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Preservation of DNA

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ABSTRACT

The preservation of DNA is of wide interest to scientists in disparate fields from biorepository management to pharmaceutical sciences. Although the term "preservation" is used by all these fields and refers to the maintenance of chemical and physical integrity of the DNA molecule, it should not be surprising that the perspectives of scientists from these distinct fields differ significantly. Most notably, the time frame for stability of a pharmaceutical product is approximately 2 years, whereas meaningful stability for an evolutionary biologist is measured in the hundreds of millions of years. Such divergent viewpoints not only have a significant effect on the time span of preservation, but also on what criteria are used to assess "stability." This review discusses the literature addressing the maintenance of DNA integrity from the perspective of developing methods that offer improved preservation. Specifically, studies on the stability of DNA in solution, frozen, and dried are discussed. The findings from these studies are compared, and the costs associated with maintaining DNA samples via contemporary methods (i.e., cold storage) are estimated. In light of the significant cost of maintaining samples in the frozen state, we conclude that dry storage at ambient temperatures would be adequate for many applications, and we suggest preservation strategies that should be investigated based on findings in the literature. In addition, we offer suggestions regarding critical studies that could be performed to compare published results from different fields using vastly different criteria to assess "stability."

INTRODUCTION

THERE IS A GROWING INTEREST in the preservation of DNA from a variety of fields including pharmaceutical science, forensics, homeland defense, biology, and biorepository management. Although each of these fields is concerned with maintenance of DNA integrity, the requirements in terms of storage time and fidelity are significantly different. Both storage time and fidelity must be carefully considered and defined when discussing strategies for "preservation." For example, the pharmaceutical scientist must be concerned with even the slightest level of degradation due to strict requirements imposed by the U.S. Food and Drug Administration (FDA). However, the shelf life required for pharmaceutical products is relatively short: typically 18–24 months. In sharp contrast to these requirements, biorepositories are most concerned with maintaining the ability to obtain DNA sequence information from stored specimens. Current sequencing technology depends on the polymerase chain reaction (PCR) to make copies of stored DNA that are ultimately used for sequence identification. As a result of this process, accurate sequence information can be obtained even from samples in which the DNA has been reduced to fragments. Although the desire for sequence identification does allow substantially greater levels of degradation to be tolerated, biorepositories typically strive to preserve samples "permanently" so that future scientists can utilize specimens for studies at some undefined point in time. In reality, some finite degrada-

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tion rate (ideally low) is occurring in any sample regardless of storage conditions, and the goal is to minimize this rate of degradation. In most instances, DNA samples are stored at −80°C or in liquid nitrogen (−196°C), but there is a significant expense associated with maintaining these conditions in hundreds of millions of samples. In contrast, dehydrated samples could be stored at room temperature, thereby greatly reducing cost and increasing convenience. Previous studies of DNA in solution have clearly shown that degradation rates can be precisely predicted, and buffer conditions can be adjusted to obtain remarkable stability at room temperature. Clearly, analogous studies with dried preparations are sorely needed. Unfortunately, periodic reports of sequences obtained from "ancient" organisms have led to the misperception that nucleic acids are inherently stable. This has undoubtedly contributed to the lack of fundamental studies concerning DNA stability in the dried state. Regardless, this review discusses relevant studies that have been performed on DNA stability, with the goal of providing insight into the critical issues that need to be addressed.

