Irradiation of Parts of Individual Cells[1]

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SELECTIVE EXPOSURE of fractions of intact individual cells to high-energy radiation is of interest for two reasons. First, it has obvious use in radiobiology as a means of gaining information about the mechanisms by which radiations produce their strikingly injurious effects on living systems. Second, and of much more general application, it can aid in analyzing the normal functions of the various cell parts by selectively altering them. In this respect it complements microsurgery.

Irradiation of parts of single intact cells has been done before, both with ultraviolet light (*1*) and with high-energy radiation (*2–7*),[3] but in each instance the success of the technique has been largely dependent on some peculiarity of cell structure, e.g., exceptionally large size (*2, 7*) or an eccentric nucleus (*2, 3*), and accordingly has been quite limited in application. Also the cell fraction irradiated has in most cases been quite large (25–50 per cent). This paper contains a brief description of a new and widely applicable method with which considerably smaller fractions of average-sized cells can be bombarded, and mention of some of the results obtained.

Procedure

The method is based upon a *microbeam* of ionizing particles arranged to traverse a selected small portion of the individual cell under study. Obviously, the cross section of such a microbeam must be small compared to the cell area perpendicular to the beam's axis, i.e., it must have dimensions of the order of microns.

In general, to produce a microbeam of high-energy radiation, one must start with a beam of macroscopic dimensions and either focus all or part of it to a microscopic cross section or allow part to pass through a microaperture in a layer of impervious material. Neutrons do not lend themselves satisfactorily to either procedure. Nor do photons (x or γ rays), except for low-energy x rays from tubes of special design (*10*). This restricts the choice of radiation type to fast charged particles (electrons, mesons, or atomic nuclei such as protons, deuterons, or alpha particles). The mesons can be disregarded because no currently available source produces a sufficiently intense macrobeam. Electrons and atomic nuclei can be focused electrically and/or magnetically. However, this procedure was not tried because it appeared that the microbeam probably would have to be not only produced but used *in vacuo*; although some types of cells withstand vacuum very well, most do not, and formidable difficulties could be foreseen in trying to protect them from vacuum without creating difficulties in design of culture chambers. Accordingly, there remained the alternative of canalizing electrons or atomic nuclei with a microaperture. The electrons were ruled out because the macroscopic beam, impinging on the layer of material containing the microaperture, would produce x rays which would superimpose a very troublesome irradiation of the entire cell upon the localized irradiation with the microbeam. Moreover, electrons are very difficult to keep canalized satisfactorily, because of their great propensity for scattering when they collide with material of any sort. Accordingly, the fast atomic nuclei remained for consideration. Deuterons were not favorably regarded because of a probable health hazard due to the neutrons and other radiations that they would generate within the accelerator. The choice between protons and alpha particles (the currently available types of nucleus which remained) was largely dictated by the accelerator available, which was a vertical Van de Graaff electrostatic generator capable of producing 2-Mev protons or 4-Mev alpha particles, whose ranges in tissue are approximately 73 and 25 μ, respectively (*11*). Since, in most experiments, the particles had to traverse two mica windows equivalent in stopping power to 25 or 30 microns of tissue, it was clear that alphas would not be feasible. The protons remained as our best practical choice.

The macroscopic beam of protons emerged from the accelerator through a mica window 2 mm in diameter and 1.5 mg/cm^2 (5 μ) thick and was allowed to impinge on a metallic shield pierced by the microaperture. Several types of microaperture were investigated, of which two (G and XS) were considered satisfactory. Type G (Fig. 1) consisted essentially of two optically flat slit jaws *A*, across one of which a microscopic groove *B* either was scratched with a diamond or was "chopped" by means of a very carefully controlled collision with an optically polished blade. The two jaws were then firmly pressed together, leaving a microaperture of approximately triangular cross section. The position of the microaperture was marked by the intersection of the index

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[3] We are here not directly concerned with certain interesting experiments (*8, 9*) in which cell parts have been separated mechanically before irradiation.

