Bifunctional Catalysis in the Nucleotide-Catalyzed Hydrolysis of (±)-7β,8α-Dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene

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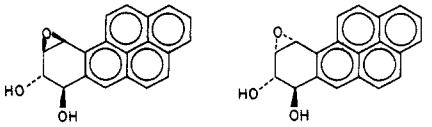
The rates of reaction of (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (DE-2) in 1:9 dioxane–water (v/v) solutions containing the 2′-, 3′-, and 5′-monophosphate derivatives of guanosine, adenosine, and cytidine have been determined. From plots of k_{obsd} vs. concentration of nucleotide at several different pH values, it could be concluded that the monohydrogen phosphate ionization state was responsible for catalyzing the hydrolysis of DE-2. It was therefore assumed that the monohydrogen phosphate group acted as a general acid catalyst in the epoxide hydrolysis reaction. The second-order rate constants for the general acid catalyzed hydrolysis of DE-2 by the nucleotides listed above were determined. The 2′- and 5′-monophosphates of guanosine and adenosine are better general acid catalysts than the corresponding 3′-isomers, although the 3′-isomers are stronger acids. The guanosine and adenosine nucleotides ae better catalysts than the cytidine monophosphates. It is concluded that not only does the monohydrogen phosphate group act as a general acid but the secondary interactions of a stacking nature between the aryl group of DE-2 and the base are important for catalytic effectiveness.

The environmental carcinogen benzo[a]pyrene is metabolized in part to a mixture of diol epoxides consisting of (+)-DE-2 (7(*R*),8(*S*)-dihydroxy-9(*S*),10(*R*)-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene) as a major component and (−)-DE-1 (7(*R*),8(*S*)-dihydroxy-9(*R*),10(*S*)-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene) as a minor component.[1]



(−)-DE-1 (+)-DE-2

These diol epoxide metabolites react in vivo with DNA to form covalent adducts[2] and are thought to be reagents responsible for the mutagenic and carcinogenic properties of the parent hydrocarbon. Noteworthy is the fact that the major 7,8-diol 9,10-epoxide metabolites ((+)-DE-2) of benzo[a]pyrene is highly tumorigenic to newborn mice, whereas its enantiomer ((−)-DE-2) and diastereomers ((−)-DE-1) and ((+)-DE-1) are essentially nontumorigenic.[3,4]

The hydrolysis reactions of DE-1 and DE-2 have been studied in detail[5] and are known to be general acid catalyzed.[6] Experiments have also shown that the rates of reaction of both DE-1[7] and DE-2[8] are accelerated in solutions containing DNA and that >90% of the DNA-promoted reactions result in hydrolysis of the diol epoxides to tetrols.

We recently reported that guanosine 5′-monophosphate (5′-GMP) acts as an efficient general acid in catalyzing the hydrolysis of DE-1 and DE-2 at pH ~7 in aqueous dioxane solutions and not as a nucleophilic reagent.[9] The catalytic effectiveness of 5′-GMP was found to be ca. 60 times greater than that for $H_2PO_4^-$ although 5′-GMP (monobasic form) and $H_2PO_4^-$ possess similar pK_a values and therefore are expected to have similar strengths as proton donors. It was suggested that the enhanced effectiveness of 5′-GMP as a general acid catalyst might be due to favorable association complexes between GMP and DE-1 and DE-2 at the transition state. A favorable transition-state complex between DE-2 and 5′-GMP could have a structure, for example, in which the monohydrogen phosphate group is positioned to donate a proton to the epoxide oxygen and the pyrene moiety is favorably located for stacking interactions with the guanine base.

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A dramatic demonstration of this mechanism for bifunctional catalysis in the hydrolysis reactions of diol epoxides was reported that described the hydrolysis of DE-2 catalyzed by riboflavin 5′-phosphate.[10] A physical complex between DE-2 and riboflavin 5′-phosphate (FMN) was detected by both spectrophotometric titration measurements and rate data at pH 6–7, and the association constant was calculated to be ca. 1400–3400 M^{-1}. Only the monoanion of FMN, in which there is a proton located on the phosphate group, is effective in catalyzing the hydrolysis of DE-2. It was determined that riboflavin formed an association complex with DE-2 but was not effective as a catalyst in promoting its hydrolysis. Therefore, both complexation and the presence of a proton-donating monohydrogen phosphate group are required for effective catalysis at pH 6–7. In order to determine the effects of individual bases at the mononucleotide level on the hydrolysis of diol epoxides, we have carried out kinetic studies on the hydrolysis reactions of DE-2 in dioxane–water solutions containing the 2′-, 3′-, and 5′-monophosphate derivatives of guanosine, adenosine, and cytidine. Our results show that the effectiveness of nucleotides as general acid catalysts in the hydrolysis of DE-2 is a function both of the nature of the base and of the location of the monohydrogen phosphate group.

