EFFECTS OF THE LUXOID GENE (*lu*) ON LIVER ESTERASE ISOZYMES OF THE MOUSE[1]

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ALTHOUGH the esterases can be separated in column chromotography or by the use of paper or agar gel electrophoresis, the introduction of starch gel and acrylamide gel systems has made it possible to conveniently sort out and quantitatively measure the multiple molecular forms (isozymes) of esterases.

In recent years evidence has been accumulating which indicates genetic control over these isozymes. In the mouse, POPP and POPP (1962) demonstrated among inbred strains of mice three esterase isozymes which appeared to be controlled by alleles at a single locus; and PETRAS (1963) presented data suggesting genetic control of another isozyme by a pair of autosomal alleles.

With the above background in mind, as well as the work of HUNTER, *et al.* (1964) which indicated the essentially progressive appearance of the esterase enzymes throughout development, a system was devised to compare the distribution of non-specific esterases in the tissues of animals whose phenotypes are manifestations of alternative alleles at a single locus. The results of these experiments have been reported briefly (CENTER and HUNTER 1966) and are presented more fully here.

MATERIALS AND METHODS

The experimental stocks used were two lines of mice which have been shown to carry the luxoid gene (*lu*), animals from the C57BL/6Sfd inbred strain and a hybrid C57BL/6-Swiss-GP line. The designation "GP" refers to a random-bred stock known to transmit recessive genes brown (*b*) and ruby-eye (*ru*). The zymograms of littermate luxoid, polydactyl and normal mice were compared by the use of both starch and acrylamide gel techniques. More than 370 electrophoretic separations were run on a total of 119 mice.

Tissue samples were removed, weighed, and homogenized (1 part tissue/3 parts water). The tissue homogenates were centrifuged at 20,000 *g* for a total of 20 minutes at a temperature of 4°C. Acrylamide gel electrophoresis was performed according to slightly modified methods of ORNSTEIN (1964) and DAVIS (1964). The acrylamide was recrystallized in chloroform and the resolving and concentrating monomers made up fresh daily. The "lower" and "spacer" gels were poured and allowed to polymerize. Ten lambda samples of the supernatant were applied directly to the upper surface of the concentrating or "spacer" gels and then covered with more concentrating gel. Table 1 provides further details regarding the components of the gel system and the composition of the substrate mixtures. Electrophoresis proceeded at 1 milliamp/gel for approximately 30 minutes until the tracking dye migrated 30 mm down the gel. The gels were then chilled, removed from the tubes, and rinsed 5 minutes in .04M Tris-HCl buffer, pH 7.0.

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TABLE 1

Reagents used in acrylamide gel electrophoresis

Resolving gel system (7.9%) (Lower gel)		Concentrating gel system (1.2%) (Spacer gel)		Substrate mixture used to illustrate esterase isozymes
2 parts	0.37M Tris-HCl buffer pH 8.7	2 parts	0.06M Tris-HCl buffer pH 6.7	(1) α-napthyl butyrate:
4 parts	30.8% recrystallized acrylamide 0.8% bis-acrylamide	4 parts	5% recrystallized acrylamide 2.5% bis-acrylamide	100 ml .04M Tris-HCl buffer (pH 7.0) 100 mg Fast Blue RR
1 part	1% Temed (N, N, N′, N′-tetramethylethylenediamine)	1 part	1% Temed	2 ml of 2% α-napthylbutyrate in acetone
8 parts	glass distilled water	6 parts	glass distilled water	(2) Napthol AS-D acetate:
1 part	0.2% ammonium persulfate	1 part	0.2% ammonium persulfate	100 ml 0.4M Tris-HCl buffer (pH 7.0)
		2 parts	0.004% riboflavin	60 mg Fast Blue RR 10 mg AS-D acetate in 1ml N, N-dimethyl-formamide

Buffer bath—0.05M Tris-glycine, pH 8.7.

