Estimation of the Growth of the T<sub>1</sub> Strain of Mycoplasma mycoides in Tryptose Broth by the Measurement of Lactate Dehydrogenase

I. Method

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A highly significant correlation coefficient \( r = 0.97, n = 18 \) was found between the concentration of lactate dehydrogenase measurable after the organisms had been disrupted and the concentration of colony-forming units during the logarithmic phase of growth of a broth culture of the T<sub>1</sub> strain of Mycoplasma mycoides var. mycoides. A concentration of \( 4.60 \times 10^7 \) milliunits of lactate dehydrogenase for each colony-forming unit was established. This relationship was used to convert the concentration of lactate dehydrogenase in the culture into an estimate of the concentration of viable mycoplasma. The lactate dehydrogenase was estimated by following the oxidation of reduced nicotinamide adenine dinucleotide, in the presence of pyruvate substrate, at 366 nm in a spectrophotometer. The nicotinamide adenine dinucleotide oxidase system probably contributed a small amount of enzyme activity to the test when lactate dehydrogenase was measured in this way. The method has been described and evaluated for the estimation of titers from \( 10^7 \) to \( 5 \times 10^9 \) colony-forming units per ml.

The methods used for following bacterial growth have been successfully applied to the growth of mycoplasma in liquid media. These methods have included dilution techniques in liquid media followed by the determination of the end point of growth after incubation (8, 13), or methods (4, 5, 13, 14, 17, 24) adapted from the colony-counting method of Miles and Misra (16). Cultures for these methods require incubation for a minimum of 72 hr and up to 7 days before assessment of the concentration of organisms in the culture undergoing test can be made.

Various methods of estimating mycoplasma concentration have been developed for the rapid evaluation of mycoplasma cultures and suspensions, including those based on turbidity (6, 13, 24), acid production (7, 18, 20, 24), dry weight (6, 19), total nitrogen (6, 15, 19), deoxyribonucleic acid (6), light scattering (25), and titrated thymidine uptake (22) measurements of the culture.

One of the first attempts to study the growth of Mycoplasma mycoides var. mycoides (M. mycoides) was based on the measurement of the lactate dehydrogenase (LDH) activity of the culture (11), but methods of establishing the viable titer of mycoplasma cultures were not available, at that time, for the correlation of the enzyme activity with the number of viable organisms.

The relationship of the LDH activity of M. mycoides organisms (strain T<sub>1</sub>) to the concentration of colony-forming units (CFU) has been given in a preliminary report (Windsor and Boarer, J. Appl. Bacteriol., in press). The present paper gives details of the method used to determine LDH, after disruption of the mycoplasma, to permit the rapid estimation of the number of viable organisms present in 10% serum Tryptose broth. These LDH results have been correlated with those results obtained by an adaptation of the method of Miles and Misra (16).

MATERIALS AND METHODS

Organism. An egg-adapted organism of the T<sub>1</sub> strain of M. mycoides was used for all cultures. This organism had been attenuated, for vaccine production, by 45 passages through embryonated eggs (23).

Growth media. For the growth of the organisms and as a diluent for the samples used for viability counts, 10% serum-Tryptose broth (10) was used. Tryptose-agar plates (9) were used for the colony-counting method, and the plates were dried for 45 min at 37°C before use.
Preparation of culture for growth curve determinations. An ampoule of freeze-dried T1, which was prepared from the second broth passage of the lyophilized egg material, was used in the preparation of a broth seed as previously described (3).

A 2-ml amount of this seed broth was mixed with 200 ml of sterile serum-Tryptose broth in a sterile 8-oz. (ca. 240 ml) screw-cap medical flat bottle. This usually produced a titer of approximately 3 x 10^6 CFU per ml of broth. The culture was then incubated at 37 C for 3 days in a static condition.

Sampling procedure. Samples were removed, during incubation, at 0, 4, 10, 21, 27, and 34 hr after inoculation, for four separate growth curve studies, to correlate LDH with the concentration of CFU. An 8-ml amount of culture, which was previously mixed by inversion, was removed aseptically and used for estimating viable CFU.

Viable counts. Each sample was diluted, in triplicate, from 10^{-3} to 10^{-6} in serum-Tryptose broth. A Vortex Junior Mixer (Scientific Industries Inc., Springfield, Mass.) was used for 10 sec to mix each stage of dilution. A sterile pipette calibrated to deliver 0.02 ml per drop of serum-Tryptose broth, at a rate of 40 drops/ min at room temperature, was used to transfer each set of dilutions onto five dried serum-Tryptose-agar plates. The plates were left at room temperature for 30 min to enable the drops to dry, and they were then incubated at 37 C for 7 days. The plates were packed in polythene bags to prevent dehydration of the medium during incubation. After incubation, colonies were counted in drop areas with up to 300 colonies per drop, by using a dissecting microscope at X 12.5 magnification. Estimations of the number of CFU/ml were based on the average count of 15 drops, i.e., 5 drops for each set of dilutions.

