A STUDY OF METHODS FOR THE PREPARATION OF PROTOPLAST CELL FORMS IN THE GENUS AGROBACTERIUM CONN.

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ABSTRACT

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Prior to these investigations, little information was available on protoplast formation in Agrobacterium spp. This study has evaluated the effectiveness of various methods in inducing protoplast formation on four species of the genus. A new method has been developed whereby a high percentage conversion consistently is available.

Photomicrographic records of the course of conversion have been included.

Studies of the effect of pH on protoplast induction in these species indicates that a similarity in cell wall composition exists between them.

INTRODUCTION

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The form of a bacterial cell is maintained by a rigid cell wall. This wall or envelope can be removed by various techniques resulting in the formation of a spherical structure known as a protoplast. Thus a protoplast may be defined as a globular cell unit enclosed by a cytoplasmic membrane but devoid of a cell wall.

Originally, protoplasts were formed by plasmoptysis of the cell. This was achieved by producing violent decreases in the osmotic pressure of the growth medium. However these techniques produced low yields. Newer more effective methods for protoplast induction were described by Weibull (25) using lysozyme, and by Lederberg (7) using penicillin. These two procedures are considered to be basic to the removal of cell wall by chemical methods. Since then, a number of modifications have been proposed. Such modifications usually involve the use of substances in addition to penicillin and lysozyme for the purpose of accelerating and stabilizing protoplast formation.

Development of these new techniques has made it

possible to gain more accurate information on bacterial cell structure and function. When used as a preparatory step to electron microscopy, these methods have added considerably to knowledge on the structure of the bacterial cytoplasmic membrane. Likewise, the accuracy of studies of enzyme localization on various cell fractions has been increased by the introduction of protoplast formation techniques.

It is generally accepted that one method for protoplast formation may not apply equally well to all bacterial species. Some cells are resistant to lysozyme or to penicillin or require additional treatment to induce protoplast formation. The available literature contained no report on a method developed specifically for protoplast formation in Agrobacterium. Gram-negative organisms do not form protoplasts readily. Accordingly the present study was carried out to investigate protoplast formation in Agrobacterium spp. It deals with three aspects of the problem: (a) methods for producing protoplasts (b) the course of protoplast formation as depicted by electron photomicrography and (c) the effect of pH on conversion to

protoplasts in the method found to be the best with these organisms.

HISTORICAL

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Probably the first recorded observation of protoplast formation by bacteria was made by Fischer (1). By suspending cells of Bacillus anthracis and Vibrio proteus in solutions of low osmotic pressure, this investigator demonstrated that, with each species, the protoplasm ejected from the cell assumed spherical form. To this phenomenon he gave the name plasmoptysis.

Other workers, Garbowski (2) and Raichel (14), confirmed in part the findings of Fischer. However, spherical forms following plasmoptic treatment were not always evident.

Subsequently, spherical cell forms were demonstrated by Stapp and Zycha (21) with Bacillus mycoides cultured in a medium containing 0.5 M magnesium sulphate. Highly refractile spherical bodies appeared in this medium.

Globular cell units in a culture of Streptobacillus moniliformis were reported by Klieneberger (5). To these he gave the name "L forms". A satisfactory distinction between the L form and the protoplast has not yet been

made.

Modern methods, using lysozyme for the induction of protoplasts, were introduced independently by Tomcsik and Geux-Holzer (22) and Weibull (25). Similar studies by McQuillen (10), Michell and Moyle (11) and Zinder and Arndt (26), have confirmed the effectiveness of lysozyme treatment in dissolving bacterial cell walls.

While induction methods based solely on lysozyme yielded significantly higher proportions of protoplasts than did any of the methods previously developed, they were successful mainly with Gram-positive bacteria. Gram-negative organisms generally gave low protoplast yields.

Repaske (15) reported that the addition of an EDTA supplement to lysozyme produced lysis in suspensions of certain Gram-negative species. Subsequently, it was demonstrated by Mahler and Frazer (8) that such lysis was prevented by the addition of 0.5 M sucrose to the treatment medium. The higher osmotic pressure afforded by sucrose stabilized the protoplasts in their spherical form.

Other modifications of the lysozyme treatment to produce protoplasts in Gram-negative bacteria have been

developed. One such method using polymyxin in addition to lysozyme was reported by Warren (24), and more recently by Tuttle and Gest (23).

A new concept in methods for producing protoplasts was introduced by Lederberg (7). This investigator reported that penicillin interfered with cell wall formation in Escherichia coli and as a result spherical forms appeared. Rubio-Huertos and Desjardins (17) prevented the development of normal cell walls in Gram-negative bacteria by the addition of glycine to the medium. The resulting spherical forms were not considered to be true protoplasts, but rather to be L form precursors. These findings were confirmed by Jeynes (4).

Hydrogen ion concentration in the treatment solutions has been reported to be an important factor in protoplast induction by lysozyme. The optimum pH for the lysozyme effect varies with the species of organism. To illustrate this the following examples are presented. Smolelis and Hartsell (20) found that lysis of Micrococcus lysodeikticus occurred optimally at pH 6.2. Grula and Hartsell (3) showed that lysozyme was still somewhat

active on this organism at pH 3.5. Repaske (16) reported an optimum of pH 8.0 with Pseudomones aeruginosa; and Zinder and Arndt (26) an optimal activity at pH 9.0 with E. coli. Numerous other reports indicate that lysozyme does not have the same pH activity optimum for different species and is effective over a wide pH range.

