MUTATION INDUCTION IN SYNCHRONOUS HAMSTER CELLS

DONNER LABORATORY

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MUTATION INDUCTION IN SYNCHRONOUS HAMSTER CELLS

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ABSTRACT

Mutagenesis of synchronous Chinese hamster cells by 5-bromodeoxyuridine (BUdR) shows pronounced cell cycle dependency. Resistance to 6-thioguanine (6-TG) and ouabain are induced maximally by BUdR at different times early in the DNA synthesis period, suggesting that the genes coding for hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and the (Na⁺K⁺)-associated ATPase of the plasma membrane are replicated early in the DNA synthesis period. Although BUdR induces mutations in specific genes only when present during their replication, the rate of mutation induction is not linearly related to the amount of BUdR incorporated into DNA. The data show a BUdR concentration threshold for mutation induction, suggesting that BUdR exerts some detrimental allosteric effect on DNA synthesis enzymes.
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"I Took a good clear piece of Cork, and with a Pen-knife sharpen'd as keen as a Razor, I cut a piece of it off, and thereby left the surface of it exceeding smooth, then examining it very diligently with a Microscope, me thought I could perceive it to appear a little porous; but I could not so plainly distinguish them, as to be sure that they were pores, much less what Figure they were of: But judging from the lightness and yielding quality of the Cork, that certainly the texture could not be so curious, but that possibly, if I could use some further diligence, I might find it to be discernable with a Microscope, I with the same sharp Pen-knife, cut off from the former smooth surface an exceeding thin piece of it, and placing it on a black object Plate, because it was it self a white body, and casting the light on it with a deep planoconvex Glass, I could exceeding plainly perceive it to be all perforated and porous, much like a Honeycomb, but that the pores of it were not regular; yet it was not unlike a Honey-comb in these particulars.

"First, in that it had a very little solid substance, in comparison of the empty cavity that was contain'd between....

"Next, in that these pores, or cells, were not very deep, but consisted of a great many little Boxes, separated out of one continued long pore, by certain Diaphragms, as is visible by the Figure B, which represents a sight of those pores split the long-ways."

"... I told several lines of these pores, and found that there were usually about threescore of these small Cells placed end-ways in the eighteenth part of an Inch in length, whence I concluded that there must be neer eleven hundred of them, or somewhat more than a thousand in the length of an Inch, and therefore in a square Inch above a Million, or 1166400. and in a Cubick Inch, above twelve hundred Millions, or 1259712000. a thing almost incredible, did not our Microscope assure us of it by ocular demonstration.... ."

Micrographia
Robert Hook, 1665
INTRODUCTION

"Cheshire Puss," she began, "...Would you tell me, please, which way I ought to walk from here?"
"That depends a good deal on where you want to get to," said the cat.

Alice in Wonderland
Lewis Carroll

Cells are truly incredible; a cubic inch of living cork contains enough DNA to wrap around the earth forty times. Each cork cell contains about 2.0 meters of DNA packed into a nucleus only 0.00001 meters in diameter. DNA helices, barely 0.000000002 meters thick, are organized into a number of distinct chromosomes visible during mitosis; these chromosomes must be duplicated prior to mitosis so that each daughter cell contains DNA identical to that of the parent. Much recent progress in understanding the organization and functioning of such vast quantities of DNA stems from the capability to culture cells in laboratories under carefully controlled conditions.

In 1907, Ross Harrison cultured frog embryo tissue in clots of lymph fluid and observed that nerve fibers grew out of such tissue into the surrounding clot. His preparations would live for about one week, although an occasional one would live for over three weeks. Claims were soon made of extended growth of chicken cells in chick embryo juice and nutritional ingredients; however, these claims are suspect since the preparation of chick embryo juice left whole viable cells in the juice. The claimed growth was no doubt due to the influx of new cells at each dilution or refeeding. Nonetheless, apparent success stimulated improvements in technology and methodology.

The digestive enzyme trypsin was discovered to detach cells from
surfaces on which they were grown and has been used to prepare subcultures of cells at lower density whenever they crowd a container. It became apparent that high density subcultures would continue to grow, but low density subcultures would die. This density dependent growth was related to the volume of medium, since even a single cell would proliferate in a capillary tube. Cells themselves were somehow conditioning the medium to support growth.

One unwieldy method devised to grow single cells into colonies involved a non-proliferating feeder layer of cells to condition the medium. Confluent monolayers of cells were X-rayed at a dose which would inhibit cell division but not cell metabolism, and low densities of healthy cells would then grow on top of such feeder layers.

Finally, after intensive trial and error search for the magic ingredients, media were developed which would support low density cell growth. Yet, these media are still somewhat mysterious in that they must contain blood serum. Only the serum proteins are necessary, and different fractions of these have been found to influence growth, flattening, migration, or permeability of cells in culture. Serum proteins are not incorporated into cell protein and therefore modulate at the cell surface.

Nutritional requirements of cultured cells are similar to those of birds, fish, and mammals, all of which need (among other factors) the same amino acids in the diet. Cultured cells additionally require: cystine, which leaks out of them faster than they synthesize it; tryosine and arginine, which are synthesized in livers of intact organisms; and histamine and glutamine, which perhaps are provided by intestinal flora in the organisms. The only carbohydrate which cultured cells will utilize is dextrose \[D-(+)-glucose\]. About 95% of dextrose is anaerobically
metabolized to lactic acid.

The first widely used line of mammalian cells was started from a cervical carcinoma biopsy and was named "HeLa" after the patient Henrietta Lacks (Scherer et al., 1953). In attempting to establish other cell lines, it was found that DNA tumor virus transformed cells would grow indefinitely but that healthy tissue cells ("primary" cultures) would generally survive only thirty to fifty generations. Occasionally, however, one of the primary cultures would attain a capability for indefinite growth which could not be explained by the growth of any particular cell in the original explant. With a few exceptions, the karyotypes of such indefinite cell lines were aberrant; therefore the culture conditions were selective for an altered genotype. During establishment of the various "3T" mouse cell lines, primary cells increased in size, many of them ended up with aberrant karyotypes, and growth was poor.

Since chromosomes are visible only during mitosis, little was known about them between mitoses (the interphase period) from classical microscopy. With radioactively labeled chemicals, Howard and Pelc (1953) found that DNA synthesis occupied only a portion of interphase. This "S" phase or period is separated from mitosis by two gaps in synthesis, "G1" and "G2". The G1 period, extending from telophase to the onset of DNA synthesis, varies widely in duration as growth rate changes (Sisken and Kinosita, 1961), whereas the durations of S, G2, and M (mitosis) are relatively constant for cells of a given species. Generally, once DNA synthesis is initiated, a cell will progress through S, G2, and M into G1, where it will remain if not destined to divide again. The resting state of non-dividing cells such as lymphocytes has been termed "G0".

Given optimal conditions, a population of cells will increase in
number exponentially. Cells in exponential growth are distributed exponentially throughout the cell cycle, there being twice as many cells leaving mitosis as there are entering. Ideally, the population would double in the same length of time as the cell cycle; in actuality, since not all cells divide, and since cell cycle times vary, the population doubling time is slightly longer than the mean cell cycle time.

During the S phase, chromosome replication in cultured cells follows a consistent pattern generation after generation (Taylor, 1959) and homologous chromosomes replicate nearly simultaneously in identical patterns (Hsu, 1964). In male Chinese hamster cells, for example, the long arm of the X chromosome and the entire Y chromosome replicate late in the S period (Hsu, 1964); in female cells of that species the heterochromatic X chromosome, the Barr body visible during interphase, is the last chromosome to replicate. Generally, in fact, heterochromatic DNA replicates later than euchromatic DNA (Lima-de-Faria, 1959). Autoradiographs of chromosome replication have also shown that there are many simultaneous sites of DNA synthesis in mammalian cells.

Painter et al (1966) have estimated the number of sites of DNA synthesis in HeLa cells to be around $10^3$ or $10^4$ at any one time in the S period. Painter and Schaefer (1969) determined that the replication of DNA at any one site proceeds at about 1 μm/minute. A detailed autoradiographic study by Huberman and Riggs (1968) suggested that replication proceeds in two directions from a common origin and that the units of replication are about 30 μm long. As mentioned earlier, eucaryotic cells contain 1 to 2 m of DNA and thus about 50,000 replicating units total. Their autoradiographs also indicate that replication occurs at fork-like, or "Y" shaped, growing points.
The current understanding of enzymes responsible for DNA synthesis in mammalian cells is reviewed by Bollum (1975). There appear to be two non-mitochondrial DNA polymerases; both are found in the nucleus and in the cytoplasm. The higher molecular weight polymerase, of the order of 200,000 molecular weight, varies in concentration with the proliferative state of the cell, whereas the approximately 40,000 molecular weight polymerase does not vary in concentration. Both polymerases require initiator-template systems, complementary deoxynucleoside triphosphates, and a divalent cation. Neither polymerase can initiate new chains in vitro; they require DNA treated with a nuclease (activated DNA). The higher molecular weight polymerase, which sediments at 7 S, displays several enzymatic activities, but not as many as E. coli polymerase I. Polymerases bind about 1000 times tighter to DNA templates than to deoxynucleoside triphosphates, yet there is preliminary evidence that polymerases dissociate from DNA after incorporation of each base. All known polymerases synthesize DNA in the 5' to 3' direction.

