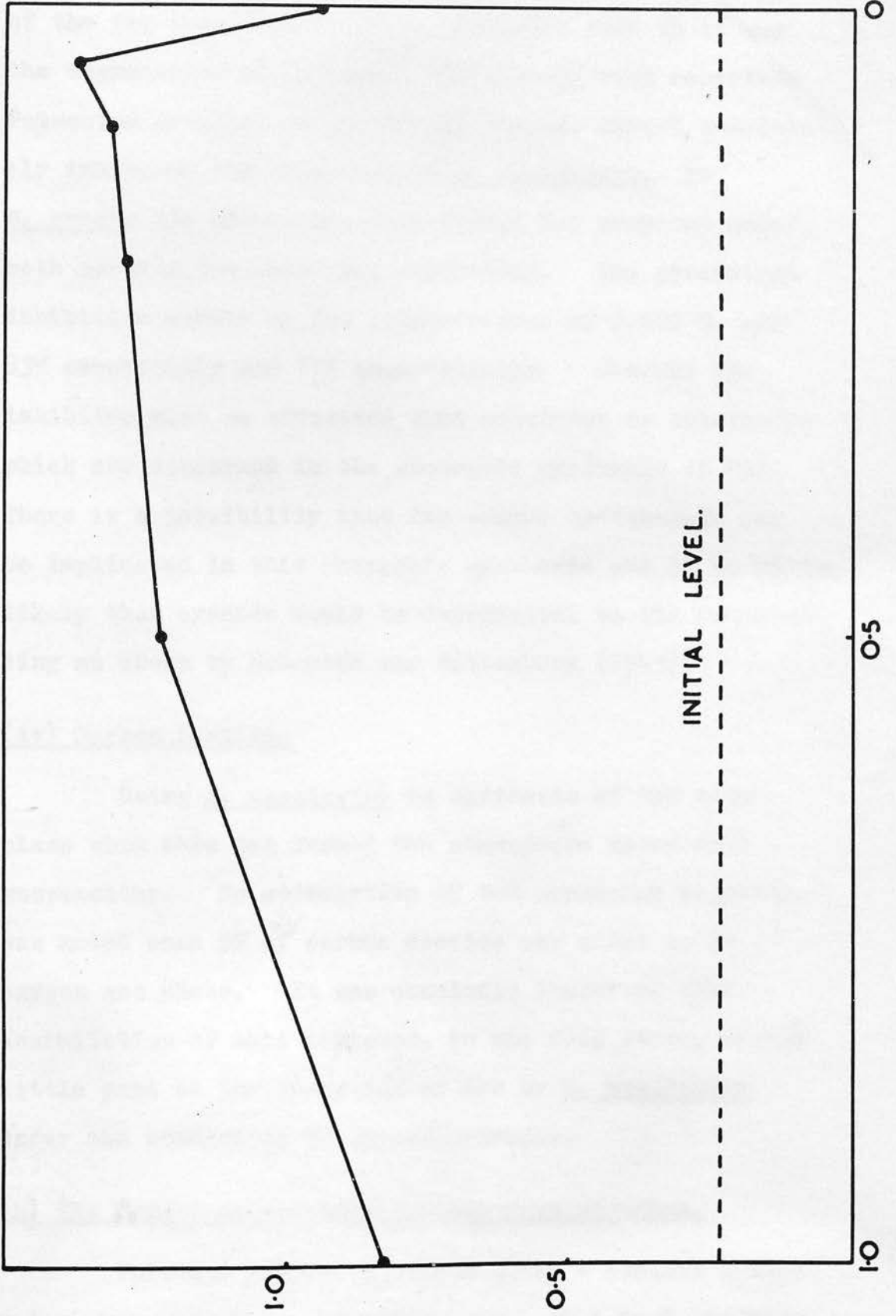


FIG. 13.

PHB/N.



P O₂

acetylmethylcarbinol production. It appears that one of the few anaerobic metabolic pathways open to it was the degradation of PHB which has already been reported. Potassium cyanide, as previously stated, almost completely inhibited PHB formation in B. megaterium. In B. cereus the effect was less marked but occurred under both aerobic and anaerobic conditions. The percentage inhibition caused by KCN concentration of 0.005 M. was 83% aerobically and 71% anaerobically. Clearly the inhibitor must be affecting some substance or substances which are concerned in the anaerobic synthesis of PHB. There is a possibility that the enzyme hydrogenase may be implicated in this anaerobic synthesis and if so it is likely that cyanide would be detrimental to its functioning as shown by Hoberman and Rittenberg (1943).

(iv) Carbon Dioxide.

Using B. megaterium no synthesis of PHB took place when this gas formed the atmosphere above cell suspensions. No stimulation of PHB producing capacity was noted when 5% of carbon dioxide was added to an oxygen gas phase. It was concluded therefore that assimilation of this compound, in the free state, played little part in the formation of PHB by B. megaterium under the conditions of the experiments.

(h) The Effect of the Hydrogen Ion Concentration.

This was studied in the glucose + acetate system using several buffers over a pH range of 5 to 9, results

being given in Fig. 14. The optimum for PHB synthesis was fairly sharp at about pH 7.5. In parallel experiments designed to follow the respiratory behaviour of the cells in relation to the ambient pH and buffer composition it was found that the oxygen uptake on glucose had similar trends of variation, with respect to the pH, as did PHB formation.

A further point arising from Fig. 14 is the inhibitory effect of tris and borate. Neither of these observations were due to a lack of phosphorus since synthesis of PHB was comparable in both phosphate and phthalate buffers at pH 6.0. Further, if phosphate was added to tris or borate buffers no increase in PHB formation was noted.

In view of this inhibition by tris an investigation was undertaken in which different concentrations of tris were added to phosphate buffer suspensions and observations on the synthesis of PHB in the glucose + acetate system, and the consumption of oxygen on glucose, were carried out. The results, which are given in Table 15, show that inhibition of PHB synthesis was quite marked and that it was roughly paralleled by the decrease in the oxygen uptake of cells metabolising glucose.

PHB/N.

FIG. 14.

