purified via column chromatography (silica gel, eluted with 1:19 ethyl acetate–hexane mixture), to recover **5** (126 mg) and pure **6** (30 mg, 80% yield based upon recovered starting material); exact mass calcd for $C_{10}H_{10}O$: M_r 146.0732, found (high-resolution mass spectroscopy) M_r 146.0732. The infrared, ^1H NMR, and ^{13}C NMR spectra of this material were in agreement with values reported previously for **6**.[8]

Acknowledgment. We thank the Air Force Office of Scientific Research (Grant AFOSR-88-0132), the Robert A. Welch Foundation (Grant B-963), the National Institutes of Health (Biomedical Research Support Grant), and the University of North Texas Faculty Research Committee for financial support of this study.

Registry No. 1, 115514-30-2; 2, 115514-31-3; 3, 102830-58-0; 4, 115514-32-4; 5, 7427-90-9; 6, 699-82-1; 7, 90991-05-2; 8, 115514-33-5; dicyclopentadiene, 77-73-6; *exo*-3-methyltricyclo-[5.2.1.$0^{2,6}$]dec-8-en-5-one, 19305-54-5; 4-methylcyclopent-2-en-1-one, 23033-96-7.

Microwave-Induced Hydrolysis of Phospho Anhydride Bonds in Nucleotide Triphosphates

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Received March 21, 1988

A large body of literature exists on the effect of microwave radiation on biosystems which debates whether the observed aberations are due to general heating of the biosystem (hyperthermia) or are a consequence of the specific absorption of microwave energy.[1-7] Except for the precipitation of proteins such as in the formation of microwave-induced cataracts due to hypothermia,[3] very few reports address molecular changes due to the absorption of microwave energy on a chemical reaction or discrete compound. In contrast to microwave irradiation, recent reports indicate that ultrasound has been successfully employed in organic synthesis. Ultrasound has been used to effect the hydrolysis of carboxylic esters,[8] induce the cleavage of carbon–halogen bonds in the presence of zinc,[9] increase the rate of formation of lithium organometallic reagents,[10] and accelerate the synthesis of thioamides.[11] In one report ultrasound and/or microwave excitation of alkali-metal vapors was found to dehalogenate organic dihalides.[12]

Due to the reported changes of ATP levels in vivo following microwave radiation of an organ or oragnism,[13-15] we subjected purine and pyrimidine nucleotide 5′-triphosphates (NTP) to continuous wave microwave radiation at 2.54 GHz with a power density of 0.16 W/cm^2. Since the temperature of the samples became elevated during the period of irradiation, controls were externally heated by convection. The rate of heating and final temperature were shown to be similar for both controls and samples exposed to microwave radiation.

In the initial experiments, ATP was exposed to microwave radiation. A rapid, time-dependent loss of ATP was observed followed initially by a concomitant rise in the level of ADP and subsequently AMP. In a radiation period of 4 min, 90.9% of the ATP was hydrolyzed, while controls heated in a dry block demonstrated only 6.9% hydrolysis over the same time period. After a total of 15 min of heating in the dry block, hydrolysis of the controls rose

