ENZYMATIC STUDIES OF THE HEMOPOIETIC DEFECT IN FLEXED MICE¹

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THE flexed mutation in the mouse causes a flexed tail, belly spotting, and a transitory siderocytic anemia which is most severe from the 13th to the 16th day of gestation and which persists into the first week of neonatal life (GRÜNEBERG 1942a,b; RUSSELL, THOMPSON and MCFARLAND 1968). Thereafter, flexed mice are hematologically normal, but when adults are made anemic, abnormalities of hemopoiesis occur, which may reflect the primary defect usually manifested only in fetal life. Recent studies using the spleen colony technique have shown that hemopoietic cells derived from either fetal liver or adult marrow of flexed mice, when injected into irradiated normal hosts, produce the same number of colonies in the host spleen as do hemopoietic cells derived from these tissues of normal mice (THOMPSON *et al.* 1966). However, colonies from flexed donors are smaller and contain fewer erythroid precursors than are found in normal colonies. In separate experiments, in which larger numbers of hemopoietic cells were transplanted, the period of high iron uptake was delayed in recipients of flexed erythroid cells, as compared with normal (FOWLER *et al.* 1967). Later when erythroid repopulation had been completed, the amount of iron incorporated returned to the same normal levels in mice which had received either flexed or normal implants. This suggests that hemopoietic cells from flexed adults still retain the metabolic defect, although it is manifested only during periods of rapid differentiation and proliferation.

Further evidence for the persistence of a hemopoietic defect into adult life comes from the studies of MARGOLIS and RUSSELL (1965), who observed that adult flexed mice were deficient in δ -aminolevulinate dehydratase (ALD), compared with normal mice from a highly congenic line of the same strain. Also, flexed mice made anemic by phenylhydrazine treatment did not exhibit the normal compensatory splenic increase in enzyme activity 24 hours after the treatment. Since ALD catalyzes the conversion of δ -aminolevulinic acid to porphobilinogen (PBG), a heme precursor, it seemed reasonable to assume that this relative enzyme deficiency, coupled with an inability of ALD to respond adequately in periods of

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hemopoietic crisis, might be the cause of the anemia which occurs in the fetal period when blood volume is increasing at an extremely rapid rate.

Tissue ALD is under the control of a single gene at the levulinate locus in mice (RUSSELL and COLEMAN 1963; COLEMAN 1966). Most strains can be classified as being either Lv^a/Lv^a with high tissue levels, or Lv^b/Lv^b with low tissue levels of enzyme. In 1965, FL/Re-*f/f* mice showed tissue levels of ALD identical to those seen in normal Lv^b/Lv^b strains such as C57BL/6J, while mice of the presumably congenic FL/Re-*+/+* line showed tissue levels identical to those seen in Lv^a/Lv^a strains of mice (MARGOLIS and RUSSELL 1965). These observations suggested that the flexed locus also controls tissue levels of ALD.

The results described in this report show that the flexed locus controls ALD only indirectly, and that the FL/Re-*f/f* line is homozygous for Lv^b while the FL/Re-*+/+* line is homozygous for Lv^a . Further, the levulinate and flexed loci are not linked and the Lv^a allele must have been retained in the congenic normal control line by chance alone. The flexed locus does control the rate of compensatory increases in ALD and uroporphyrinogen synthetase when mice are made anemic by phenylhydrazine treatment, and may act by controlling the rate of proliferation of hemopoietic cells.

MATERIALS AND METHODS

Animals. The principles of laboratory animal care as promulgated by the National Society for Medical Research are observed in this laboratory. All mice used in these studies were obtained from the research stocks maintained at The Jackson Laboratory. Male and female mice of five strains were used: the four highly congenic FL/Re strains which differ both at the flexed and levulinate loci (for actual genotypes and origin see "genetic studies" section in the RESULTS) and the unrelated C57BL/6J strain of mice, homozygous for Lv^b/Lv^b , and *+/+* at the flexed locus. This latter strain was used as a normal and nonflexed control strain for the FL/1 Re-*f/f* mice before the appropriate *+/+* congenic line was developed. When this became available, the results obtained with the C57BL/6J strain were confirmed using the *+/+* congenic line and no differences were found.

Embryonic age of mice used in studies involving fetal liver was determined by detection of vaginal plugs from timed matings.