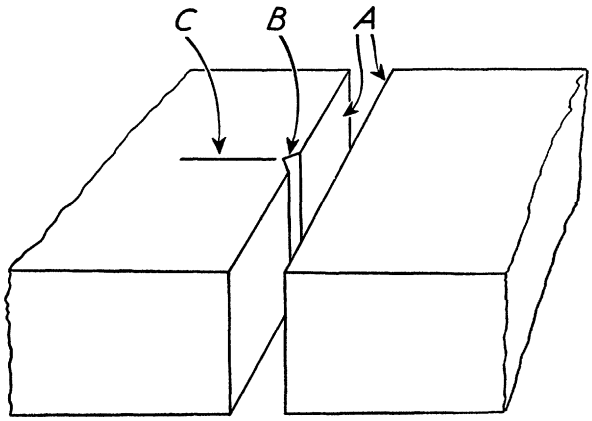

FIG. 1. Scheme of construction of a microaperture of type G. (See text.)

line C (lightly scratched on the metal) with the line of junction of the two jaws. Microapertures of type XS (Fig. 2) were formed of crossed slits, the jaws of which were polished to optical flatness. Each slit could be adjusted to any desired width from 0.5 μ to 5 mm by turning a graduated knob K.

The spatial distribution of the protons traversing each microaperture was determined by analysis of the pattern of tracks produced in Ilford C2 nuclear plates exposed at the position occupied by a cell during bombardment. Bombardment patterns for two type G microapertures are plotted in Fig. 3. The actual sizes of the microscopic holes were unknown and immaterial, but it will be noticed that, with each microaperture, when the bombarded object was 5–10 μ away from it, more than 80 per cent of the protons

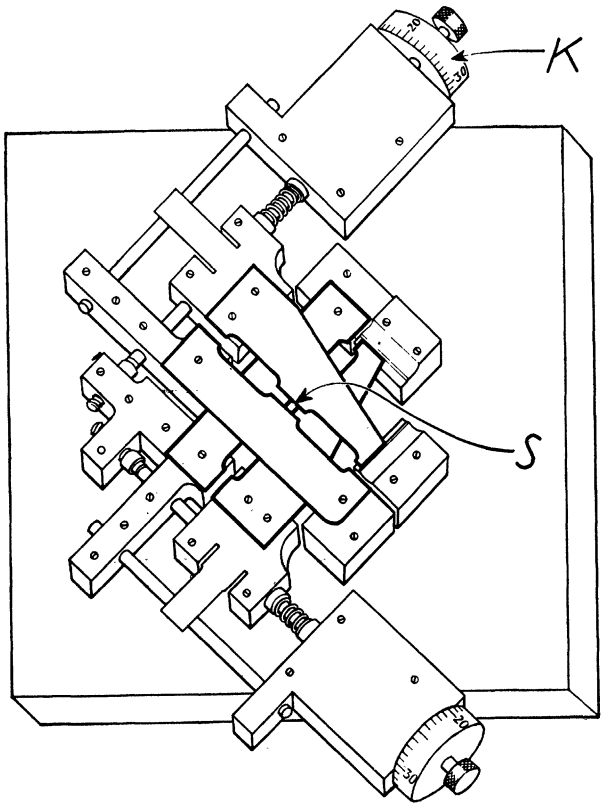

FIG. 2. Apparatus for producing variable microaperture of type XS (crossed slits). Both slits are shown open, their intersection at S. (See text.)

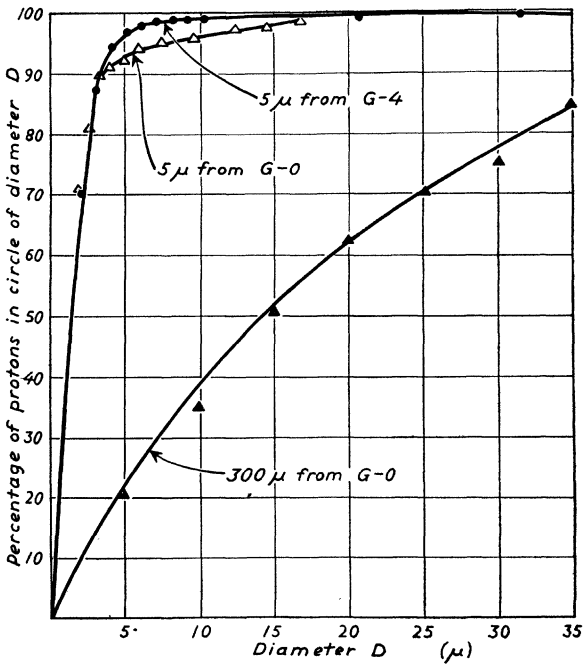

FIG. 3. Proton track distribution in nuclear plates exposed under two microapertures of type G. Note that, although the two yielded identical bombardment patterns at low angles of scatter, G-4 produced superior localization at high angles (two upper curves). Also note adverse effect of distance on localization produced by G-0.

