THE INFLUENCE OF THYMINE NUCLEOTIDE DEPLETION ON GENETIC STABILITY AND CHANGE IN EUKARYOTIC CELLS

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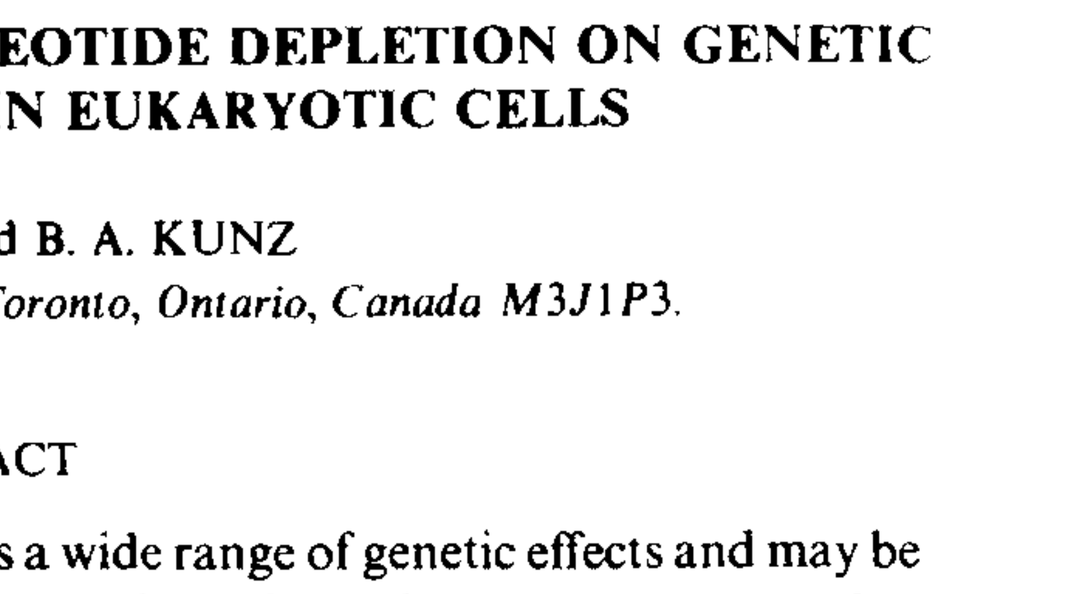
ABSTRACT

Reduction of thymine nucleotide pools elicits a wide range of genetic effects and may be an etiological factor in human cancer. Recent research on these phenomena suggests that the classical DNA damage-repair hypothesis will have to be broadened to account for the existence of non-DNA primary targets for the induction of genetic change.

INTRODUCTION

THE molecular basis of evolution resides in the intimately associated mechanisms of heredity and mutagenesis. Heredity is a manifestation of the processes involved in maintaining gene stability from one generation to the next. Mutation is but one manifestation of the instability of genes and of the fluidity of the genome. Normally, rates of mutation are low. The remarkable stability of genes could be a reflection, in principle, of two different states of affairs, one characterized by very low, the other by very high, potential rates of 'spontaneous' mutation (scheme 1). The genetic material might have been composed of atypical molecules of unusually stable, unreactive structures, capable of highly accurate self-replication. Under these circumstances, mutation rates would be controlled by physical factors and few, if any, chemicals would be expected to be mutagenic. Alternatively, and as is the case, genes could be composed of rather ordinary molecular subunits, endowed with no peculiar physical stability. Various physiochemical factors would then have the potential to generate high error rates in DNA synthesis and many chemical species might be expected to be mutagenic. Under these circumstances, genetic stability would be maintained by mechanisms capable of promoting fidelity in DNA replication, of detoxifying mutagens and of repairing induced DNA damage.

In recent years, several distinct routes to genetic change have been discovered, and much has been learned about the mechanisms of replicational fidelity and DNA repair that dynamically

