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By

Stephen Theodore Warren

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ABSTRACT

BLOOM SYNDROME AS A HUMAN MUTATOR MUTATION

By

Stephen Theodore Warren

A considerable body of evidence now exists that suggests that somatic mutations play a pivotal role in the etiology of cancer and that the rate of mutation is under genetic control. The autosomal recessive condition, xeroderma pigmentosum (XP), exemplifies this since these patients have defective DNA repair and consequently incur more UV-induced somatic mutations than do normal individuals. The ultimate result is that these patients suffer from an extremely high incidence of actinic skin cancer. XP, however, is analogous to only one of many mechanisms found in lower organisms which control their rates of mutation. For example, bacteria contain many loci which maintain the rate of spontaneous mutation to an acceptably low level and mutations in these loci (mutator mutations) result in a striking elevation of the basal rate of spontaneous mutation. It is reasonable to predict that the human genome also contains loci which function to control the rate of spontaneous mutation. Furthermore, one may expect that similar mutator mutations exist in the human population, most likely as genetic diseases with propensities to develop cancer. It was consequently hypothesized that the autosomal recessive, cancer-prone condition, Bloom syndrome (BS), may represent a human mutator mutation.

Therefore, the rates of spontaneous mutation to 6-thioguanine resistance were determined in fibroblasts derived from normal and two BS individuals (GM 2548 and GM 1492). Two methods were utilized to determine the rates. Method I obtained the spontaneous mutation rate from the increase in the mutation frequency of a cell

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population in logarithmic-phase growth over 10 days. The two BS strains had spontaneous mutation rates of 16×10^{-6} and 17×10^{-6} mutations per cell per generation, whereas two normal strains had rates of 1.5×10^{-6} and 1.1×10^{-6}. Method II utilized fluctuation analysis to measure the rate of spontaneous mutation. This method resulted in rates of 19×10^{-6} and 23×10^{-6} mutations per cell per generation in BS cells, compared to rates of 4.6×10^{-6} and 4.9×10^{-6} in control strains. These data suggest that BS may be a human mutator mutation.

Studies were then initiated to attempt to elucidate the mechanism by which BS cells were spontaneously hypermutable. Based upon studies of mutator mutations in rodent cell lines, investigation of the deoxyribonucleoside triphosphate (dNTP) pools were performed. Since one rodent mutator mutation was previously found to be resistant to the DNA polymerase inhibitor, aphidicolin, due to elevated levels of deoxycytidine triphosphate (dCTP), aphidicolin sensitivity of BS fibroblasts were determined (29). The survival curves of two normal and two BS strains, treated with various amounts of aphidicolin, were identical, suggesting that the dCTP levels were comparable. However, to determine the levels of the other three dNTPs. Measurements were performed on methanol extracts of BS and normal strains. Using an E. coli DNA polymerase I based assay, it was found that all four dNTP levels were not significantly different between BS and normal.

Based upon a report that BS fibroblasts secrete a clastogenic factor into the media in which they are grown (52), medium from seven-day old cultures of BS and normal fibroblast was collected and concentrated twenty-fold. The BS concentrates were found to be growth inhibitory and significantly more cytotoxic to V79 Chinese hamster cells than the normal concentrates. Furthermore, the BS concentrates were found to induce 6-thioguanine but not ouabain resistant mutations in the V79 cells, suggesting that BS cells secrete a mutagenic factor, in excess of 2,000 daltons, which causes nonsense but not missense mutations.

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The results of this study suggest that BS may be a mutator mutation, a previously unrecognized phenomenon in humans. Also, evidence was found suggesting that the mechanism of the hypermutability may be the production and/or lack of detoxification of a mutagenic factor. These results also lend support to the somatic mutation theory of cancer.

To Karen

for her love and support

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INTRODUCTION

There is now considerable evidence suggesting a direct relationship between somatic mutagenesis and carcinogenesis. A large mass of data has shown: (1) the clonal nature of tumors (58); (2) the mutagenicity of most carcinogens (3); (3) the correlation of *in vitro* DNA damage, *in vitro* mutation and transformation frequencies with *in vivo* tumorigenesis (104); (4) the age-related incidences of various hereditary tumors (119); and (5) the correlation between photoreactivation of DNA damage and the biological amelioration of UV-induced neoplasms (95). These findings have all been interpreted as evidence that somatic mutations play a pivotal role in the formation of malignancies.

Early studies on lower organisms, such as *E. coli* and *D. melanogastor*, have shown that the rate of mutation is under genetic control (49). For example, mutations in bacteria that result in the defective repair of DNA damage ultimately result in elevated mutagen-induced mutation frequencies. Extensive work on these DNA repair defective mutations suggests that a large number of génes may be involved in the control of mutagenesis. It would be reasonable to assume that humans also have loci which exert genetic control over the rate of mutation. Furthermore, it may also be postulated that if one of these loci were mutated, the individual carrying the mutation may have a higher predisposition toward the development of cancer.

Indeed, data accumulated on the autosomal recessive condition, xeroderma pigmentosum, support these ideas. Clinically, these patients are extremely prone to sunlight-induced skin cancers. At the cellular level, cells from the patients are defective in the repair of UV-induced DNA damage (33) and hence have a much higher frequency of UV-induced mutations than normal (134). It is therefore infered that mutagenesis is under genetic control in humans and that mutations which

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Xeroderma pigmentosum, however, is analogous to only one of many mechanisms lower organisms have genetically to control mutagenesis. It has been shown that bacteria possess a number of genes which function to maintain the spontaneous rate of mutation at an acceptably low level (41). Mutations in these genes, termed mutator mutations, greatly elevated the basal rate of spontaneous mutation. It is therefore possible that similar mutations, in the control of spontaneous mutagenesis, may be present in the human population, most likely expressed as genetic disease with predisposition to cancer.

A potential candidate for a human mutator mutation is Bloom syndrome. It is a rare, autosomal recessive condition (genotypically designated bl/bl) which is phenotypically characterized by primordial dwarfism, facial telangiectasia, and a distinct propensity to develop cancer at a relatively early age (68). Cytologically, cells obtained from patients with Bloom syndrome exhibit increased chromosomal instability, particularly breakage and homologous rearrangements (71). In addition to the chromosomal instability, these cells have a manyfold increase in sister-chromatid exchanges (SCE) over normal cells, such that a markedly elevated frequency of SCE may be considered pathognomonic of Bloom syndrome (26).

Because of the chromosomal breakage, elevated SCE frequency, and predisposition to develop cancer, Bloom syndrome frequently has been listed as a DNA repair-defective syndrome. However, studies of the DNA repair capacities of Bloom syndrome cells after DNA damage strongly suggest that the repair functions, as presently understood, are intact (78). In fact, evidence has been provided which suggest that DNA replication, rather than repair, may be abnormal (92).

Since abnormalities in DNA replication have been found to be commonly associated with mutator mutations in lower organisms, including rodent cell lines

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(41,145), it is conceivable that the bl gene, when homozygous, may have mutator activity, particularly in view of the high cancer incidence. This dissertation, therefore, is based on the hypothesis that Bloom syndrome may represent a human mutator mutation. Portions of this dissertation have been reported elsewhere (223,225).

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LITERATURE REVIEW

Somatic Mutation Theory of Cancer

There is now considerable evidence that mutations, primarily of the somatic cells, play a pivotal role in carcinogenesis. It was first suggested by Boveri in 1914 that the apparent hereditary nature of cancer growth could be explained by genetic change (16). Tyzzer, two years later, coined the term "somatic mutation" for this genetic change (214). However, it was not until the last two decades that much of the experimental evidence in support of the somatic mutation theory of cancer was obtained.

One of the major lines of evidence used for support of this theory comes from the strong correlation between the ability of a physical or chemical agent to damage DNA and cause cancer. However, not until the classic work of Miller and Miller (147,148) were investigators able definitively to make this correlation. The Millers found that many naturally occurring chemical carcinogens needed to be metabolized by the mixed-function oxidases and related enzymes to electrophilic (i.e., electron-deficient) forms in order to react with the DNA. Prior to this knowledge, the correlation between mutagenicity and carcinogenicity was weak, at best (21).

By working *in vivo* or *in vitro* with liver extracts, which contain the activating enzymes, much evidence was generated suggesting a relationship between a chemical's ability to cause cancer and to damage DNA. San and Stich, for example, demonstrated, in human cells, a strong correlation between the ability to elicit DNA repair synthesis, a cellular response to DNA damage, and the carcinogenic potential of a wide variety of chemicals (179). Likewise, Swenberg *et al.* (198) were able to correlate the carcinogenic potential of a number of chemicals and their ability to damage DNA, using alkaline elution techniques to measure the damage.

Since normal cells are able to repair some of the DNA damage induced by physical or chemical carcinogens, prior to semi-conservative DNA replication, they reduce the amount of mutational change brought about by the DNA lesions. This may be exploited to provide further evidence that DNA damage induced by carcinogens is related to their carcinogenicity. As different organs have different rates of repair, an inverse relationship has been established between the removal of specific deoxynucleotide-carcinogen complexes from the DNA of certain tissues and the potential of the carcinogen to induce tumor formation in that tissue (84,153). Similarly, the photoreactivation repair of thymine dimers from DNA, induced by ultraviolet light, has been shown by Hart et al., (95) to ameliorate ultraviolet-induced tumors in the gynogenetic teleost Poecilia formosa.

As implied above, the direct result of unrepaired DNA damage, induced by physical or chemical carcinogens, is the formation of mutations. Therefore, the correlation between carcinogenic potential and ability to damage DNA should hold true for mutations as well. Indeed, Ames and his co-workers (3) have shown that approximately 90 percent of the known carcinogens tested in the Salmonella/ mammalian microsome system are mutagenic. This relationship has now been found by numerous laboratories, such that mutagenicity in the Salmonella system is considered by many as evidence for carcinogenicity (173). Although a number of investigators, including Warren, et al., (226), have reported problems in the use of this assay for carcinogenicity testing, the relationship found by McCann et al., (141) provides ample evidence in support of the somatic mutation theory of cancer.

Carcinogen-induced mutations have also been reported in various mammalian cell lines, including human. Huberman (105) has developed a cell mediated mutation assay using V79 Chinese hamster cells which shows a good correlation between mutation induction and carcinogenic potential of various chemicals. This system has been able to demonstrate a relationship between the carcinogenic potency of

polycyclic hydrocarbons and their ability to induce mutations in mammalian cells and to identify the mutagenic metabolites of benzo(a)pyrene (104,106). These carcinogenic hydrocarbons also induce mutations in human skin fibroblasts grown in cell culture (135). Maher and McCormick have recently reviewed the data showing mammalian cell mutagenicity with a large number of carcinogenic polycyclic hydrocarbons (136). Clearly, these data strongly suggest a positive correlation between mutagenicity and carcinogenicity.

Strong inferential evidence of the role of mutations in human cancer has been documented. In an effort to explain the high stomach cancer incidence in the coastal Japanese population, Marquardt et al., (139) have found that extracts of Japanese raw fish, treated in a manner simulating the typical Japanese diet and gastric conditions, were highly mutagenic in the Ames bacterial mutagenicity assay. Likewise, Cheng et al., (30) reported mutagenic activity from an extract of pickled vegetables, which are produced and consumed in a certain region of China that also has a high incidence of esophageal cancer. The classic example of sunlight exposure and skin cancer has been recently reviewed by Scott and Straf (182). There is a clear correlation between ultraviolet light intensity, based upon latitude, climate and body exposure, and actinic skin cancers. In addition, patients injected with Thorotrast, an alpha-particle emittor introduced in 1928, have a high incidence of malignancies (43). Mays found that malignant hepatic neoplasms developed in 301 of approximately 3000 patients who were injected with Thorotrast 10 years previously (140). As judged by the natural annual frequency of hepatic malignant tumors, only about six tumors would have been expected in this group (227).

If a mutational event in a single cell is a precursor to the eventual development of a malignant focus, then all the cancer cells in that focus should be clonal in nature and reflect a common origin. Since mammalian females are natural mosaics, due to random X-chromosome inactivation, female cancer patients, hetero-

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zygous at X-linked loci, may be used to determine the clonal nature of tumors. Fialkow (57,58) has shown, using the X-linked marker glucose-6-phosphate dehydrogenase, that most malignant tumors are unicellular in origin. Further evidence for a clonal nature of cancer comes from studies of patients with chronic myelocytic leukemia, who typically have the Philadelphia (Ph^1) chromosome rearrangement present in 90 to 100 percent of their dividing marrow cells (97). The Ph^1 chromosome is a rearrangement between chromosomes 22 and 9, both of which are occasionally polymorphic and if so are distinguisable from their homologue. In patients who have polymorphic number 22 or number 9 chromosomes, the formation of the Ph^1 anomaly always involves the same number 22 (or number 9) and therefore, suggests a unicellular origin for chronic myelocytic leukemia (63,97). The above evidence suggesting a clonal nature of tumors is certainly consistent with the mutational theory of cancer. However, it is not sufficient to rule out epigenetic change in a single cell, since it would also be consistent with the evidence (29).

What may be considered the most significant evidence linking mutations and cancer has been obtained using cells derived from individuals with xeroderma pigmentosum. Affected individuals exhibit sun sensitivity, cutaneous pigmentary abnormalities, and an extremely high incidence of skin cancer (176). Soon after Regan, Trosko, and Carrier (174) demonstrated the repair of ultraviolet-induced pyrimidine dimers in DNA of normal cells, Cleaver (185), Setlow et al., (33), and Cleaver and Trosko (33) found that xeroderma pigmentosum cells failed to repair similar DNA damage. Since unrepaired DNA lesions would be expected to result in mutations after DNA replication, it is not surprising that Maher and McCormick (134) and Glover et al., (83) reported that xeroderma pigmentosum cells are hypermutable following ultraviolet irradiation. These data do, however, provide strong evidence suggesting that the actinic skin cancers in these patients is the result of somatic mutations incurred due to the lack of repair of sunlight induced

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pyrimidine dimers in their DNA. Further discussion of the relationship between DNA repair and mutagenesis will be presented below.

Epidemiologic evidence has also been used to establish a link between mutations and cancer. Knudson (119) has postulated a two mutational-event model of initiation of certain neoplasms. In this theory, non-hereditary or sporadic cases are the result of two somatic mutations while hereditary cases result from a single somatic mutation (the other necessary mutation being inherited through the germ line). Knudson predicted that the non-hereditary or sporadic cases would have solitary tumors while the hereditary cases have multiple primary sites since the germ line mutation would render many cells susceptible to the effect of a single somatic mutation. This reasoning was further extended to state that the age of onset of the hereditary cases would be earlier than the non-hereditary cases since hereditary cases require only a single somatic mutation whilst the sporadic cases require two somatic mutations. These predictions have been verified in many childhood cancers such as retinoblastoma, neuroblastoma, and pheochromocytoma (117,118). Jackson et al., (110) also found this interpretation consistent with data collected for the adult malignancy, medullary thyroid carcinoma. These clinical correlations with Knudson's theory is consistent with, and provides considerable support for, the mutational theory of cancer.

There are two major critisms of the mutation theory of cancer. The first is that not all known carcinogens seem to be mutagens (177). Many natural hormones, which appear not to be mutagens, are well known tissue specific carcinogens (112,131,183). The second criticism is that some cancer cells do not seem to have lost their genetic totipotency, as would be expected if transformation involves mutations (143,149). Mintz and Illmensee (149) have clearly implicated epigenetic mechanisms in carcinogenesis by demonstrating that teratocarcinoma cells (formed by transplanting early mouse embryos beneath the testis capsule) when injected into

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blastocysts form mosaic mice, with the cell line derived from the teratocarcinoma behaving normally. Trosko and Chang (209,210) and Chang, Trosko and Warren (28) have reconceptualized the whole process of carcinogenesis to integrate the above criticisms into a mutational theory of cancer.

The salient feature of this integrated view is that gene mutation as well as modulation of gene expression are involved in carcinogenesis. Gene mutation, in this mechanism, remains a common and important mechanism, although not the sole mechanism. Because the genes involved in carcinogenesis are most likely the very same genes involved in normal cell growth and differentiation (37), and as such are highly regulated, it is not unlikely that mechanisms disrupting normal gene control may, in certain tissues, lead to cancer. These same genes (or genes responsible for their control) may, of course, mutate leading to the same phenotype, namely cancer. Trosko and Chang (209) have proposed regulatory genes which control so-called transforming genes, which are tissue specific and operate normally in a highly orchestrated manner. When the regulatory genes are rendered inoperative, carcinogenesis is posible if the transforming genes are in a transcriable condition. Thus, epigenesis in addition to mutagenesis may lead to the same phenotype.

The overt phenotype of cancer, however, most likely involves more steps than those described above. Tumor promoters, for example, have the ability to allow a previously intiated cell (that is a cell with a single mutated or epigeneticly altered allele) to be released from the "normalizing" influences of neighboring cells and form a tumor. This may be due to the ability of tumor promoters to inhibit metabolic cooperation between cells (213,224,233). Thus a cell may be genetically changed by a mutagen (initiator) but phenotypically remain normal through the interaction with normal cells until this interaction is disrupted by a tumor promoter (or a second mutation).

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It is clear from this brief summary, that mutations play a predominant role in carcinogenesis, although other influences such as epigenesis and tumor promotion certainly have important roles in the overall progression of a malignant foci. In fact, Poste and Fidler (172) have provided evidence that cancer metastasis may involve mutational changes in cells of a primary malignant focus. The accumulation of somatic muations also has long been speculated to play a major role in the aging process (191). Hart and Setlow (94) have found a correlation between DNA excision repair and lifespan in several mammalian species, suggesting a link between mutations and senescence. Benditt (10) has speculated on the role of mutations in the origin of atherosclerosis and recently, Trosko and Chang (211) reviewed the literature and proposed a role for mutations in the development of diabetes mellitus as well as atherosclerosis. Therefore, it should be evident that the genetic control over mutation in man may influence one's predisposition to cancer as well as other major diseases.

The Genetic Control of Mutation

Mutation is a fundamental parameter of biology and is ultimately responsible for organismic diversity and evolution. Although the phenotypic impact of mutation may range from deleterious to neutral to beneficial, most mutations appear to be more harmful than beneficial (220). In higher organisms such as man, mutations in the germ cells may lead to hereditary diseases in the offspring, while mutations in the somatic cells may result in such diseases as cancer and possibly atherosclerosis (see previous section). Therefore, genetic diversity in the control of mutation may be responsible, in humans, for varying predisposition to diseases such as cancer. In fact, Neel has stated that the contribution of mutation to human disease has been significantly underestimated in past studies (151).

Mutations may be classified according to the final alteration in the DNA molecule (195). Traditionally, the first classification is molecular versus chromo-

somal, however, this may, perhaps more realistically, be divided by unobservable versus observable karyotypic changes. Despite this subjective basis, chromosomal-type mutations may be further classified on the basis of numerical changes (polyploidy, aneuploidy) and structural changes (deficiencies, rearrangements). Molecular-type mutations may be separated on the basis of base-pair substitutions (transitions, transversions) and frameshift changes (additions, deletions). The induction of mutations, and hence their control, may be broadly divided on the basis of exogenously-induced versus spontaneous.

One mechanism for the control of mutation is inherited variation of metabolism affecting the availability of environmental mutagens. The most extensively studied system in mammals where genetic control has been documented is the cytochrome P-450-dependent membrane-based monoxygenase system, which is required for the metabolism of many mutagens (carcinogens), in particular the polycyclic aromatic hydrocarbons (for reviews see Refs. 8 and 121). The P-450-dependent system exists in a variety of forms, each differing in tissue distribution, pattern of metabolites produced and in inducibility by a variety of agents (171). Therefore, genetic control over the type and level of activity as well as inducibility may modulate mutations induced by compounds requiring activation (promutagens).

Aryl hydrocarbon hydroxylase (AHH) activity has been used as a marker for the P-450-dependent system. A regulatory gene locus (the Ah locus) governing the inducibility of AHH has been defined (203). Two alleles are postulated (inducible and non-inducible) with heterozygotes showing evidence of gene dosage, thus it behaves as an autosomal codominant system (154). An individual possessing one or two copies of the inducible allele should metabolize procarcinogens to an active state much more readily than a homozygous non-inducer. Kellerman, et al., (115) and Emery et al., (154) have reported a correlation between AHH induceability and lung cancer in cigarette smokers, suggesting that genetic variation in carcinogen

metabolism influences cancer predisposition. It is likely that there are a number of loci, as yet unidentified, which similarly alter the metabolism of mutagens.

Alternatively, there may be genetic control over enzymes responsible for the detoxification of mutagens. There are various pathways which perform detoxification, including the P-450-dependent system. A number of enzymes, such as superoxide dismutase and catalase, remove cellular free radicals, which are very reactive towards DNA (127). Genetic variation in these pathways may also alter predisposition towards mutagenesis. Indeed, Nordenson (155) has found that excess superoxide desmutase can protect lymphocytes from radiation-induced chromosome aberrations and Tolmasoff et al., (206) correlated superoxide desmutase activity to primate lifespans, similar to the correlation with DNA repair (94).

Another potential mechanism which may influence genetic control over mutation is modulation of tissue availability. This mechanism has been proposed to influence predisposition towards breast cancer in humans (166). The excess incidence of breast cancer in Caucasian women, compared to Oriental women, has been correlated with wet or dry cerumen (ear wax). This trait is under genetic control with the wet allele being dominant (67). Since the cerumen type is associated with the secretion and resorption of fluid within the alveolar-ductal system, it has been proposed that women with dry cerumen have a lower secretory activity which minimizes the exposure of the breast epithelium to exogenous and endogenous mutagens (168). Petrakis and King (168) have further shown that metabolic products of cigarette smoke is rapidly secreted into breast fluids, thus indicating that the breast epithelium is in direct contact with ingested and inhaled chemical substances through the circulation.

