A Preliminary Study of the Formation, Assay and Stability of Tyrothricin

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SUMMARY: Strain selection and the addition of yeast extract to the medium have enabled high yields of tyrothricin to be produced from the culture fluids of Bacillus brevis. Contrary to the accepted view, this organism does ferment carbohydrates with the production of acid, and a description of the characters of the organism is given. A method of assaying tyrothricin has been developed and used to investigate the stability of aqueous solutions of the material. Tyrothricin produced in aerated submerged culture appears to be more stable in solution than that produced by surface culture.

The occurrence of the antibacterial agent tyrothricin in the culture fluids of an aerobic spore-forming bacillus was first recorded by Dubos (1939a, b, c). A series of further publications (Dubos, 1940, 1941; Dubos & Cattaneo, 1939; Dubos & Hotchkiss, 1941, 1942; Dubos, Hotchkiss & Coburn, 1942; Hotchkiss, 1941; Hotchkiss & Dubos, 1940a, b, c, 1941) showed that the material was of value as a chemotherapeutic agent and consisted of two clearly defined components—gramicidin and tyrocidine—both polypeptide in nature. These authors discussed the production of the antibiotic in surface culture, described its isolation and purification, and identified as Bacillus brevis the organism responsible for tyrothricin formation.

Generally, the bacillus was grown on shallow layers of culture media containing casein hydrolysate, peptone or Tryptone; yields of tyrothricin up to 0.5 g./l. culture fluid were obtained. Optimum yields were given by a strain (B.G.) grown on Tryptone media. A related product, gramicidin S, was reported by Gause & Brazhnikova (1944) as being produced in surface culture by another organism of the B. brevis type. Numerous other investigations dealing with the chemistry and clinical applications of these materials were reviewed by Hotchkiss (1944). Of these later publications two only were concerned with the conditions of formation of tyrothricin by B. brevis. Lewis, Dimick & Feustel (1945) studied the formation of tyrothricin in surface culture, particularly in media based on vegetable waste, while Stokes & Woodward (1943) reported on the production of tyrothricin under conditions of aerated submerged culture. From these and the preceding papers it is clear that under conditions of surface culture B. brevis grows well and produces tyrothricin readily in media containing complex nitrogenous nutrients of the peptone, Tryptone and vegetable waste type. The substitution of simple nitrogen sources such as inorganic ammonium salts resulted in sparse growth and poor yields of tyrothricin.

In aerated submerged culture the organism grew well in the presence of complex nitrogen sources but normally failed to produce tyrothricin. Stokes
& Woodward (1943) succeeded, however, in developing a synthetic medium containing glucose, inorganic salts, and an amino-acid as a nitrogen source, which not only supported growth of *B. brevis* but permitted production of tyrothricin in aerated submerged culture. Certain amino-acids were unsuitable for this purpose, and mixtures of amino-acids inhibited tyrothricin formation although they permitted it in surface culture. In general, with suitable single amino-acids, yields of tyrothricin of the order of 0·1–0·3 g./l. culture medium were obtained at an aeration rate of 1·5 l. air/l. medium/min. It was clear from these results that the metabolism of the organism could follow different paths in surface and in aerated submerged culture.

In view of the clinical importance of tyrothricin and the availability of appropriate equipment it was decided to investigate further the possibility of producing the antibiotic by the submerged culture technique. The results obtained are dealt with in the succeeding communication (Appleby, Knowles, McAllister, Pearson & White, 1947). Initially, using the Dubos B.G. strain, a brief survey was made of the production of tyrothricin in surface culture and, as an aid to further work, a rapid method of assay was developed. This method was used to investigate the stability of tyrothricin solutions as prepared for clinical use. The present communication describes the results of this work.

**EXPERIMENTAL**

**Characters of the organism**

The organism used in this work, the Dubos B.G. strain of *B. brevis* brought back from the U.S.A. by one of us (T. W.), does not agree in character with the incomplete description of *B. brevis* recorded by Bergey et al. (1939), nor with the accounts of this species recorded in previous literature. The most notable discrepancy is in carbohydrate fermentation, as, although *B. brevis* is generally considered to be inert in this respect, we have found that our strain, and strains from the National Collection of Type Cultures, are capable of fermenting a wide range of carbohydrates. We accordingly include a description of the characters of this species as we have found them. Unless otherwise stated, cultures were grown on Difco nutrient agar.

*Morphology.* Gram-negative motile rod, 0·5 × 2·5–5 μ., sporulating readily. Spores oval, subterminal, and considerably wider than the vegetative cell. A notable feature of cultures is the large number of free spores which stain Gram-positive and present a swollen and granular appearance. These are evidently not resting forms and may give a misleading impression of a mixed culture. They have not been seen in cultures of other strains of *B. brevis*. The organism is capable of extreme pleomorphism in aerated synthetic media.

