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The Autodegradation of Deoxyribonucleic Acid (DNA) in Human Rib Bone and Its Relationship to the Time Interval Since Death

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ABSTRACT: This research explored the feasibility of using the degradation rate of deoxyribonucleic acid (DNA) in human rib bone to determine the time interval since death. Postmortem human rib samples were surface sterilized and incubated under sterile conditions in either high or low humidity conditions at room temperature for a period of weeks. At selected times, portions of the bone were cut away, and the DNA from these samples was extracted and subjected to strand separating gel electrophoresis. The DNAs in the gels were transferred to a nylon membrane, preserving their relative positions as in the gel. and probed with radioactive total genomic human DNA. Autoradiograms produced were scanned and digitized. When the samples were incubated under identical conditions, the degradation rate of DNA in samples from different individuals appeared very similar. The DNA degradation rate may vary with temperature and humidity more than it varies between individuals.

KEYWORDS: pathology and biology, deoxyribonucleic acid (DNA), decomposition, postmortem interval, time since death, postmortem examinations

Identification may be difficult when bodies have decomposed beyond recognition. Determining the time interval since death can be an important step. Forensic scientists have traditionally relied on their experience of previous cases, which provides only a rough estimation. Techniques that estimate time since death based on specific criteria related to the decay of the body are desired. This problem has been approached by studying the decomposition of associated material (clothing, leather, paper, and so forth) as a function of time exposed to the environment. Such studies have been reported by Daily [*1*] as well as Morse et al. [*2*]. Additionally, the use of plants and related decomposition vectors of human skeletal remains were reported by Warren [*3*].

The majority of research concerning decay rates has employed an entomological ap-

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proach, often using other mammals as decomposition subjects. Payne et al. [4] found that decomposition of pig carcasses could be broken up into six stages, where each stage was characterized by a particular group of arthropods. The insect succession in relationship to weather and place of decaying dog carcasses has also been studied [5]. Recently, insect succession and decomposition of human cadavers above ground in different places and seasons has been studied [6]. Below ground, the rate of decay varied with the depth of burial and environmental temperatures [7].

Decomposition studies on unembalmed human cadavers were carried out as early as 1898, when Motter [8] discussed the order of disappearance of bodily tissues and organs. When Buromskii [9] measured the optical density of DNA and ribonucleic acid (RNA) in soft tissues (liver, kidney, and spleen) as a function of time, it was found that this approach was useful up to 8 h after death.

The research reported in this paper explores the feasibility of using the degradation rate of DNA in human rib bone to determine the time interval, in the range of weeks, since death. As a body decays, the DNA molecules in all parts of the body are presumedly degraded by nucleases (enzymes that specifically attack nucleic acids). These nucleases are presumedly released with other hydrolytic enzymes as cellular and subcellular membranes lose their integrity. As degradation continues, the average size of DNA molecules in the bone also decreases. If the degradation rate is similar enough in different individuals, and if the conditions where a corpse was found are known, and the amount of degradation is determined, the time since death might be estimated. Our results point to the feasibility of this approach under controlled conditions, and we suggest variables required to test it under uncontrolled (field) conditions.

Materials and Methods

Seven rib bone samples 7 to 13 cm in length were obtained at autopsy from cadavers where disease was not associated with death. These bone samples were donated to The University of Tennessee Department of Anthropology for the purpose of scientific research. Samples were kept at 4°C in a sterile plastic container for up to 24 h before processing. Soft tissue was removed with a razor blade, with care not to puncture the bone's surface. A 1.5-cm section of bone was cut with an X-Acto® Miter box and saw and stored in a sterile plastic vial at −70°C. The rest of the bone sample was prepared as follows.