Once a mutagen becomes activated and transported into the cell, it then interacts with the DNA, forming a lesion which, if repaired incorrectly, results in a mutation. Genetic control over the repair of DNA lesions therefore plays a pivotal

role in establishing predisposition to mutagenesis. Much information has been obtained in the last twenty years regarding DNA repair, primarily on prokaryotes. However studies examining the repair systems in man have proceeded quickly. Hanawalt et al., (91) has recently reviewed the literature on DNA repair in both bacteria and mammalian cells. The following portion of this section will be limited to genetic mutations in man influencing DNA repair capabilities.

The repair of damaged DNA may be performed by one of several pathways, each utilizing a number of enzymatic steps. The decision as to which repair pathway is used depends largely upon the type of damage, most likely on the degree of distortion the lesion causes in the chromatin-helix complex (91). Thus, different mutations in the various repair pathways may lead to dissimilarity in sensitivity to different mutagens, while different mutations within the same pathway may lead to similar mutagen sensitivities.

Xeroderma pigmentosum exemplifies this phenomenon. As previously stated, cells from patients with XP are defective in the repair of UV-induced cyclobutane dimers (32,33,185) and hence are exquisitely sensitive to UV irradiation (35). XP cells are also sensitive to a variety of chemical mutagens, such as benz(a)anthracene epoxide, 4-nitroquinoline-1-oxide, and acetylaminofluorene, all of which result in DNA damage recognizable by the excision-repair pathway also responsible for the repair of UV-induced damage (36). On the contrary, X-rays, methyl nitrosourea, and methylmethane sulfonate cause DNA damage which is repairable by XP cells and hence their cellular sensitivities to these agents are not significantly different from that of normal cells (36). Therefore, it is presumed that the repair pathway that is defective in XP is distinct from the pathway which repairs damage induced by the latter agents (which are collectively considere X-ray-like, while the former are considered UV-like). Of course, congruent to this is the understanding that those DNA lesions which are unrepairable by XP cells result in mutations after semi-

conservative replication (mutation fixation) and that those lesions repairable by XP cells (and by normal cells) are repaired in an error-free manner prior to DNA replication (134,137).

Studies on XP cells also demonstrate that different mutations within the same repair pathway result in similar mutagen sensitivities. Kraemer et al., (122) have shown that XP cells from different patients sometimes can complement each other when fused, restoring the ability to repair UV-induced DNA damage. Thus, it is suggested (but not proven) that each complementation group represents a different mutation in the same pathway. Furthermore, Lehman et al., (128) have shown that patients with a variant form of XP, which phenotypically includes neurological abnormalities, have an intact excision-repair pathway but are defective in DNA synthesis after UV irradiation (the so-called post-replication repair pathway). This then indicates that not only do different mutations in the same pathway lead to similar sensitivities but that mutations in different repair pathways, both concerned with the same type of DNA lesion, may also result in parallel sensitivities to different mutagens. It is thus clear, in the case of XP, that mutations in DNA repair can lead to enhanced mutagenesis following certain types of DNA damage. This, correlated with the striking incidence of solar-induced skin cancers in XP patients, strongly supports the idea that genetic control of mutation relates directly to cancer predisposition.

There are a few other cancer-prone genetic conditions where the experimental data, while not nearly as detailed as it is in XP, do nevertheless suggest errors in DNA repair. Ataxia telangiectasia (AT) is an autosomal recessive condition with major clinical features of progressive cerebellar ataxia, oculocutaneous telangiectasis, immune defects, elevated risk of lymphoreticular malignancy, and hypersensitivity to standard radiotherapy (164). The risk of AT patients developing malignancy is about 1200-fold greater than that of an age-matched control population and the

majority of reported neoplasms are lymphocytic leukemias, primarily the acute form, and lymphomas (163). An extensive comparison of many clinical aspects of XP and AT has been provided by Kraemer (123).

AT cells in culture, after exposure to X- or gamma-irradiation, have shown reduced survival compared to that of normal controls (164). Although the response of AT cells to various other mutagens is much less uniform, it is clear that AT cells respond normally to UV irradiation or UV-like chemicals, such as N-acetoxyacetyl-amino-fluorene (4). Therefore, it has been proposed that AT cells may have an analoguous defect, similar to XP, in the repair pathway responsible for X-ray damage. Laboratory data on the repair of AT cells have, however, been difficult to interpret. Lehmann and Stevens (129) have found that the production and repair of double strand breaks in AT cells, after gamma irradiation, are normal. Similarly, the rate of rejoining of single strand breaks appear to be the same in AT and normal cells (218). Paterson et al., (161) have shown, however, that three AT strains were defective in their ability to excise the type of gamma-ray induced base damage manifested as endonuclease-sensitive sites ("Y-lesion"). This finding however, was later found not to be consistant among different AT patients, possibly indicating genetic heterogenicity (162). Despite the inconsistancies, it is generally accepted that AT represents another mutation, distinct from XP, where the control of mutagenesis is modulated through the genetic control of DNA repair and thereby influences cancer predisposition. In fact, heterozygotes for the AT gene, who are clinically normal, may be at an increased risk of cancer because of the AT allele (199,200).

Fanconi's Anemia (FA) is another potential DNA repair defective disorder. FA, an autosomal recessive condition, is characterized by severe bone marrow deficiency, anatomical defects, growth retardation, and pigmentation changes of the skin (56). FA cells are hypersensitive to agents, such as mitomycin C, which cause

DNA cross-linking (59,180). Fujiwara et al., (62) has found that FA cells do not remove cross-linking damage as readily as normal cells. This suggests that FA may be defective in DNA repair, most probably a pathway distinct from those involved in XP and AT. However, it has been suggested recently that FA is defective in protection from free radical damage (113). It remains unclear if this finding and the cross-linking agent sensitivity is a pleotrophic manifestation of the FA gene or if it is the result of genetic heterogenicity. However, FA serves to illustrate, as do XP and AT, that genetic variation can dramatically influence the response to mutagens.

It should also be noted that FA and AT, unlike XP, have elevated incidences of chromosome aberrations (6,99). There is an unusually high frequency of chromosome aberrations in the lymphocytes of these patients, thereby classifying them as chromosome-breakage syndromes, along with Bloom syndrome, another cancer-prone syndrome which will be extensively discussed below. Since chromosome aberrations would certainly qualify as mutations, this may directly relate toward the cancer incidence. In fact, Cairns (23) has suggested the chromosomal aberrations, particularly in Bloom syndrome, may be a more efficient mechanism to cause cancer than point mutations. Cairns states that transposition (the movement of DNA segments within the genome) is more effective since XP patients do not get internal cancers. This view, as pointed out by Trosko (212), is not entirely consistant with the clinical literature (124). Nonetheless, both chromosome aberrations and point mutations, if enhanced through genetic mechanisms, certainly should influence cancer predisposition.

The data accumulated on XP provide an exquisite example of how genetic variation in the response (DNA repair) to the environment (sunlight) can influence disease susceptibility (cancer). Since it is commonly estimated that a large percentage of cancers are environmentally induced (22), genetic-environment interaction plays an important role in carcinogenesis. However, from data derived from

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prokaryotic studies, one would expect that individuals should be present in the population who intrinsically are prone to cancer because they are genetically predisposed to spontaneous mutation. The prokaryotic studies clearly demonstrate that an important mechanism of controlling mutation is genetic variation in spontaneous mutagenesis.

Genetic Control of Spontaneous Mutation

Over the course of evolution, genetic control over spontaneous mutation was acquired by organisms to maintain a low but appreciable rate of mutation (116). It was important to have some spontaneous mutations occur to maintain variability in the population. It was of equal importance that the rate be low enough so as not to interfere with genetic fitness, since, as stated previously, most mutations are deleterious. This genetic control was achieved by genes responsible for the fidelity of DNA replication and the detection and repair of occasional errors made during replication. Hence, a mutation at any one of these loci would be expected to increase the rate of spontaneous mutation within the genome. These mutations are referred to as mutator mutations or mutator genes (220).

Demerec (46), first reported the mutable gene, reddish, in Drosophila virilis and soon thereafter reported a second similar locus, miniature alpha, which like reddish, had a high rate of spontaneous mutation (47). During the ensuing 15 years, substantial data on these loci were accumulated (see Ref. 48 for review). Concurrantly, studies of spontaneous sex-linked recessive lethal mutations in Drosophila melanogaster provided evidence for the existence of mutator genes (170). However, the miniature alpha stock was inadvertently lost and the other stocks carrying mutable or mutator genes mutated themselves out of existence through frequent back mutations (87). Because these stocks were detected by chance, there was no selective technique to obtain them easily again and consequently Drosophila research with these genes came to a standstill. It was not until the early 1950's that

research with mutator genes was again stimulated, this time in different organisms. McClintock, in 1951, reported a mutable gene in maize (142) and three years later, Treffers et al., (208) reported a mutator gene in Escherichia coli. Research on these and other similar loci progressed and recent years have witnessed a sharply increased number of studies of mutator mutations in a variety of organisms. Mutator mutations are now easily obtained, either by direct selection or by search among DNA repair or replication defective mutants.

A number of genes of bacteriophage T4, whose functions in DNA replication and repair, has been at least partially identified, have been implicated in the genetic determination of both spontaneous and induced mutation rates (see Ref. 49 for review). Mutation activity has been observed with T4 mutations in genes 30 which involves DNA ligase (120); 32 which codes for the Albert's protein whose function plays a role in DNA replication, recombination, and repair (11); 42 and td whose function involves pyrimidine metabolism, specifically the synthesis of hydroxymethyl dCTP and dTTP (49); hm and v whose function is linked to DNA repair (49); and 43 which codes for DNA polymerase (196). Thus, from studies in T4 alone, it becomes apparent that mutations in one of many steps involved in DNA metabolism may lead to enhanced spontaneous mutation rates.

Extensive studies on bacterial mutator genes has been summarized by Cox (41). A number of mutator gene loci, each with several allelic variations, has been isolated and characterized gentically and biochemically (Table 1). They modify the spontaneous mutation rate from a few to 100,000 fold. These mutator genes cause transitions, transversions, and frameshifts and mostly behave as recessives. While the majority of these mutants influence the replication and/or repair of DNA via mechanisms analogous to the T4 mutators, some E. coli mutants have quite novel mechanisms. For example, Glickman and Radman (82) have presented evidence suggesting that the dam-mutator is deficient in methylation-instructed DNA mis-

Table 1. E. coli K12 Mutator Genes

Gene	Increase in Mutation Frequency	Note	References
mut T	1,000 - 10,000	AT → CG transversions, mut T^+ gene product acts at replicating fork.	40
mut D	1,000 - 100,000	transitions, transversions, frameshifts. High mutation rate turned on by thymidine; acridine sensitive	61,44
mut S	100	transitions, frameshifts	39
mut R	100 - 1,000	transitions, frameshifts	103
mut L	100	transitions, frameshifts	190
mut U (uvrE)	100 - 1,000	transitions, frameshifts. UV-sensitive involved in excision repair, possibly ligase.	189
pol A	50 - 100	frameshifts, deletions. Polymerase I mutation.	38
pol C	2 - 40	transversions. Polymerase III mutation.	90
tif-1	10 - 100	temperature dependent.	65
dam-3	7 - 50	sensitive to UV and MMC. defective methylation of adenine increases errors in repair.	82,138

Adapted from Cox (41).

match correction. As Weymouth and Loeb (230) have shown *in vitro*, DNA polymerase makes about one error for every 7,700 nucleotides incorporated into the DNA. This is much higher than the observed spontaneous mutation rate *in vivo*, suggesting that these mismatch errors are frequently repaired correctly. In order for the cell to recognize which nucleotide of the pair is the wrong one, it discriminates on the basis of base methylation, such that the template base is methylated and the newly incorporated base is not. Glickman and Radman have suggested that in the *dam*$^-$ mutant, because of the deficiency in DNA methylation, the cell cannot discriminate between the mismatched pair of nucleotides and hence randomly repairs them, frequently removing the correct base thus making an error.

Most of the mutator effects found so far in fungi have been discovered by examining radiation-sensitive strains. Game (64) has published a list of some fifty mutants in *Saccharomyces cerevisiae* along with their relative radiation sensitivities. A large number of these mutants are associated with an elevated spontaneous mutation rate (197,221). Haynes' laboratory has shown that yeast has up to four interrelated pathways for DNA repair (17,98) and Hastings *et al.*, (96) has presented evidence suggesting that mutator effects in yeast depend on blocks in these pathways and subsequent channeling of DNA lesions to other repair pathways, some of which are error-prone.

Mutator activity in *Drosophila* has been re-examined in recent years (87). The third chromosome mutator *mu* of *D. melanogastor* has been extensively characterized (85). *mu* appears to function only in females and prior to meiosis. Half of the *mu* influenced mutations are cytologically demonstrable deficiencies (86) and females carrying *mu* are more sensitive to X-rays and MMS (87). The hypothesis is favored that *mu* is associated with chromosome repair.

Examination of mutation genes in mammals has been limited to cells *in vitro* and very few studies have been undertaken. There have been only three reports of

mutator genes in mammalian cells (excluding data from this dissertation). Meuth et al., (145) has reported a mutant CHO cell line (thy$^-$) that exhibiits mutator activity. These mutants are resistant to arabinosylcytosine and auxotrophic for thymidine apparently the result of a single mutation in the gene for ribonucleoside-diphosphate reductase. These cells, which have a 5- to 10-fold increase in the dCTP pool, have a 5- to 50- fold increase in rate of spontaneous mutation.

Chang et al., (29) have isolated a V79 Chinese hamster cell line resistant to the DNA polymerase-inhibitor aphidicolin. This mutant is characterized by the following: 1) high levels of dCTP; 2) thymidine (or CdR or UdR) auxotrophy; 3) thymidine (and AdR, GdR) sensitivity; 4) slow growth; 5) cytidine sensitivity; 6) UV sensitivity and hypermutability; and 7) increased site-specific chromosome aberrations in the presence of BrdU. This cell line also has an elevated spontaneous mutation rate (Philip Liu, personal communication). Weinberg and Martin (229) have isolated a ribonucleotide reductase mutant in the S49 mouse T-lymphoma cell line that has altered pyrimidine pools. This line has a 10-fold increase in the rate of spontaneous mutation. It is suggested from these three mutator mutants that balanced nucleotide pools may be required for the fidelity of DNA replication. It has been postulated that excision repair can be rendered error-prone if the deoxyribonucleoside triphosphate pools are not balanced. Meuth (146) has demonstrated that exogenous deoxycytidine can alter the frequency of mutations induced by chemical mutagens. The effect of exogenous pyrimidine on induced mutagenesis could be attributed to enhanced base-mispairing. The perturbation of nucleotide pools may also affect spontaneous mutagenesis, as the above mutator mutants suggest. Evidence has been presented that BrdU-mutagenesis was determined by the concentration of BrdU to which the cells are exposed rather than the amount of bromouracil substitution (114). The results are consistant with the hypothesis that

BrdU triphosphate inhibits ribonucleotide reductase and decreases the dCTP pool. The pyrimidine pool imbalance then mediates the BrdU mutagenesis.

There have been no reports of mutator genes in humans to date. Undoubtedly the general difficulty in studying meiotic mutations in man has contributed to this lack of information. A seemingly simpler approach to finding human mutator genes would be the study of human fibroblast mutation rates in vitro. However, again technical difficulties have discouraged investigators. In fact, few papers have been published dealing with the rate of spontaneous mutation in human cells (Table 2). For the most thoroughly studied locus, HGPRT, the rate of mutation is generally considered to be about 4.1×10^{-6} mutation per cell per generation (45). The variance in the rate at this locus, particularly in the earlier studies is due to technical problems which were not controlled for, such as metabolic cooperation.

Table 2. Spontaneous Mutation Rates in Normal Diploid Fibroblasts

Locus	Rate[a]	Reference
Na^+/K^+ ATPase	4.0×10^{-8}	Buchwald (20)
Elongation factor-2	$5.0\text{-}6.0 \times 10^{-7}$	Gupta and Siminovitch (88)
	$1.0\text{-}3.0 \times 10^{-7}$	Gupta and Goldstein (89)
HGPRT	7.0×10^{-5}	Shapiro, et al. (186)
	$0.45\text{-}1.8 \times 10^{-6}$	DeMars and Held (45)
	5.6×10^{-6}	VanZeeland and Simons (216)
	$3.7\text{-}7.2 \times 10^{-6}$	Jacobs and DeMars (111)

[a]Mutation rate expressed as mutations per cell per generation.

Studying the rate of spontaneous mutation in human fibroblasts is much easier today, thanks to progress in the somatic cell genetics of rodent cells. Difficulties still remains. However, one may realistically begin to search for mutator mutations

in man through the use of cultured human fibroblasts. As with genetics in general, one must choose an appropriate mutant to increase the likelihood of success. In human genetics, mutants are manifested as inherited diseases. Because the above discussion has firmly linked cancer and mutations, it is possible that an inherited disease with a predisposition to cancer may be a mutator mutation. On such grounds, Bloom syndrome, an autosomal recessive condition, was chosen, for this study, as a possible candidate for a human mutator mutation.

Bloom Syndrome

Bloom syndrome was first recognized as a syndrome entity by Dr. David Bloom (14). The published transactions of the October 6, 1953 meeting of the New York Academy of Medicine provides a rare insight into the elucidation of a genetic syndrome. Earlier in the year, Bloom had listened to a case presentation by G. Machacek of a dwarf with telangiectatic erythema similar to a patient he had been following since 1941 (13). At the October 6 meeting, Bloom was present for a paper on a premordial dwarf with discoid lupus erythematosus delivered by Douglas Torre (207). The following are Bloom's comments regarding Torre's paper:

> *"This is the third such case I have seen. One was presented from the Vanderbilt Clinic by Dr. Machecek in March, 1953. At that time, I mentioned that we had such a case under observation since 1941 at the Skin and Cancer Unit. These three cases are pituitary dwarfs. One feature which makes them look very much alike is the precocious senile appearance of the face. In addition, all three of them show erythematous spots on the face, which at first glance appear as lupus erythematosus. But after long observation of our patient and after a pathological examination we came to the conclusion that this erythema may be designated as congenital telangiectasia. Our patient shows also another congenital abnormality, namely, ichthyosis-hystrix-like eruption*

on the trunk. Like the patient presented tonight, our patient shows lesions on the lips which are markedly exacerbated in the summer and consist of bullae and crusted lesions. Uroporphyrin was not found in our patient, and we believe that this bullons eruption is due to sun sensitivity. The occurrence of such a combination of symptoms in three persons justifies the assumption that we are dealing with a congenital syndrome entity."

Now nearly three decades later, 98 cases of Bloom syndrome have been documented in the world (James German, personal communication). A registry has been established at the New York Blood Center under the auspices of Dr. James German to confirm and categorize newly diagnosed cases. Numerous clinical reviews of Bloom syndrome have also been published by German (68,71,77).

In 1966, Bloom published a review of his experience with this syndrome and delineated three cardinal features: 1) congenital telangiectatic erythma; 2) sun sensitivity; and 3) stunted growth (15). The dermatologic features of Bloom syndrome are absent at birth. During the first or second summer of birth, red lesions usually appear on the cheeks and/or nose and are accentuated by sunlight (Figure 1). Exceptionally, these lesions fail to appear until later in childhood, with German reporting the latest appearance in a boy of twelve years (77). Once these acinic lesions are well established, they progress to telangiectases. The variability of these lesions is striking, ranging from absence to severe occurences in the entire butterfly area of the face as well as lower eyelids, lips, ears and neck. Some actinic erythema may be observed on the dorsa of the hand and forearm.

Skin areas other than the face, hands and forearms are not unduly sensitive to sunlight. Phototests on normal - appearing skin of both lumbar and facial areas did not show any abnormalities in erythema threshold, magnitude of response, or persistence of erythema after ultraviolet or visible light irradiation (unpublished

Figure 1. A male patient with Bloom Syndrome (15).

observations of L. C. Harber referred to in Ref. 68). The lesions may show improvement with age and are generally less severe in females.

Over half of the individuals with Bloom syndrome display a localized disturbance in dermal pigmentation (68). Irregular areas of hyper- and hypopigmentation, varying in size from one to many centimeters in diameter, may be noted. The areas of hyperpigmentation are often described as cafe-au-lait spots and are found mainly on the trunk. Occasionally, areas of both hyper- and hypopigmentation are in close juxtaposition and German has suggested that these may be "twin spots" resulting from somatic crossing-over (70).

As mentioned earlier, there is significant growth retardation in Bloom syndrome. The gestation period is normal in duration and the infant is appropriately developed and mature. However, the size of the infant at birth is much smaller than normal. The mean birth weight is 1960 g (normal 2500g) and the mean length at birth is 44cm (normal 50cm). Postnatal development is unremarkable except height and weight remain below normal. The mean adult height is 148.5 cm or 4 feet 10 inches (normal 177 cm or 5 feet 10 inches). The body habitus is normal and these patients are normally proportioned. The faces is distinctive with head being narrow and relatively long in the occipitofontal plane (dolichocephaly). Intelligence is within normal limits.

Hutteroth et al., (107) has found that patients with Bloom syndrome have at least one class of immunoglobulins in low serum concentrations. They have weak but detectable levels of humoral and cellular responses after antigenic challenge. German (77) has noted that these patients frequently suffer from upper respiratory tract infections and life-threatening complications such as pneumonia and severe ear infections, all of which have responded well to antibiotics. In fact, German has speculated that in the past many, if not most, patients succumbed to infection early in life and, therefore, the syndrome went unrecognized.

Developmental anomalies occur more commonly in Bloom syndrome patients than in normal individuals. They are very diverse and may involve the skin, teeth, eyes, bone, digits, or genitalia. Although most patients have anomaly, few patients share the same anomaly (77). Diabetes mellitus may be unusually common in Bloom syndrome, with the diagnosis having been made in six patients (76). In two cases, reversable diabetes occurred in response to a leukemia-treatment regimen.

