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Heavy-Atom Isotope Effects on the Alkaline Hydrolysis and Hydrazinolysis of Methyl Benzoate

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Abstract: A double-labeling procedure has been devised for use in measurement of heavy-atom isotope effects. This method is of particular value for measurement of isotope effects at sites which are not easily amenable to study by standard isotope-ratio techniques. Substrate is synthesized which is highly labeled at two positions—one the position of interest in the isotope effect experiment and the other a position whose isotope effect is easily measured by standard isotope-ratio methods. This labeled substrate is mixed with unlabeled substrate, and the "isotope effect" is measured for the measurable site. This apparent isotope effect is actually the product of the isotope effect at the measurable site and that at the site of interest. Separate measurement of the former isotope effect then permits calculation of the isotope effect at the site of interest. Heavy-atom isotope effects on the hydrolysis and hydrazinolysis of methyl benzoate at 25 °C in aqueous solution have been measured by this procedure, using the methyl carbon atom as the measurable site. In the alkaline hydrolysis the carbonyl oxygen isotope effect is k^{16}/k^{18} = 1.0046 ± 0.0020; the carbonyl carbon isotope effect is $k^{12}k^{13}$ = 1.0426 ± 0.0026; the ether oxygen isotope effect is k^{16}/k^{18} = 1.0062 ± 0.0006; the methyl carbon isotope effect is k^{12}/k^{13} = 1.0004 ± 0.0005. For the hydrazinolysis at pH 7.9 the values are carbonyl oxygen, 1.0184 ± 0.0014; carbonyl carbon, 1.0410 ± 0.0022; ether oxygen, 1.0413 ± 0.0028; methyl carbon, 1.0022 ± 0.0004. These isotope effects indicate that the rate-determining step in the alkaline hydrolysis is the formation of the tetrahedral intermediate. The small magnitudes of the oxygen isotope effects require that the transition state in this step be relatively reactant-like. The isotope effects on the hydrazinolysis indicate that the decomposition of the tetrahedral intermediate is rate determining and that the transition state for this step is relatively product-like.

Introduction

The alkaline hydrolysis of simple esters such as methyl benzoate is widely accepted to occur by a two-step mechanism involving a tetrahedral intermediate[1] (eq 1), although such

$$\mathrm{RCOR' + OH^- \underset{k_2}{\overset{k_1}{\rightleftharpoons}} RC(O^-)(OH)OR' \xrightarrow{k_3} RCO^- + R'OH} \quad (1)$$

intermediates have been infrequently observed. The principal evidence favoring their existence is the small amount of oxygen exchange between the carbonyl oxygen and the solvent that sometimes accompanies the hydrolysis.[2] Formation of the tetrahedral intermediate is presumably rate determining, and the small amount of exchange reflects the fact that the intermediate usually goes on to products, rather than returning to starting materials.

Isotopes have played an important role in the elucidation of the mechanism of ester hydrolysis. The pioneering work of Polanyi and Szabo[3] demonstrated that the alkaline hydrolysis of *n*-amyl acetate occurs with acyl–oxygen fission. Similar studies have been conducted with a variety of other esters.[4] Bender[2a] was the first to demonstrate that oxygen exchange between the solvent and the carbonyl oxygen accompanies the hydrolysis of benzoate esters. This phenomenon has subsequently been investigated in detail by Bender and collaborators[2b-d] and by Shain and Kirsch.[2e] The amount of exchange is always small; for example, in aqueous solution the alkaline hydrolysis of methyl benzoate is faster than oxygen exchange[2e] by a factor of about 28 at 25 °C. The measured hydrolysis to exchange ratios are all rendered rather uncertain by the small amount of exchange and by the difficulty of measuring isotopic compositions with the necessary precision.

The extent of oxygen exchange is generally taken to reflect the rate constant ratio k_3/k_2 in eq 1. However, as first pointed out by Bender,[2b] it is also possible that the small amount of exchange reflects a lack of protonic equilibria within the tetrahedral intermediate. More recently, a stereochemical rationalization for the small amount of exchange has been presented.[5]

Kinetic isotope effects have also played a role in the elucidation of the mechanism of ester hydrolysis. Carbonyl carbon isotope effects in the range k^{12}/k^{14} = 1.06–1.08 have been measured for the alkaline hydrolysis of a number of ethyl benzoates.[6a] These isotope effects indicate that extensive changes in bonding occur on going from ground state to transition state. Solvent isotope effects on the alkaline hydrolysis of a number of benzoate esters[6b] are in the range k^{H_2O}/k^{D_2O} = 0.3–0.5. Small oxygen isotope effects have been observed for the ether oxygen in the alkaline hydrolysis of acetyl tryptophan methyl ester[7] (1.007) and methyl formate[8] (1.0091). These isotope effects suggest that extensive carbon–oxygen bond breaking has not occurred in the transition states for these reactions. Carbonyl oxygen isotope effects on the methanolysis of phenyl benzoates have been shown by direct kinetic comparison[9] to be about k^{16}/k^{18} = 1.02, consistent with the idea that the carbonyl carbon–oxygen bond is somewhat weakened at the transition state.

The alkaline hydrolysis of methyl formate has been studied in detail by Sawyer and Kirsch.[8] The hydrolysis shows an ether oxygen isotope effect k^{16}/k^{18} = 1.009 at 25 °C and a formyl hydrogen isotope effect k^H/k^D = 0.95. These results suggest that the transition state in this reaction is relatively early, resembling the starting materials rather than the tetrahedral intermediate.