Bone samples were surface sterilized and incubated under sterile conditions to prevent bacterial growth. The ends of the bone were sealed with 20% sterile gelatin, and bone was surface sterilized through exposure to ultraviolet light. The sterilized bone was put in a sterile plastic bottle sealed with a foam plug, and incubated at temperatures between 19 and 25°C in a sealable plastic box containing either desiccant (low humidity conditions) or a container of water (high humidity conditions). At specific times during incubation, one end of the bone sample was cut away, and a 1.5-cm section was removed. The remaining portion of the bone was prepared as before and returned for continued incubation. The 1.5-cm bone section was sliced open with a razor blade, and the marrow was removed. Sandpaper was used to remove any traces of marrow and tissue left on the bone matrix section. These bone matrix sections were stored at −70°C in a sterile plastic vial until DNA was extracted from them.

DNA Isolation from Bone

Bone samples were removed from the −70°C freezer and kept on dry ice. Each bone sample was powdered separately using a Waring blender with a metal blender vessel half filled with liquid nitrogen. Of each powdered bone sample, 25 mg was digested with proteinase-K (50 μg/mL) in a 300-μL reaction mixture containing 90m*M* ethylenediaminetetraace-

tate (EDTA), 45m*M* Tris-acetate (pH 7.8), 0.5% sodium dodecyl sulfate (SDS), and 0.5% *N*-lauroylsarcosine at 55°C for 2 h. Samples were then extracted once with Tris buffered phenol, once with phenol chloroform (1:1, vol:vol) and twice with water saturated ether. Traces of ether were removed by blowing a stream of nitrogen gas over the samples for 5 min. Samples were stored at −20°C.

Quantitation of DNA

The quantitation of DNA in the samples isolated from bone presented a special problem since the amount of DNA obtained from each sample was small (200 to 5000 ng) in 200 μL, the size distribution of DNA fragments was extremely variable, and the SDS carryover from the isolation procedure had to be considered. These problems made the existing methods of DNA quantitation impractical. A new method [*10*] was developed to solve these problems. DNA, 3 μL, was mixed with 3 μL of 2% low melting point agarose and pipetted into preformed wells of an agarose plate where it solidified. Diffusion of SDS was accelerated by shaking the agarose plate in a container of diffusion buffer (14m*M* Tris, 3m*M* EDTA [pH 7.8]) for 3 h. The amount of DNA was then estimated by comparing ethidium bromide mediated fluorescence of samples with that of standards.

Alkaline Gel Electrophoresis

Samples of 68 μL were mixed with 10-μL loading dye [*12*], boiled for 3 min in 1.5-mL screw cap tubes (Starstedt Corp.), and immediately cooled on ice before loading. We used the NaCl-EDTA method of casting 1% alkaline agarose gels [*11, 12*]. Electrophoresis was carried out for 1.5 h at 80 V, then at 12 V for about 16 h, at which time the loading dye had migrated 7 cm from the origin.

Electrophoresis of DNA in Neutral Agarose Gels

Samples were mixed with loading dye and electrophoresed on a neutral 1% agarose gel in Tris-acetate buffer (40m*M* Tris-acetate [pH 7.8], and 2m*M* EDTA). After electrophoresis, the gel was stained with 0.5 μg/mL of ethidium bromide for 30 min. The gel was photographed on a Chromato-vue® transilluminator (Ultraviolet Products Inc., San Gabriel, California) using Polaroid® film type 55 and Kodak Wrattan filter #23A.

DNA Transfer to Membrane

After electrophoresis, alkaline gels were allowed to shake in 500 mL of neutralization buffer (1.5*M* NaCl and 1.0*M* Tris-HCl, pH 8.0) for 1 h. Transfer of DNA to a nylon membrane (Biodyne Corp.) was as described by Southern [*13*]. DNA was bound to the membrane [*14*] using exposure to ultraviolet light (General Electric G15T8. 15-W germicidal bulb) at a distance of 8 cm for 3 min.

Preparing Human DNA for Hybridization

The hybridization probe was made from human DNA, purified from an ejaculatory sample, essentially as described for bone DNA. The DNA was precipitated with sodium acetate and ethanol as described by Maniatis et al. [*15*]. The pellet was resuspended in 1 mL of TE buffer (10m*M* Tris-HCl, [pH 7.8], 1m*M* EDTA).

