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"MECHANISMS OF ABERRATION PRODUCTION IN EUKARYOTE CHROMOSOMES"

Annual Progress Report

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PROJECT ABSTRACT

Both hardware and software for a semi-automatic chromosome analysis device have been completed and the system moved from Vanderbilt University to the Johns Hopkins University during the past Contract year. Testing of the device's performance against standard manual chromosomal analysis done independently on an unselected series of clinical cases has been started.

Work on the induction of chromosomal aberrations by inducing two specific lesions in DNA at known stages in the cell cycle (under the previous Contract AT-(40-1)-4065) suggested that it might be possible to construct a general theory of aberration production by all chromosomal aberration producing agents. During the past Contract year such a model has been completed. An explanation of certain previously puzzling aspects of the segregation of newly-synthesized DNA and of the phenomenon of sister chromatid exchange as consequences of a true recombinational form of post-replication repair in eukaryotes has also resulted from this theoretical work.

Laboratory experiments were carried out in collaboration with Drs. J. S. Bedford and H. G. Griggs of Vanderbilt University in order to test the hypothesis that radiation-induced loss of reproductive integrity in mammalian cells can be attributed to chromosomal aberration production. With the same collaborators, experiments were begun to test the hypothesis that the induction of isochromatid type aberrations by lesions induced

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within a single DNA double helix results from a recombinational repair process. Finally, experiments have been carried out in collaboration with Dr. J. M. Rary of the Johns Hopkins Department of Obstetrics and Gynecology to test the hypothesis that certain genetically-determined diseases, including the Hutchinson-Gilford progeria syndrome, Fanconi's anemia, Bloom's syndrome and ataxia tetangiectasia, may involve specific DNA repair deficiencies.

BACKGROUND AND SCOPE

The present Contract actually represents a continuation of research begun under the Principal Investigator's previous Contract with Vanderbilt University (AT-(40-1)-4065). Work on two relatively independent projects, design, construction and testing of a semi-automatic system for chromosomal analysis, and research in radiation cytogenetics, was initially proposed. Though the program envisioned for the former project has remained essentially unchanged, that in the latter has evolved significantly during the past four years.

1. Semi-automatic chromosome analysis system. The development of such a system was originally proposed as a practical solution to both the relatively high cost in both time and money, and the qualitative nature of chromosomal analysis by the conventional manual "cut and paste" method. Much effort and substantial funding has been spent on attempts to develop completely automatic devices for computer chromosome analysis, but none of the resulting systems (for example FIDAC and PIQUANT) can be said to offer a practical solution to these problems. Complete automation involves digitizing the image of the chromosome spread and pattern recognition and feature extraction by computer manipulation of the individual picture elements. Very large computers and/or large amounts of CPU time are thus required, making such devices inherently very expensive. Furthermore, no one has as yet devised computer algorithms for chromosome identification and feature extraction which perform acceptably even for carefully selected spreads. In consequence, every attempt at a pattern recognizing device has been forced to incorporate provisions for supervision and correction of its performance by a human operator, thus making the operator, in fact,

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the pattern recognizer and time-limiting factor in the system. Because relatively unskilled operators can easily recognize what is or is not a chromosome and to recognize centromeres and chromosome arm ends, the problems associated with computerized pattern recognition and chromosome feature extraction can easily be avoided completely by simply using the operator to perform these functions in the first place. By simply providing a convenient means for displaying the chromosome image and entering the required chromosome features, all of the practical objectives, such as speed, quantitation, reproducibility and consistency can easily be met with the very limited capacity of a low-cost minicomputer.

At the time the construction of such a device was proposed, almost all analyses, whether qualitative or quantitative, were based on the relative chromosomal arm length in conventionally stained metaphase spreads. The one exception was analysis based on relative arm DNA contents in stoichiometrically stained preparations. Shortly thereafter, however, a variety of methods were developed for producing chromosomal banding, and chromosome analysis based upon banding very quickly became widespread. As a consequence, it might be argued that analyses based simply on arm lengths or DNA contents are no longer of value, and that no further effort should be spent on attempts at automation based on them. Such an argument seems incorrect for two reasons. First, although proponents of banding analysis frequently do not recognize this, the banding information is actually used in addition to, rather than as a substitute for, arm length information. Thus automation of arm length analysis must be a prerequisite to any attempt to automate the analysis of banded chromosomes. Second, the bulk

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of the routine chromosome analysis work now being done still is not based on banding techniques, but rather on conventional analysis of ordinary uniformly stained preparations. In part this is because of the greater time, effort and expense involved in banding analysis, but in large measure it is simply because conventional analyses provide all of the information needed to answer the question being asked.

