Tetranitromethane (TNM) was employed as an electrophilic reagent to probe for the lysyl oxidase-catalyzed processing of n-butylamine to an intermediate carbanion during the oxidation of this amine to n-butyraldehyde according to a prior description of the use of TNM to trap enzyme-generated carbanion intermediates (Christen, P. and Riordan, J. F. (1968) Biochemistry 7, 1531–1538). The addition of n-butylamine to assay mixtures containing lysyl oxidase and TNM markedly increased the background rate of nitroform release. The $K_a$ for n-butylamine was essentially the same whether determined from the rate of lysyl oxidase-catalyzed nitroform release or from the rate of n-butyraldehyde production in the absence of TNM, the latter assessed by measurements of the rate of H$_2$O$_2$ formation. The initial rate of substrate oxidation by lysyl oxidase revealed kinetic isotope effects of $K_a$ and $V_{max}/K_m$ parameters, consistent with a rate-contributing a-proton abstraction step. These and other available data are incorporated into a proposal for the mechanism of action of this enzyme.

Lysyl oxidase plays an essential role in the biosynthesis of fibrous proteins of connective tissue by oxidatively deaminating peptidyl lysine in collagen and elastin to peptidyl aminoadipic-d-semialdehyde, thereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: PQQ, pyrroloquinoline quinone; BAPN, $\beta$-aminopropionitrile; TNM, tetranitromethane.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**Substituted benzylamines and nitriles, LiAlH$_4$, Li$\cdot$AIP$_2$, tetranitromethane, and butyramide were purchased from Aldrich. Horseradish peroxidase and sodium homovanillate were products of Sigma. PQQ$^1$ was purchased from Fluka Chemical Corp., Ronkonkoma, NY.

**Enzyme Assay and Purification—**Lysyl oxidase was assayed against 184,000 dpm of an insoluble chick embryo aortic elastin substrate labeled with [4,5-3H]lysine prepared as described (13). Alternatively, the lysyl oxidase-dependent oxidation of n-butylamine was followed fluorometrically in a peroxidase-coupled assay (14). The enzymes used in these studies was purified from bovine calf aorta by a method previously described (15). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified enzyme results in a single band with a molecular weight of 32,000, although this product contains four

\[ RCH_2NH_2 + O_2 + H_2O = RCH=O + NH_3 + H_2O_2 \]
component variants of lysyl oxidase, each of which has been shown to be structurally very similar and which has apparently identical catalytic properties (16). The concentration of purified lysyl oxidase was assessed spectrophotometrically in 16 mM potassium phosphate, 6 M urea, pH 7.7, using the extinction coefficient of 2.0 absorbance units/mg⁻¹ cm⁻¹ at 280 nm as determined in these studies. The content of functional active sites in the purified enzyme was assessed by comparing the enzyme-specific activity against the tritium-labeled elastin substrate in units/mg⁻¹, where one unit is defined as one dpm of 3H₂O released by enzyme action in 2 h of assay at 37°C/0.5-ml portion of assay distillate, to the value of 4 × 10⁶ units/mg⁻¹ as the specific activity of fully active lysyl oxidase.

Assessment for a Carbamation Intermediate—The possibility that a transient carbamation is generated in the catalytic processing of amines by lysyl oxidase was assessed by the method of Christen and Riordan (9). Assays included specified quantities of lysyl oxidase, 10 mM n-butyramine, and 0.25 mM tetranitromethane in 16 mM potassium phosphate, pH 8.0. Enzyme-dependent production of the nitroform anion (λ_max, 350 nm; ε₃₆₀, 1.44 × 10⁴ M⁻¹ cm⁻¹) (9) was followed at 37°C by continuously monitoring the change in A₃₅₀ in a Perkin-Elmer Model 570 recording spectrophotometer equipped with a thermostat-controlled cuvette chamber.