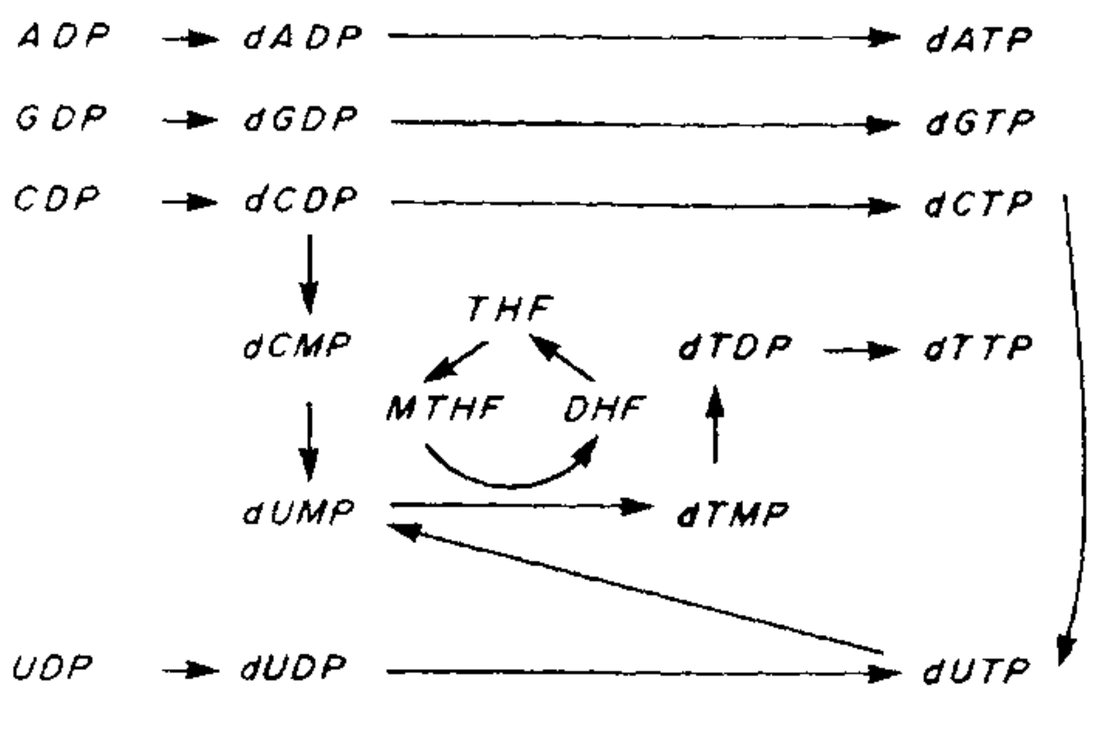


Scheme 1. Two views of the molecular basis of genetic stability and change. Left hand panel: low potential mutation rate (low P.M.R.). On this model, the potential, as well as the actual, mutation rate is very low because genes are assumed to be unusually stable molecules that are capable of extremely accurate replication. Mutations would arise from rare, quantum-statistical fluctuations between isomeric states of genetic molecules. Right hand panel: high P.M.R. This view attributes genetic stability primarily to biochemical mechanisms of fidelity, detoxification and repair. The P.M.R. is very high, but these various biochemical devices suppress it to low residual mutation rates (R.M.R.).

stabilize the genome against high mutation rates that otherwise would be expected to occur[1-4]. However, much less thought has been given to the potential genetic consequences of disturbances in DNA metabolism associated with abnormal levels of the deoxyribonucleoside triphosphate (dNTP) pools.

Biochemical experiments have shown that dNTP pool bias leads to reduced fidelity of DNA synthesis *in vitro*[4]. *In vivo* experiments suggest

that unbalanced DNA precursor pools can elicit the entire range of genetic effects commonly associated with physical and chemical mutagens[5, 6]. In mammalian cells, and recently in yeast, it has been shown that mutants harboring defects in pyrimidine dNTP biosynthesis exhibit mutator phenotypes[6a, 9]. It also has been reported that treatment with certain mutagens produces specific dNTP pool alterations in both prokaryotes and eukaryotes[10, 11]. These latter data raise the intriguing possibility that dNTP pool imbalances may mediate even the genetic effects of standard mutagens in hitherto unexpected ways. Taken together, these facts provide strong grounds for thinking that spontaneous or induced dNTP pool perturbations can be a source of genetic change, and that regulation of such pools is an important mechanism in the maintenance of genetic stability. In this article we illustrate these points by reviewing the genetic effects of *thymine* nucleotide depletion in eukaryotic cells. Also, we describe mechanisms that may account for the recombinagenicity and clastogenicity of this particular DNA precursor imbalance. Finally, we discuss the possible relations of thymine nucleotide stress to carcinogenesis, to the evolution and stability of multigene families and to gene expression.



Scheme 2. A simplified view of DNA precursor synthesis and inhibition of dTMP biosynthesis. Thymidylate synthetase methylates dUMP to dTMP and N^5, N^{10}-methylene tetrahydrofolate (MTHF) functions as the methyl donor. Fluorodeoxyuridylate (FdUMP) inhibits this reaction directly. Dihydrofolate (DHF) analogs (*e.g.* methotrexate) inhibit the reduction of DHF to tetrahydrofolate (THF) by dihydrofolate reductase. Depletion of THF, as a consequence of inhibiting DHF reduction by treatment with methotrexate, inhibits dTMP biosynthesis.

