Preliminary Observations with an Electromagnetic Method for the Noninvasive Analysis of Cell Suspension Physiology and Induced Pathophysiology

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Abstract—**A technique for the electromagnetic analysis of physiological and patho-physiological states in cell suspensions is presented. The technique is based upon high speed automatic network analysis in the HF band for measurement of complex permittivity. The results demonstrate that changes in HF-band permittivity dispersion may be related to physiological and drug-induced patho-physiological states of the cell membrane. Mechanical disruption of the cell membrane by sonication obliterates the HF-band dispersion of permittivity that is present in undisrupted cells. The effect of species, suspending medium, and temperature were systematically analyzed in erythrocyte suspensions in order to aid comparison between published studies of red cell preparations.**

I. INTRODUCTION

NONINVASIVE analysis of cell membrane physiology and pathophysiology is possible with electromagnetic measurements in the high frequency (HF) band, that is, the spectral range from 3 to 30 MHz, as conventionally described. The biophysical mechanism involved is a dielectric relaxation component in this band due to the cell membrane [1]. The dielectric relaxation is thought to result from two factors: 1) the frequency dependent electromagnetic properties of the cell membrane itself and 2) the different complex permittivities which characterize the intracellular and extracellular spaces [2]. The heterogeneous dielectric nature of cell systems is of utmost importance not only for the relaxation process [3], but also because it heralds a fundamental biological property of cell systems—namely, the creation of specialized electrolyte and molecular environments within the cell.

Dielectric relaxation is a dispersion phenomenon apparent in both homogeneous and heterogeneous dielectrics. It is explicitly a frequency dependent process [4]. Relaxation processes are typically associated with the liquid state in distinction to resonant processes which are typically associated with the gaseous state. The underlying model is that of an electric dipole with spherical shape in dilute solution with dominating friction as the dipole attempts to follow the field. In the classical Debye formulation [5], the relaxation process consists of a downward transition of the dielectric constant which is simultaneous with an upward transition of the conductivity (an expression which, in general, includes both ohmic and nonohmic losses). At the midpoint (i.e., midway between the upper and lower limiting frequencies) of this two state transition, the loss tangent peaks. Energy extraction from the field is maximized at the corresponding frequency. This frequency is known as the relaxation frequency of the process. The lumped circuit equivalent representation of this frequency dependence is a series RC with parallel C.

Heterogeneous dielectrics are characterized by relaxation spectra described the Maxwell–Wagner formulation wherein the contributing complex permittivities are volume weighted [6]. The form of the dielectric constant dispersion is the same as that in the Debye case, but the mechanism is different in that interfacial polarization combines with electronic polarization [4]. The lumped element equivalent circuit is the series combination of parallel RC networks. Interfacial polarization, of course, requires boundaries between the media. It is this fact that brings physiological interpretation to dielectric relaxation spectra according to the function of the cell membrane.

In the 1930's it became apparent that multifrequency analysis of the complex admittance of intact red blood cells suspended in electrolyte solutions could serve an important role in the analysis of membrane function as a diffusion barrier [7]. The selective permeability of cell membranes later became associated with the process of active transport and the locus of induced pathophysiology [8].

The key element in all such studies is the measurement of dispersion (frequency dependence) in the complex permittivity of a cell system in suspension. Information of this type may be presented in many forms. Lumped-element equivalent circuits, complex plane plots of the admittance, complex plane plots of impedance, complex plane plots of the permittivity, and complex plane plots of the scattering parameter(s) are some of the major variations. The locus of points traced out in the complex

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dielectric plane as frequency is varied (the so called Cole–Cole plot) provides useful information concerning the nature and number of relaxation process contributing to a given relaxation spectrum [3].

The technology which supports such studies has important implications for biomedical applications due to the need to study events in physiological time frames, i.e., on the order of seconds to minutes. Analysis time is a function of the range of dispersion to be examined and the available instrumentation. Cell suspension dispersion measurements may extend from audio to microwave frequencies. Methods vary from the impedance bridge with lumped circuit element substitution, to slotted line VSWR measurements and cavity perturbation techniques [9]. One hallmark of these methods is the comparatively slow rate of data acquisition and the relatively limited range of frequencies which could be studied with a single instrument. The result has been a paucity of physiological and pharmacological studies due to the large number of measurements which must be made within the time course of drug action. For example, a typical experiment with membrane active drugs could require frequency analysis over 6 octaves in a period of 10–20 s with as many as 50 discrete frequencies. This rate of data acquisition virtually requires high-speed computer-controlled data acquisition and data analysis. In addition, the measurements require high accuracy. For example, in a system based on admittance measurements, the magnitude and phase of the reflection coefficient must be in the order of 1 part per 1000 and 0.1–0.01°, respectively. The measurements described in the following experiments were based on error corrected amplitude and phase measurements accurate to about 3 parts per thousand and 0.25°, respectively. Accuracy of this order is necessary to detect transmembrane movements of electrolytes and water in the order of one millimole.

The physiological interpretation of cell membrane relaxation in the two decades of 1–100 MHz is based on the heterogeneous nature of the "bulk" dielectric and the capacitance of the cell membrane [10], [11]. At the low frequency limiting value, the reactance of the membrane is sufficient to attenuate current induction in the intracellular space; but at the upper frequency limiting value, the reactance is small compared to the membrane resistance. Under these conditions, appreciable currents are induced in the intracellular space. Since the intracellular space in a normal cell (variations do exist for various specialized tissues) has a different complex permittivity than that of the extracellular space, the dispersion in conductivity represents the ion permeability barrier functions of the cell membrane. If the physical integrity of the cell membrane is disrupted, the media of the intracellular space are mixed wih those of the extracellular space, and the dispersion is reduced or, in the limiting case, removed.

The location in frequency for the cell membrane dispersion is dependent on the tissue type and its physiological or pathological state. The generally recognized location for dielectric relaxation of red cell suspensions in isotonic electrolyte media is in the 1–100-MHz band. The so called β dispersion of red cell conductivity is over by 100 MHz [12]. That is, data has been presented to demonstrate an upper frequency limiting value of approximately 100 MHz [8, Fig. 15(a)]. Osswald published remarkably similar data in 1937 [13]. Schwan states that the relaxation frequency of blood is near 3 MHz [14]. However, it is prudent to recall the studies of Stuchly *et al.* [15] where simple homogeneous short-chain alcohols were studied for repeatibility of relaxation frequency determinations across various authors. For example, the range of relaxation frequencies for butylalcohol was 277 MHz–482 MHz, that for propylalcohol was 413 MHz–722 MHz, and that for alkylalcohol was 95 MHz–135 MHz. Thus, even in this comparatively simple situation, estimates of relaxation frequency vary by approximately two to one in competent studies. These authors note that a small error in the determination of the upper limiting frequency greatly affects estimates of the relaxation frequency.

II. Methods

The rabbit blood used for analysis was obtained from albino and Dutch specimens by cardiac puncture. The blood quantity taken from a single rabbit was between 20 and 30 cc. The blood was drawn into a heparinized syringe and stored in a refrigerator for periods typically between 30 min and 2 h before further preparation. Refrigeration is necessary to prevent reduction of energy stores in the cells.

Sheep blood used for analysis was obtained from mixed breed specimens. Approximately 250 cc of peripheral blood is collected into an equal volume of Alsever's solution. The blood was stored for periods between 1 and 3 days prior to preparation for the experiment.

The blood was centrifuged at 2000 rpm for 10 min in order to remove the cellular components from the serum. The serum and most of the buffy coat were removed by aspiration. The cells were then washed in the final suspending medium with three to five times volume for three washings. Final suspensions were made with hematocrits between 32 and 35 percent. The blood was returned to the refrigerator for use the next morning.

Canine peritoneal fibroplasts were obtained by peritoneal lavage. The cells were cultured and divided for seven generations (the most generations consistent with eucaryotic states). The cells were harvested with trypsin, then washed 3 times in 5 times volume of isotonic NaCl. Cell viability was assessed by typan blue exclusion. Percentage cell volume was 34 percent.

The suspending media were all potassium free, and began with isotonic saline and 10 units of heparine (10-ul) per ml. pH experiments were performed in these media plus MOPS-TRIS buffer stabilized for pH of 6.5, 7.0, and 7.5.

Those experiments performed at 37°C were thermostatically controlled (regulated to 0.1°C) by water circu-

lated around the chamber containing the specimen. Approximately 0.8 cc of suspension was used for each experiment.

The pharmacologic agents used were oubain (Calbiochem), valinomycin (Sigma), and gramicidin (Calbiochem) and collagenase (Calbiochem). The oubain was prepared by dissolving 5 mg in 5 ml of deionized water. A 100-μl sample of the stock solution was added to a 700-μl sample of blood. The final oubain concentration was approximately 10^{-4} molar (a value where the activity reaches a maximum). The collagenase was prepared by dissolving 5 mg in 10 cc of isotonic saline.

