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Quantitative Studies on Mammalian Cells *in Vitro**

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THE major advances of the science of genetics have come about through the use of two fundamental types of operations. The first and oldest consists in mating of selected, multicellular plants or animals to produce large numbers of offspring, among which the distribution of various inherited characters is examined. This procedure has produced that body of knowledge known as classical genetics and which, beginning systematically with the studies of Mendel, has delineated the processes which sustain and modulate biological heredity in all living forms. This experimental approach, which essentially examines genetic mechanisms in the germ cells, has been extremely successful in elucidating hereditary phenomena in organisms as diverse as *Drosophila* and *Zea mays*, but is difficult to apply to the slowly and less extensively reproducing organisms like the mammals. Thus, while some notable advances have been accomplished, it has not been generally possible to obtain sufficiently large numbers of progeny from selected mammalian matings to provide populations adequate for measurement of many important genetic events.

The second major class of genetic operations involves a more recent technique, which consists in study of the independent micro- and ultramicro-organisms—the free-living cells and the viruses. Here the standard unit is a single cell or particle, and the fundamental operation involves examination of the distribution of genetic traits among the progeny of asexual reproduction. The principal power of this method lies in the vast multiplication factor which it affords. Instead of hundreds or thousands, millions or billions of progeny, all arising from a single individual, can be rapidly produced and quickly scanned for a large variety of genetic characters, so that even rare events can be quantitatively examined. In the last twenty years, such genetic studies on microorganisms have produced a whole new field of knowledge. Sexual methods of genetic analysis are not excluded from these operations but can sometimes be arranged in a step preceding the clonal multiplication of the single cells. In addition, however, new methods for nonsexual genetic analysis have been found which promise to be exceedingly productive. Among the fundamental results which have issued from use of single-cell techniques applied to microorganisms during the last two decades are included: (a) the first systematic demonstration of the direct relationship between genes and enzymes; (b) delineation of many specific, metabolic pathways involved in gene-controlled biosyntheses; (c) demonstration of the direct exchange of genetic materials between cells previously considered to multiply only mitotically; (d) introduction of genes into cells by means of temperate viruses or pure deoxyribonucleic acid; (e) the most accurate measurement of gene-mutation rates; (f) demonstration and quantitative analysis of the processes involved in enzyme induction among genetically competent cells in response to specific environmental stimuli; (g) delineation of the characteristics of linear inheritance in bacteria; (h) systematic use of mitotic crossing-over in certain organisms to localize genes on their chromosomes; and (i) the most detailed mapping at the level of molecular dimensions of the linear sequence of genetic determinants, as accomplished in a bacteriophage chromosome.

An index of the fruitfulness of this latter approach to the analysis of genetics and related metabolic processes is afforded by the fact that the great majority of the discussion devoted to genetic processes in this biophysical conference has been concerned with developments arising directly from microbial genetics.

In our laboratory, a program was initiated, attempting to develop a methodology that would make possible studies of mammalian cells by means of the techniques of microbial genetics. If successful, such a methodology would provide a new avenue for exploration of mammalian genetic processes among the somatic cells of multicellular organisms, which would complement studies on the genetics of the germ cells achieved by conventional mating studies. In addition, it would afford all of the many kinds of analyses which an unlimited number of progeny provides. Such techniques might facilitate elucidation of the nature of any changes in the genome and phenome, respectively, which are responsible for the characteristic behavior of the various differentiated tissue cells; localization of different genes on the various chromosomes; provision of mutated cells containing specific biochemical blocks for the delineation of various enzymatic steps through quantitative study of specific biosyntheses, like that of hormones and antibodies in specialized cells; search for processes like sexual genetic exchange and transduction in somatic cells, and investigation of the genetic biochemistry of normal and malignant cells.

Participating in this program have been a series of devoted co-workers: Steven Cieciura, Harold Fisher, and Philip Marcus carried out studies which formed the basis of their doctoral theses; and D. Morkovin, G. Sato, and N. C. Webb joined this program in a postdoctoral capacity. Chromosome studies have been

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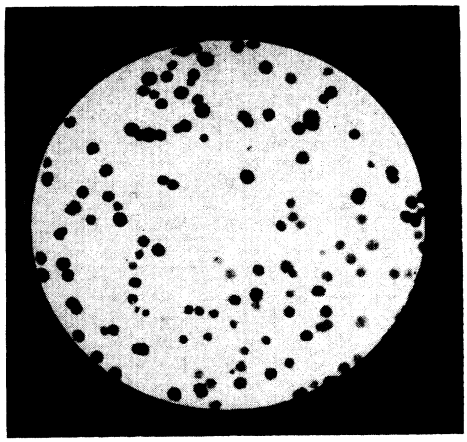
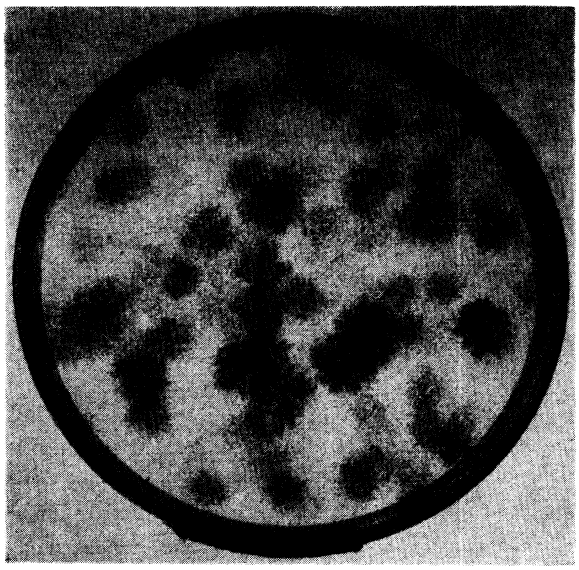


(a)



(b)

FIG. 1. (a) Petri dish which was seeded with 100 single cells of the S3 strain of the HeLa culture, an aneuploid which originated in a human cancer. (b) Petri dish seeded with single cells of a euploid human strain. These cells tend more to spread and migrate, and so the colonies tend to run together unless a smaller number of cells is plated or a larger plate is employed.

carried out initially in a collaborative arrangement with Dr. Chu and Dr. Giles at Yale University, and more recently with J. H. Tjio, who joined our laboratory last year. Dr. Arthur Robinson has conducted those aspects of these studies which involved work on human patients.

The first step in this program, which, of course, has drawn a great deal on previously existing tissue-culture methodology, was to develop means for plating single mammalian cells, under conditions such that every single cell develops into a discrete, macroscopic colony.[1] This is essential in order to quantitate growth of single cells of a large population, and to make possible isolation of mutant clones. A simple, quantitative means for growth of single cells into colonies was achieved by a procedure, identical in principle to, and only slightly more complex in practice than, the plating method which constitutes the basis of quantitative microbiology. Figure 1(a) illustrates a representative plating in which 100 cells of the S3 HeLa clone, plated in a Petri dish in nutrient medium, have attached to the glass and produced, within the limits of sampling uncertainty, a discrete macroscopic colony from each cell inoculated. The counting of such colonies is completely objective and highly reliable, so that this methodology permits all of the types of experiments characteristic of quantitative bacteriology to be applied to mammalian cells. Cells taken from organs as diverse as skin, liver, spleen, bone marrow, testis, ovary, kidney, lung, and others, from man and other mammals, and from individuals of a large variety of ages, all respond similarly. It can be concluded that at least large numbers of cells exist in virtually every organ of the mammalian body which retain the potentiality of growth as independent microorganisms, if provided with an adequate physical and chemical environment.

By and large, two general types of cellular and colonial morphologies result from such plating. That illustrated in Fig. 1(a) has been called "epithelial-like" because the cells form compact, tight colonies with relatively smooth, well-defined edges. In our experience so far, the cells that tend to grow stably in this fashion have been polyploid, and usually aneuploid. The other type of colony, which has been called "fibroblast-like" but should perhaps be referred to simply as "stretched," is illustrated in Fig. 1(b), which shows the tendency of the colonies to grow with rough edges, and of the cells to line up as elongated parallel structures. This stretched, needle-like conformation is more characteristic of the euploid cells grown by the methods developed in our laboratory. However, the molecular environment strongly influences the morphology of cells grown in this manner.[1]

The course of developments in microbial genetics has demonstrated the enormous utility of quantitative single-cell plating for the isolation of stocks with rare genetic markers that permit analytical experimentation. Unless each cell can grow in isolation to form a colony, it becomes impossible to apply the highly discriminating screening methods by which millions of cells are subjected to a stressful situation permitting growth of only occasional rare mutants, for otherwise, the exceedingly rare mutant which though potentially is capable of reproduction is not part of a large reproducing population and may not be enabled to express its growth potentiality. An effective single-cell plating technique permits ready recognition of even very rare genetic events, and quantitation of their frequency.

Isolation of mutant clones from such plates is a straightforward process for which a variety of different mechanical methods have been developed. We have established mutant clones which have arisen spontaneously or have been induced as a result of x-irradiation. Among these are included forms with divergent nutri-

tional requirements for colony formation, changed colonial morphologies, resistance to destruction by specific viruses like Newcastle disease virus, and differences in chromosomal constitutions (Fig. 2). These mutants have been grown for months or years in continuous culture, during which they have produced astronomical numbers of progeny, and have exhibited stability with respect to their identifying characteristics which is in every way comparable to that of the familiar mutants in bacteria and molds.[2,3]

Attention was next devoted to the development of a defined chemical medium which would promote growth in high efficiency of single mammalian cells, and so make available means for isolating mutants with specific nutritional markers. Many laboratories have contributed studies elucidating small molecular requirements of mammalian cells in tissue cultures utilizing massive cell inocula.[4–6] In studying the growth requirements of individual, isolated cells, we found that single cells of the S3 clonal strain of the HeLa cell form colonies with 100% plating efficiency, when a chemically defined small-molecular medium is supplemented with two macromolecular serum fractions each migrating as a single moving boundary in an electric field. The two proteins of mammalian serum which, under the conditions of these experiments, complete the growth requirements of single cells are serum albumin and an α_1-globulin with a molecular weight of approximately 45 000, which appears to be a glycoprotein in composition. Both substances are necessary for growth of single cells under the specific conditions of our procedures. The functions of these proteins are still under study, but that of the α_1-globulin has been found to be fulfilled completely by the protein fraction named fetuin, an α_1-globulin which constitutes 45% of calf fetal serum protein.[7]

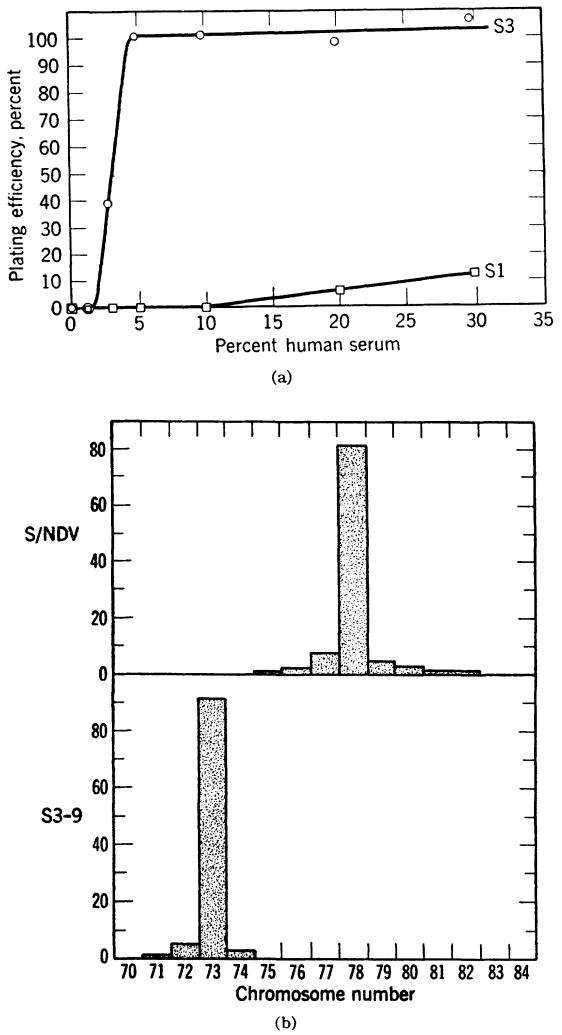


FIG. 2. Demonstration of some representative mutant strains isolated from the HeLa population. (a) Plating efficiency curves in the presence of different amounts of human serum of 2 mutants found to occur spontaneously in the original HeLa population. The cellular and colonial morphologies of these two strains are identical but their growth requirements are different. (b) Distribution of chromosome number in 2 HeLa clones. The clone at the top (*S/NDV*) has the same chromosome number as S3, but is characterized by its resistance to destruction by Newcastle disease virus.

