EXPERIMENTAL BIOLOGY

PRELIMINARY RESULTS OF A STUDY OF BACTERIAL PROTOPLASTS

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The cell wall is an important component of the bacterial cell, its basic function being to shield the cell contents from unfavorable environmental factors, as well as to maintain the characteristic shape of the cell Addition of lysozyme to a suspension of cells which are sensitive to this specific enzyme, acting on polysaccharides, leads to breakdown of the cell wall, followed by release of the cell contents, which diffuse into the medium [1, 11, 12]. It was first shown by Weibull [15, 16] that in the presence of certain "stabilizers" (various substances, such a sodium chloride or sucrose, at definite concentrations) the cell contents do not diffuse into the medium after the cell wall has been broken down by the lysozyme, but form a spherical body, which he called a protoplast. Later work showed that protoplasts are capable of performing certain vital functions: they respire, they can assimilate labelled amino acids, and they can synthesize protein, as a result of which their nitrogen content rises, as well as their weight [2, 3, 6, 7, 13], bacteriophage can multiply within them [5, 9, 12] and spores can develop within them [10]. Microscopic and electron microscope examination of protoplasts has shown the presence of mitotic figures, being evidence of their multiplication [8]. All these observations support the view that protoplasts are not merely lumps of surviving protoplasm, but that they represent a definite form of extracellular life. The question naturally arises as to whether regeneration of protoplasts, to produce secondary bacterial cultures, is possible.

The opinion has recently been expressed [14] that protoplasts are identical with L-forms. This view is supported by the observation that penicillin acts on Gram-negative bacteria (this is the usual procedure for production of L-forms) in the presence of sucrose, as a stabilizer, to give protoplasts, the morphology and mechanism of formation of which resemble those of L-forms [4].

The objects of our research were to produce protoplasts, to study their morphology and functional properties, and then to attempt to achieve regeneration of the protoplasts into secondary bacterial cultures.

EXPERIMENTAL METHODS

In our experiments we used washings of 18-20-hour cultures of B. megatherium grown on a solid synthetic nutrient medium, as well as 18-20-hour cultures in 2% peptone water to which a lysozyme preparation had been added (white of fresh hen's egg) in various proportions, from 1:100 to 1:1.

The microbes were washed from the solid nutrient cultures by means of M/30 phosphate buffer (pH 7.6) containing 10% of sucrose and lysozyme (experiment) or of buffer and lysozyme without sucrose (control 1). The second control (2) involved washing off the organisms with buffer containing sucrose, but not lysozyme. With 2% peptone water nutrient medium the experimental systems contained 10% of sucrose, control 1 was 2% peptone water with lysozyme but without sucrose, and control 2 was 2% peptone water with 10% of sucrose, but without lysozyme.

The experimental and the control suspensions were incubated at 37° for various times, for 15 minutes to

3 hours, and the organisms were then separated from the lysozyme by centrifuging (6000 rpm for 15 minutes). The residues so obtained were resuspended in the appropriate solutions not containing lysozyme, and the suspensions were examined in hanging drop and in stained preparations (fixed with 4% formalin for a number of days in a refrigerator). The organisms were stained according to Gram, and with Loeffler's methylene blue.

Respiration was measured by Warburg's method. The results were expressed as cubic millimeters of oxygen absorbed per ml of suspension in 30 minutes.

The proportions of unchanged cells to protoplasts were checked by inoculation on meat-peptone agar with and without sucrose.

EXPERIMENTAL RESULTS

Our comparative study of formation of protoplasts from B. megatherium M. lysodeicticus, and B. mesentericus cells showed that lysozyme caused total lysis of M. lysodeicticus cells, without formation of protoplasts. B. mesentericus was relatively resistant to lysozyme, only sporadic protoplasts being formed, while most of the cells remained unaffected. B. megatherium gave large numbers of protoplasts, with sporadic unaffected cells.

Microscopic Examination of Fixed Preparations

Smears from the experimental systems: intensely stained Gram-negative, homogeneous, spherical bodies, diameter 3-6 μ; sporadic bacillary forms of B. megatherium.

Control 1 (suspensions not containing sucrose): single bacillary forms of B. megatherium.

Control 2 (suspensions not treated with lysozyme): large numbers of unchanged bacillary forms of B. megatherium.

No formations resembling protoplasts could be seen in either of the controls.

Attempts at separating protoplasts from intact cells by centrifugation (3000 rpm for 15 minutes) were unsuccessful, as the larger protoplasts moved together with the larger cells, leaving the smaller protoplasts together with the smaller cells in the supernate.