The oubain was incubated with the rabbit erythrocytes in saline suspension at 37°C for a period of 75 min (oubain is a fast-acting cardiac glycoside which reaches 80 percent of its full effect within 60 min). The sample and test set were shaken every 5 min throughout the incubation period. Electromagnetic measurements were taken every 15 min after every third shaking.

Due to the osmotic shock of a 100-μl dose of oubain stock solution, a control was performed. This consisted of an identical incubation with 100 μl of deionized water.

An additional control for hemolysis due to the shaking was performed. This consisted of 0.8 cc of cell suspension without any additional reagents taken through the same shaking and measurement regimine.

The valinomycin was prepared by dissolving 5 mg in 10 ml of ethyl alcohol. Reagent activity was verified in a parallel series of erythrocytes by fluorescent measurements according to the methods of Hoffman and Laris [16]. 10 μl of this stock solution was added a sample of blood still in the chamber from the control dispersion analysis. Two minutes of incubation at 37°C were allowed with shaking just prior to measurement (according to fluorescent measurements, the valinomycin effect is complete within 90 s). Final concentration of the valinomycin was calculated to be 3×10^{-5} molar, but the exact value is uncertain due to the fact that some of the chamber contents was lost when the cap was replaced and the volume topped off with blood. All ionophore experiments were of the before-and-after type in order to minimize variability due to chamber filling. The valinomycin was spectrographically monitored for degradation after several weeks (the stock solution was stored in the dark).

The gramicidin was prepared by dissolving 1 mg in 10 cc of ethyl alcohol. 20 μl of the stock solution was added to a previously analyzed sample of blood in the same manner as for the valinomycin.

Controls for the ethyl alcohol and water solvent were performed. 10 or 20 μl of solvent with no additional reagent was introduced and incubated in the same manner as for the ionophores and collagenase.

The ionophoretic activity of the valinomycin and gramicidin was further analyzed by challenging erythrocyte suspensions with and without the ionophore for response to a 30-μl dose of 3M KCl. The procedure was to measure a blood sample, introduce the ionophore, measure the sample again, then to introduce the KCl followed by the last measurement. Experiment was then repeated with a new blood sample without the step for ionophore addition. In all cases, 2 min for incubation and shaking was allowed after any reagent was introduced prior to measurement.

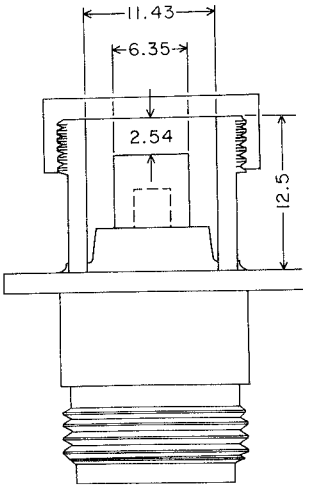


Fig. 1. The coaxial capacitor/test chamber is illustrated in a mechanical drawing with all dimensions in mm. The lower portion is a Type-*N* bulkhead connector. The sample volume is approximately 0.8 cc.

pH measurements were performed in a rapid series following both ascending and descending orders. New blood samples were used for each measurement and replications were employed to define the impact of system instability and interface equilibration. All data presented for the pH experiments were done in rapid succession with results tabulated for minimal effect, i.e., opposite to system instability and interface equilibration. Thus the results presented are diminished by these factors. System instability was diminished with use of a phase locked measurement system. The pH experiments strain the resolution of the instrumentation and stability considerations were much more important for these experiments. Also, pH affects on the electrode interface were examined with the buffer solutions for controls. Control (i.e., buffer) data were collected in the absence of cells.

Cell membrane disruption was accomplished by sonication at 70 W for 1 min with 20-kHz sound. A 40-percent duty cycle was used in order to prevent heating.

Electromagnetic analysis was performed by the technique of automatic network analysis for one port networks [17]. The instrument employed was the Hewlett–Packard model 8507B both with and without the phase locked source option. The measurements were corrected for frequency dependent components proximal and distal to the test set by reference to a set of network standards. These corrections were applied by means of a modified version of HP program AIM (accuracy improvement method). All measurements included a dc block at the test port.

The HP 8507B measured the complex reflection coefficient of a capacitive termination on port number one of

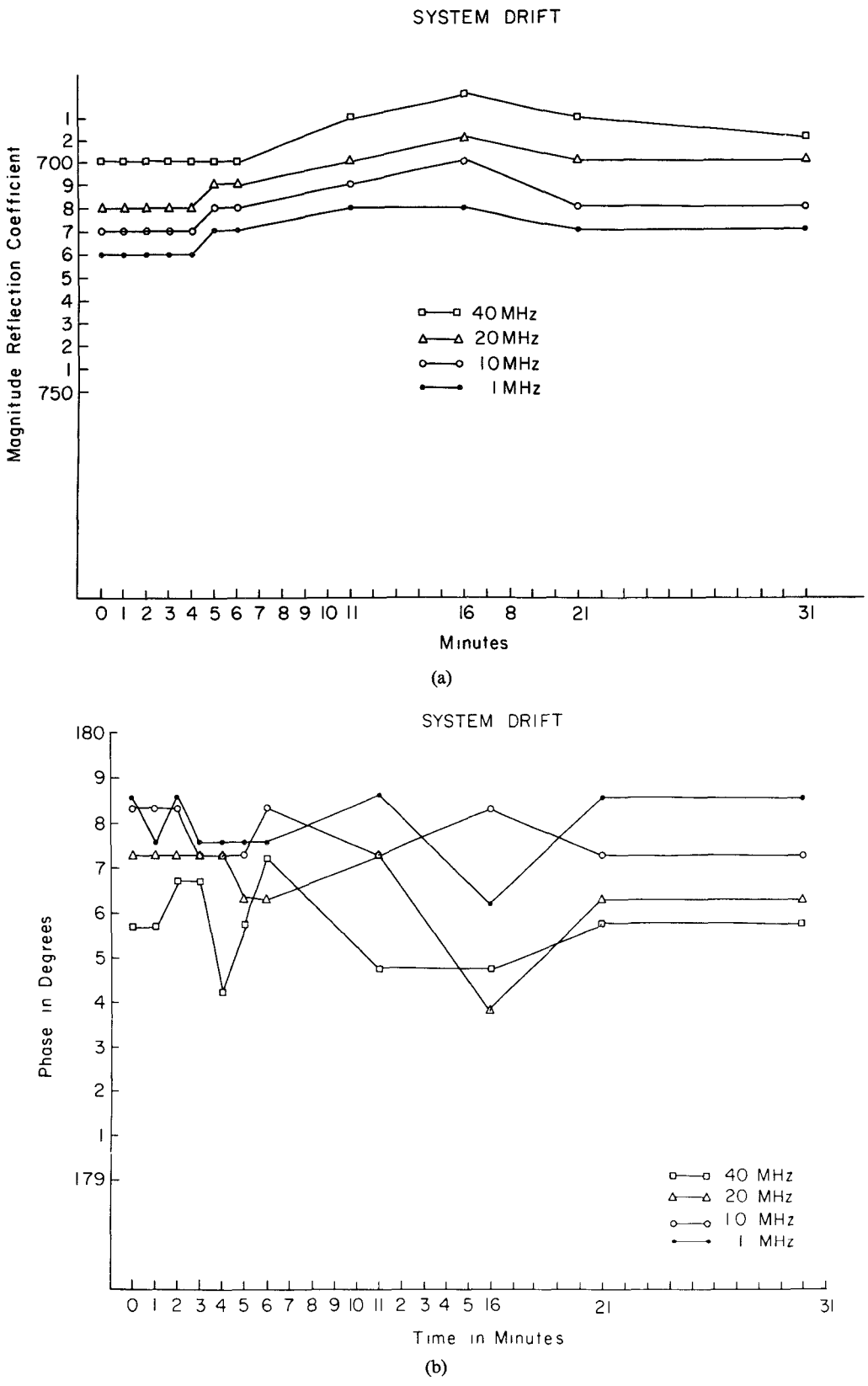
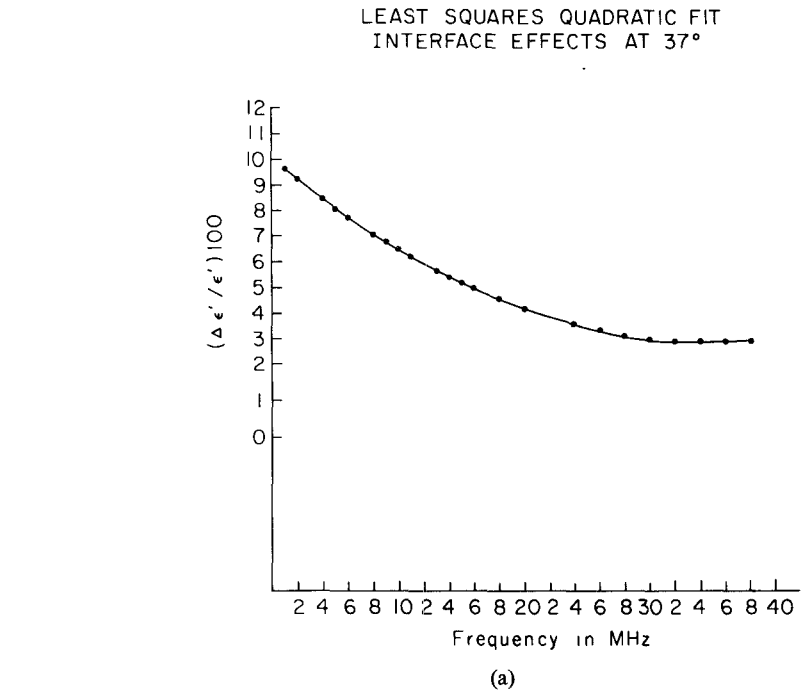
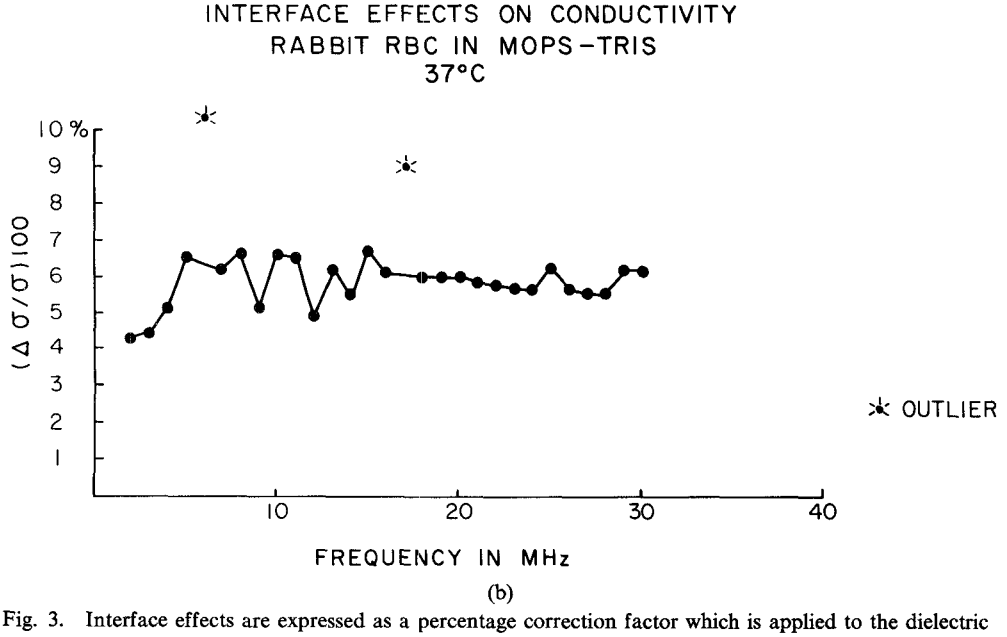


