Genetic Variations of an Oxidase in Mammals*

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SUMMARY

The genetic basis of the variation in activity of the mammalian enzyme which catalyzes the oxidation of N\textsuperscript{1}-methylnicotinamide to N\textsuperscript{1}-methyl-4-pyridone-3-carboxamide has been investigated. The data indicate that in mice the magnitude of the enzyme activity depends on a pair of autosomal alleles, one of which determines a high level, the other a low level, of enzyme activity. Differences in enzyme activity between males and females and the effect of administration of estradiol and of testosterone to intact animals indicate that the level of enzyme activity is subject to hormonal influence as well. Thus, whereas the distinction between classes with low and high enzyme activity is under genetic control, levels of enzyme activity within these classes appear to be regulated by the endocrine system.

In the course of studies on nicotinamide adenine dinucleotide metabolism, an enzyme was found in mammalian liver which catalyzes the biosynthesis of N\textsuperscript{1}-methyl-4-pyridone-3-carboxamide from N\textsuperscript{1}-methylnicotinamide (1-4). Our original investigation (1, 3) was carried out with pooled mouse liver homogenates. Further investigations, which individual mice were examined, it was noted that the activity of the enzyme was appreciable in only about one-half (36 out of 76) of the animals examined. The possibility was considered that the level of enzyme activity might be controlled by the endocrine system.

Studies with other strains of rats were found to support this idea. In the Charles River Inbred Fisher (CDF) strain, enzyme activity was low in all of the animals tested; in the Carworth Farms Wistar (CFN) and Charles River Sprague-Dawley (CD) strains, activity was high in all animals; still other strains, namely, the Carworth Farms Sprague-Dawley (CFE) and the Kalme Farms Sprague-Dawley (CD), showed polymorphism for enzyme activity, like the Royal Hart Wistar. An investigation was therefore undertaken to study the genetic basis of this variation in enzyme activity. Because of the extensive knowledge of their genetics such a study appeared most promising in mice, and became feasible when preliminary tests showed the existence of enzyme variations between genetically well defined different mouse strains. The accompanying paper by Huff and Chaykin (5) presents results of an independent study which lead to conclusions similar to those reached in the present investigation.

MATERIALS AND METHODS

Two inbred mouse strains were chosen for genetic analysis: DBA/1 and the t\textsuperscript{t}/t\textsuperscript{t} strain, both of them inbred for 32 to 37 generations.

N\textsuperscript{1}-Methylnicotinamide-7-\textsuperscript{14}C chloride having a specific activity of 7.5 mCi per mmole was obtained from New England Nuclear Corporation. N\textsuperscript{1}-Methyl-2-pyridone-5-carboxamide (2-pyridone) was purchased from Calbiochem. N\textsuperscript{1}-Methyl-4-pyridone-3-carboxamide (4-pyridone), synthesized by Wieland, Fest, and Pfleiderer (6), was generously donated by Professor T. Wieland and by Dr. M. L. W. Chang.

The 4-pyridone-forming enzyme has been shown to be located in the soluble fraction of liver homogenates of rats (1, 3) and mice (2). In the present work, the enzyme was prepared for assay as follows. Livers were homogenized with 4 volumes of 0.1 M sodium phosphate buffer, pH 7.2. The homogenates were centrifuged at 105,000 \times g for 60 min (rats) or at 48,000 \times g for 20 min (mice), and the supernatant solutions were assayed for enzyme activity. Comparison of the two types of centrifugation, with the use of pooled mouse liver homogenates, gave virtually identical results. In studies with individual mice, we did not attempt to remove the entire microsomal fraction from the liver homogenates because of the small volumes of homogenate available for centrifugation.

Enzyme activity was assayed by determining radioactive 4-pyridone formed from N\textsuperscript{1}-methylnicotinamide-7-\textsuperscript{14}C as described previously (3). The standard assay mixture (final volume of 0.75 ml) contained 100 mM sodium phosphate buffer, pH 7.2, 0.25 mM N\textsuperscript{1}-methylnicotinamide-7-\textsuperscript{14}C and, as enzyme, 0.5 ml of supernatant solution. Assay tubes were incubated at 36°C in air in a Dubnoff shaker for 10 min. It was established that enzyme activity was proportional to concentration and time under these conditions. The reaction was terminated by the addition of 1.75 ml of 14% trichloroacetic acid. Zero time control tubes were carried through the entire procedure. Following centrifugation, the trichloroacetic acid was removed from the supernatant solution by extracting three times with 2 volumes of ether. N\textsuperscript{1}-Methylnicotinamide, 4-pyridone, and 2-pyridone were separated by paper chromatography of 0.1-ml aliquots of the extracted solution, containing carrier 4-pyridone and 2-pyridone. Descending paper chromatography was carried out on
Whatman No. 2MM paper by using 1-butanol-H₂O-concentrated NH₄OH (680:114:6) as the solvent system. The compounds were located on paper by scanning for radioactivity on a Vanguard autoscanner 880 and as spots absorbing ultraviolet light. The appropriate areas were cut out and the amount of radioactivity estimated by counting directly in a Packard Tri-Carb liquid scintillation spectrometer. Enzyme activity is expressed as millimicromoles of 4-pyridone formed per 10 min per ml of incubation mixture (which contained the supernatant solution derived from 133 mg, wet wt, of liver and had an average of 18.2 mg of protein).

