PHYSICAL CHEMICAL STUDY OF MONENSIN AND ITS ALKALI METAL ION COMPLEXES

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ABSTRACT

PHYSICAL CHEMICAL STUDY OF MONENSIN
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By

Paul Gilman Gertenbach

Alkali metal ion complexes of an antibiotic drug, monensin, have been studied using potentiometric, infrared and lithium-7, carbon-13, sodium-23 and proton nuclear magnetic resonance techniques in several nonaqueous solvents. The acidity constant of monensin was determined in methanol solution. Stability constants for the monensin-sodium ion complex in methanol were determined by titrating the acid with sodium salts. Complexation titrations with several other cations revealed a monensin selectivity order for the alkali metal ions as well as for silver and ammonium. Evidence was also found for complexation of alkaline earth ions by monensin. Infrared measurements in chloroform and methanol indicated that monensin forms a complex in neutral solutions which is different from the normal sodium complex prepared from basic solutions.

Sodium-23 and lithium-7 NMR measurements in methanol solution served to confirm potentiometric evidence of monensin interaction with these ions. The proton NMR spectrum of the monensin sodium salt was interpreted. The monensin acid spectrum was monitored during sodium ion addition. Sodium ion induced conformational changes in the

monensin molecule were noted. Monensin was found to bind water molecules at two different sites. These water molecules can exchange with one another or with free water in the bulk solvent in acetone, tetrahydrofuran and chloroform solutions. Complexation of the sodium ion by monensin influences this water exchange.

A new complex of monensin with sodium perchlorate or other sodium salts was isolated from several solvents and characterized using potentiometric, infrared, Raman, proton NMR, elemental analysis and thermogravimetric techniques. Analyses indicated that the complex is made up of monensin and the sodium salt and the solvent used for preparation in a 1:1:1 mole ratio. Additional proton NMR studies revealed the possibility of fast chemical exchange of sodium ions between this new complex and the normal monensin sodium salt in chloroform solutions. Evidence for conformational equilibria between the two monensin forms was also obtained.

Experiments with a bimolecular lipid membrane (BLM) system indicated that monensin facilitates the transport of sodium ions through lipid barriers. Several tetrazole and glutarimide molecules which are also alkali metal ion complexing agents were not found to influence BLM permeability. The dilactam form of the synthetic macroheterobicyclic ligand cryptand 222 was shown to affect BLM ion transport while the cryptand 222 did not.

PHYSICAL CHEMICAL STUDY OF MONENSIN AND ITS ALKALI METAL ION COMPLEXES

By

Paul Gilman Gertenbach

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CHAPTER I

HISTORICAL

INTRODUCTION

Studies of the relatively few compounds which form strong complexes with the alkali metal cations have long been of interest to chemists. Recent discoveries that many of these agents possess biological activity have spurred a parallel interest in metal ion complexation on the part of the life scientist. Of particular note have been the compounds capable of affecting the physiological balance of sodium and potassium ions. Many complexing agents of alkali metal cations have been found to be capable of transporting these ions across semipermeable barriers. The phenomenon of selective ion transport has been extensively investigated in order to provide a model for the osmotic properties of biological membranes and to thoroughly ascertain the mechanism of complex formation.

The search for new metal ion ligands is carried on by many investigators. Initial characterization and detailed biological testing of these compounds are relegated to the life scientists. However, analytical chemistry most certainly can play an important role in the careful investigation of the physicochemical properties of these drugs. In the case to be presented here, we shall consider the application of various analytical techniques to the chemistry of a family of several interrelated drugs, with emphasis on the antibiotic monensin.

MONENSIN

The isolation of the monocarboxylic acid antibiotic monensin, a stereoisomer of 2-[2-ethyloctahydro-3'-methyl-5'-[tetrahydro-6-hydroxy-6-[hydroxymethyl]-3,5-dimethyl-2H-pyran-2-yl][2,2'-bifuran-5-yl]]-9-hydroxy-β-methoxy-α,γ,2,8-tetramethyl-1,6-dioxaspiro[4,5] decane-7-butanoic acid (Figure 1), from culture filtrates of Streptomyces cinnamonensis was first reported by Agtarap, et al. (1) in 1967. A few of the chemical properties of the molecule, then called monensic acid, and its sodium and silver salts were described. Most of the details of the isolation procedure, elaboration on the chemistry and documentation of biological testing were presented at a later date by Haney and Hoehn (2). During this period other papers appeared dealing with maximization of monensin yields by variation of fermentation broths (3) and with investigation of the molecular structure of the compound by proton nuclear magnetic resonance spectroscopy and mass spectrometry (4).

Column and thin layer chromatography studies (2,5) showed that monensin consists of four chemical species usually referred to as monensin A, B, C and D. The components were found to be very nearly identical, differing by no more than one $-CH_2-$ unit. At the present time the name monensin usually refers to the major factor, A. Thorough biological testing revealed that monensin was an excellent anti-coccidiostat for chickens (2,6). The antibiotic was found to have a dramatic influence on ion transport in mitochondria (7) as well as affecting ion transport and photophosphorolation in bacterial chromatophores (8,9). A bioautographic assay for monensin in chick tissues was also developed at that time (10).

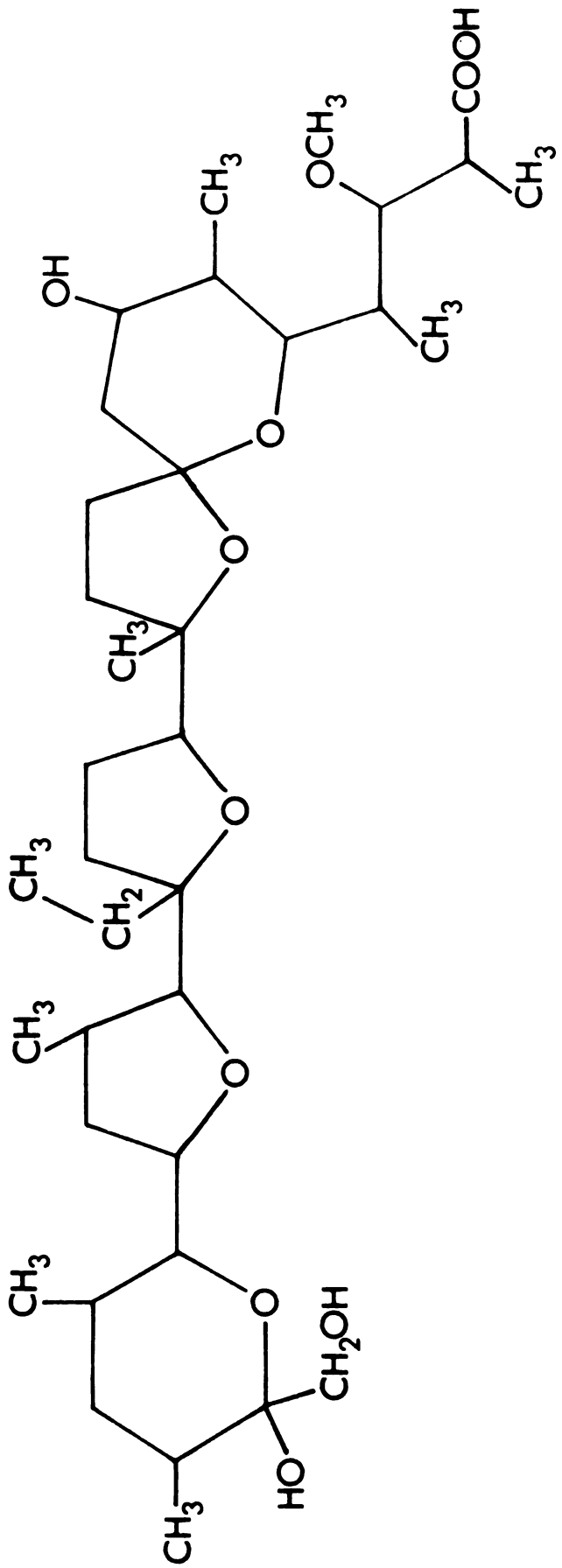


Figure 1. Monensin

After this period of discovery and initial chemical characterization in 1967 and 1968, many workers began to study the chemistry and biological activity of monensin using a wide variety of techniques. Monensin was found to belong to a general group of antibiotics known as the nigericins. Other members of this family of compounds produced by various strains of Streptomyces include dianemycin, X206, and the title compound nigericin. Nigericin had been isolated and characterized previously (11-13) and as can be seen from Figure 2, its structure is indeed quite similar to that of monensin (14-17). The structure of dianemycin was published in 1971 (18). Nigericin was known to transport alkali metal cations across lipid barriers (19) and sheep red blood cells (20). It was also found to be useful for coccidiosis control in poultry (21) and shares many of the chemical and biological properties of monensin. In 1969, Pressman and Haynes (22) reported a physicochemical study of monensin, nigericin and the cyclodepsipeptide antibiotic valinomycin as well as several other compounds. Using several analytical techniques they examined the complexing ability of monensin and reported a marked specificity for the sodium ion. Pressman also commented on the pH dependency of conformation and complexation phenomena and suggested that the monensin free acid is linear while the metal ion complex is cyclic (23). Crystallographic studies revealed the crystal structures of the silver, sodium, potassium and thallium salts of monensin (24). The solid complexes were shown to be cyclic with the metal ion coordinated by six oxygens in a nearly octahedral array (see Figure 16). This insulation of the ionic charge and the hydrophobic exterior of the complex results in a high solubility of monensin salts in organic solvents.

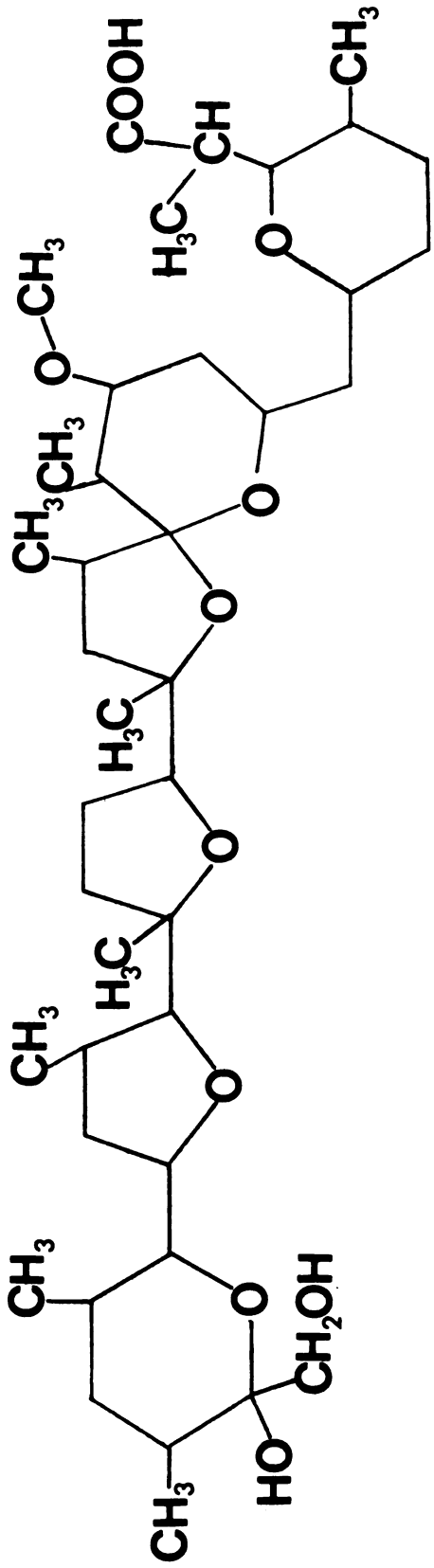


Figure 2. Nigericin

Preparation of the various solid complexes of monensin was found to be dependent on the radius of the metal ion. No lithium, cesium or ammonium complex crystals have been prepared (24). Monensin was shown to be active as an ion transporter in model membrane systems made up of simple water-chloroform barriers (25). A study of the mass spectrometry of the molecule and its factors and salts showed markedly different fragmentation patterns for the free acid and salts (26).

Some of the most definitive physicochemical work on monensin has been done by Simon and coworkers in Switzerland. They have conducted infrared studies which have shown the monensin acid and the sodium salt to be cyclic in solution (27). Electrochemical measurements with ion specific electrodes allowed the first estimates of alkali metal ion complex formation constants in basic methanol solutions which were 7.0×10^5 for sodium ion and 9.5×10^4 for potassium ion. Careful observations of model liquid membrane transport showed a monensin selectivity order of $Na^+ > K^+ > Li^+ > Rb^+ > Cs^+$. Further X-ray crystallographic studies compared the monensin structure specificity relationship to that of related antibiotics in the nigericin family (28).

Simon's group (29) also pointed out interesting differences between the structure of solid monensin salt and the acid. Although the two molecules are very similar, subtle changes in the hydrogen bonds, which hold the otherwise planar structure in its cyclic form, cause significant conformational changes on complexation. Other important differences were found in the location and the number of bound water molecules in each solid. Solid monensin free acid holds one water molecule directly in its center, hydrogen bonded at all three

atoms (see Figure 28). The salt, or complex form, however was always found to crystallize with two water molecules, now attached at the periphery of the molecule (see Figure 16). These facts led to the first model for a complexation mechanism. The hydrogen bonded water molecule was thought to function as one of the water molecules in the hydration sphere of the metal ion during the initial complexation step. Displacement of the bound water as the H_3O^+ ion from the center would complete the dehydration and deprotonation necessary to form the complex.

In 1971 Simon and coworkers presented the best available values for the thermodynamic parameters of monensin and nigericin interactions with sodium and potassium ions in methanol solution (30,31). Using solutions of the antibiotics which were made basic to ensure complete deprotonation, they reported $\Delta H°$, $\Delta G°$ and $\Delta S°$ of complexation as determined by a microcalorimetric method. These results led to values of the formation constants for the various complexes from the antibiotic anion and the metal cation. For monensin, the formation constants of the sodium and potassium complexes are 1×10^6 and 4×10^4 respectively. For nigericin the computed values are 8×10^3 for sodium and 4×10^5 for potassium.

In 1971, Haynes, Pressman and Kowalsky (32) reported the use of ^{23}Na nuclear magnetic resonance spectrometry to study the complexation of the sodium ion by monensin, nigericin, valinomycin and several other ionophores. They monitored the ^{23}Na chemical shift and line width as they titrated monensin and nigericin sodium complexes with excess sodium ion. Shifts were found to be a direct linear function of the fraction of complexed cation or a weighted average of

the chemical shift of free and complexed forms. By back extrapolation to the 1:1 complex, they were able to report a monensin sodium shift in methanol ranging from 1.5 to 3.7 ppm upfield from a 2.0 M aqueous sodium chloride reference. The nigericin sodium 1:1 complex shift was found to be between 3.8 to 5.0 ppm. Line widths in excess of 300 to 400 Hz at half-height in chloroform precluded measurements of chemical shifts in that solvent. The ^{23}Na quadrupole coupling constants for the two drugs were also given. Pressman's latest contribution (33) to monensin chemistry has been the value for the acidity constant of the free acid in a 90% ethanol-water solvent mixture which he obtained by titration of the acid with tetramethylammonium hydroxide. Using this method, he has found a pK_a of 7.95 for monensin and 8.45 for nigericin.

In a series of three papers (34-36), Cussler has shown a new potential use of monensin in possibly nonbiologically related applications (37). By constructing artificial membranes which incorporate monensin, he has devised a scheme in which a pH difference across the barrier can cause the selective transport of sodium ion against its own concentration gradient. An exact theoretical description of the system predicts the observed behavior. He also found that monensin accelerates diffusion of several solutes in mixed solvents (38). Huang (39,40) has developed a detailed kinetic theory to explain antibiotic ion carrier behavior of the nigericin group. Transport properties of monensin have also been recently examined in chloroplasts (41) and in conjunction with calcium accumulation in rat liver mitochondria (42). Sodium efflux in barnacle muscle fibers has also been found to be sensitive to monensin addition (43).

Investigation of biological properties has been of increasing interest, particularly to the Eli Lilly Company which now markets monensin sodium as an anticoccidiostat under the trade name Coban. New analytical methods for microbiological assay of monensin in chick rations have been proposed (44-47). Monensin has been investigated as an additive for increasing food utilization by ruminants in sheep (48). Studies have led to better understanding of the anticoccidial activity in chickens (49-51) as well as approval for general use in feeds by the Food and Drug Administration (52,53). Monensin has also been evaluated as a coccidiosis preventative in rabbits (54) and is currently under intensive investigation for use in improving feed efficiency and weight gain in feedlot cattle (55-58). A biosynthetic pathway for monensin has been proposed (59) and various metabolites and by-products have also been examined (60,61).

CONCLUSIONS

The questions associated with monensin solution chemistry have most certainly generated a deserved amount of interest among chemists and life scientists alike. However, detailed studies of the physical chemical properties of monensin and its complexes in pure solvents are still lacking.

BIMOLECULAR LIPID MEMBRANES

Even though the first successful attempt to make a bimolecular lipid membrane (hereafter designated as BLM, both singular and plural) was reported in 1962 (62-64), the literature now abounds with thousands of references to a legion of different experiments using this model. Several recent monographs present excellent reviews of both the

theoretical and the more important practical aspects of the system and also offer very complete narratives of BLM history (65-67).

From an oversimplified point of view, the BLM model consists only of two layers of lipid molecules in a back to back arrangement with their polar head groups exposed on each side to some aqueous solution. This membrane has been found to be an unexpectedly good mimic of many natural membrane systems and measurements of its physical properties have provided meaningful insights into the function of such systems. Of particular interest for our study are the methods by which BLM have been used to examine membrane permeability changes to alkali metal cations.

The electrical resistance of a BLM in aqueous electrolyte solution is normally very high due to low solubility of charge carrying ions in the hydrophobic membrane phase. With proper experimental technique, this resistance can be measured and monitored as a function of addition of various compounds to the BLM or to the electrolyte solution. It has been found that a large number of agents are capable of lowering the extremely high BLM resistance to one closer to that of natural systems. The most well known chemical to show this effect is the previously mentioned valinomycin. Valinomycin is a cyclic complexing agent which forms strong complexes with alkali metal cations. It is large enough to "bury" the cationic charge within its interior and thereby increase the solubility of the cation in the lipid phase. Several different types of experiments with valinomycin were designed to study this effect in BLM (68-70).

Many other compounds have since been found to complex alkali metal ions and carry them across BLM in a very similar manner.

Among the more important agents are the enniatins (71) and the actins (72). The polyether crown compounds of Pederson (73) have also been found to cause resistance changes (74). Although nigericin group antibiotics have been found to increase the permeability of natural membranes (7) and transport ions through bulk organic phases (25), their effect on BLM has remained unclear. Reports have varied from "failure of nigericin to change the electrical resistance of black lipid membranes" (75) to those in which increased conductance is reported (76). Reference (65) contradicts both reports and states that "compounds of the nigericin group, including monensin, do not appear to have been tested on BLM."

Several other compounds which could have potential as BLM resistance altering agents do not seem to have been tried in the system. One of these classes is the tetrazoles (77,78), a series of drugs whose well known physiological activity as convulsants has often been investigated in terms of membrane permeability changes (79-81). The tetrazoles (Figure 3) have been shown to possess a variable activity depending on the length and variety of various attached side chains (82,83). The molecules are known to be complexing agents (84) and have been found to form weak complexes in solution with both sodium (85) and lithium ions (86). The glutarimides (Figure 3) share many properties with the tetrazoles (78,87,88) and vary from convulsant to anticonvulsant depending on the nature of the substituent groups R_1 and R_2 (89-91). These molecules were also found to form weak alkali metal ion complexes in solution (92,93). Finally, a very new class of strong alkali metal ion complexing agents has been investigated by Lehn and

Figure 3. A Cyclopolymethylenetetrazole, A Disubstituted Glutarimide and the Cryptand 222

coworkers (94,95). These compounds, called cryptands (Figure 3), form selective cation complexes based on the size of the coordinating cavity (93,96,97). Neither biological nor membrane activity has as yet been reported for cryptands or cryptate complexes.

CONCLUSIONS

The BLM model has in the past been shown to be useful as an indicator of possible membrane permeability altering activity. However,thorough studies of the effect of monensin, tetrazoles and glutarimides, and cryptands on BLM ion transport properties do not seem to be available.

CHAPTER II

EXPERIMENTAL

MATERIALS

REAGENTS Tetrabutylammonium hydroxide (TBAH) (Eastman Kodak) was used as received as were deuterium oxide (Columbia Organic, 99.5%) and tetramethylsilane (Aldrich). Benzoic acid (Matheson Coleman and Bell) was dried at 90°C for 48 hours. Sodium perchlorate (G. F. Smith), sodium methoxide (Matheson Coleman and Bell), sodium chloride (Matheson Coleman and Bell), sodium iodide (Matheson Coleman and Bell),potassium chloride (Matheson Coleman and Bell), rubidium iodide (Alfa), cesium iodide (Alfa) and ammonium perchlorate (G. F. Smith) were dried at 110°C for at least 72 hours. Lithium perchlorate (Fisher) was dried at 190°C for several days. Beryllium sulfate (Eberbach), magnesium perchlorate (Allied), calcium chloride (Fisher), strontium perchlorate (K and K), barium perchlorate (G. F. Smith) and silver perchlorate (G. F. Smith) were also dried at 110°C for more than three days. The shift reagent $Eu(fod)_3$ (Alfa) was used as received as were thallium bromide (Alfa), thallium acetate (Alfa), thallium nitrate (Alfa) and tetraphenylarsonium chloride (G. F. Smith).

Phosphatidyl choline (egg lecithin, Applied Science) was received in the form of an ethanol solution. The solvent was evaporated at room temperature and the resulting solid was dissolved at about 1% in n-octane (Aldrich) which had been previously deoxygenated by bubbling nitrogen through it for 2 hours. The lecithin solution was stored at -5°C. Cholesterol (Sigma) was recrystallized from

ethanol. The product was suspended in n-octane and refluxed at 126°C for 6 hours during which time oxygen was constantly bubbled into the solution. The resulting solution, which appeared to be saturated with oxidized cholesterol, was also stored at -5°C. Valinomycin (Calbiochem), dicyclohexyl-18-crown-6 (E. I. DuPont), 4,7,13,16,21,24-hexaoxy-1,10-diazabicyclo[8.8.8]hexacosane (cryptand 222) (E. M. Laboratories), pentamethylene tetrazole (Aldrich), glutarimide (Eastman Kodak), 3,3-dimethylglutarimide (Aldrich) and 3-ethyl-3-methylglutarimide (Pfaltz and Bauer) were used as received. Several other tetrazole compounds and the cryptand dilactam synthetic precursor were obtained from syntheses in progress in our laboratories. The proteins protamine (Sigma) and histone (Aldrich) were used as received and stored at -5°C.

SOLVENTS Methanol (Baker-analyzed reagent) was refluxed over granulated calcium hydride (Baker) for 24 hours and fractionally distilled. Water content was found by Karl Fischer titration to be less than 70 ppm. Chloroform-d_1 (Norell, 99.8%), tetrahydrofuran-d_8 (Norell, 99%), acetone-d_6 (Diaprep-Aldrich, 99.5%) and methanol-d_4 (Diaprep-Aldrich, 99.5%) were used as received. Many solutions involving the use of these solvents were prepared in the dry box. Chloroform (Mallinckrodt), acetone (Mallinckrodt), methanol (Matheson Coleman and Bell), ethanol (Baker), 1,2-dichloroethane (Fisher), benzene (Baker-analyzed reagent), ethyl ether (Mallinckrodt), hydrochloric acid (Baker-analyzed reagent) and petroleum ether needed in various drug purification and preparation steps were used as received.

MONENSIN Monensin was received as the sodium salt (QA 166H Lot Numbers 910 AD3 and 024 FC4, hereafter MonNa) through the generous gift of the Eli Lilly Company. The salt was dissolved to saturation in boiling methanol and the solution filtered. The filtrate was cooled and the salt reprecipitated upon addition of a small quantity of water. This procedure was repeated twice and eliminated most of the brown impurity first noticed as a straw yellow color in solution. The salt was further purified by recrystallization from a 1:1 ether-petroleum ether mixture and dried at 110°C for 48 hours.

The acid form of the molecule (hereafter designated as MonH) was prepared as follows: A concentrated solution of the salt was prepared in chloroform and shaken with an equal volume of aqueous 0.1 M hydrochloric acid and the phases separated. The chloroform phase was evaporated to dryness at room temperature. The yellow solid product was then dissolved in acetone and reprecipitated with water. The now white solid was dried for 24 hours at 30°C under vacuum, then dissolved in boiling methanol. The solution was filtered and MonH was reprecipitated from the cooled filtrate by the addition of water. After drying, the acid was recrystallized from a 1:1 ether-petroleum ether mixture and again dried under vacuum for 24 hours at 30°C.

Elemental analysis of the sodium salt and acid forms gave the following results: Calculated for $C_{36}H_{61}O_{11}Na$, C, 62.70%; H, 8.87%. Found, C, 61.93%; H, 8.88%. Calculated for $C_{36}H_{62}O_{11}\cdot H_2O$, C, 62.77%; H, 9.36%. Found, C, 62.63%; H, 9.28%.

Melting points of 269°C and 117°C for the salt and acid respectively agreed with reported values of 267-269°C (2) and 117-122°C (29). Mass spectra of the two solids showed fragmentation patterns similar to those previously observed (26). Infrared spectra, taken in chloroform solutions, agreed with the available literature spectra (2) and showed a diagnostic shift of the carbonyl band from 1563 cm^{-1} to 1704 cm^{-1} in the salt to acid transformation. Carbon-13 NMR spectra, shown in Figure 4, revealed a dramatic variation in carbon environments and were especially useful in showing a change from 188.2 ppm to 177.8 ppm in the carbonyl chemical shift, which is the characteristic difference between a salt and free carboxylic acid. Tentative assignments of all of the 36 resolved carbon signals were made to the various carbon atoms in each molecule. Proton NMR spectra at ambient probe temperature in chloroform-d_1 (Figure 5) also showed many differences between the acid and the sodium salt, the most obvious one being the appearance of a previously unreported broad water peak at approximately 6 ppm in the spectrum of the acid which is totally absent in the spectrum of the salt.

On the basis of these measurements, it was concluded that the pure free acid had been prepared. No attempt was made to separate the four related factors of either the sodium salt or the acid. The acid was found to be stable in the solid form but quite susceptible to degradation in solutions.

Figure 4. The Carbon-13 NMR Spectra of Monensin and the Monensin Sodium Salt

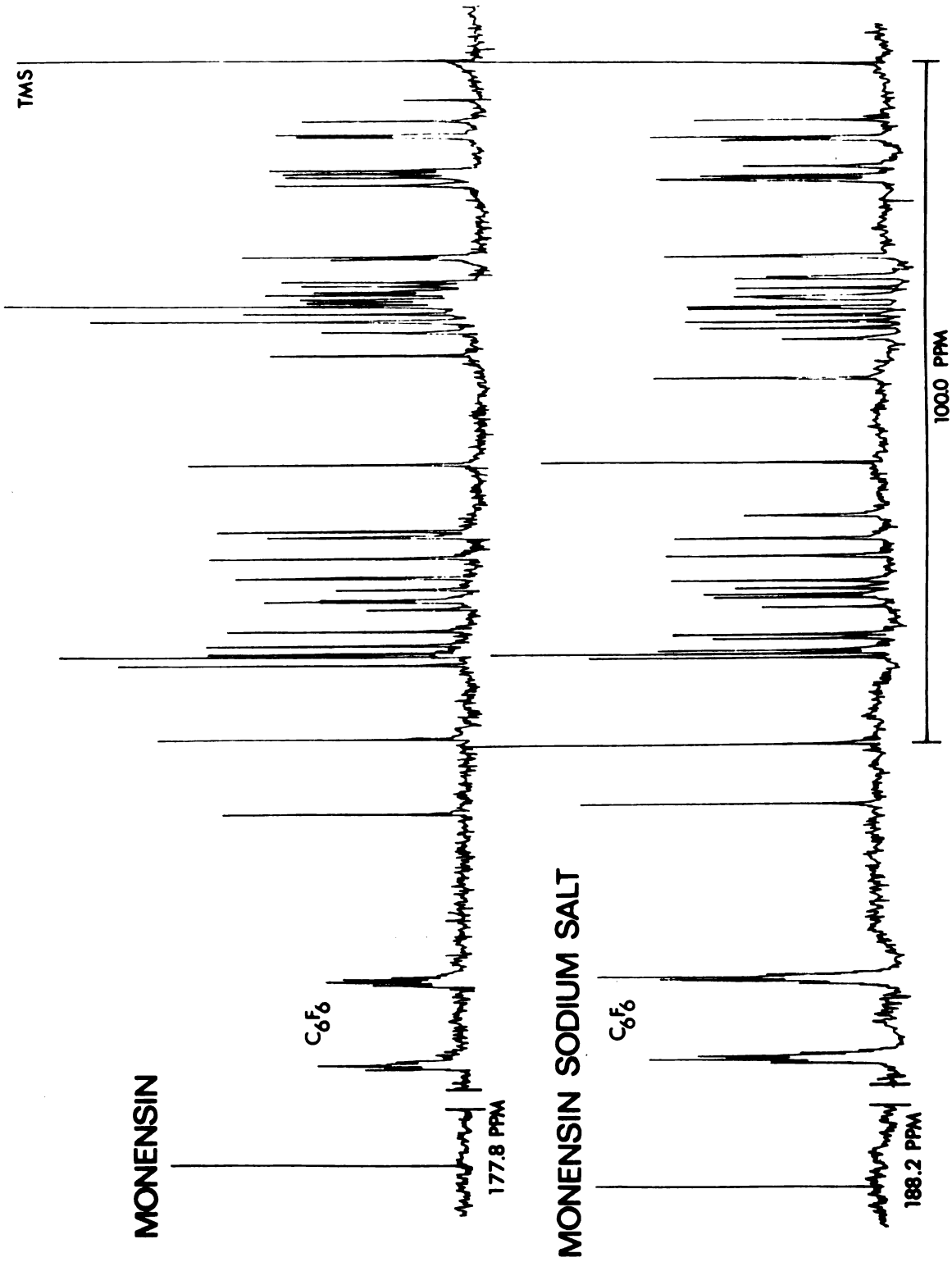
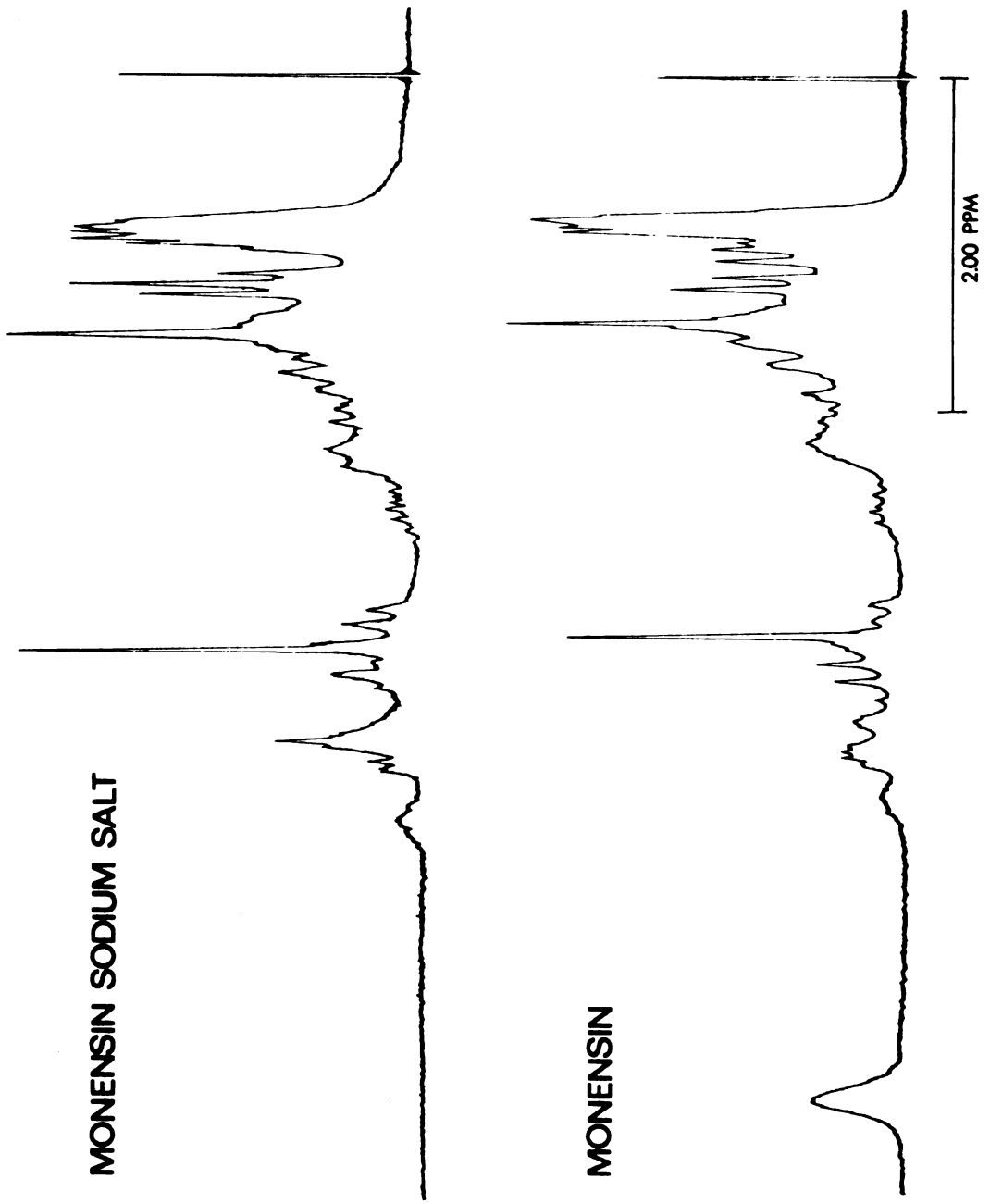

TMS
MONENSIN
C_6F_6
177.8 PPM
MONENSIN SODIUM SALT
C_6F_6
188.2 PPM
100.0 PPM

Figure 5. The Proton NMR Spectra of Monensin Sodium Salt and Monensin at 100 MHz


MONENSIN SODIUM SALT
MONENSIN
2.00 PPM

MEASUREMENTS

POTENTIOMETRIC Potentiometric measurements were made using an air tight cell constructed to accomodate a Beckman 41263 glass pH electrode, a Sargent-Welch S-30080-150 saturated calomel reference electrode and an extended tip of a 50 ml burette. The electrodes were initially soaked in methanol for 48 hours and at least for 5 hours between runs. Even this precaution still did not yield potentials whose absolute values were completely reproducible, but this fact has been accounted for in data interpretation where only changes in potential are used. In some experiments somewhat more stable potentials were obtained by replacing the aqueous saturated potassium chloride solution in the SCE by a saturated methanolic solution of this salt.

