

Mode of Action of Melinacidin, an Inhibitor of Nicotinic Acid Biosynthesis

FRITZ REUSSER

Department of Microbiology, The Upjohn Company, Kalamazoo, Michigan 49001

Received for publication 8 August 1968

Melinacidin, a new antibacterial agent, blocked the synthesis of nicotinic acid and its amide in *Bacillus subtilis* cells. The inhibitory activity of the agent was reversed by nicotinic acid, its amide, or nicotinamide adenine dinucleotides, but not by L-kynurenine, L-3-hydroxykynurenine, L-hydroxyanthranilic acid, or quinolinic acid. These properties indicated that the antibiotic interferes with the conversion of quinolinic acid to nicotinate ribonucleotide by the enzyme quinolinate phosphoribosyltransferase. However, the activity of a purified preparation of this enzyme derived from a *Pseudomonas* strain was not impaired by the antibiotic. This suggested that, in *B. subtilis*, melinacidin interferes with a reaction which occurs before the formation of quinolinic acid in the biosynthetic pathway leading to nicotinic acid. Failure of quinolinic acid to reverse melinacidin inhibition in *B. subtilis* cultures might be due to insufficient penetration of the cell membranes by quinolinate.

Melinacidin is a mixture of at least four closely related compounds isolated from the culture broth of the fungus *Acrostalagmus cinnabarinus* var. *melinacidinus*. The complex has the empirical formula $C_{16}H_{17}O_4N_5S_2$ and crystallizes in the form of colorless needles which have very limited solubility in aqueous solutions. The preparation, isolation, characterization, and biological properties of this agent will be described elsewhere (Argoudelis and Reusser, *in preparation*). The agent inhibits gram-positive bacteria *in vitro*, but in mice is ineffective in the systemic treatment of experimental infections caused by gram-positive organisms. The antibiotic is very toxic in mice (tolerated dose, 2 to 3 mg/kg).

This paper describes the effects of melinacidin on protein and nucleic acid synthesis in *Bacillus subtilis* cells and in cell-free *Escherichia coli* systems. In addition, it is demonstrated that melinacidin blocks the synthesis of nicotinic acid and its amide in *B. subtilis*. It is concluded that this property is the basis for the antibacterial activity of the antibiotic.

MATERIALS AND METHODS

B. subtilis strain 23 cells were grown in a medium containing the following ingredients, per liter: K_2HPO_4 , 6 g; K_2PO_4 , 14 g; $MgSO_4$, 0.1 g; $(NH_4)_2SO_4$, 2 g; sodium glutamate, 2 g, glucose monohydrate, 4 g; tryptone (Difco), 0.2 g. Shaken flasks containing 100 ml of medium were inoculated with 5 ml of seed derived from an overnight culture. The flasks were

incubated on a rotary shaker at 37 C. Melinacidin was dissolved in dimethylformamide and was added in 0.1-ml portions per 100 ml of culture medium. Cell growth was assessed by measuring the optical density (OD) of the bacterial suspensions at 570 nm.

The cellular protein and nucleic acid fractions of *B. subtilis* were isolated and assayed as described previously (5).

The cell-free polypeptide and nucleic acid biosynthetic *E. coli* systems were prepared as described by Reusser (5).

Total nicotinic acid in the culture was determined according to the microbiological assay procedure of Snell and Wright (6), with *Lactobacillus plantarum* ATCC 8014 as the assay organism. At cell harvest, enough concentrated H_2SO_4 was added to culture samples to make them 1 N with respect to H_2SO_4 . The samples were then autoclaved at 15 lb of pressure for 30 min to hydrolyze all of the nicotinic acid-containing compounds to nicotinic acid. After hydrolysis, the samples were cooled, neutralized with concentrated NaOH, and assayed.

Pseudomonas strain ATCC 23269 was used for the preparation of quinolinate phosphoribosyltransferase. The organism was grown in a medium containing the following ingredients, per liter of deionized water: NaH_2PO_4 , 0.5 g; K_2HPO_4 , 1.5 g; $MgSO_4$, 0.15 g; quinolinic acid, 1.5 g; the pH was adjusted to 7.0 with concentrated NaOH (4). Shaken flasks containing 100 ml of medium were inoculated with 5 ml of seed derived from a culture grown overnight. The flasks were incubated at 28 C for 18 hr. Cells were recovered by centrifugation and were stored at -20 C until used.