Baril et al (1974) raise the possibility that the 7 S mammalian polymerase exists in vivo as a membrane associated multi-enzyme complex. The other enzymes they find in isolated complexes are thymidine kinase, thymidylate synthetase, and ribonucleotide reductase, all of which participate in the DNA biosynthesis pathway. Evidence for enzyme complexes involved in DNA synthesis in bacteria has also recently been presented (Hendler et al, 1975).

Since DNA replication proceeds only in the 5' to 3' direction, replication along one strand is toward the fork and replication along the complementary strand is away from the fork. Okazaki et al (1968) observed that, during procaryotic replication, small pieces of DNA are formed.
"Okasaki" pieces are also formed during eucaryotic DNA replication, and the ratio of small to large sized pieces of newly synthesized DNA suggests that replication is continuous along one strand and discontinuous on the other (Hershey and Taylor, 1974). Interestingly, nascent DNA seems to be associated with some nuclear complex because it is inaccessible to enzymatic degradation (Weintraub, 1974); and within two minutes following replication, DNA is bound to histones. New histones combine with new DNA almost exclusively at the growing point (Weintraub, 1974).

To further characterize general patterns of DNA synthesis, synchronous cell populations are often required. Of the many methods to synchronize cells, there are but two categories: induction and selection. Induction methods use physical or chemical insult to exponentially growing cell populations, but selection methods separate cells at different stages of the cell cycle with essentially no insult. Induction methods are advantageous in that substantial fractions of, or perhaps the entire, exponentially growing population can be synchronized. However, different cells in such a synchronous population have experienced different histories and may consequently act differently. Also the entire population may be altered by the method used to synchronize it. Selection methods, on the other hand, generate smaller numbers of synchronous cells, but presumably those cells have continued unhampered through their cell cycles.

Typical of induction techniques is inhibition of DNA synthesis. Such inhibition results in large numbers of cells at the beginning of the S period; when the inhibition is released temporarily and then reinstituted, all cells in the population will end up at the beginning of the S period. These cells will not at all necessarily have ceased any aspect of growth except DNA synthesis, and therefore may be different from cells growing
Selection methods are far less disruptive to cell cycles. One widely used technique relies on the fact that cells entering mitosis round up and lose most of the surface attachments of flattened interphase cells. Simply shaking flasks of cells and decanting the medium produces a synchronous population (Terasima and Tolmach, 1963).

Braun and Wili (1970) have used synchronous populations of the multinucleate acellular slime mole Physarum to show that DNA replicated at a specific time in one S period is replicated at the same time in the next. Thus, the temporal replication of specific genes or types of DNA can be investigated. Much of the DNA of mammalian cells consists of repeated sequences, and certain families of repeated sequences replicate predominantly in the second half of the S period (May and Bello, 1974). The 300 or so copies of ribosomal genes in Chinese hamster cells replicate primarily in the first half of the S period (Stambrook, 1974). Temporal replication of individual genes has been demonstrated in bacteria (Cerdá-Olmedo et al, 1968) and in yeast (Kee and Haber, 1975) by preferential mutagenesis of the replication points with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). In mammalian cells, however, the mutagenicity of MNNG appears independent of the position of cells in the replicative cycle (Orkin and Littlefield, 1971).

Genetic characterization of cultured somatic cells has proven somewhat formidable because recessive mutations are difficult to induce in diploid cells. Only a few phenotypic variants have been easy to isolate; presumably some genes are heterozygous by chance or hemizygous by sex linkage or aneuploidy of the particular cell line. Even in female cell lines sex linked traits are functionally hemizygous due to inactivation of one X
chromosome. This inactivation is obvious in whole animals from the normal phenotype of X0 females and from the mosaic phenotype of females heterozygous for some sex-linked mutant (Lyon, 1961).

In the early years of somatic cell genetics, mutation was not the only hypothesis which could account for phenotypic variants; shifts in phenotypic expression as occur in developmental differentiation could give rise to variant cells. Some experimental evidence supported the differentiation hypothesis, particularly the observation that ploidy did not appreciably alter mutation rate (Harris, 1971). Lack of ploidy effects does not accord with expectations based on dominant, co-dominant, or recessive mutations. Additionally, a presumably mutant enzyme could reappear after fusion of cells of two different species (Watson et al., 1972). Such reappearance was a rare phenomenon.

Recent work has helped clarify experimental interpretations. Chasin (1975) has shown that a chromosome-wide event frequently accompanies expression of recessive mutations in tetraploid cells, but has not determined whether the event is chromosome loss, mitotic recombination, or X chromosome inactivation. Whatever the event(s) may be, it tends to increase the expected low mutation rate for recessive traits in tetraploid cells. The trait studied in the early experiments cited was resistance to 8-azaguanine, which has turned out to be far more complicated than expected and will be discussed later.

Somatic cell variants in culture are isolated by some selection procedure which discriminates between them and normal cells. Thompson and Baker (1973) have listed criteria that variant cells should meet if they are mutant:
1) The altered phenotype breeds true in the sense that it is stably transmitted through consecutive generations.  
2) The frequency of occurrence of the phenotype can be enhanced by the application of mutagenic agents.  
3) The phenotype can ordinarily be associated with an altered gene product (usually a protein).  
4) The phenotype can be attributed to a specific region of the genome, i.e., it can be mapped in a linkage group which behaves in a Mendelian manner.  

And, in general, somatic cell variants do show the required characteristics. Isolated mutants have maintained their phenotypes through hundreds of cell divisions in the absence of selective pressure (Chu et al, 1969). Physical and chemical mutagens do enhance mutation frequencies (Chu, 1971; Chu et al, 1968). Altered proteins have been identified by biochemical (Sharp et al, 1973) and immunological (Wahl et al, 1974) analysis. Finally, several mutants have been identified with particular loci on specific chromosomes (Ruddle, 1972).  

Notwithstanding, alterations in phenotypic expression occur without concomitant mutation. Convincing examples are the extinction (Davidson et al, 1969) and the induction (Peterson and Weiss, 1972) of differentiated cell products in hybrids formed between different types of specialized cells. Conceivably, then, genetic and epigenetic alterations of phenotype have sometimes been confused.  

A widely studied genetic marker is the resistance to cytotoxic purine analogues. Such resistance can be due to loss or alteration of the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT, E. C. 2.4.2.8). This enzyme, along with adenine phosphoribosyltransferase, salvages exogenous purines for DNA synthesis (figure 1). In humans, deficiency of HGPRT is
Fig. 1. Salvage of exogenous purines by HGPRT.
characterized by enormous de novo overproduction of purines, increased uric acid in the urine and hyperuricemia, mental retardation, spastic cerebral palsy, and aggressive self-mutilating behavior. The disease, known as the Lesch-Nyhan disorder (Nyhan, 1972), is sex-linked. Partial HGPRT deficiency leads to milder neurological problems, but gouty arthritis, increased uric acid excretion, and renal complications are common.

Due to the severity of Lesch-Nyhan syndromes, enzymatic analyses have been performed on cultured amniotic cells from antenatal examinations and on cultured fibroblasts from adult patients. Migeon et al (1968) found two clonal populations in heterozygous females; random inactivation of one X chromosome early in development leads to such mosaics. HGPRT in humans is linked to glucose-6-phosphate dehydrogenase (G6PD) and phosphoglycerate kinase (PGK); they are located on the long arm of the X chromosome (Ricciuti and Ruddle, 1973).

In Chinese hamsters the same three genes are linked (Westerveld et al, 1972) and presumably are also sex-linked. Considerable isolation and characterization of 8-azaguanine (8-AG) and 6-thioguanine (6-TG) resistant Chinese hamster cells has been reported. There are several possible biochemical bases for such resistance (Thompson and Baker, 1973):

1) decreased activity (or altered substrate specificity) of HGPRT;
2) decreased transport of analogue into the cell;
3) decreased feedback inhibition of de novo purine biosynthesis;
4) increased degradation of nucleotides by alkaline phosphohydrolases; and
5) altered substrate specificity of nucleotide phosphorylase or ribonucleotide diphosphate reductase.

Cells selected on the basis of their resistance to 8-AG do not uniformly fall into the category of HGPRT deficiency. Most 8-AG resistant cells have an appreciable percentage of wild type HGPRT activity and are not sensitive to back selection techniques for HGPRT reversion (Gillin et al., 1972). Cells resistant to 6-TG, on the other hand, generally have low levels of HGPRT; this may be related to the greater HGPRT substrate specificity for 6-TG than for 8-AG. Some isolated 8-AG resistant clones are not permanently resistant to 8-AG (Carson et al., 1974); this may be due to transient karyotype instability (Terzi, 1974). Differences between 8-AG and 6-TG resistant cells can be demonstrated by complementation between certain clones (Sekiguchi et al., 1974).