Measurements were taken at ambient temperature with a Heath EU-302A servo-digital pH/voltmeter allowing sufficient time between readings for the stabilization of the potential. A constant pressure of nitrogen was maintained in the cell at all times and the magnetic stirrer used for mixing was stopped during measurements. Voltage drift and reading error combined to give potential readings with an uncertainty of $\pm$ 0.002 V and volumes were read with an uncertainty of $\pm$ 0.02 ml. A CDC-6500 computer was used extensively for data processing in conjunction with a revised version of a program initially developed by Briggs and Steuhr (98) for the determination of equivalence points and pK_a or the FORTRAN IV KINFIT program (99). More detail on computer data analysis is presented in the appendices.

SPECTROSCOPIC Perkin Elmer 237B and 457 grating infrared spectrophotometers were used to obtain infrared spectra. A Barnes Engineering fixed pathlength (0.109 mm) potassium bromide cell was used for all infrared studies. The frequencies were calibrated using polystyrene reference peaks.

Raman spectra were obtained on the Spex Ramalog 4 Laser-Raman system employing either a Spectra-Physics Model 164 Argon-Ion Laser or the Coherent Radiation Model 52 Ion Laser. The 5145 Å green line was used for excitation with the third monochromator removed and the photomultiplier operating in the pulse counting mode. Samples were either packed or injected into 1.6-1.8 X 90 mm melting point capillary tubes and sealed.

Lithium-7 NMR spectra were obtained on a Varian Associates DA-60 spectrometer operating in the continuous wave mode at 23.3 MHz with a field of 14.09 kG. The instrument was frequency locked to a 4.0 M aqueous solution of lithium perchlorate held by Teflon spacers in a 1 mm o.d. melting point capillary positioned in a Wilmad 506 pp 5 mm o.d. polished NMR tube. Chemical shifts were measured at approximately 30°C with respect to lock with a Hewlett Packard 5245L frequency counter.

Sodium-23 NMR spectra were obtained at ambient probe temperature on a highly modified Nuclear Magnetic Resonance Specialties MP-1000 superconducting solenoid spectrometer operating at 60 MHz with a field of 53.3 kG. A Nicolet 1083 computer was used for time averaging and to drive the frequency synthesizer. Chemical shifts were measured with respect to a 3.0 M aqueous solution of sodium

chloride in a Wilmad 520-2 coaxial reference tube inserted in either a Wilmad 506 pp 5 mm o.d. or a 513A-5 pp 8 mm o.d. polished NMR tube. Uncertainties in chemical shift values were estimated individually for each spectrum and varied with sweep width and signal line width.

Thallium-205 NMR spectra were run on a modified Varian Associates HA-100 NMR spectrometer operating at 57.7 MHz with a field of 23.4 kG. Samples were run in the Wilmad 506 pp tube. Carbon-13 NMR spectra were obtained using the Bruker HFX-10 spectrometer operating at 22.6 MHz with a field of 21.1 kG. The instrument operated in the normal Fourier transform mode under control of Nicolet 1083 and 290 computers with complete proton decoupling at 90 MHz and an 84 MHz hexafluorobenzene internal lock and TMS internal reference. Samples were run in Wilmad 513-3 pp 10 mm o.d. polished NMR tubes.

Proton NMR spectra were run on three instruments. The Varian T-60 spectrometer was used in the normal mode and also for some decoupling experiments. The Varian A56/60D spectrometer with temperature controller was also used with calibrated chart paper and sideband calibrated sweep widths. The Varian HA-100 spectrometer was used for precision work with either a tetramethylsilane (TMS) or chloroform frequency lock. All chemical shifts were ultimately referenced to TMS and are given in ppm downfield.

Mass spectra were run on the Hitachi Perkin Elmer RMU-6 spectrometer.

OTHER ANALYSES Elemental analyses were done by Chemalytics Incorporated of Tempe, Arizona. Melting points were determined on the Fisher-Johns melting point apparatus. Analysis for water was accomplished with a Photovolt Aquatest II automatic Karl Fischer titration apparatus.

MEMBRANE RESISTANCE All BLM experiments were performed inside of a Faraday cage which consisted of a wooden frame covered by a double layer of copper screening in electrical contact and then covered with light shielding black cloth. Inside, a lattice of Fischer Flexframe and support stands held steady by lead weights were used for the attachment of four pieces of apparatus. A monocular zoom observation microscope (Model ZMM-1, Titan Tool Supply) was mounted at the front of the lattice to be used for inspection of BLM thinning. A spotlight illuminator (Model LS, Unitron Instrument) was used to direct a reflected light from the BLM at an appropriate angle to the microscope. In the center of the arrangement, a sturdy support held the cell in which BLM were formed and above this was the amplifier system to which electrodes were attached. The BLM were formed on a precision drilled 1 mm diameter hole in a 10 ml Teflon cup (Chemplast). This cup stood in the center of a quartz cell built by Mr. Keki Mistry of the department's glass shop. The entire volume of the cup and quartz cell, when filled with electrolyte solution, was about 25 ml. Addition and subtraction of material from either side of the membrane after formation was accomplished using a 25 µl repeating sampling syringe (Oxford). Membranes were initiated by delivering lipid

solutions with a 50 µl fixed needle syringe (Hamilton) and repeating dispenser (Hamilton) by the well known brush technique. This entire arrangement is quite similar to the common description of the BLM set-up in the literature (65).

Electrodes, inserted on either side of the Teflon cup, were of the fiber junction reference type (Beckman 39270) and were always left soaking in 0.1 M electrolyte when not in use. The two electrode connections led directly to the instrument designed by Mr. Martin Rabb of the department's electronics staff for the measurement of membrane resistance.

The instrument consists of two parts: the amplifier, and the power supply, meter and controls. The amplifier module was used inside of the cage in close proximity to the BLM and housed the circuit shown in Figure 6. Basically, this circuit consists of an inverting amplifier which produces an output voltage which is the product of the input voltage and the ratio of the feedback resistor and the input resistor. The left third of the circuit serves as a voltage divider to provide an accurate stable reference input voltage. The 200Ω variable resistor allows for setting a required 7.5 ma current through the reference zener diode at the measure link. The 1 KΩ variable resistor in the divider chain allows accurate setting of a 1.00 V input at point A. The divider and array of input resistors cause the output of the first operational amplifier to be -1.00 V for decade electrode resistances from 10^4 to $10^9\Omega$. The operational amplifier also connects to a 1 MΩ resistor to help reduce bias current and a trim pot to help

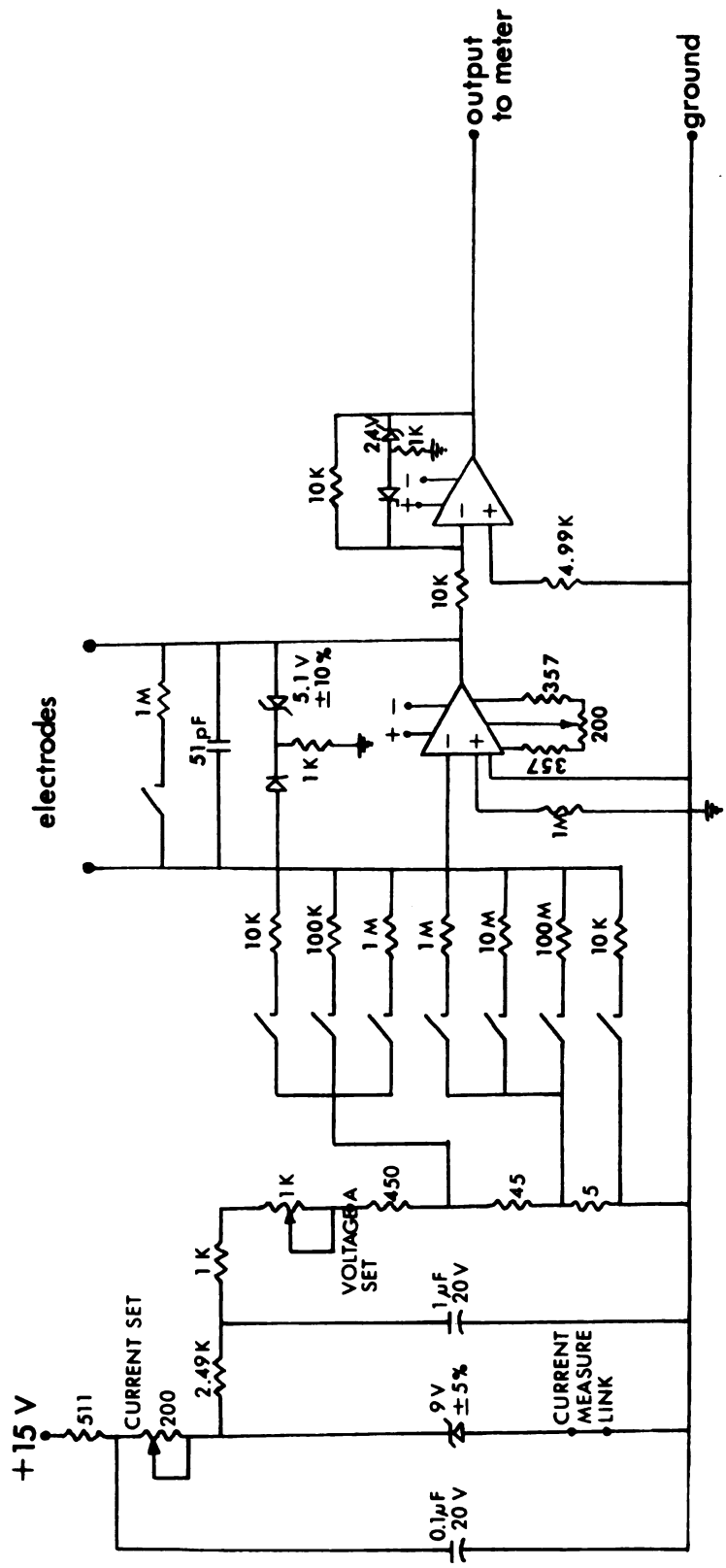


Figure 6. The Amplifier Module of the BIM Resistance Meter

compensate for offset voltage. The 5.1 V zener diode, 1 KΩ resistor and switching diode limit the output voltage of the operational amplifier to 5.1 V if the electrode terminals are open circuited. The 51 pF capacitor reduces the amplifier bandwidth for improved stability.

The second operational amplifier acts as an inverter with a gain of 1. Voltage is limited to 2.4 V to protect the 1.00 V full scale meter connected to the output. The bottom 10 KΩ input resistor and the 1 MΩ resistor of the main operational amplifier feedback loop are switched into the circuit to allow checking and setting of the amplifier to zero offset.

Cables led out of the cage to the power supply module which also contained the meter display for direct reading of membrane resistance along with the switches for changing input resistors. The instrument could measure resistances to a $\pm$ 5% tolerance in the range of 10^4 to 10^9 ohms. This was a far greater precision than necessary considering the experimentally observed reproducibility of BLM resistances. Further details on this device are found in reference 100.

CHAPTER III

POTENTIOMETRIC STUDIES OF MONENSIN

INTRODUCTION

Potentiometric measurements on monensin solutions were initiated primarily to study quantitatively the three equilibria shown below:

$$(1)\quad MonH \rightleftarrows Mon^- + H^+; \qquad K_a = \frac{[H^+][Mon^-]}{[MonH]}$$

$$(2)\quad Mon^- + M^+ \rightleftarrows MonM; \qquad K_f = \frac{[MonM]}{[Mon^-][M^+]}$$

$$(3)\quad MonH + M^+ \rightleftarrows MonM + H^+; \qquad K = \frac{[H^+][MonM]}{[MonH][M^+]}$$

where (1) is the acid dissociation of monensin, (2) the formation of the monensin metal ion complex and, (3) the overall metathesis reaction of the acid molecule complexing a metal ion and releasing a proton. In this simplified treatment, dehydration of the monensin molecule or the participation of the metal salt anion in the above equilibria are neglected.

Previous studies gave MonH acidity constant values only in mixed solvent systems (2,33). Estimates of K_f had been made by studying equilibrium (2) alone by using excessively basic solutions which ensured complete acid deprotonation (27,30,31). Equilibrium (3) has never been studied and indeed monensin had been assumed not to be a complexing agent in neutral solutions. It should be noted that if the above equilibrium scheme is correct, the

equilibrium constant for complexation of metal ions by the free acid should be equal to the product $K_a K_f$.

Neither the sodium salt nor the acid form of monensin are appreciably soluble in water, consequently all studies were carried out in nonaqueous solvent systems. Methanol was chosen as the primary solvent for the study because of previous work done with the drug in this solvent and also due to its suitability for potentiometric measurements.

DETERMINATION OF ACID DISSOCIATION CONSTANT

Before attempting a titration of the MonH molecule with a strong base, a standardization titration was designed using benzoic acid (hereafter, HBz) which has a K_a value somewhere near that anticipated for MonH. The titration curve of 1.00 mmole of HBz in 50.0 ml of solution with 0.0400 M sodium methoxide in methanol is shown in Figure 7. A smooth sigmoidal curve with a distinct equivalence point was observed. A similar titration of 1.00 mmole of MonH with 0.0400 M sodium methoxide gave the curve shown in Figure 8. It can be seen that the results indicate that MonH is a much stronger acid than HBz. This would seem to be incorrect and indeed the MonH result is fortuitious. The effect of the sodium ion in solution has been to introduce another reaction causing complexation of sodium ion by Mon^- and a consequent increase in the acid dissociation constant. For this reason, sodium methoxide or any other base with a complexable cation cannot be used to study only the acid-base reaction.

Figure 9 shows a similar titration of 1.00 mmole of MonH with 0.0400 M tetrabutylammonium hydroxide. In this case, the cation

Figure 7. Titration of 1.00 mmole Benzoic Acid in 50.00 ml of Solution with 0.0400 M Sodium Methoxide in Methanol

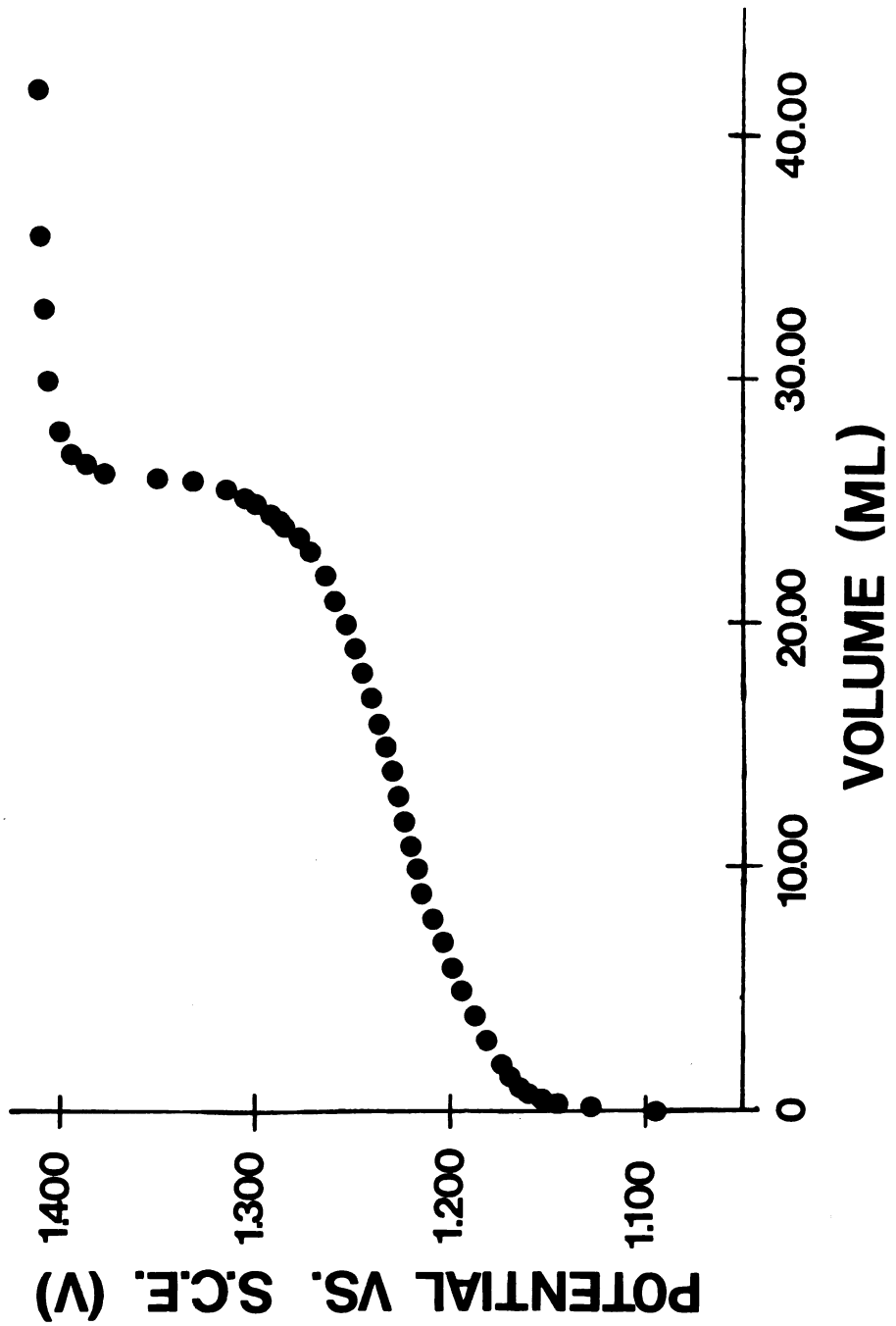

POTENTIAL VS. S.C.E. (V)
1.400
1.300
1.200
1.100
0
10.00
20.00
30.00
40.00
VOLUME (ML)

Figure 8. Titration of 1.00 mmole Monensin in 50.00 ml of Solution with 0.0400 M Sodium Methoxide in Methanol

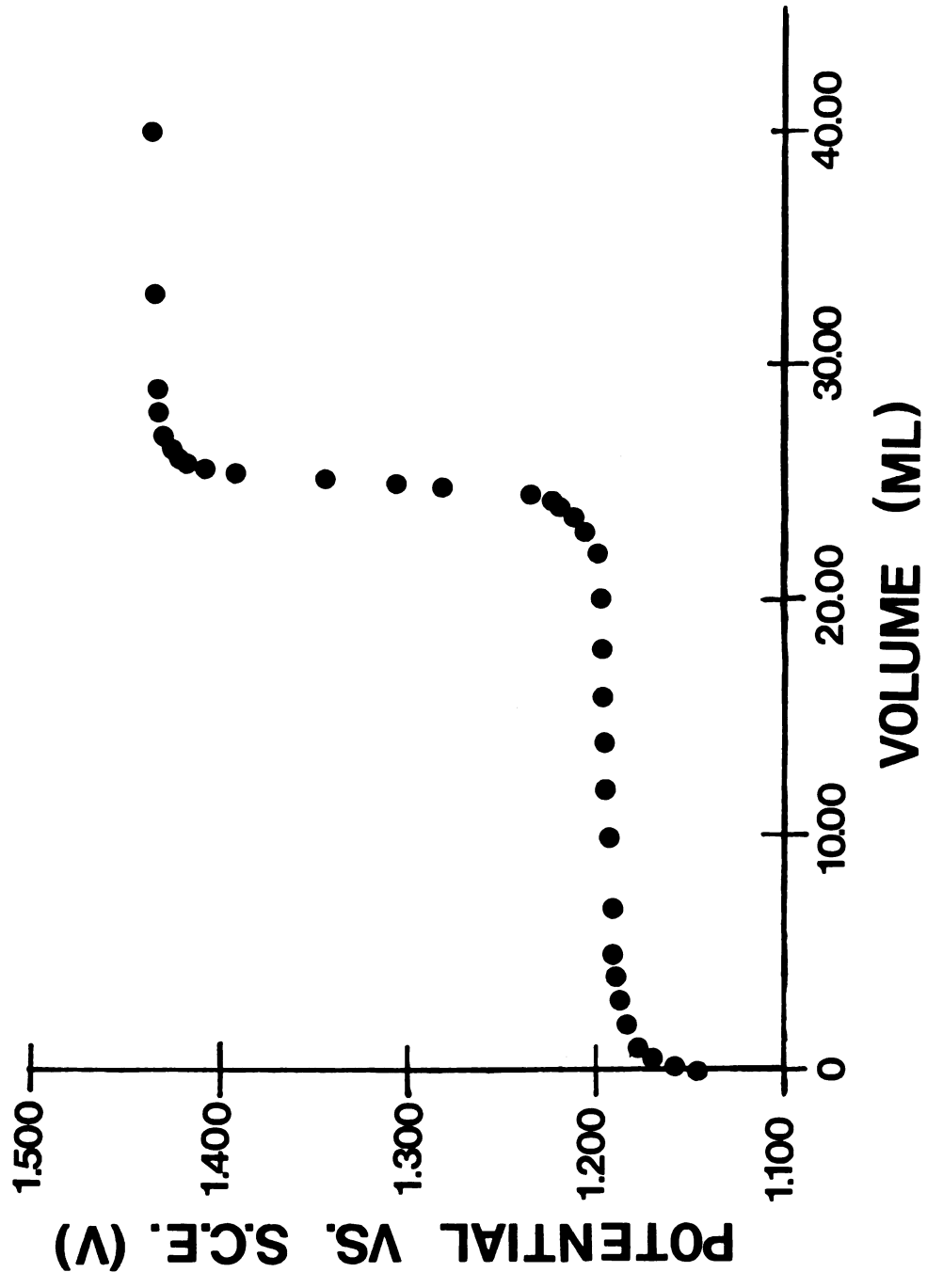
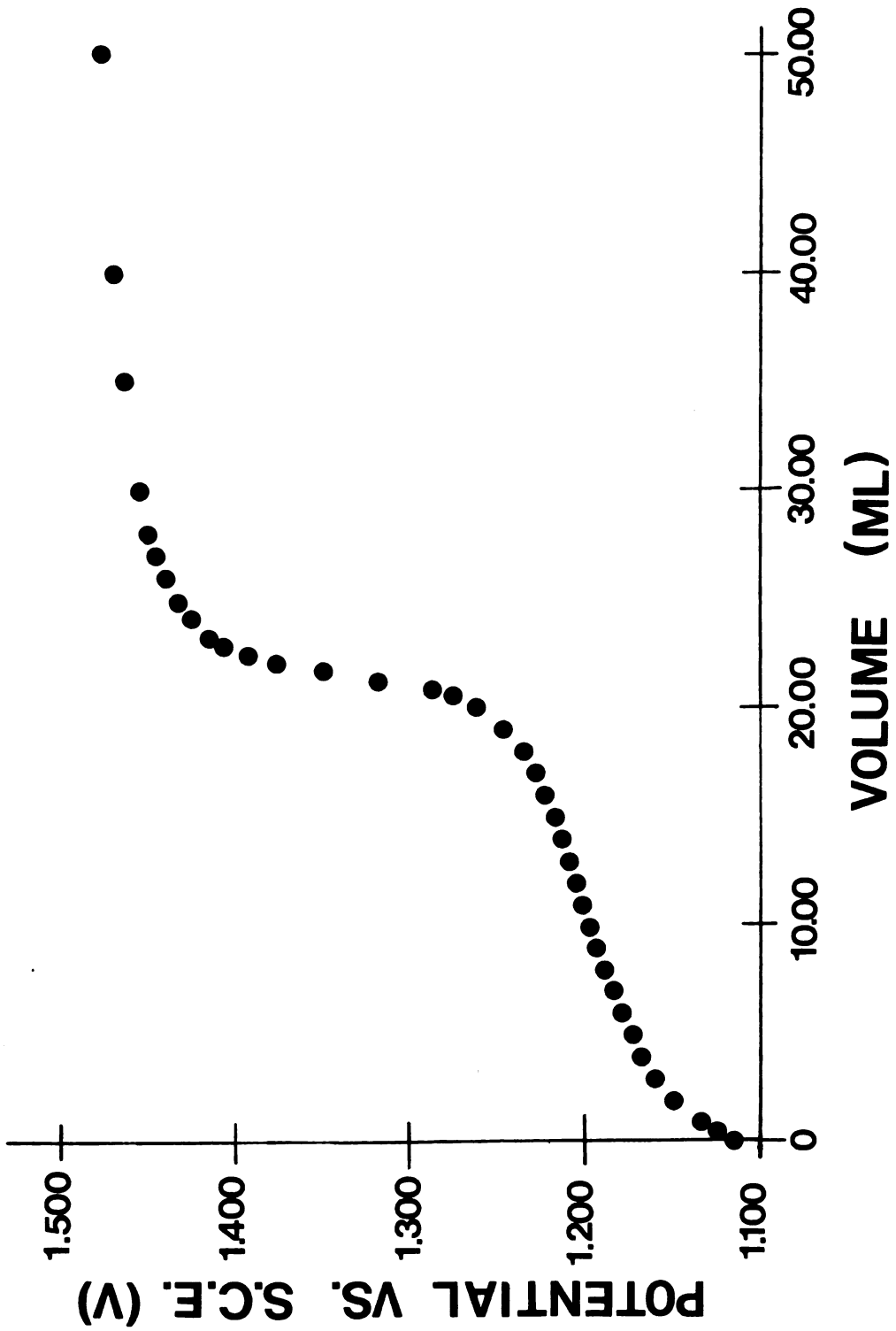

POTENTIAL VS. S.C.E. (V)
1.500
1.400
1.300
1.200
1.100
0
10.00
20.00
30.00
40.00
VOLUME (ML)

Figure 9. Titration of 1.00 mmole Monensin in 50.00 ml of Solution with 0.0400 M Tetrabutylammonium Hydroxide in Methanol


POTENTIAL VS. S.C.E. (V)
1.500
1.400
1.300
1.200
1.100
0
10.00
20.00
30.00
40.00
50.00
VOLUME (ML)

is too large to fit into the monensin cavity. The new results, as expected,indicate that MonH is a weaker acid than HBz. Several such titrations at different concentrations were performed to confirm the reproducibility of the data.

Three different methods were used in attempting to calculate a K_a value for MonH using the data from the above experiment. The first is the most well known and involves the use of the equation given below. Neglecting autoprotolysis and activity corrections, Meites and Thomas (101) show that the value of the acidity constant can be obtained from the titration curve with the expression

$$(4) \quad K_a = [H^+] \frac{fC_a \frac{V_a}{V_a + V_b} + [H^+]}{(1-f)C_a \frac{V_a}{V_a + V_b} - [H^+]}$$

where C_a is the analytical acid concentration, V_a is the initial volume of acidic solution, V_b is the volume of base titrated into solution, C_b is the base concentration and $f = V_b C_b / V_a C_a$, the fraction of the equivalent volume of base added. Potentials observed far past the equivalence point were assumed to arise only from excess base in solution thus allowing the establishment of a voltage to pH relationship. The electrodes were assumed to have a Nernstian response and calculations gave pH values in the titration buffer region. However, use of these values in equation (4) gave values of the pK_a for HBz in the area of 8.9. These were in poor agreement with the literature value of 9.27 (102).

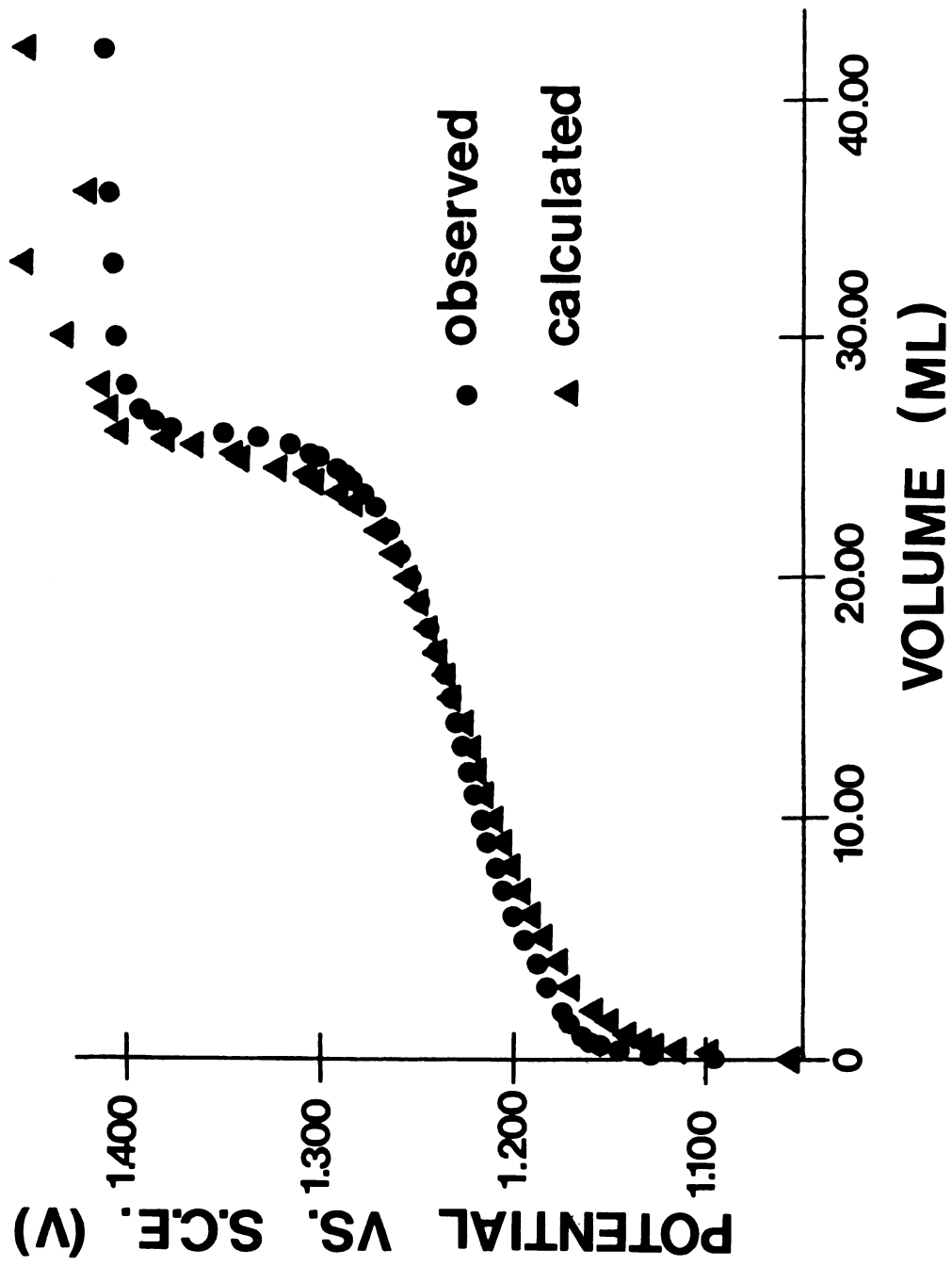
A second approach involved the use of all of the titration data rather than just those in the buffer region. The exact equation for the titration of a weak acid with a strong base with uncomplexable cation is the cubic given below.

$$(5) \quad [H^+]^3 + (C_b + K_a)[H^+]^2 + (K_a C_b - K_a C_a - K_s)[H^+] - K_a K_s = 0$$

The symbols are defined as before with K_s the autoprotolysis constant. By putting the analytical solution for a cubic equation into the KINFIT program subroutine (detailed in Appendix I), it was possible to fit the observed data to the above equation and obtain a K_a value. In this analysis, a Nernstian electrode response was again assumed, but the intercept of a Nernstian pH versus voltage plot was programmed as an adjustable parameter to give the best fit to the data. The technique proved to be extremely sensitive to the initial estimate of the adjustable K_a parameter, often causing convergence premature to reasonable data fits. After many trials, the best fit obtained using this approach is shown for HBz in Figure 10 and indicates a pK_a of about 7.4 which is unacceptably low. This method was therefore discarded.

Fortunately, a third method was successful in reproducing the literature pK_a for HBz. After investigating two other recent applications of computer analysis of multiparametric titration curve fitting (103,104), a useful method developed by Briggs and Steuhr (98) was found. These authors derived a linear equation based on exact mole balance relationships which simultaneously determines pK_a and equivalence point values and also takes into consideration the presence of any excess of strong acid or base in the titrated

Figure 10. Computer Fit of Figure 7 Data Using KINFIT and Equation (5)


POTENTIAL VS. S.C.E. (V)
1.400
1.300
1.200
1.100
0
10.00
20.00
30.00
40.00
VOLUME (ML)
observed
calculated

sample. They present equation (6)

$$(6) \quad V + R - \Delta V = -a_{H^+}(V + R - \Delta V)\frac{1}{K_a} + V_e$$

where V is the volume of base added, ΔV is the volume of this base neutralized by any strong acid present, $R = (C_{H^+} - C_{OH^-})V_t/C_b$, V_t is the total volume, V_e the equivalence volume and a_{H^+} the hydrogen ion activity. The equation was originally used in a least squares fit program which also allowed for aqueous activity corrections. A copy of this program was generously supplied by J. E. Steuhr and modifications necessary for the HBz or MonH system in methanol were made as detailed in Appendix II.

Using this modified Steuhr equation a pK_a of 9.25 $\pm$ 0.05 for HBz in methanol was calculated. This is in excellent agreement with the literature value and has been corrected for a small amount of basic impurity in the methanol. The same technique applied to MonH gave a pK_a value of 10.15 $\pm$ 0.05. The only previously determined values were 6.65 in 66% dimethylformamide-water (2) and 7.95 in 90% ethanol-water (33) mixtures. These lower values would be expected in solvent mixtures of higher dielectric constant.

CONCLUSIONS

The results indicate that MonH is a relatively weak acid in methanol and in neutral solution it is mainly in the associated form. Consequently the Mon^- anion is a relatively strong base in methanol solutions.

