Measurement of Heavy Atom Kinetic Isotope Effects by Direct Mass Spectrometric Analysis

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A method is described for the measurement of heavy atom kinetic isotope effects with high precision by direct mass spectrometric analysis of the starting material or product without conversion to a volatile derivative such as CO_2. This is accomplished with a conventional mass spectrometer by scanning the isotopic masses of the product or starting material as a function of the extent of reaction approximately 10^3 times for each sample. Applications of this technique to the measurement of oxygen-18 kinetic isotope effects on both organic (active ester aminolysis) and enzyme catalyzed (β-galactosidase) reactions are presented. This method yields isotope effects with propagated standard errors of ca. 0.2%, sufficient for the accurate determination of kinetic isotope effects for ^{13}C, ^{15}N, and ^{18}O.

The utility of the heavy atom kinetic isotope effect method for elements other than hydrogen in defining reaction mechanism and transition state structure has been severely limited in chemical and biochemical applications by the extremely rigorous technical demands of this method. For example, the magnitude of oxygen-18 kinetic isotope effects (KIEs) is expected to be at most 7%, i.e., $k_{16}/k_{18} \leq 1.07$ (*1*), so that any useful approach to the quantitation of these KIEs must have an accuracy and reproducibility of at least ±0.5%. The typical technique used has been to convert chemically the starting material or product to a gas such as CO_2, which is analyzed by isotope ratio mass spectrometry (*2*). The KIE can be calculated from the isotopic composition of the starting material or product as a function of the extent of reaction (*3*). The limitations of this technique are severe, since only gases or substances which can be quantitatively converted to gases can be analyzed.

In recent years some new methods have been developed for the measurement of heavy atom KIEs using computer-linked rapid scanning mass spectrometers (*4*), direct measurement of the rate differences due to isotopic substitution (*5–7*), and double-labeling using a second mass marker remote from the scissile bond (*8*). An additional technique, that of equilibrium perturbation (*9*) has been devised to measure KIEs for reversible enzyme-catalyzed reactions. We report in this paper a new method for the measurement of heavy atom KIEs by direct mass spectrometry without the necessity for conversion to CO_2 and which does not require a sophisticated computer-linked mass spectrometer. It is potentially applicable to any irreversible reaction where either the starting material or product has sufficient volatility to permit analysis by mass spectrometry. This method and that described in the accompanying paper (*10*) greatly reduce the difficulty and time involved in measurement of heavy atom KIEs.

EXPERIMENTAL

Apparatus. Mass spectrometric measurements were made on a Du Pont Model 21-491 mass spectrometer equipped with a Columbia Scientific Model 260/722 data system and digital printer. An automatic repetitive scan attachment facilitated the acquisition of large quantities of data. Samples were introduced into the mass spectrometer directly via the solid probe. Spectrophotometric measurements were made on a Cary Model 118C spectrophotometer which was interfaced via a microprocessor (Claremont Research) to a teletype and tape-punch.

Reagents. 2,4-Dinitrophenol-^{18}O was synthesized by the method of Gorenstein (*6*). 2,4-Dinitrophenyl acetate(DNPA)-^{18}O was prepared by the condensation of the potassium salt of 2,4-dinitrophenol-^{18}O with redistilled acetyl chloride in dry dimethylformamide. The natural abundance ester was synthesized in the same manner or was a gift from V. Zannis. Both unenriched and ^{18}O-enriched 2,4-dinitrophenyl-β-D-galactoside were prepared from the corresponding 2,4-dinitrophenol and acetobromogalactose (Sigma) by standard methods (*11*, *12*).