The possibility was considered that a substrate- and enzyme-dependent rate of nitroform accumulation might result from the reoxidation by TNM of enzyme-bound, reduced FQQ generated during amine oxidation rather than from the reaction of TNM with a substrate-derived carbamation. This was assessed by following the reaction of TNM with washed products of FQQ chemically produced from the authentic ortho-quinone, monitoring nitroform accumulation under anaerobic conditions. The quinol product of FQQ reduction (Fig. 1) was synthesized by reduction of authentic FQQ with NaBH₄ under nitrogen according to the method of Itoh et al. (17). The quinol product exhibited an absorption band at 502 nm with a molar extinction coefficient of 28,000 in 0.2 M potassium phosphate, pH 7, consistent with the reported properties of the quinol (17). The conditions used to generate a mixture of the aminophenol (Fig. 1) and quinol products of FQQ reduction by amines were similar to those of Itoh et al. (18). A solution containing 80 μM FQQ, 4 mM benzyamine, and 4 mM dodecyltrimethylammonium bromide in 0.2 M potassium phosphate, pH 7.4, was prepared from nitrogen-purged stock solutions and incubated in the absence of oxygen in an anaerobic cuvette at 37°C, following the progress of the reduction of FQQ by the increase in optical density at 320 nm, according to Itoh et al. (18). The near-UV and visible absorption spectrum of the anaerobic reaction mixture revealed two maxima at 300 and 320 nm, respectively, after 90 min of incubation, the apparent completion of the reaction. The reaction mixture was adjusted to pH 8.0, and the spectrum of an aliquot of this mixture diluted into acetonitrile displayed one prominent peak at 301 nm in the near-UV and visible spectral regions, indicative of the presence of the aminophenol as a major product of the reduction of FQQ by benzylamine and consistent with the corresponding analysis of Itoh et al. (18). The reactivity with TNM of the isolated quinol or of the mixture containing the aminophenol was assessed under anaerobic conditions, examining for nitroform production. TNM was added to a final concentration of 0.25 mM to the quinol (13 μM) in 16 mM potassium phosphate, pH 8.0, or to the unresolved mixture containing the aminophenol after adjusting the reaction mixture to pH 8.0 while maintaining anaerobiosis. In each case, the A₃₅₀ was monitored for the production of nitroform at 37°C. The A₃₅₀ was also followed in controls containing only TNM in buffer or TNM and either oxidized FQQ or benzyamine under the same conditions of pH, temperature, and anaerobiosis. The production of nitroform was negligible in each control and in the reaction mixtures containing either of the reduced forms of FQQ, with a maximum accumulation of less than 0.01 mol of nitroform/mol of quinol or aminophenol present.

Synthesis of Deuterated Compounds—1,1-Dideuterono-butyramine was synthesized by refluxing 2.6 g of n-butyramide and 1 g of LiAlH₄ in 20 ml of dry tetrahydrofuran (previously distilled over LiAlH₄) for 2 h. The amine product was extracted into ether and purified by steam distillation. Thin layer chromatography of the purified product on silica gel plates in n-butyl alcohol/acetate acid/water (4:1:1) resulted in a single ninhydrin-reactive spot with an Rᵣ (0.43) corresponding to that of authentic n-butyramine. The deuterated product boiled at 78-80°C, whereas n-butyramine boiled at 76-78°C (lit., 77°C). Melting points of derivatives of this compound were as follows (melting points of corresponding di-protio-n-butyramine are in parentheses): phenylureas, 134°C (134°C); phenylthioureas, 64°C (65°C); benzoamides, 41°C (42°C). The nuclear magnetic resonance spectrum of 1,1-dideuterono-butyramine in deuterochloroform was identical to that of n-butyramine except for the absence of a signal at 2.64 ppm corresponding to the two α-protons of n-butyramine. Nuclear magnetic resonance spectroscopy was also employed to assess the amount of the deuterated product in the distillate. Experiments utilizing 380-50 mixtures of protonated and deuterated butylamine as substrate for lysyl oxidase resulted in the predicted decrease in the magnitude of the apparent isotope effects, indicating that the deuterated substrate did not contain a contaminating inhibitor of the enzyme.

