THE PRODUCTION OF CHAINS BY DIPLOCOCCUS PNEUMONIAE IN MAGNESIUM DEFICIENT MEDIA

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The classical morphological appearance of *Diplococcus pneumoniae* in artificial culture media is that of pairs of cocci. Short chains consisting of 2 to 4 pairs of organisms, arranged linearly, are seen in young cultures. The length may vary with the serological type. Chains are relatively uncommon with Type I but are encountered frequently with Type III. It is not known if chaining in this genus is an expression of incomplete cell division or is related to the characteristics of the capsular integument.

It was observed in this laboratory that the addition of sodium citrate to a medium otherwise suited for the growth of the pneumococcus resulted in the organisms being found in chains rather than as isolated diplococci. Okamoto (1940) using quaternary ammonium compounds and Badger (1944) using ethanolamine observed like results.

Similar effects upon other organisms have been recorded. Filamentous forms of *Clostridium perfringens* were produced by Webb (1948) by growing the organisms in a medium deficient in Mg^{++} . Subculture of these organisms in a Mg^{++} rich medium caused a reversion to the normal form. This reversion could not be accomplished by the substitution of metallic ions other than Mg^{++} . Studies on the morphology of organisms grown in a Mg^{++} deficient medium were extended by Webb (1949) to include other groups of organisms such as clostridia and gram positive aerobic bacilli. Shankar and Bard (1952) investigated *C. perfringens* with respect to the production of filamentous cells in Mg^{++} deficient media and found that Ca^{++} played a role in the production of filaments even at optimal levels of Mg^{++} and other ions.

The relation of trace element concentration to the change in morphology of *D. pneumoniae* was investigated to ascertain which ions were involved in this alteration and to relate morphology and growth to metallic ion concentration.

MATERIALS AND METHODS

Diplococcus pneumoniae, Types I, II, and a rough strain of Type II (D39R), were used in this study. The strains were maintained by serial transfer in brain heart infusion broth, the cultures being checked at frequent intervals for their identity and dissociation status. The inocula were prepared from 18 to 24 hour cultures in brain heart infusion broth, grown at 37 C, washed twice, and resuspended in one-half of the original volume of sterile 0.067 M phosphate buffer, pH 7.4. Of this suspension, 0.15 ml was used as inoculum for each 5 ml test medium.

All glassware was washed thoroughly with distilled water following the usual chemical treatment with chromic acid, NaOH, and dilute HCl.

Two methods were employed routinely in this study for removal of the ions:

(1) The medium was treated with salts which had the property of complexing or precipitating the positive divalent ions. These salts were: Sodium citrate, sodium potassium tartrate, potassium oxalate, sodium hexametaphosphate ("calgon"), and the sodium salt of ethylene diamine tetraacetic acid ("versene").¹

(2) A cationic exchange column was used. A column, 24 inches long by 1 inch in diameter, was packed firmly with the resin material, "amberlite IR-120".² A volume of 200 ml of the medium (made up in a concentration 2.5 times that normally used) was passed through the column. The first effluent, 50 ml, was discarded. The remainder was collected and dispensed in chemically clean test tubes in 2 ml portions. The tubes containing the medium were brought to a final volume of 5 ml with the addition of distilled water or solutions of the salts

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² Supplied generously by the Rohm and Haas Company, Philadelphia, Pennsylvania.

of Mg^{++} , Ca^{++} , Mn^{++} , and Fe^{++} in the concentrations desired.

The resin treated medium and the distilled water were tested for the presence of free Ca^{++} and Mg^{++} ions by the method of Buckley *et al.* (1951). This method is sensitive to free Ca^{++} and Mg^{++} in amounts as small as one μg per ml. Within the limits of the method, no detectable amounts of either ion could be determined in the distilled water or the resin treated medium.

organic ions in the usual medium were varied in order to ascertain the optimum levels necessary for the production of chains. This was accomplished by adding various complexing agents to the medium or by removing the metallic ions from the medium by passing it through a cationic exchange column. Various metallic ions were resupplied in turn to the resin treated medium and the effect upon the growth and chain formation of *D. pneumoniae* observed.

