A SEX-LINKED DEFECT IN THE CROSS-LINKING OF COLLAGEN AND ELASTIN ASSOCIATED WITH THE MOTTLED LOCUS IN MICE* §

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Mice with alleles at the X-chromosomal locus, Mottled, have connective tissue abnormalities that are, in many respects, similar to those found in lathyritic animals. Like animals raised on lathyrogens, these mice have aortic aneurysms, reduced tensile strength of the skin, and bone abnormalities (1).

Data is presented to indicate that the low tensile strength is due to impaired formation of cross-links in collagen and elastin, and that cross-linking is defective because of failure to generate lysine-derived aldehydes (allysine) required for cross-link formation.

The mottled alleles are coat-color genes on the X-chromosome of the mouse (2). There are at least six alleles at this locus. Mice with the wild-type allele have a normal coat color (black) while those with the other five alleles have coats of gray and are called Tortoise (To), Dappled (Mo<sup>dp</sup>), Brindled (Mo<sup>br</sup>), Viable Brindled (Mo<sup>vbr</sup>), and Blotchy (Blo). Heterozygous females have a mosaic coat color while the hemizygous males have a uniform coat color. It was in part from study of the coat color pattern of these mice that Lyon hypothesized random X-chromosome inactivation (3).

A spectrum of viability exists among the mice carrying the five alleles in the order of Blo > Mo<sup>vbr</sup> > Mo<sup>br</sup> > Mo<sup>dp</sup> > To. Least viable is the To mouse in which many of the females die of aortic aneurysm rupture and To males die in utero with blood vessel aneurysms. The Mo<sup>dp</sup> female and the females carrying the other alleles at the mottled locus all have a near normal life expectancy; however all males have a shortened life expectancy. The Mo<sup>vbr</sup> male dies in utero. Both the Mo<sup>dp</sup> and Blo males succumb to blood vessel rupture, the former at 50–100 days after birth and the latter at more than 150 days of age (4). The Mo<sup>br</sup> male dies about 10 days after birth, exhibiting behavior of a neurological disorder (5, 6) without evidence of a connective tissue abnormality.


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1 Abbreviations used in this paper: BAPN, β-aminopropionitrile; Blo, Blotchy; Mo<sup>dp</sup>, Dappled; Mo<sup>br</sup>, Brindled; Mo<sup>vbr</sup>, Viable Brindled; To, Tortoise.
Material and Methods

The mice described here were bred at the Division of Biological and Medical Research at The Argonne National Laboratory. Of the animals bearing the five mutant alleles, we have studied the connective tissue of the B10 male and the M100 male in detail. The M0 female and the NIH stock mouse (N:NIH[S]), which do not demonstrate connective tissue abnormalities, were used as controls.

Mechanical characteristics of the skin (breaking strength and extensibility) from all mice were measured using an Instron tensiometer (Instron Corp., Canton, Mass.) as described by Sussman (7).

Extraction and purification of collagen was done at 4°C by the method of Riley and Martin (8). The skins were ground with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, N.Y.) and then extracted for 48 h in 1 M NaCl, 0.05 M Tris-HCl, pH 7.4. The insoluble residue was re-extracted twice with 0.5 M acetic acid and the acid-extracted material combined.

The percentage of collagen soluble in different extracting solvents was determined by measuring the hydroxyproline content of aliquots of the salt and acid extracts and of a weighed portion of the residue using the method of Prockop and Udenfriend (9).

The amino acid composition of purified collagen components was determined after 24 h hydrolysis in 6 N HCl at 108°C under a nitrogen atmosphere using a single column automated amino acid analyzer (10).

α-Chains were obtained by chromatographic methods according to the method of Piez et al. (11) modified to include a starting buffer of 0.05 M sodium acetate. The fractions containing α1 and α2 were pooled separately, desalted, lyophilized, and then digested with cyanogen bromide in 70% formic acid (12). Phosphocellulose chromatography was used to separate the small peptides located at the amino terminal ends of the α-chains, because it allows separation of the peptides containing the lysine-derived aldehyde (α1-CB1) from the peptides in which lysine has not been oxidized (α1-CB1) (13).

