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Enzymatic Generation of *N*-[4-Dimethylamino)phenyl]acetohydroxamic Acid by the Action of Pyruvate Decarboxylase on 4-(Dimethylamino)nitrosobenzene

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The established ability of pyruvate decarboxylase to catalyze the conversion of nitroso aromatics to hydroxamic acids was utilized to generate the previously unknown hydroxamic acid **2**. Although **2** could not be isolated in pure form from enzymatic reactions, evidence for its production is presented in this study. Under the conditions of the enzymatic reaction, **2** undergoes a slower reduction to give the corresponding acetanilide **3**, which was isolated and characterized. The isomeric hydroxamic acid **4** was synthesized and its stability compared to that of **2**. The much greater reactivity of the hydroxamic acid **2**, particularly evidenced by its facile reduction to **3**, was explained on the basis of the potential for the formation of the *N*-acylquinonediimine cation, **9**.

In previous studies we demonstrated the ability of certain thiamine-dependent enzymes to convert aromatic nitroso compounds into hydroxamic acids (*1*, *2*). Aromatic hydroxamic acids are generally very stable compounds, but certain metabolic conversions can lead to the production of highly reactive species (*3*). Current thought on the mechanism by which arylamides are metabolized to ultimate carcinogens involves an initial *N*-oxidation by a microsomal oxidase to produce the corresponding hydroxamic acid. This hydroxamic acid in turn can be subjected to further metabolic processes, some of which can produce electrophilic species that bind to cellular macromolecules. It is reasonable to assume that hydroxamic acids produced by nonmicrosomal processes (i.e., thiamine–enzyme catalyzed reactions) would be equally toxic following a final activation process. We now report evidence for the thiamine–enzyme catalyzed production of a previously unknown hydroxamic acid (**2**) that possesses high reactivity. This reactivity is of considerable interest in that further bioactivation steps may not be required in order for hydroxamic acids of this type to display necrotic properties.

EXPERIMENTAL

Yeast pyruvate decarboxylase (EC 4.1.1.1), sodium pyruvate, thiamine pyrophosphate, and 4-(dimethylamino)nitrosobenzene **1** were obtained from Sigma

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Chemical Company; *N,N*-dimethyl-*p*-phenylenediamine was obtained from Eastman Chemical Company, and 3-(dimethylamino)nitrobenzene from Aldrich Chemical Company. High-pressure liquid chromatography (HPLC)[1] was performed with a previously described system (*4*) employing μBondapak C_{18} columns (3.8 mm × 30 cm). Thin-layer chromatography (TLC) was conducted with precoated EM–silica-gel 60 plates from Brinkmann Instruments. Desferal mesylate was a gift from CIBA Pharmaceutical Company. Ultraviolet spectra were obtained with a Beckman Model 35 spectrophotometer and ir spectra were obtained with a Perkin–Elmer Model 180 spectrophotometer. Nmr spectra were obtained on a Nicolet NT 360 spectrometer and mass spectroscopy (MS) data on a Finnigan GC/MS mass spectrometry system. Melting points were obtained with a calibrated Thomas–Hoover mp apparatus and elemental analyses were performed by Galbraith Laboratories. Determination of pyruvate decarboxylase activity was done according to standard methods (*5*).

Synthesis of 4-(Dimethylamino)acetanilide 3

N,N-Dimethyl-*p*-phenylenediamine (0.1 mol) was reacted with acetic anhydride (0.11 mol) in anhydr Et_2O for 15 min, then treated with H_2O. Following neutralization with $NaHCO_3$, the mixture was extracted with EtOAc and the aqueous layer discarded. The organic solution was washed with aqueous $NaHCO_3$, dried (Na_2SO_4), and evaporated *in vacuo* to give a dark brown solid. The product was chromatographed on a EM–silica-gel 60 (70–230 mesh) column with a bed size of 50 × 2.3 cm employing in sequence: 500 ml of CH_2Cl_2, 500 ml of MeOH in CH_2Cl_2 (1:99), 500 ml MeOH in CH_2Cl_2 (2:98), and finally 2 liters of MeOH in CH_2Cl_2 (5:95). Fractions containing the major product in nearly pure form as evidenced by TLC analysis were combined and evaporated *in vacuo* to give a tan solid, which upon crystallization from benzene in hexane (6:1) gave 10 g of light tan-colored plates. Final recrystallization from benzene in hexane (9:1) gave pure **3**, mp 131–132°C; lit. (*6*), 130°C from H_2O; found: C, 67.49; H, 8.09; N, 15.74. $C_{10}H_{14}N_2O$ requires C, 67.37; H, 7.92; N, 15.72; ν_{max} (KBr) 1650 cm^{-1} with shoulders at 1640, 1660, and 1670 cm^{-1}.

