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Short title: GENETICS OF SPONTANEOUS AND INDUCED CLEFT LIP IN MICE.

GENETICS OF SPONTANEOUS AND 6-AMINONICOTINAMIDE
- INDUCED CLEFT LIP, IN MICE

BY

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GENETICS OF SPONTANEOUS AND 6-AMINONICOTINAMIDE - INDUCED
CLEFT LIP IN MICE.

ABSTRACT

The number of loci determining the difference in susceptibility to cleft lip in the inbred A/J and C57BL/6J strains of mice is probably two, with duplicate epistasis.

The evidence suggests that these are not the genes involved in the difference between the same strains in the embryonic cleft lip response to maternal treatment with 6-aminonicotinamide.

The difference between the A/J and CL/Fr strains in cleft lip frequency (10 versus 25%) appears to be determined by the maternal genotype, the A/J maternal effect trait being dominant.

Neither thyroxin nor nicotinamide maternal treatment prevents the development of cleft lip in embryos.

ETUDE GENETIQUE DE LA BEC-DE-LIEVRE DES SOURIS, SPONTANEE
ET CAUSEE PAR LA 6-AMINONICOTINAMIDE.

RESUME

Le nombre des loci qui determine la différence entre les races pures des souris, A/J et C57BL/6J, en risque de bec-de-lièvre est probablement deux, avec l'épistasie double.

L'évidence dévoile que ces gènes ne sont pas le même que ceux qui causent la différence en fréquence de bec-de-lièvre entre des embryons des races susdits après une injection de 6-aminonicotinamide à la femelle en gestation.

La différence entre les races pures, A/J et CL/Fr, en risque de bec-de-lièvre (10 en comparaison de 25%) est imputable aux génotypes maternels; la manifestation du caractère comme A/J est dominante.

Ni le thyroxine ni le nicotinamide injecté dans des femelles en gestation prevenit du bec-de-lièvre aux embryons.

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1. GENOTYPE-TERATOGEN INTERACTIONS

A. Strain Variations

(a) Examples

It has long been known that different breeds or strains may respond to teratogens in different ways and to different degrees. For Drosophila, Goldschmidt in 1935 listed the varied results of heat shock in different stocks (cited by Goldschmidt and Piternick, 1957). Among poultry stocks, also differences in teratogenic response were found (e.g., Landauer, 1954). Variation among inbred strains of mice in response to a wide variety of teratogens is known and has been reviewed frequently (e.g., Dagg, 1963; Dagg, 1966; Smithberg, 1967; Kalter, 1965, 1968a; Fraser, 1977).

"Genotype-teratogen interactions", where no known mutant gene is present, can be divided into 2 classes. First, breeds or strains that have a low frequency of spontaneous malformations tend to be more liable than others to produce phenotypically similar induced traits. Some inbred strains, but not all (Kalter, 1978), have characteristic spontaneous variants that may be attributed to multifactorial inheritance (Kalter, 1968b). Their increased teratogenic response is thought to be explained by the "quasi-continuous variant" or threshold model (Section 3). Some examples follow.

Nicotine induced malformed vertebrae in 85% of Brown Leghorn chicks that had a 0.5% spontaneous incidence, and in

only 66% of an American Leghorn stock that had no spontaneous cases (Landauer, 1960).

C57BL/6J mice, which have spontaneous microphthalmia, tend to be easily induced to higher microphthalmia frequencies (Ingalls et al., 1953; Glick, 1966; Coté, 1971). The C57BL strains also have a low spontaneous incidence of polydactyly, and gave higher frequencies of polydactyly in response to 5-fluorouracil than did other strains (Dagg, 1963).

DBA/1J embryos, which have about 2% spontaneous umbilical hernia, exhibited 14% of this anomaly in fetuses after maternal hypoxia, while A/J, Balb/cJ, and C57BL/6J mice which have no spontaneous hernias showed none after treatment (Ingalls et al., 1953). The related DBA/2J strain also showed a relative propensity to developing spontaneous and vincristine-induced umbilical hernias when compared with ICR/Ha and C3H/HeJ (Joneja and Ungthavara, 1969).

Differences between mouse strains AKR/J, DBA/2J, SWR/J, C57BL/6J, and C57L/J in susceptibility to limb and tail vertebrae malformations induced by 1-ethyl-1-nitrosourea appear to be related to their differences in spontaneous frequencies of these anomalies (Diwan, 1974).

The responses to antiheart antibody and to dexamphetamine sulphate of A/J and C57BL/6J mice were atrial septal defects and ventricular septal defects, respectively. These heart defects occur with the same specificity at a lower spontaneous frequency in those strains (Nora et al., 1968; Nora

et al., 1974).

The susceptibility to diet-induced diaphragmatic hernia in rats appeared to be related to its spontaneous occurrence in the population (Andersen, 1949).

The second class of "genotype-teratogen interactions" is represented by those breeds or strains in which the spontaneous frequencies of a given trait are extremely low and/or similar but are markedly different after treatment.

Many examples are known in fowl, involving, for example, insulin-induced rumplessness (Landauer and Bliss, 1946; Landauer, 1948a), insulin-induced micromelia and beak defects (Landauer, 1947), face and limb defects induced by boric acid (Landauer, 1954), and beak anomalies induced by acetazolamide (Landauer and Wakasugi, 1967). Unlike most examples, that involve relative sensitivities, the latter experiment found apparent total resistance of one genotype.

Mice have also shown such strain differences. The response of A/J, DBA/1J, 129/J and C57BL/6J embryos to riboflavin deficiency differed in the frequencies of structural anomalies of the brain, absent esophagus, brachygnathia, syndactyly and cleft palate induced (Kalter and Warkany, 1957). The rank of strains changed for each defect: sensitivity was not systematic, but specific for each organ. Similar results were obtained in a strain survey for susceptibility to foot, tail, and palate abnormalities induced by 5-fluorouracil (Dagg, 1963), and for rib, sternal, and thoracic vertebral

defects induced by 1-ethyl-1-nitrosourea (Diwan, 1974).

Other examples include the higher susceptibility of A/J compared with C57BL/6J for 6-aminonicotinamide-induced cleft palate and vertebral fusions (Goldstein et al., 1963) and the sensitivity of CBA/J compared with the total resistance of SWV embryos to acetazolamide-induced ectrodactyly (Biddle, 1975a).

The most highly explored example of strain differences of this kind is that of cortisone-induced cleft palate (Fraser and Fainstat, 1951; Kalter, 1954). A/J and C57BL/6J mice, which have a similar incidence of spontaneous cleft palate. (Trasler et al., 1956) were found to differ markedly in cleft palate frequencies induced by cortisone, A/J being much more sensitive.

(b) Exceptions

Despite these generalities, "genotype-teratogen interactions" are not always found in experiments where they might reasonably be expected. For example, no strain differences in type or frequency of induced congenital malformations were detected in A/J, DBA/1J and C3H/J mice after maternal hypervitaminosis A (Kalter and Warkany, 1961).

Strains having an appreciable frequency of a spontaneous malformation are not always more susceptible than others. Balb/cScott mice, with 18% spontaneous vertebral and rib malformations showed no increase in response to hypoxia, while

A/J and C57BL/6J, having a spontaneous rate of only 3 and 6%, responded with 75% and 68% respectively (Ingalls et al., 1953).

(c) Trait and teratogen specificity

Strains that are highly susceptible to induction of a given malformation by one teratogen are not necessarily more susceptible to induction of that trait through a different teratogen. For example, A/J is more sensitive to cortisone- and vitamin A-induced cleft palate than C57BL/6J but less sensitive to riboflavin-induced cleft palate, while the trait is equally sensitive to induction by both cortisone and riboflavin deficiency in DBA/1J (Kalter and Warkany, 1967; Kalter, 1959; Walker and Crain, 1960). Strain 129/Rr responded to insulin with rib and vertebral anomalies and exencephaly, while Balb/cRr was apparently refractory. A second hypoglycemia-inducing agent, tolbutamide, induced similar frequencies of rib and vertebral anomalies in these two strains, but caused 18% exencephaly in 129/Rr and none in Balb/cRr.

In chicks, Silver Grey Dorkings responded with more facial and limb defects than White Leghorns when treated with boric acid, but the breeds responded equally to insulin (Landauer, 1954).

Lastly, the relative sensitivities of genotypes to a given teratogen may differ for anomalies induced at different

periods of treatment (critical periods), as Landauer demonstrated in comparisons of poultry breeds. Thus, White Leghorn lines that differed markedly in susceptibility to insulin-induced rumplessness showed little difference in frequencies of insulin-induced micromelia and beak anomalies when treated later in development. Similarly, the high resistance to insulin of Jungle fowl, relative to other strains disappeared when later treatment times were used (Landauer, 1948a).

B. Interactions Between Mutant Genes and Teratogens

(a) Examples

Some of the attempts to demonstrate interactions between teratogens and single mutant genes have been reviewed frequently (see, for example, Landauer, 1961; Smithberg, 1967; Fraser, 1977).

A common approach to the problem of finding interactions has been to compare teratogenic responses of embryos heterozygous for mutant genes with those homozygous wild-type at that locus, in crosses where the remainder of the genome is equivalent in the two groups.

Using this approach, litters containing heterozygotes for the dominant Brachyury (T) allele in mice were found to be more sensitive than wild-type litters to early teratogenic effects of an RNA inhibitor, actinomycin D (Winfield and Bennett, 1971). The malformations observed were partial

phenocopies of lethal syndromes seen in embryos heterozygous for the T mutant and various recessive (t) alleles at this locus.

An independent study found that trypan blue induced taillessness, a phenotype of some T locus mutant genotypes, in Brachyury heterozygotes, but not in wild-type embryos (Hamburgh et al., 1970).

Heterozygosity for a series of non-allelic, semi-dominant genes of mice that all cause polydactyly has been found to increase the polydactyly response to teratogens: Paddlefoot, to 6-azauridine (Dagg and Coggins, 1976); Luxoid and Luxate, to 5-fluorouracil (Dagg, 1965, 1967); Strong's luxoid, to 5-fluorouracil and 5-fluorodeoxyuridine (Forsthoefel and Williams, 1975).

An additive effect was found between the Brachyphalangy allele (Xt^{bph}) and trypan blue on expressivity of skull, neural tube, eye and vibrissae malformations in mice (Johnson, 1970).

Polydactyly, a recessive gene, increased liability to triethylmelamine-induced polydactyly, while modifying genes differing between C57BL and CBA influenced expressivity and penetrance in the same direction for both mutant and induced traits (Kocher, 1975).

Hens heterozygous for recessive micromelia (mm^A) produced greater frequencies of limb and beak defects in their

offspring treated with 6-aminonicotinamide than did their wild type sibs whose offspring were similarly treated and who shared the same sires (Landauer, 1965).

In experiments with a similar breeding design using the recessive lethal crooked neck dwarf and nicotine, neck malformations occurred at a higher rate in chicks from "carrier" mothers (Landauer, 1960).

Outcrossed hens heterozygous for Creeper, a gene that causes multiple head and limb malformations in homozygotes, had more severe head and limb malformations in their chicks in response to excess dietary selenium than did their normal sibs (Landauer, 1940).

All of these differences were attributed to the presence of mutant alleles in the chicks, ignoring the possibility of maternal effects associated with these genes.

A controlled experiment comparing the response to sodium metaborate of Drosophila differing only at the incompletely penetrant, recessive, eyeless locus, showed increased liability to induced eyelessness in heterozygotes (Sang and McDonald, 1954). Dosage response studies indicated that penetrance and expressivity were dosage dependent and that sensitivity was greatest in homozygous eyeless flies, but that the slope of response was similar among all three genotypes. It was concluded that borate and eyeless alleles supplemented each other's actions, though not necessarily by acting on the same pathway.

Further studies on the eyeless system, comparing the borate-induced eyeless frequency in heterozygotes resulting from outcrosses to various wild-type strains indicated that the heterozygote eyeless frequency response was proportional to that of the particular parental wild-type strain used (Goldschmidt and Pitternick, 1957). It is noteworthy that this second eyeless stock, unlike the first, was refractory to borate treatment; neither expressivity nor penetrance were changed.

Both heat shock and the sex-linked major gene cvl-6b cause breaks in the posterior crossvein in the wings of Drosophila. Mohler (1965) reported that cvl-6b caused increased susceptibility to the induction of crossveinlessness, and that the same system of modifiers influenced both the spontaneous and induced trait.

Some apparent "interactions" can be explained by inequality of the background loci of the mutant states compared, as has been noted previously (Fraser, 1977, page 457) for the reported interaction of trypan blue and major genes for an anophthalmic trait (Barber, 1957; Beck, 1963, 1964).

The difference in malformation response to carbachol and sulfanilamide of wild-type and heterozygotes for Muscular dystrophy (md) in chickens (Landauer et al., 1977) is confounded with residual genotype, and in the case of carbachol, with maternal effects also.

Similarly, interpretation of data for heterozygotes and wild-types for the micromelia-producing recessive of chickens, Chondrodystrophy (ch) (Landauer, 1965) is prevented because the effects of selection for penetrance and expressivity are confounded with those resulting from changes in the ratio of wild-type and heterozygous mothers used from each selection line.

Other experiments that had potential for showing interaction were too small. For example, only 3 litters were examined in a test of hypervitaminosis A on the development of Hairless mice (Mauer, 1964).

(b) Exceptions

Not all searches for interaction between mutant genes and teratogens have produced positive results.

Neither synergism nor additivity of effects on limb malformations were found between 5-fluorodeoxycytidine, an ectrodactyly- and polydactyly-inducing agent, and homozygotes for Limb deformity (ld), which causes a low frequency of fusion and aplasia of limb and digit bones in mice (Degenhardt et al., 1968).

Heterozygosity for the recessive, Tail-kinks (tk) did not increase the frequency of tail malformations in response to cortisone, galactoflavin, or hypervitaminosis A (Kalter,

1965b).

Interaction was expected between X-linked Tabby, and heat stress, both of which reduce the number of vibrissae in mice. But treated Tabby heterozygotes were reported to have changed less from their untreated values on a probit scale than did their hemizygous wild-type sibs (Pennycuik, 1965). Further interpretation is prevented by the confounding of sex with the presence of the Tabby allele.

In a more complete experiment where X-rays were used to reduce vibrissae number (Kindred, 1964), wild-type males and females, heterozygous Tabby females, and Tabby males differed in initial vibrissae number but showed similar decreases after treatment. That is, the effect of the treatment appeared to be additive with that of the gene.

A second recessive micromelia (mm^H) in chickens, in contrast to mm^A , did not increase liability to limb and beak defects in response to 6-aminonicotinamide (Landauer, 1965). Nor did heterozygosity for short neck increase liability to neck malformations after nicotine treatment (Landauer, 1960).

Unlike cyl-6b, several other crossveinless mutants of Drosophila showed no effect on induced crossveinlessness (Milkman, cited by Mohler, 1965).

(c) Penetrance and expressivity

Genetic modifiers affecting the expressivity and penetrance of mutants may affect the liability to the comparable teratogenic response, even in the absence of the mutant allele (e.g., Landauer, 1948a; Goldschmidt and Pitternick, 1957; Mohler, 1965; Forsthoefel and Williams, 1975; Kocher, 1975). This also seems to be true for maternal effects (Dagg and Spaner, 1975). The intriguing exception, Goldschmidt and Pitternick's refractory, highly penetrant eyeless strain of Drosophila suggests that the ability to respond to a teratogen may be controlled by a genetic difference that is independent of, and epistatic to, the modifier system. A similar suggestion, that teratogenic response may be influenced by genes concerned with transport or metabolism of the teratogen, that normally have no influence on the organ disrupted by the teratogen, was made by Fraser (1977).

Mutant genes often tend to increase the expressivity of the teratogenic response trait. For example, in mice, treated heterozygotes for Paddlefoot showed extension of expression of polydactyly to forelimbs (Dagg and Coggins, 1976); treated Luxoid and Luxate heterozygotes showed involvement of the tibia, the phenotype of untreated homozygotes at these loci (Dagg, 1965, 1967). Dominant polydactyly in chickens showed increased proportions of bilaterally affected in mutant homozygotes and heterozygotes treated with insulin (Landauer,

1948b).

(d) Specificity

In general, only organs that are affected by both treatment and gene independently show increased liability to malformation when both are present. For example, trypan blue, which does not induce polydactyly, did not increase expressivity of limb malformations in Xt^{bph} hetero- and homozygous mice (Johnson, 1970); heterozygosity for Tail-kinks in mice did not increase susceptibility to cortisone induced cleft palate, galactoflavin-induced limb, brain or esophageal defects or to hypervitaminosis A-induced pinnae, orofacial or uro-recto-caudal malformations (Kalter, 1965b); the effect of Luxoid and Luxate heterozygosity did not extend to non-limb organ systems affected by 5-fluorouracil (Dagg, 1972); heterozygosity for crooked neck dwarf did not increase susceptibility to insulin-induced rumplessness (Landauer, 1961).

In summary, heterozygosity for mutant genes often, but by no means always, increases the probability of responding to a teratogen that produces a similar malformation.

Three other generalizations emerge from these studies. First, modifiers affecting expressivity and penetrance of mutants may also affect the liability to the comparable teratogenic response, even in the absence of the mutant allele. Second, mutant genes tend to increase the expressivity of

the teratogenic response trait. Third, only organs that are affected by both the teratogen and the gene independently show increased liability to malformation when both are present.

Most of these observations are consistent with the concept that developmental variation incurs threshold steps (Section 2).

The search for interactions was a stratagem for inferring a gene's function--it was thought that the metabolic pathways of gene and induced effect would have to be related in order for interaction to occur (Goldschmidt and Piternick, 1957; Landauer, 1965; Johnson, 1970). A knowledge of the specific biochemical action of the teratogen would then point to the function of the gene (Johnson, 1970; Dagg, 1972).

The usefulness of this approach has been questioned. It has been realized that the actions of teratogenic compounds are not specific (Sang and McDonald, 1954; Landauer and Waksugi, 1967; Winfield and Bennett, 1971), and that the limited variety of abnormal phenotypes produced by genes or teratogens may reflect limited alternate channels of development available to embryos, not biochemical specificity (Landauer, 1954; Dagg, 1967; Franz and Degenhardt, 1969; Winfield and Bennett, 1971; Waddington, 1975, page 77).

C. "True" Interaction

Rarely has "true" interaction between genes and teratogens (Sokal and Rohlf, page 355, 1969; Lewontin, 1974), in a strict sense been demonstrated. That is, no differences have been detected in the rates of change of response frequencies with increasing dosage given the initial difference between genotypes in spontaneous frequencies of malformation. A biological (and statistical) interaction would be present if different allelic states for a locus produced different rates of change in response to changes in dosage. That is, the dose response slopes would be different.

Frequencies of response expressed as percentages are not expected to change linearly with dosage. The implicit assumption, sometimes stated, is that underlying development there are quantitative variables ("morphogenetic substances" or Rendel's "Make", for example), that, in a given population of embryos, are normally distributed, and that must attain critical threshold levels in order for development to proceed normally (e.g., Sang and McDonald, 1954; Pennycuik, 1965; Dagg, 1966; Rendel, 1967). The effect of genes or teratogens is seen as a shifting of the distributions relative to the threshold value (Section 2). Given this model, linear relationships between dosage and malformation rate should be obtained if transformations of percentages based on the normal distribution are used (Wright, 1934; Finney, 1971; Fraser, 1977, page

453; Biddle and Fraser, 1977b). When viewed on this scale, interactions will be seen as different slopes of dose response regressions; additive effects, as shifts in the Y axis intercepts of the lines.

Few experiments tested more than 1 dosage on 2 genotypes, and interactions, if present, would not have been detected. The exceptions, the Drosophila borate response (Sang and McDonald, 1954), insulin induced rumplessness (Landauer and Bliss, 1946), and the mouse cleft palate response to cortisone (Biddle and Fraser, 1976, 1977b), and to 6-aminonicotinamide (Biddle and Fraser, 1977c), showed no evidence of interactions. That the cortisone-induced cleft palate trait may involve complex interactions between dosage, genotype, diet, and maternal weight was indicated by the data of Warburton et al. (1962), but this approach of using multiple variables has not been further pursued.

Based on the available data, it may be concluded that the higher malformation rates induced by teratogens in carriers of mutant alleles or in some strains are nearly always due to the additive effects of genes and teratogens on an underlying threshold character.

D. Genetic Analyses of Teratogenic Responses

Investigation of the genetic basis of strain differences in teratogenic response has relatively seldom been conducted. Of the studies done, three approaches have been used: comparison of reciprocal strain crosses with each other and with

parental strains for evidence of maternal effects, "dominance", or sex chromosome linkage, observation of the selection response as an indicator of the number of loci influencing the teratogenic response trait; analysis of the results from special breeding designs constructed to demonstrate the number and nature of loci controlling the response difference between two strains.

(a) Maternal effects

The contribution of maternal effects to teratogenic sensitivity in mammals has been reviewed by Biddle and Fraser (1977a). Some examples for experimental animals follow.

In mice, A/J dams, when crossed with C57BL/6J sires, were found to confer an enhancing effect on the sensitivity of hybrid offspring to cleft palate, but a protective effect against vertebral fusions, both induced by 6-aminonicotinamide (Goldstein et al., 1963). Effects of embryonic genotype were also present. In contrast, C57L/J dams conferred greater sensitivity to all malformations observed--eye, central nervous system, limb, and tail--after treatment with 1-ethyl-1-nitrosourea in crosses with AKR/J (Diwan and Meier, 1974); embryonic genotype had no apparent influence on teratogenic response differences for these strains.

In comparison of A/J and C57BL/6J crosses for cortisone-induced cleft palate, the A/J maternal environment was found to increase sensitivity (Kalter, 1954; Biddle and Fraser, 1976).

Kalter's (1954) data for individual first backcross

females suggests that the maternal effect was controlled by a few loci: the recovery of A/J-type dams observed is likely, for his relatively small sample, only if the number of loci is small. At least part of the genetic control of this maternal effect may be associated with the H-2 gene region (Bonner and Slavkin, 1975; Biddle and Fraser, 1977a).

Comparison of "C57BL" and NAW/Pr strains and crosses for sensitivity to cadmium-induced exencephaly and/or eye defects indicated that a maternal effect may explain the greater sensitivity of the C57BL strain. The data were consistent with dominant, single locus control of the maternal permissive trait, but this hypothesis was not rigorously tested (Pierro and Haines, 1977).

Maternal effects have also contributed to teratogenic response differences in poultry--for example, the greater sensitivity of White Leghorns compared with Jungle Fowl to rumplessness induced by insulin (Landauer, 1954), and the maternal plumage pigmentation influence on teratogenic response to boric acid (Landauer, 1961).

Some maternal effects in mice appear to be cytoplasmically transmitted, rather than due to maternal physiology. Examples are the maternal effect for 1-ethyl-1-nitrosourea in C57L/J, AKR/J crosses (Diwan and Meier, 1974), and that for 6-aminonicotinamide-induced cleft palate in A/J, C57BL/6J crosses (Goldstein et al., 1963). In the latter, the effect was complicated by being diet-induced and interacting with

nicotinamide "protective" treatment (Biddle and Fraser, 1977c; Verrusio et al., 1968; Pollard and Fraser, 1973).

Maternal effects, when sought, are not always found. None were found in crosses of 129/Rr and Balb/cGn mice for palate and foot anomalies in response to 5-fluorouracil (Dagg, 1963).

Maternal effects on "teratogenicity" in human beings have been documented. High maternal circulating phenylalanine (as in maternal phenylketonuria), seems to be related to multiple congenital anomalies; diabetic mothers produce a relatively high rate of congenitally deformed offspring (cited by Biddle and Fraser, 1977a). There is little data on maternal influences on embryonic response to known teratogens for the human population.

(b). Dominance

Comparison of hybrid embryo teratogenic responses with those of their parents can give a suggestion of their "dominance". No general theme emerges from the results of such experiments.

The cleft palate response to 6-aminonicotinamide of hybrids between A/J and C57BL/6J mice, in addition to showing maternal effects, showed dominance of relative tolerance or resistance (Goldstein et al., 1963; Biddle and Fraser, 1977c). The cortisone-induced cleft palate response for these two

strains exhibited a similar dominance of C57BL/6J tolerance (Biddle and Fraser, 1976). Resistances to cleft palate, to foot anomalies, and to tail malformations induced by 5-fluorouracil, however, were recessive in 129/Rr, Balb/c crosses (Dagg, 1963, Dagg et al., 1966).

(c) Sex-linked genes

Examination of the sex ratios among affected reciprocal F_1 's and/or any further discriminating crosses for evidence of sex-linked genes affecting teratogenic responses in mammals apparently has seldom been done. Francis (1973), presented evidence that there are sex-linked genes that enhance the sensitivity to cortisone-induced cleft palate of A/J and C3H/HeJ mice relative to C57BL/6J. The data of Biddle and Fraser (1976), on A/J and C57BL/6J did not show these effects.

(d) Selection

Positive responses to selection demonstrate the genetic origin of traits, and the rates of response suggest the approximate number of loci controlling them. Some teratogenic responses have been explored in this way.

Beck (1969; 1971), successfully applied divergent selection to trypan blue-induced anomalies in mice. After 3 generations of selection, a difference of 15% between the two

lines was established, the sensitive line producing 22% malformed offspring after treatment. No increase in spontaneous malformations was reported. The rapidity of the selection response was interpreted as indicating that inheritance of susceptibility was simple--perhaps as few as two loci. Considering that the "trait" selected was a variety of anomalies affecting the tail, limbs, eyes and fur, this small number suggests pleiotropy of the genes, or that the trait was actually reactivity to trypan blue dosage, independent of the anomalies used to monitor it. The finding of lysosomal acid phosphatase activity differences between the selection lines, and the demonstration in vitro that trypan blue destroys this enzyme's activity (Beck and Goldin, 1970), supports the latter interpretation.

Mice have been selected for their response to 6-aminonicotinamide also. Starting from a base population giving 2 responses, cleft lips and median facial clefts, 2 lines, one giving median clefts, the other, cleft lip have been selected (Trasler, in press). One of these, the cleft lip line, began to show spontaneous cleft lip during the course of selection. The presence of spontaneous cleft lip in one of the four strains contributing to the base population, and variation in selection pressure hampers genetic interpretation of this study.

Chickens were selected for a cleft palate response to

maternal dietary riboflavin deficiency (Roberts et al., 1974). The rapidity of the response was interpreted to be evidence for a small number of major loci influencing the response trait. The frequency of cleft palate occurring spontaneously after one generation of selection increased markedly.

The most well known experiments where selection was applied to teratogenic responses are those of Waddington and of Bateman, concerning "genetic assimilation".

Waddington's classic experiments were undertaken to investigate the means by which "acquired" (and therefore non-heritable) characteristics could be exploited by evolution. Partly as a result of these experiments, developmental plasticity and liability to environmental influence have been acknowledged to be genetically determined. The results of these genetic assimilation studies were reviewed and summarized by Waddington (1975).

The first experiment began with a Drosophila population that had no noticable spontaneous incidence of crossveinless-ness. After heat shock as pupae, a small portion of adults developed the crossveinless phenotype. These were selected to be the parents of the next generation. Treatment and selection continued over several generations until some crossveinlessness appeared in the absence of heat shock. A second phase of selection was applied, selecting these spontaneous variants, without treatment. Strains with 100% spontaneous crossveinlessness were obtained. Genetic analysis of these

stocks and of Bateman's crossveinless stocks (below) demonstrated that the trait was influenced by genes on all chromosomes and its behavior in crosses also supported a multigenic interpretation, although the loci were not necessarily of equal impact.

Bateman's similar heat shock experiment included other induced phenotypes in addition to crossveinless. She demonstrated that Waddington's second phase of selection (spontaneous variants), was not necessary to produce a line with high spontaneous incidence.

One of the phenotypes that showed assimilation was a distorted wing resembling the effect of a mutant gene, dumpy. Bateman found that the assimilated stock contained an allele of the dumpy locus, perhaps the same as the dumpy (dp^{TP}) gene, and that when this gene was removed the spontaneous variant no longer appeared.

Using a different environmental stimulus (ether treatment of eggs), Waddington selected for a different type of developmental abnormality--the conversion of the metathorax in Drosophila to a mesothorax ("bithorax"). This also showed genetic assimilation. Genetic analysis showed that an allele of the mutant bithorax ($Bx1$) had been selected, but that there were, in addition, modifiers on 2 chromosomes, plus a recessive, sex-linked maternal effect which influenced the spontaneous incidence, but not the sensitivity to ether induction.

All of these assimilated traits are threshold characters (Section 2). Waddington also experimented with genetic assimilation in a continuous character--the size of the anal papillae in Drosophila larvae. The papillae were known to function in regulation of osmotic pressure in the hemolymph. Flies were selected for survival on a salt-bearing medium.

After several generations the selected strains had larger papillae regardless of the salt content of the medium (assimilation) and showed an increased ability to respond to high salt by increasing the size of the papillae.

For all of these experiments, while the major loci may have arisen by mutation during the course of selection, the other numerous genes contributing to assimilation responses were thought to be pre-existing variations in the stocks.

It is noteworthy that when Bateman selected spontaneous crossveinlessness from her foundation stocks, a stronger major gene was found than those in the assimilated stocks.

The studies of genetic assimilation form one side of the studies of the genetic architecture of canalization (Section 3), since they involve the genetic factors that increase the modifiability of the development of a phenotype. (The other side is, of course, the studies concerned with increased stability of development.)

(e) Genetic analysis of strain differences in teratogenic response

There is little argument with the view that strain differences in teratogenic response are genetic, yet few studies have explored these genetic systems in detail.

The original mammalian example was the difference in susceptibility to cortisone induced cleft palate between the A/J and C57BL/6J mouse strains. The results of the F_1 indicated

- (1) that there was a genetically determined maternal effect;
- (2) that there was a genetically determined difference in embryonic susceptibility, and
- (3) that, since the F_1 from the resistant mother was more resistant than the resistant parental strain, there was overdominance or other heterotic effects (Fraser and Fainstat, 1951; Kalter, 1954).

This cortisone-induced cleft palate trait was re-examined by Biddle and Fraser (1976, 1977b). The experimental design was again repeated backcrossing, but the estimation of loci depended on the number of segregant classes and their mean response values rather than the mean response for each generation. Segregation of the maternal effect was avoided by use of A/J females throughout. The trait was considered to be more appropriately viewed as cortisone dosage tolerance, assayed by the cleft palate response. The strain differences

were therefore analyzed in terms of the dosages required to induce 50% cleft palate, obtained from probit analysis. The data were more compatible with a 2 to 3 independent-effect locus explanation than one locus or polygenic explanations.

The same experimental design was applied to the 6-aminonicotinamide-induced cleft palate response differences between A/J and C57BL/6J (Biddle, 1977; Biddle and Fraser, 1977c). A 3 locus, recessive epistatic system was thought to explain the data. This conclusion rested more on observation of the rate of recovery of the parental response than on the variance of segregating generations, which was used only to reject one locus and polygenic possibilities. It also was found that the loci controlling sensitivity to cortisone induced cleft palate segregate independently of those controlling the sensitivity to 6-aminonicotinamide-induced cleft palate, for this particular strain cross.

The rate of recovery of the parental strain response following an outcross and repeated backcrosses to the more sensitive of two strains was also used to analyze the genetic control of sensitivity to acetazolamide-induced ectrodactyly (Biddle, 1975b). Of the two strains of mice, CBA/J and SWV, the latter was not just relatively, but absolutely, resistant to the induced trait. The simplest explanation of the data was a 3 locus, recessive, epistatic cause of sensitivity.

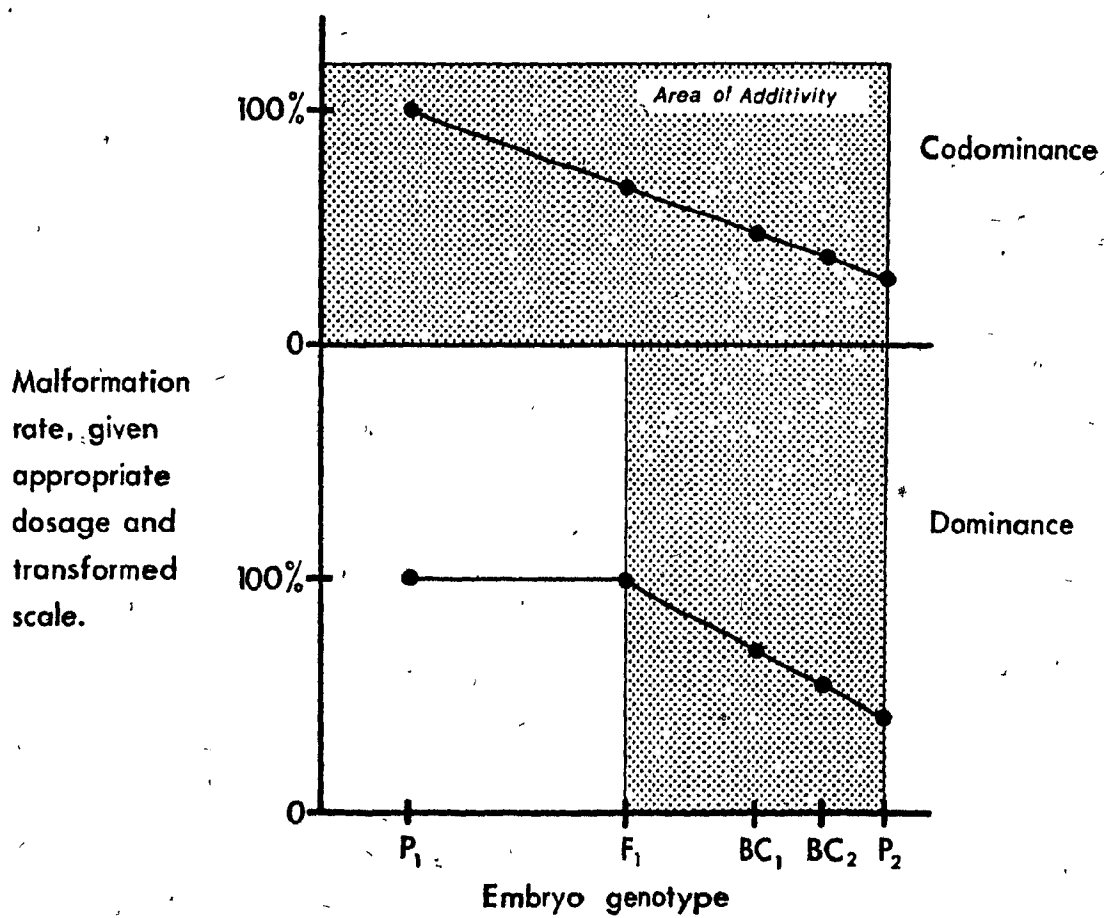
An earlier study of the control of sensitivity differences between Balb/cDg and 129/Dg mice to 5-fluorouracil-

induced cleft palate and hind limb malformations also used repeated backcrossing after a cross, but to the resistant parent (Balb/c for both traits). Sensitivity to cleft palate induction was thought to segregate independently of that for hind foot malformations, although the most critical test, correlations of the frequencies of the two traits in progenies of segregating parents was not done (see Biddle, 1975b). An estimate of the number of loci different between strains for each trait was made by use of Wright's formula, which uses the range between the parental strains and the variances of homogeneous and segregating generations. In this case the data were individual sire's progeny responses. Relative cleft palate susceptibility was estimated to be controlled by 3 to 4 loci, and malformed hind feet by 4 to 5 loci. The latter estimate was queried by Biddle (1975b) and it was suggested that 2 recessive loci of independent effect could explain the data. As was recognized by Dagg et al., (1966, page 1107), Wright's formula assumes that there is no dominance or epistasis in the genetic system observed. Apparent failure to meet these assumptions was the basis of Biddle's re-examination of the hind foot data. The discussions of both Dagg et al. (1966) and Biddle (1975b) regarding the use of Wright's formula merit further consideration.

Neither response trait showed additive inheritance. Both showed similar frequencies in F_1 's as in the parental strain 129: dominance. Surprisingly, this consideration alone does

not prevent the use of Wright's (1934) formula for the experimental design used. For a backcross of F_1 and subsequent generations to a recessive parental strain, the genetic system is "additive" within loci because only one of the 2 possible homozygous types for each locus can occur. In the absence of epistasis, Wright's formula, as used by Daggs et al. will give a correct estimate of the number of loci segregating for the trait concerned. The key to this is that the "parental" range used is that of one F_1 and one parental strain, and the formula is appropriately modified. The use of backcross breeding values for an F_1 in the numerator, and BC_1 generation backcross breeding values in the denominator reduces both values to $1/4$ those expected under the original test design, and the changes due to the new design thus cancel out. The analysis proceeds as if it involved the genotypes on one side of the F_1 for an additive trait (Figure 1). A problem arises when more than one locus with dominance influences the trait. Segregants carrying a dominant allele at only some of the relevant loci will occur, and their phenotype is important with respect to the applicability of Wright's test. Thus, for backcrossing to a "recessive parental strain", if only the genotype aabb were sensitive, while those such as Aabb or aaBb were as resistant as AaBb, the F_1 genotype, then epistasis would be present, the variance would be increased, and the estimate would be biased upwards. On the other hand, if Aabb or aaBb were sensitive, but less so than aabb, an additive system among loci

FIGURE 1. REPRESENTATION OF THE ADDITIVITY OF EFFECTS IN A BACKCROSS BREEDING PROGRAM*



*Assuming no maternal effects, no epistasis, and backcrossing to the recessive parent.

would be present and Wright's formula would apply despite the dominance within loci.

Thus the analysis by Dagg et al. was appropriate, if there was no epistasis present. The interpretation of their results is that the alleles at each locus exhibit dominance, but the effects of the loci are additive.

Biddle was correct that Wright's formula should not be applied to his acetazolamide data, because it includes evidence of epistasis.

The inheritance of, and relationship between, spontaneous and riboflavin deficiency-induced cleft palate in chickens was investigated by observation of its incidence in two segregating generations after an outcross (Jurilloff and Roberts, 1975). The spontaneous cleft palate trait was recovered at low frequencies that could be explained by 3 recessive loci with duplicate epistasis. The increase after riboflavin deficiency suggested that only 2 epistatic recessive loci were needed for cleft palate to occur under that developmental condition. If correct, this explanation suggests the existence of threshold traits whose underlying genetics is largely non-additive when the traits are at low frequencies in heterogeneous populations.

A study of the genetic basis of a "cryptocephal" phenotype induced by glucosamine in Drosophila used the elegant genetic manipulative techniques available for that species combined with statistical methods of analysis of variance

(Rapport and Sing, 1971). Stocks were created that were homozygous for 2nd and 3rd chromosomes derived from single flies. By appropriate crosses flies homozygous and heterozygous for each and both of these 2nd and 3rd chromosomes were tested with glucosamine. Three experiments, using chromosomes from different sources, were run.

Heterozygotes were usually less susceptible to the induced cryptocephal phenotype than homozygotes (heterosis). This was thought to be due to recessiveness of alleles conferring sensitivity, and the fixation of each line for dominant alleles at different loci. An exception, a combination of chromosomes that showed interaction between dominant genes at different loci to confer increased sensitivity to double heterozygotes compared with single heterozygotes was observed. It was considered to be an example of poorly coadapted genes leading to poor canalization.

Hydroxyproline, like glucosamine, induces a cryptocephal phenotype. It also induces a wing and leg defect (LWD). Using genetic manipulations of isogenic stocks similar to those used previously, Rapport (1975) found the genetic controls of liability to cryptocephal and LWD to be separate. Some recessiveness of sensitivity to LWD was found. Like the glucosamine induced trait, hydroxyproline-induced cryptocephal sensitivity was recessive and showed heterosis with some poorly coadapted exceptions. The loci controlling the cryptocephal

response to glucosamine and hydroxyproline were thought to be separate (Rapport, 1973)--lines having high sensitivity to one agent can be relatively resistant to the other.

E. Critical Periods

All references to critical periods in mice have been presented using plug day = day 0, and have been altered from their original reported format if necessary.

The cumulated experience of teratologists (reviewed by Wilson, 1965), suggests that embryos are usually resistant to induction of malformations very early in development, prior to formation of germ layers, and later in development, when organogenesis is complete. During the intervening period, preceding by hours or days its morphological differentiation, each organ shows a peak time of susceptibility to induced malformations. Some organs--for example, the palate--may show more than one peak (Dagg, 1966). This phenomenon is thought to reflect induction of the same endpoint malformation by different mechanisms (Wilson, 1965, page 254; Murakami, 1977). Because organs differentiate asynchronously, teratogenic treatments given at different times in development produce different constellations of malformations. A rich source of examples from studies of chick embryos is found in Romanoff (1972).

Agent specificity interacting with genetic susceptibility is thought to cause the observations that at a time when

several organ systems are passing through developmental critical periods, not all are disturbed by any one teratogen (Kalter, 1968a).

Both the peak time and the duration of the critical period may be influenced by the dosage used to define them. Dostal and Jelinek (1971), found that the sensitive period for cortisone-induced cleft palate was shorter and peaked later for smaller dosages. This effect may be an absorption phenomenon--large doses may reach effective blood and tissue levels sooner and stay up longer.

Critical periods may also be influenced by genotype. For example, acetazolamide-induced ectrodactyly in mice shows peak sensitivity on day 9 of gestation for C57BL/10J, and C3H/HeJ strains, day 10 for CBA, and an intermediate time for A/J (Biddle, 1975a).

2. GENETIC AND DEVELOPMENTAL THRESHOLDS

The history and application of the multifactorial threshold concept has been recently reviewed (Fraser, 1976). Usually attributed to Sewall Wright (1934), the concept explains the inheritance of traits between the genetic extremes of simple Mendelian inheritance with discrete phenotype and polygenic inheritance of quantitative traits.

Threshold traits, although discrete like Mendelian ones, are hypothesized to be expressions of traits that are quantitative at a physiological level nearer to the gene products. Variation in the quantitative trait is produced by additive effects of environmental variables, and also, but not necessarily, by additive effects of alleles and loci. Such effects are expected (mathematically) to produce an approximately normal distribution of the quantitative trait.

This quantitative variable becomes the observed alternate qualitative morphological forms through the existence of critical "threshold" values of the quantitative variables. During development these threshold values must be attained if normal morphogenesis is to proceed. Failure to obtain these values causes development to enter an alternate, abnormal course.

There seems to be considerable superficial similarity between this threshold concept, and the more recent Catastrophe Theory (reviewed by Zeeman, 1976).