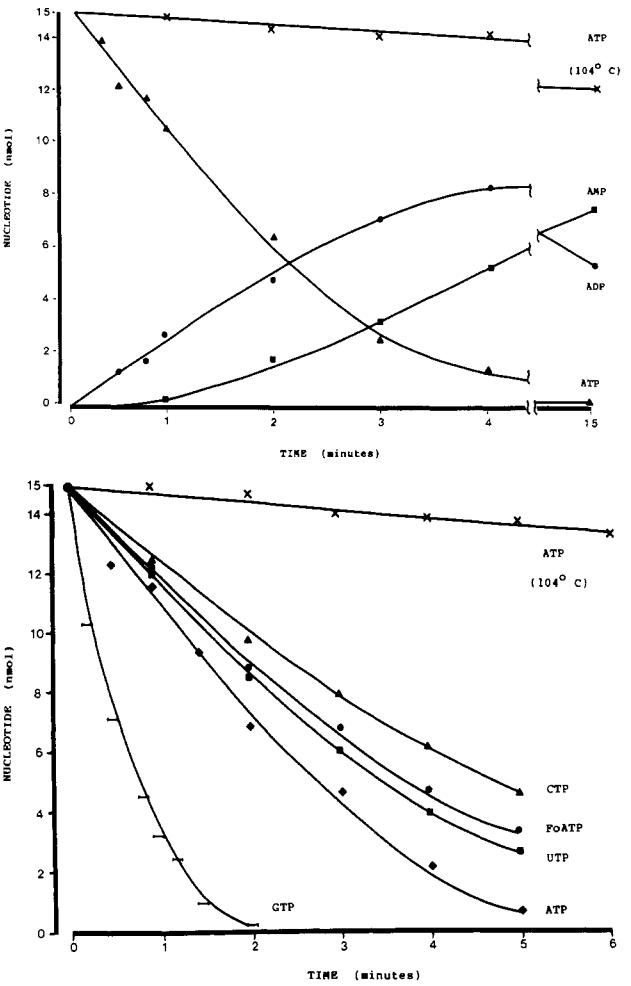

Figure 1. (a) Kinetics of the hydrolysis of ATP initiated by convection heating (X–X) and by microwave irradiation (▲–▲). ADP (●–●) and AMP (■–■) formation are from the microwave experiments. (b) Kinetics of microwave-induced hydrolysis of purine and pyrimidine nucleotide triphosphates. The control reaction is ATP (X–X) heated in a dry block.

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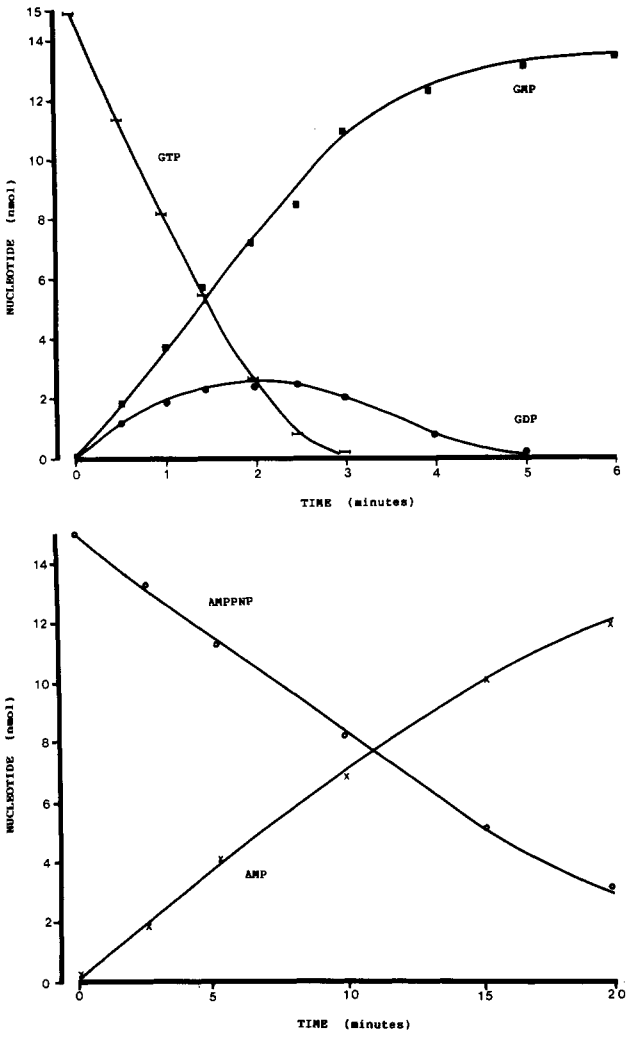

Figure 2. (a) Kinetics of the microwave-induced hydrolysis of GTP (-), yielding GDP (●–●) and GMp (■–■). (b) Microwave-induced hydrolysis of AMPPNP (O–O) forming only AMP (X–X).

to 24.5% (Figure 1a). The hyperbolic formation of ADP suggests that it is the primary hydrolytic product of the β,δ-phosphoanhydride bond of ATP. The sigmoidal formation of AMP, which coincides with the leveling of the ADP concentration, alludes to its production from ADP rather than as a primary hydrolytic product of ATP. In data not shown, ADP was shown to hydrolyze to AMP in a manner similar to that of ATP.

The studies were extended to other NTP's to examine the the generality of the microwave hydrolysis. As observed for ATP, the NTP's, uridine 5′-triphosphate (UTP), cytosine 5′-triphosphate (CTP), guanosine 5′-triphosphate (GTP), and the purine analogue formycin 5′-triphosphate (FoATP) all exhibited a rapid hydrolysis compared to controls (Figure 1b). It should be noted that while the other nucleotides appeared to hydrolyze 12–15 times faster than controls, the loss of GTP induced by microwave radiation was found to be 24 times the rate of the controls. A kinetic analysis of the microwave-induced hydrolysis of GTP showed that both GDP and GMP were formed in a hyperbolic fashion, suggesting that both nucleotides are primary hydrolytic products and that unlike the ATP hydrolysis GMP forms at a faster rate than GDP (Figure 2a). This result may be interpreted by invoking neighboring group participation of the 2-amino function of the GTP assisting in the weakening of the β,δ-phosphoanhydride bond, thereby facilitating the hydrolysis. The proximity of the 2-amino group to the 5′-phosphate is demonstrated by the fact that a variety of analogues exist with covalent bonds between the 2-amino group and the phosphate side chain.[16] In order to see if the β,δ-phosphoanhydride bond of an ATP analogue could be induced to be the primary hydrolytic product, the ATP analogue, adenosine 5′-(β,δ-imidotriphosphate) was also irradiated (Figure 2b). As expected on the basis of the strength of the P–N–P bond the only product formed was AMP, albeit at a significantly slower rate than that of the other nucleotides.

