PROGRESS REPORT

for

Period Ending December 31, 1965

NASA Grant No. - ~~NSG~~ NGR - 21-002-040

TITLE

MOLECULAR BINDING IN THE CELL SURFACE

Robert G. Grenell
Professor of Neurobiology
Psychiatric Institute
University of Maryland
School of Medicine
Baltimore, Maryland

ABSTRACT

Detailed are salient constructional features of a microwave abosrption cavity, derived from Vogelhut,[1] to be used in proposed bound-water experiments with neuroproteins. Adaptations of existing hardware are indicated where they have been applied.

An improved klystron oscillator system is proposed, and the instrumentation for digitilized readout of cavity characteristics is discussed. Appended are approximate costs and a suggested schedule for experimental stages in the proposed investigation.

Briefly discussed are the applications of current techniques in NMR and Electron Paramagnetic Resonance Spectroscopy to molecular characteristics of membrane protein and protein residues. Our tentative evaluation indicates the microwave technique as the most promising of the current spectroscopid procedures for elucidating charged relationships and binding-site activity for a variety of orientations of the macromolecule. Hence, ~~we propose~~ was proposed the construction of the absorption cavity and its initial use in an existing microwave system available on a limited basis through the courtesy of the Johns Hopkins University. Control measurements prerequisite ~~to the investigation~~ of nerve membrane would then be compared with those of Vogelhut and other workers for agreement.

We propose to continue the theoretical evaluation of NMR spectroscopy techniques, particularly with regard to high resolution NMR. With its ability to clarify simpler molecular structures, NMR may be used to catalog those residues from membrane protein which can be prepared with the purity needed for analysis.

MICROWAVE CAVITY

The major effort reported here will be the exposition of complete construction details for a microwave cavity essential to the dielectric study of dilute neuroprotein solutions. This cavity is in every respect exactly as used by Professor Paul O. Vogelhut at the University of California.[2] Information about the instrument now detailed is based on a personal interview with Dr. Vogelhut and is due in large measure to his generous cooperation. As the builder was not available, and as we could not obtain blueprints, measurements, or details for the instrument, the working drawings and adaptations of standard microwave hardware reproduced here are based entirely on our own photographs, record, and sketches. Fortunately, all critical dimensions of the cavity were know to us: # RG-52U X-band waveguide dimensions are standardized; the T-mode cavity characteristic, TE_{114}, was given;the hardware used in an adjustable short at the end of the cavity was a standard item; and the coupling iris for the reflection cavity measured to a standard dimension. Thus the dimensions indicated here are given with every confidence in their accuracy.

Figures 1-a and 1-b are reductions from the working drawings which are the basis for the construction print. Figure 1-a print is life-size in the four views of the instrument and includes an isometric projection. Figure 1-b print is a 2X rendition of sections through the cavity as indicated by the dotted lines intersecting the four views of the first figure. Dimensions are to be provided on the prints but are implicit with the scale drawings, within the accuracy of the draft angles.

Every effort was made to determine the origin in standard microwave hardware of sub-assemblies used in the instrument. To this end and for his experience

in microwave instrumentation, Mr. Thomas M. Bray, an experienced microwave engineer with the Johns Hopkins University, was retained briefly as a consultant. The variable short located at one end of the waveguide slotted section is from a Hewlett-Packard # X485B Detector Mount. The crank knob driving the short is concentric to a bracket which serves as a support to the uncoupled end of the cavity and the knob is free to rotate about the support shaft.

The origin of the slotted waveguide section, if indeed it is an adaptation, is less clear. Complete details are given for the section, as its fabrication would entail extensive modification of any commercial apparatus which could be used. It is built from free-machining, half-hard leaded brass plates, soldered around a central waveguide section, as in Figure 1-b. A slot, 1/8" x 4", is milled through both sides of the section on the centerline of the long waveguide dimension. An additional channel, 5/16" wide x 1/4" deep, is milled along the thicker side wall on the same centerline and accomodates a brass plug, 1/4" x 5/16" x 2", which shields the cavity.

