PERIPHERAL BLOOD LEUCOCYTE KARYOTYPE ANALYSIS OF TEN BREEDS OF DOGS (CANIS FAMILIARIS)

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PERIPHERAL BLOOD LEUCOCYTE KARYOTYPE ANALYSIS OF TEN BREEDS OF DOGS (*CANIS FAMILIARIS*)

By

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"Providence seldom vouschafes to mortals any more than just that degree of encouragement which suffices to keep them at a reasonably full exertion of their powers."

Certainly Hawthorne could have been referring to the research experiences of a young student. Many a student would turn away in the face of discouragement if it were not for those around him, the workers of Providence. It is indeed a pleasure, then, for the author to thank those who have helped to see this project to completion.

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To Turner

for his patient encouragement
and unlimited faith

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INTRODUCTION

Although the chromosome complement has been recognized as the means for genetic reproduction for several decades, it has been only within the past ten years that precise descriptive studies of karyotypes of man and other animals have been made.

As a result of improved techniques for studying chromosome morphology, several areas of research have been given impetus into new and exciting phases. The study of "taxonomic" karyography has opened new vistas to the evolutionist. With the normal karyotype well defined, the cytologist has been able to approach the naturally occurring or experimentally induced abnormal karyotype with a valid basis for comparison. For the pathologist, the discovery that many human abnormalities are associated with distinct chromosomal anomalies has given the first indications of the etiology of these deficiencies.

Unfortunately, the majority of descriptive mammalian cytogenetic research has been confined to man, and experimental cytogenetic research to mice. A major problem in any scientific investigation is to find the best experimental animal. Although mice are traditional experimental animals, there is a discouraging similarity

between the twenty pairs of chromosomes of its karyotype.

Extensive literature is available concerning the physiological, behavioral, genetical and anatomical variation in the domestic dog, Canis familiaris. Yet, literature concerning the cytogenetics of this mammal is noticably lacking. It has been recorded, however, that the female sex chromosome, the X, is the only non-acrocentric member of the karyotype. Such a characteristic would facilitate diagnostic analysis involving sex chromosome abnormalities.

It is the purpose of this presentation to describe adequately the normal karyotpe of a spectrum of breeds of Canis familiaris, as exhibited by peripheral blood leucocyte cultures. This may establish a basis for pathologic and experimental research and perhaps ultimately for clinical diagnostic use.

REVIEW OF LITERATURE

Moorhead (1962) defined the stock in trade of the cytogeneticist as the "correlation between the cytologically visible chromosomes and the phenotypic expression." Thus, cytogenetics is an anatomical science in that it is morphological and descriptive, but deals particularly with the morphology of genetic content itself.

Certain features of the interphase chromatin have been used as markers for differentiating and identifying specific cells or particular sublines such as the Barr bodies, sex chromatin, and nuclear size and morphology (Moorhead, 1962). But the metaphase chromatin arrangement demonstrates most dramatically the orderly and consistent distribution of the total genetic code. The metaphase chromosomes, in general, are constant in number within a species and with notable exceptions, constant within each cell of an organism (Swanson, 1961).

Photographs of dividing cells at metaphase are usually used in making a karyotype analysis of an organism. The chromosomes are cut out and arranged in homologous pairs on the basis of such "taxonomic" features as (a) position of the centromere, (b) total length of the chromosome, (c) relative length of the arms to the total length of the chromosome and to each other, and finally,

(d) secondary constrictions and satellites (Moorhead, 1962).

Thus, a standard system of classification for a particular karyotype may be developed. For man, the Denver classification (Book et al., 1960) is most widely accepted. Patau (1960), working concurrently with the Denver group statistically evaluated the chromosome complement of man and demonstrated mathematically that the Denver classification was for the most part sound. The salient feature of both studies was that, contrary to earlier claims that each chromosome was identifiable, certain pairs of chromosomes were so similar in morphology that it was necessary to consider them as a group. In particular, the X chromosome cannot be distinguished from similar sized autosomes.

By thus classifying the chromosomes of an organism, the cytologist is able to recognize the normal karyotype and distinguish it from an abnormal one. Indeed, it is possible with opportune marker chromosomes to identify cell strains not only to a species level, but occasionally even to a particular subline (Moorhead et al., 1960).

Experimentation with lower forms of life involving chromosomal anomalies has been prevalent for decades (Swanson, 1961). But only within the last ten years have chromosome counts for many of the higher mammals been

known with certainty (Ford, 1960). Only with the development of simplified techniques within the last five years, however, has the field of experimental cytogenetics with higher animals become practical (Warkany, 1963).

It would be impossible to survey completely the literature which has been produced concerning the cytogenetics of mammals. Indeed, there have been over five hundred articles on Down's syndrome (mongolism) in man (Stiles, 1964). It is pertinent, however, to illustrate the directions of research in this field by selected examples.

Down's syndrome, one of the first to be recognized as being associated with a chromosomal anomaly, is now believed to be of two types. The translocation type is often associated with a familial transmission by phenotypically normal parents (Becker et al., 1963). The type associated with triploidy of one of the chromosomes in the 13-15 group (Denver classification) is credited to non-disjunction either at meiosis in the parent or early in development of the individual with Down's syndrome (Warkany, 1963). With the knowledge of which type is present, certain conclusions may be drawn concerning the etiology of the abnormality and more meaningful medical counsel given.

By far the majority of human chromosomal anomalies

are associated with abnormal sex chromosomes. Such abnormalities are often expressed phenotypically by bizarre syndromes with various degrees of mental retardation and physical anomalies, particularly affecting the genital organs (Barr, 1963; Schutt and Hayles, 1964; Scherz and Roeckel, 1963; Patau et al., 1961). In addition to Klinefelter's syndrome (XXY), Turner's syndrome (XO), and several types of multiple X females, cases of true hermaphroditism and pseudohermaphroditism have been reported (Becker et al., 1963; Miller, 1963; Barr, 1963; Kesaree and Woolley, 1963).

Warkany (1963) reported five cases of male pseudohermaphrodites with normal karyotypes. One case of pseudohermaphroditism that he studied, however, proved to be a mosaic with 45/XO and 48/XXXY cells or a combination of Turner's and Klinefelter's syndrome.

Such mosaicism is not without precedent. It has been found in phenotypically normal individuals (Becker and Albert, 1963b) as well as in individuals with all of the previously mentioned syndromes(Ford, 1960; Hungerford, 1959; Mauer and Noe, 1964; Terresen et al., 1964). Mosaicism is believed to be a result of nondisjunction during meiotic division or in early embryonic development (Ford, 1960).

Carr (1963), studying human aborted fetuses, found that seven out of twenty-seven of them exhibited highly

abnormal karyotypes. The literature contains several other similar reports (Makino et al., 1962; Delhanty, 1961). Carr plans also to investigate the karyotypes of parents of aborted infants for etiological study.

Although leukemia is considered to be a type of malignancy, attempts are being made to associate particular types of variations in karyotype with specific kinds of leukemia (Sandberg et al., 1964).