The aminolysis of esters is more complex than the alkaline

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hydrolysis because of the widespread occurrence of buffer catalysis and because of the possibility for multiple tetrahedral intermediates. The hydrazinolysis of methyl formate has been thoroughly studied and is relatively well understood.[10] Near pH 8 the decomposition of the tetrahedral intermediate is rate determining, and an ether oxygen isotope effect $k^{16}/k^{18} = 1.06$ is observed.[8] The formyl hydrogen isotope effect under the same conditions is $k^{H}/k^{D} = 0.98$, suggesting that the transition state is quite late.[8]

Heavy-atom isotope effects have been used as mechanistic probes in a variety of organic reactions.[11] Further use of such effects for studying esters has been impeded by methodological considerations. The most common method for measurement of heavy-atom isotope effects[11b,e] makes use of the change in isotopic composition of a specific site in reactant or product during the course of the reaction. This method requires conversion of the isotopic site to a low-molecular weight, volatile compound for analysis by isotope-ratio mass spectrometry; such conversion is often inconveniently difficult. Alternatively, direct kinetic comparison of labeled and unlabeled substrates is possible,[9] but kinetic data of the required precision are extremely difficult to obtain, and the prospect for the use of this method is limited. Heavy-atom isotope effects can also be measured by the use of radioactive substrates, and this method has been widely used for determination of carbon isotope effects.[12] The lack of suitable radioisotopes of oxygen and nitrogen and the difficulty of making sufficiently precise measurements of radioactivity have limited the use of this method.

In a preliminary communication[13] we described a new double-labeling procedure for the determination of heavy-atom isotope effects. Our method takes advantage of the recent availability of a variety of isotopically labeled compounds having greater than 90% isotopic enrichment. This procedure substantially increases the number of isotope effects that can be measured by the isotope ratio method without the need for extensive chemical conversion of substrate or product prior to isotopic analysis. In this paper we report the details of the double-label method. In addition, we describe a modification of the method that makes use of isotopically depleted compounds, thereby increasing the precision of the double-label method. To illustrate the power of the method we describe studies of carbon and oxygen isotope effects in the alkaline hydrolysis and hydrazinolysis of methyl benzoate. These effects provide interesting new information regarding transition-state structure in these reactions.

Theory

The determination of heavy-atom isotope effects by the isotope ratio method (also called the competitive method) requires that the change in the ratio of isotopic abundances at the site of interest in substrate (S) or product (P) be measured over the course of the reaction.[11b,e] The isotope-ratio mass spectrometer provides ratios of abundances (P*/P or S*/S; the asterisk denotes the heavier isotope) which can be used to calculate isotope effects. When products are analyzed, the isotope ratio of the product is measured at 100% reaction $(P^*/P)_{100}$ and at a small extent of reaction $(P^*/P)_{low}$, commonly 5–30%. If the isotope ratio at low conversion is extrapolated to 0% conversion, the isotope effect is given by:

$$\frac{k}{k^*} = \frac{(P^*/P)_{100}}{(P^*/P)_{low}} \tag{2}$$

This equation is approximately true even up to 20 or 30% conversion. Methods for correcting to 0% conversion are available.[11b,e,14] Very accurate values of the isotope ratios are required, but a precision of ±1% in the extent of reaction is usually adequate.

Thus, the substrate which is used in the competitive method is actually a mixture of isotopically labeled and unlabeled materials. The small natural abundances of the heavier isotopes of carbon, nitrogen, and oxygen often suffice for use in this method, although isotopic enrichment is sometimes advantageous.

The stable-isotope double label method requires the same measurement of isotopic abundances at a single site in substrate or product as is required in the standard competitive method. However, in this case the substrate is a mixture of unlabeled and doubly labeled materials. Neglecting for the moment the complications caused by the inevitable presence of small amounts of singly labeled material, determination of the "isotope effect" on this mixture of unlabeled and doubly labeled material provides an isotope effect that is the product of the isotope effects at the two labeled sites. The isotope effect at the site being analyzed can be measured in a separate experiment using singly labeled substrate. Thus it is possible by this indirect method to measure the isotope effect at a site that is itself not amenable to direct measurements of isotopic composition. The details of this method are given in the following discussion.

The substrate being studied will be designated A–S–B. A and B represent the two sites which are isotopically labeled; A is the site which is to be analyzed; and B is the site whose isotope effect is desired. Asterisks denote the heavier isotope. The products of the reaction are A–P, which contains isotopic site A, and B–P′, which contains site B. The isotopic composition of A–P will be analyzed. The four isotopic species and their rate constants are given by:

$$\text{A–S–B} \xrightarrow{k_{AB}} \text{A–P} + \text{B–P}' \tag{3}$$

$$\text{A*–S–B*} \xrightarrow{k_{A^*B^*}} \text{A*–P} + \text{B*–P}' \tag{4}$$

$$\text{A*–S–B} \xrightarrow{k_{A^*B}} \text{A*–P} + \text{B–P}' \tag{5}$$

$$\text{A–S–B*} \xrightarrow{k_{AB^*}} \text{A–P} + \text{B*–P}' \tag{6}$$

The desired isotope effect is k_{AB}/k_{AB^*}. Isotope effect k_{AB}/k_{A^*B} must be measured in a separate experiment. According to the rule of the geometric mean:[15]

$$k_{AB}/k_{AB^*} = k_{A^*B}/k_{A^*B^*} \tag{7}$$

This equation provides the relationship between the isotope effect observed in the double-label experiment and the separate isotope effects at sites A and B.

