SUSCEPTIBILITY OF CLEFT LIP MOUSE EMBRYOS TO 6-AMINO-NICOTINAMIDE
SUSCEPTIBILITY OF CLEFT LIP MOUSE EMBRYOS TO 6-AMINONICOTINAMIDE

by

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INTRODUCTION

The fertilized egg, in its development, may take one of three possible courses. It may develop normally; it may deviate from the normal course as the result of intrinsic or external forces to produce a congenital anomaly; or it may abort or resorb. In mammalian systems the course of development is determined by the interaction between the fetal genotype and its environment. The fetal environment, which is its mother's uterus, is in turn determined by the interaction between the maternal genotype and environment.

In the highly inbred A/Jax mouse strain, spontaneous congenital cleft lip (CL) in this laboratory is found at a frequency of about 10% in embryos at term. Since litter-mates are genetically highly similar, the question arises as to why one finds one or more animals in the litter with a cleft lip, while their litter-mates are not similarly affected.

An observation of possible significance to this question was made by Goldstein et al. (1965) when treating pregnant A/Jax mice with the nicotinamide analogue, 6-amino nicotinamide (6-AN). A study of its teratogenic effects in hybrid mice had been carried out by Pinsky and Fraser (1960). Short-term exposures to the drug were achieved by injecting nicotinamide 2 hours later, in an amount which would have prevented the effects of the analogue if given concurrently. It was observed that cleft-lip, cleft-palate and hind-limb defects occurred after treatment on day 9 1/2, a high resorption rate but no
malformations occurred after treatment on day 10½, and cleft-palate occurred after treatment on day 11½. These results suggested that short-term exposures to 6-AN could greatly improve the precision with which the "critical" period, at which a teratogen acts to produce a given malformation, could be defined.

Goldstein (1964), carried this work further, investigating the effect of timed exposures to 6-AN at different stages of gestation in hybrid and inbred mice. Working with the A/Jax strain, he treated pregnant mice on one of days 8½, 9½, 10½, 11½, 12½, 13½ and 14½ of gestation with a dose of 6-AN. Embryos were examined for defects, and the frequencies observed were compared to those in a control group which had received no treatment. The data reported by Goldstein (1964) are presented in Table 1.

Those defects which did not appear among control animals were shown to have a well defined critical period, during which maternal treatment with 6-AN caused the malformation among embryos of the litter. Thus, the critical period for fused ribs and fused vertebrae was day 9½, while that for absent sacral vertebrae and hind limb defects was day 10½. Cleft palate proved to have a longer critical period, with maximal effect of the drug occurring on day 13½. Resorption was increased after treatments administered from day 8½ to day 11½, with very high frequencies occurring after maternal treatment on day 8½ or day 10½. The open eye defect and defects of the supraoccipital bones both seemed to have 3 critical periods at which 6-AN was effective as an inducer. In both cases, these might indicate 3 separate processes with
TABLE 1

Frequencies of fetal defects observed after maternal treatment at one of seven stages in gestation

<table>
<thead>
<tr>
<th>Day of treatment</th>
<th>Controls</th>
<th>8 1/2</th>
<th>9 1/2</th>
<th>10 1/2</th>
<th>11 1/2</th>
<th>12 1/2</th>
<th>13 1/2</th>
<th>14 1/2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resorption</td>
<td>23.5</td>
<td>91.5</td>
<td>40.2</td>
<td>86.6</td>
<td>39.0</td>
<td>18.2</td>
<td>21.1</td>
<td>25.6</td>
</tr>
<tr>
<td>Fused ribs</td>
<td>0.0</td>
<td>0.0</td>
<td>32.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fused vertebrae</td>
<td>0.0</td>
<td>0.0</td>
<td>89.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Open eye</td>
<td>9.9</td>
<td></td>
<td>23.6</td>
<td>27.3</td>
<td>14.0</td>
<td>33.3</td>
<td>12.7</td>
<td>25.4</td>
</tr>
<tr>
<td>Cleft lip</td>
<td>14.4</td>
<td>0.0</td>
<td>16.4</td>
<td>81.8</td>
<td>12.0</td>
<td>11.1</td>
<td>8.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Cleft palate</td>
<td>0.0</td>
<td>0.0</td>
<td>6.0</td>
<td>43.1</td>
<td>76.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent sacral vertebrae</td>
<td>0.0</td>
<td>54.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hind limb defects</td>
<td>0.0</td>
<td></td>
<td>36.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Defects of supraoccipital bones</td>
<td>24.3</td>
<td>85.7</td>
<td>32.7</td>
<td>81.8</td>
<td>36.0</td>
<td>61.1</td>
<td>64.8</td>
<td>29.9</td>
</tr>
</tbody>
</table>
which 6-amino nicotinamide is interfering.

After treating on day 10½, most of the embryos resorbed, and a high frequency of cleft lip was observed among the survivors. According to control values, the actual number of cleft lip embryos was about that expected in the observed number of implantation sites if no treatment had been administered. This suggested that the treatment spared embryos with cleft lip, rather than inducing the defect. By varying the dose of 6-AN, the resorption frequency was varied, and in each case, the number of cleft lip embryos was about that expected if all of the potential cleft lip embryos (assuming a frequency of 10% of all implantations) had survived the treatment. It was further shown that, when the 6-AN dose was reduced by half, the resorption frequency fell and the excess of cleft lip among viable embryos disappeared. One would still expect an excess of cleft lip embryos if the defect were induced, since the teratogenic dose range is generally only slightly lower than the lethal dose (Murphy, 1960).

The hypothesis was therefore presented that spontaneous congenital cleft lip embryos of the A/Jax strain are relatively resistant to a dose of 6-AN lethal to most of their litter mates on day 10½ of gestation.

This response seems to be of great significance, since it differentiates between cleft lip and non-cleft lip embryos more than 24 hours before a morphological difference is apparent (Trasler, personal communication). The difference in susceptibility in these genetically predisposed animals might, therefore, be a clue to the fundamental difference causing the cleft lip malformation.
A possible explanation for the differential susceptibility to 6-AN between cleft lip and non-cleft lip embryos is a shift in the rate of development in cleft lip embryos, causing them to be more retarded or advanced than their litter-mates. If that were the case, these embryos, if retarded, would not, at day 10\(\frac{1}{2}\), have reached the point of maximum 6-AN-sensitivity as manifested by resorption (hereafter called the 6-AN-sensitive period). If, on the other hand, they were advanced in development, they would have passed that point. Were a simple advancement or retardation of development the only point of difference between the two types of embryos, a 6-AN-sensitive period must be assumed for cleft lip embryos in which they are as sensitive to the lethal effects of 6-AN as normal embryos are on day 10\(\frac{1}{2}\). Advanced development would cause such a sensitive period to exist before day 10\(\frac{1}{2}\), while a retardation would cause it to exist after that time. Treating pregnant mice with 6-AN at a time when cleft lip embryos are selectively susceptible to its lethal action would cause more resorption among cleft lip than among normal embryos.

Goldstein (1964), in a preliminary test of this hypothesis, found that the frequency of cleft lip calculated as a percent of implantation sites was reduced to 6\% when treatments were made on day 11/0 of gestation, compared to a control value of 11\%. The difference was not statistically significant (.2 < p < .3). The cleft lip frequency was not reduced when pregnant mice were treated on day 10/0. This indication of a sensitive period for cleft lip embryos after the sensitive point for normal embryos suggested that the cleft lip embryos might be
retarded in their development, as compared to their unaffected littermates.

Experiments reported in this thesis examine the duration of the period of differential susceptibility and the possibility of a developmental retardation of cleft lip embryos as its cause.
Factors Reducing Fertility in the Maternal-Fetal Complex

Reduction in fertility may occur due to defects in any one of the components of the developing gestational system, and these defects may occur at any stage of development. They may involve a qualitative or quantitative change destroying the integrity of the system. Lethal factors may be intrinsic to the fetus, intrinsic to the mother, external to both or may involve a subtle interaction between any or all of these types. In the following discussion, they will be broadly subdivided as being genetic or environmental or not classifiable as either of these. Interactions will be included within these classifications, and mentioned where they are conspicuously evident. It can be stated as a principle, however, that no lethal effects are determined solely by either hereditary or environmental factors but are always affected to a great or small degree by an interaction of the two. A variety of dominant and recessive factors are acting in both the mother and the fetus, as well as numerous non-genetic factors, of which some are permanent and some temporary. As the temporary factors change, the response of even the genetic factors in the complex are altered, creating pliability and variety in an apparently fixed system.

Genetic Aspects of Reduced Fertility

Studies of spontaneous abortion in human females have shown
an increase in the risk of abortion in pregnancies subsequent to an abortion, and increased further as the number of aborted pregnancies increased. A number of such investigations (Rucker, 1952; Tietze et al., 1950; Warburton and Fraser, 1964) give consistent estimates. It seems that approximately 14% of all pregnancies end in detectable abortion, and that the probability is about 25% after 1 abortion, with no appreciable rise in the risk as the number of previous abortions increases. There are complicated interactions with such things as parity, parental age and gestational stage of previous abortion, and no simple genetic hypothesis could be constructed to account for the observed relationships (Warburton and Fraser, 1964).

Reports of malformations among human abortuses have suggested that as many as 60% of abortions have gross defects. The relationship of abortion to malformation in the fetus was studied by Burge (1951). Babies resulting from pregnancies in which a threatened abortion had occurred were examined at term for congenital defects. These had a frequency of 1.5%, only slightly higher than the frequency found among pregnancies which had not threatened to abort. This finding suggested that threatened abortions are not caused by fetal malformations. There does not seem to be any doubt that the frequency of abnormalities in aborted fetuses is much higher than in full-term babies, but it may be that there is a common factor causing both abortion and malformation, rather than that one gives rise to the other. The findings to be presented in the following section provide a basis for this opinion.
Chromosomal Aberrations

Human karyotyping has been developed to an extent which makes the identification of a large number of chromosomal aberrations possible. Many of them have been described among human abortuses (Sato, 1965); included among chromosomal anomalies were triploids, tetraploids, trisomies A, B, D, E and G, translocations, mosaics, monosomies XO and A, as well as some structural abnormalities.

Carr (1965) made a study of the chromosomes of 200 spontaneous human abortions. Previous studies had confirmed a reported incidence of 22.5% chromosomal abnormalities among previable human abortions, and in this work 44 such abnormalities were found. The incidence of chromosomal abnormalities at birth being 1 in 240, their frequency among abortions is more than 50 times that among live births. Of the 44 aberrations, 11 were XO monosomies. This incidence showed an increase from 1 in 5000 live births to 5.5% of abortions. It seems that only 1 in 40 XO zygotes survives to term. The probable frequency of such zygotes in man has been calculated to be 0.83% at fertilization, but raised perinatal and prenatal mortality combined with the effect of adult sterility maintains the incidence at birth at low levels. In mice, where an XO individual is a normal fertile female and where there is no evidence of high perinatal lethality, the average incidence of XO individuals is 0.7%.

Triploid abortuses were observed at a frequency of 4.5%. Among these were included 6 XXY, 2 XXX and 1 XYY individuals. Twenty-two trisomies were observed; these included 7 of a Group E trisomy, 6 Group D,
5 Group G, 2 Group C and 1 each of previously not described trisomies of Groups A and B. Trisomy D, which occurs in about 1 in 4000 live births, was here found in 3% of abortions, indicating that prenatal lethality of such zygotes is 120:1. Similarly, Trisomy 21 was observed at a frequency of 2%, while it occurs in only 1 in 600 live births, which indicates that neonates with this trisomy are only 1 in 33 of all Trisomy-21 zygotes. Two tetraploids were also observed, 1 of the XXXX and 1 of the XXYY chromosome constitution. This condition has never been reported in a living subject, but is observed only among aborted specimens.

Chromosomal abnormalities are probably a more common cause of abortion in man than the 22% reported in this series. Autosomal monosomies, which were lacking in this series, may cause early, undetected abortions which would increase the frequency of chromosomal aberrations in abortuses. Some of the abnormalities seem to be viable in at least some of the instances in which they occur. Perhaps the remainder of the zygotic genotype, as well as the maternal genotype and environmental conditions, serve to decide whether or not the abnormal zygotes will survive to term.

Triploidy seems to be one of the commonest chromosomal aberrations to arise spontaneously or by induction in mammals (Austin, 1960). As a general rule, triploid embryos are inviable, though they may survive the first half of pregnancy. Triploid eggs may form by syngamy of 1 female and 2 male haploid pronuclei (polyandry), by that of 1 male
and 2 female pronuclei (polygyny), or by the fusion of a haploid with a diploid pronucleus (aneugamy). Mammals seem to depend mainly on the prevention of entry of supernumerary sperm into the eggs as a means of discouraging triploidy; the egg has 4 lines of defense: restriction of numbers of spermatozoa reaching the site of fertilization, impedance of sperm penetration by the cumulus oophorus, the zona reaction, and the block to polyspermy.

The incidence of polyandry in rats, mice, hamsters and field voles has been cited as occurring in 1 or 2% of penetrated eggs. Differences between various estimates within the same species have been found as large as those between species, as shown by estimates of 0.3, 1.2 and 3.2% in the rat, 0.9 and 1.2% in the mouse, 1.4% in the hamster and 2% in the field vole. Differences were also found to be striking between 2 inbred strains of rats. Evidently, susceptibility to polyspermy is under genetic control to some extent, and experiments testing 2 strains of female rats with the same stock of males showed that it was the genotype of the female which was important.

Experimental situations, such as delayed mating, were found to increase polyandry in rats from two- to eleven-fold, depending on the strain tested. The incidence was chiefly attributable to ageing of the eggs which presumably resulted in reduced efficiency of sperm-blocking mechanisms. Elevation of temperature, either by local application of warm water to the Fallopian tube or by the induction of hyperthermia, also led to large increases in the frequency of polyspermy in rats, which
rose to 34% when hyperthermia was combined with delayed mating in the treatment.

Austin (1956b, cited in Austin, 1960) induced high body temperatures in mice immediately after mating, and found, in eggs recovered later, that there were significantly more sperm in the perivitelline space that had not entered the vitellus than there were in untreated eggs in which penetration had occurred. This suggested that the vitellus had been changed by the heat treatment in a way that prevented the attachment and penetration of spermatozoa. It was also evidence for a change in the vitelline cortex which spread through the vitellus after sperm penetration to prevent attachment of supernumerary sperm.

Some experimental work has also been done on polygynous triploids, which result from the fusion of a haploid sperm with a binuclear oocyte or with an egg in which the second polar body was suppressed. That polygyny is also under some genic control could be detected by the different incidences in various strains of mice: outbred strains showed a frequency of less than 0.1%. "nonsilver" strains showed about 0.25%, while "silver" females mated with non-silver CBA males showed the highest occurrence, at 5.7%.

Cold and heat shock were used by Fischberg and Beatty (1952) to suppress the extrusion of the second polar body. This was accomplished by local heating or cooling of the Fallopian tubes 2½ hours after copulation. In control mice, 0.4% of analyzable eggs were triploid; in cold-treated mice, 3.5% of eggs analysed on day 3½ were chromosomally abnormal, but
only 1 of the 85 examined was triploid. Heat treatment, however, caused 11% of all analysable eggs to exhibit triploidy, while another 4% were a variety of heteroploids. These eggs were found to survive, and appeared morphologically and histologically normal. Triploid eggs could evidently undergo a certain degree of morpho- and histogenesis, at least to the differentiation of the inner cell mass and the formation of the blastocoel and trophectoderm. It will be remembered that heat treatment in rats led to a heightened incidence of polyspermy. In that species, there was little suppression of the second polar body; this constitutes a distinct species difference. Furthermore, while triploid eggs in mice did not survive long after the blastula stage, it was observed in rats (Piko, cited in Austin, 1960) that embryos up to the eleventh day of gestation exhibited triploidy at approximately the same frequency as that of polyandry during fertilization.

Dominant Lethal Genes

Lethal genes could be responsible for death of the embryo from pre-implantation times to parturition and much later. Dominant lethal genes would, of course, arise only by mutation, but the consideration of any event which causes death of approximately half the embryos as a dominant lethal enlarges the field. Included on this category would be such conditions as heterozygosity for translocations, and single chromosomal breaks resulting in acentric and dicentric fragments. Using X-irradiation data, Bateman (1958) examined the partition of such dominant lethal events in the mouse between unimplanted and early postimplantation
stages. The relation between early post-implantation deaths and pre-implantation losses in his data excluded the possibility that some constant fraction, about one half, of all dominant lethal embryos died without implanting. Rather, his results suggested that all embryos with one lethal, and about 25% of multi-lethal embryos did not die until after implanting, while the rest failed to do so.

In the mouse, there are 13 dominant factors which are lethal when homozygous. The classic example is the yellow gene \((A^Y)\). Robertson (1942) observed that 25% of the zygotes from an \(A^Y/- \times A^Y/-\) mating, corresponding to \(A^Y/A^Y\) homozygotes, developed normally until the blastocyst stage, but became abnormal when the trophoblast made contact with the epithelium of the uterine crypt. At this stage, the end of day 5 of gestation, cell degeneration rapidly set in, and all inviable zygotes were fully resorbed 36 hours later. Ovarian transplants from yellow to non-yellow females subsequently mated to yellow males showed that the \(A^Y/A^Y\) zygotes, in a normal maternal environment, survived to a later stage, developing a small ectoplacental cone and Reichert's membrane.

Eaton and Green (1962) ascertained that all matings involving 1 or 2 yellow parents showed a direct 1:1 correlation of corpora lutea to implantation sites, showing that mortality prior to implantation was not involved in the lethality of \(A^Y/A^Y\) zygotes. They also observed that implantation crypts could be distinguished by their depths, the shallow ones containing disintegrating embryos. Implantation, which always has begun by day 5, 7 hours, was observed with homozygous yellow embryos at the age of 6 days, indicating that they evoke a normal
implantation response before dying.