The system developed under this Contract and its predecessor has two independent elements; the device which enables an operator to rapidly generate relative arm length measurements in digital form (both hardware and software), and the computer programs required to actually analyse the arm length measurements, make diagnoses, and generate the required output data (diagnoses, aberration frequencies, etc.). The latter element, it should be noted, is entirely independent of the source of its input data; any "front end" can be utilized, whether it be manual measurements made with calipers and then recorded in digital format, a semi-automatic measuring device, or even a completely automatic machine, should one which performs acceptably be developed.

The semi-automatic measuring device actually built consists of hardware which displays a composite image on a screen upon which the operator indicates the required chromosome features with a stylus. The composite display, provided by an obsolete remote computer terminal (Corning 904), consists of the chromosome spread itself, projected from a standard 35 mm negative, the image on a photochromic storage cathode ray tube, plus the image from a standard projection CRT. The projection screen is a Sylvania DT-1 graphic input device (also now obsolete) which provides 10 bit X and

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Y axis digital output data on the location of the stylus, as well as a limited sensing of the Z axis distance of the stylus from the face of the tablet. Both of these elements are interfaced to a Digital Equipment Corporation PDP 8/e 12 bit minicomputer. Software for the PDP 8/e allows it to transport the film, write on either CRT, erase the storage CRT, determine the stylus location, and calculate and store arm length information.

In operation, the system operates as a "black box". When it is turned on a small read only memory bootstraps the computer up to read a magnetic tape upon which the operating programs are stored. From then on the computer controls and checks all operations, conversing with the operator by writing messages with the projection CRT, marking the chromosomes as they are measured using the storage CRT, error-checking the operator's input, calculating arm lengths and storing the list and other information on tape for later analysis, and erasing the display and moving to the next spread as each is completed. All the operator need do is insert the roll of film and turn the machine on. No real knowledge of the system is required, only the ability to recognize chromosomes, centromeres and arm ends. Even people with no biological, much less cytogenetic background or experience can be trained to operate the device dependably in less than an hour. Once an operator gets the "feel" of the system, spreads are generally processed at the rate of about one roll of 36 negatives per hour.

It should be recognized that this particular device was built to demonstrate feasibility. Much of the hardware is obsolete, and costs for

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much of it have decreased drastically since the components were selected. Though the present design utilizes photographic negatives and optical projection, there is no reason for example that a video display could not be generated directly from the microscopic image itself, if desired. Also, it seems quite probable that should the performance of present device warrant it, a second generation analysis device could be built using state of the art components for less than \$20,000. This, it is believed, is a cost small enough to make wide utilization in cytogenetics laboratories feasible.

The problem of analysis of chromosome measurements in order to construct karyotypes, determine whether they are normal or abnormal, and make diagnoses is by no means a trivial one. Historically, workers attempting to automate chromosome analysis have started by building "front end" hardware and focusing on the problems of digital image acquisition, pattern recognition and feature extraction. The analytical software, often apparently written in ignorance of the actual nature of cytogenetic analysis, has tended to be inadequate. Frequently no thought at all appears to have been given to the need to handle the abnormal karyotype. In fact most of the pattern recognition and feature extraction algorithms written for chromosome analysis systems actually preclude the detection of abnormalities. Chromosomes are defined as having one, and only one centromere, and as having to have arm ends. Neither dicentrics nor acentric fragments fit the former, nor ring chromosomes the latter definition, of course. Similarly, the algorithms developed to construct karyotypes have usually been seriously constrained,

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often based on the notion that every spread must be normal and that the problem is simply to construct the best possible karyotype for the spread being analyzed. Where any serious testing of such systems has even been attempted, it has taken the form of determining how often the system's assignment of chromosomes in normal spreads agrees with that of a manual "cut and paste" karyotype. Worse yet, the results of such comparisons have often been expressed as "percentage of error" based on individual chromosome matches, implying a much greater degree of success than is actually the case if the desired result is a successful karyotype involving all 46 chromosomes.

