

## Effect of Copper Deficiency and Inhibitors on the Amine Oxidase Activity of Chick Tissues.\* (31458)

D. W. BIRD, J. E. SAVAGE, AND B. L. O'DELL

*Departments of Poultry Husbandry and Agricultural Chemistry, University of Missouri, Columbia*

Aortic rupture occurs in animals fed copper-deficient diets as a result of a decreased quality and quantity of elastin(1-5). Reducing agents such as ascorbic acid and isoascorbic acid tend to aggravate the pathology observed in copper-deficient chicks including a decrease in elastin content of the aorta(6,7). Beta-aminopropionitrile (BAPN), a lathyrictic agent, also decreases the elastin content of turkey aortas(8). Aortic elastin isolated from both copper-deficient and BAPN-fed chicks(5,9) has a higher than normal content of lysine and less desmosine and isodesmosine, the crosslinking components of elastin(10,11). Isotope studies (12,13) indicate that four lysine molecules serve as precursors of the desmosine isomers and show that BAPN added to *in vitro* cultures of aorta prevents the incorporation of lysine into the desmosines(9,10). The above observations led to the suggestion that a copper-containing amine oxidase in the aorta may be involved in the oxidation of lysine to form the desmosines(14) and a recent report has shown a decrease in plasma benzylamine oxidase of pigs fed a copper-deficient diet(15). Relatively little information is available concerning the amine oxidase activity of organs rich in connective tissue.

The purpose of this study was to investigate the effect of copper deficiency and dietary BAPN on amine oxidase activities of chick tissues. *In vitro* experiments were conducted to determine the effect of BAPN and ascorbic acid on aortic amine oxidase activity and an attempt has been made to correlate the results with observations on elastin biosynthesis.

*Experimental.* Groups of day-old female Vantress  $\times$  White Rock chicks were fed the copper-deficient and copper-supplemented diets previously described(5).<sup>†</sup> The food intake of the copper-supplemented group was restricted to that of the deficient group, and

both groups received distilled water *ad libitum*. A third group received a practical type diet(7) and tap water *ad libitum*. At the age of one week, 0.06% BAPN fumarate (Aldrich Chemical Co.) was added to the diet of one-half of the chicks receiving the practical diet. Tissues were removed from chicks on the copper regimens at 3 weeks and from those on the BAPN regimens at 4 weeks of age.

For the enzyme assay, tissues were homogenized with 9 or 19 volumes of 0.15 M NaCl in a ground glass homogenizer kept in an ice water bath. Individual aortas and pooled samples (3 chicks) of heart, kidney and liver were used.

Three amines, benzylamine, putrescine and N-butylamine, were tested as substrates. The oxidation of benzylamine was determined spectrophotometrically(16) and putrescine colorimetrically(17). In all assays the tubes were incubated at 37°C for 3 hours with agitation at 30-minute intervals. The assay tube for benzylamine contained 2 ml of 0.2 M phosphate buffer, pH 7.2, 10 micromoles of benzylamine, enzyme preparation (usually 50 mg of tissue), and sufficient water to make 3 ml. In the putrescine assay the tube contained 4.5 ml of 0.067 M phosphate buffer, pH 6.8, 50 micromoles of putrescine, 12.5 micromoles of o-aminobenzaldehyde, enzyme preparation, and water to make 5 ml. The assay tube for N-butylamine contained 1.5 ml of 0.1 M bicarbonate buffer, pH 7.4, 50 micromoles of N-butylamine, enzyme preparation, and water to make 2 ml.

The conversion of butylamine to butanal was determined by use of the Sawicki reaction for aldehydes(18,19). After incubation the solution was adjusted to pH 2.5 by addi-

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tion of 1 ml of 0.2 M glycine buffer, pH 1.0, and 0.05 ml of 1 N HCl. This was followed by addition of 1.0 ml of 0.6% 3-methyl-2-benzothiazolinone hydrazone hydrochloride and heating in a boiling water bath for 3 minutes. After cooling to room temperature, 1 ml of 0.5% FeCl<sub>3</sub> solution was added and allowed to stand 5 minutes. Acetone was added to a final volume of 10 ml and the absorbance measured at 670 m $\mu$ . In agreement with previously reported values(18), the molar extinction coefficient of butanal was found to be  $5.2 \times 10^4$ . Nitrogen was determined by Nesslerization(20); the enzyme activity is expressed as moles of aldehyde produced per hour per mg of nitrogen.

