SO_2 mL^{-1}. According to the guidelines for data quality evaluation in environmental chemistry (9), these data indicate a limit of detection of about 2 ng SO_2 mL^{-1} or more conveniently, 1 ppb S as S(IV). Correspondingly, limit of quantitation begins at about 6 ng SO_2 mL^{-1}.

As in the colorimetric method, added nitrite (to simulate concurrent presence of NO_2) interfered. The interference was completely eliminated by the sulfamic acid/sodium sulfamate addition step. However, unlike the colorimetric method, wherein a small but perceptible decrease in method sensitivity results due to the sulfamic acid addition, no reduction in sensitivity was apparent in this procedure.

Without any CDTA in the absorber, added Mn(II) interfered as in the pararosaniline procedure. No interference due to 400 ng Mn(II) mL^{-1} was noted in the presence of the recommended amount of CDTA.

Above 1% concentration, 1-aminonaphthalene is subject to OSHA regulations because of its reported carcinogenicity. From the known pK value of 1-aminonaphthalene, we estimate that the vapor pressure of this compound over the stock reagent (1 mM in 1.4 M HCl) is 10^{-6} times that over the solution which begins to be subject to regulatory control. The final solution involved in the analytical procedure is less acidic but is also diluted 10-fold, resulting in approximately the same vapor pressure. While these levels probably do not pose any health hazards, we are pursuing a search for alternative compounds which would be deemed safer. 4-Amino-1-naphthalenesulfonic acid and 5-amino-2-naphthalenesulfonic acid are both Schiff positive, and both display good quntum efficiencies. Also, both are much less volatile compared to 1-naphthylamine and therefore appear very promising.

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Identification of a Chromogen in the Assay of Hippuric Acid with Acetic Anhydride, Pyridine, and 4-(Dimethylamino)benzaldehyde

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A yellow chromogen, formed in the assay system for hippuric acid which consists of acetic anhydride, pyridine, and 4-(dimethylamino)benzaldehyde, was isolated, and the structure was determined to be 4-(1-acetyl-4(1*H*)-pyridylidene)-2-phenyl-2-oxazolin-5-one. The structure was confirmed by the chemical conversion to known compounds as well as by NMR spectra. The conversion was accomplished by hydrolysis of the chromogen to 4-((benzoylamino)methyl)pyridine, 4-(aminomethyl)pyridine, and benzoic acid via 5-hydroxy-2-phenyl-4-(4-pyridyl)oxazole. 4-(Dimethylamino)benzaldehyde included in the assay system increased the production of the chromogen. The formation mechanism of the chromogen via an intermediate susceptible to oxidation was proposed.

The formation of a yellow chromogen, in the reaction mixture of hippuric acid (HA) with acetic anhydride and pyridine in the presence of 4-(dimethylamino)benzaldehyde (4-DAB), is a sensitive and simple assay of HA in urine and liver homogenate (*1*). The quantitative conversion of glycine into HA is a specific method for the assay of glycine. This method has been used to determine the amount of glycine present in amino acid mixtures and biological samples (*2*).

In this paper, a predominant yellow chromogen has been isolated from the color reaction mixture, and the structure was determined to be 4-(1-acetyl-4(1*H*)-pyridylidene)-2-phenyl-2-oxazolin-5-one (APPO) which does not include the component of 4-DAB. The mechanism for the formation of APPO is proposed. This mechanism accounts for the absence of APPO as a product when a benzoylamino acid other than HA reacts with acetic anhydride and pyridine. The role of 4-DAB in the chromogen formation is also described.

EXPERIMENTAL SECTION

Chemicals and Instruments. 4-((Benzoylamino)methyl)-pyridine was prepared by the benzoylation (*3*) of 4-(aminomethyl)pyridine, purchased from Tokyo Kasei Kogyo Co. (Tokyo). Nitrogen had a purity of 99.999%. Other compounds and solvent used were of the best grade commercially available.

