FACTORS AFFECTING TERRAMYCIN ACTIVITY
IN MILK, BROTH, BUFFER, AND WATER

K. M. SHAHANI

Department of Dairy Technology and Bacteriology,
The Ohio State University, Columbus

SUMMARY

Studies were conducted to determine the effect of heat, storage, total solids content, bacterial metabolism, and various gases upon the degree of terramycin inactivation in milk, broth, buffer, and water. Terramycin activity was determined by a modified disk assay technique and the acid inhibition method. The rate of heat inactivation of the antibiotic at 143 and 160°F. for 50 min. followed the first-order reaction in milk and buffer but not in water. When autoclaved at 15 lb., the antibiotic was inactivated completely within 5 min. in all three media. The Z values for complete thermal inactivation curves for terramycin, aureomycin, streptomycin, and penicillin in milk were 48, 51.5, 36, and 49, respectively. Terramycin was more heat-susceptible than the three other antibiotics. Heat inactivation of terramycin in reconstituted milk of different solids concentrations was inversely related to the solids content. During storage terramycin lost its potency at a faster rate in the unheated than in the heated system. This may have been owing to natural microorganisms and their enzymes in the unheated media, because it was observed that the loss of terramycin potency in a broth inoculated with a raw milk bacterial suspension was directly related to bacterial multiplication. When sterile milk containing terramycin was exposed constantly to nitrogen, oxygen, or air, the antibiotic lost its potency, the greatest loss occurring with oxygen.

Terramycin (oxytetracycline), an analogue of the tetracycline antibiotics, has been shown to possess a marked effect against various microorganisms, including gram-positive, gram-negative, aerobic, and anaerobic bacteria and many viruses (5, 6). When used as a therapeutic agent, it has been shown also to possess a low degree of toxicity (4). It is, therefore, used singly or in combination with other antibiotics, for the treatment of various diseases. Cromley and Hagely (3) and Simon and Schmidt (15) have reported terramycin to be therapeutically effective against staphylococcal and streptococcal mastitis. Schipper and Petersen (10) have shown that following intravenous and intramammary administration terramycin appears in milk, blood, and urine.

Regna and Solomons (9) have reported that terramycin compares favorably with the more stable antibiotics now in common use. Previous antibiotic studies conducted at this laboratory have shown that plant practices like heat treatment and storage inactivate penicillin (13), streptomycin (12), and aureomycin (11). Information relative to the effect of heat, storage, milk solids, bacterial metabolism, and various gases upon the degree of terramycin inactivation has been obtained and is presented.

Received for publication August 5, 1957.

1 Article No. 9:57 of the Department of Dairy Technology, The Ohio State University. Supported by a grant from the U. S. Public Health Service (National Institutes of Health) and by The Ohio Dairy Products Research Fund.

2 Present address: Department of Dairy Husbandry, University of Nebraska, Lincoln.
FACTORS AFFECTING TERRAMYCIN ACTIVITY

METHODS

Throughout this study, the terramycin activity was determined by the disk assay method and the acid inhibition method. In the disk assay method, an enriched agar was used as the assay medium and *Bacillus cereus* as the test organism. The enriched agar consisted of the following gram-portions per 1,000 ml: yeast extract, 5, lactose, 5, dipotassium phosphate, 5, neopeptone, 2.5, peptonized milk, 2.5, and agar, 15. The medium was adjusted to pH 6.8 before sterilization. The principle, the details of the disk assay method, and the technique of heating and storage have been described earlier (11, 13). In all the phases of this study the antibiotic was added to milk, buffer, water, or broth in concentrations ranging 0.3 and 3.2 γ per milliliter.

RESULTS AND DISCUSSION

Effect of various heat treatments on terramycin. The initial phase of this study was to determine the effect of various heat treatments upon terramycin in milk, 0.1 M potassium phosphate buffer (pH 4.7), and distilled water. Terramycin was added to the milk, phosphate buffer, and water at the rate of 0.84 to 1.0 γ per milliliter and heated at 143 and 160 °F. The rates of inactivation of the antibiotic were determined following 10, 20, 30, 40, and 50 min. of heating. The data obtained are recorded (Figure 1). The following differential equation proposed by Prutton and Maron (8) was used in order to determine whether or not the rate of terramycin heat inactivation against time was of the first order:

\[
\log (a-x) = \frac{-k_t}{2.303} t + \log a
\]

Where \(a\) = initial concentration, \(x\) = loss, \(t\) = time, and \(k\) is a constant.

