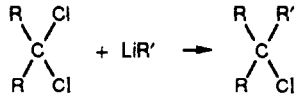

which we now report, is "unexpected". To cope with the first problem we elected to conduct the reaction at as low a temperature as possible in order to remove as much vibrational excitation as possible from the adjacent lithium and chlorine carbon bonds and minimize the possibility of elimination of lithium chloride. To minimize both reactions we selected reaction conditions with a very high concentration of *tert*-butyllithium so the reaction with the two chlorines could occur as rapidly as possible and compete with both the lithium chloride elimination reaction and the coupling reaction.

Thus, we have found at very low temperatures (−110 °C) using THF as a solvent a thermodynamic and kinetic regime where lithium–halogen exchange proceeds at a synthetic rate much more rapidly than lithium–hydrogen exchange. Temperatures are also low enough that there is a minimal problem with organolithium compounds reacting with the THF. The following new di- and trilithio compounds have been prepared:

$$Ph_2CCl_2 + 6t\text{-BuLi} \xrightarrow[THF]{-110\ ^\circ C} Ph_2CLi_2 \quad (31.0\%) \quad (1)$$

$$PhCHCl_2 + 6t\text{-BuLi} \xrightarrow[THF]{-110\ ^\circ C} PhCHLi_2 \quad (7.6\%) \quad (2)$$

$$PhCCl_3 + 9t\text{-BuLi} \xrightarrow[THF]{-110\ ^\circ C} PhCLi_3 \quad (3.2\%) \quad (3)$$

The reaction temperatures were kept as low as the solvent would allow, using a liquid nitrogen/ethanol slush with the temperature usually below −105 °C.

The reactions were quenched with $d_1$-ethanol and analyzed by $^1H$ and $^{13}C$ NMR on a GE GN-500 instrument and by GC/MS analysis on a Finnigan 4000 apparatus using a 30 m × 0.25 mm Heliflex bonded phase RSL-200 1.0-μm capillary column, with an injector temperature of 250 °C and an ionizer temperature of 150 °C. Percent deuterium incorporation was determined by mass spectroscopy.

**Dilithiodiphenylmethane (1).** Dichlorodiphenylmethane (1 mL, 5.2 mmol) was added dropwise over 5 min to a stirred solution of 21 mL of THF and 37 mL of 1.7 M *tert*-butyllithium (6:1) at −110 °C. A deep reddish-orange color became evident. Stirring was maintained for 30 min before derivatization by addition of twice as many equivalents of $d_1$-ethanol as *tert*-butyllithium at −110 °C. The reaction mixture was then allowed to warm to room temperature and stirred for several hours to insure complete derivatization and washed with $H_2O$ to remove the lithium salts. The phases were then separated and the organic layers dried overnight with magnesium sulfate. The reactions were then distilled to remove solvents. Analysis of the sample gave a yield of 51.2% of diphenylmethanes, of which 60.6% was $d_2$-diphenylmethane, for an overall yield of 31.0% of dilithiated species **1**. MS: *m/e* (%) 167 (34.52), 168 (49.75), 169 (100), 170 (96.39), 171 (12.07). $^1H$ NMR ($d_6$-acetone): 7.14 (4), 7.20 (4), 7.23 ppm (2). $^{13}C$ NMR ($d_6$-acetone): 41.7 (5), 126.6, 129.0, 129.5, 142.0 ppm.

**Dilithiophenylmethane (2).** Dichlorophenylmethane (1 mL, 7.8 mmol) was added to 6 equiv of *tert*-butyllithium as above. A dark green color was visible. The reaction mixture was stirred for 2 h. The yield was determined to be 25.6% of phenylmethanes, of which 29.5% was $d_2$-phenylmethane, for an overall yield of 7.6% of **2**. MS: *m/e* (%) 91 (48.03), 92 (100), 93 (96.50), 94 (46.21), 95 (3.00). $^1H$ NMR ($d_6$-acetone): 2.30, 7.03 ppm (5). $^{13}C$ NMR ($d_6$-acetone): 21.4 (5), 125.4, 129.1, 130.1, 137.6 ppm.