struck a central area 2.5 μ in diameter. The tails of the curves represent protons scattered by atomic nuclei in the walls of the holes. This scattering cannot be eliminated but can be considerably reduced by improvements in geometrical design and in choice of material for the jaws. (Note that G-4 is much superior to G-0 in this respect.) The effect of scattering on the bombardment pattern has been minimized by placing the bombardment object as close as possible to the microaperture (note the great spread of the bombardment pattern when the plates were exposed 300 μ away). With microapertures of type XS, the bombardment patterns are well localized by the bottom slit but poorly by the upper (as in upper and lower curves, respectively, for G-0, Fig. 3). Type XS is excellent for bombardments in which a narrow "ribbon" microbeam is desired. Type G is superior when the microbeam must be essentially isodiametric in cross section.

For each microaperture the energy spectrum of the protons incident on the cell was determined by measuring the lengths of the individual tracks produced in Ilford C2 plates after the protons had passed through mica of the same thickness as that used for the cover

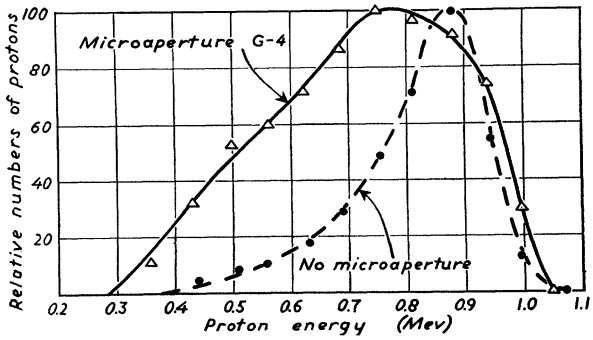

FIG. 4. Energy spectra of protons which traversed microaperture G-4 and of those which traversed no microaperture.

slips of the culture chambers. The spectrum of protons transmitted by microaperture G-4 is shown in Fig. 4, along with that of protons that passed through no microaperture.

In the microbeam bombardments the chief items of equipment were an *observation microscope* (Fig. 5) with which the cell was observed before and after bombardment; the *Van de Graaff generator* (*S*, Fig. 6); a *bombardment assembly* (Fig. 6) consisting of an *alignment microscope B*, a *bombardment stage C*, and a *microaperture assembly D*, all maintained in alignment by means of a rugged and precisely machined column *E*; a *master locator* (Fig. 7); and an *adjustable locator* (*AL*, Fig. 5) which bore the cell preparation on a small mechanical stage.

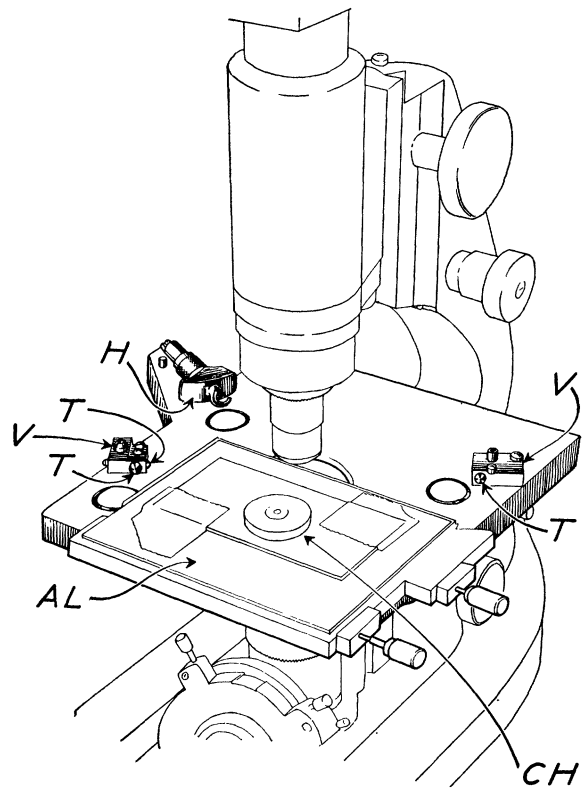

FIG. 5. Observation microscope, equipped for phase contrast. The stage is equipped with locator stops *T* and hook *H*. Adjustable locator *AL* detached from hook to show stops. Culture chamber *CH* mounted on locator.

