A CHROMOSOMAL ANALYSIS OF BONE MARROW CULTURES OF INBRED MICE

by

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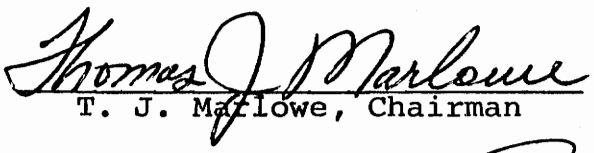

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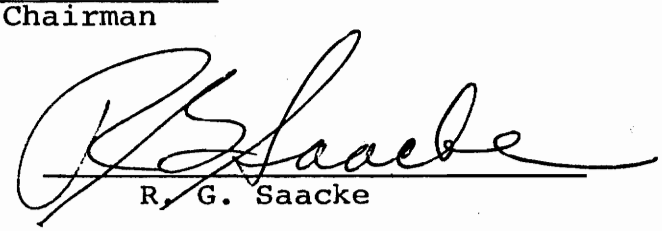

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INTRODUCTION

Near the middle of the twentieth century, cytogenetists became aware of the fact that each animal species had a constant number of chromosomes. This resulted in the study of morphological variations and the establishment of the normal karyotype. With the improvement of preparations, dyes and microscopy techniques researchers became able to study cytogenetic variations in pathological conditions. Initially, those studies were of mature animals in which the abnormalities were expressed phenotypically. Later, it was discovered that the frequency of abnormal conditions was greater in younger animals. Most important of all were the studies of the embryo during the gestation period when a high percentage of the young were still abnormal.

At the same time that cytogenetists were working to determine the extent of chromosome abnormalities, breeders were becoming concerned with inbreeding and its effects. While it was found that some inbreeding may result in superior offspring, other inbreeding produced animals that were less thrifty, less fertile and more susceptible to disease than outbred animals. It was natural that breeders should look to cytogenetists for assistance in determining the special causes and possible solutions to these problems.

There have been two basic drawbacks to the cytogenetic studies in cattle, sheep and swine. The first is the

relatively low frequency of occurrence of abnormal cytogenetic events in adults or totally and the second is the prohibitive cost of maintaining large experimental herds.

Thus, laboratory animals, such as mice, are frequently used in cytogenetic experiments. Mice are very prolific, have a short gestation period and have a low maintenance cost. In the present study, mice, at four levels of inbreeding, were used in an effort to link inbreeding with chromosomal abnormalities in the adult.

LITERATURE REVIEW

Techniques for Chromosome Analysis

During the 1950's, 1960's and early 1970's, there has been much interest in the study of human cytogenetics, especially in the areas of heredity and radiation genetics. This, in turn, has led to the study of animal cytogenetics as well. In some instances, techniques for the analysis of human chromosomes have been modified for use with animals. In other cases, it has been necessary to develop entirely new techniques for use with some animal species. Whatever the case, the renewed interest in this area and the increased importance of cytogenetic work has led to the development of numerous techniques.

Using Blood: Because of the availability of blood from most animals, many researchers have developed techniques to analyze chromosomes by using the blood (Froland, 1962; Robinson, 1964; Evans, 1965; Hungerford, 1965; Metcalf, 1965; Macario, 1966; Salzmann, 1966). Moorehead et al. (1960) collected whole blood and cultured the white blood cells in a media of TC199 with 30 to 40 percent fresh plasma. Penicillin and streptomycin were added as general broad spectrum antibiotics. Nichols and Levan (1962) modified this technique by supplementing the growth media with calf serum rather than isologous serum.

Arakai and Sparks (1963) published a microtechnique

for culturing leucocytes from whole blood. Their technique is applicable to small laboratory animals since as little as .2 ml from whole blood is required. Both Arakai and Sparks (1963) and Knight et al. (1965) used Eagles' minimal medium for culturing leucocytes; whereas Hayry et al. (1970) used a method based on the mobilization of the lymphocytes from lymphoid tissue by pertussis vaccine or supernatant fluid from B. pertussis culture, on the effective separation of leucocytes from erythrocytes by sedimentation with Plasmagel, and on the use of phytohemagglutinin as a mitogenic agent.

Experimenting with an in vitro and in vivo culture of mouse peripheral blood, Buckton and Nellsheim (1968) used a refrigerated centrifuge to break up the erythrocytes and added a .05 percent solution of pokeweek to the culture media. Also studying cells grown in vitro, Rothfels and Siminovitch (1958) used an air drying technique for flattening the chromosomes. Scherg (1962) used blaze drying to improve spreads of chromosomes in leucocytes by igniting the fixitive.

In working with bovine chromosomes, Nichols et al. (1962) used a procedure in which fibrinogen was used to separate the blood. Dextran and phytogemagglutinin were both found to be unsuccessful in this procedure.

Other researchers using bovine blood for displaying chromosomes were Biggers and McFeely (1963) and Ulbrich

et al. (1963). Biggers and McFeely (1963) separated the white blood cells from the blood sample and then cultured the cells until enough were available for examination. Although Ulbrich et al. (1963) had a similar method, more emphasis was placed on the use of Ficoll, a highly polymerized form of sucrose, to separate the red and white blood cells. Further work with bovine chromosomes was done by Basrur and Gilman (1964) and Kieffer and Cartwright (1966).

In sheep, McFee et al. (1965) undertook a study to determine the requirements for in vitro growth of sheep leucocytes and the characteristics of such cells. They found the use of whole blood to inoculate the cultures would eliminate the time and risk involved in separating the white cells and would still give satisfactory results.

Because the availability of whole blood in mice and other rodents is small, methods have been developed in which less than .5 ml of blood is needed. Ths most used site for blood collections has been the tail. Loughman (1965) cultured mouse peripheral white blood cells using only a drop or two of blood from the tail vein. Hybertson and Bryan (1966, 1967) developed a technique requiring only .2 ml of blood. In a microculture method, Doida and Sagahara (1965) used only .02 ml of blood and were still able to culture leucocytes. Using a golden hamster, Galton and Holt (1963) cultured peripheral blood for 6 days before harvesting the cells for chromosome analysis but had a high failure rate.