*Plate colonies.* At 24 hr. these are about 1 mm. diameter, round, convex, smooth and shining. After 48 hr. the surface becomes drier and finely granular, and the margin irregular. From 3 days onwards there are two distinct zones: (a) an internal zone of 1–2 mm. diameter which is raised, dry, finely granular and surrounded by (b) a flatter, smooth, moist area of growth, sometimes concentrically ringed, the margin deeply lobed or crenated. No stable colonies with a smooth surface have been obtained and colonial types have remained very constant during 18 months. Four
strains from the National Collection of Type Cultures showed a series of gradations between colony forms identical with those of our strains, and a completely smooth colony.

Growth requirements. All strains tested are obligate aerobes with a temperature optimum of 37° and a wide range for growth.

Pigmentation. Cultures are not normally pigmented but may develop a pink pigment on some media containing vitamins of the B group.

Biochemical characters. The organisms produce acid without gas from: glucose, fructose, galactose, sucrose, lactose, maltose, xylose, mannitol, arabinose, sorbitol, salicin and glycogen. Raffinose, inositol, adonitol, aesculin and starch are not fermented. One strain (N.C.T.C. no. 2611), in an unstable smooth colony phase, failed to ferment xylose, mannitol, sorbitol or glycogen. Catalase +; indole −; H₂S +; nitrate reduction +; gelatin liquefaction, saccate becoming stratiform. Litmus milk: sweet curdling and peptonization, the latter complete after about 7 days at 37°.

The basal medium for determination of the sugar reactions consisted of 0.5% (w/v) proteose-peptone with 0.5% (w/v) NaCl in tap water at pH 7.8. It was found that, from several peptones investigated, including Difco nutrient broth, the organism was able to produce small amounts of acid without the addition of any carbohydrate. In a 0.5% solution of the proteose-peptone finally used the pH decreased by not more than 0.8 unit after 3 days' incubation when inoculated from a carbohydrate-free medium. The sugar broths were accordingly made sufficiently alkaline to compensate for this. In some cases the pH was determined electrometrically after inoculation and incubation to confirm acid production. Carbohydrate solutions were sterilized by Seitz filtration (except aesculin, sterilized at 100° for 25 min.; starch and glycogen, sterilized at 110° for 10 min.) and added aseptically to the basal broth to give a final concentration of about 1% (w/v). Inoculations were made from 24 hr. peptone-water cultures.

It is known that B. brevis is actively proteolytic. It is conceivable therefore that previous failure to demonstrate saccharolysis may have been due to the simultaneous production of alkaline substances, and it would appear that the nitrogen source, initial pH, and sensitivity of the indicator may all be factors of importance in this respect. That glucose is in fact utilized has been shown by chemical analysis of cultures (Appleby et al. 1947).

Maintenance of cultures

It was found possible, by colony selection, to obtain strains varying markedly in the amount of tyrothricin they produced, either in surface or submerged culture, but with no other distinguishing feature. Thus, of six strains A–F, strain C when first isolated produced twice the amount produced by strain A; strain F was still more efficient and has remained the most satisfactory; while strain D at first produced comparatively little tyrothricin and none at all after 18 months. In order to maintain the activity of strains, bimonthly transfers were made on nutrient agar (1% (w/v) peptone, 0.5% (w/v) NaCl); after incubation for 3 days the cultures were kept at 4°.

Tyrothricin from surface cultures

Surface cultures were grown for 3 days in flat bottles each containing 100 ml. broth (1% (w/v) peptone, 0.5% (w/v) NaCl with or without the addition of 0.3% (w/v) yeast extract (Difco) at pH 6.5). For the inoculum the whole of
the growth of a 3-day slope culture, suspended in saline, was subcultured in 100 ml. broth contained in a 250 ml. Erlenmeyer flask. After 2 days' incubation 1 ml. was used as the inoculum for each bottle. These were incubated horizontally to give the maximum surface area with a depth of about 1 cm.

Tyrothricin was isolated in each experiment by pooling the contents of a batch of bottles, acidifying to pH 4.1, and centrifuging off the bacterial cells after standing at room temperature for 24 hr. The separated cells were then extracted for 24 hr. at room temperature with 95% ethanol (50 mL/L original culture fluid), the ethanolic extract clarified by centrifuging and then poured into 10 vol. 1% (w/v) NaCl solution. The precipitated tyrothricin was centrifuged off after standing for 24 hr., dried in vacuo over P$_2$O$_5$, and the dried powder defatted with dry ether (8 ml/g. crude product) at 0°. This normally removed some 15% of the weight of the crude product as fatty material. The ether was filtered off and the purified tyrothricin washed with the minimum of dry ether at 0° and again dried out in vacuo. The product had a light colour and gave brown solutions in ethanol. The addition of traces of glycerol to the ethanol markedly aided solution of the material.

