A COMPARISON OF NORMAL AND ABNORMAL CELLS USING DIELECTROPHORESIS

By

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CHAPTER I

INTRODUCTION

Research on living cells, once completely out of place in the physics laboratory, has now, due to rising demands of medicine and technology, become a very important segment of physics research. Numerous methods of studying the physical nature of cells have been used and one of the most ingenious of these is dielectrophoresis. Dielectrophoresis is defined as the motion of uncharged particles induced by a non-uniform electric field due to their induced, or permanent dipoles. Dielectrophoresis should be distinguished from electrophoresis, which is the motion of a charged particle induced by either a uniform or non-uniform field.

Over the last ten years dielectrophoresis has been used in the purification of liquids such as polymers and fuels, in the separation of mineral powder mixtures[1] and in the separation of living and dead yeast cells. Using this method it was found that live yeast cells could be separated from dead ones and collected. Also, the collected cells survived the process[2].

The dielectrophoretic force arises in the following way. Any permanent, or induced dipole has a separation of equal amounts of plus and minus charges within it. The non-uniform field will tend to produce some alignment of the dipole with it. Also, since the field is non-uniform, one end of the dipole will be in a weaker part of the field

than the other. A net force will result characterized by the migration of the dipole into the region of greatest field intensity. Also, the direction of the field can be reversed, giving rise to the original direction of travel of the dipole. Thus, in dielectrophoresis one can use either direct or alternating current and still obtain one-way motion. It is important to realize, however, that the effects of producing dielectrophoresis are in competition with the effects of conduction, thermal convection, and in some cases, even electrophoresis.

The force equation for ideal dielectrics can be shown to be:

$$\vec{F} = 2\pi R^3 K_1 \varepsilon_o \frac{K_2 - K_1}{K_2 + 2K_1} \vec{\nabla}|\vec{E}_o|^2$$

where R is the radius of the particle, K_2 is the relative dielectric constant of the particle, K_1 is the relative dielectric constant of the medium, and $\vec{E}_o$ is the applied electric field. From the equation, it is obvious that the direction of the force is influenced only by the effective dielectric constants of the two media. Thus, if we have a charged particle exposed to an alternating field, the net electrostatic force over a full cycle due to the particle's charge is zero. If the applied electric field alternates fast enough so that the particle does not move an appreciable distance on one-half of the cycle, the electrophoretic force can, for all practical purposes, be ignored. Since we are concerned with macroscopic particles (which are large when compared with atoms), frequencies of 100 Hz to 1,000 Hz or more will usually suffice to allow one to ignore the electrophoretic force.

Numerous cells possess characteristics that avail themselves to dielectrophoretic studies. Of these characteristics are the separability

of the cells into individual entities and the ability of the cells to survive in in vitro suspension media. Erythrocytes and flavobacteria possess the above characteristics to a considerable degree.

The erythrocytes used in this work were taken daily from a healthy two year old beagle. The dog was not a transmitter of, and did not have, hemophilia. The work with the erythrocytes consisted of studying their dielectrophoresis in various suspension media and studying their dielectrophoresis after exposure to various metabolic inhibitors.

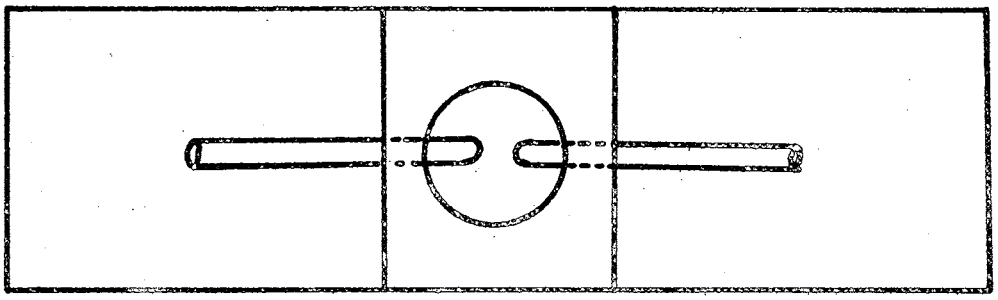
Although the force equation for ideal dielectrics contains no frequency dependent terms except for the dielectric constant, it is especially frequency dependent in the case for lossy materials (the dielectric loss components are not negligible compared to the real component). Conduction is also a frequency-dependent process. It plays an important role in the dielectrophoresis of the erythrocyte. Because of this, the frequency dependence of the dielectrophoresis of the erythrocyte was studied. In addition, the effects of applied voltage and various medium conductivities was sought. Finally, the dielectrophoresis of the erythrocyte at various values of pH of the suspension medium was examined.

The work with flavobacteria was done to determine their dielectrophoresis after being exposed to ethylene diamine tetraacetic acid (EDTA) which is thought to act upon the cell wall by chelating divalent cations.

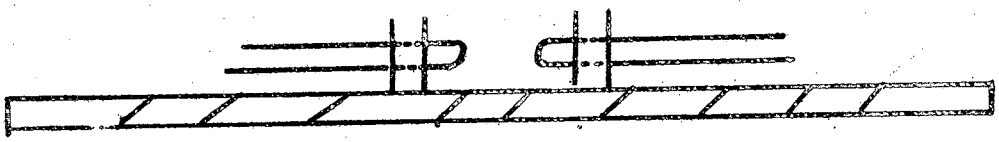
The equipment used was a pin-pin apparatus consisting of two 22 gauge platinum wire pins mounted in a well of 4.0 mm diameter with a depth of 1.7 mm. The volume of the well was 0.021 cc. The pin-pin system was mounted on a microscope slide which was three inches long and one inch wide. Figure 1 is a diagram of the top and side views of the

Figure 1. Diagram Illustrating the Pin-Pin Dielectrophoretic Apparatus.

Well Volume - 0.021 cc.



Top View



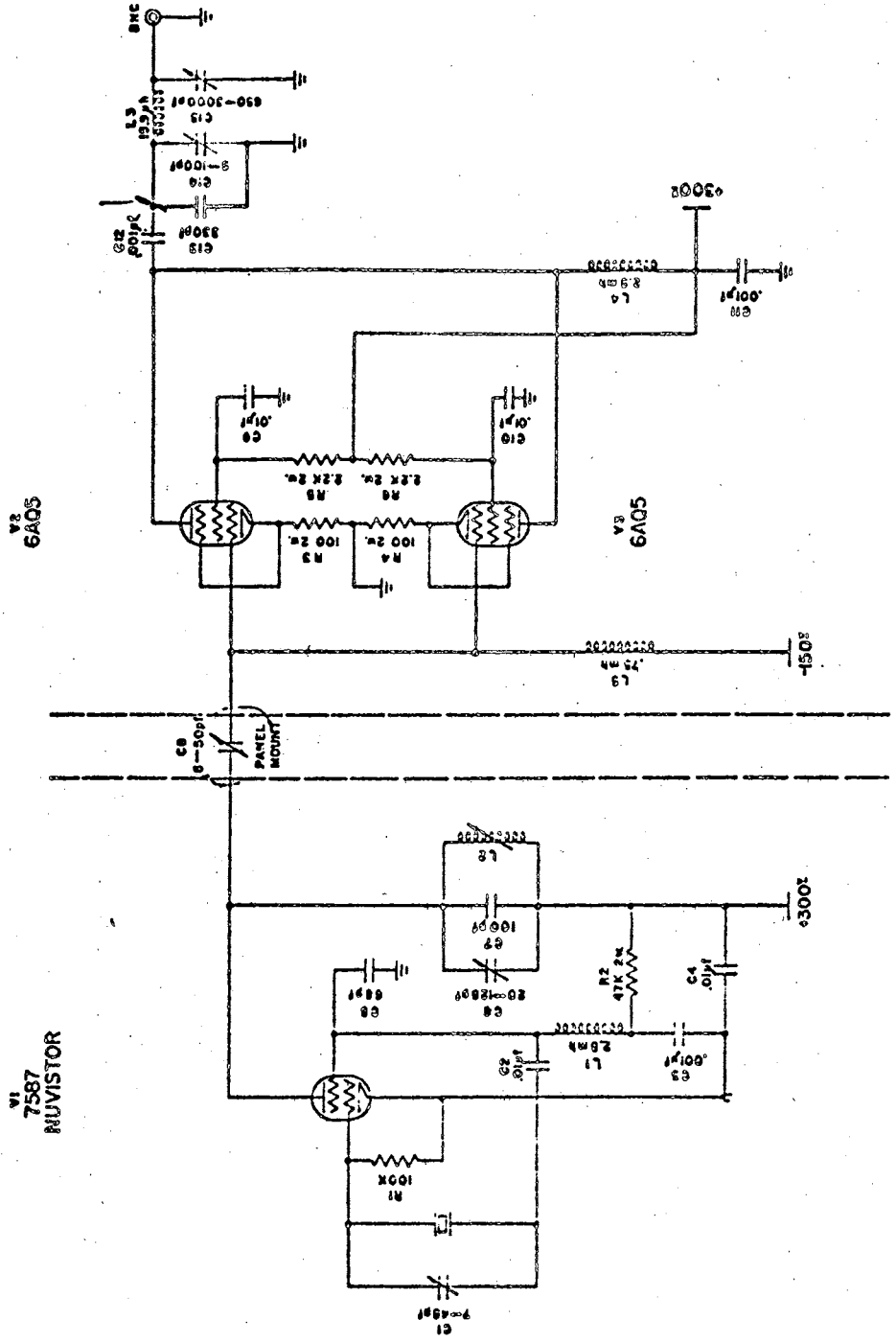
Side View

pin-pin dielectrophoretic apparatus. The pin tips were carefully rounded. A potential placed across the pins produced the non-uniform electric field.

For frequencies up to 600 KHz a Hewlett Packard model 200 CD wide-range oscillator was used. For the 2.55 MHz frequency an ultrasonic oscillator capable of producing up to 200 V, r.m.s. was used. It was designed by Dr. Harry Crawford of the Oklahoma State University Electrical Engineering Department. A circuit diagram of the apparatus is shown in Figure 2. For frequencies of 7.5 MHz to 30 MHz a Heathkit Model DX-60B transmitter was used, and an Ameco model TX-62 transmitter was used for frequencies of 50 MHz and above. Voltage was measured by a Hewlett-Packard model 410B vacuum tube voltmeter, and is indicated as rms voltage in what follows. Resistivity was measured with a General Radio Type 1650-B impedence bridge. Resistivity was determined at a frequency of 1 KHz.

Throughtout this report the results are expressed as the "Dielectrophoretic Collection Rate," or "DCR." This is merely referring to the average length of the chain of cells collected on the pin at the axis of the tip in one minute. Thus, a DCR of 1 unit/min is a chain length of 1 unit collected in 1 minute. All readings for erythrocytes were taken in a one minute time interval because the chain length is not a direct function of collection time. Although one may collect one unit in one minute he cannot expect to quite collect two units in two minutes. Hence, all DCR's represent one-minute collections.

Figure 2. Circuit Diagram of Ultrasonic Transmitter


BNC
V2
6AQ5
V3
6AQ5
V1
7587
NUVISTOR
PANEL MOUNT
+300V
+150V

FOOTNOTES

[1]Herbert A. Pohl, "Theoretical Aspects of Dielectrophoretic Deposition and Separation of Particles", Journal of the Electrochemical Society, Vol. 115, June 1968, p. 155c.

[2]Joe S. Crane and Herbert A. Pohl, "A Study of Living and Dead Yeast Cells Using Dielectrophoresis," Ibid., p. 584.

CHAPTER II

THE ERYTHROCYTE

"All living things have, to a greater or lesser extent, the properties of specific size and shape, metabolism, movement, irritability, growth, reproduction, and adaptation."[1] The above list seems very specific, however, the writer admits that the line between the living and the dead is pretty tenuous and the erythrocyte finds itself on this line. The red blood cell is about 7 or 8 microns in diameter and is 1 to 2 microns thick. Also, unlike most cells, it has no nucleus. It is not capable of moving actively and is transported by floating in the blood stream which is moved about by the pumping action of the heart. The erythrocyte does not reproduce but originates in the red bone marrow. In spite of the above statements suggesting a general state of inactivity of the erythrocyte, one must realize that it has the important job of oxygen and carbon dioxide transport within the body and that it also has distinct metabolic processes. For example, the metabolism of glucose, glycolysis, is an important part of erythrocyte metabolism. This part of erythrocyte metabolism can be inhibited by such poisons as sodium fluoride (NaF) and iodoacetic acid. Such cells are called "poisoned" cells. In addition, the respiration of the erythrocyte can be inhibited by substances such as sodium cyanide (NaCN) and sodium azide (NaN_3)[2].

The *in vivo* environment of the erythrocyte, called plasma, is a

mixture of proteins, amino acids, carbohydrates, fats, salts, hormones, enzymes, antibodies, and dissolved gases. It is slightly alkaline, with a pH of 7.4 and its chief constituent is water, approximately 90%. The most important carbohydrate in the plasma is glucose, which is metabolized to release energy.

The metabolism of the erythrocyte is extremely slow as compared with that of other tissue cells. The oxygen consumption, i.e., the physiologic respiration of the mature cell, is feeble. Also, contrary to the expectations of some, the functional activities of the energy producing metabolism and the oxygen-carrying capacity of the erythrocyte are not interdependent. It has been shown that, with stored blood, the oxygen carrying function can still be intact when the erythrocyte has ceased to utilize glucose, i.e., with all of its metabolic viability lost (1).

FOOTNOTES

[1]Claude A. Villee, Biology (Philadelphia, 1962), p. 17.

[2]Herman Passow, "Ion and Water Permeability," The Red Blood Cell, ed. Charles Bishop and Douglas M. Surgenor (New York, 1964), p. 77.

CHAPTER III

EXPERIMENTAL PROCEDURE FOR ERYTHROCYTES

In the dielectrophoresis experiments with erythrocytes a standard suspension medium consisting of 90% isoosmotic glycine (2.1%) and 10% isoosmotic glucose (5.5%) was used in all cases except in the first experiments in which a suitable suspension medium was being sought for. In all of the experiments the erythrocytes in their serum were diluted 500 fold in the suspension media. This gives an ideal cell concentration for the well dimensions that were used. The cells, suspended in the medium, were transferred from the test tubes to the well of the pin-pin apparatus with a disposable pipette and care was taken not to damage the cells by over-rapid pipetteing. When the cells were placed in the well, filling it, they were observed through the microscope to insure that there was no undue turbulence in the medium, therefore leaving the erythrocytes generally immobile. Turbulence causes poor and ragged collection of the erythrocytes. After bringing a pin into focus and the reticule of the microscope eyepiece next to the pin so that the chain length could be measured, the field was applied across the pins and was left on for one minute, at which time the cell chain length was read from the reticule (for the pin-pin system the cell chains are of relatively uniform length) and the field was then turned off. Since the cells on the pin are elongated due to the dielectrophoretic force, the reading is taken while the field is on. As soon as the field is turned

off the cells return to normal size and begin to fall away from the pin.