For reasons given below, it was essential that the cell for bombardment be directly beneath the cover slip of the cell preparation (tissue culture). When the culture was in bombardment position under the microaperture, the cell could be observed only from below, with vertical illumination. The thickness of the culture, dictated by various biological requirements, was so great that only a low-power objective could be focused on the cell. Moreover, the cell had to be observed through the entire thickness of culture medium and irrelevant cells, the result being poor optics. Accordingly, the notion of aiming the microbeam by direct observation of the cell in bombardment position was abandoned in favor of an indirect method of great flexibility and accuracy. In this pro-

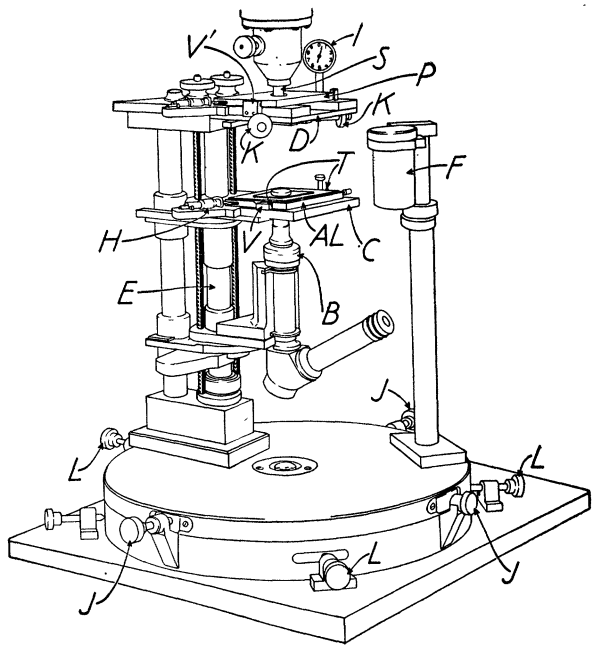

FIG. 6. Bombardment assembly. (See text.) *S*, delivery tube of Van de Graaff generator.

cedure the master locator (Fig. 7) played a central role. This device consisted of a brass plate with a hole over which was securely fastened a glass slide whose upper surface was covered with a thin deposit of chromium. A microscopic cross *Q* was scratched through this metallic layer, near the middle of the slide. The brass plate was notched in such fashion that it could be brought into contact with three rounded and polished steel *stops T* (two shown in Fig. 6, all in Fig. 5) held in two *stop assemblies V* on the bombardment stage (Fig. 6) and also on the stage of the observation microscope (Fig. 5). A spring, attached to a corner of the stage and provided with a hook *H* (Figs. 5 and 6), was used to pull the plate, with a reproducible force, against the stops and to hold it securely in place. To provide a smooth surface and

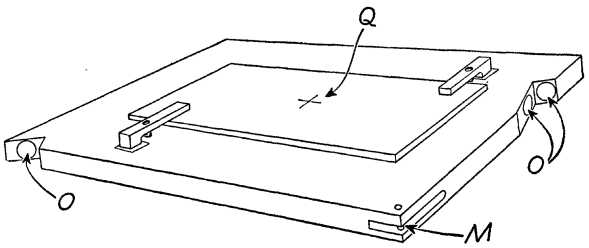

FIG. 7. Master locator. *M*, catch for hook *H* of Fig. 6. *O*, polished and hardened steel inserts. *Q*, position of microscopic cross. (Also see text.)

to prevent wear, the brass plate was provided with polished steel inserts *O* in the notches.

The master locator was hooked in place on the bombardment stage (*C*, Fig. 6), and the latter was raised to bombardment position. The alignment microscope *B* (800 × magnification) was raised and focused, through the hole in the brass locator plate, on the microscopic cross on the locator. If the image of the cross did not intersect the cross hairs in the ocular of the microscope, the stops were adjusted until it did.

The master locator was removed, and the alignment microscope was focused on the microaperture. If the cross hairs of the microscope did not intersect in the middle of the microaperture's image, the microaperture assembly, which was attached to a locator plate (*P*, Fig. 6) similar to the brass plate of the master locator, was suitably moved by adjustment of the stops *V'*. Since the cross on the master locator had also been lined up with the same cross hairs, the microbeam would have bombarded this microscopic cross if the master locator had been placed and hooked on the bombardment stage and the latter raised to bombardment position.

