Antibody Catalysis of a Disfavored Chemical Transformation

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Organic reactions are often limited by stereoelectronic constrains that appear along the reaction coordinate. An antibody has been generated that overcomes these constraints and catalyzes a highly disfavored chemical transformation. The antibody facilitates the difficult 6-endo-tet ring closure of an epoxy-alcohol to form a tetrahydropyran. The catalyzed process is in formal violation of what has become known as Baldwin's rules for ring-closure reactions. In addition to controlling the regiochemistry of the disfavored cyclization reaction, these catalytic antibodies resolve enantiomeric substrates to afford a stereochemically pure product. The principles demonstrated in this study may be applicable to other disfavored chemical processes.

The outcome of chemical transformation depends on kinetic and thermodynamic parameters. For reactions under kinetic control, where alternative products are possible, the product distribution reflects the energy barriers that the reactants encounter along the reaction coordinate (*1*). A classic example is that of intramolecular cyclization reactions where the formation of favored and disfavored products can be understood in terms of the stereoelectronic features of their respective transition states (*2*). Although the energy barriers for the favored and disfavored processes often differ by only a few kilocalories per mole, the outcome of the reaction is not easily changed because of an inability to specifically manipulate the alternative reaction pathway or pathways. Catalytic antibodies, with their exquisite specificity and ability to provide up to 20 kcal/mol of binding energy, could be ideal "reagents" for altering the outcome of chemical transformations (*3*). In essence, the issue concerns the ability of a suitably programmed antibody molecule to intercede at or near the transition state to alter the energy balance in favor of the otherwise disfavored reaction pathway. Herein we demonstrate this principle by generating antibodies that catalyze a disfavored cyclization reaction.

Ring-forming reactions are integral processes in organic chemistry (*4–7*). Consequently, a number of blueprints have been formulated to predict the outcome of such reactions (*2, 8–11*). These ring-closure guidelines apply to a variety of cyclization reactions and have been termed "Baldwin's rules" (*2, 8*). In general, favored cyclization pathways are those in which the length and nature of the linking chain enable the terminal atoms to achieve the proper geometry for reaction, whereas disfavored cases require severe distortion of bond angles. Thus, for ring closures which proceed by nucleophilic substitution at sp^3 centers, stereoelectronic constraints favor maintenance of a subtended angle of 180° between the three interacting atoms during the reaction pathway such that 3-7-exo-tet systems are favored and 5-6-endo-tet are disfavored (*11*).

We have studied the formation of O-heterocyclic rings. The tetrahydropyrans are of particular interest because they are components of many important natural products, and consequently a great deal of effort has gone into strategies for their synthesis (*5–7, 12*). One attractive synthetic strategy reduces to a regioselective 6-endo-tet ring opening of an epoxide by an internal nucleophilic oxygen atom (Fig. 1). However, overwhelming experimental evidence shows that, in accord with Baldwin's rules, the 5-exo-tet mode of cyclization is the preferred pathway yielding the unwanted tetrahydrofuran system as the exclusive product (Fig. 1) (*13*). To catalyze the formation of the disfavored product **5**, the catalytic antibody should anticipate the mechanism of epoxide ring opening and overcome the rotational entropy barrier and the strain necessary to bring the alcohol into a geometry that permits nucleophilic attack at the appropriate C–O bond.

The concept on which we relied to achieve these goals is shown in Fig. 1, which depicts a scenario for the acid-catalyzed cyclization of the epoxy-alcohol **1**. The hapten was designed to induce appropriately charged amino acid residues strategically placed adjacent to the epoxide unit to stabilize the C–O bond as it proceeds to rupture (*14*). In this scheme, endo ring closure would proceed to produce the so-called disfavored heterocycle **5**, through transition state **3**, in which the developing charges in the region of carbon atom **6′** would be stabilized by the catalyst. The alternative pathway of exo ring closure through transition state **2** to yield the smaller ring **4** would be less favored by the antibody.

