DIELECTROPHORESIS OF CANINE BLOOD PLATELETS

By

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DIELECTROPHORESIS OF CANINE

BLOOD PLATELETS

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The tedious task of typing was undertaken by a non-complaining(?) wife.

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CHAPTER I

INTRODUCTION

In recent years much effort has been expended applying the phenomenon of dielectrophoresis as a research tool in biological areas. Notable examples are the work by Hawk and Pohl (1) and by Crane and Pohl (2) with living and dead yeast cells; the research of Wiley (3), which concerned itself with the canine erythrocyte and flavobacteria; the preliminary investigation by Hawk (4) of canine blood platelets; and the study carried out by Pohl, Ting, Jolley, and Beasley (5) on the dielectrophoresis of chloroplasts.

The responses of other biological entities to the dielectrophoretic force are in the process of being studied. In all these studies, it is suggested that this phenonmenon may be a useful means by which microorganism physiology may be studied.

Dielectrophoresis is understood to mean that motion of any particle, electrically charged or neutral, caused by the application of a non-uniform electric field which acts upon the permanent or the induced dipoles of the particle. This motion is to be distinguished from electrophoresis, which is the motion of electrically charged particles in either uniform or non-uniform electric fields. The dielectrophoretic force generally is of such a magnitude that it must compete with the forces arising from conduction, thermal convection, diffusion, and from the charging/electrostatic repulsion sequence. The latter is frequently

observed at low a-c frequencies (~1000 hz).

Given this magnitude of the force, it appears reasonable that there are upper and lower bounds upon the size of the particles that will respond significantly to this force. Pohl (6) has stated that the dielectrophoretic effect should be observable most readily in relatively coarse suspensions, with particles on the order of two microns (μ) in dimension. This figure is only to indicate a range since microorganisms both larger and smaller than this have been observed responding to the dielectrophoretic force. As examples, the yeast cells studied have dimensions of six to nine microns, whereas the chloroplasts are typically 0.7 microns in dimension.

The origin of the dielectrophoretic force is most often explained in the following manner. When an electrically neutral particle is placed in a uniform electric field, the field will induce a polarization of the particle, with equal yet opposite type poles. Since the field is uniform, both poles will experience equal yet opposite forces, and the net translational force on the particle as a whole is zero. For neutral particles with permanent dipoles, the situation is identical after the torque caused by the application of the field has aligned the dipole with the field. A different situation exists if a non-uniform electric field is applied to an electrically neutral particle. The field will still induce a dipole on the particle, and particles with permanent dipoles will still align that dipole with the field and then perhaps polarize further. But now the finite separation of the two equal yet opposite charges of these dipoles will put the two poles in different regions of the electric field. Since the field is non-uniform, each pole will experience a different force so that the particle as a whole

will experience a net translational force--this force being the dielectrophoretic force.

The mathematical expression for the translational force acting on a small ideal dielectric sphere of radius $\underline{a}$ and dielectric constant $\underline{K}_2$ suspended in a medium of dielectric constant $\underline{K}_1$ is given by (7):

$$\vec{F} = 2\pi a^3 K_1 \varepsilon_o \left(\frac{K_2 - K_1}{K_2 + 2K_1}\right) \nabla (\vec{E}^2)$$

where ε_o is the permittivity of free space and $\vec{E}$ is the intensity of the applied electric field. The rationalized MKS system of units is implied.

Although the above expression is applicable only to ideal dielectrics, one may abstract certain general information from the equation that will maintain its validity when considering real dielectrics. The equation explicitly shows that the direction of the translational force can be influenced only by the difference in the dielectric constants of the particle and the medium. When the particle is more polar than the medium ($K_2 > K_1$), the particle will in general experience a force towards a region of higher electric field intensity; when it is less polar than the medium ($K_2 < K_1$), the dielectrophoretic force will urge the particle to a region of lower field intensity. The equation further indicates that the force is unaffected by a reversal in the sign of the applied field. This fact is of great particular importance to the study at hand since the canine blood platelet possesses a negative surface charge (8). If one were forced to use a uni-directional or d-c field, the electrophoretic and dielectrophoretic forces would be operational simultaneously. By applying an alternating field, however, one can eliminate the electrophoretic response. The net coulombic force acting on a charged particle over one complete cycle is essentially zero if the frequency of

the applied field is sufficiently high and/or the particle under study is sufficiently massive. Both conditions were met in this study.

It should be obvious that the force expression given is inadequate for a real dielectric. Real dielectrics experience dielectrophoretic forces that are dependent on the frequency of the applied electric field and the resistivity of the suspending medium. The expression as given contains no frequency or resistivity dependence. Dependence upon these two parameters can be brought into this expression if the complex dielectric constants for the real dielectrics are used in place of the real dielectric constants of the ideal dielectrics. With this in mind, Crane (9) has derived a general force equation for real dielectrics.

The thrust of this investigation is not primarily concerned with the experimental verification of the mathematical expressions that have been developed. The various dependencies of the amount of collection of cells at one or the other of the electrodes (high field intensity regions) creating the non-uniform field on the parameters of interest--namely, the value of the applied voltage, the amount of time the field is applied, and cell concentration--were studied by Crane and Pohl (10) using yeast cells. Rather, a comparison is to be made using blood platelets from normal dogs as a standard. Normal is taken to mean that the dog exhibits no clinical signs of any malady. These platelets are to be compared to the blood platelets of dogs which transmit Blood Platelet Factor VIII deficiencies to their offspring (hereafter referred to as transmitters) and to platelets from both male and female dogs deficient in the Blood Platelet Factor VIII.

The basis of the comparison will be the response of each of these four types of platelets to the dielectrophoretic force with the

parameters of cell concentration of the platelet suspension, time interval of collection, applied voltage (or the value of the electric field intensity), age of the platelets, and resistivity of the suspending medium, all fixed as well as possible. The response will be observed for each type as a function of the frequency of the applied electric field.

It was hoped that the platelets from the transmitters would differ strongly in their responses to the dielectrophoretic force from the responses of the platelets from the other three types. In such a case, it would then presumably be possible to determine in a dog population which members would transmit hemophilia to their offspring without having to adopt a wait-and-see position. The obvious generalization to the human population would be of considerable interest. The rationale behind this approach was the fact that other microorganisms when observed in various physiological states have shown various responses to the dielectrophoretic force.

Following this comparison was an attempt to detect changes in the dielectrophoretic response of normal platelets when the latter were subjected to various physiological conditions; to wit, doping the platelets with minute and controlled amounts of inhibitors and salts.

The age of the platelets (measured from the time the blood was withdrawn from the dog) was another physiological condition which could possibly affect the platelets' response, and hence was examined.

The dogs used in the study were supplied by Dr. Ralph Buckner of the Oklahoma State University Department of Veterinary Pathology. The members of the beagle colony from which the dogs were drawn have been carefully studied and categorized over a period of years so that when a

member is labeled a hemophiliac or a transmitter of that disease, this may be considered to be well established. The dogs are all housed together and subjected to the same environmental conditions. The diet is uniform for the colony. There are approximately 60 members of the colony. Of these, eight are "normal" dogs, 11 are hemophiliac males with the Factor VIII deficiency, 9 are hemophiliac females with the Factor VIII deficiency, and 14 are female Factor VIII deficiency transmitters.

CHAPTER II

THE MAMMALIAN PLATELET

Being smaller than the other formed elements of the blood, the platelet was quite understandably discovered last. Credit for the first observation of these "globulins" is generally given to Alfred Donné, working in 1842; but it is also suggested that George Gulliver and William Addison, working independently in England in 1841, made the first platelet drawings.

The introduction of electron microscopy and ultrathin sectioning techniques have since provided much information about the structure of the blood platelet. Platelets rapidly fixed in glutaraldehyde normally exhibit elliptical, rodlike, or disc shapes when viewed in ultrathin sections. Their dimensions are of the order of 1.5 to 4.0 μ in longitudinal diamenter and 0.5 to 2.0 μ in transversal diameter. Excrescences, or pseudopod-like projections, are rarely seen when the platelets are fixed in the above manner. When fixed after having been separated by centrifugation, or when exposed to cold (0-4°C), excrescences are frequently observed (11). Exposing the platelet to cold is also known to cause them to assume a spheric shape (12).

The water content of the platelet is found to be about 87 percent. Further, glycogen yield is observed to be 92 $\mu g/10^9$ platelets. And whereas deoxyribonucleic acid (DNA) is essentially absent, ribonucleic acid (RNA) is found in trace amounts (8).

The subcellular structures that are observed may be summarized as follows: a triple-layer surface membrane of 70 to 90 Å thick surrounded by a "fluffy" coat of up to 500 Å thick; mitochondria with their characteristic double surrounding membrane; electron dense granules which supposedly contain clotting active lipid (Platelet Factor III), lysosomal enzymes, fibrinogen, and nucleotides; vacuoles and vesicles; the golgi complex which is found only occasionally in the normal platelet and originates from the precursor cell; sidersomes; microtubules; glycogen in the form of electron-dense granules (150 to 300 Å diameter); possibly ribosomes; and occasionally lipid inclusions. An excellent and thorough survey of these structures is given by Hovig (11). The obviously missing element in the mammalian platelet is a nucleus--the platelet cannot reproduce itself, in contrast with the nucleated thrombocytes (or platelets) of lower animals. The evidence convincingly indicates that mammalian platelets originate in the cytoplasm of megakaryocytes and are released into adjacent blood capillaries by rhexis (fragmentation) of the mature cells. The location of these platelet-producing precursor cells is chiefly in the bone marrow and the lungs (13).

During the life span of the platelet in the circulatory system, metabolism is accomplished in two ways (11), glycolysis being the well-known manner. The second occurs in the mitochondria. The tricarboxylic acid cycle present there is much more efficient a mechanism for producing adenosine triphosphate (ATP--a high energy phosphate) than is glycolysis.

The function of the platelet is basically that of proper control of bleeding. Within or on the platelet one may find most, if not all, the

blood clotting factors (14).

The life span of platelets in mammals' circulatory systems has been determined by di-isopropyl-fluorophosphate isotope tagging (DFP^{32}) in vivo to be from 8 to 11 days, considerably less than the life span of the erythrocyte. Removal of the platelets is not well-established, but is possibly by phagocytosis in the spleen or by sequestration in the spleen (13).

CHAPTER III

EXPERIMENTAL PROCEDURE

The first step to be taken was the accumulation and construction of the necessary apparatus with which to observe the dielectrophoretic effect. The dielectrophoretic cell was constructed from a plain, clear microscope slide, two cover slips, epoxy glue, and 10 millimeter platinum wire. Two and a half centimeter sections of platinum wire were first rinsed in methanol to remove any large bits of foreign materials that might be present. The ends of these sections of wire were then brought close to an acetylene torch flame, resulting in expanded spherical lobes on the ends of the wires. This procedure was repeated until two lobes were produced which were not unnecessarily large and which were very similar to each other in size, both being as spherical as possible. The radius of curvature of the lobes finally produced was approximately 0.14 millimeters.

A Dremel Moto-Tool hand grinder (Dremel Manufacturing Company) and a small grindstone were used to grind a small circular hole of 4 mm. diameter in the center of each cover slip. One cover slip was glued to the microscope slide with epoxy glue. The platinum wire sections were then sandwiched into epoxy between the first and the second cover slips, the lobes extending into the circular opening and separated by a distance of 0.47 mm. The well thus produced was about 1 mm. deep. (See Figure 1.)

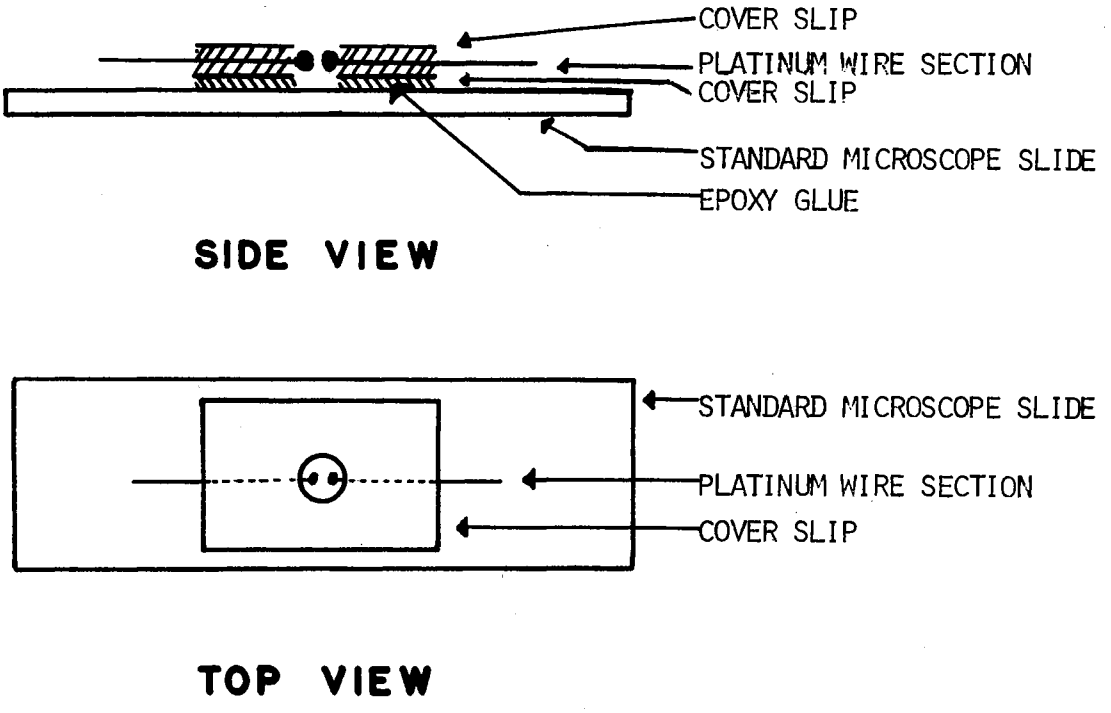

Figure 1. Details of Dielectrophoretic Cell

The lobes were positioned to lie in the same horizontal plane and to lie directly across the cell from each other. Care was taken to prevent the lobes from coming into contact with the epoxy glue. After this arrangement had dried, the loose platinum leads were lightly soldered to 20 gauge (American Wire Gauge) leads of about 6 centimeters in length. After making certain that a good connection had been made, all exposed platinum wire protruding out from under the top cover slip as well as the above connection was covered with epoxy glue to protect the rather fragile platinum wire, to protect the contact, and to give some rigidity to the 20 gauge wire leads so that the cell could be easily connected to the external electric field by alligator clips. (See Figure 1.)

The external field for frequencies 600Khz and below was provided by a Hewlett-Packard Wide Range Oscillator Model 200CD, the output being amplified by a Krohn-Hite Wide-Band DC-1Mhz 10 watt amplifier, Model DCA-10(R). For frequencies 1Mhz to 16Mhz, an oscillator designed by Dr. Bennett L. Basore of the Oklahoma State University Electrical Engineering Department was used. The oscillator provided enough voltage for the present studies and no amplifier was needed. A circuit diagram is provided in Figure 2. The voltage was constantly monitored with a Hewlett-Packard vacuum tube voltmeter (Model 410B). Occasionally, the wave form of the applied oscillating electric field at the leads to the dielectrophoretic cell was checked with a Tektronix Type 533A oscilloscope.

Suspension Preparation

To obtain the blood platelet suspension, the following procedure was established: First, all glassware used in this work had to be

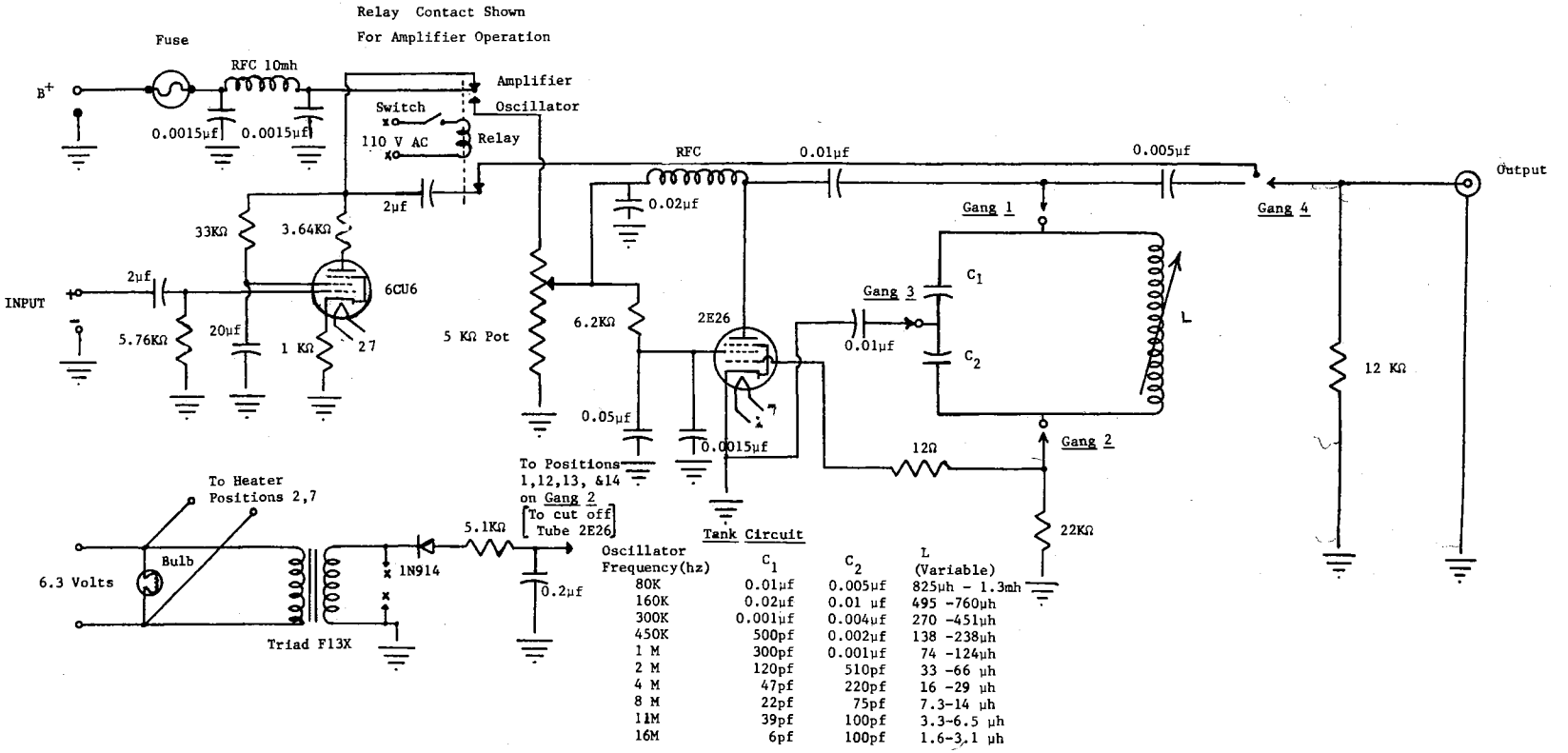

Figure 2. Schematic of Oscillator-Amplifier Unit

siliconized, including the dielectrophoretic cell and disposable pipettes. This was to prevent the platelets from adhering to the glass, which they do quite readily in the case of unsiliconized glass. Siliclad (by Clay Adams) was the product used for siliconizing glassware.

Second, the anti-coagulant used in this work (Acid-Citrate-Dextrose (ACD)) was prepared by mixing 14.70 grams anhydrous D-glucose, 13.20 grams trisodium citrate, 4.80 grams of citric acid, and 1,000 milliliters distilled deionized water. Ten percent ethylenediamine tetraacetic acid (EDTA) as an anti-coagulant gave very poor results for the present purposes, the resulting suspensions having a strong tendency to have large platelet clumps or aggregations. The use of cocaine present at a concentration of 1 percent (by weight) in the EDTA failed to prevent this clumping.

To obtain the blood, disposable plastic syringes were used. Four milliliters of ACD were added to a 20 milliliter syringe. Blood was taken from the jugular vein of the dog using a two-syringe technique. Entry was made with a 20-gauge needle (1.5 inches long) and a 2.5 milliliter syringe. One and a half to two milliliters of blood were drawn into this syringe; then the small syringe was removed, with the needle still in place in the vein, and the 20 milliliter syringe inserted into the needle. The blood was then drawn into the 4 milliliters of ACD, filling the 20 milliliter syringe. This double syringe technique was employed to assure a clean blood sample (i.e., free of body tissue fluid). Syringes equipped with Luer-Lok fittings would have been very undesirable for this technique and hence were avoided.

After making certain that the blood and the anti-coagulant had mixed thoroughly, the 20 milliliter sample was split into two samples by

gently expelling the content of the syringe into two siliconized vials. These were then covered with Parafilm "M" (American Can Company) and centrifuged at approximately 350g for ten minutes in an International Equipment Portable Refrigerated Centrifuge (Model PR-2) that had been pre-cooled to 4°C. The resulting supernatant, the platelet-rich plasma (PRP), was then pipetted off with a disposable siliconized pipette into a cold siliconized vial.

This PRP was then diluted with cold 5 percent dextrose (typical mixture: 20 grams anhydrous dextrose in 400 milliliters of distilled deionized water). The dextrose solution was approximately 0.25M and had to be used so as to form an isotonic suspension for the platelets. Once mixed, the dextrose stock solution was put into a Deeminac water purifier (Model 16-4) and kept refrigerated, the latter step being taken to prevent contamination of the dextrose; the former, to assure a high electrical resistivity for the dextrose solution. Any time dextrose was needed, it was dispersed through this deionizer.

The amount of dextrose solution added to the PRP was typically on the order of two parts dextrose (pH~6.0) to one part PRP (pH~7.1). In all cases, enough dextrose was added to drop the resulting mixture to the range of pH 6.5 to 6.8, as determined by Dual-Trace pH paper. This was found to be very important in preventing irreversible clumping of the platelets (15). It also served as something of a partial wash; since repeated centrifugation increased rapidly the chances for irreversible clumping, this dual role for the dextrose was quite helpful in that only one further centrifugation was necessary.

The PRP-dextrose mixture was covered with Parafilm and centrifuged at 4°C for 20 minutes at about 1,100g. There resulted a diluted

platelet-poor plasma (PPP) as a supernatant. All this was carefully pipetted off, and all but 2 milliliters was discarded. The two milliliters of retained PPP were put on ice for possible future use. There remained in the vial the platelet button. It should be emphasized that all PPP that could be removed was removed.

Occasionally a given dog would invariably give a final working suspension with a resistivity just a bit too low. This could sometimes be raised to a workable level by the following procedure: About 1 milliliter of cold dextrose was gently flowed in on top of the platelet button without resuspending the platelets and then pipetted off again. This helped remove the last bit of PPP, which was low in resistivity. This procedure was not used unless necessary.