In developing analytical programs for the semi-automatic chromosome analysis device, in contrast, the prime consideration has been to have as few constraints as possible. In an attempt to utilize the same strategy as that actually used by cytogeneticists, a Bayesian statistical approach to the problem was developed (Bender and Kastenbaum, 1969; Bender, Kastenbaum, Lever and Pelster, 1972). Posterior probabilities (predictive densities) are calculated for each chromosome in a spread that it is in fact a representative of the populations of each of the normal human chromosome set, thus allowing both the determination of which candidates are the "best" ones for each type of normal human chromosome, but also which are "acceptable" candidates (based on empirically determined minimum predictive densities). Karyotyping sub-routines then attempt to construct a normal karyotype using an iterative, essentially logical approach

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patterned on the strategy used in manual "cut and paste" analysis. This method has the virtue of having no more constraints than those imposed in conventional karyotype analysis while at the same time allowing the utilization of any constraining information that might be available in any given instance. That is, if for example, the sex of the individual whose spread is being analyzed is known the program is constrained, just as is the cytogeneticist, to see whether a normal karyotype for that sex can be constructed; if the sex is unknown, the program attempts both and chooses the better. It is curious that although the strategy has been published for a number of years now, and although various groups have adopted the Bayesian statistic as the basis for "forced pairing" algorithms and even for attempts at automating analysis of banding patterns, no one has adopted the simple logical approach based on the cytogeneticist's strategy.

Though it had originally been proposed to accomplish the design, construction and testing of the complete semi-automatic chromosome analysis system within three years, this project has fallen behind schedule, and the test phase has only just begun. While possibly simple overoptimism was involved, several unforeseen factors account for at least the largest part of the delay. The Principal Investigator was on leave of absence for two of the first three years, and progress was slower than it would have been had he been in day-to-day supervision of the project. Furthermore, some unexpected hardware difficulties were encountered once assembly was

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begun. These resulted largely from the collapse of an overextended data processing industry, and the consequent decisions of both Corning and Sylvania to drop the equipment that had been purchased, thus depriving the project of the support it would normally have had from these manufacturers.

2. Radiation cytogenetics. The program in this area has evolved significantly during the course of this Contract and its predecessor (AT-(40-1)-4065). Originally, rather practical work on chromosomal aberration rates in peripheral leukocytes as a function of such factors as site and size of the treated area, dose fractionation and time of sampling post-irradiation in selected radiotherapy patients had been proposed. However, as a collaborative side venture with Dr. H. G. Griggs at Vanderbilt some work was also begun on cell killing and chromosomal aberration production by ultraviolet light, and the results of this work appeared so interesting and significant that pursuit of what started out as a side venture has become the major objective of the program.

Utilizing a tissue culture cell line derived from an amphibian (A8W243) and thus possessing a competent photoreactivation DNA repair system, we first showed that pyrimidine cyclobutane dimers were the ultraviolet-induced lesion responsible for loss of reproductive integrity (Griggs and Bender, 1972). Then, using the same cell line in order to similarly exploit the known specificity of the photoreactivating enzyme for the pyrimidine cyclobutane dimer, we investigated chromosomal aberration production by ultraviolet light. Not only did we find that virtually

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all aberration production was indeed photoreactivable thus showing that the dimer is the lesion responsible, but we also found, unexpectedly, that the aberrations induced in G_1 phase cells were, unlike the case for those induced by ionizing radiation and that generally believed to hold for ultraviolet light as well, of the chromatid rather than the chromosome type (Griggs and Bender, 1973). Recognizing that the fact that dimers induced within single polynucleotide chains in G_1 chromosomes had important implications for both theories of eukaryote chromosome structure and the involvement of both normal "semiconservative" DNA synthesis and DNA repair mechanisms in aberration production, we rapidly extended our observations of ultraviolet-induced aberration production to S and G_2 phase cells and to both first and second post-irradiation mitoses. We found (Bender, Griggs and Walker, 1973; COO-2382-7) that (exactly as had been reported by Swanson as early as 1940) UV administered during the G_2 phase of the cell cycle induced achromatic lesions and some chromatid deletions, but few if any isochromatid deletions or chromatid exchanges appeared in first post-irradiation mitoses. When G_2 -irradiated cells were examined in their second post irradiation mitosis, however, or when S-irradiated cells were examined in their first mitosis, chromatid aberrations of all types were again seen. In order to account for these observations, a model was constructed containing the following major elements:

