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THE PURIFICATION AND PROPERTIES OF NICOTINE OXIDASE

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SUMMARY

Nicotine oxidase, isolated from *Arthrobacter oxydans* (strain P-34), was purified 139-fold by precipitation with $(NH_4)_2SO_4$, selective heat denaturation, and chromatography on TEAE-cellulose. The enzyme was inhibited by quinacrine and acriflavine. Riboflavin 5′-phosphate completely protected the oxidase against the effects of acriflavine inhibition, whereas flavin–adenine dinucleotide was only partially effective. The enzyme was also inhibited by potassium cyanide, α,α'-dipyridyl, and 1,10-phenanthroline. These data suggest that nicotine oxidase is a metalloflavoprotein in which the flavin component is riboflavin 5′-phosphate.

INTRODUCTION

A partially purified enzyme from *Arthrobacter oxydans* (strain P-34) was previously shown to catalyze the incorporation of oxygen from water into nicotine to form 6-hydroxynicotine (ref. 1). DECKER AND BLEEG[2] recently designated the enzyme nicotine oxidase. The reaction catalyzed by nicotine oxidase was postulated to consist of a nucleophilic attack by hydroxyl ion on the pyridine ring to yield a pseudo-base which was subsequently oxidized in a step requiring the addition of an electron acceptor[3]. Thus, either the enzyme is capable of carrying out the double function of hydration and oxidation, or nicotine oxidase consists of two moieties: one that hydrates nicotine to the pseudo-base, and one which oxidizes the resulting pseudo-base to 6-hydroxynicotine. This paper presents data on some of the properties of the latter function and describes an improved method for the purification of nicotine oxidase.

MATERIALS AND METHODS

Assay of nicotine oxidase

Nicotine oxidase was assayed by either of two methods: a colorimetric procedure or a spectrophotometric one. The latter method was employed only with the A_2

Abbreviation: PCMS, *p*-chloromercuriphenylsulfonic acid.

fraction since the other fractions contained levels of 6-hydroxynicotine oxygenase which interfered with the oxidase assay.

In the colorimetric procedure, reaction mixtures contained the following components in a final vol. of 1 ml: nicotine, 10 μmoles; brilliant cresyl blue, 0.126 μmole; sodium pyrophosphate (pH 7.9), 50 μmoles; catalase, 100 μg; and from 1 to 5 units of oxidase. Following the addition of enzyme, the reaction mixtures were shaken in a 30° water bath for 30 min. Oxidase activity was terminated by the addition of 9 ml of 5% trichloroacetic acid. Appropriate aliquots were assayed for residual nicotine by a modification of the method of McCORMICK AND SMITH[4] scaled down to a vol. of 8.5 ml. The principal changes in the procedure were: the substitution of *p*-aminobenzoic acid for β-naphthylamine; the development of the chromogen at 28°; and the measurement of the yellow chromogen at 470 mμ.

The spectrophotometric assay was carried out in a Zeiss PMQ II spectrophotometer equipped with a Zeigler thermostated cuvette chamber and a TEW convertor. The change in absorbance was transcribed on a Varian G-40 recorder. Unless stated otherwise, the assay was initiated by the addition of oxidase after the reaction mixture had reached 30°. Spectrophotometric reaction mixtures contained the following additions in a total vol. of 3 ml: nicotine, 3 μmoles; brilliant cresyl blue, 0.075 μmole; sodium pyrophosphate buffer (pH 7.9), 150 μmoles; catalase, 100 μg; and sufficient enzyme so that the change in absorbance at 295 mμ was no greater than 0.125 per min. The change in absorbance was related to the amount of 6-hydroxynicotine formed by employing 6.76 for the millimolar absorptivity coefficient.

Assay of 6-hydroxynicotine oxygenase

The oxidation of 6-hydroxynicotine to 6-hydroxypseudooxynicotine (ref. 5), catalyzed by 6-hydroxynicotine oxygenase[2], was assayed spectrophotometrically by determining the formation of 6-hydroxypseudooxynicotine.

The reaction mixture contained the following components in a total vol. of 1 ml: (—)-6-hydroxynicotine, 1 μmole; Tris–HCl (pH 8.6), 80 μmoles; catalase, 100 μg; and sufficient oxygenase to cause an absorbance change of no more than 0.1 per min at 328 mμ. The assay was initiated by the addition of oxygenase after the reaction mixture had reached 30°. The change in absorbance, recorded as previously described in the oxidase assay, was related to the amount of 6-hydroxypseudooxynicotine formed by employing 19.6 for the millimolar absorptivity coefficient.