It should be appreciated that DNA structure differs markedly from that of proteins in that noncovalent interactions (e.g., hydrogen bonds, hydrophobic interactions) in the former are readily reestablished in the absence of chemical modification. Consequently, although secondary and tertiary structures must be maintained to preserve protein function, formation of the DNA double helix is typically fully reversible upon exposure to appropriate conditions. Although a recent study by Sharma and Klibanov[1] has described some conditions in which aggregation pathways can compete with reannealing, aggregation is not considered a major concern for DNA stability. Although nuclease contamination must always be carefully avoided when handling DNA, it is chemical degradation that represents the major threat to DNA preservation.[2,3]

As is virtually always true, it is beneficial to search through the older, pre-electronic literature to learn about the insights gained from early work with DNA. Although the physical description of the double helix by Watson and Crick in 1953 elucidated many critical aspects of DNA structure critical for biological function, it was biochemical studies in subsequent decades that revealed the chemical mechanisms responsible for degradation.[4–8] Particularly relevant to preservation strategies, it was demonstrated that DNA stored in buffer was much less stable if it was denatured prior to storage.[7] Subsequent studies by Ward and Kuo[9] investigated the relative ability of single- versus double-stranded DNA to resist radiation damage in both the presence and absence of molecular oxygen. The observation that native, double-stranded DNA was more resistant to damage led the authors to suggest that "bases in intact DNA are sheltered from radical attack by virtue of their physical presence in the center of the double helix," that is, the base shielding hypothesis.[9] This hypothesis is consistent with the observation that DNA condensed in chromatin is more resistant to oxidative damage than naked DNA.[10,11] Although reaction mechanisms are beyond the scope of this review, it is important to recognize that base oxidation is known to cause mispairing during DNA replication, which can ultimately result in mutation.[12,13] Such mutations have been implicated in cancer and aging,[14,15] and these events may explain the evolution of elaborate "proof-reading" mechanisms.[16–18] This is particularly problematic because extensive modification can cause artifacts during PCR amplification, potentially resulting in inaccurate sequence identification.[16,19–21] This risk is often overlooked, and is especially germane to scientists employing more "accommodating" polymerase enzymes to sequence highly degraded, fragmented DNA.[16,22] We refer readers interested in the chemical mechanisms of DNA degradation to two excellent reviews.[23,24]

SOLUTION STABILITY

To avoid chemical and enzymatic degradation in a laboratory setting, molecular biologists commonly store DNA as a precipitate in ethanol at −80°C. Under these conditions, nucleic acids are stable for prolonged periods, but must be isolated from the ethanol, transferred to aqueous buffers, and are typically quantified

prior to use. These manipulations render ethanol precipitates undesirable for applications where samples need to be stored in a ready-to-use preparation, for example, pharmaceutical applications. In addition, the maintenance of low temperatures is exceedingly costly, and storage under ambient conditions would clearly be advantageous. Aqueous solutions of DNA would be the most convenient, but nucleic acids are sensitive to depurination,[7] depyrimidination,[8] deamination,[4] and hydrolytic cleavage,[5,6] which limit the potential for prolonged stability in aqueous media.[2,25] However, studies have demonstrated the utility of formulating DNA in alkaline conditions to inhibit these acid-catalyzed degradation mechanisms during prolonged storage in solution.[2,3] In addition, it is known that ionic strength has significant effects on both depurination and beta-elimination, and thus the use of saline (as opposed to low ionic strength buffer) is critical for maintaining DNA integrity in solution.[7] Under such conditions (pH = 8.5), the primary threat to DNA stability (assuming that nucleases are inactivated and/or absent) is oxidation.[2,3]