- a) the eukaryote chromosome is mononeme
- b) single polynucleotide chain breaks may appear at metaphase as achromatic lesions or "gaps"
- c) dimer removal (excision) may leave unrepaired single chain gaps

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- d) single-stranded DNA opposite strand gaps may be cleaved by a single-strand DNase
- e) gaps are left in newly synthesized DNA polynucleotide chains opposite dimers or single strand breaks in the template strand.
- f) double-strand breaks present following local DNA replication may "spread" to the other chromatid, possibly through a recombinational process analogous to recombinational repair in prokaryotes.

Surprisingly rapid and complete confirmation of our ultraviolet results has recently been provided by Ikushima and Wolff (1974), though they believe their data are still consistent with a polyneme structure for the eukaryote chromosome. Such an interpretation, however, is hardly to be taken seriously, particularly in the light of more recent experiments.

In collaboration with Dr. J. S. Bedford at Vanderbilt aberrations resulting from another specific DNA lesion, the single polynucleotide chain break induced by photolysis of incorporated 5-bromodeoxyuridine was also investigated. Again, the lesion was induced in G_1 , S and G_2 cells and aberrations scored in both first and second post-treatment mitosis cells. The results were very similar to those obtained in the ultraviolet experiments, except that achromatic lesion production and chromatid deletion production in G_2 cells was relatively enormously more efficient (Bender,

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Bedford and Mitchell, 1973). The difference is interpreted as confirmation of the idea, originally proposed by Taylor Haut and Tung (1962), that achromatic lesions are manifestations of single polynucleotide strand breaks.

PROGRESS DURING PRESENT CONTRACT YEAR

1. Semi-automatic chromosome analysis device. Progress on this portion of the program, though less than anticipated, has been substantial. In last year's Proposal it was indicated that the device would be moved from Vanderbilt University to the Johns Hopkins University in the fall of 1973. This turned out to be overoptimistic, for a variety of reasons, and the device was not actually shipped until February 1974. The delays were caused largely by several hardware problems which, though relatively minor, took some time to resolve. However, the device is now installed and in operation at the Johns Hopkins. Routine analysis of the proposed test series of clinical cases is just beginning, in collaboration with Dr. J. M. Rary of the Department of Obstetrics and Gynecology, and a substantial number should be completed by the end of the present Contract year. Work on additional analytical Fortran subroutines has also been started, using the Johns Hopkins Hospital IBM 370 computer.

2. Mechanisms of chromosomal aberration production. Much of the past Contract year was devoted to theoretical development of a general theory of chromosomal aberration production based upon the special models developed to explain previous ultraviolet light and 5-bromodeoxyuridine photolysis results (as already outlined), but consistent with the available

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data on aberration production by ionizing radiation and by chemical agents. This effort is viewed as highly successful. The general model is described in detail in a manuscript in press in the journal Mutation Research (COO-2382-2), and will consequently not be presented in detail here. Briefly, it was found that the available ionizing radiation data was consistent with the idea that the principal lesion leading to aberrations in G_1 is the DNA double strand break, but that single polynucleotide strand breaks also contribute to aberration production during the S and G_2 phases. The previously puzzling phenomenon of half-chromatid exchange production by irradiation of cells during the prophase of meiosis or mitosis is explained as a consequence of the production of "hairpin" double-stranded regions within single polynucleotide strands as the chromosomes contract. Hairpin formation is possible within regions where a nucleotide sequence is repeated in reverse order and as its complement, as for example the sequence "ATGCTAGCAT". Such sequences, called (somewhat incorrectly) "palindromes" have recently been demonstrated to be a consistent feature of eukaryotic DNA (Thomas, et al., 1973).