Using Eggs and Embryos: Some techniques have been developed specifically for culturing eggs and embryos for analysis. These techniques have been especially important in detecting pre-implantation and post-implantation losses due to chromosome abnormalities. Brinster (1963) developed a media to culture the mouse ova from the 2 cells stage to the blastocyst stage while Tarkowski (1966) developed an air-drying procedure for chromosome preparation from mouse eggs. Issa et al. (1968) studied rabbit blastocysts.

In order to detect chromosome abnormalities which may cause post-implantation losses, Wroblewska and Dyban (1969) developed a technique for chromosome preparations from 8 day fetuses without the use of colchicine. However, working with 14 day mice fetuses, Ford and Wollam (1963) examined the liver of the fetus and concluded that a double treatment with colchicine, once to the dam before sacrifice and once afterward to the fetal cells in suspension, gave more mitotic cells than either method used singly.

Using Tissue: Various procedures have been developed for use with tissue samples. Hsu and Pomerat (1953) were able to produce chromosome plates resulting from pre-fixation treatments of cells in tissue cultures with a hypotonic saline solution. This method proved useful with mice, guinea pigs, cotton rats, dogs, and human tumors.

Using a modification of Schultz and St. Lawrence (1949), Gardner and Punnett (1964) described an improved squash

technique for analyzing human male meiotic chromosomes. Hoyer's medium was used to eliminate the disintegration of the chromosomes which frequently does occur with conventional squashing.

Another squash technique was used by Makino and Nishimura (1952) who rinsed the slide, following staining, in H_2O and then applied a cover slide. Complete spreading was then accomplished by rubbing the surface of the cover slip with a blunt instrument. Conger and Fairchild (1953) used a quick-freeze method to make a smear slide permanent.

Sasaki and Makino (1962) used small pieces of kidney or skin collected from adult cattle or horses for tissue culture. Each tissue sample was prepared according to the monolayer technique of Younger (1954). The sample was then incubated for several days in modified Earle's balanced salt solution.

Sachs (1952) and Weleshons et al. (1962) used other squash techniques for processing and examining chromosomes from tissue cultures. Other researchers have utilized air-drying techniques for studying tissue cultures (Rothfels and Siminovitch, 1958; Fox and Zeiss, 1961).

Using Bone Marrow: Bone marrow, because of its great cellularity, its inherent high mitotic activity, the small amount required, and the lack of the necessity of maintaining sterile techniques, is often used for chromosome evaluation (Tjio and Whang, 1965). Using a colchicine, hypotonic

citrate, squash sequence, Ford and Hamerton (1956) developed a technique for evaluating chromosomes from bone marrow cells. They found that colchicine shortens the chromosomes and that the hypotonic treatment expands the cells. Because of accumulating cells in metaphase, colchicine caused the divergence of sister chromatids. Other work with colchicine and colcemid as arresting agents was done by Stubblefield and Klerieg (1965) and Tannock (1965).

While Ford and Woollam (1956, 1963) injected mice with colchicine before collecting the bone marrow, Tjio and Whang (1962, 1965) collected the bone marrow and then incubated it in colchicine for 1 to 2 hours at 20^{o} to 30^{o} C. Others who worked with procedures using bone marrow include Trowell (1959), Pilgrim (1963), Laird and Fox (1964), Bradley and Metcalf (1966), and McFee (1971).

Factors Associated with Chromosome Abnormalities

Swanson, Merz, and Young (1967) have hypothesized that if the chromosome numbers of a randomly selected group of individuals of the same species were counted it is probable that the results would be the same. This conclusion is based on the fact that species are relatively constant entities with regard to numbers and kinds of chromosomes. However, it must be remembered that just as mutations occur in genes and as there may be the loss or addition of genes the same hold true for chromosomes. Heller (1969) declared that human chromosome anomalies are relatively frequent

events and reported the occurrence of anomalies in one of every 208 newborn infants. Along the same lines, Carr (1963) documented that chromosome abnormalities are a cause of early embryonic abortuses in approximately 20 percent of the cases of early spontaneous abortions.

In recent research, O'Neill (1974) observed markedly abnormal chromosomes in more than 12 continuous cell lines derived from human, mouse, hamster, and rat tissues. He noted that such abnormalities appear usually in only about one percent of the cells and are undetectable in normal cells. They consist mainly of numerous multicentric chromosomes and scattered acentric fragments.

McKenzie and Lubs (1974) investigated the type and magnitude of human chromosomal variation in a series of "normal" individuals using a combination of banding techniques. Their findings, taken from chromosome preparations of 77 normal newborn babies, showed an average of 5.08 ± .23 variants per subject. They concluded that the magnitude of chromosomal variation in human populations is far greater than previously estimated.

Pfitzer (1971) found that polyploidy occurs in the heart muscles of swine at the frequency of about 20 percent. He postulated imcomplete mitosis with the fusion of telophase groups as the mechanism for the formation of these polyploid nuclei.

There is some evidence to indicate that not all chromosome

abnormalities are present at birth. In a study of 34 sheep, Bruere (1967) hypothesized that hyper and hypomodal conditions increase in frequency with the age of the sheep. Examining Astrakhan rams during aging, Mukhamedgaliev and Savitskii (1970) confirmed Bruere's hypothesis. They found that three year old rams possess the lowest level of chromosome aberrations in bone marrow cells and first order spermatocytes. The percentage of these abnormalities increases with the age of the animals and reaches a maximum in the eight year old rams.

According to Hafez (1968), there are three main observable types of chromosome abnormalities: (1) an abnormal number, (2) an abnormal structure, and (3) a cell population containing two or more different sex chromosomes. These variations in chromosome number, according to Swanson, Merz and Young (1967), produce two types of cells: (1) those with exact multiples of the basic haploid number, or (2) those whose somatic complements are irregular multiples of the basic number. According to Heller (1969), Swanson, Merz and Young (1967), and others, the first type of cells are termed euploid and may be haploid, diploid, triploid, etc. with the term polyploid being used to refer to any multiple chromosome number above the diploid. Cells with an irregular number of chromosomes are termed aneuploid and are designated by the notation 2N+1 or 2N-1, etc. These numerical

abberations may affect the cells of the body as a whole (heteroploidy) or only a portion of body cells (mosaicism).

