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Aging: A study of DNA microlesions and methylation

Kirsh, Marvin E., Ph.D.

City University of New York, 1986

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AGING: A STUDY OF DNA MICROLESIONS AND METHYLATION

by

MARVIN E. KIRSH

A dissertation submitted to the Graduate Faculty
in Biochemistry in partial fulfillment of the
requirements for the degree of Doctor of Philosophy,
The City University of New York.

1986

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy

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Abstract

AGING: A STUDY OF DNA MICROLESIONS AND METHYLATION

by

MARVIN E. KIRSH

Adviser: Dr. Aaron Lukton

Somatic mutation in terminally differentiated tissues may modulate the aging process. There is evidence that some DNA repair processes follow a path of decline as organisms age and correlations between lifespan and DNA repair capability have been established for several species. Although gross changes in DNA, such as single and double strand breaks, crosslinks, and chromosomal abberations and reorganizations, may be of paramount importance as reflecting the decreased stability associated with increased age, mutation in the DNA base sequence may be just as important.

The work presented here involved the measurement of altered DNA bases as they may have been generated from normal bases. Altered DNA bases which may be mutagenic and which may accumulate with age were measured. In particular, deoxyuridine (dU) (a deamination product of deoxycytidine) and 5-hydroxymethyldeoxyuridine (dHMU) (an oxidation product of thymidine), were assayed in mouse tissue DNA. This assay, using an HPLC separation, is sensitive to less than 25 picomoles in

DNA samples sized to 50 micromoles of deoxyribonucleosides. Neither dU, nor dHMU was detected in samples of mouse tissues aged 7, 11, 19, and 31 months. In chick erythrocyte DNA, however, small amounts of dU are found, varying between DNA samples of different commercial sources. The origin of dU in these samples is unknown. dHMU was also found in chick erythrocyte DNA.

In addition to measuring dU and dHMU, 5-methyldeoxycytidine (believed to have a role in regulating gene expression) was measured as a function of age in the three tissues studied. Its levels fluctuated widely in liver and brain, but remained steady in the growing and rapidly dividing small intestinal mucosa.

ACKNOWLEDGMENTS

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PREFACE

Aging can be defined as a loss of vitality over a period of time and increased susceptibility to disease. The study of aging by its nature draws on the dis iplines of philosophy, psychology, as well as biology. Time in the psychological sense is not uniform and aging in humans proceeds at different rates during the lifespan. Humans become most vulnerable to stress and disease in the later years of life, and survival curves fall steeply during this period (Comfort, 1979). Theories regarding the biology of aging are divided into separate, though not distinct, schools. Some believe that aging is as much a developmental process as is embryogenesis; that is, aging is programmed into the organism (Reviewed by Strehler, 1977a). Others (Reviewed by Strehler, 1977b) believe that senescence is, in a sense, due to accumulated damage, as if the organism were constructed to withstand so much environmental insult and then death results. These nonmutually exclusive schools of thought can be extended to include the existence of a biological clock. In the developmental school the clock is preprogrammed by instruction, whereas in the damage school the clock is a counter that sums up environmental insults and paces it against the durability of the organism.

The most striking fact to the field-of-aging-detective is the different lifespan capabilities of different organisms, and the relationship of these maximum lifespan potentials to various

biological parameters. For instance, DNA repair capability, the ratio of brain weight to body weight, and various enzymatic activities (such as superoxide dismutase levels) seem to correlate with maximum lifespan potential (Tolmosoff, 1980; Cutler, 1982). The organism that lives longer is built of better parts, but of the same building blocks (for example, the raw materials of life are better assembled in the long-living species).

The information for the assembly of macromolecules from building blocks is coded for by the DNA (Watson, 1976). Life is self-perpetuating, and so it is on the microlevel that DNA codes for the assembly of its own replicating machinery and its own protection devices, and for the form and nature of the organism to which it belongs. DNA codes for all of the macromolecules in the cell, which in turn form the cell type and the cells from which the organism is assembled. When a geneticist studies the problem of aging he first looks toward the DNA. The DNA of all organisms is composed of basically the same building blocks. However, the sequence of bases in the DNA varies significantly from organism to organism, and this is the means by which different organisms develop differently and take on different forms.

Differentiation into various cell types is believed to occur by the differential expression of genes in different cell lineages on a time programmed basis. Time in the biological sense proceeds as genes are turned on and off and, in some cells, as DNA is rearranged, reordered, and/or amplified. Changes occur quickly during early life and slow as final cell types evolve. Aging in the psychological sense

proceeds much as we age biologically. To extend this concept developmentally we ask: "As the whole organism becomes less stable, and its cells and proteins become less stable, does the DNA become less stable?"

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A. Introduction

A machine such as an automobile deteriorates with age, depending on the quality of its construction and the ability of its parts to withstand wear and tear. An organism can be viewed similarly. It is composed of parts (or molecules) such as DNA, RNA, proteins, and lipids. Aging, then, results from a deterioration or a perturbation of function. Each organism of a characteristic construction, i.e. shape, form, and distinctiveness of its parts, has a characteristic lifespan, and just as an automobile can be serviced by a mechanic, cellular components have repair capability. Then, with this capability, why aren't organisms immortal? Obviously repair capabilities themselves are not immortal, imperfect as they must be, and the fundamental question can be posed: What parts, or part, of a cell compose the weakest link in the chain? It is known, for example, that superoxide dismutase, an enzyme that destroys superoxide radicals, (Tolmasoff et al., 1980) which can cause cellular damage, and DNA excision repair, (Hart & Setlow, 1974) both have activity levels that correlate positively with lifespan.

B. Objectives

The objective of this thesis is to measure the deoxyuridine (dU) and 5-hydroxymethyldeoxyuridine (dHMU) content of DNA in aging somatic mammalian tissues, using a very sensitive HPLC assay. Uracil

introduced into DNA as a result of the deamination of cytosine presents a potential mutation. This type of lesion may, in fact, be one of the most potent and predominant changes occurring in the DNA, and thus may contribute to the aging process.

C. Background

Since there are deleterious changes in many cellular components during the aging process, there are numerous theories regarding aging. These include a membrane theory (Naggy, 1978), an error catastrophe theory (Orgel, 1963), and a somatic mutation theory (Mayneo·l & Clark, 1975). The experiments suggested and conducted in this thesis are designed to test an aspect of the somatic mutation theory of aging by taking advantage of the properties of a very sensitive HPLC assay.

1. Somatic Mutation Theory of Aging

According to the somatic mutation theory of aging, the disruption of normal cellular processes in aged cells is due to the loss of genetic information. Various authors have attempted to relate gene mutation to aging. Curtis (1967) and Prasad (1974) were able to show that doses of radiation can reduce the lifespan and it has been determined (see Huttermann et al., 1978) that radiation was mutagenic in many systems, including mammals. A theory to connect the mutagenic effect of radiation and its effect on lifespan was constructed by Mayneord and Clark (1975). Since radiation does not produce all the effects of aging, though irradiated mice die from the same spectrum of diseases as old mice (Lindop et al., 1960), the correlation between somatic mutation and aging remains somewhat nebulous. It is known that agents that induce cancers are generally mutagenic and it is also

known that cancers increase with age, but the correlation that gene mutations increase with age cannot be made. Two effects of the same agent (for radiation--aging and mutation, and for carcinogens--cancer and mutations) may not necessarily be related to each other. Further support for the somatic mutation theory of aging must be derived from experiments showing changes in the DNA or the proteins coded for by the DNA. The foundation of the somatic mutation theory of aging is that DNA, being the molecule of heredity, is the mediator of aging and eventually succumbs to excessive damage in the midst of several DNA specific repair systems.

2. Errors in Proteins as a Function of Age

Orgel (1963) proposed an error catastrophe theory of aging which stated that errors induced in proteins by the protein synthesizing machinery, amplify themselves by increasing errors, and eventually cause an error catastrophe. For instance, a mistake in synthesizing a ribosomal protein, if that protein were incorporated into a ribosome, could lead to more errors. Also to be included in this theory could be changes induced in DNA resulting from errors in proteins that replicate or interact with the DNA. This second process would also lead to error amplification, and end in error catastrophe. Although errors in proteins could arise initially from either an error catastrophe or somatic mutation, many investigators have tried to find errors in proteins to support the error catastrophe theory of aging. Holliday and Tarrant (1972) and Wulf and Cutler (1975) found increases in heat labile proteins in tissue culture. Holliday and Tarrant (1972) found an associated altered specificity with the increase in heat lability. Lewis and Tarrant (1972) found a decrease in specific

activity of glucose-6-phosphate dehydrogenase in aged fibroblasts, and Gershon and Gershon (1970) found a decrease in specific activity of isocitrate lyase in nematodes. Gershon and Gershon (1973a,b) also found a decrease in mouse liver and mouse muscle aldolase activities with age. Werner's syndrome cells that age prematurely accumulate labile glucose-6-phosphate dehydrogenase earlier than usual (Holliday et al., 1974) as well as other heat labile enzymes (Goldstein & Moerman, 1975). Linn et al. (1976) showed that DNA polymerase loses activity in aged fibroblasts and Hardin (1973) found an increase in heat labile glutathione reductase in aged human lens. Menzies et al. (1972) found differences in t-RNA turnover in rat kidney, liver, and spleen.

While the above examples may be taken as support for an error catastrophe theory of aging, they do not contradict the somatic mutation theory of aging. In fact the origin of altered enzymes from tissue culture cells is subject to question, and altered enzymes may be derived from dead or dying cells in the culture. Since all proteins apparently are not affected by aging (Rubinson et al., 1976; Barton & Yang, 1975; and Popp et al., 1976). Baird and Massie (1975) and Gershon and Gershon (1976) have concluded that an error catastrophe theory of aging is not indicated.

3. DNA Repair and Aging

Since spontaneous mutations and damage to DNA are occurring in all cells at all times (Hartman, 1980), repair processes are needed to maintain the integrity of the genetic information (see Hanawalt et al., 1979 and Lindahl, 1979, 1982 for recent reviews and discussions of DNA repair). The known repair systems for DNA are: a. excision

repair, b. post-replication repair, c. repair of single strand breaks, d. photoreactivation, and e. base repair.

a. Excision repair involves cleaving of the phosphodiester backbone, excision of the damaged region, and DNA synthesis over the excised region.

b. Post replication repair is intimately connected with semiconservative DNA replication, and apparently involves the repair of a damaged strand adjacent to an intact template.

c. The repair of single strand breaks involves limited nucleolytic activity (Dugle & Gillespie, 1975) and is rapid to repair breaks caused by various agents.

d. Photoreactivation involves the repair of pyrimidine dimers by visible light (Sutherland et al., 1974).

e. Base repair involves the excision of damaged bases by cleavage of the N-glycosidic bond between the base and the sugar. This type of damage is further repaired by the various excision repair systems.

That DNA repair is an important mechanism for the maintenance of cellular homeostasis is evidenced by the fact that several neurological degenerations (Robbins, 1983) as well as high blood pressure (Pero et al., 1976) have been linked to hypersensitivity to DNA damaging agents. Although a direct cause and effect relationship has not been established in these cases it is quite possible that defects in DNA repair may have many pleiotrophic effects on an organism.

Is there increasing DNA damage during aging? Several authors have attempted to measure single strand breaks, DNA cross links,

unscheduled DNA synthesis, and chromosome abberations in attempts to determine if DNA repair declines during lifespan. The results are generally ambiguous--some tissues, particularly liver, seem to show increased DNA damage during development but most tissues do not. (The tissues tested were peripheral lymphocytes, skin fibroblasts, bone marrow, muscle, brain, and heart.) When measuring chromosome abberations, there seems to be an increase in liver (Stevenson & Curtis, 1961) in several species (Curtis et al., 1966; Curtis & Miller, 1971; Brooks et al. 1973) but not in phytohemagglutinin stimulated leukocytes. Court-Brown et al. (1966), Tough et al. (1970), Jarvik and Kato (1970), Liniecki et al. (1971), and Ayme et al. (1976) observed a slight increase in chromosomal abberations with age where other authors have observed no such increase (Jacobs & Court-Brown, 1966; Sandberg et al., 1967; Bochkov et al., 1968; Goodman et al., 1969; and Bochkov, 1972). Price et al. (1971), and Modak and Price (1971) using the ability of nicked DNA to act as a template for DNA polymerase, found a significant increase in single strand breaks in some tissues but not in others. However, all cells examined showed increases in acid-sensitive regions. Massie et al. (1972) found a ten-fold decrease in molecular weight of DNA from old cells. Ono et al. (1976) found a decrease in molecular weight in liver, but not in spleen, thymus, or cerebellum. Other researchers have found evidence for the accumulation of single strand breaks during aging (Karran & Ormerod, 1973; Chetsanga et al., 1975; and Wheeler & Lett, 1974).