The master locator was now placed on the stage of the observation microscope (Fig. 5), which was provided with stops *T* and hooked spring *H* similar to those on the bombardment stage. If the image of the microscopic cross did not intersect the cross hairs of this microscope, the stops were suitably adjusted.

Now, if the master locator were removed and any other locator with identical notches were substituted, any small object on this latter locator would, if its image fell at the intersection of the cross hairs, be lined up with the microaperture when the locator was transferred to the bombardment stage. To eliminate any error due to slight deviations of the optical axes of the various observation microscopes, it was necessary that the microscopic cross *Q* on the master locator be the same height above the microscope stage as the object to be bombarded.[4]

Aiming was thus accomplished by an indirect method whose accuracy was determined by the precision with which the following operations could be performed: (1) alignment of the master locator with the microaperture; (2) alignment of the master locator and also of the bombarded object with the cross hairs of the observation microscope; and (3) the seating of the locators when they were shifted from one set of stops to another. All the lining-up operations could be made good to 0.2 μ. The reproducibility of the locators usually was equally good; if, as rarely happened, repeated tests of a locator's seating against the stops revealed reproducibility poorer than 0.2 μ, this was an indication that a contact point needed cleaning or that a stop assembly needed tightening; such servicing was readily accomplished and tested. The overall accuracy of aiming was accordingly no worse than 0.6 μ if all errors accumulated; since each error was random in direction, the various errors frequently compensated, and accordingly in most bombardments the aiming was better than 0.6 μ.

The microaperture was set in position in the main beam by means of adjusting screws (*L*, Fig. 6), which translated the entire bombardment assembly in two perpendicular horizontal directions, and was lined up with the beam by screws (*J*, Fig. 6), by which the axis of the bombardment assembly could be swung in two perpendicular vertical planes, the microaperture being located at the center of swing. These operations were facilitated by observing changes in proton flux through the microaperture by means of a Geiger-Müller counter with a mica window 5 μ (1.5 mg/cm²) thick (*F*, Fig. 6).

The cells were grown in tissue cultures of amphibian heart[5] (Triturus, Ambystoma). A culture chamber *CH* for microbeam bombardment is shown in Fig. 5. Since the penetration of the protons, after they had traversed the mica window of the generator, was only about 30 μ in water, the cover slip of the culture chamber was of mica 5 μ or slightly less in thickness. This left the protons with enough energy to traverse a cell, but it was essential that the cell be just below the mica cover. To ensure this, fine scratches were made on the top of the mica and selection was restricted to cells which were in focus along with a scratch in the same microscope field.

The experiments described here were limited to mitotic cells. With the observation microscope, each cell was carefully examined by medium-dark phase contrast, which clearly reveals many details in the amphibian cells, particularly chromosomes, mitochondria, nucleoli, nuclear membranes, chromatin particles, and sometimes the outline of the mitotic spindle and the cell centers. If the cell was found suitable in nature and location (not too deep in the culture, not too close to optically interfering neighboring cells, etc.), the culture was securely fastened to the adjustable locator (*AL*, Fig. 5) with Scotch tape, and a few feet of 16-mm film were exposed, usually 15 frames per minute. During this prebombardment photography, the portion of the cell to be irradiated was

[4] We have found these locators useful in many other laboratory procedures, for instance, in finding the same bombarded cell repeatedly in histological and polarization studies.

[5] Exploratory experiments have also been performed on cultured cells of warm-blooded animals and on various simple plant and animal forms.

brought accurately beneath the aiming cross hairs by means of the mechanical stage of the adjustable locator. The exact location of the bombardment region was thus permanently and quickly recorded.

Concurrently with selection and prebombardment photography of the cell, the rate at which protons were coming through the microaperture was determined. This was done with a Geiger-Müller counter (*F*, Fig. 6) which could be readily and reproducibly swung into position below the microaperture. The counter had an efficiency of about 100 per cent as determined by alternately bombarding it and Ilford C2 nuclear plates, and was corrected for natural background and for a low count due to Bremsstrahlung produced by protons impinging on the slit jaws. When a suitable rate had been established (usually a few hundred protons per minute), the time required to deliver the desired number of protons was calculated, the counter was swung out of position, the locator with the culture was transferred from the observation microscope to the bombardment stage (Fig. 6), and the latter was raised until the mica cover slip of the culture was about 5 μ from the lower slit jaws. This close approach was accomplished, rapidly and without danger of collision with the precisely adjusted microaperture, by use of a suitable height indicator (*I*, Fig. 6) which had been adjusted beforehand for the particular locator and culture chamber being used. The desired exposure was then given by opening and closing a shutter (not illustrated) in the delivery tube of the proton generator.