Reciprocal crosses between yellow and non-yellow mice of the YS/ChWf strain were tested by Wolff and Bartke (1966), who found that the mean number of young born alive was significantly less when the mother was yellow. The mean number of implanting embryos did not differ between yellow and non-yellow females, but survival was higher in the non-yellow mothers. It was further observed that there were fewer heterozygous progeny in litters of $A^Y a x aa$ than in the reciprocal cross, where the female was not yellow. It was therefore suggested that in yellow mothers, in addition to the loss of $A^Y/A^Y$ zygotes, there exists an additional loss of $A^Y/-$ embryos. Such an effect would explain the lower embryonic survival, smaller litter size and deficiency of yellow progeny that were observed. As previously mentioned, $A^Y/A^Y$ embryos survived longer in non-yellow mothers. It was also found that treating yellow mothers with progesterone at the time of trophoblast giant cell differentiation prolonged the survival of the homozygous yellow embryos. These results suggested that the uterus of yellow females does not provide as favourable an environment for $A^Y/A^Y$ embryos as that of non-yellow females, possibly due to a progesterone deficiency. It was further suggested that the metabolic differences responsible for the lethality of $A^Y/A^Y$ zygotes may decrease viability in $A^Y/-$ embryos, the difference being quantitative in nature. Homozygous embryos would fall below the survival threshold, while heterozygotes would usually develop successfully, the proportion doing so depending on the uterine environment, which could be changed by the $A^Y$ gene in the mother's
genotype, as well as by the residual genome of a given strain.

Hadorn (1961) discusses the dominant factor, Tail-short (Ts) in the mouse, which arose in a stock and had the effect of producing a shortened, bent tail when occurring in single dose in its original genic milieu. When the original stock was crossed with several other stocks, the manifestation of the Ts gene in hybrids was either diminished, unchanged or increased. In crosses where the new genic milieu enhanced the effect, Tail-short became a dominant lethal factor, causing heterozygotes to die in the embryonic stage. It was, therefore, a conditional lethal factor, its penetrance and expressivity dependent on other genetic factors in the genome. One such factor was Crooked tail (Cd), which caused an increase in general tendency for various kinds of malformations when the two mutant genes were combined. The Cd mutant itself showed several characteristic lethal phases in homozygotes. About 25% of such zygotes were eliminated soon after fertilization, while another quarter lived up to about day 5 of gestation (early post-implantation stage). The remaining Cd/Cd embryos frequently developed severe exencephaly and other central nervous system disturbances, which caused another quarter of late embryonic deaths. The rest of the homozygotes either died at birth, or survived with malformations of varying severity. Again, it was perhaps residual differences of the genome that determined the path the development of a specific Cd/Cd embryos would follow.

The Brachyury (T) locus is the only one in the mouse for which there is unequivocal evidence for an influence on gametic function. The
dominant factor, T, causes a shortening of the tail when heterozygous while homozygotes (T/T) die in utero on the 10th or 11th day of gestation. These embryos were more retarded than viable ones from the 9th day onward. It was observed that the notochord did not develop as normal from mesoderm below the neural tube. Somites were also reduced and irregular. When homozygous mutant neural tube was cultured with normal somites, growth of cartilage was induced. Homozygous mutant somites did not, however, respond to inducer from normal neural tube. Since limb buds from a T/T embryo could form cartilage in the same culture medium, the defect was in the somites. The abnormality of the archenteral mesoderm was, moreover, observed to be primary to nervous system abnormalities (Hadorn, 1961). A large number of recessive alleles at the T-locus have also been observed; those among them which show lethal effects are discussed in the next section.

Recessive Lethal Genes

The numerous recessive t alleles at the T-locus are characterized by producing viable, tailless mice when heterozygous with T, and many act as embryonic lethal factors when in the homozygous condition. It was observed that the different mutations were further characterized by different phase-specific developmental activities. The earliest lethal observed was the t^{12}/t^{12} homozygote; development was found to stop in the morula stage immediately before blastocyst formation, the day of onset being 3½. The t'/t' homozygote was similarly identified as a pre-implantation lethal, death
occurring at about day 5. Another recessive factor, \( t^0 \), had a lethal effect at the time of blastocyst implantation in the uterine wall; in the early egg cylinder there was no separation of inner ectodermal cell mass into embryonic and extraembryonic portions. The entoderm was also observed to be abnormally thick and coarse. Death occurred typically on day 6. The series of recessive lethal alleles continued with \( t^4 \) and \( t^9 \), in which homozygotes die about day 8 and day 9 of gestation, respectively. In the \( t^w^5 \) group, homozygous embryos survive to day 6½, at which time lack of differentiation was observed in the entoderm and embryonic ectoderm, accompanied by some pyknosis and degeneration (Gruneberg, 1960). Heterozygous combinations of alleles which acted as homozygous lethals were generally found to be viable and normal. The \( t^0/t' \) heterozygotes were found to be fertile if female but sterile if male. Furthermore, malformations of the head with lethal effect developed in \( t^0/t' \) embryos in percentages varying with the genetic background. The general viability of tailless heterozygotes may be ascribed to a form of complementation between the different \( t \)-mutants, in which each factor ensures normal development through the process for which its allele is mutant.

Braden (1960) reported that males carrying 2 \( t \) alleles showed a high proportion (8-48.5%) of spermatozoa with deformed heads, whether they were heterozygous for 2 lethal \( t \) alleles or homozygous for a viable allele. They also were very infertile if at least one of the \( t \) alleles was a homozygous lethal, but infertility was not correlated with the incidence of deformed spermatozoa. Females of similar genotypes were
fully fertile. It seemed that infertility was due to the inability of the spermatozoa of such males to traverse the uterotubal junction.

Other recessive lethal factors known in the mouse which cause death soon or immediately after birth include congenital hydrocephalus, dilute lethal, gray lethal, uro-rectal-caudal, and vestigial tail when in conjunction with the dominant T of Brachyury.

Lyon (1959) attempted to measure the spontaneous occurrence of recessive lethals in inbred strains of mice. It had previously been observed that only 66% of corpora lutea were represented by living young at birth, and that mean litter-sizes at birth in the DBA and C57BL mouse strains were only 58 and 84% respectively of the mean number of fertilized ova. Recessive lethals, unless very common, are only likely to be the cause of death when the parents are related, as in an inbred strain. The study utilized all reciprocal crosses between CBA/H, C3H/HeH and 101/H mice. Pre-implantation loss was measured by the difference between the numbers of corpora lutea and implantations; post-implantation loss, which could involve only embryonic death, was measured by the number of implantation sites at which no living embryo was observed. The lowest ratios of live implants to the total number of implants were observed for the within-strain crosses, supporting the hypothesis that recessive lethals caused death in within-strain but not in between-strain crosses. The difference between summed within- and between-strain crosses was 9.6%. There was no indication of any strain tending to yield different frequencies of dead implants. Within-strain crosses suggested that the frequencies of deaths due to recessive effects differed in the
3 strains. In the 101 strain, a few uteri showed an amount of fetal
death consistent with the simultaneous segregation of 2 lethals, while
no dead embryos were found in other uteri of the strain. A study of
pre-implantation loss showed no difference between the within- and
between-strain crosses, but did show large differences depending on
the types of males and females used. There was thus no evidence for
recessive lethals causing pre-implantation embryonic loss. Assuming
all excess death to be due to recessive lethals, an upper limit of
0.1 spontaneous mutations to recessive lethals per gamete over all
loci was calculated for these strains. If the average mutation rate
per locus is $1 \times 10^{-5}$, this calculation gives an estimate of 10,000 loci
mutating to lethals. However, the demonstration that the maternal
genotype makes a major contribution to the resorption frequency (Falconer,
1960) suggests that another important component of fetal mortality resides
in the maternal physiology.

Species and Strain Differences

A distinct species difference has been observed in the response
of mouse and rat eggs to localized heat treatment (Austin, 1960). Mouse
eggs responded with suppression of the second polar body to produce two
female pronuclei in the ovum, while rat eggs showed mainly a heightened
incidence of polyspermy, presumably due to a primary interference with
the ovum's defenses against this. Both responses led to a high frequency

* 4.8, 7.6 and 16.9% in CBA, C3H and 101 strains, respectively.
of triploid zygotes, which are inviable, thus reducing fertility.

Relationships of prenatal death to the age and parity of the mother animal also showed a species difference, as described by Hanly (1961). Among pigs, higher frequencies of embryonic death were observed among parous than nulliparous animals, while in cattle, heifers showed higher embryonic death than cows past their third reproductive periods.

Strain differences in fertility are also numerous, and sometimes as large as those between species. Jones and Krohn (1961) observed differences between A, CBA, their crosses and RIII mice in the rate of decline of oocyte numbers with age. The total numbers of oocytes and the percentage classed as normal differed also. Hafez (1965a), in his studies with mice, concluded there was no difference in the viability of eggs of the DBA and C57BL strains, but that the uterine environment of C57BL mothers provided a more favourable environment for development. Gametic function leading to abnormalities of maturation or development was observed to differ among inbred mouse strains (Braden, 1960). The finding that the proportion of eggs in which more than one spermatozoon had penetrated the zona pellucida was directly related to the strain of the male used indicated there was variation in spermatozoon function in the female tract. The type of female used had generally little influence. Females mated with CBA, A or RIII males contained more than one spermatozoon in 12-15% of penetrated eggs, as compared to 25% of eggs in females mated with C57BL males. The effect did not seem to be due to large numbers of
spermatozoa about the eggs of females mated to C57BL males, but rather to a greater motility of C57BL spermatozoa in the female tract.

The relative fertility of A/Jax and C57BL/6Jax mice was reported by Fainstat (1951) and Kalter (1953 and 1954). The index of fertility was calculated as the proportion of copulations that resulted in palpable pregnancies. The proportion was low in A (20.7% conception) and high in C females (58.59% conception). Reciprocal crosses were observed to vary according to the strain of the female, such that AxC matings showed 20.48% conception, typical of the low A strain conception rate, while CxA matings showed 63.08% conceptions. The Fl females (AC and CA) were of approximately the same fertility as C females at a frequency of 66.07%. This suggested that the genetic basis for fertility may be dominant to that for fertility. The back-cross females, derived from ACxA and CAxA matings, had an index of fertility much closer to that of A females, at a conception frequency of 26.53%. The rapid decrease in degree of fertility to the level of the less fertile strain seemed to confirm the contention that the character of relative fertility was not a very complex genetic phenomenon.

Hafez (1965a) investigated implantation capacity and fetal survival in two strains of rabbits, New Zealand white and Chinchilla. Implantation capacity was higher in the Chinchilla strain, but the percentage of fetal survival was lower than among New Zealand animals. The two breeds thus did not show any discrepancy in litter size, nor did they differ in fetal weights. The differences in implantation capacity were found to be due to genetic differences in the physiology and morphology.
of the uterus. Such factors as length of uterine horns, degree of
development and secretory activity of the endometrium were found to
differ in the two breeds. The variation in these factors may well
be underlying causes of some of the strain differences which were
observed.

The Albany strain of rats was studied by Wolfe and his
associates (Wolfe et al., 1940; Wolfe and Wright, 1943) who inter­
preted the low fertility characteristic of the strain in the light
of a number of related factors. Partial failure of ovulation and a
high degree of subsequent follicular atresia were noted, which led
to abnormalities of the estrous cycle. Certain histological features
of the anterior pituitary were also observed which suggested an in­
herent hypofunction.

Ehling (1964) observed that strain variation in reproductive
capacity in mice, besides existing per se, exhibited larger differences
when variation in response to an external agent increased the complexity
of the situation. He studied mice of the SEC, A, SEA, NB and DBA
strains after exposing the females to 50 r of X-rays the day before
mating. The effect of such irradiation is to induce permanent sterility
in female mice by extremely rapid destruction of oocytes in immature
follicles. Mortality during embryonic development was studied by the
counting of corpora lutea and dead and live embryos. The number of
offspring produced by each strain expressed as a percent of control
offspring were 15% for the SEC strain, 18% for A, 19% for SEA, 32% for
NB and 52% for DBA mice. It was observed that the overall breeding
period was shortened, that the time between litters increased and that the size of successive litters declined rapidly, with high prenatal mortality in some. The intraspecific variation in reproductive capacity following irradiation might have been partly due to differences in the frequencies of oocytes in later, radioresistant follicle stages present before treatment or to differential radiosensitivity of oocytes in the different strains.

Selection

The response of an unselected control line maintained with minimal inbreeding for 31 generations was reported by Falconer (1960). The rate of inbreeding was 1.25% per generation, and the litter size did not change, but remained at about 7.5 young throughout. It seems, therefore, that natural selection was effective in counteracting those effects of inbreeding which would have tended to reduce the litter size, had it been more rapid. If natural selection did indeed maintain the litter size at control values, it must have acted mainly on the viability of the young, since the breeding system used offered little opportunity for it to act on the fertility of females. Selection for increased litter size decreased the additive genetic variance, until only a negative maternal effect was left. This effect was the result of two correlations: maternal weight was positively correlated with litter size, and litter size negatively with offspring weight. Thus, large mothers had large litters consisting of small daughter that bore small litters. Selection for reduced litter size increased the genetic variance, so that genetic correlations came to outweigh environmental
ones and the daughter-dam regression (of litter size) became positive. Such an increase in genetic variance in the low selected line was compatible with expectation if low litter size were due to lethal and semi-lethal genes in the embryos. Such genes, which would have been at low initial frequencies, seem to have been brought to intermediate frequencies by selection. At those frequencies, they would be making the maximum possible contribution to the variance of litter size.

Selection was also observed to change ovulation rates in high and low lines from that found in unselected controls. The effect was evident after 16 and 17 generations of selection, and increased further by 31 generations. After 16-17 generations of selection, high lines ovulated 10.4 eggs and bore litters of 8.4 young, losing 19% of ova, while low lines ovulated 8.5 eggs, bearing 6.2 young, which was a loss of 24% of the ova. After 31 generations, the high line ovulated 13.7 eggs and bore litters of 9.2, a loss of 33% of the eggs, while the low line bore 6.0 young from 10.3 ova, a loss of 42%. The loss was thus seen to be always greater in low lines, even while the ovulation rate increased. These data fitted the lethal hypothesis. It was concluded that selection for increased litter size acted chiefly on female fertility by increasing the ovulation rate, while a proportionate increase in loss of eggs or embryos occurred. Selection for decreased litter size, on the other hand, acted chiefly on embryonic viability, with little or no decrease of the ovulation rate.

In a later study, Falconer (1965) estimated that 24% of variation in litter size is heritable, while 6% of variation is due to
permanent environmental and non-additive genetic factors. The remaining 70% of variation is due to temporary environmental variance. Previous observations that increased litter size was due to an increase in ovulation while decreased litter size was due to an increase in embryonic mortality were corroborated. The high embryonic mortality was shown to be a feature of the low line mothers and not of the embryos, since it was still present in low-line mothers bearing cross-bred embryos. Crosses between selected lines and controls showed the ovulation rate to be intermediate in F1 females, but crosses between low lines and controls showed that the high embryonic mortality disappeared in those F1 females. This particular feature of the line must therefore have been due to recessive genes acting on the mother's capacity to maintain embryos as had previously been suggested, or possibly recessive genes predisposing to non-disjunction.

Inbreeding

A series of investigations was performed by Falconer and co-workers (1960) which studied the reactions of litter size in mice of the J stock under a program of inbreeding. Litter size was taken to be the number of live young in first litters. Since litter size was reduced by inbreeding without selection, it was concluded that genes reducing litter size tend to be recessive to their alleles that increase it. The decline of litter size was in all experiments directly proportional to the coefficient of inbreeding. Selection was found to counteract the observed inbreeding depression. Crosses were made between partly inbred lines in order to separate the effects of inbreeding on the young from
those on the mother, since the reduced litter sizes might have been due partly to reduced fertility of females and partly to reduced viability of embryos. Litter sizes of inbred mothers with crossbred young were then compared with those of equally inbred mothers with inbred young. About 40% of the total inbreeding depression was found to originate from reduced fertility of females and 60% from reduced viability of the young. The inbreeding of the father did not influence the size of the litter.

The cause of reduced fertility of inbred mothers was investigated by dissecting pregnant mothers on day 16 of gestation to count corpora lutea, implantation sites and living embryos. Inbred females were mated to inbred males of another strain, and non-inbred females were compared with them. Reduced fertility of inbred females was due almost entirely to a greater pre-implantation loss of zygotes. The ovulation rate was not decreased, and post-implantation loss was only slightly increased. The difference in pre-implantation loss between inbred and non-inbred females was, however, significant at the 5% level in 2 experiments, and at the 1% level in the third. This loss was possibly due to failure of implantation from endocrine malfunction. Ovulation rate did not decline presumably because of its positive correlation with body weight. The body size of mice in these experiments did not decline, even though inbreeding should have brought this about, since the reduced litter sizes improved the maternal environment. This improvement compensated for any decline of growth rate which continued inbreeding was effecting. Thus, conclusions that inbreeding does not affect ovulation rate are valid only when body sizes remain constant.
Lyon (1959), working with CBA, C3H and 101 mice, examined post-implantation viability in between- and within-strain crosses. The post-implantation death was higher in the within-strain than in the between-strain crosses, excess death over that found in between-strain crosses averaging 9.6% when summed over all crosses.

A study of two inbred lines of mice (E albino and P chocolate), outbred mice and crosses involving the E line was made by Krzanowska (1960). The number of ova shed by outbred and E line mice was very similar, and significantly higher than that in P mice. On the other hand, the number of ova fertilized was much lower in E than in outbred mice. There were significantly more viable embryos in outbred than in either pure-line females on day 12 of gestation, and the number of young born was also significantly higher. There had been no significant differences between crosses and pure matings in the number of dead embryos, although mortality had been slightly higher in the pure matings. Peak mortality, in all groups, was found to occur at the developmental stage at which the embryo measured 3 mm.

Hanly (1961) cited a correlation found between inbreeding and increased prenatal death in cows. For outbred cows, prenatal mortality ranged from 13.3 to 19.2%, while that for inbred cows ranged from 26.7 to 28.4%. The inbred embryos also showed a tendency to higher prenatal death. In pigs, however, inbreeding lowered the ovulation rate but had no effect on embryonic death.
Immunologic Incompatibility

Experimental situations have been described in animals wherein females were immunized with some component of male ejaculate and thenceforth showed an immune response and reduced fertility upon mating or artificial insemination. Such a situation was described in female guinea-pigs sensitized with sperm. The conception rate for such animals was 34.6%, compared to 78.6% for control animals.

Behrman and Nakayama (1965) wished to elucidate the problem of why 34.6% of immunized animals were still able to conceive. They studied the relationship of antibody titre to ability to conceive in virgin rabbits after an immunization program with testis extract. When antibody titre was highest, they observed that attempts to mate the immunized rabbits never resulted in pregnancy. However, if the females were isolated until antibody was no longer detected in the serum, the rabbits mated and became pregnant in 5 days. The classic secondary response in antibody production of an immunized rabbit after mating suggested that once females are sensitized against testis tissue, fertility would be inhibited by an antigen-antibody reaction following mating. This was in fact observed if exposure to the male occurred while circulating antibodies were still demonstrable. Ejaculate seemed to be a natural form of reimmunization enhancing infertility after mating. Since dead sperm failed to elicit an antibody response, it was assumed that, rather than spermatozoal antigens themselves, the antigens of the testis, prostate or epididymis evoke the antibody response, involving the spermatozoa secondarily as a result of coating them. The value of
the experiment is, however, impaired by the lack of a more stringent investigation into the nature of the eliciting antigen and by the absence of controls.