TABLE 2

Frequency of normal, polydactylous, and hemimelic offspring in the progeny of crosses between Stanford "luxoid" and C57BL/6Sfd normal mice

Type of mating (Genotype and source)	Normal	Polydactylous	Hemimelic	Total
(1) *lu/lu* × +/+ (*lu/lu*—hybrid stock*)				
F_1	43	83	0	126
F_2 (Poly × Poly)	215	267	110	592
(2) *lu/lu* × +/+ (*lu/lu*—C57BL/6Sfd)				
F_1	12	32	0	44
F_2 (Poly × Poly)	42	63	19	124

* C57BL/6-Swiss-GP (heterogenous general purpose stock)

The multimolecular forms of esterase were demonstrated with two substrates, alpha-napthyl butyrate primarily, and napthol AS-D acetate; Blue RR salt was employed as the azo coupling agent. After staining in substrate solutions at 37°C for 20 minutes, the gels were fixed in alcohol-acetic acid (⅔ absolute, ⅓ 7% acetic) and stored in the dark in 7% acetic acid. The starch gel electrophoresis was conducted according to the previously described method of MARKERT and HUNTER (1959).

Inhibition studies were carried out with alpha-napthyl butyrate as substrate. Eserine concentrations of 1×10^{-4}, 5×10^{-4}, and 1×10^{-3} were added to a mixture of .04M Tris-HCl buffer and Fast Blue RR in which gels were incubated for 5 minutes before the substrate was added.

The resultant gels were studied visually and the density of the isozyme associated dye precipitate was measured with a model E Canalco microdensitometer. Pertinent details relative to the use of this instrument have been thoroughly described by ORNSTEIN (1964). The densitometer integrator marks below the base line of the curve were counted from the point where an isozyme associated curve began to rise to the point where it rejoins the background curve and thus the intensity of specific isozymes was expressed as mm^2 under a particular curve.

RESULTS

Genetic basis of the hemimelic mutants in the C57BL/6Sfd and the C57BL/6-Swiss-GP stocks: In a hybrid C57BL/6 × Swiss line (antecedents of the Swiss mice had been treated with the HN_2 form of nitrogen mustard and were obtained 15 years ago from another department of Stanford University), a mouse with abnormally rotated, polydactyl hind feet and shortened hind legs was first observed some years also in our laboratory. Subsequently, several of these "hemimelic" or "luxoid" females were mated to a general purpose (GP) stock and the resultant hemimelic progeny were later crossed repeatedly to C57BL/6Sfd males. The results of the outcrosses of these hybrid "luxoid" mice to normal C57BL/6Sfd mice are summarized in Table 2.

More recently, additional hemimelic mutants were found in the inbred C57BL/6Sfd stock maintained in this laboratory for approximately nine years. These "luxoid" mice, descendants of animals treated with thalidomide, were subsequently mated to normal C57BL/6Sfd mice and the segregations obtained are also presented in Table 2. It seems evident in both stocks that the "Stanford mutations" are due to a single gene which is expressed as polydactyly in the heterozygote and hemimelia in the homozygote.

Both of these lines of hemimelic mice were tested for identity with Green's

TABLE 3

Tests for identity of Stanford "luxoid" mutants with lu *and* lx

Type of mating (Genotype and source)	Normal	Polydactylous	Hemimelic	Total
1. *lu*/+ (Hybrid*) × *lu*/+ (GREEN's)	39	50	22	111
2. *lu*/*lu* (from No. 1) × +/+ (C57BL/6Sfd)	21	48	0	69
3. *lu*/+ (Hybrid) × *lx*/+	26	28	25	79
4. *lu*/+ *lx*/+ (from No. 3) × +/+ (C57BL/6Sfd	27	30	6	63
5. *lu*/+ (C57BL/6Sfd) × *lu*/+ (GREEN's)	34	38	15	87
6. *lu*/*lu* (from No. 5) × +/+ (C57BL/6Sfd)	17	44	0	61

* Hybrid—See description in Table 2.