Phenylhydrazine treatment. Mice were made anemic by three intraperitoneal injections of neutralized phenylhydrazine hydrochloride (COLEMAN 1966; LEVIN and COLEMAN 1967). Hematocrit, red cell, and reticulocyte determinations were made on each animal before the mice were killed to provide tissue for the enzyme studies.

Assays. The erythropoietic activity in the spleens and bone marrows of four mice of each genotype was measured by counting the percentage of nucleated cells which stained for hemoglobin with Lepehne's solution (FOWLER and NASH 1968). ALD and uroporphyrinogen synthetase activities were assayed by previously published methods (COLEMAN 1966; LEVIN and COLEMAN 1967). Incubations were carried out at 37°C for 1/2 hr and the enzyme activities are expressed as μ moles PBG produced per hr per g wet weight of tissue for ALD, and as $m\mu$ moles PBG consumed per hr per g wet weight of tissue for uroporphyrinogen synthetase. In the assay for ALD activity in hemopoietic tissue, some of the product PBG was converted to porphyrins because the homogenates contained other enzymes in the heme biosynthetic pathway. Corrections for this loss were made using the method described by SHEMIN (1962).

RESULTS

Genetic studies of the relation between f and Lv. Earlier data (MARGOLIS and

RUSSELL 1965) showed low levels of δ -aminolevulinic dehydratase in tissues of FL/Re-*f/f* and littermate FL/Re-*f/+* mice, but high activity (approximately 3 times that in flexed) in highly congenic FL/Re-*+/+* mice. These findings were difficult to understand because differences in activity of this enzyme, among hematologically normal mice of several different inbred strains, are known to be controlled by alleles at the levulinate locus. Three alternative hypotheses could be advanced to account for this phenomenon.

1. The flexed mutant might be a dominant allele ($L\nu'$) at the $L\nu$ locus, exerting its full repressive effect in a single dose (implied by MARGOLIS and RUSSELL 1965).

2. The flexed (*f*) locus and the $L\nu$ locus might lie close together in genetic linkage group XIV, so that in spite of the establishment of highly congenic stocks, $L\nu^b$ remained coupled with the *f* allele and $L\nu^a$ with the *+* allele of flexed (RUSSELL and McFARLAND 1966). This explanation does not account for the observed low, rather than intermediate, level of ALD in FL/Re-*f/+* heterozygotes.

3. The *f* and $L\nu$ loci might not be closely linked. The differing $L\nu$ alleles may have become fixed in the two FL/Re lines by chance, rather than through linkage with *f*; they may have been still segregating in the common ancestors of the FL/1Re-*f/f* and FL/2Re-*+/+* lines at the time of their separation (see Figure 1, RUSSELL and McFARLAND 1966). To start the FL/2Re strain, two pairs of FL/Re-*f/+* mice derived from the 11th and 13th generations of backcrosses of *f/+* individuals to mice of the latest brother-sister generation (F_{12} of the incipient FL/1Re-*f/f* strain), were mated to produce FL/Re-*+/+* offspring (genotype established by progeny test). Two proven FL/Re-*+/+* offspring of these matings formed the P_1 of the FL/2Re-*+/+* inbred line, which has been maintained by full-sib matings for 26 generations (JUNE 1968) completely independent of the FL/1Re-*f/f* inbred line (F_{39} , JUNE 1968). Chance presence of the $L\nu^a$ allele in ancestors of the new line (FL/1Re-*f/+* at b.c. 11, b.c. 13, or FL/1Re-*f/f* at F_{12}) and transmission of this allele to the P_1 of FL/2Re-*+/+*, could account for the observed strain difference.