The aortas from the mice were dissected free from adherent tissue with the aid of a dissecting microscope, lyophilized, and the dry weight and amino acid compositions determined. Lyophilized aortic samples (5–8 mg) from each group were cut into small pieces, suspended in 2 ml H2O, and an aliquot of NaBH₄ solution (specific activity 5 mCi/mg) which contained 2.5–3.3 mCi tritium, was added to each to reduce and label cross-links. The suspension was stirred at room temperature for 90 min, acidified to a pH of 3 to 4 with 50% acetic acid, washed thoroughly with H₂O, then sequentially with ethanol, ethanol-ether (1:1), and ether, and finally dried under a stream of nitrogen.

Each dried-reduced sample was treated with 0.1 N NaOH at 95°C for 45 min to remove collagen (14). The elastin residues were washed thoroughly with H₂O and then dried with nitrogen after washing with ethanol, ethanol-ether, and ether.

Analysis of reduced cross-links of aorta elastin from the animals of each group was performed after alkaline hydrolysis in 2 N NaOH at 110°C for 20 h using a Technicon amino acid analyzer (Technicon Instruments Corp., Tarrytown, N. Y.) with a split stream arrangement as described previously (15). Desmosine and isodesmosine content was determined in nonreduced samples after hydrolysis in 6 N HCl at 110°C for 20 h.

To examine lysine-derived aldehyde formation by aortic tissue, fresh aortas from three mice were minced and incubated for 24 h in 10 ml of the Dulbecco-Vogt modification of Eagle's minimal essential medium lacking lysine in the presence of 20 µCi of [¹⁴C]lysine. After this period the aortic tissue and medium were dialyzed at 4°C against 0.1% acetic acid and then homogenized with a glass homogenizer (Dounce type). The suspension was dried and then treated with performic acid for 18 h by the method of Moore (16) to oxidize lysine-derived aldehydes to α-aminoadipic acid. [¹⁴C]-α-aminoacidic acid, [¹⁴C]lysine, and [¹⁴C]hydroxylysine in the samples were separated chromatographically (10) and the column effluent collected and monitored for radioactivity.
The frequency of aortic aneurysm associated with the various alleles at the mottled locus is given in Table I (17). S-shaped and saccular aneurysms found in To, Mo\textsuperscript{obr}, and Blo mice involve the thoracic and abdominal aorta and its larger branches (Fig. 1). Even in the absence of obvious aneurysms, the aorta from Blo and Mo\textsuperscript{obr} males could be distinguished from those of control animals.

### Table I

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total no.</th>
<th>Normal</th>
<th>Aortic lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Aneurism</td>
</tr>
<tr>
<td>Blo/+ female</td>
<td>19</td>
<td>63</td>
<td>32</td>
</tr>
<tr>
<td>Blo/Blo female</td>
<td>13</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>Blo male</td>
<td>63</td>
<td>2</td>
<td>93</td>
</tr>
<tr>
<td>Blo/Mo\textsuperscript{obr} female</td>
<td>12</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Mo\textsuperscript{obr} female</td>
<td>32</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Mo\textsuperscript{obr} male</td>
<td>13</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Mo\textsuperscript{obr}/+ female</td>
<td>47</td>
<td>90</td>
<td>4</td>
</tr>
<tr>
<td>To/+ female</td>
<td>328</td>
<td>58</td>
<td>25</td>
</tr>
<tr>
<td>To male</td>
<td>26</td>
<td>19</td>
<td>81</td>
</tr>
</tbody>
</table>

Fig. 1. A postmortem arteriogram showing an aneurysm of the abdominal aorta (A), plus diffuse aneurysmal changes of the mesenteric vessels (B).
on the basis of weight (Fig. 2 only $M^{acr}$ shown). The average dry weight of a control aorta, such as shown in Fig. 2, was 1.9 mg while the weights from $Blo$ and $M^{acr}$ males were 4.9 mg and 3.9 mg, respectively. In aneurysmal aorta the weight was increased more than threefold. Amino acid compositions of control and of nonaneurysmal aortas were similar and showed that the tissue was composed in large part of collagen and elastin (not shown). In areas of aneurysm formation there was a decrease in the proportion of valine and an increase in hydroxyproline and in polar amino acids suggesting that the aneurysm was largely collagen (not shown).