THYMIDINE TRIPHOSPHATE BIOSYNTHESIS AND ITS INHIBITION

The biosynthetic pathways of the dNTPs are linked by nucleotide interconversions and controlled by various regulatory mechanisms. Among the four dNTPs that are necessary substrates for DNA synthesis, thymidine triphosphate (dTTP) occupies a unique position (scheme 2). First, its *de novo* biosynthetic pathway is longer and more complex than those of the other dNTPs. Second, it appears initially in cells as the monophosphate thymidylate (dTMP), whereas the other constituent deoxyribonucleotides of DNA are formed at the diphosphate level *via* reduction of the corresponding ribonucleoside diphosphates by the enzyme ribonucleotide reductase[12, 13]. Third, *de novo* synthesis of dTTP involves the production of another possible DNA precursor, deoxyuridine triphosphate (dUTP), which means that there are in fact three pyrimidine dNTPs potentially available for DNA synthesis. Fourth, the two purine dNTPs (dATP, dGTP) are terminal products of their biosynthetic pathways whereas dTTP is the only terminal pyrimidine dNTP: deoxycytidine triphosphate (dCTP) can be deaminated to dUTP and dUTP hydrolyzed to deoxyuridylate (dUMP) which is ultimately converted to dTTP. Finally, dNTP biosynthesis is coupled in an important way with folate metabolism: N^5, N^{10}-methylene tetrahydrofolate serves as the methyl donor in the conversion of dUMP to dTMP by thymidylate synthetase and this reaction constitutes a significant drain on intracellular tetrahydrofolate pools. This latter feature indicates that dTTP levels can be reduced not only by attack on thymine nucleotide biosynthesis, but also by attack on folate metabolism. In practice, the supply of thymine nucleotides can be diminished by: (1) starving mutants auxotrophic for thymine[14] or thymidylate[15–17]; (2) starving mutants auxotrophic for reduced folates or folate precursors[17–19]; (3) treating cells with drugs that

inhibit thymidylate synthetase[20, 21]; and (4) treating cells with drugs that limit the supply of reduced folates[20, 22, 23].

GENETIC EFFECTS OF THYMINE NUCLEOTIDE DEPLETION

A. *Cell Killing and Mutagenesis*: It is well known that growing cells die if *de novo* thymidylate synthesis is abolished. This phenomenon of "thymineless death" has been described for the yeasts *Saccharomyces cerevisiae* and *Candida albicans*[15, 22], the cellular slime mould *Dictyostelium discoideum*[24], rodent cells[14, 23, 25] and human cells[23, 26, 27]. In addition to cell killing, thymidylate depletion has been found to cause the formation of respiratory deficient mitochondrial mutants in *S. cerevisiae*[28, 29] and to induce point mutations to drug resistance in the mitochondrial DNA of yeast[28], HeLa cells and normal fibroblasts[30] and in the chloroplast DNA of *Chlamydomonas rheinhardtii*[31]. However, results for the mutagenesis of nuclear genes are less clearcut. Thymine nucleotide depletion, or agents that inhibit dTMP biosynthesis, have been reported not to cause reversion of several nuclear mutations or to induce forward mutation to adenine auxotrophy in yeast[20, 28, 32]. On the other hand, dTMP deprivation has been found to revert the *lys*1-1 allele and to increase the frequency of forward mutation to canavanine resistance in this organism[33]. These results suggest that the mutagenic effects of thymidylate depletion may be allele specific, at least with regard to reverse mutation. Inhibition of dTMP biosynthesis also has been found to have no mutagenic effect in Chinese hamster cells[34], mouse cells[35], or human cells[30]. In contrast to these findings, treatment of mouse C3H10T1/2 cells and synchronized Chinese hamster cells with fluorodeoxyuridine (FdUrd), which is converted intracellularly to fluorodeoxyuridylate (FdUMP), a potent inhibitor of thymidylate synthetase[36], is weakly mutagenic[37, 38]. Also, mild thymidylate stress mutagenizes mouse FM3A cells[38a] as does growth of Chinese hamster V79 cells in medium containing FdUrd plus deoxycytidine[38b].

Depletion of thymine nucleotides is associated with an increase in dCTP pools presumably due to reduction in feedback inhibition by dTTP on ribonucleotide reductase[7, 39]. This high dCTP:dTTP ratio might be expected to lead primarily to the enhanced production of A:T → G:C transitions in eukaryotes, as appears to be the case for bacterial cells[40, 41]. If this is the situation, the apparent allele specific response to thymidylate deprivation in yeast is not surprising. The relatively small induction of nuclear gene mutations by dTMP depletion in eukaryotic cells, as compared to the pronounced effects in bacteria[40, 41], suggests that eukaryotes and prokaryotes may differ in certain aspects of their response to thymidylate deprivation. These differences may involve mechanisms of DNA synthesis or mutagenesis or the fidelity of DNA replication.

B. *DNA and Chromosome Breakage*: It was suggested originally by McFall and Magasanik[42] that "thymineless" episodes might lead to DNA strand breakage. Alkaline sucrose gradient centrifugation has since been employed to demonstrate breakage and/or fragmentation of DNA strands during dTMP depletion of yeast[32, 44] (figure 1), mouse[45, 46] and human cells[47]. As might be anticipated from these results, treatment with agents that inhibit dTMP biosynthesis induces both chromosome and chromatid breaks and gaps, as well as chromosome shattering, in plant[48–50], mammalian[51–53] and human[10, 54–55a] cells. In addition to this generalized type of chromosome damage, breaks can be induced at specific points in human chromosomes by dTMP deprivation. These so called "fragile sites" are expressed as non-staining gaps during metaphase if cells are cultured in medium containing FdUrd, antifolate drugs or low concentrations of folic acid and thymidine[56, 57]. The fact that induction of fragile sites by FdUrd and folate antagonists can be prevented by concurrent provision of thymidine indicates that fragile site expression is a consequence of thymine nucleotide depletion. It also has been determined that culturing human cells in folic acid deficient medium results in higher frequencies of generalized spontaneous

