Effect of Microwave Radiation on Copper(II) 2,2′-Bipyridyl-Mediated Hydrolysis of Bis(*p*-nitrophenyl) Phosphodiester and Enzymatic Hydrolysis of Carbohydrates

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Introduction

The influence of microwave radiation on chemical conversions is well documented[1] but much less understood at the present time. The common opinion is that microwaved media absorb energy in two general modes, i.e., by dipole rotation and ionic movement.[2] However, these proposed mechanisms do not explain some unusually high yields and quick conversions reported in the literature.[3] Some researchers have proposed the existence of a special "microwave effect" that causes these anomalies. Others have questioned the existence of such an effect and have provided preliminary data suggesting that, if a microwave-irradiated reaction is compared to a similar thermally compensated reaction, the rates of both processes are nearly identical.[4]

The body of published work concerning heat-limited, microwave-assisted chemistry is small. Such processes (using electromagnetic radiation to amplify chemical conversions while eliminating the heating of the irradiated media) would be of great importance, if feasible, especially applied to biological reactions. This is because biological reactions typically proceed in aqueous media (water, due to its polar nature, is prone to rapid microwave heating) but have a strict temperature regime. In fact, most of the enzymes utilized in biochemical reactions will undergo denaturing at elevated temperatures.[5] Thus, the choice of an enzymatic reaction is a good one for heat-limited, microwave-assisted chemistry, because such a reaction has an inherent temperature self-check and will not yield any rate enhancement due to elevated temperature. If the heating microzones are responsible for the microwave effect, as was suggested by some researchers,[6] then the progress of an aqueous enzymatic reaction should be severly limited if not completely inhibited in the presence of microwaves. Localized zones of high temperature would irrevesibly denature the protein structure of the enzyme and thus destroy its enzymatic function. In fact, some of the previously analyzed reactions for which the microwave effect was proposed were either solid phase[7] or plainly out of the realm of biological models (high temperature and pressure).[8]

True biological reactions are very complex and can be difficult to mimic *in vitro*.[9] This led us to use model reactions of natural hydrolytic processes. In nature, the stability of phosphodiester linkages is of critical importance.[10] All organisms contain RNA and most DNA, which in turn are polymers whose backbone phosphate units link sugars. The omnipresence of microwave radiation (radio, TV, cellular phones, radar, etc.) in today's environment makes it all the more critical to understand its influence on DNA and RNA stability. These macromolecules (DNA, RNA) are prone to a variety of mutagenic factors. For example, irradiation with microwaves of the hydrogen-bonded strands of DNA could lead to modifications in chain superstructure. On the other hand, the phosphoester moiety itself, despite the fact that it possesses rather polar portions usually associated with microwave uptake, showed little or no microwave absorption over a wide range of frequencies (1000−10 000 MHz), as was reported by two independent groups of scientists at Uppsala University in Sweden and King's College in London.[11] The model reaction we have studied is the hydrolysis of bis(*p*-nitrophenyl) phosphodiester with copper(II) 2,2′-bipyridyl complex, as shown in Scheme 1. In this reaction, a hydrolysis of the bis(*p*-nitrophenyl) phosphodiester occurs with the release of *p*-nitrophenoxy anion. This reaction mimics the cleavage of the phosphodiester backbone of the DNA molecule.

The second model reaction pertains to the enzymatic backbone scission of polycarbohydrates. It utilizes cellobiose hydrolysis to model β-glycosidic bond scission (Figure 1). We have chosen cellulase ((1,4-[1,3:1,4]-β-D-glucan-4-glucanohydrolase) from *Penicillinum funiculosum*) as a model enzyme since it acts as an *exo*-β-glycosidase. Thus, despite the fact that polycarbohydrates differ immensely from a disaccharide like cellobiose, units of glucose are liberated by the action of the enzyme the same way as in the model reaction (Figure 1). The industrial importance of this process lies in the potential rapid decomposition of cellulosic waste. Annual production of cellulosic waste reaches 120 million tons.[12] Microwave enhancement of cellulose degradation reactions could lead to obvious benefits in sewage treatment and waste management in the industrial realm. From an academic point of view, the hydrolysis of cellobiose by cellulase is one of the first reports of microwave-moderated enzymatic reactions.

