TECHNICAL NOTE

Doris M. Kupfer,[1] *M.S.; Arvind K. Chaturvedi,*[2] *Ph.D.; Dennis V. Canfield,*[2] *Ph.D.; and Bruce A. Roe*[1]*, Ph.D.*

PCR-Based Identification of Postmortem Microbial Contaminants—A Preliminary Study*

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ABSTRACT: Investigation of postmortem blood can reveal the presence of significant ethanol levels. However, in some instances it cannot easily be determined if the source of ethanol is from ingestion or from postmortem endogenous fermentation by contaminating microbes. Described here is a robust polymerase chain reaction (PCR)-based method for detecting the presence of common ethanol producing microbial contaminants in human blood. A set of DNA primers were designed for use in PCR to amplify and detect the genomic DNA from humans and three test microorganisms *Escherichia coli, Proteus vulgaris,* and *Candida albicans.* A rapid and reproducible protocol was developed for isolating genomic DNA from mixed human blood-microorganism samples that yields a suitable template for PCR. The organism-specific primer pairs can detect the presence of the target microorganisms in human blood at concentrations as low as 10 colony forming units/mL. The PCR products readily can be detected after agarose gel electrophoresis. This method provides an additional means of rapidly identifying microbial contaminants in postmortem blood samples.

KEYWORDS: forensic science, forensic toxicology, postmortem ethanol production, ethanol, microbial contamination, polymerase chain reaction, *Escherichia coli, Proteus vulgaris, Candida albicans*

The presence of ethanol in postmortem blood and tissue samples often is determined in forensic investigations. However, it is well documented that a variety of microbes can produce significant amounts of ethanol under postmortem conditions (1,2). Numerous reports question the accuracy of postmortem alcohol levels when the possibility of microbial contamination of the sample exists because of tissue injury or sample age and handling (2–6).

Analysis for ethanol and other volatile organic compounds in blood, other body fluids, and tissue samples typically is determined by head-space gas chromatography (7). The composition of volatile organic compounds present in a sample can be indicators of microbial contamination (8). However, Canfield et al. (6) demonstrated that they do not always serve as reliable indicators for quantitative postmortem ethanol detection. It has been shown that determining the ethanol levels in different body fluids, for example, urine versus blood or vitreous humor versus blood also can give an indication of endogenous microbial-based production of ethanol since urine and vitreous humor rarely suffer from postmortem microbial ethanol production (9). However, endogenous ethanol production in urine has been demonstrated under laboratory conditions by Sulkowski et al. (10). Harper (9) has suggested that samples be cultured to determine if there is any microbial contamination in postmortem samples. Although this approach can be informative regarding the presence of either bacterial or fungal contamination, culturing samples is time consuming and requires a variety of growth media. Furthermore, culturing microorganisms depends upon the viability of the contaminant, which can be limited by sample storage methods such as freezing.

One of the missions of the Federal Aviation Administration (FAA) is to help determine the cause of fatal aircraft accidents. The FAA Toxicology and Accident Research Laboratory of the Civil Aeromedical Institute (CAMI) located in Oklahoma City, OK, receives tissue, blood, urine and other body fluid samples from victims of fatal aircraft accidents throughout the United States. The age, history, and handling of these samples prior to their arrival at CAMI can vary significantly. These samples are examined routinely for the presence of drugs and alcohol in the FAA laboratory, and the findings are used to assist in the accident investigation. The analyses must be highly accurate to prevent incorrect conclusions regarding the cause of an aircraft accident.

In a study conducted at CAMI, in approximately 45% of the victim samples which contained greater than 0.04% blood alcohol, the origin of the ethanol detected could not be established because of the sample condition and/or lack of additional fluid or tissue samples from the victims (6).

The polymerase chain reaction (PCR) has been used successfully in clinical settings to detect and identify a specific genomic region of both bacterial and fungal cells from microbial isolates and blood samples (11–13). However, the published methods suffer from the drawback that blood components are inhibitory to PCR (11), forcing investigators to separate the microbial cells from blood via methods such as Percoll gradients (11), antibody agglu-

[1] Department of Chemistry and Biochemistry, University of Oklahoma, Norman, OK.

[2] Toxicology and Accident Research Laboratory (AAM-610), Civil Aeromedical Institute, Federal Aviation Administration, U.S. Department of Transportation, P.O. Box 25082, Oklahoma City, OK.