Experimental Section

Materials. DE-2 was prepared by a published procedure.[11] Guanosine 5′-monophosphate (5′-GMP), adenosine 5′-monophosphate (5′-AMP), cytidine 5′-monophosphate (5′-CMP), phenyl phosphate, and 1-naphthyl phosphate were purchased as their sodium salts from Aldrich Chemical Co., Milwaukee, WI. The 2′- and 3′-monophosphates of guanosine as their sodium salts and 2′- and 3′-monophosphates of adenosine and cytidine as their free acids were purchased from Sigma Chemical Co., St. Louis, MO. The purities and apparent pK_a's for the second ionization of all phosphates in 1:9 dioxane–water solution (μ = 0.1 M, $NaClO_4$) were determined by titration with a Radiometer automatic titrator assembly. Buffer solutions of each monophosphate in 1:9 dioxane–water (v/v) were prepared by adding appropriate amounts of either perchloric acid or sodium hydroxide to solutions of the disodium salt or free acid of the monophosphate, respectively. The ionic strength of each solution was adjusted to 0.10 M by addition of $NaClO_4$. Dioxane was distilled from sodium metal.

Kinetic Procedures. The rate constants for reaction of DE-2 in series of diluted solutions of a given monophosphate in 1:9 dioxane–water (v/v) at constant pH and ionic strength (0.1 M $NaClO_4$) but varied phosphate concentrations were determined. Our reason for selecting 1:9 dioxane–water for the hydrolysis medium is that the solubility of DE-2 in wholly aqueous solutions of salt at 0.1 M or greater is marginal. The pH values of the diluted monophosphate solutions were adjusted to that of the most concentrated solution to within 0.01 pH unit when necessary. For each kinetic run, approximately 5–10 μL of a stock solution of ca. 0.1 mg of DE-2 in 0.1 mL of Me_2SO was added to 2.5 mL of monophosphate solution in the thermostatted cell compartment (25.0 ± 0.2 °C) of a Gilford 2400 or Gilford response spectrophotometer. Reactions were monitored at 348 nm, and pseudo-first-order rate constants (k_{obsd}) were calculated by nonlinear regression analysis of the absorbance vs. time data.

The second-order rate constant $k(ROPO_3H^-)$ for each monophosphate was obtained as the slope of a weighted least-squares plot of k_{obsd} vs. $[ROPO_3H^-]$, the concentration of the monohydrogen anion form of the phosphate. In those cases where $k(ROPO_3H^-)$ was determined at two or more pH values, an average value is provided.

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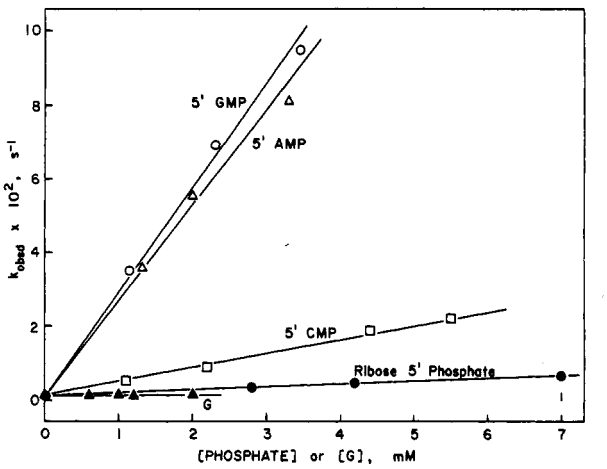
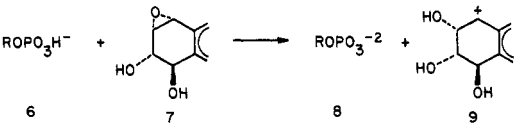


