INFECTION AND IMMUNITY, Feb. 1976, p. 425-437

Vol. 13, No. 2
Printed in U.S.A.

Comparison of Antigens in Sonic and Pressure Cell Extracts of *Mycobacterium tuberculosis*

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Received for publication 3 July 1975

Comparisons were made of the yield, chemical content, and biological activity of filtrates and extracts obtained by sonic and pressure cell disruption of bacilli from 4- and 8-week-old Proskauer and Beck cultures of the H37Rv strain (TMC no. 102) of *Mycobacterium tuberculosis*. The culture filtrates were dialyzed, freeze-dried, reconstituted in saline, and sterilized by membrane filtration. The viable bacilli were washed and resuspended in distilled water and subsequently disrupted either by sonication in the cold for 15 or 30 min or by treatment at 20,000 or 40,000 lb/in^2 in a pressure cell. The resulting extracts were clarified by centrifugation, concentrated, and sterilized by filtration. All preparations were adjusted to contain 10 mg of solids (dry weight)/ml and were analyzed quantitatively for protein, deoxyribonucleic acid, ribonucleic acid, polysaccharide, and lipid content. Separation patterns obtained by gradient acrylamide gel electrophoresis, as well as by one- and two-dimensional immunoelectrophoresis, provided the basis for qualitative comparisons of the culture filtrates and cell extracts. Three-point dose-response curves also were used to compare the preparations for skin test reactivity in BCG-vaccinated guinea pigs. It was concluded that, although there were no consistent differences in chemical content or biological activity between the preparations, a 15-min sonic treatment appeared to be the most suitable method for preparation of bacillary extracts based on yield of active components and ease of preparation.

Filtrates of mycobacterial cultures have served as source materials for the preparation of tuberculin purified protein derivative (PPD) and for attempts to isolate, identify, and characterize the biologically significant antigens of the tubercle bacillus. However, because they are autolytic products and subject to continuous enzymatic alteration and degradation during incubation, the antigenic composition of culture filtrate preparations can vary markedly, not only between strains of tubercle bacilli but also between identical cultures of the same strain (3, 21, 26). Preparations of mycobacterial cytoplasmic extracts, in contrast, have been reported to be uniform in composition (3, 6, 21, 22, 26-28). In other studies, bacillary extracts have been demonstrated to be suitable materials for immunological study. Typical delayed hypersensitive skin reactions have been produced with bacillary extracts in sensitized animals (8, 13, 15). Protoplasmic preparations have been found to be as specific and sensitive as PPD for detection of delayed hypersensitivity (4, 12, 14), and purified protoplasmic components have been claimed to be even more specific skin test antigens than PPD (24). Bacillary extracts also have been used serologically to differentiate and group mycobacterial species (9-11). To prepare biologically active cell extracts, mycobacteria have been disrupted by various procedures, including mechanical grinding (4, 8), pressure (3, 12-15, 21, 24), and sonication (5, 6, 9-11, 18, 22, 26-28). Pressures have ranged from 10,000 to 45,000 lb/in^2, and sonic treatment has been varied by intensity, time, and number of exposures. It has been difficult to determine which disruption method is the most effective on the basis of yield and activity of cytoplasmic components because prior studies did not provide the necessary comparative data. This information has been obtained recently as a preliminary step in the preparation of a bulk supply of bacillary extract for subsequent fractionation and isolation of antigens. The purpose of this report is to describe

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the results of quantitative and qualitative comparisons of the yield, chemical content, and biological activity of bacillary extracts obtained by sonic and pressure cell disruption of human-type tubercle bacilli.

MATERIALS AND METHODS

Preparation of bacterial cultures. A frozen suspension of the H37Rv strain of *Mycobacterium tuberculosis* (TMC no. 102) was obtained from the Trudeau Mycobacterial Collection (Trudeau Institute, Inc., Saranac Lake, N.Y.). The bacilli were subcultured at 2-week intervals in Proskauer and Beck medium (Difco Laboratories, Detroit, Mich.) to produce a smooth-surface pellicle; after five such transfers, seed cultures were prepared and incubated at 37 C for 2 weeks, when the pellicles were harvested, pooled, and homogenized in fresh medium. The homogeneous upper portion of the suspension was decanted and diluted in medium to serve as a seed inoculum; aliquots of the suspension were centrifuged in Hopkins tubes to determine its bacillary content. The seed suspension was used to inoculate 116 culture bottles; each contained 700 ml of medium and was inoculated with 35 mg (wet weight) of bacilli. The bottles were maintained in a high-humidity incubator at 37 C; 58 cultures were harvested after incubation for 4 weeks, and the remainder were harvested at 8 weeks.

Preparation of culture filtrates and bacillary extracts. At harvest, the culture fluids were aspirated aseptically from beneath the pellicles, pooled, and successively passed through 0.45- and 0.22-μm membrane filters (Millipore Corp., Bedford, Mass.). The filtrates subsequently were dialyzed at 5 C against distilled water and freeze-dried; the dry material was reconstituted in pyrogen-free 0.9% sodium chloride (Travenol Laboratories, Inc., Morton Grove, Ill.) to contain 10 mg of nondialyzable solids per ml, sterilized by membrane filtration, and stored at −20 C. The pellicles were resuspended in sterile, pyrogen-free distilled water (Travenol), transferred to Buchner funnels, and washed repeatedly with sterile water to remove medium components. The washed bacillary suspension was diluted with sterile water to contain 310 mg (wet weight) of bacilli per ml and divided into equal portions; one portion was reserved for future processing and was stored at −70 C, whereas the other was subdivided into four equal portions for disruption and preparation of cell extracts.

