POLYGENIC CONTROL OF THE TERATOGENICITY OF 5-FLUOROURACIL IN MICE[1]

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DROSOPHILA, chickens and mammals have frequently shown interstrain differences in the reactions of their embryos to teratogenic agents. In those cases in which such differences have been investigated, the data have been interpreted as demonstrating a polygenic basis for inheritance, but so far as we are aware, no attempts have been made to estimate the number of genes nor the contributions by individual genes.

In Drosophila, interstrain differences in developmental responses to teratogens have been reported for heat shock (GOLDSCHMIDT 1935), sodium metaborate (SANG and MCDONALD 1954) and sodium tetraborate (GOLDSCHMIDT and PITERNICK 1956, 1957a, b). GOLDSCHMIDT and PITERNICK (1957b) made crosses between Drosophila stocks of different reactivity and discovered that many, but not all, of the specific responses of both parents appeared in the hybrids. Although the experiments were not quantitative, it was concluded that some of the typical reactions of individual lines were dominant. In some of the noninbred Drosophila stocks, selection for frequency of response to sodium teraborate was possible, and the investigators suggested that a multifactorial modifier system plus a simple, perhaps monofactorial, main difference might have been the genetic bases for the selection (GOLDSCHMIDT and PITERNICK 1956, 1957a). Also, selection has been successful within noninbred Drosophila stocks for lines sensitive and resistant to the development of a break in the posterior crossvein induced by heat shock (WADDINGTON 1953) and for lines with high and low frequencies of a bithorax-like phenocopy produced by ether vapor (WADDINGTON 1956). In both reports, WADDINGTON concluded that many loci were involved.

LANDAUER (1948, 1957, 1958, 1960) has reviewed his extensive investigations on the effects of teratogenic agents on chicken embryos. Differences were found between strains, or between stocks within a strain, in the reactions of their embryos to insulin, boric acid, ethyl carbamate, thallium, and nicotine sulfate and physostigmine sulfate. By selection, lines were isolated with either high or low frequencies of certain abnormalities induced by insulin. The genetic basis for the inter- and intrastrain differences and for the differential responses after selection was concluded to be multifactorial.

In mammals, interstrain differences in frequencies of developmental abnormalities have been shown for a variety of teratogenic conditions. Unfortunately,

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in most instances in which differences between strains of rats have been reported, the strains had not been tested in the same laboratory. One exception to this situation is found in the investigation of embryonal effects of hypovitaminosis-A (ANDERSEN 1949). Much of the information on rats has been summarized (KALTER and WARKANY 1959). The extensive literature on strain differences among mice will not be presented here except to cite a few examples of the diverse conditions which have produced different percentages of abnormalities in various inbred strains: hypoxia (INGALLS *et al.* 1953), fasting (MILLER 1962), cortisone (FRASER and FAINSTAT 1951), tolbutamide (SMITHBERG and RUNNER 1963), vitamin A excess (WALKER and CRAIN 1960), galactoflavin (KALTER and WARKANY 1957) and 6-aminonicotinamide (GOLDSTEIN *et al.* 1963). KALTER (1965) has summarized his experiments on the inheritance of susceptibility to cortisone-induced cleft palate in several strains of mice. He demonstrated that both the maternal and fetal genotypes contributed to the susceptibility, and concluded that the teratogenic action of cortisone was a quantitative character influenced by the cumulative action of many genes.

DAGG (1963) found that the incidence of cleft palate and defects of the limbs and tail caused by 5-fluorouracil varied widely between inbred mouse strains. Crosses were made between two of these strains, and on the basis of certain assumptions, it was concluded that at least two genes were involved in determining reactions in the palate and hind feet.

The purposes of the investigations reported here were to make new estimates of the number of genes involved in the strain differentials in teratogenicity of fluorouracil, using a new experimental design and different statistical methods, and to determine whether the set of genes influencing cleft palate production were identical to those producing malformations of the hind feet.

MATERIALS AND METHODS

Breeding and treatment: Two inbred strains of mice were used: 129/Dg and BALB/cDg. In a previous study of embryonal responses to fluorouracil in these strains (DAGG 1963), the F_1 embryos closely resembled homozygous strain 129 embryos in incidence of malformations, and therefore, in the investigations reported here, all backcrosses were made to the BALB strain. In order to keep the maternal effects constant in each backcross generation, matings were made to BALB females, exclusively. Furthermore, in order to determine whether segregation of genetic factors occurred in BC_1 and BC_2 males, each male was mated several times and was given a score based on responses of his offspring.

The mice were mated in a constant temperature room with a reversed lighting schedule such that the dark period extended from 9 A.M. to 4 P.M. Females were kept in this room at least 2 weeks prior to mating to allow reversal of their diurnal cycle. Females showing vaginal signs of estrus were placed singly with males at 8:30 to 9:00 A.M. and were removed and examined for vaginal plugs at 4:00 to 4:30 P.M. Ten days later (240 hours after discovery of the vaginal plug), the females were given an intraperitoneal injection of 0.25 ml of a water solution of 5-fluorouracil. Pregnant females remained in the reversed lighting room until the 18th day of gestation and then were killed. The number of dead embryos and resorbing implantations were counted, and live fetuses were examined for gross external malformations.

