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Direct Isolation of Purines and Pyrimidines from Nucleic Acids Using Sublimation

Daniel P. Glavin,† Michael Schubert, and Jeffrey L. Bada*

Scripps Institution of Oceanography, University of California at San Diego, La Jolla, California 92093

A sublimation technique was developed to isolate purines and pyrimidines directly from λ-deoxyribonucleic acid (λ-DNA) and *Escherichia coli* cells. The sublimation of adenine, cytosine, guanine, and thymine from λ-DNA was tested under reduced pressure (∼ 0.5 Torr) at temperatures of >150 °C. With the exception of guanine, approximately 60−75% of each base was sublimed directly from the λ-DNA and recovered on a coldfinger of the sublimation apparatus after heating to 450 °C. Several nucleobases including adenine, cytosine, thymine, and uracil were also recovered from *E. coli* bacteria after heating the cells to the same temperature, although some thermal decomposition of the bases also occurred. These results demonstrate the feasibility of using sublimation to isolate purines and pyrimidines from native *E. coli* DNA and RNA without any chemical treatment of the cells.

Since nucleic acid bases have previously been found to have appreciable vapor pressures at temperatures above 150 °C,[1,2] these compounds will readily sublime when heated under vacuum at elevated temperatures. The sublimation of nucleic acid bases was first reported by Yamada and Fukutome in 1968 when they were able to deposit thin films of adenine, guanine, cytosine, thymine, and uracil on a cool substrate after heating the bases under a partial vacuum (∼10^{-5} Torr) at a temperature of 135−290 °C.[3] In these early experiments, the percent recoveries of the nucleic acid bases after sublimation were not quantified. Recent pyrolysis experiments carried out by Basiuk and co-workers have shown that adenine, cytosine, and uracil can be recovered at the 1−10% level after sublimation at temperatures above 400 °C.[4,5] However, it should be noted that these experiments were carried out at atmospheric pressure; therefore, the reported 1−10% nucleobase sublimation recoveries do not represent the optimum sublimation yield for these compounds at reduced pressure.

In this study, λ-DNA was heated under reduced pressure at temperatures between 150 and 750 °C in order to optimize the recoveries of adenine, guanine, cytosine, and thymine from the DNA. We also tested a sample of *Escherichia coli* to determine whether these purines and pyrimidines could be isolated directly from native bacterial DNA and RNA by sublimation. Previous experiments have demonstrated that the sublimation of thymine from DNA in various acid-hydrolyzed biological samples can be achieved under vacuum at 130 °C.[6] However, in contrast to these earlier experiments, the DNA and *E. coli* samples used in our sublimation experiments were not acid hydrolyzed prior to heating; therefore, the purines and pyrimidines were sublimed directly from the DNA. This sublimation method has also been used to isolate amino acids from *E. coli*; however, most of the amino acids in the bacteria did not sublime and were completely destroyed when the cells were heated.[7] In contrast, we found that most of the purines and pyrimidines in *E. coli* were readily sublimed from the bacteria and are apparently much more resistant to thermal degradation than amino acids.

EXPERIMENTAL SECTION

Chemicals and Reagents. Individual purine and pyrimidine standards (>99% purity) including adenine (A), cytosine (C), guanine (G), thymine (T), uracil (U), hypoxanthine (HX), and xanthine (X) were purchased from Sigma-Aldrich. A concentrated stock solution of these nucleobases (∼10^{-3} M), with the exception of guanine, was prepared by dissolving each base in double-distilled (dd) water. The guanine standard was prepared separately by filtration of a saturated solution of guanine dissolved in dd 0.01 M HCl (∼5×10^{-4} M). A λ-DNA standard (concentration, 0.467 μg/μL; length, 48 502 base pairs; guanine-cytosine content, 49.9%) was purchased from GibcoBRL and stored at 4 °C. Formic acid (minimum 95% purity) was obtained from Sigma-Aldrich.

Materials and Methods. A custom-made sublimation apparatus (SA) consisting of a quartz tube and Pyrex glass coldfinger (see Figure 1) that was designed to isolate amino acids from natural samples[7,8] was used to sublime purines and pyrimidines from λ-DNA and *E. coli* bacteria under reduced pressure at elevated temperatures. This apparatus is similar in design to one previously used for the sublimation of fatty acid methyl esters.[9] For the nucleobases and the λ-DNA, 100 μL of a standard solution was evaporated to dryness inside the quartz sublimation tube and the SA sealed at 0.5 Torr air. To initiate sublimation, the dried

* Corresponding author: (tel) 858-534-4258; (fax) 858-534-2674; (e-mail) jbada@ucsd.edu.

