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Abstract

In vivo ^{31}P NMR studies recently have shown that cocaine causes an imbalance of the free magnesium in the brain which results in pH lowering, ischemia, and even death. This direct interaction with the free Mg^{+2} in the brain also affects the Ca^{+2} balance which controls arterial and vascular contraction. This research has addressed the mechanism of the cocaine interaction with magnesium adenosine 5-triphosphate (ATP) using ^{31}P, ^{13}C, and ^{1}H NMR using a Bruker 200 MHz nuclear magnetic resonance (NMR) system. Data are presented and discussed which shows that cocaine and ATP form a complex species which directly affects the NMR spectra.

Introduction

There are numerous articles on cocaine, cocaine distereoisomers, isomeric cocaines, and tropane alkaloids involving the use of NMR for structure elucidation, syntheses conformation, detection, quantification, solvation characterization, drug differentiation, etc. A representative number are listed (Jochims et al., 1967; Stenberg et al., 1976; Baker and Borne, 1978; Taha and Rücker, 1978; Allen et al., 1981; Liu et al., 1981; Carroll et al., 1982; Valensin et al., 1985; By et al., 1988; Dawson, 1991). There has been controversy regarding the carbon-13 peak assignments, but these have been confirmed (Avdovich and Neville, 1983).

Since cocaine and its analogues are drugs of abuse, there has been great interest in the medical and forensic community regarding their psychological and medical action. Recent *in situ* observations on the rat brain have shown that reduced intracellular levels of Mg^{+2} result in rapid and progressive spasms of arterioles and venules followed by rupture of venules and capillaries leading to local hemorrhages and brain edema (Altura et al., 1991). Administered doses of cocaine have been shown to induce intracellular Mg^{+2} deficits, ischemia, and stroke as observed by *in vivo* phosphorus-31 NMR of the brain (Altura et al., 1992). These findings have been related to imbalanced Mg^{+2} gating action of Ca^{+2} necessary for contractibility in cerebral smooth muscle thereby causing cerebral vasospasm and stroke (Altura et al., 1993). Magnesium ion also stabilizes vascular endothelium and serves as an anticoagulant (Altura, 1988). Studies have shown that Mg^{+2} can prevent excessive neurotransmitter release, as well as, block the N-methyl-D-aspartate (NMDA) receptor (Watkins et al., 1990). Phosphorus-31 has demonstrated sensitivity to cerebral energy metabolism and phospholipid changes in brain regions showing decreased levels of phosphomonoester and phosphodiester in the white brain matter of polysubstance abusing subjects. These cerebral tissue effects have been linked to chronic use of cocaine (MacKay et al., 1993). Numerous medical studies led the authors to speculate whether cocaine would directly interact with ATP and if so, was the interaction with the adenosine moiety of the ATP or with only the bound Mg^{+2} ion attached to the ATP anion? To address these questions, proton, carbon, and phosphorus spectra were taken of cocaine HC1 and ATP alone and then spectra were run for mixtures of the two compounds.

Materials and Methods

Proton NMR spectra were determined in deuterium oxide using a Bruker AC 200/52 spectrometer operating at 200.133 MHz; carbon NMR spectra were recorded in deuterium oxide using the same Bruker spectrometer operating at 50.323 MHz; the phosphorus spectra likewise were determined with the Bruker instrument operating at 81.015 MHz. All pertinent acquisition parameters are shown in Table 1. The proton and carbon spectra were measured in 5-mm tubes, using approximately .075 mM and .10 mM concentrations for the ATP and cocaine HC1 solutions, respectively. The cocaine HC1/ATP mixtures were recorded in 5-mm tubes. The phosphorus spectra were obtained in 10-mm tubes with an external standard of phosphoric acid in an inserted 5-mm tube. An internal deuterium lock was used for all NMR samples. Chemical shift values are reported in parts per million

(ppm) relative to 4.63 ppm (the lower frequency of the HDO doublet due to proton exchange with the solvent D_2O) for proton spectra, whereas chemical shift values for carbon are reported in ppm values relative to acetone-d6 (29.8122 ppm relative to tetramethyl silane (TMS)), and chemical shift values for phosphorus spectra were reported relative to phosphoric acid (Aldrich Chemical Company, Milwaukee, WI 53233). All samples were pH adjusted to approximately 7.0 using $NaHCO_3$ (Aldrich Chemical Company, Milwaukee, WI 53233) prior to data collection. All NMR solvents and standards were obtained from Cambridge Isotope Laboratories, Inc., Andover, MA 01810. ATP (Adenosine 5-triphosphate magnesium from equine muscle) was obtained from Sigma Chemical Co., St. Louis, MO 63178. All spectra were run at room temperature (21°C).

