GENETICS OF SOMATIC MAMMALIAN CELLS

II. Chromosomal Constitution of Cells in Tissue Culture*

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Plates 19 and 20

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Determination of chromosome number and morphology promises to provide important cytogenetic markers for mammalian cells cultivated *in vitro*, and affords means for study of chromosomal change and stability under the action of various physical and chemical agents. Routine examination of cellular chromosomes is also important for checking the stability of clonal stocks, since spontaneous changes in karyotype of cultured animal cells have been reported to be fairly common occurrences (1). The present paper describes a simplified method for delineation of chromosomal constitution of cells grown as monolayers on glass, which is sufficiently rapid to permit continuous monitoring of tissue culture cell lines. The method has been applied to cells obtained from a variety of organs of several animals, and then grown *in vitro* for various periods. The cells discussed in this paper originated in normal and cancerous tissues of man and in normal tissues of the American opossum (*Didelphys virginiana*) and the Chinese hamster (*Cricetulus griseus*), these latter animals having been selected for their low chromosome number of 22 (2, 3). Detailed analyses of the individual chromosomes of these cell types will be presented in subsequent papers.

* Aided by grants from the National Foundation for Infantile Paralysis, Inc., the Commonwealth Fund, The Rockefeller Foundation, and the Colorado Division of the American Cancer Society, Inc. These experiments were carried out during the period, September 7 to December 5, 1957, in the course of a Visiting Professorship by Dr. Tjio in the Department of Biophysics of the University of Colorado Medical Center. While the technique for chromosome delineation here described was developed independently, it is a pleasure to acknowledge that the similar methodology recently described by Drs. Rothfels and Siminovitch (17) was developed prior to ours and that these authors kindly placed at our disposal an early version of their method and also sent us a copy of their final manuscript which was received after our own technique had been developed. There are important differences in the two techniques, and cells from different animal species were studied in the two laboratories. Contribution 65 from the Department of Biophysics, Florence R. Sabin Laboratories, University of Colorado Medical Center, Denver.

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Methods and Materials

The present method for chromosome delineation utilizes hypotonic saline for cell expansion as devised by Hsu (4) combined with colchicine pretreatment and acetic orcein staining. All the operations are performed on the cell monolayer directly attached to the glass surface on which it is grown. The epithelium-like HeLa S3 and S/NDV cells employed are standard clonal strains isolated in this laboratory (6 *a*) in 1955 from the culture originally established by Gey and his associates (7) from a human cervical carcinoma. Normal human cells were obtained from skin biopsies carried out on volunteers, or from normal tissues obtained in the course of surgical procedures on hospital patients. Biopsy specimens were also taken from a female inbred Chinese hamster and a male opossum, respectively provided by the kindness of Dr. G. Yerganian of the Boston Children's Cancer Research Foundation and Dr. Matthew Block of our own institution.

The tissue specimens, which usually amounted to 0.05 to 0.5 gm., were trypsinized, established in culture, and cultivated by the methods described elsewhere (6 *a*, 8). Growth media were employed which provided maximum reproductive rate for each cell type, and consisted of the standard mixture of amino acids, vitamins, growth factors, and salts previously described, supplemented with a mixture of human or horse serum in the case of the HeLa cells (6 *a*) and fetal calf serum (8) for the other cell types, all of which were of fibroblast-like morphology (9). All media were filtered as routine through a Selas 0.02 bacterial filter to insure absence of both bacterial and other cellular contaminants.

The procedure for chromosome delineation is as follows:—

1. Growth of Cells.—An inoculum of 2 to 5 $\times$ 10^4 cells from an actively growing culture is pipetted onto a clean, sterile coverslip in a 60 mm. Petri dish together with 5.0 cc. of the optimal growth medium for the cells employed. (Alternatively, the cells can be plated on a microscope slide.) The dish is incubated in a humidified incubator in air containing 5 per cent CO_2 at 37°C. for 48 hours or until the population has doubled or tripled.