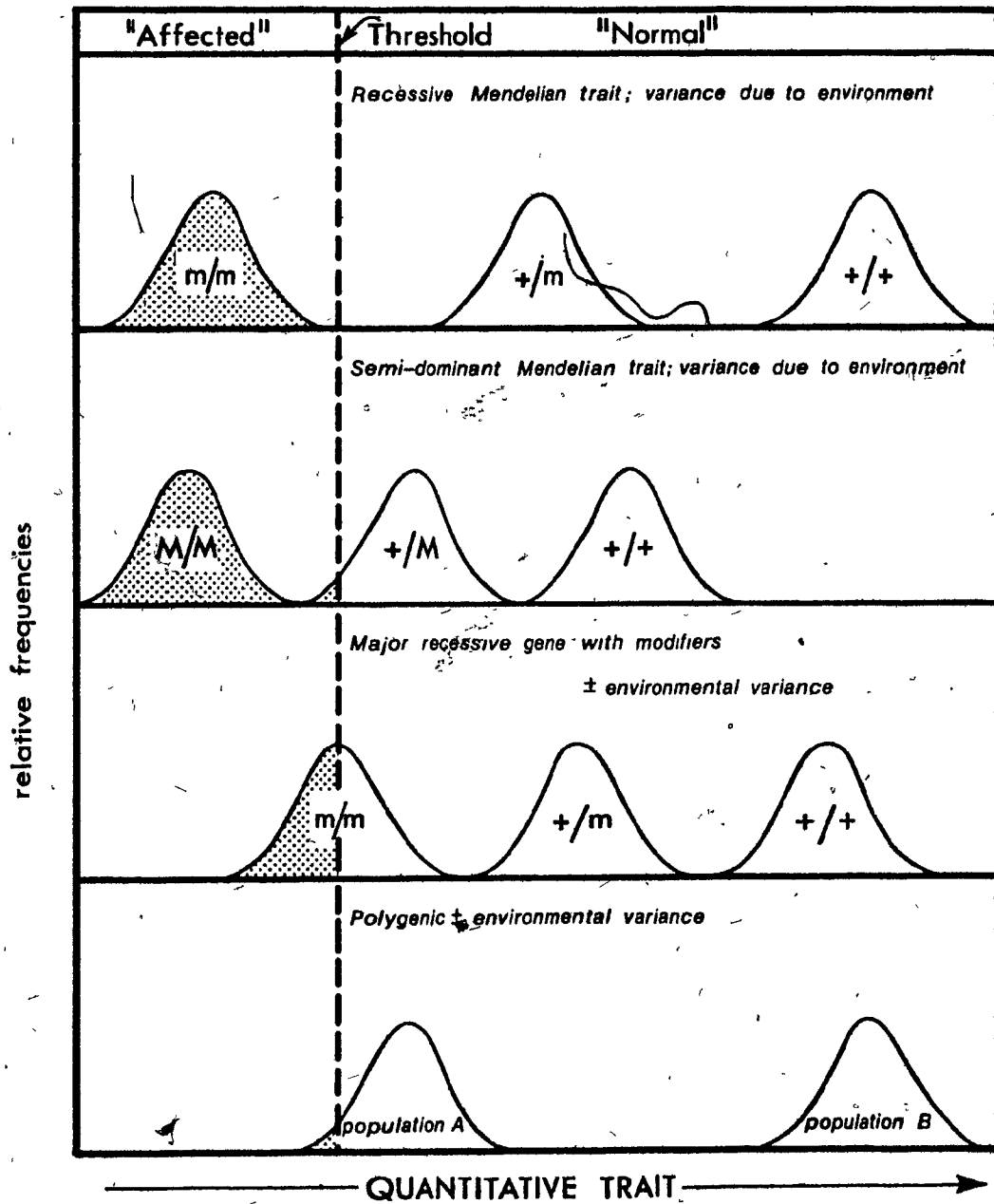
All discontinuous morphological traits are threshold

characters (Figure 2). For dominant Mendelian traits the effects of single alleles are so large relative to other effects that their carriers are placed wholly on one side or the other of the threshold. For codominant or semidominant traits, the relative effect of the alleles is less; some heterozygotes cross the threshold. If more than one locus influences the variance of the quantitative variable, the differences between a reference population and others may be expressed as the number of genes needed to move a population's distribution to the point where part of it crosses the threshold value--a threshold number of genes (Wright, 1934). While it is convenient to assume that such genes act equally and additively, there is little reason to do so. A continuum of relative effect probably exists between the additive polygenic case and single major genes with modifiers (Gruneberg, 1952a). These multigenic cases may also involve additive effects of environment, but not necessarily to an important extent.

It is the multigenic threshold case for which Gruneberg (1952a) deduced the characteristics of a "quasi-continuous" variant. Thus not all threshold traits would be expected to share the predicted qualities, which include:

- (1) more than two stable levels of a character among inbred strains;
- (2) variable behavior in crosses to different strains;
- (3) correlation between expressivity and penetrance;

FIGURE 2. THRESHOLD TRAITS



- (4) sensitivity to environmental influences;
- (5) sensitivity to genetic influences like sex;
- (6) ability of the multigenic system to act as modifiers of major genes introduced into the system.

The correlation between penetrance and expressivity has been discussed in detail (Sang, 1963). The nature of the correlation will depend on the variance of the quantitative variable. The phenomenon derives from the affected tail of the distribution obtaining more extreme values as a greater proportion of the distribution crosses the threshold, and from the additive effects contributing to the quantitative variable on both sides of the threshold.

Examples of threshold characters in mice include: cleft palate, where the threshold traits are thought to include shelf size and timing of closure--more than one threshold distribution may contribute to a trait (Fraser, 1976); cleft lip, where a threshold may be positioning of embryonic facial processes (Trasler, 1968); and absence of the third molar, where a critical mass of primordial tissue may be required (Gruneberg, 1952a).

Where the variance of the quantitative variable is largely attributable to variation at many loci, and where the resultant qualitative trait is both deleterious and relatively common (as are most multifactorial threshold traits in man), the gene frequencies at the contributing loci must be intermediate despite selection, and reasons for their continued

polymorphism become an interesting genetic problem. It is also worth noting that if most of the alleles contributing to a threshold trait in a heterogeneous population are less frequent than their alternate forms, the resultant distribution will not be normal, but, rather, skewed with the long tail extending across the threshold.

The expectation, not always obtained (Section 1), that genotypes having spontaneous malformations should be relatively easily induced to higher frequencies of those malformations by teratogens is derived from the threshold model. The effects of teratogens are expected to be additive with the others influencing the distribution of the quantitative variable, shifting it towards or across the threshold. It is possible that some teratogens act by shifting the threshold itself; this idea has not been tested (Sang, 1963). Yet another possible effect would be, in increasing the variance without affecting the means of the quantitative variables. This would be interpretable as "true interaction" (Section 1).

3. CANALIZATION AND STABILITY OF DEVELOPMENT

Canalization is a metaphor (reviewed by Spiess, 1968; Waddington, 1975, pages 103 to 126), that expresses the observation that the variation of morphology within any species is not continuous and does not encompass all possible forms, but consists of only a limited series of alternate states; that is, that development seems to follow "channels" or "canals".

Developmental thresholds (Section 2) are axiomatic to explanations of these alternate states, the thresholds being converted to walls of the "canals" in the canalization metaphor.

Canalization also refers to the capacity to produce a particular phenotype despite variability (genetic and environmental) in the initial situation from which development starts, and in conditions during its course. Thus development is said to be buffered. Such buffering is particularly characteristic of wild-type genotypes, and is usually reduced in the presence of strongly acting mutant genes.

It was observed (Waddington, 1975, page 105), that different genotypes possessed different degrees of buffering and that developmental stability could be altered by selection. Furthermore, the maximally buffered genotype could be shifted (Rendel, 1967, page 73, and see Strickberger, 1968, page 776).

Considerable effort has been made to define the genetic structure of the buffering.

Waddington suggested that the canalization of development arises from complex interactions among genes, and that there might be non-linear effects of gene action on phenotype.

Heterozygosity (Lerner, 1954, page 75); has been suggested as a contributing factor.

The multifactorial threshold model provides for differing abilities to withstand environmental and genetic perturbations by distance from the threshold on a quantitative scale. A genotype far from thresholds for all quantitative traits contributing to the final phenotype would be a well buffered one.

Major genes, regulated as under the Jacob-Monod model, in association with non-regulated minor genes (Rendel, 1967), were suggested as a way by which a fixed phenotype could be produced despite gene substitutions and environmental changes.

The scutellar bristle trait in Drosophila provides an example of the possible complexity of canalized systems. The normal bristle number has been found to result from at least two major loci each having separate sets of modifiers, each responsive to different environments, and acting antagonistically to the other and epistatically to the other's modifiers (Fraser, 1967; Fraser et al., 1968). This genetic control system overlaps with that influencing another bristle trait

so that selective changes in the second trait also affect it (Scowcroft, 1973).

The observation that for quantitative traits a larger proportion of the phenotypic variation becomes attributable to dominance and epistasis as the environment becomes more stressful (e.g., Jinks et al., 1973; Orozco and Bell, 1974), suggests that the degree of buffering may be due to largely non-additive genetic effects.

4. CLEFT LIP

A. Description

Cleft lip occurs spontaneously in many species, but has been studied most extensively in human beings and mice.

Cleft lip is an abnormality of the upper lip where a gap or cleft extends through the primary palate between the lateral border of the premaxilla and the palatine process of the maxilla. It is variable in expression, ranging from a "scar" or a "pit" in the primary palate, to a full cleft, and may be bilateral or unilateral, right or left. The cleft of the primary palate is often accompanied by a cleft of the secondary palate which is thought to result from mechanical obstruction due to the primary cleft (Trasler and Fraser, 1963).

"Cleft lip" will be used here to mean "cleft lip with or without cleft palate." The conventional designations, CL(P), meaning cleft lip with or without cleft palate, CLP, meaning cleft lip with cleft palate; CL, meaning isolated cleft lip; and CP, meaning isolated cleft palate, will be used when appropriate.

Cleft palate in the absence of cleft lip is generally agreed (but see Chabora et al., 1974) to be genetically and developmentally distinct from CL(P) (Fraser, 1970; Bixler et al., 1971; Saxen, 1975). There are some human exceptions where CL(P) or CP are alternate expressions of the same genotypes: the dominant lip pit syndrome, and the statistical association between CL(P) and CP for the Tasmanian population (cited by Fraser, 1970). Other claims of association may be based on statistical artifacts.

The A/J strain of mice, despite the fact, apparently fortuitous, of being susceptible to spontaneous CL(P), and relatively sensitive to CP induction by cortisone or 6-aminonicotinamide (reviewed by Fraser, 1977), has a rate of spontaneous CP production not unusually high, and the two spontaneous traits thus seem unrelated (Kalter, 1968b). The segregation of liability to spontaneous CL(P) and of sensitivity to cortisone- and 6-aminonicotinamide-induced-CP has indicated that they are genetically independent (Dr. F.G. Biddle, personal communication).

B. Spontaneous Cleft Lip

Cleft lip in mice is used as a developmental and genetic model for that in human beings: an approach that assumes evolutionary conservatism and gene homology between species. In neither species does cleft lip follow Mendelian segregation patterns, and the trait is therefore of interest not only as a human medical problem, but also as a genetic problem regarding the interactions among genes, and between genes and environment in determining phenotype.

This thesis reports studies made of cleft lip in mice, but some of the literature for the human trait is reviewed to demonstrate the similarities and differences between these species.

(a) Sex ratio and other associations

An excess of males among newborn CL(P) human beings has usually been found in population surveys (Fraser, 1970).

For mice, an excess of males (Reed, 1936a; Loevy, 1969), no sex difference (Steiniger, 1939; Kalter, 1968 and 1971) and an excess of females (Dagg, 1966; Kalter, 1975) among CL(P) embryos or newborns have been reported in various A substrains. An excess of affected males was reported for CL/Fr mice (Nomura, 1970). Kalter (1975) has reviewed the apparent instability of sex ratio for CL(P) and normal embryos in A substrains, and has suggested that it is due to an interaction between sex-influenced prenatal mortality and parity.

A tendency for CL(P) embryos to occur at higher frequencies at sites adjacent to the ovaries in untreated and cortisone-treated litters was observed in A/J mice (Trasler, 1960) and in a segregating generation of a cross of CL/Fr and C57BL/6J (Bornstein et al., 1970). No similar trend was found for A/J mice in a later study of comparable size, nor for segregating generations of a cross of A/J and C57BL/6J (Davidson, 1963, page 57).

A recent study (Kalter, 1975) again found a trend toward higher CL(P) frequencies among embryos at the ovarian and cervical ends of the uteri than among those at intermediate sites, in A/J mice, but the method of analysis may have been biased. Only uterine horns of two or more embryos, at least one of

which had CL(P) or an "open-eye" trait were used. For horns with 2 implants it was thus obligatory that an affected embryo be either "ovarian" or "cervical." If the number of such 2-embryo horns was large, this method of analysis may account for the observed trend.

Associations between the "open-eye" trait and CL(P) in A substrain embryos have been reported (Reed, 1936a; Kalter, 1968b), but not consistently (Davidson, 1963, page 72).

Cyanosis was early reported to be associated with CL(P) in A substrains (Reed and Snell, 1931) and with CL(P) due to the Twirler gene (Lyon, 1958). Recently, the cyanosis was found to be the result of delayed growth of the atrial septum primum, a condition found more frequently in A/J and CL/Fr CL(P) newborns than in their normal littermates (Fraser and Rosen, 1975). There is some evidence that human CL(P) may also be associated with heart defects more often than expected by chance (Mackeprang and Hay, 1972).

(b) Maternal effects

Relatively abrupt downward shifts in CL(P) frequency with increasing maternal age have been observed at 5 months of age in Strong A and 8 months of age in A/JKt mice (Loevy, 1969; Kalter, 1975). CL(P) frequency showed an increase with larger litter size in A/JKt mice (Kalter, 1975). Both of these effects were confounded with maternal parity effects (Kalter, 1975).

In man the frequency increases slightly with increasing maternal age but only in cases associated with other malformations (Hay, 1967).

The genotype of the mother also appears to influence CL(P) frequency. In a series of repeated backcrosses to A/J after an outcross to C57BL/6J, the frequency of CL(P) was consistently higher in litters from A/J mothers than in genetically equivalent litters from genetically segregating mothers (Davidson et al., 1969). The C57BL/6J genotype seemed to contribute maternal attributes that protected embryos from penetrance of the CL(P) genotype, possibly larger litter size (Davidson, 1963, page 97) in contrast with Kalter's finding of a positive correlation of these factors within the A/JKt strain (1975).

A similar maternal effect difference between CL/Fr and C57BL/6J mice was found, and the possibility of cytoplasmic inheritance was tested and rejected (Bornstein et al., 1970).

Reed (1936b) found no reciprocal cross differences between inbred and heterozygous females after an outcross of his cleft lip stock, and thus no evidence of genetic maternal effects in his studies.

For human data involving interracial crosses in Hawaii, Ching and Chung (1974) found no clear-cut maternal effects.

(c) Genetics

Two major attempts to define the inheritance of liability to CL(P) in the A strains of mice have been made. The first was made by Reed (1936a, b) who derived several inbred sub-strains from one harelip stock. At a calculated inbreeding

coefficient of 90% or more, the cleft lip frequencies produced in these substrains fell into 3 classes: 10 to 20%, 40 to 50%, and 60 to 80%. If these substrains were actually as inbred as indicated by the calculations, this variation argues for a multigenic cause of CL(P) (Gruneberg, 1952a). Natural selection against CL(P), however, would have opposed the accumulation of homozygosity for CL(P) genes, and the lower values may simply reflect continued heterozygosity. Such selection was present (Reed and Snell, 1931).

Reed also looked specifically for a "major" gene, the presence of which would be necessary for CL(P) to develop, other genetic influences being "modifiers" unable to produce the trait alone. He made an outcross of a cleft lip line to a non-cleft line, and after a second generation of outcrossing, testcrossed the segregating generation with a cleft lip line. On the basis of a major recessive gene, half would be expected to produce CL(P) and half would not (Aa compared with aa). The results were compatible with this model, but more complicated types of inheritance, with sampling error, could also give such results, as Reed noted. Among these were a 2-locus additive threshold system where the genotypes Aabb and aaBb could develop CL(P), but less frequently than aabb, and other additive threshold systems: any 2 of 3 loci, or any 3 of 5 loci homozygous for "mutant" alleles. The latter-multiple loci explanations are not compatible with the observed recovery of a substantial (6.4%) frequency of CL(P) in the F_2

after an outcross.

The CL(P)-producing tested sires produced 5% CL(P), about half the frequency expected under a major gene hypothesis. Conversely, the 6% recovered in the F_2 was predicted by the hypothesis. Since the sample sizes for both crosses are equivalent, the inconsistency cannot be resolved by emphasis on one of the crosses only. Extension of the model to more than one locus to explain the testcross data leaves the F_2 recovery of the trait incompatibly high.

In reviewing Reed's work, Gruneberg (1952b, pages 363 to 370) noted that the F_2 seemed deficient in "normal overlaps," but emphasized the testcross data in favoring the hypothesis of cumulative action of a small number of equipotent genes. He also noted that the question could not be regarded as settled.

The second major attempt to investigate the genetic basis of cleft lip in the mouse used the technique of repeated backcrossing to the "mutant" A/J strain after an outcross to C57BL/6J (Davidson, 1963). By comparison of the rate of recovery of the A/J-like CL(P) frequency with theoretical curves of recovered homozygosity, it was possible to estimate the number of loci affecting CL(P) that differed between the strains. Theoretically as the number of loci is increased, the probability of recovery of the A/J genotype is decreased, and the number of generations required to recover the A/J CL(P) frequency is increased. It was estimated that 3 to 4 genes were involved (Davidson et al., 1969). This estimate may have been

biased upward by the presence of natural selection (CL(P) newborns die) removing part of the A/J genotype as it was recovered.

The question originally posed by Reed, and summarized by Gruneberg (1952b):

Does there exist one single major essential gene for harelip, which must be present if--with the assistance of suitable modifiers--harelip is to develop. . . ?

was not answered. That is, modifiers that in themselves could not cause CL(P), but which determine the frequency of CL(P) would have been included in Davidson's estimate.

In summary, while CL(P) in mice is termed "multifactorial" (Bornstein et al., 1970; Kalter, 1975), the word is used in the sense described by Fraser (1976):

. . . determined by a combination of several genetic factors, of unspecified nature, and environmental factors.

Cleft lip in human beings (unlike the spontaneous CL(P) studied in mice, which is found in a limited range of genotypes and except for that associated with the Twirler and Dancer genes is probably of one type of cause) can be attributed to the effects of a variety of syndromes, chromosomal aberrations, teratogen and a "multifactorial" system. CL(P) of multifactorial causation is thought to make up the majority (Fraser, 1970; Burdi et al., 1972).

It has been difficult to determine the genetic basis of

"multifactorial" CL(P) in human beings. The model, recently reviewed by Smith (1977) and Curnow and Smith (1975) assumes that there is a large number of factors, both genetic and environmental, each with small effect, contributing to an underlying liability to disease. This liability is assumed to be normally distributed on some scale, and to be divided into normal and affected phenotypes by a threshold value. While the model has been useful conceptually in explaining relationships between risk and expressivity, and the non-Mendelian pattern of inheritance, and while the data fit the model well, the fit by no means proves that the underlying genetics is polygenic. It has been shown that it is extremely difficult to discriminate, using human population data, between single locus models with various degrees of dominance and penetrance, and polygenic threshold inheritance (Vogel and Kruger, 1966; Smith, 1971; Curnow and Smith, 1975).

Nevertheless, the agreement of observed recurrence frequencies in relatives of probands with those predicted from a polygenic threshold model of inheritance has been emphasized (e.g., Takana et al., 1969; Fraser, 1970; Czeizel and Tusnady, 1972; Leck, 1977). Under the polygenic model, heritability estimates of liability have been consistently high, ranging between 78% and 99% (Fraser, 1970; Czeizel and Tusnady 1972; Chung et al., 1974). This means that if CL(P) is polygenically determined, the distance from the threshold at which a given individual is found is influenced only to a minor degree, if

at all, by environmental factors.

An interesting alternative model, allelic restriction, has been proposed (Melnick and Shields, 1976; Melnick et al., 1977) but requires experimental testing. The authors find that their CL(P) recurrence data fail to fit the predictions of a polygenic threshold model in three ways.

The first, failure to detect an association between recurrence risks and number affected in a family may be attributed to inadequate sample sizes. The second, lack of increased risks in relatives of affected females only indicates that differential mortality (for example) rather than sex-dependent positioning of a threshold may cause the excess of affected males. The third, failure of first through third degree relatives' recurrence risks to meet predicted values may be partly due to the apparent assumption of 100% heritability. Agreement is improved by recalculating risks at 93% heritability (on the appropriate normal equivalent deviate scale). A residual excess of affected third degree relatives, however, does provide a challenge to the multifactorial model, assuming that it is not due to some peculiarity of the sample, which spans the social changes of 30 years.

A recent attempt was made to discriminate between single major locus inheritance and polygenic threshold inheritance of CL(P), using segregation analysis of family data and population incidences in interracial crosses in Hawaii (Ching and Chung, 1974; Chung et al., 1974). Data from the latter

indicated that the factors controlling CL(P) were additive, and lacked heterotic effects expected of rare recessive genes in hybrid populations. Data from the former, while showing several alternatives to be unlikely, could not give discrimination between a polygenic threshold model and single locus recessive inheritance with reduced penetrance and no phenocopies. The high heritability of liability (.99) calculated under the polygenic model was considered evidence that the single gene model might be the better choice.

C. Induced Cleft Lip

(a) 6-aminonicotinamide

6-aminonicotinamide (6-AN) is an analogue of the vitamin nicotinamide that is toxic (Nanni et al., 1961; Johnson and McColl, 1955) and teratogenic (Pinsky and Fraser, 1960). It is thought to have its effects as a competitive inhibitor of nicotinamide functions by becoming incorporated into the coenzymes NAD (nicotinamide adenine dinucleotide) and NADP. Through interference with these coenzymes, many biochemical pathways are thought to be inhibited, including glycolysis, respiration, the terminal electron transport system and ATP synthesis. 6-AN has been found to reduce placenta size (Turbow and Chamberlain, 1968) and it has been suggested that some teratogenic effects may reflect alterations of the placenta, although 6-AN has also been shown to be teratogenic

when injected into the amniotic cavity of rats (Chamberlain and Goldyne, 1970). Although the teratogenic effects may be prevented by nicotinamide "protective" doses a short time before or after treatment (Pinsky and Fraser, 1960), at least one 6-AN induced biochemical effect, reduced ATP levels, has been shown to be partly irreversible for at least a day after treatment, in rat embryos (Ritter et al., 1975). The teratogenicity of 6-AN is thought to be due to reduced proliferation and differentiation in ectodermal tissues (Tuchmann-Duplessis, 1975, page 112).

Production of CL(P) in mice by 6-AN treatment was first reported by Pinsky and Fraser (1959) in a variety of unspecified genotypes. F₁ hybrid embryos of C57BL/6J and A/J strains were later reported to be sensitive to CL(P) induction (Pinsky and Fraser, 1960) as was the C57BL/6J strain itself (Leong, 1973). Heterogeneous mice that included C57BL/6 among the founding strains were also sensitive (Hamly, 1970). Apart from A/J (Goldstein et al., 1965), no genotypes resistant to CL(P) induction have been reported.

The critical period reported for CL(P) induction by "protected" 6-AN treatment has varied between day 9½ for F₁ embryos of C57BL/6J and A/J (Pinsky and Fraser, 1960) and heterogeneous mice (Hamly et al., 1970) and day 10½ for C57BL/6J (Leong, 1973, page 31).

(b) A/J resistance

A/J mice treated with 6-AN on days 9½, 10½, or 11½ of gestation with various intramuscular and intraperitoneal doses of 6-AN, followed 2 hours later by nicotinamide "protection," produced increased frequencies of CL(P) over the spontaneous rate in live term embryos after the day 10½ treatment (Goldstein et al., 1965). However, the absolute number of CL(P) embryos never exceeded the expected spontaneous frequency among implantations and the A/J strain thus appeared to be resistant to CL(P) induction. It was inferred that the CL(P) embryos were less susceptible to the embryocidal effects of 6-AN than their phenotypically normal littermates. This suggested that spontaneous CL(P) embryos are physiologically different from their genetically identical normal littermates before the cleft lip develops.

The possibility remains, however, that A/J embryos did respond to 6-AN with CL(P), and that mortality may have preferentially removed spontaneous CL(P) embryos, and that the numerical matches were fortuitous.

(c) Other teratogens

Relatively few teratogenic treatments are known to induce cleft lip in mice. For these, the gestational times of treatment cited were: for aspirin and A/J mice, multiple treat-

ments over days 8 and 9 or 9 and 10 (Trasler, 1965); X-irradiation and ddN mice, day 8½ (Murakami et al., 1962); dexamphetamine sulfate and A/J mice, day 8 (Nora et al., 1965); diphenylhydantoin and A/J mice, day 10½ (Johnston and Miller, 1977); diphenylhydantoin and Swiss-Webster mice, day 10 (Harbison and Becker, 1969).

D. Embryology.

Experimental investigation of normal embryonic face development and possible mechanisms of abnormal development have been recently reviewed (Johnston et al., 1977; Trasler and Fraser, 1977).

In mice the embryonic lip forms on day 10 or 11 of gestation. Cleft lip results from a lack of fusion between embryonic medial and lateral nasal processes. The fusion step requires the convergence of facial processes at the appropriate time with correct relative position and form and with no obstruction.

Numerous possible alterations in development that could lead to cleft lip have been envisaged, and some of them observed. For example, in mice a reduced medial nasal process, possibly due to diminished migration of neural crest cells has been associated with the cleft lip due to the Dancer gene. Conversely, the lateral nasal processes are reduced by the 6-aminonicotinamide treatment that produces cleft lip (Leong,

1973). Human embryos with cleft lip were observed to have retarded mesenchymal proliferation in the nasal and maxillary prominences (Andersen and Matthiessen, 1967). Hadacidin in rats has been observed to increase rates of autolysis and phagocytosis in the fusion zone and the resultant cleft lip is thought to be due to interference with fusion, or subsequent pulling apart of the processes.

Spontaneous CL(P) in mice was thought by Reed (1933) to arise from inadequate pressure of the maxillary processes on the lateral processes.

More recently, cleft lip in A strain mouse substrains and in the derived CL/Fr and L genotypes have been explained by a "face-shape" hypothesis (Trasler, 1968). This hypothesis suggests that the relative morphology of the developing faces of embryos of these genotypes differs from those less liable to CL(P). The cleft lip causing differences can be briefly described as a location nearer the midline of the nasal pits and medial nasal processes. The interpit distance can be taken as the quantitative variable on which a threshold occurs: if the interpit distance, relative to the rest of the developing face, is reduced beyond some critical point, the lateral and medial nasal processes will be unable to make contact and fuse. The hypothesis can be extended to include the following: if the interpit distance is reduced, relatively large lateral processes will be required for contact and fusion to take place at the normal time; with the usual developmental shift

of all facial processes toward the midline, the capacity for fusion must be extended if fusion is to take place after the relatively late contact of a normal sized lateral process with the medial process.

These quantitative variables thus incorporate thresholds: a critical distance between the nasal pits, smaller than which cleft lip will result. Alternatively, a critical time of contact may occur, after which fusion cannot take place.

Evidence supporting the "face-shape" hypothesis from L-line mouse embryos has been presented (Juriloff and Trasler, 1976).

Some controversy, cited by Gruneberg (1952b, page 366) was derived from Steiniger's observations that cleft lip in his mouse stock, related to the A strains sometimes resulted from the formation and breaking of cysts at the line of fusion, and subsequent tearing apart of the processes. However Steiniger also observed other cases where no fusion was ever present, and it thus appears that the cysts may be a second manifestation of the cause of cleft lip. It is interesting to speculate that the cysts represented the fusion of lateral and medial processes at a time when the potential for fusion was being lost.

Despite the hypotheses, the embryonic cause of lack of fusion of the medial and lateral nasal processes is still not understood. Yet another hypothesis (Trasler, 1966) suggests that pressure from the developing heart is required to provide

an adequate degree of convergence between the nasal processes.

The hypothesis concerning embryonic face shape has been extended into studies of adults. Assuming that embryonic face shape differences are at least partly inherited, and that they are maintained in some form into maturity, several experiments have sought correlations between adult face shape and liability to CL(P).

Some differences in relative size of facial bones of mice differing in liability to CL(P) were found (Machado, 1978).

For human CL(P), several experiments have detected small differences between relatives of probands and other control persons in facial morphology (Fraser and Pashayan, 1970; Erickson, 1974; Kurisu et al., 1974; MacGibbon and Preus, 1977).

For both mice and human beings, these differences can be summarized as a reduction in dimensions of midface structures.



INTRODUCTION

CL(P) in mice may be studied as an example of failure of developmental buffering. It is used as a developmental and genetic model of CL(P) in human beings, and knowledge of its cause or possible prevention therefore also has practical value.

CL(P) in mice was generally said to be a multifactorial threshold trait.

A/J strain inbred mice, which have about 10% CL(P) in newborns, had been reported to be resistant to 6-AN induced CL(P). That this was not a mere chance association was suggested by the apparent relative resistance of CL(P) embryos to 6-AN induced mortality.

A novel system of developmental buffering was suggested--that liability to spontaneous CL(P), expressed only about 10% of the time, protected against induction of CL(P) by at least one adverse environment.

The association also suggested that the basic metabolic lesion causing CL(P) might involve nicotinamide metabolism, and that CL(P) would therefore not be of "multifactorial" origin.

An experiment was designed to test whether 6-AN resistance and spontaneous CL(P) were pleiotropic effects of the same gene(s). The same experiment also allowed a search for evidence of segregation of a single major gene for CL(P).

This experiment used repeated backcrossing to A/J after a cross to a 6-AN sensitive strain having no spontaneous CL(P), C57BL/6J. The first backcross generation was expected to segregate for both spontaneous CL(P) and 6-AN resistance, and their co-segregation would provide evidence of pleiotropy (or linkage). The distributions of frequencies of spontaneous CL(P) produced by first and second backcross sires were expected to be bimodal in 1:1 and 3:1 ratios if the single major gene hypothesis were correct.

Studies were done to determine the critical periods for CL(P) induction by 6-AN so that the progeny of the first backcross generation could be treated at an appropriate time. Dosage response comparisons were done to test for gene-teratogen interactions.

The possible association of 6-AN resistance and spontaneous CL(P) was tested a second way--by their joint distribution among inbred strains.

Working from the hypothesis that spontaneous CL(P) in A/J was due to a major recessive gene with modifiers, the higher CL(P) frequency in CL/Fr mice was attributable to modifiers. This hypothesis was tested by observing the frequency of CL(P) in crosses between the strains.

Based on the presumed lesion in nicotinamide metabolism, massive doses of nicotinamide were administered to pregnant A/J and CL/Fr mice in an attempt to prevent CL(P).

A second treatment, previously reported to prevent CL(P),

thyroxin, was also used. It was thought that the nature of a cure for CL(P) would illuminate the biochemical lesion.

When genetic studies are done using species that require several months to produce segregating generations, studies must be run concurrently, rather than sequentially. Such was the case here. The results were not those expected, but they did contribute to the understanding of the development of CL(P) in mice, the genetics of spontaneous CL(P) and to a growing implication of duplicate epistasis as a common mechanism of developmental buffering.

MATERIALS AND METHODS - GENERAL

A/J and C57BL/6J mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. Females and A/J males were 6 to 12 weeks old when received. C57BL/6J males were 6 to 12 weeks, or 9 months of age (retired breeders). The latter, owing to the relatively long life expectancy of C57BL/6J mice, were fertile, and were used because young C57BL/6J males matured to breeding age at McGill became sick and failed to breed.

All mice were maintained in the McGill University Stewart Biology Building, fourth floor, mouse room. They were housed in polyethylene cages with stainless steel lids, wood chip bedding, and Purina Mouse Chow or Purina Lab Chow and tap water ad libitum. The light cycle was controlled and a dark cycle of 8 hours from 11 P.M. to 7 A.M. (Eastern Standard Time), was used.

Females were 2½ to 5 months old when bred, nulliparous, and had been at McGill for at least 2 weeks. Males were caged singly. Single females were placed with males, and remained for a few days if necessary. Females used in determination of time response curves were checked each morning for vaginal plugs. In other experiments they were checked at mid-day. Females that had mated were removed to fresh cages and housed in groups up to 5. Plug day was taken to be the first day of gestation--i.e., following the McGill convention, fertilization was assumed to have taken place at 2 A.M. the previous night (Bronson et al., 1966). Nine A.M. of the morning of

the plug would thus be designated as day 0, hour 7 of gestation (day 0/7).

By the usual convention for mouse breeding, the female's genotype was designated first in all crosses. For example, the F_1 of A/J and C57BL/6J from an A/J mother, was designated A.B6.

6-aminonicotinamide (6-AN), was obtained from Sigma Chemical Company. 6-AN from one bottle was used throughout all experiments. It was dissolved, when needed, in sterile distilled water, 45 mg per 20 ml.

Nicotinamide "protection" was not used because differences between genotypes would not have been clearly attributable to 6-AN sensitivity, ability to use the "protection", or both. In addition the use of more than one agent at a time greatly complicates dosage response studies (Finney, 1971).

Some statistical tests were frequently applied: χ^2 tests of independence, heterogeneity, and goodness-of-fit were used as described in Sokal and Rohlf (1969, pages 590, 581, and 552); Fisher's Exact Test is also described in Sokal and Rohlf (1969, page 595).

The significance level used for all tests was 5%.

CHAPTER A

GENOTYPE-TERATOGEN INTERACTION: TIME RESPONSE CURVES FOR A/J, C57BL/6J AND THEIR CROSSES

Introduction

This study was intended to determine the critical periods, if any, for 6-AN-induced CL(P) for C57BL/6J and A/J mouse strain embryos, and for some crosses of these strains. In addition, it was hoped that the putative resistance and differential mortality of A/J might be clarified by the properties of the "critical period", compared with those of the other genotypes.

Materials and Methods

The origin and maintenance of the mice has been described previously under "General Materials and Methods".

Five genotypes of embryos were tested: the A/J strain ("A"), the C57BL/6J strain ("B6"), their F_1 hybrid in A/J mothers ("A.B6"), their F_1 hybrid in C57BL/6J mothers ("B6.A") and the first backcross embryos of F_1 sires and A/J mothers ("A.AB6"). The F_1 sires were produced from untreated litters from A/J mothers and C57BL/6J sires at McGill.

All females that had had vaginal plugs were weighed and treated at designated times on day 10 of gestation with 12.26

mg/kg of 6-AN solution intraperitoneally. No nicotinamide "protection" was used. A/J was retested at a lower dosage, 7.36 mg/kg.

Females were assigned to treatment times at "random". For each time/genotype combination, progeny from an average of 10, and a minimum of 4, different sires, and a total of 8 to 15 litters were collected.

The treatment times, based on a pilot study using C57BL/6J, were: 10/6 (i.e., 8 A.M. on day 10 of gestation), 10/8, 10/10, 10/12, 10/14, and 10/16. The treatment time 10/18 was added to the series when A strain embryos were retested at 7.36 mg/kg. Early in the treatment series for B6.A embryos, 10/4 was added because there were no CL(P)'s recovered at the 10/12 and later treatment time litters obtained to that point.

Treated females were killed by cervical dislocation on day 17 of gestation. The uteri were examined for pregnancy and the uterine contents of pregnant females were examined under a dissecting microscope. Visible macerated "moles" and older dead embryos were counted as resorption sites. Live embryos and those that by developmental state and condition of tissue were judged to have been live until a few hours before examination were scrutinized, and the state of the lip and palate recorded. The sex of each embryo was assigned based on the distance between the genital papilla and anus (Kalter, 1965, page 82). All embryos were preserved in Bouin's fluid. Later, B.6, A.B6, B6.A, and A (12.26 mg/kg) embryos

were resexed by internal examination and discrimination between uteri and descended (ing) testes.

A series of untreated control embryos was collected for each genotype by killing some pregnant females at day 10/12 rather than treating them. The uteri were removed and fixed in Bouin's fluid for a few days, then transferred to 70% ethanol. The embryos were later examined under a dissecting microscope and the stage of lip formation (Trasler, 1968), and number of tail somites recorded.

Data from litters of less than 5 implantation sites (38 litters, 22 from C57BL/6J sites), were discarded.

In order to avoid bias due to litter effects, if present, in detection of the changes in frequency of CL(P) and resorption with treatment time, for each litter the proportion of CL(P) embryos in day 17 embryos, and the proportion of resorption sites in implantations were transformed to their Freeman-Tukey arcsine value for the binomial distribution (Mosteller and Youtz, 1961).

The mean transformed litter values for each time were then used in polynomial regression (Draper and Smith, 1968, page 150). The programs were written by Dr. M.A. Fieldes of the McGill Biology Department. Mean litter score, the dependent variable, weighted by the inverse of the variance of the sample at that time, was regressed against time of treatment, the independent variable. The regressions, within each genotype, were of the form:

$$1^{\circ} : Y = a + b_1x \quad (\text{i.e., linear regression})$$

$$2^{\circ} : Y = a + b_1x + b_2x^2 \quad (\text{i.e., a parabola})$$

$$3^{\circ} : Y = a + b_1x + b_2x^2 + b_3x^3 \quad (\text{i.e., 2 peaks})$$

where:

Y = time of treatment in hours after 10/0 $\pm$ 2

a = Y intercept from regression

b_1, b_2, b_3 = coefficients from regression

Resorption was fit to 1° and 2° polynomials; CL(P), to 2° and 3° . No higher degree polynomials were fit because they would not have been biologically interpretable. The "best" fitting regression was chosen on the basis of the significance of the highest order regression coefficient (Draper and Smith, 1968, page 169).

The data were also analyzed for other time trends, using χ^2 statistics. CL(P) embryos were classified into "right", "left", and "bilateral", and into groupings by time of treatment within each genotype. B6 and A.B6 embryos were grouped as: day 10/6 + 8, 10/10 + 12 and 10/14 + 16. B6.A embryos were grouped as day 10/4 + 6, 10/8 + 10, 10/12 + 14 + 16. For A.B6 embryos, day 10/6 + 8 + 10 and 10/12 + 14 + 16 formed the time groupings. A embryos were grouped as day 10/6 + 8 and 10/10 + 12 + 14 + 16 (+ 18 for 7.36 mg/kg). χ^2 tests of independence of laterality and treatment time were run within each genotype. The source of these χ^2 tests, and of those in the

comparisons described below has been described under "General Materials and Methods".

Data for all treatment times were then pooled and a similar test of independence of genotype and laterality was performed.

Similarly, an effect of time of treatment on sex ratio was sought. Within each genotype and lip type (normal or CL(P)), χ^2 tests of independence of sex and treatment time were run. The internal sex data were used where available. Treatment time groupings differed from those above for some genotypes: A.B6 embryos were grouped as day 10/6 + 8, 10/10 + 12, and 10/14 + 16; A (12.26 mg/kg) were grouped as 10/6 + 8 + 10 and 10/12 + 14 + 16; A (7.36 mg/kg) embryos were grouped as 10/6 + 8, 10/10 + 12, and 10/14 + 16 + 18.

Each of the normal and CL(P) groupings separately, and then pooled, were tested against a 50:50 sex ratio within genotypes, using goodness-of-fit χ^2 tests. Internal sex was used where available.

The results of external and internal sexing were compared. Since whole litters were stored together in one vial, individual comparisons could not be made. The internal method of determination is very accurate. The internal sex data were therefore taken to be the "true" or "expected" values, and the external sex data for 4 classes (normal, male and female, and CL(P), male and female), were compared against them in goodness-of-fit χ^2 tests. Since each subclass (normal and

CL(P)) had fixed frequencies, there are 2 degrees of freedom for this test.

To test whether normals or CL(P)'s were incorrectly sexed at different rates by the external method, for each genotype a heterogeneity χ^2 was calculated (Sokal and Rohlf, 1969, page 581), using the internal sex values as those expected.

Results

Time Response Curves

All of the genotypes tested produced CL(P) (Tables Ala and Alb). Higher frequencies of CL(P) were observed at the mid-range of treatment times than at the extremes for all genotypes, although the pattern was not clear-cut for A and A.AB6 embryos.

Differences in sensitivity were found. A embryos, if induced, (50%), A.AB6 embryos (50%) and B6 embryos (41%) at their peaks were more sensitive to CL(P) induction than the reciprocal F_1 embryos (18 and 8% for B6.A and A.B6, respectively).

Resorption rates tended to increase with later treatment time for all genotypes except B6.A embryos. Again, differences in sensitivity at the maximum points were observed. A embryos (97%) were most sensitive, followed by A.AB6 (84%). A.B6 embryos (59%) were at least as sensitive as the more resistant parental genotype, B6 (51%). B6.A embryos were most resistant (32%).

TABLE Ala

EFFECT OF TIME OF TREATMENT ON RESPONSE TO 12.26 MG/KG 6-AN

Time of Treatment Day/Hour	Number of Litters	Number of Implants	% Resorbed	Number of Embryos	% CL(P) in Embryos (n)	% of Litters with CL(P)
<u>C57BL/6J × C57BL/6J: C57BL/6J embryos</u>						
10/6	12	102	20.6	81	12 (10)	33
10/8	12	105	25.7	78	24 (19)	75
10/10	12	113	38.9	69	32 (22)	75
10/12	12	98	42.9	56	41 (23)	67
10/14	13	124	50.8	61	11 (7)	46
10/16	14	118	44.1	66	11 (7)	36
<u>C57BL/6J ♀ × A/J: F₁ embryos</u>						
10/4	12	117	17.1	97	2 (2)	17
10/6	13	123	25.2	92	20 (18)	38
10/8	12	107	15.9	90	17 (15)	33
10/10	14	130	18.5	106	10 (11)	36
10/12	12	107	31.8	73	3 (2)	17
10/14	13	106	13.2	92	1 (1)	8
10/16	12	110	28.2	79	3 (2)	8
<u>A/J ♀ × C57BL/6J: F₁ embryos</u>						
10/6	11	113	23.9	86	0 (0)	0
10/8	11	114	38.6	70	9 (6)	36
10/10	12	108	39.8	65	12 (8)	42
10/12	12	106	47.2	56	13 (7)	50
10/14	13	126	58.7	52	6 (3)	23
10/16	10	88	55.7	39	3 (1)	10

TABLE Alb

Time of Treatment Day/Hour	Number of Litters	Number of Implants	% Resorbed	Number of Embryos	% CL(P) in Embryos (n)	% of Litters with CL(P)
<u>A/J ♀ × F₁(AC): BC₁ embryos</u>						
10/6	11	111	46.0	60	2 (1)	9
10/8	11	119	60.5	47	34 (16)	45
10/10	12	119	79.8	24	50 (12)	58
10/12	13	137	81.8	25	32 (8)	38
10/14	14	168	83.9	27	19 (5)	21
10/16	15	153	81.1	29	45 (13)	40
<u>A/J × A/J: A/8 embryos</u>						
10/6	13	119	58.8	49	22 (11)	62
10/8	10	100	77.0	23	39 (9)	40
10/10	9	94	90.4	9	44 (4)	44
10/12	16	153	92.8	11	36 (4)	25
10/14	13	150	97.3	4	50 (2)	15
10/16	14	146	97.3	4	25 (1)	7
<u>A/J × A/J Treated with 7.36 mg/kg</u>						
10/6	10	93	39.8	56	13 (7)	40
10/8	9	101	46.5	54	11 (6)	33
10/10	11	115	72.2	32	25 (8)	36
10/12	9	93	83.9	15	20 (3)	33
10/14	10	99	77.8	22	18 (4)	30
10/16	10	100	67.0	33	6 (2)	20
10/18	8	89	55.1	40	10 (4)	38

The high resorption frequencies led to small day 17 embryo samples for A and A.AB6 and prompted the retest of A at a lower dosage, 7.36 mg/kg. A tendency for resorption to peak at the middle treatment periods (84%) was seen in this retest.

These trends in untransformed pooled litter data were recovered for each genotype in the mean litter arcsine values for CL(P) and resorption rate for each time of treatment (Table A2).

Weighted polynomial regression of transformed litter CL(P) frequencies against time gave significant ($P < .05$) 2° or 3° regressions for B6.A, A.B6, A.AB6, and A embryos (Table A3). This fit of the data to curves with peaks suggests that for these genotypes, critical periods have been located. The fit of B6 was nonsignificant, although there was a trend toward a peak at 10/10 to 10/12. The scatter of data points about the best fitting line (Figure A1) suggests that the data are not explained by the curve specified in the regression model, but by another unimodal curve. There is no a priori reason to expect the models used to fit the data well.

Nonetheless, polynomial regression may be a useful method for testing the reality and location of peak response times. It was previously used in this way by Landauer and Bliss (1946).