Fig. 2. Isolated aortas (left to right) are from $M^{acr}$ with an aneurysm, $M^{acr}$ without an aneurysm, and NIH control.

The presence of aneurysms suggested that the tensile strength of the aorta was reduced. Since skin would provide larger amounts of material for characterization, alterations in the mechanical stability of skin were sought. The breaking force of skin from $M^{acr}$ males was markedly lower than that from the $Blo$ male and $M^{acr}$ female and the extensibility of the skin was greater (Table II).

The amount of collagen extracted from the skin of $M^{acr}$ females and $Blo$ and $M^{acr}$ males in cold neutral salt and in acetic acid solutions was estimated by their hydroxyproline content (Fig. 3). The extractability of collagen from the $M^{acr}$ female skin was similar to that from the skin of the NIH stock mice (not shown). There was a moderate increase above these control values in the
TABLE II

<table>
<thead>
<tr>
<th></th>
<th>Breaking force</th>
<th>Extensibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Mo}^{\text{br}} ) female</td>
<td>276</td>
<td>0.163</td>
</tr>
<tr>
<td>( \text{Blo} ) male</td>
<td>264</td>
<td>0.188</td>
</tr>
<tr>
<td>( \text{Mo}^{\text{br}} ) male</td>
<td>136</td>
<td>0.387</td>
</tr>
</tbody>
</table>

Fig. 3. The extractability of collagen (in per cent) from the skin of normal and affected mice.

eextractability of skin collagen from \( \text{Blo} \) males and a marked increase in that of \( \text{Mo}^{\text{br}} \) males in both salt and acid solvents. The increased extractability paralleled the decreased breaking strength of these skins.

Purified collagen extracted from the skins of \( \text{Mo}^{\text{br}} \) females and \( \text{Blo} \) and \( \text{Mo}^{\text{br}} \) males was fractionated by CM-cellulose chromatography after denaturation (Fig. 4, only \( \text{Mo}^{\text{br}} \) female and \( \text{Mo}^{\text{br}} \) male shown). Marked differences in the proportion of \( \beta^- \) (cross-linked) components to \( \alpha^- \) chains were noted. Collagen from the \( \text{Mo}^{\text{br}} \) male has a much lower proportion of cross-linked components than the \( \text{Mo}^{\text{br}} \) females or NIH stock (not shown). The proportion of the cross-linked components (\( \beta_1 \) and \( \beta_2 \)) to \( \alpha_1 \) and \( \alpha_2 \) chains in the case of the \( \text{Blo} \) male was intermediate between control and \( \text{Mo}^{\text{br}} \) male. These studies indicate that the cross-linking of collagen in the skin of the mutant male mice is reduced when compared to that of female carriers or NIH stock mice. Amino acid analysis detected no compositional differences among skin collagen from normal NIH and \( \text{Mo}^{\text{br}} \) female or the affected \( \text{Blo} \) and \( \text{Mo}^{\text{br}} \) males.

Purified \( \alpha_1 \)-chains from normal and mutant mouse skin collagen were digested with cyanogen bromide and the resulting peptides chromatographed on phosphocellulose columns. The chromatographic properties of the peptides \( \alpha_1 \)-CB1, \( \alpha_1 \)-CB1\text{old}, \( \alpha_1 \)-CB2 and \( \alpha_1 \)-CB3 were essentially those found for rat skin (13). Identification of the peptides was based on their chromatographic
properties and their amino acid content which resembled the corresponding peptides from rat skin collagen $\alpha_1$. $\alpha_1$-CB1 contains a lysine residue which undergoes oxidative deamination before cross-link formation. The aldehyde form of the peptide ($\alpha_1$-CB$^{\text{al}}$) has different chromatographic properties. $\alpha_1$-CB2 does not contain a lysine which can be converted to an aldehyde and is present in equal amounts in both normal and abnormal skin. Thus measuring the ratio of $\alpha_1$-CB1 to $\alpha_1$-CB2, we could estimate the proportion of lysine in $\alpha_1$-CB1 that had undergone oxidative deamination. Similar measurements were made on preparations from normal and lathyritic mouse skin collagen. The ratio of $\alpha_1$-CB1 to $\alpha_1$-CB2 was higher in the $\alpha$-chains from the lathyritic (0.48) and mutant (0.47) mice ($Mo^{\text{er}}$ male) than in the NIH control mouse collagen (0.29). These results indicate that there was less lysine-derived aldehyde in $\alpha$-chains from the mutant mouse than from the control animals.