The ability of the phosphate moiety of a nucleotide to sustain a variety of protonated states prompted a study of the effect of both pH and divalent metals on the rate of microwave-induced hydrolysis of ATP. Irradiation of ATP over the pH range of 4 to 9 produced maxima at both pH extremes and a minimum at a pH of 7 (Figure 3a). These data suggest that at the acidic end of the inverted parabola line the δ-phosphate of ATP is protonated (pK_a approx. 4), making it a better leaving group, while at the basic maxima water is replaced by the more reactive hydroxide ion. This behavior is mimicked in control experiments, albeit at a 15-fold reduction in rate, where samples are heated to 104 °C.

With the pH results in hand we examined the effect of divalent metals. In biological systems the NTP are generally complexed with divalent metals and the specific metal ion has a pronounced effect on the biochemical reactivity of the NTP. The effect of divalent metals with NTP's during microwave-induced hydrolysis was compared to an arbitrary standard rate obtained by setting the pH at 6.8 with the counterion being sodium during the irradiation period. It was found that Mg^{2+} exhibited a slightly elevated reaction rate while Mn^{2+} and Zn^{2+} were 1.4 and 1.7 times faster than the standard with Na^{1+} as the counterion for the phosphates. When the Na^{1+} was substituted by Co^{2+} and Cu^{2+}, the rate decreased to 9% and 22% of the control (Figure 3b). While the rate of hydrolysis is faster in both the pH and divalent metal microwave experiments, the enhancement is an effect only of the microwave radiation. The pH and divalent metals appear to have no bearing on the mechanism of the microwave-induced acceleration of the hydrolysis.

To explain the microwave-induced acceleration of the rate of hydrolysis of the phospho anhydride bond of an NTP, we invoked the concept of spectroscopic heating. In standard external convection heating, such as the dry block experiments, the externally supplied energy is dissipated into the solution by increasing the vibrational and rotational activity of the solvent. In spectroscopic heating locally excited solvent molecules absorb the externally supplied energy, in our case in the form of microwave radiation. The contrast here is that in convection heating thermal energy is transferred from the environment through the surface of the capillary to the solvent, whereas in the spectroscopic heating the increase in the kinetic energy of the solvent is due to the direct absorption of radiated energy. These excited molecules accrue excessive amounts of energy in the form of vibrational and rotational modes and dissipate it to the environment.

In this study it appears that when waters of solvation of the phospho anhydride are excited in this fashion they obtain more kinetic energy than may be derived from external convection heating, therefore accelerating the hydrolysis process. It is possible that the acceleration of the hydrolysis process is a direct result of the absorption

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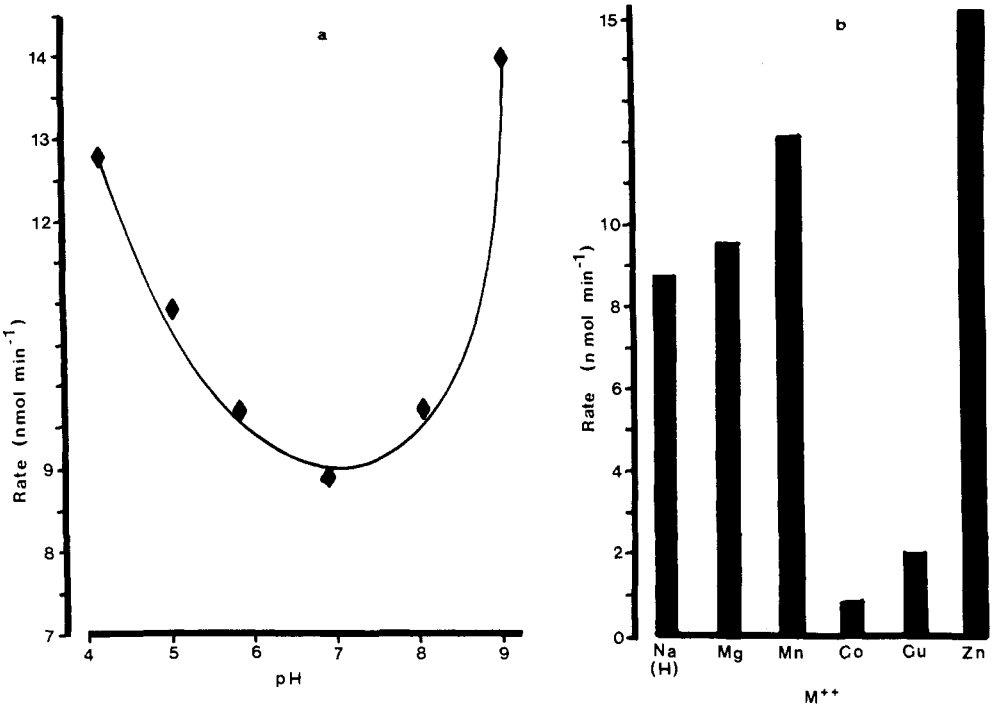