Since the literature available yielded little information on protoplast formation for members of the Agrobacterium group, the present study was initiated with three aspects in mind: the development of a reliable method for protoplast production; an evaluation of this phenomenon by electron microscopy; and, an examination of the effect of pH on the course of conversion to protoplasts as it applies to some members of this group.

MATERIALS AND METHODS

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Cultures

The cultures used were as follows: Agrobacterium tumefaciens A6 (U. of Wisc.), A. gyposophilae H38, (P.R.L., N.R.C.), A. radiobacter 426 (Dept. of Agriculture, Ottawa), and A. radiobacter 1012 (U. of Wisc.).

Culture Maintenance and Growth

Cultures were maintained routinely on mannitol - yeast extract agar of the following composition: 0.5 g KH_2PO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.2 g NaCl, 1.0 g yeast extract, 1.0 g mannitol and 15 g agar in 1000 ml distilled water.

Cells for protoplast induction by lysozyme were cultured in the medium of Smith, Payne, and Watson (19). The medium was made in three parts: (a) distilled water with glucose, (b) with $(NH_4)_2HPO_4$ and K_2HPO_4, and (c) with $MgSO_4 \cdot 7H_2O$. The total volume was 200 ml. Each portion was autoclaved separately and cooled; and the glucose solution adjusted to pH 6.6 using concentrated HCl.

The three portions were then combined aseptically to yield a medium containing 2.0% glucose, 0.6% $(NH_4)_2HPO_4$, 0.02% KH_2PO_4 and 0.025% $MgSO_4 \cdot 7H_2O$.

In each case the organism to be tested was cultured in the sugar salts medium on the rotary shaker at 28° C for 24 hours. From this culture 40 ml were transferred to 200 ml fresh medium and the culturing repeated as above for 18 hours; after which cells were harvested and washed twice in distilled water.

Cells for protoplast induction by penicillin treatment (Lederberg (7)) were cultured in Penassay broth (Difco).

Protoplast Methods

Eight methods for protoplast formation were used, as follows:

(1) Penicillin (Lederberg (7))

(2) Lysozyme (Smith, Payne and Watson (19))

(3) Combination of methods 1 and 2

(4) Lysozyme and Polymyxin B (Tuttle and Gest (23))

(5) Glycine (Rubio-Huertos and Desjardins (17))

(6) Lysozyme and EDTA (Repaske (15))

(7) Combination of methods 1 and 6

(8) A method for Agrobacterium spp.

Method 8 is a modification of the Repaske procedure. Washed cells from 200 ml of medium prepared as previously described were suspended in 15 ml 1 M Tris buffer (Sigma Chemical Co.) containing 10% sucrose, and incubated on the rotary shaker at 28° C for 15 minutes. Following this treatment, 0.3% $MgSO_4 \cdot 7H_2O$, 0.5 mg/ml of EDTA (ethylene-diaminetetracetic acid) (Sigma Chemical Co.) and 3 mg/ml of lysozyme (Nutritional Biochemical Corporation) were added in rapid succession and shaking was continued for an additional 15 minutes. One ml samples were diluted four to ten times in distilled water, and after five minutes the percent conversions were estimated by the hanging drop method.

Purification of Protoplast Suspensions

Clumping of protoplasts occurred 15 minutes after dilution of the reaction mixture. This feature permitted

the development of two techniques for separating protoplasts from unconverted cells.

(a) Settling - The suspension was stored without agitation at room temperature for two hours. Within this time the heavy clumps of protoplasts settled to the bottom. The supernatant containing whole cells was decanted, leaving a residue consisting mainly of protoplasts.

(b) Centrifugation - In this method the suspension was centrifugated at 3000 x g for 5 minutes. Protoplast aggregates were packed at the bottom of the centrifuge tube while for the most part whole cells remained in suspension and were removed by decantation.

Protoplast Enumeration

Initially the protoplast conversion was estimated by direct microscopic count using a Zeiss phase contrast microscope. Five fields were examined in each case and the ratio of protoplasts to cells was calculated on a percentage basis. In the latter part of the study the

percent conversion was calculated by comparing the plate count on a treated sample with that on an untreated or control sample.

Electron Microscope Studies

Preparation of Grids for Electron Microscopy

A precleaned and polished glass slide was immersed in a 0.175% solution of formvar in chloroform for one or two seconds, and air dried. In order to release the formvar film the margin of the slide was scored with another glass slide. By holding the coated slide at a 30° angle and slowly immersing it into distilled water, the formvar film was floated on the water by surface tension forces. Using pointed forceps, a copper grid (200 mesh) was placed shiny side up on the film surface, and a piece of Bibluous paper of the same size as the formvar membrane was dropped directly on it. The film adhered to the paper, and was dried grid side up. The grid was carbonized with Edward's Speedivac Shadowcaster,

model 12E6, and was then ready for use.

Preparation of Samples for Electron Microscopy

Using a fine-tipped Pasteur pipette a sample from the reaction medium was placed on a prepared grid and air dried. A drop of distilled water was placed on the grid with the pipette, left for one minute and then carefully withdrawn. This was repeated 4 or 5 times. This constituted the washing procedure. The washed sample was then desiccated until dry, and shadowcasted with palladium at an angle of 10° - 15° with the Speedivac Shadowcaster.

Electron Photomicrography

A Phillips Model EM 75 electron microscope was used for observation of specimens. Photographic exposures were made on 35 mm Kodak fine grain positive film and on Kodak high contrast lantern slides.