To the platelet button was now added cold 5 percent dextrose, 1 milliliter to 3 milliliters, depending upon the apparent abundance or lack of abundance of platelets present. The platelet button was gently resuspended using a wax-coated wooden applicator (2 millimeters by 15 centimeters). A 1 to 20 dilution of this suspension was made with 1 percent ammonium oxalate (one part suspension; 20 parts ammonium oxalate) in a blood-diluting pipette (certified $\pm$ 1 percent error). This was shaken for three minutes in a Burton Pipette Shaker (Model 1406); then two samples were placed in American Optical flat-bottomed counting chambers.

Fifteen minutes were allowed for settling of the platelets in the counting chambers before a visual platelet count was attempted. The count was accomplished with a binocular-type American Optical Phase Microscope (Spencer Line) which was also used in observing the platelets' response in the dielectrophoretic cell. The complete procedure for

obtaining platelets counts is given by Brecher and Cronkite (16).

Once the count had been established for the 1 to 3 milliliter suspension, enough cold 5 percent dextrose was added to result in a platelet concentration of 50,000 platelets per cubic millimeter. This concentration was chosen since at this value minimal to no clumping occurs (17). From this platelet suspension the various working samples were made. For the work comparing platelets from normal dogs, from female dogs transmitting to their offspring Blood Platelet Factor VIII deficiencies, and from dogs with Platelet Factor VIII deficiencies, the procedure was simply to add 1 milliliter of platelet suspension to 4 milliliters of cold 5 percent dextrose, resulting in a working concentration of 10,000 platelets per cubic millimeter. In the later work where chemicals were used, the chemical was added to the cold dextrose in the ratio of 1 to 40 and mixed thoroughly before introducing the 10 parts of platelet suspension. The amount of additive was kept small so as to avoid altering appreciably the platelet concentration.

In all cases, the electrical resistivity of the working sample was measured at 1Khz. This was accomplished with a General Radio Bridge Model 1650A and a probe made of hard plastic and platinum plates. Of course, a siliconized glass and platinum probe could have been used. If the measured resistivity was too low, there was no choice except to discard the entire sample and start again. The low limit for the resistivity is given in Chapter IV.

If the resistivity was exceptionally high, which did not happen frequently, PPP was added in trace amounts to the working sample to lower the resistivity to the range of resistivity which most of the samples naturally had when prepared by this procedure. Very high resis-

tivities appeared to give quite erratic results during this study.

The sample, having passed all these check points, was now ready to be observed in a non-uniform alternating electric field. The dielectrophoretic cell and a siliconized cover slip were first cleaned with a stream of distilled deionized water from a squeeze bottle. A small drop of working sample was placed in the well of the dielectrophoretic cell. The well was then covered with the cover slip. The cell was then placed on the microscope table, the 30 volts (rms) applied to the cell, and the response noted on the phase microscope at 20X. The average length of chains formed by the collecting platelets in the interval time (two minutes) was noted and recorded as units of the eyepiece reticule. Each unit as viewed on 20X corresponded to $2/3 \times 10^{-2}$ millimeters. This average length in the two-minute period was designated DCR: Dielectrophoretic Collection Rate.

DCR was also observed at a fixed frequency as a function of the time of exposure of the working suspensions to various chemicals.

In the comparison study, the sequence of applied frequencies was always the same, from the highest frequency to the lowest. The first and second trials were always begun two hours and four hours, respectively, after the blood had been taken from the dog. This information--sample age--is always included in the data to be presented, which shall be done now.

CHAPTER IV

DIELECTROPHORESIS OF BLOOD PLATELETS

The determination of a lower limit to the resistivity of the suspensions under study was established early in the study by observing the collection in a two-minute interval, or the DCR, as a function of suspension resistivity. This was typically done at one of two frequencies: 1Mhz or 450Khz. The response of the blood platelets to the non-uniform field was normally a maximum when the applied electric field had a frequency of approximately 1Mhz, the response being less at higher or lower applied frequencies.

The effect of a change in resistivity upon response was thus monitored at the frequency which gave the maximum response. The data collected with the applied frequency at 450Khz indicated that the response, or DCR, lessened with a decrease in suspension resistivity faster than it did at 1Mhz. Typical results are displayed in Figure 3. The implication was that for yet lower frequencies, the response lessened even more quickly. Cursory examination of this latter implication indicated it to be correct. A graphical display of the data is given in Figure 4. The collection of this data necessarily had to await the occurrence of a naturally high resistivity suspension as prepared by the procedure given in Chapter III. This procedure produced at times suspensions with resistivities as high as 1,500 Ω-m. Once such a suspension was obtained, its response was measured at 1Mhz and at 450Khz.

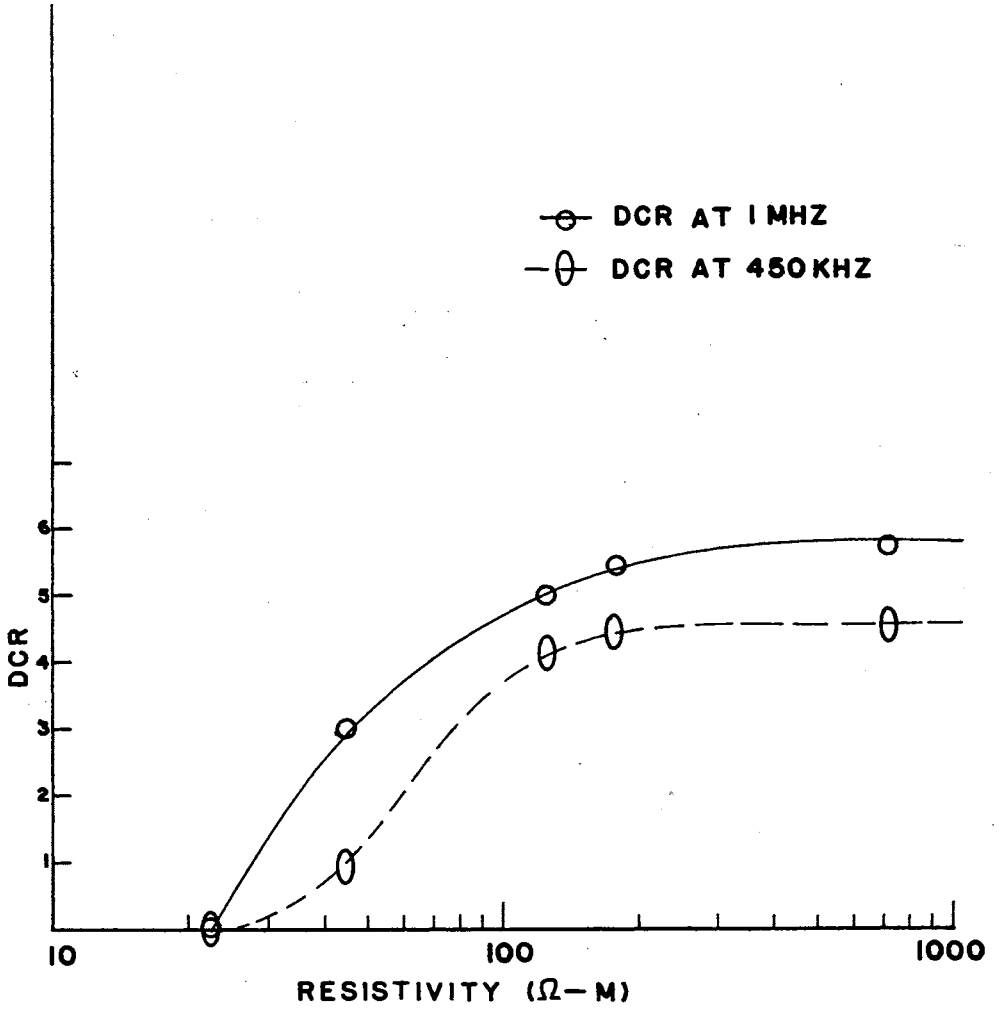

Figure 3. DCR vs Suspension Resistivity

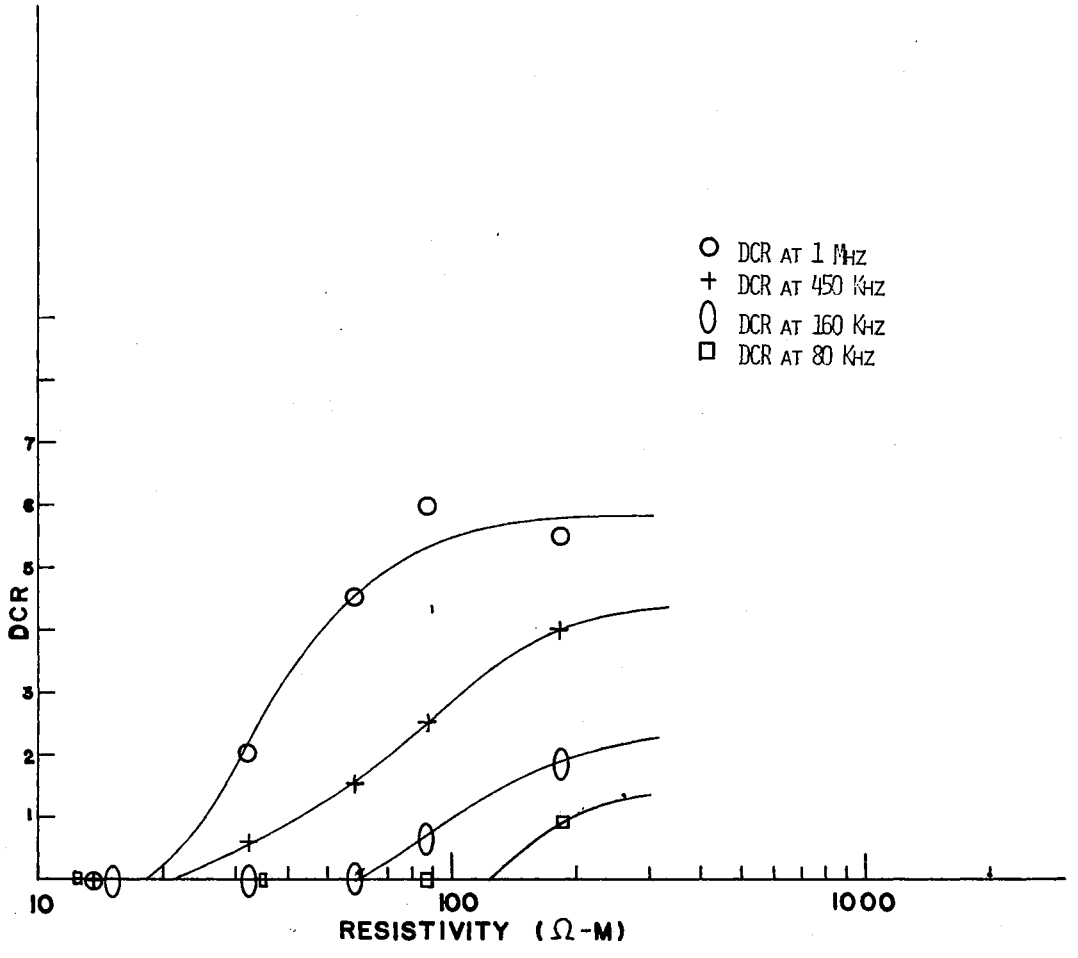

Figure 4. DCR vs Suspension Resistivity at Lower Applied Frequencies

The resistivity was then lowered by the addition of trace amounts of normal saline or PPP, and the response again noted. This was repeated until no response was observed at either frequency. It will be noted in Figure 3 that above 200 Ω-m, the response does not seem to vary drastically with suspension resistivity at either 1Mhz or 450Khz. Hence an effort was made to keep all suspension resistivities at 200 Ω-m or above. For the most part, this effort was successful.

The lower limit of 200 Ω-m being thus established, the response of normal platelets as a function of the frequency of the applied electric field was repeatedly examined. It was during this time that technique was perfected and the goal of a certain degree of consistency was attained. Out of this there arose the preparation procedure given in Chapter III.

A graphical display of this response as a function of frequency is given in Figure 5. The logarithm of the applied frequency is plotted as the abscissa. The response, or DCR, clearly exhibits a maximum when the applied field has a frequency in the neighborhood of 1Mhz. In all observations of normal platelets suspended in the dextrose solution--and there were in excess of 17 such observations or trials--the maximum response occurred in this neighborhood, the range being from 450Khz to 1Mhz. Table I gives the DCR at 1Mhz when the samples were two and four hours old for these seventeen trials. The magnitude of the response varied considerably from dog to dog, and even the same dog offered a different response on different trials in addition to differing responses of two aliquots on the same trial due to the aging of the platelets. This latter effect is obvious in Figure 5.

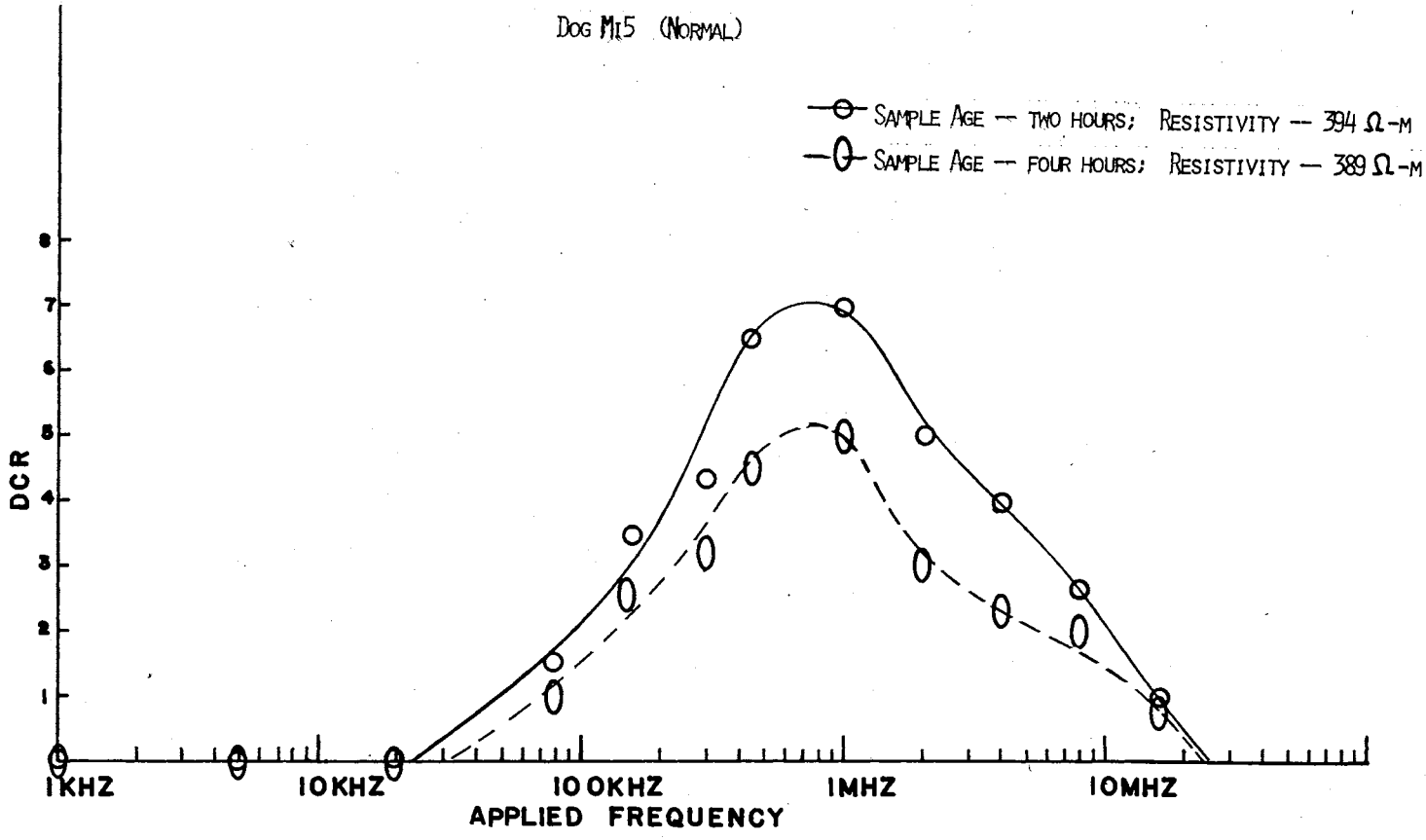

Figure 5. DCR vs Applied Frequency for a Normal Dog

TABLE I

DCR AT 1 MHZ FOR NORMAL DOGS

Dog	Trial	DCR at Two Hours	DCR at Four Hours	Average Resistivity (Ω-m)
MI4	1	5.0	4.0	298
MI4	2	6.5	6.2	410
MI4	3	6.4	5.2	300
MI4	4	4.8	4.6	320
MI4	5	15.0	9.0	400
S-6	1	11.5	8.5	378
S-6	2	10.2	5.2	400
S-6	3	5.6	4.0	397
S-6	4	7.0	3.8	296
MI5	1	5.8	5.4	560
MI5	2	10.2	6.4	354
MI5	3	9.0	7.4	424
A-4	1	5.2	5.0	351
A-4	2	7.2	6.2	406
Hank	1	6.5	6.0	334
S-5	1	7.2	5.0	270
S-5	2	7.2	6.8	290

It was now felt that the thrust of this project could be entered into with confidence and that reliable results could be obtained.

There were four biological models to be compared by the response of their blood platelets to the dielectrophoretic force. These were as follow: the normal male, the hemophilic male, the hemophilic female, and the female transmitter beagle dogs.

To arrive at unbiased results of the study, the following procedure was established: Rather than taking an active part in the collection of the raw blood, the investigator had the blood given to him and a code name given to the dog from which the blood sample had been taken. After preparation of the platelet suspension, the DCR as a function of the applied electric field frequency was observed and recorded. This procedure was repeated four times, the four observations constituting a group which contained all four different biological models. Four such groups were observed. After all the observations had been completed, the identity of the biological model to be associated with each code name was revealed. The results of one of the four groups are given in Figures 6, 7, 8, and 9. The data from the other three groups, as well as data for two additional transmitters, are presented in the appendix.

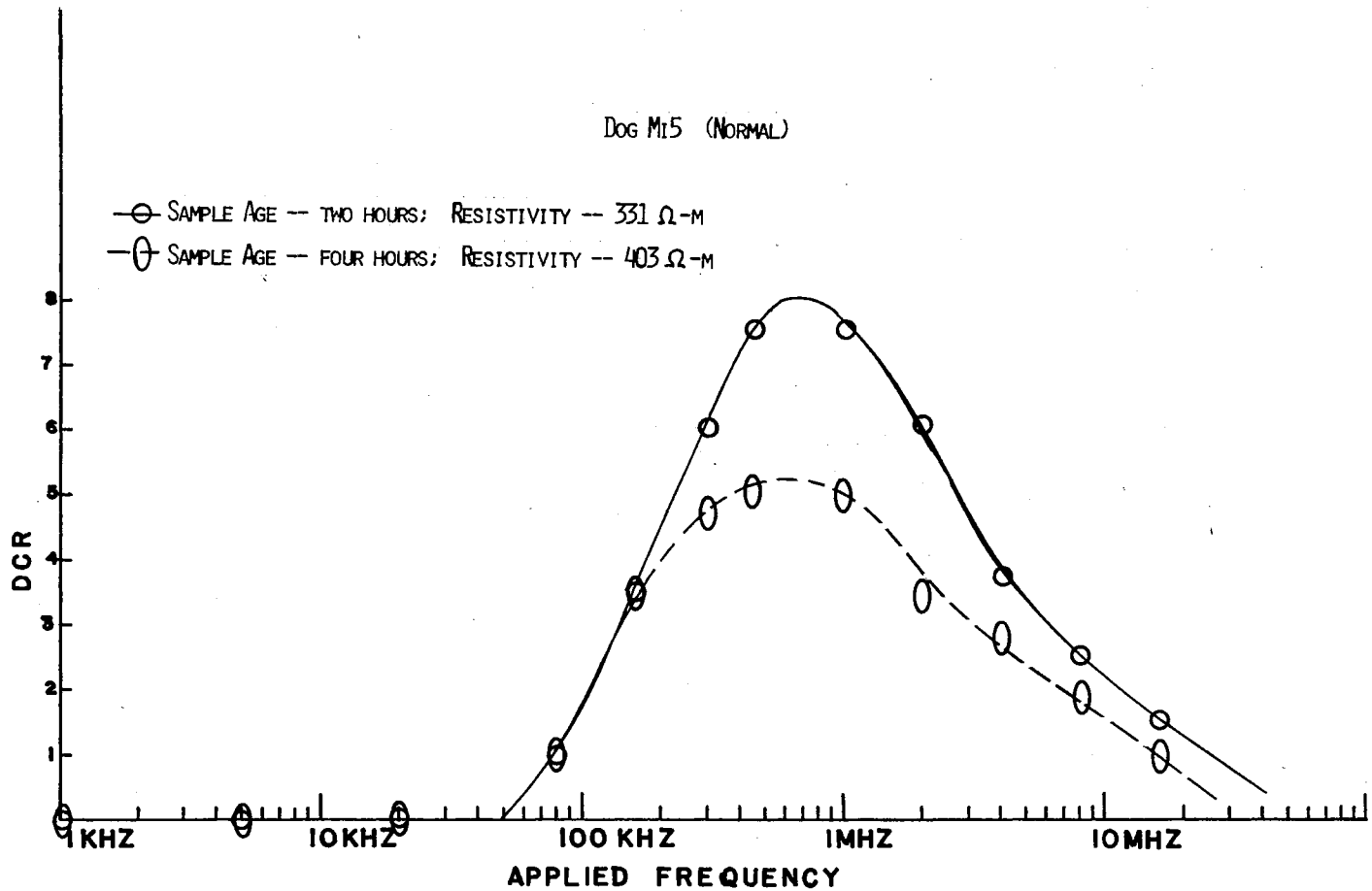

Figure 6. DCR vs Applied Frequency (Normal)