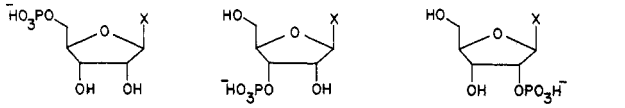
Figure 1. Plots of k_{obsd} vs. concentrations of (*f*)$[RO\textcircled{P}]_{TOT}$ ([phosphate]) or guanosine ([G]) for hydrolysis of DE-2 in 1:9 dioxane–water (v/v) solutions at 25 °C, μ = 0.1 ($NaClO_4$); ○, pH 6.32; △, pH 6.49; □, pH 6.16; ●, pH 6.06, ▲, pH 6.32. The solutions of guanosine also contained 10^{-3} M cacodylic acid buffer for maintenance of pH.

Scheme I



Results and Discussion

Plots of k_{obsd} for reaction of DE-2 vs. concentration of nucleoside monophosphates **3–5** at pH ca. 6–7 in 1:9 di-



3a, X = guanine (5′-GMP) b, X = adenine (5′-AMP) c, X = cytosine (5′-CMP)

4a, X = guanine (3′-GMP) b, X = adenine (3′-AMP) c, X = cytosine (3′-CMP)

5a, X = guanine (2′-GMP) b, X = adenine (2′-AMP) c, X = cytosine (2′-CMP)

oxane–water solutions show that the rate constants increase linearly with increase in monophosphate concentrations (Figure 1). The pseudo-first-order rate constants (k_{obsd}) were fit to eq 1, where k_u is the rate constant for

$$k_{obsd} = k(ROPO_3H^-)[ROPO_3H^-] + k_u \quad (1)$$

hydrolysis of DE-2 in the absence of phosphate at a given pH and $k(ROPO_3H^-)$ is the second-order rate constant for the phosphate-catalyzed reaction. Values of $k(ROPO_3H^-)$ for 5′-GMP, 2′-GMP, 5′-AMP, and 5′-CMP were determined at two or more pH values. In each case, the value of $k(ROPO_3H^-)$ determined at one pH was within experimental error the same as that determined at other pH values. It can therefore be concluded that the monohydrogen phosphate ionization state of each of these mononucleotides and presumably of all of the others is predominantely responsible for the phosphate kinetic term of eq 1 in the pH range listed. Product studies indicate that >94% of the products from reaction of DE-2 in 5 mM 5′-GMP (concentration of monohydrogen phosphate form **3a**) are tetrols, and covalent adducts between 5′-GMP and DE-2 were not detected by HPLC. With this information, it is reasonable to assume that the monohydrogen phosphate group acts as a general acid in the epoxide cleavage reaction (Scheme I). Consistent with this interpretation is the fact that the rate constants for hydrolysis of DE-2

Table I. Apparent pK_a Values and Second-Order Rate Constants[a] for Monophosphate and Inorganic Phosphate Catalyzed Hydrolysis of DE-2 in 1:9 (v/v) Dioxane-Water (25 °C, μ = 0.1 ($NaClO_4$))

compd	pK_a	$k(ROPO_3H^-)$, M^{-1} s^{-1}	compd	pK_a	$k(ROPO_3H^-)$, M^{-1} s^{-1}
2′-GMP	6.26	24 ± 1	2′-CMP	6.32	3.0 ± 0.2
3′-GMP	6.11	17 ± 1	3′-CMP	6.10	3.9 ± 0.3
5′-GMP[b]	6.50	30 ± 1	5′-CMP	6.49	3.8 ± 0.1
2′-AMP	6.32	26 ± 2	ribose 5-phosphate[c]	6.60	0.8
3′-AMP	6.16	13 ± 1	phenyl phosphate	6.24	2.0 ± 0.1
5′-AMP	6.64	23 ± 1	1-naphthyl phosphate	6.26	6.9 ± 0.2
			$H_2PO_4^-$[d]	6.89	0.48 ± 0.02

[a] Determined from slopes of weighted least-squares plots of k_{obsd} for reaction of DE-2 vs. concentration of monohydrogen phosphate anion ($ROPO_3H^-$) or $H_2PO_4^-$. Errors are given in standard deviation units. [b] Reference 9. [c] Reference 10. [d] Reference 6.

do not show an increase with increasing guanosine (G) concentrations (Figure 1). Therefore, guanosine does not possess a group that is sufficiently acidic to act as a general acid or sufficiently nucleophilic to cleave the epoxide group at rates that compete successfully with hydrolysis of the epoxide under the conditions.