Procedure for Studying Effect of pH on Conversion to Protoplasts Using Method 8

One hundred ml of an 18 hour culture prepared as

described previously was distributed in equal portions in each of eight centrifuge tubes. After centrifugation at 10,000 x g for 10 minutes the collected cells in each tube of the set were resuspended in 1.0 ml of 1 M Tris - 10% sucrose solution having a specific pH value. These values ranged from pH 5.0 in the case of tube #1 to pH 11.0 in tube #7. Since prior protoplast conversions were carried out at pH 8.0, this value was accepted arbitrarily as the standard and accordingly the contents of tube #8 were buffered with Tris-sucrose pH 8.0 as a reference control in the series.

The seven tubes, excluding the control, were treated using method eight following dilution 1 to 10 times with distilled water. After allowing fifteen minutes for conversion to protoplasts, the contents of each tube were washed twice with distilled water to remove traces of lysozyme.

Plating

Four replicates from each of the seven tubes of different pH values, and from the control tube were

cultured at the 10^{-5} and 10^{-7} dilutions in Trypticase-Soy Agar (Difco) at 28° C for 48 hr; and the appropriate dilution in each case counted with the aid of a Spencer colony counter. The percent conversion was estimated by comparing the count in each case with that of the control. The experiment was replicated three times with each of the four cultures.

RESULTS

Methods for Protoplast Formation

The data are presented in detail in Table I. Contrary to findings reported by Zinder and Arndt (26) conversion to protoplasts did not occur appreciably until a post-treatment osmotic shock was applied. This was done by diluting a volume of the treated sample with between four and ten volumes of distilled water. Significant protoplast conversion followed the osmotic shock treatment usually within a period of five to ten minutes. Method 8, which in the main was a modification of the method of Repaske, gave the best conversion, approximately 25% greater than by the Repaske method. Methods 2 and 4 produced essentially the same conversion, while Method 5 proved to be of little value. Method 3, a combination of 1 and 2, did not prove to be superior to Method 2. Similarly, Method 7 was not superior to Method 6.

TABLE 1

Protoplast Formation with A. tumefaciens in Relation to Total Cells;

Estimated by Direct Microscopic Counts

Methods	Number of trials	Percent conversion without dilution	Dilution for optimum conversion	Percent* conversion
1. Lederberg (7)	5	negligible	1:9	15-22-25
2. Smith, Payne, and Watson (19)	18	negligible	1:9	35-44-50
3. Combination of 1 and 2	3	negligible	1:9	45-45-45
4. Tuttle and Gest (23)	3	negligible	1:9	43-46-49
5. Rubio-Huertos and Desjardins (17)	3	5	-	3-5-6
6. Repaske (15)	24	negligible	1:4	64-72-76
7. Combination of 1 and 6	3	negligible	1:4	70-70-71
8. Modification of 6 for A. tumefaciens	7	negligible	1:4	90-95-98

*low, average, and high respectively

The Course of Protoplast Formation

The course of protoplast formation by Method 8 is shown in a series of four electron photomicrographs. A normal untreated cell is shown in Plate 1. After treatment followed by 1:4 dilution on distilled water the protoplast begins to emerge, while the cell wall appears to retain its shape. The relative height of these structures on the formvar film surface is indicated by the length of the shadows. This detail is shown in Plate 2. After complete conversion (about 15 min) the protoplasts tend to adhere to each other or to form aggregates with flattened rod-shaped cell walls as shown in Plate 3. After very high osmotic shock resulting from a second dilution treatment, the protoplasts begin to lyse. The cell contents emerge from the protoplast and are dispersed in the diluent. An early stage of this feature is illustrated in Plate 4.

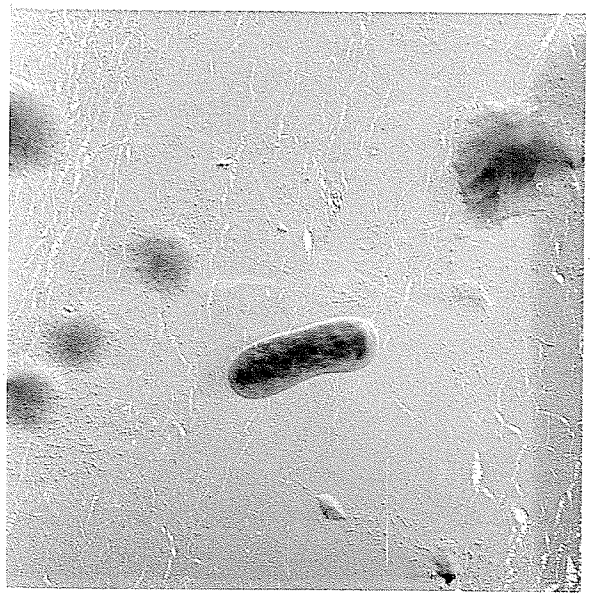


Plate 1

Electron micrograph of an untreated cell of A. tumefaciens.

