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# Cytophotometric Determinations of DNA in Karyotypes from Human Leukocyte Cultures of Ten Males and Ten Females

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CYTOPHOTOMETRIC DETERMINATIONS OF DNA IN KARYOTYPES FROM HUMAN LEUKOCYTE CULTURES OF TEN MALES AND TEN FEMALES

(TITLE)

BY

Nancy Sue Whittaker

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# INTRODUCTION

Man has long been interested in the problems of biological inheritance: the nature of the genetic determiners; the mechanism of transmission; and the mechanism of operation and control (Wilson, 1968). Underlying each of these problems are the mysteries of the chromosomes and the DNA contained in the chromosomes.

The purpose of this study, then, is to examine, through a literature review, some of the history of the investigative work performed with human chromosomes, particularly the three cytogenetic conferences on naming and classifying chromosomes. Past areas of investigation including the determination of the 2N number of man and the sequence of DNA replication, present areas of study and a prediction of the future roles of human chromosomal study are also to be reviewed.

The experimental goal is to determine the amount of DNA contained in human leukocyte nuclei for both males and females. This is to be accomplished through the culturing of leukocytes, the preparation and staining of metaphase chromosomes, and final quantative determinations of DNA values, using cytophotometry.

CYTOGENETIC CONFERENCES FOR NOMENCLATURE OF CHROMOSOMES

By the year 1960, several laboratories had begun work with human chromosomes. The rising interest in this work led to the development of several independent systems of naming the chromosomes. A group of men under the leadership of Dr. T. T. Puck met at the University of Colorado under the sponsorship of the Medical School to devise a uniform nomenclature for work with the chromosomes.

The group decided upon the following guidelines as aids in naming chromosomes, as outlined in Birth Defects, Original Article Series 2, 2, 1966:

1. In general, when possible the 22 autosomes should be numbered, 1-22, according to descending length. The sex chromosomes, X and Y, should be referred to as X and Y, rather than numbered chromosomes.

2. The 22 numbered autosomes should be grouped into seven distinctively different groups. Identification of the individual chromosome within its group should be designated by assigning its respective number when criteria are available for the positive identification of that particular chromosome.

3. The seven groups should be determined as nearly as possible according to descending chromosomal length and according to the following criteria for the groups:

   a. Group 1-3 - those chromosomes which are large and have nearly median centromeres.

b. Group 4-5 - those chromosomes which are large and have submedian centromeres.

c. Group 6-12 - those chromosomes which are medium in size and have submedian centromeres.

d. Group 13-15 - those chromosomes which are medium in size and have acrocentric (nearly terminal) centromeres.

e. Group 16-18 - those chromosomes which are medium-short and have median or submedian centromeres.

f. Group 19-20 - those chromosomes which are short and have nearly median centromeres.

g. Group 21-22 - those chromosomes which are very short and have acrocentric centromeres.

4. Distinction between individual chromosomes should be based upon the criteria of total length, arm ratio and centromeric index. The criterion of an added satellite should be excluded when computing the various measurements due to the variation in the morphology of the satellites and their connecting strands.

a. The criterion of total length of a chromosome should be expressed as relative to the sum of the total lengths of a normal haploid, X-containing set of chromosomes and expressed per thousand.

b. The criterion of arm ratio of a chromosome should be expressed as the length of the longer arm relative to the length of the shorter arm.

c. The criterion of centromeric index should be expressed as the ratio of the length of the shorter arm to the entire length of the chromosome.

5. The seven chromosomal groups should be referred to by the Arabic numbers, separated by a hyphen, of the longest and the shortest chromosomes within the group.

6. Abnormal chromosomes should be referred to by arbitrary symbols decided upon by the laboratory discovering the abnormality. The symbol should be prefixed by the name of the laboratory, thus making specific, unestablished interpretation of the symbol avoidable.

7. A fixed pattern for presenting karyotypes and idiograms should not be set forth, thus allowing some variability according to taste of the individual. The basic suggested pattern, however, should place the chromosomes in numerical order by group, with their centromeres aligned. The sex chromosomes should be placed near, but remain separated from, those chromosomes to which they are most similar.

8. The use of the term "karyotype" should be reserved for a drawing or photograph of the pattern of the chromosomes of a single cell. A karyotype from a single cell could then be used to exemplify all of the chromosomes from an individual or a species. The use of the term "idiogram" should be reserved for a diagrammatic presentation of a karyotype and may, thus, be prepared on the

basis of criteria of chromosomes from several cells.

9. Individual preferences for the nomenclature of chromosomes should be secondary to the basic system set forth so that a uniform system could be devised which would allow cross references between laboratories.

In 1963 the London Conference was called to evaluate developments made through the use of new techniques in the area of human chromosomes and to evaluate the effects of these developments upon the characterization of individual chromosomes as set forth by the Denver Conference of 1960.

Reports from several sources showed that good agreement concerning the criteria of relative chromosomal length, arm ratio, and centromeric indices could be obtained, providing clear mitotic figures free from condensation irregularities were used for study.

Variability was reported in positively identifying chromosomes using the new techniques of demonstrating chromosomal constructions and of demonstrating through the use of autoradiography the uptake of tritiated thymidine by the chromosomes. Although both of these new methods aided in describing specific chromosomes, neither method permitted positive identification because of variability in techniques and materials.

The new methods did, however, give rise to the recognition of secondardy constrictions. These secondary constrictions were found to be different from satellite stalks and were therefore defined

as achromatic regions which may sometimes appear as slender parts of the chromosome. The secondary constrictions were found in only a portion of the cells tested and were then seen only after the use of specific types of preparations. The new methods also gave rise to the recognition that different chromosomes incorporated isotopically labeled thymidine in differential patterns.

Some variability was reported in the morphology of particular chromosomes. This variability was especially notable in the Y chromosome, the difference in size occurring because of the inheritance pattern from father to son. Polymorphism due to inheritance was also noted in chromosomes bearing satellites and in No. 16.

The London Conference pointed out that the assignment of numbers to chromosome pairs according to morphological criteria was, in some cases, somewhat arbitrary and, therefore, could not ensure absolute homology.

The following characteristics were noted about some of the grouped chromosomes:

1. Group 1-3 (A) - A secondary constriction was noted on the long arm of chromosome No. 1 near the proximal region in many cells.

2. Group 6-12 (C) and the X chromosome - Four of the autosomes of Group 6-12,(Nos. 6, 7, 8, and 11) were found to be relatively metacentric. Three of the autosomes (Nos. 9, 10, and 12) were sub-metacentric. The X chromosome was believed to be that chromosome in normal female

cells which incorporated isotopically labeled thymidine over nearly its entire length later than all of the other chromosomes of this group. In all of the chromosomes contained in this group, there was found a secondary constriction in the proximal region of the longer arm of at least one chromosome of the pair.

3. Group 13-15 (D) - Satellites were noted in all of the chromosome pairs of the group.

4. Group 16-18 (E) - No. 16 was often noted to have a secondary constriction near the proximal part of the long arm.

5. Group 21-22 (G) and the Y chromosome - Satellites were often found in Nos. 21 and 22. The Y chromosome was found to be larger than Nos. 21 and 22. In this chromosome the terminal end of the long arm and the center constriction were often found to be indistinct while a secondary constriction was often noted. The long arm chromatids of the Y chromosome were seen to diverge less than the arms of the other chromosomes.

In 1966 the Chicago Conference was held at the University of Chicago to discuss a new system of nomenclature including new methods of characterizing both normal chromosomes and abnormal chromosomal sets. This Third International Congress of Human Genetics proposed the following new system of nomenclature for normal and abnormal chromosomal karyotypes, using designations

which could be easily understood, written, and coded for the exchange of data:

1. Numerical aberrations - In describing karyotypes, the total number of chromosomes, including the sex chromosomes should be recorded first and be followed by a comma. The type and number of sex chromosomes should follow the comma. For example, a normal female human karyotype would be designated "46, XX", meaning there is a total of 46 chromosomes, two of which are X chromosomes.

Particular autosomes should be mentioned in the designation only when that autosome possesses some numerical abnormality. After the designation of the sex chromosomes, the group letter of the numerical abnormality should be noted and followed by a plus (+) if an extra chromosome is present or a minus (-) if a chromosome is missing. For example, to indicate a female karyotype which has an extra chromosome in Group C, the designation "47, XX, C+" would be used. If the number of the extra or missing chromosome is known, its number should be included in the designation. If there is uncertainty, a question mark should be included in the designation.

2. Chromosome mosaics - A mosaic chromosomal pattern should be noted in numerical or alphabetical order, using a diagonal (/) to separate the types of karyotypes. For example,a mosaic pattern having both XX and XY cell lines would be designated "46, XX/46, XY."

3. Structural alterations - In discussing the structure of a chromosome, the following symbols should be used:

p - the short arm of a chromosome

q - the long arm of a chromosome

s - a satellite

h - a secondary constriction

cen - the centromere

An increase in length of a chromosomal arm should be noted by placing a plus sign after the arm designation, as "2p+". Similarly, a decrease in length should be indicated by using a minus sign.

The result of a pericentric inversion should be indicated by noting the particular arm (p or q) and using a plus sign or a minus sign to indicate increase or decrease in length, as "p+q-". This designation, enclosed in parentheses, should be preceded by the abbreviation "inv". For example, a pericentric inversion in Group D would be noted as "46, XX, inv (Dp+q-)".

A translocation should be indicated by noting the chromosomal group involved, the particular arm of the chromosome in each group involved, and the increase in chromosomal length indicated by a plus sign or the decrease indicated by a minus sign. This designation, enclosed in parentheses, should be preceded by the letter "t". Thus, a balanced reciprocal translocation between the short arm of a Group B chromosome and the long arm of a Group D chromo-

some would be designated by "46, XX, t (Bp-;Dq+)".

The abbreviations "mat" for mother and "pat" for father should be used to indicate the source of a particular chromosome if family studies make such a designation possible.

4. Duplication of a particular structure should be indicated by repetition of that particular designation. For example, the designation "46, XX, Gpss" would indicate double satellites on the short arm of a Group G chromosome.

5. Isochromosomes should be indicated by placing a letter "i" after the indication of the chromosomal arm of the particular chromosome involved. For example, the designation "46, XXqi" would indicate that one of the X chromosomes was an isochromosome involving the long arm.

6. Ring chromosomes should be indicated by placing a letter "r" after the chromosome involved. For example, the designation "46, XXr" would indicate that one of the X chromosomes was in the structure of a ring.

The Chicago Conference thus proposed that abnormal chromosomes should be named and explained by using the shorthand designation described under the new nomenclature according to their morphology rather than naming them after the laboratory of origin, as proposed by the Denver Report.

## DETERMINATION OF THE 2N NUMBER

The first attempt to determine the number of chromosomes in human cells was made by Hansemann, who in 1891 reported three cells from "normal human tissue" with 18, 24, and more than 40 chromosomes, respectively (Ford and Hameton, 1956). From that time until the appearance of de Winiwarter's classical paper in 1912, diploid numbers ranging from 16 to 36 were reported, the balance of opinion being in favor of 24. De Winiwarter claimed that there were 47 chromosomes at metaphase in spermatogonia, and 23 autosomal bivalents plus an unpaired X in primary spermatocytes.

Painter in 1921 reported the presence of a small Y chromosome in males and was the first to assert that the correct diploid number was 48 in both sexes. In the following two decades most authors supported Painter's position; but de Winiwarter and his associates adhered to the opinion that there was only a single sex chromosome at meiosis in the male and 47 chromosomes in spermatogonia. Koller's account of the behavior of the sex chromosomes in spermatocyte meiosis brought this period to an end just before the outbreak of the Second World War. From then on, the value of 2N = 48 in both males and females remained unchallenged for nearly twenty years, and it seemed that the chromosome number of man had finally been established.

However, in their paper in 1956, Tjio and Levan reported consistent counts of 2N-46 in cultures of lung tissue taken from four aborted embryos, and referred to further counts of 2N = 46

obtained by Hansen, Melander, Melander, and Kullander in preparations of liver, also from aborted embryos. This number was consistently found varying only in cells that were clearly damaged or in cell chromosome groups in which one or two extraneous chromosomes were incorporated during the cytological preparation techniques. The regular loss of two chromosomes during development of organs or growth of the cultures seemed to be most unlikely, and the implication was clear that the generally accepted figure of 2N = 48 was in error. The results of 46 diploid chromosomes per human cell were confirmed by later studies in 1956 made by Tjio and Levan using testicular preparations.

In other work done by Tjio and Puck, chromosome counts of human cells cultivated in vitro were made using cells taken from several different organs: skin, lung, prepuce, testes, cervix, myo- and endometrium, and ovary (Tjio and Puck, 1958). These studies showed that the origin of the initial biopsy had no influence on the chromosome numbers or morphologies. The ages of the subjects varied from one to forty-one years.

In later studies by Tjio and Puck (1958), they found that cells multiplying under carefully controlled conditions for as much as 8 months, during which more than 70 generations of growth occurred (equivalent to more than $10^{21}$ progeny), showed no variation in chromosome number or morphology, except for a small percentage of polyploid cells.

Deviating numbers may originate through non-disjunction, thus

representing a real chromosome number variation in the living tissue (Tjio and Levan, 1965). This kind of variation probably increases as a consequence of the change in environment for the tissue involved in the vitro explantation. Levan, (1956), studying long-carried serial subcultures, found hypotriploid stemline numbers in two cultures and a near diploid number in a third culture.

## THE STUDY OF HUMAN CHROMOSOMES - PAST ACCOMPLISHMENTS

At the beginning of the nineteenth century the study of chromosomes of any type was relatively non-existent (German, 1970). Chromosomes from plant cells were first drawn in 1848 with the study of animal chromosomes beginning shortly afterward. With an increase in the number of nineteenth century microscopists, attempts were begun to count the number of chromosomes from various cell sources. By 1882 Flemming had published some of the first drawings of human chromosomes in dividing somatic cells. In 1900 E. B. Wilson wrote his classic second edition of The Cell in Development and Heredity in which he said that "every species of plant or animal has a fixed and characteristic number of chromosomes, which regularly recurs in the division of all of its cells, and in forms arising by sexual reproduction the number is even." Once the normal appearance and behavior of chromosomes became known, attempts were begun to study variations from the norm.

Three major technical advances were responsible for much of the work that has been done with human chromosomes (German, 1970). The first technique was in the area of tissue culture, wherein cells could be removed from their natural environment and be permitted life and proliferation in vitro. Obviously, with human studies, the culture of cells, tissues, and organs made possible many investigations not otherwise possible. The field of tissue culture was begun shortly after 1900.

The second important advance was in the discovery of the

experimental value of hypotonic solutions: if a cell in metaphase is placed in a hypotonic medium, it swells causing the cell membrane to rupture, thereby allowing the chromosomes to separate and be easily seen when the cell is fixed and stained. This discovery was made accidentally in 1951 in Pomerat's laboratory in Texas. Hsu's technician by mistake washed coverships on which monolayers of mammalian cells were growing with a hypotonic rather than with a balanced saline solution. When these subsequently fixed and stained calls were examined by Hsu and Pomerat, exceptionally clear chromosomal separation and morphology were found in the metaphases. By retracing steps and repeating the error, they noted that the excessive intake of water by cells bathed in a hyposmotic medium was a useful technique for revealing chromosomes of the cells.

The third technique which was useful in study of human chromosomes was also the result of an accident. In order to separate erythrocytes from leukocytes of human blood, Peter Nowell in 1960 used phytohemagglutinin - a crude extract from ordinary beans which had been used to agglutinate erythrocytes for forty years. Upon examination of the cells including the leukocytes after several days of incubation, Nowell noted hundreds of cells in division. He thus discovered that, aside from the bean extract being of value in hemagglutination, phytohemagglutinin had the ability to induce normal small lymphocytes from circulating blood to enter DNA synthesis leading to division. Consequently in 1960 a method of

chromosomal analysis was possible with the readily available tissue, blood.