Table 1. Acquisition parameters.

	Carbon	Hydrogen	Phosphorus
Frequency (MHz)	50.323	200.133	81.015
Pulse Width (μ sec)	6	7	16
Relaxation Delay (sec)	3	2	2
Sweep Width (Hz)	10,000	4,000	10,000
Number of Scans	8,000 Cocaine 1,000 ATP 8,000 Mixture	16 Cocaine 64 ATP 16 Mixture	128 ATP 128 Mixture

Results and Discussion

Proton Spectra.--The proton spectra are shown in Fig. 1 A. The proton peak assignments for the cocaine HCl (.100 mM) and MgATP (.075 mM) are shown in Table 2. Figures 4 and 5 summarize the numbering systems for the cocaine HCl and MgATP. The proton cocaine HCl assignments were made by comparisons with the literature (Carroll et al., 1982). Slight differences are attributed to solvent, concentration, pH, and temperature affects. Our C4 protons are in a range of 2.22 ppm to 2.34 ppm and, therefore, cannot be distinguished. Further spectra run on the 300 MHz NMR would allow better resolution between the equatorial and axial protons. No assignments were made for the phenyl protons which are in the range of 7.27 to 7.77 ppm.

The MgATP assignments were made by comparisons with the literature (Davies and Danyluk, 1974; Bock, 1980; and Jochims, 1967). Higher field experiments (300 MHz) would aid greatly in resolving the proton resonances in the 4.0-6.0 ppm range. These additional experiments are planned using a GE GN 300 NMR located at UAMS. The amine protons attached at the C6 position on the adenine ring are not resolvable from the HDO peak at 4.63 ppm.

A plot of H8, H2, H1', and H(5'5") chemical shifts versus increasing concentration of both MgATP and cocaine HCl (approximately 1:1 ratios) would indicate stacking as a result of ring-current shielding. ATP self-associates in solution due to base-stacking interactions and this ATP association complex is dependent on the concentration of the ATP concentration. In the complex, the charged phosphate of one ATP molecule interacts electrostatically with the charged adenine ring of the second ATP molecule. The two adenine rings are stacked head-to-head and the ATP molecule is in the *anti* configuration. (Lam and Kotowycz, 1977). These studies are planned. Additionally, relaxation measurement studies on protons H2 and H8, both in the presence and absence of 2% EDTA, will be used to access any increased contributions to intermolecular dipole-dipole mechanism from close neighbor cocaine HCl interactions. The viscosities of the solutions would be measured so that viscosity corrections (incorporated in the reorientational correlation time, λc, via the Stokes-Einstein relation (Hawk, 1973), could be made to the measured T_1 values. Again these relaxation studies would be versus increasing concentration of both the MgATP and cocaine HCl (approximately 1:1 ratios).

Proton homonuclear NOE experiments, with irradiation of protons H2' and H3' will be done to measure signal enhancements of the H2 and H8 protons of the adenine ring versus concentration of both the MgATP and cocaine HCl (approximately 1:1 ratios). Only nuclei which are spatially close to the irradiated nucleus experience any observable intensity change since there is an inverse sixth power relationship. Any 2D NOESY experiments will be run at 300 MHz rather than 200 MHz to improve data acquisition. NOE difference experiments may be run which will aid in the study of the preferred conformations for the large flexible complex involving MgATP and cocaine HCl versus concentration ratios.