RESULTS

Reaction with Tetranitromethane—A slow rate of nitroform production was seen during a 2-min preincubation of lysyl oxidase with 0.25 mM TNM at 37°C in 16 mM potassium phosphate, pH 8.0 (Fig. 2). This likely represents a slight degree of chemical modification of lysyl oxidase by TNM since the rate of nitroform production was negligible in an
enzyme-free reaction mixture containing TNM, n-butylamine and the ammonia, H₂O₂, and n-butylaldehyde products of lysyl oxidase action on n-butylamine. Addition of n-butylamine to a final concentration of 10 mM to the enzyme-containing reaction mixture markedly increased the rate of nitroform production. The amount of nitroform accumulating in 10 min which was dependent on the presence of both the enzyme and the substrate is 80-100 times the content of enzyme active sites, consistent with a requirement for enzyme turnover to result in nitroform release. Notably, the rate of nitroform production was reduced to that seen in the absence of the amine substrate if lysyl oxidase which had been fully inactivated by prior incubation with 10 μM BAPN was used as the enzyme source (Fig. 2). Furthermore, the initial rate of substrate-dependent nitroform production was linearly related to the concentration of functional active sites of lysyl oxidase present in each assay (Fig. 3). In addition, the reciprocals of the initial rates for the substrate-dependent production of nitroform were linearly related to the reciprocals of n-butylamine concentration in a Lineweaver-Burk plot (19), consistent with saturation kinetics (Fig. 4). A corresponding plot for the oxidation of n-butylamine as assayed by following production of nitroform were linearly related to the reciprocals of n-butylamine concentration in a Lineweaver-Burk plot (19), consistent with saturation kinetics (Fig. 4). The Kₗ for n-butylamine of 16 mM calculated from the data of the peroxidase-dependent assay for H₂O₂ was identical within experimental error (±2 mM) with the value of 17 mM obtained for n-butylamine in the assay for nitroform production. The Vₘₐₓ values in the two assays differed, however, with values of 6.7 nmol of H₂O₂ produced per min⁻¹ and of 27.7 nmol of nitroform produced per min⁻¹, each obtained with 0.80 nmol of functional enzyme active sites/assay.

Although these results can be taken as evidence for reaction of TNM with an enzyme-generated carbanion intermediate derived from the substrate, the alternative possibility that the substrate-enhanced catalytic release of nitroform could reflect the reoxidation by TNM of a reduced form of the carbonyl cofactor catalytically produced during the oxidation of the amine was also considered. As noted, PQQ has recently been identified as the carbonyl cofactor in lysyl oxidase (3, 4). Model studies predict that PQQ is reduced to the corresponding quinol and/or to the aminophenol as an amine is oxidized to the corresponding aldehyde (17, 18, 20). The quinol was prepared and isolated as the NaBH₄-reduced product of PQQ, whereas the aminophenol was generated as an unresolved mixture of the quinol and aminophenol by reducing PQQ with excess benzylamine under anaerobic conditions according to Itoh et al. (18). As described under “Materials and Methods,” the addition of TNM to anaerobic solutions of the quinol or the aminophenol did not result in the reoxidation of either reduced species of PQQ or in significant quantities of nitroform production. In both cases, the amount of nitroform produced was less than 1% of the amount of quinol and/or aminophenol present in the mixture. These results argue against the conclusion that the substrate-dependent release of nitroform seen with lysyl oxidase reflects the ability of TNM to reoxidize the organic cofactor of this enzyme.

In toto, these results are consistent with the reaction of TNM with an enzyme-processed carbanionic derivative of the n-butylamine substrate, which in turn stems from the Michaelis (ES) complex, and thus with the involvement of catalytic events in the enzyme-enhanced rate of substrate-dependent nitroform production.

