

THE NIACIN-TRYPTOPHAN RELATIONSHIP IN THE METABOLISM OF *XANTHOMONAS PRUNI**

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The investigations of Starr (1) demonstrated that *Xanthomonas pruni* requires an exogenous source of nicotinic acid when grown on a medium containing vitamin-free, acid-hydrolyzed casein, methionine, or glutamic acid to provide an organic form of nitrogen. Preliminary work in this laboratory showed that tryptophan could replace nicotinic acid for growth in a similar medium. This was the first known demonstration of a relationship between these compounds in the metabolism of a bacterium. Recently Jakoby *et al.* (2) have noted that a nicotinic acid-requiring pseudomonad grows very slowly with tryptophan or anthranilic acid.

The long list of proposed biological precursors of nicotinic acid has resulted largely from the studies of a number of mutants of *Neurospora crassa*. There are still a number of unexplained findings (3), but there is good evidence for the precursor rôle of tryptophan in nicotinic acid synthesis which has also been established in animals fed isotopically labeled tryptophan (4). Kynurenine (5), 3-hydroxyanthranilic acid (6), and quinolinic acid (7) support the growth of rats receiving low tryptophan-niacin diets, and kynurenine (8) and 3-hydroxyanthranilic acid (7, 9) cause increased N^1 -methylnicotinamide excretion. Further studies of the possible rôle of quinolinic acid as an intermediate between 3-hydroxyanthranilic acid and nicotinic acid conducted with *Neurospora* mutants have shown that quinolinic acid is accumulated by the mutant strain 3416 (7, 10) and is utilized to a limited extent by mutant 4540 (7).

The results reported below show that anthranilic acid, indole, tryptophan, kynurenine, 3-hydroxyanthranilic acid, or quinolinic acid is capable of replacing nicotinic acid or its amide for the growth of *X. pruni*.

EXPERIMENTAL

A pure culture of *X. pruni*, isolated from the lesion of an infected peach twig, was maintained on a modified Emerson (11) agar medium and

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subcultured at 15 day intervals. Inocula were prepared from young cells grown for approximately 20 hours at 25° on modified Emerson (11) broth until the optical density of the culture was 0.62. The cells were washed three times, diluted 3-fold, and 0.05 ml. of this cell suspension was added to each 10 ml. culture tube.

The Starr (1) nutrient medium used in these tests was modified to include 1 per cent sucrose, 0.25 per cent vitamin-free, acid-hydrolyzed casein, iron as $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 5 mg. per cent of L-cystine. The compounds tested were sterilized by filtration through a Seitz filter. The culture tubes were incubated at 25° on a rotary shaker moving at a velocity of 200 r.p.m. in a circle of 56 mm. radius. Growth was estimated spectrophotometrically at various time intervals. The growth-promoting activity of all the compounds was determined simultaneously to eliminate variations caused by slight differences in the experimental conditions.

The kynurenine sulfate was isolated from rabbit urine by the method of Kallio and Berg¹ (8); the 3-hydroxyanthranilic acid was prepared by the method of Nyc and Mitchell (12). All compounds were shown by *Lactobacillus arabinosus* assay to contain insignificant amounts of nicotinic acid.

Results

A preliminary investigation of the nutritional requirements of *X. pruni* showed that an organic nitrogen source and tryptophan or nicotinic acid are essential for growth. While a detailed study of the type of nitrogen compounds required was not made, it was shown that, with a modified Henderson and Snell (13) medium, growth was not greatly impaired by the removal of single amino acids or vitamins other than nicotinic acid and tryptophan.

The data obtained from numerous dosage-response curves are shown in Table I. In general, the compounds used exhibited a degree of growth-promoting activity which is in agreement with the proposed biosynthetic pathway of niacin formation. Their relative activities in supporting half maximum growth in 72 hours were as follows; indole 0.25, L-tryptophan 0.35, DL-kynurenine 0.40, anthranilic acid 0.67, 3-hydroxyanthranilic acid 4.2, nicotinic acid 57, and nicotinamide 100. The growth-promoting activity of indole varied between tests, while the results with other compounds were nearly constant in all experiments.

¹ The kynurenine and kynurenine sulfate had no optical activity. Their identity was established by analysis and by melting point as DL-kynurenine or its sulfate. It has not been determined whether the racemic form was excreted or whether racemization occurred during isolation. The growth-promoting activity has been expressed as DL-kynurenine.

