Previously, many investigators have reported evidence from chemical modification studies with various esterifying agents that there are at least two catalytically essential aspartate β-carboxyl groups in the active site of pepsin.[4-8] Additionally, Erlanger et al.[9] and Gross and Morell[10] have demonstrated that an aspartate β-carboxyl group which is not crucial for catalysis is esterified by *p*-bromophenacyl bromide. It was, of course, of interest to determine the relationship of the carboxyl groups involved in hydroxamate formation to those identified as important residues in the esterification experiments. Therefore, following the procedure of Gross and Morell,[10] the hydroxamate-containing pepsin species obtained in the hydroxylamine-trapping experiments illustrated in Table I were subjected to Lossen rearrangement, followed by acid hydrolysis. With all samples studied, the amino acid analyses performed on the hydrolyzates revealed that only 2,3-diaminopropionic acid was formed. No 2,4-diaminobutyric acid was detected. Thus, the PTFS–pepsin intermediates trapped with hydroxylamine must have been formed at the β-carboxyl groups of aspartate residues.

Having demonstrated the nature of the groups involved in anhydride formation in the pepsin-catalyzed hydrolysis of PTFS, we wished to identify the particular residues taking part in catalysis. In a typical experiment a solution of the hydroxamate-containing pepsin (3.3 mol of hydroxamate per mole of pepsin) obtained from two incubations of PTFS with pepsin (mole ratios PTFS to pepsin in each incubation = 2.2, see Table I) was digested by native pepsin at pH 3.5, subjected to gel filtration through Sephadex G-25, high voltage electrophoresis (3000 V at pH values of 3.5 and 6.5), and descending paper chromatography (*tert*-butyl alcohol:methyl ethyl ketone:water, 2:2:1 by volume). Three hydroxamate-containing peptides and their amino acid compositions were determined. One peptide contained Asp_1, Thr_1, Ser_1, Gly_1, Val_1, Ile_1, Phe_1. This amino acid analysis is consistent with the composition of a fragment of pepsin (Val-29 → Ser-35) containing the active site residue Asp-32, the group modified by 1,2-epoxy-3-(*p*-nitrophenoxy)propane.[7,8,11] The second peptide had the composition Asp_1, Thr_1, Ser_1, Gly_3, Ala_1, Glu_1, Val_1, Ile_1, Leu_1 which seems to correspond to a fragment of pepsin (Gly-208 → Leu-220)[12] containing the active site residue Asp-215, the group modified by diazocarbonyl reagents.[4-6,11] The third peptide was a large one, and we still do not know its location in the amino acid sequence of pepsin.

Three principal conclusions can be drawn from our experiments. (1) The detection of hydroxamate formation in the pepsin-catalyzed hydrolysis of PTFS in the presence of hydroxylamine provides the first direct demonstration that anhydrides are intermediates in the hydrolysis reactions of sulfite esters. This finding is in good agreement with circumstantial evidence that acyl enzymes (anhydrides) may be intermediates in at least some pepsin-catalyzed transpeptidation reactions.[13,14] (2) The correspondence of two of the aspartate residues involved in hydroxamate formation to those (Asp-32 and Asp-215), the esterification of which is known to inactivate pepsin as a peptidase, not only shows that these residues are indeed direct participants in the hydrolysis of sulfite esters but also gives strong support to the hypothesis that the active site of pepsin as a sulfite esterase overlaps with that for its action as peptidase. (3) Finally, the observation that as many as 3–4 mol of hydroxamate can be incorporated per mole of pepsin shows that anhydride formation can occur at several carboxyl groups in pepsin-catalyzed sulfite ester hydrolysis. This is consistent with the earlier discovery that pepsin esterified either at Asp-32 or at Asp-215 can function as a catalytically active species toward sulfite esters.[2]

A major question which remains is to determine why pepsin in which hydroxamate groups have been introduced at the active site carboxyl groups can still act quite effectively as a catalyst for the hydrolysis of hemoglobin (see Table I). Two alternative explanations are either that a carboxyl group other than the β-carboxyls of Asp-32 or Asp-215 can participate in the peptidase action of pepsin or that the hydroxamate groups themselves are catalytically active. Experiments are now in progress in our laboratory to resolve this problem.

Acknowledgment. The support of this research by National Science Foundation Grants GB 39951X and BMS-73-01508 is gratefully acknowledged.