Weil and Roberts (1965) tested a highly antigenic component of seminal plasma which coats spermatozoa to see if sensitization against the coating would influence female fertility. They found no difference in rate of impregnation and number of embryos between those rabbits sensitized against the spermatozoa-coating antigen and the untreated controls. The antigen-antibody reaction did not impair overall fertility, although 70% of treated animals showed antibodies. It is possible that the immunization program was not intense enough, but a more likely probability is that the response observed by other workers was elicited by a more heterogeneous antigenic mixture than the one used in this experiment.

McLaren (1964) obtained evidence which suggested that the breeding performance of female mice was affected by immunization against spermatozoa. The degree to which litter size was depressed depended on the titre of agglutinating antibody in the serum. Sterility of many months' duration was observed with high antibody titres. An intense immunization program was required for good sensitization. This involved intraperitoneal injection of live spermatozoa of the random-bred Q strain in buffered saline 3 times a week for 7 weeks. The resulting serum agglutinated head or tail antigens independently of strain. Test crosses of immunized and control females with Q strain males were then set up. Females with high anti-sperm agglutinating titres showed marked reduction
in the size of the first litter and in total breeding performance over a 6 month period, those with the highest titres producing no young throughout the experiment. The contraceptive effect of immunization persisted for the major part of the treated females' reproductive life. Eggs were examined 2 days after vaginal plugs were noted in control and immunized mice; sensitized mice produced normal numbers of eggs, but the proportion of fertilization decreased rapidly as the agglutination titre in serum rose. The reduction in the rate of fertilization adequately accounted for the observed reduction in fecundity, and there was no need to postulate an increase in embryonic mortality. The pregnancy block may have been reducing fertilization by a large enough amount of antibody being secreted from the blood into the uterus to agglutinate sperm, and thus reduce the number penetrating the oviduct. The close relationship between agglutination titre and reduction of fecundity support this interpretation. In no case was agglutinating antibody found with a regimen of natural mating.

Very shortly afterwards, Edwards (1964) reported experiments that differed from those of McLaren in all but the basic observation that immunizing females with spermatozoa reduced fertility. Using out-bred mice, he embarked on 3 heavy immunization programs. The females, when sensitized, were mated with proven fertile males and killed after mating. Of 47 immunized mated females, 6 had unfertilized eggs only, 13 had more than half the eggs unfertilized with no living sperm in the perivitelline space or between cumulus cells, 2 had no eggs and one showed parthenogenetic division. The infertility observed was not
correlated with titres of circulating antibody. Most sterile mice had low titres, while the partially fertile, non-ovulating and parthenogenetic females all had high titres. Edwards did not observe typical immune spermagglutination in immune mice, but found the sperm to be motile. Infertility seemed rather to be associated with more rapid removal of spermatozoa from the uterus, with an inverse correlation between the number of sperm in the uterus and sterility. The two conflicting reports do not require that one be accepted to the exclusion of the other. Part of the difference in results may have been due to the differences in routes of immunization used and the length of the immunizing period. A point of agreement in the two reports was the presence of unfertilized eggs in the females after breeding. It is also possible that Edwards' treatment induced a tissue-bound antibody of the type involved in delayed hypersensitivity reactions, which reacts with spermatozoa as does the circulating antibody observed in McLaren's experiment.

In humans, immunological incompatibility involving the ABO blood group system has been implicated as the etiological factor in infertility of no known cause. It has been suggested that serological incompatibility between husband and wife may lead to sterility. Edwards et al. (1964) have provided evidence important to such a hypothesis. They have found the A and B blood group antigens on spermatozoa of secretors, the washed spermatozoa reacted in agglutination tests according to the ABO blood type of the individual. M, N and Tja antigens were unequivocally found on the spermatozoal membrane. In secretors, it was
inferred that the A and B antigens in the seminal plasma adhere secondarily to spermatozoa, and are not removed by 3 washings. Thus, ABO incompatibility between husband and wife could be implicated in cases of partial infertility or gametic selection. The results indicated, however, that since the antigens occur primarily in the seminal plasma, any incompatibility must be related to the secretors status of the husband.

Behrman (1961) suggested that the woman in an incompatible ABO mating secretes antibodies in her cervical, uterine or follicular fluid, and that these will agglutinate, immobilize or inactivate the incompatible sperm if her iso-antibodies are strongly agglutinating or in high titre. It was postulated that an O-type woman (ii) married to a heterozygous A man (I^A_i) would agglutinate all his I^A sperm, causing conception to be delayed, while an O- or B-type woman married to a homozygous A (I^A^A) male would have the anti-A antibody to agglutinate all the sperm he could produce, which would result in total sterility. Thus fertility could be prevented by the antigen-antibody reactions of a pre-existing and naturally occurring blood group system, as typified by the mating of an O-type woman with an A-, B- or AB-type man. Indirect statistical evidence was presented in the data of 109 cases of infertility due to unknown causes. The blood type, secretor status and presence of antigen and antibody were determined in all subjects. It was observed that, among infertile marriages, 87.3% were ABO incompatible while among 171 highly fertile control marriages, 61.4% were compatible. With regard to the 12.7% compatible matings among infertile
couples and the 38.6% incompatible matings in the fertile group, one must take into account the modifying factors in the system: the secretor status of the husband and the varying cervical antibody titre during the menstrual cycle, as well as the presence of other causes of infertility.

Of the 109 infertile marriages, 7 became pregnant after 5 years of infertility. Five couples were O women married to A men, and 2 were O women married to B men; all the children born were of the 0 blood type. This is good evidence that the presence of anti-A or anti-B in the woman's secretion did not permit A- or B-carrying sperm to fertilize any eggs, but permitted O sperm to do so.

Indirect evidence has so far been presented for ABO incompatibility as the cause of infertility. Statistical indirect evidence has also been presented to implicate ABO incompatibility between mother and fetus as the cause of abortion. Dealing with blood groups of living children, it was shown that there was a statistical dearth of A offspring in marriages of O women to A men.

Direct evidence of a connection between blood groups and abortions was presented by Allen (1964). The tissues of 39 aborted specimens were tested for ABO antigens. The blood groups of entire families of some of the abortuses were presented, and blood groups of abortuses and mothers were matched to establish compatibility status. Racial groups were analysed separately. The probability of finding 8 out of 20 abortuses with B antigen in whites and 7 out of 11 in negroes was found to be very small, while 7 out of 20 and 3 out of 11
A-type abortuses in the two populations was within the expected range. Blood group distribution of abortuses was thus very different from that of general population, supporting the hypothesis that ABO incompatibility is a cause of abortion. Presence of an unusually large proportion of ABO incompatibility among abortions was demonstrated by direct investigation of mother-abortus pairs. Thirty-seven percent of these pairs were ABO incompatible. Previous suggestions that the B antigen was of greater significance than the A antigen in abortions was supported by the data, in which the proportion of abortuses having the B antigen was notably large in both whites and negroes, and 7 out of 10 mother-abortus incompatibilities were due to antigen B.

Becker (1965) cited complete vascular obliterations (infarcts) in the villous stems of the placenta as a not infrequent occurrence in stillbirths. They have been described in some cases of diabetic fetopathy and Rh-incompatibility, 46% of all stillbirths not attributable to metabolic disorders and maternal syphilis leading to abortion. Placental infection was also described as resulting in obliterative endoarteritis when occurring during the first half of pregnancy; several months later, fetal death occurs from placental insufficiency due to vascular obliterations. Early infection of the fetus and placenta were seen to result, not in the conventional inflammatory process, but in mesenchymal proliferation of the vessel walls which resulted in narrowing and finally total obliteration of blood vessels. The scar left after the inflammatory process subsided thwarted any further vascularization in the affected area. It was observed that infectious diseases, more
than any other lesions, were responsible. Toxoplasmosis and viral infections were included as possible causes of such vascular occlusions, as were occasionally metabolic diseases, especially maternal diabetes mellitus. Burstein et al. (1965) viewed these vascular lesions with a different interpretation. They were observed in cases of erythroblastosis fetalis, a disease due to immunological incompatibility between mother and fetus, and in diabetes mellitus, where the lesions were observed to bind specifically fluorescent insulin as well as fluorescent insulin-antibody. Similarly, the lesions observed in erythroblastosis bound anti-Rh antibody. The proliferative vascular lesions were observed only on the maternal side of the placenta, where proliferation of the lining endothelium often progressed to the point of occluding the lumen. During the period covering the 4th to 12th weeks of pregnancy, 63-65% of abortions showed advanced lesions in the maternal decidua. Considering less advanced lesions also, the frequency rose to 72-94% in various groups of abortions. After 13 weeks, the frequency of proliferative lesions in all groups of abortions was 22%.

Complement binding, a definitive test for the presence of antibody, indicated its presence in these vascular lesions. The presence of lymphocytes further supported the existence of an immune reaction. These two observations were made in normal pregnancies, also. Alterations in globulins during pregnancy further supported the concept. The suggestion was therefore made that an immune reaction analogous to the homograft rejection response occurs early in pregnancy. Maternal hormones apparently suppress such a reaction sufficiently, although not completely,
so that rejection of the fetus does not normally occur until term. This hypothesis was supported by the observation that homografts are more readily accepted during pregnancy than in the non-pregnant state.

Environmental Factors Reducing Fertility

**Nutrition**

**Folic Acid Deficiency:**

Pteroyl glutamic acid (PGA), also known as folic acid, is converted to derivatives which act as co-enzymes in reactions concerned with the utilization of the one-carbon moiety, and also stimulates the synthesis and utilization of methyl groups.

A folic acid deficiency can be induced in an animal by a deficient diet or by an antimetabolite. Antimetabolites have played an important role in the study of embryonic effects of dietary deficiencies by rapidly inducing a specific virtual deficiency, wherein the metabolite is present but blocked in its activity. A deficiency of this sort can be terminated by the administration of the metabolite in sufficient amounts, thus causing a transitory deficiency very useful in studying the effects of dietary deficiencies.

Nelson and co-workers employed both the standard deficiency and that induced by antimetabolites in studying the effects of maternal folic acid deficiency on fetal development. Early experiments (Nelson and Evans, 1947; Nelson and Evans, 1949) involved placing female Long Evans rats on a PGA-deficient diet containing 1% succinylsulphathiazole (SST)
to inhibit intestinal synthesis of PGA. The deficient diet was instituted from 3 months to zero days before the day of breeding. It had no effect on reproductive performance when it was begun on the day of mating. The frequency of females completely resorbing their litters increased, however, as the interval between the time of initiation of the deficient diet and the day of breeding increased, and those females giving birth showed a reduced mean litter size. A maximum of 44% completely resorbed litters was observed when females had been on the deficient diet for 2 months previous to mating. A longer interval on the deficient diet showed no further impairment of reproductive performance.

Transitory folic acid deficiency was then induced by placing Long Evans females on a deficient diet containing SST and X-methyl folic acid, a crude folic acid antagonist (Nelson, Asling and Evans, 1952). Controls, which received the same diet supplemented with synthetic PGA, had litters showing no abnormalities. Animals were introduced to the experimental diet from day 7 to day 15 after breeding and maintained on it for the remainder of gestation until they were killed on day 21. Instituting the deficiency 7-9 days after mating produced 100% fetal resorption. When the diet was begun 15 days after breeding all the young were normal. However, feeding the diet 10-13 days after breeding, during a period of active embryonic organogenesis, resulted in multiple skeletal and visceral malformations. All the fetuses were dead at term when the deficiency was initiated on day 10 or 11, and the number of dead and abnormal young decreased as the day of initiation of the diet was advanced to day 15.
Nelson and co-workers (Nelson, Wright, Baird and Evans, 1956) also investigated the effect of a 36-hour period on the deficient diet on fetal development to determine the critical period for the deficient diet to interfere with fetal development. The peak period of susceptibility was on days 8-9½ when the deficient diet caused 65% resorption and 15% abnormalities among the fetuses. It must, however, be stressed that the period of deficiency does not correspond to the period that the deficient diet is introduced, in such experiments, as the initiation of deficiency and its length are determined by the amounts of food that experimental animals eat before, during and after the deficient diet is introduced.

Thiersch and Philips (1950) used the potent PGA antagonist, aminopterin (4-amino-PGA) which offered the advantage of inducing an immediate virtual deficiency of PGA. Pregnant Sprague-Dawley and Wistar rats and CFW mice were maintained on a normal diet throughout the experiment, and administered 3 doses of the antimetabolite during the first week of gestation, starting at the time of implantation. Much fetal death and resorption resulted, but no malformations were observed. Embryos older than 10 days were more resistant to the deficiency. Histological studies showed that the site of the drug's action was embryonic mesenchyme; the placenta and decidual tissues were normal in most cases.

The action of aminopterin administered orally was later studied by Thiersch (1952) in human patients requiring therapeutic abortions. It was suggested that a massive PGA deficiency was either teratogenic or incompatible with fetal life, and that the more advanced the pregnancy, the greater the amount of antagonist required to cause abortion.
Further studies on the effect of aminopterin on fetal development (Kinney and Morse, 1964) involved feeding Holtzman strain pregnant rats a PGA-deficient diet with 1% SST on days 9 and 10, administering aminopterin intraperitoneally on day 10. At all other times, the rats were fed a stock diet. Single doses of aminopterin, if 200 µg or above, caused complete resorption of embryos; step-by-step reduction of the analogue permitted more of the fetuses to survive and these showed a low number of congenital defects. Observations made by other workers, to the effect that fetuses either died early or survived 21 days with no conspicuous defects, or that there was a tendency for entire litters to resorb or to survive with normal development, were not confirmed. In this study, there were many instances where a fraction of the implantations yielded surviving young. When PGA was administered at various levels one hour prior to the injection of aminopterin, its effect was partially reversed. It was observed that fetal liver DNA was depressed after injection of high levels of PGA but RNA was unaffected. The possible toxic effects of large doses of PGA were also investigated; injection of 150 µg/kg or 225 µg/kg PGA caused 6.1% and 2.5% abnormal offspring, respectively, while it had been previously observed that 225 mg/kg PGA caused 50% death in mice.

Working with Long Evans rats, Johnson et al. (1963) fed pregnant females a purified PGA-deficient diet containing the antimetabolite, 9-methyl-PGA, for a 48-hour period, from days 8-10, followed by a PGA-supplemented diet from day 10 to autopsy. With a low level of the antagonist (10 mg %) in the diet, 18% of the embryos were dead on day 11,
65% on day 12, and 100% on day 13. Raising the level of 9-methyl-PGA to 80 mg % did not hasten embryonic death significantly. Among controls receiving a PGA-supplemented diet throughout, embryonic mortality was 2%. No morphological differences were observed between control and PGA-deficient embryos at 9 or 9½ days; at 10 days, deficient embryos showed retardation of growth and development and decreased mitosis, especially in the neural epithelium. By 11 days, deficient embryos showed marked retardation or anomalies in the cranial portion of the neural tubes; these were increasingly severe by day 12, and moderate retardation was observed in other areas. Furthermore, at that stage, many were already dead. It seemed, since the cranial tissues are developing rapidly at that time, that the sensitivity of a tissue to PGA-deficiency is related to the rate of its growth. Placental changes were noted only after embryonic death, and were the same as those observed after embryonic destruction by trauma on day 12. It was suggested, therefore, that the changes in the placenta were of a secondary nature.

Investigating the role of PGA in human pregnancies, Hibbard (1964) ascertained the PGA status of 87 patients who aborted at 8-25 weeks of gestation without apparent cause. Twenty-five of the 87 showed excessive excretion of FIGLU (formiminoglutamic acid), a breakdown product of amino acid histidine, which accumulates in the absence of PGA. Only 3 out of 50 control patients showed excessive FIGLU excretion. Twenty-two percent of isolated abortion cases as compared to 41% of recurrent abortion patients had positive FIGLU tests. The deficiency of PGA was found to be aggravated in patients due to its high demand in pregnancy. Dietary
intake was found to be a slight influence only. In most patients, there was an underlying defect of PGA metabolism, either preceding or precipitated by pregnancy. In some of these cases, it was observed that intestinal malabsorption was causing the inadequacy. Malutilization of available PGA was also observed: a metabolic block was indicated by a low serum folate level in some patients, indicating that conversion to the biologically active folinic acid had not taken place. Certain anticonvulsant drugs and sulphonamides were also found to be blocking the action of PGA by competitive inhibition, in a manner similar to aminopterin.

Pantothenic Acid:

Pantothenic acid is a constituent of coenzyme A, and as such is essential for the initiation of the tricarboxylic acid cycle and is involved in lipid metabolism, amino acid activation, and the conversion of acetic acid to cholesterol and steroid hormones.

When it was observed that folic acid deficiency induced a secondary pantothenic acid deficiency, the question was raised whether the effects of folic acid deficiency were actually due to a secondarily-induced pantothenic acid deficiency (cited by Kalter and Warkany, 1959). By adding graded amounts of calcium pantothenate to a pantothenic acid-deficient diet, it was found that the presence of less than 10 µg of pantothenic acid daily caused 100% embryonic resorption. As more pantothenate was added to the diet above that amount, the frequency of resorption was reduced. A minimum of 50 µg daily was required for completely
normal development.

Nelson and Evans (1946) investigated the role of pantothenic acid in reproduction of rats. Placing pregnant females on a pantothenic acid-deficient diet on day 13 of gestation had no effect on reproductive performance. If the diet was started immediately after mating, however, 33% of the females completely resorbed their litters. Starting the deficient diet 16-23 days before mating caused 33% of the females to show no signs of implantation. Of those females that did implant, 50-100% resorbed their litters, depending on the diet that was used.