To study these microwave-mediated model reactions under conditions where the temperature can be controlled

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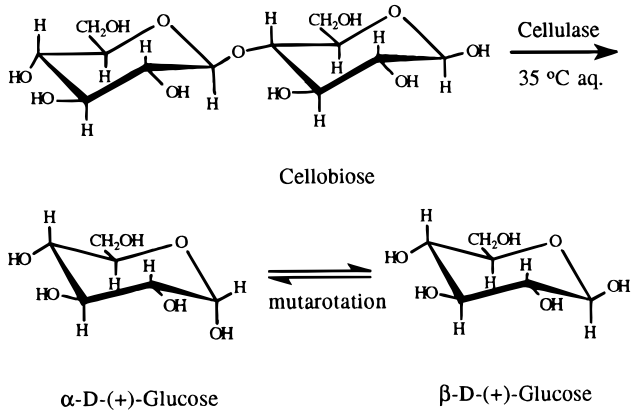


Figure 1. Enzymatic hydrolysis of cellobiose to the two anomers of glucose.

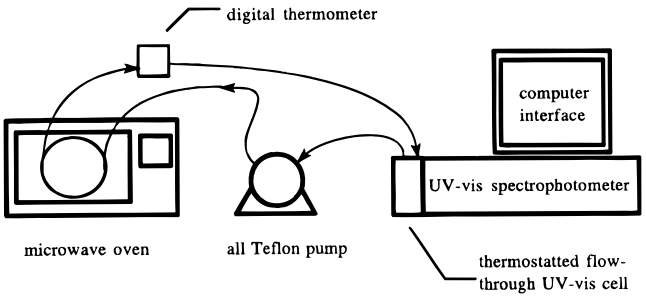


Figure 2. Continuous-flow setup for the kinetic analysis of copper(II) 2,2′-bipyridyl hydrolysis of bis(*p*-nitrophenyl)phosphate diester.

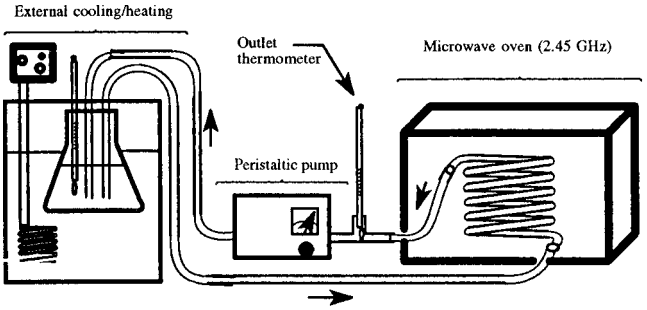


Figure 3. Experimental setup for the microwave-induced hydrolysis of biopolymers.

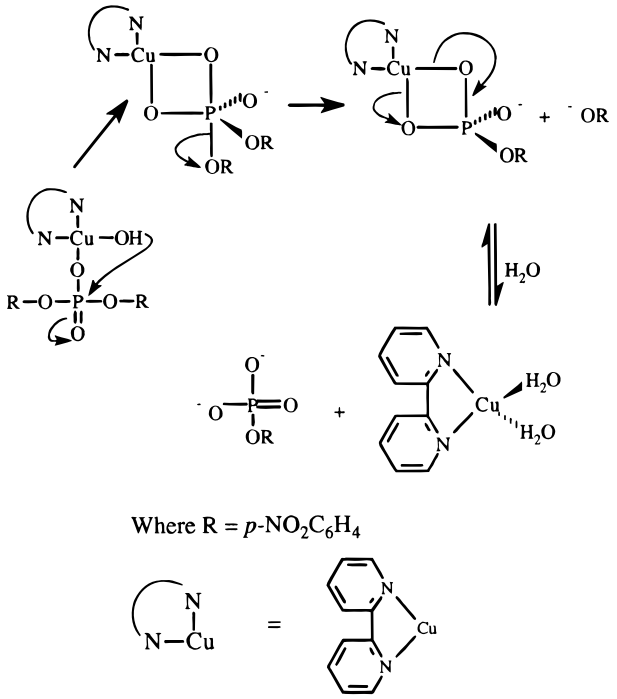
and monitored, we have developed experimental setups to follow the progress of these reactions in the presence of continuous microwave radiation while eliminating the thermal influences (see Figures 2 and 3).