Chinese hamster HGPRT has been thoroughly characterized (Olsen and Milman, 1974). Its molecular weight is 75,000 to 85,000 and it has subunits of molecular weight 25,000; thus it is probably a trimer. At least three isozymes have been detected and these may be due to post-transcriptional modifications. The $K_m$'s for hypoxanthine and guanine are 0.52 $\mu$M and 1.1 $\mu$M respectively; the $K_m$ for 5-phosphoribosyl 1-pyrophosphate (PRPP), at a $\text{Mg}^{++}$ concentration of 5.6 mM, is 5.3 $\mu$M. The HGPRT within cells under normal growth conditions could, if necessary, supply all the purines needed for DNA and RNA synthesis.

HGPRT assays of protein extracted from 8-AG or 6-TG resistant cells often reveal altered $K_m$'s or altered heat labilities (Sharp et al., 1973). Occasional mutant enzymes exhibit sharper discrimination between hypoxanthine and the selective agent. Some revertible mutant clones have neither HGPRT activity nor anti-HGPRT immunological cross reactivity (Beaudet et al., 1973);
this can be explained on the basis of premature chain termination mutations or regulatory gene mutations. Many partially 8-AG resistant clones contain decreased amounts of normal HGPRT, again suggesting the possibility of regulatory gene mutations (Littlefield, 1963).

To study mutation of the HGPRT locus, selection with 6-TG is clearly indicated. Other selection factors which need consideration are cell density, 6-TG depletion and/or degradation, HGPRT turnover time (expression time for HGPRT\textsuperscript{-} mutants), and serum influence.

High cell density may hamper selection of 6-TG cells in culture due to metabolic cooperation between cells. Transfer of material between cells is correlated with the ability to form gap, or low resistance, junctions. Via such junctions, normal cells convey a toxic substance to otherwise resistant cells. The transferred material is of molecular weight less than 1000 and is probably inosinic acid or a derivative (Sheridan, 1974); it does not diffuse through the medium (van Zeeland, 1972). Since resistant cells reestablish their phenotype immediately upon dispersion, transfer of HGPRT itself or an informational macromolecule is not involved.

High cell density may also deplete the selective medium of 6-TG by its incorporation into wild-type cells. Another way 6-TG depletion could occur is via thermal instability of the 6-TG molecule.

A cell which mutates to HGPRT\textsuperscript{-} will remain phenotypically HGPRT\textsuperscript{+} for some period of time due to stability of the HGPRT or m-RNA molecules previously synthesized. Only after a certain expression time will these molecules degrade or dilute by cell division to a low enough concentration to become ineffective.

One other influence on selection is that of the serum used in preparing growth medium. Different sera often cause different measure-
ments of mutation rate; this complication is due mostly to various concentrations of hypoxanthine in different lots of serum (Thompson and Baker, 1973). Serum dialysis or purchase of large lots of identical serum will ensure more consistency in measured mutation rates. However, reproducibility can never be assured, because the same cell line carried by serial dilutions can vary up to 1000 fold in spontaneous mutation frequency over long periods of time (Thompson and Baker, 1973). Induced mutation frequencies can be kept consistent by use of identical serum if the induced frequency is sufficiently greater than the spontaneous frequency.

A recently characterized mutation in mammalian cells is resistance to ouabain (Baker et al, 1974), a specific inhibitor of the (Na⁺K⁺)-activated ATPase of the plasma membrane. Ouabain inhibits uptake of K⁺ by normal cells and consequently is toxic. Cells resistant to ouabain presumably have an altered ATPase with either reduced binding to ouabain or reduced steric response to bound ouabain.

Resistance to ouabain behaves as a co-dominant trait (Baker et al, 1974) and can be selected for in single step assays. Selection is not complicated by permeability effects, since ouabain acts at the cell surface, or metabolic cooperation. The K⁺ concentration in growth medium does affect selection: the more K⁺, the less ouabain toxicity. The frequency of ouabain resistance is enhanced by chemical mutagens.

Induction of mutations in mammalian cells by the thymidine analogue 5-bromodeoxyuridine (BUdR) has been demonstrated by Chu et al (1972), Huberman and Heidelberger (1972), and Stark and Littlefield (1973). The molecular mechanism of BUdR mutagenesis in eucaryotic cells is not known, but it has been presumed to be similar to the known mechanism in phage and bacteria. On the basis of mutation studies with phage T4, Freese (1958)
proposed that during incorporation into DNA or during subsequent DNA replication BUdR would infrequently pair with guanine rather than adenine (figure 2). Mispairing would result in G-C to A-T transitions or vice versa, and BUdR can in fact induce reversion of BUdR induced mutations. The greater electronegativity of the bromine atom as compared to the methyl group of thymidine is thought to account for higher frequencies of enolic BUdR (Lawley and Brookes, 1962).

In vitro replication of a DNA-like polymer of alternating adenine and BUdR residues shows an unequivocal incorporation of guanine into the newly synthesized strand (Trautner et al., 1962). E. coli polymerase I, dATP, BUdR-TP, and dGTP$^{32}$ in appropriate buffers comprised the reaction mixture. The nearest neighbors of incorporated guanine would always be BUdR on the basis of the model proposed by Freese, but guanine was found to be next to guanine just as often as BUdR and less frequently next to adenine. Thus the mutagenicity of BUdR in vivo may be more complex than the proposed model.

Incorporation of thymidine or BUdR into DNA of mammalian cells begins with phosphorylation by thymidine kinase (E. C. 2.7.1.21). Two additional phosphorylations produce nucleoside triphosphate precursors for DNA synthesis. In Chinese hamster cells there is no preferential incorporation of thymidine or BUdR into DNA when both are supplied in the medium (Cleaver, 1970).

However, several factors can influence thymidine or BUdR incorporation into DNA of cells in culture (Cleaver, 1967). Thymidine kinase is inhibited by dTTP, the end product of the sequence of reactions which it begins; thus when dTTP or BUdR-TP accumulate in the cell, they begin to shut off the exogenous supply. The triphosphates also inhibit de novo thymine synthesis. Intracellular pools of dTTP complicate BUdR incorporation into DNA.
Fig. 2. Base pairing of BUDR.
The pool size and the flows in and out of the pool have been modeled mathematically and, as with many mathematical treatments, the limits provide useful information. Inflows to the pool are endogenous thymine or exogenous thymidine or BUdR; outflows from the pool are DNA synthesis or nucleoside triphosphate degradation; and the pool size is of the order of $2 \times 10^{-4}$ M for cultured mammalian cells (Burki and Okada, 1969). At low exogenous BUdR concentration, any phosphorylated BUdR will be substantially diluted into the pool; whereas at high exogenous BUdR concentration, BUdR-TP will essentially dilute the pool. Thus the substitution of BUdR for thymidine during DNA synthesis will be linearly related to the exogenous BUdR concentration only at low concentrations.

At quite high exogenous concentrations of thymidine or BUdR, around $2 \times 10^{-3}$ M, DNA synthesis is actually inhibited (Xeros, 1962) by a feedback inhibition of the deoxycytidine pathway (Gentry et al, 1965).

BUdR affects cells in numerous other ways. At the chromosomal level, bifilar labeled DNA stains differently than unifilar labeled DNA (Wolff and Perry, 1974). Either bifilar or unifilar labeling of DNA with BUdR causes delay in the spiralization of late replicating chromosome regions (Zakharov and Egolina, 1972). The delay cannot be explained solely on the basis of BUdR incorporated into DNA because there is no delay at the second metaphase even though one chromatid is still labeled with BUdR.

BUdR sensitizes cells to X-rays (Djordjevic and Szybalski, 1960) and to ultraviolet light (Greer, 1960). Fielder et al (1971) have observed extensive intramolecular migration of charge or excitation energy to BUdR substituted sites in DNA, and they postulate that subsequent release of bromide ions from those sites leaves 5' uracil radicals. Such radicals are not found in normal DNA similarly irradiated and may explain the
increased radiation sensitivity.

BUdR has a detrimental effect on cloning efficiency of cells in culture which is contingent on DNA synthesis (Kajiwara and Mueller, 1964). Interestingly, the effect is pronounced when early replicating DNA is labeled and negligible when late replicating DNA is similarly labeled. Remarkably, though, a mutant cell line has been isolated which actually grows better with total BUdR substitution for thymidine in the DNA (Bick and Davidson, 1974). The thymidine content of these cells has increased about 15%, indicating substantial G-C to A-T transitions, yet the cells are viable. It is not clear whether such transitions occurred only during selection of the cell line or whether they still occur.

RNA transcribed from BUdR-labeled DNA templates differs in base composition from RNA transcribed from unlabeled control templates (Hill et al., 1974); the uridine proportion remains similar, but the guanosine proportion increases considerably. Increased guanosine proportion is seen in RNA extracted from whole cells, isolated nuclei, or chromatin, and in RNA transcribed in vitro. The in vitro alteration is more pronounced with chromatin than with naked DNA, implying that chromatin structure is altered by the BUdR substitution. These observations do not discriminate between synthesis of altered mRNA and synthesis of normal mRNA from altered portions of the genome. Whichever the case, mutation of DNA is not responsible because the effects are reversible by growth of the same cells in normal medium without BUdR.