Another field which has been opened to the cytogeneticist is that of experimental production of chromosomal anomalies. Ohnuki et al. (1961) have worked with the induction of chromosomal anomalies by irradiation of tissues in culture. Ingalls et al. (1963) are studying the effects on the karyotype of the injection of chemicals in pregnant mice.

The use of mice as experimental animals is convenient since a great deal is known concerning their immunology, physiology and genetics. Yet, the similarity of the forty telocentric chromosomes in the mouse complement makes it difficult to distinguish the individual chromosomes (Moorhead, 1962).

So far, this discussion has centered primarily around mammalian cytogenetics in general. Emphasis has been placed on research concerning man since the greatest amount of literature in this field relates to this

species.

The species in which I am most interested is the domestic dog, Canis familiaris. Ginsburg and Slatis (1962) claimed that the use of dogs in genetic and cytogenetic research should be explored: "the range of genetic variation in the dog includes the broadest biological spectra of physical, physiological, and behavioral traits, and systematic research on the biological bases of these traits has barely scratched the surface. There is no wider potential anywhere among mammals."

Ford (1960) suggested that structural polymorphism of chromosomes may exist among various ethnic groups. Similarly, the development of distinct breeds with highly predictable phenotypes suggest a remarkable variation in the genetic content, and perhaps chromosomal polymorphism in the dog.

As a result of inbreeding to develop distinct breeds, many deleterious effects have been produced. Thus, congenital hip dysplasia is a major breeding hazard in the German Shepherd and has been recorded in thirty-six other breeds (Schnell, 1959). The anatomical appearance bred into the poodle and miniature breeds has presupposed the presence of patellar luxation (Hodgman, 1962).

Many of the syndromes seen in man are also found in the dog. Defects seen include hermaphroditism and

pseudohermaphroditism (Hoskins et al., 1962), hemophilia (Brinkhous and Graham, 1950; Graham et al., 1949; Brown et al., 1963), and leukemia (Khuen, 1947; Bradbury, 1949; White, 1946).

Studies of the cytogenetics of the dog have been relatively few, although Makino (1951) stated that as early as 1894, vom Path studied the chromosomes of the dog and claimed the diploid number to be 64. Malone (1918) studied spermatogenesis in the dog by testicular biopsy and drew the interesting conclusion that the female had 22 chromosomes and the male 21, the sex being determined by the presence or absence of an X chromosome. Makino (1951) also cited Minouchi (1928) as first correctly describing the sex constitution as X-Y, the same as in man, and the diploid number as 78.

Makino (1952) also concluded that the number 78 correct. Hsu and Pomerat (1953) and Awa et al. (1959) cultured canine embryonic lung and heart. Awa et al. (1959) also studied spleen and liver cells. Upon cytological study, both reaffirmed the 2N number of 78.

The normal karyotype of the Beagle has been described morphologically as being composed of seventy-six telocentric autosomes and two sex chromosomes, a submetacentric X and a very small Y (Jacobsen et al., 1963).

Observing the cells of a canine venereal tumor, Ta-

kayama (1958) found 60 out of 146 cells with 60 chromosomes, the others varying between 57 and 61. The cells were characterized by an increased (up to 14) number of submetacentric chromosomes.

Within the last few years, the culture of bone marrow and peripheral blood leucocytes has become popular. Although these cells cannot be cultured indefinitely (Bender and Prescott, 1962), samples are easily obtained and short term growth provides sufficient material for karyotype analyses. Moorhead et al. (1960) described the original procedure for the culture of leukocytes in man. Since that time, many workers have presented modifications of the technique (Genest, 1963; Genest and Auger, 1963; Genest, 1964; Brooke, 1962; Marshall and Capon, 1961; Punnett et al., 1962; Foft and Romero, 1963; Scherz and Louro, 1963). These modifications usually involve (a) the source and type of phytohemagglutinin, (b) the growth media requirements, (c) the length of application and concentration of colchicine, (d) the type of hypotonic solution used and the length of application, and (e) fixation and slide preparation techniques.

While it is not necessary to discuss these variations in detail, it is important to note, however, that all of these workers were using human peripheral blood, and their modifications are suited to the WBC requirements. Cells of different organisms and indeed

cells of the same organism may vary greatly in their requirements for culture (Penso and Balducci, 1963). Study of the specific requirements for canine cells in culture has been for the most part neglected (Juday, 1960).

Using modifications of the basic Moorhead procedure, Biggers and McFeely (1963), Humason and Sanders (1963) and Brown et al.,(1963), reported cultures of dog blood for observation of metaphase plates. Jacobson et al. (1963), described the normal Beagle karyotype as a basis for investigating radiation effects on chromosome morphology.

The only reported cytological study of abnormal dogs using this technique was made by Brown et al. (1963). Although he described both canine hemophilia and pseudohermaphroditism, karyotype analyses were made only on the hemophiliacs which were found to have normal karyotypes. Hemophilia in man is generally believed to be associated with the normal karyotype also (Mellman et al., 1961). Brown's study revealed the modal number of chromosomes to be 78 in all cases although cells were seen with chromosome numbers ranging from less than 78 to 80, and X chromosome numbers ranging from 0 to 2 in the females, and 0 to 1 in the male.

MATERIALS AND METHODS

A. Introduction

As was mentioned in the literature review, most researchers employing a peripheral blood culture technique, have suited their procedures to human blood. To obtain optimum results with canine blood cells, it was necessary to run several series of control cultures from a male mongrel dog. Optimum conditions determined by these tests will be included in the following discussion.

The culture and harvesting procedures were in general those of Moorhead et al. (1960).

B. Procurement of Samples

Ten ml blood samples were obtained by venipuncture from adult dogs. The region from which the blood was withdrawn was surgically prepared by scrubbing with Liquid Germicidal Detergent* as contamination was a major hazard. Blood was drawn into a sterile heparinized 10 ml syringe through a 20 gauge needle. As quickly as possible, the needle was removed and the blood trans-

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*Parke Davis, Detroit, Michigan.

ferred into a sterile, heparinized (approximately 0.5 ml ammonium heparin* per tube) 15 ml conical centrifuge tube for sedimentation.

C. Planting the Leucocyte Culture

Although some authors (Moorhead et al., 1960; Ohnuki et al., 1961) suggest mixing phytohemagglutinin with the blood before sedimentation to increase the efficiency of red blood cell agglutination, others (Genest and Auger, 1963; Foft and Romero, 1963; Becker and Albert, 1963a) find this unnecessary. With dog blood, the addition of phytohemagglutinin at this point in the procedure seemed to increase hemolysis. Therefore, phytohemagglutinin was not added at this time. The heparinized blood was allowed to stand at 4° C for 1-3 hours until sedimentation of the erythrocytes left a layer of plasma on the top.