The *N*-oxide antigen **6a** would appear to be an attractive candidate for the induction of an appropriate catalytic antibody in that it mimics the stereoelectronic features of the oxirane opening under acid conditions (Fig. 2). The use of a six-membered heterocyclic antigen with formal charges would favor the induction of antibodies that would use their binding energy to organize the reaction geometry to prefer the formation of the six-membered pyran ring **5** while allowing regiospecific induction of complementary charges in the binding pocket of the antibody. The cationic nitrogen atom is expected to induce one or more amino acid

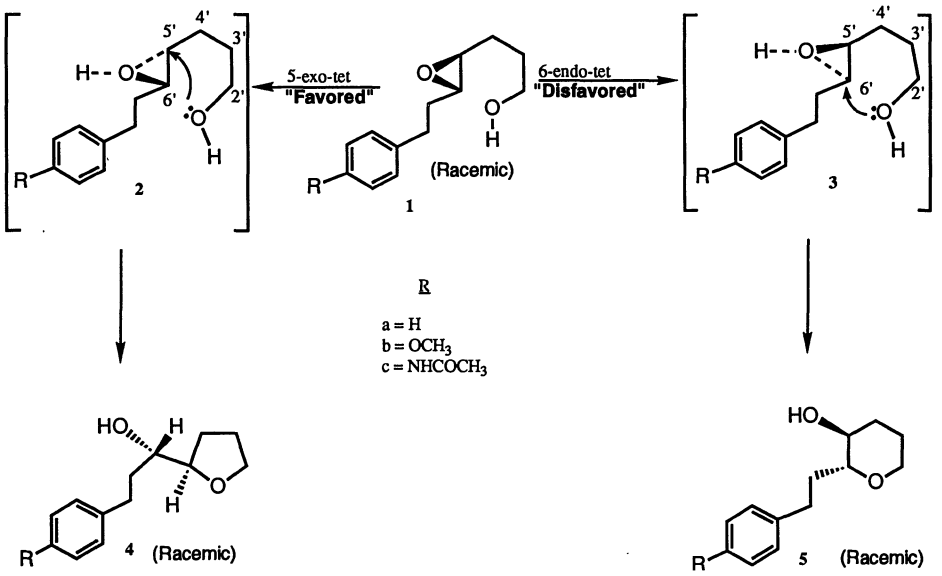

Fig. 1. Plausible mechanisms of 5-exo-tet and 6-endo-tet cyclizations of trans epoxy-alcohol **1** to form tetrahydrofuran **4** and tetrahydropyran **5**. Racemic product **4** is the "Baldwin" favored product from the uncatalyzed reaction. The antibody-catalyzed reaction provides a single enantiomer of **5**.

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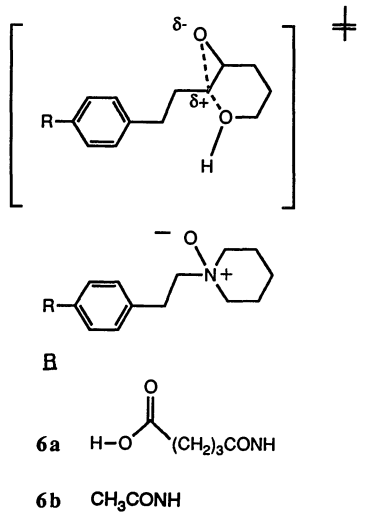

Fig. 2. A possible transition state found in the formation of **5**. Compounds **6a** and **6b** represent the *N*-oxides that were used to induce monoclonal antibodies and inhibit the reaction, respectively. The synthesis of both compounds commences with the addition of *p*-nitrophenethylbromide to piperdine, 45°C, 4 hours, 92%. This nitro adduct is then reduced with Pd/C, H_2, methanol, 99%. Hapten **6a** is obtained by the addition of glutaric anhydride, followed by oxidation of the piperdine moiety with 30% H_2O_2 (the overall yield for the last two steps was 80%). The inhibitor **6b** was obtained in a similar way except that acetic anhydride was substituted for glutaric anhydride.

