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Polycyclic Aromatic Hydrocarbons Physically Intercalate into Duplex Regions of Denatured DNA†

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ABSTRACT: We have investigated the physical binding of pyrene and benzo[*a*]pyrene derivatives to denatured DNA. These compounds exhibit a red shift in their absorbance spectra of 9 nm when bound to denatured calf thymus DNA, compared to a shift of 10 nm when binding occurs to native DNA. Fluorescence from the hydrocarbons is severely quenched when bound to both native and denatured DNA. Increasing sodium ion concentration decreases binding of neutral polycyclic aromatic hydrocarbons to native DNA and increases binding to denatured DNA. The direct relationship between binding to denatured DNA and salt concentration appears to be a general property of neutral polycyclic aromatic hydrocarbons. Absorption measurements at 260 nm were used to determine the duplex content of denatured DNA. When calculated on the basis of duplex binding sites, equilibrium constants for binding of 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene to denatured DNA are an order of magnitude larger than for binding to native DNA. The effect of salt on the binding constant was used to calculate the sodium ion release per bound ligand, which was 0.36 for both native and denatured DNA. Increasing salt concentration increases the duplex content of denatured DNA, and it appears that physical binding of polycyclic aromatic hydrocarbons consists of intercalation into these sites.

Polycyclic aromatic hydrocarbons (PAH)[1] are an important class of biologically active compounds. They are mutagenic and carcinogenic and undergo physical and covalent interactions with DNA (Heidelberger, 1975; LeBreton, 1985). Although numerous reports on the physical intercalation of charged aromatic dyes have appeared (Berman & Young, 1981; Dougherty & Pilbrow, 1984), much less attention has been given to the weaker binding of neutral PAH to DNA.

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[1] Abbreviations: PAH, polycyclic aromatic hydrocarbons; BP, benzo[*a*]pyrene; *trans*-tetrol, racemic 7*r*,8*t*,9*t*,10*c*-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; *cis*-tetrol, racemic 7*r*,8*t*,9*t*,10*t*-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; 7,8-di(OH)H_2BP, racemic *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene; 7,8-di(OH)H_4BP, racemic *trans*-7,8-dihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; BPDE, (±)-7*r*,8*t*-dihydroxy-9*t*,10*t*-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide.

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The solubility of PAH was reported to markedly increase in the presence of both native and denatured DNA (Liquori et al., 1962; Boyland & Green, 1962; T'so & Lu, 1964). However, these groups had conflicting results concerning the relative solubilities of PAH in the two forms of DNA. This issue was resolved when it was recognized that ionic strength had a differential effect on the solubilities of PAH in native and denatured DNA (Boyland & Green, 1964). Raising $[Na^+]$ from 2 to 90 mM resulted in a 5-fold decrease in the solubilities of benzo[*a*]pyrene (BP) and pyrene in native DNA, while a doubling of BP solubility and a 4-fold increase in pyrene solubility was found in denatured DNA (Boyland & Green, 1964).

On the basis of model-building studies and by analogy to the type of binding observed with proflavin, Boyland and Green (1962) proposed that PAH physically intercalate into native DNA. Observations of linear dichroism (Nagata et al., 1966) and fluorescence polarization (Green & McCarter, 1967) of PAH bound to flow-oriented DNA supported this model. More recent studies on the quenching of triplet excited states of bound PAH by molecular oxygen (Geacintov et al., 1976), the electric linear dichroism of physically bound PAH derivatives (Geacintov et al., 1978; Ibanez et al., 1980), and DNA unwinding angle measurements for PAH derivatives (Meehan et al., 1982) have essentially confirmed the intercalation model for binding of neutral PAH to native DNA. The mode of PAH binding to denatured DNA is more uncertain. Studying these complexes presents experimental difficulties due to limitations in methodology or in PAH solubility.

This investigation was undertaken to determine the nature of the interactions between neutral PAH and denatured DNA. Spectroscopic evidence and equilibrium data are used to characterize binding of neutral planar aromatic hydrocarbons to native and denatured DNA. We report that neutral PAH physically intercalate into denatured DNA at duplex sites promoted by the presence of counterions.