The isotope ratio of the product at 100% reaction, $(A^*\text{–}P/A\text{–}P)_{100}$, reflects the isotopic composition of the starting material. In the case of the double-labeling experiment:

$$\left(\frac{A^*\text{–}P}{A\text{–}P}\right)_{100} = \frac{[A^*\text{–S–}B^*]_0 + [A^*\text{–S–B}]_0}{[A\text{–S–}B^*]_0 + [A\text{–S–B}]_0} \tag{8}$$

in which the subscript zero indicates the value prior to reaction and the brackets represent relative amounts of the indicated isotopic species. The isotopic ratio of the product isolated after fraction reaction f, $(A^*\text{–}P/A\text{–}P)_f$, can be used to calculate the corresponding isotope ratio which would be obtained at 0%, $(A^*\text{–}P/A\text{–}P)_0$:

$$\left(\frac{A^*\text{–}P}{A\text{–}P}\right)_0 = (A^*\text{–}P/A\text{–}P)_{100} \times \frac{\log\,[1 - f(A^*\text{–}P/A\text{–}P)_f/(A^*\text{–}P/A\text{–}P)_{100}]}{\log\,(1-f)} \tag{9}$$

The relation of this extrapolated ratio to the various rate constants and to the abundances of the various isotopic species

Table I. Ether Oxygen and Methyl Carbon Isotope Effects on the Alkaline Hydrolysis and Hydrazinolysis of Methyl Benzoate at 25 °C in Water

	isotope ratios[a] × 10^6						
	low conversion		100% conversion				
% reaction	30/(29 + 28)	29/28	30/(29 + 28)	29/28		k^{16}/k^{18} [b]	k^{12}/k^{13} [b]
			Alkaline Hydrolysis				
11.6	2769	13 808	2785	13 821		1.0061	1.0007
12.3	2769	13 828	2788	13 837		1.0072	1.0005
12.4	2777	13 825	2793	13 829		1.0065	1.0001
12.4	2783	13 825	2798	13 842		1.0057	1.0010
12.3	2779	13 829	2793	13 827		1.0057	0.9998
					mean	1.0062 ±0.0006	1.0004 ±0.0005
			Hydrazinolysis at pH 7.9				
10.9	2699	13 765	2800	13 806		1.0403	1.0026
12.8	2690	13 794	2797	13 830		1.0427	1.0022
11.8	2697	13 786	2793	13 822		1.0379	1.0022
11.6	2683	13 791	2796	13 820		1.0443	1.0017
					mean	1.0413 ±0.0028	1.0022 ±0.0004

[a] Decade settings for *m/e* 30/(29 + 28) and 29/28, corrected to tank standard 2800, 13 800, respectively. [b] Corrected for percent reaction; k^{12}/k^{13} corrected for ^{17}O (see text).

of substrate prior to reaction is given by:

$$\left(\frac{A^*\text{-}P}{A\text{-}P}\right)_0 = \frac{[A^*\text{-}S\text{-}B^*]k_{A^*B^*} + [A^*\text{-}S\text{-}B]k_{A^*B}}{[A\text{-}S\text{-}B^*]k_{AB^*} + [A\text{-}S\text{-}B]k_{AB}} \quad (10)$$

The isotope effect at position B would be obtained from isotopic analysis of position B at 0 and 100% reaction if such analysis were possible:

$$\frac{k_{AB}}{k_{AB^*}} = \frac{(B^*\text{-}P/B\text{-}P)_{100}}{(B^*\text{-}P/B\text{-}P)_0} = \frac{(A\text{-}S\text{-}B^*/A\text{-}S\text{-}B)_0}{(B^*\text{-}P/B\text{-}P)} \quad (11)$$

This effect can be derived from the available isotope ratios by combining the above equations into:

$$\frac{k_{AB}}{k_{AB^*}} = \frac{\dfrac{(A^*\text{-}P/A\text{-}P)_{100}}{(A^*\text{-}P/A\text{-}P)_0} - \dfrac{k_{AB}}{k_{A^*B}}\left[\dfrac{1 + (A^*\text{-}S\text{-}B/A^*\text{-}S\text{-}B^*)}{1 + (A\text{-}S\text{-}B/A\text{-}S\text{-}B^*)}\right]}{\left\{\dfrac{k_{AB}}{k_{A^*B}}\left[\dfrac{1 + (A^*\text{-}S\text{-}B/A^*\text{-}S\text{-}B^*)}{1 + (A\text{-}S\text{-}B^*/A\text{-}S\text{-}B)}\right] - \dfrac{(A^*\text{-}P/A\text{-}P)_{100}}{(A^*\text{-}P/A\text{-}P)_0}\left(\dfrac{A^*\text{-}S\text{-}B}{A^*\text{-}S\text{-}B^*}\right)\right\}} \quad (12)$$

The product isotope ratios at 0 and at 100% reaction are the values obtained from the isotope ratio mass spectrometer. The isotope ratios involving labeled substrates represent corrections arising from the presence of singly labeled species. Provided that the abundances of the singly labeled species are small compared to the abundances of the unlabeled and doubly labeled species, these ratios can be obtained adequately by ordinary mass spectrometry of the substrate. The synthesis of A*-S-B* should be carried out using starting materials of at least 90% enrichment, and 97% is better. The isotope effect k_{AB}/k_{A^*B} must be determined in separate experiments using natural-abundance substrate, unless it is clear that this effect will be essentially unity.