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tination (12), or the culturing of isolates (13) prior to PCR analysis.

An investigation was begun to devise a rapid and sensitive method for detecting alcohol-producing microorganisms in blood for application to alcohol positive forensic samples. Described here is a DNA isolation procedure and a subsequent PCR-based assay that can determine the presence of microbial contamination in human blood in a species-specific manner. The microorganisms selected for the study were *Candida albicans, Escherichia coli* and *Proteus vulgaris. Candida albicans* is a yeast found on the skin, mouth, and genital mucosa, which often is present in contaminated blood and can produce large quantities of ethanol via alcoholic fermentation (2,14). The two enteric bacteria, *Escherichia coli* and *Proteus vulgaris*, are found in soil, water, and the intestinal tract and carry out fermentations which also can yield significant levels of ethanol (15). These three organisms were selected for development of the PCR assay since they are often found in forensic blood samples and potentially can be the source of endogenous alcohol (2).

To obtain PCR template, a rapid DNA isolation method was developed which requires only a small volume of sample to isolate bacterial or yeast genomic DNA directly from contaminated human blood. This method, which avoids the use of organic reagents such as phenol (16), does not require the isolation or culturing of the microbial contaminants from blood. The primers are targeted to 16S (prokaryotic) and 18S (eucaryotic) ribosomal RNA genes (rDNA). These multicopy genes increase the number of PCR target sequences per genome. Because this is a PCR-based approach, extremely low numbers of contaminating microbial cells (10–100) can be detected, regardless of their viability. The amplified PCR product does not require hybridization or fluorescent or radioactive probes (12,16) for detection. Visualization is by direct staining of the PCR product following agarose gel electrophoresis. The protocol outlined here easily could be expanded to identify other ethanol-producing microorganisms by development of additional specific PCR primer sets.

Materials and Methods

Organisms and Growth Conditions

Escherichia coli strains ATCC 8739, 11303, 25922, *Proteus vulgaris* strain ATCC 10052, and *Enterococcus faecalis* strain ATCC 19433 were grown in L broth (10 g peptone, 5 g yeast extract, 5 g sodium chloride/liter) (1.5%) (17). *Candida albicans* strain ATCC 10231 and *Saccharomyces cerevisiae* strain ATCC 18824 were grown in modified Saubouraud's medium (10 g neopeptone, 20 g dextrose/liter) or (18). All microorganisms were grown at 37°C. Expired, whole human blood was obtained from the Oklahoma Blood Bank, Oklahoma City, OK. Aliquots were stored frozen at −80°C.

Cell Concentration Determination

Microbial cells were grown with aeration for 18–20 h at 37°C. Cell pellets were collected by centrifugation and resuspended in an equal volume of 10 mM magnesium sulfate. Yeast cells were sonicated at a setting of 3 at 50% power for 60 s using a model W-380 ultrasonic processor to aid dispersion of cell clumps for an accurate cell count (Heat Systems-Ultrasonic, Farmingdale, NY). To accurately determine microbial cell concentration the viable cell count, as colony forming units/mL (cfu/mL), was determined by spreading 0.1 mL bacterial or yeast cells from a serial dilution series on L broth agar for *E. coli* and for *E. faecalis*, McConkey's Agar (Becton Dickinson Co., Cockeysville, MD) (19) for *P. vulgaris,* to prevent swarming or Modified Saubouraud's agar (18) for *C. albicans* and *S. cerevisiae*. Following incubation overnight at 37°C, the colonies from each culture were counted and the cfu/mL for the original culture was calculated. For ease of microbial cell number determination in subsequent experiments, a standard curve of A_{600} vs. cfu/mL was constructed for each microbial strain.