From the curves (Figure A1), peak response times for 6-

TABLE A2

EFFECT OF TIME OF TREATMENT ON RESPONSE TO 12.26: TRANSFORMED DATA

Time	C57BL/6J x C57BL/6J	C57BL/6J ♀ x A/J	A/J ♀ x C57BL/6J	A/J ♀ x F ₁	A/J x A/J	A/J x A/J (7.36 mg/kg)
<u>Mean Litter Freeman-Tukey Arcsine % CL(P)/Embryos ± S.E.</u>						
10/4	-	11.90±1.46	-	-	-	-
10/6	24.77±6.71	29.58±7.77	9.61±0.37	14.16±2.44	31.35± 5.34	22.55±4.65
10/8	29.28±4.69	24.51±7.60	22.68±6.32	36.84±8.79	40.06± 8.64	19.55±3.34
10/10	38.03±6.25	19.13±4.22	25.85±6.35	45.50±4.77	43.75± 8.31	28.61±5.81
10/12	37.32±5.71	14.26±2.90	26.36±4.93	36.77±6.10	51.25± 6.82	33.17±9.22
10/14	25.63±5.05	11.54±1.17	18.40±2.05	27.28±6.72	45.00±12.99	30.84±8.38
10/16	26.69±6.20	16.50±5.71	17.08±4.25	39.86±6.70	35.88±15.87	18.83±2.88
10/18	-	-	-	-	-	20.28±3.25
<u>Mean Litter Freeman-Tukey Arcsine % Resorption/Implants ± S.E.</u>						
10/4	-	24.43±3.03	-	-	-	-
10/6	24.75±5.17	29.80±6.55	30.76±5.43	43.18±5.68	51.50± 3.19	40.35±3.59
10/8	30.46±2.81	22.97±3.76	38.35±3.74	53.92±8.31	62.89± 5.46	35.62±5.97
10/10	37.53±4.33	25.36±3.28	38.83±6.12	63.69±4.53	70.71± 3.10	59.11±3.76
10/12	39.18±5.02	34.32±7.61	43.18±4.56	66.83±5.12	74.07± 2.40	67.16±5.40
10/14	45.11±3.85	21.82±2.61	52.95±3.91	68.23±5.18	78.12± 1.58	62.95±4.94
10/16	42.88±5.04	32.27±6.97	47.21±7.49	65.04±4.12	78.16± 1.78	55.87±7.19
10/18	-	-	-	-	-	48.88±9.33

FIGURE A1. EFFECT OF TIME OF 6-AN TREATMENT ON CL(P) RESPONSES

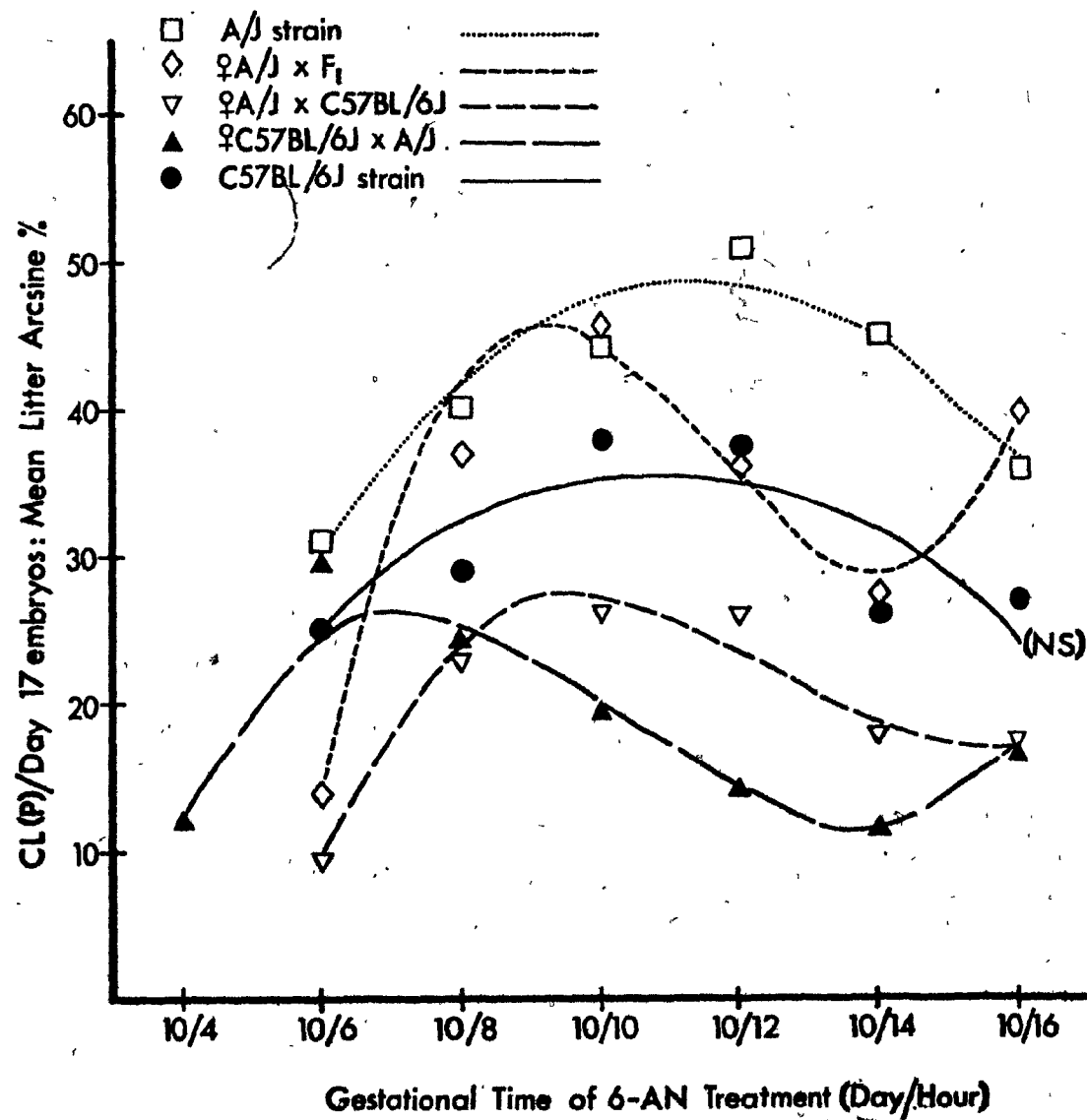


TABLE A3

WEIGHTED REGRESSIONS OF MEAN CL(P)/LIVE FREQUENCY ARCSINE AGAINST TIME OF
6-AN TREATMENT FOR A/J AND C57BL/6J AND CROSSES

Embryo Genotype	Degree of Polynomial	Intercept ± S.E.	Orthogonal Regression Coefficients ± S.E.			(df)
			b_1	b_2	b_3	
C57BL/6J	3°	29.70 ± 2.19	0.0256 ± 1.3724	-1.7839 ± 0.9636	0.2985 ± 0.6491	(2)
	2°	29.74 ± 1.88	-0.1185 ± 1.1473	-1.7709 ± 0.8270		(3)
B6·A	3°	17.96 ± 0.83	-0.7644 ± 0.2423	-0.6597* ± 0.1887	0.8307** ± 0.1375	(3)
	2°	13.48 ± 1.44	-0.8137 ± 0.7606	-0.7694 ± 0.5900		(4)
A·B6	3°	19.92 ± 0.80	0.5077 ± 0.2935	-2.0210** ± 0.2739	0.7671 ^a ± 0.2052	(2)
	2°	17.85 ± 1.33	0.9189 ± 0.6279	-1.7789 ^a ± 0.6143		(3)
A·AB6	3°	33.90 ± 1.26	2.2304 ± 0.7287	-2.2633* ± 0.4893	2.2639* ± 0.4164	(2)
	2°	32.12 ± 3.95	2.6504 ± 2.3502	-3.1084 ± 1.5049		(3)
A/J	3°	41.40 ± 1.11	1.3679 ± 0.6967	-2.3902* ± 0.4329	0.4965 ± 0.3442	(2)
	2°	41.61 ± 1.28	1.3877 ± 0.8124	-2.3339* ± 0.5028		(3)
(A/J; 7.36 mg/kg)	3°	22.17 ± 2.21	-0.1650 ± 0.9581	-0.6084 ± 0.7332	0.0973 ± 0.3534	(3)
	2°	22.18 ± 1.93	-0.2029 ± 0.8315	-0.5484 ± 0.6139		(4)

a = P < 0.1

* = P < 0.05

** = P < 0.01

$$t = b/\sigma_b$$

AN-induced CL(P) under the conditions of this experiment may be estimated to be 10/11 for A and B6, 10/9 for A.B6 and A.AB6, and 10/7 for B6.A.

The ranked order of sensitivity, from greatest to least, in order, was: A, A.AB6, B6, and, equally, A.B6 and B6.A. The relative resistance of the F_1 embryos suggests that there is some heterosis protecting face development, as well as a heterotic effect speeding developmental rate, as indicated by the earlier peak times.

The early peak of A.AB6 embryos, similar to that of A.B6, is interesting because if A is sensitive, A.AB6 would be expected to lie between A.B6 and A, being genetically intermediate to them. It suggests that the responding A.AB6 embryos are developmentally more like A.B6 than A. These A.AB6 embryos may have a larger variance of development (Table A4) than A.B6 or A embryos. This supports the view that some aspect of genetic heterogeneity contributed to the A.AB6 response.

The second, later, CL(P) response peaks suggested by fit to 3rd polynomials for A.AB6, A.B6 and B6.A embryos may be real. Only experiments extending treatment time through the area of these putative second peaks can verify them. The distribution of embryonic development is not known to be bimodal, and no evidence of bimodality is seen in the data of Juriloff and Trasler (1976) for mouse embryonic face development. Consequently, a second peak of CL(P) induction would be likely to

TABLE A4
DEVELOPMENTAL STATE OF UNTREATED EMBRYOS AT 10/12

Embryo Genotype	Number of Litters	Number of Embryos	Face Stage, Percent					Number of Tail Somites, Percent					
			Preoval	Oval	Oblong	Crescent	Comma	0	1,2	3,4	5,6	7,8	9,10
C57BL/6J	2	17	12	70	18	-	-	18	47	24	12	-	-
B6-A	4	38	-	55	24	21	-	-	16	29	29	18	8
A-B6	5	38	8	82	11	-	-	11	55	24	8	3	-
A-AB6	4	42	26	64	7	2	-	24	33	24	10	7	2
A/J	2	13	38	62	-	-	-	46	38	15	-	-	-

be due to a second mechanism, i.e., interference with a different developmental step (Wilson, 1965, page 254).

The asymmetry of the curves for A.AB6, A.B6, and B6.A is also noteworthy. It suggests that developmental variation may not be normally distributed, but rather occurs as a wave comprising the majority, followed by a trailing minority of embryos.

There is evidence of maternal-embryo-genotype interaction influencing developmental rate in that B6.A embryos and A.B6 embryos did not share the same critical period. Untreated B6.A embryos were more advanced in face development, and in overall developmental rate, as monitored by tail somite count, than other crosses at day 10/12 (Table A4). 21% had reached the step of fusion of medial with lateral nasal processes, and would be expected to be past any critical period for CL(P) induction. Its early critical period, correlated with advanced development, may also explain the lack of CL(P) in the same cross after day 10½ treatment in the experiments of Pinsky and Fraser (1960).

Resorption frequencies after arcsine transformation also showed trends similar to those in the pooled litter data (Table A2). Weighted polynomial regression of transformed litter resorption frequencies against treatment time gave significant ($P < .05$) linear or 2° regressions (Table A5) for all genotypes except B6.A (Figure A2).

All genotypes except B6.A showed a significant increase

FIGURE A2. EFFECT OF TIME OF 6-AN TREATMENT ON RESORPTION RESPONSES

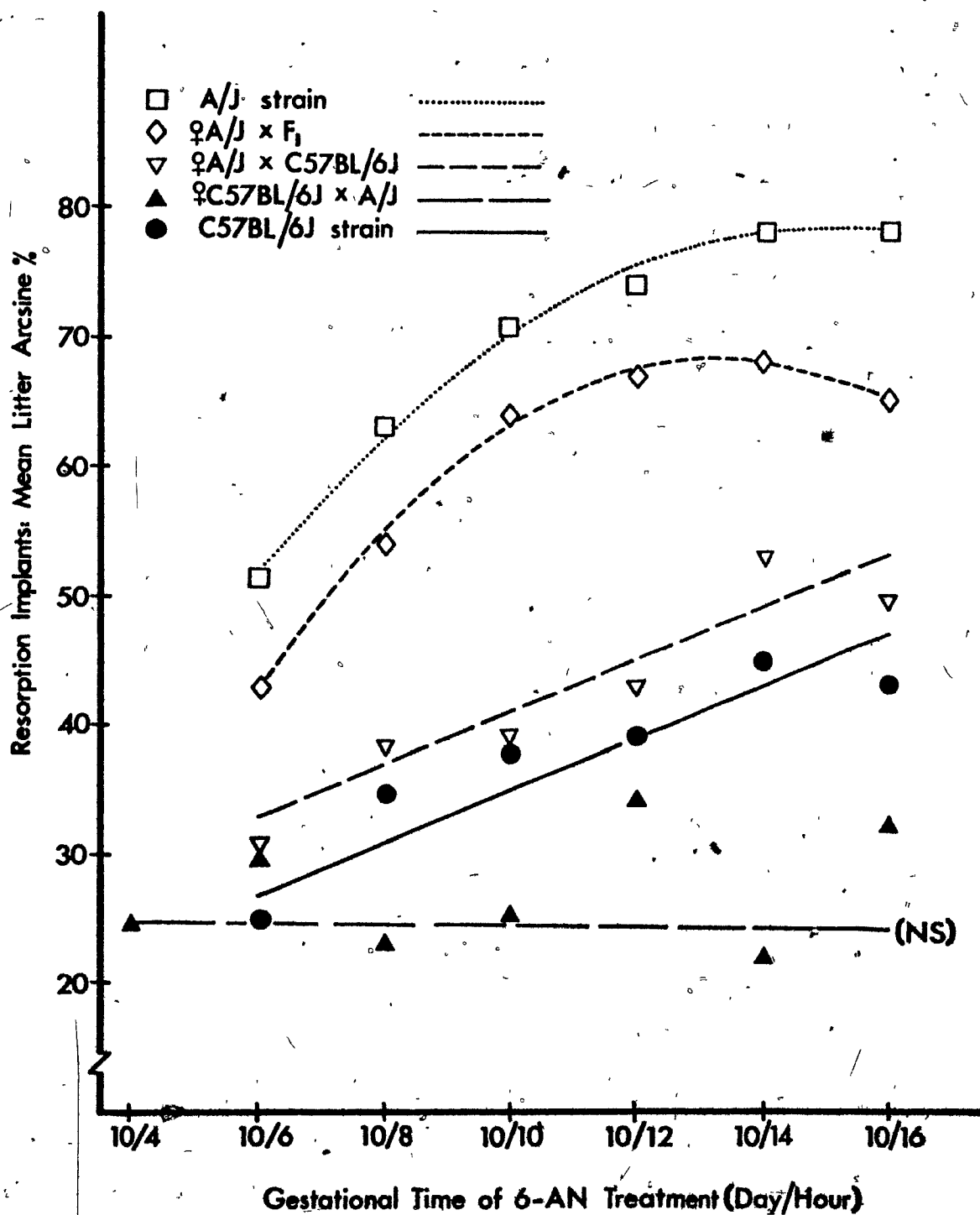


TABLE A5

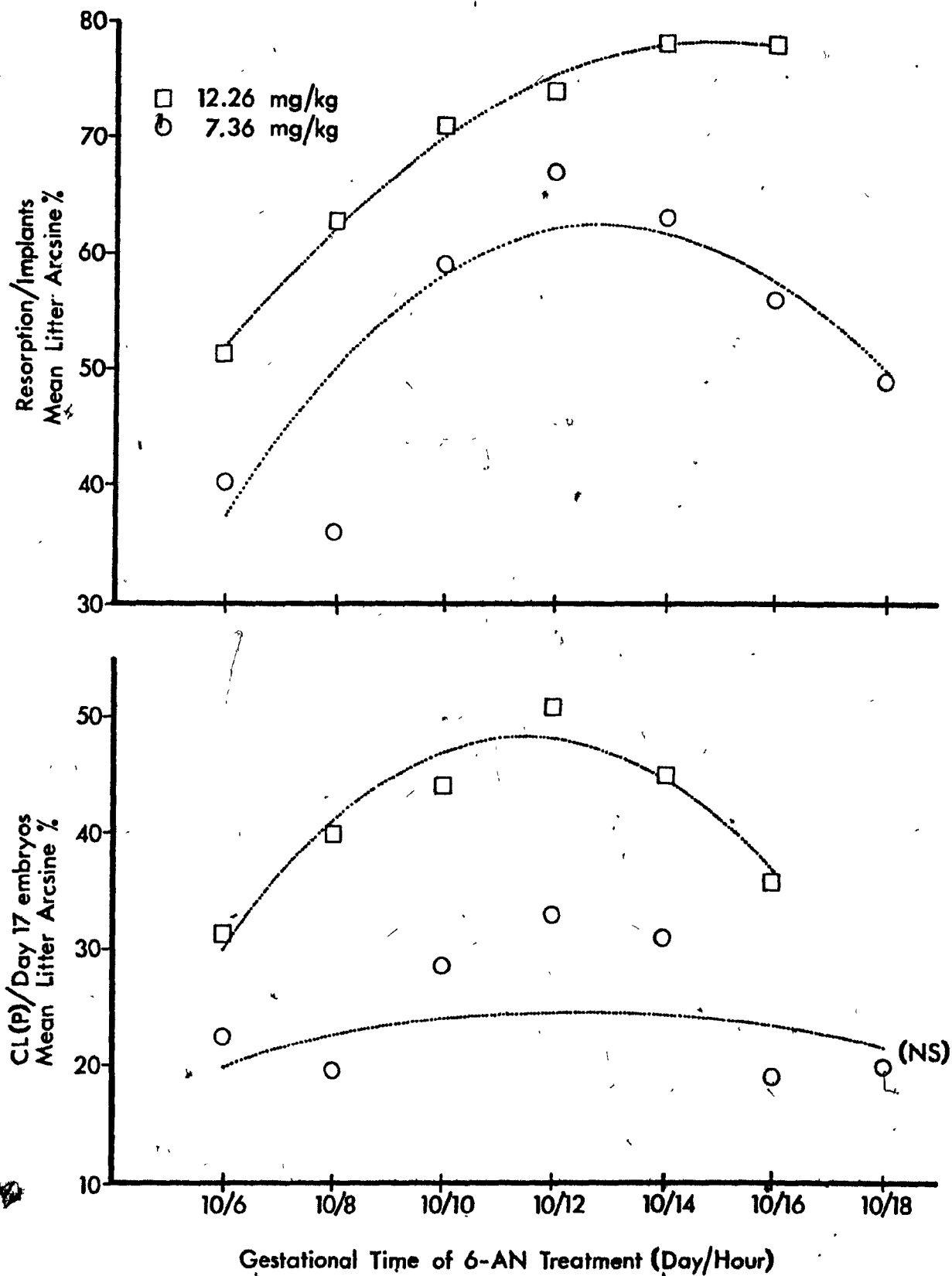
WEIGHTED REGRESSIONS OF MEAN RESORPTION FREQUENCY ARCSINE AGAINST
TIME OF 6-AN TREATMENT FOR A/J AND C57BL/6J AND CROSSES

Embryo Genotype	Degree of Polynomial	Inter- cept $\pm$ S.E.	Orthogonal Regression Coefficients $\pm$ S.E.		
			b_1	b_2	(df)
C57BL/6J	2°	36.68 $\pm$ 0.70	4.1161*** $\pm$ 0.4322	-0.7909 $\pm$ 0.3286	(3)
	1°	36.98 $\pm$ 1.02	4.1084*** $\pm$ 0.6408		(4)
B6•A	2°	24.55 $\pm$ 1.61	-0.0634 $\pm$ 0.8509	0.0426 $\pm$ 0.5055	(4)
	1°	24.55 $\pm$ 1.44	-0.0898 $\pm$ 0.7097		(5)
A•B6	2°	42.81 $\pm$ 1.62	4.0150* $\pm$ 1.0120	-0.4398 $\pm$ 0.7617	(3)
	1°	42.99 $\pm$ 1.45	4.1183*** $\pm$ 0.9093		(4)
A•AB6	2°	60.28 $\pm$ 0.28	4.3843*** $\pm$ 0.1647	-1.8681*** $\pm$ 0.1055	(3)
	1°	60.24 $\pm$ 2.51	4.0556*** $\pm$ 1.4559		(4)
A/J	2°	69.23 $\pm$ 0.35	5.2605*** $\pm$ 0.2087	-1.2716** $\pm$ 0.1312	(3)
	1°	69.45 $\pm$ 1.75	4.7370*** $\pm$ 0.9920		(4)
(A/J; 7.36 mg/kg)	2°	54.06 $\pm$ 3.00	2.1457 $\pm$ 1.6565	-2.0409 ^a $\pm$ 0.8175	(4)
	1°	55.72 $\pm$ 4.18	4.0704 $\pm$ 2.0975		(5)

a = $p < 0.1$ * = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$

$$t = b/\sigma_b$$

FIGURE A3. EFFECT OF TIME OF 6-AN TREATMENT ON CL(P) AND RESORPTION RESPONSES OF A/J AT TWO DOSAGES



in resorption frequency with later treatment times. For some (A, A.AB6), better fit was obtained to curves with levelling off or decrease at later treatment times. Deviation of A.B6 and B6 means from linear regression follow a similar pattern. The retest of A embryos, which was extended to 10/18 (Figure A3) shows evidence of a peak in resorption rate, but no significant regression was obtained. A peak in resorption frequency was seen in the response to single doses of diphenylhydantoin in Swiss-Webster mice, but on day 9 (Harbison and Becker, 1969).

Like the CL(P) response, the transformed resorption response showed evidence of differences between genotypes in dosage sensitivity. A appeared to be most sensitive, B6 less sensitive, A.B6 and A.AB6 intermediate, with A.AB6 more similar to A, as would be expected. Unlike the CL(P) sensitivity, where reciprocal F_1 's were equal and most resistant, resorption sensitivity between reciprocal F_1 's was markedly different, with A.B6 intermediate, and B6.A most resistant.

In addition to relative resistance, the B6.A embryos showed no change in resorption rate over the times tested. Recalling the more advanced development of these embryos, it is possible that their potential peak occurred prior to the treatment times used, although to do so, it would have to have occurred earlier relative to the critical period for CL(P) than in other genotypes.

Although an initial relationship based on dosage

sensitivity may be present, the changes in rank of sensitivity, and differences in shapes of the curves for the two responses, CL(P) and resorption, suggest that resorption is unlikely to be merely a more extreme expression of the teratogenic CL(P) response, as is sometimes suggested (Jusco, 1972).

Whether CL(P) was induced in A embryos remains unclear. The number of CL(P) embryos recovered never exceeded 10% of the number of implantations, the expected spontaneous frequency (Table A1). Both dosages used gave "peaks" at 10/12, though only that for the high dosage was significant (Table A3). In the face of the mortality patterns (Figure A3), differential mortality could explain the CL(P) peaks if spontaneous CL(P) embryos were more resistant than normals to resorption until about 10/10 and then became increasingly more equal to normals in resorption rate.

A.B6 embryos were also expected to have a low spontaneous CL(P) incidence: approximately 2 to 4% (Chapter F), or 13 to 26 of the 655 implantations. That only 26 CL(P) embryos were recovered is not convincing evidence that CL(P) was induced in this cross. The "resistance" trait of A strain may have been recovered, but based on genetics and the sensitivity of A.B6 embryos, a maximum of 50% of the A.B6 embryos (1 gene) would have recovered such resistance.

Other treatment time effects: CL(P) type

It was thought that the type of CL(P) (left, right, bilateral) induced might vary with treatment time (Table A6).

56% of all clefts observed were bilateral, 24% were unilateral right, and 20% were unilateral left. Within each genotype, no significant associations ($P > .05$) between laterality of CL(P) and treatment time were obtained.

No significant interaction between genotype and laterality of clefts for pooled times was found ($\chi^2_{10 \text{ df}} = 17.49$; $P > .05$). This suggests that the determinant of laterality of induced and spontaneous CL(P) is the same, since at least some in A were spontaneous.

Cleft lip was nearly always associated with cleft palate (Table A6) and the numbers of cleft lip without cleft palate were insufficient to test for time trends. All but 16 of 287 cleft lips were accompanied by cleft palate (CLP), and 12 of these were B6 embryos. This effect of the B6 genotype in "protecting" against cleft palate was significant when compared with the other pooled genotypes ($\chi^2_1 \text{ df} = 12.84$, with Yates' correction factor; $P < .05$).

Sporadic median facial clefts were observed, particularly in the B6 embryos, but at very low frequencies. Cleft palate (Table A6) was also observed at low frequencies, and it seemed

TABLE A6

EFFECTS OF TIME OF 6-AN TREATMENT ON TYPE OF CL(P) INDUCED

Embryo Genotype	Treatment Time	Number of CL(P)			% CLP in CL(P)	Number		
		Right	Left	Bilateral		CL	Median	CP
C57BL/6J	10/ 6+10/8	8	12	9	83	5	4	1
	10/10+10/12	10	8	27	87	6	11	5
	10/14+10/16	2	5	7	93	1	11	6
B6.A	10/4 +10/6	8	5	7	90	2	0	0
	10/8 +10/10	4	7	15	100	0	0	3
	10/12+10/14+10/16	1	1	3	80	1	0	2
A.B6	10/6 +10/8	0	2	4				
		*{2	3	3	100	0	0	3
	10/10+10/12	5	1	1	93	1	3	9
	10/14+10/16	{1	2	1	100	0	0	2
A.AB6	10/6 +10/8	3	1	13	100	0	0	1
	10/10+10/12	3	5	12	100	0	1	3
	10/14+10/16	4	4	10	100	0	0	5
A/J (12.26 mg/kg)	10/6 +10/8	1	7	12	100	0	0	0
	10/10+10/12	*{1	1	9	100	0	0	0
	10/14+10/16	0	0	3	100	0	0	0
A/J (7.36 mg/kg)	10/6 +10/8	1	2	10	100	0	0	1
	10/10+10/12	*{1	3	7	100	0	0	0
	10/14+10/16+10/18	2	0	8	100	0	0	2
Totals		57	69	161				
		% 20	24	56				

* Pooled for χ^2 tests

1 Associated with CL

to show a trend toward/increase at later times of treatment, although the numbers were too small to test.

Sex ratio

No significant association between treatment time and sex ratio within CL(P) and normal groupings, for any genotype, was obtained (all $P > .05$) (Table A7).

Using the internal sex data for B6, B6.A, A.B6 and A (12.26 mg/kg), and external for A.AB6 and A (7.36 mg/kg), no significant deviation from a 50:50 sex ratio was found for the pooled time data for CL(P) and normal embryos separately and pooled for B6, B6.A, A.AB6 and A (7.36 mg/kg) genotypes. A significant excess of females in the normal and pooled grouping of A.B6 embryos was found ($\chi^2_1 \text{ df} = 6.44$ and 7.35 ; $P < .05$). A significant excess of females was also found for the CL(P) and pooled groupings of A (12.26 mg/kg) embryos ($\chi^2_1 \text{ df} = 5.76$ and 6.76 ; $P < .05$), and the normals followed a similar, non-significant trend.

Unequal liability to CL(P) would result in mirror-image inequalities of sex ratio between normals and CL(P)'s. This was not seen. It therefore seems that differential mortality of the sexes, apart from CL(P), may have been present. Specifically, males bearing the A strain X chromosome may have survived 6-AN treatment less well than the other genotypes. A similar effect, expected in A.AB6 and A (7.36 mg/kg) embryos

TABLE A7
TIME TRENDS IN SEX RATIO

Embryo Genotype	Treatment Time	Normals		CL (P)	
		n	% ♂	n	% ♂
C57BL/6J	10/6 +10/8	130	55	29	72
	10/10+10/12	80	53	45	38
	10/14+10/16	113	51	14	64
B6·A	10/4 +10/6	169	55	20	60
	10/8 +10/10	170	49	26	38
	10/12+10/14+10/16	239	47	5	80
A·B6	10/6 +10/8	150	46	6	67
	10/10+10/12	106	37	15	33
	10/14+10/16	87	46	4	25
A·AB6 *	10/6 +10/8	90	51	17	59
	10/10+10/12	29	59	20	45
	10/14+10/16	38	55	18	61
A/J at 12.26 mg/kg	10/6 +10/8 +10/10	57	44	24	33
	10/12+10/14+10/16	9	22	10	20
A/J at 7.36 mg/kg *	10/6 +10/8	97	57	13	54
	10/10+10/12	36	53	11	73
	10/14+10/16+10/18	85	51	10	60

*Not internally sexed.

may have been obscured by possible systematic errors in the sexing method used there.

External and Internal Sex Determination
of 6-AN Treated Embryos

The sex ratios obtained after internal sexing differed from those obtained from the external examination for all genotypes (Table A8). Different types or rates of errors were made for normal compared with CL(P) embryos for all but the A genotype, as shown by significant heterogeneity χ^2 values (Table A8). For B6 embryos, normal males were missexed as females, while CL(P) females were missexed as males. Female B6.A embryos with CL(P) were missexed as males. For A.B6 embryos, both normal and CL(P) females were missexed as males, but CL(P)'s at a higher rate. Normal and CL(P) females of the A genotype were missexed as males at an equal rate.

These systematic errors may reflect an effect of 6-AN on relative proportions of body parts, which are a point of reference in judging sex by the relative distance between the genital papilla and anus. Further study is required, but it is clear that external sexing after 6-AN treatment is unreliable.

TABLE A8

ASSOCIATION BETWEEN CL(P) AND SEX FOR POOLED TIME CURVE DATA

Genotype	External Sex				Difference $+x^2_{2df}+$	Internal Sex				Heterogeneity x^2_{1df}
	Normal		CL(P)			Normal		CL(P)		
	♂	♀	♂	♀		♂	♀	♂	♀	
♀ First										
C57BL/6J	151	172	59	29	7.94; p<.05	171	152	51	37	6.53; p<.05
B6•A	291	287	35	16	6.42; p<.01	288	290	26	25	5.50; p<.05
A•B6	163	180	17	8	10.84; p<.01	148	195	10	15	5.48; p<.05
A•AB6	84	73	30	25						
A/J (12.26 mg/kg)	43	23	21	13	33.18; p<.001	27	39	10	24	1.91; N.S.
A/J (7.36 mg/kg)	117	101	21	13						

Summary

"Peaks" in the critical period for unprotected 6-AN induction of CL(P) were observed for all genotypes tested, and were within day 10 of gestation, but not all were statistically significant.

A.B6, B6.A and A.AB6 embryos appeared to peak 2 to 4 hours earlier than A, and B6 embryos. For B6.A and A.B6 this is probably the result of faster development. For A.AB6 embryos, the explanation may be more complex, although a portion of that segregating population that was genetically similar to F_1 embryos may also have developed relatively precociously.

Resorption responses were found to increase to the end, or near to the end, of the treatment times used. Some evidence of levelling off or decrease was found for the later times.

Based on differences in the order of relative sensitivity of genotypes to CL(P) and resorption, and on the differences in response of the two traits to varying treatment time, it was concluded that CL(P) and resorption are different responses to 6-AN; they are not part of a continuum.

The question of resistance of spontaneous CL(P) embryos to resorption after 6-AN treatment, and the possible resistance of A strain to CL(P) induction was not resolved, although the pattern of response offered little support for their being

resistant.

No effects of treatment time or genotype on the laterality of CL(P), nor on sex ratio of CL(P) or normal embryos were detected.

Although 94% of all cleft lips were accompanied by cleft palate, a significantly greater tendency of B6 embryos, compared to the other genotypes, to respond with isolated cleft lip, was found.

Significant excesses of females compared with a 50:50 sex ratio were found for normal A.B6 and CL(P) A (12.26 mg/kg) embryos. They were inferred to result from higher mortality of males carrying the A strain X chromosome, compared to other genotypes.

The usual external method of sexing embryos was found to be systematically biased under the conditions of this experiment. Females were misclassified as males at a rate dependent on genotype and an interaction between genotype and lip type.

CHAPTER B.

GENOTYPE-TERATOGEN INTERACTION: ANALYSIS OF DOSAGE RESPONSE OF A/J, C57BL/6J AND THEIR RECIPROCAL F₁'S

Probit Analysis

Although not a new technique, the treatment of dosage response data by probit analysis has recently been proposed as a means of investigating genotype-teratogen interactions, and the derived "ED₅₀" as a way of expressing response traits (Biddle, 1977; Biddle and Fraser, 1976; 1977b, c).

Probit analysis was therefore used here to attempt to determine whether heterozygotes for genes for spontaneous CL(P) show interaction with 6-AN with respect to induced CL(P), compared with C57BL/6J embryos.

All analyses were conducted as described by Finney (1971).

Probit analysis can be described as being analogous to weighted linear regression, with the exception that the data used are binary and therefore follow the binomial variance rather than being continuous and having the Gaussian (normal) distribution. While binomial data can be "normalized" by other appropriate transformations (e.g., arcsine), such transformations do not apply well to very low or very high frequencies (see, for example, Bliss, 1957 and Mosteller and Youtz, 1961).

The concepts and assumptions underlying probit analysis are as follows: that the actual trait measured is not the binomial one observed, but an underlying continuous trait, dosage-tolerance, which for a given genotype is normally distributed on a log-dose scale; that as dosage is increased, beginning at the "sensitive" end of the distribution, a part of the population reaches a critical value, and flips to its alternate state--"affected" or "responding" (this old concept seems to have much in common with that proposed in "Catastrophe Theory" reviewed by Zeeman, 1976); that as dosage increases the affected proportion of the population encroaches on a larger and larger area of the distribution; that a plot of the proportion affected with increasing dosage is equivalent to cumulating the area under a normal curve, and is non-linear; that the cumulated plot can be made linear by conversion of frequencies to units of standard deviation from the mean of the distribution; that these units of standard deviation are converted to all positive values by the addition of 5 units--such values are probits.

Thus, if dosage tolerance is normally distributed on a log scale, the relationship between dosage and percent response is expected to be linear on a probit, log dose scale. The "spread" of the distribution, its variance, will be translated into the slope of the line, while the midpoint will continue to be the dosage that causes 50% of embryos to respond, the ED₅₀. It is axiomatic that homogeneous populations that

have different tolerance variances must not have perceived dosage change in the same way--there is interaction. Therefore nonparallel probit, log dose response slopes are interpretable as interaction.

In addition to the assumption that the tolerance distribution is normal on a log scale (or can be made normal by use of another scale) are the assumptions that the probability of response for each unit (e.g., embryo) is independent of all others at that dosage and that the dosage range tested is equally spread about the ED_{50} .

Probit analysis weights each data point by sample size and by the variance expected for the frequency predicted by the incipient regression line. It offers the advantage of being able to incorporate zero and 100% response information into the determination of the best fitting regression. This regression line is a maximum likelihood fit, arrived at by a series of iterations.

Inappropriateness of the log scale is detectable as systematic deviations from linear probit regression. Non-independence of responding units (e.g., random litter effects) will be reflected in increased scatter of data points about the regression line, detected as significant heterogeneity. The presence of such heterogeneity greatly increases the calculated confidence limits about the regression line.

Previous reported use of probit analysis on the CP trait induced by cortisone (Biddle and Fraser, 1976) exhibited

evidence of heterogeneity of response. Litter effects are assumed to be widespread in mammalian teratogenic responses (Weil, 1970; Haseman and Hogan, 1975). It seems reasonable to expect that embryos sharing the same uterus at the same time will have more environmental effects in common than embryos in the same uterus at another time, or embryos in another uterus, and to expect that there are more determinants of the probability of response than simply dosage and genotype.

Despite this shortcoming, the application of probit analysis to teratogenic response has been relatively fruitful (Biddle, 1977; Biddle and Fraser, 1976, 1977b, and c).

To identify Mendelian factors underlying strain differences in teratogenic responses by analysis of segregating generations requires that no interaction be present; that the same relative difference between genotypes exists at any pair of frequencies (dosages) (Lewontin, 1974). This is of course not true of percentage data but may be true on a transformed scale. Thus, before genetic comparisons are made, the possibility of large interaction should be ruled out by fit to parallel, probit, log dose regressions. The ED_{50} then becomes a convenient way of expressing the trait. For this reason, and because of its apparent promise, the lack of demonstrably better methods, and lack of information about dosage response of the 6-AN induced CL(P) trait, probit analysis was used to attempt to find evidence of interaction between the gene(s) for spontaneous CL(P) and 6-AN induced CL(P) dosage response.

Materials and Methods

A/J and C57BL/6J mice were obtained from the Jackson Laboratory and were maintained and bred as previously described (General Materials and Methods). All were fed Purina Mouse Chow.

Females that had had vaginal plugs were treated on day 10 of gestation with an intraperitoneal injection of 6-AN solution. Assignment of females to dosage was "random".

Treatment times were chosen to be near the peak response times from the time response curves (Chapter A). C57BL/6J was treated at 10/12; B6.A, at 10/8; A.B6, at 10/10; and A/J at 10/10 (all $\pm$ 1 hour). A series of untreated controls of each genotype was collected concurrently.

The volume of 6-AN solution injected was based on the female's body weight at the time of injection.

Following the practice of conventional linear regression, a large number of dosage levels with moderate numbers of embryos at each was thought to be preferable to a few dosage points each with many embryos. Consequently, for C57BL/6J, using a range guessed to straddle the ED_{50} (from the experience of a pilot study), 12 dosages, approximately equally spaced on a linear scale, between 4.905 and 17.775 mg/kg were each tested, with a minimum of 6 litters at each. Based on the B6 response, B6.A and A.B6 litters were tested with 10 dosages between 8.595 and 17.775 mg/kg, also with a minimum

of 6 tested litters at each dosage.

Initially it had been decided that, based on the extremely high embryonic mortality at relatively low dosages in A/J (Chapter A) and the inconclusive evidence that CL(P) was induced, possibly due to the dosage that allowed survival being relatively ineffective in inducing CL(P), an A/J dosage response study would be impractical. It was later realized that non-parallelism of the A/J dosage response with those of other genotypes could be used as evidence of its resistance. Therefore, near the end of the study, the remaining stocks of A/J were used in a dosage response study. The dosage range tested was 4.905 to 10.418 mg/kg, with a goal of 6 litters at each dosage. In addition the data from the time response curves at 10/10, 12.263 and 7.358 mg/kg was incorporated.

The mice were killed on day 17 of gestation, or, rarely, on day 18. The uterine contents were examined under a dissecting microscope. Resorption sites, and the phenotype of the lip and palate of intact embryos (live until at least day 16) were recorded. Sex, as judged from the anal-genital papilla distance was also recorded.

Probit analysis (Finney, 1971) was applied to the frequencies of CL(P) observed among intact embryos and to the frequencies of resorption among implants. All embryos lacking CL(P) were classed as "normal" for this analysis. Only the 9 highest dosages for B6 were used for probit analysis of the CL(P) response. Estimates of spontaneous CL(P) and resorption frequency were obtained from control litters, except B6, whose

estimated spontaneous resorption frequency seemed unreliable. Both the pattern of a plateau at low dosages (Finney, 1971, page 127) and a previous study (Goldstein et al., 1963) indicated that 20% was a better estimate, and 20% was used.

For each genotype the maximum likelihood fit was obtained independently. Chi-square tests for heterogeneity about these lines pooled classes where expected numbers were less than 5 (Finney, 1971, pages 70, 75) and these values with their degrees of freedom were used to compute heterogeneity correction factors for the independently fitted slopes' standard errors and ED_{50} 's fiducial limits.

All genotypes were subsequently fitted to a common slope. New chi-squares for heterogeneity were computed. Differences in the heterogeneity χ^2 values between those for the best fitting lines summed, and that for the common slope were used as an estimate of the degree to which the data fit their own lines better; that is, the significance of the deviations from parallelism. (Where adjacent dosage data were pooled because expected numbers were less than 5, the pooling was done in the same way^{as} for the test of fit to the two lines within the test for parallelism.) Finney's shorter method (page 106) was also calculated so that changes, if any, due to the method of calculation could be checked. The long method (Finney, 1971, page 107) allowed a further modification, in addition to the pooling of classes. The spontaneous frequency of the trait under study had been removed from the

data, as described by Finney (page 125), with appropriate changes in the weights used during calculations. Under the modified long method, the expected frequencies of affected used χ^2 tests of heterogeneity for both the independently fitted lines, and the test of parallelism, were each the sum of the spontaneous number expected for the total embryo sample, and the expected induced number for the "corrected" (minus number spontaneous affected) sample size. Errors in the estimation of the spontaneous frequency, and random error in its occurrence would contribute to the heterogeneity of χ^2 in both tests, though not necessarily equally.

All iterations were calculated by hand and were carried to the point where expected Y values did not change by more than 0.1 between iterations, and did so nonsystematically. The number of significant digits carried during calculations were those suggested by Finney. Consequently, the values obtained for ED_{50} 's and slopes are expected to differ somewhat from those expected from precise, complete, computerized iteration, but are adequate for the purposes of this study.

Genotypes were compared by the ED_{50} 's from their independently fitted response lines and by their fit to a common slope. These, respectively, are comparisons of additive effects (if no interaction) and interaction of genotype and teratogen.

All slopes and ED_{50} 's were expressed in terms of the data with spontaneous frequencies removed, since it was the

response traits and not the final frequencies that were of interest.

The data were also examined by other methods. Having obtained the CL(P) dosage response data and observed that it did not fit the assumed range of response for probit analysis, the CL(P) data were re-analyzed using conventional linear regression (Snedecor and Cochran, 1967). Within each genotype, the percentage CL(P) in scoreable embryos for each dosages were converted to their Freeman-Tukey arcsine value (Mosteller and Youtz, 1961) and used as the dependent variable in regression against log dosage. For A/J, the values were corrected for spontaneous CL(P) and were the arcsine of $(r - nc) / (n - nc)$ where r = the number responding; n = sample size; c = spontaneous frequency. The analysis was unweighted because sample size increased as dosage decreased, thus improving reliability of the values at the same time as their theoretical variance increased. Individual litter arcsine values were not considered to be a reasonable data base from which to estimate variances for weighting because surviving litter size was very small at the high dosages (e.g., 2) while most of the low dosage litters had no CL(P) and consequently led to small calculated sample variances.

Among genotypes, homogeneity of variance of the residuals, and differences between slopes and intercepts were compared according to the methods of Snedecor and Cochran (1967,

page 435) followed by STP contrasts of slopes as shown by Sokal and Rohlf (1969; page 457).

The data were also examined for effects of dosage on sex ratio and the types of CL(P) induced. Within each genotype, dosage effects on sex ratio among normal embryos were tested by χ^2 tests of heterogeneity; where expected numbers were less than 5 data from adjacent dosages were pooled. The relatively smaller number of CL(P) embryos was tested for dosage effects on sex ratio by pooling high and low dosages to create 2 by 2 tables that were then analyzed by Fisher's Exact Test (2 tailed). The pooling of dosages that gave a response was as follows: B6: 4 highest against 4 middle; B6.A and A.B6: 6 highest against 4 lowest; A/J, 3 highest against 3 lowest.

Sex ratios within lip type and genotype, pooled across dosages, were tested against equal proportions by χ^2 tests of goodness of fit.

Interaction between sex and type of cleft lip was tested within genotype by χ^2 tests of independence. Cleft lips were classified as either "unilateral" or "bilateral".

To test the possibility that the type of CL(P) induced was influenced by dosage, within each genotype, data were pooled into the high and low dosage groups (above) and classed as bilateral or unilateral. The 2 by 2 tables were analyzed by Fisher's Exact Test (2 tailed).

Interaction between genotypes and type of cleft (bilateral, right, left) was tested by a χ^2 test of independence.

