VICTORIA UNIVERSITY OF MANCHESTER.

A THESIS

entitled

A survey of lactic acid bacteria found in association with brewery yeasts.

and

An examination of bacteria isolated from textile fabrics.

Presented for the Degree of

Doctor of Philosophy

by

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C O N T E N T S.

PART I.

A Survey of lactic acid bacteria found in association with brewery yeasts.

Note: Not included in the list of contents are forty-one tables inserted in the text, summarising some of the results.

PART II.

An examination of bacteria isolated from textile fabrics.

I N T R O D U C T I O N.

The micro-organisms belonging to the family of the Lactobacteriaceae comprise a very large group of organisms whose various roles in the dairy industry, in brewing, in the preparation of vegetable fermentation products and silage, render it of foremost importance to the well-being of man and domestic animals. Some sub-groups of lactic acid bacteria have been studied in great detail, for example the classical studies of Orla-Jensen on the bacteria associated with the dairy industry are very well known. Dr. J. G. Davis in more recent years in England and Professor Hammer in the U.S.A. have also added considerably to our knowledge of these bacteria. In Finland the work of Professor Virtanen on the lactic acid bacteria associated with the formation of silage has been of prime importance to agriculture.

Up to the present time comparatively little work has been published concerning those members of the family Lactobacteriaceae which occur in association with brewery products, namely yeast, wort and beer. These comprise a not inconsiderable number of very interesting types. Hitherto, we have for our information only the somewhat limited work of Van Laer, Henneberg, Claussen, Mees and Shimwell on the lactic acid bacteria of breweries and yeast factories. Very few types have been described by those

investigators.

It has been the purpose of the present investigator to study systematically the lactic acid-producing bacteria of brewery yeast with the object of isolating and characterising the main types to be found in this habitat. An extension of this object has been to ascertain the main facts concerning the respective nutritional requirements of some of the types which have been isolated.

Finally as regards the arrangement of the thesis the subject matter has been divided into three parts, namely, Historical Part, Practical Part and Discussion of Results, arranged in their respective order. Each part has been subdivided into two sections; Section I deals with the isolation, and characterisation of the organisms and Section II deals with the work concerning the determination of nutritional requirements of some of the organisms.

PART I.

A survey of lactic acid bacteria found in association with brewery yeasts.

HISTORICAL PART.

SECTION I.

DEVELOPMENT OF THE KNOWLEDGE OF LACTIC ACID BACTERIA.

The practical use of fermentation processes by human beings dates back to the earliest times; this surprises no one who has lived and prepared food in tropical or subtropical countries, where every sandstorm is followed by a spate of fermentation in the cooking-pot. Even to-day every tribe has its characteristic fermented food prepared according to a traditional method. Historic references to beer production originate both from the Sumerians and the Babylonians, and under the Pharaohs in Ancient Egypt the brewing of beer was a trade. Cheese and curd were prepared in Asia and in Europe several hundred years, at least, before the birth of Christ. Thus lactic acid as an unnamed component of soured milk must have been known in human experience since the days when men first had flocks and herds.

The true nature of lactic acid ($CH_3 \cdot CHOH \cdot COOH$) was discovered by Scheele (1), who isolated and identified it as the principal acid in sour milk in 1780. It was first discovered as a fermentation product by Blondeau (2) in 1847. In 1857 came the publication of Pasteur's first paper "Memoire de la fermentation dite lactique" (3), in which he claimed that the lactic fermentation was due to a living cell or ferment (cell in Pasteur's view was a ferment). His claim was proved again and again, and became one of the basic tenets in the development of microbiology.

Schultze in 1868 (4) demonstrated for the first time the presence

of lactic acid bacteria in yeast cultures of distilleries. But it was not until the year 1877 that lactic acid bacteria were isolated in pure cultures, Lister (5) having isolated Streptococcus lactis. Delbrück's name is also associated in this connection since he determined the most favourable temperature for lactic acid fermentation in distilleries. Then came in quick succession isolations of different lactic acid-forming bacteria from various divergent sources.

Kern in 1881 (6) isolated a lactic acid-forming organism from the fermented milk of the Caucasus, known as "Kefir", and gave the organism the name Dispora kaukasica, but later it was called Bacillus kaukasicus and it is now known as Lactobacillus caucasicus. A similar bacillus was isolated by Döderlein in 1892 from vaginal secretions of pregnant women. Moro (7) isolated another organism from the faeces of breast-fed infants and called it B. acidophilus; his findings were confirmed in the same year by Finkelstein (8). Tissier (9), also in 1900, isolated from the faeces of infants two new organisms of the same group, to which he gave the names B. bifidus and B. exilis. In 1905 Grigoroff (10) isolated from "Kisselo-mleko", the fermented milk of Bulgaria and better known as "Yoghurt", three organisms which he designated "A", "B" and "C"; the first of these is now known as Lactobacillus bulgaricus. Similar organisms have been found by other workers in a number of fermented milks, chiefly the Armenian "Mazun", the Sardinian "Gioddu" and the Egyptian "Leben raib" (11); they have also been isolated from ordinary market milk and from human milk (7,12,13). Heinemann and Hefferan (12) isolated such organisms from human saliva and gastric juice, from soil, and from a number of different foods, such as bran,

silage, cornmeal and olive-juice. In recent years Creœlius and Rottger (14) have found that the lactobacilli are the most prevalent bacteria in the intestinal tract of guinea pigs, while Shapiro and Sarles (15) reported that lactobacilli are most numerous bacteria in the intestinal tract of chickens. Harrison and Hansen (16) have found that anaerobic lactobacilli comprise the largest part of the flora of the cecal faeces of turkeys.

Lactic acid bacteria comprise one of the most important groups of "beer disease" bacteria, and as early as 1876 Pasteur (17) discovered their detrimental effect on fermented beverages by isolating one of them from infected beer. Later Van Laer (18) isolated from beer an organism which he named Saccharobacillus pastorianus. However, there was no advanced and systematic study of "beer infection" by lactic acid bacteria until recently, when Shimwell (19) began publishing his work on the "Saccharobacillus" question. The name Saccharobacillus has now been discarded because the organism is a lactic acid bacterium, and in accordance with the modern nomenclature it has been renamed Lactobacillus pastorianus.

Lactobacillus pastorianus is a typical beer species, and according to Shimwell (2) all the previously isolated "Saccharobacilli" from beer, including Bacterium lindneri, which Henneberg (21) isolated from a bottom fermentation beer, are only varieties of L. pastorianus, but he also pointed out that in his experience there exist other types capable of beer spoilage.

In 1941 Shimwell (22) reported that ten cultures of newly isolated beer lactobacilli had been collected. A few preliminary observations

showed that no two cultures were the same and that none of them could be regarded as identical with his strain of L. pastorianus. He also pointed out that two strains from these newly isolated lactobacilli have been found to produce the so-called "Sarcina sickness" in beer, a type of spoilage regarded as the monopoly of the beer cocci.

According to Shimwell (23), other strains of lactobacilli, apparently notably different from L. pastorianus in several characteristics, have also been isolated by him from beers from various sources, but these have not yet received the study necessary to decide whether they are distinct species or not.

The number of pure cultures of streptococci isolated from beers and pitching yeasts is considerable. During the last sixty-five years a number of investigators have given different names to their "species", very often calling them either "sarcina" or "pediococcus". Later, however, Shimwell and Kirkpatrick (24) came to the conclusion that beer cocci could not rightly be classed under "Sarcina", Pediococcus or Micrococcus, and concluded that they belong to the plant division of the genus Streptococcus.

All the various streptococci isolated from beer and described up to the present date, according to Shimwell (22), seem to belong to a single species Streptococcus damnosus, which was originally isolated from top fermentation beer by Claussen (25), and was by the latter named Pediococcus damnosus. Another similar coccus isolated from beer was described and named by Claussen (25) Pediococcus perniciosus, because of its habit of producing a growth which remains suspended in the medium. Moes (26) considered this organism to be a variety of Pediococcus damnosus, while Shimwell and Kirkpatrick (24) regard it as a mere strain of

Streptococcus damnosus. Walters (27) isolated from an Australian stout a coccus which was found to ferment pentoses. This organism is also considered to be a variety of Streptococcus damnosus and is termed as Streptococcus damnosus var pentosaceus. The other recognised varieties of Streptococcus damnosus are Streptococcus damnosus var viscosus and Str. damnosus var perniciosus, both isolated from British beers by Claussen (25).

Various semi-thermophilic cocci from mash distillery tuns were described and named by Henneberg and Lindner almost 50 years ago but have been completely overlooked in the recent literature. Of the three species described by Henneberg (28), two have been included in Shimwell's (23) table of brewery lactic acid bacteria, namely Streptococcus acidi lactici (P. acidi lactici) and Str. lindneri (P. lindneri), while P. henneberri, the third organism is regarded as a probable synonym of Str. acidi lactici.

In 1941, Shimwell (22) claimed to have isolated a new streptococcus strain from a highly hopped beer. He stated that the organism is an extremely active producer of ropiness in hopped beer, but that the war had prevented any extensive study of this culture.

In 1943 Walker and Parker (29) reported the isolation from nine different beers and one sample of yeast of 34 cultures of lactic acid bacteria, of which some are supposed to be new species, while some are very much alike in certain important characteristics and these may be physiological strains, or varieties of the same species. Further study of these organisms was postponed for some time.

More recently Kulka, Cosbie and Walker (30) reported the particulars of the isolation, characteristics and classification of a tetrad-forming

streptococcus, which was isolated from a ropy beer. The most distinctive feature of this organism is its propensity to render beer extremely viscous, not only under anaerobic but also under aerobic conditions. The principal characteristics markedly differ from the other beer streptococci already known. Thus this organism has been regarded as a new species by the authors, and has been designated Streptococcus mucilaginosus because of its ability to render certain media highly viscous.

The origin of beer lactobacilli is still uncertain. According to Davis (31), the lactic acid bacteria of milk and fermented vegetable mashes and alcoholic drinks may be regarded as "artificially produced" types of comparatively recent origin, the former arising from dead or living animal matter, the latter from plant material. It has been suggested that milk lactobacilli cannot develop in beer (32,20), but from the investigations of Walker and Parker (33) it is evident that in beers of light to medium hop content, L. casei, L. bulgaricus, L. helveticus, L. delbrückii, L. plantarum, L. brevis and L. brassicae fermentate can be induced to grow. Lactobacilli from milk and other sources are often present in the atmosphere and could easily find their way into breweries. It is not improbable that certain such strains might become acclimatized to brewery worts and develop in the course of time a considerable resistance to hop antiseptics. These bacteria could, therefore, exert detrimental effects in beer and wort.

It is quite probable that, similarly, certain streptococci from milk might become acclimatized to brewery worts and beers.

The anaerobic nature of beer streptococci and lactobacilli, and

the ability which some of them possess to adapt themselves to hop antiseptics, so that in certain cases growth can occur even in strongly hopped beers, renders the lactic acid bacteria one of the most dangerous, if not the most dangerous, group of "beer disease" bacteria.

LACTOBACILLUS AND STREPTOCOCCUS.

GENERIC NAMES OF LACTIC ACID BACTERIA.

The important group of bacteria whose members are listed as true lactic acid bacteria belong to the family of Lactobacteriaceae, and are characterised as: Gram positive, non-motile,* non-spore forming, catalase negative bacteria, requiring to varying extents a complex mixture of protein decomposition products and growth factors for their nutrition. The type of lactic acid which is produced varies with different species, being sometimes the laevo-form, sometimes the dextro-form and sometimes the mixture of the two. In addition to producing lactic acid, some species produce some volatile acids, a little alcohol, and also carbon dioxide; these are known as the heterofermentative lactic acid bacteria, as distinct from the homofermentative types which form lactic acid as almost the sole end product.

Generally speaking, lactic acid bacteria appear as cocci or rods and are divided, on the basis of morphology into two genera: Streptococcus which comprises the cocci, and Lactobacillus which comprises the rod-shaped forms. These two genera constitute a group of bacteria of great economic importance to man.

The rod-shaped lactic acid forming bacteria were discovered by Pasteur to be causes of infection in beer and other fermented products

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* Harrison and Hansen (48) have recently reported the isolation of a peritrichous motile strain of L. plantarum from faeces of turkeys.

as early as 1876 and were named by him "Bacille des Bieres Tournoes" (17). Later, Van Laer (18) and Henneberg (21) while studying lactic acid bacteria of beer, isolated and named their organisms Saccharobacillus pastorianus and Bacterium lindneri, while Orla-Jensen's (34) classification which is primarily for the use of dairy bacteriologists, subdivided the rods into the genera Thermobacterium (heat loving), Streptobacterium (chain forming), and Beta bacterium (heterofermentative). The confusion about the different names lasted for a long time until Shimwell in 1935 began publishing his work on the "Saccharobacillus question". The name Saccharobacillus or Bacterium has been dropped in recent years and in accordance with the modern nomenclature (35) rod-shaped lactic acid bacteria have been renamed, having Lactobacillus as the generic name.

Davis (36) in his classification has put all the rod-shaped lactic acid bacteria in the genus Lactobacillus but he has grouped them under three groups, Group I showing a very little respiration, which was not inhibited by monoiodoacetate and stimulated by methylene blue. Group II was characterised by a higher respiration, which could be inhibited by monoiodoacetate and stimulated by methylene blue, while Group III (heterofermentative) differed from Groups I and II in having a respiration partially inhibited by both cyanide and monoiodoacetate. For the purposes of brewing bacteriology the classification of Davis seems to offer many advantages.

The cocci, or spherical bacteria have, from the earliest times, been classified according to their morphological appearance and their method of cell division. The lactic acid-producing cocci responsible for "Sarcina sickness" of beer have been known since the condition was first described by Pasteur (17). Their relationship to the true lactic acid bacteria of the genus Streptococcus was not realised until years

later when Shimwell drew attention to it.

The spherical organisms isolated from spoiled beer were named Sarcina by Hansen (37). This name was soon adopted by brewers and brewery bacteriologists, and in spite of the fact that in later years it has been unanimously accepted that beer cocci are not Sarcina, but are the true lactic acid-forming streptococci, the name Sarcina continues to be attached to these organisms by brewing technologists.

Beer spoilage was studied in greater detail by Balcke (38), who considered that the so-called "beer sarcina" did not form cubical packets, but only tetrads and irregular masses, and he thus came to the conclusion that these organisms were not true sarcinae. Balcke applied the name "Pediococcus" to these organisms, and named his species Pediococcus cerevisiae.

Since the name Pediococcus was first used by Balcke, this has been accepted and used as a generic name in connection with other specific names by Lindner (39,40), De Toni and Trevisan (41), Richard (42), and many others. Claussen (25) during his researches pointed out that the Sarcina species are quite different from the cocci which grow in beer, and he accepted the generic name Pediococcus for the beer cocci: he applied the names P. damnosus and P. perniciosus for such organisms. Henneberg (28) also included three new organisms during his researches on the cultures isolated from potato mash, sauerkraut, grain mash, pickles and molasses. He called them P. acidi lactic, P. hennebergi and P. lindneri.

Beijerinck (43) applied the name Lactosarcina to the genus comprising the beer cocci, in line with his name Lactobacillus. Lehmann and

Neumann (44) in their classification included the names P. damnosus and P. perniciosus. No reference was made to the names proposed by Balcke or by Lindner.

In 1934 Mees (26) in his historical review of the studies on the spoilage of beer pointed out that the cultures considered to be pediococci were all non-motile, non-sporeforming, catalase negative and gram positive spherical bacteria. Mees accepted the generic name Pediococcus but used Pediococcus damnosus Claussen as his type species, with P. cerevisiae Balcke as a questionable synonym. He characterised P. perniciosus Claussen as a variety of P. damnosus and named a new variety P. salicinaceus. The names P. pentosaceus and P. halophilus were applied to organisms included by Orla-Jensen (34) as Tetracoccus in his classification.

The question of the identity of these beer spoilage cocci was reopened in 1939 by Shimwell and Kirkpatrick (24). As a result of this study, together with a critical review of the "Sarcina literature", these authors reached the conclusion that the so-called "beer Sarcina" or "beer pediococci" are neither sarcina nor micrococci. They put forward the view that it was unnecessary to keep a special genus Pediococcus, since Mees' diagnosis of that genus was virtually indistinguishable from that of the genus Streptococcus.

More recently Shimwell (45,46), while reviewing the subject of beer disease organisms, has pointed out that the streptococci are to be regarded more as a physiological than as a morphological group, and if undue emphasis is not placed on long chain formation and the beer cocci are not included because they sometimes produce tetrads in acid

media, there was no reason why these organisms should not be placed in the plant division of the established genus Streptococcus. Shimwell and Kirkpatrick (24) also pointed out that the production of lactic acid with a small amount of volatile acid and carbon dioxide, and the production under certain conditions of diacetyl, are all characteristics of streptococci. Accordingly they consider that, in the classification of lactic acid bacteria by Davis and his collaborators (31), beer streptococci would fall into Streptococcus I group, or could be classed as intermediate between this group and the Streptococcus III group, because of their resemblance to the "aroma streptococci" which produce diacetyl.

Shimwell's point of view has received considerable attention in the recent years, and it has been agreed by some bacteriologists that the correct place for beer cocci is in the genus Streptococcus. Thus the term Streptococcus is now being used as a generic name for such cocci, and in the view of the writer this is a course to be approved since it puts an end to the confusion which has lasted for a long time.

But Pederson (47) in a recent paper has again pointed out that the so-called pediococci are readily distinguishable from the species of the genus Streptococcus by their tetrad grouping and their comparatively high acid production. In his opinion these morphological and physiological characters, and the fact that they produce inactive lactic acid, seem sufficient to exclude them from the genera Streptococcus, Micrococcus and Sarcina. He has also suggested that this group should be considered as a separate genus Pediococcus with the P. cerevisiae Balcke as the type species, and this genus should

be included in the tribe Streptococceae of the family Lactobacteriaceae with the genera Diplococcus, Streptococcus, and Leuconostoc, rather than in the family Micrococcaceae as has been put in the 6th Edition of Bergey's Manual (1948).

OCCURRENCE AND ECONOMIC IMPORTANCE OF LACTIC ACID BACTERIA.

In nature, lactic acid bacteria chiefly occur on plants and vegetable matter where, amongst their other activities, they ferment certain sugars to acids, and these acids are of importance, either as carbon nutrition for a number of other micro-organisms or as means of neutralising the alkaline products formed by putrefactive bacteria. Some of these bacteria also occur on the mucous membranes of man and animals, and also in the intestinal canal.

Most of the spontaneous souring of food, milk and the like is due to lactic acid bacteria. It seems that milk and the various milk products - butter, cheese, fermented milks, etc. - have been the best source for the isolation of lactic acid bacteria species. For example, L. delbrückii, L. buchneri, L. brevis, Str. lactis and many others were isolated from milk, while Str. thermophilus, Str. cremoris, L. casei, L. bulgaricus, L. helveticus along with many other species have been found to be present in various cheeses. From time to time many aroma producing lactic acid bacteria has been also

found in milk, butter and cheeses.

Apart from the various sources mentioned in above paragraphs, lactic acid bacteria species occur in, and are closely associated with, fermented beverages and with the materials used for their manufacture. Some species of lactic acid organisms were isolated from beer, wine or cider, while some were found in malt wort.

Pressed yeast was also found to contain lactic acid bacteria, but sufficient work has not yet been done to identify all the species present. It is the writer's view that many "pitching yeasts" used for the brewing of beer in this country, on systematic investigation, might be found to possess a rich flora of lactic acid bacteria.

The above-mentioned organisms, when found in beer, wine, malt wort or in commercial yeasts, play a detrimental role as soon as the conditions allow their activities to take place. However, it has been shown in the following paragraphs that some of these organisms can be and have been usefully employed for some industrial purposes.

The very considerable and growing economic importance of the lactic acid bacteria necessitates a short reference to this subject. Apart from its uses for various purposes, lactic acid bacteria are most extensively employed in dairy industry. Milk is probably the most important article of food among many pastoral tribes of Europe and Asia. Because of the sanitary conditions under which the people live, the milk is usually fermented before it is consumed, especially during the warm seasons of the year. The consumption of soured milk preparations is widespread because of their supposedly therapeutic value. The fact that they appear under various names does not mean necessarily that each product is fermented with different organisms.

Many of these preparations result from the combined action of a mixed culture of more than one species. The well-known fermented milks are as follows.

Yoghurt is a sour milk product, which is prepared in Bulgaria and Turkey, generally from sheep milk or buffalo milk. Sometimes cow milk is also used and is then evaporated somewhat before inoculation.

Lactobacillus bulgaricus is the dominating organism found in Yoghurt, although it is a product of the mixed flora of micro-organisms. Metchnikoff introduced this product as a dietetic beverage into western Europe, as he believed that it would be possible in this manner to avoid indigestion and sclerosis, and the lives of human beings would thus be prolonged.

Kumiss is a Russian product prepared by the fermentation of mare or cow milk by lactobacilli, lactic streptococci and yeasts. The yeasts produce alcohol and carbon dioxide, and the bacteria produce lactic acid.

Kefir is a sourish, slightly alcoholic milk product originated in the Caucasus. It is extensively used as a food by the natives of the region. It is prepared by inoculating the milk of mares, cows, goats or ewes with a "seed" known as Kefir grains which usually contain L. casei, streptococci and a yeast Saccharomyces kefir. Lactic acid, alcohol and carbon dioxide are the main fermentation products.

Acidophilus Milk is prepared by inoculating sterilised fresh whole milk, or partially skimmed milk, with a pure culture

of a selected strain of L. acidophilus. This milk is employed for medicinal purposes.

Apart from the few fermented products mentioned above, there are several other fermented milks which are used in one part of the world or other.

Other important applications of lactic acid bacteria in the dairy industry are in cheese-ripening and souring of cream in butter production.

Use of lactic acid bacteria in the brewery has been well recognised and thus it is greatly employed in the brewery, in the acidification of mash where such acidification is desired and also in the production of acid and aroma in certain special beers. Acidification of the mash is especially desired when the mashing water contains considerable quantities of calcium and magnesium bicarbonates.

Employment of lactic acid bacteria in distilleries and yeast factories is increasing every day. For a biological acidification of the mash in the manufacture of air grown yeast and the production of spirit in order to suppress the bacteria (butyric acid bacteria) which might inhibit or contaminate the yeast, the use of these organisms is becoming very popular.

Another important use of these bacteria is in the manufacture of lactic acid for medicinal and technical purposes. The organisms that are used for the production of lactic acid by fermentation are L. delbrückii, L. casei, L. leichmannii, L. bulgaricus, L. pentosus and Str. lactis. All these organisms are homofermentative. The

type of organism to be selected depends primarily upon the carbohydrate being fermented and the temperature to be used. Owing to the great demand of lactic acid for various purposes, many important methods have been developed for the production of this important chemical, by fermentation, from various raw materials and by-products.

A process for producing a white calcium lactate has been also developed by Daly, Walsh and Needle (49). The special features of this process are the use of a non-denatured milk as the nutrient and the rapid drying of the calcium lactate produced.

The lactic acid obtained from the various fermentation methods is used for a variety of uses. These include uses in connection with foods, fermentations, pharmaceuticals, and the chemical industries. As an acidulent the edible grade of lactic acid is used in confectionery, fruit juices, essences, lemonades, pickles, syrups and in other products. It is also used in the curing of meat and in canned vegetable and fish products.

In the chemical industries, lactic acid is used in the dyeing of silks and other textile goods, as a mordant in the printing of woollens, in the bating and plumping of leathers. The water-white grade is used in the plastic industry.

Lastly it is of interest to point out that the use of L. acidophilus has been recognised in the therapy of various disorders of the gastro-intestinal tract. It has been found that its successful implantation in the intestines may often relieve the symptoms associated with some of these disorders.

BIOCHEMICAL ACTIVITIES OF LACTIC ACID BACTERIA.

Lactic acid bacteria are characterized by their power, when grown on suitable media, to convert glucose, and generally also certain other sugars, into lactic acid. The equation expressing this conversion in the case of homofermentative species is:-

$$C_6 H_{12} O_6 = 2CH_3 CHOH COOH.$$

For the conversion of glycogen to lactic acid in animal tissues the Embden Meyerhof (50) school proposed the phase sequence:-

A. Sugar ⟶ Hex-ose diphosphoric acid.

B. Hexose diphosphoric acid ⟶ 2 Triose phosphoric acid.

C. 2 Triose phosphoric acid ⟶ 1 α-glycerophosphoric acid + 1 phospho glyceric acid.

D. 1 Phosphoglyceric acid ⟶ 1 pyruvic acid + phosphoric acid.

E. Pyruvic acid + 1 α-glycerophosphoric acid ⟶ 1 lactic acid + 1 triose phosphoric acid (passing to C).

The processes A, B, C and D are analogous to those of alcoholic fermentation whilst E follows a course different from that of alcoholic fermentation, as lactic acid bacteria do not contain carboxylase. Therefore the pyruvic acid is not decarboxylated into acetaldehyde, but rather it is reduced to lactic acid. Neuberg and his collaborators (51) on the other hand have shown that the lactic acid fermentation may also take place via methyl glyoxal ($CH_3.CO.CHO$), which in the presence of the co-enzyme glutathione is converted by the keto-aldehyde mutase glyoxalase into lactic acid.

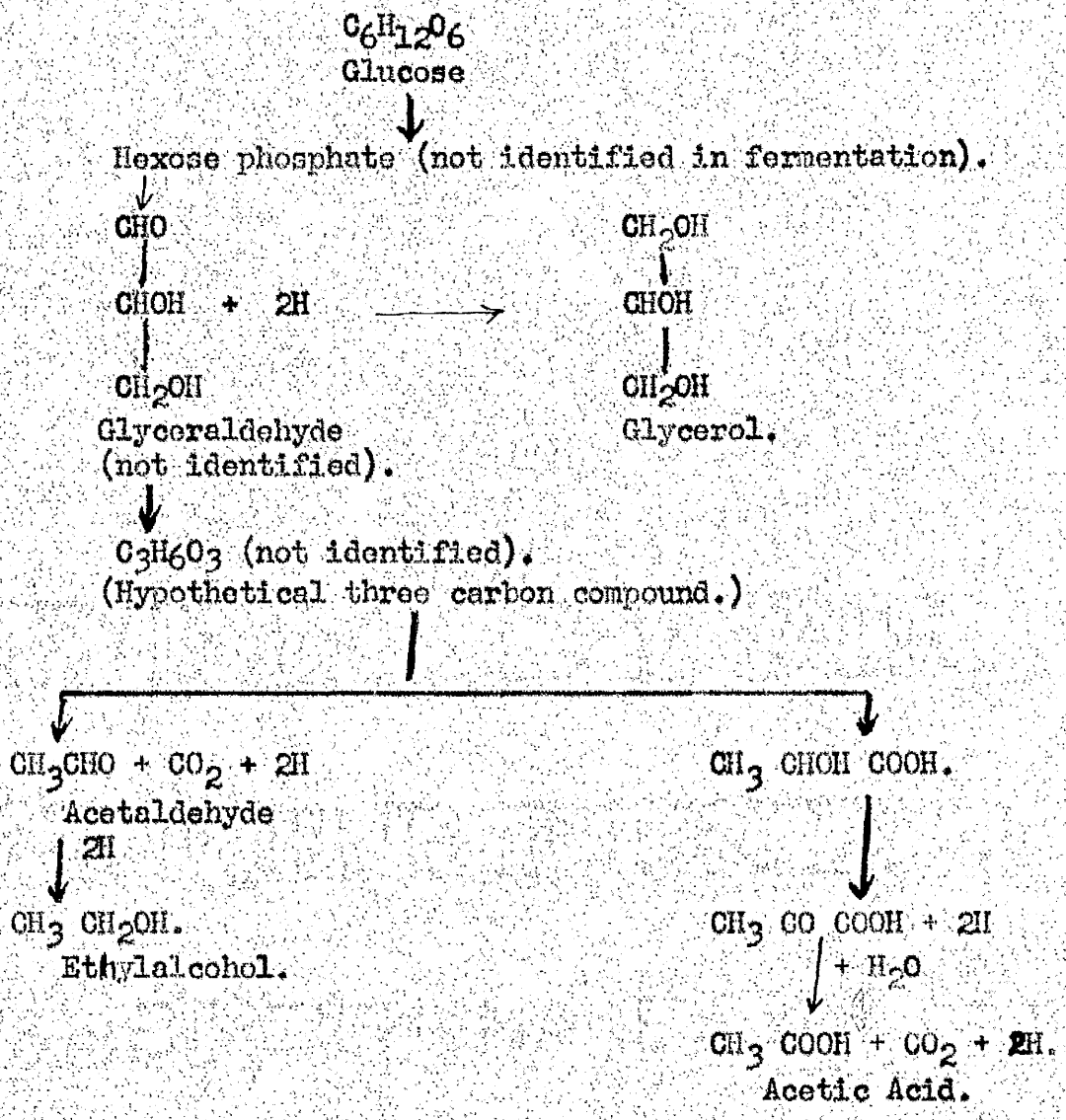
$$CH_3.CO.CHO + H_2O \longrightarrow CH_3\ CO\ CH\langle^{OH}_{OH} \longrightarrow CH_3\ CHOH\ COOH.$$

The question whether in lactic acid bacteria the process takes place via pyruvic acid or via methyl glyoxal has not yet been answered. Presumably both reactions take place side by side.

But even the earliest investigations on the action of lactic acid bacteria on sugars indicated that other fermentation products than lactic acid, notably acetic acid, were frequently formed. Subsequently, and owing primarily to the research of Kayser (52), it became customary to subdivide the lactic acid bacteria into two main groups. One which in addition to lactic acid produced volatile acids and other compounds in appreciable, though in varying, quantities, now termed as heterofermentative, while the other which gave 95 per cent or more lactic acid, as homofermentative bacteria.

Some of the first researches concerning the mechanism of the formation of the final products by heterofermentative lactic acid bacteria were carried out by Gayon and Dubourg (53), by Fred, Peterson, and Davenport (54), by Peterson and Fred (55) and by others.

Nelson and Werkman (56) carried out experiments with several heterofermentative bacteria and obtained data upon which they have suggested the following tentative scheme for the dissimilation of glucose by heterofermentative bacteria.



Even the true lactic acid bacteria, however, were shown to yield volatile acids in measurable quantities. Thus Bertrand and Weissweiler (57) obtained 1·5 per cent of acetic acid and traces of formic acid from cultures of L. bulgaricus, a type isolated by Grigoroff (10).

It is of great interest that many organisms of the group which produce appreciable quantities of volatile acids convert fructose into its corresponding alcohol, mannitol. Some writers like Bolcato (58), Sochoen and Eras (59), Kitahara and Katagiri (60) have attempted to establish the various intermediary and final fermentation

products formed by them.

Kruis and Raymann (61) isolated from malt an organism which produced from saccharose, formic and acetic acids, trace of ethylalcohol, mannitol and lactic acid. Smit (62) in 1916 and Steenberge (63) in 1920 also reported the production of mannitol by L. fermentum.

According to Steenberge the mannitol producing lactic acid bacteria possess marked reducing powers towards selenium and tellurium salts and are capable of activating these salts to function as acceptors for activated hydrogen. Among the true lactic acid bacteria the activating powers are much less marked. According to Van Steenberge they are unable to reduce selenium and tellurium salts, and fructose is converted into lactic acid by them. According to Jensen (64) all the lactic acid bacteria retain the property of reducing the methylene blue to its leuco-compound, a reaction which does not involve a preliminary activation of the molecule of the hydrogen acceptor.

Catalase is not produced by lactic acid bacteria, an observation which Beijerinck (65) made and which Jensen (64) subsequently confirmed. This indicated that hydrogen peroxide is not formed by the fermentation of carbohydrates by lactic acid bacteria, and further points to an increased sensitiveness towards oxygen by these organisms.

Myrbäck and Von Euler (66) by their researches have shown that these micro-organisms produce a co-enzyme, and that as in the case of yeast, a fermentation of glucose by these bacteria is not possible in the absence of the co-enzyme. It can be concluded, therefore,

that the first stage in the fermentation of monoses is the formation of hexose phosphoric esters, these esters are subsequently converted into methyl-glyoxal is supported by Neuberg and Gorr's researches already referred above.

It appears that a certain percentage of the methylglyoxal must be converted into acetaldehyde and formic acid, since Bertrand and Weisswiler (57) observed the formation of the latter substance in cultures of Bact. bulgaricum (L. bulgaricus).

Nelson and Werkman (67) have shown that pyruvic acid may be fermented by L. lycopersici with the production of equimolecular amounts of acetic acid, lactic acid and carbon dioxide.

(a) $CH_3\ CO\ COOH + H_2O \longrightarrow CH_3 \cdot \overset{OH}{\underset{OH}{C}} \cdot COOH \longrightarrow CH_3\ COOH + CO_2 + 2H.$

(b) $CH_3\ CO\ COOH + 2H \longrightarrow CH_3\ CHOH\ COOH.$

One molecule of pyruvic acid is hydrated to form one molecule each of acetic acid and carbon dioxide, while a second molecule is reduced to a molecule of lactic acid.

On the other hand Kakuo Kitahara (68) has pointed out that in the resting state, homofermenter (L. plantarum) and heterofermenter (Leuconostoc mesenteroides) are both unable to decompose pyruvic acid if it were substituted for glucose. But when a mixture of glucose and pyruvic acid was used as substrate, vigorous reaction was observed. From the analytical data the author proposed the following formulae:-

Homofermenter: $C_6H_{12}O_6 + 2CH_3.CO\ COOH \rightleftharpoons 2\ CH_3\ CHOH\ COOH + 2CO_2 + CH_3\ CHOH\ CO\ CH_3$.

Heterofermenter: $C_6H_{12}O_6 + 2CH_3\ CO\ COOH + H_2O = 2\ CH_3\ CHOH\ COOH + 2CO_2 + C_2H_5OH + CH_3\ COOH$.

It is very interesting to note that the main products; lactic acid and CO_2 coincide with the two types of bacteria, thus the fundamental difference between them disappears. Therefore Kitahara concluded that the key to differentiate homofermentation and heterofermentation may not be carboxylase.

In 1938 Kakuo Kitahara and Hideo Katagiri (69) observed the presence of fumarase in L. plantarum. They later on carried out experiments with dried cells of various lactic acid bacteria, in order to ascertain the occurrence of fumarase in these cells. They (69) verified the presence of fumarase in L. plantarum, L. pentoaceticus, L. brevis and L. casei, while fumarase was not detected in the cells of L. sake, Str. lactis and Leuc. mesenteroides. The occurrence of fumarase is of great interest because it does not coincide with the known natures of the lactic bacteria, particularly the optical properties of lactic acid being produced or the occurrence of racemiase. Some authors also observed that only L-component of malic acid was dehydrated by the fumarase of these bacterial cells when DL-acid was chosen for the substrate.

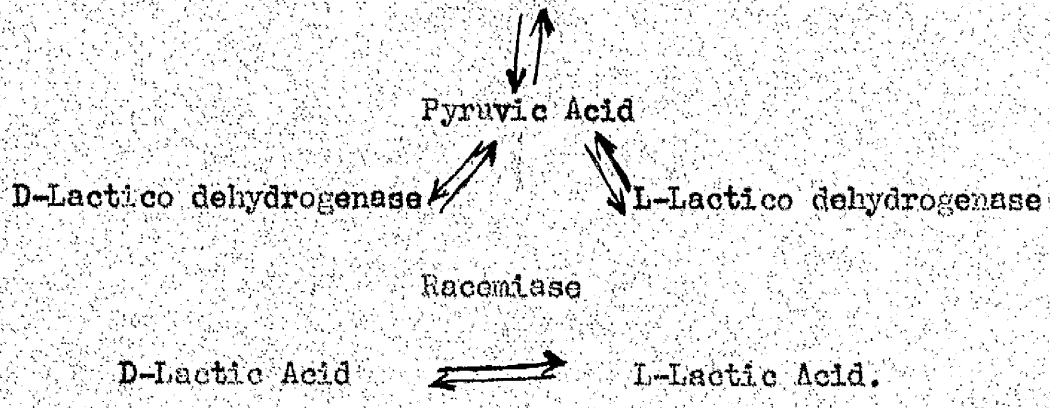
Kakuo Kitahara (70) also observed that DL-lactic acid was always dehydrogenated by all kinds of lactic acid bacteria under investigation. However, bacterial cells were found to show complete optical specificities in oxidising optically active lactic acids, as illustrated

below:-

Species.	Dehydrogenation of		
	D-Acid.	L-Acid.	DL-Acid.
L. sake (D-former).	+	-	+
Leuconostoc (L-former).	-	+	+
L. plantarum (DL-former).	+	+	+

The author, therefore, proposed that the so-called lactic dehydrogenase could be divided into D- and L-enzymes, the reason why L. plantarum attacked both D- and L- acids was attributed to the presence of racemiase. He also concluded that the lactic acid bacteria which produces D- lactic acid, possesses lactico dehydrogenase which can dehydrogenate only D-lactic acid and vice versa.

In 1949 Kakuo Kitahara (71) published a paper in which, on the basis of these results, he showed that because of the reversibility of the action of dehydrogenase, the above-mentioned characteristics of lactic acid bacteria are caused by D- and L- lactico dehydrogenases and recemiase. He thus modified the last stage of Meyerhof's scheme, as follows:-



Davis and collaborators (72) in an investigation of the metabolism of both "resting" and growing cells of lactic acid bacteria found that only faecal streptococci (Str. faecalis and Str. liquefacians) produce considerable quantities of diacetyl. No other lactic bacterium studied in this way produced diacetyl. But according to Michaelian and Hammer (73) butter cultures of the desired type always produce diacetyl and acetyl methylcarbinol in relatively large amounts. The latter substance is oxidised to diacetyl in butter cultures by citric acid-fermenting organisms such as Streptococcus citrovorus and Streptococcus paracitrovorus, while Ritter (74) has also claimed that heterofermentative lactobacilli can produce the aroma substances.

The optical properties of the lactic acid produced by bacteria has also engaged the attention of many workers. In the early investigations fermentation lactic acid was generally assumed to be inactive (75). Reports also appeared containing descriptions of lactic acid bacteria producing either dextro-acid only (76,77) or laevo-acid only (78,79), while Bertrand and Weisswiler (57) and Harden (80) stated that the lactic bacteria produce a non-compensated mixture of dextro-and laevo-acids, and that the proportion of the two acids depended in many cases on the conditions of the growth of the organisms. Mackenzie (81) brought some order into the general confusion about this problem. He showed that the reason for the divergent views could be ascribed to the methods adopted for the analysis of the acids obtained. He showed that the true nature and proportion of the lactic acid formed became entirely obscured

owing to differences in solubility of the zinc salts, that of the inactive (racemic) lactic acid being less soluble than that of the two active components. Mackenzie expressed the view that all fermentation lactic acid consists of an equal mixture of dextro- and laevo acid.

Nevertheless, the question of the optical properties of fermentation lactic acid is still obscure. Pederson, Peterson and Fred (82) also asserted that the optical nature of the acid produced by lactic acid bacteria studied by them was influenced by the presence in the culture of an infection unable to produce lactic acid.

MICRO-ORGANISMS IN GENERAL IN RELATION TO STABILITY OF FERMENTED BEVERAGES.

The micro-organisms which are responsible for the development of any harmful effect during the brewing processes or during the storage of beverages are usually called "beer disease" or beer infection organisms. The infection caused by these organisms can be divided into two main groups:-

(a) Bacterial infection, and

(b) Fungal infection,

which will be dealt with in the following two sections.

(a) BACTERIAL INFECTION.

