Biochemical Genetics, Vol. 17, Nos. 5/6, 1979

Genetics, Ontogeny, and Testosterone Inducibility of Aldehyde Oxidase Isozymes in the Mouse: Evidence for Two Genetic Loci (*Aox-1* and *Aox-2*) Closely Linked on Chromosome 1

Roger S. Holmes[1,2]

Received 19 Oct. 1978—Final 3 Nov. 1978

"Null"-activity and low-activity variants for the liver supernatant isozymes of aldehyde oxidase (designated AOX-1 and AOX-2) were observed in inbred strains and in Harwell linkage testing stocks of Mus musculus. *The genetic loci determining the activity of these isozymes (designated* Aox-1 *and* Aox-2, *respectively) are closely linked on chromosome 1 near* Id-1 *(encoding the soluble isozyme of isocitrate dehydrogenase). Linkage data of* Aox-1 *with* Id-1 *and* Dip-1 *(encoding a kidney peptidase) demonstrated that this gene coincides with or is closely linked to* Aox *(Watson* et al., *1972). Ontogenetic analyses demonstrated that liver AOX-1 appeared just before birth and increased in activity during postnatal development, whereas liver AOX-2 was observed only during postnatal development. Adult male livers exhibited higher AOX-1 and AOX-2 activities than adult female livers. Both isozymes were significantly reduced in activity by castration of adult males and increased following testosterone administration to castrated males and normal female mice.*

KEY WORDS: aldehyde oxidase genetics; testosterone inducibility; ontogeny.

INTRODUCTION

Extensive genetic variation in the activity and kinetic properties of aldehyde oxidase (AOX, E.C. 1.2.3.1) from various inbred strains of mice has been

[1] MRC Radiobiology Unit, Harwell, OX11 0RD, England.
[2] Present address: School of Science, Griffith University, Nathan 4111, Queensland, Australia.

0006-2928/79/0600-0517$03.00/0

reported (Gluecksohn-Waelsch *et al.*, 1967; Huff and Chaykin, 1967), and these activity variants have been used to localize the gene determining AOX activity (*Aox*) on chromosome 1 of the mouse (Watson *et al.*, 1972). More recently, Lush (1978) has reported an electrophoretic variant of AOX in CE inbred mice and has concluded from his genetic analyses that the locus encoding this enzyme is either the same as, or very closely linked to, the *Aox* gene.

Electrophoretic analyses of mouse AOX have also been carried out in this laboratory (Holmes, 1978), and two isozymes have been resolved, designated AOX-1 and AOX-2, which are differentially distributed in tissues of the mouse. This communication describes the independent genetic variation and control of these liver AOX isozymes among common inbred strains and Harwell linkage testing stocks of *Mus musculus*, and provides evidence for close linkage of the genetic loci involved (designated *Aox-1* and *Aox-2*) on chromosome 1. The ontogeny and androgenic hormonal control of AOX isozymes have been also investigated.

MATERIALS AND METHODS

Mouse Strains and Crosses

Sixteen inbred strains and three Harwell linkage testing stocks of *Mus musculus* were used in these studies (Table 1). Two separate series of matings were carried out: (1) Linkage testing of *Aox-1* with *Dip-1* (Chapman *et al.*, 1971), *b* (brown/chromosome 4), *wa-1* (wavy coat/chromosome 6), c^{ch} (chinchilla/chromosome 7), *d* (dilute/chromosome 9), *se* (short ear/chromosome 9), *s* (piebald/chromosome 14) (see Robinson, 1972), and *Adh-3* (Holmes, 1979); F_1 female (C57Bl/Go♀ × LIVA ♂) mice were backcrossed to male LIVA mice to yield mice for segregation analysis. (2) Linkage testing of *Aox-1* and *Aox-2* with *Id-1* (encoding the soluble isozyme of isocitrate dehydrogenase); F_1 female (CBA/Ca ♀ × SWR/J ♂) mice were backcrossed to male SWR/J mice to yield mice for segregation analysis. All backcross animals examined in this study were 6–8 weeks of age, and only male animals were analyzed in the second series of matings. C3H/He mice were used for the developmental analysis of AOX isozymes.

Chemicals

Substrates, coenzymes, enzymes, buffer chemicals, and testosterone were purchased from Sigma Chemicals (St. Louis, MO).