The slow rate of nitroform production seen upon incubation of equal amounts of active or BAPN-inactivated lysyl oxidase in the absence of n-butylamine is consistent with the modification of enzyme residues by TNM. The effect of TNM on the catalytic potential of the enzyme therefore was directly assessed. Enzyme was incubated in the absence of substrate for 4 min in the presence or absence of TNM, both samples of lysyl oxidase were dialyzed at 4 °C to remove the reagent, and the kinetic characteristics of the untreated and TNM-treated enzymes were compared. In summary, the Kᵣ values for BAPN were 3.8 and 4.6 (±0.4) μM, the Vₘₐₓ values for the n-butylamine substrate were 14 and 12 (±2) mM, and the Vₘₐₓ values for n-butylamine oxidation were 1.25 and 0.71 (±0.15) nmol H₂O₂/min⁻¹ for the unmodified and modified enzymes, respectively. Thus, although the Vₘₐₓ was reduced, the Kᵣ and Vₘₐₓ values were not changed significantly, indicating that the mechanistic course for amine oxidation does not appear to be qualitatively changed by the brief preincubation with TNM prior to the addition of n-butylamine. This seems further substantiated by the identical rates of nitroform production in the substrate-free preincubations of the native and
BAPN-inactivated enzymes since it would be expected that BAPN would protect functional residues at the active site from chemical modification (21).

Kinetic Isotope Effects—In view of the present evidence for the formation of a carbanion intermediate, the loss of two electrons from the α-methylene carbon of the substrate is likely to be initiated by abstraction of an α-proton rather than a hydride ion. The steady state kinetic parameters for the oxidation of n-butylamine and 1,1-deutero-n-butylamine were compared to determine whether α-hydrogen abstraction is a slow step in the overall rate for the oxidation of substrate by lysyl oxidase. As shown in Table I, the replacement of both α-hydrogens by deuterium resulted in significant isotope effects in both the $V_{\text{max}}$ and $V_{\text{max}}/K_m$ parameters each obtained at several assay temperatures. The values for $V_{\text{max}}/V_{\text{maxD}}$ ranged from 4 to 2.2 in a temperature-dependent fashion. The magnitudes of these values are consistent with the involvement of α-proton abstraction as a rate-contributing step of the catalytic reaction. An Arrhenius plot of these data demonstrates that the log $V_{\text{max}}$ is linearly related to $1/T$ for both the proton-bearing and deuterated substrates (Fig. 5), consistent with the same rate-contributing step(s) being involved at each temperature.

### Table I

Deuterium isotope effects in the oxidation of n-butylamine or 1,1-dideutero-n-butylamine by lysyl oxidase

| Temperature (°C) | $k_{\text{catH}}/k_{\text{catD}}$ | $(k_{\text{catH}}/K_m)/k_{\text{catD}/K_m}$ |
|-----------------|------------------|-------------------------------|
| 32              | 3.9              | 2.4                           |
| 37              | 4.3              | 2.8                           |
| 43              | 3.8              | 2.6                           |
| 48              | 2.6              | 2.6                           |
| 55              | 2.2              | 2.4                           |

**DISCUSSION**

The oxidation of primary amines by lysyl oxidase requires the breakage of a C-H bond at the α-carbon of the substrate. This can occur either by α-proton abstraction with the likely assistance of a general base of the enzyme to form a transient α-carbanion, or the α-hydrogen may be released as a hydride ion thus generating an enzyme-bound carboxonium ion form of the substrate. The substrate-dependent catalytic rate of nitroform release from assays including lysyl oxidase, n-butylamine, and TNM is consistent with the trapping of a catalytically produced carbanionic species of the enzyme-bound substrate by the electrophilic nitronium ion derived from TNM and thus with a mechanism involving α-proton abstraction. TNM has been similarly employed to trap substrate-derived carbanion intermediates generated during catalysis by glutamate dehydrogenase (9), aspartate aminotransferase (10), isocitrate lyase (11), and kynureninase (12). The apparent lack of reaction of TNM with reduced PQQ in solution argues against the alternative possibility that the catalytic release of nitroform reflects the direct reoxidation by TNM of a reduced PQQ cofactor generated as the amine is oxidized to the aldehyde at the active site of lysyl oxidase. Moreover, the relatively low oxidation potential of PQQ ($E_{\text{red}} \approx 90 \text{ mV}$) also argues against a hydride transfer mechanism in amine oxidation by PQQ-dependent enzymes, as pointed out by Itoh et al. (17). In fact, model studies of amine oxidation by PQQ reported by Itoh et al. (19) and Bruice and co-workers (20) were consistent with an α-proton abstraction mechanism.