The Dielectrophoretic Collection Rate (DCR) is defined as the length of the chain of cells collected in one minute. In these experiments it is recorded in relative values of units/minute where one unit is 0.22 mm in length

$$1 \text{ DCR} = 1 \frac{\text{unit}}{\text{minute}}$$

$$1 \text{ unit} = 0.22 \text{ mm}$$

Erythrocytes were collected daily from a two year old beagle with a trace of heparin used as an anticoagulant. When the blood was not in use it was stored at a temperature of $5^{o}C$.

After a one-minute collection, the well was emptied of its contents with a disposable pipette with an attached suction bulb, and rinsed with the next suspension to be examined. This easily removes all cells from the well and from the pin. The cells were discarded after they had been removed from the well. Each time before the well was filled, the suspension was thoroughly mixed to counteract any settling which might have occurred.

CHAPTER IV

SUSPENSION MEDIA FOR ERYTHROCYTES

The very first problem encountered in beginning the dielectrophoretic studies concerned the discovering and utilization of a proper suspension medium. The criteria were that it would be one in which the cells were relatively unrestrained in motion, that it would have the proper resistivity to keep turbulence at a minimum, and it had to be conducive to cell survival. It was quite evident that a medium which is isoosmotic to erythrocytes which possessed the above characteristics needed to be found.

Experimental Suspension Media for Erythrocytes

Since 5.5% glucose is isoosmotic to erythrocytes and glucose is instrumental in cellular metabolism, this type of suspension solution was first tried. All suspensions in this solution had a resistivity of 2.1×10^4 Ω-cm and were made by suspending the glucose in deionized, distilled water. Whole blood was diluted 500 fold in the suspension solution and was well mixed by gently tilting the test tube containing the blood and glucose suspension several times. After the cells in the suspension medium were transferred to the well of the pin-pin apparatus, the field was applied. A frequency of 600 KHz at 20 V, rms was used and readings were taken at various times after initial dilution. Time was set at zero at the moment of dilution thus allowing the determina-

tion of DCR as a function of exposure time. These results for isoosmotic glucose are given in Figure 3.

Figure 3 shows that the DCR for erythrocytes suspended in a 5.5% glucose solution is a decreasing function of exposure time. The maximum recorded DCR was read after a two-minute exposure time and had a value of 1.4 units/min. The last reading, taken at 34 minutes, was 0.2 units/min. The cells simply responded poorly after 35 minutes of suspension in isoosmotic glucose. This suggests that the erythrocytes are losing ions that are present in the cells before suspension. One can interpret this by assuming that the ions that contribute to the dielectrophoretic force have leaked out of the erythrocytes into the surrounding medium. To substantiate this conclusion, the suspension medium resistivity had dropped to 1.7×10^4 Ω-cm after the cells had been in the medium for forty minutes.

To observe the effects of doubling the concentration of glucose on the DCR of the erythrocyte, an 11% glucose solution was used. After the cells were diluted 500 fold in the 11% glucose solution its resistivity was 1.9×10^4 Ω-cm. Their DCR was taken at various exposure times and Figure 3 also shows the results of this experiment. The erythrocytes also undergo a general decrease in DCR with time when suspended in the 11% glucose solution and after 35 minutes of exposure time the collection rate and chain length of the cells became erratic and non-uniform respectively. Some of the cells underwent hemolysis and those that did survive collected very poorly or not at all. From these results, it is obvious that when a uniform DCR is desired for any period of time, a solution of pure glucose would not be a satisfactory suspension medium.

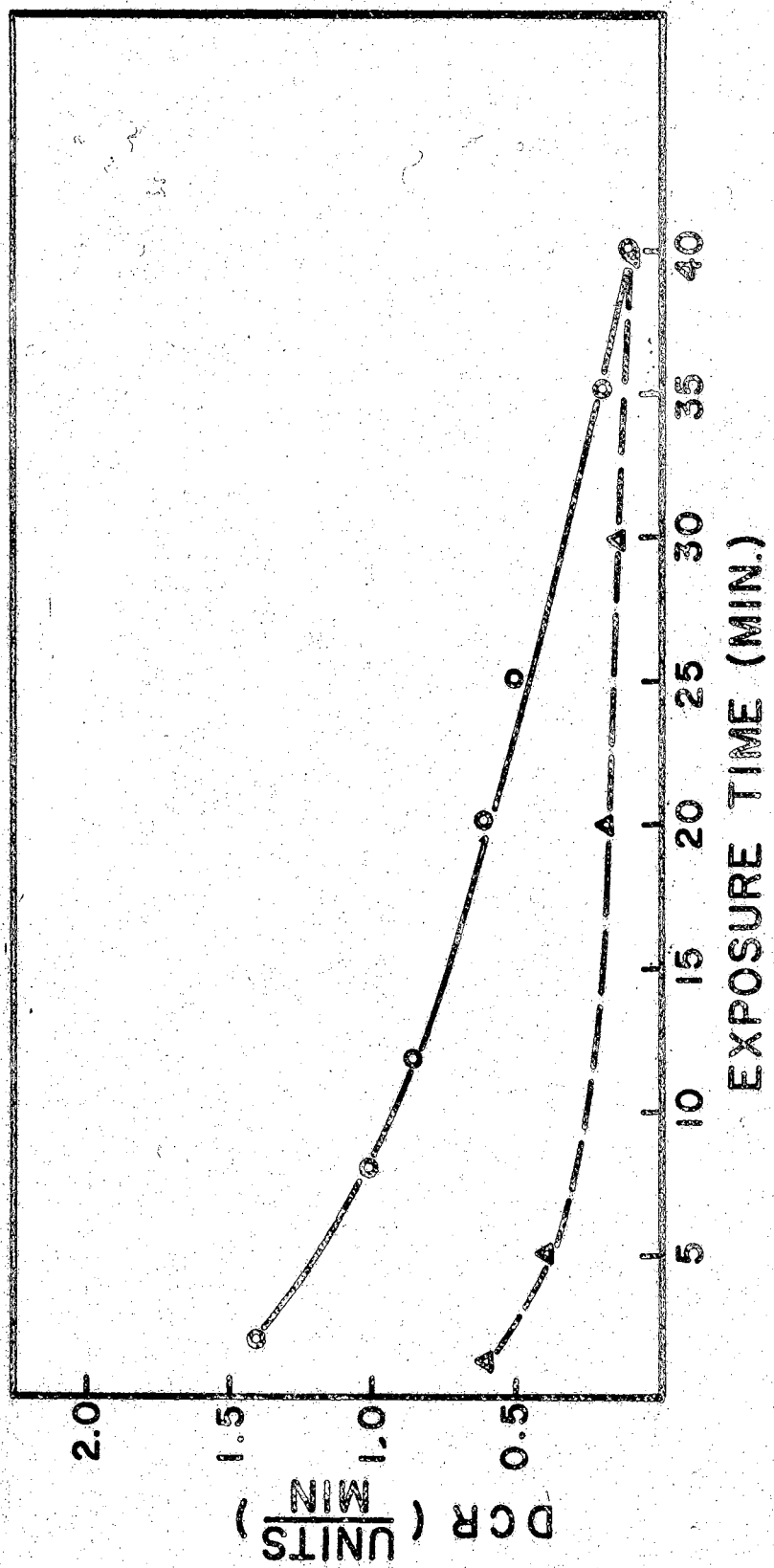
Figure 3. DCR Versus Exposure Time for Erythrocytes.

O - 5.5% Glucose Suspension Medium; Suspension Resistivity - 2.1×10^4 Ω-cm.

Δ - 11% Glucose Suspension Medium; Suspension Resistivity - 1.9×10^4 Ω-cm.

Frequency - 600 KHz

Voltage - 20 V, rms


DCR (UNITS/MIN)
2.0
1.5
1.0
0.5
5
10
15
20
25
30
35
40
EXPOSURE TIME (MIN.)

Since a dissaccharide, such as sucrose, does not easily penetrate the erythrocyte membrane, it was suspected that sucrose would prove to be a desirable suspension medium for certain types of studies, hence an 11% (isoosmotic) sucrose solution was used in exposure time experiments. The resistivity of the solution was 1.7×10^4 Ω-cm. The whole blood was diluted 500 fold into this medium. Readings were taken at 600 KHz at 20 volts. Figure 4 shows the exposure-time curve for erythrocytes suspended in an 11% sucrose medium. In addition, a 11% sucrose suspension medium with NaCl present in a 0.001$\bar{M}$ concentration was used for the exposure time experiments whose results are also shown in Figure 4.

As in glucose erythrocytes in sucrose show a decreasing DCR with respect to exposure time. The addition of NaCl decreased the overall DCR but did not change the characteristics of the collection activity. Also, in this case, the decrease in activity was due to the hemolysis of some of the cells and the decreasingly poor response of those that survived. From all visual evidence, it seems that the cells ceased their dielectrophoretic response before they underwent hemolysis. Similar results were shown using carbonates in the solution. When the 11% sucrose solution had $NaHCO_3$ present in a 0.001$\bar{M}$ concentration the resistivity was 0.60×10^4 Ω-cm. The calculated pH of 0.001$\bar{M}$ $NaHCO_3$ solution is 8.7. Figure 4 shows that 11% sucrose suspensions containing $NaHCO_3$ give approximately the same results as those containing NaCl. As a function of exposure time, their DCR's are very much alike. As with glucose, the value of sucrose as an assay medium is somewhat limited. Any studies requiring a consistent DCR for a reasonable period of time cannot be done with pure glucose or sucrose solutions used as assay

Figure 4. DCR Versus Exposure Time for Erythrocytes Suspended in an 11% Sucrose Solution.

O—O - 11% Sucrose Suspension Resistivity - 1.7 x 10^4 Ω-cm.

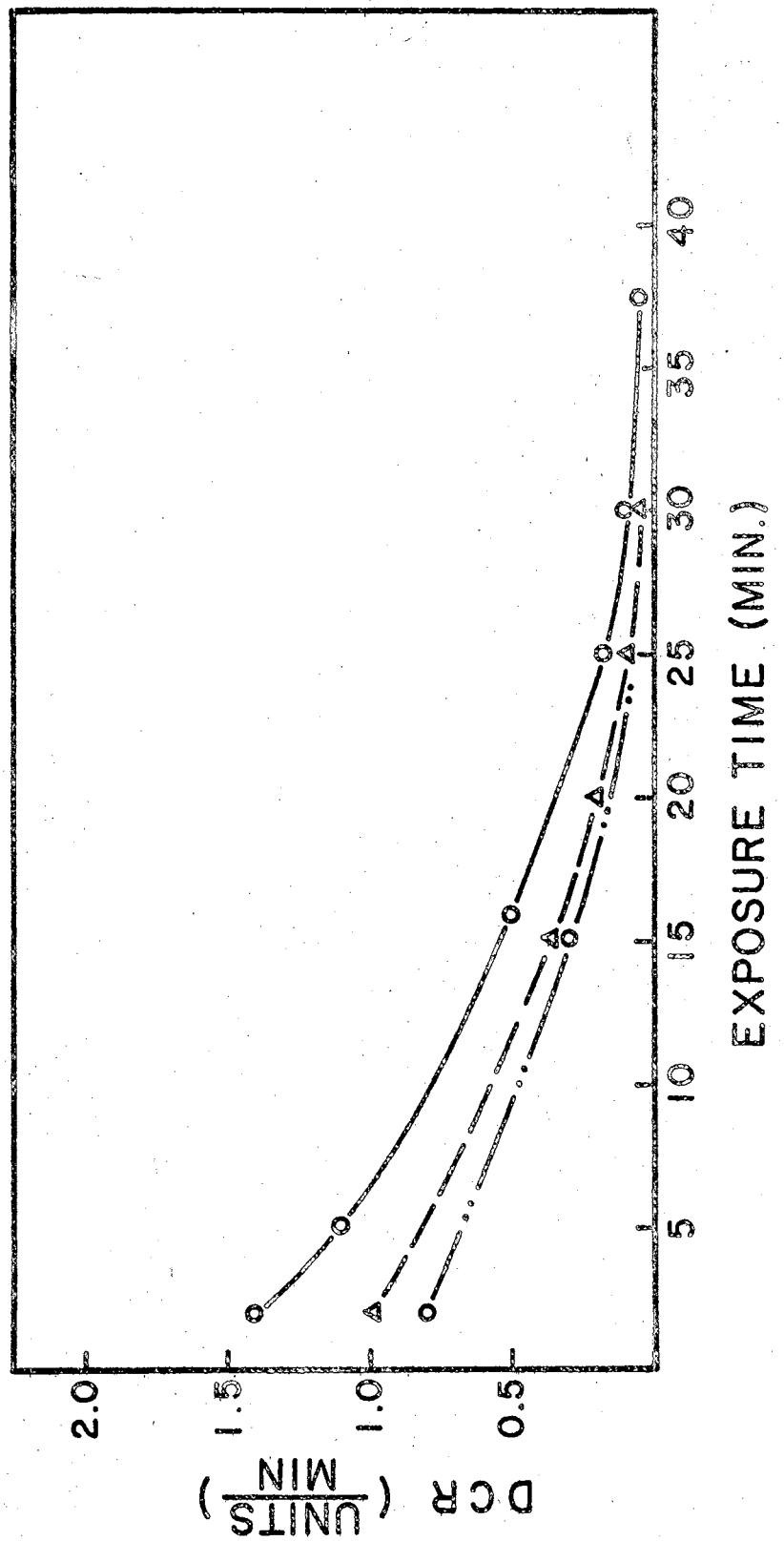
Δ---Δ - 11% Sucrose with 0.001$\bar{M}$ $NaHCO_3$ Suspension Resistivity - 0.68 x 10^4 Ω-cm.

O— ··· —O - 11% Sucrose with 0.001$\bar{M}$ NaCL.

Suspension Resistivity - 0.60 x 10^4 Ω-cm.

Frequency - 600 KHz

Voltage - 20 V, rms


DCR (UNITS/MIN)
2.0
1.5
1.0
0.5
5
10
15
20
25
30
35
40
EXPOSURE TIME (MIN.)

media. However, they avail themselves to other types of studies such as variations of salt concentrations, osmotic pressure variations, etc.

Isoosmotic glycine solution was used as a suspension medium. In it also the erythrocyte displayed a DCR which was a decreasing function of exposure time. The 2.1% glycine solution had a resistivity of 3.2×10^4 Ω-cm. Figure 5 shows the results for this particular suspension medium. Although the characteristic decrease in DCR as a function of exposure time is seen in the glycine suspension medium, erythrocytes in this medium give a high initial DCR. Note that the decrease in DCR is linear for the 2.1% glycine solution.