Albumin and fetuin have been purified by repeated precipitation to the point where preparations are approximately 98% homogeneous electrophoretically and ultracentrifugally, although the ultimate purity of such preparations is, of course, difficult to specify with certainty, particularly since fetuin has been demonstrated to be heterogeneous immunologically. A typical electrophoretic pattern is presented in Figs. 3(a), and 3(b) demonstrates the colonial development achieved from inoculation of single cells into a medium containing the purified albumin and fetuin, amino acids, growth factors, salts and glucose. The plating efficiency equals that in serum-containing medium. Experiments on mutants of S3 which exhibit different molecular nutritional requirements are now in progress.

It seems likely that fetuin and albumin may not be required as true metabolites, but rather as conditioning factors which permit establishment of the necessary physicochemical relationships between the cell and surrounding medium. Thus, the fetuin fraction exercises a specific action on the cell membrane, since trypsinized cells added to a medium, complete except for the presence of fetuin, remain as rounded spheres, unattached to the glass surface. With the addition of fetuin to such a medium, the cells rapidly attach to the glass and stretch out in a highly characteristic configuration [Figs. 4(a) and 4(b)]. As little as 1 mg/cc of fetuin can be detected by means of this action. Fetuin has been known to be a powerful antitryptic agent, and at least part of the function of fetuin seems to be antiproteolytic in nature, because this substance prevents the tryptic loosening and rounding of glass-attached cells. The participation in the attachment reaction of a protein not included in the fetuin fraction has been claimed by some workers.[8]

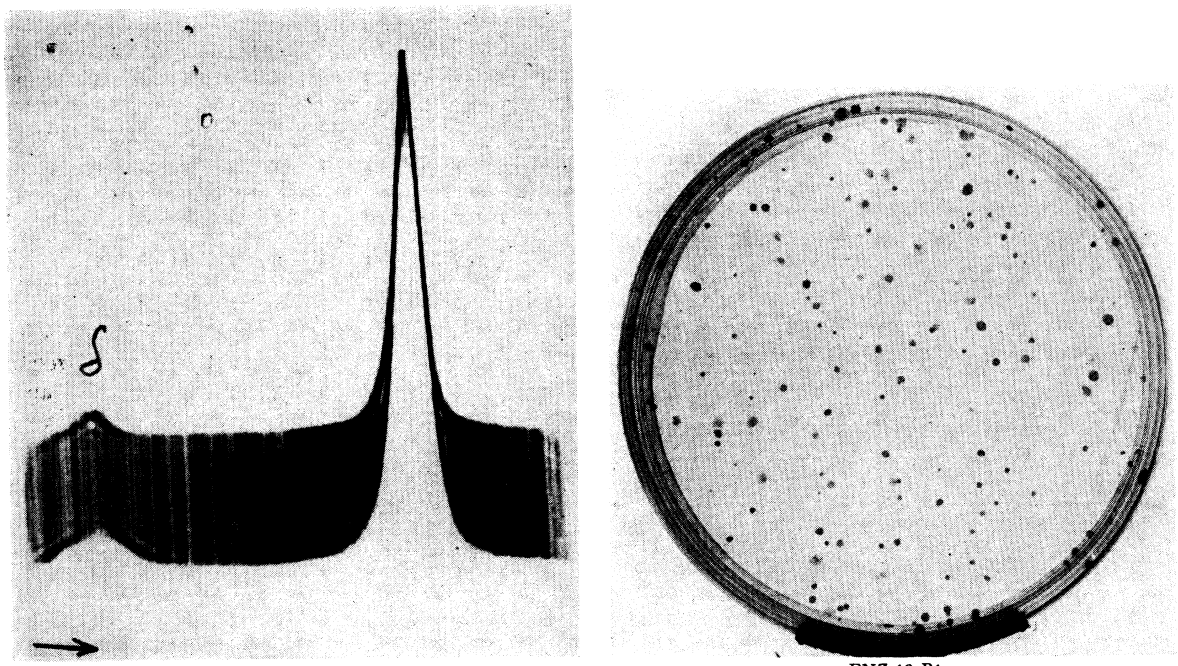
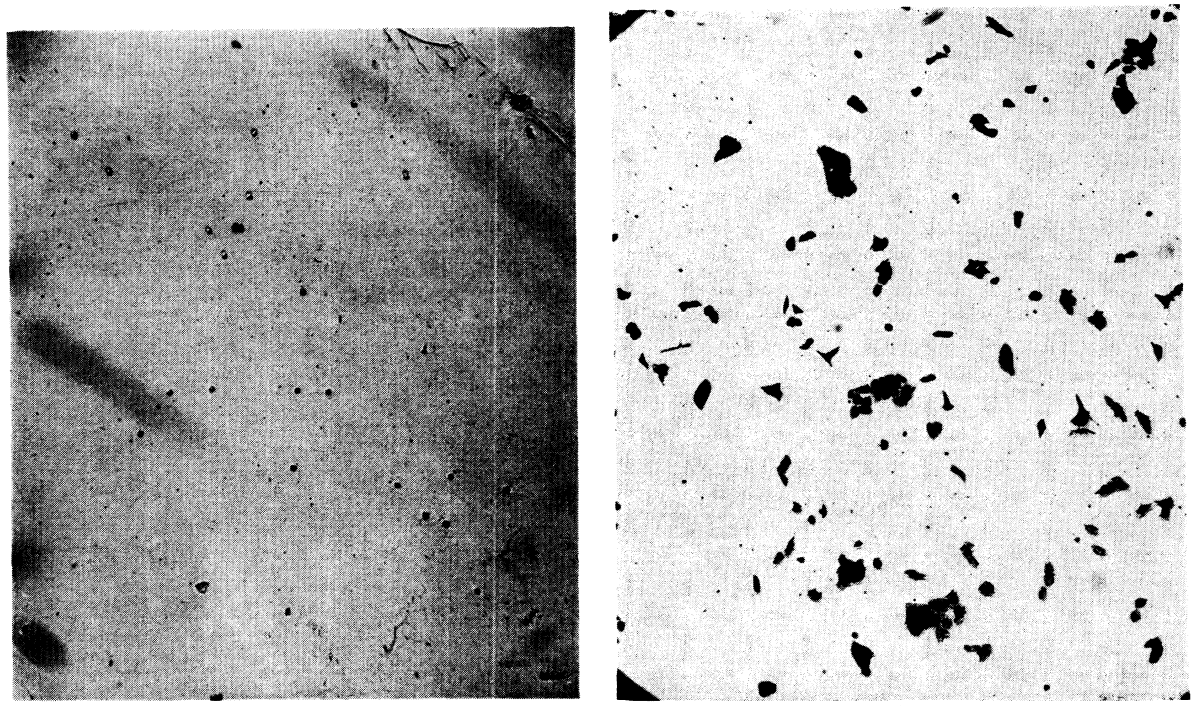


FIG. 3. (a) Electrophoretic pattern of fetuin purified by repeated, fractional $(NH_4)_2SO_4$ precipitation from fetal calf serum. (b) Colonies developing on plate seeded with 100 S3 cells, in a medium containing known small molecular components, and purified fetuin and human serum albumin.

In further development of tools for quantitative study of the growth and genetics of the mammalian cell *in vitro*, it became necessary to attempt to control the lability of karyotype which had been demonstrated to affect cells cultivated by standard tissue-culture techniques. Recent studies have shown that virtually every mammalian cell which has been established as a stable tissue-culture strain has a chromosomal constitution radically different from that of the parental species from which it originated.[9,10] Euploid cells were generally found either to stop growth after a very short period in culture, or else to proliferate, but with drastic changes in chromosome number and structure. In order to achieve conditions eliminating this proclivity for aneuploid chromosomal constitution in cell cultures (which renders genetic studies of questionable significance), methodologies were required for simple and reliable monitoring of chromosomal constitution of cells grown *in vitro*. Such methods were developed by Axelrad and McCulloch,[11] by Rothfels and Siminovitch,[12] and by Tjio and the author in our laboratories.[13] When the karyotypes of such cultures could be checked with ease and accuracy, it was possible to find growth conditions which permit karyotype integrity to be maintained. These conditions were secured by development of a screening test for media and regulation of other conditions so as to remove conditions leading to mitotic inhibition, which might induce polyploidy. By careful control of the processes involved in cell dispersion, by regulation of the *p*H and temperature during cell incubation, and by use of pretested media which incorporate all the needed nutritional requirements for cell growth and exclude toxic substances, it became possible to grow cells from normal tissues of man and other mammals, for periods now approaching a year, and for numbers of progeny which have exceeded 10^{25} without change in chromosomal constitution.[14] Figure 5 shows the chromosome complement of typical human male and female cells. These methodologies have permitted characterization of each of the human chromosomes, and particularly of the *X* and *Y* sex-determining chromosomes.[15]

In order to employ human genetic markers with biochemical, immunologic, and pathologic characteristics, a method was devised by which cells from any individual could be simply and reliably introduced into stable growth *in vitro*. A small piece of skin, approximately 10 to 50 mg in mass, is excised from the ventral part of the forearm, dispersed by trypsinization, and grown in the medium which contains fetal calf serum, which was shown to maintain stable karyotype. This method has proved itself reliable in securing actively growing cultures from any individual.[14] It has made possible confirmation of the fact that the human karyotype does, indeed, consist of 46 chromosomes, as first reported by Tjio and Levan.[16] Studies are now under way on cells of individuals with phenyl ketonuria and other genetic diseases, in an attempt to establish



(a) (b)

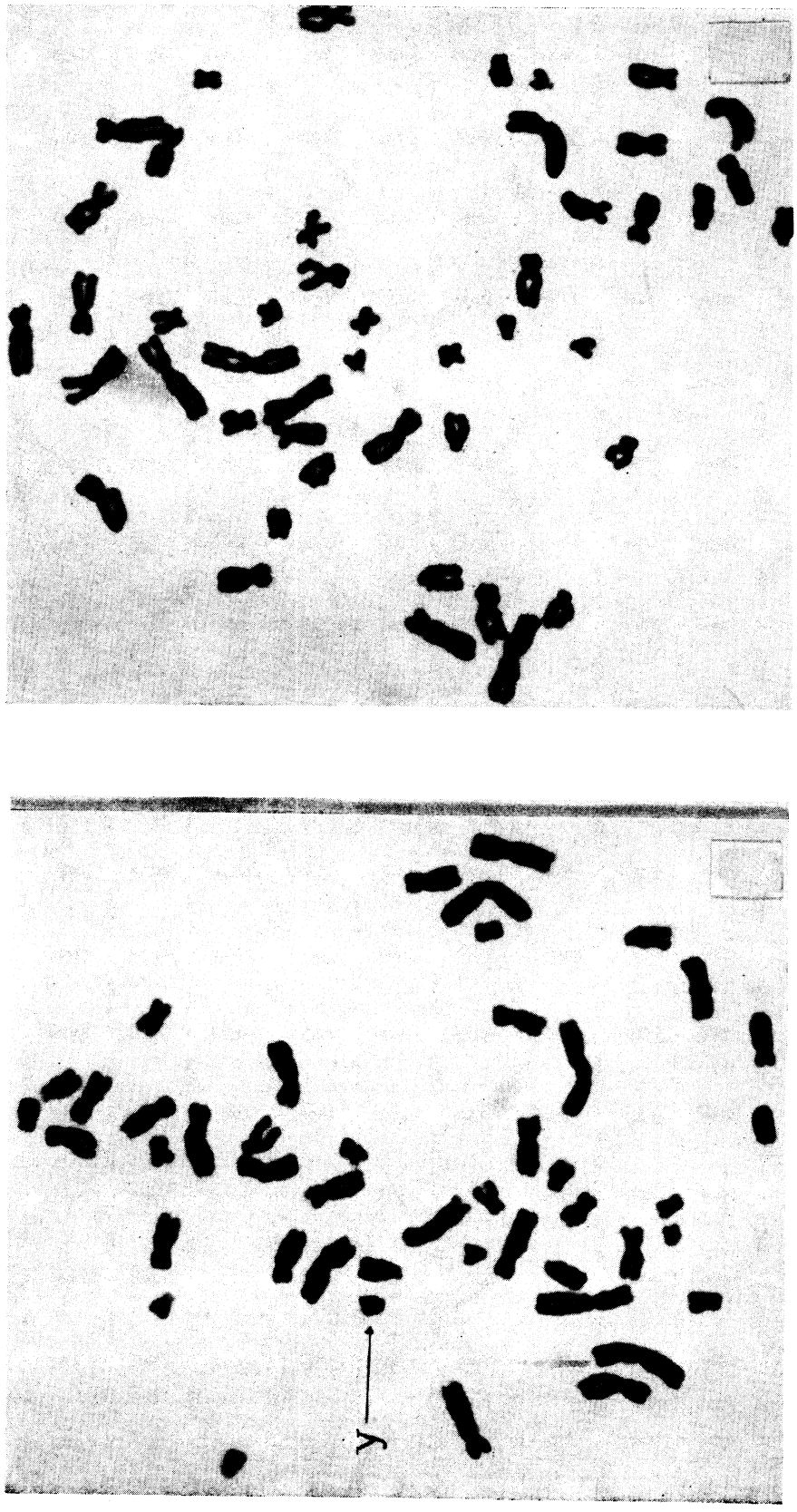
FIG. 4. (a) Photomicrograph demonstrating the rounded condition of cells, which will shortly be released from their bond to the glass in the absence of any "stretching factor." (b) Photomicrograph demonstrating the stretched condition of the cells in an adequate concentration of purified fetuin.