All females were nulliparous and were 13 to 25 weeks of age at the time of mating. The males and females were fed from weaning and during the experiments on a diet manufactured by the Emory Morse Co. for The Jackson Laboratory. Food and water were available *ad lib.*

Statistical methods: Two assumptions underlying many statistical tests are (1) a normal distribution of data and (2) independence of the mean and variance. Threshold response data, such as the proportion of offspring exhibiting a malformation, tend to violate both of these assumptions since the data are distributed binomially. The mean (np) and variance ($np(1\text{-}p)$) of a binomial variable are both related to p, the probability of an affected individual in a sample of n individuals, and the distribution will become skewed as p deviates from one half. Since the extraction of genetic information from the results of this investigation involves variances, and since skewness may result in an erroneous estimation of genetic variance, the data were transformed in an attempt to avoid these difficulties. Of the alternative choices, we selected the angular sine or "arcsin" transformation because it gave smaller distortions from the observed average percentages when the arcsin scores were converted to percentages after the calculations were completed.

Arcsin scores, representing the average proportions of malformed hind feet and average proportions of cleft palates among the offspring of each male, were calculated in two ways. One score, hereinafter called the corrected unweighted arcsin, was obtained by converting the proportion of affected fetuses to total live fetuses in each litter to arcsin degrees. BARTLETT's (1947) suggestion of substituting $1/(4k_i)$ for 0% and $100 - [1/(4k_i)]$ for 100%, where k_i is the number of fetuses in the ith litter, was applied to these extreme values before the transformations were made. This substitution mitigates the contributions of very small litters showing extreme responses. Another score, hereinafter called the corrected and weighted arcsin, was calculated by multiplying the corrected unweighted arcsin score by the number of fetuses in the litter, thereby equalizing the contribution of each offspring to the final score.

Using the corrected and weighted arcsins, the mean and variance for each male was therefore calculated as:

$$\text{mean} = \Sigma\,(k_i\,x_i)/\Sigma\,k_i$$

$$\text{variance} = \frac{\Sigma\,k_i\,x_i^2 - (\Sigma\,k_i\,x_i)^2/\Sigma\,k_i}{\Sigma\,k_i - 1}$$

where k_i is the number of fetuses in the ith litter and x_i is the proportion of affected fetuses expressed as the arcsin, employing BARTLETT's substitution where appropriate.

Means and variances of the crosses and backcross generations were based on the average corrected and weighted arcsin scores of all the males that were mated to produce the generation, and no further weighting was applied since these averages were each based on a large number of fetuses.

The number of genetic factors affecting a trait can be estimated from the means and variances of the inbred lines, their F_1, and a backcross generation. WRIGHT (1934) derived the formula

$$n = \Delta^2/[16(V_{BC} - V_p)]$$

where n is the minimum number of loci, Δ is the difference between the means of two inbred lines, V_{BC} is the variance in the backcross generation and V_p is the variance in either parental line. We replaced V_p by an average variance of the two parental lines and their F_1, accomplishing the same function as the V_p in the original formula, i.e., an estimate of the environmental variance. Estimation of n assumes that the effects of loci are additive and that there is no linkage. Violations of these assumptions will give an underestimation of the number of genes affecting the trait (WRIGHT 1952).

The degree of genetic determination of a trait can be estimated from the variances of segregating and nonsegregating populations. The phenotypic variance of the inbred lines and their F_1 will be due almost entirely to environmental variance because each of these three populations are genetically homogeneous. The variance in the backcross generation will have both environmental and genetic components, with the latter estimated by doubling the difference between the environmental variance (of the parental lines and their F_1) and the variance of the backcross generation. The ratio of genetic variance to total phenotypic variance in the backcross generation will then yield an estimate of the relative importance of genetic variation in determining the variation of the trait.

TABLE 1

Example of calculations, using F_1 sire 110

Female No.	Number MHF	Number fetuses (k)	Percent	Arcsin	Weighted arcsin	Weighted (arcsin)^2
1	1	3	33.3	35.26	105.8	3729.8
2	7	8	87.5	69.30	554.4	38419.9
3	0	4	6.3*	14.48	57.9	838.7
4	0	7	3.6†	10.89	76.2	830.1
5	5	6	83.3	65.90	395.4	26056.9
6	10	10	97.5†	80.90	809.0	65448.1
7	0	10	2.5*	9.10	91.0	828.1
8	3	4	75.0	60.00	240.0	14400.0
9	6	11	54.4	47.60	523.6	24923.4
10	5	9	55.6	48.19	433.7	20900.5
Sum	37	72	..	....	3287.0	196375.5

Percent $= 37/72 = 51.4$

Mean arcsin $= 32870/72 = 45.65$ (or 51.1%)

$$\text{Variance} = \frac{196375.5 - (3287.0)^2/72}{71} = 652.32$$

Standard error $= 3.01$

* $1/(4k)$.
† $100\% - 1/(4k)$.

As an example, the calculations for an F_1 male (No. 110) were carried out as in Table 1. The incidence of malformed hind feet in each of his ten litters are shown in columns 2 and 3. Where the incidence was neither 0 nor 100%, the percent was used directly to calculate the arcsin score for the litter. These values can be found in appropriate conversion tables (e.g., FISHER and YATES 1963) or programmed for the computer using an approximation (HASTINGS 1955, p. 159ff.). Where the incidence was zero percent the value $1/(4k)$ was substituted and the arcsin calculated for the substituted value (as in litters 3, 4 and 7). The substitution for 100% incidence is made by subtracting $1/(4k)$, as was done in litter 6. The weighted arcsin column is obtained by multiplying the arcsin values by the number of fetuses in a litter. The average arcsin for these ten litters was 45.65 or 51.1%, very near the actual overall percent of 51.4. The primary advantage of this method is that standard errors can be calculated and individual male scores statistically compared. The scores of the F_1 males were 45.65, 50.86, 43.77, 56.68, 51.66, 59.51, 47.23, and 47.50. These eight scores were then averaged without further weighting to obtain the mean of 50.35 ± 1.93 and variance of 29.88 used in succeeding calculations.