2. Colchicine Pretreatment.—Without interruption of the incubation, 0.005 per cent aqueous colchicine (Eimer and Amend Co., St. Louis) is added directly to the dish in a final concentration of 0.3 to 0.5 γ/cc. and incubation continued for another hour.

3. Osmotic Expansion.—The medium is poured off and the dish is rinsed once and flooded with 0.17 per cent saline at 37°C. to expand the cells and disperse the chromosomes. After 5 minutes at 37°C., the dish is placed at room temperature for an additional 30 to 40 minutes.

4. Fixation.—A mixture of 95 per cent ethyl alcohol, glacial acetic acid, and 40 per cent formaldehyde, mixed in the ratio 6:2:1, is used as the fixative. The coverslip or slide is first immersed for 6 seconds in a dilute solution of the fixative (0.1 cc. of fixative in 100 cc. of 0.17 per cent saline) to improve the spreading of the chromosomes within the cells, and then placed in full strength fixative for 2 to 5 minutes. The forceps used in these operations must be carefully rinsed in distilled water between successive uses, to avoid contamination of the as yet untreated cells with concentrated fixative, which may cause excessive spreading of the chromosomes and bursting of the cells.

5. Air Drying.—After fixing, the coverslip is washed twice in distilled water for 3 seconds and allowed to dry in air at room temperature. The fixed cells now form a flattened, tightly adherent monolayer on the glass and can be stored indefinitely. The chromosomes can now be studied directly with phase contrast or small diaphragm microscopy, or they can be stained whenever it is convenient and observed with conventional microscopic optics.

6. Staining.—Staining can be carried out with acetic orcein or by the Feulgen procedure with basic fuchsin, safranine or Azur A. Only the acetic orcein procedure, which was most commonly used, will be detailed. A solution of 2 per cent orcein in 45 per cent acetic acid is prepared by dissolving 2 gm. of synthetic orcein[1] in 45 cc. boiling glacial acetic acid with stir-

[1] Obtained from George T. Gurr, London, England.

ring. The solution is cooled to approximately 50°C.; 55 cc. of distilled water is added, and after cooling to room temperature, the material is filtered. The solution must be refiltered daily to eliminate precipitation.

A drop of acetic orcein is placed on a microscope slide. The coverslip is placed cell side down upon it and all excess fluid absorbed by application of filter paper to its edges. A piece of filter paper is then laid over the coverglass and pressed with the thumb so as to squeeze out the last free liquid remaining between the two surfaces. The pressure applied is not critical since the cells have already been sufficiently flattened by the previous treatment. The edges are now sealed with Krönig's cement,[2] applied with a heated spatula. The resulting preparation is now ready for examination, although storage overnight improves it by intensifying the staining. It can be stored at 2–4°C. for 3 to 6 months, but not indefinitely. It can be rendered permanent by placing the slide, coverglass down, on top of a block of dry ice. When completely chilled, the cement is scraped off with a razor blade which is then inserted gently between the slide and coverglass to separate them. The member containing the cells is then placed in the following series of 4 baths in rapid succession for 3 seconds each: absolute alcohol; absolute alcohol; a 1:1 mixture of absolute alcohol and xylol; xylol. The specimen is then placed in a last xylol bath for 5 minutes and is removed. A drop of xylol and a drop of permount[3] are placed over the cells, a new slide (or coverslip) applied, and the preparation allowed to dry at room temperature.

This procedure results in highly expanded but usually unbroken cells in which the chromosomes are clearly delineated. The spindle-inhibiting action of colchicine insures the accumulation of cells in metaphase. Often it is possible to find more than a hundred cells with clearly defined chromosomal configurations on a single slide. For scoring mitotic irregularities during anaphase and telophase, neither colchicine nor hypotonic pretreatment is applied. The coverslips are transferred directly from the growth medium to full strength fixative, rinsed, dried, and stained. Microscopic examination and photography can be carried out on either the permanent or temporary preparations.