The "Standard" Suspension Medium for Erythrocytes

For further experiments in which the cells were exposed to poisons as metabolic inhibitors, a "standard" suspension solution consisting of 9 parts of 2.1% glycine and 1 part of 5.5% glucose was used. This suspension medium met all of the aforementioned requirements and was furthermore used because the glucose stimulated the metabolism of the cells. Figure 5 shows the DCR as a function of exposure time for erythrocytes suspended in the standard suspension medium. Although the DCR drops for this suspension medium, the stability of the erythrocytes against hemolysis is good. For periods up to three hours this suspension medium did not provoke hemolysis of the cells. In almost all of the suspension media used there was some turbulence, or stirring present when the field was applied. Under this condition the cells moved in directions which were not along the lines of force of the field. At high frequencies and high resistivities (above 200 KHz and 10^3 Ω-cm) the stirring was very slight and the cells moved along the field lines.

Figure 5. DCR Versus Exposure Time for Erythrocytes.

O -- 2.1% Glycine Suspension Medium; Suspension Resistivity - 3.2×10^4 Ω-cm.

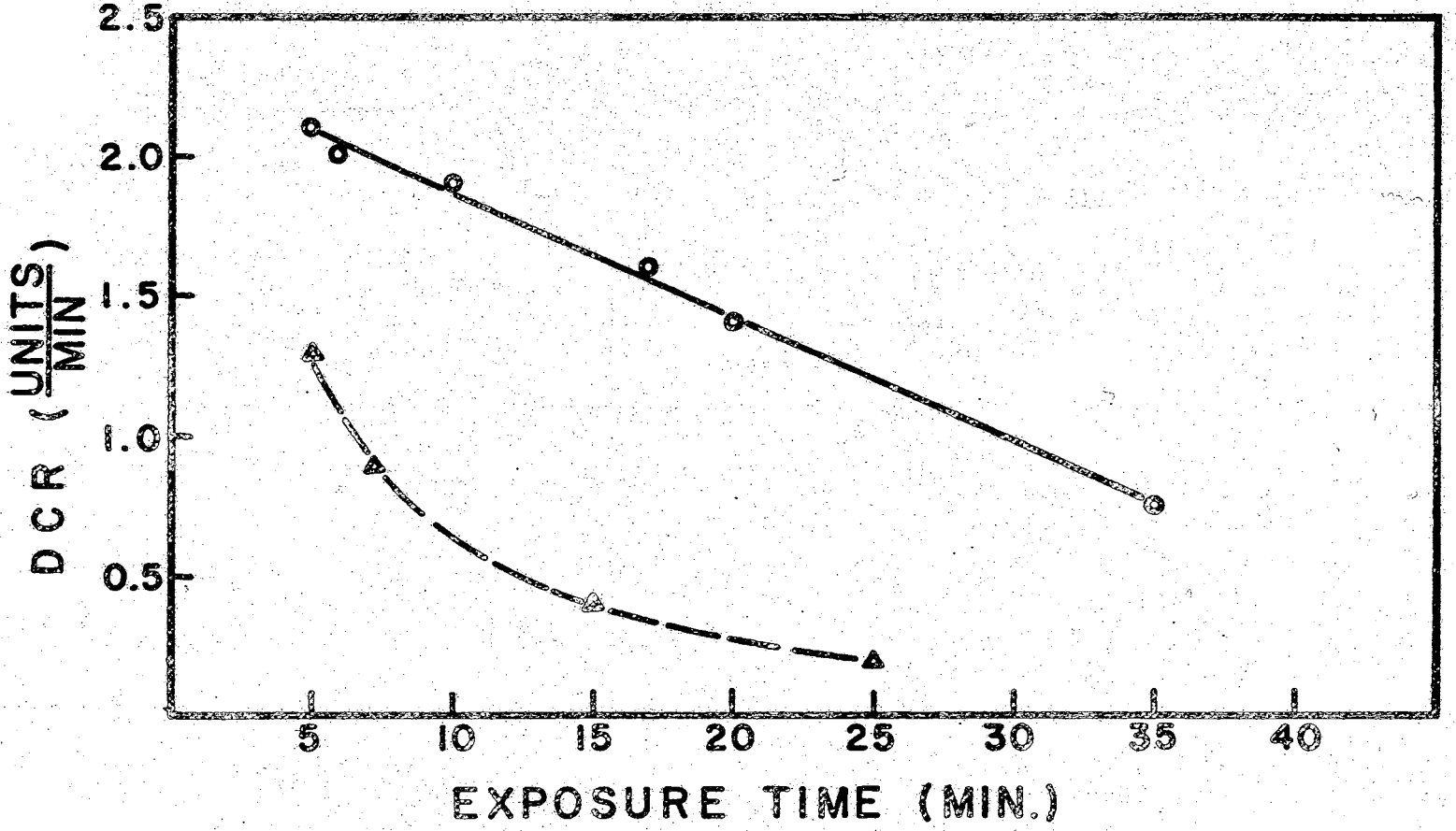
Δ -- "Standard" Suspension Medium Consisting of:

9 Parts of 2.1% Glycine
1 Part of 5.5% Glycose

Suspension Resistivity - 3.1×10^4 Ω-cm.

Frequency - 600 KHz

Voltage - 20 V_s rms


2.5
2.0
1.5
1.0
0.5
DCR (UNITS/MIN)
5
10
15
20
25
30
35
40
EXPOSURE TIME (MIN.)

At lower frequencies and lower resistivities the stirring became more pronounced and was usually in a pattern symmetrical about the pin-pin axis. All of the results reported were derived from conditions which kept this stirring at a minimum.

Another phenomenon observed was the repulsion effect on rare occasions, cells which were moving in towards a pin to be collected would suddenly move away in a direction normal to the surface. This seemed to occur just before the cell attached itself to the electrode, but there were times when a cell which had been attached to the electrode would suddenly be repulsed and leave. There were even rarer cases of a particular cell getting into a sort of cycle of motion. It would touch the pin, be repulsed a short distance, and then move back in to touch the pin. This seems to be due to charging effects on the cells, which in turn was probably caused by field emission from local spots on the metal electrode. Collected cells were never expelled from the end of the chains, only from contact or near contact with the electrode.

CHAPTER V

THE DIELECTROPHORESIS OF POISONED ERYTHROCYTES

In the following experiments, the dielectrophoresis of erythrocytes which were exposed to various poisons was examined. These poisons were sodium fluoride (NaF); iodoacetic acid, sodium salt (ICH_2COO Na); sodium cyanide (NaCN); sodium azide (NaN3); and formaldehyde (HCHO). The first four of the above mentioned poisons are metabolic inhibitors, while the last, formaldehyde, serves to denature the erythrocyte.

Sodium Fluoride-Poisoned Erythrocytes

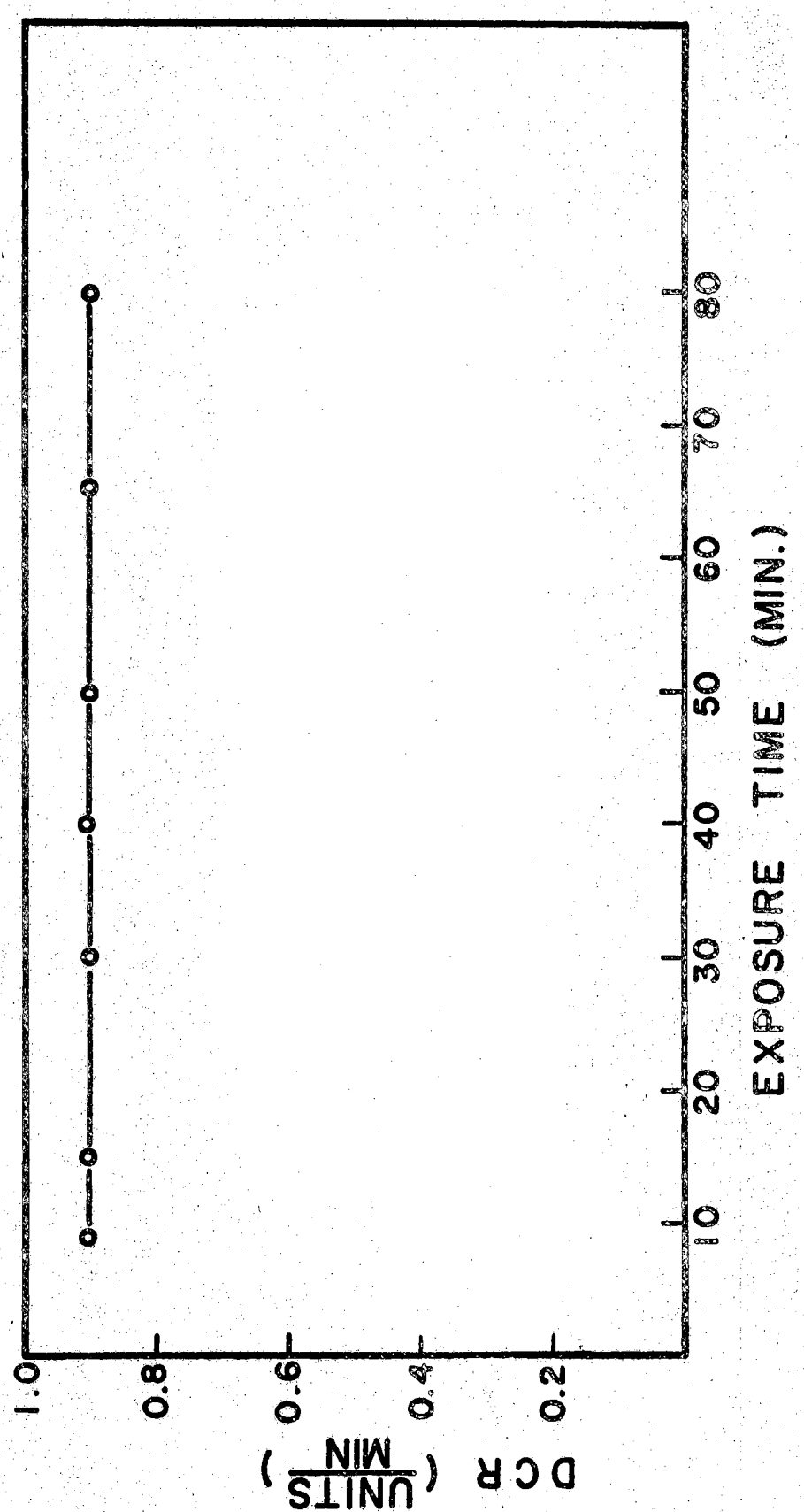
Sodium fluoride, NaF, is a well-known inhibitor of glycolysis which is a major energy-producing mechanism of the erythrocytes. To test the dielectrophoretic response of NaF-poisoned cells, they were suspended in the standard solution with NaF present in a 0.001$\bar{M}$ concentration and the resistivity of the solution was then 0.61×10^4 Ω-cm. The results of the exposure time experiment, shown in Figure 6, are striking. When NaF was present in the suspension medium in a 0.001$\bar{M}$ concentration, collection was stable for long periods. For fully eighty minutes the DCR was consistently 0.9 units/min, and at the end of a three-hour exposure time the DCR still had a value of 0.8 units/min. As stated above, the NaF-poisoned erythrocyte has ceased its energy producing functions although the oxygen-transport function of the cell is relatively undamaged.

Figure 6. DCR Versus Exposure Time for Sodium Fluoride-Poisoned Erythrocytes
Initial Calculated pH - 7.07

Suspension Resistivity - 0.61×10^4 Ω-cm.

Frequency - 600 KHz

Voltage - 20 V, rms


DCR (UNITS/MIN)
1.0
0.8
0.6
0.4
0.2
10
20
30
40
50
60
70
80
EXPOSURE TIME (MIN.)

Since the collection rate was consistent for at least 80 minutes for the NaF-poisoned cells, it was then possible to perform experiments where the DCR had to be independent of exposure time. Therefore, one can carry out various dielectrophoresis experiments on NaF-poisoned cells that he cannot carry out on normally functioning cells.

As mentioned above, the erythrocyte is not an ideal dielectric and is therefore defined as a "lossy" dielectric. Its response should be frequency dependent. For frequency response measurements, the voltage was held at 20 V, rms. The standard suspension medium was used with NaF present in a 0.001$\bar{M}$ concentration, and the DCR determined at various frequencies.

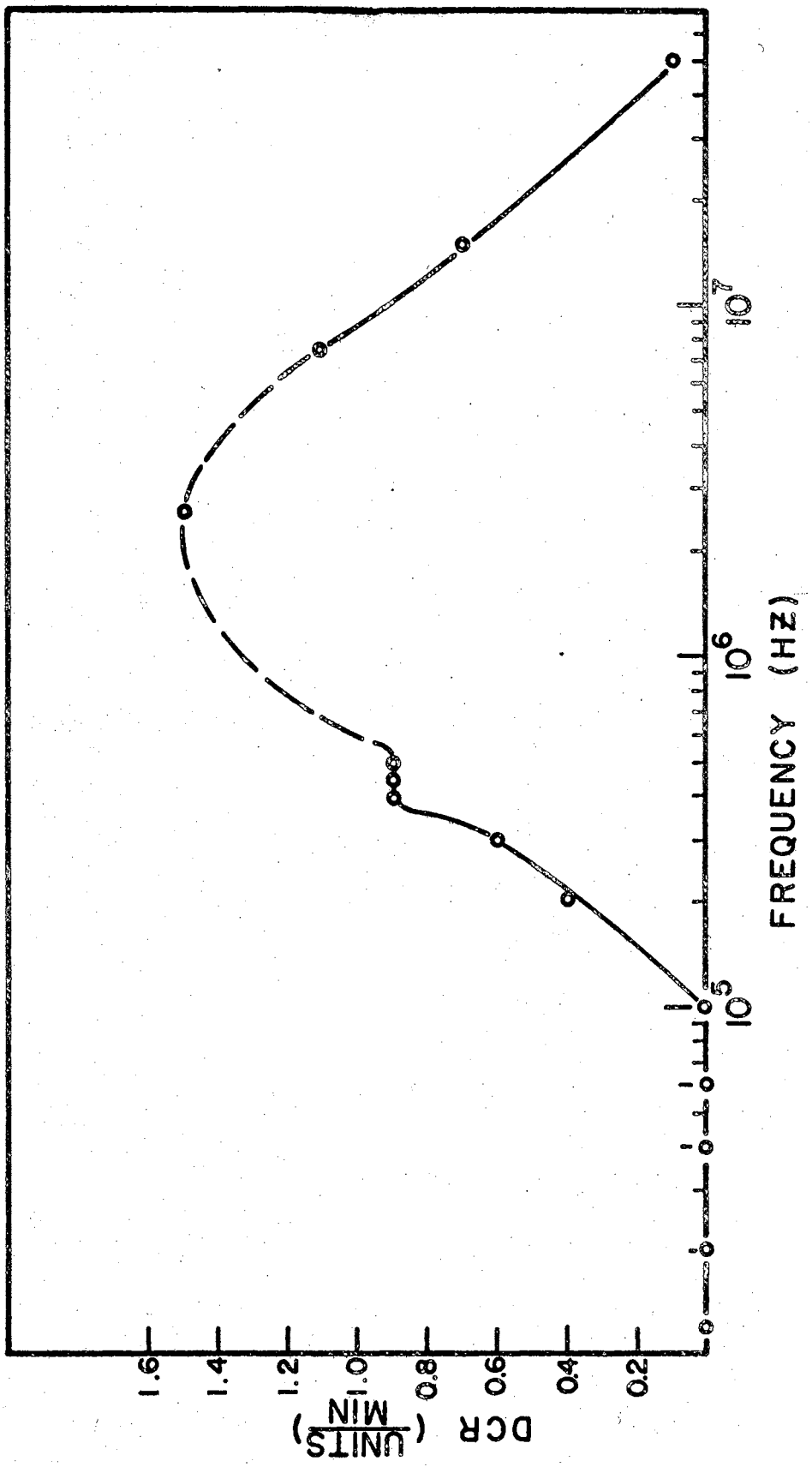
The results of the frequency response experiment for NaF-poisoned cells, Figure 7, show that at 20 V, rms there was no collection at frequencies below 100 KHz. Collection, just beginning at 100 KHz, increased until the 400 KHz mark was reached. DCR's at frequencies between 400 KHz and 600 KHz were constant. This gives a 200 KHz range in which to work without causing dielectrophoretic force changes due to frequency variations. Also, at a frequency of 300 KHz some of the collected cells began to rotate. Those near the top of the chain, farthest from the pin, rotated clockwise and those near the bottom of the chain rotated counterclockwise. There was a rapid increase in DCR with increasing frequency from 600 KHz to 2.55 MHz. After this sharp increase the collecting fell off more slowly until, at 50 MHz, the collection was hardly noticeable.