References and Notes

(1) S. W. May and E. T. Kaiser, *Biochemistry*, 11, 592 (1972).
(2) Studies on the catalytic activity toward sulfite esters of pepsin modified by α-diazo-*p*-bromoacetophenone or by 1,2-epoxy-3-(*p*-nitrophenoxy)propane suggest that only a single active site carboxylate group is necessary for the enzymatic hydrolysis of sulfite esters. Thus, whether acidic catalysis by a carboxyl group, as implied by structure II in Scheme I, is important is questionable. See H. J. Chen and E. T. Kaiser, *J. Am. Chem. Soc.*, **96**, 625 (1974).
(3) L.-H. King and E. T. Kaiser, *J. Am. Chem. Soc.*, **96**, 1410 (1974).
(4) T. G. Rajagopalan, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **241**, 4295 (1965).
(5) B. F. Erlanger, S. M. Vratsanos, N. Wassermann, and A. G. Cooper, *Biochem. Biophys. Res. Commun.*, **28**, 203 (1967).
(6) A. F. Paterson and J. R. Knowles, *Eur. J. Biochem.*, **31**, 510 (1972), and references therein.
(7) J. Tang, *J. Biol. Chem.*, **246**, 4510 (1971).
(8) K. C. S. Chen and J. Tang, *J. Biol. Chem.*, **247**, 2566 (1972).
(9) B. F. Erlanger, S. M. Vratsanos, N. Wassermann, and A. G. Cooper, *Biochem. Biophys. Res. Commun.*, **23**, 243 (1966).
(10) E. Gross and J. L. Morell, *J. Biol. Chem.*, **241**, 3638 (1966).
(11) P. Sepulveda, J. Marciniszyn, Jr., D. Liu, and J. Tang, *J. Biol. Chem.*, **250**, 5082 (1975).
(12) Although this sequence contains a cysteine residue, and a cysteine peak was observed in the amino acid analysis of the peptide fragment, the peak height was too small for computation. (The color factor for cysteine is one-half of those for other amino acids except proline.) The analysis values obtained for serine and threonine were uncorrected ones. Thus, this peptide fragment might be a somewhat longer chain than that reported here.
(13) M. Takahashi, T. T. Wang, and T. Hofmann, *Biochem. Biophys. Res. Commun.*, **57**, 39 (1974).
(14) M. Takahashi and T. Hofmann, *Biochem. J.*, **147**, 549 (1974).

Y. Nakagawa, L.-H. King Sun, E. T. Kaiser*
Departments of Chemistry and Biochemistry
University of Chicago
Chicago, Illinois 60637
Received January 5, 1976

Isotope Effects in Hydrophobic Binding Measured by High-Pressure Liquid Chromatography[1]

Sir:

We wish to report the first isotopic separations by hydrophobic high-pressure liquid chromatography. Several deuterated substances are completely separated from the corresponding protiated substances by this method. These results are of interest in four ways: (1) The possibility of quantitative analysis of isotopic content is demonstrated. (2) Extension to preparative-scale separation of isotopic species seems feasible. (3) Isotope effects on binding can be measured accurately. (4) By study of isotope effects under different conditions, the nature of the hydrophobic effect may be probed.

The ability to make such separations on a column only 30 cm long is related to the fact that the hydrophobic effect directly involves the CH(CD) bonds. Our results (Table I) show isotope effects on binding which are greater than unity, so that H is favored in the stationary phase relative to

Table I. Binding and Isotope Effects for Substances on Hydrophobic μ-Bondapak C_{18} High-Pressure Liquid Chromatographic Column (30 cm), Methanol-Water Elution, 30 °C[a]

Substance	Mobile phase, mole fraction methanol (pH)[b]	Capacity factor k'[c]	k'_H/k'_D[d]	% isotope effect per D[e]
$CH_3(CH_2)_{14}CO_2H$	0.64 (3.33)	7.289	1.076	0.24
$CD_3(CD_2)_{14}CO_2H$		6.770		
$CH_3(CH_2)_{10}CO_2H$	0.51 (3.21)	7.553	1.066	0.28
$CD_3(CD_2)_{10}CO_2H$		7.084		
C_6H_{14}	0.51 (3.21)	3.303	1.036	0.26
C_6D_{14}		3.182		
C_8H_{18}	0.51 (3.21)	8.502	1.054	0.29
C_8D_{18}		8.059		
C_6H_{14}	0.40 (3.04)	9.682	1.049	0.34
C_6D_{14}		9.249		
C_6H_{12}(cyclo)	0.40 (3.04)	5.543	1.038	0.31
C_6D_{12}(cyclo)		5.342		
C_6H_{12}(cyclo)	0.33 (2.96)	10.55	1.044	0.36
C_6D_{12}(cyclo)		10.10		
$C_6H_5CH_3$	0.33 (2.96)	3.354	1.046	0.56
$C_6D_5CD_3$		3.207		
C_6H_6	0.23 (2.79)	3.785	1.043	0.70
C_6D_6		3.628		
$C_6H_5CH_3$	0.23 (2.79)	8.238	1.057	0.70
$C_6D_5CD_3$		7.791		
C_6H_6	0.18 (2.68)	5.144	1.048	0.79
C_6D_6		4.907		
$C_6H_5CO_2H$	0 (2.51)	24.68	1.040	0.78
$C_6D_5CO_2H$		23.71		
$(CH_3)_3COH$	0 (6.93)	2.692	1.046	0.50
$(CD_3)_3COH$		2.575		