Synthesis of N-[3-(Dimethylamino)phenyl]acetohydroxamic Acid 4

A solution of 16.6 g (0.1 mol) of 3-(dimethylamino)nitrobenzene in 200 ml of EtOH was purged with N_2, then combined while stirring with a magnetic stirring bar with 10.7 g (0.2 mol) of NH_4Cl in 300 ml of H_2O. While maintaining a N_2 atmosphere and vigorous stirring, Zn dust (19.8 g, 0.3 mol) was added in the course of 2 min and vigorous stirring was continued for 30 min. The reaction was filtered and the filtrate extracted with an equal volume of 20% benzene in Et_2O. The organic extract was immediately reacted with 0.10 mol of acetyl chloride by a previously described method (*7*) to give **4** in a highly impure form as indicated by tlc analysis on silica gel with MeOH/$CHCl_3$ (5:95). Column chromatography on

[1] Abbreviations used: HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; MS, mass spectroscopy; GC, gas chromatography.

silica-gel 60 (70–230 mesh) with CH_2Cl_2 containing MeOH (0–2% v/v) resulted in the isolation of 11 g (57% yield) of **4** as a dark amber colored oil that could not be induced to crystallize. High-pressure liquid chromatography analysis indicated that the product was contaminated with the corresponding amide. For final purification, a 2-g portion of the product was dissolved in 50 ml of Et_2O, then extracted twice with 50 ml of ice-cold 0.1 *N* NaOH. The combined base extracts were washed with 100 ml of Et_2O, then the pH was adjusted to 6 with 0.5 *N* HCl. The neutralized aqueous solution was extracted twice with 200 ml of Et_2O, and the combined organic extract was dried (Na_2SO_4) and evaporated *in vacuo*. A final chromatographic purification on silica gel with CH_2Cl_2 containing MeOH (0–1%) gave 1.2 g (34% computed overall yield) of pure **4** as a viscous amber-colored oil. Treatment of silica-gel plates and solutions containing **4** with 1% $FeCl_3$ in MeOH gave an immediate violet color, suggestive of a hydroxamic acid (*8*). Found: C, 61.70; H, 7.65; N, 14.18. $C_{10}H_{14}N_2O_2$ requires C, 61.82; H, 7.27; N, 14.43. Mass spectrometry indicated the expected parent ion $[M]^+$ at m/e = 194 amu (relative abundance 100) and the $[M - 16]^+$ ion (relative abundance 36), a fragmentation which is characteristic of hydroxamic acids (*9*). Ultraviolet spectra in pH 6.0, 0.05 *M* KH_2PO_4 indicated λ_{max} 240 nm (ϵ 13,600); ν_{max} (film) 1600 and 1630 cm^{-1} with shoulders at 1640 and 1660 cm^{-1}; δ_H (CD_3COCD_3) 2.2 (3 H, br s), 2.95 (6 H, br s), 6.6 (1 H, d), 6.9 (1 H, d), 7.0 (1 H, br s), 7.15 (1 H, dd)).

Isolation of 3 from reaction of 1 with Pyruvic Acid in the Presence of Pyruvate Decarboxylase

To 90 ml of pH 6.0, 0.05 *M* KH_2PO_4 buffer was added 1.1 g (0.01 mol) of sodium pyruvate, followed by 8.2 mg (0.055 mmol) of the substrate **1** as a solution in 0.5 ml EtOH. The resulting solution was treated with 10 ml of the same buffer containing 2.2 mg (10 units) of pyruvate decarboxylase, 5 mg (0.02 mmol) of $MgSO_4 \cdot 7H_2O$ and 9.4 mg (0.02 mmol) of thiamine pyrophosphate chloride. The mixture was allowed to stand at room temperature for 20 hr, then it was treated with 25 g of NaCl and 5 g of $NaHCO_3$. The solution was extracted twice with 100-ml portions of Et_2O, and the combined ethereal extracts treated with 20 ml of benzene, dried (Na_2SO_4), then evaporated *in vacuo* to yield a yellow residue. The residue was dissolved in 1 ml of benzene and treated with 4 ml of hexane to induce precipitation of about 5 mg of a tan solid that was collected by centrifugation. Melting point 128–130°C; ir spectrum (KBr) was superimposable with that of authentic **3**; TLC and HPLC properties were also identical with those of authentic **3**.