In addition to the evidence for a-carbanion formation, it was demonstrated that substitution of both α-hydrogen positions of n-butylamine with deuterium results in apparent isotope effects on both $V_{\text{max}}$ and $V_{\text{max}}/K_m$. The magnitudes of the effects on $V_{\text{max}}$ ranging from 4.3 to 2.2 as the temperature increases are consistent with the conclusion that α-proton abstraction is rate-contributing in the overall reaction of n-butylamine oxidation. These $V_{\text{max}}$ isotope effects are also similar in magnitude to those reported for diamine oxidase (22), monoamine oxidase (23), and bovine plasma amine oxidase (24), these values ranging from 2.0 to 5.1. However, the degree to which this step is rate-determining cannot be deduced from these data alone since true isotope effects were not determined in the present study (25). Moreover, the deuterated butylamine used in these studies contain two α-deuterium atoms, one of which remains on the carbon which undergoes oxidation. Since this α-carbon is converted from a tetrahedral to a trigonal state during the catalytic reaction, a secondary isotope effect is also possible which could contribute to the magnitude of the observed isotope effect. However, secondary kinetic isotope effects seen in enzyme catalysis do not usually exceed absolute magnitude of 1.4 and more commonly are in the range of 1.05 to 1.2 (26) and thus should not have a major influence on the magnitudes of the apparent primary isotope effects noted here.

The information presently available that bovine aortic lysyl oxidase contains a PQQ carbonyl cofactor (3, 4), that rate-contributing α-proton abstraction occurs, and that the reaction mechanism follows the ping-pong kinetic pattern shown in Scheme I can be assimilated into a mechanistic proposal for the oxidation of amines by lysyl oxidase (Fig. 6). The catalytic reaction is represented as the sum of two half-reactions, the first of which yields the aldehyde and a reduced derivative of the enzyme (Fig. 6A) and the second of which involves the reoxidation of the reduced enzyme by molecular oxygen (Fig. 6B), consistent with the ping-pong kinetic pattern obtained (6). PQQ is depicted to be covalently linked to the apoenzyme through one of its (arbitrarily selected) car-
Proton Abstraction in Lysyl Oxidase Catalysis

Boxyl functions consistent with prior evidence for a covalent cofactor-enzyme bond (5). It is proposed that the first half-reaction is initiated by the formation of a substrate-cofactor imine (I) by nucleophilic addition to PQQ to form a carbonylamine intermediate (not shown) which is then dehydrated to the imine (I), consistent with results of model studies (17, 18, 20). α-Proton abstraction may then occur, presumably facilitated by a general base of the enzyme, resulting in the formation of a carbanion intermediate, the latter represented as a series of resonance-stabilized canonical forms (II). The product imine form of II is hydrolyzable by water to release the aldehyde and to produce the reductively aminated form of the enzyme-bound cofactor, III. An alternative possibility which can occur in the nonenzymatic oxidation of amines by PQQ involves the passage of an electron pair from the α-carbon of the substrate into PQQ after formation of the PQQ-substrate imine (I) with the subsequent release into solution of the oxidized substrate as the free imine and thus generating the two-electron reduced quinol form of PQQ (18). Spontaneous hydrolysis of the imine in solution would then yield the aldehyde and ammonia. This possibility is discounted from incorporation into the present proposal, however, by direct evidence previously obtained against the release of a free imine during lysyl oxidase catalysis (6).