Recent observations have established the validity of the third hypothesis. FL/1Re-*f/+* mice (from late backcross generation backcrosses of *f/+* to the inbred FL/1Re-*f/f* line) show ALD activity (Table 1, lines 2 and 5) indistinguishable from that of FL/Re-*f/f* mice (Table 1, line 4). FL/2Re-*+/+* mice have the high activity characteristic of $L\nu^a/L\nu^a$ (Table 1, line 1). These data confirm the findings of MARGOLIS and RUSSELL (1965). However, ALD activity of FL/Re-*f/+* mice produced in crosses between the two separated inbred lines, FL/1Re-*f/f* and FL/2Re-*+/+*, is intermediate and typical of $L\nu^a/L\nu^b$ individuals (Table 1, line 3). When late-backcross FL/1Re-*f/+* mice were again backcrossed to FL/1Re-*f/f*, the same low, typically $L\nu^b/L\nu^b$, levels of ALD activity were observed in both *f/+* and *f/f* offspring (Table 1, lines 5, 5a). When the outcrossed FL/Re-*f/+* mice were backcrossed to FL/1Re-*f/f*, equal numbers of *f/+* and *f/f* offspring were obtained, and in each group there were similar numbers of offspring with intermediate ($L\nu^a/L\nu^b$) and low ($L\nu^b/L\nu^b$) levels of ALD activity (Table 1, lines 6, 6a). This last population demonstrates not only segregation but also indepen-

TABLE 1

Liver δ -aminolevulinate dehydratase activity in flexed strains of mice

Strain	Genotype at flexed locus	δ -Aminolevulinate dehydratase activity*		
		High (Lv^a/Lv^a)	Intermediate (Lv^a/Lv^b)	Low (Lv^b/Lv^b)
1. FL/2Re	$+/+$	5.46 ± 0.12 (11)		
2. FL/1Re†	$f/+$			1.84 ± 0.04 (31)
3. FL/Re‡	$f/+$		3.45 ± 0.05 (26)	
4. FL/1Re	f/f			1.79 ± 0.03 (29)
5. (Class 2 $\times$ class 4)	$f/+$			1.88 ± 0.06 (19)
5a. " "	f/f			1.90 ± 0.04 (18)
6. (Class 3 $\times$ class 4)	$f/+$		3.18 ± 0.07 (9)	1.99 ± 0.10 (11)
6a. " "	f/f		3.70 ± 0.10 (10)	2.09 ± 0.16 (9)

* Figures represent μ moles of porphobilinogen produced per hr per g wet weight of liver $\pm$ S.E.M. Number of mice in parenthesis.

† Offspring of FL/1Re- $f/+$ $\times$ FL/1Re- f/f matings, from backcrosses within FL/1Re.

‡ Offspring of FL/Re- $+/+$ $\times$ FL/Re- f/f matings between FL/1 Re- f/f and FL/2 Re- $+/+$.

dent assortment of alleles at the f and Lv loci. Conclusive evidence of independent assortment of Lv and f comes from their established linkage relationships. The f locus is known to be in linkage group XIV. Recent tests show close linkage between the Lv and b loci in linkage group VIII (HUTTON and COLEMAN, unpublished).

Having established that f and Lv segregate independently, we set out to establish two new FL/Re lines. FL/3Re- f/f Lv^a/Lv^a (now at F_7 in JUNE 1968) was started by selecting f/f Lv^a/Lv^a individuals from the F_2 of a cross between FL/1Re- f/f Lv^b/Lv^b and FL/2Re- $+/+$ L^a/Lv^a . FL/4Re- $+/+$ Lv^b/Lv^b (at F_6 in JUNE 1968) was started by selecting $+/+$ Lv^b/Lv^b individuals from offspring of a cross between two FL/1Re- $f/+$ Lv^b/Lv^b mice from b.c. 30 within FL/1Re.

The typical enzyme activity values from liver and spleen of mice both homozygous and heterozygous for the flexed locus are seen in Table 2. Those strains

TABLE 2

Liver and spleen ALD activity of the highly congenic FL/Re strains which differ at the levulinate and flexed loci

Strain	Genotype	δ -Aminolevulinate dehydratase activity	
		Liver	Spleen
FL/2Re	Lv^a/Lv^a $+/+$	5.46 ± 0.12 (10)*	1.34 ± 0.28 (12)
(FL/2Re $\times$ FL/3Re) F_1	Lv^a/Lv^a $f/+$	5.12 ± 0.06 (10)	1.40 ± 0.04 (10)
FL/3Re	Lv^a/Lv^a f/f	4.87 ± 0.35 (6)	1.35 ± 0.07 (6)
FL/4Re	Lv^b/Lv^b $+/+$	1.70 ± 0.06 (8)	0.48 ± 0.06 (8)
(FL/1Re $\times$ FL/4Re) F_1	Lv^b/Lv^b $f/+$	1.88 ± 0.06 (19)	0.49 ± 0.17 (5)
FL/1Re	Lv^b/Lv^b f/f	1.71 ± 0.03 (20)	0.38 ± 0.02 (22)
C57BL/6J	Lv^b/Lv^b $+/+$	1.98 ± 0.09 (22)	0.43 ± 0.07 (12)