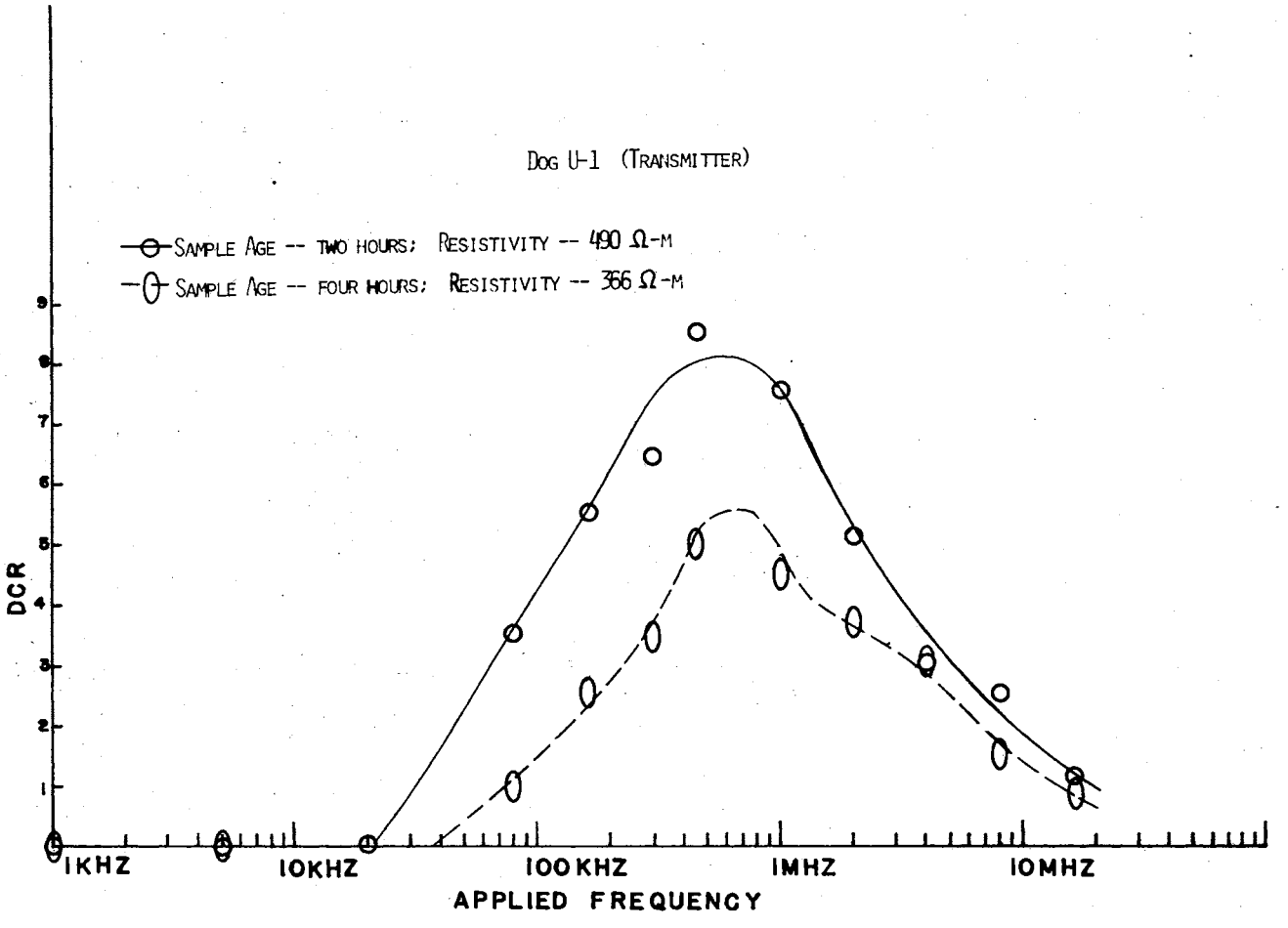

Figure 7. DCR vs Applied Frequency (Transmitter)

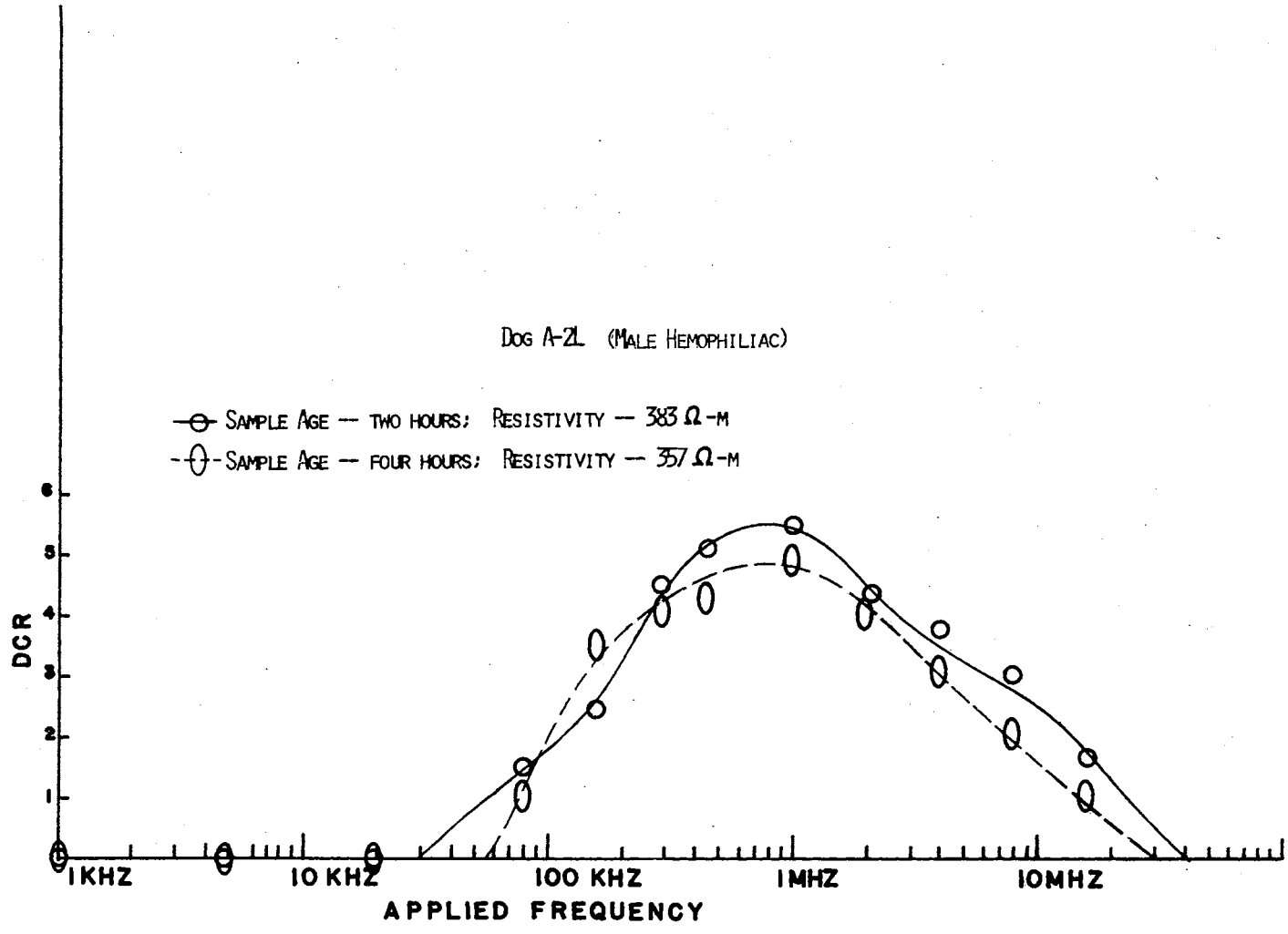

Figure 8. DCR vs Applied Frequency (Hemophilic Male)

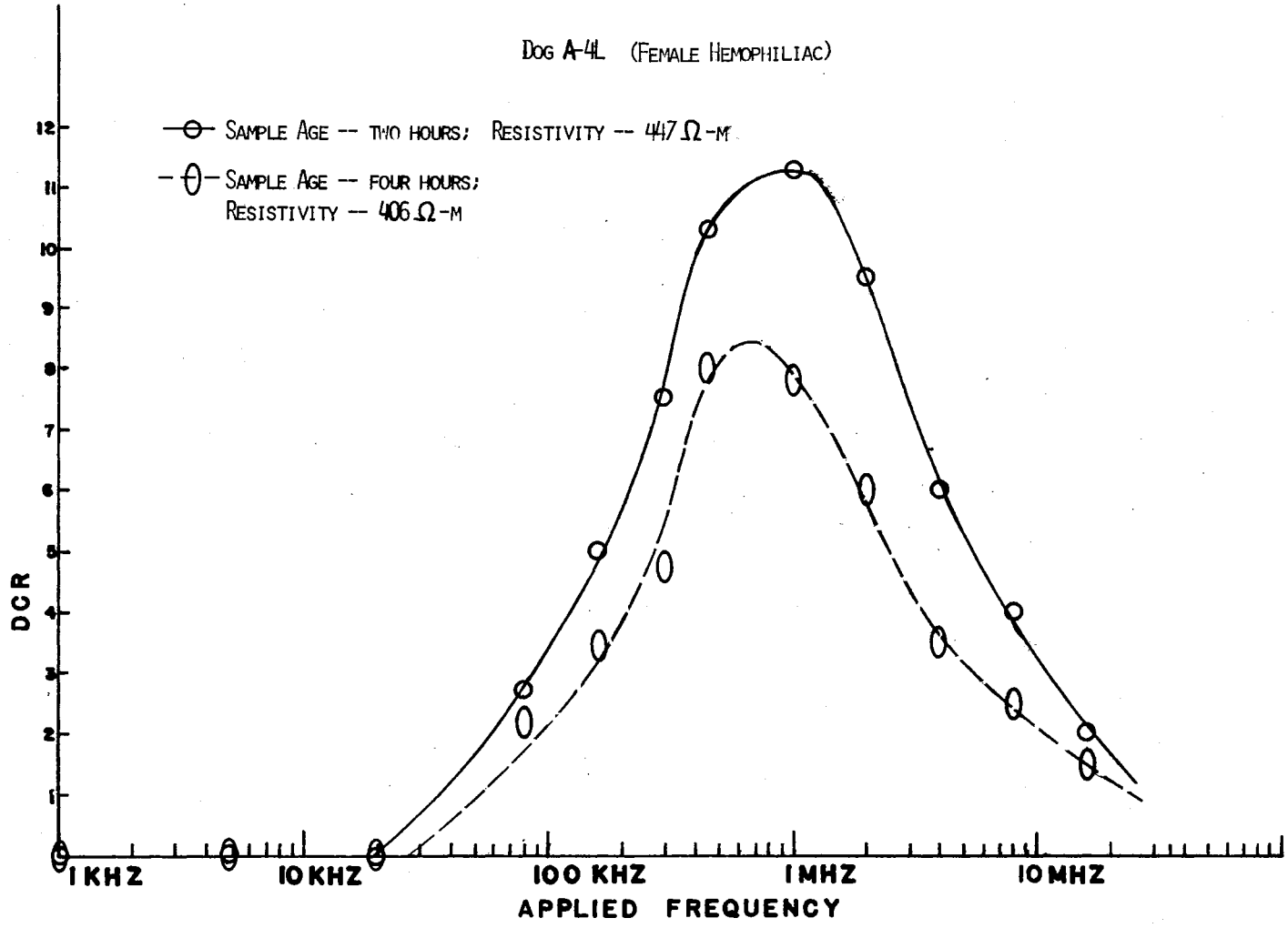

Figure 9. DCR vs Applied Frequency (Hemophilic Female)

CHAPTER V

DIELECTROPHORESIS OF NORMAL BLOOD PLATELETS UNDER VARIOUS PHYSIOLOGICAL CONDITIONS

A study was made to ascertain the effects of salts of various valences and of various metabolic inhibitors on the response of normal blood platelets. Two approaches were utilized; both employed the preparation procedure detailed in Chapter III with the slight modification noted on page 17 of that chapter. The addition of the chemical to the dextrose solution prior to the introduction of the platelets was done to prevent over-exposure of platelets to the chemicals in the local region where the chemical would otherwise be introduced. The procedure adopted assured that the platelets entered a uniform medium and that no portion of that medium received treatment unlike any other portion.

The response of the normal platelets to the dielectrophoretic force was then observed, these platelets now being suspended in a chemically doped dextrose suspension. The extent of this chemical treatment is given as a molarity in the data that is presented. A second aliquot with no chemical doping was prepared from the same 50,000 platelets per cubic millimeter concentrate that was used for the above. This sample provided a standard for comparison.

One method of ascertaining the effects of the various chemicals upon the response of the platelets was to observe the DCR of both aliquots at a fixed frequency of 1Mhz. The responses were observed as a

function of sample age, the latter parameter measured in hours with zero age being taken as the time at which the blood was withdrawn from the dog. The responses were usually monitored until the sample was ten hours old.

Typically the addition of chemicals to the platelets was accomplished when the sample age was from two to three hours old, the intervening time being required for preparation of the 50,000 platelets per cubic millimeter platelet concentrate. Results are shown in Figures 10 and 11, where the effect of KNO_3 and $Ca(NO_3)_2$ are compared with the standard.

The second mode of observation was to compare the DCR of the doped sample to that of the undoped sample as a function of the frequency of the applied electric field, similar to the procedure used in Chapter IV. Here, however, three working samples were prepared from one blood sample. First, an undoped working suspension was prepared and the DCR as a function of applied field frequency was recorded. This required approximately one and a half hours. A doped sample was then prepared, and its response observed; again, this procedure requiring about one and a half hours. Finally, another standard undoped sample was prepared, and its response observed. It was felt that this procedure would clearly point out differences in the response due only to the chemical in the doped sample, since the bracketing in time of the doped sample by undoped samples would give a very strong indication as to what the effect of time alone should be.

The nitrate salts which were used in this study were KNO_3, $NaNO_3$, $Mg(NO_3)_2$, $Ca(NO_3)_2$, $Al(NO_3)_3$, and $La(NO_3)_3$. The same ionic strength was used in all the samples. The fact that only nitrate salts were used is

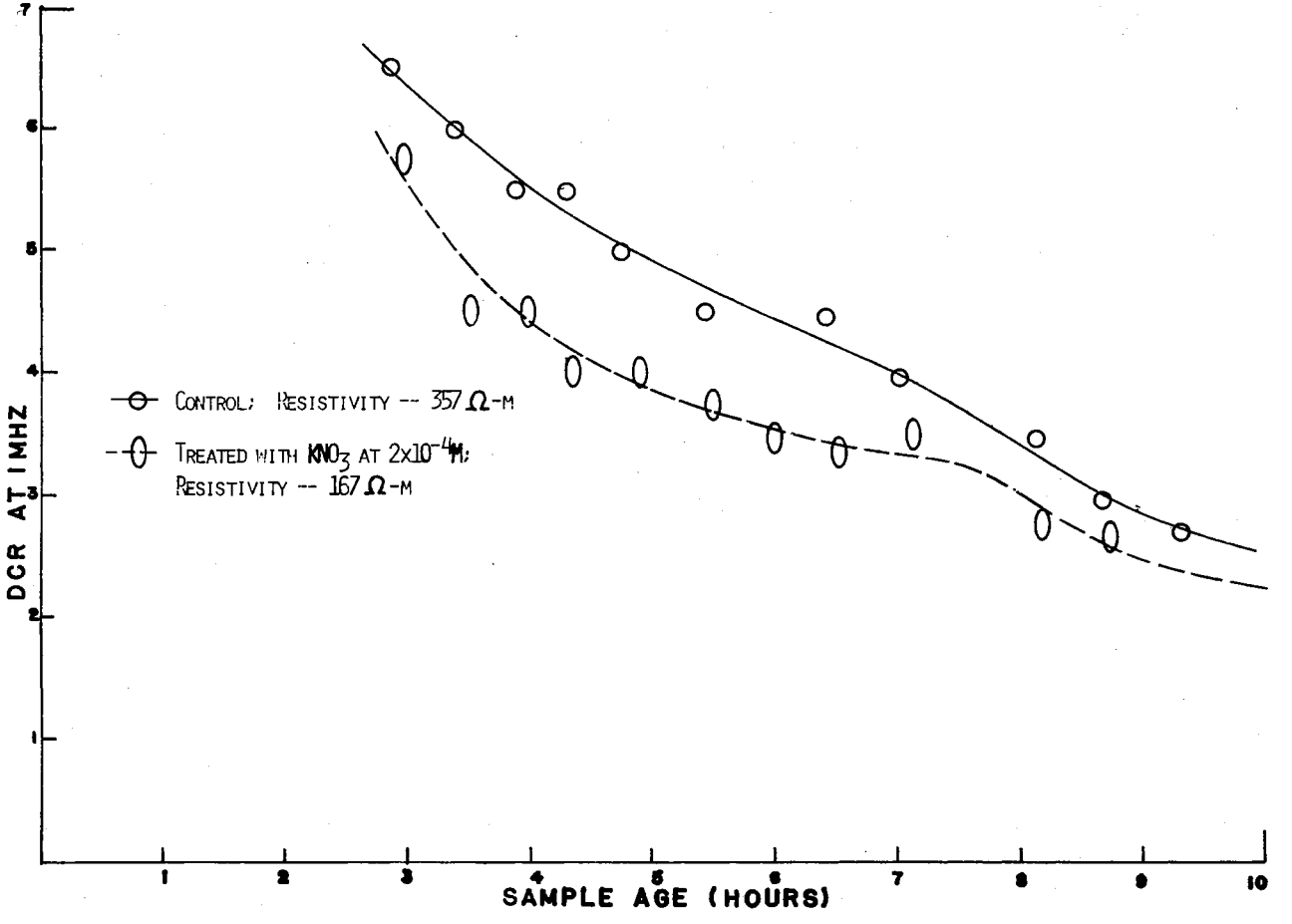

Figure 10. DCR at 1 Mhz vs Sample Age (KNO_3 Treated)

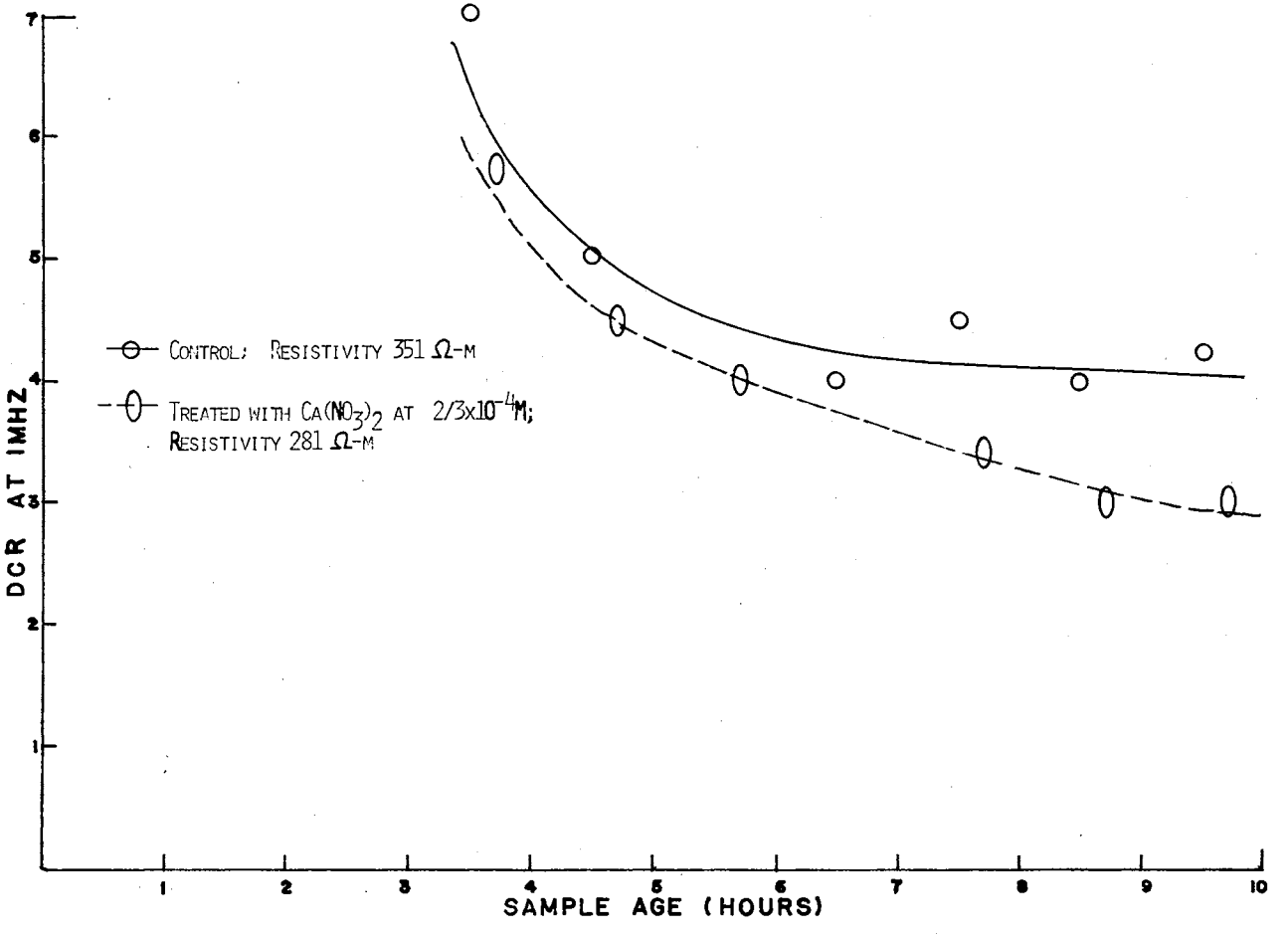

Figure 11. DCR at 1 Mhz vs Sample Age ($Ca(NO_3)_2$ Treated)

indicative of the desire to observe in this series only the effect of the cation upon the response of the platelets, and hence the effect of the cation valence upon the ionic atmosphere of the individual platelet. Data from both modes of observation are given for $NaNO_3$, $Mg(NO_3)_2$, and $La(NO_3)_3$ in Figures 12, 13, and 14, respectively.

The physiological environment of the normal platelets was also altered by the addition of K_2SO_4 and the potassium salt of mellitic acid $C_6(COOK)_6$. When this information was reviewed in conjunction with that obtained for potassium nitrate, there resulted a picture of the effect of anion upon the response of the platelets to the dielectrophoretic force and hence the effect of the anion valence upon the ionic atmosphere of the individual platelet. As the anion was held constant before, so was the cation held constant here--the potassium cation. The results for both modes of observation are given for K_2SO_4 and $C_6(COOK)_6$ in Figures 15 and 16, respectively.