Hybridization Probe Preparation

Human DNA, ΦX RF, and λ DNA were labeled in vitro by nick translation [*16*], using $^{\alpha 32}$P-labeled deoxycitidine triphosphate (ICN Corp., 3000 Ci/mMole). The other reagents used in the labeling reactions were supplied in the form of a nick translation kit (BRL Corp.), and they were used according to the directions of the supplier. The 50-μL reaction mixture was extracted with 1:1 phenol:chloroform, the aqueous phase was removed, and 5 μL of sterile glycerol was added to aid in loading the sample onto a column. To separate labeled DNA from unreacted nucleotides, the aqueous phase with glycerol was loaded onto a 6-mL column of Sephadex G-50® in an 8-mL glass column previously rinsed with elution buffer (0.25*M* NaCl, 0.05*M* Tris-HCl, pH 7.9, 0.5% SDS, 2m*M* EDTA). About twenty 250-μL fractions were collected, and the radioactivity of each fraction was estimated using a Geiger counter. Fractions that corresponded to the first peak of radioactivity were pooled and used for hybridization. The human DNA, ΦX RF DNA, and λ DNA probes were labeled with ^{32}P to specific activities of about 6 to 7 × 10^7 cpm/μg. 0.25 μg (1.5 × 10^7 cpm), 1.8 ng (1.2 × 10^5 cpm), and 1.6 ng (1.2 × 10^5 cpm) of the human DNA, λ, and ΦX RF DNA probes, respectively, were used in a 20-mL volume for hybridization of a 15- by 14-cm blot.

The nylon filters were added to individual Scotchpak® heat sealable pouches, 8.5 by 9.5 in. (21.6 by 24 cm), along with prehybridization mixture (1.0*M* NaCl, 0.50*M* Tris base [pH 7.9], 1.0m*M* EDTA, 4.5m*M* *N*-lauroylsarcosine, 2m*M* sodium pyrophosphate, 10 × Denharts solution, and 133-μg/mL alkali cleaved single stranded salmon sperm DNA). Prehybridization was carried out for 10 h at 65°C. The probes were boiled for 3 min and cooled on ice, and about 1 mL of solution containing the hybridization probe was added to the prehybridization solution in each bag using a syringe and needle. The bag was resealed and then sealed inside another heat sealable pouch. Hybridization was carried out for 4.5 days at 65°C with periodic agitation. Four blots were washed four times in the same container for 5 min with 1600 mL of 2 × SSC (0.3*M* NaCl, and 30m*M* sodium citrate, [pH 7.0]), 0.1% SDS at room temperature, then twice for 15 min at 55°C with 0.1 × SSC and 0.1% SDS. Blots were exposed to Kodak X-AR film in using Dupont Lightning Plus intensifying screens. Blots were kept at −70°C during these exposures.

Densitometry and Computer Analysis

Selected gel lanes on autoradiograms were scanned using the Beckman DU-8 spectrophotometer with the gel scanning accessories. The wavelength of the scan was set at 0 (undispersed white light), and a numerical density of the negative at 0.2-mm intervals along a gel lane was determined. This value was both graphed on the spectrophotometer's plotter and transmitted over a RS-232 data communications port to an IBM personal computer. The data were stored in an ASCII file using data communications software (X-Talk, Microstuf, Inc.).

A computer program was written in BASIC which calculated the molecular weight of DNA fragments in each position of the gel lane based on the known position and molecular weight of DNA size markers in nearby lanes. A line was drawn on the autoradiograms at the position of the bottom of the loading wells and perpendicular to the migration of DNA, to allow precise alignment of each sample with the molecular weight standards relative to the position of an initial peak (generated by the drawn line) in each scan. Molecular weight assignments were based on the method of Southern [*17*] where he determined the molecular weight of DNA fragments in a gel lane based on only three bands of known size and position. The molecular weight of DNA in each position was calculated based on the three closest molecular weight markers. After all of the positions in the densitometry scan had been assigned molecular weights, they were printed as ASCII files. These files were then read by a Pascal program which performed the analyses as discussed.