For the *in vitro* study of inhibitors, aortas were pooled from control chicks fed a practical diet. The tissue was homogenized in 0.15 M NaCl and in some experiments the soluble fraction was collected by centrifuging for 15 minutes at  $1000 \times g$ . Less than 50% of total activity was extracted by this procedure. Addition of 1% alkyl phenoxy polyethoxy ethanol (Triton X-100; Rohm and Haas) to the 0.15 M NaCl afforded more complete extraction. Three inhibitors were investigated. BAPN and ascorbic acid in buffered solution were incubated with the enzyme preparation 10 and 30 minutes, respectively, before addition of substrate. Semicarbazide was added at the same time as the substrate.

To determine the efficacy of *in vitro* addition of copper to copper-deficient tissue, cupric acetate was added to homogenates of deficient and control aortas to give a concentration of  $10^{-4}$  M. The homogenate was stirred in the cold (4°C) for 2 hours and then dialyzed for 2 hours against 0.15 M NaCl to remove excess copper. Enzyme activity was determined in non-dialyzed, dialyzed and copper-treated tissue.

**Results and discussion.** As shown in Table I, the activity of copper-deficient aortic tissue was less than one-fourth that of controls and this correlates well with the reduced concentration of elastin in the deficient aortas. Although the elastin content of aortas from normal day-old chicks is low(4), this does not appear to be directly related to benzyl-

TABLE I. Benzylamine Oxidase Activity of Tissue from Copper-Deficient and Control Chicks.\*

Tissue	Benzaldehyde/hr/mg N		Statistical significance†
	Cu-deficient	Cu-supplemented	
	10 <sup>8</sup> × moles		P
Aorta	.30 ± .03‡(6)	1.28 ± .12 (6)	<.005
Heart	1.44 ± .33 (4)	6.84 ± .07 (4)	<.005
Kidney	.65 ± .11 (4)	3.49 ± .91 (4)	<.010
Liver	1.32 ± .42 (4)	1.11 ± .35 (4)	NS
Plasma	§	§	

\* At 3 wk of age deficient and pair-fed controls weighed approximately the same, 95 and 96 g respectively; controls fed *ad libitum* weighed 255 g. Elastin content of dry fat-free aortas, as determined by the method previously described(5), averaged 39.0% for deficient chicks and 70.1% for pair-fed controls.

† Determined by "t" test.

‡ Standard error of mean.

§ No detectable activity.

( ) No. of determinations.

amine oxidase activity. In another trial the activity in a pooled sample of aortic tissue from day-old chicks was found to be  $3.10 \times 10^{-8}$  as compared with  $1.04 \times 10^{-8}$  mole per hour per mg of nitrogen for 4-week-old chicks. The benzylamine oxidase activity in heart and kidney was also markedly reduced by copper deficiency but that of liver was not affected (Table I). Failure of copper deficiency to reduce the benzylamine oxidase activity of liver indicates that the enzyme in this tissue either does not require copper or that it binds copper tenaciously. No benzylamine oxidase activity could be detected in chick plasma. The reduction in amine oxidase observed here is believed to be a function of copper deficiency *per se* and not inanition since the copper-supplemented controls were restricted to the same amount of food and gained at the same rate as the deficient animals.

Further evidence to support the concept that the aortic enzyme is copper dependent is afforded by the *in vitro* addition of copper. Addition of Cu<sup>++</sup> to control tissue had little or no effect on activity but more than doubled the activity of deficient tissue (Table II). Although the activity of the deficient tissue was not restored to the control level, the results strongly suggest that the apoenzyme is present and can be activated by ionic copper. It is possible that the benzylamine assay sys-

TABLE II. Activation of Benzylamine Oxidase from Copper-Deficient Aortas by Ionic Copper.\*

Tissue treatment	Benzaldehyde/hr/mg N	
	Cu-deficient chicks	Cu-supplemented chicks
	$10^8 \times$ moles	
None	.37	.98
Dialysis only	.26	1.18
$10^{-4}$ M copper + dialysis	.81	1.10

\* These data are representative of 4 trials in which  $\text{Cu}^{++}$  was added *in vitro*.

tem measures 2 different enzymes which act on the same substrate but this seems unlikely since similar activities were obtained using butylamine and benzylamine. In comparative tests, samples of normal aorta homogenate oxidized  $0.80 \times 10^{-8}$  mole of butylamine compared to  $1.08 \times 10^{-8}$  mole of benzylamine per hour per mg of nitrogen. No diamine oxidase activity could be detected in aortic tissue and plasma when putrescine was used as the substrate.

**Inhibitors.** The addition of BAPN to a practical diet had no significant effect upon the benzylamine oxidase activity of aortic tissue ( $.92 \times 10^{-8} \pm .04$  compared to a control value of  $1.04 \times 10^{-8} \pm .05$  mole/hr/mg N)<sup>†</sup> although death from aortic rupture and other symptoms of BAPN toxicity were observed. These data show that dietary BAPN has no effect on the synthesis of the apoenzyme or the incorporation of copper into benzylamine oxidase. However, the gross symptoms of BAPN toxicity are similar to those of copper deficiency and suggest that amine oxidase activity is in some way inhibited in the lathyratic animal.

