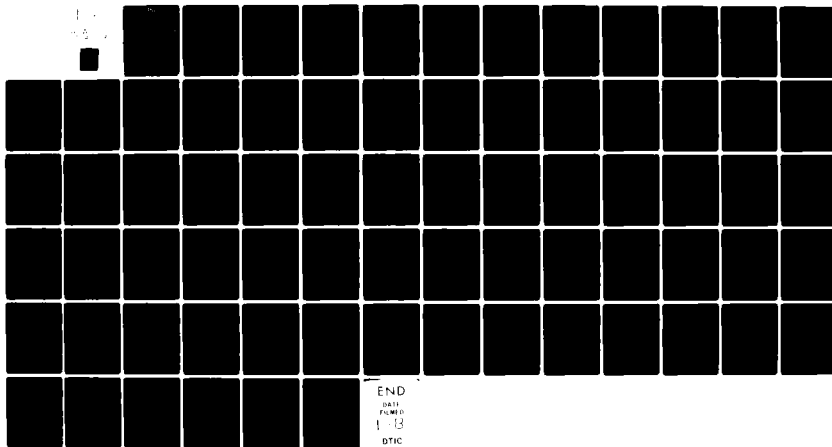


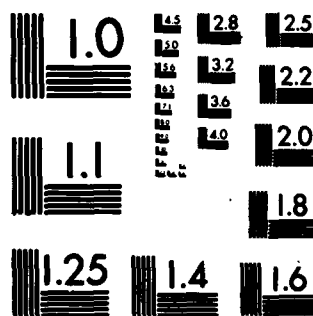
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INVESTIGATION OF THE BIOLOGICAL EFFECTS OF PULSED ELECTRICAL FI--ETC(U)
JAN 77 S F CLEARY, R HOFFMAN, L LIU N00014-75-C-0334

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6 INVESTIGATION OF THE BIOLOGICAL EFFECTS
OF PULSED ELECTRICAL FIELDS.

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I. INTRODUCTION:

Research conducted during the past contract period has involved an extension of an investigation of the physiological effects of exposure of Dutch rabbits to electromagnetic pulse (EMP) fields and the development of in vitro biological model systems to investigate the physico-chemical effects of transient electrical and electromagnetic fields. Physiological studies performed during this period have included: 1) a continuation of a study of the effect of EMP exposure on sodium pentobarbital-induced sleeping time in the Dutch rabbit which was initiated during the previous contract year; 2) investigation of EMP and heat stress effects on rabbit serum chemistry; 3) electromagnetic pulse effects on serum triglyceride levels and, 4) alterations in creatine phosphokinase (CPK) isoenzyme levels induced by EMP and nonradiation heat-stress exposures. Model systems used to investigate the mechanisms of interaction of transient electric and electromagnetic fields included artificial bilayer lipid membranes and mammalian erythrocytes. The dependent variables for the in vitro studies included dielectric breakdown and potassium and hemoglobin membrane permeabilities. The independent variables were voltage field strength, pulse duration, and exposure duration. Analysis of data from the in vitro studies has led to the formulation of a preliminary theoretical model for voltage-induced transient alterations in cell membrane permeability.

II. IN VIVO STUDIES OF EMP EFFECTS IN THE DUTCH RABBIT:

The almost total lack of data regarding the biological effects of exposure to EMP fields dictated the need to screen for various types of physiological alterations in mammalian systems. The Dutch rabbit was chosen for this study based on the availability of extensive data on the response of this species to

microwave radiation. The results of EMP exposure may be qualitatively and quantitatively compared to the effects of microwave exposure, thus providing a basis for an evaluation of the relative biological effects of these radiation types. The rabbit is also a practical choice for this study since the total blood volume of this species is large enough to permit serial serum samples to be drawn for serum chemistry and enzyme determinations.

Effect of EMP Exposure on Drug-induced Sleeping Time.

In previous studies of the effects of 2.45 and 1.7 GHz microwave exposure we have detected significant decreases in the mean duration of sodium pentobarbital-induced sleeping time in the Dutch rabbit exposed to radiation intensities of from 5 to 50 mW/cm² (1). In contrast to these findings, the initial studies of the effects of EMP exposure at 1.9 KV/cm and 24 Hz or 1.4 KV/cm and 10 Hz indicated no decrease in the duration of sodium pentobarbital sleeping time in the rabbit. In fact, the data suggested the possibility of an increase in sleeping time as a result of exposure to the 10 Hz EMP fields (2). In order to obtain more definitive data on this response variable, additional sleeping time experiments were conducted during this reporting period. The techniques used, which have been previously described in detail (1,2), consisted of determining the mean duration of sleeping (i.e. duration of the loss of the righting reflex) in animals anesthetized with 22 mg/kg of sodium pentobarbital by intravenous injection into the marginal ear vein, followed by either sham irradiation or exposure to an EMP field of 0.9 KV/cm at a pulse repetition rate (PRF) of 10 Hz. Due to the operating characteristics of the EMP simulator, it was not possible to duplicate the 1.4 KV/cm, 10 Hz exposure conditions.

The results of sham or EMP exposure under these conditions are summarized in Table 1. The mean sleeping time and standard error of the mean for five Dutch rabbits exposed to the EMP field were 63 ± 9.9 min. The corresponding values for the sham-exposed control group were 62.4 ± 6.7 min. These data

TABLE 1

Effect of Electromagnetic Pulse Exposure on
Sodium Pentobarbital Induced Sleeping Time in
the Dutch Rabbit. Pulser Voltage 0.9kV/cm; Pulse
Repetition Rate 10 Hz.

<u>Sham Irradiated Controls</u>			<u>EMP Exposed</u>	
	<u>Sleeping Time (min)</u>	<u>Rectal Temp. Change ($\Delta^{\circ}\text{C}$)</u>		
	55	-1.8	44	-1.0
	53	-0.6	101	-0.9
	52	-1.6	51	-0.9
	64	-0.9	59	-0.3
	88	-0.1	60	-0.6
Sample mean	62.4	-1.00	63.0	-0.74
Std. dev.	15.08	0.70	22.21	0.29
Std. error	6.73	0.31	9.92	0.13

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do not, therefore, provide any evidence of an alteration in sleeping time resulting from EMP exposure. This result is not inconsistent with the results obtained in studies of the effects of microwave exposure on sleeping time. The statistically significant reduction in the mean duration of pentobarbital sleeping time consequent to microwave exposure is assumed to be an indirect effect of low-level microwave-induced thermal stress, leading to redistribution of the drug from the brain to other body compartments due to increased circulation. The reduction in sleeping time in animals exposed to microwave radiation was, in general, correlated with an increase in rectal temperature, thus suggesting the involvement of thermal stress. In the case of EMP exposure, the mean and standard error of the mean rectal temperature were $-0.84 \pm 0.13^{\circ}\text{C}$ for the exposed animals and $-1.00 \pm 0.31^{\circ}\text{C}$ for sham irradiated controls. Thus, there was no evidence of thermal stress from EMP exposure of the type employed in this study. If the observed reduction of sleeping time following microwave exposure is due to thermally-stimulated drug redistribution, it is not surprising that EMP exposure did not detectably alter this response variable.

Effect of EMP on Rabbit Serum Chemistry:

In view of the fact that physiological stresses of various kinds are known to result in alterations in serum components, a study was undertaken of the effects of acute exposure of Dutch rabbits to EMP fields on the following serum components: calcium, inorganic phosphate, glucose, blood nitrogen (BUN), uric acid, cholesterol, total protein, albumin, total bilirubin, alkaline phosphatase, lactic dehydrogenase (LDH), and serum glutamic oxalacetic transaminase (SGOT).

Previous studies revealed that stress induced by exposure of rabbits to 1.7 and 2.45 GHz microwave radiation at intensities of 10 mW/cm^2 or greater resulted in transient alterations in the serum components: glucose, BUN, uric acid, bilirubin, and various serum enzymes (3,4). These results and data on the

thermal response of rabbits to microwaves suggest that such exposure may alter serum components as an indirect effect of low-level thermal stress, although other mechanisms of interaction cannot be ruled out at this time. In view of the purported effect of thermal stress on rabbit serum chemistry alterations, it was decided that nonradiation heat stress would be used as a comparison stress for EMP exposure effects. Dutch rabbits were exposed to an ambient temperature of 40°C for 2 hr, the same duration as the EMP exposure, and pre- and post-exposure samples were analyzed and compared to samples obtained from EMP exposed animals.

The procedure used for the investigation of EMP radiation effects on serum chemistry consisted of obtaining pre-exposure baseline serum samples from a group of 10 Dutch rabbits 10 days prior to exposure. Following the measurement of rectal temperatures, single animals were exposed for 2 hrs. to the EMP field. During exposure the animals were confined to the central region of the EMP simulator by the use of 2" thick styrofoam blocks with ample provision being made for free circulation of air during exposure. Although the animal was restrained to this area, there was ample room for the animal to move about within the exposure chamber. The exposures were performed using the EMP simulator at the Electromagnetic Radiation- Bio Effects Laboratory at the Naval Surface Weapons Laboratory, Dahlgren, Virginia. The staff of the Electromagnetic Radiation-Bio Effects Laboratory is hereby acknowledged for their aid in performing this phase of the study and for the use of their facilities.