Genomic DNA Isolation

Microbial cells suspended in 10 mM magnesium sulfate were added in 100 μL aliquots to 900 μL of human blood to a final concentration of 10^5, 10^4, 10^3, 10^2, 10^1, and 0 cfu/mL for each microorganism tested. 10^5 cells of the negative control bacteria *E. faecalis* were added to each sample. Mixed microbe-human genomic DNA was isolated from these mixtures by vortexing briefly and collecting the cells by centrifugation at 8000 × g for 3 min in a microfuge. A mixture of spheroplasts and white blood cells was obtained by resuspending the pellet in 500 μL Erythrocyte Lysis Solution (Qiagen Inc., Chatsworth, CA) for 10 min on ice, followed by centrifugation at 6000 × g for 3 min. This pellet was resuspended in 600 μL Cell Suspension Solution (Gentra Inc., Research Triangle Park, NC). To this solution was added 12 μL of a 4.5 mg/mL lyticase preparation and 3 μL β-mercaptoethanol to yield a final concentration of 90 μg/mL lyticase and 0.5% β-mercaptoethanol (both from Sigma Chemical Co, St. Louis, MO). The suspension was incubated for 70 min at 37°C. The resulting microbial spheroplasts and white blood cells were collected by centrifugation at 5000 × g for 5 min. The mixed genomic DNAs were isolated using the QIAamp Blood Kit (Qiagen Inc., Chatsworth, CA) modified to include a 5-min incubation at room temperature in the presence of 200 μg/mL RNase A following the cell lysis step. The samples were eluted from the silica gel spin columns into a 1.5 mL-microfuge tube by adding 150 μL of preheated (70°C) 10 mM Tris (pH 8.0), 0.1 mM EDTA to the column, incubating at room temperature for 1 min and centrifuging at 6000 × g for 1 min. This elution step was repeated with an additional 150 μL of buffer and both eluants were pooled. The typical yield of genomic DNA determined by absorbance at 260 nm was approximately 100 μg/mL human blood.

Unmixed genomic DNA samples were prepared from human blood and overnight cultures of each microbe using the above protocol. The 1 mL microbial samples were grown overnight at 37°C in the appropriate medium.

Polymerase Chain Reaction

Each unmixed template DNA was subjected to PCR with each primer pair for primer specificity testing. The following reaction conditions were used: approximately 20 ng of template DNA, 10 μL of 2 mM deoxynucleotides (dATP, dTTP, dCTP, and dGTP) (Pharmacia Biotech, Piscataway, NJ), 10 μL of 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.5, 15 mM $MgCl_2$), 5 units of Taq polymerase, and 2.5 μL 40 μM of each primer (Table 1) in a final reaction volume of 100 μL. The PCR was performed in a Perkin-Elmer 9600 thermocycler (Perkin-Elmer Corp, Norwalk, CT) with an initial denaturation for 5 min at 95°C followed by 35 cycles of 1 min at 94°C, 2 min at 50°C and 3 min at 72°C, and a final 10 min incubation at 72°C. The final reaction was held at 40°C. The resulting PCR products were visualized under long wave UV light after 10 μL of each reaction was mixed with 2 μL of agarose gel loading dye (0.02% bromphenol blue, 2 mM EDTA, 50% glyc-

TABLE 1—*PCR primers.*

Primer	Sequence	Specificity	Amplified product (bp)
FAA1	5′-GTG CCA GCA GCC GCG GTA AT	All templates tested	1097 human, yeast
FAA2	5′-GAC GGG CGG TGT GTA CAA		882 bacteria
FAA18	5′-CTC GAT GCT CTT AGC TGA GTG TCC	Human	717
FAA19	5′-TCT CGG GTG GCT GAA CGC CAC TTG		
FAA37	5′-CTT CTG GTA GCC ATT TAT G	*C. albicans*	704
FAA11	5′-CTT CCA TCG ACT TGG AGT CG		
FAA30	5′-GCT AAT ACC GCA TAA CGT TCG	*E. coli* and *P. vulgaris*	384
FAA31	5′-AAC GCT TGC ACC CTC CGT A		
FAA46	5′-CAT TTA TGA ATA GCT AAG TTT TC	*P. vulgaris*	1226
FAA45	5′-CTC CGG TTT ATC ACC GGC		
PCon1	5′-GTT GCG GCC ATA TCT AGC AG	*C. albicans* and *S. cerevisiae*	105
PCon2	5′-AGT TTC GCG TAT GGT CTC CC		

erol) and electrophoresed in parallel with a 100-basepair DNA ladder (Gibco BRL, Gaithersburg, MD) as a size standard on a 2% agarose gel containing 0.85 μg/mL ethidium bromide at 100 mA for 2 h.

Mixed templates were tested using the same PCR conditions as above. Each reaction contained approximately 20 ng human DNA and varying amounts of microbial DNA depending on the number of cells added.