BUdR incorporation into DNA has been shown to alter protein binding to such DNA (Lin and Riggs, 1972). The lac repressor of bacteria binds ten times tighter in vitro to BUdR substituted lac operator than to normal lac operator. This suggests that in vivo induction of lactose metabolizing
enzymes would require higher concentrations of inducer following BUdR labeling. In differentiated eucaryotic cells in culture, BUdR labeling lowers the concentration of certain enzymes (Stellwagen and Tomkins, 1971). Holtzer and Abbott (1968) noted that BUdR reversibly and selectively suppresses the synthesis of "luxury" molecules without grossly depressing the synthesis of essential molecules; quite conceivably, repressor proteins may exist only for differentiated functions.

Undifferentiated cells can be irreversibly affected by BUdR. Weintraub et al (1972) found that BUdR labeling of precursor erythrocytes prevents synthesis of hemoglobin by their progeny, and Levitt and Dorfman (1974) showed that chick embryo limb bud cells exposed to BUdR fail to differentiate into cartilage. In the developing limb bud cells, a most unexpected and unexplained excision of BUdR from DNA is observed; guanine is not similarly excised. BUdR, and thymidine to a lesser extent, is excised in low molecular weight pieces. Mature chondrocytes do not demonstrate such unusual metabolism. The irreversible nature of BUdR effects in early embryonic cells may be intimately related to this phenomenon.

The present study explores the mutagenicity of BUdR in cultured mammalian cells. If BUdR mutations are due simply to base mispairing with guanine, then mutants of a particular gene should be induced only when cells are labeled with BUdR at the time that gene is replicating. Additionally, the yield of mutants should be linearly related to the amount of BUdR incorporated into cellular DNA. As will be shown, BUdR induces mutations only in that portion of DNA into which it is incorporated, but by a mechanism more complex than base mispairing alone.
MATERIALS AND METHODS

"Though this be madness, yet there is method in't."

*Hamlet, Prince of Denmark*

*William Shakespeare*

Cell Lines.

The two cell lines used in these experiments were derived from the Chinese hamster, *Cricetulus griseus*, which has a diploid chromosome number of 22. The V79 cell line, described by Ford and Yerganian (1958), was derived from a male hamster lung; its modal chromosome number is nearly diploid (2n = 23). The V79-S171 strain was obtained from Sinclair, Argonne National Laboratory, in 1968. The CHO cell line was derived by Tjio and Puck (1958) from a hamster ovary; it too is pseudodiploid. CHO cells were obtained from Wolff, U.C. Medical Center in San Francisco, in 1973.

Cells have been stored frozen in this laboratory at -196°C in a cryoprotective media plus 5% dimethyl sulfoxide (DMSO). Ampoules of this frozen stock were periodically thawed and propagated for experimental use to ensure minimal changes in the characteristics of the cell lines. These cell line were free from PPLO as tested by the S.F. Medical Center PPLO Laboratory.

Culture Conditions.

Cells were grown in 90 mm Falcon plastic petri dishes at 37°C in a humid 5% CO₂ atmosphere. They were subcultured two or three times weekly to densities of $2 \times 10^6$ and $2 \times 10^5$ cells/dish; 0.03% trypsin (Worthington Biochemical) in Puck's saline A was used to detach cells; a model F Coulter Counter was used to determine cell density.
V79 cells were grown in medium composed of: 500 ml Puck's saline F; 5 ml BME amino acids (100X); 2.5 ml BME vitamins (100X); 5 ml L-glutamine (200 mM); 5 ml penicillin (10,000 units/ml) and streptomycin (10,000 μg/ml); 0.15 ml CaCl₂·H₂O (33.3% w/v); and 60 ml fetal calf serum. The V79 population doubling time in exponential growth was close to 11 hours.

CHO cells were grown in a medium of: 500 ml McCoy's Medium (modified, with glutamine); 5 ml penicillin and streptomycin as above; and 75 ml fetal calf serum. Their population doubling time in exponential growth was close to 13 hours.

CaCl₂·H₂O was obtained from Worthington Biochemical; other components came from Grand Island Biological.

### Synchrony.

V79 cells were trypsinized from two day old subcultures and innoculated into 3 oz glass prescription bottles at 2×10⁵ cells in 10 ml of medium per bottle. Generally sixteen bottles were innoculated and incubated for 36 hours. Loose cells were then detached by 2 minutes of shaking by a Burrell "Wrist-Action" mechanical shaker; bottles oscillated through roughly 3° at about 4 cycles/second. The medium was then decanted and discarded to eliminate dead and floating cells from the population. The bottles were rinsed with 6 ml Puck's saline A prior to addition of 10 ml fresh medium. After incubation for one hour, shaking was repeated and the medium was decanted into a large-mouthed sterile bottle. Stock hydroxyurea (Calbiochem) at 10⁻² M in Puck's saline A was added to a final concentration of 10⁻³ M, and replicate cultures of this cell suspension were established at 10 ml/plate. The last 10 or so ml were used for counting cells.
Generally this regimen was repeated; fresh medium was added immediately after decanting detached cells and the bottles were returned to the incubator. Thus a second set of replicate plates could be established one hour after the first set.

V79 cultures were incubated 8 hours in hydroxyurea, at which time plates were rinsed twice with Puck's saline A and fresh growth medium was substituted. All saline, medium, and chemical solutions were pre-warmed to 37°C before use, and cells were protected as best possible against temperature shock by placing bottles on 37°C slide warmers when out of the incubator, except during the shaking procedure itself.

Colcemid (Grand Island Biological), when used to test shake-off procedures, was prepared at 6 µg/ml in sterile water and added to medium in the prescription bottles for the hour prior to shake-off; the final concentration of colcemid was 0.06 µg/ml.

Synchronizing CHO cells was quite similar to the above procedure. Twelve to fourteen Corning 250 ml tissue culture flasks were inoculated at 10⁶ cells/flask. Shaking was done by hand for 10 seconds at a frequency of about 2 gentle shakes/second. The replicate cultures established as above were incubated in hydroxyurea for 10 hours.

Pulse Labeling.

Stock solutions of BUdR (Calbiochem) were prepared at 10⁻³ M and 10⁻² M in Puck's saline A. To label a synchronous culture, the appropriate stock solution was added to the medium to the desired final concentration, the plate was incubated for the desired interval, and the BUdR was removed by two rinses with Puck's saline A prior to replacement with fresh medium.

Incorporation of BUdR into cellular DNA was determined by the addition
of $^{3}$H-BUdR (27.7 C/mmole, 0.5 mC/ml) to stock $10^{-3}$ M BUdR or $^{3}$H-thymidine (13.9 C/mmole, 1.0 mC/ml) to stock $10^{-2}$ M BUdR. Since there is no preferential incorporation of BUdR or thymidine into DNA when endogenous thymine synthesis is blocked, $^{3}$H-thymidine was used only with final concentrations of BUdR that would tend to inhibit endogenous thymine synthesis. The high cost of $^{3}$H-BUdR made use of $^{3}$H-thymidine desirable with high BUdR concentrations.

$^{3}$H-hypoxanthine (12 C/mmole, 0.5 mC/ml) was obtained for determination of hypoxanthine uptake by 6-TG resistant clones and for HGPRT assays of isolated cell proteins. All radiochemicals were products of New England Nuclear.

Scintillation Counting.
Radioactively labeled cells were trypsinized, counted, and sedimented in a cold centrifuge at 1500 rpm. The pellet was resuspended in 1 ml of 10% cold trichloroacetic acid (TCA) and the acid insoluble cell constituents were sedimented as above. These were resuspended in 1 ml of 5% TCA and boiled in a water bath for 30 minutes. This suspension was decanted into a plastic counting vial (Amersham/Searle), and 10 ml of PCS scintillation fluid (Amersham/Searle) was poured into the test tube, then into the vial.

Counting was done with a Nuclear Chicago scintillation counter. The counting efficiency was determined by channel ratios calibrated with a set of quenched standards.

Selection.
Stock 6-TG was prepared at 50 $\mu$g/ml in Puck's saline A. Three drops of 1 N NaOH were added during stirring to help dissolve the 6-TG; three
drops of 1 N HCl were subsequently added. Selection was done at a final concentration of 5 μg/ml. Stock ouabain was prepared at 0.03 M in phosphate buffered saline, pH 7.2; selection was done at a final concentration of 3 mM.

Expression times were 4 days for mutagenized V79 cells and 5 days for mutagenized CHO cells. Selection for 6-TG resistance in V79 cells was maintained for 9 days, with a replenishment of the selective medium at 4 days. Selection for 6-TG or ouabain resistance in CHO cells was maintained for 8 days. Colonies were stained in 1% methylene blue for counting.

Cloning.

Colonies of 6-TG resistant V79 cells were isolated by mechanically punching small discs out of the bottom of plastic petri dishes (Todd et al, 1966). Medium was first aspirated out of the dish, the bottom was sterilized with 70% ethanol, and the colony was placed cells-down over a hole in the punch structure. The lever operated punch was pressed down and a plastic disc with the colony attached fell through the hole into a small petri dish containing trypsin solution. After the cells detached, medium was added and the cells were incubated. Colonies so isolated were later subcultured into 90 mm petri dishes in selective medium, grown for several days, and frozen in 5% DMSO medium for later analysis.