Usually a layer of white blood cells formed a film between the red blood cells and the almost clear plasma. This film contained primarily neutrophils which are considered by some to be "contaminants" as they are not believed to undergo division in culture (Hastings et al., 1961; Levine, 1956). Although these authors have devised ingenious mechanical techniques for removal of these specific cells by utilizing their phagocytic properties,

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*Ammonium heparin, aqueous solution (1 ml 1000 USP units). American Hospital Supply Corporation, Evanston, Illinois.

this author found that with careful technique using a long, large gauge needle, the two to three ml of plasma containing the remaining leucocytes were easily removed leaving most of the neutrophil layer behind. To this plasma-cell suspension, 0.4 ml Phytohemagglutinin-P* was added to stimulate mitosis.

In agreement with other authors, some form of phytohemagglutinin was found necessary to initiate division in the leucocytes. The source did not seem critical since with the use of Brooke's (1962) simple technique, a good mitotic stimulant can be prepared.

Eagle's Minimum Essential Medium (Eagle, 1959) was then added to the plasma-cell suspension in a quantity three to four times its volume. This medium proved most successful when an additional 0.2 mg arginine was added to each ml Eagle's MEM according to the method of Smith (1964). A mixture of equal parts of dog and calf serum was added to a final concentration of 20%. Antibiotic concentration appeared to be critical, and after many trials, 50 units of penicillin and 50 micro-grams of streptomycin per ml of medium was most successful.

The suspension was then inoculated into 16 mm screw cap tubes or roller tubes** in 2 ml aliquots (Foft and

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*Difco, Detroit, Michigan.
**Bellco, Vineland, New Jersey.

Romero, 1963). The unsealed tubes were placed at a 45 angle in a constant flow 5% CO_2 in air incubator at 37 C. The cultures were left undisturbed for 72 hours before any attempts were made to harvest the cells. Usually one half of the cells were harvested at 72 hours and the remainder at 84 hours incubation.

When tubes were used, the cells concentrated in a smaller area and grew in clumps. This produced better results than when the same suspension was inoculated into 2 oz. prescription bottles as suggested by Becker and Albert (1963a), Scherz and Louro (1963), and Genest and Auger (1963).

D. Harvest of cells and slide preparation

1. Colchicine pretreatment

A colchicine pretreatment is prescribed by most authors for arresting mammalian cells at metaphase. With canine leucocytes, metaphase arrests were minimal without colchicine and could be greatly increased by short application of a low concentration of colchicine. Colchicine in Hanks'(1949) BSS (Hanks and Wallace, 1949) was added to the cell suspension giving a final concentration of 2 µg colchicine/ml medium.

2. Hypotonic treatment

In order to produce swelling of intracellular volume,hypotonic treatment was first suggested by Hsu and

Pomerat (1953), who used a basic salt solution without NaCl. Various authors have preference for other solutions which probably have little advantage one over the other. This author prefers Genest's (1964) solution consisting of calf serum diluted 1:8 with distilled water.

When the colchicine pre-treatment was completed, the vials were removed from the incubator and the suspension gently pipetted with a clean Pasteur pipette. Occasionally the cells adhered to the glass necessitating a 4 minute treatment with .25% trypsin in a phosphate buffered saline.

The resulting cell suspensions were placed in a 15 ml centrifuge tube and centrifuged. The supernatant was removed with a pipette and 5 ml 1:8 serum-distilled water solution was added. A gentle pipetting resuspended the the cells in the cells in the hypotonic solution, and the tubes were incubated approximately 20 minutes at 37 C. This time allowed for incubation seemed to be critical as longer periods of treatment caused excessive breakage of cells.

The cells which had settled out, were resuspended gently with a pipette and the suspension centrifuged. The supernatant was removed.

3. Fixation

Two ml of acetic-alcohol (1 part acetic acid to 3 parts methyl alcohol) were added carefully, although a gentle breaking up of the cell pellet seemed helpful in separating the individual cells.

Fixation was allowed to proceed for at least one hour. Due to the softening effect of acetic acid, it was found best to fix for short periods, and never longer than 24 hours. Fresh fixative was made for each sample of cells. The cells were then pipetted thoroughly to break up the clumps. After centrifugation, the supernatant fixative was removed and replaced by 0.5 ml 1:1 acetic-alcohol solution and the cells were resuspended. If the suspension appeared excessively cloudy, more of the 1:1 acetic-alcohol was added. This 1:1 acetic-alcohol was not allowed to act on the cells more than five minutes.

4. Slide preparation

Meanwhile, slides were placed on a plastic tray tilted at a 45 angle in the direct current of air from an electric fan. Using a Pasteur pipette, a few drops of fixed-cell suspension were placed on one end of the slide. The circulating air provided by the fan spread the cells and dried the suspension within 60 seconds.

Another technique was used to prepare some of the

slides by placing a drop of suspension on an ice-cold slide. This was flamed and shaken gently to remove water droplets. Such a technique spread the cells better but increased the staining time from 15 minutes to six hours.

5. Staining method

The stain used most frequently was 2% aceto-orcein* although Feulgen was also tried. Cells were stained in freshly filtered aceto-orcein for 15 minutes, or six hours if flame-dried. They were then taken through 95% ethyl alcohol, 100% ethyl alcohol, and 1:1 ethyl alcohol-xylene for one minute each, followed by 100% xylene for five minutes. The preparations were mounted with #1 cover slips using Permount as the adhesive.

The air dried preparations were usually stained immediately, although they may be stored indefinitely.

E. Karyotype analysis

Slides were scanned on low power (150X) for metaphase spreads. Chromosome counts were made with the oil immersion objective (1350X), and any characteristic morphological features noted. Up to 20 cells per sample were counted. For more detailed observation of particular metaphases, photomicrographs were taken and karyo-

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*Chroma Gesellschaft, Schmid and Co, Stuttgart, Germany.

grams were constructed.

Photomicrographs for routine recordings were taken with a 35 mm Leica camera mounted by means of an Ipso adapter on a Bausch and Lomb Research microscope fitted with a 90X oil immersion apochromatic objective and illuminated by a ribbon filament lamp. A Wratten 58 filter was inserted in the light beam. The film found most satisfactory was 35 mm High Contrast Copy Film* developed with Dektol. Negatives used for photographic enlargements of metaphase spreads and interphase cells were made with 5X7 Kodalith Panchromatic film* in a Bausch and Lomb Model L camera. The chromosome photographs were then cut out, paired and arranged on a white background in order of decreasing size with the sex chromosomes last. The results of the karyogram analyses are shown in Table I.

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*Eastman Kodak Company, Rochester, New York.

RESULTS AND DISCUSSION

The development of a reliable culture technique proved to be the most difficult yet crucial aspect of this research investigation.

The maximum length of time the leukocytes remained alive was 156 hours (See Graph 1). This is a much shorter period than the 17 days Bender and Prescott (1962) were able to maintain viable human leucocytes. This is undoubtedly due to inadequate growth medium requirements for canine tissues. Juday (1960) encountered a similar problem in maintaining viable canine mast cells. The addition of more arginine (Smith, 1964) and use of 20% serum (10% dog and 10% calf) seemed to improve viability. Needless to say, this is an area of study which merits attention.