cytogenetic and biochemical differences between these and normal cells in the *in vitro* cultures. These methodologies are also being employed clinically to determine the chromosomal constitution of persons with various degrees of clinical hermaphroditism and to compare the results so obtained with those utilizing the sex chromatin method devised by Barr and his associates.[14] Thus, an anatomically female patient suffering from ovarian dysgenesis (Turner's syndrome) was shown to possess only 45 chromosomes.

In contrast with the normal human chromosome constitution shown in Fig. 5, Fig. 6 presents that of the aneuploid S3 HeLa cell, a clonal stock which we developed from the culture which Gey and his associates established from a human carcinoma of the cervix.[17] The chromosome number of this clonal strain is 78, which represents a hypotetraploid condition. Studies are in progress comparing the different morphology and behavior of cells which differ in chromosomal number and constitution. Thus, mutant clones of animals like the Chinese hamster have been isolated, which have stemline chromosome numbers of 22 and 23, respectively.

As one example of the kinds of quantitative studies made possible by these techniques, the remainder of this discussion is devoted to analysis of their application to study of the action of high-energy radiation on mammalian cells. Ionizing radiations are capable of disrupting any chemical bond in any molecule of any cell which has absorbed such energy. On the basis of such qualitative considerations alone, one might expect an enormous variety of cellular pathologic actions. Radiobiologic studies on mammalian systems carried out by many laboratories over many years have more than borne out this expectation, demonstrating a bewildering variety of pathological consequences attending various kinds of exposure to radiation of mammals, including phenomena as diverse as gastrointestinal disturbance, drop in the level of blood white cells, epilation, and carcinogenesis. One of the most critical problems in this field involves determination of the degree to which chemical disorganization attending exposure to a given dose of ionizing radiation affects the genome or the phenome, respectively, of the irradiated cell. A quantitative answer to this question would greatly simplify attempts to understand the enormously complex series of events attending irradiation of mammalian cells and of whole animals. In addition, the answer to this question is essential in order to determine the degree to which exposure of mammalian populations to various doses of ionizing radiations will result in random mutations transmissible to succeeding generations.

Physiologic damage to cells in the form of reversible lag periods in cell multiplication, changes in cell permeability and inhibition of many specific enzymatic activities, have been demonstrated in many organisms.



(a) (b)

FIG. 5. (a) Chromosome constitution of normal human male cell.[13] (b) Chromosome constitution of normal human female cell.[13]

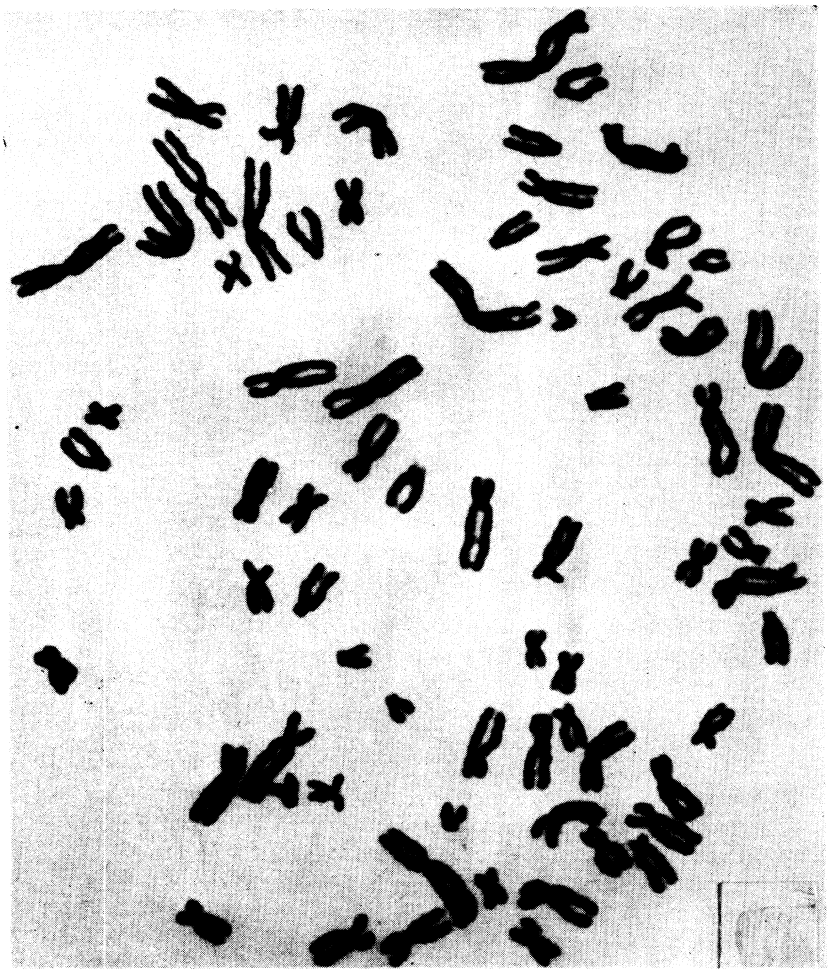


FIG. 6. Chromosome complement of a typical S39 HeLa cell, a subclone of the S3 strain, of human malignant origin, with a stemline number of 78.

In a variety of nonmammalian forms it had also been shown that the cell nucleus is much more sensitive to damage by irradiation than the cytoplasm. Genetic and cytologic examination of radiation effects, particularly in the simpler organisms, has revealed that changes of the genetic structure arising from irradiation include single gene mutations, chromosome breaks, production of areas deficient in chromatin, and occasionally elongated and amorphous chromosomes, these latter presumably arising from cells which had been irradiated during mitosis. At the site of fragmentation, a chromosome exhibits sticky surfaces which can re-attach so that the chromosomes may reconstitute themselves into a configuration more or less closely resembling the normal. In all probability, however, a defect, even though invisible, will persist at the site of breakage unless this happened to lie in a genetically inactive region. All or part of any chromosome which has suffered such a "hit" may, through imperfect restitution, fail to be incorporated in the mitotic apparatus and be lost in subsequent cell divisions. Such a condition can cause genetic imbalance that may destroy the ability of a cell exhibiting it to reproduce indefinitely. In addition, if an unrestituted chromosome divides, the two sister chromatids may unite at their broken ends to form an anaphase bridge that can prevent completion of mitosis. If multiple chromosome hits occur in the same cell, all of the foregoing damaging actions characteristic of single breaks are intensified. Moreover, abnormal restitution of the sticky, broken ends of different chromosomes may occur, producing a variety of complex translocations, such as dicentric chromosomes which can destroy the cell's reproductive capacity.[18]

Earlier studies of the relative sensitivities of the mammalian genome and phenome, respectively, to damage by high-energy radiations at first seemed to favor the thesis that for mammalian cells, in contrast to cells of other organisms, physiologic rather than genetic effects may be most important in the dose range up to and including the mean lethal dose for the whole organism, which is about 400 to 500 r. This supposition appeared to be supported by several kinds of evidence: studies in which massive cell inocula were irradiated in tissue culture with progressive doses of irradiation revealed that permanent loss of cell proliferation did

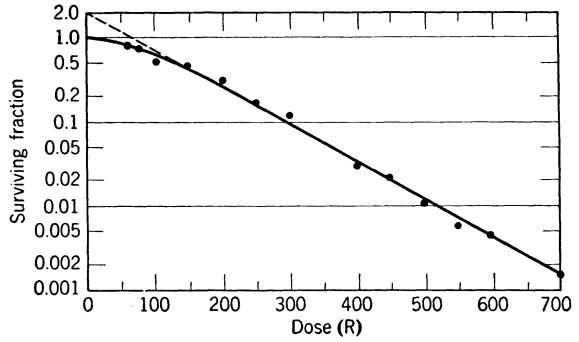


FIG. 7. X-ray survival curve of the ability of single S3 HeLa cells to form colonies as a function of the dose, D. The points can be fitted either by the equation $N/N_0=1-[1-e^{-D/96}]^2$ or by $N/N_0=e^{-D/77}(1+D/77)$, the latter relationship being preferable, since it is based on the model that reproductive death for this polyploid cell requires two or more effective hits anywhere in the chromosome complement. A more refined and comprehensive equation which expresses the contributions to genetic death of haploid, diploid, or polyploid cells of any species by single-hit and multiple-hit radiation damages is presented elsewhere.

not occur until many thousands of roentgens had been administered. Presumably because of the way in which tissue-culture thinking in the past was dominated by the concept that cell proliferation is a property of a cellular community, experiments like this were interpreted as an index of the dose needed to inactivate the cellular reproductive mechanism. Moreover, irradiation of single cells of bacteria, yeasts and protozoa, which might be considered as models for the mammalian cell, indicated that in all these forms, inactivation of reproduction required thousands of roentgens.[19] Hence, since death of a whole mammal can be effected by an exposure of only 400 to 500 roentgens, it appeared possible that mammalian-cell interaction with irradiation proceeds in a manner fundamentally different from that of other forms. While this thesis has been vigorously opposed, especially by geneticists, among whom H. J. Muller has been particularly active, it is still occasionally affirmed in the current scientific literature that very high doses in the neighborhood of many thousands of roentgens are needed to achieve irreversible damage to mammalian cells *in vitro*[20] and the ability of ionizing radiation to produce mutagenesis in mammals is still being challenged.[21]

With the development of the techniques which have been here described, it became evident that the use of cell proliferation from massive cultures cannot be used as an end point to quantitate irreversible effects of radiation on cell reproductive capacity unless the results are calculated as a survival function based on the number of cells irradiated. Since even a single cell remaining intact can proliferate eventually to produce any degree of outgrowth whatever, the dose at which a large but unspecified population fails to produce recognizable outgrowth is meaningless in terms of the dose needed on the average to inactivate the cellular reproductive apparatus. Moreover, since it is characteristic of irradiated cells to exhibit reversible mitotic lags, and to continue to multiply for one or even several generations, but then to produce a microcolony of sterile progeny, use of the mitotic index of the population as a measure of irreversible effects on cellular reproduction is not reliable. However, the use of the single-cell plating procedure makes possible precise determination of survival curves for mammalian cells by procedures exactly like those which had been used for cells of *E. coli*. In this way it is possible to measure accurately the dose required for inactivation of the reproductive mechanism of the individual cells constituting a population. The first such curve obtained is shown in Fig. 7, and approximates a two-hit relationship, with an initial shoulder, followed by a linear exponential drop, which is maintained for doses up to at least 1800 r. Of major importance is the fact that the mean lethal dose, D_0, which is obtained from the slope of the linear part of the curve, was only 96 r. The determination of this curve is completely objective and has been verified by this time in a number of laboratories.