No ^{31}P decoupling experiments were done to collapse the H(5'5") splitting patterns due to the coupling of the C(5') protons with the ^{31}P of the exocyclic phosphate group. Higher field proton spectra (300 MHz) would be required to resolve the essentially coalesced multiplet at 200 MHz for the C(5'5") protons. Spin-decoupling experiments at the proper ^{31}P frequency would collapse the C(5'5") proton multiplet. However, as a result of the near magnetic equivalence of the two C(5') protons, individual values for H(4')-H(5'),H(4')-H(5"),31P-H(5'), and 31P-H(5") couplings could not be determined, but their sums, that is, $J_{4'5'}+J_{4'5'}$ and $J_{5'P}+J_{5'P}$ could be determined. Additionally, ^{31}P decoupling experiments would simplify the multiplet at 4.3 ppm due to the C(4') proton four-bond long-range coupling to give the coupling constant

Table 2. Proton peak assignments.

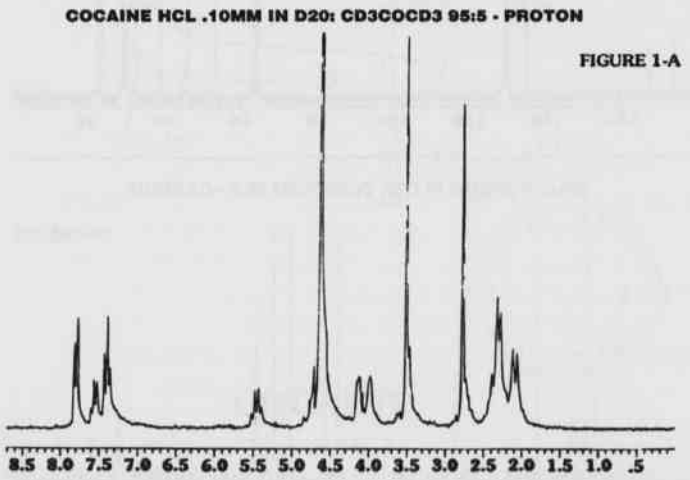
Proton Chemical Shifts for Cocaine HCl at pH 7.0

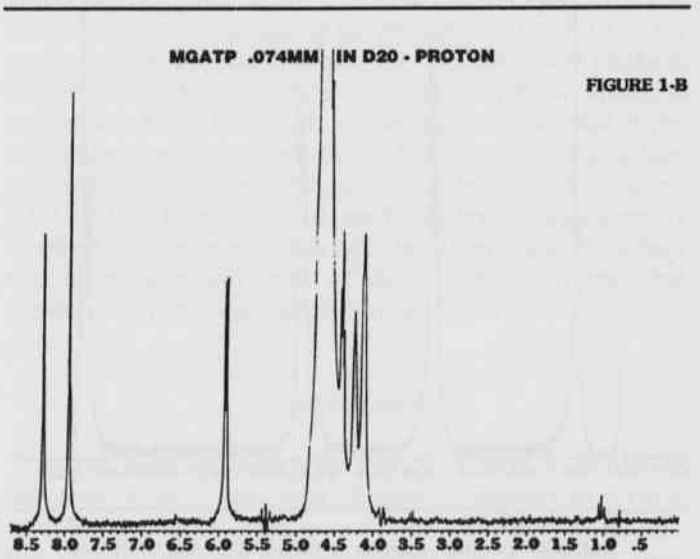
Compound	Solvent	1	2	3	4_{AX},4eq	5	CH_3N	CH_3O'	Phenyl-Protons
Cocaine HCl (.1 mM)	D_2O:CD_3COCD_3 95:5	4.09	3.41	5.37	22.22 to 2.34	3.93	2.74	3.47	7.27 to 7.77

Proton Chemical Shifts for Magnesium ATP at pH 7.0

Compound	Solvent	1'	2'	3'	4'	5'5"	2	8
Cocaine HCl (.1 mM)	D_2O	5.90	Under HDO peak	4.40	4.24	4.12	7.93	8.26

J(^{31}P,H(4')). When the 5.5-8.5 ppm regions are compared in the mixture (Fig. 1C) with cocaine HCl (Fig. 1A and MgATP (Fig. 1B), extra peaks are evident at 7.1 - 7.4 ppm. Additionally, in the region (1.0 - 4.0), the cocaine HCl peaks are shifted (compare Fig. 1A and Fig. 1C), and there is the appearance of an additional peak at 3.0 ppm (Fig. 1C).





