ADVANCES IN GENETICS

VOLUME 21

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E. W. CASPARI

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THE *Ah* LOCUS, A MULTIGENE FAMILY NECESSARY FOR SURVIVAL IN A CHEMICALLY ADVERSE ENVIRONMENT: COMPARISON WITH THE IMMUNE SYSTEM

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I. Introduction

In the past several years, a large amount of exciting information has developed with regard to the genetic regulation of certain drug-metabolizing enzymes. Most of this information has appeared in pharmacology and biochemistry journals. The purpose of this article is to introduce this subject to the geneticist. First, the drug-metabolizing enzyme systems are introduced. Second, the *Ah* system is introduced and evidence is presented for a cytosolic receptor, which is believed to be the major *Ah* regulatory gene product. Third, data are given for multiple *Ah* structural gene products; these are the various newly induced enzyme proteins, the cDNA of some of which we have recently cloned. Fourth, evidence is described for possible temporal gene(s) related to this system. Lastly, this genetic system, which aids the organism in coping with environmental adversity, is compared with other systems that also aid the organism in survival.

Many of the ideas and new data presented herein are very recent and will need corroboration and extension. We believe this research field has become extremely fascinating, however, even though there still remain many more questions than answers.

A. Phase I and Phase II Drug-Metabolizing Enzymes

At least 10^5 and possibly many more foreign chemicals exist in our environment. These chemicals are not to be confused with the approximately 10^6 antigens (proteins, or usually glycoproteins) on this planet that evoke the immune response—although antibodies have been developed against some of these foreign chemicals. Many of the foreign chemicals are highly toxic to all organisms, or to certain classes of organisms, and a growing number of these chemicals are being shown to cause mutations, cancer, and birth defects.

How are living things able to respond to this chemical adversity? Most of these foreign chemicals—also called *xenobiotics*—are so fat-soluble that they would remain in the organism indefinitely were it not for Phase I and Phase II drug-metabolizing enzymes (Goldstein *et al.*, 1974). During Phase I metabolism, polar groups (such as alcohols) are introduced into the parent molecule, thereby presenting the Phase II conjugating enzymes with a substrate. The Phase II

enzymes use the polar group as a "handle" for attaching other very water-soluble moieties such as glucuronide, sulfate, or glycine. The Phase I products (such as alcohols or quinones) and especially the Phase II conjugates are sufficiently polar to be excreted by the organism.

B. What Is "Cytochrome P-450?"

"Cytochrome," a Greek word, literally means "colored substance in the cell." The color is derived from the properties of *d* electrons of transition elements such as iron, and, indeed, cytochromes appear reddish in color when sufficient concentrations exist in a subcellular fraction.

"P-450" denotes a *p*igment with the unusual property of having its major optical absorption peak (Soret maximum) at about 450 nm, when the material has been reduced and combined with carbon monoxide (Omura and Sato, 1964). Although the name P-450 was intended to be temporary until more information about this substance became available (Omura and Sato, 1964), the terminology has persisted for 17 years because of the increasing complexity of this enzyme system with each passing year and because of the lack of agreement on any better nomenclature. The interesting history of the discovery and characterization of P-450 independently from different approaches in three laboratories—Johnson Research Foundation, University of Pennsylvania; Department of Biochemistry, University of Oregon Medical School, Portland; and Institute of Protein Research, Osaka University—between 1955 and 1962 has been recently detailed (Sato and Omura, 1978; Mannering, 1980).

Cytochrome P-450 represents a family of hemoproteins (heme-containing proteins) possessing catalytic activity toward thousands of substrates. The estimated molecular weights of various forms of P-450 range from 43,000 to 60,000 on sodium dodecyl sulfate polyacrylamide gels (Nebert, 1979). The porphyrin ring (including the iron) represents about 650 daltons; this heme group is lost during denaturating electrophoresis but under normal conditions is combined with the various apo-enzymes to form the functional holo-enzymes. The apo-enzyme portions—polypeptides ranging in length from about 390 to 550 amino acids—are believed to confer (at least some) substrate specificity toward these thousands of environmental chemicals. Some forms of P-450 appear to be glycoproteins. P-450 is ubiquitous; its presence has been studied in bacteria, plants, primitive animals, and every mammalian tissue with the possible exception of crystalline

bone. Recently, nomenclature committees have objected to the use of the term "cytochrome" for this enzyme.