Provided in Table I are the second-order rate constants $k(ROPO_3H^-)$ for reaction of DE-2 in solutions containing 2′-, 3′-, and 5′-monophosphates of guanosine, adenosine, and cytidine. From these data is can be concluded that not only does the monohydrogen phosphate group act as a proton donor but also the nature of the base and the positioning of the monohydrogen phosphate are important in determining the effectiveness of the catalyst. For example, the plots of Figure 1 and data of Table I show that **3a** (5′-GMP) and **3b** (5′-AMP) are about equally effective as catalysts in the hydrolysis of DE-2. However, **3c** (5′-CMP), which has almost the same pK_a for ionization of the monohydrogen phosphate group and would consequently be expected to be as effective as **3a** in serving as a general acid, is approximately eight times less effective than **3a** in bringing about the hydrolysis of DE-2. The value of $k(ROPO_3H^-)$ for **3c** (5′-CMP) is in turn some five times larger than that for ribose 5-phosphate, which is similar to inorganic phosphate ($H_2PO_4^-$) in catalytic effectiveness. We attribute the larger values of $k(ROPO_3H^-)$ for 5′-GMP and 5′-AMP to be due to favorable stacking associations between the aryl group and the nearby base at the transition state, with the monohydrogen phosphate group acting as a proton donor to the epoxide oxygen.

It is reported that charge transfer complexing is responsible for the enhanced solvolytic reactivity of 2,4,7-trinitro-9-fluorenyl *p*-toluenesulfonate in the presence of aromatic electron donors,[12] and this type of complexing in which the nucleotide base serves as an electron donor to stabilize the positively charged aryl moiety at the transition state may also be an important factor in enhancing the catalytic activities of nucleotides relative to that of inorganic phosphate ($H_2PO_4^-$) in promoting the hydrolysis of DE-2. Calculations of the energies of the highest occupied molecular orbitals of adenine, guanine, and cytosine[13] indicate that the abilities of these bases to act as electron donors are guanine > adenine > cytosine. The observations that k(5′-GMP) > k(5′-AMP) > k(5′-CMP) (Table I) is therefore consistent with that expected

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Scheme II

$$\text{DE-2} + \text{RO}\textcircled{P} \underset{}{\overset{K_e}{\rightleftharpoons}} \text{DE-2}\cdot\text{RO}\textcircled{P} \xrightarrow{(f)k_{cat}^{H}[\text{RO}\textcircled{P}]_{TOT}} \text{products}$$

$$\text{DE-2}\cdot\text{RO}\textcircled{P} \xrightarrow{k_u} \text{products}$$

if charge transfer complexing played a role in the nucleotide-catalyzed hydrolysis of DE-2. Another factor that may contribute to the order of catalytic activities for the nucleotides listed in table I in promoting the hydrolysis of DE-2 is the varying energy associated with hydrophobic interactions between the base moiety and the pyrene ring system of DE-2 at the transition state.

Our results (Table I) also show that the 2′- and 5′-monophosphates of GMP and AMP (pK_a 6.50 and 6.64, respectively) are very similar in their ability to catalyze the hydrolysis of DE-2. In contrast the 3′-monophosphates of GMP and AMP are only about half as effective as catalysts, even though they would be expected to be better catalysts on the basis of their lower pK_a values. Models indicate that the base moiety in the nucleotide is sufficiently close in space to both the 2′- and 5′-phosphate groups to allow favorable stacking interactions between the aryl group and the base but is further removed from 3′-phosphate group. The differences in catalytic efficiencies of the various nucleotides of Table I therefore appear best explained by a general mechanism in which the monohydrogen phosphate group acts as a general acid and secondary interactions of a stacking nature between the aryl group and the base at the transition state are important. These secondary interactions depend not only on the spacial relationship between the base and the phosphate group but also on the stacking ability of the base moiety.