The organisms responsible for the development of this type of infection are bacteria belonging to the order of Eubacteriales. Many bacterial species belonging to this order cannot develop in beer because of (a) its relatively low PH value, (b) the presence of hop antiseptics, (c) the ethanol content, (d) the oxygen tension, and (e) lack of some nutritional requirements or other factors which are determined during the brewing processes. However, other (beer disease) bacteria find beer either a suitable medium in which they can grow with ease, or a medium to which they can adapt themselves and so develop and survive.

All the beer disease bacteria, up to the present day, have been found to fall in the genera of five families in the latest classification due to Bergey et al. The families and genera are:-

Order of Eubacteriales.

(a)	Family Bacillaceae	genus:	Bacillus.
(b)	Family Micro-coccaceae	genera:	Micrococcus and Sarcinae.
(c)	Family Achromobacteriaceae	genera:	Achromobacter Flavobacterium.
(d)	Family Pseudomonadaceae	genera:	Pseudomonas Acetobacter.
(e)	Family Lactobacteriaceae	genera:	Streptococcus Lactobacillus.

Among the bacteria belonging to the above genera, the majority are very commonly found in beer, while some may only occasionally

occur as beer infections. For instance, it is considered that bacilli (aerobic, catalase positive, spore forming bacteria) are very rarely found in beer. Still Chapman (83) claimed to have isolated from the samples of English beers a number of bacilli. However, the work done in brewing bacteriology in recent years gives no evidence of similar cases. In fact Shimwell and Kirkpatrick (84) showed that an organism termed "Bacterium C", which was a spore-forming bacillus closely related to Bacillus cereus, could not be induced to grow in hopped or unhopped beer, or even in wort, if the PH of the latter was less than 5·2.

The true micrococci which are catalase positive can hardly develop in a medium like beer, although they may grow slowly in malt wort (85). Up to the present day no pure culture of a micrococcus species from beer has been isolated or described, but Heron (86) suggests that a micrococcus infection of beer may sometimes take place, while it is very doubtful if true Sarcinae were ever found in beer (24). From the early work of Claussen (25) and later of Mees (26), it is now clear that the so-called "beer sarcinae" are not Sarcina species but are true lactic acid producing bacteria. It is of interest to note that nearly all the bacteria belonging to these three genera are gram positive, which are according to Shimwell (87) sensitive towards hop antiseptics. Therefore this might be considered as one of the reasons why these bacteria are so rarely accounted as beer infections.

An interesting case of a brewery infection is Flavobacterium proteus. This bacterium was isolated by Shimwell and was studied and described by Shimwell and Grimes (88). The outstanding characteristic

of this organism is that it is extremely pleomorphic, while it is incapable of development at the PH value 4·2. Thus it is solely considered as a wort and brewery yeast bacterium. Still, according to the discoverer, "It seems possible that an excessive infection of this organism might have some influence on the character of the resulting beers." The aroma produced is of a fruity type, somewhat reminiscent of parsnips.

Another important beer disease bacteria which also belong to the same family Achromobacteriaceae as the Flabobacterium proteus is Achromobacter anaerobium. This organism was isolated by Shimwell (89) from a sample of densely turbid beer which possessed an unpleasant odour. Comrie (90) has confirmed the occurrence of this organism in English beer. It is characterised as a rod-shaped, gram negative, anaerobic, motile bacterium. Since it is not sensitive towards hop antiseptics and other growth-preventing factors of beer, it develops there, producing dense turbidity and an unpleasant odour and flavour. It is therefore considered a dangerous beer infection. However, it is fortunate for the brewer that A. anaerobium cannot utilize maltose or sucrose, but requires glucose or fructose as substrates.

One of the most important groups of beer disease bacteria is the group whose members are listed as "Acetic acid bacteria". All these organisms belong to the genus Acetobacter which has been lately placed in the family Pseudomondaceae. Acetobacter species are characterised as non-sporeforming, aerobic, gram negative bacteria, which occur as short rods, ellipsoidal or coccoid forms, with the exception of A. peroxydans they are all catalase positive (91,92).

The acetic acid bacteria are capable of growing in acid media and characteristically possess the power to attack ethyl alcohol, they may all be regarded as potentially capable of growth in beer under aerobic conditions. These species can be successfully grown on media made from brewing materials such as wort, beer or yeast. Most Acetobacter, therefore, can probably be regarded as beer-spoilage organisms. All the acetic acid bacteria are gram negative, so the hop antiseptics present in beer are no deterrent to their growth (24). From these facts it is evident how troublesome Acetobacter species can be in the brewing industry.

Broadly speaking, beer can suffer from two main types of spoilage by acetic acid bacteria, first, the development of haze, acidity or off-flavour: and second, the production of ropiness (93).

Henneberg (94,21,28) as early as 1897-1907 isolated and studied a number of acetic acid producing bacteria. His name is closely associated with Bacterium acetosum, B. acetigenum, B. ascendens, B. industrium and B. oxydans.

In this country, in 1912, Baker, Day and Hulton (95) studied the cause of ropiness in beer. The following year Day and Baker (96) described Bacterium viscosum which was found to be the cause of ropiness in beer.

The Dutch microbiologist, Beijerinck (97), isolated and described Bacterium rancens and Acetobacter melanogenum. Later working at Delft, Kluyver and de Leeuw (93,98) discovered an organism which brought about incomplete oxidation of many widely divergent organic compounds, and called it A. suboxydans. Visser't Hooft (91,99) also discovered a catalase negative acetic acid producing bacteria

and named it A. peroxydans because it formed hydrogen peroxide.

Most recently, in 1936, Shimwell isolated and described (100) an acetic acid producing bacterium which causes ropiness in beer and wort and named it A. capsulatum because it is proved to be an encapsulated strain. So also a new organism, A. turbidans, has been isolated and described by Cosbie, Tosic and Walker (101).

Some species of another genus Pseudomonas (Pseudomonas myxogenes and Pseudomonas cerevisiae) have been isolated from beer by Fuhrmann (102,103), but not much information is available in this direction, so at this stage it cannot be ascertained whether this genus is in reality responsible for the beer disease or not.

Finally, the last group of beer disease bacteria belongs to the family Lactobacteriaceae. The beer disease bacteria of this family comprise of two genera Lactobacillus and Streptococcus. The organisms of these genera and their activities were described in detail in the previous chapters.

(b) FUNGAL INFECTION.

The micro-organisms responsible for this type of infection are yeasts. They can be divided in two main groups:-

(a) Sporogenous yeasts, which are classified in genera which form the branches of the family Endomycetaceae (Saccharomycetaceae), and

(b) Asporogenous yeasts, which are represented by the three families Rhodotorulaceae, Torulopsidaceae, and Nectaromycetaceae.

The beer disease organisms which belong to Sporogenous yeasts have so far been found to belong to the single genus Saccharomyces. It seems that they have been studied for the first time by Hansen (104) when the name "wild yeast" was adopted by him in order to denote a yeast which caused the development of unusual conditions in beer, generally of an undesirable nature. From beer Hansen isolated and described three of these organisms. Saccharomyces ellipsoidous II was the name given to an organism which he regarded as a very dangerous beer disease yeast, causing turbidity in "bottom fermentation" beers, while S. pastorianus III was the designation given for an organism obtained from "bottom fermentation" Copenhagen beer, in which it gave rise to "yeast turbidity". The third organism which is a dangerous beer disease yeast occurring in "bottom fermentation" breweries was named as S. pastorianus I. The last named yeast causes a disagreeable smell and a strong bitter taste in beer, and also prevents proper clarification.

The literature concerning these and similar organisms has been very limited since Hansen's time. A "wild yeast" which in some respects is similar to Hansen's S. ellipsoidous II, was isolated by Will, who named it S. willianus. This organism propagates and ferments very strongly, giving at the beginning a sweet taste to beer which is then followed by a bitter taste and odour resembling to that of decomposing fruit. In 1929, Baker and Ward (105) isolated a "top fermentation wild yeast" which ferments sugar solution very rapidly and also produces haze in primed beverages. They named this organism Saccharomyces festinans due to the speed with which fermentation was carried out by this yeast. Two other

species were isolated which are dangerous "beer disease" yeasts - they were named S. validus and S. trubidans. But they are now considered to be the varieties of S. pastorianus III and S. ellipsoedous II respectively by Stelling Dekker (106). In the Stelling Dekker's new classification of sporogenous yeasts, S. ellipsoedous II, S. pastorianus I, S. pastorianus III, and S. festinans are renamed as S. cerevisiae var ellipsoedous, S. pastorianus, S. validus and S. cerevisiae var festinans respectively.

Asporogenous yeasts, the group of non-spore forming yeasts, are classified in the various genera of the three families: Nectaromycetaceae, Torulopsoidaceae and Rhodotorulaceae. All the known beer disease asporogenous yeasts fall into the sub-group Torulopsoideae of the family Torulopsoidaceae. Most frequently encountered asporogenous yeasts in the brewing industry fall into the following genera:-

(a) Kloeckra,

(b) Mycoderma, and

(c) Brettanomyces.

The lemon-shaped yeasts with bipolar germination, often known as apiculate yeasts, are frequently the cause of an objectionable flavour in beer. Their chief characteristic is the ability to ferment glucose but not maltose. In earlier classifications they used to be associated with the "Torula". However, the non-spore forming species now form the genus Kloeckera (107).

Another group of organisms which form a branch of asporogenous yeasts are Mycoderma species. The cells of these species are ellipsoid or lemon shaped, often arranged in branched chains. Some of these organisms under certain conditions prove troublesome to the

brewers. They are strongly aerobic, and produce a dull greyish pellicle on malt wort, wine, and beers. Some species of Mycoderma can bring about oxydation of alcohol to acetic acid and apart from the damage thus done to flavour they cause a slight turbidity. It is very probable that the species most commonly encountered in the brewing industry is Mycoderma cerevisiae.

The species of the genus Brettanomyces, which were isolated by Claussen (108) from bottled English beers, have been found to form haze which is often accompanied by an unpleasant smell. The literature regarding these organisms is not enough, but according to Custer (109) they constitute a genus of their own and cannot be considered as species of "Torula" or Torulopsis as classified by Hansen (110).

Among the early work of Hansen (110) there is to be found a group of organisms classified as "Torula". By "Torula" he understood:- yeast cells which are similar to Saccharomyces but do not form endospores. Apparently the name Torula embraced some of the organisms which are now classified as species of asporogenous yeasts.

Lastly it is worth while to mention that another class of organisms known as moulds, which are different from yeasts or bacteria, can also be a source of damage to the brewing industry to some extent. Moulds can appear on damp walls, unclean vessels, in pipes, etc., but their growth is much restricted both by mechanical and disinfectant cleaning. They are not particularly dangerous organisms as they are suppressed by the yeast in the fermentation vat and lager cask. Beer may, however, sometimes acquire an unpleasant taste, which can be ascribed to moulds. But indirectly moulds can be very harmful, because they

may harbour dangerous bacteria in their hyphae.

SECTION II.

THE DEVELOPMENT OF THE STUDY OF THE NUTRITION OF MICRO-ORGANISMS.

The study of the nutritional requirements of bacteria began with the demonstration of Pasteur (111) that bacteria were responsible for the souring of wines. Organisms responsible for various human diseases, such as the gonococcus and Johne's bacillus, were isolated by many workers, and the need then arose for media in which these could be cultivated.

In early work crude media were used to cultivate micro-organisms. For example, Kayser (112) used peptonized milk, peptones, gelatin, yeast or other protein supplements, while Henneberg (113) later used glucose, peptone, salts and infusion of yeast and prunes for cultivation of various lactic acid bacteria.

However, as early as 1860, Pasteur (114) elaborated a purely synthetic media for growth of yeast. This was apparently contradicted by Wildier's (115) in 1901, who showed that in addition to sucrose and salts yeast require an unknown factor called "bios" for growth. The

explanation that Pasteur's medium was contaminated seems to explain other early claims, to have cultured the tubercle bacillus on a chemically defined media (116), as these claims were also later contradicted.

Some success was, however, obtained and Uschinsky (117) in 1893 employed the following synthetic medium for the growth of typhoid, cholera, and certain other bacteria - ammonium tartarate or lactate, glycerol, NaCl, $CaCl_2$, $MgSO_4$, K_2HPO_4 or Na_2HPO_4 and sodium aspartate.

During the next fifty years many organisms such as E. coli (118) and Str. faecalis (119) were cultivated on media differing little from that of Uschinsky. Many organisms were found, however, for which such a medium was deficient and it was in the attempts to remedy such deficiencies that the next big strides were taken. Two lines of research were adopted: (a) extracts of cells were added to the deficient media, as by Twort and Ingram (120), who showed that Johne's bacillus (Mycobact.paratuber-culosis) would not grow on a medium supporting growth of other acid-fast bacteria, but would grow if extracts of acid-fast bacilli (e.g. Mycobact.phlei) were included in the medium; (b) the attack on the problem of bacterial nutrition through the analysis of complex media, particularly with the work of Mueller (121), who sought to fractionate complex media for growing haemolytic streptococci. Mueller did not succeed in his main object but he did discover a new amino acid, methionine. In this way the X and V factors for H. influenzae were first studied by Davis (122) in 1917.

Five years earlier, Hopkins (123) already demonstrated the existence of vitamins as the missing factors in diets which were

deficient for animals, and a year later in 1913 Osborne and Mendel (124) noted the presence in milk of a growth factor for young rats. From this time attempts were made to utilise impure animal vitamin preparations as stimulants for bacterial growth, with very uncertain results. It was also shown by animal feeding experiments that certain bacteria could synthesize B vitamins (125).

The vitamins were not isolated in a pure state for many years and there was no clear link between the nutrition of micro-organisms and higher animals until Schopfer (126) showed that thiamine (B_1) was essential for the growth of Phycomyces blakesleeanus. Later Tatum, Wood and Peterson (127) showed that it had a stimulatory effect on certain strains of lactic acid and propionic acid bacteria. Since then the number of known growth factors has rapidly increased, and the underlying unity of biochemistry has been demonstrated very clearly in the field of nutrition. The realisation of this unity and the meeting of the two lines of research - with animals and micro-organisms - acted as a catalyst in the further investigations on nutrition. The following table gives some idea of the inter-relations in the growth requirements of different classes of organisms:-

	Substance.	First recognised as essential for	Later, implicated in nutrition of
A.	Thiamine (B_1).	Higher animals.	Bacteria, fungi, protozoa, plant roots.
	Riboflavin (B_2).	"	"
	Pyridoxin (B_6).	"	"
B.	Nicotinic acid.	Staph. aureus.	Higher animals, plants and roots.
	Pyridine nucleotides.	H. influenzae.	"
	Pimelic acid.	C. diphtheriae.	"
	Inositol.	Yeasts.	Mouse, moulds, etc.
	β-Alanine.	"	C. diphtheriae.

	Substance.	First recognised as essential for	Later, implicated in nutrition of
C.	Pantothenic acid.	Yeasts.	Bacteria, higher animals, insects.
	Biotin.	Yeasts.	" " " and fungi.
D.	p-Aminobenzoic acid.	Bacteria.	Higher animals, insects and fungi.

(Adapted from Knight (128)).

Substances in Group A were new to chemistry and first discovered via animal Physiology.
" " " B were known to chemistry and first assigned roles in bacterial Physiology.
" " " C were new to chemistry and first discovered in yeast.
" " " D was discovered as antagonist of artificial inhibitor.

The first fundamental studies on the nutrition of the lactic acid bacteria were initiated by Orla-Jensen (129) in 1898. In his book "Lactic acid bacteria" he stated:-

> "The true lactic acid bacteria demand just as complicated nitrogenous food as the animals, viz:- genuine proteins or the entire complex of amino acids contained therein. Even incomplete proteins, such as gelatin (without the addition of other nitrogenous nourishment) generally prove, as in the case of animals, an extremely bad nitrogenous food."

Although this is perhaps not quite true, as lactic acid bacteria are known requiring very few amino acids, nevetheless in comparison with other bacteria, the lactic acid bacteria are very fastidious in their requirements.

In 1936 Orla-Jensen et al (130) showed the presence in milk of unknown factors necessary for growth of lactobacilli such as

L. bulgaricus, L. casei, L. helveticus and L. lactis, and that riboflavin and pantothenic acid were essential. Following this Snell, Strong and Peterson (131) studied the nutrition of lactic bacteria on a medium containing glucose, peptone, whey extract, salts, cystine, tryptophan and riboflavin. Snell and Strong (132) soon took advantage of the fact that L. casei required riboflavin for growth and forged a new tool for microbiology - the method of microbial assay of vitamins - later extended to amino acids.

Snell et al (133,134) adapted their assay procedure for determination of pantothenic and nicotinic acids, using basal media containing pyridoxin and biotin, as these had been shown to be essential for certain lactic acid bacteria by Möller (135). By 1946 Roberts and and Snell (136) were able to describe method for the assay of biotin, folic acid, nicotinic acid, pantothenic acid and riboflavin with one organism, L. casei.

Although, following the work on animal vitamins, the vitamin requirements of bacteria were first investigated, it was a natural development to examine their amino acid requirements.

Since the pioneer work of Koser and Rettger (137) in 1919, basal media containing up to 19 amino acids have been employed in nutritional studies of numerous types of bacteria, including Streptococcus (138), Escherichia (139) and other genera. Orla-Jensen (129) cultivated L. casei and other lactobacilli on a synthetic medium containing seventeen amino acids, plus a supplement of whey to provide growth factors. The following year Wood, Anderson and Werkman (140) grew six lactic acid bacteria on a medium containing 17 amino acids, glucose, sodium acetate, inorganic salts, thiamine, riboflavin and yeast

extract - a nearly synthetic medium.

The first procedure for the assay of amino-acids was described by Kuiken, Norman, Layman and Hale (141). They used L. arabinosus on a purely synthetic medium for the assay of isoleucine, leucine and valine.

The results obtained by various authors for the amino acid requirements of individual lactic acid bacteria show close agreement. However, there are some differences, whether certain amino acids are essential or stimulatory, and others stimulatory or non-essential. The reasons for such differences are mainly (a) contamination of the medium used - Hegsted and Wardwell (142) showed DL-leucine samples to contain 1-20% isoleucine; (b) differences in the responses of the organisms on different media - Shankman (143) showed phenyl-alanine and tyrosine to be essential for L. arabinosus on one basal medium, but only stimulatory on another; (c) variation in growth requirements of the organisms - a decrease in response to an added growth factor has been found with L. arabinosus and L. casei transferred for a long time on yeast extract glucose agar (144).

ROLE OF INDIVIDUAL VITAMINS IN THE FIELD OF NUTRITION OF MICRO-ORGANISMS.

Thiamine (B_1).

Schopfer (126) first showed that thiamine was required for growth by a micro-organism, namely Phycomyces blakesleeanus. Thiamine was first implicated in bacterial growth by Tatum, Wood and Peterson (127), who observed that it had a marked growth-promoting effect on certain strains of propionic acid bacteria when added to a deficient basal medium. Later in 1939 Möller (145) showed thiamine to be stimulatory for certain lactic acid bacteria. It was first shown to be essential for some lactic acid bacteria by Niven and Smiley (146). They showed that Strep. salivarius required this growth factor and that co-carboxylase (thiamine pyrophosphate) was 40% more active than thiamine. Lankford and Skaggs (147) showed that thiamine was not utilised, but co-carboxylase was essential for gonococcus. Niven later showed that Str. lactis also required thiamine.

Thiamine itself was synthesized by Williams, Clarke et al (148), and was shown to be pyrimidine-thiazole compound. It is very interesting to test the synthesizing power of bacteria by supplying them with the separate portions to see if they can provide the other portion, or link the two together if provided simultaneously. Knight (149) showed that Staph. aureus could link the two parts to give the complete vitamin, but organisms very in their synthesizing ability. Thus, Sarett and Cheldelin (15) found that L. fermenti had to be supplied with preformed thiazole.

The finding by Woods (151) that sulphonamide acted by competitive inhibition of a system involving its structural analogue PAB, stimulated attempts to find other bactericides which were analogues of growth factors. Woolley and White (152) investigated the inhibitory effect of pyrithiamine (thiazole portion is replaced by pyri-dine ring in thiamine). They showed that bacteria which required preformed thiamine were inhibited, while those which synthesized the vitamin were not. *Look up question of neopyrithiamine in Ann. Revs. of Biochemistry for 1951.*

Riboflavin.

Riboflavin was first shown to be essential for bacteria by Orla-Jensen et al (153), working with lactic acid bacteria. They observed that the bacteria would not grow on milk treated with activated carbon, but that addition of pantothenic acid and riboflavin to the treated milk resulted in supporting growth again. Three years later Snell and Strong (132) studied the requirements of eleven species of lactic acid bacteria, seven species were found to synthesize riboflavin but the remaining four required the preformed vitamin. Schütz and Theorell (154) confirmed that riboflavin was present in the cells of lactic acid bacteria, whether they require this vitamin or not. Earlier Schopfer (155) had shown Staph. aureus and Staph. flavus to synthesize riboflavin.

It is evident that various bacteria differ in their riboflavin requirements, some need it in preformed state, others can synthesize it and others being merely stimulated by it. This is of course the general picture with other vitamins too. A working hypothesis is that an organism's need for a particular factor depends on its rate of synthesis of that factor. If rapid, the factor will not be

essential; if slow, stimulatory; if so slow as to be negligible, the factor will be essential.

L. casei has been used for assay of riboflavin in natural materials by Landy and Dicken (156) measuring response by the acid produced, and by Roberts and Snell (136) using a turbidimetric method.

Vitamin B_6.

Vitamin B_6, first known as pyridoxin, was first differentiated as a member of the B- complex in experiments on rat nutrition by György (157) in 1935. Recognition of its importance in bacterial nutrition followed in 1938, with the demonstration by Möller (135) that pyridoxin was stimulatory for certain lactic acid bacteria. Later work showed that lactic acid bacteria varied in their requirements for preformed pyridoxin. The vitamin was found to be essential for L. delbrückii 3, L. lactis B1-1, but was synthesized rapidly by L. arabinosus 17-5 (158).

In 1942 Snell, Guirard and Williams (159) found evidence for a derivative of pyridoxin which was much more active for Str. lactis R. than pyridoxin itself, and later Snell (160) showed that this derivative was formed under certain conditions of autoclaving media containing pyridoxin. Bohonos, Hutchings and Peterson (161) using L. casei showed that pyridoxin and oxygen was more effective than pyridoxin itself. An explanation for this was suggested by Snell (162) who indicated that pyridoxin could be transferred to pyridoxal and pyridoxamine. The effect of oxygen in the experiment of Bohonos et al was to change pyridoxin into pyridoxal, which was more

biologically active.

Snell (163) showed the presence of the two components in natural materials, while Snell and Rannefeld (164) tested their activity on the growth of various organisms - bacteria, yeasts, moulds and rats. For the last three types, pyridoxin, pyridoxal and pyridoxamine were of equal activity; for bacteria the transformation compounds were a few fold to several thousand fold more active than pyridoxin itself.

Among lactic acid bacteria the requirements for the pyridoxin derivatives varies. Thus L. casei requires pyridoxal, pyridoxamine having no effect, but Str. faecalis and Str. lactis require both pyridoxal and pyridoxamine (164). Assay using L. casei have been described by Robinowitz, Mondy and Snell (165).

Up to the present no lactic acid organism had been found to respond solely to pyridoxamine. However, writer's strain L. plantarum G_2 has shown this type of response and should therefore provide a very useful additional tool for the assay of B_6 components.

The picture became slightly more complicated, yet at the same time more in line with other vitamins when McNutt and Snell (166) while investigating an unknown factor required for a strain of L. helveticus and one of L. acidophilus, found that pyridoxal phosphate and pyridoxamine phosphate were highly active, the latter being 6-10 times more active than the former. But the unphosphorylated compounds were quite inactive, so it is evident that with these two organisms a breakdown in the mechanism for phosphorylation has occurred. As has been pointed out earlier, this is similar to the requirement of the gonococcus for preformed thiamine pyrophosphate.

Although Bellamy Umbreit and Gunsalus (167) have shown that the

only structural form of B_6 is pyridoxal phosphate, to which the others are converted. The activity of this substance varies for different organisms; this probably is due to differences in the permeability of the cell wall to phosphates.

Pantothenic acid.

In 1939 Snell, Strong and Peterson (131) described a procedure for obtaining from an alcohol soluble liver extract an acidic, ether-extractable substance, which was essential for the growth of a number of lactic acid bacteria. The substance was thermo-labile as well as labile to both acids and alkalies. It could not be replaced by any known growth factor. They (168) later showed that the substance was pantothenic acid and a large number of lactic acid bacteria investigated required it for growth.

This was the first occasion that pantothenic acid was shown to be of nutritional importance in either animals or bacteria, although Orla-Jensen (153) had earlier suggested that pantothenic acid was one of the factors removed from milk by treatment with active charcoal.

Further work confirmed that pantothenic acid was of universal importance in lactic acid bacteria. Cheldelin, Hoag and Sarett (169) showed that the 33 species and strains of lactic acid bacteria required this vitamin, and Shankman, Camien, Block, Merrifield, and Dunn (170) showed the same with a further 23 lactic acid organisms.

β-Alanine had been reported as a yeast nutrilite by Williams and Rohrman (171) and Williams later suggested that pantothenic acid was a conjugate of β-alanine and a hydroxy acid. Woolley and Hutchings (172) found that an alkali labile factor essential for certain

streptococci would be replaced by pantothenic acid concentrates and that the alkali split substance could be recombined by methods supporting Williams' suggestion. Further work finally resulted in the synthesis of pantothenic acid (173) which was shown to be β-alanine linked to α-OH, $\beta\beta$-(Me.)$_2$ -γ- butyro lactone, or pantoic acid.

This is similar to thiamine, which is thiazole + pyrimidine, and thus attention soon becomes similarly focussed into the problem of the growth activity of the separate portions. Numerous strains of C. diphtheriae have been shown to require only β-alanine so as to synthesize complete pantothenic acid (174), and two cases have been recorded where only pantoic acid is required. These are A. suboxydans (175) and Str. haemolyticus (176). In the latter case pantothenic acid produced a quicker response, supporting the idea that it is pantothenic acid which is ultimately required.

A bound form of pantothenic acid was found in extracts of natural materials by Sarett and Cheldelin (177). β -Alanine was released by acid hydrolysis and it supported growth of A. suboxydans but not of L. arabinosus. The activity was greater than that accounted for by its pantothenic acid content. These properties agree with those of co-enzyme-A, shown by Novelli, Kaplan and Lipmann (178) to account for the bound pantothenic acid of animal tissues and many bacteria, although Cheldelin's product showed no co-acetylase activity. This was explained by Novelli, Flynn and Lipmann (179), who showed that treatment of their concentrate with an enzyme of liver extract abolished co-enzyme-A activity but not its growth supporting property for A. suboxydans. Further treatment with a phosphatase gave a substance active for both A.suboxydans and L. arabinosus. Although

pantothenic acid absorbed by L. arabinosus becomes co-enzyme-A, co-enzyme-A (containing Po_4) is itself inactive. This is similar to thiamine and thiamine pyrophosphate, pyridoxal and pyridoxal phosphate.

Biotin.

Many lines of research crossed with the demonstration that biotin was essential for certain lactic acid bacteria (145). Boas (180) in 1927 observed that rats fed on a diet containing egg-white as source of protein developed a characteristic "egg-white injury". It was found that the rats could be protected from this by being fed a "protective factor" present in potato starch. The investigation into the chemical nature of this factor, and realisation that it represented a nutritional value was due to the researches of György, who named it vitamin H.

In 1935 Kögl and Tonnis (181) isolated biotin from a variety of natural materials and showed that it was stimulatory for a yeast strain. About the same time Allison, Hoover and Burk (182) working with Rhizobia species, showed the necessity for an unknown substance which they called "Co-enzyme R". This was present in natural materials and was synthesized by Azotobacter. Comparison of co-enzyme-R with biotin showed that they were probably identical, West and Wilson (183) proving that biotin could satisfy co-enzyme-R requirements of Rhizobia.

During chemical work on vitamin H, it was suspected that it was akin to biotin. A comparison of the properties of vitamin H, biotin and co-enzyme-R showed them to be identical. Proof of their identity was followed by synthesis of biotin.

Biotin is now known to be stimulatory or essential for a great variety of bacteria, but many bacteria synthesize it and show no stimulation if biotin is added, e.g. Serratia marcescens, P. vulgaris, Aerobacter aerogenes (184).

Pimelic acid, which enters into the structure of biotin was shown by Mueller (185) to be essential for C. diphtheriae strains; however, pimelic acid was found inactive for biotin-requiring L. casei by Wright (186).

In 1944 Wright and Skeggs (187) showed evidence for the existence of a form of biotin available to L. casei but not for L. arabinosus. Bowden and Peterson (188) termed this form soluble-bound biotin, as it differed from biotin in solubility. On acid-hydrolysis, biotin is released. The conjugate is now called biocytin and has been extracted from yeast - a few mg's from several tons. Biocytin was shown to be available as a source of biotin to L. delbrückii and and L. acidophilus in addition to L. casei; but it was not available for L. pentosus, L. mesenteroides P-60 as well as L. arabinosus.

Nicotinic acid and Nicotinamide.

Nicotinic acid has been known to organic chemists since 1857, as an oxidation product of Nicotine. It was isolated from natural materials by Drummond and Funk (189) in 1914, during attempts to isolate "vitamin B". However, it was not implicated in nutrition until Lwoff and Lwoff (190) showed co-enzyme I and co-enzyme II to be identical with the "V factor" of Thjötta and Avery (191), which had been shown to be essential for growth of H. influenzae. Soon afterwards, nicotinic acid itself was demonstrated as an essential growth

factor for Staph. aureus and C. diphtheriae.

About the same time, Elvehjem, Madden, Strong and Woolley (192) working on human and canine pellagra, found that nicotinamide would protect against these diseases. It may be pointed out that it is a typical deficiency disease and it had earlier been shown that it could be cured by the administration of foods, now known to contain this vitamin. Thus nicotinic acid is another factor first shown to be important in bacterial nutrition and later in animal nutrition. Snell, Strong and Peterson (168) first demonstrated its requirement among lactic acid bacteria.

Many species of bacteria have since been shown to require nicotinic acid (or amide). Thus Str. salivarius and Leuc. mesenteroides require preformed nicotinic acid. Pasteurella species vary, some need nicotinic acid, others need nicotinamide. Lwoff and Lwoff (193) pointed out that H. parainfluenzae could not use nicotinic acid, nicotinamide or AMP, but only co-enzyme I. Schlenk and Gringrich (194) extended this by proving nicotinamide nucleoside as an active but not nicotinamide + d-ribose + Adenosine mono phosphate (AMP).

Thus the general picture of nicotinic acid utilisation is:-

nicotinic acid $\xrightarrow{\text{ii)}}$ nicotinamide $\xrightarrow{\text{iii)}}$ nicotinamide nucleoside $\xrightarrow{\text{iv)}}$ co-enzyme I.

Proteus effects i) but not ii), iii) and iv).

Pasteurella effects ii) but not iii) and iv).

Haemophilus effects iii) but not iv).

However, Johnson (195) using L. mesenteriodes for assay purposes, found that although nicotinic acid was utilised, nicotinamide was not. Koser and Kasai (196,197) investigated this problem with a number of

strains of Leuc. mesenteroides. They found that some grew well on normal amount of nicotinic acid, but on nicotinamide only when large concentrations were used. However, co-enzyme I was used readily. This contrasted with L. dextranicum strains, which utilised only nicotinic acid, and neither nicotinamide nor co-enzyme I even when high concentrations were used. Further investigations with fifteen Leuconostoc species and strains showed that 7 failed to respond to nicotinamide, of this four did not respond to co-enzyme I either. Addition of reagents such as "Tweens", sodium ricinoleate and sodium lauryl sulphate had no effect on non-utilisation of nicotinamide, thus ruling out possible membrane effects.

In all cases, the cells produced a substance active for H. influenzae which may be co-enzyme I, hydrolysis of which produced substance active for Pasteurella, which may be nicotinamide.

From this work, it is apparent that the scheme for nicotinic acid utilisation will have to be modified in some way.

p-Amino benzoic acid and Folic acid.

PAB was the first growth factor to be discovered indirectly as an antagonist of a bacterial growth inhibitor (sulphanilamide). McIntosh and Whitby (193) suggested that sulphanilamide acted by interfering with some enzymic activity within the bacterial cells, attempts were made to reverse this inhibition by the addition of various substances similar in structure to sulphanilamide, but without any success. Woods (151) succeeded with a yeast extract, Green also reversed this inhibition with an extract of Br. abortus. Woods showed that PAB had very high activity against this drug. Finally Blanchard

(199) isolated PAB from yeast.

Woods (151) on the basis that PAB was an essential metabolite in the organisms whose growth was inhibited by sulphanilamide, predicted that it would be found to be an essential growth factor for some organisms. This was verified for the first time with a lactic acid organism L. arabinosus 17-5 (200) and later for A. suboxydans. Lewis (201) elaborated a method of assay of PAB using L. arabinosus 17-5. PAB has been found to be essential for some 14 organisms, a small number compared with those for other growth factors. However, the factor has been isolated from organisms shown to synthesize it (201).

In 1940 Snell and Peterson (158) isolated from liver and yeast a factor which was essential for L. casei. This "norite eluate factor" as it was called was later known to be also essential for other lactic acid bacteria. Later another factor essential for Str. lactis R. was isolated by Keresztesy et al (202) which was inactive for L. casei but which Stokes et al (203) showed was converted to an active form by Str. lactis R. Mitchell, Snell and Williams (204) isolated a factor from spinach which was active for Str. lactis R. and called it "folic acid".

Chemical work on the nature of these factors led to the synthesis of pteroylglutamic acid (PGA) which proved to be active for L. casei but not for Str. faecalis R. The factor for the latter organism was shown to be pteroic acid (PA). Later work has shown that L. casei factor is actually pteroyl-triglutamic acid (PGTA). Pteroic acid is a pterin residue linked to p-amino benzoic acid, and it was natural therefore to suppose PAB might be a precursor in the synthesis of both pteroic acid and pteroyl triglutamic acid, or Folic acid. PAB would

then be required by those organisms for which it was essential for the synthesis of these newer factors, and sulphanilamide would act by preventing this synthesis. If this were so it would be expected that while PAB competitively inhibited the drug, folic acid would non-competitively inhibit it.

Lampen and Jones (205) reported that PA, PGA and PGTA did this in the case of strains of L. arabinosus. Nimmo-Smith and Woods (206) found the same with PGA for Sbm. plantarum 105. However, Lampen and Jones showed (205) that thymine and purines were also non-competitive antagonists of sulpha drugs. This activity of thymine and purines had been previously demonstrated for Cl. acetobutylicum and L. pentosus. Therefore, Lampen and Jones (205) proposed that PAB had two functions, the synthesis of folic acid and thence that of thymine and purines.

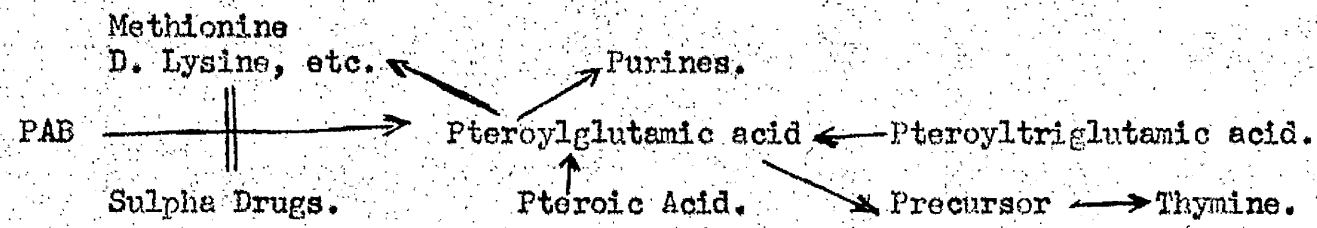
However, there is also evidence that methionine is involved. Shive and Roberts (207) found that methionine increased the anti-bacterial index - sulphanilamide. This amino acid also has anti-sulphanilamide activity for L. casei (208), though only over a limited range of drug concentration.

Other amino acids also have activity, but generally slight. Koft, Steers and Sevag (208) in a recent paper found that D-lysine showed half the activity of PAB for L. arabinosus 17-5. L-lysine was found to be inactive. Sulphanilamide inhabited D-lysine activity.

There are workers who represent a school which has challenged the general picture as presented above. They (208) claim that, not the synthesis of folic acid from PAB, but decomposition of folic acid to PAB is required for growth of L. arabinosus 17-5. This does not seem to be reconcilable with the cases of organisms requiring PGA

for growth, such as L. casei, which do not utilise PAB and is not inhibited by sulpha drugs in the presence of small amounts of PGA.

Thus, although there are many points yet to be cleared up in the story of PAB and Folic acid, the general relationship may be summarised as follows:-



Vitamin B_{12}.

Twenty-one years after Minot and Murphy (209) showed the presence in liver extracts of a factor curative for pernicious anaemia, Shorb (210) described an unknown factor present in liver extract, which was essential for the growth of L. lactis Dorner, which she called the LLD factor. Using this organism for assay Rickes et al (211) concentrated liver extracts and finally isolated a red crystalline material equivalent to 11,000 LLD units per microgram. Independently at the same time Lester-Smith and Parker (212), using chemical methods as a guide, isolated a similar crystalline material, which was highly active against pernicious anaemia. West (213) found that Rickes product was also active in this way. The factor has since been called vitamin B_{12}, and has been isolated from the fermentation liquor of Streptomyces griseus.

Vitamin B_{12} has not yet been completely chemically analysed, but has been shown to be very unusual in that it has a molecular weight of 1500, and contains one atom of cobalt per molecule (214).

In 1944 Hammond and Titus (215) found that addition of fish meal

or cow manure to diet greatly improved growth of chicks on a small vegetable ration. The "animal protein factor" responsible was later shown to be replaceable by vitamin B_{12}. The vitamin is also implicated in the nutrition of dog.

Following Shorb's (210) work, other lactic acid bacteria were discovered to respond to vitamin B_{12}. These include strains of L. lactis, L. leichmannii, L. delbrückii, and Leuc. citrovorum.

Attempts to assay liver and other natural materials for B_{12} activity using these organisms have been beset with difficulties. Often growth is found in tubes containing no (added) B_{12}. A predominant cause of this is the non-specific response of the lactic acid bacteria to the vitamin and certain other substances. Thus Shive, Ravel and Eakin (216) showed that thymidine could replace B_{12} for L. lactis. Thymidine and other desoxyribosides have been shown to be effective for L. leichmannii (217). This was also the case for most of the 18 strains of 6 species of lactic acid bacteria studied by Kitay, McNutt and Snell (218). Thus there is undoubtedly close connection between B_{12} and the desoxyribosides. It has in fact been shown by Roberts et al (219) to increase the rate of synthesis of desoxyribonucleic acid by L. leichmannii. This would seem to suggest the hypothesis that B_{12} is an essential catalyst (e.g. co-enzyme) in the formation of desoxyribosides.

Another phenomenon requiring explanation is that L. lactis required B_{12} under aerobic but not anaerobic conditions, this is also the case with L. leichmannii (217). An anti-oxidant (such as thiomalic or thioglycollic acid) is therefore included in media used for assay purposes, to ensure a greater growth of the organism in

presence of smaller amounts of B_{12}.

Inositol.

Inositol was first implicated in nutrition by Eastcott (220) for a member of the yeast growth samples. Yeasts vary greatly in their requirements, some being able to synthesize it, others being stimulated by its presence in the medium and yet others requiring it preformed, as is the case with Wildier's yeast. A method of assay of Inositol using a "typical baking yeast" has been presented by White and Munns (221).

