Biosynthesis of the Antibiotic Nisin by Whole *Streptococcus lactis* Organisms

By A. HURST*

Sub-department of Chemical Microbiology Department of Biochemistry, University of Cambridge†

SUMMARY

A reaction mixture is described consisting of a buffered solution of amino acids, salts, growth factors and glucose in which freshly harvested washed *Streptococcus lactis* incorporated radioactive tracers and synthesized nisin. Rapid nisin synthesis started after a delay of 30–60 min. but bacteria pre-incubated in the reaction mixture synthesized nisin without delay although the rate of protein synthesis remained the same as that of freshly harvested bacteria. Although growing *S. lactis* is sensitive to penicillin and mitomycin these antibiotics had no effect on nisin synthesis by washed organisms. Actinomycin D inhibited uptake of tritiated uridine immediately and inhibited nisin synthesis after a delay of about 60 min. Antibiotics which interfere with protein synthesis, e.g. chloramphenicol, puromycin and terramycin also interfered with nisin synthesis. The inhibition was immediate and occurred irrespective of whether the antibiotics were added at the beginning of an experiment or after 50 min. Nisin synthesis was more sensitive than protein synthesis. The data suggest that nisin synthesis occurs by a mechanism similar to that of protein synthesis.

INTRODUCTION

Polypeptide antibiotics frequently contain unusual amino acids; some of the constituent amino acids may be in the uncommon D configuration and at least some part of the molecule is cyclized. The physiological function of these substances is unknown. In the last few years the methods by which micro-organisms synthesize these unusual substances have interested many workers. Mach, Reich & Tatum (1963) and Mach & Tatum (1964) studied the synthesis of tyrocidine; the biosynthesis of polymyxin was studied by Paulus & Gray (1964); gramicidin S by Eikhom *et al.* (1963, 1964), Winnick, Lis & Winnick (1961); gramicidine by Okuda, Edwards & Winnick (1963); bacitracin by Bernlohr & Novelli (1963), Snoke (1961), Cornell & Snoke (1964), and Shimura, Sasaki & Sugawara (1964); mycobacillin by Banerjee & Bose (1964) and actinomycin by Katz (1960), Katz & Weissbach (1962), (1963) and Katz, Wise & Weissbach (1965). The conclusion reached by most of these workers is that polypeptide antibiotics are synthesized non-ribosomally; two recent studies with cell-free systems which synthesize gramicidin S further support this idea (Berg, Frøholm, & Laland, 1965; Yukioka *et al.* 1965).

However, Winnick and collaborators found that biosynthesis of gramicidin S,

* Formerly A. Hirsch.

† Present address: Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford.

tyrocidine and gramicidin by whole organisms was prevented by inhibitors of protein synthesis (Winnick *et al.* 1961; Winnick & Winnick, 1961; Okuda *et al.* 1963; Uemura, Bodley, Adiga & Winnick, 1965). Later work with cell-free systems showed that ribosomes were essential for the synthetic process (Uemura, Okuda & Winnick, 1963; Bodley *et al.* 1964). Recently Winnick and co-workers isolated a gramicidin S 'messenger RNA' and determined some of its physical and chemical properties (Hall *et al.*, 1965; Sedat & Hall, 1965). These results suggest that gramicidin S is synthesized by a process similar to that of normal protein synthesis.

Synthesis of the polypeptide antibiotic nisin was of interest because it is not made by a Bacillus but by *Streptococcus lactis* (Mattick & Hirsch, 1947) and it appears to have an unusually large molecular weight (Chesseman & Berridge, 1959). Nisin is used in practice as a food preservative (Hawley, 1957; Gibbs & Hurst, 1964). The name 'nisin' describes a family of antibiotics, at least four of which have been partially characterized (Berridge, Newton & Abraham, 1952). Different strains of *S. lactis* are also known which produce different antibiotics (Hirsch & Grinsted, 1951; Hirsch, 1951 b). The composition of nisin A was determined by Cheeseman & Berridge (1959); it contains 55 amino acid residues and the unusual amino acids lanthionine and β-methyllanthionine, but the optical configuration and sequence of amino acids are not known; it has no free terminal carboxyl or amino groups. Cheeseman & Berridge estimated the molecular weight of nisin A to be about 7000; this figure has recently been challenged by Bodansky & Perlman (1964). If the original estimate is correct, nisin, which is considered to be a polypeptide may be more complex than some of the small proteins, e.g. insulin, which has a molecular weight of 6000 (Sanger, 1959).