Units

In order to make the spectrophotometric assays comparable to the colorimetric assay, the changes in absorbance per min were converted to changes in μmoles per min and multiplied by 30. A unit of oxidase is defined as the amount of enzyme that causes 1 μmole of nicotine to disappear in 30 min (colorimetric oxidase assay) or 1 μmole of 6-hydroxynicotine to appear in 30 min (spectrophotometric oxidase assay). A unit of oxygenase is defined as the amount of enzyme that causes 1 μmole of 6-hydroxypseudooxynicotine to appear in 30 min.

Specific activity is defined as units per mg of protein.

Protein determination

Protein was determined by the method of LOWRY *et al.*[6], using crystalline bovine serum albumin as the standard.

Chemicals

Nicotyrine dipicrate, nornicotine, and anabasine were obtained from the Aldrich Chemical Company. Nicotine-*N*-oxide and myosmine were generously donated by Dr. T. Tso, U. S. Dept. of Agriculture, Beltsville, Md. Nicotine was obtained from Distillation Industries and used without subsequent purification; (−)-6-hydroxynicotine was prepared from 'spent' growth media *via* silicotungstic acid precipitation as reported previously[3], and purified by chromatography on Dowex-50 (ref. 1). The resulting product exhibited a 232/295-mμ ratio of 2.13 in 0.1 M HCl and a melting point of 119–122° (uncorr.). 6-Hydroxypseudooxynicotine was prepared from (−)-6-hydroxynicotine by incubating the latter with a partially purified preparation of 6-hydroxynicotine oxygenase. The oxygenase was obtained during the purification of the oxidase on TEAE-cellulose. Brilliant cresyl blue was obtained from the General Aniline Corporation; catalase from Sigma Chemical Corporation; FAD and FMN from CalBiochem; and special enzyme-grade $(NH_4)_2SO_4$ from Mann Research Laboratories.

TEAE-cellulose (Cellex-T) was obtained from the Bio Rad Corporation. It was washed sequentially with 1 M NaOH, water, 1 M HCl, and water, the process being repeated until no further color could be leached from the cellulose. The fines were removed during the washing procedure by sedimentation and decantation. The washed cellulose was equilibrated with 20 mM potassium phosphate–5 mM EDTA buffer (pH 7.0). Columns were prepared by pouring the cellulose into a chromatographic column fitted with a porous Teflon disc. After the cellulose was allowed to settle, the column was packed under slight air pressure, and equilibrated with 20 mM potassium phosphate–5 mM EDTA buffer (pH 7.0). A 2.5 cm × 19 cm column was used to chromatograph 205 mg of Fraction HA. It was operated at a flow rate of 0.75 ml/min, collecting 6 ml per fraction.

Microorganism

A. oxydans (P-34) was grown as previously described[1] and stored at −19° until used. Such cells retained their initial oxidase activity for approx. 4 months. Loss of activity was accompanied by the appearance of a purple pigment in the cell mass.

RESULTS

Purification of nicotine oxidase

Unless stated otherwise, all operations were carried out at 4°. A 15% cell suspension (w/v) in 1 mM sodium pyrophosphate (pH 7.0)–1 mM EDTA was disrupted in a Branson Sonifier for 30 min at an output of from 6.6 to 7.2 A. The resulting suspension was centrifuged for 10 min at 9800 × *g* and the supernatant decanted and saved. The sediment was made up to one-half the volume of the initial cell suspension with 1 mM sodium pyrophosphate–1 mM EDTA buffer (pH 7.0) and disrupted for an additional 30 min. The resulting suspension was centrifuged at 9800 × *g* for 10 min, the supernatant combined with the initial supernatant, and the combined supernatant fractions centrifuged for 30 min at 9800 × *g*. The clear, yellow supernatant was decanted and labeled Fraction C.

$(NH_4)_2SO_4$ fractionation[7], to yield Fraction A_1, and the subsequent selective heat denaturation of A_1, to yield Fraction H, were carried out as previously described[1].

Fraction HA was obtained by adding $(NH_4)_2SO_4$ to Fraction H so as to obtain a precipitate between 35 and 50% satn. with respect to $(NH_4)_2SO_4$. The precipitate was recovered by centrifugation at 9800 × *g* for 10 min, made up to approx. 15% of the original volume of the crude extract with 1 mM sodium pyrophosphate–1 mM EDTA buffer (pH 7.0), and labeled Fraction HA.

