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A Rapid and Sensitive Assay of Muramidase.*† (30188)

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During our investigation on the purification of muramidases (lysozymes) from human and bovine milk a need was felt for a more rapid and sensitive technique for the enzyme assay. The procedure used by Smolelis and Hartsell(1) and the modifications presented by Jolles(2) and Shahani *et al*(3) were lengthy and showed large day-to-day variations. Litwack's modification(4) which measures the change in per cent transmittance (%T) between 30 and 60 seconds after enzyme addition was found to have insufficient sensitivity for samples below 2 μg of enzyme.

The Commission on Enzymes of the International Union of Biochemistry has recommended that enzyme assays be based, wherever possible, upon measurements of initial rates of reaction(5). Therefore a continuous, sensitive and rapid technique was developed for assaying muramidase activity which allowed measurement of the initial reaction rate. This modification helps to eliminate the greatest disadvantage of earlier assay techniques, *viz.*, end-product inhibition.

Methods and materials. The reagents used in this assay are as follows: (a) a 50 mg% suspension of ultraviolet-killed and lyophilized *Micrococcus lysodeikticus* cells (Difco) in M/15 phosphate buffer, pH 6.2; (b) 0.3 M NaCl; (c) Standard muramidase solutions prepared by dissolving crystalline egg-white lysozyme (Nutritional Biochemicals Corp.) in M/15 phosphate buffer. These materials are then added to a standard 4.2 ml Beckman pyrex cuvette in the following proportions: 1.5 ml of the cell suspension, 0.5 ml of the NaCl solution, and 1.0 ml of the enzyme solution. This makes an effective concentration of 0.05 M NaCl and 25 mg% cells in the enzyme assay mixture. This mixture is momentarily stirred and rate of clearance is measured in a Beckman DB spectrophotometer with an attached Sargent SR recorder. The initial transmittance of the assay mixture is approximately 10% when water is set at 100%T at 540 mμ.

For this method, no particular time interval is necessary for making the measurements. The only requirement is that the rate of clearance of the suspension by muramidase should be linear. The linearity can be easily determined from the recorder chart.

Results and discussion. Fig. 1 presents results taken directly from the recorder at

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4 enzyme concentrations. Clearance due to settling of the cell suspension was found to be negligible, since the transmittance of cells alone (curve marked 0.0 μg) did not alter up to 20 minutes. In this assay method the initial slopes, *i.e.*, change in %T per minute, are taken while the rate is linear, generally within 2 to 4 minutes. However, with samples below 2 μg, longer time periods may be desirable to obtain greater accuracy. Linearity in the rate of lysis at the lower concentrations (curves marked 0.16 and 1.25 μg) is maintained for 20 minutes.

Extensive end-product inhibition is apparent in the assay mixtures containing 15 and 50 μg of muramidase, which must be diluted to a workable range (0.1 to 10 μg). It was found that samples containing higher than 30 μg of muramidase showed a decrease in rate of lysis or deviation from linearity after 30 to 60 seconds. It should also be noted that 15 μg of enzyme gave a higher transmittance reading after 10 minutes than 50 μg, despite the large difference in the initial reaction velocity. Therefore, in the muramidase assay system it appears to be necessary to specify enzyme concentrations and, as in the case of other enzymes, use of initial reaction velocities must be considered.

It was found that addition of salt to the assay system with egg-white muramidase did not stimulate the rate of lysis. However, it did improve the linearity of the reaction rate and also the reproducibility. It was also noted in this laboratory that NaCl is required for bovine milk muramidase activity and is an activator for human milk muramidase. Hence, salt was included in the assay system to allow better versatility when measuring muramidase from different sources.

Fig. 2 is a typical standard curve obtained with this method. In general, 1 μg of egg-white muramidase gave a Δ %T per minute of 1. This was found to vary slightly from day to day due to unknown reasons, if the same cell suspension was used over a period of 4 to 5 days. The variation did not appear to be related to temperature changes since all assays were run at the ambient temperature of an air-cooled room. It was found necessary, therefore, to prepare a standard curve daily. Also, it was observed that the cell suspension needed to be prepared at least