To establish if similar alterations in cross-linking occur in the aortic elastin of the mutant animals we examined the various lysine-derived compounds in this tissue. Table III gives the composition of selected amino acids and cross-links in various elastin preparations. The concentration of the reduced aldol condensation product (a condensation of two lysine derived aldehydes) is strikingly lower in the $Blo$ and $Mo^{\text{er}}$ males when compared to that in the $Mo^{\text{er}}$ females or NIH control mice. The lysine content of the aortic elastin is greater in the $Mo^{\text{er}}$ and $Blo$ males than in their controls indicating less conversion to lysine-derived aldehydes and cross-links. However, desmosine and isodesmosine levels in the affected animals are normal or slightly elevated. This
finding is similar to that observed in copper-deficient animals (19) and may represent a relative enrichment of the insoluble, cross-linked elastin at the expense of the more alkali soluble, non-cross-linked elastin.

A diminished cross-link content of aortic tissue was also demonstrated by determining the distribution of the lysine-derived cross-links after NaBH₄ reduction (Table IV). Fewer tritium counts were incorporated into the reduced allysine (ε-hydroxynorleucine) and the reduced aldol condensation product in both the Blø and Moⁿ⁻ male than in the Moⁿ⁻ female or NIH control. While the Moⁿ⁻ female which is a carrier of a mutant gene does not display any connective tissue abnormalities, the amount of ε-hydroxynorleucine present in the aortic tissue was less than the NIH control.

When aortas dissected from affected and control mice were incubated in tissue culture medium with [U-¹⁴C]lysine for 24 h, the relative amounts of radioactivity in lysine and α-aminoadipic acid indicated that there was less conversion of peptide-bound lysine to allysine in the tissue from mutant than from control mice (Table V).

Finally, since copper is a cofactor of lysyl oxidase, the enzyme essential for the conversion of lysine to aldehyde in collagen and elastin (20), serum

<table>
<thead>
<tr>
<th>TABLE III</th>
<th>Selecting Amino Acids and Cross-Linking Compounds Obtained from the Aortas of Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid</td>
<td>Normal</td>
</tr>
<tr>
<td>Lysine</td>
<td>2.8</td>
</tr>
<tr>
<td>Aldol</td>
<td>5.0</td>
</tr>
<tr>
<td>Isodesmosine</td>
<td>2.9</td>
</tr>
<tr>
<td>Desmosine</td>
<td>4.2</td>
</tr>
<tr>
<td>Glycine</td>
<td>374</td>
</tr>
<tr>
<td>Alanine</td>
<td>235</td>
</tr>
<tr>
<td>Leucine</td>
<td>60</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>11</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE IV</th>
<th>Lysine Derived Aldehyde (Allysine) and Aldol Condensation Product in the Alkali-Insoluble Residue of Normal and Affected Mouse Aorta Estimated from the Incorporation of Labeled Tritium after Reduction with Tritiated Sodium Borohydride</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm ε-OH-norleucine</td>
</tr>
<tr>
<td></td>
<td>μM leucine</td>
</tr>
<tr>
<td>Normal NIH</td>
<td>94,700</td>
</tr>
<tr>
<td>Moⁿ⁻ female</td>
<td>56,400</td>
</tr>
<tr>
<td>Blø male</td>
<td>26,800</td>
</tr>
<tr>
<td>Moⁿ⁻ male</td>
<td>32,500</td>
</tr>
</tbody>
</table>
copper levels were determined. No striking differences in serum copper levels were noted between mutant and control mice.