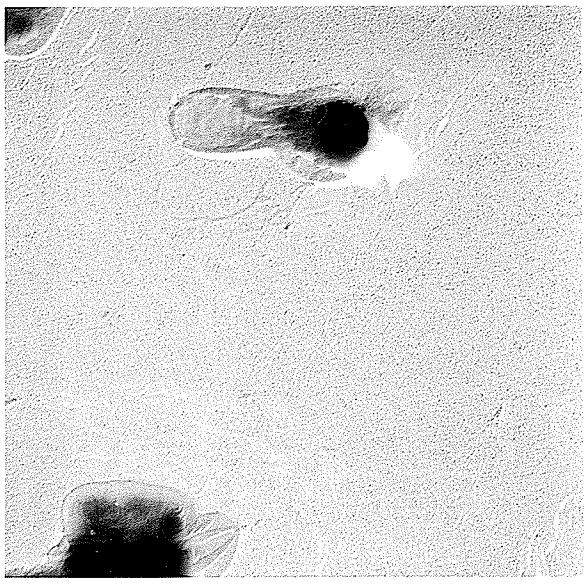


Plate 2

Electron micrograph of cell treated using Method 8 showing protoplast emerging after low osmotic shock.

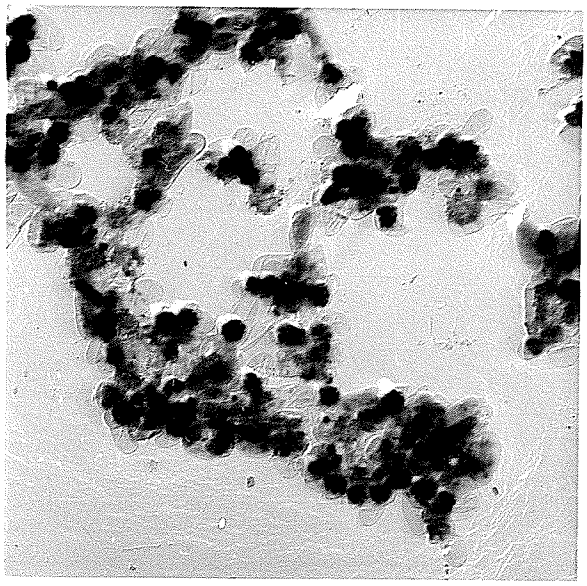


Plate 3

Electron micrograph of a field of protoplasts showing adherence of rod-shaped cell walls.

Plate 4

Electron micrograph of protoplasts undergoing lysis produced by high osmotic shock.

Effect of pH on Protoplast Formation in Agrobacterium spp.

The effect on pH on protoplast formation by Method 8 with four cultures of Agrobacterium spp. is presented graphically and in tabular form. In each case the range from lowest to highest of four replicate conversion counts is shown. The experiment was replicated three times and the overall average conversion calculated (Tables 2 to 5). The Figures represent only the overall average for each culture (Figures 5 to 8). The optimum pH for conversion of each culture was pH 8.0. Approximately the same conversion was yielded at pH 9.0. The conversion at pH 10.0 was extremely low with three of four cultures. It should be noted that a difference in the range of optimum pH values for conversion was evident even between two strains of A. radiobacter.

TABLE 2

Effect of Variation in pH on Percent Conversion* to Protoplasts for A. tumefaciens (Wisc. A6)

pH values	Trials I	II	III	Average
5.0	35-46	39-48	54-65	49
6.0	64-73	66-73	74-83	69-78
7.0	70-79	81-89	84-89	81
8.0	98-99	99-<100	99-<100	99
9.0	87-92	90-92	93-96	92
10.0	23-35	27-38	40-46	37
11.0	4-13	7-17	<1-1	7

*Values reported represent the range of four replicates within each trial.

TABLE 3

Effect of Variation in pH on Percent Conversion* to Protoplasts for A. gyposophilae (H38)

pH values	Trials I	II	III	Average
5.0	76-85	84-90	79-88	85
6.0	88-94	87-92	84-94	90
7.0	89-93	97->99	99->99	97
8.0	>99-<100	>99-<100	>99-<100	>99
9.0	>99-<100	99-<100	>99-<100	>99
10.0	26-38	23-28	15-29	26
11.0	7-16	6-11	4-11	9

*Values reported represent the range of four replicates within each trial.

TABLE 4

Effect of Variation in pH on Percent Conversion* to Protoplasts for A. radiobacter (426)

Trials

pH values	I	II	III	Average
5.0	44-49	44-53	42-53	48
6.0	41-56	49-61	54-64	53
7.0	56-65	56-64	56-65	60
8.0	88-94	85-92	89-96	91
9.0	83-89	80-86	87-94	87
10.0	21-33	16-30	17-29	25
11.0	11-17	7-20	13-22	14

*Values reported represented the range of four replicates within each trial.

Table 5

Effect of Variation in pH on Percent Conversion* to Protoplasts for A. radiobacter (1012)

Trials

pH values	I	II	III	Average
5.0	66-76	76-84	71-80	77
6.0	80-86	78-84	82-88	84
7.0	89-94	93-95	95-96	94
8.0	96-98	96-97	99-<100	98
9.0	91-94	95-97	99-99	96
10.0	76-85	88-92	83-88	88
11.0	60-67	58-67	55-63	63

*Values reported represent the range of four replicates within each trial.

FIGURE 1

Effect of Variation in pH on Percent Conversion to Protoplasts for A. tumefaciens A_6 (Wisc.)