Experimental Section

General Information. The experimental setups (Figures 2 and 3) were closed systems built of a combination of Teflon and Tygon tubing (2.0 mm i.d. for the phosphoester hydrolysis setup shown in Figure 2 and 4.5 mm i.d. for the carbohydrate hydrolysis setup shown in Figure 3). In particular, Teflon tubing was used inside the microwave cavity to ensure maximum transparency toward the microwaves. Temperature in both setups was monitored inside the tubing at the outlet of the microwave cavity. Due to the relatively small diameter of the tubing and the fast flow rate of the pump (causing turbulent rather than laminar flow), there was no need for addditional stirring of the reaction mixture. All reactants' concentrations were prepared using standard analytical dilution methods. Ultramillipure water was used in all experiments.

Ester hydrolysis reactions were run at 75 °C in aqueous media. The total volume of the system (Figure 2) was 15 mL, and the flow rate was set at 20 mL/min. UV−vis spectral data

Scheme 1. Proposed Mechanism for the Hydrolysis of Bis(*p*-nitrophenyl) Phosphodiester Using Copper(II) 2,2′-Bipyridyl Complex in Aqueous Media[13]



were acquired on Perkin Elmer 552A spectrophotometer outfitted with a Perkin-Elmer C550-0108 water-jacketed single cell holder (which allowed for temperature control) interfaced with an Omega data acquisition and control system (Model WB-ASC16).

Carbohydrate hydrolysis reactions were run at 35 °C in aqueous media in the setup shown in Figure 3. Glucose levels were measured using a commercially available glucometer (Model Accu-Chek Advantage), and the concentrations were recorded in mg/dL. The carbohydrate hydrolysis setup (Figure 3) had a total volume of 460 mL and a flow rate of 300 mL/min.

The microwave ovens used were commercially available models (General Electric JES-0601T and Daewoo KOR-611Q) operating at a maximum power level of 650 W. Each oven was equipped with a digital timer and could be operated at 10 different power levels. The domestic microwave ovens operate by alternating from maximum power output to zero power in timed cycles.[4] The frequency of the maximum power cycle is represented by a power level number. For example, power level 2 means that the magnetron is actually on for 2 s out of every 10 s, while power level 10 represents continuous irradiation.

Preparation of Copper(II) 2,2′-Bipyridyl Complex.[13] Equimolar solutions of 2,2′-bipyridyl (in methanol) and $Cu(NO_3)_2$ (aqueous) were combined. A dark blue color developed. The final concentration was adjusted to 1 mM in $Cu(bpy)^{2+}$, assuming complete complexation. This stock solution was stored refrigerated and subsequently used for all reactions.

Preparation of HEPES-Buffered Bis(*p*-nitrophenyl) Phosphodiester Stock Solution. A 1.0 mM solution of HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.0, buffer was used to prepare 0.5 mM stock solution of bis(*p*-nitrophenyl) phosphodiester, which was stored refrigerated and subsequently used for all reactions.

Bis(*p*-nitrophenyl) Phosphodiester Hydrolysis. Two samples (10 mL each) of 1.0 mM $Cu(bpy)^{+2}$ and 0.5 mM bis(*p*-nitrophenyl) phosphodiester stock were taken and added to the apparatus (Figure 2). The reaction progress was followed by monitoring the production of *p*-nitrophenoxy anion, which absorbs in the UV range at a λ_{max} of 405.0 nm. Spectroscopic data were collected continuously, at a constant wavelength of 405.0 nm, for 3 h. The microwave protocol was 10 min exposures out of every 30 min at power level 1. Also, a series of

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experiments were run at power level 2 with increased exposure times up to continuous irradiation for 3.0 h total exposure. All of these reactions were run at 75 °C, as thermostated by a water-jacketed single cell holder. Concentrations were determined by comparison to a standard curve.