Nuclear magnetic resonance spectra of samples in CF_3COOD, $CDCl_3$, and CD_3SOCD_3 solution containing internal tetramethylsilane (Me_4Si) were recorded with a JEOL FX-100 spectrometer. The quantitative spectra were obtained by using gated decoupling without NOE. Infrared spectra of crystalline samples were recorded as Nujol mulls with a Nipponbunko A-102 spectrometer. Mass spectra were obtained in a Shimadzu LKB Type 9000 spectrometer at an ionizing energy of 70 eV. Absorption

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spectra were recorded with a Shimadzu UV-180 spectrometer. Thin-layer chromatography (TLC) was performed on a glass plate precoated with silica gel 60_{254} (Merck 5729). Melting points were uncorrected. The gas–liquid chromatography (GLC) was carried out on a Shimadzu GC 4-CM with a flame ionization detector. The glass column of 1.5 m × 3 mm i.d. was packed with 5% Thermon 3000 on Chromosorb W, 80–100 mesh. The temperatures were maintained at 190 °C for the column oven and 230 °C for the injector and the detector ports. The carrier gas was nitrogen, at a flow rate of 40 mL/min.

Measurements of Absorption Spectra under Anaerobic Conditions. An anaerobic reaction with a total volume of 3 mL was carried out in a 10 × 10 mm cuvette to which an open neck with a side arm was fused. A three-way stopcock was attached to the side arm. Nitrogen was bubbled through a long needle into a solution of acetic anhydride (1.0 mL), pyridine (1.8 mL), and HA (0.15 μmol) in the cuvette for 10 min. Nitrogen was also passed through the side arm in the neck of the cuvette. The needle was withdrawn, and the neck of the cuvette was stoppered, while nitrogen continued to be flushed through the side arm. The side arm was then fused. The cuvette was incubated at 40 °C for 1 h and the spectrum was recorded. A pyridine solution (0.2 mL) containing 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) (0.15 μmol), in which oxygen was previously excluded with nitrogen bubbling, was added and mixed under a stream of nitrogen from the long needle and side arm. The cuvette was fused as described above and incubated at 40 °C for 0.5 h. The spectrum was again recorded. In another cuvette air was added, instead of DDQ, by bubbling through the long needle for 5 min.

Determination of APPO. An aliquot (0.1 mL) of the reaction mixture was charged to a TLC plate (10 × 4 cm), which was developed with a solvent system of 1,2-dichloroethane–isopropyl ether–acetic anhydride (20:5:2, v/v). After the development, a solution of acetic anhydride–pyridine (1:2, v/v) was immediately sprayed onto the plate to prevent the decomposition of APPO. The wet yellow zone was scraped off the plate and extracted with a solution (1.5 mL) of acetic anhydride and pyridine (1:2, v/v). The absorbance was measured at 458 nm. The calibration curve was prepared in the same manner described above using standard APPO dissolved in a solution of acetic anhydride and pyridine (1:2, v/v).

RESULTS

The Detection of Chromogens. The standard procedure published for the assay of HA (*1*) was followed. TLC was used for the detection of the chromogens produced: HA (1.5 μmol) in acetic anhydride (1.0 mL) and pyridine (2.0 mL) was incubated at 40 °C for 1 h in the presence of 4-DAB (60 μmol). The reaction mixture (0.1 mL) was charged to a TLC plate (10 × 5 cm) and developed with the solvent system decribed above. A predominant yellow band at an R_f value of 0.76 was observed with an another pale yellow band at an R_f value of 0. The predominant band was scraped off the plate and the yellow compound was extracted with a solution (1.5 mL) of acetic anhydride and pyridine (1:2, v/v). The spectrum of the extracted chromogen was the same as that of the original reaction mixture and has three maximal peaks at 432, 458, and 489 nm. Similar results were obtained with the reaction in the absence of 4-DAB. These spectra are shown in Figure 1. The spectra of 1 and 2 indicate those of the reaction mixture in the presence and absence of 4-DAB, respectively. Spectra of 3 and 4 indicate those of extracts from the reaction mixture in the presence and absence of 4-DAB, respectively. The reference sample was a solution of acetic anhydride and pyridine (1:2, v/v).