Fraction HA was applied to a TEAE-cellulose column. Following adsorption the column was washed with 1 mM sodium pyrophosphate–1 mM EDTA buffer (pH 7.0) (Fractions 4–30) which resulted in the elution of a large amount of protein and oxygenase. No oxidase activity was detected in these fractions. The column was then washed with 20 mM potassium phosphate buffer (pH 7.0)–5 mM EDTA–250 mM NaCl. The yellow fraction eluted with this buffer (Fractions 42–50) contained the bulk of the residual oxygenase activity (the peak fraction was used to prepare 6-hydroxypseudooxynicotine). A linear gradient, constructed with 300 ml of 20 mM potassium phosphate buffer (pH 7.0)–5 mM EDTA–250 mM NaCl in the mixing chamber and 300 ml of 20 mM potassium phosphate buffer (pH 7.0)–5 mM EDTA–500 mM NaCl in the reservoir, was subsequently connected to the column. Oxidase activity was eluted between 0.28 and 0.38 M NaCl (Fig. 1) and could be recognized by the beige color of the oxidase-rich fractions. Those fractions exhibiting the highest specific

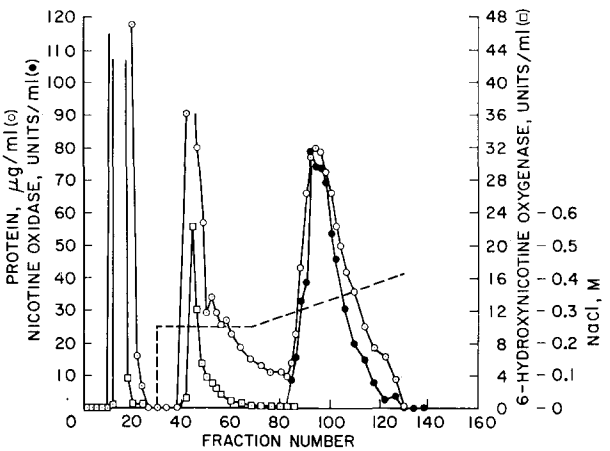


Fig. 1. Purification of nicotine oxidase by TEAE-cellulose chromatography. See text for details. ●, units/ml nicotine oxidase, determined colorimetrically; □, units/ml 6-hydroxynicotine oxygenase; ○, mg/ml protein; — — —, M NaCl.

activity were combined, saturated 80% with respect to $(NH_4)_2SO_4$, and centrifuged for 60 min at 20 200 × *g*. The brown precipitate was brought to approx. 1/50 the original volume of the crude extract with 1 mM sodium pyrophosphate–1 mM EDTA buffer (pH 7.0), yielding an orange-brown solution (Fraction A_2). The A_2 Fraction could be stored at −19° for approx. 1 month without loss of activity, provided it was not refrozen once thawed, or it could be kept at 4° for 2–3 days without significant loss of activity.

Table I represents the results of a typical isolation. The overall recovery of enzyme activity was 23%, with a 139-fold increase in purification. In 6 other isola-

TABLE I

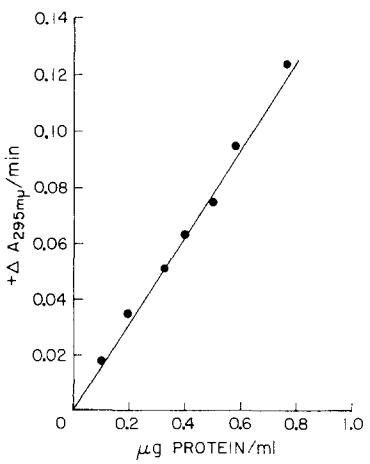
PURIFICATION OF NICOTINE OXIDASE

Nicotine oxidase was assayed colorimetrically as described in the text.

Fraction	*Total volume (ml)*	*Total protein (mg)*	*Total units*	*% recovery*	*Specific activity (units per mg)*	*Purification*	*Oxidase / Oxygenase*
C	275	3160	19 800	—	6.3	—	0.44
A_1	137	1770	18 800	95	10.6	1.7	0.81
H	114	410	14 100	71	34.4	5.5	3.9
HA	18	205	11 400	58	55.7	8.9	4.9
A_2	5	5.25	4 600	23	876	139	575

tions, overall recoveries ranged from a low of 16% to a high of 36% with overall purification from 99 to 212. A persistent low level of oxygenase activity still present in the A_2 fraction amounted to 0.17% based upon enzyme activity. At this level of contamination, oxygenase activity would contribute about 0.5% to the absorbance observed during the spectrophotometric oxidase assay.