It was found that the very large number of chemical agents reported to cause chromosomal aberrations could be sorted into four categories, according to their pattern of aberration production. A small group of compounds, typified by 8-ethoxycaffeine, have a pattern exactly like ionizing radiation (hence these are the truly radiomimetic compounds), producing chromosome types in G_1 , chromatid types in S and G_2 , and half

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chromatid exchanges in prophase. They are postulated to promptly produce DNA double strand scissions. Another relatively small group comprising compounds interfering with DNA synthesis and typified by the thymidine analog 5-fluorodeoxyuridine produces mainly achromatic lesions and chromatid deletions and only in S phase cells. These are postulated to act by causing gaps to be left in the nascent polynucleotide chains. The third group, including most active compounds and typified by, for example, nitrogen mustard, exhibit a pattern of aberration production exactly like that of ultraviolet light. The lesions they produce in DNA must, like the pyrimidine cyclobutane dimer, lead to "gap synthesis" during DNA replication. The fourth group includes miscellaneous compounds all of which probably interfere with enzymatic DNA repair enzymes. Typical are caffeine and chelating agents.

Theoretical work was also done on the possible consequences of true recombinational mode of post-replication repair in eukaryotes, resulting in a second paper, also in press in the journal Mutation Research (C00-2382-3). Briefly, it was realized that the existence of such a repair mode would explain several previously puzzling aspects of the segregation of newly synthesized DNA. Recombinational events occurring during the same cell cycle during which nascent DNA was labeled with tritiated thymidine would give rise to the "heterolabeling" phenomenon in autoradiographs of first post-labeling division cells, and to the "isolabeling" and "isonon-labeling" exceptional segregations in second division autoradiographs. Recombinational events occurring during the second cell cycle following

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labeling, however, would give rise to sister chromatid exchanges. This leads to the prediction that the stimulation of repair by radiation, for example, should have different consequences depending on whether the radiation was administered during the first or second cell cycle if the cells were all examined at the second post-labeling mitosis. Specifically, irradiation should increase the frequency of sister chromatid exchanges in second cycle cells, but not in first cycle cells, while it should increase the frequency of isolabeling, for example, during the second. It was gratifying to learn that data presently in press (Gatti, Pimpinelli and Olivieri, 1974) actually confirms these expectations!

During the past year laboratory experiments have also been carried out in several areas. Tissue culture cell lines have been obtained from Vanderbilt and cell cycle and mitotic synchrony parameters rechecked in the Johns Hopkins facility in preparation for the proposed investigation of the pattern of aberration production by the interstrand DNA crosslinking system 8-methoxypsoralin plus light. The investigation of aberration production by this specific lesion will be initiated during the remainder of the present Contract year, but not completed until next year, as outlined in the accompanying Renewal Proposal.

Work has continued on several collaborative projects with Drs. J. S. Bedford and H. G. Griggs at Vanderbilt University during the past year. Several autoradiographic experiments have been done at Vanderbilt to test whether chromosomes with ultraviolet-induced isochromatid deletions

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always also display either a proximal sister chromatid exchange or iso-labeling (depending on whether first or second post-labeling division cells are examined, of course), as is predicted by the hypothesis of their recombinational repair origin. Autoradiographs have been made at the Johns Hopkins and are presently being scored.

In a separate study, a test was made of the hypothesis that chromosomal aberrations lead directly to cell killing. In two separate experiments, cells were synchronized by mitotic shakeoff, X-irradiated in G_1 and then aliquots plated, fixed for cytogenetic scoring or again synchronized at the next division so as to provide samples again at each succeeding division. In this way the surviving fractions and the frequency of chromosomal aberrations were determined for cells that had gone through 0, 1, 2 or 3 successive post-irradiation mitoses. We reasoned that if any particular aberration type, as for example acentric fragments, were responsible directly for cell death, then as the population underwent successive divisions, the surviving fraction of those cells which had negotiated divisions should increase, and that this increase should be negatively correlated with a decrease in the frequency of that type of aberration. However, though the frequencies of all aberration types did decrease in the expected manner at each successive division, there was no detectable reciprocal increase in surviving fraction following the first mitosis. Because only cells in mitosis were selected each time, this could not have been because cells destined not to form colonies were simply delayed. Our results are reported in an abstract submitted for presentation at the 5th International Congress of Radiation Research in Seattle next July (C00-2382-5).