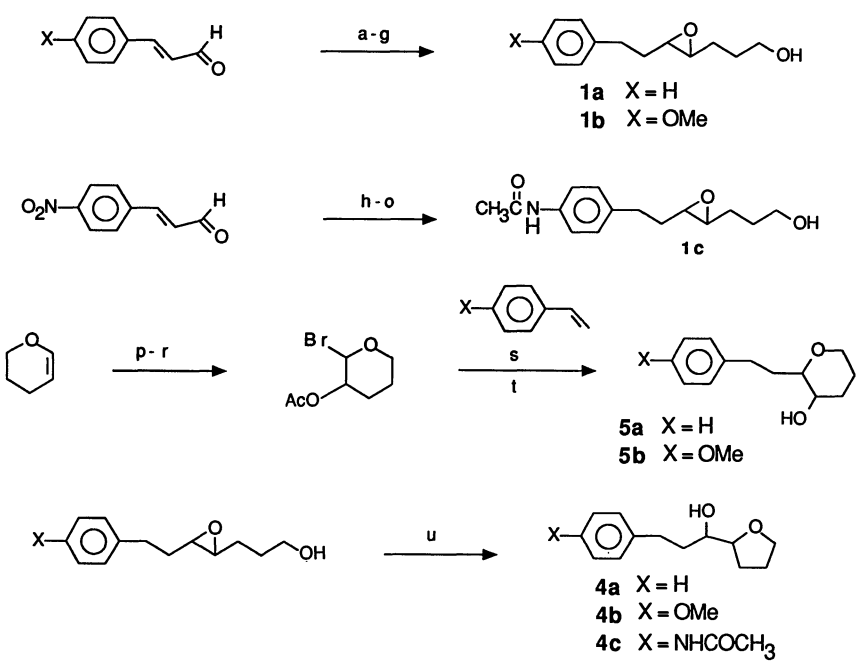

Fig. 3. Reagents and conditions for the synthesis of **1**, **4**, and **5**: (a) Montmorillonite K 10, trimethyl orthoformate and hexane; (b) H_2, 10% Pd/C, and ethyl acetate; (c) HCl and CH_3CN(aq); (d) 1 M vinyl–magnesium bromide–tetrahydrofuran (THF), −78° to 25°C, 6 hours; (e) triethyl orthoacetate and hexanoic acid, 140°C, 5 hours; (f) lithium aluminum hydride and THF, −78° to 25°C, 3 hours; and (g) dimethyldioxirane, 15 min (Me, methyl). Reagents and conditions for X = $NHCOCH_3$: (h) Montmorillonite K 10, trimethyl orthoformate, and hexane; (i) H_2, 10% Pd/C, and ethyl acetate; (j) acetic anhydride and pyridine; (k) HCl and CH_3CN(aq); (l) 2.5 equivalents 1 M vinyl–magnesium bromide–THF, −78° to 25°C, 4 hours ; (m) triethyl orthoacetate and hexanoic acid 140°C, 5 hours; (n) 2 M lithium borohydride and THF, reflux 14 hours; (o) dimethyldioxirane, 15 min; (p) *m*-chloroperbenzoic acid and ethyl ether(aq), 0° to 25°C, 2 hours (Ac, acetyl); (q) acetic anhydride and pyridine, 18 hours; (r) 30% HBr–acetic acid, 1 hour; (s) triphenyltin hydride, 2,2′-azobis(2-methylpropionitrile), and benzene, reflux 8 hours; (t) lithium aluminum hydride and THF, −78° to 25°C, 45 min; and (u) HCL (catalyst) and CH_3CN.

residues that stabilize the carbocation appearing along the reaction coordinate, while the anionic oxygen atom may induce positively charged amino acids to assist in the acid-catalyzed ring opening of the epoxide. The main design feature in this system is the spatial organization of the controlling elements in the antibody and, although we anticipate acid-catalyzed opening of the oxirane, a base-catalyzed process such as deprotonation of the alcohol by an induced base in the antibody cavity would also be allowed. Finally, the charge differences between **6** and **5** may alleviate product inhibition.

The *N*-oxide **6a** (Fig. 2) was coupled to keyhole limpet hemocyanin (KLH), and the conjugate was used to immunize 129 IX^+ mice for production of monoclonal antibodies (*15*). Twenty-six monoclonal antibodies were shown by an enzyme-linked immunosorbent assay (ELISA) (*16*) to bind to **6** conjugated to bovine serum albumin (*17*). All 26 cell lines were cloned and injected separately into mice for production of ascites fluid. Antibody from each sample of ascites fluid was purified by salt precipitation, anion exchange, and affinity chromatography (*18*).

All 26 antibodies at a concentration of 20 μM were screened initially against racemic epoxy-alcohol **1b** (synthesis shown in Fig. 3), and formation of the tetrahydrofuran **4b** and tetrahydropyran **5b**, (syntheses shown in Fig. 3) products was monitored by normal-phase high-performance liquid chromatography (HPLC) (*19*). This assay provided a facile means for identifying antibody catalysis. However, gas chromatography conducted on a chiral-phase gas-liquid chromatography column was required to cleanly separate isomers **4b** and **5b** in order to confirm the formation of the desired **5b** (*20*).