† Present address: Max-Planck-Institut für Chemie, Mainz, Germany D-55128.

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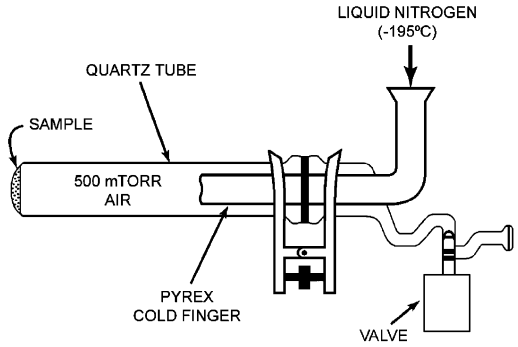

Figure 1. Diagram of the sublimation apparatus used in the heating experiments (not drawn to scale).

residues were then heated by placing the quartz tube inside a furnace set at various temperatures between 150 and 750 °C for 5 min. The interior temperature of the quartz tube where the sample was located was monitored during the sublimation using an Omega DP25-TC digital display meter and type K thermocouple. Prior to heating, the coldfinger was cooled to liquid nitrogen temperature (−195 °C). The temperature gradient inside the SA (furnace temperature to coldfinger temperature of −195 °C) during the heating experiment facilitated the vaporization of purines and pyrimidines at the bottom of the quartz tube and their condensation onto the end of the coldfinger. After sublimation was complete, the apparatus was removed from the furnace and the pressure inside the SA was brought up to 1 atm. The coldfinger was then rinsed with 1 mL of 0.01 M HCl, and the HCl extract was analyzed directly for purines and pyrimidines by high-performance liquid chromatography (HPLC) separation and UV absorption (λ = 260 nm). For the λ-DNA sample, the total concentration of adenine, guanine, cytosine, and thymine measured in the sublimed HCl extract was compared to the initial concentration of each base in the λ-DNA standard (calculated to be 3.6×10^{-4} M based on the λ-DNA concentration, the total number of base pairs, and the guanine−cytosine content) and a percent recovery was determined.

To determine the extent of λ-DNA fragmentation and chain breakage after the sublimation heating experiments, numerous agarose gel electrophoreses and ethidium bromide spot assays were carried out. After each sublimation experiment, the heated λ-DNA sample was dissolved in 1 mL of dd H_2O; the water extract was then transferred from the bottom of the sublimation tube to a capped Eppendorf tube and stored at 4 °C. For the gel electrophoresis experiment, a 10-μL aliquot of the heated λ-DNA water extract was run on 0.7% agarose gels in 0.5× TBE buffer[10] along with size standards (0.4 μg of λ-DNA cut with restriction enzyme *Hind*III and 0.5 μg of uncut λ-DNA). A staining buffer containing ethidium bromide was added to all the samples prior to electrophoresis to allow for λ-DNA recognition with short-wavelength UV light (∼320 nm). The gel was run at 30 mA (50 V) for 3 h and photographed under UV light. Ethidium bromide spot assays were conducted according to the Saran Wrap method.[10] For each spot assay, 10 μL of the heated λ-DNA water extract was added to 15 μL of ethidium bromide solution (2 μg/mL) and then photographed under short-wavelength UV light. As a control, 1 μL of the unheated λ-DNA standard was assayed in parallel. The following standards were used for λ-DNA concentration identification: 200, 100, 50, 25, 12.5, 6.25, and 0 ng.

E. coli bacteria (strain MG1655) were generously supplied by Dr. Douglas H. Bartlett (SIO). The *E. coli* cells were grown in glass tubes by shaking at 250 rpm overnight in 10 mL of Luria-Bertani (LB) medium[10] at 37 °C in a water bath. After overnight growth, the *E. coli* cells were transferred with the LB medium into Eppendorf tubes and the cells then concentrated by centrifugation at 6000 rpm for 2 min. The LB medium supernatant was then removed from the tubes, leaving behind a solid *E. coli* pellet that was rinsed with 1.5 mL of potassium phosphate-buffered saline (KPBS) and microfuged at 6000 rpm for 2 min, and the supernatant was removed. The KPBS washing procedure was repeated three more times in order to remove all residual LB medium from the bacteria pellet. Prior to sublimation, one *E. coli* pellet was sealed in a test tube with 1 mL of 95% formic acid and the test tube then placed in a heating block set at 100 °C for 24 h in order to extract purines and pyrimidines from the DNA and RNA present in the cells.[11] A separate *E. coli* pellet that had not been treated with formic acid was rinsed with potassium phosphate buffer, vacuum-dried, weighed (∼1.3 mg), and then sublimed in the SA at 450 °C for 5 min at 0.5 Torr. After heating, the coldfinger was rinsed with 2 mL of 0.01 M HCl and analyzed for the presence of purines and pyrimidines as previously described.