Two portions of the bacillary suspension were disrupted by a Branson Sonifier (model W-185E, Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) at 4 C in a sealed, 40-ml treatment chamber equipped with a continuous-flow water jacket to maintain a constant temperature. One portion was subjected to a continuous treatment at maximum output (100 to 110 W) for 15 min and the other was treated similarly for 30 min. The resulting cell extracts were labeled CE-S_{15} and CE-S_{30}, respectively, and were clarified by centrifugation at 10,000 × *g* for 30 min at 5 C. The supernatant fluids were centrifuged again at 100,000 × *g* for 60 min at 5 C, after which the clear extract layer was removed from between the particulate and lipid layers. The remaining two portions of the bacillary suspension were diluted with sterile water to contain approximately 100 mg (wet weight) of bacilli per ml and disrupted at 5 C in a Ribi cell fractionator (model RF-1, Ivan Sorvall, Inc., Newtown, Conn.). One portion was subjected to disruption at 20,000 lb/in^2 and the other was disrupted at 40,000 lb/in^2; the resulting cell extracts were labeled CE-P_{20} and CE-P_{40}, respectively, and were clarified by centrifugation as described above. After centrifugation, the extracts were sterilized by membrane filtration; representative aliquots of each extract were freeze-dried to determine total solid content. The extracts then were concentrated by partial freeze-drying, adjusted to contain 10 mg of solids per ml, resterilized by membrane filtration, and stored at −20 C. Yields of extracts were calculated from the recorded volumes and solid content and were expressed as a function of the wet weight of bacilli disrupted. Aliquots of each extract were dialyzed at 5 C against distilled water and subsequently freeze-dried to determine the content of nondialyzable solids; dialyzable solids were calculated by subtraction of nondialyzable solids from the total solid content of the sample.

Analysis of antigen preparations. Chemical, physicochemical, and biological analyses were performed at one time on the final, reconstituted preparations.

Chemical determinations. Chemical constituents were measured in triplicate by standard methods; specifically, the protein content was determined by the Lowry method (16), deoxyribonucleic acid (DNA) was assayed by the diphenylamine reaction (20), and the ribonucleic acid (RNA) content was determined from absorption measurements at 260 nm after correcting for DNA absorption. The orcinol reaction (17), corrected for RNA content, was used to measure polysaccharide I, and the anthrone method (19) was used to determine polysaccharide II. The method described by Snyder and Stephens (23) was used to measure triglycerides and lipid esters. The total content of each component in the extracts was calculated and expressed as a function of the wet weight of bacilli disrupted.

Gradient acrylamide gel electrophoresis (GAGE). Electrophoretic separation patterns in acrylamide gels provided the basis for qualitative physicochemical comparisons of the antigen preparations. The preparation of the multi-stage separating gels and the conditions for electrophoretic separation were the same as those described by Affronti et al. (1). The gel columns contained (gel concentration followed by gel height) a 3.5%, 1-cm upper gel, followed by a 4.75%, 1-cm gel, a 7%, 1.5-cm gel, and a bottom 12%, 5-cm gel. From 0.06 to 0.5 ml of sample containing 300 μg of protein in 20% sucrose was layered over the upper gel; electrophoresis was conducted in tris(hydroxymethyl)aminomethane-glycine buffer, pH 8.3, at room temperature with a constant current of 5 mA/tube until the bromophenol blue tracking dye reached a point 1 cm from the bottom of the tube. After separation, the gels

were stained for protein with Coomassie brilliant blue and photographed.

Immunoelectrophoresis (IEP). One- (IEP) and two-dimensional (2-D IEP) immunoelectrophoretic separation patterns provided the basis for qualitative comparisons of the antigenic composition of the preparations. For IEP, the antigens were separated in an agar gel, using a discontinuous barbital buffer system, pH 8.6, at room temperature for 90 min with a constant current of 1.7 mA/slide as described previously (7). After separation, the antigens were allowed to react with reference anti-H37Rv antisera to develop precipitin bands; the slides were incubated in a humid chamber at room temperature for 48 h and then photographed. (Lot no. 002 CF and anti-H37Rv antisera were obtained through the United States-Japan Cooperative Medical Science Program from the Geographic Medicine Branch of the National Institute of Allergy and Infectious Diseases, Bethesda, Md.)

Methods described previously (27) were used for 2-D IEP analyses of the antigen preparations. The first separation was conducted in agarose, using a discontinuous barbital buffer, pH 8.6, at 10 mA/slide at 10 C until the bromophenol blue tracking dye reached 0.5 cm from the anodic end of the slide. After the first electrophoretic separation, the agarose gel, 2 mm above the area where the antigen had separated, was removed and replaced with agarose mixed with the reference antisera. After the gel-antiserum mixture had solidified, the second electrophoretic separation was conducted at right angles to the first direction; the sample was separated at 12.5 mA/slide at 10 C for 2.5 h. The slide then was placed in a humid chamber and incubated at room temperature overnight to allow complete development of the precipitin bands. The nonreactive proteins were eluted from the agarose-antiserum matrix by submerging the plate in three changes of 0.9% sodium chloride containing 0.01% merthiolate and one change of distilled water. The immunoelectropherograms were dried, stained with Coomassie brilliant blue, and photographed.

Skin testing. Three-point dose-response curves were used to quantitatively compare the antigen preparations for skin test reactivity in BCG-vaccinated Hartley strain guinea pigs. Each animal was sensitized with 5 mg (dry weight) of heat-killed BCG distributed into four footpads in a total of 0.2 ml of water-in-oil emulsion; the emulsion consisted of equal parts of saline and Arlacel A-Drakeol 6VR (35:65). Six weeks after sensitization, the animals were tested with 0.016-, 0.062-, and 0.250-μg (dry-weight solids) doses of each preparation; the test materials were injected intradermally in 0.1 ml. The dose range was established by a preliminary screen in sensitized animals. Each dose level of each preparation was tested in 12 sensitized animals; 12 to 15 skin test sites were placed on each animal. Perpendicular diameters of erythema at the skin test site were measured 24 h after injection.