DETERMINATION OF THE OVERALL COMPLEXATION CONSTANT

It was thought that perhaps equilibrium (3) could be monitored simply by adding some alkali metal cation to a solution of MonH and measuring the concentration of released hydrogen ions. Various initial concentrations of MonH were titrated with various concentrations of the salt sodium perchlorate in methanol solution. Several of the titration curves obtained are shown in Figure 11. As can be observed, a gradual release of protons is indicated. Calculations reveal that the concentration of released hydrogen ions is nearly what would be expected for a 1:1 replacement of a proton of monensin with a sodium ion. It was desired to treat these data to give an estimate of the equilibrium constant for the case of the sodium ion in equilibrium (3). Since the K_a for MonH is very small, it was assumed that the concentration of Mon^- ion is negligible. With this assumption and neglecting activities and solvent autoprotolysis, we get

$$(7) \quad K = \frac{[MonNa][H^+]}{[MonH][Na^+]} = \frac{[H^+]^2}{(C_a - [H^+])(C_{Na^+} - [H^+])}$$

where C_{Na^+} is the analytical metal ion concentration. The quadratic solution to the hydrogen ion concentration was inserted in the KINFIT program and the Nernstian pH versus voltage intercept was again adjusted as well as was the value of K. Details of this program are shown in Appendix III. The values of K obtained for various sodium perchlorate titrations are shown in Table 1. Intercept values were found to vary slightly depending on the time

Figure 11. Titrations of Various Amounts of Monensin with Varying Concentrations of Sodium Perchlorate in Several Initial Volumes of Methanol

○ , 0.0975 mmoles, 0.00502 M, 50.00 ml

■ , 0.195 mmoles, 0.0100 M, 70.00 ml

● , 0.488 mmoles, 0.0400 M, 50.00 ml

□ , 0.975 mmoles, 0.0400 M, 50.00 ml

Solid lines are the computer fitted data.

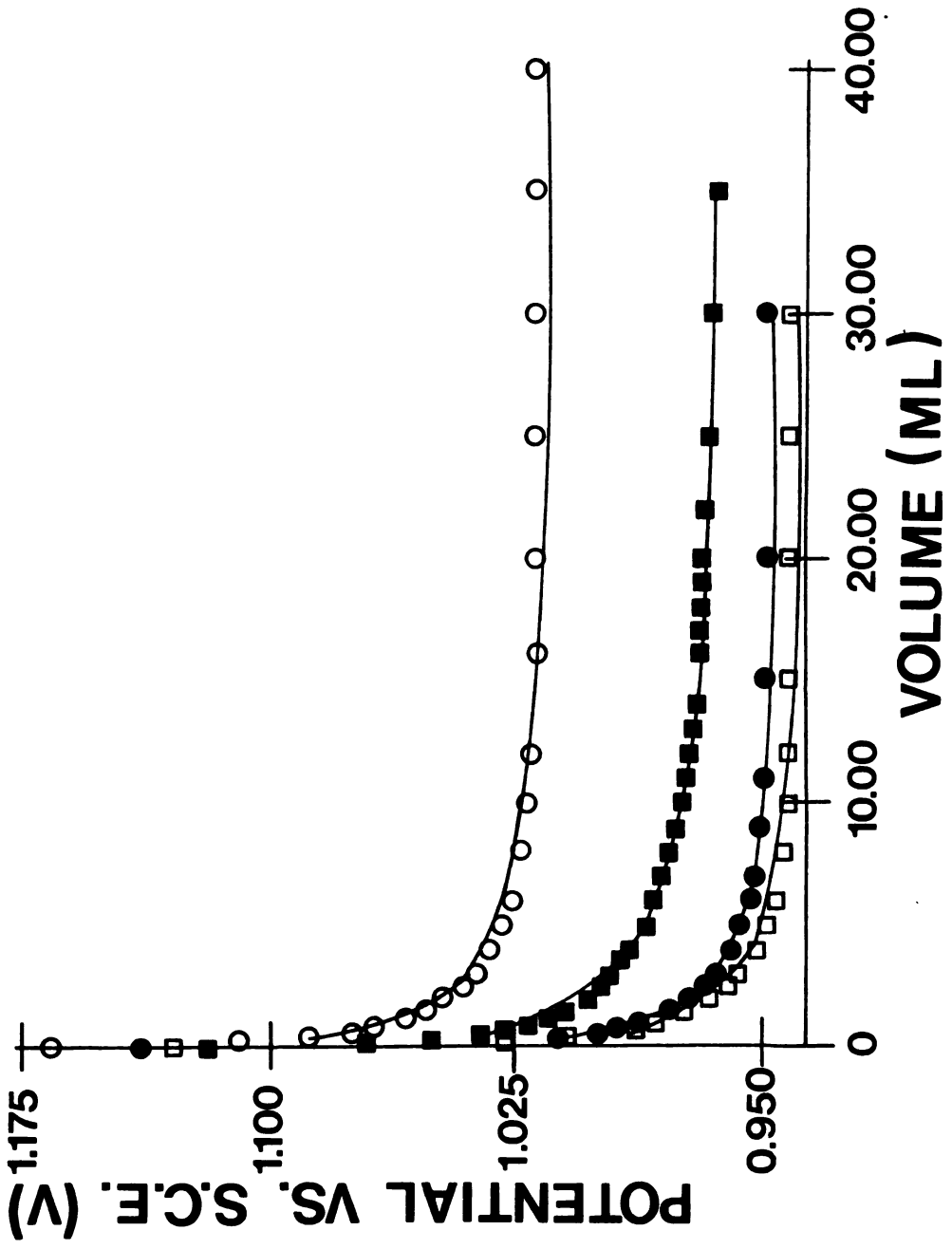

POTENTIAL VS. S.C.E. (V)
1.175
1.100
1.025
0.950
0
10.00
20.00
30.00
40.00
VOLUME (ML)

Table 1. Equilibrium Constant for the Reaction MonH + $Na^+ \rightleftarrows$ MonNa + H^+ in Methanol Solution at 25°C

MonH (mmole)	$NaClO_4$ (M)	K
0.0977	0.00100	0.96 ± 0.29
0.0975	0.00502	0.22 ± 0.07
0.195	0.0100	0.79 ± 0.04
0.488	0.0400	0.53 ± 0.11
0.975	0.0400	0.17 ± 0.07
1.50	0.0411	0.27 ± 0.09
2.00	0.0411	0.50 ± 0.31
5.004	0.1667	0.18 ± 0.14

alotted between various titrations. The solid lines shown in Figure 11 are computer fitted data. As can be seen by examining Table 1, the K for monensin acid complexation of sodium ion is very small compared to that of the anion.

Previous studies of MonH complexation ability had always been done by first adding excess base giving Mon^--sodium ion formation constants of about 10^6 in methanol. Using the previously determined K_a and the calculated K values, it should be possible to estimate K_f from the K/K_a ratio. These calculations, however, give K_f values of about 10^9 which are considerably higher than the reported values. This fact gave the first indication of the possibility that the complex formed in neutral methanol solution is not identical to that obtained under basic conditions.

Shown in Figure 12 are the results of a similar experiment with sodium iodide, sodium chloride, sodium perchlorate and potassium chloride. Also shown are the results of titration of a methanol blank into a MonH solution which, as expected, gives no potential change other than that due to dilution effects. An identical computer analysis of these data gives the K values tabulated in Table 2. As seen, variation of K with sodium counterion is not significantly outside of the experimental error of K for the titrations shown in Table 1. Potassium ion appears to form a complex quite similar in strength to that of sodium.

Further experiments showed that monensin is capable of complexing all of the alkali metal ions. Figure 13 illustrates the results obtained in titrating MonH with lithium, sodium, potassium,

Figure 12. Titration of 0.100 mmole Monensin in 50.00 ml of Solution with 0.00500 M Sodium Iodide, Sodium Chloride, Sodium Perchlorate and Potassium Chloride in Methanol

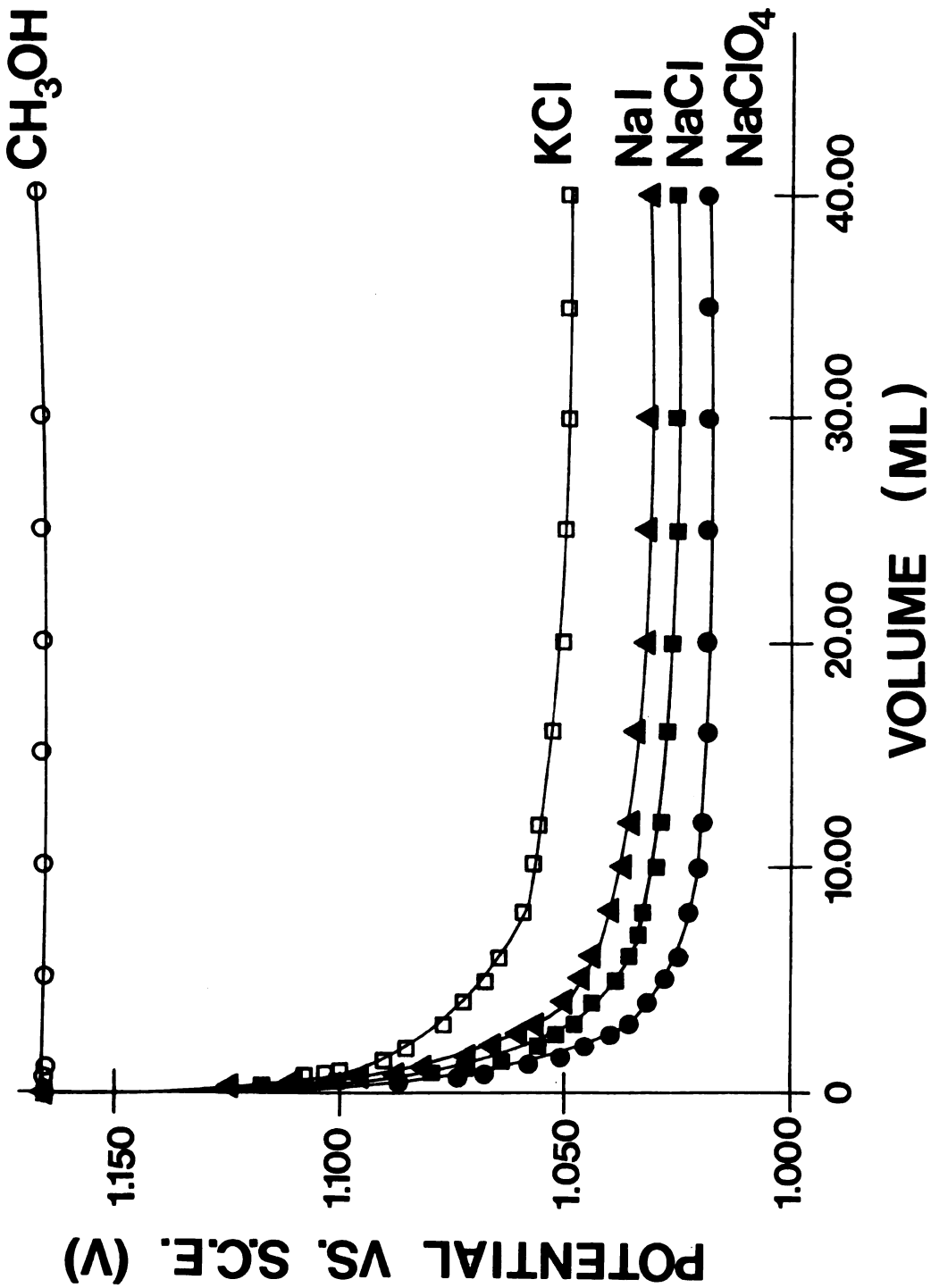
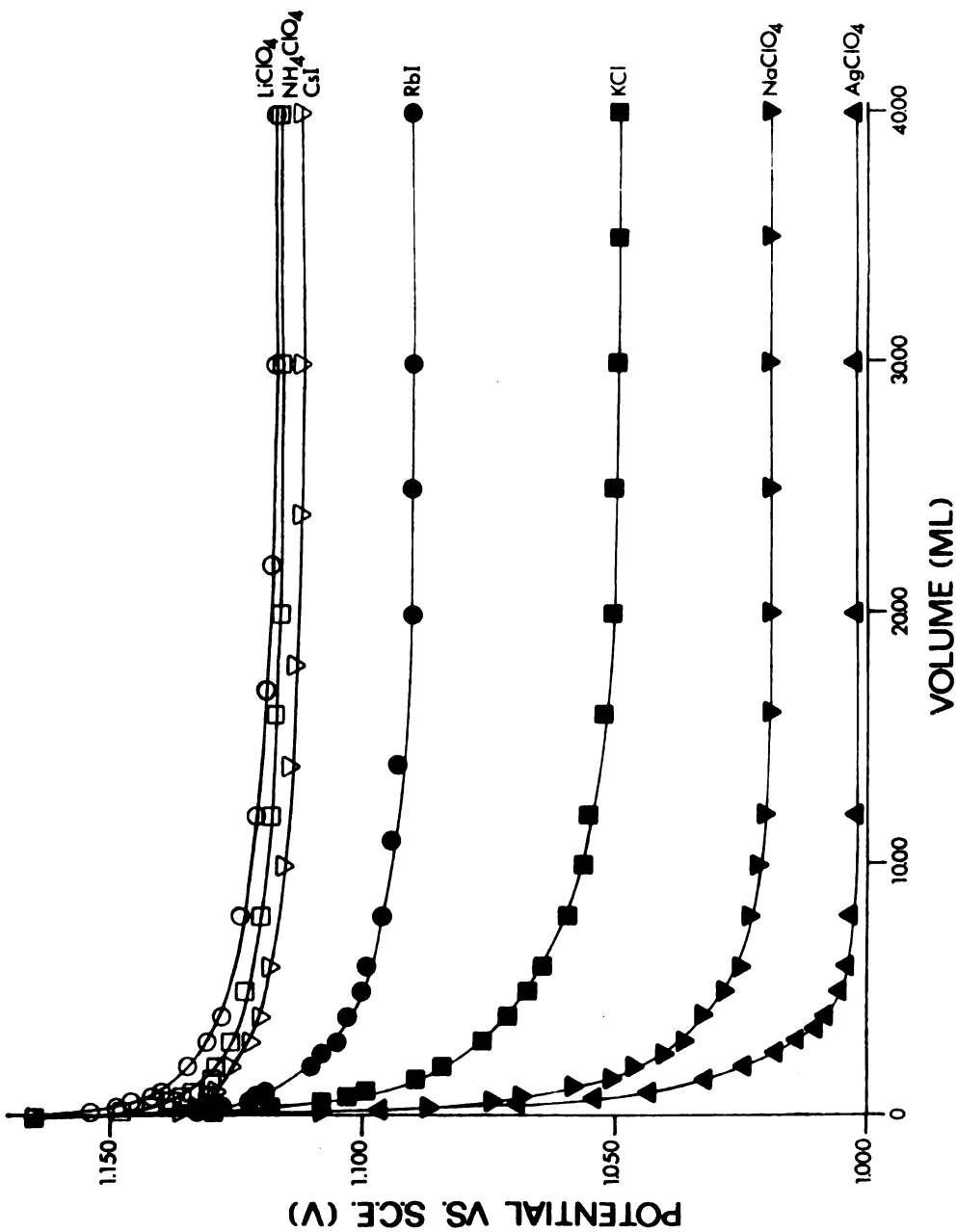

CH3OH
KCl
NaI
NaCl
NaClO4
1.150
1.100
1.050
1.000
0
10.00
20.00
30.00
40.00
VOLUME (ML)
POTENTIAL VS. S.C.E. (V)

Table 2. Equilibrium Constants for the Reaction MonH + $M^+ \rightleftarrows$ MonM + H^+ in Methanol Solution at 25°C

MonH (mmole)	Titrant (M)	K
0.0980	0.00502 NaI	0.71 ± 0.11
0.0980	0.00507 NaCl	0.48 ± 0.09
0.0975	0.00502 $NaClO_4$	0.22 ± 0.07
0.0980	0.00499 KCl	0.42 ± 0.04

Figure 13. Titration of 0.100 mmole Monensin in 50.00 ml of Solution with 0.00500 M Salts of the Alkali Metal Ions and Silver and Ammonium in Methanol


LiClO4
NH4ClO4
CsI
RbI
KCl
NaClO4
AgClO4
1.150
1.100
1.050
1.000
0
1000
2000
3000
4000
POTENTIAL VS. S.C.E. (V)
VOLUME (ML)

rubidium and cesium as well as with ammonium and silver. Anion variation was necessary due to solubility requirements. It is seen that a definite selectivity scheme has emerged. Unfortunately, the use of equation (7) and the KINFIT program does not give the equilibrium constant for lithium, rubidium, cesium, ammonium or silver ions. Although relatively small values of total residuals are calculated,convergence of the program at a best value of K and the intercept does not occur. However, if a more exact equation is used,such as

$$(8)\quad (K - 1)[H^+]^3 + (K_aK - K_a - KC_a - KC_{M^+})[H^+]^2$$

$$+ (K_aC_a - 2KK_aC_a + KC_aC_{M^+})[H^+] + K_aKC_a^2 = 0$$

which adds the consideration of the Mon^- concentration and therefore uses the constant K_a, estimates of K are obtained. Moreover, it is found that using this cubic equation as is also shown in Appendix III, all of the sodium ion data can be reinterpreted and give values of the equilibrium constant identical to those calculated previously with equation (7). In Table 3 are the values of K for the alkali metal ions and silver and ammonium. The values indicate a selectivity sequence of $Na^+ \approx K^+ > Ag^+ > Rb^+ > NH_4^+ > Li^+ \sim Cs^+$ which is in some agreement with results obtained in basic solutions and correlates extremely well with ionic size parameters with lithium ion too small and cesium or ammonium ions too large for coordination in the monensin cavity.

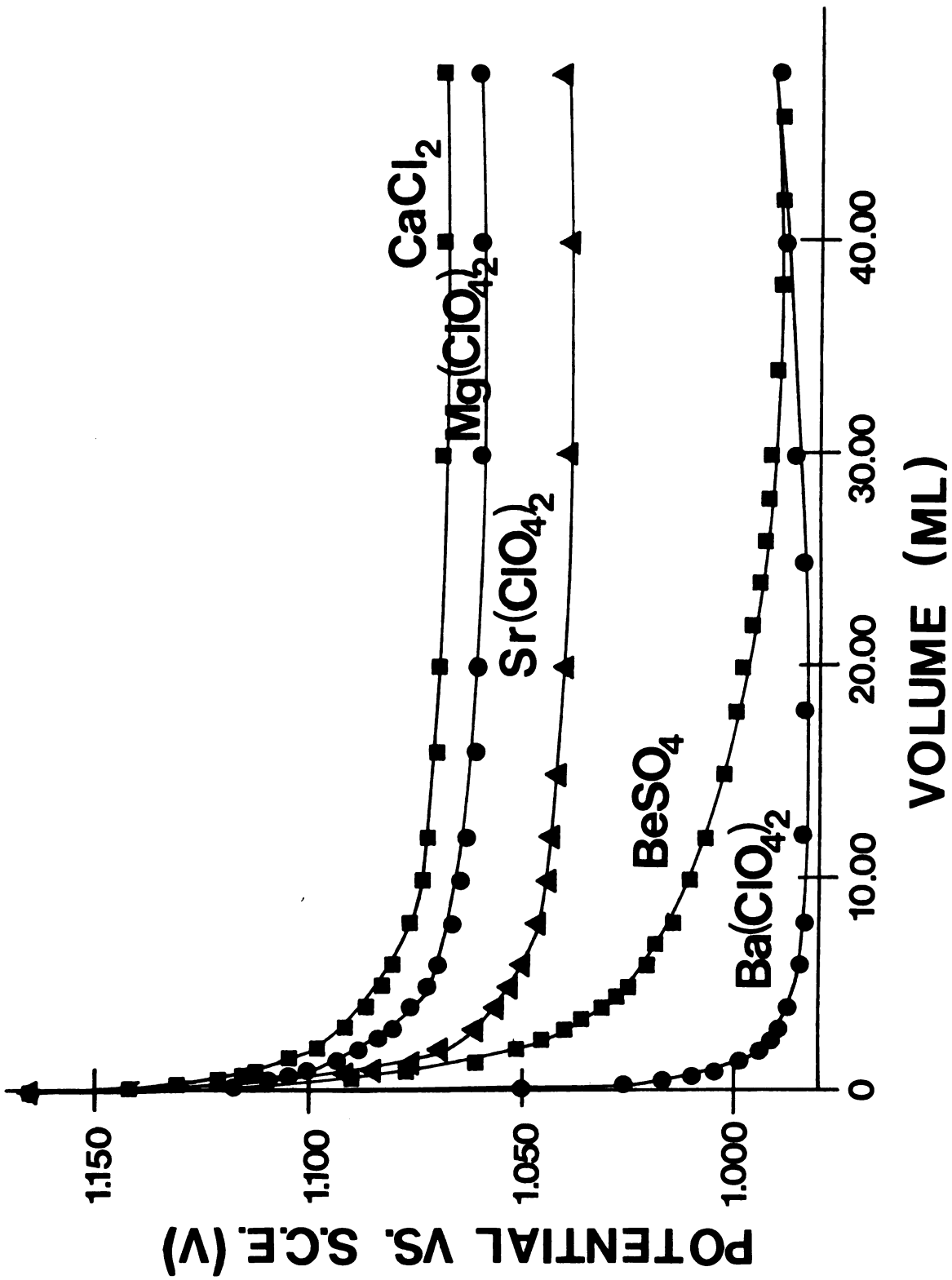
Another extension of the original experiment is obtained by titrating MonH with salts of alkaline earth ions. These titrations are shown in Figure 14 and are the first indication ever obtained

Table 3. Equilibrium Constants for the Reaction MonH + $M^+ \rightleftarrows$ MonM + H^+ in Methanol Solution at 25°C

Titration of 0.100 mmole MonH with 0.00500 M Salts

Salt	K
$LiClO_4$	$(9.3 \pm 1.2) \times 10^{-7}$
$NaClO_4$	$(2.2 \pm 0.7) \times 10^{-1}$
KCl	$(4.1 \pm 0.4) \times 10^{-1}$
RbI	$(3.3 \pm 0.8) \times 10^{-6}$
CsI	$(3.0 \pm 0.3) \times 10^{-7}$
NH_4ClO_4	$(1.5 \pm 0.5) \times 10^{-6}$
$AgClO_4$	$(6.6 \pm 8.4) \times 10^{-2}$

Figure 14. Titration of 0.100 mmole Monensin in 50.00 ml of Solution with 0.00500 M Salts of the Alkaline Earth Ions in Methanol


POTENTIAL VS. S.C.E. (V)
1.150
1.100
1.050
1.000
0
10.00
20.00
30.00
40.00
VOLUME (ML)
$CaCl_2$
$Mg(ClO_4)_2$
$Sr(ClO_4)_2$
$BeSO_4$
$Ba(ClO_4)_2$

of MonH complexation of ions of the second group. Interpretation of these data has not as yet been successful since divalent cations will not fit into the previous equilibrium scheme. Future work on the complexation mechanism involved is expected to yield alkaline earth K values.

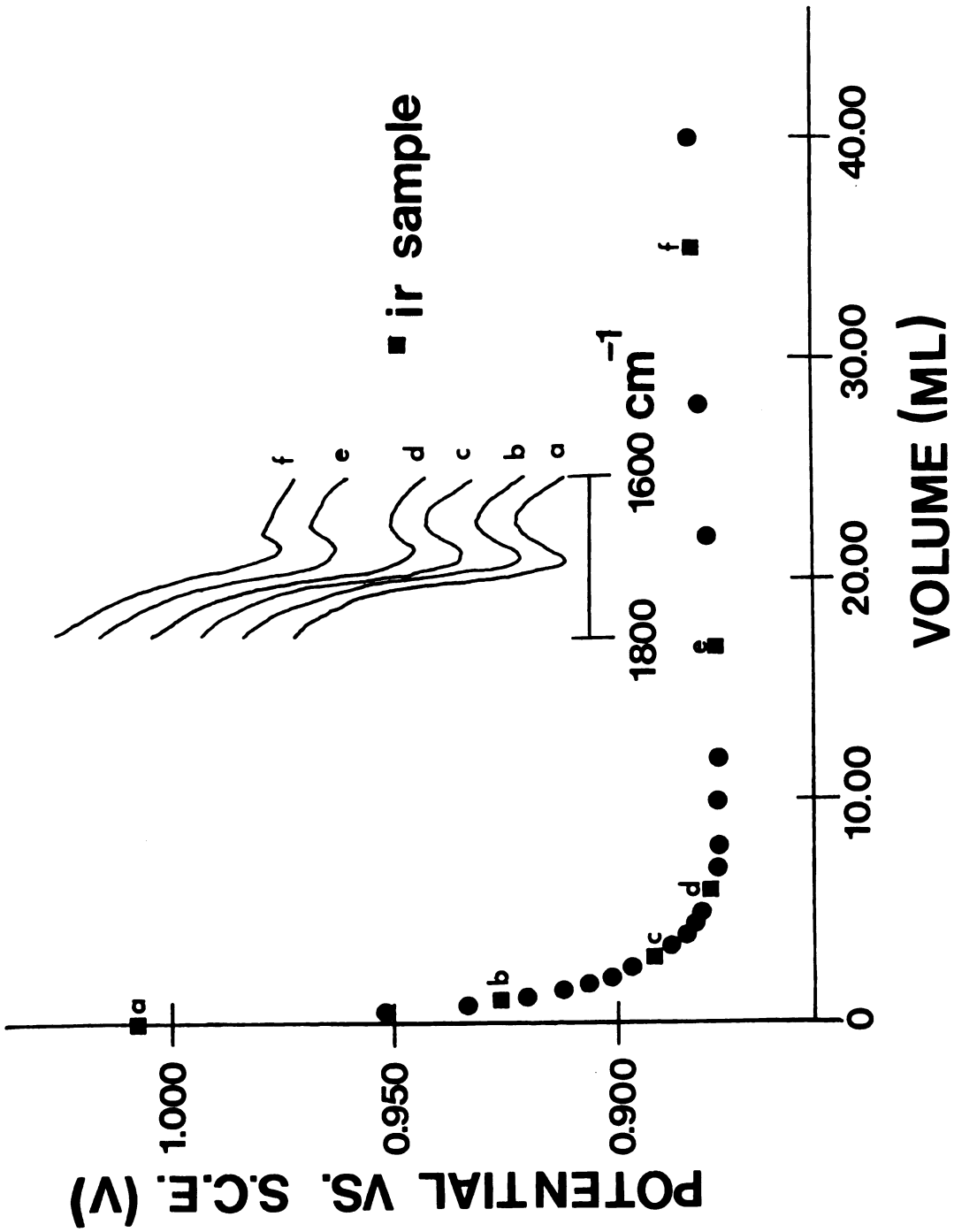
CONCLUSIONS

The monensin acid molecule has been found to form only a weak complex with sodium ion in methanol with an equilibrium constant slightly less than 1. Values of K_f calculated from K/K_a ratios do not agree with previously published values. Although the complex is only a weak one, the acid in methanol solution still exhibits a selective complexation scheme both for alkali metal and alkaline earth cations as well as silver and ammonium. Calculations of K values for alkali metal ion complexes show a good correlation with ionic size.

INFRARED STUDY OF COMPLEXOMETRIC EQUILIBRIA

Since the titrations described above had indicated varying degrees of proton release, it was desired to monitor this deprotonation in the infrared spectrum. Monensin has an easily observable carboxylic acid band at 1704 cm^{-1} in solution while the normal sodium salt has a carboxylate band at 1563 cm^{-1}. It was found that in chloroform solutions, the 1704 cm^{-1} band could easily be shifted to the salt position by an addition of ammonium hydroxide. Conversely, the 1563 cm^{-1} salt band can be caused to move to 1704 cm^{-1} by an addition of hydrochloric acid to the chloroform solutions. Shown in Figure 15 is a titration curve of MonH with sodium perchlorate in methanol and

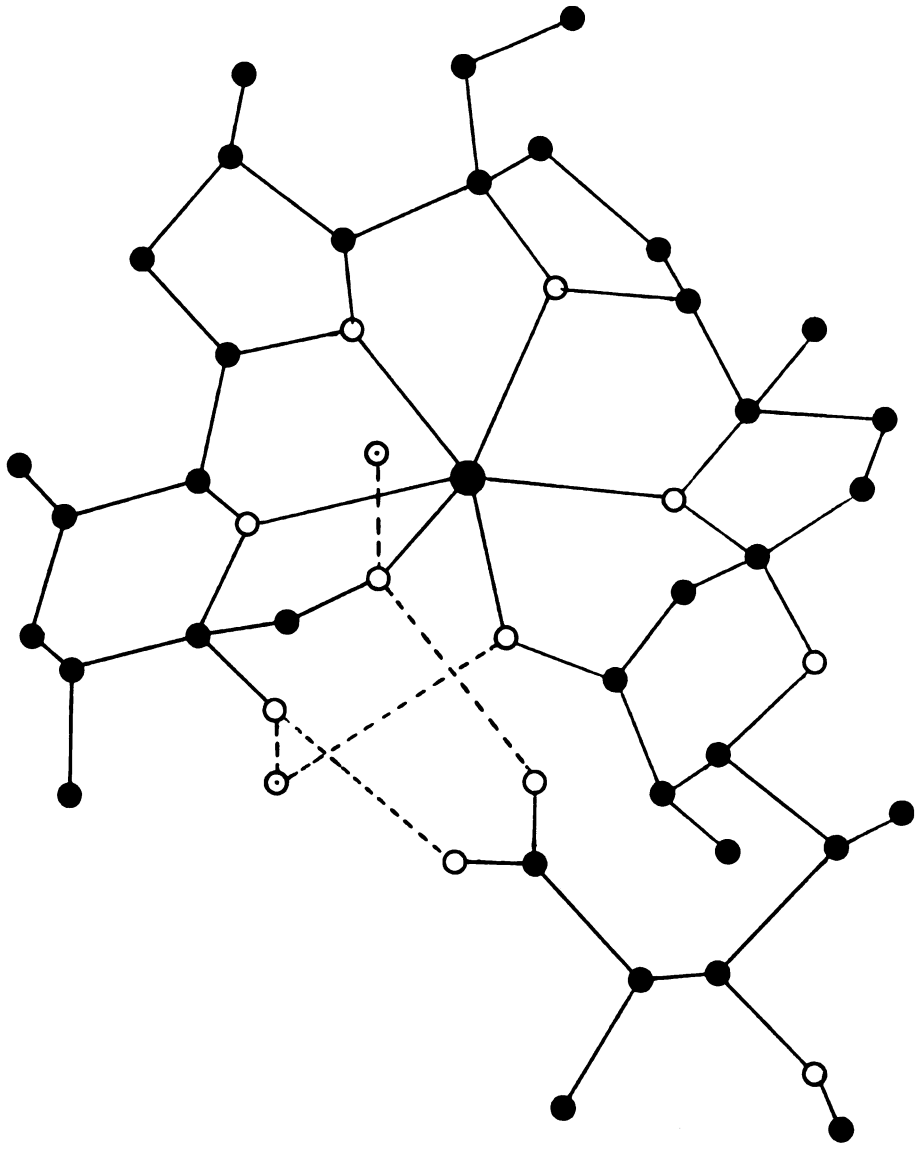
Figure 15. Titration of 5.00 mmoles Monensin in 50.00 ml of Solution with 0.1667 M Sodium Perchlorate in Methanol and Several Infrared Spectra


POTENTIAL VS. S.C.E. (V)
1.000
0.950
0.900
0
10.00
20.00
30.00
40.00
VOLUME (ML)
ir sample
1800
1600 cm⁻¹
a
b
c
d
e
f

parallel infrared spectra taken at various points along the course of the experiment. As is illustrated, no change in the position or intensity of the 1704 cm^{-1} band is noted.

Since this result was somewhat unexpected and much of the region of interest was obscured, further experiments were carried out in a better infrared solvent. It was found that MonH is capable of solubilizing sodium perchlorate in chloroform up to 1:1 mole ratios. The 1704 cm^{-1} MonH band was monitored in chloroform-d_1 solutions with increasing sodium perchlorate concentration. Again, even at 1:1 sodium ion to MonH mole ratios, no change in the intensity or position of the 1704 cm^{-1} band was observed nor did any trace of the 1563 cm^{-1} band appear. It was concluded, therefore, that if the carboxylic proton <u>is</u> lost by the acid molecule, some other proton from the hydrogen-bonded pattern near this area takes its place (see Figure 16). This is to say that in the addition of a sodium ion to monensin in neutral solution, some other proton must be more acidic than the one on the carboxylic group. This more acidic hydrogen ion must belong to one of the several hydroxyl groups. Consequently, the complex may have a lower energy state in which the negatively charged oxygen atom is somehow closer to the positive alkali metal ion than the carboxyl oxygen. The resultant zwitterionic structure may reflect a charge stabilized complex, which would give the molecule an even more hydrophobic exterior. In any case, this behavior is a second indication that the acid-sodium complex formed in neutral solution cannot be of the same structure as the ordinary salt.

Figure 16. The Crystalline Structure of Monensin Sodium Salt Closed circles are carbon atoms, open circles are oxygens with the water oxygens dotted and dashed lines indicate hydrogen bonds.



Other areas of the spectrum monitored during the sodium perchlorate addition in chloroform-d_1 gave further evidence of a complexation mechanism different from the one in basic solution. In the O-H stretching region, MonH showed a broad band at about 3300 cm^{-1} and a sharper band on its shoulder at 3520 cm^{-1}. As the sodium concentration was increased, the sharper band gradually disappeared and the 3300 cm^{-1} band increased slightly in intensity. Again,the 1:1 spectrum bore little resemblance to the MonNa infrared spectrum in chloroform-d_1. These spectral changes must result from the removal of the water molecule from the center of the monensin ring. Also noted in the above experiment was the gradual appearance of two new bands at 2025 cm^{-1} and 2220 cm^{-1} which were also present in the spectrum of the pure MonNa salt. Their growth (Figures 17 and 18) was such that at 1:1 sodium perchlorate to MonH mole ratios, this area of the spectrum was identical to the spectrum of the salt in chloroform-d_1.