It was shown (Giroud, Lefebvres and Dupuis, 1956) that increased levels of ascorbic acid partially compensate for a pantothenic acid deficiency. Pregnant albino rats placed on a deficient diet 3-14 days before mating and maintained on it throughout pregnancy showed 55.1% resorption and 22.6% abnormalities among implantations. Females receiving daily subcutaneous injections of ascorbic acid during pregnancy did not differ significantly from those which did not receive this additional treatment. When females on this regimen received 10 mgm calcium pantothenate daily from 7-18 days before mating and throughout pregnancy, there was 36.8% resorption and 20.4% abnormalities. When, in addition to the deficient diet and the analogue, females were administered 250 mgm ascorbic acid daily, the frequency of resorption was reduced to 22.7% and that of abnormalities to 15.3%. Thus, there was a strong suggestion that pantothenic acid in the presence of ascorbic acid is more beneficial to the pantothenic acid-deficient embryo than the B vitamin alone.
The teratogenic effects of a pantothenic acid deficiency in Long Evans rats were analysed by Nelson et al. (1957). A deficient diet was imposed from up to 20 days before mating until the end of gestation. A decrease in the number of females carrying litters to term and in the average litter size was observed as the interval between initiation of the deficient diet and mating increased. As the length of the deficient period increased, fetal abnormalities rose to a peak of 29% when the deficient diet was begun 4 days before mating, then dropped as resorption increased, until there was 100% resorption within litters when the deficiency began 20 days before conception. The effects of the analogue, omega-methyl-pantothenic acid, were then tested by initiating the deficient diet on the day of conception and administering the antimetabolite for 2-4 days during pregnancy. When the analogue was administered from days 9-11 or 9-12 inclusively, 27% of the pregnant females resorbed their litters and there were 15% abnormalities. When the antimetabolite was added on days 10-13 or 11-14, 25% of the litters were resorbed, compared to 0% when the antimetabolite was not added to the regimen.

Other Vitamin Deficiencies:

Several other members of the vitamin B complex besides folic acid and pantothenic acid have an effect on reproduction. Among those that will be discussed are thiamin, riboflavin and pyridoxine. Other vitamins, such as C (ascorbic acid), E (tocopherol) and A will be discussed in the category of antifertility factors.

Nelson and Evans (1948 and 1951) studied the effect on repro-
duction of shifting female rats to pyridoxine-free diets. The effects were slight when a deficient diet was introduced on the day of breeding or 1-2 months before, and were not increased when 1% succinylsulphathiazole was added to the diet to suppress intestinal production of pyridoxine. A potent pyridoxine analogue, desoxypyridoxine, was therefore added to the regimen of pyridoxine-deficient diets initiated 20, 15, 10 or 0 days before breeding. Controls were, in addition, fed 0.5 mgm% pyridoxine on the day of breeding after being fed the deficient diet and antagonist for 10-20 days. Female Long Evans rats showed 10% resorption among litters after being introduced to the experimental diet on the day of mating. The frequency of resorptions increased directly with the length of the interval prior to mating that the rats had been on the experimental diet: when the deficiency period had been 12 days, the resorption frequency was 36%, when it had been 16 days, 75%, and when 22 days, 100%. In the group that had been deficient 22 days prior to conception, failure of implantation occurred in 29% of the females. Whereas no young were born dead in the control group, a peak of 21% young born dead occurred in the group deficient for 16 days prior to breeding. Furthermore, all the deficient groups showed a tendency towards a decreased number of young per litter. A decreased food intake was associated with the pyridoxine-deficient animals, but the use of pair-fed controls showed that this decrease did not interfere with pregnancy of pyridoxine-deficient animals. Normal reproductory behaviour of rats supplemented with pyridoxine the day of conception after 2 weeks' deficiency showed that the vitamin counteracted all deleterious effects of the antagonist.
Thiamine-deficient diets were also investigated in their effect on the reproduction of female Long Evans rats (Nelson and Evans, 1955). The deficient period was initiated on the day of mating or from 6 to 22 days prior to that time. As the deficient interval before conception increased, a corresponding increase was observed in the resorption rate and the rate of failure of implantation, while the maternal weight decreased. In order to determine whether a reduction in maternal food intake had an effect in the performance of rats on a thiamine-deficient diet, pair-fed controls were run. The controls on the limited stock diet showed impaired reproductive performance. Decreased food intake may therefore have been a major factor causing reproductive impairment on the thiamine-deficient diet.

Giroud, Levy and Lefebvre-Boisselot (1950) investigated fetal response to a maternal riboflavin deficiency in rats. They measured riboflavin levels in fetal liver, maternal liver and muscle, and in the placenta. Following a maternal riboflavin deficiency, a decrease of 51.7% in the riboflavin content of fetal liver, 44% in the placenta, 34.8% in the maternal liver and 37.9% in the maternal muscle were observed. They concluded that the fetus is unable to maintain a normal level of riboflavin in the face of a maternal deficiency. A riboflavin-deficient diet initiated on the day of breeding and continued through gestation had no effect on female Long Evans rats (Nelson, Baird, Wright and Evans, 1956). Addition of galactoflavin, a competitive analogue, to the diet caused resorption, malformation and decrease in maternal weight gain in direct proportion to the amount of analogue added. In the group fed the
maximum amount of galactoflavin, 200-216 gms per kg diet, only 3% of the females produced litters. Supplementing the experimental diet with 216 gms/kg riboflavin caused all females to produce litters. Exposure of pregnant females to galactoflavin on certain days of gestation, followed by a change to a riboflavin-supplemented diet for the duration of pregnancy, showed that the number of females producing litters was inversely proportional to the length of exposure to the analogue. Of those females exposed to galactoflavin from days 7 to 9, 100% produced litters; of those exposed from days 7 to 11, 67% produced litters, while only 50% of those exposed from days 8 to 13 produced litters. In the second and third groups, 32% and 68% of fetuses exhibited abnormalities, respectively.

Pratta, Zak, Greengard and Sigg (1964) observed the effect of a diet deficient in nicotinamide, niacin and tryptophan on pregnant rats. When administered from day 1 to day 13 of gestation, such a diet caused death and resorption of almost all fetuses; only 3.9% were viable, as compared to a control value of 94.1%. It was also observed that treatment with the tranquilizers, chlorpromazine and imipramine, caused the fetal viability rate in rats on the deficient diet to increase to 81.4%. The administration of nicotinamide to pregnant animals on the deficient diet also gave normal fetal viability rates, indicating that the effect of the deficient diet was due to the lack of NAD precursors. The concentration of NAD in the livers of rats on diets deficient in NAD precursors was lower than in controls, and the fetuses failed to survive.
Treatment with tranquilizers prevented both the decrease in NAD level and the fetal mortality. These findings supported the possibility that the maintenance of near-normal NAD levels in chlorpromazine-treated rats was responsible for fetal survival.

The work of Pinsky and Fraser (1960) and Goldstein (1964) on nicotinamide deficiency caused by the analogue, 6-amino nicotinamide, has been reviewed in the Introduction. Goldstein's results following treatment of pure strain C57 BL mothers do not indicate any sensitivity of embryos in that strain to the nicotinamide analogue between days 8½ and 14½ of gestation. The difference between the A and C strains in fetal sensitivity to 6-amino nicotinamide are apparently not due to differences in available niacin and nicotinamide pools in the two strains (Rosen, J., personal communication).

Vitamin C-deficient diets in guinea pigs have been shown to prevent pregnancy when supplemented with less than 3 cc of orange or tomato juice daily. With supplements of 5 cc of juice, abortions or resorptions occurred (cited in Kalter and Warkany, 1959). No malformations were observed among the few live young that were delivered.

A deficiency of vitamin E (a compound which behaves as an antioxidant in vitro), was first recognized because of its effect on the reproductive organs of the rat. In the pregnant female, the fetuses died and were resorbed, apparently because of maldevelopment of the fetal mesoderm, after the first week of gestation (Bell, Davidson and Scarborough, 1963). Fetal resorption, it was observed, could be prevented
by giving vitamin E (tocopherol) during the first week of pregnancy.

Cheng and Thomas (cited by Kalter and Warkany, 1959) bred female rats of Sprague-Dawley origin that had been maintained on a vitamin E-deficient diet from weaning to maturity. Such animals showed 100% fetal resorption. A single dose of 1.2 mg d,l-alpha tocopherol administered to the pregnant animals by stomach tube at various stages of gestation, however, showed fetuses with abnormalities on day 21. If the vitamin was administered before day 9, all young were normal; it was only when the vitamin was given between days 9 and 12 that abnormalities were produced.

King (1964) investigated the comparative effects of feeding 3 antioxidants on gestational performance of Holtzman albino female rats fed a vitamin E-deficient diet. The 3 antioxidants tested were N, N'-diphenyl-p-phenylenediamine (DPPD), N-propyl gallate (NPG) and 1, 2-dihydro-6-ethoxyl-2,2,4-trimethylquinoline (EMQ). Animals on the vitamin E-deficient diet showed 100% fetal resorption; controls receiving 2 mg vitamin E on day 10 yielded normal young, abnormal young, dead fetuses and resorbed fetuses at 8.5, 15.0, 2.0 and 74.5%, respectively. Positive controls receiving 10 mg tocopheryl during the first 5 days of gestation yielded 100% normal live fetuses. When the antioxidants were added to the deficient diet at a 0.025% level, DPPD gave the best results, with 93.4% normal young and 6.6% resorption. When experimental groups were supplemented with vitamin E, however, EMQ gave the best results. At higher levels of antioxidants (0.05%), DPPD-supplementation
yielded better gestational performance in all groups. At lower levels of DPPD, however, there seemed to be an antagonistic action between vitamin E and DPPD. EMQ was generally found to be of intermediate potency as a substitute for vitamin E, and NPQ was the least potent. The action of vitamin E and other antioxidants was considered to be one of protecting against damage to cellular and subcellular membranes and widespread metabolic derangements, by donating hydrogen to a lipid peroxyl radical and breaking the chain reaction of peroxidation.

It has been reported that rats deficient in vitamin A alcohol always resorbed their litters. Similarly, female Hooded rats, maintained on a diet in which vitamin A alcohol had been replaced by vitamin A acid, conceived when mated but always resorbed their fetuses (Howell, Thompson and Pitt, 1964). The earliest detectable lesion was necrosis of cells in the placental labyrinth and junctional zone, which was seen on day 15 or 16 of pregnancy, and was associated with an apparently healthy fetus. The fetal surface of the placenta was next seen to become necrotic by day 17-18 of gestation, and by this time the fetus was always undergoing resorption. These lesions, being closely similar to those found in vitamin A-deficient pregnancies, were considered themselves to represent the uncomplicated lesions of vitamin A deficiency. The similarity was noted between these lesions and the lesions observed at the periphery of the placental disc and associated with infertility in the Albany strain of rats (Wolfe et al., 1940), in rats on a fat-free diet and in experimental brucellosis of rats. It was suggested that the placenta may be able to react in only a limited number of ways, and that different agents may produce a similar lesion.
Protein and Diet:

Schultz, Schultz and Conn (1960) reported the sequence of placental and embryonic changes resulting from injections of ethionine, a methionine antimetabolite, during the second week of gestation in the rat. Destruction of the allantoic mesoderm was followed by necrosis and degeneration of the labyrinth and other layers of the placenta. Necrosis of the embryo was observed next, followed by disappearance of the yolk sac and degeneration of the giant trophoblast cells. It did not seem that ethionine had a selective effect on any particular region of the embryo.

Hazelwood and Nelson (1965) observed that more than 85% of adult female rats mated and placed on diets containing less than 6% protein during the first 8 days of gestation failed to carry viable litters to term. Pregnant rats on low protein diets were shown to have reduced ovarian steroid secretion secondary to a marked reduction in gonadotrophin release from the anterior pituitary. It was observed that simultaneous injections of estrone and progesterone supported pregnancy of rats on protein deficient diets; this further supported the observation of hormonal imbalance as the result of protein deficiency. The injections of ovarian steroids, which were administered from day 3 to day 20 of gestation, probably maintained pregnancy in rats on a casein-free diet by the transfer of maternal protein from skeletal muscle to the fetus, since both the total size and protein concentration of maternal skeletal muscle were decreased in response to the injections of estrone.
and progesterone. It was further shown that protein-depleted rats required ovarian steroid injections only on days 5-9 to achieve successful littering. Since placental development in the rat occurs during days 7-9, it was suggested that the role of the exogenous steroids was closely associated with initiation of placental organization.

Drugs and Native Biological Compounds

Cortisone, an adrenal steroid with a wide range of physiological activities, was observed by Kalter (1954) to interact with genetic factors to reduce fertility. This observation was made during the treatment of pregnant mice of the A/Jax and C57BL strains. The value for fertility, taken as the proportion of copulations that resulted in palpable pregnancies, that Kalter obtained was reduced by the cortisone treatment in the A/Jax, but not in the C57BL, strain.

A detrimental effect of cortisone on pregnancy in rabbits was also reported by Courrier (1951). Daily injections of 25 mg cortisone were administered 4-7 times between days 10 and 23 of gestation. Some rabbits were observed to abort with separation of the placentas, while hemorrhage occurred in others. After treatments between days 10 to 14, fetuses were resorbed, those that were still living being reduced in size and having pale placentas. Injections between days 15 and 21 yielded only macerated fetuses, greatly reduced in size.

Another hormone, progesterone, has also been observed to be toxic to mouse fetuses in several instances (cited by Petrelli and
Large amounts administered to pregnant mice are followed by fetal death, especially when administered in later stages of gestation. Eclampsia-like conditions in pregnant rats and embryonic mortality have also been observed after progesterone treatment, while mouse blastulae have been noted to succumb when the concentration of progesterone in the culture medium was 8 μg/ml or more. Petrelli and Forbes (1964) performed experiments to investigate whether fetal death in mice was caused by an effect on the mother or placentas or by direct action on the fetus. Brown Belt stock female mice were treated on day 15 of gestation by direct injection of progesterone in sesame oil into the amniotic fluid. Control mice received one of 3 treatments: injection of oil alone into amniotic sacs, injection of oil into amniotic sacs accompanied by maternal subcutaneous injection of progesterone, or no treatment. Those fetuses receiving injections of oil alone were observed to have a 13% mortality rate, compared to 2% among untreated controls, therefore trauma, infection or the oil itself were causing some fetal resorption. Progesterone injected directly into amniotic sacs caused 61% fetal mortality, and only those embryos that had been injected died. When progesterone was injected subcutaneously into the mothers, a fetal mortality of 65% was observed, involving a large number of whole litters dying. Since the mortality rate was approximately the same whether the progesterone was injected into sacs or into mothers, it was concluded that progesterone was toxic to late mouse fetuses whether it reached the embryos directly or through the maternal circulation, and caused death by direct action on the fetus.
The response of pregnant rats to tetracycline injections was studied by Steiner and co-workers (Steiner, Bradford and Craig, 1965). Intraperitoneal injections of 85 mg/kg tetracycline were administered to Sprague-Dawley derived rats daily for 5 days beginning on day 14 of gestation. Eight out of 12 females had 1-8 abortions or dead fetuses in utero when killed 24 hours after the last injection. In aborted fetuses, the placentas showed varying degrees of degeneration and involution, and abnormal placentas were also observed in treated animals with 100% viable fetuses or attached to live fetuses with dead litter-mates. Altogether, 75% of placenta showed degenerative changes after tetracycline treatment for 5 days. In groups treated for 3 and 4 days, 60% of the placentas showed changes, and one placenta showed early changes after 2 days' treatment. Histological examination showed abnormalities of the placentas to occur chiefly in the labyrinth, and especially in the trophoblast cells. These degenerative changes in the placenta suggested that tetracycline caused abortions by a direct toxic effect on the trophoblast cells of the labyrinth and basal areas, presumably by one or more of its known biochemical effects, blocking of protein synthesis, uncoupling of oxidative phosphorylation or binding of divalent cations.

Evidence has been presented implicating histamine as a factor in the process of implantation. Its possible role was investigated further by Banik, Kobayashi and Ketchel (1963), who treated female Sprague-Dawley rats and white Swiss mice for 6 days starting on the day of conception. Eight treatments were involved: aminoguanidine (subcutaneously),
aminoguanidine plus terramycin (in drinking water), histamine (intraperitoneally), histamine plus aminoguanidine, aminoguanidine plus histidine (by feeding tube), histidine, histidine plus terramycin, or compound 48/80 (intraperitoneally). Control animals were administered the vehicle without the drug. The administration of all the treatments significantly increased the number of non-pregnant mice observed, whereas no effect was seen in rats at the same relative doses, apart from a reduction in the average number of fetuses per pregnancy at high dosage levels of aminoguanidine, histamine and histamine plus aminoguanidine. These treatments all effectively altered the uterine levels of histamine by altering the concentration of circulating histamine. Aminoguanidine plus histamine resulted in a net increase in titre of circulating histamine; systemic injection of compound 48/80 depleted the number of uterine mast cells from which uterine histamine is apparently derived, and thus reduced the level of uterine histamine. In contrast to the rat, implantation in the mouse was affected by all treatments designed to alter the animal's histamine level. The administration of aminoguanidine, histamine or histidine, all of which tended to increase histamine levels, were as effective in reducing the number of successful pregnancies as compound 48/80, which tended to deplete tissue histamine. These data indicated that, in the mouse, any change in histamine level is detrimental to the establishment of successful pregnancy, interfering specifically with the process of implantation.

A variety of other highly diverse substances have also been observed to increase fetal mortality by interfering at any one of the
susceptible sites. The carcinogenic hydrocarbon, benzpyrene, was observed (Rigdon and Rennels, 1964) to have a deleterious effect on embryonic development, specifically, and did not interfere with ovulation, fertilization or implantation, although a possible effect on lactation was observed. Its main effect after feeding to pregnant rats was death and resorption of fetuses, without causing malformations.

Administration of acetyl salicylic acid in food (1.2 gm per kg body weight) from day 6 to term was observed to cause 81.8% stillbirths in mice (Klein Obbink and Dalderup, 1964a). Of these stillbirths, 73.3% were macerated, while no maceration was observed in any of the 16.7% of young that were stillborn in the control group. Rats administered 0.6 gm acetyl salicylic acid from day 6 to term showed a striking weight loss of about 26 gm., in contrast to mice. All of the implantations in 11 treated animals were in an advanced state of resorption, while there were no fetal deaths among control animals. Klein Obbink and Dalderup (1964b) tested the theory that the embryonic mortality after treatment with acetyl salicylic acid was caused by uncoupling of oxidative phosphorylation in the maternal system. This was accomplished by injecting dinitrophenol for 1-18 consecutive days from the third to the last day of gestation. It was observed, however, that only a few fetal deaths occurred when mothers received maximum tolerated doses of DNP, and uncoupling of oxidative phosphorylation as the mode of action whereby acetyl salicylic acid caused fetal death was therefore rejected.