4. Correlation of DNA Repair with Lifespan

Hart and Setlow (1974) have attempted to correlate the DNA

excision repair capability of a species with its lifespan. They have found a linear correlation between lifespan and excision repair ability in seven species that differed several-fold in lifespan. They measured unscheduled DNA synthesis after exposure of cells (taken from the same location in animals that had completed approximately the same percentage of their lifespans) to ultraviolet light. It was estimated that about the same number of thymidine dimers was induced in the cells of each species, and that the cells had about the same DNA content. A measure of grains per nucleus after autoradiography correlated well with lifespan. However, since this correlation is not found in a group of related primates that differ in lifespan by as much as twenty-fold (Cutler, 1976) the relationship between DNA repair and lifespan found by Hart and Setlow must be viewed cautiously. The correlation between DNA damage and repair with lifespan has not been made with other forms of DNA repair on the molecular level.

5. DNA Base Repair

Until recently, it was believed that damage to DNA bases was repaired singularly by the excision repair process, but it is known that there exists a group of enzymes (glycosylases) that can release damaged or unintended bases from the DNA, leaving the phosphodiester backbone intact. These enzymes apparently act by cleavage of the N-glycosidic bond between the deoxyribose moiety and the base in a nonexchange type of reaction (Talpaert-Borle et al., 1979). Aprymidinic and apurinic sites resulting from this attack are believed to be repaired via excision repair using apurinic-aprymidinic endonucleases for the excision step (Lindahl et al., 1977; Lindahl, 1982). Glycosylase activities have been identified for uracil (the

deamination product of cytosine), hypoxanthine (the deamination product of adenine), and 3 methyl or ethyl-adenine (Lindahl, 1979; others in Lindahl, 1982). Glycosylase activities also have been identified for thymidine oxidation products including the derivative dHMU as well as other oxidation products (Hollstein et al., 1984; Demple & Linn, 1980). Glycosylase activity appears to be ubiquitous as activities have been identified in man as well as bacteria and other organisms examined (Ishiwata & Oikawa, 1979); Duncan et al., 1978; and Talpaert-Borle et al., 1979).

As an example of an altered base, consider the deamination of cytosine to uracil. It has been estimated (Hartman, 1980) that at 37°C, as many as 160 cytosines in duplex DNA may be spontaneously deaminated per cell per day in a diploid mammalian organism. The rate has also been shown to increase with temperature (Lindahl & Nyberg, 1974). Consider that if two uracils per week went unrepaired, by age 70 there would be an average of 7,240 uracils per genome in each somatic cell of the body. Since uracil introduced into DNA as a result of the deamination of cytosine is mismatched to adenine during DNA replication, each unrepaired and incorrectly repaired uracil would represent a point mutation.

Although at present there is no direct evidence for altered amino acid sequences in proteins of aged animals (any changes observed so far could have arisen from post translational metabolism and not directly from DNA microlesions), uracil in DNA may affect other vital processes. If uracil remaining in the DNA is mismatched to guanine, a local distortion of the DNA helix could interfere with DNA metabolism. For instance, such a distortion may prevent passage of an RNA or DNA

polymerase, resulting in the synthesis of an incomplete polypeptide or the inability of the cell to replicate. Repair processes intended to correct this distortion and the mismatch may make mistakes resulting in unrepaired single strand breaks, or may fix the uracil in the DNA by repairing the wrong strand. Uracil, in DNA, introduced either by misincorporation or deamination, could interfere with the binding to the DNA of regulatory proteins and may cause epigenetic alterations in cellular programming. These types of changes could lead to cellular deterioration or could cause alterations in cellular programming. DNA-glycosylase must play vital roles in DNA metabolism.

6. Uracil-DNA-Glycosylase

Uracil-DNA-glycosylase of E. coli has a molecular weight of 24,100, contains a single subunit, has no cofactor requirements, attacks single or double stranded DNA, and is noncompetitively inhibited by free uracil (Lindahl, 1979). The enzyme is specific for uracil both as an inhibitor and as substrate. The Bacillus subtilus and calf thymus enzymes closely resemble the E. coli enzyme in molecular weight and properties (Talpaert-Borle et al., 1979 and Cone et al., 1977). E. coli mutants (ung mutants) have been isolated (Duncan et al., 1978) which have low or nonexistent levels of uracil-DNA-glycosylase. Ung mutants are viable, hypermutable, and have uracil in their DNA (Duncan et al., 1978). Another type of mutant, dut, deficient in dUTPase also has uracil in its DNA, presumably because dUTP levels are higher in this mutant, and because the DNA polymerase discriminates poorly between dUTP and dTTP. Dut, ung double mutants maintain high uracil levels in their DNA and are still viable (Tye et al., 1978). However, dut, xth mutants, deficient in

the excising enzyme, are nonviable presumably because they accumulate at aprymidinic sites which cannot be repaired via excision repair. Tye et al. (1978) report no uracil in the DNA of wild-type cells. As uracil-DNA-glycosylase levels are known to increase in some cells just before DNA synthesis (Sirover, 1979; Gupta & Sirover, 1980; Gupta & Sirover, 1981) it may be that levels are low in nondividing somatic tissue and uracil may accumulate in DNA.

D. Conclusion

When one considers that there exists a specific uracil-DNA-glycosylase, it is clear that uracil in DNA, in the absence of this enzyme, would pose a serious threat to this cell's survival. One can then ask the question whether these microlesions or point mutations accumulate to any serious extent over the lifetime of the cell, considering the existing repair processes and whether they are, in fact, lesions that limit the cell's survival. These are changes in the genetic information and they can cause amino acid substitutions and subtle changes in proteins. Repair of these lesions is dependent on excision repair enzymes, whose levels are known to correlate with lifespan. E. coli mutants deficient in this enzyme are hypermutable and have uracil in their DNA and, most important, at normal body temperature these lesions occur naturally at a fixed rate. The deamination of cytosine to uracil may be one of the most common types of DNA damage which occurs. This type of damage could lead to spontaneous point mutations, distortions of the double helix, or epigenetic changes affecting the regulation and processing of information. DNA repair processes in nondividing somatic tissues may not be able to correct all this damage. The research described here

provides a simple means of testing whether or not these lesions accumulate with age.

E. 5-Hydroxymethyldeoxyuridine

In addition to measuring deoxyuridine levels in this thesis, dHMU was also examined, since it was found to elute at a position where it could be detected in chromatograms of DNA digests. Details on its biology are given in the experimental section.

II. EXPERIMENTAL CONSIDERATIONS

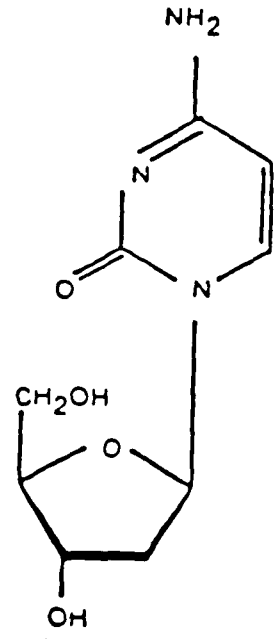
A. The Test

The structures of the pertinent deoxyribonucleosides are given in Figure 1. Uracil created in DNA is paired incorrectly with guanine. If it is not excised before DNA replication it will pair with adenine in the newly replicated DNA and a mutation results. HMU is also believed to be mutagenic, since a repair system exists for it. It can be estimated that if 20 uracils went unrepaired per week in the mouse there would be a mutation in 1 out of 32 genes by 3 years of age, and in the human 1 in every 1.4 genes by age 70.

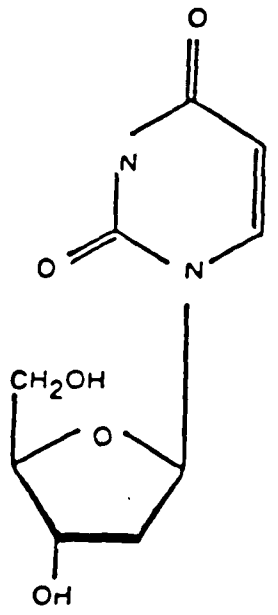
This raises the interesting question of whether or not uracil or HMU can be found in the DNA of old cells. Since Sirover (1979) reports that uracil-DNA-glycosylase is induced several fold during DNA synthesis in human lymphocytes, it might not be possible to measure any uracil in cell lineages that undergo replacement and replication, such as intestinal mucosa, but it may be possible in nondividing tissues such as brain and liver.

B. Experimental Design

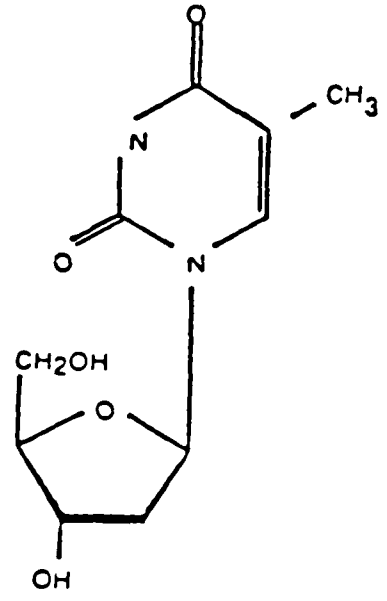
Experiments were conducted using digests of DNA from mouse tissues and from commercial samples. These digests included alkaline phosphatase, phosphodiesterase I, DNAase I, and an inhibitor of what was believed to be a trace contaminant of the alkaline phosphatase, cytidine deaminase. It was originally proposed to measure uracil by



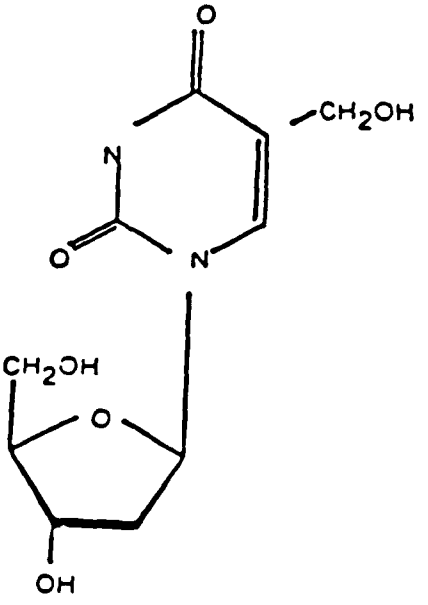
DEOXYCYTIDINE



DEOXYURIDINE



THYMIDINE



5-HYDROXYMETHYLDEOXYURIDINE

Figure 1: Structures

treating DNA with uracil-DNA-glycosylase and measuring released uracil. However, it was found to be possible to measure nucleosides in DNA digests without the use of uracil-DNA-glycosylase. With the use of triethylamine in the buffer it was possible to separate cleanly deoxyuridine and dHMU from any of the other bases that were analyzed in the DNA digests. This is done using high pressure liquid chromotography. A pH of 3.0 for the buffer was chosen because it provides the best separation of deoxycytidine from deoxyuridine. This pH is below the pK of the amino group on deoxycytidine and thus it is positively charged and very polar. The following paper, submitted to the journal Mechanisms in Aging and Development, reviews the experiments conducted on mouse DNA and summarizes the results.