It is well recognized that oxidative damage is greatly enhanced by the presence of trace metals (e.g., Fe^{3+}, Cu^{2+}) that produce hydroxyl radicals via Fenton-type reactions.[2,3,24,26,27] In their classic study,[3] it was demonstrated that contamination by transition metals caused significant levels of oxidation to occur during storage. In addition, this study showed that demetalation of all components (DNA, buffers, water) can significantly reduce degradation during storage. It should be recognized that the levels of metal impurities (<5 ppb) that are capable of compromising stability are well below the detection limits of most assays, and that the presence of transition metals (e.g., iron, copper, cobalt) *at any level* can significantly compromise storage stability. In this regard, it is important to mention that even highly purified, clinical-grade DNA can contain 30–40 ppb iron, and stabilizers (e.g., sugars, buffers) often contain considerably higher levels of metal contaminants.[27] Because it is impossible to ensure that metals are completely absent under any conditions, it seems prudent to incorporate chelators into DNA preparations to attenuate metal-catalyzed reactions. (Chelating agents generally also serve to inhibit nucleases; this can also complicate amplification via PCR in which reactions have been optimized for defined magnesium levels.) However, chelation of metals does not prevent Fenton-type chemistry, and there are distinct differences in the abilities of various chelators to attenuate oxidation via the chelated metal.[3,27,28] To further protect DNA from oxidation during storage, it may be advantageous to include antioxidants/scavengers in the storage medium. Utilizing this strategy, Evans et al.[3] combined 200 μM EDTA (a chelator) with 1% ethanol (a radical scavenger) to minimize strand breakage in plasmid DNA. This straightforward approach was capable of preserving >90% of the initial supercoil content after 2 years of storage at room temperature. However, the inclusion of components such as ethanol and salts can be incompatible with some applications, for example, maintenance of viable cells, intact tissue storage, and the use of lipids that are electrostatically associated with the DNA in a delivery vehicle.[3,29]

FROZEN DNA

Although the studies mentioned above demonstrate sufficient solution stability on a pharmaceutical time scale, maintenance of DNA integrity over prolonged periods (e.g., decades) will likely require storage conditions in which molecular mobility is more restricted, that is, low temperature and/or dehydrated. Despite the fact that DNA is routinely frozen, little is known about the effects of freezing and thawing on DNA integrity. The effects of freeze–thawing DNA were first reported by Shikama,[30] who showed that the double-helix of calf thymus DNA was preserved after freezing to temperatures as low as −192°C. Lyscov and Moshkovsky[31] later described a mechanism of "cryolysis"; DNA degradation that was dependent on the rate of cooling of the frozen sample. These authors suggested that cryolysis resulted from the formation of cracks within the ice that were more prevalent in rapidly cooled samples. A more recent study has conflicted with these earlier conclusions, and

shown that slow freezing is more damaging than rapid freezing.[32] In contrast to the typical assumption that DNA sequence has minimal effect on physicochemical properties, this study found that damage to oligonucleotides is dependent upon base composition, structure, and length. However, the study did not look at oligonucleotides longer than a 12-mer, so it is unclear how susceptible polynucleotides (e.g., plasmids, genomic DNA) are to freeze–thawing. However, previous studies have investigated plasmid stability during rapid freeze–thawing (immersion in liquid nitrogen), and demonstrated that supercoil content could be effectively preserved if sugars and EDTA were present.[33] Although the authors did not specifically address cryolysis as described by Lyscov and Moshkovsky, it seems possible that the presence of sugars reduced cracking by entrapping DNA in a glass.[31] Clearly, there is a need for fundamental studies regarding the stability of DNA during freezing and frozen storage. One would predict that enhanced stability would be observed at progressively lower storage temperatures, and such studies would be tremendously beneficial if they could provide data correlating standard measures of DNA quality (e.g., strand breaks, amplification efficiencies, and STR analysis) with storage temperature. Indeed, considering the significant expense allocated to preserving biological materials at low temperatures (−80°C or −196°C), it is surprising that such fundamental studies justifying these extreme (and costly) storage conditions have not been conducted.

A study by Eiseman and Haga[34] on human tissue storage estimated that approximately 307 million samples were stored in the frozen state in the United States. If we assume that half these samples are stored at −80°C and the other half in liquid nitrogen (−196°C), it is possible to estimate that it would cost approximately $120 million to purchase the freezers necessary to store these samples, and an additional $30 million is spent each year to maintain those samples. Furthermore, Eiseman and Haga estimated that samples are accumulating at approximately 20 million per year, and we calculate that it costs an additional $10 million for freezers and maintenance of newly generated samples *each year*.[34] Using these numbers, we estimate an annual cost in 2007 of approximately $54 million just to maintain frozen samples in the United States. It is estimated that half of the global tissue storage occurs in the United States, and thus the global cost to maintain frozen samples each year likely exceeds $100 million.