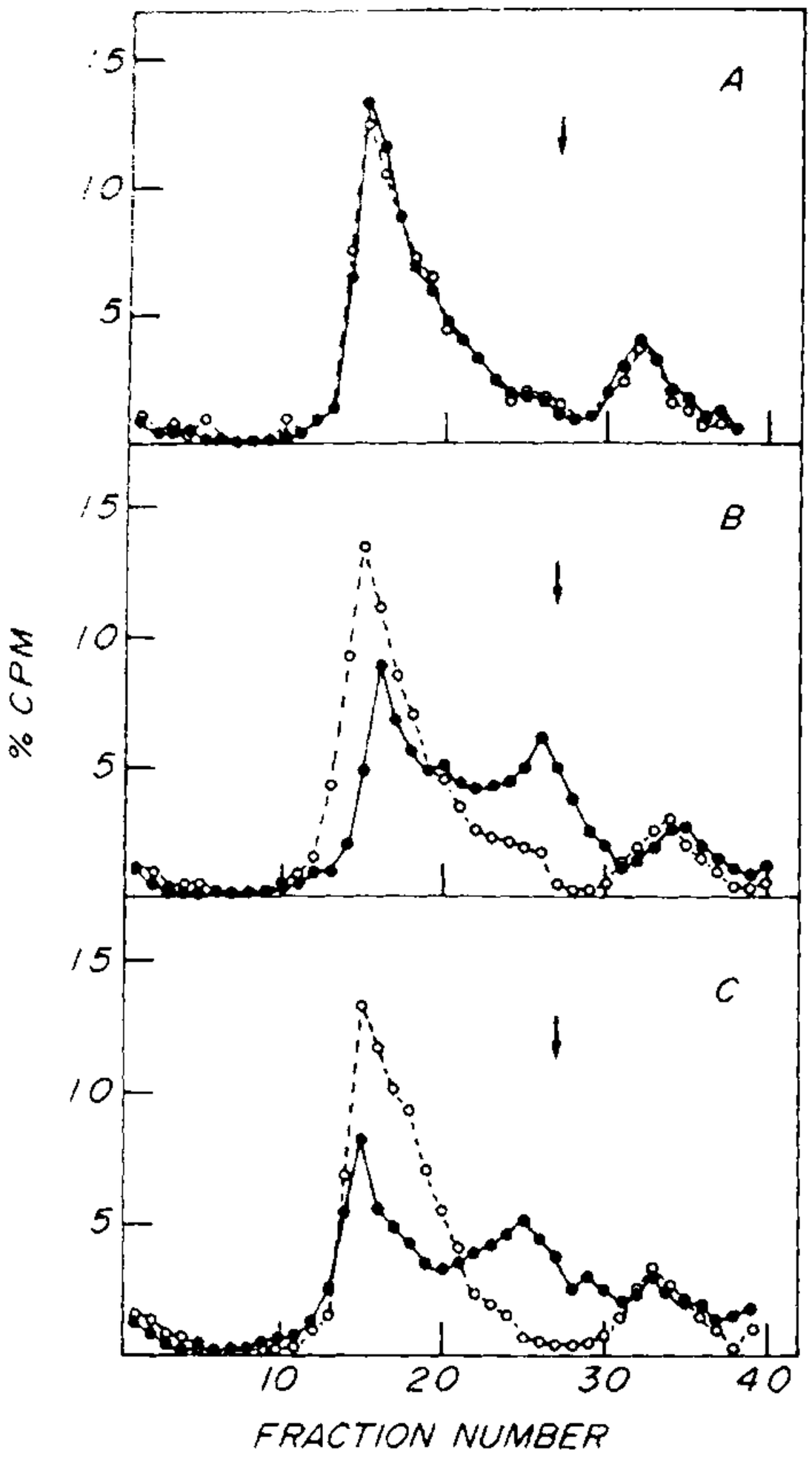


Figure 1. Sedimentation gradient analysis of DNA from FdUMP or antifolate-treated yeast cells. ^{3}H-labelled DNA (●) from treated cells and ^{14}C-labelled DNA (○) from untreated cells was subjected to alkaline sucrose gradient centrifugation[32]. **A.** control, **B.** FdUMP-treated cells, **C.** antifolate treated cells. The direction of sedimentation is from right to left. The arrows indicate the position to which bacteriophage T4 DNA sediments in these gradients. The appearance of a new peak of low molecular weight DNA in the drug-treated cells (around fraction 25 in B and C) and the decline in the peak corresponding to nuclear DNA (around fraction 15) demonstrates the production of DNA single-strand breaks as a result of FdUMP or antifolate treatment to inhibit dTMP biosynthesis.

chromosome breakage[21]. This suggests that expression of fragile sites and some other types of chromosome breakage may involve a common mechanism.