The second half of the ping-pong reaction involves the reoxidation of the cofactor by enzyme-bound molecular oxygen (Fig. 6B). It has been previously suggested that the copper cofactor, presumed to exist as Cu(II) at the active site, may interact directly with the carbonyl cofactor to mediate the transfer of two electrons from the reduced cofactor to oxygen (2). However, recent analyses based on the use of a cofactor-linked fluorescent probe indicate that the inorganic cofactor and the functional carbonyl moiety of the organic cofactor may be as much as 10 Å from each other (27). It is interesting in this regard that the model studies of Itoh et al. support the feasibility of a direct interaction of oxygen with reduced PQQ to result in the concerted two-electron reoxidation of the reduced cofactor (17, 18), thus obviating a need for an intermediary role for copper in electron transport to oxygen. Nevertheless, additional studies are required to further assess possible modes of interaction between the copper and PQQ cofactors in lysyl oxidase catalysis. In any event, the sequence of substrate binding and product release shown by steady state kinetics (6) permits the summary of events in the second half-reaction as shown (Fig. 6B). Enzyme-bound oxygen may accept two electrons from the reduced cofactor then to be released from the enzyme as H₂O₂. The resulting imine of the now reoxidized PQQ (species IV) may be hydrolyzed to release ammonia as the last product to depart, thus completing the catalytic cycle.

As noted, deuterium isotope effects were observed on \( V_{\text{max}} \) (=\( DV_{\text{max}} \)) (28) and on \( V_{\text{max}}/K_m \) (=\( DV_{\text{max}}/K_m \)) with the deuterated butylamine substrate. The values for \( DV_{\text{max}} \) exceeded those for \( DV_{\text{max}}/K_m \) at the lower temperatures examined, as occurs at 37 °C. Since isotope effects on \( V_{\text{max}}/K_m \) only reflect steps up to and including the first irreversible step (28), catalytic events after aldehyde release would not be expected to contribute to the suppression of \( DV_{\text{max}}/K_m \) since release of the aldehyde should be the first irreversible step under the initial rate conditions employed. Moreover, as indicated by the analyses of Northrop (29), the suppression of \( DV_{\text{max}}/K_m \) relative to \( DV_{\text{max}} \) should be attributable to a step with a relatively high energy barrier which precedes the isotope-sensitive α-proton abstraction step. Such early, energetically difficult steps could involve the formation of the imine intermediate (species I, Fig. 6A) and/or possibly result from rate-

**Fig. 6.** First-half (A) and second-half (B) reactions in lysyl oxidase catalysis.
contributing conformational changes occurring in the enzyme at relevant stages during catalysis. Since steps following the first irreversible step cannot suppress \( DV_{\text{max}}/K_m \) but may suppress \(DV_{\text{max}}\), it would follow that the second half of the ping-pong reaction is fast relative to the first half-reaction at the lower temperatures since \( DV_{\text{max}}/K_m \) is suppressed relative to \(DV_{\text{max}}\) under these conditions. As noted, however, \(DV_{\text{max}}\) changes as the temperature is increased, indicating that temperature alters one or more reaction components contributing to \( V_{\text{max}} \) but not to \( DV_{\text{max}}/K_m \). It is possible that this temperature-dependent effect reflects a difference in the temperature coefficients of the ground state of one or more of the catalytic intermediates in the second half-reaction. This would be consistent with the fact that \( DV_{\text{max}}/K_m \) remains relatively unchanged as the temperature is increased, indicating that temperature alters one or more reaction components contributing conformational changes occurring in the enzyme at relevant stages during catalysis.

In summary, these studies support the existence of rate-contributing steps prior to the rate-contributing proton abstraction step in the first half of this ping-pong mechanism. Furthermore, the second half of the reaction catalyzed by lysyl oxidase appears to be fast relative to the first half-reaction in the physiological range of temperatures for the alkylamine substrate used in this study. Although analyses of individual rate constants are needed to define further kinetic aspects of this mechanism, the present studies provide additional insight into the chemical and kinetic aspects of the mechanism of this enzyme which should provide a basis for the further development of mechanism-based inhibitors with antifibrotic potential.

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