Reports

Immunologic Comparison of Isolated Surface Membranes of Bacillus megaterium

Recent advances in bacterial physiology have made possible the isolation and purification of cell walls, protoplasts, and cytoplasmic membranes (*1–3*). Separation of these structures from *Bacillus megaterium* permitted an immunologic comparison, attractively simple in concept, that provided data bearing directly on the antigenic difference between the two membranes, the structural nature of surface antigens, and the question of the ability of antibody globulin to penetrate the cell wall. Although this experiment is preliminary to continuing immunocytologic work with other isolated structures of *Bacillus* spp., the results seemed of sufficient general interest to warrant reporting.

Several other studies have been concerned with the antigenic relationship between isolated cell walls and intact or ruptured cells (*4*). The continuing work of Tomcsik (*5*) is especially pertinent for its detailed immunologic analysis of capsular, cell-wall, and protoplast materials of *Bacillus* spp.

Strain KM of *Bacillus megaterium* was selected because of its sensitivity to lysozyme and its potential for the separation of several cell structures. It was grown in 2-percent peptone broth at 30°C with aeration. Microscopic examination and other studies (*6*) have indicated the improbability that capsular material is present on cells grown and washed under the conditions of the experiment.

Cell walls were isolated essentially by the procedure of Salton and Horne (*1*). Protoplasts were prepared by the method of Weibull (*2*). Membranes were obtained by resuspending an aliquot of protoplasts in phosphate-sucrose buffer containing 0.01 molar Versene and approximately 10 μg of deoxyribonuclease (DNase) per milliliter. The mixture was allowed to react for 30 minutes at 37°C. Versene disrupted the protoplasts, providing protoplast membranes that apparently were more intact than those obtainable with distilled water. The DNase prevented the suspension from becoming gelatinous. The suspension then was recentrifuged at 12,800g for 10 minutes, and the pellet was resuspended in 0.85-percent saline (which was found to facilitate removal of small particles) and finally was washed five times in distilled water. When the resulting preparation was viewed by phase microscopy, delicate membranous structures, almost completely devoid of protoplasmic particles, were observed. These protoplast membranes, probably the equivalent of cytoplasmic membranes (*3*), contained 11.0 percent ribonucleic acid (RNA) by the orcinal reaction (*7*) but no deoxyribonucleic acid (DNA) by the diphenylamine reaction (*8*), using preparations that had not been treated with DNase. The membranes were lysed by wheat-germ lipase but not by lysozyme or ribonuclease (RNase). The preparation of cell walls contained no detectable granules, DNA, or RNA and were lysed by lysozyme but not by lipase or RNase.

Cell walls, protoplasts, protoplast membranes, whole cells, and lysozyme then were used as immunizing antigens. Three or four rabbits were employed for each preparation. The animals were inoculated intravenously three times a week over a period of 3 months. Preimmune serums were obtained initially. Immune serums were obtained at intervals of 3 weeks, after allowing the animals 1 week of rest from antigen inoculations. The antibody titers reached maximum and adequately high levels in 6 weeks; at this point the serums were pooled and used in the later tests.

At first, the conventional tube agglutination reaction was used in assaying the immunologic reactions. Subsequently, the 50-percent end-point method of complement fixation (*8*) was used as a more sensitive index to confirm the agglutination titers. Although lysozyme has been reported to be antigenic (*9*), serums from rabbits injected with an amount of lysozyme equal to that used in preparing protoplasts failed to react with lysozyme, protoplasts, or protoplast membranes. Similarly, the preimmune serums were negative to all test antigens.

Table 1 includes the results of a factorial comparison of the several preparations. These data were further substantiated by conventional adsorption tests, in which the antiserum was twice adsorbed with an excess of antigen to demonstrable completion. These results are shown in Table 2. The experiment suggested the following conclusions, which are presented topically with the supporting data.

1) The cell wall and the protoplast membrane are antigenically distinct. The heterologous reactions between cell walls and either protoplasts or protoplast membranes were negative (Table 1), findings that are in agreement with those of Tomcsik (*10*). Moreover, adsorption with cell walls of whole-cell antiserum did not reduce the titer to protoplast membranes (Table 2). The data also confirmed the purity of the isolated membranes and walls.

2) Injection of the whole cell stimulates antibody to the respective component structures. Antiserum to intact cells reacted with antigens of cell walls, protoplasts, and protoplast membranes (Table 1). This observation directly confirms a basic tenet of immunology.

3) The reactive "surface antigens" of the intact cell are those of the cell wall. Although flagellar and capsular antigens have been identified with known structures, the term *surface antigens* carries a degree of ambiguity. Conceivably, such surface antigens could represent only the peripheral structure (here, the cell wall) or the underlying protoplast membrane or both. The data in Table 1 appear to

Table 1. Serological reactions of whole cells and isolated structures of *Bacillus megaterium*. Titers determined by agglutination are given in parentheses above those determined by complement fixation. Lysozyme and other controls were negative.

Test antigens	Antiserum to			
	Whole cells	Cell walls	Protoplasts	Protoplast membranes
Whole cells	(2560) 20,480	(1280) 5120	(< 10) 80	(< 10) 40
Cell walls	(1280) 2560	(640) 5120	(< 10) < 10	(< 10) < 10
Protoplasts	(320) 1280	(< 10) < 10	(320) 1280	(320) 1280
Protoplast membranes	(160) 1280	(< 10) < 10	(320) 1280	(320) 1280

Table 2. Cross adsorption of antiserums to whole cells and cell walls of *Bacillus megaterium*. Titers were determined by complement fixation.