Table I. Distribution of *Aox-1* and *Aox-2* Alleles Among Mouse Inbred Strains and Harwell Linkage Testing Stocks

Aox-1 alleles		*Aox-2* alleles	
a	*b* ("null")	*a*	*b* (low activity)
A/Ola[a]	CBA/Ca[c]	A/Ola[a]	SWR/J[a]
AKR/Ola[a]	DBA/2 Ola[a]	AKR/Ola[a]	
BALB/c[b]			
C3H/He[c]	LIVA[d]	BALB/c[b]	
C57BL/Go[c]	LVC[d]	C3H/He[c]	
C57BL/10ScSn Ola[a]		CBA/Ca[c]	
I/St[b]		C57BL/Go[c]	
LIII[d]		C57BL/10ScSn Ola[a]	
NIH/Ola[a]		DBA/2 Ola[a]	
NZB[a]		LIII[d]	
NZW[a]		LIVA[d]	
SJL/J Ola[a]		LVC[d]	
SWR/J[a]		NIH/Ola[a]	
101/H[c]		NZB[a]	
129/Da[b]		NZW[a]	
		SJL/J Ola[a]	
		101/H[c]	
		129/Da[b]	

[a] Olac Laboratories, England.
[b] Searle Diagnostics, England.
[c] Harwell inbred strain.
[d] Harwell linkage testing stock.

Homogenate Preparation

Livers, kidneys, and stomachs were excised from freshly killed mice, rinsed in cold distilled water, and homogenized in 50 mM tris–HCl, *p*H 8.0–0.1% Triton X-100 buffer (liver, 20%; kidneys and stomachs, 33%; w/v) using an Ultra-Turrax homogenizer. The homogenates were then centrifuged (45000*g*, 30 min) prior to electrophoresis. Subcellular fractions of C3H/He mouse liver were prepared according to the procedure of Hogeboom (1955): large granule fraction (containing mitochondria, peroxisomes, and lysosomes), small granule fraction (endoplasmic reticulum/cell membranes), and the cytoplasm. The granule preparations were washed in 50 mM tris-HCl buffer, *p*H 7.4, containing isotonic sucrose, extracted in 2 vol of the buffer containing Triton X-100, and centrifuged as previously described.

Cellulose Acetate Electrophoresis and Staining

Homogenate supernatants were subjected to zone electrophoresis on Titan III cellulose acetate plates (60 by 75 mm) (Helena Labs., TX) with tris-glycine buffer (25 mM tris, 192 mM glycine), *p*H 8.5. Different electrophoresis conditions were used for various enzymes: AOX-1, AOX-2, and POX-1, 25 V/cm for 15 min; stomach alcohol dehydrogenase (C_2 isozyme) and kidney dipeptidase, 25 V/cm for 20 min; kidney glucose phosphate isomerase, 25 V/cm for 25 min; and kidney isocitrate dehydrogenase, 25 V/cm for 30 min. The plates were then stained for activity, washed, dried, and photographed.

AOX isozymes were stained by an agar-overlay technique which has been previously described in detail (Holmes, 1978). The final concentrations in the agar-overlay solution were 100 mM tris-HCl (*p*H 8.0), 25 mM benzaldehyde, 0.9 mM methyl thiazolyl blue (MTT), and 0.3 mM phenazine methosulfate (PMS). A control strain containing 100 mM tris-HCl (*p*H 8.0), 0.9 mM MTT, and 0.3 mM PMS was also used to reveal oxidase activity in the absence of the substrate benzaldehyde (designated phenazine oxidase, POX).

Other biochemical loci were analyzed using the following final concentrations of buffer, substrates, enzymes, coenzymes, and histochemicals in the agar-overlay solution: (1) *Adh-3* (stomach C_2 isozyme of alcohol dehydrogenase), 100 mM tris-HCl (*p*H 8.0), 25 mM hexenol, 0.38 mM nicotinamide adenine dinucleotide (NAD^+), 0.9 mM MTT, and 0.3 mM PMS. (2) *Dip-1* (kidney isozyme of dipeptidase), 50 mM sodium phosphate buffer (*p*H 7.0), 1.6 mM L-leucyl-1-tyrosine, 0.25 mg/ml *Crotalus adamanteus* snake venom, 0.25 mg/ml type II peroxidase, 2 mM $MnCl_2$, and 1.0 mg/ml *o*-dianisidine, (3) *Id-1* (soluble isozyme of kidney isocitrate dehydrogenase), 100 mM tris-HCl (*p*H 8.0) buffer, 290 mM sodium isocitrate, 2 mM $MnCl_2$, 0.38 mM $NADP^+$, 0.3 mM PMS, and 0.9 mM MTT.

