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Cell phases during endoreduplication induced by Colcemid or radiation in cultured human lymphocytes

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CELL PHASES DURING ENDOREDUPPLICATION
INDUCED BY COLCEMID OR RADIATION
IN CULTURED HUMAN LYMPHOCYTES

A Thesis

Presented to

The Faculty of the Department of Biology
The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Master of Arts

by

Kenneth Eugene Weber

1979

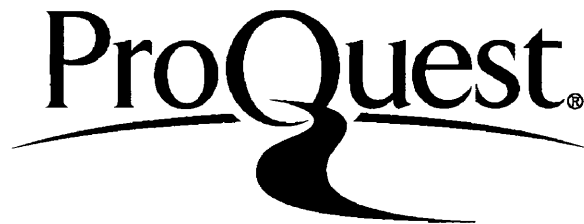
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APPROVAL SHEET

This thesis is submitted in partial fulfillment of
the requirements for the degree of


Master of Arts



Kenneth Eugene Weber

Approved, August 1979

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ABSTRACT

CELL PHASES DURING ENDOREDUPPLICATION INDUCED BY COLCEMID OR RADIATION IN CULTURED HUMAN LYMPHOCYTES

Endoreduplication was induced in human lymphocyte cultures by either (1) Colcemid at a final concentration of 10^{-4} M, or (2) 350R acute gamma radiation. Labeling with 5-bromodeoxyuridine (BUDR) and differential staining were used to reveal the timing of the phases of the endoreduplication cycle, relative to the time of application of the inducing agent. BUDR was added, and cultures were harvested, on several different schedules for each method of induction. Counts were made of the percentage of endoreduplicated metaphases which, at the different times of BUDR addition, had been in each of the distinguishable stages of the endoreduplication cycle.

The earliest endoreduplications to appear after Colcemid treatment had been in their normal G_2 at the time the agent was added. Endoreduplications which appeared at later times in continuous exposure to Colcemid had been in successively earlier stages of their normal cycle at the time of Colcemid addition. Palitti and Rizzoni (1972) and Rizzoni and Palitti (1973), studying colchicine-induced endoreduplication in Chinese hamster cells, concluded that vulnerability to endoreduplication was limited to those cells in

G_2 or very late S at the moment of colchicine addition, and that cells in earlier stages acquired a resistance or inability to respond to the inducer by the time they reached G_2 . Under the conditions of these experiments with Colcemid, however, cells which had been at periods earlier than G_2 were not excluded from the yield of endoreduplicated cells, but merely appeared at later times.

Endoreduplication induced by radiation showed a similar pattern of expression. At first, only cells which had been irradiated in their normal G_2 appeared as endoreduplications. Later, endoreduplications which had been irradiated in earlier stages appeared. The stage of endoreduplicating cells at the time of irradiation was demonstrable not only by BUdR-labeling patterns, but also by the types of chromosomal damage sustained. Both types of data make it clear that human lymphocytes in a growth mode, irradiated in any subdivision of interphase, may be subject to endoreduplication at a later time. These results differ from those obtained with Chinese hamster cells by Yu and Sinclair (1972), who showed that endoreduplications could only be induced by radiation during G_1 in their system.

Thus, human lymphocytes react differently than would have been predicted for either of these agents on the basis of work with other cells. The effects of radiation and Colcemid on human lymphocytes are similar in the lack of phase-specificity for endoreduplication induction. However, the responses of human lymphocytes to the

two agents can be distinguished. A period of transient inhibition occurs for lymphocytes induced to endoreduplicate by radiation during the S-phase. No such inhibition occurs in Colcemid-induced endoreduplications. This finding is in accord with other work on normal diploid cells.

CELL PHASES DURING ENDOREDUPLICATION
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CHAPTER 1

INTRODUCTORY REVIEW

The term "endoreduplication" may be loosely defined as the replication of chromosomes more than once in a single cell, when the resulting replicate chromosomes remain in contiguous coalignment at the succeeding metaphase. The original definition of the term (Levan & Hauschka, 1953), still routinely cited whenever it seems to apply, added to this condition of coalignment the restriction that no abortive preliminary mitotic activity appear before the extra replication. For cells polyploidising via an abortive phase of chromosome condensation, the earlier term, "endomitosis" (Geitler, 1953), was reserved. The wording of the original definition makes this second qualification the primary characteristic of endoreduplication.

Endoreduplication in the full original sense, thus, refers to a metaphase condition and an interphase process at the same time. To an extent, the term now takes on one or the other meaning exclusively as used in different contexts. For example, it is usually only the condition of coalignment that is considered when the term is applied to metaphases displaying the orderly pairing of sister chromosomes in diplochromosomes, or of sister diplochromosomes in quadruplochromosomes. Intermediate conditions are rarely found between this orderly arrangement and its alternative, the totally

random positioning of chromosomes in most polyploid metaphase spreads. Therefore, the term is employed here for convenience, to make a distinction based on metaphase morphology alone, even though it has never been rigorously demonstrated that all metaphases with this distinctive orderly chromosome arrangement do result via the same route, or that no preliminary mitotic mechanisms such as a transient condensation of chromatin are ever involved. In one study, monolayer cultures subjected to mitotic shake-off during endoreduplication showed a higher proportion of endoreduplicated metaphases, suggesting that no rounding-up occurred during interphase (Sutou & Shindo, 1975). Another study suggests that cross-attachment patterns observed in diplochromosomes would be less likely if there were any condensation of chromatin in interphase (Sutou & Tokuyama, 1974). No other evidence on this point exists to justify the most common usage.

At the other extreme of usage, the term "endoreduplication" has been made equivalent to endopolyploidisation--i.e., polyploidisation not resulting from fusion or refusion of nuclei (Mittwoch, Lele, & Webster, 1965)--with the assumption that endopolyploids characteristically display ordered pairing at the first metaphase, and that random polyploids are typically derived from an earlier orderly stage. But, although this has been assumed in many cases, it has never been shown that random polyploidy cannot arise directly from an interphase condition, and in cases to be discussed it seems likely that it does. Therefore, in mammalian cytogenetics at present, endoreduplication must be regarded as a provisional dual

term for a condition and a process which are often presumed to be inseparably linked and unitary.

In other areas of cytogenetics, this linkage scarcely exists, and the terms "endoreduplication" and "endopolyploidisation" are used interchangeably without implying anything about the arrangement of chromosomes at metaphase. Endoreduplication (or endoreplication) designates the process and endopolyploidy the condition in regard to all internal doubling. The repeated replications without division which lead to polytenisation and polyploidy in states of terminal cellular differentiation of insects and plants are termed "endoreduplications" (Nagl, 1976). Here there is no question of metaphase morphology since there is no metaphase and one arrives at a sense which is almost the opposite of the way the term is used when, for example, de novo random polyploids are distinguished from "endoreduplications" in papers on mammalian cells.

Descriptions of diplochromosomal pairing go back as early as 1910 (Stomps, 1910). Since the term "endoreduplication" was introduced, its original two-part definition has created an element of uncertainty in every instance of its use, and the various ad hoc extensions of the term since 1953 have made of it an ambiguous category. This paper will adhere to the usage in which endoreduplication means any process producing or assumed to produce diplochromosomes (or quadruplochromosomes), usually in mammalian cells, and exclusively as an abnormal process.

The phenomenon of diplochromosome formation always involves a doubling or redoubling of chromosome and centromere number.

Triploid, or other odd multiples are never found, nor cases of partial endoreduplication, with possible rare exceptions to be discussed. The number of primary entities remains constant at the stemline ($2n$) number, as sister elements remain associated at the centromeres to form diplochromosomes of four chromatids or, after double endoreduplication, quadruplochromosomes of eight chromatids. There have been two cases of octoplochromosomes reported (and pictured) in mouse and Chinese hamster cells, respectively (Levan & Hus, 1961; Yu, 1964), and spreads with $4n$ diplochromosomes as well (Schwarzacher & Schnedl, 1966; the present report).

It has been suggested that endoreduplication might represent a premature separation of chromatid subelements which achieve autonomy without the replication of DNA (Schrader & Hughes-Schrader, 1958). However, microspectrophotometric mass measurements of DNA in human leukocyte metaphases have shown that endoreduplications do contain about twice the DNA of diploid cells (Bell, 1964; Jackson & Killander, 1964) and are, thus, apparently true polyploids.

Cases have been reported of partial or selective endoreduplications of single chromosomes (de Grouchy, de Nava, Bilski-Pasquier, Zittoun, & Bernardou, 1967), or parts of chromosomes (Lejuene, Berger, & Rethore, 1966; Lejuene, Dutrillaux, Lafourcade, Berger, Abonyi, & Rethore, 1968), and of several chromosomes (Drets, Cardosa, Delfino, & Carrau, 1970; Erdogan, Aksoy, & Dincol, 1967; Houston, Levin, & Ritzmann, 1964), usually as spontaneous anomalies recurring at low frequencies in leukocyte cultures from particular probands. Cases involving regular recurrence can probably all be

alternatively interpreted as malsegregation of sticky chromosomes or fragments involving fragile sites (Sutherland, 1979). A case of a frequent triradial number 2 chromosome attributed to selective endoreduplication of the long arm was recently proved by 5-bromodeoxyuridine (BU DR) labeling to result from fragmentary non-disjunction instead of partial endoreduplication (Noel, Quack, Mottet, Nantois, & Dutrillaux, 1977). Examples of sporadic, non-recurring partial endoreduplication, as pictured, cannot be distinguished from full endoreduplications in which some randomization is accompanied by partial loss of the chromosome complement in slide-making, a frequent artifact (Erdogan et al.; Houston et al.). In light of these facts, it is necessary to conclude that as yet diplochromosomes are only known certainly to occur either in all chromosomes of a cell, or none.

Studies with tritiated thymidine show that endoreduplication occurs in two distinct S-phases, S_1 and S_2 (Schwarzacher & Schnedl, 1965; Walen, 1965). Both S-phases replicate the genome in the same order, as far as can be determined. Late replicating regions are the same in S_1 and S_2 as in normal diploid cells (Schwarzacher & Schnedl). Also, the characteristic visible morphological features of each chromosome are exactly reproduced (Schnedl, 1967; Schwarzacher & Schnedl). The incorporation and semiconservative segregation of tritiated thymidine over the two S-phases is the same as for two generations of diploid cells, with the important qualification that new DNA is always oriented to the outside with respect to centromeres

(Herrerros & Giannelli, 1967; Schnedl; Schwarzacher & Schnedl, 1966; Walen, 1965). Thus, label which is incorporated at any time during S_1 appears only on the outer two chromatids of the four, while label incorporated during S_2 appears on all four. This symmetric pattern of S_1 labeling may be lost occasionally when one sister chromosome of a pair appears reversed, and extremely rarely both chromosomes are reversed (Herrerros & Giannelli; Walen). These exceptions have been interpreted as artifacts occurring during cell spreading in slide preparation. If a doubly endoreduplicated cell is labeled in the first of its three S-phases, the resulting quadruplochromosomes are labeled on the inner chromatids of the outermost chromosomes (Herrerros & Giannelli).

What determines and preserves this characteristic orientation of chromatids is unknown. Various authors have suggested delayed division at the centromere relative to the rest of the chromosome (Schnedl, 1967; Schwarzacher & Schnedl, 1966; Walen, 1965) or linking protein strands between original chromatin (Schwarzacher & Schnedl). That the centromere is not alone responsible, however, is shown by the important finding of endoreduplicated acentric fragments with the same pattern of positioning of tritium-labeled chromatids (Herrerros & Giannelli, 1967). This and other observations of unlabeled endoreduplicated acentric fragments (Sutou & Tokuyama, 1974) and even doubly endoreduplicated acentric fragments (Bell & Baker, 1965) show further that the association between sister elements is preserved by some diffuse connection between the chromatid arms

(see also Fig. 4, this report). Still, in endoreduplicated metaphases in which chromatids are more widely separated, a relatively more durable connection sometimes appears to persist between sister centromeres.

The pattern of S_1 -labeled material to the outside can also be perturbed by the normal occurrence of sister chromatid exchanges. Exchanges occurring in the first replication appear as mirror-symmetric twin exchanges, at the same point in both chromosomes of a diplochromosome. Exchanges appearing in the second replication appear as single exchanges on one chromosome or the other. If the paired subunits, of which a single chromatid is evidently constructed, had no polarity restriction on the way they could break and rejoin, then half the sister chromatid exchanges in the first generation would disappear in the second on one chromosome or the other (Taylor, 1958). A ratio of two twin exchanges to one single exchange would, thus, support the idea of subchromatid polarity restrictions. This ratio was already indicated by careful work with tritium-labeled random tetraploids (Taylor), and has been confirmed with endoreduplications labeled with tritium (Herrerros & Giannelli, 1967) and BU DR (Dutrillaux, 1976). (The concept of the polar subchromatid includes the possibility that it is a single polynucleotide strand.)

Chromatid interchanges between sister chromosomes within diplochromosomes have been reported, as well as arch fusions between diplochromosomal chromatids, and various reports have mentioned all the diplochromosomal versions of typical radiation-induced

rearrangements found in diploid cells, including rings, dicentrics, minutes, and interdiplochromosomal G_2 -type rearrangements (Gatti, Rizzoni, Palitti, & Olivieri, 1973; Ikushima & Wolff, 1974; Sasaki, 1977; Sutou, 1973; Sutou & Tokuyama, 1974). In one report (Yu, 1964), longitudinally symmetric multicentric polyploid isochromosomes were repeatedly induced by heavy doses of X-irradiation, and a role in their formation was attributed to endoreduplication.

Endoreduplications have been induced experimentally by a wide variety of agents and treatments, and in some material they appear spontaneously at appreciable rates, or their numbers can be enhanced by modified culture conditions. Random tetraploids seem to appear also in all systems in which endoreduplications are found, although the converse is probably not true.

In human lymphocytes cultured with phytohemagglutinin, endoreduplication has been induced by X-irradiation at various doses and on various schedules (Bell, 1964; Bell & Baker, 1962, 1965; Jackson & Hill, 1967; Nasjleti & Spencer, 1968, Nasjleti, Walden, & Spencer, 1966), and also by gamma irradiation (Ohnuki, Awa, & Pomerat, 1961). Endoreduplication has also been induced in Chinese hamster cells by X-irradiation (Yu, 1964; Yu & Sinclair, 1964, 1972) and by ultra-violet (Okigaki & Rounds, 1972). Low levels of endoreduplication have been found in lymphocyte cultures after X-ray therapy (Friedman, Saenger, & Kreindler, 1964; Gripenberg, 1967; Kucerova, 1970). The majority of these reports only mention endoreduplication in the context of general radiation-induced chromosomal aberrations

and polyploidy, but two (Bell & Baker, 1965; Jackson & Hill) focus particularly on the induction of endoreduplication by radiation.