In the work described in this paper whole organisms of *Streptococcus lactis* were used to try to decide whether the synthesis of nisin is an enzymic or ribosomal process. The complex nutritional requirements and the vigorous acid production of this organism made it desirable to work with washed bacteria suspended in a reaction mixture of defined composition and, as far as possible, constant pH value. The effects produced by inhibitors of the synthesis of mucopeptides, protein, RNA and DNA (for review see Gale, 1963) suggest that the main mechanism concerned in nisin synthesis is similar to that of normal protein synthesis.

METHODS

Organisms. For nisin production *Streptococcus lactis* 354/07 (NCDO 497) was used. For nisin bioassay *S. cremoris* strain 1P5 (NCDO 495) was used.

Media and culture conditions. The organisms were subcultured daily in a medium of the following composition (%, w/v): meat extract (Lemco), 1; yeast extract (Difco), 1; tryptone (Difco), 1; glucose, 1; NaCl, 0·5; Na_2HPO_4, 0·2; pH 7·0. Incubation was at 25° in deep tubes without shaking. Stock cultures were stored at 4° on slopes of the same medium with 1·5 % (w/v) agar. New slopes were started at fortnightly intervals.

For preparing washed suspensions of *Streptococcus lactis* strain 354/07 the high nisin yielding medium of Hirsch (1951 a) was used (medium 22). It contained (%, w/v): meat extract, 1; peptone (Evans), 1; glucose, 2·5; Na acetate, 1·5; Na citrate, 1·5; Na_2HPO_4, 0·5; and Ca pantothenate 1 μg./ml.

Chemicals. A.R. grade reagents were used. (U-^{14}C)-L-glutamic acid (6,4 mc/mM), (G-^{3}H)-DL-threonine (66 mc/mM and (5,6-^{3}H) uridine (22 c/mM) were purchased from the Radiochemical Centre, Amersham, Buckinghamshire. Penicillin, chloramphenicol, puromycin, terramycin and mitomycin were gifts from members of the department. Actinomycin D was a gift from Messrs Merck, Sharpe & Dohme, U.S.A.

Reaction mixture. The synthesis of nisin, incorporation of amino acids and protein synthesis were followed in a reaction mixture containing all the known growth requirements of *Streptococcus lactis.* This mixture contained amino acid mixture A (1·0 ml.), 10 % (w/v) glucose (1·0 ml.), buffered salts solution B (1·0 ml.) purine + pyrimidine mixture C, (0·1 ml.), vitamin mixture D (0·1 ml.), tracer amino acids, bacteria equiv. 1 mg dry weight/ml. and inhibitors as required; total volume made to 10 ml. with distilled water. These various solutions had the following compositions. Amino acids, purines and pyrimidines were made up according to Gale & Folkes (1953). Amino acid mixture A contained each of the following at 2 mg./ml.: glycine, L-aspartic acid, tyrosine, tryptophan, phenylalanine, histidine, lysine, arginine, methionine proline, hydroxyproline, serine, cysteine, cystine, alanine, leucine, isoleucine and valine, the solution being adjusted to pH 7·0 with 2 N-NaOH. L-Glutamic acid and DL-threonine were made up as separate solutions at 2 mg./ml. and diluted 1/10 for use. (U-^{14}C)-L-glutamic acid was used at 50 μg./ml. with a specific activity of 2 μc/mg. (G-^{3}H)-DL-threonine was used at 100 μg./ml. with a specific activity of 30 μc/mg. Buffered salt solution B contained (%, w/v): 1, KH$_2$PO$_4$; 3·3, Na$_2$HPO$_4$; 1, NaCl; 0·7, MgSO$_4$.7H$_2$O; 15, trisodium citrate. Purine + pyrimidine mixture C contained adenine, xanthine, hypoxanthine, guanine, thymine, cytosine, uracil, each at 1 mg./ml. The vitamin mixture D was made up according to Niven (1944), and contained riboflavin, Ca-pantothenate, nicotinic acid, pyridoxine (each at 1 mg./ml.) thiamine (0·1 mg./ml.) and biotin 1 μg./ml.