CONCLUSIONS

The carboxylic acid proton on monensin, which is easily removed in basic solution, is not the proton released by sodium complexation in neutral methanol or chloroform solutions. Other information from the infrared spectrum indicates that this new type of complex is only somewhat similar in structure to the salt. This fact explains some of the discrepancy between the apparent K_f value obtained in neutral solution and the values previously determined in basic solution.

CHAPTER IV

NUCLEAR MAGNETIC RESONANCE STUDIES OF MONENSIN

Figure 17. Variation of Monensin 2025 cm^{-1} and 2220 cm^{-1} Infrared Bands with Addition of Sodium Perchlorate in Chloroform-d_1

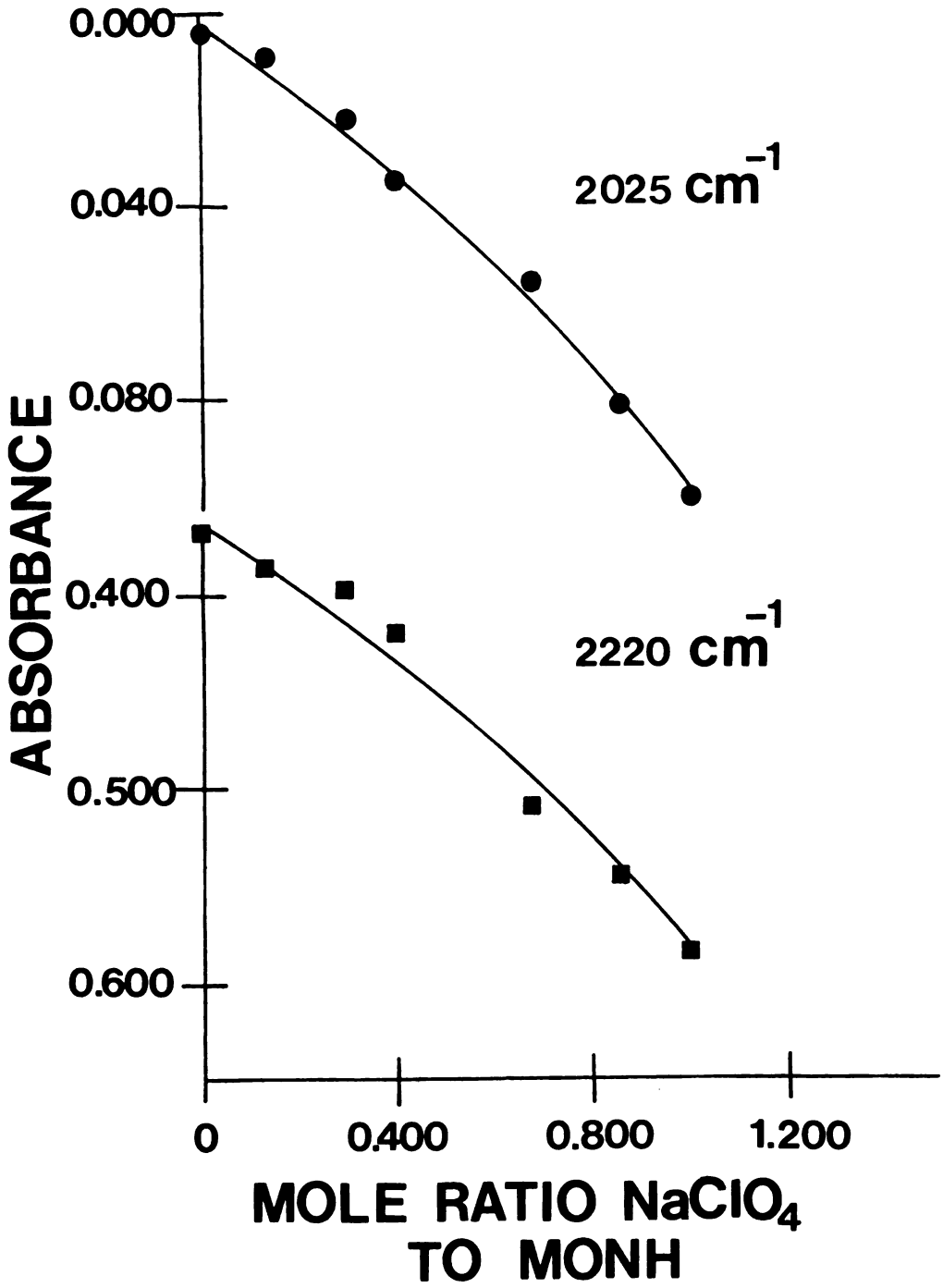
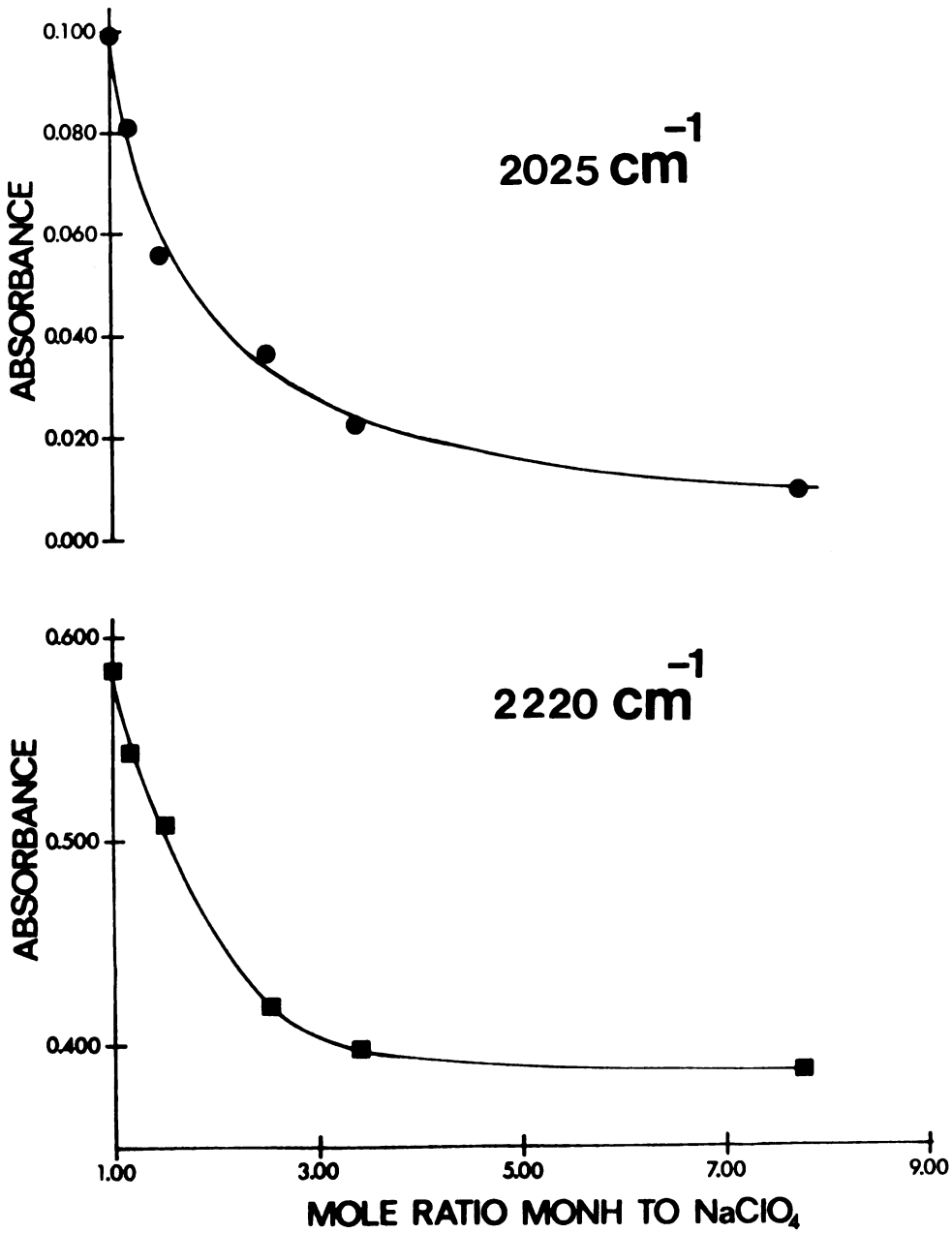

0.000
0.040
0.080
0.400
0.500
0.600
ABSORBANCE
2025 cm-1
2220 cm-1
0
0.400
0.800
1.200
MOLE RATIO NaClO4
TO MONH

Figure 18. Variation of Monensin 2025 cm^{-1} and 2220 cm^{-1} Infrared Bands with Addition of Sodium Perchlorate in Chloroform-d_1


0.100
0.080
0.060
0.040
0.020
0.000
ABSORBANCE
2025 cm-1
0.600
0.500
0.400
ABSORBANCE
2220 cm-1
1.00
3.00
5.00
7.00
9.00
MOLE RATIO MONH TO NaClO4

INTRODUCTION

Nuclear magnetic resonance (NMR) has been proven to be a very powerful tool for the examination of various types of drug interactions. Alkali metal ion NMR has already been mentioned in connection with the investigation of the monensin sodium complex (32) and both ^{7}Li and ^{23}Na NMR have been used for studying tetrazole (86), glutarimide (92), crown (105) and cryptand (93,97) complexation. Proton magnetic resonance (PMR) has been of tremendous use in the study of conformational changes which take place in drug molecules during complexation in solution. The actins have been particularly well studied in this regard by Prestegard and Chan (106,107). The technique has also been used extensively in the study of valinomycin solution chemistry (108-114).

Data obtained in the potentiometric experiments indicate that alkali ion NMR will be useful for MonH complexation studies. The many differences observed between the MonH and MonNa PMR spectra displayed in Figure 5 show that a detailed PMR study will also be of interest.

LITHIUM-7 NUCLEAR MAGNETIC RESONANCE STUDIES

Solutions of lithium perchlorate were made 0.02 M in methanol with concentrations of MonH ranging from 0.02 to 0.20 M. Even with the drug to lithium ion mole ratio of 10 to 1, a shift of only about 2 Hz from the normal resonance of uncomplexed lithium ion at

0.02 M concentration was observed. This value, while experimentally significant, indicates an exceedingly weak interaction unless the chemical shifts of solvated and complexed lithium ion are quite similar.

CONCLUSIONS

This result correlates with the data obtained in the potentiometric titrations and does confirm the evidence which shows that lithium ion may be only weakly complexed by MonH, probably because of ionic size considerations.

SODIUM-23 NUCLEAR MAGNETIC RESONANCE STUDIES

Preliminary measurements were made on a saturated solution of MonNa in methanol in an effort to locate the ^{23}Na signal for MonNa. A very broad peak was obtained (Figure 19) with a width at half-height of about 700 Hz. Because of the linewidth, it was impossible to determine the ^{23}Na chemical shift.

Several experiments were carried out in which increasing concentrations of sodium ion, in the form of sodium perchlorate or sodium tetraphenylborate, were added to MonNa solutions. As high added salt concentrations were reached, a sodium signal of reasonable linewidth allowing position measurements did eventually appear and some shifting due to averaging of the ^{23}Na environment by fast exchange was noted. However, this experiment failed to produce reliable and reproducible data and was abandoned in favor of the experiments described below.

Two studies of MonH-sodium ion interaction using ^{23}Na NMR were conducted in methanol solution. In the first, the concentration

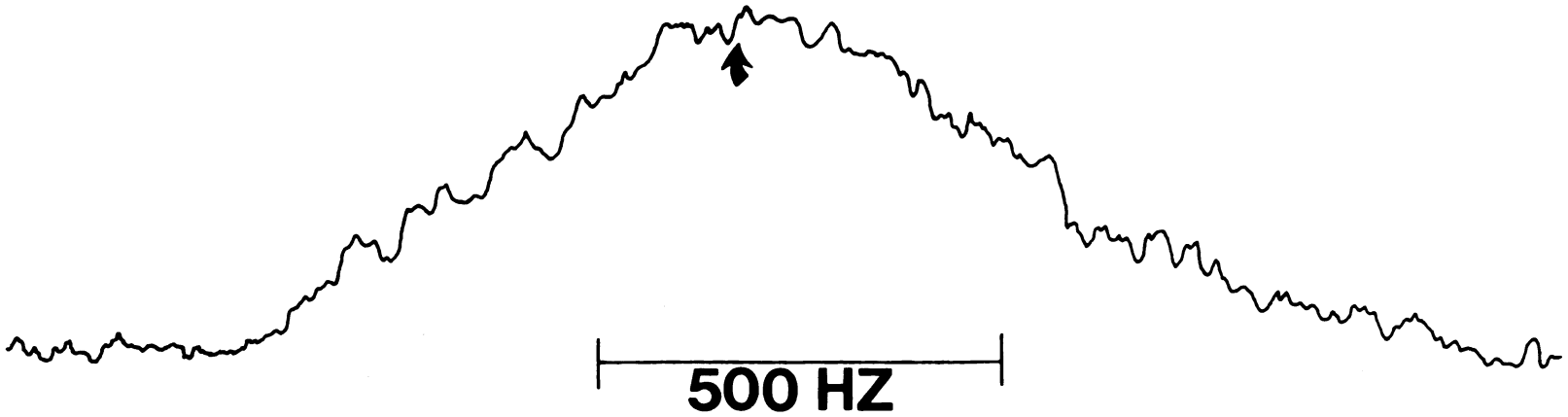


Figure 19. Sodium-23 NMR Spectrum of Monensin Sodium Salt in Saturated Methanol Solution. Arrow indicates position of a 1.0 M aqueous sodium chloride reference run separately.

of MonH was held constant at 0.200 M while the concentration of sodium perchlorate was gradually increased. Shown in Figures 20 and 21 are the results of monitoring the ^{23}Na signal until it was lost due to line broadening. As can be seen, sodium perchlorate alone has a concentration dependent chemical shift, but the behavior is definitely altered by the presence of MonH. A quantitative interpretation of these data was not attempted due to the superimposition of the sodium perchlorate shift, but evidence for the sodium ion-MonH interaction in neutral solution was definitely obtained.

A more direct and interesting approach to the problem involved the use of two constant sodium perchlorate concentrations of 0.500 M and 0.250 M in methanol. In each case, MonH was gradually added and the ^{23}Na signal monitored out to the line broadening limit of about 0.75 to 1. The data, shown in Figures 22 and 23, indicate marked ^{23}Na chemical shifts due only to the drug addition. The observation of potentially larger shifts as would be obtained with ligands such as the cryptands is precluded by line broadening. Due to this inability to go further in drug to sodium mole ratio and the fact that, considering the error involved, data of the Figure 23 yield only straight lines, an interpretation based on calculated chemical shifts as the weighted average of a complexed and free position is not possible. This quantitative treatment of the data would have allowed the calculation of a complexation equilibrium constant as was accomplished for other drugs (85).

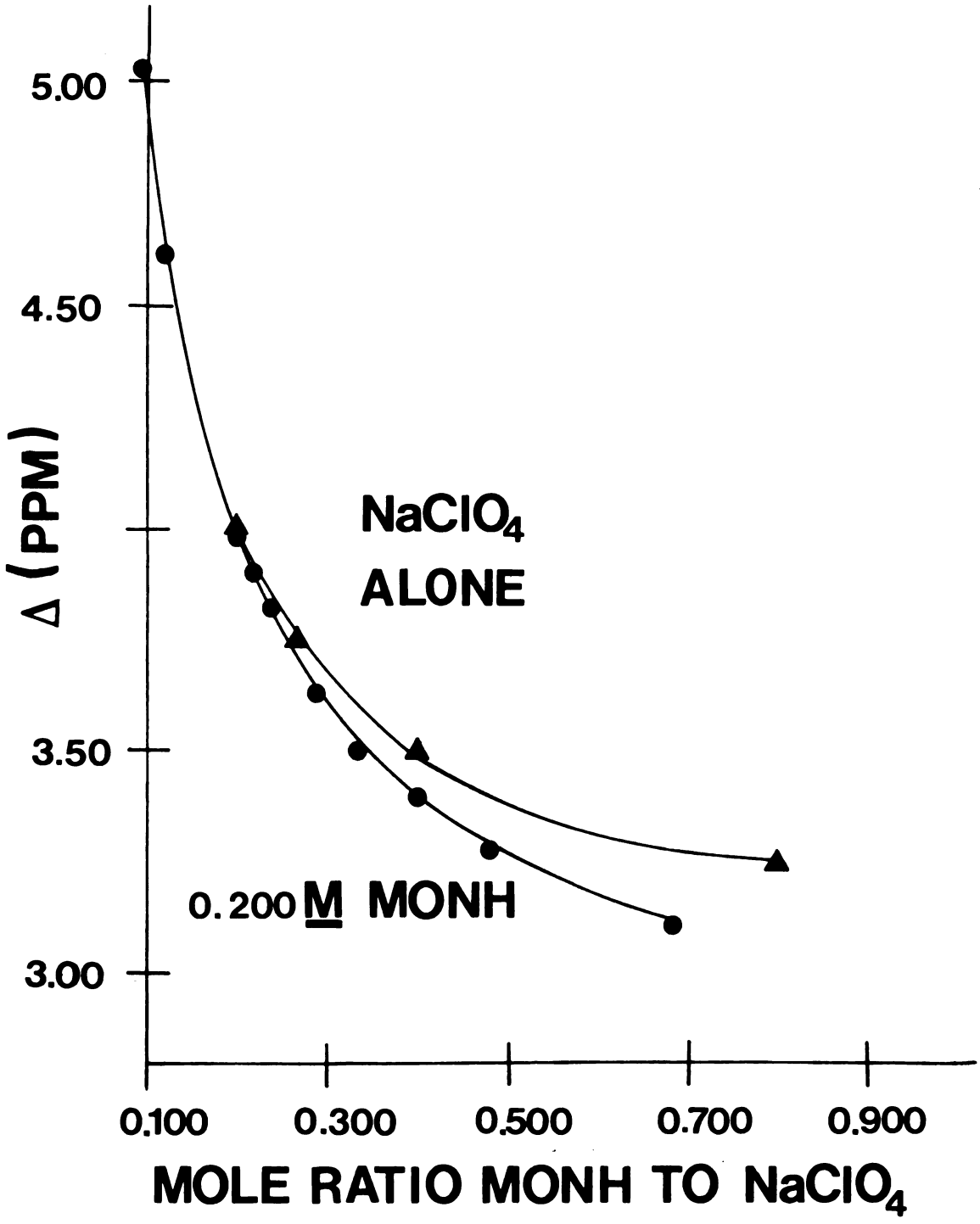


Figure 20. Sodium-23 NMR Study of 0.200 M Monensin with Increasing Sodium Perchlorate Concentration in Methanol

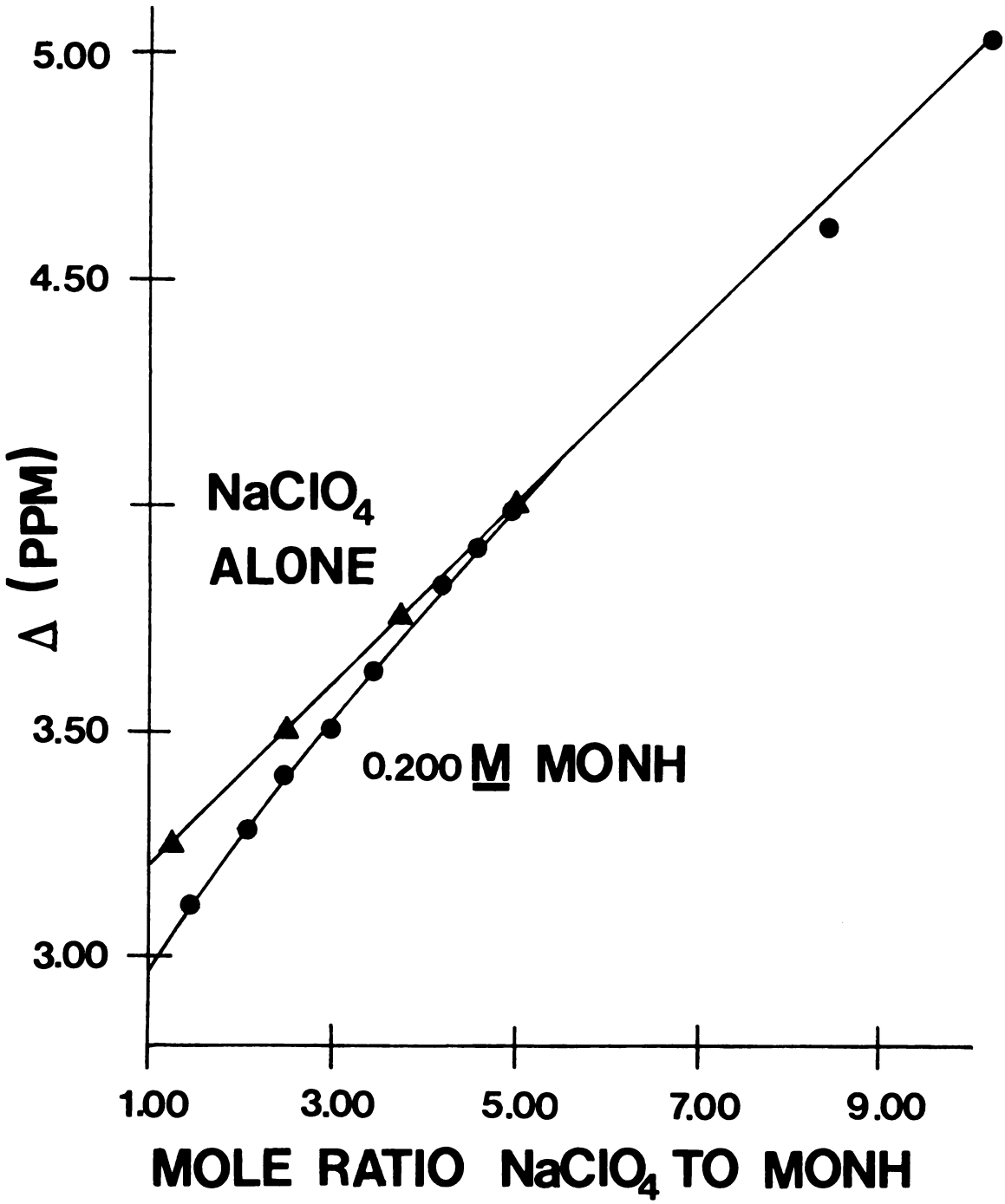


Figure 21. Sodium-23 NMR Study of 0.200 M Monensin with Increasing Sodium Perchlorate Concentration in Methanol

Figure 22. Sodium-23 NMR Study of 0.250 M and 0.500 M Sodium Perchlorate with Increasing Monensin Concentration in Methanol

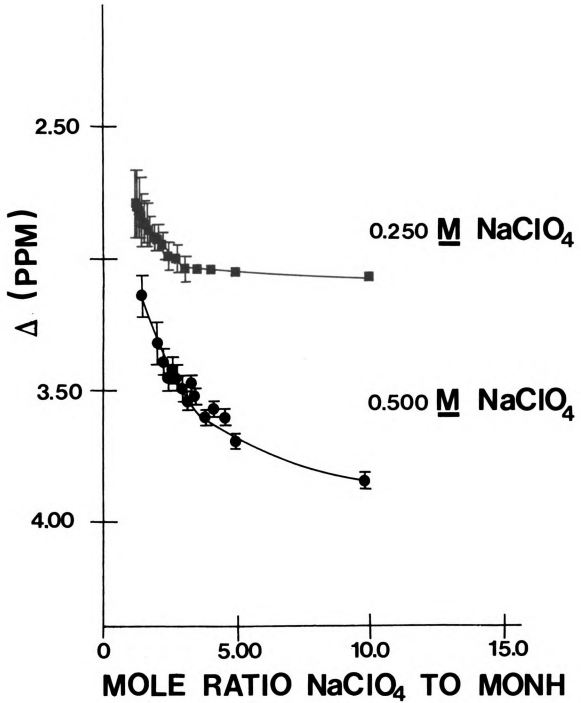
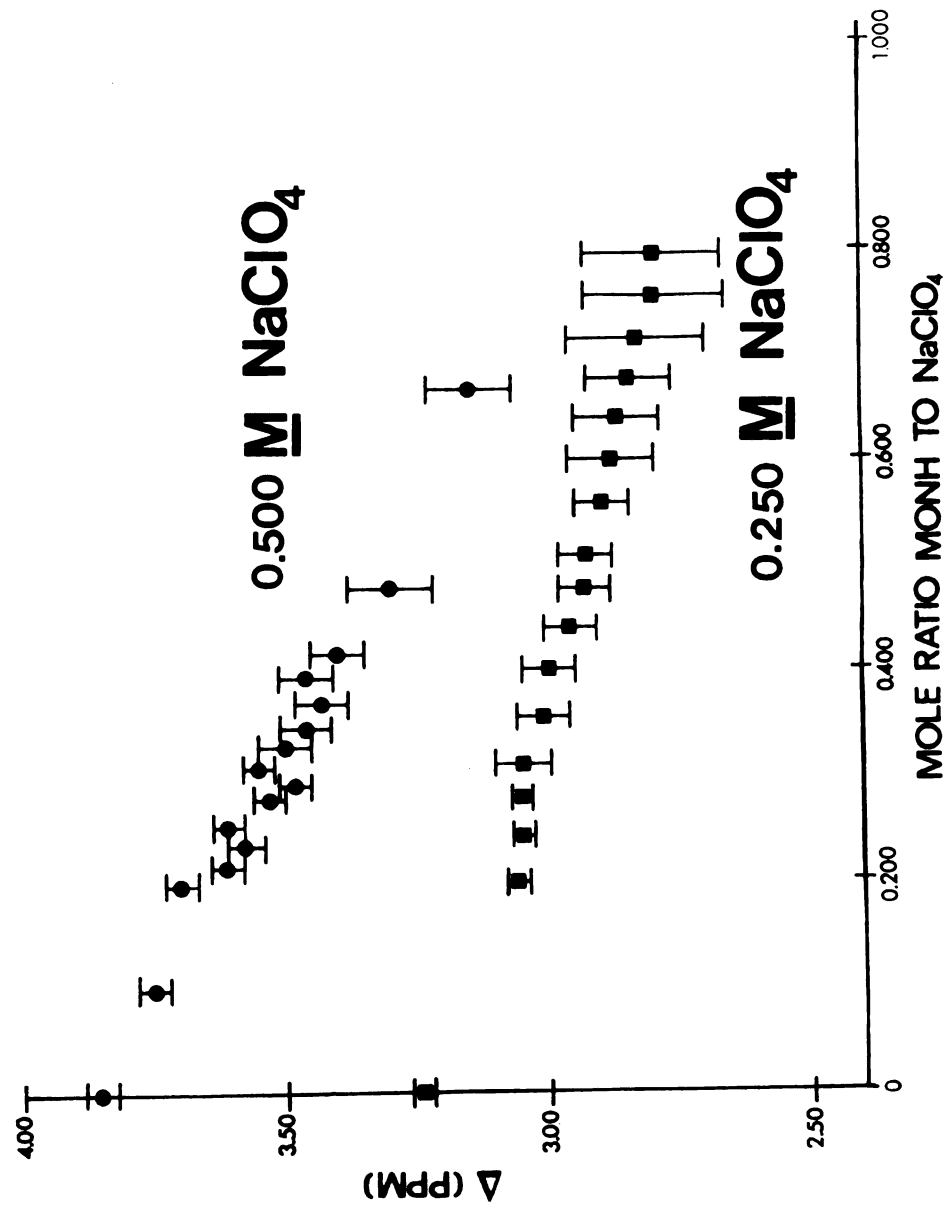

2.50
3.50
4.00
Δ (PPM)
0.250 M NaClO4
0.500 M NaClO4
0
5.00
10.0
15.0
MOLE RATIO NaClO4 TO MONH

Figure 23. Sodium-23 NMR Study of 0.250 M and 0.500 M Sodium Perchlorate with Increasing Monensin Concentration in Methanol


0.500 M NaClO4
0.250 M NaClO4
Δ (PPM)
4.00
3.50
3.00
2.50
0
0.200
0.400
0.600
0.800
1.000
MOLE RATIO MONH TO NaClO4

CONCLUSIONS

Measurements on the ^{23}Na spectrum of MonNa were somewhat inconclusive due to line broadening and other effects. The ^{23}Na NMR study of the acid MonH however, definitely confirms the potentiometric and infrared results indicating a fairly strong MonH-sodium ion interaction in methanol.

THALLIUM-205 NUCLEAR MAGNETIC RESONANCE STUDY

The monensin anion has been found to complex thallium ion (115). Several attempts were made to find the signals of various thallium salts in methanol and other solvents. Neither thallium acetate, nitrate or bromide were sufficiently soluble in methanol solutions to give detectable signals. It was found that MonH will solubilize thallium salts, but still no signal was detected.

CONCLUSIONS

Experiments in which MonH was added to thallium solutions to give ^{205}Tl chemical shifts were not successful due to lack of sufficient thallium solubility in solvents which could be used for the study of MonH equilibria.

PROTON MAGNETIC RESONANCE STUDIES

SPECTRAL ASSIGNMENT Figure 5 shows the PMR spectra of MonNa and MonH. The spectra are somewhat complex since each species has over 60 hydrogen atoms. The complete PMR spectra of MonNa or of MonH have not been intepreted in the literature, although some partial assignments of the resonances of small fragments and derivatives are given in the first reports on the drug (1,5). The MonNa spectrum in chloroform-d_1 is slightly better resolved than

that of the acid and this spectrum was chosen for an assignment attempt. The protons were numbered as shown in Figure 24.

The entire PMR spectrum of MonNa at 0.25 M concentration in chloroform-d_1 was run at the narrowest available sweep widths at both 100 MHz and 60 MHz. Sweep widths were calibrated by the side band technique and all peak positions were located within the limits of resolution and peak shape. Naturally, many peaks remained unresolved. By inspecting peak intensities, possible spin-spin coupling constant (J) values, chemical shifts and shapes, decisions were made as to whether a singlet, doublet, triplet, quartet and so forth were possible. The values of these peak positions were then recorded, an average J value calculated, and the center of the pattern either located or calculated. This center frequency was then used to predict the center frequency in the spectrum taken at the other frequency and J values used to predict the peak positions. These peak positions were compared with the actual peaks observed in the spectrum and a visual inspection of peak intensities and shapes also made. This procedure showed when an erroneous choice was made. A good fit however, did not guarantee that a true multiplet had been located since the errors in peak positions and overlap of patterns often allowed for several possibilities. An illustration of this type of analysis is shown in Table 4 for a singlet, doublet, triplet and quartet which are found in the MonNa 100 Hz spectrum. Final assignment of a particular peak or multiplet to a particular group or set of protons then proceded by examining its chemical shift and intensity with respect to the structure of

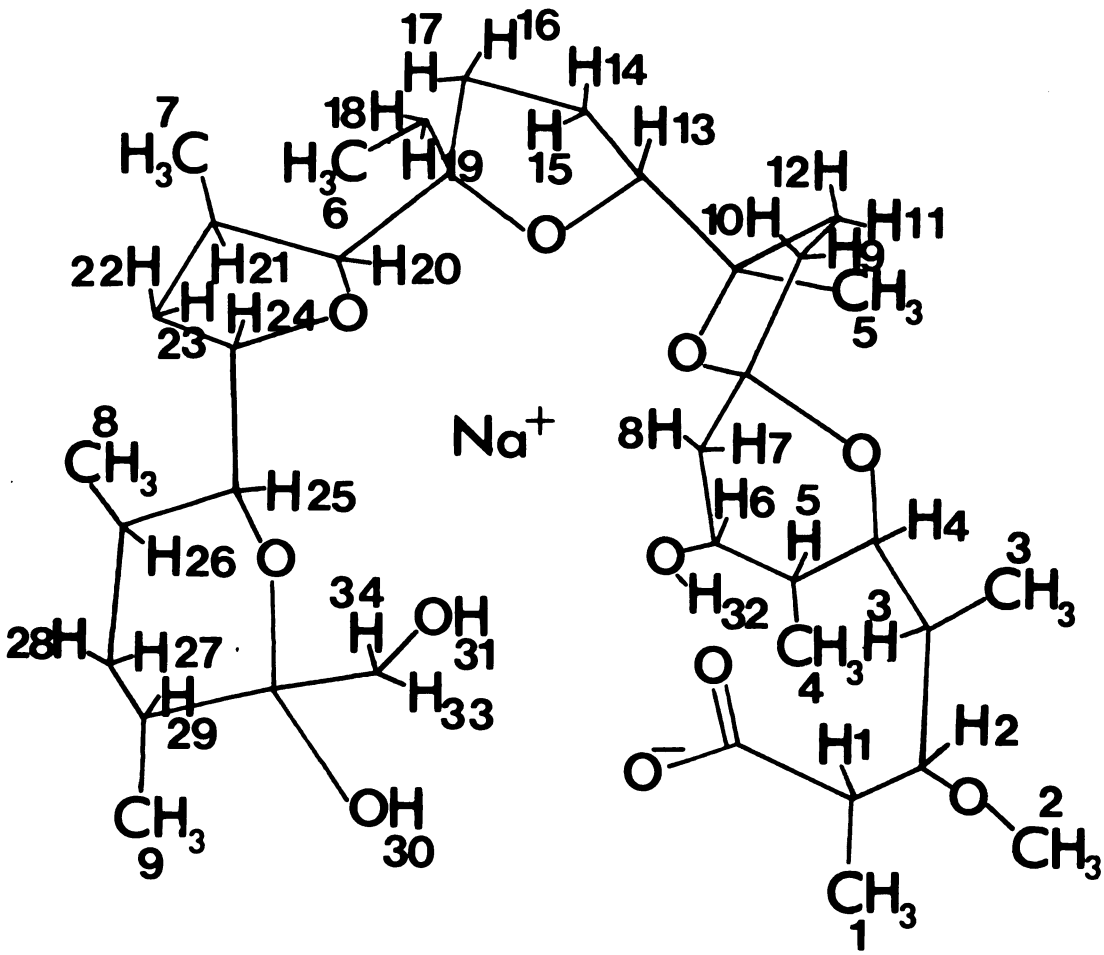


Figure 24. Numbering System for the Protons and Methyl Groups of the Monensin Sodium Salt

Table 4. Illustration of the Analysis of Several Peaks in the MonNa Proton Magnetic Resonance Spectrum

At 100 MHz			At 60 MHz		
Observed			Predicted		Observed
Peak Position	J	Center Frequency	Center Frequency	Peak Position	Peak Position
152.1	-	152.1	91.3	91.3	91.3
116	7	119.5	71.7	68	68
123				75	75
137				80	81
142	5	142	85.2	85	85
146				90	90
159				90	90
167	9	172	103	98	99
176				107	107
185				116	116

the molecule. It was here where various previous fragment assignments and general PMR references (116-119) were of assistance. No meaningful integrated PMR spectrum was obtained.

