The Assay of the Antibiotic Nisin

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SUMMARY: Nisin, which does not diffuse quickly through agar, may be assayed by dilution methods; or, because it is bactericidal and not merely bacteriostatic, it may be assayed by estimating numbers of surviving bacteria.

Nisin in low sublethal concentration simply prolongs the lag-phase of *Streptococcus agalactiae*; in higher sublethal concentrations it also induces fast-growing resistant strains. In both cases the delay can be accurately related to nisin concentration, growth being measured in terms of acid production.

The available methods for the assay of nisin are limited by the properties of the substance.

Nisin does not diffuse quickly through agar at pH 7 (Mattick & Hirsch, 1947), a character shared (Heatley, 1947) with the allied antibiotic diplococcin (Oxford, 1944). Assay by the agar-diffusion technique (Heatley, 1944) can be very accurate, and various modifications of it have been used for penicillin and streptomycin (Foster & Woodruff, 1948; Foster & Wilker, 1948; Brownlee, Delves, Dorman, Green, Grenfell, Johnson & Smith, 1948). Subtilin appears to be a borderline case since Lewis, Humphreys, Thompson, Dímick, Benedict, Langlykke & Lightbody (1947) recommend its assay by turbidimetric means, whereas Housewright, Henry & Berkman (1948) use agar-diffusion methods. According to MacMoline & Slinn (1948) subtilin does diffuse slowly through agar and if the growth of the test-organism is retarded plate assays become possible. This technique has not yet been explored with nisin, which in turbidimetric assay has an unfavourable range.

Various methods of assay for nisin were investigated, within the limitations described. Nisin solutions containing 7.5 mg./ml. (about 10,000 units/ml.) can be assayed as surface-active cationic material. Epton's (1947) method was tried but was discarded when it was found that small concentrations of nisin could not be assayed and that impurities caused marked errors.

A dilution end-point method of assay was supplemented by a methylene-blue test. As early as 1934, Cox recommended the use of methylene blue for the qualitative detection of inhibitory organisms in milk, and similar methods have been used for the assay of penicillin and streptomycin (Reid & Brewer, 1946; Sanchez & Lamensans, 1947). With nisin this test allows a reading in about 20 min. and has obvious advantages. These tests were superseded by a test based on the bacterial power of nisin and finally by the 'lag-phase assay'.

The accuracy of the dilution method and the methylene-blue test depends on the factor used in making serial dilutions. Geometrical series are usually set up, although arithmetical series (Schmidt & Moyer, 1944) are sometimes used. In the usual statistical terms the error ($\pm t \times$ standard error of the potency ratio) of a dilution method cannot be defined (Healy, 1948). The

uncertainty, however, when dealing in powers of two is $\pm 50\%$, not allowing for experimental error. The error of assay by agar-diffusion techniques is usually ± 10 to $\pm 15\%$, and it has been the aim of this work to achieve comparable accuracy. The bactericidal test has a mean accuracy of $\pm 32\%$ and the lag-phase assay one of $\pm 10\%$.

METHODS

Cultures. For the rapid methylene-blue test a fast-growing strain of *Streptococcus cremoris* (1 P 5) was subcultured daily in sterile milk using a 1% (v/v) inoculum and incubation at 22°. Before a test was begun a 10% (v/v) inoculum of the daily subculture was made in warm milk and incubated at 30° for 1 hr. (referred to subsequently as 1 P 5 milk). This culture will reduce methylene blue in about 1 min. and will clot after 1 hr. at 30°.

For all other assays the test-organism was a haemolytic strain of *Str. agalactiae*. It was subcultured daily and the fresh subculture stored in the refrigerator during working hours and incubated at 37° overnight. The organism was replaced once a month from a stock culture. Occasionally fresh cultures were started from freeze-dried material.

Media. Separated milk with or without added chalk and sterilized by steaming on three successive days was used for the propagation and maintenance of *Str. cremoris* (1 P 5).

Glucose Lemco broth (Mattick & Hirsch, 1947) was used for the propagation of *Str. agalactiae* and bullock heart medium for storage.

The assay media were as follows:

- (a) Glucose Lemco broth for the dilution-method assay.
- (b) For the bactericidal test 0.5% horse blood agar at pH 8.0.
- (c) For the lag-phase assay a medium containing $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1 part; NaCl, 1 part; glucose, 2 parts; peptone (Evans'), 2 parts; Na acetate (hydrated), 4 parts was used. These were dried, pulverized and blended, and the dry powder stored in the refrigerator. When required, 50 g. of powder were mixed with 1 l. of distilled water and sterilized at 10 lb. for 10 min. The pH was adjusted with N-NaOH. Evans's peptone gave heavier growth than British Drug Houses Ltd., Bengers', Bacto- and Neo-peptones.