**DISCUSSION**

Certain mice bearing abnormal alleles at the mottled locus of the X-chromosome have connective tissue defects. These connective tissue abnormalities are most readily observed in the mice bearing the To, Blo, and Mo<sup>vb</sup> alleles. Aortic aneurysms are observed in To female and To, Mo<sup>vb</sup>, and Blo males. The Mo<sup>vb</sup> males which die about 2 wk after parturition lack evidence of blood vessel rupture. Recent publications by Hunt and Johnson (5, 6) indicate that death in these animals is preceded by neurological abnormalities associated with decreased brain noradrenalin levels.

The observation that aortic aneurysms occur in affected animals suggested that the connective tissue is abnormal. Initially, a connective tissue defect was sought in the skin, since it is more easily studied than aortic tissue and would indicate whether there was a generalized connective tissue defect. A decrease in the strength of the skin was noted in the Mo<sup>vb</sup> male, the most severely affected mouse. Studies on the extractability of collagen from the skin of control and mutant mice indicated that there was a relation between the reduction of mechanical strength and the proportion of skin collagen that could be dissolved in aqueous solvents. The collagen extracted from the skin of normal and affected animals revealed a marked reduction in the proportion of cross-linked components (β<sub>11</sub> and β<sub>12</sub>) in the Mo<sup>vb</sup> male, intermediate values for Blo, and normal values for Mo<sup>vb</sup> females. The impression of faulty cross-linking of aortic elastin was confirmed by finding a diminution of one of the cross-link components, the aldol condensation product. In earlier studies Handrich found that the mechanical properties of skin and extractability of collagen from skin of the Blo male were not significantly different from those of control animals (21). Our studies indicate that the Blo male is the least affected carrier of the mottled allele and therefore would be the most difficult in which to detect an abnormality.

Previous studies have shown that animals administered lathyrogens have abnormal connective tissues exhibiting reduced mechanical strength (18, 22).
The decreased mechanical strength is a result of a decrease in the numbers of crosslinks in collagen and elastin fibers (19). Compounds such as β-amino-propionitrile prevent the formation of lysine-derived aldehydes by inactivating lysyl oxidase (23) (Fig. 5). Another class of compounds illustrated by penicillamine (24) and perhaps homocysteine (25) inhibit cross-linking by condensing with allysine and preventing further reactions. Localization of a defect in cross-linking at these steps can be recognized by a failure to form aldehydes (e.g. BAPN) or a build-up of excess aldehyde (e.g. penicillamine) (26).

In the aneurysm-prone mice, a reduction in aldehyde production was recognized because there was less peptidyl allysine and correspondingly more peptidyl lysine in the affected than in the control tissues. In skin collagen a specific lysine located at the N-terminal portion of the molecule is converted to allysine and then involved in cross-linking. This lysine is elevated in the collagen from affected mice to the same degree as in the collagen of animals treated with a lathyrogen, as judged by the ratio of α1-CB1 to α1-CB2. Aortic elastin was examined after aldehyde-bearing components and Schiff base cross-links were stabilized with tritium-labeled sodium borohydride. In the mutant mice the allysine levels, measured as hydroxynorleucine, were decreased while lysine was elevated. To confirm these results, we incubated aortas in culture with [14C]lysine and measured the amount of labeled allysine formed in the tissue. Again the results indicated that the conversion of lysine residues to aldehyde in elastin is impaired.
Our results indicate that the connective tissue abnormalities in these mice are due to a defect in the cross-linking of both collagen and elastin. The defect is localized to the step at which lysine residues are converted to aldehydes. Potential abnormalities in the aldehyde-generating step could be a deficiency in the enzyme lysyl oxidase or its cofactor copper, inhibitors of the enzyme and an amino acid substitution in collagen, or elastin affecting cross-linking.

To date we have not been able to measure lysyl oxidase activity in extracts of adult normal mouse skin and have not established whether this enzyme, the most likely site of a defect, is altered in the mutant mice.

