Deviation from unity of the equilibrium constants for such processes as we have discussed here (i.e., involving heavy atom isotopic substitution) is expected and indeed observed to be very small (ΔG° are very close to zero). It is obvious, then, that the ICR technique is not really accurate enough to provide quantitativelv useful information about such processes. *However, the errors in the technique should be no larger for reactions involving hydrogen-deuterium substitution where isotope effects are much larger. In these instances, the technique should be able to provide us with useful information.*

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$$(CH_3)_3N\text{-}H^+ + (CD_3)_3N \rightleftharpoons (CH_3)_3N + (CD_3)_3N\text{-}H^+$$

has been determined by ICR spectroscopy. M. Taagepera, K. D. Summerhays, T. B. McMahon, J. L. Beauchamp, and R. W. Taft, cited in ref 3.

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(12) Alfred Sloan Fellow 1974–1976.

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Organic Synthesis Using Enzymes in Two-Phase Aqueous Ternary Polymer Systems

Sir:

The practicality of enzymes as catalysts in organic synthesis often depends on the efficiency with which they can be recovered from product mixtures and reused.[1,2] Two general approaches are presently available to the design of synthetic reactors based on enzymes: either the enzymes may be immobilized on (in) an insoluble support, or they may be used in solution and reisolated by ultrafiltration, adsorption, precipitation, or other methods.[2] When applicable, immobilization is usually the preferable approach: immobilized enzymes often enjoy protection against deactivation by adventitious proteases and are not exposed to the potentially deactivating conditions encountered during isolation from solution. Immobilized enzymes are, however, not applicable in reactions involving insoluble substrates, or in sequences requiring the enzymes to associate with or dissociate from other insoluble proteins or macromolecules during reaction.[3] Further, partial or complete deactivation often accompanies the immobilization of sensitive enzymes.

We wish to describe a new strategy for utilization of enzymes as catalysts in organic synthesis based on their partition in aqueous two-phase ternary polymer systems. Many aqueous polymer solutions show low mutual solubility, and two-phase ternary polymer systems have been extensively utilized for biochemical separations.[4,5] The partition of material between the two phases depends on the composition, pH, and ionic strength of the system,[4] and is also affected by the presence of polyelectrolytes[6] or specific ligands covalently bound to one of the polymers.[7] Characteristically, the partition coefficients, K,[8] of various proteins between the phases of the system formed from dextran and poly(ethylene glycol) (PEG) in water[9] are in the range $K = 0.1$ to 10.[4] Low molecular weight substances such as inorganic salts, amino acids, sugars, and nucleotides partition almost equally between the two phases (i.e., $K = 1$). We take advantage of the difference in K between substrate, product, and enzymatic catalyst to construct a two-phase biosynthetic reactor (TPBR). An efficient TPBR should consist of two, immiscible, aqueous polymer phases in which the enzyme is partitioned predominantly into one phase. If the partition coefficient for enzyme is far from unity, and that for substrates and products is close to unity, it is possible to separate the enzymatic catalyst from products efficiently by extraction. To assess the influence of the magnitude of K of an enzyme in a two-phase system on the operation of a TPBR, it is useful to analyze a simple model. We assume that the initial quantity of the enzyme added to the TPBR is A_0, the volume of the upper (enzyme poor) phase is V_1 and its enzyme concentration is C_1, and the volume of the lower (enzyme rich) phase is V_2 and its enzyme concentration is C_2. The loss of enzyme from the lower phase in each stage of separation is described by eq 3. For

$$K = C_1/C_2 \tag{1}$$

$$A_0 = A_1 + A_2 = C_1V_1 + C_2V_2 \tag{2}$$

$$A_1 = A_0(1 + V_2(KV_1)^{-1})^{-1} \tag{3}$$

an enzyme with $K = 0.001$ in a TPBR with $V_1/V_2 = 100$, the loss of enzyme from the lower phase to the upper in one stage would be 9.1%. Since for most enzymes values of K <0.001 or >1000 are unlikely,[3] countercurrent operation with multiple partition of the product mixture is necessary to minimize loss of the enzyme from a TPBR.