The locator was then transferred from the bombardment stage back to the observation microscope (Fig. 5), and the location of the object aimed at was carefully checked against the cross hairs. If, as seldom happened, the "return" was not precise, this was due to faulty seating of the locator or, more likely, to the cell having moved. In either case, the deviation from aiming was carefully noted and the experiment was either discarded or the conclusions drawn from it were restricted in accordance with the extent and direction of the deviation. If the "return" was precise, it was assumed that the protons had been delivered as aimed, because, if either the locator or the cell produced a deviation, it appears highly improbable that an exactly compensating deviation, both in direction and extent, should have occurred.

As soon as the "return" had been checked, time-lapse photography was resumed at a suitable frequency and was continued, supplemented by long-hand notations and diagrams in the cell's individual protocol, until all events of interest had occurred or until observation had to be discontinued for other reasons.

It is clear that the foregoing bombardment method is applicable to any cell (or other microscopic object) which can be brought close to the microaperture, which does not move or can be immobilized during bombardment, and in which suitable microscopic targets can be seen in the living condition.

Observations

Observations to date have been preponderantly on cells irradiated in various phases of mitosis. As background for interpretation of the results of partial-cell irradiation, some 250 cells have been totally x-irradiated with from 50 to 4000 r. The following well-known qualitative effects have been observed and recorded: (1) temporary chromosome bridges (duration, 6 hr or less); (2) permanent chromosome bridges (if they last more than 6 hr, they usually persist for days); (3) inhibition of anaphase movement at various stages in the process; (4) complete inhibition of anaphase; (5) inhibition of metaphase

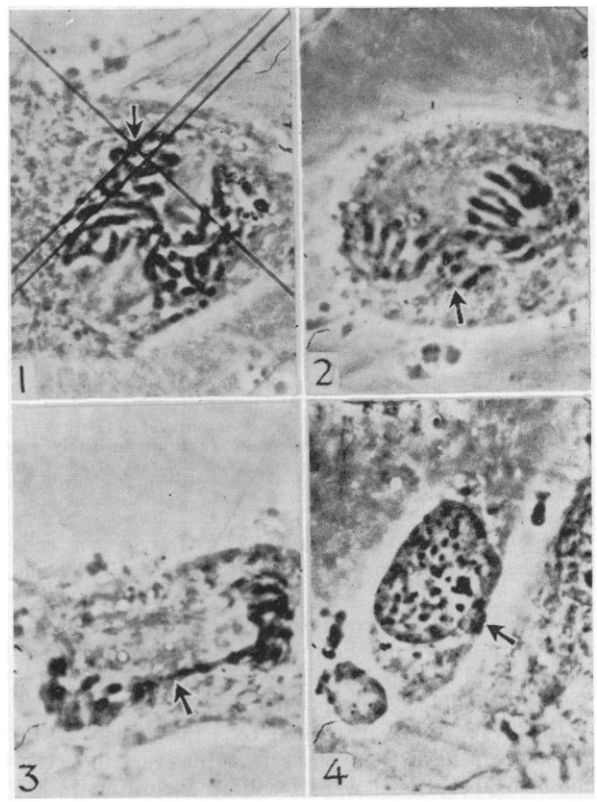

FIG. 8. Anaphase hinge. (Prints from 16-mm motion-picture film.) (1) Cell at time of bombardment. Place marked by arrow. (2) Chromosome groups form 90° hinge in anaphase because of bridge (arrow) produced by bombarded chromosomes. (3) Bridge (arrow) persists as hinge straightens out. (4) Bridge has broken, and small accessory nucleus (arrow) appears beside one of the large daughter nuclei. (Area shown in each frame is 45 × 60 μ.)

configuration (if irradiated in prophase); (6) inhibition of cytoplasmic constriction (cleavage); (7) displacement of constriction with respect to chromosomes; (8) unequal distribution of chromosomes between daughter nuclei because of abnormal anaphase movement and/or misplaced constriction; (9) interference with reconstruction of daughter nuclei; (10) rapid and repeated protrusion of abnormally large cytoplasmic "bubbles," usually beginning about the time of cell constriction. The first four of these effects