* Figures represent μ moles of porphobilinogen formed per hr per g wet weight of tissue $\pm$ standard error of the mean. The numbers in parenthesis represent the number of individual mice assayed.

homozygous for the Lv^a allele (FL/2Re-+/+, FL/3Re-f/f and crosses between them) had ALD activities in the order of 5.5 units/g in liver and 1.3 units/g in spleen while those strains homozygous for the Lv^b allele (FL/4Re-+/+, FL/1Re-f/f and crosses between them) had enzyme activities of 1.8 units/g in liver and 0.4 units/g in spleen. No effect of the flexed locus on ALD activity is apparent and the values obtained correspond to that predicted by the alleles at the Lv locus (RUSSELL and COLEMAN 1963).

Phenylhydrazine studies. Previous observations (MARGOLIS and RUSSELL 1965) suggested that flexed mice were unable to increase the level of splenic ALD in order to compensate for the severe anemic state induced by phenylhydrazine injection. The following studies were undertaken in order to fully evaluate the entire recovery period with respect to the nature and extent of the differences between flexed and normal mice. We first studied the change in peripheral blood and the rate of red blood cell formation in both genotypes after phenylhydrazine treatment. A marked anemia was seen on the first day following cessation of the treatment. Qualitatively the degree of anemia was the same in all genotypes studied (hematocrit 17 to 23% with total red blood cell count of 2.5 to 3.5 million). In all normal strains of mice, including those heterozygous for the flexed gene, the percentage of reticulocytes increased rapidly, reaching a maximum (61 to 83%) on the fourth day after phenylhydrazine treatment (Figure 1). This was followed by a drop in the reticulocyte count until a normal blood picture was seen by the 9th day post-phenylhydrazine. In contrast, flexed mice exhibited a delay in the production of reticulocytes, reaching a maximum on day 6 or later. A completely normal blood profile was not observed until day 10 or 11 after phenylhydrazine treatment. Hypertrophy of the spleen closely paralleled the rise in the reticulocyte count in mice of both flexed and normal genotypes: maximum spleen weight was observed on day 4 in normal mice and on day 6 in mice of flexed genotypes (Figure 1).

Spleen smears taken during recovery from phenylhydrazine treatment revealed a high degree of hemopoietic activity in this organ in all strains. However, the percentage of erythropoietic cells in the spleen, like the percentage of reticulocytes among the circulating red cells, rose more slowly and remained high longer in flexed mice. In normal mice the percentage of nucleated spleen cells which stained for hemoglobin rose from 2% prior to phenylhydrazine treatment, to 30 and 65% on the third and fourth day, respectively, and then rapidly dropped to a nearly normal level by day 7 after cessation of treatment. On the other hand, in flexed mice, only 5% of the nucleated spleen cells contained hemoglobin by the third day and 11% by the fourth day. The peak response (55%) was not observed until day 5 and the levels remained at 46% until day 7 post-treatment.

The response of the bone marrow to the hemolytic stress was much less striking than that observed in spleen. The percentage erythropoietic cells rose from 12% prior to treatment to only 20% by the fourth day after treatment in normal mice and to 29% on the fifth day after treatment in flexed mice. These observations are consistent with previous studies indicating that the spleen is the principal organ of erythropoietic homeostasis in the mouse (FRUHMANN 1968).

FIGURE 1.—Changes in spleen weights and reticulocyte counts in flexed and normal mice recovering from phenylhydrazine-induced anemia. Each point represents the average value obtained from at least 10 mice. No effect of the alleles at the *Lv* locus was observed and the data from the $+/+$ mice homozygous for either Lv^a or Lv^b were pooled as were the data from flexed mice of either genotype.