Cancer was first recognized as being more common in Bloom syndrome by German et al., in 1965 (66). To date 21 out of 98 individuals with Bloom syndrome have cancer (James German, personal communication). Therefore, approximately one of every 4.7 individuals with Bloom syndrome has cancer (21%) with the average age at diagnosis of 22.8 years (79). Acute leukemia is the commonest neoplasm, half being acute lymphocytic leukemia. A relatively large number of carcinomas have also occurred, usually in common sites. This is quite unusual since living patients with Bloom syndrome comprise a young group (average age in 1979 was 16.4 years; Ref. 79). Table 3 contains information on cancer deaths in Bloom syndrome. Figures 2 and 3 graphically display this data. Clearly cancer deaths are over two orders of magnitude greater in Bloom syndrome individuals than in the general population. Therefore, Bloom syndrome may clearly be considered a cancer-prone syndrome and is unique in that the cancers may occur at different sites.

The formal genetics of Bloom syndrome have been extensively reviewed by German and his colleagues (68,74,77). Szalay (201) and Wolf (231) first reported Bloom syndrome in siblings suggesting a genetic etiology. Shortly thereafter consanguinity was noted to be somewhat more frequent in the parents (15), suggesting an autosomal recessive mode of inheritance. The estimated ratio of affected to unaffected among children of heterozygotes is between 0.146 (method of discarding the propositus) and 0.255 (method of discarding the singles). German (68) has proposed that the low value of 0.146 may be due to the loss of homozygotes

Table 3. Deaths from Internal Cancers in Bloom Syndrome

Age	Patient Years	Observed/Expected	
		Leukemias Lymphomas	Carcinomas
0-14	340	1/0.013	0/0.017
5-14	640	3/0.020	0/0.019
15-24	327	2/0.011	0/0.016
25-34	108	2/0.005	2/0.013
35-44	16	NA	1/0.008
45-54	3	NA	1/0.005
TOTALS		8/0.049	4/0.078
		12/0.127	

The observed values were obtained from various clinical reports of 89 cases and the expected values were calculated from the current age-specific cancer death rates in England and Wales. Data taken from Cairns, J. (23).

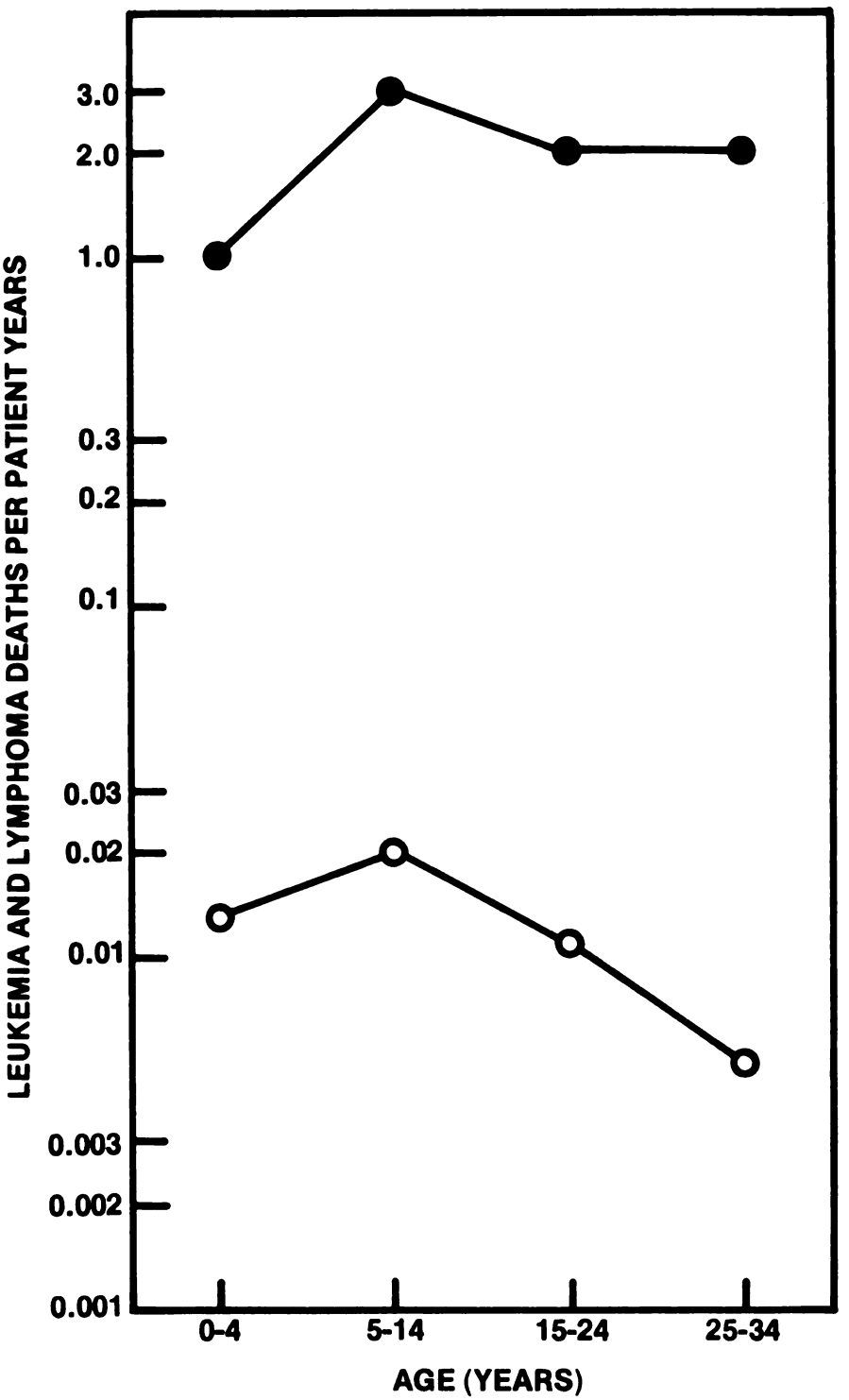

Figure 2. Leukemia and lymphoma deaths per patient years as a function of age. Closed circle, Bloom syndrome; open circle, expected values. Data from Table 3.

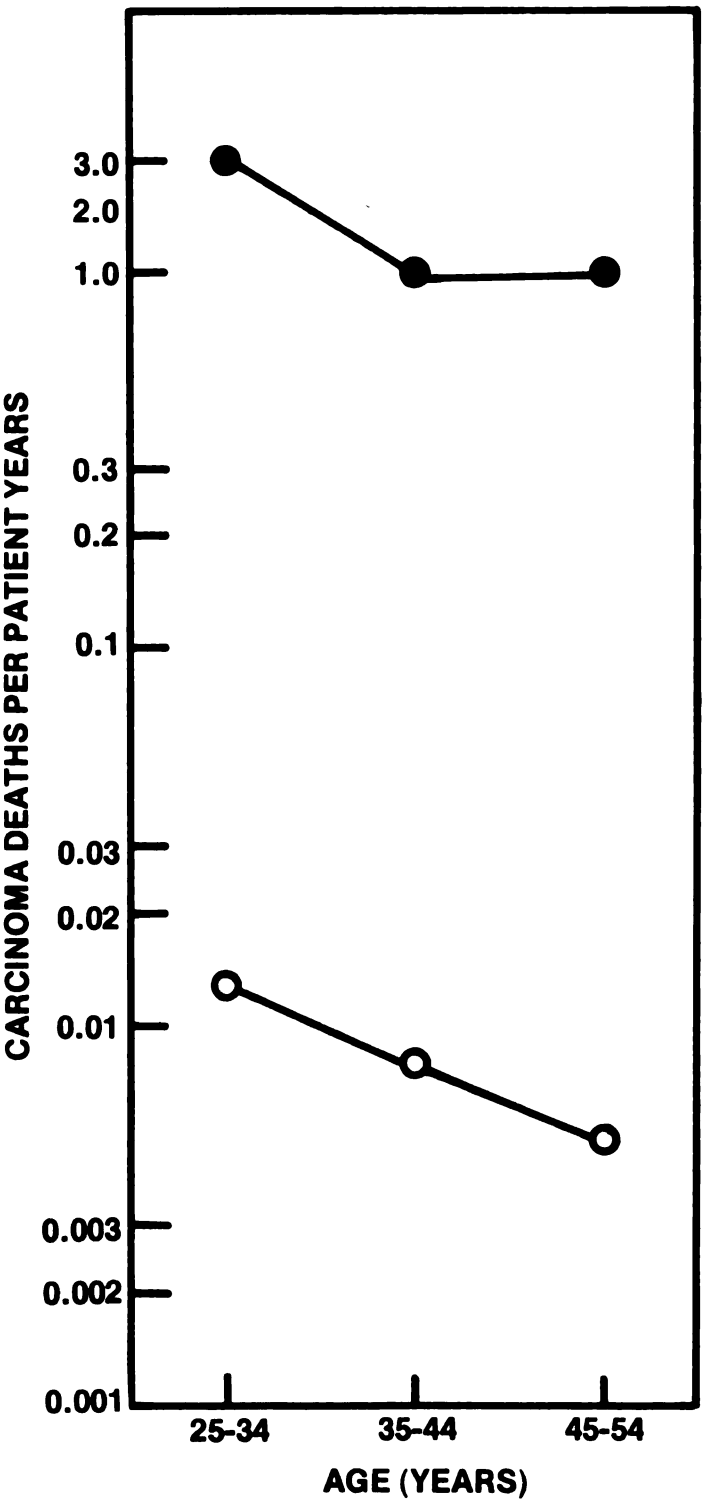

Figure 3. Carcinoma deaths per patient years as a function of age. Closed circle, Bloom syndrome; open circle, expected values. Data from Table 3.

during embryonic life. Indeed, an observed distortion of the sex ratio among homozygotes (1.6M:1F) may be a reflection of intrauterine loss of females, since affected females have lower birth weights than affected males (74). Alternatively, females may be less likely to be diagnosed since affected females more often have minimal skin involvement. Bloom syndrome is more common in Jewish populations (68) and German et al, (74) has estimated the gene frequency in Ashkenazi Jews to be 0.0042, which places the heterozygote frequency to be greater than 1 in 120. The heterozygote frequency is much lower in non-Jews since consanguinity is elevated only in non-Jewish parents of affected homozygotes (74).

German et al., (66) first reported that chromosome breakage and rearrangement is elevated in Bloom syndrome (4-27 percent of the metaphases examined). Homologous rearrangements and quadriradial formations are quite frequent as compared to normal controls (72). The most striking chromosome phenomenon in Bloom syndrome is the markedly elevated frequency of sister-chromatid exchanges (SCE) which was first noted by Chaganti et al., (26). Normal individuals have a mean of 6.9 SCEs per metaphase while patients with Bloom syndrome have a mean of 89.0 SCEs per metaphase. Elevated SCE frequencies are found in all tissues thus far examined; lymphocytes, dermal fibroblasts and bone marrow (67,187,217). However, some Epstein-Barr virus-transformed lymphoblastoid cell lines derived from patients with this syndrome do not show an elevated SCE frequency (100). A quite interesting, yet puzzling finding regarding SCEs in Bloom syndrome was reported by German et al., (73). They found that while all examined patients had elevated SCEs in peripheral blood lymphocytes, five patients (of 21) simultaneously exhibited a small proportion of cells with normal SCE values.

Because of the chromosome aberrations, sunlight sensitivity and cancer incidence, many studies dealing with the DNA repair capacities of Bloom syndrome cells have been conducted. German and Schonberg (78) have recently reviewed the

findings. Bloom syndrome fibroblasts have normal sensitivity to X-ray and gamma irradiation (7,228). The sensitivity to UV irradiation, however, has been conflicting. Most reports describe normal survival, except for strain GM 1492 which is slightly sensitive (5,109,126,184). Giannelli, et al., (80) found two Bloom syndrome strains to be UV sensitive as measured by colony-forming ability. Smith and Paterson (194) have reported that Bloom syndrome fibroblasts are hypersensitive to mid-ultraviolet light. Indeed, carefully controled experiments can show a slight but reproduceable sensitivity to UV irradiation in a number of Bloom syndrome strains (M. Wade and S.T. Warren, in preparation). Additionally, Bloom syndrome fibroblasts have been found to be sensitive to ethyl methane sulphonate and mitomycin C (5,109).

Studies of repair of DNA damage in Bloom syndrome have generally reported normal findings. DNA excision repair as well as measurements of postreplication repair have been normal following UV-irradiation (1,34,55; M. Wade and S.T. Warren, in preparation) as is repair following treatment with N-acetoxy-2-actyl-aminofluorene (175) and X-ray (219). Host-cell reactivation of UV-irradiated Herpes simplex virus-1 and adenovirus - 2 has also been within normal limits except for strain GM 1492, in which it is decreased (126,184). Apurinic-site specific endonuclease activity, as well as the enhancement of gamma irradiated DNA priming activity for DNA polymerase, has been reported as normal (108,150). Hirschi et al., (102) have reported that the formation of single-strand breaks by near-UV irradiation (313 nm) at 37°C is increased in Bloom syndrome fibroblasts, although the repair of the breaks is normal. The general trend among the above studies is that DNA repair, as presently understood, appears to be intact in Bloom syndrome. Thus, Bloom syndrome should not be classified as a repair-deficient disorder.

Attention has, therefore, turned to DNA replication rather than repair. Hand and German (92,93) have reported that DNA fork movement as measured by fiber autoradiography is retarded in fibroblasts and lymphocytes derived from patients

with Bloom syndrome. Replication unit length, incidence of bidirectional replication and the degree of initiation synchrony were all normal. These data seriously raise the question of defective DNA replication in Bloom syndrome. Ockey (157) has presented data indicating that the retarded chain growth occurs only in cells grown at low density and Giannelli et al., (80) have also shown a delay in chain maturation during replication. Crude measurements of DNA polymerase activity (α, β, γ) however, are within normal limits (12,160). The hypothesis that Bloom syndrome is replication-defective, nonetheless, has considerable attraction.

Tice et al., (205) reported in 1978 that Bloom syndrome fibroblasts when co-cultivated with control fibroblasts, increased the SCE frequency in the control cells. Furthremore, they found that medium from which Bloom syndrome fibroblasts were grown, also induced SECs in normal human lymphocytes. These investigators hypothesized that cells derived from individuals with Bloom syndrome produce an endogenous agent capable of damaging DNA. This report renewed interest in Bloom syndrome and soon other reports followed. However, unlike Tice and his co-workers, the subsequent reports found no change in the SCE frequency in normal cells either cocultivated with Bloom syndrome cells or grown in the conditioned medium. In fact some investigators found the SCE frequency in the Bloom syndrome cells to be reduced after co-cultivation (2,19,178,181,215). Schonberg and German (181) found that normal cells, when metabolically coupled to Bloom syndrome cells, do not have a change in their SCE frequency. This indicates that such a factor as reported by Tice et al., (205) may not be produced or that if it is produced it is larger than 900 daltons, the size limit for exchange through the mammalian gap junction. Also, the SCE frequency in Bloom syndrome cells is normalized by hybridization with normal cells (2,19,188), indicating two things: first, the defect in Bloom syndrome is able to be complemented by normal cells which is consistant with the presence of an autosomal recessive biochemical defect; and secondly, the defective protein is

larger than 900 daltons (see Ref. 181), which is not surprising since most proteins greatly exceed this size. These data, however, do not explain the results of Tice and his co-workers and the dilemma of a mutagenic factor in Bloom syndrome cells is still unresolved.

Emerit and Cerutti (52), however recently reported work which is supportive of that of Tice et al., (205). These investigators found that the medium of Bloom syndrome fibroblasts contain a factor which is capable of inducing chromosome aberrations and SECs in normal lymphocytes in a dose dependent fashion. This factor was further found to be between 1,000 and 10,000 in molecular weight and was inactivated by bovine superoxide dismutase. On the basis of these results Emerit and Cerrutti have postulated that Bloom syndrome has an abnormality in the formation or detoxification of active oxygen species. In addition, they have proposed that the conflicting results of the co-cultivation experiments may be due to varying activites of superoxide dismutase in different lots of fetal calf serum. These investigators further supposed that Bloom syndrome may be related etiologically to systemic lupus erythematosus.

This proposal is quite interesting since, it may be recalled, that Bloom syndrome patients were originally described as lupus erythematosus in dwarfs, thus the clinical similarity is apparent. Furthermore, it has been found recently that patients with lupus erythematosus have a serum factor, capable of clastogenic activity, that falls in the same molecular weight range as the factor from Bloom syndrome cells (51,53). The lupus factor is also inactivated by superoxide dismutase and is activated by UV light. Thus, the similarity between lupus erythematosus and Bloom syndrome deserves careful study.

In conclusion, the major hypothesis now considered in the etiology of Bloom syndrome are either an abnormality in DNA replication or the production of a clastogenic agent. Either of these possibilities may increase the rate of spontaneous

mutation, since, as shown previously, mutator mutants in lower organisms frequently have DNA synthesis abnormalities and the production of a clastogenic agent intuitively would suggest an influence on the mutation rate. Furthermore, the high cancer incidence, occurring at different sites, would be consistent with an elevated mutation rate in somatic cells, based on the somatic mutation theory of cancer. We, therefore, undertook this study to examine the hypothesis that Bloom syndrome fibroblasts have an elevated spontaneous mutation rate in vitro.

MATERIALS AND METHODS

Cell Strains

Skin fibroblast cultures, GM 1492, GM 2548, GM 3510 and GM 3402, originally derived from patients with confirmed Bloom syndrome [Bloom syndrome registry designations 44 (Ab Ru), 71 (Ha En), 47 (Ar Smi) and 9 (Em Sh), respectively] were obtained from the Human Genetic Mutant Cell Respository (Camden, New Jersey). Four fibroblast strains, NSF-1, NSF-2, NSF-791 and MSU-2, initiated in this laboratory from foreskins of four healthy male infants, served as controls. SCE determinations were performed on all strains prior to use, as previously described (165), to confirm their genotypes (bl/bl/ or +/-). All strains had male karyotypes and were used prior to passage 16.

For some experiments, an aneuyploid cell line (V79), originally derived from the lung of a male Chinese hamster (Cricetulus griseus, 2N=22), was used (60). All cells were suspended in 10% dimethyl-sulfoxide in PBS (composed of 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4 per liter distilled water), sealed in glass ampules and frozen in liquid nitrogen until needed for experimentation.

Culture Medium

Cells were grown in modified Eagle's minimal essential medium (50) with Earle's salts, supplemented with a 100% increase of all non-essential amino acids, 50% increase of all vitamins and essential amino acids except glutamine and 1 mM sodium pyruvate (Gibco, Grand Island, New York). The medium was sterilized by passage with positive pressure through Nuclepore filters (Nuclepore Corporation, Pleasanton, California), and was stored in the dark at $4^{o}C$. Prior to use, the medium was supplemented with 15% fetal calf serum or 10% fetal calf serum and 5% calf

serum (Gibco) which was stored at -20°C, thawed, and heat inactivated at 56°C for 25 minutes before addition to the medium. The medium was also supplemented with 100 units/ml penicillin G and 100 µg/ml streptomycin during the experiments (Eli Lilly and Company, Indianapolis, Indiana).

Culture Vessels and Incubation Conditions

Stock cell cultures were grown for experiments in static culture attached to the surface of sterile plastic flasks (75 or 150 cm^2; Corning Glass Works, Corning, New York). Cells were subcultured 1:2 or 1:4 using 0.01% crystalline trypsin and 0.6 mM EDTA (ethylene-diamine tetracetic acid, Sigma Chemical Company) in calcium and magnesium free PBS. All cultures were grown in water-jacketed incubators at 37°C in humid air supplied with 5% CO_2.

Cell and Colony Counts

Trypsinized individual cells in suspension were counted using a hemacytometer. Macroscopic colonies were scored visually after staining with crystal violet (2g/100 ml of 10% ethanol). Colonies were scored only if they were greater than 50 cells, microscopically.

Spontaneous Mutation Rate Experiments

Spontaneous mutation rates in human fibroblasts, were determined via two methods using resistance to 6-thioguanine (6-TG) as a genetic marker (202). Figure 4 illustrates the genetic basis of 6-thioguanine resistance. 6-TG resistant cells are presumptive mutants of the X-linked enzyme hypoxanthine/guanine phosphoribosyl transferase (HGPRT; EC 2.4.2.8).