TABLE 1

Effect of complexing agents on the growth and chain formation of Diplococcus pneumoniae showing the predominant morphologic arrangement

MOLAR CONCENTRATION	COMPOUNDS ADDED TO BRAIN HEART INFUSION BROTH ARRANGED IN ORDER OF THEIR INCREASING COMPLEXING OR CHELATING ABILITY				
	Sodium potassium tartrate	Potassium oxalate	Sodium citrate	Sodium hexametaphosphate ("calgon")	Disodium ethylene diamine tetraacetic acid ("versene")
1×10^{-1}	Long chains	No growth	No growth		
1×10^{-2}				No growth	No growth
2×10^{-2}	Short chains	No growth	No growth		
2×10^{-3}				Long chains	No growth
4×10^{-3}	Diplococci	Long chains	No growth		
8×10^{-4}	Diplococci	Short chains	Long chains		
4×10^{-4}				Long chains	No growth
1.6×10^{-4}	Diplococci	Short chains	Short chains		
8×10^{-5}				Long chains	Long chains
3.2×10^{-5}	Diplococci	Short chains	Short chains		
1.6×10^{-6}				Long chains	Long chains
6.4×10^{-6}	Diplococci	Diplococci	Diplococci		
3.2×10^{-6}				Short chains	Short chains
1.3×10^{-6}	Diplococci	Diplococci	Diplococci		
6.4×10^{-7}				Diplococci	Short chains
1.3×10^{-7}				Diplococci	Diplococci

Long chains consist of more than 10 pairs.

Short chains consist of from 4 to 8 pairs.

All measurements of growth were made using a Klett photoelectric colorimeter employing a green filter (540 $m\mu$) after the cultures had been incubated for 18 to 24 hours at 37 C.

The extent of chaining was determined by counting and averaging the number of paired cocci in 50 chains on a slide stained by the method of Gram.

RESULTS

Preliminary observations, using citrate, indicated that the phenomenon of chain formation was associated with a deficiency of metallic ions in the medium. Since citrate ions could act as a chelating agent, the concentrations of the in-

The effect of complexing compounds. The normal appearance of *D. pneumoniae* (Type I) is that of pairs of cocci (figure 1). Upon the addition of various metallic complexing compounds to the medium, however, the production of chains resulted (figures 2, 3, 4, 5). If the concentration of the complexing agent was increased above the optimal concentration required for the formation of chains, cellular multiplication was inhibited and no growth occurred (table 1). Arrangement of the compounds in order of their increased ability to complex ions as in table 1 indicates that growth, as well as chain formation, is approximately inversely proportional to the ability of the agent to bind or complex the metallic ions.

Figure 1. Typical morphology of *Diplococcus pneumoniae*, Type I, when grown in brain heart infusion broth.

Figure 2. Morphological appearance of *Diplococcus pneumoniae*, Type I, when grown in brain heart infusion broth containing "calgon" (4×10^{-4} M).

Figure 3. Morphological appearance of *Diplococcus pneumoniae*, Type I, when grown in brain heart infusion broth containing sodium potassium tartrate (0.1 M).

Figure 4. Morphological appearance of *Diplococcus pneumoniae*, Type I, when grown in brain heart infusion broth containing "versene" (8×10^{-5} M).

Figure 5. Morphological appearance of *Diplococcus pneumoniae*, Type I, when grown in brain heart infusion broth containing potassium oxalate (4×10^{-3} M).

Figure 6. Morphological appearance of *Diplococcus pneumoniae*, Type I, when grown in resin treated broth containing $0.4 \mu\text{g}$ Mg^{++} and $1 \mu\text{g}$ Ca^{++} per ml.