The maximum number of metaphases were obtained when the cells were harvested over the 72 to 84 hour incubation period (See Graph 1). Bender and Prescott (1962) found the maximum number of dividing cells could be obtained by harvest at three, seven, and ten days. They observed no mitoses at 17 and 21 days. Their cells, however, maintained a healthy appearance for two weeks, whereas the author seldom maintained canine cells for

longer than seven days before the cultures appeared moribund.

By determining colchicine time and concentration effects, optimum conditions for demonstrating metaphases were provided. Graph 2 shows the effect of colchicine over varying lengths of treatment. The percentage of mitoses increased with time, but concurrently there was an increase in aberrant mitoses. Such metaphases include colchicine-clumps with poorly staining, ill-defined chromatin. The optimum time of application should be one which would demonstrate a good percentage of metaphases, with the highest "take" of useful metaphase plates. This was four hours. By plotting treatment time against the percent of mitoses and comparing various colchicine concentrations (Graph 3), it was noted that the concentration of colchicine was not as critical as the treatment period. Two μg of colchicine per ml medium was somewhat more effective than the higher and lower concentrations, and was the concentration generally employed.

A study of the karyograms was made both microscopically and photographically. Scoring was done on well spread metaphases, and the total number of chromosomes as well as the number of X chromosomes were recorded (Table 1).

In the early stages of this scoring, the occurrence of polyploid cells was not as rare as might be expected.

Ploidy was usually of the 4N and 6N type, and in addition, a large number of bi-nucleate and tri-nucleate cells were always present. Such nuclei were most often similar in size and centrally located, although nuclei of unequal size and eccentric location were not uncommon.

It was first suspected that either or both of these phenomena were colchicine effects but controls harvested without colchicine also exhibited both polyploidy and binucleate cells. Frequent checks were made on 1000 cells which showed that the euploid metaphase to uninucleate cell ratio was no less than the polyploid metaphase to multinucleate cell ratio.

It is important to consider some conditions which could account for this multinucleation. Recall that (a) the cells were grown best in clumps, (b) they were fixed as a pellet, and (c) they were usually gently pipetted free. In addition, the acetic acid softened the cell membrane which became ill-defined when the slides were prepared. Therefore, the binucleate and trinucleate cells could result from inadequate separation of cells due to clumping, pellet formation and softened cell membrane. Polyploidy would appear to result if such a "clump" of two or three cells were dividing

concurrently. If this were true, however, one would expect to find an irregular pattern indicating more than one set of division figures, rather than the regularly scattered chromosomes usually observed. Plate III shows multinucleate interphase cells and Plate IV polyploid metaphase cells. Further observations should be recorded to determine whether a certain amount of polyploidy in canine leucocytes is truly such an artifact or a normal condition. Puck et al. (1958) found occasional polyploid cells in long term cultures which otherwise maintained a constant euploid karyotype.

Observation of canine chromosomes is complicated by the fact that all of the autosomes are telocentric. Pairing is a function of the total length of the arms. within the 76 autosomes, there appears to be little consistent variation. No secondary constrictions or satellites are seen. Thus, attempts at grouping into a standard classification at this time appears unwise.

If the chromosomes are fixed at mid-metaphase, coiling is greatest and chromosome length, minimal. At this stage (Plate V, Figure 3), they are difficult if not impossible to pair accurately. As the chromosomes begin to progress toward the interphase condition, the arms lengthen and pairing is more easily accomplished. Similarly, the early metaphase chromosome is easily paired with its mate (Plate V, Figure 2). Further, with continued

regression of the colchicine metaphase, or an early prometaphase, pairing is again difficult, as overlapping of arms makes their separation impossible (Plate V, Figures 2 and 4).

Ten breeds of dogs were represented by the 13 successfully grown cultures: 2 German Short-haired Pointers, a German Shepherd, an Italian Greyhound, a Red Bone Hound, a Toy Poodle, an Irish Setter, a Boxer, a Black and Tan Hound, a Great Dane, and three mongrels. Six were female, and the remaining 7 were male.

In al cases, chromosome number varied around a modal count of 78. Cells were observed, however, which had chromosome complements which varied from 62 to over 200 chromosomes.

The possible reasons for the existence of polyploidy of the 4N and 6N types have been discussed. The occasional occurrence of a complement in the range of 117 chromosomes or a triploidy can not be explained at this time, as in no case, were triploid cells seen frequently enough to suggest a stem-line resulting from non-disjunction. Complements of 62 to 82 were considered to be variations of the 2N condition. Such variation was also observed by Brown et al. (1963).

Variation in sex-chromosome number was also similar to that observed by Brown et al. (1963). Cells from fe-

male dogs had two submetacentric chromosomes although an occasional cell was observed with only one. In males, the rule was one submetacentric chromosome, although some cells exhibited none.

An obvious cause of variation in chromosome numbers in cells can be faulty technique (Moorhead, 1962). Certainly there could be a great tendency for the cells to burst and chromosomes to scatter after being swelled with hypotonic solutions, softened with acid and flattened on a slide.

Puck et al. (1958) suggest another source of such variation. With highly refined culture techniques developed for human tissues, they have counted 2000 mitoses finding no chromosome number other than 46 except for an occasional polyploid. Using the same technique on various animals the same consistency in chromosome number was not the case. An anlysis of the 112 cells of a male opossum revealed only the number 22 (or an occasional 44), but in culturing hamster cells, in addition to a stem line of 22 chromosomes, there were many cells with 21 or 23 chromosomes. Indeed, it was possible to produce clonal stem lines of these abnormal numbers of chromosomes in culture.

Considering this variable effectiveness of their culture technique, Puck et al. (1958) noted that the

only cell for which a medium has been definitely defined to allow infinite growth of euploid cells is the S3 Hela clonal strain. Although he described a great degree of success with human fibroblasts in culture with this medium he has not been as successful with culturing such other species as the hamster. Some of the chromosomal variation seen in this study of canine tissues was likely due to such imperfect tissue culture techniques. Certainly before extensive experimental work may be done with canine tissues, a method must be devised to maintain euploidy in cultured cells.

In culturing human peripheral blood, chromosome variation frequently occurs. If there appear to be two major complement types, mosaicism is often implicated. Minor complement types are attributed to causes already discussed. In no case studied here was there more than one major complement type, indicating a lack of mosaicism in any of the canine tissues studied.

It was noted earlier that by using particular marker chromosomes, certain cell sublines could be identified. Indeed, the natural development of variation in the gross morphology of genetic material provides the mechanism for evolution of subspecies and even species. Although no variation has yet been found in the karyograms of persons of widely separated ethnic groups, the possibility was considered. Because of

this, there was reason to assume that there may be chromosome polymorphism in the various breeds of dogs. The variation seen in the canine phenotypes is certainly expressed genetically, but variation in chromosome morphology was not recognizable in the ten breeds of this study or the four breeds of other studies made earlier (Ahmed, 1941; Jacobson et al., 1963). Such lack of chromosomal polymorphism facilitates study of pathological conditions.