[a] Flow rate was generally 0.5 ml min^{-1}; k' values are essentially independent of flow rate in this range. [b] Results are pH-independent except for carboxylic acids. At the pH values studied, data on carboxylic acids refer to the acid (not anion) form. Acid pH maintained with phosphoric acid in mobile phase, pH 6.93 with sodium phosphate buffer; concentration 0.005-0.01 M. [c] Calculated from retention times: $k' = (t_R - t_0)/t_0$, extrapolated to zero sample size, in experiments with equal-weight mixtures of protiated and deuterated species; precision ±0.5% in most cases. Values of t_0 are not corrected for the small time lag between column and detector; true k' values are therefore all 1.1% higher than those tabulated here. [d] Isotope effects are calculated for each sample size and then isotope effects averaged; precision ±0.0005-0.0015. [e] Given by $100[k'_H/k_D')^{1/n} - 1]$, where n is the number of D atoms substituted for H; precision ±0.02.

D. There is a trend toward higher isotope effects with more aqueous mobile phase using methanol-water mixtures, demonstrating that the solvent plays a role in the hydrophobic effect. If the CH(CD) bonds were not being perturbed by solvent, as would be the case if the substrate merely sat in a solvent cage, the isotope effects would be solvent independent. Finally, these hydrophobic isotope effects show little noticeable dependence on functional group or cyclic vs. open-chain structures, with two exceptions: there is a significant difference in isotope effect between alkane CH(CD) and benzenoid and benzylic; also, benzoic acid has isotope effects somewhat lower than expected.

The capacity factor k' is defined by eq 1,[2]

$$k' \equiv \frac{n_S}{n_M} = \frac{t_R - t_0}{t_0} \quad (1)$$

where n_S and n_M are the amounts of the substance present in the stationary and mobile phases, respectively, and t_R and t_0 are the retention times, at constant flow rate, of the substance and of a completely unretained substance, respectively. Plots of log (isotope effect per D) vs. mole fraction of methanol are linear for the alkyl series and also for the benzenoid-benzylic series (with the exception of benzoic acid), the slope being two times greater for the latter series. More work is needed to establish whether benzoic acid is exceptional, perhaps as a result of the polar functional group, or provides some sort of limiting case of maximum achievable isotope effect at about 0.79% per D.

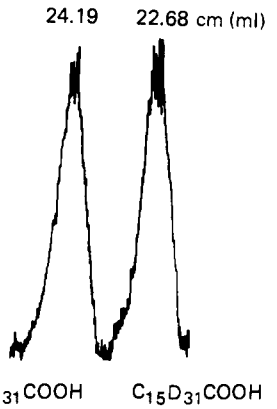


Figure 1. Chromatographic trace for 15 μl of 1:1 (by weight) mixture of carboxylic acids, on 4 mm (i.d.) by 30 cm μ-Bondapak C_{18} column: mobile phase, water-methanol; mole fraction methanol, 0.64; pH, 3.33; 30°; flow rate 0.5 ml min^{-1}; Waters Associates ALC-201 Liquid Chromatograph, Refractive Index detector, with M-6000 pump.

The columns used, μ-Bondapak C_{18},[3] are packed with 10 μ silica particles to which 18-carbon n-alkyl chains are covalently attached. Recent studies have shown the usefulness of similar types of liquid chromatographic columns for the study of hydrophobic effects.[4-7]

There have been several reports of isotopic fractionations by gas chromatography[8-10] and a few by liquid chromatography.[11,12] However, most studies of larger molecules have not had resolution sufficient to separate isotopic mixtures into two peaks. The rather good resolution[10] of the trimethylsilyl derivatives of β-glucose and β-glucose-d_7 by GLC (on a 15 m SE-30 column) probably is the closest approach to the kind of separation reported here.