By far the bulk of numerical abnormalities of chromosomes are a consequence of nondisjunction or the failure of the separation-migration mechanism. First described by Bridges (1916), nondisjunction involves a failure to synapse and cross over which results in the particular homologues arriving at the metaphase plate in an unpaired, or univalent state. The univalents fail to orient themselves properly between the poles and thus either fail to segragate, or move randomly between the poles. In some cases, they divide into their two chromatids but are unable to divide again and separate during the second meiotic division.

Errors of mitotic disjunction and meiotic disjunction appear to occur with an almost equal frequency (Stewart, 1962). The principal difference lies in the resultant chromosomal constitution, with mitotic errors producing at least two distinct cell populations in the affected individual. Valentine (1966) points out that the frequency of occurrence of abnormal cells derived from nondisjunction depends on how soon after conception a mitotic error occurred. While error at the first division leads to an equal number of normal and deviant cells; errors at the hundredth or thousandth division may produce an individual

in which the somatic and phenotypic error is inapparent.

Luthardt, Palmer, and Yu (1971) examined the increased incidence of nondisjunction with maternal age and the effect of aging in female mice on the occurrence of univalents. In a comparison of oocytes from an inbred strain and a random bred strain, these researchers found a three-fold increase in total univalents during the first nine months in the inbred strain. The random bred strain did not show as great a change.

Another chromosome error which is associated with aneuploidy is anaphase lag. Anaphase lag implies a failure of migration of a chromatid after splitting of the centromere. The result is the loss of the chromatid which either lies inert at the equatorial plate or is lost in the cytoplasm. Eggen (1965) reported that population studies of mice with aneuploidy indicate that chromosome loss is a very significant factor in the occurrence of aneuploidy in the species.

Chromosome aberrations may occur because of the processes of deletion, translocation, or nondisjunction. Moore et al. (1964) found that delection results when a chromosome breaks and the fragments are lost. Along this line, Ahnstrom and Natarajan (1966) have postulated a new theory to describe the mechanism of chromosome breakage. In translocation two chromosomes break and then mutually

exchange blocks of chromatin. Isochromosome formation, or nondisjunction, results from the transverse splitting of a centromeric region yielding two mediocentric chromosomes.

In studying fetal mouse chromosome number, Hungerford (1958) recorded chromosome counts of spermatogonial metaphases from day-old mouse testis. The cells were predominantly diploid with 0.0 percent polyploidy and 1.0 percent aneuploidy in 100 exact chromosome number determinations.

In a study of chimerism, Stewart (1968) pointed out that a chimera is a mixture of cell populations which are different in origin and composition as opposed to a mosaicism in which a mixture of cell populations are of the same genetic origin but are different only in composition. Looking at phenotypes, Stewart (1968) found that there are three types of chimerism resulting either from double fertilization, from fertilization of the first and second polar bodies, or from early fusion of two zygotes. Chromosomal chimerism has been found in human beings (Woodruff et al., 1962), in cattle (Ohno et al., 1962), and in cats (Chu et al., 1964).

Other researchers have reported mosaics in mice (Bhat, 1949; Carter, 1952; Morgan and Holman, 1955). Many of the mosaics which arise in heterozygotes are thought to be

caused by somatic crossing over as postulated in a theory by Gruneberg (1965). First developed by Carter (1952) and refined by Gruneberg (1965), somatic crossing over takes place in the four-strand stage, and the subsequent separation of the centromeres is equational.

While many researchers have studied those abnormalities which occur naturally, Beatty and Fischberg (1949) produced triploidy by subjecting fertilized mouse eggs to either heat or cold. The cold shock treatment resulted in an insignificant 3.5 percent heteroploid while the hot shock treatment resulted in a significant 35.5 percent heteroploid. Later Beatty and Fischberg (1952) used the hot shock treatment to suppress the first cleavage division and to produce tetraploidy. In that experiment, 20 percent of the experimental eggs were heteroploid as compared to only .45 percent of the controls.

In other work concerned with causing polyploids, Beatty and Fischberg (1952) were able to classify the modes of origin of polyploids into the following groups: (1) spontaneous origin, (2) temperature shock during fertilization, (3) colchicine treatment, and (4) ether and ethyl alcohol exposure. Other means of producing polyploidy could be by the use of narcotics, sulphahydral poisons, and hybridization. Edwards (1958) also found that colchicine can produce heteroploidy in the mouse when injected intrauterinely prior

to mating. In that study, Edwards (1958) also found that heteroploid embryos did not survive. This fact was further investigated by Smith and Marlowe (1971) who concluded that, in swine, chromosomally abnormal embryos are unable to survive implantation.

Turning to natural occurrence of chromosome abnormalities, Beatty and Fischberg (1951b) studied polyploid mouse eggs as 3 1/2 days after fertilization. They found the mean number of cells in polyploid mouse eggs, relative to diploids of the same age, was approximately in inverse proportion to the number of chromosome sets present.

In another study, Fischberg and Beatty (1951) took females from a stock with a high proportion of spontaneously produced heteroploid eggs at 3 1/2 days after copulation and studied them until mid-term and again concluded that the ratio of cell number in polyploid eggs to cell number in diploid eggs decreases with an increase in the number of chromosome sets present. Another of the results of their studies (Beatty and Fischberg, 1951a) was to establish that the silver factor, or a closely linked factor, is probably responsible for the production of heteroploid eggs in silver stock mice. These researchers later were able to produce experimentally the same types of heteroploidy as occurred spontaneously in the silver line by using the hot shock treatment (Fischberg and Beatty, 1952a).

In further experiments with the silver strain and other inbred strains, Fischberg and Beatty (1952b) found that crossing different strains of inbred mice raises the proportion of heteroploid embryos significantly. In silver matings, the incidence of heteroploidy was increased still further, possibly because of a separate factor.

