Formation of Spheroplasts of *Mycobacterium tuberculosis* by Lysozyme Treatment.* (28580)

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There has been much interest in the use of protoplasts and spheroplasts in biochemical and genetic studies. One of the techniques most widely used is based on the findings of Weibul(1) that protoplasts may be formed by controlled treatment with lysozyme in a medium containing sucrose. Not only has this method proved applicable for production of protoplasts of most Gram positive organisms, but when ethylenediamine tetraacetic acid (EDTA) and hydroxymethyl aminomethane (tris) are added to the system Gram negative species are also rendered susceptible(2).

There have been several reports of the tuberculostatic and tuberculocidal effects of serum and tissue extracts, and an attempt to equate this inhibitory effect with the lysozyme content of animal tissues and fluids(3). There has been no reference, however, to formation of protoplasts or spheroplasts of *Mycobacterium tuberculosis* when exposed to lysozyme, or any other chemical agent.

The finding that lysozyme converts cells of *M. tuberculosis* to osmotically fragile spheroplasts under conditions of growth is reported here.

Materials and methods. The H37Ra strain of *M. tuberculosis,* maintained by weekly subculture on Tween 80 liquid medium without albumin, was used. Cells used for this experiment were harvested after 3 days growth and washed once with Tween® 80-albumin medium containing albumin (1 μg/ml) and glucose (10 μg/ml). They were resuspended in a small volume of this medium to give a final optical density reading of 0.03 at 450 mμ when included in the final reaction system. The complete reaction mixture was contained in 10.0 ml of Tween-albumin medium at pH 7.1-7.2. When sucrose was used it was at a concentration of 0.34 M, and Mg^{++} (as $MgSO_4$) at 1.1 μg/ml. Concentrations of lysozyme from 50 to 200 μg/ml and EDTA from 1000 to 4000 μg/ml were tried. Optimal results were obtained with 100 μg/ml lysozyme and 2000 μg/ml EDTA. The tubes were incubated at 37°C, shaken gently daily to resuspend the cells and the optical density read on a Coleman Junior spectrophotometer at 450 mμ. Cultures were examined at 24-hour intervals with dark phase optics, for changes in morphological structure.

Results. Effect of lysozyme. In the absence of sucrose and Mg^{++}, lysozyme (100 μg/ml) had a tuberculocidal effect, with approximately 70% inhibition of growth (Fig. 2). During the first 24 hours following exposure to lysozyme, no change was observed in the morphology of the organism. Most cells continued to appear normal through the third day although a small number of cells in the system did show a slight amount of swelling, either in the middle of the rod or on the ends. During the next 4 days of incubation many of the cells became thinner and more transparent; many were without swelling but contained dark granules within the cytoplasm. By the ninth day most of the cells had become smaller than the control cells, were thinner, transparent, and subsequently lysed, releasing their cytoplasmic contents. The surviving cells of the system remained unchanged and continued to grow.

When sucrose (0.34 M) and Mg^{++} (1.1 μg/ml) were added to the system, 100 μg/ml lysozyme caused approximately 50% inhibition of growth. The morphological changes were slow in appearing and lysis was delayed. The optical density rose steadily and although some of the cells were swollen and transparent on the ninth day following inoculation, most of the cells appeared normal. On the tenth day some lysis occurred, and a few thin degenerate cells were seen.

Effect of EDTA. In the absence of sucrose and Mg^{++}, EDTA (2000 μg/ml) did not

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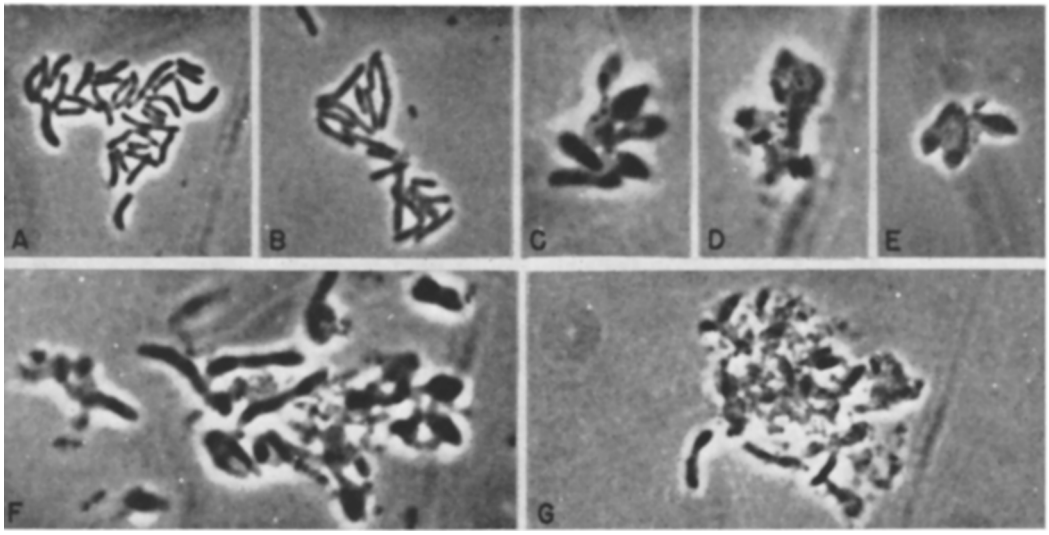