Inhibition of dTMP biosynthesis leads to an increase in the dUTP pool and to misincorporation of uracil into DNA[47, 58, 59]. Uracil moities in DNA can be removed by a specific excision-repair system that involves the concerted action of uracil-DNA glycosylase, apyrimidinic endonuclease, repair polymerase and DNA ligase[60]. Under conditions of thymidylate deprivation, uracil nucleotides also would be inserted into DNA by the repair polymerase. Uracil inserted into repair patches would be itself a substrate for further rounds of excision repair. On this basis, it has been proposed that a process of reiterative uracil incorporation and removal, during dTMP depletion, would hinder ligation and the completion of uracil excision repair; this could give rise to the DNA strand breakage detected following inhibition of dTMP biosynthesis[20, 32].

Another mechanism also could account for the formation of DNA strand scissions in thymidylate starved cells. High dTTP levels signal ribonucleotide reductase to commence reducing guanosine diphosphate (GDP) to deoxyguanosine diphosphate (dGDP). In turn, high dGTP levels stimulate reduction of adenosine diphosphate (ADP)[61]. Thus, depletion of dTTP also leads to diminution of the dGTP and dATP pools as DNA synthesis continues. This limitation of the purine dNTPs would inhibit replication of DNA[62] causing gaps to form between points of DNA synthesis inhibition.

C. *Recombination*: Interchromosomal recombination occurs primarily between two homologous regions of DNA located on homologous chromosomes. Both reciprocal (crossing-over) and non-reciprocal (gene conversion) exchanges of genetic information arise spontaneously and can be induced by physical and chemical agents[63, 64]. As mentioned in the previous section, dTTP depletion leads to the formation of DNA strand breaks and the presence of strand gaps is known to stimulate recombination in yeast[65]. Accordingly, it has been found that inhibition of thymidylate synthetase activity by genetic lesion, FdUMP treatment or antifolate-

induced limitation of reduced folates stimulates both mitotic crossing-over and gene conversion in *S. cerevisiae*[20, 32, 33, 44, 66]. In nucleotide permeable strains, these effects are abolished by concurrent provision of dTMP (figure 2). This demonstrates that the recombinagenicity of the