A detailed description of the EMP simulator has been previously given (2). The exposure conditions for the study of serum chemistry changes were a field strength of 1.5 KV/cm, pulse repetition rate of 38 ± 2 Hz, and an exposure duration of 2 hrs. The total number of pulses to which the animal was exposed was $2.73 \times 10^5 \pm 1.44 \times 10^4$. The variation in the pulse repetition rate of the EMP simulator was due to the operating characteristics of the "free-running" spark

TABLE 2 EFFECT OF EMP EXPOSURE AND HEAT STRESS ON SERUM CHEMISTRY OF DUTCH RABBITS
RESPONSE VARIABLE (MEAN ± STANDARD ERROR OF MEAN)

Treatment	Number of Animals	Ca ⁺⁺ (mg%)	Inor Phos (mg%)	Glucose (mg%)	BUN (mg%)	Uric Acid (mg%)	Chol (mg%)
pre-exposure baseline	10	13.7 ± 0.19	5.0 ± 0.2	191.0 ± 8.2	16.0 ± 1.3	0.6 ± 0.03	39.7 ± 5.2
immed. post 2 hr sham irradiation	2	14.2 ± 1.3	5.1 ± 0.05	159.5 ± 0.5	18.5 ± 0.5	0.6 ± 0.05	41.5 ± 13.5
immed post 2hr EXP exp 1.5KV/cm; 38Hz	3	14.1 ± 0.5	4.5 ± 0.6	153.7 ± 2.9	15.7 ± 2.9	0.63 ± 0.1	41.6 ± 6.0
1 day post sham irradiation	2	13.8 ± 0.1	5.1 ± 0.3	150.5 ± 0.5	18	0.55 ± 0.2	30 ± 4.0
1 day post EXP exp 1.5KV/cm; 38Hz	3	14.1 ± 0.6	4.0 ± 0.7	145.0 ± 8.7	17.3 ± 4.9	0.63 ± 0.1	43.6 ± 5.2
immed post 2 hr. sham heat stress at 22° C	4	14.4 ± 0.2	4.3 ± 0.1	157.3 ± 5.1	18.8 ± 2.5	0.80 ± 0.04	33.8 ± 3.8
immed. post 2hr heat stress at 40° C	4	13.7 ± 0.1	3.8 ± 0.3	152.8 ± 6.8	15.8 ± 1.6	0.68 ± 0.11	31.5 ± 3.1

TABLE 2 EFFECT OF EMP EXPOSURE AND HEAT STRESS ON SERUM CHEMISTRY OF DUTCH RABBITS
RESPONSE VARIABLE (MEAN + STANDARD ERROR OF MEAN)

Treatment	Number of Animals	Tot. Protein (gm%)	Alb(gm%)	Tot Bili(mg%)	Alk Phos(mU/ml)	LDH (mU/ml)	SGOT (mU/ml)
Pre-exposure baseline	10	6.3±0.1	4.4±0.1	0.2±0.02	88.5±13.6	79.9±10.0	31.7 ±6.8
immed. post 2 hr sham irradiation	2	6.8±0.1	4.5±0.1	0.2±0.1	61.5± 3.5	56±8	35.5±17.5
immed post 2hr EMP exp 1.5KV/cm; 38Hz	3	6.6±0.3	4.3±0.1	0.3±0.1	70.7±11.1	54.7±10.2	54.3± 16.8
1 day post sham irrad.	2	6.0±0.1	4.0±0.2	0.2	130.5±7.5	83.5±34.5	24.5±0.5
1 day post EMP exp. 1.5KV/cm; 38Hz	3	6.3±0.3	4.2±0.2	0.2±0.03	75±27.1	55.7±11.5	22.3±2.3
immed post 2 hr sham heat stress at 22°C	4	6.4±0.2	4.6±0.1	0.2±0.05	39.3±7.8	46.3±9.0	18.8±2.3
immed post 2 hr heat stress at 40°C	4	6.2±0.2	4.3±0.0	0.2±0.04	49.0±6.1	56.5± 19.3	27.3±6.1

See long notes

gap used to trigger the pulser. The simulator pulse may be described as a exponentially decaying cosine wave, the amplitude of which decreases to one-half maximum voltage in 4 cycles. The characteristic frequency of the EMP simulator is 23.5 MHz and the simulator pulse duration is thus approximately 0.4 μ sec. The rise time of the pulse is less than 0.1 μ sec. All exposures were at normal room temperature ($22 \pm 1^\circ\text{C}$).

Immediately following exposure, the rectal temperature was determined and a 5 ml blood sample was obtained from the marginal ear vein of the experimental animal. In an attempt to detect exposure effects with longer latencies, another blood sample was taken 24 hours post EMP exposure. Sham-irradiated control animals were treated identically except that the pulser was not energized during the 2 hr sham exposure. Serum chemistry analyses were performed by the use of an SMA 12/60 autoanalyzer. The sampling procedure for the investigation of the effects of nonradiation heat stress was as previously described. In this case, the animals were exposed singly for two hours to an ambient temperature of 40°C in an environmental chamber.

The results of EMP and nonradiation heat stress exposure on rabbit serum components are summarized in Table 2. No statistically significant alterations in the serum chemistry of Dutch rabbits were detected as a result of EMP exposure. Although the levels of certain response variables such as the enzymes alkaline phosphatase and SGOT appear to have been elevated in the immediate post exposure samples, the variability of the data is such that no statistical significance can be attached to these findings. It should be noted that the levels of alkaline phosphatase, LDH, and SGOT were increased as a result of nonradiation heat stress, whereas no significant alterations in other serum components were detected except for an increase in calcium. Increased levels of serum enzymes

are generally attributed to cell death or to alterations in cell membrane permeability.

It may be concluded that the EMP exposure under the condition of this experiment did not result in significant alterations in serum components compared to sham irradiated controls. Exposure to EMP radiation for 2 hrs resulted in a 15% increase in serum alkaline phosphatase, whereas heat stress for the same duration caused a 25% increase. The levels of SGOT were increased by 53% following EMP exposure as compared to a 45% increase following heat stress. Nonradiation heat stress led to a 22% increase in LDH in contrast to a 2% decrease in the EMP exposed animals. The mean and standard error of the mean rectal temperature change were $0.28 \pm 0.21^{\circ}\text{C}$ for the EMP exposed animals and $0.10 \pm 0.26^{\circ}\text{C}$ for the sham irradiated controls. Exposure to an ambient temperature of 40°C produced a mean rectal temperature elevation of $2.1 \pm 0.4^{\circ}\text{C}$.

The significance of these basically negative findings is limited by the small sample sizes employed which, in view of the inherent inter- and intra-animal variability in serum components, reduces the sensitivity of this study. Additional serum chemistry, studies, especially of enzyme levels, in which larger sample sizes are used are needed to evaluate the effects of EMP exposure.

Serum Triglyceride Study

The effects of acute stress on the mammalian system produces a generalized response mediated by the neuroendocrine system. One reported consequence of this stress response is the activation of the pituitary gland leading to the release of growth hormone which in turn causes an inhibition of cellular glucose uptake leading to increased serum glucose. Growth hormone also causes the release of free fatty acids from tissue storage deposits resulting in elevations of serum triglycerides. We have determined that low-level microwave exposure results in elevations in both serum glucose and triglycerides. Consequently we have investigated the effects of EMP exposure on these response variables. The results of the serum glucose study were indicated

in Table 2. No increase in serum glucose was detected in animals exposed to EMP for 2 hr periods as described in the previous section of this report. Serum triglyceride levels were determined in a group of 6 Dutch rabbits immediately following a 2 hr exposure to EMP radiation using the same exposure parameters as described in the preceding section. The mean and standard error of the mean serum triglyceride level were 38.3 ± 4.1 mg/dl as compared to a mean level of 40.4 ± 5.9 obtained from a sample of 5 sham irradiated rabbits. There was thus no evidence that EMP exposure of the type employed in this study altered serum triglyceride levels.

Effect of EMP and Heat Stress on CPK Isoenzymes

Creatine phosphokinase, an enzyme which catalyzes the conversion of creatine phosphate to creatine, resulting in the synthesis of ATP, exists as three distinct isoenzymes in body tissues. The MM isoenzyme is found primarily in skeletal muscle, MB is located in heart muscle, and the BB isoenzyme is associated with brain tissue. Cell death or increased cell membranes permeability is thought to account for increased levels of these enzymes in the serum. Analysis of CPK isoenzyme levels thus provides a means of detecting tissue-specific effects of physiological stress such as that induced by EMP radiation exposure. CPK isoenzyme levels were determined by the method described by Nealon and Henderson (5). The procedures used for the exposure of Dutch rabbits to EMP radiation were as previously described. Serum samples were taken immediately upon cessation of EMP exposure and at 24 hrs post exposure. For purposes of comparison of EMP effects with a well known physiological stress, a group of rabbits was exposed for 2 hrs. to nonradiation heat stress in an environmental chamber maintained at 40°C . The mean rectal temperature change in the heat stressed animals was $2.1 \pm 0.4^{\circ}\text{C}$.

Creatine phosphokinase isoenzyme concentrations are affected by storage time between sampling and analysis as well as by the age and general metabolic status.

of the experimental animals. For these reasons and due also to limitations on the availability of the EMP exposure facility and animal holding facilities, CPK isoenzyme studies were conducted with three groups of experimental animals at three different times during the contract year. Due to the aforementioned sampling problems it is not feasible to pool the results of these experiments, consequently the results are analyzed in each case by comparing the EMP exposed group values with the sham-exposed group values obtained at the same time (ie. within the same period of experimentation).