Several interesting comparisons can be made of the kinetic parameters for the FMN-catalyzed hydrolysis of DE-2 with the kinetic parameters for the nucleotide-catalyzed hydrolysis of DE-2 if it is assumed that there is a common mechanism for these reactions as outlined in Scheme II. In this reaction scheme, K_e is the apparent association complex between DE-2 and FMN or mononucleotide, f is the fraction of FMN or mononucleotide that exists in the monohydrogen phosphate ionization state, and $k_{cat.}^{H}$ is the apparent first-order rate constant for the reaction of the physical complex of DE-2 and the monohydrogen phosphate ionization form of FMN or mononucleotide. The rate expression for this mechanism is given by eq 2, where k_u is the first order rate constant

$$k_{obsd} = (k_u + K_e(f)k_{cat.}^{H}[\text{RO}\textcircled{P}]_{TOT})/(1 + K_e[\text{RO}\textcircled{P}]_{TOT}) \quad (2)$$

for reaction of diol epoxide in the absence of phosphate and is equal to $k_{H^+}[H^+] + k_0$, the sum of the pseudo-first-order rate constant for the acid-catalyzed hydrolysis of DE-2 and the first-order rate constant for the spontaneous hydrolysis of DE-2, respectively. The total concentration of FMN or mononucleotide is represented by $[\text{RO}\textcircled{P}]_{TOT}$. For k_{obsd} to increase linearly with nucleotide concentration, $K_e[\text{RO}\textcircled{P}] \ll 1$. The slope of a plot of k_{obsd} vs. $(f)[\text{RO}\textcircled{P}]_{TOT}$, the concentration of the monohydrogenphosphate ionization state of the nucleotide, is therefore equal to $K_e k_{cat.}^{H}$. If it is assumed that the limiting rate constant for a given nucleotide is at least twice as large as that determined in the most concentrated phosphate solution with $(f)[\text{RO}\textcircled{P}]_{TOT}$ = 0.005 M, since no curvatures in rate plots similar to those of Figure 1 are detected for phosphate concentrations up to these values, then it can be estimated that $k_{cat.}^{H} > 2\ K_e(f)k_{cat.}^{H}[\text{RO}\textcircled{P}]_{TOT}$ and $K_e <$

Table II. Apparent Values of K_e and $k^H_{cat.}$ for Hydrolysis of DE-2 in 1:9 (v/v) Dioxane–Water (25 °C, μ = 0.1 ($NaClO_4$)) Catalyzed by FMN, 5′-Mononucleotides, and Ribose 5-Phosphate

phosphate	pK_a	K_e (M^{-1})	$k^H_{cat.}$ (s^{-1})
FMN[a]	6.46	3.4×10^3	0.06
5′-GMP[b]	6.50	<100	>0.30
5′-AMP[b]	6.64	<100	>0.27
5′-CMP[b]	6.49	<100	>0.035
ribose 5-phosphate[a,b]	6.60	<25	>0.032

[a] Reference 10. [b] Values of K_e and $k^H_{cat.}$ are estimated from the values of $k(ROPO_3H^-)$ listed in Table I and the fact that rate plots of k_{obsd} vs. (f) $[RO\textcircled{P}]_{TOT}$ show no curvature at $(f)[RO\textcircled{P}]_{TOT}$ < 0.005 M for 5′-GMP, 5′-AMP, and 5′-CMP and no curvature at $(f)[RO\textcircled{P}]_{TOT}$ < 0.02 M for ribose 5-phosphate (ref 10).

$^1/_2(f)[RO\textcircled{P}]_{TOT}$. The minimum values for $k^H_{cat.}$ and maximum values for K_e can therefore be estimated and are provided in Table II for the 5′-mononucleotides and ribose 5-phosphate. The published values of K_e and $k^H_{cat.}$ for the FMN-catalyzed reaction of DE-2 are also given for comparison.

It is clear from the data of Table II that the equilibrium constants for association of DE-2 with mononucleotides and ribose 5-phosphate are very much less than that for association of DE-2 with FMN. However, calculated minimum values of $k^H_{cat.}$ for 5′-GMP and 5′-AMP are substantially larger than $k^H_{cat.}$ for FMN. For concentrations of 5′-GMP and 5′-AMP in the monohydrogen phosphate ionization state greater than 2 mM, the catalytic effectiveness of these nucleotides in promoting the hydrolysis of DE-2 exceeds that of FMN. The pK_a values of 5′-GMP and 5′-AMP are very similar to that of FMN, and therefore the larger values of $k^H_{cat.}$ for these nucleotides is not due to greater acidities of their monohydrogen phosphate groups relative to that in FMN. Rather, a combination of proton-donating ability of the monohydrogen phosphate group and favorable stacking interactions at the transition state must account for their greater catalytic effectiveness. It should be possible to design other phosphate catalysts in which there is proper orientation of a phosphate group with a second group capable of complexing with the diol epoxide such that $k^H_{cat.}$ is comparable to or greater than that for 5′-GMP or 5′-AMP, but in addition K_e is equal to or greater than that for FMN. Such catalyst, without being any more acidic than FMN, would be far more effective than FMN at promoting the hydrolysis of DE-2 at low catalyst concentrations.