Isolation of APPO. Isolation of APPO was carried out from the reaction system in the absence of 4-DAB. HA (1 g) was suspended in a mixture of acetic anhydride (3.0 mL) and pyridine (6.0 mL), and the suspension was stirred at room temperature for 3 h. The resultant brown solution was allowed to stand at 4 °C overnight. A brown precipitate was collected by filtration and washed with chloroform to give APPO (170 mg) in a yield of 11% on the basis of HA. Recrystallization from chloroform produced a sample of analytical quality. A single spot was observed with R_f values of 0.55 and 0.76 in two different solvent systems. The first R_f value was obtained in the benzene–dimethylformamide (5:1, v/v) solvent, and the second value was obtained in the 1,2-dichloroethane–isopropyl ether–acetic anhydride (20:5:2, v/v) solvent. Analytical data obtained for APPO are as follows: mp 217–220 °C [lit. mp 227 °C (*4*)]; mass spectra *m/e* (relative intensity) 281 (4), 280 (18) (calcd for $C_{16}H_{12}N_2O_3$, 280), 239 (9), 238 (93), 194 (7), 106 (10), 105 (100), 78 (5), 77 (22), 51 (10), 43 (11), 28 (6); IR 1720, 1640, 1550, 1260, 1200, 1080, 1050, 1000, 950 cm^{-1}; ^{13}C NMR δ (CF_3 COOD) 21.5 (q), 100.3 (s), 115.4 (broad d), 121.1 (s), 129.8 (d), 131.7 (d), 137.6 (d), 138.1 (d), 147.1 (s), 158.3 (s), 162.4 (s), 170.0 (s); ^1H NMR δ (CF_3COOD) 2.88 (s, 3), 7.4–8.4 (m, 7), 8.7 (d, 2, J = 8 Hz); λ_{max} acetic anhydride–pyridine (1:2, v/v), 489 (38 300), 458 (52 200), 432 nm (38 200). Anal. Calcd for $C_{16}H_{12}N_2O_3$: C, 68.57; H, 4.29; N, 10.00. Found: C, 68.59; H, 4.18; N, 10.02.

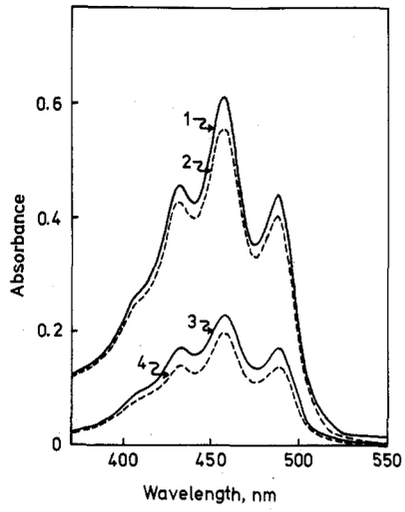

Figure 1. Spectra of chromogens produced by the reaction of HA with acetic anhydride and pyridine.

Conversion of APPO to 5-Hydroxy-2-phenyl-4-(4-pyridyl)oxazole (HPPO). A suspension of APPO (150 mg) in 2 N sodium hydroxide (7 mL) was heated at 90 °C for 15 min and the solution was acidified with 3 N hydrochloric acid. A bulky solid was collected by filtration and washed with water. HPPO was recrystallized from an acetone–water (2:1, v/v) solution with a yield of 73%. A single spot was observed with an R_f value of 0.40 on a TLC plate in a solvent system of benzene–dimethylformamide (5:1, v/v). Analytical data obtained for HPPO are as follows: mp 207–218 °C; IR 3400, 1670, 1620, 1590, 1520, 1430, 1200, 900, 820, 780, 700 cm^{-1}; ^{13}C NMR δ (CD_3SOCD_3) 107.3 (s, 1), 113.3 (d, 1), 114.0 (d, 1), 126.3 (d, 2), 129.2 (s, 1), 130.3 (d, 2), 130.6 (d, 1), 138.2 (d, 2), 147.6 (s, 1), 150.6 (s, 1), 166.8 (s, 1); ^1H NMR δ (CF_3COOD) 7.6–7.9 (m, 3), 8.0–8.5 (m, 6). Anal. Calcd for $C_{14}H_{10}N_2O_2$: C, 70.58; H, 4.23; N, 11.76. Found: C, 70.31; H, 4.14; N, 11.53.