Suspensions of organisms. Medium 22 was inoculated (10 %, v/v) with an overnight culture of *Streptococcus lactis* 354/07, incubated at 30°, and the growth of the culture followed by its acid production. When the pH value had decreased to 6·0–5·8 (after about 6 hr) the organisms were harvested on the centrifuge, washed once with buffered salts solution B and finally suspended in distilled water at equiv. 10 mg. dry wt/ml.; 1·0 ml. of this suspension was used in 9 ml. reaction mixture. The dry weight of bacterial suspensions was estimated turbidimetrically with a Hilger spectrophotometer calibrated for the organism. Incubations were done at 30°.

Preparation of samples. For estimating nisin by bioassay the reaction was stopped by adding 10 N-HCl to bring the sample to pH 1·8–2·0; the tubes were then placed in a boiling-water bath for 5 min. and then centrifuged at 5000g for 10 min.; at pH 1·8–2·0 nisin is stable and remains entirely in the supernatant fluid (Hirsch, 1951 *a*).

For estimating radioactivity and protein, the reaction was stopped by diluting with an equal vol. of cold 10 % trichloroacetic acid (TCA).

For radioactivity estimations with a 'Panax' end-window Geiger counter (taking at least 300 counts) and the samples were prepared as described by Park & Hancock (1960). For estimations with a Nuclear Chicago liquid scintillation counter (System 724) TCA precipitates were filtered on 2-cm. membranes (Oxoid, London), washed

successively with 5 and 1 % acetic acid and finally with distilled water. The membranes were put into glass vials ($\frac{1}{2}$ in. × 2 in.), dried at 105° for 2 hr and then covered by 2·5 ml. toluene scintillation fluid (3·5 g. PPO, 2:5-diphenyloxazole, and 50 mg. POPOP, 1:4-bis-2-(4-methyl-5-phenyloxazolybenzene) in 1 l. toluene). The vials were then closed, placed in carrier bottles and counted to 10,000 counts or 4 min., whichever was quicker.

Fractionation of bacteria was done according to the method of Park & Hancock (1960).

Protein was estimated by the Lowry modification of the Folin & Ciocalteau method (Lowry *et al.* 1951).

Bioassay of nisin. The turbidimetric method of Berridge & Barrett (1952) modified to increase its sensitivity was used as the basis of the present method with which 1 mμg. nisin/ml. could be estimated with an accuracy of ± 15 %. Each result reported in this paper represents the average of at least three independent assays. The method was as follows.

Overnight cultures of the test organism *Streptococcus cremoris* (1 P5) were diluted with fresh medium and incubated at 30° for 1 hr. This step was repeated 2–3 times to get a vigorously growing culture; when a 1/10 dilution of such a culture had an extinction value of 0·1 at 600 mμ (in the Unicam S.P. 500 spectrophotometer) it was used to inoculate (1 %, v/v) a 1-l. volume of the same medium which was incubated for 1 hr at 30° (culture A). A volume (0·1 ml.) of sample containing nisin was added to 10 ml. of culture A and further dilutions in culture A were made as quickly as possible; the tubes were then incubated for 2$\frac{1}{2}$–3 hr at 30°. Growth was stopped by injecting into each tube 0·1 ml. of 0·004 % solution of thiomersalate (Berridge & Barrett, 1952). The extinction was measured in an S.P. 500 Unicam spectrophotometer at 600 mμ. Sterile precautions were unnecessary after the culture stages. A series of standards were set up in triplicate for each assay and values for the unknown samples were read from the curve. Figure 1 shows an example of the growth response curve obtained.

Nisin standard. A gift of purified nisin was received from Dr J. Tramer (United Dairies Central Laboratories, London). Dr B. Giles (Unilever Research Laboratory, Sharnbrook, Bedford) examined this material in a Spinco Analytical Ultracentrifuge Model E and reported that it behaved as a single substance. This purified nisin was further examined by high voltage paper electrophoresis (Shandon, London). Up to 1 mg. nisin/spot was used; the nisin spot did not give a ninhydrin reaction but the histidine residue which nisin contains was used to locate it with Pauly's reagent (Dent, 1947). The nisin spot was also located by bio-electrophoretograms. Neither test showed more than one spot. On the basis of the ultracentrifuge and electrophoretic data the sample was assumed to be pure nisin and the results of assays were expressed as μg./ml. in terms of this standard. The relationship of this material to the nisins A, B, C and D of Berridge *et al.* (1952) is not known. The nisin standard was dissolved at 100 μg./ml. in 0·05 N-HCl, dispensed in 0·1 ml. volumes in 15 ml. vials and stored frozen. For assay 10 ml. of 0·01 N-HCl was added to each vial and 0·1 ml. of the solution added to 10 ml. of culture A; further dilutions were then made. The first tube of the standard series thus contained 10 mμg. nisin/ml. and usually did not become turbid during the assay period.