One of the first major accomplishments made with human chromosomes was the determination of the 2N number (in 1956). In 1921 Painter had examined testicular tissue and was uncertain whether the number for man was 46 or 48 but by 1923 he had decided it was 48. Although there was much interest in man's chromosomal complement, little work was done since the study was difficult and viewed to be of very little practical importance. And it was not until 1956 that Tjio and Levan established the correct 2N number as 46 instead of 48.

In 1932, J. B. S. Haldane stated a definite need for "a technique for counting of human chromosomes without involving the death of the person concerned." He was the first to postulate "that satisfactory mitoses might be observed in a culture of leukocytes." This technique was finally available for use in 1964 upon Nowell's discovery of the mitogenic effect of the crude extract from beans.

In 1932, Waardenberg correctly surmised that mongolism is caused by an extra chromosome: ". . . in mongolism lies an example of specific chromosomal aberration in man . . . perhaps . . . a chromosomal deficiency through nondisjunction or the reverse, a chromosomal duplication."

Chrustschoff and Berlin in 1936 successfully cultured and studied human blood cells in mitosis, leading the way for Andres and Navaschin in 1936 to make an accurate description of the first ten chromosomal pairs of the human complement.

Based on the accurate photographs and descriptions of all 46 chromosomes published in 1956, Jerome Lejeune in 1958 discovered that mongolism was the developmental consequence of aneuploidy, the trisomy being of chromosome # 21.

The discovery of the aneuploidy causing mongolism stimulated many laboratories to initiate the study of human chromosomes in various pathological conditions. During 1959, aneuploidy was found to be the cause of several disturbances in sexual development. For example, Turner's syndrome was found to be the result of an abnormal chromosomal complement 45, X, with only a single sex chromosome. Klinefelter's syndrome was shown to result from a Y-chromosome in addition to the extra X sex chromosome, the complement being 47, XXY. More extensive aneuploidy (for example, 49, XXXXY) resulted in a more seriously affected condition. In other abnormal cases, extra Y chromosomes (for example, 47, XYY) or extra X chromosomes (for example, 47 XXX) were found. Although the presence of extra Y's is not easily determined, the presence of extra X's can easily be demonstrated by the presence of extra chromatin masses of Barr. Mosaicism, a condition of a mixture of cell types, was discovered in 1959 by Charles Ford.

Trisomy 13 and trisomy 18 were discovered by Klaus Patau in 1960. These two trisomy syndromes and trisomy 21 have been the only syndromes of autosomal trisomy found in liveborn humans. Man does not have 23 trisomy syndromes since any other trisomy other than the 13, the 18, or the 21 produces a lethal genetic imbalance.

Duplications or deficiencies of chromosomal segments were found to be compatible with life. Such chromosomal rearrangements were found as translocations, inversion, duplications and deletions. The first structurally abnormal chromosome to be found was discovered in 1959 in a child with multiple bone anomalies. In 1963 Lafourcade, Lejeune and their colleagues defined a recognizable syndrome resulting from the deletion of a segment of the short arm of a No. 5 chromosome. This condition of 5p- has been named the cri du chat syndrome. Deletions resulting in 4p- and 18q- have subsequently been recognized. Countless chromosomal rearrangements and structural chromosomal aberrations have since been observed.

## THE STUDY OF HUMAN CHROMOSOMES - PRESENT INVESTIGATIONS

At the present time much work is being done in the area of chromosome study by cytogeneticists. Such careful investigations of a patient's chromosomal complement combined with careful observations and descriptive study is providing interesting and very pertinent data. These results are being correlated with embryonic development in determining the effects of duplications and deficiencies of various autosomal regions (German, 1970).

Many karyotype-phenotype correlations are being made. For example, in recent years correlations have been made between variations in the sex chromosomal makeup and clinically recognizable disturbances in sex differentiation. This work is providing a clarity never before possible in the understanding of human sex-determining mechanisms. Classifications of disturbances of sexual differentiation are proving important in the areas of endocrinology and developmental biology.

Surveys are being conducted in various human populations using several different approaches (German, 1970). Some studies are being made of the general population by serially examining the complement of each infant born at a given hospital. At the present time the first large-scale study of the general population is being done in Scotland through sampling of persons of various ages. New, streamlined methods are being developed to make these studies more accurate, easier, and more rapid. The use of mechanized scanning of slides, analysis of cells, and mechanized computation

of results is now being employed.

Genetic counseling is becoming more important, particularly in the screening of families or selected groups of people in which an abnormal chromosome is known to be segregating. The search for chromosomal aberrations is involving the examination of both germ and somatic cells in many laboratories.

Techniques are now being developed to sample the chromoeomal complement of developing embryos. The field of diagnostic cytogenetics is becoming more important in detecting chromosomal abnormalities early enough to permit the induction of abortion.

Diagnostic cytogenetice is being integrated into the law profession. For example, the legal statue of aneuploid individuals who break the law is now being debated by human cytogeneticists and lawyers.

Because of the frequency and importance of chromosomal abnormalities as causes of abortion, birth defects, and mental retardation, much interest is developing in the origin of these aberrations. Numerous hypotheses are being presented to explain chromosomal nondisjunction in older mothers.

Studies are being conducted on agents which are known to cause structural aberrations, such as X-rays, viral infections, various drugs, and commonly ingested chemicals. In this area genes which may predispose an individual to chromosomal rearrangements or abnormal behavior are becoming more intriguing to geneticists.

Work is being done in the mapping of the human chromosomal complement. Over 60 X-linked genes, that is, genes located on the

X chromosome, are known. The relative positions of a few of these have been determined so that a rudimentary map of one arm of the X has been developed, though it is uncertain whether it is the long or the short arm. In the area of chromosomal mapping, individuals with chromosomal deficiences and duplications are becoming increasingly important. At present only a few autosomal genes have been located but additional techniques, such as cell hybridization, is providing information to aid in the location of other genes.

## DNA REPLICATION SEQUENCE

Experimental studies of human leukocyte chromosomes require information concerning the timing of DNA synthesis, time of the onset of mitosis, duration of mitotic cycle, and the number of mitoses undergone by individual leukocytes (Bender and Prescott, 1962). The use of a tritium labeled precursor of deoxyribonucleic acid, such as H-thymidine, has made possible high resolution autoradiographic studies of chromosome duplication and has, thus, proved to be useful in the study of the cell cycle and of DNA synthesis (Bader, Miller, and Mukherjee, 1963).

Studies of the DNA labeling patterns of the chromosomes have shown that duplication may occur within comparable regions of homologous chromosomes either during the same interval or during different intervals of the DNA synthetic period (Bader, Miller, and Mukherjee, 1963). Asychrony in the duplication of human chromosomes was initially described by Lima de Faria et. al.(1961). In cultured human leukocytes, asychronous chromosome duplication has generally been observed.

Because leukocytes in blood cultures behave asynchronously, the combination of pulse and continous treatments with thymidine-$H^3$ reveals more details about the chronology of the DNA synthesis (Blanchi and de Blanchi, 1965).

In a cell population the DNA synthesis period (S period) is referred to as the stage in interphase when most of the cells begin DNA replication up to the stage when most of them finish the

process. The $G_2$ period is the time lapse between the completion of the S period and the beginning of the mitosis. Blanchi and de Blanchi (1965) suggest that the S period begins 15 to 20 hours and finishes 5 to 3 hours before the cells reach the metaphase stage. The S period can be subdivided into the four phases $S_1$ to $S_4$ (Blanchi and de Blanchi, 1965).

$S_1$ group:

Chromosome complements included in this group present the following characteristics: a) one or more unlabeled chromosomes, b) predominant labeling over one or both telomeric regions of the chromosomes Nos. 1, 2, 3, and over several chromosomes of Group 6-12 X, c) one chromosome each of pairs tentatively identified as 4 and 5 labeled in the telomeric region of the long arm whereas the other chromosome number 4 labeled in the short arm and the other number 5 in the short arm and in the telomeric region of the long arm, d) the chromosome Y unlabeled and all or several chromosomes of Group 21-22 labeled, e) asynchrony between homologues.

All the complements in the $S_1$ group belonged to cells pulse labeled 25, 20, 15 hours before harvesting, and therefore they are considered as representative of the initial S period ($S_1$ stage).

$S_2$ group:

The pattern of labeling in this group is the following: a) labeling over all the complement, b) distribution of the silver grains over the entire length of every chromosome, c) absence of asychrony between homologues.

Metaphases with the mentioned pattern stemmed from pulse thymidine H treatments performed at 25, 20, 15 and 10 hours before harvesting. These metaphases were considered as examples of the intermediate S period ($S_2$ stage) in accordance with a) their occurence in a moment of the S period in which metaphases from the initial S stage ($S_1$ stage) were no longer observed (10 hours before harvesting) and b) the more homogeneously spread pattern of labsling over the chromosome in comparison to the chromosome complement groups.

$S_3$ group:

Complement included in this group show the following characteristics: a) more than the half of the complement labeled b) a predominant labeling over the centromeric region of chromosomes Nos. 1, 2, and over several chromosomes of Group 6-12 X, c) bands of radioactivity in the telomeric and centromeric regions of one chromosome No. 3 while its partner is labeled only in the centromeric region, d) both chromosomes tentatively identified as Nos. 4 labeled throughout their length while chromosomes No. 5 are preferentially labeled in the short arm, e) one chromosome No. 16 labeled over the long arm, f) the Y chromosome labeled and one or several unlabeled chromosomes of Group 21-22, g) asynchrony between homologues.

With the exception of some complements scattered at the beginning and at the end of the S period, the majority of metaphasss in this group arose from cells labeled 10 and 5 hours before

harvesting. Therefore, they were considered as belonging to a late stage of the S period ($S_3$ period). As a result of this interpretation, unlabeled zones over the chromosomes indicated areas which had finished their replication at the moment of labeling.

The results obtained with the continous labeling with thymidine-$H^3$ showed a 20 to 30% of leukocytes which were finishing chromosome replication 20 or even 25 hours before harvesting.

In accordance with this, the few metaphases of the present group found at earlier stages of the S period were interpreted as cells which duplicated asynchronously in respect to others.

$S_4$ group:

Chromosome complements in this group exhibit the following characteristics: a) less than ½ of the complement labeled, b) a pattern of labeling similar to the one observed in the $S_3$ period with the exception of both chromosomes No. 4 which showed patches of radioactivity over the short arm and the telomeric region of the long arms, c) very often observed are labelings of the Y chromosome and unlabeling of the four chromosomes of group 21-22, d) in more than 1/3 of the metaphases from females only one chromosome, tentatively identified as X, show radioactivity, e) asynchrony between homologues more conspicuous than before.

In summary, the first chromosomes to replicate are Nos. 1, 3, 5, and X followed by the Nos. 2, 4 and several chromosomes of Groups 6-12, 13-15, and 19-20. Later the pairs 16, 17, 18 and the chromosomes of groups 21-22 replicate. Chromosome Y in the male is

the last to replicate, beginning its duplication when all the other chromosomes have reached the intermediate S stage.

The earliest chromosomes to finish the duplication are Nos. 19, 20, and 21 followed by Nos. 16, 17, 18, 22 and the chromosomes of Group 13-15. Afterward and at about the same time the replication of pairs 2, 4, 6, 8, and X and Y chromosomes in the male and one X chromosome in the female conclude. The other X chromosome in the female is the last to end its duplication being totally labeled until the final stage of the S period. (This may be evidence for the Lyon hypothesis of 1961: In mammals one X chromosome is inactivated in some embryonic cells and their descendants, that the other is inactiviated in the rest, and that mammalian females are consequently X chromosome mosaics (King, 1965).

Duplication of the long and medium size chromosomes begins at localized regions and then extends over the total length of the chromosome and at the end of the S stage takes place only in small zones different from those replicating early.

Asychrony between homologous chromosomes is observed at the beginning and at the end of the S period.

## THE ROLE AND IMPLICATIONS OF HUMAN CHROMOSOME STUDY

The knowledge of many species of organisms has been advanced through an accurate study of the characteristics and behavior of the species' chromosomes. There is, therefore, good reason to assume that the genetic study of man's chromosomes will yield valuable and reliable information about his own species.

A thorough examination of the chromosomes in a range of human groups could make a significant contribution in the studies of many of the problems confronting man today. The unique features of the human genetic milieu - the alternations in man's genetic composition, the changing selection pressures, and the possible influence upon mutation rates of the environmental changes of the past two or three centuries - are all of great need of study and measurement in order to be able to estimate their effect upon the genetic structure of future human populations. A more complete comprehension and knowledge of "normal" human chromosomes would undoubtedly provide a necessary baeis for future studies.

# METHODS AND MATERIALS

The ultimate goal of the experimental methods was to produce a random series of karyotypes from leukocyte cultures from ten females and from ten males from which DNA determinations could be made. The entire procedure involved obtaining and culturing white blood cells, preparing and staining of metaphase chromosomes, photometrically measuring DNA, and photographing the karyotypes.

## OBTAINING AND CULTURING OF WHITE BLOOD CELLS

The culturing and preparation of leukocytes in obtaining metaphase chromosomes were accomplished using the methods and materials supplied in the "TC Chromosome Culture Kit" purchased from the Carolina Biological Supply Company, Burlington, North Carolina. The kit included the following preparation: (Difco Technical Information, 1969)

1. Blood Separation Vial

   TC Chromosome Blood Separation Vial which contains 2 ml. of a sterile anticoagulant, provides a convenient means for the maximum sedimentation of erythrocytes from 10 ml. whole blood within 30 minutes. The supernatant plasma containing the suspension of lymphocytes may be used for the direct inoculation into 2 (or 3) bottles of TC Chromosome Medium which have been rehydrated with TC Chromosome Reconstituting Fluid.

2. TC Chromosome Medium

   Ingredients per liter:

| | |
|---|---|
| L-Arginine | 70 mg |
| L-Histidine | 20 mg |
| L-Lysine | 70 mg |
| L-Tyrosine | 40 mg |
| DL-Tryptophane | 20 mg |
| DL-Phenylalanine | 50 mg |
| L-Cystine | 20 mg |
| DL-Methionine | 30 mg |
| DL-Serine | 50 mg |
| DL-Threonine | 60 mg |
| DL-Leucine | 120 mg |
| DL-Isoleucine | 40 mg |
| DL-Valine | 50 mg |

| | |
|---|---|
| DL-Glutamic Acid | 150 mg |
| DL-Aspartic Acid | 60 mg |
| DL-Alanine | 50 mg |
| L-Proline | 40 mg |
| L-Hydroxyproline | 10 mg |
| Glycine | 50 mg |
| L-Cysteine | 0 mg |
| Adenine | 10 mg |
| Guanine | 0.3 mg |
| Xanthine | 0.3 mg |
| Hypoxanthine | 0.3 mg |
| Thymine | 0.3 mg |
| Uracil | 0.3 mg |
| Thiamine Hydrochloride | 0.01 mg |
| Riboflavin | 0.01 mg |
| Pyridoxine Hydrochloride | 0.025 mg |
| Pyridoxal Hydrochloride | 0.025 mg |
| Niacin | 0.025 mg |
| Niacinamide | 0.025 mg |
| Calcium Pantothenate | 0.01 mg |
| Biotin | 0.01 mg |
| Folic Acid | 0.01 mg |
| Choline | 0.5 mg |
| Inositol | 0.05 mg |
| p-Aminobenzoic Acid | 0.05 mg |
| Vitamin A | 0.1 mg |
| Calciferol | 0.1 mg |
| Menadione | 0.01 mg |
| a-Tocopherol Phosphate | 0.01 mg |
| Ascorbic Acid | 0.05 mg |
| Glutathione | 0.05 mg |
| Cholesterol | 0.2 mg |
| L-Glutamine | 100 mg |
| Adenosinetriphosphate | 1 mg |
| Adenlic Acid | 0.2 mg |
| Ribose | 0.5 mg |
| Desoxyriboss | 0.5 mg |
| Bacto-Dextrose | 1 g |
| Tween 80 | 5 mg |
| Sodium Acetate | 50 mg |
| Iron (as Ferric Nitrate) | 0.1 mg |
| Sodium Chloride | 8 g |
| Potassium Chloride | 0.4 g |
| Calcium Chloride | 0.14 g |
| Magnesium Sulfate | 0.2 g |
| Disodium Phosphate | 0.06 g |
| Sodium Bicarbonate | 0.35 g |
| Bacto-Phenol Red | 0.02 g |
| Carbon Dioxide | to pH 7.2 |
| Triple Distilled Water | 1000 ml |

TC Chromosome Medium contains a sterile, dried, pre-tested formulation which, after rehydration with TC Chromosome Reconstituting Fluid, provides an inter-standardized, complete medium for initiating mitosis of lymphocytes for chromosomal analysis.

