*ACTION OF RADIATION ON MAMMALIAN CELLS III. RELATIONSHIP BETWEEN REPRODUCTIVE DEATH AND INDUCTION OF CHROMOSOME ANOMALIES BY X-IRRADIATION OF EUPLOID HUMAN CELLS IN VITRO**

By Theodore T. Puck

UNIVERSITY OF COLORADO MEDICAL CENTER, DENVER

Communicated by H. J. Muller, June 18, 1958

In earlier papers, quantitative determination of survival curves for the reproductive function of single mammalian cells exposed to X-irradiation was described.[1, 2, 3] A variety of human and animal cells displaying both normal and aneuploid karyotypes was studied and found to possess surprisingly low values for the mean lethal dose for colony formation, (D_0), all the different values obtained lying within the range of 50–160 r for mammalian cells and approximately 300–400 r for those of the chick. Of the human strains, aneuploid, epithelial-like cells were generally somewhat more radio-resistant than euploid, fibroblast-like cells, but the duration of the period of in vitro culture and the organ of origin of the cell lines examined had no discernible effect on the shape of the survival curve. Most of the animal cell lines unequivocally exhibited a hit number of approximately 2; some cells yielded curves whose extrapolation to zero dose was sufficiently uncertain as to fit a hit number anywhere between 1 and 3; and at least one animal cell studied by us appears to approximate a 1-hit curve.[4]

From analysis of these curves and other data, it was concluded that the primary event leading to loss of cellular ability to multiply indefinitely is most commonly chromosomal damage.[3] By analogy with chromosomal radiation damage in other living systems, [5, 6] it was considered that the chromosomes of mammalian somatic cells, too, could exhibit either a generalized stickiness if the irradiation occurred during mitosis[7] or chromosome breaks which could be followed either by approximately normal restitution or by the various rearrangements which have been documented in *Drosophila*, *Tradescantia*, and other forms. Reproductive death of irradiated mammalian cells whose curves are truly 1-hit might in some cases result from the genic and chromosomal imbalance attending the loss of chromosomal material as a result of an unrestituted break and in other cases, perhaps more frequently, from the formation of a mitotic bridge, disorganizing in its effect on subsequent divisions, by the dicentric isochromatid fragment resulting from a break. In the cells exhibiting multiple-hit survival curves, reproductive death would occur through the intensification of both these factors. This interpretation, while demanding a relatively high yield of chromosome mutations per roentgen, explained the general shape of the survival curves; accounted for the low magnitude of the observed lethal doses for the mammalian cells studied; correctly predicted that most of the survivors of irradiation with 5–7 lethal hits would form new clones with mutant characteristics; and permitted various aspects of the radiation biology of the mammalian somatic cell to be understood in terms of the principles already established for other living forms.[5] Further support of this interpretation was obtained from examination of the chromosomal constitution of several clonal strains of cells surviving irradiation equivalent to about 6 lethal hits. All the four cell

lines so far studied have exhibited chromosome constitutions markedly different from that of the original, unirradiated clonal stock.[4, 8]

If the D_0 value as here described is indeed a measure of the sensitivity to radiation damage of mammalian chromosomes, chromosomal aberrations in mammalian cells should be visualized in considerable numbers after X-irradiation with doses in the vicinity of the D_0 value for the particular cell employed. However, it is not possible to predict the expected frequency of such anomalies in stained mitotic preparations resulting from irradiation of large cell populations. This follows because of several complications which tend to upset the proportion of mitoses among cells damaged by radiation, as compared with those that have escaped with little or no deleterious effect: cells damaged slightly by radiation exhibit a lag in reproduction; hence their proportion may be reduced in subsequent harvests of mitoses;[1] mammalian cells which have suffered reproductive death as a consequence of irradiation may reproduce for only one or a few cell generations and, hence, may not be adequately represented in the sample of mitoses which is collected at a given period;[2] and, finally, following a time distribution not yet defined for mammalian cells but undoubtedly influenced strongly by the culture conditions, chromosome breaks tend to become restituted, so that evidence of damage remains only for those hits which have taken part in readily recognizable, gross rearrangements or which have formed chromatid or isochromatid fragments.