Among chemical inducers of endoreduplication, the most well-defined category may be the sulfhydryl compounds. The chemical most frequently used has been β -mercaptoethanol (Jackson, 1963; Jackson & Killander, 1964; Sasaki, 1977; Schnedl, 1967) or α -mercaptoethanol (Schwarzacher & Schnedl, 1966). Other sulfhydryls used include cysteamine (Jackson & Hill, 1967; Jackson & Lindahl-Kiessling, 1964) and the natural metabolites β -mercaptopyruvate (Jackson & Lindahl-Kiessling, 1963, 1964) and L-cysteine (Jackson & Lindahl-Kiessling, 1964; Sutou & Arai, 1975) and L-cystine (Sutou & Arai). Several chemicals which react with sulfhydryls are inducers by themselves, e.g., captan (Sutou & Tokuyama, 1974) and 4-nitroquinoline-1-oxide (4NQO) (Sutou, 1973; Sutou & Arai; Sutou & Tokuyama). It has been found that L-cysteine and 4NQO can counteract the effect of each other in the induction of endoreduplication (Sutou & Arai). However, when the sulfhydryl cysteamine (which may be the most effective radioprotective agent known against other effects) was used in various combinations with X-irradiation, the effects of the combined agents on frequency of polyploidy were found to be additive and perhaps synergistic (Jackson & Hill). Most of the cited work with sulfhydryls was done with human lymphocytes, primary human fibroblasts (Sasaki, 1977), or an established Chinese hamster fibroblast line (Sutou; Sutou & Arai; Sutou & Tokuyama).

A second broad category of chemicals which can induce endoreduplication comprises chemicals which directly affect DNA in

various ways. For example, a number of base analogues have been found to induce endoreduplication, among them BUDR (Dutrillaux, Fosse, Prieur, & Lejeune, 1974) and 6-mercaptopurine (Nasjleti & Spencer, 1966) in human lymphocytes; cytosine arabinoside in Chinese hamster cells (Sutou & Arai, 1975); and 8-azaguanine in pea root meristems (Nuti Ronchi, Avanzi, & D'Amato, 1965). The DNA-specific dyes acridine orange and acridine yellow (Sutou & Tokuyama, 1974) and 33258-Hoechst (Kusyk & Hsu, 1979) were found to be inducers in Chinese hamster cells. Actinomycin D, which binds to DNA, was an inducer in Indian muntjac cells (Pathak, McGill, & Hsu, 1975), but not in Chinese hamster cells (Palitti, Ricordy, Perticone, D'Andrea, & Rizzoni, 1976). Belonging in the same broad category may be miscellaneous mutagens such as 4NQO (as mentioned) which also binds to DNA; hydroxylamine sulfate (Lin & Walden, 1974) which hydroxylates cytosine residues in DNA (Freese, 1971); Cytosin (Sutou & Tokuyama); and sodium nitrite (NaNO_2) (Tsuda & Kato, 1977), known to produce lymphoma in rats (Newberne, 1979). These chemicals all act in different ways. For example, the acridine dyes are also point mutagens and one of the base analogues mentioned is also a sulfhydryl (6-mercaptopurine). Also, some potent mutagens have failed to induce endoreduplication (Sutou & Tokuyama).

Treatments which cause gross chromosome aberrations, such as breaks and translocations, quite often induce random polyploidy and endoreduplication as well. In this category belong not only some of the mutagens mentioned, but also various anticancer

chemotherapeutics, including N, N'-bis-(3-bromopropionyl) piperazine (Nasjleti, Walden, & Spencer, 1965), nitrogen mustard (Nasjleti & Spencer, 1966), streptonigrin, and cyclophosphamide (Nasjleti & Spencer, 1967). It might be expected that most clastogens and radio-mimetic drugs would produce some endoreduplication.

Endoreduplications have been induced by the mitotic poisons colchicine (Palitti et al., 1976; Palitti & Rizzoni, 1972; Rizzoni & Palitti, 1973), Colcemid (Herrerros & Giannelli, 1967; Herrerros, Guerro, & Romo, 1966; Hux & Tegenkamp, 1975; Ikushima & Wolff, 1974), and, also, by the plant lectins phytohemagglutinin (Sutou & Arai, 1975) and concanavalin-A (Sutou & Shindo, 1975). Prolonged treatments of various established cell lines at temperatures a few degrees above 0 C have produced endoreduplications (Cerny, Baudysava, & Holeckova, 1965; Hampel & Levan, 1964). Endoreduplicated bivalents consisting of synapsed homologous diplochromosomes have been reported after heat disturbance of meiosis in pollen mother cells of Fritillaria meleagris (Barber, 1940). Several studies have identified specific culture conditions tending to increase endoreduplication frequency in Chinese hamster cells, including refeeding in the plateau phase and growth in stoppered vs. 5% CO₂-buffered flasks (Gatti & Olivieri, 1976; Gatti, Pecci, & Olivieri, 1976). Many spontaneous cases have been reported in tumor cells (e.g., Ising & Levan, 1957) and in cultured lymphocytes of cancer patients and patients with hormonal imbalance, e.g., testicular feminisation (Aspillaga, Neu, & Gardner, 1964). Thyroxine has induced

endoreduplication in lymphocytes in vitro (Bishun, Morton, & Rashad, 1964).

Much variability in frequency has been noted in studies of endoreduplication. Significant differences were reported between the susceptibilities of lymphocytes from different individuals to BUDR (Dutrillaux et al., 1974), radiation (Bell & Baker, 1965; Ohnuki et al., 1961), and β -mercaptoethanol (Jackson, 1963). In various reports, instances may be discovered of widely fluctuating yields of endoreduplication even with established cell lines under similar test conditions (Hampel & Levan, 1964; Sutou, 1972; Walen, 1965) or of variable results at different times with lymphocytes from the same individual under similar test conditions (Bell & Baker). Particular mention was made in one report of wide variation between yields in parallel cultures from the same biopsy (Schwarzacher & Schnedl, 1965).

As yet, few definite statements can be made about the biochemical and ultrastructural mechanisms for the induction of endoreduplication. The chemical activities of individual inducers are manifold, so that no common avenue of action emerges. Several hypotheses have been proposed.

The disruption of spindle fibers has been suggested as an important factor (Jackson & Hill, 1967; Jackson & Lindahl-Kiessling, 1963). The threshold concentration of colchicine required to completely suppress mitosis is near the threshold required to induce endoreduplications (Rizzoni & Palitti, 1973; Sutou & Arai, 1975). But, several authors have shown that S_2 can begin earlier in

interphase than the construction of the spindle apparatus. The disruption of interphase cytoskeletal microtubules is therefore a possible cause (Rizzoni & Palitti; Sutou & Arai).

The cell membranes has also been proposed as a site of initiation of endoreduplication (Sutou & Arai, 1975). In support of this, it has been shown that several lectins previously mentioned are inducers, with the effect of concanavalin-A reversed by α -methyl-mannoside and enhanced by trypsinization (Sutou & Arai). Colcemid and colchicine, as well as the acridine dyes, do bind to membranes and can be related to this hypothesis, but not all other inducers (see Sutou & Arai for references and discussion).

The centriole has frequently been mentioned in connection with endoreduplication as a possible regulatory site of nuclear synthesis and division (Howell, Hsu, & Block, 1977; Jackson, 1963; Jackson & Hill, 1967; Jackson & Killander, 1964; Kusyck & Hsu, 1979; Schmid, 1965). If the replication of the centriole is delayed or disturbed, the synchrony of the two cycles--centriolar and nuclear--might not be restored until after a supernumerary replication. High frequencies of multipolar mitoses (tripolar, tetrapolar, and even pentapolar) have been observed in endoreduplications both spontaneous (Schmid) and induced (Gatti et al., 1976; Palitti & Rizzoni, 1972), terminated without Colcemid. Of course, the centriole is not a candidate as a target in the induction of endoreduplication in higher plants (Lin & Walden, 1974; Nuti Ronchi et al, 1965).

As mentioned, several Chinese hamster strains were found to yield high frequencies of spontaneous endoreduplications under

particular culture conditions (Gatti & Olivieri, 1976; Gatti et al., 1976). These cells had the capacity to accumulate in G_2 at the plateau stage, rather than G_1 , which is atypical but not unique. This tendency could be greatly enhanced by refeeding cells in the plateau phase and delaying subculture for about 3 more days. Cells in G_2 were then likely to undergo a second round of DNA synthesis after subculture, leading to diplochromosome formation. During the interval in the plateau phase, the refeed cells not only tended to pass through S, but also subsequently continued to grow in quantity of protein per cell. Plateau growth in a sealed atmosphere, without CO_2 buffering, enhanced both the amount of protein per cell and the yield of endoreduplications appearing in subcultures. What is especially interesting is that a minimum interval of 48 hours between refeeding and subculturing was required to produce endoreduplications, and their appearance as this interval was varied was fairly abrupt within a limited range. All this suggests a mechanism for the initiation of DNA synthesis based on the cytoplasm/nucleus ratio with a rather distinct threshold.

In work with Zea mays root tips and hydroxylamine sulfate (Lin & Walden, 1974), endoreduplication has been attributed to interaction of the drug with a "transition factor" or control protein, which functions to stop replication as it reaches the end of a replicon or genome while the cell is in a DNA-synthetic mode. To the extent that other considerations may implicate some initiating event in synthesis, this model may be unparsimonious, and the idea of a

replicon-by-replicon system which can be controlled from outside the system seems excluded by the apparent restriction of endoreduplication to integral multiples of the genome. The induction of endoreduplication by azaguanine in Pisum sativum root meristem (Nutti Ronchi et al., 1964) demonstrates an induction system in which the initiation (but not the continuation) of normal DNA synthesis is blocked for the duration of the treatment so that growing cells are all ultimately 2c or 4c. The initiation of mitosis is also blocked. The extra synthesis takes place after the drug is removed. In this system, the relative numbers of endoreduplicated metaphases and their positions in the root tip indicate that the cells which endoreduplicate may be limited to those which were exiting from the dividing line into a differentiated, nondividing state. This type of endoreduplication may be a special case.

The foregoing touches on the main ideas which have been raised in the literature with regard to the mechanism of endoreduplication induction. Several additional points might be suggested, however. To the knowledge of the writer, the possible indirect role of sulfhydryls in the action of ribonucleotide reductase has not been considered. The source of reducing power in the generation of deoxyribonucleotides from ribonucleotides in vivo is unknown. Thioredoxin and glutathione, two small sulfhydryl polypeptides, play a role in vitro. These two hydrogen donors must in turn be reduced (White, Handler, Smith, Hill, & Lehman, 1978).

On another line of thought, references can be found linking various inducers of endoreduplications which are otherwise chemically

dissimilar to a common effect in the localized despiralization of chromosomes. Despiralization, or enhancement of secondary constrictions, has been noted as an effect of Colcemid (Zakharov & Egolina, 1968), Actinomycin D (Arrighi & Hsu, 1965; Pathak et al., 1975; Viegas-Pequignot & Dutrillaux, 1976), Cytosan (Hsu, Pathak, & Shafer, 1973), various base analogues including 5-bromodeoxycytidine (Zakharov, Baranovskaya, Ibraimov, Benjusch, Demintseva, & Oblapenko, 1974), and BUDR (Viegas-Pequignot & Dutrillaux; Zakharov et al.). These effects, which depend on proper dose and timing of administration, may indicate that small packaging defects in replicated chromatin, induced by various mechanisms (even by radiation), can trigger a new round of synthesis by putting chromatin into an S-phase level of condensation, or keeping it there. This can be related to studies of premature chromosome condensation in S-phase (Rao, Wilson, & Puck, 1977).

A full explanation of endoreduplication must ultimately explain random tetraploidy as well, and the relationship between the two phenomena. It has been proposed that endoreduplication represents the first mitosis after the induction of polyploidy, and that cells with randomly oriented chromosomes are descended from these (Mittwoch et al., 1965; Schwarzacher & Schnedl, 1965). A study of "spontaneous" endoreduplication found no statistical correlation between the numbers of endoreduplications and random tetraploids appearing simultaneously under varied conditions, but did find that when a wave of endoreduplication appeared, it was followed by an increased frequency of random tetraploids (Gatti et al., 1976).

Similarly, in cultures polyploidized by cold treatment (Cerny et al., 1965) and radiation (Bell & Baker, 1962; Yu & Sinclair, 1964), a high relative frequency of endoreduplication appeared first after treatment, but was ultimately replaced almost entirely by random tetraploids. In some systems, random tetraploids and endoreduplication first appear simultaneously with polyploid metaphases becoming mainly random thereafter (Palitti & Rizzoni, 1972).

But, opposing results have been obtained. In one study, the appearance of radiation-induced random tetraploids regularly preceded that of endoreduplications leading the authors (Bell & Baker, 1965) to reverse their own earlier conclusions. Apparently, random tetraploids were being induced directly and de novo from interphase cells. It still appeared that these endoreduplications later gave rise to random tetraploid descendants, however. A reversed order of first appearance was also noted in lymphocyte cultures treated with β -mercaptoethanol (Jackson, 1963; Jackson & Lindahl-Kiessling, 1964); at first all polyploids were random, but the percent of endoreduplications slowly increased with time. Limited observations, without mitotic poisons, of diplochromosomes at anaphase indicate that randomization occurs during division. Whole chromosomes are sometimes seen to move in opposite directions, but only at the beginning of anaphase (Levan & Hauschka, 1953; Nuti Ronchi et al., 1965), and no association between chromatids persists through anaphase (Schwarzacher & Schnedl, 1965).

The time required for the various phases of endoreduplication has been a subject of several investigations. Most of the extra

time required is apparently taken up by synthesis in the second round according to several labeling studies (Lin & Walden, 1974; Rizzoni & Palitti, 1973; Sutou & Tokuyama, 1974). In Chinese hamster cells, there is a minimum interval between S_1 and S_2 of about 3 hours, and a maximum of perhaps 7 hours (Rizzoni & Palitti; Sutou & Arai, 1975; Sutou & Tokuyama). S_1 appears to be a normal S-phase, but S_2 may take twice as long (Schwarzacher & Schnedl, 1965), or even longer than a whole normal cycle (Rizzoni & Palitti). In human lymphocytes, up to several days have been required for some cells to endoreduplicate, with a wide variation in times (Herrerros & Giannelli, 1967; Schnedl, 1967; Schwarzacher & Schnedl, 1966). A study of cultured human lymphocytes claimed to have found endoreduplicated metaphases produced by Colcemid within a mere 2 hours of treatment time (Hux & Tegenkamp, 1975), but this report is hardly to be taken seriously.

The point in the normal cell cycle at which certain agents can induce endoreduplication has been found to be variously restricted in studies to date. Root tip cells of Zea mays, pulsed with hydroxylamine sulfate, only undergo endoreduplication if they are in S at the time of treatment (Lin & Walden, 1974). It has been reported that Chinese hamster cells of a particular line (CHEF-125) will not endoreduplicate in response to colchicine unless they are in late S or G_2 at the moment they first encounter the agent (Palitti & Rizzoni, 1972; Rizzoni & Palitti, 1973). Exposure to colchicine before late S/ G_2 prevents these cells from endoreduplicating later.

In contrast, it has been demonstrated for Chinese hamster cells of a different line (V79-S171) (Yu & Sinclair, 1972) that the induction of endoreduplication by X-radiation is strictly limited to cells irradiated in G_1 .