The first protocol, Method I, was a modification (216) of the technique originally used by Newcombe (152) in bacterial cultures to estimate the spontaneous mutation rate from the increase in the spontaneous mutation frequency during growth period of a cell population (Figure 5). Into five 150 cm^2 plastic flasks,

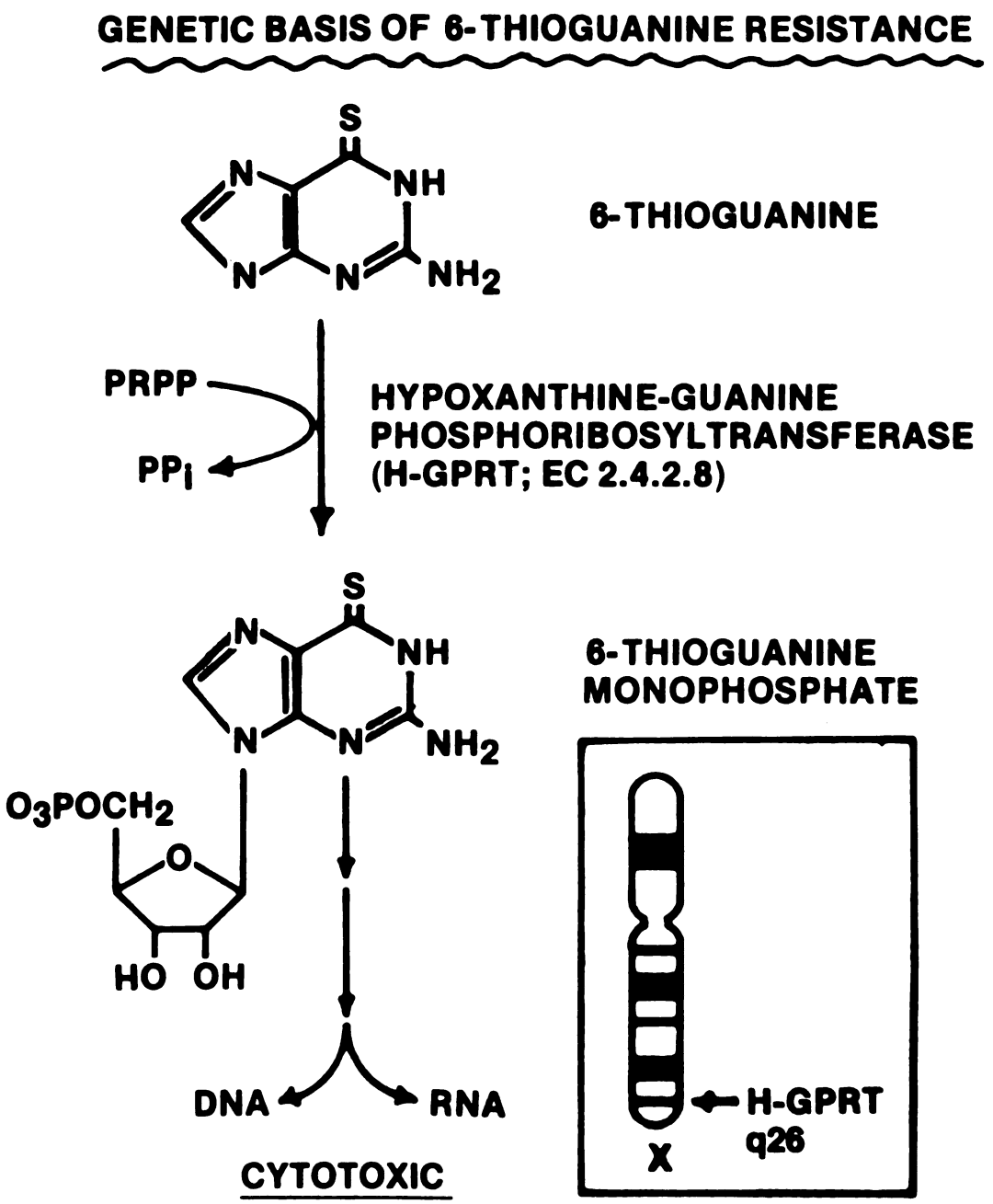

Figure 4. The genetic basis of 6-thioguanine resistance in human cells.

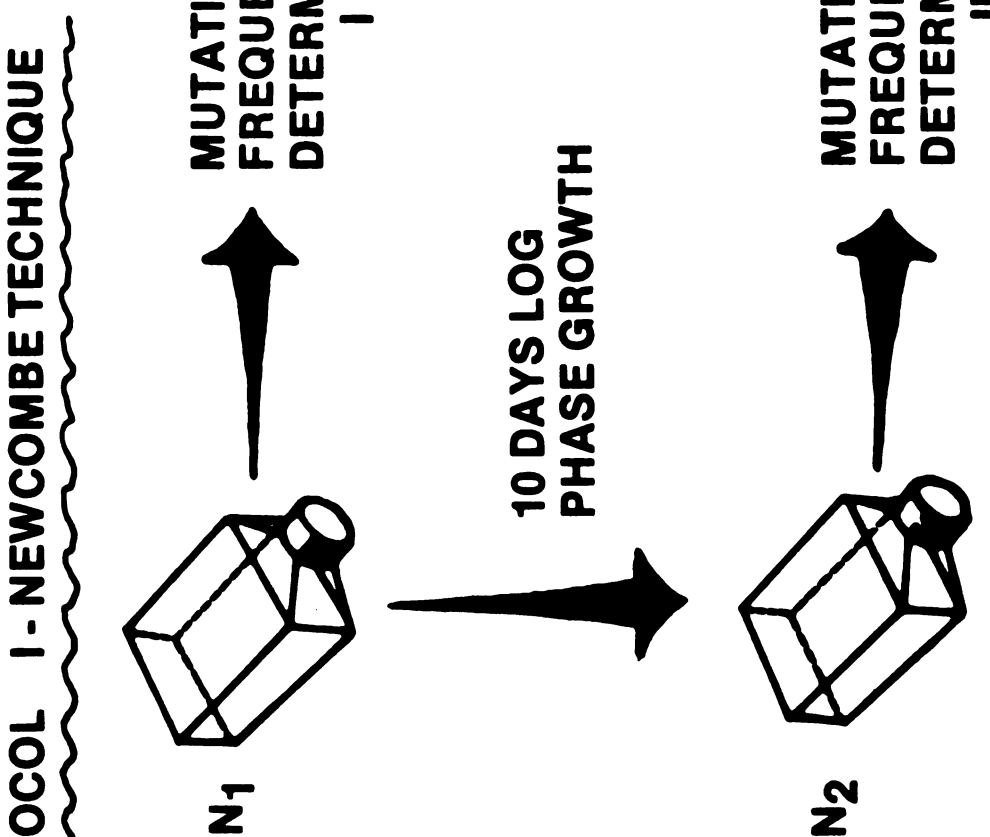

Figure 5. Protocol for the determination of the spontaneous mutation rate based upon that of Newcombe (152; Method I).

approximately 4 x 10^6 cells per cell strain were inoculated in growth medium; this initial cell population is designated N_1. Cell attachment was complete for mass culture of these cell strains. From the same initial population, 4.8 x 10^6 cells per strain were inoculated into 120 plastic dishes (9cm) and treated with 6-TG (10 µg/ml; Sigma Chemical Company) for the determination of the initial mutation frequencies, MF_1. This procedure is diagramatically shown in Figure 6. Six additional dishes per strain were each inoculated with 200 cells in growth medium for an estimation of the cloning efficiencies of each strain. The medium in the selection dishes was changed every third or fourth day with fresh medium containing 6-TG. The dishes were stained, as above, on the 25th day and the colonies scored. The cells in the flasks were allowed to grow for 10 days, with the medium changed twice during this period. At the end of 10 days, the cells of each strain were rinsed with PBS, trypsinized and pooled for the determination of cell number, designated N_2. The pooled cells from each strain were plated, as described, for the determination of the second mutation frequencies, MF_2. N_1 and N_2 were determined from hemacytometer counts on the pooled cells without correction for cloning efficiency.

The second protocol, Method II, was a modification (45) of the fluctuation analysis of Luria and Delbruck (133) and is shown in Figure 7. For each strain, cells (50 cells per dish for control strains and 100 cells per dish for the Bloom syndrome strains, to compensate for the lowered plating efficiency in Bloom syndrome fibroblasts) were inoculated into each of 96 dishes (9cm) and grown in nonselective medium for 15 days with a single medium change at day 8. At the end of this growth period, three dishes per strain were stained, and colonies were counted to determine the initial cell number, N_o, that was plated into the dishes. Another five dishes were separately rinsed with PBS, and the cells were trypsinized and counted to determine the final cell number, N_t. The cells from these dishes were pooled for each strain, recounted, and diluted to inoculate six culture dishes per strain with 200

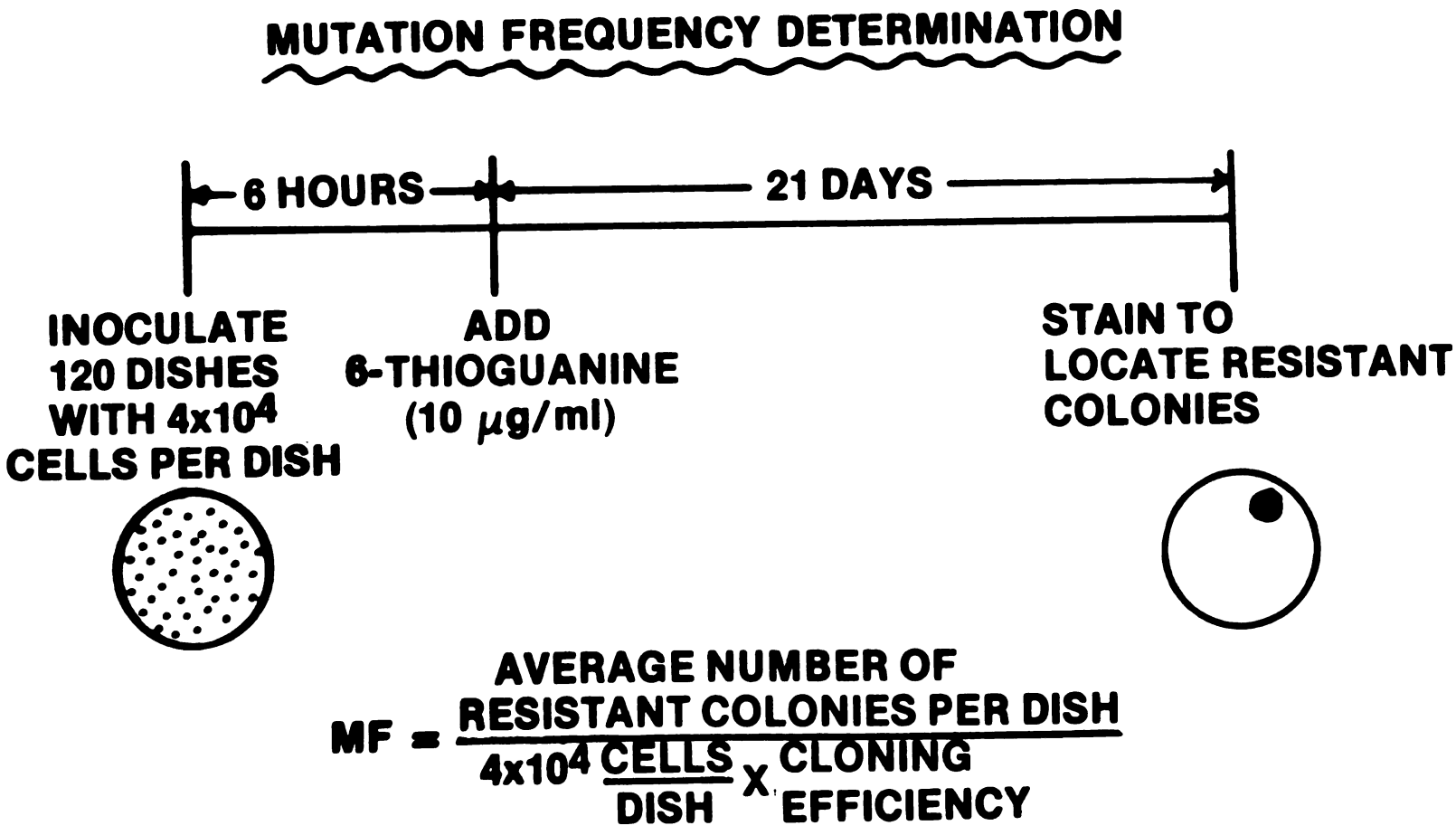

Figure 6. Protocol for the determination of the mutation frequencies (MF_1 and MF_2) used in Method I.

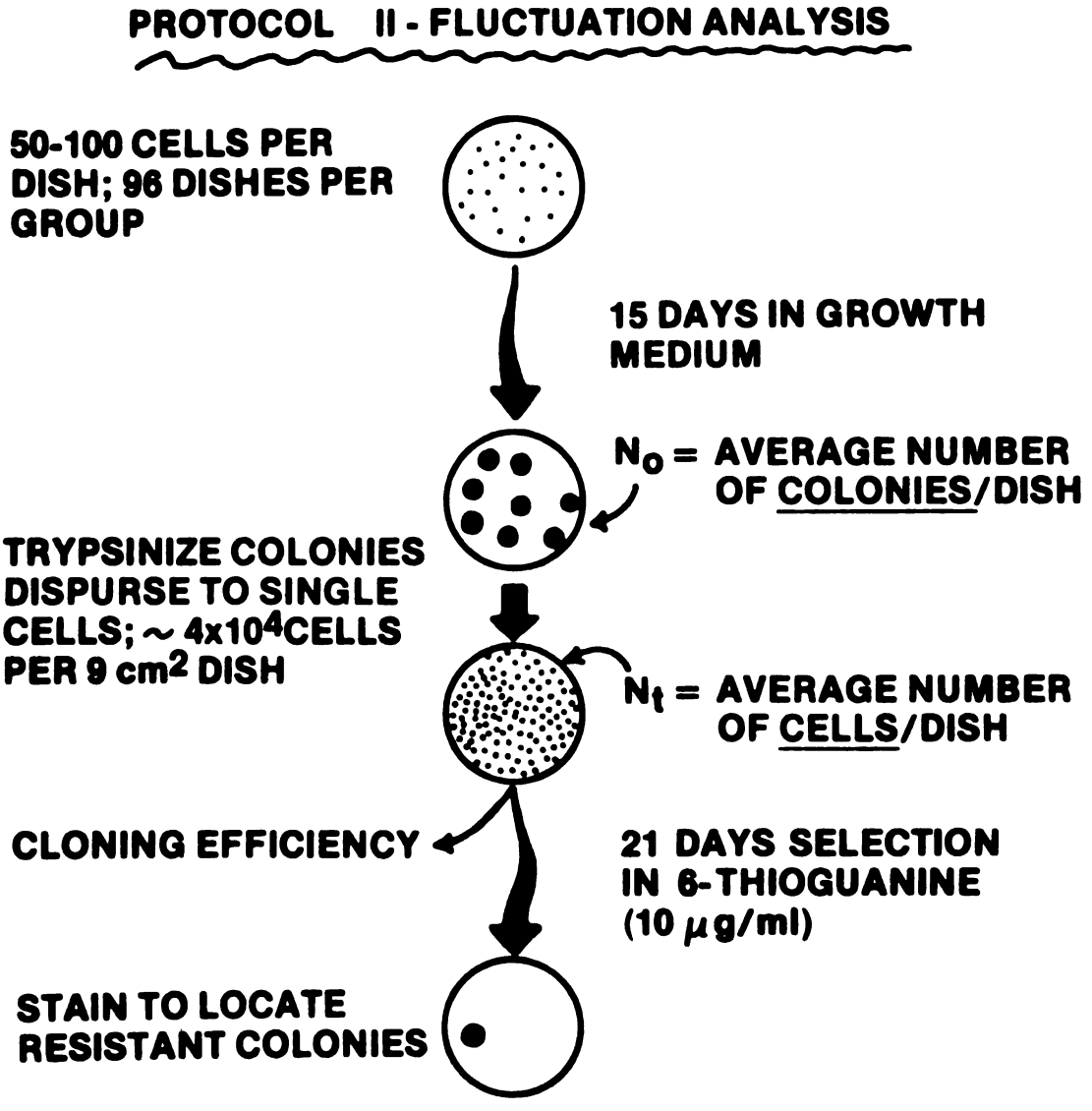

Figure 7. Protocol of the fluctuation analysis used to determine the spontaneous mutation rate (Method II).

cells per dish in order to determine the cloning efficiencies. The cells in the remaining dishes were each rinsed once with saline containing 0.6 mM EDTA, treated for 8 minutes with 1 ml of 0.01% trypsin (without EDTA), dispersed with Pasteur pipettes (dispersion monitored microscopically), and suspended in the original dishes with 10 ml of medium containing 10 µg per ml. It was estimated that each dish contained less than 4.1×10^4 cells. The medium was changed 16 hours later with fresh medium containing 6-TG and was changed thereafter every third day for 22 days. At that time the plates were stained and colonies were counted as described above.

Mutation Rate Calculations

The number of cell divisions was determined from the formulae $(N_2 - N_1)/\ell n 2$ and $(N_t - N_o)/\ell n 2$ for Methods I and II, respectively. Calculations of mutation frequencies and rates were based on the mean number of mutant colonies per dish, $\underline{m}$, which was determined in two ways: (i) $\underline{m} = \ell n\ 1/P_o$, where P_o equals the percentage of plates with zero 6-TG resistant colonies; and (ii) the arithmetic mean, where $\underline{m}$ equals the total observed 6-TG resistant colonies per total dishes.

For Method I the mutation frequencies, MF_1 and MF_2, were determined by dividing $\underline{m}$ by the number of cells inoculated per dish (4×10^4) multiplied by the cloning efficiency (Figure 6). The spontaneous mutation rate, $\underline{a}$, was then determined using Equation 1. The derivation of Equation 1 is as follows:

Let: m_i = Number of mutants in generation i

N_i = Total cells in generation i

a = Mutation rate (mutations/cell/generation)

x = Any integer

The mutation frequency (MF) may be described as m_i/N_i-1, since $\underline{m}$ mutants arose in one or more prior generation(s). Therefore, the mutation rate, a, may be calculated

from the difference between two mutation frequency determinations performed X generations apart. Thus:

$$a = \frac{\frac{m_i}{N_i-1} - \frac{m_i-x}{N_i-1-x}}{\ln \frac{N_i-1}{N_i-x}} \quad (\ln 2)$$

Since $N_i-1 = \frac{1}{2} N_i$ and $N_i-1-x = \frac{1}{2} N_i-x$, we have:

$$a = \frac{\frac{m_i}{\frac{1}{2} N_i} - \frac{m_i-x}{\frac{1}{2} N_i-x}}{\ln \frac{\frac{1}{2} N_i}{\frac{1}{2} N_i-x}} \quad (\ln 2)$$

which equals:

$$\frac{2\frac{m_i}{N_i} \quad \frac{m_i-x}{N_i-x}}{\ln \frac{N_i}{N_i-x}} \quad (\ln 2)$$

by substituting MF_2 and MF_1 for m_i/N_i and m_i-x/N_i-x, respectively; and N_2 and N_1 for N_i and N_i-x, respectively; we have:

$$a = \frac{2(MF_2-MF_1)}{\ln \frac{N_2}{N_1}} (\ln 2) \qquad (1)$$

For the fluctuation analysis (Method II), the spontaneous mutation rate was calculated in two ways. The first was derived from equation four of Luria and Delbruck (133):

$$a = \frac{mC}{(N_t - N_o) \ln 2} \qquad (2)$$

in which C is the number of replicate cultures (dishes). The second calculation of a is based on equation 8 of Luria and Delbruck (133) as formulated by Capizzi and Jameson (24):

$$a = \frac{(CaN_t)}{CN_t} \qquad (3)$$

Equation 3 may be solved by first determining Cr, in which r is the mean number of 6-TG resistant colonies per dish corrected by the cloning efficiency; thus, r = m/CE. Therefore, the numerator of Equation 3, (CaNt), may be found, based on the quantity Cr, in tables provided by Capizzi and Jameson (24). Note that Equation 3 is thus corrected for the cloning efficiencies of the cell strains, whereas Equation 2 is not.

Fibroblast Medium Collection and Concentration

For each strain, two 150 cm^2 plastic flasks were inoculated with cells from a 1:2 split of a confluent flask. The medium was changed 24 hours later (50 ml medium per flask) and the cells incubated for 7 days. After this growth period, the medium was collected and pooled for each strain (100 ml total per strain) and concentrated to 20 ml using Amicon DC-2 hollow fiber dialyzer/concentrator set-up with a H1P2 hollow fiber cartridge (pore size cutoff of 2,000 MW; Amicon Corporation, Lexington, Massachusetts). The concentrator was set at a power level of 4 and a restriction number of 5. The reservoir of the concentrator was modified using a 150 ml separatory flask in order to work with small volumes. The resulting 20 ml volumes, then devoid of molecules below 2,000 MW, was lyophilized to dryness. The dried material was dissolved in 5 ml of PBS resulting in a 20X concentration of the original conditioned medium. Protein determinations were performed on all samples and aliquots were then adjusted to 50 mg/ml protein. The concentrates were then sterilized by passage through a 0.22 micron filter under

vacuum (Falcon, Oxnard, California). The filtered aliquots were frozen at -20°C until use (less than one week).

Cell Survival Determinations

Cell survival determinations, both for human fibroblasts and Chinese hamster cells, were based upon colony-forming ability after treatment with aphidicolin for the human cells, and medium concentrate for the hamster cells.

To determine the sensitivity of Bloom syndrome and control fibroblasts to aphidicolin (supplied by the Developmental Therapeutics Program, National Cancer Institute), cells were plated in 9 cm plastic dishes (Corning Glass Works) containing 10 ml of medium at such a cell density as to expect about 50 colonies per dish. The aphidicolin was added to each dish at various concentrations after cell attachment (about 6 hours) and the dishes were incubated for 5 days. The medium was then removed and fresh medium was added to each dish. After an additional 10-15 days incubation, the colonies were stained with crystal violet and counted. The results were expressed as percent survival relative to the untreated group for each cell strain.

The hamster cell survival studies with the medium concentrates were performed in essentially the same manner. To dishes containing appropriate numbers of hamster cells, various amounts of the medium concentrates (based on mg protein per 10 ml medium) were added such that a total medium volume of 10 ml was maintained. After 48 hours of incubation, the medium was removed and replaced with fresh medium without the concentrate. After an additional 7-10 days growth, the colonies were stained and scored as above.

Chinese Hamster Cell Mutagenesis

Resistance to 6-thioguanine and the cardiac glycoside ouabain were used as selective markers to determine mutation frequencies in V79 cells as has been

previously described (27,31,204). Sufficient numbers of cells were plated such that at least 10^6 survived the treatments. Treatment with the medium concentrates was accomplished as described above and the treatment duration was kept at 48 hours. In later experiments, the hamster cells were pretreated in HAT medium (5 µg/ml hypoxanthine, Sigma; 4 µg/ml thymidine, Sigma; 3.2 µM amethopterin, National Biochemical Corporation, Cleveland, Ohio) for 10 days prior to treatment with the media concentrates to lower the frequency of 6-TG resistant cells. After treatment, the cells were allowed to grow in complete medium, with subculturing when appropriate, for an expression time of 3 days (4 cell divisions) for ouabain selection and 8 days for 6-thioguanine selection. Mutation frequencies were determined for both markers on each treatment flask. Selection plates contained 5 x 10^4 cells per 9 cm dish (21 dishes per point) for 6-thioguanine resistance (10 µg/ml; Sigma Chemical Company) and 2 x 10^5 cells per 9 cm dish (12 dishes per point) for ouabain resistance (1 mM; Sigma Chemical Company). Additional plates were inoculated with 100 cells/dish in nonselective medium to determine the cloning efficiencies. After 10-14 days, resistant colonies were stained and counted (colonies were scored if they contained greater than 50 cells microscopically). Mutation frequencies were expressed as mutants per million surviving cells.