PCR Primers

Synthetic oligonucleotide primers were designed to hybridize to either the 16S or 18S rDNAs. The GCG program BESTFIT (Version 9.0, Genetics Computer Group, Madison, WI) was used to align the rDNA sequences and identify regions of low homology. The GenBank entries examined were accession numbers J01695 for *E. coli* rDNA, M10098 for human rDNA, X53497 for *C. albicans*, and X07652 for *P. vulgaris*. FAA1 and FAA2 are universal sequences with exact homology regions in all known 16S or 18S rDNAs. As a landmark, FAA1 matches position 611–630 and FAA2 is from 1708–1691 of the human 18S sequence. FAA18 is from human position 759–782 and FAA19 is from position 1476–1453. The *C. albicans* specific primers are from position 695–714 for FAA37 and position 1399–1381 for FAA11. FAA30 matches position 168–187 and FAA31 matches position 552–527 of *E. coli*. The *P. vulgaris* specific primer FAA46 is from position 374–396 and primer FAA45 is from position 1178–1161. The PCon1,2 primer set developed by Holmes et al. (11) amplifies a 5S rDNA region of most yeast species. The primer sequences and their specificities are listed in Table 1. The above primers were synthesized on a Beckman Oligo 1000M DNA synthesizer (Beckman Instruments, Houston, TX).

Results

The goal of the study reported here was to identify sets of PCR primers that could discriminate between *C. albicans*, *E. coli*, and *P. vulgaris* DNA in human blood at a concentration of 10^3 cells/mL for *C. albicans* and 10^5 cells/mL for *E. coli* and for *P. vulgaris*. It has been determined that these are sufficient cell numbers to provide detectable levels of ethanol in postmortem samples (2). Successful use of a PCR-based assay required the development of a rapid, reproducible protocol for isolating sufficiently pure quantities of microbial genomic DNA from mixed human blood and microbial samples at or below the cell concentrations mentioned above that could be amplified and detected by visualization after agarose gel electrophoresis.

Identification of Specific Primer Sets

Initially a series of potential primer sets were tested via the PCR detailed in Materials and Methods using DNA isolated from pure cultures or human blood. The predicted PCR product size and specificity were verified by electrophoresis on agarose gels. Once a primer pair passed this first set of tests, it was used with DNA isolated from human blood mixed with various concentrations of the target microbial cells (see Material and Methods). A universal primer pair, FAA 1, 2, which was positive with all DNAs tested (Fig. 1a, lanes 1, 2, 3, 4), yielded a 1097 basepair (bp) PCR product with the eucaryotic DNAs and a 882 bp product with the prokaryotic DNAs. However, this primer pair could not discriminate between human and microbial DNA in mixed blood microbial samples due to the excess number of human priming sites compared to bacterial or yeast sites. In addition, the *C. albicans* PCR product was identical in size to that produced from the human DNA (Fig. 1a, lanes 1, 2). Therefore, the additional sets of primers listed in Table 1 were developed. The size of the amplified PCR products resulting from each primer set and corresponding microbial or human genomic DNA template are shown in Fig. 1a and b. These results indicate that the primer pair FAA37, 11 designed to detect *C. albicans*, did not cross-react with either *S. cerevisiae* (Fig. 1b, lanes 5, 6) or any of the other DNAs tested (data not shown), while primer pair PCon1,2 designed by Holmes et al. (11) as a yeast specific primer set, was positive for both *C. albicans* and *S. cerevisiae* (Fig. 1b, lanes 5, 6). Primer pair FAA46, 45 was specific for *P. vulgaris* 16S rDNA (Fig. 1a, lane 7). Primer pair FAA30, 31, designed from a region conserved by the enteric bacteria (16), gave a positive signature with both *E. coli* and *P. vulgaris* (Fig. 1a, lane 6 and data not shown). Only the universal primer pair FAA1, 2 and the human specific pair FAA18, 19 were positive with human DNA (Fig. 1a, lane 1, 5).

Finally, *E. faecalis*, a non-ethanol producing bacteria, commonly found in contaminated blood samples was used as a negative control microorganism in the spiked blood samples to simulate more closely forensic samples. The *E. faecalis* genomic DNA was negative for PCR when tested with the microbial and human specific primers (data not shown). It was positive for PCR with the universal primers (Fig. 1b, lane 1).