In an attempt to elucidate the metabolic effect of BAPN it was added *in vitro* to normal aorta homogenates. As shown in Table III, BAPN at concentrations of  $10^{-3}$  and  $10^{-4}$  M markedly inhibited benzylamine oxidase. The nature of the inhibition was determined by use of a soluble enzyme preparation. A double reciprocal plot of the data indicates a competitive type inhibition (Fig. 1). Competition of BAPN with the normal substrate for the active site on the aortic

‡ Mean  $\pm$  standard error of 10 individual determinations per group.

amine oxidase may well explain the effect of this lathyrigen upon the biosynthesis of the desmosines in aortic elastin.

Since ascorbic acid also appears to decrease elastin biosynthesis, its *in vitro* effect upon the aortic amine oxidase system was determined and the results are shown in Table III. Ascorbic acid was nearly as potent an inhibitor as BAPN, but it proved to be a non-competitive inhibitor (Fig. 2) as might be predicted if it inhibits by changing the oxidative state of copper in the enzyme. Inhibition of the aortic enzyme by semicarbazide (Table III) suggests the presence of a carbonyl group.

Lysine is a precursor of the desmosines in elastin and its incorporation into these compounds is inhibited by copper deficiency and by BAPN. Biosynthesis of desmosine must involve the oxidation of the  $\epsilon$ -carbon of lysine after formation of the peptide chain. Such oxidations are catalyzed by amine oxidases and these enzymes are known to contain copper (21). Although there is no conclusive proof that the aortic amine oxidase described here is directly concerned with desmosine biosynthesis, the results are highly suggestive of such a relationship. The activity of the enzyme was severely depressed in copper-deficient aortas and competitively inhibited *in vitro* by BAPN. It appears that copper de-

TABLE III. *In vitro* Inhibitors of Aortic Benzylamine Oxidase.

Inhibitor	Activity benzaldehyde /hr/mg N		Inhibition %
	$10^8 \times$ moles		
BAPN*	None	1.11 (3)	
	$10^{-4}$ M	.84 (3)	24.3
	$10^{-3}$ M	.64 (3)	42.3
Ascorbic acid <sup>†</sup>	None	1.06 (3)	
	$10^{-4}$ M	.94 (2)	11.3
	$10^{-3}$ M	.61 (3)	42.5
	$10^{-2}$ M	.28 (2)	73.6
Semicarbazide <sup>‡</sup>	None	.76 (1)	
	$10^{-4}$ M	.53 (1)	30.3

\* BAPN was incubated with homogenate 10 min before addition of substrate.

<sup>†</sup> Ascorbic acid was incubated with homogenate 30 min before addition of substrate.

<sup>‡</sup> Semicarbazide was added at same time as substrate.

( ) No. of determinations.

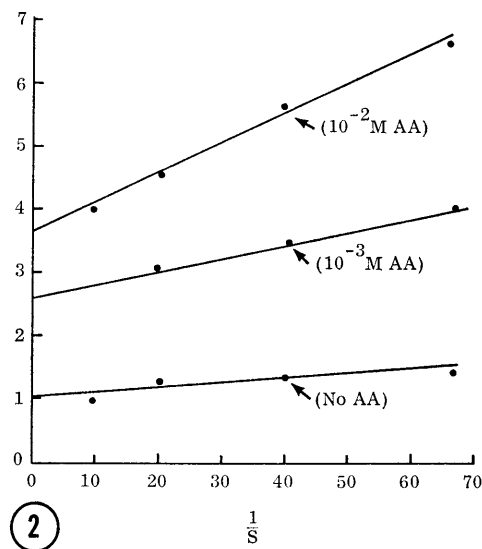
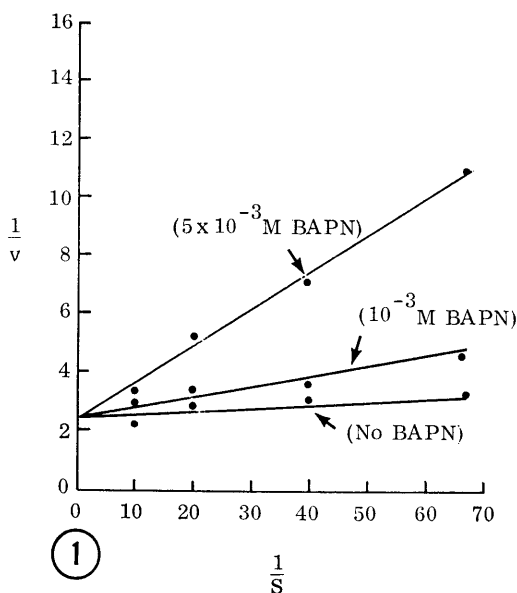


FIG. 1. Inhibition of benzylamine oxidase by beta-aminopropionitrile (BAPN). The units of  $v$  are  $10^8 \times$  moles benzaldehyde/hr/mg N and  $S$  is given in molar concentration  $\times 30$ . Enzyme preparation was a Triton X-100 extract of aortas from chicks fed the practical diet.