A dovetail section of brass or stainless steel is attached to the top of the slotted section perpendicular to the slot axis. Its accomodation of a gear rack requires the milling of a recessed groove, 1/8" wide x 0.0468?" deep, along its entire length. The gear rack is a 48-pitch, 20° pressure angle section of brass, 1/8" x 1/8", and in the original instrument it is clamped in place by the shoulders on each of two heat-treated # 6-32, Allen-head machine screws. If brass were used to construct the dovetail ways, the gear rack could be soldered. The yoke surrounding the slotted section slides along the 60° dovetail faces, any play in its fit adjusted out through a bearing plate, 1/16" x 5/16" x 2", with its own adjustment screws. The four pieces of brass which make up the yoke frame are butted together and

fastened with recessed # 6-32, Allen-head screws. Recessed # 4-40, Allen-head screws are used to fasten 1/4" brass end plates to the yoke sides at the slot centerline axis. The yoke is then bored through on this axis, and the 5/16" bore is milled out at the reflection end of the cavity. Brass plugs, 1/2" x 7/8" x 5/16" fit in the outer slots, securing in place the 5/16" spherical balls which are bored to receive the quartz sample tube.

Mounted normal to the slot axis on top of the yoke is the spur gear which drives it along the engaged rack and thus through nodes of absorption maxima of the cavity. Gear specifications for the spur gear are: 48 - pitch; 20° pressure angle; pitch diameter of 0.5833", face of 1/8", bore of 0.1875", Class 1 precision. The gear hub is machined flat, the gear is heated, and a chilled shaft, 0.375" x 1 7/8" is inserted for a shrink fit. The shaft arbor, a 1/4" x 3/4" x 3/4" brass plate, is secured to the yoke with # 2-56 machine screws. When an absorption maximum has been found, the assembly may be locked in place by tightening the adjusting screws for the bearing plate. This will probably not be needed for the planned horizontal mounting of the cavity.

A standard # UG - 39U cover flange, MS 90059, is soldered to the protruding # RG - 52/U waveguide. The flange incorporates an iris to couple the reflection cavity to the transmittion waveguide from the Klystron oscillator. The coupling iris is fabricated from brass shim-stock. 0.002" thick to minimize phase-shift, and is sweat-soldered to the flange mating surface on the waveguide axis. At frequencies in the neighborhood of 10×10^9 cps, $\frac{\lambda}{a}$ is near unity, where a is the long dimension of the waveguide. At this ratio, $\frac{-B}{Y_o}$, the susceptance of the 1/4" coupling iris hole, is approximately 12 mhos, indicating the balance chosen between good coupling and isolation from cavity perturbations.[3] Empirical corrections of the adjustable short for cavity length and of the sample tube for maximum sample absorption give a maximum displacement of the cavity characteristic due to the sample.

A sample holder design is detailed in Figure 2. End spacers are bored to slip easily over the tube ends, compressing the sample holder in place under pressure from the end plugs at the 5/8" bore. These plugs are made from a styrene plastic in the original model. However, with a low loss dielectric for the capillary tube, they could be made from slotted brass or aluminum, perhaps improving cavity shielding.

The capillary encloses a sample volume of 10^{-4} ml. As the tube is located at an E-max. node, the loss associated with the capillary material has a smaller effect on the total dielectric constant than would be the case if an I-max. node were used. Perhaps for this reason, other workers have used glass, and as relative measurements of, the complex dielectric constant, examine only a difference in cavity Q and frequency in the relationships,[4]

$$\delta \epsilon' = \epsilon'_w - \epsilon'_s = \frac{2c}{f_o} (f_s - f_w), \quad (1)$$

$$\delta \epsilon'' = \epsilon''_w - \epsilon''_s = C (\frac{1}{Q_s} - \frac{1}{Q_w}), \quad (2)$$

the loss associated with glass cancels out. The above expressions were derived in our previous report and have the significance assigned to them there.[5]

It is instructive to examine tan δ, the dissipation factor, for both glass and quartz at 2.5×10^{10} cps. For glass tan δ is typically > 0.01. Quartz is an excellent material at this frequency, tan $\delta < 0.0003$.[6] These values indicate that if there is appreciable current induced in the tube, it will heat up. The temperature rise will increase time-dependent shifts in the sample dielectric constant, associated with structural changes in the sample polypeptides. Quartz tubing blanks are available from:

General Electric Co., Lamp Glass Div.