RESULTS AND DISCUSSION

The DBA/1 strain showed low activity of the 4-pyridone producing oxidase in all its animals. High enzyme activity was found in all animals of the t³ strain. Females varied less in enzyme activity than did males, and the genetic analysis was therefore carried out on the data obtained from females.

Twenty-two DBA/1 females, 2 to 6 months of age, had a mean enzyme activity of 1.2 (Table I). The mean enzyme activity of 29 t³ females was 10.9. Reciprocal crosses of mice of these two strains were made to obtain F₁ females for tests. There was no difference in the enzyme values of F₁ hybrids from the two crosses and the data were therefore treated together. Fifty-five F₁ females had a mean enzyme activity of 6.0, i.e. intermediate between the low and the high strains (Table I). The results obtained with parent strains and F₁ hybrids are presented as a histogram in Fig. 1.

The three populations, DBA/1 (low), t³ (high), and F₁ (intermediate) proved to be statistically different with respect to their enzyme activities. No overlapping occurred between the values of DBA/1 on the one hand and those of the F₁ generation or the t³ strain on the other hand. However, there was overlapping between the values of the F₁ and those of the t³ strain in spite of the significant difference between their means. The overlap region ranges from 6.25 to 10.0 and includes 36 values, 14 of the 29 t³ (high) strain and 22 of the 55 F₁ (intermediate) group. These data show that approximately 48% of all t³ animals and 40% of the F₁ generation have enzyme activity levels within the overlap region, so that the nonoverlap values represent 52% of t³ and 60% of F₁, respectively. For subsequent experiments reported below a crude correction factor was calculated from these figures; accordingly animals with values in the overlap region were assigned proportionally to the t³ and to the F₁ category.

Backcross generations were obtained by crossing F₁ hybrids to the t³ (high activity) and DBA/1 (low activity) strains, and F₁ animals were bred with each other for the F₂ generation (Table I). Twenty-four females of the backcross to t³ gave enzyme values of two groups, high and intermediate, and not a single animal had enzyme activity in the low range. Seven of the twenty-four fell into the nonoverlapping portion of the intermediate range, and nine into the nonoverlapping portion of the high range. The remaining eight values were found in the overlap region. With the use of the crude correction mentioned above for values in the overlap area, three of these eight animals were assigned to the F₁ group, and five to the t³, and the following backcross ratio was obtained: 0 low to 10 intermediate to 14 high.

The backcross of F₁ to DBA/1 yielded 20 females with enzyme values in the low and intermediate ranges only; not a single animal was found with high enzyme activity. The ratio for the backcross to DBA/1 was 9 low to 11 intermediate to 0 high.

The 56 females of the F₂ generation were tested for enzyme activity and distributed as follows: 11 low, 21 intermediate not overlapping high (3.0 to 6.0), 10 high (10.0 to 14.0). Fourteen

### Table I

<table>
<thead>
<tr>
<th>Strain and generation</th>
<th>n</th>
<th>Mean ± S.D.</th>
<th>Ratio</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Low</td>
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<tr>
<td>DBA/1</td>
<td>22</td>
<td>1.20 ± 0.42</td>
<td>22</td>
</tr>
<tr>
<td>t³</td>
<td>29</td>
<td>10.67 ± 2.07</td>
<td>0</td>
</tr>
<tr>
<td>F₁</td>
<td>35</td>
<td>6.00 ± 1.11</td>
<td>0</td>
</tr>
<tr>
<td>F₂</td>
<td>56</td>
<td>11.20 ± 1.16</td>
<td>11</td>
</tr>
<tr>
<td>BC (F₁ × t³)</td>
<td>24</td>
<td>10.87 ± 2.97</td>
<td>0</td>
</tr>
<tr>
<td>BC (F₁ × DBA/1)</td>
<td>20</td>
<td>9</td>
<td>11</td>
</tr>
</tbody>
</table>

**TABLE II**

**Strain difference in formation of 4-pyridone by using K₂Fe(CN)₆ as electron acceptor**

Assays of liver preparations from female mice were carried out in Thunberg tubes by using the conditions described under "Materials and Methods," except for the indicated modifications. Where indicated, K₂Fe(CN)₆ was present in a final concentration of 10⁻³ M. The results of three experiments, in each of which three mice were analyzed individually, were similar, and the data were therefore pooled. The residual activity present under anaerobic conditions may have been due to incomplete removal of oxygen.

