AN INVESTIGATION OF THE BACILLUS PASTEURI GROUP

II. SPECIAL PHYSIOLOGY OF THE ORGANISMS

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In the first accounts of *Urobacillus Pasteurii* by Miquel (1889, 1898) the organism was described as having been cultivated in bouillon and peptone media containing various concentrations of urea. Beijerinck (1901) found that the bacillus would not grow on the ordinary laboratory media, but developed in bouillon containing 10 per cent urea, or an agar and gelatin to which 2 per cent urea and 0.3 per cent ammonium carbonate were added. He was unable to obtain growth of the organism in urea peptone solutions unless Chapoteau peptone, which was used by Miquel, was employed. Simple media containing, in addition to urea, asparagine and glucose, or ammonium salts of organic acids, did not support growth. Löhnis and Kuntze (1908) used bouillon, agar, and gelatin, each containing 2 per cent urea, for the cultivation of the organism, and confirmed Beijerinck's observations concerning the unsuitability of media which contained no urea or protein. It became generally accepted that the most important criterion for the recognition of *B. Pasteuri* was its inability to grow on ordinary media without added urea. Later work by Viehoever (1913), however, cast doubt on the necessity for urea, and this author observed growth of the organism in much simpler media than those used by Beijerinck and Löhnis and Kuntze. Viehoever described good growth in an ammonium carbonate mineral solution containing glucose and asparagine as the only organic compounds. In the same medium without glucose the organism was still capable of growing, but less vigorously. Viehoever added either urea, ammonium carbonate, or sodium carbonate to all the media he used.

There appears, therefore, to be considerable uncertainty concerning the environmental and nutritive requirements of *B. Pasteuri*, especially with regard to the necessity for urea. The function of urea in the metabolism of the organism has not been examined experimentally. It has, however, been established that the bacillus requires alkaline media, and a consideration of the literature suggests the possibility that urea merely serves to produce a strongly alkaline reaction through its breakdown to ammonium carbonate. This change would occur to some extent during the heat sterilisation of artificial media, and would later be completed by the urease activity of the cell.

THE EFFECTS OF UREA AND AMMONIA ON GROWTH

In this investigation it was observed that most of the strains usually failed to develop from light inoculations in media prepared from one per cent peptone and one per cent meat extract, and adjusted to pH 7.0 to 7.5. If the media were heavily inoculated, many strains initiated growth after incubation for several days, or several weeks, and having started, the organisms multiplied rapidly and the quantity of macroscopic growth frequently exceeded that formed on the same medium to which urea had been added. Evidently the heavy seeding enabled the cells to produce conditions suitable for their multiplication. When the organisms succeeded in growing in ordinary bouillon or agar, the cultures became distinctly alkaline, and the possibility that these substrates might be suitable for growth if made sufficiently alkaline was investigated. Tests were made with bouillon varying in alkalinity up to pH 8.5, but many strains failed to initiate growth at any reaction. Tests were then carried out with samples of bouillon of different reactions, to which 2 per cent urea was added. For this purpose, the urea in concentrated aqueous solution was sterilised by filtration and added aseptically to the tubes of bouillon, thus avoiding complications arising from the decomposition of urea by heat. Small and fairly uniform inoculations were made by thoroughly emulsifying growth from agar in M/15 phosphate buffer of pH 8, so that a definite but faint turbidity was produced, and transferring a loopful to each

tube of bouillon. The results were unexpected. Some strains failed to grow and others grew only after a long interval in the urea bouillon at pH 7.5 to 7.7. In the more alkaline bouillons growth occurred with less difficulty, but only after an appreciable lag, and certain strains failed to develop in any sample. In all cases, if growth started it progressed rapidly. Control inoculations of urea bouillon sterilised by intermittent steaming were made at the same time, and these invariably yielded prompt growth. It became evident that urea bouillon was converted into a satisfactory medium for *B. Pasteuri* only as a result of heating, and that the beneficial effect of steaming was only in part attributable to the alkaline reaction arising from a partial hydrolysis of urea. The use of different peptones (Bacto, Proteose and Witte), and of meat infusion instead of meat extract for preparing bouillon, did not influence the results.