Several features of the behavior of such irradiated cells pointed to damage to the genome as the primary process responsible for reproductive death.[19] The small value of the mean lethal dose for the S3 HeLa cell made it possible to rule out of consideration single-gene mutation as the dominant radiobiological process. The hypothesis was advanced that cell death is a consequence of chromosomal damage resulting from irradiation, and this proposal appeared to give good quantitative fit with the behavior of the system. This interpretation was supported by the following facts. After x-irradiation of single cells with approximately 6 mean lethal doses, the survivors were allowed to form colonies which were then picked and grown into new cell stocks. Examination showed at least 4 out of 5 such strains to be mutated from the original form, exhibiting morphological or nutritional differences, which persisted after long-term growth. Moreover, each of these four strains was revealed to possess a chromosomal constitution grossly altered from that of the original, unirradiated strain (Fig. 8). The 2-hit nature of the curve for the S3 cell was explained on the basis that reproductive death in such cells requires, in the main, at least two independent hits, and therefore appears to be a result of aberration produced by interaction between two separately damaged chromosomes. Other aneuploid cells of human origin, like S3, and with chromosome numbers far in excess of the normal 46, gave x-ray survival curves similar to that of S3.[22]

The fate of the cells whose ability to multiply indefinitely has been destroyed by irradiation is of interest. While some may multiply for a few generations, all appear to retain the ability to metabolize effectively and to carry out complex biosyntheses of macromolecular structures like that of a specific virus, added to the system after irradiation. Under proper environmental conditions, cells of either aneuploid or euploid

constitution may, after irradiation, produce giant forms. Thus, these cells which have been destroyed reproductively by irradiation, have maintained intact a large proportion of their other metabolic activities. At least small distortions of these functions probably also exist, but may require subtle means for their demonstration.

A pattern of events similar in some ways, but different in others, was obtained on irradiation of human cells with normal chromosome complements. All of these cells originated in normal human tissues and possessed the typical, elongated configuration commonly associated with euploid human cells.[14] Determination of chromosome constitution on several of these revealed them to have the normal diploid number. All of them behaved identically, and exhibited a mean lethal dose of about 50 r, corresponding to an even greater radiosensitivity than the HeLa cell. The hit number of these survival curves is less than that of the aneuploid HeLa cell, and lies somewhere between 1 and 2, a greater accuracy not yet being available for these cells because of their great radiation sensitivity. Hence, the conclusion may be drawn that euploid human cells are even more radiosensitive than the malignant, aneuploid HeLa.

As a further test of the hypothesis that cell reproductive death, as a result of x-irradiation, arises directly from chromosomal damage, a series of experiments was carried out in which euploid human cells were irradiated, after which the chromosomes were delineated and examined cytologically for direct evidence of aberrations. A preliminary report by Bender,[24] counting

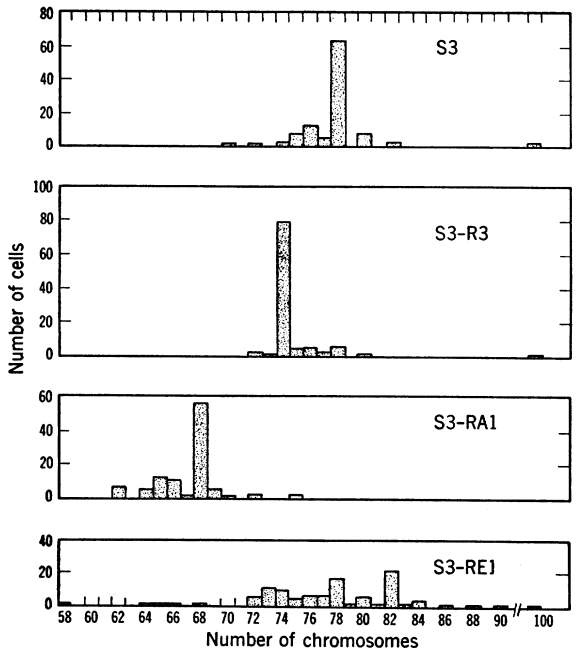


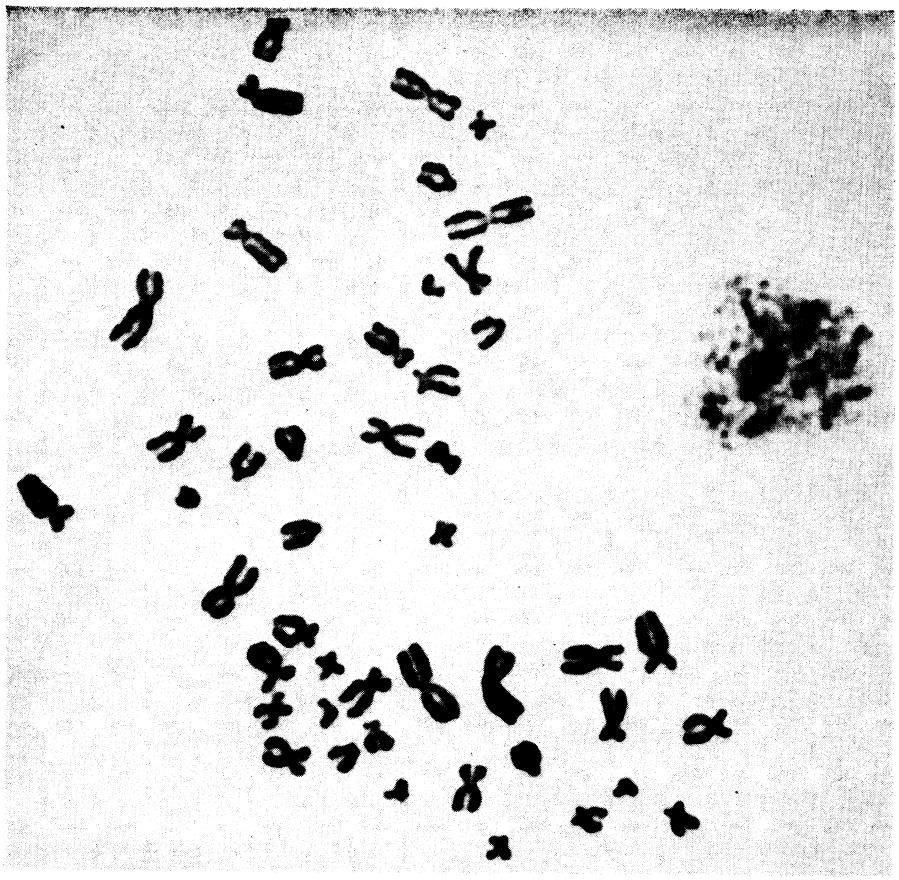
FIG. 8. Chromosome number distribution of the clonal strain S3 (top), and of 3 typical subclones isolated from among the survivors of radiation with 500–700 r (S3R3, S3RA1, and S3RE1). These chromosome analyses were carried out by Chu and Giles[23] in a collaborative study with our laboratory.

TABLE I. Chromosome aberrations at various radiation doses. Single-hit aberrations are chromosomal defects caused by a single ionizing event, and include a complete break in one chromatid only; a break in both chromatids at the same point presumably reflecting a break in the chromosome before it has doubled or a traverse of both chromatids by the same ionizing particle; an achromatic region in which the continuity of the chromosome is uninterrupted, but chromatin has disappeared from a particular area; or the presence of one or more greatly elongated chromosomes trailing "sticky" streamers. Multihit complexes comprise chromosomal aberrations involving interaction between two or more independent hits to 1 or more chromosomes, such as translocated dicentrics and ring chromosomes.

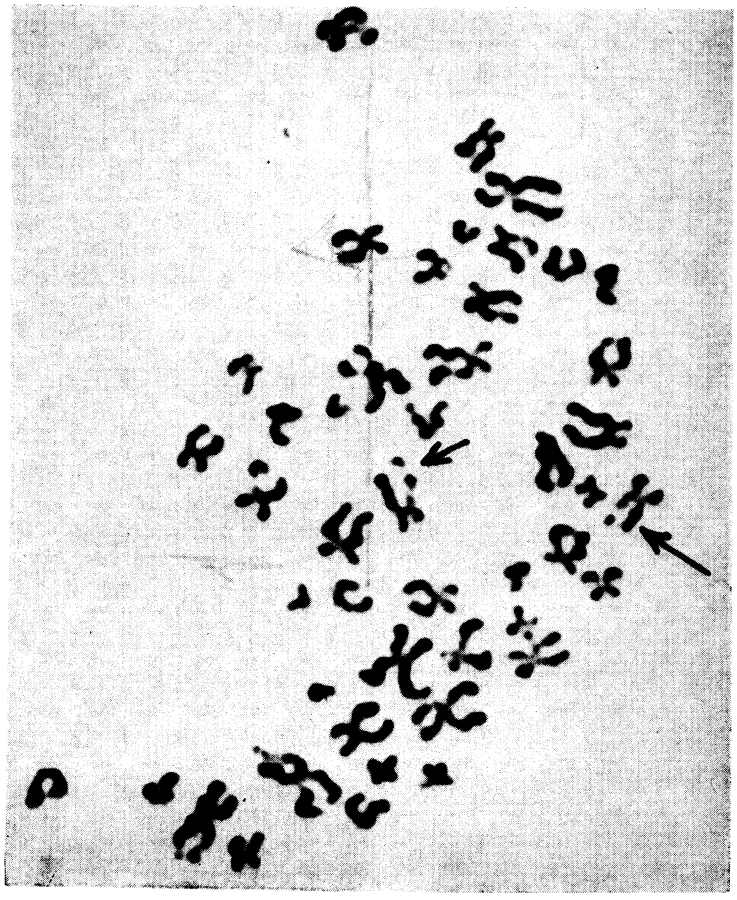
		Single-hit aberrations				
Dose (roentgens)	No. of mitoses scored	Single chromatid break	Double chromatid break	Achromatic regions	Presence of one or more "sticky" chromosomes	Multihit aberrations
0	116	22	1	1	1	1
10	3	0	0	0	0	0
20–25	33	6	1	0	0	0
40–50	20	37	4	0	2	1
75	101	113	23	7	2	14
150	26	26	5	8	4	10
Totals	299	204	34	16	9	26

chromosome breaks induced in euploid human cells grown *in vitro*, suggested that approximately 300 r were required to produce an average of one visible chromosome break per cell. This figure is almost six times higher than the mean lethal dose for these cells. In an attempt to obtain an estimate of the roentgen efficiency of chromosome breaks for euploid human cells, valid under our conditions of study, experiments were carried out in our laboratory in which the conditions of growth of the cells were brought as nearly as possible to optimal values in order to minimize some of the uncertainties owing to mitotic lags. In addition, however, an independent method of arriving at the chromosome-breaking efficiency was employed, based on the fact that the appearance of aberrations due to abnormal chromosome restitutions, can occur only in appreciable numbers at doses beyond the mean lethal dose. Such abnormal restitutions will not disappear, as can single chromosome breaks which can reseal without leaving any visible trace.[25]