Disruption of the organisms by ultrasonic waves. Dilutions of the culture sample were made with distilled water. A 20-ml volume of the diluted sample was treated with ultrasonic waves in a sterile universal bottle with an ultrasonic disintegrator (60 w; Measuring & Scientific Equipment Ltd., Manor Royal, Crawley, Sussex) tuned to give a peak output of 1.1 to 1.4 amp (12). The bottle was placed in an ice bath at 0 C during this treatment, and the duration of treatment was varied to assess the time interval of treatment necessary to ensure that each mycoplasma was disrupted.

Disruption of the organisms by freezing and thawing. A 2.5-ml sample of culture, which was diluted (usually 1 : 10) in distilled water, was placed in a 6 by 3/4 inch (ca. 15.2 by 1.6 cm) Pyrex test tube and then tightly stoppered to prevent ingress of acetone. This sample was frozen by placing the tube in an acetone-solid carbon dioxide mixture, at a temperature of less than -60 C for 1 min, followed by thawing in a water bath at 37 C for 1 min. Repetive freezing and thawing was carried out to establish the number of cycles required to ensure that each mycoplasma was disrupted.

LDH estimation. LDH was estimated by measuring the oxidation rate of reduced nicotinamide adenine dinucleotide (NADH) at 366 nm in a Beckman D.U. Spectrophotometer. Tests were read in 1-cm cuvettes at a controlled temperature of 25 ± 0.5 C, and the rate of reaction was measured at 1-min intervals for a total period of 5 min. The method used was essentially that recommended in the manual of the Boehringer Corp. for serum LDH (2). The phosphate buffer-substrate of this method was prepared at three times the concentration of that recommended in the Boehringer method, i.e., 0.15 M potassium phosphate buffer (pH 7.5) containing 9.0 x 10^{-4} M sodium pyruvate. This permitted greater flexibility of the volume of sample which could be added to the cuvette with the reaction mixture. The reaction cuvette contained 1 ml of phosphate buffer-substrate, sample volume, distilled water to make a total volume of 3.1 ml and 0.05 M of 9.0 x 10^{-4} M NADH. The concentration of sodium pyruvate substrate was varied between 2.3 x 10^{-4} and 18 x 10^{-4} M, in the phosphate buffer to determine the optimal concentration of substrate in the reaction mixture.

Two LDH estimations were made on each sample: the level of LDH was estimated in the culture before disruption of the organisms (free LDH) and again after disruption of the organisms (total LDH).

The volumes of culture sample placed in the cuvette for measuring free LDH were 0.5 and 0.2 ml for the incubation time 0 to 45 and > 45 hr, respectively. The volumes of dilute (1:10) culture sample placed in the cuvette for measuring total LDH were 2.0, 0.5, 0.1, and 0.05 ml for culture incubation times of 0, 21, 45, and 69 hr, respectively.

RESULTS

It was found that the LDH in the culture samples originated from three sources. A small quantity was detectable before treatment of the culture. This originated from the pig serum used in the preparation of the medium and also from a small number of mycoplasma, which lysed as the culture passed from the logarithmic phase and entered the stationary phase of growth. The third source of LDH was from the physically disrupted organisms, which resulted from the treatment of the culture with ultrasonic waves or by freezing and thawing. The LDH figure used for quantifying the mycoplasma was derived by subtracting the value for free LDH from that found for total LDH.

The LDH results given in Tables 1 and 2 have had the free LDH subtracted. Mean free LDH figures for these cultures were 25 and 30 milliunits of LDH/ml of culture for incubation times of 0 to 45 and 69 hr, respectively.

The oxidation of NADH was optimal when the sodium pyruvate content of the buffer-substrate reagent was 9 x 10^{-4} M. At this concentration of sodium pyruvate, 75% of the rate of oxidation of NADH was induced by the addition of pyruvate substrate to the reaction cuvette. This was a constant proportion of the total activity at different points of the growth curve. No effort was made to determine the amount of non-LDH activity in the
TABLE 1. Effect of dilution on the efficiency of disrupting Mycoplasma mycoides (T1 strain) organisms and the keeping properties of lactate dehydrogenase activity at 4 C