In 1931 Buston and Pramanik (222) found that inositol was essential for the growth of the fungus Nematospora gossypii. Ten years later its absence was shown by Woolley (223) to be conducive to a alopecia in rats. It is known to be present in high concentrations in the brain and heart muscle of higher animals. Inositol is therefore of wide biological importance. Up to the present, it has not been implicated in bacterial nutrition. A number of bacteria, however, has been found to synthesize inositol in fairly large amount and Kluyver, Hof and Boezaardt (224) showed that Ps. beijerinckii Hof produced a purple pigment from inositol, when growing on a simple medium.

Choline.

Choline has long been studied in mammalian metabolism, for its lipotropic role and function in relation to the sulphur containing amino-acids in particular. It is also important in avian nutrition (225). Rane and SubbaRow (226) in 1940 found choline essential for the growth of a strain of pneumococcus. Three years later (227) a

mutant strain of Neurospora was also found to require choline. Although there is no correlation between the activity of choline for the pneumococcus and mammal, there is a correlation between activity in the Neurospora mutant and mammal. Work in this field may help determining the role of choline in animals.

Vitamin Interrelationships.

Although the bacterial vitamins have been discussed individually, this is not because they are mutually exclusive. As has been indicated several times, bacteria often show a requirement for a factor in one medium but not in another, which contains some additional or different components. Thus biotin is required by some bacteria in the absence of aspartic acid, but not in its presence; the relation between PAB, folic acid and purines has been already discussed in some detail.

The B-vitamins are concerned in many enzymic reactions. Thus thiamine pyrophosphate enter into metabolism of pyruvic acid, riboflavin and nicotinic acid into dehydrogenation mechanisms; pyridoxal phosphate is implicated in amino acid decarboxylation. It is to be expected, therefore, that the vitamins will exert influences on each other by their absence or predominance. It has in fact been shown that a massive dose of one B-vitamin lead to a disequilibrium among the others. Thus in treatment of pellagra with nicotinic acid, symptom of thiamine riboflavin deficiency developed. Conversely, Bhagvat and Devi (228) found that lack of thiamine resulted in an increase of riboflavin in the liver and concomitant increase in activity of lactic and succinic dehydrogenases.

It is well known that tryptophan and nicotinic acid are closely

linked. Beadle, Mitchell and Nye (229) have shown that tryptophan is the precursor of nicotinic acid in a mutant of Neurospora. Lepkowsky and Nielsen (230) have shown that pyridoxin-deficient rats have an abnormal tryptophan metabolism, so that pyridoxin is also linked with nicotinic acid. It is therefore very important precisely to state the condition of test under which an organism shows any particular growth requirement. Failure to do this may give in quite misleading results.

PURINES AND PYRIMIDINES AS GROWTH FACTORS.

The first purine found essential for the growth of micro-organisms was uracil. Richardson (231) growing Staph. aureus anaerobically in a medium of amino acids, glucose, pyruvic acid, thiolacetic acid and an unknown organic factor, found that uracil was essential. A trace of oxygen allowed growth in the absence of uracil, thus the organism still metabolised uracil, synthesizing it. The uracil requirement was very specific, neither adenine, guanine, xanthine nor a large number of other related compounds being at all active.

Möeller (145) in 1939 found adenine necessary for growth of L. plantarum. This, together with Richardson's (231) observation indicated that breakdown products of nucleic acids might be important in bacterial nutrition. Shortly afterwards, Snell and Mitchell (232) reported that for various lactic acid bacteria conditions could be

devised in which each of the four purines and pyrimidines - adenine, guanine, uracil and thymine - was essential for rapid growth. Growth occurred in the absence of these compounds after a long period of incubation. The requirement was not very specific and furthermore in the case of L. arabinosus PAB abolished any purine or pyrimidine requirement. This was extended by the authors (233) to show that xanthine, hypoxanthine and methionine would also replace the substances already mentioned.

This effect of PAB and methionine has already been mentioned when discussing PAB and folic acid. It does not occur with all the lactic bacteria. For example, Leuc. mesenteroides requires guanine, xanthine or hypoxanthine for growth even in the presence of PAB and folic acid (233). Snell (234) writes "Survey of a large number of bacteria of this (lactic) group would probably show great many additional examples of organisms differing slightly in the specificities of their requirements for the substances."

ROLE OF AMINO ACIDS IN THE FIELD OF NUTRITION OF MICRO-ORGANISMS.

Amino Acid requirements.

It has been mentioned previously that Uschinisky (117) was one of the first investigators to use a synthetic medium for the growth of bacteria. One of the constituents of his medium was Sodium-aspartate, which with Am. lactate or tartrate provided a source of nitrogen. Following him, Lowenstein and Pick (235) incorporated aspartic acid in a medium designed to support growth of the tuberculosis bacillus and other workers also used aspartic acid when culturing this bacillus.

Other amino acids were later used in synthetic or semi-synthetic media. Koser and Rettger (137) grew 24 organisms on a synthetic medium containing 8 amino acids in addition to glucose and salts. Gladstone (236) later employed 18 amino acids for the cultivation of B. anthracis. In 1936 Orla-Jensen et al (130) found that L. casei, L. helveticus and many other lactobacilli could be cultivated on a synthetic medium containing 17 amino acids provided a supplement of whey were added, equivalent to one quarter of the nitrogen-content of the medium. Wood, Anderson and Werkman (140) similarly grew 6 lactic acid bacteria on a medium of glucose, salts and 17 amino acids and yeast extract.

Orla-Jensen (130) found that for several of his organisms the following amino acids were essential for growth:- arginine, aspartic acid, glutamic acid, histidine, lysine and phenyl-alanine.

But the detailed studies of amino acid nutrition had to wait until pure vitamins were available. By 1942 this was so, and many

investigators entered the field, largely prompted by the possibility of using bacteria to assay amino acids as they were used for vitamins. The lactic acid bacteria in particular were examined; in view of their known complex vitamin requirements, it was thought quite likely to be equally exacting in their amino acid requirements. Shankman (143) found that L. arabinosus required 8 amino acids, while Hutchings and Peterson determined the 10 amino acids essential for the growth of L. casei. The organisms were found to vary greatly in their requirements, and the acids varied just as greatly in their indispensability. The most fastidious lactic acid organism known so far is Leuc. mesenteroides p-60, which requires no less than 18 amino acids for growth (237). The amino acids which were not essential were hydroxy-proline, nor-valine and nor-leucine. All but one of the 18 essential amino acids have been assayed in natural materials by various workers (238).

At the other end of the scale is Leuc. mesenteroides 8293 which requires only glutamic acid and valine plus glucose, salts and vitamins. This is an interesting illustration of its difference in nutritional requirements between different strains of the same species. It is unlikely, therefore, that determination of their requirements will be of any great help in the classification of the lactic acid bacteria. However, Dunn et al (239) on the basis of nutritional requirements, decided that L. casei and L. delbrückii were one and the same organism.

Specificity towards dextro- or laevo-amino acids.

Amino acids found as components of (bacterial) proteins are normally the L-isomers. It was always assumed that bacteria could utilise only the L-isomers and the work described was done either with the L-amino acids or the DL-racemic mixture. When an amino acid has

been said to be essential, it is the L-form which has been considered.

However, it has been known for some time that some lactic acid bacteria can use the D-isomer of a variety of amino acids, particularly alanine, glutamic acid (240), lysine and methionine (241). Mac, Snell and Williams (242) while investigating the relationship between glutamic acid and glutamine as nutrients for L. arabinosus 17-5 found that D-glutamic acid permitted growth of this organism when supplied in high concentrations. Camien and Dunn (240) confirmed this and showed that aspartic acid and to some extent asparagine, inhibited the activity of the D-acid. These authors (241) also examined the utilisation of D-methionine by L. arabinosus 17-5, on a synthetic medium. They found that D-methionine was converted to L-methionine but only in the presence of 1 µgm% of pyridoxal or 10 µgm% pyridoxamine, pyridoxin was ineffective in this respect. In a later paper, they demonstrated the activity of D-methionine for 5 other lactobacilli; and that DL-ethionine inhibited this activity, but had no effect on the organism's response to L-methionine. They suggested that DL-ethionine inhibited the conversion of the D-isomer to the L-isomer.

Spies and Chambers found that L. arabinosus 17-5 would not utilise D-methionine on a synthetic medium. The medium used differed from that of Camien and Dunn (241) in that it contained only pyridoxin as representative of the B_6 group.

These findings implicated pyridoxal and pyridoxamine as being concerned in the conversion of D-methionine to L-methionine and possibly other amino acids too. Holden, Furmen and Snell (243), and Holden and Snell (244), reported very significant results from their work on the relationship between D-alanine and vitamin B_6 (as pyridoxal) in the

growth of lactic acid bacteria. It had been earlier shown that D-alanine could replace B_6 in the nutrition of L. casei and Str. faecalis in an otherwise complete medium. It was not known whether D-alanine was a precursor of B_6 or whether B_6 was essential for the synthesis of D-alanine. Holden et al (243) showed that L. casei grown in presence of D-alanine, without B_6, synthesized very small amounts of the vitamin, smaller than when the organism was grown with limiting quantities of B_6. Thus D-alanine was not primarily a precursor of pyridoxal. Later, Holden et al showed that when the cells were grown with B_6, D-alanine was produced in appreciable amounts. Thus B_6 is concerned in the synthesis of D-alanine, and in the absence of B_6 this amino acid becomes essential for growth. This was the first time that an "unnatural" amino acid had been shown to be essential.

The most likely precursor of D-alanine would appear to be L-alanine, and L-alanine does to a slight extent substitute for D-alanine in growth of Str. faecalis, though not L. casei, in the absence of B_6. The necessity for B_6 in the absence of the D-isomer suggests that it has a "racemizing" role, as indicated above. This is supported by Lyman and Kuiken's findings that certain lactic acid bacteria could use the D-isomer in the presence of high concentration of B_6.

Utilisation of peptides.

It has been known for some time that L. casei develops slowly on a synthetic medium containing all the usual vitamins and amino acids. Usually little or no growth appeared during the first 16-24 hours incubation. Various substances have been shown to reduce this lag phase, including a factor found in trypsinized casein by Wright and Skeggs (245). They suggested that asparagine and glutamine were involved.

Sprince and Woolley named the factor "Strepogenin" and identified it with that required by L. casei. Other workers also implicated glutamine and glycine. Contrary to these and other simple substances, "Strepogenin" is thermo-stable. This, with the fact that all active peptide compounds contained glutamic acid, indicated that Strepogenin was a peptide.

On this basis, Woolley (245) synthesized a number of peptides containing glutamic acid. The most active was seryl-glycyl-glutamic acid, which had an activity of one as compared with a standard liver preparation. A concentrate of insulin had an activity of 40, so it is clear that strepogenin is more complex than seryl-glycyl-glutamic acid. Rickes, Koch and Wood (247) showed that on a synthetic medium, asparagine, serine and glutamic acid increased the rate of growth of L. casei. Thus asparagine also plays a role in a strepogenin synthesis.

In an extension of these observations, Dunn and McClure (248) examined the response of L. casei to partial hydrolysates of protein. They found that acid production on a bound amino acid was often much greater than on the free amino acid. This suggested that L. casei utilised some acids much better if they were bound than they were free.

Agren (249) investigated the utilisation of leucine and valine with ten lactic acid bacteria. He found that some organisms only assimilated these two amino acids in peptides when they were incorporated in certain order within the peptides. L-leucine of glycyl-DL-leucyl-DL-alanine was least active of the tri-peptides used; L-leucine of DL-alanyl-DL-leucine and DL-leucyl-DL-alanine was less active than leucine combined with glycine.

The suggestion has been made by Simonds, Tatum and Fruton (250)

that with many peptides, utilisation for growth is preceded by hydrolysis. If this is so, the inactive peptides alone may be resistant to the peptidases of the lactic acid bacteria employed. (The suggestion does not agree with the findings of Dunn and McClure (248).)

Ågren (249) makes the interesting proposal that further investigation into the utilisation of peptides by lactic acid bacteria may be useful in the examination of the order in which amino acids occur in natural peptides.

Amino Acid Inter-relationships.

Inter-relationships between amino acids in the bacterial nutrition have been mentioned previously. Thus methionine and lysine in folic acid metabolism; glutamic acid and serine in streptogenin; and D-glutamic acid and aspartic acid in nutrition of L. arabinosus 17-5.

This last case is an example of amino acid antagonism, i.e. the presence of one amino acid preventing the utilisation of another by a micro-organism. This phenomenon was first shown by Gladstone (236) in 1939, while investigating the growth of B. anthracis. He found that certain amino acids prevented growth when added singly to a medium capable of supporting growth without them. Thus valine inhibited leucine utilisation and vice versa. Similar mutual antagonisms were found between valine and threonine and threonine and serine. Later Snell and Guirard (251) reported a more complex case, where glycine, serine, β-alanine and threonine inhibited the utilisation of alanine. Camien and Dunn (252) have shown that DL-serine and glycine inhibit assimilation of D-alanine.

Solhjell and Elvehjem (253) reported that the growth of L. arabinosus

and Leuc. mesenteroides p-60 was adversely affected by an unbalance in the concentrations of leucine, isoleucine, valine and methionine. The growth of Leuc. mesenteroides p-60 was also inhibited by aspartic acid when the glutamic acid content was limiting.

Threonine-serine antagonism in lactic acid bacteria has been studied in some detail by Meinke and Holland (254). They found that at a given level of serine increasing amounts of threonine caused greater decreases in the growth. Under the conditions used the inhibition ratios threonine to serine were 150, 75, 1100 to 1400 for L. delbrückii, L. casei and Leuc. mesenteroides P. 60 respectively. Serine is essential for growth of all the three. Conversely, serine inhibited utilisation of threonine by Str. faecalis R. and L. arabinosus 17-5, which require threonine for growth.

Referring to amino acid antagonisms, Hegsted (255) says "If such relationships are general at the level of amino acid used, the term essential amino acid may have significance only in relation to the composition of the basal medium".

This is well illustrated by the work of Washburn and Niven (256). They showed that Str. bovis grew well on a medium of salts, sugar, vitamins and L-arginine. Single additions of isoleucine, leucine, threonine, nor-leucine, or alanine inhibited growth; further single additions of cystine, glutamic acid, methionine or valine reversed this inhibition and allowed full growth. Also a mixture of phenylalanine and tyrosine was inhibitory and the addition of tryptophan counteracted this inhibition.

Relatively little work has been done on the problem of synthesis of amino acids by lactic acid bacteria, but it is clear that inter-

relations between amino acids exist in this field too. Thus Hood and Lyman (257) showed that omission of arginine from a synthetic medium led to an increased requirement of glutamic acid for rapid growth of L. arabinosus 17-5. Methionine sulphoxide inhibited this growth but glutamine or better citrulline reversed this inhibition. It was suggested that glutamine provided - NH_2 group for the formation of citrulline from δ-carbamino-L-ornithine. The subsequent formation of arginine is supported by the work of Srb and Horowitz with Neurospora crassa mutants.

Cystine has been said to be essential for a number of lactic acid bacteria (255,258). However, a study by Riesen, Spengler, Robblee, Hankes and Elvehjem (259) showed that six lactobacilli and one streptococcus could utilise other sulphur compounds such as glutathione and cysteine in place of cystine; Leuc. mesenteroides P-60 was the only organism to show a specific requirement for cystine. Hift and Wallace (260) using strains of L. arabinosus and Leuc. mesenteroids, which required cystine but not methionine for maximal growth found that homocystine in the presence of serine was also effective. They concluded that these strains could link homocystine and serine to form cystine, but could not produce cystine from methionine.

Amino Acid - Vitamin Inter-relationships.

It has been seen that nutrients required by lactic acid bacteria are not constant, but may vary according to the presence or absence of other nutrients. Thus one vitamin may influence the requirement for another. Bearing in mind the role of many of the vitamins as co-enzymes, one should expect to find inter-relationships between them and amino acids.

Among the best known such examples are those between biotin and aspartic and oleic acids; pyridoxal and a number of amino acids; and folic acid, PAB and certain amino acids.

The variation in folic acid and PAB requirements of lactic acid bacteria due to methionine and lysine has already been described in some detail. Serine has now been involved, too. Holland and Meinke (261) while investigating the serine requirements of Str. faecalis R. found that it varied with the basal medium employed. In addition to threonine, folic acid, adenine, guanine and uracil altered the requirements for serine. The purines and pyrimidine (in presence of folic acid) inhibited growth in the presence of limiting quantities of serine, having greatest effect when added together. Increase in the folic acid concentration partially reversed this inhibition. This also resulted in good growth in the absence of serine (and presence of purines and pyrimidine). This is indicative of serine synthesis due to folic acid, and lends weight to the remark of Hegsted quoted earlier.

The work of Holden et al (244) on the connection between pyridoxal and D-alanine has already been discussed. They pointed out that the substances are mutually replaceable nutrients for L. casei only in media containing all other vitamins and amino acids. Layman, Moseley, Wood, Butler and Hale (262) showed that L. arabinosus which in complete media grows without B_6, requires this vitamin in the absence of any of the following amino acids:- threonine, lysine, alanine, phenyl alanine, arginine, tyrosine, serine, histidine and aspartic acid. The mechanism governing this relationship is unknown, though it is probable that the vitamin functions as a catalyst in the synthesis of the amino acids.

More direct evidence that this is so is provided by the work of

Speck and Pitt (263). They, while attempting to assay cystine with L. arabinosus 17-5 found that pyridoxal or pyridoxamine eliminated the organisms need for this amino acid. Similar results were found with Str. faecalis and L. casei.

Work linking PAB, serine and pyridoxal has been reported by Lascelles and Woods (264). They found that with growing cultures of Leuc. mesenteroides P-60 serine may be partially replaced by glycine plus pyridoxal and that the replacement is almost total by the further addition of PAB.

In 1942, Koser, Wright and Dorfman showed that aspartic acid exerted a biotin sparing effect for Torula cremoris. This connection between aspartic acid and biotin was investigated by Stokes, Larsen and Gunness (265) for several lactic acid bacteria. They found that if the biotin level was less than 0·001 µgm/10 ml. of medium, aspartic acid was required for growth. When the biotin level was increased to 0.005 µgm/10 ml. of medium, the need for aspartic acid was eliminated. Assay of cells grown in the absence of aspartic acid demonstrated the synthesis of this amino acid. Thus biotin functions in the formation of aspartic acid.

Landy, Potter and Elvehjem (266), studying biotin and L. arabinosus found that oxaloacetate stimulated growth on a biotin and aspartic acid deficient medium, and that bicarbonate stimulated growth in a biotin rich medium. This indicated role of biotin in carbon dioxide fixation, as also shown by Lichstein and Umbreit (267) using cell suspensions of E. coli. They found that biotin mediated the release of carbondioxide from oxaloacetate by oxaloacetic decarboxylase. These workers also suggested a function of biotin in deaminases of aspartic acid, serine

and threonine.

Oleic acid is another substance which has been shown to have a biotin-sparing effect for some lactic acid bacteria. Oleic acid did not induce synthesis of biotin and the stimulation is not nullified by avidin. Later (268) oleic acid was shown to be essential for the growth of a number of lactic acid bacteria, and it was suggested that biotin affected the synthesis of oleic acid in other organisms. Some organisms such as L. bulgaricus and L. acidophylus require oleic acid even in the presence of biotin; more often, as with L. arabinosus and L. casei oleic acid is only required in the absence of biotin.

Potter and Elvehjem (269) studied biotin and the metabolism of L. arabinosus. They showed that oleic acid and aspartic acid could not replace each other, but that together they could replace biotin. They also showed that oleic acid was not a precursor of biotin. This work strengthens the hypothesis that biotin mediates in the synthesis of both aspartic acid and oleic acid.

A discordant note was sounded by Williams and Williams (270) that a number of non-ionic detergents, the most effective being those containing oleic acid, could replace biotin requirement of L. casei. They suggested that biotin and oleic acid acted by exerting a surface effect. They measured the electrophoretic motility of L. casei in the presence of (a) biotin, (b) oleic acid, and (c) anionic, cationic and non-ionic detergents. The results indicated that both oleic acid and biotin were absorbed on to the surface of the cells. These findings do indicate a surface-active nature for biotin.

Amino Acid Assays.

Just as the lactic acid bacteria have been used for assay of vitamins of the B-complex group, so they have been used to determine the amino acid content of natural materials. All the naturally occurring amino acids have been assayed with the exception of hydroxyproline, nor-leucine and nor-valine. This is because as yet no organism has been found to require any of the three acids for growth.

Many lactic acid bacteria have been used for assay purposes. With Leuc. mesenteroides P-60, fifteen amino acids can be assayed. But it is probably better to use a particular organism for particular amino acid. Comprehensive review on microbiological assay of amino acids have appeared by Dunn (271) and Snell (272).

PRACTICAL PART.

SECTION I.

I. APPARATUS.

Glassware. Glass apparatus previously used for chemical purposes has never been employed subsequently for bacteriological work.

Culture tubes used for bacteriological work were:- (a) 5" x $\frac{1}{2}$" or 6" x $\frac{3}{4}$" "lipless" bacteriological tubes, and (b) 6" x 1" "boiling tubes". Durham fermentation tubes were used for detection of gas formation in aerobically growing liquid cultures.

Petri Dishes employed in all experiments were of the following size:- 100 mm. x 15 mm. in height.

Graduated and Pasteur pipettes were plugged with cotton wool prior to sterilising them in the oven or autoclave.

Cotton wool of non-absorbent type was used for plugging purposes, while for filtration of media the absorbent type was employed.

Inoculating loops and needles were made of platinum or nicron wire (gauge No. 25): the diameter of the loops was about 4 mm.

Incubators employed during these investigations were of the Hearson's electric type as well as ordinary types.

II. STERILISATION.

(a) Flaming.

Inoculating needles, "mouth" of culture tubes and flasks, and other objects were sterilised by passing through the Bunsen flame several times.

(b) Dry Heat.

All glass apparatus employed in the aseptic work was sterilised for one hour at 160°C. in a dry heat oven.

(c) Steaming.

Sugar containing media as well as gelatin containing media were steamed for 20 minutes at 100°C. on each of the three consecutive days.

(d) Autoclaving.

All non-sugar containing media were autoclaved under 15 lb. steam pressure for 15 minutes.

(e) Disinfection.

All infected pipettes, petri dishes, etc., were autoclaved under 20 lb. pressure for half an hour before they were washed for further use. Slides were placed in a disinfectant solution, such as 50 per cent alcohol.

III. MEDIA AND THEIR PREPARATION.

(A) Standard beer medium.

Freshly ground pale ale malt (650 g.) was mashed with 1,500 c.c. of water at 155° to 160°F. The mash was allowed to stand for 2½ to 3 hours at 145° to 150°F. till no starch remained (test by iodine in KI). The mash was filtered through muslin, on a pump, and the residue was washed (sparged) with 2,500 c.c. of water at 160°F. The washings were added to the extract and the lot was boiled in a beaker over a burner for 1½ hours. The wort was

allowed to cool overnight and filtered through absorbent cotton wool. The filtered wort was diluted by using distilled water to the specific gravity 1040, steamed for 30 minutes and was cooled down to the room temperature. Pressed pitching yeast (4 g. per litre of wort) was dissolved in a portion of the wort and was then thoroughly mixed with the whole of the wort. The flask containing the pitched wort was allowed to stand in a cool cupboard for four days, a periodic good shaking being given every 24 hours. At the end of four days, when the fermentation was found to be completed, the beer produced was filtered through the cotton wool, steamed for half an hour, cooled and refiltered through coarse filter paper. It was dispersed in 1 litre flasks and the flasks were sterilised for 20 minutes in free steam on each of the three consecutive days. This gave the "stock" beer and was kept in the dark.

When required it was clarified, by adding powdered egg albumin (5 g. albumin dissolved in cold water and added to 1 litre of beer), and heating in free steam for 30 minutes. After allowing it to cool overnight it was filtered through a coarse filter paper, mixed with 1 per cent of glucose and dispersed in the tubes as required. The tubes were sterilised for 15 minutes in free steam on each of the three consecutive days. Prepared in this way the beer remained clear and did not give deposit on standing. This medium was also used for the preparation of beer-ager and beer-gelatin. Morphological, physiological and most of the cultural characteristics were all determined in this beer medium and it was termed as <u>standard beer medium</u>.

(B) Standard beer-agar media.

(a) For plating out purposes was made by adding 2 per cent agar to the standard beer medium.

(b) For cultural characteristics and morphological characteristics was made by adding 1.5 per cent agar to the standard beer medium.

(c) For catalase reaction as described under (a), with an addition of 2 per cent sterile $CaCo_3$.

(C) Standard beer-gelatin was prepared by adding 15 per cent of gold-leaf gelatin to the standard beer medium.

(D) Standard broth media.

Nutrient Broth (standard broth) was prepared as described in "Laboratory Manual" by Salle (273) and PH was adjusted to 5 to 5.2 using NaOH and HCl. by the help of Johnson's PH papers.

Glucose Broth was prepared by adding 0.5 per cent of glucose to the nutrient broth as described by Salle (274).

(E) Standard malt wort was prepared by the method described in (A) for standard beer medium, but the wort, after diluting to 0°G 1040, was not pitched with yeast, instead it was treated with egg albumin, as was done in the case of standard beer medium. After filtering the medium was dispersed in tubes and sterilised for 15 minutes in free steam on each of three consecutive days. Wort tubes prepared in this way remained brilliant and did not give any deposit on standing. Such a medium was called standard malt wort.

(F) Litmus Milk. Fresh milk was centrifuged for about 30 minutes at 4,500 revolutions per minute to separate the cream. The cream

was then removed and 1 g. of Azolitmin dissolved in 10 ml. of distilled water with the aid of a few drops of $N/_2$ NaOH was added to 1 litre of skimmed milk. The milk was dispensed into test tubes which were sterilised for 20 minutes in free steam on three successive days.

(G) Casein double digest + yeast autolysate medium.

Casein double digest as well as yeast autolysate, both were prepared as described by Davis (276). These two media were mixed in the ratio of 100 ml : 5 ml to give the required suitable medium. Nitrogen content determined by Kjeldahl method was found to be 5 mg. per ml.

(H) Other media and Reagents.

(a) Medium for the acetyl-methyl-carbinol test was prepared as described in the booklet "Difco Mannual" (275) and the PH value was adjusted to 5·2 to 5·3 as usual. (The acetyl-methyl-carbinol test is termed the Voges-Proskauer test only if performed in the inorganic medium described by Voges-Proskauer).

(b) Nitrate broth was prepared as described by Salle (273) and the PH was adjusted to 5·2 to 5·3. This medium was used to test for the nitrate reduction by the organisms.

(c) Peptone broth was also prepared as described by Salle. PH was adjusted to 5·2 to 5·3 with the help of Johnson's PH papers. This medium was used as the standard medium for the test of indole production.

(d) Hydrogen peroxide solution used for the catalase reaction, was diluted to $N/_2$ strength by using distilled water.

(e) The saline dilution medium used was 0·9 per cent solution of sodium chloride in distilled water.

THE STANDARD INOCULATION PROCEDURE.

The inoculation procedure was standardised throughout the work described in this thesis. The media, in most of the experiments contained standard beer medium, was inoculated with young and vigorous standard beer cultures. These in turn were prepared as follows:-

A tube containing 8 ml. of standard beer was inoculated with two loopsful of the culture, and this subculture was incubated at the appropriate optimum temperature for the organism in question for 24 hours after the minimum time, when the growth appeared in the tube. A second subculture in same medium was prepared in a similar manner from the first. This subculture then served as a standard inoculation culture. For inoculation of slopes (streaks) and stab-cultures the same above-mentioned standard inoculation cultures were used, the inoculation having been done with a platinum needle.

In case of the big experiments where hundreds of tubes had to be

inoculated simultaneously, the inoculation was carried out by means of small sterile Pasteur pipettes in place of platinum loop. The Pasteur pipettes were prepared from narrow glass tubes by stretching one end of the tube in the form of a narrow thin dropper. The other end of the pipette was plugged with cotton wool, sterilised in steam, and a rubber teat (dropper) was used for dropping. One drop was used to inoculate each tube.

THE PROCEDURE FOR ISOLATION OF LACTIC ACID BACTERIA SPECIES.

The Sources.

The isolation of lactic acid bacteria species can be successfully attempted from various materials employed in the brewing industry and also from the finished products of the industry, the best sources being:-

(a) Beers and ales.

(b) Cider.

(c) Compressed yeast.

(d) Brewer's yeast.

The present work had been limited to the isolation of lactic acid bacteria species from various samples of brewer's yeast which are employed for the brewing of different types of British beers and ales. In order to obtain strains of lactic acid bacteria species which might prevail at different times of the year, the various samples were plated out between September, 1949, and March, 1950. The summary of

the information concerning the samples of various brewery yeasts employed and the organisms isolated is given in Table I, whilst the information concerning the organisms obtained from other people is given in Table II.

The Technique employed for the Anaerobic Cultivation of the Organisms.

Methods of using petri-dishes alone for the anaerobic cultivation of bacteria had been reviewed by Snieszko (277). He recommended a method which combined the best ideas of the former workers. Writer has employed this method of Snieszko with very satisfactory results and had isolated a number of different bacteria.

Pairs of petri-dishes were selected, the lower plates of which were of exactly equal diameter, so that when one plate was superimposed on the other the ground glass edges were coincident. Into one lower plate of each pair sterile beef-extract peptone agar was poured. The covers were replaced and the dishes were allowed to stand inverted for two days at 30°C. to allow excess moisture to evaporate. The other lower plate of each pair of dishes received the medium for anaerobic organisms; in the present case this was standard beer gelatin (containing 20 per cent of gelatin). Before the medium was poured on those plates, 1 ml. of the suspension of the bacteria to be separated was placed on each plate. After replacing the covers those plates were kept for two days at room temperature (18° to 19°).

At the end of two days the broth agar plates were streaked with an active culture of Serratia marcescens, a highly aerobic organism. It was found very desirable to confine the streaks to an area in the centre of the plate, keeping at least half an inch from the edge of

the plate all the way round. The lower plates of the pairs of petri-dishes were now sealed to each other with strips of plasticine, as follows:-

> Plasticine was first rolled to thickness of lead pencil, laid out straight on a clean smooth glass piece 12" square, and the pair of plates held firmly together (taking care not to apply too great a pressure) was rolled along the plasticine, which was pressed firmly to the plates and made a firm and air-tight union.

The sealed pairs of dishes were now placed in a cool cupboard (temperature 18° to 20°C.) with the plate containing the Serratia marcescens at the bottom in each case, so as to prevent contamination should any liquid separate by syneresis. It was for the same reason that the streaks of the latter organism were restricted to the centre of the plates only, else culture might gain access to the top plate by following a path made by condensed or separated liquid.

Technique employed for the preliminary and subsequent platings.

In each case 1 g. of yeast sample was added to a bottle containing 100 ml. of sterilised beer, and was mixed thoroughly. The anaerobic conditions were attained by putting a $\frac{1}{8}$" layer of liquid paraffin on the surface of the beer. The bottles were placed in a cool cupboard for three weeks and were given a good shaking once a week. At the end of the third week bottles were shaken thoroughly (care being taken not to disturb the paraffin layer) so as to spread the infection uniformly throughout the medium, and were then allowed to stand for one hour to settle the yeast. At the end of that period 1 ml. of supernatent

beer was drawn off from each bottle by a sterile pipette, from the middle of the bottle and without disturbing the deposit, for plating out purposes.

Samples of the beers taken for plating were diluted with sterile saline (0·9% Nacl). The appropriate degree of dilution to be effected was judged in each case from the appearance of the sample; those which were very turbid or which had deposited sediment were diluted to a greater extent than those which showed less evidence of bacterial growth. The dilutions chosen for plating purposes covered the range 10^{-5} to 10^{-10} and usually four or five plates were prepared from each beer. Thus, if the sample showed strong infection, dilutions 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} were plated; while in other cases the dilutions 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} were employed.

In each plate 1 ml. of the appropriate diluted sample was mixed with 15 ml. of clarified unhopped beer gelatin (containing 20 per cent of gold leaf gelatin). The plates were sealed with the Serratia marcescens plates by means of plasticine as described before and were incubated at room temperature (18°-20°C.). Those plates which showed no growth at the end of 14 days were placed in an incubator maintained at 20°C.

In some cases colonies were observed at the end of four days, in other cases no development was noticed until after 18-20 days of incubation. A further period of 4 to 7 days of incubation was allowed in each case from the time of the first appearance of the colonies. Only those plates which showed relatively few colonies, and those well spaced, were selected to furnish cultures.

Visually dissimilar colonies from such plates were picked off,

inoculated into sterile standard beer tubes and incubated at 25°C. Almost all the colonies picked off were sub-surface ones.

After the primary plating of the different samples had been completed and all the selected cultures were found growing in tubes of standard beer medium, replating of all those cultures was commenced in order to ensure that pure strains were secured for purposes of characterisation and future work. Those cultures which were found to be the most rapid growers were used first for replating by using standard beer + 2% agar. Agar was used in order to permit incubation at 25°C. It was found that the medium, though quite satisfactory for some of the organisms, did not permit the free and satisfactory development of others as was the case with standard beer gelatin. Accordingly, the later platings were carried out with standard beer gelatin, such as had been employed for the initial isolations. In each case three to four platings were found essential to ensure pure cultures. Each time platings were carried out as in the initial case, but no aerobic culture was used to attain anaerobic conditions in these subsequent platings. Anaerobic incubation of the replated cultures was carried out in desiccators, in atmosphere of carbon dioxide, which was attained first by evacuating the desiccator on the pump and then filling it with carbon dioxide.

THE ORIGIN OF ORGANISMS STUDIED AND DESCRIBED IN THIS THESIS.

The organisms which have been studied and described in this thesis fall into two groups:-

(a) Cultures given to the writer for study.

(b) Newly isolated cultures of different lactic acid bacteria species.

Cultures mentioned under (a) were obtained from Dr. T. K. Walker. Their origin is given in Table II.

The origin of the cultures which were independently isolated by the writer is given in Table I.

TABLE I.

Sample of yeast.	Source.	Date of receipt.	Organism isolated.
A.	A Manchester Brewery.	September, 1949.	A_1, A_2, A_3, A_4, A_5, A_6, A_7.
B.	A Bolton Brewery.	October, 1949.	B_1, B_2, B_3, B_4, B_5.
C.	A Manchester Brewery.	November, 1949.	C_1, C_2, C_3, C_4, C_5, C_7, C_8.
D.	A Manchester Brewery.	December, 1949.	D_1, D_2, D_4, D_5.
E.	A Manchester Brewery.	December, 1949.	E_1, E_2, E_3, E_4, E_5, E_6.
F.	A Manchester Brewery.	December, 1949.	F_1, F_2, F_3, F_6.
G.	A Blackburn Brewery.	January, 1950.	G_1, G_2, G_3, G_4, G_5, G_6.
H.	A Sheffield Brewery.	February, 1950.	H_1, H_2, H_3, H_4, H_5, H_6.

Note: Yeasts from Manchester were collected from the various breweries all situated far apart from each other.

TABLE II.

Organisms.	Received from	Isolated from	Date of isolation.
W_1, W_4, W_5.	Norman Blakebrough.	A Manchester Brewery.	1946.
W_D & W_L.	Norman Blakebrough.	Another Manchester Brewery.	1946.
W_7 & W_{10}.	Dr. T. K. Walker.	A Manchester Brewery.	1946.

THE STANDARDISED TECHNIQUE ADOPTED IN THE PRESENT WORK FOR THE CHARACTERISATION OF THE LACTIC ACID BACTERIA SPECIES.

MORPHOLOGICAL CHARACTERISTICS.

(A) Size and Shape: Organisms were examined for their size and shape in (a) hanging drop preparations, and (b) methylene blue stained preparations.

For the hanging drop preparations the organisms in question were grown in standard beer medium, at their respective predetermined optimum temperatures until they reached the logarithimic phase of growth; at that stage their hanging drop preparations were examined under microscope.

Methylene blue stained preparations were made from the same liquid cultures mentioned above, as well as from cultures grown on slopes of standard beer agar, as described later under the heading "Staining". The size and shape of many cultures grown on slopes was found to be different from their respective liquid cultures and thus, for the sake of comparison with the hanging drop preparations, the staining preparations from the liquid cultures alone were described. The information obtained from those preparations was in great conformity with the results obtained from the hanging drop preparations, when the film on the slides were completely air-dried before fixing on the flame. But when sufficient time was not allowed for the air-drying of the films before they were fixed, the size of the cells were

found slightly changed (smaller) than those from the hanging drop preparations.

(B) Arrangement: This was examined in hanging drop preparations as well as in the stained preparations as described above (A); in latter cases the fields which were not crowded were examined. Similar results were obtained in both cases.

(C) Motility: This characteristic was sought for in the hanging drop preparations. Such preparations were made from unhopped beer cultures, one set incubated at 20°C. and the other set incubated at the respective optimum temperatures. The cultures kept at both the temperatures were examined for motility at their early stages of the logarithmic phase of growth, which in most cases reached after an incubation period of 20 to 30 hours, depending on the temperature of incubation and the species under examination.

None of the organisms examined showed this characteristic.

(D) Staining: This characteristic was divided into two groups for the sake of convenience: (a) special stainings, when formation of endospores, capsules, etc., were sought for, and (b) ordinary staining with reagents like Loeffler's methylene blue and carbol fuchsin were carried out to obtain information about the size, shape and arrangement of cells.

(a) Special Stainings.

Spores Staining.

Formation of endospores was sought for by Gray's method (279), and also by Schaffer and Fulton's method (278). The organisms in

question were grown on standard beer agar slopes and were incubated for seven days at their respective predetermined optimum temperatures and subsequently for seven days at the room temperature (18°-20°C.), after that treatment the cells from the slopes were stained by both the above-named techniques and were examined for spores.

None of the organisms in question were found to form spores.

Gram Stain.

Hucker's modification of the Gram stain was employed (280) throughout the present work. The organisms were grown on standard beer agar slopes as well as in standard beer tubes at their respective optimum temperatures until they reached the logarithmic phase of growth. At that point the cells were stained and examined. Identical results were obtained with the cells from the solid as well as the liquid medium.

All the organisms in question were found to be gram positive.

Capsule Staining.

For the detection of capsules Plimmer and Paine's method (281), as well as Welch's modified method (282), was employed. Organisms were grown as in the case of Gram staining and were stained and examined when they reached logarithmic phase of growth.

None of the organisms in question showed the presence of capsules.

(b) Ordinary Staining.

The organisms were grown as described in the previous stainings and were then stained with Loeffler's methylene blue and carbol fuchsin staining reagents, respectively, as described by Salle.

TABLE III.

Description of Morphological Characteristics.