Preparation of Partially Labeled Reactants. A potentially major source of deterministic error in these experiments is contamination of the reactant with the phenolic product. This problem is most severe if the isotopic enrichment of the contaminant differs substantially from that of the reactant. The most effective method for minimizing this problem, in addition to the rigorous purification of reactants, is to synthesize the reactant with the substituted phenol of the final desired isotopic enrichment, usually 50%. The procedure adopted was to recrystallize appropriate amounts of highly enriched and natural abundance dinitrophenol together, yielding 50% enriched material, which was used in the subsequent synthetic steps. Alternatively, if this was impractical, labeled and unlabeled reactants were mixed in the appropriate proportions and recrystallized together to minimize contamination by the phenol. The use of these procedures makes the only likely dinitrophenol contaminant one which has the same isotopic enrichment as the substrate. Small impurities of this type have a very small effect on the isotope ratios and, hence, on the observed KIE.

Monitoring of Kinetics and Determination of the Extent of Reaction. Since the KIE is measured by the variation of the isotope ratio of partially labeled substrate or product with the extent of reaction, the time course of the reaction must be accurately determined. One typically determines the isotope ratio of the product by mass spectrometry at ca. 10% reaction and at the completion of the reaction (*2*). Typically, a solution of 250–1000 mL containing partially enriched reactant, the nucleophile, or enzyme was equilibrated in a flask at the designated temperature (usually 25 °C) in a thermostated water bath. A second flask was temperature equilibrated in the same water bath. The relative stabilities of the reactant in the absence of enzyme or nucleophile determined whether the reactant was preincubated or added to initiate the reaction. Two cuvettes were placed in the cell compartment of a Cary 118C spectrophotometer, balanced at the appropriate wavelength used to follow the reaction, and temperature equilibrated to the same temperature as the solution in the water bath. The temperature was checked in the cell compartment and the water bath using the same thermometer. The microprocessor linked to the spectrophotometer was set at the appropriate time interval, and an overhead stirrer was engaged to mix the solution in the water bath. The sample cuvette was then removed from the cell compartment and placed next to the reaction flask.

The reaction was initiated by the addition of a small volume of concentrated reactant or enzyme with a syringe or micropipet. A stopwatch was started at this time (time zero). After mixing was complete (ca. 15–30 s after time zero), ca. 3 mL of the solution was removed with a Pasteur pipet and transferred to the sample cuvette. This was placed in the cell compartment of the spectrophotometer, and the time of the first data point (absorbance reading) recorded by the teletype, linked via the microprocessor

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to the spectrophotometer, was noted. Another aliquot, corresponding to ca. 10% of the total volume of the reaction mixture was transferred to the second flask in the water bath.

The remainder of the reaction mixture (ca. 90%) was quenched at a time or absorbance previously determined, which corresponded to approximately 10% reaction. This coincided with an absorbance reading recorded by the spectrophotometer data system, so that the exact time of the quenching was known. The aliquot which had been transferred to the second flask was allowed to react to completion.

The extent of reaction at which the initial aliquot was quenched was calculated from the observed absorbance at the time the aliquot was quenched, and the absorbance at 100% reaction. The latter was determined from a computer analysis of the progress curve for the reaction fit to either a single exponential for reactions run under pseudo-first-order conditions, or to the integrated form of the Michaelis–Menten equation for enzyme-catalyzed reactions. Both types of reactions were fit to the appropriate functions by unweighted nonlinear regression analysis.

Reaction Quenching and Product Isolation. The quenching of the reaction of interest requires in addition that no side reactions occur in the quenched solution. Quenching the reactions discussed here involved acidifying the reaction mixture so that the enzyme was denatured or inactive, or that the nucleophile was no longer in its reactive form. The acid quench converts the dinitrophenol product completely to its acidic form.