Testosterone Treatment

Six littermate male and three littermate female C3H/He mice were used for this analysis. At 12 weeks of age, five of the males were castrated and allowed to recuperate for 3 weeks. At 15 weeks, the castrated males and the females were given 0.5-ml subcutaneous injections of peanut oil (control animals) or peanut oil with 10 mg/ml testosterone on days 0, 1, 2, 4, and 6. On day 7, the animals were killed and liver AOX isozymes were examined electrophoretically as previously described.

RESULTS

Figure 1 illustrates a cellulose acetate zymogram of mouse liver aldehyde oxidase (AOX) isozymes at different stages in development. Xanthine oxidase

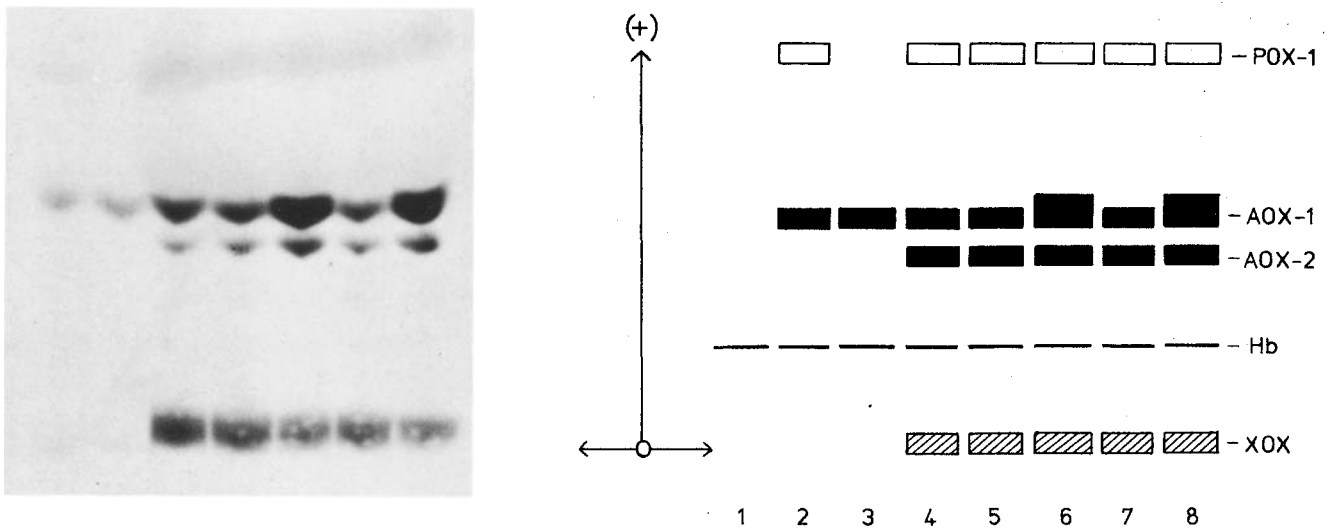

Fig. 1. Cellulose acetate zymogram and diagrammatic illustration of C3H/He mouse liver AOX isozymes at various stages in development. Phenazine oxidase activity is designated by open bars, xanthine oxidase (XOX) activity by hatched bars; Hb designates a hemoglobin zone. Developmental stages: (1) 18-day fetus; (2) 20-day fetus; (3) newborn; (4) 3-week-old ♂; (5) 6-week-old ♀; (6) 6-week-old ♂; (7) adult ♀; (8) adult ♂.

(XOX) and phenazine oxidase (POX) are also stained by this procedure; however, these are readily distinguished by the use of a control stain from which the benzaldehyde substrate has been omitted, thus revealing POX and XOX activity only, or by the use of hypoxanthine as a specific substrate (Holmes 1978). AOX-1 appeared in the 20-day fetus and increased in activity following birth to give highest activity in the maturing and adult male. AOX-2 was observed only in postnatal animals and also gave some indication of higher activity in maturing and adult males. POX-1 and XOX exhibited readily observable zones of activity in livers of animals of 3 weeks of age or older.