Organisms.	Shape and Size.	Arrangement.
A_1, A_2, A_3, A_4, E_5.	Uniform rods. 0·5 to 0·7 μ by 1·8 to 3·5 μ.	Mostly single cells, but a few pairs, clumps and short chains were also observed. Heavily granulated cells.
A_5, A_6, A_7, E_4, E_1, E_2, E_3.	Rods of various sizes. 0·5 to 0·7 μ by 2·5 to 5·5 μ. Some cells were up to 7 μ long.	Single cells and pairs were very common, but some short chains were also detected. In most cases cells were situated parallel to each other. Mostly rods were straight, but a few cases of bent rods were also detected. Granulation was observed in some rods.
D_1, D_5, F_3, E_6.	Rods of varying sizes. Average size of the rods was 0·5 to 0·6 u by 2 to 6 μ. But many rods were up to 10 u long.	Single cells, pairs and short chains were very common. Rods of all shapes were present - U shaped, Y shaped and shape of Chinese letters. No granulation.
B_1, B_2, B_5, C_1, W_D, W_L, W_1, C_4.	Rods of various sizes. 0·6 to 0·9 μ by 2·5 μ to 8 μ. A few rods were up to 11 to 12 μ long.	A few single cells, but mostly cells were in pairs, united at angles. A few chains of 6 to 12 cells were also detected.
B_3, B_4, D_4, H_4, D_2.	Rods of various sizes. 0·7 to 1 μ by 3·5 to 7 μ.	Mostly cells were in pairs, united at angles, but a few single cells and short chains were also detected.
C_5, W_{10}, W_7, W_5.	Long thin rods of various sizes. 0·5 to 0·8 μ by 3·5 to 10 μ.	Single cells, pairs and small irregular clumps were detected, but no chains could be found. Pairs of cells were united at obtuse angles.
C_7, C_8, C_2, G_6.	Rods of various sizes, with rounded ends. 0·7 to 1·1 μ by 2·5 to 7 μ.	Mostly single cells and some small chains were detected. No groups or pairs observed. Mostly cells were long but some were very short and fat in appearance.
H_1, H_2, H_3, H_5.	Uniform short fat rods, ellipsoidal with rounded ends. 0·7 to 0·8 μ by 1 to 1·2 μ.	Single cells, pairs as well as small irregular clumps were present. A few chains of four to six cells were also detected.
C_3, H_6, G_2, G_4.	Uniform rods with somewhat rounded ends. 0·3 to 0·5 by 0·9 to 3·5 μ. Some threads of 15 to 20 u were also observed.	Single cells, pairs and long and short chains were present. But only a few small irregular clumps were detected.
F_6, F_1.	Uniform small spherical cells 0·6 to 0·7 μ in diameter.	Mostly the cells are in tetrads, but a few diplos and single cells were also observed. Some clumps of tetrads and eights and a few chains of tetrads were also detected.

Description of Morphological Characteristics (continued).

Organisms.	Shape and Size.	Arrangement.
G_3, G_5.	Uniform small spherical shaped cells. 0·5 to 0·6 μ in diameter.	Mostly the cells are in diplos, but a few single cells and tetrads were also detected. A few clumps of diplos were also observed.
G_1, F_2.	Spherical shaped small cells. 0·5 to 0·7 μ in diameter.	Mostly in diplos but some single cells were also detected. Some cases where diplos were so arranged so as to give the impression of tetrads. No clumps were detected.

Note:

1. All the organisms described above were found to be non-motile and non-sporeforming.
2. Capsules were not detected in any case.
3. All were found to be gram positive.
4. All the organisms took good stain from Carbol fuchsin and methylene blue.

In connection with the technique of the preparation of slides for various stainings the writer would like to point out that when the culture films were completely air-dried before fixing them on the flame, the crowded fields and clumps could be very successfully avoided, and fields with evenly distributed cells were obtained, which provided reliable results concerning morphological characteristics.

The results obtained with the 52 organisms employed by the writer, with regard to their morphological characteristics and the various staining techniques, has been summarised in Table No. III. The organisms which showed similar characteristics were placed in one column rather than describing all the organisms individually.

CULTURAL CHARACTERISTICS.

For the observation of cultural characteristics of the organisms, the temperature of incubation used was either the respective optimum temperature of the organism in question, or a temperature from its optimum range. But the experiments with the gelatin media were carried out at the room temperature (18°-20°C.). In all those experiments the following observations were reported: the minimum time after which the growth became evident and the description of growth at the time when the organisms entered their logarithmic phase and also at an interval of four days after that period. The cases where descriptions of single colonies were recorded, the organisms were incubated for further period of 4 to 5 days, after the first appearance of the

growth, before they were examined. The meanings of the terms used in describing the type of growth are given in the glossary of terms (this thesis, page 119).

In connection with the use of solid media, it must be mentioned that it was noticed that growth was sometimes delayed, and in some cases slightly different in character when old slopes were used. Thus, the preparation of slopes was standardised by sloping them 24 hours before they were inoculated. The inoculation procedure was also standardised as described before. All the cultures on solid media were incubated in the atmosphere of carbon dioxide except the stabs.

The results of the experiments have been summarised as follows.

A. Description of Growth on/in Solid Media.

(a) The Description of Single Colonies was given from standard beer gelatin plates. The plating out procedure employed was as follows: in each case 1 ml. of the suitably diluted culture medium was plated out with 15 ml. of sterile standard beer gelatin (20% gelatin). On setting the medium the plates were incubated in inverted position in the atmosphere of carbon dioxide in desiccators at room temperature (18°-20°C.). The description of the colonies was carried out from the respective plates which contained between 20 to 40 colonies.

The detailed description of size and shape, elevation, surface, structure, margin, colour, opacity, consistency, emulsifiability and the rate of growth of these colonies is given in the Table (IV). For the sake of convenience the organisms which showed the similar characteristics were

TABLE IV.

Description of Single Colonies on Beer Gelatine Plates at room temperature (18-20°C.) in Co_2 atmosphere.

Organisms.	Size and Shape.	Surface Structure.	Elevation.	Margin.	Opacity.	Colour.	Spreading.	Consistency.	Emulsifiability.	First appearance of growth.
A_1, A_3, A_5, A_6, A_7.	Round. 1 to 2 m.m.	Subsurface smooth and dull.	Flat.	Entire.	Opaque.	Brownish tinge, mainly white.	Not observed.	Somewhat cohesive.	Slow.	Fast (3 to 4 days).
A_2, A_4, E_4, E_5.	Mostly round and some ellipsoidal. 1·5 m.m. to 2 m.m.	Subsurface smooth and dull.	Swollen up. Cushion shaped (pulvinate).	Entire.	Opaque.	Mainly white; slight brownish tinge when old.	Not observed.	Coherent.	Fair.	Moderate (5 to 6 days).
B_1, B_3, B_2, WL, WD, W1.	Round. 1 to 1·5 m.m. at the most.	Subsurface smooth and glistening.	Convex.	Entire.	Translucent.	Greyish white.	Not observed.	Butterlike.	Fair.	Moderate (6 to 7 days).
B_4, B_5.	Round. 1·5 to 2 m.m. in diameter.	Just below the surface smooth and shining.	Flat.	Entire.	Opaque.	Colourless.	Not observed.	Somewhat cohesive.	Fair.	Slow (10-12 days).
C_1, C_5, C_7, C_8.	Round and punctiform. 0·7 m.m. to 1 m.m.	Subsurface smooth, thick and glistening.	Slightly convex.	Entire.	Translucent.	Colourless.	Not observed.	Butterlike.	Fast.	Slow (10-13 days).
C_3, H_6, G_4, G_6, G_2.	Slightly ellipsoidal (practically round). 1·5 m.m. to 2 m.m.	Just below the surface smooth and dull.	Flat.	Entire.	Opaque.	Slightly yellowish due to reflection.	Not observed.	Somewhat cohesive.	Slow.	Moderate (6 to 7 days).
C_2, C_4, W_7, W_{10}.	Round. 1 to 2 m.m.	Subsurface smooth and glistening.	Flat.	Entire.	Opaque.	Colourless.	Not observed.	Butterlike.	Fair.	Slow (11-13 days).
D_1, D_5, H_4, E_2, F_3.	Round. 1 m.m. to 1·5 m.m.	Smooth and dull.	Flat.	Entire.	Opaque.	Slightly yellowish. May be due to reflection.	Not observed.	Somewhat cohesive.	Fair.	Slow (10-12 days).
D_2, D_4, F_3, W_4, W_5.	Slightly ellipsoidal (more like round). 2 m.m. to 3 m.m.	Subsurface smooth and glistening.	Convex.	Entire.	Opaque.	Colourless.	Not observed.	Butterlike.	Fast.	Moderate (6 to 7 days).
E_1, E_3, E_6.	Ellipsoidal. 1 m.m. to 1·5 m.m.	Subsurface smooth and dull.	Flat.	Entire edged.	Opaque.	Colourless.	Not observed.	Butterlike.	Fast.	Moderate (6 to 7 days).
G_1, F_2.	Round, small. 0·7 to 1 m.m.	Deep colonies smooth and dull.	Flat.	Entire.	Opaque.	Brownish tinge.	Not observed.	Butterlike.	Fast.	Slow (12 to 13 days).
H_1, H_2, H_3, H_5.	Round. 1 m.m. to 1·5 m.m.	Just below the surface smooth, compact and dull.	Convex.	Entire.	Translucent.	Colourless.	Not observed.	Somewhat cohesive.	Fair.	V. slow (16 to 17 da
G_3, G_5.	Round. 1 to 1·5 m.m.	Deep colonies. Smooth and dull.	Swollen up. Cushion shaped.	Entire.	Opaque.	Greyish white (off white).	May be slightly.	Ropy consistency (spreads like threads).	V. difficult.	Slow (10 to 12 days).
F1, F6.	Round, small. 0·5 to 0·8 m.m.	Deep colonies smooth and dull.	Flat.	Entire.	Opaque.	Brownish tinge.	Not observed.	Butterlike.	Fair.	Slow (12 to 13 days)

placed in one group.

(b) The description of growth on slopes was recorded from the standard beer agar slopes and is given in Table V. It was of interest to notice the entirely different type of growth in case of some organisms, while in others the striking similarity.

The appearance of growth of stab cultures (standard beer agar) was very indistinct in case of all the species because of the colour of the medium. It could be observed that the growth appeared along the line of inoculation, but the description of the type of growth could not be carried out.

(c) The description of growth in standard beer gelatin stabs was recorded as described above in the paragraph (a) dealing with cultural characteristics. It was of interest to note that the growth was much stronger as compared with the growth on standard beer agar slopes as well as stabs. The details of the characteristics of gelatin stabs are given in Table VI, but they could not be of any great help in identification of cultures because of the similarity of growth in case of almost all the organisms under examination. In all the cases there was good beaded growth along the line of inoculation, and in a few cases slight film was observed on the surface of the medium, but there was no growth on the surface in case of any of the organisms.

(d) The description of growth on/in glucose broth agar. It was noticed that growth on slopes and in stabs of glucose broth agar was considerably weaker and in some cases almost negligible as

TABLE V.

Description of growth on Standard Beer Agar Slopes.

Organisms.	Incubation Temp. °C.	Incubation Time. Days.	Description of Growth.
A1, A3, A5, A6, A7.	30°	3	Abundant, beaded, flat, opaque white and entire edged colonies (0·3 to 0·5 m.m. diameter).
E1, E5, B5, WL.	30°	5	Fair, beaded, flat, opaque white and entire edged colonies (0·25 to 0·4 diameter).
B2, B4, C7, C1, C5, C8, F3.	25°	7	Scant, isolated colonies, dull, opaque white and entire edged (0·25 to 0·5 m.m.).
B1, B3, C2, C4.	25°	7	Fair, in single isolated colonies, dull, opaque white and entire edge (0·3 to 0·5 m.m.).
C3, H6, D2.	30°	4	Abundant, filiform (on the line of streaks), smooth, dull and white appearance.
D1, D4, D5, H4, A2, A4, G4, G6.	25°	7	Abundant, filiform (on the line of streaks) as well as single colonies, opaque, dull and entire edged (0·25 m.m.).
E4, W10, E6, WD, W7, W5.	25°	6	Abundant, round, flat, entire edged, opaque white colonies (0·4 to 0·6 m.m.).
H1, H5, H3, H2, G2.	25°	8	Scant, isolated colonies, round, dull and entire edged (0·25 to 0·6 m.m.).
E3, E2, W1, W4.	25°	6	Fair, filiform (on the line of inoculation), whitish, smooth and dull.
F1, F6.	25°	8	Fair, growth throughout in single isolated colonies, round, dull and entire edged (0·25 to 0·5 m.m. diameter).
G3, G5.	25°	8	Scant, isolated colonies, round, dull and entire edged (0·5 to 0·8 m.m.).
G1, F2.	25°	8	Fair, beaded as well as isolated colonies, but very small, may be round (0·25 to 0·3 m.m. diameter).

TABLE VI.

Description of growth in Standard Beer Gelatin Stabs.

Organisms.	Incubation Temp. °C.	Time Days.	
A1, A2, A3, A4.	18-20°	10	Abundant, filiform, beaded, uniform growth throughout, not spreading, no film at the surface.
A5, A6, A7, C3, H6.	18-20°	10	Abundant filiform, beaded, v. good just below the surface but good throughout. Somewhat spreading sideways, no surface film.
B1, B4, B5.	18-20°	12	Scant, filiform, beaded, more near the surface and decreases slightly in descending order, slight film at the surface. Not spreading.
B2, B3, E2, E5, W7.	18-20°	12	Moderate, filiform, beaded, more near the surface and decreases slightly in descending order. No film, not spreading.
C1, C2, C7, C8.	18-20°	12	Moderate, filiform, beaded, slightly more near the surface but also quite good throughout, not spreading. No film.
C4, C5, F1, F6.	18-20°	12	Scant, filiform, beaded, some growth near the surface but decreases markedly below X2" depth, may be slightly spreading sideways. Slight film at the surface.
D1, D4, W10, H2.	18-20°	10	Abundant, filiform, beaded, very good near the surface and decreases slightly in the descending order, fairly spreading sideways. Moderate surface film.
D2, D5, E1, E3, E4, E6, G1, F2.	18-20°	10	Abundant, filiform, beaded, good throughout but slightly more near the surface. Slightly spreading sideways. No surface film.
G2, G4, G6, H4, W1, W4, W5, WD, WL.	18-20°	12	Abundant, filiform, beaded, very good near the surface and slight decrease in the descending order. Slight film at the surface and not spreading.
G3, G5.	18-20°	12	Abundant, filiform, beaded, very good growth throughout. Not spreading and no film.
H1, H3, H5.	18-20°	12	Moderate, filiform, beaded, growth scant near the surface and increases in the descending order, slightly spreading. No surface film.

compared to that on standard beer agar. Moreover, all the species exhibited a very similar type of growth, so that it was hardly possible to distinguish between the different organisms in question. On slopes there was some beaded to nodose growth along the line of streaks. In stabs there was slightly more growth throughout, along the line of inoculation.

B. Description of Growth in Liquid Media.

(a) Description of growth in standard beer medium. In most of the cases the description of growth was recorded after 3 & 7 days of incubation, but the times of incubation were altered in case of the week growers. Complete description of growth at different times is recorded in Table VII. For the sake of convenience, the organisms showing the similar type of growth were placed in the same groups. It was particularly of interest to note that all the organisms grew well in that medium and none of the organisms formed any surface film or other surface type of growth.

(b) Description of growth in standard malt wort is given in Table VIII. The description of growth is recorded exactly as described in the case of standard beer medium.

It was of interest to notice that apart from a few organisms, all the rest did not grow so well as they did in standard beer medium. The growth in this medium was very much identical to the growth in the standard beer medium.

TABLE VII.

Description of Growth in Standard Beer Medium.

Organisms.	Incubation Temp. °C.	Time Days.	
A_1, A_3, A_5, A_6, A_7, C_3, H_6, F_3.	30°	3	No film, medium clear, moderate powdery deposit. Good silky turbidity on shaking.
		7	Exactly as above.
A_2, A_4, B_1, B_2, B_3, B_4, B_5, W_4, W_D, W_L.	30°	3	No film, medium slightly turbid, moderate deposit, good silky turbidity on shaking.
		7	As above, except that medium became clear.
C_2, C_4, C_5, C_7, C_8, H_1, H_3, H_5.	25°	4	No film, medium slightly turbid, scant deposit, fair silky turbidity on shaking.
		7	No film, medium clear, scant deposit, fair silky turbidity on shaking.
D_1, D_4, E_1, G_1, H_2, H_4, W_1, W_5.	25°	3	No film, medium clear. Fair deposit. On shaking good silky turbidity.
		7	As above, except that there was slight increase in the deposit.
D_2, D_5, E_3, E_6, G_2, G_4, G_6.	25°	3	No film, medium moderately turbid, scant deposit, good silky turbidity on shaking.
		7	As above, except that medium became clear and deposit slightly increased.
E_2, E_4, E_5, W_7, W_{10}.	25°	3	No film, medium clear, moderate flaky deposit. On vigorous shaking produced fairly silky turbidity.
		7	As above.
F_1, F_6.	25°	5	No film, medium turbid (deep haze), scant deposit, fair non-silky turbidity on shaking.
		9	As above.
F_2, G_1.	25°	6	No film, medium slightly hazy, scant deposit. Poor turbidity (non-silky) on shaking.
		10	As above, but turbidity was slightly increased on shaking.
G_3, G_5.	25°	3	No film, medium slightly turbid, fair ropy deposit which did not break on shaking nor the turbidity increased.
		7	No film, medium clear, good ropy deposit as before, medium was highly viscid.

TABLE VIII.

Description of Growth in Standard Malt Wort.

Organisms.	Incubation Temp. °C.	Time Days.	
A1, A3, A4, A5, A_6, C_3, H_6.	30°	3	Medium clear, good powdery deposit, gives heavy silky turbidity on shaking.
		7	As above.
A_2, A7, B1, B_2, B_3, W_D, W_L.	30°	3	Medium slightly turbid, fair deposit and adherence to walls, gives moderate silky turbidity.
		7	Medium became clear, rest as above.
B_4, C_6, W7, G_2, G_4, D_2, W_1), W_4.	25°	3	Medium clear, scant deposit. In some cases suspension adhering to walls, fair silky turbidity on shaking.
		7	As above, except that deposit slightly increased.
B_5, E_1, E_2, E_3, D_5, C_1, E_4, E_5, E_6.	25°	3	Medium clear, good deposit, no suspension, etc. Produced good silky turbidity on shaking.
		7	As above.
C_2, C_4, C_5, C_7, C_8, H_1, H_3, H_5.	25°	4	Medium slightly turbid (hazy). No deposit. On shaking fair weak silky turbidity.
		7	As above, except that medium became clear.
D1, D_4, F_3, W1, W_5, H_2, H_4.	25°	3	Medium clear, moderate deposit and some adhering to the sides. Produced good silky turbidity.
		7	As above.
F_1, F_6.	25°	5	Slight turbid medium, some viscid deposit, fair non-silky turbidity on shaking.
		8	As above, except that medium became nearly clear.
F_2, G_1.	25°	6	Medium clear, scant viscid deposit, mere haziness arose on shaking.
		8	As above.
G_3, G_5.	25°	4	Medium slightly turbid, fair sticky ropy deposit, on shaking medium became viscid but no turbidity increased.
		7	As above, but deposit increased.

(c) The description of growth in glucose broth. The details of growth in this medium are summarised in Table IX.

It is interesting to note that again the similar types of growths were exhibited by the organisms as they did in beer medium and standard malt wort medium respectively, though the amount of growths in the case of all the organisms was decidedly decreased to a considerable extent, and which pointed out the fact that glucose broth medium did not contain some basic factor which could promote growth of the species to the extent the beer medium could do.

(d) The description of growth in nutrient broth (standard broth). The growth was greatly decreased in case of most of the organisms and some of the organisms did not show any sign of growth even after long periods of incubation. Wherever growth appeared it was identical with the growth in glucose broth and thus it was not described in detail.

(e) The description of growth in litmus milk. Litmus milk was prepared as described on page 74. The litmus milk tubes were inoculated by the standard inoculation procedure and were incubated at the respective optimum temperatures of the organisms in question. The tubes were examined at an interval of 24 hours. It was very interesting to note that, except two, none of the organisms showed any growth even after the incubation period of 21 days. Except these two tubes, none of the tubes showed any acidity or any other change.

TABLE IX.

Description of Growth in Glucose Broth.

Organisms.	Incubation Temp. °C.	Time Days.	
A_1, A_3, A_5, A_6, A_7, C_3, A_2, A_4.	30°	3	Medium clear, moderate deposit, good silky turbidity on shaking.
		7	As above, except that deposit increased slightly.
B_1, B_2, B_3, B_4, B_5, F_3, W_1, W_4, W_D, W_L, G_6.	30°	3	Medium clear, scant deposit, on shaking slight turbidity and silkiness produced.
		7	Medium clear, fair deposit, on shaking moderate silky turbidity produced.
C1, C2, C4, C5, C7, C8, H1, H2, H3, H5, W7, W10.	25°	4	Medium slight turbid (hazy), scant deposit, on shaking fair silky turbidity. In some cases, suspension and adherence to sides of the tubes.
		7	As above, except that on shaking, moderate turbidity was produced.
D1, D4, D5, H6, E2, E4 E5.	30°	3	Medium clear, some suspension and adherence to the walls, fair deposit, on shaking good silky turbidity.
		7	No change, except that there was no suspension or adherence.
E_1, E_3, E_6.	25°	3	Medium clear, good powdery deposit, good silky turbidity on shaking.
		7	As above.
G_2, G_4, H_4, W_5.	25°	3	Medium clear, fair deposit, fair turbidity on shaking (slightly silky).
		7	As above, except that silkiness increased considerably.
F1, G5, G1, F2, F6, G3.	25°	5	Medium clear, scant deposit, on shaking a mere haziness was produced.
		8	As above, except that haziness increased considerably.

PHYSIOLOGICAL CHARACTERISTICS.

The study of the physiological characteristics was undertaken by means of the standard technique. The data resulting from those examinations had been summarised as follows.

A. Relation to Temperature.

This characteristic was investigated in standard beer of PH 5·2 adjusted with the help of Johnson's PH Papers. The following temperatures were employed to incubate the cultures in question: 14°-16° (average 15°C.), 20°C., 25°, 30°, 35°, 38°, 43° and 48°C. and 50°C. The incubation period was continued up to 14 days. In each case two loopfuls of the young growing cultures in standard beer medium were added to each tube containing 8 ml. of unhopped beer medium, and the inoculated tubes were incubated at various temperatures mentioned above. The observations were made as frequently as possible. The time required for the organisms to develop viable growth at the respective temperatures was recorded in Table X. In all the cases time is expressed in hours. In Table XI is recorded the optimum temperature range and optimum temperature for the organisms examined.

From the above experiments it became evident that the growth of majority of the organisms, except a few, was somewhat delayed at 20°C., as compared to the development of the respective organism at a temperature in its optimum temperature range. At 15°C. growth was even more delayed, but in all the cases it

TABLE X.

The relative shortest time of Incubation, in hours, necessary for the development of the organisms at different temperatures.

Organisms.	15°C.	20°C.	25°C.	30°C.	35°C.	38°C.	43°C.	48°C.	50°C.
A_1, A_3, A_5, A_6, A_7, C_3, H_6.	72	36	24	22	17	19	30	36	-
A_2, A_4, E_4, E_5.	96	42	30	24	21	22	36	-	-
E_1, E_2, E_3, E_4, D_5.	96	48	36	22	22	24	48	-	-
F_3, D_1.	96	30	24	24	24	30	72	-	-
B_1, B_2, B_3, B_4, D_5, D_2, D_4, W_1, C_1, W_D, W_L.	120	42	36	24	24	30	-	-	-
H_4, W_4, W_5.	96	46	36	30	30	36	-	-	-
C_2, C_4, C_5, C_7, C_8, G_2, G_4, G_6, W_7, W_{10}.	144	54	48	48	96 very weak.	-	-	-	-
H_1, H_2, H_3, H_5.	192	96	96	144	-	-	-	-	-
F_1, F_6.	192	96	120	168	-	-	-	-	-
F_2, G_1.	192	144	144	-	-	-	-	-	-
G_3, G_5.	192	100	78	144	-	-	-	-	-

became evident within 9 days of incubation. It was also of interest to notice that none of the organisms grew at 50°C. and only 7 organisms grew at 48°C.

As regards the optimum temperatures of those organisms, only 11 out of the 52 organisms have an optimum of about 37°C., while the optimum temperature range for the majority of organisms was between 25° and 30°C.

When the results at 25°C. were scrutinised it could be seen that at that temperature all the organisms developed with fair speed, in fact the difference between the time required at 25°C. and that required at the respective optimum temperature was in each case small. But it is the opinion of the writer that the experiments concerning the morphological and cultural and physiological characteristics should be carried out, wherever possible, at the respective optimum temperature, or at the temperature from the optimum temperature range. For the preliminary experiments, it was found advisable to use 25°C. as the incubation temperature, as at higher temperatures some organisms would bt not only discouraged to develop but some might be altogether prevented from growing due to the relatively high temperature of incubation.

Apart from the variation in the character of growth between different organisms, the character of growth of any one individual organism was, broadly speaking, of the same type, irrespective of incubation temperature, differing only in minute details of growth. Generally speaking, growth was much weaker at higher temperatures, though not delayed to the same degree

TABLE XI.

Optimum temperatures, and the range of optimum temperatures of the organisms.

Organisms.	Optimum Temp. in °C.	Optimum Temp. range.	Temp. range of growth.
A_1, A_3, A_5, A_6, A_7, C_3, H_6.	37°C.	35 - 38°C.	15 - 48°C.
A_2, A_4, E_4, E_5.	37°C.	35 - 38°C.	15 - 43°C.
E_1, E_2, E_3, E_6, D_5.	33°C.	30 - 35°C.	15 - 43°C.
F_3, D_1.	33°C.	25 - 35°C.	15 - 43°C.
B_1, B_2, B_3, B_4, B_5, D_2, D_4, W_1, W_D, W_L, C_1.	33°C.	30 - 35°C.	15 - 38°C.
H_4, W_4, W_5.	33°C.	30 - 35°C.	15 - 38°C.
C_2, C_4, C_5, C_7, C_8, G_2, G_4, G_6, W_7, W_{10}.	25-26°C.	25 - 30°C.	15 - 35°C.
H_1, H_2, H_3, H_5.	23°C.	20 - 25°C.	15 - 30°C.
F_1, F_6.	22-23°C.	20 - 25°C.	15 - 30°C.
F_2, G_1.	22-23°C.	20 - 25°C.	15 - 25°C.
G_3, G_5.	25°C.	20 - 25°C.	15 - 30°C.

Note: Cultures were not grown below 15°C.

as at lower temperatures.

The optimum temperatures proved of considerable value for differentiating some of the organisms in question, but that should not be generalised as there were some organisms which showed very little difference in the speed of development within a relatively broad temperature range.

B. Relation to Hydrogen Ion Concentration (PH).

This characteristic was examined in the standard beer medium. The PH values of this medium were adjusted with the help of Johnson's PH papers, so as to be as near as possible to the following figures: 7·3, 6·9, 6·6, 5, 5·6, 5, 4·8, 4·5 and 3·7, respectively.

The tubes of beer medium adjusted to the respective PH values were inoculated with the standard inoculum of the respective organisms and then incubated at the optimum temperatures of the organisms in question. The development of the growth was regularly observed at an interval of 12-13 hours. The data collected during the examination are summarised in Tables XII and XIII, showing the minimum time of incubation required for the perceptible viable growth by the individual organisms, and the time after which the growth became strongest as well as the relative strength of growth.

From the data in Tables XII and XIII, it became evident that the lactic acid bacteria species described in this thesis had their optimum range between PH 5·6 to PH 5. Therefore, the species examined definitely preferred an acid medium. At PH 7·3 only half of the organisms in question developed,

TABLE XII.

Minimum time required, in hours, for perceptible growth at various PH values.

ORGANISMS.	3·6	4·2	4·6	5	5·6	6	6·6	6·9	7·3
A1, A3, A5, A6, A7, C3, H6, D4.	24	24	24	24	24	24	24	24	24
H4, E4, WL, D1, D2, D5, E1, E2, E3, E5, A2, E6, A4, WD, F3.	36	36	24	24	24	24	24	24	24
B1, B3, W4.	96	36	24	24	24	24	24	36	72
B2, B4, B5, C1.	96	72	24	24	24	36	72	96	96
W1, W5.	72	72	36	24	24	24	24	36	72
C8, H1, H2, C2, C7, H3, H5.	-	156	156	96	96	96	96	156	-
G2, G4, G6.	108	108	96	96	72	72	96	144	-
W7, W10, C4, C5.	144	144	72	36	36	36	36	32	-
F1, F6.	156	156	144	96	96	120	156	-	-
G1, F2.	-	156	156	144	144	144	156	-	-
G3, G5.	-	144	96	96	96	96	96	156	-

TABLE XIII.

Description of the time required, in days, to attain the maximum growth at various PH values.

(Time described in days.)

	3·6	4·2	4·6	5	5·6	6	6·6	6·9	7·3	Optimum range.
A1, A3, A5, A6, A7, C3, H6, D4.	3 days. Moderate.	3 days. Moderate.	3 days. Strong.	2 days. Strong.	2 days. Strong.	3 days. Strong.	4 days. Strong.	6½ days. Strong.	6½ days. Moderate.	4·6 - 5·6
H4, D1, D2, E4, D5, WL, E1, E2, E3, E5, E6, F3, A2, A4, WD.	4 days. Fair.	4 days. Moderate.	3 days. Strong.	3 days. Strong.	3 days. Strong.	3 days. Moderate.	4 days. Moderate.	4 days. Moderate.	4 days. Moderate.	4·6 - 5·6
B1, B3, W4.	6 days. Fair.	4 days. Moderate.	4 days. Moderate.	4 days. Moderate.	4 days. Strong.	5 days. Strong.	3 days. Moderate.	4 days. Moderate.	4 days. Fair.	4·6 - 6
B2, B4, B5, C1.	6 days. Fair.	4 days. Moderate.	4 days. Strong.	4 days. Strong.	4 days. Strong.	5 days. Strong.	6 days. Moderate.	6½ days. Moderate.	6 days. Fair.	4·6 - 6
W1, W5.	6½ days. Moderate.	5 days. Moderate.	4 days. Moderate.	4 days. Strong.	4 days. Strong.	5 days. Strong.	5 days. Strong.	4 days. Moderate.	6 days. Moderate.	4·6 - 6
C8, H1, H2, H3, H5, C2, C7.	-	7½ days. Fair.	7½ days. Moderate.	6 days. Moderate.	6 days. Moderate.	7 days. Moderate.	7 days. Moderate.	8 days. Fair.	-	5 - 5·6
G2, G4, G6.	6½ days. Fair.	6½ days. Fair.	6½ days. Moderate.	5 days. Moderate.	5 days. Moderate.	6½ days. Moderate.	7½ days. Moderate.	8 days. Fair.	-	5 - 5·6
C4, C5, W7, W10.	7½ days. Fair.	7 days. Fair.	4 days. Moderate.	6½ days. Strong.	6½ days. Strong.	6½ days. Strong.	8 days. Strong.	7 days. Moderate.	-	5 - 6
F1, F6.	9½ days. Fair.	9 days. Fair.	8 days. Moderate.	7½ days. Moderate.	7½ days. Moderate.	7½ days. Moderate.	8½ days. Moderate.	-	-	5 - 6
G1, F2.	-	9 days. Fair.	8 days. Fair.	8 days. Moderate.	8 days. Moderate.	8 days. Moderate.	9 days. Moderate.	- -	- -	5 - 6
G3, G5.	-	6½ days. Fair.	10½ days. Moderate.	7½ days. Moderate.	7½ days. Moderate.	7½ days. Moderate.	8 days. Moderate.	10 days. Moderate.	-	5 - 6

while others did not grow even after the prolonged incubation period of four weeks. At PH 3·6 there was delay in development with a large number of organisms, but after continued incubation as much as 45 cultures showed the growth, though the growth in some cases was very weak as compared to the others, while the remaining 7 cultures did not show any growth at all.

The behaviour of organisms at the various PH values provided with valuable information which could used as an additional feature, in conjunction with other properties, for the differentiation among the species within the lactic acid forming bacteria group.

C. Relation to Oxygen Requirements.

This characteristic was not investigated by any standard technique, but was sought for, in the types of growth in the liquid medium, in the stabs, on the slopes and on the plates. It was observed that none of the organisms in question formed any film or ring on the surface of the liquid medium. The growth was always better in all the cases when the medium in the test tubes was taken more (8-10 ml.). Except in a few cases there was no trace of film or growth at the surface of the stab cultures, and the growth in stabs was far greater than the respective slopes which were incubated aerobically. The plate and slope cultures grew very poorly when incubated aerobically, but the growth considerably increased in all the cases when the cultures were incubated in the atmosphere of carbon dioxide. In most of the cases the plate cultures formed subsurfaced colonies. All those observations lead to

the conclusion that the organisms in question required very small amount of oxygen or none at all.

D. Heat Resistance.

This experiment was performed in the standard beer medium cultures as follows: organisms in question were subcultured and were incubated at their respective optimum temperatures. When the cultures were in their logarithmic phase of growth, two loopfulls of the inoculum from these cultures was inoculated in 6 ml. of fresh sterile medium. The tubes were shaken well so as to mix the inoculum, were heated in a water bath at 64° to 66°C. for 15 minutes, and were instantly cooled in the cold water bath to the room temperature before being incubated at their respective optimum temperatures. The tubes were examined every day for growth. The minimum time required for viable growth, and the time when the growth became strongest was noted for every organism which showed growth. The organisms which did not grow during that period were incubated for three weeks and then the incubation was abandoned.

This experiment divided the organisms in two major groups, those which grew after that treatment (heat resistant) and those which failed to grow (heat labile). The heat resistant group could be subdivided into the various groups according to the time taken in growing after the heat treatment, that is, whether the time taken for growth was the same as before treatment or was delayed by that treatment. If the delay was observed, then the cultures were grouped according to the time required for the appearance of growth.

TABLE XIV.

Heat Resistance shown by the Organisms.

(Heated at 64-65°C. for 15 minutes.)

Organisms.	Time taken for the appearance of growth after heat treatment.	Usual time for growth.
A_1, A_3, A_5, A_6, A_7, D_1, D_5, A_2, A_4.	3 days.	1 day.
D_2, D_4, E_1, E_4, E_5, E_6, G_2, G_4, W_1.	5 days.	1 day.
W_4, W_5, C_3, W_L, B_1, H_6, B_3, E_3, E_2.	7 days.	1 day.
F_3, W_D, B_2, B_5, W_7, H_4.	10 days.	1 day.
C_4, C_1, C_7, H_1, H_5, H_2, C_2.	11 days.	3-4 days.
B_4, W_{10}.	13 days.	1-2 days.
C_5, C_8, F_1, F_2, F_6, G_1, G_3, G_5, G_6, H_3.	Did not show any growth even after incubation of 21 days.	most of them grow after 4-5 days.

The information collected by the above experiment, summarised in Table XIV, was proved to be of considerable value in conjunction with the other properties, in differentiating the species among themselves.

E. Resistance towards Hop Antiseptics.

This characteristic was sought for in the standard beer medium. Fairly pure humulone was employed in the following concentrations, 0·00025 per cent, 0·0005 per cent, 0·001 per cent, 0·002 per cent and 0·003 per cent. The alcoholic solution of humulone in the required concentrations was added to the sterile beer tubes and tubes were again steamed for five minutes. On cooling, tubes were inoculated by the standard inoculation technique (dropping pipettes were used), using young vigorous cultures in each case, and were incubated at their respective optimum temperatures. A complete set of unhopped beer tubes was also inoculated at the same time and was placed with the above tubes for control purposes. One uninoculated tube, each of the various humulone concentrations was also incubated with the above tubes, to be used as controls. The tubes were examined after the incubation period of 1, 3, 4, 6, 8, 10, 17 and 24 days.

From this experiment it was found that most of the organisms could not grow in 0·003 per cent concentration of humulone, even after the incubation period of 24 days, while only one-third of the total number of organisms could grow in 0·002 per cent concentration after the prolonged incubation for 17 to 24 days. The results are summarised in Table XV.

TABLE XV.

Influence of Humulone Concentration on the development of the Organisms.

(Time expressed in days.)

Organisms.	0·00025%		0·0005%		0·001%		0·002%		0·003%		No Humulone.	
	First appearance of growth.	Maximum Growth.	First appearance of growth.	Maximum Growth.	First appearance of growth.	Maximum Growth.	First appearance of growth.	Maximum Growth.	First appearance of growth.	Maximum Growth.	First appearance of growth.	Maximum Growth.
B_1, B_2, B_3, B_4, B_5, W_1, W_4, W_D, W_L, W_5, D_2, D_4.	1	4	1	8	3	10	8	10	21	No change.	1	3
C_1, H_1, H_2, H_3, H_5, G_2, G_4, G_6.	4	8	4	8	5	9	5	9	-	-	3	7
W_{10}, W_7, D_1, F_3, C_7.	3	6	3	6	-	-	-	-	-	-	1	3
D_5, E_1, E_2, E_3, E_6, C_3, H_6, H_4, C_8.	3	6	4	8	21	-	-	-	-	-	1	3
A_1, A_2, A_3, A_4, A_5, A_6, A_7, E_4, E_5.	3	6	6	8	-	-	-	-	-	-	1	3
C_2, C_4, C_5.	4	9	4	9	-	-	-	-	-	-	4	8
F_1, F_6.	4	6	4	6	8	10	17	No change.	-	-	4	6
F_2, G_1.	17	Same after 21 days.	-	-	-	-	-	-	-	-	6	8
G_3, G_5.	3	6	3	6	4	10	-	-	-	-	3	6

The type of growth in hopped and unhopped tubes was practically the same. The experiment had shown that the lactic acid bacteria species in question were much less resistant to hop antiseptics as compared to Acetobacter species and less than ½ lb. per barrel, of hops, were sufficient to prevent their growth completely.

In the opinion of the writer, this characteristic of the organisms can be employed as a genus characteristic.

F. Resistance towards Ethyl Alcohol.

This characteristic was investigated in the usual beer medium. Different concentrations of absolute ethyl alcohol were added aseptically to the sterile beer tubes containing 10 ml. medium in each tube. The various concentration of alcohol employed were following: 2%, 4%, 6%, 8% and 10%. The tubes were inoculated by the standard technique, using vigorous and young organisms, and were incubated at 25°C. so as to minimise the evaporation of alcohol.

It was very interesting to note that except 14 organisms all the rest grew in 10% concentration of alcohol, though after a lag period of 2-4 days in most cases, as compared to the growth in alcohol free medium, while as many as 46 organisms grew in 8% concentrations. The results of this experiment are summarised in Table XVI.

From the above experiment it was noted that lactic acid bacteria species are fairly tolerant towards alcohol, and this characteristic could be employed as an additional feature in characterisation of species within the lactic acid bacteria

TABLE XVI.

Influence of Ethyl Alcohol Concentration on the Development of Lactic Acid Bacteria Species.

(Time expressed in days.)

Organisms.	2%		4%		6%		8%		10%		No Alcohol.	
	First appearance of growth.	Maximum Growth.	First appearance of growth.	Maximum Growth.	First appearance of growth.	Maximum Growth.	First appearance of growth.	Maximum Growth.	First appearance of growth.	Maximum Growth.	First appearance of growth.	Maximum Growth.
A_1, A_3, A_5, A_6, A_7, C_3, D_1, D_5, H_6, E_2, F_3.	1	3	1	3	1	3	1	3	3	4	1	3
B_1, B_3, B_4, W_D, B_2, B_5, C_1.	1	3	1	3	1	4	3	6	4	8	1	3
C_2, C_4, C_5, C_7, C_8, W_7, W_{10}.	3	6	3	6	3	6	-	-	-	-	3	6
D_2, D_4, H_4, W_1, W_4, E_3, E_4, A_2, W_5, E_1, E_5, E_6, W_L, A_4.	1	3	1	3	1	3	1	3	1	4	1	3
G_2, G_4, H_3, H_5, G_6, H_1, H_2.	3	6	3	6	3	6	17	-	-	-	3	6
F_1, F_6.	4	6	4	6	6	8	6	8	-	-	4	6
G_1, F_2.	6	8	6	8	6	8	-	-	-	-	6	8
G_3, G_5.	3	4	3	6	3	6	3	6	-	-	3	6

group.

BIOCHEMICAL CHARACTERISTICS.