The double-label method described above requires that isotope effect measurements be conducted with an isotopically mixed substrate containing principally unlabeled and doubly labeled components. The best results are obtained when the doubly labeled material is 20–50% of the total. Under these conditions, the isotope ratios used to determine the isotope effect are very different from natural-abundance ratios, and the possibilities for error due to sample contamination are much greater than in measurements conducted with natural-abundance materials. A modified double-labeling method, the "pseudo-natural-abundance" method, overcomes this problem and permits measurements to be made with isotope ratios at or near natural abundance.

In order to use the pseudo-natural-abundance method it is necessary to synthesize substrate which is depleted in the heavier isotope at position A, the position being analyzed (depletion at position B is not necessary). This depleted material is then mixed (about 90:1 for carbon) with the doubly enriched material to produce a pseudo-natural-abundance synthetic isotopic mixture in which the isotopic abundance at position A is essentially natural abundance, but nearly every molecule having the heavier isotope at position A also has the heavier isotope at position B. The isotope effect is determined exactly as described above, but the experimental errors are often smaller in this procedure than when the isotope ratios at position A are far from natural abundance.

Results

Ether Oxygen and Methyl Carbon Isotope Effects. Ether oxygen and methyl carbon isotope effects on the alkaline hydrolysis and hydrazinolysis of methyl benzoate were measured by comparing the oxygen and carbon isotopic compositions of methanol produced after approximately 10% reaction with those produced after complete reaction. Natural abundance methyl benzoate was used. The methanol was isolated, purified, and pyrolyzed to carbon monoxide. The isotope effects were calculated from the *m/e* 29/28 and 30/28 isotope ratios in the usual way.[11e]

The results of these measurements are summarized in Table I. The validity of the procedure was shown by several tests: (1) The isotope ratios of all 100% samples were the same and the isotope ratios for the low conversion samples were constant for alkaline hydrolysis and for hydrazinolysis. (2) The isotope effects were constant from experiment to experiment, within the precision of the analytical procedure. (3) No methanol could be detected if either substrate or nucleophile was omitted from the reaction mixture. (4) Methanol of known isotopic composition could be carried through our entire procedure with no significant change in isotopic composition.

Carbonyl Oxygen Isotope Effects. [*methyl*-^{13}C,*carbonyl*-^{18}O]methyl benzoate was synthesized with 90% enrichment at both positions. This compound was mixed with natural abundance ester in the approximate proportions of 1:4. This substrate was then subjected to alkaline hydrolysis and hydrazinolysis. The methanol produced at 30 and 100% reaction was isolated and converted to CO_2 by pyrolysis at 1200 °C in

Table II. Carbonyl Oxygen Isotope Effects on the Alkaline Hydrolysis and Hydrazinolysis of Methyl Benzoate at 25 °C

% reaction[a]	isotope ratios[b] × 10^4 low conversion	isotope ratios[b] × 10^4 100% conversion		k^{16}/k^{18} [c]
	Alkaline Hydrolysis			
30.0	3164	3174		1.0043
28.9	3164	3169		1.0019
30.0	3150	3161		1.0048
30.7	3164	3179		1.0065
20.0	3156	3163		1.0028
35.0	3168	3184		1.0071
			mean	1.0046
				±0.0020
	Hydrazinolysis at pH 7.9			
33.7	3132	3179		1.0179
32.5	3131	3175		1.0163
36.3	3137	3186		1.0185
33.1	3129	3179		1.0197
31.6	3130	3182		1.0195
			mean	1.0184
				±0.0014

[a] Determined by spectrophotometric monitoring at 282 nm for alkaline hydrolysis and at 258 nm for hydrazinolysis. [b] Decade settings for *m/e* 45/44, corrected to tank standard = 3150. [c] The isotope effects are corrected for percent reaction and for the presence of singly labeled ester (see text), as well as for the presence of ^{18}O in the ether oxygen.

Table III. Carbonyl Carbon Isotope Effects on the Alkaline Hydrolysis and Hydrazinolysis of Methyl Benzoate at 25 °C

% reaction[a]	isotope ratios[b] × 10^6 low conversion	isotope ratios[b] × 10^6 100% conversion		k^{12}/k^{13} [c]
	Alkaline Hydrolysis			
30.0	10 421	10 725		1.0433
29.5	10 390	10 717		1.0465
29.9	10 428	10 710		1.0401
30.0	10 421	10 722		1.0428
30.3	10 432	10 716		1.0404
			mean	1.0426
				±0.0026
	Hydrazinolysis at pH 8.0			
34.7	10 398	10 724		1.0429
34.2	10 384	10 712		1.0431
32.8	10 395	10 707		1.0402
33.9	10 396	10 710		1.0409
29.5	10 410	10 714		1.0378
			mean	1.0410
				±0.0022

[a] Determined by spectrophotometric monitoring at 282 nm for alkaline hydrolysis and at 258 nm for hydrazinolysis. [b] Decade settings for *m/e* 45/44, corrected to tank standard = 1140. [c] The isotope effects are corrected for percent reaction, for the presence of singly labeled esters, and for 5% [*ether*-^{18}O]methyl benzoates (see text).

the presence of water. The isotopic composition of this methanol was used to calculate the carbonyl oxygen isotope effects by use of eq 12. In addition to the corrections involved in eq 12, a small additional correction was required because of the presence of 5% ^{18}O in the [^{13}C]methanol used for the synthesis of methyl benzoate.

The results of these experiments are summarized in Table II. The same criteria previously applied were used to show the adequacy of the isotope effect experiments.