The product isolation procedures were slightly different for each reaction, but several general themes are apparent. Identical procedures were used for the samples quenched early in the reaction and at completion to minimize artifacts introduced by any isotope effects on these procedures. The isolation problem involved the separation of 1–2 mg of the phenolic product from a large volume of solution (typically 200–900 mL). These solutions contained a large excess of starting material at 10% reaction. Advantage was taken of the acid–base properties of the dinitrophenol products, which are relatively insoluble in aqueous acid and soluble in alkaline solution. A typical procedure involved extracting the quenched acidified reaction solution twice with ether after making sure by pH measurement that all of the dinitrophenol was in its acidic form. The ether extracts were combined and extracted twice with dilute (about 0.1 M) sodium bicarbonate or sodium carbonate solutions. The dinitrophenol was separated from the remaining reactant by this step. The combined aqueous layers were acidified to at least 2 pH units below the pK_a of the phenol, and re-extracted with ether. The ethereal solution was taken to dryness by rotary evaporation. The usually crystalline residue was dissolved in aqueous bicarbonate, acidified, and extracted once more with ether. This solution was dried with sodium sulfate and the ether removed in a vacuum oven at relatively low temperature (40–60 °C). The resulting dinitrophenol samples were used to measure isotope ratios by mass spectrometry.

The yields of product were typically in the range of 50–75% of the theoretical. Thus, each sample at ca. 10% reaction and at the completion of the reaction contained 500–1000 μg of dinitrophenol.

Preparation of Samples for Mass Spectrometry. Samples were prepared for mass spectrometric analysis by dissolving the product in 100–500 μL of dry chloroform. This solution was carefully transferred to a mass spectrometer sample tube using 5-μL micro-caps (Drummond Scientific). Care was taken never to touch the sample tubes or to contaminate the outside of the tubes with the solution of dinitrophenol. Sample tubes were either melting point capillaries or Pyrex capillary tubes, 1.9-mm outside diameter and ca. 1.5 cm in length. These tubes were cleaned several times in acetone and baked in a vacuum oven at 140 °C overnight. Prior to use they were placed in a furnace at 400 °C for ca. 6 h. A typical tube holds 20–30 μL of solution. After addition of the solution of dinitrophenol, the solvent was removed by heating the tubes in a vacuum oven at 40–60 °C. Typically 3–5 samples of the product at both 10% and 100% reaction were prepared and analyzed by mass spectrometry.

Mass Spectrometric Analysis. Samples were introduced into the mass spectrometer via the solid probe at a source temperature of 70–100 °C. The sensitivity and ion gain settings employed were set as low as possible to reduce instrument noise and yet obtain a sufficient number of counts for accurate results. Thus, fairly large samples containing 50–250 μg of dinitrophenol were used. This led to a maximum sample pressure of $2–4 \times 10^{-6}$ Torr. The parent peaks of the dinitrophenol were scanned repetitively at ca. 5-s time intervals for the lifetime of the sample in the spectrometer. This ranged from 5–30 min. The source temperature slowly rose after the introduction of the sample, reaching a maximum of 130 °C. The source intensity was kept low to reduce fragmentation while the ionizing voltage was maintained at 70 eV. Attempts at using lower voltages led to greatly reduced numbers of counts, insufficient for the accuracy needed. After a sample was depleted, the source temperature was increased to ca. 150 °C for about 10 min to remove any unvolatilized dinitrophenol. The sample tube was then removed from the solid probe, and the next sample was introduced after the temperature decreased. The 3–5 product samples from 10% and 100% reactions were run alternately, to reduce deterministic error due to machine drift and to randomize small memory effects.

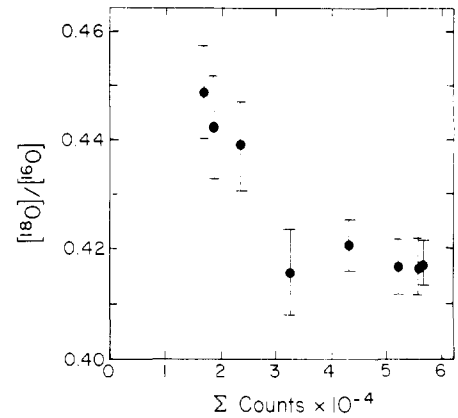


Figure 1. Dependence of the observed isotope ratio of partially enriched 2,4-dinitrophenol on the sample size. 2,4-Dinitrophenol, approximately 31% enriched with oxygen-18 in the phenolic oxygen, was analyzed by mass spectrometry as described in the text. The isotope ratio is defined as the ratio of the number of counts in the 186 and 184 m/e peaks, the molecular ions of the labeled and unlabeled 2,4-dinitrophenol. Each point represents 20 individual determinations of the isotope ratio, and the error bars are ± one standard error