A 6-g amount of cells (wet weight) was suspended in 24 ml of 0.1 M potassium phosphate buffer (pH 7.0),

containing 5 mm glutathione. The cells were disrupted by sonic treatment for 4 min with a Biosonik sonicator. Cell debris and unbroken cells were removed by centrifugation at $30,000 \times g$ for 20 min. The supernatant solution was made 2 mM with respect to sodium quinolate; it was then heated at 60 C for 10 min and again centrifuged at $30,000 \times g$ for 45 min. Solid $(\text{NH}_4)_2\text{SO}_4$ was then added to the recovered supernatant solution to 50% saturation at 2 C. The precipitate formed was recovered by centrifugation at $30,000 \times g$ for 15 min and was dissolved in 2 ml of 0.01 M potassium phosphate buffer (pH 7.15) containing 5 mm glutathione. This solution was applied to a diethylaminoethyl (DEAE)-cellulose column (2 by 28 cm) equilibrated with the same buffer mixture. The enzyme was eluted from the column by increasing the NaCl concentration in the eluting buffer; the mixing vessel contained 250 ml of the buffer used for the preparation of the column, the reservoir contained 250 ml of buffer containing 0.5 M NaCl. The active fractions were pooled, dialyzed, and lyophilized. Transferase activity was assayed as described by Packman and Jakoby (4).

Crude cell extracts of *B. subtilis* were prepared by sonic treatment and centrifugation of the cell homogenate at $30,000 \times g$ for 20 min as described for the *Pseudomonas* cells above. Quinolate phosphoribosyltransferase activity was assessed as described previously (4).

RESULTS

Experiments with *B. subtilis* cells. The effect of different concentrations of melinacidin on exponentially growing *B. subtilis* cells is shown in Fig. 1. At a concentration of 5 $\mu\text{g}/\text{ml}$, cell growth was impaired almost immediately after the addition of the drug. However, complete cessation of growth was not observed, even at 2 hr. Antibiotic concentrations of 1 or 0.1 $\mu\text{g}/\text{ml}$ did not cause any inhibition during the first hour after

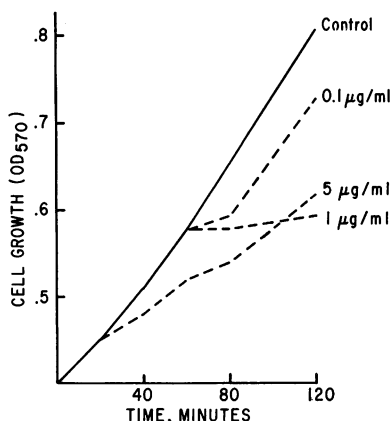


FIG. 1. Effect of melinacidin on *B. subtilis* cell growth. Antibiotic was added at the onset of exponential growth.

addition of the agent. After this time, 1 μg of melinacidin per ml caused virtually complete cessation of cell growth; 0.1 μg of melinacidin per ml caused less inhibition.

Partial inhibition of cellular protein synthesis was apparent immediately after addition of melinacidin at all concentrations (0.1, 1, 5 $\mu\text{g}/\text{ml}$) tested (Fig. 2). After approximately 50 min of exposure of the cells to 1 μg of drug per ml, protein synthesis ceased completely; however, some synthesis persisted in the presence of either 5 or 0.1 $\mu\text{g}/\text{ml}$ for up to 2 hr.

Partial inhibition of cellular deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis took place immediately after addition of 5 μg of melinacidin per ml (Fig. 2). Drug concentrations of 1 or 0.1 $\mu\text{g}/\text{ml}$ stimulated nucleic acid synthesis slightly during the first 40 min after addition of the antibiotic. After this period, both DNA and RNA synthesis became gradually impaired. Complete cessation occurred after 40 to 60 min in the presence of 1 μg of antibiotic per ml; 5 or 0.1 μg of drug per ml did not block nucleic acid synthesis completely during the duration of the experiment (2 hr).

Effects of melinacidin on macromolecular biosynthetic processes in cell-free systems. Antibiotic concentrations ranging from 5 to 40 $\mu\text{g}/\text{ml}$ did not interfere significantly with the polynucleotide-mediated incorporation of ^{14}C -phenylalanine or ^{14}C -proline in the amino acid incorporation systems prepared as described by Reusser (5). The DNA-directed RNA polymerase system remained unaffected by antibiotic concentrations of 5 to 40 $\mu\text{g}/\text{ml}$. By contrast, the DNA polymerase reaction was stimulated in the presence of relatively high antibiotic concentrations. A drug concentration of 40 $\mu\text{g}/\text{ml}$ caused approximately 100% stimulation of this reaction (Table 1). Lower concentrations of melinacidin caused less stimulation.