General Method for Study of the Action of Pyruvate Decarboxylase on 1

For analytical determinations made on this reaction, the final reaction concentrations were generally 82 m*M* sodium pyruvate, 0.05–0.25 m*M* nitroso substrate **1**, 0.20 m*M* thiamine pyrophosphate, 0.20 m*M* $MgSO_4$ and 23 μg/ml (0.3 units/ml) of pyruvate decarboxylase in pH 6.0, 0.05 *M* KH_2PO_4 buffer. The enzymatic reaction was initiated by the addition of 1 vol of the enzyme in buffer, which also contained the thiamine pyrophosphate and magnesium cofactors at 2 m*M* concen-

trations each (*5*) to 9 vol of buffer containing 91 m*M* sodium pyruvate and the nitroso substrate **1** at its desired concentration. The nitroso substrate was added to pyruvate-containing buffer as a solution in EtOH such that the volume of EtOH added did not exceed 0.1% of the final reaction volume. The studies were generally conducted at 25°C by the use of water baths or temperature-controlled cuvette holders in the uv spectrophotometer.

Analytical Methods

Ultraviolet spectrophotometry was employed to follow the initial reaction of the nitroso substrate **1** with the enzymatic system. This was achieved by following the decrease in optical absorbance at 405 nm with time in 1.0-cm-path cuvettes. The molar absorbtivity (ϵ_{405}) of the substrate **1** in pyruvate-containing pH 6.0 buffer was determined to be 17,000. The initial rate of reaction was computed by graphical methods for substrate **1** concentrations ranging from 0.05 to 0.1 m*M*.

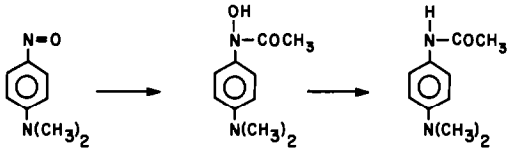
High-pressure liquid chromatography was employed to follow the rate of appearance of the two products **2** and **3**, while varying the concentration of **1** from 0.05 to 0.25 m*M*. The solvent employed was 30% MeOH in H_2O buffered to pH 6.0 with KH_2PO_4 (0.01 *M*), and which contained 0.01% desferal mesylate as a conditioning agent (*4*). The flow rate was 1.5 ml/min through a μBondapak C_{18} column with uv detection at 254 nm. Aliquots of 10 μl each were directly analyzed by introduction into the HPLC system through a septumless injector. Both peak height and area measurements were employed to quantitate the amount of product present at any selected time.

RESULTS

We observed that the reaction of 4-(dimethylamino)nitrosobenzene **1** with pyruvic acid in the presence of yeast pyruvate decarboxylase gave 4-(dimethylamino)acetanilide **3** as the final product. Upon careful examination of this reaction with HPLC and TLC we observed the production of a free intermediate for which all evidence indicates the hydroxamic acid structure **2**. Attempts to isolate **2** for structural characterization were unsuccessful due to the instability of this intermediate. The preparation of **2** by synthetic methods was equally unsuccessful, and even the synthesis of the meta isomer **4** was found to be a very difficult task due to the instability of the intermediate hydroxylamine. There is no literature precedence for the synthesis of aromatic hydroxylamines and hydroxamic acids that also contain an amino substituent on the same ring. The hydroxamic acids **2** and **4** have never been reported previously in the chemical literature.

Preparative-scale reactions of **1** with pyruvic acid and pyruvate decarboxylase provided the first evidence for our conclusion that the conversion of **1** to **3** proceeds through the hydroxamic acid **2**. Prolonged reactions (16–20 hr) at ambient temperature showed only the presence of **3** upon TLC analysis; whereas shorter reaction times (1–4 hr) indicated the major product to be a material of similar polarity and chromatographic characteristics to those of **3**, but which gave an

immediate and intense violet color upon spraying TLC plates with 1% ferric chloride in methanol. Such a colorimetric test does not occur with **3**, but is a well-known test for the hydroxamic acid functional group (*8*). The final product **3** could be readily isolated from prolonged preparative-scale reactions, and its structure was conclusively established by comparison to synthetic **3**.