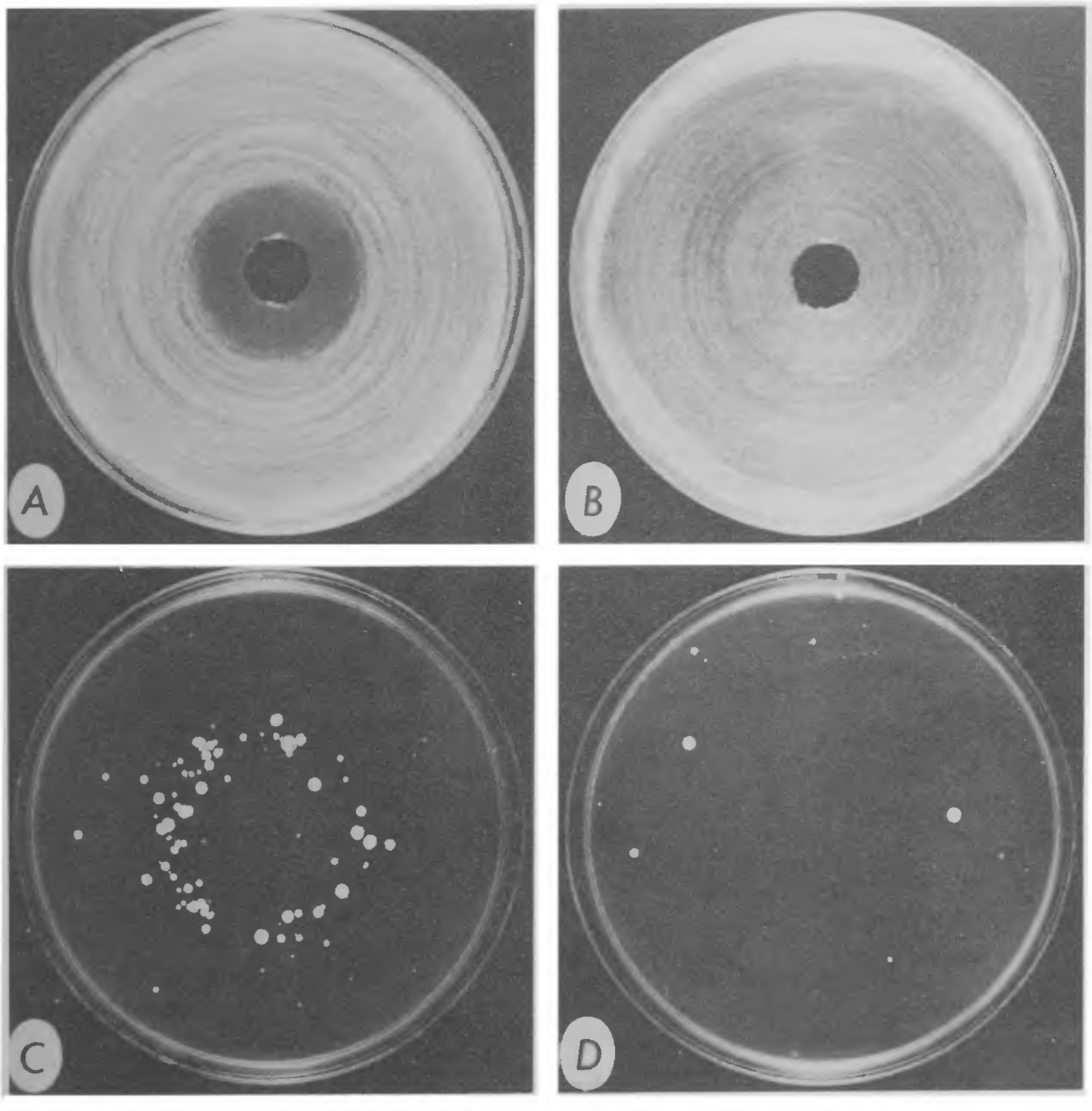


Figure 2. Induction of mitotic recombination in yeast by FdUMP and the abolition of this effect by provision of dTMP. The strain used is a nucleotide-permeable diploid heterozygous for a recessive defect conferring resistance to cycloheximide an inhibitor of protein synthesis. Plate A shows growth of the strain on nutrient medium with FdUMP added to the centre well; note the zone of growth inhibition around the well. Plate **B** dTMP in the medium and FdUMP in the well; the zone of growth inhibition around the well is eliminated. Plate **C** is a replicate of **A** on cycloheximide medium; the ring of colonies indicates the induction of interchromosomal recombination by FdUMP. The recombinagenic effect is abolished in **B**, as no such ring of recombinants appears when it is replicated onto cycloheximide medium (Plate **D**).

FdUMP and antifolate treatments are a consequence of thymine nucleotide depletion. Folate antagonists also have been shown to induce mitotic recombination in *Aspergillus nidulans*[67, 68] and *Drosophila melanogaster*[69] and both antifolate drugs and FdUrd enhance meiotic recombination in *D. melanogaster*[70]. Cytological manifestations of interchromosomal recombination, *i.e.* chromosome and chromatid exchanges, have been detected following exposure to drugs that inhibit dTMP biosynthesis in rodent[51, 52, 71] and human[72, 73] cells.

Intrachromosomal recombination is the term applied to the transfer of genetic information between repeated genes on the same chromosome or chromatid or between sister chromatids[63, 64]. Antifolate treatment has been found to enhance the formation of leucine prototrophs in a haploid strain of yeast carrying two different mutant *leu2* alleles in tandem on the same chromosome[75]. That this effect is the result of thymine nucleotide depletion was verified by the finding that provision of thymidylate eliminated the increased production of leucine prototrophs (figure 3). DNA hybridization analysis revealed that recombination, including reciprocal exchange, gene conversion and unequal sister chromatid crossing-over, between the duplicated genes gave rise to the induced prototrophs. Inhibition of dTMP biosynthesis by FdUMP or antifolate treatment also was shown to enhance unequal sister chromatid recombination within the ribosomal DNA gene cluster of yeast[76]. In addition, yeast mating-type switching, which is thought to involve a highly directional form of intrachromosomal gene conversion, can be stimulated by FdUMP-induced dTMP deprivation[77]. To date, intrachromosomal recombination has not been demonstrated unequivocally in any other eukaryotic organism. However, treatment with the antifolate methotrexate has been found to induce the loss of a stably integrated copy of the human thymidylate synthetase gene from mouse cells[35] possibly *via* intrachromosomal exchange. The various findings outlined in this section suggest that in actively growing cells, thymine nucleotide depletion may create a metabolic condition under