RESULTS OF THE EXPERIMENTS

1. The Chromosomal Constitution of Cells from Normal Human Subjects.— Cells were established in culture from 7 different human subjects, of which 3 were males and 4 females. Their ages varied between 1 and 41 years, and the biopsies were taken from various organs including skin, prepuce, cervix, uterine myo- and endometrium, and testis, but specific tissues were not isolated in any case. All the cells grew with typical fibroblast-like morphology (9) in the medium employed. The chromosome number in all cultures of normal human cells was always 46, as described by Tjio and Levan (10), and the incidence of polyploidy was small. Except for the expected morphological difference in the sex chromosomes between the male and female cells no variation either in number or morphology has been observed in the chromosomes of all these cultures.

In Figs. 1 to 3 typical photomicrographs are presented showing the kind of results which can be obtained with cells from a variety of normal human male and female tissues grown *in vitro*. Figs. 1 and 2 show the chromosome complements of a human male and female tissue respectively. Both have $2n = 46$ chromosomes.

[2] Obtained from George T. Gurr, London, England.

[3] Fisher Scientific Co., St. Louis.

Fig. 3 shows a tetraploid metaphase plate with $4n = 92$ chromosomes from a normal uterine cell culture. The Y chromosome can be easily recognized in the male cells (Fig. 1). It is similar in size to the smallest autosomal chromosomes of which there are 2 pairs. One of these autosomal pairs has a tiny satellite, which in some individuals shows a heterozygosity, like that in *Allium cepa* (16) and other plant species. While these two autosomal pairs are clearly subterminal, the Y chromosome apparently possesses an almost terminal kinetochore and displays a fairly large heterochromatic region close to the centric end. The X chromosome seems to be approximately double the size of the Y and is subterminally attached. A closer study of this chromosome is being made. An additional autosomal pair, which has an almost terminal kinetochore, also possesses a satellite. Heterozygosity, with respect to the size of the satellites of this pair, has been observed in cells of 2 female individuals. A detailed analysis of the human chromosome complement will be published later. Slides prepared as here described permit identification of chromosomal sex in human subjects.

Some idea of the constancy of the chromosome number in these preparations can be obtained from the fact that, in the examination of 274 cells selected at random of a culture from the cervix, 250 cells with 46 and 24 cells with 92 chromosomes were counted, a result fairly representative of all the normal human cell lines examined. Cells cultivated from normal human skin, cervix, prepuce, and uterus unequivocally displayed the same chromosome number of 46, with about 4 to 10 per cent of tetraploid forms. Aneuploid cells have not been found in these cultures.

No changes in chromosomal numbers or morphology occurred throughout 5 months of intensive cultivation *in vitro*, during which such cells underwent approximately 20 successive harvests involving transfer to new Petri dishes and more than 40 generations of reproduction (equivalent to a total progeny of 2^{40} individuals).

2. Cells from the Female Inbred Chinese Hamster and Male American Opossum. —The relatively large number of the human chromosomes, together with their relatively small range of size (5), makes difficult the quantitation and localization of cytogenetic changes. Hence, the cells of the opossum and Chinese hamster, possessing considerably smaller numbers of chromosomes, were examined by this technique in the hope of using such cell lines as model systems in the quantitative study of the genetic action of various agents on cells cultivated *in vitro*.

Fig. 4 shows a typical picture of the chromosomes from cultured cells of the female inbred Chinese hamster lung. The larger and more varied sizes of these chromosomes, their smaller number, and the distinctive morphology of each pair constitute great advantages in cytogenetic studies of cell cultures. However, in contrast to the human cells, in every culture originating from the various organs of this animal, cells with chromosomal irregularities were found. Thus, in a typical series of 568 unselected cells from a spleen culture, 3 had a chromosome number of 21, 511 had 22 chromosomes, 4 displayed 23, and 50 cells possessed a tetraploid configura-

tion with 44 chromosomes. Fig. 5 presents a tetraploid spleen cell in prophase. Virtually the same distribution was obtained for chromosomes of cell cultures established from the spleen, kidney, lung, and ovary. These irregularities in number appeared in the very first cells examined on the 20th day after biopsy. Their distribution in uncloned cultures has not changed materially after months of cultivation.