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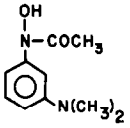

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Direct analysis of the reaction mixture by HPLC also suggested that an intermediate hydroxamic acid was produced during this enzymatic reaction. In earlier work we had noted the difficulty associated with attempts to analyze hydroxamic acids in general by HPLC, and subsequently we developed a specialized solvent system that permitted hydroxamic acid analyses by HPLC (*4*). The addition of the aliphatic trihydroxamate, desferal mesylate, to HPLC solvents results in a pronounced sharpening of peaks due to aromatic hydroxamic acids, but has little effect upon nonhydroxamate peaks (*4*). In the present reaction, we found that an HPLC peak attributable to **2** was observed only when desferal mesylate was present in the elution solvent. This finding provided us not only suggestive evidence for the intermediacy of **2** in the reaction, but also enabled us to follow the enzymatic formation of **2** and its slower reduction to **3**.

The two HPLC chromatograms in Fig. 1 illustrate the ease with which the intermediate **2** can be detected even in the presence of the final product **3**. The acetanilide product **3** had a retention time of 11.1 min, and the peak we assigned to **2** had a slightly longer retention time of 12.5 min. The close elution times of this amide/hydroxamic acid pair is similar to how other such pairs behave in this HPLC system (*4*). The isomeric hydroxamic acid **4** had a rentention time of 13.6 min in this HPLC system, and also displayed a strong dependency on the presence of desferal mesylate in the elution solvent. The amide corresponding to **4** had a rentention time of 14.5 min in this HPLC system. The two chromatograms in Fig. 1 were obtained by identical sampling of the same enzymatic reaction mixture at different times (45 and 90 min). Most noteworthy was the decrease in size of the peak assigned to **2**, with a corresponding increase in the size of the peak due to the final product **3** in going from 45 to 90 min of reaction time. At the time of the first sampling (45 min) all of the nitroso substrate **1** had been consumed, as evident by

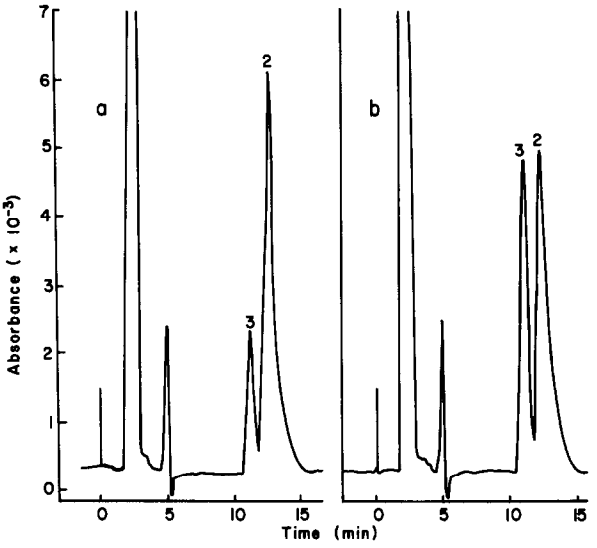


FIG. 1. High-pressure liquid chromatography analysis of the title reaction. The reaction mixture contained the substrate **1** at 0.15 m*M*, sodium pyruvate at 40 m*M*, and pyruvate decarboxylase at 0.3 units/ml of pH 6.0, 0.05 *M*, KH_2PO_4 buffer. Aliquots of 10 μl each were analyzed by direct injection into the HPLC system at (a) T = 45 min and (b) T = 90 min following the start of the reaction. Chromatographic conditions are described under Experimental. Peak assignments correspond to the chemical structures in the text.

the absence of any color due to the highly chromophoric **1** in the reaction solution. When present, **1** appeared as a peak with a retention time of 13.1 min, but due to low optical absorbance at 254 nm, it gave relatively small peaks in HPLC chromatograms.

Figure 2 demonstrates our finding that the action of pyruvate decarboxylase on **1** resulted in a relatively rapid conversion of this substrate to **2**, which then underwent a slow reduction to the final product **3**. Incubations that lacked either pyruvic acid or pyruvate decarboxylase had no effect upon the substrate **1**. The HPLC data of Fig. 2 demonstrate that within 15 min the nitroso substrate **1** had been converted to the proposed intermediate **2** to the extent of at least 75%. Spectrophotometric data obtained by following the decrease in absorbance at 405 nm due to the consumption of **1** also indicate an initial rapid reaction with a pseudo first-order rate constant of 0.47 min^{-1} relative to a pyruvate decarboxylase concentration of 1.0 unit/ml and sodium pyruvate at an enzyme saturating concentration (*10*). As expected, the maximum rate of formation of **3** occurred at the time just when, and shortly after, the concentration of **2** was greatest. The biphasic appearance of the curve for the concentration of **3** in Fig. 2, particularly the initial lag, is consistent with the proposal that **1** is converted to **2** in a rapid enzyme-catalyzed process, which is followed by a slower enzyme-dependent conversion of **2** to **3**.