Methods of counting. Plate counts and the roll-tube method were used (Hirsch, 1948).

Methylene-blue solution was prepared by dissolving one tablet (standard tablet, British Drug Houses Ltd.) in 200 ml. of water and stored in a dark bottle; 0.8 ml. of this solution was used per 10 ml. milk.

Units of nisin have already been defined by Mattick & Hirsch (1947).

Standards. The standard preparation of nisin was a batch of powder stored at -25° . Fresh standard solutions containing 10,000–50,000 units/ml. were prepared monthly. A solution in 0.02 N-HCl was made and sterilized by placing in a boiling water-bath for 5 min. This solution when stored at $0-2^\circ$ deteriorated only slowly. Dilute solutions required for assay are, however, unstable and cannot be stored.

Samples for assay were acidified with N-HCl and if necessary sterilized by placing in a boiling water-bath for 5 min. At pH 8 nisin is heat stable and small quantities of acid have a negligible effect on the assay.

Diluent. The diluent for the dilution and bactericidal assays was acidified distilled water or saline. For the lag-phase assay the standard diluent used was glucose Lemco-broth in which *Str. cremoris* (1 P 5) had grown overnight, the cells being then removed by centrifuging and the supernatant broth sterilized by steaming.

Cleaning of glassware. Nisin is adsorbed on glass and traces of it are extremely difficult to remove. Test-tubes thus contaminated may yield up to 2 units of nisin per ml. when sterile fresh broth is placed in them. Two consecutive boilings in the detergent 'Hexo' (Boro' Dairy Laboratories, London) will usually destroy residual nisin, but even after this procedure it is advisable to test each batch of test-tubes for growth inhibition.

For the preparation of master solutions in the lag-phase assay it is advisable to use new test-tubes cleaned in chromic-sulphuric acid mixture.

DILUTION METHODS OF ASSAY

The procedure with *Str. agalactiae* has been described by Mattick & Hirsch (1947).

For the rapid methylene-blue assay with *Str. cremoris* (1 P 5), the following procedure was adopted:

Unknown solution (ml.)	1.00	0.50	0.30	0.20	0.13	0.10
Distilled water (ml.)	0	0.50	0.70	0.80	0.87	0.90
1 P 5 milk (ml.)	9	9	9	9	9	9
Final dilution	1/10	1/20	1/33	1/50	1/77	1/100

After the addition of the 1 P 5 milk the tubes were placed in a 30° water-bath for 10–15 min. The methylene blue was then added and the tubes were inverted to mix and reincubated for a further 5 min. when the colour of tubes containing sufficient nisin for the inhibition of the test organism was unchanged, whereas the other tubes were colourless. *Str. agalactiae* and *Mycobacterium phlei* both behave erratically towards methylene blue.

Although *Str. agalactiae* is about four times less sensitive to nisin than *Str. cremoris* (1 P 5), there was reasonable concordance between the two dilution-method assays as shown in Table 1.

Table 1. *The reproducibility of methylene-blue assay and its concordance with the dilution end-point assay*

Sample	Units/ml.	
	Methylene-blue assay	Dilution end-point assay
1	60, 60, 40	—
2	100, 120, 100	—
3	150, 150, 133	—
4	100, 100	100
5	233, 200	200
6	150, 133	100
7	60, 100	80
8	120	100

THE BACTERICIDAL METHOD OF ASSAY

The survival of Streptococcus agalactiae in solutions of nisin. The bacterial death-rates with nisin are typical of a disinfectant (Hirsch & Mattick, 1949). Since it is usually possible to arrive at a simple linear relationship between the concentration of a disinfectant and its biological activity, it was hoped that this would facilitate the assay of nisin. However, since *Str. agalactiae* usually grows in chains, there was doubt about the possibility of an accurate count. Contrary to expectation, it was found that the strain of *Str. agalactiae* used could be counted with considerable accuracy. This can probably be attributed to the fact that the old laboratory culture used occurs largely in the form of diplococci, and that the experiments were carried out by one observer only. The control chart of Hannay (1946) was used. Eighty-eight sets of replicate colony counts showed that with four degrees of freedom $x^2=5.917$ and the probability of an agreement between expected and observed readings is within 10 %.