Reversal of melinacidin inhibition by cellular metabolites (vitamins, amino acids, etc.). The results discussed thus far indicated that the addition of melinacidin at first causes only partial inhibition of cell growth. This phenomenon was apparent even though relatively high antibiotic concentrations were used. Substantial net synthesis of protein and nucleic acid occurred during the phase immediately after addition of drug. None of the cell-free macromolecular biosynthetic systems was inhibited by the drug. The limited continuation of cell growth after addition of melinacidin suggested that the culture is gradually depleting an essential cell metabolite whose de novo synthesis is blocked by the antibiotic.

Mixtures of amino acids, purines, pyrimidines, or vitamins were thus added to cultures contain-

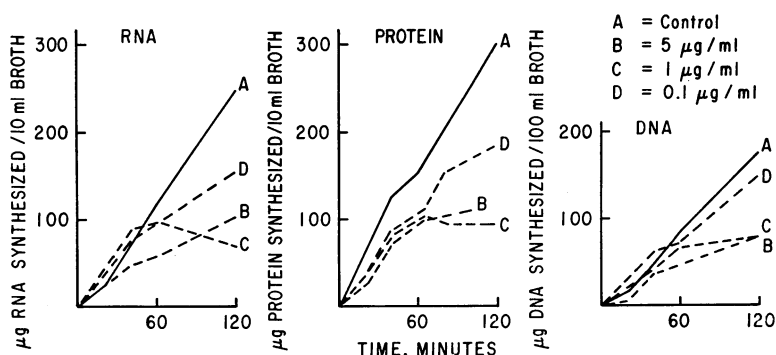


FIG. 2. Effect of melinacidin on protein and nucleic acid synthesis in *B. subtilis* cells. Protein and nucleic acid fractions were isolated and quantitated as described by Reusser (5).

TABLE 1. Effect of melinacidin on cell-free DNA-dependent DNA polymerase system^a

Sample	Radioactivity (counts/min)	Per cent of control
Less DNA.....	95.5	3.8
Control, 0 min.....	37.7	1.5
Control, 2,506.9	100.0	
Melinacidin, 5 µg/ml.	3,305.9	131.8
Melinacidin, 10 µg/ml.	3,919.7	156.3
Melinacidin, 20 µg/ml.	3,643.5	145.3
Melinacidin, 40 µg/ml.	5,450.6	217.4

^a The assay mixtures (0.5 ml) contained: glycine buffer (pH 9.2), 500 µmoles; MgCl₂, 50 µmoles; mercaptoethanol, 5 µmoles; denatured calf thymus DNA, 6.6 µg; deoxyadenosine, deoxycytidine, and deoxyguanosine triphosphates, 0.03 µmole each; ³H-deoxythymidine triphosphate, 0.03 µmole, 0.22 µC; and polymerase, 291 µg. Reactions were run at room temperature for 30 min, tubes were then chilled in ice, and 0.1 ml of 2.5 N perchloric acid containing 2 g of Celite per 100 ml was added. The acid-insoluble product was collected on 0.45-µm filters and was washed extensively with cold water; the discs were then assayed for radioactivity.

ing melinacidin in an attempt to reverse the inhibitory activity of melinacidin. Of all the metabolites tested, only nicotinamide reversed the antibacterial activity of melinacidin in *B. subtilis* (Fig. 3). A concentration of 1 µg of nicotinamide per ml caused substantial reversal of the growth inhibition induced by 5 µg of melinacidin per ml. When nicotinamide was added after cessation of cell growth following drug addition (3.5 hr after addition of the drug in the experiment shown in Fig. 3), growth of the

culture resumed. Nicotinic acid reversed inhibition by melinacidin to the same extent as did nicotinamide. Nicotinamide adenine dinucleotide (NAD) or reduced nicotinamide adenine dinucleotide were slightly less effective than nicotinic acid or its amide. At concentrations of 1 µg/ml, L-kynurenine, L-3-hydroxykynurenine, 3-hydroxyanthranilic acid, or quinolinic acid did not cause any reversal of melinacidin inhibition of *B. subtilis* cell growth.

