The Oxidation of Nicotinic Acid by *Pseudomonas fluorescens*

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The ability of certain bacterial species to oxidize nicotinic acid was first reported by Allinson (1943) who used suspensions of a *Pseudomonas* culture originally isolated by Dubos & Miller (1937). Nicotinic acid was degraded by suspensions prepared from cultures grown only in the presence of this compound. These suspensions failed to attack nicotinamide, coramine or nicotinuric acid. Allinson concluded that this oxidation was an adaptive feature of the organism.

A short time later, Koser & Baird (1944) isolated strains of *Pseudomonas fluorescens* and *Serratia marcescens* which were able to grow in a nicotinic acid medium. These strains did not attack isonicotinic acid, picolinic acid or pyridine. There was no growth or poor growth on nicotinamide, quinalinic acid or ethyl nicotinate. Nicotinethylamide and nicotindithylamide supported growth to a very slight extent.

Nichol & Michaelis (1947) suggested that an oxidase was responsible for the destruction of the nicotinic acid. Certain inhibitors were able to interfere drastically with the ability of bacterial suspensions to utilize nicotinic acid. These included sodium azide, hydroxylamine hydrochloride, octan-2-ol, laurylpyridinium chloride and toluene.

The experiments reported below were designed to elucidate further, by means of Warburg respiration technique, the utilization of nicotinic acid as a source of carbon and nitrogen by *Ps. fluorescens*. A number of nicotinic acid analogues were tested with a view to finding intermediates and thereby outlining the possible metabolic route. In addition, inhibitors were used to characterize the enzyme system.

EXPERIMENTAL

Organism. The organism used was *Ps. fluorescens* 30 of Koser & Baird (1944), which was found by Nichol & Michaelis (1947) to be the most active of the strains tested.

Media and suspensions. The broth medium, the agar slopes and suspensions of resting bacterial cells were prepared as outlined by Nichol & Michaelis (1947). In the media, apart from the agar, the only source of C and N was nicotinic acid.

Measurement of oxidation. Conventional Warburg respiration technique was used for the measurement of O_2 uptake at 27°. The total volume of reacting liquid in each flask was 2.3 ml., consisting of 0.3 ml. of cell suspension in distilled water, 0.1–1.0 ml. of the substrate under test, 0.5 ml. of 0.2M-phosphate buffer, pH 7.0, and distilled water to make up to volume. The centre well contained 0.2 ml. of 20% (w/v) KOH. The rate of shaking was 120–150 complete excursions/min. The gas space was filled with air and readings were taken at 15 min. intervals.

Determination of nicotinic acid. Nicotinic acid was determined by the method described by Nichol (1946). This is essentially a modification of Allinson's (1943) method. The difference in chromogen content (compounds reacting with CNBr) of an enzyme-treated sample as compared to one untreated, gives an accurate measure of the nicotinic acid content. The following procedure was used. Two liquids were treated in the same manner, except that one sample had 0.1 ml. distilled water replacing 0.1 ml. of cell suspension used in the other sample. Cell suspension (0.1 ml.), prepared as described, or distilled water and 0.5 ml. of 0.2M-phosphate buffer, pH 6.0, were added to 5 ml. of test solution. After standing at room temperature for 30 min., the mixtures were centrifuged at high speed. Samples (1 ml.) of CNBr (freshly prepared by shaking successive portions of powdered KCN with saturated bromine water until the colour disappeared) were added to 5 ml. of the above supernatant and held at 60° for 7–10 min. The test tubes were cooled to 20° and held at this temperature for 5 min. after which 1 ml. of 5% (w/v) aqueous *p*-phenylenediamine was added. The solutions were mixed by inversion and 0.5 ml. of 20% (w/v) HCl was immediately added. Readings were made in a Coleman Universal spectrophotometer at 420 m μ . The difference in optical densities of the enzyme-treated and the enzyme-untreated samples formed the basis for the calculation of the nicotinic acid content.