Willoughby Quartz Plant

Euclid Avenue & Campbell Road

Willoughby, Ohio

To avoid these temperature increases consequent from the use of glass, we propose to use quartz tubing in this application.

NMR SPECTROSCOPY

New ferromagnetic materials, cryogenic environments for superconduction, and advances in winding techniques have all contributed to the improved resolution of frequency - associated nuclear spin-spin couplings which now characterizes the molecular spectra of those molecules which possess magnetic moments. High Resolution NMR has clarified the multiplet peaks of relaxation - induced voltage for molecular nucleii whose size and field complexity has heretofore defied analysis with less refined techniques. (7) Currently, Varian Associates have developed instruments for which H_o, the principal magnetic field, may reach 47×10^3 gauss, using niobium - alloy soleniods at liquid helium temperatures.(8) Field energies of this order, which produce precessional frequencies of approximately 10^8 cps, and spin-decoupling techniques allow both spectral simplification and increased sensitivity. High-Resolution NMR has been applied to a large number of organic molecules, and the Varian Spectra Catalog details the identifying characteristics of the amino acids and of many of those peptide residues presumed to be associated with specific proteins.(9)

Modern analytical methods in biochemistry(10) are capable of producing residues which are structure - characteristic in their derivative proportions and in their species. Ultrapurification techniques could yield these residues in fractions which ideally would be sufficiently homogeneous for a unique spectral record, thus making identification possible by either direct superposition on the chart of a known molecule or by analysis of multiplet patterns, relating these to the multiplets for known sub-groups. "Tickling" the spin-spin coupling peaks with a low-level rotating field has been used to indicate the type of nuclear attraction observed.(11) The CAT (Computer of Average Transients) has come into use

to retrieve spectral peaks of very low level signals, such as would be found with dilute solutions or well-shielded nucleii.[12] Mentioned here are only those developments which have been publicized and which are generally known among spectroscopists. The field is rife with new developments and seems on the verge of a breakthrough, perhaps in pulsed NMR, which will allow the technique to cope with macromolecules as large as the conjugated peptides.

Not withstanding these developments, we do not consider that at this time NMR is capable of yielding the unambiguous structural information about neuroproteins which is sought in our research. The requirements for molecular simplicity and for ultrapurity preclude the examination of intact polypeptides as they are found in vivo. The microwave method places no such restriction on molecular size; indeed, suspensions of minced tissue will be placed in the cavity. It is possible that a monolayer might be simulated by molecular anisotropy. The decision to precede with the construction of the cavity and to make control measurements is predicated on these considerations.

The continuing influx of new refinements in High-Resolution techniques makes NMR an exciting field to watch. We will keep abreast of new developments as they are reported, continuing our theoretical evaluation in an attempt to uncover methods for its application in nerve membrane research.

ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY

Considerations of Electron Paramagnetic Resonance methods to elucidate neuroprotein structure appear to meet with much the same limitations as found in NMR. New developments for NMR carry over into this allied field, with consequences for improved resolution and increased sensitivity. The retrieval of discrete spectral information again hinges on the simplicity of the electronic structures encountered, with transitions from electronic splitting appearing as a smeared signal when adjacent fields influence the quantized spin-coupling.[13] As this technique has been given only a brief examination, its critical appraisal must follow a more intensive study. We propose this study as a correlate to continued NMR research.

IMPROVED KLYSTRON OSCILLATOR

Laboratory For Electronics, Inc, offers a new Klystron oscillator, the Series # 841 Phase-Locked Oscillator. Stability is given as 1 part in 10^8/sec; power output is to 200 mw. This oscillator has provision for crystal frequency monitoring and would be locked to an external crystal as a reference standard if greater stability were needed and could be achieved. PIN diode leveling of the output could give additional power stability if this were needed. The cavity would be swept in the minimum time compatible with the detection circuitry time constants.