Experimental Procedures

Chemicals. (±)-*trans*-7,8-Dihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [7,8-di(OH)H_4BP] and (±)-7*r*,8*t*-dihydroxy-9*t*,10*t*-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE) were synthesized essentially as described previously (Meehan et al., 1982). BPDE was hydrolyzed to its 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (tetrol) derivatives by mild acid treatment. The *cis*- and *trans*-tetrols were separated by high-performance liquid chromatography (Yang et al., 1977). The trans isomer was used exclusively in these studies. Stock solutions of *trans*-tetrol were made with 25% dimethyl sulfoxide (DMSO) in methanol and stored at −20 °C. Samples of BP derivatives were shielded from light. Pyrene (Aldrich, Milwaukee, WI) was used as supplied.

DNA Purification. High molecular weight, calf thymus DNA was obtained from commercial sources and purified by RNase treatment, phenol extraction, and dialysis. DNA concentration was determined with ϵ_{260} = 6550, in 10 mM NaCl.

DNA Denaturation. DNA was denatured by boiling in water 30 min in tightly capped vials and quenching in ice–water. It was stored frozen. When needed for experiments, it was thawed, boiled for 5 min, and quenched in ice–water. These solutions were kept at 0 °C until used. In experiments where high levels of sodium chloride were used (>20 mM), the salt was added just prior to measurement in order to minimize renaturation. Hyperchromicity was determined by diluting the denatured DNA in water or dilute (2 mM sodium phosphate, pH 7.5) buffer and measuring absorbance at 260 nm. Hyperchromicities ranged from 32 to 36%.

Instruments. Absorbance measurements were performed on a Cary Model 118 spectrophotometer equipped with a thermostatted cell holder. For intercalation studies the temperature of the cell was maintained at 21 ± 0.2 °C. Absorption of denatured DNA at 260 nm as a function of salt concentration was measured at room temperature. Fluorescence measurements were made with a Perkin-Elmer Model 650-10S fluorescence spectrophotometer. Emission of the tetrol was measured at 401 nm with excitation set at 344 nm. All measurements were made at room temperature with 1×10^{-7} M tetrol.

Determination of Equilibrium Constants. Equilibrium constants were determined with modifications to previously reported procedures (Meehan et al., 1982). When *r*, the ratio of bound ligand to DNA base pairs, is near zero, the equilibrium binding constant *K* can be determined from a double-reciprocal plot of the change in the apparent extinction coefficient of the ligand vs. DNA concentration (Schmechel & Crothers, 1971). The equation representing this relationship is

$$\frac{1}{\Delta\epsilon_{ap}} = \frac{1}{\Delta\epsilon KD} + \frac{1}{\Delta\epsilon}$$

where $\Delta\epsilon_{ap} = |\epsilon_a - \epsilon_F|$, $\Delta\epsilon = |\epsilon_B - \epsilon_F|$, and ϵ_a, ϵ_F, and ϵ_B are the apparent, free, and bound ligand extinctions, respectively. *D* is the DNA concentration in base pairs for native DNA and in potential base pairs (half the concentration of bases) for denatured DNA. Multiplying by *D* puts the equation in the half-reciprocal form:

$$\frac{D}{\Delta\epsilon_{ap}} = \frac{1}{\Delta\epsilon}D + \frac{1}{\Delta\epsilon K} \quad (1)$$

A plot of $D/\Delta\epsilon_{ap}$ vs. *D* will have a slope of $1/\Delta\epsilon$ and a *y* intercept equal to $1/(\Delta\epsilon K)$. *K* is then given by the ratio of the slope to the *y* intercept. ϵ_B is obtained from $\Delta\epsilon$ and a measured value of ϵ_F. Since a double-reciprocal plot gives excessive weight to data points obtained at low *D*, the half-reciprocal plot should generally be more accurate.

Results

Changes in the absorption spectrum of *trans*-tetrol in the presence of native DNA as a function of sodium chloride concentration are shown in Figure 1A. The peak at 343 nm represents free *trans*-tetrol, while the peak at 353 nm represents the red-shifted intercalation complex between the hydrocarbon and native DNA (Meehan et al., 1982). Salt concentration was varied between 0 and 1 M. As salt concentration is increased, the amount bound is reduced (decreased absorbance at 353 nm and increased absorbance at 343 nm).