In the absence of antibody, only **4b** was formed, in accordance with Baldwin's rules. In contrast, two of the 26 antibodies (17F6 and 26D9) were regioselective catalysts for the formation of the anti-Baldwin product, **5b**. The enantioselectivity of product formation was studied with a normal-phase HPLC column of chiral preparation (*21*). The enantiomers of **1b** as well as those of **4b** and **5b** were separable on the column (*22*). Antibody 26D9 was the most stereoselective. It exclusively utilized only one of the two enantiomers of **1b** and thereby produced only one of the enantiomers of **5b**.

The initial rate of ring closure catalyzed by antibody 26D9, when measured as a function of substrate **1b** concentrations followed Michaelis-Menten kinetics (*23*). The Michaelis constant K_m, the maximum rate V_{max}, and the catalytic rate constant k_{cat} values were 356 μM, 4.6×10^{-6} M min^{-1}, and 0.91 min^{-1}, respectively. Comparison of k_{cat}/k_{uncat} was not possible because in the uncatalyzed reaction formation of the six-membered ring **5b** was negligible under our assay conditions (*8*). The failure to form **5b** in the absence of antibody illustrates the potential of the catalytic antibody to facilitate an otherwise undetectable reaction.

To determine the stereochemistry of the antibody-catalyzed reaction products, the antipodes of the starting epoxy-alcohols were prepared. The olefin was asymmetrically dihydroxylated by the method of Sharpless *et al.* (*24*) and subsequently converted to the enantiomerically pure epoxide (*25*). With both optically pure isomers in hand, it is now possible to determine the absolute stereochemical course of the catalyzed reaction. Antibody 26D9 used only the *S*, *S* epoxide as a substrate to catalyze formation of the pyran ring which, as determined by nuclear magnetic resonance of the isolat-

ed product **5**, has the *S*, *R* configuration. Thus, the antibody controls both the regio and stereochemistry of this reaction.

Antigen inhibition and substrate specificity assays were undertaken to investigate binding affinity and substrate fidelity of 26D9. The hapten **6b** (Fig. 2) was a potent inhibitor of the reaction (*26*). Potent inhibition by the immunogen been observed in other catalytic antibody systems (*27*). The overall specificity of 26D9 has yet to be examined in detail. However, our initial studies show that this antibody is rather indifferent to substitution at the 4-position of the phenyl ring in that epoxy-alcohols **1a** and **1c** were also competent substrates. This binding promiscuity in the region where the hapten is linked to the carrier protein is similar to that seen with other catalytic antibodies (*28*) and presumably reflects an insensitivity of some antibodies to this region of the immunogen.

Much of the potential of classical organic chemistry goes wanting but for the ability to selectively direct only a few kilocalories of energy. In this study, we have overcome this problem by using an antibody molecule to specifically address multiple parameters that appear along the reaction coordinate and thereby "reroute" a chemical reaction. These antibodies, which have no enzymatic or synthetic equivalents, catalyze a highly disfavored chemical process while also providing a chiral environment for the kinetic resolution and processing of stereochemically impure molecules. If these concepts and results can be generalized to other disfavored chemical processes, antibody catalysis may offer the chemist an additional way to control the outcome of many reactions.