FIG. 1. Cells of *Mycobacterium tuberculosis* (strain H37Ra) grown in Tween-albumin medium in absence of sucrose and Mg^{++} in a system containing 100 μg/ml lysozyme and 2000 μg/ml EDTA. A: Control at 3 days. B: Control at 8 days. C and D: 3 days. E: 4 days. F: 5 days. G: 8 days.

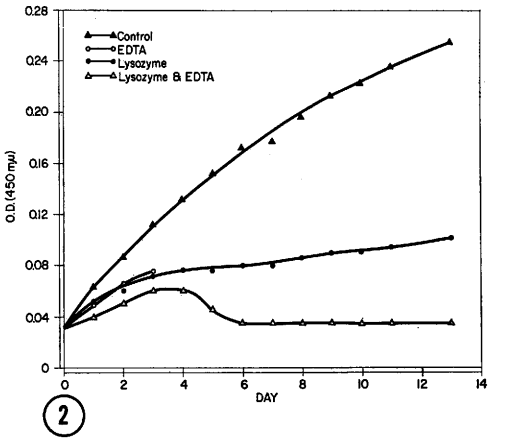
cause lysis of cells although some cells did show a slight amount of swelling, either in the middle or at the end of the rods. There appeared to be less inhibition of growth as compared with lysozyme, but accurate optical density readings could not be obtained because in the presence of EDTA alone, the cells adhere to the walls of the tubes and cannot be resuspended by vigorous shaking.

When 2000 μg/ml EDTA was tested in the presence of sucrose and Mg^{++}, inhibition of growth was minimal; no lysis was observed although a few enlarged cells were seen after the second day of incubation and increased in size until the second week. The swelling was usually terminal. Most cells, however, remained normal in appearance.

Effect of lysozyme and EDTA. In a system containing both lysozyme and EDTA at optimal concentrations, as determined by prior testing of various combinations, more inhibition of growth and lysis of cells were observed than when each was used singly. A slight amount of swelling was observed after 24 hours of incubation. This increased in degree and number of cells affected until by the fourth day practically all of the cells in the system were swollen in varying degrees, transparent, and contained dark granules (Fig. 1 C, E). The cytoplasm in many cells appeared vacuolar. On the fifth day there was evidence of lysis, with the release of cytoplasmic contents including very small dark granules (Fig. 1 D, F). Lysis was observed to continue until by the end of the first week only broken cell wall remnants and granular material were seen (Fig. 4 G). A viability test at this time was negative.

Microscopic findings paralleled very closely the optical density readings (Fig. 2). There was a steady rise in optical density during the first 3 days, followed by a leveling off between the third and fourth day, and a marked drop between the fourth and sixth days. A Gram stain of cells at this stage showed beaded forms which did not stain uniformly and which did not take the stain as intensely as the control cells. When stained with Ziehl-Neelsen's stain, the organisms were either non-acidfast or stained very faintly when compared with control cells.

When sucrose and Mg^{++} were added to the system containing both lysozyme and EDTA the sharp drop in optical density was prevented (Fig. 3). After an initial rise during the first 3 days, at which time swelling of the cells was first observed (Fig. 4 D), optical density readings leveled off and remained stationary until the tenth day when they showed a small drop. During this period, be-



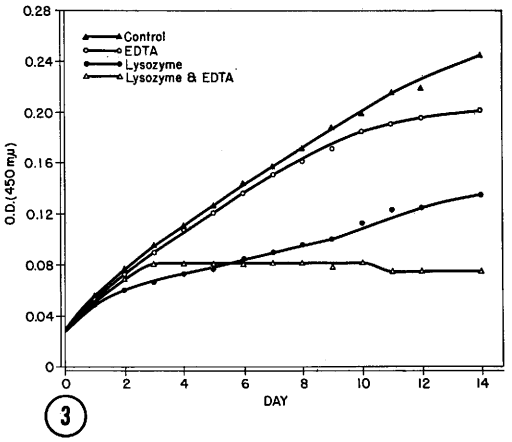


FIG. 2. Effect of 100 μg/ml lysozyme and 2000 μg/ml EDTA alone and in combination on growth of *Mycobacterium tuberculosis*.