Results

Probit dosage responses

Dosage-dependent frequencies of CL(P) were obtained in all genotypes (Tables B1a and B2b). Resorption also increased with dosage, so that sample sizes for surviving embryos at the high dosages were small, and individual dosage data therefore were relatively unreliable. At 17.775 mg/kg, the highest dosage tested, approximately 70 to 90% of B6 and A.B6 embryos and 50 to 70% of B6.A embryos were resorbed. A much lower dosage, 12.263 mg/kg, killed more than 90% of A/J embryos.

Concomitant with these high resorption rates, CL(P) frequencies among surviving embryos remained relatively low. While 100% of 5 surviving B5 embryos at the highest dosage were affected, only 38% at the second highest dosage were affected. Only 17 and 27% of A.B6 and B6.A were affected at the highest dosage. Including, or perhaps totally due to, spontaneous CL(P), 44% were affected at the highest dosage tested for A/J.

CL(P)

Independently fitted probit, log dose regressions of CL(P) for each genotype are shown in Figure B1. There was

TABLE Bla

6-AN DOSAGE RESPONSE DATA; A/J AND C57BL/6J

Dose (mg/kg)	Number of Litters	Total Implants	% Resorbed	Number Embryos	% CL(P)
<u>A/J</u>					
12.263*	9	95	91	9	44
10.418	5	56	100	0	-
8.595	6	57	84	9	22
8.010	6	61	89	7	14
7.358*	16	173	66	59	24
4.905	5	57	40	34	6
0.000	17	171	27	125	10
<u>C57BL/6J</u>					
17.775	6	49	90	5	100
16.875	6	56	71	16	38
15.953	6	53	72	15	80
15.030	6	55	76	13	38
14.108	6	54	31	37	24
13.185	6	49	22	38	29
12.263	5	50	44	28	11
11.340	7	68	31	47	9
10.418	6	49	37	31	0
8.595	6	50	20	40	0
6.750	6	53	21	42	0
4.905	6	44	20	35	0
0.000	4	31	10	28	0

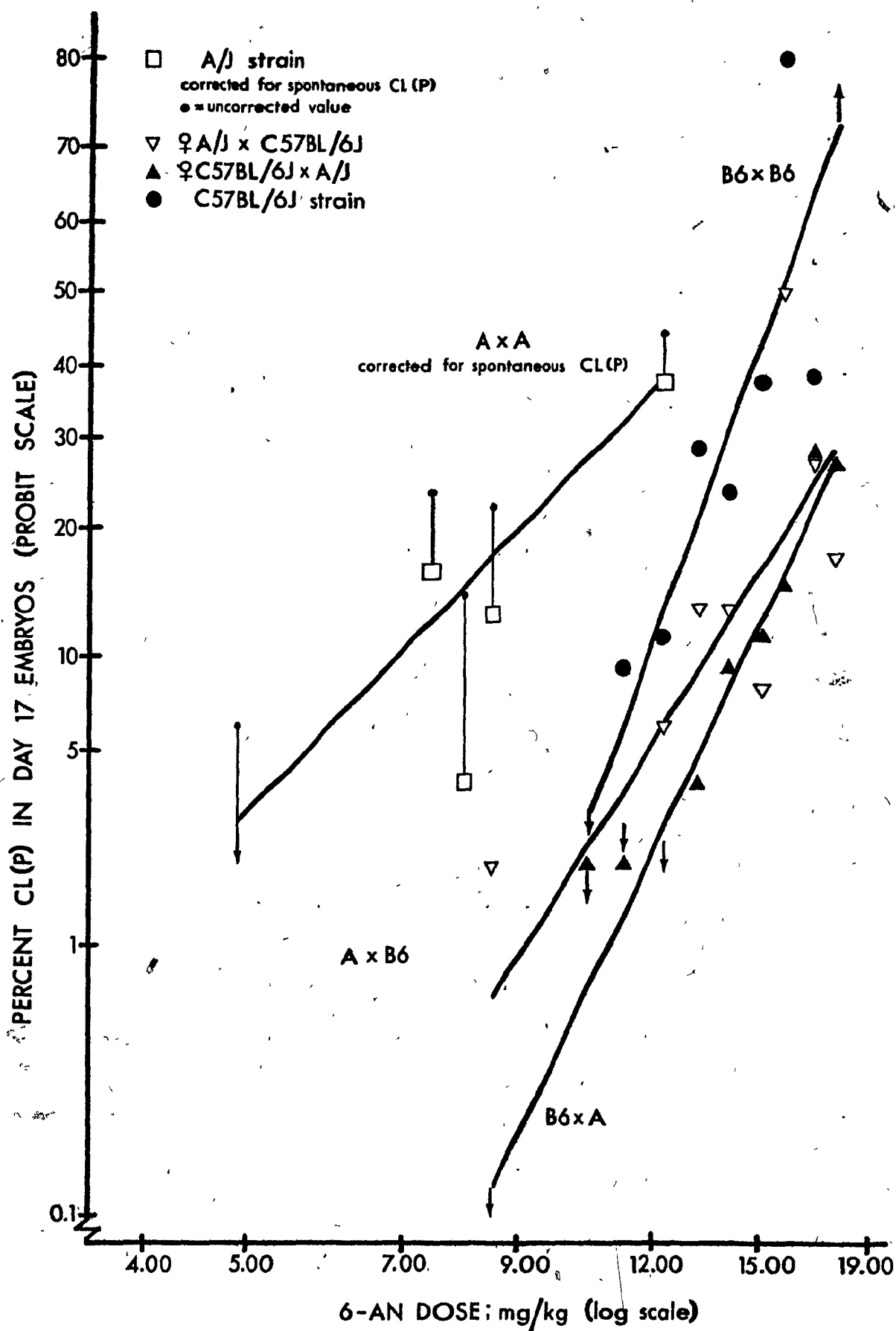
*Includes data from Time Response curves at 10/10.

TABLE Blb

6-AN DOSAGE RESPONSE DATA, RECIPROCAL
CROSSES OF A/J AND C57BL/6J

Dose (mg/kg)	Number of Litters	Total Implants	% Resorbed	Number Embryos	% CL(P)
<u>A.B6</u>					
17.775	8	74	84	12	17
16.875	8	67	84	11	27
15.953	7	63	81	12	50
15.030	7	70	49	36	8
14.108	6	55	44	31	13
13.185	7	64	39	39	13
12.263	7	73	56	32	6
11.340	6	64	38	40	0
10.418	6	62	27	45	0
8.595	6	67	25	50	2
0.000	13	132	11	118	0
<u>B6.A</u>					
17.775	6	53	72	15	27
16.875	6	51	43	29	28
15.953	6	55	53	26	15
15.030	6	50	28	36	11
14.108	6	58	24	44	9
13.185	6	59	14	51	4
12.263	6	51	6	48	0
11.340	6	66	17	55	2
10.418	6	55	5	42	2
8.595	6	54	15	46	0
0.000	18	160	11	143	0

FIGURE B1. INDEPENDENTLY FITTED PROBIT DOSE RESPONSES OF CL(P)
FOR A/J, C57BL/6J AND RECIPROCAL CROSSES



considerable nonsystematic scatter of the data points about their regression lines and C57BL/6J showed significant heterogeneity (χ^2 , 4 df = 9.74; $P < .05$). A (χ^2 , 1 df = 2.01), A.B6 (χ^2 , 1 df = 1.77) and B6.A (χ^2 , 1 df = 0.52) did not. The observed scatter for the latter three genotypes is therefore probably a reflection of the small samples at each dosage, although the significant heterogeneity of B6 suggests that litter effects may influence the response also.

Although the independently fitted slopes appeared to differ somewhat, they did not deviate significantly from parallelism (Tables B2 and B3). Thus there was no significant evidence of interaction between the genes that cause spontaneous CL(P), in heterozygous state (B6.A and A.B6) or in homozygous state (A), and 6-AN dosage tolerance for induced CL(P). Furthermore, in sharing the common slope, A could not be shown to be resistant to 6-AN induced CL(P).

The test of A was not particularly powerful, but the original question of a negative relationship between spontaneous and induced CL(P) was answered by observation of other genotypes (Chapters C, D, and E).

The relative dosage tolerances for CL(P), the ED_{50} 's for A.B6 and B6.A, were very similar to each other, being, respectively, 21.6 and 20.9 mg/kg (Table B2). No evidence of a maternal effect was present. This was different from the 6-AN induced CP response for the same strains, where a protective maternal effect of B6 mothers was observed (Goldstein

TABLE B2

ED₅₀'s AND COMMON&INDEPENDENTLY FITTED SLOPES OF PROBIT REGRESSIONS FOR 6-AN-INDUCED
CL(P) AND RESORPTIONS IN A/J, C57BL/6J AND RECIPROCAL CROSSES

Embryo Genotype	Constrained to Parallelism		Independently Fitted			
	Y intercept	slope	Y intercept	slope $\pm$ S.E.	ED ₅₀ (mg/kg)	(95% fiducial limits)
A/J*	-2.990	7.700	0.197	4.162 $\pm$ 2.178	14.257	(all dosages**)
A.B6	-3.656	7.700	-3.352	6.237 $\pm$ 1.474	21.849	(18.589 to 33.074)
B6.A	-5.248	7.700	-5.907	8.266 $\pm$ 1.678	20.869	(18.647 to 26.704)
C57BL/6J	-4.371	7.700	-7.425	10.393 $\pm$ 2.540	15.686	(14.200 to 21.942)
<u>Resorption*</u>						
A/J	-0.737	6.846	0.247	5.770 $\pm$ 1.568	9.981	(8.259 to 39.819)
A.B6	-2.782	6.846	-2.493	6.594 $\pm$ 1.450	13.689	(12.459 to 15.031)
B6.A	-3.574	6.846	-12.028	13.858 $\pm$ 3.084	16.939	(16.441 to 17.786)
C57BL/6J	-3.065	6.846	-7.746	10.846 $\pm$ 2.540	14.968	(13.785 to 16.236)

* Corrected for spontaneous trait.

** g > 1.0

TABLE B3

TESTS OF PARALLELISM; CL(P) RESPONSE OF A/J,
C57BL/6J AND RECIPROCAL CROSSES

Finney's Short Method

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>
Parallelism	3	6.332	2.111	1.35 ^{N.S.}
Heterogeneity	26	40.518*	1.558	.
Total	29	46.850		

Modified Long Method

<u>Source</u>	<u>df</u>	<u>SS</u>
Parallelism	3	1.784 ^{N.S.}
Heterogeneity	7	13.047 ^{N.S.}
Total	10	14.831

* $P < .05$

et al., 1963). That the maternal effect is not generalized may indicate that it operates on the underlying threshold traits, rather than on dosage tolerance of the embryo as a whole. That is, it probably is not due simply to differences in the amount of 6-AN reaching the embryos.

Had the reciprocal F_1 embryos been treated at the same chronological time, e.g., 10/10, B6.A, in having matured past CL(P) inductability in many cases, due to a maternal effect on developmental rate, would have appeared to be relatively resistant. Hence, differences in developmental rate may account for apparent differences in dosage tolerance.

The ED_{50} 's of CL(P) for A and B6 were 14.3 and 15.7 mg/kg, respectively, and were therefore relatively similar to each other and lower than those for F_1 embryos. These results suggest that F_1 embryos exhibit heterosis, expressed as an additive effect on dosage tolerance: the F_1 embryos require more 6-AN to get CL(P) than either parental strain does.

These interpretations of ED_{50} data should be viewed as tentative, owing to the large overlapping 95% fiducial limits surrounding values for all genotypes, and in particular, A. These wide ranges are one of the consequences of failure to obtain response frequencies equally above 50% as below.

One clear conclusion could be made, however, and it answered one of the original questions posed. Heterozygosity for the genes for spontaneous CL(P) did not increase liability

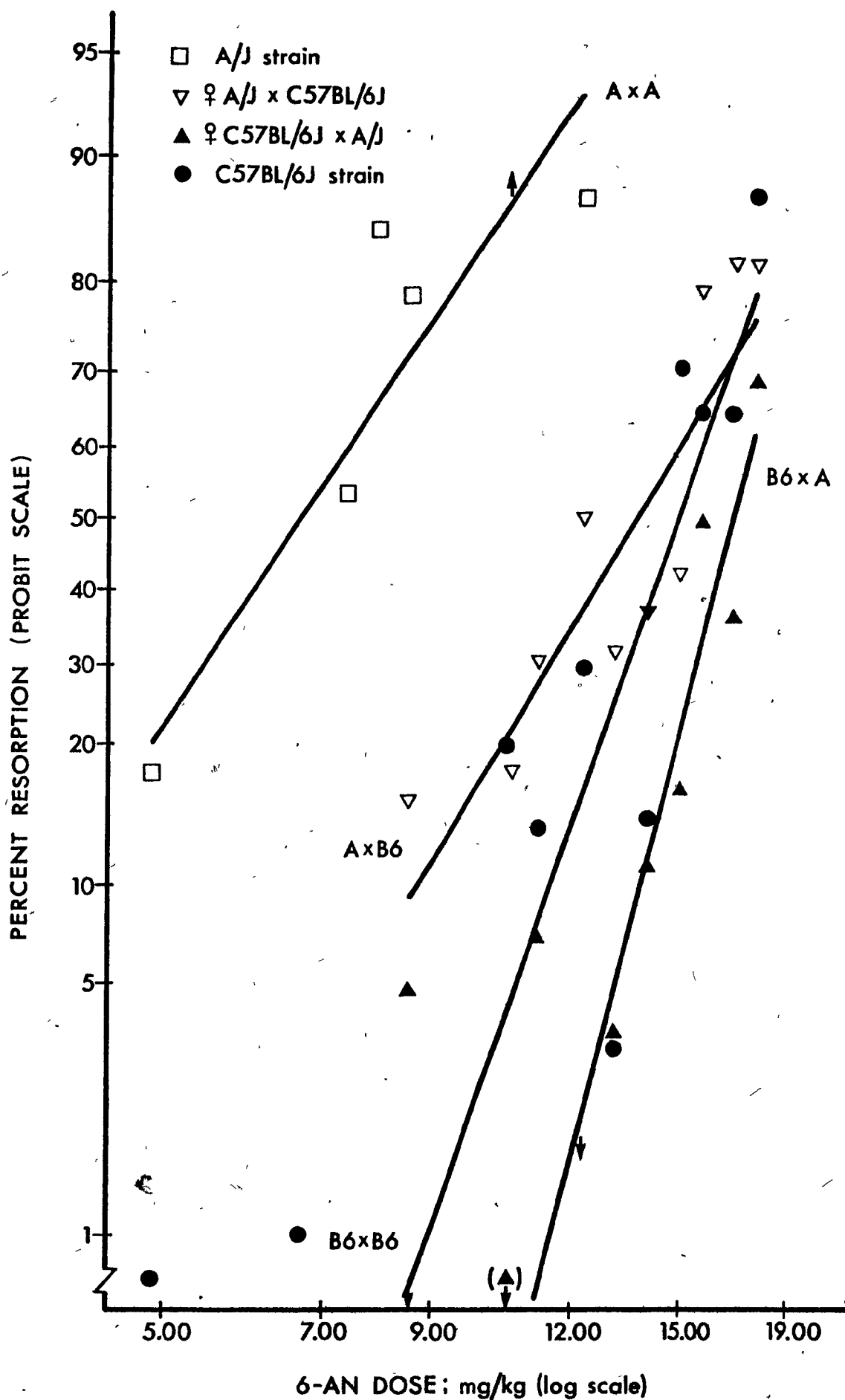
to 6-AN induced CL(P); they did not exert detectable additive effects on the threshold distribution underlying the 6-AN inducible trait.

Resorption

Independently fitted, probit, log dose regressions for the 6-AN induced resorption response are shown in Figure B2. The data points were scattered widely about their best fitting regressions, but no systematic departures were evident except a "plateau" of two B6 values at low dosages. These were probably instances where the spontaneous resorption frequency had by chance slightly exceeded the 20% estimated, at dosages where the induced response rate expected is near zero. Their effect on the regression line was minimal, owing to the small expected frequency and consequent weight in the analysis, and they had little effect on the heterogeneity χ^2 when pooled with adjacent cells. Nonetheless, the deviations from the best fitting line for B6 were significant (χ^2 , 10 df = 30.24; $P < .05$) as were those for A (χ^2 , 3 df = 10.37; $P < .05$) and A.B6 (χ^2 , 8 df = 29.72; $P < .05$), while that for B6.A approached significance (χ^2 , 8 df = 14.45; $P < .1$).

Bearing in mind Finney's admonition (page 72) that "temptation simply to use the heterogeneity factor must be resisted unless the evidence is clear that no sign of systematic deviation from linear regression appears. . .", the pattern

FIGURE B2. INDEPENDENTLY FITTED PROBIT DOSE RESPONSES OF RESORPTION FOR A/J, C57BL/6J AND RECIPROCAL CROSSES (CORRECTED FOR SPONTANEOUS RESORPTION)



of deviations was examined closely, and no systematic pattern, apart from that for B6, was evident. Heterogeneity factors were therefore used.

Litter effects were the most likely source of the heterogeneity. As a typical example, the 6 B6 litters treated at 15.030 mg/kg contained the following results: 2 litters (7 and 10 implants) resorbed completely; 2 litters (10 and 11 implants) had one survivor each; one litter (9 implants) contained 3 survivors, and one litter (8 implants) totally survived. Variation in volume of 6-AN injected did not seem to account for the effects. In this example, the highest dosage of a range of 176 to 198 μ l per mouse, had been administered to the litter with no resorptions, but the implied inverse relationship did not extend to the other data.

The resorption response slopes seemed to fall into two classes: values of about 6.0 for A and A.B6 and values of about 11.0 for B6.A and B6 (Table B2 and Figure B2). While the short approximate test did not detect deviations from parallelism, the long modified version did (Table B4). It appears that the embryos, under the influence of a maternal effect, die by a different mechanism--that is, for a different reason. This situation is interesting, but not surprising; maternal effects influencing qualitatively the predominant teratogenic response have been noted before (e.g., Goldstein et al., 1963), and mortality may be a nonspecific endpoint of one or more anomalies.

TABLE B4

TESTS OF PARALLELISM; RESORPTION RESPONSE OF
A/J, C57BL/6J AND RECIPROCAL CROSSES

Finney's short method

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>
Parallelism	3	11.596*	3.865	0.903 ^{N.S.}
Heterogeneity	30	128.415*	4.281	
Total	33	140.011		

Modified long method

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>
Parallelism	3	31.034*	10.345	3.539*
Heterogeneity	29	84.782*	2.924	
Total	32	115.816		

* $P < .05$

The slope differences prohibit meaningful genetic interpretation of the ED_{50} 's, because the relative distances between genotypes are dosage dependent. The ED_{50} 's are presented in Table B2. It is clear (Figure B2) that over the entire response range tested, A embryos were most sensitive. Over the range up to about 70% resorption B6.A were least sensitive, in good agreement with the time response data (Chapter A). A.B6 and B6 embryos were intermediate, also as observed in the time response data.

For these two strains and their hybrids at least, there was a gross correspondence between 6-AN CL(P) dosage tolerance and 6-AN resorption dosage tolerance, taking into account the fiducial limits (Table B2). Superimposed on heterotic effects, maternal effects, and the genetics of dosage-tolerance for specific traits, there may be a heritable trait of general sensitivity to 6-AN per se, as might be expected from strain differences in nicotinamide pool size, for example.

Reanalysis of CL(P) data

Probit analysis emphasizes the comparison of potencies of compounds, and thus the accurate estimation of ED_{50} 's. The assumption that responses were equally spread around the ED_{50} is a consequence of this purpose; the confidence band about the ED_{50} is minimized by such data. Secondly, the weighting system places the most emphasis on data values near

the ED_{50} . Slopes derived from data where most values lie on one side of the ED_{50} will have large confidence limits, and can be visualized as lines affixed to the page at the ED_{50} and allowed to swing free at the extreme frequency end.

While the maximum likelihood line may be taken as "true" for the data, the latitude allowed in slope will greatly reduce the sensitivity of tests of parallelism with other lines.

One of the purposes of this study was to compare the slopes of the dosage responses of the various genotypes. CL(P) responses greater than 50% are unlikely to be found in experiments of reasonable size for these genotypes because of the high and increasing concomitant resorption frequencies at dosages above the ED_{50} for CL(P).

Conventional linear regression emphasizes estimation of slopes, and makes no assumptions about the range of response. The major difficulty in its application to binomial data is in meeting the assumptions of normality of variance, particularly when response frequencies are very high or very low. The analysis is said to be "robust" however, with respect to violation of this assumption: that is, empirical experience suggests that moderate failure to meet the assumption does not greatly alter the outcome of the analysis. Therefore, the slopes of the arcsine transformed CL(P), log dose response were obtained from linear regression and compared.

All regressions were significant ($P < .05$) (Table B5). The responses of reciprocal F_1 embryos were similar in both

TABLE B5

INDEPENDENTLY FITTED LINEAR REGRESSIONS OF ARCSINE CL(P) FREQUENCY AGAINST
LOG DOSAGE OF 6-AN FOR A/J, C57BL/6J AND RECIPROCAL CROSSES

Genotype	Intercept	Regression Coefficient $\pm$ S.E.	df
A/J ¹	-52.861	81.631* $\pm$ 22.249	3
A.B6	-87.936	96.156* $\pm$ 28.433	8
B6.A	-91.609	96.114* $\pm$ 16.272	8
C57BL/6J	-248.382	248.648* $\pm$ 50.199	7

* $P < .05$ from t-test b/σ_b
¹ Spontaneous CL(P) removed.

Y intercept value (-87.9 and -91.6 for A.B6 and B6.A respectively) and slope (96.16 and 96.11), and they were intermediate in both parameters to those of the parental strains. The calculated slope of A was somewhat flatter (81.63) and its intercept was somewhat higher (-52.7), suggesting a higher sensitivity to dosage. The response slope of B6 was markedly steeper than the other genotypes' (248.65) and the intercept was the lowest (-248.4).

Residual variances from regression were not significantly different among the genotypes (Bartlett's Test χ^2 , 3 df = 6.82; $P > .05$) while significant differences among the slopes and among the intercepts were indicated (Table B6).

By the STP test, the slopes of A, A.B6 and B6.A responses did not differ significantly from each other, while that of B6 differed significantly from each of the 3 other genotypes (each $P < .05$). An interaction was thus found to be present between genotype and dosage. The genotype having no genes for spontaneous CL(P), B6, had a significantly steeper rate of response than did those heterozygous or homozygous for spontaneous CL(P) genes. If these results may be extrapolated back to the threshold scale, they indicate that the variance of B6 would be smaller than that of the other 3 genotypes. This result would be compatible with the concept that mutant genes (here, those for spontaneous CL(P)) destabilize development of the face and thereby allow greater developmental variance (Section 2 of Literature Review).

TABLE B6

COMPARISON OF LINEAR REGRESSIONS OF CL(P) AGAINST DOSAGE FOR
A/J, C57BL/6J AND RECIPROCAL CROSSES¹

	df	Σx^2	Σxy	Σy^2	Regression Coefficient	Deviations from Regression		
						df	SS	MS
Within								
• A/J	4	.08192	6.68720	667.5346	81.631	3	121.651	40.550
A.B6	9	.09176	8.82332	1441.8777	96.156	8	593.463	74.183
B6.A	9	.09176	8.81938	1042.0359	96.113	8	194.370	24.296
C57BL/6J	8	.05080	12.63127	4036.8226	248.647	7	896.102	128.015
						26	1805.586	69.446
Pooled, W	30	.31624	36.96117	7188.2708	116.877	29	2868.362	98.909
						3	1062.776	354.259
Between, B	3	.23360	5.67149	2086.3127				
W+B	33	.54984	42.63266	9274.5835		32	5968.997	
						3	3100.635	1033.545
Comparison of slopes:								
Comparison of elevations:								

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¹According to the method of Snedecor and Cochran, 1967, page 435.

Due to the slope differences, the relative rank of sensitivity of B6 compared with the other genotypes depended on dosage. Of the genotypes sharing similar slopes, A would be most sensitive, followed by A.B6 and B6.A that would be similar to each other. "Heterotic" effects for the latter would depend on the dosage tested and the dependent relative rank of B6; at high dosages "heterosis" would appear.

Dosage effects on sex ratio

Keeping in mind the biases inherent in sex assignment after 6-AN treatment (Chapter A), the data were examined for evidence of overriding interactions with dosage (Tables B7a and B7b).

No trend of change in sex ratio among normal embryos was observed for increasing dosages, and none of the genotypes showed significant heterogeneity of sex ratio across dosages ($\chi^2 =$ A/J: 1.47, 2 df; A.B6: 9.95, 7 df; B6.A: 5.64, 9 df; C57BL/6J: 1.96, 8 df).

When normal embryos were pooled across dosages, slight deficiencies of males, compared with a 50:50 ratio, were observed for A/J (45/95), A.B6 (135/282) and B6.A (161/374), while somewhat more than expected were obtained in C57BL/6J (154/292). Only B6.A deviated significantly from a 50:50 ratio (χ^2 , 1 df = 7.23; $P < 0.05$; all other $\chi^2 < 0.9$). These trends for A/J, A.B6 and C57BL/6J were consistent with the

TABLE B7a

EFFECTS OF 6-AN DOSAGE ON SEX AND TYPE OF CL(P)

Embryo Genotype	Dose (mg/kg)	Number Normal ¹		Number		Number CL(P) ²					
		M	F	CP	Median	M			F		
						B	R	L	B	R	L
A/J	4.905	15	17	1F	none	1	-	-	1	-	-
	7.358	20	25	1M		4	-	2	6	2	-
	8.010	4	2			1	-	-	-	-	-
	8.595	3	4			-	-	-	1	1	-
	10.418	-	-			-	-	-	-	-	-
	12.263	4	1			1	1	-	1	-	1
		46	49			7	1	2	9	3	1
C57BL/6J	0	59	53	1M; 1F		5	1	3	2	0	2
	4.905	17	18			-	-	-	-	-	-
	6.750	25	17	1F		-	-	-	-	-	-
	8.595	19	21			-	-	-	-	-	-
	10.418	15	16			-	-	-	-	-	-
	11.340	24	19			-	1(1)	1	2(1)	-	-
	12.263	13	12			1	1	-	-	-	1
	13.185	15	12		1F	2	-	3	2(1)	1	3(1)
	14.108	14	14		1F	2	1	2	3	-	1
	15.030	5	3		2M; 1F	1(1)	-	1(1)	1	2(1)	-
	15.953	2	1	1F	3M; 1F	7	1	1	1	-	2
	16.875	5	5	2M; 2F		2	-	2(2)	1	1(1)	-
	17.775	-	-			1	2(1)	1	1	-	-
		154	138			16	6	11	11	4	7
	0	14	14			-	-	-	-	-	-

¹ Includes CP and median clefts.² Bracketed numbers indicate isolated CL included.

TABLE B7b

EFFECTS OF 6-AN DOSAGE ON SEX AND TYPE OF CL(P)

Embryo Genotype	Dose (mg/kg)	Number Normal ¹		Number		Number CL(P) ²					
		M	F	CP	Median	M			F		
						B	R	L	B	R	L
A.B6	8.595	21	28			-	-	-	-	-	1(1)
	10.418	21	24			-	-	-	-	-	-
	11.340	21	19			-	-	-	-	-	-
	12.263	10	20	1F		1	-	1	-	-	-
	13.185	17	17	1M; 1F	1M; 1F	3	-	1	-	-	1
	14.108	16	11			2	-	-	-	1	1
	15.030	17	16	1M; 2F	2M	-	2	-	1(1)	-	-
	15.953	4	2	1F		3	1	1	-	-	1
	16.875	5	3		1F	1	-	-	2	-	-
	17.775	3	7		1F	1	-	-	-	1	-
		135	147			11	3	3	3	2	4
	0	59	59								
B6.A	8.595	18	28	1M; 1F		-	-	-	-	-	-
	10.418	20	31	1F		-	-	1	-	-	-
	11.340	27	27	1F		-	-	1(1)	-	-	-
	12.263	17	31			-	-	-	-	-	-
	13.185	23	26	1F		2	-	-	-	-	-
	14.108	17	23			1	2	-	-	-	1
	15.030	16	16			2	-	1	-	1	-
	15.953	7	15	1F		-	1	1	-	2	-
	16.875	10	11	2M		3	3	-	-	1	1(1)
	17.775	6	5	2M		1	2	-	1	-	-
		161	213			9	8	4	1	4	2
	0	72	71								

1,2 as in Table B7a

time response data (Chapter A). That for B6.A was not. The previous data showed no systematic error in the sexing of normal embryos and no strong excess of normal females. In the absence of dosage dependent trends, the disagreement cannot be attributed to increased dosage and its cause is unknown.

Control A.B6, B6.A and B6 embryos, collected concurrently with those treated showed no significant departures from 50:50 sex ratios, 59 of 118 A.B6, 72 of 143 B6.A, and 14 of 28 B6 being male. All had normal lips. Similarly 59 of 112 normal A, and 9 of 13 CL(P) A embryos were male, and no significant departures from a 50:50 sex ratio were obtained. This finding suggests that the biases of external sexing may be limited to treated embryos.

None of the genotypes demonstrated significant interaction between dosage and sex ratio among CL(P) embryos (all $P > .28$ from Fisher's Exact Test, 2 tailed).

A, B6 and A.B6 embryos with CL(P) had no significant excess of either sex. Slightly more than half (12/23) of affected A embryos were female (χ^2 , 1 df = 0.39) while affected B6 and A.B6 embryos were more frequently male: B6, 33/55 (χ^2 , 1 df = 2.20); A.B6, 17/26 (χ^2 , 1 df = 2.46). This trend toward an excess of affected males obtained statistical significance in B6.A embryos where 21/28 were male (χ^2 , 1 df = 7.00; $P < .05$). All of these trends toward excess numbers of males may be explained by the systematic biases described in

Chapter A.

Dosage effects on type of CL(P)

No significant changes in the proportion of bilaterally affected among CL(P) embryos were found. Among affected A embryos from "high" dosages, 76% of 17, compared with 50% of 6 embryos from "low" dosages were bilaterals ($P = .32$). Among B6 embryos, 54% of 28 and 44% of 27 embryos, from high and low dosages respectively, were bilaterals ($P = .59$). Similarly, among A.B6 embryos, 50% of 12 high dosage and 57% of 14 low dosage embryos were bilaterals, and among B6.A embryos, 38% of 8 high dose and 35% of 20 low dose embryos were bilaterals (both $P = 1.0$).

Based on a threshold model, if bilaterally affected were a more severe expression of CL(P) than unilaterals, the proportion of bilaterals would be expected to increase with dosage and higher CL(P) frequency. That it did not suggests that laterality is not a measure of severity, on the threshold scale, for induced CL(P) in mice.

Sex ratio and type of CL(P)

No significant associations between sex and type of CL(P) (unilateral or bilateral) were observed. Approximately 70% of affected A were bilateral in both sexes; 50% of each sex in B6

were bilateral. While 64% of 17 affected male and 33% of 9 affected female A.B6 were bilateral, the apparent difference was not significant ($P = .22$). A similar trend in B6.A, 42% of 18 male, and 16% of 7 female affected being bilaterals, also was not significant ($P = .36$).

Laterality of CL(P)

Among unilateral clefts, 4/7 in A, 10/28 in B6, 5/12 in A.B6 and 12/18 of B6.A were on the right side. Of the total of 65, 31, or 48% were on the right side, suggesting that there was no side preference.

Genotypic effects on the type of CL(P) induced

All 23 CL(P) embryos in the A strain had associated cleft palates. Of 54 affected F_1 embryos, 93% had CLP, there being 2 isolated CL's in each reciprocal cross. Of these, 3 were unilateral left; one was bilateral; 3 of the 4 were female. In contrast, B6 embryos showed a tendency to resist CP induction by CL. Only 80% of 55 affected had CLP. Half (6) of the 11 CL's were male. This excess of CL's in B6, compared with the data pooled for the other 3 genotypes, was significant (χ^2 , 1 df = 5.74, with Yates' correction factor; $P < .05$), and was also found in the time curve data (Chapter A). A tendency for CL's in B6 to be unilateral was observed, only 3 cases

(27%) being bilateral compared with the overall rate of 49% bilaterals, but it was not statistically significant ($P = .18$, Fisher's Exact Test, 2 tailed).

The 4 genotypes varied in the proportions of bilateral, right and left types of CL(P) produced. B6 and B6.A were affected unilaterally more frequently than bilaterally, (28/55 and 18/28 respectively) but left clefts were slightly more frequent in B6 (18/55) than in B6.A (6/28). A and A.B6 embryos were affected bilaterally as often as unilaterally (16/23 and 14/26 respectively) and both sides were equally often affected (left = 3/23 and 7/26 respectively). These differences among genotypes approached statistical significance (χ^2 , 6 df = 11.33; $P < .08$), and, if real, imply a maternal influence on cleft type, as well as an embryonic influence.

Other facial clefts

Median facial clefts do not occur spontaneously in any of the genotypes used, but have been induced by 6-AN previously (e.g., Leong, 1973). None were observed in A/J or B6.A embryos after treatment, but 9 B6 and 6 A.B6 embryos with median clefts were observed. Of these, 8 were male and 5 (3 male) were associated with lateral cleft lip.

The resistance of A, and sensitivity of B6, to induction of median clefts is well known (Trasler, 1968). While the treatment times used probably were generally later than the

peak critical period for median facial cleft induction, it is interesting, in view of the "face shape hypothesis", that at least one of the F_1 's between the two strains, is, like B6, sensitive to both CL(P) and median cleft induction.

Cleft palate occurred sporadically in all genotypes over most of the dosage ranges used (Tables B7a and B7b). For all genotypes its frequency was greater than 0.3%, the spontaneous frequency expected, (Trasler et al. 1956), suggesting that it was induced by the 6-AN treatment.

Summary and Discussion

Using probit analysis, no evidence of genotype-teratogen interaction in the CL(P) response was obtained. The A/J strain appeared to respond to 6-AN with induced CL(P) and was the most sensitive genotype tested. Heterozygosity for the genes for spontaneous CL(P) had no additive effect on the ED_{50} ; the F_1 embryos were no more sensitive than the C57BL/6J strain, and may have had some additional tolerance (heterosis).

From probit analysis, the resorption response to 6-AN was found to show genotype-teratogen interaction, an effect that seemed to be maternally mediated. The rank order of sensitivity to resorption was roughly the same as that for CL(P).

With respect to the induced CL(P) trait for the genotypes used, probit analysis was not a sensitive technique for detecting interaction, due to 2 factors: heterogeneity, possibly

due to litter effects and inability to extend the CL(P) response beyond 50 to 60% because of very high concomitant resorption frequencies. Both of these factors expanded markedly the confidence limits about the lines obtained. Failure to detect deviations from parallelism does not mean that the response slopes were the same, only that the differences were not large enough to be detected in the samples tested.

Methods for distinguishing between litter effects and chance effects in the proportions affected within litters do not seem to have been established. Robertson's test of binomial variance (1951 and see James, 1976), seems applicable to very large sets of data treated with one dosage, or to data where an independent estimate of the response frequency has been obtained. A conservative test for small samples and unknown response frequencies has been described (Potthoff and Whittinghill, 1966).

The use of probit analysis on the kind of data found for the genotypes used here, that is, with high resorption frequencies and probable litter effects, could probably be made more sensitive by using a minimal number of dosages (e.g., 3 or 4) with as many litters as possible at each dosage.

In contrast to probit analysis, which gave no significant interaction between genotype and dosage tolerance for CL(P), conventional regression analysis produced a significant interaction, attributable to the lack of CL(P) genes in C57BL/6J.

How can one choose between these divergent interpretations of the same data? Within the response range of 10% to 90% one would have expected the results by both analyses to be similar. The probit analysis was, for reasons discussed previously, probably highly conservative with respect to detecting slope differences, and it probably attempts to predict the slope for the entire possible response range. Conversely, the conventional linear regression used produced results applicable only to the data range tested, and made no consideration of sample size (beyond that inherent in the transformation), depending upon the internal consistencies of the data sets to determine the significances and residual variances of the lines. Thus, while the lines best explain the data, they may not explain the phenomenon, and the experiment should probably be repeated before accepting the slope differences found as genotype-teratogen interaction. In general, when litter effects and mortality are expected, and the response is therefore expected to be mostly below 50%, conventional regression, despite being less easily interpreted in terms of variance of dosage tolerance, is possibly a more powerful method of detecting genotype-teratogen interaction.

The multifactorial threshold model applied to CL(P) would tend to predict that heterozygotes for spontaneous CL(P) genes would show additive effects increasing their sensitivity to induced CL(P). The data, by either analysis, did not show this. A/J, if sensitive, was most sensitive to induced CL(P),

and would thereby show a possible additive effect of homozygosity for CL(P) genes: that they are recessive. The mathematical models used to predict recurrence risks for multifactorial traits do not incorporate such dominance effects.

Although the average sensitivity to 6-AN was not increased by heterozygosity for CL(P) genes, based on the second analysis, the probability of having CL(P) was increased for a proportion of the embryos, as indicated by the (inferred) increased variance of the heterozygotes compared with C57BL/6J. This possible effect on variance is not incorporated into existing multifactorial mathematical models.

A different type of genotype-teratogen interaction was noted: the relative resistance of C57BL/6J embryos with CL to accompanying CP. This is presumably an effect of the increased "shelf force" or other factor that protects C57BL/6J mice from CP induced later in development (Fraser, 1977).

Using the mouse as an animal model, it might be predicted that data for human populations would show a higher frequency of isolated CP in relatives of CLP probands than in relatives of CL probands. This would not imply any obligatory genetic relationship between CL(P) and CP, but rather the situation could be viewed as a natural probe locating genotypes at relatively different risks for CP. The frequency of CP in CL families would also be expected to be slightly lower than that in "normal" families.

CHAPTER C

GENOTYPE-TERATOGEN INTERACTION: TEST OF INDEPENDENCE OF SEGREGATION OF SPONTANEOUS CL(P) AND INDUCIBILITY TO CL(P) BY 6-AN

Introduction

It has been reported that CL(P) was not induced by 6-AN in A/J mice, which produce spontaneously about 10% CL(P) in newborns (Goldstein et al., 1965). The inbred strain (C57BL/6J) that responded to 6-AN with CL(P) did not have spontaneous CL(P). (The information in Chapters A and B was not yet known.) These facts did not conform to expectations from the multifactorial threshold model where A/J would be expected to be most liable to increased CL(P) induction if the A/J genes for CL(P) and 6-AN affected the same system. The additional observation (Goldstein et al., 1965), that A/J embryos with CL(P) resisted 6-AN induced embryonic death better than normal littermates suggested that nicotinamide metabolism was in some way associated with spontaneous CL(P) and suggested that the presence of the 2 traits in A/J was more than coincidence.

The following experiment was designed to test the possibility that spontaneous CL(P) and resistance to 6-AN induced CL(P) were pleiotropic effects of the same genes.

Materials and Methods

To observe independence of the segregation of the two traits, a segregating generation was required that could produce measurable frequencies of spontaneous CL(P) and a liability to 6-AN induced CL(P) among its progeny.

First backcross sires from a cross of A/J and C57BL/6J backcrossed to A/J, were chosen. They were backcrossed to A/J to test for the independence of spontaneous and induced CL(P). Two sets of litters were collected for each sire: one treated with 6-AN; one untreated. It was expected that, under the maximum rate of recovery of the A/J "resistance", the single locus case, half of the sires would produce sensitive litters in which half of the embryos would be sensitive --a situation that would allow detection of the transmission of sensitivity. These sensitive embryos would be for that particular locus genetically similar to F_1 embryos, which were known to be sensitive (Pinsky and Fraser, 1960).

The first backcross sires (" BC_1 ") were generated as described in Chapter F. They were caged singly and bred with individual A/J females. Approximately 15 untreated litters were collected before pregnancies were assigned to treatment and up to 12 treated litters were collected. Of 17 sires whose breeding persisted long enough to begin the treated litter set, 16 produced more than 5 treated litters.

Treated litters received 7.36 mg/kg 6-AN, based on

maternal body weight at treatment time, day 10/10. The females were killed on day 17 of gestation (or, rarely, day 16 or 18), and litters were observed under a dissecting microscope. (Untreated, nonpregnant females were sometimes rebred rather than killed.) Resorption sites and the lip type and sex of intact embryos that had been live until at least day 16 were recorded.

Associations between CL(P) frequencies in the treated and untreated embryos were sought. Segregation, and consequent non-normal distributions of the variables were expected, and therefore statistics not affected by distributions were used. Spearman's Rank Correlation (Siegel, 1956, page 214) was applied to treated and untreated scores for each sire for the following: CL(P) frequencies in live embryos and CL(P) frequencies in implants. It was also used in correlating resorption frequencies and CL(P) among embryos in "treated" data from each sire.

Fisher's Exact Test, one tailed, was used to test the significance of increases in CL(P) frequencies among implants from treated, compared with untreated, pooled litters for each BC₁ sire.

Results

Low CL(P) frequencies, ranging from zero to 29% of embryos, were obtained in the 6-AN treated litters. The upper

end of this range was much higher than the maximum of 7.3% obtained in untreated litters. Resorption, too, was increased, from a range of 10 to 26% to a range of 38 to 79% (Table C1).

Owing to the presence of spontaneous CL(P) in both, a correlation between CL(P) frequencies in treated and untreated samples among sires was expected. No correlation was found, however, for CL(P) in embryos ($r_s = 0.369$; $P > .05$) or for CL(P) in all implants ($r_s = 0.276$; $P > .05$).

The progeny of sires 46, 47, 85 and 96 showed significant increases of CL(P) among implants after 6-AN treatment ($P < .05$ from Fisher's Exact Test, one tailed), while the progeny of sire 110 showed an increase that approached significance ($P = .087$). These sires ranked 13.5 (tied), 6, 3, 15.5 (tied) and 9, respectively, among the 16 sires in rate of CL(P) production among untreated embryos. There does not appear to be any evidence of genetic correlation between spontaneous CL(P) liability and susceptibility to 6-AN induced CL(P) in the data.

The frequency of CL(P) in embryos showed no correlation with resorption in the 6-AN treated data among sires ($r_s = -0.076$; $P > .05$).

Discussion

Reading from the plot of probit dose response regression (Chapter B, Figure B1), the dosage used, 7.36 mg/kg, chosen

TABLE C1

SPONTANEOUS AND POST-6-AN TREATMENT CL(P) AND RESORPTION FREQUENCIES IN
LITTERS FROM BC₁ SIRES TESTCROSSED WITH A/J FEMALES

Sire Identifi- cation Number	Untreated Litters						6-AN Treated Litters					
	Number of Litters	Number of Implants	Number Resorbed	(%)	Number CL(P)	(% in Live)	Number of Litters	Number of Implants	Number Resorbed	(%)	Number CL(P)	(% in Live)
31	15	159	23	(14.5)	3	(2.2)	10	75	37	(49.3)	1	(2.6)
32	16	179	26	(14.5)	2	(1.3)	5	54	31	(57.4)	0	(0)
39	16	181	40	(22.1)	5	(3.6)	10	104	68	(65.4)	1	(2.8)
40	14	154	30	(19.5)	9	(7.3)	8	81	51	(63.0)	3	(10.0)
44	18	195	32	(16.4)	8	(4.9)	10	106	84	(79.2)	1	(4.5)
46	16	181	43	(23.8)	2	(1.5)	12	113	53	(46.9)	6	(10.0)
47	16	185	27	(14.6)	7	(4.4)	11	110	53	(48.2)	12	(26.0)
54	14	160	17	(10.6)	3	(2.1)	9	68	40	(58.8)	2	(7.1)
56	19	199	37	(18.6)	7	(4.3)	9	98	47	(48.0)	4	(7.8)
85	16	147	33	(22.5)	6	(5.3)	9	93	52	(55.9)	12	(29.3)
88	18	186	31	(16.7)	9	(5.8)	9	96	36	(37.5)	6	(10.0)
96	18	201	49	(24.4)	2	(1.3)	10	114	71	(62.3)	7	(16.3)
108	15	158	32	(20.3)	6	(4.8)	11	109	66	(60.6)	6	(14.0)
110	17	172	44	(25.6)	3	(2.3)	9	87	57	(65.5)	5	(16.7)
117	15	167	36	(21.6)	2	(1.5)	11	103	68	(66.0)	2	(5.7)
122	16	161	39	(24.2)	2	(1.6)	7	64	38	(59.4)	1	(3.8)

to give intermediate resorption frequencies (the data in Chapter B had not yet been completed), would be expected to induce CL(P) in less than 1% of F_1 -like embryos, and in about 10% of A/J-like embryos. That 4/16 of the sires produced significantly higher CL(P) frequencies among implants after treatment, indicates that the responding embryos were more sensitive than F_1 -like embryos and that therefore A/J must have contributed genes for higher sensitivity. When sample size is taken into consideration, rates of induction of CL(P) less than 10% would probably not have been detected, and the 4 sires were therefore probably very much like A/J at the relevant loci. To recover 1/4 of BC_1 sires like A/J is the prediction for a 2 locus genetic model, and it is likely that the difference in sensitivity between A/J and F_1 embryos in A/J mothers is due to between 1 and 3 loci.