luxoid (*lu*), and the hybrid stock was mated also with luxate (*lx*) heterozygotes (Table 3). We have found hemimelic young in the F_1 of the crosses between mice heterozygous for *lu* and those heterozygous for the Stanford mutations. As GREEN (1955) observed, this could result from the interaction of two genes, such as *lu* and *lx*, to produce hemimelia in the double heterozygote. However, when these F_1 hemimelic animals were crossed to normal C57BL/6Sfd mice (Matings 2 and 6, Table 3), no hemimelic animals were observed in the progeny from either the hybrid or inbred Stanford lines. This indicates that the genetic basis for the hemimelic mutants in both of these stocks is Green's luxoid gene or *lu*. If two genes were involved, segregation would have occurred and some hemimelic animals (double heterozygotes) would have resulted as was the case when the F_1 hemimelic mice from the matings of hybrid line heterozygotes with carriers of *lx* were outcrossed to normal C57BL/6Sfd animals (Mating 4, Table 3).

The deficiency in the incidence of polydactylous mice (Tables 2, 3) agrees with GREEN's (1955) conclusion that in the heterozygote, the penetrance of the luxoid gene is limited and dependent on genetic background. It would also seem that in both of the Stanford stocks, especially in the inbred line, the penetrance in the homozygote is incomplete.

The phenotype of the Stanford mutants is very similar to that described for Green's luxoid homozygotes and heterozygotes (GREEN 1955; FORSTHOEFEL 1958). In homozygous Stanford mice both forelimbs frequently show hyperphalangism or polydactylism. In the hind limbs the tibia is more slender than normal and shortened while the fibula is enlarged (Figure 1). In some cases there is virtual loss of the tibia and concomitant shortening and thickening of the fibula. The effect on the tibia and fibula in the two hind limbs is usually quite

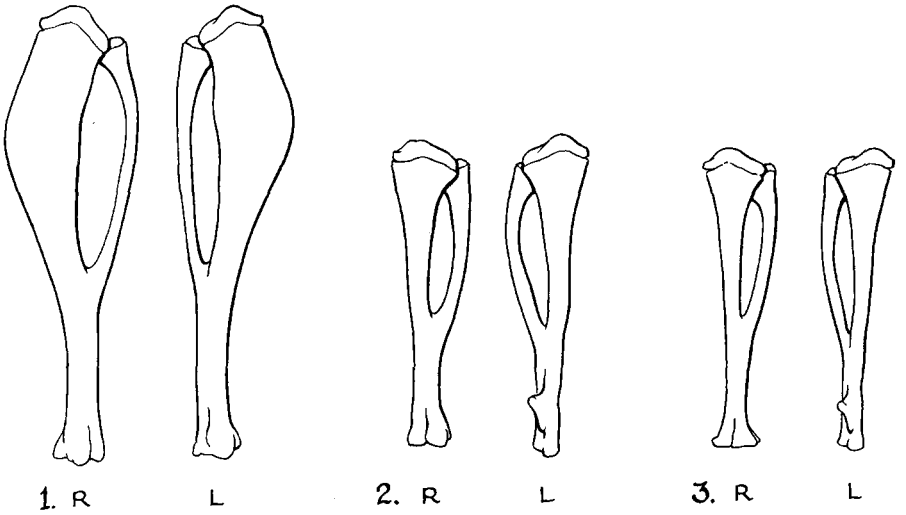

FIGURE 1.—Tibia and fibula from the right and left hind legs of (1) normal C57BL/6Sfd, (2) luxoid (*lu/lu*) C57BL/6Sfd and (3) luxoid (*lu/lu*) C57BL/6-Swiss-GP adult males (8 weeks old; alizarin preparations; approximately $\times 3\frac{1}{2}$.

symmetrical. The hind feet show a range of modifications from hyperphalangism through polydactylism to ectrodactylism. In the hybrid stock, the caudal vertebrae are often abnormally formed resulting in tail kinks; however, in our C57BL/6Sfd strain no tail deformities affecting the homozygote have been recorded. Homozygous males have been found to be sterile in both lines under discussion.

Heterozygous males and females of both Stanford stocks are characterized by full fertility and morphologically by hyperphalangism or polydactylism on one or both hind feet; the forefeet have been uniformly normal. Except for the absence of tail abnormalities in the C57BL/6Sfd hemimelic mice, in all the above respects the morphology of the Stanford mutants parallels that of *lu* homozygotes or heterozygotes with a C57BL/10 background.