3. TC Chromosome Reconstituting Fluid

TC Chromosome Reconstituting Fluid is a sterile, inter-standardized, pre-tested solution especially formulated for rehydrating TC Chromosome Medium to provide a complete medium for initiating mitosis of lymphocytes for chromosomal analysis.

Reconstituting Fluid contains penicillin, streptomycin, and Bacto-Phytohemagglutinin M.

4. TC Chromosome Arresting Solution

TC Chromosome Arresting Solution is a sterile, pre-tested, inter-standardized solution formulated for the termination of mitosis of lymphocytes cultured in the TC Chromosome Medium previously rehydrated with TC Chromosome Reconstituting Fluid.

5. TC Hanks Solution

Ingredients per liter:

| | |
|---|---|
| Bacto-Dextrose | 1 g |
| Sodium Chloride | 8 g |
| Potassium Chloride | 0.4 g |
| Calcium Chloride | 0.14g |
| Magnesium Sulfate | 0.1 g |
| Magnesium Chloride | 0.1 g |
| Monopotassium Phosphate | 60 mg |
| Disodium Phosphate | 60 mg |
| Sodium Bicarbonate | 0.35g |
| Phenol Red | 20 g |
| Triple Distilled Water | 1000 ml |

TC Hanks Solution contains 12 ml. of sterile Hanks

Balanced Salt Solution. This volume is sufficient for rinsing the resuspended button of centrifuged, cultured lymphocytes, and for the subsequent soaking of the cells in hypotonic Hanks solution, (obtained by dilution with 3 volumes of water), for two cultures.

6. Bacto-Giemsa Stain

Bacto-Giemsa Stain is supplied as 1 ml. of a stable, stock Giemsa stain which is to be diluted with 20 ml. of water just before use. The 20 ml. of stain is sufficient for covering 6 - 8 slides for the staining of lymphocytes and chromosomes.

The following methods were used in obtaining and culturing white blood cells:

1. The services of the Eastern Illinois University Health Service were used to obtain blood samples from each of ten females and ten males.
2. Ten ml. of blood were withdrawn from the arm of each subject under sterile conditions at the University Health Service and transferred to the Blood Separation Vial.
3. The blood was mixed by inversion and allowed to stand 1-3 hours until at least 4 ml. of plasma-leukocyte suspension had separated.
4. The bottle of Chromosome Reconstituting Fluid ($37^{0}$ C.) was added to the Chromosome Medium and mixed.
5. 1.5-2.5 ml. of plasma-leukocyte suspension from the top of the Blood Separation Vial were added to the mixed culture medium using a sterile Pasteur pipette and bulb for the addition process.
6. The inoculated medium bottle was incubated in a vertical position for 3 days at $37^{0}$ C.
   a. During this incubation period, the proper pH (approximately 7.3) of the medium was maintained through the use of a color indicator in the medium.
   b. During this time the indicator in the medium was not allowed to become more yellow than a light amber and no more red than a light pink.

c. If the medium became too yellowish, the cap of the bottle was loosened slightly to allow excess carbon dioxide to escape.

## PREPARING AND STAINING METAPHASE CHROMOSOMES

The following methods were used in preparing and staining metaphase chromosomes:

1. After 3 days, the vial of Chromosome Arresting Solution was added to the medium bottle and mixed thoroughly.
2. The medium containing the Chromosome Arresting Solution was incubated for 3 - 6 hours at 37° C.
3. At the end of the incubation period, the cells were re-suspended with a Pasteur pipette.
4. The medium was transferred to a 15 ml. graduated conical centrifuge and centrifuged for 12 minutes at a low speed (800 rpm).
5. The culture medium was poured off gently and 5 - 6 ml. of warm (37° C.) Hanks Solution were added.
6. The cells were resuspended in the centrifuge tube with a pipette and centrifuged for 5 minutes at 800 rpm.
7. All but 0.5 ml. of the Hanks Solution was carefully pipetted off and the packed cells were resuspended in the remaining 0.5 ml. with a pipette.
8. A hypotonic solution was produced by slowly adding 1.5 ml. of warm (37° C.) deionized water while shaking the tube.
9. The hypotonic suspension was incubated for 10 minutes at 37° C.
10. The suspension was centrifuged for 5 minutes at a low speed (600 rpm) and the fluid was drawn off.

11. The Glacial Acetic Acid was added to the Methanol and mixed thoroughly.
12. 3 - 4 ml. of this fixative were added slowly to the centrifuge tube without disturbing the button of cells.
13. The cells were allowed to soak in this fixative at room temperature for 30 minutes.
14. After the 30 minutes, the cells were resuspended with a pipette and centrifuged slowly (600 rpm) for 5 minutes.
15. The fixative was drawn off carefully and 3 - 4 ml. of fresh fixative were added.
16. The cells were resuspended with a pipette and allowed to stand 5 minutes before being centrifuged slowly (600 rpm) for 5 minutes.
17. The fixative was drawn off and 0.3 - 0.5 ml. of fresh fixative were added to the button of cells and the cells were resuspended with a pipette to get a hazy suspension.
18. Excess water was shaken from slides which had been previously cleaned with ethanol and chilled in deionized water in a refrigerator.
19. 1 - 3 drops of the cell suspension were added to each slide with a Pasteur pipette, the slide being immediately tipped several times to spread the suspension.
20. The fixative was ignited on the slides by momentarily touching them to a flame.
21. As soon as the fixative had been burned off, the slides

were waved vigorously or passed quickly through a flame to speed drying, taking care that the slides did not become hot.

22. The dried slides were stained with Giemsa Stain diluted with 20 ml. of deionized water for 15 minutes.

23. The slides were then rinsed with deionized water and allowed to air-dry.

24. A drop of Kleermount and a cover glass cleaned in ethanol were added to each slide to make the slides permanent.

## MEASURING DNA

The quantitative measurement of DNA is based upon its light absorption. As stated by Wilson and Morrison (1966), "in all photometric studies involving the quantitative measurement of cell substances by virtue of their light absorption, it is essential to show that the amount of light absorbed by the substance is proportional to the number of absorbing molecules present per unit thickness." The linear relationship between the amount of light absorbed and the number of absorbing molecules is expressed as Beer-Lambert's law. The amount of light absorbed is usually a reasonably accurate measurement of the amount of DNA (Wilson and Morrison, 1966).

In order to measure the amount of light absorption by the karyotypes of the twenty subjects, a cytophotometer at the University of Illinois, Department of Animal Sciences, was used. In this procedure of photometric analysis, the intensity of light transmitted by the stained chromosomes ($I_s$) compared with that transmitted by a blank part of the slide ($I_o$) is measured by means of a photoelectric cell. The ratio $I_s/I_o \times 100$ represents the per cent of light transmitted by the chromosomes (Wilson and Morrison, 1966). From this information the absorption or extinction coefficient (E) can be calculated. The value of E can then be used to calculate the amount of nuclear DNA in arbitrary units.

In measuring the DNA, fifty karyotypes were chosen at random under the microscope from each of the twenty individuals. The

readings were then averaged to obtain the individual mean amount of DNA for the subjects.

At the Data Processing Center of Eastern Illinois University, the data of the ten female subjects and the data of the ten male subjects were averaged, respectively, to ascertain the mean amount of DNA for females and the mean amount for males. The amount of variance within the fifty readings of each individual was calculated and the amount of variance between the entire group of females and the entire group of males was determined and compared.

## PHOTOGRAPHING OF KARYOTYPES

Representative karyotypes of each of the ten male subjects and the ten female subjects were photographed using a Beseler camera mounted on a Zeiss phase-contrast microscope. The karyotypes were photographed under bright-field illumination using the oil-immersion objective (ph 3 Neofluar 100/1.30) to give magnifications of 1000X.

Panatomic X film (Kodak) was exposed to light for a time period ranging from approximately .5 second to 3 seconds, depending on the darkness of the stain on the slide. The film was then developed, using the following procedure as suggested by the Eastman Kodak Company and chemicals supplied from Walt's Camera Shop in Mattoon, Illinois:

1. The film was developed in total darkness in a small developing tank.
2. Microdol-X (75° F.) was added to the tank and agitated for approximately 6 minutes.
3. The film was rinsed.
4. Kodak Indicator Stop Bath was added and agitated in the tank for 15 seconds.
5. Kodak Rapid Rixer was added to the tank with agitation for 2-4 minutes.
6. After the film was washed, Quix Wash was added with agitation for 1-2 minutes.
7. The film was rinsed and then rinsed in Kodak PhotoFlo.

In order to print pictures of the karyotypes, the negatives were placed in an enlarger and AGFA # 3 paper was exposed to light for times ranging from 5 to 15 seconds, depending on the darkness of the negatives. The exposed paper was then developed and fixed according to the following schedule:

1. Dektol (dilution 1 Dektol / 2 water) until desired darkness was obtained
2. Stop bath
3. Rapid Fixer for 5 - 10 minutes
4. Rinse for 30 seconds
5. Quix Wash for 2 minutes
6. Rinse for 20 minutes
7. PhotoFlo rinse

The pictures were then dried and pressed and trimmed to the desired size.

Chart I

Means, Variances, and F Ratios for Five Groups of Determinations* for Ten Human Male Individuals

| Individual | Group I | | Group II | | Group III | | Group IV | | Group V | | Calculated F Ratio Between Groups | Expected F Ratio Between Groups |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | Variance | Mean | Variance | Mean | Variance | Mean | Variance | Mean | Variance | | |
| 1 | 9.02 | -0.015 | 9.01 | -0.007 | 8.91 | 0.004 | 8.77 | 0.014 | 8.79 | 0.017 | 1.378 | 2.6** |
| 2 | 8.75 | -0.023 | 7.91 | 0.182 | 8.25 | 0.190 | 8.06 | 0.200 | 8.19 | 0.327 | 3.528 | 2.6 |
| 3 | 7.72 | 0.122 | 7.92 | 0.070 | 7.63 | 0.048 | 8.46 | -0.006 | 8.43 | -0.058 | 11.943 | 2.6 |
| 4 | 8.25 | -0.038 | 7.83 | 0.306 | 8.02 | -0.033 | 8.12 | -0.036 | 7.85 | 0.106 | 2.039 | 2.6** |
| 5 | 7.91 | 0.208 | 8.06 | -0.012 | 7.62 | 0.075 | 7.87 | 0.161 | 8.26 | 0.146 | 2.596 | 2.6** |
| 6 | 7.92 | 0.214 | 7.85 | 0.084 | 7.90 | -0.007 | 7.85 | -0.010 | 7.92 | 0.014 | 0.089 | 2.6** |
| 7 | 7.91 | 0.142 | 7.87 | 0.159 | 7.81 | 0.067 | 7.57 | 0.080 | 7.76 | 0.183 | 0.779 | 2.6** |
| 8 | 7.53 | 0.213 | 8.06 | 0.056 | 7.52 | 0.104 | 7.96 | 0.001 | 7.52 | 0.104 | 3.767 | 2.6 |
| 9 | 7.89 | 0.030 | 7.56 | 0.065 | 7.74 | 0.215 | 7.51 | 0.116 | 7.68 | 0.167 | 1.051 | 2.6** |
| 10 | 7.56 | 0.147 | 7.24 | 0.346 | 6.48 | 0.131 | 7.03 | -0.020 | 7.57 | 0.276 | 7.381 | 2.6 |

* Each group of determinations based upon means of ten determinations

** F Ratios indicate no significant difference between groups

Chart II Means, Variances, and F Ratios for Five Groups of Determinations* for Ten Human Female Individuals

| Individual | Group I | | | | | | | | | | Calculated F Ratio Between Groups | Expected F Ratio Between Groups |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | Variance | Mean | Variance | Mean | Variance | Mean | Variance | Mean | Variance | | |
| 1. | 9.01 | 0.031 | 9.24 | 0.024 | 9.68 | -0.017 | 9.37 | 0.146 | 9.56 | -0.015 | 4.926 | 2.6 |
| 2 | 9.00 | 0.014 | 9.16 | 0.001 | 9.20 | -0.066 | 8.96 | 0.069 | 9.11 | -0.006 | 1.037 | 2.6** |
| 3 | 9.07 | 0.043 | 9.13 | 0.073 | 8.98 | 0.042 | 9.10 | 0.053 | 9.12 | 0.041 | 0.249 | 2.6** |
| 4 | 9.07 | 0.037 | 8.86 | 0.020 | 8.91 | 0.078 | 9.20 | 0.002 | 9.08 | 0.171 | 1.140 | 2.6** |
| 5 | 9.03 | 0.126 | 9.08 | 0.043 | 9.06 | 0.060 | 8.93 | 0.007 | 9.02 | 0.053 | 0.216 | 2.6** |
| 6 | 8.85 | 0.156 | 9.00 | 0.014 | 8.89 | 0.016 | 9.02 | 0.017 | 8.81 | 0.075 | 0.535 | 2.6** |
| 7 | 8.93 | 0.065 | 9.12 | 0.000 | 8.84 | 0.018 | 8.96 | 0.065 | 8.70 | 0.057 | 1.671 | 2.6** |
| 8 | 8.81 | 0.071 | 9.10 | -0.005 | 8.80 | 0.092 | 8.94 | 0.011 | 8.90 | -0.031 | 1.154 | 2.6** |
| 9 | 8.91 | 0.102 | 8.89 | 0.024 | 8.93 | 0.105 | 8.77 | 0.022 | 9.00 | 0.004 | 0.460 | 2.6** |
| 10 | 8.44 | 0.136 | 8.84 | 0.020 | 8.68 | 0.035 | 8.65 | 0.010 | 8.85 | 0.024 | 1.931 | 2.6** |

* Each group of determinations based upon means of ten determinations

** F Ratios indicate no significant difference between groups

Chart III Grand Mean DNA per 50 Karyotypes and Grand Variances Within the 50 Karyotypes of Ten Human Female Individuals

| Individual | Grand Mean DNA per 50 Karyotypes (mg X $10^{-9}$) | Grand Variance Within 50 Karyotypes | Calculated F Ratio Between Indiv. | Expected F Ratio Between Indiv. |
|---|---|---|---|---|
| 1. | 9.38 | 0.090 | | |
| 2 | 9.09 | 0.011 | | |
| 3 | 9.08 | 0.054 | | |
| 4 | 9.03 | 0.078 | | |
| 5 | 9.03 | 0.061 | | |
| 6 | 8.92 | 0.063 | | |
| 7 | 8.91 | 0.061 | | |
| 8 | 8.91 | 0.040 | | |
| 9 | 8.90 | 0.058 | | |
| 10 | 8.70 | 0.068 | 10.333 | 2.0 |

Chart IV Grand Mean DNA per 50 Karyotypes and Grand Variances Within the 50 Karyptypes of Ten Human Male Individuals

| Individual | Grand Mean DNA per 50 Karyotypes ($mg \times 10^{-9}$) | Grand Variance Within 50 Karyotypes | Calculated F Ratio Between Indiv. | Expected F Ratio Between Indiv. |
|---|---|---|---|---|
| 1 | 8.90 | 0.015 | | |
| 2 | 8.24 | 0.257 | | |
| 3 | 8.04 | 0.158 | | |
| 4 | 8.02 | 0.087 | | |
| 5 | 7.95 | 0.161 | | |
| 6 | 7.89 | 0.061 | | |
| 7 | 7.79 | 0.141 | | |
| 8 | 7.72 | 0.154 | | |
| 9 | 7.68 | 0.137 | | |
| 10 | 7.18 | 0.339 | 41.349 | 2.0 |

Chart 5 Mean Amount of DNA for Ten Human Females And for Ten Human Males (mg X $10^{-9}$)

| | Females | Males |
|---|---|---|
| Amount of DNA | 9.00 | 7.94 |

# DISCUSSION

The goal of this study was the quantitative determination in man of the mean amount of DNA in karyotypes from males and females. Leukocytes were cultured, slides were prepared, and the amount of DNA per karyotype was measured by cytophotometry.