A direct-reading microwave counter, the Systron-Donner Model # 1037 with # 1292 Transfer Oscillator, could be used to read out digitally the cavity half-power bandwidth, giving an accuracy to frequency information which would be limited only by the flex point triggering accuracy.[14] As a calibration device, this counter would be an invaluable time-saver. If triggering circuits could be devised which were superior to graphic methods for determining the half-power points, measurement accuracy would increase directly with the

sensing ability of these circuits. Solid State circuitry with this sensitivity may have been developed, but we have not uncovered it as yet.

TENTATIVE EXPERIMENTAL SCHEDULE

1. 1/1/66 - 4/1/66: Construction and testing of the microwave cavity

2. 4/1/66 - 7/1/66: Establishment of control values, with bovine serum albumen, horse haemoglobin, lysozyme, gelatin, β-lactoglobin. These will be examined for their agreement with values of Buchanon, et al, PROC. ROY. SOC. LON., 213, p. 379, 1952, and with Haggis, et al, J. CHEM. PHYS., 20, p. 1452, 1952.
   A concurrent project will be to determine cell box characteristics for the environmentally-controlled neuroprotein measurements to follow. Present plans call for the use of existing microwave equipment at the Johns Hopkins University; however, X-Y Plotter will be needed.

3. 7/1/66 - 12/31/66: Final cell design will be determined, using the Vogelhut cell as a guide. Temperature control and humidity control systems will be built and used to stabilize the cell. Initial neuroprotein experiments will be made at this time, using the method of Haggis and Vogelhut. No anisotropic measurements will be attempted in this phase.

4. 1/1/67 - 6/30/67: Anisotropic effects on sample dielectric constant will be examined. Hardware will be proposed for this purpose and its construction begun. Both control proteins and experimental neuroproteins will be measured, and the results will be analysed in relation to possible molecular and group structures, suggested by dielectric anisotropy.

REFERENCES

1. Vogelhut, P.O., Nature, 203, p. 1169, 1964
2. Personal communication with Dr. Vogelhut
3. "Microwave Engineer's Handbook and Buyer's Guide", Horizon House Microwave, Inc., Brookline, Mass., p. T-43, 1963.
4. Vogelhut, P.O., op cit, p. 1170
5. R.G. Grenell, D. McCulloch, NASA PROGRESS REPORT, February, 1964
6. Von Hippel, A.G., "Dielectric Materials and Applications"
7. Bovey, F.A., CHEMICAL AND ENGINEERING NEWS, 43, p. 102, 1965
8. Ibid, p. 101
9. "High Resolution NMR Spectra Catalog", Vols. I and II, Varian Associates, Palo Alto, California, 1963
10. Fruton, J.S., "General Biochemistry", J. Wiley & Sons, New York, 1958, pp. 129-161.

    Oncley, J.L., "Chemical Characterization of Proteins, Carbohydrates and Lipids", In J.L. Oncley, et al, "Biophysical Science, A Study Program", J. Wiley & Sons, New York, 1959.

    Pauling, L., "The Nature of the Chemical Bond", Cornell University Press, Ithaca, New York, 1960, p. 289, pp. 498-504
11. Bovey, F.A., opere citato, p. 104
12. Silverstein, R.M., Bassler, G.C., "Spectrometric Identification of Organic Compounds", J. Wiley & Sons, New York, 1963, p. 82