Enzyme studies. Having established both the importance of the spleen in the compensatory hemopoiesis which follows phenylhydrazine treatment and the involvement of the flexed locus in delaying the rate at which the spleen can participate, it was of interest to examine the enzymatic changes in spleen which occurred during the recovery period. The endogenous activity of δ -aminolevulinate dehydratase is controlled in liver, spleen, and kidney by the levulinate locus (COLEMAN 1966). The normal level observed for mice homozygous for Lv^b is 0.39 units per g wet weight of spleen, while that for mice homozygous for Lv^a is 1.35 units per g wet weight of spleen (Table 2 and Figure 2). In normal mice this activity is doubled by the first day after phenylhydrazine treatment, while in flexed mice no change is observed until day 2 or 3 after the induction of the anemia (Figure 2a and 2b). These results are in agreement with earlier studies, which were terminated after day 1 (MARGOLIS and RUSSELL 1965). If studies are extended throughout the entire anemic period, it is seen that peak levels of splenic ALD are achieved in the f/f spleens about 3 days after the peak occurs in the $+/+$ spleens; and further, this delay is common to $Lv^a/Lv^a f/f$ (Figure 2a) and $Lv^b/Lv^b f/f$ (Figure 2b) mice. Thus the slower appearance of maximum ALD activity is directly dependent upon the presence of the flexed mutation, and independent of the action of the *Lv* alleles.

On the other hand, the flexed locus seems to have no effect upon the magnitude of the enzyme peaks achieved, since the peak values in $Lv^a/Lv^a f/f$ (6.6 units/g) and $Lv^a/Lv^a +/+$ (6.4 units/g) mice are approximately equal, as are those in

FIGURE 2.—Effect of phenylhydrazine treatment on δ -aminolevulinate dehydratase activity in spleen from flexed and normal mice homozygous for the Lv^a -gene (2A) and homozygous for the Lv^b -gene (2B). Units of activity are μ moles of porphobilinogen produced per hour per g of spleen. Each point represents the average value obtained from 4 to 8 separate assays performed on individual spleens.

Lv^b/Lv^b f/f (1.8 units/g) and Lv^b/Lv^b +/+ (1.9 units/g). However, the peaks in mice of both Lv^a/Lv^a genotypes are 3 to 4 times greater than those in mice of the corresponding Lv^b/Lv^b genotypes. This indicates that although the flexed gene controls the delay in reaching peak enzyme activity, it is the Lv allele which controls the magnitude of this response.

In both genotypes, the pattern of increase in ALD activity closely parallels the increase in spleen weight, percentages of erythropoietic cells in spleen, and reticulocyte count. This increase in enzyme activity is not due to increase in the size of the spleen, since activity levels are expressed in units per g of tissue, and must be multiplied by the organ weight to get total activity. Thus a 4-fold increase in

FIGURE 3.—Effect of phenylhydrazine treatment on uroporphyrinogen synthetase activity in spleens from flexed and normal mice homozygous for the Lv^b -allele. Each point represents the average value obtained from 4 to 6 separate assays. Each assay was run on the combined homogenates of spleen from 2 or 3 treated mice.

activity of the ALD per gram of spleen, coupled with a 6-fold increase in spleen size represents a 24-fold increase in enzyme activity per spleen. This is consistent with the observation that the spleen cells actively producing hemoglobin multiply 30 times as rapidly as do other cell types, increasing from 2% to 60% of the total after phenylhydrazine treatment.

These data further suggest that the flexed locus does not have a role in controlling ALD activity directly, but instead may control the differentiation or proliferation of the hemopoietic cells which contain this enzyme. On the basis of this hypothesis, other enzymes in the heme-biosynthetic pathway should be altered in spleens of stressed flexed mice. Therefore, studies were initiated on the next enzyme in the pathway, uroporphyrinogen synthetase. Spleens from normal non-anemic mice do not have detectable amounts of this enzyme under the assay conditions, but by day 3 or 4 after phenylhydrazine treatment some synthetase is present. The changes in synthetase in flexed and normal mice homozygous for Lv^b are summarized in Figure 3. Again a 2- to 3-day delay in response is observed in flexed mice, with maximal activity on day 4 in normal mice but not until day 7 in flexed mice.