Isolated clones were identified with the date of the experiment and the number of the interval labeled with BUdR; i.e., clone 3-18-3d was isolated from the third interval (1.0 - 1.5 hours) of an experiment started March 18. When two or more colonies were isolated from one plate, they were identified by a lower case letter following the interval. Colonies isolated from control plates were identified with a "C" rather than an interval number.
HGPRT Assay.

The formation of inosinic acid (figure 1) was measured in a radioisotope assay (Olsen and Milman, 1974). Cells were trypsinized and counted, then sedimented for 5 minutes at top speed in a desk-top clinical centrifuge. The medium was removed and the cells were lysed by suspending in 200 µl of distilled water and quick freezing in powdered dry ice. The lysate was immediately thawed and 15 µl of 75 mM dithiothreitol and 25 µl of buffer (0.5 M Tricine, pH 7.5, 0.5 M KCl, 0.2 M MgCl₂, and 1.0 mM ethylenediaminetetraacetic acid) were added. The debris was removed by centrifugation at 19,000 x g for 10 minutes in a Sorvall SE 12 rotor.

The assay mixture contained 50 mM Tris-HCl, pH 7.8, 7 mM MgCl₂, 2 mM dithiothreitol, 1 mM PRPP tetrasodium salt, and 0.06 mM hypoxanthine with 10 µC/ml ³H-hypoxanthine. Reaction mixtures of 50 µl assay mixture and 50 µl enzyme sample were incubated for 15 minutes in a 37°C water bath; incubation was terminated by adding 150 µl of cold 50 mM Tris-HCl, pH 7.8, and placing the mixture on ice. A 200 µl aliquot was added to a Pasteur pipette column (3.5 x 0.5 cm) of DEAE-cellulose (capacity 0.95 meq/g) equilibrated with 50 mM Tris-HCl, pH 7.8. Unreacted hypoxanthine was washed out with 5 ml of the same buffer, and the inosinic acid was eluted with 1.5 ml of 1 N HCl. The nucleotide was counted in 10 ml of PCS scintillation fluid.

Protein extracts from 2 to 3 x 10⁶ 6-TG resistant cells were assayed along with control extracts from 10⁵ and 10⁶ V79 cells and a blank enzyme buffer.
RESULTS

"In anything at all, perfection is finally attained not when there is no longer anything to add, but when there is no longer anything to take away."

*Wind, Sand and Stars*
Antoine de Saint Exupery

Early experiments were performed with V79 cells. Later, CHO cells were used exclusively due to the greater ease in obtaining large numbers of synchronized cells. Data on synchrony of V79 and CHO cells are presented together; all other data are grouped according to cell line.

**Synchrony.**

Several preliminary selection experiments were performed using cells synchronized by hydroxyurea alone (Sinclair, 1965 and 1967). Hydroxyurea is toxic to cells in *S* period and inhibits other cells from initiating DNA synthesis. Thus, hydroxyurea-synchronized populations contain about 60% metabolically active, but reproductively dead, cells which make such populations inappropriate for metabolic studies. Although hydroxyurea synchronization is quite simple, it was abandoned so that selection and metabolic studies could be compared directly.

**Hydroxyurea.** Growth of V79 cells exposed to hydroxyurea is retarded relative to exponentially growing control cells (figure 3). During the first hour and a half of hydroxyurea exposure, cell number increased due to division of *G₂* cells; subsequently, there was no further increase for the duration of exposure. The increase in cell number between 5 and 7 hours after rinsing hydroxyurea off the plates corresponds to mitosis of half the cells on each plate. This level of mitosis following hydroxyurea
Fig. 3. Effect of hydroxyurea on asynchronous V79 cells. Replicate cultures were established at 168,000 cells/plate. Open circles show growth of cells exposed to 1.0 mM hydroxyurea; closed circles show growth of controls.
treatment reflects the reproductive death of cells which were in S period.

**Shake-off.** Cells selected from asynchronous populations by shake-off were found to have a mitotic index less than 50%. If colcemid were added to the medium for an hour prior to shake-off, the cells then selected had about a 90% mitotic index. A qualitative comparison of cell sizes was made by observing Coulter counter oscilloscope pulses: cells selected after colcemid exposure produced large pulses, whereas cells selected without colcemid produced predominantly small pulses. Since colcemid prevents cells from completing mitosis (Stubblefield et al., 1967), it also depletes a cell population of small, early G₁ cells. Thus, shake-off selects mitotic and early G₁ cells, both of which are rounded and loosely attached to the culture flasks (Peterson et al., 1968).

To determine the initiation time of DNA synthesis following shake-off, replicate cultures so established were sequentially exposed to ³H-thymidine. Incorporation of ³H-thymidine into DNA does not begin for some time after shake-off (figure 4); in fact, the G₁ period is extended, particularly in CHO cells. The delay may be due to temperature shock or other traumas associated with shake-off techniques.

**Shake-off into hydroxyurea.** To obtain good synchrony during the S period, cells selected by shake-off were blocked from DNA synthesis with hydroxyurea. After release of the hydroxyurea block, cells synchronously completed S phase and mitosis (figures 5 and 6). Completion of S phase is marked by an increased tolerance to a second hydroxyurea treatment. Completion of mitosis is marked by an increase in cell number; the cell number does not double, but does correspond to the 70 to 80% plating efficiency of these cell lines.
Fig. 4. Initiation of DNA synthesis following shake-off. Replicate cultures were established at 40,800 CHO cells/plate and 37,200 V79 cells/plate. Cultures were sequentially exposed to 0.2 µCi/ml (CHO) or 0.1 µCi/ml (V79) of $^3$H-thymidine for one hour intervals. At the end of each interval, labeled DNA was isolated for scintillation counting.
Fig. 5. Synchrony of V79 cells. Triangles show the ratio of cell number at subsequent times to cell number at rinsing off the hydroxyurea; data are the averages from shake-offs yielding 49,100 cells/plate, 36,200 cells/plate, and 19,800 cells/plate. Circles show survival of cells following a second 8 hour exposure to 1.0 mM hydroxyurea starting at the times plotted. Innoculations were 200 cells/plate; control plates received no second hydroxyurea exposure. The average survivals obtained from two shake-offs are shown.
Fig. 6. Synchrony of CHO cells. Triangles show the ratio of cell number at subsequent times to cell number at rinsing off the hydroxyurea; data are the averages from shake-offs yielding 113,600 cells/plate and 84,000 cells/plate. Circles show survival of cells following a second exposure to hydroxyurea for 6 hours starting at the times plotted. Innoculations were 200 cells/plate; control plates received no second hydroxyurea exposure. The average survivals from two shake-offs are shown.
V79 cells complete the S period somewhat more rapidly following shake-off into hydroxyurea than they do in asynchronous growth. This may be due to their having been blocked at the transition into S phase for several hours, during which time aspects of cell growth other than DNA synthesis proceed (Rosenberg and Gregg, 1969).

Studies With Male V79 Cells.

Medium for all experiments with V79 cells was made with serum from one manufacturer's lot. The 4.5 hour S period of synchronized populations was arbitrarily considered at sequential 30 minutes intervals. BUDR pulse labeling was done at $10^{-4}$ M.

**BUDR toxicity.** Under these experimental conditions, toxicity due to BUDR exposure does not vary significantly during the S phase (Table 1). Relative to unlabeled controls, between 80 and 90% of cells survive to form colonies. The amount of BUDR incorporated into DNA varies moderately during the S phase (figure 7, bottom). Maximum incorporation occurs between 2.5 and 3 hours, and there is a sharp decline in incorporation toward the end of the S period.

**Induction of 6-TG resistance.** The number of 6-TG resistant colonies resulting from BUDR incorporation during different portions of the S phase varies considerably (figure 7, top). The data of four independent experiments (Table 1) are normalized to $10^5$ surviving cells. There is large uncertainty in these data, but the combined results clearly show the temporal response evident in each experiment separately.

From a comparison of BUDR incorporation with induction of 6-TG resistant colonies under exactly the same labeling conditions, it is clear that induction of 6-TG colonies is not dependent on the amount of BUDR incorporated into DNA.
Fig. 7. BUdR uptake and induction of 6-TG resistance in V79 cells. Triangles show $^3$H disintegrations in DNA after labeling various portions of the S period with 2.0 $\mu$C/ml $^3$H-BUdR in $10^{-4}$ M total BUdR. Six hours after release of the hydroxyurea block, cells were trypsinized off plates and DNA was isolated for scintillation counting. Data are the averages from shake-offs yielding 65,100 cells/plate and 42,500 cells/plate. Circles show the number of 6-TG resistant colonies selected following $10^{-4}$ M BUdR labeling and a 4 day expression time. Error bars show standard deviations of the means of the four experiments detailed in Table 1. The dashed line indicates the number of colonies resistant to 6-TG on control plates.
Hours following release of hydroxyurea block
TABLE 1. BUdR toxicity and induction of 6-TG resistance.

