tures had a third property, inhibition of proteolysis. The activator described here resembles "tissue activator" in that it is not proteolytic in the absence of serum and can be dissolved by KSCN. It also resembles activator from excised rat lung(16) in that it is the size of microsomes. The interpretation that release of activator from cell culture is a metabolic process and not from cell breakdown seems warranted since only a small fraction of the activity found in the supernatant can be extracted from the cells.

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Enzymatic Determination of *in vitro* Lysis of Leukocytes by Tuberculin. (25230)

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In vitro lysis of leukocytes by tuberculin, described by Favour(1), generally is determined by microscopic counts of leukocytes before and after incubation in presence and absence of tuberculin. The sensitivity of this method depends on the accuracy of cell counts. O'Neill and Favour(2) reviewed the factors affecting leukocyte counts and concluded that the mechanical errors inherent in the method can be controlled so that an overall error of less than 10% may be obtained. In practice, however, a cytolysis of less than 10% is not considered significant(3,4). Another limiting factor in the method is the time involved in performing large numbers of cell counts. For these reasons, we have been interested in finding a method for determining leukocyte lysis which is more rapid and more sensitive than microscopic cell counts. Kerby (5) described a method to demonstrate leukocyte injury and lysis, based on the fact that certain cellular components are released into the surrounding medium when leukocytes are damaged. Using release of a lysozyme-like enzyme as a measure of cell damage, Kerby demonstrated a harmful effect of certain bacterial derivatives on human leukocytes(6), and later showed that ACTH and cortisone therapy protected human leukocytes against injury by piromen(7). This paper describes our attempts to use release of this intracellular enzyme as a measure of lysis of leukocytes by tuberculin.

Materials and methods. The technic for assay of lysozyme-like enzyme was similar to that described by Kerby(5). However, it

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was necessary to use M/15 phosphate buffer at pH 7.0 in place of pH 6.2 buffer because the latter caused precipitation in some samples of guinea pig plasma. A 200 mg % suspension of *Micrococcus lysodeikticus* (Difco) in the phosphate buffer was used as substrate for purified egg-white lysozyme (Difco) to prepare a standard curve in the range of enzyme found in plasma of normal and tuberculin-positive guinea pigs(8). One-tenth ml aliquots of various enzyme concentrations were diluted in 0.9 ml of phosphate buffer and 1.5 ml of substrate. Optical density of suspensions was read with an electrophotometer (Fisher) at 525 mμ before and after 20 minutes incubation at 37°C after standardizing the instrument with a buffer blank. The per cent lysis of substrate was calculated as the difference between initial and final optical density divided by initial optical density. Normal male albino guinea pigs (400 g) were sensitized to tuberculin by intramuscular injection of 5 mg of heat-killed *M. tuberculosis* (H37Rv) suspended in 1 ml of sterile paraffin oil. Cytolysis tests were performed on day of bleeding, using heparinized (0.4 mg/ml) blood samples collected by cardiac puncture from fasted animals. Four siliconized serology tubes, each containing 0.8 ml of whole blood, were prepared for each sample. To 2 tubes was added 0.2 ml of pyrogen-free saline (Baxter) containing 10 μg of PPD (Sharp and Dohme); to the 2 remaining tubes was added 0.2 ml of saline. Tubes were sealed with silicone stoppers and rotated at 37°C on roller drum (5 rpm) for 2 hours. After rotation, samples were removed for total leukocyte counts. Both chambers of a standard hemocytometer (AO Spencer) were counted for each sample. The per cent PPD lysis for each sample was calculated as the difference between average cell counts of duplicate saline and PPD tubes divided by average cell count of the saline tubes. After samples were removed for leukocyte counts, all tubes were centrifuged at 1,000 x g for 20 minutes and assays for lysozyme-like enzyme were performed on 0.1 ml aliquots of the supernatant fluid. To each sample was added phosphate buffer and substrate as described above. Per cent lysis of substrate was converted to lysozyme equivalents by reference to standard curve. In addition, the lysozyme Δ was calculated for each sample by subtracting the average per cent lysis of substrate by saline tubes from that obtained with PPD tubes. Positive values for lysozyme Δ were considered indicative of tuberculin cytolysis since the higher values of the PPD tubes were assumed due to increased enzyme levels originating from lysed leukocytes. Preliminary tests showed that PPD in the concentration used had no effect on enzyme or substrate.