In the case of the carbonyl oxygen isotope effect on the alkaline hydrolysis of methyl benzoate, it is conceivable that the occurrence of oxygen exchange during the hydrolysis[2e] might create an error in the observed isotope effect. That such an error is negligibly small is shown by the following argument: The isotope analysis of the 100% reaction sample depends only upon the isotopic composition of the carbon atom in question; oxygen exchange during hydrolysis is of no consequence because the reaction is carried to completion. The isotopic composition of the system at 30% reaction would be slightly affected by the oxygen exchange because of the presence of a slightly larger than expected amount of [^{13}C]methyl benzoate and a correspondingly smaller amount of [^{13}C,^{18}O]methyl benzoate. This increases the size of the correction for singly labeled substrates (eq 12). However, oxygen exchange is at least 20 times slower than hydrolysis under our reaction conditions,[2e] and the change in isotopic composition at 30% reaction will cause a negligible change in the magnitude of the correction.

Carbonyl Carbon Isotope Effects. Pseudo-natural-abundance methyl benzoate containing a 90:1 mixture of [*methyl*-^{12}C]methyl benzoate and [*carbonyl,methyl*-$^{13}C_2$]-methyl benzoate was prepared, and carbon isotope effects were measured for the alkaline hydrolysis and hydrazinolysis of this material. Experimental data are shown in Table III. The carbonyl carbon isotope effects were calculated as described above for the carbonyl oxygen isotope effects. The same criteria of accuracy were applied as before.

Discussion

Traditional methods for studying heavy-atom isotope effects using stable isotopes have often been limited by the difficulty of the degradation required to make accurate isotope ratio measurements on the position in question. The stable-isotope double label method eliminates the necessity for this degradation in many cases, provided that some other atom in the substrate in question is easily susceptible to isotopic analysis.

Although a difficult analytical problem is circumvented by the double-label method, the experimental problem now becomes a sometimes formidable synthetic problem. The method requires synthesis of a substrate labeled at two sites to the extent of at least 90%. Even higher levels of labeling are preferable. The increasing availability of compounds labeled with carbon, oxygen, or nitrogen at levels near 97% makes this approach feasible. If the pseudo-natural-abundance method is used, then the total amount of the doubly labeled material needed is small, and cost is not an important consideration.

In addition to isotope effect measurements using the doubly labeled substrate, it is necessary to measure the isotope effect at the position being analyzed by the use of singly labeled (ordinarily natural abundance) substrate, unless the position being analyzed is sufficiently remote from the reaction center that the isotope effect at that position is very much smaller than the isotope effect at the position of interest. Under these conditions, it suffices in eq 12 to let $k_{A^*B}/k_{AB} = 1.00$. In any case, the method used for measurement of the isotope effect at the position being analyzed is the same as that for measurement using the doubly labeled material, so this ordinarily does not represent a significant limitation. The one exception to this arises in the case of reactions in which the isotope effect at the position being analyzed is comparatively large and that at the position of interest is small; under those circumstances the uncertainty in the value of the isotope effect at the position of interest may be distressingly large.

It should be emphasized that the position being analyzed serves only as an isotopic tracer, and it is perfectly possible for the two isotopic sites to be quite far apart, provided only that they are in the same molecule. There is also no requirement that the site being analyzed be cleaved from the remainder of the molecule during the reaction in question, provided that an analytical method for isolating the site is available. Isotopic analysis of starting material or product works equally well.

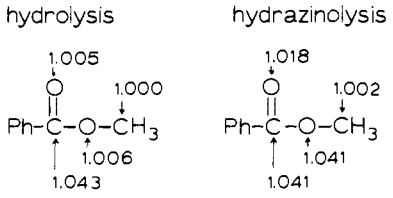

Figure 1. Summary of isotope effects on the hydrolysis and hydrazinolysis of methyl benzoate in aqueous solution at 25 °C.

Once a measurement method is established, it can be used to measure as many isotope effects in a system as desired. For example, we have used the double-label method to measure carbonyl carbon and carbonyl oxygen isotope effects in reactions of methyl benzoate. The same method could be used to measure carbon isotope effects for the ring carbons or the small hydrogen isotope effects for one or more aromatic hydrogens. This method may prove useful in making accurate measurements of small secondary hydrogen isotope effects. The principal limitation is in the willingness of the investigator to synthesize the appropriately labeled compound.

In the first application of the double-label method, we mixed doubly labeled ester with natural abundance ester to produce a mixture which contained about 25% of the heavier isotope at each of the two positions. This material gives satisfactory isotope effects, but, as mentioned previously, the problems of measuring isotope ratios on enriched samples are often substantially more difficult than on natural abundance materials. Subsequently we synthesized substrate which was isotopically depleted at the position being analyzed and we mixed that material with enriched material to give a pseudo-natural-abundance substrate with a high isotopic correlation. Experimentally, we find that accurate isotope effects are much more easily obtained by this procedure. It should be emphasized that in the isotopically depleted material only the site being analyzed has to be isotopically depleted; failure to deplete at the other site increases the amount of singly labeled substrate, but the error due to that introduction is very small.

The control experiments which are needed in the double-label method are more or less the same as those in the ordinary isotope-ratio method. Essentially, it is necessary to demonstrate that sample preparation and purification show no isotope effects which might interfere with the determination of isotope ratios. In addition, it is necessary to know the extent to which singly labeled species are present.