Later, Beatty (1957) concluded that the spontaneous incidence of heteroploidy in mammalian embryos is apparently under genetic control, basing this conclusion on the great difference in percentage of spontaneous heteroploidy between silver stocks of mice and a variety of non-silver strains. Braden (1957) also presented evidence that the susceptibility of eggs to many aberrations of the normal processes of maturation and fertilization is under genetic control. Reports of genetic control of the spontaneous incidence of heteroploidy were also made by Russell (1962) and Uchida (1963). Furthermore, Miller (1963) stated that genetical factors were important in the etiology of many types of abnormal differentiation. Krzanowska (1969) was able to show that sperm head abnormality is polygenically determined with one of the genes being located on the Y chromosome of mice.

Looking at the causes of hyperploidy in opossums, Sinha (1967) found the two major factors to be non-disjunction and accidental chromosome loss in tetraploid leucocytes

which were believed to be formed from endomitosis in bone marrow or from two diploid cells fusing to form a primordial tetraploid cell. Tetrapolar mitosis explained the somatic recombination and segregation in tetraploid hybrid cells which subsequently gave rise to near haploid daughter cells by a process of double reduction division. A significant tendency for non-disjunction to be inherited in certain human families has been reported by Hamerton et al. (1961) and Hauschka et al. (1962).

Triploidy is one of the commonest chromosomal aberrations arising either spontaneously or through experimental induction, such as the work done by Beatty and Fischberg (1949) or Beatty (1957). In classifying the conditions that exist at syngamy which lead to triploidy, Austin (1960) found that triploidy may arise from any of three anomalous forms of syngamy: (1) polyandry, one female and two male pronuclei; (2) polygyny, one male and two female pronuclei; or (3) aneugamy, a haploid pronucleus together with a diploid pronucleus, either being the male and the other the female. Polyandry and polygyny are both under some genetic control and are the two most important controlling sources for triploidy with the incidence of both of these classes of anomaly increasing in aging eggs. The two anomalies, according to Austin (1960), may surpass 25

percent and even approach 50 percent of the aged eggs undergoing fertilization.

Polyandry arises from polyspermy, an event which Austin and Braden (1953) investigated in the rat and rabbit. Their results were along the same line as Austin's (1960) above mentioned study, for Austin and Braden (1953) found that susceptibility to polyspermy increases rapidly after ovulation with between one-quarter and one-third of all eggs that have a chance to become polyspermic actually doing so within two to three hours after ovulation. Little increase in susceptibility occurred after the first three hours. These findings were confirmed in part by Butcher and Fugo (1967) in their study of delayed ovulation and chromosome abnormalities in which they linked overripe ova with chromosome abnormalities and embryonic death.

Few reports deal with the question of chromosome number variability in germ-line cells. One researcher who did work in this area was Feckheimer (1961), who made chromosome counts on 431 spermatogonia and 400 primary spermatocytes from 49 mice in order to determine the type and the frequency of heteroploid spermatogonia and their fate. He found the chromosome number was hypodiploid or hyperdiploid in 18 percent of the spermatogonia with 7.2 percent of the spermatogonia being polyploid. Only 4.7 percent of the primary spermatocytes were heteroploid and

all of these heteroploid cells of the primary spermatocytes were close to diploid.

While examining 5,000 mouse oocytes in vitro, Donahue (1970) found three types of errors of the first polar body. These included: (1) twenty (.4 percent) oocytes with a very large polar body possessing an organized metaphase II spindle instead of the usual scatter and degenerate polar body chromatin, (2) four (.08 percent) oocytes in which two normal-sized polar bodies shared equally all the egg chromatin, and (3) one (.02 percent) oocyte with all the chromatin in one group, probably representing a failure of polar body formation.

In a later study, Donahue (1972) made chromosome counts of 338 mouse zygotes in late prophase and metaphase of the first cleavage division and found 96.4 percent diploidy, 1.8 percent hypodiploidy, 1.2 percent triploidy and .03 percent tetraploidy. This led to the conclusion that, under normal conditions, male and female gametes transmit few chromosomal abnormalities.

Under ideal conditions, Austin and Braden (1953) found 1.2 percent polyspermy in rats and 1.4 percent in rabbits. Hungerford (1958) found 1.0 percent polyploidy and 2.0 percent aneuploidy in 100 mouse fetuses that were ten days old. However, when Austin and Braden (1953) delayed matings after ovulation, the incidence of polyspermy

in both rats and rabbits increased rapidly to a high of 8.8 percent.

Some researchers have studied chromosomal abnormalities in developing embryos to determine the relationship with embryonic mortality. Bomsel-Helmreich (1961) made observations on sows which had been served more than 44 hours after the onset of estrus. Results showed that 26 percent of the gestating sows had heteroploid embryos at 17 days of gestation. The heteroploid products were either triploid or mosaic. However, at 26 days, no chromosomal abnormalities were present. Hofsaess (1969) reported a total of 12 percent abnormal rabbit embryos in his study of California and Dutch-Belted does. Smith and Marlowe (1971), in studying 25 day old pig embryos, found no polyploids but did find one monosomic embryo. They postulated that the mosaic embryo could have arisen from anaphase lagging or nondisjunction.

In studying humans, Heller (1969) found that, in most cases, the result of chromosome breaks is unknown. Many agents have, however, been demonstrated to produce breaks experimentally such as ionizing radiation, alkylating agents, nitroso-compounds, antibiotics and DNA precursors. The incidence of human chromosome anomalies are relatively frequent events occurring in .48 percent of all newborn infants. At least 25 percent of all spontaneous miscarriages

result from gross chromsome errors (Carr, 1963). The etiology of numerical aberrations are believed to arise from unequal chromosome distribution or nondisjunction. This can happen at the first, second, or both meiotic divisions.

Heller (1969) summarized the syndromes related to human autosome abnormalities as: Down's syndrome, E trisomy syndrome, trisomy 17 syndrome, trisomy 18 syndrome, D syndrome, trisomy 22 syndrome, Cri-du-chat syndrome, and Philadelphia chromosome. Syndromes related to sex chromosome aberrations include Klinefelter's syndrome, Turner's syndrome, triplo-X syndrome, and the YY syndrome.