RESULTS

A comparison of total, nondialyzable, and dialyzable solids in cell extracts obtained from 4- and 8-week-old cultures by different disruption methods is presented in Fig. 1. Review of these data permitted two generalizations. First, 4-week-old cultures yielded more extractable solids than 8-week-old cultures, regardless of the disruption procedure. Second, prolonging the sonic exposure beyond 15 min or increasing the pressure above 20,000 lb/in^2 did not result in a greater yield of extracted material, regardless of the age of the cultures. Also, the proportions of dialyzable solids to total solids appeared relatively constant as sonic exposure or pressure was increased. The efficiency of disruption did not appear to be consistent; the 15-min sonic exposure yielded the most material from 4-week-old tubercle bacilli, whereas treatment at 20,000 lb/in^2 provided the greatest yield from 8-week-old cultures. However, the 40,000-lb/in^2 treatment was least productive with either type of culture.

The results of the chemical analyses of the extracts (Fig. 2) agreed well with those of the yield measurements. Extracts prepared by 15-min sonic treatment of 4-week-old bacilli generally contained the most protein, nucleic acids, polysaccharides, and lipids, whereas in 8-week-old cultures the extracts prepared by exposure to 20,000 lb/in^2 had the greatest concentrations of these components. Also, as noted previously, increasing the sonic exposure or pressure treatment did not result in an increase in the chemical composition of the extracts.

No marked qualitative differences between the extracts were noted on examination of either the GAGE or the conventional IEP separation patterns. The GAGE patterns of the culture filtrates and the various cell extracts are shown in Fig. 3. The conventional IEP separation patterns for the 4- and 8-week cell extracts are shown, respectively, in Fig. 4 and 5; IEP patterns obtained with the culture filtrates using both reference antisera are presented in Fig. 6. Other than differences in band intensity, which may reflect differences in concentration of the components, the patterns of the extracts were practically identical. However, regardless of the disruption method, the extracts consistently differed from the respective culture filtrate preparations in that their components were more clearly resolved.

The separation patterns obtained by 2-D IEP are shown in Fig. 7 through 9. This analytical method was more discriminating and sensitive than conventional IEP and detected striking quantitative differences between extracts of 4- and 8-week-old cultures. Extracts prepared from 4-week-old cultures (Fig. 7) contained greater antigen concentrations than those of 8-week-old cultures (Fig.

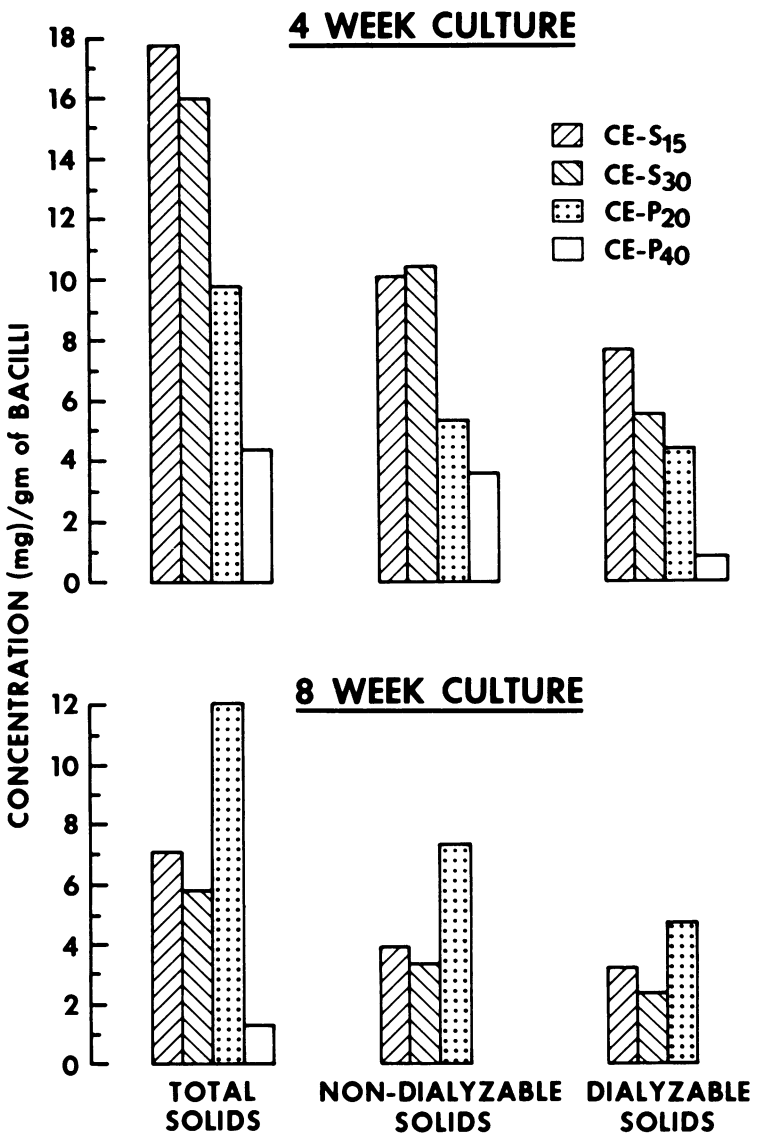

FIG. 1. *Influence of disruption method on yield of mycobacterial extracts.*

8) as reflected by the higher, and more intensely staining, precipitin peaks of their major components. When the disruption methods were compared, no significant differences were noted in extracts of 4-week-old cultures. However, both quantitative and qualitative differences were observed in the patterns obtained with 8-week-old extracts, especially in the 20,000- and 40,000-lb/in^2 patterns (Fig. 8). Nevertheless, as also detected by GAGE and conventional IEP, the extracts, regardless of the disruption method, consistently differed from their respective culture filtrate preparations (Fig. 9) in that their components were more clearly resolved.