Proportion of S-Phase Cells

Fibroblasts, which were held at confluency for at least 24 hours, were trypsinized and counted. 5 x 10^4 cells per 30 mm plastic dish were inoculated into 2 ml of medium. Each dish contained one 22 x 22 mm sterile, ethanol washed coverglass. After incubation for various times (5-75 hours), 20 µCi of ^{3}H-thymidine (specific activity of 25 Ci per mmole; Amersham Corporation, Arlington Heights, Illinois) was added to each dish one hour prior to harvesting. After the one hour incorporation, the medium was removed and discarded, the dishes rinsed three times in PBS and then Bouin's fixative (75 ml saturated aqueous picric acid, 25 ml 40%

formaldehyde and 5 ml glacial acetic acid) was added to each plate. After 20 minutes, the fixative was removed, the plates rinsed once with water and the coverslips removed, rinsed with distilled water and dried. The coverslips were then mounted, cell side up, on microscope slides with clear nail polish and allowed to dry. The slides were then dipped, in the dark, in NTB-2 liquid emulsion which was diluted 1:1 with distilled water, (Eastman Kodak Company, Rochester, New York), dried 30 minutes, placed into desicated light-tight boxes and incubated 4 days at $4^{o}C$. Slides were then developed and fixed, rinsed for 10 minutes under running tap water and stained in 1% toluidine blue in citric acid (21 g/ml, pH 5.0) for 5 minutes. The slides were then rinsed twice in distilled water, dried, and a second coverslip was mounted with Pre-texx (Scientific Products, McGraw Park, Illinois). The slides were then examined at 400 x magnification and a total of 50 labelled and unlabelled cells were counted within a linear microscopic field. The proportion of labelled cells (those in S-phase) was directly calculated.

Deoxyribonucleoside Triphosphate Measurements

The extraction of dNTP pools from cells was accomplished by a modification of the procedure of North et al., (156). Fibroblasts that were held at confluency for 24 hours were trypsinized, counted, and 5×10^{6} cells were inoculated into five 9 cm plastic dishes for each cell strain. After 24 hours of incubation in growth medium, the cells were harvested with a rubber policeman in ice-cold 60% methanol. The five plates per strain were pooled into a test tube and incubated overnight at $-20^{o}C$. The tubes were then centrifuged and the resulting supernatant removed from the methanol precipitate. The amount of DNA in the precipitate was determined as described below and the supernatant was taken to dryness in a lyophilizer. To the dried material, 0.5N perchloric acid (3ml) was added and the tubes were incubated 30 minutes at $4^{o}C$. The tubes were then neutralized with 1.5N potassium hydroxide (0.705ml) at $4^{o}C$ for 30 minutes. The tubes were then centrifuged and the

supernatant again taken to dryness in a lyophilizer. The dried material was dissolved in 1 ml distilled water, centrifuged and the resulting supernatant was isolated for dNTP determinations.

Measurements of dNTP pools were accomplished using the defined copolymers poly d(I-C)-poly d(I-C) and poly d(A-T)-poly d(A-T) (PL Biochemicals, Milwaukee, Wisconsin) and E. coli DNA polymerase I (Boehringer Mannheim, Indianapolis, Indiana) as previously described (130,192). Briefly, one volume of sample was added to nine volumes of a solution containing 50 mM Tris-HCl, pH 8.0, 5.0 mM $MgCl_2$; 1 mM dithiothreitol; 0.0011 mM of the appropriate [^{3}H]-labelled complementary dNTP at 0.01 mCi/ml; 1.1 units/ml of E. coli DNA polymerase I; 0.035 mg/ml of an alternating copolymer template, either poly d(A-T) or poly d(I-C); and 0.22 mg/ml of bovine serum albumin. The poly d(A-T) was used for the assays of dATP and dTTP, while poly d(I-C) was used to assay dCTP and dGTP. As an example, the assay for dATP would measure the incorporation of [^{3}H]dTTP into poly d(A-T). For each set of assays a standard curve was obtained from known amounts of the dNTP in question.

Samples and reaction components were mixed together at 4°C and reactions were begun by transferring this mixture (in a volume of 0.1 - 0.2 ml) to a 37°C waterbath. After a reaction time of 40 minutes, the tubes were placed on ice and 3ml of ice-cold 10% trichloroacetic acid with 1% sodium pyrophosphate was immediately added to each tube. After 30 minutes, the mixtures were filtered, under vacuum, through Whatman GF/C filters (2.4 cm; Whatman Ltd., England) that were presoaked with the above TCA-Na pyrophosphate solution. The filters were then washed three times with the TCA-Na pyrophosphate solution, once with 5ml of ice-cold 95% ethanol and dried under a heat lamp. The dried filters were placed in scintillation vials containing a toluene-based counting fluid. Radioactivity was

measured in a Beckman LS 9000 liquid scintillation counter. The results were expressed as pmoles dNTP per µg DNA.

DNA and Protein Determinations

DNA measurements were performed according to the procedure of Giles and Myers (81). The methanol precipitates, from the dNTP assays, were resuspended in 1ml of distilled water and sonicated using a Branson sonifier at a power level of 25 for 5 seconds. 0.5 ml of the suspended samples were mixed with 0.5 ml of 20% perchloric acid and to this 1 ml volume, 1 ml of 4% diphenylamine (prepared in glacial acetic acid) and 0.05 ml of acetaldehyde (1.6 mg/ml, prepared fresh) were added. This reaction mixture was vortexed and incubated at 30°C overnight. The optical density of each sample was then read at 595 and 700 nm against a reagent blank on a Gilford Stasar II spectrophotometer. A standard curve was generated simultaneously by using calf thymus DNA (Sigma Chemical Company, St. Louis) in concentrations from 5 to 50 µg. Using the differences in optical densities (595-700 nm) the results were expressed as µg DNA.

Protein was measured according to Lowry et al., (132). Briefly, 0.2 ml sample was mixed with 1.0 ml of an alkaline copper solution (prepared by mixing 25 ml of 2% Na_2CO_3 and 0.02% Na tartrate in 0.1N NaOH with 0.5 ml of 0.5% $CuSO_4$ - 5 H_2O). After 10 minutes of incubation, 1.0 ml of Folin reqagent (1.0N Folin phenol reagent, Fisher Scientific Company, Pittsburgh) was added to each sample, vortexed and incubated at least 30 minutes. 2.0 ml of distilled water was added to each tube and the optical density read at 595 nm. A standard curve was generated simultaneously with bovine serum albumin (50 to 500 µg/ml) and the results expressed as µg protein.

RESULTS

The Spontaneous Rate of Mutation From Method I

Four cell strains were used in the following experiments; GM 1492, GM 2548, NSF-1 and NSF-4. The first two strains were derived from patients with Bloom syndrome while the latter were obtained from normal individuals to serve as controls. From a large cell population, for each strain, an initial mutation frequency was obtained and, following 10 days of log-phase population growth, a second mutation frequency was obtained. From the increase in the frequency of mutants, which had arisen through spontaneous mutations, coupled with the increase in the cell population growth, a rate of spontaneous mutation was determined.

Table 4 summarizes the cell population growth in the five 150 cm^2 flasks over the 10-day period for each cell strain examined. The two Bloom syndrome strains GM 2548 and GM 1492 started at a population size of 4.0×10^6 and 4.2×10^6 cells, respectively, and increased to 15.5×10^6 and 17.7×10^6 cells at the end of the growth period, corresponding to 16.6×10^6 and 19.5×10^6 cell divisions for the respective populations (calculated as described). The two normal strains, NSF-1 and NSF-4, began at a populataion size of 4.4×10^6 and 4.0×10^6 and ended at 86.0×10^6 and 82.2×10^6 cells, respectively. This represents 118×10^6 and 113×10^6 cell divisions for the normal strains, considerably more than the Bloom syndrome strains, which are known to have slower growth rates (157,217). Table 4 also shows a reduced cloning efficiency for Bloom syndrome fibroblasts as compared to normal human fibroblasts, which again is consistent with the observations of earlier investigators (80, 157). Also it should be noted that, ideally, the protocol of Method I should be carried out beyond the 10-day growth period, with multiple mutation frequency determinations. However, our experience indicates that Bloom syndrome

Table 4. Cell Population Growth Parameters for Method I

Cell Strain	N_1	N_2	CE_1(%)	CE_2(%)	Cell Divisions
GM 2548	4.0×10^6	15.5×10^6	7.9	7.9	16.6×10^6
GM 1492	4.2×10^6	17.7×10^6	13.3	7.8	19.5×10^6
NSF-1	4.4×10^6	86.0×10^6	32.6	33.2	118×10^6
NSF-4	4.0×10^6	82.2×10^6	27.3	39.2	113×10^6

N_1, the cell number at beginning of the growth period, and N_2, the cell number at the end of 10 days growth, were determined from hemacytometer counts uncorrected for cloning efficiencies (CE_1 and CE_2). Cell divisions calculated as described in Materials and Methods.

cells senesce early in culture, as manifested by continually lower cloning efficiencies and slower growth rates, thus precluding the extention of the experiment.

Data for mutation frequency determinations at two different times (one performed on the cell populations at the start of the experiment and the second performed on the populations after 10 days of growth) are in Table 5. The mutants were selected at a cell density of 4×10^4 cells per 9 cm dish. Under these conditions, metabolic cooperation, which may lower the recovery of 6-TG resistant mutants, should not be an influence (111, 234). NSF-4 had the lowest number of observable 6-TG resistant colonies, 3 out of 120 dishes, whereas GM 1492 had the highest number of 6-TG resistant colonies with 137 out of 119 plates. Some groups had less than the initial 120 dishes because of loss from contamination. The mean number of mutants per dish, m was calculated either from the direct arithmetic mean observed or from the P_0 number, which assumes the resistant colonies were in a Poisson distribution among the dishes. Using the P_0 number, to calculate m, eliminates a potential bias of satellite colony formation, which arises from a cell(s) disassociating from a resistant colony and reattaching elsewhere on the dish forming a secondary colony. However, the arithmetic m and P_0 calculated m, in Table 5, were all reasonably close to one another for each strain, which indicate that satellite colony formation did not occur to a significant degree. The mean number of 6-TG resistant colonies per dish (m) did not necessarily show an increase during the 10-day growth period because of differences in the total number of plates and/or cloning efficiencies between MF_1 and MF_2.

However, the mutation frequencies (Table 6), which are calculated using the number of plates and the cloning efficiencies as well as other data from Tables 4 and 5, do increase over the 10-day growth period. The two Bloom syndrome strains showed the greatest increase, despite data in Table 4 which showed that the Bloom syndrome strains had undergone significantly fewer cell divisions over the 10-day

Table 5. Spontaneous Mutation Data for Method I

Cell Strain	Number of 6-TG^R Colonies	Number of Dishes	Po(%)	m(mean)	m(Po)
Mutation Frequency 1					
GM 2548	40	120	72.5	0.33	0.32
GM 1492	137	119	34.5	1.15	1.06
NSF-1	126	120	37.5	1.05	0.98
NSF-4	3	120	97.5	0.03	0.03
Mutation Frequency 2					
GM 2548	44	115	68.7	0.38	0.38
GM 1492	87	120	48.3	0.73	0.73
NSF-1	130	117	46.2	1.11	0.77
NSF-4	10	120	92.5	0.08	0.08

Po, percentage of dishes, from total, containing zero colonies; m(mean), mean number of 6-TG^R colonies per dish directly calculated from the data; m(Po), mean number of 6-TG^R colonies per dish calculated from the Po number as described in Materials and Methods.

Table 6. Spontaneous Mutation Frequencies and Rates for Method I

Cell Strain	MF_1	MF_2	Spontaneous Mutation Rate
GM 2548	104×10^{-6}	120×10^{-6}	16×10^{-6}
GM 1492	216×10^{-6}	234×10^{-6}	17×10^{-6}
NSF-1	80.5×10^{-6}	83.6×10^{-6}	1.5×10^{-6}
NSF-4	2.8×10^{-6}	5.1×10^{-6}	1.1×10^{-6}

MF_1 and MF_2, spontaneous mutation frequencies to 6-TG^R of the cell population at the beginning and end of the 10 day growth period, respectively, expressed as mutations per cell corrected for cloning efficiencies. The spontaneous mutation rate was calculated from Equation I using m(mean) of Table 5, and expressed as mutations per cell per generation.

period. In addition, the mutation frequencies for GM 2548 and GM 1492 were quite high (1-2 x 10^{-4} mutants per cell) which would be consistent with an elevated mutation rate. However, this alone is sufficient to justify a high mutation rate since a great deal of variance in mutation frequencies may be observed owing to random drifts in the population. Indeed, considerable variance was observed in the mutation frequencies of the two normal strains. NSF-1 had a frequency of about 8 x 10^{-5} mutants per cell while NSF-4 had a frequency of about 4 x 10^{-6} mutants per cell.

The rate of spontaneous mutation, however, being under genetic control (see Literature Review), should not show such random variance. As can be seen in Table 6, the two normal strains had spontaneous mutation rates quite close to each other (1.5 x 10^{-6} and 1.1 x 10^{-6} mutations per cell per generation for NSF-1 and NSF-4, respectively). These rates for the normal fibroblast strains are in close agreement with previously published rates for normal diploid human fibroblasts at the HGPRT locus (111). As opposed to the rates of the two normal strains as well as previously published rates, the two Bloom syndrome strains had spontaneous mutation rates which were over 10-fold greater. GM 2548 and GM 1492 had spontaneous mutation rates of 16 x 10^{-6} and 17 x 10^{-6} mutations per cell per generation, suggesting that Bloom syndrome fibroblasts have an elevated rate of spontaneous mutation.

The Spontaneous Rate of Mutation From Method II

In order to help validate the above results, the spontaneous rates of mutation for two normal and two Bloom syndrome strains were determined via a second method, Method II. This protocol was based upon the fluctuation analysis of Luria and Delbruck (133), published in 1943 for bacterial studies. The design of this experiment is based upon the assumption that if one plates few cells into multiple dishes, the chance that any of the originally inoculated cells, in any single dish, is a mutant is quite small (< 0.05%). Thus, when these cells are grown, under non-

selective conditions, to a large population size and then exposed to 6-TG, the observed resistant cells in each dish arose, through spontaneous mutation, during the growth of the initial, small cell population per dish. The rate of mutation can thus be established. If, however, a single mutant cell was, by chance, initially inoculated into a dish, that dish would exhibit a great number of resistant colonies, since all cell progeny would share the resistant phenotype. Such a dish could be easily discriminated from the rest of the dishes and not be included in the rate calculations.

The results of the fluctuation analysis for the same fibroblast strains used in Method I are shown in Table 7. For NSF-4, NSF-791, GM 1492 and GM 2548 cell strains, 8, 15, 13 and 16 cells per dish, respectively attached and formed colonies. After 15 days growth, these initially seeded cells grew to populations of 2.0×10^4, 4.1×10^4, 2.4×10^4, 3.5×10^4 and 2.5×10^4 cell divisions per dish for strains NSF-4, NSF-791, GM 1492 and GM 2548, respectively. Table 6 expresses these data for the total populations in the total replicate dishes. The number of replicate cultures were different for the cell strains due to loss from contamination, which is a considerable problem in these experiments as the dishes are kept in culture for periods exceeding 37 days in total with multiple manipulations of the dishes during this time. Nevertheless, greater than 60 replicate cultures were examined for each strain, more than a sufficient number.

After selection, 10 6-TG resistant colonies from 75 replicate cultures (dishes) and 20 6-TG resistant colonies from 68 cultures were observed in control strains NSF-4 and NSF-791, respectively. In contrast to this, 58 6-TG resistant colonies from 88 cultures and 43 6-TG resistant colonies from 79 cultures were observed in Bloom syndrome strains GM 1492 and GM 2548, respectively. The distribution of these resistant colonies among the cultures and the increase of mutant colonies among the Bloom syndrome strains is seen in Figure 8. These polygons are

Table 7. Determination of Spontaneous Mutation Rates Through Fluctuation Analysis

	NSF-4	NSF-791	GM 1492	GM 2548
Replicate cultures tested	75	68	88	79
Initial cell number (No)	600	1020	1144	1264
Final cell number (Nt)	1.5×10^6	2.8×10^6	2.1×10^6	1.3×10^6
Cell divisions (Nt-No/ℓn2)	2.2×10^6	4.1×10^6	3.0×10^6	1.9×10^6
Number of 6-TG^R mutants per culture:				
Range	0-2	0-5	0-8	0-7
Mean	0.133	0.294	0.658	0.546
Variance	0.142	0.766	2.326	1.836
Po	0.880	0.853	0.709	0.739
m(Po)	0.128	0.159	0.344	0.303
Cloning efficiencies	0.21	0.18	0.05	0.09
Mutation rate (m(Po))	4.4×10^{-6}	2.7×10^{-6}	10×10^{-6}	13×10^{-6}
Mutation rate (mean)	4.6×10^{-6}	4.9×10^{-6}	19×10^{-6}	23×10^{-6}
Mutation rate (Eq. III, mean)	12×10^{-6}	12×10^{-6}	103×10^{-6}	80×10^{-6}

Mutation rates, expressed as mutations per cell per generation were calculated using mean 6-TG^R colonies per dish either directly calculated or from the Po number and using Eq. II. Another calculation of the spontaneous mutation rate was performed using Eq. III and the directly calculated mean 6-TG^R colonies per dish corrected for cloning efficiencies. Cloning efficiencies were determined at the time of selection as described in Materials and Methods.

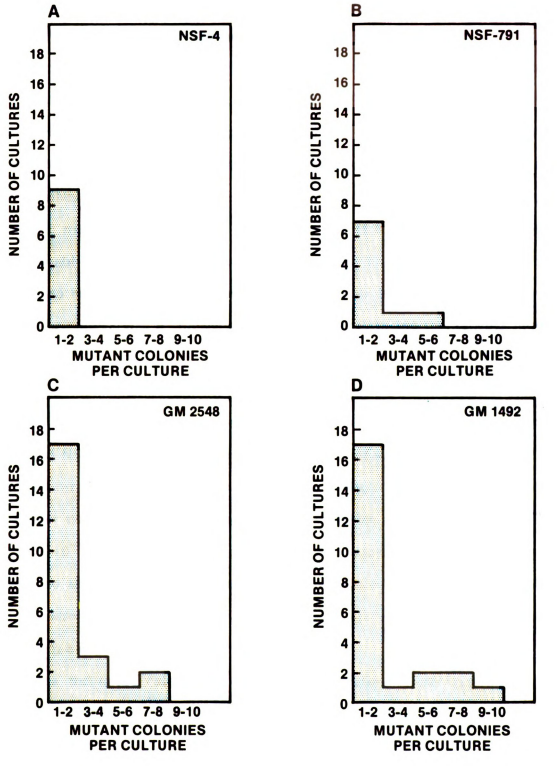

Figure 8. Distribution of 6-TG resistant colonies among replicate cultures from the fluctuation analysis (Method II). (A) Control strain NSF-4. (B) Control strain NSF-791. (C) Bloom syndrome strain GM2548. (D) Bloom syndrome strain GM 1492.

consistant with a Poisson distribution with a mean less than one. The observed mean number of resistant colonies per dish was 0.133 and 0.294 for the normal strains and 0.658 and 0.546 for the two Bloom syndrome strains. The variance, in all strains, was greater than the means, indicating the colonies arose from random, spontaneous, and independent events (i.e., mutations). The means calculated from the P_o number (percentage of dishes without colonies) was, without exception, lower than the arithmetic means. This is because using the P_o-generated mean technique, one counts dishes with more than one colony as a single mutation while using the arithmetic mean, one counts each resistant colony as a single mutation. The true mean probably lies somewhere between, since an early mutation in a single plate can give rise to multiple resistant colonies as can multiple later mutations. Therefore, both the arithmetic and P_o means were used to calculate the spontaneous mutation rates.

The spontaneous mutation rates were first calculated according to Equation 2 using the arithmetic means and the P_o-generated means. The two normal strains, NSF-4 and NSF-791, had spontaneous mutation rates of $4.4 - 4.6 \times 10^{-6}$ and $2.7 - 4.9 \times 10^{-6}$ mutation per cell per generation, respectively. GM 1492 and GM 2548, the two Bloom syndrome strains, had spontaneous mutation rates of $10 - 19 \times 10^{-6}$ and $13 - 23 \times 10^{-6}$ mutations per cell per generation, respectively. Thus, the normal strains had an average rate of about 4×10^{-6} mutations/cell/generation while the Bloom syndrome fibroblasts had an average rate of about 17×10^{-6} mutations/cell/generation, a clearly elevated rate.

Since the above calculation does not compensate for cloning efficiencies at the time of selection and the Bloom syndrome strains have lowered plating efficiencies (see Tables 4 and 6), the rate was again calculated according to Equation 3 which may be manipulated to compensate for cloning efficiencies (Robert DeMars, personal communication). Using the cloning efficiencies in Table

6, which were determined at the time of selection, and the arithmetic means, NSF-4 and NSF-791 both had spontaneous mutation rates of 12×10^{-6} mutations/cell/generation while GM 1492 and GM 2548 had rates of 103×10^{-6} and 80×10^{-6} mutations/cell/generation. The Bloom syndrome strains again had an elevated rate of spontaneous mutation. Thus, the data from both Method I and Method II clearly indicate that Bloom syndrome fibroblasts have an elevated rate of spontaneous mutation in vitro when compared to normal strains at the HGPRT locus.