These three factors operate to decrease the number of visible breaks over those that may actually contribute to cell reproductive death. In *Tradescantia* it has been estimated that because of these factors only one-twentieth of the breaks produced are visualized.[9] Hence the most that can be expected from counting of simple chromosomal abnormalities induced by various radiation doses is a minimum value for the yield of chromosomal aberrations per roentgen, and this number would vary with the conditions of cell growth and the incubation time allowed between irradiation and fixation of the cells and with the physiologic state of the culture employed. Moreover, since the operation of each of these distorting factors is itself dose-dependent,[1] this technique may be expected to produce considerable scatter when the number of abnormalities per roentgen per mitosis is measured at different radiation doses.

The first such estimate for euploid human cells was recently published by Bender,[10] who demonstrated 11 chromatid deletions, 41 isochromatid deletions, and 4 chromatid exchanges in a survey of almost 1,000 mitoses gleaned from epithelial-like cells originating in a human kidney and irradiated in vitro with 0, 25, or 50 r. The D_0 value for these cells was not specified. An average yield of 0.003 chromosome breaks per cell per roentgen (or about 300 roentgens per hit per mitosis) was calculated for these cells.

Similar experiments to evaluate an upper limit for the dose needed to produce chromosomal damage were undertaken in this laboratory with cells of a type whose D_0 had been determined. The following consideration was used as a basis for improvement of this estimate. For irradiation less than the mean chromosomal-damaging dose, single hits should be mainly in evidence. At exposures beyond this value, however, complex chromosomal rearrangements indicative of interactions between multiple chromosomal hits in the same cell should appear in appreciable numbers. Hence, by noting the lowest dose at which dicentric and

similar translocations begin to appear in noticeable numbers, a minimum estimate for the mean chromosome-damaging dose can be secured which is independent of that obtained by scoring the total number of hits of all kinds.

In the experiments here to be reported, euploid, human, fibroblast-like cell strains were employed. The D_0 for colony formation by such cells had been shown to lie between 50 and 60 r;[2] so attention was devoted to irradiation with doses on either side of this figure, in order to determine whether the distribution of single- and multihit chromosomal abnormalities would follow the course to be expected on the basis of the foregoing considerations. Moreover, in order to minimize the complications due to radiation-induced lag periods, an attempt was made to provide maximally favorable medium and growth conditions to permit cells damaged by the irradiation experience to divide with a rate as nearly as possible approaching that of their undamaged and presumably more self-sufficient neighbors. Methods were devised for growth of fibroblast-like human cells in culture under conditions which produce a maximum rate of cell division (generation time of approximately 18 hours) and which permit unlimited cell growth in vitro without development of the chromosomal anomalies which heretofore were considered inevitable for normal mammalian cells in tissue culture.[11] Cell cultures originating from normal skin of four different individuals were used in the present experiments. Some of these cells were previously grown in vitro for more than 4 months and 50 generations, while others were used within 2–3 weeks of the initial biopsy. All these cell lines possessed normal karyotypes of exactly 46 chromosomes, and all exhibited similar patterns of chromosome aberrations on irradiation. Examination of the chromosomal constitution of these cells with and without irradiation was carried out by means of the method recently developed in which the cells are fixed on the glass in the same positions in which they have grown.[12] The incidence of polyploidy in these cells was less than 3 per cent.

Replicate inocula of about 2×10^4 cells were plated as described,[12] in pretested medium fortified with fetal calf serum.[11] The plates were incubated for a period of 48 hours or more and then irradiated with doses of X-rays varying from 0 to 150 r. Test and control plates received identical treatment except for the specific radiation exposures. The latter were administered by a constant-voltage Westinghouse machine operated at 230 KVP and 15 m.a. and filtered by means of 0.5 mm. of copper, 1 mm. of aluminum, and 2 mm. of glass. The dose rate was usually 143 r/minute, although a few points were taken at 50 r/minute, without any apparent change in result. After irradiation, incubation at 37° C. was resumed for periods varying from a minimum of a few minutes to a maximum of 7 days, although most of the cells were fixed within 72 hours. The factors which affect the yield of various chromosome aberrations respond differently to differences in this post-irradiation incubation period; hence it seemed advisable to use a variety of incubation periods in this initial survey.