Development of cells from protoplasts plated on meat-peptone agar containing sucrose was not observed. Growth of lysozyme-resistant cells took place, as in the control systems.

Comparison of respiration of protoplast suspensions with that of the initial culture gave no evidence of respiration of protoplasts.

Since we were unable to obtain suspensions of protoplasts reasonably free of unaffected cells we proceeded to the production of protoplasts from Gram-negative bacteria by treatment with penicillin.

Preparation of Protoplasts by the Action of Penicillin on Gram-Negative Bacteria (B. Coli 844; Salmonella Gallinarum 7979; Salmonella Gallinarum 398, Proteus X_{19})

Lederberg [4] showed in 1956 that regeneration of bacteria from their protoplasts was possible. His experimental material consisted of B. coli cultures. He used penicillin for breaking down the cell wall, with sucrose as the stabilizer. Lederberg followed the process of gradual formation of protoplasts, and of the subsequent transformation into cells in a penicillin-free medium.

EXPERIMENTAL METHODS

We used 18-20-hour cultures of Salmonella gallinarum in meat-peptone broth, and also 3-4-hour cultures for some experiments. The cultures were either not aerated, or were aerated by means of a magnetic stirrer. After incubation, 1 ml of the cultures was added to 10 ml of meat-peptone broth containing 10% sucrose, 0.2% of magnesium sulfate, and varying amounts of penicillin (from 100 to 2000 units/ml).

Control 1: the same, without sucrose; Control 2: the same, without penicillin.

The cultures were incubated for 6 hours at 37°, with or without aeration.

Samples of the cultures were plated out at hourly intervals on meat-peptone agar containing 10% sucrose, or without sucrose, and preparations were taken for microscopic examination (see techniques for examination of Gram-positive bacteria).

The optimum conditions for production of protoplasts were as follows: 1) concentration of microbial bodies; 1 ml of culture to 10 ml of meat-peptone broth; 2) final concentration of penicillin 1000 units/ml; 3) incubation conditions, 3 hours without aeration.

EXPERIMENTAL RESULTS

Formalin-fixed preparations for cultures containing penicillin and sucrose showed the presence of homogeneous spherical bodies 2-5 μ in diameter, giving fairly intense Gram-negative staining. Groups of 2, 4, or 6 protoplasts, diameter 1-2 μ, could be seen (Figs. 1, 2).

The controls without sucrose showed single unchanged Salmonella gallinarum bacilli, and clumps of amorphous material (evidently lysed cell debris). Large numbers of Salmonella gallinarum bacilli were seen in the control cultures without penicillin.

Formations resembling protoplasts were not found.

Tables 1 and 2 present the results of inoculating the experimental and the control cultures on meat-peptone agar containing sucrose and meat-peptone-agar not containing sucrose.

TABLE 1

Growth of Salmonella gallinarum 7979 on Meat-Peptone Agar With and Without Sucrose, after Different Durations of Exposure to Pencillin

Initial culture	Nutrient medium	Duration of action of penicillin		
		1 hr	2 hr	3 hr
Salmonella gallinarum in meat-peptone broth containing sucrose and 1000 units/ml of penicillin	Meat-peptone agar containing sucrose	+++	++	+
	Meat-peptone agar	++	++	–
Salmonella gallinarum in meat-peptone broth without sucrose, containing 1000 units/ml of penicillin	Meat-peptone agar containing sucrose	++	++	–
	Meat-peptone agar	+++	++	–
Salmonella gallinarum in meat-peptone broth containing sucrose	Meat-peptone agar containing sucrose	Over 50 colonies		
	Meat-peptone agar			

Explanation of symbols: + single colonies; ++ over 15 colonies; +++ over 30 colonies; – no growth.

Examination of stained smears taken from colonies grown on meat-peptone agar containing 10% sucrose (fixed in 4% formalin) supported the view that these colonies consisted of cells which had developed from protoplasts: the cells were polymorphous, and the preparations contained sporadic protoplasts 2-3 μ in diameter, as well as smaller ones (0.5-1 μ) distributed in groups of three (Fig. 3).

Comparative studies were made of the respiration of the cells and of their protoplasts (Tables 3 and 4).

The respiration of the cells always considerably exceeded that of the protoplasts. Very low respiration, much less than in suspensions of protoplasts containing sucrose, was found in the control suspensions containing penicillin but not sucrose (due evidently to cells on which penicillin had not acted). Only in a few of the experiments did penicillin totally abolish respiration.