RESULTS

The stoichiometric relationship between oxygen consumption and nicotinic acid breakdown. By the use of Warburg technique, a series of experiments was carried out with varying amounts of nicotinic acid as substrate. It was assumed that respiration on nicotinic acid came to an end at that point where the curve of oxygen consumption against time flattened out. The total oxygen consumptions at these points were plotted against the μ moles of nicotinic acid originally in the substrates. The results are shown in Fig. 1. The points obtained lie close to a straight line and show that the degradation of 1 mole of nicotinic acid was accompanied by the

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uptake of 3.9 moles of oxygen. This value is distinctly higher than that of 2.7 reported by Nichol & Michaelis (1947).

Fig. 1. O₂ consumption by *Pseudomonas fluorescens* with varying quantities of nicotinic acid as substrate. The side arms of each manometer vessel contained the amounts of nicotinic acid listed. The main chamber contained 0.5 ml. 0.2 M-phosphate buffer, pH 7.0, 0.3 ml. cell suspension, and water to make up 2.3 ml. total volume excluding KOH. The centre well contained 0.2 ml. 20% (w/v) KOH. Temp., 27°; gas, air. Values shown were obtained after the substrate was exhausted.

As shown subsequently (Table 1), the ratio of oxygen consumed to nicotinic acid degraded gradually rose and did not reach the maximal figure until all of the nicotinic acid had disappeared.

Nichol & Michaelis (1947) determined the stoichiometric ratio before the autorespiration stage had been reached and when nicotinic acid was still present in the substrate. Therefore they could not have obtained the final stoichiometric relationship reported here. It appears likely that they would have obtained the higher ratio had they allowed the oxidation to go to completion.

The relationship between oxygen consumption and degradation of nicotinic acid. In the following Warburg experiment, the nicotinic acid in the substrate was 2.17×10^{-3} M. At intervals, a pair of flasks was removed and one drop of a 1% (w/v) aqueous solution of anhydrous mercuric chloride was added to each flask in order to stop any further reaction. The suspension in each flask was centrifuged at 10 000 rev./min. and 1 ml. of the supernatant was diluted to 100 ml. with distilled water. The nicotinic acid content of the resultant solution was determined as previously described. Table 1 gives the results obtained.

The results show that the molar ratio between oxygen consumed and nicotinic acid oxidized gradually increased during the time the cell suspension was respiring, and the maximum was obtained only when the substrate was completely oxidized. Apparently the experiment was stopped before all the intermediates were oxidized so that even though no nicotinic acid was detectable, the molar ratio had not reached 3.9 as obtained in other experiments.

We may also conclude from these results that the first stage in the degradation of nicotinic acid requires less than 1 mole of oxygen per mole of nicotinic acid. The first determinations were made after 5 min. but it seems likely that had a determination been made earlier, a ratio of one atom of oxygen per mole of nicotinic acid would have been obtained.

Hale, Davis & Baldwin (1942) have shown that the cyanogen bromide reaction is given by all α -unsubstituted pyridine compounds. The failure of the nicotinic acid residue to react with cyanogen

Table 1. *Ratio of oxygen consumed to nicotinic acid destroyed by Pseudomonas fluorescens as respiration proceeds*

(The main chamber of the manometer vessels contained 0.5 ml. 0.2 M-phosphate buffer, pH 7.0, 0.3 ml. cell suspension and 1.3 ml. water. The centre well contained 0.2 ml. 20% (w/v) KOH; 0.2 ml. 0.025 M-nicotinic acid was in the side arm. Temp., 27°; gas, air.)

Nicotinic acid destroyed (μmoles)	Percentage of total nicotinic acid	Corresponding O ₂ consumption (μmoles)	Percentage of ultimate O ₂ consumption	Molar ratio O ₂ consumed to nicotinic acid oxidized
2.46	49	1.92	11	0.78
2.70	54	2.22	12	0.82
3.94	79	4.64	25	1.18
4.61	92	5.99	33	1.30
4.67	93	10.42	57	2.23
4.67	93	14.42	79	3.09
5.00	100	18.25	100	3.65

bromide is therefore due to α -substitution or the opening of the pyridine ring. Experiments described subsequently indicate that carbon dioxide is produced early in the oxidation. In addition, several analogues of nicotinic acid where the pyridine ring was intact were relatively inert. The evidence, therefore, strongly suggests that the initial oxidation involves opening of the pyridine ring. The major part of the oxygen consumption apparently involved the oxidation of products of degradation.