In addition to variations in chromosome number occasional structural irregularities, such as translocations (Fig. 6, arrow), and centric and acentric fragments, also were observed. In cultures which were not treated with colchicine, anaphases with bridges, with or without fragments, were encountered. These irregularities also appeared even in the first subcultures after biopsy. Such instability of the mitotic mechanism was not found in the cultures of normal human or opossum cells grown under identical conditions.

The American opossum, also possessing $2n = 22$ chromosomes, may offer the same advantage as the Chinese hamster for cytogenetic studies in cell cultures. In order to obtain a continuing record of the chromosome constitution of such cells after their separation from the animal, chromosome preparations were made of testicular material *in situ* by the technique of Tjio and Levan (5). A biopsy from the other testis of the same animal was then cultured by the standard technique. The chromosomal picture of the growing cells is being examined in a continuing study to follow closely the appearance of deviations from the original pattern, none of which have yet been observed.

In Figs. 7 and 8 are represented respectively the chromosomes of a mitotic metaphase from the testis *in situ* and a late prophase of a cell originated from the testis, from the same animal, grown *in vitro*. As can be observed from the micrographs, the chromosome complement of this marsupial is less favorable cytologically than that of the Chinese hamster. The autosomes have subterminal or terminal kinetochores and the variation in size is less distinct in both groups. The sex chromosomes, however, are readily distinguishable, as they are smaller than any of the autosomal pairs. The Y is the smallest chromosome and is terminally attached, while the X has a submedian kinetochore. Thus, these 2 chromosomes appear very useful as cytological markers.

Table I shows the chromosome number distribution among the cells of cultures established from the testis of the American opossum and of the ovary of the Chinese hamster, in which the heterogeneity of the latter contrasts strongly with the high uniformity of the former.

3. Clonal Strains of HeLa Cells Cultivated for Long Periods In Vitro.—A number of clonal cell strains were isolated in this laboratory from the HeLa culture of a human cervical carcinoma and were characterized on the basis of differences in nutritional requirements for single cell growth (S1 and S3) (11), and differences in virus resistance (S3 and S/NDV) (6 *b*, *c*, *d*). These cells have been cultivated intensively for approximately 3 years, during which 5-fold increases in population and transfer to new bottles occurred on the average of once a week, so that these cultures have now passed through hundreds of

generations. Routine recloning has been instituted on an annual or semiannual basis, exactly as in microbiological genetic practice. During this period these cell strains have preserved intact the differences in genetic characters which served as the basis of their original isolation (6 *d*).

The chromosomal constitution of the S3 strain has been examined by Hsu (12) and more recently both it and the S1 strain were studied by Chu and Giles (13). The clonal lines were found to be much more uniform in karyotype than the highly heterogeneous HeLa parental culture from which they were

TABLE I

Representative Distributions of Chromosome Numbers in Cells Cultured from the Male American Opossum and the Female Chinese Hamster

Cell origin	Total cells examined	Cells with chromosome numbers as follows:						
		<21	21	22	23	24-43	44	>44
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Opossum testis	112	0	0	99.1	0	0	0.9	0
Chinese hamster ovary	531	0	2.6	83.8	1.90	0	11.3	0.4

TABLE II

Distribution of Chromosome Numbers in Cells of the HeLa S/NDV Clone, a Strain Distinguished from HeLa S1 and S3 by Its Resistance to Newcastle Disease Virus

The S/NDV strain has been in continuous cultivation in this laboratory for 3 years. Counts were made on 139 cells.