DEHYDRATED DNA

In contrast to freezing, drying offers a more practical alternative by eliminating the need for cold storage. In addition to reducing molecular mobility, dehydration also removes water that can participate in hydrolytic reactions.[5,6] There are several methods of removing water from liquid preparations to produce dehydrated DNA formulations, for example, spray drying, spray freeze drying, air drying, or lyophilization. Although concerns about shear stress[33,35–38] have led investigators to avoid spraying DNA, more recent studies have clearly elucidated the mechanisms of shear-induced damage and concluded that only very long DNA strands (i.e., genomic) are susceptible to shear stress.[39,40] Regardless of the dehydration methods used, systematic studies of dry DNA stability are lacking. Incidental reports on this topic are contradictory; some suggest that dehydration might be stabilizing,[41,42] while others show that drying induces damage.[33,43–45] Studies that utilize spectroscopic methods have reported that lyophilization causes perturbation of the native helical structure.[46] This finding is consistent with earlier work by Lindhal,[47] who observed that DNA stored over phosphorous pentoxide does not retain its native structure, which renders it more vulnerable to oxidative damage during storage. Considering that many organisms routinely survive extended bouts of dehydration, it is interesting to note that some bacteria are known to respond to environmental stress by synthesizing proteins that cocrystallize with DNA such that oxidation is greatly reduced.[48] Similarly, reports have shown that spores that survive prolonged periods in a quiescent state synthesize proteins that bind to the dehydrated A conformation, reducing rates of depurination by at least 20-fold.[10] As mentioned previously,

it has been suggested that chromatin may also play a protective role in mammalian cells.[11]

There is considerable interest in studying "ancient" DNA to assess both DNA stability and phylogenetic relationships. A study by Shirkey et al.[49] on cyanobacteria samples dried for up to 139 years indicated that survival was limited to samples <60 years old, but that some sequence amplification was possible from the oldest samples.[49] In addition to the complications inherent in analyzing highly fragmented samples, the authors point out concerns regarding oxidation via free radicals and the potential problems associated with base modification. Previous studies of an extinct horse species have also indicated that although significant fragmentation occurs in dried museum samples, it is possible to obtain some sequence information that can be used to establish phylogenetic relationships.[50] Similar studies with mummified human tissue[51] and maize[52,53] have obtained sequence information from dried samples as old as 4700 years. In all these cases, DNA was extracted from museum samples that had been dried and stored at ambient temperatures. Considerably older DNA samples have been recovered from permafrost cores dating as far back as 400,000 years;[54] presumably, the maintenance of continuously cold temperatures contributes to enhanced DNA stability. More recent studies of cryogenic seed storage have concluded that the although low temperatures extend shelf-lives, it is ultimately the reduction in molecular mobility that is crucial for preservation.[55,56] The implication of molecular mobility as the definitive parameter that ultimately determines stability is consistent with the exceptional preservation of DNA in amber.[57–60] Although some skepticism has been expressed concerning the validity of DNA sequences preserved in amber,[61,62] theoretical calculations taking into account the rate of amino acid racemization in amber have concluded that DNA could be preserved for 100 million years under these conditions.[63] As astonishing as these time scales may seem, there are reports of even older DNA being preserved in salt crystals.[64,65] In the case of Vreeland et al.,[64] the authors claim to have isolated *viable bacteria* from salt crystals that are 250 million years old! As with the studies in amber, concerns about contamination and the true age of the samples have raised questions about the authenticity of sequence information from "ancient" samples.[20,66,67] Even if the shelf-life of dehydrated samples is a mere 1000 years, a minimal time frame from a geological/evolutionary perspective, it should be recognized that such stability should be more than adequate for even the most conservation-minded biorepository. It is interesting to note that scientists have argued that DNA crosslinking, not oxidation, is ultimately what limits access to "ancient" DNA sequences.[67,68] Furthermore, Shirkey et al.[49] described problems associated with crosslinking in samples that had been dried for decades.