On the basis of the above genetic and morphological evidence, it would appear that the hemimelic mice in both the hybrid and inbred Stanford stocks are the result of the homozygous expression of *lu* (luxoid) and that the polydoctylous animals in the same stocks are the expression of this gene in the heterozygote.

It should perhaps be noted that in any mouse colony the possibility of undiscovered migration exists and it may be that mice of the hybrid line which transmitted the mutant gene were introduced into the C57BL/6Sfd stock. The significance of the history of treatment with nitrogen mustard or thalidomide with reference to the appearance of the trait is also questionable. Since polydactylous and hemimelic mice were found and subsequently lost in the C57BL/6 strain maintained in our laboratory more than ten years ago, and all Stanford *lu* mice have C57BL/6 ancestry, it does seem probable that C57BL/6 mice which were normal "overlaps" carried *lu* in heterozygous condition and that this has been the origin of our hemimelic mice in all instances, the original mutation having occurred some years ago in a C57BL/6 mouse.

Starch gel electrophoresis: Our work with 38 serum, kidney, and liver samples on starch gel did not reveal any consistent differences between luxoid and nonluxoid littermates.

Acrylamide gel electrophoresis: Acrylamide gel esterase zymograms made from 34 serum samples did not show any constant variations between luxoid and nonluxoid control animals. Similar experiments involving mouse kidney were complicated by a sexual dimorphism and were not useful in revealing esterase differences in the mice examined. Sex-related esterase isozyme variations have been reported in the mouse kidney by SHAW and KOEN (1963) and by RUDDLE (1966). In contrast to our earlier experiences, we did find consistent differences between the liver isozymes of luxoid and normal mice using the acrylamide system. The normal liver zymogram of the esterases was established with samples from both the hybrid and inbred animals (Figure 2). A total of 24 isozymes was found in both of these stocks with the use of the two substrates. There was a tendency for isozyme or band 50 to split in some of the hybrid animals, but since this was not always found, we would state that the number of isozymes in the livers from normal animals of both mouse stocks was found to be the same. However, differ-

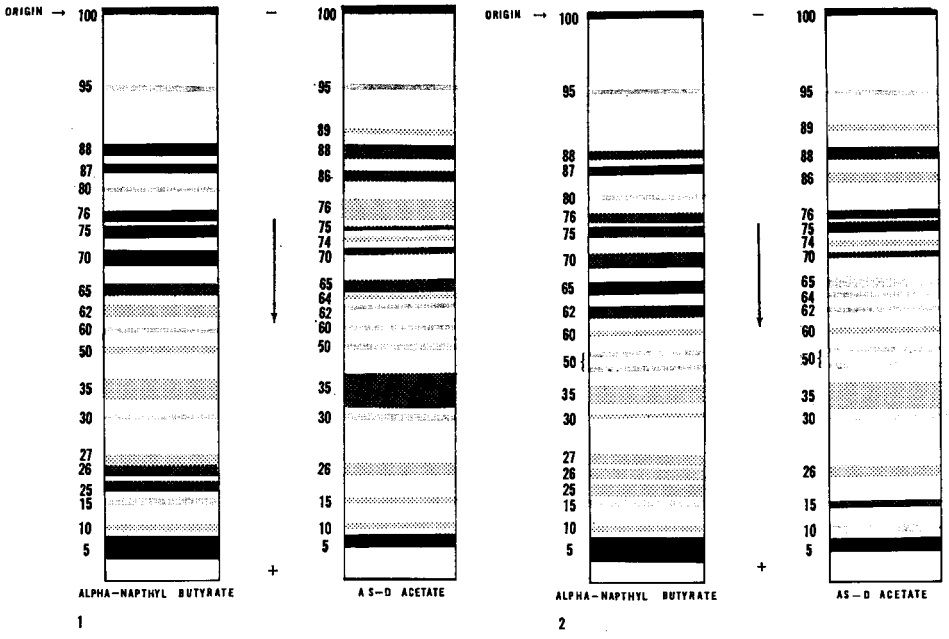

FIGURE 2.—Diagram of the esterase zymogram of liver homogenates from (1) normal C57BL/6Sfd and (2) C57BL/6-Swiss-GP 8 week old female mice using two substrates, alpha-napthyl butyrate and napthol AS-D acetate. (Approximately $\times 2\frac{1}{2}$).

ences in the intensities of specific isozymes were shown between liver samples from the hybrid and inbred lines.