Copper is a cofactor for lysyl oxidase. Dietary copper deficiency in pigs is known to produce multiple connective tissue abnormalities, including aortic aneurysms, due to deficient cross-linking (27). Low serum copper levels as a result of faulty intestinal absorption of copper have been found in a sex-linked human disease, Menke's kinky hair syndrome. Patients with this disorder have retarded mental development and blood vessel aneurysms which are said to resemble the abnormalities found in experimental copper deficiency (28, 29). Because of the low serum copper levels, the cross-linking of collagen and elastin might be impaired although this has not yet been demonstrated. The serum copper levels in our aneurysm-prone mice are normal. However, Hunt and Johnson (5, 6) indicate that the most likely cause of the low norepinephrine and elevated dopamine content in the brain tissue of the M0b and M0vb males is an abnormality in dopamine β-hydroxylase, a copper requiring enzyme. An abnormality in copper metabolism could explain the different defects observed in the mottled mice.

In preliminary experiments endogenous inhibitors of lysyl oxidase were sought in the serum of the affected mice as the cause of the deficient aldehyde formation. No reduction in lysyl oxidase activity derived from chick cartilage occurred when sera from normal or affected mice were added. Similarly a substrate abnormality would seen unlikely since the same cross-linking defect is found in two distinct proteins—collagen and elastin.

When comparisons of similar mutations are made between animals species, all mutations that are located on the X-chromosome in one species have also been located on the X-chromosome in other species (30). For example, mutations in glucose-6-phosphate dehydrogenase and hypoxathine-guanine phosphoribosyl transferase are X-linked traits in all mammals studied to date. Although Marfan's syndrome might appear to be the human counterpart to our aneurysm-prone mice, it would seem unlikely because Marfan's syndrome is inherited in an autosomal, dominant manner. Thus for these mice to be a model of a human syndrome the syndrome should be sex linked. One such example would be Menke's kinky hair syndrome discussed previously. Another X-linked connective tissue disorder is a form of Ehlers-Danlos described by Beighton (31). No biochemical studies have yet been reported in these patients.

A number of genetic disorders are attributed to alterations in the cross-linking of collagen (Fig. 5). Collagen from skin and bone of patients with one
form of the Ehlers-Danlos syndrome have low levels of hydroxylysine due to a defect in the enzyme lysyl hydroxylase (32). These patients have scoliosis and lax joints which have been attributed to a defect in cross-linking. They lack the hydroxylysine-derived cross-links and their collagen is more extractable (32, 33).

Another defect, failure to convert procollagen to collagen, has been observed in cattle (34), sheep (35), and most recently in still another variant of the Ehlers-Danlos syndrome in humans (36). Collagen fibers have been found to be poorly structured and although aldehydes are present in the collagen, cross-links do not form between the adjacent groups perhaps because of steric factors (37).

The aneurysm-prone mice are the first animals identified to have a heritable defect in the aldehyde producing step of cross-linking. It is known that the extent and rate of collagen and possibly elastin cross-linking varies in different tissues (38, 27). Since there is a spectrum of severity of connective tissue defects among mice carrying different alleles, these animals may be useful in identifying the genetic, hormonal, and enzymatic factors that account for tissue variability in cross-linking.

SUMMARY

A genetic abnormality in collagen and elastin cross-linking resembling experimental lathyrism has been identified in mice. The defect is an X-linked trait, attributed to the mottled locus which also influences coat color. The affected mice have aneurysms of the aorta and its branches, weak skin, and bone deformities in a spectrum of severity varying with the alleles at the mottled locus.

A defect in the cross-linking of collagen was demonstrated in the skin of the affected animals by a marked increase in collagen extractability and a reduced proportion of cross-linked components in the extracted collagen. A decrease in lysine-derived aldehyde levels was found in both skin collagen and aortic elastin similar to that found in lathyritic tissue. Furthermore the in vitro formation of lysine-derived aldehyde was reduced. Thus the cause of the connective tissue abnormalities in these mice appears to be a defect in cross-link formation due to an impairment in aldehyde formation.

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