C. Absence of Deoxyuridine and 5-hydroxymethyldeoxyuridine in the DNA from Three Tissues of Mice of Different Ages

INTRODUCTION

In view of the result that DNA excision capacity correlates with lifespan (Hart & Setlow, 1974), aging may result from the accumulation of genetic damage in somatic cells. There is evidence that DNA repair processes decline in late passage cells (Hartman, 1983; Failla, 1960; Hart et al., 1979), and that unscheduled DNA synthesis declines in somatic tissue upon terminal differentiation (Hart et al., 1979). There is also evidence that during aging in the rat unscheduled DNA synthesis declines rapidly in hepatocytes (Kennah et al., 1985). In addition, other types of DNA repair in various tissues are low in old rats (Niedermuller et al., 1985). In this study we examined two possible types of DNA damage, dU and dHMU, during the aging process.

dU and dHMU in DNA are the products of deamination of deoxycytidine and oxidation of thymidine, respectively. If mutational damage increases during aging, these two compounds would be suspect in DNA samples from old mice. It has been estimated (Hartman, 1980) that about 160 deamination events of deoxycytidine occur in DNA in the nucleus of a diploid mammalian cell at 37°C per day. It can be calculated that if two of these went unrepaired per cell per week, then by age 70 there would be a mutation in one out of 14 genes (assuming there are 100,000 genes in a diploid nucleus). It also has been estimated from the levels found in urine (Ames & Saul, 1985) that the rat excretes one molecule of HMU per 16 genes per day. Mattern and Cerutti (1975) have found that late passage WI38 cells lose the ability to excise gamma-ray induced thymidine products in DNA. This gamma-ray induced damage includes dHMU.

The two compounds, dU and dHMU, if incompletely repaired, should be detectable in cellular DNA by sensitive assays. We therefore used a sensitive HPLC assay to look for the presence of dU and dHMU in enzyme hydrolysates of DNA from the tissues of mice of various ages.

MATERIALS AND METHODS

Chemicals

Deoxyuridine was purchased from Aldrich Chemical Co. Other deoxynucleosides and ribosides as well as alkaline phosphatase (calf intestine-type XXX) were purchased from Sigma Chemical Co. Triethylamine (HPLC grade) was purchased from Fisher Scientific and was stored under nitrogen to prevent oxidation. Deoxyribonuclease I (DPFF) and venom diesterase (VPH) were purchased from Worthington Biochemical Corp. Tetrahydrouridine was purchased from Behring

Diagnostics.

DNA extraction

DNA was extracted from liver, brain, and small intestinal mucosa tissues from C57BL/6J male mice housed in the animal facility at the Gerontology Research Center. Tissues were removed, weighed, and minced in ice-cold 0.15 M NaCl, 0.1 M EDTA buffer, pH 8.0, washed extensively, and then homogenized by five full passes with a close-clearance (0.01 inch) teflon homogenizer. The homogenate was incubated with pronase B, nuclease-free, at 500 micro g/ml for 3 hours at 40°C and then brought to 1 M $NaClO_4$. Deproteinization was achieved with an equal volume of chloroform-isoamyl alcohol (20:1) and vigorous shaking of the lysate for 30 minutes. This was followed by centrifugation at 10,000 RPM, 25°C for 15 minutes. The top phase was carefully removed and the DNA was spooled onto glass rods after addition of 2 volumes of ethanol at -20°C. DNA was dissolved in 0.1 x SSC (0.15 M NaCl, 0.0015 M NaCitrate) and then brought to 1 x SSC. RNAase was added at 50 micro g/ml, followed by a 1-hour incubation. Nuclease-free pronase B was added to 50 micro g/ml, followed by another 1-hour incubation. The mixture was then extracted with an equal volume of phenol, centrifuged at 10,000 RPM at 25°C for 15 minutes, followed by a chloroform-isoamyl extraction step and centrifugation at 10,000 RPM for 15 minutes. The top phase was removed and added to 2 volumes cold ethanol. The DNA was centrifuged at 12,000 RPM at 4°C for 20 minutes and the pellet dissolved in 0.01 x SSC. The absorbance at 260 and 280 nm was measured and the concentration of DNA determined, using 1 OD at 260 as 50 micro g/ml DNA. A typical 260/280 ratio was 1.90.

DNA digestion

DNA from liver, brain, or mucosa of C57/BL6 mice of different ages was lyophilyzed, weighed, and dissolved in 100mM Tris 50mM $MgSo_4$ pH 8.0 (digestion buffer) to 1.0 mg/ml by stirring in the cold. In digestions 2.0 ml (for liver and mucosa) and 0.5 ml (for brain) of DNA was used. The DNA was mixed with 8×10^{-7} moles of tetrahydrouridine (see results), 2 units of phosphodiesterase I, 200 units of DNAase I, and 9 units of calf intestine alkaline phosphatase to a total volume of 2.5 ml in digestion buffer for liver and mucosa, 1.0 ml for brain. Incubation was carried out at 37°C for 9 hours, and the reaction was stopped by addition of 1/2 volume methanol. The precipitate was removed by centrifugation and samples dessicated or lyophilized and stored at -20°C.

HPLC Analysis

A Waters Model 510 HPLC pump equipped with a model 710 WISP automatic sample injector, model 440 UV detector and model 730 Data Module was used. The running buffer was 0.5% triethylamine pH 3.0 with phosphoric acid, which causes large retention time for the nucleosides and cleanly separates dU and dHMU from the common deoxyribonucleosides. Samples were dissolved in 0.3 ml running buffer and filtered through Millipore 0.45 micron filters. 100 microliters of each sample was injected. The column used was a Waters micro bondapak C_{18} column fitted with a Waters C_{18} Guard Pak precolumn. Absorbance was monitored at 254 nm at the highest sensitivity scale (0.005 AUFS). The flow rate was 1.0 ml/minute with a 1.0 hour. equilibration time.

RESULTS

E. coli alkaline phosphatase preparations cannot be used for DNA digestions of the type reported here because they contain significant cytidine deaminase activity that generates dU upon incubation of DNA digests. In contrast, the calf intestine alkaline phosphatase preparation used here was found to contain minimal cytidine deaminase activity. In the presence of tetrahydrouridine, a potent inhibitor of cytidine deaminase (Wentworth & Wolfenden, 1978), this contaminating activity was completely eliminated. This allowed us a sensitive assay for the occurrence of dU in DNA digests of mouse DNA. We simultaneously examined our HPLC profiles of enzyme hydrolyzed DNA (see Methods) for the presence of dHMU.

Neither dU nor dHMU was detected in any of 12 DNA samples prepared from mouse embryos and from brain, liver, and small intestinal mucosa of animals of various ages (Table 1). The Table (last column) gives an indication of the sensitivity of the test for each sample. For example (top line), embryo DNA is estimated to contain less than one molecule of dHMU and less than one molecule of dU for every 76,000 deoxynucleotides in the sample.

The samples, as judged by the peak height of ribosides in the chromatograms, contained up to 30% RNA. However, none of the ribosides coelute with dU or dHMU; since the presence of RNA was judged not to interfere with the assays, the DNA was not further purified.

A chromatogram of 19 month small intestinal mucosal DNA spiked with 25 picomoles each of dU and dHMU is shown in Figure 2A. 19 month mucosa, unspiked, has no discernable dU or dHMU peak (data not shown).

TABLE I

SENSITIVITY OF THE HPLC ASSAY FOR DETECTION OF dU AND dHMU IN MOUSE SAMPLES[a]

DNA	Age (months)	micro moles deoxycytidine[b]	micro moles deoxyribo-nucleotides	Sensitivity: moles deoxyribonucleotides in DNA per mole of dU or dHMU[c]
Embryo		0.47	1.9	76,000
Brain	7	0.065	0.26	10,400
	11	0.12	0.49	19,600
	19	0.025	0.10	4,000
	31	0.10	0.40	16,400
Liver	7	0.05	0.24	9.600
	11	0.20	0.80	32,000
	19	0.12	0.48	19,200
	31	0.28	1.10	44,000
Mucosa	11	0.14	0.56	22,400
	19	0.14	0.56	22,400
	31	0.13	0.50	20,000

[a]Based on one injection of each sample.

[b]Amount injected in sample as deduced from peak height in diluted samples.

[c]Assuming that 25 picomoles is the limit of detection.

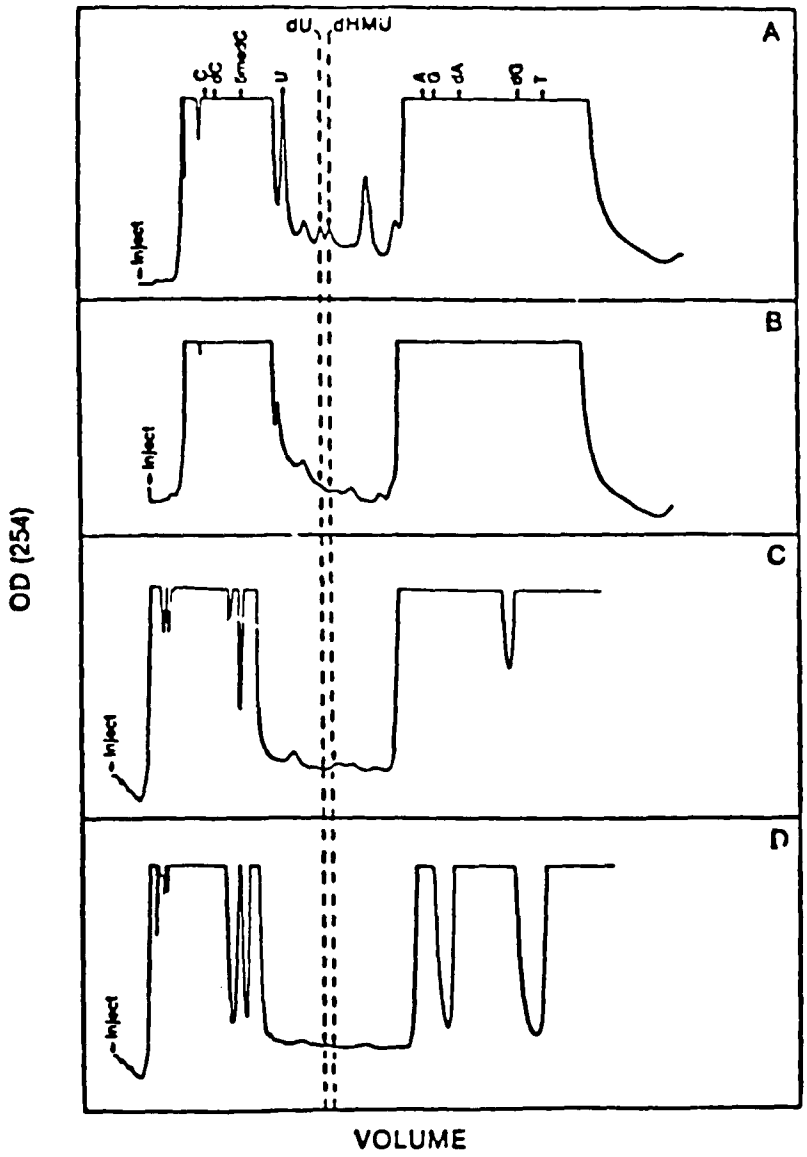


Figure 2: HPLC chromatograms of mouse tissue DNA digests. (A) 19 month small intestinal mucosa spiked with 25 picomoles each of dU and dHMU; (B) 31 month small intestinal mucosa; (C) 31 month liver; (D) 31 month brain; Full scale is 0.005 absorbance units. dU and dHMU elute between 13-15 ml as indicated by the dotted line. Abbreviations: dC, deoxycytidine; C, cytidine; A, adenosine; dA, deoxyadenosine; G, guanosine; dG, deoxyguanosine: T, thymidine; 5medC, 5-methyldeoxycytidine; U, uridine.

It is clear from this chromatograph that 25 picomoles of either compound are easily detected, and this value was used as the limiting amount in the calculations generated in Table I. Twenty-five picomoles of dU added to DNA digests are completely recovered. This excludes the possibility that du was present in the DNA and was modified to another compound. Figures 2B, C, and D are chromatograms of DNA digests from small intestinal mucosa, liver and brain, respectively, of 31 month old mice. Although there are several peaks that remain unidentified in these chromatograms, no significant amount of ultraviolet absorbing material was detected at retention times characteristic of added dU and dHMU.