Subcellular fractionation studies demonstrated that AOX-1 and AOX-2 are predominantly localized in the extraparticulate cytoplasm of liver isotonic sucrose extracts (see Materials and Methods for details).

The electrophoretic patterns for variants of AOX-1 as well as the hybrid phenotype for this enzyme are shown in Fig. 2. Two phenotypes were observed among the 16 inbred strains and three Harwell linkage testing stocks

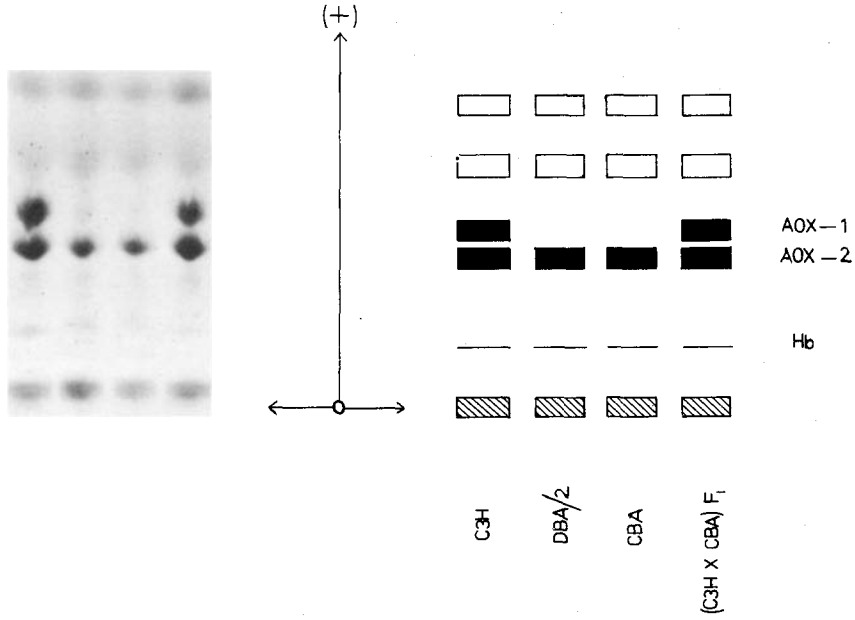

Fig. 2. Cellulose acetate zymogram and diagrammatic illustration of the genetic variation of the liver AOX-1 isozyme from C3H/He, DBA/2 Ola, CBA/Ca, and F_1 (C3H/He × CBA/Ca) hybrid male mice. Phenazine oxidase activity is represented by open bars, xanthine oxidase activity by hatched bars; Hb designates a hemoglobin zone. Note the absence of AOX-1 in CBA/Ca and DBA/2 Ola liver extracts.

of mice examined (Table I). In most inbred strains liver AOX-1 exhibited high activity in adult males (e.g., C3H/He) whereas in CBA/Ca and DBA/2Ola adult males no detectable AOX-1 activity was observed. Hybrid (C3H/He × CBA/Ca) adult males, however, exhibited intermediate levels of AOX-1 activity. Genetic analysis showed that these variants were determined by two codominant alleles at one locus (designated *Aox-1*^{a} and *Aox-1*^{b} for the presence or absence of AOX-1 activity, respectively, which is consistent with the terminology introduced by Watson *et al.*, 1972) and the patterns observed in Fig. 2 for CBA/Ca and hybrid mice are representative of those obtained for individuals of a (C57BL/Go ♀ × LIVA ♂) F_1 ♀ (proposed genotype *Aox-1*^{a} *Aox-1*^{b}) × LIVA ♂ (*Aox-1*^{b} *Aox-1*^{b}) backcross. Eighty-three of these progeny of the above backcross were typed with respect to the eight segregating loci (see Materials and Methods for details). Linkage was detected between *Aox-1* and *Dip-1*. Twenty-five of the progeny were recombinants, giving a recombination frequency of $30.1 \pm 6\%$ between these two loci. *Aox-1* segregated independently of the other seven loci.