Spector (1961) investigated the effect of compound 1275, an
inhibitor of monoamine oxidase of low toxicity and with anti-inflammatory properties, on fertility in female Wistar rats. Subcutaneous injection and administration in drinking water were both found to reduce fertility by interfering with implantation of the fertilized ovum. Since implantation involves vascular changes that resemble those occurring in acute inflammation, the suggestion was made that the drug's antifertility action is related to its anti-inflammatory properties, especially the suppression of increased capillary permeability by inhibition of the inactivation of a vasoconstrictor amine.

Treating Wistar rats with trypan blue during pregnancy, Beck and Lloyd (1963) clarified the relationship between fetal death and fetal malformation that exists in relation to this and probably certain other teratogens. Injections were made on day 8½ of gestation, and animals were killed on either day 11½, 14½ or 20½. Controls showed a fetal resorption rate of 4%. It was found that, for all 3 times of killing, the percent of normally developed animals was fairly constant, while the frequency of resorption, which had been about the same as control values when animals were killed on day 11½, rose sharply as gestation advanced. The rise in resorption was reflected by a corresponding fall in detectable malformations. It was also found that the same doses of trypan blue administered after the period of fetal organogenesis resulted in less than 10% embryonic mortality. The results indicated that resorption as a result of teratogenic doses of trypan blue at any stage of gestation was secondary to fetal malformation. Since experimental malformations produced by any means are generally accompanied by increased
embryonic death, it was suggested these conclusions are widely applicable.

**Radiation**

Matings of male mice during an interval of a few weeks following X-irradiation yield litters of reduced sizes. Bateman (1958), in the discussion of his X-ray data, regarded this as the effect of dominant lethals involving heterozygosity for translocations and single unrestituted chromosomal breaks. The time of death of such dominant lethal embryos arising after paternal irradiation indicated that all embryos with one lethal break died after implantation, while about one quarter of embryos with more than one lethal implanted before dying, the rest dying before implantation.

Peters (1963) investigated the effect of a 20r dose of X-irradiation during early postnatal stages on subsequent fertility in female mice. The reproductive performance of treated mice, all of which showed impairment relative to controls, were observed throughout the reproductive life span. Mice irradiated on the day of birth produced 72% of the normal complement of young, and this was reduced after treatments 7 or 14 days after birth until females irradiated at 21 days of age showed maximum impairment of reproduction by producing only 1% of the normal number of young during their reproductive life span. Treatments at the age of 28, 35, 42 or 49 days showed that radiation sensitivity of the ovary decreased after maximal sensitivity at 21 days, since mice
irradiated at 42 days of age or later again produced about 72% of the normal number of young. A close correlation was demonstrated between the number of growing and large oocytes in the ovary at the beginning of reproductive life after irradiation, and the reproductive capacity subsequently seen. No correlation was observed between reproductive capacity and total oocyte population in the ovary, indicating that X-irradiation impairs reproduction by arresting oocytes in the small and immature stages.

The effect of 50 r of X-rays on female mice the day prior to mating was studied by Ehling (1964) in 5 strains of mice. A degree of permanent sterility was caused by rapid destruction of oocytes in immature follicles. There were marked strain differences in the number of offspring produced during the reproductive span of females of the different strains. The differences possibly reflected strain differences in the frequency of oocytes which are present in an immature stage in the ovary and/or differential radiosensitivity of oocytes. A shortening of the breeding period was evident in treated mice, as well as an increase in the time between litters and a rapid decline in the size of successive litters, due to a high prenatal mortality in some strains.

Substerilizing irradiation of 800 r was applied to the exteriorized left ovary, to both ovaries, or to the left ovary after right ovariectomy in rats in a study on implantation by Krehbiel and Plagge (1963). The females were either mated immediately after irradiation or 10-30 days later, and were killed on day 8 or 9 after breeding. In
animals with one normal and one irradiated ovary, a normal number of implantations, 84.7%, was observed in the first 7 pregnancies, with the normal ovary compensating for the loss of ovulation by the irradiated ovary. Bilateral irradiation yielded only 21.6% fertile matings, with a marked decrease of implantations evident in the second and third pregnancies. Irradiating the left ovary in the absence of the right one showed a compensation effect by producing more implantations; however, the total period of production was limited to 31 days after treatment, and there was only an 8.6% frequency of fertile matings.

Rönnbäck (1965) observed that continuous very low irradiation during gestation had no effect on mice of the CBA strain. A dose of 5 r on day ½ after conception had been observed to delay cleavage, but gamma-irradiation begun within the first 12 hours of the zygotes' life, and continued through gestation to a total dose of 170 r over 20 days did not affect litter size, intrauterine death, or the subsequent fertility of the young.

Ohzu (1965) in his experiments involving low-dose X-irradiation in early mouse embryos, obtained different results. He exposed Fl hybrids from a dd/Mk x CBA/Mk cross to 5, 15 or 25 r of X-rays half a day or one and a half days after mating had occurred. Autopsies on day 18½ of gestation showed an increase of resorbed fetuses in irradiated groups, the frequency varying with the dose, and slightly lower after irradiation on day 1½ (20.5 - 27.2%) than on day ½ (25.6 - 38.8%). The number of malformations not being statistically different from that found in
in control animals, it was concluded that the effect of low-level irradiation on early mouse embryos was manifested mainly by an increase in embryonic mortality.

X-irradiation of rabbit blastocysts in one uterine horn was carried out with 150 or 200 r at 3½ days of gestation by Inman and Markivee (1963), and comparisons were made at 9½ days with litter-mates in the untreated horn. Pre-implantation mortality was increased by 11.1% in the irradiated horns after both doses of radiation.

The effect of small radiation doses at a later stage of development was studied in the mouse by Jacobsen (1965). Whole-body irradiation was carried out with doses of 5, 20 or 100 r on day 7½ of gestation. Litter size was observed to be fairly constant in controls and those mice receiving 5 or 20 r doses, while treatment with 100 r caused reduced litter size and a high incidence of abortion. A seasonal variation in radiation susceptibility was evident such that abortion occurred in all groups during the winter, while in the summer, it was observed only among mice receiving 20 or 100 r doses of radiation.

Wilson (1954) employed the technique of direct irradiation of certain segments of the uterus to study the reaction of Wistar rat embryos to radiation. After irradiation on the ninth, tenth or eleventh day, mortality increased with dose, but this relationship was not apparent after eighth-day irradiation. At that stage, there was no increase in mortality until a 200 r dose was used; this treatment killed all embryos within 3 days. It appears that some threshold phenomenon must be involved
in the resistance of eight-day embryos to radiation that is not present in older embryos. Another observation was that susceptibility to the lethal effect of X-rays decreased with advancing age of the embryo, such that 200 r killed all day 8 embryos in 3 days, while 400 r was required to kill day 9 and 10 embryos and 600 r to kill day 11 embryos. If, however, embryos were examined more than 3 days after treatment, it was observed that lower doses effected total mortality.

The changing radiation response of developing mouse embryos was analyzed through gestation by Russell and Russell (1954). Single doses of total body irradiation ranging from 25 to 400 r were given to C57BL females, previously mated with NB males, on day \( \frac{1}{2} \) after fertilization or at one of the successive 24-hour intervals. Irradiation during the first phase of gestation, the pre-implantation period of the embryo, caused a high incidence of prenatal mortality but showed an almost complete absence of abnormalities among survivors. The second phase of gestation was marked by a decreasing susceptibility to prenatal death and peak sensitivity to neonatal death and congenital abnormality. This period corresponded to the time of major embryonic organogenesis, lasting from about day 5\( \frac{1}{2} \) to day 13\( \frac{1}{2} \) of gestation. After that point of development, susceptibility to X-rays, measured by pre- or neonatal death and abnormalities, dropped to low levels. Prenatal loss of litters was observed after 200 r and perhaps 100 r delivered between days \( \frac{1}{2} - 4\frac{1}{2} \). Irradiation after implantation (day 5\( \frac{1}{2} \)) did not show any significant difference between irradiated litters and controls with doses up to 200 r during organogenesis and up to 400 r later in gestation.
Infectious and Metabolic Diseases

Some diseases cause fetal death as an added effect of a severe pathological process in the maternal animal, but other infections interfere preferentially in the reproductive process. Diseases of the latter type are caused by infectious organisms such as Vibrio fetus, Trichomonas fetus, and Brucella abortus. These organisms frequently cause death of the fertilized ovum and embryo (Hanly, 1961). Vibrio fetus is a common cause of abortion in sheep, goats, and cattle. The disease process interferes with placental circulation, resulting in fetal damage. Infection has been found to occur through ingestion of contaminated food and water, but transmission, which is strictly venereal, occurs during mating. The male, who is the carrier, may maintain the organism indefinitely in the testis; during pregnancy, the transmitted organism causes suppuration and necrosis of the fetal membranes. The resultant abortion probably results from fetal asphyxia due to inflammation of the chorion. Vibrio fetus was recently recognized as a human pathogen, and 8 cases isolated during pregnancy were documented by Eden (1966). Of these 8 cases, two pregnancies terminated in spontaneous abortions and 4 in neonatal deaths, three of which had meningoencephalitis, one being a premature delivery. Two children survived; one of these was also premature. Five of the 8 placentas were examined; all were described as necrotic. These histories of vibriosis therefore suggested that the pathogen, when infecting human pregnancies, produced some placental lesions similar to these found in domestic farm animals which resulted
in cases of spontaneous abortion and prematurity.

Experimental brucellosis of rats was described by Howell et al. (1964). Intraperitoneal injection of *Brucella abortus* on day 12 or 13 of gestation was followed two to three days later by large areas of necrosis in the placenta. The maternal placenta was normal except where it adjoined to fetal tissues, indicating that the preferential target of the organism was embryonic tissue. Many organisms and migrating polymorphonuclear leucocytes were observed in the area. Abortion was usually observed shortly after necrosis had become well established. Yet another organism well recognized as acting against embryonic development is a virus of the psittacosis-lymphogranuloma venereum (PLV) group, which is known as the cause of enzootic abortion in sheep (Wilson and Dungworth, 1963).

Metabolic diseases, such as diabetes, have been shown to be associated with excessive intrauterine and neonatal deaths. When the disease and pregnancy were unattended, such deaths occurred in 30-40% of all diabetic pregnancies. Even with the most detailed attention, 10-20% of such pregnancies ended in perinatal death. Some reports suggested high fetal loss to commence during pregnancies occurring several years before the diagnosis of diabetes was made in the mother, but conflicting studies (Malins and Fitzgerald, 1965) have indicated that stillbirth rates more than 5 years before diagnosis (3.9%) were not significantly different from control values (4.1%). An excess of stillbirths, 13.8%, was observed during the 5 year period preceding diagnosis, but this may
have been due to delays in diagnosis, rather than representing a pre-diabetic effect.

Other studies on the effect of maternal diabetes on the developing embryo (Mayer and Camara, 1964) reported an increased incidence of toxemia, hydramnios and congenital abnormalities, as well as high rates of abortion and stillbirth. The etiology of all these changes is deranged maternal metabolism. Hypoglycemia in the first half of pregnancy and ketoacidosis in the second half lead to fetal endocrine imbalance. The severity of the maternal disturbance and the genetic and environmental situation of the fetus probably determine whether the combined effect will be severe enough to result in fetal death.

Maternal illness caused by acute viral infections may result in fetal death in the absence of overt fetal infection when the disease process is marked by high fever, systemic toxemia and a reduction in arterial oxygen saturation (Hardy, 1965). Older reports implicated smallpox as a cause of increased abortion, stillbirth and premature labour, and recent prospective studies suggest that vaccination during the first trimester may itself cause a significant increase in fetal loss by causing abortion or fatal transplacental infection of the fetus. Conflicting reports have been made with regard to varicella, which some studies have indicated as a cause of premature deliveries, stillbirths and neonatal deaths, while others have not. During the pandemic of 1918, pregnancy ended in abortion or stillbirth in 26% of uncomplicated influenza cases and in 52% of those complicated by pneumonia. The measles virus is another which is implicated in fetal loss in some reports and not in others.
Abortion and stillbirth have been reported with great regularity following mumps infection during pregnancy. The frequency of congenital abnormalities following maternal mumps is, however, not significantly increased above that of controls. Infection with the polio virus during pregnancy has also been reported to result in fetal wastage, as may fetal infection with rubella during the second trimester. Certain studies (Remington, Newell and Cavanaugh, 1964) have also indicated that chronic toxoplasmosis may also be a significant cause of abortion, prematurity and early postnatal mortality. Further evidence of the role of Toxoplasma gondii in fetal wastage was offered by the findings that 26 of 34 women seropositive for Toxoplasma had one or more abortions, and that 61% of 379 women with various histories of perinatal mortality had a positive skin test for toxoplasmin, compared with 30-39% in the normal population.

"Stressing" Factors

Olfactory block

Bruce (1959), studying conception after mating in non-inbred albino mice, found that pregnancy was blocked and implantation inhibited in nearly 30% of the females after the introduction of a strange male mouse within 24 hours of coitus. This inhibition of pregnancy was not found upon exposure of the mated females to their original mates or to strange females. Pregnancy was also blocked if the strange male was confined to a small cage within the female stock cage. Implantation was inhibited in over 70% of the females when wild-type males were used as pregnancy-blocking agents. In 15 females that mated with a strange male,
it was observed that all the young of the litter were sired by the second male.

Bruce and Parrot (1960) found that the implantation block to the first mating was virtually abolished after removal of the olfactory bulbs of the female mice.

A more extensive study, using 230 females, was designed to observe time relations in the pregnancy block (Bruce, 1961). It was found that nearly 80% of mated females experienced the block when exposure to strange males began within 48 hours of coitus, and that this frequency was reduced to less than 40% on day 5, while the reaction had totally disappeared by day 7 of gestation. The use of cages soiled by alien males was also observed to vary in effectiveness of pregnancy block (Parkes and Bruce, 1962). When such cages were used to house newly mated females and changed twice daily for 3 days, 83% of the females returned to oestrus within a week. Reducing the length of this treatment caused a decrease in pregnancy block. These results indicated the evanescent nature of the active substances, and supported earlier experiments which suggested that it was the smell of the alien males which blocked pregnancy. Bruce and Parkes (1961) found that this olfactory block had the maximum effect when the second male was of a different strain from the stud male, regardless of the female's strain.

Further study of this effect was carried out by Bruce (1963a), using P strain, Dutch, TO and CBA mice. More efficient blocking by CBA than by P males when they were tested with TO females suggested that the difference between odour "spectra" is greater between CBA and P strains
than between TO and P. TO females showed greater resistance to disturbance of pregnancies than females of other strains similarly exposed.

Stress imposed by olfactory stimulation was then compared to that imposed by fasting (Bruce, 1963b). Females of the P albino strain were mated to P or TO males. Those with vaginal plugs were housed 5-8 per cage, and subdivided into 3 groups. Controls were kept in a clean box with food; those testing olfactory stimulation were transferred to a box containing males of a different strain from the stud male, and those testing nutritional stress were transferred to a clean box containing water but no food. Test conditions were maintained for 1, 2 or 3 days. It was observed that olfactory stimulation on days 1, 2 and 3 following mating blocked 63% of all pregnancies. On the other hand, 48 hours' fasting blocked 92% of the pregnancies. Five of the 6 fasted females bearing litters delivered them 12-24 hours later than control mice, of which only 11% failed to become pregnant. Twenty-four hours' exposure to the two regimens tested revealed further differences. Olfactory stimulation on day 1 blocked 46% of pregnancies and fasting blocked 28%, while olfactory stimulus on day 3 blocked only 11% (the control value) and fasting blocked 54%. Mice fasted on day 3 that bore litters showed no effect on litter size and no prolongation of gestation. Olfactory stimulation of 24 hours' duration, in contrast to fasting, was found to have no effect on implantation and pregnancy on day 3 of gestation. From these observations, it was apparent that two distinct reactions were concerned, differing in chronology and perhaps in certain physiological aspects also. Olfactory block could therefore be seen not to involve
nutritional stress. It was slower to take effect than fasting, requiring 3 days of exposure to olfactory stimulation to show maximal effect, compared with 2 days required for fasting. Furthermore, maximum sensitivities to the two effects occurred at different times. The only direct effect of olfactory stimulation seemed to be inhibition of pituitary luteotrophic activity.

The stress of crowding was also examined (Bruce, 1963a). Increasing the number of strange males to which the mated female was exposed did not increase the incidence of pregnancy block, but the presence of other females reduced it proportionally to their numbers. Under inadequate conditions brought about by crowding, pregnancy failure was more than 3 times as frequent than under standard control conditions. Even though the presence of other females reduced the effect of strange males on pregnancy, as many as 8 females grouped together in the presence of a male did not maintain the pregnancy rate at the level found among controls. The olfactory influence of the male was therefore partially effective when competing with the odour of other females.

The mutual protection afforded by the presence of other females against the influence of the male was strong presumptive evidence that the mated females' response was the same as that of unmated females. That reaction is an increase in the production of prolactin by the adenohypophysis, presumably as the result of inhibition of the hypothalamus.

Fasting

As mentioned previously (Bruce, 1963b), nutritional stress in
pregnant P strain mice caused pregnancy block. Fasting 48 hours during the first 3 days of gestation allowed only 8% of mated females to bear young, and in 5 of the 6 litters born gestation was prolonged. Twenty-eight percent of all pregnancies were blocked by fasting 24 hours on day 1 of gestation, and the effect was increased on day 3 to block 54% of pregnancies after the same period of nutritional stress. Grouped females were observed to withstand fasting better than those caged singly; the presence of other females increased luteotrophic activity by the pituitary gland.

McClure (1961) also observed early embryonic mortality caused by fasting pregnant mice and rats for short periods. Pregnancy might have been terminated by hypersecretion of corticosterone or by a failure in gonadotrophic function of the hypophysis caused by the induced stress. Albino rats were used in experiments testing corticosterone hypersecretion as the cause of reduced pregnancy. Half the animals were adrenalectomized and administered desoxycorticosterone. Two 72-hour periods of fasting after mating separated by 48 hours of feeding reduced fertility in the animals to zero. A species difference was noted here, as mated mice gave that response after 2 48-hour periods. Adrenalectomy did not affect the proportion of rats that became pregnant, which suggested that the adrenal cortex was not involved to any great extent in the pathogenesis of infertility.