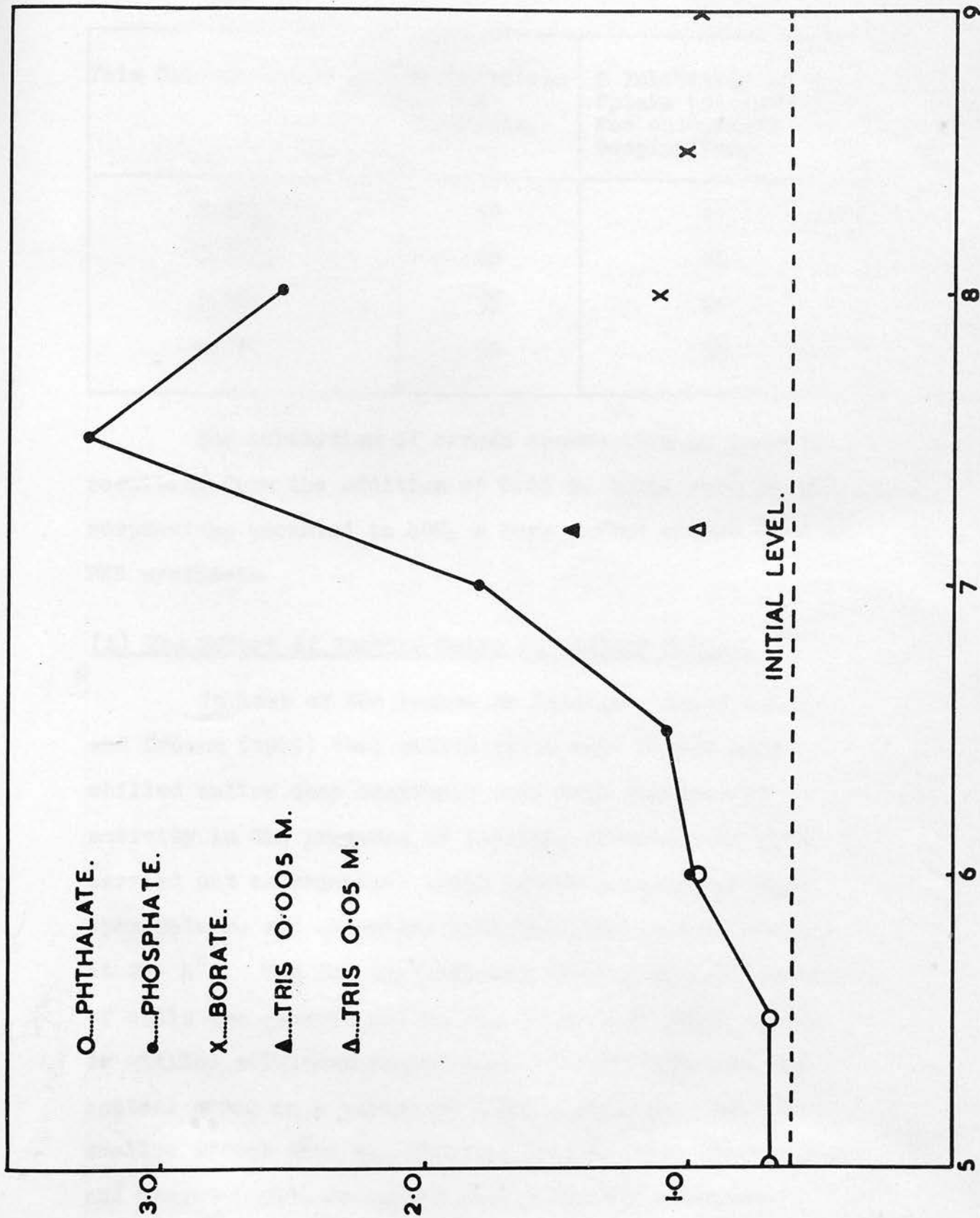


Table 15.

Tris Concentration (M).	% Inhibition of PHB Synthesis.	% Inhibition of O ₂ Uptake (corrected for endogenous respiration).
0.005	10	11
0.0125	20	14
0.025	35	29
0.05	50	56

The inhibition of oxygen consumption on glucose resulting from the addition of 0.05 M. boric acid to cell suspensions amounted to 40%, a less marked effect than on PHB synthesis.

(1) The Effect of Washing Cells in Chilled Saline.

In view of the report by Lemoigne, Péaud-Lenoël and Croson (1949) that unless cells were washed with chilled saline they displayed only weak respiratory activity in the presence of glucose, observations were carried out on organisms which had been washed at room temperature, and organisms from the same culture washed at 2 - 4°. The PHB synthesising ability of both groups of cells was studied and it was found that those washed in chilled solutions formed some 23% more PHB than the control group in a period of 4 hr. This is a much smaller effect than was observed by Lemoigne, Péaud-Lenoël and Croson (1949) on oxygen uptake and the previous studies reported above seem to bear out the contention

that little inhibition of metabolic capacity is caused with the present organisms by washing in solutions at room temperature.

(j) Conversion of β -hydroxybutyrate to Acetoacetate during Synthesis of PHB.

In experiments in which assays were made of the levels of PHB, β -hydroxybutyrate and acetoacetate, it was found that the organisms accumulated quantities of acetoacetate whilst bringing about the formation of PHB from β -hydroxybutyrate. Table 16 gives the numerical results (in μ . mole) of such an experiment in which the cells were shaken in air at 30° in the presence of a final concentration of 0.1 M. sodium β -hydroxybutyrate.

Table 16.

Sample	PHB (μ . Mole)	β -hydroxybutyrate (μ . Mole.)	Acetoacetate (μ . Mole.)
Initial	284	910	0
3 hr.	307	360	178
Difference	+23	-550	+178

Thus the increase in PHB and acetoacetate account for only 37% of the decrease in the β -hydroxybutyrate level. It seemed likely that this excess of β -hydroxybutyrate was further metabolised as was suggested in the case of the aerobic degradation of PHB. The conversion of β -hydroxybutyrate to acetoacetate was

repeated and is in accord with the observations of Lemoigne, Péaud-Lenoël and Croson (1949). These workers concluded, however, that with non-proliferating cells the degradation of β -hydroxybutyrate ceased at the acetoacetate stage and they did not allow for the possibility of PHB synthesis taking place. The results given above, and those from experiments of a similar nature, do not support these conclusions.

6. Studies Using Cell-free Bacterial Extracts.

(a) Preparation of Extract.

In view of the limited usefulness of experiments using washed suspensions of whole cells where problems of impermeability complicate the interpretation of experimental results, it was felt advantageous to carry out studies with cell-free systems.

The KM strain of B. megaterium was chosen in this study in view of its sensitivity to lysozyme which held out the possibility of producing cell-free bacterial extracts very easily. After some preliminary research to discover the most suitable conditions for the dissolution of the cell wall with lysozyme the following procedure was adopted.

The cells were harvested, washed in the normal manner and resuspended in 0.002 M. $MgCl_2$ + 0.05 M. KH_2PO_4 /NaOH buffer pH 7.2 to give a final density of 10 - 15 mg./ml. dry cell matter. To this suspension 10%(w/v) polyethylene glycol (M.Wt. 4000) was added and, when completely dissolved, 0.4 mg./ml. crystalline lysozyme (Armour) was tipped in. The mixture was vigorously shaken for 15 sec. and incubated at 30° for 30 min. in which time the formation of protoplasts was complete as determined by phase contrast microscopic observation. The protoplasts were sedimented by centrifuging at 5000 g. for 15 min. and the supernatant discarded. A small quantity of the phosphate buffer suspending fluid was added together with

0.1 $\mu\text{g.}/\text{ml.}$ crystalline desoxyribonuclease (DNA-ase, Light), the protoplast pellets being lysed by this treatment. The addition of DNA-ase was found to be helpful in reducing the viscosity of the lysate which otherwise rendered manipulation extremely difficult. When the lysate had liquefied all further operations were performed at $0 - 4^{\circ}$. Normally this preparation was used without further treatment apart from dilution with buffer to about 3 mg.N/ml. Where other manipulations were carried out the details are given in the requisite section.

(b) Synthesis and Degradation of PHB.

As already reported in these Results p. 62 there was a discrepancy between the turbidities obtained after hypochlorite treatment of whole and lysed cells with the same PHB content. It was thought, however, that the hypochlorite method would nevertheless indicate changes in the PHB level. Experiments were conducted with aliquots (1.1 mg.N) of a cell-free extract to which were added 1 μ mole CoA (Light), 5 μ mole adenosine triphosphate (ATP, dipotassium salt, Pabst) and 500 μ mole β -hydroxybutyrate, where indicated. The total volume was 5 ml. and incubation was in air at 30° . Table 17 shows the turbidities found after treating initial, $1\frac{1}{2}$ hr. and 4 hr. samples with hypochlorite under standard conditions.

Table 17.

Additions to Extract.	"Spekker" Scale Reading.		
	Initial	1½ hr.	4 hr.
β-OHb., CoA, ATP	0.536	0.442	0.456
β-OHb.	"	0.440	0.435
CoA	"	0.413	0.422
None	"	0.418	0.407

The sharp fall in turbidity between the initial and 1½ hr. samples, the reason for which is not understood, complicates the interpretation of the results. Other experiments of a similar nature gave comparable results indicating that it is uncertain if PHB is formed by the extract from β-hydroxybutyrate or degraded in the absence of an external carbon and energy supply.

Since the hypochlorite method did not appear wholly satisfactory in estimating PHB in cell lysates, repeated attempts were made to develop a speedy and reliable chemical procedure for its measurement. Many different concentrations of acid and alkali and various times of digestion at 100° were tried to bring about hydrolysis of PHB with a consistent yield of β-hydroxybutyrate, which could then be estimated by the alkaline salicylaldehyde reaction. These efforts met with no success, however, and other chemical methods are too lengthy and insensitive to be of any value.