Results and Discussion

The reproducibility of the procedure for DNA extraction from ground bone samples is shown in Fig. 1. Two DNA isolations were carried out in parallel using five ground bone samples. Lanes containing DNA from the duplicate extractions are nearly identical, verifying that degradation during extraction is not likely to be a problem.

Preliminary experiments using neutral gels appeared to indicate variation in the amount of degradation in samples from different individuals incubated for the same amount of time. This led us to take an additional precaution concerning the method of gel electrophoresis being used. Since it is probable that the degradation of the DNA in the bone samples occurred, at least in part, by single stranded breaks, more reliable results would be expected from measurement of the single stranded length of DNA fragments rather than their double-strand length. For some types of analysis, neutral gels may be useful. However, alkaline strand separating gels were used for further analysis, so that the potential for variation as a result of this effect could be ignored.

The decision to use alkaline gels generated another technical problem. The amount of bone available to us was small, and consequently the amount of DNA obtained from each DNA isolation was small (200 to 5000 ng). If 500 ng of DNA is needed for each lane of a neutral agarose gel, approximately 1500 ng is needed on an alkaline gel, since the intensity of fluorescence of ethidium bromide stained double stranded DNA is about three times as great

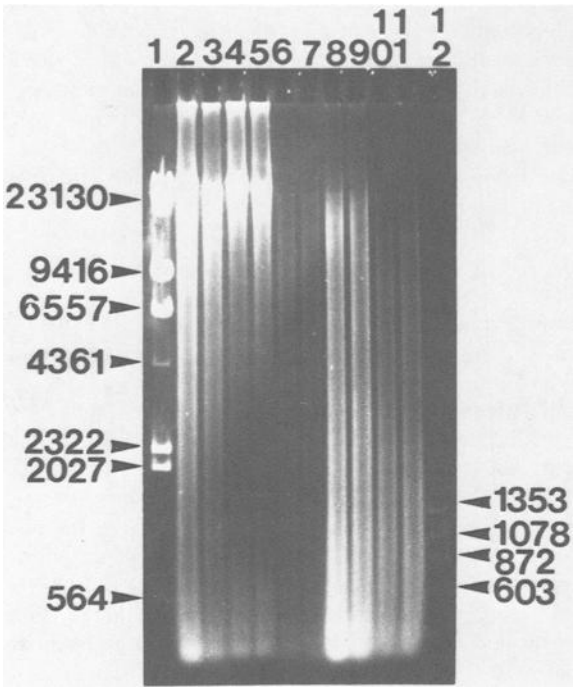

FIG. 1—*Reproducibility of DNA extractions from bone. Two DNA isolations were done in parallel from each of five ground bone samples. Samples were resolved on a 1% agarose gel and stained with ethidium bromide. DNA was visualized on an ultraviolet transilluminator, and it was photographed onto Polaroid film. Sizes for the molecular weight markers are given in base pairs at the left and right. Samples:* (1) *Phage* λ *DNA/*Hin *dIII digest,* (2 and 3) *bone Sample 1,* (4 and 5) *bone Sample 2,* (6 and 7) *bone Sample 3,* (8 and 9) *bone Sample 4,* (10 and 11) *bone Sample 5,* (12) ΦX *RF DNA/*Hae *III digest.*

as that of single stranded DNA. Thus a more sensitive method of detecting DNA is needed. We decided to use Southern blotting and hybridization to a labeled human probe, since only nanogram quantities of DNA are needed for this type of analysis.

Results of the 21 samples, from 3 time periods from 7 individuals, are shown in Fig. 2. The data were treated in a number of ways to generate a statistic which would summarize the amount of apparent degradation. The statistic (I) which most nearly matched our judgment of the relative degree of degradation in the various samples is defined as:

$$I = \frac{\Sigma[\text{MW} \times (\text{ABS-MIN})]^2 / \Sigma[\text{MW} \times (\text{ABS-MIN})]}{\Sigma[(\text{ABS-MIN})/\text{MW}]^2 / \Sigma[(\text{ABS-MIN})/\text{MW}]} \times 10^{-5}$$

where MW is the molecular weight and ABS the absorbency at each (0.2-mm) position in the autoradiogram resulting from the gel, and MIN is the minimum absorbance for a sample. The term in the numerator is relatively more sensitive to high molecular weight DNA while the term in the denominator is relatively more sensitive to low molecular weight DNA; the ratio appears to be a good measure of DNA integrity, taking into account the entire molecular weight distribution.