Table 2. *Respiratory quotients of Pseudomonas fluorescens on a nicotinic acid substrate*

(Manometric technique involving the 'direct method' for CO_2 was employed. The side arm of each vessel contained 0.2 ml. 0.025 M-nicotinic acid. The main chamber contained 0.5 ml. 0.2 M-phosphate buffer, pH 7.0, 0.3 ml. cell suspension and 1.3 or 1.5 ml. water. The centre well contained 0.2 ml. 20% (w/v) KOH or was empty. Temp., 27°; gas, air.)

Time (min.)	O_2 consumed ($\mu\text{l.}$)	CO_2 produced ($\mu\text{l.}$)	R.Q.
15	66.9	38.2	0.57
30	151.9	89.2	0.59
45	251.9	181.8	0.72
60	366.6	294.5	0.80
75	489.9	419.9	0.86
100	534.1	477.6	0.89
115	543.3	490.6	0.90

Respiratory quotient. The respiratory quotient of a cell suspension on nicotinic acid substrate was determined by manometric technique using the 'direct method' for carbon dioxide. The results obtained are listed in Table 2. The calculations were made on the basis of the substrate remaining at pH 7.0. Since the bicarbonate errors in the calculations of carbon dioxide output are large and increase as the pH rises, the values obtained are most probably too low, especially for the later parts of the experiment. It has been found that under the

experimental conditions, respiring suspensions of *Ps. fluorescens* on nicotinic acid become alkaline, the pH rising beyond 7.8. The final respiratory quotient value of 0.90 most probably approaches unity.

From the above data, the relationship at maximum oxidation, of 3.5 moles of carbon dioxide produced per mole of nicotinic acid destroyed, is obtained. It must again be emphasized that this value is minimal and most likely approaches 3.9. The results therefore agree approximately with those expected if four of the six carbon atoms of the nicotinic acid molecule form carbon dioxide.

The evolution of carbon dioxide at an early stage in the oxidation cannot be attributed to decarboxylation because pyridine will react with cyanogen bromide whereas the product obtained failed to react.

Anaerobic activity. Measurements of anaerobic activity were made by the method of Quastel & Wheatley (1938). There appeared to be no anaerobic degradation of nicotinic acid, thus corroborating the work of Nichol (1946) who used Thunberg technique. It is clear, therefore, that the initial attack on the nicotinic acid molecule does not take place in the absence of oxygen, and this phase of the degradation of nicotinic acid must be a specific oxidative action.

The effects of cell suspensions on other compounds in the presence and absence of nicotinic acid. The compounds used included those with the pyridine ring, indole derivatives and aromatic diketones. Table 3 gives the results obtained with the pyridine analogues used.

The effect of the organism on compounds containing the indole nucleus, especially tryptophan, was determined because of the numerous references in the literature that tryptophan was a precursor of nicotinic acid in the metabolism of a number of genera (Haskins & Mitchell, 1949; Rosen, Huff & Perlzweig, 1946). Table 4 gives the results obtained with indolylpropionic acid and with L-tryptophan.

Table 3. *Effects of suspensions of Pseudomonas fluorescens on various pyridine derivatives*

(The side arms of each manometer vessel contained either one of the compounds listed below or 0.2 ml. 0.025 M-nicotinic acid or both. The main chamber contained 0.5 ml. 0.2 M-phosphate buffer, pH 7.0, 0.3 ml. cell suspension and water to make up 2.3 ml. excluding KOH. The centre well contained 0.2 ml. 20% (w/v) KOH. Temp., 27°; gas, air. All values corrected for autorespiration.)