These means and variances were then used to obtain estimates of the number of loci and the degree of genetic determination. The environmental variance V_E was calculated by averaging the variances of the sires of the two inbred strains and their F_1. For example, the variances for the incidence of malformed hind feet were 12.58 (129/Dg), 8.40 (BALB/c Dg) and 29.88 (F_1) for an average of 16.95 as shown in Table 6. This value replaces V_P in WRIGHT's (1934) formula. This 16.95 is subtracted from the variance of the backcross generation (50.44) and the difference of 33.49 is one half the genetic variance, V_G. The denominator of WRIGHT's equation is obtained by multiplying the 50.44 by 16. The numerator is the difference between the means of the two inbred lines (50.4) squared. The estimate of the minimum number of loci is then 2540.16/535.84 or 4.7 loci. The estimation of the degree of genetic determination of the trait uses these same values of V_E and V_G as $V_G/(V_G + V_E)$ or $66.98/(66.98 + 16.95) = 80\%$.

RESULTS AND DISCUSSION

Photographs of typical malformations of the hind feet and palate produced in these experiments with fluorouracil are shown in Figure. 1.

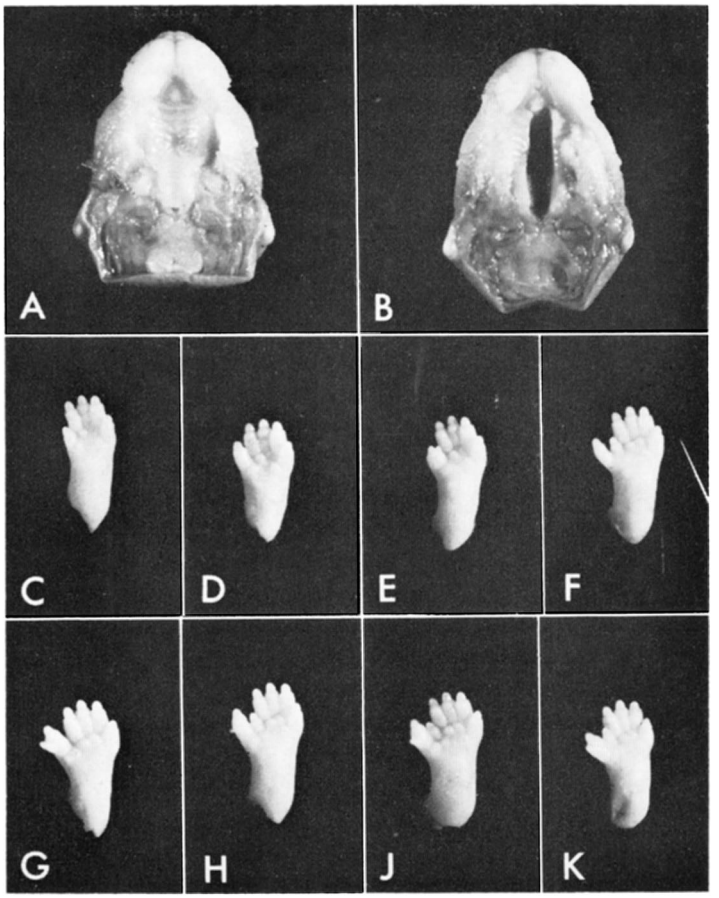

FIGURE 1.—Cleft palate and polydactylous hind feet in 18-day-old fetuses from BALB/c females mated to backcross males and treated with 500 μg of 5-fluorouracil at 10 days after mating. All specimens were taken from treated litters. A: normal palate. B: cleft palate. C-K: left hind feet. C: normal foot. D: triphalangous hallux. E-K: varying degrees of polydactylism.

In previous investigations of interstrain differences in response to fluorouracil treatment at 10 days after mating (DAGG 1963), the frequencies of polydactylous hind feet and cleft palate were much higher in strain 129 than in strain BALB, and the F_1 fetuses from the reciprocal crosses were indistinguishable from homozygous strain 129 fetuses. The conclusion was drawn that factors promoting high frequencies of abnormalities appeared to be dominant to those for low frequencies under those experimental conditions. For making the comparisons, data were available only for responses to a single dose, 20 mg/kg, approximately 600 μg/mouse. In the experiments reported here, 500 μg/mouse was selected as the test dose, because preliminary trials indicated that it might cause fewer embryonal deaths and still produce large numbers of deformities in strain 129. In addition, even lower doses of fluorouracil were used to determine whether F_1 embryos always responded like homozygous strain 129 embryos.

Fluorouracil at 375 and 500 μg/mouse caused a lower proportion of embryonal deaths in the (BALB × 129)F_1 embryos than in 129 × 129 embryos (Table 2). The frequencies of polydactylous hind feet were not different at 375 μg/mouse, but the response was higher in F_1 than in homozygous strain 129 embryos at 500 μg/mouse. A different relationship was found for cleft palate. At 375 μg/mouse, the higher incidence of cleft palates was found in the homozygous embryos, whereas at 500 μg/mouse the difference was not as pronounced. It is apparent that simple statements regarding dominance or recessiveness cannot be made from these data alone. However, by making certain assumptions concerning differences in relative degrees of developmental homogeneity and differences in average developmental stages of the hybrids and inbreds, the data can be interpreted as showing that dominance, if it existed, was not complete but only partial.