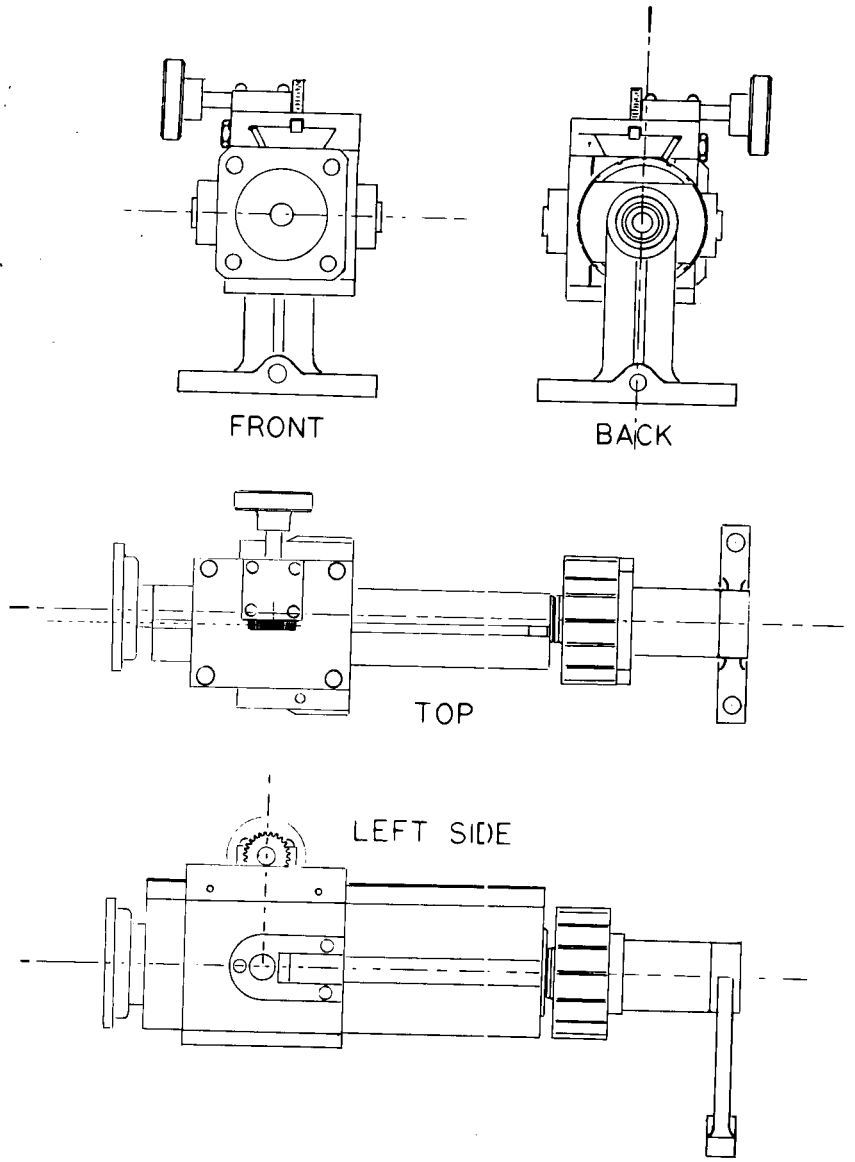
    Bovey, F.A., op cit, p. 111

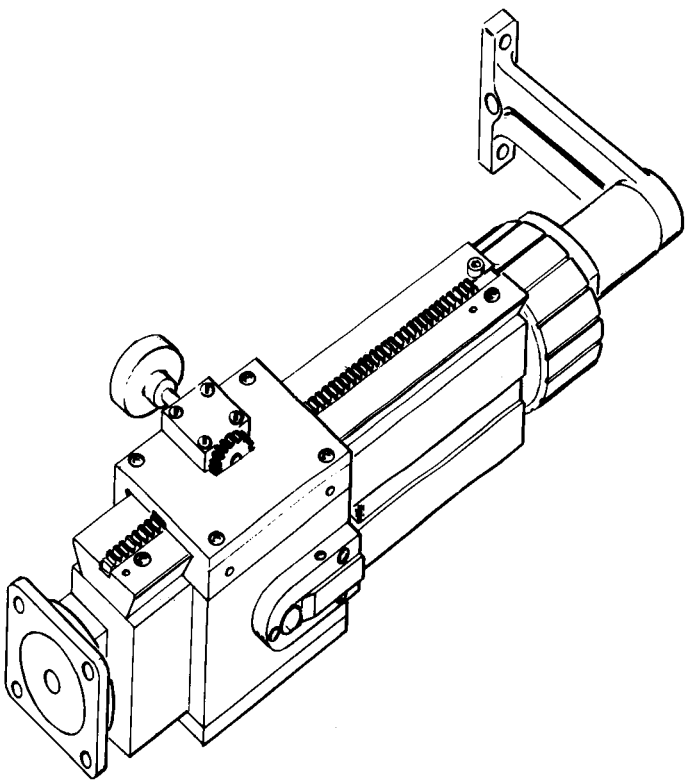
    COMPUTER OF AVERAGE TRANSIENTS, Series 400, Mnenotron, Inc., White Plains, New York, 1964, Section VI.
13. Sandorfy, C., "Electronic Spectra and Quantum Chemistry", Prentice Hall Inc., Englewood Cliffs, New Jersey, 1964, p. 337, 140

REFERENCES (Cont'd.)