The data were insufficient for a critical assessment of the true nature of the dose-response curve. Approximate linear relationship was given by a number of functions. Thus the logarithm of concentration could be used either against the logarithm of percentage survivors or against probits, but the logarithm of percentage survivors could also be plotted against nisin concentration. These three cases are illustrated with the data of one experiment in Fig. 1. Besides these, however, other possibilities also existed, but despite these apparently contradictory results the impression was formed that the true linear relation was between the logarithm of concentration and probits, as suggested by Withell's work (1942*a, b*).

The procedure for the bactericidal assay

In preliminary experiments it was established that the range which could be covered by the assay was always about fivefold. When the cell concentration was about 800,000/ml. an average of 2-10 units of nisin/ml. could be assayed. This cell concentration was chosen for further work, since the cells were not too numerous to make dilutions for plating unduly laborious. One difficulty was the elimination of carried-over nisin, since 0.4 unit/ml. has a marked bactericidal action when the number of cells is below 5000/ml. The count usually entailed plating dilutions from 1/100 to 1/1000, thus rendering the effect of carry-over negligible. As nisin is alkali-labile, the high pH of the plate medium was an added precaution.

The procedure was as follows. A 24 hr. culture of *Str. agalactiae*, containing 200-400 million viable particles per ml. was diluted 100-fold and 1 ml. added to 8 ml. of sterile glucose Lemco broth (contact tube); 1 ml. quantities of the 20-100 units/ml. nisin solutions were added to the contact tubes. At least two standard solutions and one control containing no nisin were set up with each assay. The two nisin standards were usually 2 and 10 units/ml. to give about 80 and 10 % survivors respectively.

Fig. 1. Some dose-response curves for the bacterial assay of nisin.

The inoculated contact tubes were placed in a water-bath accurately controlled at 37°. After 5 min. necessary to bring the tubes to 37°, 1 ml. of the nisin solutions and the control saline were added every 1½ min. from 0 to 9 min. (seven solutions, three controls and four unknowns). After 10 min. the first contact tube was sampled; from 10 ml. of medium samples were withdrawn with 1 ml. pipettes. When the count fell to below 20,000 colonies/ml. there was no correlation between loop and pipette counts. During the next 1½ min. a series of 10-fold dilutions up to 1/10,000 was made. The blood-agar was poured on the plates while the next series was warming to 37°. The plates were counted after 24 hr. incubation at 37°.

Using the four-point assay design of Bliss (1944) on the data of one experiment the accuracy of this method was determined. A nisin solution containing 7.2 units/ml. was assayed as an unknown against another standard, and with only one sample or only one plate per sample results from 5.1 to 8.6 units/ml. for the solution were obtained. Under the most favourable conditions (three samples each with three plates) the estimated potency of this solution was 7.04 units/ml. with approximate 95 % limits of ± 2.2 units or ± 32 % ($P=0.95$).

When this assay is made as accurately as possible, it is more accurate than the dilution end-point method, but when only one sample of the unknown is tested, even with replication, its accuracy is approximately that of the dilution end-point method.

THE LAG-PHASE METHOD OF ASSAY

The growth of Streptococcus agalactiae in the presence of small concentrations of nisin

The observation that with prolonged incubation the titres obtained in a dilution end-point method of assay decrease, suggested a relationship between the time required for a culture to start growing and nisin concentration. At the end-points of dilution assays the concentration of nisin is constant, irrespective of the original strength of the sample and thus this relationship suggested itself for assaying. It was realized that a measurement of time was difficult to make as a routine. Instead, pH measurements were taken, since the amount of acid produced by the organisms in a buffered medium is related to the time when growth began. An approximately linear relationship was found between pH and the logarithm of the nisin concentration.

The assay technique first used thus consisted in plotting a standard line and reading off the nisin concentration from this (Fig. 2). Wood (1946) gave the name of 'single curve' method to this assay technique which does not involve the plotting of a curve for the unknown. The accuracy of this method was tested by assaying one standard solution, divided into two parts, and expressing the activities of the solutions in terms of each other. Discrepancies up to 30 % were obtained and it was felt that, apart from the unfavourable slope of the standard line, some other factors must be affecting the assay.

Further observations showed that when the curves were divided into two portions a statistically significant increase in accuracy was obtained.

Comparisons between the ranges 1-5 and 5-50 units were not possible without loss of accuracy. The standard curve as shown in Fig. 2, where each point is the average of sixty-three readings, was found to be heterogeneous, i.e. the responses in the two ranges (1-5 and 5-50 units/ml.) were due to different causes.