The present procedure differs from the previously reported method[1] in that TEAE-cellulose was substituted for DEAE-cellulose; NaCl rather than potassium phosphate was used to elute the enzyme; the concentration of EDTA in the elution buffers was increased from 1 mM to 5 mM; 5 mM EDTA was substituted for GSH in order to stabilize the A_2 fraction. Although the revised procedure did not markedly



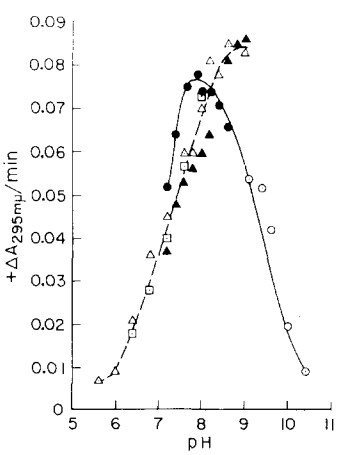


Fig. 2. The relation between nicotine oxidase activity and enzyme concentration. Nicotine oxidase activity was assayed spectrophotometrically as described in the text except that the concentration of the A_2 fraction was varied.

Fig. 3. The pH optimum of nicotine oxidase. The reaction mixtures were identical to those described in the text, except that where indicated 150 μmoles of the following buffers were employed: □, potassium phosphate; △, Tris phosphate; ▲, Tris chloride; ●, sodium pyrophosphate; ○, sodium glycinate. Nicotine oxidase was assayed spectrophotometrically.

improve the oxidase to oxygenase ratio of the final product, it did result in a far more stable A_2 fraction and a higher overall yield of final material.

Effect of enzyme concentration

The rate of the reaction was linear with protein concentration up to at least 0.8 μg protein/ml, which represented approx. 0.55 unit of oxidase (Fig. 2). The observed specific activity was 710 compared to a specific activity of 876 as determined by the colorimetric assay.

Effect of pH

When nicotine oxidase was assayed in sodium pyrophosphate buffer, the pH optimum ranged from 7.6 to 8.2 (Fig. 3). An entirely different response was observed when Tris chloride, Tris phosphate, or potassium phosphate buffers were used. Below pH 8.2, oxidase activity in these buffers was less than that observed with pyrophosphate buffer. At pH 8.2, the rate of oxidase activity in the presence of both Tris buffers equaled the rate observed with pyrophosphate buffer, and continued to increase until it reached a maximum at pH 8.6. At this point the rate remained essentially constant up to pH 9.0, the last pH at which the Tris buffers were assayed. Over this same region (pH 8.2–9.0) nicotine oxidase activity decreased when pyrophosphate or glycine buffers were employed.

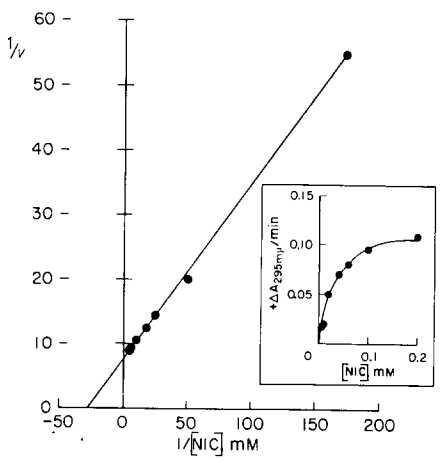


Fig. 4. The dependence of the reaction rate on the nicotine (NIC) concentration. Nicotine oxidase activity was assayed spectrophotometrically as described in the text except that the concentration of nicotine was varied.

The effect of nicotine and brilliant cresyl blue concentrations

The K_m for nicotine was determined to be 0.037 mM (Fig. 4). The rate of nicotine oxidation was independent of the nicotine concentration above 0.1 mM nicotine, and was not inhibited when the concentration was increased to 50 mM.

The K_m for brilliant cresyl blue was determined, by the spectrophotometric assay, to be 0.008 mM (Fig. 5). The concentration of the dye employed in the assay was critical as the dye proved inhibitory at concentrations exceeding 0.04 mM. The

range over which the rate of oxidase activity was independent of the dye concentration was small (from approx. 0.02 mM to 0.04 mM). When the oxidase was assayed colorimetrically, the rate of oxidation was proportional to the concentration of brilliant cresyl blue up to approx. 0.2 mM. Nicotine oxidase activity was inhibited at greater concentrations of brilliant cresyl blue.

Substrate specificity

The enzyme oxidized nornicotine, nicotine-*N*-oxide, myosmine, and anabasine. The following compounds were not oxidized: nicotyrine, 6-hydroxynicotine, 6-hydroxypseudooxynicotine, nicotinic acid, nicotinamide, *N'*-methylnicotinamide, pyridine, quinoline, and α,α'-dipyridyl. Nor did any of these compounds act as competitive inhibitors of nicotine oxidation.