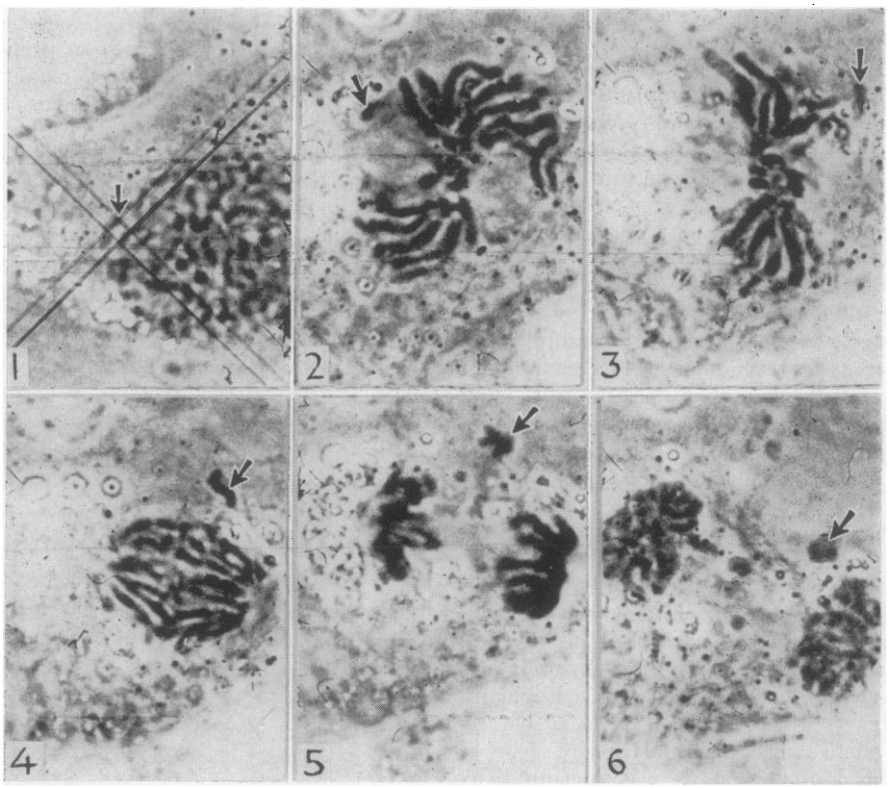

FIG. 9. Development of an accessory nucleus. (Prints from 16-mm motion-picture film.) (1) Bombardment (arrow). (2) Position of bombarded chromosomes (arrow) in early metaphase. (3–5) A chromatin clump consisting of one or more chromosomes (arrows) is detached from the rest of the chromosome group. (6) Membrane (arrow) has formed around this clump. Two other accessory nuclei lie between the two large nuclei. (Area shown in each frame is 45 × 60 μ.)

were smoothly dose-dependent.[6] A few dozen cells have been totally proton-irradiated, with similar results.

In some 120 nonirradiated cells, most of these abnormalities have not been observed at all. Two temporary and one permanent bridge occurred, and a few cells "bubbled" as specified in item 10 above.

In 300 partially irradiated cells the same qualitative effects have been observed. However, the chromosome aberrations were localized according to the spot of bombardment. A few dozen[7] protons through the middle of a metaphase plate or of a prophase chromosome configuration regularly produced bridges; a few hundred protons produced so many bridges that anaphase was stopped early. A few dozen protons to one corner of the "butterfly" configuration in metaphase (Fig. 8) regularly produced a bridge near one side of the spindle; if the bridge was strong enough, its tension and the normal forces of anaphase movement (whatever they may be) produced an anaphase "hinge" (2, Fig. 8).

[6] We shall describe in another place the dependence of these effects on dose, stage of mitosis, irradiation, etc., as well as modification of the mitotic time schedule.

[7] Partial-cell bombardments are here stated as numbers of individual protons which traverse the cell part. In partial-cell irradiation the concept of dose (energy transferred per unit mass of cell) is complicated and will be discussed elsewhere.

Although even a few dozen protons to the chromosomes[8] regularly produced unambiguous abnormalities (chiefly bridges), hundreds and even a few thousands to extrachromosomal regions (general cytoplasm, spindle, centriolar region) produced no detectable effects, if no more than a few protons were scattered into the chromosomes.

Occasionally it was possible, because of an outspread configuration of the chromosomes in early metaphase, to bombard a single chromosome with fair selectivity and produce nondisjunction of the two daughter chromosomes, both going to the same pole in anaphase.