<table>
<thead>
<tr>
<th></th>
<th>hours following release of hydroxyurea block</th>
<th>initial cells/plate</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0-1/2</td>
<td>1/2-1</td>
</tr>
<tr>
<td>Colonies, % control (error)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>92</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>(1)</td>
<td>(5)</td>
</tr>
<tr>
<td>6-TG resistant colonies per plate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
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<td></td>
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<td>0</td>
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<tr>
<td></td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>
Characterization of 6-TG resistant clones. During the course of preliminary selection experiments in which synchrony was induced solely by hydroxyurea treatment, 24 clones resistant to 6-TG were isolated. These preliminary experiments showed the same temporal induction of resistant colonies as those for which data are shown.

The first three clones isolated, 8-1-1, 8-1-2, and 8-1-3, were subcultured in normal medium for three months, at which time their plating efficiency was essentially unaffected by 6-TG at the selective concentration of 5 μg/ml. The phenotypic stability of other clones was not tested, but many of the plating efficiencies were tested several weeks after thawing cells from stocks.

For the most part, plating efficiencies are unaffected by 5 μg/ml of 6-TG (Table 2). The ratio of plating efficiency in 6-TG to plating efficiency in normal medium is 1.00 ± 0.07 for 15 clones. Most of the clones incorporate very little tritiated hypoxanthine into DNA and have very little measurable HGPRT activity (Table 2). The clones with substantial HGPRT activity, 3-18-2a, 3-25-2b, and 3-25-8, are among those which exhibit lower plating efficiency in 6-TG than in normal medium. Uptake of hypoxanthine by these clones is less than would be expected on the basis of their high HGPRT activity, and thus their resistance to 6-TG may be due to altered membrane transport or increased purine degradation.

All isolated clones have growth rates similar to V79 cells: colonies stained for plating efficiencies are the same size.

Clones 3-18-3d and 3-25-2b and V79 cells were plated in varying concentrations of 6-TG (figure 8). Different sera affected plating efficiency, an effect partly or wholly due to different concentrations of hypoxanthine, which competes with 6-TG for HGPRT binding. Clone 3-25-2b,
TABLE 2. Characterization of 6-TG resistant clones.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Plating Efficiency</th>
<th>HGPRT activity</th>
<th>Hypoxanthine uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent 6-TG</td>
<td>V79 = 1</td>
<td>V79 = 1</td>
</tr>
<tr>
<td></td>
<td>Control Ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V79</td>
<td>0 75 0</td>
<td>1.000</td>
<td>1.00</td>
</tr>
<tr>
<td>8-1-1</td>
<td>41 51 .80</td>
<td>.005</td>
<td>.03</td>
</tr>
<tr>
<td>8-1-2</td>
<td>63 72 .87</td>
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<td>.01</td>
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<tr>
<td>8-1-3</td>
<td>44 45 .97</td>
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<td>.02</td>
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<tr>
<td>1-26-5</td>
<td>66 68 .96</td>
<td>.003</td>
<td>.01</td>
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<td>1-28-C</td>
<td>66 61 1.07</td>
<td>.013</td>
<td>.04</td>
</tr>
<tr>
<td>3-18-2a</td>
<td>46 62 .74</td>
<td>.51</td>
<td>.01</td>
</tr>
<tr>
<td>3-18-2b</td>
<td>64 67 .96</td>
<td>.012</td>
<td>.02</td>
</tr>
<tr>
<td>3-18-3a</td>
<td>88 86 1.02</td>
<td>.005</td>
<td>.01</td>
</tr>
<tr>
<td>3-18-3b</td>
<td>63 66 .96</td>
<td>.009</td>
<td>.03</td>
</tr>
<tr>
<td>3-18-3c</td>
<td>58 66 .89</td>
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<td>.003</td>
<td>.02</td>
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<td>3-25-3a</td>
<td>79 81 .98</td>
<td>.010</td>
<td>.02</td>
</tr>
<tr>
<td>3-25-3b</td>
<td>73 68 1.06</td>
<td>.007</td>
<td>.01</td>
</tr>
<tr>
<td>3-25-5a</td>
<td>69 73 .95</td>
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<td>.03</td>
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<tr>
<td>3-25-5b</td>
<td>70 81 .86</td>
<td>.008</td>
<td>.02</td>
</tr>
<tr>
<td>3-25-8</td>
<td>65 74 .88</td>
<td>.36</td>
<td>.20</td>
</tr>
<tr>
<td>3-25-C</td>
<td>56 59 .95</td>
<td>.017</td>
<td>.02</td>
</tr>
<tr>
<td>4-1-1</td>
<td>72 76 .96</td>
<td>.011</td>
<td>.02</td>
</tr>
<tr>
<td>4-1-2</td>
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</tr>
<tr>
<td>4-1-4</td>
<td>67 68 .99</td>
<td>.017</td>
<td>.04</td>
</tr>
</tbody>
</table>
Fig. 8. 6-TG sensitivity of V79 cells and resistant clones. Replicate cultures at 200 cells/plate were established for V79 cells and clones 3-18-3d and 3-25-2b. Except for controls, plates received various final concentrations of 6-TG, and colonies were stained one week later. Open circles show survival of clone 3-18-3d and closed circles show survival of clone 3-25-2b. Open triangles show survival of V79 cells for two independent experiments; closed triangles show survival of V79 cells in medium prepared with serum from a different lot.
as mentioned above, shows a decreased plating efficiency under selective conditions, whereas clone 3-18-3d shows no decrease at three times the selective concentration of 6-TG.

**Metabolic cooperation.** A small number of 6-TG resistant cells were plated along with varying numbers of V79 cells in selective medium, and the number of resistant colonies obtained was plotted against total cell number (figure 9, top). It is apparent that a cell density of $3 \times 10^6$ cells/plate adversely influences selection of resistant cells; this density is approximately half that of a confluent plate.

When isolating 6-TG resistant cells from mutagenized V79 populations, selection began after a four day expression time during which cells could proliferate into small colonies. Thus, other reconstruction experiments were done in which selection began at various times after plating of resistant and V79 cells (figure 9, bottom). Small colonies are relatively unaffected by metabolic cooperation with surrounding cells; in fact, survival actually increases at higher cell density if the selective medium is not replenished.

The above reconstruction experiments probably represent two extremes of metabolic cooperation in selection experiments, because a colony containing 6-TG resistant cells at the end of expression time may also contain sensitive cells. Sectoring of colonies could result from different transcriptional events in daughter cells of the mutagenized population, or from different transcriptional events on complementary strands of DNA in parent cells.
Fig. 9. Effect of cell density on selection for 6-TG resistance. Circles show survival of clone 3-25-2b and triangles show survival of clone 3-18-3d.

Top. 200 resistant cells were plated with various numbers of V79 cells in selective medium; control plates had resistant cells only.

Bottom. 200 resistant cells were plated with $10^5$ to $2 \times 10^5$ V79 cells in normal medium and incubated for various lengths of time before adding 6-TG. Cell number at the time 6-TG was added was determined by trypsinizing and counting one of the replicate cultures. The closed figures show survival on plates which did not receive fresh selective medium on the fourth day of selection. Controls were allowed no growth prior to selection.
Studies With Female CHO Cells.

CHO cells adhere much less tenaciously to growing surfaces during mitosis than do V79 cells; healthy cultures actually contain many floating cells which plate at nearly the same efficiency as cells detached by trypsinization. This property of CHO cells is advantageous in that large numbers of synchronous cells can be obtained by shake-off, but disadvantageous in that any motion of a plate results in establishment of numerous secondary colonies. These considerations necessitate subculturing of CHO cells during and at the end of the expression time to keep cells from growing to confluency and from forming secondary colonies.

Since selective medium could not be replenished without establishing secondary colonies, the thermal stability of 6-TG was tested. Stock 6-TG at 50 µg/ml was incubated at 37°C for 12 days, then added to plates of V79 cells at a final concentration of 5 µg/ml. None of the $2 \times 10^4$ cells/plate survived. Data of figure 8 indicate that 0.5 µg/ml of fresh 6-TG is toxic to V79 cells, so 6-TG must have a half life of at least 3.6 days at 37°C.

**BUdR toxicity.** Incorporation of BUdR into DNA varies during the S period (figure 10, top). Although colony formation is not significantly impaired by one hour pulses of BUdR, colony size is reduced. Thus, the number of cells obtained at the end of five day's expression time is substantially reduced compared with unlabeled control cells. The plating efficiency of cells at the end of expression time is essentially the same as that of controls.

The kinetics of cell growth subsequent to BUdR exposure was monitored through four mitoses by cell count (figure 11). Cells labeled with 0.1 mM BUdR grew as well as controls for 30 hours, whereas cells labeled with 1.0 mM BUdR were delayed in reaching the second mitosis (Simon, 1961). Despite
Fig. 10. BUDR uptake and toxicity in CHO cells. Various hour portions of the S period were labeled with BUDR.

Top. The label contained 1.0 μCi/ml 3H-BUDR in 10^-4 total BUDR. Cells were trypsinized 8 hours after rinsing off the hydroxyurea and DNA was isolated for scintillation counting. Data are the averages from shake-offs yielding 90,800 cells/plate and 110,900 cells/plate.