As stated above, at frequencies below 100 KHz there was no collection whatsoever but at low frequencies cells did become active. At 3 KHz the cells were attracted towards the pin but were stopped a short

Figure 7. DCR Versus Frequency for Sodium Fluoride-Poisoned Erythrocytes.

Suspension Resistivity - 0.61×10^4 Ω-cm.

Voltage - 20 V, rms


DCR (UNITS/MIN)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
10^5
10^6
10^7
FREQUENCY (HZ)

distance from it as if some type of barrier were present.

To determine the effects of voltage variations on NaF-poisoned erythrocytes, the cells were suspended in the standard medium with a 0.001$\bar{M}$ concentration of NaF present. The frequency was 2.55 MHz for the voltage range of 0 to 140 V, rms. The resistivity of the suspension solution was 1.0×10^4 Ω-cm. The general shape of the curve in Figure 8 shows agreement with the theoretical prediction that the force on the cells is proportional to the square of the voltage, and that the DCR is proportional to the first power of the applied voltage (6).

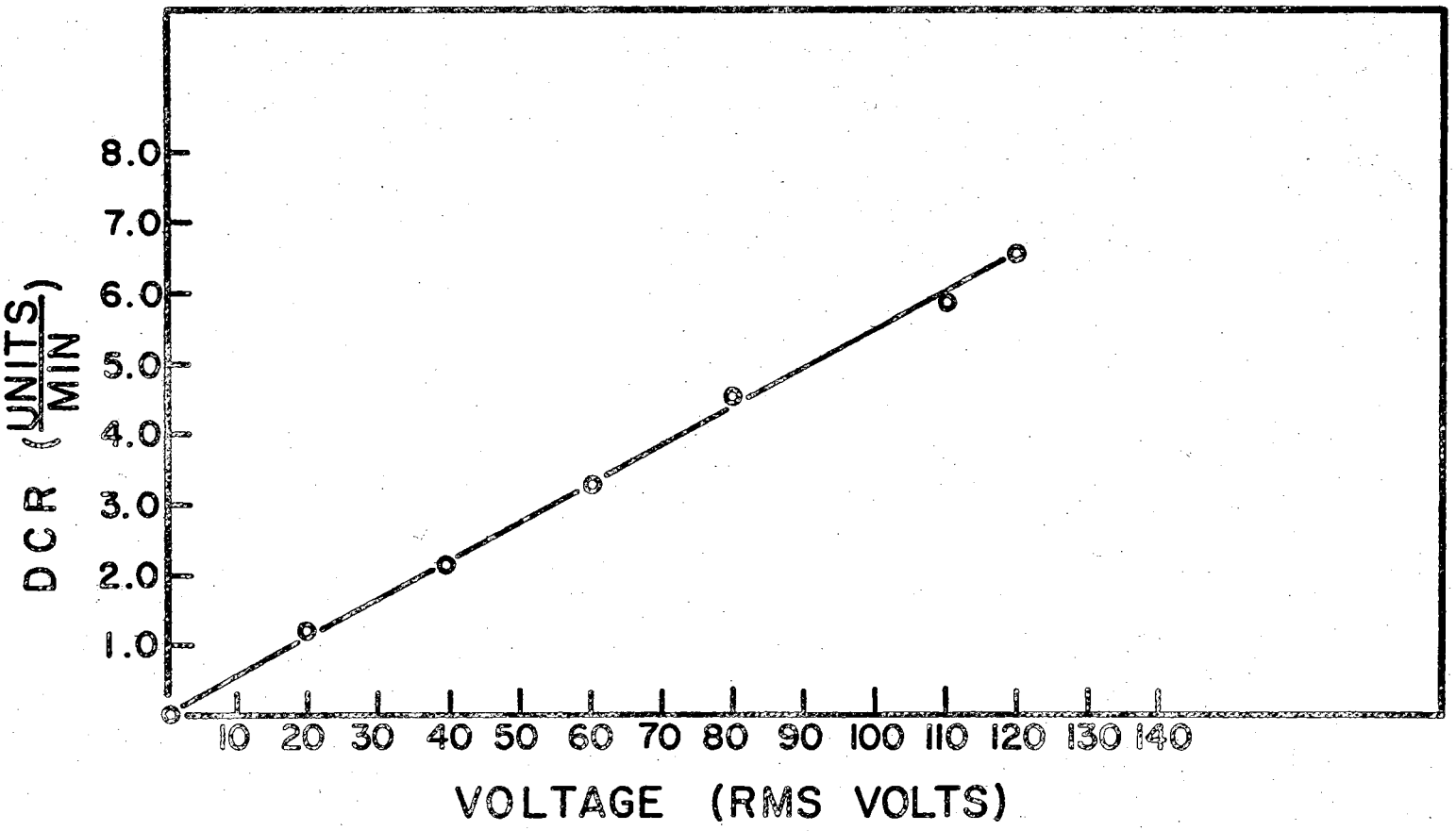
The metabolic inhibitors used in the preceeding experiments were added to the standard suspension medium before the cells were placed into the medium. To check the effects of the method of poisoning, a different type of exposure was tried with the sodium fluoride. In this method, the whole blood was exposed to NaF before adding it to the standard suspension medium. Equal amounts of whole blood and 0.5$\bar{M}$ sodium flouride were mixed and this solution was diluted 500 fold into the basic suspension medium after 2 minutes of exposure to NaF. The results of the exposure time experiments as well as the frequency and voltage variation experiments were the same as the results for the first experiments on sodium fluoride-poisoned cells. After exposure for one hour, the DCR for the cells was still consistent and even after three days at room temperature the DCR was half of its original value.

In conclusion, the dielectrophoresis of the NaF-poisoned erythrocyte shows unique features when compared to the dielectrophoresis of normal cells. The poisoned cells showed a consistent DCR for long periods (exceeding two hours). The frequency studies show that the red blood cells were most responsive at 2.55 MHz. The DCR was a linear function

Figure 8. DCR Versus Voltage for Sodium Fluoride-Poisoned Erythrocytes

Suspension Resistivity - 1.0×10^4 Ω-cm.

Frequency - 2.55 MHz


DCR (UNITS/MIN)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
10 20 30 40 50 60 70 80 90 100 110 120 130 140
VOLTAGE (RMS VOLTS)

of voltage.

Sodium Iodoacetate-Poisoned Erythrocytes

Another substance that inhibits the glycolysis of the erythrocyte is iodoacetic acid. To study the dielectrophoretic character of cells exposed to this poison, the standard suspension medium was used with iodoacetic acid, sodium salt present in a 0.001M concentration.

The first experiment was done to study the DCR as a function of exposure time. Data was taken at a frequency of 600 KHz at a voltage of 20 V, rms. The resistivity of the suspension medium was 1.06×10^4 Ω-cm.

Figure 9 shows that for cells whose glycolysis was inhibited by sodium iodoacetate, the DCR as a function of exposure time was constant. The DCR for these cells was, in fact, even more consistent and uniform than that of the NaF-poisoned cells. For 60 minutes the cells maintained this consistent collection without any disturbing variations. Therefore, it was again possible to carry out the experiments in which the DCR could not be a varying function of time. These experiments were the ones in which the frequency and voltage affects on the DCR were determined.

Figure 10 shows the DCR as a function of frequency for iodoacetic acid-poisoned cells. From the graph one sees that there was no collection for frequencies below 10^5 Hz. Upon reaching 10^5 Hz the DCR increased rapidly with increasing frequency until it reached a plateau which began at 4.0×10^5 Hz and lasted until a frequency of 6.0×10^5 Hz was reached. Thus, for iodoacetate-poisoned cells there was also a 200 KHz range in which experiments could be done where the DCR was not a varying function of frequency. The highest DCR occurred at a frequency

Figure 9. DCR Versus Exposure Time for Sodium Iodoacetate-Poisoned Erythrocytes
Initial Calculated pH - 7.0.

Suspension Resistivity - 1.06×10^4 Ω-cm.

Frequency - 600 KHz

Voltage - 20 V_p rms

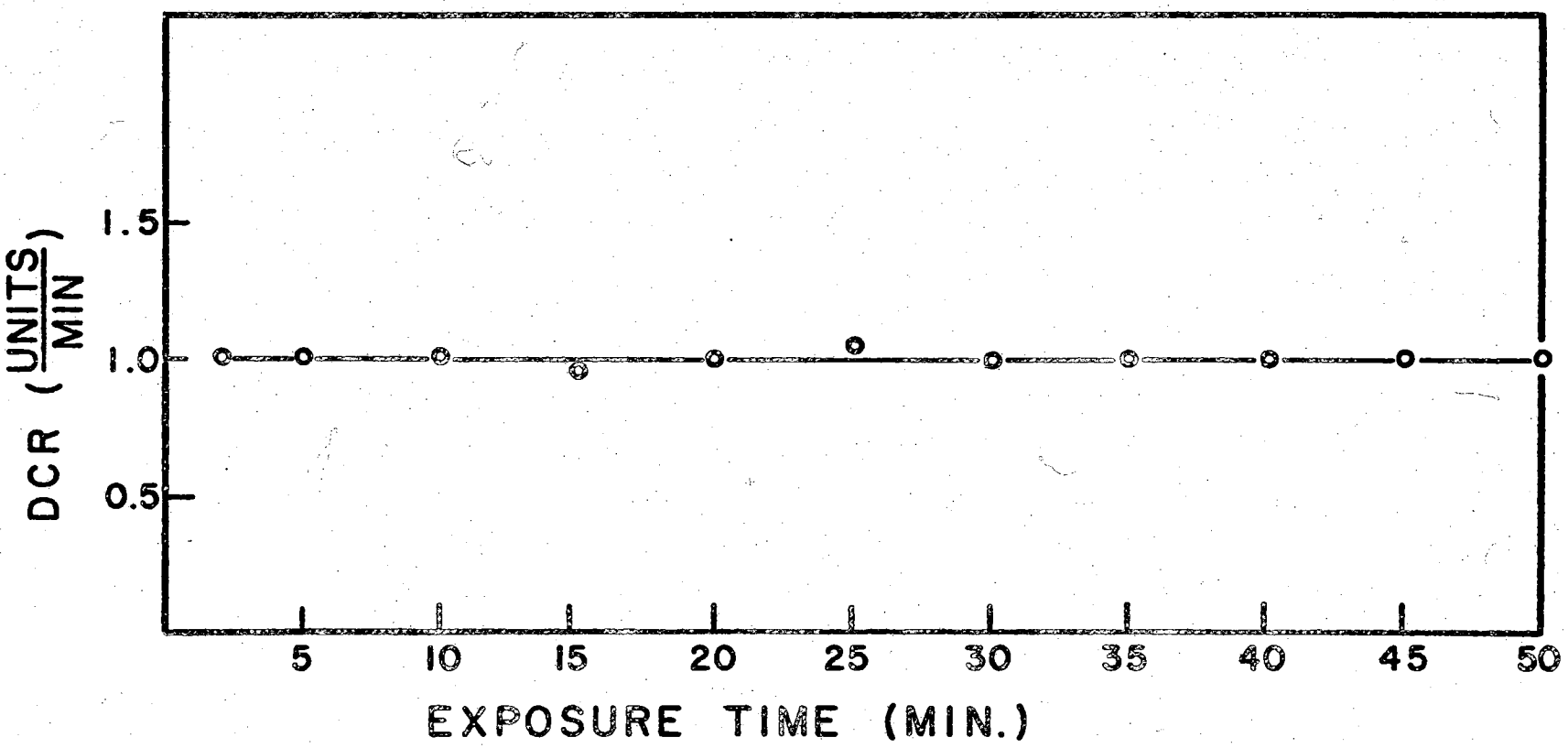
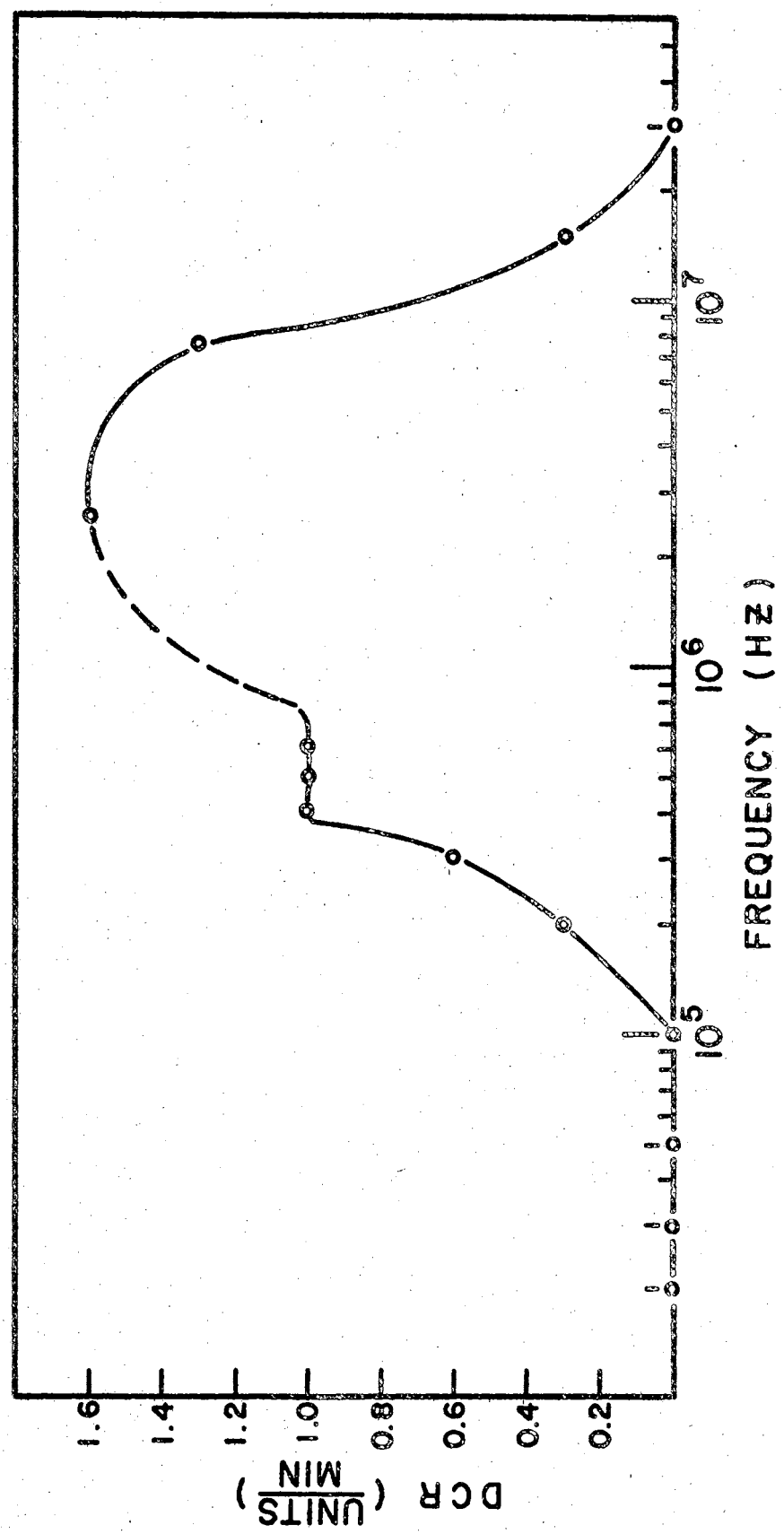

DCR (UNITS / MIN)
1.5
1.0
0.5
5
10
15
20
25
30
35
40
45
50
EXPOSURE TIME (MIN.)