The next experiments were planned to show whether the beneficial effects of heating urea bouillon arose from an alteration of the urea, the meat extract, or the peptone. Ordinary bouillon, consisting of 1 per cent peptone and 1 per cent meat extract, was prepared in the usual way, and the reaction was adjusted to pH 7.5 by the addition of NaOH. The bouillon was divided into three portions. The first was untreated, the second was adjusted to pH 8.8 by adding Na_2CO_3, and to the third 0.5 per cent commercial ammonium carbonate was added. The three portions were distributed in tubes and sterilised at 22 pounds. A 50 per cent aqueous solution of urea was also prepared. Of this, one portion was sterilised by filtration and a second was autoclaved at 22 pounds. Another urea solution of the same concentration was prepared by weighing the crystals aseptically, dissolving them in sterile water and placing the solution in flowing steam for fifteen minutes. The urea solutions were pipetted aseptically into tubes of the different bouillons in amounts which brought the concentration of urea to 2 per cent in each case. The reaction of all the media was then adjusted to pH 7.8 by adding sterile Na_2CO_3 or H_3PO_4. The different media were inoculated by transferring to each tube a loopful of a faintly turbid suspension of cells in phosphate buffer. Table 1

gives the essential features of one of the experiments in which an incubation temperature of 30°C. was employed, the intensity of growth at twenty-four hours being indicated by the number of + symbols. The table shows that heating the bouillon which had been made alkaline with Na_2CO_3 did not improve the medium. The beneficial effect obtained by the use of ammonium carbonate can therefore be attributed to the presence of ammonia in the medium and not to an alteration of the bouillon constituents produced by sterilisation at an alkaline reaction. The table also shows that heating the urea apart from the bouillon resulted in an improved medium, and that the most drastically heated urea gave the best result. This effect can be attributed to a partial conversion of the urea to ammonium carbonate at the high

TABLE 1

Growth in 24 hours at 30°C. in media containing 1 per cent peptone, 1 per cent meat extract and 2 per cent urea; pH 7.8

BOUILLON	UREA		
	Filtered	Steamed 15 minutes	Autoclaved
1. Ordinary; pH 7.5 before sterilisation...	−	−	+
2. Adjusted to pH 8.8 with Na_2CO_3 before sterilisation.....	−	−	+
3. Ammonium carbonate (0.5 per cent) added before sterilisation.....	+	++	+++

temperatures. The urea solutions were all tested for cyanates with $AgNO_3$ but the results were negative. These experiments were carried out with several strains, some of which grow more readily than others on media without urea. The results were approximately the same in the case of each strain except one. The latter, an organism which is exceptional in its ability to grow easily on ordinary media, was not influenced definitely by the presence of ammonia. The results of these experiments were confirmed by using agars instead of bouillons. Agar media produced clearer distinctions than bouillons for, in addition to showing the total amount of growth, they gave an indication of the relative number of cells in the inoculum which initiated growth on each medium.

It appeared to be certain that the ammonia requirement of the organisms is unusually high. Experiments were accordingly carried out to test the effect of adding ammonium salts to bouillon or agar. In the first tests the media, adjusted to pH 7.8 to 8.2, were made up to contain when completed 1 per cent peptone, 1 per cent meat extract, 2 per cent urea sterilised by filtration, and varying proportions of $(NH_4)_2SO_4$. The last two ingredients were added to the remainder after sterilisation. The tests again demonstrated that growth was considerably delayed in the absence of added ammonia, and they further showed that the addition of 0.2 per cent $(NH_4)_2SO_4$ to the media containing unheated urea permitted growth as readily as did urea bouillon or urea agar sterilised by steaming.

In the succeeding experiments, media containing different concentrations of $(NH_4)_2SO_4$ or NH_4Cl but no urea, were used. The ammonium salts in concentrated aqueous solution were sterilised separately and added to the alkaline media immediately before inoculation. Growth occurred most readily when about 1 per cent of either salt was added, and if the reaction was close to pH 8 the organisms developed almost as quickly as in the usual urea media. The optimum concentration of ammonia was not clearly defined: most of the strains examined appeared to grow with equal facility at any concentration between 0.5 and 2 per cent. The optimum for certain strains which decompose urea slowly appeared to be between 0.5 and 1 per cent of either the sulphate or the chloride. The maximum concentration of ammonia tolerated by the organisms is high, but in attempting to elucidate this factor the results were erratic and inconclusive. Wide differences were obtained with a single strain when different amounts of inoculum were used.