Aphidicolin Sensitivity of Bloom Syndrome Fibroblasts

Having now obtained evidence suggesting that the homozygous state of the Bloom syndrome mutation (bl/bl) may have mutator activity associated with it, attempts were made to determine the mechanism of the enhanced spontaneous mutation rate. Initially, experiments were designed, using Bloom syndrome fibroblasts, to examine phenotypes associated with mutator mutations in rodent cell lines. One such phenotype is resistance to the tetracyclic diterpenoid, aphidicolin (18). Aphidicolin has been shown to be a specific inhibitor of DNA polymerase α and this inhibition is competitive with dCTP (158,159). Chang et al., (29) have described a mutant V79 Chinese hamster cell line which is resistant to aphidicolin at a concentration greater than 1 μM and has abnormally high levels of dCTP (greater than a 3-fold increase over normal). This cell line has an elevated spontaneous mutation rate at two loci (Philip Liu, personal communication). The sensitivity of Bloom syndrome fibroblasts to aphidicolin was, therefore, assessed.

Figure 9 shows the survival curves, based on colony forming ability, of two Bloom syndrome strains and two normal strains exposed to aphidicolin. The two Bloom syndrome strains were not significantly different from normal for aphidicolin sensitivity. These data suggest that the mechnism of the mutator activity in Bloom syndrome fibroblasts is distinct from the mechanism of the cell lines described by Chang et al., (29).

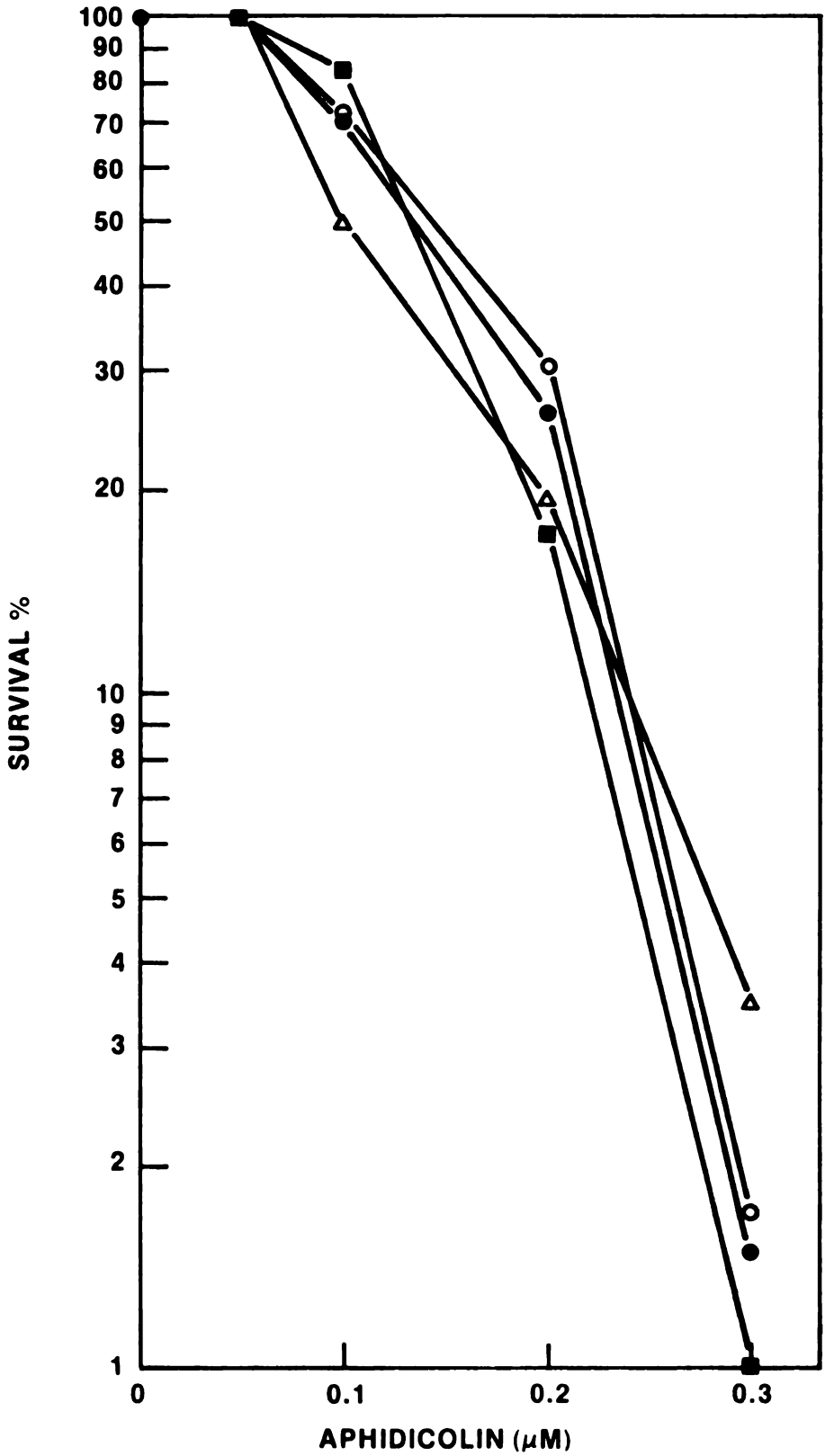

Figure 9. Survival of Bloom syndrome and normal fibroblasts in medium containing aphidicolin. ●, NSF-4; O, NSF-791; ■, GM 1492; △, GM 2548.

Also, it should be noted that besides aphidicolin resistance, some of the rodent cell lines with mutator activity reported by Meuth et al., (145) were resistant to 1-β-D-arabinofuranosylcytosine (araC). This laboratory has observed that, similar to the aphidicolin-sensitivity described above, Bloom syndrome firbroblasts do not have signficiantly different senstivity to araC when compared to normal fibroblasts (M.H. Wade and S.T. Warren, unpublished observations).

Deoxyribonucleoside Triphosphate Pools in Bloom Syndrome Fibroblasts

The aphidicolin and araC studies described above suggest that Bloom syndrome fibroblasts do not have altered DNA precursor pools. However, measurements of the deoxyribonucleoside triphosphate pools in Bloom syndrome fibroblasts were performed since, to date, all mutator mutations in mammalian cells have altered DNA precursor pools (29,145,229). Furthermore, normal aphidicolin sensitivity implies only that dCTP is present in normal amounts and does not necessarily infer that the other three deoxyribonucleoside concentrations are normal (159).

Since the deoxyribonucleoside triphosphate pools are maximal only during the S period of the cell cycle and are degraded quickly (222), the cell culture parameters were first established for the human fibroblasts. In order to determine at what point in culture the fibroblasts should be harvested to maximize the recovery of the pools, the following experiment was performed. Fibroblasts from a normal and a Bloom syndrome strain were held at confluency for 24 hours to accumulate cells in G_0. They were then trypsinized and plated into dishes containing coverslips. At various intervals, up to 76 hours after trypsin release, the cells were pulsed with radioactive thymidine and subsequently examined autoradiographically to determine the percentage of cells in S-phase. The results are presented in Figure 10. For both the normal and Bloom syndrome strains, the maximal percentage of cells in S-phase was approached at about 24 hours after release from confluency. In addition, Figure 10 shows that in the Bloom syndrome strain there was approximately half the

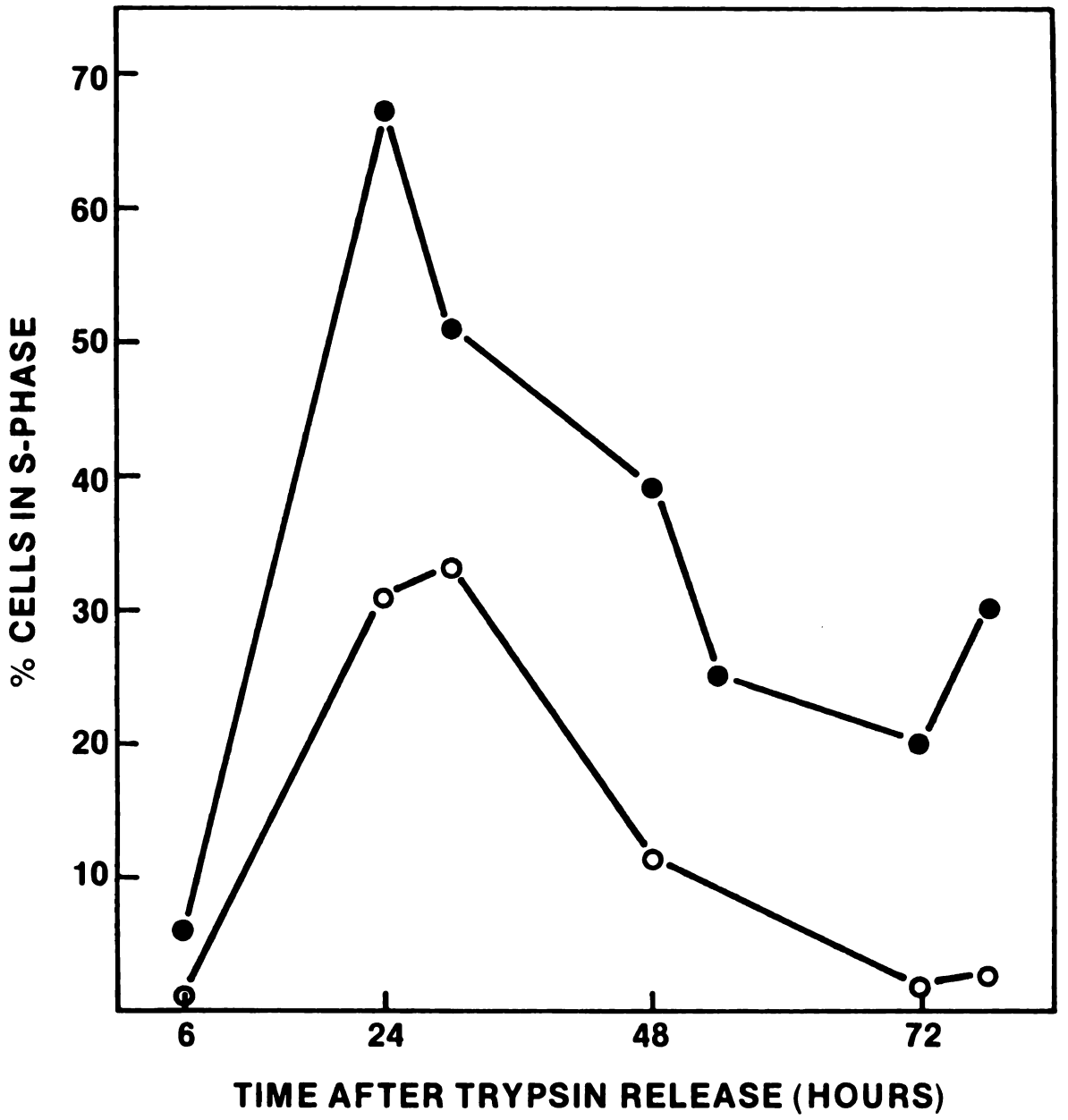

Figure 10. Percentage of cells in S-phase at various times after trypsin release from confluency. ●, NSF-791; O, GM 1492.

number of cells in S-phase when compared to the normal strain. This is consistent with the observations of earlier investigators (80,217) who found that there is an abnormally high number of non-cycling cells in Bloom syndrome cell cultures.

From the information gleaned in Figure 10, cells were harvested 24 hours after release from confluency to measure the dNTP pools. The pools were extracted and measured as described above and the results are shown in Table 8. Although there is considerable variation among and between the two normal strains and the two Bloom syndrome strains, there does not appear to be a significant difference in the relative pool sizes. Figure 11 shows the relative dNTP pools of the Bloom syndrome strains' average amounts compared to the average concentrations of the two normal strains. Although the dCTP concentration is about 50% higher and the dATP concentration about 60% lower in the Bloom syndrome fibroblasts relative to the normal strains, it was felt that this was within experimental error. Besides the well-known difficulties in measuring dNTP concentrations leading to rather large error estimates, it was felt that this conclusion was justified since aphidicolin and araC sensitivities of the Bloom syndrome strains were normal. As stated above, aphidicolin sensitivity is influenced by dCTP concentrations as is araC sensitivity, which also may be influenced by dATP (145). Thus, if these pools were significantly different from normal in Bloom syndrome, it would be expected that the aphidicolin and araC sensitivities would likewise be different. Furthermore, rodent mutator mutants exhibit dNTP pools in excess of three times the normal concentrations and often are in as much as a 13-fold excess (29,145,229). Although it may be argued that the pools were extracted from only half as many dNTP-producing cells (S-phase cells) in the Bloom syndrome cultures relative to the normal cultures (Figure 10), thus doubling the relative amounts of dNTPs in the Bloom syndrome cells, again, one would expect different survival curves for aphidicolin exposure. Therefore, it was

felt that the mutator activity in Bloom syndrome fibroblasts was not due to abnormal deoxyribonucleoside triphosphate pools.

Table 8. Deoxyribonucleoside Triphosphate Pools in Bloom Syndrome and Normal Fibroblasts

	pmol/μg DNA			
CELL STRAIN	dCTP	dTTP	dATP	dGTP
NSF-791	9.7	7.9	3.6	5.2
MSU-2	5.6	6.7	7.4	4.5
GM 1492	9.5	3.5	1.7	3.1
GM 2548	13.3	4.6	2.7	4.3

The Effect of Bloom Syndrome Fibroblast Conditioned Medium Concentrate on V79 Cells

Since the DNA precursor pools do not appear to be involved in the spontaneous hypermutability of the Bloom syndrome fibroblasts, another, quite different mechanism was considered based upon the observations of Emerit and Cerutti (52). These investigators found that a concentrate of media, in which Bloom syndrome fibroblasts had been grown, contained clastogenic activity. This suggests that Bloom syndrome cells may produce an enodogenous mutagen, a hypothesis previously put forth by German (75) and Tice et al., (205). In order to test this hypothesis, media were collected from fibroblast cultures of Bloom syndrome and normal strains and concentrated 20X as described in Materials and Methods. Figure 12 shows the effect of the media concentrates on the survival of V79 Chinese hamster cells, in terms of colony-forming ability. The hamster cells were treated with the concentrate on the basis of final protein concentration per ml of growth medium in

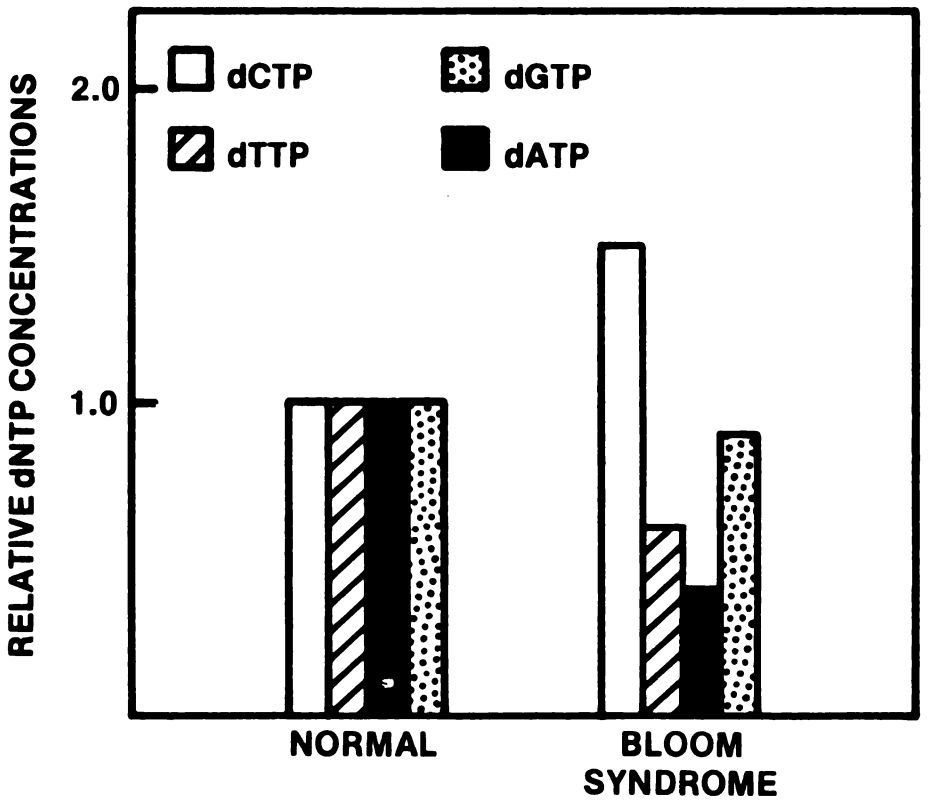

Figure 11. Intracellular deoxyribonucleoside triphosphate pools in Bloom syndrome fibroblasts relative to control fibroblasts. Based upon the average value presented in Table 8.

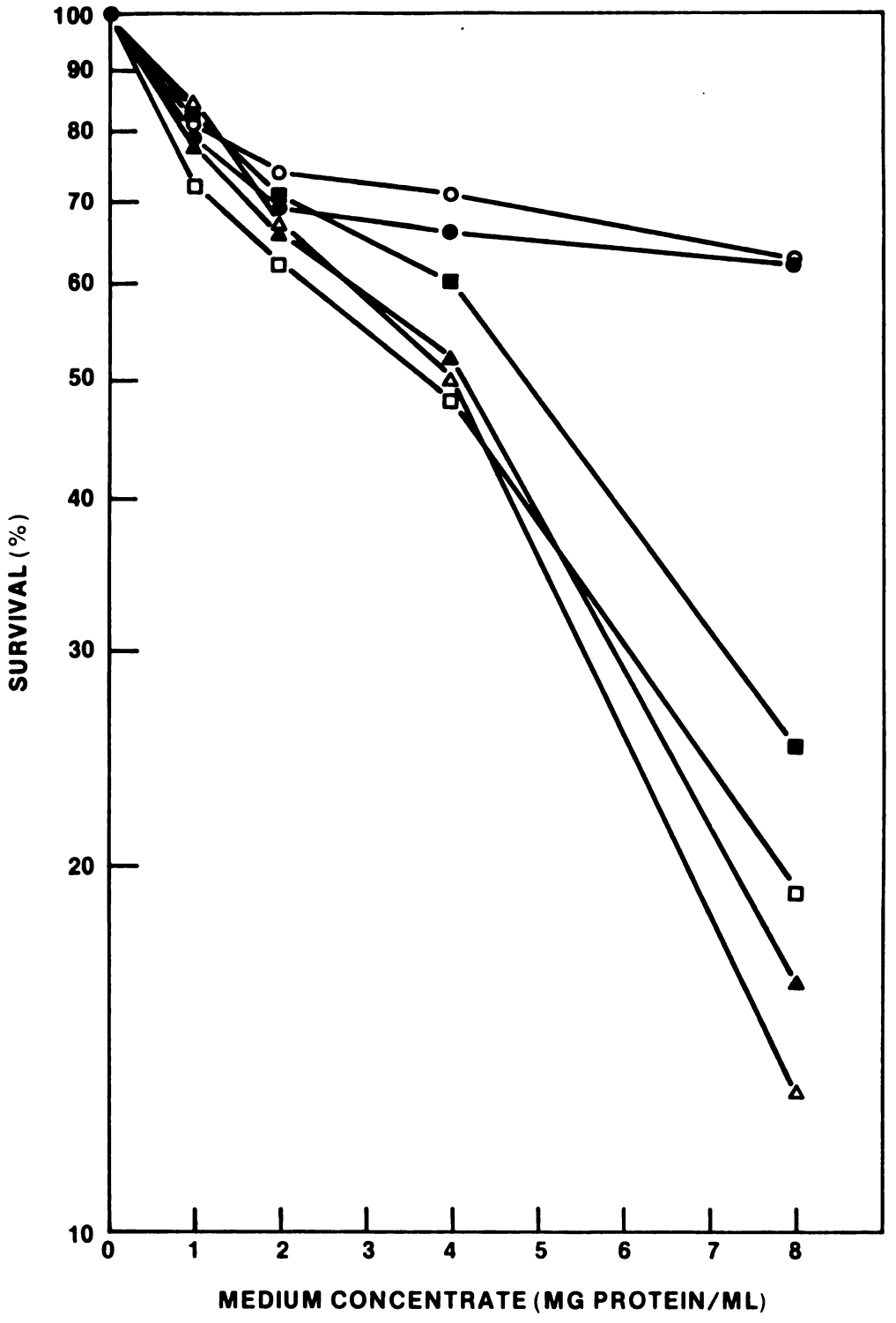

Figure 12. Survival of V79 cells following treatment with concentrated media obtained from Bloom syndrome and normal fibroblasts. Twenty-fold concentrated media was prepared from the following fibroblast strains: ●, NSF-791; O, MSU-2; □, GM 3510; ■, GM 2548; ▲, GM 3402; △, GM 1492.

the dishes. This was not meant to imply that the clastogenic factor is a protein, rather was used only as a convenient method of quantitating the concentrates. As can be seen, the concentrates obtained from the Bloom syndrome fibroblast-conditioned media were significantly more toxic to V79 cells at higher concentrations than were the concentrates derived from the normal fibroblast-conditioned media. At a concentration of 8 mg protein/ml, the Bloom syndrome fibroblast media concentrates resulted in an average survival of 16% while the normal concentrates resulted in an average survival of 64%, thus the Bloom syndrome-derived concentrates were 4x as toxic to the hamster cells. Furthermore, it was noted that the hamster cell colonies that survived the treatment with the Bloom syndrome-derived concentrates were considerably smaller in size than the colonies that survived the normal strain-derived concentrates (Figure 13). These data suggest that not only are the Bloom syndrome-derived concentrates more toxic to hamster cells but they are growth inhibitory as well. Furthermore, since the hollow fiber cartridge used to concentrate the media allows the passage of molecules with a molecular weight below 2,000, it may be assumed that the toxic factor(s) in the Bloom syndrome fibroblast-conditioned media is of a size in excess of 2,000 daltons.