Figure 10. DCR Versus Frequency for Sodium Iodoacetate-Poisoned Erythrocytes.

Suspension Resistivity - 1.06×10^4 Ω-cm.

Voltage - 20 V, rms


DCR (UNITS/MIN)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
10^5
10^6
10^7
FREQUENCY (HZ)

of 2.55 MHz. At frequencies higher than 2.55 MHz the DCR was a decreasing function of frequency. At 20 MHz all cell collection had ceased and there was not even a feeble response observed from the erythrocytes.

For voltage dependence studies, the frequency was held at 600 KHz and the voltage was varied from 0 to 20 V, rms. In this case the power put into the cell did not cause appreciable heating of the suspension medium and therefore most of the thermal activity which affected the DCR was eliminated. Figure 11 shows the results of the experiment. The results do not agree well with the theoretical prediction that the dielectrophoretic force is a function of the square of the voltage, i.e., with the yield directly proportional to the voltage. Activity was observable even at very low voltages. The first recordedable DCR occurred at 4 volts. At 2 volts the cells began to align themselves with the lines of force of the field.

Sodium Cyanide-Poisoned Erythrocytes

Like sodium fluoride and sodium iodoacetate, sodium cyanide is also a metabolic inhibitor for the erythrocyte. However, instead of inhibiting the glycolysis of the cells, NaCN poisons their respiration. The standard suspension medium was used with the sodium cyanide present in a 0.001M concentration. Care must be taken because of this assay solution's capability of releasing toxic HCN. The first experiment determined the DCR as a function of exposure time. Figure 12 shows the results.

In this figure a DCR unlike any heretofore reported is shown. For fully 15 minutes the DCR was consistently 1.0 units/min. Afterwards,

Figure 11. DCR Versus Voltage for Iodoacetate-Poisoned Erythrocytes.

Suspension Resistivity - 1.06×10^4 Ω-cm.

Frequency - 600 KHz.

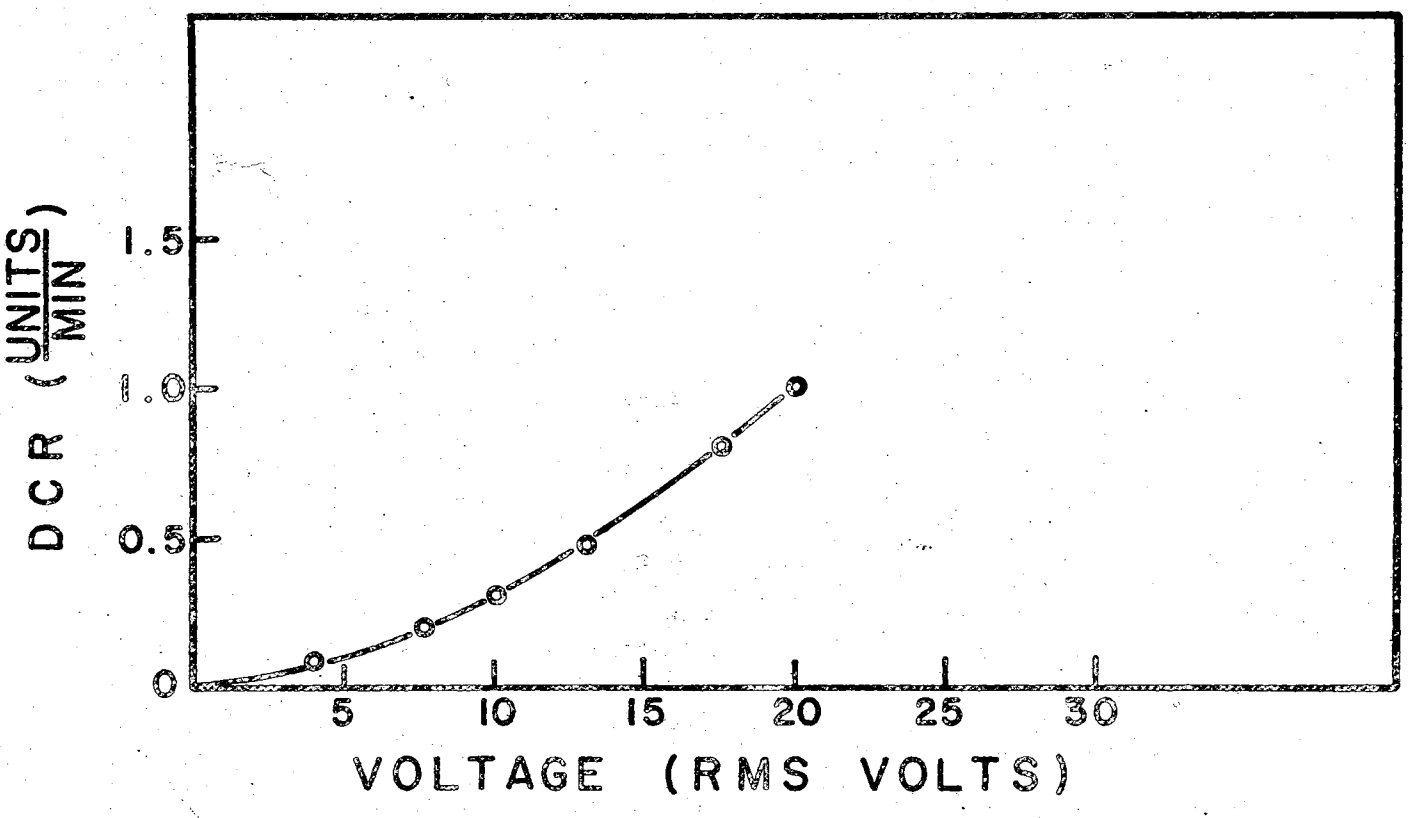

DCR (UNITS/MIN)
1.5
1.0
0.5
0
5
10
15
20
25
30
VOLTAGE (RMS VOLTS)

Figure 12. DCR Versus Exposure Time for Sodium Cyanide-Poisoned Erythrocytes.

O — 0.001$\bar{M}$ NaCN

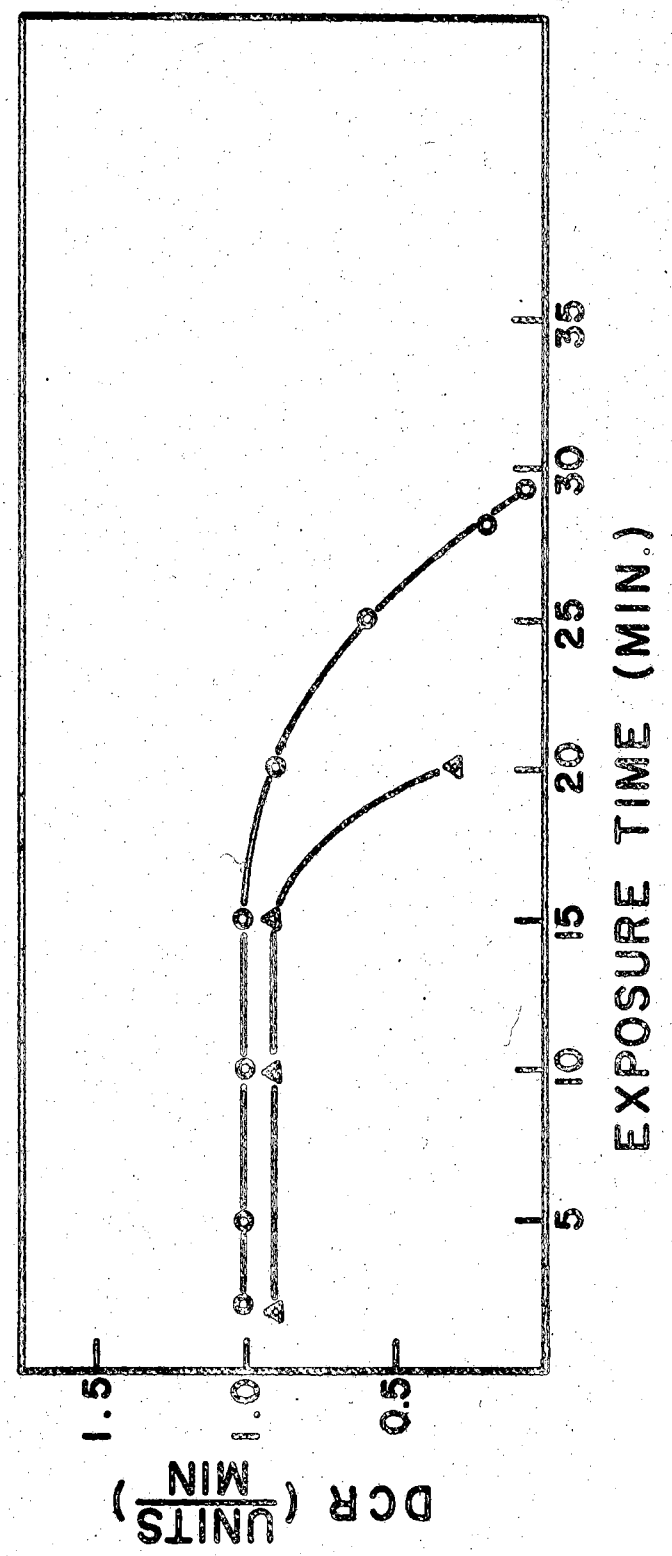
Suspension Resistivity - 1.76×10^4 Ω-cm. Initial Calculated pH = 10.07 for 0.001$\bar{M}$ NaCN.

Δ - 0.002$\bar{M}$ NaCN

Suspension Resistivity - 0.78×10^4 Ω-cm. Initial Calculated pH = 10.22 for 0.002$\bar{M}$ NaCN.

Frequency - 600 KHz.

Voltage - 20 V, rms.


DCR (UNITS/MIN)
1.5
1.0
0.5
5
10
15
20
25
30
35
EXPOSURE TIME (MIN.)

however, a sharp decrease in DCR was observed until, at 30 minutes, the collection rate was practically zero. Unlike some previous experiments, the DCR decrease was not accompanied by erythrocyte hemolysis. It is obvious that to carry on any studies of NaCN-poisoned erythrocytes where the DCR is not a function of exposure time, the experiments should be carried out well within the fifteen minute time limit in which the DCR is stable. Interestingly, after one hour of exposure to NaCN the cells had ceased to collect completely but there were still no signs of hemolysis.

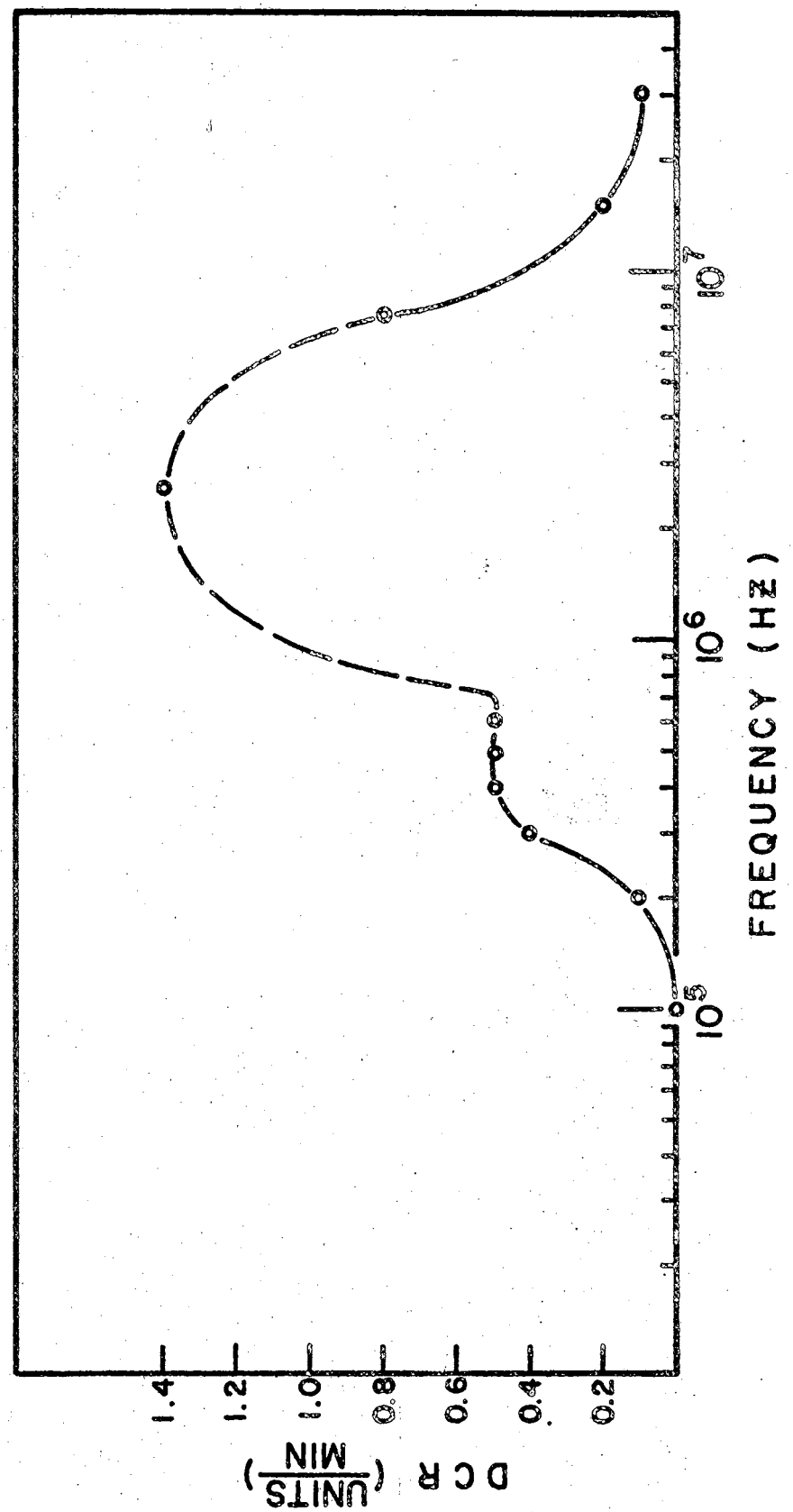
Experiments were also carried out with the concentration of NaCN doubled to $0.002\bar{M}$. A frequency of 600 KHz at 20 V, rms was used and the resistivity of the solution was 0.78×10^4 Ω-cm. Figure 12 shows that these erythrocytes had a consistent DCR for fifteen minutes. This was followed by a sharp drop in activity for erythrocytes in the standard suspension medium with NaCN present in a $0.002\bar{M}$ concentration.