Six other chemicals were used and their effects upon the response of the normal platelets to the non-uniform field were observed. One was sodium fluoride (NaF), an inhibitor of glycolysis. It will be seen from Figure 17 that this agent was quite successful in slowing down the decay of the DCR with time relative to the untreated control sample. This relative stabilization was also found by Wiley (3) when he treated the canine erythrocyte with the same agent. This treated sample and the control were retained and examined on subsequent days, the doped sample continuing to give a response significantly larger than the control. The response of the control was nil after the sample was on the order of 30 hours old, whereas the response of the treated sample continued to be significant for up to 72 hours or more. The DCR as a function of

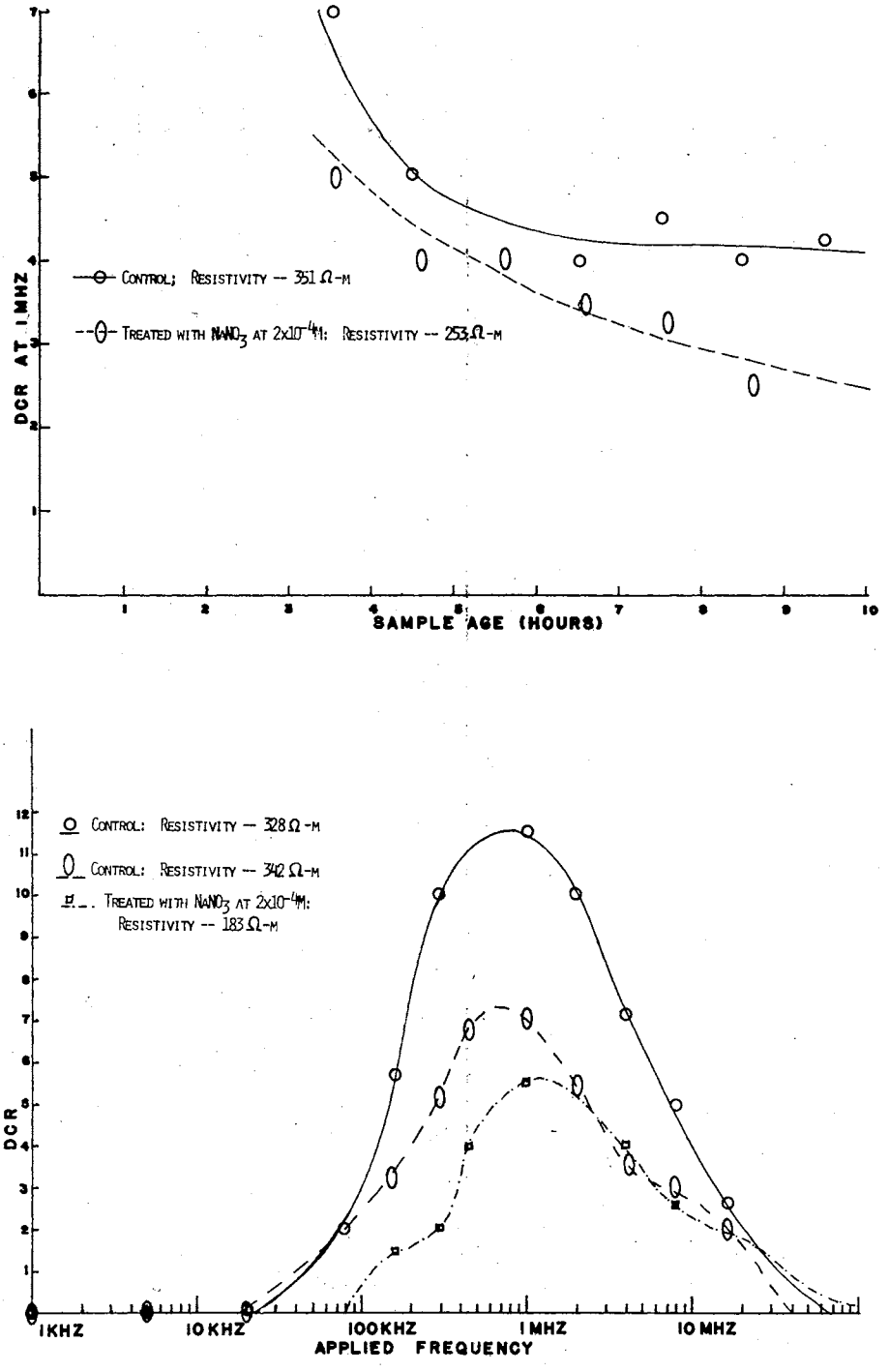

Figure 12. DCR of $NaNO_3$ Treated Platelets vs Sample Age and vs Applied Frequency

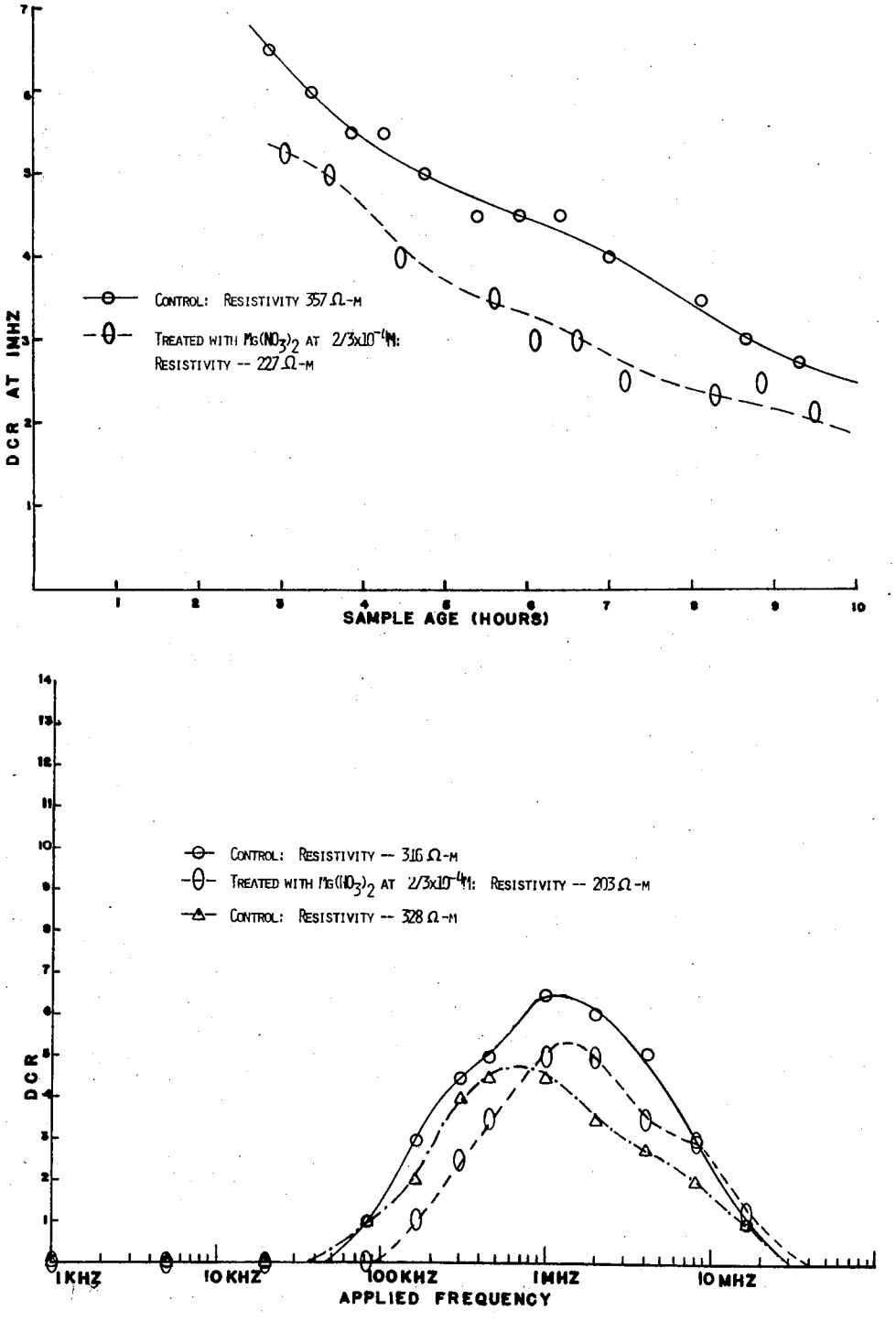

Figure 13. DCR of $Mg(NO_3)_2$ Treated Platelets vs Sample Age and vs Applied Frequency

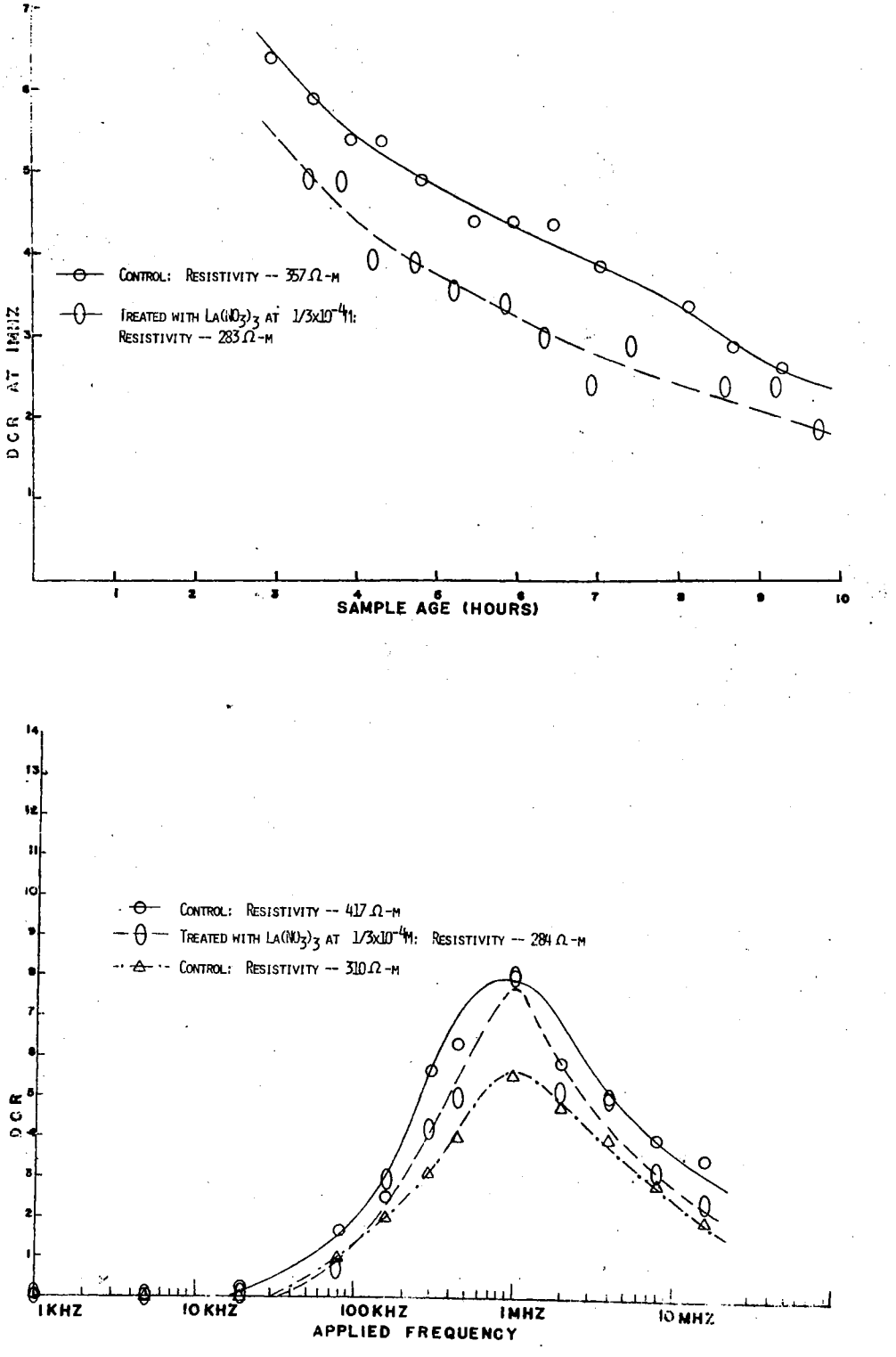

Figure 14. DCR of $La(NO_3)_3$ Treated Platelets vs Sample Age and vs Applied Frequency

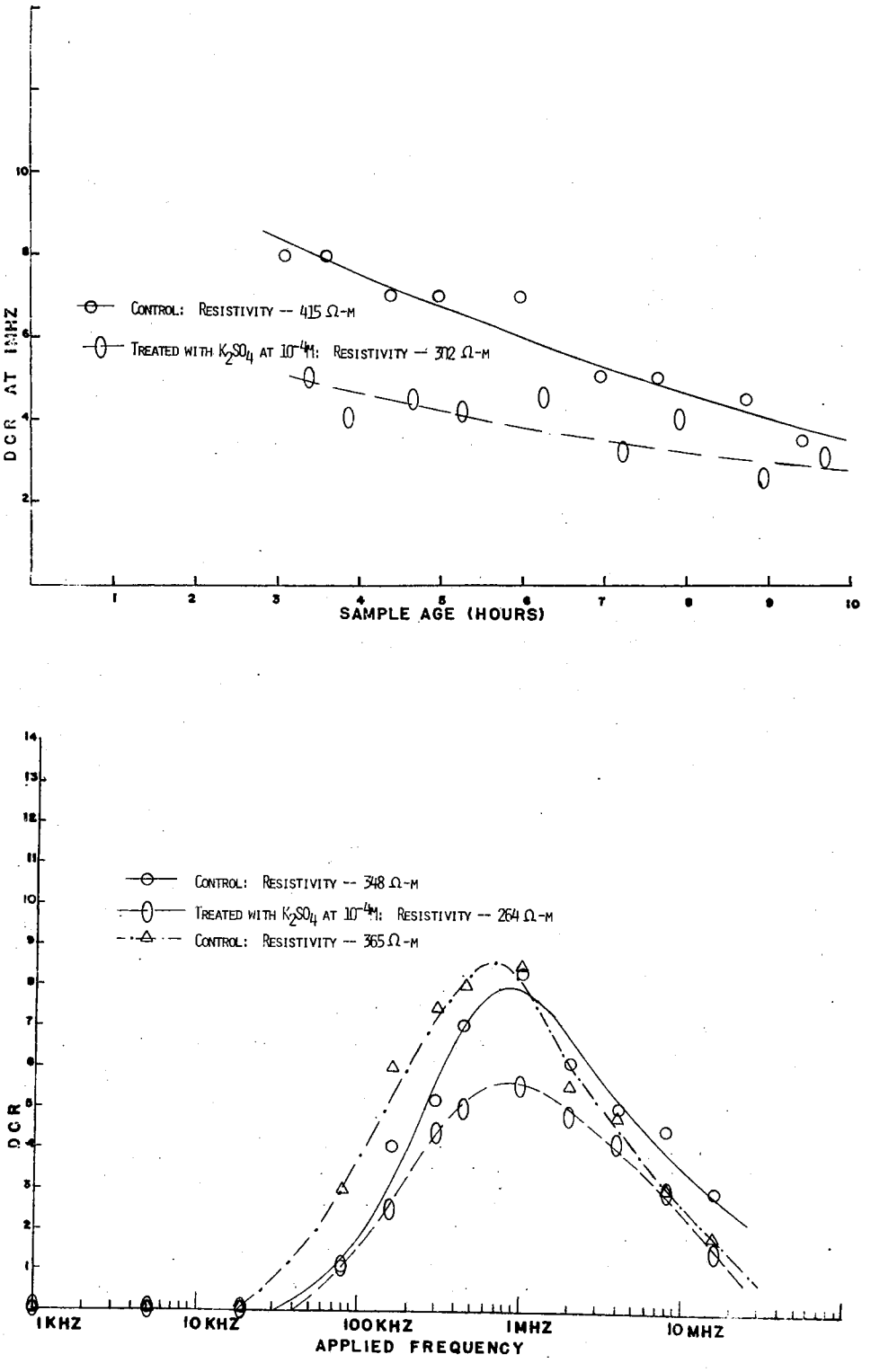

Figure 15. DCR of K_2SO_4 Treated Platelets vs Sample Age and vs Applied Frequency

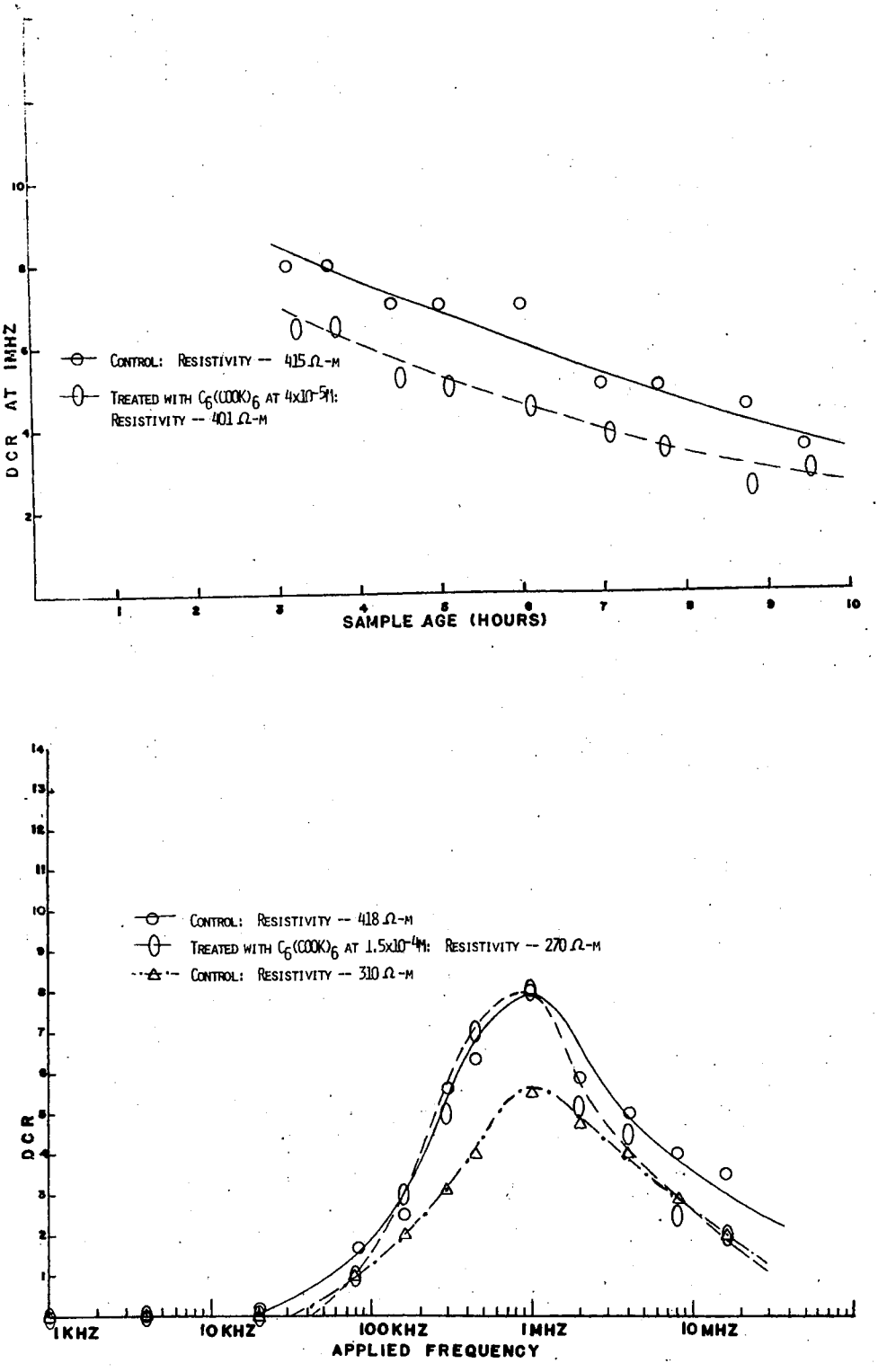

Figure 16. DCR of $C_6(COOK)_6$ Treated Platelets vs Sample Age and vs Applied Frequency

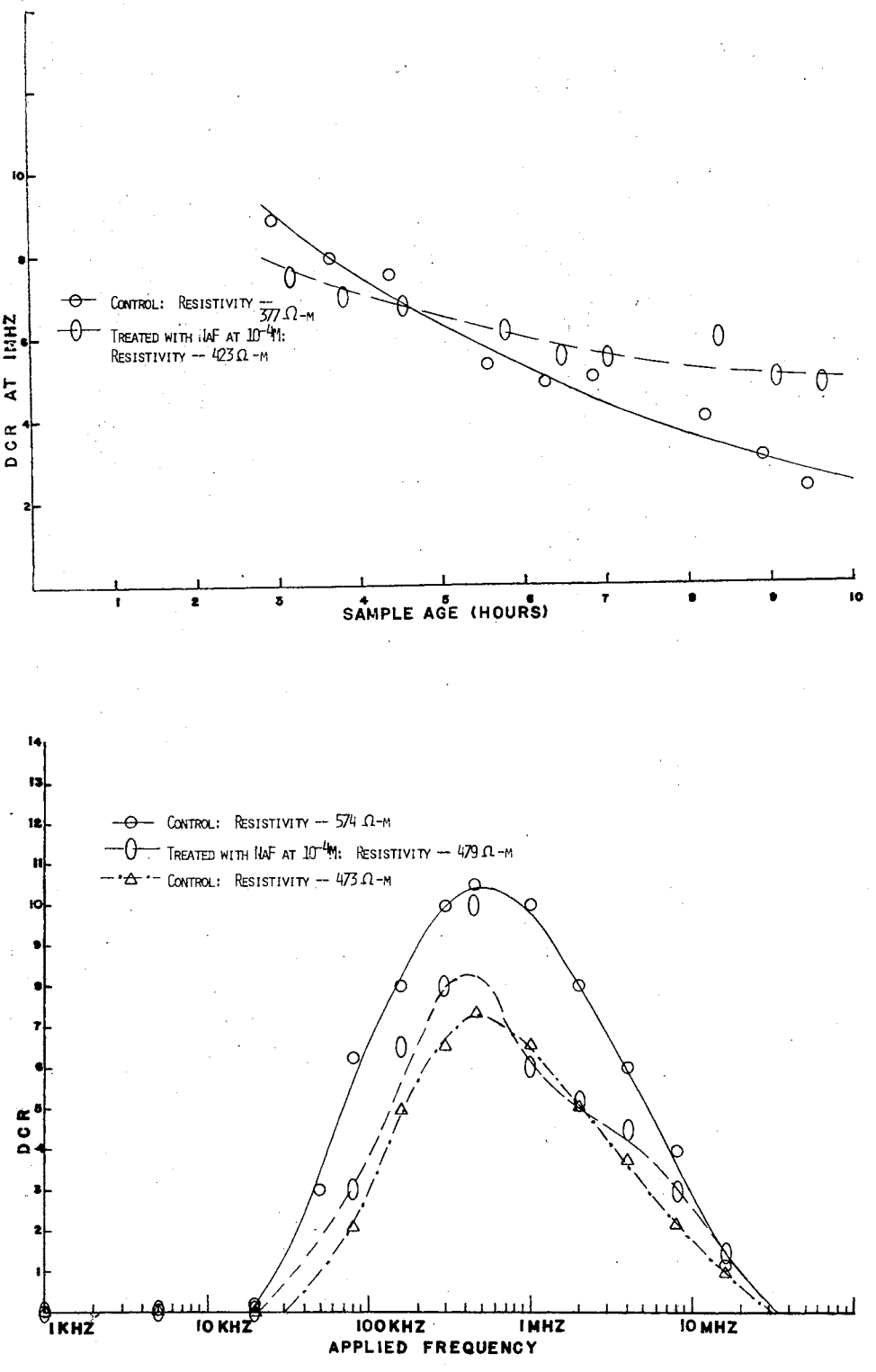

Figure 17. DCR of NaF Treated Platelets vs Sample Age and vs Applied Frequency

applied field frequency was not particularly illuminating.