This statistic, to be useful, should be relatively insensitive to the amount of DNA in a lane and the intensity of the exposure within some usable range. To test whether or not this was the case, the same sample (the sample from Individual 6 before incubation) was run in two gels; three different exposures of the sample in one of the gels and one of the exposures from the other gel were used to generate values for I. The results of this and the other calculations are shown in Table 1. These statistics are also displayed in Fig. 3. When three different exposures of a blot were made, the resulting statistics were similar. The largest variation is seen in the lightest exposure, where the regions of the gel lane with the least DNA are too weakly radioactive to expose the X-ray film. Because of this observation, only longer exposures were used for the analyses presented here.

Our data support the hypothesis that temperature has a large effect on degradation rate of DNA. Although the samples were incubated at temperatures between 19 and 25°C, even this much fluctuation in temperature may cause large differences in degradation over a period of weeks or months. Samples 3 and 4 were incubated simultaneously; Samples 6 and 7 were also incubated simultaneously, but at a different time from Samples 3 and 4. The rate of degradation in these matched pairs is very similar. This would be expected if temperature were a major factor in DNA degradation, since samples that experienced the same temperature variations have very similar degradation rates. The third time point (84 days) of Sample 7 yields a higher value than expected, probably because of a problem in autoradiography. The lane to its right on the gel in which it was electrophoresed gave a very intense signal (it was the same DNA as was used to generate the radioactive hybridization probe and was serving as a positive control), and in Fig. 2, it can be seen how some of this signal "leaked" over to the lane of Sample 7.

Sample 1 was the only sample incubated under low humidity conditions. It was also incubated during the same time as Samples 3 and 4. From these data, it appears that the DNA in bone is degraded faster in samples incubated in high humidity conditions than in low humidity. These variations were also analyzed in terms of sex, race, and age of the individual, and no correlations were found.

Conclusion

The results from this research can be summarized in the following way. It was possible to isolate DNA from bone samples and measure the amount of degradation in the isolated DNA. The amount of degradation appeared to be reproducible for multiple isolates from the same sample. The DNA in the sample incubated in low humidity conditions was degraded