| Conc of culture | Lactate dehydrogenase (milliunits per ml of culture) |
|-----------------|---------------------------------------------|
|                 | 0 hr | 1 hr | 2 hr | 3 hr | 4 hr | 24 hr |
| 3-Min treatment with ultrasonic waves |     |      |      |      |      |      |
| Undiluted       | 1,223|      |      |      |      |      |
| 1:5             | 1,220|      |      |      |      |      |
| 1:10            | 1,200|      |      |      |      |      |
| 1:20            | 1,036| 1,178| 1,187| 1,169| 1,155| 1,137 |
| Frozen and thawed four times |     |      |      |      |      |      |
| Undiluted       | 275  | 1,160| 1,187| 1,148| 1,213| 1,116 |
| 1:5             | 1,160|      |      |      |      |      |
| 1:10            | 1,213| 1,160| 1,187| 1,148| 1,213| 1,116 |
| 1:20            | 1,129|      |      |      |      |      |

* Time of storage of disrupted culture at 4 C before analysis for lactate dehydrogenase.

TABLE 2. Comparison of the effectiveness of treatment with ultrasonic waves with that of freezing and thawing, on the release of lactate dehydrogenase (LDH) from Mycoplasma mycoides (T1 strain)

| Pretreatment of culture | Cellular LDH (milliunits/ml) | LDH titer (= CFU/ml) | Residual plate viability (CFU/ml) |
|-------------------------|-----------------------------|----------------------|----------------------------------|
| Ultrasonic treatment for |                             |                      |                                  |
| 0 sec                   | 1,873                       | 4.06 \times 10^9     | 4.25 \times 10^9                 |
| 30 sec                  | 2,061                       | 4.47 \times 10^9     | 5.55 \times 10^9                 |
| 60 sec                  | 2,209                       | 4.79 \times 10^9     | 2.42 \times 10^9                 |
| 90 sec                  | 2,188                       | 4.75 \times 10^9     | 1.74 \times 10^9                 |
| 120 sec                 | 2,150                       | 4.67 \times 10^9     | 1.17 \times 10^9                 |
| 150 sec                 | 2,209                       | 4.79 \times 10^9     | 5.20 \times 10^9                 |
| 180 sec                 |                             |                      |                                  |
| Frozen and thawed       |                             |                      |                                  |
| 0 times                 | 1,972                       | 4.28 \times 10^9     | 4.25 \times 10^9                 |
| 1 time                  | 2,241                       | 4.86 \times 10^9     | 1.95 \times 10^9                 |
| 2 times                 | 2,397                       | 5.20 \times 10^9     | 4.42 \times 10^7                 |
| 3 times                 | 2,604                       | 5.65 \times 10^9     | 1.06 \times 10^7                 |
| 4 times                 | 2,378                       | 5.16 \times 10^9     | 2.85 \times 10^6                 |
| 5 times                 |                             |                      | 5.39 \times 10^5                 |
| 6 times                 |                             |                      |                                  |

* Culture diluted 1:10 with distilled water.

test system. For the purpose of this paper, total oxidation of NADH has been recorded as caused by LDH activity in milliunits per ml at 25 C.

Four growth curves were studied in four different preparations of serum-Tryptose broth. The LDH/ml values obtained showed a highly significant correlation coefficient \( r = 0.97, n = 18 \) with the number of CFU/ml found. The regression line calculated by the method of least squares was \( \log_{10} y = 0.95 \log_{10} x - 5.975 \), with a residual standard deviation of 0.205 \( (y = \text{LDH}, x = \text{CFU}) \). A value of \( 4.60 \times 10^{-7} \) milliunits of LDH per organism was found at the mean point of \( x \) and \( y \). This mean value has been used throughout the paper for relating LDH to CFU to provide an equivalent titer (LDH titer).

The results in Table 1 show the efficiency of releasing LDH, at various dilutions of the culture, by the two methods used to disrupt the mycoplasma. The method of freezing and thawing was preferred because a larger number of samples could be handled at the same time. This method of disruption was not always complete at a dilution of 1:5 and was routinely performed at a dilution of 1:10. Results showed that LDH was completely released from the organisms when undiluted culture was subjected to ultrasonic treatment, whereas freezing and thawing released only...
25% of the LDH from the mycoplasma in the undiluted culture sample. The LDH activity of the prepared culture was constant for 4 hr when stored at 4°C.

Table 2 shows the increasing effect of treatment by the two methods of disrupting the mycoplasma when the culture samples were diluted 1:10. Cells treated with ultrasonic waves released LDH, which reached a maximum concentration after 90 sec. Freezing and thawing produced a similar maximum concentration after three cycles. The numbers of CFU/ml fell concurrently with the release of LDH during freezing and thawing, but the loss of viability after treatment with ultrasonic waves lagged behind the release of LDH from the mycoplasma. Thus, when preparing the samples for the estimation of total LDH, portions of the culture, which were diluted 1:10, were routinely treated with ultrasonic waves for 3 min or recycled four times through the freezing and thawing procedure to disrupt the organisms. This ensured that 99% of the organisms were disrupted in the treated sample.