Bottom. Circles show colony formation following 1.0 mM BUDR labeling. Controls were not labeled; inoculations were 200 cells/plate. Triangles show cell number 5 days after 0.5 mM BUDR labeling; cells were subcultured to 10% density 3 days after labeling. Controls were not labeled; initial platings were 75,700 cells/plate and 102,000 cells/plate.
Hours following release of hydroxyurea block

$^{3}H$ disintegrations/cell/minutes ($\times 10^2$)

Survival (% of control)
Fig. 11. Growth of BUdR labeled cells. Circles show growth of controls; closed circles additionally show growth of cells labeled with 0.1 mM BUdR for the first hour of the S period. Triangles show growth of cells labeled with 1.0 mM BUdR for the first hour of the S period. One set of replicate cultures was monitored from 4 to 30 hours after BUdR labeling; a second set was monitored from 26 to 53 hours after labeling. Initial platings were 79,000 cells/plate and 53,300 cells/plate respectively.
this division delay, cell number increased to the same level as controls at completion of three mitoses; only at the fourth mitosis did fewer cells divide.

Expression time. The number of 6-TG resistant colonies obtained from mutagenized populations depends on the expression time (figure 12). No resistance is expressed for two days following the mutagenic insult. The number of resistant colonies obtained increases dramatically as expression time increases from three to four days, but there is little change as expression time increases beyond four days. Five days was picked as the expression time for selection experiments with CHO cells.

Mutagenic mechanism of BUdR. Initial experiments selecting for 6-TG resistance in CHO cells, labeling cells with 0.1 mM BUdR at different times in the S period, yielded resistant colonies at a rate indistinguishable from controls. Labeling of V79 cells with 0.1 mM BUdR had induced significantly more resistant colonies than background, but a comparison of figures 7 and 10 indicates that V79 cells incorporated more BUdR into DNA under the same labeling concentration. Thus, various concentrations of BUdR were applied to replicate CHO cultures during the second hour of the S period and the induced 6-TG resistant colonies were subsequently selected (figure 13, bottom). Incorporation of BUdR into DNA under identical labeling conditions was not a linear function of BUdR concentration.

Toxic effects of BUdR increase with BUdR concentration (figure 13, top). As previously discussed, cell growth is more markedly affected than is colony formation. At the end of expression time, having been subcultured once, cells plate at essentially the same efficiency as controls regardless of the concentration of previous BUdR exposure.
Fig. 12. Expression time for 6-TG resistance. Replicate cultures were labeled with 0.5 mM BUdR during the third hour of the S period. Circles show the number of 6-TG resistant colonies selected from plates which were subcultured at 3 days and 5 days after BUdR labeling; initial plating was 60,000 cells/plate. Triangles show the number of 6-TG resistant colonies selected from plates subcultured on alternate days; initial plating was 77,500 cells/plate. In both experiments, a minimum of 200,000 cells were transferred at each subculture. The plating efficiency at day 2 was about half the plating efficiency at day 4, whereas the plating efficiency at day 6 increased only 15% over that of day 4.
Fig. 13. BUdR dose responses in CHO cells. Replicate cultures were labeled with BUdR during the second hour of the S period.

**Top.** Triangles show colony formation after BUdR labeling. Controls were not labeled; inoculations were 200 cells/plate. Circles show cell number 5 days after BUdR labeling; cells were subcultured to 10% density 3 days after labeling. Controls were not labeled; initial platings were 90,200 cells/plate and 103,000 cells/plate.

**Bottom.** Open circles show $^3$H decays in DNA when 100 $\mu$C/ml $^3$H-thymidine was added to stock 10 mM BUdR prior to the various dilutions; closed circles show $^3$H decays in DNA when 10 $\mu$C/ml $^3$H-BUdR was added to stock 1.0 mM BUdR prior to dilution. Cells were incubated three hours following the labeling, then were trypsinized to isolate DNA. Only 1 to 2% of the counts were in acid soluble cell constituents. Plating was 109,700 cells/plate. Triangles show induction of 6-TG resistant colonies as a function of concentration of the BUdR label; initial plating was 107,000 cells/plate.
Data of figures 10 and 13 show that approximately $10^{-2}$ dpm/cell result from labeling the second hour of the $S$ period with 1.0 $\mu$C/ml of $^3$H-BUdR in $10^{-4}$ M total BUdR. The ratio of BUdR molecules to $^3$H-BUdR dpm is $2.7 \times 10^{10}$, which means that each cell has incorporated $2.7 \times 10^8$ BUdR molecules into DNA, barring any unlikely isotope effect (Appendix). A Chinese hamster cell contains $5 \times 10^{-12}$ grams of DNA (Mahler and Cordes, 1971), or $9.3 \times 10^9$ nucleotide bases assuming an average nucleotide molecular weight to be 326. Nearly 30%, or $2.7 \times 10^9$, of these bases are thymidine (Mahler and Cordes, 1971). Perhaps 20% of these thymidines are replicated during the BUdR pulse, implying that BUdR substitutes for half of the thymidines under these labeling conditions. Certainly the rate of substitution is greater than 10%.

Considering that higher concentrations of BUdR will lead to nearly full substitution for thymidine during the pulse, it is expected that a plateau of incorporation as seen in figure 13 would be reached. If intracellular pools of pyrimidine precursors get too large, presumably degradation of precursors increases and/or the exogenous supply is shut off by inhibition of thymidine kinase activity. Concentration of the dTTP pool in the absence of exogenous labeling is of the order of $2 \times 10^{-4}$ M.

It is interesting that there is a threshold effect to the induction of 6-TG resistant colonies by BUdR and that few resistant colonies are induced until BUdR concentrations exceed that required to substantially substitute BUdR for thymidine.

To further characterize this threshold effect, BUdR and thymidine at a combined total concentration of 1.0 mM were simultaneously applied to CHO cells during the second hour of the $S$ period. Incorporation of thymidine, or conversely of BUdR, into DNA is linearly related to external thymidine
concentration under these labeling conditions (figure 14). Induction of 6-TG resistance by BUdR, however, is markedly reduced by concurrent addition of thymidine to the label (figures 13 and 14).

Induction of 6-TG resistance. CHO cells, like V79 cells, exhibit a pronounced S phase dependency for induction of 6-TG resistant colonies by BUdR. Whereas BUdR is incorporated into DNA maximally between hours 3 and 4 of the S period, 6-TG resistance is induced maximally between hours 1 and 2 (figure 15, top).

Induction of ouabain resistance. Resistance to ouabain is induced at a much lower frequency than is 6-TG resistance (Table 3). Comparison of the numbers of 6-TG resistance colonies versus ouabain resistant colonies selected from sister cultures indicates that maximum induction of ouabain resistance occurs later in the S period than the maximum induction of 6-TG resistance (figure 15, bottom).
Fig. 14. Effect of thymidine on BUdR dose responses. Replicate cultures were labeled with BUdR and thymidine during the second hour of the S period. The ratio of BUdR to thymidine was varied, but the combined total concentration was always 1.0 mM.

Top. Triangles show colony formation after BUdR + thymidine labeling. Controls were not labeled; initial plating was 200 cells/plate. Circles show cell number 5 days after BUdR + thymidine labeling; cells were subcultured to 10% density 3 days after labeling. Controls were not labeled; inoculations were 62,700 cells/plate and 133,000 cells/plate.

Bottom. Squares show $^3$H disintegrations in DNA when 100 µC/ml $^3$H-thymidine was added to stock 10 mM thymidine prior to the various dilutions. Following labeling, cells were incubated for three hours before trypsinizing to isolate DNA. Initial plating was 70,500 cells/plate. Triangles show induction of 6-TG resistant colonies as a function of the proportion of BUdR in the 1.0 mM label; initial plating was 133,000 cells/plate.
Fig. 15. BUdR induction of 6-TG and ouabain resistance. Replicate cultures were labeled with 0.5 mM BUdR during sequential hours of the S period. After 5 days expression time, sister subcultures were exposed either to 5 μg/ml 6-TG or to 3.0 mM ouabain.

**Top.** Circles show induction of 6-TG resistance; the closed circles represent colonies selected in medium made with serum from a different lot. Triangles show induction of 6-TG resistance following 0.2 mM BUdR labeling.

**Bottom.** Circles show induction of ouabain resistance. Error bars show standard deviations of the means of the two experiments detailed in Table 3. Dotted line shows relative position of induction of 6-TG resistance.
Hours following release hydroxyurea block
TABLE 3. Induction of ouabain resistant clones.

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DISCUSSION

Upon this gifted age, in its dark hour,
Rains from the sky a meteoric shower
Of facts... they lie unquestioned, uncombined.
Wisdom enough to leech us of our ill
Is daily spun; but there exists no loom
To weave it into fabric... .

Wine from These Grapes
Edna St. Vincent Millay

The 6-TG resistant clones characterized in the present study exhibit traits expected of mutants: the resistant phenotype is stable and its frequency of occurrence is enhanced by the mutagen BUdR. Further, the phenotype is associated with a low activity of HGPRT as measured in vitro, although enzyme assay does not discriminate between an altered gene product and a nonexistent gene product.