The present study was undertaken to investigate the timing of the development of diplochromosomes in human lymphocytes. Radiation and Colcemid were used as inducing agents. The progression of cell phases was traced by the recently developed technique of BU DR-labeling with sister chromatid differentiation (Bibliography, Appendix B). Some preliminary experimentation with the variety of protocols now available was required. The simplified system outlined here (Appendix C) is one of the minor results of this study. The logic of BU DR differentiation is rather simple: DNA with one or both strands unsubstituted is dark, and DNA with both strands substituted is pale. The already classic progression of diploid labeling patterns (Dutrillaux & Fosse, 1976) may be seen in Appendix D (as well as a new advance in intermediate staining). The chemical mechanisms which are responsible for this differentiation are not yet fully agreed upon.

The rationale of the present experiments was to reconstruct the sequence of events in culture, under a given set of conditions, by growing simultaneous cultures under identical conditions and labeling each with BU DR at a different time. The labeling patterns of diplochromosomes reveal the part of the cell cycle during which label was administered (Fig. 1). There were six series of experimental

cultures grown, each involving the action of either Colcemid or radiation. In all, the inducing agent was administered at 48 hours. In the first four, BUdR was added at different times and the cultures were terminated either early (2 series) or late (2 series). In the last two, BUdR was added to all cultures simultaneously with the inducing treatment at 48 hours, and cultures were terminated sequentially.

CHAPTER 2

MATERIALS AND METHODS

In each experiment, a set of simultaneous cultures, undergoing identical treatment, was used to reveal the sequence of events in endoreduplication by the addition of BU DR (to 20 $\mu\text{g}/\text{ml}$ final concentration) to individual cultures at increasing intervals of time after initiation. Cells were cultured in Ham's F10 with 20% fetal calf serum, phytohaemagglutinin, and penicillin-streptomycin. Blood from the same male donor was used in all experiments. About 0.4 ml of plasma, including some erythrocytes, was added to 5 ml of medium in 30 ml plastic culture flasks. Cultures were kept tightly capped at 37 C with several complete resuspensions daily.

Endoreduplications were induced by either (1) Colcemid at a final concentration of 10^{-4} M, or (2) Cesium-137 gamma radiation with an exposed dose of 350 Roentgens. The radiation system, utilizing a cast lead shield fitted to receive a culture flask, was calibrated at a dose rate of 0.6 Roentgens/second by thermoluminescent dosimetry. Colcemid or radiation was always administered at 48 hours after culture initiation. The radiation was given in a single dose requiring about 10 minutes per culture. Since a cumulative time of more than an hour was thus required to irradiate all cultures within a set, the serial irradiation of cultures was begun ahead of the nominal 48-hour treatment time, so that the culture labeled with BU DR

at 48 hours was in fact irradiated at that time, while cultures labeled at times shortly before or after were irradiated within 10 minutes or 20 minutes of the nominal irradiation time. In the radiation-treated cultures, Colcemid was used to collect cells in metaphase during the final hour of culture. In the Colcemid-treated cultures, the Colcemid remained in the medium until termination.

Simultaneous termination of cultures within a series was initiated by the rapid injection of distilled water at 37 C into each culture flask to a 3:1 dilution of the medium. After 6 minutes, the cells were concentrated by centrifugation and fixed in 3:1 methanol-acetic acid. Air- or flame-dried slides were placed for 1 hour in 20 $\mu\text{g/ml}$ 33258 Hoechst in distilled water; irradiated with UV (254 nm) for 1 hour while lying face-up, without coverslips, under 2XSSC in a white enameled pan; placed in 2XSSC at 75 C for 15 minutes; and transferred to warm tap water before staining. Separate stock solutions of 0.1% Eosine Y and 0.1% Azure B, in 0.05M NaH_2PO_4 adjusted to pH 6.8, were then diluted for immediate use with the same buffer in the volume ratio of 2:5:30, respectively, with a staining time of 5.5 minutes. Staining techniques for sister chromatid differentiation are outlined in more detail in Appendix C.

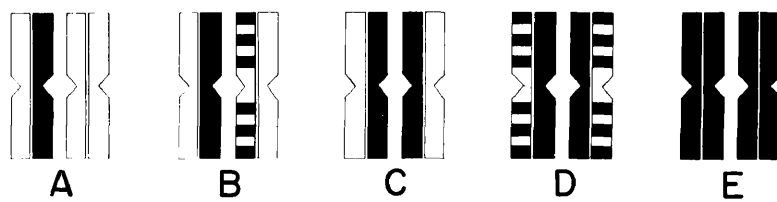
CHAPTER 3

RESULTS

In total, approximately 1,000 endoreduplicated metaphases were analysed and scored. The slide coordinates of all endoreduplications were recorded for reference (data not included in this report). A progression of different staining patterns appeared in diplochromosomes as individual cultures were labeled with BUdR at advancing intervals during the process of endoreduplication. The progressive BUdR-labeling patterns of normal diploid cells, published elsewhere (Dutrillaux & Fosse, 1976) and in Appendix D are combined symmetrically in diplochromosomes. The original DNA of diplochromosomes lies in the two inner chromatids, while the DNA made in the first S-phase lies in the two outer chromatids. In all four chromatids, this DNA is paired with DNA made in the second S-phase. Wherever these combinations give complementary labeled strands in the same double helix, pale staining appears. The main distinguishable staining patterns are diagrammed and explained in Fig. 1.

Cells of type E in Fig. 1 were labeled after S_1 . Some variability in this category exists, since faint differentiation may occur between areas which are single-stranded in labeled DNA and areas which are not. This differentiation is obscured by the faint R-banding which it often resembles, and which is induced even in unlabeled spreads, to some extent, by the treatments involved in

Fig. 1. Diagram of major BUdR-labeling patterns of diplochromosomes--types A, B, C, D, and E. These were the five main labeling patterns found in cultures. Pattern A results when the endoreduplicating cell and its parent cell undergo all (three) replications in the continuous presence of BUdR. When the label was introduced during the S-phase of the parent cell, Pattern B results. Pattern C indicates label was introduced later, but before the beginning of the first S-phase in the endoreduplicating cell. Pattern D indicates labeling sometime during the first S-phase of endoreduplication. Pattern E results when label is introduced after the first S-phase. These diagrams show the pattern of staining as it actually appears on the slide. The photos of chromosomes in this report are negative images.



differentiation. Hence, more accurate subdivisions of the post- S_1 period are not reliably distinguishable by these techniques. Cells of type D, however, display a sharp contrast between light and dark areas with very little ambiguity in any part. These cells can be assigned not only to a particular phase (S_1), but can also be given a relative position in S_1 , from "very early" to "very late." These assessments were recorded for each cell, but only summary statements are included in this report. Cells of type C are absolutely undifferentiable from each other by labeling pattern, and can indicate labeling at any time during G_1 of the endoreduplicating cell, or G_2 or M of its precursor. The three patterns--C, D, and E--represent the sequence of principal distinguishable phases within the endoreduplicating cell itself, and comprise the vast majority of endoreduplications which were found. Only these categories were graphed, but cells of types A and B are included in the tables and treated in the discussion.

The series of photographs (Fig. 2 through Fig. 18) includes the aforementioned and various other patterns of labeling, with explanations. These prints were made from positive transparencies so that the shades are reversed from the appearance of the cells on the slide. This corresponds exactly to the appearance of BUdR differentiation when it is visualized with UV-fluorescent staining and reproduced with black and white photography. By this method, some details are seen with more clarity, and others with less, than in the positive image. It is impossible to get all chromosomes in

Fig. 2. Endoreduplication, unlabeled.



Fig. 3. Double endoreduplication, unlabeled. Focus is averaged over these thick groupings.

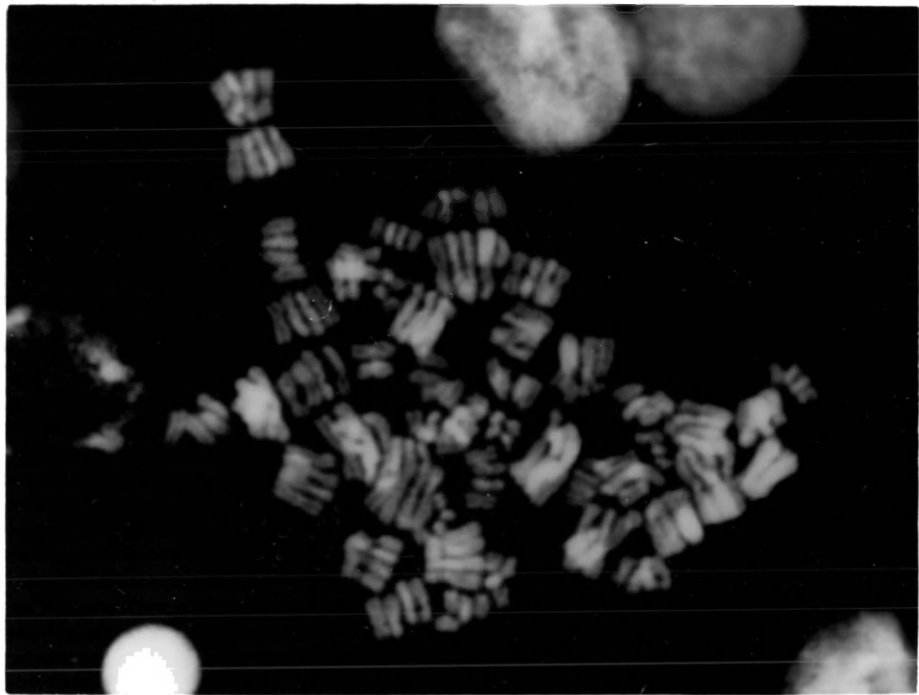


Fig. 4. Endoreduplication, type E, with G_1 damage. Note endoreduplicated acentric fragments. Some obscure R-banding is visible in this spread, due to labeling during S_2 .

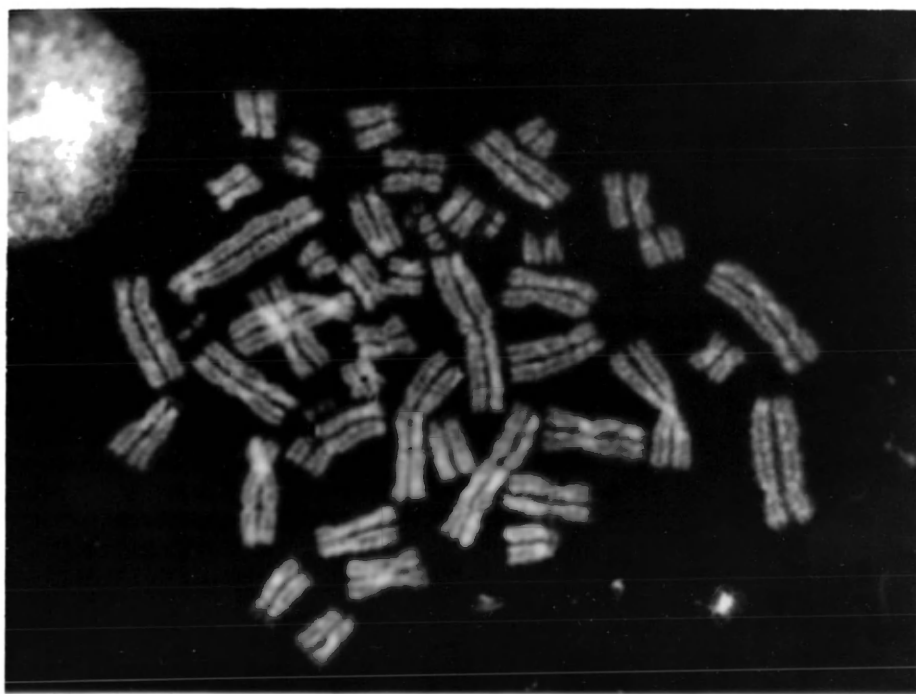


Fig. 5. Endoreduplication, late type D. This is the latest stage of S_1 which can be distinguished. Note small symmetric gaps on outer chromatids, especially 5 p's, 13 q's, and C-band regions of the 9's.

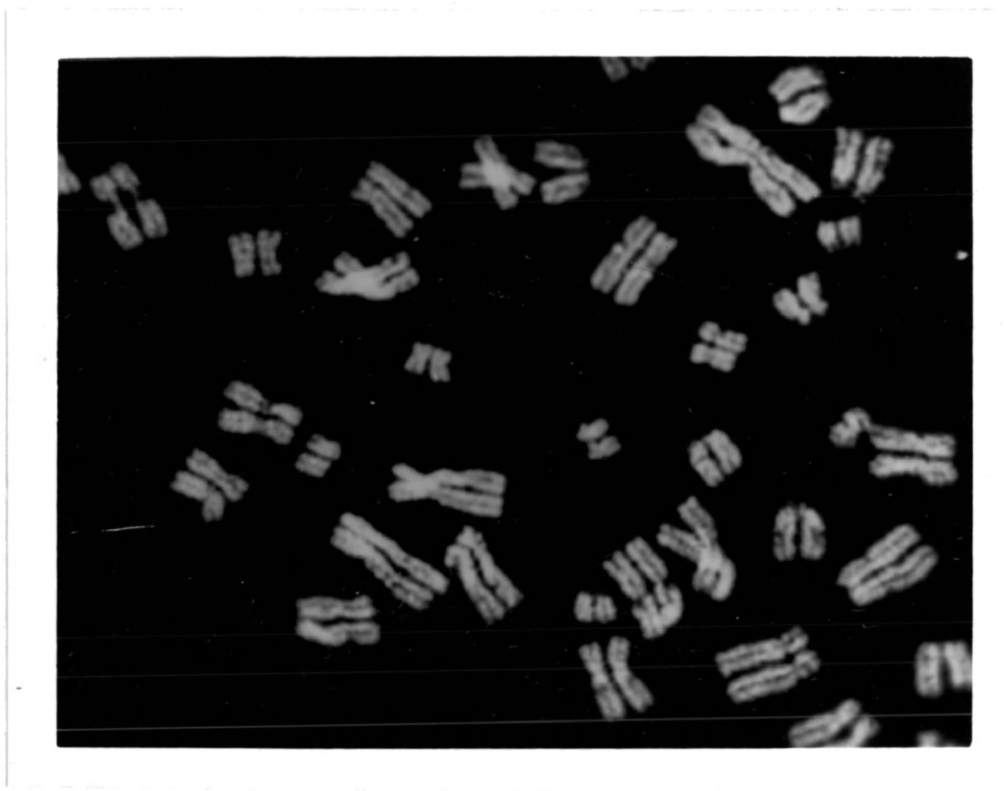


Fig. 6. Endoreduplication, mid-type D. Labeled from near the middle of S_1 . Large gaps in staining appear symmetrically on outer chromatids. Note several G_2 -type rearrangements.