At 375 μg, 50% of the inbred and hybrid embryos had malformed hind feet. At the highest dose, 500 μg, a greater proportion of hybrid than inbred embryos had malformed feet indicating that the dose-response curve was steeper for the hybrids, which in turn may have been a reflection of their greater degree of developmental homogeneity. For cleft palate, strain 129 responded with higher frequencies of affected embryos at all doses, but again, the dose-response curve was steeper for the hybrids. Thus, for both malformations the hybrids appeared to be more homogeneous, but whereas the average inbred and hybrid embryos were alike so far as the dose producing malformed hind feet, i.e., the ED_{50}'s were identical, the inbred embryos had greater frequencies of cleft palate at all doses. This dissimilarity of response for the hind feet and palate may have been due to differences in average developmental ages of the two types of embryos. In the previous study (DAGG 1963), F_1 embryos appeared more advanced, developmentally, than homozygous strain 129 embryos. Furthermore, although the frequencies of fluorouracil-induced polydactyly and cleft palate varied with developmental age, the responses of the feet and palate did not vary in a parallel fashion with time. For example, in strain 129 embryos, the incidence of cleft palate remained unaltered while the incidence of malformed hind feet increased as the time of treatment was changed from 10 to 10½ days after mating. If, as is likely, the same relationships hold for the F_1 embryos, then the differences in incidence of cleft palates in the hybrid and inbred embryos would not be affected by the difference

TABLE 2

Teratogenicity and lethality of 5-fluorouracil in mouse embryos

Parental strains		Fluorouracil	Dead conceptuses	Live fetuses	Malformed hind feet	Cleft palate
Females	Males	μg	percent	No.	percent	percent
129/Dg	129/Dg	250	35	92	10	3
129/Dg	129/Dg	375	34	101	50	71
129/Dg	129/Dg	500	49	92	64	91
BALB/c	129/Dg	375	4	190	51	2
BALB/c	129/Dg	500	13	550	83	84

Pregnant females received a single intraperitoneal injection of 5-fluorouracil at 10 days after mating. The fetuses were examined at 18 days.

in developmental ages, and therefore the palates in the hybrid embryos would be, as the data show, less responsive to fluorouracil than the palates of homozygous strain 129 embryos. For the hind feet, however, the frequency of malformations would be increased in the hybrids owing to their more advanced stage of development and, therefore, the hind feet in the hybrids would be expected to be less responsive to fluorouracil than the hind feet of homozygous strain 129 embryos if equivalent developmental stages were treated. If these arguments are correct, the data shown in Table 2 are not inconsistent with assumptions employed in calculating the minimum number of genes involved in the differences between strains 129 and BALB in response to fluorouracil, i.e., the loci were additive and equal and dominance was absent.

The frequencies of cleft palate and malformed hind feet in fetuses from BALB females mated to strain 129, F_1, BC_1, or BALB males and treated with 500 μg of fluorouracil are shown in Table 3. These figures were obtained by pooling all the results for every male in each class. Although genes governing responses to fluorouracil would begin to segregate among the BC_1 sires, the results from these males were pooled because it was believed that in the 18 BC_1 males the distribution of genes pertinent to this study would be representative of the distribution in a large unselected population. The incidence of malformed hind feet dropped steadily with each backcross toward the level seen in homozygous BALB fetuses, but the frequency of cleft palate dropped precipitously in the offspring of F_1 sires and remained relatively unchanged in the next generation. One noteworthy feature is the wide range of values for individual males. A large variation was expected for BC_1 males, where the offspring of one male had only 9% cleft palate while 62% of the offspring of another were affected. In contrast, the variations found among the strain 129 and F_1 males were comparatively small.

When polydactyly developed on only one hind foot, it was present more often on the left side in the offspring of F_1, BC_1, BC_2, and BALB males. In 561 cases of unilateral polydactyly, the left hind foot accounted for 60.6% of the total. For the offspring of 129 $\times$ 129 and BALB $\times$ 129 the figures were reversed, with unilateral left-sided polydactyly in 33/80 (41.2%). No conclusions are drawn regarding possible strain influences on laterality of this defect.

Data for offspring of BC_2 males are not shown in Table 3 because these males were sired by a small group of BC_1 males selected on the basis of frequencies of abnormalities in their fluorouracil-treated litters.

In Table 4 the incidence of dead conceptuses, malformed hind feet, and cleft palate are presented as corrected, unweighted arcsin scores. These scores, when transformed to percentages for comparison with the figures in Table 3, show that the arcsin transformation does not greatly distort the data in samples of this size. The average body weight of females mated to F_1 males were significantly different from those of females mated to BC_1 and BALB sires. This difference had very little effect on the estimation of the number of genes involved in responses of the hind feet to the teratogen, but it did have an effect on the average values for cleft palate. These points will be discussed in more detail later. There were no great differences in the average number of conceptuses per female in the various groups.

TABLE 3

Frequencies of dead and malformed mouse embryos produced by injecting 500 μg of 5-fluorouracil at 10 days after mating into BALB/cDg dams mated to sires of the types: 129/Dg, BALB/cDg, (BALB/c × 129)F_1 and (BALB/c × F_1)BC_1

	Sires			
	129/Dg	F_1	BC_1	BALB/cDg
Sires (number)	6	8	18	11
Litters (number)	67 (10–14)	94 (10–13)	284 (12–21)	116 (9–13)
Dead embryos (percent)	12.6 (8–21)	23.5 (14–33)	29.6 (9–44)	27.4 (18–35)
Live fetuses (number)	550 (78–111)	691 (75–101)	1794 (100–119)	798 (59–82)
Malformed hind feet (percent)	83.3 (77–90)	58.3 (48–70)	35.3 (19–58)	7.9 (3–18)
Cleft palate (percent)	84.2 (75–90)	37.5 (26–51)	33.7 (9–62)	4.1 (0–7)

The figures not enclosed in parentheses are the grand totals or percentages based on grand totals for all litters. The figures in parentheses are the ranges of values in totals or percentages for individual sires.