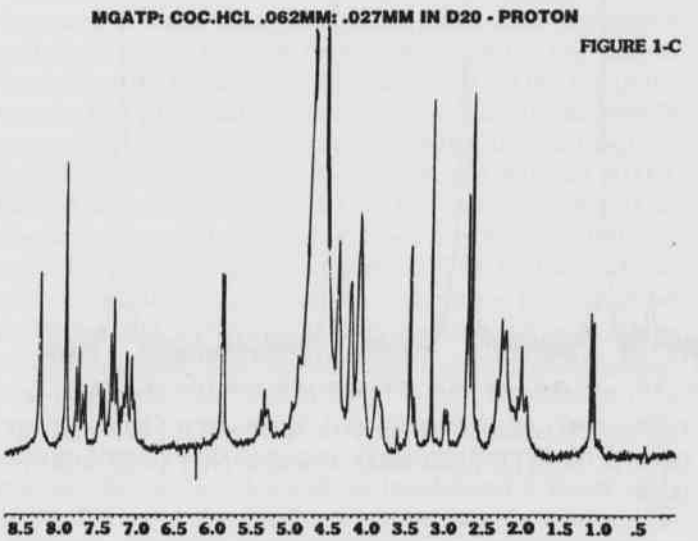
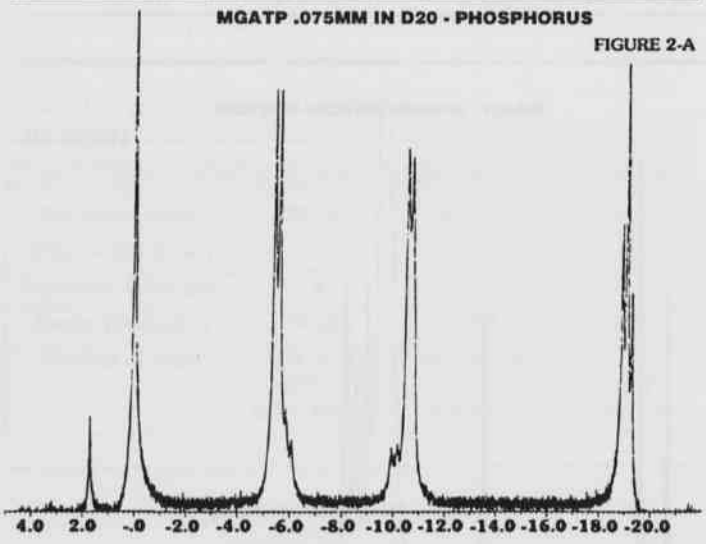


Fig. 1. Proton Spectra A. Cocaine HCl (.100 mM) in D_2O:CD_3COCD_3(95:5); B. MgATP (70 mM) in D_2O; C. MgATP (.062 mM): Cocaine HCl (.027 mM) in D_2O;.

Phosphorus Spectra.--The effect of the interaction of cocaine HC1 and MgATP is shown in Fig. 2 where the splitting of the β triplet and γ doublet are greatly affected. This indicates an overlap of the two molecular species which influences the electron distribution in the phosphate groups. Plots of the ^{31}P chemical shifts for the alpha, beta, and gamma resonances versus increasing concentration of both MgATP and cocaine HC1 (approximately 1:1 ratios) would indicate stacking affects. It is anticipated that there will be insignificant changes in the ^{31}P coupling constants between the phosphorus nuclei. The cocaine N and phenyl groups could interact with the adenine and phosphate groups of ATP through a similar base stacking association.