Figure 1B displays the absorption spectrum of *trans*-tetrol in the presence of denatured calf thymus DNA. With no added salt the spectrum represents primarily that of the free hydrocarbon (long-wavelength peaks at 327 and 343 nm). As the ionic strength is increased, the absorbance at 343 nm decreases and the absorbance at 352 nm increases, indicating the formation of a binding complex between *trans*-tetrol and denatured DNA.

Isosbestic points are observed near 347 nm for *trans*-tetrol binding to native and denatured DNA. These results suggest that for each form of DNA there is a single observable bound form of *trans*-tetrol.

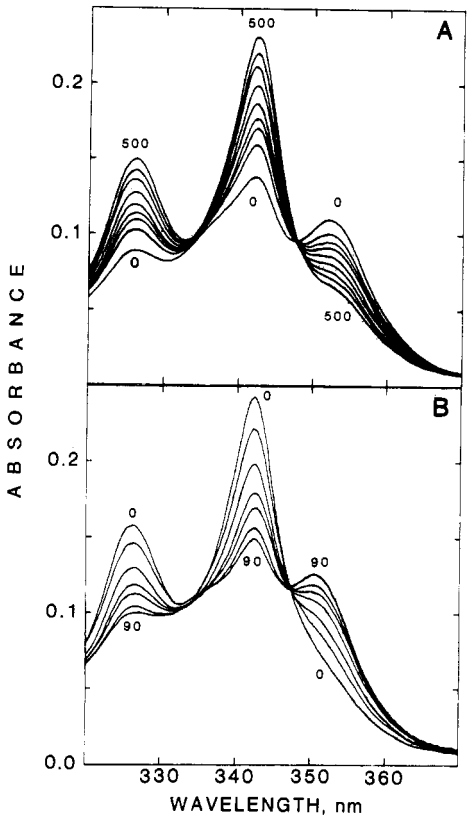

FIGURE 1: Absorption spectra of *trans*-tetrol (8×10^{-6} M) physically bound to native or denatured DNA (0.75×10^{-3} M in base pairs or potential base pairs, respectively) at varying levels of NaCl (extremes are indicated). Buffer was 2 mM phosphate (pH 7.5) containing 0.1 mM EDTA, 1.25% DMSO, and 0.75% methanol and contributed 5.7 mM sodium in addition to the indicated salt. (A) Native DNA and 0, 10, 20, 30, 50, 90, 150, 240, and 500 mM NaCl. (B) Denatured DNA and 0, 3.75, 10, 20, 30, 50, and 90 mM NaCl.

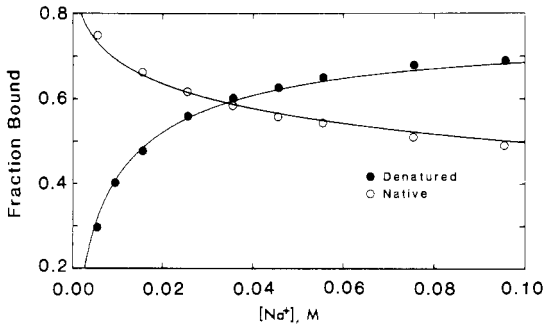

FIGURE 2: Binding isotherms of *trans*-tetrol and native (○) or denatured (●) DNA. Fraction bound was determined from the apparent extinction (Figure 1), the extinction of free *trans*-tetrol, and the calculated extinction of bound *trans*-tetrol. Curves were calculated from the corresponding line or curve in Figure 3. Conditions are the same as indicated in Figure 1.

The red shift of the absorbance peak of bound *trans*-tetrol and the other PAH tested is about 1 nm greater with native DNA (at low salt) than with denatured DNA (at high salt), suggesting that while the complexes are similar they are not identical. The fluorescence of tetrol (Ibanez et al., 1980; Meehan et al., 1982) and pyrene (Geacintov et al., 1976) when physically intercalated into native DNA is completely quenched. The fluorescence of the *trans*-tetrol and pyrene when bound to denatured DNA is similarly quenched (data not shown).