TABLE 4

Average litter scores with standard errors for maternal body weight, dead conceptuses and malformed fetuses produced by injecting 500 μg of 5-fluorouracil at 10 days after mating into BALB/cDg dams mated to sires of the following types: 129/Dg, BALB/cDg, (BALB/c × 129)F_1, (BALB/c × F_1)BC_1 and (BALB/c × BC_1)BC_2

	Sires				
	129/Dg	F_1	BC_1	BC_2	BALB/cDg
Maternal body weight, g	27.9±0.3	28.3±0.2	27.7±0.1	27.6±0.1	27.3±0.2
Conceptuses, No./litter	9.8±0.3	9.9±0.1	9.9±0.1	10.0±0.1	9.6±0.2
Dead conceptuses, arcsin (%)	19.3±1.3 (10.9)	23.1±1.3 (15.4)	27.8±0.9 (21.8)	25.8±0.8 (18.9)	28.1±1.2 (22.2)
Live fetuses, No./litter	8.6±0.3	8.2±0.3	7.4±0.2	7.8±0.1	7.3±0.2
Malformed hind feet, arcsin (%)	64.7±2.3 (81.7)	49.0±2.3 (57.0)	34.0±1.2 (31.3)	.....	16.7±0.9 (8.3)
Cleft palate, arcsin (%)	68.1±2.3 (86.0)	37.2±2.3 (36.6)	34.1±1.4 (31.4)	.....	14.4±0.8 (6.2)

For dead conceptuses, malformed hind feet and cleft palate the figures not enclosed in parentheses are the corrected unweighted arcsin scores per litter. The figures enclosed in parentheses were obtained by transforming the arcsin scores to percentages.

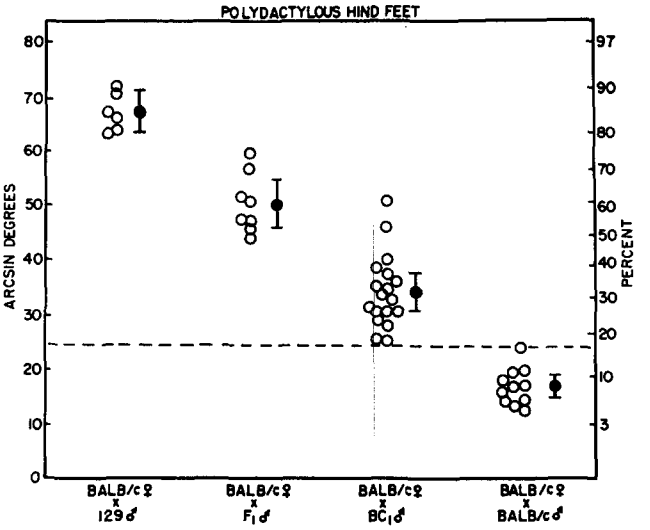

FIGURE 2.—The incidence of polydactylous hind feet in the 18-day-old fetuses from BALB/cDg females mated to 129/Dg, F_1, BC_1 or BALB/cDg males, and injected with 5-fluorouracil at 10 days after mating. The left ordinate is given in corrected and weighted arcsin degrees. The calculation of these values is described in the text. The open circles are values for individual males. The solid circles are the average of the individual scores with the standard error indicated by the vertical line. The horizontal broken line is the upper bound of the 95% confidence limits for the distribution of scores for the BALB/c males.

Figure 2 shows the distribution of scores for polydactylous hind feet for individual 129/Dg, F_1, BC_1 and BALB sires. The left-hand ordinate is given in corrected and weighted arcsin degrees, while the right-hand ordinate represents the arcsin scores transformed to percentages. The mean and its 95% confidence limits are shown for each generation. The dashed line represents the upper bound to the distribution of male scores in the BALB × BALB cross below which 95% of the scores are expected to fall, i.e., the 95% confidence limits of the male scores in that generation. It is immediately evident that the distribution in the BC_1 generation lacked the bimodality that one would expect on the basis of a single gene determining the response. For a single gene difference, in the first backcross generation half the circles should have fallen near the F_1 values and half near the BALB values. In fact, no male score resembled the original BALB scores sufficiently to be assignable to that population. In the second backcross the mean response continued to decrease and some male scores fell below the upper limit of the BALB scores (Table 5).

Figure 3 shows similar results for cleft palates. The mean tended to decrease with each succeeding generation. Again there was no clear indication of bimodality in the distribution of BC_1 males. One of the BC_1 males fell within the 95% confidence limits for the distribution of BALB males. The manner in which the mean responses to the teratogen decreased and the corresponding changes in variance as the alleles had an opportunity to segregate were indicative of a trait inherited as a multifactor genetic characteristic.