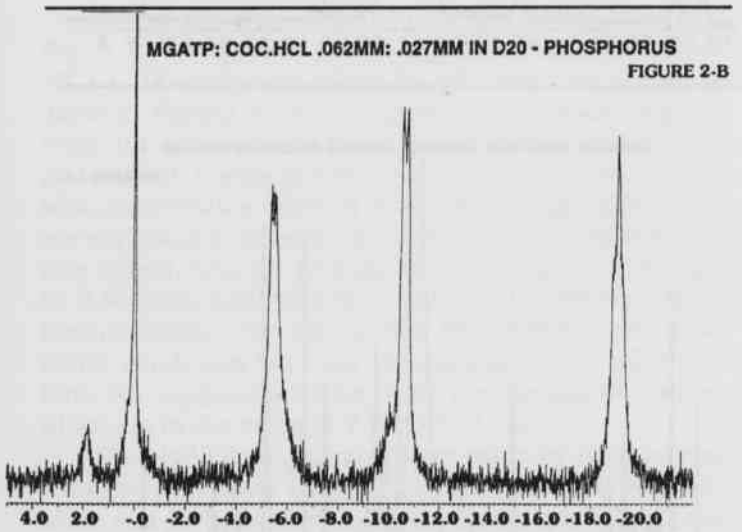
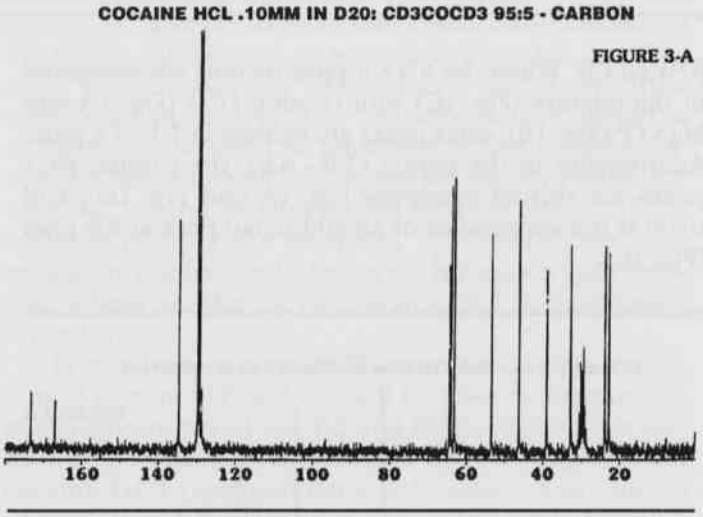
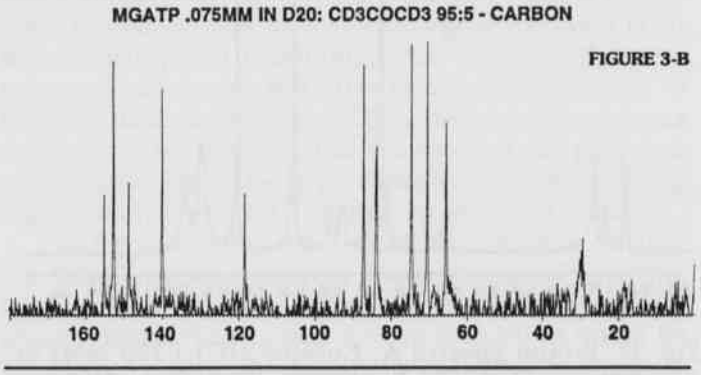


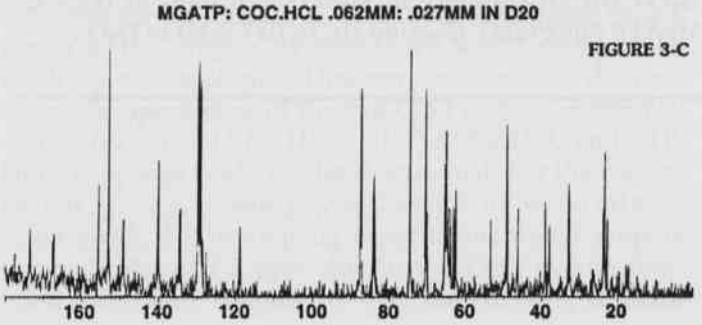
Fig. 2. Phosphorus-31 Spectra A. MgATP (.075 mM) in D_2O; B. MgATP (.062 mM); Cocaine NC1 (.027 mM) in D_2O.

Carbon Spectra.--I. *Cocaine HC1.* The carbon-13 spectrum as shown in Fig. 3-A for .1 mM cocaine HC1 in a solvent system, D_2O:CD_3COCD_3 (95:5), was compared to the literature (Avdovich and Neville, 1983) where slight ppm differences were attributed to solvent, concentration, and temperature affects. Additionally, the pH of the literature system may not have been adjusted to pH 7.0 as in our system. All peak assignments are listed in Tables 3 and 4.