Since it was not possible to maintain a constant flux of ions in the mass spectrometer during the entire lifetime of a sample, it was necessary to know if there was any dependence of the isotope ratios on the number of counts. The direct analysis of ca. 30% oxygen-18 labeled 2,4-dinitrophenol is shown in Figure 1. The isotope ratio, i.e., the ratio of the intensities of the parent ions at m/e 184 and 186, does exhibit a slight dependence on the number of counts. These data show about a 10% change in the isotope ratio as the number of counts increases by a factor of 5. This was due to background contamination, and, therefore, all isotope ratio determinations were made in the plateau region above 3×10^4 counts. No such dependence of the isotope ratio upon sample size was observed when 50% oxygen-18 labeled 2,4-dinitrophenol or p-nitrophenol was analyzed. The use of 50% labeled substrates is optimal for the measurement of heavy atom KIEs because the two peaks are of near equal intensity.

Data Analysis. A complete experiment, consisting of 3–5 samples of the product at 10% and 100% reaction, generated between 1000 and 2000 measurements of the isotope ratios. No allowance was made for the natural abundance of oxygen-18 or other stable isotopes, as this correction is smaller than the experimental error with the relatively high isotopic enrichment used. Thus, only the P and $P + 2$ peaks were included in the data analysis, corresponding to m/e 184 and 186 for 2,4-dinitrophenol. These pairs of values were analyzed with the aid of an off-line computer (CDC 6400) in sets of ca. 20 scans to yield the mean and standard error of the isotope ratio ($^{18}O/^{16}O$) and the average number of counts in these isotope ratio determinations. The isotope ratios for these sets of 20 scans were plotted as a function of the number of counts in each set for each sample. If no dependence of the isotope ratio on the sample size was apparent, the weighted mean of the isotope ratio and its associated standard error were calculated from the isotope ratios for each set using

normal statistical methods (*13*). The procedure was followed for each product sample at 10% and 100% reaction, and the resulting isotope ratios at these two extents of reaction were used to yield the final isotope ratios and their associated standard errors by weighted averaging.

The kinetic isotope effect was calculated from the isotope ratios at ca. 10% and 100% reaction and from the exact fraction of reaction at the time of quenching. This value is given by Equation 1 (*3*), where f is the fraction of the reaction (usually about 10%); R is the isotope ratio of the product at f; and R_{100} is the isotope ratio of the product at 100% reaction. The analogous relationship for mass spectrometric analysis of reactant is given by Equation 2 in which R_0 is the initial isotope ratio of the starting material.

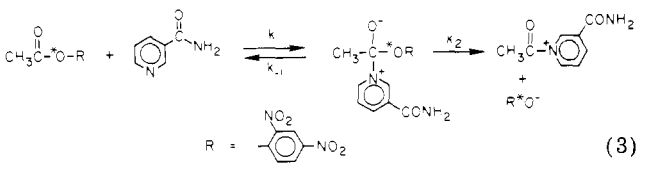
$$\mathrm{KIE} = \ln\left(1 - \frac{f(R_{100} + 1)}{R + 1}\right) \Big/ \ln\left(1 - \frac{fR(R_{100} + 1)}{R_{100}(R + 1)}\right) \quad (1)$$

$$\mathrm{KIE} = \ln\left(\frac{f(1 + R_0)}{1 + R}\right) \Big/ \ln\left(\frac{f(1 + 1/R_0)}{1 + 1/R}\right) \quad (2)$$

The propagated standard error of the KIE was calculated from the standard errors of the isotope ratios and fraction of reaction by a computer program using standard statistical methods, assuming that the errors in each parameter are independent.