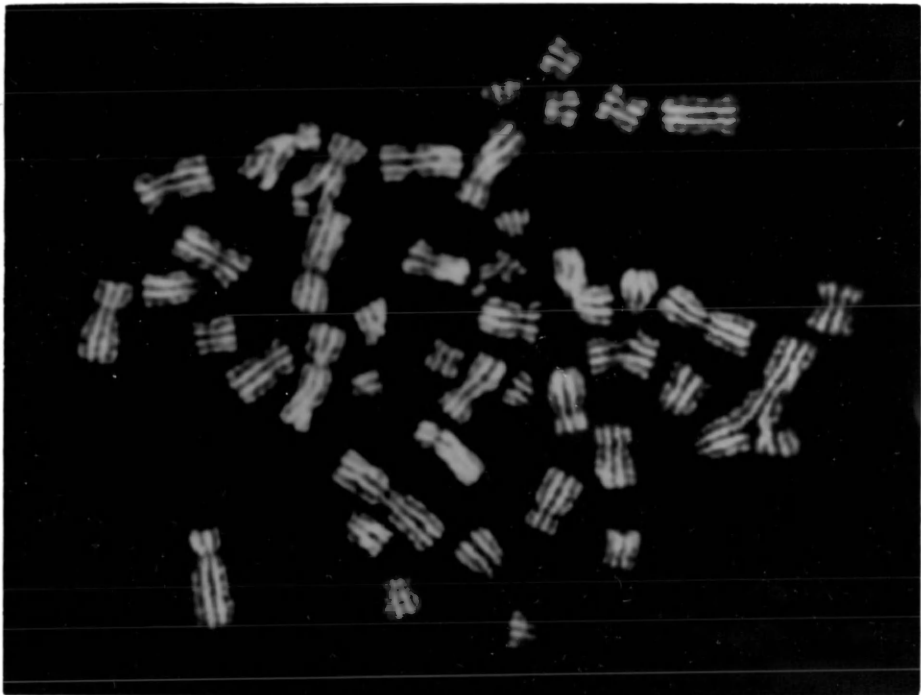


Fig. 7. Endoreduplication, early type D. Labeled from the very beginning of S_1 . Slight irregularities of staining appear in the outer chromatids which are not quite completely labeled.



Fig. 8. Endoreduplication, type C. Outer chromatids are uniformly labeled. Both twin and single sister chromatid exchanges are visible in this spread, sometimes twisted out of their normal orientation.

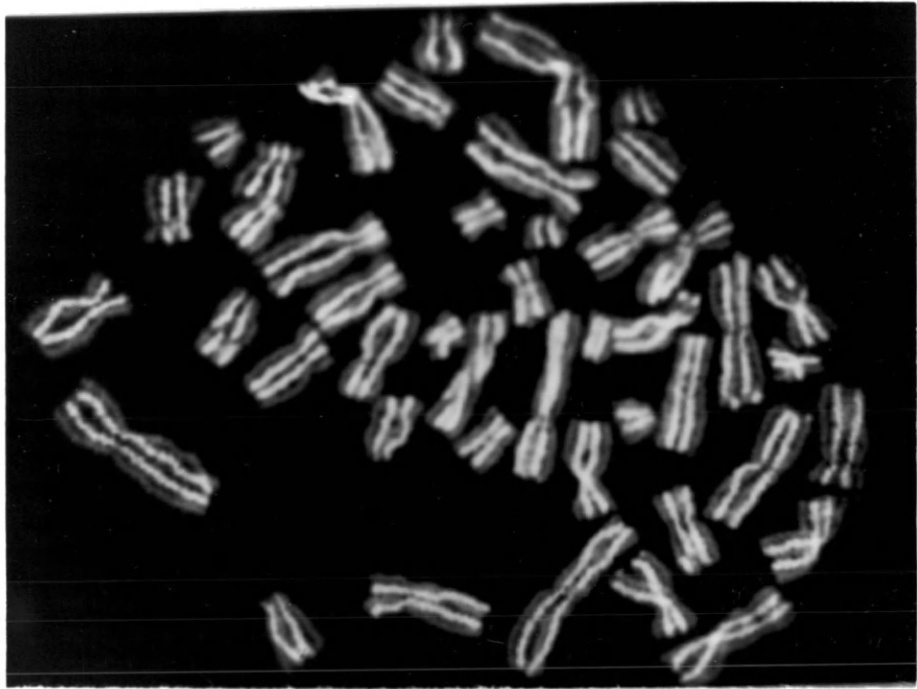


Fig. 9. Endoreduplication, type C, with G_2 damage.

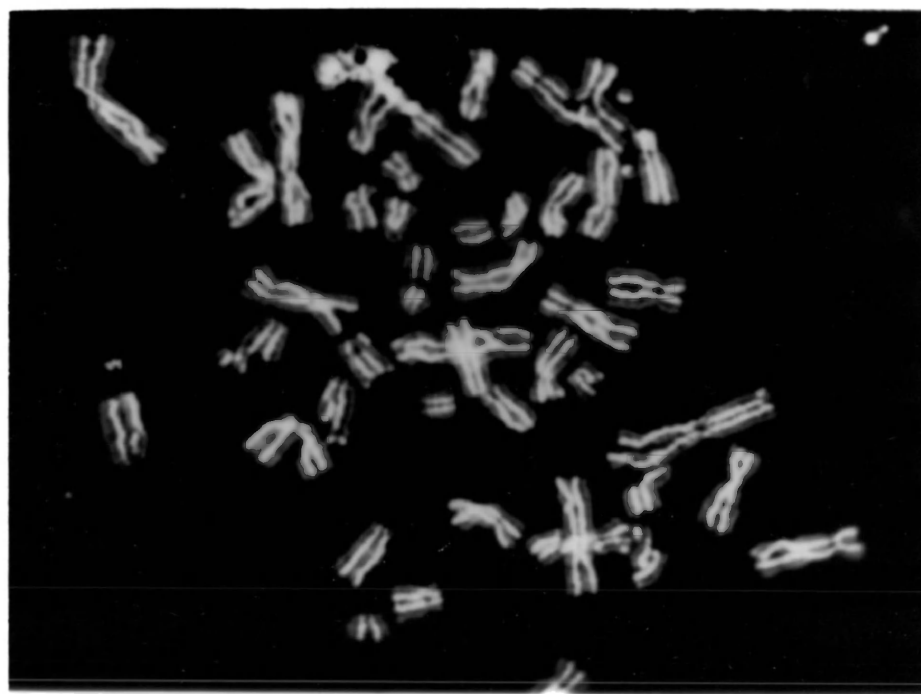


Fig. 10. Endoreduplication, type C, with G_1 damage. Note dicentric and ring diplochromosomes.

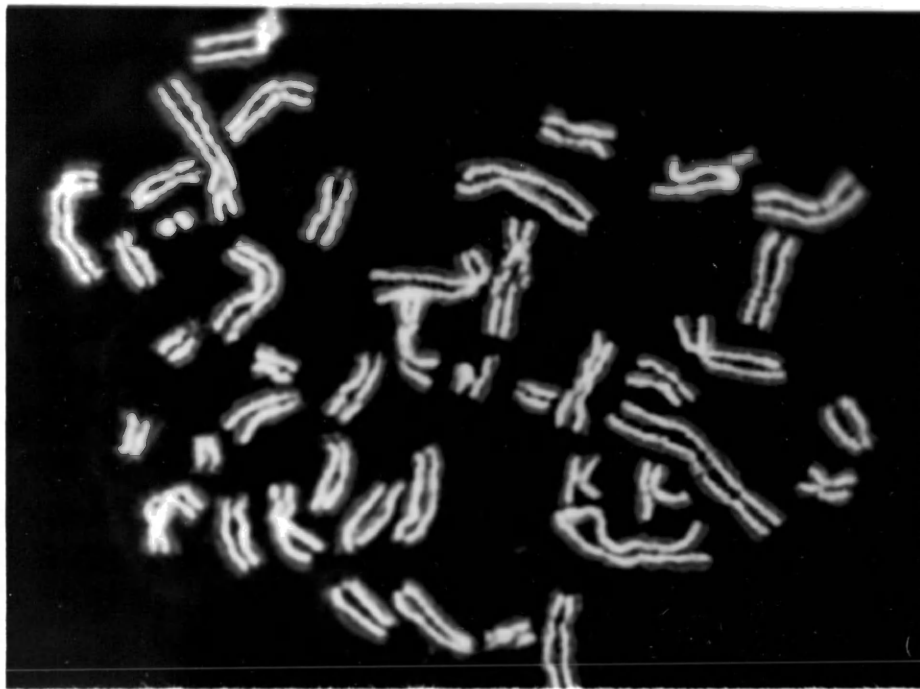


Fig. 11. Endoreduplication, type B. Both outer chromatids are uniformly labeled, while one inner chromatid is unlabeled and one is partially labeled.

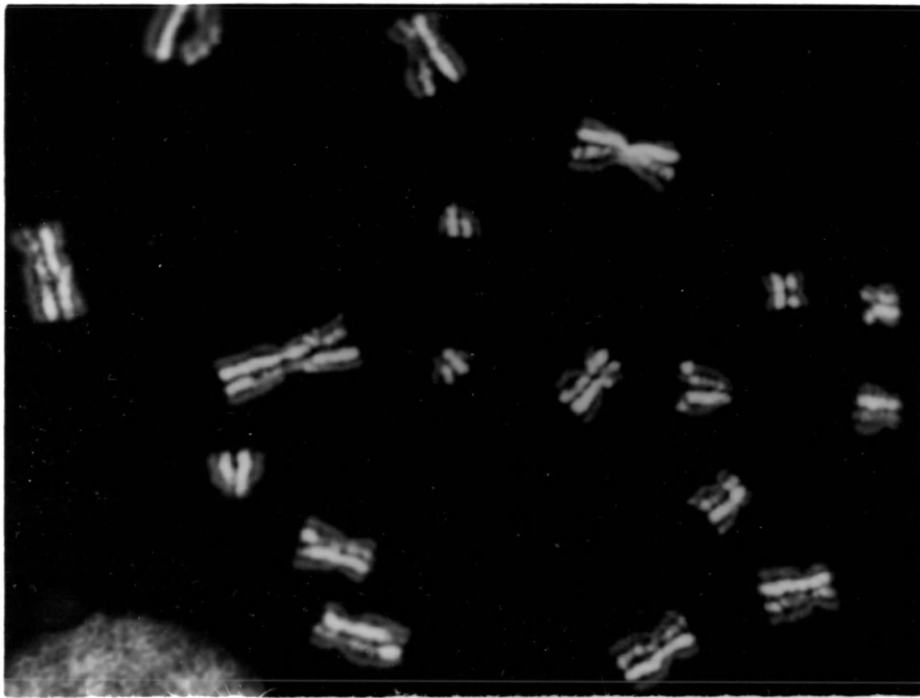


Fig. 12. Endoreduplication, type B (partial group).



Fig. 13. Endoreduplication, type A. An inner chromatid in each diplochromosome remains unlabeled.

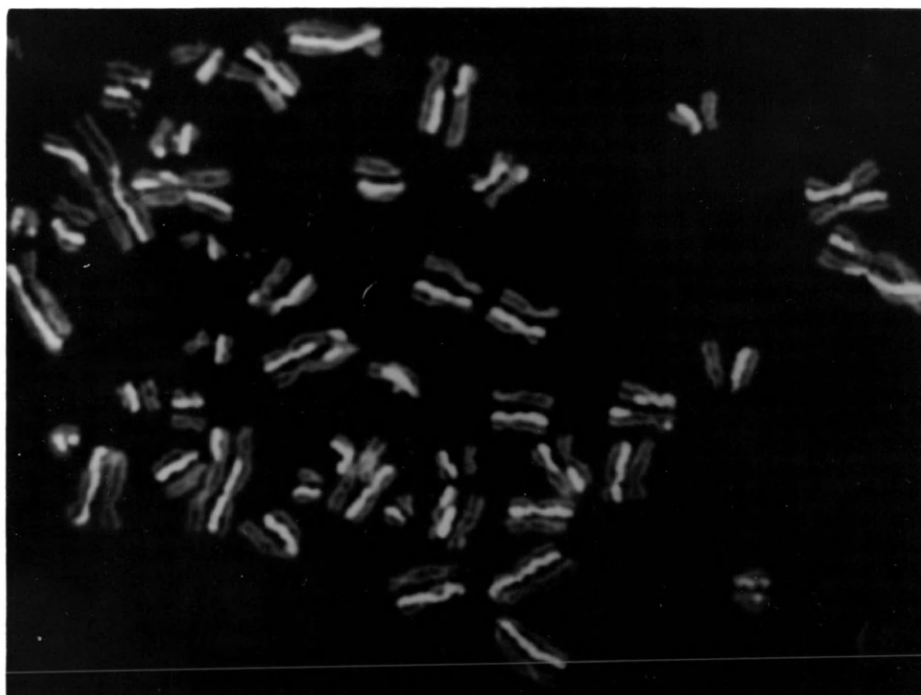


Fig. 14. Endoreduplication, labeled grandparent cell.

In this spread, one inner chromatid is entirely unlabeled in approximately half the diplochromosomes, while the rest are labeled on all four chromatids uniformly. Label was introduced before the S-phase of the grandparent cell of the endoreduplicating cell.

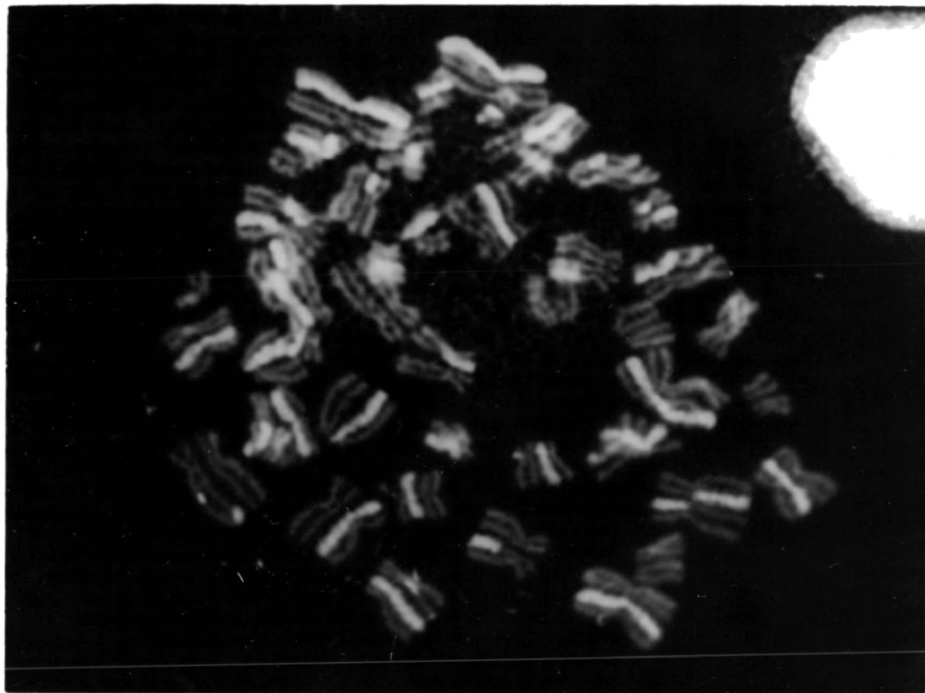


Fig. 15. Endoreduplication, labeled great-grandparent cell.
From control culture, 114 hours in BUdR.