In some cases the radiation survivors were grown out into new stocks, from which clones were picked by the methods previously described.[11]

In scoring chromosomal abnormalities on irradiated plates, only those were counted which were unequivocal. In the unirradiated plates, however, even questionable anomalies were often counted, to insure that, if any subjective element were involved, it could only distort in the direction of minimizing the number of chromosome aberrations attributable to the radiation.

Experimental Results.—A summary of all the experimental data collected is presented in Table 1. At doses well below 50–60 r, the D_0 value for cell multiplica-

TABLE 1*

Dose (Roentgens)	No. of Mitoses Scored	Single-Hit Aberrations: Single Chromatid Breaks	Double Chromatid Breaks	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
Total	299	204	34	16	9	26

* 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; 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 comprised chromosomal aberrations involving interaction, between two or more independent hits to one or more chromosomes, such as dicentrics and ring chromosomes.

tion, virtually the only observable aberrations are 1-hit in type, i.e., simple chromosome breaks involving either one or both chromatids and achromatic regions. As the dose is increased, the number of such anomalies increases. A representative picture of the chromosomal constitution of a normal cell is shown in Figure 1 and is contrasted in Figure 2 with that of a cell irradiated with 50 r. Figure 3 demonstrates a cell from a slide irradiated with 75 r, containing many such anomalies. Photographs of the translocations, dicentrics, and ring chromosomes, indicative of interaction between multiple sites of damage in the chromosomal complement which arise after irradiation with doses in the neighborhood of 75–150 r are illustrated in Figures 4 and 5.

TABLE 2*

Calculation of Frequency of Chromosome Hits per Mitosis per r at Various Doses for Which Significant Numbers of Mitoses Were Scored

Dose (r)	Chromosome Hits/Mitosis/r
40–50	0.045
75	0.020
150	0.015
Average	0.027

* Each of the single-hit aberrations of Table 1 was scored as one, and each of the multihit complexes was scored as two. The number of hits per mitosis at each dose was diminished by 0.23 to correct for the aberrations encountered in the unirradiated group.

In Table 2 the calculated values of the total number of hits per mitosis are presented for each of the dose ranges at which a significant number of mitoses was studied. The values obtained are as constant as could be expected in view of the inherent uncertainties discussed, and they yield a maximum value of 40 r as the mean dose needed to produce one chromosome hit per mitosis.

Inspection of the last column of Table 1 reveals that the complex, multihit aberrations are vanishingly small at doses less than 40–50 r but appear in approximately 14 per cent of the mitoses of the cells irradiated with 75 r and to an extent of 39 per cent of the cells which have received 150 r. Clearly, these figures indicate the mean chromosomal damaging dose per euploid cell to be less than 75 r, and the upper limit demanded by these experiments may safely be set at 40–60 r.