Two other techniques were helpful in assigning various areas of the spectrum. Five of the nine MonNa methyl groups show up as a large clump of only partially resolved peaks in the chemical shift region less than about 1 ppm. Even at narrow sweep widths, these peaks cannot be assigned. Figure 25 shows this area of the PMR spectrum in chloroform-d_1 solution as well as the effect of adding first a small amount, then a larger amount of shift reagent $Eu(fod)_3$. As can be seen, aside from the broadening effect of the diamagnetic europium, the pattern has been selectively shifted downfield making some of its fine structure visible. Additional resolution was provided by diluting this final solution which lowered the $Eu(fod)_3$ concentration and sharpened the peaks somewhat. The actual positions of the peaks were estimated from their positions in the original unshifted spectrum and will be presented below.

Another useful technique in assigning methyl doublets and the protons to which they were coupled was nuclear magnetic double resonance or proton decoupling. Figure 26 shows a portion of a normal MonNa 60 MHz PMR spectrum and the effect of irradiation of proton signals at 128 Hz and 150 Hz from TMS respectively. Two separate doublets are caused to collapse indicating methyls coupled to single protons.

Using the information obtained from the 100 MHz and 60 MHz narrow sweep spectra, shift reagent studies, various decoupling experiments and general trial and error methods and calculations

Figure 25. Illustration of the Effect of the Shift Reagent $Eu(fod)_3$ on the Methyl Region of the 100 MHz Proton NMR Spectrum of the Monensin Sodium Salt in Chloroform-d_1

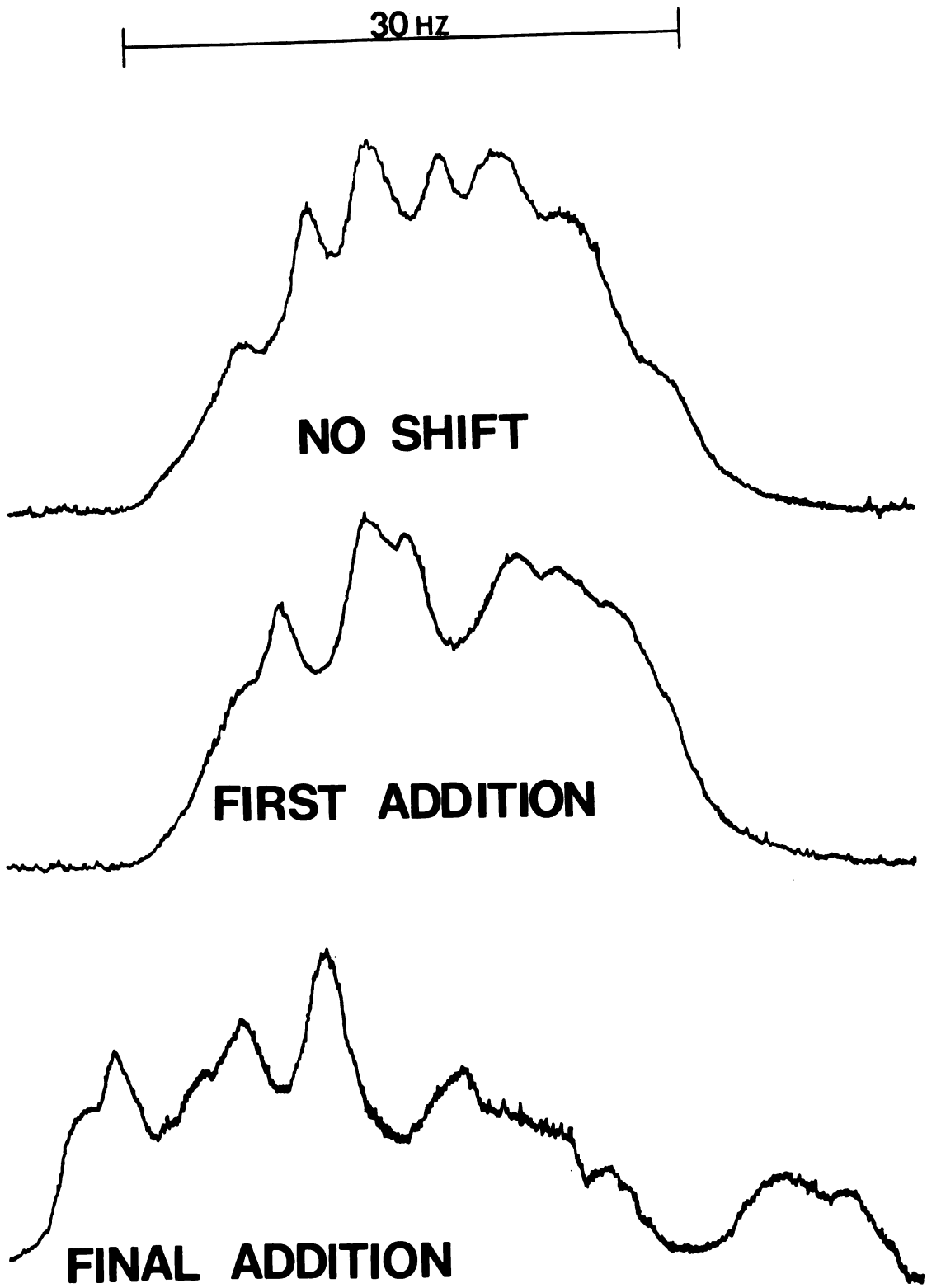
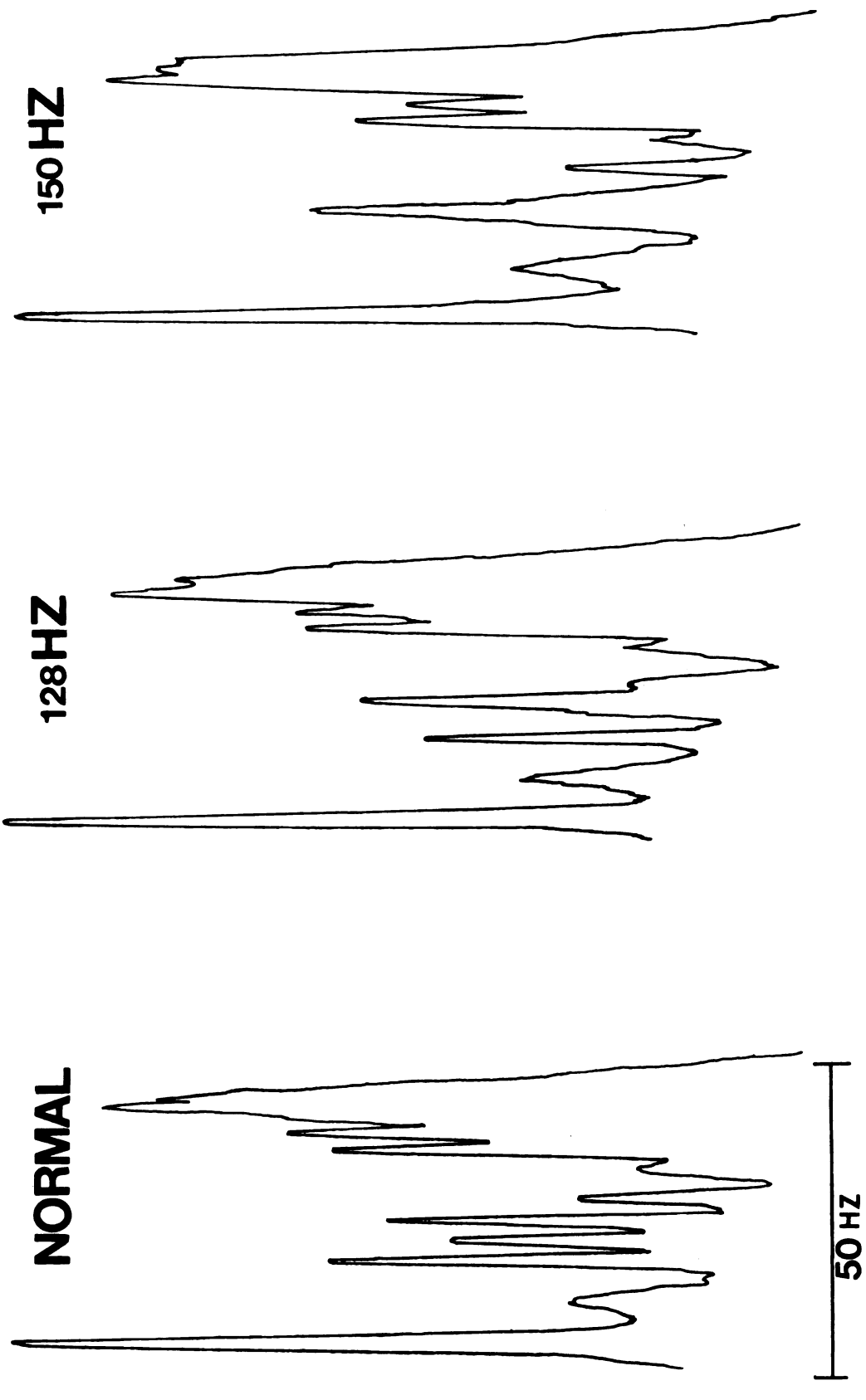

30 HZ
NO SHIFT
FIRST ADDITION
FINAL ADDITION

Figure 26. Illustration of the Effect of Irradiation at 128 Hz and 150 Hz on the Methyl Region of the 60 MHz Proton NMR Spectrum of the Monensin Sodium Salt in Chloroform-d_1


150 HZ
128 HZ
NORMAL
50 HZ

supplemented by PMR literature whenever possible, tentative proton assignments were made. As a general rule, assignments became more tenuous as analysis progressed downfield due to degradation of the resolution of peaks and the increase in the number of possible protons assignable to a particular chemical shift area. The analysis began at the high field end of the 100 MHz spectrum. For convenience, in the discussion below, all chemical shift values are given in Hz from TMS with no units included. Coupling constants are also in Hz.

The shift reagent study indicates the presence of three doublets at 79 and 85, 80 and 86 and 81 and 87, each with a J of 6. These are assigned to the methyl groups 7, 8 and 9 as a group. The coupling constant for the methyl group in methyltetrahydrofuran which the 7 methyl environment resembles has been given as 6.6 (120). The analogous values for the methyl and dimethyl tetrahydropyran do not seem to be available, although a study of substituted methoxy tetrahydropyrans (121) does seem to indicate that similar coupling constants should be expected for the 8 and 9 methyls.

Both the 60 MHz and 100 MHz spectra bear out the fact that the 102, 95 and 88 peaks are a triplet with J = 7. Since there is only one methyl group which may appear as a triplet, this is assigned to methyl 6. Reference (115) gives the shift of a $CH_3-CH_2-\overset{|}{\underset{|}{C}}-$ methyl as between 0.9 and 1.1 ppm and the 1:3:1 intensities are approximately correct in both spectra.

Peaks at 91 and 98 definitely form a doublet with J = 7 which was clearly confirmed by decoupling experiments. This doublet has been assigned to methyl 4. Its chemical shift would be expected to be similar to the dimethyltetrahydropyran analogues and methyls 8

and 9, but a slight downfield shift may be caused by its proximity to the 32 O-H group. The doublet moves significantly with addition of the shift reagent which may indicate its nearness to the acidic part of the molecule in contrast with the 7, 8 and 9 methyls whose shift positions were little changed.

The peaks at 116 and 123,and 122 and 128 have been found to be doublets with J values of 7 and 6 respectively. Both move quite rapidly during shift addition. Using trends predicted for methyl groups neighboring ethers and carboxylic acids (116), these doublets are assigned to methyls 1 and 3 respectively. Decoupling experiments also provided confirmation that these four peaks are indeed doublets.

The sharp peak at 152 is definitely a singlet due to methyl 5. It is the only methyl which can appear as a singlet and its position agrees with that of a $CH_3-\overset{|}{\underset{|}{C}}-$ methyl (115).

In one of the most poorly resolved areas of the spectrum there seems to be a possibility of two J = 5 triplets at 137, 142, 146 and 152, 157 and 162. Most of this region is obscured in both the 100 MHz and 60 MHz spectra, but these triplets have been tentatively assigned to protons 9 and 10 and 11 and 12. A triplet with J = 7 at 180, 187 and 194 may be due to protons 16 and 17.

The peaks at 159, 167, 176 and 185 most likely represent a pure quartet with J of 8. As the region beyond the methyl groups is considered, agreement between the 60 MHz and 100 MHz spectra becomes a much more important criterion for deciding on a particular multiplet. All of those given here can be assumed to be in substantial agreement. There is only one <u>pure</u> uncoupled quartet possible in the spectrum, and although other seemingly pure quartets were

naturally observed, this grouping was assigned to protons 18 and 19. Irradiation in this area produces a change in the signal for the 6 methyl and the coupling constants of 7 and 8 are within experimental error of one another.

Peaks at 190, 197, 205 and 212 have been assigned as a quartet with J = 7 and are due to proton 1. The best decoupling of the 1 methyl signal is obtained in the center of this region and the coupling constant value for the 1 methyl doublet is identical.

Several peaks in the 205 to 245 area contain what are thought to be coupled quartets. For example, the first multiplet is at 207, 215, 223, 231 and 239. If proton 5 gave a signal at 223, it could be split by methyl 4 with a coupling constant of about 8 to give a 1:3:3:1 quartet at 211, 219, 227, and 235. If each of these peaks were split by some adjacent hydrogen, say 6 or 4, each signal in the quartet would be further split to a doublet. In this case, if the second coupling constant is also on the order of 8, a quintet rather than an octet is obtained with a 1/2:2:3:2:1/2 intensity pattern at 207, 215, 223, 231 and 239. This type of analysis also seems to indicate that there are coupled quartets at 211, 218, 227, 234, and 242 and also at 205, 213, 221, 229 and 237 both with J values of 8. Irradiation at 223 does effectively decouple the signal assigned to the 4 methyl and seems to provide some validity for this type of reasoning and the assignment of the first multiplet to proton 5. The other two quintets probably belong to protons 21, 26 and 29 with the likelihood that 26 and 29 are nearly coincident. These quartets would be split in a somewhat more complex fashion, but since resolution here could not distinguish a six or twelve peak multiplet, no detailed analysis is possible.

The peaks at 269, 263, 256 and 251 appear to be grouped as a quartet with an average J of 6. This quartet is most likely due to proton 3 which was confirmed by the decoupling of the 3 methyl doublet caused by irradiation in the 263 to 256 region. The doublet and quartet both have the J = 6 values.

Signals at 315, 316, 324 and 326 can be analyzed as arising from a doublet of doublets with J values of 1 and 9. This signal is due to proton 2 and has been observed in monensin fragments. Proton number 4 should also show up as a doublet of doublets and the peaks at 336 and 324 tend to point to a doublet with J = 12 and another J less than 1.

The sharp singlet at 340 is due to the 2 methyl or methoxy group. This is the only MonNa peak which has been unequivocally assigned in the literature (1) and falls exactly where a methoxy signal is classically expected.

The signals at 354 and 356 are tentatively assigned to a pure doublet of J = 2 belonging to protons 7 and 8, while apparent doublets at 382 and 388 as well as 400 and 406 with J values of 6 are ascribed to protons 22 and 23,and 27 and 28. Here again, poor resolution and the increased number of possible protons which may give rise to each signal make assignments more tentative. A sharp peak at 394 is thought to be a singlet due to protons 33 and 34. It is downfield far enough to be on a carbon bound to oxygen and rapid exchange of the hydroxy proton 31 would cause it to be a singlet.

The 397 and 409 peaks seem to fit as a pure doublet with J = 12 which is assigned to proton 20. There is some evidence to indicate

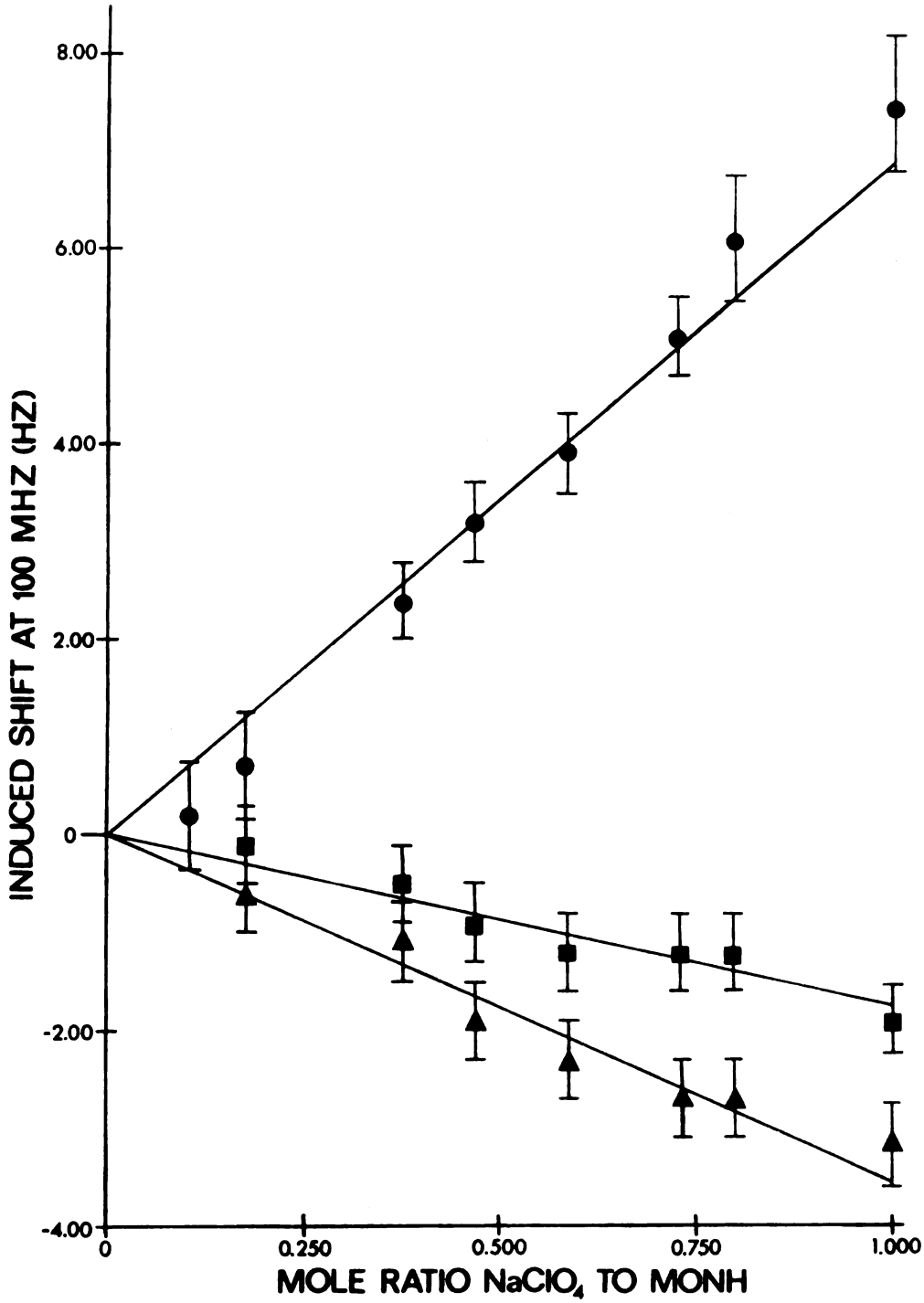
that a 389, 400, 411 grouping may be a triplet with a J of 11 due to proton 13. Coupling constants of this magnitude for a proton in a bridgehead position coupled to ring protons are shown to be reasonable by the assignment and J values for similar protons in some MonNa fragments (1).

A broad peak past 420 cannot be meaningfully resolved at any sweep width. Its position and width give some reason to believe that it may be due to the hydroxyl protons 30, 31 and 32. The two MonNa water molecules do not appear in the spectrum at room temperature as will be seen later.

An explicit assignment of all of the peaks in the MonH spectrum has not been made. As Figure 5 shows, many similarities exist. The most important difference in the MonH spectrum is the previously unreported broad peak at about 6 ppm which has been assigned to the bound water molecule of the acid. This assignment was confirmed by an experiment in which MonH in concentrated methanol solution was reprecipitated twice with deuterium oxide. The product gave a PMR spectrum in which the water peak had lost 50% of its intensity. Likewise,the infrared spectrum of this compound showed that intensities of the 3300 cm^{-1} and 3520 cm^{-1} bands also decreased by about 50% and new bands characteristic of deuterium oxide appeared at 2450 cm^{-1} and 2600 cm^{-1}.

EFFECTS OF COMPLEXATION As is shown in Figure 27, the 1, 3 and 5 methyl signals undergo induced shifts in 0.25 M MonH as sodium perchlorate is added in chloroform-d_1 solutions. Approximately linear shifts with different slopes are observed as the complex is formed indicating definite molecular conformation changes as MonH

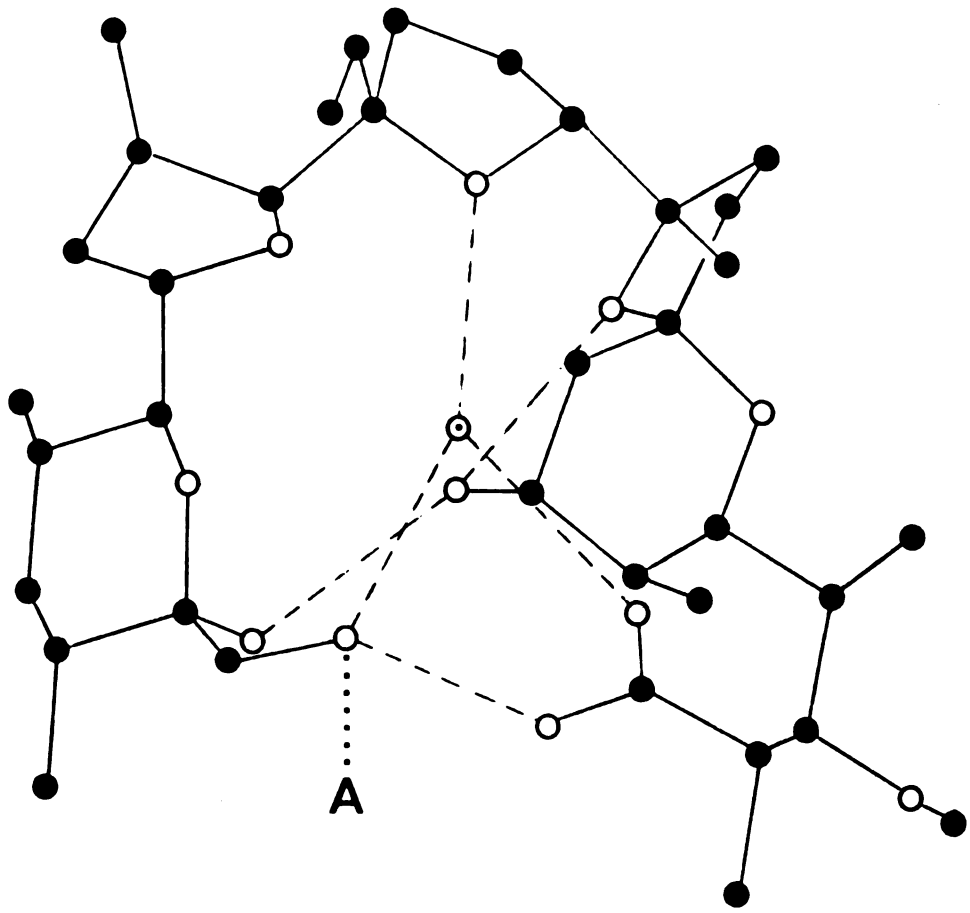
Figure 27. Proton NMR Study of 0.250 M Monensin with Increasing Sodium Perchlorate Concentration in Chloroform-d_1. Methyl 3 ●, methyl 1 ■, and methyl 5 ▲.


8.00
6.00
4.00
2.00
0
-2.00
-4.00
INDUCED SHIFT AT 100 MHZ (HZ)
0
0.250
0.500
0.750
1.000
MOLE RATIO NaClO4 TO MONH

and the sodium ion interact. The limiting shifts at 1:1 MonH to sodium perchlorate mole ratios do not appear at the frequencies of the same signals in the MonNa PMR spectrum. Carbon-13 NMR and PMR had already predicted conformational differences between acid and salt forms. This evidence indicated conformational changes also occur in the formation of the sodium complex with MonH in neutral solution.

The frequency of the bound water signal has been found to be solvent dependent, even though, as shown in Figure 28, the water molecule itself seems to be somewhat isolated from the solvent environment. Shifts of 6.03 ppm in chloroform-d_1, 4.83 ppm in tetrahydrofuran-d_8 and 4.98 ppm in acetone-d_6 were observed for 0.250 M MonH solutions. Attempts to obtain a reasonable PMR spectrum in methanol-d_4 were unsuccessful due to the interference of the normal methanol hydroxyl peak. The deuterium oxide experiment did indicate that the water molecule was labile in methanol which explains the interference of the OH signal as the OD exchange of methanol-d_4 with the water available from MonH. The variation in peak position in the other three solvents is probably also due to exchange of the hydrogen bonded water molecule with free water in the solvent. All three peaks are in a position downfield of the shift of pure free water. In tetrahydrofuran-d_8 the peak is closest to that of free water (4.72 ppm). The bound water signal in acetone-d_6 is slightly further downfield and in chloroform-d_1 it is very distant from the free position. The positions are compared to the shift of pure liquid water at 4.72 ppm (122). The same relationship noted can also be observed by comparison with the infinite dilution shifts of water in each solvent

Figure 28. The Crystalline Structure of Monensin. Closed circles are carbon atoms, open circles are oxygens with the water oxygen dotted, dashed lines indicate hydrogen bonds and A is a possible position for associated exchangeable water.


A

which are 2.42 ppm for tetrahydrofuran (122), 2.40 ppm for acetone (123) and 1.99 ppm for chloroform (124). This evidence would tend to support a simple model of free and bound exchange based primarily on water solubility in each solvent.

Lowering the temperature produces a downfield shift of the water peak in all solvents as is shown in Figure 29 for the chloroform-d_1 case. In addition, the peak splits to give a broader line at lower field and an upfield sharper peak. Both peaks migrate further downfield at approximately constant separation with further decrease in temperature. Figure 30 graphically exhibits this behavior in the three solvents. This splitting seems to indicate that the water molecule can be attached to MonH at two different sites. The separations and temperatures of coalescence, shown in Figure 30, indicate that the exchange is easiest in tetrahydrofuran-d_8 and most difficult in chloroform-d_1. The further downfield shift reaffirms the previous model of exchange with water in the solvent in that the decrease in temperature seems to favor an equilibrium towards the bound water molecules with fewer and fewer free water molecules in the solvent. There is little indication of the signals ever reaching a frequency characteristic of completely bound water molecules before line broadening and solvent freezing make measurements impossible.

Figures 31 and 32 show the behavior of the bound water peak in acetone-d_6 and chloroform-d_1 at ambient temperature as sodium perchlorate is gradually added to the MonH solutions. In both cases, a steady decrease in peak intensity is observed and the peak disappears as the 1:1 ratio is approached. However, in acetone-d_6 solution a downfield shift is noted while in chloroform-d_1 the peak moves upfield.

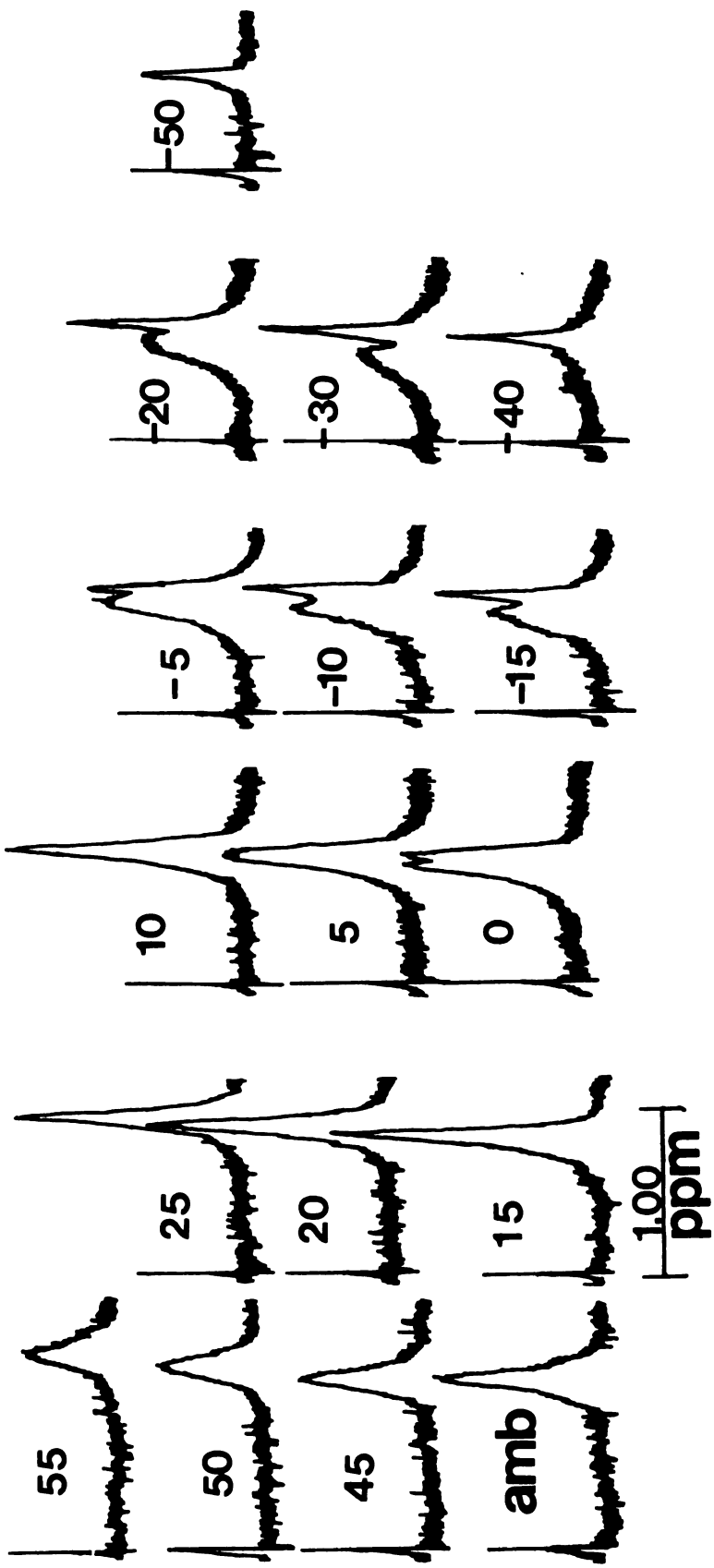


Figure 29. Proton NMR Spectra of the Bound Water Region of 0.250 M Monensin in Chloroform-d_1.

Temperatures are °C. A chloroform reference peak is also shown.

Figure 30. Proton NMR Study of 0.250 M Monensin in Tetrahydrofuran-d_8, Acetone-d_6 and Chloroform-d_1. Signal separations and coalescence temperatures are ●, tetrahydrofuran-d_8, 18 Hz, 20°C; □, acetone-d_6, 15 Hz, 10°C; and ▲, chloroform-d_1, 8 Hz, 0°C.