The sodium salt of iodoacetic acid was used, again as a glycolysis inhibitor. Contrary to the findings of Wiley with the erythrocytes, the platelets failed to stabilize at a relatively constant DCR as a function of time in the presence of this agent. As Figure 18 shows, the effect of $CH_2ICOONa$ upon the response of the platelets was to enhance the decay of the DCR with time relative to the control. Figure 18 explicitly shows that the DCR for the doped sample was less than the DCR of the controls at all frequencies, but that the trend of the response was about the same in both cases.

Sodium cyanide is a respiratory inhibitor rather than a glycolysis inhibitor. When platelets were exposed to this agent, the DCR at 1Mhz applied field frequency for the platelets remained relatively constant after a transition period of what appeared to be about three hours. (See Figure 19.) That is, the rate of the decay of the DCR with time was slower for the NaCN treated sample than for the control. As with the NaF sample, the response of this sample was checked on subsequent days. It was found that the NaCN sample continued to respond even better than the NaF sample did.

The DCR of this NaCN doped sample as a function of applied field frequency showed a very marked variation from all previous and subsequent observations. As can be seen in Figure 19, the maximum DCR occurred at a much lower frequency for the NaCN treated sample than for either of the two controls. The magnitude of the response for the treated sample at its maximum lay between the magnitudes of the maximum of the controls, indicating that the variation was not due only to a sample age difference.

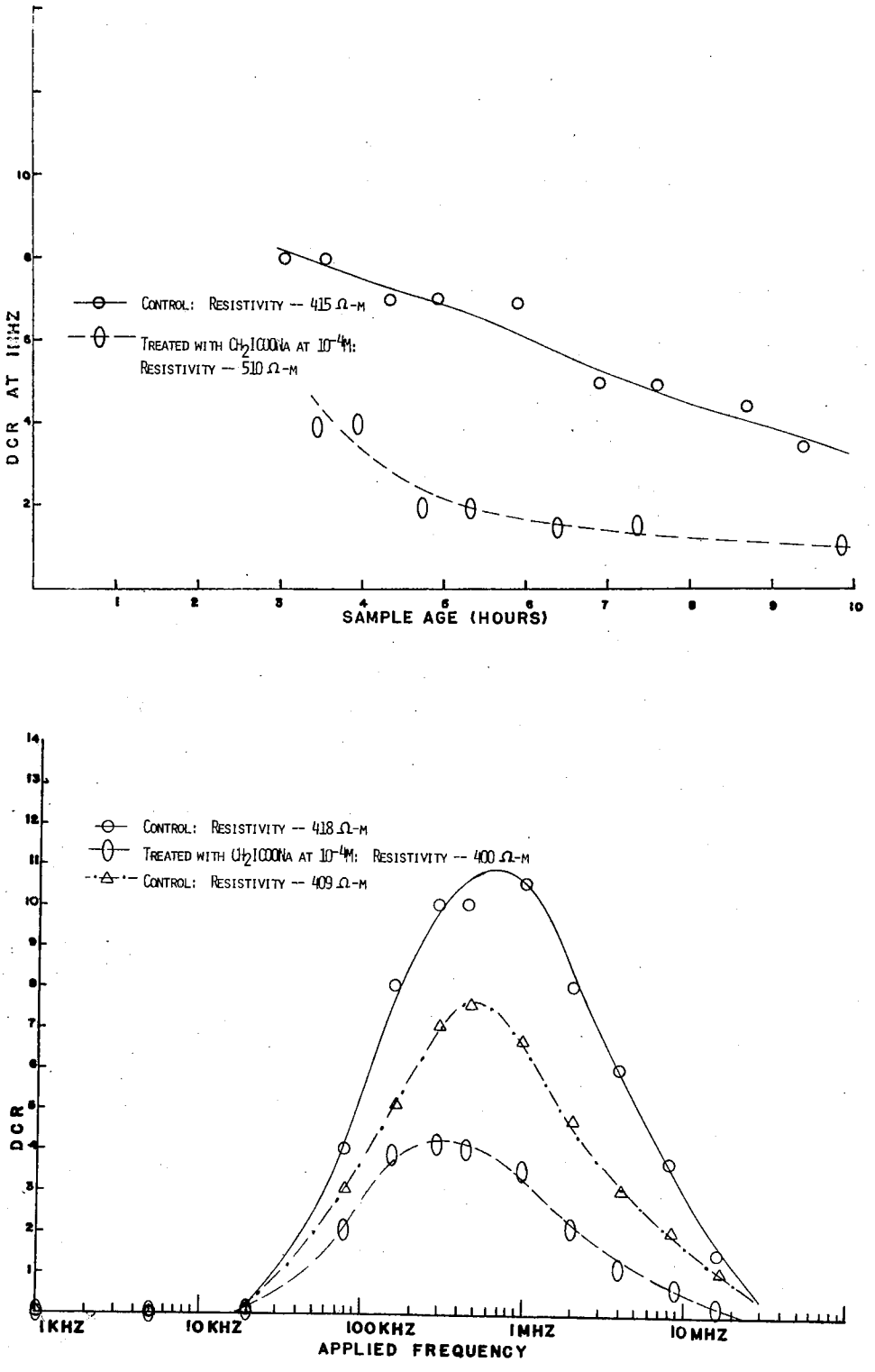

Figure 18. DCR of $CH_2ICOONa$ Treated Platelets vs Sample Age and vs Applied Frequency

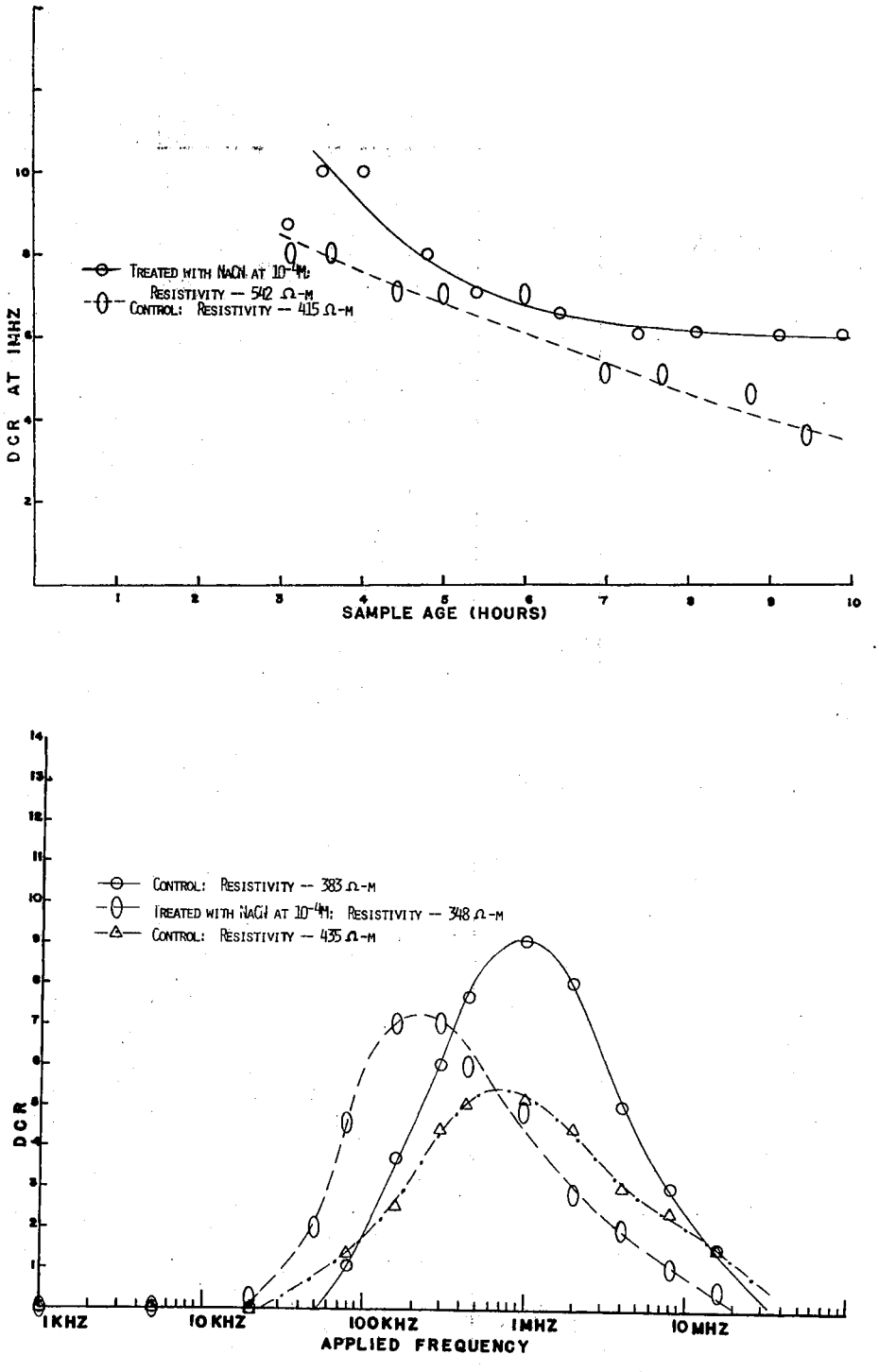

Figure 19. DCR of NaCN Treated Platelets vs Sample Age and vs Applied Frequency

Sodium azide, another respiratory inhibitor, appears to have caused a very slight acceleration in the decay of the DCR with time, as can be seen in Figure 20. Obviously the magnitude of the response was also less. From Figure 20 it will be seen that this latter observation maintained its validity at all applied frequencies.

Dinitrophenol, DNP, was found to greatly accelerate the decay of the DCR with time. After four to five hours of exposure to this agent, the response of the platelets was negligible. (See Figure 21.) Again, at all frequencies the response of the DNP treated platelets was less than that of the control platelets, as is evident in Figure 21.

The sixth chemical used was formaldehyde, HCHO. Although the initial response of the HCHO treated platelets was less than that of the control, the rate of decay of the DCR with time appears (as seen in Figure 22) to be less than that of the control. Further, within the time interval observed, the HCHO treated platelets reached a somewhat stable value for the DCR, whereas the DCR of the control platelets continued to decrease in a steady fashion. Figure 22 displays the response of the HCHO treated platelets as a function of the frequency of the applied field; the effect was simply to lessen slightly the response relative to the control platelets, but again the trend of the response was essentially the same.

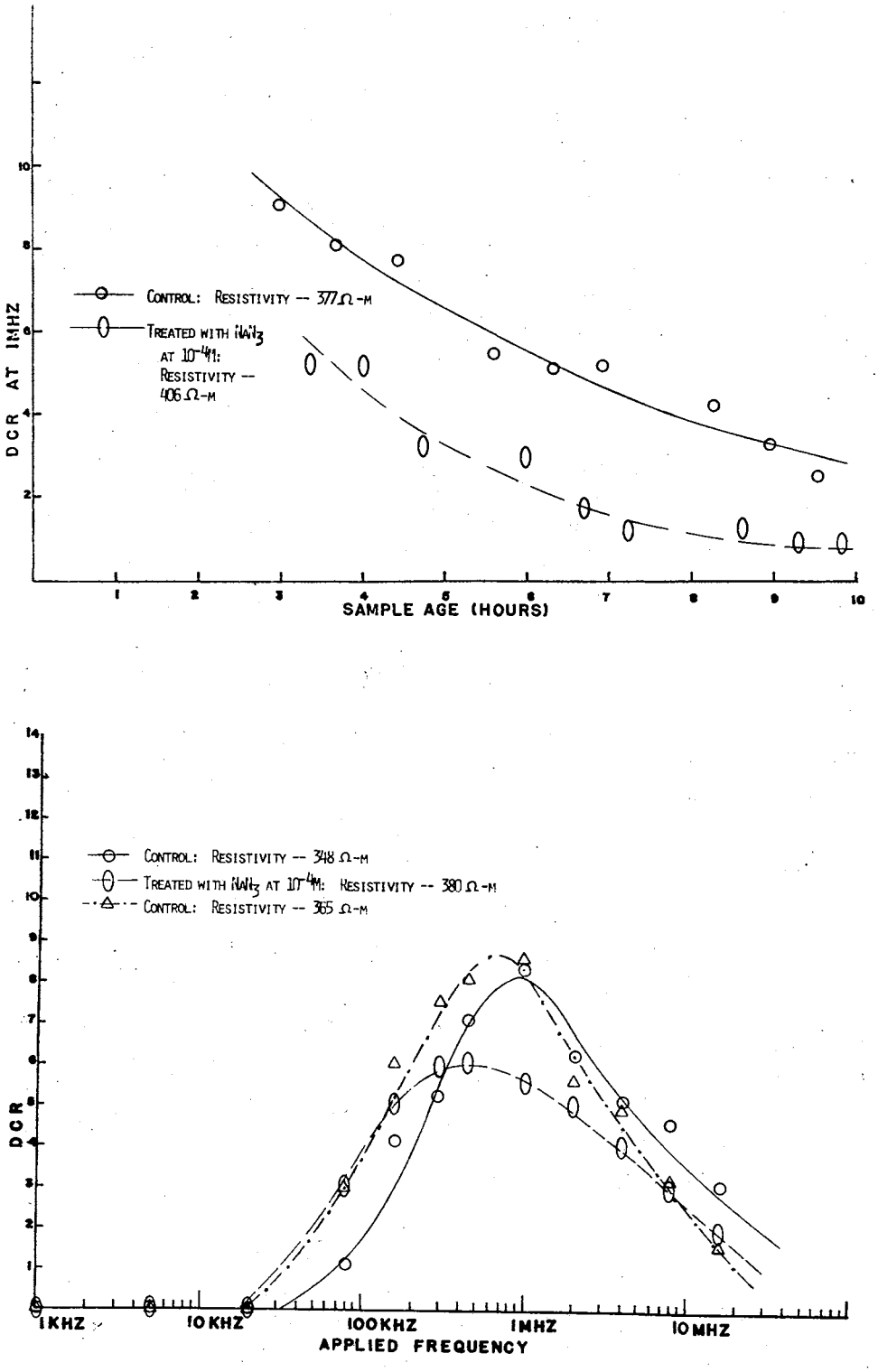

Figure 20. DCR of NaN_3 Treated Platelets vs Sample Age and vs Applied Frequency

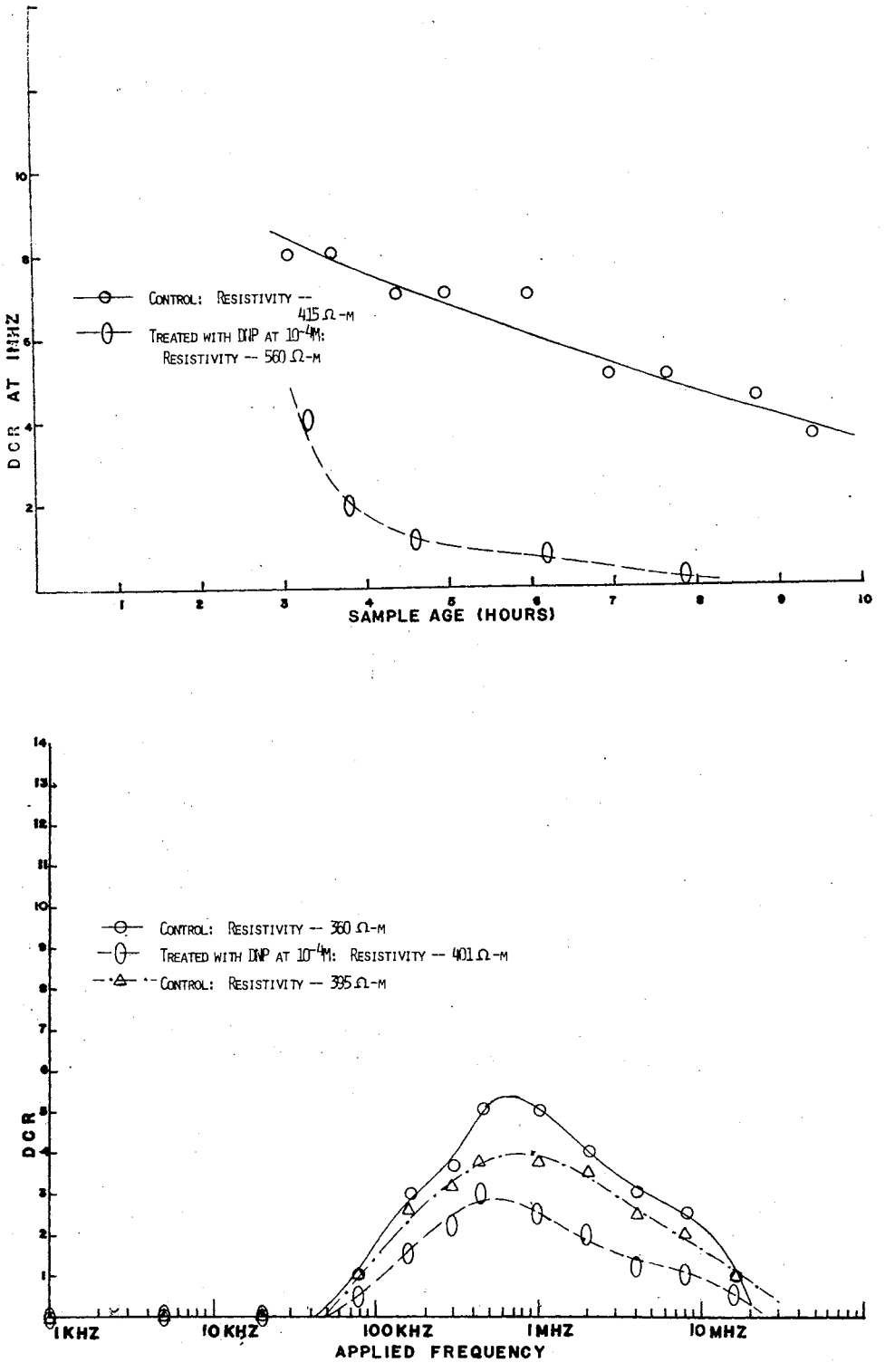

Figure 21. DCR of Dinitrophenol Treated Platelets vs Sample Age and vs Applied Frequency

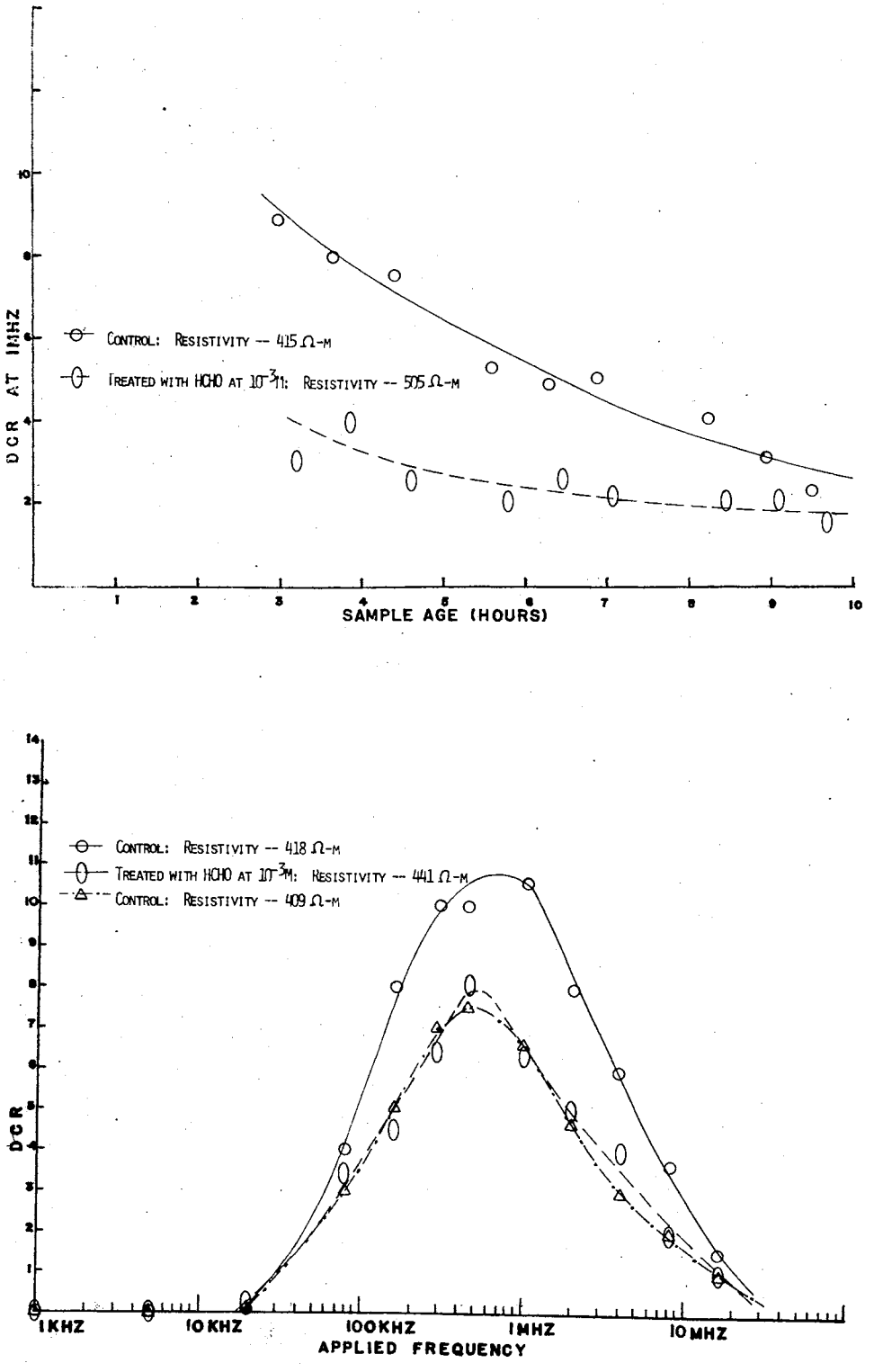

Figure 22. DCR of HCHO Treated Platelets vs Sample Age and vs Applied Frequency

CHAPTER VI

CONCLUSIONS

Once the preparation procedure for the platelet suspensions had been established, their response to the dielectrophoretic force was both apparent and consistent. The search for the proper procedure began with the search for an acceptable anti-coagulant. As mentioned in Chapter III, 10 percent EDTA, which was the first anti-coagulant used, gave very poor results as pertained to this study. The platelets appeared to aggregate readily and response of those few platelets that did not enter into aggregates was at best poor. The suggestion by Dr. Ralph Buckner that cocaine be used at a level of 1 percent (by weight) in the EDTA failed to either enhance the response or reduce the clumping of these platelets. When it was finally established that EDTA would not suffice under any circumstances, the acid-citrate-dextrose (ACD) anti-coagulant was tried successfully. The resulting suspensions lacked the large aggregates so prevalent in the EDTA-prepared suspensions, and the response was consistently significant.