Like the results of Chapters A and B, the data were more compatible with A/J sensitivity to 6-AN induced CL(P) than resistance.

Induced resorption at 7.36 mg/kg (Chapter B, Figure B2), would be expected to fall between 60% (like A/J) and 5% (like F_1). When the spontaneous resorption was removed from the 6-AN treated data by Abbott's formula (Finney, 1971, page 125), the induced resorption rates were between 75% and 25%. Were a single allele responsible for the difference between F_1 and A/J embryos, the BC_1 sires would be expected to fall into 2 equal classes: about 60% and about 33% ($5 + 60/2$).

Only one sire value exceeded 57%. Four were in the range 25 to 40%, 5 between 40 and 50%, and 6 between 51 and 57%. That most of the values were intermediate to the F_1 to A/J range suggests that either more than one relevant locus was segregating, or that litter effects on resorption had increased the variance of the sire values. The fact that 7 of 16 sire's values were near 60% suggests that the number of loci may be small, if litter effects were not large.

CHAPTER D

GENOTYPE-TERATOGEN INTERACTION: STRAIN SURVEY OF SENSITIVITY TO 6-AN INDUCED CL(P)

Introduction

Apart from the apparent resistance of A/J mice to 6-AN induced CL(P) and sensitivity of hybrid and C57BL/6J mice, little was known at the beginning of this study about the genetic variation of the CL(P) response to 6-AN.

In mice, the large number of available inbred strains makes possible tests of independence of traits by observation of their joint distribution among the strains. To find inbred strains resistant to CL(P) induction by 6-AN, as A/J was believed to be, but without the liability to spontaneous CL(P), and also strains having sensitivity and spontaneous CL(P), would be strong evidence that the traits are independent.

Materials and Methods

Mice of SWV (F_{46} to F_{50}) and CL/Fr (F_{36} to F_{42}) inbred strains were obtained from McGill colonies. CBA/J mice were obtained from Dr. F.G. Biddle at McGill, but were of Jackson Laboratory origin. C3H mice were derived from breeding pairs from Dr. D.G. Trasler's McGill stocks. SWR/J, DBA/2J and PL/J were derived by inbreeding and random mating from mice

obtained from the Jackson Laboratory in 1976, and were no more than 5 generations removed from the original imported animals. Of these strains, only CL/Fr produces spontaneous CL(P).

All mice were 2 to 5 months old when bred. They were maintained, bred, and their day 17 embryos collected as described previously (e.g., Chapters A and B). Various treatment times and dosages were used. CL/Fr were fed Purina Mouse Chow from 6 weeks of age. Other strains were fed Purina Lab Chow. Some females of PL/J, DBA/2J, and SWR/J strains were fed Purina Mouse Chow from the plug day.

Results

Three strains, CL/Fr, SWR/J, and CBA/J, responded to 6-AN treatment on day 10 of gestation with induced CL(P). Four strains, PL/J, DBA/2J, C3H and SWV, were apparently resistant (Table D1). CL/Fr responded to 9.81 mg/kg 6-AN with 100% CL(P) and low embryonic mortality in the first 3 litters tested, and was therefore studied extensively (Chapter E). SWR/J and CBA/J produced 30% CL(P) at dosages causing 25 to 52% resorption. For the resistant strains, no CL(P) was found in survivors of 50 to 95% resorption, and it seems improbable that CL(P) is inducible, for the time range tested, at dosages allowing embryonic survival.

Cleft palate was induced by day 10 treatment in both

TABLE D1

STRAIN SURVEY OF SENSITIVITY TO 6-AN INDUCED CL(P)

Strain	Treatment Time	Dosage Range (mg/kg)	Number of Litters	Number of Implants	% Resorbed	Number			
						Live	CL(P)	CP	Median
SWR/J	9/12- 9/20	14.1-14.6	4	31	16	26	1	1	0
	10/10-10/12	16.9	5	35	43	20	10	5	0
	10/10-10/12	14.1	4	31	26	23	7	8	0
	10/14-10/16	14.6-16.9	3	22	14	19	0	14	0
CBA/J	10/12	12.3	2	21	52	10	3	1	1
	10/12	7.4	3	26	35	17	0	0	0
PL/J	9/12- 9/20	13.5-14.1	6	34	85	4	0	0	0
	10/ 9-10/15	14.1-14.6	11	73	82	13	0	0	0
DBA/2J	9/10- 9/14	14.1-14.6	4	18	89	2	0	0	0
	10/10-10/14	11.3-16.0	13	100	68	32	0	6	0
	11/15	14.1	1	7	57	3	0	0	0
SWV	9/10- 9/19	12.3	13	171	27	124	0	2	0
	9/18	17.2	1	15	73	4	0	0	0
	10/12	12.3	9	108	31	74	0	20	0
	10/12	16.0-17.2	6	79	95	4	0	1	0
	11/12	12.3	2	28	18	23	0	9	0
C3H	9/10- 9/15	12.3-15.0	7	40	83	7	0	0	0
	10/8 -10/19	12.3-16.0	12	87	49	44	0	0	0
	11/10-11/16	14.6-16.0	5	35	46	19	0	0	0
CL/Fr	10/12	Presented in Table E1							

resistant (SWV, DBA/2J) and sensitive (SWR/J, CBA/J) genotypes at frequencies between 10 and 27%. Resistance therefore was organ specific, not generalized.

Discussion

It is possible that critical periods for CL(P) induction were missed for the "resistant" genotypes. If so, the critical periods must be relatively short, and embryonic variance relatively small for no "outliers" to have responded among the cumulated resistant strain sample. In contrast, the sensitive strains responded at a variety of times and dosages.

Whether resistance to 6-AN induced CL(P) was relative or absolute, if A/J was resistant, as originally believed, the sensitivity of CL/Fr, and the existence of both sensitive and resistant strains that do not have spontaneous CL(P), demonstrated that spontaneous CL(P) and resistance to 6-AN induced CL(P) were unrelated traits.

Taking the revised point of view that A/J is sensitive, the possibility arises that sensitivity may always accompany spontaneous CL(P), in addition to occurring independently. That this association is not probable was shown in Chapter C.

The genetic variation revealed by this strain survey provides material for future genetic studies to determine whether sensitivity and resistance are controlled by the same loci in all strains, and for testing for association with other loci known to vary among them (e.g., the enzyme polymorphisms).

CHAPTER E

GENOTYPE-TERATOGEN INTERACTION: DOSE RESPONSE STUDIES OF CL/Fr AND CROSSES WITH C57BL/6J

Introduction

The finding that CL/Fr clearly responded to 6-AN with induced CL(P) in addition to the 25% that occurs spontaneously provided an opportunity to test for interaction between the genes for CL(P) in the homozygous state, and 6-AN. It was also of interest to compare the response of the hybrid of CL/Fr and C57BL/6J with that of the latter strain and A/J.

Materials and Methods

CL/Fr mice were obtained from the McGill colony derived from Dr. F.C. Fraser's stocks at F_{34} and were at F_{36} to F_{42} . C57BL/6J mice were obtained from the Jackson Laboratory.

The mice were maintained and bred as described previously (General Materials and Methods). All were fed Purina Mouse Chow from at least 2 weeks prior to breeding. Females were between 2 and 5 months old when bred.

Females were treated intraperitoneally with one of several 6-AN dosages at 10/12. The dosages, based on treatment time body weight, were: for CL/Fr, 4.905, 6.143, 7.358, 8.550, 9.810, or 12.263 mg/kg; for CL/Fr females bred with

C57BL/6J males (CL.B6 embryos), 9.810, 12.263, 14.108, or 15.953 mg/kg; for C57BL/6J females bred with CL/Fr males (B6.CL embryos), 12.263, 14.625, 15.953 and 16.875 mg/kg. Assignment of females to dosage was not strictly random, but all dosages were spread over the time interval of the experiment. C57BL/6J data from Chapter B was used.

The females were killed on day 17 (or, rarely, day 16 or 18) of gestation, and the uterine contents examined. Six litters with less than 5 implants were removed from the data. Numbers of resorptions and numbers of live or newly dead embryos and their lip and palate type and sex were recorded. Sex was assigned on the basis of the anal-genital papilla distance.

Interactions were sought by comparisons of the slopes of the dosage response by probit analysis, which has been discussed previously (Chapter B). CL/Fr CL(P) frequencies and resorption frequencies for all genotypes were corrected for the spontaneous rate as previously described because it was the response to treatment and not the absolute frequency that was of interest. No control CL.B6 embryos were observed, and the spontaneous resorption frequency was not known. An estimate of 6%, the CL/Fr frequency, and similar to the frequency observed at the lowest dosage, was used. This estimate was also supported by data for other hybrid embryos in CL/Fr mothers (Chapter F).

Within each genotype, the sex ratio for normal and for

CL(P) embryos from all dosages pooled was tested against a 50:50 ratio by χ^2 tests of goodness-of-fit.

Effects of dosage on the laterality of the CL(P) induced were tested within each genotype by pooling dosages to "high" and "low" groups, and pooling lip types to "bilateral" and "unilateral". The resulting 2 by 2 tables were tested for independence by Fisher's Exact Test (2 tailed). For CL/Fr the top 3 dosages and the bottom 3 dosages made up the 2 dosage groups. For CL.B6, the upper and lower 2 dosages each formed a group. B6.CL data were insufficient for analysis, and C57BL/6J was analyzed previously (Chapter B).

Differences in the ratio of bilateral to right to left types of CL(P) between CL/Fr and CL.B6 for pooled dosages were compared by a χ^2 test of independence.

Results

The CL/Fr strain and the reciprocal F_1 embryos from crosses with C57BL/6J were sensitive to CL(P) induction by 6-AN and the response showed dosage dependence (Table E1). Resorption, also, was caused by 6-AN treatment, and approximately 50% of CL/Fr implants were resorbed after treatment causing near 100% CL(P) in survivors.

Unlike the case of A/J, there is no question of differential mortality and resistance leading to the increased CL(P) frequency among survivors of 6-AN treatment. At each

TABLE E1

RESPONSE OF CL/Fr AND RECIPROCAL CROSSES
WITH C57BL/6J TO 6-AN

Genotype of Embryos	Dose (mg/kg)	Number of Litters	Total Implants	% Resorbed	Number Embryos	% CL(P)
CL/Fr	12.263	10	83	72	23	100
	9.810	9	84	32	49	98
	8.550	8	79	42	46	63
	7.358	12	113	27	83	69
	6.143	8	87	29	62	52
	4.905	6	59	19	48	31
	0	13	120	6	113	21
GL.B6	15.953	5	48	81	9	67
	14.108	5	39	26	29	55
	12.263	5	45	51	22	23
	9.810	5	42	7	39	8
B6.CL	16.875	3	26	81	5	20
	15.953	3	22	55	10	20
	14.625	3	30	80	6	67
	12.263	2	21	13	18	17

C57BL/6J: Table B1

of the 5 highest dosages, the observed frequency of CL(P) among implants exceeded the spontaneous 25% expected.

If the genes for spontaneous CL(P) are the same in A/J and CL/Fr (Chapter G), this response of CL/Fr would have demonstrated that if A/J were resistant to CL(P) induction by 6-AN, it would be a trait independent of spontaneous CL(P).

The data for CL.B6 and B6.CL embryos were limited and showed large fluctuations, but CL.B6 had a clear dosage response with 67% CL(P) at the highest dosage tested, 15.953 mg/kg. Approximately 80% of implants were resorbed at this dosage. B6.CL embryos also produced CL(P) after treatment, but the samples were small, and they were not used in probit analysis.

No untreated CL.B6 or B6.CL embryos were observed. Despite its having been made before, (Bornstein et al., 1970), no data are available for this cross. Therefore it is possible that some of the CL(P) observed was spontaneous. This is thought to be unlikely, in view of the "recessive" behavior of the CL(P) trait in outcrosses (Reed, 1936a, b; Loevy, 1964; Davidson et al., 1969).

Probit analysis of CL(P)

Independently fitted regressions of CL(P) responses against log dosage from probit analysis are shown in Figure E1. The CL.B6 response had no significant heterogeneity

FIGURE E1. INDEPENDENTLY FITTED PROBIT DOSE RESPONSES OF CL(P)
FOR CL/FR, C57BL/6J AND F_1

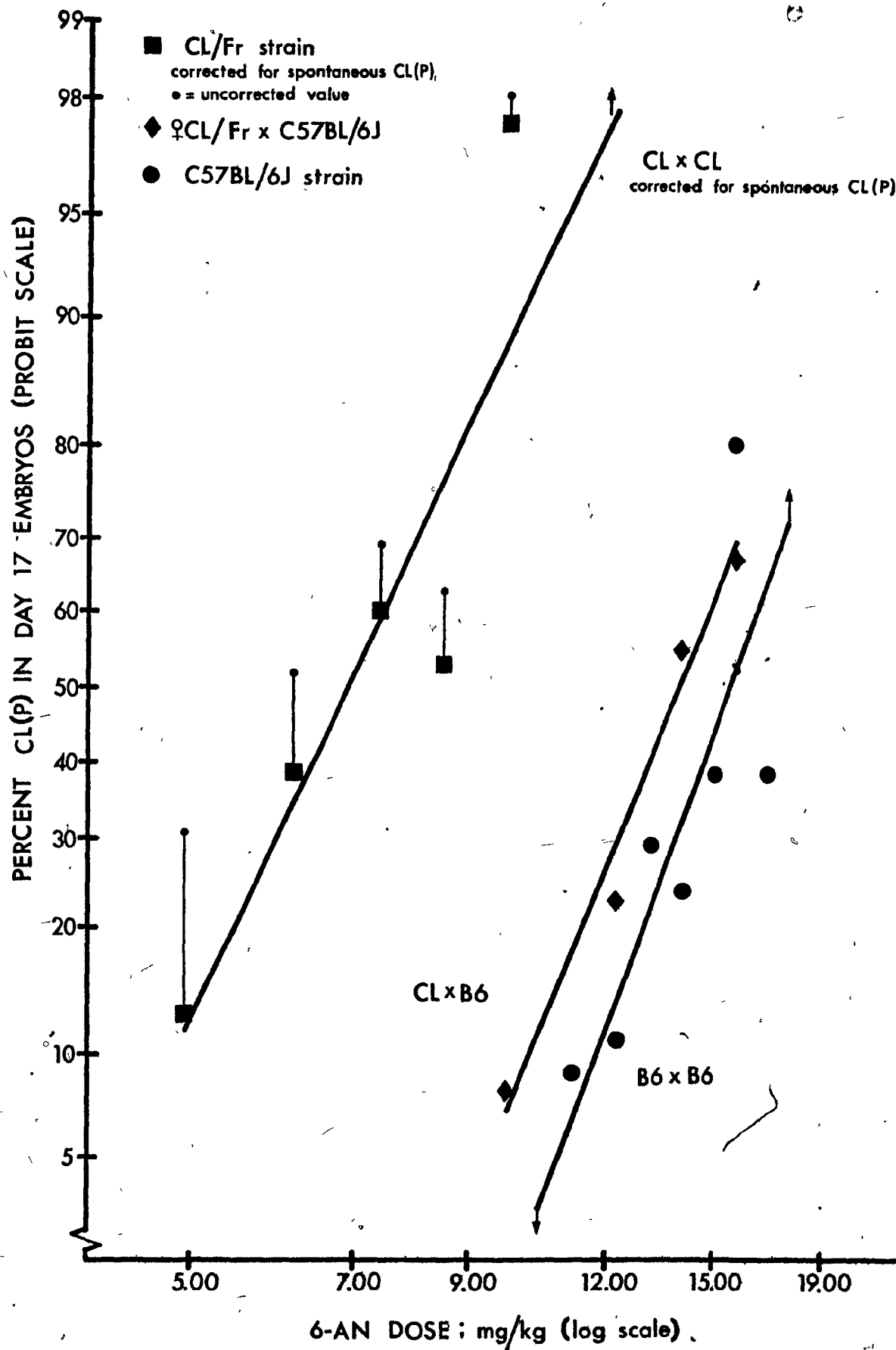


TABLE E2

ED₅₀'s AND COMMON AND INDEPENDENTLY FITTED SLOPES OF PROBIT REGRESSIONS FOR 6-AN INDUCED CL(P) AND RESORPTION IN CL/Fr, C57BL/6J AND CL.B6 EMBRYOS

CL(P) Embryo Genotype	Constrained to Parallelism		Independently Fitted			
	Y intercept	slope	Y intercept	slope	S.E.	ED ₅₀ (mg/kg) (95% fiducial limits)
CL/Fr	-2.662	9.038	-1.673	7.918 ± 3.214	6.955	(2.080 to 11.137)
CL.B6	-5.382	9.038	-5.783	9.401 ± 2.104	14.030	(13.362 to 15.803)
C57BL/6J ¹	-5.887	9.038	-7.425	10.393 ± 2.540	15.686	(14.200 to 21.942)
<u>Resorption</u>						
CL/Fr	-0.934	5.944	1.097	3.792 ± 1.369	10.707	(all dosages**)
CL.B6	-1.885	5.944	-6.251	9.824 ± 7.620	13.976	(all dosages**)
C57BL/6J	-2.033	5.944	-7.746	10.846 ± 2.540	14.968	(13.785 to 16.236)

¹ Same data as in Table 3B

** g > 1.0

TABLE E3

TESTS OF PARALLELISM; CL(P) RESPONSE OF
CL/Fr, C57BL/6J AND CL.B6

Finney's short method

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>
Parallelism	2	1.434	0.717	0.296 ^{N.S.}
Heterogeneity	13	31.374*	2.413	
Total	15	32.808		

Modified long method

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>
Parallelism	2	-1.055	-0.527	-0.527 ^{N.S.}
Heterogeneity	8	23.901*	2.988	
Total	10	22.846		

* $P < .05$

(χ^2 , 1 df = 0.93; $P > .05$), but CL/Fr (χ^2 , 3 df = 13.23; $P < .01$) and C57BL/6J, as previously noted, (χ^2 , 4 df = 9.74; $P < .05$) did. The C57BL/6J heterogeneity had previously been concluded to be nonsystematic. That for CL/Fr was limited to the higher dosages, but did not seem to have a pattern.

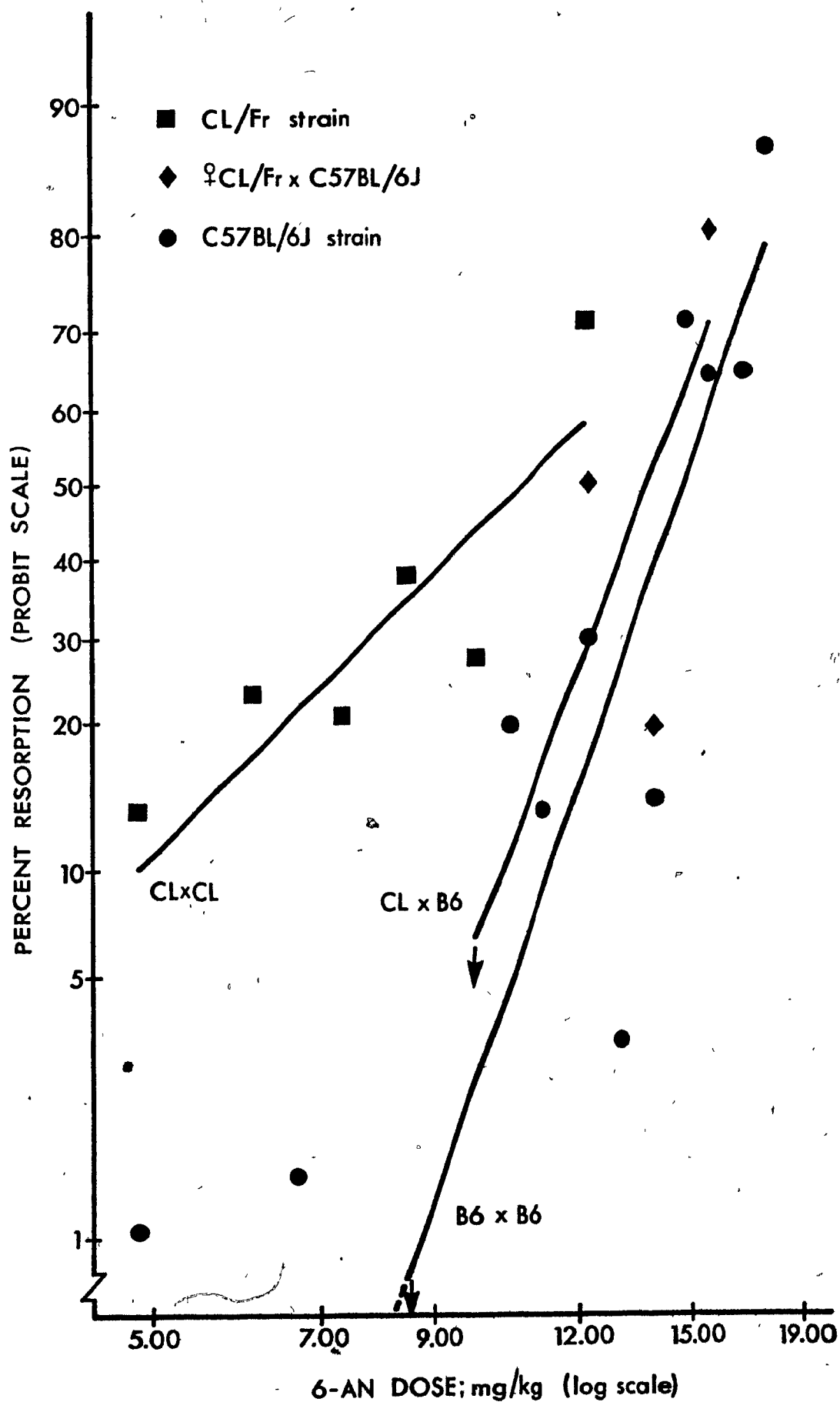
The slopes of the regressions appeared to be similar graphically and ranged between 8.0 and 10.5 (Table E2). They did not differ significantly from parallelism (Table E3). The negative value obtained in the modified test of parallelism probably resulted from the property that maximum likelihood fit does not always minimize the χ^2 value for heterogeneity (Finney, 1971, page 67), and it is possible for the heterogeneity to be reduced in the fit to the common slope.

The lack of significant deviation from a common slope indicates that there was no detectable interaction between 6-AN treatment and homozygosity or heterozygosity for the genes for spontaneous CL(P). Unlike the data for crosses between A/J and C57BL/6J, the genotypes in this experiment more nearly met the assumption that the response range was spread about the ED_{50} . Thus the possibility of finding slope differences was improved, and no advantage was expected from use of classical regression analysis.

Despite the lack of interaction, there were large differences in dosage tolerance. CL/Fr embryos were much more sensitive than CL.B6 or C57BL/6J embryos to CL(P) induction.

The 95% fiducial limits of the CL/Fr ED_{50} (6.96 mg/kg) had no

FIGURE E2. INDEPENDENTLY FITTED PROBIT DOSE RESPONSES OF RESORPTION FOR CL/FR, C57BL/6J AND F_1



overlap with the lower edges of the 95% fiducial limits for CL.B6 and C57BL/6J, 14.03 and 15.69 mg/kg respectively. CL.B6 and C57BL/6J were similar and the 95% fiducial limits of each included the ED₅₀ of the other. Because CL.B6 embryos, which had a CL/Fr maternal environment, were more like C57BL/6J in response, the sensitivity of CL/Fr cannot be attributed to a maternal effect.

Probit Analysis of Resorption

Independently fitted, probit, log dose regressions for the 6-AN induced resorption response are shown in Figure E2. There was considerable scatter of the data points about their best fitting lines, and all 3 genotypes had significant heterogeneity (CL/Fr χ^2 , 4 df = 21.27; CL.B6 χ^2 , 2 df = 28.85; C57BL/6J χ^2 , 10 df = 30.24 (as reported in Chapter B); all $P < .05$.

The calculated slopes ranged between 3.8 for CL/Fr to 10.8 for C57BL/6J. CL.B6 was similar to C57BL/6J at approximately 9.8. The effect of removing spontaneous resorption from the data is to increase the slope, and the minimum slope possible for CL.B6, based on a zero natural resorption frequency, 8.78, was still more similar to C57BL/6J.

Despite the apparent flatter slope of the CL/Fr resorption response, no significant deviations from parallelism could be demonstrated (Table E4), and the 3 genotypes fit a common slope of 5.9.

TABLE E4
TESTS OF PARALLELISM; RESORPTION RESPONSE OF
CL/Fr, C57BL/6J AND CL.B6

Finney's short method

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>
Parallelism	2	24.569*	12.285	2.665 ^{N.S.}
Heterogeneity	16	73.755*	4.610	
Total	18	98.324		

Modified long method

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>
Parallelism	2	13.399*	6.670	1.328 ^{N.S.}
Heterogeneity	16	80.362*	5.023	
Total	18	93.761		

* $P < .05$

The 95% fiducial limits for the resorption ED_{50} 's of CL/Fr and CL.B6 could not be calculated because the heterogeneity of the data contributed to "g" values greater than 1.0 (Finney, 1971, page 79). Nevertheless, CL.B6 (14.0 mg/kg) and C57BL/6J (15.0 mg/kg), ED_{50} values were probably not different. CL/Fr, at 11.0 mg/kg, showed no overlap of data values at dosages shared with the other genotypes, being consistently more sensitive.

Other dosage effects

No trends in sex ratio among normal embryos within genotypes with increased dosage were observed (Table E5). The data for C57BL/6J were presented in Chapter B. For all dosages pooled, about half of the normal embryos were male in CL/Fr (57/107), CL.B6 (32/69) and B6.CL (17/29) and none differed significantly from a 50:50 ratio (all χ^2 , 1 df < 0.9). Similarly, CL(P) embryos had no significant deviations from 50:50 sex ratios, males being 112 of 204 CL/Fr (χ^2 , 1 df = 1.96), 17 of 30 CL.B6 (χ^2 , 1 df = 0.53), and 5 of 10 B6.CL.

The deficiencies of males obtained in treated crosses of A/J and C57BL/6J due to biases inherent in the external method of sexing were not seen in this data. Perhaps the biasing factor is genotype specific. This possibility is enhanced by the interaction of the bias with genotype noted in Chapter A.

For CL/Fr embryos, within each sex, a significant

TABLE E5

EFFECT OF 6-AN DOSAGE ON SEX AND TYPE OF CL(P)

Embryo Genotype	Dose (mg/kg)	Number Normal		Number CL(P)					
		♂	♀	♂			♀		
				B	R	L	B	R	L
CL/Fr	4.905	19 ¹	14	5	0	2	3	2	3
	6.143	12	18	5	2	6	13	2	4 ¹
	7.358	13	13 ¹	23	5	6	18	1	4 ¹
	8.550	12 ²	5 ¹	17	1	0	10	1	0
	9.810	1	0	22	0	3	20	2	1
	12.263	0	0	14	1	0	8	0	0
		57	50	86	9	17	72	8	12
				= 112			= 92		
CL·B6	9.810	17	19	1	0	0	0	0	2
	12.263	10	7 ¹	1	0	1 ¹	1	1	1
	14.108	5 ¹	8 ³	1	1	7 ¹	2	1	4
	15.953	0	3 ¹	3	0	2	0	0	1
		32	37	6	1	10	3	2	8
				= 17			= 13		
B6·CL	12.263	9 ³	6 ¹	0	1	0	0	1	1
	14.625	1 ¹	1 ¹	2	0	0	2	0	0
	15.953	5 ¹	3 ¹	0	0	1	0	0	1
	16.875	2 ¹	2 ¹	1	0	0	0	0	0
		17	12	3	1	1	2	1	2
				= 5			= 5		

¹ Superscripts denote number of CP included.¹ Subscripts denote number of CL.

interaction of dosage and type of cleft lip was found. The proportion of bilateral clefts among CL(P) increased with dosage. Thirty-nine percent of 54 low dosage, but only 9% of 58 high dosage CL(P) males were "unilaterals" (Fisher's Exact Probability, 2 tailed = .0002). Similarly 32% of 50 low dose and only 10% of 42 high dose females were "unilaterals" (Fisher's Exact Probability, 2 tailed = .01). A reverse non-significant trend was observed for the pooled sexes in CL.B6 (Fisher's Exact Probability, 2 tailed = 0.67) where 63% of 8 low dosage and 73% of 22 high dosage affected embryos were "unilaterals". There were insufficient B6.CL for testing.

One possible explanation of the results for CL/Fr is that induced CL(P) are more often bilateral in CL/Fr than are spontaneous CL(P), and that as the relative proportion of induced CL(P) in the sample increased with dosage, so did the proportion bilateral. Alternatively, bilaterals may be a more severe expression of CL(P) and their dosage dependence may reflect additive effects of dosage on the threshold trait, although there is little evidence to support this from other genotypes (see also Chapter B).

For pooled dosages and sexes, of 204 affected CL/Fr, the majority, 77%, were bilaterally affected, while 8% and 14% were unilateral right and left, respectively. In contrast, the majority of 30 CL.B6 affected embryos were unilaterals, 60% being "left" and 10% being "right". This difference between genotypes in the type of CL(P) was significant (χ^2 2 df

= 35.63; $P < .05$). It may be at least partly due to the different dosage tolerances of the genotypes--i.e., a true difference would exist if similar results were obtained when dosages giving the same frequency of CL(P) in both genotypes were used.

No evidence of associations between sex and type of CL(P) (unilateral or bilateral) was found. 77% of male, and 78% of female, CL/Fr were bilateral; 15% of males and 13% of females were unilateral left. Among CL.B6, 65% of males and 77% of females were unilateral, but the interaction was not significant (Fisher's Exact Probability, 2 tailed = 0.69).

Unlike the data for crosses with A/J, where no side preference among unilaterals was noted, both CL/Fr and CL.B6 embryos showed a left side preference among unilaterals, 65% (37/57) being on the left (χ^2 , 1 df = 5.07; $P < .05$, for goodness-of-fit to 50:50 ratio).

29 of 30 CL.B6 affected embryos had cleft palate associated with the cleft lip; 203 of 204 CL/Fr were similarly CLP. The exceptions were isolated left CL in one male and one female.

Other face and palate defects

A few isolated CP were observed at all dosages (Table E1), and being in excess of the 1% expected spontaneously, were

probably induced by treatment. No median clefts were observed.

Discussion

CL/Fr was the most sensitive of the strains or genotypes tested for CL(P) inducibility. It also had the highest spontaneous CL(P) frequency. It is tempting to interpret this correlation as a meaningful one, despite the failure to find genetic association between the two traits in testcrosses (Chapter C).

Unlike A.B6 and B6.A embryos, CL.B6 showed no heterosis, but like them, did show dominance of the relative resistance of B6 to induced CL(P).

As was noted for the A/J and C57BL/6J crosses, the variation in dosage tolerance for CL(P) among genotypes again seemed grossly correlated with that for resorption, the rank order again being the same.

CHAPTER F

SEARCH FOR A MAJOR GENE FOR SPONTANEOUS CL(P) IN MICE

Introduction

Previous genetic studies (Reed, 1936a, b; Davidson, 1963), could not reject the possibility that a single major locus determined whether or not CL(P) occurred in the mouse strains compared.

In the course of generating genotypes to use to test the genetic relationship between 6-AN induced and spontaneous CL(P), genotypes were obtained that made it possible to look specifically for evidence of segregation of a major gene of this kind.

The experimental strategy was as follows: after a cross of A/J (10% CL(P)) and C57BL/6J (0% CL(P)) to conduct 3 generations of repeated backcrossing to A/J, and to compare the distribution of breeding values (CL(P) production) of first and second backcross sires to the distributions predicted by a single major locus model. By using A/J females throughout, maternal effects would be held constant.

It is well known, e.g., (Bloom and Falconer, 1964), and can be developed from simple Mendelian principles, that for segregation of a single major locus under repeated backcrossing to the recessive parent, or for a codominant trait, the

distribution of first backcross breeding values will be bimodal. The degree of bimodality will depend on the range between parental strains and the ability to discriminate between genotypes despite sampling error and environmental influences. If two or more loci of relatively equal additive effect control the trait, then the distribution will tend to become less spread and more "normal" as the number of overlapping modes is increased. Recessive epistasis will be expressed as ranges of breeding values like single locus inheritance but with different relative proportions leading to lower means in each generation than under the additive or single gene cases. Some of these possibilities are represented in Figure F1.

Materials and Methods

A/J and C57BL/6J mice were obtained from the Jackson Laboratory. They were maintained and bred as described previously (General Materials and Methods). All mice were fed Purina Mouse Chow.

Experimental Design and Breeding Tests

C57BL/6J males were bred with A/J females. Their F_1 (A.B6) male progeny were backcrossed to A/J females to produce the BC_1 (A.AB6) generation. BC_1 males were backcrossed to A/J females to produce the BC_2 generation (A.AAB6) (Figure F2).

FIGURE F1. EXPECTED DISTRIBUTIONS OF SIRE BREEDING VALUES, UNDER REPEATED BACKCROSSING, FOR VARIOUS GENETIC MODELS

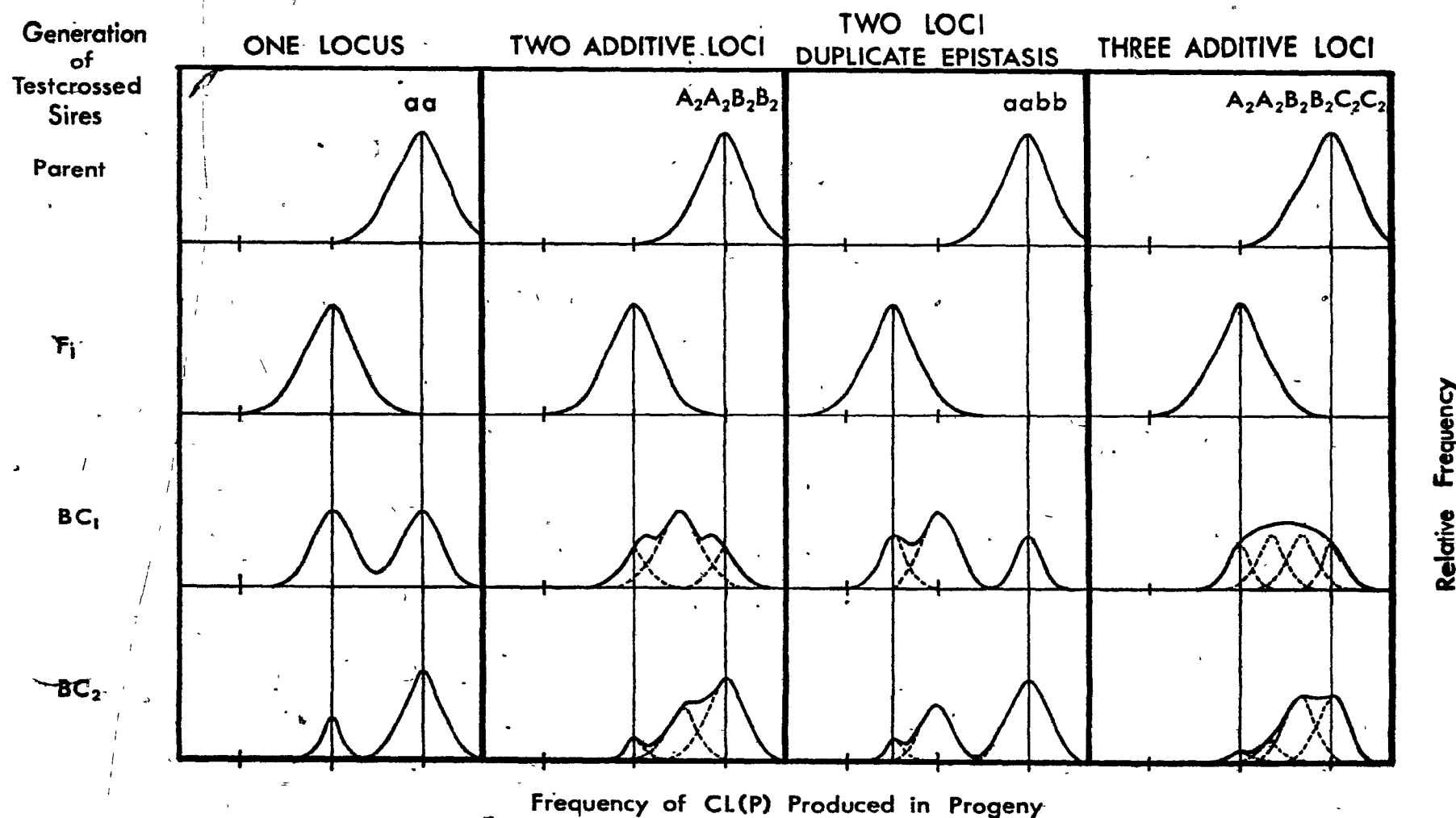
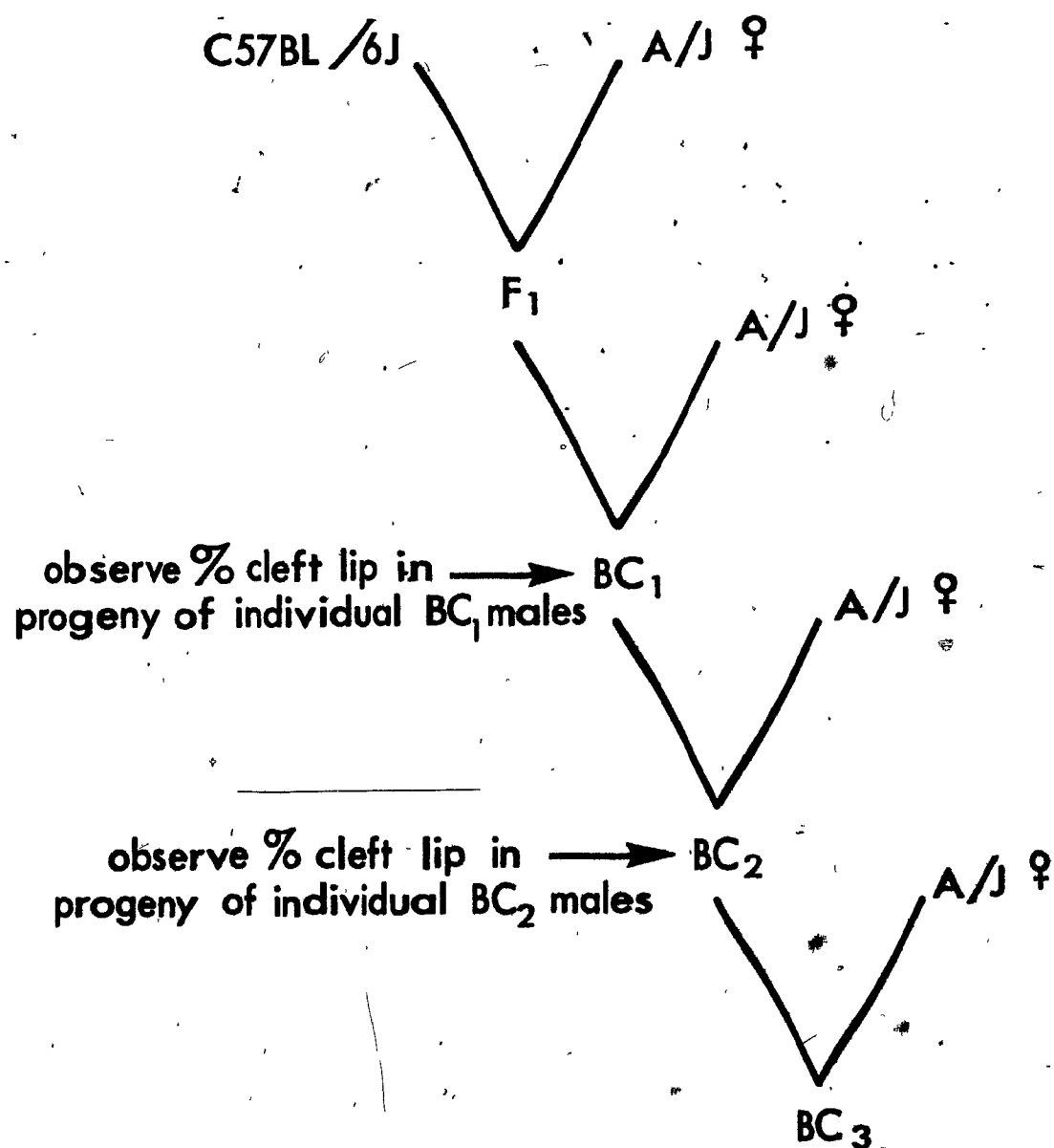


FIGURE F2. BREEDING DESIGN OF SEARCH FOR MAJOR GENE



Determinations of CL(P) breeding values for a series of 35 BC_1 and 28 BC_2 males, randomly chosen, were attempted. Each BC_1 male was bred with a series of A/J females until at least 15 litters were collected or the male ceased to breed; each BC_2 male was bred until 12 litters were collected, if possible.

In addition 28 A.AB6 litters (F_1 sire testcrosses), from several sires were collected, and data from 13 A.B6 litters and 17 A/J litters (Chapter B), also from several sires, was used.

Pregnant females were killed on day 17 of gestation (or, rarely, day 16 or day 18), and the uterine contents were examined under a dissecting microscope. The number and uterine position of embryos and resorptions were recorded. An estimate of the time of embryonic death (pre- or post-day 10) was made for resorptions. The condition of the lip and palate of intact embryos (live to at least day 16) was recorded, and the embryos were also sexed by anal-genital papilla distance. The "open-eye" trait was not recorded because by day 17 some normal eye closure may not yet be completed (Kalter, 1975). Non-pregnant females were rebred.

Derivation of the BC_1 and BC_2 sires

The final sample of BC_2 sires for which data was collected was derived from 8 original C57BL/6J males and 8 A/J females.

No more than 2 BC_2 sons were used from any one BC_1 sire, and the 24 BC_2 sires used were progeny of 14 different BC_1 sires. The 28 BC_1 sires used for testcrosses were derived from 15 F_1 sires.

Individual A/J females were placed with singly caged males and were removed when a vaginal plug was found. They were then caged alone until parturition. If they proved to be nonpregnant they were sometimes rebred.

Attempts were made to raise BC_2 progeny from all BC_1 sires. Those obtained represent the successes, usually after more than one attempt. Of 104 pregnant A/J females from all types of breeding crosses, 22 did not suckle the young, and 29 died after delayed parturition (see also Kalter, 1975). Two "runty" litters were discarded.