RESULTS AND DISCUSSION

Purines and Pyrimidines. To optimize the sublimation recoveries of adenine, guanine, cytosine, and thymine under reduced pressure, pure mixtures of these compounds were heated for 5 min at several different temperatures up to 750 °C. After heating to 150 °C, we found that less than 1% of the bases tested sublimed onto the coldfinger. The low nucleobase sublimation recoveries at this temperature were not surprising given that the vapor pressure of adenine and cytosine, reported to be ∼1 mTorr at 150 °C,[2,12] was much lower than the internal SA pressure at 500 mTorr; therefore, the sublimation of these bases at this temperature was not feasible. We did not test for the sublimation of these compounds at pressures lower than 500 mTorr. To our knowledge, the vapor pressures of guanine and thymine have not been reported.

In contrast to the low sublimation yield at 150 °C, we found that the optimum sublimation recoveries of adenine (99%), cytosine (81%), and thymine (98%) were achieved after heating the SA at 450 °C (see Figure 2), which indicated that the vapor pressure of these bases at this temperature is higher than 500 mTorr. We were unable to sublime any guanine (less than 1%) from the mixture at 450 °C, and although there are no apparent structural reasons for the low sublimation recoveries of guanine relative to other purines such as adenine, this finding is consistent with an earlier report.[4] Even though guanine did not sublime at 450 °C, we did not detect any xanthine on the coldfinger, which suggests that the thermal deamination of guanine did not occur during the experiment. It is also important to note that, after heating a mixture of all of the bases at the same time to 450 °C, a much

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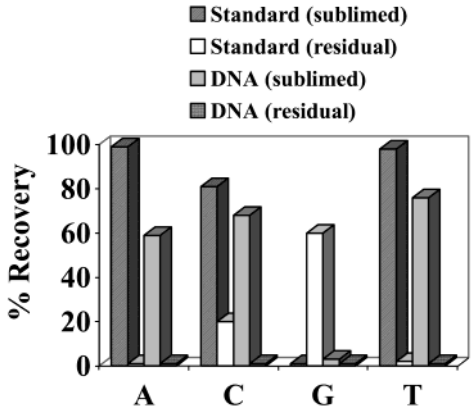

Figure 2. Sublimation recoveries of adenine (A), cytosine (C), guanine (G), and thymine (T) from pure standards and from dry λ-DNA after heating in the sublimation apparatus at 450 °C for 5 min (sublimed). The percent recoveries were calculated by dividing the number of moles of each base detected on the coldfinger after sublimation by the total moles of each base present in the samples prior to heating. A percent recovery for the nucleobases that did not sublime during the experiment, but were detected in the sublimation tube after heating, was also determined (residual). The uncertainty for the recoveries was ~±5% based on the standard deviation of the average value of two separate measurements.

lower yield of adenine (61%), cytosine (26%), and thymine (79%) was recovered on the coldfinger, compared to the recoveries when these bases were sublimed individually at this temperature (81−99%). This result could indicate that molecular interactions among these bases in the mixture (possibly including van der Waals' forces between stacked bases, base pairing, or both) partially inhibited the sublimation of these compounds from the dry state. Experimental studies on the molecular interactions between nitrogen bases of nucleic acids have previously shown that G−C, C−C, A−T, and T−T dimers will form in the crystalline phase at ~25 to 60 °C.[13] Therefore, it is possible that these base pairs formed in the dry crystalline state in our heating experiments.