Test antigens	Antiserum to whole cells adsorbed with			Antiserum to cell walls adsorbed with		
	Whole cells	Cell walls	Control	Whole cells	Cell walls	Control
Whole cells	< 20	< 20	20,480	< 20	< 20	2560
Cell walls	1280	< 20	2560	1280	< 20	5120
Protoplast membranes	1280	1280	1280	< 20	< 20	< 20

identify the structural locus of such surface antigens with the cell wall alone. It was observed that antiserum to protoplasts or protoplast membranes did not react significantly with intact cells or cell-wall antigens, but that antiserum to cell walls did react with whole-cell antigens. Moreover, intact cells adsorbed only a portion of the antibodies to cell walls (Table 2).

4) Antibody globulin does not penetrate the cell wall. The literature on immunology and data on the apparent impermeability of bacteria to globulin molecules (*11*) have implied that antibodies to bacterial cells react only with antigens on the exterior of the intact cell and do not penetrate to deeper lying structures. However, experiments with fixed animal cells and tagged antibodies have indicated that penetration of antibody molecules into such cells may occur (*12*). In our experiment, antiserum to protoplasts or protoplast membranes in reactions with whole-cell antigens fixed complement to only a minimal degree (Table 1), suggesting that the cell wall is impermeable to antibody (if the assumption is valid that complement is at least as capable of penetration as antibody). The parallel agglutination tests appear to be inapplicable because of the necessity of spatial proximity for bonding of the reactants; this may not be realized because of the thickness of the cell walls. The conclusion is further substantiated by the observation that adsorption of whole-cell antiserum with whole cells or cell walls failed to change the titer against protoplast membranes, and that adsorption of cell-wall antiserum with whole cells removed only a portion of the antibodies to cell walls (Table 2).

Similarly, it was observed that lipase, although lytic to protoplasts or protoplast membranes, had no apparent effect on whole cells or on protoplast formation from lipase-treated, washed cells. Thus, both antibody- and enzyme-protein molecules, each having a specific affinity for the protoplast membranes, apparently are unable to penetrate the cell wall of this bacterium (*13*).

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References and Notes

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Algae as Sources of Lysine and Threonine in Supplementing Wheat and Bread Diets

A shortage of protein in many parts of the world has stimulated studies of possible additional resources. Algae have received considerable attention because they can be grown with simple inorganic nutrients in mass culture, and many have a high nitrogen content (*1*). However, their nutritional value has received scant study. Combs (*2*) found that supplements of *Chlorella pyrenoidosa* improved growth and feed efficiency in chicks, an effect attributed to riboflavin, carotene, and perhaps other vitamins contributed by *Chlorella*. Henry (*3*) fed *Chlorella* to young rats and reported a protein efficiency ratio somewhat superior to peanut meal, about equal to brewers' yeast, but considerably inferior to dried skim milk. Bender *et al.* (*4*) found a variable but generally low protein value for several marine algae in rat studies.

Published values on the amino-acid composition of algae indicate a surprisingly good spectrum of essential amino acids, except perhaps for cystine-methionine (*1, 2, 5–7*). The threonine and lysine content of *Chlorella* is about the same as that of hen eggs (*6*). These facts prompted us to test algae as supplements in wheat diets, for lysine, threonine, and valine have been shown to improve wheat flour (*8*), and lysine (*9*) or lysine and threonine improve the nutritional value of enriched white bread.

In the first experiment, a green algae *Scenedesmus obliquus* WH-50 was mass cultured as described elsewhere (*10*), harvested, dried, ground to a powder and fed to weanling rats as indicated in Table 1. The results show that *Scenedesmus* improved growth significantly with both flour and bread diets. These effects can be attributed almost certainly to amino-acid supplementation. The flour diets were adequately fortified with all known required minerals and vitamins except vitamin B_{12}, which had no growth-promoting effect in parallel experiments. Although the bread diet was not so fortified, other experiments showed that vitamin and mineral supplements produced only a slight growth response with such diets. It is also clear that *Scenedesmus* was an excellent source of threonine (group 4 versus groups 7 and 3). A comparison of group 2 versus groups 1 and 3 suggests that *Scenedesmus* was contributing considerable lysine. However, a comparison of group 6 with groups 7, 2, and 3 indicates that the lysine content of *Scenedesmus* was not sufficient for a complete growth response. This may be due to the fact that lysine probably is more deficient than threonine in these diets. The supply of *Scenedesmus* was insufficient to test it at levels higher than 4 percent. The nitrogen content of this lot of *Scenedesmus* was not determined but was assumed to be equivalent to 50 percent protein, based on experience with other lots that were cultured under the same conditions (*11*). The growth effects produced by *Scenedesmus* cannot be explained as due simply to the addition of a source of amino nitrogen, for group 5 showed no growth response. Furthermore, in parallel experiments, supplementation with amino acids other than lysine and threonine did not produce a growth response with these diets.

Another green algae, *Chlorella pyrenoidosa*, which was cultured and prepared in the same way as *Scenedesmus*, and a different strain of weanling rat (Osborne and Mendel) were used in a second series of experiments. The nitrogen content of two different lots of *Chlorella* was 7.38 and 7.92 percent (dry basis). One test (five rats per group and