It was decided that as PHB could not easily be

estimated it might be possible to demonstrate the utilisation of β -hydroxybutyrate by the cell lysate. Accordingly, an experiment was carried out using two aliquots (1.85 mg.N) of the supernatant of a cell-free extract previously centrifuged 5 min. at 25,000 g. To one aliquot a final concentration of 0.1 M. β -hydroxybutyrate was added, the total volume was 10 ml. and incubation was at 30° for 3 hr. in air. Initial and final samples were removed for the assay of β -hydroxybutyrate and acetoacetate. In the test to which the β -hydroxybutyrate had been added no change was noted in the level of this substance after incubation and no acetoacetate accumulated. In the absence of an added substrate neither β -hydroxybutyrate nor acetoacetate accumulated. It will be recalled that whole cells normally utilise β -hydroxybutyrate. Thus in an experiment using whole cells (4.88 mg.N) under similar conditions to the above 63% of the available β -hydroxybutyrate was metabolised after 3 hr. incubation.

(c) Manometric Observations.

Estimations were carried out on the production of acid by the extract (0.68 mg.N) incubated 70 min. at 30° in bicarbonate buffer in the presence of 5% carbon dioxide in nitrogen. No significant quantities of acid were formed in these conditions thus corroborating the results reported above.

Studies were conducted using several substances as substrates at the final concentrations shown and observing the gas exchange upon incubation in the presence of the

extract (pH 7.2) in air at 30°. Glucose (0.5 M.) was found to bring about an uptake of oxygen ($Q_{O_2}(N) = 31$) without any carbon dioxide being evolved. When a similar lysate was centrifuged at 5,000 g. for 10 min. the supernatant also absorbed oxygen when glucose was supplied. ($Q_{O_2}(N) = 59$). It will be noted that the specific activity of the supernatant of the lysate is greater than the total lysate which confirms the observations of Storck and Wachsmann (1957) who found most of the glucose oxidising system to be "soluble".

When pyruvate (0.05 M.) was tested with the total lysate both oxygen uptake ($Q_{O_2}(N) = 12$) and CO_2 output ($Q_{CO_2}(N) = 13$) were noted. Under anaerobic conditions the extract was still active in forming CO_2 from pyruvate ($Q_{CO_2}(N)$ anaerobic = 8). This aerobic and anaerobic decarboxylation was enhanced by the addition of 0.05 mg./ml. diphosphothiamine (DPT, Light) thus:

$Q_{O_2}(N)$ aerobic + DPT = 16; $Q_{CO_2}(N)$ aerobic + DPT = 21;
anaerobic + DPT = 16. The presence of 0.1 mg./ml.

co-enzyme A (CoA) did not increase anaerobic CO_2 formation. If DPT (0.05 mg./ml.), CoA (0.1 mg./ml.), $MgCl_2$ (0.01 M.), lipoic acid (0.02 mg./ml.) and DPN (0.5 mg./ml.) were added to the extract + pyruvate system in air the $Q_{O_2}(N)$ was raised to 51 and $Q_{CO_2}(N)$ to 55.

Confirmation that the gas evolved in these experiments was CO_2 was obtained by complete absorption with NaOH.

Since it seemed possible that acetaldehyde might accumulate during this anaerobic decarboxylation,

parallel observations in phosphate buffer pH 7.2 were made on the amount of CO₂ formed from pyruvate and the quantity of acetaldehyde produced. The results given in Table 18 show that there was reasonably good agreement between the amount of acetaldehyde expected from the CO₂ output, if a simple decarboxylation was being effected, and the quantity of acetaldehyde found by duplicate chemical assays.

Table 18.

System. (Total vol. 3.0 ml.)	Acetaldehyde calculated from CO ₂ output (µg.).	Acetaldehyde found (µg.).
Extract (3.16mg. N) + pyruvate (0.05M.) +DPT (0.17mg.).	600	696
Extract + DPT	20	13

Pyruvate was found not to interfere in the assay method used for acetaldehyde. Since the acetaldehyde was steam distilled it is quite possible that it existed not as the free substance but as some heat-labile conjugate.

Studies conducted using acetaldehyde (0.1 M.) as a substrate indicated that this material was not oxidised by the extract.

Neither β-hydroxybutyrate, acetoacetate, acetate nor formate caused oxygen uptake by the extract. In the case of β-hydroxybutyrate and acetate this was so even after the addition of CoA and ATP to the system. The negative results obtained with acetate are similar to the findings of several workers, e.g. Kogut and Podoski (1953)

Beck and Lindstrom (1955) and Moyle (1957) who noted that rupturing the cell inactivated the acetate metabolising mechanism.

(d) Studies on Dehydrogenases.

The hydrogen acceptors employed were diphosphopyridine nucleotide (DPN, Light), triphosphopyridine nucleotide (TPN, Light), 2 : 6- dichlorophenol indophenol (DCP, B.D.H.) and 2:3:5-triphenyltetrazoleum chloride (TAS, Pal Chemicals). Readings were taken in the spectrophotometer at 340 m μ with DPN and TPN, 600 m μ with DCP and 375 m μ with TAS. Table 19 shows the results obtained with different substrates.

Table 19.

Substrate	Hydrogen Acceptor.			
	DPN	TPN	DCP	TAS
Glucose	+	+	+	+
β -hydroxybutyrate	+	-	\pm	-
pyruvate	-	-	+	+
acetaldehyde	\pm	-	\pm	-

+ reduction of acceptor.

- no reduction of acceptor.

\pm weak or doubtful result.

Using glucose as substrate a short lag of about 4 min., which increased as the extract became older, was exhibited in the case of DPN and TAS. This lag could not be shortened by adding KCN, cysteine or gassing with

CO. The DPNH formed in the reaction could be re-oxidised by shaking in air but this could not be demonstrated with TPN. These observations suggested that possibly some factor, which insured the re-oxidation of TPNH was present in subliminal quantities only, or had been inactivated. Reduction of DCP and TAS was extremely rapid.

Extracts prepared by grinding the cells with alumina (McIlwain, 1948) showed similar behaviour to the above as did those obtained from cells ruptured by shaking with glass beads (Mickle, 1948).

The supernatant derived from any of these extracts by centrifuging for 10 min. at 25,000 g. was also active in reducing DPN and TPN in the presence of glucose. Other hydrogen acceptors were not tested.

It had been hoped that much useful information might be obtained from a study of the system oxidising β -hydroxybutyrate. Initial experiments on the reduction of DPN seemed negative, however, until it was noticed that reduction of this co-enzyme did occur after a lag period. This lag lengthened speedily after the initial preparation of the extract, the effect being particularly rapid at room temperature but also occurring during storage in the deep freeze at -25° . With β -hydroxybutyrate as the hydrogen donor some slight increase over the rate of the endogenous reduction of DCP was observed.

The effects of adding co-factors to the extract - β -hydroxybutyrate-DPN system were uniformly disappointing

as neither CoA nor ATP, singly or together, stimulated any increase in the rate of DPN reduction or shortening of the lag period. The addition of KCN was also without effect on this latter property.

Extracts prepared by the alumina grinding method were also active but those from "Mickle"-disintegrated cells were not so. These results suggest that the enzyme system responsible for the oxidation of β -hydroxybutyrate is probably very sensitive to molecular oxygen and possibly to mechanical agitation. Thus almost all the activity may be lost during manipulation and under conditions of experiments designed to show the utilisation of β -hydroxybutyrate or uptake of oxygen upon addition of this substrate. The anomaly of the demonstration of a β -hydroxybutyric dehydrogenase without also being able to show the utilisation of β -hydroxybutyrate or oxygen uptake upon its addition to the extract might be explained in this way.

The oxidation of DPNH by acetoacetate in the presence of the lysozyme extract could not be demonstrated by observations at 260 m μ .

When pyruvate was used as the substrate in these studies DCP and TAS were reduced quite readily but little increase in rate was displayed on the addition of DPT.

Results with acetaldehyde were erratic but suggested some slight reduction of DPN and DCP.

Using DPN as the hydrogen acceptor no activity was shown with formate, malate, glutamate, lactate or ethanol. Neither did the acetate + ATP system cause the reduction of DCP.

7. Experiments Using Isotopically Labelled Substrates.

(a) Aims and Design of Experiments.