C. What Is "Monooxygenase Activity?"

Monooxygenases are enzymes that insert one atom of atmospheric oxygen into their substrates (Mason *et al.*, 1955; Hayaishi *et al.*, 1955). The various forms of P-450 represent a large subset of all monooxygenases. To perform this monooxygenation, the P-450 hemoprotein receives two electrons from the cofactors NADPH and/or NADH, and these electrons are received one at a time, usually via reductases (flavoproteins). In certain bacteria such as *Pseudomonas* (Tanaka *et al.*, 1976), the entire electron chain—NADH, reductase, an iron-sulfur protein, and P-450—is in the cytosol. In certain fungi (Yoshida, 1978; Kato, 1979; Cerniglia and Gibson, 1979) the P-450 appears to be rather easily dissociated from microsomal* membranes. In most organisms, however, the electron chain is deeply embedded in the endoplasmic reticulum, inner mitochondrial membrane, and perhaps the nuclear envelope. The microsomal electron chain contains reductase and P-450, but the mitochondrial electron chain includes reductase, iron-sulfur protein ("adrenodoxin"), and P-450.

P-450-mediated monooxygenases therefore represent a large number of Phase I enzymes. These enzymes very often appear to be stimulated—or *induced*—when the organism is exposed to a particular P-450 substrate or to other inducing chemicals (Nebert *et al.*, 1981). The thousands of foreign chemicals and normal body substrates for these enzymes include polycyclic hydrocarbons such as benzo[*a*]pyrene (ubiquitous in the combustion of coal and in city smog, cigarette smoke, and charcoal-cooked foods), anthracenes, and biphenyl; halogenated hydrocarbons such as polychlorinated and polybrominated biphenyls, defoliants, insecticides, and ingredients in soaps and deodorants; certain fungal toxins and antibiotics; many of the chemotherapeutic agents used to treat human cancer; ethanol; almost all drugs; almost all commonly used laboratory reagents; strong mutagens such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and nitrosamines; various chemicals found in cosmetics and perfumes; numerous aromatic amines, such as those found in hair dyes, nitro aromatics,

*Microsomal membranes denote the pellet formed when the postmitochondrial supernatant fraction (following 9,000 or 15,000 *g* × 15 min) is centrifuged again at 105,000 *g* for 60 min. Whereas the majority of the microsomes represent the endoplasmic reticulum, this crude fraction usually contains plasma membranes, nuclear and mitochondrial membrane fragments, Golgi bodies, and lysosomal membranes.

aminoazo and diazo compounds, and heterocyclics; *N*-acetylarylamines and nitrofurans; most plant phytoalexins and wood terpenoid derivatives; epoxides; carbamates; alkyl halides; safrole derivatives; antioxidants, other food additives, and many ingredients of foodstuffs and spices; both naturally occurring and synthetic steroids; prostaglandins; and other endogenous substrates such as biogenic amines, indoles, thyroxine, and fatty acids.

Monooxygenase activities (Fig. 1) therefore require the integrity of an electron flow between the cofactor NADPH (in some cases, NADH) and the oxygenated form of P-450. More than three-fourths of the liver microsomal reductase molecule is believed (Vermilion and Coon,

