with steroids had significantly lesser cross-sectional growth in the tibias than their companion control rats whereas the length of the femurs and the widths of the tibial epiphyseal lines were not significantly reduced. However, in the 35-day-old rats the length of the femurs were significantly shorter in the treated rats than in the control rats.

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The Partial Characterization of an Amine Oxidase in Bone Tissue* (33740)

R. B. RUCKER, J. C. ROGLER, AND H. E. PARKER
(Introduced by W. R. Featherston)

Departments of Biochemistry and Animal Science, Purdue University, Lafayette, Indiana 47907

The role of amine oxidase in the cross-linking of collagen and elastin has now been established (3, 4, 7, 12). In nutritional copper deficiency the level of this enzyme is decreased in vascular and connective tissue with a concommitant decrease in the intramolecular cross-linking of elastin (5, 7, 10) and collagen (3, 11, 15). The steps in the cross-linking mechanism are presumed to include the oxidative deamination of lysyl residues contained in collagen and elastin to residues of α-amino adipic-Δ-semialdehyde followed by aldol condensation (12). The research reported herein involves the partial characterization of an amine oxidase from bone which could catalyze the initial step of this sequence.

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Materials and Methods. The femurs from 14-day-old chicks were removed, broken and immediately cleaned of adhering tissue and marrow. The bones were then placed in 0.1 *M* phosphate buffer (pH 7.4) and homogenized for 2 min at full speed in a Servall homogenizer. The homogenate (2 g of bone/10 ml buffer) was differentially centrifuged as outlined in Table I. All procedures were performed at 4°. Amine oxidase was assayed by the method of Gorkin *et al.* (4) using benzylamine as substrate, but incubated in the present experiments at 41°.

Columns of Sephadex G-100 (5× 100 cm) and Sephadex G-200 (1 × 15 cm) were calibrated for gel filtration studies as described by Andrews (1). The proteins used for calibration were purified preparations of catalase, bovine serum albumin, ovalbumin, and lysozyme (Sigma Chemicals). After calibration and equilibration of the column, 10 and 3 ml of the 110,000*g* supernatant layer

TABLE I. Fractionation of Bone Amine Oxidase.

Fraction	Procedure	Relative activity[b]	Total activity[c] (%)
1. 700*g* residue	Centrifugation for 30 min (4°); washed with buffer, 2×; sonicated;[a] assayed	1.0	80
2. 700*g* supernate	Assayed directly	10.0	20
3. 7000*g* residue	Centrifuged 700*g* supernate for 30 min (4°); washed with buffer; sonicated;[a] assayed	10.0	
4. 7000*g* supernate	Assayed directly	13.8	19
5. 110,000*g* residue	Centrifuged 7000*g* supernate for 90 min (4°); sonicated; assayed	8.2	
6. 110,000*g* supernate	Assayed directly	13.1	15

[a] Two hundred W, 10,000 cps for 2 min at 4°.
[b] Relative activity = (Specific activity of indicated fraction/sp act. of 700*g* residue).
[c] Total activity in 700*g* residue + 700*g* supernate expressed as 100%.

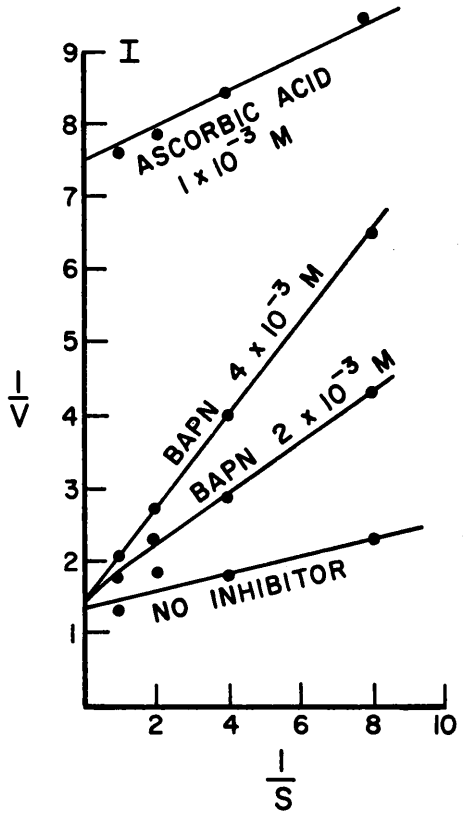
from bone homogenates (Table I) were applied to the Sephadex G-100 and Sephadex G-200 columns, respectively. The proteins in the supernatant layer were eluted from the column with 0.1 *M* phosphate buffer (pH 7.4) containing 0.1 *M* NaCl and 0.1 ppm of copper.