**Trilithiophenylmethane (3).** Trichlorophenylmethane (1 mL, 7.1 mmol) was added to 9 equiv of *tert*-butyllithium under the conditions stated above. A deep purple color was detected. The reaction mixture was stirred for 1.75 h. The yield was determined to be 14.9% of phenylmethanes, of which 21.7% was $d_3$-phenylmethane, for an overall yield of 3.2% of **3**. MS: *m/e* (%) 91 (30.68), 92 (55.86), 93 (100), 94 (92.04), 95 (41.17), 96 (2.43). $^1H$ NMR ($d_6$-acetone): 7.03 ppm (5). $^{13}C$ NMR ($d_6$-acetone): 21.3 (7), 125.4, 129.2, 130.1, 137.7 ppm.

One may follow the progress of the lithium–halogen exchange reaction by quenching at various time intervals with $d_1$-ethanol. We have readily observed the increase of lithium substitution (lithium–halogen exchange) with time. For example, within the first 15 min after initiating reaction 1, $Ph_2CDCl$ is obtained as a prominent hydrolysis product, whereas after 30 min no $Ph_2CDCl$ is observed.

We believe the remaining metal–halogen exchanges to be stepwise, because upon using 1 equiv of *t*-BuLi for every halogen, the vibrant colors that are produced in these reactions disappear at around −90 °C before derivatization. Use of 3 equiv/halogen permits the polylithiated compounds to form. The excess *t*-BuLi is believed to react with the *t*-BuCl formed by the exchange reaction thus decreasing the reaction of *t*-BuCl with the newly lithiated compound.[5]

Presently the best solvent system we have found has been 4 equiv of THF/1 equiv of *t*-BuLi. This allows the *t*-BuLi to react; however, it is believed that a reaction also occurs between THF and lithium compounds at elevated temperatures.[6] This results in lower yields.

We think these experiments are the beginnings of a general synthesis for polylithium organic compounds. We expect higher yields to be forthcoming as a result of increased experience and more careful selection of conditions.

We have enough additional work in progress studying a variety of classes of multiply halogen substituted starting materials to state that this technique will be a more widely applicable synthesis. For example, we can convert hexachlorobenzene to hexalithiobenzene in over 60% yield.[7] Each reaction requires somewhat different conditions.

**Acknowledgment.** We are grateful to the National Science Foundation (CHE-9012405) and the Robert A. Welch Foundation (F-700) for support of this work.

(5) Seebach, D.; Neuman, H. *Chem. Ber.* **1974**, *107*, 847.
(6) Wakefield, B. J. *The Chemistry of Organolithium Compounds*; Pergamon: New York, 1976; p 199. See references within.
(7) Baran, J. R., Jr.; Lagow, R. J., to be published.

## Crystal Structure of *N*-Methyl-*N*-phenylretinal Iminium Perchlorate: A Structural Model for the Bacteriorhodopsin Chromophore[1]

Bernard D. Santarsiero and Michael N. G. James

*Department of Biochemistry*
*474 Medical Sciences Building, University of Alberta*
*Edmonton, Alberta, T6G 2H7 Canada*

Malivaganan Mahendran and Ronald F. Childs*

*Department of Chemistry, McMaster University*
*Hamilton, Ontario, L8S 4M1 Canada*

*Received July 6, 1990*

Bacteriorhodopsin (BR), one of a family of related pigments, is a membrane-bound polypeptide that acts as a light-driven proton pump.[2] The chromophore of BR consists of a retinal molecule covalently bound as a protonated Schiff base to the ε-amino group of lysine 216.[2] Despite the importance of this chromophore, no reliable X-ray structure of a retinal iminium salt has yet been reported.

*N*-Methyl-*N*-phenylretinal iminium perchlorate (**1**) was obtained by treating retinal with *N*-methylanilinium perchlorate. The phenyl substituent on **1** was selected in order to reduce the formation of various isomers and/or conformers during the

(1) This work was supported by the Natural Science and Engineering Research Council of Canada.
(2) Becker, R. S. *Photochem. Photobiol.* **1988**, *48*, 369–399.