<table>
<thead>
<tr>
<th>Addition</th>
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<tr>
<td></td>
<td></td>
<td>DBA/1</td>
</tr>
<tr>
<td>None</td>
<td>Air</td>
<td>12.0</td>
</tr>
<tr>
<td>K₂Fe(CN)₆</td>
<td></td>
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</tr>
<tr>
<td>None</td>
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<td>1.3</td>
</tr>
<tr>
<td>K₂Fe(CN)₆</td>
<td>N₂</td>
<td>0.3</td>
</tr>
</tbody>
</table>

**Figure 1.** The formation of 4-pyridone (4-PC) by liver preparations from female mice.
values were found in the overlap region. After correction according to the proportions reported above, 8 of the 14 values were assigned to the $F_1$ category, and 6 to the $t^2$ (high) genotype. This resulted in the following ratio for the $F_2$: 11 low to 20 intermediate to 16 high, which is a good fit to the 1:2:1 ratio expected.

The possibility was tested that the differences in enzyme activity might be due to an activator or inhibitor. For this purpose, the 48,000 $\times$ g supernatant solutions of liver homogenates of a strain with high enzyme activity and of the DBA/1 (low) strain were compared singly and in combination. The results obtained by combination were additive, indicating that the enzyme activity differences do not result from a difference in level of an activator or inhibitor.

Homogenates of liver catalyze the formation both of 4-pyridone and of 2-pyridone. The available evidence suggests that in the mouse the synthesis of 4-pyridone and that of 2-pyridone are catalyzed by a single enzyme or by two enzymes with similar properties (2, 3). In the present investigation of individual animals from various mouse strains, a close parallelism was observed between the 4-pyridone- and the 2-pyridone-forming activities. Moreover, in the extensive genetic experiments involving $t^2$ and DBA/1 mice, results obtained for 2-pyridone formation were qualitatively the same as for 4-pyridone formation. This would be expected if 4-pyridone and 2-pyridone formation were catalyzed by the same enzyme or by two genetically closely related enzymes.

Determinations of enzyme activity in males of the parent strains and crosses gave results qualitatively similar to those obtained with females. However, the male enzyme values were considerably higher and more variable than those of the females. For instance, 4-pyridone formation by liver homogenates of male DBA/1, $t^2$, and $F_2$ mice was, respectively, $2.17 \pm 1.01 (n = 10)$, $30.8 \pm 12.2 (n = 20)$, and $22.2 \pm 6.3 (n = 33)$ (means ± standard deviations). In view of the high variability, the present data on males of the $F_2$ and the backcrosses do not lend themselves to further genetic analysis.

A comparison of activity in males with that in females indicates hormonal effects on enzyme activity. Thus, whereas the distinction between classes with low and high enzyme activity is under genetic control, levels of enzyme activity within these classes seem to be subject to hormonal influence. Homogenates of livers of male and female mice gave additive results in combination experiments, indicating that the sex difference in enzyme activity did not result from a difference in level of an activator or inhibitor. Variations of enzyme activity may possibly be correlated with variations of hormone levels.

In two experiments, the effect on enzyme activity of administration of estradiol to male mice and of testosterone to female mice was investigated. For comparison, dexamethasone, a potent synthetic glucocorticoid, was also studied. The steroids were given in a dose of 1 mg per mouse per day subcutaneously for 3 days. One hour after the final steroid injection, animals were sacrificed and enzyme activity determined. (Three animals were used per group.) Estradiol was found to reduce enzyme activity of male mice by 60% in the first and by 76% in the second experiment. Testosterone had a slight (23%) but definite stimulatory effect on the enzyme activity of female mice. Dexamethasone caused a decrease of enzyme activity of about 50% in both male and female mice.

It was shown previously that in the presence of potassium ferricyanide the enzymatic oxidation of $N^7$-methylisocitramide to 4-pyridone occurs in the absence of molecular oxygen (2, 3) indicating that the oxygen introduced into position 4 of the substrate is derived from $H_2O$ and not from $O_2$. In the present investigation the formation of 4-pyridone, when using potassium ferricyanide as electron acceptor, has been compared in females of the $t^2$ (high) and DBA/1 (low) strains of mice. As shown in Table II, a difference between the $t^2$ and DBA/1 strains of mice with respect to the formation of 4-pyridone, similar to that observed in the presence of $O_2$, was seen when potassium ferricyanide was used in place of oxygen as electron acceptor. The fact that, in the presence of potassium ferricyanide, the activity of preparations from DBA/1 mice did not increase to the level observed with preparations from $t^2$ mice indicates that the enzyme step exhibiting genetic variation occurs at an early stage in the transport of electrons between substrate and oxygen.

REFERENCES

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