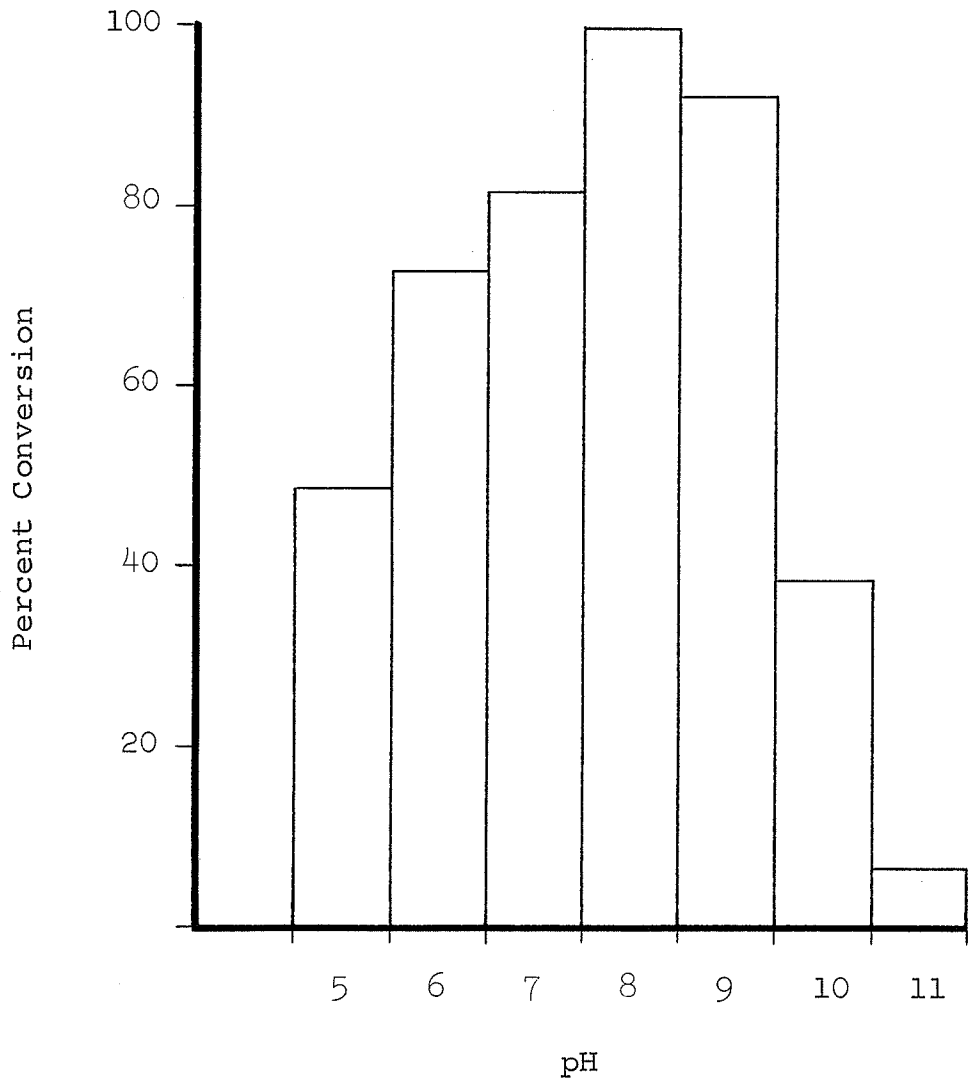


FIGURE 2

Effect of Variation in pH on Percent Conversion to Protoplasts for A. gyposophilae (H38)