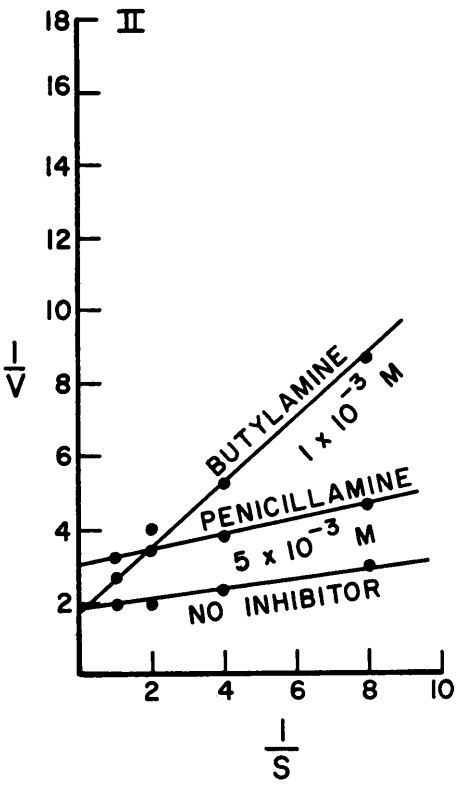
In other studies, the bone homogenates were centrifuged at 30,000*g* for 30 min. Solid ammonium sulfate was then added slowly to the supernatant fraction to 40% saturation, and the resulting suspension was centrifuged at 30,000*g* for 120 min. A precipitate which floated on the top of the solution was suspended in 0.1 *M* phosphate buffer (pH 7.4) and was dialyzed overnight against 5 liters of 0.05 *M* phosphate buffer (pH 7.4) containing 0.5 ppm of Cu and 10^{-4} *M* pyridoxal phosphate. The cloudy dialyzed solution was centrifuged at 10,000*g* for 30 min and the precipitate was discarded.

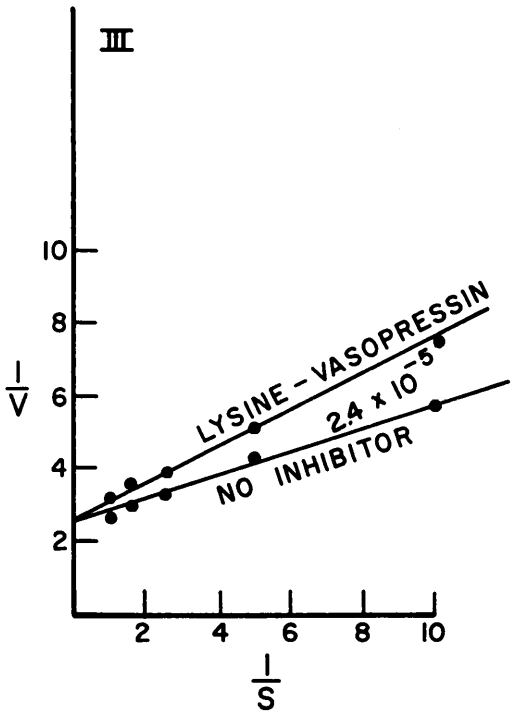
Assay mixtures for kinetic studies consisted of 1 ml of enzyme preparation and 2 ml of 0.1 *M* phosphate buffer (pH 7.4) containing benzylamine plus inhibitor or cofactor (see Tables and Figures for concentrations). In all cases amine oxidase activity was proportional to incubation time and protein concentration. Incubations were stopped at fixed times by the addition of 0.1 ml of concentrated HCl. The benzaldehyde which was formed during the incubations was extracted with cyclohexane and measured at 254 mμ. Blanks were obtained by extracting the incubation mixtures at zero time.