Compound	Final concentration (mM)	Duration of exp. (min.)	O_2 uptake ($\mu\text{l.}$)			Change (%)
			With compound	With nicotinic acid	With both	
2-Aminopyridine	2.17	100	3.7	401.7	467.9	16.5
3-Aminopyridine	2.17	115	3.7	523.6	483.4	-7.7
Coramine	2.17	75	8.3	389.1	453.7	16.6
Nicotinamide	8.54	140	13.8	388.6	391.6	0.8
Nicotine	0.677	135	0.9	366.4	414.6	13.2
Picoline	5.04	170	7.6	380.3	422.4	11.1
Pyridine	2.16	150	10.3	378.7	432.4	14.2
Pyridoxin	1.28	90	1.0	352.3	387.9	10.1
Sodium pyridine-3-sulphonate	4.35	90	7.2	430.3	430.3	0
Trigonelline	2.17	100	12.6	401.7	491.7	22.4
Sodium 3-sulphanilamidopyridine	3.43	90	2.1	430.3	388.9	-9.6

3.2

It may be seen that the oxygen consumption of the suspension in the presence of any of the above compounds (pyridine and indole analogues) when used as the sole substrate, was negligible. In all cases where the oxygen consumption in the presence of any of the above compounds and nicotinic acid was greater than the oxygen consumption on the latter substrate alone, the increase was only evident after the nicotinic acid had been completely exhausted.

than the autorepiration. Whereas the diphenol was inert, the corresponding diketone was a very effective inhibitor.

The above effect was eliminated by the addition of D-cysteine hydrochloride. With 1.85×10^{-3} M-benzoquinone and 8.35×10^{-4} M-cysteine the oxygen consumption was 83 % of normal. When the benzoquinone was added 15 min. before the cysteine, the inhibition was not neutralized. In the concentrations used, the cysteine appeared to neutralize the

Table 4. *Effects of suspensions of Pseudomonas fluorescens on L-tryptophan and indolylpropionic acid*

(The side arms of each manometer vessel contained either one of the compounds listed below or 0.2 ml. 0.025 M-nicotinic acid or both. The main chamber contained 0.5 ml. 0.2 M-phosphate buffer, pH 7.0, 0.3 ml. cell suspension and water to make up 2.3 ml. excluding KOH. The centre well contained 0.2 ml. 20 % (w/v) KOH. Temp., 27°; gas, air. All values corrected for autorepiration.)

Compound	Final concentration (mM)	Duration of exp. (min.)	O ₂ uptake (μl.)			Change (%)
			With compound	With nicotinic acid	With both	
L-Tryptophan	2.17	115	8.9	523.6	519.1	0.9
Indolylpropionic acid	2.17	90	13.2	356.1	424.8	19.3

Table 5. *Influence of cyclic diketones and quinol on the oxygen consumption of Pseudomonas fluorescens on a nicotinic acid substrate*

(The side arms of each manometer vessel contained either one of the compounds listed below or 0.2 ml. 0.025 M-nicotinic acid or both. The main chamber contained 0.5 ml. 0.2 M-phosphate buffer, pH 7.0, 0.3 ml. cell suspension and water to make up 2.3 ml. total volume excluding KOH. The centre well contained 0.2 ml. 20 % (w/v) KOH. Temp., 27°; gas, air.)

Compound	Final concentration (M)	Duration of exp. (min.)	O ₂ consumption (μl.)				Inhibition (%)
			Auto-respiration	With compound	With nicotinic acid	With both	
Benzoquinone	4.10×10^{-3}	105	20.3	19.3	473.5	20.6	99.9
Benzoquinone	8.60×10^{-4}	120	5.1	—	427.5	307.9	28.3
Quinol	3.06×10^{-3}	105	20.3	15.4	473.5	490.1	0
Quinhydrone	8.93×10^{-4}	105	20.3	9.8	473.5	27.6	98.4
1:2-Naphthoquinone	1.30×10^{-4}	120	5.1	—	427.5	67.7	85.2
1:4-Naphthoquinone	3.30×10^{-4}	120	5.1	—	427.5	17.0	97.2

Quinones. The first diketone tested was isatin which was selected because it had the indole nucleus in its structure. This compound inhibited the respiration of a cell suspension on a nicotinic acid substrate when added in a concentration of 1.75×10^{-3} M. When the isatin was added before the nicotinic acid, inhibition was more marked. This inhibition seemed to be of a competitive nature since an increased concentration of nicotinic acid raised the oxygen consumption when the isatin concentration remained constant.