Precautions. Many of the assays described below involved the estimation of nisin

in the presence of other antibiotics. Penicillin, oxamycin and terramycin introduced no complications since they were inactivated by the treatment (5 min. at 100° at pH 1·8–2·0) used for the extraction of nisin. Other antibiotics, particularly chloramphenicol, resisted this treatment and preliminary experiments were run to determine the maximum concentration at which these substances could be used without subsequently affecting the nisin assay. For example, the maximum permissible concentration of chloramphenicol in a reaction mixture was 20 μg./ml.
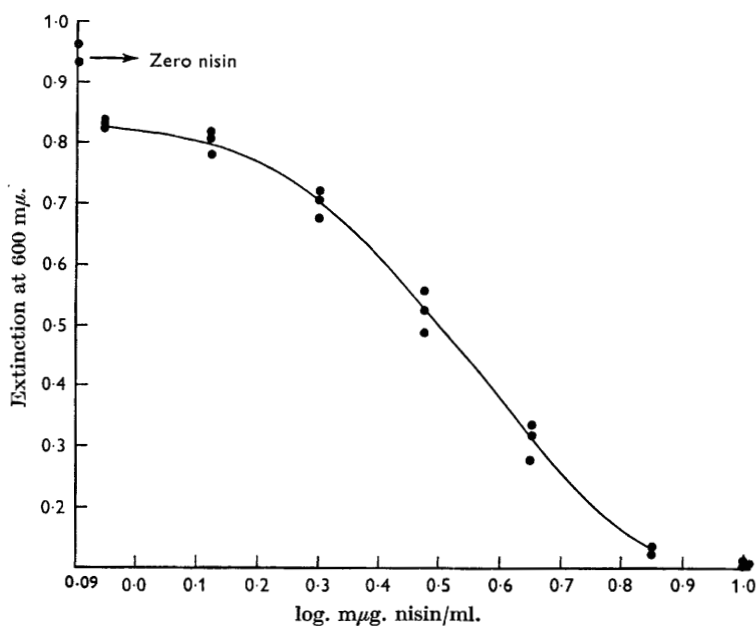
Fig. 1. Growth/response of standard in bioassay of nisin.

RESULTS

Time course of nisin synthesis

Washed *Streptococcus lactis* organisms grew slowly and synthesized nisin at an accelerating rate in the reaction mixture (Fig. 2). During the 120 min. experimental period the pH value remained above 6·0, dry wt. increased from 1·0 to 1·55 mg./ml., protein increased from 555 to 853 μg./ml., and the protein content of the bacteria remained approximately constant at 55 %. The nisin content increased from about 1 μg./ml. to about 15 μg./ml. during the same period. The rates of protein synthesis and nisin synthesis were not linear; the rates increased particularly after 60 min. (Fig. 2). The total amount of nisin synthesized by suspensions of organisms prepared on different days varied considerably; at the end of the experimental period (120 min.) the nisin content ranged from 0·2 to 5 % of the dry wt. of organism.

In another experiment a suspension of bacteria was divided into two equal volumes; one portion was incubated for 100 min. in the reaction mixture and then resuspended in fresh reaction mixture. Nisin synthesis and the incorporation of tritiated threonine of the pre-incubated and freshly harvested organisms were then

compared. The results (Fig. 3) showed that threonine was incorporated at the same rate by the two suspensions, but that nisin synthesis was more rapid in the pre-incubated bacteria and occurred without lag.
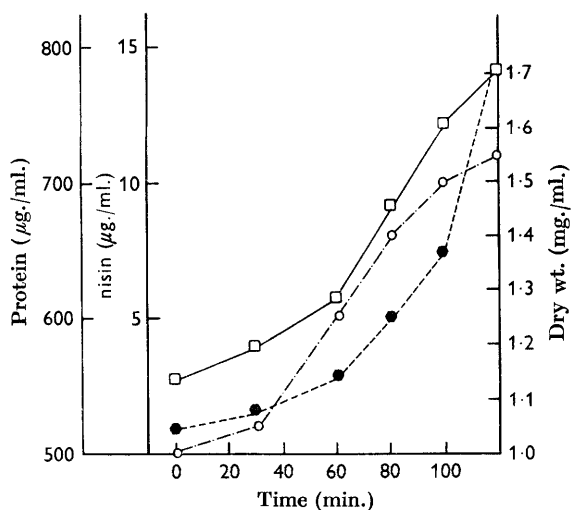