The effects of exposure to the EMP field and nonradiation heat stress on CPK isoenzyme levels are summarized in Table 3 and the statistical analysis of these data, using the Student's t test is presented in Table 4. Exposure to either heat stress or EMP radiation, in all cases, led to an increase in the serum levels of total CPK isoenzymes and in each separate isoenzyme fraction as shown in Figure 1. A mean isoenzyme elevation of 65% occurred for the MB fraction of EMP exposed animals whereas heat stress produced a maximum elevation of 95% in the MM fraction. Statistical analysis of the data obtained from individual experiments revealed, however, that with the exception of the elevation in the MB fraction for the EMP exposed group 8 compared to its control group (ie. group 7), the differences were not statistically significant at the 5% level. This may be attributed to the inherent inter-animal variability in CPK isoenzyme levels and to the relatively small sample sizes employed. Exposure to nonradiation heat stress resulted in elevations in all CPK isoenzyme levels which again were not statistically significant at the 5% level. The overall significance of the consistent increase in CPK isoenzyme levels following EMP exposure may alternatively be evaluated by use of the binomial or sign test applied to the twelve independent determinations of CPK isoenzyme levels (ie 3 enzyme fractions in 4 separate experiments; excluding the total CPK values which, being a sum of the MM, MB, and BB fractions, are not

TABLE 3 EFFECT OF EMP EXPOSURE AND HEAT STRESS ON CPK ISOENZYMES

Group	Number of animals	Treatment	Mean rectal temp change (°C)	CPK ISOENZYME LEVELS U/1 (mean ± std. error)			
				MM	MB	BB	Total
1	4	sham heat stress 2 hr @ 22°C immed. post sample	0.1 ± 1.5	55.7 ± 6.5	13.0 ± 1.5	50.7 ± 22.6	119.4 ± 18
2	4	heat stress 2 hr @ 40°C immed post sample	2.1 ± 0.4	108.9 ± 34.7	18.8 ± 5.5	80.3 ± 49.1	208.1 ± 60.2
3	5	sham EMP exposure, 2hr.. immed. post sample	0.3 ± 0.4	55.7 ± 10.1	16.8 ± 5.7	47.5 ± 17.1	119.8 ± 20.2
4	6	EMP exposed 2 hr; 1.5 KV/cm 50 Hz, 0.28 ma immed. post sample	-0.1 ± 0.2	70.9 ± 15.5	24.1 ± 11.0	82.0 ± 53.8	177.0 ± 70.9
5	4	sham EMP exposure, 2hr; immed. post sample	0.0	72.2 ± 11.4	11.6 ± 2.4	44.9 ± 15.9	128.4 ± 15.5
6	6	EMP exposed 2 hr; 1.5 KV/cm 50Hz, 0.28 ma immed. post sample	-0.2 ± 0.4	173.9 ± 57.5	23.6 ± 5.9	49.2 ± 11.3	246.8 ± 59.0
7	4	sham EMP exposure, 2hr immed post sample	0.2 ± 0.1	48.9 ± 11.5	6.8 ± 1.0	12.1 ± 3.2	67.8 ± 14.1
8	5	EMP exposed 2 hr; 2KV/cm 42 Hz, 0.36 ma; immed. post sample	0.2 ± 0.6	58.1 ± 8.1	12.4 ± 2.8	17.0 ± 5.8	87.4 ± 12.2

TABLE 3 EFFECT OF EMP EXPOSURE AND HEAT STRESS ON CPK ISOENZYMES
CPK ISOENZYME LEVELS U/1 (MEAN \pm STD. ERROR)

Group	Number of animals	Treatment	Mean rectal temp change ($^{\circ}$ C)	MM	MB	BB	Total
9	3	sham EMP exposure, 2hr, 24 hr post exposure sample	-	87.8 \pm 38.4	11.6 \pm 2.2	12.9 \pm 3.4	112.2 \pm 39.2
10	4	EMP exposed 2 hr; 2KV/cm 45 Hz, 0.36 ma 24 hr. post exposure sample	-	114.8 \pm 6.8	15.4 \pm 2.2	22.2 \pm 6.0	152.4 \pm 6.6

Fig

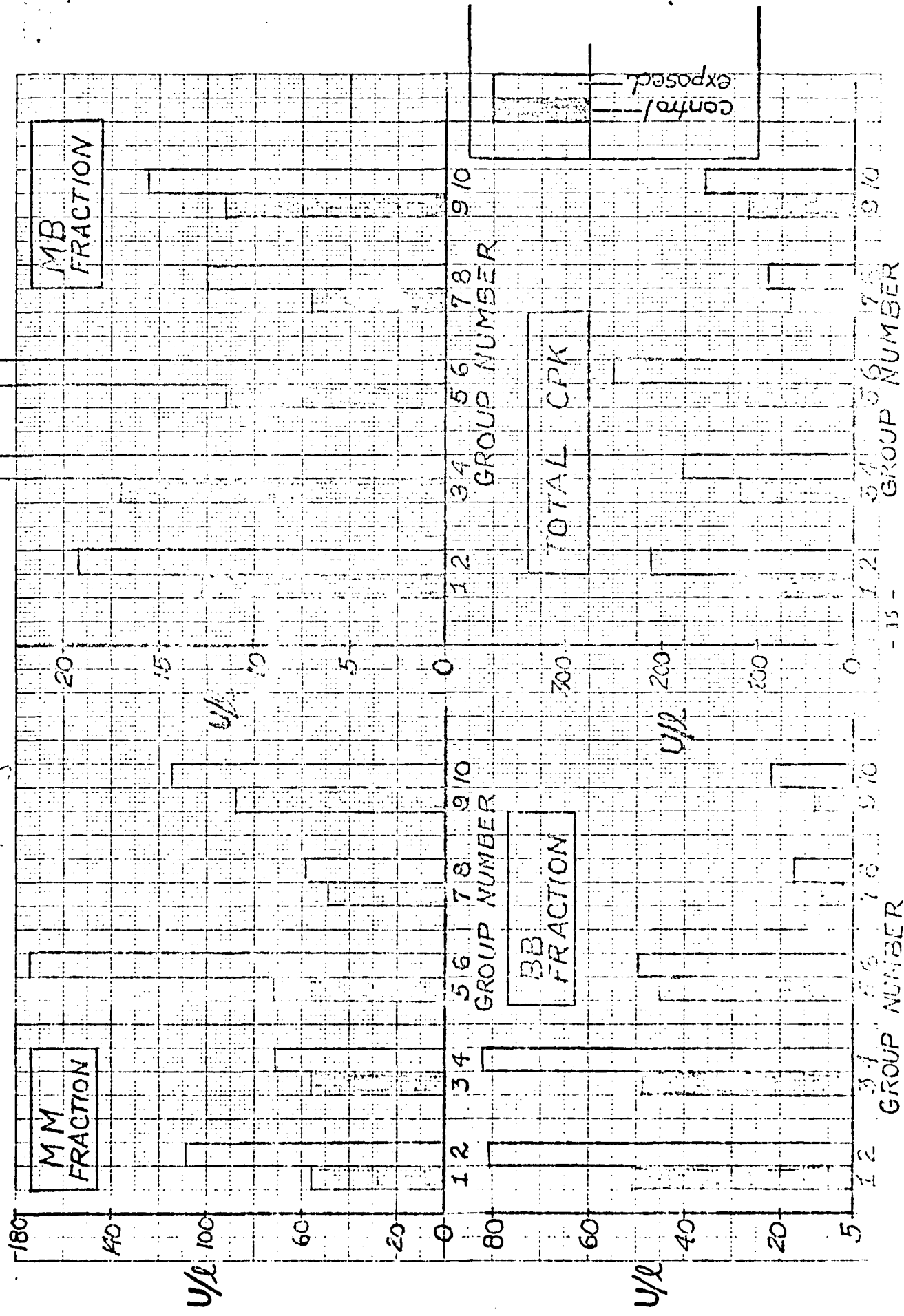


FIGURE 1 CPK ISOENZYME LEVELS FOR FMD EXPOSED RABBITS

(independent measures of enzyme concentration). The probability of the CPK enzyme level being greater in all twelve determinations following EMP exposure is, in fact there is no treatment effect (ie. the probability that the enzyme levels following EMP exposure, on any trial, exceeds the control value is equal to the probability that the control value exceeds the EMP value; both probabilities being equal to $1/2$) is $(1/2)^{12} = 0.00024$. The combined results of the CPK isoenzyme determinations from the 4 experiments thus suggest that exposure to EMP fields results in a consistent and statistically significant ($p < 0.01$) increase in enzyme levels. The response patterns of the CPK isoenzymes do not indicate any obvious tissue specific effects of EMP exposure although there is some suggestion of heightened sensitivity of cardiac muscle. Additional data is required to substantiate these findings.