Specificity of the dye requirement

In the absence of an added electron acceptor, nicotine oxidase catalyzed the formation of a small amount of 6-hydroxynicotine. Nicotine oxidation was stimulated by the addition of suitable redox dyes such as brilliant cresyl blue, methylene blue, and 2,6-dichlorophenolindophenol. Naphthoquinones, such as 5-hydroxy-1,5-naphthoquinone and menadione, as well as 1-hydroxy-2-methyl-4-aminophthalene (vitamin K_5), also stimulated nicotine oxidation (Table II). No activity was observed when the

TABLE II

THE ELECTRON ACCEPTOR SPECIFICITY OF NICOTINE OXIDASE

The reaction mixtures were those described in the text for the spectrophotometric assay except that where indicated brilliant cresyl blue was replaced with variable quantities of the indicated electron acceptors. The oxidase was preincubated for 2 min with the appropriate acceptor prior to initiating the reaction by the addition of nicotine. The data reported in the second column were obtained at an acceptor concentration of 0.025 mM.

Acceptor	$+\Delta A_{295m\mu}$ *per min*	$v_{max.}$	K_m *(mM)*
None	0.0005	—	—
Brilliant cresyl blue	0.078	0.125	0.008
Methylene blue	0.066	0.133	0.027
2,6-Dichlorophenolindophenol	0.006	0.040	1.24
Menadione	0.034	0.082	0.49
5-Hydroxy-1,4-naphthoquinone	0.058	0.118	0.037
Vitamin K_5	0.026	0.067	1.0

dyes were incubated with nicotine in the absence of the oxidase. No stimulation of nicotine oxidation was observed with the following compounds: potassium ferricyanide, FAD, FMN, cytochrome *c*, phthiocol, vitamin K_1, 2-hydroxy-1,4-naphthoquinone, and H_2O_2.

The use of artificial electron acceptors made the addition of catalase necessary. Catalase at concentrations up to 25 μg/ml stimulated the rate of nicotine oxidation as shown by spectrophotometric measurements (Table III). At greater concentrations, the activity was independent of the catalase concentration. Bovine serum albumin did not substitute for catalase, indicating that the action of catalase involved more than some non-specific protective effect. An even greater dependence upon the catalase

TABLE III

THE EFFECT OF CATALASE ON NICOTINE OXIDASE ACTIVITY

The spectrophotometric assay as described in the text was employed, except that the catalase concentration was varied, as indicated, or replaced with crystalline bovine serum albumin.

Addition (μg)		$+\Delta A_{295\ m\mu}$ *per min*	*% stimulation*
Catalase	(0)	0.060	—
	(8)	0.065	8
	(17)	0.068	13
	(25)	0.075	25
	(33)	0.075	25
Bovine serum albumin	(33)	0.057	0

concentration was observed in the colorimetric assay in which the rate of oxidation was proportional to the catalase concentration up to 100 μg/ml.

The effect of inhibitors

As indicated in Table IV, nicotine oxidase was sensitive to cyanide, a concentration of 1 mM resulting in a 95% inhibition. A slight inhibition was observed when nicotine oxidase was incubated with 1,10-phenanthroline and α,α′-dipyridyl, whereas 8-hydroxyquinoline (1.2 mM), azide (10 mM), EDTA (10 mM), and diethyldithiocarbamate (1.3 mM) were not inhibitory.

The oxidase was inhibited by quinacrine, a concentration of 0.1 mM producing a 49% inhibition (Table IV). The inhibition by quinacrine was partially reversed by FMN and to a lesser extent by FAD. The data were complicated by the presence of

TABLE IV

THE INHIBITION OF NICOTINE OXIDASE ACTIVITY

The standard spectrophotometric assay as described in the text was employed, except that the oxidase was preincubated for 2 min with the appropriate inhibitor prior to the addition of nicotine in order to start the reaction.

Addition	*Concn. (mM)*	$+\Delta A_{295\ m\mu}$ *per min*	*% inhibition*
None	—	0.075	—
KCN	0.1	0.050	33
	1.0	0.004	95
1,10-Phenanthroline	1.0	0.060	20
	2.0	0.040	47
α,α′-Dipyridyl	1.0	0.065	13
	2.0	0.055	27
None	—	0.078	—
Quinacrine	0.1	0.040	49
Acriflavine	0.1	0.068	13
	0.2	0.050	36
PCMS	0.1	0.055	29
	1.0	0.030	62
$HgCl_2$	0.1	0.003	96
Iodoacetamide	10	0.060	23