Many of the chromosomal aberrations in man are associated with defects in genital organs and are attributed to X chromosome abnormalities. It has been difficult, however, to ascertain whether the abnormal (triploid or translocation or satellite, for example) chromosome was the X or another of the similar sized autosomes. In a dog with such a congenital anomaly, it would be obvious whether the X or an autosome was involved. It is interesting that Takayama (1958) working with canine venereal tumor cells, found the most grossly affected chromosome to be the submetacentric one, the X.

SUMMARY AND CONCLUSIONS

1. Modifications of the Moorhead et al. (1960) technique were described for optimum culture of canine peripheral blood cells.
2. The chromosome complement of ten breeds of dogs was described. The modal euploid 2N number was found to be 78 in all cases.
3. The chromosome morphology of all breeds studied was similar. The 76 autosomes were found to be telocentric. The male sex chromosomes were a submetacentric X and a very small telocentric Y. The females had two submetacentric X chromosomes.
4. Reasons for variation in chromosome number in individual cells of the same organism were discussed.
5. Although the normal complement appeared to be 78, valid experimental work will be limited until euploid strains can be maintained in culture. Further study of medium requirements was indicated.

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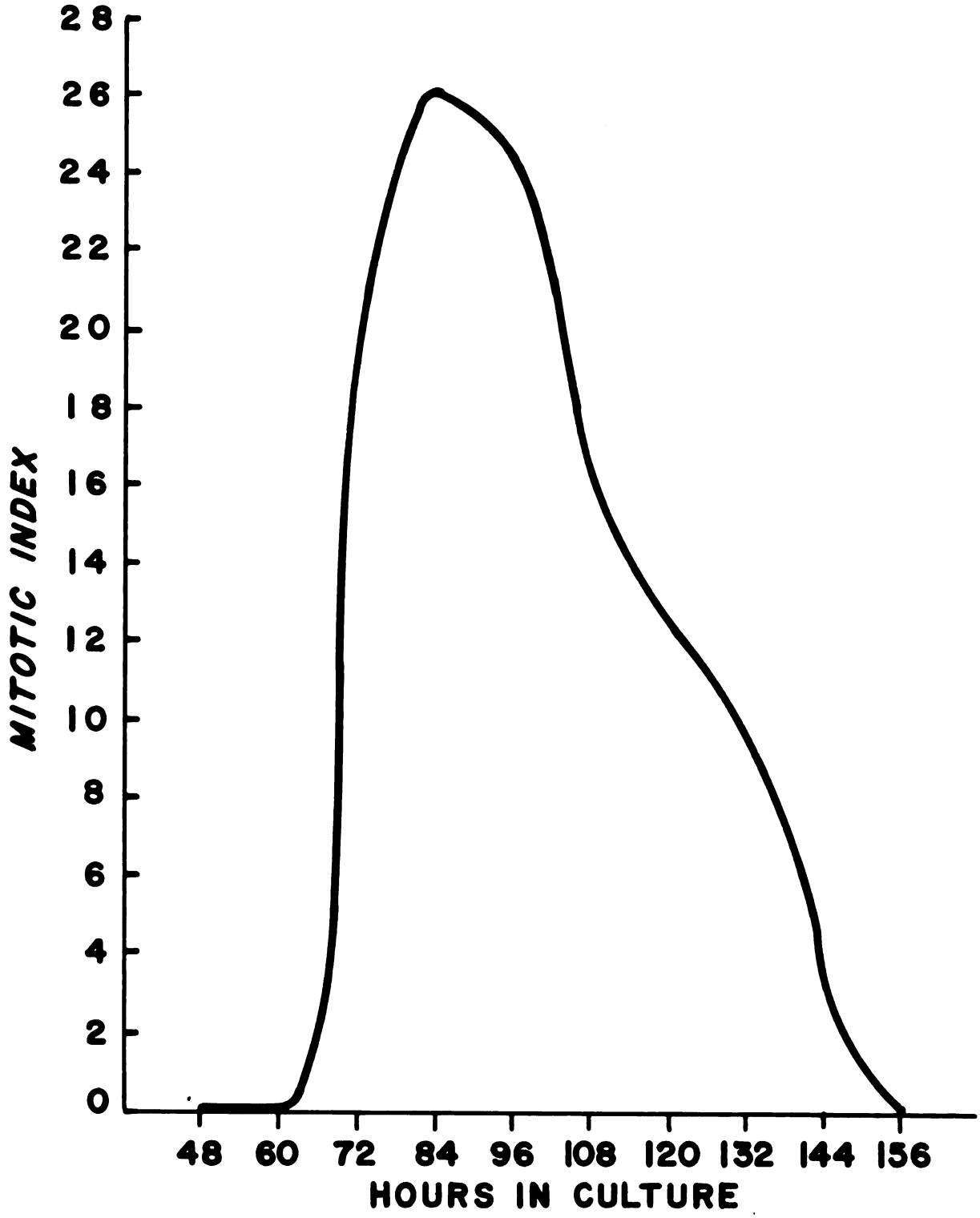
TABLE I. CHROMOSOME COUNTS