Fetal liver studies. Although adult flexed mice show no hematological abnormalities unless stressed by an agent such as phenylhydrazine, the most conspicuous defect caused by the flexed gene is siderocytic anemia, which occurs only during fetal life, being especially pronounced between the 13th and 16th days of gestation (RUSSELL *et al.* 1968), when very active hemopoiesis occurs in the liver, rather than in bone marrow or spleen. Studies of ALD activity in livers from 13- to 17-day fetuses were undertaken in the hope of shedding light on the nature of the flexed hemopoietic defect as manifested in fetal erythropoiesis (Table 3). Again ALD activity in fetal liver is seen to be controlled by the Lv

TABLE 3

Activity of δ -aminolevulinatase dehydratase and uroporphyrinogen synthetase in normal and flexed fetal liver

Fetal age	δ -Aminolevulinatase dehydratase activity			Uroporphyrinogen synthetase activity	
	Lv^a/Lv^a +/+	Lv^b/Lv^b +/+	Lv^b/Lv^b f/f	Lv^b/Lv^b +/+	Lv^b/Lv^b f/f
Days	μ moles PBG produced/hr/g of liver			m μ moles PBG utilized/hr/g of liver	
13	4.80* (1)	1.67 $\pm$ 0.03 (3)	1.76 $\pm$ 0.19 (3)	145 $\pm$ 2.9 (4)	95 $\pm$ 16.9† (4)
14	6.43 $\pm$ 0.15 (4)	2.05 $\pm$ 0.07 (5)	1.93 $\pm$ 0.13 (4)	157 $\pm$ 4.2 (8)	140 $\pm$ 6.3 (6)
15	6.15 $\pm$ 0.17 (4)	1.99 $\pm$ 0.07 (6)	1.90 $\pm$ 0.07 (5)	180 $\pm$ 9.2 (5)	161 $\pm$ 4.3 (3)
16	5.60 $\pm$ 0.17 (5)	2.16 $\pm$ 0.09 (5)	1.90 $\pm$ 0.09 (4)		
17	5.40 $\pm$ 0.18 (8)	1.77 $\pm$ 0.07 (6)	1.85 $\pm$ 0.08 (5)		

* Figures represent units of enzyme activity $\pm$ standard error of the mean. The numbers in parenthesis refer to the number of determinations made on each genotype.

† This value for synthetase activity in flexed fetuses is significantly lower than the normal value ($P < 0.05$).

locus, in agreement with previous observations (RUSSELL and COLEMAN 1963). Fetal liver from normal mice homozygous for the Lv^a allele had ALD activities about 3 times those seen for normal mice homozygous for Lv^b at each age studied. Peak enzyme activity occurred on days 14 and 15 and coincided with the probable period of maximum heme biosynthesis. Superimposition of the flexed gene on mice homozygous for Lv^b had no effect on the developmental profile.

Since ALD is a normal constituent of adult liver, which does not participate in hemopoiesis, it was felt that the endogenous non-hemopoietic level of this enzyme in fetal liver might be sufficient to obscure possible changes in ALD activity. Therefore, studies were extended to uroporphyrinogen synthetase, an enzyme which is not found in significant quantities in adult liver, and is more specific to hemopoietic tissue. The developmental pattern of this enzyme from 13 to 15 days is also shown in Table 3. No deficiency of enzyme was seen in days 14 and 15 in flexed mice but a moderate deficiency was found on day 13. This suggests that the developmental pattern of hemopoietic cells in livers from flexed fetuses may be delayed. Confirmatory studies were not feasible at 12 days because of the unavailability of sufficient amounts of fetal liver from flexed embryos.

DISCUSSION

Enzyme studies in spleens made hemopoietic by phenylhydrazine treatment confirmed the persistence of a hemopoietic defect in adult flexed mice. This defect only becomes apparent under conditions demanding rapid differentiation and proliferation of erythropoietic tissue, a conclusion in agreement with that of THOMPSON *et al.* (1966) and FOWLER *et al.* (1967). These investigators analyzed growth of flexed *vs.* normal hemopoietic cells implanted in lethally irradiated recipient mice, a situation which demands rapid differentiation and proliferation of erythroid tissue. Both studies compared behavior of FL/Re-f/f Lv^b/Lv^b and FL/Re-+/+ Lv^a/Lv^a cells, but it now seems probable that all of the differences

they observed may safely be attributed to flexed gene action. Although the same number of colonies developed from flexed as from normal implanted cells, those from *f/f* cells "were found to be small in size and to possess a decreased ability to incorporate radio-iron into haem" (THOMPSON *et al.* 1966). Peak Fe^{59} incorporation into heme was delayed following implant of *f/f* as compared with *+/+* cells. FOWLER *et al.* (1967) demonstrated by radioautography that these defects in *f/f* iron incorporation were caused by delay in proliferation of erythroid cells. They also showed the flexed hemopoietic defect was specific for erythropoiesis, since proliferation of myeloid cells was normal.