TABLE 5

Malformed hind feet and cleft palate in offspring of BALB/c females mated to BC_1 or BC_2 males and treated with 500 μg of fluorouracil at 10 days after mating. The scores for the BC_2 males are listed below their respective BC_1 sires

Malformed hind feet												
BC_1	(1)	24.3	(2)	31.4	(3)	33.7	(4)	34.9	(5)	40.7	(6)	50.7
BC_2	(1–1)	33.0	(2–1)	32.1	(3–1)	24.1*	(4–1)	36.7	(5–1)	31.4	(6–1)	43.1
BC_2	(1–2)	31.7	(2–2)	24.5	(3–2)	22.8*	(4–2)	24.4	(5–2)	26.5	(6–2)	29.6
BC_2	(1–3)	27.9	(2–3)	18.7*	(3–3)	22.4*	(4–3)	20.3*	(5–3)	22.5*	(6–3)	22.1*
BC_2	(1–4)	26.8					(4–4)	18.8*	(5–4)	21.2*		
BC_2	(1–5)	23.4*										
BC_2	(1–6)	21.2*										
Cleft palate												
BC_1	(1)	28.2	(2)	24.2	(3)	41.6	(4)	36.7	(5)	50.9	(6)	43.6
BC_2	(1–1)	22.4	(2–1)	20.3	(3–1)	17.5*	(4–1)	28.8	(5–1)	20.1	(6–1)	28.7
BC_2	(1–2)	20.4	(2–2)	11.5*	(3–2)	23.8	(4–2)	17.8*	(5–2)	15.2*	(6–2)	14.9*
BC_2	(1–3)	16.6*	(2–3)	12.3*	(3–3)	23.2	(4–3)	17.6*	(5–3)	25.5	(6–3)	23.7
BC_2	(1–4)	20.8					(4–4)	21.2	(5–4)	22.2		
BC_2	(1–5)	18.4*										
BC_2	(1–6)	19.4										

* These values lie below the 95% confidence limits for the distribution of scores for the BALB/c males. The upper bounds of the 95% confidence limits for the BALB/c males were 24.2 and 18.7 degrees, respectively, for malformed hind feet and cleft palate.

The number of each male is given in parenthesis, followed by the corrected and weighted arcsin scores, not in parentheses.

Table 6 gives the parameters used to calculate estimates of the number of genetic factors ($\hat{n}$) and the degree of genetic determination of the traits. The F_1

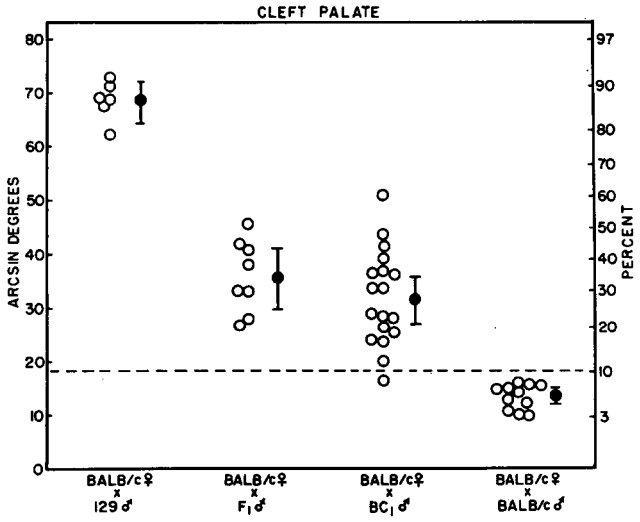

FIGURE 3.—Like Figure 2, but showing the values for cleft palate.

TABLE 6

Means and variances of scores of males in corrected and weighted arcsins for the inbred, F_1, and backcross-one generations, and estimates of the minimum number of loci, $\hat{n}$, and the degree of genetic determination for response to 5-fluorouracil

	Malformed hind feet		Cleft palate	
Sire	Mean	Variance	Mean	Variance
129/Dg	67.1	12.58	68.7	13.76
BALB/cDg	16.7	8.40	14.4	6.45
F_1	50.4	29.88	35.9	46.34
(averaged)		(16.95)		(22.18)
BC_1	34.1	50.44	31.9	78.08
V_E		16.95		22.18
$V_G/2$		33.49		55.90
n		4.7		3.3
Genetic determination		80%		83%

males showed a larger variance than either of the inbred sires. The three variances were averaged to obtain a conservative estimate of the environmental variance. Use of the F_1 variance, alone, may have been justified by the heterozygosity of that generation and the first backcross generation, and by the possibility of truncation at the lower end of the BALB/cDg distribution and at the upper end of the 129/Dg distribution. However, with no *a priori* knowledge of the underlying distribution of this threshold trait, we felt that the use of a conservative estimate was justified. At least four or five loci were involved in the response to 5-fluorouracil as measured by the incidence of malformed hind feet, and three or four loci as measured by the incidence of cleft palate.

In the BC_2 males, the probability of the occurrence of an allele from the 129 and BALB strains at one locus was 0.125 and 0.875 resspectively. The probability of genotypes homozygous for BALB strain alleles at 3, 4, 5 and 6 loci were 0.67, 0.59, 0.51, and 0.45 as calculated by the binomial expansion. For hind foot malformations,approximately half of the BC_2 males could be classified as belonging to the BALB strain as can be seen in Table 5, and consequently an estimate of four or five genes is not unreasonable. For cleft palate 14 out of 23 BC_2 males were within the BALB confidence limits. The actual number of genes that determined the trait may be greater if the effects at each locus were not additive and if there were two or more genes closely linked that would tend to segregate as a unit in four generations.

The difference in the number of genes affecting the response as measured by the incidence of cleft palate and that by the incidence of polydactylous hind feet indicates that these two responses were not necessarily controlled by the same set of genes. It appears that one gene more may have affected the incidence of malformed hind feet than affected cleft palates. Evidence from succeeding backcrosses does in fact show a separation of the incidence of these two malformations, but not enough data has accumulated for this to be clearly demonstrated.