The rate of growth obtained with each compound was measured by determining the turbidity at frequent intervals during the early stages of incubation. The intermediates fell into two classes with respect to their effect upon growth rates. Anthranilic acid, indole, and L-tryptophan gave slow growth at concentrations which were adequate for optimum growth after 72 hours. The growth rate was so rapid with nicotinic acid, nicotinamide, and 3-hydroxyanthranilic acid that nearly maximum development had occurred in 36 hours. The rate of growth with DL-kynurenine sulfate fell between that of the two groups, supporting slow growth during the first 24 hours, but very rapid development between 24 and 48 hours after inoculation. This phenomenon of slow growth is distinct from the lag in the dosage-response curves described below.

TABLE I
Comparative Growth-Promoting Activity of Various Intermediates for *X. pruni*

Incubation time.....	Per cent nicotinamide activity, molar basis								
	24 hrs.			48 hrs.			72 hrs.		
	77	54	31	77	54	31	77	54	31
Transmission, %									
Nicotinic acid.....	18.0	26.5	28.0	33.3	47.0	60.0	45.0	57.0	73.0
3-Hydroxyanthranilic acid.....	2.7	6.1	10.2	2.0	4.2	8.2	2.0	4.2	8.2
Anthranilic acid.....				0.25	0.20		0.45	0.67	0.85
DL-Kynurenine.....				0.11	0.28	0.56	0.15	0.40	1.0
L-Tryptophan.....				0.083	0.19	0.27	0.13	0.35	0.77
Indole.....				0.04	0.09		0.095	0.25	0.48
Quinolinic acid.....							0.018	0.033	0.067

Although the behavior of anthranilic acid resembled that of indole and L-tryptophan in that growth was slow, the anthranilic acid curve did not show the lag noted with the other two compounds.

The data in Table I demonstrate another point in connection with the utilization of these compounds by *X. pruni*. At three arbitrary growth levels (77, 54, and 31 per cent transmission), the nicotinamide activity of all compounds, except 3-hydroxyanthranilic acid, increased as the incubation period was extended. This increase is to be expected if it is assumed that they are utilized after conversion to nicotinamide. The failure of 3-hydroxyanthranilic acid to behave this way, especially at low concentrations, may be the result of its slow destruction during incubation (6).

Relatively high concentrations of indole, L-tryptophan, and DL-kynurenine were required to initiate growth. This is demonstrated in the 72 hour dosage-response curves (Fig. 1). A slight lag in the 3-hydroxyanthranilic acid curve was also observed, especially in the early phases of

growth. In contrast, anthranilic acid was relatively more effective at low than at high concentrations (Fig. 1). Nicotinic acid and nicotinamide dosage-response curves likewise had no lag.

The difference in the dosage-response curves of anthranilic acid on the one hand and those of indole, L-tryptophan, and DL-kynurenine on the other suggests that anthranilic acid might not be serving as a precursor of indole, as has been proposed for *Neurospora* (14) and *L. arabinosus* (15). Further evidence bearing on this point was sought.

The relative decrease in activity of anthranilic acid, as the concentration was increased, was shown to be the result of slight toxicity of this acid. Indole was also toxic at high concentrations (100 to 150 μM) both

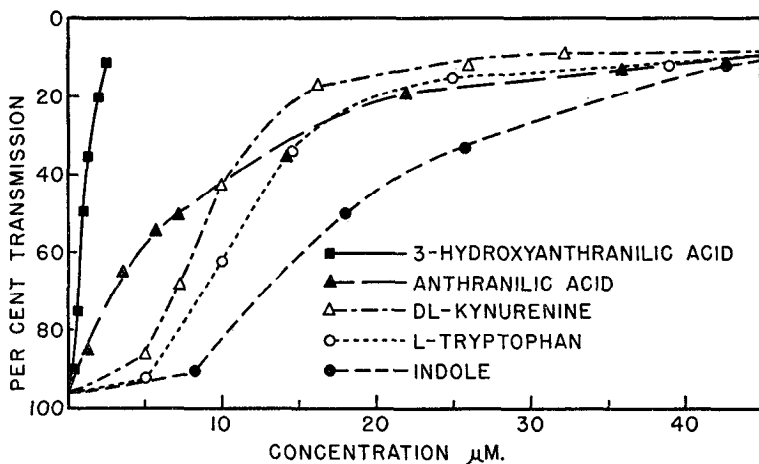


FIG. 1. Comparison of dosage-response curves at 72 hours

with and without added nicotinic acid, while tryptophan was not. The growth-suppressing effect of both anthranilic acid and indole was overcome in longer periods of growth. That the greater activity of anthranilic acid was not alone the result of its greater effectiveness in initiating growth was shown by experiments in which suboptimum levels (0.08 μM) of nicotinic acid were added to the basal medium. Under these conditions anthranilic acid was more active than indole or tryptophan at 10 μM , but about equally active at 90 μM after 24 to 36 hours of incubation.