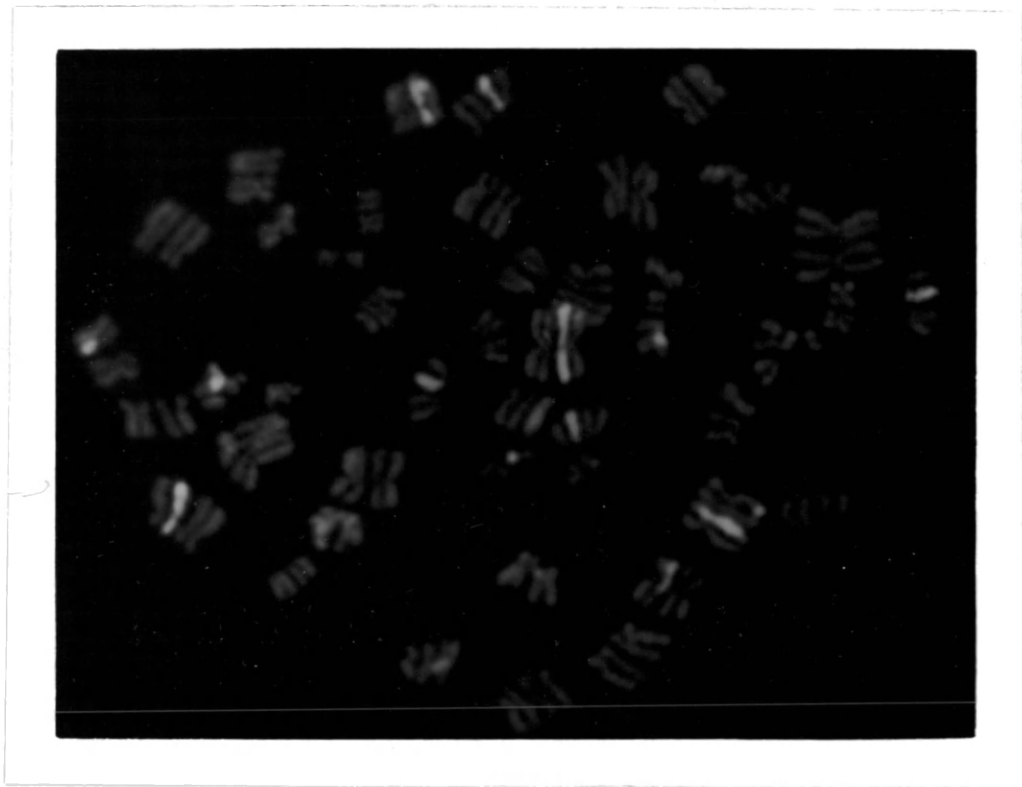


Fig. 16. Cell with $4n$ diplochromosomes. Each is labeled with type C pattern. Outer chromatids are barely visible in negative image.

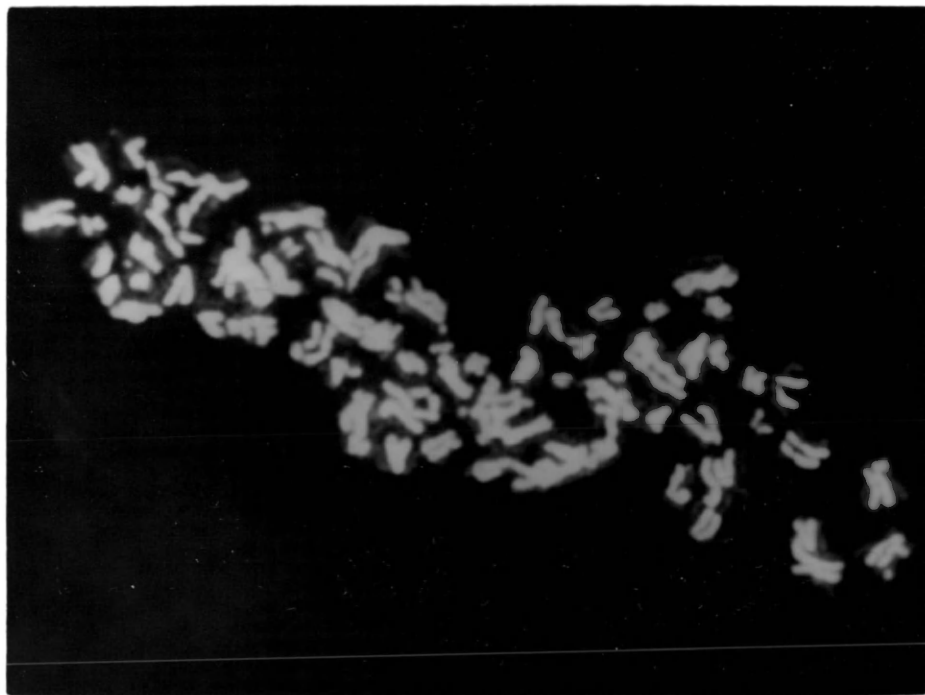


Fig. 17. Random tetraploid, undifferentiated. Note twin pair of long dicentrics.



Fig. 18. Random tetraploid in second post-labeling division.
Note rings, dicentrics; one ring is missing from spread.



focus, especially in polyploid spreads. If one point is in perfect focus, the rest is badly blurred, and, if the best average focus is used, everything is slightly blurred.

Series I. Colcemid--Short Schedule

(Fig. 19)

In the first series, eight simultaneous cultures were treated with Colcemid at 48 hours after initiation and terminated at 78 hours. The relative frequencies of the three staining patterns show that almost all cells which were endoreduplicated on this schedule were somewhere in G_2 at the time Colcemid was administered. Some had entered G_2 as long as 9 hours before the time of Colcemid treatment, and some were just completing S. Members of this population underwent a second S-phase and appeared as endoreduplicated metaphases 30 hours later.

Endoreduplications accounted for only 0.9% of metaphases on this schedule. After 30 hours in Colcemid, endoreduplications were actually beginning to appear in some quantity, but a great accumulation of diploid metaphases remained. The situation was somewhat reversed after longer times.

Series II. Colcemid--Long Schedule

(Fig. 20)

Again at 48 hours, 11 simultaneous cultures were treated with Colcemid, but termination was delayed until 114 hours. BU DR was added to individual cultures at various times from initiation to

Fig. 19. Graph of Series I results. At each time given on the abscissa, BU DR was added to one culture. Below each time is given the total number of endoreduplicated metaphases (EM) which were later scored from each culture in the categories C, D, and E of Fig. 1. The ordinate is the percent of this total in each category--C (open circles), D (triangles), and E (filled circles). The same remarks apply to Fig. 20 through Fig. 22.

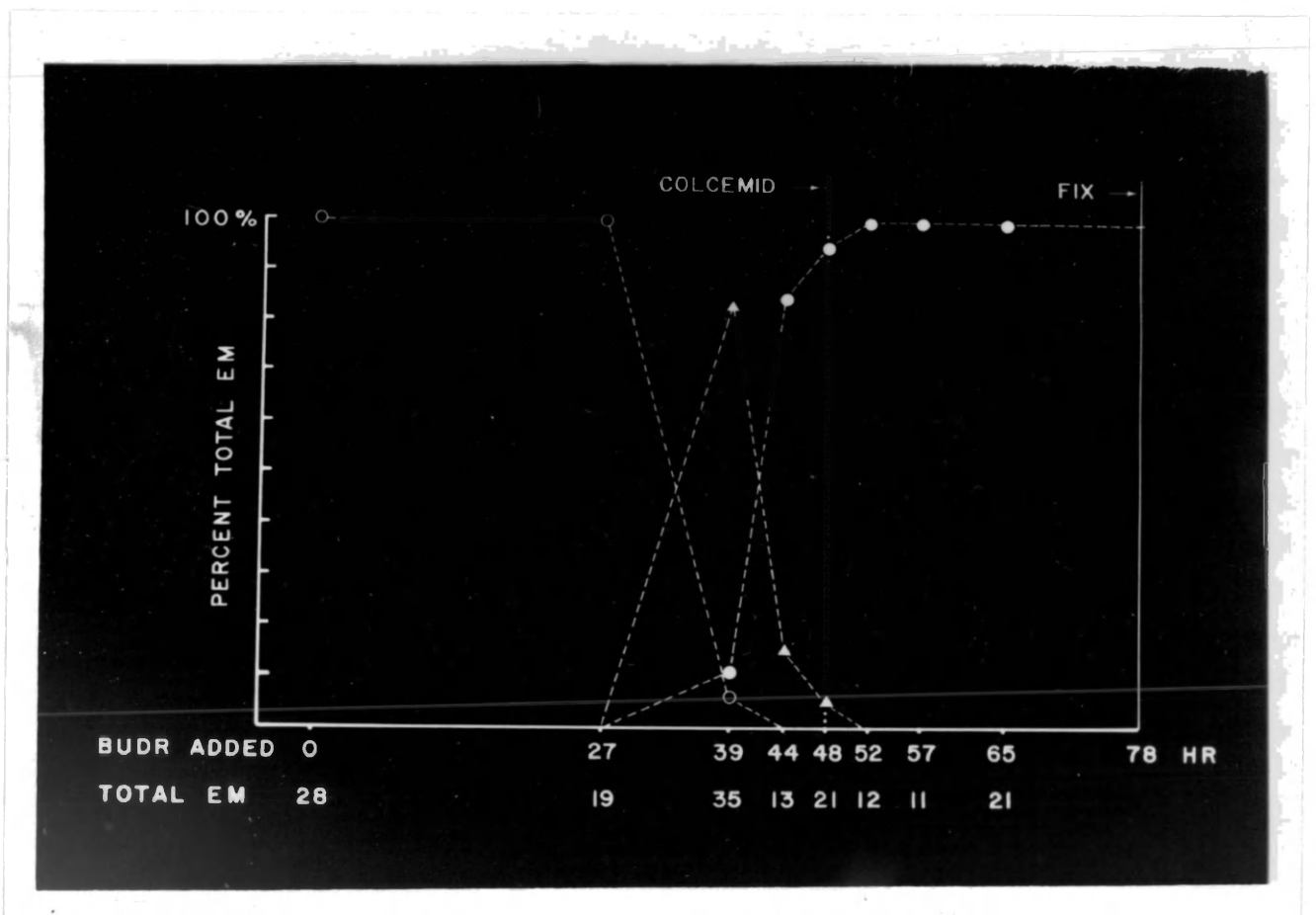
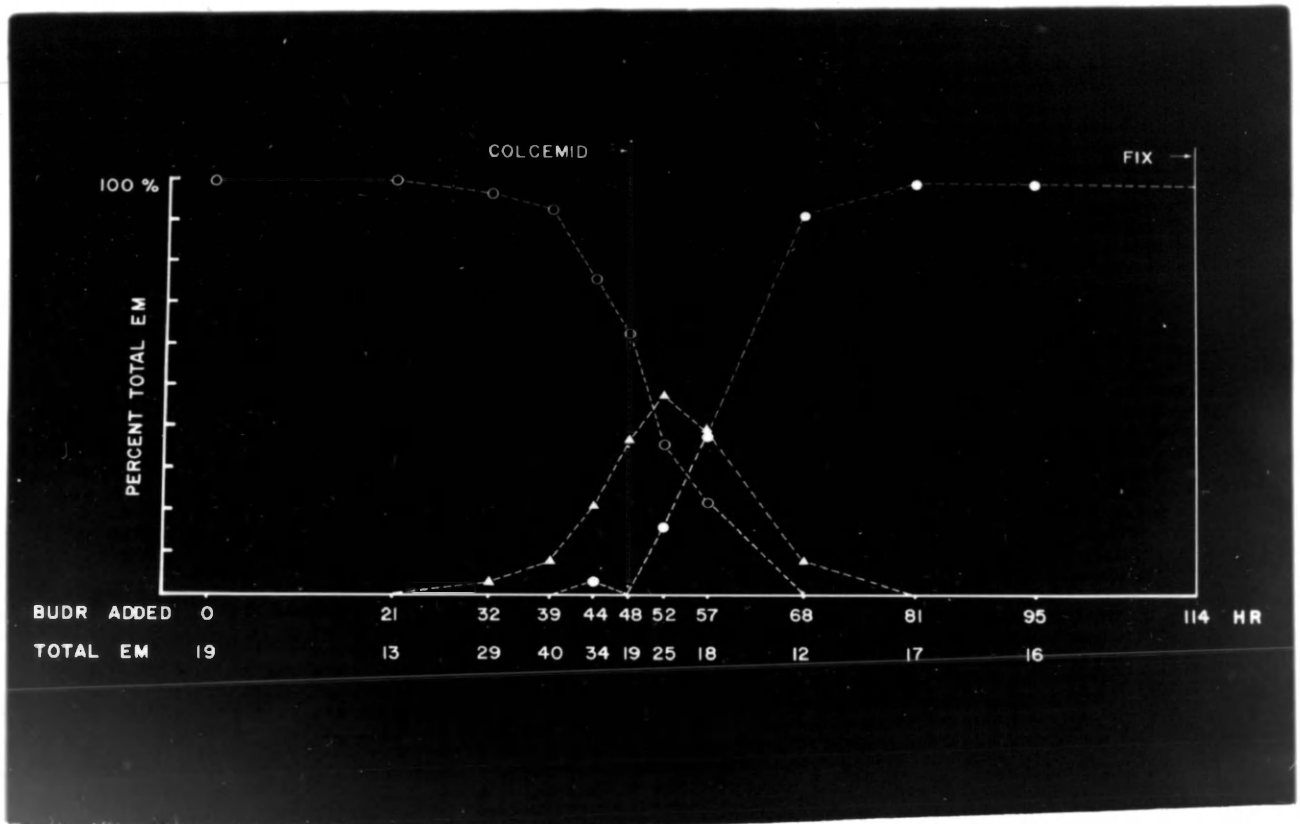


Fig. 20. Graph of Series II results.



95 hours of culture, not always at the same times as in the previous series.

After 66 hours in Colcemid, endoreduplications represented 4.6% of metaphases. These endoreduplications present a different history from those of Series I when the cell phase frequencies at the time of Colcemid addition are compared. Of these cells, a majority was in G_1 at 48 hours. Many of these were evidently still at an early point in G_1 since significant numbers were still found in G_1 9 hours later. Many cells were also in S at 48 hours, and these were frequently in early S. Only a few were in G_2 .