Figure 2 shows the salt dependence for the binding of *trans*-tetrol to native and denatured DNA, as determined by absorbance measurements. We have also used fluorescence

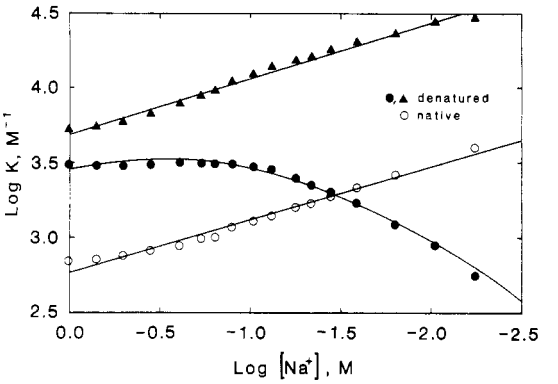

FIGURE 3: log K vs. − log sodium ion concentration for binding of *trans*-tetrol to native (○) or denatured (●, ▲) DNA. Binding constants were calculated from the fraction of *trans*-tetrol bound (Figure 2) and either the total (○, ●) DNA concentration or the concentration of duplex in denatured DNA (▲). Lines for native DNA (○) and duplex denatured DNA (▲) are from least-squares regression. The curve for denatured DNA (●) was calculated from the regression line and the expression (eq 2 under Results) for duplex content of denatured DNA as a function of sodium ion concentration. Conditions are the same as indicated in Figure 1.

to monitor the amount of bound hydrocarbon as a function of salt concentration and find the same general trends as obtained with the absorbance data shown in Figure 2. Other neutral PAH tested [BPDE, 7,8-di(OH)H_4BP, and pyrene] show the same relationship between binding levels and salt concentration as *trans*-tetrol. Binding of charged intercalators (ethidium bromide and propidium iodide) to denatured DNA is reduced with increasing salt concentration (data not shown). Thus, salt concentration has opposite effects on the binding of these neutral PAH and charged dyes to denatured DNA, and the results appear to reflect general properties of these two classes of compounds.

The equilibrium constants for *trans*-tetrol binding to native and denatured DNA as a function of salt concentration are given in Figure 3. From a theoretical viewpoint, the slope of the log K vs. − log [Na^+] plot is expected to equal the number of condensed cations released from the polynucleotide per bound ligand (Record et al., 1976a). If the mechanism of ligand binding does not change with salt concentration, the plot should be linear. For binding of *trans*-tetrol to native DNA, the expected linear plot is obtained. However, when calculated on the basis of a constant number of binding sites, the plot for *trans*-tetrol binding to denatured DNA is curved. Thus, the assumption that there are a constant number of binding sites in denatured DNA may be incorrect.

One possibility is that *trans*-tetrol binds only to the duplex portion of denatured DNA, which increases with salt concentration. This change in duplex content is due primarily to short-range, intrastrand base pairing, which results in the formation of hairpin loops (Doty et al., 1959; Studier, 1969; Walz, 1972). The duplex content of denatured DNA can be estimated from the decrease in absorption at 260 nm with increasing salt concentration. We have found (unpublished data) that the mole fraction of duplex (χ_{ds}) in denatured DNA can be expressed as

$$\chi_{ds} = \frac{0.69K_d[Na^+]}{1 + K_d[Na^+]} \qquad (2)$$

The value of the equilibrium constant K_d was determined to be 5.9 M^{-1} at room temperature. The extrapolated duplex fraction of denatured DNA at infinite salt concentration is 0.69. If *trans*-tetrol binds only to the duplex portion of de-

natured DNA, then the number of binding sites in denatured DNA changes with $[Na^+]$, and the factor χ_{ds} must be inserted in the expression for the equilbrium constant, giving

$$K = \frac{C_B}{C_F(\chi_{ds}D - C_B)} \quad (3)$$

where C_B and C_F are concentrations of bound and free *trans*-tetrol, respectively. $\chi_{ds}D - C_B$ is the concentration of unoccupied duplex binding sites.