OBJECTIVES

The objectives of this study were:

(1) To compare the frequency of abnormal chromosome number in bone marrow cells of 14 week old mice with 0, 50, 73, and 90 percent levels of inbreeding.

(2) To identify the general types of abnormal chromosome number in bone marrow cells of 14 week old mice with 0, 50, 73, and 90 percent levels of inbreeding.

MATERIALS AND METHODS

Experimental Animals

The experimental animals were from the mouse colony of Dr. J. M. White, Department of Dairy Science, Virginia Polytechnic Institute and State University. Dr. White's inbred lines of mice were developed from a large outbred popluation of ICR albino mice which were obtained from the Institute of Cancer Research, Philadelphia, Pennsylvania. In the production of these inbred lines, litters were sexed and randomly standardized to eight offspring with the remainder being discarded. The young mice were identified by toe clipping, weaned at twenty-one days, and randomly assigned to cages with the restriction that each cage must contain mice from more than one litter. The mousery was maintained at a constant temperature of 72° F. and a relative humidity between 50 and 60 percent, with equal hours of day and night on twelve hour shifts. The mice were kept in single pen mouse boxes and were fed a commercial mouse breeding and growth ration and water ad. libitum.

The experimental males and females were first put together at eight weeks of age and were checked daily for the vaginal plug. After mating, females were separated from the other mice and placed in individual boxes. Following the birth of a litter of mice, all siblings were numbered for identification. At three weeks of age the siblings were

weaned and the males and females placed in separate boxes. The experimental mice, regardless of level of inbreeding, were sacrificed at approximately fourteen weeks of age. Animals of various lines and levels of inbreeding were sacrificed on the same day in an attempt to eliminate procedural variations caused by sacrificing all mice at a given level of inbreeding on the same day.

Bone Marrow Procedure

The procedure used was a modification of the technique reported by Ford and Wollam (1963). An animal to be sacrificed at approximately 14 weeks of age was injected intraperitoneally with .03 ml of 1 mg/ml stock solution of Velban[1] one and one half hours prior to sacrifice. After the mouse was sacrificed, the femur was cut out, the muscle removed from the bone, and the proximal and distal ends of the femur removed with a pair of scissors to allow good entrance to the bone marrow. Three ml of a 1.0 percent sodium citrate solution were used to flush out the bone marrow into a small test tube. The contents of the test tube were thoroughly and carefully mixed by agitating the test tube which was then incubated for twenty minutes at 37° C. After incubation, the test tube was centrifuged for two to three minutes at 200 to 300 r.p.m. and the supernatant removed and disgarded.

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1 Venblastine sulfate obtained from Eli Lilly and Company, Indianapolis, Indiana.

Next, 1 to 2 ml of fresh methanol:acetic acid (3:1) were added to the test tube, the contents shaken gently, and allowed to stand for thrity minutes, after which the contents were again mixed gently and then centrifuged for five minutes at 300 r.p.m. All of the supernatant was discarded and 1 ml of fixative (consisting of one part of glacial acetic acid to three parts absolute methyl alcohol) was added. Drops of this suspension were placed on clean slides at 54^{o} C. and allowed to air dry. The dried slides were stained with a stock solution of Giemsa[2] (made up by mixing 7 ml Giemsa plus 3 ml of .15N NH_4OH plus 90 ml distilled H_2O) for fifteen minutes and then run through a series of five baths of which the first three were absolute acetone and the last two were zylene. The slides were kept in each bath for three seconds and then air dried. After the slides had dried, a cover slip was mounted with permont. A total of three slides was made for each animal in the experiment.

Scanning of Slides

All slides were carefully and systematically scanned under a Nikon microscope at 100X magnification. The exact location of each chromosome spread was recorded by the use of a graduated mechanical stage. Chromosome counts were made on cells with an apparent integrity of the cell wall,

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2 Giemsa stain pH 6.8 obtained from Grand Island Biological Company, Grand Island, New York.

dispersion of the chromosomes, and distinctness of chromosome number. The counts were made on the first twenty-four cells fitting the above criteria in each animal. If twenty four countable spreads were not found, the animal was not included in the statistical analysis. If a count other than normal (2N=40) was observed, additional counts were made on the same cell to eliminate errors. At the control level and at each level of inbreeding, twenty animals were selected at random and the slides of these animals were scanned. An exception to this was at the 73 percent level where only thirteen animals were available. The term polyploid was used to refer to any multiple chromosome number above the diploid. Cells with an irregular number of chromosomes such as 2N+1 or 2N-1, etc. were termed aneuploid.

Statistical Analysis

The number of normal cells from each of the twenty four cells counted from each of the seventy three mice were divided by twenty four to obtain the percent of normal cells for each mouse at each level of inbreeding. The arc-sin of the square root of the precentage values were then used for analysis of variance between and within levels of inbreeding and the analysis of variance run on a Monroe Model 1860 desk computer.

RESULTS

A modal class of 40N chromosomes was found for all mice even though there was considerable variation within each group. Of the 480 cells studied from the control lines with no inbreeding (Table I) there were 367 cells with chromosome counts of 2N=40, 105 aneuploid cells and 8 polyploid cells or 23.5 percent anomalies. The range of abnormalities per animal was from 8.3 to 50.0 percent.

Mice with 50 percent inbreeding produced 298 cells with chromosome counts of 2N=40, 169 aneuploid cells and 14 polyploid cells based on a total count of 480 cells or 37.9 percent irregulars. The range of chromosomal abnormalities per animal was from 20.0 to 54.0 percent (Table II).

Results from the 73 percent inbred line are shown in Table III. At this level, only thirteen animals with 24 countable cells per animal were available. Of the 312 counts, 222 were normal and 90 were abnormal, resulting in a 28.8 percent abnormality. The range of abnormalities per mouse was from 12.5 to 45.8 percent.