Fig. 2. The heterogeneous response of *Str. agalactiae* in the lag-phase assay.

Table 2. *The effect on the production of resistant cultures by growth of Streptococcus agalactiae in various concentrations of nisin*

Organism grown in nisin (units/ml.)	Strain no.	*Organisms at the end of growth, inhibited by	
		Units/ml.	Average units/ml.
0	1	1	1
	2	1	
	3	1	
1	1	1	1
	2	1	
	3	1	
2	1	1	1
	2	1	
	3	1	
5	1	20	9
	2	2	
	3	5	
10	1	10	23
	2	50	
	3	10	
20	1	50	27
	2	10	
	3	20	
100	1	50	40
	2	20	
	3	50	

* Sensitivity test carried out by a dilution end-point method.

Experiments were therefore carried out in which the pH of inoculated broths containing varying amounts of nisin was measured at intervals of time. From Fig. 8 it can be seen that growth of the normal culture (0) is delayed, and the length of lag is related to the nisin concentration. The resulting straight lines

Fig. 8. The effect of nisin on the acid production of *Str. agalactiae*.

are, however, of different slopes; in all cases the eventual growth of those cultures which contain nisin is much more rapid (curves 1, 2). All the cultures appeared to be stimulated by nisin, but from 5 to 50 units/ml. the curves obtained converged. This observation has been repeatedly made. An explanation was required and accordingly the sensitivity to nisin of the test organisms at the end of the assay was tested. From Table 2 it can be seen that 1 and 2 units/ml. did not produce resistant organisms; other experiments have shown that this is true of up to 4–4.5 units/ml. Concentrations greater than 5 units/ml. invariably yielded nisin-resistant organisms at the end of the assay.

The range 1–5 units of nisin/ml. may thus be regarded as causing a true increase in the length of the lag-phase. In the range of 5–10 units/ml. secondary

growth appears, which, because nisin is bactericidal, is undoubtedly preceded by the killing of a large proportion of sensitive cells. This point was confirmed by plate counts. It appears that, proportionally with increasing nisin concentration, increasingly resistant cultures result which, once growth commences, grow faster than the sensitive cells.

When samples taken at 30 min. intervals were examined it was found that the sensitivity of the few cultures which could be isolated at the beginning did not change during the experiment; from the start only resistant forms could be isolated from tubes containing 75 units/ml. It thus appears that there is a selection of naturally occurring resistant cells.

The observations recorded in Fig. 3, in which the amount of stimulation appeared to be proportional to the nisin concentration, suggested that the resistant cells might utilize nisin. Accordingly, resistant and normal cultures were grown in the presence of various concentrations of nisin, the optical densities and the pH were measured hourly. Contrary to expectation it was found that nisin did not now stimulate growth, but whereas the growth of normal cultures in nisin was delayed, resistant cultures grew in the presence of nisin at the same rate as the controls without nisin.

The line $T_3-T'_3$ in Fig. 3 represents the moment when the experiment is stopped and pH readings taken. It is obvious that this moment has to be carefully selected in order to obtain maximum accuracy.

Choice of dose-response relationship

The response due to the formation of resistant cultures (approximate assay range 5–50 units/ml.) was preferred to the lag-phase response (range of 1–5 units/ml.), not only because of the range, but also because of the more favourable slope. The following convenient linear relationships exist in this case:

- (a) Range of 5–15 units: units $\times$ pH = a constant.
- (b) Range of 5–50 units: log of units $\times$ pH = a constant.
- (c) Range of 5–50 units: log lag period $\times$ pH = a constant.

Titration of the acid formed, instead of pH measurements, were tried and discarded. They were no better than pH readings and were more laborious.

The lag period is determined quite simply when two pH readings are taken at intervals of time. It was found that the logarithm of the time is linearly related to pH, so that the line joining the two pH readings may be extrapolated and the length of the lag determined. Cases (a), (b) and (c) are shown in Fig. 4.

Whereas case (a) appears to be the most accurate, the range is very small. Occasionally data were obtained, which for no apparent reason, could not be fitted to case (c). The preferred relationship was therefore that of case (b), but before further experiments could be usefully undertaken it was necessary to test the effect of impurities on the assay. Nisin of varying purities, prepared from culture fluids with and without various peptones were used. The results presented in Fig. 5 show that the response is specifically due to nisin since the same slopes were obtained. Further experiments were now undertaken on the preferred method.

Fig. 4. Some dose-response curves for the lag-phase assay of nisin.