III IN VITRO STUDIES OF PULSED ELECTRICAL FIELDS

The complexity of mammalian systems and the limitations imposed on the exposure facilities capable of providing the desired range of field strengths, pulse durations and pulse repetition rates, provides the rationale for investigations of pulsed electrical field effects in biological model systems. We have therefore initiated an investigation of the effects of pulsed fields on artificial bilayer lipid membranes and mammalian erythrocytes. The lipid bilayers are the most elementary model systems available and since such systems closely simulate biological membranes in many of their properties, they offer a means for investigating basic mechanisms of interaction of membranes with electrical fields. In recognition, however, of the differences in the physical properties of artificial and biological membranes there are limitations on the usefulness of artificial bilayers for the interpretation of pulsed field effects in more highly organized biological systems. For this reason

mammalian erythrocytes have also been used as a cell model system. Erythrocytes offer the advantages of being a relatively simplified cell model that can potentially provide information on both basic interaction mechanisms as well as data of direct physiological relevance in the evaluation of the effects of EMP radiation on mammalian systems. The somewhat limited data on the in vivo effects of EMP exposure of the Dutch rabbit obtained in this investigation suggest that such exposure alters cell membrane permeability as reflected in the release of intracellular enzymes. This finding indicates the need for an investigation of pulsed field effects on membranes, with special emphasis being directed toward the mechanisms for the alteration of cell membrane permeability.

Due to differences in the modes of coupling of pulsed electrical fields to biological systems under different exposure conditions, it is necessary to consider effects of both inductive and conductive fields. In recognition of the differences in the magnitude of such coupling, it is evident that significantly larger external field strengths are required in the case of inductive field interactions than conductive interactions to induce a given field strength in a model system such as a cell membrane. Preliminary theoretical and experimental studies have thus been undertaken of both inductive and conductive field exposures in an attempt to establish the conditions under which each type of field alters biological systems. Since the basic interaction mechanisms at the membrane level should be dependent only upon the field characteristics at the membrane, the results of experiments with conductive or inductive fields should be directly relateable, even though the external field parameters differ. Limitations on the presently available apparatus for the exposure of model systems to inductive or capacitive fields in our laboratory have precluded a direct comparison of such fields with conductive fields. The preliminary inductive field exposure of erythrocytes reported here were at both lower field strengths and shorter pulse durations than the conductive field exposures. Experiments were also initiated to determine the effect of the transient voltage waveform on cell

permeability by the use of voltage square waves and exponentially decaying pulses. Again, the available square wave pulse generating equipment did not provide sufficient voltage output to provide a direct comparison of waveform effects. The future availability of such equipment would permit a direct comparison of both inductive and conductive field effects as well as waveform comparisons.

In order to provide a comparison of pulsed electric or electromagnetic fields with a more widely studied and somewhat better characterized form of nonionizing radiation, erythrocyte suspension were exposed to 2.45 GHz microwaves in a waveguide irradiation chamber. The effects of such exposure on erythrocyte membrane permeability have been studied by determining the potassium and hemoglobin efflux in irradiated and control cells.

Artificial Membrane Studies

One of the primary functions of the cell membrane is its selective permeability to various molecules and ions which enables the cell to obtain and retain substances which it requires and to prevent the entry of undesirable substances. It is clear that the introduction of large pores into the cell membrane would allow substances to pass through the membrane thus destroying the important permselective function. The major question we have sought to answer in this investigation is whether electric field pulses can induce pores which alter membrane permeability.

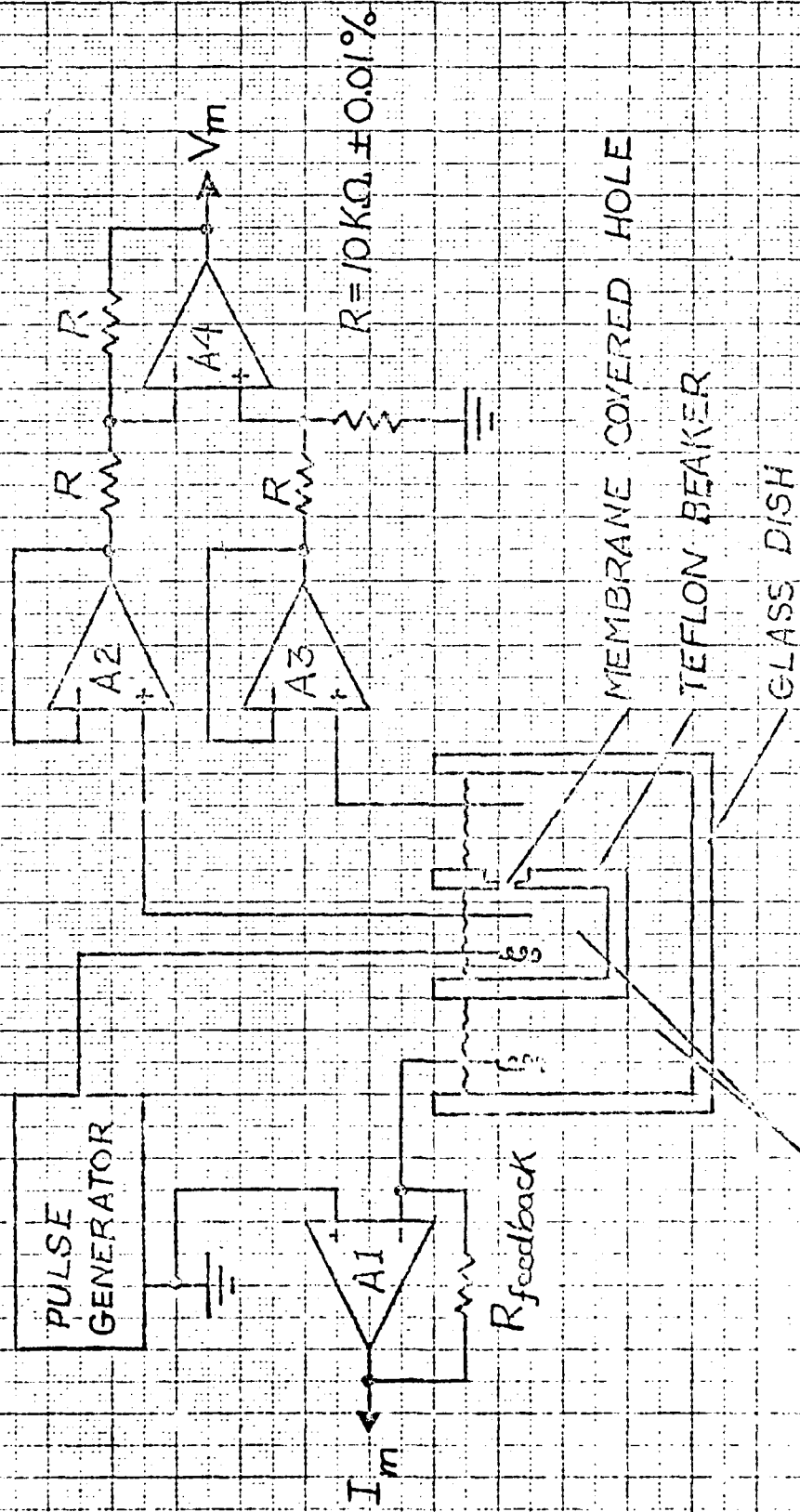
The possibility that long lasting pores might exist is suggested by the observations of Yafuso et al. (6) on aged, oxidized cholesterol lipid bilayer membranes. In these membranes step changes in current while the membrane was held at a constant voltage suggested the formation of pores or channels in the membrane. The membranes were not permselective to Na and K. Although moderate voltages caused conductance increases in the membranes, higher voltages were often observed to cause a conductance decrease in stepwise fashion, indicating that the higher voltages caused the channels to close. We have

confirmed these basic observations in a previous investigation (7).

A more drastic type of pore formation is the actual breakdown of the membrane which is referred to as "dielectric breakdown". In the case of a single bilayer lipid membrane such breakdown causes the complete destruction of the membrane. However, in a cell or in a discontinuous arrangement of a matrix of very small membranes, dielectric breakdown may be localized. The dielectric breakdown of cells has been investigated by Zimmerman et al. (8,9,10) while Ohki (11) has observed localized breakdown in a grid-like system of membranes. Crowley (12) has studied the theoretical aspects of dielectric breakdown of both artificial and cellular membranes. Previous investigators have determined the voltage which produces dielectric breakdown under D.C. conditions (11,13). Although it is recognized that the breakdown voltage increases as the duration of the voltage pulse decreases (14), this phenomenon has not to our knowledge been investigated quantitatively. In the present work we have determined the breakdown voltage of an artificial bilayer lipid membrane as a function of the voltage pulse duration. In addition we report on attempts to observe conductance changes in the membrane following the application of voltage pulses approaching the breakdown level.

Oxidized cholesterol was prepared according to the method described by Tien (15), and the dried product was dissolved in decane to form a saturated solution. Membranes were formed by applying the cholesterol solution with a brush or micropipet (16,17) across a hole in a teflon beaker immersed in 0.1 M KCl. Figure 2 shows the major components of the experimental apparatus. The membrane was viewed under reflected light with a 40 X microscope. The standard procedure was to wait a minimum of two minutes after the membrane had become black, which

APPARATUS FOR THE STUDY OF ELECTRICAL PROPERTIES OF ARTIFICIAL BILAYER LIPID MEMBRANES



A1 = AD615K CURRENT TO VOLTAGE CONVERTER
A2 = TELEDYNE PHILPICK 1029-01
A3 = NE 536
A4 = AD741K ELECTRONETER DIFFERENTIAL AMP

Figure 2

indicates the transition to a bilayer, before making any electrical measurements. The experiments were conducted at normal room temperature of $22 \pm 2^\circ\text{C}$.