DISCUSSION

dU and dHMU in DNA are repaired by glycosylases (Lindahl, 1979; Hollstein et al., 1984). The presence of dU and dHMU in DNA may pose a threat to the integrity of the genetic information in somatic cells. Their absence in the described experiments indicates that although they may be present at levels below the detectability in these experiments, neither dU nor dHMU is present in high levels. If it is assumed that the coding region of a typical gene is 1,000 base pairs and that any dU or dHMU in the DNA is randomly distributed, then one lesion may have occurred per 38 genes and not have been detected in embryonic DNA. In DNA from 31 month old liver, where the sensitivity of the assay is lower, one lesion out of 22 genes may have occurred and escaped detection. This is a very conservative estimate. By comparison of Figures 2B, C, and D with Figure 2A, it is clear that the amount of altered base does not even approach the 25 picomoles set

as an arbitrary limit of detection.

The absence of dU and dHMU in these DNA samples implies that DNA repair must be very efficient. Although there may exist areas of chromatin which are relatively inaccessible to DNA repair enzymes (Hart et al., 1979; Yielding, 1974) our results in which total DNA was assayed indicate that neither dU nor dHMU accumulates substantially in any major portions of the genetic material. It is interesting to note that Green and Deutsch (1984) found no dU in Drosophila DNA, even in DNA isolated from stages when there is no apparent uracil repair enzyme. Weiss et al. (1983) also found no dU in DNA from aging human brain, although their method was much less sensitive than the one presented here.

Although no dHMU was found in the mouse tissues studied, a small amount of dHMU (about 1 molecule per 100,000 bases) was detected in chick erythrocyte DNA as a peak that coeluted with added dHMU. This DNA sample, purchased from California Biochemicals, may not be representative of native avain erythrocyte DNA in situ; for example, the DNA sample may have suffered metal ion catalyzed oxidative DNA damage during work-up. On the other hand, it is possible that nucleated erythrocytes are deficient in release of dHMU from DNA or suffer excessive amounts of oxidative damage in vivo.

D. Materials and Methods

Chemicals

Salmon testes DNA and chick erythrocyte DNA were purchased from P.L. Biochemicals. Another sample of chick erythrocyte DNA was purchased from California Biochemicals. Herring testes DNA and calf

thymus DNA were purchased from Sigma. For the origin of other reagents see Section C.

HPLC

A Waters HPLC system equipped with a data module was used. In these experiments calibration was performed by injecting standards, the data module automatically reads peak height vs elution volume of standards. Standards were injected separately on each day. The data module then, after a run, calculates the concentration of unknowns. Peak height is read using a baseline established by the data module. Peak height calibration was used as opposed to area because it seemed more reliable and reproduceable. A line is determined by reading the portion of the chromatogram preceding the peak, where the slope change vs time is negligible. In the case of multiple peaks the line is extrapolated from the last preceding "flat" portion of the curve and in the case of one peak superimposed on another the peak height is determined from the point of slope change. In some cases, where peaks are very small, no concentration measurement is made.

Purification of dU for mass spectometry

Material from a peak from chick erythrocyte DNA, that co-chromatographs (on a reverse phase column) with dU was collected, concentrated, and passed through an anion exchange column. dU is not retained on this column and elutes as a single peak within the void volume, whereas contaminating compounds are retained. This material was then passed through the reverse phase C_{18} column with pure H_2O. The collected and concentrated material was used for mass spectometry.

This work was performed using a digest that did not contain tetrahydrouridine. It was later shown that, although the material was

confirmed to be deoxyuridine, it was an artifact of the DNA digestion. However, it was established that the artifactual material eluting at this position was an authentic sample of dU. Along with mass spectometry UV spectral ratios of the purified material confirmed that the material was dU. Subsequent identification of dU in samples was made by elution volume.

DNA digestion

For details on the DNA digestion see Section C of this Chapter. Digestion was judged to be complete when no further increase was observed in the peak height of the four major bases on extended digestion with calf thymus DNA. Four hours seemed suitable for complete digestion. However, digestion had to be carried out for nine hours due to the presence of several slowly disappearing minor peaks. Their identity is unknown. They could be slowly digested dinucleotides and trinucleotides. The same time course of digestion was also demonstrated for chick erythrocyte DNA (CalBiochem) and nine hours of digestion time was used routinely.

E. Results

Initial tests were done with commercial DNA samples. Samples used were chick erythrocyte DNA from two different sources, calf thymus DNA, and herring and salmon testes DNA. Initially large amounts of deoxyuridine were found in chick erythrocyte DNA, samples purchased from California Biochemicals (as much as 1 deoxyuridine per 600 nucleosides was detected). This deoxyuridine was isolated in near homogeneity and examined on the mass spectrometer (Figure 3). From UV spectral ratios and mass spectrometric data the material was confirmed

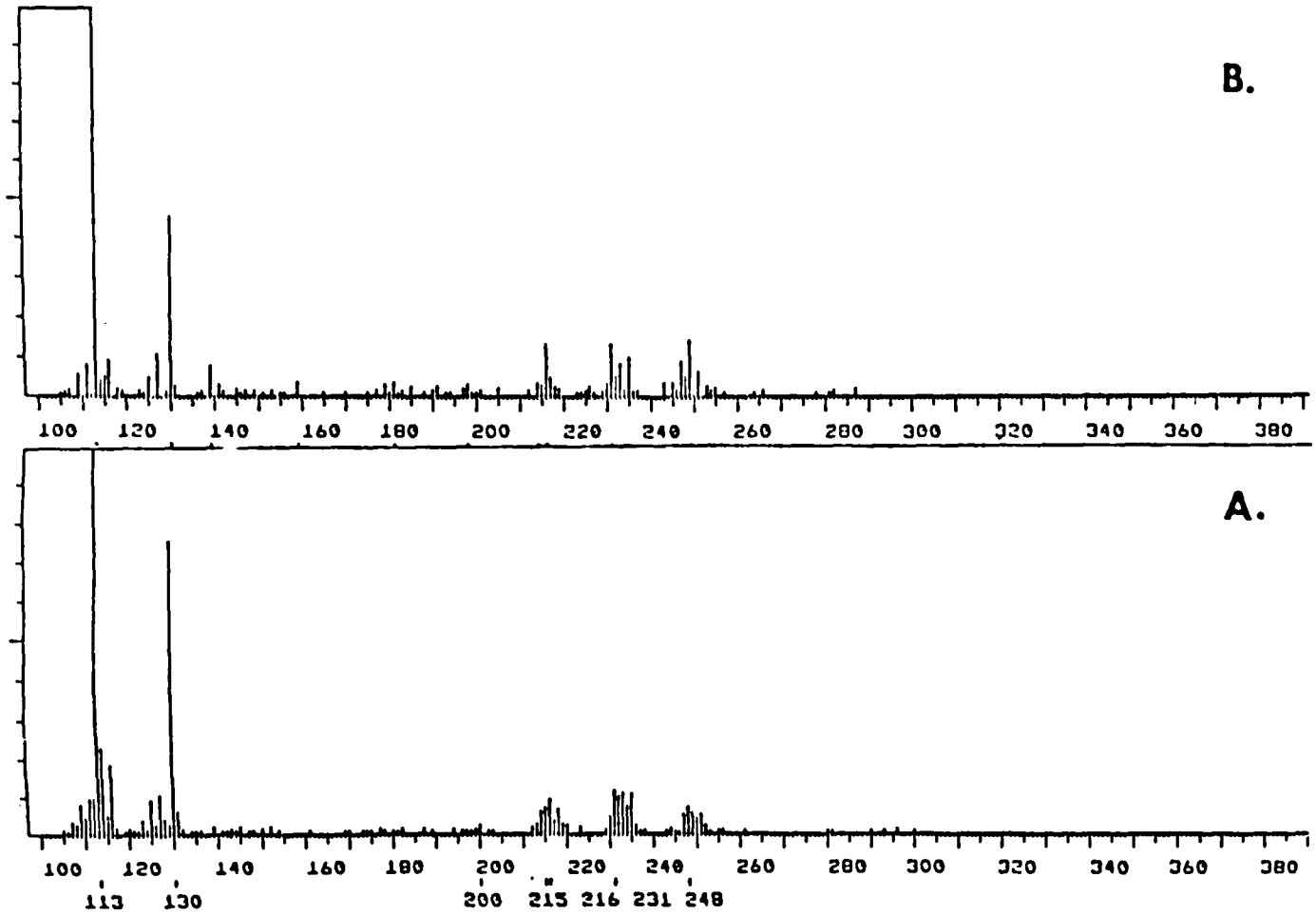


Figure 3: Chemical desorption ionization fragmentation pattern of deoxyuridine from chick erythrocyte, B, and control deoxyuridine, A. I acknowledge the chemistry department of Columbia University for the use of their facilities.

to be deoxyuridine. With extended digestions, however, increases in the ratio of dU to dC were detected (.75, initial, to 1.25, final, by peak height). These extended digestions involved recurrent additions of enzyme every 24 hours and the ratios given are those obtained with values determined between 24 and 74 hours. However, when the same DNA was dissolved and hydrolysed with new enzyme preparations much smaller amounts of deoxyuridine were detected. An enzyme contaminant of the alkaline phosphatase (cytidine deaminase) was suspected and an inhibitor of this enzyme, tetrahydrouridine (Wentworth & Wolfenden, 1978), was added. After tetrahydrouridine addition to the digestion near zero but detectable levels were found (17 to 32 picomoles). Extended digestions (18 hr.) showed a large dU peak with tetrahydrouridine (Figure 4). The large amount in the 18 hour digestion may be partially accounted for by the lability of tetrahydrouridine. In contrast, chick erythrocyte DNA from P.L. Pharmaceuticals had large amounts of dU upon digestion and analysis even in the presence of tetrahydrouridine (with a 9 hour digestion time) (Figure 5). Figure 6 shows the same digest spiked with dHMU. dHMU appears as a shoulder on the dU peak and is clearly distinct from dU. It was not possible to detect or measure dHMU in chick erythrocyte P.L. due to the large interfering dU peak. Salmon testes, herring testes, and calf thymus all had measurable dU peaks but little or no dHMU was detected; each analysis contained approximately 5mg of DNA, (one-third of the digest was loaded). Chick erythrocyte DNA (CalBio) was found to contain 36 picomoles of dHMU in runs containing 170 nanomoles of deoxycytidine. Figures 7, 8, and 9 show chick