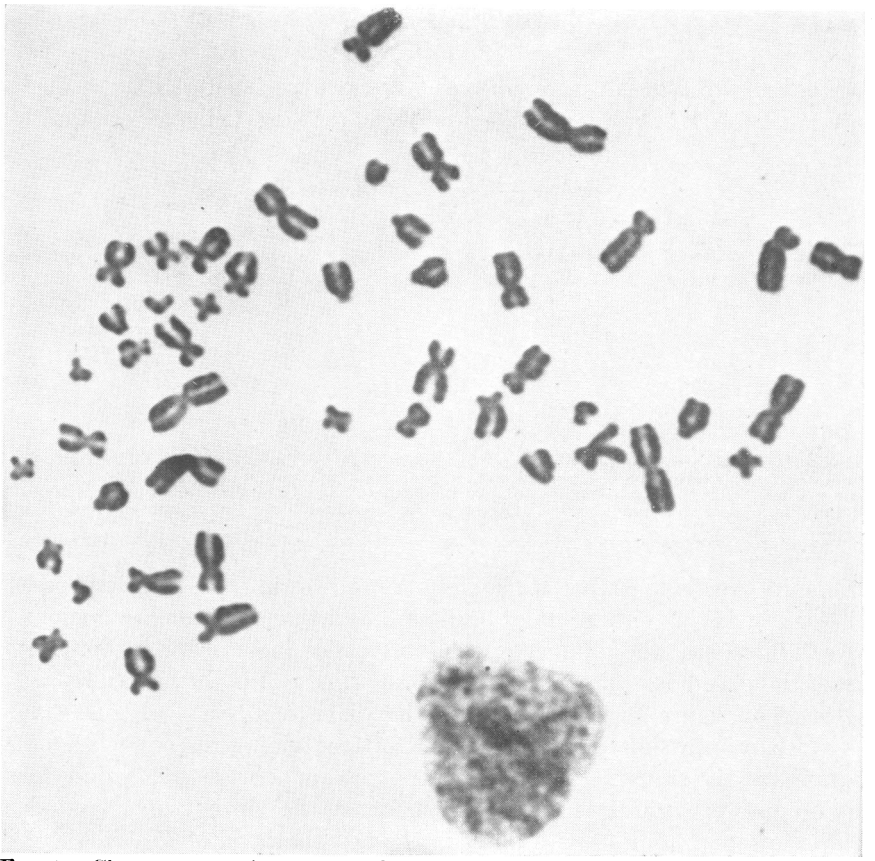

FIG. 1.—Chromosomes of unirradiated, fibroblast-like cells from normal, human male skin. × 2500.

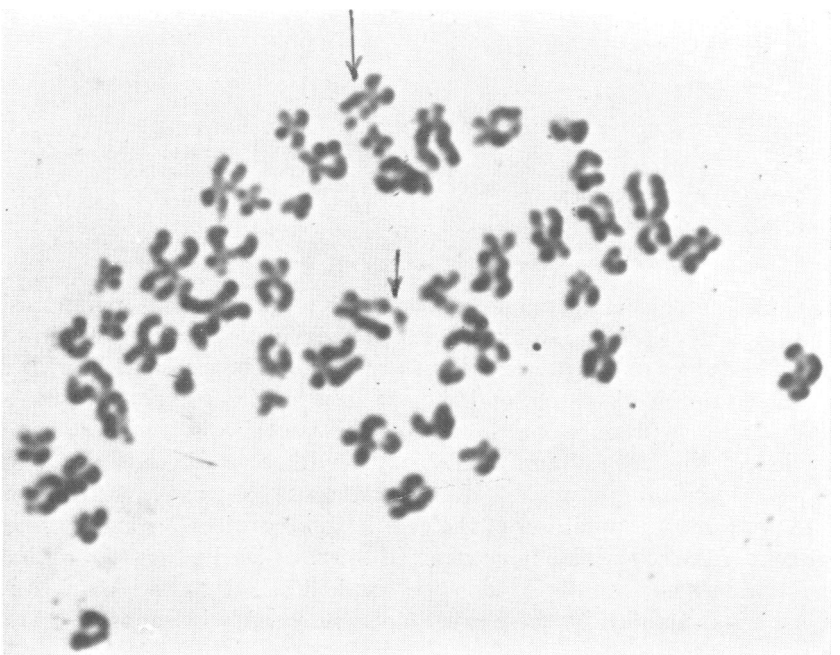

FIG. 2.—Chromosomes of cell from same culture as in Fig. 1, after irradiation with 50 r. Arrows indicate two single breaks. × 2500.