Fig. 5. The effect of impurities on the assay of nisin by the lag-phase method. x = crude nisin made from peptone medium; ▲ = crystalline nisin; △ = nisin preparation made from peptone medium in 1946; ● = nisin preparation made from peptone medium in 1948; ○ = nisin preparation made from yeast medium.

Variables of the preferred method

Apart from improvement in accuracy, one of the reasons which prompted investigation of the preferred method was that when two identical standard solutions were assayed, the responses did not always coincide. The causes of this disconcerting effect may be summarized as follows:

(a) The most important single factor is the age of the test-organism. When the inoculum was 6–10 hr. old instead of 18–24 hr. much better slopes were obtained (cf. Figs. 2, 5), the upper range of linearity was extended from 50 to 100 units/ml. and the accuracy and usefulness of the technique much improved.

(b) The nature of the diluent used was important. Markedly different slopes, suggesting almost that different substances were being assayed, were obtained when the standard was made up in distilled water instead of broth.

(c) A progressive shift of the standard line was observed with assays lasting more than a few hours, those standards which were kept longer usually giving a lower reading.

(d) The method of preparing the dilution was of slight importance; the number of pipettes used affected the answer but slightly. The temperature of the diluent was unimportant.

(e) Fatty acids from cotton-wool plugs did not appear to affect the assays. Cleanliness of the glassware was of prime importance and the critical master dilution tubes (see below) had to be scrupulously cleaned. The test-tubes had also to be of even size.

Method of assay

(a) *Preparation of the standard.* A solution containing 10,000–80,000 units of the standard/ml. was serially diluted in 10 ml. lots of diluent, preferably using a fresh pipette for each concentration. The concentrations were ten times higher in these master dilutions than in the final tubes. Each dilution was then distributed in 1 ml. lots using only one pipette to make ten replicates for each concentration. In routine practice the method is sufficiently reproducible to make it unnecessary to use more than four tubes for each concentration. To the tubes containing 1 ml. of nisin 9 ml. of inoculated broth were added as rapidly and accurately as possible.

(b) *Preparations of unknowns.* Solutions of chemically concentrated nisin preparation are not difficult to assay. They may be sterilized by heat and then diluted. Culture fluids containing nisin are, however, very much more difficult to assay. According to various treatments the same culture fluid can give different answers. To obtain consistent results it is necessary first to adjust to pH 8.0. The broth is then boiled to extract more nisin from the cells, which are then centrifuged down. Filtration through Seitz-type filter-pads is not suitable as nisin is adsorbed by the filter-pads.

The choice of assay design

Wood (1946) and Wood & Finney (1946) have defined the best assay design for microbiological assays of growth substances and there seems no reason why their definitions should not hold for the assay of antibiotics; the principles

involved are the same. The upper end-point of the graded response is that concentration of the antibiotic at which growth is no longer possible. In the same way in microbiological assays, the lower end-point is that concentration of the essential nutrients which does not permit growth. The most accurate design is the 'common-zero 5-point' assay. They recommend 'that, if the linear relationship does not ordinarily hold down to zero dose for the particular assay procedure in use, a small quantity of the factor being assayed is added to the basal medium for all tests, in order to make the relationship linear...'. Since in the preferred method of assay the linear relationship ends at concentrations less than 5 units/ml., experiments were carried out in which the tubes contained 5 units added nisin per ml. The results were not encouraging, probably because contact between nisin and cells in the inoculated broth lead to an unpredictable condition of the inoculum.

The remaining practicable design is that of parallel lines. Whereas for the same amount of labour this method is less precise than the 'common-zero 5-point' assay the experimental arrangement is easier and the parallelism of the lines indicates that the response to standard and unknown are due to the same substance. It is thus possible to obtain fairly reliable assay results by simple graphical procedures as suggested by Wood. It is important that the dilutions should be prepared as geometrical series so that when a plot of log nisin concentration against pH is made, an equally weighted line is obtained.

Bliss (1944) described a simplified method of calculation for the assay of penicillin. Later he developed graphic control charts which he used for the assay of penicillin (Bliss, 1946). By comparison with nisin, the penicillin assay is a well-established method, and it was felt that the 4-point assay design was not suitable for this comparatively new substance. A 6-point assay which could test curvature as well as parallelism was preferable, and Healy (1949) has developed this method to enable its use in routine assay. Applying this method to data collected over a period of time the accuracy of the lag-phase assay varies from ± 7 to $\pm 15\%$.

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