A. Acid Formation.

This characteristic was investigated in double casein digest + 5% yeast autolysate medium, using the various carbohydrates and alcohols, each in 2% concentration separately. The carbohydrates and alcohol used were the following: xylose, arabinose, glucose, fructose, galactose, raffinose, lactose, mannose, maltose, sucrose, mannitol, salicin and inulin. Bromo Cresol green indicator (PH range:- yellow $<3\cdot6$, green $5\cdot2<$blue) was used for the detection of acid formation. The tubes were inoculated by the standard technique and were incubated at the respective optimum temperatures of the organisms in question. The time after which the change of colour took place was recorded. Often the incubation period was continued for 14 days. The data obtained through this experiment is summarised in Table XVII.

It was interesting to note that considerably varied results were obtained with most of the organisms. Glucose was attacked by all the organisms, with the formation of acid. Fructose, maltose and arabinose were next to glucose most readily attacked by the majority of the organisms. Lactose was attacked by only two organisms, while the rest of the carbohydrates and alcohol were attacked by some organisms, while the others did not attack them at all.

TABLE XVII.

Description of Growth and Acid production by the organisms with various carbohydrates.

Organisms.	Xylose.	Arabinose.	Glucose.	Fructose.	Mannose.	Galactose.	Sucrose.	Maltose.	Lactose.	Raffinose.	Mannitol.	Salicin.	Inulin.
A1, A3, A5, A2, A4, A6.	– –	– –	++ oo	++ oo	++ oo	++ oo	++ oo	++ oo	– –	– –	++ oo	++ oo	++ oo
E4, E5, A7, E6.	++ oo	– –	++ oo	++ oo	++ oo	++ oo	++ oo	++ oo	– –	– –	++ oo	++ oo	++ oo
E1, E2, E3, F3, D1, D5.	++ oo	++ oo	++ oo	++ oo	++ oo	++ oo	++ oo	++ oo	+ –	++ oo	+ o	++ oo	++ oo
B1, B2, B3, B4, WD, WL, H4.	++ o	++ oo	++ oo	++ oo	– –	++ oo	– –	++ oo	– –	– –	++ oo	+ –	(+) –
D2, D4, W1, W4, W5, C1, B5.	– –	++ oo	++ oo	++ oo	++ oo	++ oo	– –	++ oo	– –	++ oo	+ o	++ oo	++ oo
G2, G4, G6.	– –	++ oo	++ oo	++ oo	++ oo	++ oo	+ o	++ oo	+ o	++ oo	– –	++ oo	++ oo
C7, C8, C2.	– –	++ oo	++ oo	++ oo	++ oo	++ oo	– –	++ oo	+ o	++ oo	++ oo	– –	++ oo
C5, W7, W10, C4.	++ oo	++ oo	++ oo	++ oo	– –	++ oo	– –	++ oo	– –	(+) o	+ –	++ –	– –
C3, H6.	++ oo	++ oo	++ oo	++ oo	++ oo	++ oo	++ oo	++ oo	++ oo	++ oo	++ o	++ oo	++ o
H1, H2, H3, H5.	++ oo	++ oo	++ oo	++ oo	++ oo	(+) –	++ oo	++ oo	– –	– –	+ –	++ –	+ –
F1, F6.	– –	(+) (o)	++ oo	++ oo	++ oo	++ oo	++ oo	++ oo	– –	– –	– –	++ oo	++ oo
F2, G1.	– –	++ oo	++ oo	++ oo	++ oo	– –	– –	++ oo	– –	+ o	+ –	++ oo	++ oo
G3, G5.	– –	– –	++ oo	++ oo	++ oo	+ –	– –	++ –	– –	(+) –	(+) –	++ oo	++ –

Symbols used:

Good growth	++
Poor growth	+
Strong acidity	oo
Weak acidity	o
No acid or growth	–

( ) Indicates doubtful

Note. No gas production was observed in any of the cases.

In connection with the above results, it may be of interest to mention that occasionally it might happen that two strains of the same species may not behave identically as regards the formation of acid from a particular substance (31,34). Thus this characteristic should not be employed singly for the characterisation of the lactic acid bacteria species, but if used in addition to other features of a species, it may prove to be one of the most useful characteristics which could be employed for the differentiation of lactic acid bacteria species.

Production of acid was also tested in beer medium. In all cases lactic acid was detected with the other acids.

B. Gas Formation.

This characteristic was sought for in conjunction with the above experiment carried out for the determination of acid formation by various carbohydrates and alcohols. Durham tubes were put in the test tubes prior to the sterilisation, so as to remove all the air from the Durham tubes.

There was not a single case among the species examined in which gas evolution in Durham tubes was noted.

C. Liquefaction of Gelatin.

This characteristic was sought for during the examination of growth of the species in the gelatin beer stabs, and gelatin beer slopes and plates.

During these examinations it was found that there was no liquefaction of gelatin by any of the species examined.

D. The presence of acetyl methyl carbinol was tested for by the latest Barritt's modification (283), which offers a high sensitivity, enabling detection of one part of diacetyl and acetyl methyl carbinol in 2,500,000 parts of the substrate. The medium used is described on page 75 of this thesis. The inoculated tubes were incubated at the optimum temperatures of the respective organisms in question, and were examined after growth had become strong. It was interesting to note that about half of the organisms in question gave the positive reaction, while the other half showed the negative reaction.

Catalase Reaction. Standard beer agar slopes, prepared as described under (C) page 74 of this thesis, were inoculated with the organisms in question and incubated, at 25°C. in the atmosphere of carbon dioxide (anaerobically), till the logarithmic phase had been reached individually by all the cultures to be examined. The slopes were then flooded with the hydrogen peroxide solution (page 75) and left standing for about 15-20 minutes. In none of the cases did the evolution of gas (effervescence) take place. It was thus noted that all the organisms in question were catalase negative.

Indole production was tested by the Ehrlich-Böhme method (284). The organisms were grown in peptone broth containing trytophan. When the fair or good growth appeared in the tubes, the organisms were shaken with 1 ml. of xylol so as to concentrate the indole at the surface, which is soluble in xylol. After allowing the tubes to stand for a few minutes, 2 ml. of Ehrlich reagent was then gently added to each tube in such a way to allow the reagent to remain

between xylol and the medium. A development of pink colour at the junction of xylol and reagent would indicate the presence of indole.

It was interesting to note that a negative result was shown by all the organisms in question. Even when the organisms were grown in the presence of glucose (to ensure better growth), negative reaction was indicated.

Nitrate Reduction. This characteristic was investigated as described below. The organisms were grown in nitrate broth, which was prepared as described on page 75, at their respective optimum temperatures. 1 ml. of each culture was transferred to another tube, and to each of these tubes (containing 1 ml. medium) 2 drops of sulphanilic acid solution and 2 drops of α-napthylamine solution were added and the contents were allowed to stand after shaking. The presence of nitrate would be indicated by a pink or red colour.

None of the organisms in question showed the nitrate reduction test.

Determination of volatile and non-volatile acidity.

For this experiment the cultures were grown in 100 ml. conical flasks containing 50 ml. of double casein digest + yeast autolysate medium at initial PH 5·1 to 5·3, to which glucose (5% W/V) was added and incubated at 25°C. for fourteen days. At the end of the incubation period 10 ml. of culture was diluted to 40 ml. with distilled water and titrated with N/100 barium hydroxide, using bromothymol blue as indicator. The titre obtained was termed total acidity. 10 ml. of uninoculated medium was similarly titrated

after the dilution. This titre was subtracted from the total acidity to give the final total acidity.

Another 10 ml. of culture was diluted as before and was steam distilled, 250 ml. of distillate being collected, and the whole was titrated as described above. This titre was termed total volatile acidity. The control medium (10 ml.) was treated exactly as the culture medium. The titre obtained by titrating 250 ml. distillate in this case was subtracted from the total volatile acidity to give the final volatile acidity.

On subtracting final volatile acidity from the final total acidity the value for the non-volatile acidity was obtained.

Molecular ratio of non-volatile acidity to volatile acidity was calculated in terms of lactic acid to acetic acid respectively.

For the purpose of classification it has been assumed that an organism giving the molecular ratio of lactic acid to acetic acid of less than 100 : 10 was heterofermentative, while an organism giving the ratio greater than 100 : 10 was homofermentative, thus, for example, 100 : 16 would be regarded as indicative of heterofermentation and 100 : 6 would be considered to be homofermentation.

The results of these experiments have been summarised in Tables XVIII and XIX.

TABLE XVIII.

Description of other biochemical characteristics.

Acetyl methyl carbinol producing.	Acetyl methyl carbinol not producing.
A_1, A_2, A_3, A_4, A_5, A_6, A_7, E_1, E_2,	B_1, B_2, B_3, B_4, B_5, C_1, C_3,
E_4, E_3, E_5, E_6, D_5, C_2, D_1, F_1, F_6,	C_4, C_5, D_2, D_4, H_1, H_2, H_3,
G_1, F_3, F_2, G_2, G_4, C_7, C_8, G_6.	H_5, H_6, H_4, W_1, W_4, W_5, W_7,
	W_K, W_D, W_L, G_3, G_5.

1. All the organisms in question neither reduced nitrate nor produced Indole.
2. All showed catalase negative reaction.
3. Gelatin was not liquefied by any of the organisms.

TABLE XIX.

Details of Acid Production.

Organisms.	Total acidity.	Volatile acidity.	Non-volatile acidity.	Molecular ratio of non-volatile and volatile acids in terms of lactic acid and acetic acid respectively.	Type of Fermentation.
A1	114	8·4	105·6	100 : 7·5	Homo.
A_2	111	9	102	100 : 8·8	"
A_3	115	8·7	106·3	100 : 8·4	"
A_4	108	9	99	100 : 9·9	"
A_5	116	8	108	100 : 8·3	"
A_6	111	9	102	100 : 8·8	"
A_7	116	7·5	108·5	100 : 6·7	"
B_1	77	13·7	63·3	100 : 21·6	Hetero.
B_2	76.2	13·2	63	100 : 21	"
B_3	52	15	37	100 : 40·5	"
B_4	30	8·5	21·5	100 : 40	"
B_5	44·5	12·5	32	100 : 39·5	"
C_1	58	6	52	100 : 11·5	"
C_2	67	3·5	63·5	100 : 5·6	Homo.
C_3	67·2	10·2	56	100 : 18·1	Hetero.
C_4	57·7	6·7	51	100 : 13·1	Hetero.
C_5	55·5	6·5	49	100 : 13·2	Hetero.
C_7	41·5	3·5	38	100 : 9·1	Homo.
C_8	58·8	3·8	55	100 : 7·5	"
D_1	121	17	104	100 : 16·4	Hetero.
D_2	74	18	56	100 : 32	"
D_4	65	17	48	100 : 35·4	"
D_5	52	10	42	100 : 23·8	"
E_1	45·5	75	38	100 : 18·6	"
E_2	115	13	102	100 : 12·9	"
E_3	35	4	31	100 : 12·6	"
E_4	106·5	9	97·5	100 : 9·7	Homo.

Details of Acid Production (continued).

Organisms.	Total acidity.	Volatile acidity.	Non-volatile acidity.	Molecular ratio of non-volatile and volatile acids in terms of lactic acid and acetic acid respectively.	Type of Fermentation.
E_5	107·5	9·5	98	100 : 9·6	Homo.
E_6	36·5	4·5	32	100 : 14	Hetero.
F_1	79.5	6·5	73	100 : 8·8	Homo.
F_2	64	2	62	100 : 3·3	"
F_3	60	13	47	100 : 27·6	Hetero
F_6	83	1·5	81·5	100 : 1·9	Homo.
G_1	26	2	24	100 : 8·2	"
G_2	79	7	72	100 : 9·6	"
G_3	64·8	2·8	62	100 : 4·5	"
G_4	82	5·5	76·5	100 : 7·2	"
G_5	64	2	62	100 : 3·3	"
G_6	76	5	71	100 : 7·1	"
H_1	52	6	46	100 : 13·1	Hetero.
H_2	64·5	11·5	53	100 : 21·6	"
H_3	62	10	52	100 : 19·5	"
H_4	114	44	70	100 : 61	"
H_5	54	8	46	100 : 16·5	"
H_6	136	38	98	100 : 38	"
W_1	75·8	18·8	57	100 : 32	"
W_4	68	20	48	100 : 41	"
W_5	80	25	55	100 : 45	"
W_7	63	11	52	100 : 21	"
W_{10}	70	18	52	100 : 34·5	"
W_D	55	17	38	100 : 43·6	"
W_L	60	14	46	100 : 30·5	"

IDENTIFICATION AND NAMING OF THE ORGANISMS.

After all the characteristics described in the above paragraphs were noted down, the 52 cultures described in this thesis were separated into two groups, one comprising the rod shaped lactobacilli and the other the coccoid shaped organisms, i.e. the streptococci.

All the lactobacilli were then regrouped by taking into account the following major characteristics - namely, the maximum temperature allowing growth, optimum temperature, the homo- or hetero-fermentative nature of the culture and the production of acetyl methyl carbinol.

From the Table XXA it can be seen that the lactobacilli fall into seven different groups. The organisms within the each group were found to have the same optimum PH values as well as maximum and minimum PH values allowing growth. In addition to that they showed similar response to humulone concentration, and identical cultural, morphological characteristics, with very little differences. This may be seen from the Tables III to XIX.

The six streptococci were also regrouped as above. Table XXB shows that the cocci fall into three different groups. The organisms within each group showed similar morphological, cultural and biochemical characteristics with very small differences.

Hence all the 52 organisms in question were found to fall in ten different groups as follows:-

TABLE IV A.

Temperatures.

Grew at 48°C. but not at 50°C.
A1, A3, A5, A6, A7, C3, H6.

- Homo: A1, A3, A6, A7, A5
  - \+ Acetyl methyl carbinol: A1, A3, A6, A7, A5. Optimum temp. 37°C. PH range 4·6-5·6.
  - − None.
- Hetero: C3, H6.
  - \+ None.
  - − C3, H6. Optimum temp. 37°. PH range 5-5·6. Gp. 7 C3, H6.

Similar morphological and cultural characteristics.

Gp.1
A1, A3, A6, A7, A5
A2, A4, E4, E5
Similar morphological and cultural characteristics.

Grew at 43°C. but not at 46°C.
A2, A4, E4, E5, E1, E2, E3, E6, D5, D1, F3.

- Homo: A2, A4, E4, E5.
  - \+ A2, A4, E4, E5. Optimum temp. 37°. PH range 4·6-5·6.
  - − None.
- Hetero: E1, E2, E3, E6, D1, D5, F3.
  - \+ E1, E2, E3, E6, D1, D5, F3. Optimum temp. 33°C. PH range 4·6 to 5·6. Gp. 2 E1, E2, E3, E6, D1, D5, F3. Similar morphological and cultural characteristics.
  - − None.

Grew at 38°C. but not at 40°C.
B1, B2, B3, B4, B5, C1, D2, D4, H4, W1, W4, W5, WD, WL.

- Homo: None.
  - \+ None.
  - − None.
- Hetero: B1, B2, B3, B4, B5, C1, D2, D4, H4, W1, W4, W5, WD, WL.
  - \+ B1, B2, B3, B4, B5, C1, D2, D4, H4, W1, W4, W5, WD, WL. Optimum temp. 33°C. PH range 4·6 to 6. Gp. 3 B1, B2, B3, B4, B5, C1, D2, D4, H4, W1, W4, W5, WD, WL. Similar morphological and cultural characteristics.
  - − None.

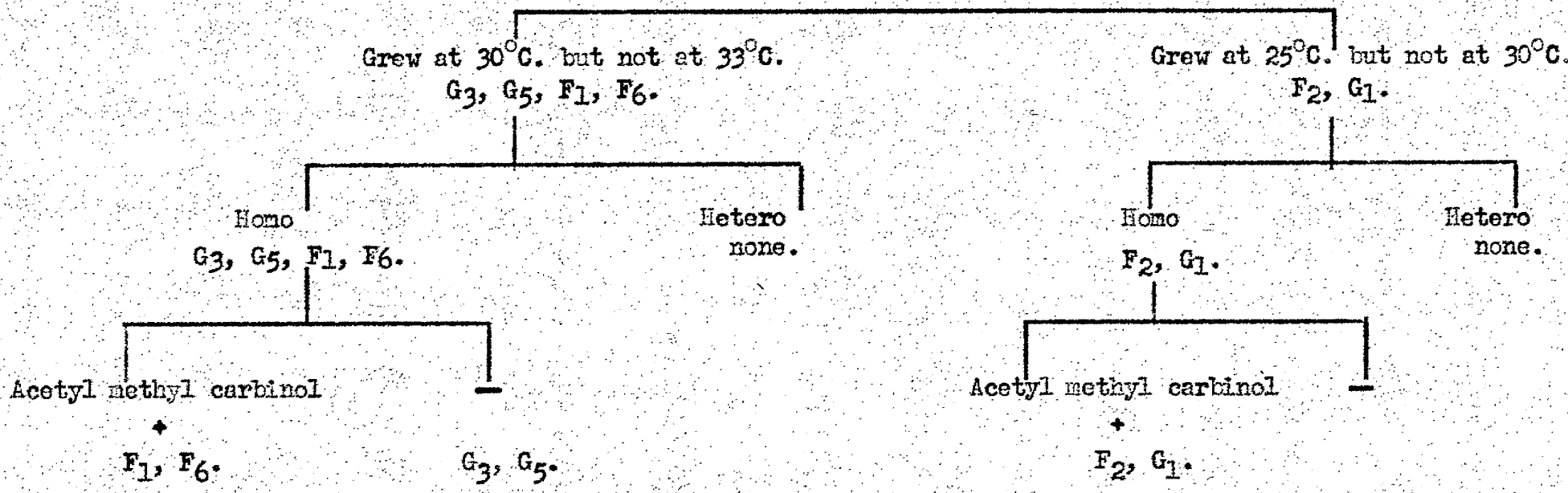
Grew at 35°C. but not at 38°C.
C2, C4, C5, C7, C8, G2, G4, G6, W7, W10.

- Homo: C2, C7, C8, G2, G4, G6.
  - \+ C2, C7, C8, G2, G4, G6. Optimum temp. 25°C. PH range 5-5·6. Gp. 4. C2, C7, C8, G2, G4, G6. Similar morphological and cultural characteristics.
  - − None.
- Hetero: C4, C5, W7, W10.
  - \+ C4, C5, W7, W10. Optimum temp. 25-26°C. PH Range, 5-6. Gp. 5 C4, C5, W7, W10. Similar morphological and cultural characteristics.
  - − None.

Grew at 30°C. but not at 33°C.
H1, H2, H3, H5.

- Homo: None.
  - \+ None.
  - − None.
- Hetero: H1, H2, H3, H5.
  - \+ H1, H2, H3, H5. Optimum temp. 23°C. PH range 5-5·6. Gp. 6 H1, H2, H3, H5. Similar morphological & cultural characteristics.
  - − None.

TABLE XXB.



1. Optimum temp. 23°C.
2. Does not produce viscid medium.
3. Similar morphological and cultural characters.

Gp. 8
F_1, F_6.

1. Optimum temp. 25°C.
2. Produce highly viscid medium.
3. Similar morphological and cultural characters.

Gp. 9
G_3, G_5.

1. Optimum temp. 21-22°C.
2. Does not produce viscid medium.
3. Similar morphological and cultural characters.

Gp. 10
F_2, G_1.

Group 1. A_1, A_2, A_3, A_4, A_5, A_6, A_7, E_4, E_5.

Group 2. E_1, E_2, E_3, E_6, D_1, D_5, F_3.

Group 3. B_1, B_2, B_3, B_4, B_5, C_1, D_2, D_4, H_4, W_1, W_4, W_5, W_D, W_L.

Group 4. C_2, C_7, C_8, G_2, G_4, G_6.

Group 5. C_4, C_5, W_7, W_{10}.

Group 6. H_1, H_2, H_3, H_5.

Group 7. C_3, H_6.

Group 8. F_1, F_6.

Group 9. G_3, G_5.

Group 10. G_1, F_2.

A complete survey of the literature available was carried out to identify these organisms. This survey showed that in most of the cases members of one particular group had similar morphological, cultural, physiological and biochemical characteristics, with very little differences, from those of one particular Lactobacillus species already known and described in the literature. In this way members comprising six groups out of the ten groups could be identified as follows:-

Group 1. A_1, A_2, A_3, A_4, A_5, A_6, A_7, E_4, E_5 as varieties of or as strains of L. leichmannii, Bergey et al. (For references see 23,35.)

Group 2. E_1, E_2, E_3, E_6, D_1, D_5, F_3 as varieties of or as strains of L. bifidus, Tissier. (For reference see 35,285.)

Group 3. B_1, B_2, B_3, B_4, B_5, C_1, D_2, D_4, H_4, W_1, W_4, W_5, W_D, W_L as varieties of or as strains of L. pastoriamus, Van Laer. (For references see 23,35.)

Group 4.	C_2, C_7, C_8, G_2, G_4, G_6	as varieties of or as strains of *L. plantarum*, Orla-Jensen. (For references see 35,286,287.)
Group 7.	C_3, H_6	as varieties of or as strains of *L. buchneri*, Henneberg. (For reference see 35.)
Group 9.	G_3, G_5	as varieties of or as strains of *Str. mucilaginosus*, Kulka, Cosbie and Walker. (For reference see 30.)

Cocci comprising group 8 (F_1 and F_6) in most of their cultural, physiological and biochemical characteristics resembled *Str. damnosus*, but were found to be smaller in size as compared to *Str. damnosus* (0·6 to 0·7 μ against 1 to 1·5 μ).

Characteristics of *Str. damnosus* (23).	Characteristics of Group 8 (F_1, F_6) organisms.
Spheres of 1 to 1·5 μ in diameter.	Spherical cells of 0·6 to 0·7 μ in diameter.
Growth at 15°C., no growth at 33°C.	Growth at 15°C. and 30°C., but not at 33°C. or above.
Optimum temperature 21-25°C.	Optimum temp. range 20-25°C., optimum temp. 23°C.
Facultative anaerobe.	Facultative anaerobe.
Acetyl methyl carbinol produced.	Acetyl methyl carbinol produced.
Homofermentative.	Homofermentative.
Acid and growth with glucose, fructose, maltose and sucrose. Arabinose, xylose, lactose and salicin are not attacked.	Acid and growth with glucose, fructose, maltose, sucrose, mannose, and galactose. Salicin, arabinose, xylose, lactose, raffinose and mannitol were not attacked.
Optimum PH value 5 to 6, no growth at PH 7.	Optimum PH value 5 to 6, no growth at 6·9.

Characteristics of Str. damnosus (23) (continued).	Characteristics of Group 8 (F_1, F_6) organisms (continued).
In glucose broth: scanty white, flocculent growth.	In glucose broth: medium clear, scant white flocculent deposit, produces some haziness and no turbidity.
Grows in hopped beer, but growth is delayed.	Grows in beer containing 0·002% humulone, but not in the higher concentration. Growth is delayed above 0·001% concentrations or above.

Identical growth in gelatin beer stabs and malt wort.

The above comparison shows the great resemblance of Str. damnosus with the organisms of Group 8, apart from the smallness in size of the cells of these organisms as compared to those of Str. damnosus. Hence the name Str. damnosus var minimus is proposed for this particular group, the culture F_6 being named as the type culture and culture F_1, which is very similar to F_6, being named Str. damnosus var minimus strain 1.

Lactobacilli comprising Group 5 (C_4, C_5, W_7, W_{10}) showed in several of their characteristics resemblances to those of L. pastorianus, but differed in other characteristics. However, from the comparison (as below) it became evident that the differences between these organisms and L. pastorianus were not considered to be sufficiently wide to indicate clearly that the organisms in question should be considered as new Lactobacillus species.

L. pastorianus (for reference see 35.23).	Organisms of Group 5 (C_4, C_5, W_7, W_{10}).
Rods 0·6 to 1 μ by 5 to 10 μ and even up to 35 μ.	Rods 0·5 to 0·8 μ by 3·5 to 10 μ.
Single cells, pairs united at obtuse angle and a few chains.	Single cells, pairs and chains present. Cells were united at various angles.
Maximum temp. for growth 38°C. and optimum temp. 29-33°C.	Grew at 15° and 35°C. but not at 38°C. Optimum 25-26°C., while optimum temperature range was 25-30°C.
Grew at PH values 3·4 to 8 and even more.	Grew at PH 3·6 and 6·9, but not at 7·3.
Optimum PH range not reported.	Optimum PH range 5 to 6.
Increasing hop rates increases the initial stationary phase but were able to grow even in 4 lb. per barrel hop concentration.	Could not grow at all in the humulone concentrations above 0·0005%.
Heterofermentative.	Heterofermentative.
Acetyl methyl carbinol not produced.	Acetyl methyl carbinol not produced.
In yeast extract medium growth and acid occurs with arabinose, glucose, fructose, galactose, sucrose and maltose. Lactose may or may not be attacked.	In casein double digest + yeast autolysate, growth and acid is produced with glucose, fructose, xylose, arabinose, galactose, maltose, raffinose and salicin. Lactose, sucrose, mannose and inulin are not attacked.

Very similar type of growth on various solid as well as in liquid media in both the cases.

The principal differences are thus shown to be a lower optimum temperature, slightly lower maximum temperature allowing growth, and slightly more restricted range of PH.

In the opinion of the author these differences are not wide enough to consider these organisms different from L. pastorianus. Hence, Group 5 members (C_4, C_5, W_7, W_{10}) are considered as the strains or varieties of L. pastorianus.

The organisms comprising Groups 6 and 10 respectively were found to differ in their characteristics from all the species described in the literature, and were thus considered to be new species. Complete descriptions of their characteristics and classification are given in the following pages.

Description of the characteristics of Streptococci Group 10 (G_1 & F_2).

Morphological characteristics.

Shape, size and arrangement.

In standard beer medium cultures at 25°C., cells appeared to be spherical, elongated forms being absent. They were of 0·5 to 0·7 μ in diameter and were mostly arranged in diplo-forms and some as single cells. Some cases were detected where diplos were so arranged, as to give an impression of tetrads.

Motility	Not detected.
Endospores	Not detected.
Capsules	Not detected.
Gram stain	Positive.
Staining	Carbol fuchsin and Loeffler's methylene blue stained the cells fairly well.

Cultural characteristics.

In liquid media at 25°C.

With free excess to air the organisms developed best in the following media of initial PH value 5·2 to 5·3:-

1. Standard beer medium.
2. Standard malt wort.
3. Casein double digest + yeast autolysate + 1% glucose.

In each of these medium, scant deposit developed, but medium remained clear and no film on the surface developed. On shaking non-silky turbidity developed.

Growth in nutrient broth and glucose broth was poor, and no sign of growth at all in litmus milk even after three weeks incubation.

On solid media (in the atmosphere of CO_2).

Description of single colonies on beer gelatin plates at room temp. (19-20°C.).

These were from 0·5 to 0·8 m.m. in diameter. Colonies were subsurface with a brownish tinge and were opaque, dull, smooth, flat entire and had a butterlike consistency.

Beer gelatin stabs. Growth was very good and was beaded along the line of stab. No growth on the surface.

Beer agar slopes. Fair growth, colonies were very small (0·25 to 0·3 m.m.).

Physiological Characteristics.

Oxygen requirement.	Facultative anaerobes.
Temperature relations.	Optimum temperature 21-22°C. in standard beer medium. The organisms grow at 15°C. and 25°C. but not at 30°C.
PH relations.	Optimum PH value between 5 to 5·6. Grew at 4·2 and 6·6, but not at 3·6 and 6·9.

Biochemical Characteristics.

Catalase reaction.	Negative.
Diacetyl and acetyl methyl carbinol.	Positive.
Reduction of nitrate.	Not detected.
Indole formation.	Not detected.

Growth and Acid from various Carbohydrates, in casein double digest + yeast autolysate.

Growth and acid took place with glucose, fructose, mannose, maltose, salicin, inulin and very little with raffinose. Xylose, arabinose, lactose, sucrose and galactose were not used at all by the organisms. Gas was not detected with any of the carbohydrates used.

Resistance towards ethyl alcohol.

The organisms developed without delay in standard beer medium containing 6 ml. of absolute alcohol per 100 ml. of medium. No growth occurred at all in the tubes with the higher alcohol concentrations (8 ml. and 10 ml. per 100 ml. of medium respectively).

Resistance towards humulone.

Growth was greatly delayed even when 0·00025% humulone was employed. No growth occurred at the higher humulone concentrations.

Homo- or hetero-fermentative. Homo-fermentative.

Classification.

It will be seen that the characteristics of these cocci (G_1 & F_2) as described above permits their allocation to the genus Streptococcus. However, in order to ascertain whether these organisms are new species or merely strains of one of the species already described, the characteristics of these organisms were compared with the characteristics of all the Streptococcus species as described in the literature.

The new cocci were seen to differ from Str. damnosus in the following features:-

Str. damnosus.	Species G_1 and F_2.
Spheres of 1 to 1·5 μ in diameter, as single cells, diplos and tetrads.	Spheres of 0·5 to 0·7 μ in diameter mostly as diplos and some as single cells. No tetrads at all.
Growth at 15°C. but not above 30°C.	Growth at 15°C. and 25°C. but not at 30°C.
Optimum temperature 21-25°C.	Optimum temperature 21-22°C.
No growth at PH 7 but took place at 3·6.	No growth at PH 3·6 and 6·9.
Grows in hopped beer, but is delayed.	No growth in hopped beer at all.
No growth or acid from arabinose and salicin, but acid and growth in sucrose.	No growth and acid with sucrose, but acid and growth from arabinose and salicin.

It was thus clear that these streptococci were different from all

the known Streptococcus species, and were thus considered as a new species. Since one of the main features of this species is its small size (0·5 to 0·7), it is proposed to name these two very similar organisms Streptococcus parvus, the culture designated G_1 being named as the type culture and culture F_2, which is very similar to G_1 being named Str. parvus strain 1.

Description of the characteristics of Lactobacilli Group 6 (H_1, H_2, H_3, H_5).

Morphological Characteristics.

Shape, size and arrangement.

In standard beer cultures at 25°C., cells were seen chiefly as short fat rods, of which some appeared almost coccoid in shape. Size, 0·7 to 0·8 u by 1 to 1·2 u. Cells were observed as single, in pairs and small clumps and chains of four to six cells.

Motility. Non-motile.

Endospores. Not detected.

Gram stain. Positive.

Capsules. Not detected.

Staining. Good staining with carbol fuchsin and Loeffler's methylene blue.

Cultural Characteristics.

In liquid media at 25°C.

With free excess to air the organisms developed fairly well in

the following media with an initial PH value 5·2 to 5·3:-

1. Standard beer medium.
2. Standard malt wort.
3. Glucose broth.
4. Casein double digest + yeast autolysate + 1% glucose.

In each of the above media the organisms developed well, producing fair silky turbidity at the early stages, but the liquid became clear after 5 to 6 days incubation period. No film was formed in any medium, while fair deposit accumulated in each medium.

Growth in nutrient broth was very poor and no growth occurred at all in litmus-milk even after 3 weeks incubation.

On solid media (in the atmosphere of CO_2).

Poor growth was found to occur on the beer agar slopes, but very good beaded growth appeared in beer gelatin stabs.

Description of single colonies on gelatin plates at room temp. (19-20°C.).

These were from 1 m.m. to 1.5 m.m. in diameter and were situated just below the surface. They appeared smooth, dull, convex, entire, colourless and somewhat coherent.

Physiological Characteristics.

Oxygen requirements.	Facultative anaerobes.
Temperature relations.	Optimum temperature 22-23°C. in standard beer medium. The organisms grew at 15°C. and 30°C., but not at 33°C.
PH relations.	Optimum PH was found to be between 5 to 5·6. The organisms grew at PH 4·2 and 6·9, but not at 3·6 or 7·3.

Biochemical Characteristics.

Catalase Reaction.	Negative.
Diacetyl or acetyl methyl carbinol.	Not detected.
Reduction of nitrate.	Not detected.
Indole formation.	Not detected.
Hetero- or Homo-fermentative.	Hetero-fermentative.

Resistance towards ethyl alcohol.

Organisms developed without delay in standard beer medium to which 6 ml. of absolute alcohol per 100 ml. of medium was added. Growth was greatly delayed (14 days) in the medium containing 8 ml. of alcohol per 100 ml. of medium. No growth occurred at all in the medium containing 10 ml. of alcohol per 100 ml. of medium.

Resistance towards humulone.

In standard beer growth was delayed by one day in the presence of humulone up to 0·0005% concentration, while there was delay of two days in the presence of humulone up to 0·001% concentration. No growth occurred at all in higher concentrations even after prolonged incubation.

Growth and Acid from carbohydrates in casein double digest + yeast autolysate medium.

Good growth and acid from xylose, arabinose, glucose, fructose, mannose, sucrose and maltose. Signs of growth but no acid from mannitol, salicin and inulin. No growth or acid from galactose, lactose and raffinose. Gas formation was not detected in any of the above cases.

Classification.

From the above characteristics it is apparent that the organisms in question should be allocated to the genus *Lactobacillus* according to the classification elaborated by Bergey *et al* (35). Therefore, a search has been carried out in the literature, for an identical or closely similar *Lactobacillus* species, in order to identify our organisms with one of the species already described in the literature.

The only species which showed some resemblance with these organisms was *L. pastorianus*, but the differences between them were still too wide to consider them the strains of varieties of *L. pastorianus*. The differences are shown in the following table:-

L. pastorianus (35,23).	Group 6 members (H_1, H_2, H_3, H_5).
Rods 0·6 to 1 μ by 5 to 10 μ and even up to 35 μ.	Rods 0·7 to 0·8 by 1 to 1·2 μ.
Maximum temp. for growth 38°C. and optimum temp. 29-33°C.	Maximum temp. for growth 30°C. and optimum temp. 22-23°C.
Grew at PH 3·4 and 8.	Grew at PH 4·2 and 6·9 but not at 3·6 and 7.
Acid from galactose, lactose, raffinose and salicin. No acid from xylose.	No acid from galactose, lactose, raffinose and salicin. Acid from xylose.
Grows in high concentration of humulone.	Failed to grow even in 0·001 per cent humulone concentrations.

It thus became evident that these *Lactobacillus* strains of Group 6 were different from all the known *Lactobacillus* species and were thus considered to belong to a new species hitherto undescribed. Since one of the main features of this group is that the optimum growth occurs at 22-23°C. and that the maximum temperature which allows

growth is 30°C., it is proposed to name this group, Lactobacillus frigidus, H_5 being the type species and H_1, H_2 and H_3 being strains of the same.

Glossary of some Terms used for recording characteristics of Bacteria.

The following terms were found helpful for recording the characteristics of the organisms described in this thesis:-

Abundant: Should be thought of in connection with the terms strong, moderate and slight.

Beaded: Consisting of loosely placed or disjointed colonies.

Butterlike: Similar to butter in consistency.

Capitate: Surface hemispherical (for colonies).

Chains: Three or more bacterial cells attached end to end.

Clumps: Irregular groupings of closely joined cells.

Contoured: Having an irregular, smoothly undulating surface, like that of a relief map.

Convex: Surface like the segment of a circle, but very flatly convex. Should be thought of in connection with the terms pulvinate and capitate.

Dull: Not glistening.

Entire: With an even margin.

Filiform: A uniform growth along line of inoculation (in streak or stab cultures).

Film: Thin, often delicate, surface growth on liquid media.

Flaky: That which resembles flakes (for describing deposits).

Flocculent: Containing adherent masses of various shapes floating in culture media.

Glistening: Shining, not dull.

Moderate: Should be thought of in connection with the terms abundant, strong and slight.

Nodose: Consisting of closely aggregated colonies.

Oil-like: Similar to oil or glycerine in consistency.

Opaque: Impervious to light.

Punctiform: Very small colonies (pin point).

Pellicle: Thick continuous surface growth formed on liquid cultures.

Pulvinate: Decidedly convex. Should be thought in connection with the terms convex and capitate.

Raised: Thick growth (on solid media), with abrupt or terraced edges. Should be thought in connection with the term flat.

Ring: Growth at the upper margin of liquid cultures, adhering to the glass.

Ropy: Growth follows the needle when touched and withdrawn.

Round: Of a more or less circular outline.

Scanty: Should be thought of in connection with the terms moderate and heavy (for deposit).

Slimy: Resembling slime.

Smooth: Surface even.

Spreading: Growth extending much beyond the line of inoculation.

Tough: Strongly cohesive.

Translucent: Faintly transparent.

Transparent: Transmitting light.

Turbid: Cloudy, with suspended matter; not clear.

Viscid: For sediment which on shaking rises as a coherent swirl.

SECTION II.

Apparatus.

Culture tubes used throughout these investigations were of 4" x ½" size. New tubes were ordered for these investigations and were reserved exclusively for this work only.

Conical flasks of all capacities employed during these investigations were mostly new, and those not new were treated with chromic acid (as mentioned below) before being used for these investigations.

Graduated and Pasteur pipettes were plugged with cotton wool prior to sterilising them in the oven or autoclave.

Standard procedure employed for cleaning the glassware.

The used tubes, flasks, pipettes, etc., all the glassware employed during these investigations, were washed with tap water, drained, and submerged in the chromic acid (prepared as mentioned below) and kept on the steam bath over night. The chromic acid was drained and the apparatus rinsed several times with tap water, and lastly with distilled water. Finally the apparatus was filled with distilled water or submerged in distilled water in covered beakers and autoclaved at 20 pounds pressure for 20 minutes. Flasks, pipettes, etc., were drained and air dried, while the tubes were dried in gas oven before used for these investigations.

Chromic acid used throughout these investigations was prepared

as follows:- A hot solution of equal weights of sodium dichromate and water was prepared in a porcelain basin and to this solution was added, with stirring (and great care), approximately eleven times its weight of concentrated sulphuric acid (100 gm. chromate : 100 c.c. water : 1200 c.c. H_2SO_4 of specific gravity 1.84). This solution was used repeatedly until the green colour of reduced chromium became noticeable.

Sterilisation.

(a) Inoculating needles and "mouth" of the culture tubes were sterilised by passing through the Bunsen flame several times.

(b) Pipettes, etc., employed in the aseptic work were sterilised by autoclaving at 20 pounds pressure for 20 minutes.

(c) Media containing low concentrations of sugar (up to 1%) were autoclaved at 10 pounds pressure for 10 minutes, while the tubes containing synthetic medium (4" tubes containing 3 ml. of medium) were autoclaved at 10 pounds pressure for $2\frac{1}{2}$ minutes. This technique has proved to be completely adequate, and no growth has been observed in uninoculated control tubes at any time.

(d) Flasks containing stock solutions of individual amino acids, and purines were sterilised in free steam for 15 minutes on each of the three consecutive days.

(e) The growth was arrested by steaming the tubes for 20 minutes at 100°C. (free steam), before the volume in them was made

to 8 ml., required for reading on the specker.

The standard inoculation procedure adopted for this work.

The centrifuge tubes containing 4 ml. of yeast extract peptone medium (described below) were given a heavy inoculum (2 to 3 loopfulls) and incubated at 25°C. till a vigorous growth appeared in the tubes. The tubes which showed growth earlier than they were needed, were put in the refrigerator till they were required for further use.

When needed the tubes were centrifuged at 4000 r.p.m. for 10 minutes and the cells were resuspended in 9 ml. of sterile saline solution (.9% NaCl in distilled water). The tubes were again centrifuged as above and after decanting the medium, the cells were resuspended in 9 to 12 ml. or even more saline solution, so that there was uniformly same turbidity in all the tubes.

Each tube containing the basal medium (complete or deficient) was inoculated with approximately 0·05 ml. of the above cell suspension by using Pasteur pipettes (one drop was added to each tube).

This standardised procedure adopted throughout these investigations gave very satisfactory results.