Similarly, no significant differences were noted when the skin test reactivity of the cell extracts was examined (Fig. 10). Each bar represents the mean of 12 values; the analysis was designed so that all preparations from the same culture interval were tested within the same group of sensitized animals. As detected previously when the yields were examined, regardless of the disruption method, cell extracts prepared from 4-week-old cultures contained more skin test-reactive material

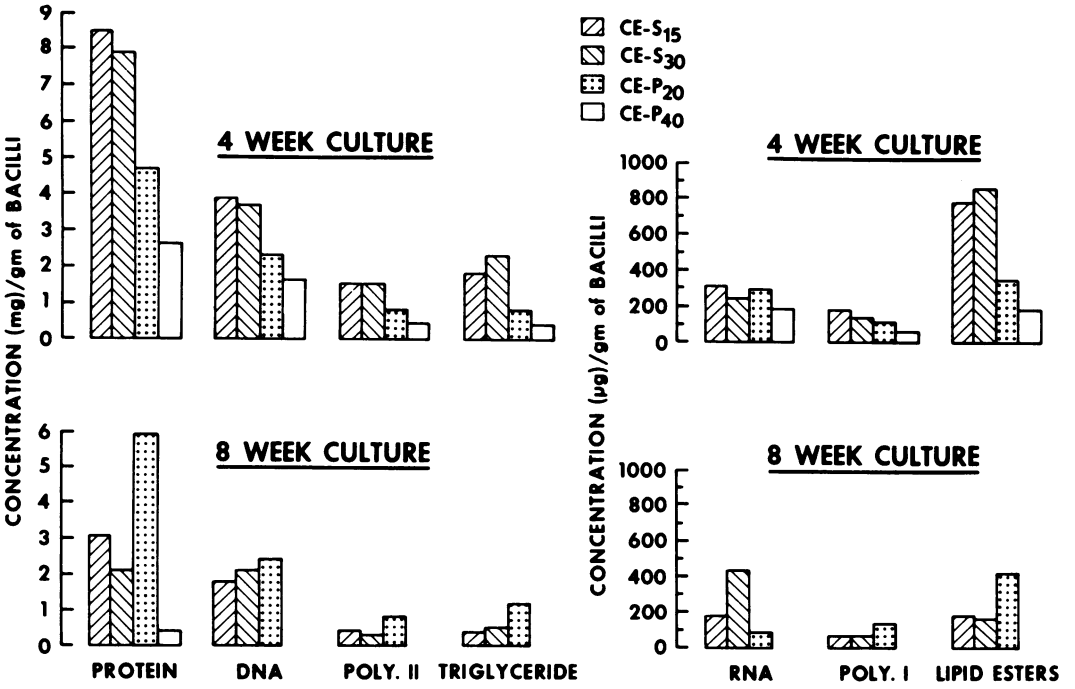

FIG. 2. *Influence of disruption method on chemical composition of mycobacterial extracts.*

than similar preparations from 8-week-old cultures. All of the extracts of 4-week-old tubercle bacilli were significantly more reactive than the culture filtrate preparation. Extracts of 8-week-old cultures, however, were no more reactive than the culture filtrate, probably because the culture filtrate preparation increased in reactivity whereas the extracts decreased slightly from the levels obtained with 4-week-old cultures.

DISCUSSION

When considered collectively, the results of this study indicate that 4-week-old cultures of human-type tubercle bacilli are a more appropriate source for preparation of bacillary extracts than 8-week-old cultures, based on antigenic composition. This observation is consistent with the findings of Castelnuovo et al. (3), who showed that the IEP patterns of bacillary extracts were constant for a strain of tubercle bacilli regardless of the age of the culture. Similarly, the results of previous studies from one of our laboratories have indicated that sonic extracts prepared from different strains of a single mycobacterial species had essentially the same GAGE and 2-D IEP patterns; in contrast, the patterns of their culture filtrates varied markedly in most cases from strain to strain (1, 22, 26–28). Also, as shown in the present study, the antigenic composition did not differ significantly when the disruption method was varied. Thus, based on these collective results, it appears that cell extracts may be more suitable source materials than culture filtrates for the isolation and purification of tuberculin antigens. Comparison of the GAGE and the conventional and 2-D IEP patterns of the culture filtrates and cell extracts suggests that the component antigens are better defined and more homogeneous in the bacillary extracts and, therefore, may be more readily isolated by preparative gel electrophoresis, for example, than from culture filtrates.

It also was observed in the present study that more biologically active material could be extracted from 4-week-old tubercle bacilli than from 8-week-old cultures. This observation is unexplained at present. However, it is likely that significantly more autolysis occurred in the older cultures; thus, the decreased yield of extractable cytoplasmic material may have reflected a marked reduction of intact bacilli in the pellicles. This view is consistent with the findings of Turcotte and Des Ormeaux (25), who also noted that, as cultures of the H37Rv strain aged, the yield of extractable cytoplasmic material decreased, whereas the concentration of culture filtrate components correspondingly increased.