***λ*-DNA.** For the λ-DNA standard, we also found that adenine, cytosine, and thymine could be sublimed directly from the sample at temperatures above 150 °C. The sublimation recovery of adenine, guanine, cytosine, and thymine from λ-DNA is shown in Figure 2. The optimum sublimation yield of these bases was obtained after heating the λ-DNA at 450 °C, where ~60−75% of adenine, cytosine, and thymine and less than 3% of guanine were recovered on the coldfinger. In addition to adenine, cytosine, guanine, and thymine, trace levels of uracil and possibly hypoxanthine (the identification of hypoxanthine is uncertain because this compound could not be separated from guanine using this HPLC method) were also identified by HPLC analysis of the coldfinger HCl extract after sublimation at 450 °C (see Figure 3a). Although uracil and hypoxanthine were not present in the λ-DNA standard, they could have been produced from the thermal decomposition of cytosine and adenine after heating. The deamination of cytosine residues in single-stranded DNA has been demonstrated in aqueous solution at temperatures above 65 °C.[14] This reaction would proceed by the hydrolysis of the amine group followed by a keto−enol tautomerization. However, xanthine,

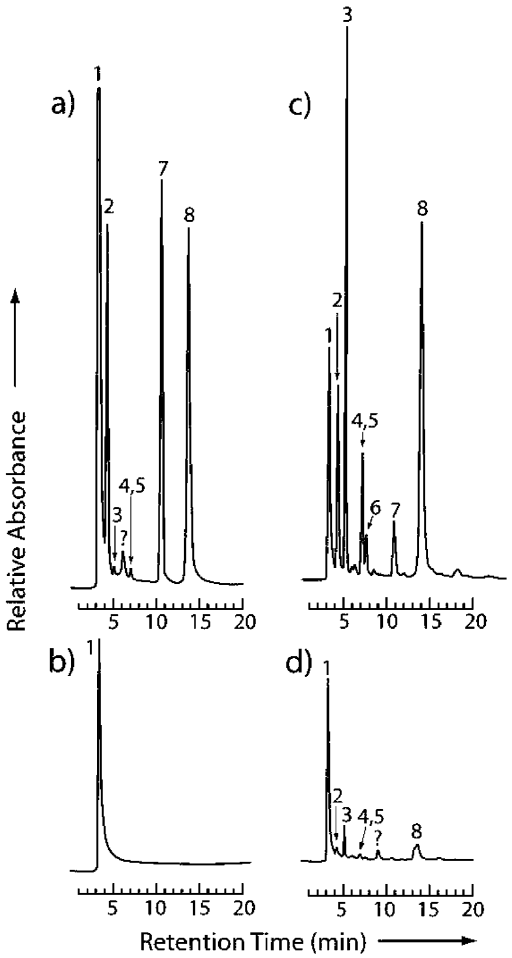

Figure 3. The 0−20-min region reversed-phase HPLC chromatograms of the sublimed HCl extracts from λ-DNA (a) and *E. coli* (c) after heating the samples in the SA at 450 °C for 5 min. For comparison, chromatograms from a blank carried through the same processing procedures as the DNA and *E. coli* are also shown (b + d). Peaks were identified by comparison of the retention times with those of a standard run in parallel. Peak identifications: (1) HCl front; (2) cytosine; (3) uracil; (4/5) guanine/hypoxanthine; (6) xanthine; (7) thymine; and (8) adenine.

which can also be produced from the thermal deamination of guanine by a similar pathway, was not identified (Figure 3a). We were unable to detect any purines or pyrimidines in an HCl blank that was carried through the identical procedure as the λ-DNA (see Figure 3b). The very low recovery of nucleobases in an HCl extract of the interior of the quartz sublimation tube after heating the λ-DNA at 450 °C (see Figure 2) suggested that all of the nucleobases either sublimed from the λ-DNA or were completely destroyed during the sublimation procedure.

Overall, the sublimation recoveries of the bases from the λ-DNA were lower than the recoveries from the pure nucleobase standards. This finding is not surprising because the glycosidic bonds that connect the bases to the λ-DNA strand must first be broken before the bases can sublime onto the coldfinger. When these bonds are broken, the stability of the λ-DNA is reduced and melting and fragmentation can occur.[15] In fact, ethidium bromide spot assays of the residual λ-DNA sample indicated that

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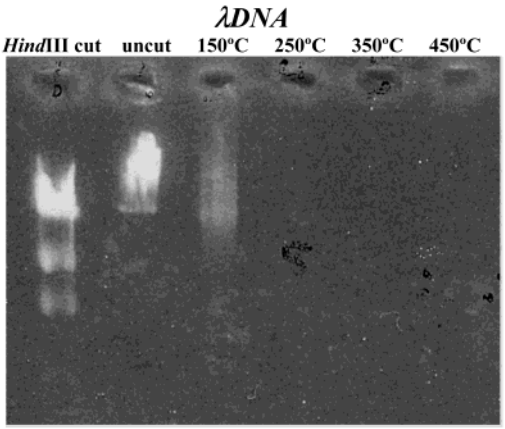

Figure 4. Agarose gel electrophoresis of λ-DNA after heating in the SA at elevated temperatures (lanes 3−6). For comparison, a 0.4-μg λ-DNA size standard cut with restriction enzyme *Hind*III (lane 1) and a 0.5-μg uncut λ-DNA sample (lane 2) that had not been heated are also shown.