The fundamental requirements for good preparations of karyotypes from leukocytes of human peripheral blood were a sufficiency of blood containing leukocytes, a sufficiency of cell divisions, good fixation, suitable staining, and adequate dispersion of the chromosomes (Ford, 1960).

While the directions supplied in the Blood Chromosome Kit were generally adhered to, no one step seemed especially critical. The culturing temperature varied at times from 35° C. to 40° C. without apparent adverse effects. Although the suggested time for leukocyte separation from whole blood was 1 - 3 hours, the suspension was allowed to stand for the time required for approximately 4 ml. of plasma-leukocyte suspension to separate.

The preparation of the culturing medium involved merely the mixing of the Chromosome Medium and the Chromosome Reconstituting Fluid. The Chromosome Medium was composed of a desicoated phytohemagglutinin which is recommended for the isolation of viable leukocytes by the selective agglutination and sedimentation of mature erythrocytes (Difco Technical Information, 1969). The Chromosome Reconstituting Fluid was composed of a hemagglutination buffer recommended for rehydrating phytohemagglutinin providing an

isotonic medium for the culturing of blood cells.

After the leukocytes had been inoculated into the medium bottle, it was suggested that the cells be incubated for 3 days. Again, the time did not seem too critical, since incubation time varied from 65 - 76 hours with no vast difference in the number of karyotypes obtained.

With the pH indicator included in the culture medium, the general range of the pH of the culture was watched in order to insure a phyeiologically proper environment for cell division. In none of the cultures did the indicator become more yellow than amber nor more red than a light pink. Therefore, the factor of pH did not become critical during culturing.

While the addition of the Chromosome Arresting Solution itself was a very important step in chromosome preparation, the suggested time of incubation of 3 - 6 hours after addition did not seem to make a great difference in the number of karyotypes. This time was varied among the cultures merely as a matter of convenience.

The Chromosome Arresting Solution was composed of colchicine, an alkaloid derived from the autumn crocus, used to disrupt spindle formation during metaphase. The use of colchicine and other agents capable of arresting mitosis at metaphase has held an important place in the preparative techniques used by cytogeneticists for a long time. Colchicine is frequently referred to as a spindle inhibitor because of its action in bringing about the arrest of mitosis by causing the failure of spindle formation (Ford, 1960). When cells are exposed to colchicine before immersion in a hypotonic solution,

it is the action of the drug that disperses the chromosomes. The hypotonic treatment is useful in that the chromosomes are better dispersed than if drug treatment alone had been used.

Colchicine treatment also has two other consequences which are of value when the chromosomes are less dispersed: increased chromosomal contraction and divergence of sister chromatids (Ford, 1960). Extra shortening of the chromosomes reduces the amount of overlapping, and the divergence of the chromatids aids interpretation by emphasizing the bilateral symmetry of each chromosome.

In the harvesting of the cells, resuspension of the cells with a Pasteur pipette did seem to be rather important. Better slides were prepared when the clump of cells was vigorously and completely broken up each time the directions called for resuspension.

Like the colchicine, the addition of deionized water to the medium was an important prerequisite in the good karyotype preparation. The water produced a hypotonic solution which was very desirable. Hypotonic solutions are used to uncoil and dissociate human chromosomes by disrupting spindle organization and permitting the chromosomes in metaphase plates to become well separated from one another (Hungerford, 1965). Hypotonic solutions swell human cells in tissue cultures, disturbing the spindles of the dividing cells, consequently causing the prometaphasic and metaphasic chromosomes to be widely spread apart (Hsu and Pomerat, 1953). Cells swell considerably in hypotonic fluids, and the improved dispersion is due to lowered viscosity and increased apparent volume of cytoplasm.

In the preparation of slides, the slides (usually nine were sufficient) were cleaned with ethanol instead of the suggested methanol. Ethanol was used since it was more convenient and the difference between the alcohols was not significant as cleaning agents. The cleaned slides were stored in deionized water in a freezer so that they were frosted when they were used. Better slides seemed to be produced when the slides were colder than just chilled as suggested in the directions because the cold aided in the rupturing of the cell membranes.

Three drops of the cell suspension were added to the slide and the slide was tipped from side to side to spread the suspension. Since spreading was desirable, the amount of fresh fixative added to the cells in the last steps, while generally from 0.3 - 0 5 ml., was determined by the number and concentrstion of cells. The suspension was best when it was fluid enough to spread easily.

As suggested, the drying of the slides was accomplished both by waving the slides in the air and by rapidly passing the slides through a flame. The air-drying of the cells on the slides has recently been introduces by Tjio and Puck (Ford, 1960). As the fixative evaporates, and the preparation dries, each cell spreads into a thin sheet adherent to the glass, the chromosomes of the mitotic cells being dispersed in the process.

Although most of the details of the cytological protocol are not considered critical, consistently well-spread metaphase plates appear to be dependent upon the surface-tension forces involved during drying on a wet slide (Moorhead, et. al., 1960). Conse-

quently, successive changes of fresh fixative, well-cleaned slides and the speed of drying are important in achieving good flattening and attachment of the cell membranes to the glass surface. Air-drying of metaphase cells cultured on glass provides well-spread chromosomes in one focal plane with a minimum of overlay and ecattering.

The Giemsa stain supplied in the kit was used. The time of staining was increased to approximately 15 minutes in order that the chromosomes were stained darkly enough to be photographed.

The results of the DNA determinations for the ten female subjects and for the ten male subjects are presented in charts I and II respectively. For each individual, the fifty determinations taken were divided into five groups of ten determinations and the mean of each group was calculatsd, as shown in the charts. The variances between the determinations in each group are shown and were used to calculate the F ratios between the groups. The low F ratios, as compared to the expected F ratioa, obtained in most cases (9 out of 10 females and 6 out of 10 males) show there is little statistical difference between the groups for each individual.

However, the high F ratios, as compared to those expected, for the mean determinations of the females collectively and of the males collectively show there are statistical differences between the amounts of DNA between individuals of the same sex (charts III and IV). In both the females and the males, a rather large

range was found for the mean amount of DNA. The range of mean DNA content for females was from 9.38 mg X $10^{-9}$ to 8.70 mg X $10^{-9}$ giving a variation of .68 mg X $10^{-9}$ among the sex group. The range found among the males was from 8.90 mg X $10^{-9}$ to 7.18 mg X $10^{-9}$ giving a higher variation of 1.72 mg X $10^{-9}$ among the group.

Chart V, which gives the mean DNA values for the females as a whole and for the males as a whole shows the amount of DNA found in female karyotypes to be considerably higher than the amount found in male karyotypes (9.00 mg X $10^{-9}$ as compared to 7.94 mg X $10^{-9}$). This 2.06 mg X $10^{-9}$ difference may be explained by the difference in size of the X sex chromosome of females as compared to the smaller Y sex chromosome of males. The X chromosome, being nearly three times as large as the Y chromosome, could be expected to have a greater DNA contnet. The greater DNA content, consequently, can be said to confer a substantially richer genetic capacity upon the female (Tjio and Puck, 1958).

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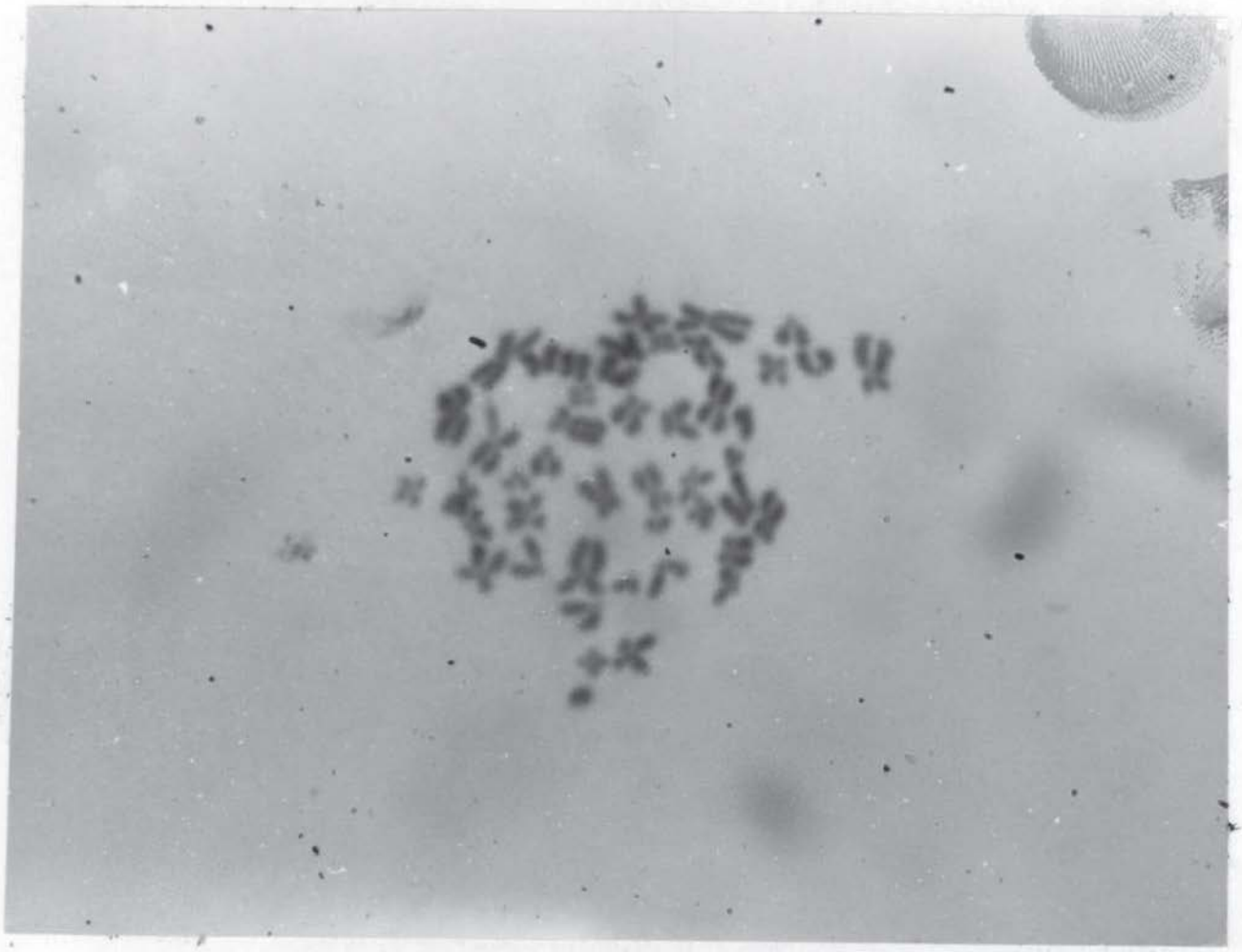
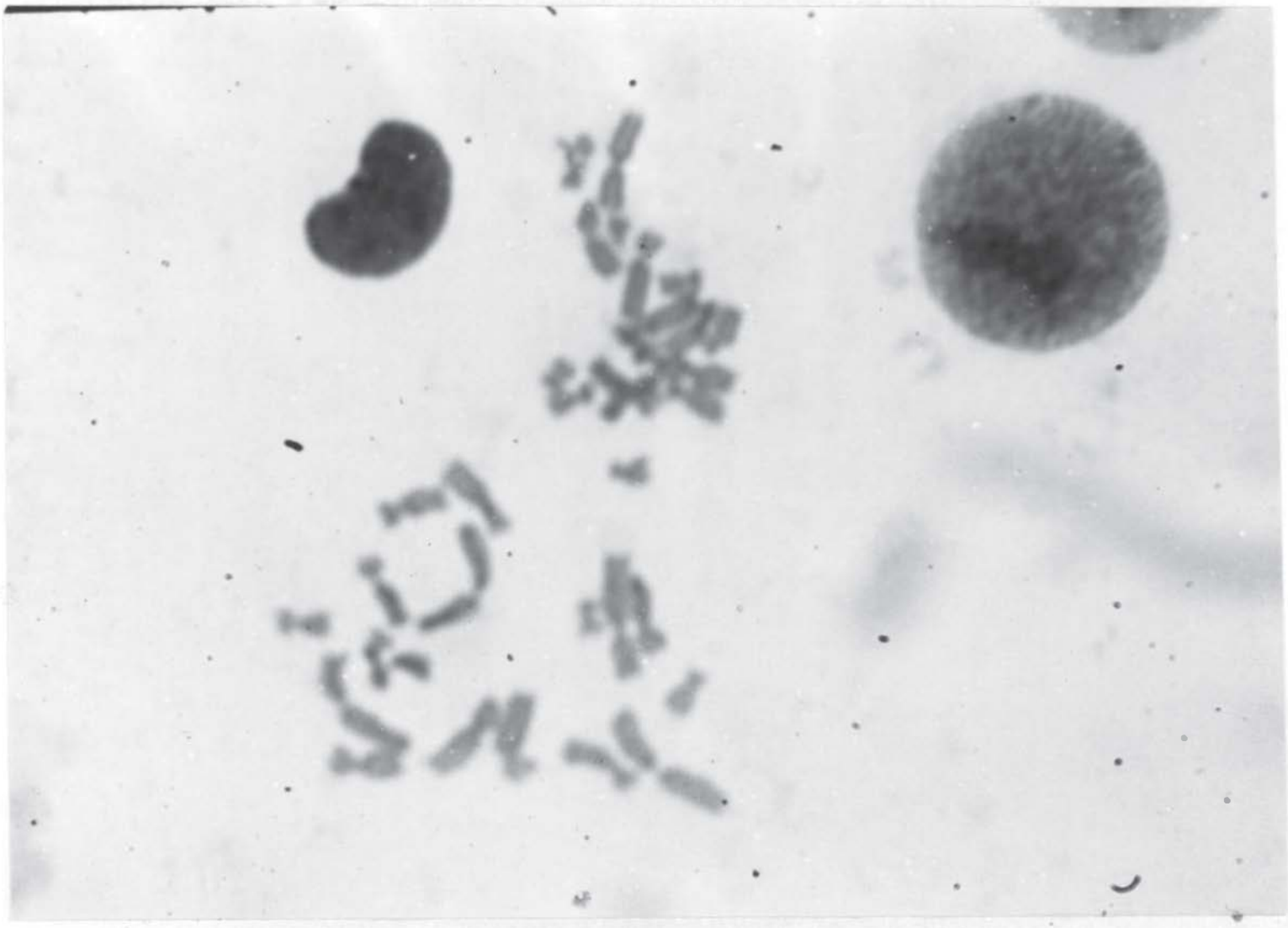
# Photographs of Karyotypes of Normal Human Male Subjects