II. *ATP.* The carbon-13 spectrum (Fig. 3-B) for .075 mM in D_2O:CD_3COCD_3 (95:5) shows the normal number of peaks (10 carbon environments) for the molecule. The solid sample was kept at approximately 0°C prior to dissolution in the solvent system to insure minimum degradation.







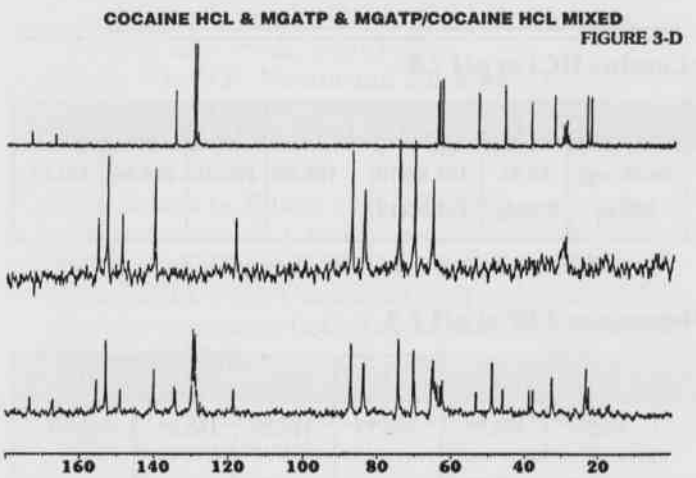


Fig. 3. Carbon-13 Spectra A. .1 mM Cocaine HCl in D_2O:CD_3COCD_3 (95:5); B. .075 mM MgATP in D_2O:CD_3COCD_3 (95:5); C. MgATP (.062 mM): Cocaine HCl (.027 mM) in D_2O; D. Overlayed spectra of A, B, and C.

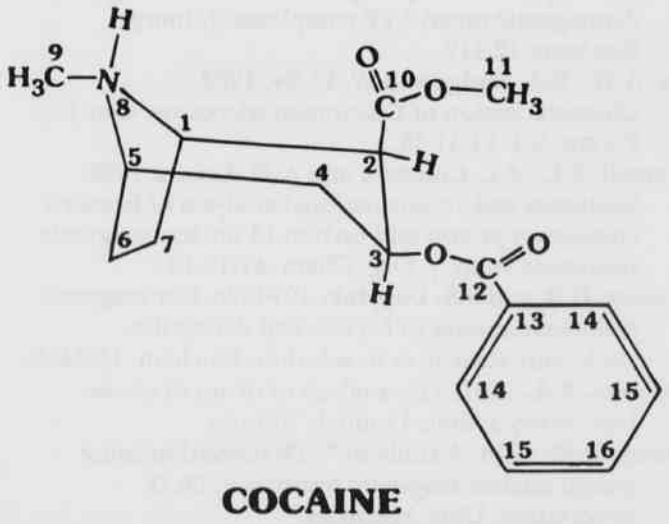


Fig. 4. Structure of Cocaine HCl.

III. *Mixture of Cocaine HCl and ATP.* The carbon-13 spectrum (Fig. 3C) for .027 mM cocaine HCl and .062 mM ATP in D_2O was compared to the carbon-13 spectra of .075 mM ATP and of .100 mM cocaine HCL (both in D_2O:CD_3COCD_3 (95:5)). The peaks are listed in Table 4. Five extra peaks at 23.565, 38.012, 49.189, 62.699, and 65.171 ppm were observed. All peaks were shifted 0.3 to 1.5 ppm away from the assigned peaks of C6 (or 7), NCH_3, C2, C5, and C3 of cocaine alone and are of interest. The four extra peaks at 128.470, 129.059, 129.572, and 134.218 ppm that are shifted 0.1 to 0.4 ppm from the assigned aromatic peaks of C3',5': C2'6': C1': and C4' for cocaine alone are also noteworthy. The two extra peaks at 70.212 and 84.003 ppm are in the aliphatic spectral region for ATP. This leads to the supposition that three molecular species are present in the mixed solution: free cocaine HCL, free ATP, and a complex of cocaine HCl/ATP. The areas of each molecule apparently involved in this interaction are the phenyl and 7 carbon ring of cocaine and some of the aliphatic carbons (and possibly the triphosphate group) of the ATP.