Figure 3. (a) Effect of pH on the microwave-induced hydrolysis of ATP. (b) Microwave-induced hydrolysis of ATP in the presence of divalent metals. The standard reaction is the hydrolysis of ATP in the presence of Na^{+1}.

of the microwave energy or that the waters of hydration of the phospho anhydride bonds are selectively excited compared to the bulk solution. In the absence of a microwave spectrograph of the NTP these possibilities cannot be excluded.

While nonionizing radiation in the range of microwaves is generally not thought to impinge on chemical and biochemical systems, our data suggest that this form of radiation may induce significant changes in the rate of hydrolysis of some biomolecules. In our studies the temperature of the solutions became elevated; however, it is conceivable that the phenomenon of localized spectroscopic heating could occur withtout a significant increase in the temperature of the system. An examination of the literature on the biological effects of microwaves suggests that there are two types of microwave-induced damage, one thermal and one nonthermal. In the cases considered to be nonthermal many of the observed effects, may be explained by a total or transient loss of nucleotides.[13-15] We are now examining the possibility that nonthermal spectroscopic heating of the biosystem may account for the observed microwave-induced abnormalities.

Experimental Section

All nucleotides, oligonucleotides, and buffers were purchased from Sigma Chemical Co. (St. Louis, MO). Solvents for the HPLC were from Fisher Scientific (Medford, MA). Product analysis was performed on an LKB 2152 high-performance liquid chromatograph equipped with an LKB 2158 Uvicord detector set at 254 nm. Separations were obtained on a 3-μm, reverse phase C_{18} Column and quantitation was obtained with an LKB 2220 recording integrator.

General Microwave Irradiation and Thermal Procedures. Samples were subjected to microwave radiation in a microwave oven (General Electric III) that provides 2.45 GHz (continuous wave) with a power density of 0.16 W/cm^2. Sealed capillaries of the samples to be irradiated were degassed and irradiated in identical positions in the microwave for varing amounts of time. each time point was assayed by HPLC and run in triplicate. The temperature inside a sealed capillary containing the buffer was measured to be 101 °C after 5 min as measured by a microthermal probe. Samples that were subjected to convection heating were placed in a Fisher dry block that was preequilibrated to 150 °C. The control reactions were identical with the microwave capillaries. With use of the microthermal probe, the maximum temperature in these samples rose to 104 °C, and the heating rate was similar for both microwave-treated and dry block treated samples. The dry block samples were also run in triplicate and analyzed by HPLC. The curves were fitted to the data by using a Simplex program.

Microwave-Induced Hydrolysis of Nucleotides. Samples of the nucleotides were dissolved in a 10 mM phosphate buffer (pH 6.8) to a final concentration of 1 mM. Aliquots of 50 μL were sealed in capillary tubes, degassed, and irradiated in identical positions in the microwave for varing amounts of time. For each time point a capillary was removed and analyzed by HPLC with a mobile phase consisting of 65 mM phosphate buffer (pH 5.4), 1 mM tetra-butylammonium phosphate (ion-pairing reagent), and 10% methanol. In this system base-line separation was obtained with the triphosphates being retained the longest and the monophosphates the shortest. Standard nucleosides were also run, but none were observed in the experiments.

Effect of pH and Divalent Metal Ions. The solutions were prepared in an identical fashion with those described above. The pH of the buffer was changed to adjust the pH. The metal salts were added to the solution to a final concentration of 10 mM and the pH readjusted to 6.8 to examine the effect of divalent metals. The samples were run and analyzed as described above.

Acknowledgment. This work was supported in part by funding from NIH grants DE 07280 (E.G.E.J.) and DE 04657 (E.F.R.). We also acknowledge the technical assistance of Robert Sirois and support from the University of Lowell Seed Money Grant administered by Dr. Leon Beghian, Associated Vice President for Research at the University of Lowell.