Among the most highly inbred mice, 90 percent or eleven generations of half-sib matings, there were 347 normal, 122 aneuploid and 11 polyploid cells among the 480 counts. This resulted in a 27.7 percent level of abnormalities. The range in abnormalities per animal was from 0.0 to 58.3 percent. These results are shown in Table IV.

A graphic representation of the relationship between the percent of abnormalities and the percent of inbreeding is shown in Figure I. The relationship is not linear but indicates a particularly high incidence of variation in chromosome number at the 50 percent level of inbreeding.

Tables V, VI, and VII illustrate the distribution of normal, aneuploid, and total abnormal cells, respectively, at each of the levels of inbreeding. Data on the tables also include means and standard deviations of the samples. The percent normal cells were 76.5, 62.1, 71.1, and 72.3 respectively for 0, 50, 73 and 90 percent levels of inbreeding.

Table VIII shows the results of an analysis of variance based on the arc-sin transformation of the percentage of normal cells. The F-Ratio was 4.38 which indicates that the differences between the levels of inbreeding were beyond chance expectations, being significant at the .01 level of probability.

The Duncan's Multiple Range Test showed that the 50 percent level of inbreeding was significantly different from each of the other three levels but that the other levels were not significantly different from each other.

Table I. Distribution of Cells from Bone Marrow Cultures of Mice at Control Level.

Line	Mouse number	Normal	Aneuploid	Polyploid	Total abnormal
01	38M	19	5	0	5
	41M	22	1	1	2
	43M	22	2	0	2
	40M	16	8	0	8
	211M	18	6	0	6
	195M	17	7	0	7
	12M	18	6	0	6
	59M	18	5	1	6
	146M	16	8	0	8
	154M	19	4	1	5
	52F	15	9	0	9
	7F	12	12	0	12
	61F	21	2	1	3
	8F	21	3	0	3
	5F	19	4	1	5
02	133F	19	3	2	5
	136F	22	2	0	2
	134F	17	7	0	7
	128F	20	4	0	4
	141F	16	7	1	8
Total		367	105	8	113

Percent of total abnormalities to total cells = 23.54 percent

Table II. Distribution of Cells from Bone Marrow Cultures of Mice at 50 percent Inbreeding.

Line	Mouse number	Normal	Aneuploid	Polyploid	Total abnormal
60_1	52F	19	5	0	5
	54F	17	4	3	7
	61F	13	9	3	11
	62F	18	5	1	6
	37F	18	4	2	6
	34M	13	11	0	11
	41M	13	10	1	11
	42M	13	11	0	11
	51M	5	19	0	19
	19M	14	9	1	10
50_3	28M	17	7	0	7
	20M	15	7	2	9
	18M	17	7	0	7
	9M	19	5	0	5
	440M	13	11	0	11
	731M	12	12	0	12
	25M	15	9	0	9
	730M	11	13	0	13
	17M	19	5	0	5
	11M	17	6	1	7
Total		298	169	13	182

Percent of total abnormalities to total cells = 37.92 percent

Table III. Distribution of Cells from Bone Marrow Cultures of Mice at 73 percent Inbreeding.

Line	Mouse number	Normal	Aneuploid	Polyploid	Total abnormal
59_1	24F	21	3	0	3
	9M	15	9	0	9
	19M	13	11	0	11
	11M	15	9	0	9
	27M	19	5	0	5
	18M	17	7	0	7
	10M	17	6	1	7
	12M	16	8	0	8
	3M	15	9	0	9
	15M	17	5	2	7
	25M	17	7	0	7
	17M	20	4	0	4
	31M	20	4	0	4
Total		222	87	3	90

Percent of total abnormalities to total cells = 28.85 percent

Table IV. Distribution of Cells from Bone Marrow Cultures of Mice at 90 percent Inbreeding.

Line	Mouse number	Normal	Aneuploid	Polyploid	Total abnormal
58_1	323M	16	6	2	8
	516M	10	12	2	14
	313M	19	3	2	5
	518M	17	7	0	7
	517M	17	7	0	7
	465M	16	8	0	8
	450M	15	9	0	9
	315M	20	4	0	4
	513M	19	5	0	5
	471M	24	0	0	0
	321M	17	6	1	7
	328M	20	2	2	4
	586M	12	12	0	12
	441M	18	5	1	6
	381M	17	7	0	7
	489M	16	8	0	8
	449M	21	3	0	3
	401M	21	3	0	3
	361M	16	8	0	8
	280M	16	7	1	8
Total		347	122	11	133

Percent of total abnormalities to total cells = 27.71 percent

Table V. Distribution of Normal Cells in Four Levels of Inbred Mice

	Levels of Inbreeding				Total
	0	50	73	90	
Observations (of Normal Cells)	19	19	21	16	
	22	17	15	10	
	22	13	13	19	
	16	18	15	17	
	18	18	19	17	
	17	13	17	16	
	18	13	17	15	
	18	13	16	20	
	16	5	15	19	
	19	14	17	24	
	15	17	17	17	
	12	15	20	20	
	21	17	20	12	
	21	19		18	
	19	13		17	
	19	12		16	
	22	15		21	
	17	11		21	
	20	19		16	
	16	17		16	
Total Number of Observations	367	298	222	347	1234
Number of Animals	20	20	13	20	73
Means	18.35	14.90	17.08	17.35	
Standard Deviation	2.62	3.46	2.36	3.15	

Table VI. Distribution of Aneuploid Cells in Four Levels of Inbred Mice

	Levels of Inbreeding				Total
	0	50	73	90	
Observations (of Aneuploid Cells)	5	5	3	6	
	1	4	9	12	
	2	9	11	3	
	8	5	9	7	
	6	4	5	7	
	7	11	7	8	
	6	10	6	9	
	5	11	8	4	
	8	19	9	5	
	4	9	5	0	
	9	7	7	6	
	12	7	4	2	
	2	7	4	12	
	3	5		5	
	4	11		7	
	3	12		8	
	2	9		3	
	7	13		3	
	4	5		8	
	7	6		7	
Total Number of Observations	105	169	87	122	483
Number of Animals	20	20	13	20	73
Means	5.25	8.45	6.69	6.10	
Standard Deviation	2.80	3.75	2.43	3.09	