In order to obtain more direct evidence that PHB was being synthesised from the various active substrates employed and to try to discover something of its mode of formation experiments were conducted using isotopically labelled compounds. In particular it was hoped to find out how acetate brought about the formation of PHB in the presence of glucose and to gather more information about the acetate + pyruvate substrate system. Two radioactive compounds were used, sodium 1 - C¹⁴ acetate (CH₃.C¹⁴OOH) and sodium 2 - C¹⁴ pyruvate (CH₃.C¹⁴O.COOH). The substrate systems employed were, glucose + C¹⁴ acetate, pyruvate + C¹⁴ acetate and acetate + C¹⁴ pyruvate, and the organisms were allowed to form PHB in air as previously described.

(b) Chemical Degradation of PHB.

After the experimental period, which was of 4 hr. at 30° the PHB was recovered and degraded in the following manner:

The organisms were treated with "Standard" hypochlorite reagent for 90 min. and the granules sedimented by centrifuging at 5,000 g. for 15 min., the supernatant being discarded. Washing of the granules was effected by centrifugation and resuspension in three changes of distilled water. They were then dried at 95° and, after weighing, were exhaustively extracted with

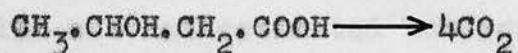
boiling ether. The residue was again weighed, diluted with non-isotopic PHB and taken up in hot chloroform in which it was completely soluble. This solution which contained 350 - 400 mg. of PHB was evaporated to a small volume and the PHB precipitated with ether. The flocculent precipitate of PHB was washed with ether, dried, placed in a small round bottom flask and 15 ml. 25%(w/v) Na_2CO_3 added. The mixture was then refluxed at 100° until complete dissolution of the PHB had occurred, which normally took about 2 hr. At this stage the hydrolysate which contained crotonate as well as β -hydroxybutyrate, was acidified to pH 2 with concentrated HCl using thymol blue as indicator. After filtration, evaporation under reduced pressure at 45° removed the crotonic acid which is more volatile than β -hydroxybutyric acid. Further purification of the product was brought about by dissolving in ether in which β -hydroxybutyric acid is very soluble. The ether solution was decanted from the sodium chloride formed during acidification and the ether removed by evaporation, leaving a syrupy residue of β -hydroxybutyric acid which was neutralised with NaOH. The yield of β -hydroxybutyrate from PHB was about 50%, though this may vary as discussed previously in connection with a possible chemical method for the assay of PHB.

The neutralised β -hydroxybutyrate was made up to a standard volume (10 ml.) and submitted to a chemical degradation by the following methods:-

(In all reactions carried out in combustion/diffusion vessels the type described by Katz, Abraham

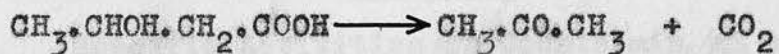
and Baker (1954) was used).

Firstly, persulphate oxidations (Katz et al. 1954) were performed on a portion of the material which brought about complete wet combustion of all four carbon atoms of the β -hydroxybutyrate to CO_2 .

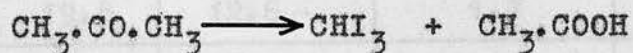


This was then trapped in NaOH and precipitated as BaCO_3 in the standard manner.

Next a chromic acid oxidation was performed with another portion of the β -hydroxybutyrate using the same concentrations of reagents as in the Thin and Robertson (1952) method for the assay of this material. The CO_2 was trapped in the usual way and represents the carbon from the carboxyl group of the β -hydroxybutyrate, viz.:-



The next stage was the degradation of acetone, which was steam distilled into chilled alkali. The addition of iodine solution brought about the formation of iodoform and acetate, viz.:-



The iodoform, which contains carbon atoms 2 and 4 was removed by filtration and dried. It was then taken up in ether and the ethereal solution transferred to the outer compartment of a combustion/diffusion vessel. The solvent was removed by evaporation and the iodoform oxidised to CO_2 by the Van Slyke and Folch (1940) method

viz. :-



This gave BaCO_3 derived from a mixture of carbon atoms 2 and 4.

The acetate remaining in the filtrate from the iodoform reaction was not estimated but could have been degraded according to the procedure of Katz, Abraham and Chaikoff (1955).

(c) Results from PHB Synthesis Experiments.

The specific activities of the BaCO_3 samples, derived from the carbon atoms indicated, are given in Table 20, which shows the respective substrate systems employed.

Table 20.

Specific Activities in Counts/min./mg. BaCO_3

C Atom	Substrate System.			
	Glucose + C^{14} acetate		pyruvate + C^{14} acetate	acetate + C^{14} pyruvate
	Exp. 1	Exp. 2		
1, 2, 3, 4	19.4	19.4	2.6	3.0
	19.4	13.4	1.7	2.6
	19.5	12.6	1.7	3.3
1.	26.2	21.7	4.9	6.2
	27.3	21.6	4.4	7.5
		21.3	5.5	6.3
2, 4	-	nil	nil	nil

It is significant that the material is radioactive which confirms that acetate and pyruvate are assimilated into PHB. It should be noted that in all samples carbon atoms 2 and 4 do not show any radioactivity and cannot therefore have been derived from the carboxyl group carbon of acetate nor the keto group carbon of pyruvate. Thus only carbon atoms 1 and/or 3 have become labelled and comparison of the specific activity of BaCO_3 from carbon atoms 1, 2, 3 and 4 and BaCO_3 from carbon atom 1 shows that probably both these have been affected. This follows from the fact that the BaCO_3 from carbon 1 is approximately twice as radioactive as that from carbon atoms 1, 2, 3 and 4. Four times the degree of activity should have been shown if only carbon 1 had been labelled and no activity if only carbon 3 had been so affected. Thus the labelling pattern of PHB is similar in the three substrate systems studied.

The information gained in the present study on the metabolism of PHB may be considered in two main aspects. The first is the general significance of PHB as a metabolic product, its functional importance from the point of view of the ecological survival of the organism. The other, more particular aspect, is the biological mode of synthesis and of degradation of this material, which in view of its unique occurrence is especially interesting.

The functional significance of the sudanophilic granules, which have been shown to be composed mainly of PHB, has been the source of repeated controversy as described in the Introduction. Although differing antithetically from the views held by Knaysi (1945, 1951c) or Sparrow (1933), results obtained in this work indicate that PHB functions as a reserve carbon and energy source. Broadly, this conclusion receives support from three considerations, (a) PHB is synthesised rapidly in conditions of carbon and energy excess, (b) it is dissimilated speedily when the supply of carbon and energy is depleted, (c) on dissimilation it provides energy and/or intermediates which are useful to the cell. These three criteria may now be discussed.

(a) In order to substantiate the claim that any material functions as a carbon and energy storage substance, it is necessary to show that it can be accumulated in relatively large quantities when suitable nutrients are available in excess of immediate growth requirements. For example in a strain of Bacillus mycoides a PHB level of 60% of the dry weight has been

reported (Lemoigne, 1947). Williamson (1956) noted that when B. cereus, strain AC was grown on solid media, which were deficient in the potassium or phosphorus sources, large quantities of PHB were formed. If growth was limited by a scarcity of the sulphur, nitrogen or carbon and energy sources, the amount of PHB produced was very small. The results of these experiments are open to question, however, since control of environmental conditions using solid growth media is very difficult. The use of agar as the solidifying agent introduces the problem of trace minerals and perhaps other impurities of an organic nature. Further, since the surface of the agar was inoculated, the cells would be incompletely immersed in the medium with consequent dissimilarities in the amount and concentration of nutrients available to them. In the later stages of growth, which were those most intensively studied, many of the cells must have been heaped upon their fellows and therefore out of contact with the medium altogether. On the other hand the cells underneath would be denied the same degree of aeration as those at the surface. Williamson was aware of these difficulties but was unsuccessful in his attempts to grow the organism in liquid media. This was possibly due to a manganese deficiency or inadequate aeration as in the present work B. cereus, strain AC, was found to grow quite satisfactorily in the liquid medium developed for the growth of B. megaterium. In the present growth experiments with the latter it was demonstrated quantitatively that excess of glucose