In Figure 1, the logarithm of the per cent of residual terramycin has been plotted on the ordinate and the time in minutes on the abscissa. The loss of the antibiotic was found to be related directly to the heat treatment. The rate of terramycin inactivation owing to heat was faster in water than in buffer or milk. In general, these results are in close agreement with those of Bohonos et al. (2), who have reported that during heating, aureomycin, terramycin, and tetracycline are less labile at a lower pH than under neutral or alkaline conditions.

It was observed that the rate of heat inactivation of terramycin at 143 and 160 °F. for 50 min. in milk and buffer followed the first-order reaction, because a straight-line curve was obtained when the logarithm of the concentration of the residual antibiotic was plotted against time. In water, the rate of inactivation followed the first-order reaction at 143 °F. When heated at 160 °F., the inactivation rate of terramycin followed the first-order reaction up to the first 30 min. of heating only, following which the inactivation rate was progressively faster and resulted in a complete loss of the antibiotic activity in 50 min.

Studies were also conducted to determine the rate of terramycin inactivation when heated at 15 lb. (approximately 250 °F.) in an autoclave. An empty autoclave was heated with steam to remove air, quickly opened, the samples inserted,
and pressure increased quickly. As soon as the pressure reached 15 lb., the time was recorded. At the end of the desired period the pressure was reduced rapidly and the samples were withdrawn and cooled in an ice-bath. Milk, buffer, and water samples, inoculated with known amounts of terramycin, were subjected to the above treatment for 5, 10, and 15 min. and assayed for the antibiotic activity. Separate lots of the test samples were used for each time interval. It was observed that under the conditions of the above experimental procedure the antibiotic lost its potency completely within 5 min. in all three media.

**Complete heat inactivation of terramycin in milk.** The next phase of this study was the determination of the time-temperature relationships necessary for the complete inactivation of small concentrations of terramycin in milk. Fresh, raw, mixed-herd milk was obtained from the University dairy, and terramycin was added at the rate of 0.5 to 0.55 γ per milliliter and heated at various temperatures until the antibiotic potency was lost completely. The assay procedure included the disk assay method and also the starter activity method. As used in the previous studies (11), the nondevelopment of a zone of inhibition by the first

![Graphs showing thermal inactivation of terramycin in milk, buffer, and water at 143 and 160°F.](image)
method and no retardation in the acid development in the latter method were taken as criteria for the complete inactivation of the antibiotic. Average results of from three to six trials conducted at each temperature have been presented (Figure 2). The temperature of heating is plotted on the abscissa and the logarithm of time required for complete inactivation is plotted on the ordinate. To compare the heat stability of terramycin with that for other antibiotics, the thermal inactivation curves for penicillin, streptomycin, and aureomycin reported previously also have been included in the same figure.

Fig. 2. Complete thermal inactivation of various antibiotics in milk. P=Penicillin, S=Streptomycin, A=Aureomycin, T=Terramycin.

Terramycin was inactivated completely in milk when heated at 160°F. for 190 min., at 175°F. for 92 min., or at 185°F. for 60 min. A linear relationship was observed between the logarithm of time and the temperature, and by using the formula proposed by Ball (1) the Z value for the thermal inactivation curve for terramycin was calculated to be 48.

The data revealed that terramycin was more susceptible to heat than were the three other antibiotics. It was observed that when heating at 160°F., 190 min. were required to inactivate terramycin, compared to 280 for aureomycin, 1,320 for streptomycin, and 1,705 for penicillin. Compared to the values of 51.5, 36,
and 49 for the thermal inactivation curves for aureomycin, streptomycin, and penicillin, respectively, the Z value for terramycin was 48.