The extent of reversal of antibiotic activity caused by nicotinamide in the presence of a constant amount of melinacidin was dependent upon the concentration of nicotinamide present in the

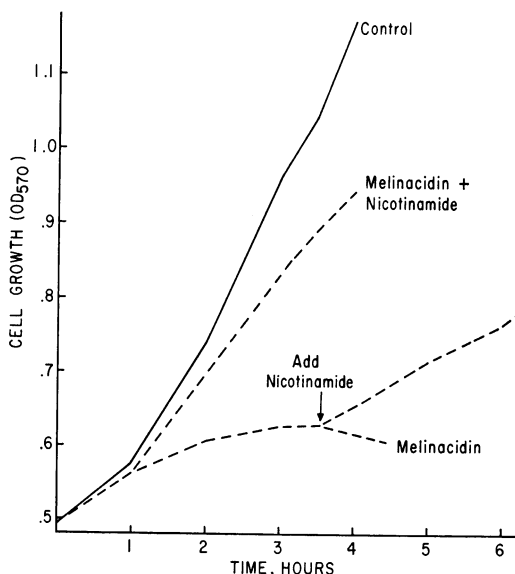


FIG. 3. Reversal of melinacidin activity in *B. subtilis* by nicotinamide. The concentration of melinacidin was 5 µg/ml; the concentration of nicotinamide was 1 µg/ml.

culture medium (Fig. 4). It is of interest to note that, at a concentration of $0.001 \mu\text{g/ml}$, nicotinamide was able to induce some reversal of melinacidin inhibition when the antibiotic was present at a concentration of $5 \mu\text{g/ml}$ (ratio of nicotinamide to melinacidin was 1:5,000). Maximal reversal was achieved at a nicotinamide concentration of $0.1 \mu\text{g/ml}$. Cell growth at this concentration was still not quite as abundant as in the control culture.

When the concentration of melinacidin varied in the presence of a constant amount of nicotinamide, the extent of reversal was found to be much greater at 0.1 or $5 \mu\text{g}$ of melinacidin per ml than at $1 \mu\text{g}$ of melinacidin per ml (Fig. 5). Thus, reversal of the antibacterial activity is not inversely proportional to the antibiotic concentration.

Effect of melinacidin on nicotinic acid synthesis in B. subtilis. The results discussed thus far implied that melinacidin prevents synthesis of nicotinic acid and nicotinamide. This assumption was further substantiated by assaying cell growth and net nicotinic acid synthesis simultaneously in the presence and absence of melinacidin. In the presence of $5 \mu\text{g}$ of melinacidin per ml, cellular growth continued at a reduced rate (Fig. 6). On the other hand, nicotinic acid synthesis ceased immediately upon addition of the drug. Total nicotinic acid present in the culture at the time of antibiotic addition was $14 \mu\text{g}/100$

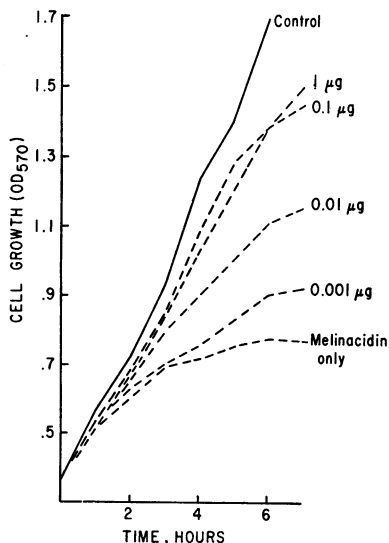


FIG. 4. Effect of different concentrations of nicotinamide on reversion of melinacidin activity. Concentration of melinacidin was $5 \mu\text{g/ml}$.

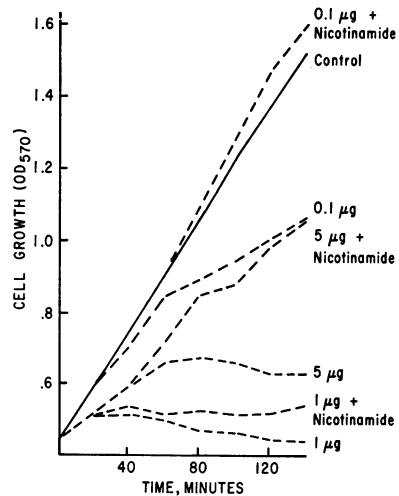


FIG. 5. Effect of different concentrations of melinacidin on *B. subtilis* cell growth in the presence and absence of nicotinamide. Nicotinamide concentration was $1 \mu\text{g/ml}$.