relative to ammonium chloride in the growth medium led to the formation of large amounts of PHB. Sall, Mudd and Payne (1957) have recently noted that sudanophilic material was more abundant in cells grown on nitrogen-deficient media but no assays of PHB content were carried out. The reasons for the discrepancy between the above observations and those of Williamson (1956), who found that PHB formation did not continue extensively in nitrogen deficiency, are not understood. The quantity of PHB formed depends to some extent on the nature of the carbon and energy source. Lemoigne, Grelet and Croson (1950) found variations in PHB content between 21 and 44% of the cell dry weight when B. megaterium was grown on the ammonium salts of different organic acids as the carbon and energy source. Their observations were only roughly quantitative, however, no attempt being made to control the effects of pH value, rate of growth or age of culture. When cultures were supplied with acetate as well as glucose in the present study, very high levels of PHB could be attained, often amounting to 50% of the mass bacterial dry matter. Although useful in demonstrating the organisms' ability to store PHB, these experiments suffer from the disadvantages inherent in growth studies, where other vectors previously mentioned such as pH value, physiological age of culture, rate of growth and availability of nutrients may complicate interpretation of the results. These various factors are notoriously difficult to control and the washed suspension

experiments were designed to overcome these disadvantages. Hitherto it had not been possible to demonstrate PHB synthesis with non-proliferating cells (Lemoigne, Grelet and Croson, 1950) but the present observations show that the strains of B. cereus and B. megaterium employed were quite capable of forming PHB in these conditions. This synthesis may be extremely rapid and under optimal conditions the organisms were found to raise their content of PHB from 5 to 20% of the mass of dry matter, in an experimental period of 4 hr.

(b) The next criterion, which should be satisfied by a storage material, is that it can be readily dissimilated by the cell when the external food supply is depleted. Lemoigne and Roukhelman (1940) and Lemoigne, Grelet, Croson and Le Tries (1945) previously observed the diminution of PHB in old cultures, but their experiments were conducted in complex media of unknown constitution in which the levels of carbon and energy and nitrogen sources were not controlled. Further, these experiments were often prolonged, sometimes lasting two weeks, with the consequent presentation of a virtually uninterpretable picture. This is particularly so when the complications introduced by autolysis are considered whereby cannibalisation of the constituents of one cell by another may take place. Further difficulties were presented by the introduction of inaccuracies into the PHB assay procedure in which autolytic losses of PHB to the supernatant may not have been satisfactorily measured. The present growth experiments have shown that

when the environmental glucose is exhausted, the internal PHB is gradually lost. This process is not due to autolysis for the cell nitrogen content is maintained at a stable level. In contrast to the synthesis of PHB, its degradation has been demonstrated previously in washed suspensions by Tinelli (1955b,c). This observer noted a loss of PHB as spore formation proceeded in a strain of B. megaterium and she was able to show the uptake of sufficient oxygen to account for the complete oxidation of the amount of PHB depleted. On the other hand asporogenous variants of this organism did not fully oxidise their reserves of PHB but underwent an autolysis with the liberation of undegraded granule material into the medium. In the present work, using asporogenous strains of B. cereus and B. megaterium, it has been shown that in washed suspensions in the absence of an external carbon and energy source the organisms dissimilate their stores of PHB very rapidly, for example, the level may fall by 60% of the initial value in an experimental period of 8 hr. That oxidation of this material has taken place aerobically is substantiated by the demonstration of the uptake of sufficient oxygen to bring this about, coupled with the observation that only small amounts of acetoacetic and β -hydroxybutyric acids accumulate in the surrounding medium. Anaerobically, it will be recalled, these acids appear in quantitative yields from the amount of PHB degraded. These findings of complete aerobic degradation of PHB in asporogenous strains are at variance with the

observations of Tinelli cited above.

(c) The foregoing criteria of rapid accumulation of PHB in the presence of an excess food supply and depletion of this substance when the external carbon and energy supply is inadequate, do not by themselves clinch the argument that PHB acts as a carbon and energy reserve. It might simply be a "metabolic shunt" which became operative when the correct environmental conditions were satisfied. As an example one might consider the extensive synthesis of extracellular polysaccharide by Aerobacter aerogenes (Duguid and Wilkinson, 1953) when growth is halted by the depletion of certain nutrients. Although this polysaccharide might amount to several times the dry mass of the organisms it is apparently not utilised as a carbon or energy reserve nor is it broken down by the cells. The crucial point which must be demonstrated is that any reserve material must make available energy and/or intermediates which are valuable to the cell metabolism. This is perhaps the most difficult of the three criteria to demonstrate satisfactorily. It was decided that the problem could be studied along three main lines of enquiry which were, (i) the rate of endogenous respiration, (ii) the rate of autolysis and (iii) the assimilation of an environmental nitrogenous material in the absence of an extraneous carbon and energy source. A convincing demonstration of the correlation between the PHB content of cells and their rate of endogenous oxygen consumption has been carried out. Cells with a high content of PHB have the

highest rate of endogenous activity and therefore of metabolic potentiality and viability. It is important to note that this high rate is apparently largely independent of the polysaccharide level as this has been shown to be only slightly lowered in an experiment where the PHB fell markedly. This means that the energy requirements of the organisms are probably being met largely by PHB dissimilation and is strong evidence for regarding the possession of high levels of PHB as useful to the cell, especially in adverse environmental conditions. Three stages of endogenous respiration were observed in washed suspensions of B. cereus by Ingram (1939). These were, (a) a 2 hr. period of greatly enhanced respiration, attributed to asphyxia caused by the washing procedure, (b) a linear phase which occupied 5 hr. and (c) a decline phase when respiration fell exponentially with time. From the R.Q. (0,7) it was concluded that "fat" (sic) was being oxidised but no characterisation of this fat was made and the extraction procedures were unsuitable for the measurement of PHB, the existence of which the author was apparently unaware. The present results which showed a steady fall in the rate of endogenous respiration cannot be reconciled with these findings.

Hinging upon this greater endogenous respiratory activity it would be expected that other signs of enhanced viability would be apparent. A suitable index suggested itself in the rate of autolysis as measured by the decrease in the non-dialysable nitrogen content of cell

suspensions. Again, it was possible to show that a high level of PHB bestowed an advantage on the organisms in that autolytic processes were delayed when compared with PHB-poor cells. Thus organisms rich in PHB would tend to survive for a longer period than those which had a low content of this material. Insofar as this increased time of survival would enhance the opportunities of an organism of withstanding intermittent starvation, it is evidence for regarding PHB as a reserve of evolutionary significance. (This contention, however, is modified below by the remarks on sporulation). A useful adjunct to these observations would be the demonstration that cells could divide and grow in the absence of an external carbon and energy source if they had sufficient reserves of PHB. Accurate viable counts of the organisms are precluded by chain formation, whereby the daughter cells do not separate after binary fission and thus give rise to only one colony by the usual counting techniques. As an indication of growth, therefore, it was decided to measure the cell nitrogen level of suspensions which were supplied with an external nitrogen source in the form of ammonium chloride. As reported, however, no increase in the nitrogen content could be observed even with cells very rich in PHB and in the presence of high concentrations of ammonium chloride. In these circumstances it must be concluded that, either other factors are necessary for the assimilation of cell nitrogen from environmental sources, or PHB is incapable

of acting in such a manner as to enable the cell to bring about such assimilation. The rationale of this finding may possibly be found in the fact that in choosing asporogenous variants of organisms which normally produce spores, and survive ecologically largely due to this property, one is dealing with somewhat artificial biological entities. The power of survival of these asporogenous strains in comparison to the naturally occurring spore-forming parent would in all probability be very poor in their normal environments. The work of Tinelli on the connection between PHB and sporulation has already been described and, although the present research does not support her observations on asporogenous variants, it does seem feasible that PHB is concerned largely, in natural conditions, with furnishing a repository of carbon and energy for spore formation. This is reinforced by the knowledge that the depletion of the external carbon and energy source may provide the trigger for sporulation, (Grelet, 1951, 1952).