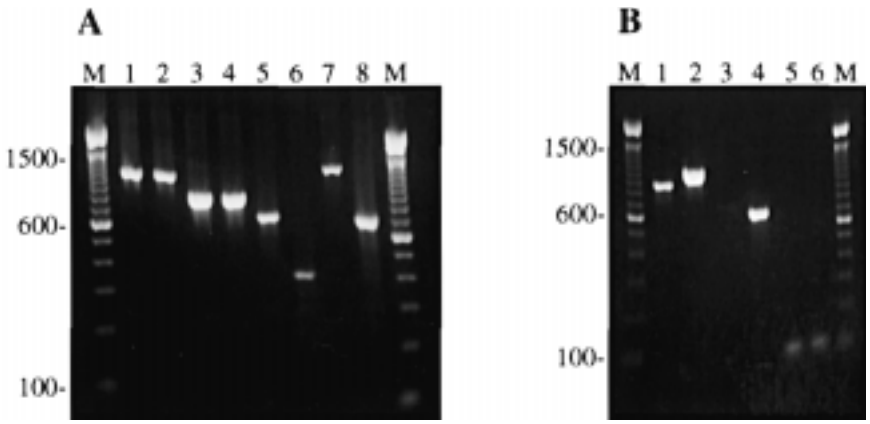

FIG. 1—*PCR amplified products obtained with specific primer pairs and 20 ng unmixed template DNAs.* A. *Human and test microorganisms. Lanes 1, 2, 3, 4 primer pair FAA1, 2; lane 5 primer pair FAA18, 19; lane 6 primer pair 30, 31; lane 7 primer pair FAA46, 45; lane 8 primer pair FAA37, 11. Lanes 1 and 5 human DNA, lanes 2 and 8* C. albicans *DNA, lanes 3 and 6* E. coli *ATTC 11303 DNA, lanes 4 and 7* P. vulgaris *DNA. Marker lanes (M) contain a 100 bp DNA ladder (Gibco BRL).* B. *Control microorganisms. Lanes 1, 2 primer pair FAA1, 2; lanes 3, 4 primer pair FAA37, 11, lanes 5, 6, primer pair PCon1, 2 Lane 1* E. faecalis *DNA, lanes 2, 3, 5* S. cerevisiae *DNA, lanes 4, 6* C. albicans *DNA for comparison. Marker lanes (M).*