Since indole was inactive, it appeared that the inhibitory effect of the isatin might be attributed to the ketone groups in the molecule. In order to verify this assumption, experiments were conducted using a number of aromatic diketones as well as quinol as listed in Table 5.

The oxygen consumption, when any one of these compounds was the sole organic substrate, was less

inhibiting effect of the benzoquinone when the molar ratio of the former to the latter was 1:2. Displacement of this equilibrium either way caused pronounced inhibition. When cysteine was 4.5×10^{-3} M and benzoquinone was 3.28×10^{-3} M, the resulting inhibition was 68.7 %.

The effects of other inhibitors. A number of other inhibitors were tested for their effects on the nicotinic acid-oxidizing ability of the cell suspension and gave the results listed in Table 6.

The 2:2'-dipyridyl was dissolved in 10 % (v/v) ethanol because it was insoluble in water. Other experiments have shown that the ethanol itself has no inhibitory action but supports cell respiration to a considerable extent. The inhibition caused by the 2:2'-dipyridyl is therefore solely due to this compound.

From the results it is evident that the enzyme studied involves the participation of a metal.

DISCUSSION

The stoichiometric relationship between nicotinic acid oxidized and oxygen consumed indicates that the oxidation of the nicotinic acid molecule proceeds to over 70 % of total theoretical oxidation. The relationship found was 3.9 moles of oxygen consumed per mole of nicotinic acid degraded. By calculation, if the nitrogen of the nicotinic acid molecule forms ammonia; the hydrogen, water; and the carbon, carbon dioxide; 11 atoms of oxygen would be required for complete oxidation. The value of 7.8 atoms indicates over 70 % total oxidation. Experiments in this laboratory have shown that ammonia is produced early in the reaction so that the nitrogen of the nicotinic acid molecule undoubtedly forms this product.

strongly supports the assumption that this initial attack involved the opening of the pyridine ring.

The work with the nicotinic acid analogues has clearly emphasized the specific nature of the oxidase. If the carboxyl group of the nicotinic acid molecule is treated to form the amide, or replaced by a sulphonic acid group, an amino group or a sulph-anilamide group or removed entirely, the enzyme is inactive on the resulting compound. The work of Koser & Baird (1944) indicates that the carboxyl group must be in the 3 position.

The interpretation of the results according to Stanier's (1947) theory of simultaneous adaptation showed that none of the pyridine or indole compounds tested was involved in any of the intermediate steps in the degradation of nicotinic acid. Here we have additional evidence for the conclusion

Table 6. *Effects of certain inhibitors on the oxygen consumption of Pseudomonas fluorescens on a nicotinic acid substrate*

(The side arms of each manometer vessel contained either one of the inhibitors listed below or 0.2 ml. 0.025 M-nicotinic acid or both. The main chamber contained 0.5 ml. 0.2 M-phosphate buffer, pH 7.0, 0.3 ml. cell suspension and water to make up 2.3 ml. total volume excluding KOH. The centre well contained 0.2 ml. 20 % (w/v) KOH. Temp., 27°; gas, air.)

Compound	Final concentration (M)	Duration of exp. (min.)	O ₂ consumption (μl.)				Inhibition (%)
			Auto-respiration	With compound	With nicotinic acid	With both	
Dichloroacetic acid	2.60×10^{-2}	90	18.6	—	406.2	17.4	100
2:2'-Dipyridyl	2.44×10^{-3} in 10 % (v/v) ethanol	90	7.2	107.0	364.5	187.3	68.8
Iodoacetic acid	2.17×10^{-4}	90	7.0	—	227.4	63.2	74.5
8-Hydroxyquinoline	1.10×10^{-3}	135	11.6	15.7	378.0	66.5	85.0
Sodium cyanide	4.80×10^{-3}	90	18.6	—	406.2	53.4	91.0
Disodium hydrogen arsenite	1.87×10^{-4}	120	29.3	—	541.8	331.7	41.0
Sodium fluoride	2.17×10^{-3}	75	18.3	—	329.7	325.7	1.3
Sodium diethyl-dithiocarbamate	2.50×10^{-3}	75	48.7	29.8	292.5	99.1	83.4
Sodium azide	6.69×10^{-2}	90	13.3	—	286.5	47.8	87.4

The respiratory quotient was 0.90 in the later stages of the respiration. As previously indicated, this value is minimal and most probably approached unity. Therefore only two carbon atoms of the nicotinic acid molecule form unknown products; the other four form carbon dioxide.