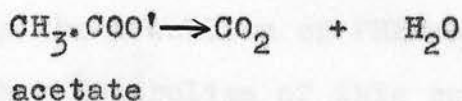
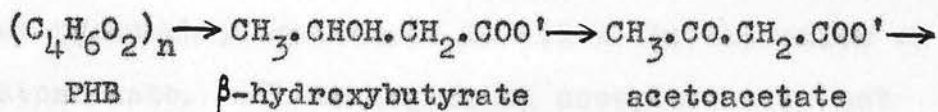
These latter suggestions are necessarily tentative but much more information could be obtained by a study of spore formation in Bacillus species with particular reference to PHB metabolism.

As the formation of PHB causes the elimination of the carboxyl groups of its constituent monomer the neutralisation of this acidic material is brought about. It might be considered, therefore, that the primary

significance of PHB production was that of a "neutralisation mechanism". In the growth experiments the pH value never fell below 7 but the level of PHB obtained was often very considerable. If PHB production had subserved a neutralisation function, as for example acetylmethylcarbinol formation, it would be expected that the optimum for the production of PHB synthesising enzymes would be on the acid side of neutrality, possibly in the region of pH 6. In view of the fact that the internal and external pH optima for formation of PHB may be markedly different, the results of washed suspension experiments, which showed maximal PHB synthesis at pH 7.5, have perhaps less value than the growth studies. It does seem unlikely, however, that the main significance of PHB formation is that of a neutralisation mechanism.

The evidence presented indicates that PHB must be considered together with polysaccharides, triglycerides and possibly volutin as a major storage compound in bacterial metabolism. It is well suited for this purpose since it is insoluble in water, inert, high in carbon content and only very weakly ionic.

Turning to the possible biochemical degradative pathway of PHB, a simple scheme (omitting co-enzyme) based on work with animal tissues could be:-



The evidence uncovered in support of this sequence of intermediates derives from experiments on the products liberated by the organisms during aerobic and anaerobic breakdown of PHB. Quantitative yields of β -hydroxybutyric and acetoacetic acids could be obtained from the amount of PHB degraded under anaerobic conditions. The identity of these compounds was confirmed by paper partition chromatography and since they were the only products found it seems unlikely that other substances interfered with the assay procedure. In a study of the fermentation products of B. cereus and B. anthracis grown in glucose containing media, Puziss and Rittenberg (1957) make no mention of β -hydroxybutyrate, acetoacetate or PHB. From the data obtained in the present study it seems unlikely that neither β -hydroxybutyrate nor acetoacetate would have been formed and it is possible that since they identified many of the products by chromatographic analysis, their results may have been misinterpreted. This is particularly so since no standard samples of these two compounds were run with their test chromatograms.

Quite how the formation of acetoacetate can take place under anaerobic conditions is uncertain. No gaseous hydrogen is formed so other hydrogen acceptors

must presumably be reduced to allow the formation of acetoacetate. These suggested acceptors were not identified. Aerobically the amount of β -hydroxybutyric acid found after the breakdown of PHB was very small suggesting further metabolism of this compound.

Acetoacetic acid was identified but no unequivocal proof of the presence of acetate was obtained. This may indicate simply that acetate is not released into the surrounding medium but is further metabolised intracellularly, directly it is formed.

The reason for the greater degrees of breakdown under aerobic conditions as compared with anaerobic conditions can be explained in the light of these results. Anaerobically the accumulation of β -hydroxybutyric acid would, by its mass action effect, tend to depress the dissimilation of PHB. Aerobically, on the other hand, the β -hydroxybutyric acid is removed from the system by further oxidative processes and thus exerts little braking effect on PHB degradation.

Looking more closely at the biochemical mechanisms which are involved in the process, one may speculate on what co-enzymes are implicated. In the initial hydrolysis reaction it is not clear if any co-enzymes are concerned at all. Without any data on the energy changes involved in the formation of PHB from β -hydroxybutyrate it is difficult to decide, from theoretical considerations, whether any co-enzyme complexes are likely to be formed. It seems improbable

that any large changes in free energy occur in this reaction since the β -hydroxybutyric acid units are bound by ester bonds. Further PHB is insoluble in water and, presumably, also in the internal milieu of the cell. Because of its removal from the system, therefore, this would result in the formation of PHB even if the equilibrium of the reaction;

β -hydroxybutyrate \rightleftharpoons PHB, were greatly in favour of β -hydroxybutyrate. From the fact that no breakdown of PHB, or formation from it of β -hydroxybutyrate could be demonstrated in cell-free extracts, it appears that the structural integrity of the cell is possibly essential for the working of the enzyme or enzymes concerned. When it is borne in mind that PHB occurs as discrete granules in the cytoplasm of the organisms it is perhaps not surprising that disruption of the cell structure inactivates the PHB forming system. It would be expected that the synthesising system would be located in very close proximity to the granules themselves, possibly as an enzymic coating. If this were not so it is difficult to imagine how PHB would progressively accrete to form such large granules as, for example, those of 1 μ in diameter which are found. The disorientation of any enzymic membrane, which is likely to occur when the cells are disrupted, perhaps renders the prospect of deriving cell-free preparations which actively hydrolyse PHB as rather unpromising. If these prognostications are correct it means that, since cell impermeability precludes the use of whole cells in

co-enzyme moiety with the terminal carboxyl group of the PHB molecule. Whatever the acylating mechanism the product of the subsequent hydrolysis of PHB would be β -hydroxybutyryl-CoA. It will be recalled that the presence of CoA, with or without ATP, did not accelerate the reduction of DPN by the enzyme system in the presence of β -hydroxybutyrate. This does not rule out the possibility that CoA is concerned, it may only reflect the fact that the enzymes necessary for the formation of β -hydroxybutyryl-CoA from β -hydroxybutyrate were inactivated in the preparation of the cell-free system. It would have been most interesting to study a system which consisted of DPN and reagent quantities of β -hydroxybutyryl-CoA in the presence of the extract. Insofar as probably some quantity of long chain fatty acid is synthesised by the organisms, one is reluctant to believe that this synthesis does not occur by the well-authenticated sequence outlined by Green (1954) for animal tissues and reviewed by Barker (1956) for micro-organisms. This system implicates β -hydroxybutyryl-CoA as an intermediate and requires the presence of dehydrating and dehydrogenating enzymes active against this compound. In passing it may be noted that the dehydration product, crotonyl-CoA, may by de-acylation yield the free acid which has the same empirical formula ($C_4H_6O_2$) as PHB. The possibility of some enzymic isomerising and polymerising reaction is perhaps excluded by the reported occurrence (Képès and Péaud-Lenoël, 1952) of a terminal secondary alcohol group on

the PHB molecule. Nevertheless, it would be interesting to discover if infra-red analysis revealed any terminal double bonds in PHB which had been carefully extracted from freeze-dried, disintegrated organisms.

If one accepts that the hydrolysis product of PHB breakdown is β -hydroxybutyryl-CoA, then under aerobic conditions this may be further degraded by oxidation to acetoacetyl-CoA with the mediation of DPN as described above. Re-oxidation of the DPNH formed in this reaction would provide energy to the organisms. Acetoacetyl-CoA has been shown to be degraded in animal tissues by a thiolase reaction (Stern, Coon and del Campillo, 1956) which yields two molecules of acetyl-CoA. This may then be introduced into the tricarboxylic acid cycle by condensing with oxalacetate to yield citrate. Complete oxidation of the acetyl-CoA may be effected by this cycle thus providing further supplies of energy to the cell. Alternatively the increased levels of tricarboxylic acid cycle components may lead to the synthesis of intermediates useful to the cell metabolism.

If co-enzyme A is not concerned in the depolymerisation of PHB and the initial product is β -hydroxybutyric acid, then this may be oxidised to acetoacetate by the DPN-linked dehydrogenase reaction which has been demonstrated in the present research and which is known to occur in animal tissues (Green, Dewan and Leloir, 1937). From other experiments with animal tissues it seems likely that for further degradation of acetoacetate to occur it must be esterified to

acetoacetyl-CoA (Stern, Coon and del Campillo, 1956). After the formation of this complex further degradation would be similar to that described above.