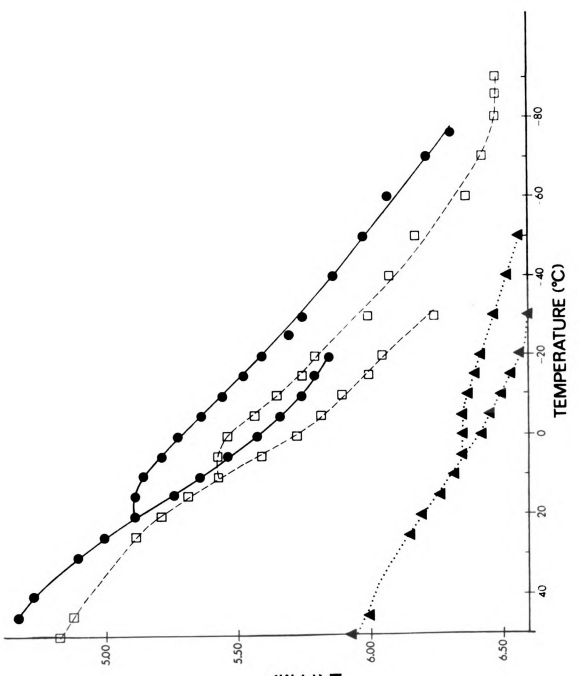

Δ (PPM)
5.00
5.50
6.00
6.50
40
20
0
-20
-40
-60
-80
TEMPERATURE (°C)

Figure 31. Proton NMR Study of 0.250 M Monensin with Increasing Sodium Perchlorate Concentration in Chloroform-d_1 and Acetone-d_6

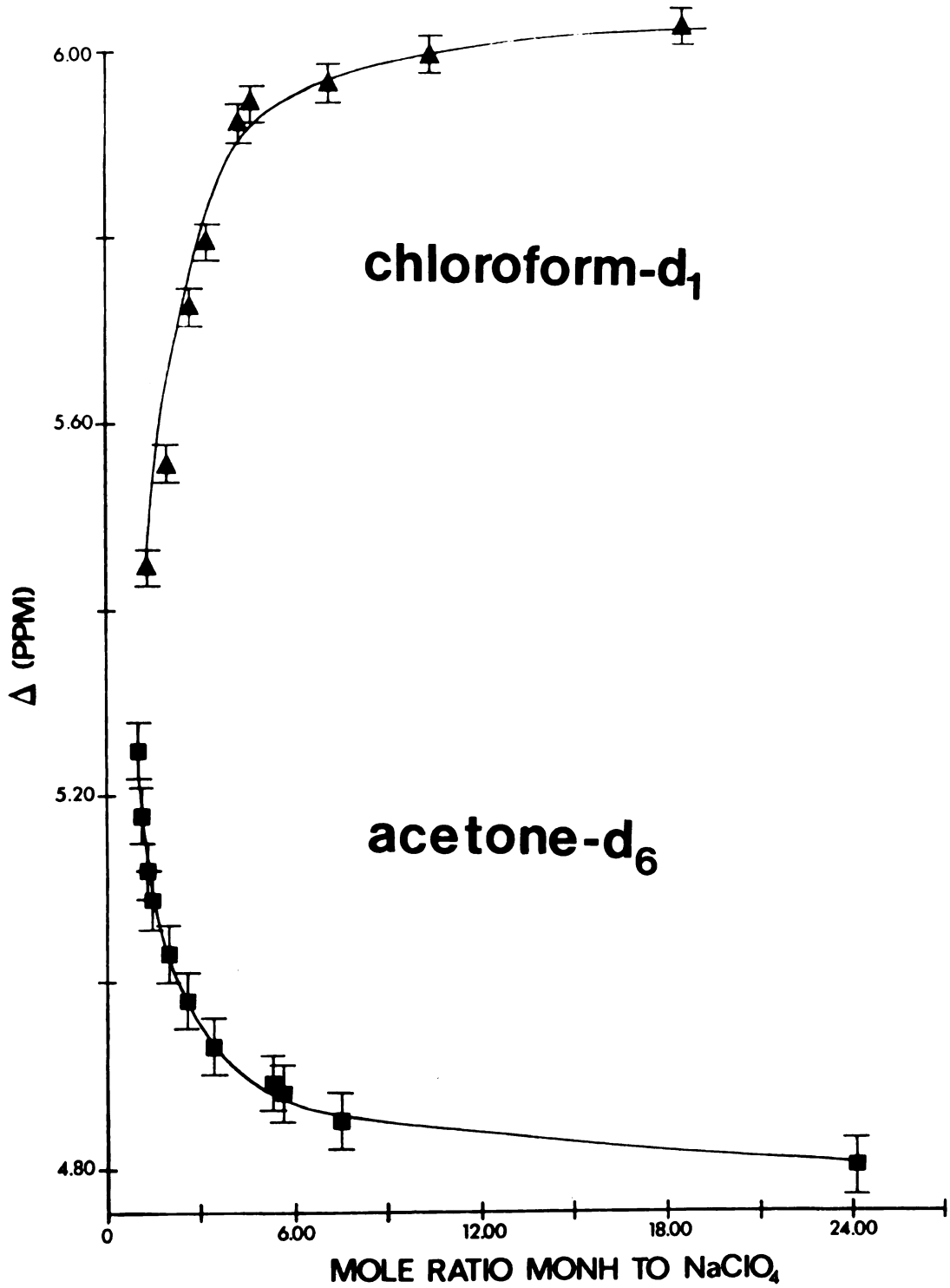

6.00
5.60
5.20
4.80
Δ (PPM)
chloroform-d1
acetone-d6
0
6.00
12.00
18.00
24.00
MOLE RATIO MONH TO NaClO4

Figure 32. Proton NMR Study of 0.250 M Monensin with Increasing Sodium Perchlorate Concentration in Chloroform-d_1 and Acetone-d_6

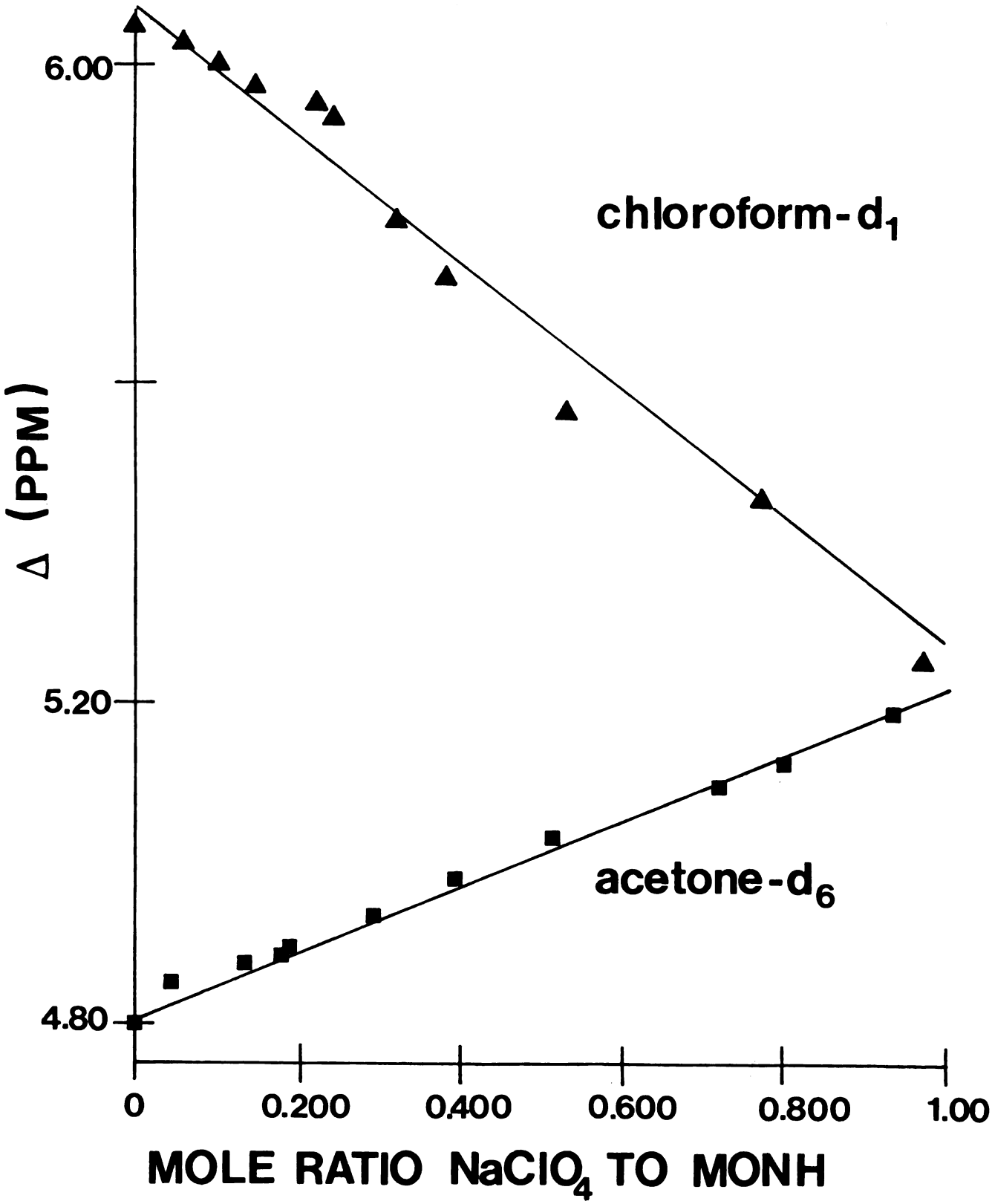

6.00
5.20
4.80
Δ (PPM)
chloroform-d1
acetone-d6
0
0.200
0.400
0.600
0.800
1.00
MOLE RATIO NaClO4 TO MONH

Figure 32 shows that an approximate common position can be extrapolated for the 1:1 solutions in both solvents. A simple displacement of the water molecule in the monensin cavity by the sodium ion fails to account for the observed behavior since in this case increasing amounts of free water would cause only upfield shifts to be observed. It may be necessary to invoke a model in which water is exchanged into the solvent by way of two sites, each site being affected differently by the interaction of MonH with the sodium ion. The sharper signal is thought to arise from a water in position A as illustrated in Figure 28, rather than in the center of the MonH species. This is a more loosely hydrogen bonded water, bound only through its own oxygen, and is in fact in a position occupied by a water in the solid MonNa salt (see Figure 16). The broad signal may come from the water molecule in the center of the MonH ring. The water molecule at A would be easier to release to the solvent since only one hydrogen bond would need to be broken. It could also remain on the site even when sodium ion occupied the monensin cavity. This situation may have been represented in the infrared spectrum by the sharp peak at 3520 cm^{-1} and the broad band at 3300 cm^{-1}. Further differential behavior between acetone-d_6 and chloroform-d_1 is noted when sodium perchlorate addition experiments are conducted at temperatures below the coalescence temperature of the two water signals. In chloroform-d_1, as sodium ion is added, the sharper peak, due to associated water, gradually decreases in intensity without change in position. The bound water peak, after a small shift at low sodium concentrations, shows the same behavior. As mentioned previously, the sharp peak in the infrared spectrum also slowly decreases upon addition of sodium

ion. In the acetone-d_6 case however, the sharp PMR peak immediately disappears even at very low sodium ion concentration and the broader peak stays in position and gradually loses intensity as 1:1 mole ratios of MonH and sodium ion are reached.

It would seem that further analysis and other types of experiments may be necessary to clarify the observations presented here. However, the data obtained above and below the coalescence temperature seem to warrant several speculative conclusions. At the higher temperatures, the addition of sodium ion to MonH in acetone-d_6 solution causes an increase in the population of the more tightly held water molecules, perhaps by way of depleting the associated molecules which causes a relative increase in the bound population as equilibrium between associated and bound is reestablished and free water available from the solvent is taken up. At lower temperatures however, this pathway is blocked by the lack of exchange between bound and associated positions and only the sudden drop in the intensity of the peak due to associated water with no chemical shift is observed. In chloroform-d_1, at higher temperatures, sodium addition causes an increase in the free water population, perhaps by simple gradual displacement of water from the cavity into the bulk solvent. Low temperature results confirm this idea in that associated and bound peaks decrease in intensity at approximately the same rate.

CONCLUSIONS

The monensin PMR spectrum is somewhat complex, but a reasonably self-consistent assignment has been proposed. The shifts of the MonH methyl peaks upon sodium ion interaction indicate conformational

changes occur during complexation even in neutral solution. The chemistry of the MonH water molecule may not conform to simple models of gradual sodium ion displacement. The existence of three sites for the water molecule and the dramatic differences observed upon sodium ion interaction in a hydrophobic and hydrophilic solvents indicate that the behavior of MonH in various solvents is much more complex than expected from previous studies.

CHAPTER V

STUDIES OF A NEW MONENSIN COMPLEX

INTRODUCTION

Potentiometric measurements have indicated that the monensin sodium complex which formed in neutral methanol was not the same as the salt usually isolated from basic solutions. This conclusion was confirmed by infrared and PMR studies. In both cases, marked dissimilarities were observed between the 1:1 MonH to sodium perchlorate spectra and those of the MonNa salt. In an effort to further elucidate the nature of this new monensin complex, attempts were made to isolate and characterize it and then study its behavior in the presence of the normal MonNa complex.

ISOLATION AND CHARACTERIZATION

Several methods for the preparation of the new complex were investigated. The first approach was to prepare the 1:1 MonH to sodium perchlorate solution in a solvent such as methanol and then evaporate the solvent. This procedure gave a powdery white solid compound different from either MonH, MonNa or sodium perchlorate. The evaporation can be carried out quickly under vacuum or slowly in an evaporating dish in the atmosphere with the same end result. Proton NMR spectra of the solid product dissolved in chloroform-d_1 gave the following results. The methyl region of the spectrum was not that of MonH or MonNa but was rather identical with that of a solution of MonH and sodium perchlorate in 1:1 mole ratio. Further, no bound water peak at 6 ppm was observed. However, the new PMR spectrum

indicated the presence of methanol in concentrations equal to that of the complex.

It was found that extended periods of solvent evaporation via long vacuum drying, even at elevated temperatures, did not effect the intensity of the methanol PMR signal. The complex could also be isolated from many other solvents such as chloroform, acetone, benzene, ethanol and 1,2-dichloroethane. These solids were also found to give PMR spectra indicating the presence of associated solvent molecules. All signals appeared in the correct positions for each solvent dissolved in chloroform-d_1 which implies that in solution the solvent is probably not bound to the complex.

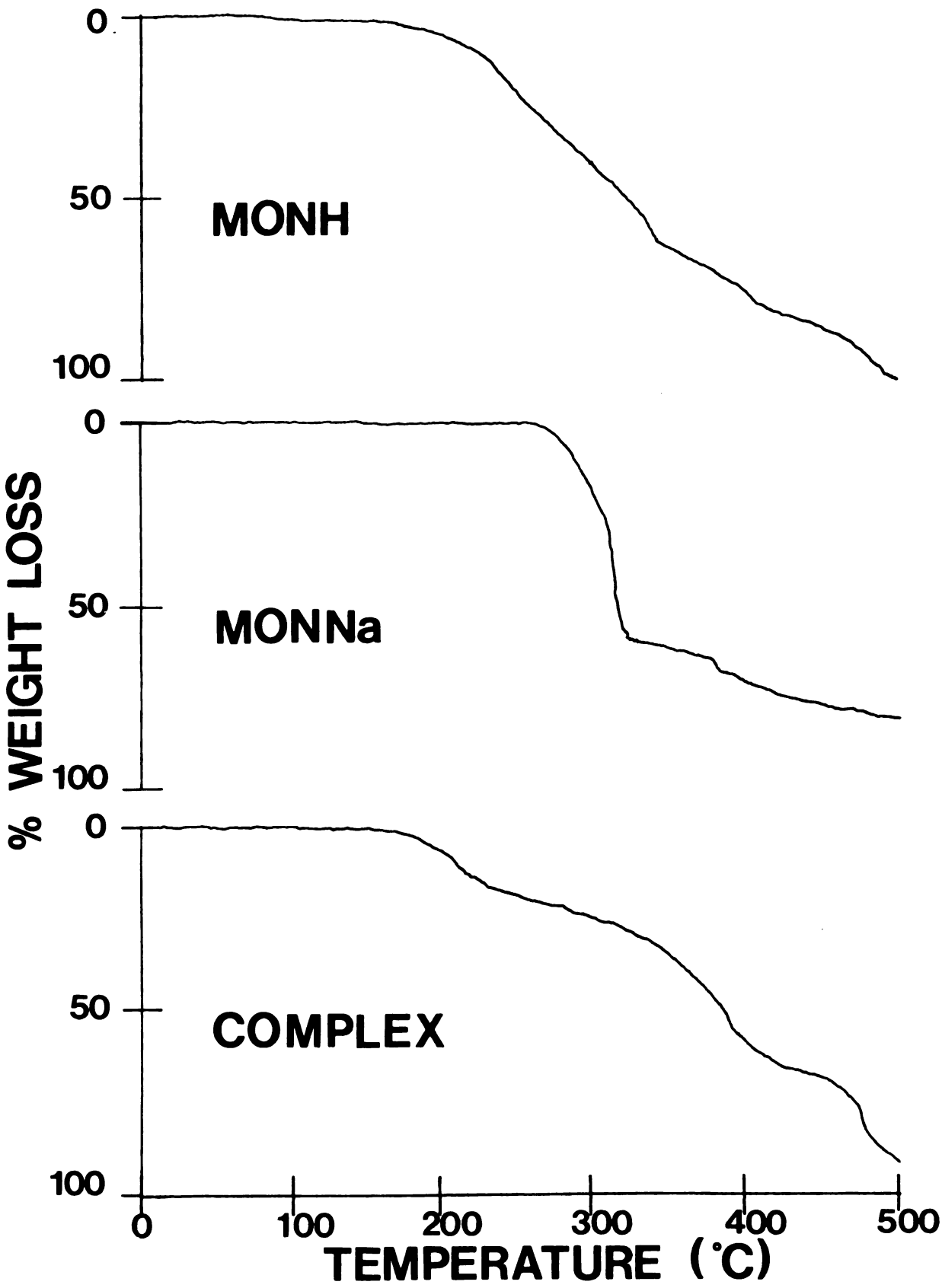
Several attempts were made to eliminate the solvent <u>after</u> the complex had been prepared. The solid isolated from chloroform was washed with a hot 1:1 ethyl ether-petroleum ether mixture and then dried. The result was a compound in which solid-liquid exchange caused the total release of the chloroform and PMR signals due to the ether mixture appeared. Washing the methanol complex with water gave a solid which, when analyzed as detailed below, was shown to be a mixture of the methanol complex and the normal MonH free acid. Attempts to precipitate the complex with water directly from a methanol solution of 1:1 MonH to sodium perchlorate yielded only the acid. There seems to be little possibility of preparing the complex with water as the associated solvent. In addition, no matter how bulky the solvent molecule, some amount of solvent seems to be necessary for the formation of the solid complex.

None of the above methods gave crystalline products. Recrystallization attempts from various solvents and solvent mixtures were also unsuccessful. It was thought that a change in the anion of the sodium salt might facilitate crystal formation. A 1:1 solution of MonH to sodium iodide in methanol was found to give a colorless crystalline solid upon solvent evaporation which turned yellow after exposure to air. This method may eventually give crystals suitable for X-ray crystallographic studies. It was also found that the new complex could be made with other cations such as silver and potassium, but these compounds have not been studied thus far.

The melting points of the complexes from any of the solvents were substantially above that of MonH (117°C), with the most important methanol and chloroform complexes melting at about 140°C with rapid decomposition. The fact that the complex is neither MonH, MonNa or sodium perchlorate was further confirmed by the thermograms shown in Figure 33. Although the curves are not as featured as might be desired, it appears that MonH exhibits a plateau from 0°C to 150°C at about 2% weight loss, which probably corresponds to the loss of its bound water which is 2.6% of the molecule by weight. The MonNa thermogram is even less informative, but the curve for the new complex isolated from chloroform shows inflection points at about 2% and 14%. This would approximately coincide with a crude formulation of the complex as $MonH \cdot NaClO_4 \cdot H_2O \cdot CHCl_3$ which would be 2% water and 13% chloroform. In fact, as will be shown below, this may be a reasonable representation.

Infrared spectroscopy had shown that the sodium perchlorate complex formed in methanol or chloroform solutions did not have a

Figure 33. Thermogravimetric Analysis of Monensin, the Monensin Sodium Salt and the Monensin Sodium Perchlorate Complex Prepared from Chloroform Solution


0
50
100
MONH
0
50
100
MONNa
0
50
100
COMPLEX
% WEIGHT LOSS
0
100
200
300
400
500
TEMPERATURE (°C)

deprotonated carboxylic acid group. The spectra of the new solid complexes obtained from methanol or chloroform solution, when redissolved in chloroform, also exhibited the 1704 cm^{-1} band with no trace of the salt peak. Therefore, it was presumed that the new complexes were still acidic and titrations of some of the various solids in methanol with TBAH were carried out. Figures 34 and 35 show the titration curves of 0.100 gram in 50.00 ml of solution of two of the new complex preparations titrated with standardized 0.0046 M TBAH. In Figure 34, it is seen that the complex as isolated from chloroform is indeed a weak acid, with a pK_a estimated to be about 7, but it is considerably stronger than MonH. A rationale for this difference is not immediately obvious. Certainly the presence of the sodium ion will affect the MonH acid-base equilibria as has been demonstrated previously. An equivalent weight of greater than 850 gm eq^{-1} is indicated, again pointing to a formulation of the complex which includes not only MonH and the sodium ion, but also perchlorate and a chloroform molecule.

Figure 35 shows the titration curve for the methanol complex after soaking in water and confirms the fact that a mixture of two different acids is obtained. Two different equivalence points are observed, the second of which does not occur at twice the volume of the first. It does therefore seem that a complex-MonH mixture exists and this is confirmed by PMR which shows only a small methanol peak and the return of the MonH water peak. Two distinct melting points of 110°C and 150°C are also observed for the solid mixture. Titrations of other complexes from benzene, ethanol and the like also give indications of weak acids

Figure 34. Titration of 0.100 gram Monensin Sodium Perchlorate Complex in 50.00 ml of Solution with 0.0046 M Tetrabutylammonium Hydroxide in Methanol. Complex prepared from chloroform solution.

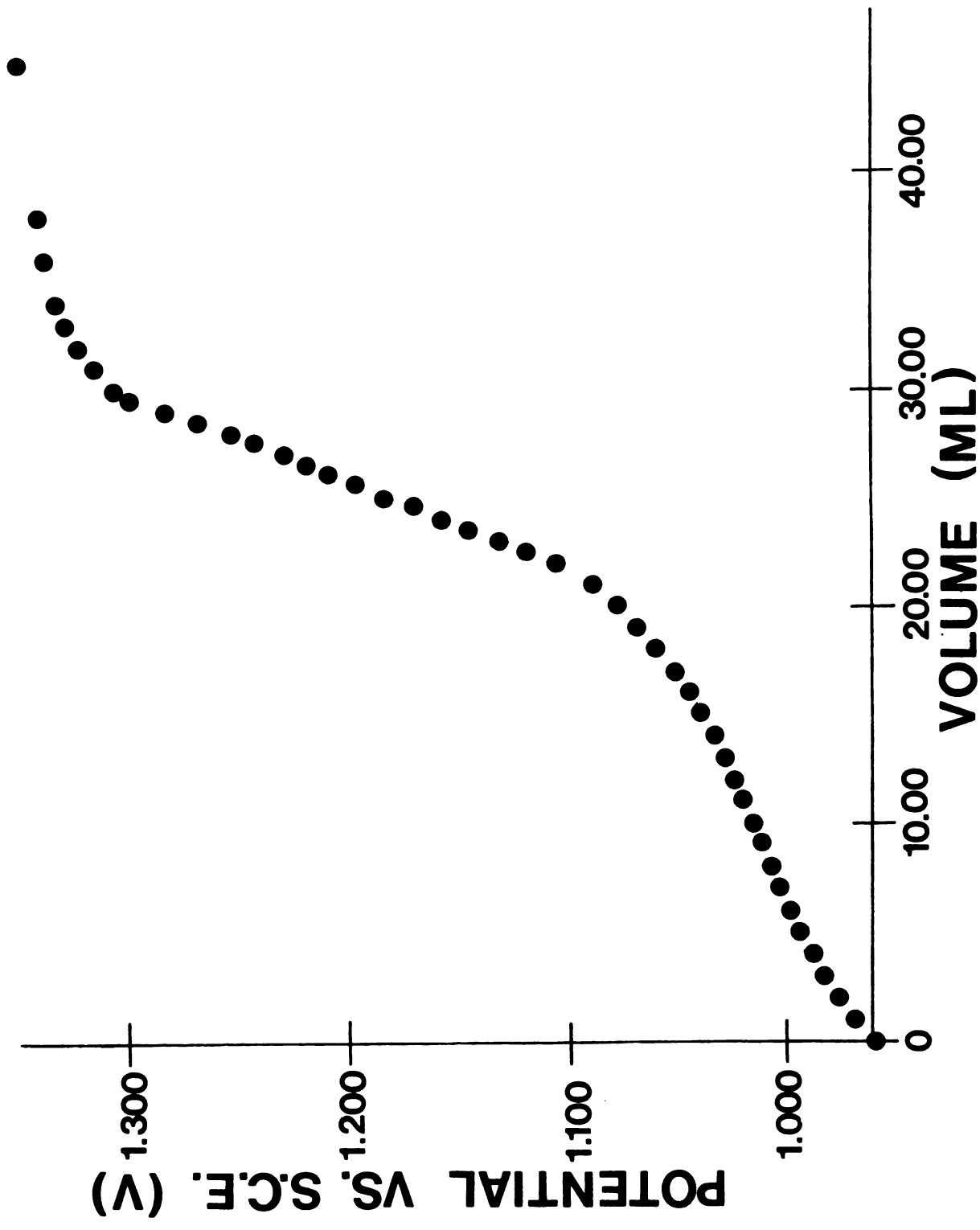
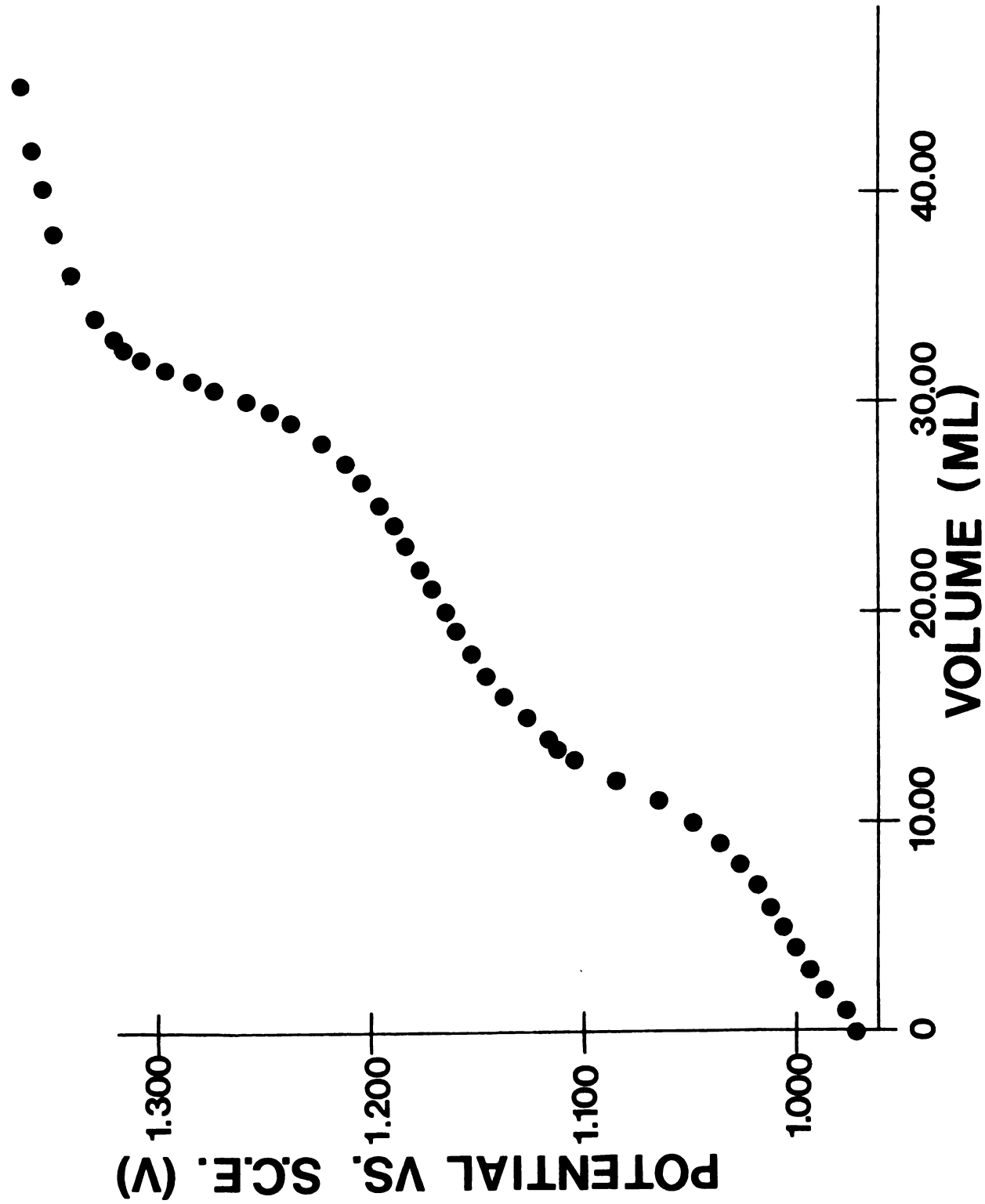

POTENTIAL VS. S.C.E. (V)
1.300
1.200
1.100
1.000
0
10.00
20.00
30.00
40.00
VOLUME (ML)

Figure 35. Titration of 0.100 gram Monensin Sodium Perchlorate Complex in 50.00 ml of Solution with 0.0046 M Tetrabutylammonium Hydroxide in Methanol. Complex prepared from methanol solution and soaked in water.


POTENTIAL VS. S.C.E. (V)
1.300
1.200
1.100
1.000
0
10.00
20.00
30.00
40.00
VOLUME (ML)

with equivalent weights roughly corresponding to the formula $MonH \cdot NaClO_4 \cdot S$ where S is an attached solvent molecule.

It was of some interest to obtain positive proof that the perchlorate anion is indeed incorporated in the new solid complex. Efforts were made to determine whether perchlorate as perchloric acid might escape in the solvent evaporation during complex preparation. No evidence of perchloric acid was found in trapped portions of the evaporated solvent. However, if any of the solid complexes were dissolved in methanol, several drops of methanolic tetraphenylarsonnium chloride were found to cause a flocculant white precipitate diagnostic of the presence of the perchlorate ion.

In an effort to further confirm this evidence and perhaps begin to explore the perchlorate ion surroundings, Raman spectroscopy was employed. Shown in Figure 36 is the extremely compressed Raman spectrum of solid MonNa. As can be seen, the spectrum is quite complex below about 1500 cm^{-1} as would be expected from the large number of carbon-carbon and carbon-hydrogen bonds. (Table 5 lists the exact positions of the various bands as obtained from this and other better resolved spectra.) The large group of peaks from 2800 cm^{-1} to 3000 cm^{-1} and the one from about 1400 cm^{-1} to 1500 cm^{-1} are typical of MonNa, MonH and the new complex from chloroform in the solid form. There are very few differences between the MonNa and MonH spectra. The complex, however, shows two very strong bands at 1862 cm^{-1} and 1961 cm^{-1}. These have not been assigned. It also has a unique pattern of bands at 462 cm^{-1}, 611 cm^{-1} to 634 cm^{-1} and 927 cm^{-1}. These have been attributed to perchlorate ion vibrations on the basis of literature values of 460 cm^{-1},

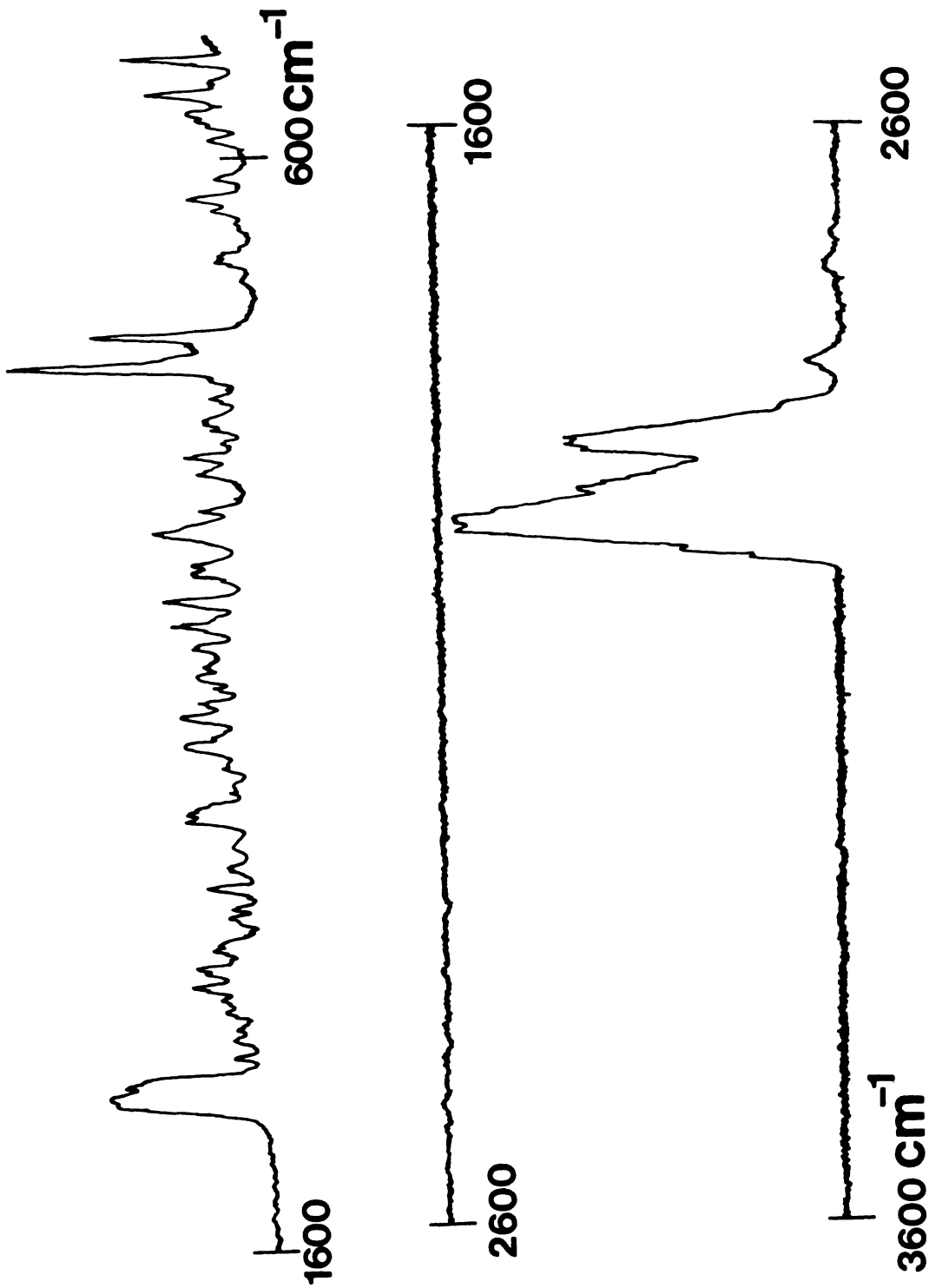


Figure 36. The Raman Spectra of the Solid Monensin Sodium Salt

Table 5. Band Positions in the Raman Spectrum of the Solid Monensin Sodium Salt (cm^{-1})

512 m	849 w	1151 w	1140 m
520 w	860 w	1158 w	1446 m
533 w	873 m	1192 m	1453 s
545 w	888 m	1197 m	1458 s
563 m	911 w	1206 m	1464 s
569 w	922 m	1227 w	1470 w
574 w	942 m	1248 w	2817 w
587 m	970 m	1268 m	2855 m
621 m	978 m	1286 w	2873 m
640 m	980 m	1293 w	2876 s
652 m	1004 m	1317 w	2887 s
665 w	1026 m	1323 w	2894 w
690 m	1045 m	1329 m	2916 w
695 m	1063 w	1335 m	2933 s
713 w	1074 w	1356 m	2938 s
764 s	1083 w	1368 w	2965 m
791 s	1095 w	1380 w	2975 m
803 w	1110 m	1393 w	2987 m
819 w	1128 w	1405 m	2998 w
840 m	1136 m	1420 w	

626 cm^{-1} and 932 cm^{-1} (125). The region of the 1110 cm^{-1} band was obscured.