Reactions of Methyl Benzoate. The isotope effects which have been obtained in this study are summarized in Figure 1. For purposes of interpretation, we will assume that both the alkaline hydrolysis and the hydrazinolysis of methyl benzoate occur by way of a tetrahedral intermediate, as shown in eq 1. Although it is possible, particularly in the hydrazinolysis reaction, that more than one species of tetrahedral intermediate exists, our data can be interpreted without considering more than one intermediate.

In the general case, all three rate constants in eq 1 might show significant isotope effects. The observed isotope effect at any position of methyl benzoate is then given by:

$$\text{obsd}\ \frac{k}{k^*} = \frac{k_1/k_1^*}{k_2/k_2^*}\,\frac{[k_3/k_3^* + (k_3/k_2)(k_2/k_2^*)]}{[1 + k_3/k_2]} \qquad (13)$$

The observed isotope effect thus depends on the individual isotope effects on the three rate constants and on the partitioning ratio k_3/k_2, which indicates the fate of the tetrahedral intermediate. It is useful to note that the first term on the right side of eq 13 is the equilibrium isotope effect on formation of the tetrahedral intermediate.

Hydrazinolysis. Although the hydrazinolysis of an ester is inherently more complex than alkaline hydrolysis because of the potential for a greater number of intermediates and because of possibilities for buffer catalysis of hydrazinolysis, analogy with studies of methyl formate[8] suggests that under the conditions chosen for our study of the hydrazinolysis of methyl benzoate the rate-determining step in the hydrazinolysis is the decomposition of the tetrahedral intermediate.

The ether oxygen isotope effect on the hydrazinolysis of methyl benzoate ($k^{16}/k^{18} = 1.041$) is large compared to oxygen isotope effects that have been observed in most other reactions. The hydrazinolysis of methyl formate shows an oxygen isotope effect of 1.062 under conditions where decomposition of the tetrahedral intermediate is rate determining.[8] The magnitude of this effect and the secondary deuterium effect in the same reaction[8] indicate that the transition state is productlike. Preliminary calculations of Buddenbaum and Shiner[16] indicate that oxygen isotope effects for very productlike transition states in systems such as these might be as large as 1.08. Thus the ether oxygen isotope effect on the hydrazinolysis of methyl benzoate indicates that the decomposition of the tetrahedral intermediate is rate determining and that the transition state is moderately late. Whether the difference in magnitude between this isotope effect and that in the methyl formate case reflects a difference in partitioning ratio k_3/k_2 or a difference in transition-state structure is unknown.

According to eq 13 the carbonyl oxygen isotope effect reflects the equilibrium isotope effect on formation of the tetrahedral intermediate and the kinetic isotope effect on decomposition of this intermediate. The equilibrium isotope effect is expected to be large in this case because a double bond is being converted into a single bond. Calculations of Buddenbaum and Shiner[16] suggest that this effect is near 1.03. The kinetic isotope effect on the decomposition step may be either inverse or normal because a bond is being formed to the isotopic atom in this step; if the transition state is early, the isotope effect will be normal, whereas with an increasingly late transition state the isotope effect will tend toward being inverse. The observed oxygen isotope effect and the equilibrium isotope effect estimated by Buddenbaum and Shiner suggest that k_3^{16}/k_3^{18} is near 0.99. This is indicative of a late transition state, consistent with the conclusion based on the ether oxygen isotope effect.

The large carbonyl carbon isotope effect indicates that there is a substantial change in bonding to this atom on going from ground state to transition state. Because of the similar structures for the transition states before and after the tetrahedral intermediate, the magnitude of this isotope effect does not help distinguish which step is rate determining. Theoretical studies in progress should provide useful insight into the interpretation of this effect.

Thus, isotope effects on the hydrazinolysis of methyl benzoate indicate that at pH 8 the rate-determining step is the breakdown of the tetrahedral intermediate. The transition state for the breakdown is relatively late. These conclusions are similar to the conclusions derived from studies of kinetics and isotope effects in the hydrazinolysis of methyl formate.[8,10]

Alkaline Hydrolysis. Benzoate esters often show a small degree of exchange between the carbonyl oxygen and water during hydrolysis.[2] Provided that the lifetime of the tetrahedral intermediate is not too short, the extent of oxygen exchange reflects the fate of the tetrahedral intermediate:

$$\frac{k_{\text{hydrolysis}}}{k_{\text{exchange}}} = \frac{2k_3}{k_2} \qquad (14)$$

and thus indicates whether formation or decomposition of the intermediate is the rate-determining step. If the lifetime of the intermediate is too short for protonic and conformational equilibria to be established, then the ratio k_3/k_2 will be smaller than the value calculated from eq 14. The ratio k_3/k_2 appears

to be about 14 in the alkaline hydrolysis of methyl benzoate in water[2e] at 25 °C. Thus, formation of the tetrahedral intermediate is rate determining.

The ether oxygen isotope effect on the alkaline hydrolysis of methyl benzoate (k^{16}/k^{18} = 1.006) is substantially smaller than those observed in the hydrazinolysis of methyl benzoate (1.041) and in the hydrazinolysis of methyl formate[8] (1.062), reactions in which the decomposition of the tetrahedral intermediate is rate determining. Small oxygen isotope effects have been observed in reactions of hydroxide ion with methyl formate[8] and with *N*-acetyl-L-tryptophanamide.[7] Presumably in these cases the formation of the tetrahedral intermediate is the rate-determining step. For methyl benzoate this conclusion is, of course, nicely consistent with the results of the oxygen exchange studies.