Carbohydrate Hydrolysis. A 2.00 g sample of cellobiose and 10 mL of pH 7.41 buffer were placed in a 500 mL Erlenmeyer flask. These reagents were diluted with 450 mL of ultramillipure water. To start the reaction, 0.10 g of cellulase (from *Penicillinum funiculosum*, Sigma) was added. This solution was placed in the apparatus (Figure 3). The reaction progress was monitored by taking 5 mL aliquots of reaction solution at 1 h intervals for 6 h and filtering through a 5 μm Teflon filter to remove particulates (including the enzyme). These aliquots were tested for the presence of glucose using the Accu-Chek Advantage glucometer. In irradiated reactions, the microwave was run continously at power level 6. Temperature was held at 35 °C. To keep the temperature at 35 °C, the reaction mixture had to be cooled externally by chilling the water bath (Figure 3) with copious amounts of ice.

Results and Discussion

Bis(*p*-nitrophenyl) Phosphodiester Hydrolysis by Copper(II) 2,2′-bipyridyl Complex. The nonenzymatic hydrolysis of phosphate esters is complicated by a variety of factors.[14] For example, such reactions are prone to influence of the ionizable hydroxyl groups present on the phosphate moiety itself. As mentioned in the introduction, the model we chose has similarities to the DNA hydrolysis, but it also has deficiencies, as it utilizes a small molecule rather than a biopolymer. The mode of copper(II) 2,2′-bipyridyl complexation to the substrate is drastically simplified. However, the fundamental interest in the phosphate diester hydrolysis suggests that such a study would be of value. Factors other than the nonpolymeric nature of the substrate may play an even more important role in affecting the reaction. For example, these reactions have previously been shown to be very sensitive to both pH and temperature.[13] In fact, in our preliminary experiments performed in insufficiently buffered media, the reaction failed to proceed to completion. We settled on monitoring the reaction's pH at the end of each run.

As shown in Scheme 1, the proposed mechanism involves a direct nucleophilic attack by the hydroxyl bound to the copper(II) 2,2′-bipyridyl onto the central phosphorus atom. In principle, the reaction rate might have accelerated with a direct microwave input. Absorption of energy by an attacking hydroxyl moiety could serve to amplify the reaction progress. However, both microwaved and non-microwaved reactions showed similar kinetics (Figures 4 and 5).

Figure 4 shows real-time kinetic plots of the production of the phenoxy anion at power level 1 and without microwave radiation. Both plots exhibit good pseudo-first-order kinetics for the phosphoester hydrolysis, particularly in the first 100 min of the reaction. After ~140 min, the production of the phenoxy anion approaches equilibrium. This may be due to an autoinhibitive role of the produced phenoxy anion or a result of the limited buffering capacity of HEPES present in the reaction mixture.[13] The leveling of the kinetic plot after the first 140 min could also be attributed to the possible instability of the copper complex itself. However, our control reaction (1 h of continuous irradiation at power level 2) indicated the outstanding stability of copper(II) 2,2′-bipyridyl in aqueous medium, as monitored by UV−vis spectroscopy.

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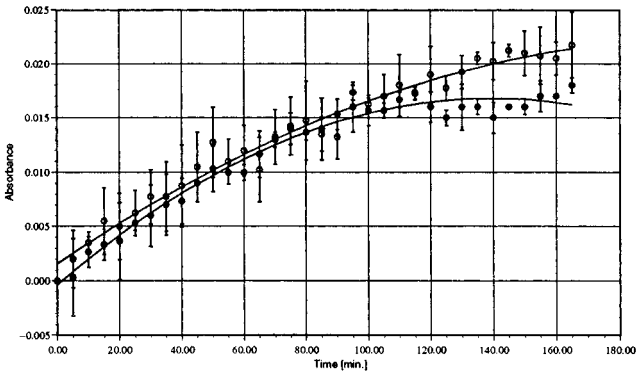

Figure 4. Comparison of nonmicrowaved (○) hydrolysis of bis(*p*-nitrophenyl) phosphodiester with power level 1-irradiated reaction (●). All reactions were monitored by UV−vis spectroscopy at a constant λ_{max} = 405 nm at 75 °C. Error bars represent 1 standard deviation for an average of three experiments.