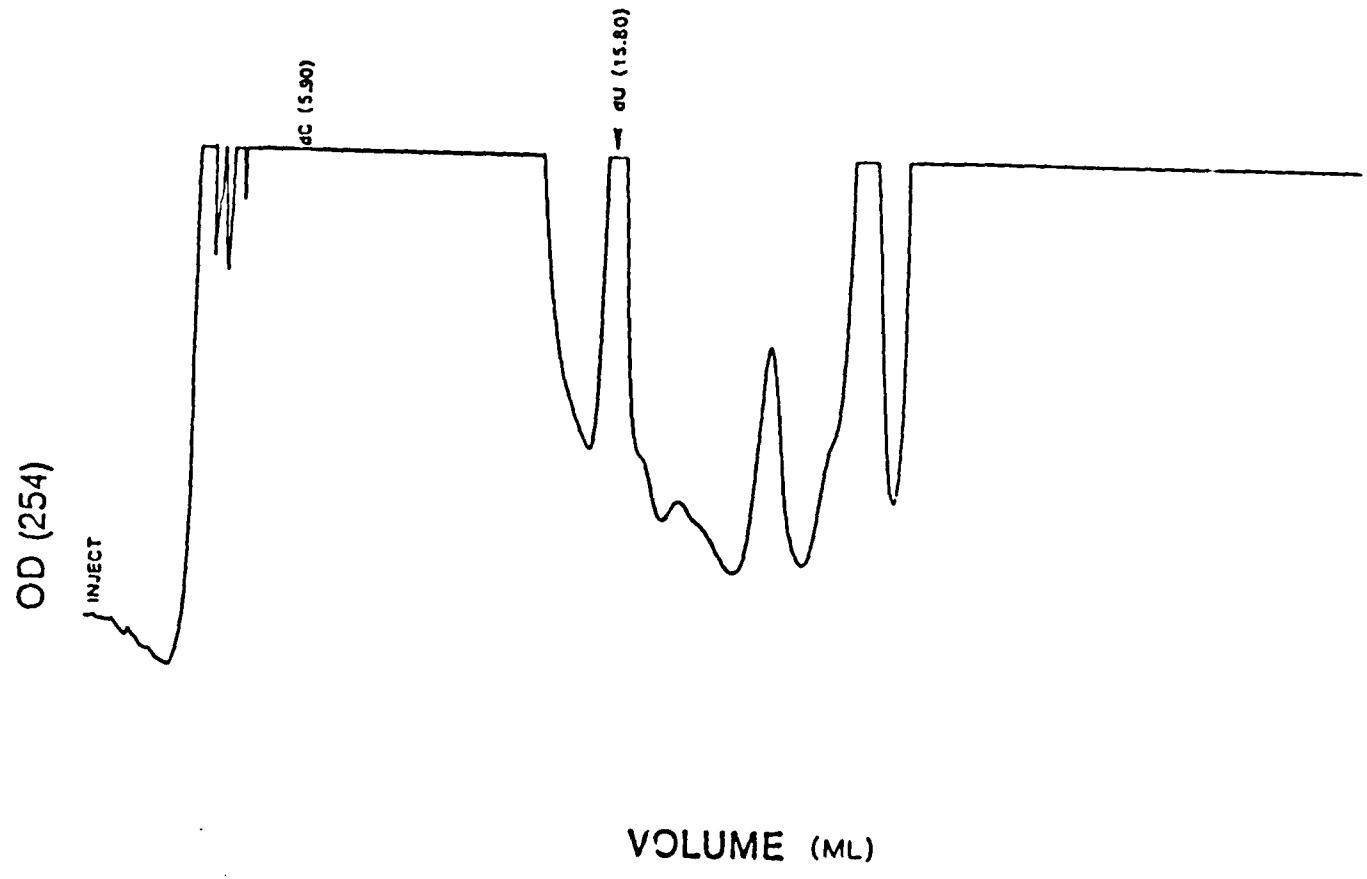


Figure 4: HPLC chromatogram of a chick erythrocyte DNA (CalBiochem) digest, digested for the extended time of 18 hr. Full scale on all chromatograms is 0.005 absorbance units (unless otherwise noted).

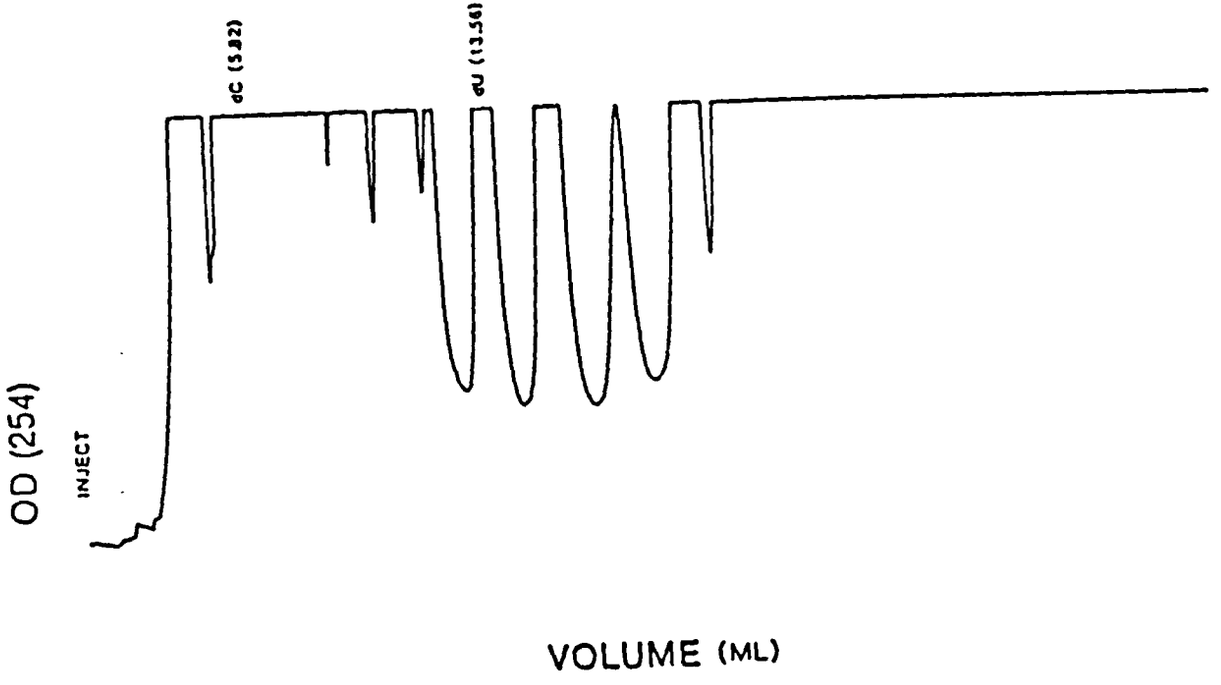


Figure 5: HPLC chromatogram of a chick erythrocyte DNA (P-L) digest (unspiked).

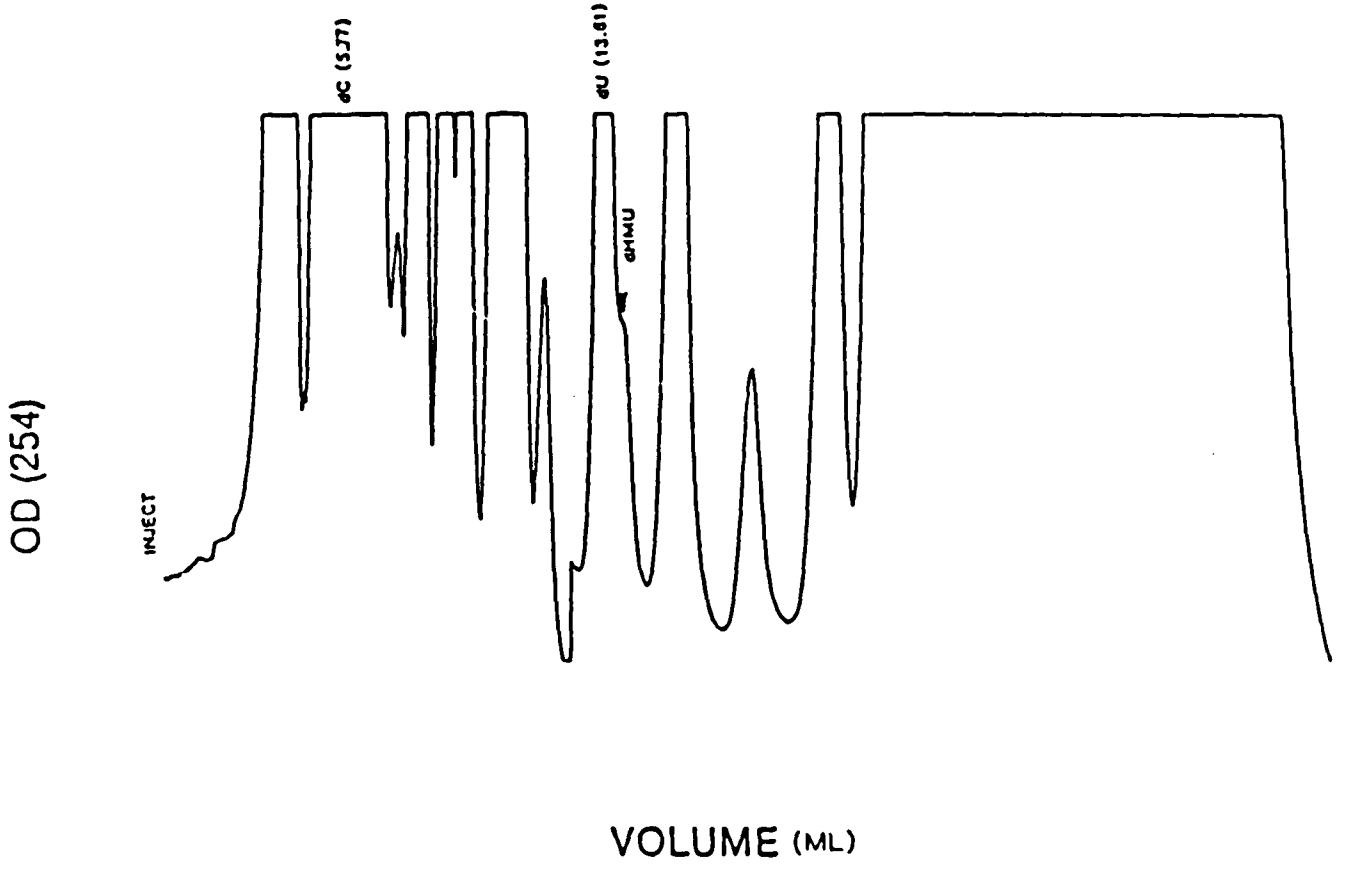


Figure 6: HPLC chromatogram of a chick erythrocyte DNA (P-L) digest spiked with dHMU.

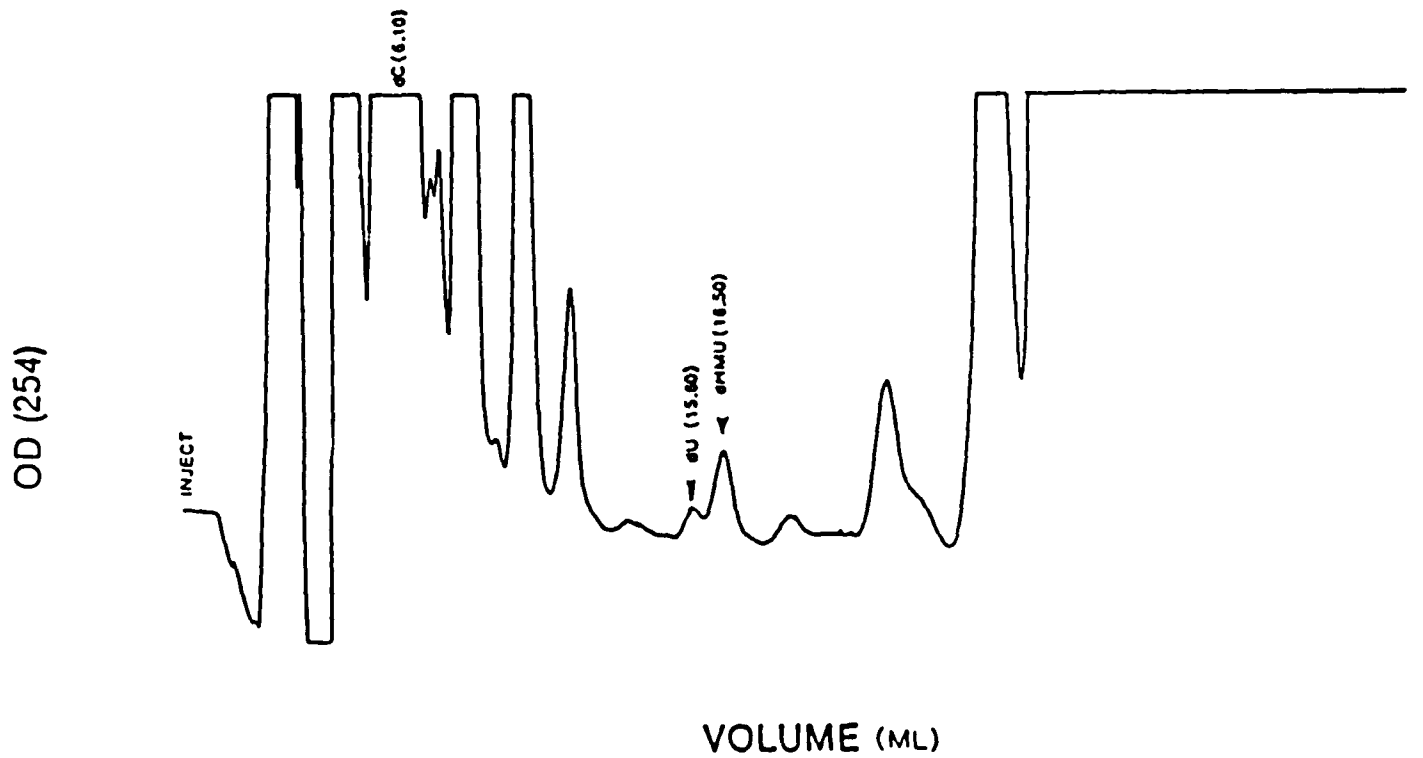


Figure 7: HPLC chromatogram of a chick erythrocyte DNA (CalBiochem) digest (unspiked).