On the other hand, we found that the meta isomer **4** was not converted to the corresponding amide product by this enzymatic system. This observation estab-

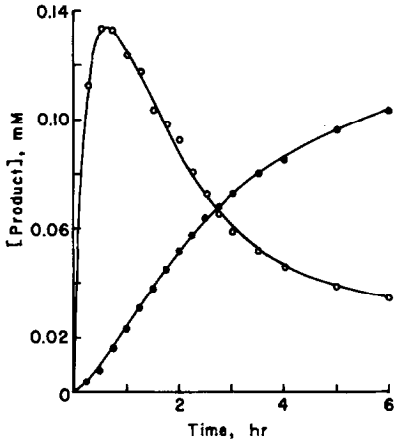


FIG. 2. Product distribution as a function of time. The reaction conditions are described in the legend to Fig. 1. O, hydroxamic acid 2; ●, final anilide product 3. The peak height response for 2 was calculated from material balance studies.

lished that the conversion of the hydroxamic acid 2 to the amide 3 under mild reducing conditions is dependent upon the $-N(CH_3)_2$ substituent being in a position whereby resonance interaction with the hydroxamic acid nitrogen can occur.

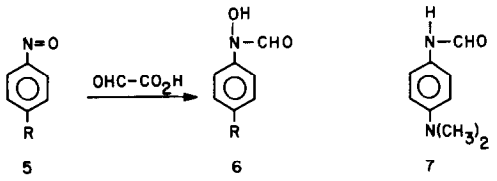
Additional studies were conducted that further demonstrated the necessity of pyruvate decarboxylase in order for the conversion of 1 to 2 to occur. The initial rate of formation of 2 was linearly dependent upon the enzyme concentration in the range of reactant concentrations that were employed in these studies. The optimal rate of formation of 2 occurred at pH 6.0, which is the pH optimum for the normal function of pyruvate decarboxylase (*5, 10*). Saturation of the enzyme by the nitroso substrate 1 was not observed at the maximum concentration of 1 employed, which was 0.30 m*M*. The rate of disappearance of nitroso substrate was found to be about 6 times faster for 4-chloronitrosobenzene than for 1 under identical conditions and with both nitroso substrate concentrations at 0.10 m*M*.

It was possible to extract 2 from preparative-scale enzymatic reactions with Et_2O. Upon such removal of the hydroxamic acid product 2 from the enzymatic system, no further reduction to 3 occurred. Evaporation of these ethereal extracts gave 2 in a highly impure form, but which allowed for preliminary investigations on the stability of 2. Most notable was the tendency of 2 to undergo decomposition to several products, with 1 being a major product. This decomposition was greatly facilitated by contact of 2 with silica gel. On the other hand, the meta isomer 4 was quite stable under similar conditions. In phosphate buffer solutions, 2 was relatively stable over the pH range 3–8 for at least 4 hr at ambient temperature. Upon incubation of the crude 2 under the usual enzymatic reaction conditions, the conversion to 3 proceeded in the same manner, but only in the presence of both pyruvic acid and pyruvate decarboxylase.

DISCUSSION

The results from this investigation indicate that the pyruvate decarboxylase catalyzed conversion of **1** to **3** proceeds through an initial production of the hydroxamic acid **2**. This conclusion is further supported by two other previous investigations that were conducted by us. One investigation was our discovery that thiamine-dependent enzymes (*1, 2*), including pyruvate decarboxylase (*2, 11*), can effectively catalyze the conversion of nitroso aromatics to aromatic hydroxamic acids. Two possible mechanisms for such conversions have been proposed (*2*). With the exception of the present case, all other nitroso aromatics that have been investigated gave the corresponding hydroxamic acid as the final product.