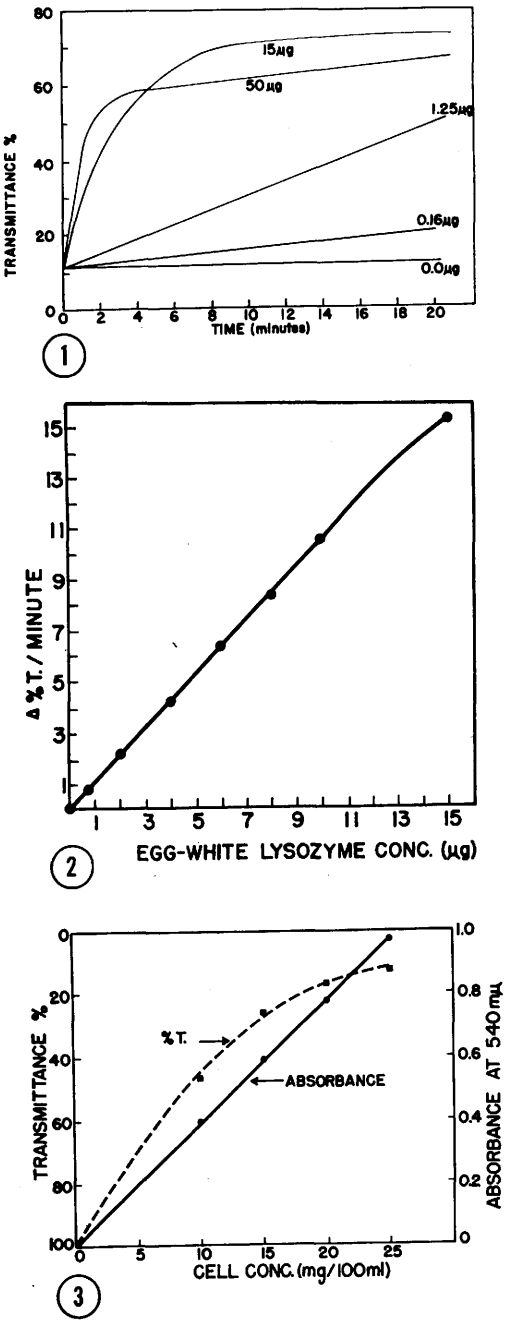

FIG. 1. Rate of cell lysis at various concentrations of egg-white muramidase.

FIG. 2. Standard curve for determination of muramidase activity.

FIG. 3. Per cent transmittance and absorbance at various concentrations of *M. lysodeikticus* cells at 540 mμ.

6 hours prior to use because better reproducibility could be obtained after the cells had been allowed to equilibrate with the buffer for this period. The suspension of *M. lysodeikticus* should be refrigerated (3 to 4°C) when not in use to minimize autolysis of the cells and to prevent microbial growth.

Fig. 3 is a plot of the per cent transmittance and absorbance at 540 mμ of various concentrations (ranging between 10 and 25 mg%) of *M. lysodeikticus* cells containing no muramidase. A straight line Beer's law relationship was observed between absorbance and concentration of cells. During the assay, on the other hand, one obtains a straight line in %T units between 10% and 40%T (Fig. 1). Therefore, if one were only measuring decrease in cell numbers as a result of muramidase activity, the results should resemble Fig. 3, in that there should be a linear relationship between absorbance and muramidase activity, and not between %T and muramidase activity. Consequently, the determination of the dissolution of bacterial cells by muramidase is a poor representation of the true enzymic reaction due to the complex system involved, and therefore its application to the study of muramidase kinetics(7) should be viewed with caution. This limitation cannot be overcome until a method can be devised to measure the appearance of end-products or disappearance of substrate, employing a pure well-defined substrate.

Despite this limitation, turbidimetric muramidase assays do offer a rapid quantitative method which is readily applicable to assay the enzyme from many sources. However, it is important to use enzyme concentrations ranging from 0.1 μg to 10 μg and measurement of the initial reaction velocities. This problem may be resolved by continually recording the rate of clearing in the cell suspension in per cent transmittance units within a defined range of muramidase activity.

This method has been used successfully during our current work on the isolation of human and bovine milk muramidase. The bovine milk contains very low concentrations (13 μg/100 ml) of muramidase(3). This necessitates removal of the casein from the milk by isoelectric precipitation at pH 4.6, adjusting the pH to 6.2 and assaying 1 ml of the clear whey. Human milk muramidase, on the other hand, may be assayed by making a 1:100 or greater dilution since the average content is 39 mg/100 ml(8). The method has also been applied successfully to determination of the muramidase activity in human serum and plasma using 0.5 ml of the sample. In all the above cases analyses can be completed within 3 minutes.

Summary. An improved method has been developed for assay of muramidase in several biological systems. This method involves measurement of the rate of lysis of a 25 mg% suspension of *M. lysodeikticus* in 0.05 M NaCl and M/15 phosphate buffer, pH 6.2. The rate of lysis is measured in a Beckman DB spectrophotometer at 540 mμ with an attached recorder. This modification allows continuous measurement of the initial reaction velocity for samples containing from 0.1 to 10 μg of muramidase.

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