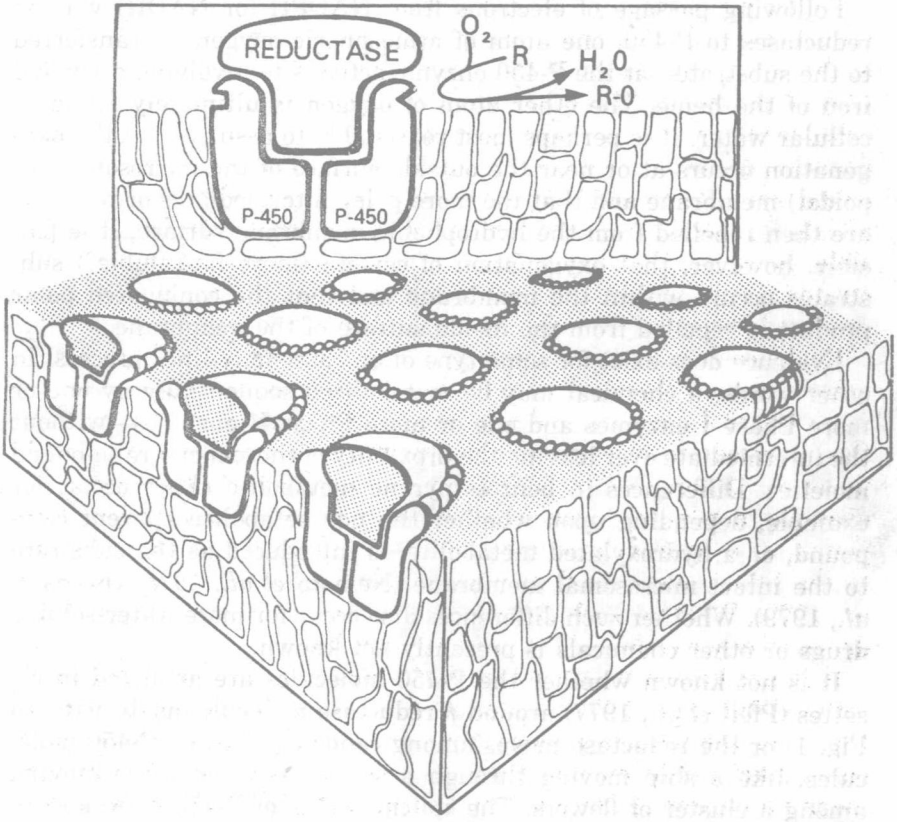

FIG. 1. Hypothetical diagram of the relationship between flavoprotein reductases and forms of cytochrome P-450 embedded in cellular membranes (Nebert, 1979). R, substrate (such as a drug); R-O, oxygenated intermediate or product. Reproduced with permission from Dr. W. Junk Publishers.

1978a) to sit free of the lipid bilayer, whereas P-450 molecules are believed to be usually deeply embedded in the membrane, thereby making solubilization of "pure" forms of these hemoproteins extremely difficult. Detergent treatment involving micelle formation often interferes with normal function (rate of catalytic activity in intact microsomes or in perfused liver), so that "reconstituted" activity sometimes may differ from "intact" microsomal catalytic activity (Lu and Levin, 1974). The functions of membrane-bound FAD- and FMN-containing flavoproteins (Vermilion and Coon, 1978b), mitochondrial P-450 (Mason *et al.*, 1973), cytochrome b_5 (Enomoto and Sato, 1973; Noshiro *et al.*, 1979), and cyanide-sensitive fatty acid desaturase (Ohba *et al.*, 1978) are beyond the scope of this article.

Following passage of electrons from NADPH (or NADH) via the reductases to P-450, one atom of atmospheric oxygen is transferred to the substrate—at the P-450 enzyme-active site involving activated iron of the heme. The other atom of oxygen is ultimately found in cellular water. It is perhaps most reasonable to assume that the oxygenation occurs at or near the outside surface of the microsomal (lipoidal) membrane and that the more polar intermediates or products are then repelled from the hydrophobic membrane surface. It is feasible, however, that oxygenation of certain (more fat-soluble?) substrates occurs within the membrane and that the conjugated polar product is repelled from the inside surface of the membrane.

Evidence does exist for some type of an "assembly-line" process. In other words, a chemical may be metabolized sequentially by one or more Phase I enzymes and one or more Phase II enzymes—without the intermediate ever leaving the proximity of these membrane-bound moieties. Differences in benzo[*a*]pyrene metabolite ratios occur, for example, depending upon whether the nonmetabolized parent compound, or a hydroxylated metabolite, is introduced as the substrate to the intact microsomal membrane (Nemoto *et al.*, 1978; Owens *et al.*, 1979). Whether such differences also occur for more water-soluble drugs or other chemicals is presently not known.