We have thus confirmed that breakdown of the cell wall of bacteria by specific agents (lysozyme, penicillin) in the presence of stabilizer (sucrose) is associated with the formation of spherical bodies, analogous to Weibull's protoplasts.

Fig. 1. Formation of protoplasts from Salmonella gallinarum cells.

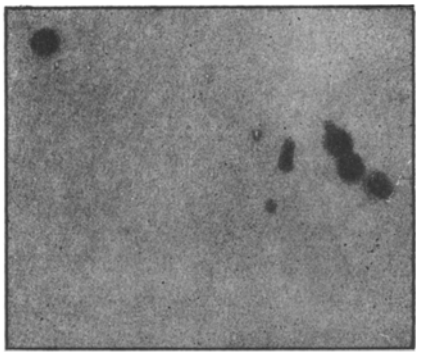

Fig. 2. Division of Salmonella gallinarum protoplasts.

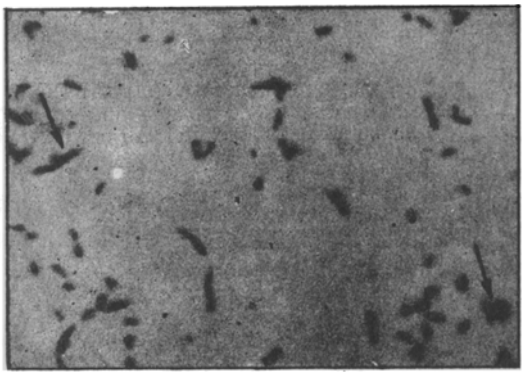

Fig. 3. Protoplasts in a smear from a colony of Salmonella gallinarum grown on meat-peptone agar containing 10% sucrose.

TABLE 2

Growth of Salmonella gallinarum 7979 and of Salmonella gallinarum 398 after Exposure to the Action of Penicillin for 3 Hours.

Nutrient medium	Initial culture					
	microbial culture in meat-peptone broth containing sucrose and 1000 units/ml of penicillin		microbial culture in meat-peptone broth containing sucrose and 1000 units/ml of penicillin		microbial culture containing 1000 units/ml of penicillin, without sucrose	
	strain of Salmonella gallinarum					
	7979	398	7979	398	7979	398
Meat-peptone agar containing sucrose	++	++	–	+	Over 50 colonies	
Meat-peptone agar	–	–	–	–		

Explanation of symbols: as in Table 1.

Mitotic figures were seen in fixed smears of B. megatherium and S. gallinarum protoplasts, showing that these may have the capability of development. Respiratory activity was also observed with S. gallinarum protoplasts; it was quite pronounced, although less than with suspensions of the intact organisms.

The best material for preparation of protoplasts was S. gallinarum subjected to the action of penicillin in the presence of 10% sucrose as stabilizer. In conclusion, we would like to thank Prof. G. P. Kalina for supervizing this research.

TABLE 3

Comparison of Respiration of Various Bacteria and of Their Protoplasts

Microbial species	Initial culture		
	microbial culture in meat-peptone broth containing sucrose	microbial culture in meat-peptone broth containing sucrose and 1000 units/ml of penicillin	microbial culture containing 1000 units/ml of penicillin, without sucrose
Salmonella gallinarum 7979	278	50	–
Salmonella gallinarum 398	248	37	9
B. coli 844	169	56	10
Proteus X_{19}	269	190	127

TABLE 4

Relation Between Respiration of Salmonella gallinarum 7979 Cells and Protoplasts and the Concentration of Penicillin (cubic millimeters of oxygen absorbed per ml of suspension in 30 minutes)

Initial culture	Concentration of penicillin					
	0 units/ml	2000 units/ml	1000 units/ml	500 units/ml	250 units/ml	100 units/ml
In meat-peptone broth containing sucrose	68	–	32	50	50	55
In meat-peptone broth	61	–	10	16	23	25

SUMMARY

Spherical bodies resembling Weibull's protoplasts are formed when certain bacteria (B. megatherium Salmonella gallinarum, B. mesentericus) are incubated with lysozyme or penicillin in the presence of 10% sucrose, as a stabilizer. The presence of mitotic figures in some of the protoplasts suggests that they are capable of development and regeneration.

The S. gallinarum protoplasts showed some respiratory activity, although much less than the intact cells. B. megatherium protoplasts did not respire.

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