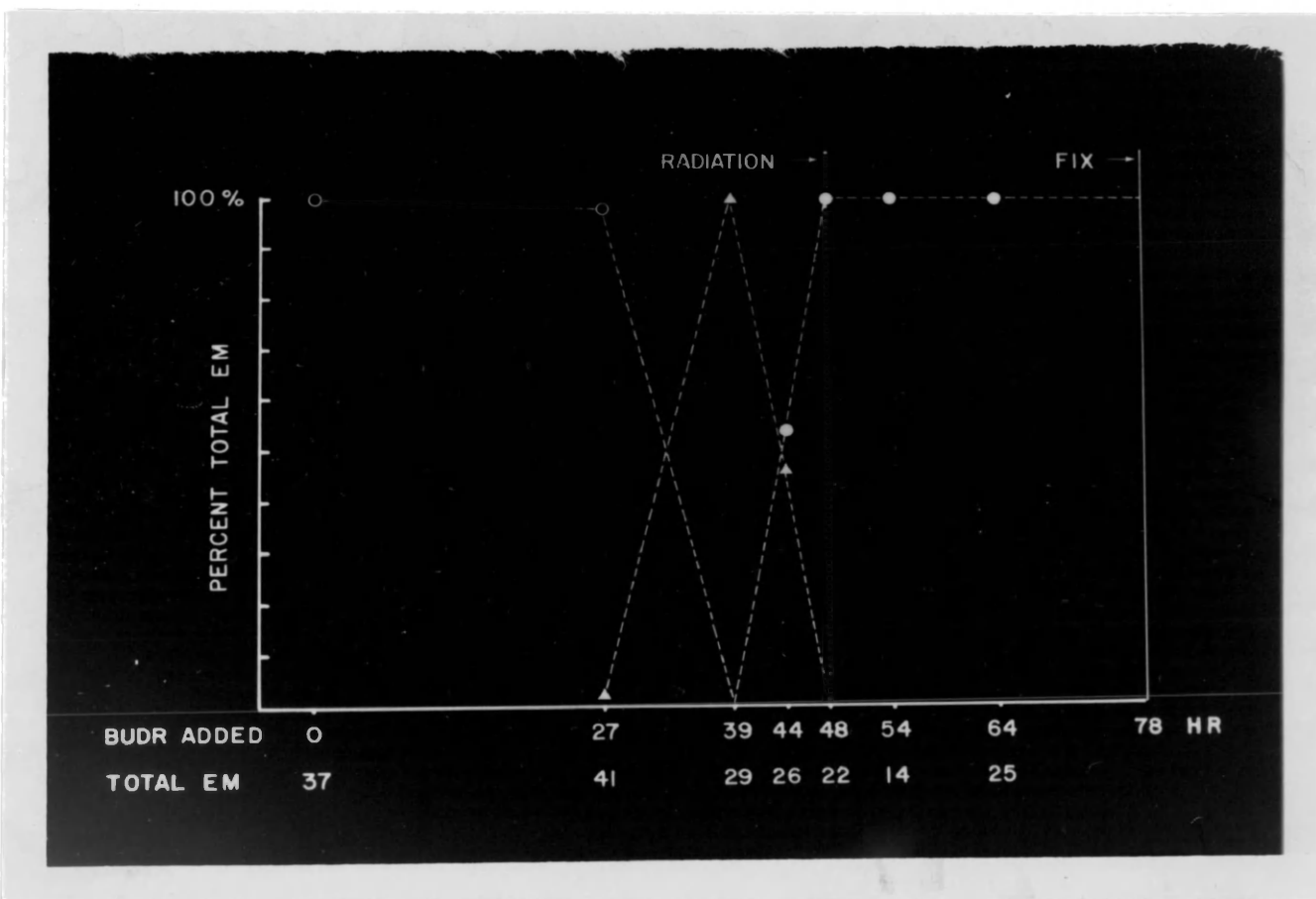
It is evident that the induction of endoreduplication in this system has no dependence on the point in the cell cycle at which a cell first encounters Colcemid, nor does a cell become unable to endoreduplicate during a long exposure prior to G_2 . If the time from Colcemid treatment to termination is extended sufficiently, even cells which were in early interphase at the time of treatment appear as endoreduplications.

Series III. Radiation--Short Schedule

(Fig. 21)

In this experiment and the next, the times of culture treatment and termination were the same as in the preceding experiments, but radiation, instead of Colcemid, was administered at 48 hours. The times of BUdR addition were also varied slightly and fewer cultures were used. Endoreduplications accounted for 4.2% and 4.6%

Fig. 21. Graph of Series III results.



of metaphases, respectively, in these two experiments.

Endoreduplications appearing 30 hours after irradiation exhibited much the same history of development as with Colcemid on the same schedule. The main difference was a higher degree of apparent synchrony with radiation. For example, there was no overlap between the last cells in G_1 and the first cells in G_2 : at 39 hours, 100% were in S. At 44 hours, about 50% were still in S, but all of these were in the latest stage of S which can be distinguished by these techniques (Fig. 5). By contrast, the cells in S at 44 hours in the comparable experiment with Colcemid, though fewer in proportion, still showed some diversity in their positions within the S-phase, as indicated by variable staining in the general pattern of Fig. 1, type D.

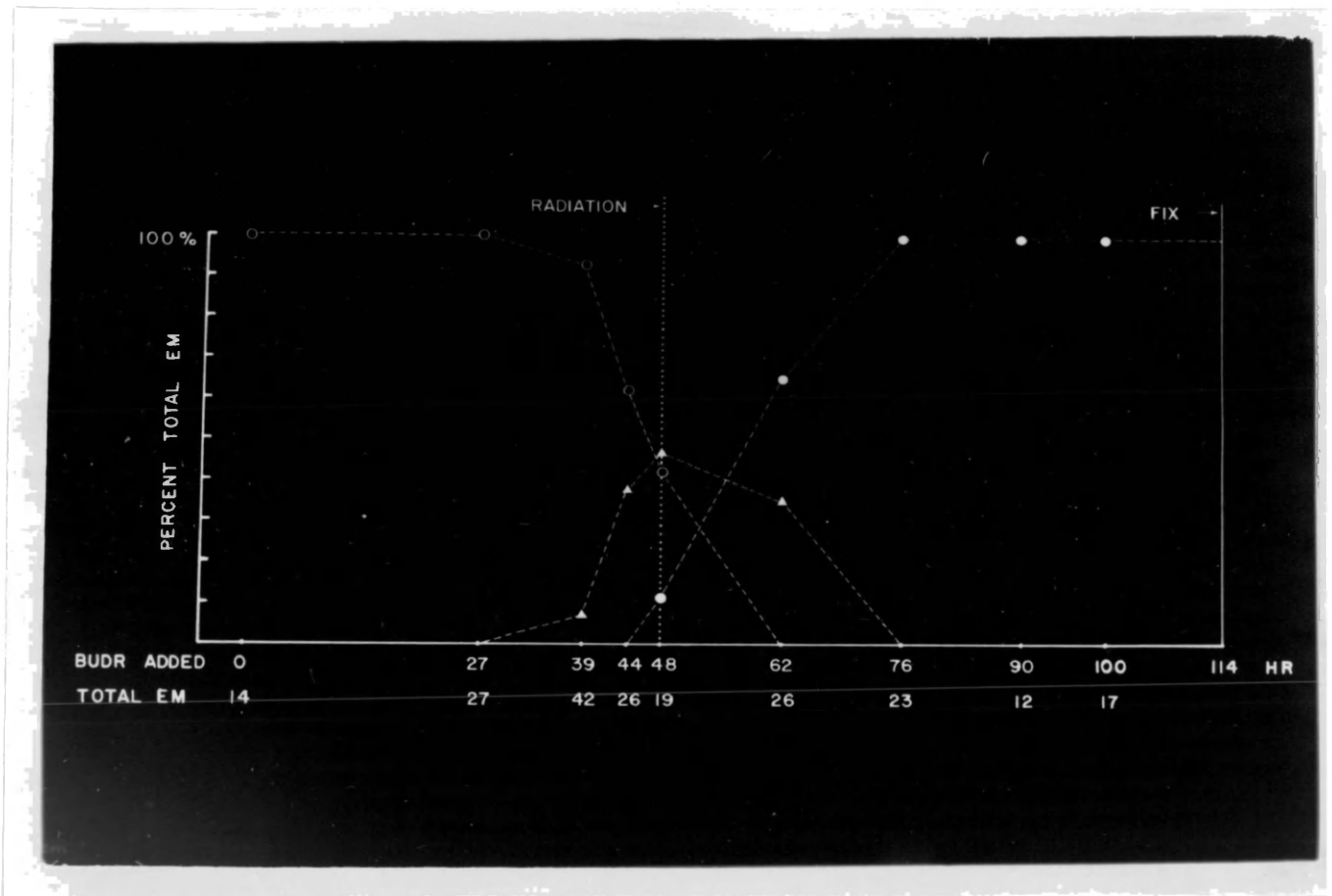
At the time radiation was administered, all these cells appear to have been in G_2 according to the data in Fig. 21. This point is also supported by an analysis of the types of radiation damage found in these cells. Of these endoreduplications, 24, or about 12%, exhibited clear G_2 -type (chromatid) damage (Fig. 6); one endoreduplication showed possible G_1 -type (chromosome) damage.

Series IV. Radiation--Long Schedule

(Fig. 22)

In this experiment, cells were again irradiated at 48 hours, with termination delayed until 114 hours. With respect to the cell phase at the time of irradiation, these cells again showed

Fig. 22. Graph of Series IV results.



displacement to the right of their position on the short schedule, similar to the displacement between Series I and Series II. The curves for Colcemid and radiation on both schedules are roughly congruent and synchronous.

Again, analysis of radiation damage supports the labeling data. Of endoreduplications scorable for radiation damage from this series, 28 cells, or about 13% of total endoreduplications, showed G_1 -type damage; three showed G_2 damage.

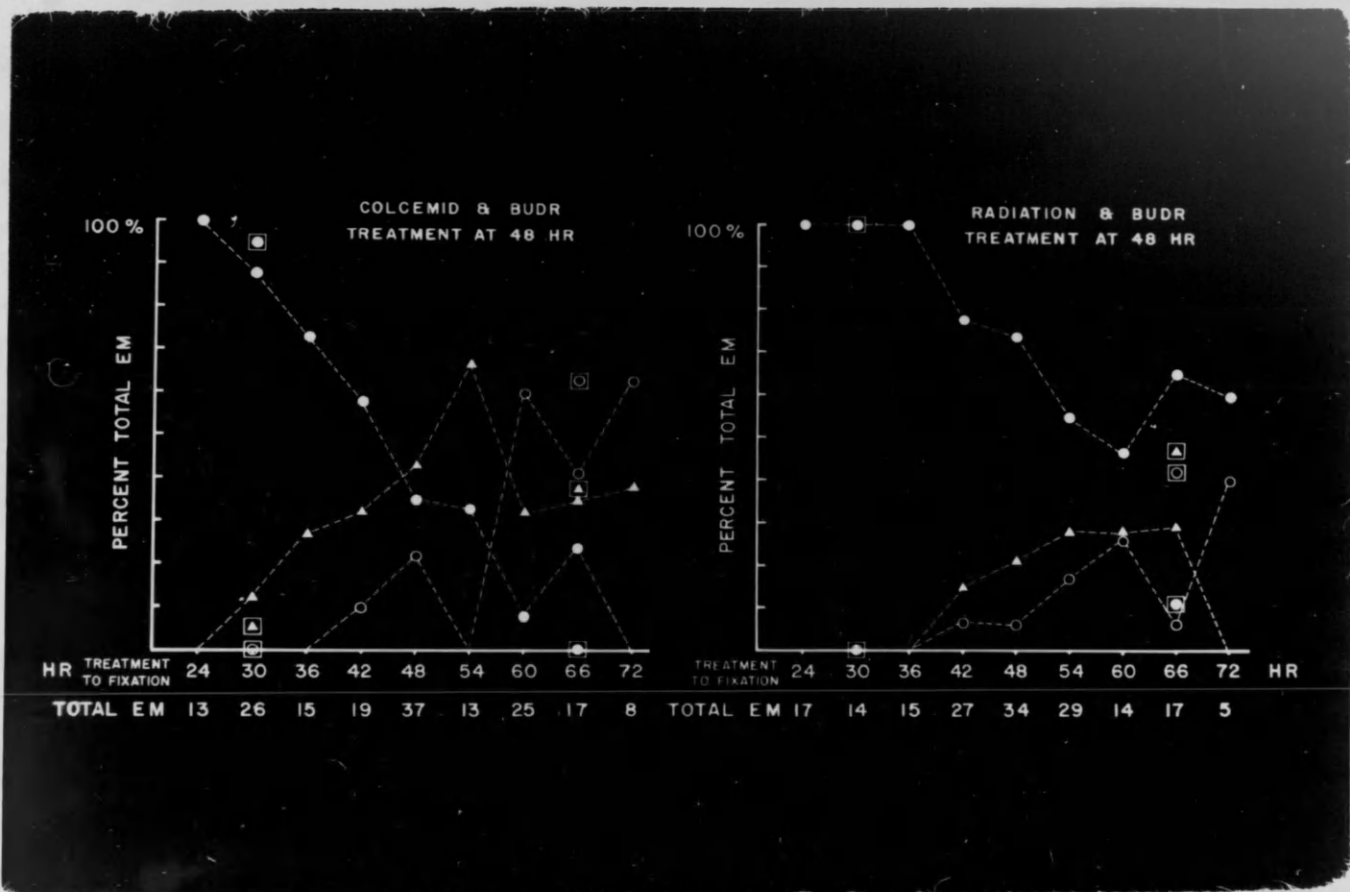
Series V. Colcemid--Variable Schedule

(Fig. 23A)

In the last two experiments (Series V and VI), the inducing treatment was again administered to all cultures in a series at 48 hours. A culture was then terminated every 6 hours, from 24 hours to 72 hours after the combined treatment with inducer and label. This change of plan allows the phase frequencies at the time of inductive treatment to be determined for populations of cells which endoreduplicate on different schedules over a range of intervals between treatment and fixation.

The inducing agent in Series V was Colcemid. The same graph symbols are used for the same phases of the cell. The comparable data points from Series I and II are also entered on the same graph (enclosed in squares) for comparison. Despite some fluctuations, the general trend is clear. Cells which were endoreduplicated after 24 hours in Colcemid had all been in G_2 at the time of treatment. As the time from treatment to fixation was extended, the population

Fig. 23. Graphs of Series V and VI results. There were two series of cultures grown concurrently. At 48 hours, one was treated with Colcemid (23A) and the other with radiation (23B). All cultures were also labeled at this time. The time from treatment to fixation is given on the abscissa, and the number of cells scored is below each time. The symbols for data points have the same meaning as in Fig. 19 through Fig. 22. The data points from those figures, where they represent cultures treated in the same way, are also entered on these graphs for comparison, enclosed in squares.



of endoreduplications changed to cells which had been in earlier and earlier stages of their normal cell cycle at the time of treatment.

Series VI. Radiation--Variable Schedule

(Fig. 23B)

Series VI follows the same plan as Series V, with radiation at 48 hours instead of Colcemid. Again, the comparable data points from Series III and IV are entered on the same graph.

Both Series V and VI were set up from the same blood sample at the same time, with medium from the same batch, and grown concurrently in the same incubator. Therefore, these two series should be exactly comparable as far as variables can be controlled. Some unknown factor caused severe fluctuation in the results of both these series in cultures terminated 54 hours or more after the inducing treatment. Data up to 54 hours are clearly trending toward the expected values obtained in the first four experimental series. After 54 hours, the data are no longer consistent within or between series.