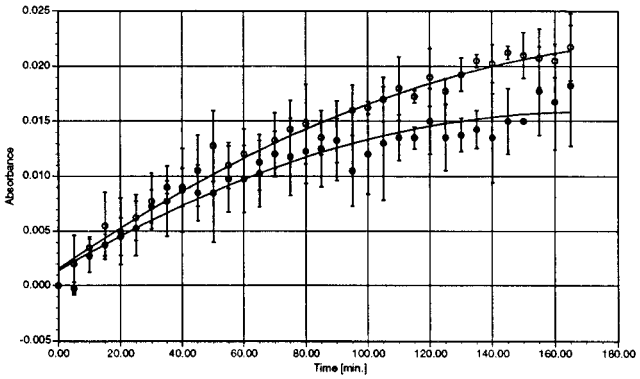

Figure 5. Comparison of nonmicrowaved (○) hydrolysis of bis(*p*-nitrophenyl) phosphodiester with power level 2-irradiated reaction (●). All reactions were monitored by UV−vis spectroscopy at a constant λ_{max} = 405 nm at 75 °C. Error bars represent 1 standard deviation for an average of three experiments.

Even at the confidence level of 1 standard deviation, the plots of the irradiated and nonmicrowaved reactions are comparable. The irradiated reactions seem, in fact, to have the same or a slightly lower rate constant than the control (nonmicrowaved) ones. The similarity is greatest particularly in the initial portion of the kinetic plots. The disparity of the two kinetic graphs starts ~100 min into the reaction. It is possible that, perhaps, buffer stability in the later stages of the reaction is affected by microwave radiation.

In order to further study the influence of heat-limited microwave irradiation on our model reaction, it was decided to double the microwaving time (power level 2). The results are presented in Figure 5. Again, the average of three control reactions, represented by a best-fit solid line, is plotted along with the average of three microwaved reactions. The same trend as in Figure 4 can be observed. We have successfully kept the reaction temperature at 75 ± 5 °C, as monitored by a digital thermometer at the microwave outlet shown in Figure 2. To ensure that the microwave radiation levels were sufficient, we have tried a hydrolysis reaction with continuous irradiation at power level 2 for 180 min. This trial showed almost exactly the same kinetics as presented in Figures 4 and 5 (kinetics not shown). Table 1 shows the pseudo-first-order kinetic data obtained for the three types of hydrolytic reactions. As can be seen, all of the rate constants are very similar for the linear

Table 1. Pseudo-First-Order Rate Constants for the Hydrolysis of Bis(*p*-nitrophenyl) Phosphodiester by Copper(II) 2,2′-Bipyridyl

reaction type	rate constant[b] (k, $\times 10^{-4}$ s^{-1})
nonmicrowaved	2.3
microwaved (power level 1)[a]	2.2
microwaved (power level 2)[a]	2.0

[a] Power levels 1 and 2 mean that the reaction mixture was actually irradiated for 1/10 and 2/10 of the total time, respectively, that it was in the microwave cavity. [b] The rates were calculated for reactions run at 75 °C and for the linear portion (first 100 min) of the reaction progress.

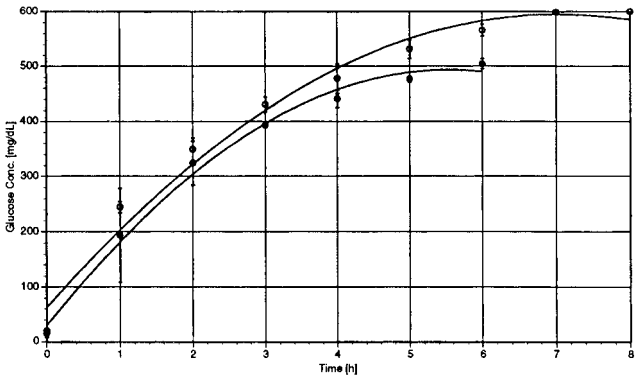

Figure 6. Comparison of nonmicrowaved (○) enzymatic hydrolysis of cellobiose by cellulase with a microwaved reaction (●). All reactions were run in aqueous media at 35 °C. Microwaved reactions were performed at power level 6. Error bars on both kinetic plots represent 1 standard deviation. The plots represent averages of three data sets for nonmicrowaved and two data sets for irradiated reactions.