Electrical connection to the membrane was made by a pair of Ag - AgCl₂ current delivering electrodes and a pair of potential measuring calomel electrodes connected to either side of the membrane by KCl-agar salt bridges. The time constant for charging the membrane capacitance was 7 μs . Current flow through the membrane was measured with an electrometer operational amplifier in the current-to-voltage conversion mode. With the feedback resistance $10^9 \Omega$, the 10% to 90% rise time was 18 nS and decreased to 7 μs with a feedback resistance of $10^6 \Omega$. Generally the most sensitive setting with the feedback resistance at $10^9 \Omega$ was used. The voltage across the membrane was measured with a differential electrometer amplifier with an input impedance of at least $10^{13} \Omega$ at either input and a 10% to 90% response time to a 250 mV step change of less than 1 μs . Square pulses were obtained either from a HP214A pulse generator, or a pulse generator of our own design.

This arrangement was suitable for D.C. or slowly varying signals, but when fast rising pulses are applied to the membrane the current charging the membrane capacitance ($\approx 5 \text{ nF}$) saturates the current to voltage converter, which requires 100 nS or longer to recover. To overcome the effect of the capacitance charging current two methods were used. In the first method, a relay was activated during the pulse and was used to short out the feedback resistance in the current-to-voltage converter. In the second method, adapted from Sargent (18,19), a current equal to but opposite in sign from the capacitance charging current was added to the membrane current, effectively nulling the capacitance charging current. Using either method produced a transient artifact at the output of the current-to-voltage converter which occurred after the applied voltage pulse. The worst case condition using the relay method produced an artifact pulse with an amplitude of $2 \times 10^{-10} \text{ A}$ and a half width of 8 nS, while with the current nulling method the transient artifact was a damped oscillation with a maximum of several hundred

picoamperes and a decay constant of about 8 mS. The worst case condition was with a feedback resistance of $10^9 \Omega$ in either method. The electrical noise of the current-to-voltage converter using the relay method was 10 pA peak-to-peak and 40 pA peak-to-peak using the current nulling circuit.

For the determination of membrane breakdown voltage as a function of pulse width, the membrane voltage was held at 50 mV between pulses by using the D.C. offset of the pulse generator. One or two pulses were applied at each voltage increment with a one or two second delay between pulses. The pulse voltage was increased in approximately 25 mV increments until the membrane was broken. Membrane current and/or voltage were recorded on a Tektronix storage oscilloscope for each sequence of pulses.

The storage oscilloscope was also used for the determination of membrane current following the application of a voltage pulse in our attempt to determine whether or not long lasting conductance changes were produced by the voltage pulses. In these experiments, the current through the membrane was recorded following the application of a voltage pulse across the membrane. The voltage pulse was added to a normal holding voltage of 50 mV, so that following the voltage pulse one would expect the current to immediately return to the normal value produced by the 50 mV holding voltage. A change in membrane conductance would be indicated by a change in the current produced by the holding voltage following the application of the voltage pulse.

The results of the determination of membrane breakdown voltage for various pulse widths are shown in Fig. 3. The results represent experiments on 13 membranes for both the 30 μ S and 300 μ S pulses and 5 membranes for the 10 mS pulses. The error bars shown in Fig. 3 denote standard deviations. A definite dependence of breakdown voltage on pulse width is demonstrated, and the experimental results indicate the following relationship between the breakdown voltage (V_b) and the pulse duration (T).

$$V_b = 465 - 109 \log \{ T \}$$

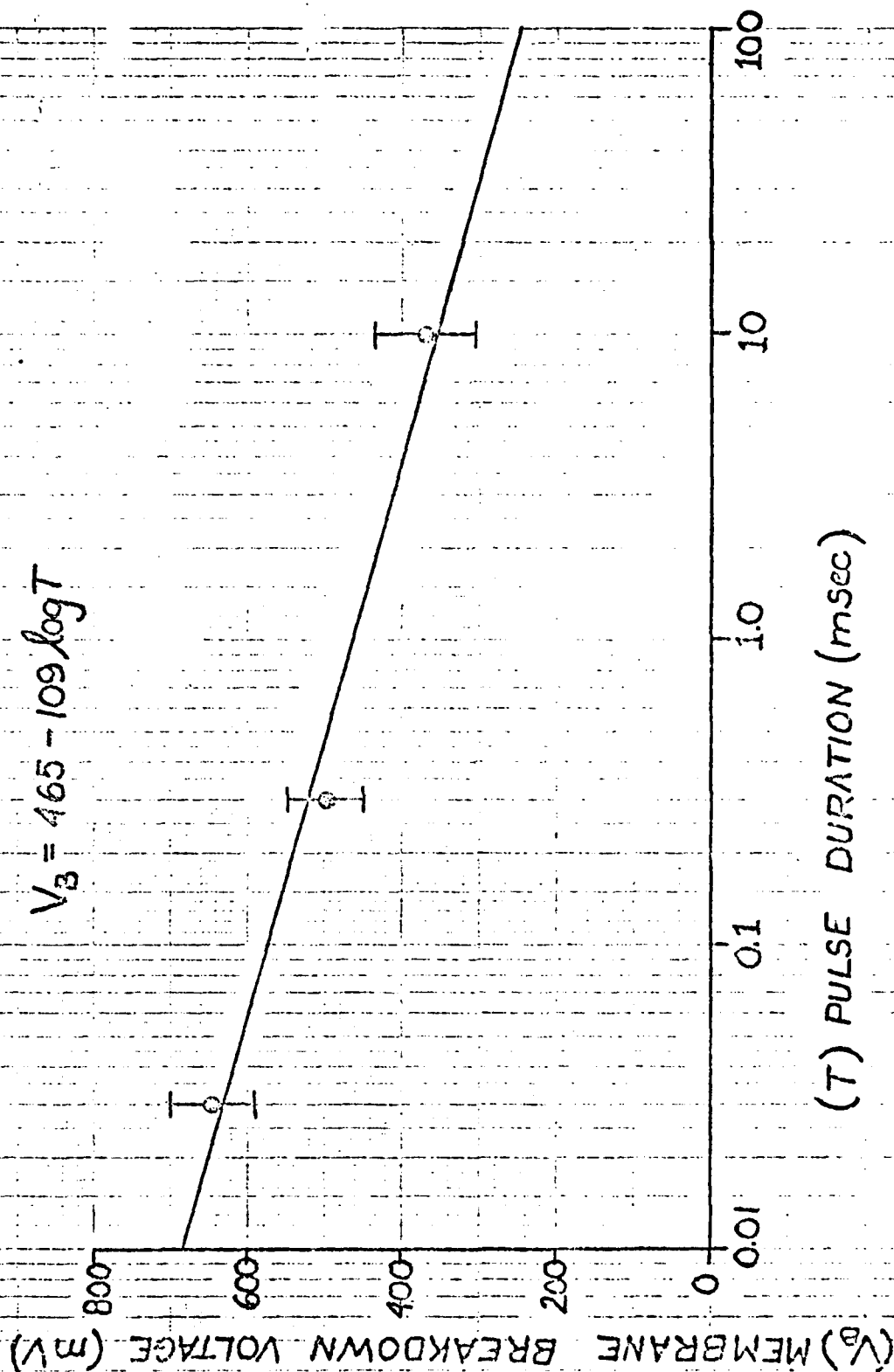


Figure 3

where the pulse width, T , is in msec, and the breakdown voltage, V_b , is in mV. The breakdown voltage increases by 109 mV for each decade decrease in the pulse width.

It must be emphasized that these results apply only to oxidized cholesterol membranes at 24°C in 0.1 M KCl and that the membrane breakdown voltage depends on many factors including the composition of the membrane, temperature, pH, and the bathing salt solution (11, 13, 20). However, if the mechanism for membrane breakdown is the same in other membranes as in the oxidized cholesterol membranes, then one would expect a qualitatively similar, though not necessarily quantitatively identical, dependence of breakdown voltage on pulse width. This may help explain why the determination of the breakdown voltage of cell membranes using voltage pulses yields breakdown voltages of 0.8 to 1.6 volts (9,10), while the breakdown voltages of artificial membranes for D.C. voltages is usually not greater than 0.4 to 0.6 volts (13) and often less than 0.3 V (11). There are, of course, many other factors which might contribute to this difference such as actual differences between artificial and biological membranes.

Our attempts to observe persistent conductance increases using either the relay method or current nulling technique have yielded no positive results, and we tentatively conclude that voltage pulses below the breakdown voltage do not produce persistent conductance changes of durations greater than 10 ms in oxidized cholesterol membranes under our experimental conditions. These results do not contradict results reported by Sargent (19) on oxidized cholesterol membranes. Sargent was not seeking, nor did he discuss, persistent conductance changes following step changes in the membrane voltage. Rather he sought and observed transient currents apparently due to membrane capacitance relaxation phenomena. To observe persistent conductance changes due to a voltage pulse would require the observation of the current following a voltage change from a higher (in magnitude) voltage to a lower non-zero voltage. Unfortunately Sargent does not report any experiments of this type. We must also note that oxi-

dized cholesterol membranes are among the most stable artificial lipid membranes. Phospholipid membranes have much lower breakdown voltages (11) and may be more likely to show a long lasting effect of voltage pulses on membrane conductance. Additional studies using other types of membranes are thus required before conclusions can be drawn regarding the generality of the relationship between breakdown voltage and voltage pulse duration as well as the effects, or lack thereof, of transient voltages on membrane conductance. Based on the results obtained with erythrocyte membranes, to be discussed in the next section, it is suggested that in view of the general correlation between the pulse duration and dielectric breakdown in artificial membranes, on the one hand, and permeability alterations in erythrocyte membranes, the two phenomena may be related mechanistically.