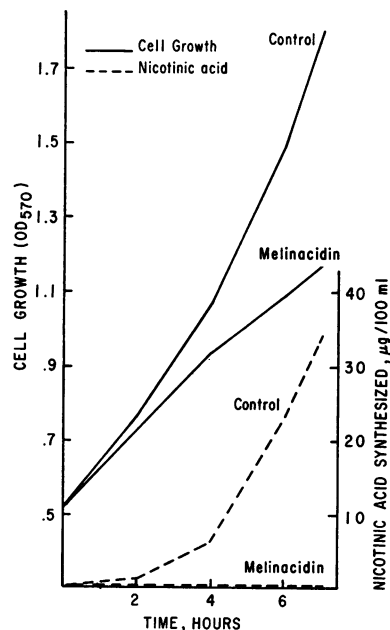


FIG. 6. Effect of melinacidin on cell growth and nicotinic acid synthesis in *B. subtilis*. Melinacidin concentration was $5 \mu\text{g/ml}$. Total nicotinic acid in the cultures at time-zero was $14 \mu\text{g}/100 \text{ ml}$. In the cultures containing melinacidin, this amount remained constant for the duration of the experiment.

ml. This amount remained constant for the duration of the experiment. Thus, no destruction of nicotinic acid in the presence of the drug took place. This observation proved that melinacidin acts as a very efficient blocking agent of nicotinic acid biosynthesis and that cessation of cell growth occurs only after the culture is apparently depleted of nicotinic acid.

Effect of melinacidin on quinolinic acid phosphoribosyltransferase. In most organisms, quinolinic acid occurs as an intermediate in the biosynthetic pathway leading to nicotinic acid (1-3). Thus, it was of interest to study the effect of melinacidin on quinolinic acid phosphoribosyltransferase. This enzyme catalyzes the formation of nicotinate ribonucleotide from quinolinate (see Discussion). An initial attempt was made to study this reaction with crude *B. subtilis* extracts. However, such cell-free extracts did not show any detectable quinolinate phosphoribosyltransferase activity during incubation times up to 90 min. The enzyme preparation used in this study was thus derived from a *Pseudomonas* strain grown on quinolinic acid as the sole carbon and nitrogen source (4). The results indicated that melinacidin had no effect on the formation of nicotinate ribonucleotide from quinolinate (Table 2). In addition, cell growth of the *Pseudomonas* strain remained unaffected in the presence of 5 μ g of antibiotic per ml.

DISCUSSION

B. subtilis cell growth and cellular protein and nucleic acid synthesis cease only after prolonged exposure of the culture to melinacidin. This observation suggests that de novo synthesis of a low molecular weight, essential cell metabolite is blocked by the antibiotic in the organism.

None of the cell-free macromolecular biosynthetic processes investigated (DNA, RNA, polypeptide biosynthesis) is inhibited by the antibiotic. The DNA polymerase reaction is sometimes stimulated. For example, a twofold increase was observed in the presence of 40 μ g of antibiotic per ml (Table 1). Stimulation was less or did not occur with other preparations of DNA polymerase. The significance of this stimulation is difficult to assess at this time.

Addition of nicotinamide or nicotinic acid to *B. subtilis* cultures in the presence of melinacidin effectively reverses the inhibitory activity of the antibiotic. An optimal concentration of melinacidin with regard to toxicity exists, since 1 μ g of melinacidin per ml consistently proved more inhibitory than either 5 or 0.1 μ g of the drug per

TABLE 2. *Effect of melinacidin on purified quinolinate phosphoribosyltransferase*^a

Sample	Change in OD at 315 nm	Per cent of control
Control, 0 min.....	0	0
Control.....	0.343	100.0
Melinacidin, 10 μ g/ml.....	0.353	102.8
Melinacidin, 5 μ g/ml.....	0.350	102.0
Melinacidin, 2.5 μ g/ml.....	0.353	102.8

^a Assay mixtures contained, in a total volume of 0.5 ml: tris(hydroxymethyl)aminomethane-chloride buffer (pH 7.2), 40 μ moles; magnesium acetate, 2.5 μ moles; quinolinic acid, 2 μ moles; PRPP, 0.4 μ moles, enzyme, 89 μ g of protein. The reactions were run at room temperature for 15 min and were stopped by the addition of 2.5 ml of 1 M NaCN. OD was measured at 315 nm.