portions of kinetic plots (Figures 4 and 5). Even though these reactions' conditions vary from those of earlier studies, our rate constants are quite comparable.[13]

Carbohydrate Hydrolysis. We have studied the enzymatic hydrolysis of cellobiose as a model reaction of *in vivo* cellulose hydrolysis. The hydrolysis of cellulose *in vivo* is a multistep process. It involves steps like solvation (typically complex for macromolecules), followed by β-exoglycosidase-catalyzed hydrolysis that results in intermediate production of cellobiose, which in turn is digested further to glucose. All of the steps described above are prone to the competitive inhibition by the generated intermediates and products, thus making it all the more complex.[15] Our model system focuses on the more academic question of the β-glycosidic linkage stability under the influence of electromagnetically moderated enzymatic reactions. Reactions performed with and without the influence of microwaves showed comparable rates. As can be observed in Figure 6, glucose production was nearly identical between the microwaved and nonmicrowaved reactions in the initial (linear) portion of the graph and remained comparable throughout the experiment.

In fact, the accuracy of the glucometer that we used in this study is best below 500 mg/dL, which could account for the disparity of the two curves in the higher range of glucose concentration. The rate of reaction was also studied. To obtain rate data, we analyzed the initial portions of the kinetic plots (Figure 6). The data are presented in Figure 7. The rate constants were determined from the slopes of logarithmically replotted data and were found to be 0.56 h^{-1} for the control (nonmicrowaved) reaction and 0.45 h^{-1} for the irradiated one. These plots are an average of three experiments each. Due to the large reaction volume and high flow rate, excellent temperature control was achieved. In fact, despite the aqueous nature of the reaction medium, microwave power levels up to 6 were utilized with temperature control ±1 °C.

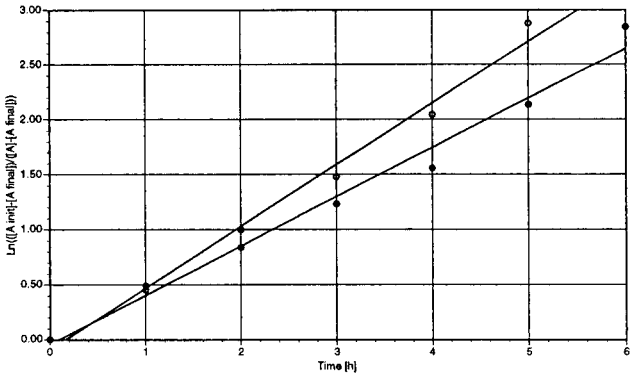

Figure 7. Comparisons of rate constants for microwave-moderated (●) and control (○) hydrolyses of cellobiose in aqueous media at 35 °C. For the control reaction, $k = 0.56$ h^{-1}, while for the irradiated reaction, $k = 0.45$ h^{-1}.

Conclusions

In conclusion, we found the two model reactions to show no particular microwave enhancement. This does not necessarily discount the notion of the alleged "microwave effect" phenomenon, but it rather implies that 2.45 GHz radiation had no influence on these two reactions if thermal heating was eliminated as a factor in such conversions. Also, it was established that microwave radiation does not affect the activity of the cellulase enzyme and does not diminish its biological activity. Despite the possibility of the existence of small, untraceable, localized heating zones, it was found that, by controlling the overall temperature of the reaction, it was possible to achieve similar results for both microwaved and nonmicrowaved reactions. The pursuit of these alleged heating microzones seems to be of no benefit, as the most thermally sensitive reactions, like these of a biological nature, were virtually unaffected in the microwave environment. There is clearly a need for further study of the microwave effect phenomenon. Other types of reactions should be investigated: for example, nonaqueous reactions are good candidates to test the existence and nature of such an effect. Such reactions could be judiciously designed to be run in low microwave absorbing media, e.g., hydrocarbons. The intrinsicly low absorptivity of electromagnetic radiation by the reaction solvent would then direct the power input to the actual reactants rather than the media used as a solvent. Such selectivity would be helpful in finding the true uniqueness of microwave-enhanced chemistry over the traditional methods. Studies directed to this question are currently underway in our laboratory.

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