Since these data were consistent with the presence of a clastogenic factor that Emerit and Cerutti (52) observed in the media from Bloom syndrome fibroblasts, the possibility of a mutagenic factor being released by these fibroblasts was pursued. V79 Chinese hamster cells, in log-phase growth, were treated with various amounts of media-concentrates derived from both Bloom syndrome and normal fibroblasts for 48 hours. This media was then removed and replaced by fresh media until four cell divisions had occurred (approximately 40 hours post-treatment). The cells were then trypsinized, counted and replated into dishes for selection in ouabain (1mM) containing medium. The remainder of the cells were returned to flasks with growth medium and allowed to grow for an additional 4 days. These cells were then

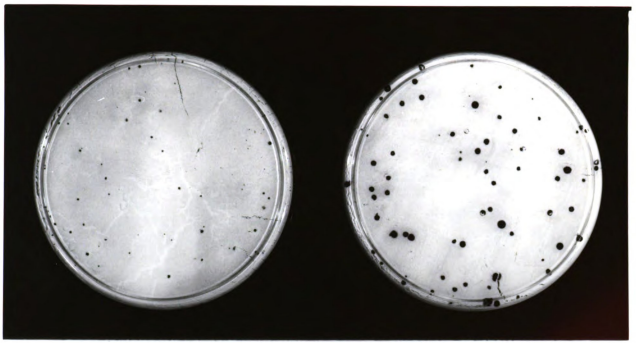

Figure 13. The effect of concentrated media on V79 colony growth. Left dish treated with media concentrate of GM 1492; Right dish treated with media concentrate of MSU-2.

trypsinized, counted and replated into dishes for selection in 6-thioguanine (10 μg/ml) containing medium.

Figures 14 and 15 show the results of this experiment. Clearly, Figure 14 shows no change in the frequency of ouabain-resistant colonies in any of the treatment groups. Figure 15, however, shows that the media-concentrate derived from two Bloom syndrome strains increased the frequency of 6-TG resistant colonies 3-and 4-fold above background at the highest treatment dose. Media-concentrate from a normal fibroblast strain did not alter the mutation frequency at any dose. Furthermore, the media-concentrates from the Bloom syndrome strains appeared to increase the mutation frequencies in a dose-dependent fashion with saturation occurring at the higher doses.

However, because of the rather high background 6-TG resistant mutant frequency (5×10^{-5} mutants/survivor) and the limited number of strains tested, the experiment was repeated. Media was collected from four Bloom syndrome strains and two normal strains as described above. The ouabain and 6-TG mutation protocols were repeated in an identical fashion as the first experiment except that the hamster cells were grown in HAT medium for 10 days followed by 3 days growth in growth medium prior to the experiment in order to lower the background of 6-TG resistant cells.

Figures 16 and 17 show the results of the second experiment. As observed in the preceding experiment, there was no change in the frequency of ouabain-resistant cells in any treatment group (Figure 16). At all points, the frequency of ouabain resistance was between one and four resistant cells per million surviving cells. The frequency and variation observed were within normal limits for ouabain resistance in this laboratory. The data for 6-TG resistance is shown in Figure 17. Again, the media-derived from Bloom syndrome fibroblasts increased the frequency of 6-TG resistance 3-to 4-fold over background at the highest dose (one group was lost due to

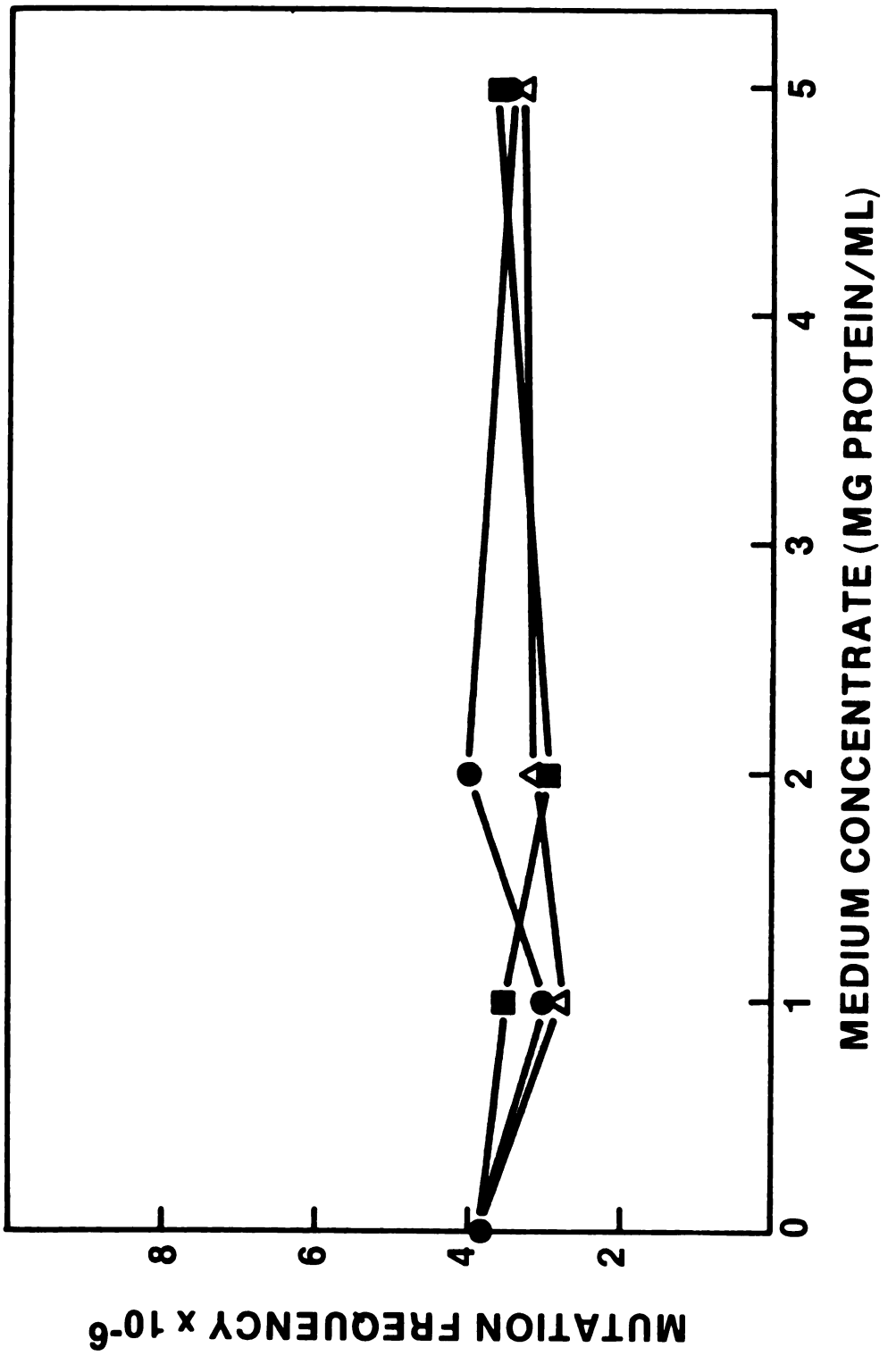

Figure 14. Ouabain-resistant mutation frequencies in V79 cells following treatment with concentrated media. Concentrate prepared from media of the following fibroblast strains: ●, NSF-791; ■, GM 2548; Δ, GM 1492.

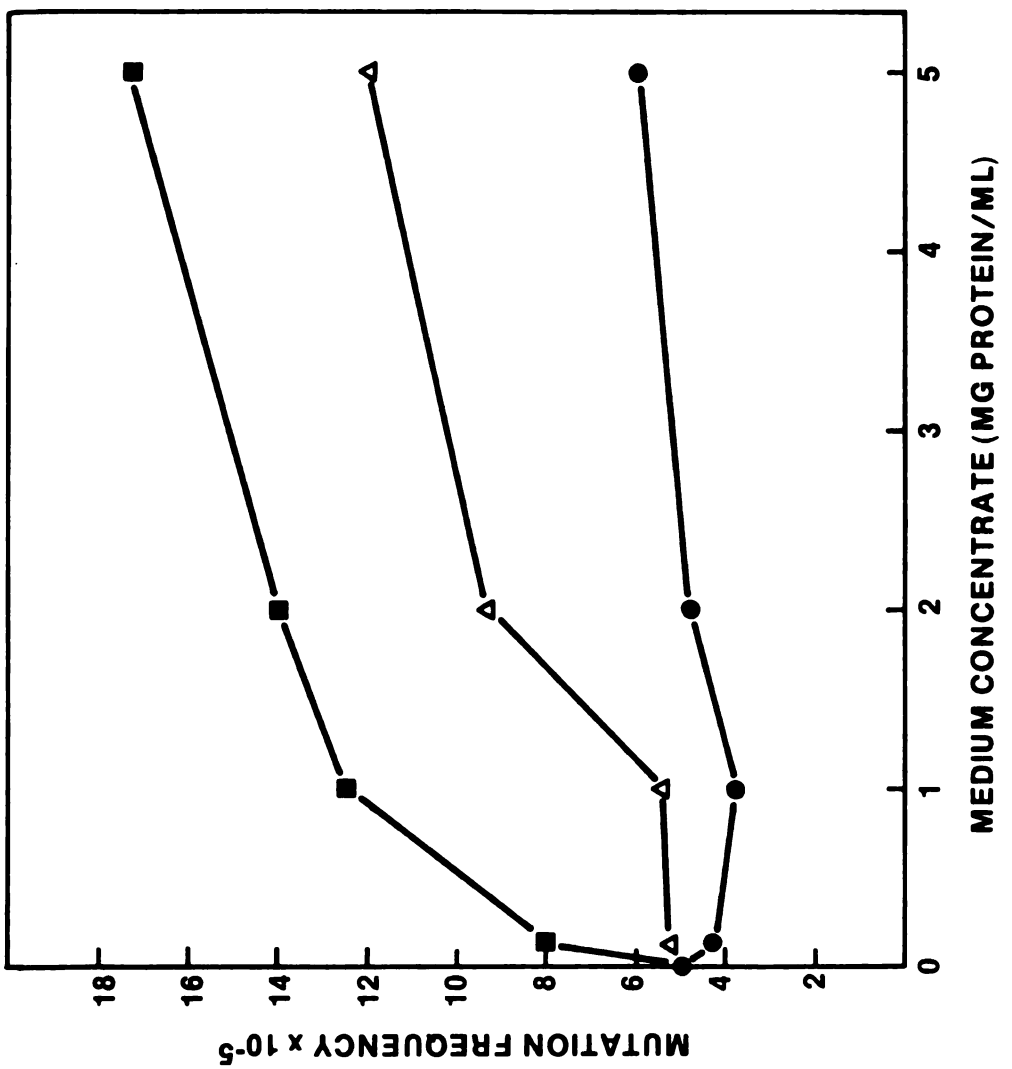

Figure 15. 6-Thioguanine-resistant mutation frequencies in V79 cells following treatment with concentrated media. Concentrate prepared from media of the following fibroblast strains: ●, NSF-791; ■, GM 2548; Δ, GM 1492.

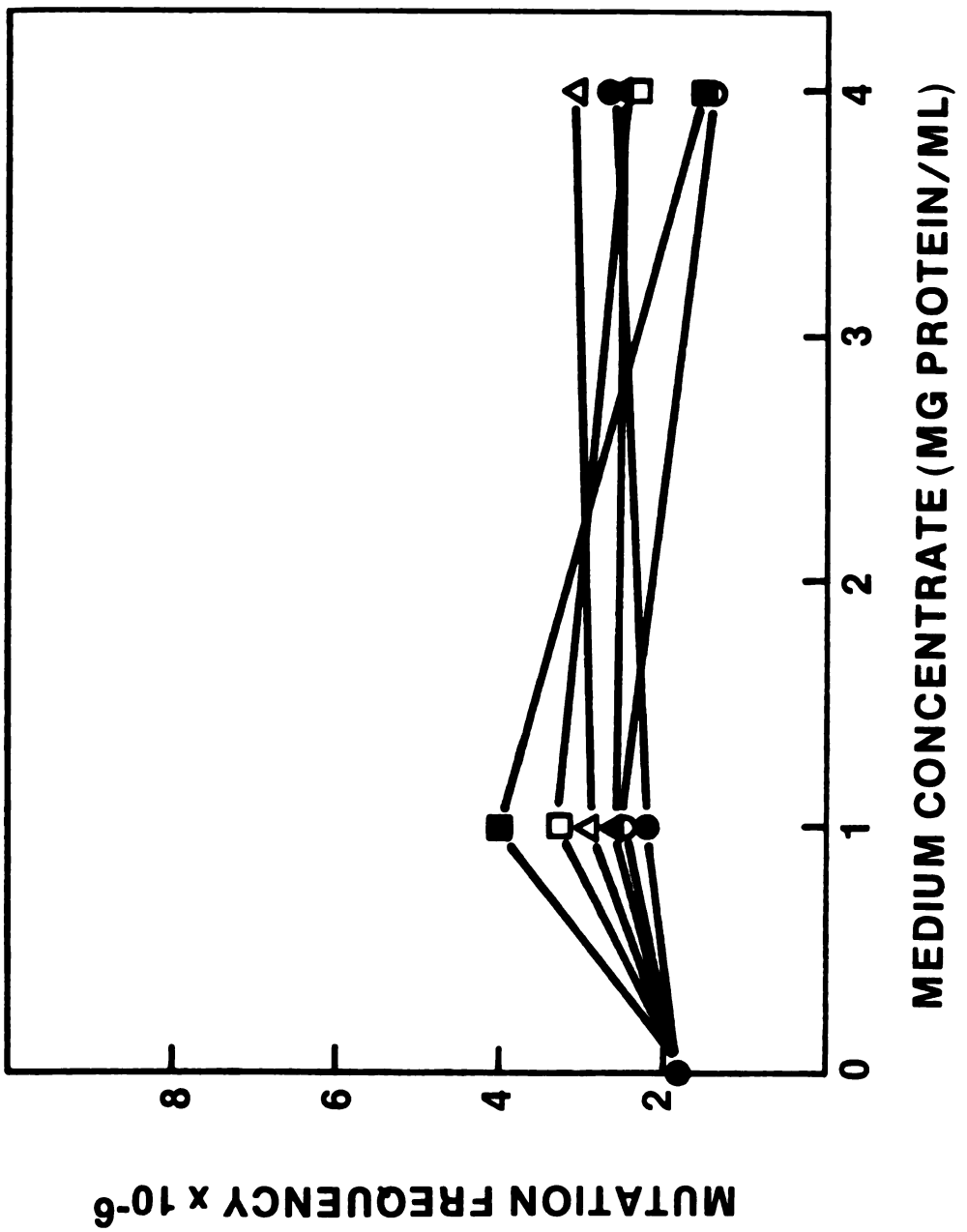

Figure 16. Ouabain-resistant mutation frequencies in V79 cells following treatment with concentrated media. Concentrate prepared from the media of the following fibroblast strains: ●, NSF-791; O, MSU-2; □, GM 3510; ■, GM 2548; ▲, GM 3402; △, GM 1492.

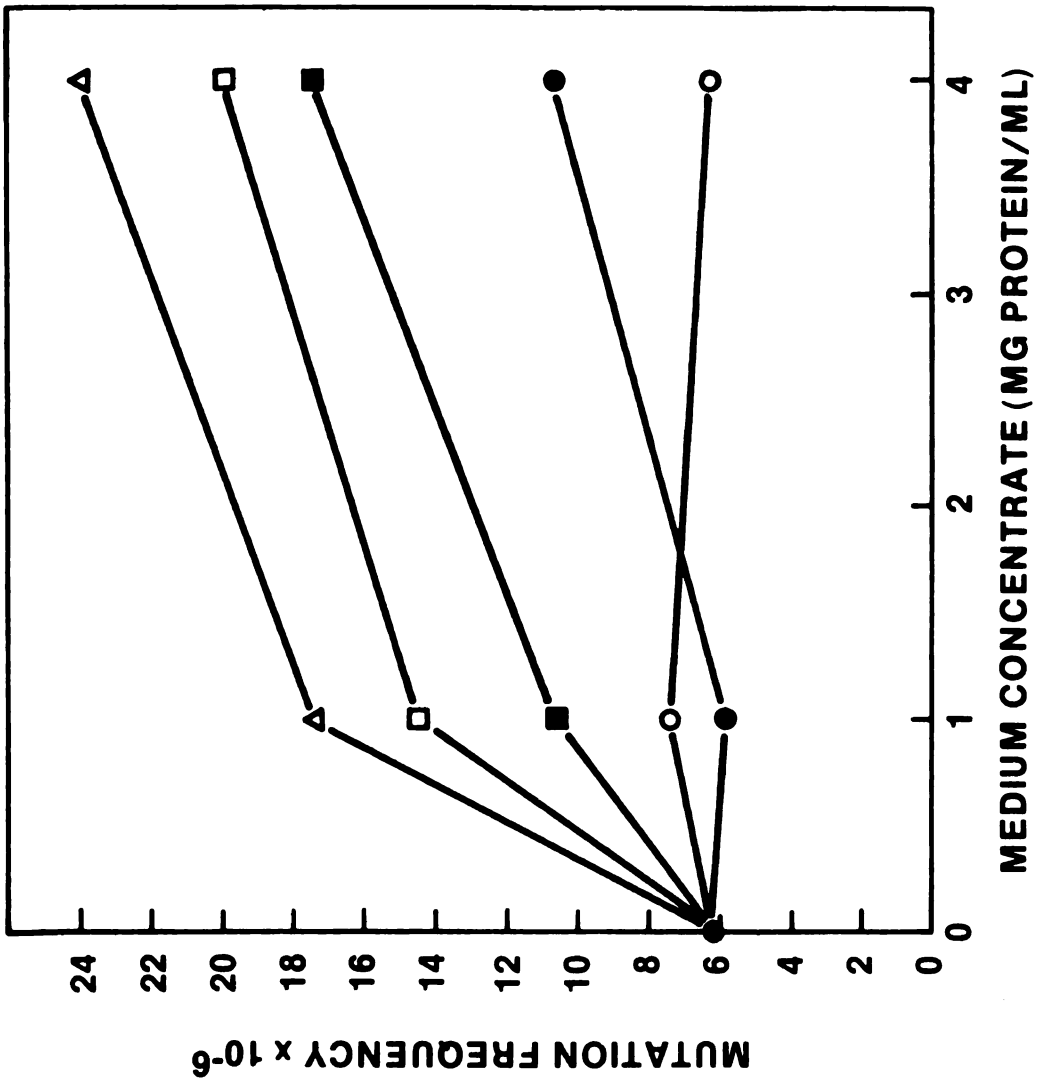

Figure 17. 6-Thioguanine-resistant mutation frequencies in V79 cells following treatment with concentrated media. Concentrate prepared from the media of the following fibroblast strains: ●, NSF-791; ○, MSU-2; □, GM 3510; ■, GM 2548; △, GM 1492.

contamination between the ouabain and 6-TG selection times). The two concentrates obtained from normal fibroblasts did not significantly change the mutation frequencies. Note that the background frequency of 6-TG resistant cells was lowered by the HAT pretreatment. As in the previous experiment, there was a dose relationship with the mutation frequencies and the curves saturated at the higher doses in those groups treated with concentrate from Bloom syndrome medium.

Since ouabain resistance is induced, almost exclusively, by point mutations (9), a clastogenic factor would not be expected to alter the mutation frequency of this marker. Resistance to 6-TG however, may be induced by both missense and nonsense mutations (25,42) and thus agents which cause chromosomal damage would be expected to increase the mutation frequency of this selective marker. Therefore, these data are consistent with the work of Emerit and Cerutti (52) and support the concept that Bloom syndrome fibroblasts secrete a clastogenic (mutagenic) factor into the growth medium.

DISCUSSION

The hypothesis which generated the experiments reported above was that cells obtained from patients with Bloom syndrome may have an elevated mutation rate. The rationale for this hypothesis was deduced in two steps. First, it is clear that somatic mutations play a major role in the initiation of malignant neoplasias. Thus an excess of mutations incurred by the somatic cells of an individual should result in an elevated risk of cancer. Since it is also known from data on various organisms that the rate of mutation is genetically controlled, there should be human mutants that have an elevated rate of somatic mutations, phenotypically expressed as a strong predisposition toward the development of cancer. Indeed, the autosomal recessive condition, xeroderma pigmentosum, is an excellent example. These individuals are extremely prone to actinic skin cancers, a direct result from an excess accumulation of somatic mutations due to defective repair of sunlight-induced DNA damage. As the defect in xeroderma pigmentosum parrallels similar defects observed in bacteria, it therefore is likely that other human genetic diseases may be similar in principle to mutant bacteria that have excess mutation accumulation secondary to defects in mechanisms other than DNA repair.

A major mechanism controlling mutation accumulation in lower organisms is the genetic control of the spontaneous mutation rate. Cox (41), for example, has recently reviewed the numerous mutations in E. coli, termed mutator mutations, which can elevate the spontaneous rate of mutation up to a hundred-thousand fold. Similarly, there are some mammalian mutants, isolated from rodent cell lines, which also have high rates of spontaneous mutation (29,145,229). It was, therefore, reasoned that there ought to be analogous mutator mutants in the human population, most likely expressed as cancer-prone genetic diseases.