Fig. 2

Fig. 3

Fig. 2. Changes in dry weight, protein and nisin synthesis of a washed suspension of *Streptococcus lactis* in the reaction mixture. Protein, □—; dry weight, ○-·—·; nisin, ●- - -.
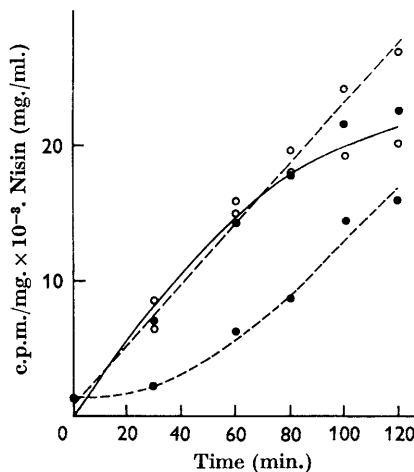
Fig. 3. Nisin synthesis and incorporation of [G-³H]-DL-threonine in freshly harvested and pre-incubated *Streptococcus lactis* organisms. Radioactivity, ——; nisin - - -; fresh organisms, ●; pre-incubated organisms, ○.

Incorporation of amino acids

The reaction mixture with organisms was incubated for 60 min. and the bacteria then fractionated by the method of Park & Hancock (1960). With (U-^{14}C)-L-glutamic acid 62% of the radioactivity was found in the fraction liberated by trypsin digestion; 28 % was in the insoluble residue; and the remaining 10 % was distributed between the nucleic acid and ethanol-soluble fractions. Glutamic acid does not specifically label the protein of *Streptococcus lactis* and was used only in early experiments on time-course studies with inhibitors of protein synthesis (e.g. as in Fig. 4). In later experiments protein was estimated by the Lowry *et al.* method or by incorporation of (G-^{3}H)-DL-threonine. Fractionation of bacteria incubated with labelled threonine showed that 88% of the radioactivity was in the trypsin-digestible fraction.

Effect of the antibiotics penicillin and cycloserine

Growing cultures of *Streptococcus lactis* are sensitive to penicillin; 0·05 µg./ml. causes inhibition in overnight growth tests (minimal inhibitory concentration, m.i.c., = 0·05 µg./ml.). Concentrations of penicillin up to 2 µg./ml. were without effect on nisin synthesis and incorporation of DL-threonine when tested in the reaction mixture; the time of addition of the antibiotic was also unimportant. Oxamycin which was tested up to 100 µg./ml. was also without effect.