It was noted also that the length of the chains appeared to increase with the proportional increase of the complexing ability of the agent used.

Various complexing agents were able to produce the same result. This suggested that the action in each case was similar, namely that the availability of the critical inorganic ion or ions became a limiting factor and thereby resulted in the production of long chains.

The effect of the addition of salts to resin treated media. In an attempt to demonstrate which of the metallic ions was responsible for the growth and chain formation, various concentrations of salts were added to the resin treated medium. The medium, after passage through the column, was unable to support the growth of *D. pneumoniae* unless both Mg^{++} and Ca^{++} were returned to the resin treated broth. In the absence of one or both of these, growth did not occur even when Fe^{++} or Mn^{++} was used singly or in combination. Growth in this medium was dependent therefore upon the concentrations of Ca^{++} and Mg^{++} .

When the Mg^{++} concentration was increased in the presence of a constant concentration of Ca^{++} ($1 \mu\text{g}$ per ml), which was in excess of the growth requirement, growth would increase in a manner directly proportional to the Mg^{++} level present in the medium as shown in figure 15. At a concentration of 0.7 to $0.8 \mu\text{g}$ per ml of Mg^{++} or greater, the amount of growth would approach the maximum for this system. Growth would occur, but no chains would be formed when the concentration of Mg^{++} was below $0.4 \mu\text{g}$ per ml. A higher concentration, 0.4 to $0.6 \mu\text{g}$ per ml, would result in the production of chains (figure 6). Above this level of Mg^{++} the cells would revert to their normal appearance of diplococci and the chaining phenomenon would be lost (figure 10).

Cellular growth was dependent also upon the Ca^{++} concentration present in the culture medium even in the presence of an excess of Mg^{++} . Although the growth of the organism was related to the amount of Ca^{++} present in the medium, the variation of the Ca^{++} concentration did not effect an alteration of the morphology of the organisms from that of diplococci to that of chains (figure 16). When Ca^{++} was added to a system which contained a level of Mg^{++} (0.4 to $0.6 \mu\text{g}$ per ml) which is in the range of the formation of chains, the cellular morphology would

Figure 7. Conditions the same as in figure 6 but with the Ca^{++} raised to $1.4 \mu\text{g}$ per ml.

Figure 8. Conditions the same as in figure 6 but with the Ca^{++} raised to $2.0 \mu\text{g}$ per ml.

Figure 9. Conditions the same as in figure 6 but with the Ca^{++} raised to $5 \mu\text{g}$ per ml.

Figure 10. Morphological appearance of *Diplococcus pneumoniae*, Type I, when grown in resin treated broth containing $2 \mu\text{g}$ Mg^{++} and $5 \mu\text{g}$ Ca^{++} per ml.

Figure 11. Normal appearance of a rough strain of *Diplococcus pneumoniae*, Type II, when grown in brain heart infusion broth.

Figure 12. Morphological appearance of a rough strain of *Diplococcus pneumoniae*, Type II, when grown in brain heart infusion broth containing 0.2 per cent choline.

Figure 13. Morphological appearance of *Diplococcus pneumoniae*, Type I, when grown in brain heart infusion broth containing 0.2 per cent choline.

Figure 14. Conditions the same as figure 13

remain that of chains regardless of the amount of Ca^{++} present (figure 16) (figures 6, 7, 8, 9).

The Mg^{++} requirement for the production of diplococcal forms was not met by the substitution of Ca^{++} , Fe^{++} , or Mn^{++} either singly or in combination. However, it was observed that the number of cells which contributed to making up the chain length appeared to increase when Mn^{++} was added to the medium which contained a level of Mg^{++} optimal for chain formation (0.4 to $0.6 \mu\text{g}$ per ml) (similar to figure 2). The Mn^{++} did not shift the range of the Mg^{++} action for chain formation, but rather its effect seemed to be upon the length of individual chains.