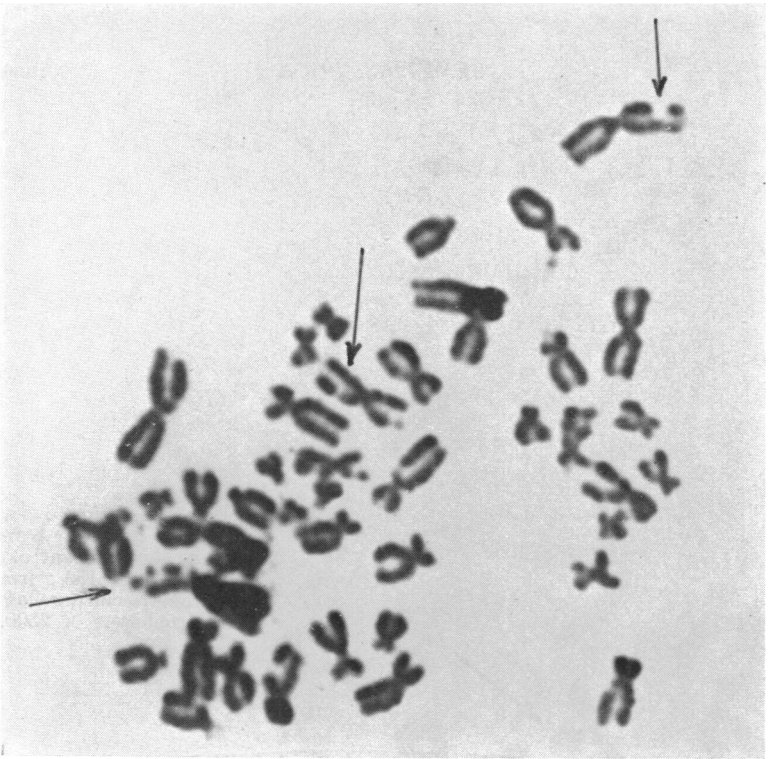

FIG. 3.—Chromosomes of a normal human fibroblast-like cell irradiated with 75 r. Many broken chromosomes are present, a few of which are indicated by the arrows. × 2500.

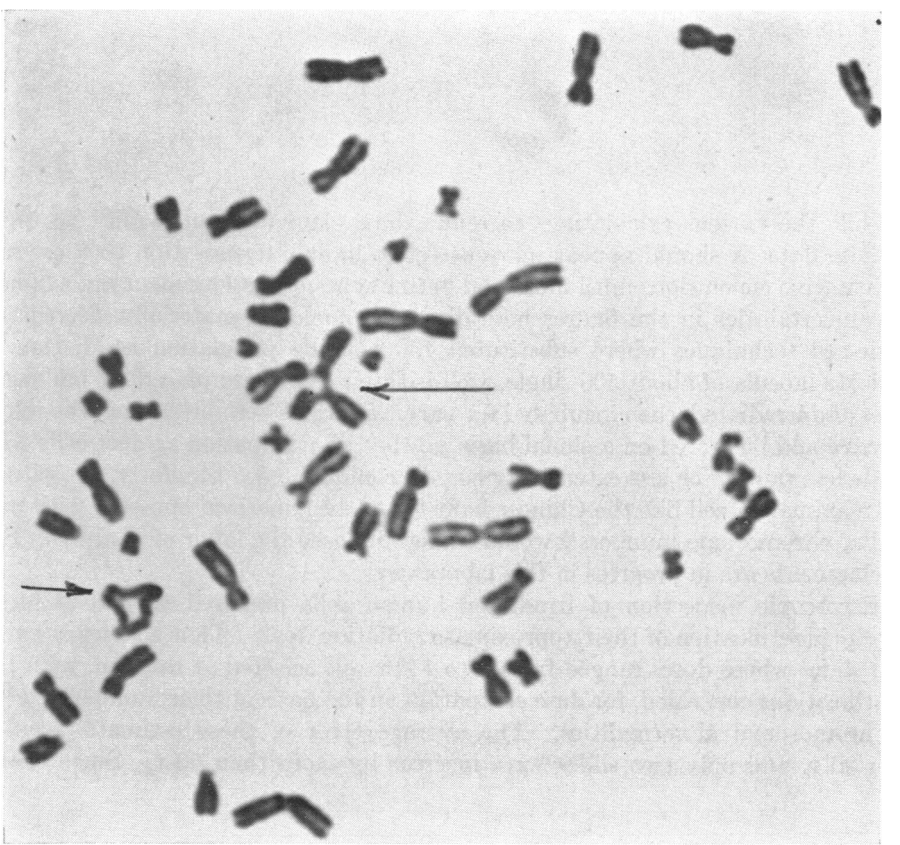

FIG. 4.—Chromosomes of cell from same culture as in Fig. 1, after irradiation with 75 r, with a translocation showing in the center and a dicentric in the lower left. × 2500.