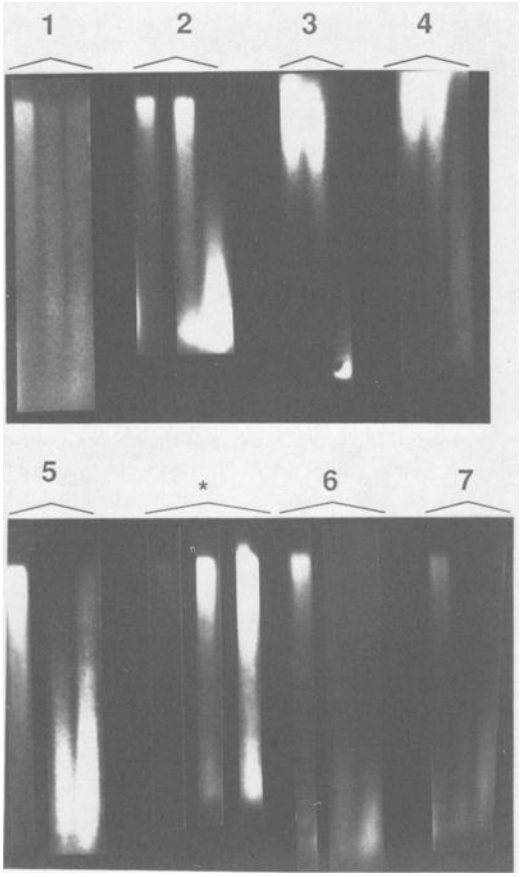

FIG. 2—*Autoradiograms of hybridized bone DNAs. Bone DNAs of various incubation times and markers were resolved on an alkaline strand separating gel, blotted to a nylon membrane, hybridized with appropriate nick translated probes, and exposed to Kodak X-AR film.* (1-7) *From Individuals 1 through 7, with increasing times of incubation from left (no incubation) to right. The three lanes marked with an asterisk are three exposures from the same sample as Individual 6, before incubation, but electrophoresed, originally, in a different gel. These exposures were 12, 48, 96 h, from left to right. All the samples shown were generated using the same autoradiograms as were scanned, with the photographic reproductions being done in an identical fashion, to preserve the relative intensity, with the exception of the lanes shown for Individuals 1 and 4. The autoradiograms for individuals 1 and 4 that were scanned were lost, and only briefer, lighter exposures were available. These were exposed to bring out the signal in each lane, but these lanes cannot be directly compared with the other lanes. Table 1 contains the data for the exposure times for the other individuals.*

TABLE 1—*Results of DNA integrity study.*

Person	Days	Hours of Exposure	*I*
1	0	90	310
	21	90	100
	42	90	81
2	0	48	520
	3	48	270
	14	48	8.4
3	0	48	1100
	21	48	8.0
	42	48	3.6
4	0	48	210
	21	90	9.3
	42	90	5.7
5	0	48	480
	14	48	32
	21	48	25
6	0[a]	11	1200
	0[a]	48	390
	0[a]	96	250
	0	48	350
	42	48	17
	84	48	6.1
7	0	48	390
	42	48	14
	84	48	14

[a]These samples were electrophoresed on a different gel than the other samples from this same individual.

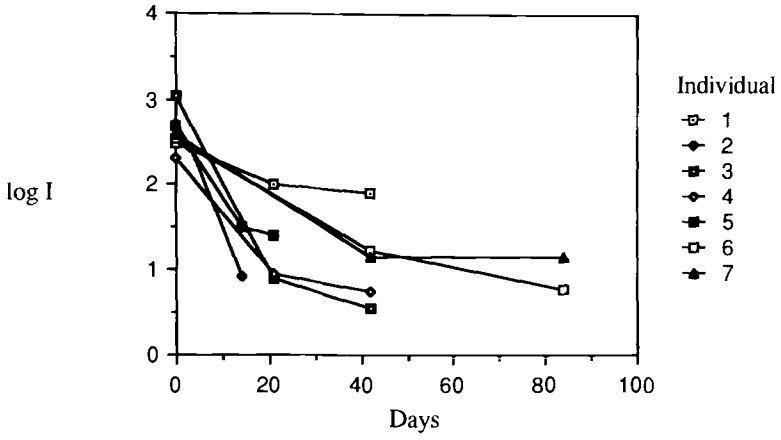

FIG. 3—*Graphical display of DNA integrity statistic. The data from Table 1 are displayed, with the integrity statistic* I *being displayed as its common logarithm.*

more slowly than those in high humidity conditions. Variations in temperature may have had the largest effect on the degradation rate, which would be consistent with previous studies [5-7]. There was some evidence that the DNA degradation rate is similar among different individuals. This is especially apparent when samples from different individuals were incubated together and similar decay rates were found. Different bones may have different DNA decay rates. When degradation of DNA in clavicle bone was compared with the DNA degradation in a rib bone from the same individual (data not shown), the clavicle bone DNA seemed to be degraded more slowly, but autoradiographic artifacts made a detailed comparison impossible. Early in this project, analysis of the DNA degradation in the bone marrow portion of the bone seemed to indicate that the bone marrow DNA was degraded more quickly than the bone matrix DNA. This analysis was not continued, because a large section of bone was needed to obtain a small amount of marrow for analysis, and the sample size was limited.

There are still other questions related to DNA degradation in bone that need to be addressed if this approach is to become a practical field tool. What role do bacterial nucleases play in DNA degradation in the bones of a decaying body? How do environmental factors such as rain and sunlight affect degradation? Does the position of the bone in the body near other decaying organs in the body affect the degradation rate? Do the different parts of the bone have different degradation rates? We believe that our study has established the feasibility of addressing these questions experimentally.

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