These results were obtained from observations upon the growth and morphology of *D. pneumoniae*, Type I. However, identical results were observed with Type II. In order to determine whether this chaining phenomenon was the result of some property of the capsular material of the organism, the experiments were conducted using a rough strain Type II (D39R) pneumococcus. It was demonstrated that again chaining occurred when the Mg^{++} level was suboptimal (figure 11, 12). Since the chaining effect could be produced with this organism, it was felt that the chaining phenomenon was associated with the cell and its mechanism for disengaging the paired cocci rather than with the capsular substance.

The effect of the addition of choline to normal and resin treated media. In light of the above, it was desired to confirm the observation of Okamoto (1940) that the presence of an excess of choline promoted long chain formation of *D. pneumoniae*. The results presented in figure 17 demonstrate that in a concentration of 0.20 per cent choline chloride, in the brain heart infusion medium, extremely long chain formations of the pneumococci occurred (figure 13). The concentration of the choline was increased up to one per cent and long chain formation persisted (figure 14). Also, the addition of choline to the medium, up to the amounts used above, had a stimulatory effect upon the growth of *D. pneumoniae* (figure 17). The same results were obtained when a rough strain derived from *D. pneumoniae*, Type II, was used (figure 12).

but with the choline concentration raised to one per cent.

Figures 1 to 14 represent a $\times 1,200$ magnification of gram stained preparations.

When varying concentrations of choline chloride were added to resin treated medium fortified with 0.4 to 0.6 μg Mg^{++} and 1 μg Ca^{++} per

the salt level was doubled, the amount of growth increased, but in this system one per cent choline still exhibited an inhibitory effect upon the growth of the organism (figure 18).

In order to determine whether choline had the property of complexing metallic ions, excess choline (one per cent) was added to a water solution which contained 1 μg Mg^{++} and 1 μg Ca^{++} per ml. A test for free Mg^{++} indicated that the salts remained ionized in the presence of the choline. This result was in contrast to similar experiments using oxalate or versene in which it

Figure 15. The effect of Mg^{++} on the growth and chain formation of *Diplococcus pneumoniae* in resin treated brain heart infusion broth.

Figure 16. The effect of Ca^{++} on the growth and chain formation of *Diplococcus pneumoniae* in the presence of a limited magnesium concentration.

ml, the optimal amount of Mg^{++} for chain formation, long chains of the cocci were observed. However, in contrast to untreated medium, concentrations of one per cent choline chloride tended to inhibit the growth of the organism. When

Figure 17. The effect of choline on the growth and chain formation of *Diplococcus pneumoniae* in brain heart medium.

was apparent that the ions were free in the solution. These findings do not exclude the possibility that choline is a weak complexing agent, for it is likely that the reagents used in the test could compete successfully for the Mg^{++} in such a manner as to remove it from the choline and thus give the appearance that the Mg^{++} had not been bound. However, the extreme length of the chains obtained when choline was used (figure 14) as compared to the relatively short ones received when a complexing agent such as potassium oxalate was used (figure 5) would make it seem that the organism was less able to remove Mg^{++} from choline (if it were bound) than from oxalate which forms a sufficiently stable complex with Mg^{++} when tested by the colorimetric method. Therefore, the choline does not appear to bind or complex the metallic ions when it promotes the formation of long chains in a growth system.

Figure 18. The effect of choline on the growth and chain formation of *Diplococcus pneumoniae* in resin treated media with Mg^{++} and Ca^{++} added.

DISCUSSION

The present investigation has been concerned primarily with the morphological changes of *D. pneumoniae* produced by alteration of cationic levels in the growth medium, rather than with the optimal growth conditions for the organism although the two properties are interrelated.