0002-7863/90/1512-9416$02.50/0 

**Table I.** Selected Bond Lenghts (Å) and Angles (deg)

| | | | | | |
|---|---|---|---|---|---|
| C(5)–C(6) | 1.349 (6) | C(6)–C(7) | 1.458 (5) | C(7)–C(8) | 1.336 (6) |
| C(8)–C(9) | 1.455 (5) | C(9)–C(10) | 1.354 (6) | C(10)–C(11) | 1.429 (5) |
| C(11)–C(12) | 1.357 (6) | C(12)–C(13) | 1.419 (5) | C(13)–C(14) | 1.368 (6) |
| C(14)–C(15) | 1.399 (5) | C(15)–N | 1.324 (6) | N–C(22) | 1.428 (5) |
| C(4)–C(5)–C(6) | 124.2 (4) | C(5)–C(6)–C(1) | 121.4 (3) | C(5)–C(6)–C(7) | 118.0 (4) |
| C(6)–C(7)–C(8) | 129.7 (4) | C(7)–C(8)–C(9) | 125.6 (4) | C(8)–C(9)–C(10) | 117.8 (4) |
| C(9)–C(10)–C(11) | 127.7 (4) | C(10)–C(11)–C(12) | 121.1 (4) | C(11)–C(12)–C(13) | 127.3 (4) |
| C(12)–C(13)–C(14) | 116.2 (3) | C(13)–C(14)–C(15) | 125.0 (3) | C(14)–C(15)–N | 124.2 (3) |

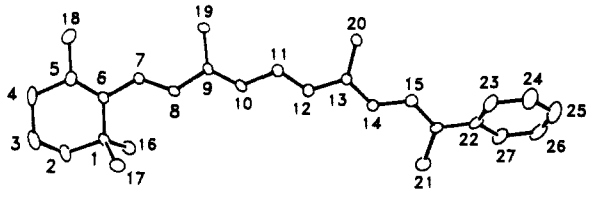


**Figure 1.** Structure of the cation of **1** showing atom numbering. Hydrogen atoms are omitted. Gaussian amplitudes are drawn at 20% probability level.

preparation and crystallization of retinal iminium salts.[3] Previous studies on simple iminium salts have shown that the replacement of an *N*-alkyl by an aryl group does not alter the structure significantly.[4]

The salt **1** was recrystallized from acetonitrile/ether mixtures and its structure determined by using single-crystal X-ray crystallography, Table I, Figure 1.[5] Some disorder was present in the cyclohexene ring of **1**, as is commonly found with other neutral retinal derivatives.[6,7] The perchlorate anion was rotationally disordered, and no attempt was made to model this disorder. Despite these difficulties, the structure of the unsaturated portion of **1** is well defined and the various interatomic distances and angles have been obtained with good precision.

The dihedral angle between the phenyl ring and the polyene fragment is 46.2 (5)°. This large dihedral angle, coupled with the relatively long N–C(22) bond distance [1.428 (5) Å], indicates that there is little delocalization of the positive charge into the aryl ring of **1**. This means that the structure of the unsaturated portion of **1** can serve as a good model for the BR chromophore.

The cation **1** has a trans configuration about each of the C–C single and double bonds of the unsaturated chain, Figure 1. In particular, **1** has an s-trans conformation about the C(6)–C(7) bond [C(5)–C(6)–C(7)–C(8) torsion angle 175.7 (4°)] as has been suggested for the chromophore in BR.[8] The C(6)–C(7)–C(8) bond angle in **1** [129.7 (4)°] is large as a result of the steric interactions between the methyl groups on C(1) and the hydrogen atom on C(8).

The polyene chain of **1** [C(6)–C(15) and N] is essentially planar. The largest displacements are at C(12), 0.190 Å, and N, 0.051 Å. Successive torsion angles along the chain are uniformly large, clustering in magnitude about an average value of 176.6 (11)°. The polyene chain of **1** exhibits a substantial in-plane bending, or curvature, Figure 1. The bond angles opposite the methyl groups on C(9) and C(13) are significantly compressed as compared to the other bond angles along the chain.