RESULTS AND DISCUSSION

An example of a reaction studied by the procedure described is the aminolysis of DNPA by nicotinamide shown in Equation 3:



(3)

It has been shown by Jencks and co-workers that the rate determining step in ester aminolysis is sensitive to the relative acidities of the nucleophile and leaving group (*14*, *15*). The formation of a tetrahedral intermediate (k_1) is rate determining for strongly basic amines, whereas the breakdown of this intermediate (k_2) is the slow step for weakly basic amines. It has been shown by structure-reactivity correlations for esters closely related to DNPA that this change in rate determining step occurs when the amine pK_a is ca. 4 pH units more basic than that of the leaving alcohol or phenol (*16*, *17*). Since the pK_as of 2,4-dinitrophenol and nicotinamide are both close to 4.0 (*18*), the decomposition of the tetrahedral intermediate should be rate determining for this reaction. Thus, cleavage of the carbon–oxygen bond will occur in the rate limiting step, and a substantial oxygen-18 KIE should be observed if bond breaking is sufficiently advanced in the transition state.

The kinetics of the reaction of nicotinamide with DNPA enriched with 31% oxygen-18 are shown in Figure 2, as monitored at 400 nm. An aliquot of the reaction mixture was quenched at an absorbance of 0.0879, which corresponds to 9.9% of the total reaction. The remainder of the reaction mixture was allowed to react to completion. The product, 2,4-dinitrophenol, was isolated and subjected to mass spectrometric analysis as described in the Experimental section. Some typical data are shown in Table I and the complete collection of data is given in Figure 3. It can be seen in Table I that there is no dependence of the observed isotope ratio on sample size above 40 000 counts. These data yield an isotope effect of 1.043 ± 0.007. This result shows conclusively that the C–O bond is broken in the transition state and that breakdown of a tetrahedral intermediate must be rate determining as predicted by Jencks and co-workers.

We have also used this method of direct mass spectrometric analysis to measure ^{18}O leaving group KIEs for enzyme-

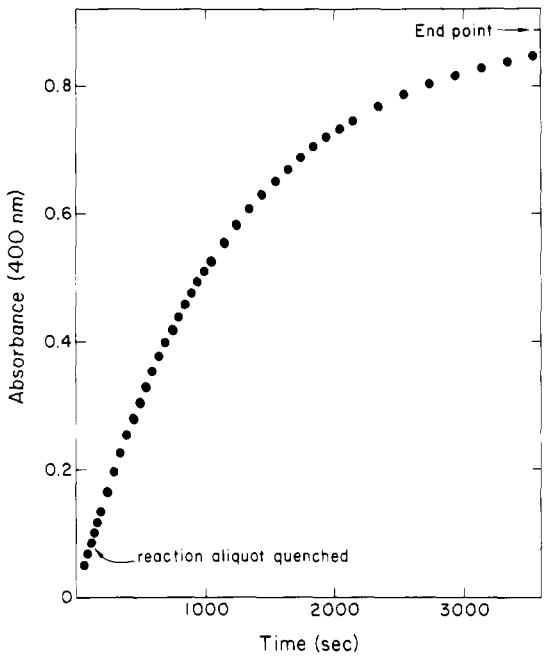


Figure 2. Time course of the reaction of 2,4-dinitrophenyl acetate partially enriched with oxygen-18 in the ether oxygen with nicotinamide at pH 4.35 in 3% (v/v) acetonitrile, 25 °C, I_c = 1.0 (KCl), monitored at 400 nm. The initial concentrations of ester and amine were 0.115 mM and 0.02 M, respectively. The observed pseudo-first-order rate constant is 8.583 ± 0.007 × 10^{-4} s^{-1} and the end-point absorbance is 0.8890 ± 0.0002 as determined by computer analysis. The calculated curve is superimposable on the data points at the scale shown. An aliquot of the reaction was quenched at an absorbance of 0.0879 corresponding to 9.9% of the total reaction