In general, the results obtained conformed with those reported by earlier workers. In media containing only peptone and NaCl, average strains of _B. brevis_ produced c. 0.25 g. tyrothricin/l. culture fluid when grown for 72 hr. at 37°. We were able, however, to increase this yield appreciably by isolating strains from the parent B.G. culture as mentioned above, and by using media containing 0.3% Difco yeast extract in addition to the peptone; under these conditions one of the strains (F) gave 0.8 g./l. in 72 hr. Generally the isolated strains appeared each to maintain a characteristic level of tyrothricin yield in a given medium, and in later experiments strain F was found to be particularly good in submerged culture also. The stimulating influence of yeast extract on tyrothricin was, of course, not unexpected, but it does not appear to have been recorded previously, and from the work reported later it appears to be due mainly to the presence of biotin in the extract. This is in agreement with Landy, Dicken, Bicking & Mitchell (1942), who observed that biotin stimulated the growth of _B. brevis._

**Method of assay**

The above experiments provided sufficient material to permit the development of a method of assaying tyrothricin. Tishler, Stokes, Trenner & Conn (1941) estimated the separated gramicidin and tyrocidine components by determining the quantities of each required to destroy 50% of the cells of a _Micrococcus_ in 2 hr. under standard conditions, the number of surviving cells being determined by plating. Stokes & Woodward (1943) used a modified form of this method. Dimick (1943) assayed tyrothricin solutions by estimating photoelectrically the degree of haemolysis of rat erythrocyte suspensions under controlled conditions. It was felt desirable, however, to develop a method which was rapid and based on the antibacterial activity of tyrothricin rather than on some other property, which could be used without adopting a rigid aseptic technique, and which could be applied for following the course of submerged culture fermentations. These criteria were ultimately realized.
Tyrothricin, assay and stability

In general, solutions for assay were made up by dissolving 1 g. tyrothricin in 40 ml. 95% ethanol and diluting with sterile distilled water to a concentration of 25 μg./ml. In the case of culture fluids, 50 ml. samples were acidified to pH 4.1 in centrifuge tubes, the cells centrifuged down after 2 hr. and, after removal of the supernatant, triturated with 5 ml. 95% ethanol. The suspensions were kept at room temperature for 30 min., recentrifuged, and 1 ml. aliquots of the clear ethanolic supernatants added to 50 ml. sterile distilled water to give the preliminary dilutions for assay.

The test organism was a member of the Streptococcus lactis group, supplied by Prof. Raistrick for diplococcin production. After a few weeks in the laboratory, this strain grew well at 37°C. It was markedly inhibited by tyrothricin, and the latter was conveniently estimated by its suppression of acid production from glucose by the above organism.

The medium used for assay purposes was a papain digest of ox heart, diluted to contain 0.5% oxidizable matter, with 1% (w/v) glucose and sufficient bromocresol-purple to give a distinct colour, at pH 7.0. Stock cultures of the test organism in chalk-litmus milk were kept at 4°C and subcultured at fortnightly intervals. From these, further cultures were prepared in litmus milk, incubated at 28°C until growth was shown by reduction of litmus with little or no acid production (approx. 16 hr.), and used immediately. With the culture thus in the early logarithmic growth phase, 1 ml. added to 40 ml. heart broth served as the inoculum for assays.

Into each of a series of test-tubes (3 x ½ in.) were delivered in this order: (a) a known amount of tyrothricin solution, (b) sufficient sterile distilled water to bring the volume to 1.0 ml., (c) 1.0 ml. inoculated broth. This was easily accomplished by using specially designed dropping burettes, each calibrated to deliver 25 drops/ml. of a particular fluid. After mixing, the tubes were immersed in racks in a thermostatically controlled water-bath at 37°C. If, as was generally arranged in practice, the difference between the tyrothricin content of two successive tubes of a series was one drop of a solution containing c. 25 μg./ml. tyrothricin, the strength of an unknown solution (by comparison with a standard solution) could be estimated to within 0.5 μg./ml. Two control tubes containing (a) no tyrothricin, (b) 25 μg. tyrothricin were included in each set.

The end-point was taken as the first tube in which the purple colour remained after incubation at 37°C for 4 hr., the readings being made at the time when the control series first gave clear-cut results. This did not indicate complete bacteriostasis but provided a means of assessing the bacteriostatic value of an unknown solution by comparison with one of known strength. Incubation below 37°C or at a temperature not sufficiently uniform, or failure to incubate the assay tubes immediately after mixing the inoculum with the tyrothricin, tended to result in intermediate colour changes; the end-point under these conditions was less clear-cut. By adhering rigidly to a standardized technique it was found that assay results by this method were surprisingly constant for a solution of known strength, not only over a long period, but also with different workers. Differences in the treatment of the test culture did cause some variation in assay values, so a control of standard strength was always included. Using the above technique, inhibition of acid production was usually caused by 7.0 μg. tyrothricin per tube.