The degree of genetic determination of these two traits was about 80%. However, the variance for the two types of inbred males was much smaller than that of the F_1 males and the degree of genetic determination based on calculations using the F_1 variance as the best estimate of the environmental variance was about 60%. The genetic differences among individuals of a population were responsible for 60 to 80% of the variation in response to the teratogenic effects of 5-fluorouracil. Since the genetic influences via uterine environment were held constant the response could be considered moderately sensitive to external environmental influences.

Correlations between maternal body weight at the time of treatment and the teratogenic and embryocidal effects of fluorouracil were made for each class of males and for the litters of all males combined (Table 7). In all of the crosses, except those involving F_1 males, there were significant negative correlations between maternal body weight and the frequency of dead conceptuses per litter. A similar inverse relationship was found for body weight and cleft palate for the BC_1 and BALB males, but not for the others. Since both the frequency of dead fetuses per litter and the frequency of cleft palate per litter in surviving fetuses tended to increase as the maternal weight decreased it was to be expected that the incidence of dead conceptuses would be positively correlated with the incidence of cleft palate in the survivors. Such a relationship was found for all except matings with strain 129 males. Partial correlation coefficients for maternal body weight, dead fetuses and cleft palate were calculated. The results showed slightly lower correlations in all groups, but the significance levels were not changed except for the correlation between body weight and cleft palate in matings with BALB males. Therefore, only in crosses involving BC_1 males, was there a significant correlation between body weight and cleft palate when the frequency of dead fetuses was held constant.

When the results for all litters from all males were combined, significant inverse relations between maternal body weight and dead conceptuses and between maternal body weight and cleft palate were found, while the frequency of dead fetuses and cleft palate per litter for all groups combined were positively correlated. The partial correlation coefficients indicated that maternal body weight was negatively correlated with cleft palate or dead conceptuses when either of the latter variables was held constant.

Since the average maternal body weights varied between groups, the estimated minimum number of genes was recalculated after adjusting the arcsin score of each litter according to the mother's body weight. After making these adjustments the minimum number of genes influencing palate development was estimated as 3.7, in comparison to 3.3 for the unadjusted scores.

In the offspring of mice treated with cortisone, an inverse relation between maternal body weight at conception and the frequency of cleft palate has been found in fetuses of three different genotypes (KALTER 1965). Similarly, maternal body weight is inversely related to the frequency of cleft palate produced by a given dose of 6-aminonicotinamide (PINSKY and FRASER 1959). KALTER (1965) also found that the frequency of cortisone-induced cleft palate in newborn mice

TABLE 7

Correlations between maternal body weight, dead conceptuses, malformed hind feet, and cleft palate

	Sires					
	129/Dg	F_1	BC_1	BC_2	BALB/cDg	All
Number of litters	63	82	247	279	107	778
Body weight and dead conceptuses	—.269*	—.151	—.249**	—.149**	—.179*	—.204**
Body weight and cleft palate	—.180	—.135	—.272**	—.111	—.241*	—.102*
Body weight and malformed hind feet	.179	.057	—.034	.131*	.122	.116**
Cleft palate and dead conceptuses	.069	.249*	.419**	.468**	.381**	.214**
Malformed hind feet and dead conceptuses	—.243	.045	.123	.067	.079	—.058
Cleft palate and malformed hind feet	.553**	.429**	.508**	.397**	.265**	.631**

* $P < .05$, ** $P < .01$ or smaller.
In each cross the dams were BALB/cDg. To calculate the correlations, embryonal mortality was expressed as the corrected, unweighted arcsin transformation of the ratio of number dead to total number of implantations per litter. For malformed hind feet and cleft palate, the same transformation was used for the ratio of affected live fetuses to total live fetuses per litter.

was inversely related to maternal age and to litter size. A multiple regression analysis revealed that the cleft palate frequency depended upon maternal weight, but that maternal age and litter size appeared to be effective only because they were correlated with maternal weight. There was no evidence that maternal age and litter size had independent influences on cleft palate frequency.

In the fetuses from fluorouracil-treated mice, the frequency of malformed hind feet was not correlated with the frequency of dead conceptuses (Table 7). For the separate classes of males, the incidence of foot malformations was correlated, positively and significantly, with the maternal body weight in the offspring of BC_2 males, only. However, the combined data of all groups, also showed a positive correlation between foot defects and the mother's weight. This relationship is the reverse of that found for cleft palate. This difference between the two types of malformations can be interpreted as showing that the set of physiological processes that influenced the development of one of the abnormalities was not completely identical with the set of processes involved for the other. It would follow that, since these physiological processes are probably genetically influenced, the set of genes involved in the development of malformed hind feet after fluorouracil treatment are not entirely identical with the set of genes involved in the development of cleft palate. Additional indirect evidence for this interpretation is provided by four other correlations.

First, the magnitude of the correlation between malformed hind feet and cleft palate per litter tended to decrease with successive backcrosses to the BALB strain (Table 7), as would be expected if trait-specific genes were segregating.

Second, the results for the 23 BC_2 males (Table 5) show that for seven of these the scores for both cleft palate and malformed hind feet lay outside the 95% confidence limits for the distribution of scores for the BALB males. For five others the scores for malformed hind feet, but not for cleft palate, lay outside the confi-

dence limits; while for another seven the scores for cleft palate, but not for malformed hind feet, were outside. Accordingly, if the factors determining an "outside" score for cleft palate were independent of those determining an "outside" score for malformed hind feet, then the number of BC_2 males showing an "outside" score for both defects would be expected to be 7.3 ($12/23 \times 14/23$), which is in remarkably good agreement with the observed value of 7.