Table VII. Distribution of Total Abnormal Cells in Four Levels of Inbred Mice

	Levels of Inbreeding				Total
	0	50	73	90	
Observations (of Total Abnormal Cells)	5	5	3	8	
	2	7	9	14	
	2	11	11	5	
	8	6	9	7	
	6	6	5	7	
	7	11	7	8	
	6	11	7	9	
	6	11	8	4	
	8	19	9	5	
	5	10	7	0	
	9	7	7	7	
	12	9	4	4	
	3	7	4	12	
	3	5		6	
	5	11		7	
	5	12		8	
	2	9		3	
	7	13		3	
	4	5		8	
	8	7		8	
Total Number of Observations	113	183	90	133	518
Number of Animals	20	20	13	20	73
Means	5.56	9.10	6.92	6.65	
Standard Deviation	2.62	3.46	2.36	3.15	

Table VIII. Analysis of Variance Based on Arc-Sin Transformations of the Percentages of Normal Cells

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F-Ratio
Among Levels of Inbreeding	3	961.60	320.52	4.38**
Within Levels of Inbreeding (Error)	69	5052.01	73.22	

**P<.01

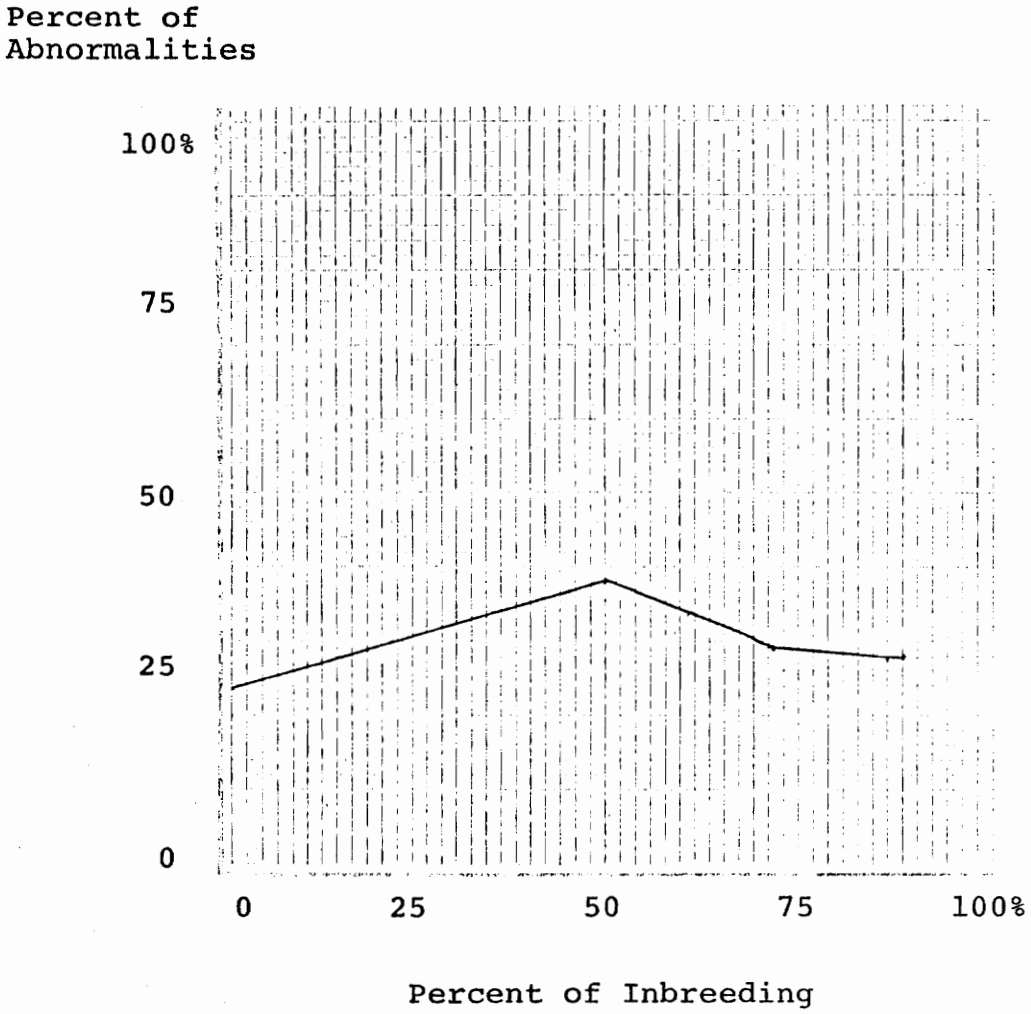

Figure I. A Graphic Presentation of the Relationship between percent of Chromosome Abnormalities and percent of Inbreeding.

DISCUSSION

Chromosome Number and Morphology

The normal modal class number of 40 chromosomes for the mouse was noted in all the lines of mice in this study. The normal complement was characterized by 20 pairs of chromosomes varying in length but all teleocentric. In the males, one pair of chromosomes were of unequal size. These were the sex chromosomes with the smaller one being the Y chromosome. These findings are in general agreement with Cox (1926), Painter (1928), Cutright (1932), Beatty and Fischberg (1951a, 1951b), Griffin and Bunker (1964), and Doida and Sagahara (1965).

Sources of Error in Chromosome Number

Variability in the chromosome number in the bone marrow cells can, in many instances, be traced to the limitations of the procedure which was used. Such steps as suspending the cells in hypotonic solution and fixitive can result in the rupturing of the cell walls, leading to errors in chromosome counts (McConnell et al., 1963).

Chromosome Analysis of Bone Marrow Cultures

In the last two decades, tremendous advancements have been made in the techniques used in the study of cytogenetics. Although initial research in the field dealt mainly with the human, it prompted the investigation into animal cytogenetics, especially in species with disease or chromosome abnormalities.

One important technique used throughout this research was the bone marrow cell culture. Bone marrow has been frequently used because of its great cellularity, its inherent high mitotic activity, the small amount of material required, and the lack of the necessity to maintain sterile techniques (Tjio and Whang, 1965).