A more quantitative approach to the ether oxygen isotope effect can be made using eq 13. If we assume for the moment that there is no oxygen isotope effect on k_1 or k_2, then the measured isotope effect and isotope exchange data predict a value of approximately 1.09 for k_3^{16}/k_3^{18}. Although an isotope effect of this magnitude is theoretically possible (it is near the extreme of values predicted by Buddenbaum and Shiner[16]), no oxygen isotope effect this large has ever been observed in any reaction.

The flaw in this approach probably lies in the failure to consider an isotope effect on k_1. The carbonyl carbon–ether oxygen bond in methyl benzoate is significantly stronger than an ordinary single bond. Estimates based on Pauling's rule give a bond order of approximately 1.3. This extra strength is lost on going to the tetrahedral intermediate, thus giving rise to a small oxygen isotope effect on k_1. The change in bond order on going to the transition state is probably not larger than 0.1–0.2; thus, the isotope effect on k_1 is expected to be small. Buddenbaum and Shiner[16] have estimated a value of approximately 1.01 for the equilibrium isotope effect on formation of the tetrahedral intermediate, and it is likely that the kinetic isotope effect on k_1 is smaller than this.

Thus, the ether oxygen isotope effect on the alkaline hydrolysis of methyl benzoate is a composite of the isotope effects on k_1 and k_3. A unique dissection of these two effects is not possible at this point, but, by way of illustration, if k_3^{16}/k_3^{18} in the alkaline hydrolysis is about the same as that in the hydrazinolysis, then k_1^{16}/k_1^{18} would be approximately 1.004; this is a reasonable value for this effect.

The carbonyl oxygen isotope effect on the alkaline hydrolysis of methyl benzoate provides additional useful insight into the structure of the transition state. This isotope effect is different from unity, indicating that some degree of carbon–oxygen bond breaking has taken place at the transition state, but it is substantially smaller than corresponding effects observed in the hydrazinolysis of methyl benzoate and in the hydrolysis of phenyl acetates.[9] Because both the nucleophile and the leaving group in the alkaline hydrolysis of methyl benzoate are oxygens of similar basicity, we expect that the carbonyl oxygen isotope effects on k_2 and k_3 should be very similar. Under those circumstances the observed isotope effect becomes equal to the isotope effect on k_1. The equilibrium carbonyl oxygen isotope effect for formation of the tetrahedral intermediate is about 1.03. The kinetic isotope effect for a transition state similar to the tetrahedral intermediate will be somewhat larger than this value. Earlier transition states give correspondingly smaller kinetic isotope effects. Thus, the small observed kinetic isotope effect reflects an early transition state.

Both oxygen isotope effects on the hydrolysis of methyl benzoate indicate that the transition state is relatively early. The most surprising aspect of the present results is the large carbon isotope effect on the hydrolysis. Although a quantitative discussion of this effect is not currently possible, the effect qualitatively indicates that extensive bonding changes have taken place at the transition state. A more coherent picture of this reaction awaits additional experimental and theoretical studies.

Experimental Section

Materials. Methyl benzoate of natural isotopic abundance was obtained from Aldrich. This compound, as well as those synthesized with isotopic enrichment, was purified by fractional vacuum distillation. [*methyl*-^{13}C]Methanol, 92.1 atom %, [*methyl*-^{12}C]methanol, 99.9 atom %, and [*carbonyl*-^{13}C]benzoic acid, 91.3 atom %, were obtained from Prochem. [^{18}O]Water, 90 atom %, was obtained from Mound Laboratories. Norit was purified by heating at 1000 °C in a quartz tube under high vacuum. All other chemicals were of high purity and were used without further purification.

Water for the ether oxygen isotope effect experiments was purified by a Millipore Super Q filtration system. The resistance of this water was at least 18 MΩ. For the carbonyl oxygen and carbon isotope effects, water purified by the Millipore system was further purified by distillation from alkaline permanganate.

[*methyl*-^{13}C,*carbonyl*-^{18}O]Methyl Benzoate. Benzoyl chloride–piperidinium chloride was synthesized as described by Eilingsfeld et al.[17] This material was reacted with $^{13}CH_3OH$ at −5 °C in acetonitrile under nitrogen. After 2 min the imino ester so formed was reacted with $H_2{}^{18}O$ in the presence of dry NaOAc for 1 h. The solution was extracted several times with dilute HCl in saturated NaCl, then with aqueous $NaHCO_3$, after which the organic layer was dried and evaporated at reduced pressure. The product ester was purified by distillation.

[*methyl*-^{13}C,*carboxyl*-^{13}C]Methyl Benzoate. [*carboxyl*-^{13}C]-Benzoic acid was converted to benzoyl chloride by reaction with $SOCl_2$. After purification this compound was reacted with $^{13}CH_3OH$ and the product was distilled.

[*methyl*-^{12}C]Methyl Benzoate. This compound was prepared by reaction of benzoyl chloride with $^{12}CH_3OH$, followed by distillation.

Methods. Kinetic measurements were made on a Gilford Model 222 spectrophotometer with a thermostated cell compartment at 25 ± 0.2 °C. Gas chromatography was done on a Varian Model 90P-3 equipped with a thermocouple detector and a 20% 1,2,3-tris(2-cyanoethoxy)propane (TCEP) on Chromosorb P column (15 ft × 3/8 in.). All pH measurements were made on a Radiometer Model 26 pH meter calibrated by the two-buffer method. Pyrolysis of methanol to either carbon monoxide or carbon dioxide was done using the apparatus described by Borowitz et al.[18] Isotopic analyses of carbon monoxide or carbon dioxide were done on a Nuclide Associates RMS 6-60 isotope ratio mass spectrometer with a dual inlet system. Alternate readings were taken for tank standard and sample gases to correct for instrumental drift. Prior to using the mass spectrometer two controls were done: (1) the background was scanned; (2) tank standard gas was introduced into both inlet systems and the isotope ratios were shown to be the same.