Fig. 2. The system drift is expressed as temporal variation in the magnitude (a) and phase (b) of reflection coefficient at 4 frequencies with isotonic saline in the test chamber.

the test set. The termination consisted of a coaxial chamber fabricated from a male type-*N* bulkhead connector as shown in Fig. 1. The space between the center conductor and the shell-cap formed a capacitor into which the blood samples and dielectric standards were introduced by means of a removable cap which was shorted to the shell. The chamber was calibrated for capacitance according to the methods of Jacobi by measurement of the reflection



(a)



(b)

Fig. 3. Interface effects are expressed as a percentage correction factor which is applied to the dielectric constant (a) and conductivity (b) obtained with bright copper electrodes to obtain the complex permittivity that would be obtained with platinum/platinum black electrodes.

coefficient at each frequency for air and deionized water [18]. Measurements at 40 discrete frequencies were accomplished in 15 s in 1-MHz steps over the range 1 – 40 MHz.

At frequencies in the lower range of the 8507B, the phase angles were obtained by analytic continuation and interpolation. This procedure was applied in such a way that very small corrections were obtained at frequencies above 4 or 5 MHz. At 3 MHz and below, the corrections were significant in that they amounted to as much as 0.8 degree. The corrected reflection coefficient data was processed according to the methods of Jacobi to yield the relative dielectric constant and conductivity at each measured frequency.

Calibration steps also included the use of platinum/platinum black electrode coating to evaluate polarization effects at the electrode-electrolyte interface [19]. Other surface treatments tested were polished copper and bright platinum. Bright platinum electrodes were produced by platinum plating (approximately 100 μin) over the copper with an intermediate layer of W nickel (approximately 100 – 200 μin) over the original copper surface. Platinum black was produced by the method of Kohlrausch. That is, 2-percent lead acetate was added to the platinum salt solution. The thickness of the platinum black over the platinum plate was determined by the tables of Jones and Bollinger [20] (approximately 50 – 200 C). The underlying

TABLE I
INTERFACE AND SUSPENDING MEDIA EFFECTS ON CONDUCTIVITY

FREQUENCY IN MHz	SALINE, COPPER 26 °C	SALINE, PtB 26 °C	MOPS-TRIS, PtB 26 °C
1	1.36	1.67	1.59
2	1.49	1.68	1.76
3	1.55	1.73	1.82
4	1.55	1.73	1.81
5	1.57	1.75	1.83
6	1.55	1.73	1.81
7	1.56	1.75	1.83
8	1.54	1.72	1.80
9	1.55	1.74	1.81
10	1.55	1.74	1.82
11	1.55	1.74	1.82
12	1.55	1.74	1.82
13	1.54	1.73	1.82
14	1.55	1.75	1.83
15	1.56	1.75	1.84
16	1.56	1.75	1.84
17	1.56	1.76	1.84
18	1.56	1.76	1.84
19	1.57	1.76	1.85
20	1.56	1.75	1.85
21	1.56	1.76	1.84
22	1.56	1.75	1.84
23	1.56	1.76	1.84
24	1.56	1.75	1 84
25	1.57	1.77	1.86
26	1.57	1.76	1.84
27	1.57	1.77	1.86
28	1.58	1.77	1.87
29	1.58	1.77	1.86
30	1.57	1.78	1.85
31	1.56	1.76	1.85
32	1.57	1.77	1.85
33	1.57	1.78	1.86
34	1.58	1.77	1.85
35	1.58	1.78	1.86
36	1.57	1.77	1.85
37	1.57	1.79	1.87
38	1.57	1.78	1.87
39	1.58	1.79	1.87
40	1.58	1.79	1.88

platinum plating was roughened by etching in order to maximize surface area for greatest polarization capacitance and minimal polarization resistance as recommended by Schwan [21]. All plating solutions were obtained from the Electrometalics Division of Englehard Industries in Union, N J.

The electrode interface was characterized by an equivalent polarization impedance in series with the sample impedance. That is, identical ionic conditions including cells, buffer, temperature, and species were measured in both bright copper and platinum black chambers to establish a set of equivalences. Correction factors were thereby generated to accommodate use of the polarizable system for selected later experiments.

Additional data were collected at both 26°C and 37°C with both rabbit and sheep blood in both saline and MOPS-TRIS suspensions in the platinum/platinum black chamber in order to assist comparability to other studies. Other workers vary in the use of buffer, pH, hemotocrit, temperature, and species which makes comparison difficult, even for a comparatively "simple" tissue such as blood.

III. RESULTS

A study of the measurement system's stability is presented in Figs. 2(a) and 2(b) wherefrom it was estimated that phase measurements are accurate to about 0.25° and amplitude measurements are accurate to about 3 parts per 1000. Note that data are presented for the two extreme frequencies used in the measurements as well as for two intermediate frequencies.

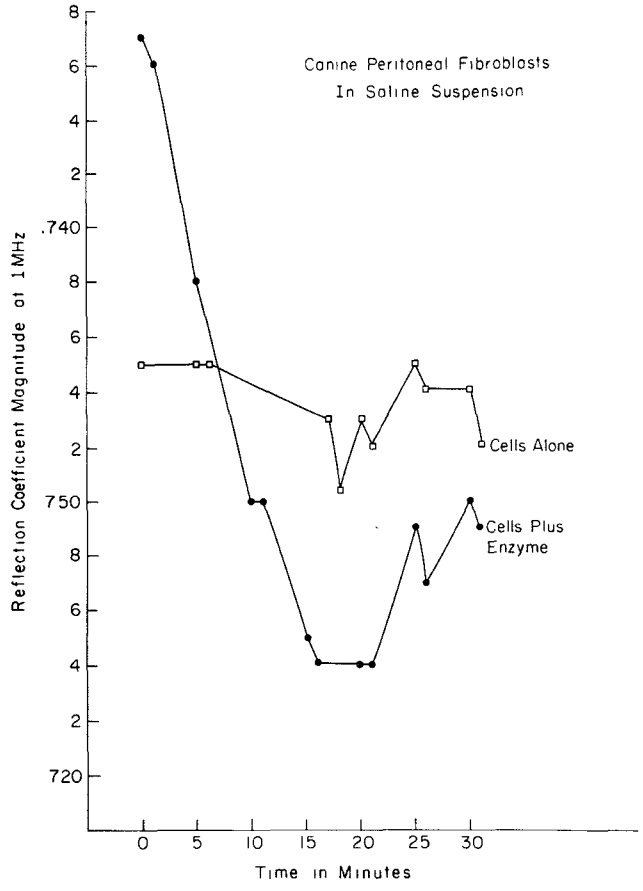


Fig. 4. The magnitude of the complex reflection coefficient for a saline suspension of cultured canine peritoneal (34-percent cell volume fraction) is displayed as a function time since the origin with and without incubation with collagenase.