Table 1. Summary of the Recovery of Nucleobases from *E. coli* in Formic Acid and Sublimed Extracts[a]

	E. coli cells		
nucleobase	formic acid[b]	sublimed[c]	untreated[d]
adenine	123 ± 20	99 ± 28	~100
cytosine	26 ± 4	42 ± 11	~100
thymine	14 ± 4	22 ± 7	~10
guanine	91 ± 13	<1 ± 1	~100
uracil	5 ± 4	64 ± 24	~90
hypoxanthine	<1 ± 1	31 ± 1	
xanthine	<1 ± 1	7 ± 1	

[a] All concentrations are reported as nanomoles per milligram of *E. coli* cell. The uncertainties are based on the standard deviation of the average value of two separate measurements. [b] Sample treated with formic acid at 100 °C for 24 h. [c] Sample heated inside the SA at 450 °C for 5 min. [d] Calculated values based on an average weight of a single *E. coli* cell of 9.5×10^{-13} g, and assuming the total concentration of DNA and RNA in *E. coli* to be 9×10^{-15} and 95×10^{-15} g/cell, respectively.[16] For this calculation, the average length of DNA in *E. coli*, estimated to be $\sim 3.9 \times 10^6$ and $\sim 1 \times 10^3$ base pairs for RNA was also taken into account.

the initial concentration of unheated λ-DNA (~500 ng/μL) decreased to 80 ng/μL after heating at 250 °C, to 8 ng/μL at 350 °C, and to less than 0.5 ng/μL at 450 °C (data not shown). Although the entire λ-DNA strand remained essentially intact at 150 °C (some minor fragmentation was observed at this temperature), strand breakage began to occur rapidly at a temperature of 250 °C and higher as indicated by agarose gel electrophoresis of the λ-DNA after heating (see Figure 4).

In summary, these results indicate that both DNA strand breakage and nucleobase sublimation will start to occur rapidly when λ-DNA is heated to temperatures above 150 °C at 500 mTorr. The optimum sublimation recovery of nucleobases from the λ-DNA was achieved when the temperature of the sublimation tube reached 450 °C and the λ-DNA was completely destroyed.

***E. coli* Cells.** Because high concentrations of nucleic acids are known to be present in *E. coli* cells,[16] this type of bacteria was used to investigate whether sublimation could be used to isolate purines and pyrimidines from bacterial DNA and RNA. The *E. coli* cells were heated in the SA at 450 °C for 5 min (the optimum sublimation temperature for the recovery of nucleobases from λ-DNA). HPLC analysis of the SA coldfinger HCl extract, after heating a solid *E. coli* pellet under reduced pressure inside the SA at 450 °C for 5 min, indicated that several purines and pyrimidines including adenine, cytosine, thymine, uracil, hypoxanthine, and xanthine had sublimed directly from the *E. coli* without any prior chemical treatment of the cells (see Figure 3c). Guanine did not sublime from the cells at this temperature. Some of these nucleobases, specifically adenine, cytosine, and thymine, were probably sublimed directly from native *E. coli* DNA based on the λ-DNA sublimation results (see Figure 2 and Figure 3a). Furthermore, since uracil is found in RNA, but not in DNA (thymine replaces uracil in DNA), the presence of high levels of uracil relative to thymine (U/T ~ 3) in the *E. coli* sublimed extract (see Table 1) suggests that a large fraction of the bases present on the coldfinger were derived from RNA. Only trace levels (<5 nmol/mg) of adenine, cytosine, guanine, and uracil were detected in the procedural blank (Figure 3d), which indicates that the purines and pyrimidines recovered on the coldfinger after sublimation were derived entirely from the native *E. coli* DNA and RNA and were not associated with any remnants of the LB growth medium. We were unable to detect any purines or pyrimidines in a formic acid extract of the heated cell pellet after sublimation at 450 °C; therefore, these compounds either sublimed from the cells or were completely destroyed in the course of the sublimation procedure.