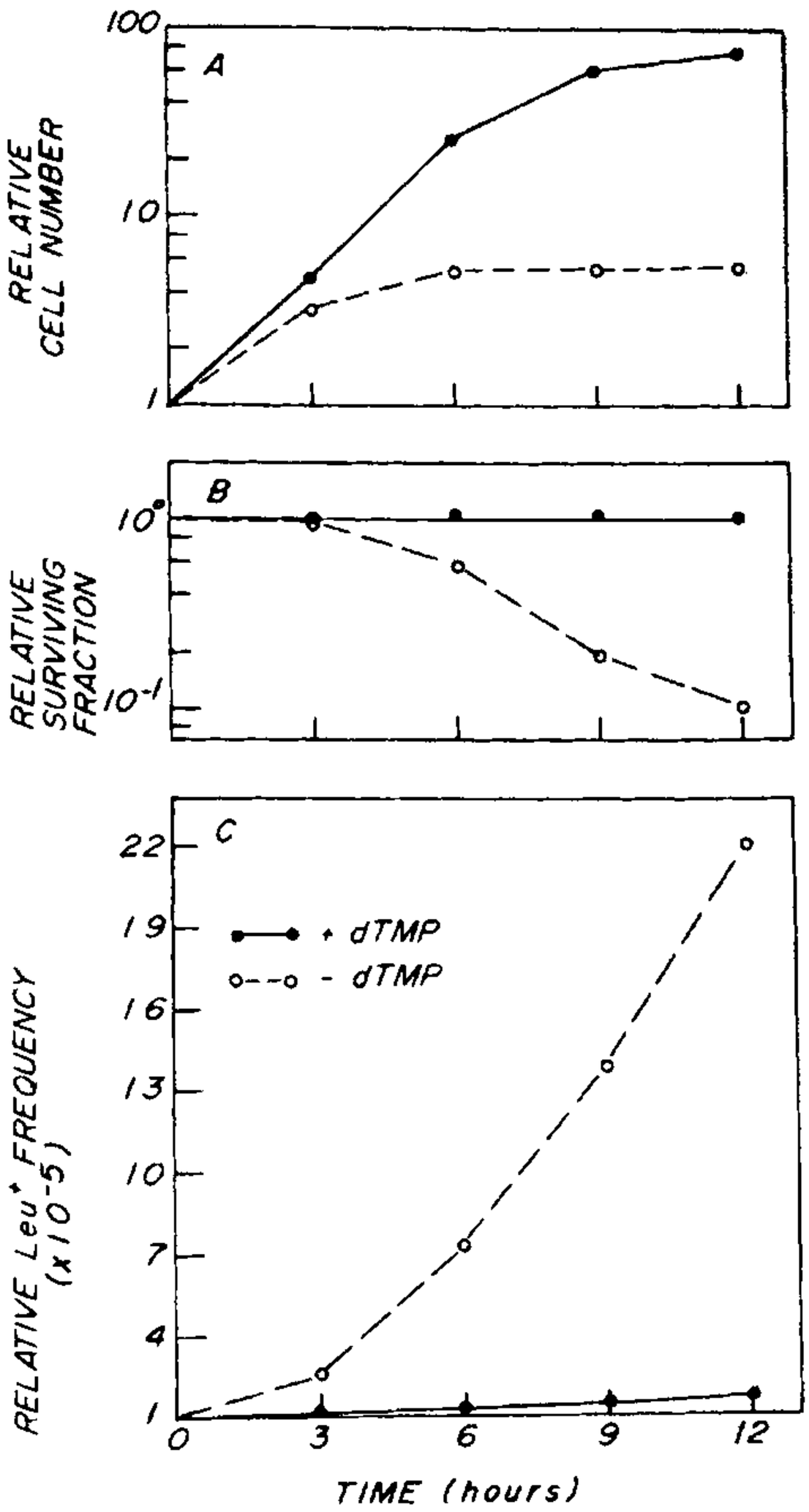


Figure 3. Induction of leucine prototrophs (Leu$^+$) by antifolate treatment of a nucleotide-permeable haploid strain carrying two different mutant *leu2* alleles in tandem. The strain was treated with the folate antagonists methotrexate plus sulfanilamide in the presence (●) or absence (o) of dTMP. Panel **A.** cell titres; note that inhibition of cell growth by the drugs is prevented by dTMP. Panel **B.** cell survival; note that antifolate treatment is not lethal in the presence of dTMP. Panel **C.** frequencies of leucine prototrophs; dTMP eliminates antifolate-induced intrachromosomal recombination that generates leucine prototrophs.

which all forms of mitotic recombination are induced to elevated frequencies.

Finally, it should be noted that in yeast, mutants defective in double strand break repair have reduced frequencies of both inter- and intrachromosomal recombination under conditions of thymidylate depletion[44, 76]. In diploid strains, this response is associated with chromosome loss and agents that inhibit dTMP biosynthesis also have been found to cause aneuploidy in *A. nidulans*[67], *Neurospora crassa*[78] and cells of developing mouse embryos[52].

D. *Teratogenesis and Oncogenic Transformation*: In view of the ability of agents that provoke dTMP deprivation to induce DNA strand scissions, mutation, recombination and chromosome breakage, exchange and loss, it is not surprising that antifolates have been found to be teratogenic in humans[79-81]. Similarly, the induction of chromosome damage and exchange may be involved in the transformation of mouse C3H/10T1/2 cells by methotrexate and FdUrd[51, 82]. The fact that the oncogenic transformation caused by FdUrd treatment was decreased markedly by concurrent provision of thymidine indicates that this transformation was related to dTMP depletion.

FURTHER CONSIDERATIONS

A. *Multigene Families and Gene Expression*: It has been suggested that both unequal sister chromatid crossing-over and intrachromosomal gene conversion might play roles in the evolution and stability of multigene families[83, 84]. Both types of event have been demonstrated in yeast[83, 84]. Furthermore, there is evidence for intrachromosomal gene conversion between duplicated thymidine kinase genes in cultured mouse cells[85]. In addition, experimental data suggests that intrachromosomal gene conversion may occur among the gamma and alpha globin genes[86, 87] the immunoglobulin heavy and light chain genes[87a-88] and certain of the heat shock genes in *D. melanogaster*[89]. If intrachromosomal recombination is involved in the maintenance of sequence homogeneity and/or replacement of existing information by a variant sequence in multigene families, then such phenomena may be influenced by thymine nucleotide pool depletion which can induce various types of intrachromosomal exchange.