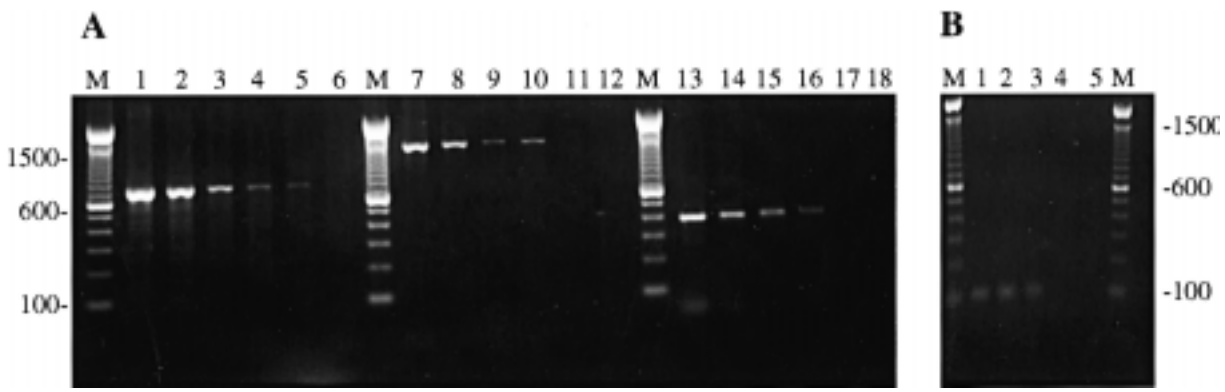

FIG. 2—*Titration of PCR amplified products. PCR amplified product obtained from 10^5, 10^4, 10^3, 10^2, 10^1, and 0 cfu/mL microbial cells in 1 mL human blood and 10^5 cfu/mL* E. faecalis.. A. *16S and 18S rDNA based primers. Lanes 1–6* C. albicans, *(10^5, 10^4, 10^3, 10^2, 10^1, 0 cfu/mL) primer set FAA37, 11; lanes 7–12* E. coli, *(10^5, 10^4, 10^3, 10^2, 10^1, 0 cfu/mL) primer set FAA30, 31; lanes 13–18* P. vulgaris, *(10^5, 10^4, 10^3, 10^2, 10^1, 0 cfu/mL) primer set FAA46, 45. Marker lanes (M).* B. *5S rDNA based primers. Lanes 1–5* C. albicans *(10^5, 10^4, 10^3, 10^2, and 0 cfu/mL), primer set PCon1, 2 Marker lanes (M).*

Isolation of Genomic DNA from Microbe-containing Blood Samples

Existing methods did not yield reproducible quantities of genomic DNA from the mixed microbe and human samples. During the course of this work, several DNA isolation methods were investigated. The Chelex 100 boiling method of Walsh et al. (22) proved difficult to reproduce. The Purgene kit from Gentra Systems yielded a pigmented DNA product that was minimally soluble. The QIAamp Blood Kit from Qiagen Incorporated had very low yields of template, especially with the *Candida* cells, which was not suitable for PCR. However, pretreatment with a hypotonic solution to lyse the erythrocytes and remove heme-containing material and incubation in the presence of lyticase and β-mercaptoethanol allowed the successful use of the QIAamp kit. Genomic DNA with the signal strengths shown in Fig. 2a and b could be detected by probe-specific PCR even in the presence of human and *E. faecalis* genomic DNAs. PCR products from three strains of *E. coli* showed equal signal strength with gel electrophoresis (data not shown) and *P. vulgaris* and *E. coli* were detected at a cell concentration lower than that required for production of significant levels of ethanol (2) (Fig. 2a). Similar acceptable results were obtained for *C. albicans* using the FAA37, 11 primer pair (Fig. 2a) and the yeast-specific pair PCon1,2 (Fig. 2b). Note that detection of ten cells of *E. coli* or *Candida* in 1 mL of human blood and 10^5 *E. faecalis* cells is equivalent to detection of 0.05-0.14 pg microbial DNA in the presence of 100 μg human and 5 ng *E. faecalis* DNA.

Discussion

A DNA isolation method was developed as a rapid means of directly quantitating the presence of selected bacterial or yeast genomic DNA in mixed human blood samples using a series of microbe and human specific PCR primers. The inclusion of a hypotonic erythrocyte lysis step allows the removal of heme-containing material. Preparing spheroplasts by incubation of the heme-free sample with lyticase in the presence of β-mercaptoethanol prior to DNA isolation by a commercially available protocol, significantly improves the cell lysis and yield of both yeast and bacteria DNA. The DNA obtained from this protocol appears to be free

of PCR-inhibitory blood components allowing preparation of template DNA directly from blood. The microbe-specific PCR primer pairs developed during the course of this study could detect microbe contamination of human blood samples in the 10^1–10^5 cell/mL range. In addition, one of the primer pairs, FAA30, 31, has significant homology with *Klebsiella, Yersinia* and *Salmonella* 16S rDNA in addition to the *Escherichia* and *Proteus* 16S rDNA as determined by BESTFIT analysis. Although not tested in this present work, this primer pair may be useful in detecting these additional ethanol-producing enteric bacteria.

The sensitivity of the PCR detection method reported here exceeds the levels of microbial cells judged to have significant ethanol producing activity in postmortem samples (2). This degree of sensitivity assures that minimal levels of microbial contamination can be detected, thereby reducing the possibility of a false indication of imbibed alcohol.

Finally, because the DNA isolation method described above is rapid, large numbers of samples can rapidly be processed and prepared for PCR, another feature desirable in protocols for clinical and forensic laboratories. This method has a potential for being implemented in forensic settings to rapidly test if microorganisms are present in ethanol-containing postmortem samples. Implementation of this assay is being considered by the FAA. Currently, the assay is being tested at CAMI with ethanol positive postmortem samples.

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Additional information and reprint requests:
Bruce A. Roe, Ph.D.
George Lynn Cross Professor
Department of Chemistry and Biochemistry
620 Parrington Oval
University of Oklahoma
Norman, Oklahoma 73019-0245