Growth as well as the resulting morphological form of *D. pneumoniae* is dependent upon the level of Mg^{++} in medium deficient in the ion. There are three distinct morphological phases demonstrable when the Mg^{++} available to the organisms is provided at concentrations conducive to the production of each of the three phases. At the lower concentration (below $0.4 \mu g$ per ml Mg^{++}) diplococci were observed, but the total population was small. If the concentration was brought to a range of from $0.4 \mu g$ per ml to $0.6 \mu g$ per ml Mg^{++} , the growth approached the maximum obtainable with the medium but the cocci were found to be arranged in chains instead of having divided into pairs. At concentrations of Mg^{++} higher than $0.6 \mu g$ per ml the classical form of diplococci was found and the amount of growth was not greatly increased above that of the intermediate concentration. It would seem as if the Mg^{++} requirement for the production of protoplasm, i.e. cells, has priority over the demand for Mg^{++} to be utilized for formation of typical diplococci.

Under the conditions of this investigation it was found that the effect upon morphology was specific for the Mg^{++} and directly related to its concentration. Neither Ca^{++} , Fe^{++} , nor Mn^{++} was able to substitute for Mg^{++} . Calcium was essential for growth, but increased Ca^{++} levels did not affect the chaining phenomenon. The addition of Mn^{++} to a medium containing an optimal level of Mg^{++} for the formation of chains appeared to increase the length of the chains. However, the optimum range of Mg^{++} concentration for producing the chaining phenomenon did not shift upon addition of the Mn^{++} .

These findings are in agreement with the results of Webb (1948) and Shankar and Bard (1952) on the effect of metallic ions upon the cell division of the genus *Clostridium* and the genus *Bacillus*. Webb reported (1949) that he was unable to demonstrate chain formation when the coccid forms of the genera *Micrococcus*, *Sarcina*, and *Neisseria* were grown in a Mg^{++} deficient medium. In the Mg^{++} medium used in the present investigation, chains of cocci have been demonstrated. There was no evidence of filaments (elongated cells without cross walls) as described by Webb (1948), but this might be due to the fact that Webb utilized rod shaped organisms.

The phenomenon of organisms growing in chains was produced also using *D. pneumoniae*, Type II, and a rough strain of Type II (D39R). It may be postulated, therefore, that the chaining property is a general phenomenon for more than one type of pneumococcus. The phenomenon then is not dependent upon the presence of the capsular polysaccharide material surrounding the pneumococci but rather seems related to incomplete separation of morphologic units.

The experiments with choline chloride demonstrated that chain formation could be produced by other means than by a deficiency of Mg^{++} . The Mg^{++} level is apparently not the only factor which may result in the production of chains. Thus, Tunnicliff (1939) showed that filamentous forms of *Streptococcus viridans* occurred when it was grown in the presence of sulfonamide. Gardner (1940) observed the production of filaments and chains when *Clostridium welchii* was grown in the presence of penicillin. Whether these compounds act in the same manner as choline is not understood.

Although our results show that choline apparently does not affect directly the function

of the Mg^{++} in its relationship to the growth and cell division of the organism, it is interesting to note that the presence of the choline in the growth system is capable of producing the same effect upon the morphology of *D. pneumoniae* as that of a Mg^{++} deficiency. It might be that the choline exerts its influence upon some metabolic process related to mechanisms for the disengagement of the diplococcal unit with which Mg^{++} is also closely associated, that is, that the sites of action of each lie along a common metabolic pathway. Such a relationship would help explain why choline does not demonstrate any complexing ability in the presence of Mg^{++} and yet has the same result as that of a Mg^{++} deficiency.

SUMMARY

The controlled production of long chains of *Diplococcus pneumoniae* was achieved by the use of Mg^{++} deficient media. It was shown that chain formation is a function of the Mg^{++} concentration and that the progressive changes in morphology are exhibited by the population as a whole, dependent upon critical gradients of the ions available after the requirements for cellular synthesis have been met.

A comparison has been made with the effect of suboptimal concentrations of Mg^{++} on the morphology of *D. pneumoniae* to the same morphologic effect produced by the addition of choline to brain heart infusion. Although the morphological appearance is identical, the mechanism resulting in chaining evidently is dissimilar.

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