In addition to sublimation, a separate *E. coli* pellet was extracted using a standard formic acid treatment commonly used to isolate purines and pyrimidines from bacterial DNA[11] and then analyzed for nucleobases by HPLC. In Table 1, we report the nucleobase recoveries for adenine, cytosine, thymine, guanine, uracil, hypoxanthine, and xanthine in the formic acid and sublimed extracts of *E. coli*. The theoretical concentration of nucleobases in an untreated *E. coli* sample based on the average concentration of DNA and RNA in a single *E. coli* cell is also shown for comparison. We found that the recoveries of adenine, cytosine, and thymine from *E. coli* after formic acid and sublimation extraction were similar within the uncertainties of the measurements. The most notable difference in recoveries between the two extraction methods was that high levels of uracil, hypoxanthine, and xanthine were detected in the sublimed extract of the cells but were found only in trace amounts in the formic acid extract (see Table 1). Thermal decomposition of adenine into hypoxanthine and guanine into xanthine during the sublimation heating is the most likely explanation for the presence of these two nucleobases in the sublimed *E. coli* extract. Even though hypoxanthine and xanthine were not observed in any of the previous sublimation experiments with dry λ-DNA, the presence of a significant amount of water in *E. coli* (~ 70 wt %)[16] could accelerate the deamination of adenine and guanine at elevated temperatures. Uracil could have been produced from the decomposition of cytosine in the *E. coli* DNA and RNA,[14] as well as from the sublimation of uracil directly from RNA. Because the sublimation yield of uracil from an RNA standard was not tested in this study, we are unable to estimate what fraction of sublimed uracil was derived from the *E. coli* RNA.

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The ratio of uracil to thymine (U/T) in *E. coli* extracts could potentially be used to estimate the ratio of RNA to DNA present in the cells. Formic acid treatment of *E. coli* at 100 °C for 24 h prior to sublimation yielded a U/T ratio of ∼0.4 while a much higher ratio (U/T ∼3) was measured in the sublimed *E. coli* extract (see Table 1). One explanation for the high U/T ratio in the sublimed extract relative to the formic acid extract is that some of the cytosine originally present in the cellular RNA and DNA decomposed to uracil by deamination during the sublimation heating. This is consistent with a significantly higher yield of uracil as well as hypoxanthine and xanthine in the sublimed cell extract (Table 1). Because we were unable to estimate how much cytosine decomposed to uracil during the *E. coli* sublimation heating, the U/T ratio of sublimed *E. coli* extracts is not an accurate estimation of the ratio of cellular RNA to DNA. Furthermore, it should be pointed out that since the concentration of RNA in *E. coli* is known to be ∼10 times higher than DNA,[16] the U/T ratio in *E. coli* should be much higher than the U/T ratios calculated for both formic acid and sublimed extracts of *E. coli*.

CONCLUSIONS

The recovery of intact purines and pyrimidines from λ-DNA and *E. coli* bacteria was achieved at elevated temperatures under reduced pressure. With the exception of guanine, approximately 60−75% of the bases were sublimed from a λ-DNA standard and collected on a coldfinger after heating at 450 °C for 5 min at a pressure of 500 mTorr. Guanine did not sublime from the λ-DNA at this temperature. This result suggests that the vapor pressure of guanine at this temperature is much lower than the internal SA pressure; therefore, pressures lower than 500 mTorr, temperatures higher than 450 °C, or both are required to sublime guanine from λ-DNA. Although some of the nucleobases in the λ-DNA were clearly destroyed after heating at 450 °C, we did not detect any thermal decomposition products of adenine, guanine, or cytosine on the coldfinger. Preliminary experiments have also demonstrated that it is possible to isolate adenine, cytosine, thymine, and uracil from DNA and RNA in *E. coli* cells by sublimation at 450 °C; however, some deamination of the bases occurred at this temperature. In contrast to the traditional protocol for isolating purines and pyrimidines from nucleic acids in bacteria that requires heating the bacteria in formic acid, sublimation does not require any wet chemical treatment of the cells and can be carried out in less than 3 h compared to formic acid treatment, which requires more than 1 day. In order for this technique to be used to quantify the amount of DNA and RNA in biological samples, additional experiments on the sublimation of *E. coli* cells and RNA standards will be required.

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