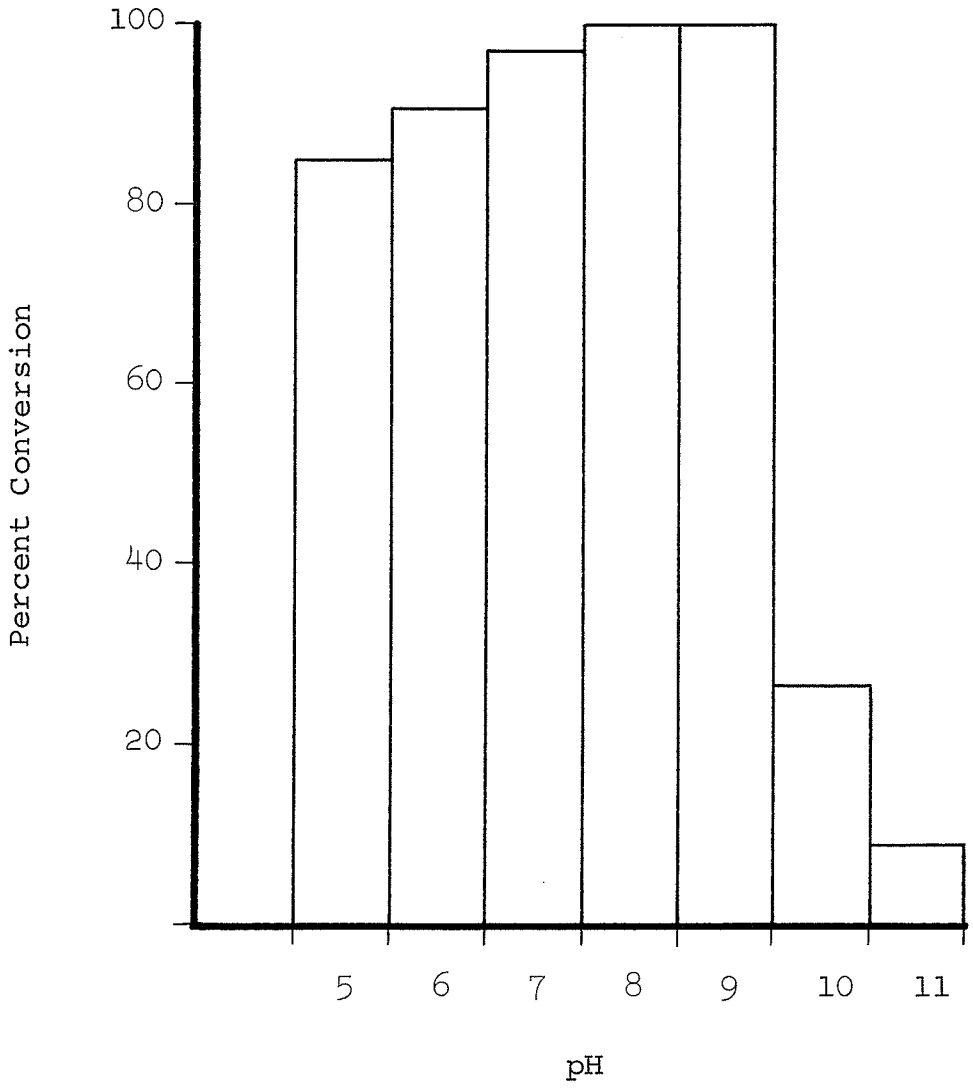


FIGURE 3

Effect of Variation in pH on Percent Conversion to Protoplasts for A. radiobacter (426)

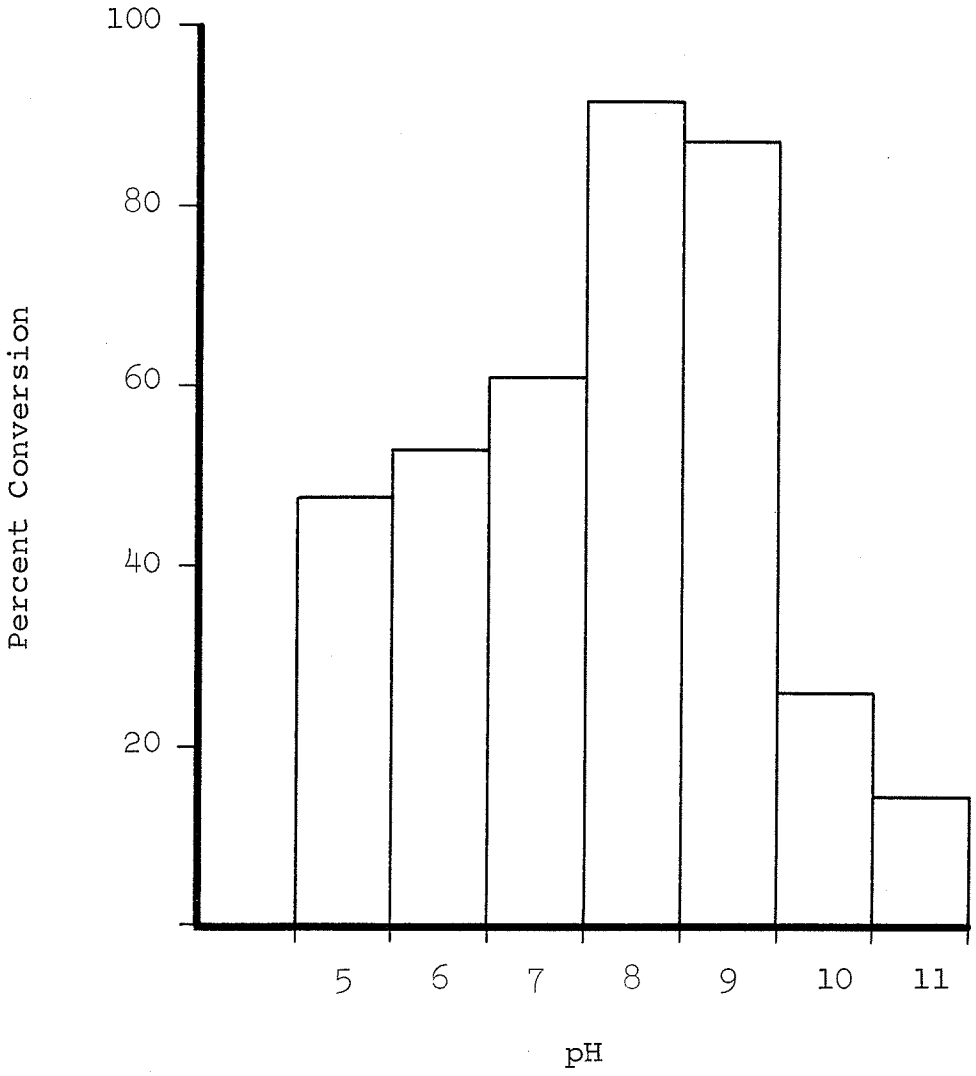
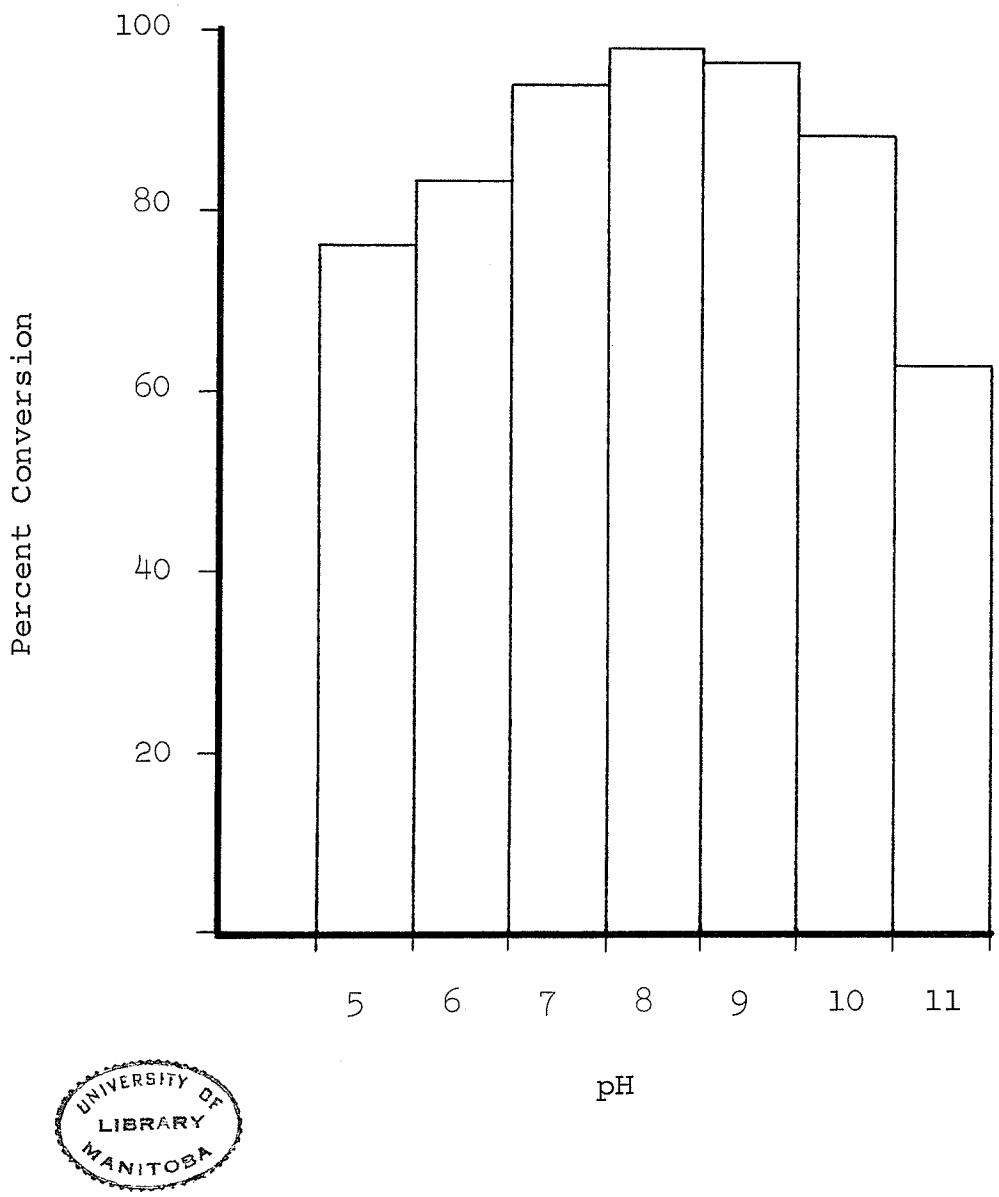