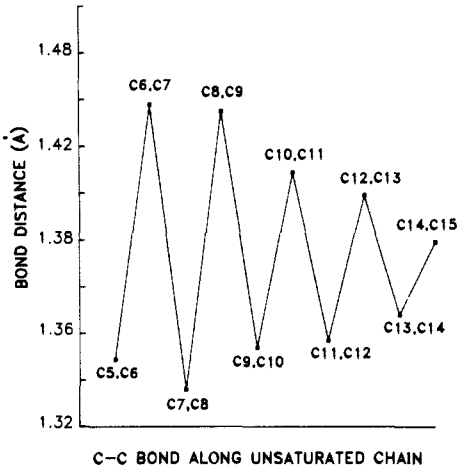


**Figure 2.** C–C bond distances along the unsaturated chain.

While the overall conformation found here for **1** is similar to that found for neutral retinoids,[6,7] some very significant differences are observed in internuclear distances. The C—C single bonds of the unsaturated chain of **1** are shortened and the C=C bonds lengthened as compared to those in neutral systems. This effect is the most pronounced with the bonds close to N. Thus C(14)–C(15) [1.394 (5) Å] is significantly shorter while C(13)–C(14) [1.368 (6) Å] is longer than the corresponding bonds of *all-trans*-retinal (1.455 and 1.344 Å, respectively[6]) or those calculated for the *N*-methyl imine of *all-trans*-retinal (1.474 and 1.354 Å, respectively[9]). The C(15)–N distance of **1** [1.324 (6) Å] is longer than that of a neutral imine[10] and in the range expected for an unsaturated iminium salt.[4]

These changes in bond distances in **1** are fully consistent with delocalization of the positive charge from N to the unsaturated chain. However, it is also clear that the effect of this delocalization falls off with distance from the nitrogen atom. Progressively larger alternations in bond distances are observed the further the bond in question is removed from the N atom, Figure 2. To the best of our knowledge, this systematic change in degree of bond alternation provides for the first time clear-cut structural evidence for progressive dimunition of $\pi$-electron delocalization with distance in a cationic system. These structural changes in **1** are fully consistent both with NMR data of protonated retinal Schiff bases[11] and with changes in the ease of isomerization about the formal C=C double bonds of the unsaturated chain.[12]

There have been several reports of theoretical calculations of the struture of retinal iminium salts.[8,13] While the results of these