Isotope effects in chromatography[8] are subject to interpretation according to the theory of Bigeleisen for isotope effects on vapor pressure.[13-16]

A typical chromatographic trace (Figure 1) shows the baseline separations possible. The results (Table I) are highly reproducible. The constancy of isotope effects with sample size indicates that the small number of polar SiOH sites remaining on the column are not contributing to the isotope effects. The pH has no effect on k' values except for acids; the values in Table I are for the acidic, not the anionic, form.

In gas chromatography, Van Hook found stronger retention of protiated molecules for nonpolar columns, but stronger retention of deuterated molecules for water-coated columns.[8] These observations were interpreted in terms of a red shift (decreased vibrational frequency) upon adsorption from the gas phase onto the nonpolar column and a blue shift upon adsorption from the gas phase onto the very polar water-coated column. The present results do not distinguish red from blue relative to the gas phase. However, unlike gas chromatography, liquid chromatography can be used to study ionic and other involatile species. The aqueous mobile phases studied all produce blue shift effects *relative* to the hydrophobic stationary phase. Water does not appear to be unique in producing hydrophobic effects. Rather, the hydrophobic effects seem to be gradually attenuated as methanol is added.

The difference between alkyl CH(CD) isotope effects and benzenoid-benzylic could possibly result from the high-

er acidity of both of the latter types. Another possibility is that the latter types have a more exposed surface area of the H(D) atom simply for steric reasons. Models suggest this might be so. It has been shown that hydrophobic binding effects (not isotope effects) in phase transfer equilibria are proportional to the surface area of the molecule.[17]

If the isotope effects only involve more freedom of motion in the hydrophobic phase, then in more ordered systems such as the current models for biomembrane structure,[18,19] isotope effects would be attenuated. On the other hand, if a real lipophilic effect produces red shifts, perhaps through van der Waals interactions, more ordered systems ought to produce increased isotope effects. Therefore, further experiments to explore the effect of order in the stationary phase, and in particular to produce columns which will serve as closer models for biomembrane structure and binding, are in progress.

Acknowledgment. Support of this research by the National Science Foundation is gratefully acknowledged.

References and Notes

(1) Supported by the National Science Foundation through Grant MPS72-04818 A03.
(2) See B. L. Karger, L. R. Snyder, and C. Horvath, "An Introduction to Separation Science", Wiley, New York, N.Y., 1973, pp 30, 130–131.
(3) Manufactured by Waters Associates, Milford, Mass.
(4) R. B. Sleight, *J. Chromatogr.*, **83**, 31 (1973).
(5) W. J. Haggerty, Jr. and E. A. Murrill, *Res./Dev.*, **25**, 30 (1974).
(6) R. M. Carlson, R. E. Carlson, and H. L. Kopperman, *J. Chromatogr.*, **107**, 219 (1975).
(7) J. M. McCall, *J. Med. Chem.*, **18**, 549 (1975).
(8) W. A. Van Hook, *Adv. Chem. Ser.*, **No. 89**, 99 (1969).
(9) D. MacNaughtan, Jr. and L. B. Rogers, *Sep. Sci.*, **7**, 307 (1972).
(10) R. Bentley, N. C. Saha, and C. C. Sweeley, *Anal. Chem.*, **37**, 1118 (1965).
(11) P. D. Klein, *Adv. Chromatogr.*, **3**, 3 (1966); P. D. Klein, V. Cejka, and P. A. Szczepanik, *Adv. Tracer Methodol.*, **4**, 105 (1968).
(12) H. Kakihana, *J. Chromatogr.*, **102**, 47 (1974).
(13) J. Bigeleisen, *J. Chem. Phys.*, **34**, 1485 (1961).
(14) J. Bigeleisen, S. V. Ribnikar, and W. A. Van Hook, *J. Chem. Phys.*, **38**, 489 (1963).
(15) J. Bigeleisen, M. J. Stern, and W. A. Van Hook, *J. Chem. Phys.*, **38**, 497 (1963).
(16) M. J. Stern, W. A. Van Hook, and M. Wolfsberg, *J. Chem. Phys.*, **39**, 3179 (1963).
(17) J. A. Reynolds, D. B. Gilbert, and C. Tanford, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 2925 (1974).
(18) C. Tanford, "The Hydrophobic Effect: Formation of Micelles and Biological Membranes", Wiley, New York, N.Y., 1973.
(19) S. J. Singer, *Annu. Rev. Biochem.*, **43**, 805 (1974).