Consistent differences between normal and luxoid littermates were apparent in the liver zymogram in the region between isozymes 35 and 70. In the case of both the hybrid and inbred lines there was a diminution in isozymes 50 and 65 in the luxoid (*lu/lu*) mouse as compared with its normal sib (Figure 3). These alterations were demonstrated both by visual inspection and by the study of densitometric tracings. The differences between the esterase zymograms of luxoid and normal sibs were consistently found in 17 litters of mice two weeks of age or older (Table 4). In all age groups the values recorded for luxoid mice were significantly lower ($P<.001$) than for normal mice in regard to isozymes 65 and 50 (Table 4B). Isozyme 62 was chosen as a control band and did not show consistent significant diminution in luxoid animals in comparison with normal mice.

The summarized data (Table 4) indicate differences between polydactylous and normal littermates and between polydactylous and luxoid sibs. However, these relationships were inconsistent (Table 4B) while concentration of liver isozymes 50 and 65 in the luxoid mice was uniformly lower than that of their normal littermates in all mice two weeks of age or older. The lower concentration of isozymes 50 and 65 was evident in both sexes in luxoid mice of the inbred strain and hybrid line but was most easily recognized visually in C57BL/6 females (Figure 4). It would seem that with the use of AS-D acetate as the sub-

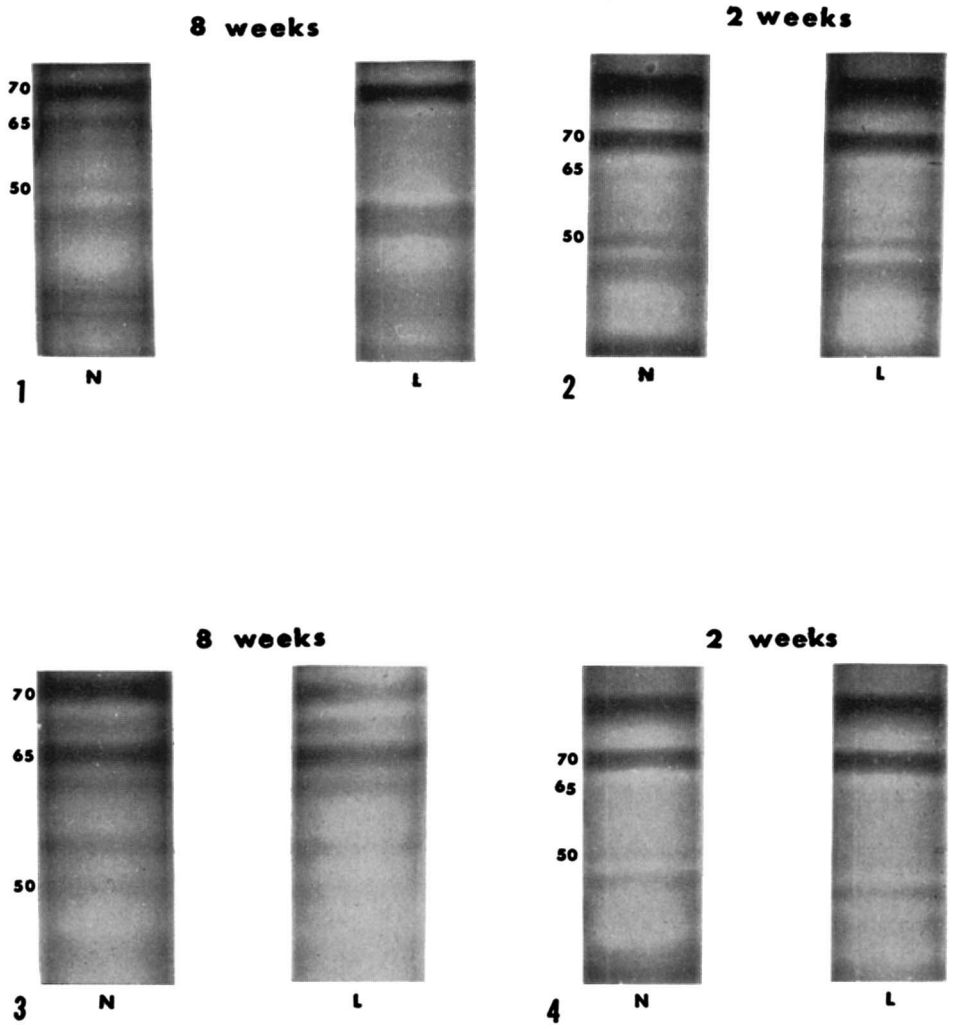

FIGURE 3.—Composite photographs of acrylamide gels showing approximately the region of isozymes 35 to 70 of liver homogenates from (1) and (2) C57BL/6Sfd, and (3) and (4) C57BL/6-Swiss-GP normal (N) and luxoid (L) littermates at eight weeks and at two weeks. (Substrate: alpha-napthyl butyrate; approximately ×2½ to 3.)

strate there was also a lower intensity of isozyme 64 in luxoid mice (Figure 4); this band was not apparent when alpha-napthyl butyrate was used as the substrate. Eserine inhibition studies showed that isozymes 50 and 65 are not cholinesterase.