Virgin Ruakura mice were employed in testing failure of gonadotrophic function of the hypophysis as the cause of embryonic mortality after fasting. Animals fasted for 48 hours after the end of the third
day of gestation showed pregnancy to have terminated within the next 48 hours. Experimental fasted animals were administered progesterone (0.5 mg) or chorionic gonadotrophin (5 i.u.) daily during the period of fasting. These treatments protected most of the embryos for 2 days after those of fasted mice showed signs of resorbing and dying. Many of these pregnancies terminated between days 8 and 11 of pregnancy. When progesterone or chorionic gonadotrophin were administered in the same doses from day 3 to day 10 of gestation, pregnancies were normal till day 11, but most had degenerated by days 12 and 13. The dose of progesterone used in these treatments was less than that generally considered necessary for maintenance of pregnancy. When high doses of these substances (progesterone, 1.5 mg or chorionic gonadotrophin, 15 i.u.) were administered from day 3 to day 17 in the fasted mice, it was observed that none of the animals were normally pregnant, but that all embryos were in a state of degeneration. The observation that both progesterone and chorionic gonadotrophin administered separately reduced the effect of fasting supported the hypothesis that the embryonic mortality was caused by depression of hypophyseal function. However, they were not able to maintain normal pregnancy to term, indicating that the doses and methods used had not established the normal placenta-ovary relationship.

Audiogenic stimulation:

The effect of auditory stimuli before mating and during gestation was studied by Zondek and Tamari (1964) in rats. Exposure of male and female rats to auditory stimuli for 9 days prior to mating caused a decrease
in fertility from 70-80% in controls to 10-20% in experimental animals. The deleterious effect was not reduced 2-4 days after treatment was halted, but by 8 days, fertility was 50% of the normal, and was fully restored after 12 days.

The reduction in fertility occurred in males as well as in females. Treated males mated with untreated females were 11.2% fertile and treated females were 18% fertile.

Auditory stimulation was also found to disturb the course of gestation, acting as a pregnancy-block. Maximal effect was observed after stimulation between days 8 and 10 of gestation, when a 94% decrease in pregnancy rate was observed. After pregnancy was well established, auditory stimulation had no effect.

The infertility induced by stimulation before mating and the pregnancy block induced by stimulation during gestation could not be prevented by hormonal treatment during the period of auditory stimulation. Administering pregnant mare's blood containing FSH and chorionic gonadotrophic containing LH did not change the results, nor did treatment with hydrocortisone or vitamin E.

Temperature

Barnett (1961 and 1962) studied the effect of cold on the breeding of mice. Two inbred strains, A2G and C57BL, and a mixed stock arising from matings of 4 strains (A2G, C57BL, A and GFF) were bred for a number of generations at -3°C. Controls from the same strains were raised at 21°C. The only deliberate selection was for fertility in the hybrids raised
at the cold temperature. Higher fertility was observed in hybrids in both environments and all strains were less fertile at cold than at warm temperatures. Litter size at birth increased in hybrid mice over 12 generations in both environments, but no such change was observed in the inbred strains. Higher fertility was also observed in A2G and hybrid mice reared at control temperatures and transferred to the cold environment at 5 weeks of age than among mice of the same strain reared in the cold. It was also observed that, in both the A2G and C57BL strains, breeding began later and intervals between litters were longer in the cold environment than in the warm. Approximately the same number of offspring were produced and reared by A2G mice at the two temperatures, but breeding of control mice ended at about 40 weeks of age, while mice in the cold continued to breed until 80 weeks. C57BL mice, on the other hand, produced only half as many offspring in the cold as in the warmth: their breeding period ended at approximately 52 weeks of age in both environments.

Increase in temperature, as well as decrease, has been observed to affect fertility and the general stability of the maternal-fetal complex. Hanly (1961) cited the case of early season infertility in sheep, which was found to be largely caused by an increase in body temperature which included among its effects an increase in fetal mortality. The induction of hyperthermia in pregnant Wistar rats (cited by Kalter and Warkany, 1959), by placing them in incubators at several high temperature levels at various stages of gestation and for different lengths of time was also found to cause a high frequency of embryonic resorption.
During the disease process, it has been recognized (Hardy, 1965) that basal metabolism is increased in the presence of fever, and that oxygen requirements are increased by approximately 10% for each degree of elevation of body temperature. As a result, a body temperature of 104°F would result in an increase of 50% or more in oxygen requirements. Thus, the severity of any maternal illness would carry a proportional risk of fetal mortality.

**Intrauterine Conditions**

The effect of uterine crowding was investigated by Hafez in mice (1965a) and rabbits (1965b). It was observed that the uterine environment of C57BL mice was more favourable for development than that of DBA mice. Each uterine horn, however, was capable of maintaining only a limited number of viable embryos successfully to term. The two strains differed little in this respect; increased crowding by transplantation of ova caused a sharp rise in embryonic death. Most of the prenatal loss was found to occur immediately following implantation. The effect of overcrowding was reduced later in gestation, indicating that the result was not a simple mechanical effect of crowding.

Hafez' study on intrauterine crowding in rabbits also utilized the embryo transfer technique, transferring 2-day embryos to the uterine tubes of recipients. Controls, which had an average number of 11 implantations among 2 uterine horns yielded an average of 7.6 viable fetuses on day 9 of gestation. The first experimental group had 33 implantations in the two horns as a result of excessive transfer, but on day 9, only
7.2 viable embryos were observed in the average litter. The three-fold increase in the number of implantations was not reflected in the number of viable embryos. A second experimental group received an excessive number of implantations (21) in a single or one half of a single horn. On day 9, the excess of embryos transferred was reflected by a proportionate reduction in the number of viable fetuses, which averaged 4.5 to a litter. The third experimental group consisted of the normal number of implantations limited to the anterior half of the uterine horn. It was observed on day 9 that all these implantations had degenerated; there was no explanation for this result. It was concluded that placental development was influenced by the availability of space and the vascular supply within the uterus. As the number of implantations rose, the vascular supply to each site was diminished, resulting in restriction of placental development.

By ligating the main uterine vessels at one end of a pregnant uterine horn, Wigglesworth (1964) was able to obstruct the blood supply to rat fetuses and to run a natural control in the untreated horn. This operation was performed on albino rats on day 17 of gestation, and the animals were killed 4 days later to examine fetal death in experimental and control horns. A variable number of fetuses were found dead and partially resorbed at the lower end of the horn. The degree of maceration was in direct proportion to the proximity to the ligated vessels. In most cases, the placenta was necrotic, but the placentas of some dead fetuses were still healthy. It was observed that fetal death had, in the majority of cases, occurred shortly after the operation as an effect
of utero-placental ischemia resulting from experimental inadequate blood flow.

**Atmospheric Changes**

It has been observed (Goldstein, 1965) that carbon monoxide in pregnant rabbits readily crosses the placenta and results in fetal damage or death. The amount of gas inhaled by the mother and the length of exposure were found to determine the degree to which carbon monoxide saturation of fetal blood occurred. A lag in saturation of fetal hemoglobin compared to that of the mother was apparent. A longer exposure time therefore caused fetal damage more readily than shorter ones. It was, however, believed that fetal death did not occur as a result of placental introduction of carbon monoxide but rather from fetal anoxia arising from a lack of oxygen in the blood in the presence of high levels of maternal carboxyhemoglobin. This interpretation was based on analyses of maternal and fetal blood after maternal exposure to carbon monoxide resulted in the death of both.

Experimental maternal hypoxia was induced in pregnant rabbits to ascertain the effect on foetal survival (McNicholl, 1965). Pregnant does were subjected to a 24-hour period of hypoxia commencing on day 27 of gestation. On day 28, the litters were delivered by section. Four out of seven does yielded dead litters. Hypoxia had been induced rapidly, the chamber oxygen being below 9% for some hours before delivery. It was concluded that approximately 9% oxygen was the critical level breathed by the mother relative to fetal survival at this stage of gestation.
Other Factors Reducing Fertility

Decreased fertility has generally been observed with increasing maternal age, but as maternal age increases, so, commonly, does parity, and in order to define clearly the effects of each, they must be separated by some statistical or experimental technique.

Finn (1963) observed that the reproductive performance of mothers unmated until 9 months of age did not differ significantly from the performance of mothers bred continuously from puberty when judgment was made over an equivalent period of time. Earlier studies on the variation of litter size with parity (Biggers, Finn and McLaren, 1961) had shown that the rate of ovulation remains consistently high throughout the reproductive life span, indicating that the observed reductions in litter size were due to increased embryonic loss. The collagen content furthermore did not differ in horns which had borne fetuses and those which, as a result of unilateral ovariectomy before sexual maturity, had remained barren (Finn, Fitch and Harkness, 1963). Such barren horns had, however, been subject to hormonal changes in the blood which had taken place during pregnancy. However, study of a group of old virgins confirmed the impression that the increase of collagen content with age was independent of previous pregnancies. The results of these experiments all indicate that parity has no effect on fertility, and any changes observed with increasing maternal age can therefore be attributed to the effect of age.

A study of the reproductive power of female mice of different
ages was carried out by Sugiyama (1961). Three age groups were examined - young, middle-aged and old. Litter size was highest in the middle-aged group, but the frequency of dead fetuses was found to vary directly with the maternal age. Young mice, which had a low rate of fetal death, yielded intermediate-sized litters, indicating a lower ovulation rate.

In humans, also, it has been established that the risk of abortion increases with increasing age of the mother (Rucker, 1952; Tietze et al., 1950) and father and with parity (Warburton and Fraser, 1964). A possible cause of this trend may be an increased incidence of placental insufficiency, which has been found to occur more frequently in aged mothers than in young ones (Browne, 1962).

Jones and Krohn (1961), working with female mice of the A, CBA and RIII strains, counted oocytes in the ovaries of mice of various ages from birth to 933 days. It was observed that the numbers of oocytes gradually declined with age in all strains investigated. No relation could, however, be formed between the level of fertility and oocyte number, or between the rate of decline of fertility and the reduction in numbers of Graafian follicles or corpora lutea.

A study of embryonic death in aged mice (Finn, 1962) confirmed earlier evidence that embryonic losses were much greater in old than in young mice. The total number of implantations was found to be comparable to the number of offspring per litter that the same mice had produced earlier in their reproductive lives. It was considered that the implanted embryos may have failed to develop due to structural changes in the uterus,
changes in the endocrine environment or lethal factors in the eggs.

Finn (1963) confirmed that the reduced litter size and high level of embryonic loss in old animals was associated with post-implantation failure of development. The disparity between numbers of corpora lutea noted and litter size indicated loss after ovulation, and the large numbers of moles evident during the phase of declining litter size provided direct evidence. Since the numbers of implantations of old mice were similar to the plateau litter size for these mice, it was assumed that implantation rates had not decreased with age, but that most of the fetal loss occurred after implantation. In the light of a number of experimental observations, uterine changes were implicated as the most likely explanation of reduced fertility. It was noted (Hafez, 1965) that embryos transferred from young mothers to the uteri of old females failed to develop. This indicated that the loss of fertility in old females is caused mainly by anomalies extrinsic to the embryo. Studies on the collagen content of uterine horns (Finn, Fitch and Harkness, 1963) carried out by estimation of hydroxyproline indicated that, in two year old mice that had ceased breeding, the total collagen content was three times and the collagen concentration was 1.5 times that found in 2 month old mice. These increased amounts of collagen in old uteri were confirmed histologically. It was also observed that collagen content increased in the uterus during pregnancy and decreased rapidly at parturition; after pregnancy, the collagen content was reduced below the original level, eliminating the possibility that residual increases with successive pregnancies caused the ultimate high levels in old mice.
The observation that uterine collagen increased during pregnancy raised the possibility that collagen synthesis may be essential during gestation. In vitro experiments showed that the capacity of uterine tissue to synthesize collagen was an inverse function of the donor's age. The gradual loss of this synthetic ability was therefore suggested as the factor interfering with maintenance of pregnancy in aging mice.
MATERIALS AND METHODS

I. Maintenance of Mice

All mice used in these experiments were of the A/Jax strain, obtained at 6-7 weeks of age from the Jackson Laboratory in Bar Harbor, Maine. The A/Jax strain is a highly inbred line of albino mice, bred from over 70 generations of brother-sister matings (Dickie, 1961), of medium fertility and having the coat colour genetics a/a; b/b; c/c. About 10% of A/Jax mice in this laboratory exhibit a cleft lip before term.

In this laboratory, the mice were on a light cycle of 8 hours' darkness (from 10:00 p.m. to 6:00 a.m. E.S.T.) and 16 hours' light, and maintained at a temperature of 70°- 72°F. Two to five nulliparous females were kept in each translucent polypropylene cage. Their diet consisted of Mouse Breeder Chow and water ad libitum.

Approximately 4 times a week, a single A/Jax male was placed in each cage overnight. The following morning, the males were returned to their cages and the females were examined for vaginal plugs. The vaginal plug, which is formed from the coagulated secretion of the seminal vesicles of the male a few minutes after successful copulation, is usually extruded within 24 hours of its formation (Fischberg and Beatty, 1952; Gruneberg, 1952).

Females having a vaginal plug and, therefore, known to have mated during the night, were weighed and removed from their breeding cages. Ten days later, they were palpated, and those found to be pregnant were treated.
II. Treatment of Mice

Experimental Animals:

The treatment consisted of an intraperitoneal injection of 6-amino nicotinamide, followed two hours later by a similarly administered injection of nicotinamide. The dose of nicotinamide given was one that had been found to abolish the teratological effects of 6-AN when given concurrently (Pinsky and Fraser, 1960). Embryos were thus exposed to the action of the analogue for a period of two hours.

The times of treatment were based on the calculation of Snell et al. (1940) that the modal time of all matings is 2:00 a.m. Considering 2:00 a.m. of the night before the vaginal plug was observed as time 0 of gestation, pregnant females were treated on one of the following times of gestation: day 10/8 (day 10 and 8 hours of gestation, i.e. 10:00 a.m. of day 10), 10/12, 10/16, 10/20, 11/0, 11/4, 11/8 or 11/12. To eliminate bias, each pregnant mouse was assigned to any one treatment time at random, by means of a statistical table of random numbers.

Based on doses used by Pinsky and Fraser (1960), 19 mg/kg 6-amino nicotinamide and 7.3 mg/kg nicotinamide, according to maternal weight when the vaginal plug is observed, are considered the standard "single dose" by intramuscular injection and other doses will be referred to as functions of these. When administered intraperitoneally, however, 3/4 and 7/8 doses of 6-AN (14.25 mg/kg and 16.67 mg/kg, respectively) were found sufficient to elicit the same response. In certain experiments, where higher dosages were required, single and double (38 mg/kg) doses of 6-AN were used.
Both substances were injected in a sterile distilled water solution, using a Hamilton microliter syringe #750, with the Chaney attachment.

Controls:

In each experiment, control groups and treatments were carried on concurrently. Pregnant females were assigned at random to the control group, and then randomized for control treatment at any one of the treatment times involved in that particular experiment. The control treatment comprised an intraperitoneal injection of distilled water equal in volume to the dose of 6-AN which would have been administered in a treated group, followed two hours later by the protective injection of nicotinamide.

The control animals were handled exactly as the experimental animals, barring exposure to 6-amino nicotinamide, making the control groups' responses the criterion for measuring the effect of that drug.

III. Length of Period of Differential Susceptibility

Six treatment groups and a control group were included in this study, each group consisting of 15 litters, except the day 10/8 group, which included 3 litters. For each stage of treatment, the dose was adjusted to give a high level of resorption, so that sparing of cleft lip embryos would be detected, if present.

Treatments were (i) a single dose on day 10/8, (ii) a 3/4 dose on day 10/12, (iii) a 3/4 dose on day 10/16, (iv) a 7/8 dose on day 10/20, (v) a 3/4 dose on day 11/0, and (vi) a double dose on day 11/12. Two
hours after the injection of 6-AN, the corresponding protective dose of nicotinamide was administered. Controls received the previously mentioned treatment.

On day 18/12 of gestation, the pregnant females were killed and the embryos were removed by caesarean section. The position in the uterus of each embryo and of resorptions were noted. Anything from a recently dead embryo showing little maceration to a small, barely recognizable remains of an implantation was scored as a resorption. Viable embryos were classified as cleft lip or non-cleft lip.

IV. Effect of Dose Dilution on Fetal Response

Pregnant mice were treated on day 10/12 or day 11/12 of gestation. Females treated on day 10/12 were given a 2/3 dose of 6-amino nicotinamide (12.67 mg/kg) or a 3/4 dose, while those treated on day 11/12 were given either a single or a double dose. These 4 treatment groups were further subdivided by administering the drug from a dilute solution (22.5 mg/10 ml HOH) or from a concentrated solution (45 mg/10 ml. HOH). A dose from the dilute solution was thus given in a volume twice that of the same dose from the concentrated solution.

All animals were injected with a corresponding dose of nicotinamide 2 hours after treatment with the teratogen. On day 18/12, they were killed and their uteri were removed and examined.

V. Test for Developmental Retardation of Cleft Lip Animals

Treatment groups in this experiment were spaced 4 hours apart,
starting at day 10/12 and continuing through days 10/16, 10/20 and 11/0 to day 11/4. All these groups consisted of 15 litters. The object was to see if, at any one of those times, 6-amino nicotinamide would exert its lethal effect preferentially against cleft lip embryos.

The experiment was repeated three times. In the first trial, all treatment times from day 10/12 to day 11/4 were represented, and 3 litters were treated at day 11/8. The second trial extended only to day 11/0, and the third to day 10/20. Each trial had its own control group.

The first and third trials employed a dilute 3/4 dose (14.25 mg/kg) at each treatment time, while the second employed a dilute 7/8 dose (16.67 mg/kg). The higher dose was used to increase the resorption frequency slightly and thus make the sparing effect more distinct.
RESULTS

I. Length of Period of Differential Susceptibility

Goldstein (1964) showed that A/Jax mouse embryos with spontaneous congenital cleft lip are resistant to the lethal effect of 6-amino nicotinamide at day 10/12 of gestation. The levels of resorption at earlier and later treatment times were, however, too low to show whether there was an excess of cleft lip embryos among the survivors.

As can be seen in Table 2, if it is assumed that cleft lip embryos are not killed by doses of 6-AN that increase the resorption rate of the litter as a whole, there will be an excess of cleft lip embryos among the survivors of the treatment, though the frequency of cleft lip among implantation sites remains constant. This excess varies in direct proportion to the level of resorption. As the level of resorption drops, the excess due to a sparing effect decreases rapidly to a point where it becomes negligible (Table 2A, line 5).

For this reason, dosages of 6-AN causing close to 80% resorption were employed in order to see whether or not the differential susceptibility was present at the various stages of gestation. Results are shown in Table 3.