It is not known whether the P-450 molecules are arranged in rosettes (Pfeil *et al.*, 1977) around a reductase molecule (as depicted in Fig. 1) or the reductase moves among randomly located P-450 molecules, like a ship moving through a sea of rocks or a bee moving among a cluster of flowers. The stoichiometry of P-450 molecules to reductase molecules ranges between 10:1 and 100:1 (Estabrook *et al.*, 1971; Sato and Omura, 1978; Mannering, 1980). It is entirely possible that one P-450 molecule is able to donate electrons directly to another

P-450 molecule; the mitochondrial oxidative phosphorylation chain is an example for such oxidation–reduction occurring among different cytochromes. P-450 may comprise between 3 and 12% of the total microsomal protein.

D. Multiple Forms of P-450

In summary of the previous sections, therefore, the metabolism of thousands of foreign (and endogenous) chemicals by P-450 is rivaled in complexity perhaps only by the immune response to about one million unique antigens, and cognitive processes, in response to the thousands of unique sensory stimuli. The stimuli leading to monooxygenase induction likewise may be complex and may result in a large number of induced forms of P-450.

It is common knowledge that many chemicals (e.g., ethanol, benzene, most tranquilizers, antiseizure medications, etc.) administered chronically will require increasing doses to maintain the same "effect." This phenomenon includes the mechanism of drug addiction and tolerance. It is also common knowledge that many chemicals administered chronically influence the effects of a second chemical. For example, cigarette smokers require several times more coffee to feel the same caffeine effect. The bones of children on chronic antiseizure medication may become osteoporotic (decreased calcium content due to changes in vitamin D metabolism caused by the drug). The egg shells of birds exposed chronically to various insecticides may become brittle, presumably due to the interference by these environmental chemicals in normal sex steroid metabolism. Conney's extensive review 14 years ago (Conney, 1967) listed more than 200 drugs, carcinogens, other environmental chemicals—and even normal body steroids—that induce their own metabolism and/or that of other substrates via P-450 induction.

More than five dozen inducers have now been described in sufficient detail to suggest that each may be inducing its own unique form of P-450 (Nebert *et al.*, 1981). In other words, one or more of the 390 to 550 amino acids may be different so that substrate specificity (and perhaps even molecular weight) may be unique. It has been suggested (Nebert, 1979) that organisms possess the genetic capacity to synthesize as many new forms of P-450 as there are chemicals capable of being inducers. What makes one chemical a better inducer than another? Why are there differences among species? How is this induction process evoked, and what are the steps in the response? What

is the significance of P-450 having overlapping substrate specificity? Some aspects of these questions can be answered by our recent investigation of the murine *Ah* locus.

II. The *Ah* System

A. Early Studies

A far better understanding than ever before about the genetic control of P-450 induction resulted from the exciting discovery (Nebert and Bausserman, 1970; Nebert *et al.*, 1971) that certain forms of inducible P-450 differ among inbred strains of mice. This heritable trait has been named the *Ah* locus. Wild mice and the majority of inbred strains are *a*romatic *h*ydrocarbon "responsive," meaning that new forms of P-450 (such as P_1-450 and P-448) are easily induced by such polycyclic aromatic compounds as 3-methylcholanthrene, β-naphthoflavone, benzo[*a*]pyrene, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Induced aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase (AHH) activity (Fig. 2) is regarded as an accurate assessment of the appearance of induced P_1-450. C57BL/6 is the prototype strain (B6, responsive, Ah^b).

DBA/2 was the first mutant characterized (D2, nonresponsive, Ah^d).

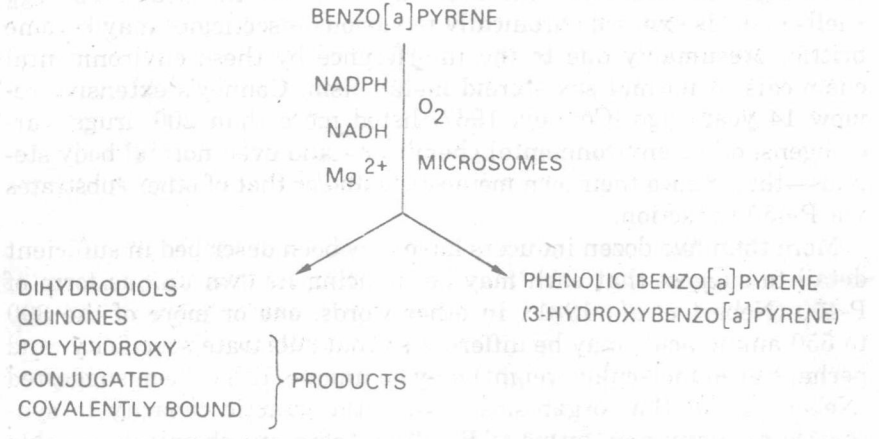

FIG. 2. Diagram of the assay for AHH activity. The substrate benzo[*a*]pyrene is metabolized most specifically by P_1-450 to various phenols; the 3- and 9-phenols have the strongest fluorescence. Other oxygenated products of benzo[*a*]pyrene, such as dihydrodiols and quinones, are not measured by this assay (discussed further in Nebert and Jensen, 1979).