Several of the new solid complexes have been subjected to elemental analysis for carbon and hydrogen. Results of these determinations are summarized in Table 6 for the sodium perchlorate complex as isolated from methanol, ethanol and chloroform and also the sodium iodide and chloride complexes from methanol. Also shown are the calculated carbon and hydrogen compositions of various formulations of the complex based on combinations of MonH and the salt and solvent used. By comparison with the calculated compositions of MonH and MonNa which are also shown, it is quite obvious that none of the new complexes are simply the acid MonH or normal salt MonNa. Choosing a composition with or without associated water or solvent solely on the basis of the elemental analysis data would be quite tenuous. The addition of these relatively small molecules simply does not significantly change the percentage of carbon and hydrogen. In four cases, <u>all</u> of the various calculated compositions are in fair agreement with the values observed.

In the sodium perchlorate complex as isolated from chloroform, a rather high observed result has occurred. However, in this case, the analysis itself is somewhat suspect. It is known from other determinations that chloroform is indeed present in the complex, but additional analysis of the solid for elemental chlorine gave a value of 4.02%. If associated chloroform had been detected, the value would be approximately 15%. Indeed, the 4% figure corresponds to the presence of the sodium perchlorate alone, as does the carbon and hydrogen analysis. The results indicate that the chloroform was simply missed in the analysis, perhaps due to its volitility.

Table 6. Elemental Analysis of New Monensin Complexes

$NaClO_4$ Complex From Methanol			$NaClO_4$ Complex From Ethanol			$NaClO_4$ Complex From Chloroform		
	%C	%H		%C	%H		%C	%H
OBSERVED	55.34	7.91		53.59	7.62		53.88	7.62
$MonHNaClO_4$	54.51	7.88	$MonHNaClO_4$	54.51	7.88	$MonHNaClO_4$	54.51	7.88
$MonHNaClO_4$ H_2O	53.29	7.95	$MonHNaClO_4$ H_2O	53.29	7.95	$MonHNaClO_4$ H_2O	53.29	7.95
$MonHNaClO_4$ CH_3OH	53.84	8.06	$MonHNaClO_4$ CH_3CH_2OH	54.37	8.17	$MonHNaClO_4$ $CHCl_3$	48.69	6.96
$MonHNaClO_4$ H_2OCH_3OH	52.69	8.13	$MonHNaClO_4$ $H_2OCH_3CH_2OH$	53.23	8.23	$MonHNaClO_4$ H_2OCHCl_3	47.75	7.04

NaI Complex From Methanol			NaCl Complex From Methanol					
	%C	%H		%C	%H			
OBSERVED	52.28	7.36		58.04	8.45			
MonHNaI	52.68	7.61	MonHNaCl	59.28	8.57			
MonHNaI H_2O	51.54	7.69	MonHNaCl H_2O	57.86	8.63	$MonH \cdot H_2O$	62.77	9.36
MonHNaI CH_3OH	52.11	7.80	MonHNaCl CH_3OH	58.37	8.74	MonNa	62.70	8.87
MonHNaI H_2OCH_3OH	51.03	7.87	MonHNaCl H_2OCH_3OH	57.02	8.79			

CONCLUSIONS

All analyses led to the conclusion that a new monensin-sodium complex has been prepared. Indications are that in addition to a molecule of MonH, molecules of sodium salt and the solvent used for preparation are also involved. Neither the exact stoichiometry nor the structure of the new complex have been determined, however it is assumed that the sodium ion must be at least near the center of the MonH moiety due to the ionic solubilizing effect of MonH in hydrophobic solvents.

EXCHANGE STUDIES

Studies of possible exchange involving the new complex and the normal salt were begun using the sodium perchlorate preparation from chloroform and again employed PMR spectroscopy. Most spectra were run in chloroform-d_1 at varying temperatures. Four temperature dependent spectra were considered. The temperature dependent spectrum of the MonH water region in chloroform-d_1 has been shown previously (Figures 29 and 30). The MonNa spectrum exhibits no bound water peak at temperatures above 30°C, but does develop a distinct peak at 9.6 ppm at about -20°C which at -70°C seems to split to two peaks with a separation of about 18 Hz. The spectrum of a 1:1 solution made up of MonH and sodium perchlorate in chloroform-d_1 and the spectrum of the solid new complex as isolated from chloroform and then redissolved in chloroform-d_1 were found to be in every way identical with the exception of the associated solvent bands. Since the exact molecular weight and, therefore, the concentrations of the solutions of the new solid complex were unknown, all preparations of the new complex for this study were made in solution and not isolated.

The PMR spectrum of the complex does not show any peaks downfield from 5 ppm at normal probe temperatures. However, as the solution is cooled to 0°C, a broad peak does appear at about 5.8 ppm. In addition, at -40°C, where this new peak is quite sharp, another peak begins to grow in at 10.4 ppm. The 5.8 ppm peak is thought to be due to bound water in the complex and seems to split to give two peaks with a separation of 14 Hz at about -70°C. Efforts to assign the 10.4 ppm peak have so far been unsuccessful.

Exchange experiments centered around the anticipated interaction between the 9.6 ppm signal of the salt and the 5.8 ppm signal of the complex. It was found that a 1:1 mixture of the complex and salt produced only one peak at about 7 ppm which appeared even at the ambient probe temperature of approximately 30°C but had a somewhat temperature dependent position. This led to the suspicion that fast sodium ion exchange from one environment to the other might be creating an average chemical shift for the water molecule and motivated the study below.

Solutions were made up in chloroform-d_1 which were 0.100 M in complex with concentrations of MonNa ranging from 0 to 0.150 M. Although the complex itself showed no water peak at ambient temperature, addition of 0.010 M MonNa caused a peak to appear in the 7 ppm region and further MonNa addition caused this peak to grow and gradually shift downfield towards the MonNa position. Repeating the experiment at -10°C yielded a very similar pattern which was transposed upfield. These results are illustrated in Figures 37 and 38 which show the observed chemical shifts as a function of complex to salt mole ratio

Figure 37. Proton NMR Study of 0.100 M Monensin Sodium Perchlorate Complex with Increasing Monensin Sodium Salt Concentration in Chloroform-d_1

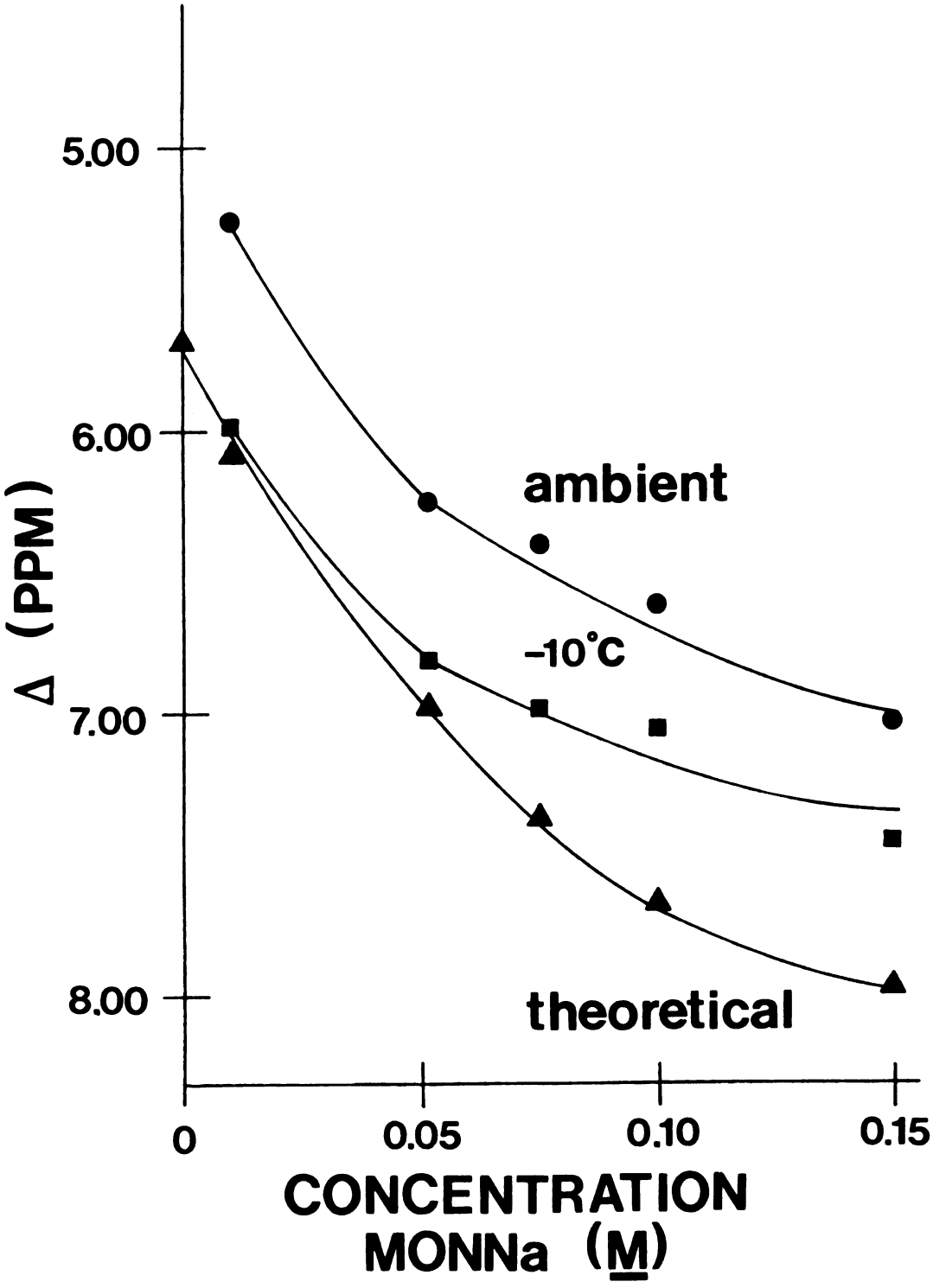
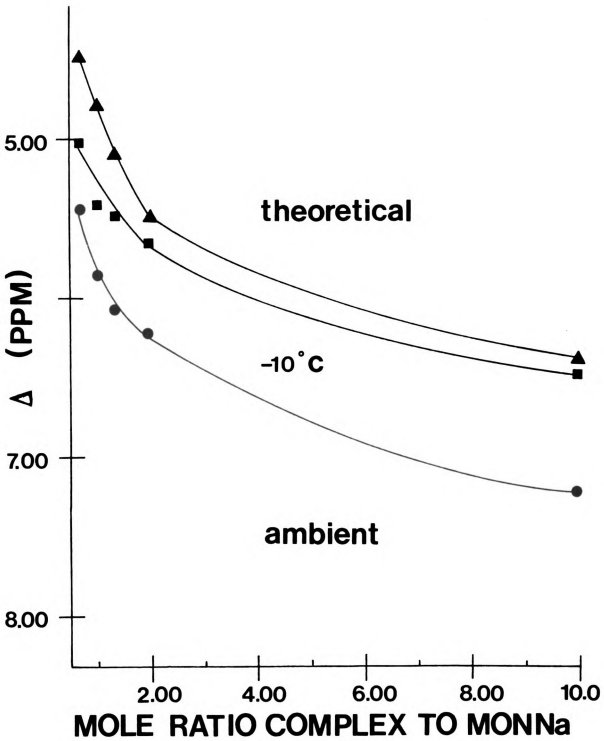

5.00
6.00
7.00
8.00
Δ (PPM)
ambient
-10°C
theoretical
0
0.05
0.10
0.15
CONCENTRATION
MONNa (M)

Figure 38. Proton NMR Study of 0.100 M Monensin Sodium Perchlorate Complex with Increasing Monensin Sodium Salt Concentration in Chloroform-d_1


5.00
7.00
8.00
Δ (PPM)
theoretical
-10°C
ambient
2.00
4.00
6.00
8.00
10.0
MOLE RATIO COMPLEX TO MONNa

and added salt concentration. The analogous experiment in acetone-d_6 could not be carried out due to solubility limitations.

As mentioned previously, a shift due to the averaging of the sodium ion environment was expected. This behavior should be predicted by the equation below:

$$(9) \quad \delta_{obs} = X(5.8 \text{ ppm}) + Y(9.6 \text{ ppm})$$

where 5.8 and 9.6 ppm are the temperature independent positions of the exchanging peaks for the complex and salt respectively and X and Y are the respective fractions of each form (32,125). Using analytical concentrations of the complex and MonNa present in the chloroform-d_1 solutions and the observed temperature independent chemical shifts for the complex and salt alone in solution, it was possible to calculate values for a hypothetical shift of the peak with increasing concentrations of MonNa. This curve is shown in both of the figures and can be seen to be in general agreement with the shapes of the experimental plots. However, the predicted behavior is far from accurate. The equation uses temperature independent shifts and also assumes that the fraction of each form as calculated from analytical concentrations is temperature independent. The observed plots tend to indicate that the fractions of each form are temperature dependent, with different behavior at -10°C than at ambient probe temperature. At very high complex to MonNa mole ratios or at low salt to complex ratios, the positions of the observed and theoretical peaks should agree. Visual extrapolation indicates that they do not, with the ambient result falling almost 1 ppm too far upfield. Even an unknown effect of the unassigned 10.4 ppm peak could not cause the observed chemical shift to move upfield.

The only explanation which seems to be reasonable is that the fractions of complex and salt are affected by their own interaction, perhaps exclusive of sodium ion exchange. This interaction, possibly of the conformational equilibrium type, would of course have a temperature dependent equilibrium constant. Calculations show that the -10°C data can be made to fit equation 9 if an equilibrium constant for interaction is incorporated. However the actual values obtained for such a constant cannot be meaningfully interpreted considering that no value of an equilibrium constant could ever suitably account for data at the ambient temperature. Yet the results are of interest in that they do point towards exchange which is temperature dependent and possible interconversion of monensin forms.

Other evidence obtained in this same experiment by monitoring the methyl region confirms this idea of conformational equilibration. The peaks which have always been most sensitive to conformational changes are those due to methyls 1 and 3. Their shifts are tabulated below.

Table 7. Positions of the 1 and 3 Methyl Groups in Several Monensin Solutions in Chloroform-d_1

	Chemical Shift from TMS at 60 MHz (Hz)			
	Methyl 1		Methyl 3	
Complex	66	82	59	75
1:1 Complex-MonNa	69	79	62	72
MonNa	68	75	72	79

The shifts in this range at -10°C are identical to those above, within experimental error of locating the peaks. Examination of the complex and 1:1 mixture reveals that the doublets in each case are both centered at 74 and 67 Hz respectively. The complex however, has a coupling constant of 16 Hz while the 1:1 mixture has a reduced J value of 10 Hz. This change in the direction of the 7 Hz value for MonNa is reasonably close to an average value of the constants calculated for a complex and salt mixture. Again, conformational changes giving rise to an average spectrum of both conformers seems to be indicated.

CONCLUSIONS

Average chemical shifts and coupling constants observed for the water molecules and methyl groups in the complex-MonNa mixtures indicate the possibility of the occurence of fast sodium ion exchange between the two types of monensin complexes or perhaps fast interconversion between the two monensin forms.

CHAPTER VI

BIMOLECULAR LIPID MEMBRANE STUDIES

INTRODUCTION

The bimolecular lipid membrane (BLM, singular and plural) system has often been used to test the ionic transport capabilities of various new synthetic and naturally occurring complexing agents. It was of interest to us to determine the ion transport properties of monensin as well as of some other compounds such as the tetrazoles, glutarimides and cryptands referred to previously.

INITIAL TESTS OF THE SYSTEM

It was found that the most stable BLM were formed with a 1:1 mixture of oxidized cholesterol and lecithin dissolved in normal octane. The electrical resistance of these BLM is usually slightly greater than 10^9 ohms with aqueous 0.10 M sodium chloride solutions. It was noted from the very outset of the experiments that many variables seemed to affect the formation of BLM and measurements of their properties. Among the more important factors to be considered were the mechanical stability of the system and the electrical shielding of the apparatus. Temperature also appeared to be a crucial variable although temperature control of the system was not maintained. Age of the various lipid solutions seemed to be of the utmost importance. Older lipid solutions often did not make BLM or made membranes of low or irreproducible resistance. Even with many precautions, on occasion, the formation, the stability or the resistance of a BLM would not follow the expected pattern.

Due to the fact that the system often behaved in a rather chemically ill-defined manner, all experiments took on a unique character and much repetition was an absolute necessity. Basically, the only justifiable conclusions regarding the testing of various ion transport compounds were that they either changed the BLM resistance or did not. Although quantitative estimates of resistance changes were available, they were, for the most part, not reproducible and only continued indications of resistance changes in many experiments allowed the qualitative conclusion that ion transport was occurring.

Initial testing of the system to observe its response to ionic permeability changes was accomplished with aqueous solutions of valinomycin, dicyclohexyl-18-crown-6 and the triodide ion. Several methods are often used for the introduction of modifying agents: direct addition to BLM via insertion in BLM forming solutions or addition to electrolyte before or after BLM formation. The second method was found to be the most reproducible and easiest and was used in all experiments.

Valinomycin in approximately 10^{-6} M concentrations in 0.10 M sodium chloride was found to change the BLM resistance by several orders of magnitude, often lowering it from approximately 10^{9} ohms to approximately 10^{5} ohms. The crown compound in more concentrated solutions of 10^{-3} M in the same electrolyte also showed this same resistance change characteristic of ion transport. Solutions of triodide ion made up with iodine and iodide in 0.10 M sodium chloride also lowered the BLM resistance 3 to 4 orders of magnitude. This effect is suspected to be due to the delocalization of the ionic

charge on I_3^- and on higher aggregates such as I_5^- and I_7^- which causes higher solubility of the ion in the lipid phase (66).

CONCLUSIONS

The BLM system responded to the addition of compounds known to cause ion transport through membranes. However, BLM experiments were found to be influenced by a host of variables, some of them unknown, and data were often found to offer only qualitative evidence of ionic transport.

MONENSIN AND OTHER ION COMPLEXING AGENTS

Although monensin has a reported water solubility of only about 0.1 mg/ml (1), this concentration (10^{-4} M) is more than sufficient for BLM testing. Saturated solutions of MonNa and MonH were prepared in aqueous 0.10 M sodium chloride and BLM formed in these solutions usually did show a decreased resistance. The change from the normal BLM resistance was not as great as the 4 orders of magnitude drop observed for valinomycin and crown, but usually exceeded a change of 2 orders of magnitude. Thus transport of sodium ions by monensin as had been previously observed in bulk solutions (25), natural (7) and synthetic membranes (34-38) also seems to occur through BLM.

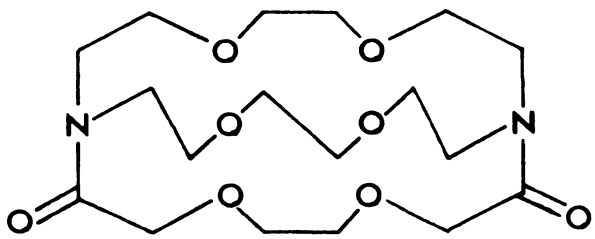
Various tetrazoles were also tested in the system. Solutions of 0.10 M trimethylenetetrazole, pentamethylenetetrazole (PMT), hexamethylenetetrazole and heptamethylenetetrazole were each made up in 0.10 M sodium chloride and BLM were formed in these solutions. No changes of the BLM resistances were ever observed in these experiments. Replacement of the sodium chloride with a 0.10 M potassium chloride electrolyte did not effect the BLM resistances.

Several BLM were formed from normal octane solutions which also contained either the protein histone or protamine in addition to the cholesterol-lecithin lipid mixtures. This produced BLM with the usual resistance (greater than 10^9 ohms) which were designed to be better models of the true biological membrane. Again, addition of tetrazoles had no detectable effect on the 10^9 ohm BLM electrical resistance. Similar tests were made with the convulsants glutarimide, 3,3-dimethyl-glutarimide and 3-ethyl-3-methylglutarimide with the same result. None of these compounds showed any resistance changes.

Although all of these molecules are weak ion complexing agents and are also highly biologically active, it would seem that they are not capable of transporting ions through BLM. Tetrazoles are known to be surface active (87) and have been shown to effect ion transport in isolated biological preparations (126). However, the complex which tetrazoles form with sodium ion is not of the charge insulating type, such as that of valinomycin or monensin, and the ionic solubilizing effect is not present. Glutarimides are also surface active drugs, but again, the weak complex with sodium ion does not change its solubility in the lipid phase.

The cryptand 222 (Figure 3) was also tested in the system, again using 0.10 M sodium or potassium chloride solutions. The ligand was found not to cause any resistance change at any concentration up to 10 mg/ml. Even though the cryptand is a strong complexing agent, it is quite basic in aqueous solutions. At pH 7, the nitrogen atoms are protonated and alkali ion complexes are not formed. However, experiments with the synthetic precursor to cryptand 222, the dilactam shown below,

Figure 39. The Dilactam Form of Cryptand 222



did cause a resistance drop of 2 to 3 orders of magnitude. The dilactam also forms alkali metal ion complexes and has been found to be much less basic in solution than cryptand 222 (127). Thus the metal ion is insulated and little charge remains on the outside of the molecule allowing for increased lipid solubility and BLM permeability changes.

CONCLUSIONS

Both MonNa and MonH have been found to facilitate ion transport across BLM. Tetrazoles and glutarimides, although they do form ion complexes, do not influence BLM permeability to sodium and potassium ions. The cryptand 222 does not affect BLM resistance in neutral solution, however the dilactam form is able to complex and transport ions through the BLM.

APPENDICES

APPENDIX I

APPLICATION OF COMPUTER PROGRAM KINFIT AND SUBROUTINE EQUATION FOR THE DETERMINATION OF ACID DISSOCIATION CONSTANTS

APPLICATION OF COMPUTER PROGRAM KINFIT AND SUBROUTINE EQUATION FOR THE DETERMINATION OF ACID DISSOCIATION CONSTANTS

As described in Chapter III, one approach to the determination of acid dissociation constants was to fit the observed titration data to the exact cubic equation for a weak acid-strong base system. These calculations were accomplished using the CDC 6500 computer and program KINFIT (99) by manipulation of SUBROUTINE EQN. This appendix details the manner in which the analysis was performed and lists the input necessary for proper operation of the program.

Equation (5),

$$(5) \quad [H^+]^3 + (C_b + K_a)[H^+]^2 + (K_a C_b - K_a C_a - K_s)[H^+] - K_a K_s = 0$$

with symbols as previously defined, can be solved by several analytical methods. A trigonometric solution was found to be the most useful. In the general cubic equation

$$(10) \quad y^3 + py^2 + qy + r = 0$$

if y is allowed to have the value x - p/3, the equation is reduced to the form

$$(11) \quad x^3 + ax + b = 0$$

where

$$(12)\quad a = \frac{1}{3}(3q - p^2) \qquad b = \frac{1}{27}(2p^3 - 9pq + 27r)$$

Then by computing the value of the angle ϕ in the equation below,

$$(13)\quad \cos\phi = -\frac{b}{2} \div \sqrt{(-\frac{a^3}{27})}$$

x is found to have the values

$$(14)\quad 2\sqrt{-\frac{a}{3}}\cos\frac{\phi}{3},\quad 2\sqrt{-\frac{a}{3}}\cos(\frac{\phi}{3} + 120°),\quad 2\sqrt{-\frac{a}{3}}\cos(\frac{\phi}{3} + 240°)$$

This solution was inserted into the SUBROUTINE EQN as shown in the listing. Thus a value of the calculated hydrogen ion concentration was obtained.

Since the hydrogen ion concentration is not the observed variable, it was necessary to convert this value to a voltage, which was accomplished via the equation

$$(15)\quad E = -0.05916 \log_{10} [H^+] + V_b$$

which is the general form of the Nernst equation with V_b serving to incorporate all constant terms. The equation appears in the program as

```
(16)  EE = -(CONST(5)*ALOG10(ABS(DD))) + U(1)
```

where EE should be the observed voltage, CONST(5) is the RT/nF term at 25°C with n = 1 and V_b has been made the unknown, U(1). The value of U(1) is the intercept of a Nernstian pH versus voltage plot and

was allowed to vary to give the best fit to the data. As mentioned previously, since the absolute values of the observed potentials are arbitrary, the value of U(1) is also arbitrary and does indeed vary slightly from run to run.

The other unknown parameter is the value for the acid dissociation constant, U(2). The variables, titrant volume and observed voltage, were assigned as shown. Values of V_b and K_a are varied in the program to give the best fit at each volume of the calculated voltage to that observed. Five constants were necessary for these calculations and were named as illustrated in the listing. Some improvements in the fit were noticed by allowing the initial basic titrant concentration also to be an unknown.

The format and structure of the control card and data deck for KINFIT have been exhaustively described previously (99,125,93) and detailed instructions as to their operation will be omitted. The normal ordering was always used with the control card first, giving the maximum number of iterations allowed, number of constants and convergence tolerance. A title card followed, after which came the card containing the values of the constants and fourth,the initial estimates of the unknowns. Voltage versus volume data completed the deck with relative variances attached in the usual manner.

This particular subroutine was found to be extremely sensitive to initial guesses of the K_a and V_b values, often converging very near the original estimates. Therefore, variation of the estimated values produced changes in the calculated values and also variation in the value of the total residuals calculated at convergence. This phenomenon

may have been due to local minima in the function. In any case, poor fits and poorer estimates of the known HBz K_a value caused this method to be abandoned.

```
ATTACH(KINFIT,KINFIT)
LOAD(KINFIT)
LGO.
00000000000000000000
      SUBROUTINE EQN
      COMMON KOUNT,ITAPE,JTAPE,IWT,LAP,XINCR,NOPT,NOVAR,NOUNK,X,U,ITMAX,
     1WTX,TEST,I,AV,RESID,IAR,EPS,ITYP,XX,RXTYP,DX1I,FOP,FO,FU,P,ZL,TO,E
     2IGVAL,XST,T,DT,L,M,JJJ,Y,DY,VECT,NCST,CONST
      DIMENSION X(4,100),U(20),WTX(4,100),XX(4),FOP(100),FO(100),F
     1U(100), P(20,21), VECT(20,21), ZL(100), TO(20), EIGVAL(20), XST(10
     20), Y(10), DY(10), CONST(16)
      CALL NOBLANK
      GO TO (2,3,4,5,1), ITYP
    1 CONTINUE
      ITAPE=60
      JTAPE=61
      WRITE (JTAPE,6)
    6 FORMAT(*WEAK ACID IN METHANOL TITRATIONS*)
      NOUNK=2
C     THE UNKNOWNS ARE U(1) THE PH VS V INTERCEPT
C                      U(2) THE ACID DISSOCIATION CONSTANT
      NOVAR=2
C     THE VARIABLES ARE XX(1) THE TITRANT VOLUME IN LITERS
C                       XX(2) THE OBSERVED VOLTAGE
C     THE CONSTANTS ARE CONST(1) THE NUMBER OF MOLES ACID
C                       CONST(2) THE INITIAL VOLUME
C                       CONST(3) THE TITRANT CONCENTRATION
C                       CONST(4) THE AUTO-PROTOLYSIS CONSTANT
C                       CONST(5) THE PH VS V SLOPE
      RETURN
    2 CONTINUE
```

```
C       GG IS THE ACID CONCENTRATION AT EACH TITRANT VOLUME
        GG=CONST(1)/(XX(1)+CONST(2))
C       S IS THE BASE CONCENTRATION AT EACH TITRANT VOLUME
        S=(XX(1)*CONST(3))/(XX(1)+CONST(2))
C       IN THE GENERAL FORM OF THE CUBIC EQUATION
C       A IS THE COEFFICIENT OF THE H SQUARED TERM
        A=S+U(2)
C       B IS THE COEFFICIENT OF THE H TERM
        B=(U(2)*(S-GG))-CONST(4)
C       C IS THE TERM WITH NO H
        C=-(U(2)*CONST(4))
C       THE FOLLOWING IS THE GENERAL SOLUTION OF THE CUBIC EQUATION
        AA=(1.0/3.0)*((3.0*B)-(A*A))
        BB=(1.0/27.0)*((2.0*(A**3))-(9.0*A*B)+(27.0*C))
        F=(-BB/2.0)/(SQRT((-AA**3)/27.0))
        FF=ACOS(F)
        FFF=FF/3.0
C       CC CHOOSES WHICH OF THE THREE ROOTS TO USE
        CC=2.0*SQRT(-AA/3.0)*COS(FFF)
C       DD IS THE HYDROGEN ION CONCENTRATION
        DD=CC-(A/3.0)
C       EE SHOULD BE THE OBSERVED VOLTAGE
        EE=-(CONST(5)*ALOG10(ABS(DD)))+U(1)
        RESID=EE-XX(2)
        RETURN
      3 CONTINUE
        RETURN
      4 CONTINUE
        RETURN
      5 CONTINUE
        RETURN
        END
```

```
00000000000000000000
   45      100                          50.00001
                    BENZOIC ACID WITH SODIUM METHOXIDE
0.0010    0.05000   0.0400    3.3E-16   0.05916
0.450     4.0E-08
0.00000   4.0E-04   1.096     1.0       0.00020   4.0E-04   1.129   1.0
0.00040   4.0E-04   1.145     1.0       0.00060   4.0E-04   1.154   1.0
0.00080   4.0E-04   1.161     1.0       0.00100   4.0E-04   1.165   1.0
0.00150   4.0E-04   1.170     1.0       0.00200   4.0E-04   1.175   1.0
0.00300   4.0E-04   1.182     1.0       0.00400   4.0E-04   1.188   1.0
0.00500   4.0E-04   1.194     1.0       0.00600   4.0E-04   1.200   1.0
0.00700   4.0E-04   1.205     1.0       0.00800   4.0E-04   1.208   1.0
0.00900   4.0E-04   1.214     1.0       0.01000   4.0E-04   1.217   1.0
0.01100   4.0E-04   1.220     1.0       0.01200   4.0E-04   1.223   1.0
0.01300   4.0E-04   1.227     1.0       0.01400   4.0E-04   1.230   1.0
0.01500   4.0E-04   1.234     1.0       0.01600   4.0E-04   1.237   1.0
0.01700   4.0E-04   1.241     1.0       0.01800   4.0E-04   1.245   1.0
0.01900   4.0E-04   1.249     1.0       0.02000   4.0E-04   1.253   1.0
0.02100   4.0E-04   1.259     1.0       0.02200   4.0E-04   1.264   1.0
0.02300   4.0E-04   1.272     1.0       0.02350   4.0E-04   1.278   1.0
0.02400   4.0E-04   1.284     1.0       0.02420   4.0E-04   1.286   1.0
0.02450   4.0E-04   1.291     1.0       0.02500   4.0E-04   1.300   1.0
0.02520   4.0E-04   1.305     1.0       0.02550   4.0E-04   1.315   1.0
0.02580   4.0E-04   1.333     1.0       0.02600   4.0E-04   1.350   1.0
0.02650   4.0E-04   1.377     1.0       0.02700   4.0E-04   1.394   1.0
0.02800   4.0E-04   1.401     1.0       0.03000   4.0E-04   1.407   1.0
0.03300   4.0E-04   1.408     1.0       0.03600   4.0E-04   1.410   1.0
0.04200   4.0E-04   1.412     1.0
00000000000000000000
00000000000000000000
```

APPENDIX II

APPLICATION OF COMPUTER PROGRAM STEUHR FOR THE DETERMINATION OF ACID DISSOCIATION CONSTANTS

APPLICATION OF COMPUTER PROGRAM STEUHR FOR THE
DETERMINATION OF ACID DISSOCIATION CONSTANTS

As explained in Chapter III, a successful method for the computation of acid dissociation constants was developed using a modified version of a program written by Briggs and Steuhr (98,128). In this appendix a short description of program STEUHR is presented as well as a listing including a sample data deck.

The exact expression for the titration of a weak acid with a strong base can be arranged as

$$(6) \quad V + R - \Delta V = -a_{H^+}(V + R - \Delta V)\frac{1}{K_a} + V_e$$

with parameters previously defined in Chapter III. This equation is in the form $y = mx + b$ with slope $1/K_a$ and intercept V_e. The program simply uses a least squares fit to this linear equation, adjusting the value of ΔV to give the best straight line and computing the values of the acid dissociation constant and the equivalence point volume. The original version of the program is also capable of making activity corrections for titrations performed in aqueous solutions in a medium of high ionic strength. This option has not been used. Instead, another option has been inserted which allows the program to read in voltage versus volume data rather than pH versus volume data. Conversion to pH is then accomplished internally via an equation which

subtracts the intercept V_b from each voltage and then divides by the Nernstian slope, 0.05916.

Other parameters are calculated in the program as the listing shows. The meaning of these calculations and other operational details are fully explained in the Briggs and Steuhr articles (99,129). Photocopies of the program are available from the authors, but many changes must be made for use on the CDC 6500 since FORTRAN IV has not been used. In the program shown in the listing, the minimum number of alterations have been made.