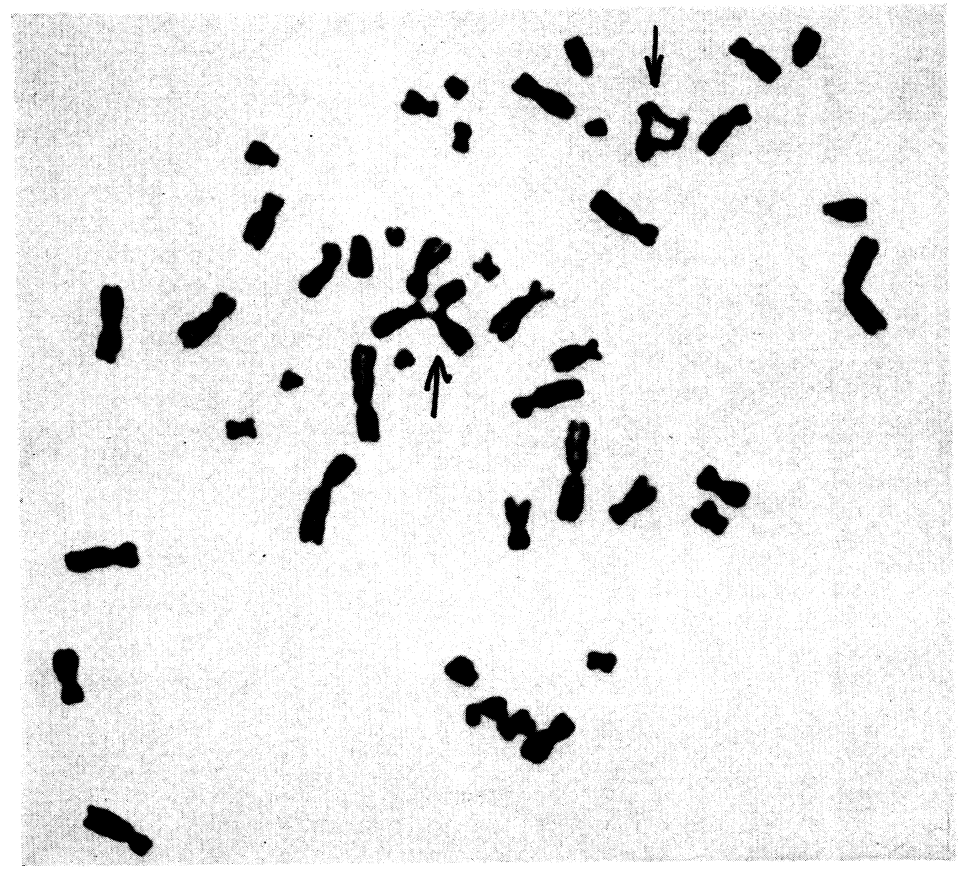
Figure 9 presents typical sets of mitotic figures obtained when cells with normal chromosome constitution taken from various tissues of normal human subjects are irradiated *in vitro* at different dose levels. It is obvious that, with cells irradiated with 50 r, single chromosome breaks are readily evident [Fig. 9(b)]. When the dose is increased to 75 r, two effects appear: the number of single breaks is increased; in addition, however, new types of aberrations appear which indicate that, at this dose, multiple chromosome hits in individual cells have become a frequent occurrence. Examples of such complex anomalies formed through interaction of several radiation-damaged chromosomal sites are presented in Figs. 9(c) and 9(d). A summary of this data is presented in Table I. From these figures,



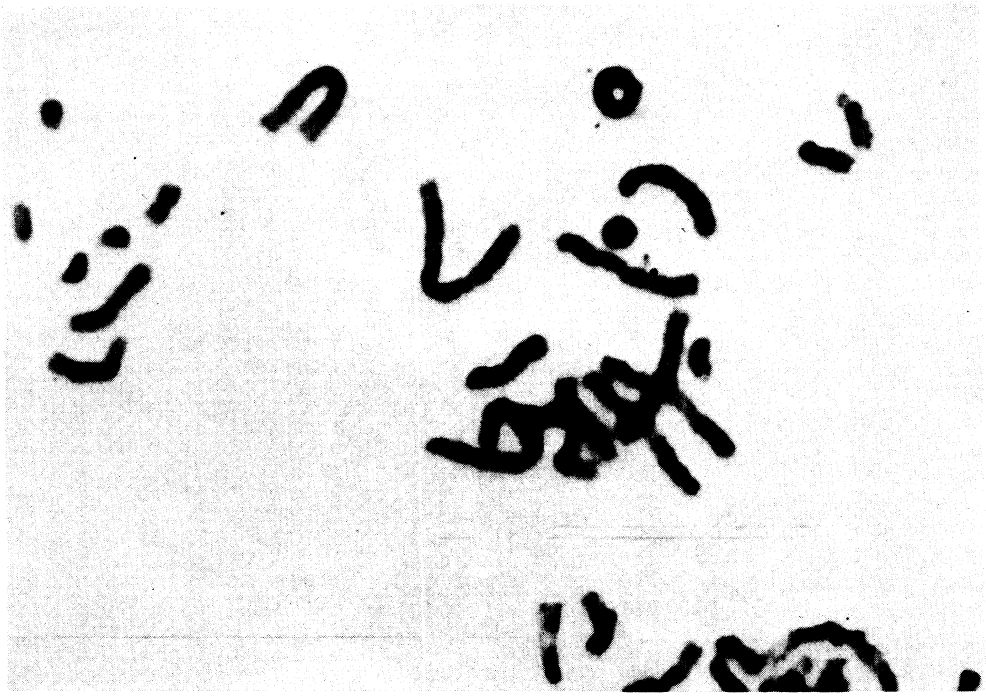
(a)



(b)



(c)



(d)

FIG. 9. Typical kinds of chromosome abnormalities observed when euploid human cells are irradiated *in vitro* with 230-kv x-rays. (a) Unirradiated cell. (b) Cell irradiated with 50 r. Breaks and deletions appear as indicated by arrows. (c) Chromosomes of cell irradiated with 75r, showing a translocation in the center, and a dicentric. (d) Portion of a mitotic figure of a cell after 150-r irradiation, demonstrating formation of ring chromosomes.

one can safely conclude that the dose needed to introduce an average of one visible chromosome break per cell is no more than 40 to 60 r, a value which agrees within the limits of experimental uncertainty with that obtained as the mean lethal dose for colony formation of these cells.

The conclusion may be drawn that the genome of the euploid human cell is extraordinarily susceptible to chromosome damage by ionizing radiation, and that the average dose needed to introduce one chromosome break per cell is similar to the mean lethal dose to the cell's reproductive function, i.e., approximately 50 r. Since this value is far less than that required for the inactivation of various enzymatic activities in mammalian cells,[26] it appears to establish the fact that the cellular genome of man is far more sensitive to radiation damage which is, at least in part, presumably irreversible, than are other of its structures. While studies are currently in progress to determine whether cells *in vivo* will behave in the same way, experiments with radioprotective agents added to the medium suggest that only relatively small changes in the cellular D_0 value can be accomplished by changes in the cellular environment.

These considerations afford an understanding of the action of ionizing radiation on mammalian cells in terms of a primary effect—the initiation of a chromosome break, which requires on the average an exposure of a normal human cell to 50 r or less, and a secondary effect—the production of complex chromosomal aberrations through abnormal restitution of the fragments resulting from multiple breaks occurring within the same cell. The first of these involves a variety of alternative possibilities: The cell may continue reproduction with no recognizable cytogenetic change, though with perhaps a gene mutation at the site of the original chromosome break; it may suffer loss of all or part of a chromosome which could impair its ability to reproduce; or it may undergo formation of a chromosome bridge at anaphase which will certainly end its reproductive ability. When multiple chromosome fragmentations leading to complex translocations occur, the cell is much more likely to be incapacitated with respect to reproduction. Different cells should display 1-hit, multiple-hit, or intermediate types of survival curves, depending on the degree to which these different processes operate in causing destruction of the ability to multiply.

This analysis appears to offer an explanation for the difference in radiobiologic response exhibited by various mammalian cells. For example, normal diploid cells of different species, differing in their total mass of chromosome material, should be expected to exhibit differences in radiation sensitivity roughly though not exactly paralleling their chromosomal volumes, since the cell with greater chromosomal mass will offer greater opportunity for a given radiation dose to produce ionization directly or indirectly effective within this region. In accordance with this expectation, we have found that cells of the chick, whose DNA content is less than one-quarter that of human cells, possess a mean lethal dose for x-rays approximately 4 to 8 times greater than that of euploid cells of man. In Table II is presented a comparison of the D_0 values and DNA content of diploid cells from a variety of different species. Despite relatively large uncertainties in the individual determinations of the mean lethal dose and of the DNA content, and the large range of values encompassed, it is evident that these very different diploid cells exhibit a remarkably constant value for the product of these 2 variables. Other aspects of these relationships are considered elsewhere.

These considerations afford insight into the effects of various chromosome conditions like polyploidy on the radiosensitivity of mammalian cells from the same species. With increasing numbers of chromosome sets within a cell, its sensitivity to killing by a 1-hit process should decrease. Thus, a haploid cell will lose its ability for indefinite multiplication as a result of damage to any single gene essential to replication. Diploid cells which have a double gene set presumably will require loss or aberration of larger portions of a chromosome before the genetic imbalance necessary for inhibition of multiplication results. Since such chromosome losses readily occur as a result of 1-hit processes following exposure to ionizing radiations, such cells will often exhibit curves with a hit number close to unity, but may in some cases be multiple hit, if the gene distribution among the chromosomes is such that death rarely follows a single-hit process. The D_0 value of such curves will be influenced by the volume of each chromosome, the magnitude of the breaks which are produced, and the degree to which 1-hit lethal and 2-hit lethal processes occur when cells are exposed to various types of irradiation under various conditions. Cells with higher degrees of ploidy will be much less susceptible to reproductive killing by loss of part or all of any chromosome because of the smaller imbalance produced when larger multiples of the chromosome set are present. In such cells, the major lethal process will involve interaction between

TABLE II. Comparison of radiation and the DNA content for diploid cells of three different living forms. The data indicate that, despite individual variations in D_0 values and DNA contents of over a hundred-fold, the product of these two variables remains reasonably constant, varying only by a factor of about two.

Diploid cell type	D_0 value[a]	DNA content (in picograms per cell)	Product: $D_0 \times$DNA content (roentgens $\times$picograms)
Yeast	about 8000 r[b]	0.05[b]	400
Chick	about 300–400 r[c]	2.5[d]	900
Man	50–60 r[e]	8.3[d]	500

[a] The D_0 value is taken as the dose needed to reduce the reproducing fraction of the cell population to 37%, in the region where the survival curve is linearly exponential.
[b] See reference 27.
[c] See reference 25.
[d] See reference 28.
[e] See reference 22.

two or more chromosomes to produce malformations like anaphase bridges. Hence, such cells will usually display a hit number in the neighborhood of 2. While the larger volume of the chromosome set in such cells might at first be considered to make them more vulnerable than diploid cells to the accumulation of hits that can lead to cell death, this factor may be more than counterbalanced by the relatively low probability with which two simultaneously broken chromosomes will restitute abnormally, so as to form a nondividing configuration (like a dicentric) as opposed to a union still permitting normal mitosis. Thus, the HeLa S3 cell, an aneuploid human malignant cell with 78 chromosomes, exhibits a survival curve which is approximately 2-hit, as opposed to the more nearly 1-hit curve of the euploid cell, and exhibits a D_0 value of 96 r instead of 50 r characteristic of the normal diploid cell. These parameters—the degree of cell ploidy; the distribution among the different chromosomes of regions whose loss may lead to cell death through imbalance; and the separate probability of formation of chromosomal aberrations which mechanically prevent unlimited division by 1-hit and 2-hit processes distributed among the various chromosomes—would appear to afford explanation of the difference in radiosensitivities of different cells. Quantitative evaluation of these parameters in selected normal and malignant cell systems is now under study.

These considerations also can offer explanation for our recent findings that the S3 cell which is enormously more sensitive than bacterial cells to killing by x-rays, is much more resistant to ultraviolet irradiation. Ultraviolet, while a highly effective lethal and mutagenic agent for bacteria, is known to be much less efficient than x-rays in producing chromosome breaks. While haploid bacterial cells can be killed effectively by point mutations and so are readily inactivated by ultraviolet light, the need for chromosome breakage to occur before the multiploid animal cell is inactivated would render it resistant to the action of ultraviolet irradiation.

Application of these aspects of the action of ionizing radiation at the cellular level, to analysis of the underlying mechanism involved in the pathologies arising from acute, whole body irradiation of animals leaves little doubt that many of the most important features of the mammalian radiation syndrome are due to the cumulative actions on the individual body cells of the same kind as those described in the preceding paragraphs:

(a) Knowledge of the D_0 value for individual cells for the first time provides clear explanation why the mean lethal dose for total body irradiation of mammals should lie in the range of 400 to 600 r. Exposure of the whole body to a dose of this magnitude would inactivate the reproductive mechanism of more than 99% of the cells with radiosensitivities like those of the cells studied *in vitro*. The body might recover from smaller doses which would still leave sufficient intact cells which could reproduce at a rate rapid enough to compensate for the losses. The mean lethal dose to the whole animal represents a point of loss of reproducing cells so extensive as to provide widespread damage to the integrities of physiological function which depends on cell reproduction and which constitutes a stress which cannot ordinarily be withstood.