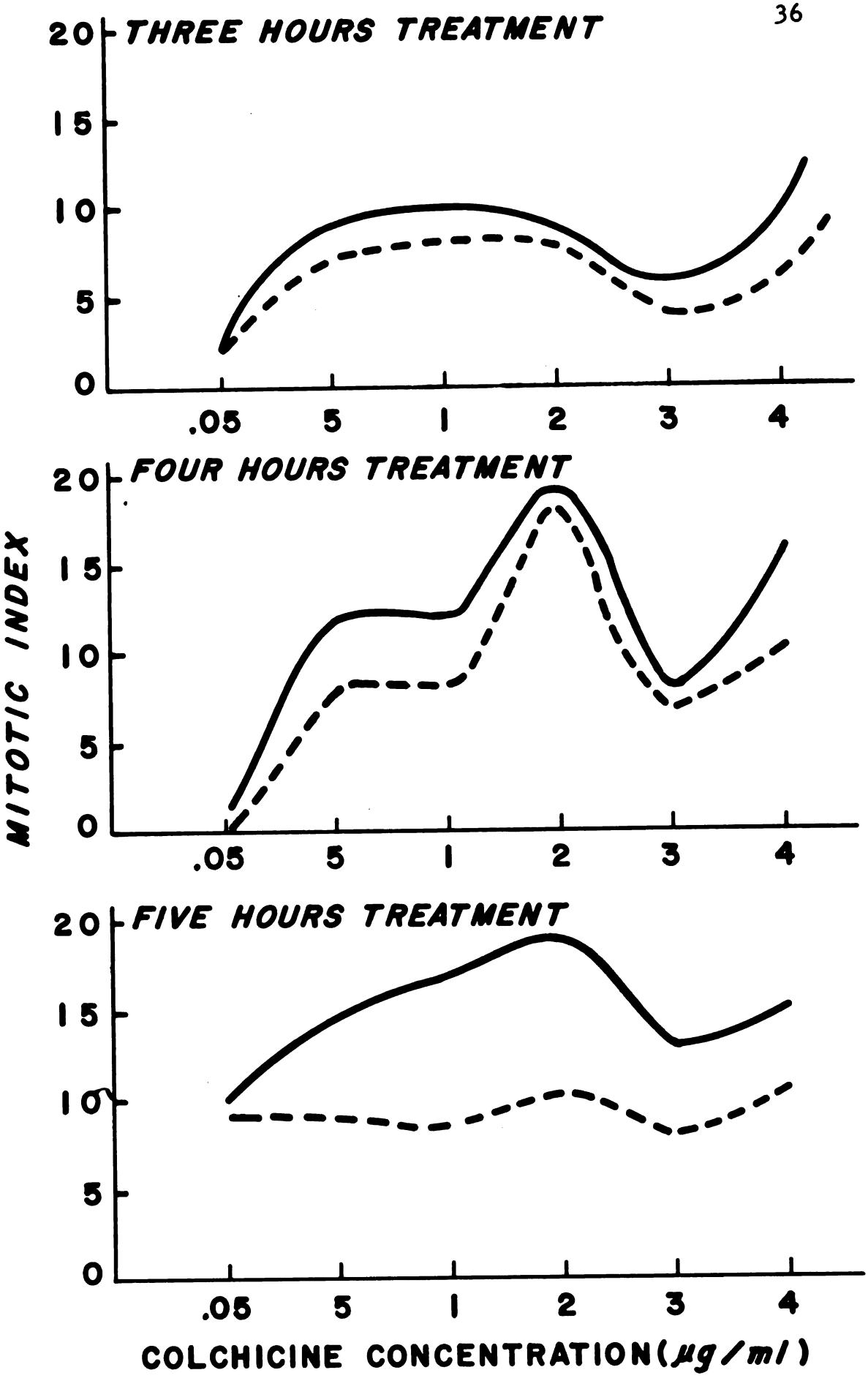
BREED	SEX	CONDITION	TOTAL CELLS COUNTED	CHROMOSOME NUMBER								#chromosomes in polyploid cells	X-CHROMOSOMES*		
				<74	74	75	76	77	78	79	>79		0	1	2
German Short-haired Pointer	F	Normal	15	1	1	0	2	0	11	0	0	150 ; 200	0	0	15
Italian Grey-hound	M	Normal	20	2	0	0	0	0	13	0	4	123	2	17	0
Red-bone Hound	M	Normal	16	2	2	2	0	1	8	1	0		1	15	0
Boxer	M	Normal	11	0	1	0	0	1	5	0	3	112	0	9	1
Black and Tan Hound	F	Normal	7	2	1	0	0	0	4	0	0		0	0	7
Great Dane	F	Normal	18	2	2	0	0	0	10	0	4		0	4	14
German Shepherd	M	**	20	2	0	1	0	0	13	0	3	156	1	17	1
Toy Poodle	F	***	6	1	0	0	0	0	4	0	1		0	1	5
German Short-haired Pointer	M	Normal	12	1	0	1	1	0	5	1	0	104; 156; 192	0	9	0
Mongrel	M	Normal	20	3	6	0	1	1	8	1	0		1	19	0
Mongrel	F	Normal	19	1	3	0	1	0	11	2	0		0	1	18
Mongrel	M	Normal	12	7	1	0	2	0	7	0	0	144	1	10	0
Irish Setter	F	Normal	41	1	3	1	3	5	25	1	3	126	0	7	33

*This column does not include the number of X chromosomes in the polyploid cells.
**Dysplasia of the elbow joint
***Bi-patellar luxation

Graph I. Growth curve for canine leucocytes in culture.


MITOTIC INDEX
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
48
60
72
84
96
108
120
132
144
156
HOURS IN CULTURE

Graph II. Effect of colchicine treatment time on the mitotic index of canine leucocytes in culture. The solid line represents the total number of dividing cells. The dotted line represents the percentage of the total number of dividing cells which are useful for chromosome study.


THREE HOURS TREATMENT
FOUR HOURS TREATMENT
FIVE HOURS TREATMENT
MITOTIC INDEX
0
5
10
15
20
.05
5
1
2
3
4
COLCHICINE CONCENTRATION(μg/ml)

Graph III. Effect of colchicine concentration on mitotic index of canine leucocytes in culture.