In a second investigation we reported the discovery of a unique chemical reaction between nitroso aromatics **5** and glyoxylic acid that resulted in the quantitative production of formyl-derived hydroxamic acids **6** (*12*). In the case of **1**, however, the hydroxamic acid [**6**, R = $-N(CH_3)_2$] underwent a reductive reaction with glyoxylic acid to produce the formanilide **7** as the final product (*13*). In the present study, we did not observe the purely chemical reduction of **2** to **3** by acetaldehyde, formaldehyde, or glyoxylic acid. The conversion of **2** to **3** was mediated by pyruvate decarboxylase, a reasonable process since the "active acetaldehyde" form of this enzyme is known to serve as a reducing agent toward exogenous chemicals (*2*). An explanation for the occurrence of a purely chemical reduction of the formyl-derived hydroxamic acid **6** (*13*), but no detectable chemical reduction of **2**, is not obvious.



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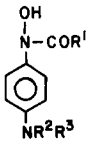

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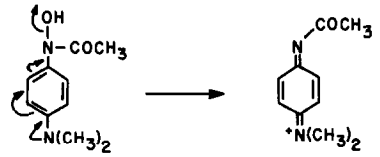
The reactivity of **2**, as evidenced by its enzymatic reduction to **3**, is apparent from a consideration of the ability of the *p*-dimethylamino group to donate an electron pair by a resonant interaction. Similar properties are to be expected for hydroxamic acids of the general structure **8**. Heterolytic cleavage of the hydroxamate N—O bond in **2** could be a favorable process since stabilization of the resultant cation by resonant structure **9** should be very strong. The *N*-acyl-

quinonediimine cation **9** is closely analogous to the quinonediimine cation **11**, which was recently proposed as the initial product of another enzymatic reaction (*14*). Alcohol dehydrogenase was reported to convert **1** to the enzyme-bound arylhydroxylamine **10**, which, because of a Lewis acid (Zn^{2+}) at the enzyme active site and also the resonant contribution from the *p*-dimethylamino group, rapidly loses ^-OH to give **11** (*14*). The reaction of **1** with pyruvate decarboxylase differs in that the hydroxamic acid product **2** produced at the enzyme active site does not undergo an immediate loss of ^-OH, but is actually released into solution. The very facile conversion of **10** to **11** at the active site of alcohol dehydrogenase, contrasted to the relative stability of **2** following its production by pyruvate decarboxylase, can also be explained on chemical grounds. A theoretical consideration of all possible resonant structures for **9** and **11** does suggest that **11** is more stable than **9**; therefore, it is reasonable to expect **10** to react faster than **2** with regards to heterolytic N—O bond cleavage.



2 9

10 11

One unusual feature of the present reaction was the apparent lack of conversion of **1** to free 4-(dimethylamino)phenylhydroxylamine **10**. In our previous study we found that simple $2e^-$ reduction of 4-chloronitrosobenzene to give 4-chlorophenylhydroxylamine predominated over hydroxamic acid production for the case of pyruvate decarboxylase (*2*). To explain simultaneous production of both arylhydroxylamines and hydroxamic acids by the action of pyruvate decarboxylase on most nitroso aromatics, we proposed a redox mechanism for pyruvate decarboxylase (*2*). The oxidation of enzyme-bound "active acetaldehyde" by a nitroso functional group generates enzyme-bound "active acetate" and an arylhydroxylamine in the vicinity of the active site. Condensation of the two products at the enzyme active site would explain hydroxamic acid production, while diffusion of the arylhydroxlamine away from the active site would explain its presence in enzymic incubations. A possible explanation for the failure to detect **10** as a product during the reaction of **1** with pyruvate decarboxylase is that **10** is much more nucleophilic than other arylhydroxylamines investigated. The active-site generation of **10** by a redox reaction of **1** with enzyme-bound "active acetalde-

hyde" (*2*) might be followed by immediate reaction of the highly nucleophilic **10** with enzyme-bound "active acetate" (*2*) to produce **2**. The lower nucleophilicity of 4-chlorophenylhydroxylamine might allow more for the escape of this hydroxylamine from the enzyme active site.

The ease with which **2** (but not **4**) undergoes enzymatic reduction to **3** is explainable on the basis of reaction intermediates such as **9**. One, however, can propose reasonable additional reactions for an intermediate such as **9**. Of greatest interest is the possibility that other nucleophiles might react either at a nitrogen or ring carbon of **9** to give covalent adducts. Such an interaction is an attractive explanation for the toxicity of acetaminophen (*15*). The possibility that **2** or **9** might form covalent adducts with certain DNA bases without the requirement for further metabolic activation is under investigation.

ACKNOWLEDGMENTS

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