The protein in the residues and supernates was determined by the method of Lowry *et al.* (8). The 700*g* residue was solubilized by gelatination at 100° in sealed tubes 24 hr before determination. All other samples could be analyzed directly. Serum albumin was used as the standard.

Lysine-vasopressin (NIH) was used as the source of a lysine-containing peptide in one series of enzyme studies. The peptide was used as an inhibitor for oxidation of benzylamine and was separated after incubation in the following manner. The acidified incubation media after cyclohexane extraction were pooled and the protein was removed by centrifugation. The resulting solution was filtered through a fine porosity glass filter and treated with 2 ml of 0.5% (w/v) 2,4-dinitrophenylhydrazone (DNP) in 2 *N* HCl for 2 hr. The excess DNP was extracted with toluene and the toluene layer was removed. The solution was then dried, dissolved in 3 ml of 0.15 *M* acetic acid and applied across the center of Whatman no. 3 MM paper (46 × 57 cm). The paper was wetted with buffer containing pyridine:acetic acid:water (50:2:950), pH 6.4, in preparation for high voltage electrophoresis (6). After electrophoresis (3000 V, 40 min) the components which migrated towards the cathode were eluted, hydrolyzed, and chro-


I
ASCORBIC ACID
1 x 10⁻³ M
BAPN 4 x 10⁻³ M
BAPN 2 x 10⁻³ M
NO INHIBITOR
1/V
1/S


II
BUTYLAMINE
1 x 10⁻³ M
PENICILLAMINE
5 x 10⁻³ M
NO INHIBITOR
1/V
1/S


III
LYSINE-VASOPRESSIN
2.4 x 10⁻⁵
NO INHIBITOR
1/V
1/S

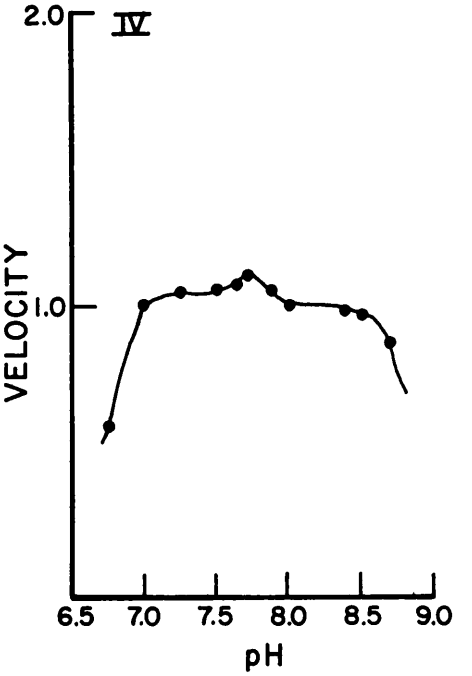

IV
VELOCITY
pH

FIG. 1. Inhibition studies and pH optima for bone amine oxidase: velocity is expressed as μmoles of benzyaldehyde/hr/mg of protein × 100; substrate concentration is expressed as M × 1000 of benzylamine. Assay mixtures contain 1 ml of enzyme preparation (I and II, 30,000g supernate; III and IV, ammonium sulfate fraction), 1 ml of benzylamine and 1 ml of inhibitor in 0.1 M phosphate buffer (pH 7.4). Incubations were stopped at 30 and 60 min and benzaldehyde was then determined. The pH optimum was determined using phosphate buffer (0.1 M).

matographed in a solvent of 1-butanol:acetic acid:water (3:1:1).