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17. After 2 weeks, the antibodies produced by wells containing macroscopic colonies were assayed by ELISA for binding to **6**. Colonies that initially produced antibodies that bound **6** were subcloned twice, after which 26 remained active. The subtype distribution of the 26 monoclonal antibody was 6 immunoglobulins G1 (IgG1), 13 $IgG2_a$, 6 $IgG2_b$, and 1 IgG3. All 26 monoclonal antibodies were injected into pristane-primed 129G1X$^+$ × BALB/c mice to generate ascitic fluid.
18. The globular fractions from ascitic fluid were precipitated by dropwise addition of saturated ammonium sulfate at 4°C, pH 7.2, to achieve a final concentration of 45%. The ammonium sulfate was removed by dialysis against 10 mM tris, pH 8. The concentrated antibodies were next purified by anion exchange chromatography on DEAE-Sephacel and eluted with a stepwise salt gradient (50 to 500 mM NaCl). The antibodies eluted in the 100 mM NaCl fraction and were concentrated by ultrafiltration prior to affinity purification on a protein G–Sepharose column. The antibody was loaded onto the column, and nonadherent material removed by extensive washings (20 to 30 column volumes). The column was eluted with 0.05 M citric acid, pH 3.0, and fractions were immediately neutralized by collecting into 1 M tris, pH 9.0. All antibodies were then concentrated and dialyzed into 50 mM Pipes, pH 6.6, and assayed by HPLC.
19. The preliminary screening assays were performed by HPLC (Si-60, 5-μm column) with heptane–ethyl ether on an isocratic program of 20/80 at 1 ml/min. An external standard of 2,5-dimethoxybenzylnitrile was used to calculate the amount of product formed. Antibody stock solutions were diluted into 500 μl of Pipes buffer (50 mM, pH 6.6) to give a final protein concentration of 20 μM. All reactions contained 5% cosolvent acetonitrile in which the substrate **1b** (1.2 mM) was dissolved. Aliquots of 200 μl of the reaction mixture were removed after 3 hours, extracted with two 250-μl portions of methylene chloride, and brought to dryness under a stream of nitrogen. The residue was then diluted with 100 μl of a stock solution (4×10^{-4} M) of the standard in acetonitrile and analyzed. Resolution of the five- and six-membered rings was not possible with the above system and consequently was used only to identify potential catalysts of the six-membered ring.
20. The substrate **1b** was transformed to product with the protocol described in (*19*). An aliquot was analyzed by gas chromatography (Chrompac CP cyclodextrin, 50 m by 0.25 mm, 220°C). Coinjection with independently synthesized **5b** (Fig. 3) confirmed its presence in the reaction mixture.
21. We used a DAICEL chiral pak AD with an hexane-isopropanol isocratic program of 97/3 at 1 ml/min at a wavelength of 278 nM.
22. Again, racemic epoxy-alcohol **1b** was used as the substrate. However, to facilitate the detection of products, 95% hexane–5% Pipes buffer solution was used as the assay medium. This type of aqueous-organic biphasic assay system has been investigated previously and was found not to affect an antibodies substrate specificity or performance [J. A. Ashley and K. D. Janda, *J. Org. Chem.*, in press]. To insure that this was the case here, reactions were also run in aqueous media and shown to give similar results.
23. The kinetic assays were performed with an HPLC assay (column and conditions described in (*21*). An external standard of 4-methoxyphenethyl alcohol (5×10^{-4} M) was used to calculate the amount of product formed. An aliquot of antibody stock solution (so that its dilution into 1200 μl gives a 5 μM solution of protein) was placed into a 2-ml polypropylene vial, rapidly frozen in a bath at −78°C, and lyophilized. The residue was rehydrated with 60 μl of Pipes buffer (50 mM, pH 6.6) and diluted with hexane so that the total volume of reaction was 1200 μl. The resulting biphasic mixture was then vibrated for 5 min at 1000 rpm (IKA-VIBRAX-UXR). The reactions were initiated by addition of varying amounts of a 0.044 M solution in hexane–methylene chloride (80/20)

of 1b to give a substrate concentration of 330 to 2000 μM, and the reaction mixture was immediately brought to 1000 rpm. Initial linear rates were measured at <5% consumption of 1b. Thus, in a typical analysis, the organic phase was removed from the reaction vial and stripped of solvent in vacu. The residue was diluted with 100 μl of a stock solution (5×10^{-4} M) of the standard in hexane-isopropanol (97/3) and analyzed.

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Transient Turing Structures in a Gradient-Free Closed System

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Transient, symmetry-breaking, spatial patterns were obtained in a closed, gradient-free, aqueous medium containing chlorine dioxide, iodine, malonic acid, and starch at 4° to 5°C. The conditions under which these Turing-type structures appear can be accurately predicted from a simple mathematical model of the system. The patterns, which consist of spots, stripes, or both spots and stripes, require about 25 minutes to form and remain stationary for 10 to 30 minutes.

The symmetry-breaking, stable, stationary structures predicted by Turing (*1*) in 1952 to result from coupling diffusion with a set of appropriate chemical reactions have attracted considerable attention as a mechanism for morphogenesis, not only in biology (*2*, *3*) but also in such diverse fields as astrophysics (*4*) and economics (*5*). The first unambiguous evidence of Turing structures emerged only recently, 38 years after Turing's remarkable theoretical work, in experiments (*6*) on the chlorite–iodide–malonic acid (CIMA) reaction in an open gel reactor. These and subsequent experiments (*7*, *8*) have used an experimental configuration in which different reactants are fed into the system from opposite ends of the reactor, producing concentration gradients. Turing's model (*1*) envisions a system without imposed gradients in which the key reactants are maintained at uniform concentrations throughout the medium. In fact, nearly all mathematical analysis of Turing structures is based on such a picture, although for practical reasons all experiments to date have used imposed gradients. We report a set of experiments in which Turing-type structures have been generated in a closed system without externally imposed gradients. Because the system is closed, the patterns are necessarily transient.