TABLE V

THE REVERSAL OF ACRIFLAVINE INHIBITION

The standard colorimetric assay as described in the text was employed. In experiment 1, nicotine oxidase was incubated for 2 min prior to the addition of nicotine to start the reaction. In experiments 2, 3, and 4, nicotine oxidase was incubated for 2 minutes with 1 μmole acriflavine, 2 μmoles FMN, or 2 μmoles FAD, as indicated, prior to the addition of nicotine. In experiment 5, the oxidase was incubated for 4 minutes prior to initiating the reaction by the addition of nicotine. In experiments 6 and 7, nicotine oxidase was preincubated for 2 minutes with either 2 μmoles FMN or 2 μmoles FAD, as indicated, and then incubated an additional 2 minutes with 1 μmole acriflavine prior to initiating the reaction by the addition of nicotine. In experiments 8 and 9, the oxidase was preincubated with 1 μmole acriflavine for 2 minutes followed by a 2-minute incubation with either 2 μmoles FMN or 2 μmoles FAD, as indicated, before initiating the reaction by the addition of nicotine.

Experiment No.	*Addition*	*Total preincubation time (min)*	*Units of oxidase activity*	*% inhibition*
1	Complete	2	2.5	—
2	+ acriflavine	2	0.9	64
3	+ FMN	2	2.3	8
4	+ FAD	2	2.5	0
5	Complete	4	2.2	—
6	+ FMN, acriflavine	4	2.4	0
7	+ FAD, acriflavine	4	1.4	36
8	+ acriflavine, FMN	4	2.1	5
9	+ acriflavine, FAD	4	1.3	41

ethanol so that complete reversal of quinacrine inhibition was neither observed nor approached. Acriflavine also inhibited the oxidase and the inhibition could be reversed by flavins. As indicated in Table V, 1 mM acriflavine inhibited oxidase activity 64%. Little or no inhibition was observed when the enzyme was incubated with FMN or FAD for 2 min, or when the enzyme was incubated for a total of 4 min. Preincubation of the oxidase with FMN prior to the addition of acriflavine resulted in complete protection of activity. On the other hand, FAD only partially protected the oxidase. The two flavin coenzymes also reversed the inhibition produced by preincubation of the oxidase with acriflavine.

The oxidase was inhibited by *p*-chloromercuriphenylsulfonic acid (PCMS),

TABLE VI

THE INHIBITION OF NICOTINE OXIDASE BY COPPER

Nicotine oxidase was assayed by the spectrophotometric method described in the text. Where indicated, the oxidase was preincubated for 2 minutes with 3.3 mM cupric sulfate, 3.3 mM ascorbic acid, or a solution 3.3 mM with respect to cupric sulfate and ascorbic acid. The reaction was initiated by the addition of nicotine.

Addition	$+\Delta A_{295\ m\mu}$ *per min*	*% inhibition*
None	0.103	—
Cu^{2+}	0.083	19
Ascorbate	0.087	15
Cu^{2+} + ascorbate	0.005	95

$HgCl_2$, and iodoacetamide (Table IV). The inhibition by PCMS was partially reversed by the subsequent addition of GSH. Iodoacetic acid (10 mM) and copper sulfate (1 mM) failed to inhibit the enzyme. When the concentration of copper sulfate was increased to 3.3 mM, a 19% inhibition of oxidase activity was observed (Table VI). The inhibi-

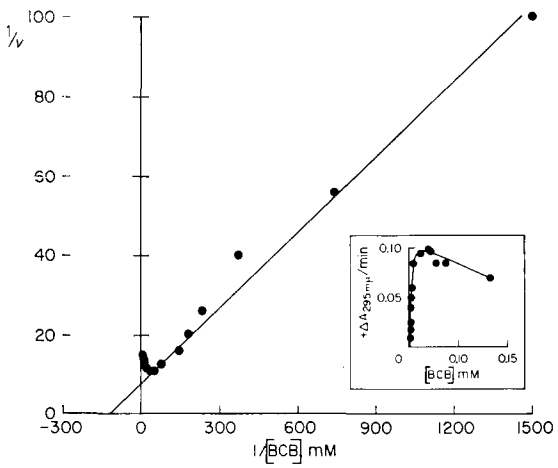


Fig. 5. The dependence of the reaction rate on the concentration of brilliant cresyl blue (BCB). Nicotine oxidase activity was assayed spectrophotometrically as described in the text except that the concentration of brilliant cresyl blue was varied.