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In collaboration with Dr. J. M. Rary at the Johns Hopkins, a number of experiments have been done to test the chromosomal sensitivity of several genetically determined human diseases either reported to be DNA repair deficiencies or suspected to involve such deficiencies because of characteristic high spontaneous chromosomal aberration frequencies. Peripheral blood lymphocytes from patients with Fanconi's anemia and ataxia telangiectasia (Louis-Barr syndrome) have been X-irradiated in either G_1 or G_2 and aberration frequencies compared with those from appropriate controls. Though scoring of these experiments is as yet far from complete, it is already clear that cells from persons with both diseases display higher induced aberration frequencies than those from normal individuals. This confirms and extends the reports of others (Higuroshi and Conen, 1973; Sasaki and Tonomura, 1973). When both the G_1 and G_2 data are complete, analysis of the relative frequencies of aberrations of the various types in the light of the general chromosomal aberration theory should, it is hoped, provide clues as to which DNA repair mechanism (if any) is involved.

Similar G_2 irradiation experiments were carried out with cells from the same Hutchinson Gilford progeria patients reported by Epstein, Williams and Little (1973) to be DNA strand break repair deficient. It was reasoned that if achromatic lesion production by G_2 irradiation results from the production of single polynucleotide chain breaks then the patients' cells should be much more sensitive to their induction. Also, a strand break repair deficiency would be expected to be reflected in an increased

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spontaneous aberration frequency. Neither expectation proved true, however, and we are forced to conclude either that the general aberration theory is incorrect, or that progeria is not in fact strand break repair deficient. We believe the latter possibility to be the case (J. D. Regan, personal communication). These results have been submitted as an abstract for presentation at the 5th International Congress of Radiation Research also (C00-2382-4).

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DEVIATIONS FROM PROPOSED ACTIVITY LEVELS

None: the Principal Investigator has devoted 100% of his time and effort to work under this Contract during the past year.

PUBLICATIONS

Three papers were published during the past Contract period. "Uniformity of radiation-induced mutation rates among different species", by S. Abrahamson, M. A. Bender, A. D. Conger and S. Wolff, appeared in the journal NATURE. A reprint is submitted as Report No. COO-2382-6. Two papers, "Mechanisms of chromosomal aberration production. I. Aberration Induction by ultraviolet light", by M. A. Bender, H. G. Griggs and P. L. Walker, and "Mechanisms of chromosomal aberration production. II. Aberrations induced by 5-bromodeoxyuridine and visible light", by M. A. Bender, J. S. Bedford and J. B. Mitchell, appeared in the journal MUTATION RESEARCH. Reprints are submitted as Reports No. COO-2382-7 and COO-2382-8, respectively (It should be noted that preprints of these latter two papers were submitted with the Progress Report last year under the former Vanderbilt Contract AT-(40-1)-4065 as Reports No. ORO-4065-73-6 and ORO-4065-73-7, respectively).

Two abstracts for papers to be presented at the 5th International of Congress of Radiation Research were also submitted during the past Contract Period. "Spontaneous and x-ray-induced chromosomal aberrations in progeria", by M. A. Bender and J. M. Rary and "Chromosome aberrations and cell death in V79 Chinese hamster cells", by J. S. Bedford, H. G. Griggs, J. B. Mitchell and M. A. Bender are submitted as Reports No. COO-2382-4 and COO-2382-5, respectively.

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Finally, two manuscripts, "Mechanisms of chromosomal aberration production. III. Chemicals and ionizing radiation," by M. A. Bender, H. G. Griggs and J. S. Bedford, and "Recombinational DNA repair and sister chromatid exchanges," by M. A. Bender, H. G. Griggs and J. S. Bedford, have been accepted for publication in the journal MUTATION RESEARCH. Preprints are submitted as Reports No. COO-2382-2 and COO-2382-3, respectively.