TABLE II
RANGE OF PERMITIVITY CONDITIONS IN ERYTHROCYTE SUSPENSION

FREQUENCY IN MHz	RABBIT RBC IN MOPS-TRIS at 37 °C		SHEEP RBC IN SALINE at 26 °C	
	σ	ε	σ	ε
1	0.81	2471	0.83	1756
2	1.03	1601	0.95	1062
3	1.19	1394	1.01	788.2
4	1.24	1033	1.03	592 9
5	1.31	915.0	1.07	507.5
6	1.39	842.4	1.07	412.6
7	1.37	701 0	1.10	372.1
8	1.40	615.3	1.10	314.3
9	1.43	578.4	1.11	289.3
10	1.45	529.1	1.13	263.9
11	1.48	490.1	1.14	236 2
12	1.50	456.3	1.15	216.5
13	1.53	417.6	1 15	200.2
14	1.53	384.9	1.15	185.5
15	1.57	379.8	1.17	189 9
16	1.57	353.2	1 17	175.9
17	1.57	336.2	1.17	167.4
18	1.59	311.7	1.18	158.8
19	1.60	310.3	1 18	162.0
20	1.60	289.2	1 18	155.7
21	1.61	283.9	1.19	154 5
22	1.62	277.5	1.19	142.2
23	1.64	259 7	1 19	137.9
24	1.63	247.2	1.19	135.7
25	1.66	251.2	1.20	143.0
26	1.65	245.2	1.20	134.0
27	1.67	233.1	1.20	132 8
28	1.66	228.1	1.21	132 0
29	1.64	222.3	1.21	131.2
30	1.68	207.8	1.21	129.1
31	1.68	211.9	1.21	129 2
32	1.68	203.8	1.21	124 4
33	1.70	212.9	1.22	127.9
34	1.69	203.8	1.22	122.9
35	1.70	203.2	1.22	124.8
36	1.70	192.5	1.22	120 6
37	1.70	192.2	1.23	121.4
38	1.72	183 6	1.22	116.4
39	1.72	182.6	1.23	117.1
40	1.73	188.6	1.23	118.8

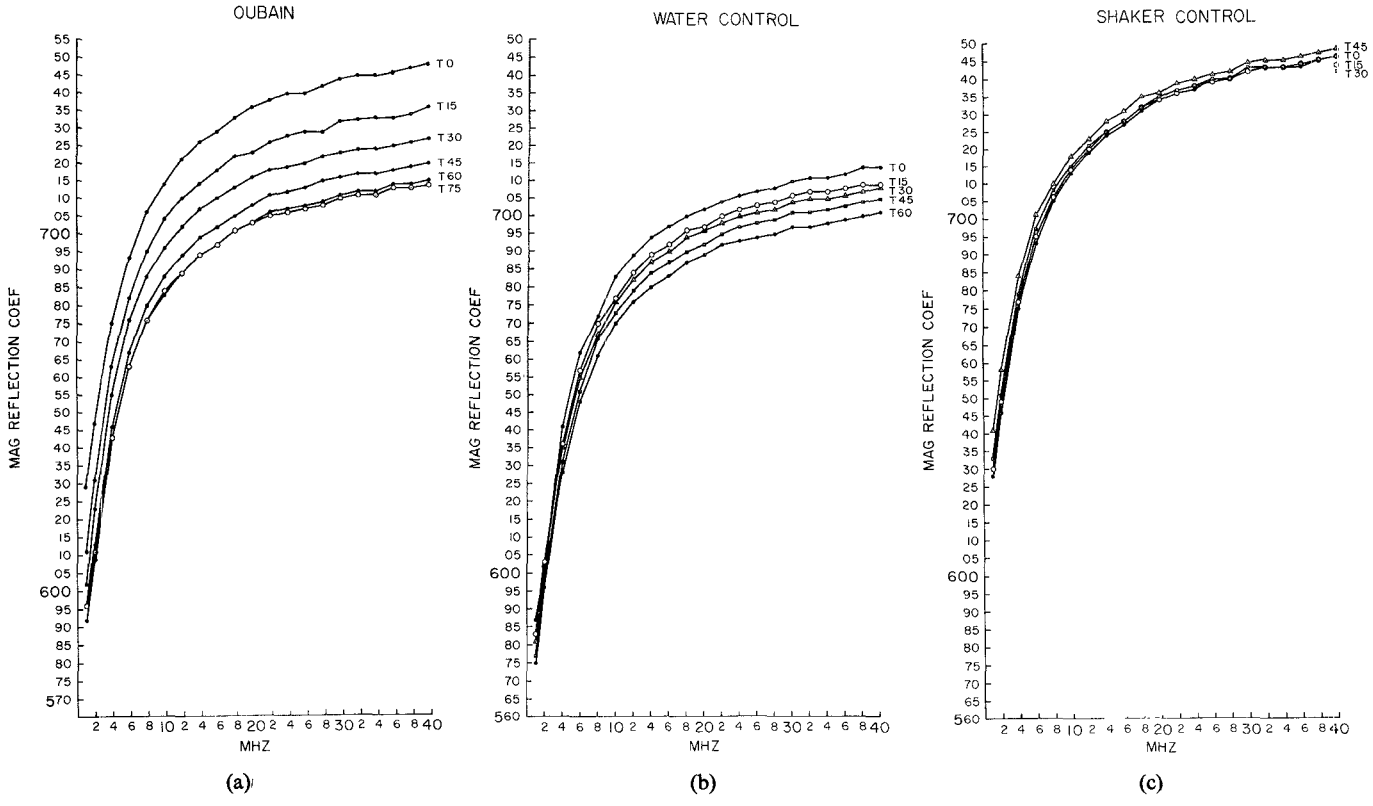


Fig. 5. The dispersion of the magnitude of the reflection coefficient for a rabbit erythrocytes in saline suspension (34-percent Hct) is expressed as a function of time in minutes since the origin with oubain incubation (a), with water incubation of an equal volume (b), and with no treatment except periodic shaking as above (c).

Electrode interface effects are demonstrated in Table I for the simple and reproducible situation of saline and buffer conductivity at 26°C with platinum/platinum black and bright copper surfaces. An upward trend in these measured values with increasing frequency is apparent. The effect of a polarizable interface is seen to cause a downward bias in the measured values. The MOPS-TRIS buffer also affects the interface and/or conductivity inasmuch as the conductivities are uniformly higher in buffered than in nonbuffered isotonic saline when measured in the platinum black chamber.

Correction factors which relate measured complex permittivity with the platinum black to measured complex permittivity with the bright copper surface are shown in Figs. 3(a) and 3(b). The use of a copper interface results in a downward bias in estimates of both dielectric constant and conductivity when compared to the "nonpolarizable" platinum black interface. Such differences were consistent across suspending media, temperature, and species.

The effects due to cell preparation are tabulated in Table II for the two extreme circumstances of rabbit erythrocytes in MOPS-TRIS at 37°C and sheep erythrocytes at 26°C in saline suspension. Various pair-wise comparisons (expressed as the average-over-frequency for percent change at each frequency) are displayed in Table III for media, temperature, and species. All measurements reported in the Tables were collected with the platinum black surface treatment. Clearly, the two types of cell preparation differ substantially in absolute values of permittivity, but the relaxation spectra have very similar shapes.