Results. The results presented in Table I show release of lysozyme-like enzyme from leukocytes following exposure to tuberculin *in vitro*. Each value represents the mean of duplicate samples from 4 sensitized animals, tested for cytolysis from 7 to 12 days following injection of heat-killed tubercle bacilli. By the leukocyte count method, each group showed significant cytolysis as indicated by the fact that 10% or more of the cells were lysed by PPD. Actual number of cells lysed was calculated as the difference between total number of leukocytes of the saline and PPD tubes and is shown as lysed cell contents/tube. The lysozyme equivalents represent enzyme originating from the lysed leukocytes in excess of that in plasma of surrounding medium. The concentration of enzyme in tubes containing PPD was significantly higher ($P = 0.025$) than that in saline tubes. The content of enzyme in guinea pig peripheral blood leukocytes was calculated from the experimental results.

The application of the lysozyme assay method for detecting leukocyte lysis is further illustrated in Fig. 1 which shows development of leukocytic susceptibility to tuberculin following injection of guinea pigs with heat-killed tubercle bacilli as measured by total leukocyte counts and by the lysozyme assay method. Each point represents the mean of duplicate samples from 4 animals. Statistically significant cytolysis ($P = 0.025$), as reflected by positive values of the lysozyme Δ, occurred at 10 and 12 days. By the leukocyte count method, significant cytolysis also occurred at 10 and 12 days.

Discussion. Our results indicate that release of a lysozyme-like enzyme is an accep-

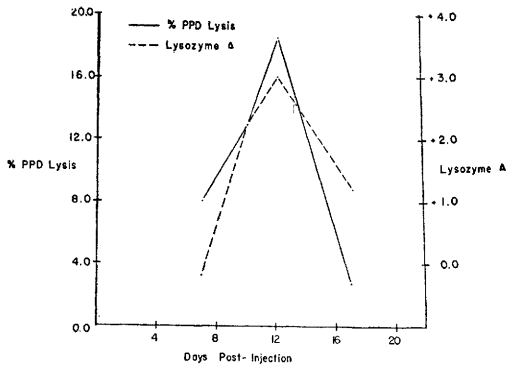

FIG. 1. Leukocyte cytolysis following injection of guinea pigs with heat-killed tubercle bacilli.

table substitute for microscopic cell counts in measuring *in vitro* lysis of leukocytes by tuberculin. It is of interest that, in previous studies with guinea pigs using the leukocyte count method, 10% cytolysis was accepted as significant(3,4). Using the method described here and assuming that the guinea pig has a total leukocyte count of approximately 7,000/mm^3, this degree of cytolysis would indicate lysis of 560,000 leukocytes. These cells would release 0.070 μg of enzyme (Table I) which, by the lysozyme assay method, would produce a lysozyme Δ of approximately + 2.0. This value appears the minimum necessary for significance since a mean lysozyme Δ of + 0.4 with a standard error of the mean of 0.6 was obtained when leukocytes from 16 normal guinea pigs were exposed to PPD under similar conditions. These calculations are supported by the correlation between the cytolysis measured by both methods (Fig. 1). This study and several subsequent experiments have convinced us that the lysozyme assay method is an adequate measure of leukocyte lysis by tuberculin. The relative speed and simplicity of the method adds to its applicability. It seems possible that the method may be useful in other systems involving leukocyte lysis provided the reagents employed are carefully investigated for activity against enzyme and substrate. We observed that markedly hemolysed plasma and serum interfered in the assay method. Similarly, as reported by Kerby and Eadie(9), heparin in concentrations greater than 7 mg/ml was inhibitory in the system. However, this is far in excess of the 0.4 mg heparin used for anticoagulation in the present study.

TABLE I. Leukocyte Cytolysis and Release of a Lysozyme-Like Enzyme.