Isotope Effect Procedure. Alkaline Hydrolysis. For the ether oxygen isotope effect experiments in water, a 250-mL solution of 3 mM methyl benzoate was prepared. For the carbonyl oxygen and carbon experiments 325 mL of a 4.25 mM solution was prepared. Each of these solutions was then split into a large and a small portion. All solutions were stored in glass-stoppered flasks and incubated at 25 ± 0.02 °C for 1 h.

To the larger portion was added an amount of freshly prepared potassium hydroxide solution (titrated with primary standard potassium hydrogen phthalate) that was equimolar with the initial ester. A small aliquot was withdrawn for spectrophotometric monitoring at 282 nm. At the proper time the reaction was stopped by addition of the reaction mixture to a flask containing 2 g of Norit and enough H_2SO_4 to neutralize the remaining base. The Norit was removed by filtration through Whatman No. 42 filter paper. A second portion of Norit was added, the flask was swirled, and the Norit filtered off again.

To the smaller portions, enough base was added to make its concentration ten times the initial ester concentration. These reactions were allowed to proceed beyond 10 half-lives. They were then treated as described above.

At this point in the procedure both the large and small portions were treated in an identical manner. The methanol was concentrated by careful fractional distillation.

The 2 mL of distillate containing the methanol was injected onto the gas chromatography column in five successive injections (approximately 30 s apart). The column temperature was 80 °C; the helium flow was 90 cm^3/min. Methanol eluted first with a retention time of nearly 15 min; the water, which was not collected, had a retention time of nearly 35 min. The methanol was collected in a U-tube equipped with two vacuum stopcocks (2 mm bore), a 12/30 standard tapered inner ground glass joint on one end, and a 10/30 inner joint on the other. A pretrap was attached to the gas chromatograph on one end and to the U-tube on the other. The pretrap was immersed in an ice-salt bath.

In subsequent steps both portions were treated identically. The aqueous solution containing a small amount of methanol was carefully fractionally distilled. The first 2 mL of distillate, containing all of the methanol, was subjected to gas chromatography. The methanol was collected and transferred to the pyrolysis apparatus described by Borowitz et al.[18]

For the ether oxygen isotope effects this methanol was pyrolyzed at 1360 °C. It was determined by Fourier transform infrared analysis of the volatile pyrolysis products that CO was the only product of the pyrolysis under these conditions. Repeated measurement of the oxygen isotopic composition of a standard sample of methanol by this procedure gave consistent results. At lower temperatures substantial amounts of CO_2 and other volatile products were produced and isotope ratio analysis of the CO produced gave erratic results.

For the carbonyl carbon and carbonyl oxygen isotope effects the pyrolysis was conducted in the presence of an approximately twofold excess of H_2O. Under these conditions CO_2 is the only product of the pyrolysis.

Hydrazinolysis. The procedure in the hydrazinolysis experiments was basically the same as that in the hydrolysis experiments. The same quantities and ester concentrations were used. Complete reaction in the 100% reaction samples was achieved by conducting the hydrazinolysis at pH 13. The isotope ratios for these samples were, as expected, the same as those observed in the 100% reaction samples in the alkaline hydrolysis.

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Naphtho[1,8-*c,d*:4,5-*c',d'*]bis[1,2,6]selenadiazine

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Abstract: The synthesis and chemical and physical properties of the title compound (**1**) are reported. With one notable exception the spectroscopic properties of naphtho[1,8-*c,d*:4,5-*c',d'*]bis[1,2,6]selenadiazine (**1**) closely resemble those observed for naphtho[1,8-*c,d*:4,5-*c',d'*]bis[1,2,6]thiadiazine (**2**). In particular, the compounds show ^{1}H NMR chemical shifts of δ 5.91 (**1**) and 4.74 (**2**) in dimethyl-d_6 sulfoxide. The discrepancy in the ^{1}H NMR chemical shifts of **1** and **2** is ascribed to differences in the magnitude of the induced paramagnetic ring currents. Theoretical analysis of these results leads to two important conclusions: (1) The magnitude of paramagnetic ring currents in structurally similar molecules is primarily determined by the spectrum of orbital energies and, in particular, the HOMO–LUMO energy gap. (2) The difference between the nitrogen–chalcogen resonance integrals in **1** and **2** is the dominant factor in determining the relative magnitude of the HOMO–LUMO energy gaps (and thus the intensity of the paramagnetic ring currents). Evidence is presented to show that the most important excitation for the development of the paramagnetic ring current in **1** and **2** is from the HOMO (B_{1u}) to the LUMO (A_u), whereas the long-wavelength band in the electronic spectrum involves the HOMO (B_{1u}) → second LUMO (B_{2g}) transition.

We report here the preparation and properties of naphtho[1,8-*c,d*:4,5-*c',d'*]bis[1,2,6]selenadiazine (**1**), the first member of that class of compounds containing the =N—Se—N= ↔ -N=Se=N linkage in a six-membered ring. By analogy with the previously prepared naphtho[1,8-*e,d*:4,5-*c',d'*]bis[1,2,6]thiadiazine (**2**),[2] we expected **1** to exhibit the

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