FIG. 3. Effect of sucrose (.34 M) on growth of *Mycobacterium tuberculosis* in a system containing no lysozyme or EDTA (control) and in systems containing 100 μg/ml lysozyme, 2000 μg/ml EDTA alone and in combination.

tween the third and tenth day, there was an increase in number and degree of swelling and transparency of the cells. Swelling was observed either in the middle or at one or both ends of the cell (Fig. 4 E, F, G). From the eighth day on, an increasing number of spherical forms or spheroplasts were observed in the system, most of which contained 2 dark granules (Fig. 4 H-R). Although a minimal amount of lysis occurred on the tenth day, most of the cells were stabilized as spheroplasts.

Mg^{++} was found to be necessary for the formation and stabilization of spheroplasts. In the presence of 0.34 M sucrose and optimal concentrations of lysozyme and EDTA, cells lysed on the third day of incubation in the absence of Mg^{++}. In the presence of 0.07 μg/ml Mg^{++}, lysis was delayed until the fifth day, and few spheroplasts observed among the survivors during the second week. With the optimal concentration of Mg^{++} tested, 1.1 μg/ml, very little lysis was observed and a large number of spheroplasts formed. Spheroplasts were still observed in the culture on the nineteenth day, at which time the experiment was terminated. With concentrations of 1.6 μg/ml and higher, swelling of cells was suppressed and no spheroplasts observed.

Discussion. The finding that *Mycobacterium tuberculosis* can be converted into osmotically fragile spheroplasts by lysozyme treatment in an appropriate system suitable for growth, is of interest in view of the importance of lysozyme to the body's natural defenses.

Lysozyme is present in the tissues and body fluids of many animals and man. Not only has lysozyme been shown to be tuberculostatic(4) but the possibility has emerged from the studies of Myrvik and his associates(3,5) of its playing an important role in resistance to tuberculosis and in acquired resistance to the disease. There has been, however, no clear-cut indication of the mode of action of lysozyme on tubercle bacilli.

Salton(6,7), Weidel(8) and a number of other workers have demonstrated that the mucopeptide rigid layer of the cell wall is the substrate for the enzyme in those organisms tested. Experiments in our laboratory(9) and in others(10) have demonstrated that tubercle bacilli also possess this basal layer which can be digested by lysozyme. In tubercle bacilli, however, the mucopeptide layer accounts for a much smaller percentage of the total wall substance than in Gram positive organisms. The outer layers, probably lipoprotein and lipopolysaccharide in nature, no doubt offer a considerable degree of protection against destruction of the inner rigid layer by lysozyme. This has also been shown in our laboratory by the finding that organisms first treated with chloroform and other fat solvents are more readily lysed by lysozyme than untreated cells in a resting cell buffer system.