(3) Childs, R. F.; Shaw, G. S. *J. Am. Chem. Soc.* **1988**, *110*, 3013–3017.
(4) Childs, R. F.; Shaw, G. S.; Lock, C. J. L. *J. Am. Chem. Soc.* **1989**, *111*, 5424–5429.
(5) Crystallographic information: $P\bar{1}$, $a$ = 7.547 (1) Å, $b$ = 8.652 (3) Å, $c$ = 21.190 (6) Å, $\alpha$ = 93.76 (2)°, $\beta$ = 93.00 (2)°, $\gamma$ = 106.38 (2)°, $V$ = 1321.0 (6) Å$^3$, $Z$ = 2, $D_{calcd}$ = 1.192 g/cm$^3$, $F(000)$ = 254 e. Diffractometry: CAD4, Cu K$\alpha$ (Ni filter) radiation, $\lambda$ = 1.5418 Å, $T \approx$ 295 K, 7980 reflections collected, $\theta \leq 55°$, $(-h,\pm k,\pm l)$, $\theta$ scan width 1.0° plus dispersion, $\theta$–$2\theta$ scans, no decay with five standards, absorption correction via two azimithal $\psi$ scans. Structure solution and refinement: 3298 averaged intensities, solution by GENTAN (XTAL2.4 Crystallographic Computing System, S. R. Hall and J. M. Stewart, 1987), full-matrix least-squares refinement on $F$ with $F \geq 3\sigma$ data, weights $w = (\sigma_F)^{-2}$ with instability constant of 2%, anisotropic Gaussian displacement parameters for non-hydrogen atoms, hydrogen atoms idealized with riding model on C, C–H 1.00 Å, and $B$ = 9 Å$^2$. Final cycle: GOF = 2.74 (2892 observations, 298 parameters), $R$ = 0.073, $R_w$ = 0.064.
(6) Hamanaka, T.; Mitsui, T.; Ashida, T.; Kakudo, M. *Acta Crystallogr.* **1972**, *B28*, 214–222.
(7) Stam, C. H. *Acta Crystallogr.* **1972**, *B28*, 2936–2945. Simmons, C. J.; Liu, R. S. H.; Denny, M.; Seff, K. *Acta Crystallogr.* **1981**, *B37*, 2197–2205.
(8) Harbison, G. S.; Smith, S. O.; Pardoen, J. A.; Courtin, J. M. L.; Lugtenburg, J.; Herzfeld, J.; Mathies, R. A.; Griffin, R. G. *Biochemistry* **1985**, *24*, 6955–6962. van der Steen, R.; Biesheuvel, P. L.; Mathies, R. A.; Lugtenburg, J. *J. Am. Chem. Soc.* **1986**, *108*, 6410–6411.
(9) Tavan, P.; Schulten, K.; Oesterhelt, D. *Biophys. J.* **1985**, *47*, 415–430.
(10) Santarsiero, B. D.; Moore, E. J. *Acta Crystallogr.* **1988**, *C44*, 433–435. Allen, F. H.; Kennard, O.; Watson, D. G.; Brammer, L.; Orpen, A. G.; Taylor, R. *J. Chem. Soc., Perkin Trans 2* **1987**, S1–S19.
(11) Shriver, J.; Abrahamson, E. W.; Mateescu, G. D. *J. Am. Chem. Soc.* **1976**, *98*, 2407–2409.
(12) Lukton, O.; Rando, R. R. *J. Am. Chem. Soc.* **1984**, *106*, 4525–4531.
(13) Poirer, R. A.; Yadav, A. *Chem. Phys. Lett.* **1989**, *156*, 122–124. Kakitani, T.; Kakitani, H. *J. Phys. Soc. Jpn.* **1975**, *38*, 1435–1463. Salem, L.; Bruckmann, P. *Nature* **1975**, *258*, 526–528.

calculations show trends similar to those found here for **1**, the calculations tend to overestimate C-C bond lengths along the unsaturated chain. The most significant difference occurs with the C(13)-C(14) bond, for which the calculated distances are ca. 0.04 Å larger than those measured here.

In conclusion, **1** should serve as a good model for the chromophore of BR and provide detailed information on the structure of this important natural chromophore.

**Supplementary Material Available:** Tables containing atomic coordinates, bond lengths and angles, selected torsion angles, anisotropic Gaussian displacement parameters, hydrogen atom coordinates, and least-squares planes and a figure showing the stereoview of the molecular packing in the unit cell for the title compound (7 pages); listing of observed and calculated structure factor amplitudes of all reflections for the title compound (13 pages). Ordering information is given on any current masthead page.

## Kinetic Isotope Effect Investigation of Enzyme Mechanism in Organic Solvents

Kenneth A. H. Adams,† Sook-Hyun Chung,‡ and Alexander M. Klibanov*

*Department of Chemistry*
*Massachusetts Institute of Technology*
*Cambridge, Massachusetts 02139*
*Received July 16, 1990*

Enzymatic catalysis in anhydrous organic solvents (instead of water) has revealed exciting mechanistic phenomena[1] and led to new synthetic methodologies.[2] Nevertheless, our mechanistic understanding of enzyme action in such media, required to take a full advantage of these novel opportunities, is still in its infancy. Although recent studies of enzyme structure by solid-state NMR,[3] and mechanism by means of linear free energy correlations,[4] suggest profound similarities of those in water and in anhydrous solvents, no single method is sufficiently penetrating and only their repertoire can be truly conclusive. To this end, in the present work we have used a kinetic isotope effect approach to compare the mechanistic behavior of enzymes in various organic solvents.