Hydrolysis of HPPO with Hydrochloric Acid. HPPO (500 mg) was refluxed with concentrated hydrochloric acid (5 mL) for 10 min. The reaction mixture was filtered while warm and allowed to stand for 2 days in a refrigerator. Crystals (10 mg) were collected on a filter and recrystallized from 3 N hydrochloric acid, mp 121 °C. The melting point and IR spectrum of this product were the same as those of the authentic benzoic acid, and the mixture melting point of the two products was 121–122 °C. The filtrate from the reaction mixture was dried under reduced pressure. The residue was dissolved in 10% sodium carbonate (10 mL) and extracted with ether. The extract was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness. Recrystallization of the residue from ethyl acetate

Table I. The Yield and Color Contribution of APPO in the Color Reaction

additive	yield of APPO, %	color contribn,[a] %
none	36	97
4-DAB	43	96
DDQ	53	106

[a] (Absorbance at 458 nm of the whole reaction mixture after the reaction)/(absorbance at 458 nm of APPO standard solution) × 100. The standard solution was an acetic anhydride–pyridine (1:2, v/v) solution (3.0 mL) containing each amount of 1.5 × 0.36 (none), 1.5 × 0.43 (4-DAB), and 1.5 × 0.53 μmol (DDQ) of standard APPO.

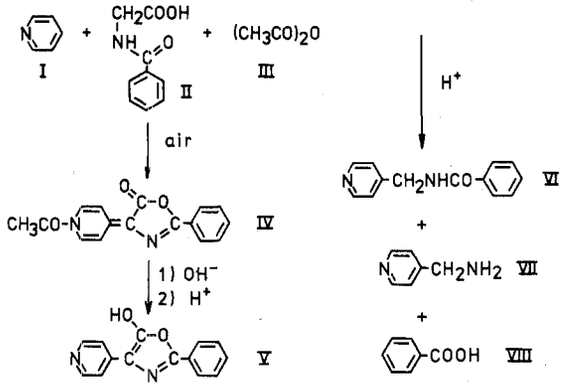

Figure 2. The hydrolytic cleavage of APPO formed by the reaction of HA with acetic anhydride and pyridine.

gave 4-((benzoylamino)methyl)pyridine (200 mg) in a yield of 52%, mp 111–114 °C. The IR spectrum and melting point of this sample were identical with those of the authentic compound. The sodium carbonate solution and washing from the ether in the above procedure were combined and evaporated to dryness. The residue from the evaporation process was derivatized with trifluoroacetic anhydride. The resultant (trifluoroaminomethyl)pyridine sample was chromatographed on GLC with a retention time of 9 min, which was identical with the retention time of the authentic sample.

The Yield and Color Contribution of APPO. The above standard procedure for the assay of HA was carried out in the presence of 4-DAB (60 μmol) or DDQ (3 μmol), and APPO produced was determined with TLC. The addition of 4-DAB and DDQ increased the yield of APPO on the basis of HA. The color contribution of APPO to the reaction was calculated from the yield and the absorbance of the reaction mixture. The results showed that APPO greatly contributed to the reaction (Table I).

DISCUSSION

The yellow chromogen isolated in the color reaction of HA with acetic anhydride and pyridine was APPO (IX), and a conjugated compound, which included pyridine, benzene, and 2-oxazolin-5-one rings. This chromogen was the same as the compound previously prepared by acetylation of HPPO (V) (*4*). The direct identity of APPO by comparison with the reported data was impossible because the melting point did not agree with the reported data. The reported ^1H NMR data and ours, although obtained in the same manner, were not sufficient to support the gross structure of APPO because of the lack of proton in the 2-oxazolin-5-one ring. The identity of this compound was determined by chemical conversion (Figure 2) and ^{13}C NMR spectra. APPO was demonstrated by conversion to compound V which is hydrolyzed to VI, VII, and VIII. Compounds of VI and VIII were confirmed by comparison with authentic samples, and that of VII was confirmed by GLC analysis after the trifluoroacetylation.