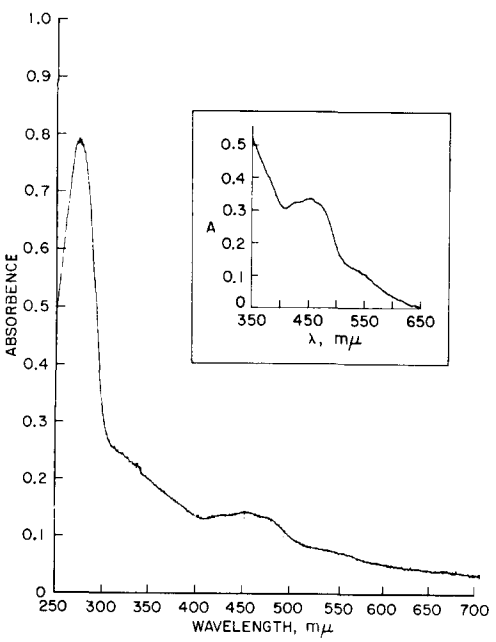


Fig. 6. The absorption spectrum of nicotine oxidase. The absorption spectrum was obtained by diluting 0.211 mg of Fraction A_2 to 1 ml with 0.1 M sodium pyrophosphate–5 mM EDTA buffer (pH 7.9) and determining the spectrum in a Cary 14 spectrophotometer against a blank consisting of 1 ml of 0.1 M sodium pyrophosphate–5 mM EDTA buffer (pH 7.9). The spectrum contained in the insert was obtained at an A_2 concn. of 0.844 mg protein/ml.

tion by copper sulfate was potentiated by the addition of ascorbic acid (which by itself was slightly inhibitory) suggesting that cuprous ion was the inhibitory form of the metal.

Spectral properties of nicotine oxidase

The absorption spectrum of the enzyme (Fig. 6) was characterized by a maximum at 450 mμ, on either side of which were located shoulders at approx. 425 and 475 mμ. Considerable absorption was observed beyond 500 mμ and a characteristic shoulder was located at 575 mμ. Aside from a sharply defined absorption maximum at 273 mμ, no other maxima were observed.

The absorption maximum located at 450 mμ could be bleached when the enzyme and nicotine were incubated anaerobically in the absence of brilliant cresyl blue.

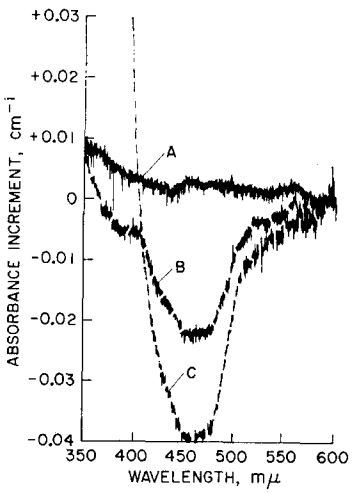


Fig. 7. The nicotine-dependent reduction of nicotine oxidase. 1.28 mg of A_2 were made up to 6.0 ml with 0.1 M sodium pyrophosphate buffer (pH 7.9) and 2.9 ml of the resulting solution were placed in each of two Thunberg-type cuvettes. The cuvette destined to be employed as the sample cuvette was evacuated and flushed with argon, the process being repeated 3 times. After determining the base line (Trace A), 0.1 ml of 30 mM nicotine was tipped into the sample cuvette and 0.1 ml of 0.1 M sodium pyrophosphate buffer (pH 7.9) was tipped into the blank cuvette. A series of difference spectra were obtained representing nicotine-reduced enzyme *vs.* 'oxidized' enzyme. Trace B represents the spectrum obtained 9 min after the addition of nicotine. No further change was observed upon further incubation. In order to obtain Trace C, a few mg of sodium dithionite were added to the sample cuvette and the resulting difference spectrum was traced. The spectra were run at room temperature (22°) in a Cary spectrophotometer using the sensitive (0–0.1) slidewire.

Fig. 7 represents data taken from a typical experiment in which the spectrum of the nicotine-reduced enzyme was compared with the spectrum of nicotine oxidase which was incubated aerobically in buffer. The enzyme was rapidly reduced upon the addition of nicotine as evidenced by the appearance of a trough extending from 450 mμ to 470 mμ (Fig. 7, Trace B). Not all the flavin in the A_2 Fraction was reduced by nicotine since adding sodium dithionite to the nicotine-reduced enzyme caused a further bleaching of the trough as well as a slight decrease in the absorbance in the region from 500 to 600 mμ (Fig. 7, Trace C).