FIG. 2. Inhibition of benzylamine oxidase by ascorbic acid (AA). The units of  $v$  are  $10^8 \times$  moles benzaldehyde/hr/mg N and  $S$  is given in molar concentration  $\times 30$ . Enzyme preparation was an aortic homogenate from chicks fed the practical diet.

ciency and BAPN produce similar gross defects because they impinge upon the same biosynthetic site, copper being essential for enzymic activity and BAPN inhibiting the same enzyme. This may also explain the failure of copper supplementation to overcome the toxicity of BAPN (8) since the lathyrogen competitively inhibits the intact amine oxidase without affecting its copper content. It is significant that ascorbic acid aggravates the symptoms of copper deficiency. Ascorbic acid inhibition of amine oxidase may explain its effect on elastin biosynthesis (6) but there is no evidence that this inhibition is responsible for its effect on other aspects of copper deficiency pathology (7).

Copper deficiency and dietary BAPN affect crosslinking in collagen as well as in elastin (8). Recent evidence (22) suggests that the  $\epsilon$ -carbon of lysine in tropocollagen is oxidized to an aldehyde which then condenses with another similar group to form the primary crosslink between alpha chains.

It appears that the crosslinks in both collagen and elastin are formed by a similar mechanism involving amine oxidase and that

copper deficiency and lathyrogens impair the function of this enzyme system.

**Summary.** The effect of copper deficiency and a dietary lathyrogen (BAPN) on the amine oxidase activity of various tissues in growing chicks was investigated. The benzylamine oxidase activity of aorta, heart and kidney was decreased in copper-deficient chicks compared to copper-supplemented chicks while the activity of liver was not affected. Copper added *in vitro* partially restored the activity of deficient aortic tissue. Dietary BAPN had no effect on aortic benzylamine oxidase activity but *in vitro* addition of BAPN competitively inhibited the enzyme. Ascorbic acid *in vitro* also inhibited the activity of aortic benzylamine oxidase but in a non-competitive manner.

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### Chronic Toxicity of Methyl Linoleate Hydroperoxide for the Rabbit. (31459)

M. G. KOKATNUR, J. G. BERGAN, AND H. H. DRAPER

*Division of Nutritional Biochemistry, Department of Animal Science, University of Illinois, Urbana*

Although the toxicity of oxidized fats for animals is well known(1-6), the explanation of their toxic effect is still unclear. There is evidence that the oral toxicity of air-oxidized oils is associated with their peroxide concentration; however, since peroxides apparently are destroyed in the intestine their toxicity presumably is exerted at this site(5,6). Whether fatty acid peroxides are formed in the tissues of animals in general, and of vitamin E deficient animals in particular, is a subject of current controversy. Clearly, if peroxides are formed *in vivo* they must be rapidly decomposed, as no appreciable accumulation occurs and their continued presence would have serious implications for the structural integrity of cell membranes and subcellular particles(7).

The muscular dystrophy which results from a deficiency of vit E may be attributable to peroxidation of lipids in the cellular and subcellular membranes which proceeds in the absence of this vitamin(8). If this explanation

is correct, chronic parenteral administration of small amounts of preformed hydroperoxides might be expected to enhance the appearance of the disease by increasing the rate of vit E consumption in the tissues for decomposition of the peroxide or for destruction of additional free radicals whose formation may be catalyzed in its presence. This hypothesis was tested in the present study by determining the effects on the occurrence of vit E deficiency of administering small doses of purified methyl linoleate hydroperoxide to rabbits over protracted periods of time.

*Experimental.* New Zealand White weanling rabbits weighing 1100-1600 g were housed individually in metal cages and maintained on a vit E deficient diet for 10 days to deplete their tocopherol reserves. The composition of the diet was as follows(%): Labco casein 20.0, glucose (Cerelese) 20.4, starch 40.0, distilled lard 7.0, salts 4164(9) 4.0, cellulose 5.0, cod liver oil 3.0, choline chloride 0.1 and vit premix(9) 0.5. Methyl linoleate hydroperox-