TABLE III

SUSPENDING MEDIA DIFFERENCES WITH RABBIT	
37 °C	26 °C
Saline is 8.2% lower than MOPS-TRIS	Saline is <1% lower than MOPS-TRIS
TEMPERATURE DIFFERENCES	
Rabbit in MOPS-TRIS	Sheep in Saline
26 °C is 17.6% lower than 37 °C	26 °C is 19.3% lower than 37 °C
SPECIES DIFFERENCES IN SALINE	
37 °C	26 °C
Sheep is 24.8% lower than rabbit	Sheep is 26.6% lower than rabbit

The canine peritoneal fibroblasts in saline suspension are shown in Fig. 4 wherein control and collagenase treated cells are compared by the time course of change in the magnitude of the complex reflection coefficient (rho) at 1 MHz. The time origin corresponds to introduction of the cells into the chamber. It is notable that at all

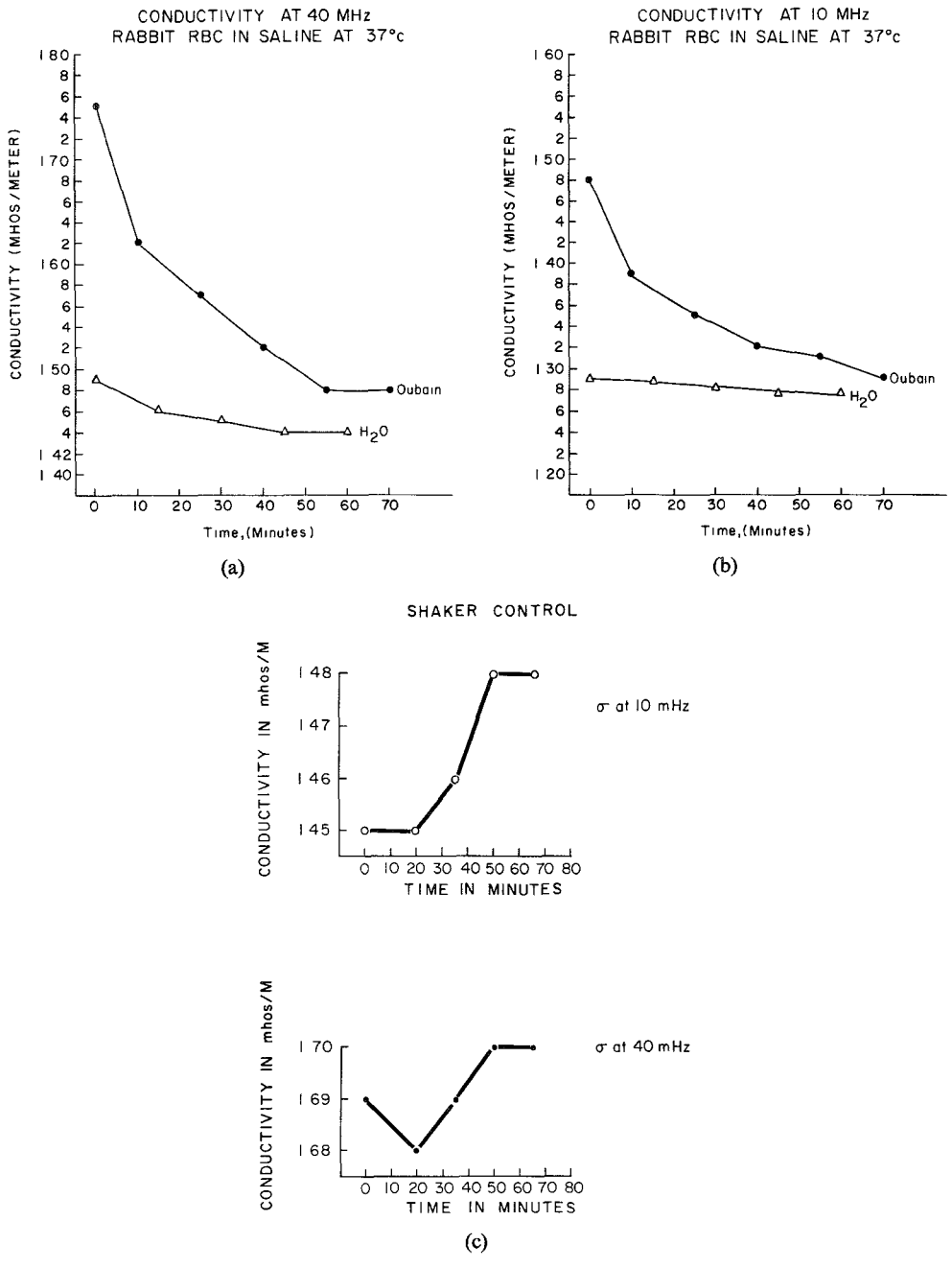


Fig. 6. The time course of conductivity at 40 MHz (a) and 10 MHz (b) is displayed for the oubain and water incubated rabbit erythrocytes. The time course of conductivity for the shaker control at 40 and 10 MHz is displayed in (c).

frequencies the treated and untreated cells differed in the time course of rho, but the greatest differences were observed at 1 MHz. At the end of the time course, approximately 10 percent of the treated cells no longer excluded dye. In the untreated group, approximately 1 percent failed to exclude dye.

The results of the oubain experiment are shown in Figs. 5(a),(b), and (c) for the complex reflection coefficient magnitude and in Figs. 6(a),(b), and (c) for conductivity at the two selected frequencies of 10 MHz and 40 MHz. Fig. 5(a) represents the time course of changes in the dispersion of rho for a saline suspension of rabbit erythrocytes during incubation with oubain. Fig. 5(b) represents rho as a function of time for the osmotic control, whereas 5(c) presents rho as a function of time with only periodic shaking of the chamber. Likewise, Fig. 6(a) is the time course of changes in conductivity with oubain incubation; Fig. 6(b) is the time course with water incubation; and Fig. 6(c) is the time course for cell shaking. Conductivity values are presented at the same location in time as for the reflection coefficient data, but only two frequencies are displayed because the greatest differences between treated and control samples were at high frequencies with effects substantially decreasing below 10

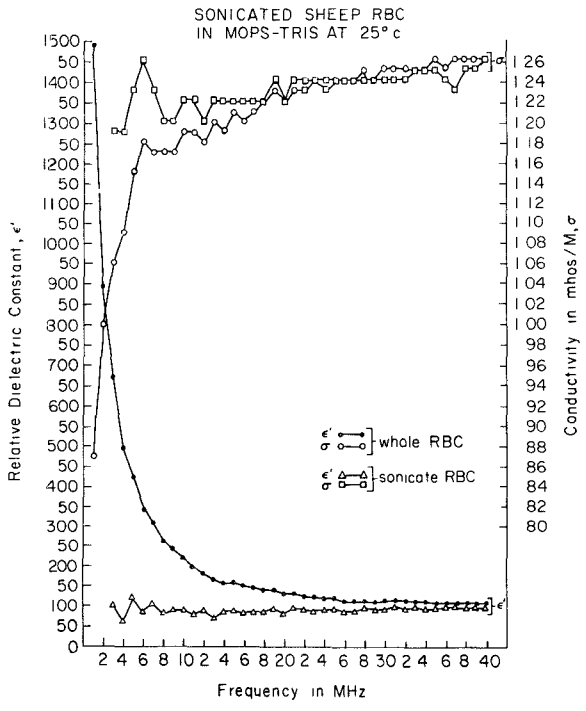


Fig. 7. The effect of cell disruption by sonication on the dispersion of complex permittivity is displayed for a MOPS-TRIS buffered (pH 7.0) suspension of sheep erythrocytes (Hct 35 percent).

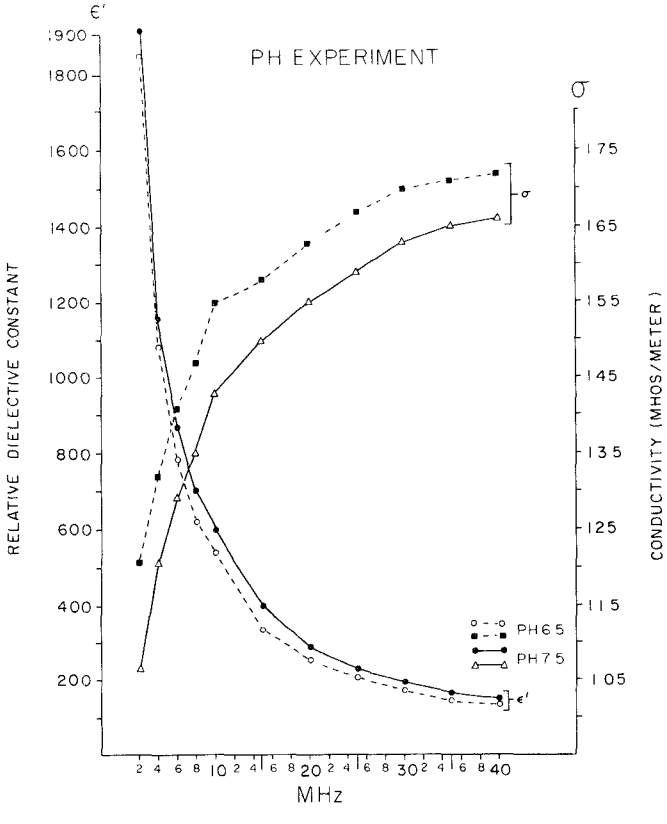


Fig. 8. The effect of pH on the complex permittivity is displayed for a rabbit erythrocyte suspension (34-percent Hct) in isotonic saline buffered with MOPS-TRIS at pH 6.5 and pH 7.5.