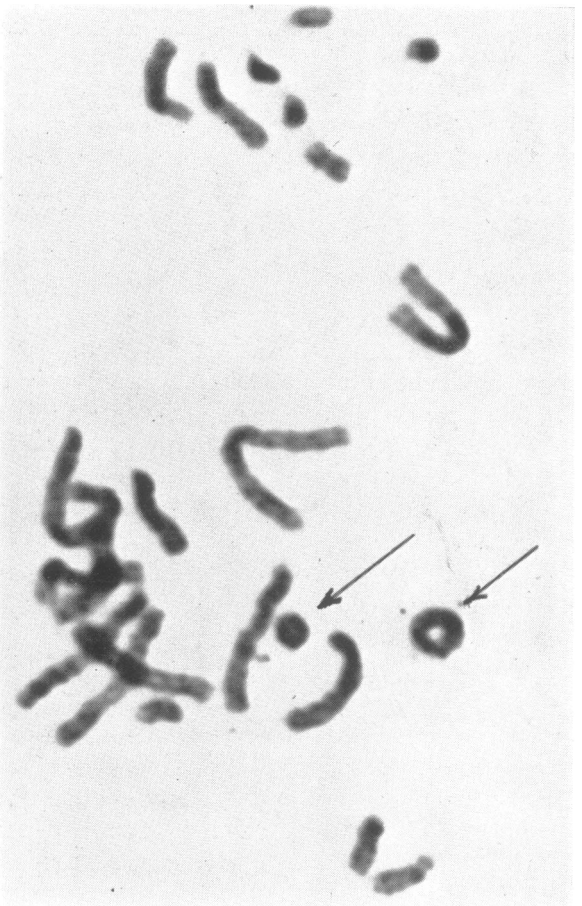

FIG. 5.—Enlarged section of a portion of a mitotic figure, demonstrating development of ring chromosomes after 150 r irradiation of cells from the culture shown in Fig. 1. Late prophase. × 2500.

While the various calculations to refine these estimates can readily be applied to these data, it should appear more useful to limit interpretation to these semi-quantitative conclusions until more and better types of measurement are available. The uncertainties in the figures here discussed could be materially decreased if single-cell techniques where substituted for massive population irradiation. If replicate inocula of about 500 single, well-isolated cells were placed on microscope slides and irradiated, then incubated for varying periods before staining, the mitotic figures could be scored on a clonal basis, so that discrimination against cells which divide less rapidly or less extensively could be eliminated. Similarly, substitution of a mammalian cell like the Chinese hamster or the American opossum with much smaller chromosome numbers[12] would materially ease the labor of analysis. Such developments are in progress in this laboratory.

Microscopic inspection of irradiated human cells prepared as here described permits identification of their approximate radiation dose. Thus a series of twenty such slides whose doses ranged from 0 to 150 r was selected at random, with their identifications concealed, for dose estimation on the basis of their number and kind of chromosomal abnormalities. The average error of these estimates was less than 30 r, and only two slides were in error by more than 50 r. Since the ab-

normalities described here are relatively gross, it may be expected that searching cytological analysis will reveal evidence of more subtle chromosome damage at lower dose ranges.

Chromosomal changes and anomalies continue to be exhibited in cells from cultures which have survived various radiation exposures. Clonal stocks of such cells have been developed and are under study. Search is under way for qualitative and quantitative relationships between specific chromosomal abnormalities and metabolic markers, like resistance to destruction by Newcastle disease virus which we have induced in S3 HeLa cells by X-irradiation.

The uniformity of the results of such diverse kinds of experiments as survival-curve analysis, scoring of chromosomal aberrations, and isolation of mutant cell strains with changed karyotype leaves no doubt of the chromosomal localization of radiation injury in these cells. The fact, too, that the doses needed to accomplish these effects are so much smaller than those needed to produce significant changes in enzymes, specific proteins, and other cellular constituents[13] testifies to the uniqueness of the DNA-containing structures of the cell. In conformity with the general interpretation developed throughout this series of papers, it has been demonstrated that fibroblast-like animal cells containing much smaller quantities of DNA, like those of the chick, display an increased radiation resistance of the predicted magnitude. Details of such studies analyzing cellular radiation sensitivity as a function of chromosome number, DNA content, and ploidy in a variety of animal cells will be described in a forthcoming report.

These studies reveal that the human cell, at least when grown under the conditions here specified, is extraordinarily sensitive to chromosomal damage by ionizing radiation—perhaps more so than any other cell yet studied. The minuteness of the dose needed to produce chromosomal aberrations and cell reproductive death makes this ideal material for separation of genetic and physiological actions of ionizing radiations.[14]

Summary.—X-irradiation of euploid human cells under conditions of stable growth in tissue culture produces chromosomal anomalies whose nature and frequency depend on the dose. Below 50 r—the approximate mean lethal dose for cell reproduction in the strains employed—practically the only anomalies obtained are those characteristic of single hits. Above this dose range, the incidence of simple breaks is increased, and complex breaks indicative of interaction between two or more hit chromosomes appear and increase with dose.