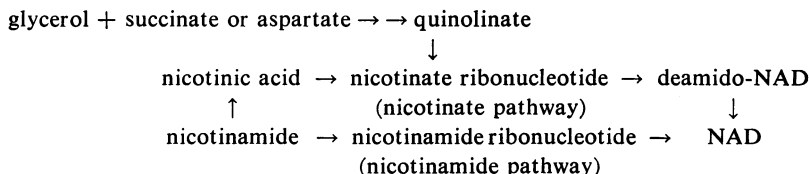
ml. This indicates that the antibiotic blocks the synthesis of nicotinic acid or its amide and does not merely serve as an antimetabolite of the vitamin.

Quantitative assays of the nicotinic acid formed in the cultures established that the synthesis of this vitamin ceases immediately upon addition of melinacidin. No destruction of nicotinic acid is apparent.

In mammals and fungi, nicotinic acid arises from tryptophan as a precursor. In bacteria, nicotinic acid is formed via the condensation of a 4-carbon dicarboxylic acid (succinic acid, aspartic acid) with a 3-carbon compound (glycerol). One or several unknown steps lead to the formation of quinolinic acid which is then converted to nicotinate ribonucleotide. Thus, quinolinic acid occurs as a common precursor in both the bacterial and mammalian pathway (1, 2). Several intermediates occurring within the mammalian pathway (L-kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid) as well as quinolinic acid did not cause reversal of melinacidin inhibition. This suggests that the antibiotic interferes with nicotinic acid synthesis at a point after the formation of quinolinate but before the formation of nicotinate ribonucleotide. Specifically, these results suggest that the antibiotic inhibits the function of quinolinate phosphoribosyltransferase. This enzyme catalyzes the reaction quinolinate + PRPP \rightarrow nicotinate ribonucleotide. Inhibition of this reaction may account for the toxicity of the antibiotic in mammals and in

bacteria. However, this reaction step, studied with a purified enzyme preparation derived from a *Pseudomonas* strain, remained totally unaffected by the antibiotic.

The proposed pathways leading to the synthesis of NAD in bacteria are shown in the scheme below (2, 3):



The fact that both nicotinic acid and its amide are equally active in reversing melinacidin inhibition suggests that the nicotinate pathway and probably also the nicotinamide pathway are functional in *B. subtilis*, unless nicotinamide enters the nicotinate pathway via deamination by nicotinamide deaminase. Thus, I postulate that melinacidin blocks nicotinic acid synthesis in susceptible bacteria at a reaction step occurring before the formation of quinolinic acid. Failure of quinolinic acid to reverse melinacidin inhibition in *B. subtilis* may be due to the inability of quinolinate to penetrate the cell membranes in an amount sufficient to cause effective reversal. A similar lack of effective penetration of quinolinate has been observed by Hayaishi et al. (2) in liver.

It must also be considered that the two quinolinate phosphoribosyltransferases present in *B. subtilis* and *Pseudomonas* may differ sufficiently in their structure to render only the *B. subtilis* enzyme susceptible to inhibition by the antibiotic. Because of the undetectable amounts of enzyme

activity in crude *B. subtilis* extracts, this possibility cannot be entirely ruled out.

In addition, synthesis of nicotinic acid in *B. subtilis* and other bacteria susceptible to the antibiotic may proceed via slightly different pathways than those postulated for most bacteria and a yet unknown step may be blocked.

ACKNOWLEDGMENTS

The technical assistance of Mary A. Conklin is gratefully acknowledged.

LITERATURE CITED

1. Chaykin, S. 1967. Nicotinamide coenzymes. *Ann. Rev. Biochem.* **36**:149-170.
2. Hayaishi, O., H. Ijichi, and A. Ichiyama. 1967. Studies on the biosynthesis of NAD by a direct method of tracing metabolism *in vivo*, p. 9-21. *In* G. Weber (ed.), *Advances in enzyme regulation*, vol. 5. Pergamon Press, New York.
3. Ohtsu, E., A. Ichiyama, Y. Nishizuka, and O. Hayaishi. 1967. Pathways of NAD biosynthesis in nicotinic acid or nicotinamide requiring microorganisms. *Biochem. Biophys. Res. Commun.* **29**:635-641.
4. Packman, P. M., and W. B. Jakoby. 1965. Quinolinate phosphoribosyltransferase. *Biochem. Biophys. Res. Commun.* **18**:710-715.
5. Reusser, F. 1967. Mode of action of antibiotic U-20,661. *J. Bacteriol.* **93**:65-70.
6. Snell, E. E., and L. D. Wright. 1941. A microbiological method for the determination of nicotinic acid. *J. Biol. Chem.* **139**:675-686.