It seemed possible that the unexpected high biological activity of anthranilic acid was the result of more effective uptake of this compound from the culture medium. To check this possibility, the uptake of L-tryptophan and anthranilic acid by *X. pruni* was determined and related to growth. L-Tryptophan was determined in the medium with *L.*

arabinosus (13) and anthranilic acid fluorometrically after dilution with a large volume of *n*-butanol by using the Coleman photofluorometer with Filters B-1-S and PC-1. Though this method was only roughly quantitative, chiefly because of the formation of fluorescent compounds by the bacterium in the later stages of growth, it was adequate for the purposes of this experiment since only gross changes were important. At high concentrations (100 μM) the uptake of anthranilic acid was more rapid than of L-tryptophan, as was growth.

The growth-promoting activity of quinolinic acid at pH 6.8 varied from 0.018 per cent of that of nicotinamide at low concentrations (77 per cent transmission) to 0.067 per cent at high concentrations (31 per cent transmission) after 72 hours of growth. It was not possible to determine its activity at low pH values, since the organism would not develop well in this medium below pH 6.0. The slight biological activity could not have been the result of nicotinic acid contamination, since *L. arabinosus* assay indicated only 0.009 per cent nicotinic acid in the sample of quinolinic acid used. Quinolinic acid at concentrations of approximately 240 μM inhibited growth. When *X. pruni* was grown in the basal medium, supplemented with tryptophan, no quinolinic acid accumulated in the culture medium. The cells contained a small amount of a compound which became active for *L. arabinosus* after autoclaving with acetic acid. These results indicated that there is no extensive accumulation of quinolinic acid such as has been found with *N. crassa* mutant 3416 (7).

The diamide of quinolinic acid and anthranilic acid amide were shown to possess no biological activity. Phenylalanine and tyrosine, supplied in the form of additional casein hydrolysate, likewise did not support growth.

DISCUSSION

These studies with a non-mutant bacterium have further emphasized the universal nature of the biosynthetic scheme previously proposed for nicotinic acid formation. With the exception of anthranilic acid, the relative molar activities of these precursors are in accord with the postulated steps. Some of the differences in activity between these intermediates can be reconciled with the findings of other investigators.

The difference in activity of nicotinic acid and nicotinamide for *Neurospora* has been explained as impermeability of the cell membrane to the dissociated form of the acid (16). Furthermore the acid may first be converted to the amide before incorporation into the coenzymes. The nicotinamide activity of L-tryptophan is in the same range as that found for the rat (7, 17), but falls below that obtained with *Neurospora* mutant 65001 (18). The variability in the growth-promoting activity of indole for *X. pruni* is in agreement with the findings of other investigators (19,

20) in their studies with *L. arabinosus*. The low biological activity of quinolinic acid could be the result of failure of the dissociated form of the acid to be absorbed from the medium. The growth-promoting activity of this compound was tested at pH levels where dissociation would be essentially complete, and where the activity for *Neurospora* mutant 4540 is very small (7). Another possibility is that quinolinic acid is not an intermediate. The inactivity of quinolinic acid diamide and anthranilic acid amide suggests that the organism is incapable of deamidating these compounds for further utilization.

An explanation of the anomalous behavior of anthranilic acid is not apparent. Its nicotinamide activity, distinctly greater than that for indole, L-tryptophan, and DL-kynurenine at lower concentrations, is not only inconsistent with the accepted steps in niacin synthesis, but stands in sharp contrast to reports that it is approximately one-third as active as tryptophan for *L. arabinosus* (19, 21, 22). The observed toxic nature confirms the findings of others (15, 22, 23).

These experiments have indicated that *X. pruni* has the ability to synthesize nicotinic acid when tryptophan is supplied. On the other hand, this organism has an adequate capacity to produce tryptophan in the presence of nicotinic acid. This observation is in agreement with the results of Beadle *et al.* (18), who suggested that nicotinic acid might act catalytically in the synthesis of tryptophan by *Neurospora* mutant 65001.

SUMMARY

Xanthomonas pruni can use tryptophan and a number of other postulated niacin precursors to satisfy its requirement for this vitamin. The relative molar activities of indole, tryptophan, kynurenine, 3-hydroxy-anthranilic acid, nicotinic acid, and nicotinamide conform with the pathway of biosynthesis indicated by studies with *Neurospora* mutants. However, anthranilic acid exhibits greater activity at low concentrations than does indole, L-tryptophan, or DL-kynurenine. Quinolinic acid possesses small but measurable activity at pH 6 to 7. High levels of indole or anthranilic acid are inhibitory in the absence or presence of nicotinic acid. The complete interchangeability of nicotinic acid and tryptophan for growth has led to the suggestion that nicotinic acid is required for tryptophan synthesis by this bacterium.

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