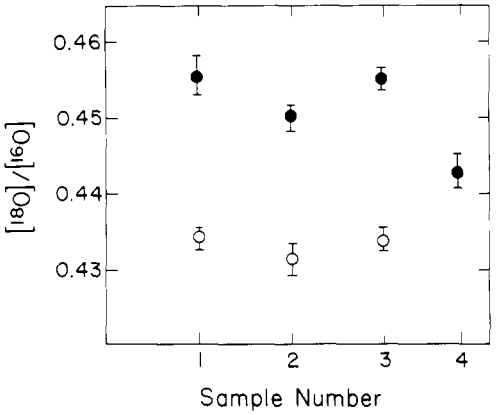
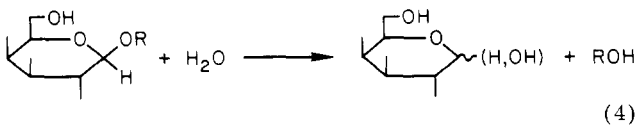


Figure 3. Results of mass spectrometric analysis of the product, 2,4-dinitrophenol at 9.9% (O) and 100% (●) reaction. Each point represents the weighted mean of between 80 and 290 determinations of the isotope ratios, and the error bars represent ± one standard error. The final isotope ratios for this complete set of data are 0.4331 ± 0.0008 and 0.4509 ± 0.0027 at 9.9% and 100% reaction, respectively. These data yield a KIE of k_{16}/k_{18} = 1.043 ± 0.007

catalyzed reactions. β-Galactosidase from *Escherichia coli*, which catalyzes the hydrolysis of a wide variety of β-galactosides (Equation 4), has been the subject of intense study (*19*).



(4)

The mechanism of this reaction has been recently pursued by Sinnott and co-workers (*20*, *21*), who have proposed a scheme involving a conformational change and a galactosyl carbonium ion which is in equilibrium with a covalent ga-

Table I. Representative Mass Spectrometric Data for the Determination of the Oxygen-18 Kinetic Isotope Effect on the Reaction of Nicotinamide with 2,4-Dinitrophenyl Acetate

	Sample					
	1[a]			2[b]		
set[d]	isotope ratio[c]	S.E.	Σ counts × 10^{-4} [e]	isotope ratio	S.E.	Σ counts × 10^{-4}
1	0.4558	0.0089	3.46	0.4556	0.0057	4.62
2	0.4428	0.0052	4.60	0.4572	0.0053	6.26
3	0.4316	0.0050	6.08	0.4507	0.0062	7.51
4	0.4338	0.0045	7.39	0.4494	0.0029	7.89
5	0.4296	0.0040	8.29	0.4438	0.0049	8.38
6	0.4278	0.0037	8.95	0.4426	0.0037	8.76
7	0.4260	0.0045	9.00	0.4534	0.0043	9.07
8	0.4256	0.0044	8.55	0.4401	0.0031	7.73
9	0.4320	0.0042	7.95			
10	0.4361	0.0050	7.31			
11	0.4241	0.0056	6.56			
12	0.4352	0.0043	5.73			
13	0.4394	0.0068	4.66			
14	0.4385	0.0066	3.60			
wtd. mean	0.4338	0.0014		0.4495	0.0017	

[a] Sample 1 was obtained by quenching at 9.9% reaction and isolating the 2,4-dinitrophenol as described in the Experimental section. [b] Sample 2 was an aliquot of the product, 2,4-dinitrophenol, obtained at 100% reaction. [c] The ratio of the molecular ion peak intensities at *m/e* 186 and 184. [d] Each set consists of 20-22 determinations of the isotope ratios. [e] The average of the sum of the counts in the molecular ion peaks for the labeled and unlabeled 2,4-dinitrophenol.