Effects of Pulsed Electric Fields on Erythrocytes

The effects of pulsed electric fields on erythrocytes have been the subject of a number of investigations. In general, electric field strengths of 2 to 30 kV/cm and pulse durations of 1 to 100 μ s have been used, and suspensions of erythrocytes have been exposed to one or a few pulses. Hemolysis (8,9,21-24), potassium release and sodium uptake (23), glucose uptake (24), light scattering relaxations (25,26), and enzyme uptake (27) are among the reported effects. Erythrocytes lysed by electric field pulses have been examined by electron microscopy and the cell membrane appears to remain intact. (28) Microorganisms and phospholipid vesicles have also been investigated (23,28-32). Some investigators have reported that the primary effect on cells is dielectric breakdown of the cell membrane (8-13,21-23,27, 33-34), while others suggest that the effects may be due to both the electric current and temperature increase (25) . A mechanism (or mechanisms) which satisfactorily describes all the data available in the literature has not been presented.

With one exception (25), previous investigators have exposed cells to only one or a few pulses. Baranski et al. (35) have reported that low level microwave irradiation of 1 to 10 mW/cm² produced significant leakage of potassium

and hemoglobin from erythrocytes after exposures of up to 3 hours. It has also been reported (36) that pulsed microwave fields are often more effective than continuous wave fields in producing alterations in biological systems. In view of these reports and the results of the in vivo experiments discussed in the preceding section of this report, we decided to investigate the effects of exposure of erythrocytes to relatively low intensity electric field pulses using long exposure times. In the present work we report on the results of such exposures using both induced and conductive electric fields. We also report on an attempt to confirm the results obtained by Baranski et al. (35) by exposing erythrocytes to low intensity microwave radiation.

The procedures used in this study included the drawing of blood from apparently healthy dogs, rabbits, or humans into a heparinized syringe (20 I.U. per ml of blood). The blood was immediately centrifuged for 10 minutes at 1,500 g and the plasma, buffy coat, and top layer of cells were removed. The packed cells were then washed twice in cold, potassium-free, buffered saline containing 90g NaCl, 13.6g Na_2HPO_4 , 2.15 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ per liter of solution. The pH of the buffer was 7.4 and it was osmotically equivalent to 0.9% NaCl. The washed cells were resuspended in the buffer in a concentration ratio which varied from 1:9 to 1:1 packed cells: buffer.

Exposure of Erythrocytes to Pulsed Capacitive Field

In these experiments current did not flow directly from the voltage source through the cell suspension, but rather an electric field and thus a current was induced in the cell suspension by the changing electric field between a pair of capacitor plates. The capacitor plates were 25 cm x 25 cm and were spaced 4 cm apart. The capacitor thus formed was part of the energy storage capacitor of a commercial high voltage discharge circuit (TRW Model 31B). Measurement

of the capacitor voltage during the discharge of the circuit was limited by the response speed of the Tektronix Model P6015 high voltage probe used. It was determined that the major part of the capacitor discharge had a 90% to 10% fall time of ≤ 4 nsec. Assuming for simplicity that the pulse was approximately exponential, this corresponds to a decay time constant of ≤ 1.8 nsec. The manufacturer's specifications for the TRW Model 31B indicate that the duration of the pulse is 4 nsec so that a time constant of 1.8 nsec for the decay of the voltage across the energy storage capacitor is reasonable. The rise time for charging the capacitor was 120 μ sec. Three milliliters of the cell suspension were placed in a small vinyl bag and suspended by a nylon string in the center of and between the capacitor plates. Assuming that the volume of the cell suspension is spherical, the field induced in the sample (E_i) by a changing, initially uniform electric field (E_e) is calculated in Appendix I to be

$$E_i = \omega_0^{-1} dE_e/dt \quad (1)$$

where ω_0 is a constant which is dependent upon the conductivity of the sample. For a decaying exponential external field with time constant τ the maximum field induced in the cell suspension is

$$E_i^{\max} = E_e^{\max} / \omega_0 \tau = 0.019 E_e^{\max}$$

under the conditions $\omega_0 = 2.96 \times 10^{10}$ radians/sec and $\tau = 1.8$ nsec. No appreciable field is induced in the sample during the charging of the capacitor due to the long rise time of the pulse. The maximum capacitor voltage was varied between 4 kV and 5.5 kV so that the induced field varied from approximately 75 to 104 V/cm. The pulse repetition rate was 1KHz, and the exposure time was 3 or 6 hours. Control samples were treated exactly as the exposed samples except that they were not exposed to the field. For each exposed sample and its control, the supernate remaining after centrifugation for 10 minutes at 1,500 g was analyzed for potassium concentration using an Instrumentation Laboratory Model 343 flame photometer and for hemoglobin by determining the absorbance at 540 nm by means of a Beckman Model 25 spectrophotometer.

The results of exposure of dog, rabbit, and human erythrocytes to pulsed capacitive electric fields are summarized in Table 5. No consistent or statistically significant alterations in potassium or hemoglobin efflux from dog or rabbit erythrocytes were detected. Exposure of human erythrocytes resulted in a 52% increase in potassium release with no increase in hemolysis. Although the data for human erythrocytes indicates an effect of pulsed induced electrical fields on potassium release, additional data is required in order to establish the statistical significance of this result. It should be noted, however, that the results of exposure of erythrocytes to pulsed conductive fields, discussed in the next section, suggest that of the three species that were studied, human cells appear to be the most sensitive to the electric field effects. The induced field strength in these experiments was 75 V/cm with the exception of experiment 4 in which case the field strength was 104 V/cm.

Exposure to Pulsed Conductive Field

The chamber for the exposure of erythrocytes to pulsed conductive fields consisted of a pair of flat platinum foil electrodes separated by a 3.3 mm thick rubber insulator containing a cavity into which the cell suspension was placed. The volume of the sample cavity was 0.5 ml. For most experiments the exposure chamber containing the sample was the discharge resistor for a high voltage discharge circuit, shown in Fig. 4, controlled by a needle point spark gap. The charging resistor R_c (100 M Ω) determines the time required to charge the energy storage capacitor C to the critical voltage V_c at which the spark gap discharges the charge stored in C through the cell suspension resistance R_s in the exposure chamber. The cell suspension is thus exposed to an exponentially decaying electric field with a time constant determined by $R_s C$ and maximum voltage V_c . For most experiments R_s was 100 Ω , so that the ratio of charging time to discharge time was 10^6 and was independent of the value of capacitor C, which ranged from 0.0043 μF to 0.06 μF . The time

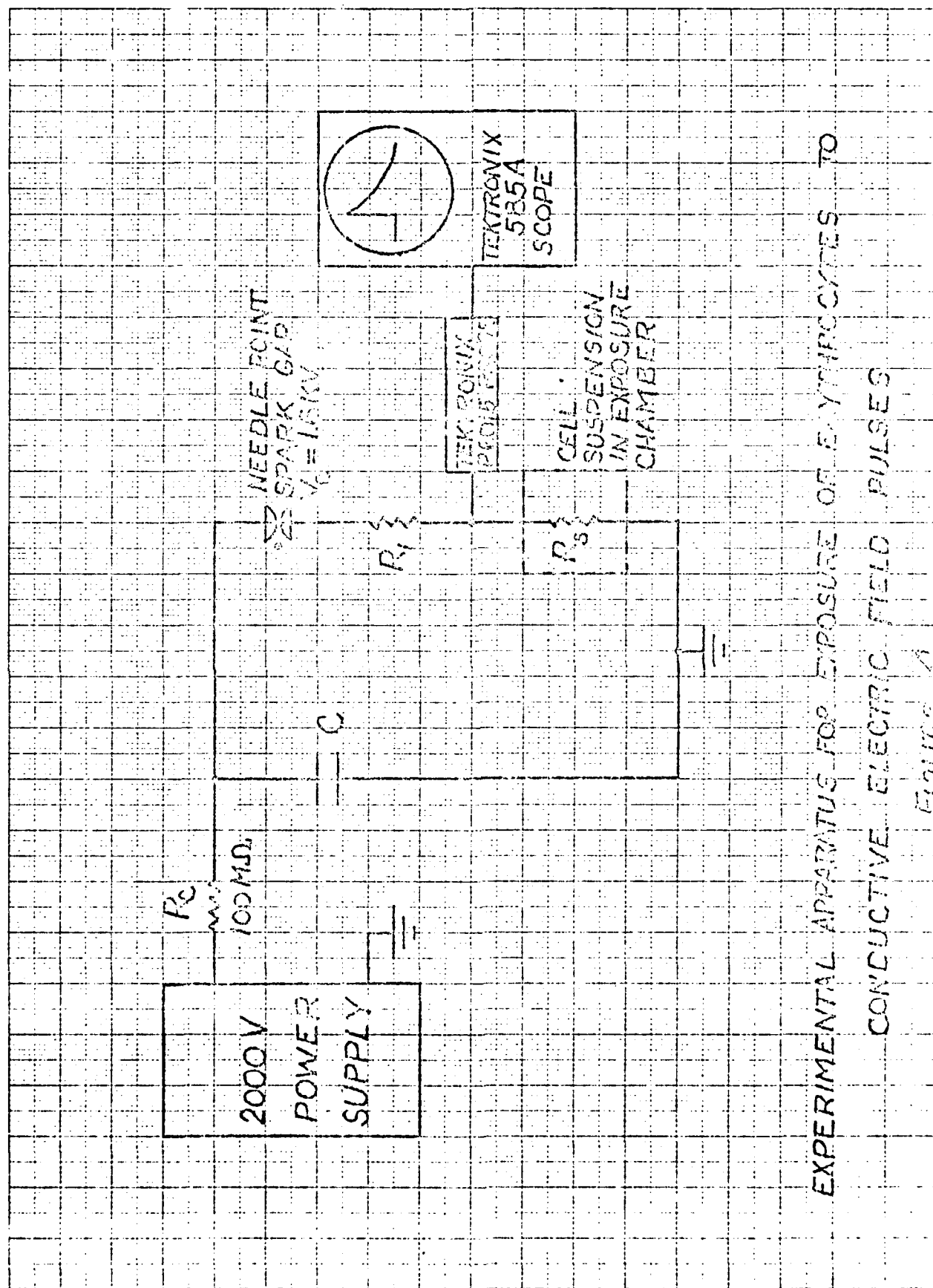
TABLE 5 EXPOSURE OF ERYTHROCYTES TO CAPACITIVE FIELD VOLTAGE PULSES
TIME CONSTANT OF PULSE = 1.8nsec, Pulse Repetition Rate = 1KHz