However, the suspensions still had some aggregates. The finding by Flatow and Freireich (15) was utilized, with dextrose used in place of the citric acid to eliminate the remaining aggregates in the final working suspensions by lowering the pH of the platelet suspensions to 6.8 or lower. This realization of an upper acceptable level to the pH was one of the more important stages in the quest for the proper preparation procedure.

From the beginning an isotonic solution of 5 percent by weight dextrose was utilized here as the standard suspension. This was an easy solution to prepare, and it was found quite satisfactory for all phases of this study.

From Figure 5 of Chapter IV it is obvious that once the platelet preparation procedure had been perfected, the response of the platelets to the dielectrophoretic force was readily observable. The variation of the response with the frequency of the applied electric field which is evident in this figure has invariably been observed in other dielectrophoretic studies. The maximum response of the platelets occurs when the frequency of the applied field is somewhere in the range from 450Khz to 1Mhz, with the response dropping off at frequencies higher and lower than this range. In contrast to the response versus frequency of the yeast cells studied by Crane, the platelets failed to show any significant response at low frequencies. Response was observed to be nil for the platelets in the frequency range from 20Khz down to 100hz, whereas the yeast cells exhibited an increase in the response in this same range, with the response increasing as frequency decreased (10).

An examination of the data presented in Chapter IV and the appendix is presented in Table II and Figure 23. The averages given in Table II are the mean values of the DCR at 1Mhz when the sample is two and four hours old. It is obvious that there are differences in these mean values, the response for the transmitter and female hemophilic dogs being less than that for the normal dog; the response for the male hemophilic dog being significantly greater than that for the normal.

It would be hoped that by various improvements in technique these noted distinctions could be heightened. Additionally, a larger number

TABLE II

MEAN VALUES FOR DCR AT 1 MHZ APPLIED FREQUENCY

Number Trials N	Model	Two Hour			Four Hour		
		Mean	$\frac{\sigma}{\sqrt{N-1}}$	Standard Deviation σ	Mean	$\frac{\sigma}{\sqrt{N-1}}$	Standard Deviation σ
21	Normal	7.33	$\pm$0.57	2.53	5.59	$\pm$0.31	1.39
6	Transmitters	6.38	$\pm$0.52	1.16	5.05	$\pm$0.21	1.48
4	Hemophiliac (females)	7.05	$\pm$1.54	2.66	5.42	$\pm$1.09	1.88
4	Hemophiliac (males)	9.07	$\pm$1.89	3.28	5.90	$\pm$0.47	0.82

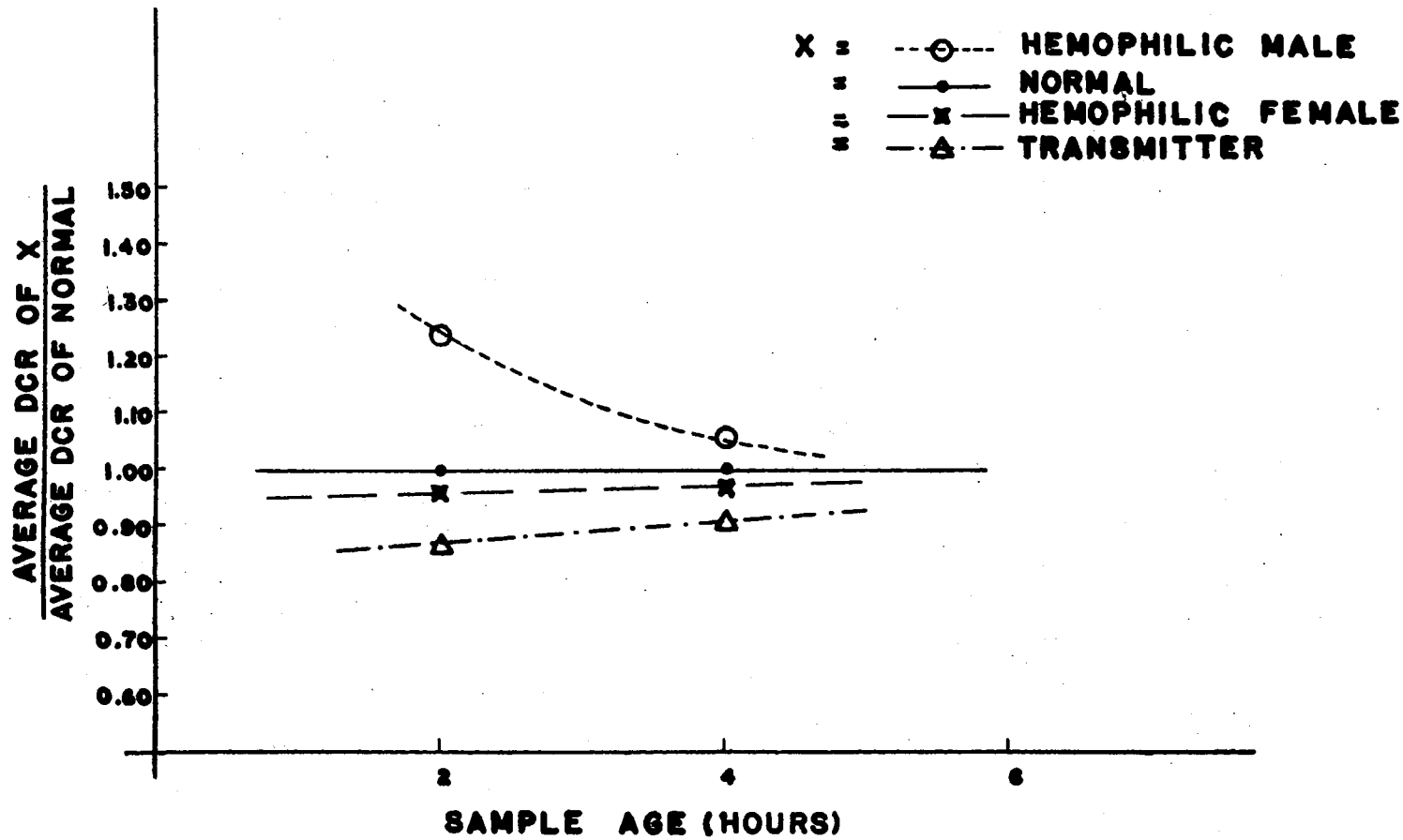

Figure 23. Average DCR at 1 Mhz of X ÷ Average DCR at 1 Mhz of Normal vs Sample Age

of trials for each biological model should serve to fix the means at much more precise values. Study of other colonies of dogs would serve as a comparison to the work done here and as a reference in future study.

The DCR of normal blood platelets at 1Mhz as a function of time was displayed in a number of figures in Chapter V; for example, Figure 10. The decrease in the response as a function of time is evident. This same fact is also seen in Chapter IV, since the response of the old aliquot was invariably less than that of the other. It is known that the age of a platelet-rich plasma determines how effective it will be in elevating the platelet count of a recipient to a PRP transfusion. It has been found that PRP stored 24 and 48 hours is 62 percent and 37 percent, respectively, as effective as fresh PRP in elevating the platelet count in a recipient one hour after a transfusion (18).

It is contended here that the physiological state of the platelets deteriorates in a stored plasma and in a dextrose solution. This deterioration of the platelet is reflected both in the decrease of its response to dielectrophoresis and the decrease of its effectiveness in a transfusion.

Figures 10, 11, 12, 13, and 14 of Chapter V demonstrate the effect, or better yet, the lack of effect of the dilute cation upon the response of the platelets as a function of time. Figures 12, 13, and 14 give the response of these doped platelets as a function of the frequency of the applied field. Again, there appeared no significant effect. The response of the platelet, then, is not necessarily dependent upon the surrounding ionic atmosphere at the dilute concentrations used in this study.

The anion effect was demonstrated in Figures 10, 15, and 16 of Chapter V. The NO_3^{-1} anion effect was insignificant, as was the hexavalent $C_6(COO^{-1})_6$, even when the latter was tried at differing concentrations. From these results and the results of the cation study, it appears again that the response of the platelet does not depend strongly upon the surrounding ionic atmosphere at the concentrations used. However, the remaining anion studied, the sulfate ion SO_4^{--}, did appear to produce an effect, even if slight. It appears from Figure 15 of Chapter V that the rate of decay of the response of the K_2SO_4 treated sample is less than that of the untreated or standard suspension. Further, Figure 15 shows that the response of the K_2SO_4 doped sample is less than the controls at all frequencies. Since the KNO_3 and the $C_6(COOK)_6$ samples failed to show an effect, the potassium ion is probably not the basis for the observed effect in the K_2SO_4 sample. Rather, the effect must be due to some interaction of the sulfate ion with the platelet itself.

Of the remaining studies done, the platelets treated with iodoacetic acid (sodium salt thereof), $CH_2ICOONa$, and dinitrophenol, DNP, failed to respond as well as the control platelets. (Figures 18 and 21 of Chapter V) Since these two agents interact with the platelet itself, this would further tend to indicate that the response of the platelet to the dielectrophoretic force is due to the platelet itself and not its surrounding ionic atmosphere.

Sodium azide (Figure 20) also produced an effect already mentioned in Chapter V, the effect being perhaps a slight acceleration in the decay of the DCR with time as compared with an untreated sample. Otherwise, the effect of NaN_3 was uninformative. Similarly, the results of the study with formaldehyde created no great sensations.

Perhaps the most important phase of this study was with the sodium cyanide and sodium fluoride treated samples, Figures 19 and 17, respectively. The relative stabilization of the DCR of these treated platelets probably represents a "freezing" of the platelets in their physiological state. However, Figure 19 clearly shows that the NaCN treated platelets are no longer normal in their response to the dielectrophoretic force, whereas 17 shows that the NaF treated platelets are. Clearly, the cyanide ion, CN^-, was the only agent studied that was capable of affecting the frequency at which the maximum DCR occurred. This follows since $NaNO_3$ or any of the other sodium compounds failed to produce this same effect, ruling out the possibility that the sodium ion was responsible.

The relative stabilization of the platelets due to the addition of the inhibitors NaCN and NaF represents a general "freezing" of at least the electrical properties of the cells under study, and perhaps even the "freezing" of some portions of the physiological state of the platelets. This now can be considered a general effect since Ting, Jolley, Beasley, and Pohl (5) have shown a very similar relative stabilization of spinach chloroplasts treated with 3-(3,4-dichlorophenyl)-1; 1-dimethylurea (DCMU); and Wiley (3) has shown a similar stabilization of the response of the canine erythrocyte when these are treated with the F^-, CN^-, iodoacetate, and azide, N_3^- ions.

We can infer, then, that stabilization of the electrical polarization characteristics of cells and organelles can be accomplished with chosen metabolic inhibitors. How far this can be carried over into the broader aspects of physiological stabilization has yet to be shown. If, for example, a given cellular system, together with a given inhibitor

shows steady degradation, yet exhibits "stabilization" as far as its dielectrophoresis (and hence its electrical polarizability), then we can be certain that factors other than those involved in the electrical polarization aspects are involved. This could be a powerful tool in the analysis of metabolic changes.

Here the relative stabilization of the DCR in time was of interest for the following reason. Perhaps either of the agents NaCN or NaF could be utilized to increase the effectiveness of stored platelets in transfusions of PRP for the purpose of elevating platelet counts in recipients. Markus and Zucker (8) have stated that the "metabolic inhibitors such as cyanide, fluoride, and azide do not inhibit clumping by ADP, thrombin, ATP, or 5-HT when added to PRP at concentrations of 2.5 - 10 x 10^{-4}M." Further, they state that "NaCN had little to no effect on the viscous metamorphosis" of the platelet. Hence, platelets treated with either inhibitor should clot normally. Of the two chemicals, NaCN proved to give a longer stabilization, whereas NaF failed to alter the shape of the DCR versus frequency curve from that found for untreated platelets. The questions to be asked are (1) can the recipient receive these treated platelets safely and (2) can either of these two chemicals be used to enhance the effectiveness of stored PRP transfusions.

As in other dielectrophoretic studies, the phenomena of rotation of the cells that had collected was observed. Further uniform motion of the suspending medium was observed at low frequencies (~ 1Khz) even though the platelets were not present. A basic understanding of these phenomena is yet to be had.

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APPENDIX

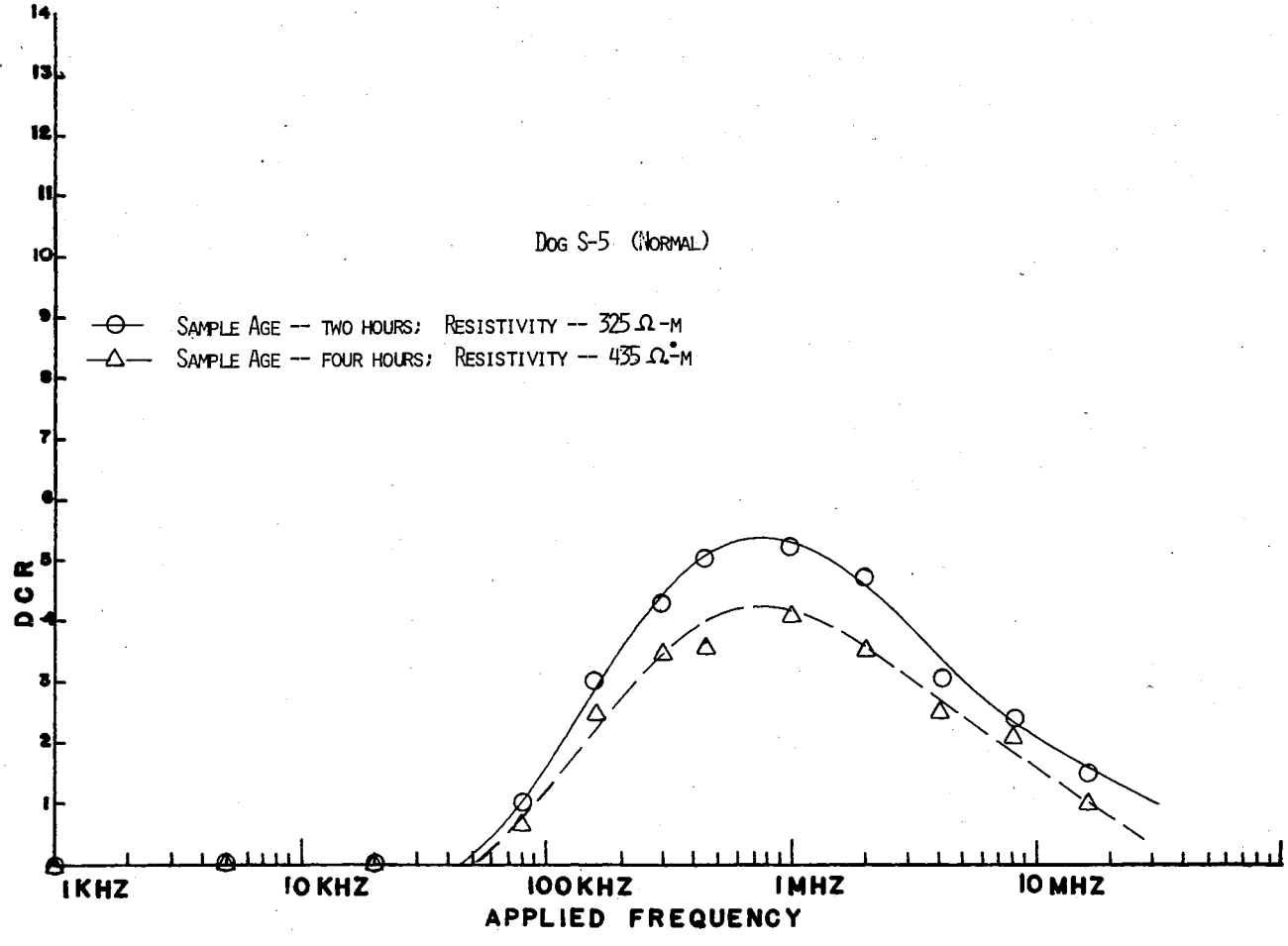

Figure 24. DCR vs Applied Frequency (Normal)

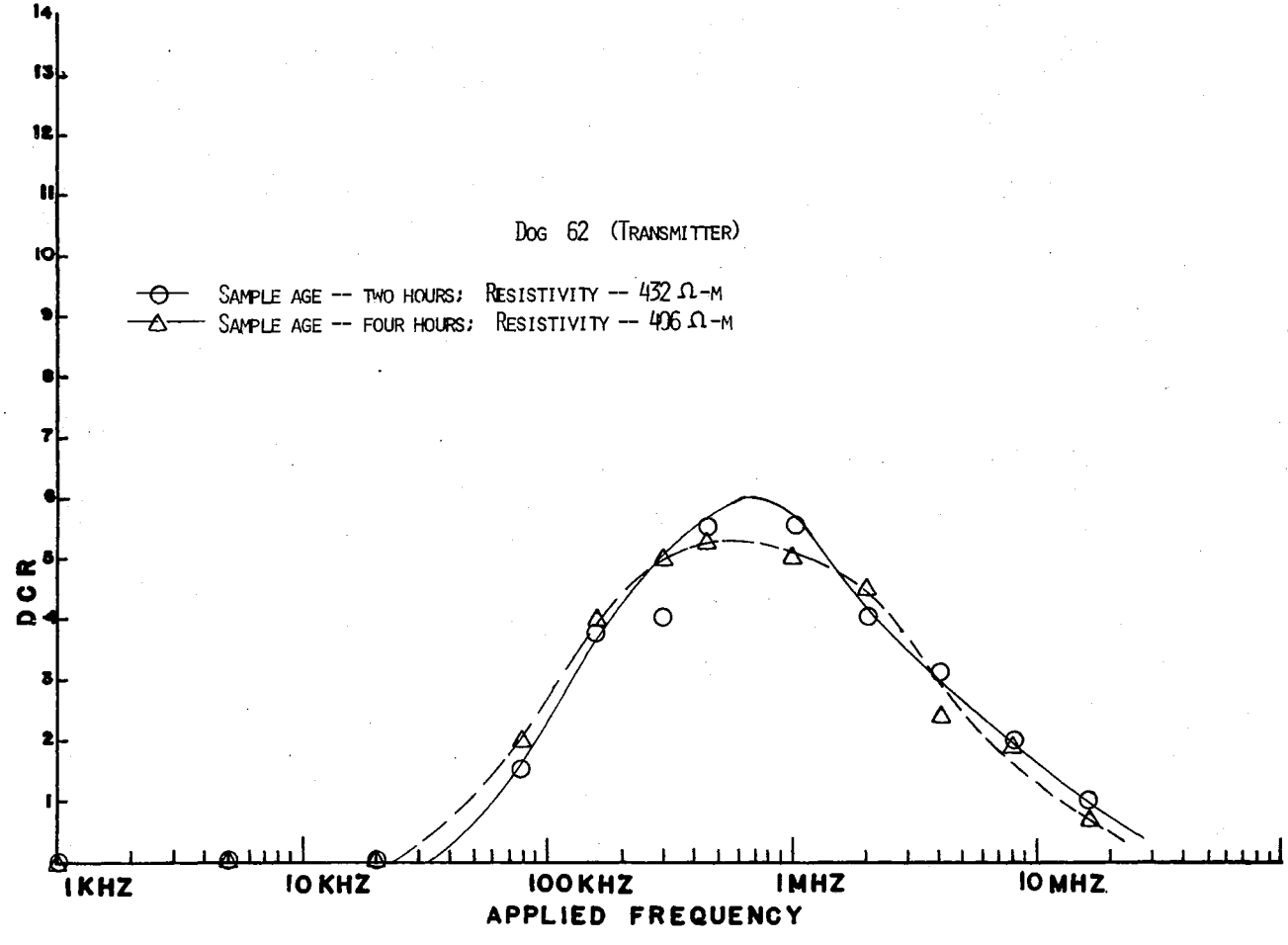

Figure 25. DCR vs Applied Frequency (Transmitter)

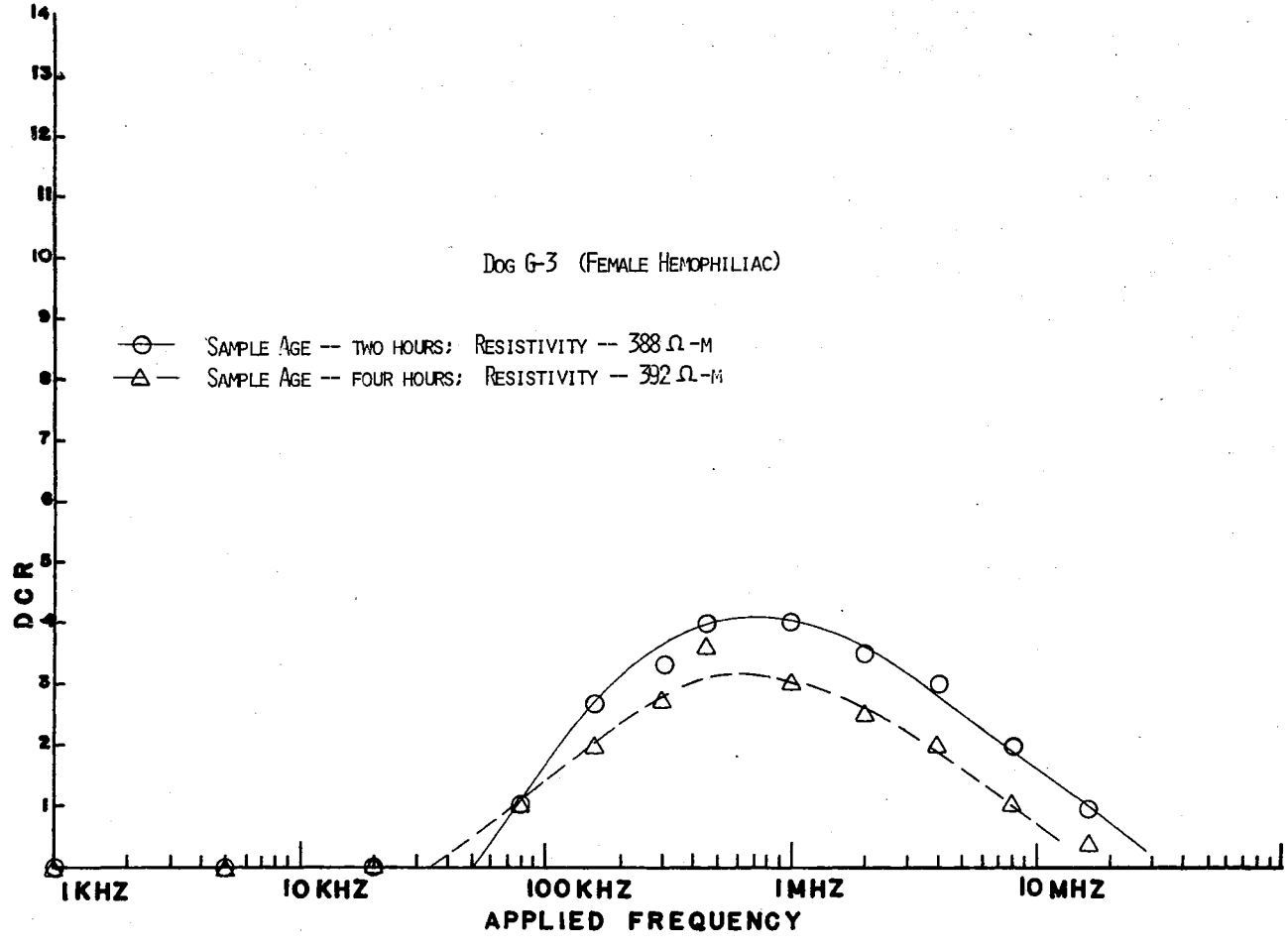

Figure 26. DCR vs Applied Frequency (Female Hemophiliac)