A comparison of the extraction of soluble

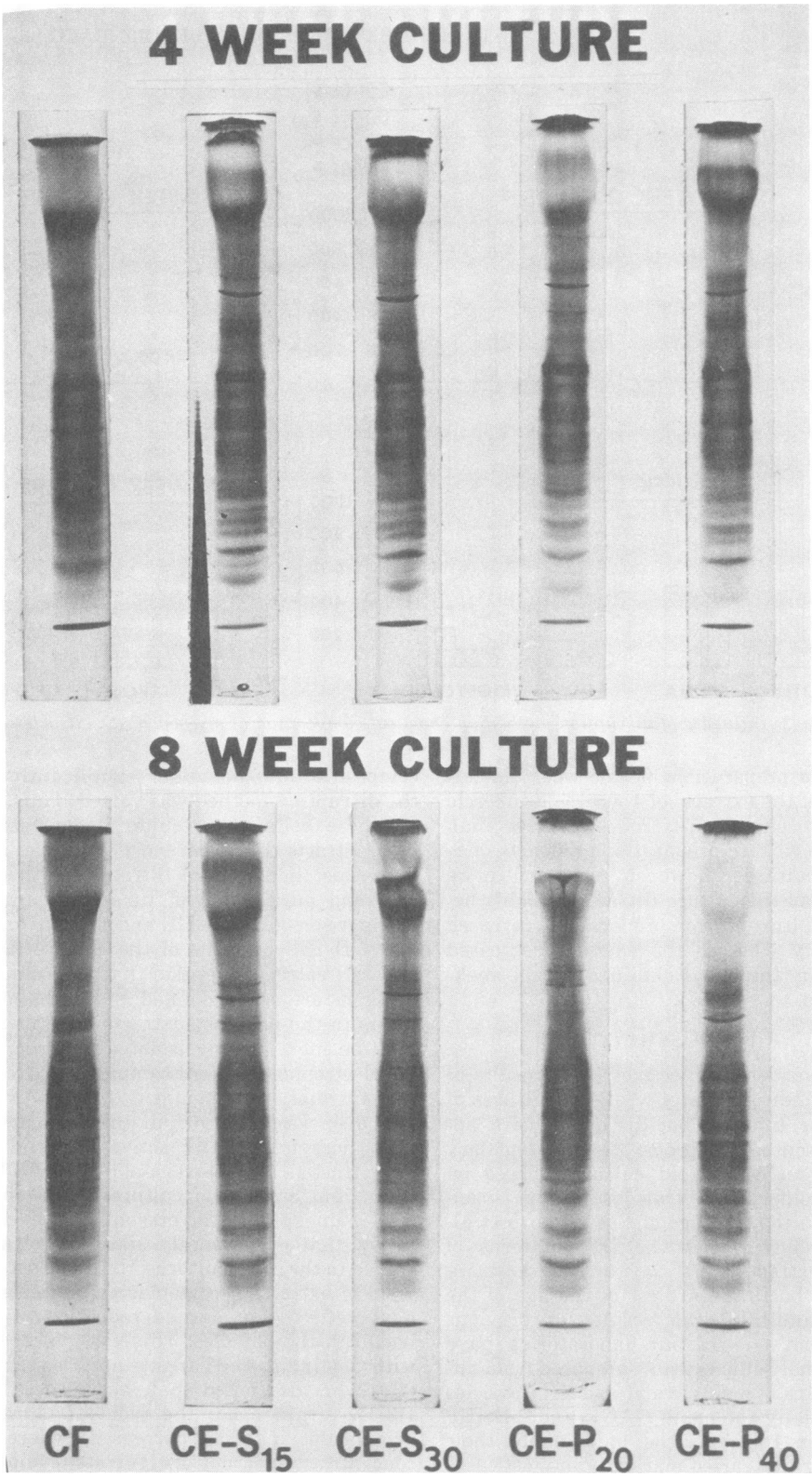

FIG. 3. *GAGE separation patterns of mycobacterial extracts.*

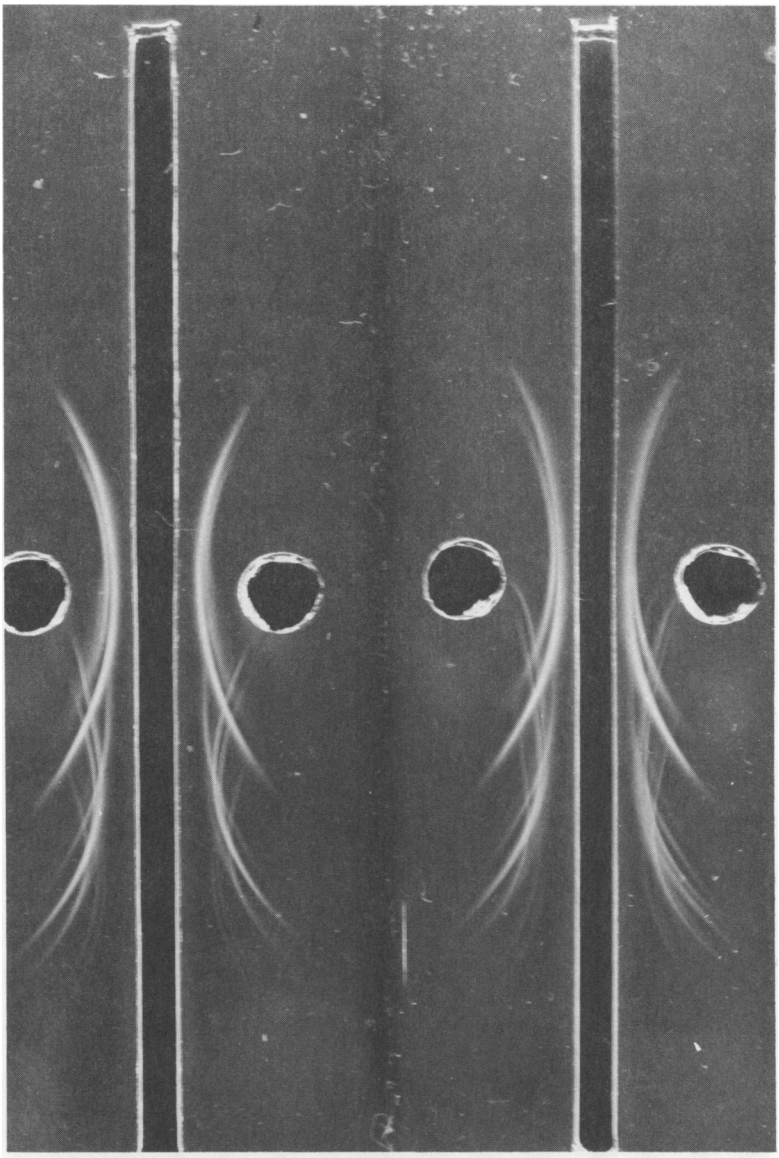

FIG. 4. *Immunoelectrophoretic separation patterns of mycobacterial extracts prepared from 4-week-old cultures. Wells contained, from top down, CE-S_{15}, CE-S_{30}, CE-P_{20}, and CE-P_{40}; troughs contained lot no. 002 anti-H37Rv cell extract reference antiserum.*

antigens by sonic treatment with pressure cell disruption indicated that a continuous 15-min sonic exposure was the most effective method for the preparation of the bacillary extracts. Moreover, sonication has the advantage of being technically simple, and relatively large volumes of bacillary suspension can be processed conveniently in 40-ml batches within a day. Also, the operating temperature can be adequately controlled to minimize denaturation of antigenic components, and safety to personnel can be maintained by placing the sonic probe in a biohazard hood.