FIGURE 4

Effect of Variation in pH on Percent Conversion to Protoplasts for A. radiobacter (1012)



DISCUSSION

DISCUSSION

It should be noted that the work reported herein was limited to species of Agrobacterium, whereas the methods examined were developed for studies in other species. Evidently, the usefulness of a method for producing protoplasts in one species is not necessarily applicable to other species. This evidence tends to support the widely held contention that considerable difference in cell wall chemical structure exists between species. Indeed, the difference in pH optimum range for lysozyme noted between strains of A. radiobacter suggests the structural difference may even be considerable between closely related species. This may be true with respect to the mucopolysaccharide structure in particular. Such strain differences are known to exist between closely related members of the Rhizobiaceae with respect to bacteriophage sensitivity.

The substrate acted upon by lysozyme and penicillin in Gram-negative cells has been discussed by Salton (18). His experiments show that both lysozyme and penicillin act on

the same type of muco-complex structure in the cell. This was not borne out in the present study. Table 1 shows 22% conversion to protoplasts by the penicillin treatment. However, when penicillin was used in conjunction with lysozyme, Method 2, and the lysozyme-EDTA method, Method 6, no greater number of protoplasts were formed than when penicillin was omitted. This strongly suggests that penicillin acts upon a different structure, in the case of protoplast induction, than does lysozyme.

It was noted that the protoplasts prepared using the lysozyme treatment, Method 2, were lysed by mild osmotic shock. However, the protoplasts prepared using the lysozyme-EDTA treatment, Method 8, required a very high osmotic shock to induce lysis and even then lysis was not complete. Lees (6) put forth the suggestion that lysozyme sometimes contains lipase which could help to break down a lipid fraction of the cell wall during lysozyme treatment. The presence of EDTA may inactivate the lipase, leaving a covering of lipid material on the "protoplast", giving it a more hardy structure. Since the role of EDTA in the

production of protoplasts, according to Repaske (16), is to rupture co-ordinate bonds between metals and the cell, the theory advanced by Lees could be applicable. More recently, however, Noller and Hartsell (13) reported that the function of EDTA is thought to be that of a lipid dissociant working by simple detergent-like action. If such were the case, then any lipid material in the cell wall would be acted upon and removed.

The photographs (Plate 1, 2, 3, 4) appear to show that true protoplasts formed. A circular body seemed to leave the cell wall which retained its rod shape. This is in opposition to an opinion expressed by Robinow* in that the cell wall loses its shape when it is empty. The photograph of the lysing protoplast, Plate 4, suggests that there is a cytoplasmic membrane present in Gram-negative species - a belief that is not too widely held. Absence of residual cell wall fragments around the protoplasts suggests that they are true protoplasts.