Inhibitors of protein synthesis

Incorporation of (U-¹⁴C)-L-glutamic acid and nisin synthesis were measured in time-course experiments, with and without inhibitors. Chloramphenicol has an m.i.c. of 5–10 μg./ml. and was tested at 5, 10 and 20 μg./ml. Results of a typical experiment in which chloramphenicol was added at time 0 or at 50 min. are shown in Fig. 4. Addition of chloramphenicol at time 0 caused a 94 % inhibition of glutamic acid incorporation and 100 % inhibition of nisin synthesis. When chloramphenicol was added at 50 min. further nisin synthesis was completely halted, but incorporation of glutamic acid continued at a slower rate. In similar experiments puromycin (m.i.c. about 30 μg./ml.) at 50 μg./ml. and terramycin (m.i.c. 0·5–1 μg./ml.) at 1 μg./ml. gave results similar to those obtained with chloramphenicol.
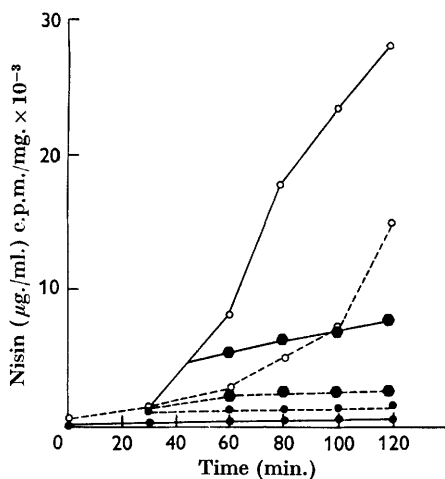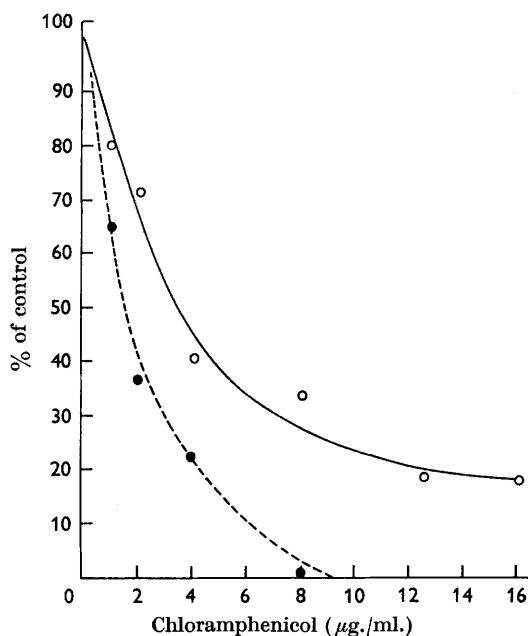

Fig. 4. The effect of chloramphenicol (20 μg. ml.) on nisin synthesis and incorporation of [U-¹⁴C]-L-glutamic acid by *Streptococcus lactis*. Radioactivity, ——; nisin; - - -; control, O; chloramphenicol added at time zero, ●; at 50 min. ◖.

Fig. 5. Protein and nisin synthesis by *Streptococcus lactis* in the presence of various concentrations of chloramphenicol, as % of control without antibiotic. Protein, O——; nisin, ●- - -.

The controls shown in Figs. 2–4 all have the common feature that the rate of nisin synthesis increased after a lag of 30–60 min. To compare the action of antibiotics on protein and nisin synthesis, inhibitors were added after 60 min. to avoid this lag period: 20 ml. of the reaction mixture were incubated for 60 min. at 30° and then subdivided into 2 ml. portions containing a range of concentrations of inhibitors or with distilled water as a control. Incubation at 30° was continued for another hour and the protein (by method of Lowry *et al.*) and the nisin content

estimated. The increase in protein and nisin between 60 and 120 min. in the control tube was taken as 100 % and the effect of inhibitors was expressed as a % of the control. Typical results obtained with the same three inhibitors are shown in Figs. 5, 6 and 7. In each case concentrations of inhibitor well below the m.i.c. were used. In all three cases the antibiotic was a more effective inhibitor of nisin synthesis than of protein synthesis. At an antibiotic concentration of one-tenth m.i.c. nisin synthesis was more sensitive than protein synthesis by a factor of 2 for chloramphenicol, 3·3 for terramycin and 10 for puromycin.
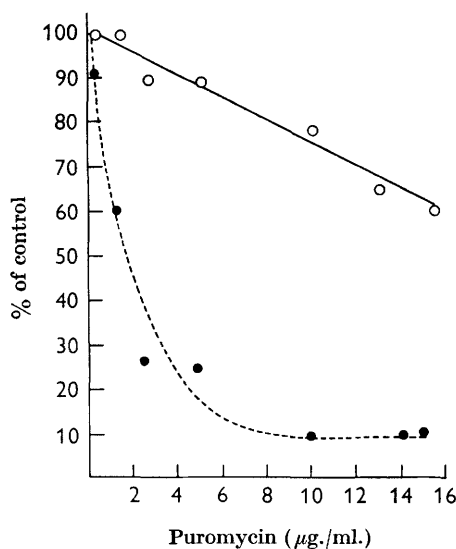