These data support the previously advanced interpretation that the primary process leading to destruction of the reproductive ability of cultured single mammalian cells by X-irradiation is a damage to the chromosomes, and they place an upper limit of 40–60 r on the value of the dose needed to produce an average of one chromosome abnormality in an euploid, fibroblast-like human cell under these conditions.

Pictures of various radiation-produced chromosome breaks, translocations, dicentrics, and ring chromosomes in reproducing, euploid human cells are presented.

* Contribution No. 74 from the Department of Biophysics, Florence R. Sabin Laboratories, University of Colorado Medical Center, Denver. This work was supported by grants from the Commonwealth Fund and the Rockefeller Foundation.

[1] T. T. Puck and P. I. Marcus, *J. Exptl. Med.*, **103**, 653, 1956.

[2] T. T. Puck, D. Morkovin, P. I. Marcus, and S. J. Cieciura, *J. Exptl. Med.*, **106**, 485, 1957.
[3] T. T. Puck, *Advances in Biol. and Med. Phys.*, **5**, 75, 1957.
[4] T. T. Puck, *J. Cell. Comp. Physiol.* (in press).
[5] H. J. Muller, in *Radiation Biology*, ed. Alexander Hollaender (New York, Toronto, London: McGraw-Hill Book Co., Inc., 1954), pp. 351–626.
[6] Lea, D. E., *Actions of Radiations on Living Cells* (Cambridge: Cambridge University Press, New York: Macmillan Co., 1947), p. 189.
[7] *Ibid.*
[8] E. H. Y. Chu and N. M. Giles, *J. Natl. Cancer Inst.*, **20**, 383, 1958.
[9] Lea, *op. cit.*
[10] M. A. Bender, *Science*, **126**, 974, 1957.
[11] T. T. Puck, A. Robinson, and S. J. Cieciura, in preparation.
[12] J. H. Tjio and T. T. Puck, *J. Exptl. Med.*, **108**, 259, 1958.
[13] A. Pirie, in *Ciba Foundation Symposium on Ionizing Radiations and Cell Metabolism* (Boston: Little, Brown & Co., 1956), pp. 38–58.
[14] T. T. Puck, in preparation.

SEISMIC SURFACE WAVES AT PALISADES FROM EXPLOSIONS IN NEVADA AND THE MARSHALL ISLANDS

BY JACK OLIVER AND MAURICE EWING

LAMONT GEOLOGICAL OBSERVATORY, COLUMBIA UNIVERSITY, PALISADES, NEW YORK

Communicated June 18, 1958

Introduction.—The United States Atomic Energy Commission recently declassified precise times and locations of fifteen nuclear explosions, ten in the Marshall Islands and five in Nevada, in order to facilitate research in various fields of science, particularly seismology. This information had been requested by seismological groups, since it was already well known that body waves from the large hydrogen bombs are detected by seismic stations throughout the world; hence precise data on the source would assure valuable results on the structure of the earth's interior.

The Palisades station, which specializes in long-period surface-wave instrumentation, detected surface waves from almost all these explosions. Surface waves are the only seismic effect at Palisades. The station is in the shadow zone (distance 104°–106°) of the earth's core for body waves from the Marshall Islands region and is apparently too far (33°) from Nevada to detect the relatively weak body waves generated by the explosions there, which are much smaller than those at the Pacific site.

The greater range of the surface waves is probably due, at least in part, to their inherent geometric advantage over body waves. Surface waves spread in only two dimensions as opposed to three for body waves, so the amplitudes decay as $r^{-1/2}$ rather than r^{-1}, where r is the distance from the source, as a result of this effect.

With the exception of the underground explosion Rainier, no yields were released by the Atomic Energy Commission. Newspaper reports suggest, however, that the Marshall Islands explosions were all in the megaton range and that the four Nevada tests other than Rainier were some of the larger of the 1957 series, probably 10 kilotons or greater.