For each BC_1 and BC_2 sire, the frequencies of CL(P) in embryos and resorptions in implants were transformed to their arcsine equivalent (Rohlf and Sokal, 1969). A midpoint formula technique for smoothing and differentiating frequency distributions (Stewart, 1969), was applied to the transformed data.

The transformed BC_1 sire breeding value distributions were also compared to a normal distribution by the Kolmogorov-Smirnov One Sample Test (Sokal and Rohlf, 1969, page 573).

The CL(P) breeding values were also examined by the graphical method of rankit plots (Sokal and Rohlf, 1969, page 125).

This method causes normally distributed data to fit a linear regression (by eye) while other distributions will show

characteristic systematic deviations from linearity. An attempt to quantify the rankit graphical method by applying linear regression to the rankit (plus a constant 2.02 to make all values positive), transformed CL(P) breeding value plot. The order of the signs (+ or -) of the deviation of each data point from the regression line was then tested for randomness by the Runs Test (Siegel, 1956, page 52). A normal distribution is expected to give nonsystematic variation about a straight line, and detection of systematic order in the deviations should be a test of the presence of systematic deviations from normality. A graphical method, similar to rankit plots, whereby theoretical plots may be generated from the data if the ratio of underlying distributions may be predicted, was also used (Harding, 1949).

For the Kolmogorov-Smirnov Test and the linear regressions of rankit values, zero CL(P) frequencies were converted to $1/4n$ (Bartlett, 1947), and then transformed to arcsine values.

Other influences on CL(P)

The effect of the ovarian site on CL(P) frequencies was tested by comparison of the frequencies of CL(P) and non-CL(P) embryos at ovarian and non-ovarian sites, within each type of cross, using χ^2 tests of independence. Similar tests were used to compare the frequencies of resorption and scored embryos at ovarian and non-ovarian sites. Where the total sample was less than 25 or expected numbers were less than 5, Fisher's Exact Test (2 tailed) was used.

An association between BC_1 sire color and production of CL(P) was tested by one-way analysis of variance (Sokal and Rohlf, 1969, page 211), using the arcsine values for each sire (above).

For each generation, sex ratios within normal, CL(P) and CP embryos were tested against 50:50 sex ratios by χ^2 tests of goodness-of-fit, where sample sizes exceeded 25.

The independence of genotype and type of CL(P) (bilateral, right, left), was tested for BC_2 , BC_3 and A/J embryos by a χ^2 test of independence.

The proportion of CL(P) that was isolated CL was tested for independence of genotype for the BC_2 and BC_3 embryos by Fisher's Exact Test, 2 tailed.

Skull Measurements

When A/J, C57BL/6J, F_1 and BC_1 sires were discarded from breeding, at an approximate age of 9 months or older, their skulls were collected and cleaned by papain digestion (Luther, 1949). Two dimensions were measured using an ocular micrometer in a microscope with fixed magnification (ensured by use of an external standard). These dimensions were: the distance between the posterior edge of the incisive foramen and the anterior edge of the anterior palatine foramen (PL); the distance across the maxillae, just posterior to the sutures with the premaxilla, at the widest point, in the basal aspect of

the skull (PW2). These measurements had previously been suggested to be associated with predisposition to CL(P) in between-strain comparisons (Machado, 1978). The arbitrary units from the micrometer were analyzed. One arbitrary unit equalled 0.1053 mm.

Data for 34 C57BL/6J, 17 F₁, 27 BC₁, and 20 A/J sires was collected.

An association between the BC₁ sires' breeding values for CL(P) and their skull measurements was sought by Kendall's Rank Correlation (τ). Rank correlation was used because clustering due to segregation was expected for the CL(P) trait. It was not thought reasonable to attempt to correct the skull values for body size by use of body weight because several of the males were very fat.

BC₂ sire correlations were not tested because the more limited range of phenotype expected, and the smaller number of sires, would reduce the likelihood of finding associations, if present.

Results

Cleft lip was first recovered in the A.AB6 embryos, as has been observed previously (Table F1). The overall frequency of CL(P) recovered was, for all crosses, including the A/J strain itself, lower than those obtained by Davidson (1963), for the same genotypes. The change may be attributable

TABLE F1

RECOVERY OF CLEFT LIP FREQUENCY IN REPEATED BACKCROSSES TO
A/J FEMALES AFTER AN OUTCROSS TO C57BL/6J

Embryo Genotype	Sire Genotype	Number of Litters	Number of Implants	% Resorbed	Number Live	% CL(P) in Live
A-B6*	C57BL/6J	13	132	10.6	118	0.0
A-AB6	F ₁	28	306	12.4	268	0.7
A-AAB6	BC ₁	400	4260	19.3	3438	3.4
A-AAAB6	BC ₂	266	2730	23.5	2139	4.9
A*	A/J	17	171	26.9	125	10.4

* same data as Table D1

to genetic changes in the inbred strains during the decade between these studies, or to the difference in diet or other environmental factors, or to the later day of embryo collection used here, or to differences in parity (Kalter, 1975), Davidson's study having used second litters on day 15 of gestation.

Resorption frequency showed a progressive increase from a low of 11% in A.B6 embryos, through 12%, 19%, and 24% for the first through third backcross generation embryos, approaching the 27% resorption frequency of A/J. This approach toward the A/J resorption frequency with recovery of the A/J embryonic genotype suggests that resorption is at least partly a function of embryonic genotype. This result is in contrast to that of Davidson et al. (1969), who found resorption in A/J mothers to be approximately constant at 20% and independent of embryonic genotype. The lower recovery of CL(P) in the present study does not seem to have been due to increased resorption of CL(P) embryos, since it occurred at comparatively lower frequencies of resorption as well as higher ones.

Individual BC_1 and BC_2 sire breeding values for CL(P) and resorption are presented in Tables F2 and F3. Eight BC_1 sires and 4 BC_2 sires for whom samples were begun failed to produce 50 live embryos and were not included in the data. Their CL(P) production did not appear to be unusual: for the pooled BC_1 sires it was 4.5% in 31 litters; for the pooled BC_2 sires it was 1.5% in 8 litters.

TABLE F2

CL(P) AND RESORPTION FREQUENCIES IN LITTERS FROM BC₁
 SIRES CROSSED WITH A/J FEMALES

BC ₁ Sire ID No.	Color	Number of Litters	Number of Implants	% Resorbed	Number Live	% CL(P) in Live
31	blk	15	159	14.5	136	2.2
32	brn	16	179	14.5	153	1.3
36	brn	15	158	20.3	126	4.8
39	alb	16	181	22.1	141	3.6
40	alb	14	154	19.5	124	7.3
44	blk	18	195	16.4	163	4.9
46	brn	16	181	23.8	138	1.5
47	alb	16	185	14.6	158	4.4
48	brn	7	77	28.6	55	7.3
54	brn	14	160	10.6	143	2.1
56	blk	19	199	18.6	162	4.3
57	blk	13	146	13.7	126	0.8
85	alb	16	147	22.5	114	5.3
88	blk	18	186	16.7	155	5.8
91	blk	11	122	24.6	92	7.6
96	brn	18	201	24.4	152	1.3
107	brn	13	136	14.0	117	1.7
108	alb	15	158	20.3	126	4.8
110	brn	17	172	25.6	128	2.3
111	brn	11	128	15.6	108	4.6
117	brn	15	167	21.6	131	1.5
118	alb	18	179	26.8	131	6.9
120	blk	13	132	12.1	116	2.6
121	blk	15	154	16.2	129	0.0
122	alb	16	161	24.2	122	1.6
124	blk	8	68	14.7	58	0.0
125	blk	7	63	17.5	52	0.0
127	brn	10	112	26.8	82	4.9

TABLE F3

CL(P) AND RESORPTION FREQUENCIES IN LITTERS FROM BC₂
 SIRES CROSSED WITH A/J FEMALES

BC ₂ Sire ID No.	Color	ID No. of BC ₁ Sire	Number of Litters	Number of Implants	% Resorbed	Number Live	% CL(P) in Live
201	blk	88	13	134	17.9	110	3.6
202	blk	88	11	116	24.1	88	3.4
207	alb	56	11	102	28.4	73	8.2
210	alb	56	14	166	30.7	115	0.9
214	alb	39	12	113	29.2	80	3.8
215	alb	39	13	115	20.9	91	1.1
216	alb	96	12	125	22.4	97	0.0
218	alb	96	12	134	22.4	104	6.7
221	alb	47	11	114	26.3	84	14.3
224	alb	47	12	112	17.9	92	5.4
226	brn	107	9	95	27.4	69	10.1
228	brn	107	13	125	23.2	96	9.4
231	alb	122	12	136	19.9	109	8.3
232	alb	122	11	102	15.7	86	15.1
233	alb	31	10	97	15.5	82	2.4
234	alb	118	9	94	19.2	76	5.3
235	alb	118	7	72	13.9	62	4.8
236	alb	126	12	129	21.7	101	3.0
238	alb	126	10	94	18.1	77	1.3
240	alb	125	13	135	14.8	115	1.7
243	alb	108	9	109	22.9	84	1.2
247	alb	123	9	99	25.3	74	0.0
248	alb	123	11	104	13.5	90	3.3
250	alb	46	10	108	22.2	84	7.1

The association between CL(P) and resorption

BC₁ sires that produced relatively high frequencies of CL(P) also tended to produce relatively high resorption rates. Since genetic segregation was expected to be present and to cause clustering, a distribution-free test of association, Spearman's Rank Correlation (r_s) was used (Siegel, 1956, page 212), and the calculated correlation was 0.42 ($P < .05$). No such evidence of association between CL(P) and resorption was found for the BC₂ sire group ($r_s = 0.05$; $P > .05$).

When resorption was subdivided into "early" (pre-day 10) and "late" (post-day 10), and each type was correlated with the CL(P) frequency, the following Kendall rank correlations (τ) were obtained: BC₁ sires, early, $\tau = 0.09$; $P > .05$; BC₁ sires, late, $\tau = 0.30$; $P < .05$; BC₂ sires, early, $\tau = -0.12$; $P > .05$; BC₂ sires, late, $\tau = 0.09$; $P > .05$. (Kendall's Rank Correlation shares the same sensitivity as Spearman's, and was obtained from the McGill "MUSIC" statistical Computer Programs). While the BC₂ correlation was not improved, it was evident that the correlation between CL(P) and resorption for BC₁ sires arose at least mainly from the post-day 10 mortality.

The association between CL(P) and resorption production by BC₁ sires indicates that the traits are linked or pleiotropic effects of the same gene(s). The failure to find a similar association for BC₂ sires is not surprising if one or

both traits are due to segregation at a very few loci, since little genetic variance is expected to remain ($F = .75$), and for a small number of sires the majority could all be of one genotype, and most variation present be due to environment or chance.

Segregation of Resorption

It was of interest, in view of the above, to try to detect evidence of segregation of the high resorption trait among the BC_1 and BC_2 males. The pattern of recovery of the A/J resorption frequency is shown in Figure F3. The A/J strain does not have a resorption frequency markedly higher than C57BL/6J (20%, Chapter B), and the lower rates in heterozygotes may be due to heterosis.

The mean resorption frequencies produced by F_1 , BC_1 and BC_2 sires were all relatively low compared with those expected for additive inheritance, or loci with independent effects, and indicate that epistasis may have been present. Yet approximately half of the BC_1 sires produced resorption in the A/J range, the expectation for a single locus. The epistatic factors contributed by C57BL/6J, if any, may therefore have been modifiers that buffered the resorption frequency of A/J-like segregants and heterozygotes at a major locus downward, but did not obliterate the differences between them.

The BC_1 sire distribution (Figure F4) generated by

FIGURE F3. RECOVERY OF RESORPTION FREQUENCIES WITH REPEATED BACKCROSSING TO A/J

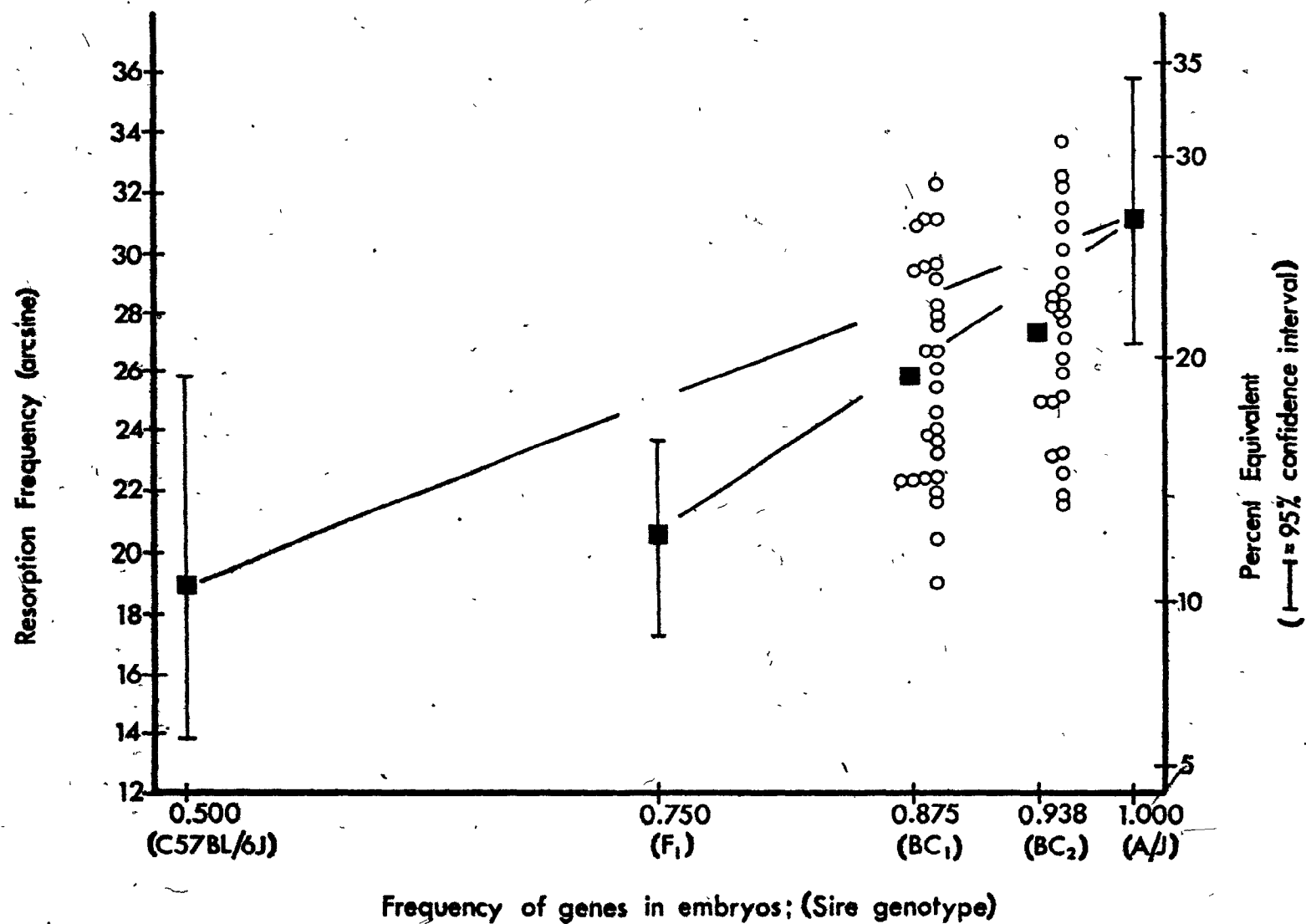
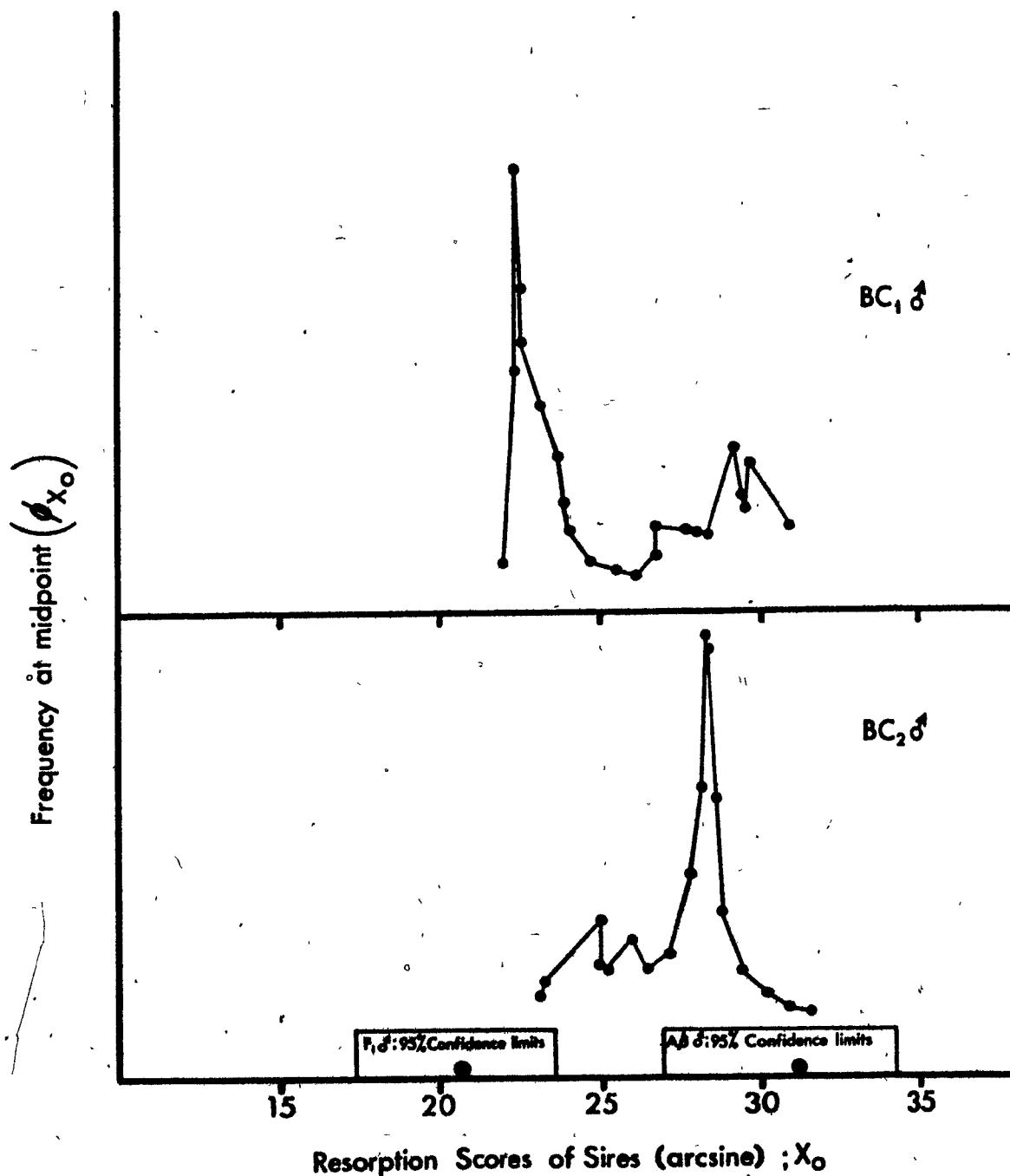


FIGURE F4. SMOOTHED FREQUENCY DISTRIBUTIONS OF RESORPTION BREEDING VALUES



application of Stewart's (1969) midpoint formula agrees well with the bimodal, equal proportion distribution expected for the segregation of a single major locus; the major discrepancy was in the BC_2 distribution: a deficiency of sires like the F_1 class, which seems best explained by sampling error. Two loci with recessive epistasis could also explain the data.

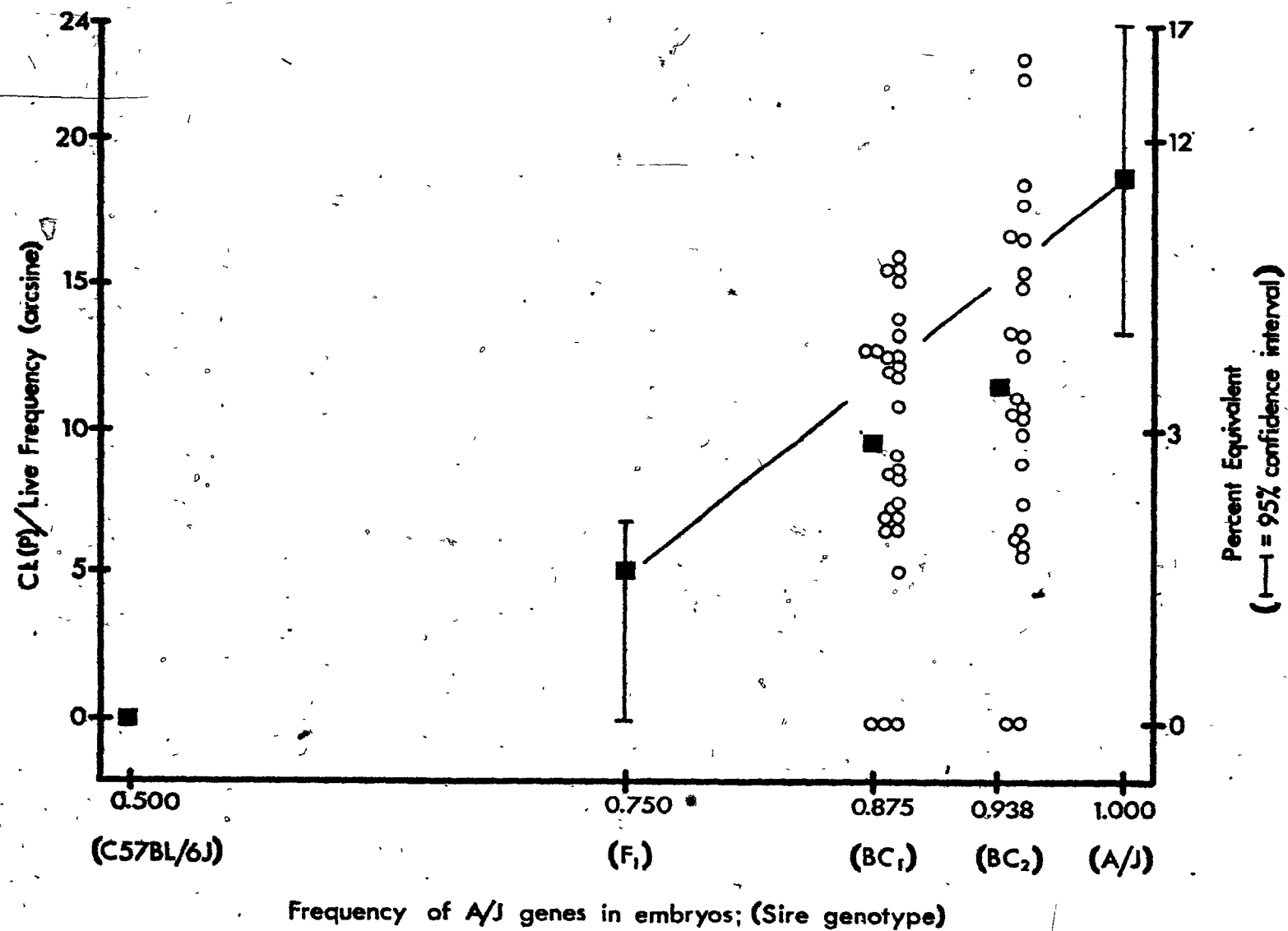
Despite the bimodality of the graphical presentation, no significant deviation from one normally distributed population could be detected by the Kolmogorov-Smirnov One-Sample Test for the BC_1 sires.

Nevertheless, the most likely explanation of the recovery of A/J-like high resorption frequencies with repeated backcrossing seems to be the recovery of homozygosity at one recessive locus, or two with duplicate epistasis. This locus (or loci) would be linked to, or identical with (a) gene(s) causing spontaneous CL(P).

Segregation of CL(P)

The rate of recovery of CL(P) with repeated backcrossing to A/J is presented in Figure F5. No CL(P) was recovered in A.B6 embryos, the scale is therefore truncated at zero, and no useful genetic information can be obtained from that cross, apart from the "recessiveness" of the trait. Davidson (1963) observed similar results.

FIGURE F5. RECOVERY OF CL(P) FREQUENCIES WITH REPEATED BACKCROSSING TO A/J

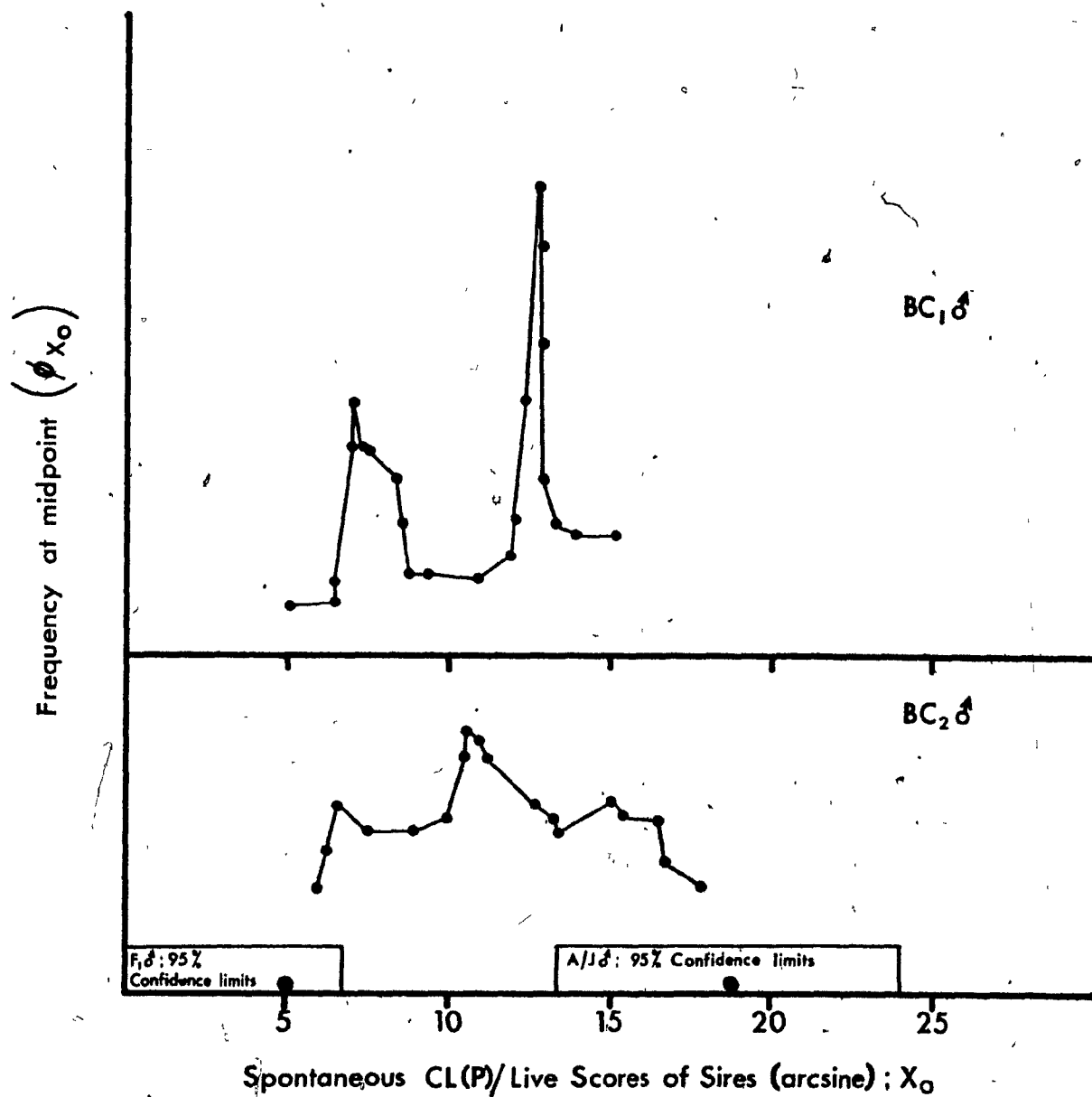


The mean CL(P) frequencies produced by BC₁ sires and BC₂ sires, were lower than those expected for genetic additivity and suggest that epistasis by "protecting" alleles from C57BL/6J may have been present (Mather and Jinks, 1971, pages 177 and 278; Lush, 1945, page 81). Either the number of such "protective" loci or their effects must be small, for several BC₁ sires reached the A/J range of CL(P) production. If two recessive loci with duplicate epistasis caused CL(P), then 1/4 of the BC₁ sires ("aabb") would be expected to be like A/J males; whereas, if three such loci were involved ("aabbcc"), their frequency would drop to 1/8.

The number of major loci contributing to liability to CL(P) must therefore be small. If the epistatic effects of C57BL/6J alleles were confined to being modifiers that shift the frequencies of CL(P) downward among all liable embryos, then the shapes of the BC₁ and BC₂ sire distributions of breeding values would remain unchanged; only their means would be shifted.

Smoothed differentiated frequency distributions (Stewart, 1969), for BC₁ and BC₂ sires (Figure F6), indicate that 2 groups of BC₁ sires in roughly equal proportions were recovered, one group like F₁ sires, and one group like A/J sires, as predicted by a single major locus hypothesis. The 25:75 ratio expected among two similar modes for BC₂ sires predicted by the hypothesis was not so clearly matched. The discrepancies may be due to chance, but if the means of the BC₁ and BC₂ sire

FIGURE F6. SMOOTHED FREQUENCY DISTRIBUTIONS OF CL(P) BREEDING VALUES



breeding values and their distributions are considered jointly, a two locus model with duplicate epistasis which also predicts a grossly "bimodal" BC_1 distribution (Figure F1), seems to fit better.

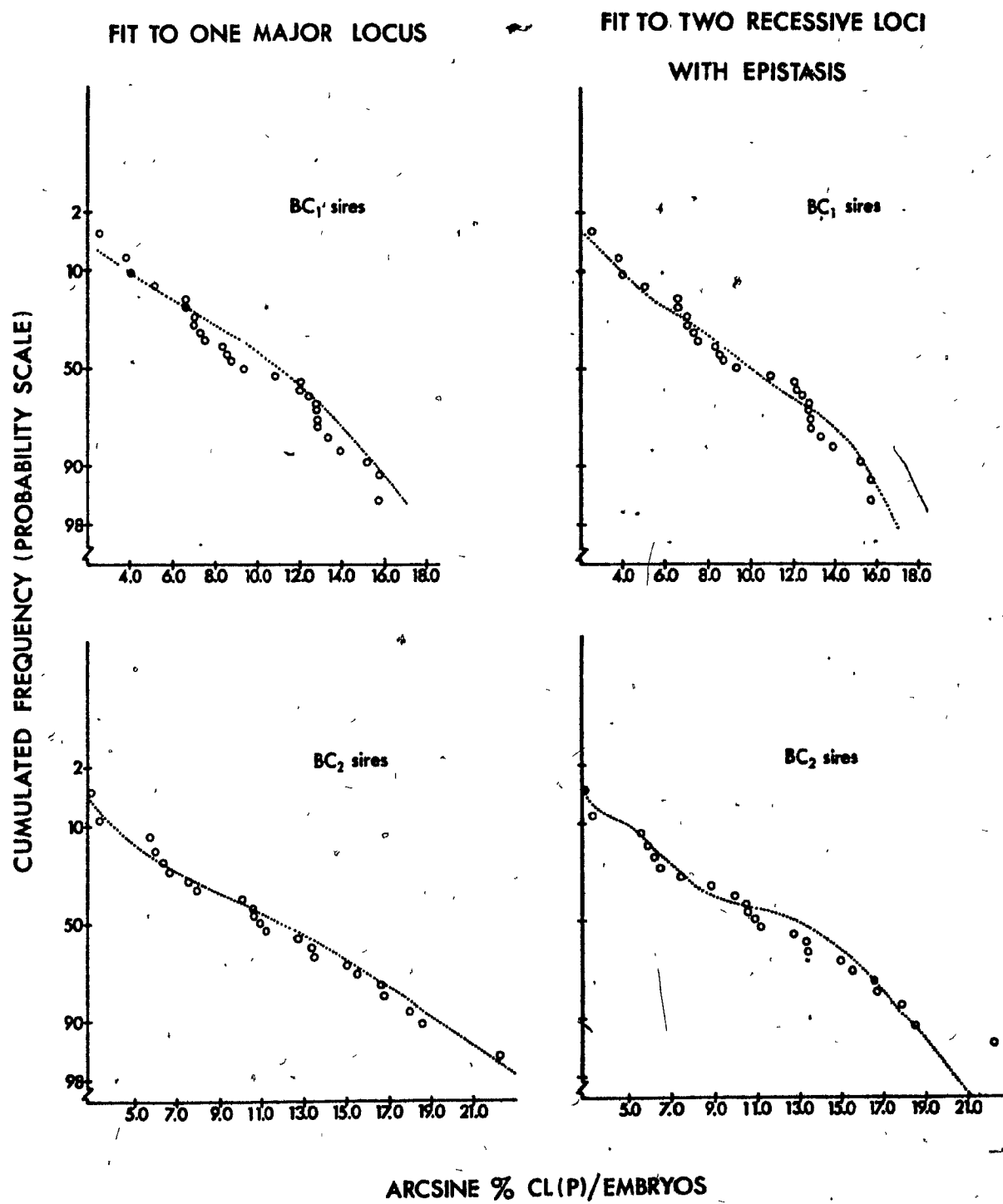
The apparent bimodality of the BC_1 sire distribution argues against "threshold" inheritance, where, for example, the genotypes Aabb and aabb could both have CL(P) but the latter more frequently. Such inheritance would lead to several (e.g., 3) modes, with the largest peak intermediate to the A/J and F_1 CL(P) breeding values.

The Kolmogorov-Smirnov One Sample Test could not reject the hypothesis ($P > .05$) that all BC_1 sires' CL(P) breeding values belonged to one normally distributed population. The test, however, is probably relatively insensitive for small numbers.

Deviations of the BC_1 sire values from a straight line fit by linear regression to a "rankit" plot were found to be significantly systematic ($P < .05$) by the Runs Test. That is, systematic deviations from a normal distribution were indicated. The pattern of deviations (3+, 1-, 2+, 8-, 8+, 2-, 2+, 2-) was compatible with both the major locus and the 2 duplicate epistatic loci hypothesis.

Cumulated probability plots similar in principle to rankit plots (Harding, 1949) for the BC_1 and BC_2 sire breeding values are shown in Figure F7. The fit of the data to the theoretical plots for a duplicate epistasis model may be slightly better than that for a single major locus.

FIGURE F7. CUMULATED PROBABILITY PLOTS OF CL(P) BREEDING VALUES



The maximum likelihood method of Elston and Stewart (1973), for distinguishing among genetic models for quantitative traits in BC_1 generations could be applied to BC_1 sire breeding values for threshold traits. It could not be applied to this data, however, because no individual F_1 and A/J sire breeding values were obtained. This deficiency of the experimental design also precluded the use of a distribution free test of goodness-of-fit to single locus inheritance for the BC_1 sires' breeding values (Mode and Gasser, 1972). This latter test does not seem applicable to more complex genetic models, while the former would not incorporate the BC_2 sire breeding data, the critical generation for testing models derived from BC_1 observations (Wright, 1934).

The most likely explanation of CL(P) differences between A/J and C57BL/6J seems to be two major loci, with duplicate epistasis. If one major locus is the cause, then CL(P) penetrance is reduced downward by dominant C57BL/6J modifiers.

Correlations between generations

There were no detectable correlations between BC_1 sire breeding values for CL(P) or resorption and their BC_2 son's mean breeding values, on the arcsine scale. Spearman's Rank Correlations were 0.05 for CL(P) in embryos and -0.04 for resorption. This is compatible with the view that dominance and epistasis have large influence on the traits, and with the segregation of a small number of loci. Sampling error, in that the most extreme BC_1 sires failed to provide BC_2 sons,

may also have contributed. Most notable was the ability of low scoring BC_1 sires (CL(P)) to produce A/J-like BC_2 sons. In a small sample this argues strongly for segregation at a small number of loci.

The ovarian site

Approximately 20% of all implantations were at the most ovarian ends of the uteri, the "ovarian sites" (Table F4).

The frequencies of CL(P) in the two largest samples were higher at the ovarian sites (4.4%) than at other sites (3.2%) in BC_2 embryos and lower at the ovarian sites (4.1%) than at other sites (5.1%) in BC_3 embryos. Neither of these trends was significant (χ^2 1df = 2.34 and 0.54, respectively; $P > .05$). Nor were significant differences found among A/J embryos where 7% of 27 ovarian site embryos and 11% of 98 non-ovarian site embryos had CL(P) (Fisher's Exact Probability = 0.73). Similarly, while 2% of 50 BC_1 ovarian sites and 0.4% of 217 non-ovarian sites had CL(P), the trend was not significant (Fisher's Exact Probability = 0.34).

Resorption frequencies for the relatively small A/J and BC_1 samples showed little evidence of site effects, (Fisher's Exact Probability = 0.40 and 0.50, respectively). The trend for both was toward lower resorption frequencies at the ovarian site: 21% compared with 28% in A/J, and 9% compared with 13% in BC_1 implants. This trend obtained statistical

TABLE F4

EFFECTS OF THE OVARIAN SITE (O.S.) ON CL(P) AND RESORPTION FREQUENCIES

Genotypes of Embryos	Total Implants	Observed Frequencies/Implants				CL(P)			Normal*			Resorption		
		Resorp- tion	CL(P)	Left O.S.	Right O.S.	Left O.S.	Right O.S.	Other	Left O.S.	Right O.S.	Other	Left O.S.	Right O.S.	Other
A/J	171	.2690	.0760	.0994	.0994	2	0	11	11	14	87	4	3	39
A.AAB6 (BC ₃)	2730	.2354	.0385	.0945	.0949	8	10	87	211	208	1615	39	41	511
A.AAB6 (BC ₂)	4260	.1930	.0275	.0923	.0916	15	15	87	318	329	2674	60	46	716
A.AB6 (BC ₁)	306	.1242	.0065	.0915	.0882	1	0	1	26	23	217	1	4	33

* Includes CP

significance among BC_2 and BC_3 implants (χ^2 , 1 df = 19.98 and 13.89, respectively; $P < .05$). Among BC_2 implants at the ovarian sites 13.5% were resorbed; at other sites 20.6% were resorbed. 15.5%, compared with 23.1% of BC_3 ovarian and non-ovarian site implants were resorbed.

In summary, no consistent effect of the ovarian site on CL(P) frequency was detected, but a consistent and often significant trend towards fewer resorptions being found at the ovarian sites was observed.

Test of association with coat color

The mean arcsine CL(P) breeding values for the 10 black, 11 brown, and 7 albino BC_1 sires were 8.10, 9.56, and 12.46 respectively. The differences between these means were not significant ($F_{2,25} = 2.23$; $P > .05$). There were only 2 black and 2 brown BC_2 sires, and they therefore were not tested. Previously, Reed (1936a, b), also found no associations between the albino, brown, or agouti loci and spontaneous CL(P).

Test of association with sex

None of the 3 backcross generations of embryos gave evidence of deviation from 50:50 sex ratios among CL(P) embryos (Table F5). No deviation from equal risks for both sexes was found for the relatively small numbers of spontaneous isolated CP.

TABLE F5

SEX RATIO OF EMBRYOS

Embryo Genotype	<u>Number normal</u>		<u>Number CL(P)</u>		<u>Number CP</u>	
	♂	♀	♂	♀	♂	♀
A·B6 (F ₁)	59	53	9	4	1	1
A·AB6 (BC ₁)	138	128	2	0	1	0
A·AAB6 (BC ₂)	1760	1561	65	52	18	29
A·AAAB6 (BC ₃)	1006	1000	50	55	17	11

An excess of males among normal embryos in the BC_2 generation was significant (χ^2 , 1 df = 11.92; $P < .05$). All other generations of normal embryos also showed similar but nonsignificant trends.

The failure to detect a sex ratio difference among CL(P) embryos agrees with some previous findings for the A/J strain (Literature Review). No sex ratios for similar segregating generations were reported by Davidson (1963), probably because of the relatively early examination time used.

If the method of sexing can be assumed to be accurate for untreated embryos, then the deficiency of females among normal embryos suggests that they have differential mortality. Since mortality and CL(P) seem to be related (above), it is possible that a disproportionate number of these females had CL(P). The apparent instability of sex ratio among normal and CL(P) embryos has been discussed (Literature Review).

Types of CL(P)

The numbers of CL(P) embryos from individual BC_1 and BC_2 sires were small, and showed no evidence of heterogeneity (Tables F6 and F7) of type. There was little evidence of litter effects on spontaneous CL(P) occurrence. Most affected were the only ones in their litter, as would be predicted from the low CL(P) frequency.

The proportions of bilateral to right to left CL(P) did

TABLE F6

TYPES OF CL(P) AND RESORPTION IN LITTERS FROM
BC₁ MALES CROSSED WITH A/J FEMALES

BC ₁ Male Number	% of Resorptions Before Day 10	Number of CL(P)			Number CL	Number of Litters with CL(P)	Number CP	Number of Litters with CP
		Bilateral	Right	Left				
31	52	2	-	1	-	3	2	2
32	46	-	-	2	-	2	4	3
36	56	1	1	4	-	5	3	3
39	68	4	1	-	-	5	5	4
40	60	5	3	1	-	5	1	1
44	56	6	1	1	-	6	2	2
46	51	-	2	-	-	2	3	2
47	48	2	2	3	2	6	3	3
48	32	1	3	-	1	4	1	1
54	35	1	-	2	1	2	1	1
56	51	5	2	-	1	6	2	1
57	70	1	-	-	-	1	1	1
85	64	3	2	1	-	5	1	1
88	52	4	2	3	-	6	1	1
91	43	7	-	-	-	6	1	1
96	65	2	-	-	-	2	1	1
107	47	-	1	1	-	2	1	1
108	66	2	1	3	-	5	3	3
110	41	2	-	1	-	2	1	1
111	85	4	-	1	-	4	2	2
117	53	1	-	1	-	2	1	1
118	65	4	2	3	-	7	2	1
120	81	1	2	-	-	3	3	3
121	76	-	-	-	-	-	1	1
122	54	2	-	-	-	2	1	1
124	60	-	-	-	-	-	-	-
125	64	-	-	-	-	-	-	-
127	50	3	-	1	-	4	-	-
		63	25	29			47	
Females		32	14	16				
Males		31	11	13				

TABLE F7

TYPES OF CL(P) AND RESORPTION IN LITTERS FROM
BC₂ MALES CROSSED WITH A/J FEMALES

BC ₂ Male Number	% of Resorptions Before Day 10	Number of CL(P)			Number CL	Number of Litters with CL(P)	Number CP	Number of Litters with CP
		Bilateral	Right	Left				
201	67	3	1	-	-	4	6	2
202	43	2	-	1	-	3	1	1
207	72	5	-	1	-	5	-	-
210	67	1	-	-	-	1	3	2
214	70	1	1	1	-	3	1	1
215	58	-	-	1	-	1	1	1
216	57	-	-	-	-	-	2	1
218	63	4	2	1	-	5	2	2
221	60	8	1	3	-	8	1	1
224	55	1	2	2	-	5	-	-
226	65	4	2	1	-	4	1	1
228	28	6	1	2	1	6	1	1
231	37	8	-	1	-	5	1	1
232	25	8	4	1	-	8	1	1
233	73	2	-	-	-	2	2	2
234	67	3	-	1	-	3	-	-
235	10	3	-	-	-	2	-	-
236	54	-	1	2	-	3	-	-
238	53	1	-	-	-	1	2	1
240	55	2	-	-	-	1	-	-
243	52	1	-	-	-	1	-	-
247	52	-	-	-	-	-	2	1
248	57	3	-	-	-	3	-	-
250	46	3	1	2	-	4	1	1
		69	16	20		78	28	
Females		35	6	9			17	
Males		34	10	11			11	

not differ significantly among BC_2 , BC_3 and A/J embryos (χ^2 , 4 df = 5.57; $P > .05$) (Table F6). For all of these genotypes and BC_1 embryos pooled, 59% were bilateral, 18% were unilateral right, and 22% were unilateral left.