In another experiment, the assay solutions containing lysine-vasopressin were again clarified and taken to dryness. The residue was then dissolved in 4 ml of 0.1 M glycine (pH 4.0) and 1 ml was treated with 1 ml of 0.04% (w/v) n-methylbenzothiazolone (MB TH) as described by Paz *et al.* (11) to determine the presence of aldehyde. A 1 ml portion of the solution was also treated with 1 ml of 0.1% (w/v) DNP in 2 N HCl and the spectra determined.

Results and Discussion. Although most of the amine oxidase activity remained in particulate fractions, a substantial amount of the activity could be solubilized (Table I). The activity in the crude mitochondrial (7000g) and microsomal fractions (110,000g) was partially latent and was only fully released after sonication. Since none of the supernatant fractions from bone homogenates were sonicated or treated with detergent, it was not likely that the final supernate (110,000g) represented large amounts of polydispersed particulate material. Other investigators (2) have shown, however, that the enzyme from vascular tissue is more extractable if prepared in the presence of detergent.

After gel filtration of the 110,000g supernate on both Sephadex G-100 and G-200, three broad peaks of protein were obtained which corresponded to $\log_{10}$ molecular weights of approximately 5.40, 5.15, and 4.70. The protein in the forepeak (approx mol. wt., 250,000) contained the amine oxidase activity. Two hundred fifty thousand is in the range of molecular weights determined for other amine oxidases (16).

Fractionation with $(NH_4)_2SO_4$ enhanced activity 8-fold when compared with the original 30,000g supernatant fraction (Table II). The apparent K_m calculated for benzylamine with both the original supernate and ammonium sulfate fraction was 0.15 mM.

Competitive and noncompetitive inhibition of benzylamine oxidation by a variety of compounds are indicated in Fig. 1 and Table III. *Beta*-amino proprionitrile (BAPN) has been demonstrated to inhibit the enzyme in vascular tissue competitively (2, 13). In this study the same appears true for the bone enzyme. It is possible that the marked effect of BAPN *in vivo* on collagen and elastin cross-linking (12) may be related to the inhibition of amine oxidase. Penicillamine, a copper chelator, isoniazid, an inhibitor of pyridoxal-containing enzymes, and ascorbic acid inhibited the benzylamine oxidation noncompetitively. Compounds such as butylamine acted as competitive inhibitors in these studies.

TABLE II. Ammonium Sulfate Fractionation.

Procedure	Volume[a] (ml)	Protein (mg/ml)	Sp act.[b]	Recovery (%)	Purification (-fold)
1. Centrifugation (30,000g; 30 min)	200	2.7	2.3	100	(—)
2. Ammonium sulfate (40% saturation)					
a. Pellet	40	4.6	1.8	27	0.78
b. Supernate	225	1.3	1.0	24	0.43
c. Floating precipitate	20	1.5	18.2	44	7.90

[a] Volume after dialysis and suspension in 0.1 M phosphate buffer (pH 7.4).

[b] (μmoles of benzaldehyde/hr/mg of protein × 100).

TABLE III. Inhibition of Benzylamine Oxidation.[a]

Inhibitor	Concentration (M)	Inhibition (%)
1. L-Lysine	2.5×10^{-3}	1.0
	2.5×10^{-2}	4.7
2. ϵ-aminocaproic acid	2.5×10^{-3}	0.0
	2.5×10^{-2}	2.5
3. Putrescine	1×10^{-3}	1.0
	2×10^{-3}	2.0
4. KCN	2.5×10^{-4}	46.0
	2.5×10^{-3}	84.0
5. p-Hydroxymercuri-benzoate	1×10^{-5}	3
	2.5×10^{-5}	10.0
	2.5×10^{-4}	10.0
	2.5×10^{-3}	21.0

[a] Assay mixtures contained $1 \times 10^{-3} M$ benzylamine, enzyme preparation and inhibitors as indicated (total volume, 3 ml).