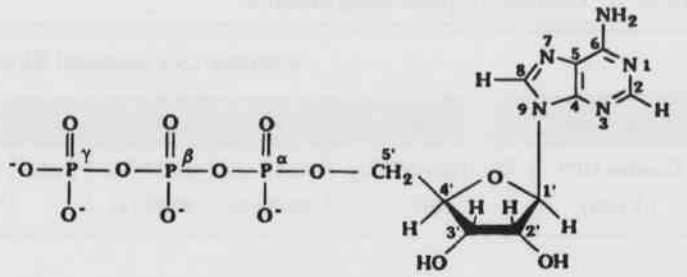


Fig. 5. Structure of MgATP

Conclusions

NMR spectra (shown in Figs. 1, 2, and 3) evidence that cocaine HCl and MgATP directly interact to form a complex species, at least under these concentrations, pH, and solvent system. Research (2D NMR-NOESY and COSY) is underway to determine connectivities between the proton and carbon environments. Samples will be degassed 5 times using the freeze-pump-thaw technique and then sealed in their respective tubes for the proton homonuclear NOE experiments or 2D NOESY experiments. The pD of all solutions will be 7.0. All nucleotide concentrations are determined using uv techniques. Samples will be prepared both with and without EDTA (2% as a mole fraction of the ATP concentration) to check for any effects arising from any trace metal ion impurities (Wasylishen and Cohen, 1974). Proton and carbon chemical shifts, as well as, relaxation times will be determined at 25°C. Commercial samples of nucleotides contain amounts of bound H_2O, and exchangeable acidic, base-ring, and hydroxyl protons. These contribute to a residual HDO peak which has obscured the ribose H2' peak in the MgATP spectrum shown in Fig. 1B. In future studies, the nucleotides will be lyophilized 5 times with 99.8% D_2O and the final lyophilized sample will be dis-

Table 3. Carbon-13 peak assignments.

Carbon-13 Chemical Shifts for Cocaine HCl at pH 7.0

Compound	Solvent	1,5	2,4	3	6,7	9	11	C=0	1'	2',6'	3',5'	4'
Cocaine HCl (.1 mM)	D_2O:CD_3COCD_3 95:5	64.10 (1) 63.29 (5)	46.31 (2) 32.82 (4)	64.55	23.89* 22.79*	39.06 (eq) NCH_3	53.44 OCH_3	173.38(10) 167.50(12)	128.69	129.71	129.24	134.67

Carbon-13 Chemical Shifts for Magnesium ATP at pH 7.0

Compound	Solvent	1'	2'	3'	4'	5'	2	4	5	6	8
Mg ATP (.075 mM)	D_2O:CD_3COCD_3 95:5	87.17	70.50	74.66	83.79	65.54	152.77	148.77	118.26	155.23	140.00

* Denotes interchangeable pairs of chemical shifts.

solved in 100% D_2O. If additional water suppression is required, presaturation will be the desired starting point.

Additionally, enriched ^{25}Mg NMR studies are being pursued to ascertain whether cocaine HCl will directly bind to free Mg^{+2}.

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Table 4
Comparison of the ^{13}C spectral peaks for Cocaine HCl, MgATP, and the mixture of Cocaine HCl/MgATP

Mixture Peaks	Cocaine HCl	Assignment	MgATP	Assignment
173.50	173.38	C=O		
167.24	167.50	C=O(Me)		
155.57			155.23	6
152.45			152.77	2
149.07			148.77	4
140.07			140.00	8
134.54	134.67	C4'		
*134.22				
129.75	129.71	C1'		
*129.57				
129.16	129.24	C2',6'		
*129.06				
128.93	128.68	C3',5'		
*128.47				
118.63			118.26	5
87.22			87.17	1'
#84.00				
83.84			83.79	4'
74.57			74.66	3'
70.51			70.50	2'
#70.21				
65.51			65.54	5'
*65.17				
64.61	64.55	C3		
64.08	64.10	C1		
63.27	63.29	C5		
*62.70				
53.50	53.44	OCH_3		
*49.19				
46.29	46.31	C2		
39.07	39.06	NCH_3		
*38.01				
32.92	32.82	C4		
23.86	23.89	6 (or 7)		
*23.57				
22.81	22.79	7 (or 6)		

* Extra Cocaine peak # Extra ATP peak