THE EFFECT OF REACTION

Having obtained a suitable medium which is not subject to violent changes in reaction as a result of growth, a study was made of the effects of hydrogen ion concentration. Bouillon with a high buffering capacity, containing 2 per cent peptone, 2 per cent meat extract and 1 per cent NH_4Cl, was used. In

order to obtain a medium which would remain clear when made alkaline, the bouillon was prepared by adjusting the reaction to approximately pH 9, autoclaving, filtering, and bringing the reaction back to neutrality, prior to adding the ammonium salt and sterilising. By adding sterile Na_2CO_3 or HCl aseptically to samples of the bouillon, several series of media varying in reaction were obtained. These were inoculated with dilute suspensions of the organisms, and observations were made for the appearance of growth during incubation.

For most strains the optimum reaction proved to be in the region of pH 9, but in the case of the least active urea-decomposing organisms it was nearer to pH 8. The active strains seldom grew at pH values below 7.5, while the inactive types usually produced visible growth at pH 7. In these experiments few strains developed on the acid side of pH 7; but on other occasions a number of strains were observed to grow in media as far from neutrality as pH 6.2 to 6.4, if heavy inoculations were made. The alkaline limit for growth appears to be inconstant even for individual strains: the vigour of growth, the composition of the medium, the temperature, and probably other factors seem to produce variations.

NUTRITIVE AND OTHER ASSOCIATED REQUIREMENTS

The growth of the organisms was tested in a considerable number of media. The most suitable substrates were prepared from bouillon containing comparatively high concentrations of peptone and meat extract. The use of at least 1 per cent peptone is especially important in the case of urea-containing media, in order to prevent cultures from becoming excessively alkaline. Optimal conditions for growth in bouillon were produced by adding 0.5 to 1 per cent NH_4Cl, and adjusting the reaction to between pH 8.5 and 9 after sterilisation. The organisms grew more profusely in this medium than in 2 per cent urea bouillon. They also developed earlier than in urea bouillon, especially if the latter had been allowed to stand for several weeks after preparation, so that its ammonia content was reduced. Alkaline ammonia-containing media cannot be sterilised

without deterioration, and urea media are therefore more suitable for general use. The comparative values of ammonia and urea suggest that the latter compound does not serve as an energy source in the metabolism of the organisms. It appears to be impossible, however, to investigate this question satisfactorily with the complex media which were found to be necessary in this work. Söhngen (1909) and Christensen (1910) stated that certain bacteria which decompose urea may utilise energy liberated by the reaction, but their findings were not supported by any decisive proof.

In nature, bacteria of this group seldom encounter media comparable in nutritive value to bouillon, and their ability to grow in poorer substrates was tested. In autoclaved soil, soil extract and dilute peptone solutions (0.1 to 0.01 per cent) the organisms were capable of growing, provided the reaction and the content of ammonia were suitable, but the amount of growth produced was meagre in comparison with that in bouillon cultures. A visible growth was never observed in synthetic solutions which contained glucose or salts of organic acids as carbon sources, and asparagine with the addition of either ammonia or urea as nitrogen sources. No further efforts were made to confirm the findings of Viehoever (1913) and Rubentschik (1925, 1926) that organisms of this group are capable of multiplication in synthetic media, for if bacteria are unable to produce a macroscopic growth in a transparent test solution, evidence of their proliferation obtained by counting methods is generally of uncertain value.

Observations which indicate how bacteria of the Pasteuri group may find in nature conditions suitable for growth were made from experiments in bacterial association. In protein-containing media to which neither ammonia nor urea were added, organisms of this group, while incapable of multiplying in pure culture, were able to proliferate actively in the presence of other bacteria which decompose the protein. Solutions of casein and gelatin (1 per cent) adjusted to pH 7.5 to 7.7 were inoculated with *B. mycoides* and with a small number of cells from a dilute emulsion of a Pasteuri agar culture. Several strains of the urea-decomposing organisms produced, after

incubation for some days, a densely turbid growth, distinguishable from the flocculent growth of *B. mycoides*, and by plating on urea agar, the number of viable cells of the former was found to be of the order of 10^{10} per ml. of culture. Growth of the urea decomposers took place more readily if they were inoculated into cultures of *B. mycoides* which had been incubated previously for seven to fourteen days so that the protein decomposition was already well advanced. In the tests with casein and gelatin the strains of *B. Pasteuri* which are especially dependent on an alkaline reaction and a high concentration of ammonia did not initiate growth easily, but these strains proved to be capable of developing on ordinary agar in association with other bacteria, such as organisms of the Coli group. When agar was used, the liquefied medium was inoculated with the urea decomposer, poured into a Petri dish, allowed to solidify, and then inoculated at one point with the second organism. After a period of incubation, the length of which depended on the strain used, colonies of the urea-decomposing bacillus appeared in the agar around and beneath the growth of the other organism, but not on the remainder of the plate. It seems probable that the ability of organisms of the Pasteuri group to grow in the presence of other bacteria depends on the production of ammonia by the latter.