Furthermore, the observation gained by Beam et al. (2) from microscopic examination of pressure cell-disrupted mycobacteria that pressures in excess of 30,000 lb/in^2 resulted in complete fragmentation of cell walls was not reflected in the present study. Neither yield measurements nor electrophoretic and im-

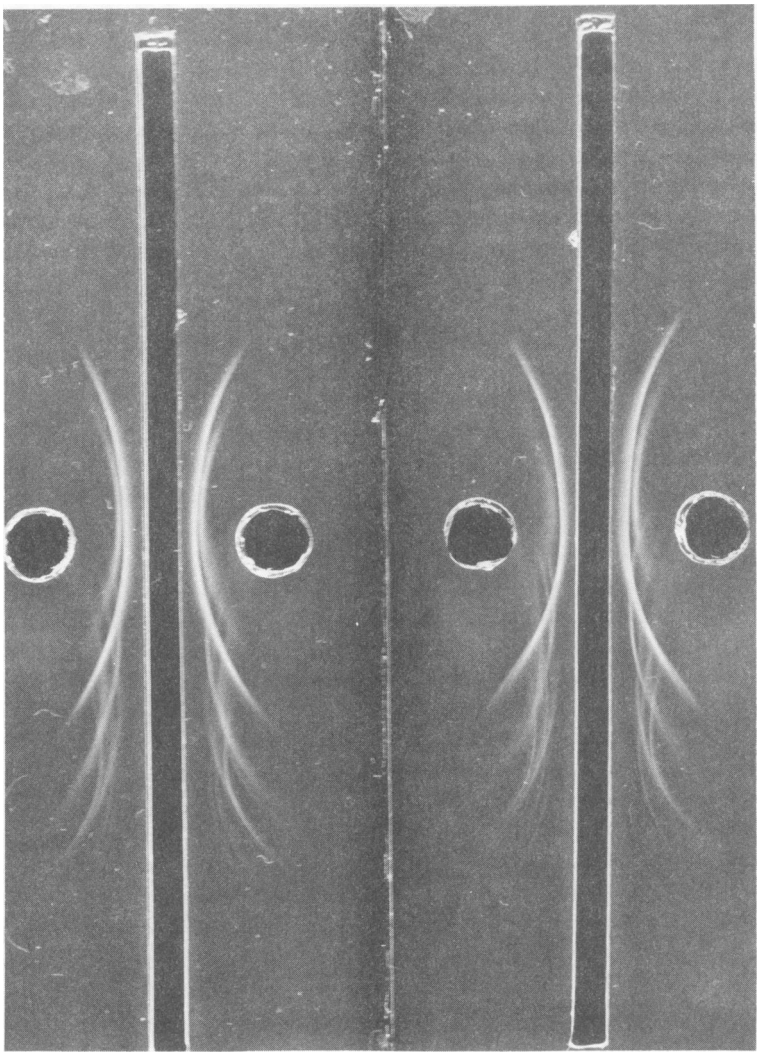

FIG. 5. *Immunoelectrophoretic separation patterns of mycobacterial extracts prepared from 8-week-old cultures. Wells contained, from top down, CE-S_{15}, CE-S_{30}, CE-P_{20}, and CE-P_{40}; troughs contained lot no. 002 anti-H37Rv cell extract reference antiserum.*

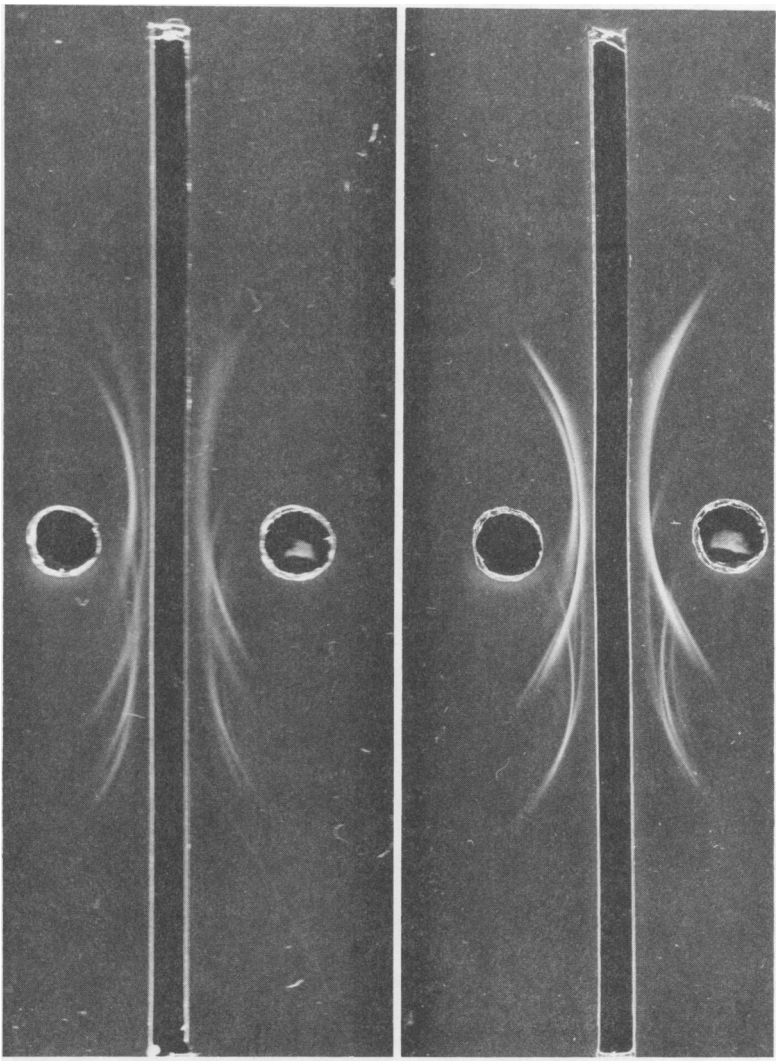

FIG. 6. *Immunoelectrophoretic separation patterns of mycobacterial culture filtrates. In upper slide, trough contained lot no. 002 anti-H37Rv culture filtrate reference antiserum; upper well contained filtrate from 4-week-old cultures, and lower well contained culture filtrate from 8-week-old cultures. In lower slide, trough contained lot no. 002 anti-H37Rv cell extract reference antiserum; content of wells was the same as in upper slide.*