More recent studies with forensic samples have also focused almost exclusively on obtaining sequence information. Unfortunately, most of these studies merely report how samples were stored and whether sequencing was possible.[69–72] In most cases, sequence information can be obtained even from highly degraded samples, and thus such studies lack the more quantitative information that might be used to determine the usable "shelf-life" for DNA. In some cases, different storage temperatures were employed, but "survival" is only qualitatively described.[73–75] In order to compare the stability of samples stored under a given set of conditions, and thereby identify optimal strategies for preservation, studies need to be conducted wherein DNA degradation is quantified over time under different storage conditions. In addition, the storage conditions need to be clearly characterized with regard to moisture content, access to molecular oxygen, presence of light, etc. Clearly, the state of purification (e.g., whole tissues, intact cells, presence of histones), can have a significant effect because some components, for example, lipids, may facilitate chemical degradation.[76–78] Because such studies typically involve extended time scales, the use of "forced degradation" or "accelerated stability" conditions (i.e., high storage temperature) might enable researchers to differentiate between different storage strategies in a practical timeframe (e.g., 1–2 years). Of course, the use of higher storage temperatures would not allow a direct comparison of dried DNA with the current practice

involving frozen storage, but one could utilize elevated temperatures to optimize conditions (e.g., moisture content, state of purification) for storage in the dried state before conducting prolonged studies at normal (e.g., ambient) temperatures. However, it may take decades for significant degradation to occur under optimized conditions, and stability would need to be quantitatively compared to that achieved with conventional, frozen storage.

Unfortunately, the only published study regarding the prolonged storage stability of dried, isolated ("naked") DNA was conducted by Kolobov and Vainberg[44] over 30 years ago. These authors demonstrated that dry DNA stored at 0–4°C undergoes significant changes in molecular weight (i.e., strand breakage) within 6 months. Studies incorporating sugars to stabilize purified, dried DNA reported that lactose, glucose, and sucrose were able to preserve biological activity for 3 weeks of storage at 75°C.[46] This study also monitored structural changes in the DNA upon rehydration, and reported that sugars partially prevented dehydration-induced structural changes. Although the latter study suggests that the addition of sugars may be sufficient to preserve DNA during prolonged storage, other studies by Shirkey et al.[49] have shown that purified plasmid DNA desiccated in the presence of trehalose and stored at room temperature is initially protected against aggregation and light-induced damage, but that degradation is clearly observed after 8 weeks. Similarly, our recent studies have investigated the storage stability of purified plasmid DNA in highly purified trehalose (undetectable iron and copper content) that had been lyophilized to achieve very low water contents (≈0.5%). Our results indicate that strand breakage (i.e., loss of supercoil content) is observed even under these "ideal" conditions (Fig. 1). Furthermore, degradation is clearly observed after only 2 weeks of storage at 20°C! We point out that single-strand breakage (as monitored in Fig. 1) is a much more sensitive measure of damage than is sequence identification, and thus our data should not be directly compared with the studies utilizing amplification/sequence recovery as an indicator of stability. Regardless, the data suggest that additional studies are needed with both purified DNA and intact cells to identify the degradation mechanism(s) that are active under such conditions. In this regard, it is noteworthy that studies with lipid-based DNA formulations have implicated free radical formation in the dried state as a likely contributor to strand breakage during prolonged storage.[79] As such, strategies that minimize exposure to molecular oxygen (e.g., storage under nitrogen or argon gas) and/or attenuate free radical formation (e.g., the incorporation of chelators) should be considered when developing optimized strategies.