The inability of *B. Pasteuri* to grow in the ordinary bacteriological media adjusted to neutrality has been considered an important diagnostic feature. The Pasteuri group does, however, comprise a series of types varying from the most active urea-decomposing strains which cannot be cultivated in ordinary neutral bouillon, to feebly ureaclastic organisms which grow readily from small inocula. A test with ordinary media does not therefore permit of a clear differentiation of the group, and it is further complicated by variations due to the amount of inoculum and, especially in the case of agar, by the evolution of ammonia from other cultures in an incubator. Neutral one per cent peptone is of greater differential value than bouillon. Of the strains examined in this work only three develop easily in a peptone solution if the medium is lightly inoculated. Milk and potato, probably on account of their reaction, do not support the growth of any strain unless they are inoculated fairly heavily.

THE FORMATION OF NITRITES

In seeking an explanation of the unusually high ammonia requirement, the possibility that the organisms utilise ammonia as a source of energy was considered. Viehoever (1913) has claimed that bacilli of this group are capable of oxidising ammonia to nitrous acid, and that they may multiply to some extent in a solution in which ammonium carbonate constitutes the only source of carbon and nitrogen. Should this claim be substantiated, it would be of more than theoretical interest, for the invariable occurrence of these organisms in farm manure would account for a loss of nitrogen through nitrite formation and subsequent denitrification. Several of the strains used in this work, when cultivated in peptone water containing 0.5 per cent ammonium sulphate, produced appreciable quantities of nitrites as shown by using the Griess-Ilosvay reagents, while other strains did not. The strains which formed nitrites are all nitrate reducers and those which gave negative or doubtful tests do not reduce nitrates. It seemed probable, therefore, that the nitrites were formed by the reduction of traces of nitrates in the medium. There are numerous records of the formation of small amounts of nitrous acid from ammonia or from organic matter by heterotrophic microörganisms, but it should be pointed out that experimental work on this problem is particularly subject to error. The chief difficulties arise from the use of highly sensitive reagents for nitrites, the lack of comparably sensitive nitrate reagents, the frequent occurrence of nitrates as impurities in the ingredients of culture media, and the presence of oxides of nitrogen in the atmosphere, especially in the proximity of Bunsen burners. Without describing the exploratory work, it may be stated that after trying a considerable number of media and after preparing the purest ammonium salts, it was found that strains of *B. Pasteuri*, which readily formed nitrites when tested in the ordinary way in ammonia peptone solutions, could be cultivated at 30°C. for periods up to seven days under conditions permitting of excellent growth, without a trace of nitrous acid being formed. In the final experiments media were used immediately after preparation, the usual flaming of culture con-

tainers was omitted, and the cultures were incubated in a stream of purified air. It was concluded that *B. Pasteuri* is incapable of oxidising ammonia. The experiments also suggest that similar work with other heterotrophic bacteria requires the most rigid control.

SUMMARY AND CONCLUSIONS

Most of the organisms which may be placed in the *Bacillus Pasteuri* group require alkaline media containing ammonia. In the case of strains which bring about the most rapid decomposition of urea and are incapable of growing in ordinary neutral media, optimal conditions for growth in bouillon are produced by adding about 1 per cent NH_4Cl and adjusting the reaction to about pH 9. Strains which produce a weaker action on urea are less dependent on ammonia and an alkaline reaction, but few of them grow readily from small inocula on the ordinary bacteriological media. The most suitable substrates for cultivating the organisms contain urea from which the necessary ammonia is formed during sterilization and later by the urease activity of the bacteria. The organisms grow most readily in media containing comparatively high concentrations of peptone and meat extract. A visible growth has not been observed in synthetic solutions. In media containing ordinary bouillon or a protein, the organisms, while incapable of growth in pure culture, may proliferate actively in the presence of other bacteria. The appearance of nitrites in ammonia-containing cultures may be attributed to one or more of several circumstances, but not to an oxidation of ammonia by the organisms.

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