Electrophoretic patterns illustrating the independent phenotypic variation of AOX-1 and AOX-2 in CBA/Ca and SWR/J inbred mice, and their F_1 hybrids, are shown in Fig. 3. Similar variants for AOX-1 were observed in

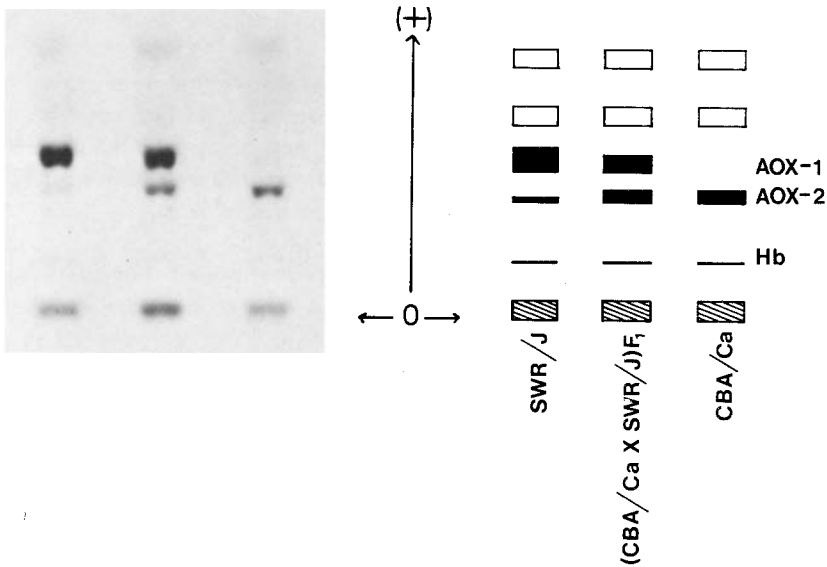

Fig. 3. Cellulose acetate zymogram and diagrammatic illustration of the genetic variation of liver AOX-1 and AOX-2 isozymes from SWR/J, CBA/Ca, and F_1 hybrid male mice. Phenazine oxidase activity is represented by open bars; xanthine oxidase by hatched bars; Hb designates a hemoglobin zone.

these animals as were shown for the CBA/Ca, C3H/He, and F_1 hybrid mice (Fig. 2). In contrast, phenotypic variation of AOX-2 was observed only in SWR/J animals for which a low-activity variant was observed that did not appear to involve AOX-1. CBA/Ca inbred mice, however, showed normal levels of AOX-2 activity, also observed in all of the other inbred strains examined with the exception of SWR/J mice; in addition, F_1 (CBA/Ca × SWR/j) ♂ revealed levels of activity which were readily distinguished from those of the SWR/J ♂ AOX-2 phenotype. Genetic analyses demonstrated that these variants behaved as normal codominant alleles at a single locus for AOX-2 (designated *Aox-2*^{a} and *Aox-2*^{b} for the normal-activity and low-activity phenotypes, respectively). The patterns observed in Fig. 3 for SWR/J and hybrid mice are representative of those obtained for male individuals of a (CBA/Ca ♀ × SWRJ ♂) F_1 ♀ (proposed genotype (*Aox-1*^{a} *Aox-1*^{b}/*Aox-2*^{a} *Aox-2*^{b}) × SWR/J ♂ (*Aox-1*^{a} *Aox-1*^{a}/*Aox-2*^{b} *Aox-2*^{b}) backcross. Phenotypes were examined electrophoretically using SWR/J and (CBA/Ca × SWR/J) F_1 male liver extracts as standards on each cellulose acetate zymogram to ensure that the correct phenotypes were assigned independently to the AOX-1 and AOX-2 isozymes. Sixty-two of these progeny were examined for segregation at the *Aox-1*, *Aox-2*, and *Id-1* loci (Table II). No recombinants were observed between the *Aox-1* and *Aox-2* loci, which demonstrates that these are closely linked genes. In contrast, both *Aox* loci exhibited a recombination frequency of $8.2 \pm 3.6\%$ with *Id-1*, thus indicating that all three genetic loci are on the same chromosome.