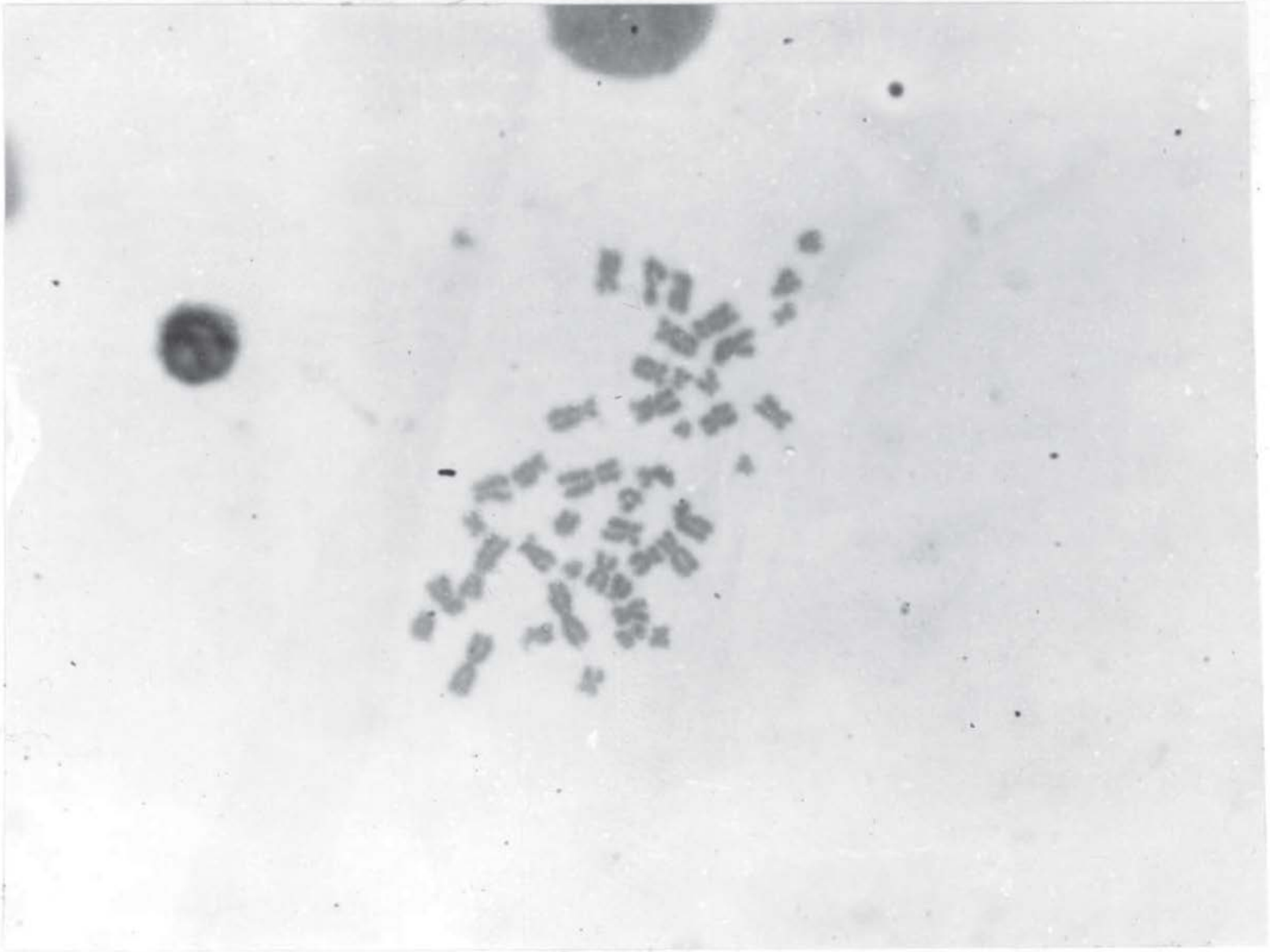
## (Photographed at magnifications of 400X)