Yeast-extract-peptone medium was prepared as follows:-

5 gm. Peptone (Evans).
10 gm. Yeast extract (Difco).
10 gm. Glucose.
12 gm. Sodium Acetate.
6 gm. Ammonium chloride.
0·5 gm. KH_2PO_4.
0·5 gm. K_2HPO_4.
0.2 gm. $MgSO_4 . 7H_2O$.
0·35 gm. NaCl.
0·01 gm. $MnSO_4 . 4H_2$ and $FeSO_4 . 7H_2O$ each.

The above quantities were dissolved in 1 litre of distilled water on the water bath. The PH of the medium was adjusted to 5·1 to 5·3 using Johnson's PH Papers by means of dilute HCl and dilute NaOH. The flask was again heated on steam bath for 10 to 15 minutes to ensure complete dissolution of the various constituents. The medium was then dispensed into tubes as required and sterilised as mentioned in the previous paragraphs.

Procedure adopted for the anaerobic cultivation of the inoculated tubes.

The inoculated tubes were placed in desiccators and the desiccators were evacuated on the pump, to which a manometer was attached. Evacuation was carried up to 65 to 70 cm. Carbon dioxide was then passed from a CO_2 cylinder through a wash bottle containing water, till the desiccators were full (indicated by the non-appearance of bubbles in the bottle). The covers were again pressed securely and the desiccators were incubated at 25°C. for the periods required for the particular experiments.

Description of the basal medium employed.

The composition of the basal medium (239) employed during these investigations is given in the Table No. 1. All the amino acids, vitamins purines, and pyrimidines used for these purposes were either Roche or Light products and were of high quality. Glucose and all the salts except sodium acetate were AR quality, while sodium acetate was of ordinary CP quality.

The PH of the final solution was adjusted to 5·1 to 5·3 before it was dispensed into the tubes.

TABLE I.

Composition of Basal Medium.

Constituent.	mg. per litre.	Constituent.	mg. per litre.	Constituent.	mg. per litre.	Constituent.	mg. per litre.
DL-Alanine.	667	DL-Nor Leucine.	667	Dextrose.	20,000	Riboflavin.	2
Asparagine (natural).	667	DL-Nor Valine.	667	Sodium Acetate.	12,000	Nicotinic Acid.	2
L-Arginine-HCl.	667	DL-Phenyl Alanine.	667	Ammonium Chloride.	6,000	Biotin.	0·005
L-Cysteine-HCl.	667	L-Proline.	667	KH_2PO_4.	500	P. Amino Benzoic Acid.	0·10
L-Glutamic Acid.	667	DL-Serine.	667	K_2HPO_4.	500	Folic Acid.	0·005
Glycine.	667	DL-Threonine.	667	$MgSO_4 . 7H_2O$.	200	Choline chloride.	10
L-Histidine.	667	DL-Tryptophan.	667	$FeSO_4 . 7H_2O$.	10	Inositol.	25
L-Hydroxy Proline.	667	L-Tyrosine.	667	$MnSO_4 . 4H_2O$.	10		
DL-Isoleucine.	667	DL-Valine.	667	NaCl.	350		
L-Leucine.	667	Adenine Sulphate $2H_2O$.	13·8	Thiamine HCl.	1		
L-Lysine-HCl.	667	Guanine HCl $2H_2O$.	13·0	Pyridoxin.	1·6		
DL-Methionine.	667	Uracil.	12·0	Pyridoxamine di HCl.	0·10		
		Xanthine.	12·0	D-Calcium Pantothenate.	2		

Experiment No. 1.

Determination of suitable time of incubation for each organism.

This experiment was carried out in the complete synthetic medium (described in Table I) to determine the best suitable incubation period for individual organisms employed during these investigations.

Cell suspensions of all the organisms were prepared as described in the previous paragraph (page 123) and each organism was inoculated in nine tubes, each tube containing 3 ml. of the medium. All the inoculated tubes were divided into three batches, each batch containing three tubes of each organism, and were placed in the desiccatore with some uninoculated control tubes. The desiccators were evacuated, filled with carbon dioxide and incubated at 25°C.

Each batch of tubes was removed from the desiccators after an incubation period of 3, 5 and 7 days respectively. The growth in all the tubes was arrested by steaming them for 20 minutes and the volume in each tube was made to 8 ml. by adding 5 ml. of distilled water to each tube from a burette. All the tubes were given good shaking so as to produce homogenous turbidity throughout the medium before being transferred to the cells, to be read on the Specker. Turbidity produced in each inoculated tube was then read on the Specker against the uninoculated medium as control.

The incubation period after which a particular organism produced the greatest amount of turbidity (shown by the readings on the Specker) was considered as the best suitable period of incubation for that particular organism.

It may be pointed out that the three organisms which did not show

any signs of growth after seven days' incubation were not used for further investigations.

It may also be pointed out that, for the sake of simplicity, only the average of the three readings (tubes in triplicate) obtained for each organism have been given, in the following tables, summarising the results of this experiment. To avoid decimals the writer also found it convenient to multiply each reading by a constant 1000.

Table IIa summarises the results of the entire experiment, while in Table IIb is given the most suitable time selected for the incubation of every individual organism.

Table IIa.

Growth at different periods of Incubation at 25°C.

Organism.	3 days.	5 days.	7 days.
B_4	445	625	749
C_3	545	743	936
E_4	31	70	499
A_4	371	562	666
D_5	408	547	687
E_1	522	555	808
D_1	551	554	563
B_1	170	181	190
G_2	278	991	1300
W_{10}	225	352	375
H_5	51	284	642
F_6	46	125	494
G_1	12	11	12
W_5	219	277	228
W_D	212	270	196
W_4	177	258	224
W_1	543	707	618
C_5	Nil	Nil	Nil
F_3	Nil	Nil	Nil

Table IIb.

Organism.	Incubation period selected.
B_4	7 days.
C_3	7 days.
E_4	8 days.
A_4	7 days.
D_5	8 days.
E_1	8 days.
D_1	5 days.
B_1	7 days.
G_2	5 days.
W_{10}	7 days.
H_5	8 days.
F_6	7 days.
W_5	6 days.
W_D	6 days.
W_4	6 days.
W_1	6 days.

Experiment No. 2.

Effect of omitting single amino acid from the complete basal medium.

From the Experiment No. 1 it became evident that 3 out of the 19 organisms examined would not grow in the complete basal medium, so also the best suitable period of incubation for each of the 16 growing organisms was obtained (Table IIb).

In this experiment each of the 16 organisms in question was grown on the basal medium from which each of the twenty-one amino acids had been omitted in turn, so as to determine the effect of the missing amino acid on the growth of each of the 16 organisms.

Stock solutions of the required concentrations of individual amino acids were prepared in distilled water by heating on steam bath. All the amino acids except tyrosine and tryptophan dissolved easily to give clear solutions, while these two were dissolved by adding a few pellets of sodium hydroxide.

The other constituents of the basal medium, except the 21 amino acids were dissolved in a suitable amount of distilled water. The solution was equally divided into 22 flasks. To one flask all the 21 amino acids were added (from the stock solutions, in the required concentrations) to give the complete basal medium. To the remaining 21 flasks, 20 amino acids were added to each flask, leaving out one amino acid in turn. PH of all the solutions was adjusted to 5·1 to 5·3 by the help of Johnson's PH Papers, using dilute hydrochloric acid and sodium hydroxide. The volumes of all the solutions were adjusted to bring about the desired concentrations of all the constituents.

TABLE III.

Maximum turbidity production in media lacking amino acid.

	Now designated as L. leichmanni. A_4	Now designated as L. pastorianus. B_1	Now designated as L. pastorianus. B_4	Now designated as L. buchneri. C_3	Now designated as L. bifidus. D_1	Now designated as L. bifidus. D_5	Now designated as L. bifidus. E_1	Now designated as L. leichmanni. E_4	Now designated as Streptobacterium damnosus var. minimus F_6	Now designated as L. plantarum. G_2	A new species designated L. frigidus. H_5	Now designated as L. pastorianus. W_1	Now designated as L. pastorianus. W_4	Now designated as L. pastorianus. W_5	Now designated as L. pastorianus. W_{10}	Now designated as L. pastorianus. D
Complete.	791	154	103	468	288	442	678	528	997	385	855	525	196	855	430	535
Alanine.	205	59	0	40	129	360	172	48	505	11	270	360	24	374	37	696
Arginine.	438	48	39	72	102	101	135	97	98	149	154	92	48	940	135	165
Asparagine.	5	100	23	346	152	313	507	950	550	142	441	54	21	905	258	182
Cystein.	503	131	57	450	516	525	666	375	761	63	882	587	203	713	540	550
Glycine.	5	20	14	300	29	65	130	614	101	30	206	88	42	854	106	78
Glutamic Acid	41	13	0	12	14	36	20	20	0	15	29	14	44	50	102	45
Histidine.	199	100	55	132	477	549	608	692	563	446	620	172	233	860	425	538
Hydroxy Proline.	551	127	74	385	337	604	822	920	968	90	685	210	242	854	426	523
Iso leucine.	438	118	72	866	344	468	782	921	1035	408	805	66	223	875	391	398
Leucine.	5	2	3	0	10	15	26	29	0	27	22	0	0	0	10	15
Lysine.	595	273	60	475	206	37	373	852	546	144	318	775	0	836	857	534
Methionine.	718	127	41	321	152	530	706	644	815	274	718	580	128	728	415	588
Nor leucine.	603	131	81	486	218	435	438	554	868	421	861	501	180	810	361	546
Nor valine.	652	99	86	575	375	768	892	758	645	305	960	395	166	923	384	570
Phenyl Alanine.	40	142	74	119	211	449	657	43	729	53	727	330	176	590	299	438
Proline.	476	41	10	252	20	49	27	250	10	33	39	290	105	932	100	300
Serine.	592	5	0	629	10	18	25	752	0	158	35	250	57	785	469	111
Tryptophan.	73	25	0	20	46	20	46	23	60	28	38	20	3	854	5	22
Tyrosine.	460	127	40	99	104	177	50	128	152	334	123	265	75	859	264	240
Threonine.	593	5	15	248	23	27	17	346	17	33	25	0	23	931	719	12
Valine.	69	23	19	479	21	50	148	589	26	26	305	47	8	430	16	14

TABLE IV.

Amino acid requirements of the organisms.

	Now designated as L. plantarum. G_2	Now designated as L. pastorianus. B_4	Now designated as L. bifidus. D_5	Now designated as L. bifidus. E_1	Now designated as Strep. damnosus var minimus. F_6	Now designated as L. pastorianus. W_4	A new species designated L. frigidus. H_5	Now designated as L. pastorianus. W_1	Now designated as L. bifidus. D_1	Now designated as L. leichmanni. A_4	Now designated as L. pastorianus. B_1	Now designated as L. leichmanni. E_4	Now designated as L. pastorianus. W_{10}	Now designated as L. pastorianus. W_D	Now designated as L. buchneri. C_3	Now designated as L. pastorianus. W_5	Number of organisms requiring the each Amino Acid.
Glutamic Acid.	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	16.
Leucine.	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	16.
Tryptophan.	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	N	15.
Glycine.	E	E	E	E	E	E	E	E	E	E	E	N	E	E	N	N	13.
Valine.	E	E	E	E	E	E	N	E	E	E	E	N	E	E	N	N	12.
Threonine.	E	E	E	E	E	E	E	E	E	N	E	N	N	E	N	N	11.
Arginine.	N	N	E	E	E	E	E	E	N	N	N	E	N	N	E	N	8.
Proline.	E	E	E	E	E	N	E	N	E	N	N	N	E	N	N	N	8.
Serine.	N	E	E	E	E	N	E	N	E	N	E	N	N	E	N	N	8.
Alanine.	E	E	N	N	N	E	N	N	N	N	N	E	E	N	E	N	6.
Tyrosine.	N	N	N	E	E	N	E	N	N	N	N	E	N	N	E	N	5.
Asparagine.	N	E	N	N	N	E	N	E	N	E	N	N	N	N	N	N	4.
Phenyl Alanine.	E	N	N	N	N	N	N	N	N	E	N	E	N	N	N	N	3.
Lysine.	N	N	E	N	N	E	N	N	N	N	N	N	N	N	N	N	2.
Hydroxy Proline.	E	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	1.
Iso leucine.	N	N	N	N	N	N	N	E	N	N	N	N	N	N	N	N	1.
Cystein.	E	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	1.
Number of Amino Acides essential for each organism.	11	10	10	10	10	10	9	9	8	7	7	7	7	7	6	2	

Note: Histidine, Methionine, Nor leucine and Nor valine were not found to be essential for any of the organisms.

Symbols used: E = Essential.
N = Not essential.

The solutions were dispensed in the tubes (3 ml. in each tube) and the tubes were sterilised as usual.

Three tubes of each medium were inoculated by using the cell suspension of individual organism and all the tubes were incubated at 25°C. in the desiccators filled with CO_2. Along with those tubes some uninoculated control tubes were also placed.

The tubes inoculated with an individual organism were incubated for the period which was already found to be suitable for that organism.

At the end of incubation period the tubes were steamed, volume made to 8 ml. by using distilled water and were read individually against the control, as described before.

The results of this experiment are summarised in Tables III and IV. The underlined values in Table III are less than one-fourth the maximum control values (readings shown by the complete media tubes) and are considered to be indicative of essential amino acids.

While in Table IV organisms are arranged in order of their fastidiousness for the amino acids, and the latter are listed approximately in order of their indispensability for the strains tested.

Experiment No. 3.

Effect of omitting single vitamin from the complete basal medium.

This experiment was carried out in a similar way to the previous experiment (Exp. 2). Stock solutions of the individual vitamins in desired concentrations were prepared in 50 per cent. alcohol.

The constituents of the basal medium other than vitamins were

TABLE V.

Maximum turbidity production in media lacking vitamin.

	Now designated as L. leichmanni. A_4	Now designated as L. pastorianus. B_1	Now designated as L. pastorianus. B_4	Now designated as L. buchneri. C_3	Now designated as L. bifidus. D_1	Now designated as L. bifidus. D_5	Now designated as L. bifidus. E_1	Now designated as L. leichmanni. E_4	Now designated as Strep. demnosus var minimus F_6	Now designated as L. plantarum. G_2	A new species designated L. frigidus. H_5	Now designated as L. pastorianus. W_1	Now designated as L. pastorianus. W_4	Now designated as L. pastorianus. W_5	Now designated as L. pastorianus. W_{10}	Now designated as L. pastorianus. W_D
Complete.	606	154	103	468	288	442	678	528	997	385	855	315	213	883	375	212
Biotin.	681	123	102	485	170	503	722	556	952	390	812	246	228	773	292	288
Choline.	744	153	71	318	248	549	714	364	839	391	888	341	291	898	304	189
Folic Acid.	683	145	81	286	240	501	755	651	932	387	914	250	176	813	302	276
Inositol.	721	135	68	489	239	550	749	1080	918	228	901	260	215	980	367	208
Nicotinic Acid.	324	10	19	24	69	55	74	65	8	48	31	153	47	69	107	73
Pantothenic Acid.	37	22	21	57	55	66	15	40	17	47	41	78	38	74	93	16
PAB.	616	124	73	496	454	464	960	922	862	378	833	379	14	793	302	268
Pyridoxin.	612	135	71	322	187	452	760	520	966	250	843	242	180	954	456	231
Pyridoxal.	592	112	93	651	456	435	664	914	763	304	775	232	135	650	399	225
Pyridoxamine.	608	129	92	485	281	445	817	647	1144	25	737	306	185	791	450	241
Pyridoxal & Pyridoxin.	658	130	67	749	494	462	711	640	743	371	923	269	177	772	365	196
Pyridoxin & Pyridoxamine	666	150	74	474	223	637	792	658	1039	38	938	275	198	883	370	185
Pyridoxal & Pyridoxamine	619	153	84	384	181	454	1000	927	849	32	830	220	188	740	362	213
Pyridoxal, Pyridoxin & Pyridoxamine	621	146	36	351	330	527	745	517	519	52	633	271	238	908	396	270
Riboflavin.	509	100	52	95	110	400	735	125	1101	35	819	380	129	898	270	105
Thiamine.	509	252	76	252	213	311	477	338	753	382	476	311	225	901	332	196

TABLE VI.

Vitamin requirements of the organisms.

	Now designated as L. plantarum. G2	Now designated as L. buchneri. C3	Now designated as L. leichmanni. E4	Now designated as L. pastorianus. W4	Now designated as L. pastorianus. B1	Now designated as L. pastorianus. B4	Now designated as L. bifidus. D1	Now designated as L. bifidus. D5	Now designated as L. bifidus. E1	Now designated as Strep. dannosus var minimus. F6	A new species designated L. frigidus. H5	Now designated as L. pastorianus. W5	Now designated as L. leichmanni. A4	Now designated as L. pastorianus. W1	Now designated as L. pastorianus. W10	Now designated as L. pastorianus. WD	Number of organisms requiring each vitamin.
Pantothinic.	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	16
Nicotinic.	E	E	E	E	E	E	E	E	E	E	E	E	N	N	N	N	12
Riboflavin.	E	E	E	N	N	N	N	N	N	N	N	N	N	N	N	N	3
Pyridoxin.	E	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	1
PAB.	N	N	N	E	N	N	N	N	N	N	N	N	N	N	N	N	1
Number of vitamins essential for each organism.	4	3	3	3	2	2	2	2	2	2	2	2	1	1	1	1	

Note: The remaining of the vitamins were not found to be essential for any of the organisms.

Symbols used: E = Essential.
N = Not essential.

dissolved in required quantities in a suitable volume of distilled water and the solution was equally divided into seventeen flasks. To one of these flasks all the twelve vitamins were added to give the complete basal medium, while to each of the other twelve flasks eleven vitamins were added, leaving one vitamin in turn.

To one of the remaining four flasks all the vitamins were added except three, namely, pyridoxin, pyridoxal and pyridoxamine, while to the other three flasks all the vitamins were added, leaving out from each in turn two out of the above three vitamins.

After adjusting the PH and volumes, the solutions were tubed and sterilised as usual. Three tubes of each of the seventeen solutions were inoculated with individual organism in question and the tubes were incubated in the desiccators at 25°C. for the predetermined periods (Table IIb).

The results of this experiment are summarised in Tables V and VI. As in Table III of the previous experiment, the underlined values in Table V are also less than one-fourth the maximum control values and are considered to be indicative of essential vitamins.

In Table III organisms are arranged in order of their fastidiousness for the vitamins, and the vitamins are listed approximately in their indispensability for the organisms tested.

Experiment No. 4.

Effect of omitting single purine or pyrimidine from the complete basal medium.

The details of this experiment were identical to the Experiment No. 2. The stock solutions of adenine, guanine and uracil were

TABLE VII.

Maximum turbidity production in media lacking purine & pyrimidine.

	Now designated as L. leichmanni. A4	Now designated as L. pastorianus. B1	Now designated as L. pastorianus. B4	Now designated as L. buchneri. C3	Now designated as L. bifidus. D1	Now designated as L. bifidus. D5	Now designated as L. bifidus. E1	Now designated as L. leichmanni. E4	Now designated as Strep. damnosus var minimus. F6	Now designated as L. plantarum. G2	A new species designated L. frigidus. H5	Now designated as L. pastorianus. W1	Now designated as L. pastorianus. W4	Now designated as L. pastorianus. W5	Now designated as L. pastorianus. W10	Now designated as L. pastorianus. W
Complete.	172	154	103	468	288	442	678	528	997	385	855	525	528	650	217	391
Adenine.	29	542	13	871	523	634	745	1107	695	345	755	557	0	640	10	10
Guanine.	61	155	62	578	505	416	850	1138	839	398	799	587	325	625	181	370
Uracil.	23	22	10	667	12	55	35	794	4	211	255	10	466	610	19	10
Xanthine.	154	113	65	646	544	367	693	977	717	232	934	534	476	660	233	366

prepared in 0.5 N. hydrochloric acid, while that of xanthine was prepared in 0.5 N. sodium hydroxide. The constituents of the basal medium other than the three purines and one pyrimidine were dissolved in a suitable volume and the solution was equally divided into five flasks. To one of these flasks all the purines and pyrimidine were added to give complete basal medium, while to each of the remaining four flasks three of the four factors were added, leaving out one each in turn.

The rest of the details are similar to those described in Experiments 2 and 3.

The results of this experiment are summarised in Table VII by adopting the same procedure as has been adopted in the case of Table III for amino acids or Table V for vitamins.

The author wishes to point out that the investigations into the nutrient requirements of the sixteen organisms were carried out in two stages, with slightly different techniques. In the first stage organisms L. pastorianus W_1, W_4, W_5, W_D and W_{10}, and L. leichmannii A_4 were used. 8 media - 1 complete and 7 deficient - were made up and inoculated with all these six organisms; then another 8 media - 1 complete and 7 deficient - were inoculated and so on, until all the 41 deficient media had been tested. There were thus 6 occasions on which the complete media was used.

Now although the complete media used at different times was of constant composition, and the methods of inoculation and incubation were the same in each case, growth response of the individual organisms was not constant. That is to say, the Specker readings for the complete medium inoculated at six different occasions, by the same organism, were different. For example, W_1 grown in complete medium

TABLE VIII.

Purine and pyrimidine requirements of the organisms.

	Now designated as L. leichmanni. A_4	Now designated as L. pastorianus. B_4	Now designated as L. pastorianus. W_{10}	Now designated as L. pastorianus. D	Now designated as L. pastorianus. B_1	Now designated as L. bifidus. D_1	Now designated as L. bifidus. D_5	Now designated as L. bifidus. E_1	Now designated as Strep. damnosus var liquefaciens. E_6	Now designated as L. pastorianus. W_1	Now designated as L. plantarum. W_4	Now designated as L. buchneri. C_3	Now designated as L. leichmanni. E_4	Now designated as L. plantarum. G_2	A new species designated L. frigidus. H_5	Now designated as L. pastorianus. W_5	Number of organisms requiring each Purines or Pyrimidenes.
Uracil.	E	E	E	E	E	E	E	E	E	E	N	N	N	N	N	N	10
Adenine.	E	E	E	E	N	N	N	N	N	N	E	N	N	N	N	N	5
Number of Purine and Pyrimidenes required by each organism.	2	2	2	2	1	1	1	1	1	1	1	None.	None.	None.	None.	None.	

Note: Guanine and Xanthine were not found to be essential for any of the organisms.

at six different occasions gave six different readings (315, 290, 129, 227, 512 and 543).

However, by the use of a complete medium along with deficient mediums, the ratio of growth in deficient medium to that in complete medium was a reliable estimate of the essentiality or otherwise of the substance omitted from the deficient medium.

To eliminate this need for numerous experiments with the complete medium, a new technique was adopted for the second stage. The organisms examined were L. pastorianus B_1 and B_4, L. buchneri C_3, L. bifidus D_1, D_5 and E_1, L. leichmanni E_4, L. plantarum G_2, L. frigidus H_5 and Str. damnosus var minimus F_6.

In this technique, instead of examining all organisms at a time in a few deficient media, only two organisms were examined in all the deficient media, plus the complete medium. In this way, the growth response of the organism in the complete medium was constant for all the deficient media employed. This procedure effected a great saving of time and media.

In the Tables III, V & VII, the growth response in the complete medium of each of the six organisms used in the first stage is represented by one figure, generally the highest obtained. The figures for growth in deficient media have been adjusted so that the ratio of growth in deficient media to that in complete media remain the same as actually found.

TABLE IX.

Increase in Turbidity on Omitting individual Amino Acid.

	L. Pastorianus.	L. Fructivorans.	L. Pastorianus.	L. Pastorianus.	L. Pastorianus.	L. Hansenii.	L. Buchneri.	L. Plantarum.	L. Viridescens.	L. Bifidus.	L. Bifidus.	L. Pastorianus.	L. Leichmannii.
CONTROL	154	855	525	196	535	430	468	385	678	288	442	855	528
	B_1	H_5	W_1	W_4	W_D	W_{10}	C_3	G_2	E_1	D_1	D_5	W_5	E_4
NORVAL.	-	115	-	-	-	-	107	-	214	87	326	68	130
ISOLEU.	-	-	-	-	-	-	398	23	104	56	26	-	393
HYDROXY PROL.	-	-	-	46	-	-	-	-	144	49	162	-	392
LYS.	119	-	250	-	-	427	-	-	-	-	-	-	324
HIST.	-	-	-	-	-	-	-	61	-	189	107	-	164
CYST.	-	-	-	-	-	110	-	-	-	288	83	-	-
METH.	-	-	-	-	-	-	-	-	28	-	82	-	116
ASP.	-	-	-	-	-	-	-	-	-	-	-	50	422
ALA.	-	-	-	-	161	-	-	-	-	-	-	-	-
ARG.	-	-	-	-	-	-	-	-	-	-	-	85	-
PROL.	-	-	-	-	-	-	-	-	-	-	-	77	-
SER.	-	-	-	-	-	39	161	-	-	-	-	-	224
THREO.	-	-	-	-	-	289	-	-	-	-	-	76	-
NORLEU.	-	-	-	-	-	-	-	36	-	-	-	-	-
GLY.	-	-	-	-	-	-	-	-	-	-	-	-	86

DISCUSSION OF RESULTS.

SECTION I.

BACTERIOLOGICAL PART.

It has long been known that certain species of lactic acid bacteria may be obtained from sour beers, thus Van Laer (18) isolated Lactobacillus pastorianus, and Claussen (25) isolated Streptococcus damnosus from this source. In several other countries one or two species of lactic acid bacteria have been isolated from sour beers, but no systematic survey has hitherto been conducted with the purpose of surveying the types to be found in pitching yeast. Shimwell, as already mentioned, has stated that a number of species of lactobacilli and streptococci are to be found in yeast, but he has not identified more than two species.

During the course of these studies of bacterial infection of pitching yeasts the present author has isolated 52 specimens of lactic acid bacteria comprising both lactobacilli and streptococci.

The procedure adopted for isolating and purifying the cultures has been described in detail in the practical part (Section I), but it is worth mentioning that the method using petri-dishes alone as reviewed by Snieszko (277), for the anaerobic cultivation of organisms has been employed by the author very successfully and with satisfactory results, thereby eliminating the use of bulky vacuum vessels and the oxygen removers such as pyrogallol.

After obtaining the organisms in pure cultures, the question arose whether these were already known or whether they were new

species hitherto not reported. The detailed examination of their characteristics was carried out to settle the question of their identities. The results of the experiments have been summarised in the Tables III to XIX. An attempt was then made to classify and name these cultures by comparing them with the lactic acid bacteria already known and described in the literature.

Forty-four out of the 52 organisms in question have been identified as follows:-

9	specimens were found to be strains or varieties of	L. leichmannii Bergey et al.
7	" " " " " " " "	L. bifidus Tissier.
18	" " " " " " " "	L. pastorianus Van Laer.
6	" " " " " " " "	L. plantarum Orla-Jensen.
2	" " " " " " " "	L. buchneri Henneberg.
2	" " " " " " " "	Str. mucilaginosus Kulka, Cosbie and Walker.

The nine strains of L. leichmannii isolated and examined by the author have been found to have characteristics similar to those of L. leichmannii as described by Bergey et al. (35) and by Shimwell (23). All the author's strains have optimum temperature 37°C., and their respective maximum temperatures are between 43-48°C., as compared to the optimum 30-36°C. and the maximum 40-46°C, for different strains of L. leichmannii as described in the literature. In their homo-fermentative nature and in their morphological and cultural characters, they also strongly resembled those of L. leichmannii as described in the literature. The writer's strains produced acid (in casein double digest + yeast autolysate) with glucose, fructose, maltose, galactose,

xylose, inulin and salicin, but not from lactose, arabinose and raffinose. This behaviour with various carbohydrates also closely resembled the behaviour of L. leichmannii as described by Bergey et al. and by Shimwell.

Seven strains identified as L. bifidus have been found to have similar cultural, physiological and biochemical characteristics to those described under this name, in the literature by Tissier (9), Bergey et al (35) and Norris et al (285). The writer's strains on microscopic examination, shortly after the isolation, showed some cells which were like Chinese letters - that is, the cells were of very different shapes - but after continued subculturing the cells lost that property and appeared as straight rods of varying sizes. It is noteworthy that none of the writer's strains showed any branching or "antler-like" cells as described by Orla-Jensen (34), and which is supposed, by many workers, to be a characteristic of L. bifidus. It may also be pointed out that some workers (48,285) during their investigations have observed that L. bifidus species after prolonged subculturing exist as straight rods, without any branching. The writer's strains in the early stages of purification were found to be strict anaerobes, but on prolonged subculturing they behaved more like facultative anaerobes, which is quite consistent with the observations of the previous workers (48,285).

Out of the eighteen strains of L. pastorianus now isolated by the author, fourteen were very similar in their morphological, cultural, physiological and biochemical characteristics to those described in Bergey et al (35), by Shimwell (23) and originally by Van Laer (18).

The remainder of the four strains had a lower optimum temperature, slightly lower maximum temperature and somewhat restricted PH range, as compared to those described in the literature. Apart from these differences, the four strains showed great resemblance in all their other characteristics to those described in the literature. These minor differences in some of the characteristics are not inconsistent with the observations of the previous workers. It was thus thought fit to classify them as varieties of L. pastorianus.

Six specimens now identified as L. plantarum varied in morphology from strain to strain as far as the size and arrangement of the cells was concerned, but the cells of all the six strains had rounded ends. The writer's strains also formed acid from glucose, fructose, mannose, galactose, arabinose, sucrose, maltose, raffinose and salicin, and L. plantarum is known to behave in a similar manner. Lactose, though utilised, allowed less growth and acid formation in comparison with the other sugars. Xylose and inulin were not utilised by the writer's strains but some strains of L. plantarum have been observed to use these two substances. Apart from minor differences in morphology and such small differences in the power to utilise certain carbohydrates the writer's strains showed strong resemblance in all of their characteristics to L. plantarum as described by Orla-Jensen (34), later by Pederson (286) and by Bergey et al (35).

The two strains, classified as strains of L. buchneri were observed under microscopic examination as thin medium-sized rods, a few filamentus forms of 15 to 20 μ length were also detected. These strains produced acid from arabinose, xylose, glucose, fructose, mannose, sucrose, lactose, maltose and raffinose. Mannitol, salicin

and inulin were not fermented by these strains. The above-mentioned characteristics as well as other cultural, physiological and biochemical characteristics of these strains strongly resembled to those of L. buchneri as described in the literature (35).

The two strains of a Streptococcus species identified as strains of Str. mucilaginosus, itself a newly-described rope-forming coccus (30), have been found to show close similarities in their morphological, cultural, physiological and biochemical characteristics to those described for Str. mucilaginosus by the authors responsible for its isolation and characterisation. That these strains are entirely different from Str. damnosus var viscosus described by Shimwell (23), can be seen by comparing the details of their characteristics (Tables III to XIX) with those of Str. damnosus var viscosus.

Two other streptococci designated as Streptococcus damnosus var minimus have been found to show characteristics similar to those of Str. damnosus, except that the writer's strains have smaller cells compared to those of Str. damnosus, hence the designation var minimus. A detailed comparison of the characteristics of writer's strains with those of Str. damnosus has been given in the practical part (page 107).

There remained two other strains of a Streptococcus species which were found to be widely different from all of the streptococci already known and described in the literature. Along with many other differences the cells of these two specimens were of considerably smaller size (0.5 to 0.6 μ) than those of other cocci isolated from yeast, except those of Str. mucilaginosus. These organisms were designated Streptococcus parvus. The complete details of their

identification and naming are given in the practical part (pages 110-114).

Four specimens of lactobacillus, all of which were very much alike, were found to be very widely different from all the Lactobacillus species described in the literature. The complete details of their identification and naming have been described in the practical part (pages 114-118). Because of their low maximum temperature (for allowing growth) these specimens have been grouped together under the new name Lactobacillus frigidus.

As far as the writer's knowledge goes, it is the first time that strains of L. bifidus have been isolated from brewery pitching yeasts, though these have been normally found in the intestines of the warm-blooded animals.

The presence of L. buchneri, L. plantarum, L. leichmannii and L. pastorianus in pressed yeasts, distillery yeasts and in various fermenting substances have been reported in the literature from time to time, but apart from the last named species all the other species have been reported to be unable to grow in beer (23). Shimwell (20) has mentioned it more than once that L. leichmannii and L. plantarum, normally the typical milk bacteria are able to grow in wort and mash but cannot grow in hopped or unhopped beer (32).

The isolation of these species from brewery yeasts now clearly demonstrates that L. leichmannii, L. plantarum and L. buchneri can develop in beer with the same ease as can L. pastorianus. Further the isolation of these species, under the conditions mentioned above, has confirmed the results of Walker and Parker (33), who pointed out that some typical milk bacteria can develop well in beer on

prolonged subculturing in that medium.

It is noteworthy that most of the Lactobacillus species isolated during these investigations have the ability to grow at 15°C. and produce heavy silky turbidity and in many cases strong honey-like odour. These qualities have persisted even after the continued subculturing over a period of 13 to 14 months. These facts indicate that these bacteria can be as dangerous as L. pastorianus, which has been supposed to be the only Lactobacillus species capable of damage to beer.

The medium employed (casein double digest + yeast autolysate) throughout these investigations for determining the growth and acid production by the organisms with various carbohydrates has been recommended and employed by Davis (276) for the identification and classification of lactic acid bacteria in dairy bacteriology. The writer's organisms thus could conveniently be compared in this characteristic with those described by Davis (31).

Lastly it ought to be pointed out that it was found during the course of these investigations that most of the brewery pitching yeasts examined were strongly contaminated with lactic acid bacteria species. It will be apparent from the following details that the pressed brewery yeast did not become contaminated by the lactic acid bacteria since leaving the brewery, but these organisms were present in the yeast at the time it was pressed in the brewery.

The samples of yeast employed to pitch the sterile beer were always picked up from the interior of the lump of pressed yeast by breaking it into two or more pieces. Further, the large number of lactic acid bacteria colonies which always appeared amongst the

colonies of yeasts on the plates showed that the infection must have been heavy in the yeast at the time it was collected and pressed.

Moreover, this contamination by lactic acid bacteria was in the majority of cases a contamination by more than one species, for in most of the yeasts two or more types of lactic acid bacteria species were found to be present together. Further, it was found that two similar strains were present in the yeast from two breweries situated far apart, and which were known never to have exchanged their "pitching yeasts".

It has been a supposition that lactic acid bacteria infection in beer is derived from the atmosphere or arise by keeping beers under unclean conditions. However, from these investigations it is evident that the source of the majority of lactic acid bacteria infections in fermented beverages is the yeast used for their production.

These observations are significant in regard to the use of brewery yeast in biological enquiries concerned with the problems of intermediate metabolism. Yeast has been employed for a wide variety of purposes in studies of enzymic activity and thus the rich bacterial flora associated with brewery yeast may prove to be a source of grave error in such cases where delicate metabolic changes are being studied by the enzymes of brewery yeast. The bacterial infections are not easily detected unless microscopic and biological methods are employed, and thus could easily escape the notice of a worker unless he suspects their presence.

On the other hand the growing importance of the lactic acid

bacteria species in various industries, as has been pointed out in the early part of this thesis, has made them even more important.

All these facts indicate that continued study of the lactic acid bacteria is likely to be of considerable value both from the academic as well as the industrial point of view.

SECTION II.

NUTRITIONAL PART.

During the present investigations it was desired to determine the nutrient requirements of some of the lactic acid bacteria species isolated from the brewery yeasts. The composition of the basal medium employed in the present investigations is given in Table I in Section II of the practical part. From the experiences of the previous workers it was expected that this medium would be reasonably satisfactory.

That this medium does not fully satisfy the requirements of all strains of lactic acid bacteria, however, is evident since some of the strains employed by the writer for the preliminary investigations did not show any sign of growth even after an incubation period of ten days. From an examination of Table IIA it is evident that the turbidity produced over incubation periods ranging from 3 to 7 days was relatively high for C_2 and C_3 only, while it was moderately good

for all the other strains except B_1, W_4 and W_5.

The maximum turbidity produced by each of the sixteen strains was measured in the basal media from which each of the twenty-one amino acids, twelve vitamins, three purines and one pyrimidine had been omitted in turn. The results of the experiments with the amino acids, vitamins and purines and pyrimidines are summarised in Tables III, V and VII respectively, in Section II of the practical part. Apart from those experiments an extra experiment was carried out with the three members of the B_6 group, namely, pyridoxin, pyridoxal and pyridoxamine: two out of those three vitamins were omitted each time from the complete basal medium, and in one experiment all the three were omitted. The results of this experiment have also been included in Table V, comprising the results with various other vitamins.

In Tables IV, VI and VIII in Section II of the practical part, the micro-organisms are arranged in order of their fastidiousness for the amino acids, vitamins and purines respectively, while the latter are listed, in the respective tables, in order of their indispensability for the strains tested.

Before discussing results of these investigations in detail it may be pointed out that the technique adopted by the writer during these investigations was different from that of Dunn et al (239). Dunn et al, measured the growth by titrating the acid produced, while the writer measured the growth by turbidimetric measurements on the Hilger Specker using neutral filters.

It may also be pointed out that Dunn et al (239) apparently did not take account of the possibility of variation of the maximal

growth response of an organism in the complete basal medium. Thus, although they estimated growth of an organism in the various deficient media after different periods of incubation (e.g. for L. dextranicum 8359 periods of 50, 72, and 114 hours), they compared the responses to one single response in the complete medium which in this particular case was after 72 hours.

It is quite likely that an organism showing slight growth in a deficient medium when grown for 50 hours may show better growth if incubated for further 22 hours. In fact many cases are known of slow synthesis of a necessary factor finally giving rise to almost normal growth on prolonged incubation.

It follows that at 50 hours the necessary factor would be considered essential, while it may not at 72 hours. Although in most cases examined by Dunn et al, growth in absence of essential substance was extremely small, in some "border cases" this was not so. Thus for L. dextranicum 8359 growth in complete medium at 72 hours was 9·3 ml., whereas in methionine deficient medium it was 2·2 ml. at 50 hours. 9·3/4 is 2·3. It would thus appear quite feasible that a further 22 hours incubation of methionine-deficient medium might give a further 0·1 ml. or more of acid, and thus would transfer the amino acid from the "essential" to the "stimulatory" category.

With these points in view the writer adopted the technique (described in the Section II of the practical part) which eliminated the above sources of error. It is worthwhile to point out that during these investigations it was confirmed again and again that the growth response in the complete medium of identical concentration,

inoculated and incubated under identical conditions, could fluctuate to a considerable extent.

Table V shows that L. pastorianus W_5, the least fastidious of all the strains in question, required only two amino acids - glutamic acid and leucine - for its growth, while L.plantarum G_2 the most fastidious of all the strains required as many as eleven amino acids. Glutamic acid and leucine were found to be essential for all the organisms, while tryptophan was essential for all but one and glycine for all but three strains. The number of strains which required each of the following amino acids is shown in parentheses: valine (12), threonine (11), arginine (8), proline (8), serine (8), alanine (6), tyrosine (5), asparagine (4), phenyl alanine (3), lysine (2), hydroxyproline (1), isoleucine (1) and cystëine (1). Histidine, methionine, norleucine and norvaline were not found to be essential for the growth of any of the organisms.

An examination of the Tables IV and V reveals that strains of individual species have very different requirements. Thus L. pastorianus W_5 requires but 2 amino acids, glutamic acid and leucine, whereas L. pastorianus B_4 requires 8 amino acids in addition to these two. Further, although both L. leichmanni A_4 and L. leichmannii E_4 require 7 amino acids, these differ as follows:-

Strain A_4 - glutamic acid, leucine, tryptophan, glycine, valine, threonine and serine,

Strain E_4 - glutamic acid, leucine, tryptophan, arginine, alanine, tyrosine and phenyl alanine,

i.e. only 3 requirements in common. (The strain used by Schweigert, Guthneck and Scheid (288) required only alanine.)