Although lysine and terminally charged analogs of lysine, such as putrescine and ϵ-aminocaproic acid, showed no inhibition of benzylamine oxidation, a lysine-containing peptide, lysine-vasopressin, acted as a competitive inhibitor in the reaction (Fig. 1). Evidence that lysine-vasopressin was reacted upon by the enzyme was obtained from electrophoresis and spectral data. A DNP derivative was obtained after enzyme incubation which had reduced migration towards the cathode in an electrical field when compared with nonaltered lysine-vasopression.[1] After hydrolysis and chromatography of the amino acids in this peptide, the following residues were obtained: glycine, proline, cystine, glutamic acid, aspartic acid, phenylalanine, tyrosine and a compound which absorbed intensively in UV light. Hydrolysis of nonaltered lysine-vasopression resulted in the same amino acids, but lysine was obtained in place of the intensely absorbing compound. The DNP and MBTH derivatives of the altered peptide demonstrated spectral properties almost identical with DNP and MBTH derivatives from enzymatic digests of lathyritic and control elastin preparations as reported by Miller and Fillmore (9). The hydrazone after DNP reaction absorbed maximally at 387 mμ[2]. The incubated peptide in another study formed an azine with MBTH which absorbed maximally from 305–310 mμ at pH 4 and shifted to 295 mμ at pH 1. These data are characteristic of MBTH and DNP derivatives of saturated aldehydes (9, 11).

That the enzyme requires pyridoxal phosphate could be demonstrated by the fact that with addition of pyridoxal phosphate, inhibition by isoniazid was completely overcome (Table IV). The KCN also markedly inhibited the enzyme (Table III). Furthermore, inhibition due to the addition of p-hydroxymercuribenzoate suggests that sulfydryl groups may also be important in maintaining the integrity of the enzyme. The benzylamine oxidation was optimal between pH 7.0 to 8.2 with a peak of pH 7.7 (Fig. 1).

From this study, it is evident that the bone amine oxidase is copper-containing and requires pyridoxal phosphate for activity. The inhibition of benzylamine with a lysine-containing peptide also indicates that the use of benzylamine as substrate when studying amine oxidase in connective tissue may be partially justified, since the primary role of this enzyme appears to be the oxidative deamination of lysyl residues contained in elastin and collagen.

Summary. Evidence is presented which demonstrates that chick bone contains an

TABLE IV. Effect of Pyridoxal-5-phosphate on Isoniazid Inhibition.[a]

Isoniazid ($M \times 10^{-4}$)	Pyridoxal-5-phosphate (M)	Inhibition (%)
1.67	0.00	37.5
1.67	1.67×10^{-4}	17.5
1.67	8.34×10^{-4}	10.5
1.67	1.67×10^{-3}	1.0

[a] Assay mixtures contained $1 \times 10^{-3} M$ benzylamine, 1.5 mg of ammonium sulfate fraction and the above compounds in a total volume of 3 ml.

1 Lysine-vasopressin

```
               NH2 NH2
               /   /
Cy–Tyr–Phe–Glu–Asp–Cy–Pro–Lys–Gly–NH2.
|_________S–S_________|
```

2 We have observed that acidic aqueous solutions of DNP hydrazones of some saturated aldehydes show a 10–20 mμ shift in the τ_{max} toward the visible as compared with acid aqueous solutions of DNP alone (see Ref. 14).

amine oxidase which could function in the oxidative deamination of collagen lysyl residues to α-amino adipic acid-Δ-semialdehyde residues. This function would be directly related to the process of collagen cross-linking. The enzyme was slightly soluble, had an apparent molecular weight from gel filtration studies of 250,000, and could be partially purified by ammonium sulfate precipitation. When benzylamine was used as the substrate in kinetic studies, a K_{mapp} of 0.15 m*M* was obtained. The enzyme required copper and pyridoxal phosphate and was competitively inhibited by *beta*-amino propionitrile, butylamine, and the peptide, lysine-vasopressin. Benzylamine oxidation was noncompetively inhibited by ascorbic acid, penicillamine, and isoniazid. The lysine in lysine-vasopressin was oxidized to the semialdehyde. The pH optimum for oxidations was approximately pH 7.7.

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