Input for the program, read in the formats shown, consists of the number of curves to be analyzed, name of the acid, experiment date, name of supporting medium (here, none), the initial incrementing value of ΔV, the initial volume of acid solution, the concentration of basic titrant, the temperature, the number of voltage versus volume points, the maximum number of iterations allowed, the Nernstian intercept, V_b, and the concentration of the medium (here, 0). These values are shown for a typical run of HBz data in the listing. All volumes are read consecutively (F10.0) and then corresponding voltages are inserted (F10.0). The authors recommend using data between 5% and 90% of the equivalence volume where the highest accuracy is obtained and this was found to work quite well.

```
      PROGRAM STEUHR (INPUT, OUTPUT, TAPE60=INPUT, TAPE61=OUTPUT)
C     THIS PROGRAM FINDS THE BEST VALUES OF THE PK, VE AND DV FOR SINGLE
C     PK SYSTEMS
    1 FORMAT(I5)
    2 FORMAT (4A10)
    3 FORMAT (2A10)
    4 FORMAT (2A12)
    5 FORMAT (8F10.0)
   11 FORMAT (4F10.0,2I10,2F10.0)
   12 FORMAT (8F10.0)
   13 FORMAT (8F10.0)
   40 FORMAT (1H1, 14X, 15HMIXED PKA,S OF , 25X, 12H EXP. DATE. , 2A7)
   42 FORMAT (1H+, 29X, 4A6///)
  171 FORMAT (2X, F10.4, 8X, F10.3, F20.3, 4X, F20.5)
  191 FORMAT (1HO, 6X, 6HVE = F10.6, 4X, 6HDV = F12.6)
  195 FORMAT (1HO, 6X, 26HNO. OF ITERATIONS    = I5)
  196 FORMAT (1HO, 6X, 26HSTANDARD DEVIATION   = F12.6)
  197 FORMAT (1HO, 6X, 26HACT. COEFF. OF H+    =F12.6)
  198 FORMAT (1HO, 6X, 26HCOEFFICIENT OF CORRELATN = F12.6)
  199 FORMAT (1HO, 6X, 26HCOEFFICIENT OF VARIATION = F12.6, 3H P )
  200 FORMAT (1HO, 6X, 26HCONC. OF WEAK ACID       =F12.6)
  201 FORMAT (1HO, 6X, 26HCONC. OF STRONG BASE     =F12.6)
  210 FORMAT (1HO, 7X, 3HVOL, 15X, 2HPH, 17X, 4H PKA, 20X, 4HDPKA/)
  225 FORMAT (1H1, 1X)
  230 FORMAT (1HO, 6X,6HPKA1 =F9.5)
  290 FORMAT (7X, 6HTEMP = F5.1,6X,6HBO   =F8.5,3X,5HVO  =F6.2,5X,
     B5HMED =A11,14X,8HINTER  =F5.3/)
      DIMENSION VOL(30), PH(30), H(30), X(30), Y(30), PK(30),V(30)
      DIMENSION T(30), F1(30), XZ(30)
      DIMENSION DATE(2), MED(2), LIG(4)
      REAL K1,KC,KB1,KM,KW,LOGF1,LN,MDEV,MDV
      INTEGER TAPE60, TAPE61
```

```
TAPE60=60
TAPE61=61
READ (TAPE60,1) NSYSTM
DO 1600 NS=1,NSYSTM
READ (TAPE60,2) LIG(1), LIG(2), LIG(3), LIG(4)
READ (TAPE60,3) DATE(1), DATE(2)
READ (TAPE60,4) MED(1), MED(2)
READ (TAPE60,11) DV,VO,BO,TEMP,JB,NW,BB,CMED
READ (TAPE60,12) (VOL(I), I=1, JB)
READ (TAPE60,13) (V(I), I=1,JB)
WRITE (TAPE61, 40) DATE(1), DATE(2)
WRITE (TAPE61, 42) LIG(1), LIG(2), LIG(3), LIG(4)
WRITE (TAPE61, 290) TEMP, BO, VO, MED(1), BB
WRITE (TAPE61,210)
KW = 3.3E-16
GH = 1.00
NIT=0
DO 803 I = 1, JB
TV = VO+VOL(I)
SL = 0.05916
PH(I) = (V(I) - BB)/SL
H(I) = 10.0**(-PH(I))
CH = H(I)/GH
COH = KW/CH
R = ((CH-COH)*TV)/BO
T(I) = VOL(I) +R
CS = (CMED*VO)/TV
CB = (VOL(I)*BO)/TV
U = CH+CB+CS
ROOTU = SQRT(U)
LOGF1 = -0.50*(ROOTU/(1.0+ROOTU)-0.3*U-0.21025)
F1(I) = 10.0**(LOGF1)
```

```
803 CONTINUE
    LN = 1.0E+20
    MDEV = LN
    DEV = LN
    NCD = 0
    DDV = 0.002
    DO 1308 NA = 1, NW
    NIT = NIT+1
    IF (NCD.EQ.NW) DV = MDV
    DEV1 = DEV
    DO 890 I = 1, JB
    Y(I) = T(I)-DV
    X(I) = -H(I)*Y(I)
890 CONTINUE
    SUM3 = 0.00
    SUM4 = 0.00
    SUM5 = 0.00
    SUM6 = 0.00
    DO 895 I = 1, JB
    SUM3 = SUM3+Y(I)
    SUM4 = SUM4+X(I)
    SUM5 = SUM5+X(I)*Y(I)
    SUM6 = SUM6+X(I)*X(I)
895 CONTINUE
    RR = JB*SUM6-SUM4*SUM4
    KM = (JB*SUM5-SUM4*SUM3)/RR
    KC = (SUM3*SUM6-SUM4*SUM5)/RR
    K1 = KM
    VE = KC
    SUM1 = 0.00
    DO 940 I = 1, JB
    XZ(I) = (Y(I)-VE)/X(I)
    SUM1 = SUM1+XZ(I)
```

```
 940 CONTINUE
     KBI = SUM1/JB
     SUM2 = 0.00
     DO 970 I = 1, JB
     G = (XZ(I)-KB1)
     SUM2 = SUM2+G*G
 970 CONTINUE
     DEV = SUM2/(KB1*KB1)
     IF (NCD.EQ.NW) GO TO 1310
     IF (DEV.GT.MDEV) GO TO 1015
     MDEV = DEV
     MDV = DV
1015 IF (DEV.LT.DEV1) GO TO 1021
     DDV = -0.45*DDV
1021 DV = DV+DDV
     IF (ABS(DDV).GT.0.00005) GO TO 1308
     NCD = NA
1308 CONTINUE
1310 SD = SQRT(SUM2/(JB-1))
     COV = (100.00*SD)/K1
     XB = SUM4/JB
     YB = SUM3/JB
     SUM1 = 0.00
     SUM2 = 0.00
     SUM3 = 0.00
     DO 1350 I = 1, JB
     XX = X(I) - XB
     YY = Y(I) - YB
     SUM1 = SUM1+XX*XX
     SUM2 = SUM2+YY*YY
     SUM3 = SUM3+XX*YY
1350 CONTINUE
     COC = SQRT((SUM3*SUM3)/(SUM1*SUM2))
     SUM3 = 0.00
     DO 1450 I = 1, JB
```

```
      IF (XZ(I).LT.1.0) GO TO 1450
      PK(I) = ALOG10(XZ(I))
      SUM3 = SUM3+PK(I)
1450  CONTINUE
      PKA1 = SUM3/JB
      DO 1460 I = 1, JB
      G = PK(I)-PKA1
      WRITE (TAPE61, 171) VOL(I), PH(I), PK(I), G
1460  CONTINUE
      SUM7=0
      DO 1470 I = 1,JB
      G = PK(I) - PKA1
      SUM7 = SUM7+ (G*G)
1470  CONTINUE
      SDP = SQRT(SUM7/(JB-1))
      WRITE (TAPE61, 230) PKA1
      WRITE (TAPE61, 191) VE, DV
      WRITE (TAPE61, 197) GH
      WRITE (TAPE61, 196) SDP
      WRITE (TAPE61, 199) COV
      WRITE (TAPE61, 198) COC
      A = (VE*BO)/VO
      B = (DV*BO)/VO
      WRITE (TAPE61, 200) A
      WRITE (TAPE61, 201) B
      NIT = NIT-1
      WRITE (TAPE61, 195) NIT
1600  CONTINUE
      WRITE (TAPE61, 225)
      STOP
      END
00000000000000000000
```

```
    1
HBZ
7.23.74
NONE
     -1.25      50.00      0.0430      25.0        20      5000      0.595       0.0
      2.00       2.50        3.00      4.00      5.00      6.00       7.00      8.08
      9.00      10.03       11.00     12.00     13.00     14.00      15.00     16.00
     17.02      18.04      19.00      20.00
     1.095      1.100      1.104      1.110     1.116     1.121      1.127     1.132
     1.136      1.141      1.145      1.149     1.154     1.158      1.163     1.168
     1.174      1.180      1.189      1.202
00000000000000000000
```

APPENDIX III

APPLICATION OF COMPUTER PROGRAM KINFIT AND SUBROUTINE EQUATION FOR THE DETERMINATION OF COMPLEXATION CONSTANTS

APPLICATION OF COMPUTER PROGRAM KINFIT AND SUBROUTINE EQUATION FOR THE DETERMINATION OF COMPLEXATION CONSTANTS

The computer was again employed in fitting voltage versus volume data to theoretical equations in calculation of the overall monensin complexation constant. The KINFIT program was used in a manner analogous to that explained in Appendix I. Additional information necessary to fit the complexometric titration data is included here as well as listings of the two SUBROUTINE EQNS used.

As mentioned previously, equation (7)

$$(7)\quad K \cong \frac{[H^+]^2}{(C_a - [H^+])(C_{Na^+} - [H^+])}$$

is easily solved for hydrogen ion concentration in the quadratic below.

$$(17)\quad [H^+] = \frac{-(KC_{Na^+} + KC_a) + \sqrt{(KC_{Na^+} + KC_a)^2 + 4(1 - K)(KC_aC_{Na^+})}}{2(1 - K)}$$

The general solution for the quadratic was inserted in the subroutine and hydrogen ion concentration was converted to voltage in the manner previously described. Again, U(1) served as the unknown V_b and here U(2) was the overall complexation constant. As shown in the listing, necessary constants for this calculation were the number of moles of acid, the initial solution volume, the concentration of the titrant and the Nernstian slope.

The control card and deck structure were strictly analogous to that used previously and are shown in the list. Although the program was somewhat sensitive to the initial estimate of the equilibrium constant, once a reasonable value was chosen convergence occurred quite rapidly.

```
ATTACH(KINFIT,KINFIT)
LOAD(KINFIT)
LGO.
00000000000000000000
      SUBROUTINE EQN
      COMMON KOUNT,ITAPE,JTAPE,IWT,LAP,XINCR,NOPT,NOVAR,NOUNK,X,U,ITMAX,
     1WTX,TEST,I,AV,RESID,IAR,EPS,ITYP,XX,RXTYP,DX1I,FOP,FO,FU,P,ZL,TO,E
     2IGVAL,XST,T,DT,L,M,JJJ,Y,DY,VECT,NCST,CONST
      DIMENSION X(4,100), U(20), WTX(4,100), XX(4), FOP(100), FO(100), F
     1U(100), P(20,21), VECT(20,21), ZL(100), TO(20), EIGVAL(20),XST(10
     20), Y(10), DY(10), CONST(16)
      CALL NOBLANK
      GO TO (2,3,4,5,1), ITYP
    1 CONTINUE
      ITAPE=60
      JTAPE=61
      WRITE (JTAPE,6)
    6 FORMAT(*MONENSIN COMPLEXATION IN METHANOL*)
      NOUNK=2
C     THE UNKNOWNS ARE  U(1) THE PH VS V INTERCEPT IN VOLTS
C                       U(2) THE OVER-ALL EQUILIBRIUM CONSTANT
      NOVAR=2
C     THE VARIABLES ARE XX(1) THE TITRANT VOLUME IN LITERS
C                       XX(2) THE OBSERVED VOLTAGE
C     THE CONSTANTS ARE CONST(1) THE NUMBER OF MOLES ACID
C                       CONST(2) THE INITIAL VOLUME
C                       CONST(3) THE TITRANT CONCENTRATION
C                       CONST(4) THE PH VS V SLOPE
      RETURN
```

```
    2 CONTINUE
C     GG IS THE ACID CONCENTRATION AT EACH TITRANT VOLUME
      GG=CONST(1)/(XX(1)+CONST(2))
C     S IS THE METAL ION CONCENTRATION AT EACH TITRANT VOLUME
      S=(XX(1)*CONST(3))/(XX(1)+CONST(2))
C     IN THE APPROXIMATE SOLUTION TO THE MULTIPLE EQUILIBRIA PROBLEM
C     A IS THE COEFFICIENT OF THE H SQUARED TERM
      A=1.0 - U(2)
C     B IS THE COEFFICIENT OF THE H TERM
      B=(U(2)*S)+(U(2)*GG)
C     C IS THE TERM WITH NO H
      C=-(U(2)*S*GG)
C     DD IS THE H ION CONCENTRATION
      DD=(1.0/2.0*A)*(-B+SQRT((B**2)-4.0*A*C))
C     EE SHOULD BE THE OBSERVED VOLTAGE
      EE=-(CONST(4)*ALOG10(ABS(DD)))+U(1)
      RESID=EE-XX(2)
      RETURN
    3 CONTINUE
      RETURN
    4 CONTINUE
      RETURN
    5 CONTINUE
      RETURN
      END
```

```
00000000000000000000
   20       100                          40.00001
                                 NACLO4
9.753E-05 0.05000   5.016E-03 0.05916
1.0       5.0E-02
0.00040   4.0E-04   1.087     1.0       0.00060   4.0E-04   1.074     1.0
0.00080   4.0E-04   1.068     1.0       0.00120   4.0E-04   1.058     1.0
0.00150   4.0E-04   1.051     1.0       0.00200   4.0E-04   1.046     1.0
0.00250   4.0E-04   1.040     1.0       0.00300   4.0E-04   1.036     1.0
0.00400   4.0E-04   1.032     1.0       0.00500   4.0E-04   1.028     1.0
0.00600   4.0E-04   1.025     1.0       0.00800   4.0E-04   1.023     1.0
0.01000   4.0E-04   1.021     1.0       0.01200   4.0E-04   1.020     1.0
0.01600   4.0E-04   1.019     1.0       0.02000   4.0E-04   1.019     1.0
0.02500   4.0E-04   1.019     1.0       0.03000   4.0E-04   1.019     1.0
0.03500   4.0E-04   1.019     1.0       0.04000   4.0E-04   1.019     1.0

00000000000000000000
```

In using the KINFIT program with equation (8) several changes were necessary. The general solution for the cubic equation, as shown in Appendix I, was again inserted into the program replacing the quadratic solution. Variables and unknowns remained the same, but the addition of the constant K_a was necessary bringing the number of constants to five.

This modified approach to the problem worked successfully with the lithium, cesium, rubidium, ammonium and silver data where equations (6) and (17) would not converge. The listing of this program with a sample data deck is shown below.

```
ATTACH(KINFIT,KINFIT)
LOAD(KINFIT)
LGO.
00000000000000000000
       SUBROUTINE EQN
       COMMON KOUNT,ITAPE,JTAPE,IWT,LAP,XINCR,NOPT,NOVAR,NOUNK,X,U,ITMAX,
      1WTX,TEST,I,AV,RESID,IAR,EPS,ITYP,XX,RXTYP,DX1I,FOP,FO,FU,P,ZL,TO,E
      2IGVAL,XST,T,DT,L,M,JJJ,Y,DY,VECT,NCST,CONST
       DIMENSION X(4,100), U(20), WTX(4,100), XX(4), FOP(100), FO(100), F
      1U(100), P(20,21), VECT(20,21), ZL(100), TO(20), EIGVAL(20), XST(10
      20), Y(10), DY(10), CONST(16)
       CALL NOBLANK
       GO TO (2,3,4,5,1), ITYP
     1 CONTINUE
       ITAPE=60
       JTAPE=61
       WRITE (JTAPE,6)
     6 FORMAT(* MONENSIN COMPLEXATION IN METHANOL*)
       NOUNK=2
C      THE UNKNOWNS ARE U(1) THE PH VS V INTERCEPT IN VOLTS
C                       U(2) THE OVER-ALL EQUILIBRIUM CONSTANT
       NOVAR=2
C      THE VARIABLES ARE XX(1) THE TITRANT VOLUME IN LITERS
C                        XX(2) THE OBSERVED VOLTAGE
C      THE CONSTANTS ARE CONST(1) THE NUMBER OF MOLES ACID
C                        CONST(2) THE INITIAL VOLUME
C                        CONST(3) THE TITRANT CONCENTRATION
C                        CONST(4) THE ACID KA VALUE
C                        CONST(5) THE PH VS V SLOPE
```

```
      RETURN
    2 CONTINUE
C     GG IS THE ACID CONCENTRATION AT EACH TITRANT VOLUME
      GG=CONST(1)/(XX(1)+CONST(2))
C     S IS THE METAL ION CONCENTRATION AT EACH TITRANT VOLUME
      S=(XX(1)*CONST(3))/(XX(1)+CONST(2))
C     IN THE EXACT SOLUTION TO THE MULTIPLE EQUILIBRIA PROBLEM
C     D IS THE COEFFICIENT OF THE H CUBED TERM
      D=U(2)-1.
C     A IS THE COEFFICIENT OF THE H SQUARED TERM
      A=(CONST(4)*U(2)-CONST(4)-U(2)*GG-U(2)*S)/D
C     B IS THE COEFFICIENT OF THE H TERM
      B=(CONST(4)*GG-2.*U(2)*CONST(4)*GG+U(2)*GG*S)/D
C     C IS THE TERM WITH NO H
      C=(CONST(4)*U(2)*GG**2)/D
C     THE FOLLOWING IS THE GENERAL SOLUTION OF THE CUBIC EQUATION
      AA=(1.0/3.0)*((3.0*B)-(A*A))
      BB=(1.0/27.0)*((2.0*(A**3))-(9.0*A*B)+(27.0*C))
      F=(-BB/2.0)/(SQRT((-AA**3)/27.0))
      FF=ACOS(F)
      FFF=FF/3.0
C     CC CHOOSES WHICH OF THE THREE ROOTS TO USE
      CC=2.0*SQRT(-AA/3.0)*COS(FFF)
C     DD IS THE HYDROGEN ION CONCENTRATION
      DD=CC-(A/3.0)
C     EE SHOULD BE THE OBSERVED VOLTAGE
      EE=-(CONST(5)*ALOG10(ABS(DD)))+U(1)
      RESID=EE-XX(2)
      RETURN
    3 CONTINUE
      RETURN
    4 CONTINUE
      RETURN
    5 CONTINUE
      RETURN
      END
```

```
00000000000000000000
   14      100                          50.0001
                                  LICLO4
9.796E-05 0.05000   5.029E-03     7.1E-11 0.05916
1.0       5.0E-10
0.00040   4.0E-04   1.149      1.0       0.00060   4.0E-04   1.146    1.0
0.00080   4.0E-04   1.142      1.0       0.00100   4.0E-04   1.140    1.0
0.00200   4.0E-04   1.135      1.0       0.00300   4.0E-04   1.131    1.0
0.00500   4.0E-04   1.128      1.0       0.00800   4.0E-04   1.124    1.0
0.01200   4.0E-04   1.121      1.0       0.01700   4.0E-04   1.119    1.0
0.02200   4.0E-04   1.118      1.0       0.03000   4.0E-04   1.117    1.0
0.04000   4.0E-04   1.117      1.0       0.04800   4.0E-04   1.117    1.0

00000000000000000000
```

LITERATURE CITED

LITERATURE CITED

(1) A. Agtarap, J. W. Chamberlin, M. Pinkerton and L. K. Steinrauf, J. Am. Chem. Soc., 89, 5737 (1967).

(2) M. E. Haney and M. M. Hoehn, Antimicrob. Agents Chemother., 1967, 349 (1968).

(3) W. M. Stark, N. G. Knox and J. E. Westhead, Antimicrob. Agents Chemother., 1967, 353 (1968).

(4) A. Agtarap and J. W. Chamberlin, Antimicrob. Agents Chemother., 1967, 359 (1968).

(5) M. Gorman, J. W. Chamberlin and R. L. Hamill, Antimicrob. Agents Chemother., 1967, 363 (1968).

(6) R. F. Shumard and M. E. Callender, Antimicrob. Agents Chemother., 1967, 369 (1968).

(7) S. Estrada-O., B. Rightmire and H. A. Lardy, Antimicrob. Agents Chemother., 1967, 279 (1968).

(8) A. Thore,D. L. Keister, N. Shavit and A. San Pietro, Biochemistry, 7, 3499 (1968).

(9) M. Nishimura and B. C. Pressman, Biochemistry, 8, 1360 (1969).

(10) A. L. Donoho and R. M. Kline, Antimicrob. Agents Chemother., 1967, 763 (1968).

(11) R. L. Harned, P. H. Hidy, C. J. Corum and K. L. Jones, Antibiotics Chemother., 9, 594 (1951).

(12) J. Shoji, S. Kozuku, S. Matsutani, T. Kubota, H. Nishimura, M. Mayama, K. Motokawa, Y. Tanaka, N. Shimaoka and H. Otsuka, J. Antibiotics, 21, 402 (1968).

(13) T. Staron, C. Esteve, A. Kergomard, H. Veschambre, P. Gachon, M. Alleaume and D. Hickel, Ann. Phytopathol., 2, 555 (1970).

(14) L. K. Steinrauf and M. Pinkerton, Biochem. Biophys. Res. Commun., 33, 29 (1968).

(15) T. Kubota and S. Matsutani, J. Chem. Soc. (C), 695 (1970).

(16) P. Gachon, A. Kergomard and H. Veschambre, Chem. Commun., 1421, 1422 (1970).

(17) A. J. Geddes, B. Sheldrick, W. T. J. Stevenson and L. K. Steinrauf, Biochem. Biophys. Res. Commun., 60, 1245 (1974).

(18) E. W. Czerwinski and L. K. Steinrauf, Biochem. Biophys. Res. Commun., 45, 1284 (1971).

(19) B. C. Pressman, E. J. Harris, W. S. Jagger and J. H. Johnson, Proc. Nat. Acad. Sci. U.S., 58, 1949 (1967).

(20) D. C. Tosteson, T. E. Andreoli, M. Tieffenberg and P. Cook, J. Gen. Physiol., 51, 373S (1968).

(21) M. Gorman and R. L. Hamill, U. S. Patent 3,555,150, 12 Jan 1971.

(22) B. C. Pressman and D. H. Haynes, "The Molecular Basis of Membrane Function", Prentice-Hall, Englewood Cliffs, N.J., 1969, p. 221.

(23) B. C. Pressman, Fed. Proc., Fed. Am. Soc. Exp. Biol., 27, 1283 (1968).

(24) M. Pinkerton and L. K. Steinrauf, J. Mol. Biol., 49, 533 (1970).

(25) R. Ashton and L. K. Steinrauf, J. Mol. Biol., 49, 547 (1970).

(26) J. W. Chamberlin and A. Agtarap, Org. Mass Spectrom., 3, 271 (1970).

(27) W. K. Lutz, H. K. Wipf and W. Simon, Helv. Chim. Acta, 53, 1741 (1970).

(28) L. K. Steinrauf, E. W. Czerwinski and M. Pinkerton, Biochem. Biophys. Res. Commun., 45, 1279 (1971).

(29) W. K. Lutz, F. K. Winkler and J. D. Dunitz, Helv. Chim. Acta, 54, 1103 (1971).

(30) W. K. Lutz, P. U. Früh and W. Simon, Helv. Chim. Acta, 54, 2767 (1971).

(31) a) P. U. Früh, J. T. Clerc and W. Simon, Helv. Chim. Acta, 54, 1445 (1971).
b) W. E. Morf and W. Simon, Helv. Chim. Acta, 54, 2683 (1971).
c) W. Simon, W. E. Morf and P. C. Meier, Struct. Bonding (Berlin), 16, 113 (1973).

(32) D. H. Haynes, B. C. Pressman and A. Kowalsky, Biochemistry, 10, 852 (1971).

(33) B. C. Pressman, Fed. Proc., Fed. Am. Soc. Exp. Biol., 32, 1698 (1973).

(34) E. L. Cussler, D. F. Evans and M. A. Matesich, Science, 177, 377 (1971).

(35) E. L. Cussler, AIChE J., 17, 1300 (1971).

(36) E. M. Choy, D. F. Evans and E. L. Cussler, J. Am. Chem. Soc., 96, 7085 (1974).

(37) Chem. Eng. News, 52, 35 (1974).

(38) E. L. Cussler and M. M. Breuer, AIChE J., 18, 812 (1972).

(39) H. W. Huang, J. Theor. Biol., 32, 351 (1971).

(40) H. W. Huang, J. Theor. Biol., 32, 363 (1971).

(41) N. Shavit, D. Hadassa and A. San Pietro, Biochim. Biophys. Acta, 216, 208 (1970).

(42) S. Estrada-O., C. De Cespedes and E. Calderon, J. Bioenerg., 3, 361 (1972).

(43) H. Walkowiak, Life Sci., 15, 1353 (1974).

(44) F. W. Kavanagh and M. Willis, J. Ass. Offc. Anal. Chem., 55, 114 (1972).

(45) H. L. Breunig, R. M. Kline and H. Bikin, J. Ass. Offc. Anal. Chem., 55, 718 (1972).

(46) T. Golab, S. J. Barton and R. E. Scroggs, J. Ass. Offc. Anal. Chem., 56, 171 (1973).

(47) W. J. Begue and R. M. Kline, J. Chromatogr., 64, 182 (1972).

(48) A. P. Raun, Fr. Demande 2,169,201, 12 Oct 1973.

(49) W. M. Reid, L. Kowalski and J. Rice, Poultry Sci., 51, 139 (1972).

(50) R. E. Hurst, E. J. Day and B. C. Dilworth, Poultry Sci., 53, 434 (1974).

(51) J. Biely, Avian Dis., 17, 362 (1973).

(52) Anon, Fed. Regist., 39, 37056 (1974).

(53) Anon, Fed. Regist., 38, 21921 (1973).

(54) P. R. Fitzgerald, J. Protozool., 19, 286 (1972).

(55) P. R. Fitzgerald and M. E. Mansfield, J. Protozool., 20, 121 (1973).

(56) E. L. Potter, C. O. Cooley, A. P. Raun, L. F. Richardson and R. P. Rathmacher, Proc., West. Sect., Am. Soc. Anim. Sci., 25, 343 (1974).

(57) A. P. Raun, C. O. Cooley, R. P. Rathmacher, L. F. Richardson and E. L. Potter, Proc., West. Sect., Am. Soc. Anim. Sci., 25, 346 (1974).

(58) H. Brown, L. H. Carroll, N. G. Elliston, H. P. Grueter, J. W. McAskill, R. D. Olson and R. P. Rathmacher, Proc., West. Sect., Am. Soc. Anim. Sci., 25, 300 (1974).

(59) L. E. Day, J. W. Chamberlin, E. Z. Gordee, S. Chen, M. Gorman, R. L. Hamill, T. Ness, R. E. Weeks and S. Stroshane, Antimicrob. Agents Chemother., 4, 410 (1973).

(60) D. R. Brannon and D. R. Horton, Ger. Offen. 2,404,958, 22 Aug 1974.

(61) E. J. Corey, K. C. Nicolaou and L. S. Melvin, J. Am. Chem. Soc., 97, 653 (1975).

(62) P. Mueller, D. O. Rudin, H. T. Tien and W. C. Wescott, Nature, 194, 979 (1962).

(63) P. Mueller, D. O. Rudin, H. T. Tien and W. C. Wescott, Circulation, 26, 1167 (1962).

(64) P. Mueller, D. O. Rudin, H. T. Tien and W. C. Wescott, J. Phys. Chem., 67, 534 (1963).

(65) H. T. Tien, "Bilayer Lipid Membranes (BLM) Theory and Practice", Marcel Dekker, New York, N.Y., 1974.

(66) M. K. Jain, "The Bimolecular Lipid Membrane: A System", Van Nostrand Reinhold, New York, N.Y., 1972.

(67) G. Eisenman (ed.), "Membranes - A Series of Advances", Vol. 1,2, Marcel Dekker, New York, N.Y., 1972.

(68) P. Mueller and D. O. Rudin, Biochem. Biophys. Res. Commun., 26, 398 (1967).

(69) B. C. Pressman, E. J. Harris, W. S. Jagger and J. H. Johnson, Proc. Nat. Acad. Sci. U.S., 58, 1949 (1967).

(70) T. E. Andreoli, M. Tieffenberg and D. C. Tosteson, J. Gen. Physiol., 50, 2527 (1967).

(71) D. C. Tosteson, P. Cook, T. E. Andreoli and M. Tieffenberg, J. Gen. Physiol., 50, 2513 (1967).

(72) G. Szabo, G. Eisenman and S. M. Ciani, Fed. Proc., Fed. Am. Soc. Exp. Biol., 27, 1289 (1968).

(73) C. J. Pederson, J. Am. Chem. Soc., 92, 391 (1970).

(74) G. Szabo, G. Eisenman and S. M. Ciani, J. Memb. Biol., 1, 346 (1969).

(75) P. J. F. Henderson, J. D. McGivan and J. B. Chappell, Biochem. J., 111, 521 (1969).

(76) S. M. F. Ferguson, S. Estrada-O. and H. A. Lardy, J. Biol. Chem., 246, 5645 (1971).

(77) F. R. Benson, Chem. Rev., 41, 1 (1947).

(78) F. Hahn, Pharmacol. Rev., 12, 447 (1960).

(79) E. A. Swinyard, D. O. Schiffman and L. S. Goodman, J. Pharmacol. Exp. Ther., 114, 160 (1955).

(80) J. W. Prichard, Brain Research, 27, 414 (1971).

(81) E. D. Robertis, M. Alberici and G. R. De Lores Arnaiz, Brain Research, 12, 461 (1969).

(82) E. G. Gross and R. M. Featherstone, J. Pharmacol. Exp. Ther., 87, 291, 299 (1946); 88, 353 (1946); 92, 323, 330 (1948).

(83) W. E. Stone, Pharmacology, 3, 367 (1970).

(84) A. I. Popov, Coord.Chem. Rev., 4, 463 (1969).

(85) R. L. Bodner, M. S. Greenberg and A. I. Popov, Spectrosc. Lett., 5, 489 (1972).

(86) E. T. Roach, P. R. Handy and A. I. Popov, Inorg. Nucl. Chem. Lett., 9, 359 (1973).

(87) A. S. Buchanan, G. M. Laycock and A. Shulman, European J. Pharmacol., 7, 60 (1969).

(88) A. Shulman, Proc. Roy. Austral. Chem. Inst., 31, 41 (1964).

(89) G. M. Laycock and A. Shulman, Nature, 200, 849 (1963).

(90) F. H. Shaw, S. E. Simon, N. Cass, A. Shulman, J. R. Anstee and E. R. Nelson, Nature, 174, 402 (1954).

(91) T. C. Somers, Nature, 178, 996 (1956).

(92) Y. M. Cahen, R. F. Beisel and A. I. Popov, J. Inorg. Nucl. Chem., in press.

(93) Y. M. Cahen, Ph.D. Thesis, Michigan State University, East Lansing, Michigan (1975).

(94) B. Dietrich, J. M. Lehn and J. P. Sauvage, Tetrahedron Lett., 34, 2885 (1969).

(95) B. Dietrich, J. M. Lehn and J. P. Sauvage, Tetrahedron Lett., 34, 2889 (1969).

(96) J. M. Lehn and J. P. Sauvage, Chem. Commun., 440 (1971).

(97) Y. M. Cahen, J. L. Dye and A. I. Popov, Inorg. Nucl. Chem. Letters, 10, 899 (1974); J. Phys. Chem., 79, 1289 (1975).

(98) T. N. Briggs and J. E. Steuhr, Anal. Chem., 46, 1517 (1974).

(99) J. L. Dye and V. A. Nicely, J. Chem. Educ., 48, 443 (1971).

(100) M. Rabb, "The Resistance Meter", Department of Chemistry, Michigan State University, East Lansing, Michigan, 1973.

(101) L. Meites and H. C. Thomas, "Advanced Analytical Chemistry", McGraw-Hill, New York, N.Y., 1958, p. 89.

(102) J. Juillard, Bull. Chim. Soc. Fr., 1727 (1966).

(103) K. Waldmeier and W. Rellstab, Z. Anal. Chem., 264, 337 (1973).

(104) D. M. Barry, L. Meites and B. H. Campbell, Anal. Chim. Acta, 69, 143 (1974).

(105) E. Shchori, J. Jagur-Grodzinski, Z. Luz and M. Shporer, J. Am. Chem. Soc., 93, 7133 (1971).

(106) J. H. Prestegard and S. I. Chan, Biochemistry, 8, 3921 (1969).

(107) J. H. Prestegard and S. I. Chan, J. Am. Chem. Soc., 92, 4440 (1970).

(108) V. T. Ivanov, I. A. Laine, N. D. Abdulaev, L. B. Senyavina, E. M. Popov, Y. A. Ovchinnikov and M. M. Shemyakin, Biochem. Biophys. Res. Commun., 34, 803 (1969).

(109) M. M. Shemyakin, Y. U. Ovchinnikov, V. T. Ivanov, V. K. Antonov, E. I. Vinogradova, A. M. Shkrob, G. G. Malenkov, A. V. Evstratov, I. A. Laine, E. I. Melnik and I. D. Ryabova, J. Membrane Biol., 1, 402 (1969).

(110) D. H. Haynes, A. Kowalsky and B. C. Pressman, J. Biol. Chem., 244, 502 (1969).

(111) D. F. Mayers and D. W. Urry, J. Am. Chem. Soc., 94, 77 (1972).

(112) D. J. Patel and A. E. Tonelli, Biochemistry, 12, 486 (1973)

(113) D. J. Patel, Biochemistry, 12, 496 (1973).

(114) D. W. Urry and N. G. Kumar, Biochemistry, 13, 1829 (1974).

(115) G. Cornelius, W. Gärtner and D. H. Haynes, Biochemistry, 13, 3052 (1974).

(116) P. Laszlo and P. J. Stang, "Organic Spectroscopy", Harper and Row, New York, N.Y., 1971.

(117) R. M. Silverstein and G. C. Bassler, "Spectrometric Identification of Organic Compounds", Wiley, New York, N.Y., 1967.

(118) J. R. Dyer, "Applications of Absorption Spectroscopy of Organic Compounds", Prentice-Hall, Englewood Cliffs, N.J., 1965.

(119) J. A. Pople, W. G. Schneider and H. J. Bernstein, "High-resolution Nuclear Magnetic Resonance", McGraw-Hill, New York, N.Y., 1959.

(120) J. Bogner, J. C. Duplan, Y. Infarnet, J. Delmau and J. Huet, Bull. Chim. Soc. Fr., 3616 (1972).

(121) G. Descotes, D. Sinou and J. C. Martin, Bull. Chim. Soc. Fr., 3730 (1970).

(122) C. J. Clemett, J. Chem. Soc. A, 455 (1969).

(123) W. Köhler, P. Frölich and R. Radeglia, Z. Phys. Chem., 242, 200 (1969).

(124) L. Ödberg, A. Löfvenberg, E. Hogfeldt and F. Fredlund, J. Inorg. Nucl. Chem., 34, 2605 (1972).

(125) M. S. Greenberg, Ph.D. Thesis, Michigan State University, East Lansing, Michigan, 1974.

(126) G. J. Gross and D. M. Woodbury, J. Pharmac. Exp. Ther., 181, 257 (1972).

(127) A. Hourdakis, private communication.

(128) L. Meites, J. E. Steuhr and T. N. Briggs, Anal. Chem., 47, 1485 (1975).