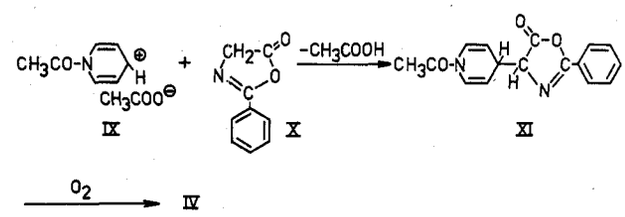

Figure 3. The proposed mechanism of formation of APPO.

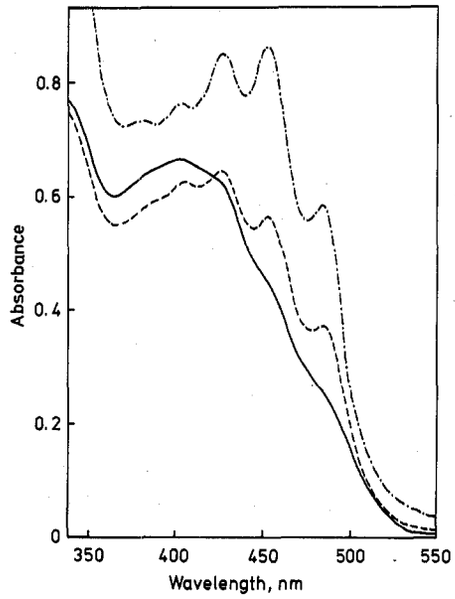

Figure 4. Oxidation of the product produced by anaerobic reaction of HA with acetic anhydride and pyridine. HA was reacted with pyridine and acetic anhydride under anaerobic conditions (—) and air (- - -) or DDQ (-·-) was added to the anaerobic mixture.

Gross structures of IV and V were supported by ^{13}C NMR data at 100 MHz assisted with off-resonance decoupling and gated decoupling without NOE, respectively.

The mechanism for the direct formation of APPO by the reaction of HA with acetic anhydride and pyridine is proposed in Figure 3. 2-Phenyl-2-oxazolin-5-one (X), formed by the action of acetic anhydride on HA, undergoes a nucleophilic substitution by *N*-acetylpyridinium cation (IX) at the 4-position to produce a condensation product (XI) which converts into APPO by oxidation. Such a substitution by the cation had been observed in the reaction of acetophenone or dimethylaniline with pyridine and benzoyl chloride (*5*, *6*). When the color reaction was carried out under anaerobic conditions, no formation of APPO was observed. The addition of air or DDQ to the anaerobic solution produced APPO, as seen in the spectral change with three characteristic peaks (Figure 4). These findings indicate that oxygen dissolved in the reaction mixture initiates the oxidation of XI and plays an important role in the color development of HA.

The sensitivity in the assay of HA was improved by the addition of 4-DAB to the mixture of acetic anhydride and pyridine (*1*, *2*). The role of 4-DAB produced no additional chromogens but enhanced the production of APPO by 7% (Table I). This finding indicates that the sensitivity increased with the addition of 4-DAB is due to the enhanced production of APPO. The participation of 4-DAB in the reaction mechanism is uncertain, because the addition of 4-DAB to the above anaerobic solution did not cause any spectral change to APPO; however, the successive addition of air did (data not shown). This fact indicates that 4-DAB requires oxygen for the enhanced production of APPO. The autoxidation of benzaldehyde and its derivatives produce peracids which can take place in the dehydrogenation of several reductants (*7*,

8). The peracid from 4-DAB and oxygen can promote dehydrogenation of IX and lead to enhanced production of APPO. These data are supported by the fact that the addition of DDQ as a hydrogen abstractor, instead of 4-DAB, produced more APPO (Table I).