If the equilibrium constant for *trans*-tetrol binding to denatured DNA as a function of salt is recalculated with eq 3, a linear plot is obtained that is parallel to the plot for native DNA (Figure 3). The success of the correction supports this model. Conversely, the linearity of the uncorrected plot for native DNA indicates that the number of *trans*-tetrol binding sites in native DNA does not change with salt concentration.

The slopes of the log K vs. $-\log [Na^+]$ plots are 0.35 for native DNA and 0.37 for denatured DNA. Thus, the same number of condensed cations are released upon binding of *trans*-tetrol to both forms of DNA. However, the equilibrium constants for binding of *trans*-tetrol to denatured DNA are approximately 9 times greater than for binding to native DNA.

Discussion

Duplex formation in denatured DNA is facilitated by sodium ions because counterion shielding is needed both to overcome repulsion between strands and to allow the axial intrastrand distance between phosphates to decrease in the coil-to-helix transition. The great majority of the helices formed should be due to short-range intrastrand interactions, which are favored from a kinetic, entropic, and electrostatic standpoint (Studier, 1969). Single-strand base stacking also occurs to some degree in denatured DNA, but this type of stacking is not significantly affected by changing salt concentration (Studier, 1969).

The differential effect of salt on PAH solubilization by native and denatured DNA has been known for some time (Boyland & Green, 1964; Kodama et al., 1966; Lesko et al., 1968; Chen, 1983). Early workers assumed that salt acted by changing the number of binding sites in both native and denatured DNA (Liquori et al., 1962; Boyland & Green, 1964; Lesko et al., 1968); our results indicate that this idea is correct only for denatured DNA. When denatured DNA is treated with formaldehyde, which reacts reversibly with the amino groups of bases so as to prevent base pairing (Haselkorn & Doty, 1961), the salt-induced increase in PAH solubility is abolished (Boyland & Green, 1964; Kodama et al., 1966), which suggests that duplex DNA is required for binding.

The measurements of triplet excited state lifetimes of DNA-bound BP (Geacintov et al., 1976) also support intercalative binding to denatured DNA. In degassed solutions, quenching of the excited state presumably occurs via stacking interactions between the BP and DNA bases. The lifetime of BP is significantly shorter in denatured DNA compared to native DNA, suggesting that denatured DNA·BP stacking interactions are stronger. Triplet excited states of PAH are strongly quenched by molecular oxygen; this quenching is reduced 10–20-fold by binding to native DNA. Denatured DNA is only slightly less effective in preventing quenching by O_2. The inaccessibility of bound BP to O_2 suggests that the hydrocarbon is intercalated within duplex regions of native or denatured DNA.

The counterion release that accompanies binding of a ligand to DNA (given by the slope of the log K vs. $-\log [Na^+]$ plot) provides an entropic driving force for the reaction. For an uncharged intercalator the expected salt release (Wilson & Lopp, 1979) is

$$\frac{-\delta \log K}{\delta \log [Na^+]} = 2n(\psi_1 - \psi_2) \quad (4)$$

where n is the number of phosphates on each strand affected by the conformational change due to binding of the intercalator and ψ_1 and ψ_2 are the number of Na^+ ions thermodynamically bound per affected DNA phosphate before and after ligand binding, respectively. Using the relation $\psi = 1 - b/1.42$ (Record et al., 1976a,b), where b is the axial distance between phosphates in nanometers, the expected number of sodium ions released upon binding per neutral intercalator to native DNA is 0.24. Our measured value of 0.36 with both native and denatured DNA is 50% larger. The reason for the disparity is unclear; however, the similar counterion release shows that the change in phosphate–phosphate distances and the number of phosphates affected are nearly the same for *trans*-tetrol binding to native and denatured DNA. This implies that the DNA conformational change is nearly the same for the two processes.