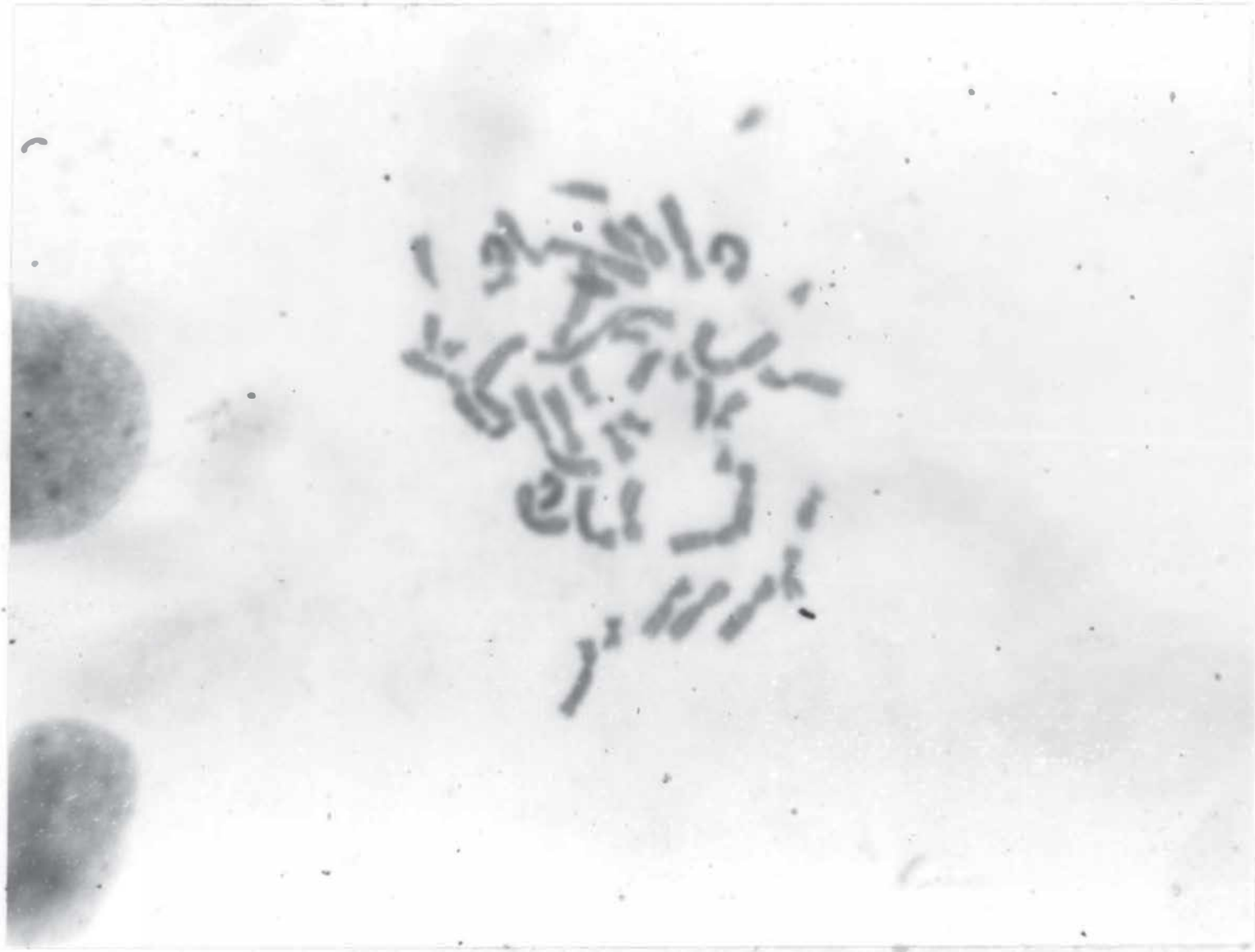
Male I



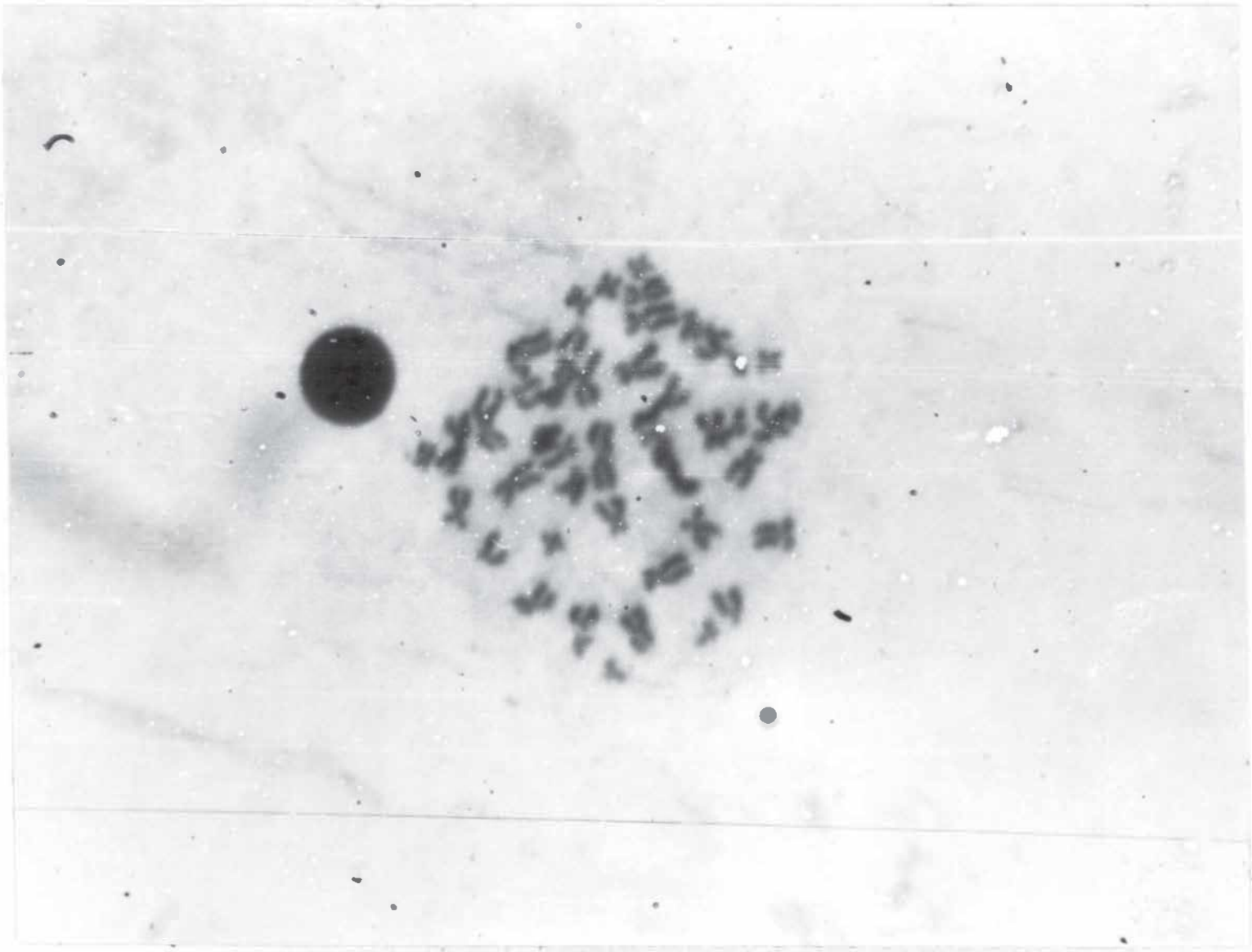
Male II



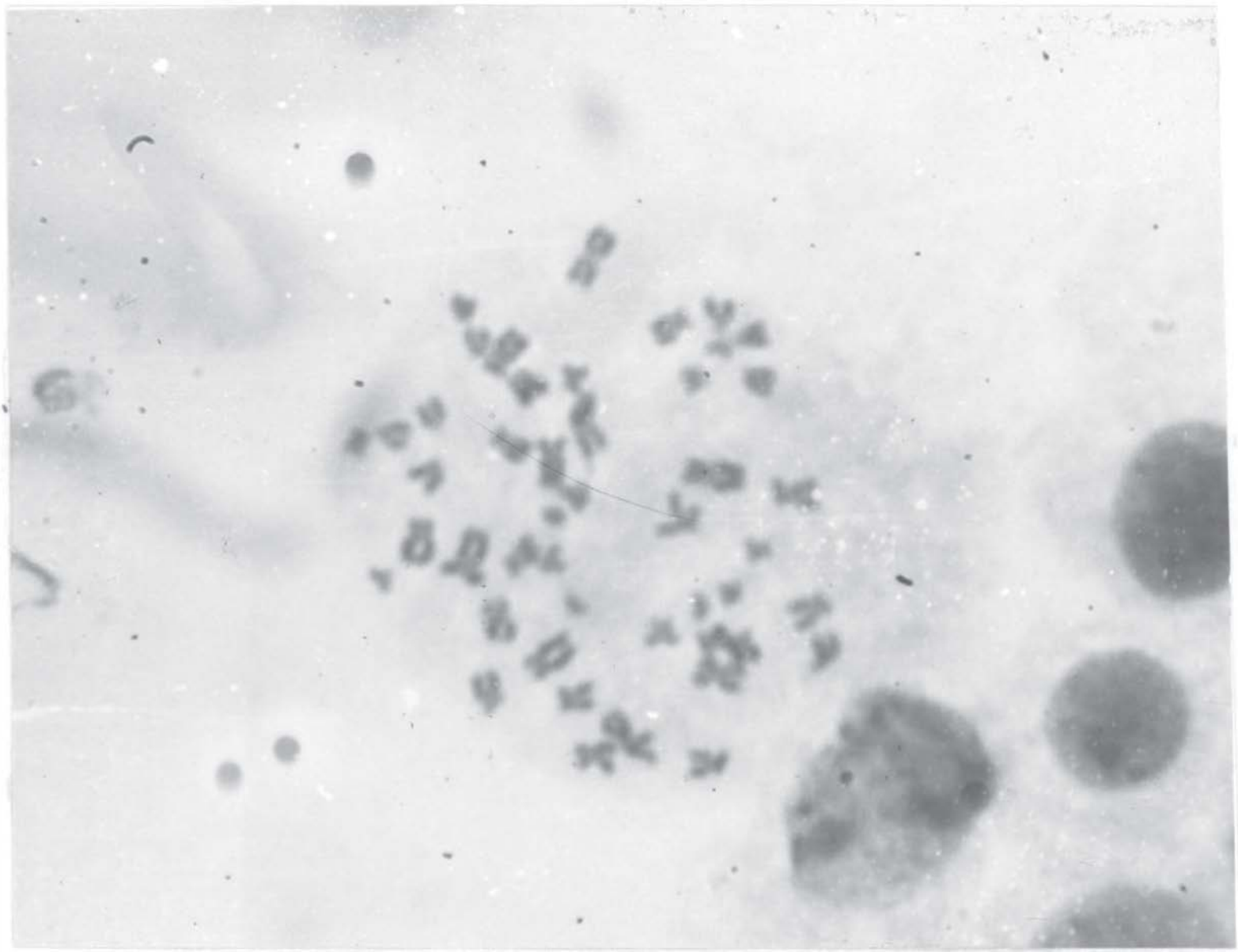
Male III



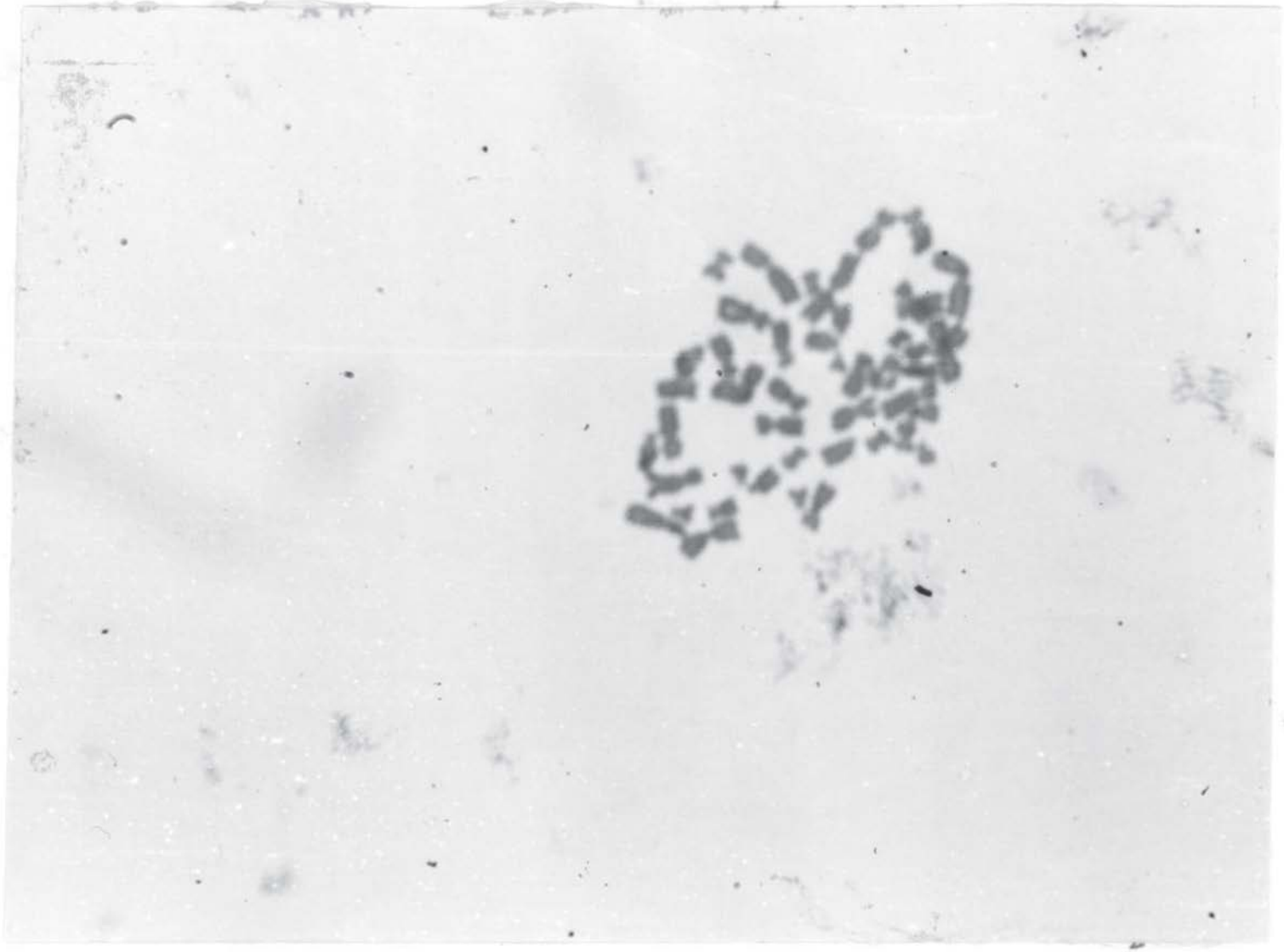
Male IV



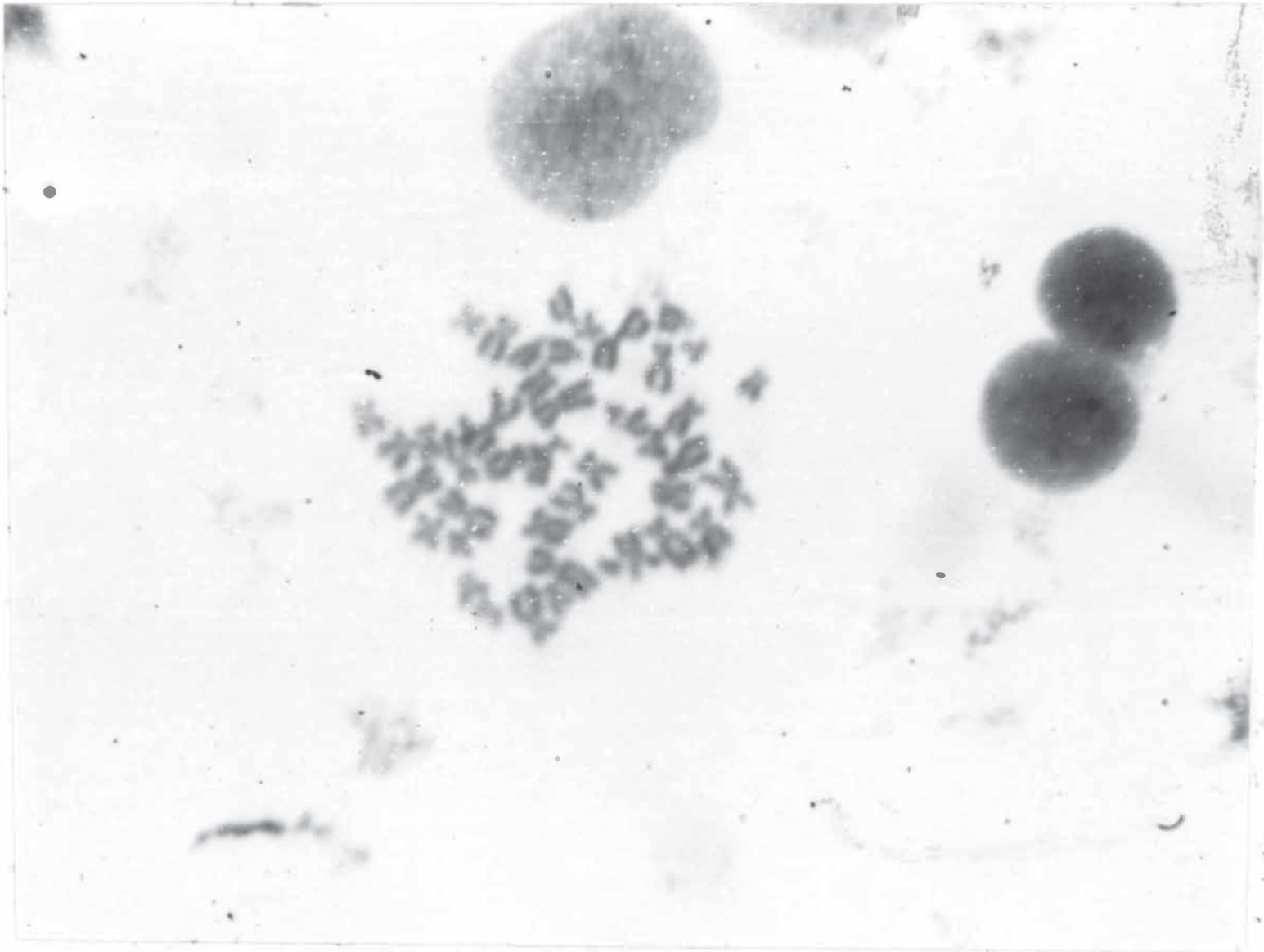
Male V



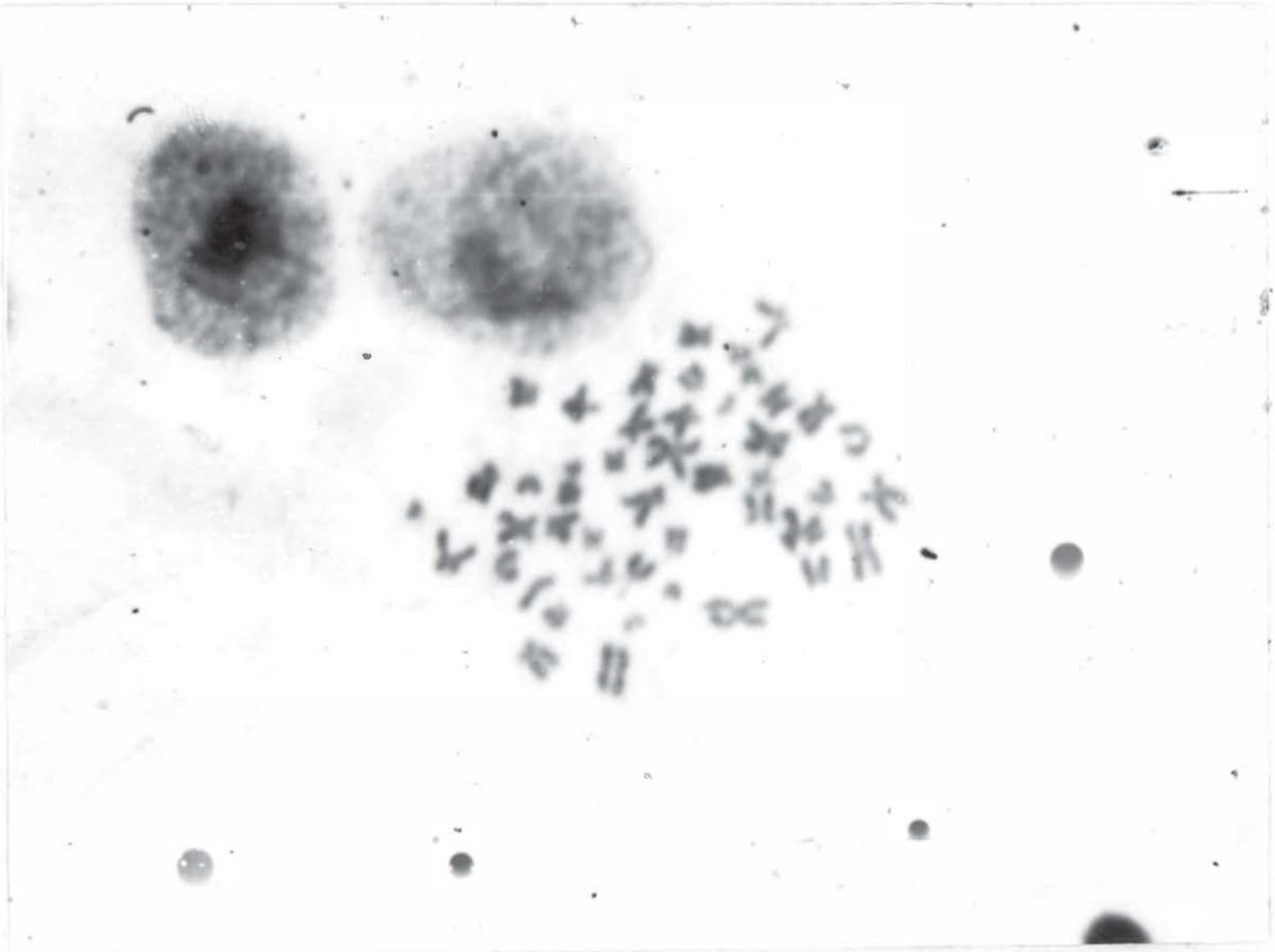
Male VI