A chromogen other than APPO was found. The TLC showed the presence of another pale yellow chromogen with an R_f value of 0. The contribution of this chromogen to the color reaction, however, was negligible, since that of APPO was nearly complete (Table I). It was reported that a colored condensation compound of 4-DAB and X, 2-phenyl-4-(*p*-dimethylaminobenzal)oxazol-5-one, was produced in the reaction system containing HA, 4-DAB, and acetic anhydride but no pyridine (9). In our system, no formation of the chromogen was confirmed with TLC using the authentic compound. This indicates that pyridine interferes in the formation of the above condensation compound.

The above mechanism accounts for the absence of APPO formation with benzoylamino acids other than HA. In these compounds oxidation of the condensation compounds such as XI to conjugated compounds such as APPO is not possible. In fact, benzoylated compounds of amino acids other than glycine, were reported to produce no color reaction (2). On the other hand, peptides having a glycine residue at the C-terminus produced a yellow chromogen with a spectrum similar to that of APPO (2). Work is in the progress to identify the structure of this product.

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Immobilized Enzyme Chemically Modified Electrode as an Amperometric Sensor

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The construction and response of the immobilized enzyme chemically modified electrode as an amperometric sensor is described. Glucose oxidase and L-amino acid oxidase have been covalently bonded to a graphite electrode via a cyanuric chloride linkage. Various response characteristics as well as kinetic parameters have been evaluated and compared to previously reported amperometric enzyme electrodes.

Highly specific methods for biochemical analysis have been reported by coupling an immobilized enzyme layer with an electrochemical sensor. This technique combines the specificity of enzyme catalysis with the sensitivity of potentiometric and amperometric electrodes to yield devices capable of repetitive, low cost assays. Amperometric enzyme electrodes which consume a specific product of the enzymic reaction usually display an expanded linear response range and larger apparent Michaelis–Menten constant (K_m) than their potentiometric enzyme electrode counterparts because the reaction product is removed from solution and its concentration profile in the active layer is altered. For thin enzyme membranes, Mell and Maloy (1, 2) have shown, through digital simulation, that the steady-state current is determined, in part, by the effective electrode area, membrane thickness, and enzyme concentration per unit volume. Electrodes with high surface area (i.e., semiporous) would be expected to give steady-state currents of greater magnitude than metallic electrodes (e.g., Pt, Ag) commonly employed as amperometric sensors (3). Increased steady-state current can result from the use of thick membranes. The response time, however, will be undesirably long, making the use of thick membranes impractical. Clearly the use of high surface area electrodes covered with thin enzyme membranes should result in optimized response characteristics.

Considering the available immobilization techniques, covalent attachment of an enzyme onto an electrode surface would satisfy the above requirements. This technique can produce an extremely thin (ca., monolayer), permanently bound, enzyme layer in a convenient and reproducible fashion. Enzymes are usually anchored to an insoluble support by covalent attachment via an intermediate linkage (4). The variety of linkages available make immobilization attractive, provided the support surface can be functionalized. Unlike metal electrodes, carbon and graphite can be conveniently functionalized to serve as amperometric enzyme electrodes. Recently, chemically modified carbonaceous materials (5–8) have been employed as rotating amperometric enzyme electrodes. While promising, these electrodes have shown smaller response ranges and relatively low steady-state currents.

We wish to report the construction and response of the immobilized enzyme chemically modified electrode (IECME) as a stationary amperometric sensor. Glucose oxidase (EC 1.1.3.4) and L-amino acid oxidase (EC 1.4.3.2) have been covalently bonded to chemically modified graphite electrodes via the cyanuric chloride linkage to yield glucose and L-phenylalanine sensors, respectively. These enzymes catalyze the oxidation of their respective substrates in the presence of O_2 to yield H_2O_2 as one of the products. The hydrogen peroxide formed is then electrochemically consumed at +0.9 V vs. a saturated calomel electrode (SCE) to give a measurable steady-state current. Response time, linearity, temperature, and pH response characteristics have been examined. In an attempt to optimize the steady-state current response, we also found it necessary to fully investigate the anodic decomposition of H_2O_2 at the bare graphite electrode.

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