As a result of partial-cell irradiation, small accessory nuclei (sometimes called "micronuclei") were frequently produced (4, Fig. 8), although this has never been unequivocally observed in our totally irradiated cells.[9] Usually these accessory nuclei appeared late, presumably having been obscured previously by the large neighboring nucleus. However, in one case we traced the full development of one of several accessory nuclei that resulted from the localized irradiation of a few chromosomes in prophase. A few frames from the motion picture are shown in Fig. 9.

[8] Of course, in chromosome bombardments a small amount of cytoplasm was also irradiated.

[9] In some material, accessory nuclei are copious after total-cell irradiation (e.g., Politzer, *12*).

Details of the observations and also of the apparatus will be published elsewhere.

A widely applicable method has been developed for high-energy irradiation of cell parts by use of proton microbeams with diameters as small as 2.5 μ. In mitotic cells of newt heart cultures, a few dozen protons to chromosomes produce severe aberrations, whereas a few thousand to cytoplasm, spindle, or centriolar region are without detectable effect. Some of these types of chromosome aberrations are readily localized and help in the study of certain aspects of mitotic movement.

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News and Notes

Southern Society of Cancer Cytology

JANUARY 28th marked the second meeting of the Southern Society of Cancer Cytology, the inaugural meeting having taken place in Miami last November during the Southern Medical Association meeting. The 88 members in this group represent 13 of the southern states and the District of Columbia, and include many leading clinicians, cytologists, pathologists, and researchers, all having a special interest in cancer cytology.

The aims of the organization, as set forth in the by-laws, are as follows:

1. To stimulate the development of standards of nomenclature, cell classification, techniques, education, etc.
2. To stimulate research, both practical and theoretical, on problems related to cancer.
3. To stimulate education in cancer cytology.
4. To provide a forum for discussion of cytological problems.
5. To promote sound statistical studies based on uniform and standard methods.
6. To act as a regional advisory group covering the aforementioned objectives.

Honorary members of the society include the following: Emil Novak, Herbert F. Traut, Charles Read, Wilton R. Earle, Edgar R. Pund, and Robert Chambers.

The following officers were elected to serve for two years from the date of the inaugural meeting: president, F. Bayard Carter; vice president, M. Y. Dabney; secretary, J. Ernest Ayre; treasurer, Joseph K. Cline.

This new society is organized around three councils and two committees, which include:

Pathology Advisory Council: C. C. Erickson, chairman, associate director, Institute of Pathology, University of Tennessee; Virgil H. Moon, professor of pathology, University of Miami; J. M. Blumberg, pathologist, Walter Reed Army Hospital, Washington, D. C.; Paul Kimmelstiel, pathologist, Charlotte Memorial Hospital, Charlotte, N. C.

Clinical Advisory Council: F. Bayard Carter, chairman, head, Department of Obstetrics and Gynecology, Duke University, Durham, N. C.; Ralph W. Jack, Miami, Florida; M. Y. Dabney, editor, *Southern Medical Journal*; John E. Dunn, chief, Field Investigation Center Cancer Control Branch, National Cancer Institute; R. W. TeLinde, Johns Hopkins Hospital, Baltimore, Md.

Cytology Advisory Council: J. Ernest Ayre, chairman, director, Dade County Cancer Institute, Miami, Florida; Robert E. Seibels, Columbia, South Carolina; H. E. Nieburgs, Department of Clinical Cytology, Medical College of Georgia, Augusta; L. I. Platt, Washington, D. C.; E. E. Siegler, U. S. Public Health Service, Memphis, Tenn.

Research Advisory Committee: J. K. Cline, chairman, director of cancer research, Medical College of Alabama, Birmingham.

Educational Advisory Committee: Homer L. Pearson, chairman, Miami, Fla.; Jay F. W. Pearson, president, University of Miami, Fla.; Chauncey Leake, vice president, University of Texas Medical Branch.

This group is planning to hold the first scientific session at Atlanta, Ga., in November of this year, simultaneously with the sessions of the Southern Medical Association. Application is being made by this society to cooperate in establishing a Section on Cytology of the Southern Medical Association.

Regular membership in the society is open to physicians, scientists and other research workers, interested in the furthering of cytology, and associate membership is open to technicians in the field. The territorial scope of the Southern Society of Cancer Cytology includes the states covered by the Southern Medical Association.

J. ERNEST AYRE

Dade County Cancer Institute, Miami, Florida