Mechanistic investigation of the CIMA system (*9*) led to the discovery that chlorine dioxide (ClO_2) and iodine (I_2) play key roles in the dynamical behavior of this reaction and that Turing structures can also be obtained in a system containing these two species and malonic acid (MA) (*10*). The behavior is well described by a simple two-variable mathematical model (*9*). Analysis of this model and of more general models shows that it is possible to calculate the position and depth of quasi–two-dimensional Turing structures in the usual experimental configuration by taking into account the gradient, thereby making the model parameters position-dependent (*11*). The success of this approach suggests that, just as one can treat imposed gradients by allowing the concentrations of "constant" reagents to vary in space, one should be able to predict the emergence of transient Turing structures in a gradient-free, closed system from knowledge of the concentrations at which a Turing instability occurs in a gradient-containing open system.

In Fig. 1A we show for an open system the spatial dependence of the concentrations of the reactants, where $x = 0$ is the boundary at which ClO_2 and MA enter the gel and I_2 enters at $x = 1$. The starch (Fisher, soluble) concentration is uniform throughout the gel. The range of Turing instability, which occurs whenever the functions of concentration K', H_1, and H_2 are related by $K' > H_1 > H_2 > 0$ (*12*), is shown in Fig. 1B. Turing structures appear only in a narrow range along the spatial coordinate. Initial concentrations in this range should be capable of generating Turing patterns in a batch reactor. In earlier open-system experiments, polyacrylamide gel was used to provide a convection-free medium. However, its presence is not essential. Agladze *et al.* (*13*) obtained Turing structures without gel in the presence of starch an open capillary tube reactor.

In an open spatial reactor, Turing structures in the ClO_2-I_2-MA reaction develop over several hours. During their development, their qualitative appearance (hexagons or stripes or both) does not change, but they move in the medium. Eventually, their motion stops, and they remain stationary until the concentration of input reactants leaves the region of Turing behavior. Structures can develop more rapidly in a batch system, because it is not necessary to wait until a stationary concentration gradient is established. In a closed system, zero flux boundary conditions apply, because there is no mass exchange at the boundaries. The structures, however, cannot be truly stationary, because the reactants are consumed.

Several other factors are crucial for gen-

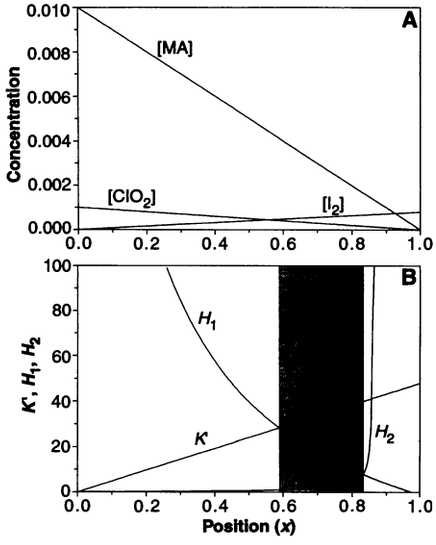

Fig. 1. (**A**) Concentration gradients imposed by the boundary conditions and (**B**) the range of Turing instability in the presence of this gradient. $[ClO_2]_0 = 1 \times 10^{-3}$ M, $[I_2]_0 = 8 \times 10^{-4}$ M, $[MA]_0 = 1 \times 10^{-2}$ M, rate constants (*9*): $k_1 = 6.2 \times 10^{-4}\ s^{-1}$, $k_2 = 9.0 \times 10^{2}\ M^{-1}\ s^{-1}$, $k_3 = 9.2 \times 10^{-5}\ s^{-1}$, $h = 10^{-14}\ M^2$, $D_u = 7.0 \times 10^{-6}\ cm^2\ s^{-1}$, $D_v = 7.0 \times 10^{-6}\ cm^2\ s^{-1}$, $K'[S] = [SI_3]/([I][I_2]) = 6.0 \times 10^{4}\ M^{-1}$ at 4°C.

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