DISCUSSION

The nicotine oxidase of *A. oxydans* (strain P-34) was released from whole cells following sonic disruption. All of the oxidase activity remained in the supernatant fraction following centrifugation at 9800 × *g* for 30 min and, although not reported here, centrifugation at 96 000 × *g* for 120 min also failed to sediment the enzyme. As isolated, nicotine oxidase catalyzed an extremely slow conversion of nicotine to 6-hydroxynicotine equivalent to 0.5 μmole/30 min per mg protein. The addition of certain electron acceptors greatly stimulated the rate of 6-hydroxynicotine formation. Brilliant cresyl blue was the most effective acceptor, although methylene blue and 5-hydroxy-1,4-naphthoquinone were nearly as effective. Nicotine oxidase was inhibited by these dyes at relatively low concentrations and the range over which the rate was independent of the dye concentration was rather narrow.

The marked inhibition of the oxidase by cyanide as well as the inhibitions by 1,10-phenanthroline and α,α′-dipyridyl is consistent with the notion that a metal is essential for enzyme activity. The oxidase was inhibited by the following sulfhydryl reagents: PCMS, Hg^{2+}, iodoacetamide, and Cu^{+}. Glutathione protected the enzyme against PCMS inhibition but only partially restored PCMS-inhibited oxidase activity.

Several lines of evidence suggest that nicotine oxidase is a flavin-containing enzyme: the absorption spectrum of the enzyme was reminiscent of metalloflavoproteins, such as the xanthine oxidase from *Micrococcus lactilyticus*[8] and hepatic aldehyde dehydrogenase[9]; the inhibition produced by acriflavine and quinacrine; the reversal of the acriflavine inhibition by FMN; the substrate-dependent reduction of the absorption maximum located at 450 mμ during the anaerobic incubation of the enzyme with substrate. The ability of FMN to completely reverse acriflavine inhibition (and to partially reverse quinacrine inhibition), whereas FAD was less effective, suggests that FMN is the functional flavin in nicotine oxidase.

Nicotinic acid hydroxylase[10,11], like nicotine oxidase, has been shown to incorporate the oxygen derived from water during the oxidation of nicotinic acid to 6-hydroxynicotinic acid[12]. Unlike nicotine oxidase, nicotinic acid hydroxylase activity resided in a particulate fraction which sedimented with the cell wall and protoplasmic membrane fraction[13]. Furthermore, nicotinic acid hydroxylase activity could be coupled to oxygen[13] as well as to ferricyanide and redox dyes[14]. These differences between nicotine oxidase and nicotinic acid hydroxylase may reflect the harsh treatment required to disrupt *A. oxydans* since prolonged sonication would be expected to destroy membranous processes, such as those associated with nicotinic acid hydroxylase activity. Indeed, prolonged sonication solubilized the particulate nicotinic acid hydroxylase[14].

No evidence has as yet been obtained to indicate that two separate enzymes are associated with nicotine oxidase activity (*i.e.*, hydration and oxidation activity). Indeed all the current evidence suggests that both activities are associated with a single component. At the present time nicotine oxidase is conceived as a two-headed enzyme carrying out the dissimilar functions of hydration followed by oxidation. Verification of this model depends upon the isolation of greater quantities of nicotine oxidase and is currently under investigation.

REFERENCES

1 L. I. HOCHSTEIN AND B. P. DALTON, *Biochem. Biophys. Res. Commun.*, 21 (1965) 64.
2 K. DECKER AND H. BLEEG, *Biochim. Biophys. Acta*, 105 (1965) 313.
3 L. I. HOCHSTEIN AND S. C. RITTENBERG, *J. Biol. Chem.*, 234 (1959) 156.
4 W. E. MCCORMICK AND M. SMITH, *Ind. Eng. Chem.*, 18 (1946) 508.
5 L. I. HOCHSTEIN AND S. C. RITTENBERG, *J. Biol. Chem.*, 234 (1960) 795.
6 H. D. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
7 A. A. GREEN AND W. L. HUGHES, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, New York, 1955, p. 67.
8 V. ALEMAN, S. T. SMITH, K. V. RAJAGOPALAN AND P. HANDLER, in A. SAN PIETRO, *Non-Heme Iron Protein: Role in Energy Conversion*. The Antioch Press, Yellow Springs, Ohio, 1965, p. 327.
9 K. V. RAJAGOPALAN, I. FRIDOVICH AND P. HANDLER, *J. Biol. Chem.*, 237 (1962) 922.
10 D. E. HUGHES, *Biochem. J.*, 60 (1955) 303.
11 E. J. BEHRMAN AND R. Y. STANIER, *J. Biol. Chem.*, 228 (1957) 923.
12 A. L. HUNT, D. E. HUGHES AND J. M. LOWENSTEIN, *Biochem. J.*, 69 (1958) 70.
13 D. E. HUGHES, *Biochem. J.*, 60 (1955) 303.
14 A. L. HUNT, A. RODGERS AND D. E. HUGHES, *Biochim. Biophys. Acta*, 34 (1959) 354.