**Effect of milk solids upon heat inactivation of terramycin.** To determine whether or not the solids content of milk was the factor responsible for the antibiotics being more heat-labile in water and buffer than in milk, a study was conducted to determine the rate of heat inactivation of terramycin in milk of various solids concentrations. Using skim milk powder, reconstituted milk was prepared containing 10 and 25% total solids. Terramycin was added to the milk at the rate of 0.71 and 1.06 γ per milliliter, and loss of antibiotic potency was determined following heating at 160 and 185° F. for 30 min. The data obtained are presented (Table 1). Upon heating at 160° F. for 30 min., 26% of the anti-

<table>
<thead>
<tr>
<th>Antibiotic inoculated (γ/ml)</th>
<th>10% T.S. Milk</th>
<th>25% T.S. Milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ant.</td>
<td>Loss</td>
<td>Ant.</td>
</tr>
<tr>
<td>0.71</td>
<td>58</td>
<td>18.3</td>
</tr>
<tr>
<td>1.06</td>
<td>72</td>
<td>32.1</td>
</tr>
<tr>
<td>Average loss</td>
<td>26.0</td>
<td>63.1</td>
</tr>
</tbody>
</table>

biotic was lost in milk with 10% solids, compared to a 16% antibiotic loss in milk containing 25% solids. A similar trend was obtained when the samples were heated at 185° F. In reconstituted milk with 10% total solids there appeared to be a direct relationship between the terramycin content of the samples and the extent of heat inactivation of the antibiotic. However, this was not true in the 25% total solids milk.

Attempts were made to determine the loss of antibiotic activity in 40% solids milk, also. However, the samples could not be assayed accurately, because the milk became too viscous upon heat treatment and could not be pipetted. Data revealed that heat inactivation of terramycin in reconstituted milk of different solids concentrations was inversely related to the solids content, indicating that milk solids perhaps exert a protective effect upon the antibiotic during heating.

**Effect of storage upon terramycin in raw and heated samples.** To determine the effect of storage upon terramycin in raw and heated milk, phosphate buffer, and water, the samples were inoculated with the antibiotic at the rate of 0.32 to 3.22 γ per milliliter. Using the same experimental procedure described in previous reports (10, 11), the milk, buffer, and water samples were heated at 143 and 160° F. for 30 min. The raw and processed samples were then stored at from 2 to 4° C. and assayed every week for 5 wk. The per cent losses of terra-
FACTORS AFFECTING TERRAMYCIN ACTIVITY

mycin in the three media upon heating and storage are presented (Table 2). These data represent average results of from two to six trials.

Similarly to what was observed in a previous experiment (Figure 1), the heat inactivation of the antibiotic was found to be related directly to the extent of heat applied, and the rate of loss of terramycin in milk, buffer, and water was in an increasing order. There were considerable variations between the results obtained in various trials. As observed in previous studies (11–13), there was no apparent relationship between the concentration of the antibiotic and the percentage loss of terramycin upon heating.

<table>
<thead>
<tr>
<th>Heat treatment</th>
<th>No. of trials</th>
<th>Terramycin inoculated</th>
<th>Loss upon heating</th>
<th>Cumulative loss upon storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(γ/ml)</td>
<td></td>
<td>1 wk. 2 wk. 3 wk. 4 wk. 5 wk.</td>
</tr>
<tr>
<td>Milk</td>
<td></td>
<td>143° F. for 30 min.</td>
<td>Milk</td>
<td></td>
</tr>
<tr>
<td>Raw</td>
<td>6</td>
<td>0.32-1.29</td>
<td>\ldots</td>
<td>13.9 21.8 28.2 33.0 41.9</td>
</tr>
<tr>
<td>143° F. for 30 min.</td>
<td>3</td>
<td>0.32-0.90</td>
<td>22.6 5.8 8.6 10.8 12.5 20.2</td>
<td></td>
</tr>
<tr>
<td>160° F. for 30 min.</td>
<td>3</td>
<td>0.49-1.29</td>
<td>35.6 1.5 6.4 \ldots</td>
<td>12.8 16.3</td>
</tr>
<tr>
<td>Buffer</td>
<td></td>
<td>143° F. for 30 min.</td>
<td>Buffer</td>
<td></td>
</tr>
<tr>
<td>Unheated</td>
<td>6</td>
<td>0.43-3.22</td>
<td>\ldots</td>
<td>18.0 21.4 25.8 28.9 33.9</td>
</tr>
<tr>
<td>143° F. for 30 min.</td>
<td>3</td>
<td>1.00-3.22</td>
<td>32.4 12.3 14.1 16.9 17.4 24.7</td>
<td></td>
</tr>
<tr>
<td>160° F. for 30 min.</td>
<td>2</td>
<td>0.43-1.28</td>
<td>48.4 \ldots</td>
<td>15.2 21.2 22.6</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td>143° F. for 30 min.</td>
<td>Water</td>
<td></td>
</tr>
<tr>
<td>Unheated</td>
<td>6</td>
<td>0.43-3.22</td>
<td>\ldots</td>
<td>19.3 32.1 34.4 39.0 48.9</td>
</tr>
<tr>
<td>143° F. for 30 min.</td>
<td>3</td>
<td>1.00-3.22</td>
<td>41.4 4.7 10.6 12.7 19.0 25.1</td>
<td></td>
</tr>
<tr>
<td>160° F. for 30 min.</td>
<td>3</td>
<td>0.43-1.28</td>
<td>66.4 \ldots</td>
<td>26.7 27.8</td>
</tr>
</tbody>
</table>