Comparing the frequencies of cleft lip in columns 3 and 4 to those for 80% resorption in Table 1, it can be seen that the differential resistance of cleft lip embryos to resorption by 6-AN is acting at day 10/12 and day 10/16. The frequencies of 38.2% and 32.0% for cleft lips per viable embryo are significantly above 10% (p < 0.005), the value expected when no differential response exists,
TABLE 2

Expected cleft lip frequencies as the level of resorption varies

A. Cleft lip embryos resistant to lethal effect of 6-AN

<table>
<thead>
<tr>
<th>(1) No. of implantations</th>
<th>100</th>
<th>100</th>
<th>100</th>
<th>100</th>
<th>100</th>
<th>100</th>
<th>100</th>
<th>100</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2) % resorptions</td>
<td>90</td>
<td>80</td>
<td>70</td>
<td>60</td>
<td>50</td>
<td>40</td>
<td>30</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>(3) No. of viable embryos</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
<td>60</td>
<td>70</td>
<td>80</td>
<td>90</td>
</tr>
<tr>
<td>(4) No. of embryos with CL</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>(5) % CL in viable embryos</td>
<td>100</td>
<td>50</td>
<td>33</td>
<td>25</td>
<td>20</td>
<td>17</td>
<td>14</td>
<td>13</td>
<td>11</td>
</tr>
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</table>

B. Cleft lip embryos not resistant to lethal effect of 6-AN

<table>
<thead>
<tr>
<th>(1) No. of implantations</th>
<th>100</th>
<th>100</th>
<th>100</th>
<th>100</th>
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<th>100</th>
<th>100</th>
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<tbody>
<tr>
<td>(2) % resorptions</td>
<td>90</td>
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<td>70</td>
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<td>50</td>
<td>40</td>
<td>30</td>
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<td>10</td>
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<tr>
<td>(3) No. of viable embryos</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
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<td>70</td>
<td>80</td>
<td>90</td>
</tr>
<tr>
<td>(4) No. of embryos with CL</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
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<td>(5) % CL in viable embryos</td>
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<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

* Assume 10% frequency of cleft lip among implantations
+ Assume sparing of cleft lip embryos absolute
** Assume no sparing of cleft lip embryos

---

87
**TABLE 3**

*Cleft lip frequencies from day 10/8 to day 11/12 at high levels of resorption*

<table>
<thead>
<tr>
<th>Dose of 6-AN (mg/kg)</th>
<th>1 Control</th>
<th>2 19.0 10/8</th>
<th>3 14.25 10/12</th>
<th>4 16.67 10/16</th>
<th>5 14.25 10/20</th>
<th>6 38.0 11/0</th>
<th>7 11/12</th>
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<tbody>
<tr>
<td>Day of treatment</td>
<td></td>
<td>10/8 10/12 10/16 10/20 11/0 11/12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of litters</td>
<td>15 3 15 15 15 15 15 15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of implantations</td>
<td>153 30 158 143 153 134 162</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% resorptions</td>
<td>16.3 40.0 78.5 82.5 78.4 61.2 79.6</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% viable embryos</td>
<td>93.7 60.0 21.5 17.5 21.6 38.8 20.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of embryos with CL</td>
<td>12 2 13 8 6 8 17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% CL in viable embryos</td>
<td>9.4 11.1 38.2 32.0 18.2 15.4 10.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
but, falling short of 50%, the value expected with sparing of all cleft lip embryos, they indicate that either the percentage of cleft lip embryos among implantations is less than 10%, or that the sparing effect is not complete, and that some cleft lip embryos were resorbed.

Treating four hours later, at day 10/20, it can be seen in column 5 that the sparing effect has been reduced, since, even with the dose increased to cause 78.4% resorption, the frequency of cleft lip embryos dropped to 18.2%. Comparison of this value with 10%, expected when no differential resistance exists, gave a chi-square of 2.78, $P = .10$ for 1 d.f. The chi-square found when 18.2% was compared to 50% was highly significant at 22.5, $P < .005$ for 1 d.f. This indicates that the differential resistance is largely gone at day 10/20, but that some sparing of cleft lip embryos may be present, though not demonstrable with the sample size obtained.

Column 6 shows the results of maternal treatment with a 3/4 dose of 6-AN on day 11/0. The resulting 61.2% level of resorption and 15.4% cleft lip coincide more nearly with the results expected when the sparing effect is absent. The cleft lip frequency of 15.4% gives a chi-square of 1.08, $.25 < P < .50$ for 1 d.f., showing it to have no statistically significant difference from 10%. It would have been more satisfactory, however, to have achieved a higher resorption frequency.

At day 11/12, as shown in column 7, a double dose was required to bring the resorption level to 78.9%, since the embryos, at that time of gestation, have lost the extreme sensitivity to 6-amino nicotinamide shown around day 10/12. At that level of resorption, the presence of
the sparing effect would cause the cleft lip frequency to range upward of 30%, while the frequency observed, 10.5%, coincides exactly with the frequency expected when the cleft lip embryos respond to 6-AN as their normal litter-mates do.

II. Effect of Dose Dilution on Fetal Response

In the previous experiment, mice treated on day 11/12 of gestation were administered a double dose of 6-amino nicotinamide in order to achieve a high level of resorption. To keep the volume of the double doses in the same range as other dosages, a solution of 6-AN twice as concentrated as the usual solution was used for animals receiving high doses after some had already been treated with the more dilute solution. The resorption frequencies observed among litters were found to differ according to whether treatment had been administered in the dilute or concentrated form. An experiment was then designed to investigate further the effect of dose dilution.

Pregnant mice were treated on day 10/12 with one of two low doses in a dilute or concentrated form, or on day 11/12 with one of two high doses of 6-amino nicotinamide. The concentrated doses were given in a volume one half that of the same doses in the dilute solution. The results are shown in Table 4.

The response to the dilute 3/4 dose on day 10/12 showed resistance of cleft lip embryos to resorption, with a cleft lip frequency of 33.3% at an 84.2% resorption rate. At the same dose in concentrated
**TABLE 4**

The effect of dose dilution on fetal response to the lethal action of 6-amino nicotinamide on days 10/12 and 11/12 of gestation

<table>
<thead>
<tr>
<th>Day of treatment</th>
<th>Dose of 6-AN</th>
<th>Controls</th>
<th>10/12</th>
<th>11/12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2/3</td>
<td>3/4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dilute</td>
<td>Conc.</td>
</tr>
<tr>
<td>No. of litters</td>
<td>16</td>
<td>5</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>No. of implantations</td>
<td>161</td>
<td>55</td>
<td>36</td>
<td>76</td>
</tr>
<tr>
<td>% resorption</td>
<td>18.7</td>
<td>67.3</td>
<td>97.2</td>
<td>84.2</td>
</tr>
<tr>
<td>No. viable embryos</td>
<td>131</td>
<td>18</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>No. of embryos with CL</td>
<td>11</td>
<td>1</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>% CL in viable embryos</td>
<td>8.4</td>
<td>5.6</td>
<td>0.0</td>
<td>33.3</td>
</tr>
</tbody>
</table>
form, no cleft lip embryos were viable; the 3 embryos surviving concentrated doses on day 10/12 were all non-cleft lip. The sparing effect was not seen after the dilute 2/3 dose treatment, but the sample size was so small that the expected value if sparing was complete was near the upper confidence limit (UL = 28.9%) of the observed value (5.6%). A sparing effect, therefore, cannot be ruled out in this experiment.

The frequency of cleft lip found after a single dilute dose on day 11/12 was 14.8%, but the resorption frequency at 37.8% does not permit a conclusion to be drawn as to the presence of the sparing effect. The concentrated single dose and both the double dose treatments caused cleft lip frequencies indicating its absence, however, in the presence of resorption frequencies of 64.2%, 79.8% and 82.8%. This confirms the impression from the previous section (I) that the sparing effect has disappeared by this stage of gestation.

It can be seen that, in each time-dose treatment pair, the concentrated dose caused a higher frequency of resorption than the dilute dose. This effect was statistically significant only at the low dose treatments on both days. On day 10/12, the dilute 2/3 dose caused 67.3% resorption, while the concentrated 2/3 dose caused 97.2% (p < 0.005). Similarly, on day 11/12, the concentrated single dose caused 64.2% resorption, compared to a frequency of 37.8% caused by the dilute dose (p < 0.005).

The high doses on both days did not show such a large increase in resorption frequency when treatments were made with concentrated solutions.
Indeed, they could not, since the dilute concentrations were themselves giving high resorption frequencies. Neither of the dilute dose responses are significantly differently from the concentrated dose responses at the high dose levels.

III. Developmental Retardation as the Cause of Differential Susceptibility

This experiment was designed to test the hypothesis that the cleft lip embryos spared by the treatment on day 10/12 were relatively resistant because they were lagging behind their normal litter-mates in developmental stage, and had not reached the point of maximum susceptibility by the time of treatment. 6-amino nicotinamide was administered to pregnant mice, spacing treatment groups at 4 hour intervals. For reasons to be discussed later, 3 sets of experiments were run. All 3 trials had their own control group. The results are shown in Table 5.

Resorption:

Days 10/12 and 10/16 showed the highest sensitivity to the lethal effect of 6-AN. The first and third trials, using a 3/4 dose of the drug, showed resorption frequencies of 78.5% and 78.4% at day 10/12 and frequencies of 85.0% and 82.5% at day 10/16. The higher dose, used in Trial II, gave 90.9% and 88.7% resorption after treating on days 10/12 and 10/16. These two treatment times were therefore considered to be of comparable and maximum sensitivity to 6-amino nicotinamide.

Fig.1 illustrates graphically the effect of 6-AN on the frequency of resorption in the three trials. The resorption rate after maternal
TABLE 5

Results of three trials in which 6-AN was given as maternal treatment at day 10/8, 10/12, 10/16, 10/20, 11/0, 11/4 or 11/8 of gestation

<table>
<thead>
<tr>
<th>Day of treatment</th>
<th>Controls</th>
<th>10/8</th>
<th>10/12</th>
<th>10/16</th>
<th>10/20</th>
<th>11/0</th>
<th>11/4</th>
<th>11/8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial</td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>No. of litters</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>3</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>No. of implantations</td>
<td>151</td>
<td>153</td>
<td>141</td>
<td>30</td>
<td>158</td>
<td>154</td>
<td>139</td>
<td>160</td>
</tr>
<tr>
<td>% resorptions</td>
<td>14.6</td>
<td>16.3</td>
<td>20.6</td>
<td>40.0</td>
<td>78.5</td>
<td>90.9</td>
<td>78.4</td>
<td>85.0</td>
</tr>
<tr>
<td>No. of viable embryos</td>
<td>129</td>
<td>128</td>
<td>112</td>
<td>18</td>
<td>34</td>
<td>14</td>
<td>30</td>
<td>24</td>
</tr>
<tr>
<td>No. of embryos with CL</td>
<td>11</td>
<td>12</td>
<td>15</td>
<td>2</td>
<td>13</td>
<td>4</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>% CL of viable embryos</td>
<td>8.5</td>
<td>9.4</td>
<td>13.4</td>
<td>11.1</td>
<td>38.2</td>
<td>28.6</td>
<td>33.3</td>
<td>16.7</td>
</tr>
</tbody>
</table>
Fig. 1 Frequency of resorption in A/Jax embryos following maternal treatment with 6-amino nicotinamide between days 10/8 and 11/4 of gestation.
treatment on day 10/8 is included in all 3 graphs for comparison. In each of the trials, the resorption frequency was much higher after treatment on day 10/12 than on day 10/8.

In trial I, treatment on day 10/12 caused the resorption frequency to rise to 78.5%, an increase of 38.5% above that found after treating on day 10/8. The frequency rose to 85.0%, then dropped to 61.6% in the groups treated on days 10/16 and 10/20 respectively. A continuing decrease in susceptibility to resorb was not evident after treating on day 11/0, which showed a frequency of 61.2%, but was evident on day 11/4, with an observed frequency of 43.3% resorption.

Trial II showed a much more regular response to the lethal effect of 6-AN. The resorption frequency rose sharply to 90.9% on day 10/12, was maintained at a similarly high frequency of 88.7% at day 10/16, then declined to 78.4% and 55.6% at days 10/20 and 11/0.

In trial III, the decline in susceptibility to resorption did not begin at day 10/20, and we can only ascribe this to "random" variation, since the other two trials did show an increase in resistance at this stage. Contingency chi-squares showed all frequencies of resorption in the three trials to be significantly different from their corresponding control values (p < 0.005).

The results show a sudden increase in susceptibility to the lethal effect of 6-amino nicotinamide between day 10/8 and day 10/12. The highest average frequency of resorption following a 3/4 dose, 85.4%, is found following maternal treatment on day 10/16 though this value
is not significantly higher than the values for day 10/12. This period will be discussed in detail in the following section. The susceptibility is reduced at day 10/20, and continues to decline through days 11/0 and 11/4. By day 11/8, it has returned to the same level observed following treatment 24 hours earlier.

Cleft lip:

The offspring of females treated on day 11/8 of pregnancy exhibited cleft lip in 11.1% of viable embryos. This figure does not differ significantly from the control values, which average 10.4% for the 3 trials. Graphs of the cleft lip frequencies can be seen in Fig. 2.

In trial I, the cleft lip frequency dropped to 16.7% (0.25 < p < 0.05) after treatment on day 10/16 from a day 10/12 frequency of 38.2% (p < 0.005). This was followed by a second increase at day 10/20 to 32.8% (p < 0.005), after which the frequency dropped through the next two treatment times. Neither of these values, 15.4% (0.10 < p < 0.25) and 11.3% (0.5 < p < 0.75) were significantly different from the control value at the 5% probability level.

Since the second peak at day 10/20 was unexpected and difficult to account for, a second trial was run to see if it might have been an artifact. In the second trial, treatments on days 10/12, 10/16, 10/20 and 11/0 gave cleft lip frequencies of 28.6% (0.025 < p < 0.05), 41.2% (p < 0.005), 18.2% (0.10 < p < 0.25) and 15.5% (0.10 < p < 0.25). These results differed from those of the first trial by having a high frequency on day 10/16 and a low one on day 10/20, instead of vice versa. The
Fig. 2 Frequency of cleft lip in viable embryos following maternal treatment with 6-amino nicotinamide between days 10/8 and 11/4 of gestation.
bimodality of the first curve was, therefore, suspect, and a third trial was made to settle the question.

In the last repetition, the bimodal curve was rejected, as cleft lip frequencies of 33.3% (.01 < p < .025), 32.0% (.01 < p < .025), and 19.2% (.25 < p < .50) were obtained following treatments on days 10/12, 10/16 and 10/20. The low frequency of day 10/16 in the first trial was possibly due to the large number of litters which resorbed completely in that group, in which only 7 of the 15 mothers bore viable embryos. This effect will be discussed more fully in the following section of the Results and in the Discussion.

The results indicate that the sparing effect is present both on day 10/12 and day 10/16. On day 10/20, trials II and III indicate that the sparing effect is reduced to the point where the cleft lip frequencies are not significantly different from control values. Frequencies of cleft lip after treatment on days 11/0 or 11/4 are not significantly different from controls.

The hypothesis that cleft lip embryos differ from normal ones in their response to 6-AN due to a developmental retardation assumes that, at some time after day 10/12, cleft lip embryos will have an increased susceptibility to the drug. The results were therefore examined for a treatment time which showed fewer cleft lip embryos than expected if no differential susceptibility existed. The results appear in Table 6.

It can be seen that there is no differential susceptibility on day 11/4 (see Table 1). All other cleft lip frequencies, being greater
TABLE 6

Observed frequencies of cleft lip in viable embryos and values expected when a differential susceptibility is sparing a) cleft lip and b) non-cleft lip embryos

<table>
<thead>
<tr>
<th>Day of treatment</th>
<th>10/12</th>
<th>11/16</th>
<th>11/20</th>
<th>11/0</th>
<th>11/4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% CL expected if CL embryos are spared*</td>
<td>50</td>
<td>50</td>
<td>25</td>
<td>25</td>
<td>17</td>
</tr>
<tr>
<td>% CL expected if non-CL embryos are spared*</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>% CL in viable embryos observed</td>
<td>38.2</td>
<td>16.7</td>
<td>32.8</td>
<td>15.4</td>
<td>11.3</td>
</tr>
<tr>
<td>Trial II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% CL expected if CL embryos are spared*</td>
<td>100</td>
<td>100</td>
<td>50</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>% CL expected if non-CL embryos are spared*</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>% CL in viable embryos observed</td>
<td>28.6</td>
<td>41.2</td>
<td>18.2</td>
<td>15.5</td>
<td></td>
</tr>
<tr>
<td>Trial III</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% CL expected if CL embryos are spared*</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% CL expected if non-CL embryos are spared*</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% CL in viable embryos observed</td>
<td>33.3</td>
<td>32.0</td>
<td>19.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* refer to Table 1
than 10%, indicate at least a partial differential susceptibility to be
operating in favour of cleft lip embryos from day 10/12 to day 11/0. At
none of these treatment times was a frequency of less than 10% cleft lip
observed, indicating the cleft lip embryos do not pass through a stage
where they are preferentially susceptible to the lethal action of 6-amino
nicotinamide.

IV. A Period of Susceptibility of Entire Litters to Resorption

It was noted, in the experiment that tested whether or not
developmental retardation was the cause of differential susceptibility,
that at each treatment time, some complete litters were resorbed, such
that no viable embryos were found among the implantations.

As can be seen in Table 7, more litters resorbed entirely after
maternal treatment on day 10/16 than after treatment at any other time.

TABLE 7

The number of litters, out of fifteen, in which no embryos survived
maternal treatment, based on 3 trials including controls and five
treatment times.

<table>
<thead>
<tr>
<th>Day of treatment</th>
<th>Controls 10/12</th>
<th>10/16</th>
<th>10/20</th>
<th>11/0</th>
<th>11/4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial I</td>
<td>0</td>
<td>3</td>
<td>8</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Trial II</td>
<td>0</td>
<td>4</td>
<td>7</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Trial III</td>
<td>0</td>
<td>5</td>
<td>6</td>
<td>3</td>
<td>-</td>
</tr>
</tbody>
</table>
Since so many litters were wholly resorbed after maternal treatment on day 10/16, the data was analyzed to see if more litters were resorbed than would be expected when each embryo in a litter has an 85.0%, 88.7% or 82.5% chance of resorbing. The mean litter size for all animals treated on day 10/16 was 10.05; calculations were based on a litter size of 10 and a resorption frequency of 85%.