MHz. Either set of data indicates that the osmotic control moves the permittivity in the same direction as oubain treatment, and that the effect of shaking is in the opposite

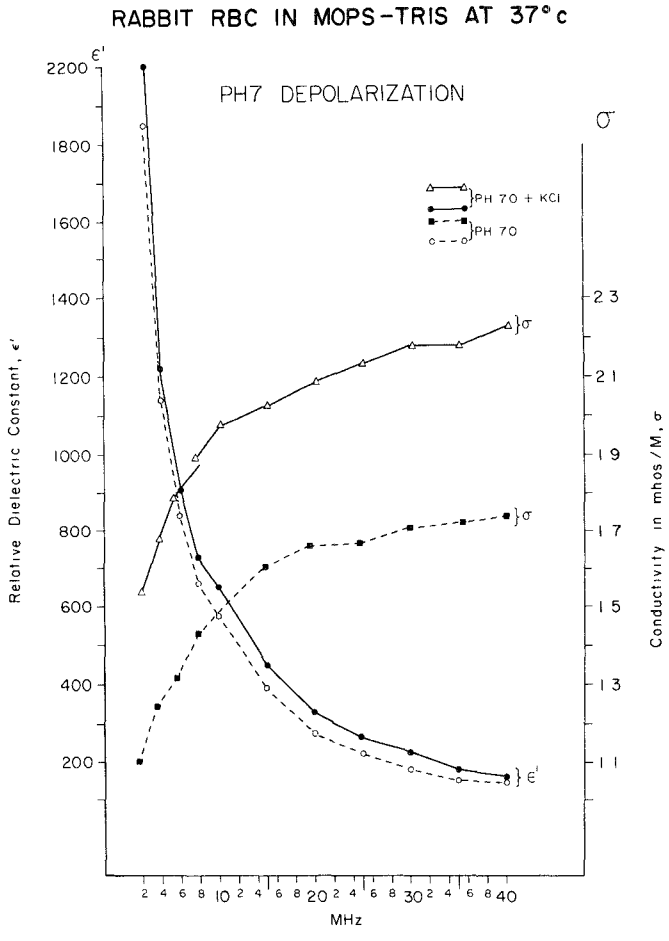


Fig. 9. A MOPS-TRIS buffered rabbit erythrocyte suspension depolarized with extracellular dose of KCl expressed as an effect on the dispersion of complex permittivity.

direction. However, the water and oubain treatments differ in their time course and final values.

The data in Fig. 7 demonstrate a classical dielectric relaxation in the dispersion of complex permittivity for a buffered suspension of intact sheep erythrocytes. This is manifest by decreasing dielectric constant and increasing conductivity as the interrogating frequency is increased from 1 MHz to 40 MHz. The sonciated sample is markedly different with relatively frequency independent values of dielectric constant and conductivity. At the lower frequency, the sonicated and intact cells depart most markedly. The intact cell suspension demonstrates progressively lower conductivity and a progressively higher dielectric constant in comparison to the sonicated cells as the interrogating frequency approaches 1 MHz. At the upper limiting frequency of 40 MHz the two treatments have differences in conductivity and dielectric constant which are minimal. Dispersion of permittivity in the sonicated cells, while greatly diminished in comparison to the intact cell suspension, is not reduced to that observed with the buffered saline alone. Neither do the conductivity values of the sonicated sample approach that of the buffer.

The results of the pH experiments are shown in Fig. 8. The three pH's are distinguishable in that increasing the

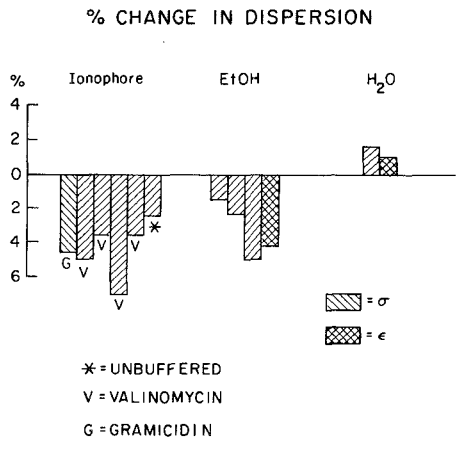


Fig. 10. The effects of ionophore alone and ionophore solvents alone on the dispersion of complex permittivity in a rabbit erythrocyte suspension with and wihout buffers.

pH from 6.5 to 7.5 decreases conductivity at all frequencies with the greatest effect at 1 MHz. Conversely, higher pH increases dielectric constant, again with the greatest effect at low frequency. The results with pH 7.0 are intermediate between those of pH 6.5 and 7.5. pH does alter the interface, but the direction of its effect is opposite in the buffer series alone than in the buffered cell suspensions.

Depolarization of the erythrocyte suspension with extracellular KCl has a marked effect on the dispersion of complex permittivity as shown in Fig. 9. The action on conductivity is, of course, to elevate greatly the values at all frequencies. There is also a small upward shift in the dielectric constant at all frequencies, and a change in dispersion which is to be presented in the following paragraphs.

The results of the ionophore experiments are presented in Figs. 10 and 11(a), (b), (c), and (d). The ionophore alone has little effect except at the lowest frequencies. The direction of this effect is to increase conductivity differentially at the frequencies thereby decreasing dispersion as shown in Fig. 10. In addition, Fig. 10 illustrates that most of the effect due to the ethylalcohol solvent alone is on conductivity unlike the effect of an equal volume (10 μl) of water solvent which increases the dielectric constant rather than the conductivity. There is little evidence to suggest that gramicidin is different than valinomycin in that the direction and magnitude of its effect is similar to valinomycin.

When the cell suspension is challenged with an extracellular KCl dose before and after treatment with ionophore, it is apparent that the control and treatment groups respond differently. Specifically, Figs. 11(a) and (b) illustrate that the KCl challenge increased the dispersion in conductivity in the untreated erythrocytes. On the other hand, pretreatment with valinomycin in one case reserved the effect, and in the other case, greatly diminished the effect. In other words, pretreatment with ionophore diminished or prevented the increase in conductivity dispersion produced with extracellular KCl challenge.

IV. Discussion: Instrumentation

A statement of absolute system accuracy is a complicated matter due to the interplay between interfacial effects, instrumental limitations, and the range of permittivities to be studied. Well-documented difficulties exist in conductive media wherein the imaginary part of the permittivity greatly exceeds (i.e., by 4 orders of magnitude) the real part. Since the conductivity is markedly temperature sensitive, this problem is further compounded by the need for microdegree temperature control to prevent temperature-dependent conductivity effects similating 1-percent changes in dielectric constant [22]. Since with this system it is not possible to measure phase with millidegree accuracy, nor is it possible to obtain microdegree temperature control, the method must be limited to samples wherein ϵ' and ϵ'' are within two orders of magnitude. This is no problem with biological samples containing cells, but the analysis of electrolytes with concentration above 2×10^{-3} M in the absence of cells must be limited to conductivity since the dielectric constant is not reliably obtainable. Note, for example, that the estimated dielectric constant in the hemolysed red blood cell sample (Fig. 7) is close to the expected value of 78 with little dispersion; however, the values are consistently about 10 percent high. In any event, accuracy in the lower frequency range decreases for frequencies below 4 or 5 MHz, with greatest errors at 1 MHz.

The role of interfacial effects deserves additional discussion. At the frequency ranges we have studied, the error introduced by polarizable electrodes (Fig. 3) is in the order of 6 percent for conductivity and a maximum of 10 percent for dielectric constant at the lower limiting frequency of 1 MHz. The effect of a polarizable electrode–electrolyte interface is to insert a polarization impedance in series with the sample impedance [21]. As a result, the effect is reproducible for a given set of ionic conditions and suitable for a frequency dependent correction factor.

The reason for correction rather than use of the platinum black surface treatment throughout was the difficulty in obtaining reproducible platinum/platinum black coatings, the short time such coating remained stable and most importantly the very lengthy equilibration times required for this surface. Furthermore, the platinum/platinum black system is markedly pH dependent [23]. As a result, pH variable runs required separate controls which further depreciated the value of this surface treatment as compared to the extra calibration step.