The operation of a single stage of this type of reactor has been demonstrated using a model system based on glucose 6-phosphate dehydrogenase (G-6-PDH, D-glucose 6-phosphate:$NADP^+$-oxidoreductase, EC 1.1.1.49) isolated from Torula yeast.[10] The partition coefficient of G-6-PDH in the commonly used dextran-PEG two-phase system is $K = 0.20$ (Table I); this value indicates that the protein partitions to the extent of 83% in the lower (dextran rich) phase at a phase volume ratio, $V_1/V_2 = 1$. Because this partition ratio

Table I. Partition Coefficients, K, for G-6-PDH, Its Conjugate with Modified Polyethylene Glycol, Substrate, Cofactor, and Product in Dextran-PEG and Ficoll-UCON Two-Phase Systems

Substance	Partition coefficient, K[a]	
	Dextran-PEG[b]	Ficoll-UCON[c]
G-6-PDH (Torula yeast)	0.20	0.0080
G-6-PDH (bakers yeast)	0.073	0.0083
G-6-PD-PEG conjugate	27.0	1.0
Glucose 6-phosphate	0.90	0.68
6-Phosphogluconate	0.99	0.81
$NADP^+$	0.78	0.44
NADPH	0.84	0.73

[a] Determined at 25°. [b] Two-phase system of the total composition: dextran 7.0% (w/w), poly(ethylene glycol) 4.0% (w/w), and triethanolamine buffer (20 mM, pH 7.0) 89% (w/w). [c] Two-phase system with total composition: Ficoll 10.0% (w/w), UCON 50 HB 5100 10.0% (w/w), and *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid buffer (100 mM, 50 mM potassium chloride, 10 mM magnesium chloride, 2 mM EDTA, 4 mM β-mercaptoethanol, 1 mg/ml bovine serum albumin (Sigma, fraction V, pH 7.5) 80.0% (w/w).

would be unsatisfactory for efficient "immobilization" of the enzyme in a TPBR without several countercurrent extraction stages, other two-phase systems were explored which were expected to minimize or maximize K. Systems incorporating ionic polymers were avoided, on the (untested) belief that they might interact with the enzyme, substrate, or product in undesirable ways. The most useful system seemed to be that which involved neutral polymers having the largest possible difference in hydrophilicity; we employed Ficoll (a synthetic polymer of sucrose) and UCON 50 HB 5100 (a copolymer of ethylene glycol and propylene glycol). The partition coefficient for G-6-PDH in this system was 0.008.[11]

A representative experimental procedure for the conversion of glucose 6-phosphate to 6-phosphogluconate illustrates the synthetic application of this two-phase system. Reaction was carried out in a 150-ml polyethylene centrifuge tube, equipped with a Teflon-coated magnetic stirring bar and a pH electrode. The UCON phase (90.0 ml) was added into the tube, and, with gentle stirring, 0.855 g (1.00 mmol) of $NADP^+$ and 0.300 g (1.05 mmol) of glucose 6-phosphate were dissolved in this solution. The solution was adjusted to pH 7.51 by adding a few drops of 50% aqueous sodium hydroxide. G-6-PDH (220 U.) was dissolved in 10.0 ml of the Ficoll phase, and added to the well-stirred UCON phase. The pH of the heterogeneous reaction mixture decreased sharply during the first 5 min, and became constant at pH 7.25 after 30 min at 25°. The two liquid phases were separated by centrifugation. Appropriate assays indicated the presence of 203 U. (92%) of G-6-PDH in the Ficoll phase; the experimental recovery of the enzyme was in excellent agreement with value of 93% calculated using the experimental partition coefficients. The yields of 6-phosphogluconate[10] and NADPH spectrophotometrically determined in the UCON phase were 81 and 84%, respectively; both yields are consistent with the partition coefficient and phase volume ratio used. Further separation and purification of the products by DEAE cellulose anion exchange chromatography yielded the pure ammonium salts of 6-phosphogluconate and NADPH in 57 and 59% isolated yields, respectively.