Experiment Number	Erythrocyte Type	External Field (kV/cm)	Exposure Time (hours)	Concentration of Cells (%)	Potassium Release		Con't % * Difference
					mEq/L Exposed	mEq/L Control	
1	Dog	1.0	3	10	0.12±0.03	0.10±0.05	20
2	Dog	1.1	3	10	0.11±0.01	0.10±0.02	10
3	Dog	1.0	3	10	0.09±0.01	0.11±0.02	-18
4	Dog	1.4	6	33	0.27±0.02	0.21±0.02	29
5	Rabbit	1.0	3	10	0.32±0.07	0.47±0.10	-32
6	Rabbit	1.0	6	10	0.43±0.04	0.45±0.11	-4
7	Human	1.0	3	10	0.41±0.12	0.27±0.01	52

$$\% \text{ Difference} = \frac{\{K\}_{\text{exposed}} - \{K\}_{\text{control}}}{\{K\}_{\text{control}}} \times 100$$

TABLE 5 EXPOSURE OF ERYTHROCYTES TO CAPACITIVE FIELD VOLTAGE PULSES
TIME CONSTANT OF PULSE = 1.8nsec, Pulse Repetition Rate = 1KHz

Experiment Number	Erythrocyte Type	External Field (kV/cm)	Exposure Time (hours)	Concentration of Cells (%)	Hemolysis		
					Exposed Absorbance	Control Absorbance	% Difference
1	Dog	1.0	3	10	1.658	1.648	+0.6
2	Dog	1.1	3	10	1.180	0.827	+42.7
3	Dog	1.0	3	10	0.080	0.181	-50.8
4	Dog	1.4	6	33	2.450	2.425	+1.0
5	Rabbit	1.0	3	10	0.143	0.186	-23.1
6	Rabbit	1.0	6	10	0.225	0.192	+17.2
7	Human	1.0	3	10	0.121	0.123	-1.6



EXPERIMENTAL APPARATUS FOR EXPOSURE OF ERYTHROCYTES TO
 CONDUCTIVE ELECTRIC FIELD PULSES

Figure 1

between pulses ranged from approximately 1 second using the 0.0043 μF capacitor to approximately 12 seconds using the 0.06 μF capacitor. In Appendix II it is shown that under these conditions the average energy dissipated in the sample was less than 10 mW. The sample was a 1:1 suspension of cells in buffer and the volume of sample in the exposure chamber was typically 0.2 ml. Thus the average specific heating rate of the sample was less than 50 mW/cm^3 . The volume of sample was very small compared with the size of the exposure chamber, and the average temperature increase measured with a miniature thermistor was found to be less than 0.3°C . The temperature jump produced by a single pulse is calculated in Appendix II and found to be less than 0.1°C . In experiments where less than the total voltage V_c was to be applied to the sample, a resistor R_1 was added in series with R_s . The resistance R_s was adjusted so that the total resistance $R_1 + R_s$ was 100 Ω . The voltage pulses across R_s were monitored with an oscilloscope and gave a V_c of 1.6 kV with a variation of ± 0.3 kV. This was in agreement with the voltage calculated on the basis of the charging time of capacitor C. For the shortest pulses ($R_s C = 0.43 \text{ } \mu\text{sec}$) oscilloscope triggering was difficult and erratic and some observations gave apparent values for V_c of only 1.0 kV for these pulses. However, based on the time required to charge capacitor C we calculated a V_c of 1.6 kV. In those experiments in which square electric field pulses of 300 volts/cm were used, the pulses were produced by a Hewlett Packard model 214 A pulse generator. In this case the sample volume was adjusted so that the sample resistance was equal to the 50 Ω characteristic impedance of the connecting cable. The rise time of these pulses was 15 nsec. Controls for these experiments were kept at room temperature. For both control and exposed samples the potassium release and absorbance were determined as for the samples exposed to pulsed capacitive fields. In addition, osmotic fragility of the cell membranes was measured in some experiments. For this determination a 10 μl aliquot of the exposed or control cell suspension was added to 2 ml of 0.5% saline and maintained at room temperature for 30 minutes. The suspension

was then centrifuged to remove the cells, and the absorbance of the supernate was determined at 540 nm.

The results of the exposures of rabbit erythrocytes to exponential voltage pulses are shown in Figures 5 a, b and 6, and the results for human erythrocytes exposed to exponential pulses are summarized in Figures 7 to 10. Exposure of dog erythrocytes to exponential pulses in a series of preliminary experiments gave results similar to those observed for rabbit erythrocytes. In another series of experiments rabbit erythrocytes were exposed for 2 hours to square wave electrical pulses of 300V/cm with pulse widths of 0.50 and 2.5 μ sec. No significant difference was noted in potassium and hemoglobin release between exposed samples and controls. For rabbit and human erythrocytes, potassium release occurs much more readily than does hemoglobin release following exposure to conductive exponential voltage pulses. This has also been observed by Riemann et al. (23) in human erythrocytes exposed to single pulses. The more rapid release of potassium through the cell membrane is not surprising since the ion is significantly smaller than the hemoglobin molecule. These results suggest that less pronounced membrane alterations are sufficient to permit the passage of potassium than are required for the release of hemoglobin.

A second obvious feature of the results obtained in this study is the strong dependence of both potassium and hemoglobin release on the decay time constant of the pulse. It is shown in Appendix II that under the conditions of this experiment the average power delivered to the sample is independent of the time constant of the pulse. It is also shown that the average time that the voltage across the cell suspension is greater than a given value is independent of the pulse decay time. Figure 11 shows the effect of temperature on human erythrocytes. A 3-hour incubation at 37°C results in potassium and hemoglobin release that is small compared with that which occurs due to pulsed field exposure. Thus, temperature and average power can be ruled out as possible causes for the dependence of the effects on the pulse time constant. This