The fact that there is apparently no reaction under anaerobic conditions supports the work of Nichol & Michaelis (1947). We may conclude that the initial attack on the nicotinic acid molecule is essentially an oxidative action and that the enzyme responsible is a true oxidase. The relationship between nicotinic acid oxidation and oxygen consumption—as the oxidation proceeds—supports the above assumption. A ratio of 0.78 mole of oxygen per mole of nicotinic acid was obtained in the first 5 min. Most likely 1 atom of oxygen was used to degrade the nicotinic acid molecule to the point where it no longer gave the cyanogen bromide reaction. The evidence

that the first step in the oxidation of the nicotinic acid molecule is the opening of the pyridine ring.

Any increased oxygen consumption when the substrate was nicotinic acid and any one of the above compounds, apparently was due to interaction between these compounds and degradation products of the nicotinic acid molecule. There could have been no adaptation to any of these substrates because the compound alone supported virtually no cell respiration and active cell suspensions were used in all cases.

The enzyme system studied here appears to have no connexion at all to the system in some species which converts tryptophan to nicotinic acid.

The experiments where known inhibitors were used indicate that a metal took part in the enzyme action.

Attempts to isolate active cell-free preparations were unsuccessful. No components could be

dialysed away when cell suspensions were dialysed in a cellophan sausage skin in running tap water and in distilled water. Ultrasonic irradiation at 350 kcyc./sec. under an atmosphere of air, carbon dioxide or nitrogen resulted in inactive suspensions. The decrease in activity apparently paralleled the rupturing of the cells. Allinson (1943) and Nichol & Michaelis (1947) were also unsuccessful in obtaining active cell-free enzyme preparations.

SUMMARY

1. Cell suspensions of *Pseudomonas fluorescens* oxidized nicotinic acid, consuming 3.9 molecules of oxygen per molecule of nicotinic acid attacked.

2. The respiratory quotient was low at the start of the oxidation but rose to 0.90 towards the end of the respiration.

3. The degradation of the nicotinic acid did not take place under anaerobic conditions. The enzyme involved in the initial attack on the molecule is therefore a true oxidase.

4. The evidence obtained very strongly supports the conclusion that the first step in the degradation of the nicotinic acid molecule is the opening of the pyridine ring.

5. The specificity of the enzyme has been studied. If the carboxyl group of the nicotinic acid

molecule is treated to form the amide, or replaced by a sulphonic acid group, an amino group, or a sulphanilamide group, or removed entirely, the enzyme is inactive on the resulting compound.

6. Indolylpropionic acid and L-tryptophan were not attacked.

7. Aromatic diketones and cysteine inhibited nicotinic acid oxidation. The effect of one could be neutralized by the other at specific concentration ratios.

8. The enzyme studied contains a non-dialysable metal.

9. Attempts to obtain active cell-free enzyme preparations were unsuccessful.

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o-Phenylenediamine and 1:2-Diamino-4-nitrobenzene as Reagents for α -Keto Acids

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It is possible to isolate and characterize as 2:4-dinitrophenylhydrazones many of the keto acids formed by *Penicillium chrysogenum* and thus to study the mould's intermediary metabolism. These hydrazones are, however, sometimes neither adequately stable nor chromatographically separable.

The condensation of *o*-phenylenediamine (OPD) with α -ketonic acids has been shown to yield stable

quinoxalinols (cf. Hinsberg, 1887), which were found to be separable by paper chromatography. These derivatives were most useful, but it was thought that the introduction of a chromogenic grouping might make chromatography easier. We chose 1:2-diamino-4-nitrobenzene (DANB) in preference to a diazo derivative, as this might involve an increase in molecular size such as to swamp the effect of differences in the original