Figure 1 shows an individual growth curve constructed from the CFU and LDH data.
DISCUSSION

LDH data have been compared with plate titers because the ability of mycoplasma to form colonies has been shown to provide an accurate basis for estimating the titer of broth cultures of these organisms (1, 5, 6, 24). Any alternative method, which employs principles other than actual growth for evaluating the concentration of mycoplasma in liquid media, must ideally be sensitive, rapid, and accurate. The results obtained should correlate well with the numbers of CFU found at all stages of the growth curve. Such alternative methods are valuable for growth curve studies, assessing volumes of seed for inocula and for quality control procedures for vaccine production.

The estimation of titers assessed by dry weight, total nitrogen, and deoxyribonucleic acid concentrations are only useful near to the maximum concentration of organisms because of the small size of the mycoplasma (6). An increase in the sensitivity of these methods cannot be achieved by centrifugation of the culture because, even at high speeds, it has been shown that there is a loss of organisms in the supernatant fluid and this will introduce inaccuracies (24). An estimation of the titer made directly on the culture will reduce this type of inaccuracy. Measurements of the turbidity of mycoplasma cultures are not sensitive (24, 25) and maximal changes of the absorption do not always correspond to the peak growth of the mycoplasma (13). The uptake of tritiated thymidine by mycoplasma cultures has disadvantages for application in some laboratories because it is necessary to contain radioactivity within designated areas. Consideration of the alternative methods, above, which are used for evaluating mycoplasma growth, show that they all have one or more limitations for application in the laboratory. A frequent limitation is that they are not sufficiently sensitive.

The measurement of the light scattering properties of mycoplasma cultures has been claimed to have the best sensitivity for detecting changes of growth in liquid medium (25). This method detects upwards of $10^5$ CFU/ml in medium without serum. However, this threshold was reduced to $10^5$ CFU/ml after the addition of 10% serum to the medium. The authors intimated that the lower titers are only measurable on the more expensive range of spectrophotofluorimeters. The expense of apparatus which is sufficiently sensitive limits the application of the method, if such an instrument is not already available.

The highly significant correlation between LDH and CFU, during the logarithmic phase of growth of *M. mycoides*, indicated that LDH estimations of the cultures could be interpreted to give an assessment of growth. The estimation of LDH has, therefore, been used to quantify the growth of *M. mycoides* for the production of T1 vaccine. For this purpose, it is necessary to cover a range of titer from $10^4$ to $5 \times 10^6$ CFU/ml, and the method described here is suitable for this range. The sensitivity of the method can be extended to cover the range from $10^4$ to $10^7$ CFU/ml by measuring the optical density changes at 340 nm, maintaining the reaction temperature at 37 C, and performing the test on undiluted culture which has been treated with ultrasonic waves. The amount of LDH in the broth from the pig serum is a limiting factor when considering accuracy at these lower titer levels.

The relationship between the concentration of LDH and CFU has been described in a preliminary report (Windsor and Boarer, J. Appl. Bacteriol., *in press*). This was assessed for LDH values and CFU values extending beyond the logarithmic growth phase (0 to 69 hr). In the cultures investigated, the logarithmic growth phase was short (about 25 hr); therefore, the relationship of LDH to CFU was reestablished for the period 0 to 34 hr, i.e., before the growth of the culture deviated too greatly from the logarithmic phase. As growth of the culture passed from the logarithmic phase and entered the stationary phase, the LDH method gave a greater estimate of the concentration of mycoplasma, as compared with the CFU data (Fig. 1). This suggested the existence of intact but nonviable organisms at this late stage of growth, or friable whole organisms which were damaged during the plating procedure and subsequently lost their ability to form colonies. Alternatively, each mycoplasma could have increased its LDH content as it entered the stationary phase of the growth curve.

NADH oxidase activity is present in organisms of the V5 strain of *M. mycoides* (21), and it is probable that a small proportion of the LDH activity, recorded by this method, was contributed by NADH oxidase.

The measurement of mycoplasma growth by estimating LDH has a similar sensitivity to the method, which uses the light scattering properties of the culture for making such measurements; it is, therefore, a very useful addition to methods which have already been developed for assessing mycoplasma growth. The application of colorimetric methods for determining LDH for the further simplification of growth measurement of *M. mycoides* in broth is under investigation.

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