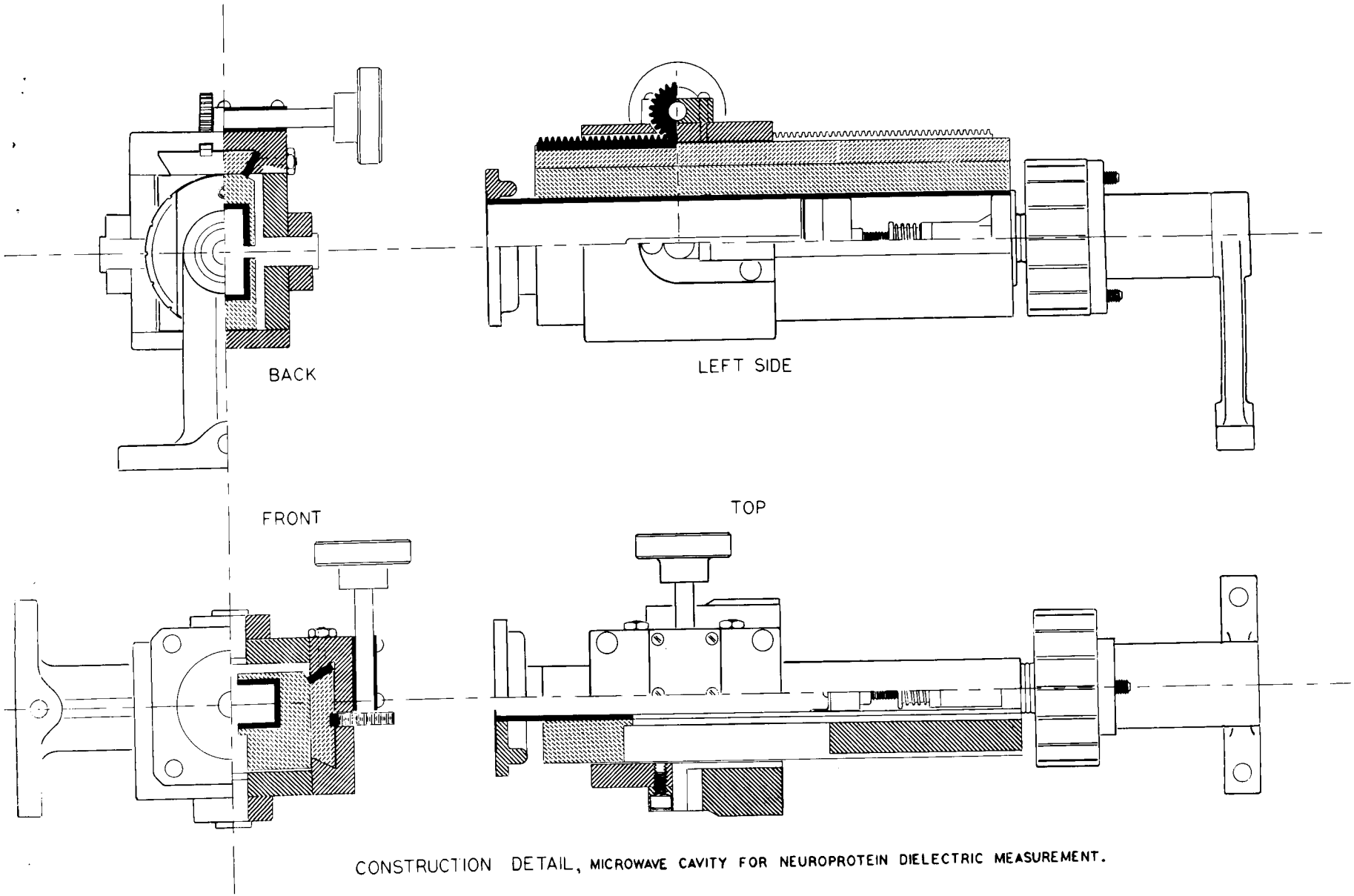
13. (cont'd) Dyer, J. R., "Applications of Absorption Spectroscopy of Organic Compounds", Prentice Hall Inc., Englewood Cliffs, New Jersey, 1965, p. 97.