Isotope effects on enzyme action have proven to be an insightful diagnostic tool.[5] Herein, we have initially applied it to a model reaction between vinyl butyrate and 1-butanol (BuOH or BuOD) catalyzed by subtilisin Carlsberg (protease from *Bacillus licheniformis*) in anhydrous acetonitrile. Such enzymatic transesterifications follow the compulsory order mechanism without ternary complexes:[6]

$$\text{E} + \text{ester} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{E·ester} \xrightarrow{k_2} \text{acyl-E} + \underset{\text{P}_1}{\text{alcohol}} \underset{k_{-1'}}{\overset{k_{1'}}{\rightleftharpoons}}$$

$$\text{acyl-E·alcohol} \xrightarrow{k_3} \text{E} + \text{P}_2 \quad (1)$$

where E is the enzyme, ester and alcohol are vinyl butyrate and butanol, respectively, and $P_1$ and $P_2$ are the reaction products. By measuring the dependence of the initial rate of the subtilisin-catalyzed transesterification on the concentration of the alcohol at a fixed ester concentration, we have determined the values of $V$ and $K_m^{\text{alcohol}}$ for BuOH and BuOD. As one can see in the first line of Table I, both the maximal velocity and the Michaelis constant for BuOH are 3-4-fold greater than those for BuOD. Thus enzymatic reaction 1 in acetonitrile exhibits a pronounced deuterium kinetic isotope effect.

**Table I.** Primary Deuterium Kinetic Isotope Effects in the Enzymatic Transesterification Reaction between Vinyl Butyrate and 1-Butanol (BuOH or BuOD) in Various Anhydrous Organic Solvents[a]

| enzyme | solvent | $V^H/V^D$ | ${}^HK_m^{\text{alcohol}}/{}^DK_m^{\text{alcohol}}$ |
|---|---|---|---|
| subtilisin | acetonitrile | 3.7 ± 0.7[b] | 3.2 ± 0.7[c] |
| subtilisin | tetrahydrofuran | 4.6 ± 0.8[b] | 3.8 ± 0.6[c] |
| subtilisin | ethyl acetate[d] | 4.6 ± 0.7[b] | 3.1 ± 0.4[c] |
| lipase | acetonitrile | 1.9 ± 0.2[b] | 1.8 ± 0.2[c] |
| lipase | cyclohexane | 1.5 ± 0.1[b] | 1.9 ± 0.2[c] |

[a] For both subtilisin- and lipase-catalyzed transesterifications, the ester concentration was 200 mM and the alcohol concentrations were varied in the range from 2 to 400 mM; the enzyme concentration was 1 mg/mL. Reaction mixtures (1 mL) were shaken at 300 rpm and 30 °C; periodically, 0.5-μL aliquots were withdrawn and analyzed by gas chromatography.[6] Organic solvents were dried prior to use to bring their water content below about 0.01% (for procedures, see ref 6). Subtilisin (Sigma Chemical Co., type VIII) was prepared by dissolving 5 mg/mL enzyme in 20 mM aqueous potassium phosphate buffer (pH 7.8), followed by lyophilization. Lipoprotein lipase from *Pseudomonas fluorescens* (Amano International Enzyme Co., type P 30) was merely extensively dried under vacuum prior to use. All reactions were initiated by the addition of the solid enzyme to the substrate solutions, followed by a 10-s ultrasonication of the resultant suspensions to homogenize them. The values of $V$ and $K_m$ were determined from the kinetic data by using a nonlinear regression analysis computer program (R. J. Leatherbarrow, "Enzfitter", Elsevier-BIOSOFT). Sample standard deviations were calculated on the basis of the average of at least three independent experiments. Error limits of the isotope effects were estimated by standard statistical techniques (Bevington, P. R. *Data Reduction and Error Analysis for the Physical Sciences*; McGraw-Hill: New York, 1969; pp 61, 113, 117). [b] The mean individual values for $V^H$ and $V^D$ were (in mM/min, from top to bottom) 0.081 and 0.022, 0.43 and 0.093, 0.55 and 0.12, 0.051 and 0.027, and 0.83 and 0.56, respectively. [c] The mean individual values for ${}^HK_m$ and ${}^DK_m$ were (in mM, from top to bottom) 200 and 63, 300 and 80, 470 and 150, 42 and 23, and 13 and 7, respectively. [d] No appreciable subtilisin-catalyzed reaction was observed between 1-butanol and ethyl acetate in the latter (i.e., in the absence of vinyl butyrate).