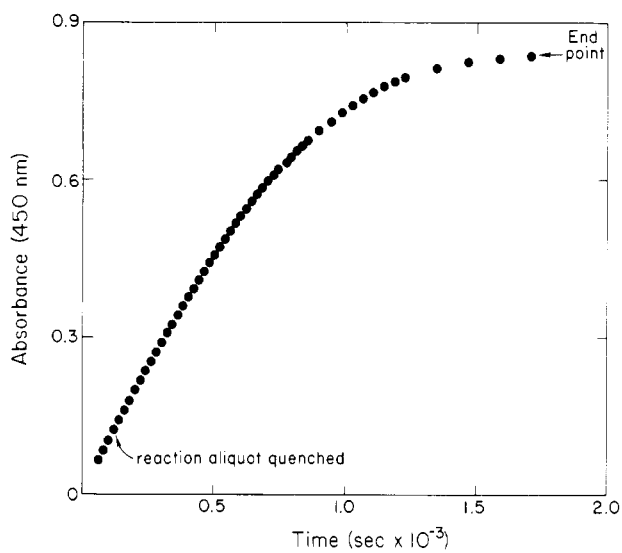


Figure 4. Time course of the reaction of β-galactosidase with 2,4-dinitrophenyl-β-D-galactoside at pH 7.0 as monitored at 450 nm. The reaction was carried out in 0.1 M sodium phosphate buffer containing 1 mM $MgCl_2$. The substrate was 49% enriched with oxygen-18 in the glycosidic oxygen atom. An aliquot of the reaction was quenched at an A_{450} = 0.1130 which corresponds to 13.4% of the total reaction. The maximum deviation of any data point from the calculated curve is <0.001 absorbance unit

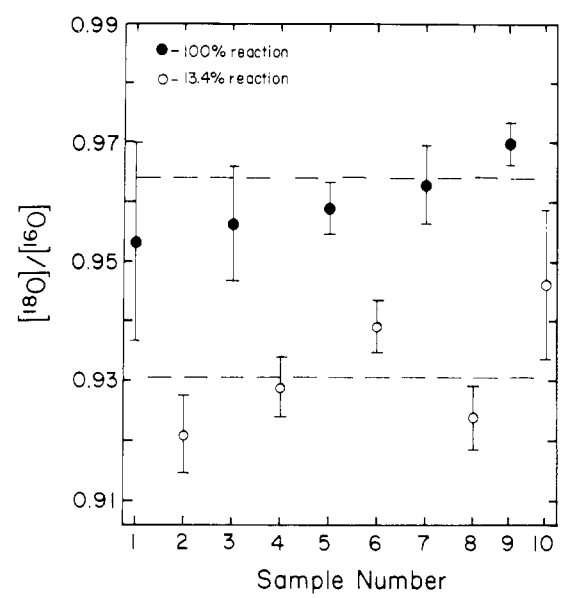


Figure 5. Results of mass spectrometric analysis of the product, 2,4-dinitrophenol, at 13.4% (○) and 100% (●) reaction from the reaction of β-galactosidase with 2,4-dinitrophenyl-β-D-galactoside. Each point represents between 40 and 200 determinations of the isotope ratios, and the error bars are ± one standard error. The final mean isotope ratios from these data, shown as the dashed lines, are 0.9307 ± 0.0038 and 0.9642 ± 0.0028 at 13.4% and 100% reaction, respectively. These data yield a KIE of $(V/K)_{16}/(V/K)_{18}$ = 1.039 ± 0.006

lactosyl–enzyme intermediate for aryl galactoside substrates. We have measured ^{18}O leaving group KIEs for this reaction in order to elucidate the mechanism further (22). The time course of the reaction of 2,4-dinitrophenyl-β-D-galactoside with this enzyme is shown in Figure 4. This substrate was enriched with about 49% oxygen-18 in the glycosidic oxygen atom.

An aliquot was quenched at an absorbance of 0.1130 corresponding to 13.4% of the total reaction, and the remainder of the reaction mixture was allowed to react to completion. Mass spectrometric analysis of the product, 2,4-dinitrophenol, yielded the results shown in Figure 5. The observed isotope effect from this experiment is $(V/K)_{16}/(V/K)_{18}$ = 1.039 ± 0.006, where V/K is defined as the second-order rate constant for an enzyme-catalyzed reaction extrapolated to zero substrate concentration. The result requires that the C–O bond is substantially broken in the first irreversible step in the reaction sequence.