To measure the frequency response of erythrocytes exposed to NaCN a $0.001\bar{M}$ concentration of NaCN was used in the standard suspension medium. Due to the 15 minute time limit on the DCR stability of the NaCN-poisoned erythrocytes, all frequency measurements were taken within 10 minutes after the cells were suspended. For the DCR versus frequency experiments a voltage of 20 V, rms was used. The resistivity of the suspension medium was 0.9×10^4 Ω-cm. In Figure 13 one sees that there was no collection at frequencies below 1.0×10^5 Hertz. However, at 2.0×10^5 Hz the DCR increased rapidly with increasing frequency until a frequency of 4.0×10^5 Hz was reached. At frequencies between 4.0×10^5 Hz and 6.0×10^5 Hz the DCR was constant. When frequency was increased from 6.0×10^5 Hz to 2.55 MHz the DCR underwent a rapid in-

Figure 13. DCR Versus Frequency for Sodium Cyanide-Poisoned Erythrocytes.

Suspension Resistivity = 0.9×10^4 Ω-cm.

Voltage - 20 V, rms.


DCR (UNITS/MIN)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
10^5
10^6
10^7
FREQUENCY (HZ)

crease until it reached its maximum value of 1.4 units/min at 2.55 MHz. At frequencies higher than 2.55 MHz the DCR became a decreasing function of frequency.

We conclude that the DCR for NaCN-poisoned erythrocytes is different from that of the other poisoned cells. Instead of having a consistent DCR for well over one hour, their DCR was consistent for only fifteen minutes. This decrease in DCR was not accompanied by hemolysis. One must keep in mind that the physiologic function which the presence of NaCN affects is not the oxygen-carrying capacity of the hemoglobin, but the action of respiratory enzymes which catalyze the utilization of oxygen. Using dielectrophoresis one can tell whether or not erythrocytes have been exposed to NaCN as opposed to sodium fluoride or iodoacetic acid.

Sodium Azide-Poisoned Erythrocytes

Like cyanide, azide is also a substance which poisons the respiration of the erythrocyte. When the cells were exposed to sodium azide in a 0.001$\bar{M}$ concentration in the standard suspension medium a very consistent DCR was observed. For well over one hour the DCR remained at 1.0 units/min. Readings were taken at a frequency of 600 KHz at 20 V, rms. The resistivity of the suspension medium was 0.8×10^4 Ω-cm. Thus, the NaN3-poisoned erythrocytes show the same consistency in DCR as did the sodium fluoride and iodoacetic acid-poisoned cells.

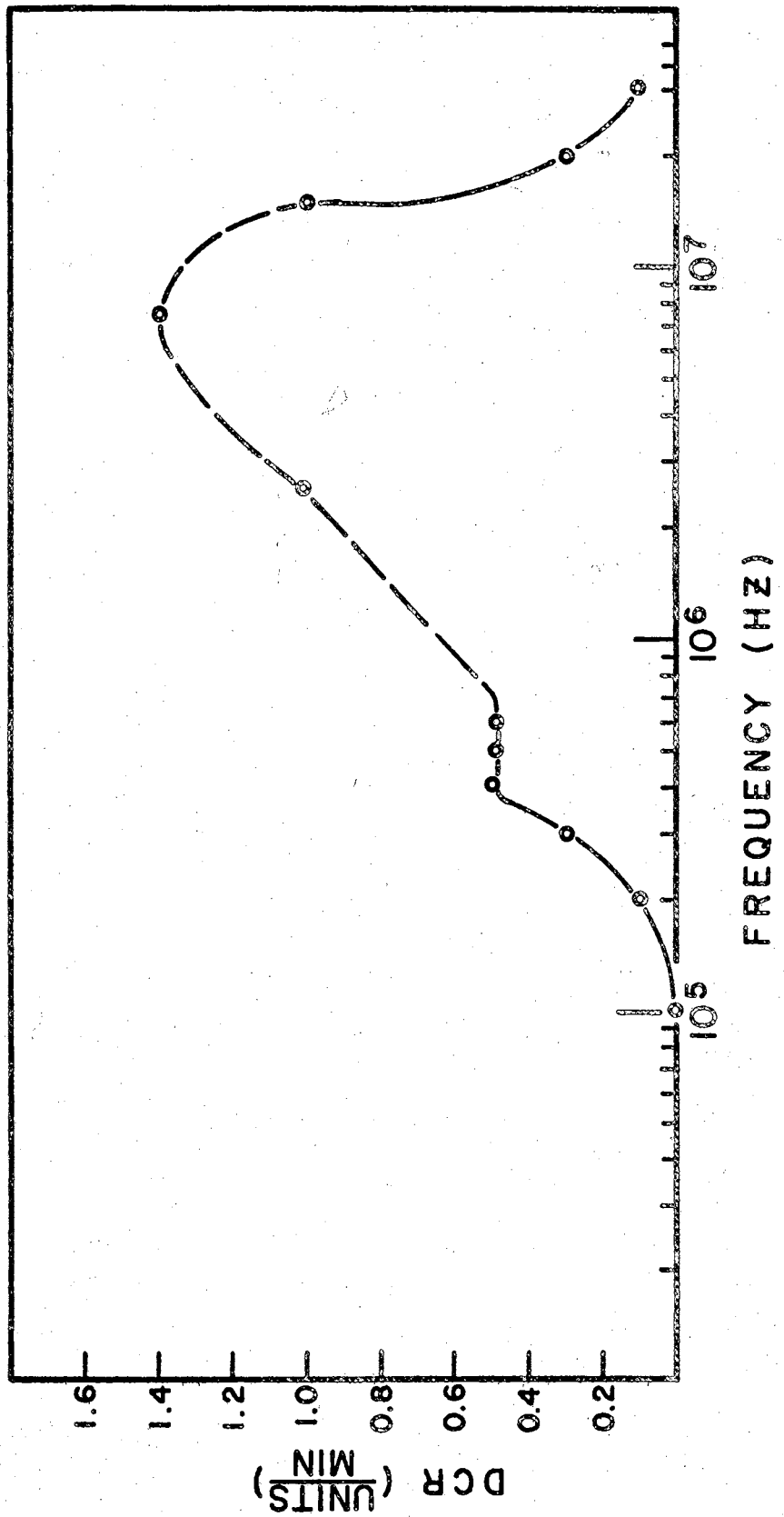
Figure 14 shows the DCR vs frequency curve for NaN3-poisoned cells. The graph shows the DCR plateau between 400 and 600 KHz. At the 600 KHz mark there was a rapid increase in DCR with increasing frequency until a maximum DCR was reached at 7.5 MHz. At frequencies higher than 7.5

Figure 14. DCR Versus Frequency for Sodium Azide-Poisoned Erythrocytes.

Calculated Initial pH - 7.8.

Suspension Resistivity - 0.35×10^4 Ω-cm.

Voltage - 20 V, rms.


DCR (UNITS/MIN)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
10^5
10^6
10^7
FREQUENCY (HZ)

MHz the DCR became a decreasing function of frequency. The readings were taken at 20 V, rms. The resistivity of the suspension medium was 0.35×10^4 Ω-cm. There was no collection at frequencies below 100 KHz.

Since the DCR for erythrocytes is a function of the applied electric field (theoretically, proportional to E for ideal dielectrics) experiments were carried out to determine the DCR as a function of voltage for the NaN3-poisoned erythrocyte. The cells were collected at 600 KHz. Figure 15 shows that the DCR for NaN3-poisoned erythrocytes is approximately a bit higher than first power.

Formaldehyde-Poisoned Erythrocytes

One must keep in mind that the solutions of sodium fluoride, sodium cyanide, sodium azide, and iodoacetic acid only serve to inhibit certain metabolic functions of the erythrocyte and do not necessarily destroy its viability. The cells can not strictly be considered dead after being exposed to these substances. Formaldehyde, however, can serve to completely denature the erythrocyte. Therefore an experiment was done to determine the dielectrophoresis of the formaldehyde-poisoned cells as a function of exposure time. The formaldehyde was present in the standard suspension medium in a 0.001$\bar{M}$ concentration. The exposure-time results are shown in Figure 16. The results show a decreasing DCR and, after 45 minutes of exposure time the DCR was down to the very low value of 0.2 units/min. When the concentration of formaldehyde (HCHO) was doubled to 0.002$\bar{M}$ the DCR was practically zero within six minutes. However, fairly good collection was evident for the first three minutes.

Thus, the formaldehyde-poisoned cells do not exhibit a consistent DCR as do the cells that have their metabolic functions inhibited.

Figure 15. DCR Versus Voltage for Sodium Azide-Poisoned Erythrocytes.

Suspension Resistivity - 0.35×10^4 Ω-cm.

Voltage - 20 V, rms.

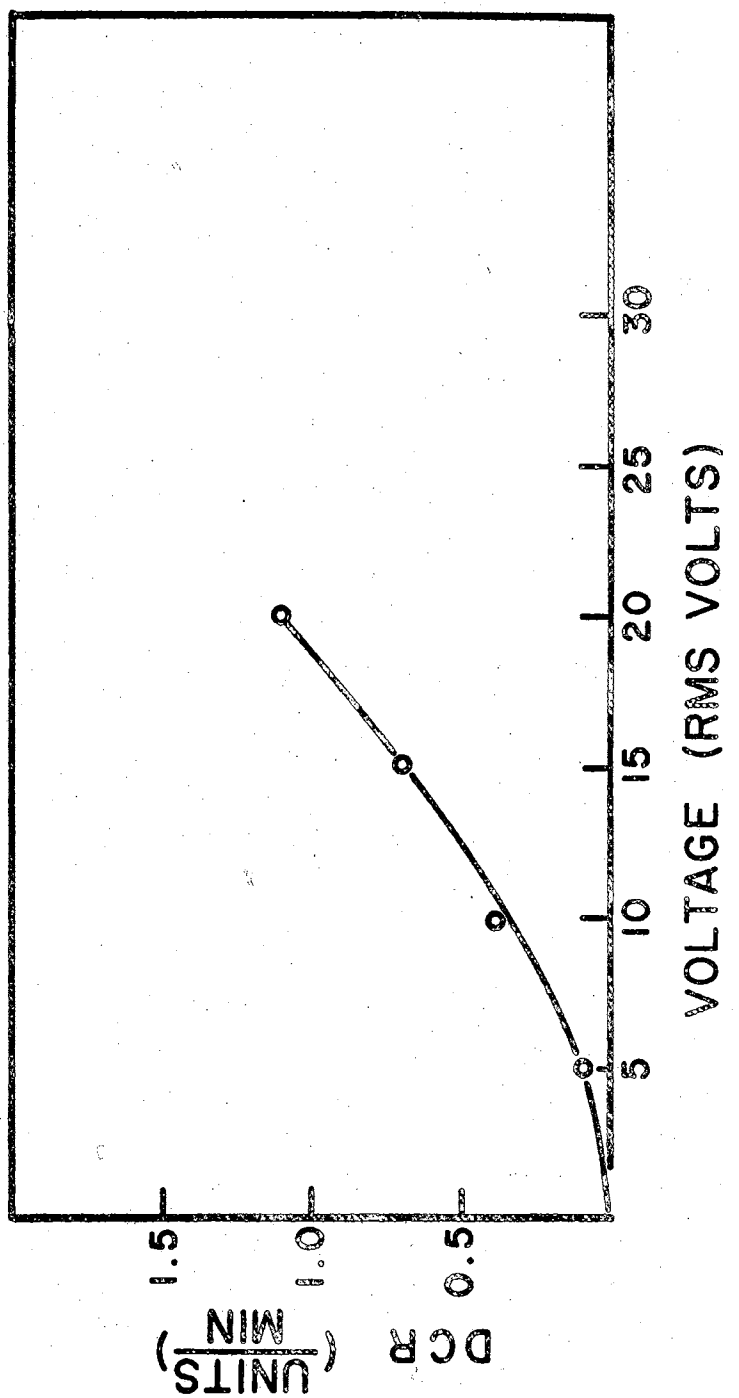
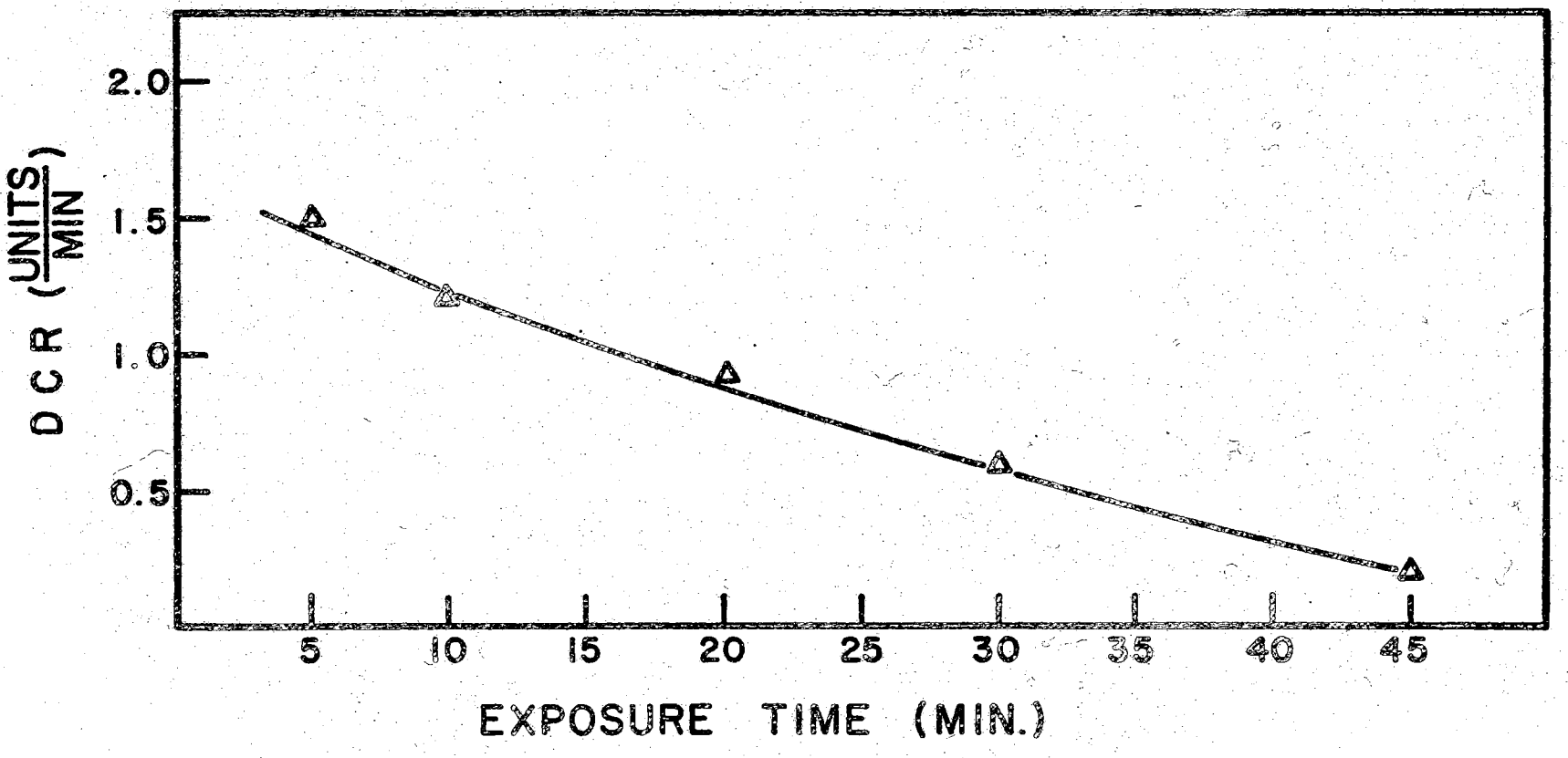