Letting \( a = 0.85 \) be the probability of resorption per embryo, \( a^{10} \) was calculated to find the frequency with which one could expect to find litters with resorption of all implantations. This frequency was multiplied by 15 (the number of litters in each treatment group) to give the expected number of resorbed litters. This number subtracted from 15 gave the expected number of litters which would have one or more viable embryos.

A chi-square analysis was carried out on each of the three sets of day 10/16 observations to compare the numbers of resorbed litters observed to those expected. The results are shown in Table 8. The overall chi-square values of 11.12, 7.19 and 4.10 show that, in each trial, the pattern of resorption within litters differed significantly from that expected with an average resorption rate of 85.0%. Although only 3(2.9) litters in 15 were expected to have no surviving embryos after maternal treatment, 8 were found in the first trial, 7 in the second and 6 in the third. The corrected individual chi-squares indicate that it was the excess of resorbed litters which caused the significant deviation from expected values. The individual chi-squares for trials I and II were significant \((.005 < p < .01 \text{ and } .025 < p < .05, \text{ respectively})\).
TABLE 8

Chi-square analysis of observed numbers of litters resorbed entirely
and partially after treatment on day 10/16

<table>
<thead>
<tr>
<th>Trial</th>
<th>Number of litters</th>
<th>Overall Chi-square</th>
<th>Individual Chi-square</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expected</td>
<td>Observed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial I</td>
<td>Entire resorption</td>
<td>2.9</td>
<td>8</td>
<td>7.30</td>
</tr>
<tr>
<td></td>
<td>Partial</td>
<td>12.1</td>
<td>7</td>
<td>1.75</td>
</tr>
<tr>
<td>Trial II</td>
<td>Entire resorption</td>
<td>2.9</td>
<td>7</td>
<td>4.47</td>
</tr>
<tr>
<td></td>
<td>Partial</td>
<td>12.1</td>
<td>8</td>
<td>1.07</td>
</tr>
<tr>
<td>Trial III</td>
<td>Entire resorption</td>
<td>2.9</td>
<td>6</td>
<td>2.33</td>
</tr>
<tr>
<td></td>
<td>Partial</td>
<td>12.1</td>
<td>9</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Chi-square analysis of observed numbers of litters resorbed entirely
and partially after treatment on day 10/16
When corrected, the value for the third trial was not significant (.10 < p < .25). The same analysis carried out on the data obtained after treatment on day 10/12 did not reveal a similar excess of resorbed litters.

Although the method of statistical analysis is rather crude, it seems reasonably clear that in each trial, there was an excess of wholly-resorbed litters after maternal treatment on day 10/16.

The fact that complete resorption appeared more frequently than expected indicates that, on day 10/16, there may be two different mechanisms involved in 6-AN-induced resorptions, one acting on individual embryos and one acting on whole litters, presumably through a maternal effect. If so, the frequency of resorption on day 10/16 due to the individual effect (which seems to be operating alone at days 10/12 and 10/20) would be less than the observed 85%.

The data were next examined for the differences in cleft lip frequency that would arise by eliminating resorbed litters. Calculations were based only on those litters which had at least one viable embryo just before term. The results are shown in Table 9.

In each treatment group, the reduction in resorption frequency caused by eliminating resorbed litters varied according to the number and sizes of litters which had no viable embryos. The frequency of cleft lip in viable embryos was, naturally, not affected by the change in calculations. Removal of resorbed litters from the data did, however, affect the cleft lip frequency calculated as a percentage of implantations.
<table>
<thead>
<tr>
<th>Day of treatment</th>
<th>Controls</th>
<th>10/12</th>
<th>10/16</th>
<th>10/20</th>
<th>11/0</th>
<th>11/4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial I</td>
<td>Trial II</td>
<td>Trial III</td>
<td>Trial I</td>
<td>Trial II</td>
<td>Trial III</td>
</tr>
<tr>
<td>1. No. entirely resorbed litters (of 15)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>2. No. of implantations in remaining litters</td>
<td>151</td>
<td>153</td>
<td>141</td>
<td>105</td>
<td>99</td>
<td>93</td>
</tr>
<tr>
<td>3. % resorption (15 litters)</td>
<td>14.6</td>
<td>16.3</td>
<td>20.6</td>
<td>78.5</td>
<td>90.9</td>
<td>78.4</td>
</tr>
<tr>
<td>4. % resorption (excluding resorbed litters)</td>
<td>14.6</td>
<td>16.3</td>
<td>20.6</td>
<td>67.6</td>
<td>85.6</td>
<td>67.7</td>
</tr>
<tr>
<td>5. % CL in viable embryos</td>
<td>8.5</td>
<td>9.4</td>
<td>13.4</td>
<td>38.2</td>
<td>28.6</td>
<td>33.3</td>
</tr>
<tr>
<td>6. % CL in implantations (15 litters)</td>
<td>7.3</td>
<td>7.8</td>
<td>10.6</td>
<td>8.7</td>
<td>2.6</td>
<td>7.2</td>
</tr>
<tr>
<td>7. % CL in implantations (excluding wholly resorbed litters)</td>
<td>7.3</td>
<td>7.8</td>
<td>10.6</td>
<td>12.3</td>
<td>4.0</td>
<td>10.7</td>
</tr>
<tr>
<td>8. % increase in CL/implantations by excluding resorbed litters</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>41.4</td>
<td>53.8</td>
<td>48.6</td>
</tr>
</tbody>
</table>
As seen in line 6 of Table 9, the frequency of cleft lip among implantations in the uncorrected data was puzzlingly low (less than 6%) in 6 cases. They raise the possibility that cleft lip embryos were being resorbed (refer to Table 2), while the frequencies calculated as a percent of viable embryos indicated a sparing effect operating (on days 10/12 and 10/16) or declining (on day 10/20). When the correction was introduced (line 7, Table 9), all the frequencies were increased in proportion to the number of litters resorbed, and the low values, especially as observed in the results of day 10/16 treatments, were brought closer to the 8% expected on the basis of control values. The low frequencies had apparently been due mainly to the bias introduced by the resorption of whole litters, and not to the selective killing of cleft lip embryos.

The low frequencies of cleft lip among implantations observed concurrently with large numbers of wholly-resorbed litters on day 10/16 indicated that spontaneous cleft lip embryos had been resorbed among other implantations in the resorbed litters. The further observation that, among litters with viable embryos, the cleft lip frequency among implantations was near control values (day 10/16, line 7), strengthens the suggestion that some of the resorptions among the resorbed litters had been cleft lip embryos.
DISCUSSION

The experiments described were designed to investigate the period of sensitivity to 6-amino nicotinamide as evidenced by fetal resorption, and to determine the limits of the period of differential susceptibility of cleft lip embryos. The question of whether or not the differential susceptibility resulted from a developmental delay on the part of cleft lip embryos was considered. These topics, as well as a period of susceptibility to whole-litter resorption and an effect of dose dilution observed during the course of experimental work will be discussed in the following sections.

I. Susceptibility to Resorption by 6-Amino Nicotinamide and the Differential Response of Cleft Lip Embryos

It was found that, between day 10/8 and day 10/12 of gestation, A/Jax embryos develop a high susceptibility to resorption after maternal treatment with 6-amino nicotinamide. The sensitivity was maintained through day 10/16 at approximately double the frequency seen after treatment on day 10/8. On day 10/16, it was also observed that more treated mothers resorbed the whole litter than expected if resorptions were independently distributed among mothers. By day 10/20, the resorption frequency was somewhat reduced, and continued to decline slowly through day 11/0 until, at day 11/4, following the dose of 6-AN which caused high resorption on days 10/12 and 10/16, it fell to the level observed after treatment on day 10/8. Similar resorption frequencies, of about 40%, were found after treatments on days 11/8 and 11/12.
At the same time that the sensitivity to 6-AN suddenly increased, it was observed that cleft lip frequency rose also. On day 10/8, it was apparent that cleft lip embryos succumbed to the treatment in the same proportions as non-cleft lip ones, since the proportion of cleft lips in the surviving embryos did not differ from that in controls. On days 10/12 and 10/16, however, cleft lip frequencies indicated that 3-4 times as many cleft lip embryos as non-cleft lip ones were surviving maternal treatment. (It had been shown by Goldstein et al. (1965) that a sparing of cleft lip embryos was operating rather than an induction of the defect.) It was at those two treatment times that the sparing effect was greatest. Even then, however, it did not seem that the cleft lip anomaly was associated with an absolute resistance to 6-AN, for, such a case, assuming the 10% cleft lip frequency indicated by the controls, would result in a 50% cleft lip frequency at an 80% resorption rate, while the cleft lip frequencies observed range from 16.7% to 41.2%. It is possible that a more complete sparing of cleft lip embryos occurs prior to day 10/12, but since the sparing effect seems to be of comparable magnitude on both days 10/12 and 10/16, and since on day 10/8 there is no differential effect operating, it seems unlikely that a period of complete resistance for cleft lip embryos occurs between days 10/8 and 10/12. On the other hand, it is possible that, by random fluctuation, the frequencies of spontaneous congenital cleft lip embryos in the litters treated on days 10/12 and 10/16 were less than 10%, and that the sparing effect was thus more complete than judgment based on a 10% frequency would indicate.
By day 10/20, the resistance of cleft lip embryos was much reduced, and, on day 11/0, if it was there at all, the effect was too small to be statistically significant with the numbers used. From day 11/4 on, the responses of both classes of embryos were comparable, and no differential susceptibility to the lethal effect of 6-AN was apparent, even at very high doses. This shows clearly that the specific response of the cleft lip fetuses is not a permanent difference from non-cleft lip fetuses, but is due to a transient difference lasting for a period of 4-8 hours. Since the cleft lip embryos were at no later treatment time killed in larger proportions than the other embryos, this difference cannot be, as previously suggested, merely a developmental retardation. The low frequency observed by Coldstein (1960) following treating on day 11/0 which suggested the retardation hypothesis may have been due to some whole-litter resorptions in the data.

It is worth noting that the period of differential susceptibility of the cleft lip embryos is shorter than the period of high sensitivity to the lethal effects of 6-AN, since the former is nearly gone by day 10/20, at which time the latter is still maximal. Even though the susceptibility to resorption and the sparing effect appear at the same time, they are reduced at different rates. Since the two phenomena do not have the same time span, it seems they are determined by differing mechanisms.

From the rapidity with which susceptibility to resorption after exposure to 6-AN makes its appearance on day 10/12 (less than 4 hours), it can be inferred that a specific process is being attacked.
Further evidence of this specificity lies in the differentiation between cleft lip and normal embryos.

II. A Period of Susceptibility of Entire Litters to Resorption

It was observed that, following maternal treatment on day 10/16, more resorbed litters were noticed than could be explained by the frequencies of resorption assuming resorption occurred at random. This indicated a heightened susceptibility to the lethal effect of 6-AN in certain litters at that stage of gestation. Excluding resorbed litters from the calculations gave some insight into the cause of otherwise inexplicably low frequencies of cleft lip among implantations. When only those litters which had viable embryos, thereby giving information on the sparing effect, were examined, it was found that the cleft lip frequencies in 3 trials on day 10/16 were 6.0%, 8.7% and 9.5%, instead of 2.5%, 4.7% and 5.6%, the frequencies when all litters were included. Thus, the resorption of cleft lip embryos which the low frequencies indicated was due only to loading of the data by wholly resorbed litters. In litters with viable embryos, the sparing effect was in operation; however, since control litters indicated about one cleft lip embryo to be expected in each litter, it is unlikely that the litters which resorbed entirely had no spontaneous cleft lip embryos among all the implantations. Since wholly resorbed litters comprised almost half of all litters treated on day 10/16, it seems likely that there were almost as many cleft lip embryos in the resorbed litters as in those where some embryos survived. The resorption frequency observed after treatment
on day 10/16 would, in that case, result from two causes: a) the sensitivity to 6-AN to which cleft lip embryos are more resistant and b) a second transient sensitivity to the drug, perhaps a maternal effect, which overrides the differential susceptibility.

III. Effect of Dose Dilution on Fetal Response

On both days 10/12 and 11/12, diluted doses were seen to cause lower resorption frequencies than concentrated doses. The differences were significant when low doses were administered, as the 2/3 dose on day 10/12 and the single dose on day 11/12 indicated. The increase in the former treatment when the dose was concentrated was from 67.3% to 97.2%, and in the latter was from 37.8% to 64.2%. The concentrated 3/4 dose on day 10/12 showed an increase from 84.2% to 94.3% only, and the concentrated double dose on day 11/12 an increase from 79.8% to 82.8% only.

At high doses, even the dilute solutions produced high resorption rates, so the possibility of seeing a large dilution effect was reduced. It seems that high doses cause resorption of embryos by a severe generalized 2-hour deprivation of nicotinamide, which is separate from the effect underlying the specific susceptibility that predisposed animals show at certain stages. High doses, even when dilute, would seem to introduce enough of the teratogen to cause resorption. On day 10/12, the general effect would be working in conjunction with the specific sensitivity, but on day 11/12, when the specific sensitivity has passed, it would be operating alone. Lower
doses of 6-AN apparently do not introduce enough of the competitive analogue to cause a depletion of nicotinamide lethal to the majority of embryos unless it is administered in a concentrated form or unless a predisposition to its lethal effects exists among embryos.

This experiment showed that a unit of 6-amino nicotinamide delivered in 1 volume has a greater effect, in terms of fetal response, than the same unit in 2 volumes. It is possible that the larger volume causes the teratogen to be more diluted in the maternal circulation by simply increasing the volume of the maternal blood. It would then take a longer time for the dose of the analogue to reach the fetal circulations, the rate of maternal circulation remaining constant. It would be more possible that the diluted dose does not permit the drug to enter the maternal circulation as rapidly as does the concentrated dose by an osmotic pressure difference. In this case, again, the effect would be to expose the embryo to less 6-AN per unit time when maternal treatment is delivered in a dilute dose.

The suggestion is therefore made that the extent of the fetal response to treatment depends on the length of time it takes for the embryos to be exposed to the full teratogenic dose. The effect of dose dilution on fetal response would then result from a change in the rate of exposure.

Treating with concentrated solutions of 6-amino nicotinamide on day 10/12, 3 viable embryos were recovered. They were all non-cleft lip animals, even though the mothers were treated at a period when cleft lip embryos showed resistance to the drug's lethal effects. Since
almost all litters in control groups had a cleft lip embryo, it is unlikely that this observation was due to random effects. This is some evidence that the difference between cleft lip and normal animals which determines the differential susceptibility is neither an absolute nor a single one, but that several factors interrelate to determine whether or not an embryo will survive the treatment.

The effect that dose dilution has here been shown to exert on the response to a treatment should be mentioned in regard to experiments which showed poor repeatability. While all other aspects of the treatment were identical, the slight difference of the volume in which the dose was administered was found in certain cases to exert a sizable influence. This should impress the necessity of exact duplication of detail in repeated experiments, especially when dealing with the complexity of mammalian experimental systems.

Since the results of this study provide evidence against developmental retardation as the basis of the cleft lip embryos' resistance to 6-amino nicotinamide, the problem of the etiology of differential susceptibility remains unsolved.

The observations presented on resorption may, however, find analogies in those on fetal death in rats caused by maternal treatment with tetracycline (Steiner et al., 1965) and by maternal vitamin A deficiency (Howell et al., 1964). In both cases, it appeared that the direct
effect of the treatment was on the placenta. The earliest lesions appeared at the periphery of the placental disc in these experiments, as well as in experimental brucellosis and dietary fat deficiency (cited by Howell et al., 1964); resorption of the embryo occurred secondarily to the resulting placental insufficiency. Histological experiments are presently being conducted in this laboratory to ascertain whether 6-amino nicotinamide treatment effects primary pathological changes in the placenta or in the embryo. Primary changes in the embryo would imply a specific essential organ or tissue sensitivity to 6-AN, which should be manifested histologically.

The differential resistance of cleft lip embryos to the resorbing effect of 6-AN provides a challenge in interpretation. A number of factors, some of them interacting, apparently determine the production of cleft lip in certain embryos in a litter; these may be related to, or identical to some extent, with factors determining the response of individual embryos to 6-amino nicotinamide.

Cleft lip frequencies within untreated litters have been observed to follow the binomial distribution, indicating that cleft lip is not determined by the segregation of major genetic factors (Trasler, D.C., unpublished). However, in the A/Jax strain, as in any product of inbreeding, complete genetic homozygosity is never reached, but at the most approximated. The possibility thus exists that a certain number of genes with minor effects are segregating in the line.

If such conditions may be postulated, the results of Doolittle's
experiments (1961) concerning the effects of single-locus differences on radiosensitivity would apply, with interesting implications. Segregation at the short ear, agouti, hairless or albino locus was observed in 5 strains of mice, and the effects of different genotypes on radiosensitivity were noted. Alleles at the short ear locus showed genotype differences in radiosensitivity which differed in different strains. The dominant gene imparted resistance in SEA males and sensitivity in SEC males. In both strains, females showed associations of radioresistance and genotype opposite to those of the males. Moreover, in the SEC strain, genetic segregation at the locus did not affect radioresistance until sexual maturity had been attained. Various relationships of genotype difference to radioresistance were observed in the other locus-strain pairs investigated. These also showed interactions with sex and sexual maturity.

If such minor genetic segregants as were suggested do indeed exist, they might similarly increase 6-AN-resistance in certain genotypic combinations in the A/Jax strain, influenced, perhaps, by factors predisposing to cleft lip, as certain genotypes at the short ear locus increased radioresistance in animals after sexual maturity in the SEC strain.
SUMMARY

Pregnant A/Jax mice were treated with 6-amino nicotinamide between days 10/8 and 11/12 of gestation, with protective doses of nicotinamide administered 2 hours later limiting the period of embryonic exposure to the drug analogue.

Fetal sensitivity to resorption after maternal treatment was maximal for a period of at least 8 hours, from day 10/12 to day 10/20 of development.

On days 10/12 and 10/16, a differential resistance to 6-amino nicotinamide was present sparing cleft lip embryos from the lethal effect of the drug. This sparing effect was largely gone by day 10/20, and was not observed at other stages of development.

A transient sensitivity to whole-litter resorption was observed on day 10/16, which manifested itself in a large proportion of litters yielding no viable embryos. This effect did not differ between cleft lip and normal embryos.

At no treatment time were cleft lip embryos resorbed in greater proportions than their normal litter-mates. This is evidence against the hypothesis that cleft lip embryos differ from normal fetuses only by a retardation in development.

The dilution of low 6-amino nicotinamide doses was found to affect fetal response, such that dilute doses caused lower frequencies of resorption than concentrated equivalent doses.
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