If one looks at the origins of the older inbred strains of mice (Staats, 1966) and a list of *Ah*-nonresponsive strains (Kouri and Nebert, 1977), it appears that nonresponsive mutations have arisen independently at least three or four times between 1909 and 1950 in different parts of the world (DBA, Furth's A and R stocks, European white mice, and SJL from Webster Swiss). P_1-450 and AHH activity in "nonresponsive" inbred strains (Fig. 3) can be induced by a dose of TCDD 12 to 18 times larger than that needed in responsive mice (Poland *et al.*, 1974). This difference in sensitivity has been found for numerous induced monooxygenase activities in virtually every tissue of the mouse. The untreated control mouse has no detectable liver P_1-450 (Negishi *et al.*, 1981a).

The Ah^b/Ah^d heterozygote is generally responsive, indicating that the trait of P_1-450 and AHH induction is autosomal dominant (Fig.

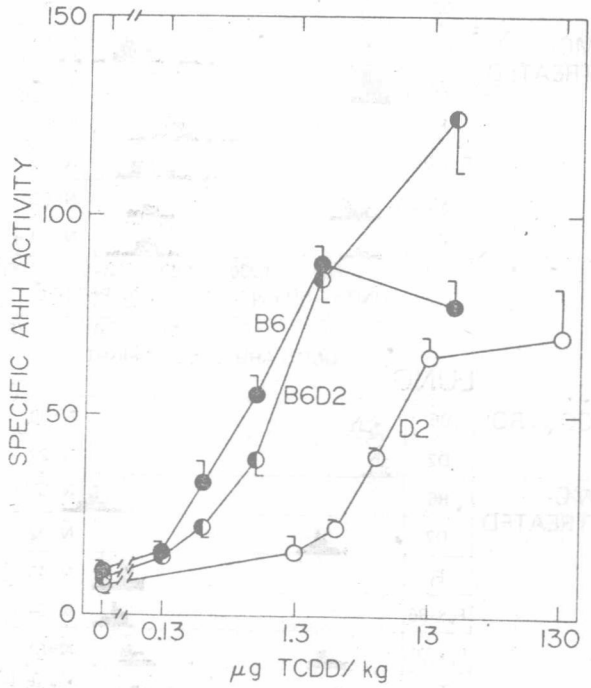

FIG. 3. Dose–response curve for B6, D2, and B6D2F$_1$ mice (Niwa *et al.*, 1975). Dose on the abscissa represents intraperitoneal TCDD; response on the ordinate represents liver microsomal AHH activity 48 hours later. Specific activity of AHH denotes units per mg of wet weight of liver; brackets represent standard error ($N = 6$). Induced AHH activity predominantly reflects induced P_1-450. Reproduced with permission from Academic Press.

4). AKR/N and AKR/J strains are nonresponsive, and C57BL/6N and C57BL/6J strains are responsive. The (C57BL/6N)(AKR/N)F_1 is *Ah*-nonresponsive, however, whereas the F_1 derived from either B6 strain crossed with AKR/J and from either AKR strain crossed with C57BL/6J is *Ah*-responsive (Fig. 5). Among F_2 progeny when certain responsive strains are the progenitors (Fig. 6), some mice are nonresponsive. Among F_2 progeny when certain nonresponsive strains are the progenitors (Fig. 7), some mice are responsive. Hence, it is clear that regulation of the P_1-450 induction process is complicated. It has been estimated that the P_1-450 induction process must involve at least two