DCR (UNITS/MIN)
1.5
1.0
0.5
5
10
15
20
25
30
VOLTAGE (RMS VOLTS)

Figure 16. DCR Versus Exposure Time for Formaldehyde-Poisoned Erythrocytes.

Suspension Resistivity - 2.2×10^4 Ω-cm.

Frequency - 600 KHz.

Voltage - 20 V, rms.


DCR (UNITS/MIN)
2.0
1.5
1.0
0.5
5
10
15
20
25
30
35
40
45
EXPOSURE TIME (MIN.)

CHAPTER VI

THE DIELECTROPHORESIS OF THE ERYTHROCYTES AS A FUNCTION OF SUSPENSION MEDIUM RESISTIVITY AND pH

The accepted pH of blood serum is 7.4 and is therefore slightly alkaline. The pH of the standard suspension medium was controlled here with a potassium hydroxide (KOH) solution. The conductivity was controlled with a solution of sodium chloride (NaCl). The pH of the suspensions was read from a Beckman Zeromatic II pH meter with a glass electrode and a silver-silver chloride electrode. The suspensions were at a temperature of $24^{o}C$ when pH was recorded. The frequency used was 600 KHz at a voltage of 20 V, rms. All readings were taken after a two minute exposure time because the DCR for normal erythrocytes is a decreasing function of exposure time.

At low resistivities the field causes so much turbulence in the suspension medium that the dielectrophoretic aspects cannot be observed. After repeated experiments at various solution resistivities at a pH of 7.4 the lowest resistivity at which collection was observed was determined. This resistivity was 1.36×10^{3} Ω-cm. Nevertheless, even at this resistivity, there was still some field-engendered turbulence in the suspension medium. Using the method of adjusting pH with KOH and varying the resistivity with NaCl it was possible to construct graphs of DCR versus resistivity for different pH values. Figure 17 shows this

Figure 17. DCR Versus Resistivity for Erythrocytes in Standard Suspension Medium.

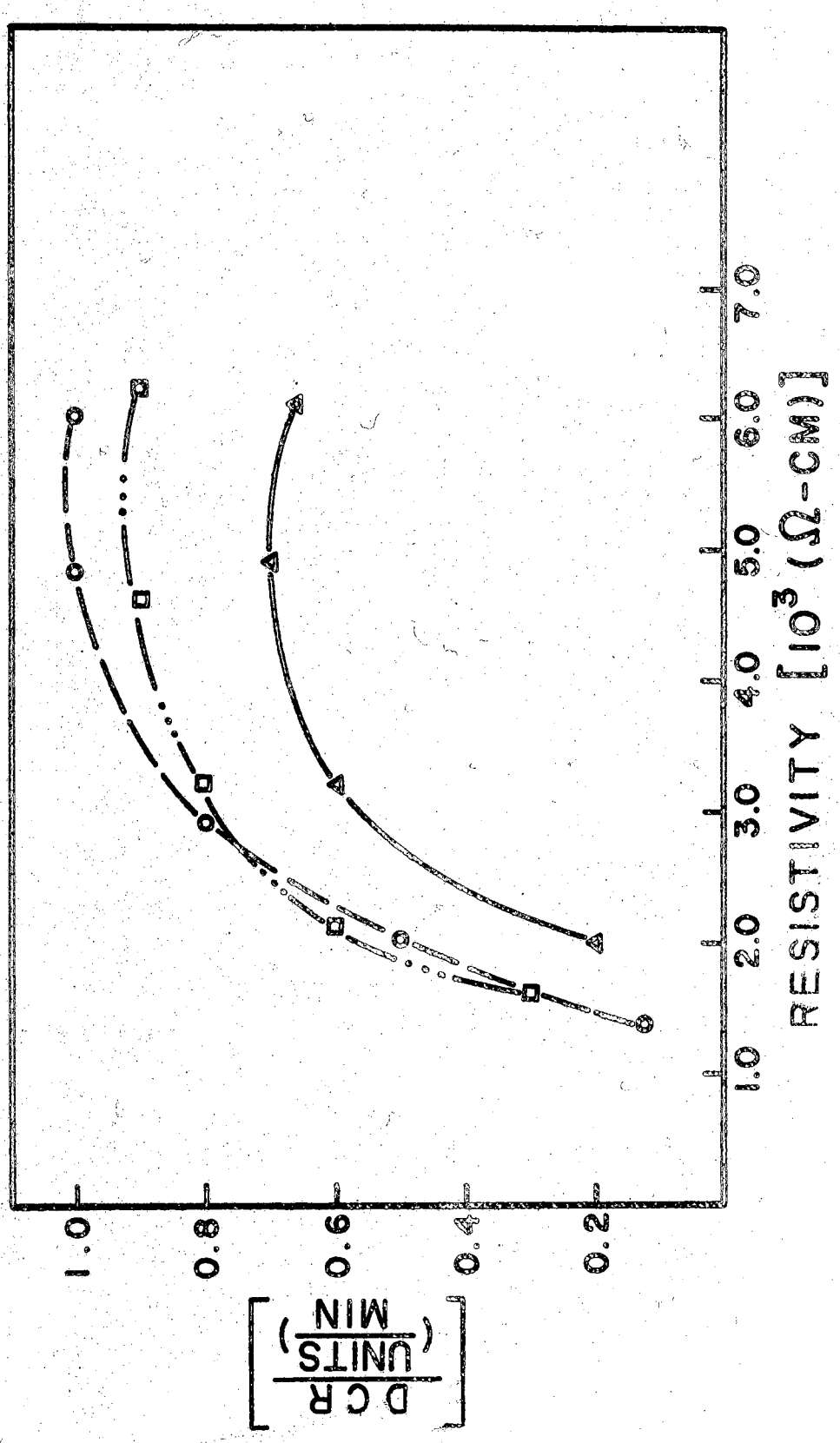
Δ - pH 5.4

□ - pH 6.4

O - pH 7.4

Frequency - 600 KHz.

Voltage - 20 V_s rms.


1.0
0.8
0.6
0.4
0.2
DCR (UNITS/MIN)
1.0
2.0
3.0
4.0
5.0
6.0
7.0
RESISTIVITY [10^3 (Ω-CM)]

relation for the three pH values of 7.4, 6.4 and 5.4. These graphs show that even at a specific resistivity the DCR was dependent upon the pH of the suspension medium. For cells whose suspensions pH's were 7.4 and 6.4 the DCR's were relatively close until a resistivity of 2.6×10^3 Ω-cm was reached. At this point the pH 6.4 curve leveled off at a lower value than that of the pH 7.4. The graph stops at a resistivity of 6.2×10^3 Ω-cm because keeping the pH constant demands the presence of a certain amount of hydrogen or hydroxyl ions in the suspension. This puts an upper limit on the resistivity of the standard suspension medium. Also the pH range was limited to the spread listed for dependable results. When the pH was 8.5 or higher and 4.0 or lower, the erythrocytes underwent immediate hemolysis as soon as they were introduced into the suspension medium.

We conclude that the dielectrophoresis of canine erythrocytes is well-behaved for pH lying in the range 6.4 to 7.4 (or possibly slightly higher) and if the "standard" (glycine-glucose) medium has a salt content such that the resistivity is above about 3×10^3 ohm-cm.

CHAPTER VII

THE DIELECTROPHORESIS OF FLAVOBACTERIA

Flavobacterium is a gram-negative rod-shaped organism that can be found in soil and water. The cell wall of the flavobacterium is a three-layered structure. The major chemical component of the outermost layer is lipopolysaccharide, that of the center wall is lipoprotein, and that of the innermost wall is mucopeptide. The cell membrane is within the cell wall and it therefore forms a boundary between the cytoplasm and the cell wall.

It has been suggested that ethylene diamine tetraacetic acid (EDTA) acts upon the cell wall of flavobacteria by chelating divalent cations, principally Mg^{++} and Ca^{++}. These cations are thought to have a neutralizing effect on the difference in electrical charges on different subunit layers of the cell wall. By removing these cations, EDTA removes their "neutralizing presence" and the layers of the wall are not as tightly bound. Under these conditions it is said that the wall has lost its "integrity". If the cells are then re-exposed to an abundance of cations, as by resuspension in a growth medium, the available cations will exert their neutralizing effect, and the cell wall will regain its integrity. The purpose of the following experiments was to determine if the wall breakdown and repair could be observed by using dielectrophoresis.

Cells of flavobacterium were grown on a slant of succinate salts

agar for 12 to 15 hours at a temperature of 37°C. The slant was harvested with sterile deionized water and the harvest suspension was used to inoculate succinate salts agar plates with 0.5 mil suspension per plate. These were incubated at 37°C for 12-15 hours. The plates were harvested with phosphate buffer (0.01M, pH 6-7) and centrifuged at 10,000 RPM for ten minutes. The pellet was washed in phosphate buffer and centrifuged as before. Finally the washed pellet was suspended in sterile, deionized water to a final optical density (O.D.) of 1.0 measured on a Coleman Jr. II Spectrophotometer. The cells were then ready for the treatments and studies.

The first experiment was done to determine the DCR of flavobacteria when suspended in deionized water. These cells, at a frequency of 600 KHz at 20 volts, gave a consistent DCR of 1.0 for very long periods. The resistivity of the suspension was 0.85×10^4 Ω-cm. In a 5.5% glucose solution with NaCl present to adjust the resistivity to 3.0×10^3 Ω-cm the cells also gave a consistent DCR of 0.5 units/min. for very long periods. Again, the operating frequency was 600 KHz at 20 volts.

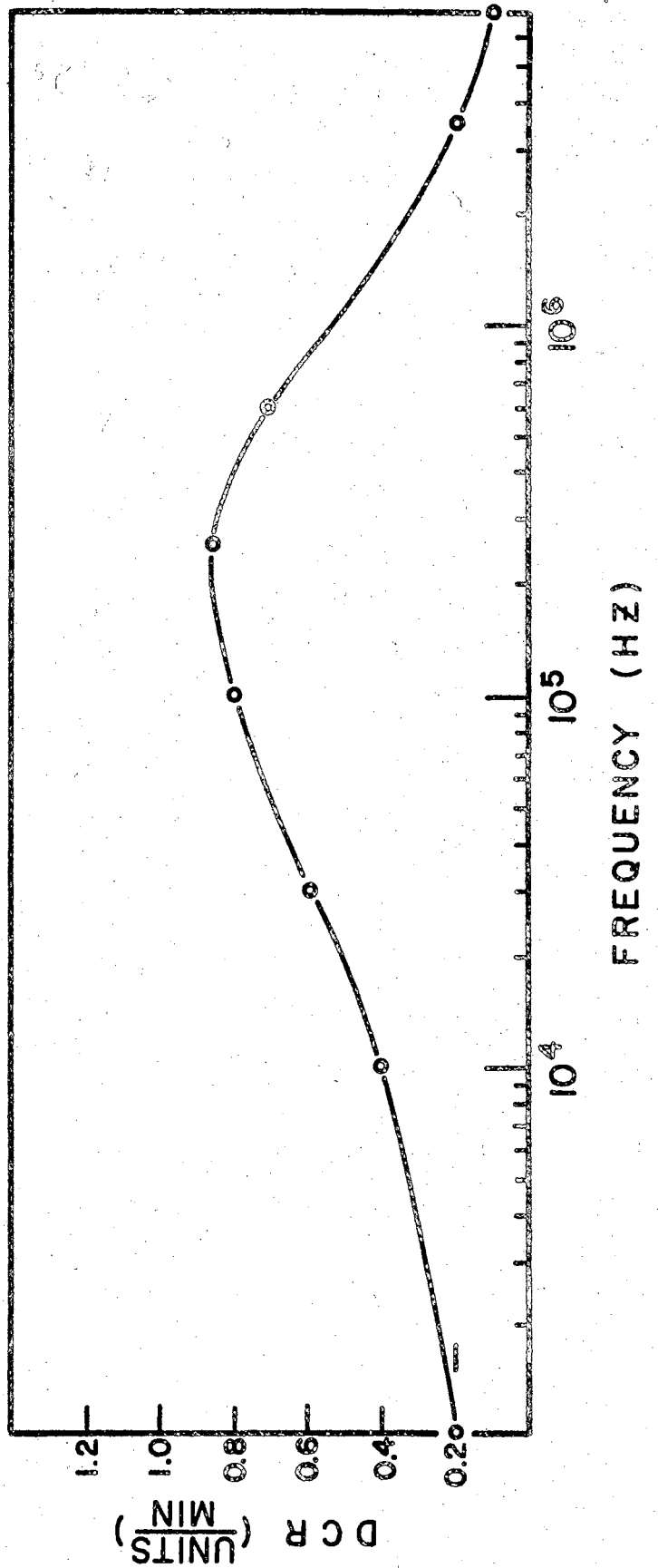
To determine the DCR as a function of frequency the cells were suspended in distilled deionized water which had a resistivity of 6.0×10^4 Ω-cm. All readings were taken at 20 volts and the results are shown in Figure 18. This curve shows that flavobacteria can be collected at much lower frequencies than can erythrocytes. Although for erythrocytes all collection ceased at 10^5 Hz one sees that for flavobacteria there is still some collection at 10^3 Hz.