Puromycin (μg./ml.)
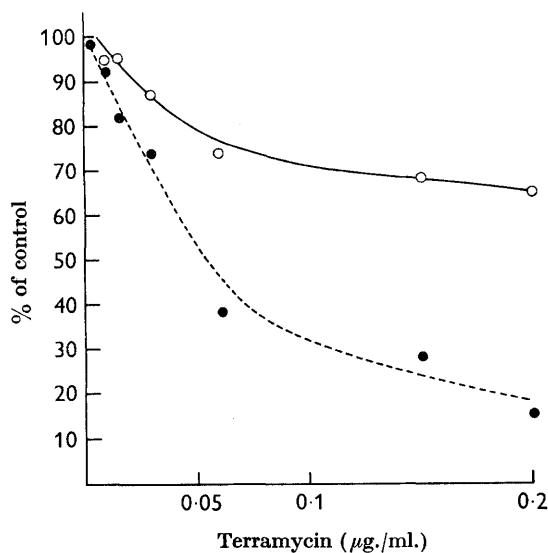
Fig. 6

Terramycin (μg./ml.)

Fig. 7

Fig. 6. Protein and nisin synthesis by *Streptococcus lactis* in the presence of various concentrations of puromycin, as % of control without antibiotic. Protein, O—; nisin, ●- - -.

Fig. 7. Protein and nisin synthesis of *S. lactis* in the presence of various concentrations of terramycin, as % of control without antibiotic. Protein, O—, nisin, ●- - -.

Inhibitors of nucleic acid synthesis

Further time-course studies were made with mitomycin which inhibits DNA strand separation (Iyer & Szybalski, 1963). Nisin synthesis was insensitive to mitomycin (0·5 μg./ml.) although growth was inhibited by 0·03 μg./ml., the lowest concentration tested.

Actinomycin D, an inhibitor of RNA synthesis (Kirk, 1960; Hurwitz *et al.* 1962) was used at 10 μg./ml. In the 120 min. experimental period it had no effect on nisin synthesis when added at 60 min., but nisin synthesis was halted after 80 min. when the antibiotic was added at zero time. This experiment was repeated with tritiated uridine (1 μc./ml. reaction mixture) to assess the inhibition of RNA synthesis. In the first of such experiments (Fig. 8) the added isotope was all taken up by the control bacteria in the first 20 min. During the same period there was insignificant incorporation by the actinomycin-treated bacteria. Nisin synthesis in the control followed a linear course for 80 min., as in the previous experiment, and then increased. The actinomycin-treated culture synthesized nisin at a rate 60 % that of

the control for 60 min., after which time nisin synthesis ceased altogether. In another similar experiment no nisin was synthesized during the first 40–60 min. and actinomycin completely blocked subsequent nisin synthesis; uptake of tritiated uridine was also inhibited (Fig. 9).
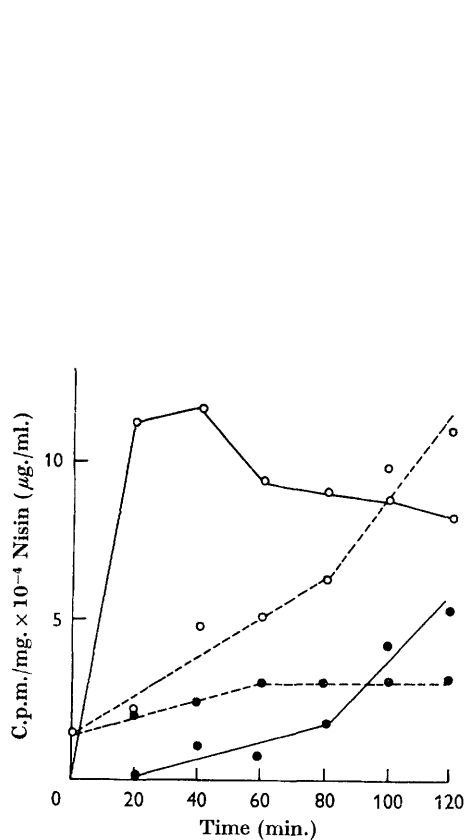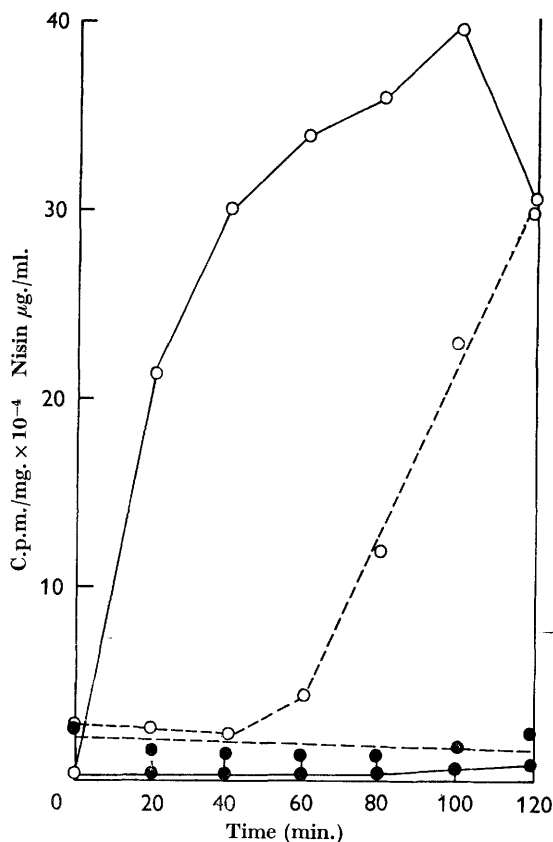