Third, the results for fetuses themselves indicated some independence of factors for cleft palate or malformed hind feet responses. For example, in the BC_2 fetuses, sired by BC_1 males, cleft palate and malformed hind feet would be expected to occur together in the same fetus 10.8% of the time if the factors for these malformations were completely independent of each other. The observed value for concurrence was 18.0%. In the offspring of the BC_2 males, the calculated value for concurrence was 3.7% in comparison to the observed value of 7.5%. Furthermore, in F_1 fetuses cleft palate occurred alone, without malformed hind feet, in 11.4% of the fetuses with cleft palate, whereas in the BC_2 and BC_3 fetuses, this defect occurred alone in 44 and 49% of the fetuses with cleft palate. Likewise, in the F_1 fetuses malformed hind feet appeared alone in 9.7% of the fetuses with hind foot defects, while in BC_2 and BC_3 fetuses these abnormalities occurred alone in 46 and 69% of the fetuses with foot defects.

Fourth, polydactyly appeared with equal frequencies in both sexes in litters that had both males and females and in which one but not all fetuses had the defect. This was true for all crosses and therefore this trait was not sex-influenced. Because of the manner in which each generation of test males was produced, all Y chromosomes, except of course in the cross BALB × BALB were from strain 129. It seems unlikely, therefore, that any of the genes postulated in this study were located on the Y chromosome. The X chromosomes of F_1 males and all female and male offspring of this and succeeding generations were from strain BALB. With reference to palate defects, however, in litters containing both males and females in which at least one but not all fetuses had cleft palate, the frequency of this defect was slightly but significantly higher in female fetuses than in males (676/1508 for females, 489/1337 for males).

Because of the difference in frequency of cleft palate in male and female fetuses, the estimated minimum number of loci was recalculated using data for male and female fetuses, separately. For males the estimated minimum number was 3.6, as compared to 2.9 for females. These values do not appear to differ appreciably from each other nor from the combined value of 3.3 (Table 6).

Thus the evidence that, following fluorouracil treatment, some of the genes influencing the development of cleft palate are different from some influencing the development of foot malformations comes from several sources: (1) the estimated minimum number of loci is different for the two defects, (2) the defects are not similarly correlated with maternal body weight, incidence of embryonal mortality or sex of the embryos, and (3) trait-specific genes appear to be segregating in the offspring of BC_1 and BC_2 males.

It is of interest that LANDAUER (1947) found that after selection for modifiers of a recessive gene which produced a short upper beak and shortened extremities,

the modifiers were more effective in protecting the extremities than the beak from damage by insulin. GOLDSTEIN *et al.* (1963) found that the frequency of 6-aminonicotinamide-induced cleft palate was higher in F_1 embryos from crosses between A/J females and C57BL/6J males than in the reciprocal cross, whereas the frequency of vertebral fusions in F_1 embryos was higher in C57BL/6J than in A/J females. Thus, the hereditary factors influencing susceptibility appeared to be organ specific.

With regard to the disassociation of factors for hind foot and palate deformities, KALTER (1965) has proposed a quite different approach for studying 10 distinct malformations produced in newborn mice by riboflavin-deficient, galactoflavin-containing diets. All defects, regardless of their incongruity and variety, were considered parts of a single syndrome. It is obvious that such an interpretation for the fluorouracil-induced abnormalities would have been misleading.

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SUMMARY

The intraperitoneal injection of pregnant females with 5-fluorouracil produced higher frequencies of cleft palate and malformed hind feet in the fetuses of inbred mouse strain 129/Gg than in strain BALB/cDg. The number of genetic factors involved in the interstrain difference was estimated in the following manner. The strains were crossed and two successive backcrosses were made to the BALB/c strain. 129/Gg, F_1, BC_1, BC_2 and BALB/c males were mated repeatedly with BALB/c females, who were given a single dose (500 μg) of fluorouracil at 10 days after mating. The fetuses were examined 8 days later. A score was computed for each litter based on the ratio of cleft palate or malformed hind feet to total live fetuses. The ratio was transformed to the arcsin, corrected for litters in which zero or 100% responded, and then weighted by the number of live fetuses per litter. Each male sired several litters and was given an average score based on the incidence of malformations in his offspring.—The incidence of malformed hind feet and cleft palate decreased with each succeeding cross. The frequency distribution for the males of the first backcross generation was not bimodal as would be expected if a single gene determined the response to fluorouracil. The means and variances of the parental strains, and the variance of the first backcross generations were used to estimate that a minimum of four loci played a role in determining the incidence of malformed hind feet. The degree of genetic determination of this response was estimated at 80%. There was a low but significant correlation between the frequency of malformed hind feet and the body weight of the mother. Malformed hind feet occurred with nearly equal frequencies in male and female fetuses.—Similar estimations for cleft palate gave a minimum of three loci and a degree of genetic determination of 83%. Estimates for cleft palate are somewhat less reliable since there was a significant negative correlation between the body weight of the dam and the incidence of cleft palate among her offspring. There was also a significant positive correlation between the

incidence of dead or resorbed conceptuses per litter and the frequency of cleft palate in the surviving fetuses. Cleft palate tended to occur slightly, although significantly, more often in female than in male fetuses.—The set of genetic factors that influenced the incidence of malformed hind feet were apparently not completely identical with those influencing cleft palate in response to fluorouracil, because (1) the estimated minimum number of loci is different for the two defects, (2) the defects are not similarly correlated with maternal body weight, incidence of embryonal mortality or sex of the embryos, and (3) trait-specific genes appear to be segregating in the offspring of BC_1 and BC_2 males.

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