(b) This formulation is in accord with the failure to find any specific biochemical lesion resulting from animal irradiation in the mean lethal range. Significant depression of individual enzyme functions have been found to require much higher doses than that needed to kill the whole animal. While no specific enzyme function could be implicated, many studies showed that DNA synthesis was greatly reduced as a result of irradiation in the dose range here considered.[29] The theory here considered would predict such behavior. DNA synthesis can proceed normally only when cell reproduction progresses. Destruction of this function through production of chromosome aberrations must eventually depress DNA synthesis, even though no enzyme system of the cells or body fluids has been significantly altered by the irradiation.

(c) Similarly, the failure to find any accumulation of toxic material in the body fluids after exposure to radiation in the mean lethal range is to be expected. While at higher doses significant amounts of toxic products might be produced as a result of chemical structural alterations resulting from ionizations produced in the tissue, doses of several hundred roentgens only will alter significantly structures with a size of the order of magnitude of the chromosomes, and will produce biological changes only if the uninterrupted integrity of such structures is vital to normal body operations as is indeed the case with the genetic apparatus of the individual cells.

(d) It follows that one would expect those tissues to be most radiosensitive which must maintain the highest rate of cell mitosis, since these will suffer the most immediate loss of their specific functions, on which the body depends. This correlation between radiosensitivity and mitotic rate has been recognized as one of the earliest generalizations to emerge from radiobiologic experience. However, while the rapidly dividing tissues are indeed most radiosensitive from the point of view of their immediate contribution to embarrassment of the whole organism through failure to maintain their specific functions, it is necessary to regard virtually all of the body cells as equally sensitive to chromosomal damage. Each normal cell nucleus has an equal probability of accumulating similar chromosomal injuries. Such aberrations will remain largely latent in each cell until it comes to reproduce. Hence, the cells of the more rapidly dividing tissues are simply the first to make evident the injuries which must be regarded as equally numerous in the more slowly multiplying

regions of the body. This fact is of vital importance in understanding genetic transmission of radiation damage, and the ability for tumors to develop many years after a radiation experience.[30]

(e) Similarly, our picture accounts in straightforward fashion for the characteristic lag period between radiation exposure and the development of symptoms, which has long been recognized as typifying mammalian injury by ionizing radiation. The cell which has suffered damage which is primarily only chromosomal, will continue to function effectively for a period, since its full complement of enzymes and other structures presumably remains largely unaltered. Not until the cell reaches the point where it should normally divide will it begin to exhibit grossly deviant behavior from the normal. Moreover, we have shown that such cells may even multiply for several generations, before they and their progeny cease reproduction.[19]

(f) The sequence of events which ultimately leads to death of any acutely irradiated animal may thus be explained as follows. Chromosomal aberrations introduced among all of the cells exposed will first reveal themselves in those cells of the most actively mitotic tissues. Division will be prevented in most of such cells irradiated with several hundred roentgens, with the result that the functions supplied by these tissues will fail and the body will be threatened. As other more-slowly dividing tissues reach the point where they too should produce cell proliferation, they will add the results of their failure to those which have already introduced distortion of normal body physiology. In contrast to the actively dividing structures like the bone marrow, and the epithelial linings of the gastrointestinal tract, tissues like nerve and muscle in which cell division rarely occurs, can continue to exhibit normal function even after exposure to many thousands of roentgens. It is of interest that cell reproductive death has also been identified as the major factor in the mammalian radiation syndrome by Quastler and his co-workers, using experimental approaches and techniques completely different from those employed here.[31]

In this connection, an alternative hypothesis has been proposed that the more-rapidly dividing tissues are more radiosensitive only because cells in mitosis are in a more sensitive condition and hence are damaged more readily by irradiation. This proposal does not fit the facts, since even in the most rapidly dividing tissues, no more than 3% of the cells are in mitosis at any one time. Hence, if this were the true explanation, it could account at most for a negligible reduction in cell viability of such tissues. However, in early embryonic development, where high mitotic frequencies often are achieved and where phased cell reproduction may occur to bring many cells into mitosis simultaneously, this factor might easily play an important role.

(g) These considerations also explain quite naturally how it is possible to save animals irradiated with doses in the mean lethal range, by injection of viable bone-marrow cells, though not by cellular fragments or extracts. Such cells recolonize the irradiated tissue which is being depleted through impaired cell-reproductive function. While other tissues have presumably suffered equal diminution in the percent of cells capable of reproduction, these require replenishment less rapidly and hence provide more time for the surviving cells to recolonize the tissue, provided the bone-marrow functions can be maintained. It may be expected that animals might be saved from lethal effects of even higher doses, by additional inoculation of viable cells from other tissues which, as the dose increases progressively, would become critical in their failure to maintain functional integrity of the body.

(h) Similarly, the interesting experiments first carried on by Patt and his co-workers,[32] in which animals were subjected to low temperatures immediately after irradiation, become explicable. Such animals failed to develop any of the symptoms of radiation injury until after their temperature was restored, after which the entire sequence of pathogenesis was initiated as though the irradiation had just occurred at the time their body temperatures had been raised back to normal. At the low temperature, mitosis is inhibited so that the body does not produce the conditions which can bring into expression the latent damage to the cellular genetic apparatus. On rewarming, normal cell reproduction is again initiated, so that each time a genetically damaged cell comes to mitosis its injury becomes functional and contributes to embarrassment of a normal function.

(i) One might expect, then, to find some rough correlation between the D_0 obtained for euploid cells of different animals as measured by the survival curves here described, and the mean lethal dose for the whole animal. While the latter figure must, of necessity, be influenced by many complex interactions of an unpredictable kind, it is of distinct interest to find that such a correlation is at least suggested by the small amount of currently available data, as shown in Table III.

Studies of the action of radioprotective agents on x-ray survival curves of S3 cells are completely consistent with the interpretations here developed. The compound, 2-mercaptoethyl guanidine, which has been demonstrated to raise the MLD for mice from 900 to a maximum of 1450 r,[35,36] also exercises significant radio-

TABLE III. Data demonstrating that possibly some parallelism may exist between the mean lethal dose for whole warm-blooded organisms (LD_{50}) and the D_0 values of their single, euploid cells as determined *in vitro*.

	X-ray LD_{50} for whole animal	X-ray D_0 of euploid cell *in vitro*
Man	400– 500 r	50– 60 r
Chinese hamster	825–1190 r[a]	160 r
Fowl	1000 r[b]	300–400 r

[a] See reference 33.
[b] See reference 34.

protection on S3 HeLa cells plated *in vitro*. The shape of the survival curve remains approximately 2-hit as it was in the absence of the compound, but D_0, the mean lethal dose for cell reproduction, is raised from 96 r to a maximum of 160 r, a value which agrees far better than perhaps could be expected with the degree of protection achieved in the whole animal. All of the data on the kinetics of the radioprotective action of this compound on single plated cells are consistent with the interpretation that the presence of this material lowers the effective dose of x-rays which reaches the sites within the cell whose inactivation results in loss of the ability to reproduce indefinitely.

(j) Finally, these data support the cellular-genetic interpretation of the great effectiveness of relatively low doses of radiation in suppressing antibody formation in the mammalian body. The dose range needed to inhibit antibody production significantly lies in the region of 50 to 150 r,[37] a value which, through its close correspondence with the mean lethal dose for euploid cell reproduction, suggests this action to reflect the effect of radiation in preventing multiplication of antibody-producing cells. By contrast, specific macromolecular biosynthetic mechanisms appear essentially undamaged even after irradiation of mammalian cells with thousands of roentgens.[15] Thus, the great radiosensitivity of antibody production suggests that the mechanism of new antibody production involves the need for cell multiplication, rather than simply antibody synthesis by pre-existing cells.

The fact that some mammalian cells exhibit survival curves for the reproductive function which are multiple-hit in character must not be taken as an indication that a threshold exists for the induction of mutations by high-energy radiations. On the contrary, all of the evidence here presented leads to the conclusion that, while a cell displaying a 2-hit survival curve does display a threshold for killing by high-energy radiation, the production of single chromosome breaks, and the attendant alteration of genetic material at the site of such a break, goes on even when doses insufficient to kill are applied. From the point of view of the entire organism and its progeny, it may be far better for any cell to be permanently inactivated by multiple chromosomal aberrations than to suffer only a single chromosomal change, which then may be handed on to the offspring, usually as a recessive genetic defect.

The data here presented make possible an estimation as to whether background radiation may contribute to the processes of aging in man. Since 50 r is the average dose needed to produce 1 visible chromosome break per cell by the techniques here employed, one may well expect that damage on a smaller scale which cannot be seen by ordinary microscopy results from even smaller doses. Thus, one can calculate that, with a background exposure of 0.1 r per year, in 70 years the accumulated dose is sufficient to have produced gross chromosome damage in one-seventh of all of the body cells, and probably more subtle effects in a much larger number. This would appear not to be a negligible process, and thus makes it probable that the gradual attrition of body functions which constitute aging, may be, to a very significant degree, the reflection of accumulation of cellular genetic injuries in cells and their descendants originating from background irradiation. By extension of the methodologies here discussed, it is proposed to attempt analysis of physiologic and genetic differences in the behavior of cells taken from aging animals, and to examine as well the effects of the molecular constituents of body fluids from such subjects on physiologic and genetic behavior of standard cell strains *in vitro*.

Discussion of the use of quantitative methods in measuring mammalian cell growth and genetics would be incomplete without at least mention of the outstanding development by Dulbecco and his associates[38] in achieving a plaque technique for precise enumeration of single particles of mammalian viruses exactly as has been current in bacteriophage studies. Thus, it becomes possible also to quantitate virus and cell interaction in animal systems, so that one may expect equally profound insights to arise from such studies as has been the case in the interaction of bacteriophages with their bacterial hosts.

The thesis which has been the purpose of this discussion is that newer developments promise to provide well-defined systems for quantitative exploration of physical and physicochemical mechanisms involved in growth, genetics, and differentiation in mammalian-cell systems. There is every indication that in the space of a few years it will be possible to come to grips with specific molecular aspects of these basic mechanisms in as intimate a fashion as is now current in the biophysical approach to microbial systems.