This example indicates the feasibility of carrying out enzymatically catalyzed organic synthesis in a two-phase aqueous ternary polymer system, and demonstrates the ease of recovery of enzyme from product in such a system. Although the partition coefficient of native G-6-PDH from Torula yeast was satisfactory for direct use in the Ficoll-UCON system (Table I), the partition coefficients of other enzymes of interest in synthesis are not, and it is not necessarily the case that manipulation of polymer compositions will generate ternary two-phase systems in which some arbitary enzyme will show both satisfactory partition coefficients, good activity, and long operating lifetime. We have briefly explored the practicality of modifying the enzyme instead of the ternary polymer system to influence its partitioning characteristics. α,ω-Di-*p*-nitrobenzoxypoly(ethylene-glycol) (**1**) was synthesized by the reaction of the potassium salt of PEG with *p*-nitrobenzyl bromide in DMSO. Compound **1** was reduced with alkaline aqueous hydrosulfite to α,ω-di-*p*-aminobenzoxypoly(ethylene glycol), to which G-6-PDH was coupled by treatment with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride in a phosphate buffer at pH 4.80. The crude G-6-PDH–PEG conjugate (22% yield, calculated on the basis of enzymatic activity), had K = 3.0 in the dextran–PEG two-phase system, and was assumed to be a mixture of conjugates with different K values. It was purified by successive partition between PEG and dextran phases until a constant K = 27 was reached (9.5% yield, based on original activity). Thus, conjugation of G-6-PDH with a functionalized polyethylene glycol results in a 135-fold increase in partition coefficient (Table I), with the G-6-PDH–PEG conjugate partitioning, as expected, predominantly into the more hydrophobic PEG phase in a PEG–dextran two-phase system. This result emphasizes the potential flexibility of the TPBR; it is possible to manipulate the partition coefficients of enzymes between the two phases by choice of polymers, by changing ionic strength and composition and pH, and by chemical modification of the enzymes; control of the partition coefficients of starting materials, products, and cofactors should also be possible. Judicious adjustment of all of these partition coefficients should make it possible to separate enzymatic activity from products efficiently in many fully developed systems.

A number of questions must be explored before a large-scale TPBR based on cell-free enzymatic catalysis can be constructed routinely. First, how do the polymers that compose the two phases influence substrate, product, and cofactor binding and substrate turnover at the enzyme active site? Second, what are the characteristics of diffusion of substrates and products within a single phase and between two phases of the ternary polymer system, and how does this diffusion influence the productivity of a TPBR? Third, what are the most efficient methods of dispersing the phases in one another, and of separating them after reaction? Fourth, how are enzymatic and cofactor lifetimes in these systems maximized? Fifth, what are the most efficient methods for separating polymers from products? Work on these problems is in progress. Although these questions cannot presently be answered, it is clear that two-phase ternary aqueous polymer systems already provide a useful basis for utilizing enzymes in bench-scale organic synthesis, and offer a potentially practical approach to large-scale synthesis with enzymes whose constitution or mechanism of action preclude conventional immobilization methods.

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(9) Dextran (M_w = 500 000)–poly(ethylene glycol) (M_w = 6000–7500) two-phase system of the total composition: dextran 7% (w/w), poly(ethylene glycol) 5% (w/w), water 88% (w/w).[4] This two-phase system is one of those most commonly used.
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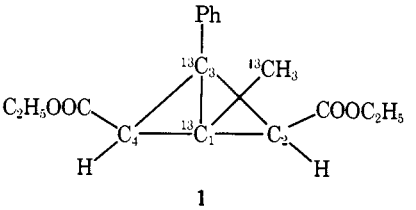
The Sign of the Bridgehead–Bridgehead ^{13}C–^{13}C Coupling Constant in a Bicyclobutane[1]

Sir:

Our interest in the chemical and physical properties of bicyclobutane and derivatives, coupled with our recent successful synthesis of diethyl 1-methyl-^{13}C-3-phenylbicyclo[1.1.0]butane-*1,3*-$^{13}C_2$-*exo,exo*-2,4-dicarboxylate (**1**) has allowed us to determine and herein report signs of several C–C and C–H coupling constants. The most significant of these is the bridgehead–bridgehead ^{13}C–^{13}C coupling constant which we now have determined to be *negative* by a series of off-resonance heteronuclear decoupling experiments as described below.



1

The interest in the sign of this coupling constant stems from the notion that this bond contains a very high degree of p character[3] and, as a result, should have a very small coupling constant. This derives from the assumed relationship between the Fermi contact contribution to the coupling constant and the product of the s character of the orbitals comprising the bond.[4] The first such postulated relationship (eq 1)[4] could not accommodate a negative coupling constant. Further refinement by Pople and Santry[5] led to the ability to predict negative coupling constants.