At least one of the clones selected for its resistance to 5 µg/ml of 6-TG is able to grow normally in 15 µg/ml of 6-TG. Such a clear change in phenotype is typical of mutational events and atypical of shifts in phenotypic expression induced by alteration of the environment. Additionally, the 6-TG resistant clones survive in 100 times the concentration of 6-TG which is toxic to normal cells. In sum, it is clear that the methods used in the present study are selective for mutant cells.

Induction of mutations in synchronized Chinese hamster cells by BUdR is dependent on the replicative state of the cells. Resistance to 6-TG in both male V79 cells and female CHO cells is induced by BUdR early in the S period, and resistance to ouabain is induced by BUdR later in the S period of CHO cells than resistance to 6-TG (figure 15). The simplest interpretation of these data is two-fold: genes in mammalian cells replicate in a temporal sequence consistent from generation to generation, and BUdR
mutates genes only when present during their replication.

Replication of the HGPRT gene in synchronous cell populations thus occurs over an interval of about two hours (figures 7 and 15). Cell division in synchronous cell populations is also spread over an interval of about two hours. Thus, either the spread in synchrony observable at mitosis is present within the population early in S period, or the population consists of heterogeneous cell types which replicate the HGPRT gene at slightly different times. Mutation induction in a recently cloned population would discriminate between these alternatives.

Evidence for the orderly replication of mammalian DNA has been discussed earlier. Since BUdR toxicity is greater in early replicating DNA and since the genes coding for ribosomal proteins, HGPRT, and (Na⁺K⁺)-activated ATPase are replicated early, the early-replicating euchromatic DNA probably contains the bulk of information necessary for growth and reproduction of cells (Stubblefield et al, 1967). Messenger RNA is, in fact, transcribed predominantly in euchromatic DNA (Littau et al, 1964).

A general pattern of intrachromosomal replication from telomere to centromere has been noticed (Schmid, 1963). Centromeres contain much redundant DNA and are important structures for chromosomal attachment to the mitotic spindle, so it is unlikely that they would contain genes coding for metabolic enzymes. Thus the early replication of specific genes correlates with the late replication of heterochromatin and centromeres.

The location of the presumably sex-linked HGPRT locus on the X chromosome of Chinese hamster cells is unknown. Autoradiographic localization of DNA synthesis at the time of maximum mutation induction might indicate the HGPRT gene location; but if DNA synthesis occurs at more than one location on the X chromosome at that time, the HGPRT gene could be at
any of them. However, the long arm of the X chromosome of V79 cells replicates late in the S period, so a sensitive compilation of autoradiographs of many X chromosomes might show that no DNA synthesis occurs on the long arm at the time of maximum induction of 6-TG resistance.

While the present study demonstrates temporal replication of two mammalian genes, there are few genes for which temporal replication could so easily be shown: with most mutations there is not any straightforward way to select for the mutant and against the wild-type cell. Since most selection procedures work the other way, temporal studies of gene reversion are possible but, of course, require many more synchronized cells since reversion of a mutant gene to the wild-type gene is quite infrequent.

Another possibility for temporal replication study is the use of heterozygous cell lines to obtain adequate mutation rates for autosomal recessive traits. Chasin (1974) has selected revertants of adenine phosphoribosyltransferase (APRT) deficient cells and shown them to have a much higher mutation rate to diaminopurine resistance than do wild-type cells. Thus the genotype of APRT− revertants is bound to be APRT+/APRT−.

Even though few markers are suitable for temporal replication study, the temporal mutation assay for HGPRT gene replication can be used to test any mutagen's molecular mechanism: if a mutagen affects primarily the replicating regions of the genome, then a temporal pattern of 6-TG resistance will be obtained from synchronized populations, whereas if a mutagen affects all portions of the genome, then mutations will be obtained regardless of the replicative state of cells. An interesting study would be a test of the temporal induction of mutations by tritium located at different positions on thymidine; conceivably, a tritium decay in the methyl group would be less mutagenic to DNA than a decay in the ring structure which could alter hydrogen bonding with the complementary adenine.
Mutation induction by BUdR, although contingent upon DNA synthesis, cannot be explained simply on the basis of occasional base mispairing with guanine. The data do not rule out base mispairing as the ultimate source of mutation; but they do indicate that if base mispairing occurs, the probability of any particular BUdR molecule mispairing during replication is enhanced by a high concentration of other BUdR molecules. Thus, substantial substitution of BUdR for thymidine is not in itself very mutagenic, whereas concentrations of BUdR higher than that necessary to substantially substitute BUdR for thymidine do lead to numerous mutations (figure 13). The observations of Trautner et al suggest that BUdR interferes with DNA synthesis in some way that causes occasional mispairing of guanine with adenine, but their data do not discriminate between BUdR in the template strand or BUdR as a precursor for synthesis as the source of replicative errors. In CHO cells, since mutations increase without concomitant increase in BUdR incorporation into DNA (figure 13), precursor BUdR is indicated as the source of replicative errors.

A simple explanation for these phenomena is that BUdR exerts some allosteric effect on DNA polymerase which renders it more error prone. Such an allosteric effect, noticeable at high concentrations of BUdR, could be mediated via thymidine kinase complexed to DNA polymerase or via DNA polymerase directly. Such an allosteric effect is depressed by thymidine (figures 13 and 14).

It is interesting that toxicity does not increase with the sharp increase in mutation induction (figure 13). Huberman and Heidelberger similarly observed a four-fold increase in BUdR-induced mutations with only a 25% increase in toxicity. Apparently much of the toxicity of BUdR in mammalian cells is due to its nonmutational effects. This demonstrates
the value of ploidy to organisms; when the mutation rate for sex-linked traits or dominant traits is of the order of $10^{-3}$, the mutation rate for autosomal recessive traits will be only $10^{-6}$, in diploid organisms. Since inactivation of an enzyme by mutation is likely to be lethal, the number of functional genes on the X chromosome can be estimated. Lethal mutations appear to contribute only an additional 25% toxicity (figure 13) and their frequency should be the same as HGPRT$^-$ mutations, about $8 \times 10^{-4}$ following 0.5 mM BUdR labeling of the second hour of DNA synthesis. Therefore, not more than 300 genes coding for necessary enzymes could be replicating on the X chromosome during the second hour of the S period, and perhaps 1000 or so during the entire S period.

The mechanism postulated here for the mutagenicity of BUdR at high concentrations correlates with the existence of mutant cells which grow well with total substitution of BUdR for thymidine. Davidson and Bick (1974) postulate that the DNA synthesizing system of such mutant cells has been altered. It could be either BUdR or BUdR-TP that interferes with DNA replication; if it is BUdR, then it should be possible to demonstrate BUdR induced mutations in thymidine kinase deficient cells which do not incorporate BUdR in DNA at all.

Hopefully the observations and speculations presented here will stimulate further experimentation on these phenomena.
EPILOGUE

Believing, as I do, in the continuity of nature, I cannot stop abruptly where our microscopes cease to be of use. Here the vision of the mind authoritatively supplements the vision of the eye. By a necessity engendered and justified by science, I cross the boundary of the experimental evidence, and discern in that Matter which we, in our ignorance of its latent powers, and notwithstanding our professed reverence for its Creator, have hitherto covered with opprobrium, the promise and potency of all terrestrial Life.

Speech to the British Association for the Advancement of Science, John Tyndall, 1874

Man is a machine by birth but a self by experience. And the special character of the self lies in its experience not of nature but of others. A man enters the lives of other men more directly than he can enter nature, because he recognizes his own thoughts and feelings in them; he learns to make theirs his own, and to find in himself a deeper self that has the features of all humanity. The knowledge of nature teaches him to act, and makes him master of the creation. The knowledge of self does not teach him to act but to be; it steeps him in the human predicament and the predicament of life; it makes him one with all the creatures.

The Identity of Man
Jacob Bronowski
APPENDIX

Calculation of BUdR substitution for thymidine.

\[ 1 \text{ Curie} = 3.7 \times 10^{10} \text{ dps} \]
\[ 1 \mu\text{Ci/ml} = 3.7 \times 10^6 \text{ dps/ml} = 2.22 \times 10^6 \text{ dpm/ml} \]
\[ 1 \text{ M BUdR} = 6.02 \times 10^{23} \text{ molecules/l} \]
\[ 10^{-6} \text{ M BUdR} = 6.02 \times 10^{19} \text{ molecules/l} = 6.02 \times 10^{16} \text{ molecules/ml} \]

Thus:

\[ \frac{\text{BUdR molecules}}{\text{tritium decays}} = \frac{6.02 \times 10^{16} \text{ molecules/ml}}{2.22 \times 10^6 \text{ dpm/ml}} = 2.7 \times 10^{10} \frac{\text{molecules}}{\text{dpm}} \]

Since each cell contains \(10^{-2}\) dpm of \(^3\text{H}-\text{BUdR}\) following labeling of the second hour of the S period (figures 10 and 13), it contains

\[ (2.7 \times 10^{10} \text{ molecules/dpm}) \times (10^{-2} \text{ dpm}) = 2.7 \times 10^8 \text{ BUdR molecules.} \]
LITERATURE CITED


Harris, M. Mutation rates in cells at different ploidy levels. J. Cell. Physiol. 78: 177 (1971).