The main observations these data allow, however, concern the first part of the series from 24 hours to 54 hours after treatment. In comparing this portion between Series V and Series VI, one notes that cells in both series which were treated in G_1 or G_2 appear on similar schedules, but there is a difference in the timing of cells treated in S. Cells which were irradiated in S took 12 hours longer to appear as endoreduplications than cells which were Colcemid-treated in S.

CHAPTER 4

DISCUSSION

Palitti and Rizzoni (1972) and Rizzoni and Palitti (1973), studying colchicine-induced endoreduplication in Chinese hamster cells labeled with tritiated thymidine, found an induction-phase relationship identical to that in Series I (Fig. 19). They also noted that endoreduplications appeared and disappeared in a single wave during a defined period following the application of colchicine, whether or not the colchicine was subsequently removed from the medium. They concluded that only cells which were in late-S/G₂ at the time the agent was administered could be induced to endoreduplicate and that cells which were at other points in the cycle when first exposed to the agent either built up a resistance to this particular effect of colchicine, or otherwise lost the ability to respond by endoreduplicating, by the time they too reached late-S/G₂.

Again, Sutou and Tokuyama (1974) and Sutou and Arai (1975) reported the same phase relationship for Chinese hamster cells stimulated to endoreduplicate by a pulse of 4-nitroquinoline-1-oxide. In these reports, autoradiographic monitoring yielded the same curve Rizzoni and Palitti (1973) had obtained, and further experiments with synchronized cells confirmed that few or no endoreduplications could be induced in cells pulsed with this agent outside the specific

inducible period. It was not determined whether continuous exposure to the inducer before the inducible period limited the subsequent ability of the cells to endoreduplicate.

It can be seen from Series II (Fig. 20) that continuous exposure during G_1 and S does not render human lymphocytes unable to endoreduplicate in response to Colcemid. Cells anywhere in interphase at the time Colcemid is added seem equally able to endoreduplicate, but necessarily require more time to appear as endoreduplications when they are earlier in their normal interphase.

It is interesting that a single dose of radiation acts on lymphocytes with the same phase independence as a continuous exposure to Colcemid. Apparently the radiation-induced damage which causes the extra synthetic phase can persist in its effect throughout G_1 and S. The yield of endoreduplications in the two radiation experiments, in percent of metaphases, was about the same (4.2% and 4.6%), even though the cells in each population had been irradiated in different parts of interphase. These results may indicate that radiation induces eventual endoreduplication by causing, in some part of the cell, a change which is irreversible, or at least very long-lived, but which is not especially detrimental to cell function during the time before the supernumerary replication begins, and for which the target is present during all stages of interphase. An example of such a target might be the centriole (Howell et al., 1977).

Yu and Sinclair (1972) found a quite different radiation response in Chinese hamster cells. In their system, using

synchronized cells, random tetraploids and endoreduplications were only induced in cells irradiated in G_1 . However, the critical target in these cells is not necessarily different from that in human lymphocytes. In both cell types, the effective radiation damage can occur at an early point in interphase only to be expressed after some time has elapsed during which the cell goes through one complete normal S-phase.

In all series, a few endoreduplications were found (0.1% to 0.6% of metaphases) which displayed the first and second labeling patterns shown in Fig. 1 (types A and B), indicating that BUdR had been added before or during the S-phase of the parent cell of the endoreduplicated cell. Most of these can be explained as cells which were able to complete one mitosis before treatment at 48 hours. These cells appear only in the cultures which were labeled first, second, or third in each series, except in Series IV where three were found in the culture labeled at 44 hours. If these three endoreduplications are to be attributed to the radiation at 48 hours, then the specific damage would seem to have been transmitted through one normal mitosis after the radiation before causing endoreduplication in a daughter cell. The same explanation suggests itself in regard to three endoreduplications found in Series VI (two of type A, and one as in Fig. 14--see Table 6, Appendix A) which were also of a more advanced labeling type than the circumstances would seem to permit. More probably, these cells represent part of a background of endoreduplications not caused by the agents tested. Control

cultures of 78 hours and 114 hours duration, labeled from the beginning with BU DR but not treated with Colcemid or radiation, showed frequencies of endoreduplication high enough to account for these anomalies; i.e., 0.2% and 0.1% of metaphases, respectively. Some low levels are to be expected spontaneously or as side effects of both PHA (Sutou & Arai, 1975) and BU DR (Dutrillaux et al., 1974).

In the same paper in which they demonstrated a phase-specific action of colchicine in endoreduplication induction, Rizzoni and Palitti (1973) showed that some cells with quadruplochromosomes could be obtained by treating cultures a second time with colchicine. Interestingly, they were able to obtain quadruplochromosomes even when the second treatment came 24 hours after the (single) endoreduplications from the first treatment had disappeared. This proved that there were some endoreduplications induced by a single treatment which normally remained permanently blocked in interphase in their system. Rizzoni and Palitti do not note that these nonmanifesting endoreduplications may represent the "lost" parts of the cell cycle in their experiments. The cells which are in G_1 and S when colchicine is added may indeed endoreduplicate, but be unable to enter metaphase. Thus, their results may be more similar to the present results than would appear as far as phase-specific induction is concerned.

In general, random tetraploids and endoreduplications are often induced by the same treatments, but their relative frequencies can be unaccountably variable even within the same series of

experiments (Bell & Baker, 1965). In the present study, random tetraploids occurred in all four experiments at lower frequencies than endoreduplications. In Series IV, random tetraploids in their second post-labeling division (similar to Fig. 18) were found in cultures labeled at 62 hours and 76 hours, good evidence that there was some propagation of tetraploid lines after their induction. It is likely that some of these random tetraploids were the descendants of endoreduplicated cells. It also appeared that some random tetraploids represented endoreduplications in which most of the original diplochromosomal associations had become unrecognizable, especially in the two Colcemid series. Random tetraploids were found in which diplochromosomal organization was not apparent morphologically, but in which a regularity in the distribution of dark and light chromatids seemed to reveal pairs of chromosomes which had originally been diplochromosomes.

That diplochromosome formation in some human lymphocytes could require up to several days was already shown by Herreros and Giannelli (1967), Schnedl (1967), and Schwarzacher and Schnedl (1966). Therefore, the demonstration here of some cells which require as long as 66 hours to go from G_2 through one supernumerary S-phase to mitosis is not surprising, although it was not clear previously in what phases the main delays occurred. The cells involved in the longest delays in Series II and IV do not appear to represent a distinct lymphocyte subpopulation, but only part of a continuous range of variation. In fact, endoreduplications above control levels

have been found in lymphocyte cultures as long as 96 hours after Colcemid or radiation treatments at 48 hours (Weber, unpublished results).

The data from Series V and VI show a pronounced, selective inhibition of cells irradiated in S, compared to cells Colcemid-treated in S. This finding agrees well with the results of a study on radiation-induced mitotic delay in the cell cycle of diploid Chinese hamster fibroblasts (line V79-285B) (Yu & Sinclair, 1967). These cells were most strongly inhibited, relative to controls, when irradiated in S. Endoreduplications were not observed in this study, but the results are clearly applicable since during the S_1 when radiation was administered, the lymphocytes in the present study were normal diploid cells. However, in the cited study, the maximum delay for cells at a comparable radiation dose would have been about 2 hours, not 12 hours as in the present study. Lymphocytes are especially sensitive to radiation, and would not survive doses routinely administered to some Chinese hamster cell lines. The extreme delay of lymphocytes irradiated in S may reflect this sensitivity.

Series V and VI, therefore, demonstrate that the close correspondences between the graphs for Colcemid and radiation induction, on the short schedule and the long schedule, result from a fortuitous choice of timing. The correspondence between Colcemid and radiation induction would not be as close for all post-treatment periods between 30 hours and 66 hours because of this transient delay

in the appearance of endoreduplications from lymphocytes which were irradiated in S.

The study of endoreduplication and its induction can help in understanding normal cell cycle controls. At present, the various methods of inducing endoreduplication may offer the only convenient experimental approach capable of inducing an S-phase "on command," as distinguished from block-reversal methods. It might be interesting to test whether normal S-phases are ever triggered in G_1 cells by any of the treatments which induce endoreduplication. In any case, the mechanisms of normal and abnormal initiation of synthesis are likely to be understood simultaneously.

APPENDIX

APPENDIX A

TABLES

TABLE 1. Series I. Colcemid--short
schedule

Time	Type	Type	Type	Type	Type
label	E	D	C	B	A
added					
0			28		3
27			19		
39	4	29	2		
44	11	2			
48	20	1			
52	12				
57	11				
65	21				

TABLE 2. Series II. Colcemid--long
schedule

Time	Type	Type	Type	Type	Type	Mis-
label	E	D	C	B	A	cella-
added						neous
0			19		1	
21			13		1	
32		1	28		4	
39		3	37			
44	1	7	26			
48		7	12			
52	4	12	9			1 ^a
57	7	7	4			
68	11	1				
81	17					
95	16					

^aChromosome pulverization; not certainly scorable--apparent endore-
duplication.

TABLE 3. Series III. Radiation--short
schedule

Time	Type	Type	Type	Type	Type
label	E	D	C	B	A
added					
0			37		1
27		1	40	1	
39		29			
44	14	12			
48	22				
54	14				
64	25				

TABLE 4. Series IV. Radiation--long
schedule

Time	Type	Type	Type	Type	Type	Mis-
label	E	D	C	B	A	cella-
added						neous
0			14		2	
27			27		2	
39		3	39		1	
44		10	16	2	1	
48	2	9	8			
62	17	9				
76	23					1 ^a
90	12					
100	17					

^aOctoploid cell with quadruplochromosomes, unlabeled--type E
(Fig. 3).

TABLE 5. Series V. Colcemid--variable
schedule

Post-treatment time	Type E	Type D	Type C	Type B	Type A	Mis-cellaneous
24	13					
30	23	3				
36	11	4				
42	11	6	2			1 ^a
48	13	16	8			1 ^b
54	5	8				
60	2	8	15			
66	4	6	7			
72		3	5			

^aOctoploid cell with quadruplochromosomes composed of paired type C diplochromosomes. This cell was in normal G_2 when label was added.

^bOctoploid cell containing 4N diplochromosomes labeled in type C pattern (Fig. 16).

TABLE 6. Series VI. Radiation--variable
schedule

Post-treatment time	Type E	Type D	Type C	Type B	Type A	Miscellaneous
24	17					
30	14					
36	15					
42	21	4	2			
48	25	7	2			
54	16	8	5			
60	7	4	4		1	
66	11	5	1		1	1 ^a
72	3		2			1 ^b

^aCell which had gone through two post-labeling divisions before endoreduplicating (Fig. 14).

^bOctoploid cell with quadruplochromosomes composed of paired diplochromosomes labeled in type C pattern. This cell was in normal G₂ when label was added.

APPENDIX B

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The bibliography has been selected for techniques of BUdR-labeling, in order of publication.

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APPENDIX C

TECHNIQUES FOR SISTER CHROMATID DIFFERENTIATION (SCD) AS USED IN THIS STUDY

Growth of Cultures

The proper concentration of BU DR depends on the methods of culture. The cultures in this study, containing approximately 0.4 ml of plasma in 5 ml of medium, were labeled with 100 μg BU DR/culture, delivered in 0.1 ml of calcium-magnesium-free Hanks' balanced salt solution (CMF-HBSS), for a final concentration of roughly 20 $\mu\text{g}/\text{ml}$. This is a good average dose. The degree of SCD can be varied, within a certain range, by varying the concentration of BU DR. Slide treatment must be adapted to different concentrations used. If a properly adjusted concentration is used with a heavily inoculated, rapidly dividing culture, distinctly different intermediate shades of staining will appear in chromatin synthesized at different times. For example, with an inoculum of ~ 1.5 ml of plasma (including ~ 0.5 ml from the red blood cell fraction) added to 5 ml of culture medium, good three-way and four-way differentiation can be obtained at a dose of 100 μg BU DR per culture (see photos, Appendix D). If this dose is raised to 150 μg , labeling becomes much more uniform. SCD itself is detectable at final concentrations of only 2 $\mu\text{g}/\text{ml}$. On the other hand, final concentrations of 2,000 $\mu\text{g}/\text{ml}$ have been used by the writer and others for late-labeling techniques.

Making stock solutions of BUDR with CMF-HBSS, instead of HBSS, helps prevent formation of precipitates, especially when solutions are kept frozen. BUDR solutions may be stored frozen and thawed for use. They seem to be effective indefinitely if protected from light.

Preparation of Slides

Dropping cells on dry slides or on water-dipped slides has opposite effects on chromosome morphology. With increasing ratio of fixative to water on the slide, chromatids tend to appear longer, thicker, more diffuse, and more widely separated. These effects are most pronounced when cells are dropped on a dry slide. At the other extreme, a single small drop of cells in fixative may be applied to a wet, undrained slide. The morphological result will be the opposite: tight, narrow, unseparated, small chromatids. Between these extremes, the best point may be found for a particular batch of cells. In most material, two or more full drops, partly overlapping each other, on a dipped and drained slide, will give good results. These considerations are important for the clarity of structure required for the best SCD.

Slides may be air- or flame-dried for SCD. The only difference may be that flame-drying, when very rapid, promotes spreading. In any case, slides should be thoroughly dry before proceeding.

Treatment of Slides

A solution of 20 µg/ml 33258 Hoechst in distilled water can

be reused for at least a month if kept clean and free of bacteria. Bacteria can ruin a solution in 24 hours. It will then appear cloudy, clog new Millipore filters almost at once, and cover slides with bacteria. Solutions should, therefore, be Millipore-filtered immediately after each use, then either frozen solid or poisoned with a few drops of CHCl_3 . Note that CHCl_3 instantly dissolves the plastic platform in some Millipore holders if it goes through in undissolved droplets.

Slides may be treated in 33258 Hoechst for 1 hour. After Hoechst staining, they can be transferred directly, without rinsing, to 2XSSC (0.3M NaCl + 0.03 M Na-citrate, pH adjusted to 7.0 with citric acid solution). The slides should be laid flat, face up, in an enameled pan with just enough 2XSSC to cover the slides a few mm. They may also be attached to various kinds of flat frames for ease of handling. The slides are then irradiated for 20 minutes to 60 minutes with UV from a mercury vapor lamp. This must be either a germicidal lamp or a mineral-fluorescence lamp with mica window. Phosphate-barriered mercury-vapor lamps meant for illumination will not work well, nor will "black-lights." The radiation must be strong at 254 nm. The amount of irradiation required will depend on the dose of BUdR incorporated by the cells, and, in some material, too much irradiation will cause light regions to disappear completely. The 2XSSC solution, like all solutions through which the slides pass, should be millipore-filtered before use to eliminate bacteria from the slides.

After irradiation, the slides may be transferred without rinsing to 2XSSC at 75 C; it is good to make sure that no residue of stain contaminates this hot salt solution, as might result, for example, from use of the same equipment for salt treatment and staining. The temperature should be kept between 70 C and 80 C for 15 minutes to 20 minutes. Then, the slides are transferred directly to hot tap water. This should be done rapidly to prevent salt crystal formation on the slides. Once in tap water, the slides may be left for hours, or even removed and dried, before staining.