Consideration may now be given to those aspects of the synthetic pathway which have not already been covered in the above discussion. Glucose is presumably dissimilated via the glycolysis or hexose monophosphate pathways to pyruvate which may then be decarboxylated and acylated to form acetyl-CoA (e.g. Reed, 1953, Rodwell and Rodwell, 1954). The acetyl-CoA may then follow the reverse of either of the pathways outlined above for PHB degradation. From the point of view of energy conservation it would seem more profitable to the organism, if PHB is not formed by the condensation of β -hydroxybutyryl-CoA units, to bring about deacylation of acetoacetyl-CoA by a transferase reaction rather than a simple hydrolysis which would result in the loss of the thiol bond energy. Acetate would seem to be the most likely CoA acceptor as in its co-enzyme form it could condense to give further quantities of acetoacetyl-CoA viz. (Stern, Coon and del Campillo, 1956).

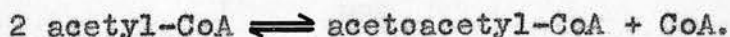
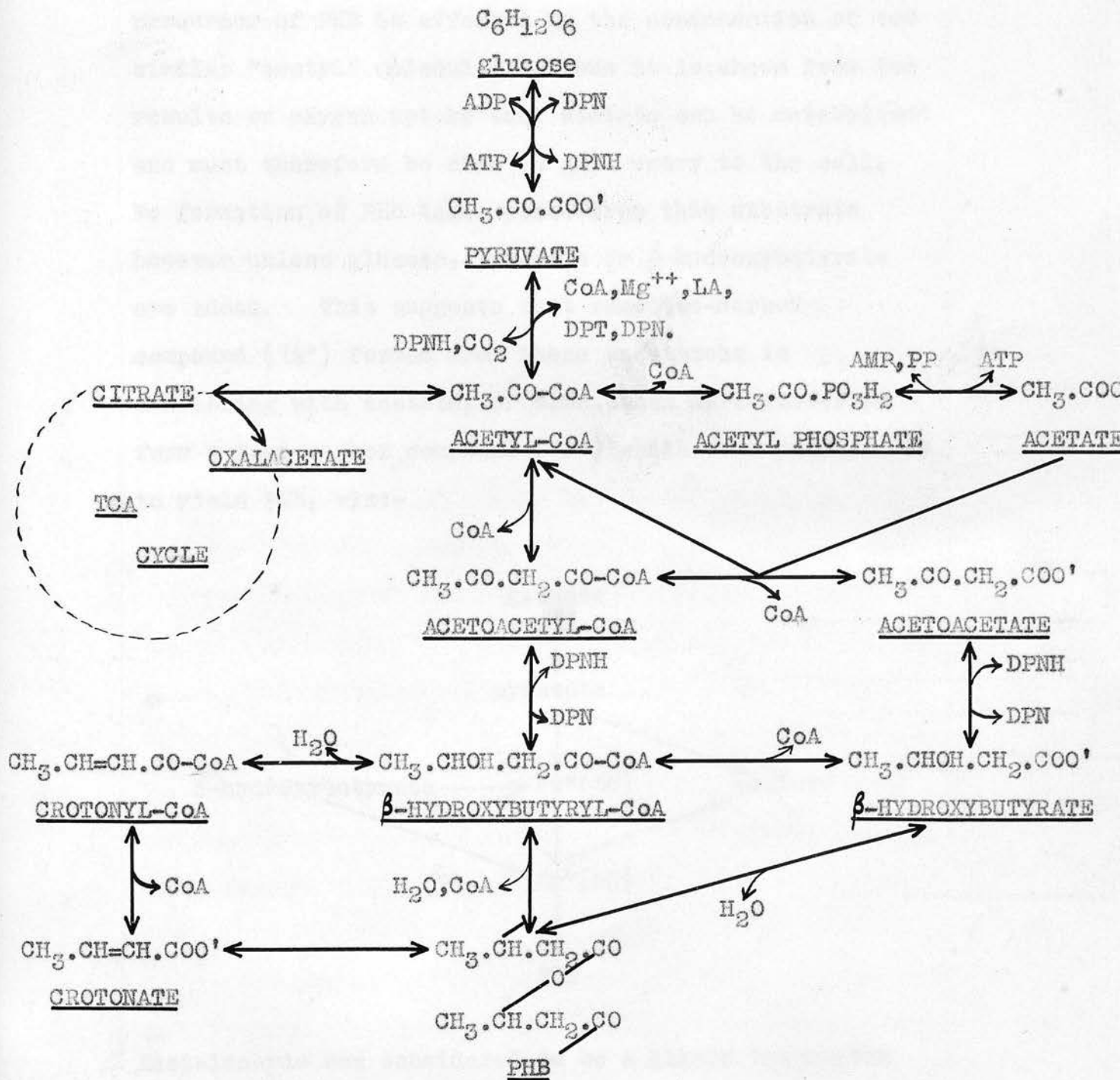


Diagram "A" summarises the possible pathways discussed above.

Cogent analysis of the results of PHB synthesis using different substrate systems questions the validity

DIAGRAM "A"

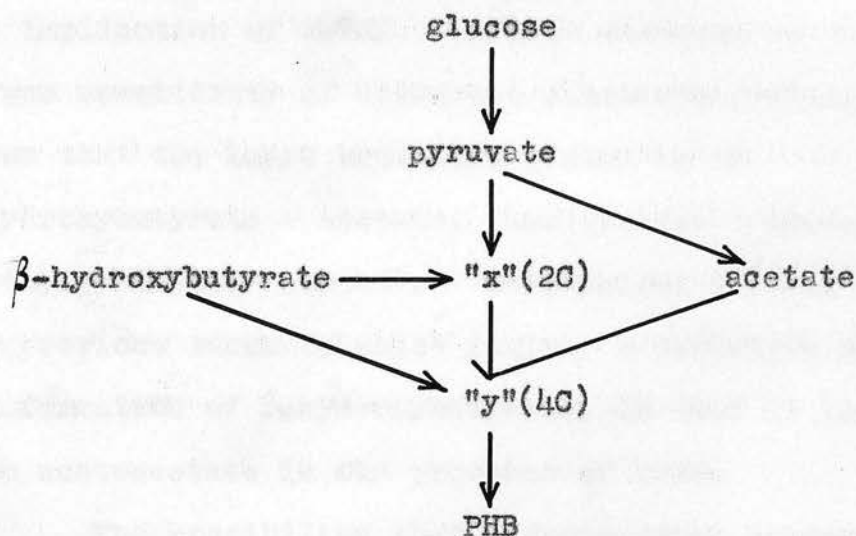


KEY:

AMP...adenosine monophosphate
 ADP... " diphosphate
 ATP... " triphosphate
 CoA...co-enzyme A

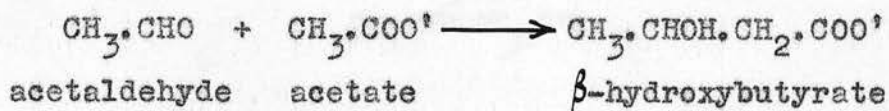
DPN...diphosphopyridine nucleotide
 DPNH.. " (reduced)
 DPT...diphosphothiamine
 LA....lipoic acid
 PP....pyrophosphate

of the assumption that the synthesis of a four-carbon precursor of PHB is effected by the condensation of two similar "acetyl" molecules. Thus it is shown from the results on oxygen uptake that acetate can be metabolised and must therefore be able to gain entry to the cell. No formation of PHB takes place from this substrate however unless glucose, pyruvate or β -hydroxybutyrate are added. This suggests that some two-carbon compound ("x") formed from these substances is condensing with acetate, or some close derivative, to form a four-carbon compound ("y") which then polymerises to yield PHB, viz:-



Acetaldehyde was considered to be a likely two-carbon intermediate in this respect but in the free state it caused inhibition of PHB synthesis (and breakdown) even with the addition of acetate. Nevertheless acetaldehyde, or some heat-labile conjugate, accumulates when pyruvate is incubated anaerobically with the enzyme

extract, which suggests that a close derivative of this compound may be concerned. Thus a condensation reaction between acetate and acetaldehyde might yield β -hydroxybutyrate:



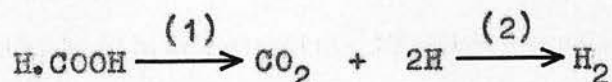
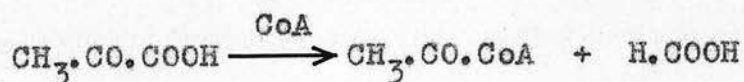
The experiments conducted to determine the effect of oxygen on synthesis from glucose + acetate have shown that at high oxygen tensions PHB formation is depressed. This suggests that some reductive stage occurs in the synthesis of PHB precursors from acetate, possibly with the implication of DPNH. Further studies, on the oxygen sensitivity of different substrate systems, have shown that the least sensitive system is of β -hydroxybutyrate + acetate, then glucose + acetate and lastly pyruvate + acetate. More weight is thus given to the previous schemata which include a reductive stage in the formation of β -hydroxybutyrate, in that it is formed from acetoacetate in the presence of DPNH.