An attempt is being made to determine at what stage in ontogeny the above differences in liver esterase zymograms of luxoid and normal sibs are first discernable. Our first results with both substrates, alpha-napthyl butyrate and AS-D acetate, did not show differences between the liver isozymes of newborn and luxoid mice in the zymogram area under discussion. However, more recent

TABLE 4

A. *Densitometric readings and standard errors of isozymes 50, 62, and 65 of liver homogenates from a total of 69 mice, and* B. *Significance of the differences between mean readings among these progeny of luxoid heterozygotes, using a t-test*

A.

Age (weeks)	Genotype	Number of animals	Mean densitometric value*		
			Isozyme 65	Isozyme 62	Isozyme 50
2	+/+	8	177±20.0	100±7.6	145±15.1
	lu/+	6	127±6.8	97±11.0	117±8.2
	lu/*lu*	8	107±15.7	85±11.9	97±9.7
8	+/+	8	192±17.5	137±19.2	175±13.6
	lu/+	8	172±10.8	122±13.5	165±18.5
	lu/*lu*	7	143±12.6	117±9.3	109±8.7
16 to 47	+/+	10	170±15.7	104±7.9	112±8.0
	lu/+	8	160±20.6	125±5.4	122±8.6
	lu/*lu*	6	100±12.9	93±10.1	77±12.9

B.

Age (weeks)	Phenotypes compared	t-values		
		Isozyme 65	Isozyme 62	Isozyme 50
2	Normal and luxoid	5.51(P<.001)	2.13(P<.10)	5.39(P<.001)
	Normal and polydactylous	4.20(P<.005)	.47(P<.70)	2.98(P<.02)
	Polydactylous and luxoid	2.10(P<.10)	1.45(P<.20)	3.17(P<.01)
8	Normal and luxoid	4.54(P<.001)	1.82(P<.10)	8.05(P<.001)
	Normal and polydactylous	1.98(P<.10)	1.29(P<.25)	.88(P<.40)
	Polydactylous and luxoid	3.62(P<.005)	.61(P<.60)	5.30(P<.001)
16 to 47	Normal and luxoid	6.36(P<.001)	1.94(P<.10)	5.15(P<.001)
	Normal and polydactylous	.80(P<.50)	4.12(P<.001)	1.70(P<.20)
	Polydactylous and luxoid	4.58(P<.001)	6.15(P<.001)	6.16(P<.001)

* Density of the isozyme associated dye precipitate recorded in mm² under the curve (substrate—α-napthyl butyrate).

studies have indicated that the pattern is not the same in all luxoid and normal newborn littermates.

Preliminary investigations of heart and skeletal muscle from eight-week old mice also indicated differences between the esterases present in the tissues from luxoid and normal littermates.