Fig. 8. The effect of actinomycin D (10 μg./ml.) added at time 0 on nisin synthesis and incorporation of [5,6-³H]-uridine by a suspension of *Streptococcus lactis* already synthesizing nisin. Radioactivity, ——; nisin, - - -; control, ○; actinomycin-D treated, ●.

Fig. 9. As Fig. 8 but this suspension of *S. lactis* was not synthesizing nisin during the first 40 min. Radioactivity, ——; nisin, - - -; control, ○, actinomycin-D treated, ●.

DISCUSSION

The evidence put forward in this paper favours the view that the major part of nisin synthesis occurs by a mechanism similar to that of normal protein synthesis. This view is based on the effect of inhibitors of protein synthesis (chloramphenicol, puromycin, terramycin) which also inhibited nisin synthesis. The effect observed might have been due either to nisin being synthesized ribosomally or to an indirect effect of the inhibitors on the formation of the enzymes concerned in nisin formation. In every case, therefore, the inhibitors were also tested under conditions when it

might be expected that the nisin-synthesizing enzymes were already formed. However, under these conditions there was also an immediate halting of nisin synthesis.

Actinomycin D when added early in the experiment inhibited RNA synthesis and nisin synthesis, suggesting that, as in the normal protein synthesis mechanism, messenger RNA is also involved in nisin synthesis. The results shown in Fig. 8 suggest that this RNA may have an unusually long half-life. The lack of effect of mitomycin suggests that the synthesis of nisin does not depend on newly formed DNA and that the code was already in the bacteria when they were harvested for experiment.

Penicillin did not inhibit nisin synthesis; cycloserine (oxamycin), an antibiotic known to interfere specifically with wall mucopeptide synthesis in *Staphylococcus aureus* (Strominger, 1962) was without effect on nisin synthesis. It does not appear likely that nisin is connected with the wall mucopeptide of the organism.

At present there is controversy about the mechanism of synthesis of gramicidin S which may be made ribosomally (Hall *et al.* 1965) or by some other means (Berg *et al.* 1965; Yukioka *et al.* 1965). However, the molecular weight of gramicidin S is only about 1350 according to Bodansky & Perlman (1964). A final proof that nisin is made ribosomally must await the development of a cell-free system derived from *Streptococcus lactis* as has been done with *Bacillus brevis* (Uemura *et al.* 1963; Okuda *et al.* 1964; Berg *et al.* 1965; Yukioka *et al.* 1965). The state of the organisms at the time of harvesting may be critical for preparations of this nature. For example, Fig. 3 here shows that freshly harvested bacteria and bacteria which had been incubated in the reaction mixture made protein at the same rate; the rate of nisin synthesis by the two suspensions was, however, very different. In the first 30 min. the pre-incubated bacteria made 6 μg. nisin/ml. reaction mixture and the freshly harvested bacteria made 1·2 μg. nisin/ml. reaction mixture. There is no simple explanation for this observation. It is also difficult to understand why, if nisin is made as other proteins on ribosomes, its synthesis should be more sensitive to chloramphenicol, puromycin and terramycin than other proteins (see, for example, Figs. 5, 6, 7). However, similar results have already been reported, for example, the preferential inhibition of β-galactosidase synthesis in *Escherichia coli* by chloramphenicol (Sypherd & Strauss, 1963) and puromycin (Sells, 1965) and of ornithine transcarbamylase synthesis by chloramphenicol (Browne & Rogers, 1963).

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