In an effort to further demonstrate that secondary stacking abilities of groups within a general acid molecule also play a role in determining its effectiveness as a catalyst in the hydrolysis of DE-2, we have determined the second-order rate constants for the hydrolysis of DE-2 catalyzed by $H_2PO_4^-$, phenyl hydrogen phosphate ($C_6H_5OPO_3H^-$), and 1-naphthyl hydrogen phosphate ($C_{10}H_7OPO_3H^-$) (Table I). Phenyl hydrogen phosphate and 1-naphthyl hydrogen phosphate have almost identical pK_a's and on this basis should be comparable as general acid catalysts. Dihydrogen phosphate anion ($H_2PO_4^-$) has a somewhat higher pK_a value but is a diprotic acid. Consequently it should be almost as efficient as phenyl hydrogen phosphate and 1-naphthyl hydrogen phosphate anions in promoting the hydrolysis of DE-2. Yet the data of Table I show that $k(ROPO_3H^-)$ for 1-naphthyl hydrogen phosphate anion is more than three times larger than that for phenyl hydrogen phosphate anion, which is in turn four times greater than that for $H_2PO_4^-$. We conclude, therefore, that favorable stacking interactions between DE-2 and general acids at the transition state can contribute significantly to enhanced reaction rates.[14]

Acknowledgment. This investigation was supported in part by Public Health Service Grants No. CA-26086 and CA-17278 from the National Cancer Institute. Helpful discussions with Dr. Jane M. Sayer (National Institutes of Health) are appreciated.

Registry No. DE-2, 58917-67-2; **3a**, 85-32-5; **3b**, 61-19-8; **3c**, 63-37-6; **4a**, 117-68-0; **4b**, 84-21-9; **4c**, 84-52-6; **5a**, 130-50-7; **5b**, 130-49-4; **5c**, 85-94-9.

(14) DE-2 is much more reactive toward ellagic acid (ref 15) than it is toward other general acids of comparable pK_a values (ref 6). It was postulated that favorable stacking interactions between DE-2 and ellagic acid at the transition state accounted for the enhanced reaction rate.

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Kinetic (^{18}O and ^{14}C) and Magnetic (^{13}C) Isotope Effects in the Photo-Fries Rearrangement of 4-Methoxyphenyl Acetate

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Kinetic isotope effects (KIE) were measured for the photorearrangement of 4-methoxyphenyl acetate (1) into 2-acetyl-4-methoxyphenol (2) in ethanol solution. The KIE for labeling the phenolic oxygen atom with ^{18}O was 1.0000 ± 0.0023. The KIE for labeling with ^{14}C at the α-carbon atom of the acetyl group was measured in two ways: with recovered 1 (0.9988 ± 0.0051) and with isolated 2 (1.007 ± 0.008). Labeling with ^{13}C at the α-carbon atom led to a magnetic, inverse isotope effect (0.9511 ± 0.0042). The results show that there is not a detectable activation barrier for breaking the ester bond and that 2 is formed by recombination of a caged radical pair which originates from an excited singlet state. Surprisingly, labeling of 1 with ^{14}C in the ortho position led to a KIE, measured with recovered 1, of 1.0286 ± 0.0021. We attribute this to a reaction of 1, as yet unknown, which is not associated with rearrangement into 2. It is noteworthy that rearrangement is not the major reaction pathway. The larger part (over 60%) of 1 is converted into polymeric material. The origin of the polymeric material lay in the scission product, 4-methoxyphenol (3), which was itself not obtained during the lengthy irradiations of the KIE work. Whether the KIE for ortho labeling is connected with polymer formation is not now known.

The photo-Fries rearrangement is the conversion of an aryl ester into an acylphenol.[2] It is exemplified, for example, by the conversion of phenyl acetate into *o*- and *p*-acetylphenol or of *p*-tolyl acetate into 2-acetyl-4-

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