In some cases it was not possible to prepare an extract of tyrothricin from a B. brevis culture, but was necessary to estimate the approximate amount of antibiotic in the culture fluid itself. The sample of culture was then centrifuged to remove the majority of the cells, and the supernatant itself assayed as above but using a medium containing cysteine hydrochloride. The presence of 0.1% (w/v) of this substance usually prevented growth of B. brevis under the test conditions (especially if the surface of the culture was sealed with sterile liquid paraffin), and 0.5% inhibited B. brevis completely but had no adverse effect on the test organism.
Stability of tyrothricin solutions

Certain early publications give the impression that tyrothricin solutions gradually lose antibacterial activity through temperature inactivation and, in consequence, it is generally advised that for clinical application freshly prepared solutions should be used. Dubos & Hotchkiss (1942) showed, however, that although aqueous tyrothricin solutions lost activity on heating or long standing, this was not a true inactivation but was due to colloidal aggregation followed by precipitation of the antibiotic. The full activity could be restored by redissolving the precipitated material in ethanol and resuspending in colloidal form by adding this extract to appropriate quantities of distilled water.

Table 1. Percentage loss of antibacterial activity of surface-culture tyrothricin solutions upon heating

<table>
<thead>
<tr>
<th>Concentration of solution (µg./ml.)</th>
<th>Temperature of heating (°)</th>
<th>% loss of activity after heating for 10 min.</th>
<th>60 min.</th>
<th>24 hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>37</td>
<td>12.5</td>
<td>22.3</td>
<td>36.3</td>
</tr>
<tr>
<td>500</td>
<td>50</td>
<td>12.5</td>
<td>30.0</td>
<td>68.2</td>
</tr>
<tr>
<td>5000</td>
<td>37</td>
<td>12.5</td>
<td>22.3</td>
<td>41.7</td>
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<tr>
<td>5000</td>
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<td>12.5</td>
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<tr>
<td></td>
<td></td>
<td>22.3</td>
<td>30.0</td>
<td>63.2</td>
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<tr>
<td></td>
<td></td>
<td>41.7</td>
<td>75.0</td>
<td>82.5</td>
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</table>

Since a tendency to loss of activity is clearly of importance in clinical treatment and the way in which the material is used, some investigation of this matter was carried out by comparing the surface-culture material already discussed with material produced by submerged culture (Appleby et al. 1947). Aqueous solutions of both types of products from surface and submerged cultures at 25 µg./ml. were prepared by appropriate dilution of solutions (1.25 g. tyrothricin/100 ml. ethanol), and maintained in sealed Pyrex tubes at room temperature for several months with periodic readings of turbidity. A clear distinction emerged in that the initial turbidities (Spekker, 1 cm. cell) were: ‘surface’ material, 0.136; ‘submerged’ material, 0.054; indicating presumably a smaller particle size or greater water solubility of the ‘submerged’ product, since no difference was found in the gramicidin/tyrocidine ratio for the two products. It was also found that the initial assay figure for the ‘submerged’ product was some 10% higher than that of the ‘surface’ product. On storage, the surface-produced material showed a gradual colloidal aggregation and increased turbidity leading to complete precipitation in less than 3 months. The submerged-culture material, however, showed no sign of precipitation over a period of 6 months, the turbidity during this period increasing to 0.001, which was still well below the initial value for the ‘surface’ material. In accord with this it was found, by assaying an aqueous solution (25 µg./ml.) of submerged-culture tyrothricin at intervals for 6 months, that solutions prepared as for clinical use showed no detectable loss of antibacterial activity.
activity if maintained at 4°C. This is clearly a fact of practical value and indicates some difference between the ‘surface’ and the ‘submerged’ products.

Some investigation was also carried out of the effect of heat on the inactivation of aqueous tyrothricin solutions of concentration 25,500 and 5000 μg./ml. Such solutions were maintained at 37°C and 50°C up to 24 hr. and assayed at intervals for bacteriostatic activity; unheated controls were included in each case. The latter showed no detectable inactivation at room temperature during the period of test. The results of the higher temperature experiments, presented in Table 1 for the ‘surface’ material, show that the extent of inactivation by heat increases both with the time of heating and with increasing concentration of tyrothricin. Similar results were obtained with submerged-culture tyrothricin, such differences as existed suggesting, if anything, that the latter material was the more stable. The distinction, however, was not pronounced and was certainly not as clear as the findings in the other stability tests.

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