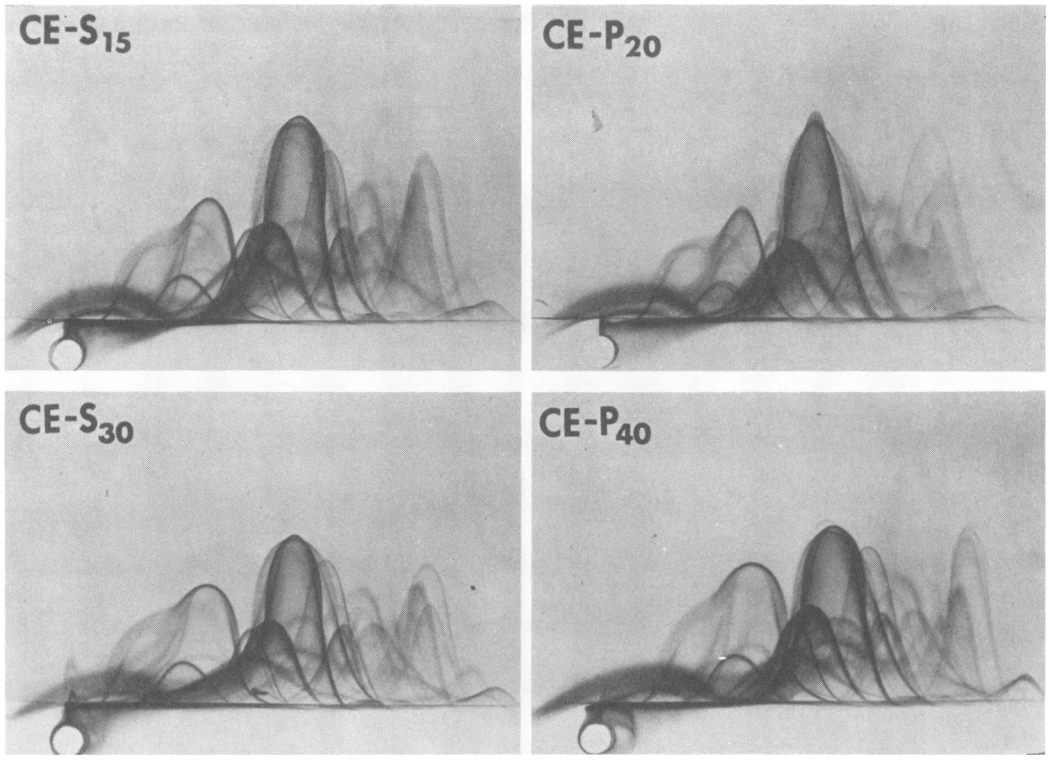

FIG. 7. *Two-dimensional immunoelectrophoretic patterns of mycobacterial extracts prepared from 4-week-old cultures.*

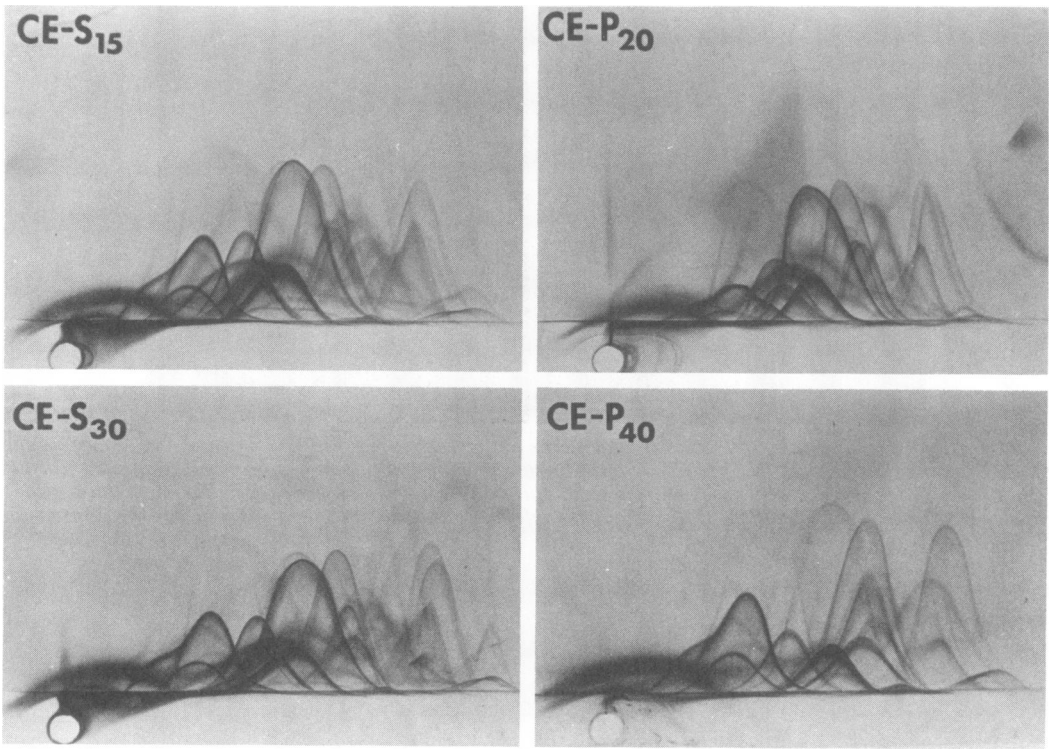

FIG. 8. *Two-dimensional immunoelectrophoretic patterns of mycobacterial extracts prepared from 8-week-old cultures.*