The possibility that a four-carbon precursor for PHB formation was produced by the condensation of acetate with another two-carbon compound, formed from the other substrate in dual systems, was further discredited by the information obtained from the isotopic tracer studies. This showed that the PHB formed from 1-C¹⁴ acetate + glucose or pyruvate; or 2-C¹⁴ pyruvate + acetate, was labelled in the 1 and 3 positions of the

system, augmented with other co-enzymes, led to increased oxygen uptake. It is possible therefore that pyruvate can maintain a high level of DPNH, which cannot be established with acetate alone, and that high levels of DPNH are necessary for the formation of PHB.

Another difficulty which is presented by the results is the synthesis of PHB which is effected by B. cereus in pure hydrogen, especially since formation of PHB does not take place in nitrogen. A simple explanation of anaerobic synthesis from glucose and acetate would be to assume that the glucose was being fermented and giving rise to energy and perhaps intermediates necessary to cause PHB synthesis from acetate. Certainly B. cereus can ferment glucose anaerobically but if the suggested explanation were correct it would be expected that PHB formation would be similar in hydrogen and in nitrogen. Since it is not so other factors must be implicated. A possible clue is afforded by the inhibitory action of cyanide on synthesis in hydrogen. Aerobically the suppressory influence of cyanide can be attributed to the inactivation of the cytochrome system but under hydrogen this system would in any case be inoperative. As has been suggested in the Results it may be that hydrogenase is implicated and is being affected by the cyanide. Further weight is given to this hypothesis when the work of Fisher, Krasna and Rittenberg (1954) is considered. These observers showed that hydrogenase

could act in a manner similar to haemoglobin inasmuch as it could be oxidised or oxygenated. Both of these complexes were enzymically inactive but under hydrogen an autocatalytic reduction was initiated resulting in the reactivation of the oxygenated complex. As this did not take place in nitrogen, these findings may have considerable relevance to the present observations if a similar hydrogenase is concerned in PHB formation in a hydrogen atmosphere. The manner in which hydrogenase could be implicated is far from clear. It might be supposed that some pyruvate was dissimilated in a phosphoroclastic reaction similar to that occurring in Escherichia coli with the resultant formation of formic acid and acetyl CoA viz:-



Step (1) would be catalysed by formic dehydrogenase and step (2) by dehydrogenase. (These reactions have recently been unequivocally separated from the composite formic hydrogenlyase system by Peck and Gest, 1957).

In the presence of high levels of pyruvate derived from glucose oxidation, hydrogenase would tend to accelerate the formation of acetyl-CoA which is possibly concerned in PHB synthesis. Against this suggested pathway is the observation that hydrogen gas is not formed from any sugars fermented by B. cereus, though some suggestion of

hydrogen formation has been obtained in the present research when the organisms are supplied with glucose and acetate in an atmosphere of hydrogen. Further research aimed at demonstrating the presence, or otherwise, of the enzymes concerned in this pathway in cell-free extracts of B. cereus might clarify the position.

Thus the results described in this Thesis have provided strong evidence for believing that PHB acts as a carbon and/or energy reserve. Possible biochemical pathways concerned in the synthesis and degradation of this compound have been suggested and a lead has been given to further research which should help in establishing with greater certainty the mechanisms involved.

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SUMMARY.

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1. Experiments have been conducted with asporogenous strains of Bacillus cereus and Bacillus megaterium.
2. Methods and chemically defined media have been developed for the bulk culture of the organisms. Manganese was found to be an essential additive to the growth medium.
3. A major constituent of the "lipid" granules in these species is a polymer of β -hydroxybutyric acid (PHB). The metabolism of PHB has been studied under various conditions.
4. PHB was formed rapidly during growth and in washed suspensions in conditions of carbon and energy excess and dissimilated in the absence of a utilisable substrate. The formation of PHB has not previously been demonstrated in washed suspensions.
5. A high level of PHB in a cell conferred a high rate of endogenous respiration and delayed autolysis.
6. The formation of PHB showed an optimum around pH 7.5 and growth studies confirmed that it is unlikely to be a "neutralisation mechanism".
7. It was concluded from the above (4 - 6) that PHB acted primarily as a reserve of carbon and energy.
8. Washed suspension experiments showed that PHB was dissimilated more rapidly aerobically than anaerobically. More oxygen was absorbed than would have been required for the complete oxidation of the PHB lost aerobically and more acid was formed anaerobically than was accounted for by a simple hydrolysis of the degraded PHB to β -hydroxybutyric acid.

9. Quantitative studies showed 90% of the theoretical yield of β -hydroxybutyric acid and acetoacetic acid from the amount of PHB broken down anaerobically. Little β -hydroxybutyric acid or acetoacetic acid accumulated aerobically and it appeared that almost complete oxidation of the degraded PHB took place.
10. Chromatographic analyses of the products of PHB breakdown confirmed the presence of β -hydroxybutyric and acetoacetic acids.
11. The organisms formed PHB in the presence of glucose, pyruvate or β -hydroxybutyrate.
12. Acetate did not induce synthesis of PHB on its own but caused an extensive increase in the amount formed from the above substrates. With a fixed glucose concentration (0.05 M.) and varied acetate concentrations, PHB formation was proportional to the acetate concentration up to 0.05 M.
13. Various other organic substances were tested as substrates for PHB synthesis. No formation of PHB was effected but several of the substances inhibited its breakdown to some extent.
14. Neither a nitrogen nor a magnesium source was a necessary additive for PHB synthesis.
15. PHB formation from glucose and acetate was strongly inhibited by cyanide and dinitrophenol and less strongly by the co-enzyme A antagonist pantoyl-tauryl anisidide.
16. Low concentrations of fluoroacetate stimulated

PHB formation from glucose and acetate, the oxygen consumption being slightly inhibited. The β -hydroxybutyrate analogue 2-hydroxy-1-propane sulphonate caused a slight increase in PHB synthesis without affecting the oxygen uptake.

17. Pure oxygen inhibited the formation of PHB, though synthesis was optimal with 5% oxygen in nitrogen as the gas phase. Neither B. cereus nor B. megaterium formed PHB under nitrogen but the former organism did so under hydrogen. Carbon dioxide did not stimulate PHB formation.

18. While utilising β -hydroxybutyrate for the synthesis of PHB the organisms converted much of the substrate to acetoacetate.

19. The hypochlorite method was unsuitable for the estimation of PHB in lysates derived from lysozyme treatment of whole cells. Evidence is led that the ether soluble component from hypochlorite isolated granules may be derived, at least partly, from other cell constituents.

20. Synthesis or degradation of PHB in lysozyme prepared cell-free extracts could not be demonstrated.

21. Neither utilisation of β -hydroxybutyrate nor initiation of oxygen uptake upon its addition could be shown with the extract.

22. A DPN-linked β -hydroxybutyric dehydrogenase could be demonstrated in carefully prepared extracts after a lag period which lengthened on storage. Neither CoA, ATP nor KCN enhanced DPN reduction in these circumstances.

23. Pyruvate was decarboxylated anaerobically with the formation of quantitative amounts of acetaldehyde. The addition of DPT accelerated this process but CoA was inactive.

24. Studies using isotopically labelled substrates showed that acetate was certainly incorporated into PHB during synthesis in glucose + acetate, and both pyruvate and acetate during synthesis in pyruvate + acetate.

25. Possible biochemical pathways of synthesis and degradation of PHB are discussed in the light of these experimental findings.

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