Chromosome number	<75	75	76	77	78	79	80	81	82
Per cent of cells	0	0.7	2.2	7.2	81.5	4.3	2.9	0.7	0.7

In a count of 3210 cells, 6.4 per cent displayed two or more times the stemline number of chromosomes.

taken. Chu and Giles found a stemline chromosome number of 78 to be displayed by more than 60 per cent of both the S1 and S3 strains after 2 years of cultivation in our laboratories. We have repeated chromosomal analysis of these strains and have also examined the S/NDV HeLa clone which was isolated at the same time and from the same parental culture as the other 2, and which differs metabolically from them in exhibiting a high degree of resistance to, and the ability to associate stably with, a strain of Newcastle disease virus which destroys completely all other HeLa strains we have studied (6 *b*, *c*). More than 80 per cent of the cells of the S/NDV clonal line have a chromosome number of 78, as shown in Table II. A typical photomicrograph of the chromosomes from such a cell is shown in Fig. 9.

While the frequency of occurrence of the same stemline number of 78 in the 3 clonal lines isolated from the same parental HeLa culture is probably not mere coincidence, it should not be inferred that this number represents the only karyotype in this culture capable of indefinite cell division. Thus, of several subclones of S3 picked and developed into standard stocks by means of at least 2 consecutive single cell isolations, other stemline numbers have also appeared. One of these, designated S3-9, has an advantage over S3 in producing colonies more uniform in morphology and with more closely packed cells. This subclone displays a stemline number of 73. In a count of 108 cells performed within 2 to 3 months after establishment of the subclone, 91 per cent of the cells exhibited the stemline chromosome number, 1 per cent had 71, 5 per cent had 72, and 3 per cent of the cells had 74 chromosomes. In a survey of over 1000 cells, the incidence of forms with approximately double the stemline number was somewhat less than 1 per cent. This line is so far the most uniform of all the HeLa strains of our experience and hence affords special advantages for use as a standard strain. Fig. 10 presents the chromosomes of a typical cell of the S3-9 subclone.

Data of this kind demonstrate that even highly aneuploid malignant cells can be cultivated *in vitro* practically indefinitely with reasonable chromosomal stability.

DISCUSSION

The availability of rapid and precise means for visualization of chromosomal constitution of animal cells grown *in vitro* makes possible continuous monitoring of tissue cultures to insure against overgrowth by a new karyotype, and permits ready identification and quantitative study of chromosomal variations introduced into clonal cell strains by physical and chemical agents. In the method here described the cells are fixed and stained without ever becoming detached from the glass surface on which they have grown, so that it becomes possible even to plate single cells on a slide, and permit them to grow into colonies of thousands of cells, whose chromosomal constitution within these clones may then be examined as a primary genetic marker.

This demonstration that both normal and aneuploid animal cells can be cultivated for long periods without any recognizable change in karyotype or in specific genetic markers is particularly important in view of the reports of transformation of cultured animal cells which have recently appeared (1). We have found that under certain conditions of growth, probably associated with toxic media, new chromosomal variants may suddenly appear in clonal cultures. However, such spontaneous changes in genotype characterize every living population, and since such changes are usually unitary events, recloning of the culture and screening the re-isolated stocks for the desired characters would appear to be as effective in animal cells as it is in microbiology. While experience alone can determine how long a given cell line can be safely cultivated under specified conditions before the inevitable appearance of divergent

genotypes threatens its integrity, we have found that semiannual recloning suffices, even for the highly aneuploid HeLa cells of carcinomatous origin. The colonies which are so isolated are checked for conformance to the parental type with respect to cellular and colonial morphology; virus resistance; plating efficiency and growth rate of single cells in standard media; x-ray sensitivity; and chromosomal constitution. The simplicity of the cloning technique makes these procedures only slightly more laborious than the corresponding bacteriological routines (6 *a*).

The nature of the chromosomal lability in the cell cultures of the inbred Chinese hamster presents an extremely important field for study, particularly since human and opossum cells grown under identical conditions in this laboratory thus far fail to exhibit similar abnormalities. Quantitative study of these abnormalities in clonal strains of the Chinese hamster cells is proceeding.