Cleft lip was nearly always associated with cleft palate (Table F8). Although a higher proportion of BC_2 embryos with CL(P) had isolated CL (5/117) than BC_3 embryos (1/105), this trend was not significant ($P > .05$).

Neither laterality nor the rate of association with CP appeared to be correlated with average liability to CL(P), which theoretically increased with repeated backcrossing to A/J. Neither, therefore seem to be measures of severity of CL(P) on the underlying threshold scale.

Tests for association with adult "face shape"

As was found previously (Machado, 1978), A/J males tended to have smaller PL and PW2 values than did C57BL/6J. Intermediate values were obtained for F_1 and BC_1 sires (Table F9). The two traits were apparently partly or completely independent, for PL tended to regain the A/J phenotype in at least half of the BC_1 sires, while PW2 for these sires remained similar to C57BL/6J. The rapid return to A/J-like values for PL suggests that the difference between strains may be largely due to a single locus.

The individual BC_1 sire skull values are presented in

TABLE F8

PROPORTIONS OF BILATERAL, RIGHT AND LEFT CL(P) AND
CL AMONG BACKCROSSES

Embryo Genotype	Number of Embryos			Number CL	(% of CL(P))
	Bilateral	Right	Left		
A•AB6 (BC ₁)	2	0	0	0	-
A•AAB6 (BC ₂)	63	25	29	5	4.3
A•AAAB6 (BC ₃)	69	16	20	1	1.0
A/J	7	1	5	0	-

TABLE F9
SIRE SKULL MEASUREMENTS

Genotype	(N)	Number, of Mice Having Each Value																											
		PL ¹												PW2 ²															
		6	6.5	7	7.5	8	8.5	9	9.5	10	40	40.5	41	41.5	42	42.5	43	43.5	44	44.5	45	45.5	46	46.5	47	47.5	48	48.5	
C57BL/6J	(34)				1	3	8	8	9	5							1		5	5	7		6	3	3	3			
F ₁ (A·B6)	(17)			5	5	5	1	1											1	4	6	2		2		1	1		
BC ₁ (A·AB6)	(27)	1	7	10	6	2		1								1	1	5	1	4	4	6	2	1	2				
A/J	(20)	7	9	4							2		1	2	4	2	2	4	1			1							

¹ distance from the posterior edge of the incisive foramen to the anterior edge of the anterior palatine foramen.

² distance across the maxillae, just posterior to the sutures with the premaxilla, at the widest point, in basal aspect of the skull.

Table F10. No evidence of association between PL or PW2 and CL(P) breeding values was detected. Kendall's Rank Correlations (τ) were -0.095 and 0.043 respectively, and were non-significant ($P > .05$). The ratio, PW2/PL, an attempt to remove differences due solely to head size, was also not significantly correlated with CL(P) production ($\tau = 0.077$; $P > .05$).

BC₁ sires that produce "high" CL(P) frequencies are expected to be genotypically more like A/J sires at the relevant loci than "low" CL(P) producers, which are expected to be more like F₁ sires. The range of variation of PL in BC₁ sires included F₁- and A/J-like values, and if the PL dimension reflected genetic liability to CL(P), a correlation between the PL value and CL(P) should have been detected. It is possible that the advanced age of the males obscured the type of difference Machado (1978), found between strains for much younger mice, or that PL must be corrected for other differences in skull dimensions.

To have found an association between PW2 and CL(P) would have been less likely. Few BC₁ sires, some of whom neared A/J CL(P) breeding values, recovered the A/J PW2 value, although there is an interesting hint of bimodality in the data (Table F9).

The use of segregating generations is a most powerful genetic test of the correlations suggested by study of small numbers of inbred strains. The failure of this study, which used relatively insensitive measures of face-shape (but the

TABLE F10

KENDALL RANK CORRELATIONS (τ) BETWEEN CL(P) PRODUCTION AND
EACH OF TWO SKULL DIMENSIONS, FOR BC₁ SIRES

BC ₁ Sire Identification Number	% CL(P) in Live Embryos	Skull Parameters, in Arbitrary Units ¹		
		PL	PW2	$\frac{PW2}{PL}$
125	0	7.0	44.0	6.3
124	0	7.0	46.0	6.6
121	0	7.5	44.0	5.9
57	0.8	6.5	45.5	7.0
32	1.3	7.0	45.0	6.4
96	1.3	7.0	45.0	6.4
46	1.5	7.5	44.5	5.9
117	1.5	7.5	46.0	6.1
122	1.6	6.5	44.0	6.8
107	1.7	7.5	44.0	5.9
54	2.1	6.5	46.5	7.2
31	2.2	9.0	46.0	5.1
110	2.3	6.0	46.0	7.7
120	2.6	7.0	47.0	6.7
39	3.6	7.0	46.5	6.6

193

.....Continued

TABLE F10
(continued)

56	4.3	7.5	46.0	6.1
47	4.4	7.0	47.5	6.8
111	4.6	8.0	46.0	5.8
108	4.8	7.0	44.0	6.3
36	4.8	6.5	45.5	7.0
127	4.9	7.0	43.0	6.1
44	4.9	no data	-	-
85	5.3	7.5	45.5	6.1
88	5.8	8.0	45.0	5.6
118	6.9	7.0	45.0	6.4
40	7.3	6.5	47.5	7.3
48	7.3	6.5	45.5	7.0
91	7.6	6.5	43.5	6.7
		$\tau = -0.095$	0.043	0.077
		$P = 0.243$	0.377	0.287

¹ Defined in Table F9

same ones as used for the inbred strains) to find the expected correlations may be due to the presence of other variables (e.g., old age), or to the insensitivity of the measurements combined with increased variation due to segregation. On the other hand, the data offered no support for the view that liability to CL(P) is reflected in these skull parameters.

Summary and Discussion

The difference between A/J and C57BL/6J in liability to spontaneous CL(P) seems best explained by 2 recessive (in A/J) major loci with duplicate epistasis and no modifiers of important effect. An alternative that may also explain the data is a single major recessive locus (or linkage group) that determines that CL(P) can occur, and some modifiers that enhance its penetrance.

In terms of genetic thresholds, the threshold number of genes (not loci) required to cause CL(P) in at least some embryos would be whatever are the same in A/J and C57BL/6J plus 2 (1 recessive locus) or 4 (2 loci with epistasis).

One or both of the two major loci (or the major locus), may have pleiotropic effects causing embryonic mortality, or one may be linked to a gene with such effects.

The ovarian site of the A/J uterus was not observed to have detectable effects on CL(P) frequencies, but did consistently show smaller proportions of resorption. This

result cannot be explained by the prevailing view that the ovarian site is relatively deprived (Trasler, 1960).

Neither laterality of CL(P) nor rate of association of CL with CP appear to be related to CL(P) liability. If these traits are taken to be measures of expressivity, it is not related to risk, contrary to what would be expected from the multifactorial threshold model.

While genetic differences between 2 inbred strains must be taken as a minimum estimate of the genetic variation contributing to a trait, there is little evidence to indicate that crosses to other strains would give different results. Loëvy (1964) obtained 5% CL(P), compared with 0.7% in the present study, in BC_1 embryos from crosses between Strong A and C3H mice. Similarly, the BC_1 progeny from F_1 mothers from a cross of CL/Fr and SWV had 6.7% affected among 342 newborns (personal observations). The higher frequencies observed suggest that these strains contributed higher penetrance or had in common one of the major genes. More major loci being involved would have given lower CL(P) frequencies. In neither cross were F_1 embryos affected. These additional strain comparisons suggest that other major loci affecting CL(P) liability in embryos are at least not common.

As pointed out by Thoday (1966; Thoday and Thompson, 1976), to assume that a continuous variable underlies a trait is not an assumption that the number of genes influencing it

need be large and:

whenever serious attempts have been made to study polygenic variation in terms of specific genes, a few handleable genes have proved to mediate a large part of the genetic variance under study.

In support of this argument was the finding (Stewart and Elston, 1973), that genetic variation in 11 of 14 continuous variables differing between 2 strains of mice could be adequately explained in terms of only one or two loci each. Similar findings for Drosophila were reviewed by Thompson (1975).

If the finding of major genes when sought (and which identify major steps in development) can be extended to developmental threshold traits, then an interesting genetic structure emerges. Rather than a given developmental step being buffered by being polygenic (as can be generalized from Waddington's genetic assimilation and canalization concepts, and Gruneberg's (1952) quasi-continuous variant studies), the genetic structure can be seen as being hierarchial (as in Rendel's major gene concept, where only major genes and not modifiers are regulated) and "buffering" may arise from non-additive interactions between major loci (e.g., duplicate epistasis, as suggested by Lerner, 1954, page 34).

CHAPTER 6

CROSSES BETWEEN A/J AND CL/Fr

Introduction

One of the characteristics predicted for multifactorial threshold traits is the existence of inbred strains having different stable frequencies of the trait in question (Gruneberg, 1952a). This prediction would hold for the range of types of inheritance from a "major" gene whose penetrance is controlled by modifiers, to polygenic inheritance where the impact of all loci is roughly equal. For all cases, the modifiers or the many genes are expected to act largely additively.

The difference in CL(P) frequency produced by A/J (10 to 15%) and CL/Fr (20 to 25%) in term embryos seemed to fit this prediction.

The CL(P) of CL/Fr is assumed to share the same etiology as that in A/J because CL/Fr was derived by selection for CL(P) and inbreeding from an outcross of A/J to a non-CL(P) heterogeneous stock (Staats, 1972).

It was of interest to test the prediction of the multifactorial threshold model that F_1 embryos of a cross between CL/Fr and A/J, on an appropriately transformed scale, should have a CL(P) frequency intermediate to those of the two parental strains. Additional generations after this cross were

available from another study and were therefore examined also.

In the light of the maternal effect suggested by the results of the crosses between A/J and CL/Fr, it was of interest to test whether the effect would apply to CL(P) segregating in the progeny of A.AB6 sires (Chapter F), and whether there would be a proportionate increase in CL(P) frequencies in the progeny of these sires when conceived in CL/Fr mothers, or whether there would be interactions.

Since an apparent correlation between resorption and CL(P) had been found in the A.AB6 sires' breeding values with A/J females, it was also sought in the testcrosses with CL/Fr.

Materials and Methods: Crosses of A/J and CL/Fr

A/J mice were obtained at 6 to 12 weeks of age from the Jackson Laboratory, and fed Purina Mouse Chow. CL/Fr mice were derived from Dr. F.C. Fraser's stocks at F₃₄ and were between F₃₅ and F₄₂ of brother-sister inbreeding; they were fed Purina Mouse Chow from at least 2 weeks prior to breeding. F₁ and segregating generation mice were fed Purina Mouse Chow from weaning (by their mothers). Females used were nulliparous.

F₁ and segregating genotypes were produced by M. Joseph's study of the segregation of a nickel-dependent dermatitis that was observed to be a strain specific interaction of CL/Fr mice about 12 days after birth with the Mouse Chow diet of lactating

CL/Fr mothers--the trait did not appear in CL/Fr mice fed Purina Lab Chow or in A/J mice fed either diet (D. Juriloff and M. Joseph, unpublished data). These litters were observed but not treated, and being of no further use to the dermatitis study, were raised and used to extend the breeding design of the present study. Some additional breeding pairs were set up specifically for the present study, and like the former, were allowed to raise as many litters as they would produce.

The maintenance and breeding of mice used in the experimental crosses was as described in the General Materials and Methods section.

The males used for each type of cross were not all the same individuals; at least 6 different males were used for each. The pedigree relationships between males and females were not considered.

Females that had had vaginal plugs were killed on day 17 of gestation (or rarely, day 16 or 18). The uterine contents were examined under a dissecting microscope, and the number of resorptions and the phenotype of examinable (live until at least day 16) embryos was recorded for the lip, palate and sex. Sex was designated according to the anal-genital papilla distance.

Four litters of less than 5 implants were removed from the data as being atypical. Two were of size 2 and 3 and were in F_1 mothers crossed to CL/Fr males; two were in A/J mothers crossed to CL/Fr males, and had 4 implants in each.

The breeding design is summarized in Figure G1. Data for tested reciprocal F_1 , BC_1 and RBC_1 females were each pooled because none showed evidence of differing in CL(P) or resorption production.

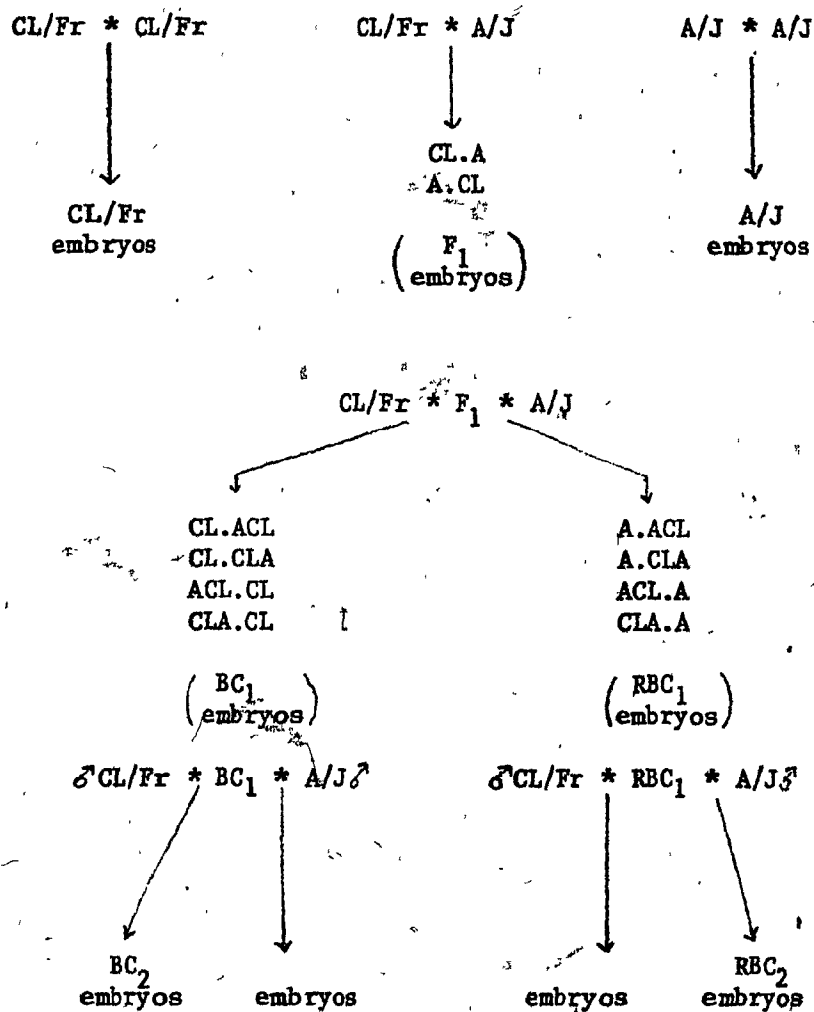
The frequencies of CL(P) among embryos and resorption among implants were transformed to their Freeman-Tukey arcsine values (Mosteller and Youtz, 1961), for each litter. The transformation was necessary because, under the threshold model of development and genetics assumed, the frequencies of CL(P) observed represent areas under a normal distribution curve, beyond a threshold. Their values would be expected to change in a non-linear (sigmoid) way as the distribution was shifted through the assumed threshold. Several transformations exist, but seem not to differ substantially in application (Naylor, 1964). Freeman-Tukey arcsines were chosen because they have been designed for small samples.

Differences among the crosses were sought by a one-way-analysis of variance using the transformed litter data for each trait (Sokal and Rohlf, 1969, page 211), followed by Student-Newman-Keuls Test (Sokal and Rohlf, 1969, page 243), to identify which genotypes differed from each other.

Spearman's Rank Correlation (Siegel, 1956, page 212), between mean CL(P) frequencies and mean resorption frequencies, on the arcsine scale, for the 12 types of crosses, was calculated.

The cumulated numbers of embryos within each genotype

FIGURE G1. BREEDING DESIGN FOR CROSSES BETWEEN A/J AND CL/FR



were used to test for deviations from 50:50 sex ratios within normal embryos by χ^2 tests of goodness-of-fit. Similar χ^2 tests were used to test against 50:50 sex ratios the data for normal embryos pooled, and the data for CL(P) embryos pooled.

Crosses of A.AB6 and CL/Fr

A.AB6 sires that were available for breeding, and 4 A.B6 sires were testcrossed with CL/Fr females. Pregnancies were terminated on day 17 of gestation and data on CL(P), resorption, and sex recorded as usual (above). Between 4 and 7 litters were obtained for each of 13 A.AB6 sires, and a total of 8 litters were obtained for each of 13 A.AB6 sires, and a total of 8 litters were collected from A.B6 sires. The percentage of CL(P) among total scoreable embryos, and the percentage resorption among total implants for each sire were used as the "breeding values" for each trait. Spearman's Rank Correlation (Siegel, 1956; page 212), was calculated for the association between A.AB6 sires' breeding values for: CL(P) in CL/Fr and A/J mothers; resorption in CL/Fr and A/J mothers; CL(P) and resorption in CL/Fr mothers.

Results

Crosses of A/J and CL/Fr

Significant differences ($P < .05$) in CL(P) frequency and resorption were observed among the crosses of A/J and CL/Fr (Table G1). A significantly higher frequency of CL(P) in CL/Fr and F_1 embryos in CL/Fr mothers than in other embryos, and a significantly lower frequency of CL(P) in RBC_2 embryos were observed.

The difference between reciprocal F_1 embryos in CL(P) frequency indicates the presence of a maternal effect, and a clear pattern emerged when the data were plotted (Figure G2). The frequency of CL(P) in CL/Fr mothers was like that of the CL/Fr strain regardless of embryonic genotype, and the frequency of CL(P) in A/J mothers was always at least as low as A/J, despite variation in embryonic genotype (Figure G2a). When hybrid and segregating mothers were used, the frequency of CL(P), regardless of embryo genotype, was at least as low as that in A/J for F_1 and RBC_1 mothers. This fact suggests that the alleles from A/J that influence the maternal trait are dominant. If so, the BC_1 females would be expected to segregate for such factors and to recover the CL/Fr recessive maternal trait in a maximum of half of the females if one locus were involved, and in fewer females if more than one locus influenced the trait. The two sets of BC_1 females'

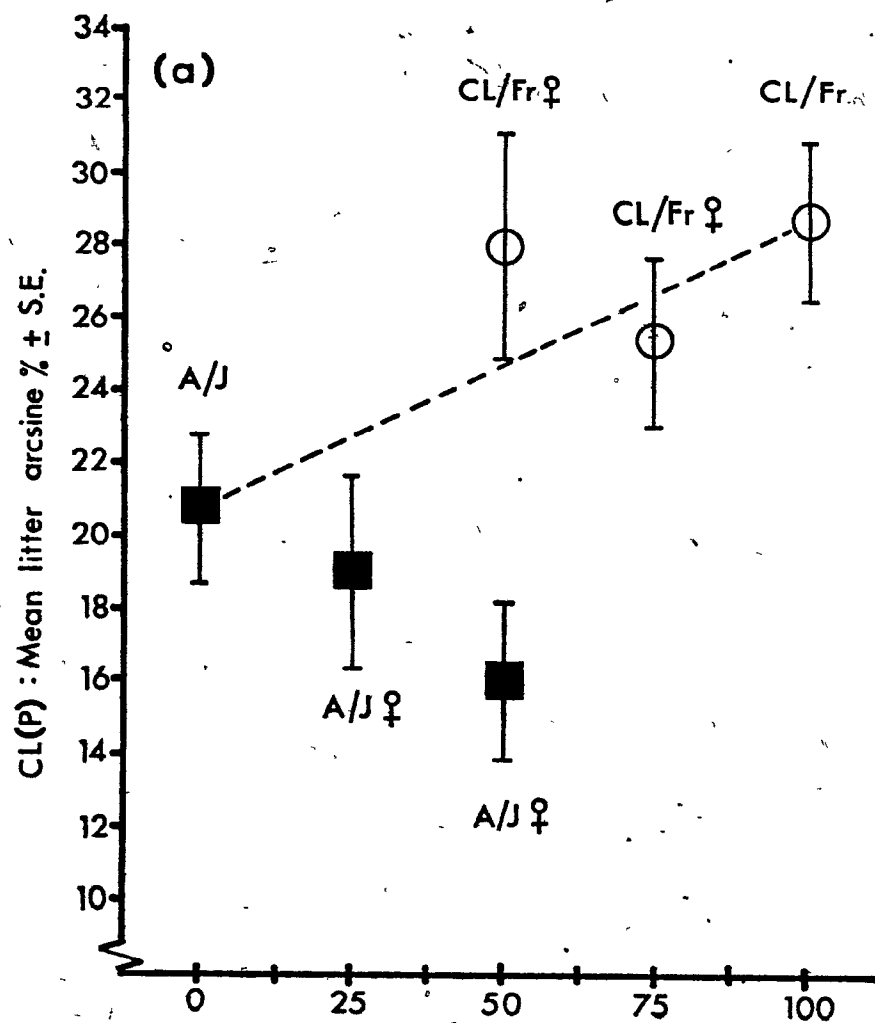
TABLE G1
RESULTS OF CROSSES BETWEEN A/J AND CL/FR

% CL/Fr genes in embryos	Maternal Genotype	Number of Litters	Number of Implants	(Mean Litter size)	Number Resorbed	Number CL(P)	Mean Litter Arcsine* % CL(P)/Live±S.E.	Mean Litter Arcsine** % Resorbed±S.E.
100.0	CL/Fr	13	120	(9.2)	7	24	28.69±2.23 ^a	15.43±2.45 ^{bc}
87.5	BC ₁	24	263	(11.0)	44	37	24.03±2.36 ^{ab}	24.51±1.88 ^{abc}
75.0	CL/Fr	13	126	(9.7)	12	20	25.41±2.34 ^{ab}	18.68±3.32 ^{abc}
75.0	F ₁	13	140	(10.8)	30	9	17.33±2.72 ^{ab}	27.17±4.38 ^{abc}
62.5	RBC ₁	15	181	(12.1)	30	12	18.07±2.51 ^{ab}	24.38±2.80 ^{abc}
50.0	CL/Fr	13	127	(9.8)	7	26	27.92±3.13 ^a	14.82±2.24 ^c
50.0	A/J	11	99	(9.0)	11	6	16.23±2.53 ^{ab}	19.76±3.12 ^{abc}
37.5	BC ₁	23	250	(10.9)	31	33	23.22±1.90 ^{ab}	21.12±1.83 ^{abc}
25.0	F ₁	12	137	(11.4)	16	12	18.59±3.13 ^{ab}	21.09±1.91 ^{abc}
25.0	A/J	13	126	(9.7)	22	8	18.99±2.68 ^{ab}	26.43±3.18 ^{abc}
12.5	RBC ₁	15	180	(12.0)	34	7	14.70±1.97	25.25±2.75 ^{abc}
0	A/J	17	171	(10.1)	46	13	20.72±2.10 ^{ab}	31.24±3.70 ^a

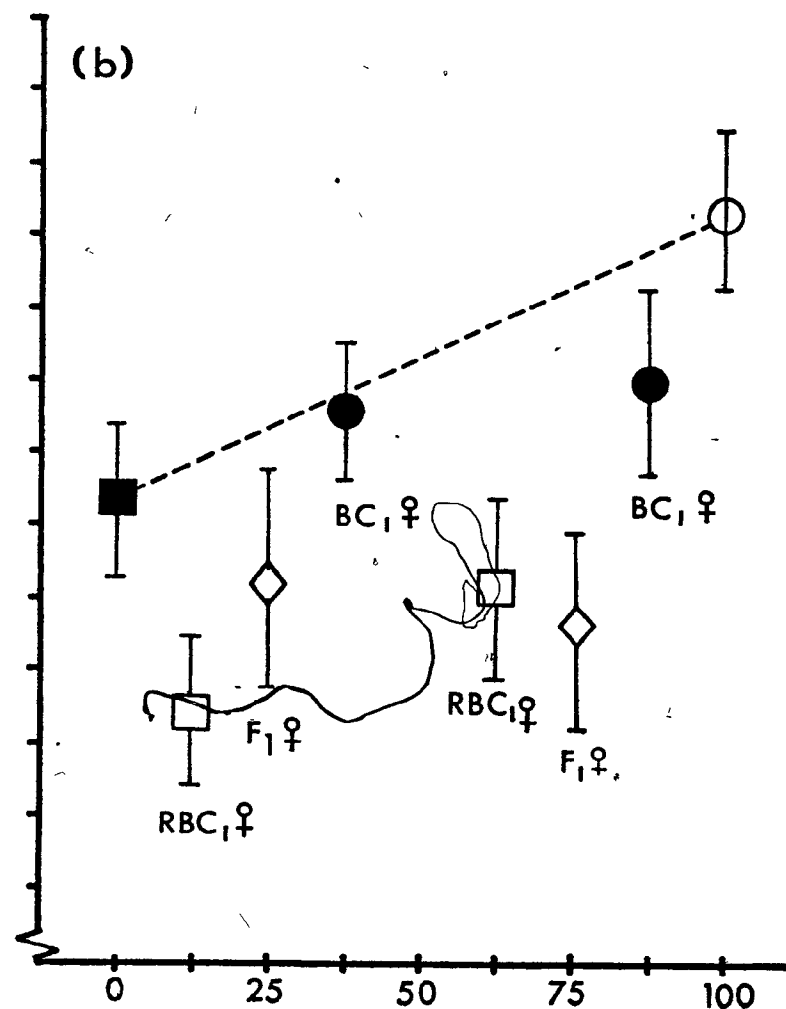
*F_(11,174) = 3.18 ; P < 0.01; **F_(11,174) = 2.90 ; P < 0.01; means sharing the same superscript do not differ at P < .05 by Student-Newman-Keuls Test;

FIGURE G2. CL(P) FREQUENCY IN CROSSES OF A/J AND CL/FR AND SEGREGATING GENERATIONS

RECIPROCAL F_1 AND BACKCROSSES



F_1 AND SEGREGATING FEMALES



CL(P) production, again showing no influence of embryonic genotype, each fell approximately halfway between the A/J and CL/Fr female values, the result expected on the basis of segregation of a single dominant locus. A second expectation from the segregation of a single locus, increased variance among litter frequencies of CL(P), was not observed, the standard deviations for BC_1 females being 9.1 and 11.6 and the range for those of the other genotypes being 7.7 to 11.3. This failure may be attributable to the arcsine transformation being near the limit of its variance-stabilizing abilities, much of the variance in each sample being due to the transformed values of zero frequencies.

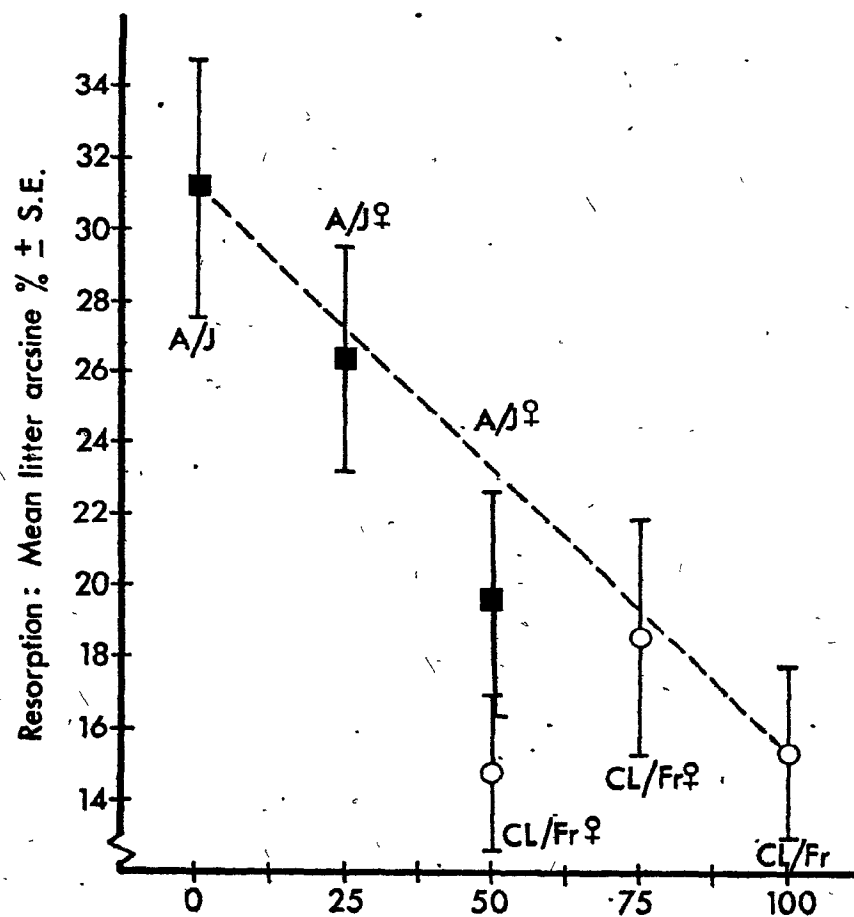
Many of the crosses gave lower CL(P) frequencies than the A/J strain, including some in A/J mothers. Although only the RBC_1 mothers were significantly low, these observations suggested that some heterotic effects may have been expressed in the A/J-like maternal environment or that there had been preferential mortality of CL(P) embryos in that environment.

Comparing the A/J and CL/Fr strains (Table G1) resorption frequency followed the reverse pattern to CL(P) frequency, suggesting that perhaps the higher frequency of CL(P) in CL/Fr mothers was due to its increased survival. Spearman's Rank Correlation between CL(P) and resorption among the 12 crosses was -0.52, which was in the expected direction, and was significant ($P < .05$).

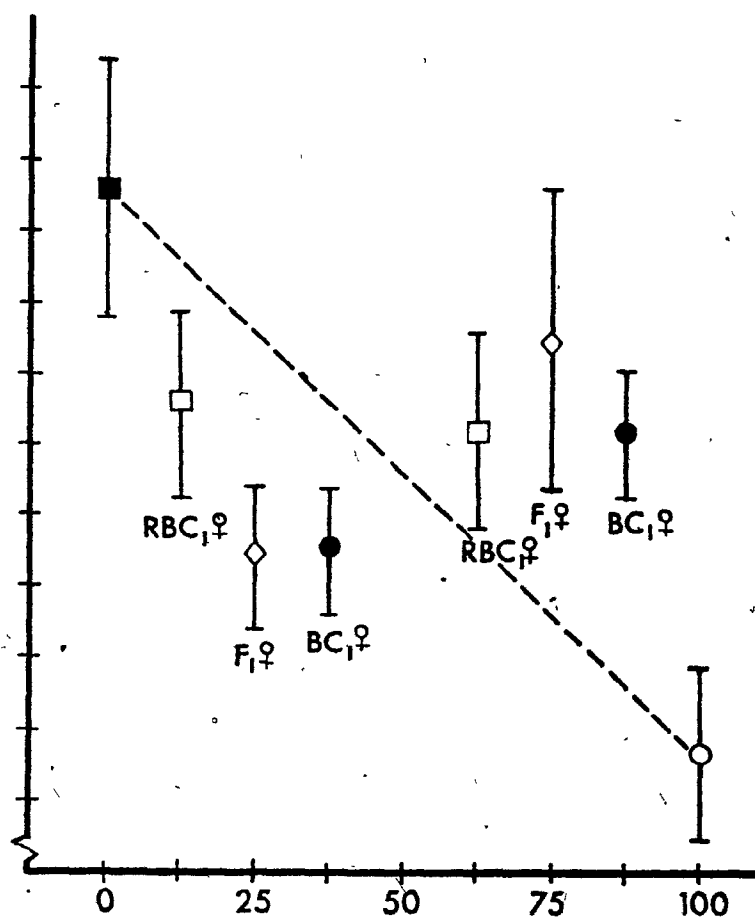
Resorption frequency appeared to be influenced by maternal

FIGURE G3. RESORPTION FREQUENCY IN CROSSES OF A/J AND CL/FR AND SEGREGATING GENERATIONS

(a) RECIPROCAL F_1 AND BACKCROSSES



(b) F_1 AND SEGREGATING FEMALES



genotype, as the correlation implied. The 3 lower rates were for embryos in CL/Fr mothers, and the F_1 in CL/Fr mothers and the CL/Fr strain were significantly lower than other genotypes (Table G1). The A/J strain resorption frequency was significantly higher than all other genotypes.

The pattern of variation of resorption frequency (Figure G3) was not as clear-cut as that for CL(P). F_1 embryos, particularly in CL/Fr mothers, seemed to have heterosis; other genotypes of embryos seemed to show additive effects and no influence of the genotype of the mother in A/J and CL/Fr mothers (Figure G3a). Yet F_1 and segregating mothers seemed to cause maternal effects. Regardless of the genotypes of the embryos, resorption frequencies in F_1 mothers were intermediate; RBC_1 females seemed to regain more of the resorption-inducing property of A/J. BC_1 females, expected to partially recover the CL/Fr low resorption trait, also produced intermediate resorption frequencies.

The crosses varied in mean litter size (implantations) between 9.7 and 12.1 (Table G1). No significant correlation between mean litter size and arcsine resorption ($r_s = 0.42$; $P > .05$) was detected, but, among the crosses, mean implantation number and mean arcsine CL(P) frequency were negatively correlated ($r_s = -0.58$; $P < .05$). This statistical relationship may be partly attributable to the consistently smaller litter sizes in CL/Fr mothers, coupled with their high production of CL(P) regardless of embryonic genotype.

Sex ratio

The sex ratio for normal embryos did not differ significantly (all χ^2 1df < 3.0) from 50:50 for the 12 crosses taken separately or pooled (Table G2). The numbers of CL(P) embryos for each cross were small, but they showed no pattern of deviation with genotype, and pooled, gave a 50:50 ratio.

Laterality of CL(P)

47% of the 207 CL(P)'s observed were bilateral; 21% were unilateral right, the remainder, unilateral left. No associations of laterality with proportions of CL/Fr genes in embryos, or with sex, were evident (Table G3). 10% of the cleft lips observed were not associated with cleft palate; all were from crosses containing at least 37.5% CL/Fr genes in embryos. Of these 20 isolated CL's 90% were unilateral; this suggests that unilateral clefts offer less resistance to palate closure than do bilaterals.

Isolated CP

A uniform, low (1 to 2%) spontaneous occurrence of CP was observed in all crosses.

TABLE G2
SEX RATIOS FOR CROSSES OF A/J AND CL/FR

% CL/Fr genes in embryos	Maternal Genotype	CL(P); Number		Normal; Number	
		♂	♀	♂	♀
100.0	CL/Fr	13	11	42	47
87.5	BC ₁	17	20	86	96
75.0	CL/Fr	10	10	44	50
75.0	F ₁	4	5	48	53
62.5	RBC ₁	7	5	72	67
50.0	CL/Fr	12	14	50	44
50.0	A/J	4	2	38	44
37.5	BC ₁	14	19	104	82
25.0	F ₁	8	4	53	56
25.0	A/J	2	6	53	43
12.5	RBC ₁	4	3	73	66
0.0	A/J	<u>9</u>	<u>4</u>	<u>59</u>	<u>53</u>
		104	103	722	701

TABLE G3

TYPES OF CL(P) OBSERVED IN CROSSES OF A/J AND CL/FR

% CL/Fr genes in embryos	Maternal Genotype	Number CL(P)						Number CL		Number CP	
		Bilateral		Right		Left					
		♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
100.0	CL/Fr	9	4	0	2	4	5	-	2	-	1
87.5	BC ₁	7	11	2	4	8	5	-	3	-	1
75.0	CL/Fr	5	3	2	2	2	6	-	1	-	1
75.0	F ₁	3	2	0	1	1	2	1	-	1	-
62.5	RBC ₁	3	4	2	0	2	1	1	1	2	-
50.0	CL/Fr	6	5	4	2	3	6	2	3	-	1
50.0	A/J	1	0	2	1	1	1	2	-	-	2
37.5	BC ₁	8	8	3	7	3	4	3	1	1	1
25.0	F ₁	2	3	3	1	3	0	-	-	-	-
25.0	A/J	1	5	1	1	0	0	-	-	1	1
12.5	RBC ₁	0	1	2	0	2	2	-	-	1	2
0	A/J	5	2	1	0	3	2	-	-	1	1
		50	48	22	21	32	34	9	11	7	10

Results of testcrosses of A.AB6 sires

The maternal effect enhancing CL(P) frequency in CL/Fr mothers extended to testcrosses of A.AB6 sires. Of 13 sires, 8 produced CL(P) frequencies higher than 7.6%, the highest frequency observed among all A.AB6 sires testcrossed with A/J (Table G4). The highest CL(P) frequencies produced in CL/Fr mothers by 4 sires were 16 to 17%, and were somewhat lower than the 21 to 25% expected if these males were identical with A/J males at the relevant loci. This result is consistent with there being some dominant negative modifiers contributed by the C57BL/6J in the crosses or with duplicate epistasis of two major loci. These 4 "high" sires had produced intermediate (4-6%) CL(P) frequencies in testcrosses with A/J and their putative genotypes under the 2 locus model would be aaBb and Aabb, with half of their offspring liable to CL(P). None of the highest CL(P) producers with A/J, by chance, were tested with CL/Fr.

The resorption frequencies were consistently lower in the crosses to CL/Fr mothers (2 to 11%) than for the crosses to A/J mothers (12 to 29%).

There was good agreement, within the sample of 13 sires, between their production of CL(P) in A/J and in CL/Fr mothers ($r_s = 0.57$; $P < .05$), but no detectable correlation between their production of resorption in the 2 kinds of mother ($r_s = -0.07$; $P > .05$). No significant correlation between CL(P) "

TABLE G4

BREEDING DATA FOR A-AB6 (BC₁) SIRES TESTED WITH CL/FR FEMALES

Sire Identification Number	Number of Litters	Number of Implants	% Resorption	Number Live	% CL(P) in Live
A-B6 (F ₁)	8	77	6.5	72	0.0
39	6	60	10.0	54	9.3
44	6	55	1.8	54	11.1
46	7	61	9.8	55	7.3
47	5	48	10.4	43	16.3
56	5	52	1.9	51	0.0
88	5	48	8.3	44	15.9
96	5	43	4.7	41	4.9
108	7	66	3.0	64	7.8
110	4	39	7.7	36	16.7
117	7	71	8.5	65	4.6
118	7	71	7.0	66	16.7
121	5	53	7.5	49	8.2
122	5	41	2.4	40	0.0

production and resorption within A.AB6 sires tested with CL/Fr females was detected ($r_s = 0.35$; $P > .05$), although this may be partly due to the small sample size.

Summary and Discussion

The internal consistencies of the data suggest that a maternal trait, with Mendelian single locus inheritance, dominant in A/J, may account for the difference in CL(P) frequency between A/J and CL/Fr mice. The data did not offer support for additive embryonic effects influencing the strain difference.

The maternal effect seemed to be related to resorption frequency: in the crosses between A/J and CL/Fr, and in the crosses of A.AB6 sires with A/J and CL/Fr females, high CL(P) frequency was associated with lower resorption. Resorption frequency in turn seemed to depend on litter size, being lowest in the genotype (CL/Fr) with the smallest number of implantations. Thus it seems that the maternal effect of CL/Fr on CL(P) frequency may be attributed to its lower implantation rate, lowered severity of embryonic competition and consequent increased survival of embryos that have, or will develop, CL(P).

The data indicated that a third maternal effect difference had been found for CL(P). Previous reports had established that in reciprocal crosses, CL(P) was higher in A/J

mothers than in A.B6 and segregating mothers (Davidson et al., 1969), and higher in CL/Fr mothers than in CL.B6 and segregating mothers of that cross (Bornstein et al., 1970). The order of magnitude of maternal effects may now be summarized, in order of CL(P) frequency, as CL/Fr > A/J > C57BL/6J or hybrids.

While the data of Davidson et al. (1969) are consistent with the maternal effect being attributable to implantation rate and improved CL(P) survival in small litters, the data of Bornstein et al. (1970) cannot be explained in this way.

That more is involved than a simple relationship between implantation rate and CL(P) survival is also suggested by the failure of the resorption frequency to follow the pattern of inheritance observed for the maternal effect on CL(P) in crosses of A/J and CL/Fr.

CHAPTER H

THE THYROXIN EXPERIMENTS

Introduction

Almost two decades ago, the prevention of spontaneous CL(P) in Strong A mice by thyroxin treatment of the mothers on days 10 and 11 of gestation (plug day=0) was described (Woollam and Millen, 1960). If repeatable, this observation would be of great importance for understanding the etiology of CL(P) and as a possible means of preventing CL(P) in human beings.

Few attempts to repeat this experiment have been published.

Using A/J mice treated with the same dosage of thyroxin as used by Woollam and Millen (0.1 mg), Chaudrey et al. (1966) obtained no significant changes in the incidence of CL(P) or resorption. The interpretation of this negative result is uncertain because the treatment times used (day 11 and 12) were later than those of Woollam and Millen and later than the usual time of lip formation of A/J mice, days 10 and 11 (Trasler, 1968).

A more recent test of A/J mice with thyroxin (0.1 mg) used day 9 and 10 treatments (Brown et al., 1974). Increased embryonic death and decreased CL(P) incidence were observed. Preferential death of embryos that would have developed CL(P) was inferred. It is conceivable, however, that thyroxin prevented CL(P) and independently caused increased embryonic mortality.

It was therefore of interest to repeat the study of Woollam and Millen once again.

Materials and Methods

CL/Fr mice, which have approximately 25% CL(P) in day 17 embryos, were used. They were derived from Dr. F.C. Fraser's stock at F₃₄, and were between F₃₇ and F₄₃ of brother-sister inbreeding. Females were nulliparous and 2 to 5 months old. They were placed with singly caged males and checked daily for vaginal plugs. Females with plugs on any given day were removed to a fresh cage and housed in groups up to 5. Males were replaced at approximately 6 months of age or when breeding performance declined. Their pedigree relationship to the females was not considered. A total of 38 males contributed to the data.

Embryos were assumed to be 0 days, 12 hours old at 2 P.M. of the day the plug was found (d 0/12).

During the experiment all mice were fed Purina Mouse Chow. Females had received Mouse Chow from at least 6 to 8 weeks of age, or from at least 2 weeks prior to breeding. The previous diet was Purina Lab Chow.

Other aspects of maintenance have been described earlier (General Materials and Methods).

A series of 4 experiments was run in chronological order, each having been suggested by the results of the preceding study.

Thyroxin (3, 3', 5, 5'-Tetraiodo-L-Thyronine; sodium salt) was obtained from the Sigma Chemical Company. Sterile distilled water was used as a carrier (1.0 mg/3 ml). Mice were treated by subcutaneous, interscapular injection.

Propranolol hydrochloride ("Inderal", from Ayerst Labora-

tories, 1 mg/ml) was administered intraperitoneally.

Experiment One

Each female was treated on both day 9/12 and day 10/12 (± 3 hours) with one of the following thyroxin dosages: 0.025, 0.05, 0.075, 0.1, 0.15, or 0.2 mg/mouse/day. All were killed by cervical dislocation on day 17 of gestation and their uteri removed. The uterine contents were examined under a dissecting microscope. Resorption sites and dead embryos were recorded, with their approximate day of death. The sex (externally determined), condition of the lip and palate, and other external malformations were recorded for live embryos.

Experiment Two

✓ Each female was treated on both day 9/12 and day 10/12 (± 3 hours) with one of the following dosages: 0.075 or 0.15 mg thyroxin/mouse/day. All were killed by cervical dislocation on day 13 or 14 of gestation, and the uteri removed immediately into physiological saline. The uterine contents were examined under a dissecting microscope. Puncture of the amniotic sac was avoided. Resorptions of less than 12 days' gestation were recorded. Embryos, living or dead, of greater than 12 days' development were examined initially through the amniotic sacs, which are nearly transparent. The structural integrity of dead embryos is well maintained by the amniotic fluid cushion at this stage of pregnancy, despite the increased fragility due to decomposition.