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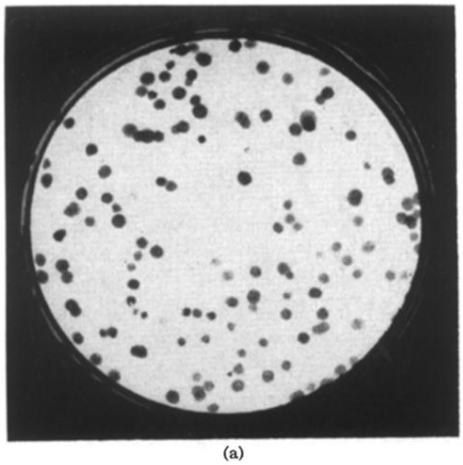

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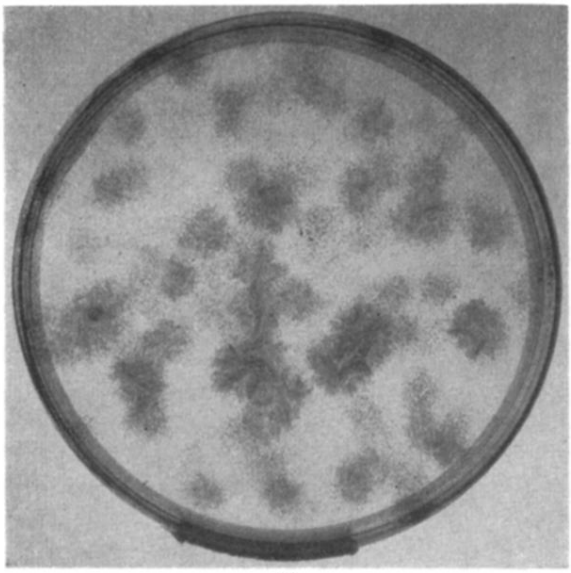
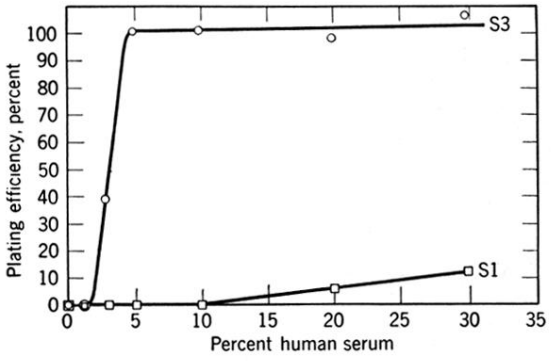
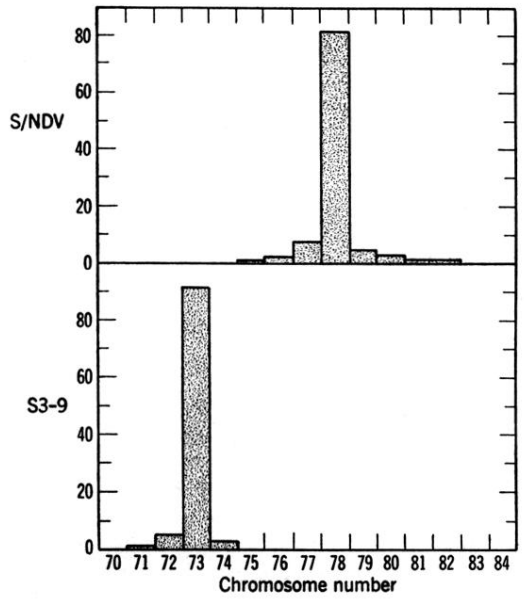


(b)

FIG. 1. (a) Petri dish which was seeded with 100 single cells of the S3 strain of the HeLa culture, an aneuploid which originated in a human cancer. (b) Petri dish seeded with single cells of a euploid human strain. These cells tend more to spread and migrate, and so the colonies tend to run together unless a smaller number of cells is plated or a larger plate is employed.

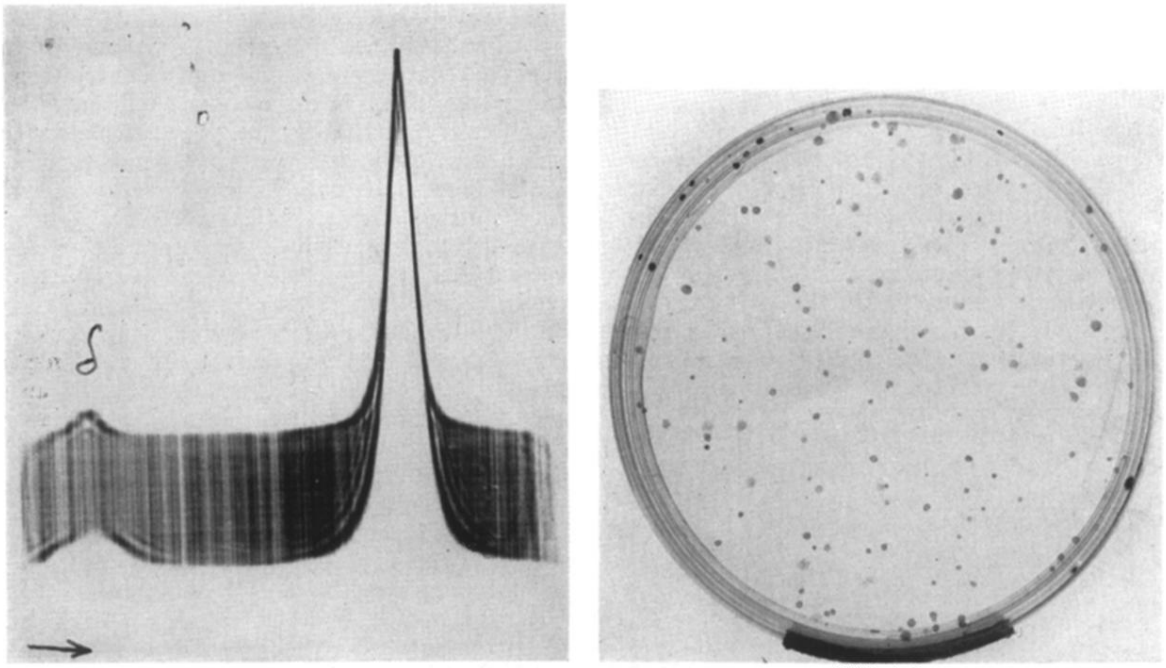


(a)



(b)

FIG. 2. Demonstration of some representative mutant strains isolated from the HeLa population. (a) Plating efficiency curves in the presence of different amounts of human serum of 2 mutants found to occur spontaneously in the original HeLa population. The cellular and colonial morphologies of these two strains are identical but their growth requirements are different. (b) Distribution of chromosome number in 2 HeLa clones. The clone at the top (*S/NDV*) has the same chromosome number as S3, but is characterized by its resistance to destruction by Newcastle disease virus.



(a)

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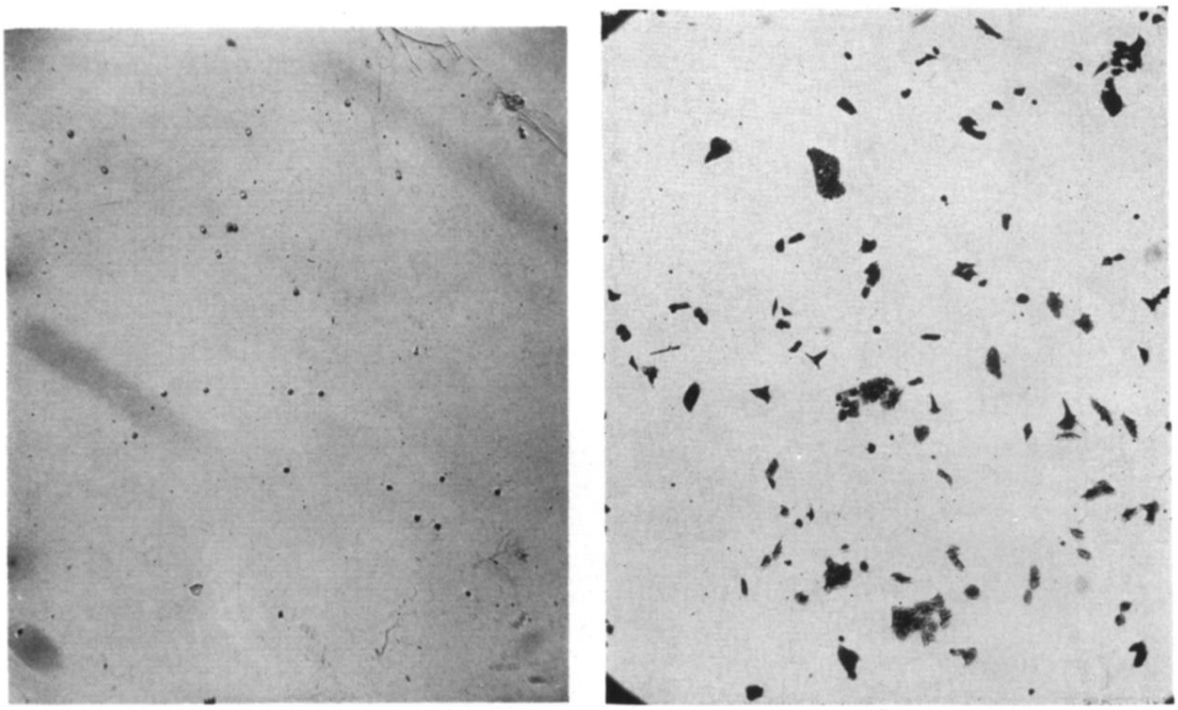
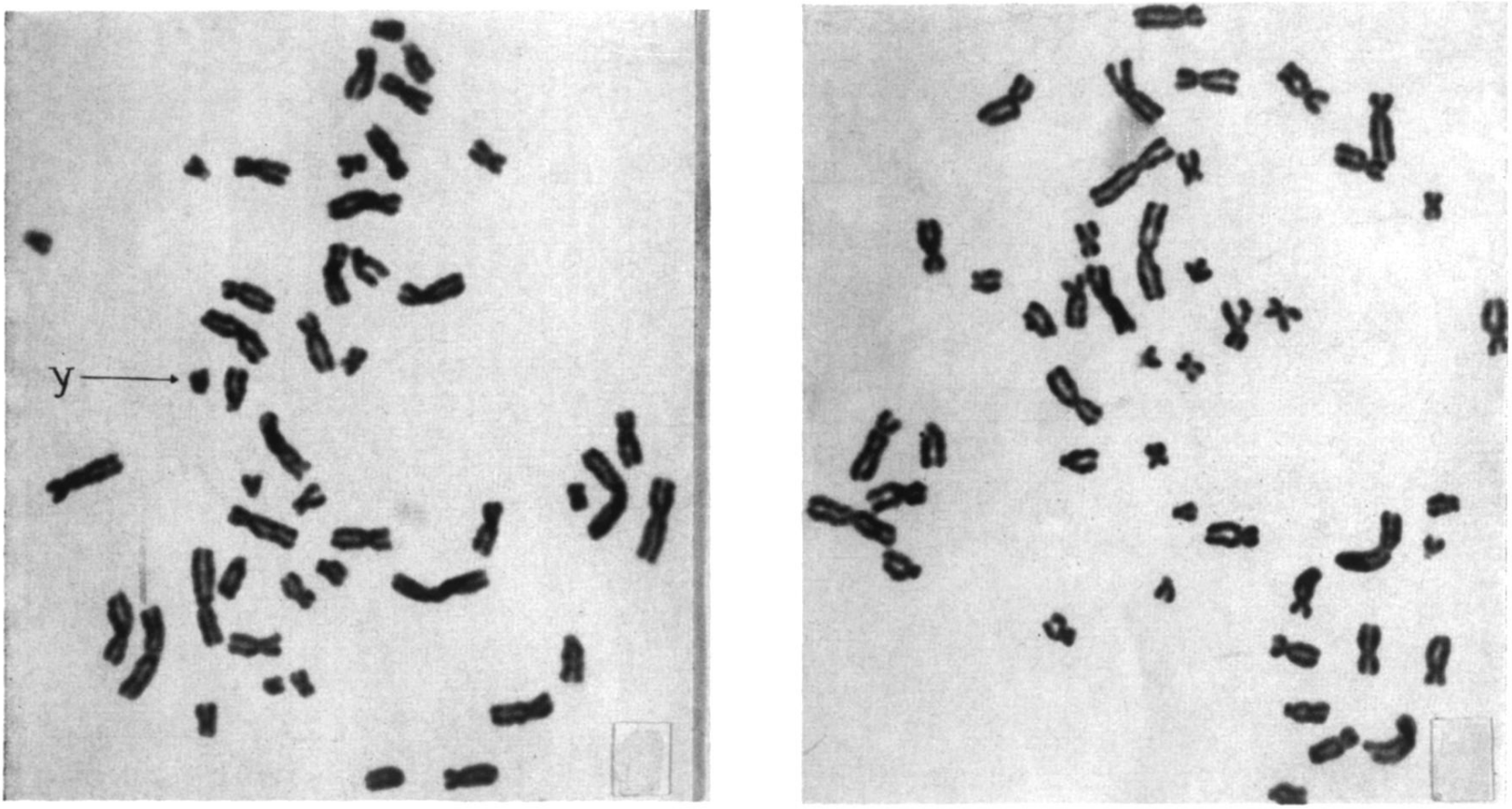
(b)

FIG. 3. (a) Electrophoretic pattern of fetuin purified by repeated, fractional $(NH_4)_2SO_4$ precipitation from fetal calf serum. (b) Colonies developing on plate seeded with 100 S3 cells, in a medium containing known small molecular components, and purified fetuin and human serum albumin.



(a) (b)

FIG. 4. (a) Photomicrograph demonstrating the rounded condition of cells, which will shortly be released from their bond to the glass in the absence of any "stretching factor." (b) Photomicrograph demonstrating the stretched condition of the cells in an adequate concentration of purified fetuin.