Group	% PPD lysis	Lysed cell contents/tube	Lysozyme equivalents μg/tube	Lysozyme equivalents μg/10^6 cells
		× 1000		
1	10.0	660	.077	.117
2	14.7	782.56	.104	.133
3	12.9	619	.077	.124
4	18.5	814	.104	.128
Mean				.126

Kerby and Chaudhuri(10) were unsuccessful in demonstrating *in vitro* tuberculin cytolysis of leukocytes from tuberculous humans by a lysozyme assay method. However, this failure does not detract from the applicability of the method since it is possible that the cells from tuberculous subjects were not susceptible to lysis by tuberculin. Their study would have been more valuable if actual tuberculin sensitivity of the cells were ascertained by the leukocyte count method. Favour and his coworkers(11) reported that leukocytes from tuberculin hypersensitive individuals vary markedly in their sensitivity to tuberculin. The demonstration of tuberculin cytolysis of leukocytes from sensitized guinea pigs (Fig. 1) agrees with the results by Waksman(3) and closely parallels the presence of a circulating "plasma factor" responsible for the cytolysis phenomenon as reported previously from our laboratory(4).

Summary. A method based on release of an intracellular, lysozyme-like enzyme from lysed leukocytes was an acceptable substitute for microscopic cell counts in measuring tuberculin cytolysis of leukocytes from guinea pigs injected with heat-killed, human type, *M. tuberculosis*.

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Calcification XXVII. Collagen Transformation under Influence of Calcium and Phosphate Ions.*† (25231)

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Earlier studies have shown that fibers of acid-soluble collagen reconstituted with chondroitin sulfate mineralize *in vitro* with formation of apatite crystals(1-4). Such fibers have the non-striated form(5,6). The collagens occurring in animal tissues generally have a periodicity of 640 Å, although in preosseous cartilage which mineralizes, no periodicity is discernible under electron microscopic examination. Glimcher, *et al.*(7), have reported that, of the various types of reconstituted collagen fibers, only those with 640 Å spacings are able to nucleate hydroxyapatite. The question arose whether non-striated collagen fibers undergo any structural changes during incubation in calcifying solution.

Methods. Solutions of acid soluble collagen in 0.04% acetic acid were prepared from rat tail tendon as previously described(4). Collagen fibers were reconstituted by mixing samples of 5 ml of the collagen solution with 0.25 ml of a 1% solution of whale tracheal chondroitin sulfate‡ in 0.04% acetic acid. Fibers were further reconstituted from the collagen solution by adding a 30% NaCl solution up to a final concentration of 1% NaCl. The precipitates obtained with chondroitin sulfate were washed with distilled water, and those obtained with NaCl were washed with a solution of 1% NaCl. The washed precipitates were employed for further studies.

The basal salt solution used in the experiments was a carbonate buffer containing in mE/L, sodium: 95.0; chloride: 80.0; bicarbonate: 22.0; and potassium: 5.0. This solution was diluted 10 times with doubly-distilled water. If needed, Na_2HPO_4 and/or $CaCl_2$ stock solutions were added to the diluted basal salt solution, resulting in concentrations of 5 mg % P and 15 mg % Ca. The pH of the solutions was adjusted to 7.3 ± 0.05, as described earlier(8). The ionic strength of the solutions employed was about 0.10.

Collagen fibers reconstituted with chondroitin sulfate were incubated in diluted basal salt solution containing calcium or phosphate or both calcium and phosphate (calcifying solution) at pH 7.3 ± 0.05 and 37°C. Time of incubation in the various solutions was 1 and 2 days. Incubation in calcifying solution was also carried out for 6 hours. At the end of incubation the fibers were washed with distilled water until, after adding a few drops of 3% $AgNO_3$ solution, no precipitation of AgCl occurred in the washing fluid. The fibers were then stored in distilled water in the refrigerator for use in electronmicroscope studies. Collagen fibers reconstituted with NaCl were used without further pretreatment.

Samples used for the electronmicroscope studies§ were cut in fine segments with surgi-

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‡ We wish to thank Prof. Erik Jorpes for this preparation of chondroitin sulfate, prepared according to method of Strandberg (*Acta Physiol. Scand.*, 1950, v21, 222) and probably consists of a mixture of chondroitin sulfate A and C.

§ Electronmicroscopy done by Ladd Research Industries, Roslyn Heights, L. I., N. Y.