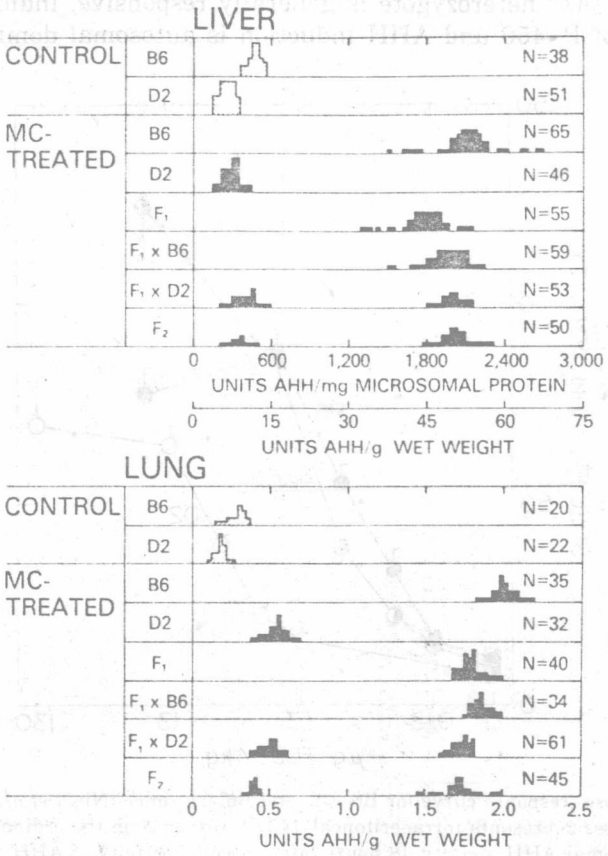

FIG. 4. Genetic variance in liver (top) and lung (bottom) AHH activity in control and 3-methylcholanthrene-treated (MC) offspring from appropriate crosses between B6 and D2 inbred strains (Kouri and Nebert, 1977). MC was given 24 hours before the AHH assay. The number of mice examined individually is given at the right for each group. Reproduced with permission from the Cold Spring Harbor Laboratory Press.

independent loci with at least three alleles each (Robinson *et al.*, 1974).

B. Linkage

The *Ah* system is believed to comprise regulatory genes, structural genes, and probably temporal genes. This classification of genes has been introduced by Paigen and co-workers (Lusis and Paigen, 1977). The *Ah* locus has not been mapped, although chromosomes 8, 9, 17, and 19 are most suspect (C. Legraverend, D. W. Nebert, and P. Lalley, in preparation).* Whether all these genes even reside on a single chromosome is not known. Chromosome 17 is of particular interest, because this chromosome not only has the *H-2* complex with its *K*, *D*, and *L* loci and the Ia antigens (which may be identical with the Ir immune-response product) (Klein, 1979) but also has the *T* locus. The *T* locus is a region of chromosome identified by sets of dominant and recessive mutations, some of which have profound effects on embryonic development, sperm production and function, and even genetic recombination with parts of the *H-2* region; the *T* locus is responsible for surface antigens specific for early development, affecting also tail length, and has recessive lethal alleles present in wild populations at high frequencies (Bennett, 1975).

C. Pleiotypic Response of the *Ah* Locus

The actions of a particular gene in more than one organ have been called "pleiotropic" (Plate, 1910) (*pleiōn* meaning "more, various, or several" and *tropē* meaning "turning, orientation toward, or changing response to external stimuli"). Pleiotropic gene action (Caspari, 1952) denotes morphologic and phenotypic changes occurring in multiple organs. We propose to use the more specific term "pleiotypic" (*typikos* meaning "distinctive features of any type"), when referring to discrete biochemically detectable changes such as those associated with the *Ah* system. Accordingly, we suggest that "pleiogenic" would be the best term for describing specific intranuclear interactions between genes in response to a stimulus.

After certain chemicals (Fig. 8) bind avidly to a cytosolic receptor, the inducer–receptor complex is believed to translocate to the nucleus in a temperature-dependent step (Okey *et al.*, 1979, 1980), and a pleiotypic response ensues. Induction-specific mRNA (Nebert and Gielen, 1971; Negishi and Nebert, 1981) and protein (Haugen *et al.*, 1976)

*The Ah^b allele appears to be linked with one major allele associated with resistance to audiogenic seizures (Seyfried *et al.*, 1980).