The rapid rate of advance in understanding of genetic processes in bacteria was made possible by the possession of certain key operations. Some of the most basic of these are now available in animal cell systems: the quantitative growth of single cells into discrete, macroscopic clones (6 *a*); the ability to isolate mutant clones readily (11); and the availability of a defined medium in which every plated cell can form a discrete colony of any desired size (14). Other operations, like the ability to transform a cell's genetic constitution with the aid of a temperate virus, appear imminent (15). Availability of means for routine examination of chromosomes lends to studies of animal cells grown *in vitro* a tool which has never been available in bacterial genetics. Mammalian somatic cells may eventually afford an even more versatile system than microorganisms as a tool for fundamental genetic analysis.

SUMMARY

A convenient, reliable method for chromosome delineation of animal cells grown as monolayers on glass has been applied to human, opossum, and Chinese hamster cells.

Tissue cultured cells from 5 different, normal organs of 7 different human subjects uniformly displayed the expected chromosome number of 46 and showed no variations in morphology or number other than the expected sex differences and a small incidence of polyploidy.

The chromosomes of normal cells from the American opossum were as uniform as those of human cells. Cells of the inbred Chinese hamster demonstrated appreciable karyotype variability, the cause of which is under investigation.

The chromosome number and morphology of cells from normal human tissues have remained constant after more than 5 months of continuous, rapid growth in tissue culture involving scores of vessel transfers and a number of generations equivalent to many billions of progeny.

By the use of routine recloning, even cells of malignant, aneuploid constitution have been maintained in active growth for 3 years and hundreds of generations, with stable chromosomal and metabolic characteristics.

The cells of the American opossum and Chinese hamster which possess only 22 chromosomes have been established *in vitro* and are especially suitable for genetic studies. The readily recognizeable Y and X chromosomes of the male opossum are particularly favorable as cytological markers.

Photomicrographs of the chromosomes of the various cells employed are presented.

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EXPLANATION OF PLATES

PLATE 19

FIG. 1. Metaphase of a human male cell, grown *in vitro*; $2n = 46$. The biopsy tissue was obtained from an adult and grown *in vitro*, in a medium supplemented with fetal calf serum for about 3 weeks, at a generation time after the 1st week of approximately 20 to 25 hours. Acetic orcein stain. × 1320.

FIG. 2. Metaphase chromosome of a human female cell, grown *in vitro*. The biopsy was taken from the cervix and grown as described above. Acetic orcein stain. × 1320.

FIG. 3. Metaphase of a tetraploid human female cell, grown *in vitro*; $4n = 92$. The biopsy was taken from the myo- and endometrial uterine tissue. Acetic orcein stain. × 1320.

FIG. 4. Metaphase of a female Chinese hamster cell, grown *in vitro*; $2n = 22$. The biopsy was taken from the lung. Acetic orcein stain. × 1320.

FIG. 5. Late prophase of a tetraploid female Chinese hamster cell grown *in vitro*; $4n = 44$. The biopsy was taken from the spleen. Acetic orcein stain. × 1320.