The condition of the lip of all scoreable embryos was recorded. The relatively less fragile living and newly dead embryos were removed from the amniotic sacs and examined for additional malformations. Embryos showing a heart beat were recorded as live. All were examined within 10 minutes of maternal death.

Experiment Three

Each female was treated on days 9/12, 10/12, 11/12, and 12/12 (± 3 hours) with 0.075 mg thyroxin/mouse/day. The embryos and resorptions were examined and recorded on day 13 or 14 of gestation by the same methods as in Experiment Two.

Experiment Four

Each female was treated on days 9/12 and 10/12 with 0.1 mg thyroxin/mouse/day, and on days 11/12 and 12/12 (± 3 hours) with propranolol, a β -adrenergic receptor blocking agent, at one of the following dosages: 20 μ l, 40 μ l, 60 μ l, or 80 μ l/mouse/day. Embryos and resorptions were examined and recorded on day 13 or 14 of gestation, as in Experiment Two. The rationale for this experiment is presented in the Discussion section.

Data Analysis

The dosage response of resorption in Experiment One was analyzed using probit analysis, corrected for spontaneous resorption by Abbott's formula (Finney, 1971, pages 61 to 75 and 125 to 126). Control data from an independent study (Chapter E)

was used to provide natural resorption (6%) and CL(P) (21%) frequencies.

χ^2 tests of goodness-of-fit were used to compare CL(P) frequencies after thyroxin treatment to the expected value of 21%. Yates' correction for continuity was used.

χ^2 tests of independence were used to test the independence of sex ratio and dosage within normal embryos (Experiment One), of day of examination and incidence of dead embryos for pooled lip types (Experiment Three), of propranolol dosage and mortality for pooled lip types (Experiment Four), and of laterality of cleft lips in day 17 embryos and thyroxin dosage (Experiment One).

Fisher's Exact Test (2-tailed) was used to test the independence of normal versus cleft lip and mortality, and of unilateral versus bilateral cleft lip and mortality for Experiments Two, Three and Four.

To combine probabilities, Fisher's method was used (Sokal and Rohlf, 1969, page 623).

Results

Preliminary results of these experiments have been reported (Juriloff and Fraser, 1977).

Experiment One

Two effects of increasing dosages of thyroxin on days 9 and 10 of gestation were found in day 17 pregnancies (Table H1). First, resorption frequency increased with dosage to 80% at the highest dosage tested. Second, CL(P) frequency decreased as dosage increased, and no CL(P) embryos were found among survivors of the 3 highest dosages used.

The embryonic $ED_{50} \pm S.E.$, corrected for the natural resorption rate, was calculated to be 90.5 ± 13.1 $\mu\text{g}/\text{mouse}/\text{day}$. The slope of the probit, log-dose regression was 1.24 ± 0.28 (S.E.); resorption was dosage-dependent.

Probit analysis was not applied to the limited CL(P) data.

The 36% CL(P) frequency in live embryos obtained at the lowest thyroxin dosage (.025 mg/mouse/day) was not significantly greater than an expected value of 25%, but did significantly exceed the 21% predicted from controls ($\chi^2_{1df} = 4.00$; $p < .05$). The 19 and 16% CL(P) produced at the second and third lowest dosages (.050 and .075) were not significantly different from 21%. The four highest dosages (.075, .100, .150 and .200) pooled showed a significant deficiency of CL(P) compared with 21% expected. ($\chi^2_{1df} = 10.08$; $p < .05$).

Several females aborted litters between day 14 and 16 of gestation. This response seemed dosage dependent: one abortion occurred at each of the 3 lowest dosages, and 3 at each of the 3 highest. Two females died after treatment, at .025 and .075 mg dosages.

TABLE H1

EFFECT OF THYROXIN GIVEN ON DAYS 9½ AND 10½ ON DAY 17 CL/Fr LITTERS

Daily Dose (mg/mouse)	Number of Litters	Number of Implants	% Resorbed	% CL(P) in Live	% of Resorptions 12<day<14	% of CL(P) Bilateral
.025	6	56	36	36	20	69
.050	8	73	42	19	90	50
.075	6	58	36	16	86	33
.100	6	61	52	0	78	-
.150	4	40	63	0	84	-
.200	4	40	80	0	72	-

A trend was noted that the proportion of bilateral clefts among all CL(P) decreased from 69% to 33% with dosage.

The majority of embryonic death, 70 to 90% of mortality, occurred on day 12 or later, i.e., after the lip had formed. It was clear that the question of differential mortality versus cure of CL(P) embryos could be answered simply by examining lips of the treated dead embryos. Most mortality appeared to have occurred on day 13 or 14. Therefore, for Experiment Two the embryos were examined on those days.

No significant association between sex ratio and dosage was found ($\chi^2_{5df}=2.30$) for normal embryos, half (70/140) of which were male. About half (12/27) CL(P) were male, and no trend in sex ratio across dosages was noted. All but 2 of these 27 CL(P)'s observed had associated CP.

Experiment Two

The results (Table H2) demonstrated that thyroxin treatment on days 9 and 10 did not prevent CL. (Since the palates had not closed on day 13, their fate is unknown). In pooled living and dead scoreable embryos neither any of the treatment groups, nor the controls, differed significantly from 21% CL (all $\chi^2_{1df}<0.5$) expected for day 17 embryos.

Thyroxin-induced preferential mortality of CL embryos was strongly indicated. At .075 and .150 mg thyroxin dosages, 50% or more of CL embryos, compared with less than 15% of normal embryos, were dead. No dead embryos of either type occurred in

TABLE H2
EFFECTS OF THYROXIN ON DAY 13 OR 14 CL/FR EMBRYOS

Days Treated	Daily Dose (mg/mouse)	Day Examined	Number of Litters	Number of Implants	% Resorbed Before Day 12	Number of Live Embryos	Number of Dead Embryos	% of Normals Dead	% of CL's Dead	% CL in all Embryos	% of all CL's Bilateral	% of Dead CL's Bilateral
<u>Experiment 2</u>												
d9+10	0	13	5	52	10	47	0	0	0	17	88	-
d9+10	.050	13 14	4 1 }	48	4	46	0	0	0	26	67	-
d9+10	.075	13 14	4 1 }	45	7	36	6	3	50	24	80	100
d9+10	.150	13 14	6 2 }	77	19*	47	15	14	62	21	69	88
<u>Experiment 3</u>												
d9,10, 11+12	.075	13	5	43	12	29	9	14	56	24	78	80
d9,10, 11+12	.075	14	4	43	14	17	20	37	100	27	90	90

*Includes 9 embryos from one litter

the control and lowest thyroxin dosage (.050 mg) groups, suggesting dosage dependence of mortality. The probabilities of independence of lip type and mortality for .075 and .150 mg dosages, from Fisher's Exact Test, were less than .004 and .033, respectively.

The dead embryos had died between 3 and 30 hours prior to their examination, as judged by their state of maceration and embryonic development. It is likely that more embryos would have died if gestation had extended beyond day 13. For example, only 62% of CL's were dead on day 13 for the .150 mg dosage, yet by day 17 in Experiment One none had survived at that dosage.

No additional external malformations were seen, and no cause of death could be discerned.

Because the death of the embryos lagged at least two days behind the administration of thyroxin, the question arose of whether the lethal effects might be a withdrawal effect. It was thought that the exogenous thyroxin might have caused the maternal thyroid to cease functioning temporarily, and a period of hypothyroidism to result. Accordingly, Experiment Three was conducted.

Experiment Three

Embryonic death was not prevented by extended thyroxin treatment (Table H2); excess, rather than withdrawal of thyroxin was therefore its cause. Significantly more ($\chi^2_{1df}=7.30$; $p<.05$) embryos were found dead in litters checked on day 14 than on day

13. Much of the mortality takes place during this time.

The frequency of CL among examinable embryos did not differ significantly from an expected value of 21% (χ^2_{1df} both <0.8).

Fewer normal embryos (14 and 37%) were dead compared with CL's (56 and 100%) for days 13 and 14 respectively. The probabilities of lip type and mortality being independent, from Fisher's Exact Test were $<.05$ for day 13 data and $<.00001$ for day 14.

As in previous experiments, no obvious cause of death or additional external malformations were discerned.

Experiment Four

Propranolol did not appear to affect the post-day 12 mortality response to thyroxin. Of 163 scoreable embryos, 13% were dead (Table H3). This is in the range expected for thyroxin treatment alone predicted from Experiment Two (Table H2).

No significant effect of propranolol dosage on frequency of mortality for pooled lip types was found ($\chi^2_{3df}=1.09$).

The frequency of CL did not differ significantly from 21% at any propranolol dosage (all $\chi^2_{1df}<1.3$).

While the percentage of death among CL's exceeded that for normals at all propranolol dosages, mortality and lip type were significantly associated ($P<.05$) only at the 80 μ l propranolol dosage, by Fisher's Exact Test. The combined probability, however, of obtaining the data values of the 3 lower dosages was less than 0.2. It therefore seems likely that preferential

TABLE H3

EFFECTS OF THYROXIN (DAYS 9 AND 10) FOLLOWED BY PROPRANOLOL (DAYS 11 AND 12) ON DAY 13 CL/Pr EMBRYOS

Daily Dose Thyroxin	Daily Dose Propranolol	Number of Litters ¹	Number of Implants	% Resorbed Before Day 12	Number of Live Embryos	Number of Dead Embryos	% of Normals Dead	% of CL's Dead	% CL in All Embryos	% of All CL's Bilateral	% of Dead CL's Bilateral
.100 mg/mouse	20 µl	7 ²	64	11	48	9	10	31	28	75	100
	40	5	38	18	29	2	0	25	26	88	100
	60	5	48	2	41	6	10	25	17	50	50
	80	4	40	30	23	5	5	66	21	66	75

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¹Superscript denotes number of litters checked on day 14.

mortality continued despite propranolol treatment.

Two additionally malformed embryos were observed: one had exencephaly (80 μ l dosage) and the other, a failure of midline closure of the thorax and abdomen (20 μ l).

Laterality of Clefts

A consistent tendency for a larger proportion of dead CL's to be bilaterally affected than the proportion of all CL's to be bilateral was observed for all experiments.

In Experiment One (Table H1) the proportion of bilateral clefts among living day 17 CL embryos dropped from 69% (9/13) to 50% (4/8) to 33% (2/6) as the frequency of surviving CL's dropped from 36% to 16% with increasing thyroxin dosage. This trend was not statistically significant (χ^2_{2df} for independence of dose and proportion of CL's bilateral = 2.27).

In Experiment Two (Table H2) for the .075 and .150 thyroxin dosages pooled, 67% (12/18) of bilateral CL's were dead, compared with 20% (1/5) of unilaterals. This association between type of CL and mortality was not statistically significant ($P = .18$, Fisher's Exact Test).

In Experiment Three, for day 13 and 14 data pooled, 81% (13/16) of bilateral CL's, versus 67% (2/3) of unilaterals, were dead, but no significant association between laterality within CL's and mortality was found ($P = 1$, Fisher's Exact Test).

In pooled data from Experiment Four, 41% (11/27) of bilateral CL's and 22% (2/9) of unilateral CL's were dead. From

Fisher's Exact Test, the excess of dead bilaterals was not significant ($P=.34$).

If the probabilities associated with Experiments Two through Four, or One through Four are combined by Fisher's method, the association between laterality of cleft lip and mortality is not statistically significant ($\chi^2_{6df}=5.60$; $\chi^2_{8df}=7.67$; $p>0.5$ and >0.3). The trend observed in these experiments will therefore require verification by additional testing with larger sample sizes before preferential mortality of bilateral cleft lips can be considered a reality.

Discussion

The experiments demonstrated that thyroxin treatment induces preferential mortality of CL(P) embryos after the lip has formed, in CL/Fr mice. The inference that CL(P) embryos preferentially die after similar treatment in A/J mice (Brown et al., 1974) is thus supported. It is likely that this phenomenon extends also to Strong A mice, since CL/Fr, A/J and Strong A share common ancestry (Staats, 1968, page 4; 1972). It is unlikely that spontaneous CL(P) can be prevented by maternal thyroxin treatment in mice during the time of embryonic lip formation.

Considerably more work must be done before it can be known whether differential mortality is specific to thyroxin, or is a generalized response to some kinds of maternal metabolic upset. The thyroxin treatment was simply a probe that brought to light some difference between CL(P) and normal embryos. The thyroxin

dosages were very large, being 100 to 800 times the daily requirement (W. Layton, personal communication). It is likely that the treatment caused maternal hyperthyroidism (Schayer and Reilly, 1975) and that some of the thyroxin reached the embryos as it does in rats (Sweney and Shapiro, 1975) and guinea pigs (Peterson and Young, 1952). It is interesting to note that no evidence of changes in palate closure were found in Experiment One, since thyroxin has been found to localize in the palates of embryonic rats (Sweney and Shapiro, 1975).

If the preferential mortality of CL(P) embryos is caused by a specific effect of thyroxin, it will be necessary to determine whether it is due to thyroxin activity in the embryos or to maternal hyperthyroidism and its attendant changes.

Propranolol was viewed as a chemical probe to test the hypothesis that one of the many effects of hyperthyroidism, tachycardia leading to cardiac decompensation, might cause the death of thyroxin-treated embryos. Tachycardia in hyperthyroid states has been prevented by propranolol, a β -adrenergic blocking agent (De Groot and Stanbury, 1975, page 139). Propranolol has been shown to be effective in day 11 rat embryos (Robkin *et al.*, 1974) in vitro, as measured by heart rate. It has also protected against caffeine teratogenicity (thought to act through catecholamines and their β -adrenergic action) in mice when given intravenously to the mothers on day 12 of gestation (Fujii and Nishimura, 1974).

The failure of propranolol to protect embryos from death

after thyroxin administration may reflect a non- β -receptor mediated cause of death. It is also possible that the dosages used were inadequate -- the highest dosage of Fujii and Nishimura was 3 times larger than the highest used here -- or that the time or route of administration were inappropriate.

It was known previously that CL(P) embryos in the A/J and CL/Fr strains tend to have a relative immaturity of heart development compared to normal littermates from at least as early as day 11 of gestation (Fraser and Rosen, 1975), and that they may also have small right ventricles (Trasler, 1966).

A functional, rather than malformational, cause of death was hypothesized for the following reasons: no additional external malformations were seen; severely malformed embryos in other experiments (personal observations) including those with severe heart malformations (Dr. W. Layton, personal communication), tend to survive to near term.

It remains possible that structural heart malformations were induced by thyroxin since other β -adrenergic active substances are known to do so (Jaffee, 1977).

The clear finding of these thyroxin experiments is that CL(P) embryos are different from their normal littermates in more than lip development. The association, within the CL/Fr or A/J genotypes, between atrial septal defects (Rosen and Fraser, 1975), CL(P), and increased liability to mortality after maternal thyroxin treatment suggests that they may be part of one chain of events. Since it is possible to compare, on day

13 prior to the death of CL(P)'s, at dosages known to kill all CL(P)'s, treated CL(P) and normal embryos, it may be possible to identify an earlier link.

Preferential death of CL(P) embryos has been reported after other treatments. A significant reduction in CL(P) frequency in A/J was found after cortisone treatment on days 11 to 14 of gestation (Walker and Crain, 1959). This treatment also seemed to reduce the bilateral portion of CL(P)'s preferentially. Vitamin A treatment in the same set of experiments did not affect CL(P) frequency, nor did riboflavin deficiency in an earlier study (Kalter and Warkany, 1957), although both caused increased resorption. (Also, CL(P) embryos were believed to show preferential survival when exposed to 6-AN (Goldstein et al., 1965), at the time when these experiments were conducted.) In summary, as Walker and Crain observed, CL(P) embryos are not more susceptible than normals to a generally adverse environment, but seem to react to some specific environments with increased mortality.

Thyroxin treatment does not, as thought by Woollam and Millen, cure CL(P) in mice, but it can prevent its occurrence in term fetuses, and therefore can be cited as an example of "Terathanasia" (Warkany, 1978).

CHAPTER I

THE NICOTINAMIDE EXPERIMENTS

Introduction

As an off-shoot from the main course of the project, a series of attempts were made to prevent formation of spontaneous CL(P) in CL/Fr or A/J embryos by the administration of nicotinamide to the pregnant mothers.

The rationale for these attempts follows. A nicotinamide antagonist, 6-AN, was known to induce CL(P) in mice (Pinsky and Fraser, 1960). The A/J strain, which has a liability to spontaneous CL(P), appeared to resist induction of more CL(P) by 6-AN, and furthermore, the spontaneously affected embryos were apparently relatively resistant to the lethal effects of 6-AN (Goldstein et al., 1965). If this association between genetic liability to CL(P) and resistance to 6-AN induced CL(P) and mortality were causal, then one possible explanation would be that liability to CL(P) is a result of a deficiency (transient or otherwise) in cellular uptake of nicotinamide or its analogues. Consequently, an approach similar to the treatment of vitamin-responsive inborn errors of metabolism (Scriver, 1973), "megadoses" of the vitamin, was tried. The daily requirement for nicotinamide for adult mice was taken as 1.8 mg/kg (Hoag and Dickie, 1966), and multiples of this quantity were administered to pregnant females. It was assumed that some of this excess would reach the embryos. A variety of dosages, times, and

routes of treatment were tried in these exploratory experiments.

Materials and Methods

Mice

CL/Fr mice used in Experiments One, Two and Three were obtained from M. Baldwin's colony, were parous and up to 8 months old, and had been brother-sister inbred for at least 20 generations. The CL/Fr mice used in Experiment Four were derived from Dr. F.C. Fraser's colony, were nulliparous, between 2 and 3 months old, and at the 38th to 40th generation of inbreeding. With each experiment the mice were assigned at random to treatment and control groups, and to CL/Fr males of similar origin.

A/J mice were obtained from the Jackson Laboratory, Bar Harbor, Maine at 6 weeks of age. Females were used at 3 to 4 months of age and were assigned to A/J males and treatment or control groups at random. All were nulliparous.

Maintenance

All mice were maintained, bred, and their matings timed in the usual manner (General Materials and Methods). Mice in Experiments One, Two, and Three were fed Purina Lab Chow. Those in Experiment Four received Purina Mouse Chow beginning at least 2 weeks prior to breeding. All A/J mice (Experiments Five and Six) received Purina Mouse Chow from receipt at McGill.

Nicotinamide

The nicotinamide used in Experiments One to Three had been obtained from Eastman Organic Chemicals and was in general use in the laboratory. That used in Experiments Four to Six was obtained newly from the Sigma Chemical Company.

The nicotinamide was dissolved in sterile distilled water at concentrations ranging between 18.75 and 1406 mg/25 ml (Table II).

Dosages and Treatment Times

The nicotinamide dosages and times of administration used for the 6 experiments are summarized in Table II.

Mice in Experiments One and Two were treated intraperitoneally on alternate days from day 1 through day 15 of gestation. The daily dosages were approximately 5 and 100 times the daily adult requirement, respectively and were based on plug day weight. Controls received equivalent volumes of sterile distilled water.

For Experiments Three to Six, day 10/12 (± 2 hours) of gestation was used as the time of treatment, and the dosage was approximately 500 times the daily requirement, based on day 10/12 body weight. All treatments were intraperitoneal except those in Experiment Five, which were subcutaneous (interscapular).

Untreated controls were collected for Experiments Three, Four and Six. Sterile distilled water-treated (equivalent

TABLE II

SUMMARY OF TREATMENT TIMES, DOSAGES AND
ROUTES OF ADMINISTRATION OF NICOTINAMIDE

Experiment Number	Nicotinamide Solution Concentration (mg/25 ml)	Day Treated	Daily Dose (mg/kg)	Route of, Treatment	Genotype
One	18.75	1,3,5,7, 9,11,13,15	9	intraperitoneal	CL/Fr
Two	375	1,3,5,7, 9,11,13,15	180	intraperitoneal	CL/Fr
Three	1875	10	900	intraperitoneal	CL/Fr
Four	1406	10	900	intraperitoneal	CL/Fr
Five	1406	10	900	subcutaneous (intrascapular)	A/J
Six	1406	10	900	intraperitoneal	A/J

volumes) controls were also used in Experiment Three.

Embryo Collection

Experiment One pregnant females were killed on day 16 of gestation. Nonpregnant control females were rebred and retreated as controls. Experiment Two females were allowed to give birth over wire. They were then rebred and retreated according to their initial assignment. All females in the remaining experiments were killed on day 17 of "gestation", whether pregnant or not.

Uteri were removed from killed females, and the number of embryos and resorption sites recorded. Embryos were examined under a dissecting microscope, and their sex and malformations of the head, limbs, and tails, recorded. The "open-eye" trait was not recorded because it might have been confused with the slow end of normal developmental variation. Newborns collected from females over wire were similarly examined.

Analysis

Frequencies of CL(P) and resorption in control and experimental groups were compared using χ^2 tests of independence, and frequencies of CL(P) and resorption in treated A/J mice were compared with theoretical frequencies of 10% and 27% from independent controls (Chapter C) using χ^2 tests of goodness-of-fit. χ^2 tests of independence of sex and group were run within lip type for pooled treatment versus pooled control data for each

genotype. For all tests Yates' correction for continuity was applied. Fisher's Exact Test was used to test independence of ectrodactyly and CL(P).

Data from Experiments Three and Four were pooled. They comprised 4 untreated, 2 water control and 4 treated litters (45% CL(P)) and 2 untreated and 3 treated litters (43% CL(P)) respectively.

Results

CL(P). No evidence of any preventative effect of nicotinamide on CL(P) formation was found. For every experiment the frequency of CL(P) in the treated group was higher than that of the controls (Table I2).

For CL/Fr mice the CL(P) frequencies of the pooled data of Experiments Three and Four, the highest nicotinamide dosage used, were significantly greater than controls' ($\chi^2_{1df}=5.39$; $p < .05$). This observation suggests that high dosages of nicotinamide may induce CL(P) in CL/Fr mice.

While the CL(P) frequency from A/J mothers in Experiment Five (S.C.) was significantly greater than the 10% expected theoretically ($\chi^2_{1df}=7.11$; $p < .05$), no such excess over theoretical frequency or controls was found for the much larger sample of A/J in Experiment Six (I.P.).

It is possible that CL/Fr and A/J have different sensitivities to CL(P) induction by nicotinamide.

Resorption. No change in resorption frequency after

TABLE 12

EFFECT OF NICOTINAMIDE ON CL(P) AND OTHER MALFORMATION FREQUENCIES

Experiment Number	Genotype	Dosage (mg/kg)	Treatment Time	Route	Number of Litters	Number of Implants	% Resorbed	Number Live	% CL(P) in Live	Other Malformations
One	CL/Fr	9	d1,3,5,7,9,11,13,15	ip	4	33	9	30	27	-
		H ₂ O control	"	"	5	45	9	41	22	-
Two	CL/Fr	180	"	ip	3	-	-	18	28	1:CP, micromelia, syndactyly, bent tail
		H ₂ O control	"	"	4	-	-	29	10	-
Three+Four	CL/Fr	900*	d10	ip	7	62	5	59	44	-
		H ₂ O control	"	"	2	17	-	17	24	-
		* control	-	-	6	51	12	45	22	-
Five	A/J	900	d10	sc	5	50	50	25	28	1:CP
Six	A/J	900	"	ip	18	154	44	87	12	1:CP 1:exencephaly 9:forelimb ectrodactyly
	A/J	control	-	-	9	82	22	64	6	-

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*Pooled for χ^2 tests.

nicotinamide treatment in CL/Fr mice was observed; it was below 13% in all groups.

The A/J resorption frequency was, on the other hand, increased significantly at 44% in treated mice in Experiment Six over 22% in controls ($\chi^2_{1df}=9.82$; $p<.05$). For the more limited data of Experiment Five, when one wholly resorbed litter was deleted as being atypical, the remaining 36% resorption frequency was not significantly greater than a theoretical 27% value but did follow the trend of Experiment Six.

The results from Experiment Six suggest that in A/J, but not CL/Fr mice, nicotinamide (or handling) may increase embryonic mortality.

Types of CL(P) Observed

Nicotinamide treatment had no apparent effect on the laterality or rate of palate involvement of CL(P) (Table I3). Cleft lip was nearly always accompanied by cleft palate in both A/J and CL/Fr embryos, and a tendency toward a preponderance of bilateral clefts in CL/Fr was noted. The limited data agree well with those from other sets of A/J and CL/Fr untreated embryos (Chapter G).

Sex

Within the CL/Fr strain, pooled by treatment, 43% of 68 nicotinamide treated, and 48% of 106 control normal embryos were male. 59% of 39 treated and 65% of 26 control CL(P)

TABLE 13

TYPES OF CLEFTS OBSERVED AFTER NICOTINAMIDE TREATMENT

Genotype	Group	Number			Number	
		B	R	L	CLP	CL
CL/Fr	treated	32	3	4	38	1
	control	15	4	5	22	2
A/J	treated	6	3	8	17	0
	control	3	1	0	4	0

embryos were male.

Within A/J, 49% of 95 treated, and 52% of 60 control normal embryos were male. 71% of 17 treated, and 50% of 4 control CL(P) embryos were male.

No significant associations between sex ratio and treatment within genotype and lip type were found.

Other Malformations

Forelimb postaxial ectrodactyly was induced in A/J, but not CL/Fr embryos (Table I2). Nine embryos from 6 litters showed an absence of the fifth digit on one or both forelimbs. Two exhibited, in addition, absence or reduction of the fourth digit. The majority, 7/9 had only the right forelimb affected; the remaining two had both forelimbs affected. 3/9 also had CL(P); the probability of this and all greater rates of association from Fisher's Exact Test (two-tailed) was 0.13, suggesting that CL(P) and ectrodactyly occurred independently. 6/9 were males.

If the frequency of ectrodactyly is taken to be 0.1 (9/87), the probability of the observation of none among 64 control embryos is $(1-.1)^{64} = 0.0012$. Thus the ectrodactyly may be attributed to the nicotinamide treatment.

Discussion

The goal of these experiments, prevention of CL(P) by administration of megadoses of nicotinamide during pregnancy, was

not achieved. In view of the observations in this thesis that A/J may not be resistant to 6-AN induced CL(P) (Chapters A, B, and C), the rationale for these experiments has become untenable, and the negative results unsurprising.

Of greater interest are the observations that very large dosages of nicotinamide may be teratogenic and toxic, causing increases in CL(P) frequency in CL/Fr mice, and increased resorption and ectrodactyly in A/J mice.

That nicotinamide is a teratogen has been suggested previously. It was reported to induce beak defects while protecting other organs against teratogenesis by insulin, sulfanilamide and para-aminobenzoic acid in chick embryos (Landauer, 1948; Zwilling and De Bell, 1950). In strain 129 mice, high dosages of nicotinamide were reported to induce malformations, and to enhance the teratogenicity of tolbutamide (Smithberg, 1961; Smithberg and Runner, 1963; Nogami and Ingalls, 1967). The specific effects of nicotinamide alone, except spinal defects, were not described.

An association between pooled human birth defects and maternal nicotinamide supplementation has been reported (Nelson and Forfar, 1971).

Considering the forelimb ectrodactyly induced in A/J mice in the present study, it is interesting to note that NAD levels may determine muscle and cartilage differentiation of limb buds, and that exposure to nicotinamide has been shown to inhibit chondrogenic expression in chick limb buds (Caplan and Rosenberg, 1975).

The apparent forelimb, right side, predominance of induced ectrodactyly seen in Experiment 6 has also been observed for the acetazolamide-induced ectrodactyly trait (Biddle, 1975a).

Whether the effects in the present experiments were the result of nicotinamide per se, or the result of contaminants of the nicotinamide source, or a physiological response to the introduction of a large volume of hypertonic fluid, or to other unknown variables, remains to be tested.

GENERAL SUMMARY AND DISCUSSION

Summary of Results

The major new findings of the studies reported here were:

- (1) A/J mice were sensitive to induction of CL(P) by 6-AN. This finding contradicted a previous study (Goldstein et al., 1965) that formed the basis of some of the hypotheses tested (Chapters C and I). Sensitivity, however, is the best explanation of the observed CL(P) response of A.AB6 embryos to a dosage too low to induce detectable frequencies of CL(P) in A.B6- or B6-like embryos (Chapter C). A/J sensitivity also explains the finding of a critical period (Chapter A) and a dosage response regression (Chapter B) similar to those of other genotypes.
- (2) The critical periods for CL(P) induction by 6-AN in the genotypes tested were found to span most of day 10 of gestation. The maximum sensitivities of A/J and B6 were at midday treatment, while those of A.B6 and A.AB6 were about 2 hours earlier, and that of B6.A was about 4 hours earlier.

A/J, B6, A.B6, and A.AB6, but not B6.A, had markedly increased embryonic mortality with later treatment time on day 10.

- (3) The order of relative sensitivities from probit analysis of dosage responses, and from the maximum frequencies obtained from the critical period study, agreed. From most to least sensitive, the orders were: for CL(P) -- A/J, B6, A.B6 and B6.A; for resorption--A/J, A.B6, B6 and B6.A.
- (4) The differences in pattern of response to changes in treatment time and in relative sensitivity to dosage indicated that CL(P) and resorption were different traits: resorbed embryos were probably not severely affected CL(P)'s.
- (5) Interpretation of probit analysis of CL(P) dosage responses of A/J, B6, A.B6, B6.A, CL/Fr, and CL.B6 indicated that relative tolerance was an embryonic trait, and not due to maternal effects; the higher tolerance of 6-AN dosage of B6 embryos was "dominant", A.B6 and B6.A embryos may also have some heterosis contributing to their tolerance. The lower tolerance of A/J-like embryos having been recovered in the A.AB6 progeny of 4 of 16 A.AB6 sires, the number of loci causing the tolerance difference between A/J and B6 was probably less than 3.
- (6) The similarity of B6.A, A.B6 and CL.B6 embryos to B6 embryos in dosage tolerance indicated that heterozygosity for the genes causing spontaneous CL(P) had no detectable additive effects on liability to 6-AN induced CL(P).

No response slope differences and thus no evidence

of gene-teratogen interaction was detected by probit analysis.

A/J-like sensitivity to 6-AN induced CL(P) and liability to spontaneous CL(P) did not appear to segregate together in A.B6 sires.

These findings suggest that 6-AN induced CL(P) and spontaneous CL(P) were probably largely independent, genetically and developmentally. Two different, mostly independent, threshold traits each producing the same phenotype, CL(P) are suggested.

- (7) A strain survey indicated that PL/J, DBA/2J, SWV, and C3H strain mice were resistant, possibly absolutely, to 6-AN induced CL(P), while SWR/J, CBA/J and CL/Fr, in addition to A/J, and C57BL/6J were sensitive.
- (8) Excesses of normal and CL(P) female A/J and A.B6 embryos (internal sexing method) in the study of critical periods of 6-AN induction of CL(P) suggested that there may be an X-linked factor in A/J that confers increased liability to 6-AN induced mortality in males.
- (9) The liability to spontaneous CL(P) in A/J, compared with B6 was attributed to two loci, having duplicate epistasis and their recessive alleles in A/J.

Evidence of segregation of one or two recessive loci causing resorption was also detected. Furthermore, a genetic correlation between resorption and CL(P) was found, and therefore one or both of the CL(P) genes

probably had pleiotropic effects causing mortality or were linked to genes with such effects.

- (10) A maternal trait, possibly due to a single locus with a dominant allele in A/J was found to explain the spontaneous CL(P) frequency difference between A/J (10%) and CL/Fr (25%). This difference between inbred strains, an expected attribute of polygenic threshold traits, was thus reduced to Mendelian terms.

The maternal effect seemed to be related to implantation rate--CL/Fr had consistently lower implantation numbers, lower resorption, and higher CL(P) frequencies than A/J in crosses with A/J and subsequent generations, and compared with A/J in crosses with A.AB6 sires. Enhanced survival of CL(P) embryos in CL/Fr mothers due to reduced embryonic competition was thus suggested.

That the maternal effect involved more than a linear relationship between implantation rate and mortality was indicated by the failure of resorption frequency to follow the same pattern of inheritance as CL(P) in crosses between A/J and CL/Fr.

- (11) A trend significant in A.AB6 embryos, toward deficiency of normal females was also observed in A/J, and A.AB6, embryos, and in crosses between A/J and CL/Fr.
- (12) C57BL/6J embryos from studies of critical periods and dosage responses for 6-AN induced CL(P) (Chapters A and B) had more isolated cleft lip than did other genotypes

where nearly all CL was accompanied by CP. This observation was attributed to the stronger "shelf force" of B6, that is thought to contribute to its resistance to induced CP later in development (Fraser, 1976). There seemed to be a tendency for isolated CL's to be unilateral more often than expected, and the combined probability from Chapters A and B, from Fisher's Exact Test, one tailed was less than 0.1 (χ^2 , 4 df = 7.85).

- (12) Thyroxin treatment of mothers during the time of embryonic lip formation was demonstrated to cause the preferential death of CL(P) embryos, compared with their genetically identical normal CL/Fr littermates.
- (13) Large dosages of nicotinamide were found not to prevent CL(P) in A/J or CL/Fr mice, but to be in themselves teratogenic.

The relationship between 6-AN induced-
and spontaneous-CL(P)

One of the initial purposes of these studies was to examine what appeared to be a negative relationship between liabilities to spontaneous and 6-AN induced CL(P). From the experiments, however, it may be concluded that the genes causing spontaneous CL(P) in homozygous or heterozygous state probably do not influence to any important degree the liability to 6-AN induced CL(P). Neither additivity of effects nor interaction could be unequivocally demonstrated. The maternal effects known to influence spontaneous CL(P) frequencies in the genotypes used also did not influence liability to 6-AN induced CL(P).

Spontaneous CL(P) and 6-AN induced CL(P) seem to result from two independent threshold distributions that do not interact with each other. That is, the liability to CL(P) on one scale does not influence the liability on the other.

The threshold trait underlying liability to 6-AN induced CL(P) may be embryonic face shape. Evidence supporting this is as follows: genotypes having relatively high liability to 6-AN induced CL(P), the A/J and CL/Fr strains, and the L selection line, tend to have reduced and less divergent medial nasal processes and a smaller "interpit" distance than genotypes having a relatively lower liability, the C57BL/6J strain and the M and C selection lines (Trasler, 1968 and personal

communication; Juriloff and Trasler, 1976); 6-AN has been shown to reduce the size of the lateral processes in treated mice (Leong, 1973); the cytotoxic and antimitotic effects of 6-AN have been reported to be specific to cells of ectodermal origin (Tuchmann-Duplessis, 1975), as the neural crest cells that proliferate to form the facial processes are. Thus, normal genetic variation in embryonic face shape and 6-AN dosage tolerance may act additively on the face shape threshold scale.

An independent threshold character not involving cells of endodermal origin is required for spontaneous CL(P). There has been recent interest in a glycoprotein of "glue-like" appearance on the surfaces of the opposed epithelia of the nasal pit just before they meet and fuse (unpublished; e.g., Dr. T. Steffek). The genes for spontaneous CL(P) may be hypothesized as changing the quantity or stickiness of this glycoprotein, perhaps leading to reduced contact between epithelia and the induction of breakdown and fusion.

The epithelial cysts (Literature Review) observed in the fusion zone of closed lips of CL(P) liable embryos possibly represent areas where insufficient contact was made.

CL(P) in mice as a multifactorial threshold trait

The best explanations of the A/J, C57BL/6J mouse strain difference in liability to spontaneous CL(P) seem to be 2 recessive loci with duplicate epistasis, or a single major

recessive locus with modifiers. There must also be at least one locus with multiple alleles, or 2 loci that control maternal effect traits. These maternal effects may influence embryonic survival rather than CL(P) liability.

While certainly meeting Fraser's (1976) definition of "multifactorial", the known genetic variation contributing to spontaneous CL(P) can hardly be said to be "polygenic", nor can the effects be considered to be largely additive on the threshold scale, since dominance, epistasis and maternal effects on survival are not.

With the exception of CL/Fr, the proportions of CL(P)'s after 6-AN treatment that were bilateral were not found to increase with dosage. In general there was little evidence that (bilateral) CL(P) represented more extreme values on a threshold scale than did unilaterals.

Similarly, the proportion of bilateral CL(P)'s showed little evidence of increasing with increased spontaneous CL(P) liability, as would be expected if they represented the more extreme values on that threshold scale.

The rate of association of CP with CL also did not show evidence of association with 6-AN dosage or liability to spontaneous CL(P). Two genotypes, treated C57BL/6J and untreated CL/Fr, had a higher proportion of isolated CL's than did other genotypes, although the majority affected (at least 80%) were CLP. For C57BL/6J, the protection against CP may be related to that strain's relative resistance to induced CP by other

treatments (Literature Review). It is interesting to speculate that CL/Fr may also have a relative resistance to CP. In general the rate of palate involvement in CL(P) seems best explained as a separate liability rather than as an expression of position on the threshold scales for CL(P).

For spontaneous CL(P), the evidence that two loci with duplicate epistasis can account for most of the difference between virtually never having CL(P) and having it 10% of the time, points to a possibility of finding a cause of CL(P) at a biochemical level, and developing a metabolic intervention to prevent it. Duplicate epistasis indicates that the two loci perform the same function, though not necessarily in the same way. In contrast to this, a multigenic cause, being the cumulated action of diverse and interchangeable loci on a threshold trait would be less easily elucidated; at a metabolic level, many different systems would be found to be slightly deficient, and they would not necessarily have any common pathway.

This illustrates the importance of testing multifactorial threshold traits for the presence of major genes. Such genes identify important steps in normal development, and enable it to be described in functional, biochemical terms rather than morphological descriptive ones. From that point, cure and prevention of anomalies of similar cause in man become possible.

Buffering of development, genetic control of teratogenic responses, and backup systems

It is a commonplace to say that development is buffered against environmental and genetic perturbations, and it is widely thought that the buffering is derived from the interconnections of metabolic pathways and interactions among genes (e.g., heterosis). It is also accepted that the degree of buffering of a particular developmental system depends on genotype, and that the genes contributing to a spontaneous malformation often contribute to the liability to induction of that malformation. How the buffering is brought about is, however, not well understood.

Although numerous experiments have attempted to study the genetic architecture of developmental stability of Drosophila, and some more biochemically oriented work has been done using chickens, mammalian systems have been little studied.

The "theoretical models" of developmental buffering derived from experiments have tended to be either generalized descriptions of the data (e.g., Lerner, 1954; Waddington, 1975), or highly specific constructs that do not explain all of the observations (e.g., Rendel's (1967) major gene model or the multifactorial threshold model).

Taking into account the results of the present studies of CL(P) in mice and the results of other studies reported in the Literature Review, the developmental stability of a

particular developmental morphological trait, or its liability to malformation caused by environment may be described by the following formalized hierarchial system:

- (1) the genotype may be absolutely resistant to the teratogen used; such resistance has been observed for, e.g., borate-induced eyelessness in Drosophila, acetazolamide-induced beak defects in chickens, acetazolamide-induced ectrodactyly in mice, and possibly 6-AN induced CL(P) in mice.
- (2) if the genotype is not absolutely resistant, then genes controlling its relative dosage tolerance are expressed as the frequency of malformation at a given dosage--e.g., as shown for the cortisone-induced CP trait in mice, and 6-AN induced CL(P) in mice.
- (3a) if the liability to spontaneous malformation and the teratogen affect the same threshold trait, then the genes for the spontaneous trait, and the relative dosage tolerance will act additively to produce an increased liability to malformation--e.g., as shown for a nonresistant stock with borate-induced and genetic eyelessness in Drosophila, and perhaps for cortisone-induced CP in mice (Vekemans and Fraser, 1977).
- (3b) a final possibility, not unequivocally described in the published literature, is that of synergistic or antagonistic interactions between teratogens and genes producing the same malformations.

Where genetic differences controlling relative tolerance to teratogens have been studied, variation at a small number of loci has usually been found. The variation at these loci may be polymorphic (e.g., the H-2 locus and its contribution to cortisone-induced CP liability), and may be maintained by mechanisms independent of development.

There is a recurring theme of epistatic interaction in the genetic control of both absolute and relative resistance to teratogenesis (Literature Review).

It has been customary to assume that the alleles (or their products) identified by such studies may contribute to the position of the genotype relative to a threshold. Alternatively, the allelic differences may interact with a teratogen to produce the observed sensitivity difference. For example, structural alterations in an enzyme molecule may allow it to compete better with a foreign compound, or there may be different rates of inducibility of drug metabolizing enzymes (e.g., Nebert and Felton, 1976).

While these models may explain relative sensitivity difference, another model to explain absolute resistance is proposed here, and follows. The studies of acetazolamide-induced ectrodactyly in mice (Biddle, 1975a, b), will be used as an example.

It was found that the absolute resistance of SWV and the sensitivity of CBA mice to acetazolamide-induced ectrodactyly was explained by 3 recessive loci with duplicate epistasis.

That is, embryos had to be simultaneously homozygous "mutant" at three loci, like CBA, before they could develop acetazolamide-induced ectrodactyly.

It is hard to envision a threshold trait or a difference in metabolic turnover where increasing dosage would not eventually produce an effect, and where one single wild-type allele could handle the entire load of teratogen.

As an alternative, it may be proposed that all genotypes were affected in the same way by the acetazolamide, and that the same metabolic pathways were effectively blocked in all. The three loci may control other biochemical pathways not influenced by acetazolamide, that by other routes each produces a substance necessary for normal limb development. CBA carried mutant, nonfunctional alleles at these loci, which would not normally have mattered. But in the presence of acetazolamide this hidden variation became important and CBA had no "backup system" to produce the necessary product, and thus developed a malformation. These backup systems were duplicated--a normal allele at any one of the three loci would have been sufficient, and in that sense development was buffered.

A riboflavin deficiency-induced CP trait in chickens (Jurilloff and Roberts, 1975), may be similarly explained. There, 3 recessive loci with duplicate epistasis were required to produce spontaneous CP. Under riboflavin deficiency only 2 such loci were needed. The "lost" locus may have contributed

to a riboflavin-dependent pathway; the wild-type, dominant alleles at the remaining two loci were its backup system.

Returning to spontaneous CL(P) in mice, where there is evidence that to be affected, embryos must be homozygous recessive mutant at two loci simultaneously, once again dominance of the wild-type alleles and duplication of gene functions would act as buffering systems--each locus would be a backup system for the other.

It may be possible to determine the metabolic pathways used by backup systems by experiments comparing the genetic control of sensitivity for individual teratogens and then combinations. For example, the metabolic effect of a second teratogen that reduced the number of loci required in homozygous recessive state to give ectrodactyly when co-administered with acetazolamide would point to the metabolic pathway controlled by the locus that would "drop out".

The question arises of the difference between single mutant genes that cause spontaneous malformations and the hidden mutant genes uncovered by response to teratogens in the model above. Overt mutant genes must function in unduplicated pathways (e.g., of recent evolutionary origin or proximate to a final necessary product), while hidden genes must function in duplicated pathways.

It was suggested (e.g., Lerner, 1954), that duplicate epistasis might account for developmental stability. The data observed over the intervening years strongly support

this conjecture. The genetic architecture of "buffering" may depend heavily on duplication of gene function and dominance of the wild-type allele: duplicate epistasis.

This type of epistasis would be relatively easily established by evolution through gene duplication followed by mutational base changes and acquisition of new regulation.

It is now possible to examine individual genes and their products in vitro. If phenotype is largely the cumulated activity of genes, then the study of individual genes, cumulated, will explain phenotype. But if interactions between genes and environment and among genes are common, then gene activity can only be viewed within the context of the environment and genetic constellation it is found. Then the phenotype, the gestalt, will indeed be different than the sum of its parts:

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