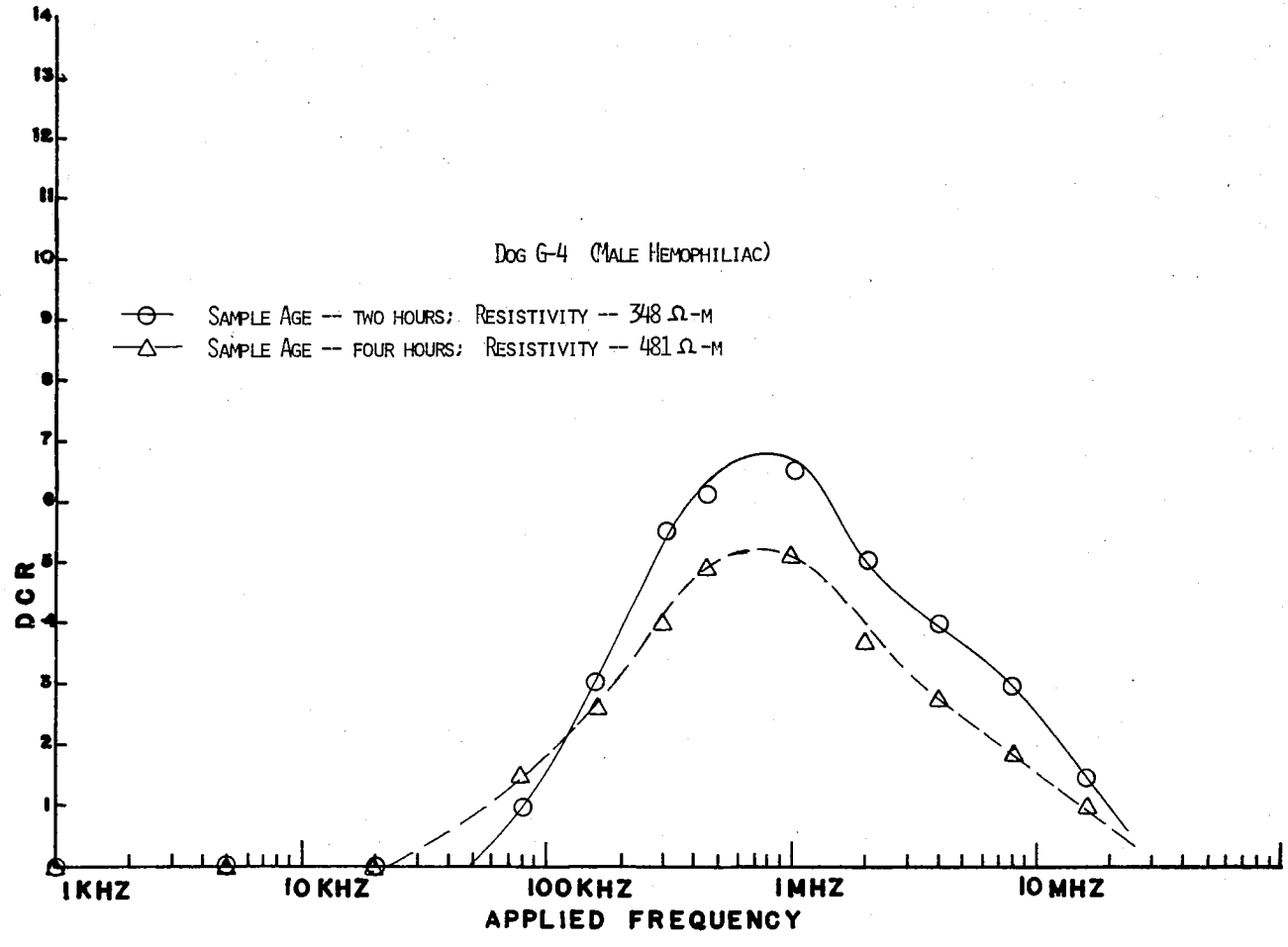

Figure 27. DCR vs Applied Frequency (Male Hemophiliac)

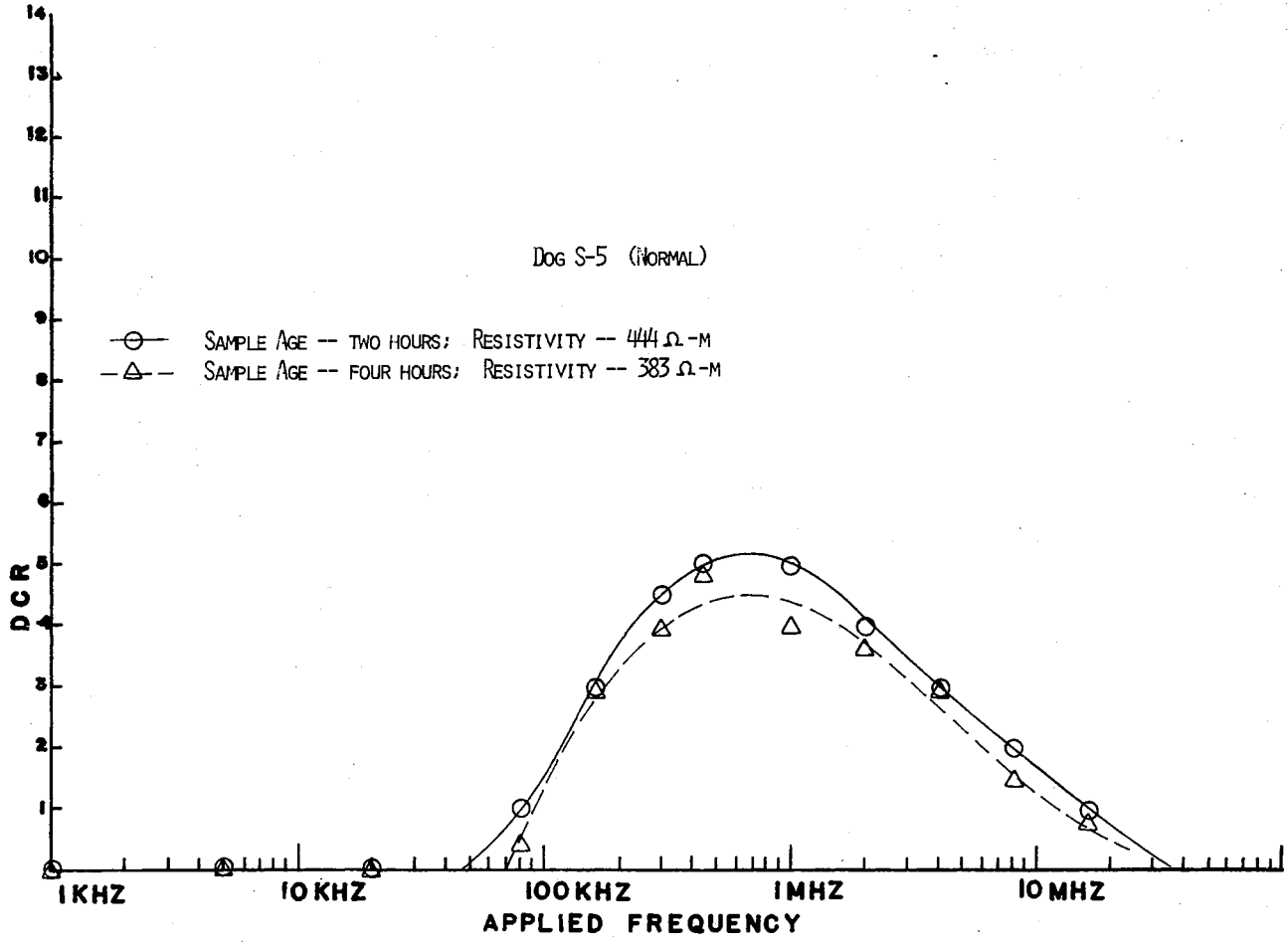

Figure 28. DCR vs Applied Frequency (Normal)

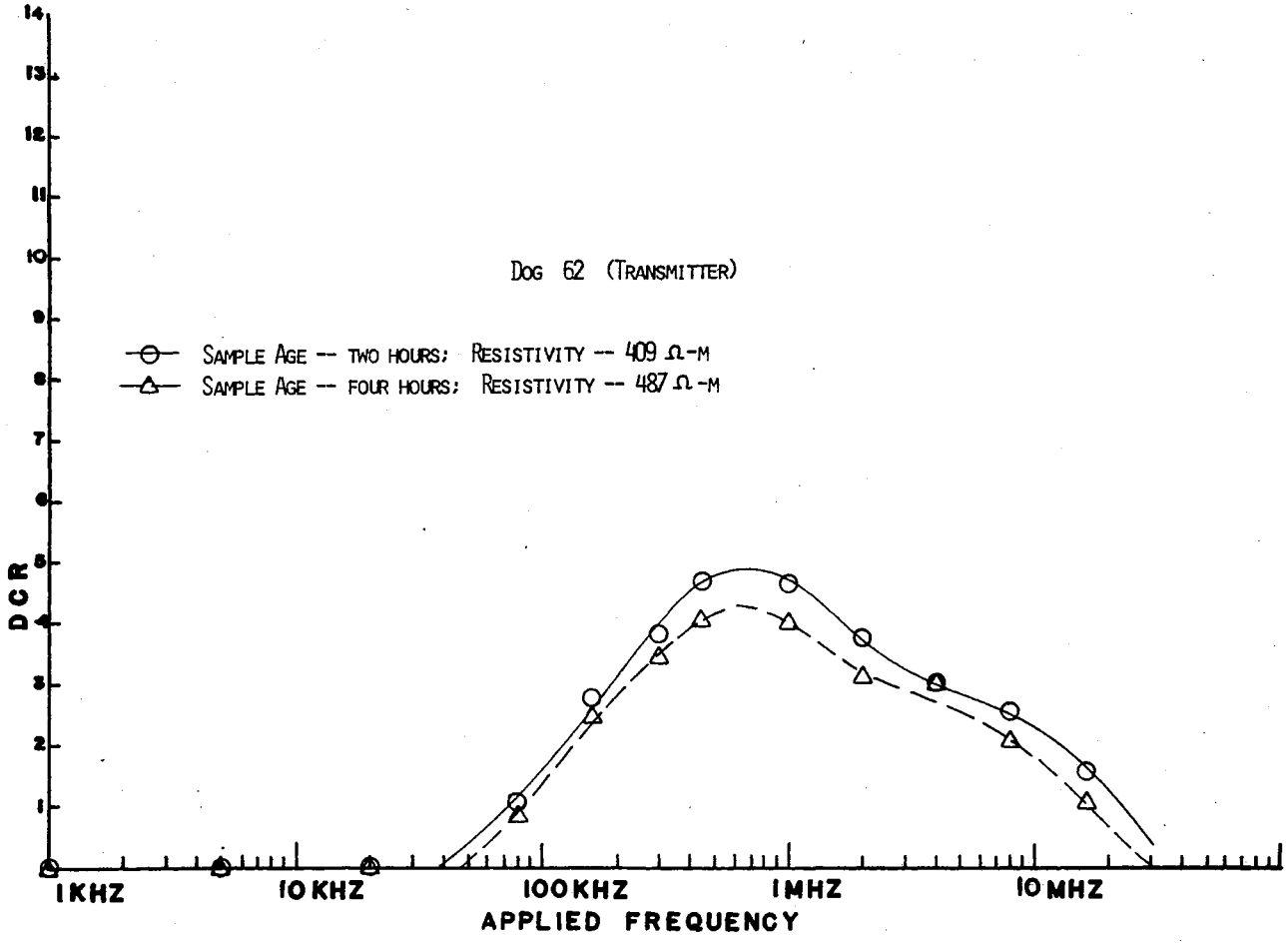

Figure 29. DCR vs Applied Frequency (Transmitter)

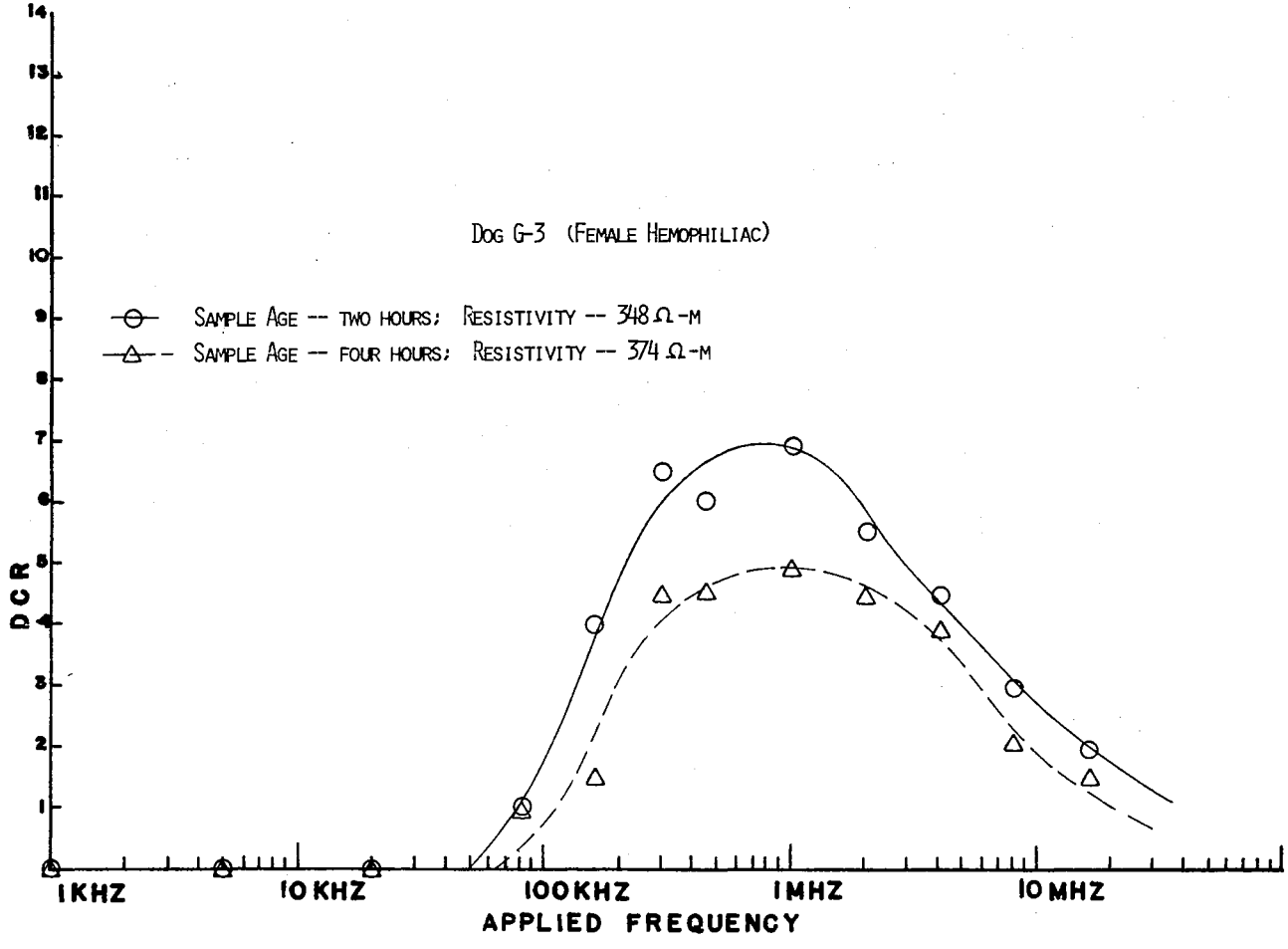

Figure 30. DCR vs Applied Frequency (Female Hemophiliac)

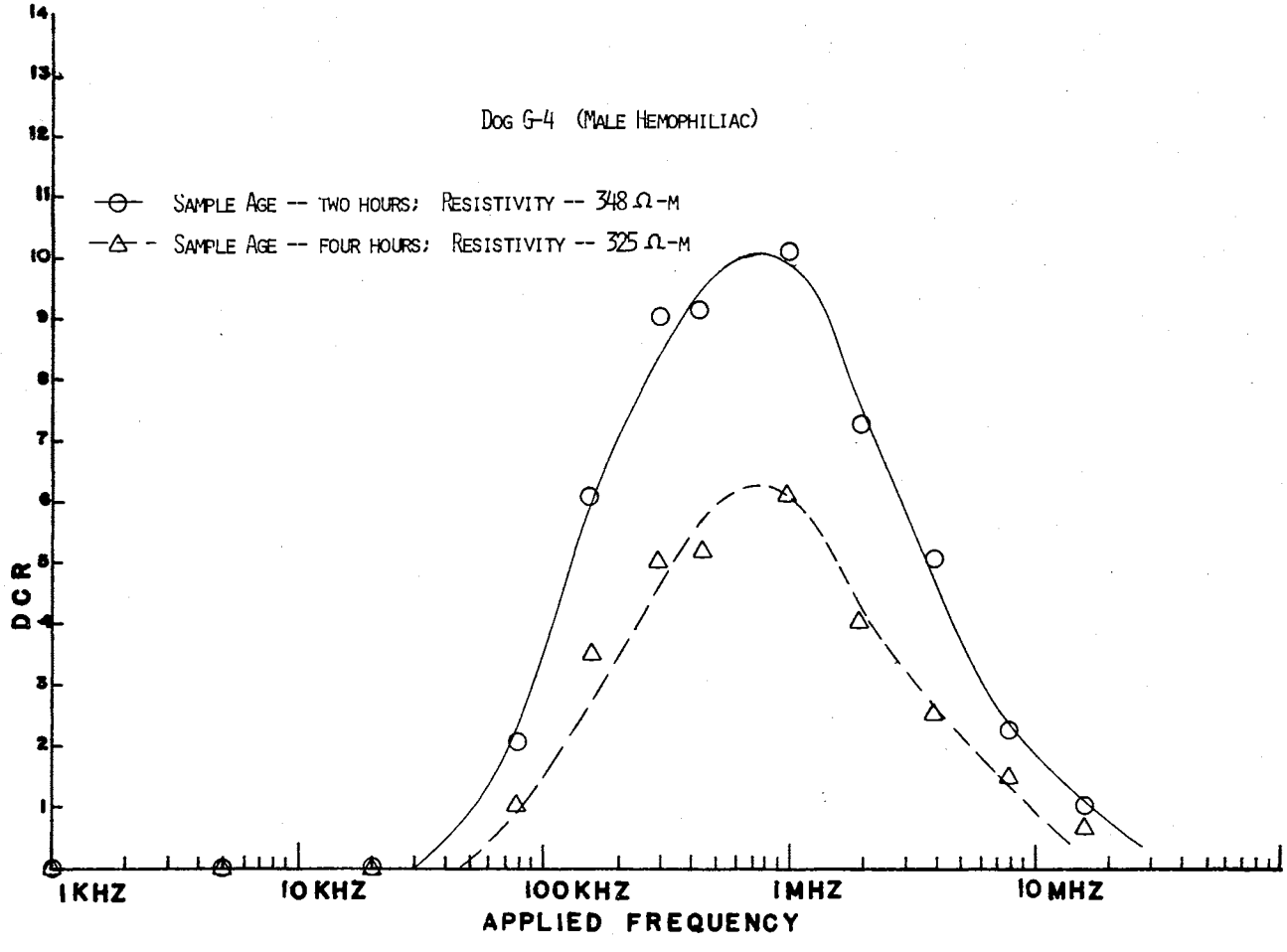

Figure 31. DCR vs Applied Frequency (Male Hemophiliac)