Through its effect on recombination, reduction of dTTP levels also may play a role in the control of gene expression, at least in certain instances. For example, it has been determined that inhibition of dTMP biosynthesis can enhance excision of a transposon situated 5′ to an alcohol dehydrogenase gene in yeast (B. A. Kunz, unpublished observations). This occurs *via* intrachromosomal reciprocal exchange between the repeated sequences that flank the transposon and leads to inactivation of the alcohol dehydrogenase gene. As mentioned above, mating-type switching in yeast, which results in the expression of new information at the mating-type locus, also can be provoked by thymine nucleotide depletion[77]. Finally, interchromosomal recombination, which also can be induced by dTTP pool imbalance[20], can give rise to the expression of recessive genetic markers.

B. *Carcinogenesis*: Recent work on DNA alterations that activate cellular proto-oncogenes has indicated that such changes can arise through at least five different mechanisms: one involves single base pair substitutions whereas three involve chromosomal rearrangements and the fifth is associated with gene amplification[90], which can result from unequal sister chromatid recombination. Many cancers are associated with chromosomal defects, the most common being band or segment deletions and reciprocal translocations. Because these structural defects are clonal in nature and are present throughout the disease process, it has been suggested that chromosomal alterations might represent a common step in neoplastic development[91-93]. As indicated in a preceeding section, depletion of the dTTP pool can lead to the formation of various types of structural rearrangements in chromosomes and there is *in vitro* evidence to suggest that dTTP pool imbalances may be carcinogenic, or at least play a role in carcinogenesis. First, inhibition of dTMP biosynthesis sensitizes HeLa cells to the lethal effects of x-rays[94], and mouse cells to the lethal and mutagenic effects of

ultraviolet light and chemical mutagens[95]. Thus, it is possible that alterations in dTTP pools may mediate even the genetic effects of standard mutagens/carcinogens. Second, the ability of dTTP pool depletion alone to induce chromosome and chromatid aberrations, including exchange, in cultured cells, suggests that it also may provoke the specific translocations that have been found to be associated with various types of cancer. In certain cases, the activation of viral-associated cellular oncogenes appears related to these translocations[93] and it has been shown that inhibition of dTMP biosynthesis can induce viral DNA synthesis in polyoma transformed mammalian cells[96]. Third, at least 26 fragile sites coincide with break points involved in chromosomal defects found in leukemias, lymphomas and malignant solid tumors[97, 98]. Such sites are expressed under conditions of thymine nucleotide deficiency. Therefore, dTTP depletion may enhance the formation of specific chromosomal rearrangements by inducing fragile site expression. Fourth, drugs that inhibit dTMP biosynthesis cause oncogenic transformation of cultured mammalian cells[51, 82]. Taken collectively, these findings argue that reductions in the dTTP pool may induce, or predispose, cells to oncogenesis.

As outlined in scheme 2, the biosynthesis of dTMP requires a tetrahydrofolate cofactor. Agents or conditions that limit the supply of reduced folates block dTMP production. In human cells folate is an essential vitamin and necessary precursor of tetrahydrofolate. *In vivo* evidence supporting a role for thymine nucleotide depletion in the etiology of human cancer involves the association between folate metabolism and thymidylate biosynthesis. First, the folate deficiency symptomatic of megaloblastic anemia leads to extensive chromosome damage in human cells[55]. In addition, administration of the antifolate drug methotrexate results in the induction of chromosome aberrations including fragmentation, breaks, gaps and exchanges[99]. Thus, folate deficiency, and hence thymine nucleotide depletion, may give rise to the chromosomal alterations associated with various types of cancer. Second, the use of methotrexate for the treatment of non-malignant disease, *e.g.* psoriasis, or as an immunosuppressant agent in humans, has been linked with the occasional development of lymphomas, leukemias, carcinomas and Kaposi's sarcoma[99, 100]. Similarly, cancer patients have been found to develop secondary tumors subsequent to antifolate therapy for a primary neoplasm[99, 101, 102]. Finally, epidemiological associations have been found between several disorders that lead to folate deficiency (*e.g.* pernicious anemia, celiac disease, alcoholism, malaria) and increased cancer incidence[103–107]. These various findings are consistent with the hypothesis that altered dTTP levels in human cells, as a consequence of folate deficiency, may lead to neoplastic transformation.

CONCLUDING REMARKS

All known forms of genetic alteration, from point mutations to oncogenic transformation, have been observed subsequent to induced dTTP pool reductions in appropriate eukaryotic assay systems. These effects increase with exposure to the inducing agent, and their magnitudes are not insignificant in comparison with those of mutagens that attack DNA directly. The drugs used to decrease dTTP levels specifically inhibit certain enzymes involved in dTTP biosynthesis. This indicates that eukaryotic cells contain non-DNA primary targets for the induction of genetic change. Furthermore, it emphasizes the need to develop more sophisticated models of mutagenesis than those based solely on the DNA damage-repair hypothesis. There is strong evidence that the genetic effects resulting from thymine nucleotide depletion are not merely curiosities that arise from the production of highly artificial dTTP pool disturbances in cultured cells. Folate deficiency, which leads to the inhibition of dTMP biosynthesis, is one of the most common vitamin deficiencies in the world[107a, 108] and has been linked to human carcinogenesis. In addition, antifolate drugs are known to be teratogenic in humans. These findings argue that dTTP pool perturbations are of significance in genetic disease and may be widespread in the human population.

ACKNOWLEDGEMENTS

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