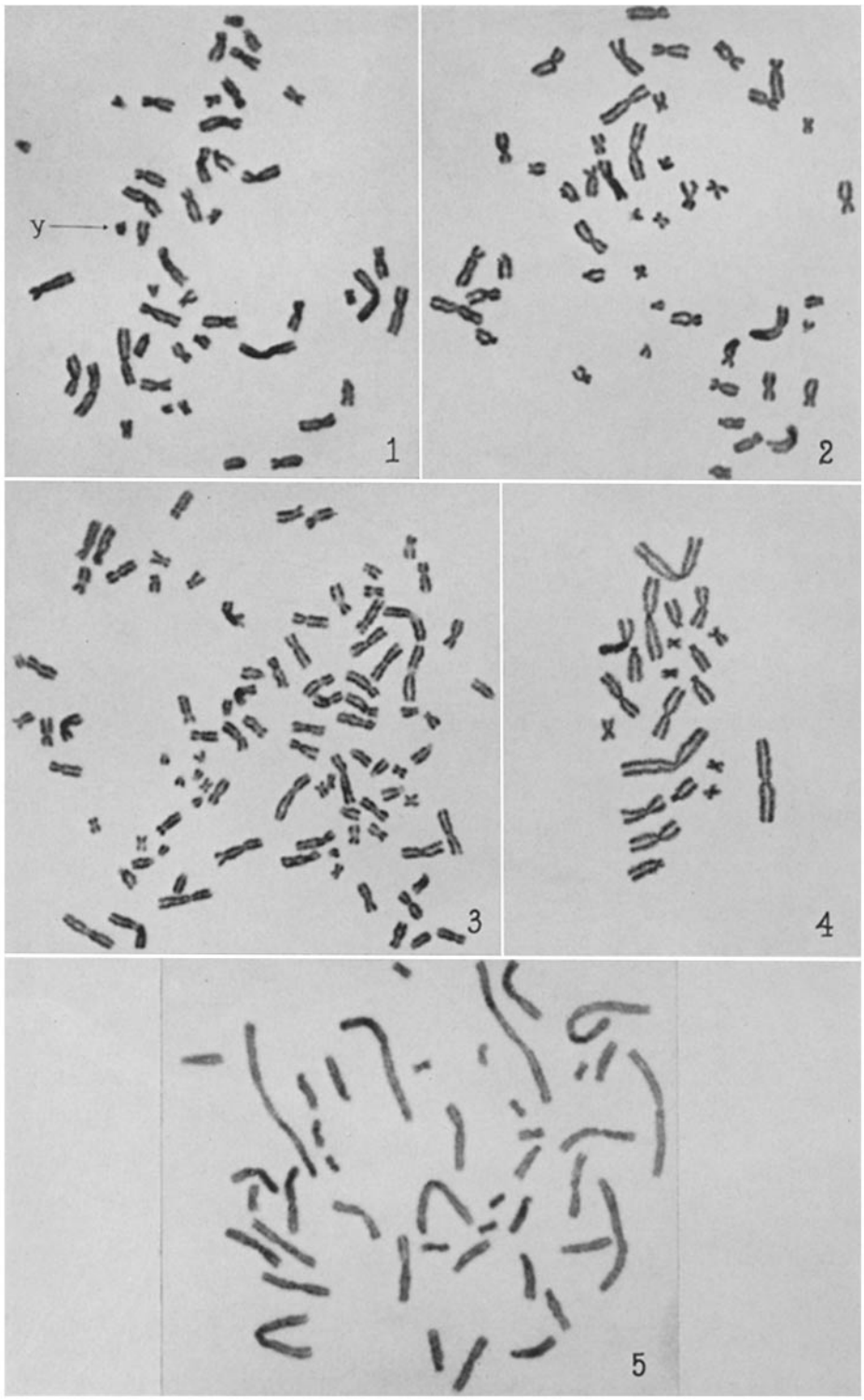

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PLATE 20

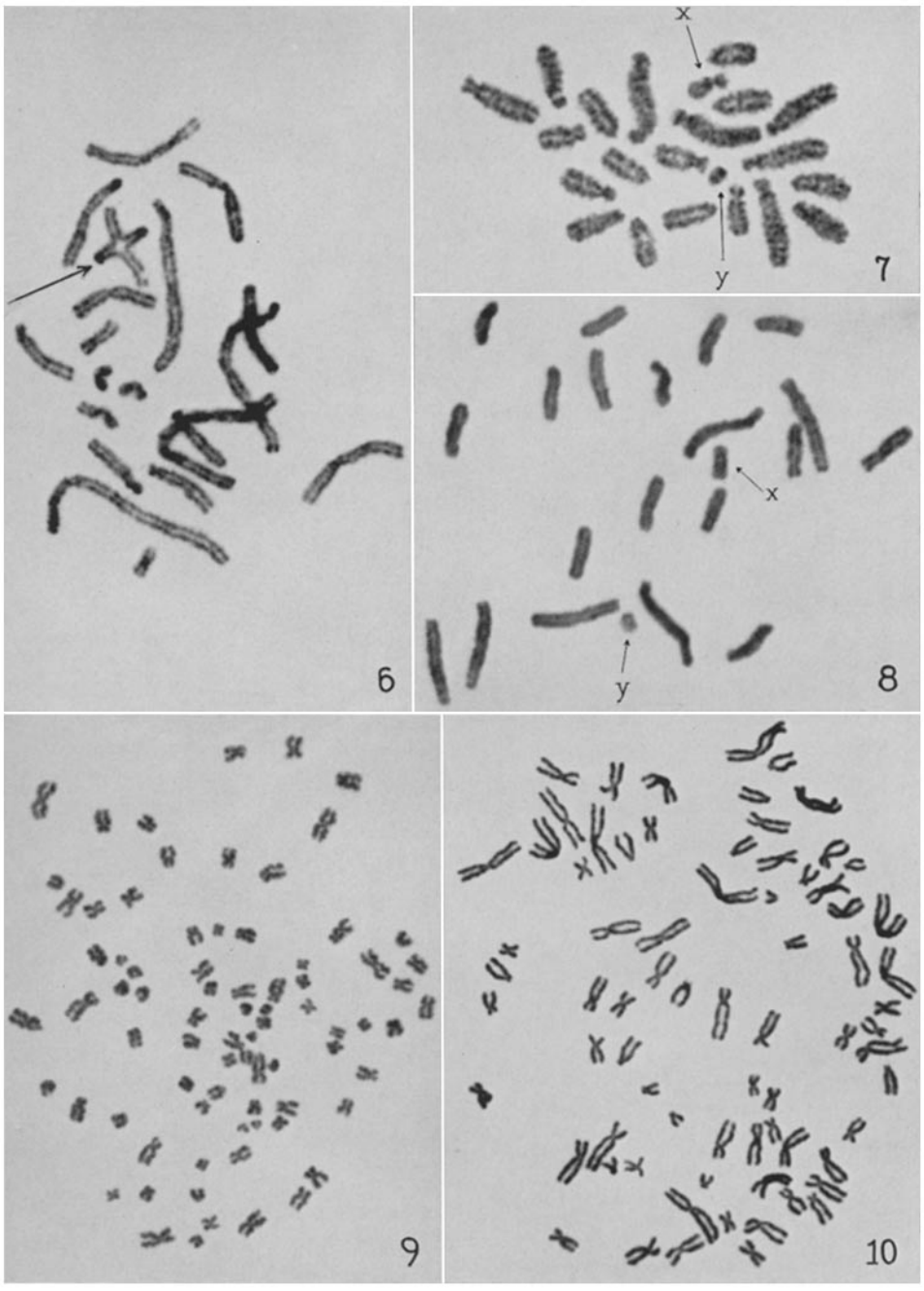
FIG. 6. Late prophase of a female Chinese hamster lung cell grown *in vitro*, showing structural and numerical changes, which are occasionally found in this culture. Note the chromosomal translocation in the form of a quadriradiate. Acetic orcein stain. × 1320.

FIG. 7. Metaphase of spermatogonial mitosis *in situ* of the male American opossum; $2n = 22$. Note the X and Y chromosomes. Acetic orcein stain. × 1440.

FIG. 8. Late prophase of a male American opossum cell grown *in vitro*. The biopsy was taken from the testis of the same animal as in Fig. 7. Acetic orcein stain. × 1440.

FIG. 9. Metaphase chromosomes of an S/NDV cell, a clonal strain isolated from the HeLa cervical carcinoma culture, and distinguished by its resistance to destruction by Newcastle disease virus. Acetic orcein stain. × 1150.

FIG. 10. Early metaphase of a subclone of the S3 HeLa strain with 73 chromosomes. Acetic orcein stain. × 1440.



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