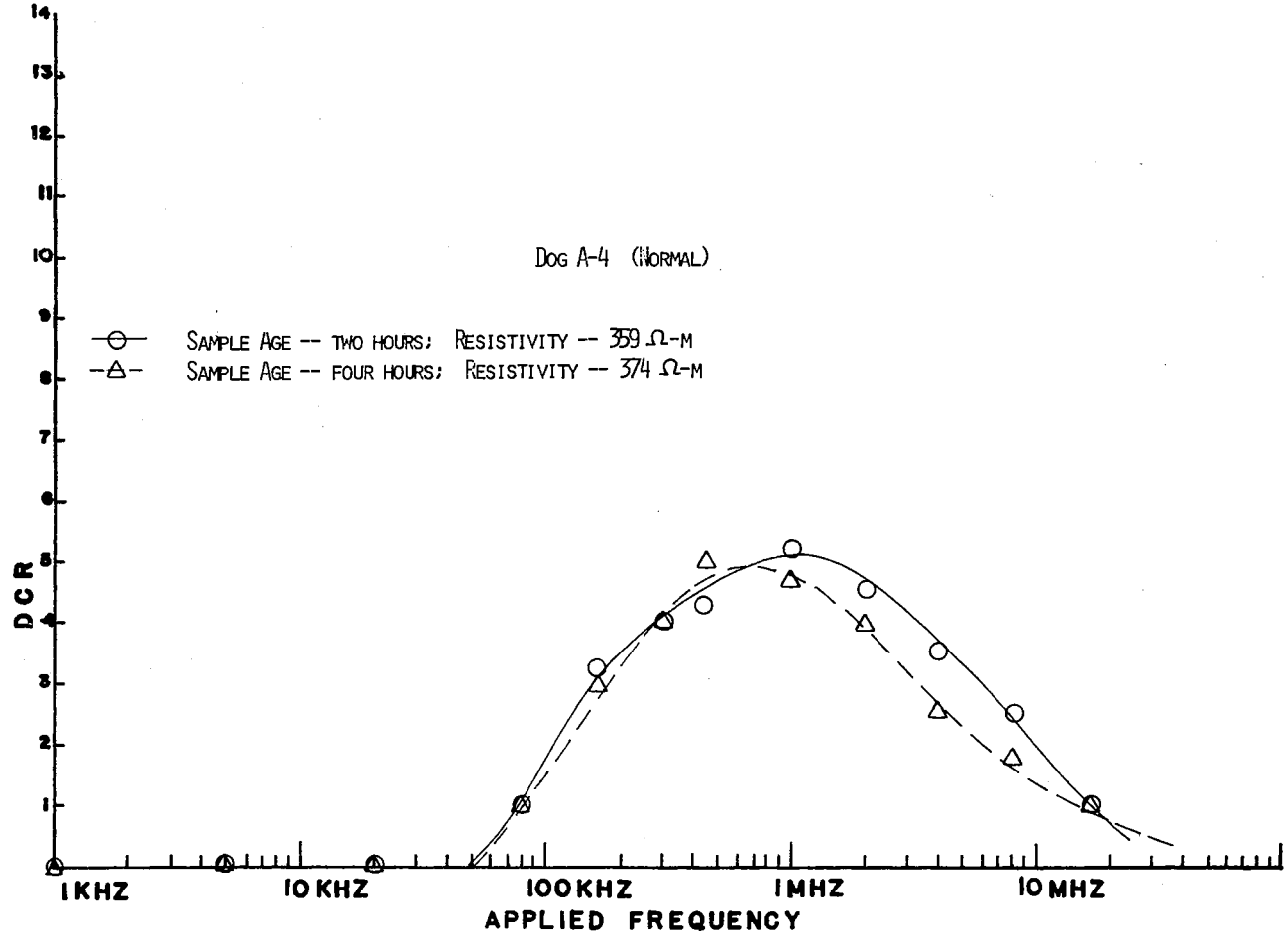

Figure 32. DCR vs Applied Frequency (Normal)

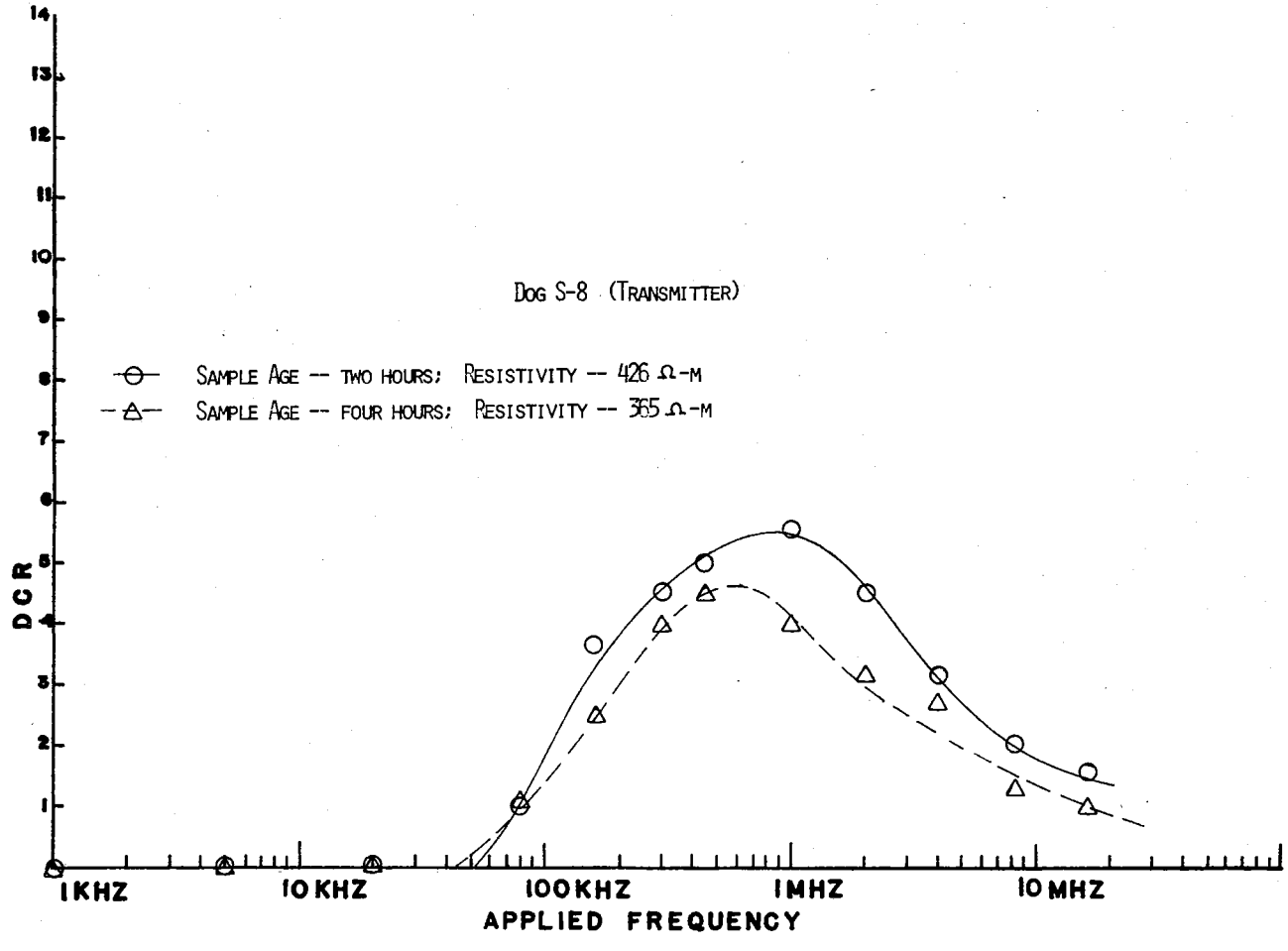

Figure 33. DCR vs Applied Frequency (Transmitter)

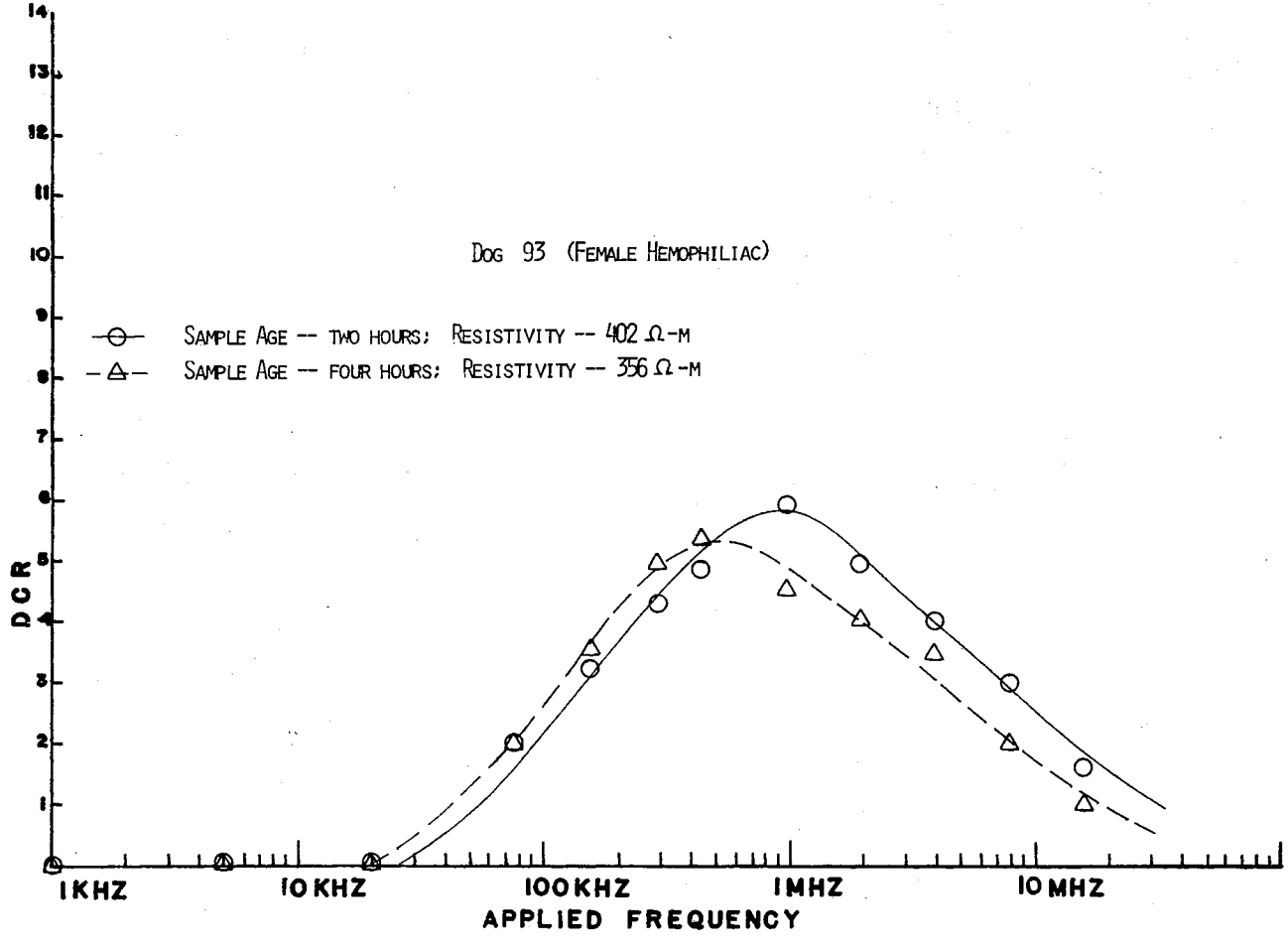

Figure 34. DCR vs Applied Frequency (Female Hemophiliac)

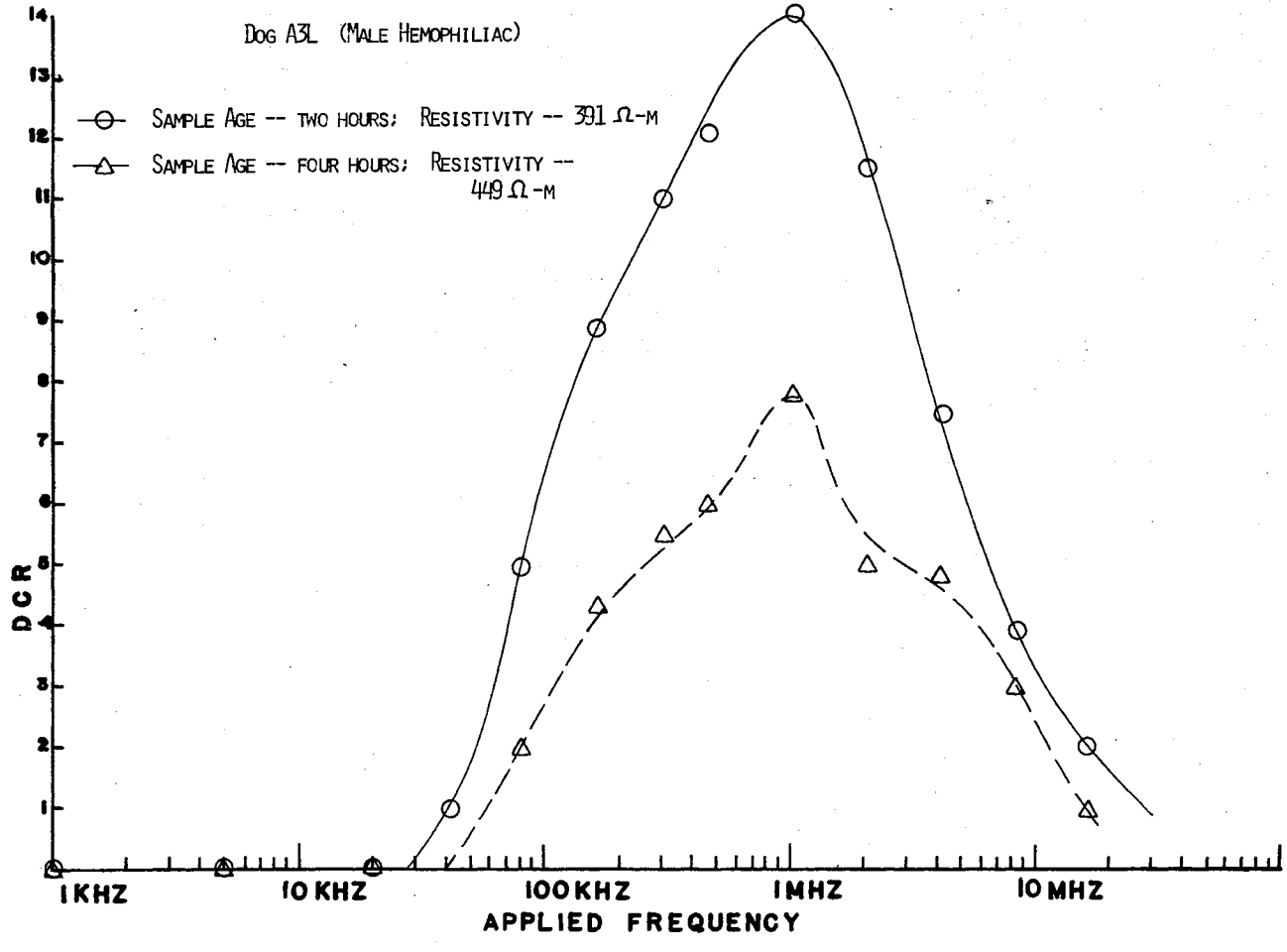

Figure 35. DCR vs Applied Frequency (Male Hemophiliac)

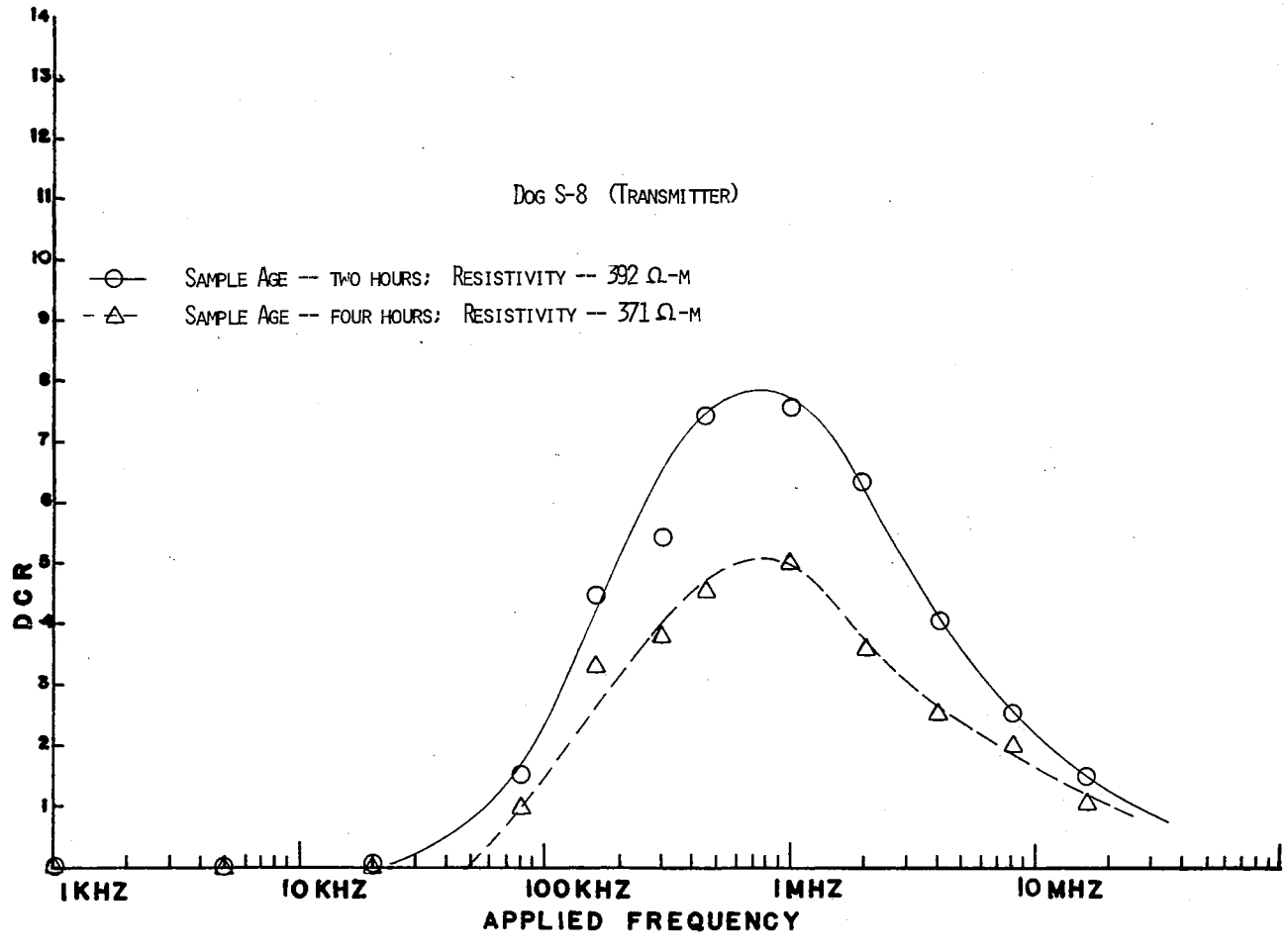

Figure 36. DCR vs Applied Frequency (Transmitter)

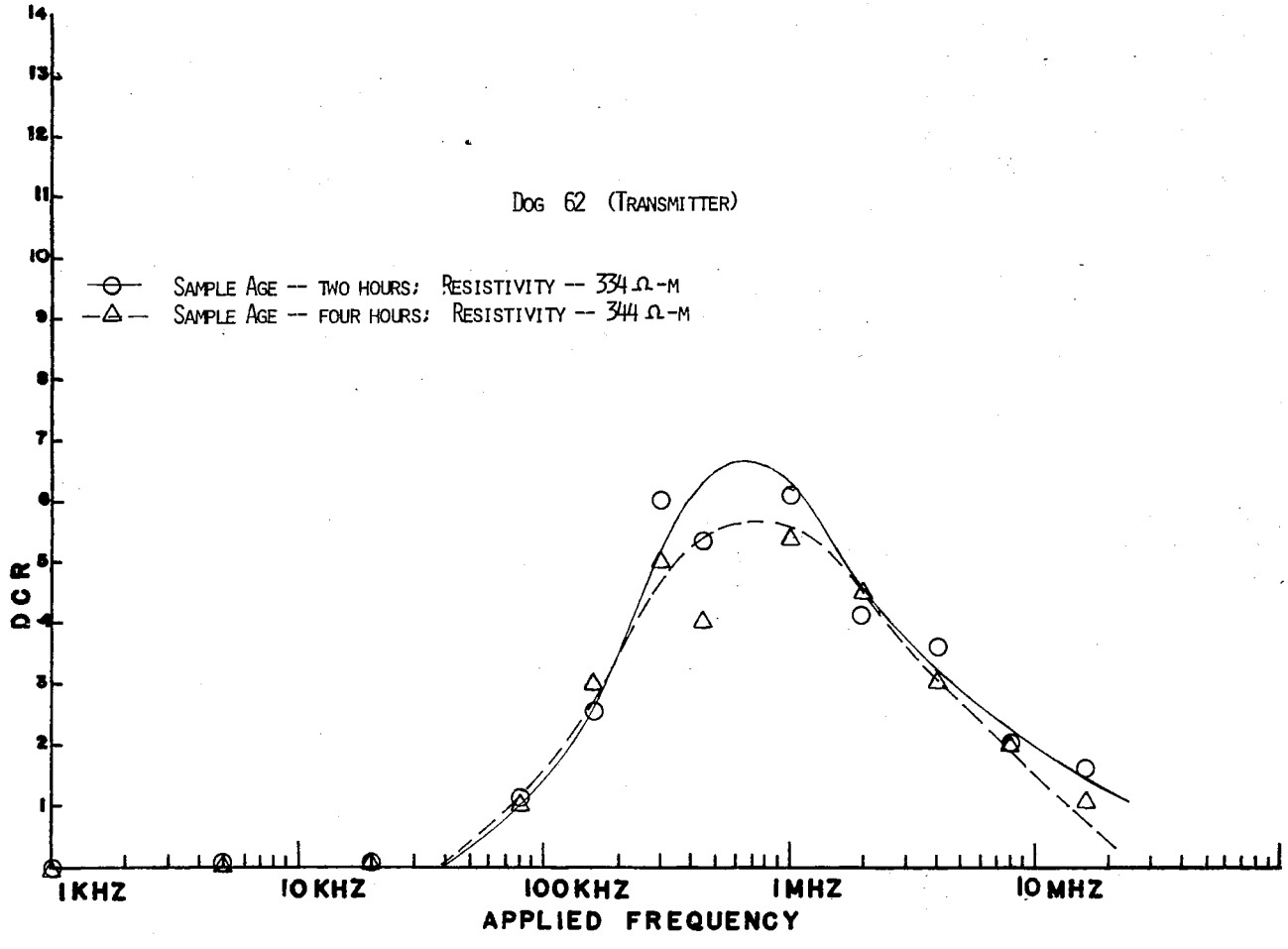

Figure 37. DCR vs Applied Frequency (Transmitter)

VITA

John Everett Rhoads

Candidate for the Degree of

Master of Science

Thesis: DIELECTROPHORESIS OF CANINE BLOOD PLATELETS

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Biographical:

Personal Data: Born in Decatur, Texas, July 13, 1946, the son of Mr. and Mrs. John L. Rhoads.

Education: Graduated from Wichita Falls High School, Wichita Falls, Texas, in May, 1964; received Bachelor of Science degree with a major in physics and a minor in mathematics from Midwestern University, Wichita Falls, Texas, in 1968; received Hardin Award, was selected outstanding freshman and senior physics student, was a member of Alpha Chi (national honorary fraternity), and was named to Who's Who in American Colleges and Universities while attending Midwestern University; completed requirements for the Master of Science degree at Oklahoma State University in May, 1973.

Military Service: U. S. Army, 1968 to 1970 (Vietnam service, 1969-1970); recipient of Army Commendation Award.