The present preliminary studies have limitations which must be understood and circumvented before the method may be more widely applied. The most significant of these limitations are a consequence of the range of operating frequencies. In the present case, the low frequency limit is determined by inaccuracies and irreproducibility in the HP 8507B when working in the lower frequency range of 500 kHz to 4 or 5 MHz. These problems are compounded

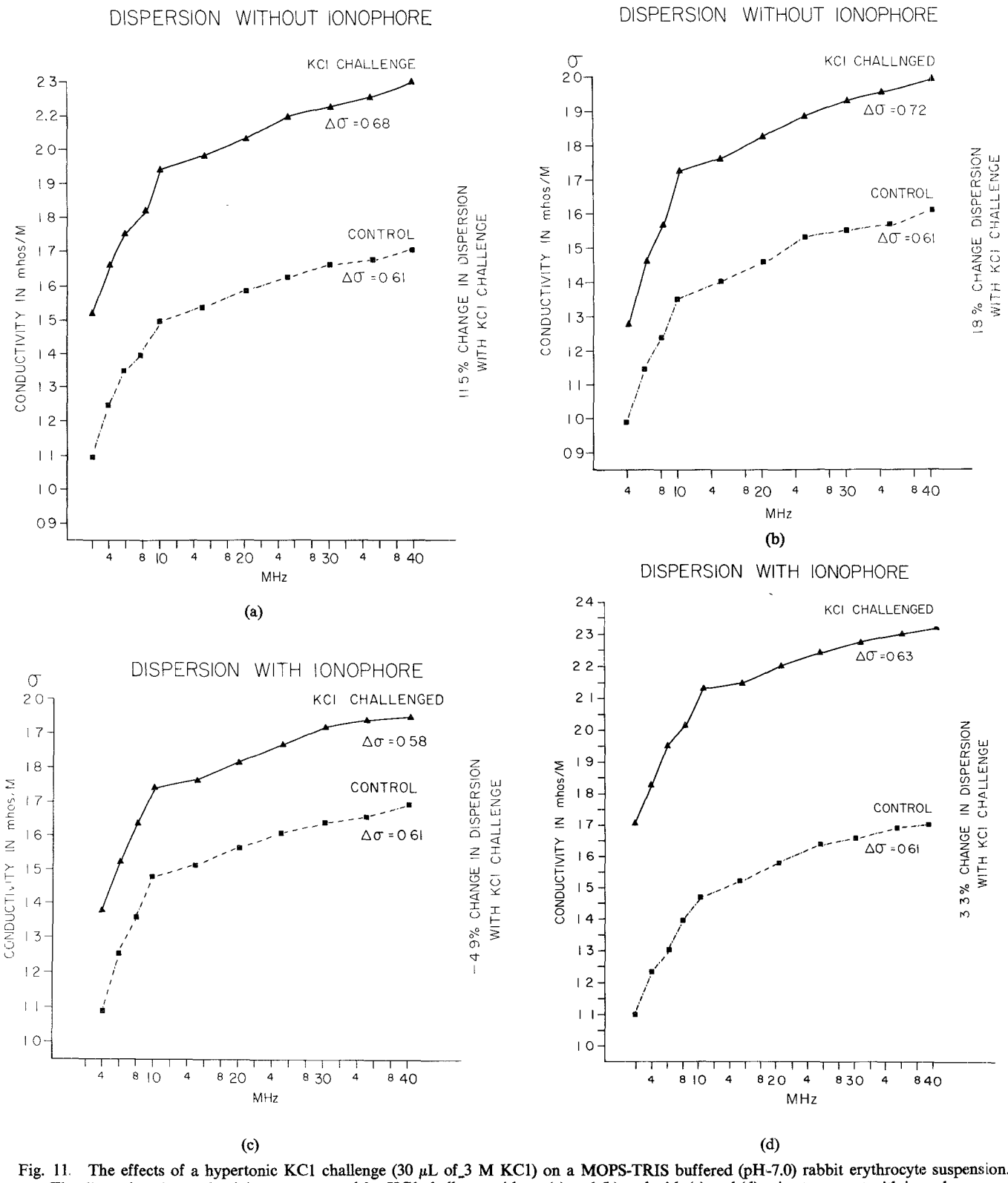


Fig. 11. The effects of a hypertonic KCl challenge (30 μL of 3 M KCl) on a MOPS-TRIS buffered (pH-7.0) rabbit erythrocyte suspension. The dispersions in conductivity are presented for KCl challenge without (a) and (b) and with (c) and (d) prior treatment with ionophore.

by the very high dielectric constant of blood at these frequencies. In other words, the inherent lower range inaccuracy is multiplied by the high ϵ' of biological samples. In addition, electrode polarization becomes more troublesome as the frequency is reduced below 1 MHz, even with platinum/platinum black surface treatment. The combination of these features for measurements below the HF band indicates that high speed, automatic network analysis methods must be adapted to 4 terminal designs. Likewise, a different network analyzer will be required to operate at frequencies significantly below 1 MHz. In the HF band, however, the instrumental inac-

curacies in phase measurement below 4 MHz greatly exceed the effects due to interfaces. The accuracy of phase measurements further deteriorates as the phase angle approaches 180° at low frequencies.

Lower frequency measurements are especially important in order to determine the low frequency limits of the HF band dispersion. This will permit the estimation of relaxation frequency which we cannot do with the present system. As a result, we cannot comment on previous estimates of the relaxation frequency. We can only comment on the upper limiting frequency.

At the high frequency limit, the present system is confined to frequencies below 200 MHz for high ϵ' materials such as biological samples due to the analytical model and the design of the capacitance chamber. The method has been used with low ϵ' organic solvents at frequencies up to L band.

The major advantages of the present system are very high speed measurements, relative simplicity of operation, and the ease with which error correction for components, interfaces, etc. can be made. These advantages speak most loudly in physiological, pathophysiological, and pharmacological applications where high-speed noninvasive analysis is necessary.

V. Discussion: Biological Results

The decrease of dispersion with sonication of the cell suspension (Fig. 7) is inconsistent with previous studies of osmotically hemolysed blood [7],[24]. Previous studies state that chemical destruction of the membrane ghosts is necessary before the dispersion is materially changed except at audio frequencies. The present experiment differs from these earlier reports in the method of hemolysis. The prior studies used osmotic hemolysis with the addition of as much as 7 times volume of distilled water. As a result, the conductivities are substantially decreased and the ionic concentrations are markedly diluted. In addition, there are grounds to question the degree of membrane fragmentation with osmotic hemolysis. Since the agent is hydrostatic force, a single tear in the cell membrane would be sufficient to release hemoglobin, but the ghosts or vesicles may reseal into smaller effective volumes which commence to concentrate potassium and extrude sodium [25],[26]. The greater membrane fragmentation with sonication would effectively diminish any such volume containment. Also it is important to measure the dispersion immediately after sonication since vesicles seem to form within a few hours after sonication.

The incomplete loss of dispersion for the sonicated cells in comparison with the buffer and failure of convergence of conductivity values in the sonicated sample and buffer may well represent the resistivity of the cell membrane fragments or vesicles which still exist in the sonicated sample. The conductivity as a function of frequency demonstrates a small consistent upward trend with frequency. This may represent the Debye–Falkenhagen effect [27], but the combination of wavelength and concentration is not easily related to such studies in the physical chemistry literature of aqueous dielectrics.

Sonication, of course, mechanically destroys the cell membrane. The result is that the low-frequency conductivity of the sonicated sample is greatly elevated in comparison to that obtained with intact cells (Fig. 7). The case with intact cells represents lower conductivity at low frequencies because the reactance of the cell membrane prevents current induction in the intracellular space. The cells, therefore, represent regions of lower conductivity which are in a sense volume averaged with the region of high conductivity (the extracellular space) to reduce the "bulk" conductivity at low frequencies. As the frequency of analysis is increased, the cell membrane reactance is progressively diminished and the "bulk" conductivity approaches that of the hemolysed sample. Similarly, the dielectric constant in the sonicated sample is only that due to the H_2O solvent. The very large values of dielectric constant with intact cells is completely abolished with the sonicated sample. The interpretation of this finding depends upon the interface between the extracellular and intracellular spaces. This interface is, of course, destroyed with sonication. In other words, the heterogeneous dielectric of intact cells in suspension is converted to a homogeneous dielectric by means of sonication. In the present studies, we find the upper frequency to be about one octave lower than that reported by Schwan [12]. In general, this aspect of the present work is more consonant with Fricke and Curtis [7]. The differences are probably not beyond what other experience indicates in far simpler systems [15]. In addition to measurement methods, there are many biological factors which must be controlled in order to make meaningful comparisons. This includes temperature, suspending medium, pH, and species of animal. Osswald has shown a marked temperature dependence in the ϵ' of blood [13]. We have verified this observation with measurements at 20°C and 37°C for otherwise identical cell suspensions. The result (Tables II and III) is a marked increase in ϵ' at the higher temperature. Likewise, the choice of species effects complex permittivity. Sheep blood cells consistently have lower ϵ' and σ than rabbit blood cells under identical conditions. Similarly, MOPS-TRIS buffer apparently increases dielectric constant and conductivity at 37°C. The exact mechanism is unclear, since the MOPS-TRIS concentration is so low. It is obvious that the ionic environment has a profound effect on cell surface charge. What is not so obvious is that different species have a potassium–sodium dimorphism. That is, certain ungulates have the inverse of the "normal" high extracellular Na^+, low extracellular K^+. The question of species-specific drug responses is even more complex, especially in carnivores.

Another consequence of the absence of the lower limiting value of conductivity is the inability to calculate an equivalent intracellular resistance. In any event, such a calculation is sensitive to the underlying assumption of spherical cell shape. In that regard, it is interesting to note that Schwan [10] found better conformity to square law

predictions (i.e., spherical cell shape) for lysed than for intact erythrocytes, especially as frequency departs downward from the relaxation frequency. Since normal erythrocytes are well known to be biconcave disks, the discrepancy in this case is not surprising. However, the spherical shape of lysed cells probably represented resealing or vesical formation rather than swollen and spherical but apparently intact cells [10, Fig. 8]. Both Schwan [10, p. 163] and Fricke and Curtis [7, p. 827] seem to be thinking of hemolysed blood with intact but leaky erythrocyte membranes. "It was shown that hemolysis with distilled water does not destroy the membrane but merely increase its permeability to release hemoglobin..." "The measurements, therefore, establish that the addition of distilled water to a suspension of erythrocytes merely increase the permeability of the plasma membrane..." "The large value of capacitance and general similarity of these curves (hemolysed blood) to those for normal corpuscles give evidence of the presence of 'cells' in the hemolysed suspension."

