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Acid Hydrolase Activity of Granulomatous Tissue in the Lathyritic Rat* (33352)

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The pathogenesis of the experimental connective tissue disease, lathyrism, and the mode of the resultant disruption of collagen metabolism has been the focus of much recent attention. Characteristically, lathyritic collagen shows a marked increase in soluble molecules and a decrease in mature, insoluble collagen. Numerous theories on the mechanism of this phenomenon have been proposed, but none at present is totally reconcilable with all of the data. Lathyrogens appear, at least in part, to exert their effect on connective tissues by inhibiting the production of covalent cross-links. Bornstein and Piez (1) have presented evidence that aldehyde intermediates are involved in intramolecular cross-link formation and have proposed that lathyrogens inhibit the process by which specific lysyl residues of the tropocollagen molecule are oxidatively deaminated to aldehydes. Page and Benditt (2) have shown that pig plasma amine oxidase, an enzyme believed to be similar to the oxidase functional in aldehyde formation in collagen, is competitively and reversibly inhibited by the lathyrogen β-aminopropionitrile (BAPN).

Although investigations have centered mainly on the inhibition of cross-link formation in the collagen of lathyritic animals, some attention has been paid to the catabolic aspects of the disease. Tanzer and Gross (3) have presented evidence that there is some degradation of insoluble collagen and have postulated the possible involvement of a protease in lathyrism. Holzmann *et al.* (4) have demonstrated a significant increase in serum catheptic activity in lathyritic rats, and it has been shown (5) that protease-induced alterations of tropocollagen yield a material that will no longer form cross-links. Dense granules in cartilage matrix of chick embryos, that are believed to be protein–polysaccharide complexes, have also been shown to disappear shortly after the injection of BAPN (6).

To further evaluate the degradative aspects of lathyrism, and to investigate the possible role of lysosomal enzymes in this process, the activities of five acid hydrolases, of the lysosomal type, were determined in granulomatous tissue from normal and lathyritic rats.

Methods. Subcutaneous granulomas were induced by implanting 2 or 3 polyvinyl plastic sponges (weighing 120 $\pm$ 5 mg) subdermally on the dorsum of male rats of the Holtzman strain (initial weight 140–150 g). Beta-aminopropionitrile (BAPN) was either incorporated in the diet at a level of 0.4%, or was administered by intraperitoneal injection (144 mg/day). There were 6–9 rats in each group, and the control rats were pair fed. The rats on the dietary BAPN, and their controls, were sacrificed on day 18, and

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TABLE I. Collagen Content of Sponges.

Administration	Group	Collagen (mg/sponge) NaCl	Citrate	Insoluble
BAPN diet	Lathyritic (9)[a]	5.4	1.8	12.5
	Control (9)	1.4	1.1	15.5
BAPN injected	Lathyritic (6)	3.1	1.9	5.3
	Control (6)	1.1	0.9	6.8

[a] Numbers in parentheses represent number of rats per group.

those injected with BAPN, or with saline as a control, were sacrificed on day 14.

Excised sponges were sequentially extracted with 1 *M* NaCl and 0.5 *M* citrate (pH 3.6), and the extracts and insoluble residue analyzed for collagen hydroxyproline as described by Nimni and Bavetta (7). A second sponge from each animal was extracted for the acid hydrolases with 0.15 *M* NaCl following the procedure of Woessner and Boucek (8).

All enzyme assays were carried out at 37°. Cathepsin activity was determined at pH 3.6 by the method of Anson (9). β-Glucosaminidase was determined with 1.1×10^{-3} *M* *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide as substrate (0.2 *M* citrate-phosphate buffer, pH 4.4); β-glucuronidase with 1.2×10^{-3} *M* *p*-nitrophenyl-β-D-glucuronide (0.2 *M* acetate buffer, pH 4.5); β-galactosidase with 2.5×10^{-3} *M* *p*-nitrophenyl-β-D-galactopyranoside (0.2 *M* citrate-phosphate buffer, pH 3.5); and acid phosphatase with 1.4×10^{-3} *M* *p*-nitrophenyl phosphate (0.2 *M* acetate buffer, pH 4.5). Liberated *p*-nitrophenol was measured at 400 mμ at pH 12.