The electrophoretic phenotypes for liver AOX-isozymes in normal adult C3H/He males, castrated C3H/He males, castrated C3H/He males treated over a period of 1 week with testosterone in peanut oil, and adult female C3H/He mice similarly injected with peanut oil with and without testosterone were examined. The results clearly demonstrated that AOX-1 activity was significantly lowered after castration and that following subsequent testosterone treatment approximately adult male levels of activity were attained. Moreover, female liver AOX-1 activities were also induced to near adult male levels of activity by testosterone administration. Although the effect on AOX-2 was less pronounced, similar changes in activity were observed for this isozyme following castration and treatment with hormone.

DISCUSSION

There have been a number of reports that mouse liver aldehyde oxidase activity is controlled by a codominant autosomal gene. The following characteristics of the variant AOX forms among inbred strains of mice have been observed (Huff and Chaykin, 1967; Gleucksohn-Waelsch *et al.*, 1967): (1) a tenfold lower enzyme activity in the DBA/1 and DBA/2 strains compared

Table II. Results of the Test for Linkage Between *Aox-1*, *Aox-2*, and *Id-1*[a]

Parents (♀×♂)	Parentals		Recombinants	
	$\frac{Aox\text{-}1^a\ Aox\text{-}2^b\ Id\text{-}1^a}{Aox\text{-}1^a\ Aox\text{-}2^b\ Id\text{-}1^a}$	$\frac{Aox\text{-}1^b\ Aox\text{-}2^a\ Id\text{-}1^b}{Aox\text{-}1^a\ Aox\text{-}2^b\ Id\text{-}1^a}$	$\frac{Aox\text{-}1^a\ Aox\text{-}2^b\ Id\text{-}1^b}{Aox\text{-}1^a\ Aox\text{-}2^b\ Id\text{-}1^a}$	$\frac{Aox\text{-}1^b\ Aox\text{-}2^a\ Id\text{-}1^a}{Aox\text{-}1^a\ Aox\text{-}2^b\ Id\text{-}1^a}$
(CBA/Ca × SWR/J) × SWR/J	27	30	1	4
Total	57		5	

[a] No recombinants between *Aox-1* and *Aox-2* were observed in these animals, indicating close linkage.

with other inbred mice, (2) a higher K_m for the DBA/2 variant, and (3) genetic inheritance of these variants consistent with codominant alleles at a single locus determining AOX activity in these animals. Subsequently, Watson *et al.* (1972) mapped the *Aox* locus on chromosome 1 near *Id-1* (encoding soluble isocitrate dehydrogenase) (Hutton and Roderick, 1970), and Lush (1978) reported three electrophoretic phenotypes for liver AOX among 26 inbred strains: (1) mice with a single high-activity zone of AOX activity (most inbred strains examined) (designated A phenotype), (2) mice with no detectable AOX activity (A2G, CBA, DBA/2, and I/St inbred strains) (B phenotype), and (3) mice with an extra AOX activity zone (CE strain) (C phenotype). Genetic analyses showed that the alleles determining the AOX-A and AOX-C phenotypes were both localized at approximately the same distance from the *Id-1* locus, and were considered as alleles at a single *Aox* locus.

This current investigation, however, provides conclusive evidence that at least two genetic loci, designated *Aox-1* and *Aox-2*, are involved in the determination of AOX activity in mouse liver. This proposal is based on the following lines of evidence: (1) the independent genetic variation of liver AOX-1 and AOX-2 observed among the mouse inbred strains (Table I), (2) the independent genetic inheritance of these isozymes as demonstrated by the AOX phenotypes observed for progeny of a (C57BL/Go × LIVA) F_1 × LIVA backcross (Fig. 2), and (3) the differential tissue distribution of AOX-1 and AOX-2 in C3H/He mice (Holmes, 1978). Moreover, the linkage data in Table II, which examined the segregation of *Aox-1* and *Aox-2* loci in a (CBA/Ca × SWR/J) × SWR/J backcross, showed that these genes are closely localized on chromosome 1 near *Id-1*. Linkage data of *Aox-1* with *Dip-1* ($30.1 \pm 6.0\%$) and of *Aox-1* and *Aox-2* with *Id-1* ($8.2 \pm 3.6\%$) corresponded to the results of Watson *et al.* (1972) for *Aox*. Consequently, it would appear that this latter locus actually comprises two separate but closely linked genes, determining AOX activity in the mouse. It is significant to note that a similar conclusion has been recently proposed for the genetic control of AOX in *Drosophila melanogaster*, although evidence for close linkage of the two genes responsible has not, as yet, been obtained (David *et al.*, 1978). Using "null"-activity variants as a biochemical marker for one of the loci, these workers have established gene duplicity for this previously considered single locus (*Aldox*) (Dickinson, 1970).