In contrast to the results obtained by MARGOLIS and RUSSELL (1965), in our study no direct effect of the flexed gene was observed on the resting level of ALD in liver, kidney, or spleen. Their results can be explained by genetic studies which established that the highly congenic FL/2Re-*+/+* strain used as a normal control in their studies was homozygous for the *Lv^a* allele while the flexed strain was homozygous for the *Lv^b* allele at the levulinate locus. Their failure to observe a compensatory increase in ALD activity 24 hr after phenylhydrazine treatment was caused by the delay in response of this enzyme in spleens from flexed mice. A similar delay occurs in the rise of splenic uroporphyrinogen synthetase. These parallel changes in two enzymes involved in heme biosynthesis confirm the lag in proliferation of heme-synthesizing cells which was suggested by the actual cell counts in LEPEHNE's stained preparations.

It was hoped that by studying the developmental profile of hemopoietic enzymes in fetal liver a similar delay in the development of maximal specific activity would be observed. This was not the case with ALD activity, and with uroporphyrinogen synthetase, only a marginal deficiency in 13-day old fetal liver was observed. However, the observed normal enzyme complement of fetal liver from flexed mice was based on a gram wet weight of tissue. RUSSELL *et al.* (1968) have shown that flexed mice at all ages are smaller than normal and our experience indicates that the liver-weight of flexed fetuses is only 50 to 60% that of normal fetuses. Thus, the actual enzyme activity per liver is decreased in flexed mice even though the amount per gram is normal. This decrease in the relative size of the fetal liver, which is composed mainly of hemopoietic cells, is in keeping with the hypothesis that the flexed locus in some fashion regulates the rate of proliferation of erythropoietic cells.

RUSSELL *et al.* (1968) also demonstrated anemia and decreased weight of flexed fetuses when compared with normals on the 12th day of gestation, indicating that alleles at the flexed locus affect hemopoiesis and growth rate prior to the development of the liver as the major hemopoietic site. This suggests that the flexed locus may influence differentiation or proliferation of tissues other than hemopoietic tissue. The reduced body weight observed in flexed mice during post-natal growth is compatible with this hypothesis (RUSSELL and McFARLAND 1966). Another pleiotropic effect of *f/f* gene substitution is ventral white-spotting, involving absence of melanocytes from many hair follicles and probably representing reduced migration of melanoblasts. Flexed mice also usually show tail flexure through fusion of vertebrae due to anomalies of intervertebral disks. Al-

though both of these pleiotropic effects may be secondary to flexed fetal anemia, they may equally well represent independent action of the flexed genes in other tissues (RUSSELL and McFARLAND 1966).

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SUMMARY

The activity of δ -aminolevulinate dehydratase (ALD) has been investigated in liver and spleens from flexed (*f/f*) and normal strains of mice known to be homozygous for either the *Lv^a* or *Lv^b* allele at the levulinate locus. The level of this enzyme was controlled solely by the levulinate locus, and no effect of the flexed locus was observed. Similarly, in tissues actively undergoing hemopoiesis, such as fetal liver or spleens of mice made anemic by phenylhydrazine, both the basal level and the maximal rise of enzyme activity was dictated by the levulinate locus. However, a delay in the normal compensatory increase in ALD activity was observed in spleens from homozygous flexed mice after phenylhydrazine treatment when compared with normal mice. The parallel delays in the rate of spleen hypertrophy in the increase of nucleated cells staining for hemoglobin, and in the development of a second enzyme in the heme biosynthetic pathway, uroporphyrinogen synthetase, suggest that the flexed locus affects hemopoiesis by controlling the rate of proliferation of hemopoietic cells. This delay was not observed in the development of the hemopoietic capacity of fetal liver when based on a gram wet weight of tissue, but the smaller size of the livers from flexed fetuses when compared with normal fetuses indicates that a deficiency in blood-forming capacity exists throughout the period when liver is the major hemopoietic site.

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