Limitations of the Method. The use of direct mass spectrometric analysis requires that isotope ratios must be determined with a precision of ca. 0.1% for meaningful quantitative analysis of heavy atom KIEs. In order to accomplish this there must be an intense parent ion peak. Conditions which induce substantial fragmentation such as high temperature are to be avoided. A facile method of synthesis and purification of the labeled compound of interest is necessary, and fairly high isotopic enrichment of optimally 50% is required.

We have measured oxygen-18 leaving group KIEs by this method with a propagated standard error of ±0.2%. This

degree of precision, although considerably less than that which can be achieved under the best conditions using isotope ratio mass spectrometry, should be sufficient to measure KIEs due to ^{13}C and ^{15}N as well as ^{18}O. When used in concert with the direct rate method described in the accompanying paper, this technique should continue to prove versatile in helping to probe both organic and enzyme reaction mechanisms.

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Direct Spectrophotometric Measurement of Small Kinetic Isotope Effects

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A method is described for the measurement of heavy atom kinetic isotope effects by directly determining the rate differences due to isotopic substitution. This is accomplished by measuring the rates of reaction of highly enriched reactants compared with compounds of natural abundance using a high precision spectrophotometer interfaced to a teletype via a microprocessor for automated digital readout. The spectrophotometric data are fit directly to the relevant rate expression by nonlinear least squares regression analysis. Applications of this method to the measurement of oxygen-18 leaving group kinetic isotope effects on the reaction of nicotinamide with 2,4-dinitrophenyl acetate and on the β-galactosidase catalyzed hydrolysis of *p*-nitrophenyl-β-D-galactoside are given.

Heavy atom kinetic isotope effects (KIEs) have usually been measured by a competitive technique involving the determination of the isotopic composition of reactants or products as a function of the extent of reaction. This has been done in most cases by isotope ratio mass spectrometry following chemical conversion of the products or starting materials to a gas. A few workers have succeeded in measuring heavy atom KIEs by determining directly the rate differences engendered by isotopic substitution. Mitton and Schowen were the first to show the feasibility of this technique. They determined the carbonyl oxygen, ^{18}O KIE for the methanolysis of phenyl benzoate (*1*). Subsequently, Gorenstein and co-workers (*2*, *3*) utilized a similar approach to analyze the detailed mechanism of phosphate ester hydrolysis. The measurement of heavy atom KIEs by this method for enzyme-catalyzed reactions has been questioned (*4*).

It is important to develop the direct method to as high a degree of accuracy and precision as possible because of the ease of application compared to mass spectrometric procedures which require larger amounts of isotopically enriched materials and are more time consuming; and because this method offers the only apparent possibility to obtain isotope effects on V_{max} (the rate at saturating substrate concentration) for enzyme-catalyzed reactions.

The recent development of high precision spectrophotometers such as the Cary 118 and the interfacing of these instruments via microprocessors to yield data in digital form without intervention of the experimenter led us to explore this system for the direct measurement of heavy atom KIEs. The photometric accuracy of such a spectrophotometer is better than 0.001 absorbance unit, representing an error of only 0.1% for a change in absorbance of 1.0. We have found that this degree of precision allows KIEs to be determined to ±0.1%. Although considerably less than that obtainable by isotope ratio mass spectrometry, this precision is sufficient for all but the most exacting studies using heavy atom KIEs.

EXPERIMENTAL

Apparatus. All spectrophotometric measurements of reaction kinetics were made on a Cary 118C Spectrophotometer interfaced via a microprocessor (Claremont Research) to an ASR 33 teletype and tape-punch. The microprocessor can be set to sample the absorbance data at intervals from 1–999 s. Both the cell holder and cell compartment were maintained at constant temperature with a Lauda Model K2 circulating water bath. A cell holder capable of holding up to 5 cuvettes was used for all isotope effect experiments. The original design of the microprocessor was modified so that up to 5 reactions could be monitored simultaneously. A Radiometer Model PHM4c pH meter equipped with

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