Analogous experiments have been carried out in two other, unrelated organic solvents. As can be seen in Table I, in anhydrous tetrahydrofuran and ethyl acetate, as in acetonitrile, subtilisin catalysis displays very similar deuterium kinetic isotope effects (whether for $V$ or $K_m^{\text{alcohol}}$, as expected from the analysis of kinetic expressions for reaction 1[7]). It is worth mentioning that their magnitude is in the same range as those for hydrolysis reactions catalyzed by proteases and other hydrolases in water.[8]

In the simplest case,[7] the kinetic isotope effect observed (both $V^H/V^D$ and ${}^HK_m^{\text{alcohol}}/{}^DK_m^{\text{alcohol}}$) equals $k_3{}^H/k_3{}^D$, i.e., corresponds to the reaction of acyl-subtilisin with the nucleophile (1-butanol). This deacylation process consists of at least four distinct steps:

† On leave from the Department of Chemistry, Mount Allison University, Sackville, New Brunswick E0A 3C0, Canada.
‡ On leave from Doosan Research Laboratory, Seoul, Korea.

(1) Klibanov, A. M. *Trends Biochem. Sci.* **1989**, *14*, 141-144.
(2) Klibanov, A. M. *Acc. Chem. Res.* **1990**, *23*, 114-120. Dordick, J. S. *Enzyme Microb. Technol.* **1989**, *11*, 194-211. Chen, C.-S.; Sih, C. J. *Angew. Chem., Int. Ed. Engl.* **1989**, *28*, 695-707.
(3) Burke, P. A.; Smith, S. O.; Bachovchin, W. W.; Klibanov, A. M. *J. Am. Chem. Soc.* **1989**, *111*, 8290-8291.
(4) Kanerva, L. T.; Klibanov, A. M. *J. Am. Chem. Soc.* **1989**, *111*, 6864-6865.
(5) (a) Cleland, W. W., O'Leary, M. H., Northrop, D. B., Eds. *Isotope Effects on Enzyme-Catalyzed Reactions*; University Park Press: Baltimore, 1976. (b) Gandour, R. D., Schowen, R. L., Eds. *Transition States of Biochemical Processes*; Plenum Press: New York, 1978. (c) Cleland, W. W. *Bioorg. Chem.* **1987**, *15*, 283-302. (d) Cook, P. F. *Enzyme Mechanism from Isotope Effects*; CRC Press: Boca Raton, FL, 1990.
(6) Zaks, A.; Klibanov, A. M. *J. Biol. Chem.* **1988**, *263*, 3194-3201.
(7) See supplementary material. It should be pointed out that we were concerned about the possibility of D/H exchange among BuOD, adventitious water, and the enzyme, but ruled it out for the following reasons. In all cases, we observed perfectly linear initial rates (i.e., product concentration vs time dependences) for BuOD (as well as BuOH) as the nucleophile. Thus the putative D/H exchange is either almost instantaneous or does not occur to any significant extent (otherwise, the enzymatic transesterification would have accelerated with time for BuOD). The former possibility, however, is inconsistent with a large kinetic isotope effect observed for both $V$ and $K_m$ in different solvents. In addition, simple calculations (taking into account the water content in our system) indicate that the aforementioned D/H exchange, if occurring, may have significantly affected the BuOD concentration at 2-20 mM but not at 100-400 mM. Consequently, such an exchange would have resulted in deviations from linear Michaelis-Menten dependences which, in fact, were not observed.
(8) Schowen, R. L. In ref 5a, pp 64-99. Schowen, K. B. J. In ref 5b, pp 225-283.

0002-7863/90/1512-9418$02.50/0 