Table I.

Coupling constant[a]	Value	Coupling constant[a]	Value
$^1J_{C_1C_3}$	−5.4 ± 0.5 Hz	$^2J_{C_3C_M}$	−2.4 ± 0.5 Hz
$^1J_{C_1C_M}$	+53.2 ± 0.5 Hz	$^2J_{C_1H_M}$	−7.3 ± 0.5 Hz
$^1J_{C_MH_M}$	+129 ± 1 Hz	$^3J_{C_3H_M}$	+2.7 ± 0.5 Hz

[a] See text for explanation of subscripts.

$$^1J_{^{13}C-^{13}C} = 0.0550(\%S_A)(\%S_B)\ \text{Hz} \quad (1)$$

This has not been useful, however, because it requires knowledge of energies and wave functions of excited states. Recent developments in spin–spin coupling theory by Pople, McIver, and Ostlund[6] have eliminated this requirement. Using localized orbitals Schulman and Newton have derived eq 2[7,8]

$$^1J_{^{13}C-^{13}C} = 0.0621(\%S_A)(\%S_B) - 10.2(\pm 2.4)\ \text{Hz} \quad (2)$$

and have predicted *negative* values for the bridgehead–bridgehead coupling constants in bicyclobutanes.[3b,7-9] Their perturbation calculations[7] show the negative value to be the result of small negative values of all three terms—the Fermi contact, orbital-dipole, and spin-dipolar contributions. In fact bicyclobutanes may be unique in this regard and might be the only compounds to show a negative $^1J_{CC}$.[7]

The observation of a negative $^1J_{CC}$ therefore is consistent with the idea of a negative Fermi contact term and a high degree of p character in the bridgehead–bridgehead bond. From eq 2, the value of −5.4 Hz for $^1J_{CC}$, and the assumption that the C_1–C_3 bond in **1** is symmetrical, one can calculate that the carbon orbitals which make up this bond are hybridized $sp^{10.4}$.

Table I lists the appropriate coupling constants,[2] including signs, as determined by the method of off-resonance heteronuclear decoupling. We will designate the nuclei as: C_3, C_1, C_M (methyl carbon), and H_M (hydrogens on the methyl group). All signs are relative to the C_M–H_M sign which is taken as positive.[10]

The carbon magnetic resonance spectrum of **1** consisted of overlapping spectra of triply labeled material, three types of doubly labeled material, and three types of singly labeled compound. This was a result of 90% ^{13}C labeling in C_1 and C_3 and 65% ^{13}C labeling in C_M.[2] In addition the proton spectrum of the methyl group similarly showed overlapping spectra due to these same molecules.

The off-resonance heteronuclear decoupling technique utilized extensively by Jakobsen and co-workers[11] has subsequently been used successfully to obtain the relative signs of ^{13}C–^{31}P and ^{31}P–^1H coupling constants.[12] Our experiments involved irradiating the protons at various frequencies in steps as small as 4 Hz around the selective decoupling condition while observing the ^{13}C spectrum. The observations consisted of noting that, for example, low frequency proton irradiation enhanced the low frequency ^{13}C doublet of C_1 whereas high frequency proton irradiation enhanced the high frequency ^{13}C doublet of C_1 (in the triply ^{13}C labeled material). This requires that $^1J_{C_1C_M}$ and $^1J_{C_MH_M}$ have the same sign; that is, since $^1J_{C_MH_M}$ is positive, so is $^1J_{C_1C_M}$. The ^{13}C doublet due to C_1, where C_1 and C_3 are labeled, showed the high frequency ^{13}C peak enhanced at lower proton frequency whereas the low frequency ^{13}C peak was enhanced at high frequency proton irradiation. Thus $^1J_{C_1C_3}$ and $^3J_{C_3H_M}$ have opposite signs. By observation of the methyl carbon atom we could determine similarly that $^1J_{C_1C_M}$ and $^2J_{C_1H_M}$ are of opposite sign and $^2J_{C_3C_M}$ and $^3J_{C_3H_M}$ are also of opposite sign. Because of the small couplings of C_3 to the aromatic hydrogens resulting in broad peaks we were not able to use this technique further.