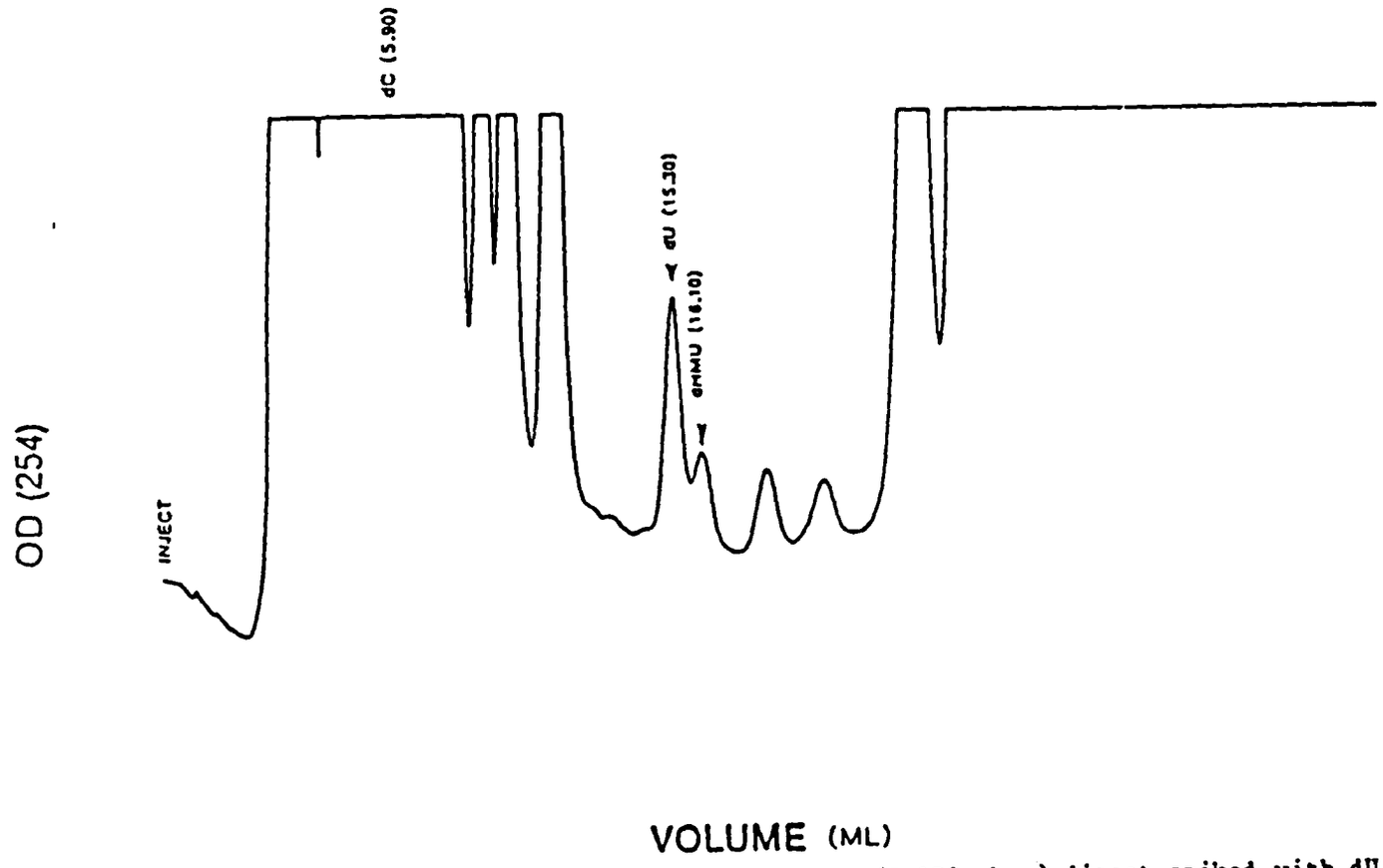


Figure 8: HPLC chromatogram of a chick erythrocyte DNA (CalBiochem) digest spiked with dU.

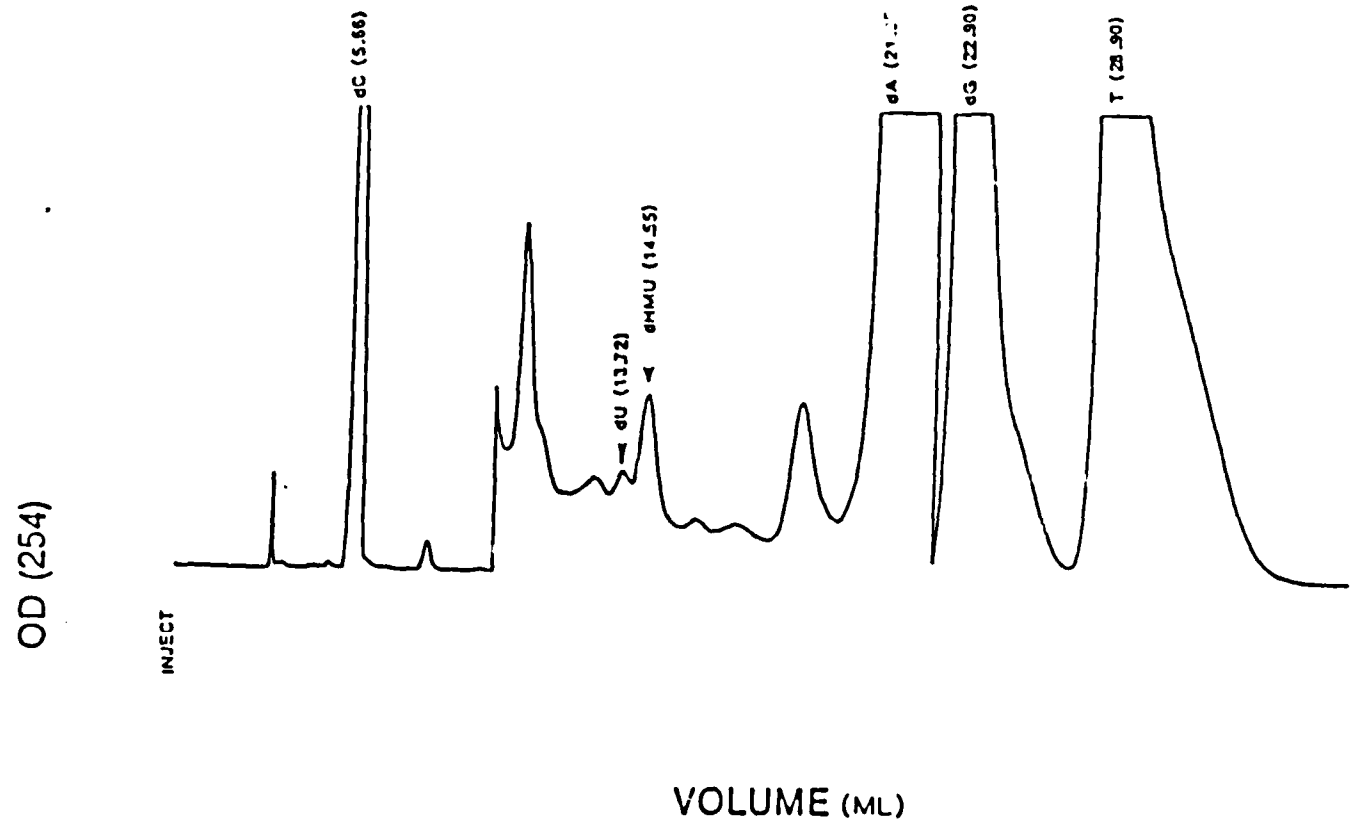


Figure 9: HPLC chromatogram of a chick erythrocyte DNA (CalBiochem) digest spiked with dHMU. Full scale is 2.0 absorbance units except for the central portion with dU and dHMU. Here the full scale absorbance is 0.005.

erythrocyte DNA digests (CalBio) chromatogrammed alone and spiked with either dU or dHMU. Figures 10, 11, and 12 show herring textes, salmon testes, and calf thymus digests. Table II gives the levels of dU found in each of the samples. There remain several unidentified peaks (Figures 5, 7, 10, 11, 12), but a dU peak is detectable in all the chromatograms and salmon testes as well as chick erythrocyte (CalBiochem) appear to have a slight dHMU peak. I believe that the dU in these chromatograms is an artifact of digestion since levels are exceedingly small and increase significantly in extended digestions. Small amounts of dU (as determined by elution volume) can always be seen in controls and when tetrahydrouridine is omitted levels increase substantially. The levels in controls greatly exceed values in samples. This may be possible since during the digestion very little dC is accessible to enzymes until the digestion has progressed. The presence of other major bases may also interfere with cytidine deaminase. However, chick erythrocyte purchased from P.L. appears to contain deoxyuridine in high levels. The inert chick erythrocyte nucleus may be able to tolerate mutagenic components. Alternately the treatment of DNA during commerical preparation may have generated uracil. In addition, very little was found in the chick erythrocyte preparation from CalBiochem.

Mouse DNA--C57/BL6 mouse DNA was isolated from liver, brain, and intestinal mucosa of mice of either 7, 11, 19, or 31 months old. Embryo DNA was also isolated. When digestion was conducted in the presence of tetrahydrouridine, it was shown not to contain detectable levels of either dU or dHMU (Table 1), but did contain large amounts of RNA (as much as 30%). Several large peaks remain unidentified.

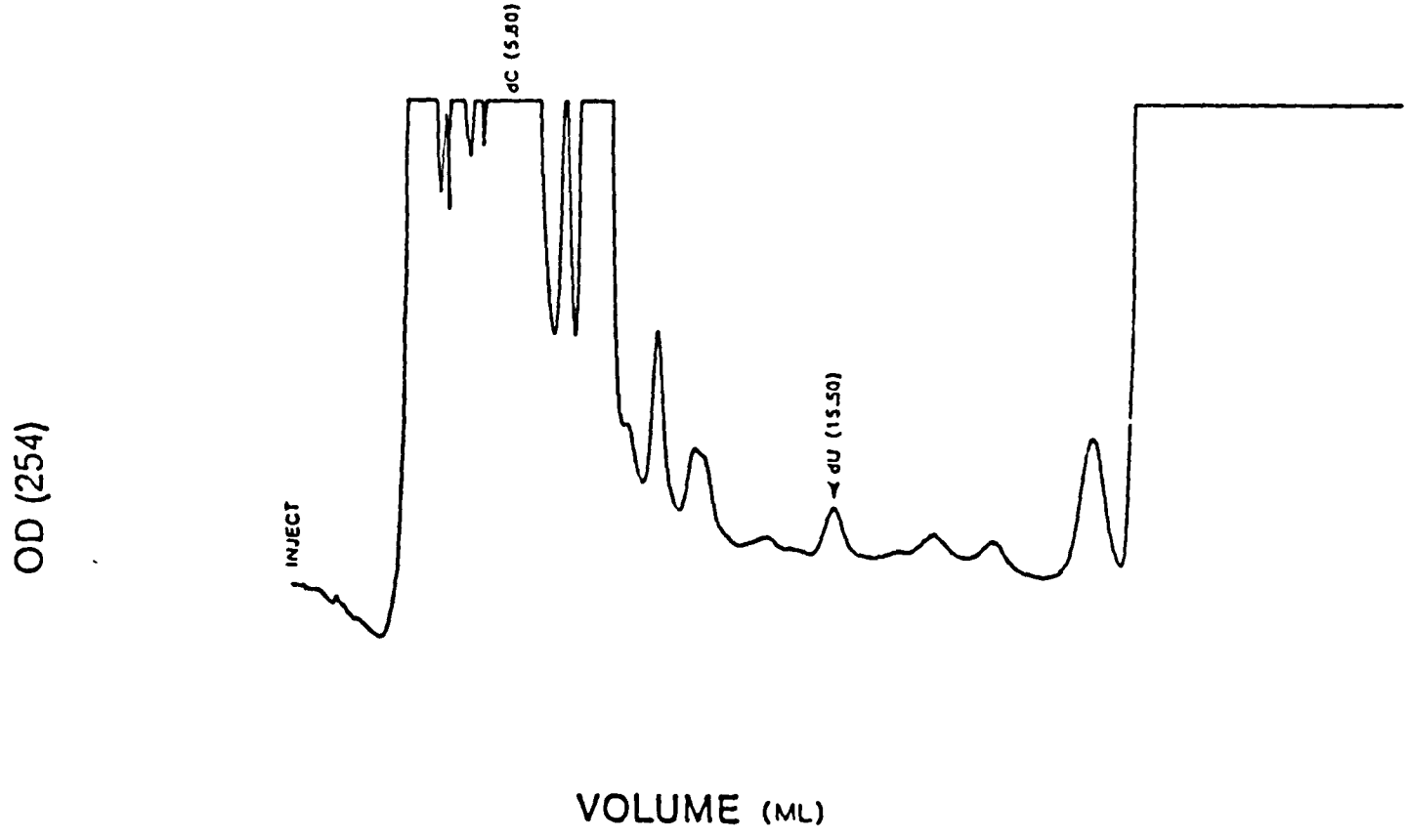


Figure 10: HPLC chromatogram of a herring testes DNA digest.