These multiple genes in the mouse are apparently differentially active during tissue differentiation. Ontogenetic analyses of liver AOX isozymes (Fig. 1) demonstrated that AOX-1 appeared earlier in development than the alternative isozyme, exhibiting low activity just prior to birth and increasing to a maximum in the adult male, whereas AOX-2 was not observed in the animals studied until 3 weeks after birth and exhibited little subsequent change in activity during maturation. The androgenic control of liver AOX

isozymes has been also investigated, and the results were consistent with previous studies by Huff and Chaykin (1967, 1968), who established that the levels of N^1-methylnicotinamide (a substrate of AOX) oxidase in mouse liver are regulated by testesterone administration.

ACKNOWLEDGMENTS

I am grateful to Dr. Mary Lyon and other members of the genetics section of the MRC Radiobiology Unit at Harwell for their advice concerning this project and for supplying the mice; to Dr. J. T. Jones and Dr. B. M. Cattanach for their assistance in the testosterone induction experiments; to Dr. E. Eicher for making available details of the cellulose acetate electrophoretic procedure prior to publication; to Dr. I. Lush for providing details of his studies on AOX prior to publication; to Dr. J. Vennart for providing the research facilities; and to Mrs. Diane Holmes for drawing the diagrams.

REFERENCES

Chapman, V. M., Ruddle, F. H., and Roderick, T. H. (1971). Linkage of isozyme loci in the mouse: phosphoglucomutase-2 (*Pgm-2*), mitochondrial NADP malate dehydrogenase (*Mod-2*) and dipeptidase-1 (*Dip-1*). *Biochem. Genet.* **5**:101.

David, J., Bocquet, C., Van Herrewege, J., Fouillet, P., and Arens, M.F. (1978). Alcohol metabolism in *Drosophila melanogaster*: Uselessness of the most active aldehyde oxidase produced by *Aldox* locus. *Biochem. Genet.* **16**:203.

Dickinson, W. J. (1970). The genetics of aldehyde oxidase in *Drosophila melanogaster*. *Genetics* **66**:487.

Gluecksohn-Waelsch, S., Greengard, P., Quinn, G. P., and Teicher, L. S. (1967). Genetic variations of an oxidase in mammals. *J. Biol. Chem.* **242**:1217.

Hogeboom, G. H. (1955). Fractionation of cell components of animals tissues. *Methods Enzymol.* **1**:16.

Holmes, R. S. (1978). Electrophoretic analyses of alcohol dehydrogenase, aldehyde dehydrogenase, aldehyde oxidase, sorbitol dehydrogenase and xanthine oxidase from mouse tissues. *Comp. Biochem. Physiol.* **61B**:339.

Holmes, R. S. (1979). Genetics and ontogeny of alcohol dehydrogenase isozymes in the mouse: Evidence for a *cis*-acting regulator gene (*Adt-1*) controlling C_2 isozyme expression in reproductive tissues and close linkage of *Adh-3* and *Adt-1* on chromosome 3. *Biochem. Genet.* **17**:461.

Huff, S. D., and Chaykin, S. (1967). Genetic and androgenic control of N^1-methylnicotinamide oxidase activity in mice. *J. Biol. Chem.* **242**:1265.

Huff, S. D., and Chaykin, S. (1968). Kinetics of testosterone action, *in vivo*, on liver N'-methylnicotinamide oxidase activity in mice. *Endocrinology* **83**:1259.

Hutton, J. J., and Roderick, T. H. (1970). Linkage analyses using biochemical variants in mice. III. Linkage relationships of eleven biochemical markers. *Biochem. Genet.* **4**:339.

Lush, I. E. (1978). Genetic variation of some aldehyde-oxidising enzymes in the mouse. *Anim. Blood Groups Biochem. Genet.* **9**:85.

Robinson, R. (1972). *Gene Mapping in Laboratory Mammals*, Part B, Plenum Press, New York.

Watson, J. G., Higgins, T. J., Collins, P. B., and Chaykin, S. (1972). The mouse liver aldehyde oxidase locus (*Aox*). *Biochem. Genet.* **6**:195.