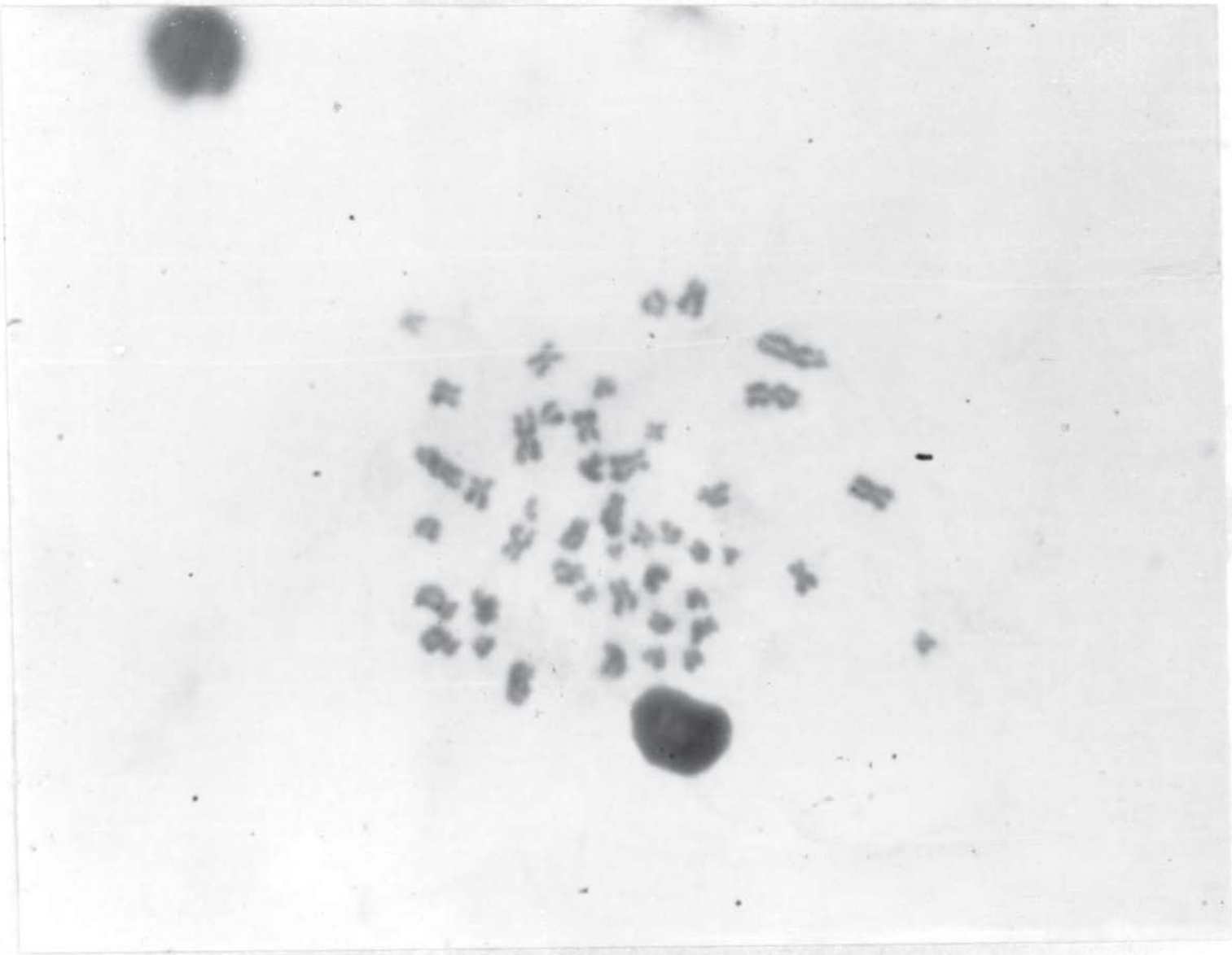
Male VII



Male VIII



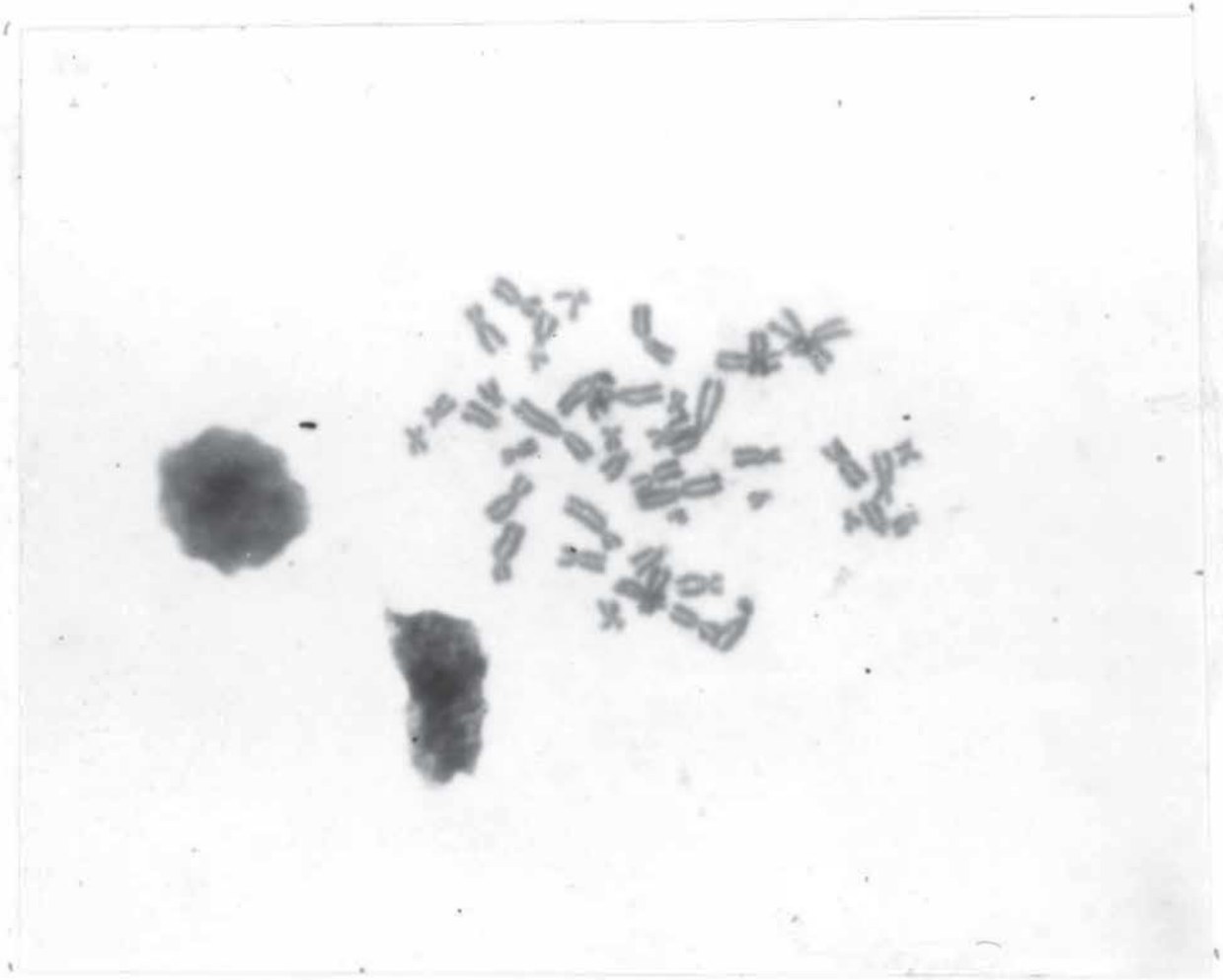
Male IX



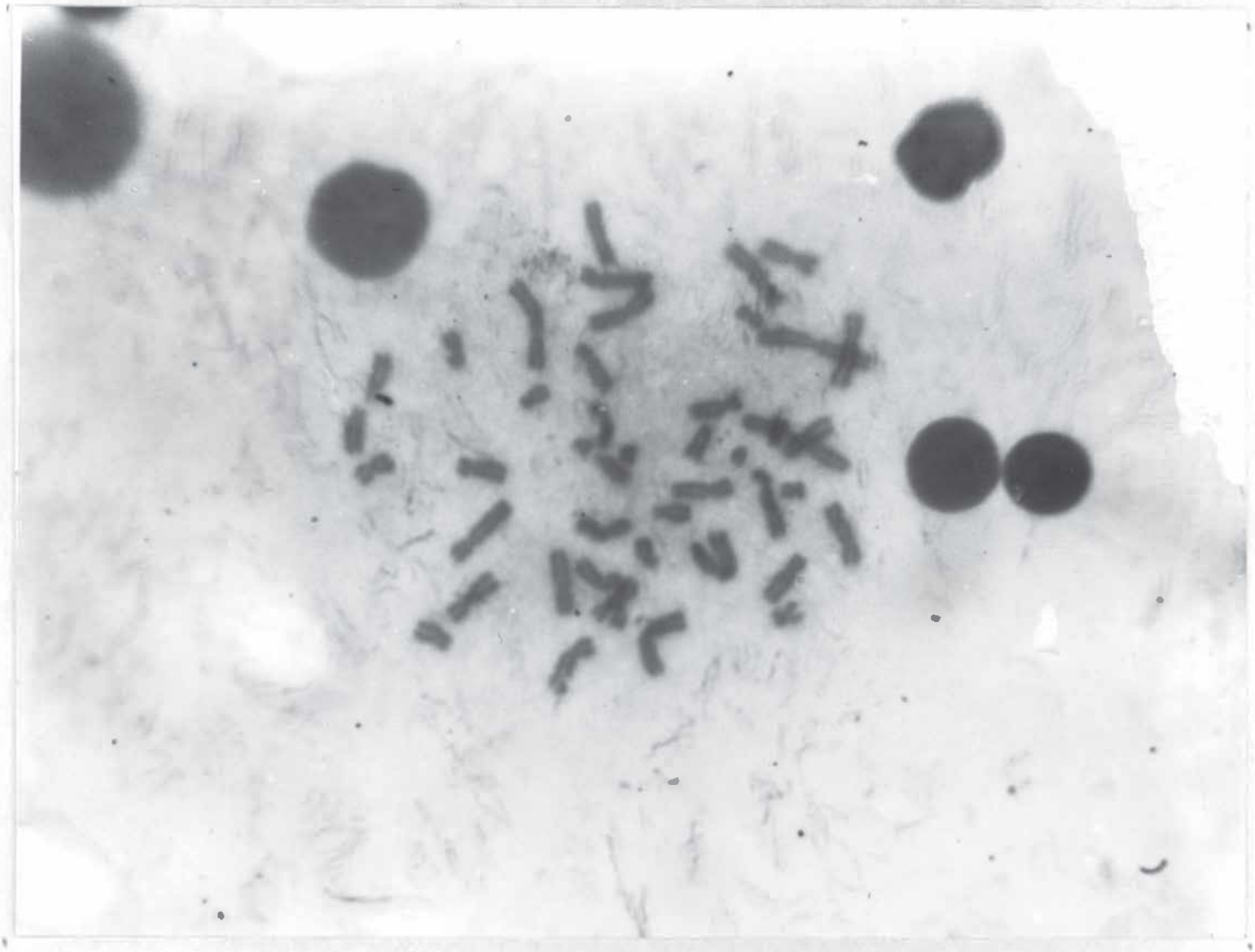
Male X

# Photographs of Karyotypes of Normal Human Female Subjects

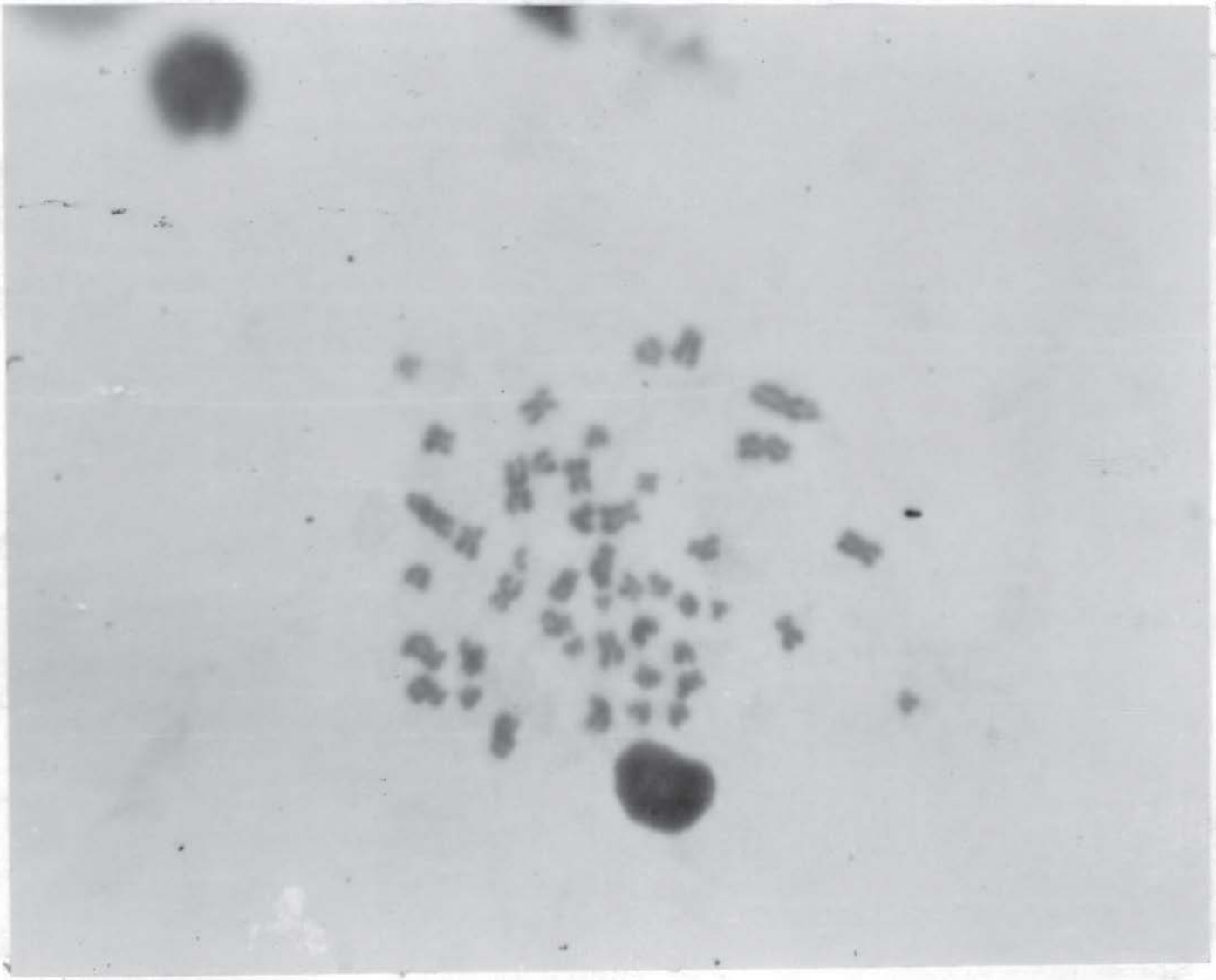
## (Photographed at magnifications of 400X)