The stain that is most effective is a totally aqueous solution made from separate Eosine Y and Azure B stocks, rather than the commercial Giemsa stain in methanol/glycerol. (This is not true for all other applications, however. For example, C-banding is much better with commercial Giemsa.) The stain stocks should be 0.1% solutions in 0.05 M NaH_2PO_4 adjusted to pH 6.8. These should be diluted for use with the same buffer in a 2:5:30 ratio (Eosine Y: Azure B:buffer). After these components are mixed, the stain will begin to lose its strength noticeably after 1 hour, but could still be used for several hours. With fresh stain, slides should be stained about 5 minutes. The best time depends on the material.

Immediately before the slides are placed in the stain, the surface of the stain solution should be wiped clean of the film which rapidly forms, and, before slides are removed, the surface should be wiped again. Slides should not be drained, but rapidly rinsed. Only distilled water should ever be used as a rinse. Then, the slides

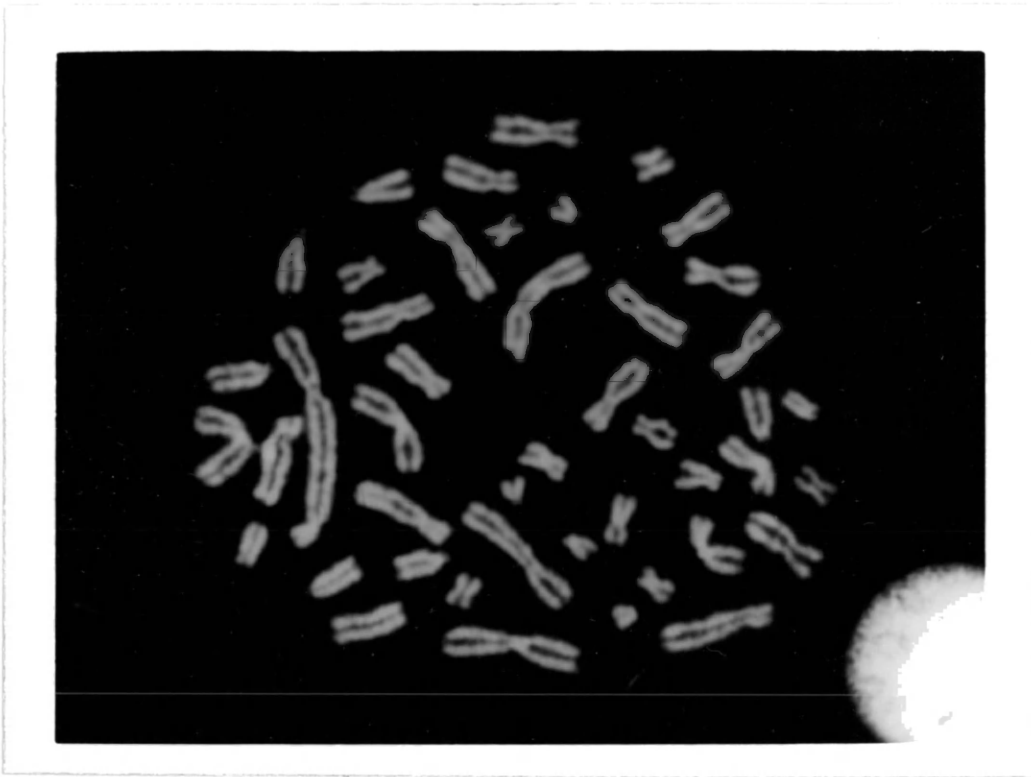
should be air-dried, perhaps with a fan, but never with heat as spotting will develop.

While the slides are still wet, one slide should be checked by making a wet mount with a large cover slip and very little water. Under oil, the quality of differentiation can be monitored though it will not appear as clearly as in a dry mount. This is one of the most critical steps. Either the chromosomes will be plainly visible and well differentiated, or they will be understained (pale areas invisible) or overstained (differentiation tending to disappear). If understained, the slides should be returned to the stain solution briefly. If overstained, the slides can be rapidly destained by soaking a few minutes in plain distilled water. If faster destaining is desired, a few drops of ethanol may be added. Acid should not be used. By these methods, slides can be adjusted up or down in staining intensity very easily, until the proper contrast and clarity are achieved. These techniques are effective even several days after the slides were first stained.

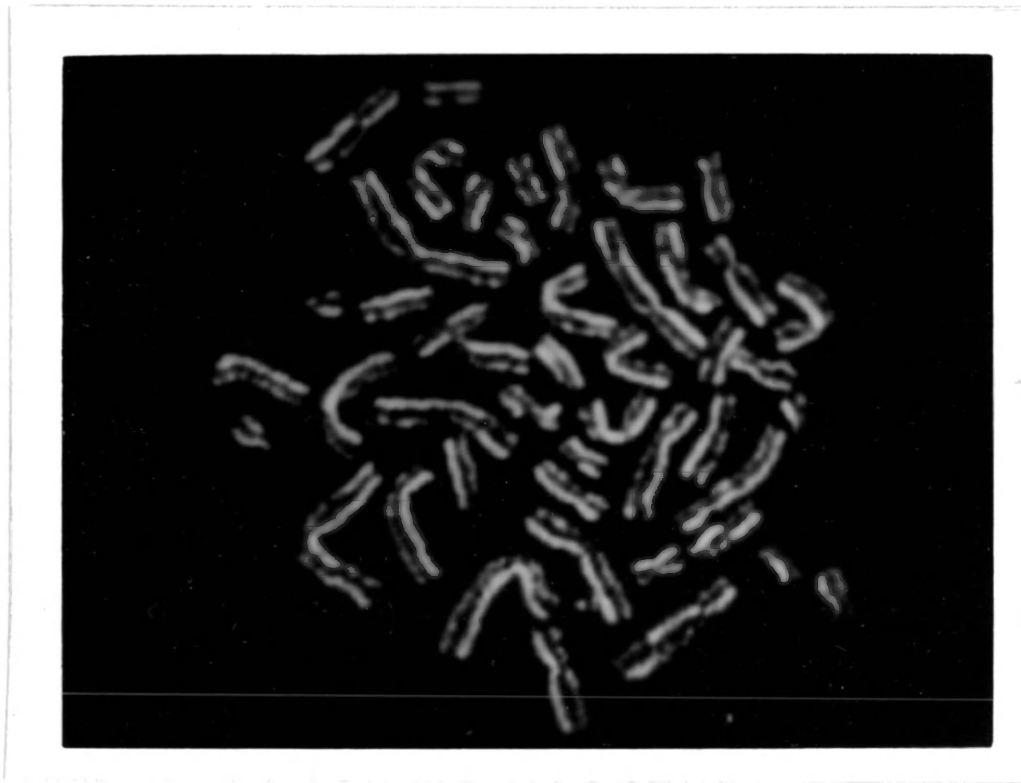
APPENDIX D

PHOTOS OF DIPLOID CELLS WITH SISTER
CHROMATID DIFFERENTIATION

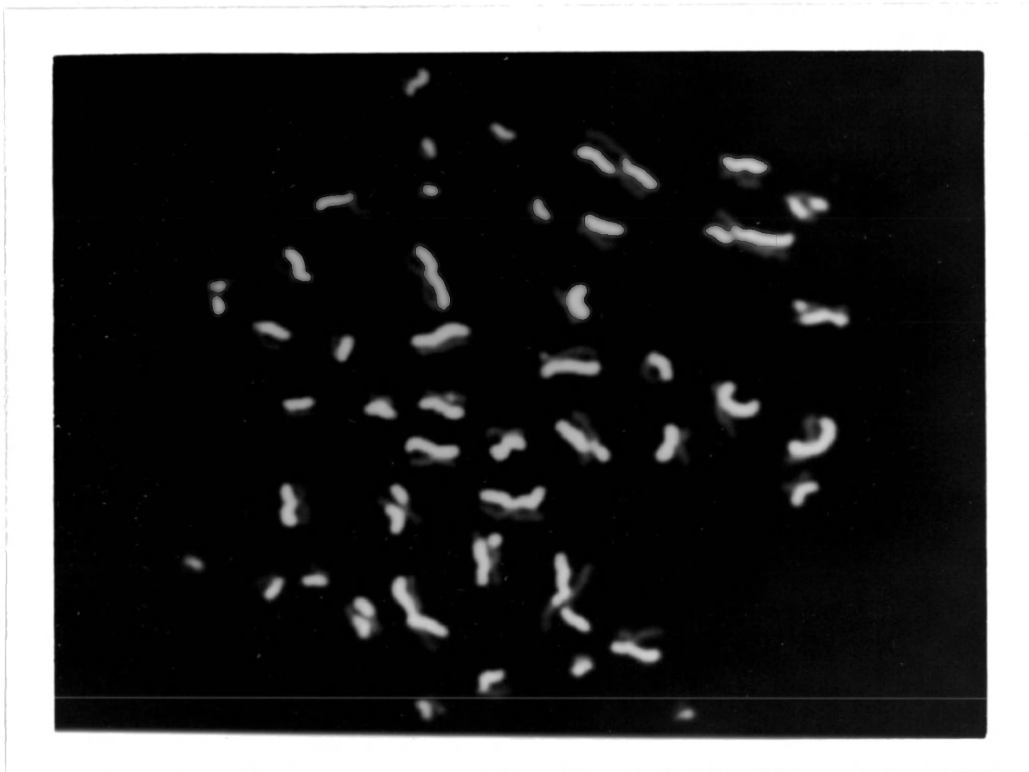
After one replication in BUdR, no differentiation is visible
(note long dicentric in this spread).



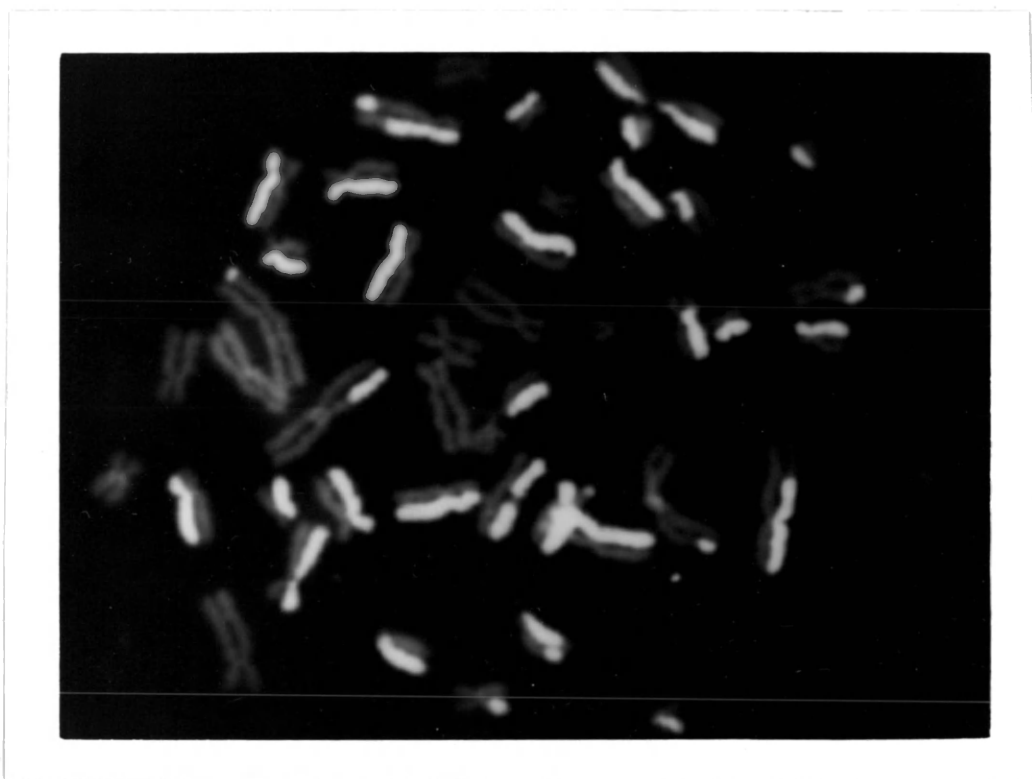
After one and a half replications, one chromatid is unlabeled while the other is intermittently labeled. Here the label was added during the S-phase of the next-to-last cell cycle.



After two full replications, one chromatid is dark and the other light.



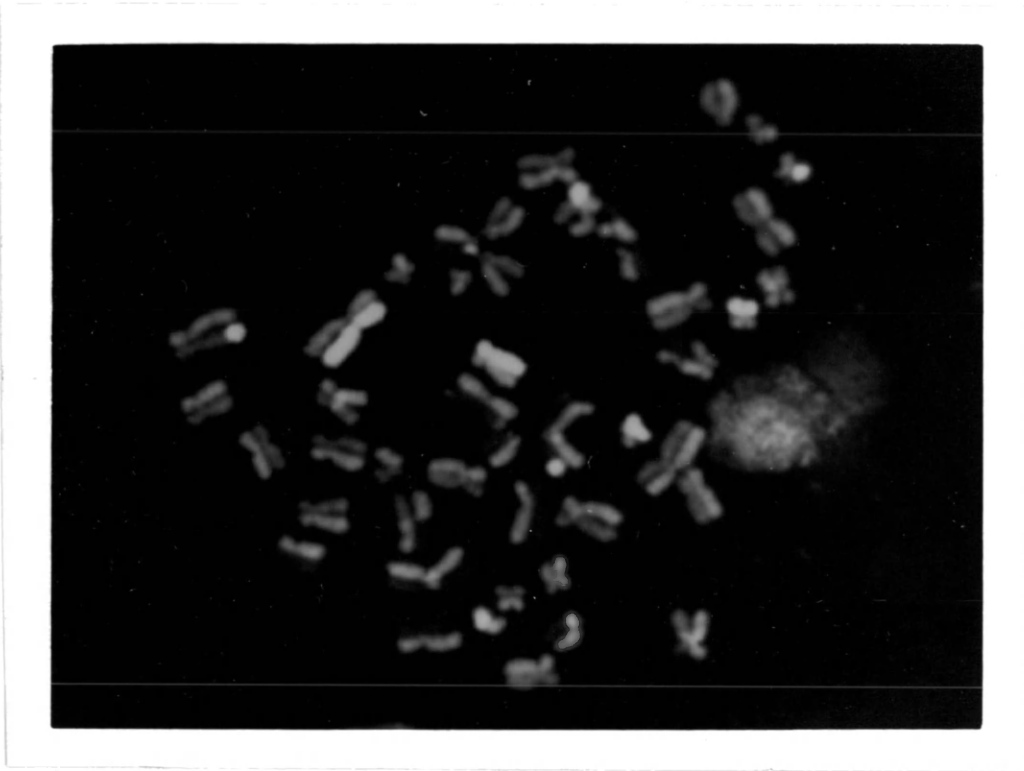
After three replications, some chromosomes are completely labeled.



After many replications, only a few areas of unlabeled chromatin appear.



In this spread, four different shades of differentiation appear. This is caused by decreasing incorporation of label in each generation. Where less label is incorporated, regions appear more and more like unlabeled chromatin. The original unlabeled chromatin still stands out the most strongly.



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