The postulated "cells" must have been resealed ghosts. Since many hours were allowed for "complete" hemolysis, there was sufficient time for some resealing and reestablishment of ionic gradients across the membrane of the vesicle ghosts. Sonication, of course, can take place in a matter of seconds. Multifrequency analysis within a few minutes of sonication produces the lack of dispersion shown in Fig. 2. If several hours elapse between sonication and measurement, resealing will introduce a dispersion intermediate between whole blood and freshly hemolysed blood.

Collagenase produces hydrolytic cleavage of the collagen molecule [28]. Collagen is a prominent component of fibroblasts. As a result, collagenase affects cell surface charge states and membrane structure. Predictably, these actions are most apparent at 1 MHz as shown in Fig. 4. The early large differences may reflect this effect. The latter differences correspond with a minor percentage of cell death (approximately 10 percent) as judged by dye exclusion.

Oubain blocks the activity of the membrane transport mechanisms responsible for concentrating K^+ in and extruding Na^+ from the intracellular space [29],[30]. The effect of this agent is to hyperpolarize the cell membrane by a movement of the transmembrane potential away from the chloride equilibrium toward a more negative value. This is the result of passive diffusion of cations along their concentration gradients. K^+ moves outward and Na^+ moves inward. The effective permeability increase of Na^+ exceeds that of K^+ since Na^+ movement is enhanced by the transmembrane potential whereas K^+ movement is retarded by the same mechanism [31],[32]. The result is that water enters the cell. This is a plausible explanation of why water movement alone acts in the same direction as oubain as shown in Figs. 5 and 6. In both cases, the intracellular conductivity is decreased by dilution. Thus the upper limiting frequency value of conductivity decreases from the control values. The upward shift in conductivity with shaking represents slight homolysis with the presence of additional K^+ in the extracellular space due to extravisation of cell contents. This conclusion is supported by the relatively frequency independent upward off-set of reflection coefficient seen in Fig. 5(c).

The ionophore results (Fig. 10) demonstrate the effect of increased cation conductance. In the case of valinomycin, the effect is specific for the K^+ ion [33]. Gramicidin increase conductivity for all cations (Na^+, K^+, and H^+) [34]. A comparison of the change in dispersion from the control situation with a KCl does in the presence and absence of valinomycin shows that KCl alone increases the dispersion more than KCl with valinomycin as seen in Fig. 11. The interpretation we place on this result is that KCl alone effects the extracellular space to a larger extent that KCl plus valinomycin since the ionophore will allow more of the extracellular dose to reach the intracellular space. The result would be a more uniform increase in conductivity and transmembrane depolarization. Similar arguments apply to the gramicidin results, but Na^+ and H^+ movements complicate the interpretation. In general, the interpretation of the ionophore and depolarization experiments (Figs. 9–11) is made more difficult due to the fact that changes in cell volume cannot be separated from changes in transmembrane potential [34]. Ion specific electrodes are necessary in order to further distinguish the possibilities since interpretation becomes more difficult when multiple ion species are involved. Obviously, our methods cannot distinguish ion species so long as dispersion analysis is limited to the HF band.

Alcohol alone (Fig. 10) appeared to produce effects similar to the ionophore dose. Temperature effects also complicate the situation. This is why a KCl challenge is necessary to affirm the permeability alterations. The ionopphore alone does decrease dispersion by increasing conductivity in the extracellular space.

The pH experiments (Fig. 8) are interesting because of the fact that transmembrane potential in the erythrocyte is determined by a combination of the chloride equilibrium potential and the polyanionic state of hemoglobin which is a function of intracellular pH. As the pH is shifted toward 6.5, the charge on the hemoglobin becomes more negative and the transmembrane potential moves toward depolarization. Conversely, as the pH moves toward 7.5, the charge on the hemoglobin becomes less negative and the transmembrane potential moves toward hyperpolarization [4]. The lengthly equilibrium period and pH stabilized washing presumably buffers the intracellular pH to the same value as the extracellular pH, but no direct confirmation of this condition could be performed. It is reassuring that pH 6.5 moves both the lower and upper frequency limiting value of conductivity toward depolarization (i.e., upward), whereas pH 7.5 moves it toward hyperpolarization (i.e., downward).

In addition to the effects of pH alterations on transmembrane potential, there is also an effect on the surface

charge of the cell membrane [36]. There is good reason to believe that hydophylic proteins of the exterior leaflet of the red cell membrane (so called exoproteins) are pH dependent polyanions [36],[37]. As a result, there is a pH dependent potential which exponentially decays with radial separation from the cell membrane. This potential undoubtedly interacts with the zeta potential due to counter ions at the phospholipid layer of the dielectric interface [38]. We speculate that the prominence of low frequency effect with pH alteration is due to changes in cell surface charge. Further, since the effect is less prominent at the upper limiting frequencies, we speculate that membrane-bound endoproteins (which are chemically distinct from the membrane attached exoproteins) are in a hydrophobic environment where pH cannot affect charge [37]. An alternative interpretation is that transmembrane potential alterations consequent to changes of the hemoglobin-charge, effects the outer cell membrane surfaces more than the cytoplasmic surface. In other words, the bulk pH in combination with the surface potential alters surface pH on exterior surfaces, but no similar relationship has been demonstrated for the interior on cytoplasmic surfaces of the membrane [39],[40].

VI. Conclusions

A method for the high-speed noninvasive electromagnetic analysis of cell suspension physiology and induced pathophysiology has been presented. The method is especially suitable for pathophysiological applications where a high rate of data acquisition is necessary. This requirement is met by the technique of automatic network analysis and off-line processing to derive complex permittivity from the error-corrected complex reflection coefficient.

The present system offers some freedom from the interfacial effects that plague all permittivity measurements in electrolytes insofar as platinum/platinum black is used only in a calibration step. Due to limitations in the absolute accuracy of phase measurements, interface equilibration, overall system accuracy is acceptable only under specific conditions. These conditions exclude measurements above 200 MHz, below 1 MHz, and require ϵ''/ϵ' to be within 0.01 and 100. Nevertheless, proper procedural controls do permit reliable, high-speed comparative measurements. Absolute measurements, subject to the above limitations, appear to be within the range of variation in the published literature once all contributing factors such as temperature, pH, volume proportions, species, etc. are considered.

The method appears to have useful sensitivity to small movements of water and electrolytes. A system for the interpretation of cell suspension dispersion was presented along with experimental evidence to support the interpretative scheme.

This method offers the potential for noninvasive measurement whereby the contribution of intracellular and extracellular spaces may be separately analyzed. It was asserted that the physiological foundation for the observed dispersion is the cell membrane and that its activity may be inferred without the need for membrane disruption.

Acknowledgment

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Microwave-Induced Hyperthermia Dose Definition

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***Abstract*—*In vitro* thermal data on cytotoxicity are consistent with the simple picture of chemical reaction kinetics as governed by an activation energy. These kinetics are used to calculate, for any arbitrary heating profile used in clinical hyperthermia, the corresponding percentage of cells killed by such treatment in *in vitro* tissue cultures. The quantity calculated, which incorporates biological response to thermodynamic parameters, is suggested as a measure of hyperthermal dosage. Alternative dosage measures are discussed. Doses, defined by thermal cytokinetics, are derived for current clinical practice in whole-body and local hyperthermia. Both types of treatment, although superficially very different, are shown to employ comparable dose magnitudes, and these magnitudes are found to be in quantitative accord with the thermal cytotoxic basis for dosage measurement.**

I. Introduction

WHEN ANTIBIOTICS were first introduced, physical and chemical assays of their potency were found to be poorly correlated with treatment efficacy. The problem, of course, was that it was not until years afterward that it was discovered which of the closely related derivatives and isomers were effective. In order to quantitate dose for research and clinical trials, a system of "units" was adopted. The units of penicillin, for example, were related to the area of a petri dish that would be cleared of a trial organism after innoculation with a measured quantity of a given batch of "penicillin." The problems of assessing efficacy and toxicity of hyperthermia are similarly plagued by the lack of a definition of hyperthermal dosage. In the absence of a dose–response measurement procedure, hyperthermal dosage has been assigned by a variety of schemes.

One class of methods is based upon observed sequelae to hyperthermia. To this class belong such units as dose to produce a certain percentage decrease in liver function [1], dose to produce an arbitrary erythema score [2], [3], dose to produce various serum enzyme elevations [1], [4], etc. Although these methods beg the question of hyper-

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