DISCUSSION

We have demonstrated differences in the esterase isozymes of liver samples from luxoid (*lu*) and normal littermates, which differ genetically at a single locus. These changes in the amount of non-specific esterases present are interpreted as pleiotropic effects of *lu* and indicate control of certain aspects of juvenile and adult physiology of the mouse by a gene which earlier had a marked effect on embryonic development (FORSTHOEFEL 1959). In this connection it is of consequence to note as GRÜNEBERG (1962) pointed out that the question of genetic control of less obvious functions in adult physiology by genes which have obvious effects during development is a basic consideration.

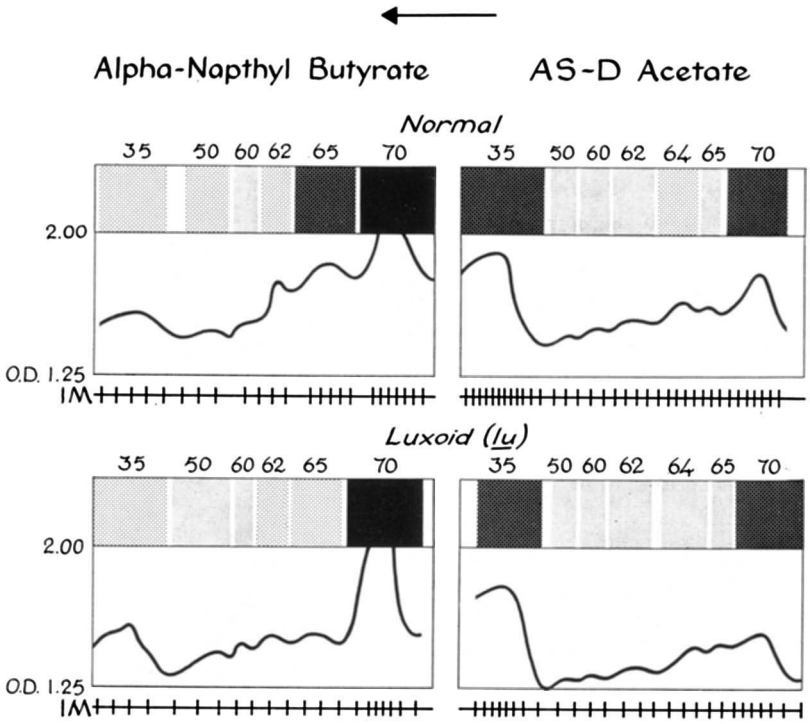

FIGURE 4.—Diagrammatic reproduction of densitometric tracings and esterase zymograms of liver homogenates of normal and luxoid 8 week old, C57BL/6Sfd female littermates; two substrates: alpha-napthyl butyrate and AS-D acetate. The abscissa is the millimeters of sample migration in acrylamide gel while the ordinate is the optical density (O.D). The base line below the curve was set at an optical density of 1. Each integrator mark (I.M.) represents 40 mm² of chart area with alpha-napthyl butyrate and 20 mm² of chart area with AS-D acetate.

Several studies, including that of RUDDLE and RODERICK (1965), have shown that the presence or absence of the esterases in mouse tissues is under genetic control. The present experiments indicate that the amount of an isozyme present in a tissue is also under genetic control. It is apparent that changes in the esterases of one tissue do not portend comparable alterations in these enzymes in any other specific tissue of the same animal. The results of these experiments also emphasize the advisability of testing tissue samples with more than one type of electrophoresis. The superior resolution of plasma esterases with acrylamide gel in comparison with starch gel has been indicated previously (ALLEN, POPP, and MOORE 1965). In addition, our results illustrate the importance of quantitative estimation of the isozymes in cases where the change in the relative amount of isozyme present is the effect produced by gene action. Subtle changes such as those reported here could not have been reported with confidence without the use of the microdensitometer.

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SUMMARY

Consistent differences between the liver esterase isozymes of luxoid (*lu/lu*) and normal sibs were found in two-week old and older mice. These changes in the esterases are interpreted as pleiotropic effects of *lu* and indicate expression in the juvenile and adult mouse of a gene which earlier had a marked effect on embryological development.

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