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*A discussion of a paper "Spheroplast formation in the genus Agrobacterium, Conn" given before the annual meeting of the Canadian Society of Microbiology at Kingston, June 1961.

In Method 8 the sequence of addition of lysozyme and EDTA into the reaction medium proved to be instrumental in obtaining maximum conversion. This is in accordance with the findings reported by Repaske (16) who used different addition sequences to obtain maximum conversion for different species.

In this study Mg^{++} added to the reaction mixture did not stabilize the protoplasts formed, as was reported by Zinder and Arndt (26). Instead, Mg^{++} appeared to increase the activity of lysozyme.

Results from the determinations of pH for optimum conversion to protoplast indicated that four members of the genus Agrobacterium studied probably have similar cell wall compositions. The fact that the lysozyme-EDTA treatment worked optimally at pH 8.0 for all four cultures, suggests that it is acting on the same substrate. Salton (18) could not show this consistency in members of the genus Bacillus using lysozyme treatment. Instead, he obtained a "spectrum of sensitivities" for the different species.

REFERENCES

REFERENCES

1. Fischer, A. Die empfindlichkeit der bakterienzelle und das baktericide serum. Z. Hyg. Infekt Kr. 35, 1 (1900).

2. Garbowski, L. Gestaltsanderung und plasmoptyse. Arch. Protistenk 9, 53 (1907).

3. Grula, E. A. and Hartsell, S. E. Lysozyme action and its relation to the Nakamura effect. J. Bacteriol. 68, 302 (1954).

4. Jeynes, M. H. Growth and properties of bacterial protoplasts. Nature 180, 867 (1957).

5. Klieneberger, E. The natural occurrence of pleuro-pneumonia-like organisms in apparent symbiosis with Streptobacillus moniliformis and other bacteria. J. Path. Bact. 40, 93 (1935).

6. Lees, H. Personal communication (1961).

7. Lederberg, J. Bacterial protoplasts induced by penicillin. Proc. Natl. Acad. Sci. U.S. 42, 574 (1956).

8. Mahler, H. R. and Frazer, D. Reproduction of bacteriophage T_3 in protoplasts of Escherichia coli strain B. Biochim. et Biophys. Acta. 22, 197 (1956).

9. McQuillen, K. Bacterial protoplasts in "The Bacteria". (I. C. Gunsales and R. Y. Stanier eds.) p. 249 Academic Press (1960).

10. McQuillen, K. Bacterial protoplasts. I. Protein and nucleic acid metabolism in protoplasts of Bacillus megaterium. Biochim. et Biophys. Acta. 17, 382 (1955).

11. Mitchell, P. and Moyle, J. Liberation and osmotic properties of the protoplasts of Micrococcus lysodeikticus and Sarcina lutea. J. Gen. Microbiol. 15, 512 (1956).

12. Noller, E. C. and Hartsell, S. E. I. Lysis by four

lytic systems utilizing lysozyme. J. Bacteriol. 81, 482 (1961a).

13. Noller, E. C. and Hartsell, S. E. II. Pre- and co-lytic treatments potentiating the action of lysozyme. J. Bacteriol. 81, 492 (1961b).

14. Raichel, B. Uber den einfluss osmotisch wirksamer mittel auf die bakoterienzelle. Arch. Protistenk. 63, 333 (1928).

15. Repaske, R. Lysis of gram-negative bacteria by lysozyme. Biochim. et Biophys. Acta. 22, 189 (1956).

16. Repaske, R. Lysis of gram-negative organisms and the role of versene. Biochim. et Biophys. Acta. 30, 225 (1958).

17. Rubio-Huertos, M. and Desjardins, P. R. Microscopia electronica de las formes "L" y filterables del Agrobacterium tumefaciens. Microbiol. Espan. 9, 375 (1956).

18. Salton, M. R. J. The lysis of microorganisms by lysozyme and related enzymes. J. Gen. Microbiol. 18, 481 (1958).

19. Smith, B. S. W., Payne, J. I. and Watson, R. W. Preparation of spheroplasts of Aerobacter cloacae. Can. J. Microbiol. 6, 485 (1960).

20. Smolelis, A. N. and Hartsell, S. E. Factors affecting the lytic activity of lysozyme. J. Bacteriol. 63, 665 (1952).

21. Stapp, C. and Zycha, H. Morphologische untersuchungen an Bacillus mycoides; ein beitrag zur frage des pleomorphismus der bakterien. Arch. Mikrobiol. 2, 493 (1931).

22. Tomcsik, J. and Guex-Holzer, S. Änderung der strukturder bakterien zelle im verlauf der lysozym-einwirkung. Schweiz. Z. allg. Path. Bakt. 15, 517 (1952).

23. Tuttle, A. L. and Gest, H. Subcellular particulate

systems and the photochemical apparatus of Rhodospirillum rubrum. Proc. Natl. Acad. Sci. U. S. 45, 1261 (1959).

24. Warren, G. H., Gray, J. and Yurchenco, J. A. Effect of polymyxin on lysis of Neisseria catarrhalis by lysozyme. J. Bacteriol. 74, 788 (1957).

25. Weibull, C. The isolation of protoplasts from Bacillus megaterium by controlled treatment with lysozyme. J. Bact. 66, 688 (1953).

26. Zinder, N. D. and Arndt, W. F. Production of protoplasts of Escherichia coli by lysozyme treatment. Proc. Natl. Acad. Sci. U. S. 42, 586 (1956).