This lack of correlation between nutritional requirements of

different strains of the same species has been remarked upon earlier. Dunn et al (239) found similar results with the 23 organisms they investigated (all but one of which were of different species from those used in the present work).

In three cases an amino acid is required by only one organism. Thus isoleucine is required only by L. pastorianus W_1, cysteine by L. plantarum G_2 and hydroxyproline by L. plantarum G_2. Dunn et al (239) found 20 out of 23 organisms required isoleucine, 12 required cysteine and none required hydroxyproline.

This lack of essentiality of isoleucine is surprising, especially in view of the unanimous requirement of leucine. It is very probable that the explanation lies in contamination of the leucine used, by isoleucine. Hegsted and Wardwell (142) showed that commercial DL-alanine satisfied the leucine and isoleucine requirements of L. arabinosus, and isolated isoleucine as the Cu salt of the tosyl derivative.

The fact that only one organism, L.plantarum G_2, requires cysteine is interesting in view of the findings of Speck and Pitt (263), discussed earlier in this thesis. They showed that for some organisms pyridoxal and pyridoxamine eliminated the cysteine requirement. As the medium used in the present work contained these B_6 representatives, this may account for the findings with cysteine. A further fact is that L. plantarum G_2 is the only organism to require any of the B_6 group - pyridoxamine - for growth. The situation is somewhat similar to those organisms which require both oleic acid and biotin - which mediates the synthesis of oleic acid - for growth (268).

Further work is obviously desired to determine the response of

these bacteria in media deficient in (a) leucine and isoleucine, and (b) cysteine and B_6. It is probable that many of the organisms would not grow in these conditions.

Hydroxyproline has not previously been shown to be required by any lactic acid bacteria. The drop of 295 units or 76% makes it probable that this organism - L. plantarum G2 - could be used to assay hydroxyproline. This is made more likely by the fact that the organism synthesises the amino acid very slowly, if at all, the above determination being made after 5 days incubation.

There are many instances in the literature of inhibition of bacterial growth by amino acids, though that is not so frequent on such rich media as has been used in the present work. Amino acids reported inhibitory are threonine and serine (254), arginine (143), valine, leucine and isoleucine (236), alanine, asparagine and glycine (239) and cysteine.

From Table IX (in Section II) it can be seen that of these amino acids, 8 have been found inhibitory to, for one to six organisms. However, in addition to these, 7 other amino acids show inhibitory properties. These include primarily norvaline, which apparently inhibits the growth of seven organisms, i.e. 3 strains of L. bifidus and 1 each of L. frigidus, L. pastorianus, L. buchneri and L. leichmannii. This amino acid has not been previously reported active in this manner, although data presented by Dunn et al (239) reveals that it inhibited growth of L. gayonii 8289 and also L. citrovorum 8081 quite appreciably. Inhibition by this amino acid sets quite a problem, as it is not known to play any part in protein synthesis. It is perhaps significant that all L. bifidus strains require valine for growth.

L. leichmannii E_4 is inhibited by any one of the following 9 amino acids:- asparagine, glycine, histidine, hydroxyproline, isoleucine, lysine, methionine, norvaline and serine, while L. pastorianus strains B_1, W_1 & W_{10} are all inhibited by one amino acid, namely lysine.

L. pastorianus W_{10} is also strongly inhibited by threonine and at the same time requires valine for its growth. This implies the possibility of threonine-valine antagonism as first demonstrated by Gladstone (236).

In a number of cases, although omission of an amino acid did not result in a drop in turbidity to $^1/_4$ of the control value, nevertheless the drop was considerable. The amino acids and organisms showing this type of response may be said to show a stimulatory relationship. Those amino acids which when omitted result in a drop of one-half of the maximum or more are considered to be stimulatory. The number of organisms for which individual amino acids are stimulatory is shown in parentheses:- alanine (6), tyrosine (4), arginine (3), histidine (3), asparagine (2), proline (2), serine (2), hydroxyproline (1), lysine (1), methionine (1), phenylalanine (1), valine (1). Only two organisms - L. bifidus D_5 and and Str. damnosus var minimus F_6 - show no "stimulation" responses.

The relationship between alanine, phenylalanine and tyrosine are worthy of attention. Three organisms require simply alanine, one simply phenylalanine and three simply tyrosine; one requires alanine and phenylalanine, and one alanine and tyrosine, and one organism, L. leichmannii E_4, requires alanine, phenylalanine and tyrosine. As far as the writer is aware, no other lactic acid organism has been

shown to require these 3 amino acids together (Lymen et al (262) stated Str. faecalis R. requires them, but Groenhut et al (289) stated the amino acids to be only stimulatory for this organism).

A study of the Table IV shows that usually the drop in turbidity resulting from the omission of a particular amino acid is very large. Although a drop to $^1/_4$ has been taken as the criterion for essentiality, very often the drop is to $^1/_{10}$ or even less of the total value. This suggests that these organisms should be useful for assay purposes.

Threonine is one amino acid which has been assayed with difficulty, because generally it is synthesized slowly by the assay organism (assay organisms used have been Str. faecalis R. (238) and L. fermenti 36 (290)). However, the 11 organisms requiring threonine give very little growth in its absence, even after 5-8 days incubation. It is therefore likely that threonine in particular should be assayed successfully with a number of these organisms.

Results of the experiments with various vitamins are summarised in Tables VI and VII in Section II of the practical part. An examination of these tables reveals that pantothenic acid was found to be essential for all the 16 organisms examined, while nicotinic acid was required by 12 organisms. The organisms for which nicotinic acid was not found to be essential are the three strains of L. pastorianus W_1, W_{10} and W_D and a strain L. leichmannii A_4. But it is noteworthy that with these four strains the drop in the turbidity readings of nicotinic acid deficient medium was very considerable, though not so much as to make this vitamin essential. The drop in these cases was from $^1/_2$ to $^1/_3$ of the complete values, thus nicotinic acid has stimulatory effect for these four organisms.

These findings with regard to the needs of pantothenic acid and nicotinic acid are in conformity with the observations of the previous workers (239).

The number of organisms for which other vitamins are found essential for growth are given in parentheses:- riboflavin (3), pyridoxamine (1) and PAB (1). The omission of biotin, choline, folic acid and pyridoxin had no marked effect on the growth of any of the organisms. Inositol was also not found to exert any effect on the growth of these organisms, except that it was found to inhibit considerably the growth of one strain, namely, L. leichmannii E_4.

Thiamine was not found to be essential for any of the organisms tested in the present work, but it, as well as the other vitamins, probably had a stimulatory effect for many of the organisms in question. The number of organisms for which individual vitamins have shown stimulatory relationship is shown in parentheses:- thiamine (5), nicotinic acid (4), and riboflavin (3).

Although similar vitamin requirements were found in other laboratories, a number of differences may be noted. Some of the vitamins reported in the literature to be essential were found to be non-essential in the present case. That these vitamins were synthesized under the present experimental conditions was not unexpected, since the synthesis of growth factors including folic acid (291), riboflavin (292) and pyridoxin (161) has been reported on the relatively incomplete media employed by other workers.

$\underline{p}$ -Amino benzoic acid (PAB) has been found to be essential for only one strain, namely, L. nastorianus W_4 in the present experiments. Though Dunn et al did not find PAB essential for any of their 23

organisms, it has been reported to be essential for L. arabinosus 17-5 (200) and essential under restricted conditions for Leuc. mesenteroides P-60. On the other hand, Snell and Mitchell (233) have reported that the requirements for L. arabinosus and L. pentosus is non-specific for PAB, since other nutrients (methionine, adenine, guanine and xanthine) were also effective in promoting growth of these organisms.

It is probably for the first time that pyridoxamine alone of the B_6 complex has been found to be essential for any organism (L. plantarum G_2), though the essentiality of pyridoxin and pyridoxal has been reported by previous workers. The specific requirement of pyridoxamine for this organism is evident by the fact that the presence of pyridoxin and pyridoxal in appreciable quantities made no difference to the essentiality of pyridoxamine. This organism L. plantarum G_2 can thus serve an extra tool for assaying vitamin B_6 in natural materials.

As pointed out before, riboflavin has been found to be essential for the following three organisms, L. buchneri C_3, L. leichmannii E_4 and L. plantarum G_2. This vitamin was also reported essential for these species by previous workers.

Apart from the stimulatory and essential nature of the various vitamins observed in these investigations, some cases were observed where vitamins seemed to exert inhibitory effects. The number of organisms for which individual vitamins have been found to show inhibitory relationship is shown in parentheses:- inositol (1), PAB (3), pyridoxal (3), pyridoxamine (3), riboflavin (1), thiamine (1).

Pyridoxin, pyridoxal and pyridoxamine were reported slightly inhibitory for L. brevis by Dunn et al (239). PAB has also been

reported to be inhibitory to L. casei (239).

Lastly it may be pointed out that uracil was found to be essential for 10 organisms and adenine for 5 organisms (Tables VII and VIII in Section II of the practical part). Guanine and xanthine was not found to be essential in any of the sixteen cases examined. On the other hand, adenine was found to inhibit, to some extent, growth of 6 organisms, guanine inhibited 4 organisms, uracil inhibited 2 organisms and xanthine inhibited 3 organisms.

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PART II.

An examination of bacteria isolated from textile fabrics.

INTRODUCTION.

In spite of the fact that the textile industry has developed to an enormous extent in the last 50 years little work has been done in the investigation of bacteria which attack textile fabrics. Most of the researches have been confined to the study of moulds which attack fabrics and many of these have been identified, but little is known of the other microbic-forms.

The object of this research was to isolate pure cultures from the three specimens which have been picked off the grey cloth from a textile factory, and subsequently to characterise and identify them.

As to the arrangement of this part of the thesis, the subject matter has been divided and arranged in their respective order in three parts, namely, Historical Part, Practical Part and Comments.

HISTORICAL PART.

THE COURSE OF DEVELOPMENT OF KNOWLEDGE OF THE ORGANISMS AFFECTING TEXTILES.

The earliest investigations on the mildewing of cotton goods are recorded in publications by Witz (1) in 1875, who investigated samples of printed cotton goods. He observed that goods lying in damp places became marked with white or yellow mould spots; this also occurred when wet goods were stretched on wooden frames. He also pointed out that glycerine and sizing increased the rate of growth of moulds. Similar observations were recorded by Thomson (2) in 1877. Three years later Davis, Dreyfus and Holland (3) published their well known book "Sizing and mildew in cotton goods." They pointed out that mildew is due to the growth of the fungi on raw cotton and cotton goods, and that spores are constantly present in the atmosphere, thus any cloth exposed to moisture and warmth is liable to become mildewed. According to them sized yarns mildewed much more readily than unsized, unless antiseptics were present in sufficient quantity. They also observed that bleached cotton was attacked less easily than unbleached, the resistance depending on the removal of food constituents necessary for growth, such as nitrogen and mineral salts. An attempt was also made by these authors to name various types of fungi producing mildew and a list of 27 different species was presented, but the accompanying illustrations are insufficient for botanical confirmation of the correctness of the identification, and it is impossible for the most part to state what those fungi represent in present day nomenclature.

In 1891 Williams (4) performed significant experiments upon the

$ZnCl_2$ content of grey cloth and liability to mildew. He observed that samples containing 2·39% or over $ZnCl_2$, mildewed slowly or not at all, while those containing less than 0·73% mildewed readily. In 1898 Gruene (5) published a method designed to prevent mildew of sails. He attributed growth of moulds on sails to the presence of sizing materials and excessive moisture and recommended that the size can be removed by use of malt enzymes, or boiling in sodium hydroxide or sodium silicate. This desizing treatment was followed by impregnation with aluminium acetate, drying, and fixing of the alumina with a dilute solution of sodium silicate. In the same year Von Holle (6) noted that raw cotton submerged in sea water developed green and brown colourations and a disagreeable odour, and he found it to be unfit for spinning. Colourless and brown coloured fungi were isolated. He attributed the acceleration of fibre destruction in the presence of sizing to the acids developed by mildewing of the starchy materials used.

Trotman (7) in 1909 reported the discovery and isolation of an organism which caused a pink discolouration in cotton after a fortnight of incubation of the infected material. He also pointed out that sizing and slightly acid reaction favoured growth of the organism. Osborne (8) tested various types of cloth by placing them on potato agar using sterile material and apparatus. He found that none of the cloth used was free from infecting organisms. The principal genera isolated by him were Penicillium, Mucor, Fusarium, Aspergillus, and several unidentified bacteria and hyphomycetes. Broughton Alcock (9) in 1919 made some observations on canvas destroying fungi in Malta and Italy. He concluded that the principal agents causing the destruction of canvas made from cotton were fungi of the genera Macrosporium and Stemphyllium. Levine

and Veitch (10) recorded the finding of species of Alternaria, Cladosporium and Mucor upon fabrics injured by mildew. They suggested that some changes in the fabric may be due to a symbiotic condition when more than one species is present.

In 1920 a paper was published by Corrigan (11) in which he discussed in outline the relationship between bacteria and moulds in the vegetable kingdom and their specific application in the textile industry. He found the following organism impregnating flax taken from crates during the retting process: B. subtilis, B. megatherium, B. coli, B. fluorescens putridus, the last appeared to be the most active. He pointed out that the bacteria exert a decomposing action upon cellulose and starch matters which results in the tendering of cloth, and suggested that the destruction of bacterial growths on finished goods may be brought about by heat, light, and antiseptic substances such as KNO_3, $ZnCl_2$, H_3BO_3, and various phenols.

Doree (12) in 1920 observed that fabrics of cotton and silk were destroyed by immersion in sea water for three weeks, wool lasted somewhat longer. This destruction was due to an aerobic growth of micro-organisms. He also suggested that acetylated cotton or cellulose acetate is much more resistant and may last for months under similar conditions. In the same year Fleming and Thaysen (13) pointed out that deterioration of cotton in damp storage was due to Streptothrix and and a Schizomycete. Deterioration was said to be retarded when the moisture content was reduced below 9%.

In 1922 Sidebotham (14) examined a sample of dyed cotton cloth, showing discolouration. It was found that a species of Botrytus was the infecting organism. Growth was rapid at 90°-100°F. and cellulose

was quickly destroyed in both dyed and uncoloured areas. Armstead and Harland (15) observed the occurrence of mildew in black bordered dhooties, mould was found to develop under the black border, and A. niger and a Penicillium were isolated. The border was not attacked by the moulds, dye in the border was thought to be inhibiting. Trotman and Sutton (16) performed tests with B. subtilis and B. mesentericus which indicated that they were able to grow upon and destroy the fibres in the presence of moisture.

In 1923 Thaysen and Bunker (17) published a paper "Bacterial Decomposition of Cellulose", in which they stated that cellulose decomposing bacteria becomes active in raw cotton samples containing more than 10% moisture. According to them flax is similarly attacked, and although it undergoes strenuous treatment in course of manufacture it is liable to reinfection at all times.

It will be noted that though the damage caused by the development of fungi and bacteria was already realised to be serious, a systematic investigation of mildewing was not undertaken and could not perhaps have been undertaken at that time owing to lack of technical facilities.

The first attempt to study mildew in its various aspects was made by Bright, Morris and Summers (18) as recently as 1924. By their detailed study on the question of the origin of the infection they came to the conclusion that mildew might in some cases be the result of contamination of the raw cotton, while in others, where the fabrics have undergone such drastic processes as bleaching or mercerization, the original microflora of the cotton hairs would have been destroyed. In such cases a subsequent infection would therefore be responsible for mildewing. They mention Penicillium, Aspergillus, Rhizopus as the

fungi likely to cause mildewing and which may be frequently met with in cotton warehouses and mills. They also recommended for creating conditions during the manufacture of textiles which would render it difficult for fungi to develop, and of preventing the spread of an already existing infection by introducing improved methods of cleanliness in textile mills. In the same year Thaysen (19) presented an interesting non-technical paper on the subject of mildew in cellulose fibres, in which he pointed out that destruction of non-lignin encrusted fibres occurs in the presence of more than about 10% of moisture and is due to moulds and bacteria. Preventive measures suggested by him were use of starch substitute in sizing, elimination of moisture, development of resistant fibres and the development of treatments such as acetylation of fibres. In 1925 Department of Scientific and Industrial Research (20) published a review covering the deterioration of fabrics by light and micro-organisms. It was pointed out that untreated linen was observed to deteriorate faster than untreated cotton and moulds as well as bacteria were found to be responsible for the deterioration. The most frequently occurring organism was a brown mould, while seven types of bacteria were isolated from the damaged areas. Of these a few were facultative anaerobes.

Allyn (21) in 1926 during his researches pointed out that bacterial infection seemed to be principal source, in laundry goods and laundry men troubled with this type of damage were urged to leave the wet wash slightly acid rather than alkaline or neutral. In the same year Thaysen and Bunker (22) attempted to determine whether B. subtilis or B. mesentericus could visibly damage the fibres. Using the strains formerly employed by Trotman and Sutton (16), as well as other strains

it was found that no fibre destroying action could be proved even after long incubation periods.

Dubos (23) and Coolhas (24), respectively, pointed out the decomposition of cellulose by aerobic and thermophilic bacteria, but neither of them experimented on the cellulose fabrics directly.

It will be noted that from time to time some attempts were made to identify the responsible organisms. Smith (25) was among the first of those who tried to identify and classify systematically the individual species of organisms responsible for mildewing the cellulose fabrics. He has discussed the general morphology and characteristics of growth of Aspergillus species. The species described by him were divided into two groups: (1) Those found growing on fabrics under trade conditions, and (2) those found as chance contamination but not growing on fabrics. In 1930 Galloway (26) after his extensive researches published a paper on "The fungi causing Mildew in Cotton Goods". In this paper he pointed out that mildew occurs most commonly in grey cloth. According to him most of the infection seems to come from raw cotton and some, such as A. niger, may even remain from an infection of the cotton boll itself. Other sources of infection are the air and the machinery in course of manufacture. It has also been stated that samples of cloth with a moisture regain as high as 9% showed no mildew, but cloths with a regain of 9·5% to 10% mildewed quite readily. High percentage of CO_2 were said to inhibit mould growth. He also pointed out that the source of infection cannot be determined or even approximated by identifying the organisms involved because of the diverse conditions under which they grow. The results of the fungus growth described are as follows: musty smell, acid production (oxalic, citric, etc.),

protein degradation and cellulose digestion.

Galloway has also given a list of about 180 moulds together with their sources, cloth staining ability, acid production and cellulose digestion power. In the same year Roberts and Herbert (27) discussed thoroughly the mildew problem from an economical rather than a chemical or biological view point.

In spite of the so many published researches quoted above, it may be said with respect to the microbiology of cotton fibre, that the published results of research were far from complete: Prindle realised this fact and commenced his extensive researches on the textile microbiology to make a comprehensive and systematic survey of microbiological and biochemical relationships between various micro-organisms and the textile fibres with which they are associated and to correlate this information, if possible, with the strength and usefulness of the fibres. The results of his researches were published in a series of papers in Textile Research (28,29). Prindle (29) by his microbial analysis of twelve samples of cotton fibre had shown that commercial raw cotton is usually highly infected with moulds and bacteria, and that fresh samples of raw cotton fibre have yielded from 4 to 58 million bacteria and from 120,000 to 400,000 moulds per gram. The moulds and bacteria found on raw cotton by Prindle (29) were largely of the soil type, or the type found on fresh plant tissues. Moulds found by him on unstored samples include species of Hormodendrum, Fusarium, Alternarium, Sporotrichum and Monilia-like organisms, with small numbers of the genera Aspergillus and Penicillium, while the stored samples of raw cotton contained Aspergilli and Penicillia and soil types of spore-forming bacteria. Bacteria of the genera Bacillus and Flavobacterium

predominated.

According to Prindle (29), one would expect unstored samples to deteriorate much more rapidly than samples that had been stored. The bacteria found in raw cotton by Prindle were usually protein digesters, which did not demonstrate much action toward carbohydrate media. Prindle also pointed out (29) that bacterial spores present in samples of raw cotton may survive the manufacturing processes and that there is evidence to show that a high percentage of the microbic population of the cotton boll may be carried over into the cleaned fibre, or even into the spun and woven fabrics.

It will be noted from the literature mentioned above that the more evident effects of the growth of moulds upon fibres have been well studied, and many of the organisms positively identified, but little is known of the other microbic forms, such as bacteria.

That bacteria in certain circumstances can be responsible for the decay of fibres and fabrics without the assistance of fungi is certain (11,12,16,20,21,24). Thus Cross and Bevan (30) found no signs of fungi in the sample of heart-damaged jute which they investigated, and Doree (12), who studied the decay of fabrics in sea water, describes it as the action of bacteria. In other cases described by Doree (31) and Osborne (8), bacteria appear to be associated with fungi in the decay. The relative importance of bacteria and fungi cannot at present be definitely circumscribed, since the investigation of the activity of the former in this direction has only recently been seriously considered and still remains incompletely studied. There can be no doubt, however, that the importance of bacteria is very great, particularly in the case of such materials as raw cotton, linen and fabrics subjected

to soil contamination or to exposure under water (8,11,12,17,20,21,23,24). Goulding (32) also described the black arm cotton rot as due to the activity of Bact. malvacearum. The decay due to bacterial infections has also been investigated by Von Gescher (33) and Ruschmann (34). Ruschmann found that scutched flax which had been kept for fourteen days lost 25 per cent of its tensile strength. He found Bact. mesentericus and Bact. megatherium extensively represented on scutched flax, while Von Gescher paid particular attention to the cellulose destroying bacteria affecting flax, among which he describes a type which is either identical with, or closely related to, Spirochaeta cytophaga.

The earlier attempts to study the microbiological decay of fibres and fabrics met with the serious difficulty that it was found impossible to determine the extent of the participation of cellulose fermenting bacteria. There is still no known method by which these organisms can be isolated and enumerated and deductions made as to the extent of their activity.

Itano, Arao and Satiyo Arakawa (35) isolated a new organism from dry farm soil, described and named it as Cellvibrio calida. It reduced nitrate to nitrite in cellulose synthetic liquid culture, but the action of this new isolated organism was not carried out on fibres and fabrics.

Prindle (29), by his researches on textile microbiology, had thrown sufficient light on the fungal infection of the cotton fibres. But like other textile microbiologists he has only touched the problem of bacterial infection to a very small extent. He no doubt pointed out the predominance of the bacteria belonging to the genera Bacillus and Flavobacterium, with some other types in raw cotton, but still the combined researches of all the workers mentioned above have not

amounted to a great deal in comparison with the amount of work which has been done on the topic of mould infections in cloth.

PRACTICAL PART.

THE SOURCE OF THE ORGANISMS.

Three impure organisms were provided by Mr. F. L. Barrett, Director of Research of the Bleachers' Association Ltd., at Bolton. These organisms were picked up off the grey cloth from a textile factory.

PROCEDURE EMPLOYED FOR ISOLATION OF THE PURE ORGANISMS.

Each of the three impure organisms was inoculated in two tubes containing glucose broth and glucose yeast extract respectively, and all the tubes were incubated at 25°C. At the end of one week it was noticed that all the three organisms grew best in glucose broth. The organisms were thus subcultured in fresh glucose broth tubes from the original glucose broth tubes and the tubes were incubated at 30°C. At the end of two days incubation a vigorous growth appeared in all the three tubes. 1 ml. of culture from each tube was diluted to 10^{-2}, using 0·9% saline solution, and 1 ml. of the diluted culture in each case was plated out separately with 15 ml. of glucose agar broth (2% agar was employed). The plates were incubated at 30°C. At the end of 24 hours' incubation period many colonies were found developing on all the plates and were quite suitably spaced.

Five, visually dissimilar, colonies were picked off in the glucose broth tubes from the three plates, and the tubes were incubated at 30°C. for two days. At the end of two days, 1 ml. from each of the five tubes was diluted to 10^{-3} as before, and 1 ml. of each of the diluted

culture was plated out separately with 15 ml. of glucose agar broth and the plates were incubated at 30°C. as before.

After 24 hours' incubation some colonies developed and were found suitable for picking. Eight colonies were thus picked off in the 8 glucose broth tubes and the tubes, as well as the plates, were incubated as usual. At the end of another 48 hours some more colonies, dissimilar in appearance, appeared on the plates and thus three more colonies were picked off in three glucose broth tubes.

All the eleven tubes were incubated at 30°C. for three days and then another set of eleven tubes was inoculated from them. To ensure perfect purity all the cultures were replated exactly as described before and after the incubation of the plates at 30°C. for 3 days, one colony was picked from each of the eleven plates into the glucose broth tubes and the tubes were incubated as usual. Microscopic examination of these eleven cultures showed that each of them was perfectly pure.

One set of the freshly growing cultures was put aside under $CaCO_3$, as stock cultures, in a cool place, while the other set was kept for the further examination of their characteristics.

The writer wishes to point out that the examination of morphological, cultural, physiological and biochemical characteristics of these organisms were carried out in a similar manner as described in the first part of this thesis, and thus neither the description of the media, apparatus, inoculation procedure, etc., nor the references for the other various techniques applied in this part have been repeated.

MORPHOLOGICAL CHARACTERISTICS.

These characteristics were examined with cultures grown in glucose broth medium or on glucose broth agar medium, prepared as described on page 74. The cultures were always examined when they used to reach logarithmic phase of growth at their respective optimum temperatures.

Size, shape and arrangement was examined in (a) hanging drop preparations, and (b) in stained preparations. Preparations (a) were made from liquid cultures alone, but preparations (b) were made from liquid as well as solid medium. The results obtained from Gram stained slides or methyle blue stained slides were identical in the case of respective organism. The descriptions given in Table I are from the Gram stained slides for all the organisms.

(B) Motility was sought for in (a) semi-solid media cultures, and (b) in hanging drop preparations. The first way of examination provided useful preliminary information which had been always confirmed by the latter method.

The method of Tittsler and Sandholzer (36) was used for detection of motility under (a). Glucose broth agar was specially prepared for this purpose (0·5% agar medium was used) and was inoculated with the organisms in question, and one set of tubes was kept at 20°C., whilst the other set was kept at the respective optimum temperatures of the organisms. The observations for the "Zone of diffused growth" were made as frequently as possible.

The definite conclusion concerning the motility of the organisms was obtained from hanging drop preparations of the liquid cultures grown at 20°C. as well as at their respective optimum temperatures.

(C) Staining. This characteristic was divided into two groups for the sake of convenience: (a) ordinary staining with Loeffler's methylene blue, carbol fuchsin, etc., and (b) special staining for Gram stain, spores and capsules, etc.

(a) Ordinary Staining.

The organisms in question were grown on the solid media slopes and incubated at their optimum temperatures. The cells were then stained with Loeffler's methylene blue and carbol fuchsin staining reagents, respectively.

(b) Special Stainings.

Gram stain. The cultures were grown on the solid media slopes, and Hucker's modification of Gram stain was employed.

Spore staining. Slope cultures were employed for this experiment after the incubation of 8 days at their respective optimum temperatures. Formation of endospores was sought for by Grey's method as well as by Schaeffer and Fulton's method.

The results obtained for the morphological characteristics have been summarised in Table No. I.

TABLE I.

Morphological Characteristics (observed from the gram stained slides of liquid cultures).

Organism.	Shape and Size.	Arrangement.	Motility.	Spores.	Gram Stain.	Methylen Blue Stain.
D_1	Long uniform rods with rounded ends as sausages. 1·9 to 2u by 4 to 4·5u.	Mostly in chains of three to five, but some single cells also. A few clumps also detected.	Non-motile.	Not detected.	Gram negative.	Fair stain.
D_3	Long uniform rods with rounded ends. 1·6u to 2u by 4 to 4·5u.	Singles, in chains and a few small clumps also detected.	Non-motile.	Not detected.	Gram negative.	Fair stain.
D_4	Spheres, slightly ellipsoidal. Diameter 1·4 to 1·8u.	Occurring as single cells, in pairs, chains and clumps. A few tetrads also detected.	Non-motile.	Not detected.	Gram positive, good staining.	Good stain.
D_5	Long uniform rods with rounded ends. 1·8u to 2·1u by 4 to 4·5u.	Mostly in chains of three to six. Some single cells also. But only a few small clumps.	Non-motile.	Not detected.	Gram negative.	Good stain.
D_6	Long rods with slightly rounded ends. ·6 to ·9u by 2·4u to 3·5u.	Mostly single cells as well as short chains. No groups observed.	Motile.	Detected ·6 to ·8 by 1 to 1·3u.	Gram positive, very weak staining.	Good stain.
D_7	Long uniform rods with rounded ends like sausages.	In chains, clumps, singles as well as pairs.	Non-motile.	Not detected.	Gram negative.	Fair stain.
D_2	Long rods with somewhat rounded ends. ·7 to ·9u by 2·5 to 3·8 u.	Mostly single cells, some short chains, but only a few clumps.	Motile.	Detected size ·6 to ·7 by 1 to 1·2u.	Gram positive, very weak stain.	Good stain.
D_2b	Rods of varying sizes, with round ends. 1 to 1·2 by 3 to 4u.	Mostly as single cells. But some short chains and clumps were also observed.	Non-motile.	Not detected.	Gram negative.	Fair stain.
C_4	Spheres slightly ellipsoidal, diameter 1·5 to 1·9u.	Mostly in single cells, but some pairs, chains and clumps also observed. No tetrads observed.	Non-motile.	Not detected.	Gram positive.	Good stain.
C_5	Oval shaped cocci. Diameter ·8 to 1u.	Mostly in pairs. But single cells, chains and clumps were also present.	Non-motile.	Not detected.	Gram positive.	Good stain.
C_5b	Uniform rods slightly rounded at the end. 1 to 1·2u by 2·5 to 3·2u.	Single cells, pairs, short chains and clumps all were present to the same extent. (Slightly elongated on staining.)	Non-motile.	Not detected.	Gram negative.	Good stain.

CULTURAL CHARACTERISTICS.

These characteristics were examined with the organisms grown at their optimum temperatures, or at the temperature from the optimum range. Only in the experiments with gelatin media, were the cultures incubated at 20°C. In all the experiments with the liquid cultures description of growth after 2 and 7 days was recorded for the organisms in question, while the solid cultures were described after 1 day and 3 days incubation period.

Throughout these experiments 24 hour old solid media tubes (stabs and slopes) and plates were used because old slopes, etc., were found to hinder and delay the growth of the organisms.

The meaning of the terms used in describing the type of growth are given in the glossary of the terms (this thesis page 119).

A. Description of growth on/in solid media.

(a) The description of growth on glucose broth agar slopes and stabs was recorded as described above in the first paragraph dealing with the cultural characteristics.

The results of the experiment are summarised in Tables II & III.

(b) The description of growth on glucose broth gelatin slopes. Gelatin slopes were prepared by using 15% gold leaf gelatin. The tubes after inoculation were incubated at 20°C. and the description of growth was recorded as mentioned above.

The summary of the results is given in Table IV.

(c) The description of the growth of the giant colonies on the special glucose broth agar medium.

The glucose broth agar medium used for this experiment was specially

TABLE II.

Description of Glucose Agar Broth Slope Cultures.

Organism.	Incubation Temp. °C.	Time. Day.	
D_1	35°C.	1	Moderate growth, yellowish-white, glistening, raised, opaque, round, feathery edged.
		3	Growth slightly increased, distinct broken edges and size, and the yellow colour of the colony also increased.
D_3	35°C.	1	Moderate growth, pale yellow, glistening, round, opaque, raised in centre and feathery edged.
		3	Growth and size increased. No other change, but yellow tinge increased.
D_4	25°C.	1	Moderate growth, colonies punctiform, appears to be round, opaque, glistening, entire edges, colour somewhat dirty white.
		3	No change except brownish-yellow tinge appeared.
D_5	35°C.	1	Abundant growth throughout, yellow, glistening, raised, opaque, round with some broken edges.
		3	Size and growth increased to some extent. Some yellow tinge also increased.
D_6	37°C.	1	Abundant growth throughout, dull, light brownish colour, opaque, round, entire edged.
		3	Size increased. Brownish colour greatly increased, colonies nearly flat and somewhat dried appearance.
D_7	35°C.	1	Moderate growth, yellowish-white, glistening, raised, opaque, round, distinctly feathery edged.
		3	Growth and size increased and yellow tinge greatly increased.
D_2	37°C.	1	Abundant growth throughout, colonies dull, light brown colour, opaque, rough, round and entire edged.
		3	Brownish colour greatly increased, colonies looked quite flat, dried and wrinkled.
D_2b	30°C.	1	Moderate growth, very small white colonies, beaded, opaque, raised, glistening, round and entire edged.
		3	No change except the size and growth increased slightly.
C_4	25°C.	1	Fair beaded growth, colonies punctiform, raised, glistening, dirty white colour.
		3	Growth slightly increased, colonies still small, light yellowish brown tinge appeared.
C_5	25°C.	1	Moderate growth, colonies deep yellowish, glistening, opaque, round, flat and entire edged.
		3	Size considerably increased, colonies distinctly flat, entire edged and opaque. Some light brownish tinge also appeared along with deep yellow colour.
C_5b	30°C.	1	Poor growth, colonies punctiform, beaded, shining, but no other indication possible.
			Growth increased, colonies still very small, but are round, glistening and entire edged.

TABLE III.

Description of growth in Glucose Agar Broth Stabs.

Note: There was very faint growth in all the tubes at the end of 3 days, but not sufficient for characterization.

Organism.	Incubation: Temp. °C.	Incubation: Time Days.	
D_1	35°C.	7	Abundant growth at the surface and just below it. But decreased markedly below the surface and became irregular.
D_3	35°C.	7	Moderate beaded like growth on the surface; below the surface it was markedly decreased and irregular.
D_4	25°C.	7	Fair regular growth at the surface and just below it. But greatly decreased and became irregular below that part.
D_5	35°C.	7	Thick regular growth on the surface and some around the walls. But below the surface there were scattered patches only.
D_6	37°C.	7	Thick wrinkled growth throughout the surface and below it (up to 1 c.m.), but after that it decreased markedly and became scattered.
D_7	35°C.	7	Fair growth at the top, while below it was very faint along the line of inoculation.
D_2	37°C.	7	Thick regular growth at the surface and much below the surface (2 c.m. deep), but decreased markedly below it and became scattered.
D_2b	30°C.	7	Slight surface growth, but absolutely none below it.
C_4	25°C.	7	Slight growth at the surface and below it. But markedly decreased after that.
C_5	25°C.	7	Moderate surface growth and slight below it. But very faint and irregular throughout the stab. Yellowish tinge throughout.
C_5b	30°C.	7	Slight surface growth, but faint below the surface which decreased to absolutely nil at 2 c.m. depth.

TABLE IV.

Description of growth on Glucose Broth Gelatin Slopes.

Note: At the end of 1 day there was very faint growth in D_6, D_2, D_1, D_3 and D_7, but no sign of liquefaction.

Organism.	Incubation: Temp.	Time.	
D_1	19–20°	3	Almost all colonies are liquefying (crateriform), so cannot make out the shape, etc., of colonies, but size very small. Most of the gelatin liquefied.
D_3	"	3	Same as D_1 except that the colonies appear to be slightly bigger than D_1.
D_4	"	3	Abundant growth, colonies are very small, but appears to be round, smooth and glistening with a yellow centre. No sign of liquefaction even after 10 days incubation.
D_5	"	3	Abundant growth and the colonies are very small (punctiform), may be round and dull. Most of the gelatin is liquefied.
D_6	"	3	Moderate growth in the form of streaks only. Gelatin was mostly liquefied.
D_7	"	3	Abundant growth, but colonies very small (punctiform). Colonies seem to be round, dull and entire edged. Most of the gelatin is liquefied.
D_2	"	3	Abundant growth, but it was in the form of streaks and not the separate colonies. Nearly half of the gelatin was liquefied.
D_2b	"	3	Poor beaded growth and very small colonies. No liquefaction even after 10 days incubation.
C_4	"	3	Moderate growth. Small colonies nearly round shaped, smooth glistening, with a yellow centre. No sign of liquefaction even after 10 days incubation.
C_5	"	3	Moderate growth, colonies were yellowish-brown, round, raised in the centre, opaque, dull and entire edged. About half the gelatin liquefied.
C_5b	"	3	Poor beaded scattered growth. No sign of liquefaction even after 10 days incubation.

TABLE V.

Description of Giant Colonies (on 5% Agar, Glucose Broth Medium, grown at room temperature (19–20°) for 14 days).

Organism.	General appearance of growth.	Shape.	Size.	Elevation.	Consistency.	Opaque or otherwise.	Shiny or dull.	Colour.	Description of the Zonal Area.
D_1	Abundant growth and has various zones of growth. Four to five zones.	Not perfectly circular, but is more round than ellipsoidal.	4·5 by 5·2 c.m.	Flat.	Coherent consistency.	Opaque.	Shining.	Outermost and innermost zones colourless but the remaining zones were deep yellow.	The growth was decreasing regularly from the inner to the outer zone, but growth in each zone was evenly spread to cover the marginal areas. Has slightly broken edges.
D_3	Ditto.	Slightly ellipsoidal.	4·4 to 5·4.	Almost flat except that the centre was slightly raised.	Ditto.	Ditto.	Ditto.	Faintly yellow throughout, but deep yellow at the edges.	Same as D_1, and has distinctly broken edges.
D_4	Very compact and thick growth throughout the colony. No zones uniform throughout.	Practically circular.	6 m.m. diameter.	Perfectly flat.	Butter-like consistency.	Ditto.	Ditto.	Milky white.	Zone less regular and even growth with entire edges.
D_5	Abundant growth and has five distinct zones.	Not entirely circular.	4·5 by 5·3.	Flat, centre was slightly raised.	Coherent consistency.	Opaque.	Ditto.	Yellowish tinge throughout, but central part was deep yellow.	Growth was decreasing from the innermost zone to the outermost zone. Distinctly feathery edges.
D_6	Abundant wrinkled growth and has three zones, but not very distinct.	Practically circular.	2·5 to 2·7 c.m.	Almost flat, except slightly raised in the centre.	Dried and hard appearance.	Opaque.	Dull.	Light brown tinge throughout, but yellowish in the centre.	Growth was nearly uniform throughout, but there appeared to be some diffused growth at the outermost zone. May be motile.
D_7	Abundant growth with various zones.	Ellipsoidal.	4 c.m. by 5·3 c.m.	Flat.	Coherent consistency.	Opaque.	Shining.	Deep yellow throughout.	Growth was markedly reduced in the outer zones, except the first. Distinctly feathery edges.
D_2	Abundant growth.	Circular.	2·6 to 2·8 c.m.	Almost flat except slightly raised in the centre.	Dried and hard.	Opaque.	Dull.	Dirty brown tinge throughout, centre yellow.	Uniform growth in all zones, but there was distinctly diffused growth also.
D_2b	Abundant growth with many zones.	Nearly circular.	3·6 to 3·8 c.m.	Flat. The centre, a distinct point, is slightly raised.	Butter-like consistency.	Opaque.	Dull.	Faint pink-brown-tinge throughout.	Uniform growth in all zones except the last, which has a weaker growth. A distinct centre. Entire edges. No diffused growth.
C_4	Compact and thick growth throughout. No zones.	Nearly circular.	6 m.m. diameter.	Flat.	Butter-like consistency.	Opaque.	Shining.	Milky white.	Zone less regular and even growth with entire edges. No diffused growth.
C_5	Moderate growth. May have a faint zone.	Nearly circular.	8 m.m. diameter.	Flat.	Butter-like consistency.	Opaque.	Shining.	Shining sulphur yellow with a brown centre.	Nearly even growth with entire edges. No diffused growth.
C_5b	Abundant growth with three distinct zones.	Nearly circular.	2·4 to 2·5 c.m.	Flat.	Coherent consistency.	Opaque.	Shining.	Faint dirty brown tinge throughout.	Even, circular growth throughout, except in the outermost zone where growth is weak. Entire edges. No diffused growth.

prepared by using 0·5% agar only. Inoculation was performed by means of a platinum wire such as is used for making stab cultures, but with the difference that the wire was bent at an angle near to its free point. This was dipped into an active young liquid culture and was allowed just to touch the surface of the agar medium, which previously had been poured into a petri-dish and allowed to set there. Great care was taken to avoid penetration of the wire below the surface. Should the wire penetrate the agar the resultant colony will not be of typical character.