(a) (b)

FIG. 5. (a) Chromosome constitution of normal human male cell.[13] (b) Chromosome constitution of normal human female cell.[13]

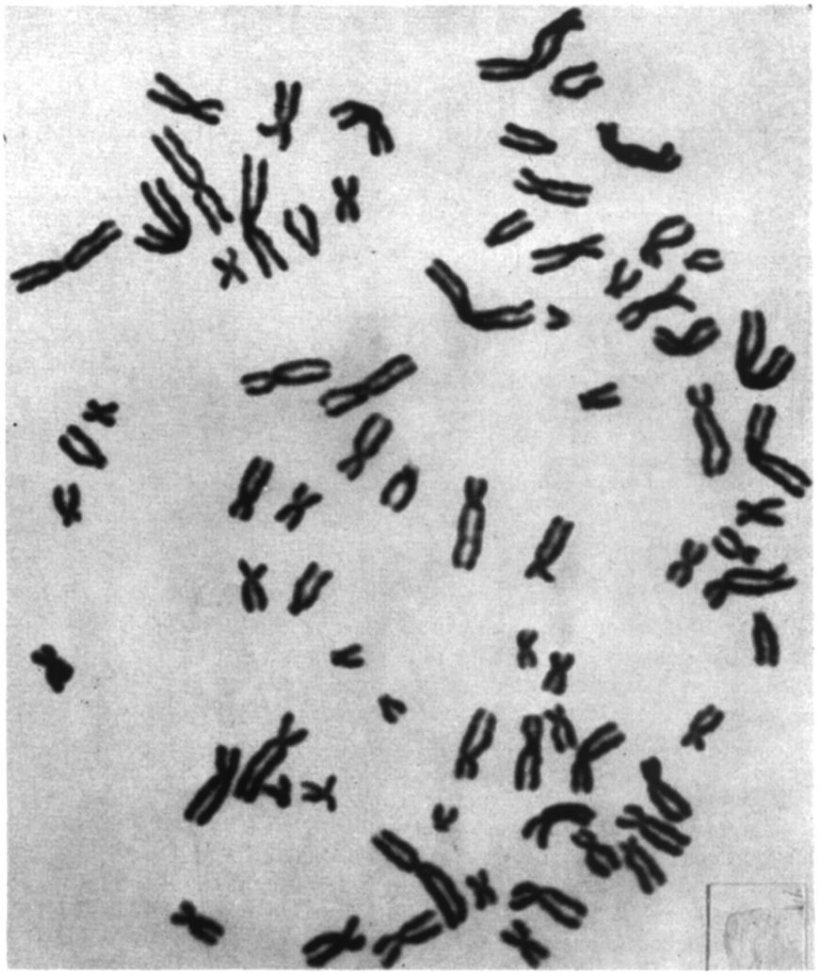


FIG. 6. Chromosome complement of a typical S39 HeLa cell, a subclone of the S3 strain, of human malignant origin, with a stemline number of 78.

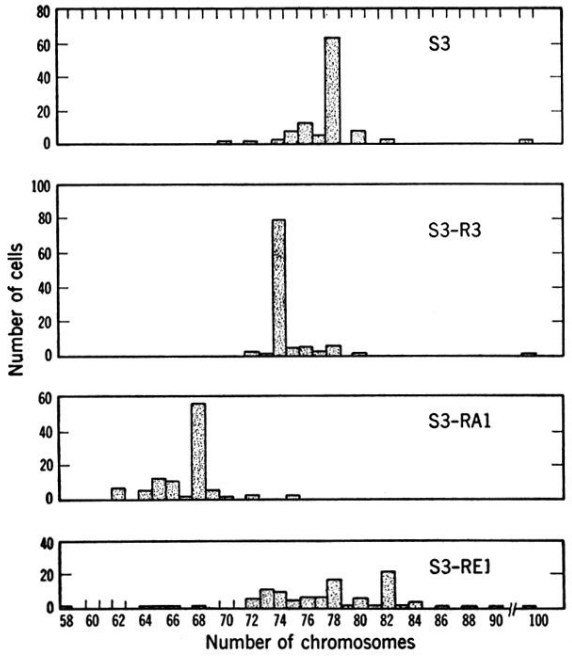
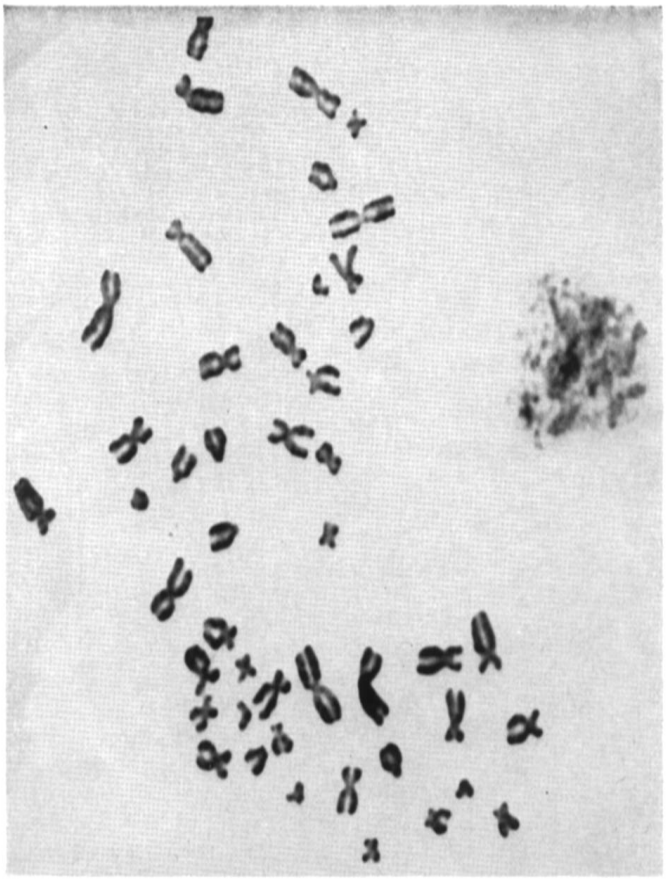
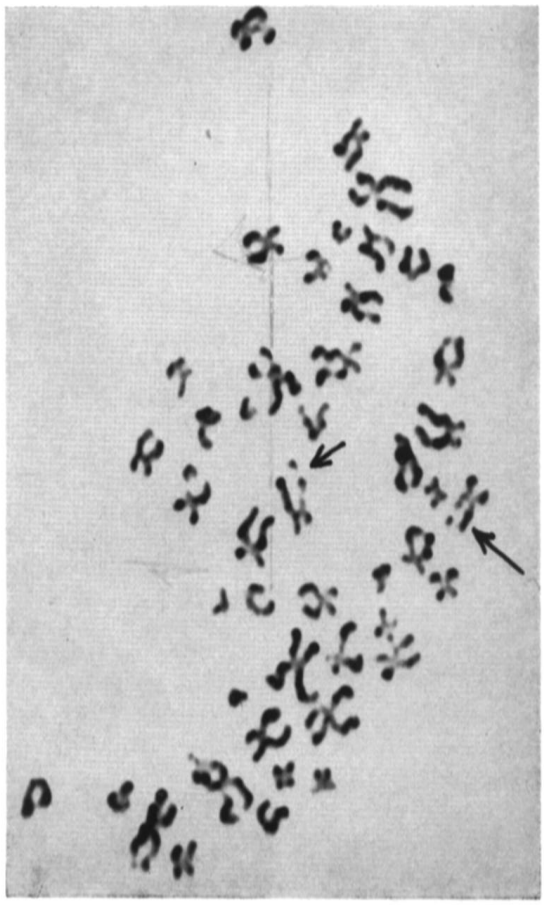


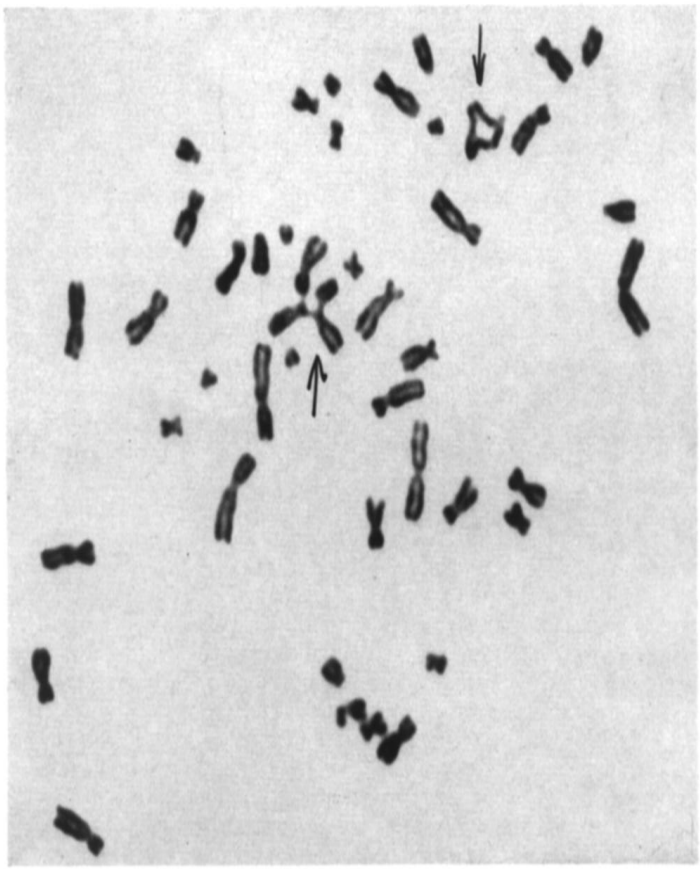
FIG. 8. Chromosome number distribution of the clonal strain S3 (top), and of 3 typical subclones isolated from among the survivors of radiation with 500–700 r (S3R3, S3RA1, and S3RE1). These chromosome analyses were carried out by Chu and Giles[23] in a collaborative study with our laboratory.



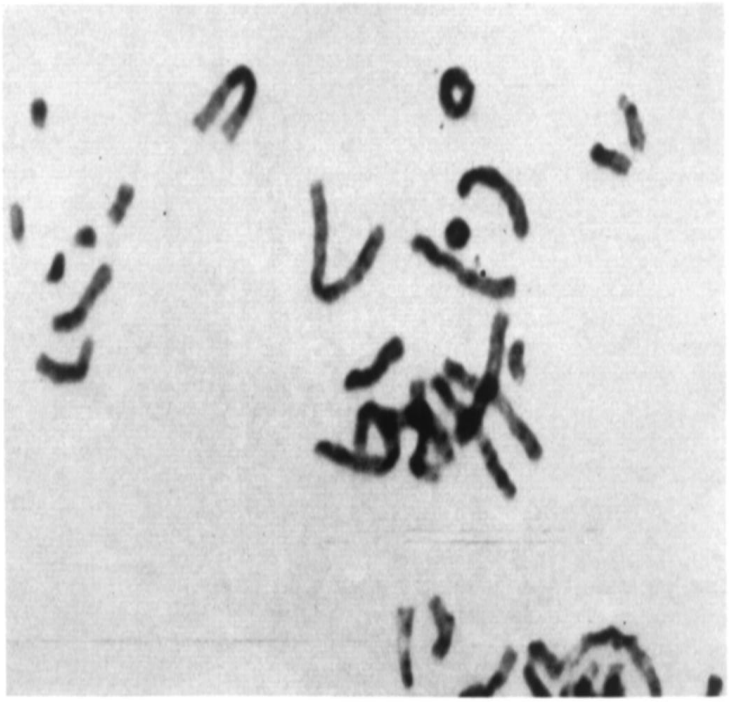
(a)



(b)



(c)



(d)

FIG. 9. Typical kinds of chromosome abnormalities observed when euploid human cells are irradiated *in vitro* with 230-kv x-rays. (a) Unirradiated cell. (b) Cell irradiated with 50 r. Breaks and deletions appear as indicated by arrows. (c) Chromosomes of cell irradiated with 75r, showing a translocation in the center, and a dicentric. (d) Portion of a mitotic figure of a cell after 150-r irradiation, demonstrating formation of ring chromosomes.