On the basis of the similarities in their absorption, fluorescence, and counterion-release properties and the requirement for duplex content in binding to denatured DNA, it may be concluded that the geometries of the binding complexes between PAH and native and denatured DNA are very similar. Since intercalation has been established for native DNA, it appears to be the mode of PAH binding to denatured DNA as well. Given this similarity, the 9-fold greater affinity of *trans*-tetrol for duplex denatured DNA over native DNA is striking. The intrastrand helices in denatured DNA are much shorter than in a typical sample of native DNA and probably have a much higher mismatch content (Studier, 1969). These changes may increase the flexibility of the helix, leading to more favorable stacking interactions between the PAH and bases or decreasing the energy needed to unwind the helix. Similar factors may be responsible for the increased affinity of tetrol binding to protonated native DNA, which is destabilized relative to native DNA at neutral pH (Chen, 1984). There are indications that, in RNA, psoralens bind preferentially to ends of double-stranded regions, where base pairs tend to fray easily (Thompson & Hearst, 1983).

The differential effect of salt on physical binding levels of PAH in native and denatured DNA helps to explain a number of previously reported observations. The binding of 7,8-di-(OH)H_2BP to single-stranded ϕX174 DNA has been shown to reduce its infectivity (Hsu et al., 1979, 1981). Binding of the hydrocarbon was noncovalent and greater to single-stranded ϕX174 DNA than to the double-stranded RF form. While the 7,8-di(OH)H_2BP was incubated with the DNA at low salt concentration, prior to ethanol precipitation the salt was raised to ~80 mM, well above the point where binding to denatured DNA is favored. On the other hand, it has been reported that native DNA quenches fluorescence of bound PAH to a much greater degree than denatured DNA (LeBreton, 1985). These studies were carried out in 1 mM buffer without added salt (Zegar et al., 1984), so physical binding to denatured DNA would be expected to be weak. We have also reported that binding to denatured DNA is weak under low ionic strength conditions (Meehan et al., 1982).

The effect of 7,8-di(OH)H_2BP on ϕX174 indicates that the biological activity of single-stranded DNA can be altered by physical binding of PAH. In addition, physical intercalation has been proposed as a prerequisite for covalent binding of activated PAH to DNA (Meehan & Straub, 1979; Meehan

& Bond, 1984; Lin et al., 1980). It is believed that DNA alkylation is responsible for the mutagenicity and tumorigenicity of PAH. Steric restrictions imposed by intercalation may lead to differences in the accessibility of the exocyclic amino group of guanine to the epoxide rings of (+)- and (−)-*anti*-BPDE, which would explain the enantioselectivity of the covalent binding to DNA (Meehan & Straub, 1979; Meehan & Bond, 1984; MacLeod & Zachary, 1985). Shahbaz et al. (1986) have shown that *anti*-BPDE physically intercalates into DNA to a greater extent than *syn*-BPDE. After alkylation of dG by either diastereomer, only the anti form rearranges, ultimately residing on the outside of the DNA helix. Thus, intercalation levels and conformations of the complexes may play a crucial role in the relative carcinogenicity of BPDEs and other PAH.

Several reports indicate that the decrease in physical binding of activated PAH to native DNA with added monovalent or divalent cations is associated with a decrease in covalent binding to DNA (Gamper et al., 1980; MacLeod et al., 1982; Geacintov et al., 1984; Meehan & Bond, 1984). It has been pointed out that if physical binding of BPDE occurs prior to covalent binding, then there must be a correlation between the two types of binding under conditions where DNA-catalyzed hydrolysis is minimal (Geacintov, 1986). Consistent with this analysis, we find that under high ionic strength conditions covalent binding of BPDE occurs to a greater extent with denatured than with native DNA (unpublished data). This observation supports the suggestion that physical intercalation is a prerequisite to covalent binding (Meehan & Straub, 1979). It has been reported that the ability of various PAH to physically intercalate correlates with their carcinogenic potency (Harvey et al., 1985). Thus the effect of DNA secondary structure on physical intercalation levels of PAH may have a significant influence on the carcinogenicity of these hydrocarbons.

Registry No. BPDE, 58917-67-2; 7,8-di(OH)H_4BP, 64314-00-7; *trans*-tetrol, 62697-19-2; pyrene, 129-00-0; sodium, 7440-23-5.

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