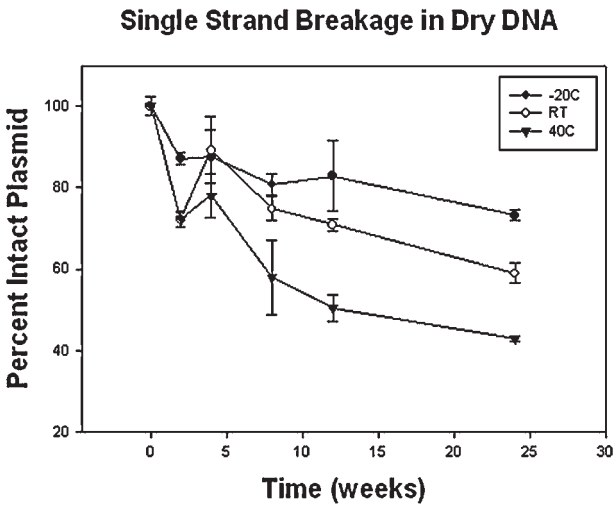

FIG. 1. Plasmid DNA samples were lyophilized in trehalose and stored at −20°C (closed diamonds), room temperature (open circles), or 40°C (closed triangles). Triplicate samples were rehydrated at the indicated timepoints and analyzed for single strand breaks by agarose gel electrophoresis according to the methods described in Molina *et al.* (2004). Subsequent analysis showed that moisture content was approximately 0.5% in these samples, and the glass transition temperature of the dried cake was 113°C, indicating that these samples remained in the glassy state during storage. Each symbol and error bar represents the mean ± one standard error, respectively, of the triplicate samples.

CONCLUSIONS

Considering the lack of studies concerning the long-term storage stability of DNA, there is an obvious need for systematic storage studies on both purified DNA and intact cells. Upon the completion of these studies, more informed decisions could be made regarding the optimal, most cost-effective method for the storage of

genetic material. Regardless of storage conditions, it is important to question whether storage for indefinite periods (e.g., millennia) is truly necessary, even if it is attainable under certain storage conditions. It is often stated that samples must be preserved indefinitely for "generations of future scientists." But given the number of samples that are archived each year, it seems highly improbable that investigators in subsequent millennia will choose to access the billions (literally) of preserved samples. Furthermore, given human life spans, we must consider whether it is necessary to preserve forensic samples for more than a century in order to solve cases in which all potential perpetrators and victims are deceased. What is the probability that future generations will be interested in "cracking" *very cold* cases? Such practical questions bear heavily on the stabilization strategies pursued and the costs incurred. Clearly, we must get beyond the "preserve everything indefinitely" mentality that is prevalent at many biorepositories and forensic archives. More specifically, the data suggest that sequence information can be recovered from DNA that has been preserved in the dried state for at least decades, if not thousands of years. Archiving such specimens would not require refrigeration, and would thereby eliminate the approximately $100 million associated with maintaining frozen samples each year.

Although the optimal conditions used in preparing samples for storage have not been clearly identified, the emphasis has clearly been on recovering "activity"; typically measured by sequence identification or gene expression (e.g., transfection of cells in culture). With regard to the former measure of activity, archivists should be aware of the emerging field of epigenetics that has identified heritable changes in gene expression that are not due to changes in DNA sequence.[80] Considering the revelations from this burgeoning field of study, it may be prudent to consider stabilization strategies that reliably preserve chemical modifications to DNA, proteins, and RNA in addition to the DNA sequence. With regard to the preservation of gene expression, it should be recognized that the loss of the supercoiled form (single-strand breaks) does not prevent gene expression, and thus storage stability studies should not rely solely on gene expression in assessing the effectiveness of various preservation strategies. Clearly, sufficient numbers of single strand breaks will result in inactive/nonfunctional DNA, and thus loss of the supercoiled form may be used as a more sensitive assay (compared to sequence identification or gene expression) to test the effectiveness of different methods of preservation on a more practical time scale. As stated above, these approaches could be used to compare stabilization strategies and would likely identify more cost-effective preservation methods.

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