14. This technique was suggested by Dr. Vogelhut as an alternative method for frequency measurements.



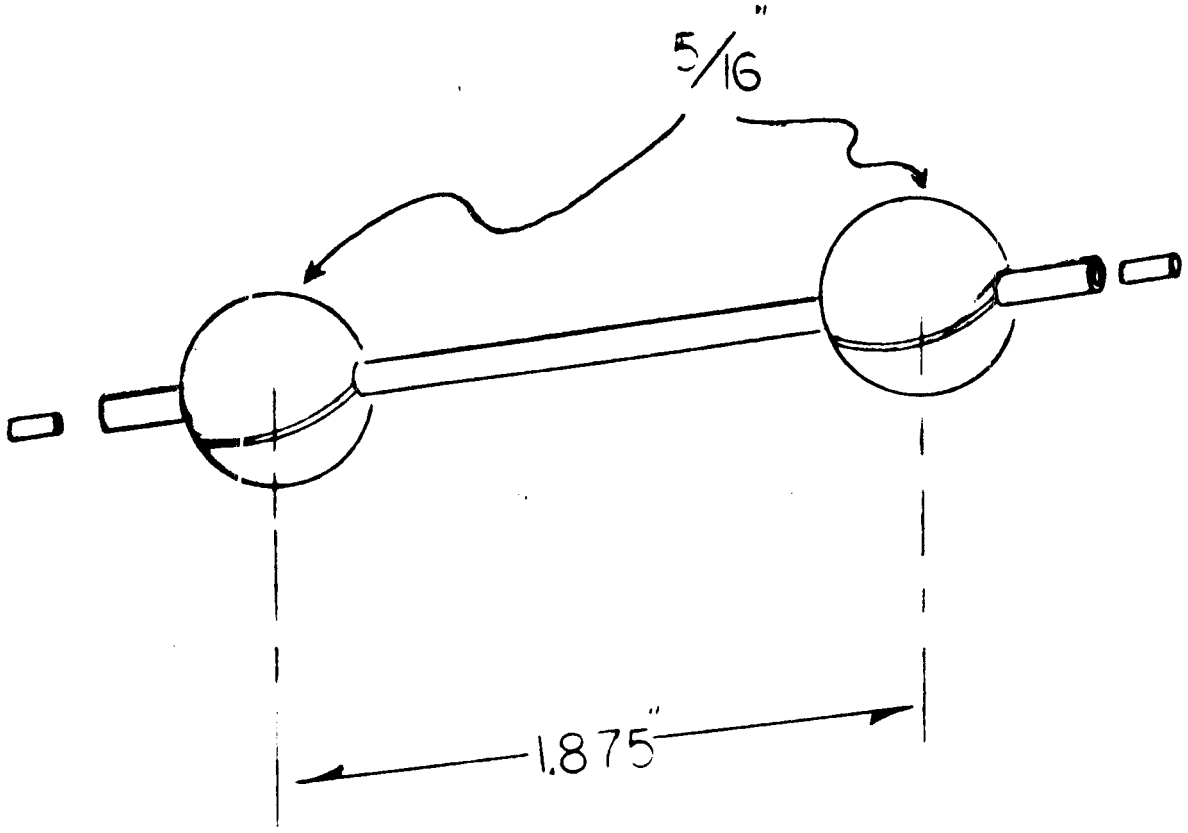


MICROWAVE CAVITY, FOR DIELECTRIC "BOUND-WATER" EXPERIMENT.
AFTER P. O. VOGELHUT



CONSTRUCTION DETAIL, MICROWAVE CAVITY FOR NEUROPROTEIN DIELECTRIC MEASUREMENT.

FIGURE 2



CAVITY SAMPLE HOLDER