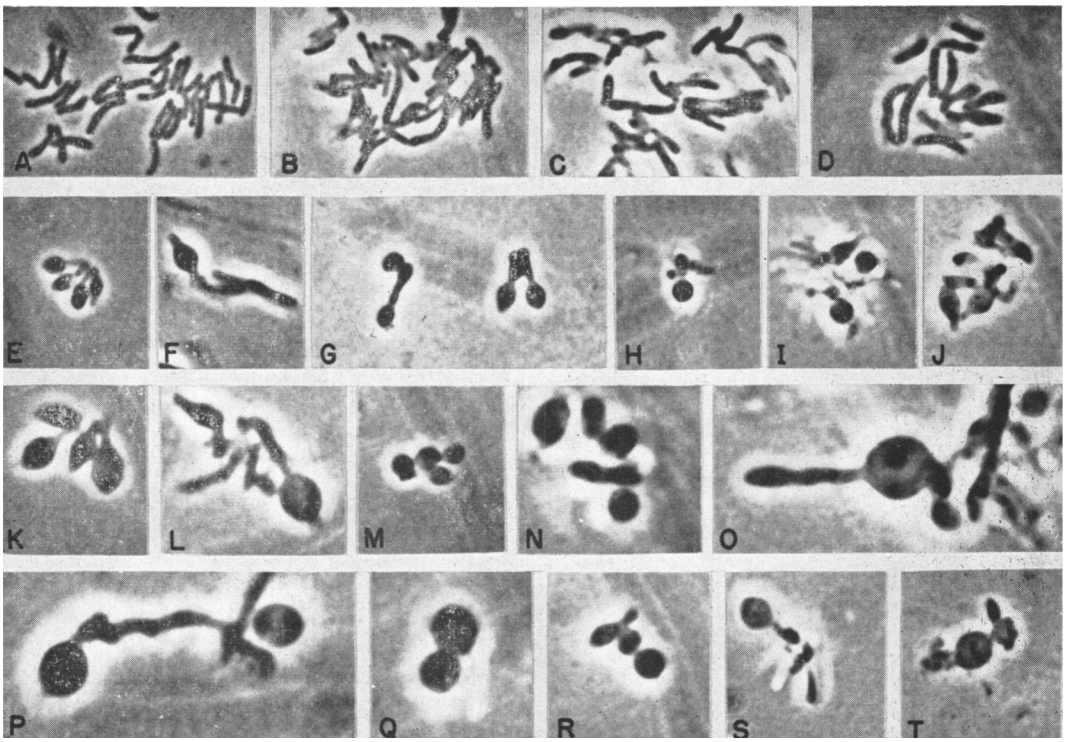


FIG. 4. Changes in morphology of cells of *Mycobacterium tuberculosis* (strain H37Ra) grown in a Tween-albumin medium containing .34 M sucrose and 1.1 μg/ml Mg^{++} in a system containing 100 μg/ml lysozyme and 2000 μg/ml EDTA alone and in combination. A: Control at 4 days. B: Control at 10 days. C: Lysozyme omitted, at 10 days. D: 3 days. E: 6 days. F-L: 10 days. M-P: 11 days. Q and R: 12 days. S and T: 14 days.

This protection provided by the surface lipids of the wall of the organism, together with the slow rate of growth of tubercle bacilli and the consequent building up of a high osmotic pressure within the cell explain the apparent delay in spheroplast production by this organism.

The role of EDTA has not been completely explained, although it may be assumed to function by making the essential β 1-4 linkages between N-acetylmuramic acid and N-acetylglucosamine more available to the lytic enzyme.

The finding of the mode of action of lysozyme *in vitro* suggests a similar action *in vivo*. The elevation of a lysozyme-like substance in immunized animals has been demonstrated by Janicki and Patnode(11). Although the mechanism of this increase is unknown, it is suggested that the lysozyme is released into the plasma subsequent to leukocyte damage. The presence of other factors in the tissue fluids, their identity now unknown, may play an equivalent or similar role to that of EDTA in making the tubercle bacillus more susceptible to lysozyme.

Summary. *Mycobacterium tuberculosis* (strain H37Ra) has been converted into osmotically fragile spheroplasts by growth in a medium containing lysozyme and EDTA. These spherical forms were stabilized in the presence of sucrose and Mg^{++}.

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Effect of Drugs on Goldfish Scale Homograft Survival. (28581)

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Hildemann has shown(1,2,3) that the goldfish *(Carassius auratus)* is immunologically competent and develops antibodies to scale homografts. Such scale (skin) homografts are rejected following a predictable immune response, while autografts are permanently successful and elicit little or no inflammatory reaction.

Recently there has been great interest in the effect of drugs on homograft rejection. In mammalian systems analogs of nucleic acid such as 6-mercapto purine, delay homograft rejection(4,5). Even in pure immunological systems these analogs have been shown to be effective depressants of immunological response(6,7,8). However, there have also been reports that these compounds have no effect on homograft rejection(9). On the basis of the previous findings, it was assumed that the goldfish *(Carassius auratus)* could be a useful tool in evaluating the effect of drugs on homograft reactions. This report describes some of the effects on scale homografts observed when the fish were treated with 6-mercapto purine (6-MP) and other compounds.

Method. Fish skin (scale) transplants were made according to the method of Hildemann(1). The fish were obtained locally from random bred populations at the Pacific Goldfish Farm in Westminster, Calif. All fish received an autograft as well as numerous homografts from at least 2 other fish. Drugs were given by intraperitoneal injection on different time schedules. The most frequently used procedure was injection of drug on the day of transplant and on each of 3 successive days. The transplanted scales were observed and scored on days 3, 5, 7, 9 and 11 after transplantation depending upon the treatment and rate of rejection. Observations were made using a B&L dissecting stereoscope with magnification up to 30 power. The criteria used for evaluation of scale graft acceptance were restoration and maintenance of blood flow and survival of pigment cells (xanthophores) in the scale. According to Hildemann(1) those scales which still have a substantial number of intact chromatophores are viable. The fish were kept in aquaria at 26 $\pm$ 1°C except for periods of transplantation, drug injection or observation. The fish were anesthetized with 60 mg/l of Tricaine prior to manipulation.

Results. A dose-response relationship for 6-MP is presented in Fig. 1. It can be seen that 5 mg/kg has practically no effect upon the scale chromatophore clearing or rejection and 10 mg/kg has a slight effect. Doses of 25 and 50 mg/kg definitely delay homograft rejection. The data are represented as per cent of average control scale chromatophore clearing because all scales do not clear at exactly the same rate. Each point represents the average of at least 5 scales. It should also be noted that the first observation (Fig. 1) is on the 3rd day after transplantation which is also the last day of drug injections and the treated as well as non-treated fish do not show the graded differences. Usually after the 7th day following transplantation the control scales or those scales in fish which have not received the drug are almost completely free of pigment (Fig. 2). In the fish treated with 6-MP in increasing doses, the scales are depigmented less. Blood flow was seen in