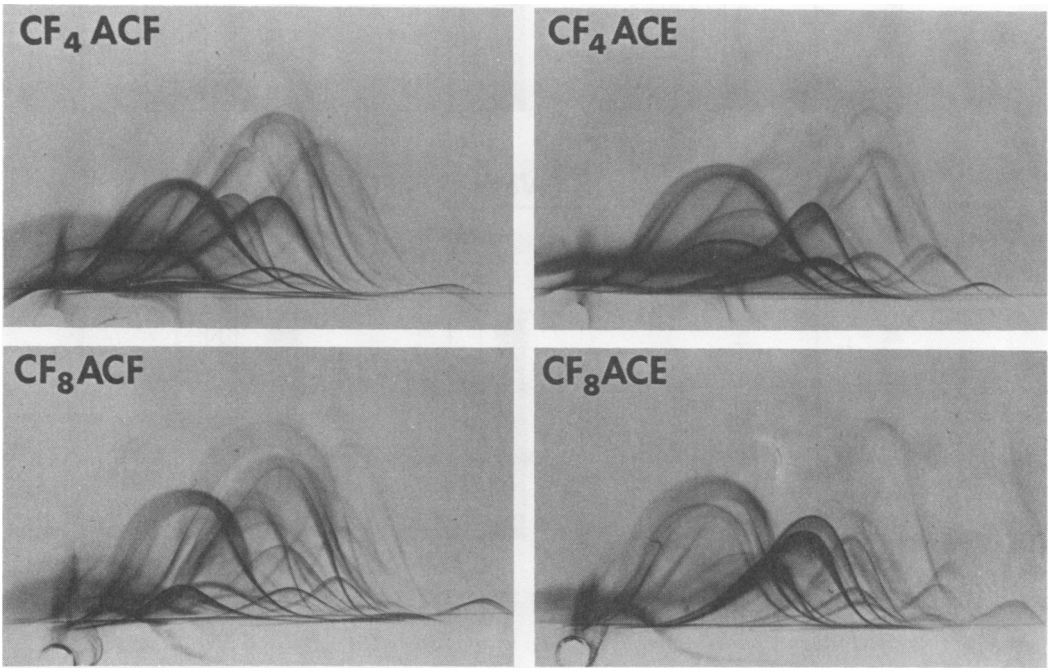

FIG. 9. *Two-dimensional immunoelectrophoretic patterns of mycobacterial culture filtrates. CF_4 and CF_8 represent filtrates from 4- and 8-week-old cultures, respectively. ACF represents lot no. 002 anti-H37Rv culture filtrate reference antiserum, and ACE represents lot no. 002 anti-H37Rv cell extract reference antiserum.*

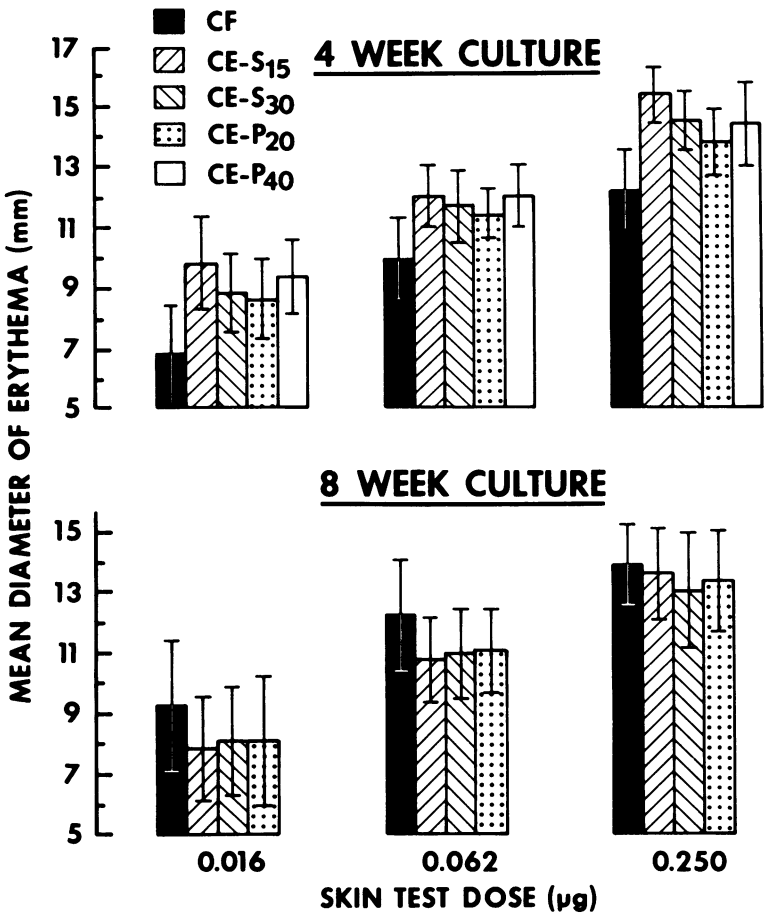

FIG. 10. *Influence of disruption method on skin test reactivity of mycobacterial extracts.*

munoelectrophoretic separation patterns indicated the appearance of additional components in cell extracts prepared from bacilli treated at 40,000 lb/in^2. It is possible that cell walls, although most likely fragmented by the vigorous treatment, remained insoluble and were contained in the particulate layer or were localized in the lipid layer during ultracentrifugation. Similarly, no evidence was found for the appearance or disappearance of antigenic components in extracts of bacilli subjected to prolonged sonic treatment.

Based on the above observations, a bulk supply of cell extract has been prepared from 4-week-old cultures of the H37Rv strain of *M. tuberculosis* by a single 15-min sonic treatment. It is planned that this extract will be made available as a source material to qualified investigators (direct inquiries to: Dr. Paul D. Lambert, Geographic Medicine Branch, National Institute of Allergy and Infectious Diseases, Bethesda, Md. 20014); an IEP reference system has been developed for this material and will be described in a subsequent report.

ACKNOWLEDGMENTS

We are grateful to Edgar Ribi and James Simmons of the Rocky Mountain Laboratory for their valuable assistance in conducting the pressure cell disruptions.

This investigation was supported partly by the United States-Japan Cooperative Medical Science Program through a Public Health Service Contract (NO1 AI 2-2534) administered by the National Institute of Allergy and Infectious Diseases.

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