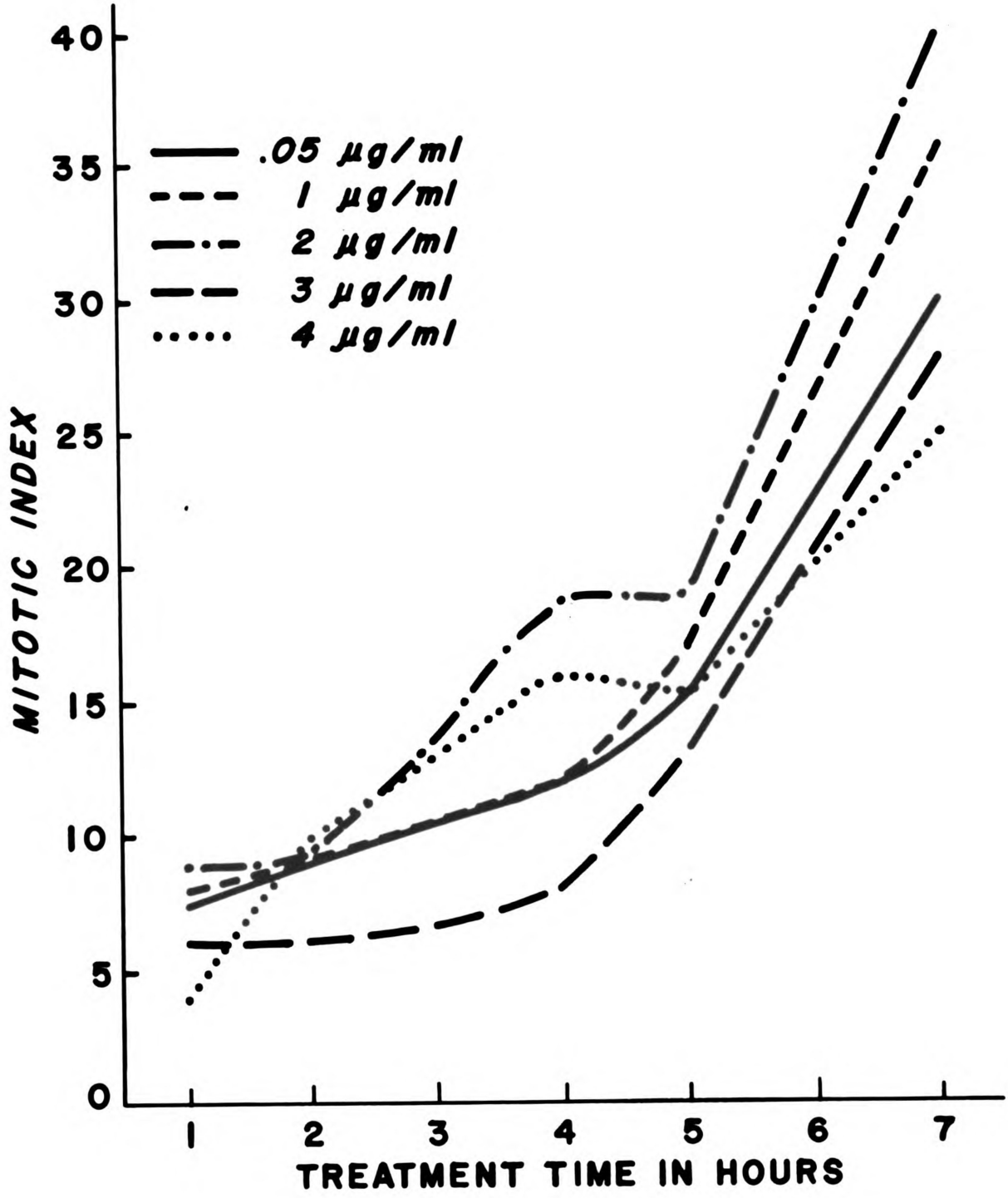

40
35
30
25
20
15
10
5
0
MITOTIC INDEX
.05 µg/ml
1 µg/ml
2 µg/ml
3 µg/ml
4 µg/ml
1
2
3
4
5
6
7
TREATMENT TIME IN HOURS

PLATE I

Figure 1. Colchicine metaphase of a male dog. The X chromosome is indicated by an arrow (2,345 X).

Figure 2. Karyogram of the chromosome complement of a male dog. Constructed from the metaphase shown in Figure 1 (2,178 X).

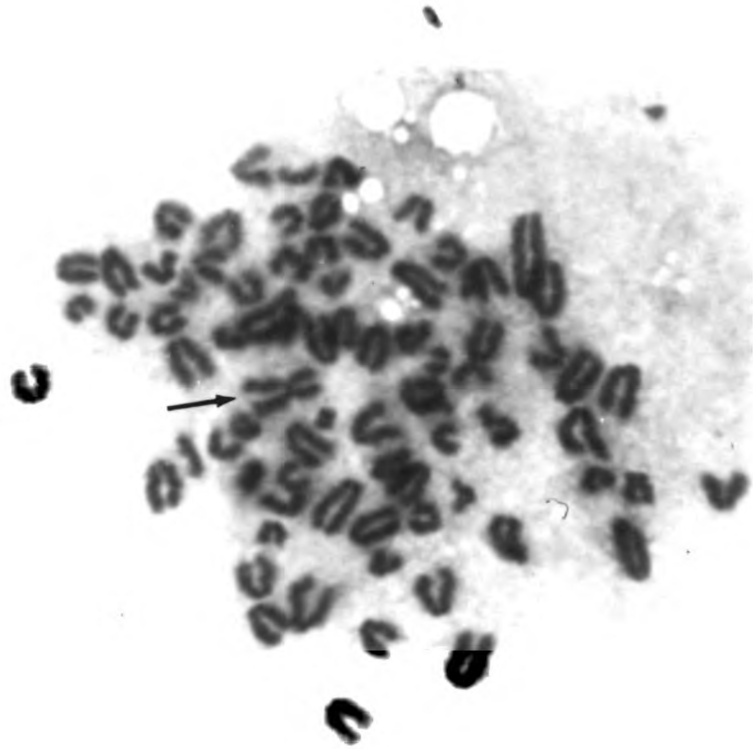


PLATE II

Figure 1. Colchicine metaphase of a female dog. The X chromosomes are indicated by arrows (1,340 X).

Figure 2. Karyogram of the chromosome complement of a female dog. Constructed from the metaphase shown in Figure 1 (1,340 X).

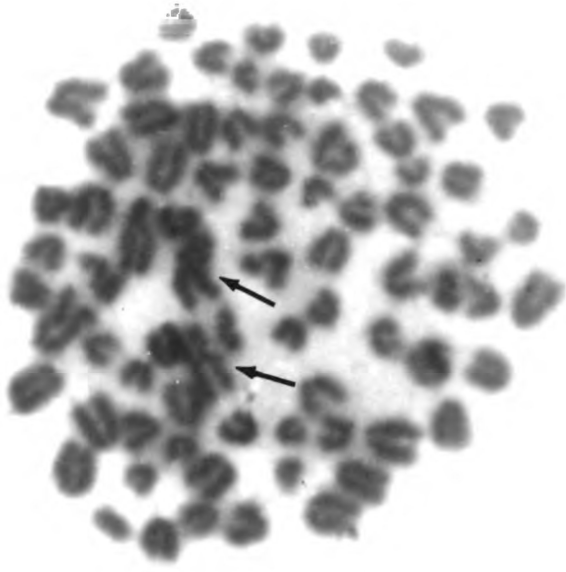
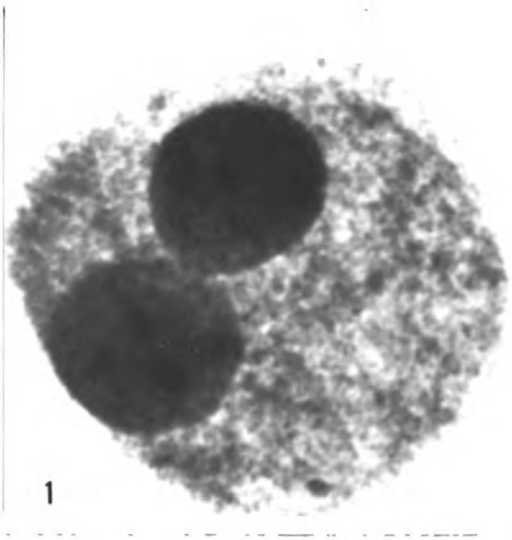