The results of the experiment are summarised in Table V. The plates showing the giant colonies (actual size) are also attached.

B. Description of Growth in Liquid Media.

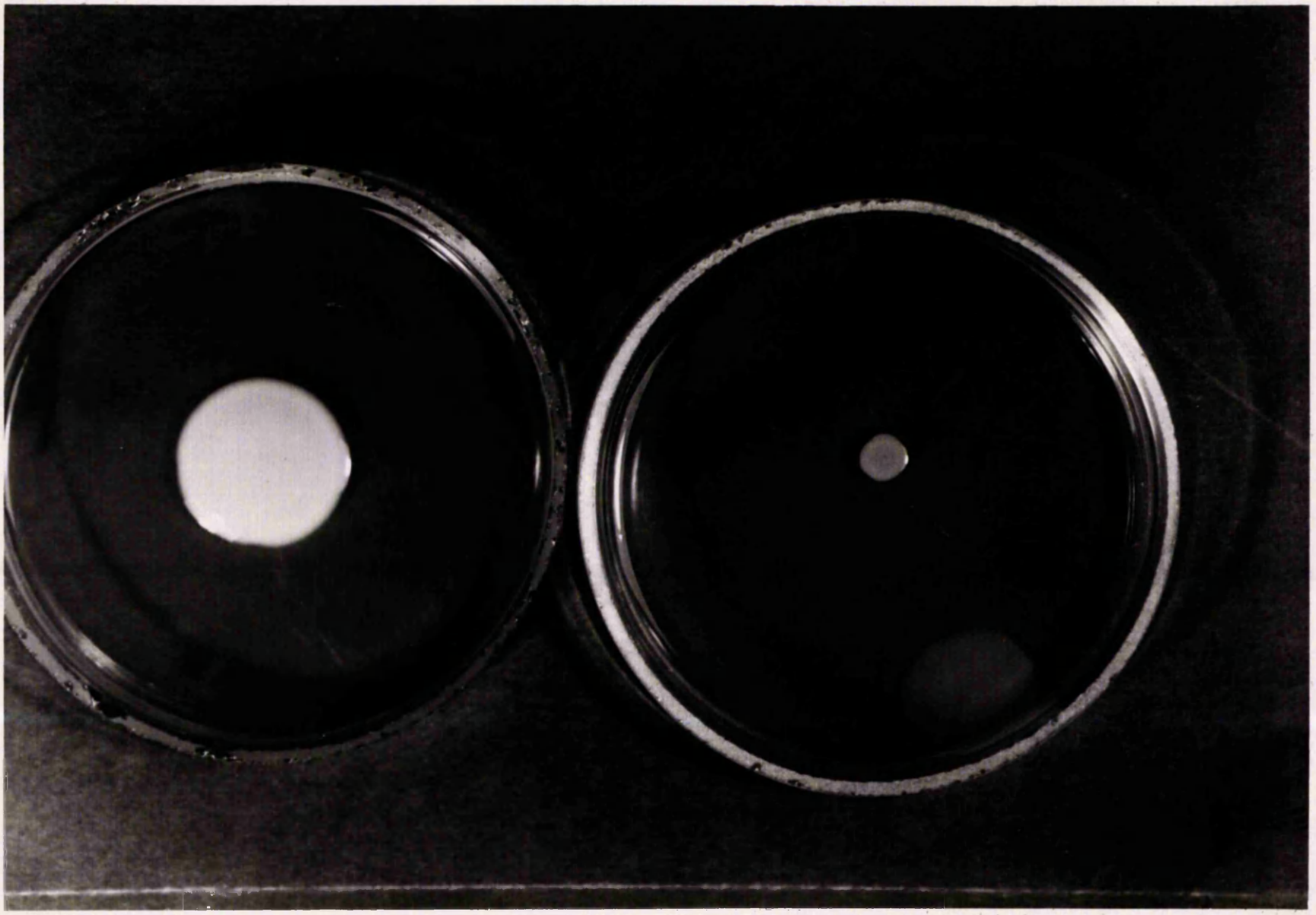
The description of all the organisms in question was recorded in the following liquid mediums, as described in the first paragraph dealing with cultural characteristics:-

(a) Nutrient broth.
(b) Glucose broth.
(c) Peptone water.
(d) Litmus milk.

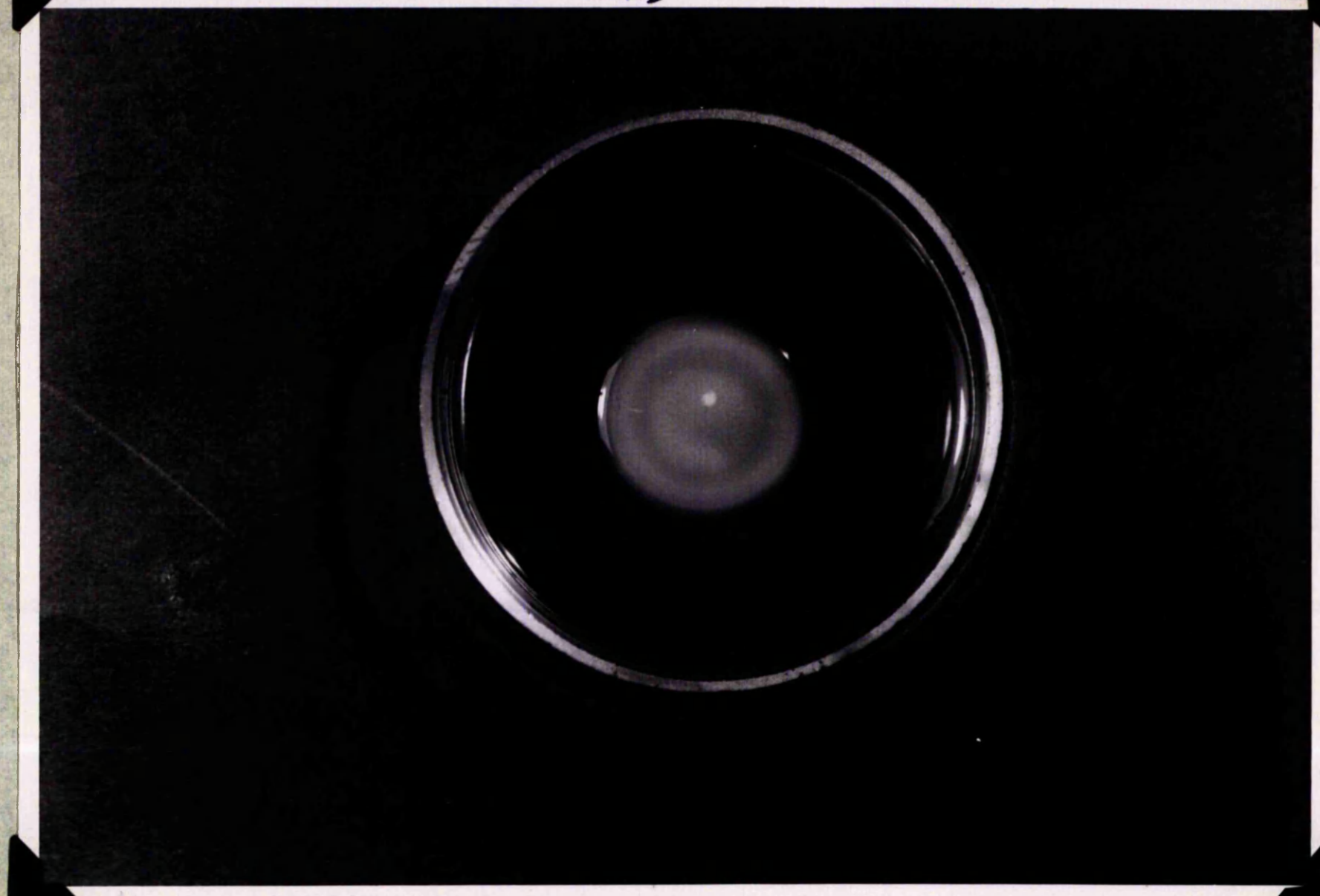
All the media were prepared as described on pages 73-75 of this thesis.

The results of these experiments are summarised in the Tables VI, VII, VIII and IX respectively.

D_2 C_5

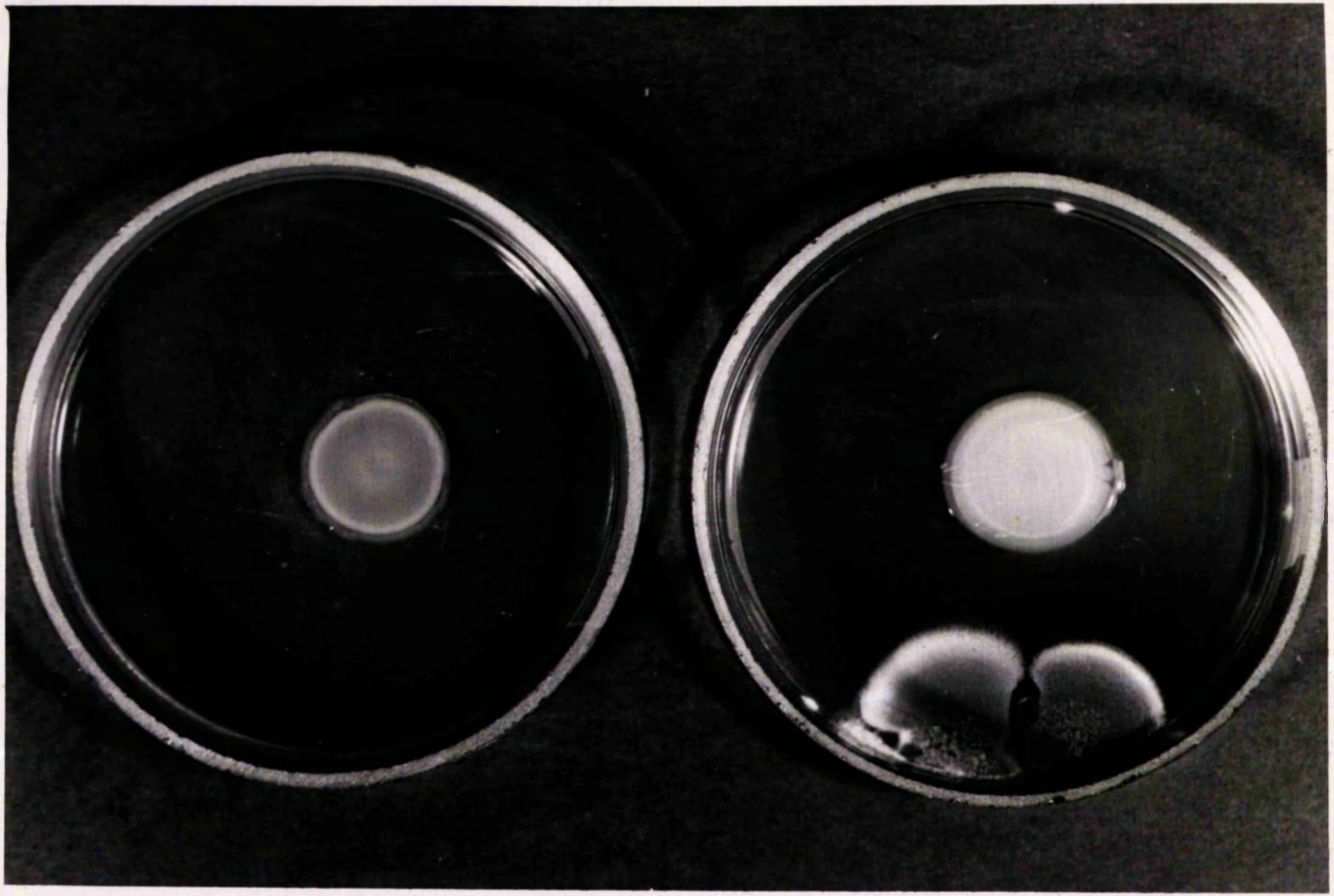


D2b.



Esb

D6



D_5

C_4

TABLE VI.

Description of Growth in Nutrient Broth.

Organism.	Incubation Temp. °C.	Time. Day.	
D_1	35°	2	Medium clear, no surface ring, fine particles clinging to the walls of the tube. Scant coherent deposit.
		7	Medium clear, no surface ring, deposit increased considerably, which on vigorous shaking produced slight silky turbidity.
D_3	35°	2	Medium clear, no surface ring. Some particles clinging to the walls. Scant coherent deposit.
		7	No change.
D_4	25°	2	Medium hazy. A thin surface film. No deposit.
		7	Medium slightly turbid. A uniform thin film. Scant deposit, which produced faint turbidity.
D_5	35°	2	Medium slightly turbid, no surface ring. Fair coherent deposit. Produced silky turbidity on shaking.
		7	Medium became clear, no surface ring. Slight increase in the deposit.
D_6	37°	2	Medium fairly turbid. A thin ring adhering to the walls of the tube. Some deposit.
		7	Medium clear. A tough fairly thick pellicle formed. Deposit slightly increased.
D_7	35°	2	Medium clear, no surface ring. Scant white deposit.
		7	Medium clear, no surface ring. Moderate deposit appeared, which on shaking produced uniform heavy turbidity.
D_2	37°	2	Medium slightly turbid. Patches of thin friable ring. Some deposit.
		7	Medium clear. A tough wrinkled pellicle formed and slight increase in deposit.
D_2b	30°	2	Medium slightly turbid, no surface ring. No suspension. Fair deposit.
		7	Medium fairly turbid. A thin film on the surface appeared. No suspension but moderate deposit which produced uniform silky turbidity.
C_4	25°	2	Medium slightly hazy. Isolated patches of surface film. No deposit.
		7	Medium slightly turbid. A uniform film on the surface. Scant deposit.
C_5	25°	2	Medium turbid, yellow surface ring. Scant coherent cheesy deposit.
		7	Medium clear, yellow surface ring. Moderate deposit which produced uniform turbidity on shaking.
C_5b	30°	2	Medium slightly turbid, no surface ring. Fine silky suspension and scant coherent deposit.
		7	Medium moderately turbid. A thin film on the surface appeared. Moderate deposit which produced uniform silky turbidity.

TABLE VII.

Description of Growth in Glucose Broth.

Organism.	Incubation Temp. °C.	Time. Day.	
D_1	35°	2	White friable surface ring, medium clear. Scant powdery deposit.
		7	A fairly thick friable ring, medium clear. Fair powdery deposit which produced fair turbidity on shaking.
D_3	35°	2	No surface ring. Medium clear. Coherent, fair powdery deposit and some suspension.
		7	A fair friable ring, medium clear. Moderate deposit and fair suspension which produced fair turbidity on shaking.
D_4	25°	2	Medium slightly turbid. A thin pellicle. No deposit.
		7	Medium fairly turbid. A thin pellicle on the surface. Some deposit which produced moderate turbidity (non-silky).
D_5	35°	2	No surface ring, medium clear. Heavy powdery deposit producing silky turbidity.
		7	Moderate, friable surface ring. Medium clear. No increase in deposit or turbidity.
D_6	37°	2	Medium moderate turbid. A fair amount of film on the surface. No deposit.
		7	Medium clear. A thick tough pellicle formed and scant deposit appeared.
D_7	35°	2	No surface ring, medium clear. Fair powdery deposit and some suspension.
		7	A fair friable ring, medium clear. Moderate powdery deposit and fair suspension. A fair uniform turbidity on shaking.
D_2	37°	2	Medium moderate turbid. A fair film throughout the surface. Scant deposit.
		7	Medium clear. A tough pellicle formed and slight increase in the deposit.
D_2b	30°	2	No surface ring, medium clear. Fair deposit produced fair silky turbidity.
		7	A fair pellicle-like layer clinging to the walls appeared. Slight increase in deposit.
C_4	25°	2	Medium fairly turbid. A thin pellicle. No deposit.
		7	Medium fairly turbid. A thin pellicle on the surface. Scant deposit which produced moderate turbidity.
C_5	25°	2	White wavy surface ring, falling entire when disturbed. Powdery particles in suspension and some deposit.
		7	A thick surface ring, fell entirely when disturbed. A thick cheesy deposit and moderate suspension. Thick turbidity when shaken.
C_5b	30°	2	No surface ring, medium clear; scant deposit produced fair silky turbidity.
		7	A thin pellicle-like layer clinging to the walls appeared. No increase in deposit or turbidity.

TABLE VIII.

Description of growth in Peptone Water.

Organism.	Incubation. Temp. °C.	Time. Days.	
D_1	35°	2	No surface ring turbidity, fine powdery deposit and suspension.
		7	Some patches of ring, no turbidity. Heavy deposit and fair suspension; on shaking moderate silky turbidity was produced.
D_3	35°	2	Some patches of ring clinging to walls, no turbidity. Fine powdery deposit and some suspension.
		7	A thin surface ring, broken when disturbed. No turbidity. Heavy deposit which produced silky turbidity.
D_4	25°	2	No surface ring, no turbidity. Scant deposit and some particles clinging to the walls.
		7	A slight increase in deposit only.
D_5	35°	2	No surface ring, slight turbidity. Some coherent deposit and fine silky suspension.
		7	Fair surface ring which broke to pieces when disturbed. Moderate deposit and fair suspension. Silky turbidity on shaking.
D_6	37°	2	No sign of growth.
		7	May be very faint growth, cannot be described.
D_7	35°	2	No surface ring, no turbidity, some deposit and suspension.
		7	A thin surface ring, broken down when disturbed. No turbidity. Moderate deposit producing moderate silky turbidity.
D_2	37°	2	No growth.
		7	Very faint growth, not sufficient for description.
D_2b	35°	2	No surface ring, no turbidity. Scant deposit and no suspension.
		7	Very fine surface ring, no turbidity. Fair coherent deposit which produced dense silky turbidity.
C_4	25°	2	No surface ring, no turbidity. Some deposit and some particles clinging to the walls.
		7	A slight increase in deposit only, but faint turbidity.
C_5		2	A fair yellow surface ring, which breaks to pieces when disturbed, no turbidity. Some powdery deposit.
		7	Fair yellow surface ring, falling entire on disturbing. No turbidity. Moderate coherent deposit. No turbidity on shaking.
C_5b	35°	2	No surface ring, no turbidity. Scant deposit and suspension.
		7	No further change except some increase in deposit. Produced silky turbidity on shaking.

TABLE IX.

Description of the growth in Litmus Milk.

Organism.	Incubation: Temp. °C.	Incubation: Time Days.	
D_1	30°	7	Medium acidic, no other change.
		14	No further change.
D_3	30°	7	Medium weakly acid only.
		14	No further change.
D_4	25°	7	Fairly acid, but no coagulation even after
		14	incubating for 14 days.
D_5	30°	7	Medium moderately acid.
		14	No further change.
D_6	30°	7	No change appeared at the end of 7 days, but at
		14	the end of 14 days a good deal of deposit appeared and medium became definitely alkaline.
D_7	30°	7	Medium faintly acidic.
		14	Acidity increased considerably. No other change.
D_2	30°	7	Appeared to be neutral, but some coagulation appeared
		14	to be going on. At the end of 14 days, medium was definitely alkaline and good amount of peptonization took place.
D_2b	30°	7	Medium unchanged.
		14	No further change.
C_4	25°	7	Fairly acid, but no coagulation even after the
		14	incubation period of 14 days.
C_5	25°	7	Medium moderately acidic, coagulation took place slowly.
		14	No further change.
C_5b	30°	7	Medium unchanged.
		14	No further change.

Physiological and Bio-chemical Characteristics.

(A) Relation to temperature. The basic temperatures employed in determining this characteristic were: 20°C., 25°C., 30°C., 37°C., 42°C. and 45°C. In more detailed examinations, wherever it was necessary, the temperature range was further subdivided and temperature effects were observed at 33°C. and 40°C.

Results of this experiment are summarised in Table X.

(B) Thermal Death Point. This characteristic was examined as follows: 1 ml. of fresh young liquid culture was heated in each case for 11 minutes at 50°C., 55°C., 60°C., 65°C., 70°C., 80°C. and 90°C. Tubes were then rapidly cooled down to room temperature and each tube was plated out separately, using 15 ml. of nutrient agar. The plates were incubated at the respective optimum temperatures of the organisms employed. The plates in which growth did not appear, the thermal death point (temperature and the time given for heating) was obtained.

Results of this experiment are also summarised in Table X.

(C) Relation to oxygen requirements. No specific experiment was performed for this characteristic, but the information was gathered for this characteristic during the examination of growth in liquid and solid cultures.

(D) Relation to Hydrogen Ion Concentration (PH). This characteristic was examined in the usual medium, the PH value of which was adjusted so as to be as near as possible to the following figures: 3·5, 4, 4·6, 5, 5·7, 6·4, 7, 7·5, 7·8 and 8·7. The cultures were

TABLE X.

Description of Temperature Relations.

	Temp. range of growth. (Examined from 20-45°)	Optimum range.	Optimum temp.	Thermal death point.
D_1	20-45°	33-42°	35°	Growth up to 65° C. and not above.
D_3	"	33-42°	35°	Growth up to 65° C. and not above.
D_4	"	25-30°	25° C.	Growth up to 70° C. and not above.
D_5	"	33-42°	35-37°	Growth up to 65° C. and not above.
D_6	"	33-42°	37° C.	Fair growth even at 90° C.
D_7	"	33-42°	35-37°	Growth up to 65° C. and not above.
D_2	"	33-42° C.	37°	Fair growth even at 90° C.
D_2b	"	30-35°	33° C.	Growth up to 55° C. and not above.
C_4	"	25-30°	25° C.	Growth up to 70° C. and not above.
C_5	"	25-30° C.	25° C.	Fair growth even at 90° C.
C5b	"	30-35°	33° C.	Growth up to 55° C. and not above.

incubated at their optimum temperatures for a period of time which extended in some cases up to two weeks.

Results of this experiment are summarised in Table XI.

(E) Acid and gas formation was sought for in the nutrient broth. Various carbohydrates were employed for these characteristics, and each carbohydrate was separately used in the concentration of 2%. Groves' modification of the Durham fermentation tube was used for the detection of gas. Bromothymol blue was used for the detection of acid formation. Incubation was carried out at the optimum temperature of the organism in question. Often, the incubation period was continued as long as 14 days.

(F) Liquefaction of Gelatin. This characteristic was recorded from the gelatin stab cultures in which growth was established. Cultures were incubated at 20°C. Prolonged incubation was necessary owing to the relatively low temperature of incubation.

(G) Voges - Proskauer Reaction was tested by the latest Barritt's modification. The medium used is described on page 74 under heading D. of this thesis. The cultures were examined for this reaction after 3 to 4 days' incubation when the growth became strong.

(H) Catalase Reaction. Young and vigorous nutrient agar slope cultures were flooded with hydrogen peroxide solution (page 76) and allowed to stand for some time. Positive reaction was denoted by the evolution of effervescences. It was of interest to notice that all the organisms in question showed positive reaction.

TABLE XI.

Description of Hydrogen Ion Concentration Relations.

	PH Range where growth appeared.	Optimum PH or PH range.
D_1	5·7 - 7·8.	7.
D_3	5·7 - 7·8.	7.
D_4	5·7 - 8·7.	7·5 to 7·8.
D_5	5·7 - 8·7.	7 to 7·5.
D_6	5·7 - 8·7.	7·5 to 7·8.
D_7	5·7 - 7·8.	7.0
D_2	5·7 - 8·7.	7·5 to 7·8.
D_2b	4·6 to 7·8.	5 to 5·5.
C_4	5·7 - 8·7.	7·5 to 7·8.
C_5	5·7 - 7·8.	7 to 7·5.
C_5b	4·6 to 7·8.	5 to 5·5.

(I) Reduction of Nitrate. For the detection of this characteristic the organisms were grown in nitrate broth (as described on page 75). When good growth appeared in the nitrate broth tubes, 1 ml. of each culture was separately mixed with 2 drops of sulphanilic acid solution and 2 drops of α-naphthylamine solution. The contents were allowed to stand after shaking. The presence of nitrite was indicated by a pink or red colour.

(J) Indole production was tested by Ehrlich Bohme method (37). Organisms were grown in peptone broth containing extra tryptophan. The above test was performed when good growth appeared in the tubes. A development of pink colour at the junction of the medium and zylol, on standing, would show the presence of indole.

None of the organisms in question was found to produce indole.

Results of the experiments for biochemical characteristics are summarised in Table XII.

TABLE XII.

Description of the Various Biochemical Characteristics.

	Oxygen requirement.	Liquefaction of gelatin.	Voges Proskauer Reaction.	Reduction of Nitrate.	Starch hydrolysis.	Acid and gas formation with various carbohydrates.			
						Glucose.	Fructose.	Lactose.	Mannitol.
D_1	Aerobic.	Crateriform rapid liquefaction.	Negative.	Not reduced.	Hydrolysed.	++ oo –	++ oo –	++ o –	++ o –
D_3	Aerobic.	Liquefaction.	Negative.	Not reduced.	Hydrolysed.	++ oo	++ oo	++ oo	++ oo
D_4	Aerobic.	No liquefaction.	Negative.	Nitrate reduced.	Not hydrolysed.	++ oo –	++ oo –	++ oo –	++ oo –
D_5	Aerobic.	Crateriform liquefaction.	Negative.	Not reduced.	Hydrolysed.	++ oo –	++ oo –	++ oo –	++ oo –
D_6	Aerobic (facultative).	Rapid liquefaction.	Positive Reaction.	Nitrate reduced.	Hydrolysed.	++ oo –	++ oo –	+ – –	++ oo –
D_7	Aerobic.	Liquefaction.	Negative.	Not reduced.	Hydrolysed.	++ oo –	++ oo –	++ oo –	++ oo –
D_2	Aerobic (facultative).	Rapid liquefaction.	Positive reaction.	Nitrate reduced.	Not hydrolysed.	++ oo –	++ oo –	(+) – –	++ oo –
D_2b	Aerobic.	No liquefaction.	Negative.	Nitrate reduced.	Not hydrolysed.	++ oo –	++ oo (✓)	– – –	– – –
C_4	Aerobic.	No liquefaction.	Negative.	Nitrate reduced.	Not hydrolysed.	++ oo –	++ oo –	++ oo –	++ oo –
C_5	Aerobic.	Liquefaction.	Negative.	Not reduced.	Not hydrolysed.	++ oo –	++ oo –	++ oo –	– – –
C_5b	Aerobic.	No liquefaction.	Negative.	Nitrate reduced.	Not hydrolysed.	++ oo –	++ oo (✓)	– – –	– – –

Note: (1) All the organisms were catalase positive, and none of them were found to produce Indole.

(2) Gas formation was not detected with any of the carbohydrates used.

Symbols used:

Good growth.	++
Moderate acid.	oo
Gas formation.	✓
No gas or acid or growth.	–

IDENTIFICATION OF THE ORGANISMS.

After the above characteristics had been noted, a detailed study of the tables describing all the characteristics of the organisms in question was made so as to group together the identical strains. Organisms which showed the similarity in their Morphological, Cultural and Biochemical Characteristics, with very little differences, were placed in one group. All the eleven organisms were found to fall in five separate groups, as follows:-

1. C_4 and D_4.
2. C_5.
3. D_2 and D_6.
4. D_2b and C_5b.
5. D_1, D_3, D_5 and D_7.

A survey of the literature (Bergey and Breed, 6th Edition) was then carried out to identify these organisms. This survey showed that members of Group 1 (C_4 and D_4) resembled in most of their characteristics with very little differences, with Micrococcus aurantiacus, member of Group 2 (C_5) resembled in a similar manner with Micrococcus flavus and members comprising Group 3 (D_2 and D_6) showed identical characteristics to that of Bacillus subtilis.

Group 4 (D_2b and C_5b) as well as Group 5 members (D_1, D_3, D_5 and D_7) were found to belong to the genus Flavobacterium. Group 4 members (D_2b and C_5b) showed very great resemblance in their characteristics to that of Flavobacterium proteus, but the members of Group 5 could not be identified as the strains of any of the known species described under

the genus Flavobacterium.

It thus became evident that the members of the respective groups were the physiological strains of the respective organisms, with which they showed great resemblance in their characteristics. Hence the organisms were classified and renamed as follows:-

C_4, D_4.	as varieties or strains of					Micrococcus aurantiacus, Schroeter.
C_5	"	"	"	"	"	Micrococcus flavus, Trevisan.
D_2, D_6	"	"	"	"	"	Bacillus subtilis, Cohn.
D_2b, C_5b	"	"	"	"	"	Flavobacterium proteus, Shimwell and Grimes.

Strains D_1, D_3, D_5 and D_7 were assigned to the genus Flavobacterium, but no attempt was made to name them. A short description of their important characteristics is given below.

All the four strains showed the following characteristics:-

Morphological characteristics.

Moderately long fat rods, 1·5 to 2·1 u by 2·5 to 4·5 u, were found as single cells in short chains and small irregular clumps. Gram negative, non-motile and non-spore forming.

Cultural characteristics.

Colonies on different solid media were found to be round, dull, yellow to deep yellow, opaque, with definitely broken edges (feathery).

Biochemical and Physiological characteristics.

Aerobic, catalase positive and Voges-Proskauer negative.

Showed crateriform rapid liquefaction of gelatin.

Hydrolysed starch fairly rapidly.

Did not reduce nitrate. Did not produce indole.

Optimum temperature was 35°C. Optimum PH was 7 and the PH range was 5·7 to 7·8.

COMMENTS.

In the early part of the year 1949 the author was provided with three impure organisms which were picked up off the grey cloth from a textile factory. By subsequent platings and sub-culturing eleven pure strains were isolated. The object of this research was to identify these strains.

A survey of the literature on the textile microbiology indicated that, though the causes of mildew growth on textiles had been investigated to a considerable extent and the more evident effects of the growth of moulds upon fibres had been well studied and many of the organisms positively identified, very little work had been done on the bacteria which were responsible for the damage of textiles. Early research workers in textile microbiology had made passing references to the bacteria found in association with raw cotton or finished product. No serious study on this subject was undertaken by anyone until Prindle (28,29) during his researches on textile microbiology, isolated a large number of bacteria from raw cotton and finished products. He pointed out that the bacteria belonging to the genus Bacillus and Flavobacterium were present in abundance in raw cotton as well as the finished products, but he did not go farther than this.

During the present investigations two strains of Flavobacterium proteus and four other strains belonging to the genus Flavobacterium have been found but the latter could not be related to any particular strain belonging to that genus. It is very interesting

to note that Flavobacterium proteus reported to exist mainly in brewery yeasts, had been found to be present on cotton textiles.

Two strains of Bacillus subtilis which had been isolated were in conformity with the observations of workers like Smith (25) and Prindle (29). However, as far as the writer's knowledge, it is the first time that three strains belonging to the genus Micrococcus had been isolated from cotton textiles, though strains of this genus had been reported from wool and woollen goods. Two of those three Micrococcus species were found to be strains of Micrococcus aurantiacus while the remaining one was identified as a strain of Micrococcus flavus.

It is noteworthy that most of the species in question gave characteristic giant colonies (plates given) and the information obtained from them served as an aid for their differentiation.

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Note: References marked with an asterisk have not been read in original.

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ABSTRACT.

During the course of these studies of bacterial infection of "pitching yeasts" the author has isolated 52 specimens of lactic acid bacteria comprising both lactobacilli and streptococci.

The procedure adopted for isolating and purifying the cultures has been described in detail in the practical part (Section I). The method reviewed by Snieszko (277), for the anaerobic cultivation of organisms has been employed by the author very successfully and with satisfactory results.

After obtaining the organisms in pure cultures, the detailed examination of their morphological, cultural, physiological and biochemical characteristics was carried out to settle the question of their identities. The results of those experiments are summarised in Tables III to XIX in Section I of the practical part.

A complete survey of the literature available was then made to identify these organisms. This survey showed that most of the organisms in question had very similar characteristics, with little differences, to those of one particular Lactobacillus or Streptococcus species already known and described in the literature.

Thus 46 out of the 52 organisms in question were identified as follows:-

A_1, A_2, A_3, A_4, A_5, A_6, A_7, E_4, E_5.	as varieties or strains of L. leichmannii, Bergey et al.
E_1, E_2, E_3, E_6, D_1, D_5, F_3.	as varieties or strains of L. bifidus, Tissier.

B_1, B_2, B_3, B_4, B_5, C_1, D_2, D_4, H_4, W_1, W_4, W_5, W_D, W_L, C_4, C_5, W_7, W_{10}.	as varieties or strains of L. pastorianus, Van Laer.
C_2, C_7, C_8, G_2, G_4, G_6.	as varieties or strains of L. plantarum, Orla-Jensen.
C_3, H_6.	as varieties or strains of L. buchneri, Henneberg.
G_3, G_5.	as varieties or strains of Str. mucilaginosus, Kulka, Cosbic and Walker.

Streptococcus species F_1 and F_6 in their cultural, physiological and biochemical characteristics resembled closely to Str. damnosus, except that the cells of these specimens were considerably smaller in size as compared to those of Str. damnosus (0·6 to 0·7 μ against 1 to 1·5 μ). Thus these species have been named by the author Str. damnosus var minimus, F_6 being named as type culture and F_1 as Strain I of the same.

The remaining 6 organisms, two Streptococci and four lactobacilli were found to differ considerably in their characteristics from all the known species described in literature, and were thus considered to be new species.

Complete descriptions of their characteristics and naming of these organisms are given in the practical part (page 114-120). A short description of their characteristics and classification is given below.

The species Str. parvus and L. frigidus is now formally proposed as new.

Streptococcus parvus Bhandari and Walker, n. sp.

Cells spherical, 0·5 to 0·7 μ in diameter. Non-motile. No endospores. Capsules not detected. Gram positive. Single colonies on beer gelatin plates were 0.5 to 0.8 m.m. in diameter and were opaque,

dull, smooth, flat, entire with a butterlike consistency and had a brownish tinge.

Facultative anaerobes. Optimum temperature 21-22°C. Grow at 15°C. and 25°C. but not at 30°C. Optimum hydrogen ion concentration 5-5·6.

Good growth in beer, malt wort and casein double digest, with scant deposit and no film at the surface. Medium generally clear, but on shaking fair non-silky turbidity developed.

Catalase negative. Indole formation and nitrate reduction not detected but produced diacetyl and acetyl methyl carbinol. Growth and acid with glucose, fructose, mannose, maltose, salicin, inulin and very little with raffinose. No growth in xylose, arabinose, lactose, sucrose and galactose. Homofermentative.

Isolated as a contaminant of brewery pitching yeast.

Lactobacillus frigidus Bhandari and Walker, n-sp.

Cells as short fat rods, sometimes almost coccoid, 0·7 to 0·8 μ x 1 to 1·2 μ. Non-motile. No endospores. Capsules not detected. Gram positive. Single colonies on gelatin beer plates (19-20°C.) were 1 to 1·5 m.m. in diameter. Colonies were subsurface, smooth, dull, convex, entire and colourless.

Facultative anaerobes. Optimum temperature 23°C. Grow at 15°C. and 30°C. but not at 33°C. Optimum hydrogen ion concentration 5-5·6. No growth at 3·6 and 7·3.

Good rapid growth in beer, malt wort, casein double digest and glucose broth. Pronounced silky turbidity and powdery deposit. No film at the surface.

Catalase negative. Indole formation and Nitrate reduction and acetyl methyl carbinol formation not detected. Growth and acid with xylose, arabinose, glucose, fructose, mannose, sucrose and maltose. No growth in galactose, lactose and raffinose. Heterofermentative.

Isolated as a contaminant of brewery pitching yeast.

It ought also to be pointed out that, during the course of these investigations, it was found that most of the pitching yeasts examined were strongly contaminated with lactic acid bacteria species. Moreover, this contamination by lactic acid bacteria was in the majority of cases a contamination of more than one species, for in most of the yeasts two or more types of lactic acid bacteria species were found to be present together. Further, it was found that two similar strains were present in the yeast from two breweries situated far apart.

These observations are significant in regard to the use of brewery yeast in biological enquiries, concerned with the problems of intermediate metabolism.

On the other hand, the growing importance of the lactic acid bacteria species in various industries has made them even more important. All these facts indicate that continued study of the lactic acid bacteria is likely to be of considerable value both from academic as well as the industrial point of view.

Nineteen strains and species from the 52 specimens examined by the author were then selected for further investigations in their nutrient requirements. It is noteworthy that the complete basal medium selected for the purpose of growing the lactic acid bacteria

species was not satisfactory for all the organisms, because some of the organisms did not show any signs of growth even after prolonged incubation periods. Thus only sixteen strains and species were selected for further work which showed growth in this medium.

Complete details of these investigations have been described in Section II of the practical part. But it may be pointed out that the technique employed during these investigations was different from that of Dunn *et al*.

Glutamic acid and leucine was found essential for all the sixteen organisms, while tryptophan was essential for all but one and glycine for all but three strains. Number of strains which required each of the following amino acids are shown in parentheses: valine (12), threonine (11), arginine (8), proline (8), serine (8), alanine (6), tyrosine (5), asparagine (4), phenylalanine (3), lysine (2), hydroxyproline, isoleucine and cysteine, each was essential for one organism. Histidine, methionine, norleucine and norvaline were not found to have any pronounced effect on the growth of any of the organisms.

The amino acid requirements of strains of same species have been found to have very different requirements. *L. pastorianus* W_5 was found to be the least fastidious strain, requiring only two vitamins. On the other hand, the most fastidious of all the strains examined (*L. plantarum* G_2) required as many as eleven amino acids.

L. plantarum G_2 has been found to require hydroxyproline for its growth, probably it is the first organism to show such specific requirements for hydroxyproline, and thus can be usefully employed for assaying this vitamin.

Apart from the specificities of the amino acids for certain organisms for their growths, many instances were observed where the certain amino acids showed stimulatory effect on the growth of organisms. The number of organisms for which individual amino acids are stimulatory is shown in the parentheses:- alanine (6), tyrosine (4), arginine (3), histidine (3), asparagine (2), proline (2), serine (2), hydroxyproline (1), lysine (1), methionine (1), phenylalanine (1) and valine (1).

Some instances of inhibition by certain amino acids have been also observed thus, norvaline apparently inhibited the growth of seven organisms, i.e. 3 strains of L. bifidus and 1 each of L. frigidus, L. pastorianus, L. buchneri and L. leichmannii. L. leichmannii E_4 was inhibited to certain extent by many amino acids and L. pastorianus W_{10} was strongly inhibited by threonine.

Pantothenic acid was found to be essential for all the 16 organisms, while nicotinic acid was essential for 12 organisms and stimulatory for the remaining four organisms. Riboflavin was required by 3 organisms and was also stimulatory for another 3 organisms. p-Aminobenzoic acid and pyridoxamine each was essential for one organism.

It is probably the first time when pyridoxamine has been found to be essential for any organism. Writer's strain L. plantarum G_2 which has shown great specificity towards pyridoxamine can thus be employed for assaying this vitamin in natural materials.

The vitamins other than mentioned above have not found to be required by any of the organisms for their growth, but some of them have no doubt shown a stimulatory relationship towards many organisms,

while a few others have also shown inhibitory relationship for some organisms tested.

Uracil was found to be essential by 10 organisms and adenine by 5 organisms, while guanine and xanthine was not required by any of the organisms. Quite a pronounced inhibition was shown by all these four substances in a number of cases.