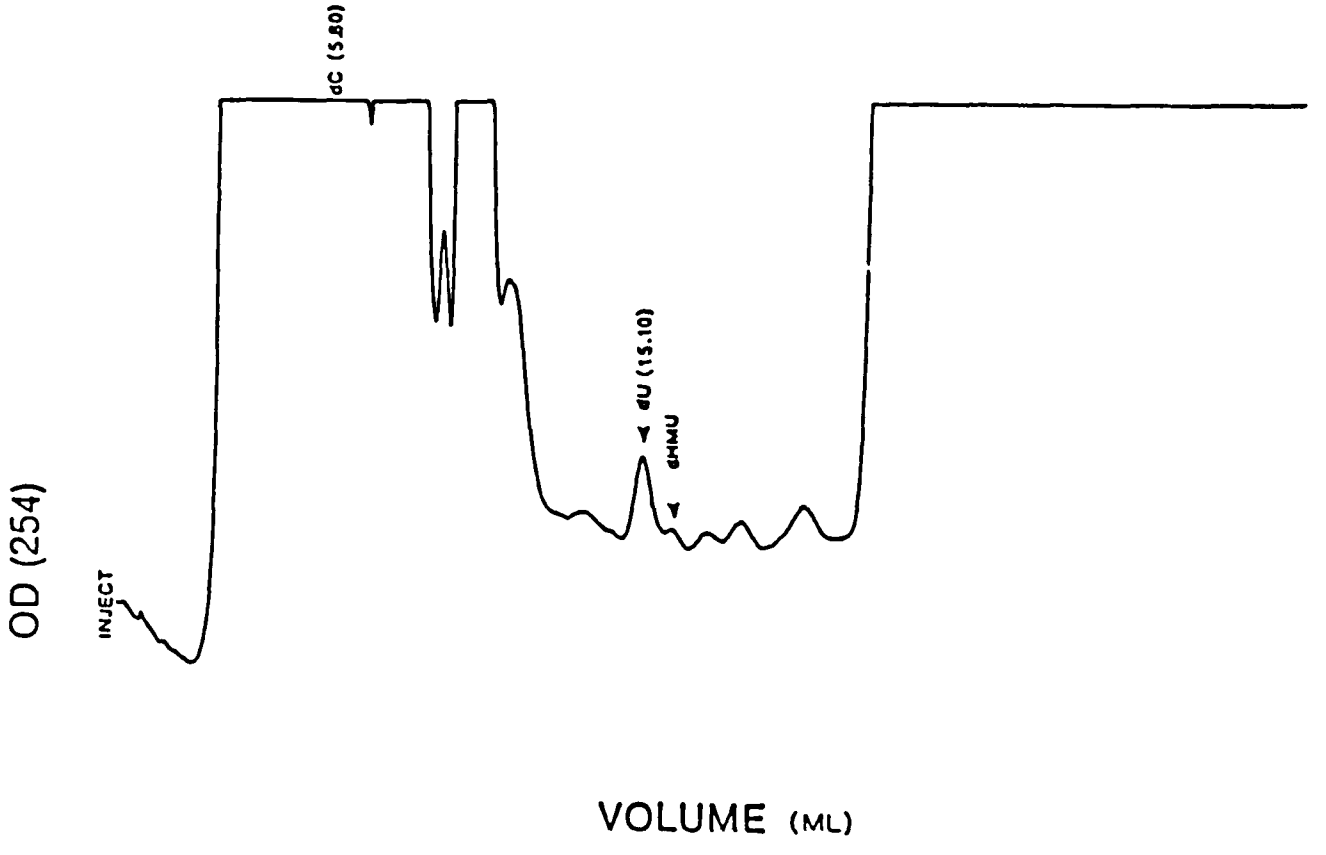


Figure 11: HPLC chromatogram of a salmon testes DNA digest.

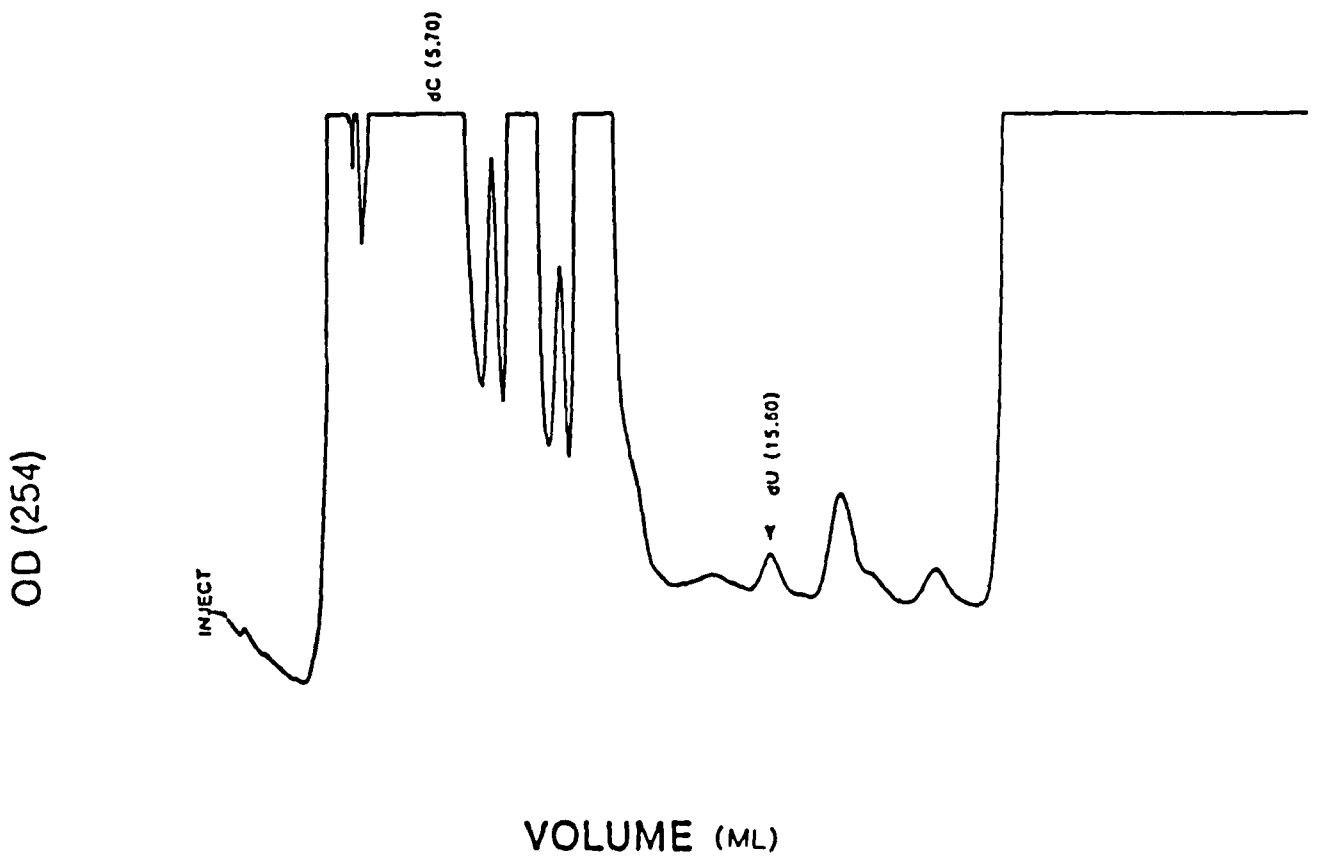


Figure 12: HPLC chromatogram of a calf thymus DNA digest.

Most of the major peaks could be ascribed to either DNA or RNA digestion products, and I believe that several of the unidentified peaks can be attributed to minor RNA bases.

F. Discussion

Enzymatic DNA digests appear to be complex as trace amounts of contaminating enzymes can generate minor components. In the experiments described, a contaminating cytidine deaminase activity appears to generate deoxyuridine at appreciable rates during digestion. However, some dU is found in samples of commercial DNA when a potent inhibitor of this enzymatic activity is included. The origin of the dU remains uncertain, but a possible explanation is the presence of another enzyme, deoxycytidalyte deaminase, for which an inhibitor, deoxytetrahydrouridine, was not included. However, levels are still small and increase substantially when inhibition is excluded. Secondarily, the treatment and isolation procedures of commercial DNA are not known. The absence of any detectable deoxyuridine in mouse DNA implies that the inhibitor does work. Small amounts of deoxyuridine in controls that use fresh frozen deoxycytidine with all of the standard enzymes and tetrahydrouridine implies that there is indeed some "leakiness" of dC to dU in these experiments. Controls were typically run with 100 nanomoles of dC and the levels of dU found were unmeasurable but visible (Figure 13). As given in Table II there was substantial generation of dU in controls--at levels greater than found in the mouse DNA digests. Mouse DNA samples contained about 100 nanomoles of dC (range was 50 to 280) and no dU was detected although there were some slight peaks on the

TABLE II

LEVELS OF d'' IN COMMERCIAL DNA DIGESTS[a]

DNA source	picomoles dU[b]	moles deoxyribonucleosides[c] / moles dU	Figure #
chick erythrocyte 18 hr. digestion (CalBiochem)	858	5,880	4
chick erythrocyte (P-L) 9 hr.	916	5,480	5
chick erythrocyte (CalBiochem) 9 hr.	17	307,000	7
herring testes 9 hr.	22	235,000	10
salmon testes 9 hr.	32	160,000	11
calf thymus 9 hr.	26	190,500	12
control: dC plus enzymes 9 hr.	12[d]	11,250	13