PLATE III

Figure 1. Binucleate interphase cell (1,661 X).
Figure 2. Trinucleate interphase cell (1,830 X).
Figure 3. Quadranucleate interphase cell (1,340 X).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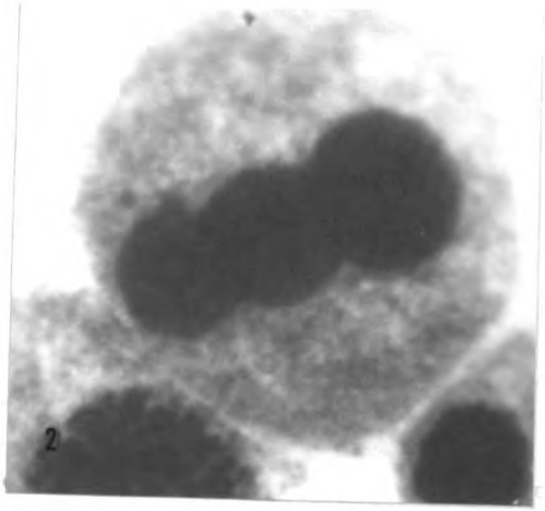
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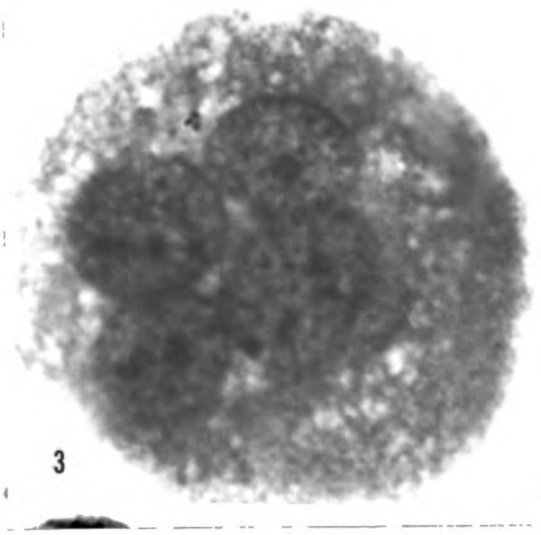
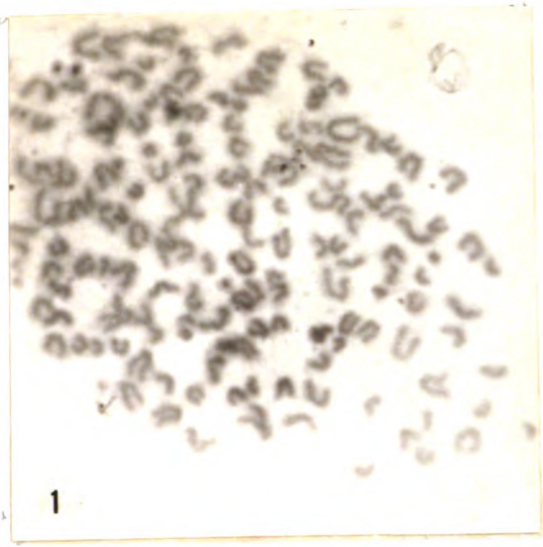
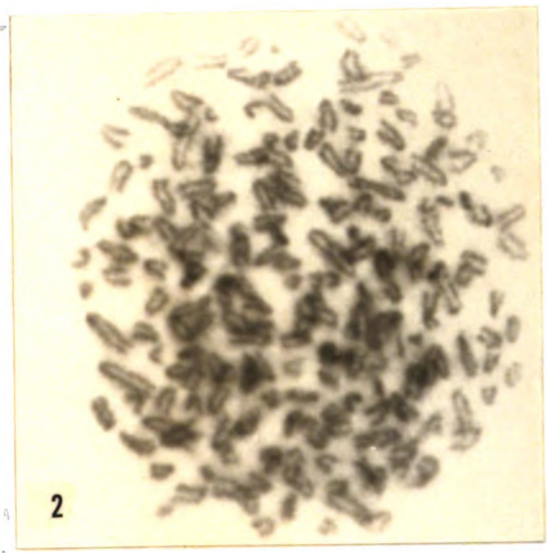

3

PLATE IV

Figure 1. 4N metaphase cell (782 X).
Figure 2. 6N metaphase cell (1340 X).
Figure 3. Possible 8N metaphase cell (1589 X).



1



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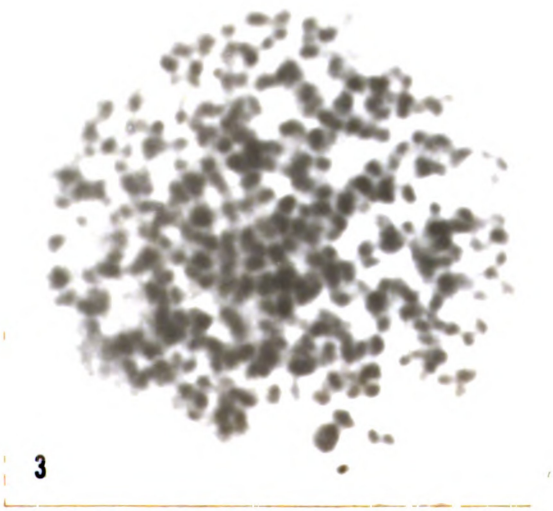


3

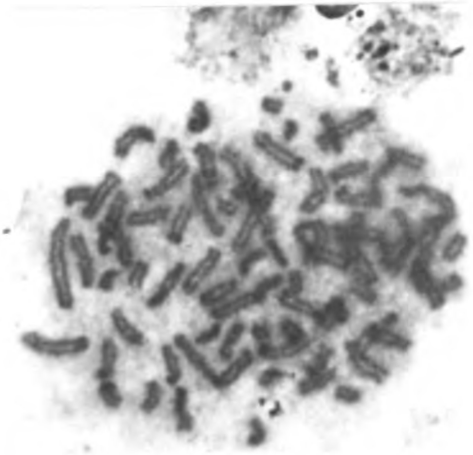
PLATE V

Figure 1. Prometaphase chromosome complement (1,340 X).

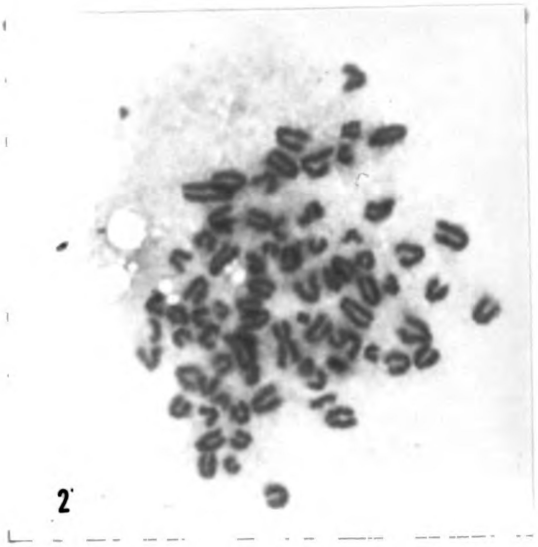
Figure 2. Early metaphase chromosome complement (1,340 X).

Figure 3. Midmetaphase chromosome complement (1,628 X).

Figure 4. Post-metaphase chromosome complement (2,010 X).



1



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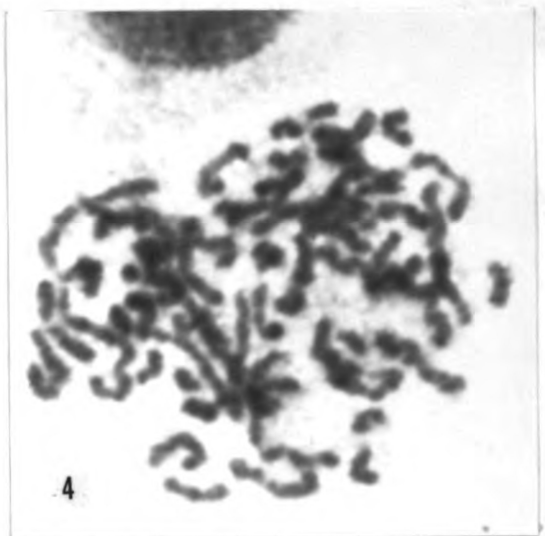
3



4