Results. Table I summarizes the changes in sponge granuloma collagen resulting from the administration of BAPN. The amount of soluble collagen, especially that soluble in 1 *M* NaCl, is considerably increased in lathyrism, while there is a significant decrease in insoluble collagen. These data are in agreement with those reported in the literature (10).

The activities of the 5 acid hydrolases in sponge granuloma extracts from the lathyritic rats are significantly higher than the activities in extracts from the control animals, as shown in Tables II and III. Thus, there is an apparent positive correlation between the increase in soluble forms of collagen in lathyrism and the increase in acid hydrolase activity.

TABLE III. Enzyme Activities in Lathyrism Relative to Control.

Hydrolase	Dietary BAPN	Injected BAPN
Cathepsin	2.1	2.2
β-Glucuronidase	1.7	1.9
β-Galactosidase	1.8	1.4
β-Glucosaminidase	2.2	2.1
Acid phosphatase	1.7	1.7

Discussion. There are a number of possible explanations for the increases found in lathyrism of acid soluble and neutral salt soluble forms of collagen, and for the concomitant decrease in insoluble collagen. First of all, mature, insoluble collagen could be de-

TABLE II. Enzyme Activities.[a]

Hydrolase	Dietary BAPN Control	Dietary BAPN Lathyritic	Injected BAPN Control	Injected BAPN Lathyritic
Cathepsin	5.6	11.5	6.0	13.3
β-Glucuronidase	36.8	62.8	31.6	60.7
β-Galactosidase	29.6	53.0	20.8	28.4
β-Glucosaminidase	18.0[b]	40.2[b]	78.0[c]	161.2[c]
Acid phosphatase	41.0	69.7	53.7	91.2

[a] *p*-Nitrophenol (μmoles) (or tyrosine for cathepsin)/60 min/sponge.
[b] Acetate buffer.
[c] Citrate-phosphate buffer.

graded to soluble forms either by the rupture of the actual covalent cross-links, or by removal of the nonhelical, telopeptide portions of the tropocollagen molecule in which at least one of the cross-links is known to occur. It has been shown *in vitro* that under certain experimental conditions several proteases do in fact depolymerize collagen in this manner (11, 12). There is also evidence that collagen depolymerization, or solubilization, occurs to at least a limited extent in lathyrism (3), and probably occurs to an appreciable extent in other connective tissue disorders such as rheumatoid arthritis (13). Secondly, enzymes involved in cross-link formation could be inhibited, and recent evidence indicates that this may be a major factor in lathyrism. It has been shown that oxidative deamination, presumably enzymatic, of the specific lysyl residues involved in intramolecular cross-linking is considerably reduced in lathyritic animals (1, 2). Thirdly, cross-link formation might be prevented by a number of structural modifications of the tropocollagen molecule. Proteolytic removal has effectively prevented its *in vitro* polymerization (5). Also, the small amount of carbohydrate covalently bound to collagen is believed by some to play a role in cross-linking (14), and if this is the case, its removal would also be expected to reduce polymerization. Fourthly, enzymatic modification of ground substance glycoproteins and mucopolysaccharides, which have been shown to play an important role in fibril formation and stability (15), could also account in part for the increased collagen solubility in lathyrism.