Thus the second step in the hypothesis formulation was the selection of an appropriate genetic disease in which the clinical and experimental data would be reasonably consistent with the possibility of a mutator mutation. Because there are relatively few known genetic diseases with an unusually high cancer incidence, this task was fairly straight forward. Bloom syndrome came immediately to mind for the following reasons. It is an autosomal recessive condition with over a hundred-fold increased risk of cancer (73,76). The cancers are of common types and involve more than one system in the body. Although the patients are sunlight-sensitive, no defect in DNA repair has been elucidated. However, abnormal DNA replication, a common defect in bacterial mutator mutations (41), has been described (92,93). Finally, the cells from patients with Bloom syndrome have greatly elevated rates of chromosomal aberrations and sister chromatid exchanges (71), thus there was cytological evidence for an elevated rate of spontaneous chromosomal mutations. Indeed, German had previously suggested that Bloom syndrome cells may spontaneously incur more mutations than normal cells (75).

In order to determine the spontaneous mutation rate in Bloom syndrome fibroblasts, an appropriate selective marker had to be chosen. Since the measurement of the spontaneous mutation rate depends upon the accumulation of spontaneous mutants within a growing cell population under non-selective conditions, the marker had to be one that did not confer a selective disadvantage to the mutant cells, lest they be lost prior to selection. Resistance to 6-thioguanine was chosen since the mutation (loss of HGPRT activity) was not in a vital function and hence no selective pressures were placed upon the mutant cells (234). An additional benefit was that this marker had been extensively characterized in human cell culture and had previously been used to measure the spontaneous rate of mutation in normal human fibroblasts (111,126). Also, unlike other available selective markers, resistance to 6-TG could result from both point and frameshift (including deletion-

type) mutations (25). A disadvantage to 6-TG resistance is that mutant cells may be killed due to metabolic cooperation, however this could be overcome by inoculating dishes at a low cell density (27). Therefore, two separate protocols were designed to measure the rate of spontaneous mutation to 6-TG resistance in Bloom syndrome fibroblasts.

Both protocols, originally conceived for bacterial studies (133,152), had been previously employed using human diploid fibroblasts (111,126). Although these experiments were laborious because of the large number of dishes required (for example, over 1000 dishes were simultaneously incubated for Method I), they would provide reasonably accurate comparative rates. The first protocol (Method I) revealed approximately a 10-fold greater rate of mutation in the Bloom syndrome fibroblasts compared to normal. Method II resulted in a mutation rate approximately a 4-fold greater in Bloom syndrome fibroblasts (Figure 18 graphically displays the average rates for both methods). The calculation of the spontaneous mutation rate is very dependent upon the number of cell divisions. This may bias the results if a low mutation rate is observed in a strain with a low number of cell divisions, since mutations occur per cell division. In this case, however, a <u>high</u> mutation rate was observed in the Bloom syndrome strains that had a low number of cell divisions. Therefore, the observed rate is not elevated because of the cell division number, in fact, the true rate may be even higher than that observed.

The difference in the increased rates in Bloom syndrome cells between the two methods likely results from factors intrinsic to the two protocols. For example, the expression times were different, thus lower rates were obtained from Method I which had a shorter expression time. The important concept, however, is that rates of spontaneous mutation in the two Bloom syndrome strains were consistently higher than the two normal strains. Thus, the data obtained via both methods support the hypothesis that Bloom syndrome strains have an elevated rate of spontaneous

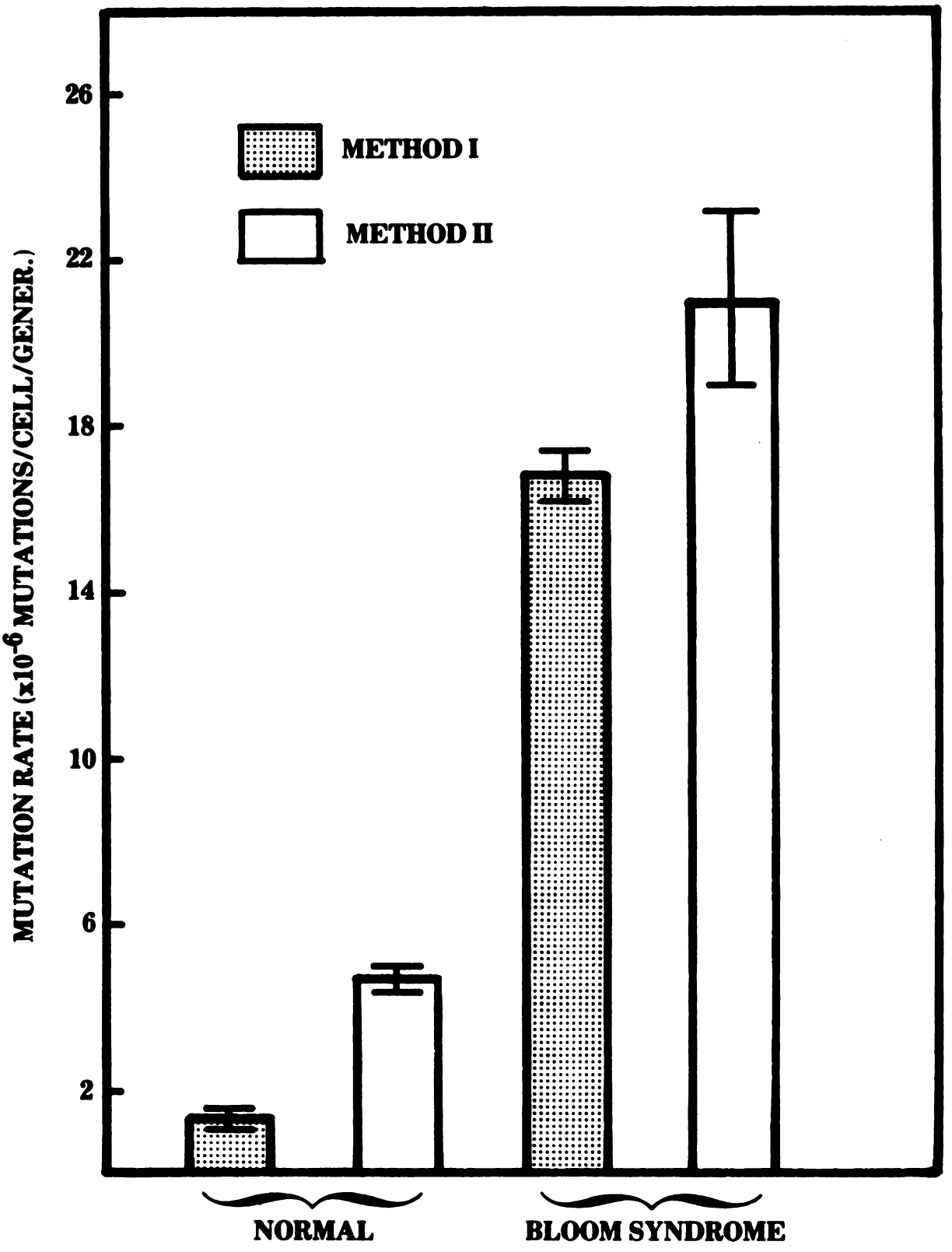

Figure 18. Comparison of the spontaneous mutation rates obtained by both methods. Data presented is the mean values (± S.E.M.) of the two Bloom Syndrome and the two normal strains studied.

mutation. After the completion of the above experiments, Grupta and Goldstein (89) reported that a Bloom syndrome fibroblast strain (GM 1492) had an eight-fold elevation, over other cell strains, of the rate of spontaneous mutation to diphtheria toxin resistance. Therefore, the findings of Grupta and Goldstein (89), in conjunction with the data reported here, strongly suggest that Bloom syndrome may be a mutator mutation, a previously unrecognized phenomenon in humans.

The second phase of this study was aimed at elucidating the mechanism of the elevated spontaneous mutation rate. As previously stated, the abnormality in Bloom syndrome may reside in DNA repair, DNA replication, or the production of a mutagenic metabolite. Since numerous studies by others, as well as studies in this laboratory, have failed to detect a consistent defect in DNA repair the other two mechanisms were pursued. However, it still remains a possibility that Bloom syndrome cells are defective in an as yet unrecognized mode of DNA repair. Nevertheless it was assumed during this study, perhaps prematurely, that DNA repair was ostensibly normal in Bloom syndrome.

Defective DNA replication has long been considered a viable possibility in Bloom syndrome since Hand and German reported a retarded rate of DNA chain growth in cells derived from these patients (92,93). However, the activity of the DNA polymerases has been reported to be normal (160). It was then thought that perhaps the DNA precursur pools were aberrant in Bloom syndrome cells, particularly since all the mammalian mutator mutations thus far described have abnormal levels of deoxyribonucleside triphosphates (29,145,229). One of these mutants, discovered in this laboratory, was resistant to the DNA polymerase inhibitor aphidicolin, most probably due to high levels of dCTP (29). Therefore, aphidicolin-sensitivity in Bloom syndrome cells was investigated. However this was found to be normal as was ara-C sensitivity, suggesting that dCTP levels were not abnormally elevated. Still, the possibility of abnormal levels of dNTPs in Bloom syndrome was

intriguing, and therefore the decision was made to actually measure the dNTP pools utilizing an E. coli DNA polymerase based assay.

Although dNTP pools have never been measured in human fibroblasts to our knowledge, we had some success after numerous attempts. After determining that the cells had to be, at least partially synchronized by trypsin-release from confluency in order to maximize the cellular dNTP pools, no major abnormalities were found in Bloom syndrome cells as compared to normal. The level of dCTP was found to be approximately 50% greater in Bloom syndrome cells. However, because of the experimental variability and the normal aphidicolin-sensitivity, this was interpreted as normal. Also it must be kept in mind that the pool abnormalities in the rodent mutators was very signficant, showing up to 13-times normal levels. In addition, the data may be interpreted in terms of the percentage of Bloom syndrome cells in S-phase at the time of harvest. Since half as many Bloom syndrome cells were in S-phase, the point in the cell cycle when dNTPs are present, as compared to normal, perhaps the pools measured in the Bloom syndrome cultures were extracted from half as many cells as normal. This would effectively double the dNTP pools in the Bloom syndrome cells relative to the normal cells. However, again, one would then predict that aphidicolin- and araC- sensitivities would be different, which was not observed. Thus, these data were interpreted as normal, although further studies of dNTP pools are certainly warranted, perhaps using lymphoblastoid cultures to obtain larger cell numbers and hence higher and more significant dNTP levels. Additionally, it may be best to express the dNTP values as pmoles per μg replicating DNA in order to control for the different cell cycling parameters observed in Bloom syndrome fibroblasts.

The focus of the experiments was then turned toward the possibility of an endogenous mutagen being produced by Bloom syndrome cells. This would not only account for the elevated spontaneous mutation rate but also the high levels of

chromosomal aberrations and SCEs. Although there is no precedent in any other organism for such a mechanism, it was intellectually intriguing. Furthermore, the report of Emerit and Cerutti (52) provides strong support for this contention. These investigators found, as previously mentioned, a factor secreted by Bloom syndrome cells with a molecular weight between 1,000 and 10,000, that induced chromosomal aberrations and, to a lesster extent, SCEs in normal human lymphocytes. The activity of this factor was suppressed by the free-radical scavaging enzyme, superoxide dismutase. These investigators postulated that Bloom syndrome cells secreted a radical-containing molecule because of a defective detoxification enzyme.

To investigate this possibility, media was collected from Bloom syndrome fibroblast cultures and normal cultures, concentrated twenty-fold, and used to treat V79 hamster cells. Both ouabain and 6-TG resistant mutations were then measured in the hamster cells. As shown above, the media concentrates from all Bloom syndrome strains tested induced 6-thioguanine resistant mutants but not ouabain-resistant mutatns. The media-concentrates from the normal fibroblast strains induced neither ouabain- nor 6-TG-resistant mutants. Furthermore, the media-concentrates of the Bloom syndrome fibroblasts were more cytotoxic to V79 cells than the normal concentrates and were growth inhibitory as well. 6-Thioguanine-resistant cells were induced by the Bloom syndrome concentrates in a dose-dependent fashion with saturation occurring at the higher doses. The saturation phenomenon could conceivably be the result of saturation of a transport mechanism on the membrane of the hamster cells. Since the mutagenic component of the Bloom syndrome concentrate is of a molecular weight in excess of 2,000, it is likely that it is actively, rather than passively, transported into the hamster cells. Thus, saturation of the transport mechanism may explain the mutation curves observed.

Some data were obtained relating to the molecular nature of the mutagenic factor. It can be deduced that the factor must induce deletion- or frameshift-type mutations rather than point mutations since ouabain-resistant mutants were not induced. As previously mentioned, ouabain-resistance is induced by point mutations only since Na^+/K^+ ATPase, the target of ouabain, is an essential enzyme. HGPRT however is not essential, hence deletions or frameshift mutations that result in the loss of enzymatic activity do not interfere with the cell viability, they only confer resistance to 6-TG. Therefore, the observation of only frameshift-or deletion-type mutations being induced by the Bloom syndrome concentrates is entirely compatible with Emerit and Cerrutti's clastogenic factor, since clastogenic agents would be expected to induce only such mutations. In addition, the molecular weight range of the clastogenic factor and of the mutagenic factor found in these studies is compatible. Indeed, it is likely that the findings of Emerit and Cerrutti and those of this study are the result of the same factor.

Based upon the findings of this study incorporated with the observations of Emerit and Cerutti (52), a mechanism may be postulated for the error in Bloom syndrome. Although a purely speculative endeavor, a scenario may be constructed to explain most of the experimental data obtained here as well as in other laboratories.

As German (75) has previously speculated, the defect in Bloom syndrome may not be in DNA repair or replication, but rather in an excess of endogenous DNA damage occurring spontaneously. The spontaneous mutation rate studies reported here support this. Obviously, if the DNA is incurring spontaneous damage at a high rate, the basal mutation rate as well as the spontaneous rate of chromosomal aberrations would be elevated. Similarly, the high SCE frequency would be cellular response to the DNA damage.

Because of the additional DNA damage, the DNA repair systems, which presumably are intact, would be operating closer to saturation levels than they are in normal cells. Indeed, Selsky et al., (184) and Henson et al., (101) have both reported unusually early saturation of DNA repair, after mutagen treatment, in Bloom syndrome fibroblasts as compared to normal. Furthermore, as the repair systems may be saturating earlier after mutagen treatment, this would leave more DNA lesions unrepaired per unit dose. Since unrepaired DNA lesions are thought to trigger SCEs (232), more SCEs should be formed per unit mutagen dose in Bloom syndrome cells than in normal cells. This is exactly the observation made by Krepinsky et al., (125) using ethyl methanesulfonate.

The mechanism causing the elevated rate of DNA damage in Bloom syndrome cells is certainly poorly understood. However, based upon the data of this study and that of Emerit and Cerutti (52), certain features may be concluded. It appears that the endogenous damage may be the result of a mutagenic metabolite being produced by Bloom syndrome cells. This factor has a molecular weight between 2,000 and 10,000, although it is unknown if it is a protein, lipid, or other structure. Since the activity of the factor is inhibited by superoxide dismutase, it must exert its action via a radical (electron-deficient atom) which is highly reactive towards DNA. The type of DNA damage this factor causes apparently does not result in point mutation but rather in frameshift or deletion mutations, both at the chromosome and gene level.

In keeping with the autosomal recessive inheritance of Bloom syndrome as well as the cell hybridization studies, the defect must be a loss of normal function. Since radical-containing molecules are normally produced in cells either enzymatically, spontaneously, or through irradiation (e.g., 313 nm ultraviolet or X-ray; 144), one may speculate that the Bloom syndrome factor is a normal metabolite, but present in excess amounts due to the genetic loss of enzymatic activity responsible for its

detoxification. Actually, this would be a rather classic explanation for a human biochemical defect; loss of enzyme function and substrate accumulation.

Figure 19 visually displays the above concept. First, it is hypothesized that some resonant molecule (toluene is used here only as a simple example of such a structure) is induced to lose an electron from one of its atoms via a mechanism that occurs in normal as well as Bloom syndrome cells. This radical molecule is relatively stable because of the resonance of the ring structure (or as in the case of lipids, because of multiple double bonds). Normally, this radical is detoxified by an as yet unknown enzyme. In Bloom syndrome, it is further speculated that this enzyme is absent and therefore the substrate, the Bloom syndrome factor, accumulates and hence damages DNA. The lesions on the DNA, which also are uncharacterized, may be repaired or, if the cell is undergoing DNA replication, induce mutations and chromosomal abberations. Also, since DNA lesions are known to slow or stop DNA replication, the slower DNA chain growth observed (92,93) may be a response to these lesions. Of course, it must be emphasized that this is a purely hypothetical scheme which may be completely or partially incorrect. However, it will serve to generate future experiments, whose results may or may not be consistent with the above theory, but will produce more data aimed at the elucidation of the basic defect in Bloom syndrome.

It is tempting to correlate the possible mutator activity found in this study with the clinical phenotype of Bloom syndrome patients. The major clinical findings are: primordial short stature, sunlight-induced facial telangiectasia, and a distinct propensity to develop relatively common types of cancer at an early age. Because the rate of growth of an individual is fundamentally determined by cell number (193), cell loss caused by inviable somatic mutations in addition to a slow mitotic rate may play an important role in the dwarfism observed in these patients. Since all studies on the repair of UV-induced DNA lesions are reported as normal in Bloom

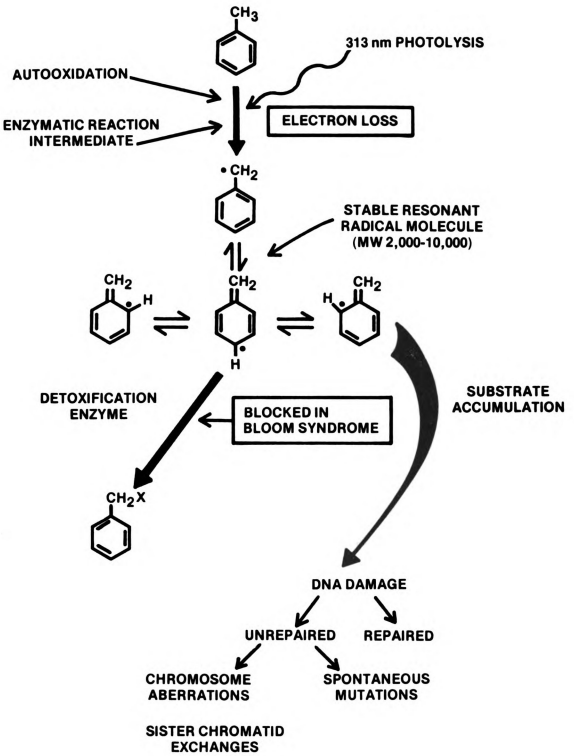

Figure 19. A hypothetical explanation for the molecular defect in Bloom syndrome. Note that toluene only serves as an example of a simple resonant structure and is not meant to imply a benzene ring is involved in the actual molecular defect.

syndrome, the actinic telangiectasia may be caused by the clastogenic/mutagenic factor which was found to be secreted by the fibroblasts of these pateints. This may be mechanistically similar to the UV-activation of a circulating factor found in patients with systemic lupus erythematosus (193,194). These patients have very similar actinic telangiectasia about the malar region, so similar in fact, that Bloom syndrome was originally described as lupus erythematosus in dwarfs. Finally, the high cancer incidence in these patients may be directly explainable because of the elevated spontaneous mutation rate as would be predicted from the somatic mutational theory of cancer. Since the yield of spontaneous mutants is intimately correlated with the number of cell divisions, it is interesting that the majority of cancers in Bloom syndrome patients are found in tissues of high cellular proliferation, such as the bone marrow and gastro intestinal tract (69). Furthermore, the observation of German et al., (73) that in some patients with Bloom syndrome a small proportion of lymphocytes exhibit a normal SCE frequency could be explained by a somatic back mutation to +/bl in a clone of progenitor stem cells.

In conclusion, the studies reported in this dissertation suggest that the bl/bl genotype of patients with Bloom syndrome may have mutator activity associated with it. Further data were presented suggesting that this mutator activity was mediated by a small mutagenic factor secreted by the fibroblasts of these patients. This is the first report of a potential mutator mutation in man and provides additional support for the somatic mutational theory of cancer.

SUMMARY

Considerable data accumulated over the past two decades have indicated that somatic mutation is a primary etiological event in carcinogenesis. Thus, the genetic control over somatic mutation should modulate cancer predisposition. Past experience has shown that, for at least one group of loci which control mutagenesis, analogous mutations exist between bacteria and humans (xeroderma pigmentosum). This dissertation reports evidence which suggest another similar mutation existing between bacteria and man, mutator mutations.

Mutator mutations in lower organisms signficantly increase the rate of spontaneous mutation and generally involve loci concerned with DNA replication. Evidence is presented here which indicate that the genes responsible for the cancer-prone condition, Bloom syndrome, may be mutator genes. Fibroblasts from patients with Bloom syndrome were found to spontaneously mutate at a rate 4-10 times the normal rate. This observation was repeatable using two different methods of estimating the in vitro rate of spontaneous mutation and suggests that the elevated incidence of cancer in patients with Bloom syndrome may be due to an excess accumulation of spontaneous somatic mutations.

Further data were presented which indicate that the mechanism of the mutator activity in Bloom syndrome cells may be distinct from the mechanisms of mutator mutants in lower organisms. Unlike mutator mutants in rodent cell lines, Bloom syndrome cells appear to have normal levels of deoxyribonucleoside triphosphates. However, it was found the Bloom syndrome cells secrete a mutagenic factor which may cause nonsense but not missense mutations. This factor is probably identical to the clastogenic factor found to be secreted by Bloom syndrome cells by Emerit and Cerutti (52). Based upon these data, a mechanism is proposed that Bloom syndrome cells are deficient in a radical detoxifying enzyme which allows for the accumulation of a clastogenic/mutagenic substrate.

The significance of the results of this study are two-fold. First, it suggests the existance of a mutator mutation in humans, a previously unrecognized phenomenon. Second, the results provide further support for the somatic mutation theory of cancer.

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