It was observed that during storage terramycin lost its potency at a faster rate in the unheated samples than in the heated ones. Similar results were obtained with penicillin, streptomycin, and aureomycin in previous studies. In the milk group, during storage for 5 wk., an average of 41.9% of terramycin was lost in the raw samples, compared to 20.2% in the samples that had been heated at 143° F. and to 16.3% in the samples heated at 160° F. Essentially similar trends were observed in the buffer and water samples. Under the conditions of this study there was no relationship between the terramycin content and the loss of its activity during storage. The fact that the antibiotic activity decreased during storage is in contrast to the reports of Regna and Solomons (9), who observed that an aqueous solution of terramycin hydrochloride at pH 3.0–9.0 did not show detectable loss in activity during storage at 5° C. for one month.

In another study relative to the preservation of milk with antibiotics (14), it was observed that during storage raw milk samples inoculated with terramycin, aureomycin, streptomycin, or penicillin developed putrefaction, proteolysis, and microbial deterioration in from ten to 14 days. In milk inoculated with terramycin or aureomycin and then pasteurized, no microbial spoilage was observed for from 3 to 4 wk. Although in the present study no attempts were made to determine bacterial numbers in the raw and heated milk, on the basis of the
study just mentioned (14) it may be hypothesized that during storage there was greater bacterial metabolism in raw milk than in heated milk.

Effect of bacterial metabolism on terramycin activity. Because the inactivation of terramycin during storage was faster in raw milk than in heated milk, studies were made to determine the effect of the microorganisms present in raw milk upon the antibiotic activity.

Raw milk was inoculated with terramycin and held at three temperature ranges—at 2–4, 22–26, and 35°C. Terramycin activity was determined following one, two, and three days of storage. The samples, held at from 2 to 4°C, did not show any appreciable loss of antibiotic activity. The loss of antibiotic activity could not be determined in the samples held at the higher temperatures, because the samples could not be assayed, owing to coagulation of milk. Attempts were made, therefore, to determine the effect of the microorganisms present in raw milk upon the antibiotic activity.

A raw milk bacterial suspension was prepared by inoculating raw milk in the enriched broth (composition the same as used in the enriched agar, except for agar) and incubating it at 30°C, for from 14 to 16 hr. The broth culture was transferred daily in fresh broth and was used in these trials after it had been transferred from six to seven times. Two 100-ml lots of enriched broth containing 0.60–0.62 γ of terramycin per milliliter were inoculated with the broth suspension of raw milk bacteria at the rate of 1 and 5%, respectively. Each lot was divided into two parts. One part of each lot was incubated at 35°C and the other part was held at 2°C in a refrigerator. The antibiotic content and total bacterial counts were determined immediately and following one, two, and three days of incubation. The samples were stored for a maximum of three days, in contrast to 5 wk. in the immediately preceding phase of the work (Table 2), because the preliminary trials revealed that the antibiotic loss was considerably faster in the broth inoculated with a bacterial suspension than in the unheated milk, buffer, or water. Also, because the medium composition and incubation temperature influence the type and numbers of bacteria in a medium, the present phase of work was conducted not to duplicate but to investigate what might happen in raw milk.