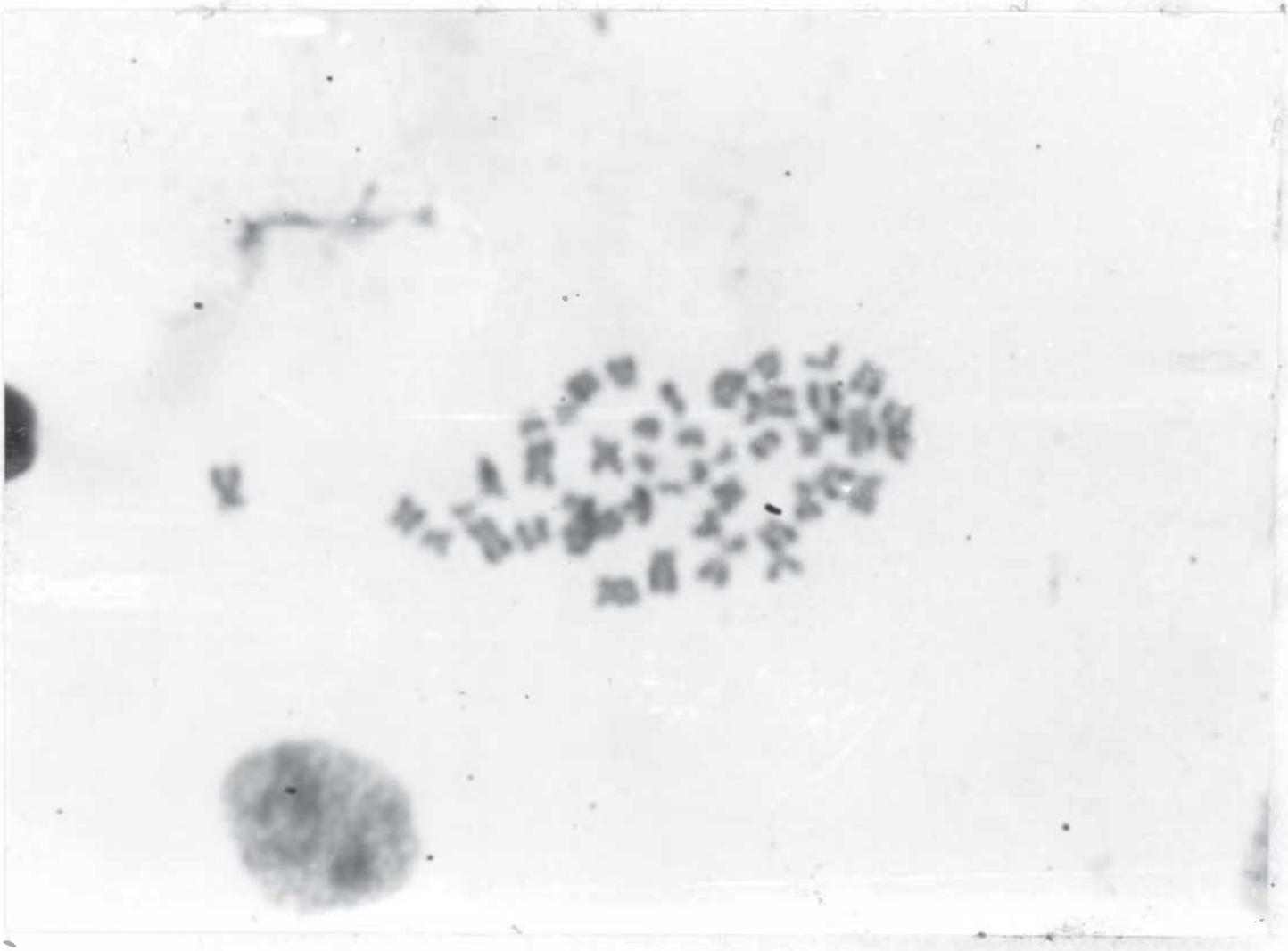
Female I



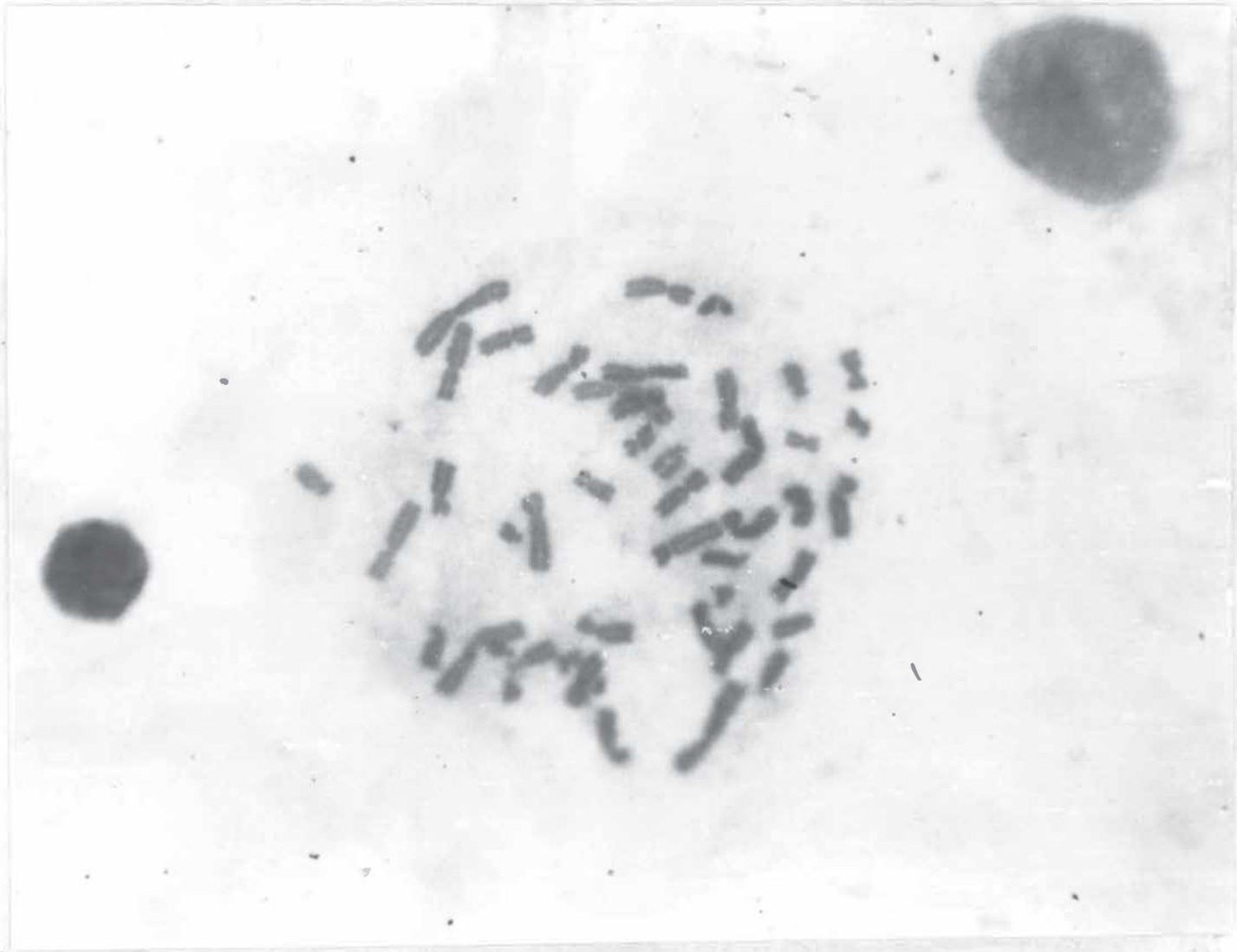
Female II



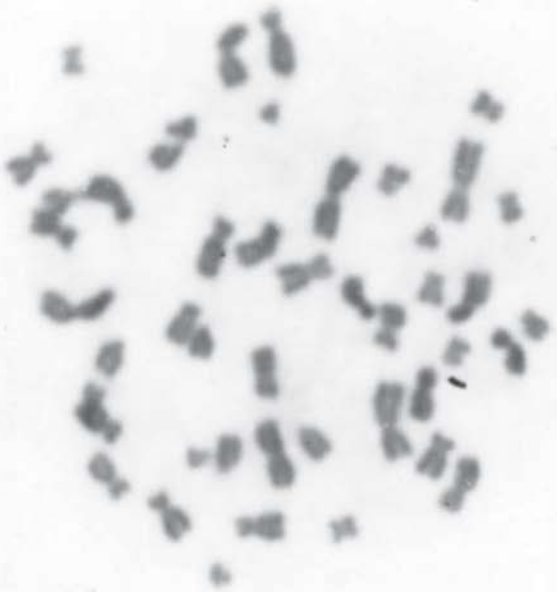
Female III



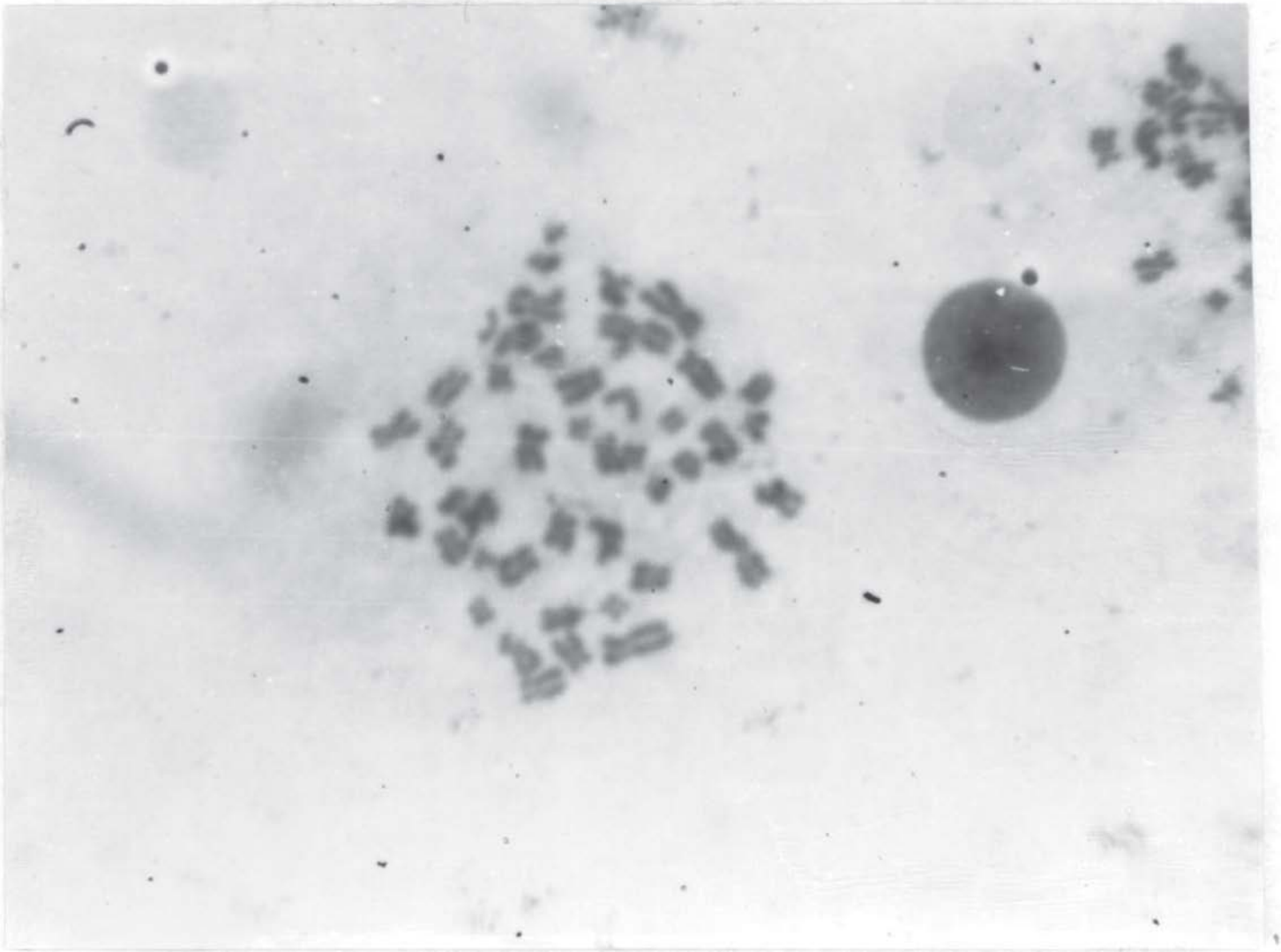
Female IV



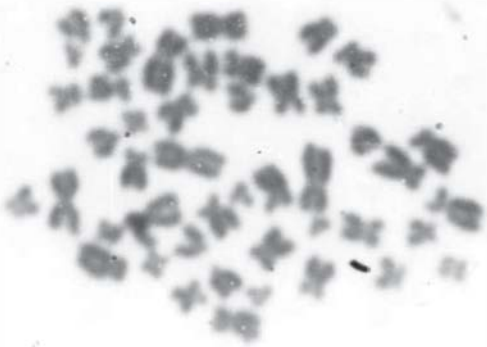
Female V



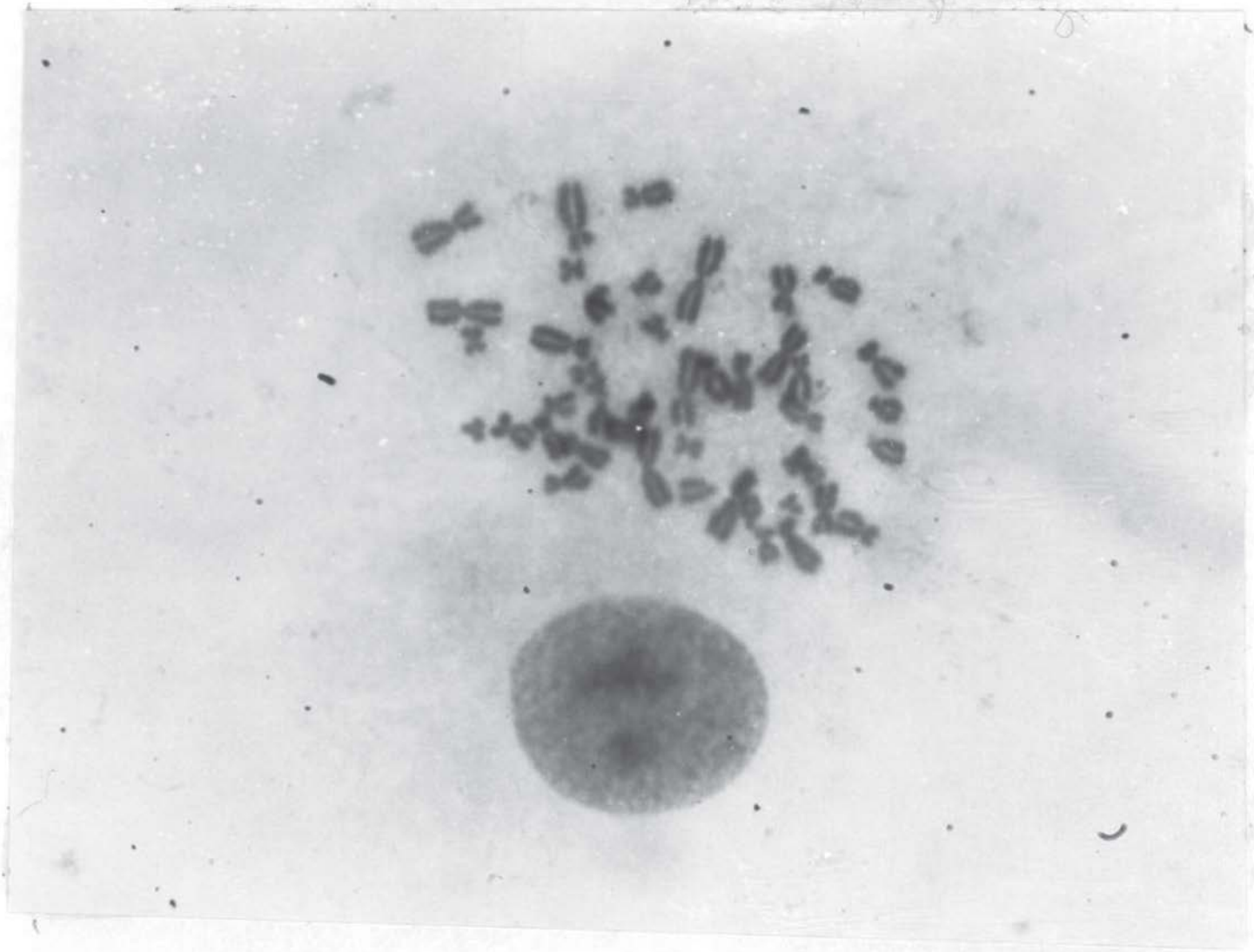
Female VI



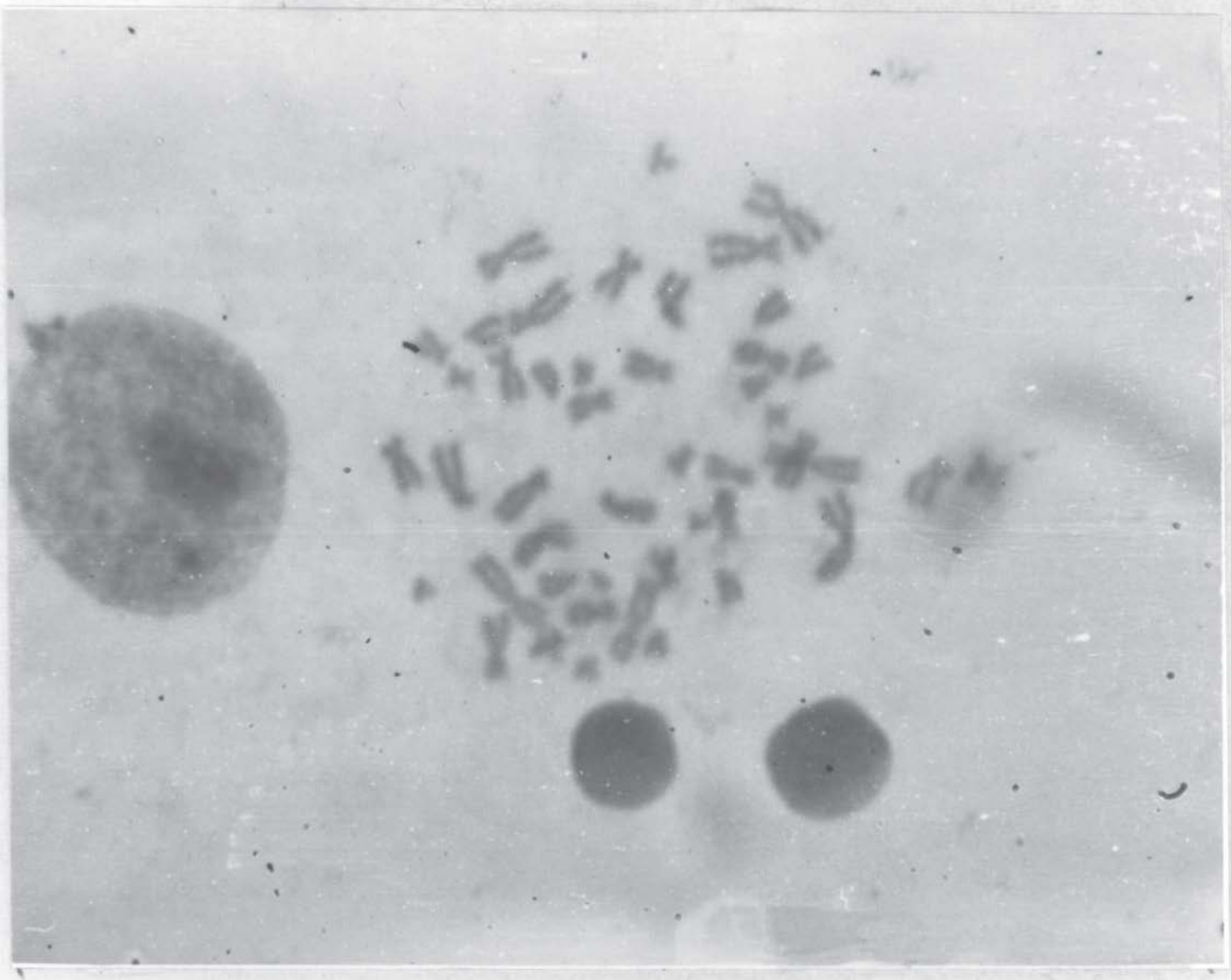
Female VII



Female VIII



Female IX



Female X