In this present experiment, the over-all mean of the aneuploid cell frequency per animal was 28.7 percent. This is higher than Feckheimer's (1961) findings of 18 percent in non-inbred mice spermatogonia and about three times as high as Herschler's et al. (1962) counts of somatic chromosomes of non-inbred cattle. Most of the aneuploids fell within the range of 38 to 42 chromosomes. The analysis of variance of the present data shows a highly significant difference of total normal to abnormal cells at the 50 percent level of inbreeding ($P<.01$).

It appears that there were inherent factors transmitted that enhance the probability for certain types of mitotic aberrations giving rise to aneuploid cells of the bone marrow studied. Also, there are indications that inbreeding enhances these characteristics. Two researchers, Russell (1962) and Uchida (1963) reported that spontaneous incidence of heteroploidy in mammalian embryos is under some genetic control. Miller (1963) concluded that genetic factors were important in the etiology of many of the types of

abnormal differentiation through their effects on steriod metabolism.

In order to determine more presicely where the significant variations of chromosome counts were found, a Duncan's Multiple Range Test was applied to the means of the four levels of inbreeding and the significant difference was found at the 50 percent level of inbreeding. Therefore, the 50 percent level of inbreeding appears to be the major contributing factor to the significant F-test in the analysis of variance.

It appears that genetics influence chromosome number in the mice studied in this experiment. Hamerton et al. (1961) and Hauschka et al. (1962) have reported a significant tendency for nondisjunction to be inherited in certain human families. Hamerton et al. (1965), in studying lymphocyte cultures from isolated human populations, found an increase in the frequency of aneuploid cells as the doners aged. The maximum rate of increase in aneuploid cells was from ages 45 to 64 years. Females were more susceptible than males.

Bomsel-Helmreich (1965) hypothesized that heteroploid mortality was apparently not the result of an inability to establish a normal fetal-maternal circulation, but that death occurred at some stage independent of implantation. Hofsaeses (1969) hypothesized that an abnormal chromosomal

constitution in the rabbit embryo could exhibit its effect in two ways: first, an abnormality arising during oogenesis could affect some or many templates necessary for cellular division thereby causing death before implantation, and secondly, abnormal embryos which develop from normal gametes at or after fertilization would probably survive through the blastula stages; however, when gastrulation begins and new proteins are needed for cell differentiation death would occur because of the abnormal constitution.

The initial objective of this research was to use the developing mouse ova prior to implantation to study chromosomal abnormalities. Two hours before the eggs were to be flushed, the mouse was injected intraperitoneally with velban to arrest the cells in metaphase. Following flushing of the oviducts, the eggs were put in a hypotonic solution, then in fixative, and then air dried. However, a problem occurred in getting the cells to spread sufficiently to allow the counting of the chromosomes. Although repeated attempts were made over many weeks, sufficient spreading was not achieved, and the experiment was modified to the use of the bone marrow procedure.

From the present study, the exact reason for the significant number of chromosomal abnormalities is not clearly understood. However, at some level of inbreeding equal to or lower than the 50 percent level, there is a

significant increase in chromosomal abnormalities with a decreased number at the higher levels of inbreeding. It is postulated that during the early generations of inbreeding the embryos with abnormal complements of chromosomes carrying deleterious genes die off leaving only those with few or no abnormal chromosomes for future generations; or the animals surviving at the higher levels were actually less highly homogeneous because of chance combinations of genes.

SUMMARY

Adult CBA mice at 0, 50, 73, and 90 percent levels of inbreeding were used to study variations in chromosome number from bone marrow cells. Chromosome counts were made from 20 animals at each level of inbreeding except at the 73 percent level where only 13 animals were available. The chromosome number was determined for 24 countable cells per animal at each level, and used to determine if there were significant differences in the variation of chromosome number among the various levels of inbreeding.

A modal class of 40N chromosomes was noted for the mice in all groups. However, there was considerable variation within each group. The average percentages of abnormal counts were 23.5 for the controls, 37.9 for the 50 percent inbred level, 28.3 for the 73 percent inbred level and 27.7 for the 90 percent inbred level.

An analysis of variance indicated a significant difference between the various levels of inbreeding for the percentage of normal cells. Duncan's Multiple Range Test indicated that the major coutribution to the significant F-ratio came from the 50 percent level of inbreeding. Consequently, the relationship is not linear, but the higher level of abnormal cells occurred at or below the 50 percent level of inbreeding with a decreasing number of abnormal cells at the higher levels.

The reason for the significant variation in chromosome number at the 50 percent level of inbreeding was not indicated in this research. However, it can be postulated that during the early generations of inbreeding embryos with abnormal complements of chromosomes carrying deleterious genes die off, leaving only those with few abnormal chromosomes for future generations.

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Glenn Allen Bucher

A CHROMOSOMAL ANALYSIS OF BONE MARROW CULTURES OF INBRED MICE

by

Glenn Allen Bucher

(ABSTRACT)

Adult CBA mice at 0, 50, 73, and 90 percent levels of inbreeding were used to study variations in chromosome number from bone marrow cells. Chromosome counts were made from 20 animals at each level of inbreeding except at the 73 percent level where only thirteen animals were available. The chromosome number was determined for 24 countable cells per animal at each level of inbreeding.

A modal class of 40N chromosomes was noted for the mice in all groups. The average percentages of abnormal counts were 23.5 for controls, 37.9 for the 50 percent inbred level, 28.3 for the 73 percent inbred level and 27.7 for the 90 percent inbred level.

An analysis of variance indicated a significant difference between the various levels of inbreeding for the percentages of normal cells. A Duncan's Multiple Range Test indicated that the major contribution to the significant F-ratio came from the 50 percent level of inbreeding. Consequently, the relationship is not linear, but the higher level of abnormal cells occurred at or below the 50 percent level of inbreeding with a decreasing number of abnormal

cells at the higher levels. From the data it can be postulated that during the early generations of inbreeding the embryos with abnormal complements of chromosomes carrying deleterious genes die off, leaving only those with few abnromal chromosomes for future generations.