From the results presented in this paper, it is tempting to speculate that acid hydrolases, presumably of lysosomal origin, play a significant role in the pathogenesis of experimental lathyrism. If the 5 enzymes measured are indeed of lysosomal origin, it is to be expected that other lysosomal enzymes, such as hyaluronidase, that could either directly or indirectly affect collagen maturation and degradation, would also show increased activities. The individual or combined action of lysosomal proteases and glycosidases under favorable conditions could conceivably bring about most of the structural changes in collagen and ground substance discussed above. Collagen synthesis is not impaired in lathyrism (10), but is possible that the newly synthesized tropocollagen, due to modifications of the ground substance, may not be able to align itself properly into fibrils, thus becoming more susceptible to denaturation and enzymatic attack. It is also possible that for enzymatic oxidative deamination to occur the collagen molecules must be properly oriented, and that this proper orientation is hindered by changes brought about by the action of lysosomal enzymes, or even that the amine oxidase itself is destroyed by such enzymes.

Summary. The activities of the acid hydrolases, cathepsin, β-glucuronidase, β-galactosidase, β-glucosaminidase, and acid phosphatase have been shown to increase significantly in granulomatous tissue of lathyritic rats. These enzymes are of the type normally found in lysosomal particles. An apparent correlation between the increase in acid hydrolase activity and the increase in soluble forms of collagen was demonstrated.

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Ethanol Effects on Methionine Metabolism in Rat Liver (33353)

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Dietary supplements of methionine and choline can partially reverse the hepatic steatosis caused by chronic ethanol administration of rats (1). This finding can be interpreted as an increase in the dietary lipotrope requirement secondary to alcohol feeding (2). In order to define the enzymatic basis for this phenomenon, we have studied the effect of ethanol feeding on four enzymes of methionine metabolism in rat liver—methionine-activating enzyme (ATP:L-methionine-*S*-adenosyltransferase, E.C.2.5.1.6); cystathionine synthase; cystathionase (L-homoserine hydro-lyase (deaminating), E.C.4.2.1.15); and betaine–homocysteine methyltransferase (E.C.2.1.1.5). The four enzymes catalyze reactions (1) through (4), respectively:

Methionine + ATP ⟶ *S*-adenosylmethionine + PP_i + P_i, (1)

Serine + homocysteine ⟶ cystathionine, (2)

Cystathionine ⟶ α-ketobutyrate + cysteine + NH_3, (3)

Betaine + homocysteine ⟶ methionine + dimethylglycine. (4)

Experimental Procedures. Male, Sprague-Dawley rats (weight 150–200 g) maintained on Wayne Lab Blox were used routinely. At the time of sacrifice, the animals were stunned and then exsanguinated by carotid transection. The tissues were removed, chilled immediately, and tested either individually or as pooled specimens. Homogenization was performed in ice-cold 0.03 *M* potassium phosphate buffer, pH 6.9, in a Sorvall Omni-Mixer at half speed for 2 min. The extracts were centrifuged at 8000*g* for 15 min at 4°. The supernatant was used for all assays which were performed on the day of sacrifice.

We used our usual assay methods (3, 4). Each is a specific and direct measurement of product formation. Since each of the assay systems is unsaturated with respect to one of the substrates, direct comparisons between the different enzyme activities cannot be made. The results are expressed as mμmoles of product/time/mg of protein. No significant differences in interpretation occurred when we employed units/g of wet weight of liver; units/animal; or units/g of body weight.

All chemicals were of reagent grade and were obtained from commercial sources. Merck, Sharp and Dohme generously supplied the actinomycin D.

Results. Chronic administration of ethanol. The specific activities of methionine-activating enzyme and cystathionase in rat liver were increased significantly in rats given ethanol (4.4 g/kg daily for 14 days) as a supplement to a normal laboratory ration (Table I). The alcohol feeding did not affect the levels of cystathionine synthase and betaine–homocysteine methyltransferase. Ethanol-treated animals gained weight at the same rate as the glucose-supplemented control rats and the alcohol caused no significant change in liver weight, liver fat, or extractable protein per gram of liver. The increase in specific activities therefore reflects a net increase in methionine-activating enzyme and cystathionase. The increase in methionine-activating enzyme occurred within 5 days following the beginning of the ethanol feeding and was unchanged thereafter.

The enzyme levels also were measured in