Average results of from two to three trials are presented (Figure 3). It was observed that during holding at both the temperatures there was an increase in the bacterial count and a concomitant decrease in the antibiotic activity. As should be expected, there was greater bacterial multiplication at 35 than at 2°C. The rate and amount of the antibiotic loss were greater in the samples inoculated with 5% suspension or incubated at 35°C than in the samples inoculated with 1% suspension or incubated at 2°C. The results clearly indicate that the loss of the antibiotic potency was directly related to the extent of bacterial metabolism. The present results are in close agreement with observations made in a previous study (12), in which it was observed that in 2 wk. there occurred an appreciable loss of streptomycin activity in milk stored raw, whereas no apparent loss of the antibiotic occurred in sterilized milk. These results are not in complete agreement with the findings of Jurtshuk et al. (7). They observed that zones
Fig. 3. Per cent loss of terramycin and rate of bacterial multiplication during incubation of broth inoculated with raw milk bacterial suspension. Inoculum: A=5%, B=1%, C=5%, D=1%, and incubation temperatures, 35, 35, 2, and 2°C, respectively.

Effect of different gases upon terramycin activity in milk. In the search for causes and mechanisms involved in the inactivation of antibiotics, a study was conducted to determine the effect of various gases in air upon terramycin activity in milk. Twenty-five milliliter aliquots of fresh sterile milk were inoculated with from 0.96 to 1.1 γ terramycin per milliliter and dispensed in 25 × 200 mm pyrex test-tubes with a side opening near the top of the tube. The tube was fitted with a rubber stopper with a narrow glass tubing running from the top to the bottom of the tube. Through the center glass tube, the milk was subjected to constant exposure to air, nitrogen, or oxygen by connecting the glass tubing to the
gas supply and constantly bubbling the gas through the milk system. The flow of the gas was controlled so as to produce about 50 to 60 bubbles per minute. All of these trials were conducted at room temperature (22 to 27°C). An extra tube not containing the center tube and the side opening, and through which no gas was bubbled, served as a control. At the end of 4, 24, 36, and 48 hr. of exposure, aliquots of milk were withdrawn and assayed for antibiotic activity.

Average results of three trials are presented (Figure 4), which show the per cent loss of the antibiotic in milk upon constant exposure to various gases.

![Figure 4. Effect of different gases upon terramycin in milk. O---Oxygen, A---Air, N---Nitrogen, C---Control.](image)

It was observed that the control samples not exposed to any gas lost about 13% of their antibiotic content in 48 hr. When milk containing terramycin was exposed to nitrogen, oxygen, or air, the samples lost part of their antibiotic activity and the loss was directly related to the time of exposure. Over a long period of exposure, oxygen was found to be more detrimental than air or nitrogen. At the end of 24 hr. of exposure to nitrogen, air, or oxygen, there resulted a loss of 8.4, 17.7, and 12.5%, respectively. Upon additional exposure of 24 hr., or at the end of a total exposure of 48 hr., 23.3% terramycin was lost in milk when exposed to nitrogen, 28.9% when exposed to air, and 64.5% when exposed to oxygen. The greater inactivation of the antibiotic when exposed to oxygen might be owing to oxidation of the antibiotic. The control milk samples in rubber-stoppered tubes, through which no gas was bubbled, also lost about 13% of their antibiotic content. These results suggest that the loss of antibiotic activity during storage might have been owing partly to the air present in the empty part of the tube. These observations may have significance to the dairy industry, because milk is often transported in cans or bulk tanks with air space.
FACTORS AFFECTING TERRAMYCIN ACTIVITY

CONCLUSIONS

Terramycin was found to be more heat-labile than penicillin, streptomycin, and aureomycin. The rate of the antibiotic loss was directly related to the extent of heat applied, and its heat-lability in milk, buffer, and water was in an increasing order. Heat inactivation of the antibiotic in reconstituted milk was inversely related to its total solids content. Storage loss of terramycin in milk might be owing partly to the presence of naturally present microorganisms and their enzymes in milk. Also, it is believed that the inactivation of the antibiotic during storage might be owing in part to oxidation.

ACKNOWLEDGMENTS

Sincere thanks are extended to Drs. I. A. Gould, H. H. Weiser, and W. L. Slatter for their interest and counsel in this study. Also, acknowledgment is made to Dr. R. C. Ottke, Chas. Pfizer and Co., Inc., for the generous supply of terramycin used in this study, and to Dr. H. L. Schoenlein of Difco Laboratories for supplying the B. cereus spore suspension used in part of this study.

REFERENCES