When flavobacteria were suspended in a 5.5% glucose solution and EDTA was added in a 0.001M concentration the resistivity of the suspension was 3.0×10^3 Ω-cm. An exposure-time experiment was carried out at

Figure 18. DCR Versus Frequency for Flavobacteria Suspended in Distilled Deionized Water.

Suspension Resistivity - 6.0×10^4 Ω-cm.

Voltage - 20 V, rms.


DCR (UNITS/MIN)
1.2
1.0
0.8
0.6
0.4
0.2
10^4
10^5
10^6
FREQUENCY (HZ)

a frequency of 600 KHz at 20 volts. Figure 19 shows the results for the EDTA treated cells and the results for untreated cells suspended in 5.5% glucose with NaCl added to adjust the resistivity to 3.0×10^3 Ω-cm. After 2 minutes of exposure time the EDTA treated cells have a DCR of 0.5 units/min. At 5 minutes of exposure time the DCR drops to 0.1 units/min. and then begins a slow rise up to the value of 0.4 units/min. at the end of 30 minutes. These cells never regain their DCR value of 0.5 units/min. One must realize, however, that the EDTA is still in this solution and that the cells are not in an ideal growth medium. One should therefore probably never expect the cells to completely regain the DCR that they had before the EDTA was added.

Experiments were also done to expose the cells to EDTA and then wash them by centrifuging and resuspending them in water and in succinate salts. The cells which were resuspended in water after EDTA exposure did not show a much different DCR from that of the cells suspended in water that had not been exposed to EDTA. It appears from this that cell repair can occur if re-exposure to a good growth medium is allowed. The problem is that much time is consumed in centrifuging the cells from EDTA solutions (10 minutes). The same problem was found with the succinate salts growth medium. It had too low a resistivity to be used directly in the dielectrophoresis experiments and therefore the cells had to be washed and resuspended in deionized water. This delay introduces a slight uncertainty perhaps.

One cannot then say, for certain, that cell wall repair has been observed. There are still variables that may be affecting the DCR. For example, pH changes with solution may be affecting the DCR. Further refinements will have to be initiated before a final conclusion can be

Figure 19. DCR Versus Exposure Time for Flavobacteria Suspended in 5.5% Glucose.

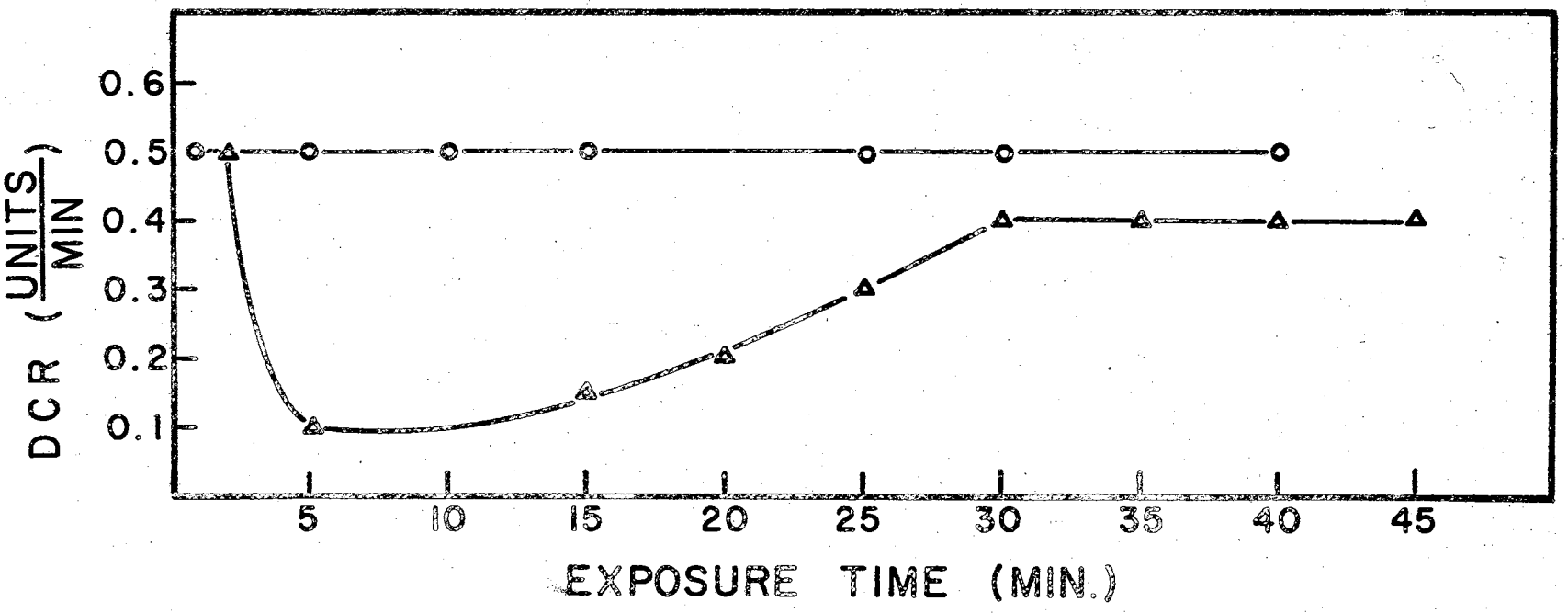
Δ - 5.5% Glucose with 0.001M EDTA.

Suspension Resistivity - 0.3×10^4 Ω-cm,

O - 5.5% Glucose with NaCl Added to Adjust Resistivity to 0.3×10^4 Ω-cm.

Frequency - 600 KHz.

Voltage - 20 V, rms.


DCR (UNITS / MIN)
0.6
0.5
0.4
0.3
0.2
0.1
5
10
15
20
25
30
35
40
45
EXPOSURE TIME (MIN.)

reached. In the meantime, we provisionally conclude that repair from EDTA effects can occur.

CHAPTER VIII

CONCLUSIONS

The erythrocyte has proved to be a very responsive cell in its dielectrophoresis. This is encouraging since the erythrocytes carry on the vital function of oxygen transport in the body.

The *in vitro* suspension media used proved to be quite satisfactory for allowing the cells to have a dielectrophoretic response. However, a non-toxic medium which would allow the cells to collect and yet allow them to maintain a consistent DCR for periods of, say, one hour, was not found. For normal (unpoisoned) erythrocytes, the DCR has proved to be a decreasing function of exposure time. All of the evidence so far shows that the cells leak out ions into the suspension medium and this affects the DCR in two ways. First, it lowers the resistivity of the suspension medium which serves to decrease the DCR. Second, and most important the ion concentration in the cell is lowered and the dipole which responds to the non-uniform field is therefore weaker and this causes the dielectrophoretic force to be smaller. These two factors seem to be the main reason that the DCR of the normal erythrocytes drops so rapidly in the *in vitro* suspension media used so far. Addition of substances such as NaCl, buffers, and carbonates seem to only affect the DCR as they determine the resistivity of the suspension medium. Although the DCR was taken with all of these substances present, the general shape of the curves of the DCR vs. exposure time for these

suspension media were all similar.

When the poisons sodium fluoride, sodium cyanide, sodium azide, and sodium iodoacetate were present in the suspension medium in a 0.001$\bar{M}$ concentration an altogether different type of DCR was observed. Except in the case of NaCN-poisoned erythrocytes, the DCRs remained constant for long periods of time. For the fluoride-poisoned cells the DCR maintained the constant value of 0.9 units/min for approximately three hours. There was also no considerable drop in the resistivity of the suspension medium after a one hour exposure time. This indicates that the cells are not leaking ions into the medium and are therefore able to maintain their dielectrophoretic response. In addition, the frequency response of the sodium fluoride-poisoned erythrocytes as well as the cells poisoned by sodium cyanide, sodium iodoacetate and sodium azide show similar characteristics. At frequencies below 100 KHz at 20 volts none of these poisoned cells have a dielectrophoretic response. Since 20 volts does not cause considerable heating in a one minute reading for suspension media with the range of resistivities used, it can be considered an optimum voltage. Therefore it is not possible to collect cells at frequencies below 100 KHz using this method of experimentation. Also, all of the cells exhibited a constant DCR for frequencies between 400 and 600 KHz. A possible explanation for this is that there are two types of dipoles in action in an erythrocyte. At values between 400 KHz and 600 KHz a decrease in the DCR of one dipole is negated by an increase in the DCR of the other which has the same magnitude at those frequencies. This, therefore, produces what might be called the frequency plateau. At very high frequencies (15-30 MHz) the cells practically cease their dielectrophoretic response. Therefore, there is an

upper and a lower limit in which one can effectively work. The lower limit is about 400 KHz and the upper limit is about 7.5 MHz.

Some of the DCR vs. voltage curves do not show exact agreement with the theoretical prediction that the DCR is proportional to the first power of the applied voltage. However, one must remember that the dielectrophoretic force is in competition with many other forces such as electrophoresis, convection and thermal effects.

It seems that for the sodium cyanide poisoned-erythrocytes, the poison loses its DCR stabilizing effects after a 15 minute exposure time. Clinical studies have shown that the presence of methylene blue renders cyanide relatively non-toxic and decomposes it, however, this is not the case in this suspension medium. It is not yet known why the cyanide loses its effect of stabilizing the DCR of the erythrocyte.

In all, the erythrocyte has proved to be a very rewarding cell with which to carry out dielectrophoretic studies. The groundwork for these studies has been laid and it has been shown that the condition of the erythrocyte is mirrored in its dielectrophoretic response. These initial experiments have been done in in vitro suspension media. Extensions of this work could be channeled to the cells suspended in their own serum, thus eliminating the strange environment in which the cells have to respond. It is highly unlikely that the cells can really respond as normal cells when suspended in an in vitro medium. Some type of continuous flow system in which the cells in their serum are run through a non-uniform field is needed for these studies. Then, either the number of collected cells or the reduced concentration of the serum can serve as a DCR indicator. Such studies and extensions of the dielectrophoresis of the erythrocyte promise to be very rewarding.

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APPENDIX A

SUCCINATE SALTS GROWTH MEDIUM FOR FLAVOBACTERIA

Succinate Salts Solution

0.2% NH_4Cl

0.2% NaCl

0.2% Sodium Succinate

0.32% KH_2PO_4

0.42% K_2HPO_4

Adjust pH to 7.0 With 1.0M KOH

Growth Medium

Dissolve the following in 100 milliliters of distilled water:

$MgSO_4 \cdot 7H_2O$ - 5.0 gm.

$MnSO_4$ ——— 0.1 gm.

$FeCl_3$ ——— 1.0 gm.

$CaCl_2$ ——— 0.5 gm.

Use 0.1 milliliters of succinate salts solution in 100 milliliters of growth medium to prepare the succinate salts growth medium.

APPENDIX B

CALCULATION OF pH FOR THE STANDARD SUSPENSION MEDIUM CONTAINING A 0.001$\bar{M}$ CONCENTRATION OF H_2CO_3

If the acid HA is weak, the conjugate base A^- will be fairly strong, and interaction with the solvent, acting as an acid, will take place to a definite extent. It follows, also, that when the salt of a weak acid and strong base, e.g., NaA, is dissolved in water the A^- ions produced on dissociation will establish an equilibrium with water molecules (3):

$$HA + OH \rightleftharpoons HOH + A^-$$

and

$$H(HCO_3) \rightleftharpoons H^+ + (HCO_3)^-$$

$$Na^+ + (HCO_3) + HOH \rightleftharpoons Na^+ + H_2CO_3 + OH^-$$

is equivalent to

$$Na^+ + A^- + HOH \rightleftharpoons Na^+ + HA + OH^-$$

$$- Na^+ \rightleftharpoons - Na^+$$

$$HA + OH^- \rightleftharpoons HOH + A^-$$

$$K_h^{-1} = \frac{a_{H_2O} \cdot a_{A^-}}{a_{HA} \cdot a_{OH^-}} = \frac{a_{H_2O} \cdot C_{A^-} \cdot F_{A^-}}{C_{HA} \cdot F_{HA} \cdot C_{OH} \cdot C_{OH} \cdot F_{OH^-}}$$

where K_h is the hydrolysis constant

$$fa \cong f_{OH^-} \quad a_{H_2O} = 1; \; f_{HA} = 1$$

$$K_h^{-1} = \frac{C_{A^-}}{C_{HA} \cdot C_{OH^-}}$$

Let X = fraction of total acid in HA form

$$C_{HA} = CX$$

$$C_{OH^-} \cong CX$$

$$C_{A^-} = C(1-x)$$

$$K_h^{-1} = \frac{C(1-x)}{C^2X^2} = \frac{1-X}{CX^2}$$

$$X^2 = \frac{K_h(1-x)}{C} \cong \frac{K_n}{C}$$

$$X = \sqrt{\frac{K_h}{C}}$$

$$K_h^{-1} = \left(\frac{a_{H_2O}}{a_H + a_{OH^-}}\right)\left(\frac{a_H + a_{A^-}}{a_{HA}}\right) = \left(\frac{1}{K_w}\right) K_a$$

$$X = \sqrt{\frac{K_w}{K_a C}}$$

$$C_{OH^-} \cdot C_{H^+} = K_w = \frac{(a_{H^+})(A_{OH^-})}{a_{H_2O}}$$

$$C_{H^+} = \frac{K_w}{C_{OH^-}} \qquad C_{OH^-} = \sqrt{\frac{K_w C}{K_a}}$$

$$C_{H^+} = \frac{K_w\sqrt{K_a}}{\sqrt{K_w C}} = \sqrt{\frac{K_w K_a}{C}}$$

$$K_w = 10^{-14}; \; K_a = 4.31 \times 10^{-7}; \; C = 10^{-3}$$

$$-\log C_{H+} = pH = \tfrac{1}{2} \log C - \tfrac{1}{2} \log K_w - \tfrac{1}{2} \log K_a$$

$$= -\frac{3}{2} + 7 + \frac{6.37}{2}$$

$$\boxed{pH = 8.68}$$

For the other substances

Substance	pK_a
H_2CO_3	6.37
HCN	9.14
ICH_2COO	2.87
HN_3	4.59
HF	7.07

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