[a]All digestions were carried out in the presence of tetrahydrouridine

[b]Determined automatically by peak height

[c]Based on the weight of DNA added to digests

[d]Determined manually by peak height

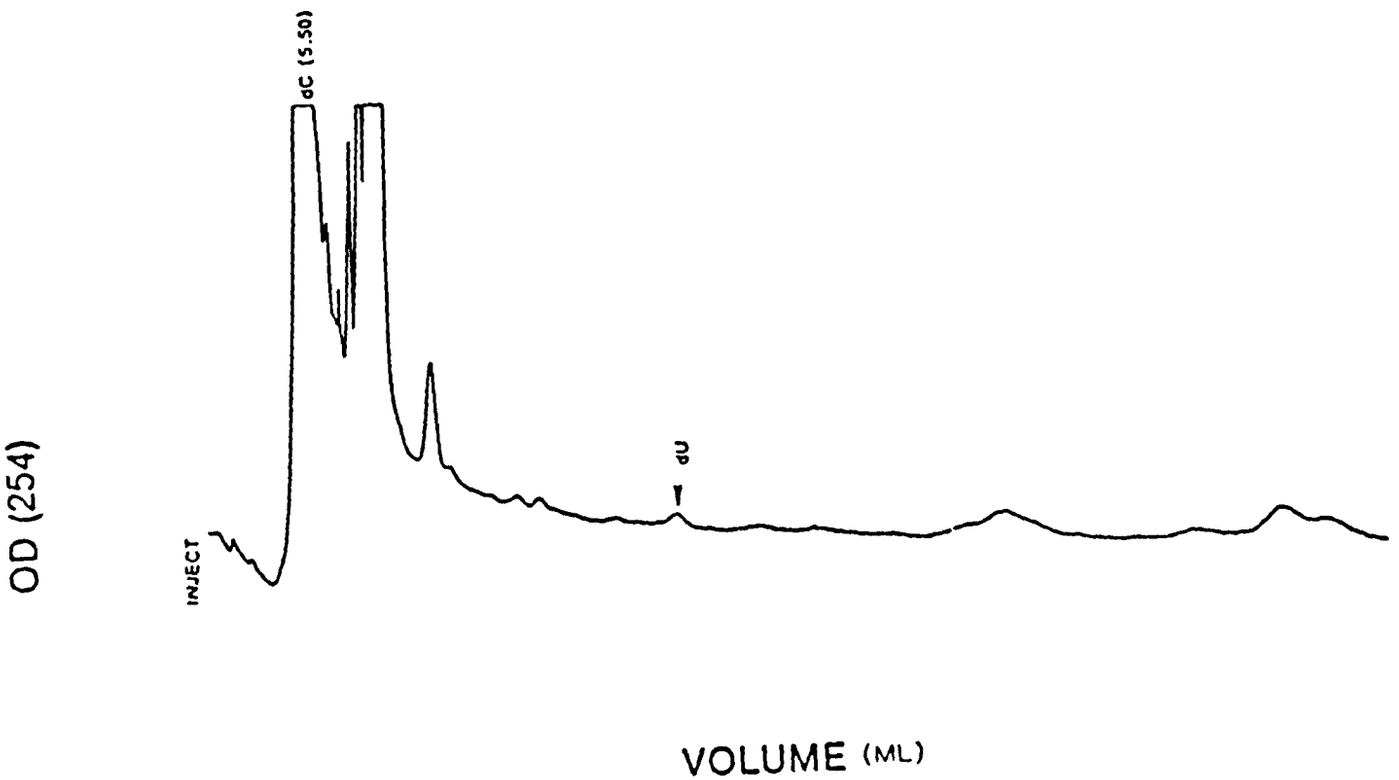


Figure 13: HPLC chromatogram of dC incubated with DNA digestion enzymes and tetrahydrouridine.

chromatograms. This implies that any significant contribution from the DNA itself would have been clearly distinguished from background levels and the amount found is indeed close to zero. Five milligrams of commercial DNA was used in each assay and the amount of dC in each sample was 3.0 micromoles, 30 times the amount in the mouse samples. It would be expected that some deoxyuridine would be generated. In fact, chick erythrocyte DNA (CalBiochem) on 18 hours of digestion has a very large deoxyuridine peak (Figure 4) as compared to 9 hours. This background generation of deoxyuridine poses a significant problem to the interpretation of results of experiments where dU was detected following DNA digestion. It can be said, however, that the levels in mouse DNA are near zero in these experiments and that there may be some deoxyuridine in chick erythrocyte DNA calf thymus, salmon and herring testes DNA, but only chick erythrocyte and possibly salmon testes were found to contain dHMU.

There remain several unidentified peaks in all the chromatograms. A possible source of future research might be the identification of some of these peaks and their correlation (if any) with the aging process. The presence of dHMU in chick erythrocyte DNA indicates that possibly nucleated erythrocytes are deficient in the repair of this lesion. It is also possible that the amount of dHMU found was generated during workup by oxidation caused by metal ions in the preparation.

III. 5-METHYLDEOXYCYTIDINE IN DNA SAMPLES

A. Introduction

5-methyl 2' deoxycytidine (5-methyl dC) in DNA is believed to control gene expression through an unknown mechanism. Tumor cells seem to be deficient in 5-methyl dC, and it has been reported that methylation levels decrease with aging (Hoopes, 1985). It also has been reported that DNAase I sensitive sites (the sites of putative, active genes) are hypomethylated with respect to total DNA (Naveh-Many & Cedar, 1981). Also, 5 azacytidine, when incorporated in the DNA and which cannot be methylated seems to activate some genes. These reports seem crucial to the assignment of a regulatory role to DNA methylation, but not all methylations appear to turn off genes, and it seems that specific sites are involved. It is believed that 5-methyl dC, located on the five prime end of housekeeping genes in sequences GGCC, represent key control elements. Tissue specific sites of methylation also have been reported (Vanyushin et al., 1973). Since 5-methyl dC in DNA is found in daughter strands at positions where it was present in the parental DNA (Doefler, 1983), there must be a control of methylation and of demethylation at particular sites.

The levels of 5-methyl dC have been measured mostly with restriction enzymes that recognize the sequence CCGG (Hoopes, 1985). It has been reported that levels decrease in oncogenic tissues and

during aging. It is also believed that dC in sequences CGCC represent only 6% of the CG sequences in the DNA. Levels have not been measured in toto during the aging process by the accurate method of HPLC.

B. Method

5 Methyl dC was quantitated by measuring the peak height at its elution position and comparing it to standards injected prior to the run. Values in Table II were derived from diluted samples (1:6) using the same triethylamine buffer as used to analyze dU and dHMU. Mucosal values were derived from undiluted samples.

C. Results

5 methyl 2' deoxycytidine (5-methyl dC) has been cited as the regulatory agent in gene expression (Wilson & Jones, 1983; Hoopes, 1985). It has been reported that levels decrease during aging and that they are very high in the embryo. My data show (Table III) that levels do indeed decrease between 7 months and 31 months in liver and brain, but not in mucosa, and that levels are not exceedingly high in the embryo. The levels found also do not either consistently rise or fall between embryo and death, the points are scattered but do follow the same fluctuations with age in liver and brain. In this regard in the DNA of cultured human fibroblasts methylation of GGCC sequences seems to have a variable pattern (Shmookler Reis & Goldstein, 1982). In studies of bovine thymus and heart 5-methyl dC fell with age in reiterated DNA sequences (Romanov & Vanyushin, 1981), but this was shown not to be the case in some tissues from young and old humans (Ehrlich et al., 1982). Cells cloned even in tissues from which total

TABLE III

5-METHYL 2' DEOXYCYTIDINE LEVELS IN DNA FOR MOUSE TISSUE OF DIFFERENT AGES*

Tissue	Nanomoles dC	Nanomoles 5 medc	5 medc/dC
Embryo	39.2	2.11	.053
Brain			
7 month	5.4	1.55	.29
11 month	10.0	0.63	.063
31 month	8.46	1.12	.13
Liver			
7 month	4.9	1.17	.24
11 month	16.75	1.12	.067
19 month	10.0	1.06	.10
31 month	22.9	1.4	.061
Small Intestine			
11 month	140	3.98	.028
19 month	138	2.91	.021
31 month	125	2.66	.021

*as determined by peak height with one injection of each sample.

DNA methylation declines show a range of high to low methylation patterns (Shmookler Reis & Goldstein, 1980, 1982). It thus seems that total methylation patterns mostly reflect heterogeneity from cell to cell within a tissue. It has also been reported that different organs have different methylation patterns in non-unique sequences and that with respect to age, changes in methylation patterns are tissue specific (Romanov & Vanyushin, 1981). Although most 5 methyl dC resides in GC sequences (Doefler, 1983), not all of these sequences may be crucial regulatory targets. Thus the measurement of total 5-methyl dC contents may not represent events occurring at age specific and developmentally critical sites.

Dr. Richard Cutler, who supplied the mouse DNA for these studies, determined 5-methyl dC levels in the same DNA by thin layer chromatography. We are in close agreement on the values for small intestinal mucosa. My values on the brain and liver, however, are exceedingly high and random compared to his. Literature values for percent of methylated cytosines range from about 2-3% (Hoopes, 1985) whereas my value for liver and brain greatly exceed these known values. It is possible that other UV-absorbing material in these samples has been coeluted with the 5-methyl dC in my studies. The mucosal values were determined on a separate day than those of brain and liver. The mucosal DNA may have been cleaner or, as it happens occasionally, elution patterns vary from day to day, resulting in better separations of the DNA digest from intestinal mucosa. Varying elution patterns do not affect the calibration, however, since the data module is recalibrated with fresh standards each time the HPLC is used.

IV. DISCUSSION

DNA is not an inert genetic element that is active only in its own replication and in the transcription of RNA. It is constantly undergoing changes, and rearrangements. From the damage it suffers due to its instability, DNA is consistently undergoing repair. The data presented in this paper indicate that the most suspected common type of DNA damage, cytosine deamination, is repaired throughout the lifespan of the mice studied. This occurs in each of the three different tissues examined. The same high efficiency of repair also seems to be true temporally for dHMU. This efficiency of repair, along with the stable status of DNA during aging with respect to these types of lesions, suggests that these types of lesions are not coherently involved in the aging process. However, it may be that development proceeds as long as the elements (proteins, lipids, etc.) that make up the cell can protect and endure in the threat of constantly incurring damage and much shorter lived species as compared to mice might have poorer repair systems. Within the sensitivity of my assays it seems that the mechanisms that protect the DNA from damage are sufficient to maintain the stability of the DNA complex. The type of damage studied, deamination, or other types such as oxidation, is not poorly repaired. On the other hand, it is possible that deoxyuridine was present in the samples from older animals but was beyond the limit of detection in these experiments (Given in

Table 1). For example, it is conceivable that dU or dHMU in only one in 500 genes could be extremely deleterious and lead to aging.

Some authors have attempted to find uracil in the DNA of various organisms, by methods other than the one described in this thesis. Weiss et al. (1983) used the DNA cloned from human cells of various ages. They sequenced the DNA and found no uracil. This method, however, is not very sensitive (a 340 base pair segment was examined) and the authors state that even 1% substitution of uracil for cytosine in this assay would have been barely detectable. Green and Deutsch (1984) used a radioassay to detect uracil in Drosophilia DNA isolated from organisms of several developmental stages. Their assay involved the digestion of DNA samples to 3' monophosphates and 5' end-labeling the monophosphates with ^{32}P and T4 polynucleotide kinase. The diphosphates were then converted to 5' end-labeled monophosphates by P1 nuclease digestion. This was followed by two-dimensional chromatography, autoradiography, and scintillation counting. dUMP was found only in bisulfite treated samples. None was found in Drosophilia DNA. The assay is very sensitive as it can detect 1×10^{-16} moles of dUMP. Although the assay is much more sensitive than the one described here, only 1-2 nanomoles of nucleotides were analyzed as compared to the 1-15 micromoles routinely used in my experiments. Their assay is still quite sensitive (one uracil in 10^7 bases can be detected) and yet even though Drosophilia contains no uracil-DNA-glycosylase and some stages of development are deficient in dUTPase, no uracil was found in their DNA samples.

Goulian et al. (1980) found no uracil in the DNA of a human

lymphoid line--less than one femtomole per micromole of DNA. However, when cells were treated with methotrexate which interferes with thymidylate synthetase (leading to increased levels of dUTP and subsequent incorporation into DNA), there is $\geq 10^3$ increase in the amount of uracil in DNA. In light of this fact, it is interesting that Luzzatto et al. (1981) found absurdly high levels of uracil--ranging from 10 to 75% of the cytosine residues in the DNA of bone marrow cells from patients suffering from megoblastic anemia. This disease is believed to result from a deficiency in folate, a deficiency which interferes in the conversion of dUMP to dTMP. However, they also found about 10% uracil per cytosines in the DNA of normal cells. The method involved the use of [^{3}H-uridine] as a tracer. The DNA was digested with the standard enzymes. This value of 10% substitution is erroneous compared to my results. It is possible that somehow their [^{3}H-uridine] was contaminated with [^{3}H-cytidine] which was deaminated by contaminating cytidine deaminase in their digestion mixtures.

If uracil is generated in DNA and remains unrepaired, two or several "hits" of the right gene or genes in the right cell in a tissue containing millions of cells could be all that is needed to start a malignancy or cause aging. Such levels of uracil would not be detectable by my experimental approach. It indeed might be possible that a cytidine deamination occurs, goes unrepaired, and is fixed in the DNA as thymidine in dividing cells. This may be especially crucial when a gene is "hit" before it is expressed during development. Are new genes expressed in old age? It is possible that

"old age" genes are sequestered in unrepaired regions during development. The fall in 5-methyl dC with age does suggest that new genes are being turned on. The transformation of deoxycytidine to dU may provide the mutational force for species and individual diversity and faulty repair systems in somatic cells may have deleterious effects on survival after the reproductive years.

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