Nobuo Tanaka, Edward R. Thornton*
Department of Chemistry, University of Pennsylvania
Philadelphia, Pennsylvania 19174
Received November 4, 1975

Cesium-133 Nuclear Magnetic Resonance Study of Crown and Cryptate Complexes of Cs^+ Ion in Nonaqueous Solvents

Sir:

In recent years the study of complexes of alkali metal ions with macrocyclic polyether ligands became a popular and important field of research. We have shown recently that alkali metal NMR is a powerful technique for the exploration of the formation and the solution properties of alkali cryptates with lithium[1] and sodium[2] ions. The extent of complexation is strongly dependent on the cationic and macrocyclic dimensions as well as on the solvent in which the reaction takes place. The polyethers used in this research were 18-crown-6 (I) (18C6) and monobenzo-222-cryptand (II) (C222B).

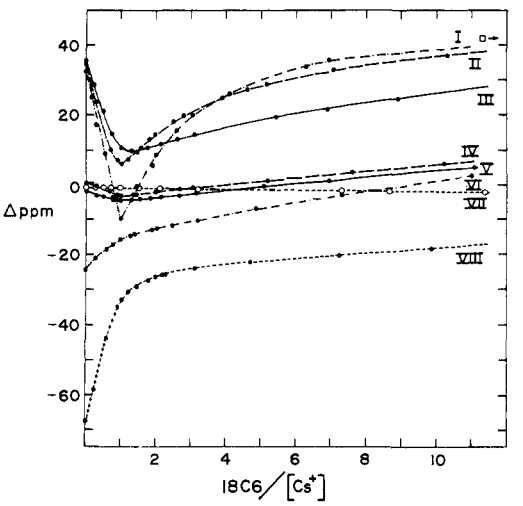
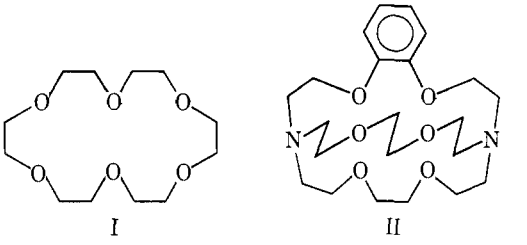


Figure 1. Plot of cesium-133 chemical shift vs. 18-crown-6/Cs^+ mole ratio in different solvents. Concentration of cesium salts is 0.01 M in all cases: (I) $CsBPh_4$ in PY, (II) $CsBPh_4$ in Me_2CO, (III) $CsBPh_4$ in PC, (IV) $CsBPh_4$ in DMF, (V) CsI in DMF, (VI) $CsBPh_4$ in MeCN, (VII) CsI in H_2O, (VIII) $CsBPh_4$ in Me_2SO.



Most inorganic cesium salts are only sparingly soluble in nonaqueous solvents. Cesium tetraphenylborate and, to some extent, cesium iodide, however, are sufficiently soluble to permit an NMR investigation. Measurements were carried out in propylene carbonate (PC), pyridine (PY), acetone (Me_2CO), *N,N*-dimethylformamide (DMF), dimethyl sulfoxide (Me_2SO), and acetonitrile (MeCN). These solvents covered a dielectric constant range from $D = 12.4$ to $D = 65$. The solvents were purified by previously described techniques.[1] Karl Fischer titrations and gas chromatographic analysis showed that they contained less than 100 ppm of water.

The measurements were made at 7.8709 MHz in the pulsed Fourier-transform mode. The field was locked with a home-built lock probe[3] which used the Varian DA-60 console to lock on a proton resonance. The NMR spectrometer is interfaced to a Nicolet 1083 computer for time averaging of spectra and also for on-line Fourier transformation of data. A 0.5 M cesium bromide solution was used as external reference. All ^{133}Cs chemical shifts are referred to an infinitely dilute aqueous Cs^+ solution as reference and are corrected for the differences in the bulk diamagnetic susceptibility of the solvents. A positive value of Δ indicates a shift to higher field.

The variation of the ^{133}Cs chemical shift as a function of the 18C6/Cs^+ mole ratio in different solvents is shown in Figure 1. It is immediately obvious that the solvent plays an extremely important role in the complexation process. The behavior in pyridine, acetone, and propylene carbonate solutions is especially interesting in that the ^{133}Cs resonance shifts *linearly downfield* until a 1:1 ligand/Cs^+ mole ratio is reached and then gradually shifts *upfield* as the concentration of ligand is increased. The data seem to indicate a two-step reaction, first the formation of a stable 1:1 complex and then the addition of a second molecule of the li-