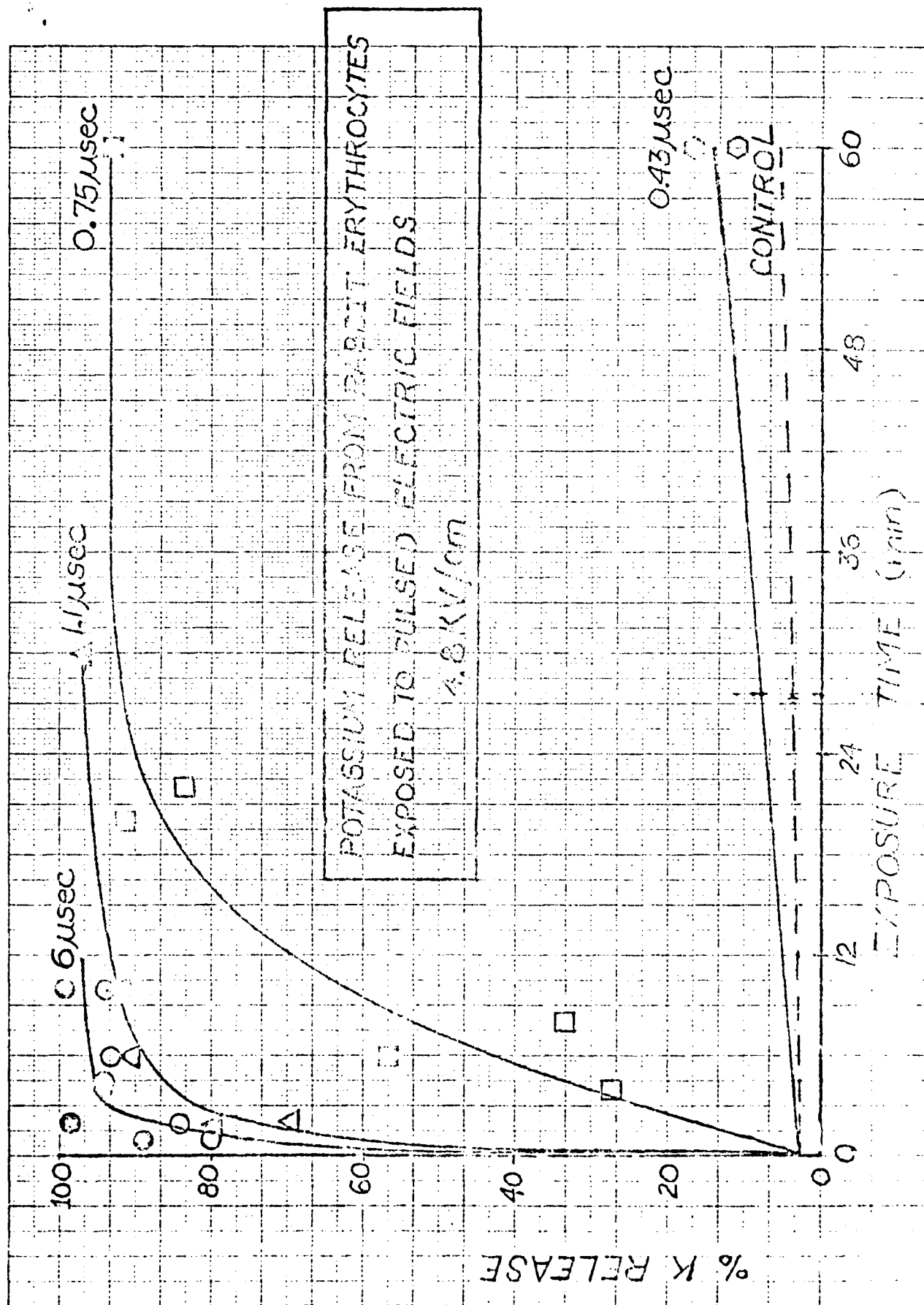


Figure 5a

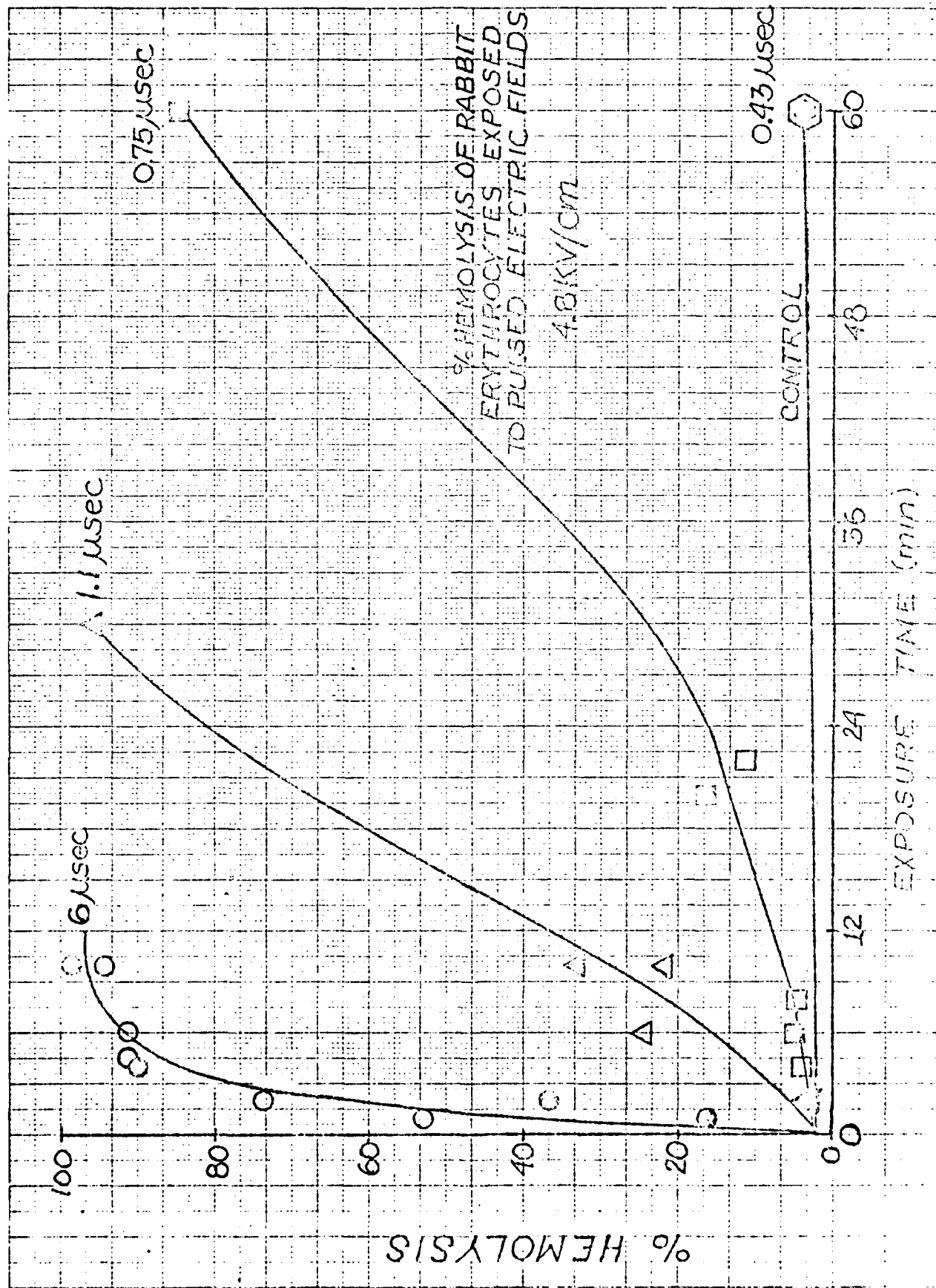


Figure 50

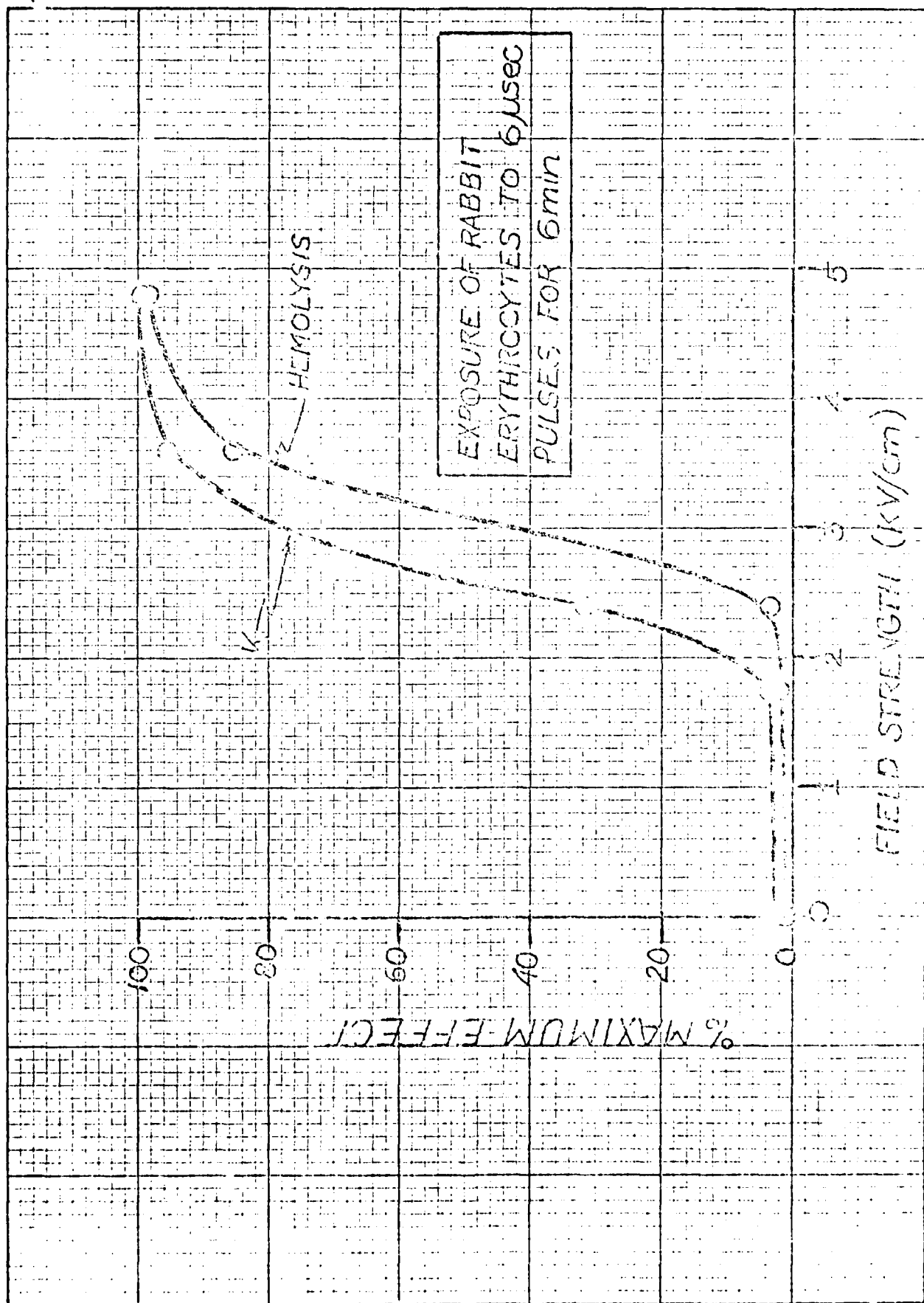


Figure 6

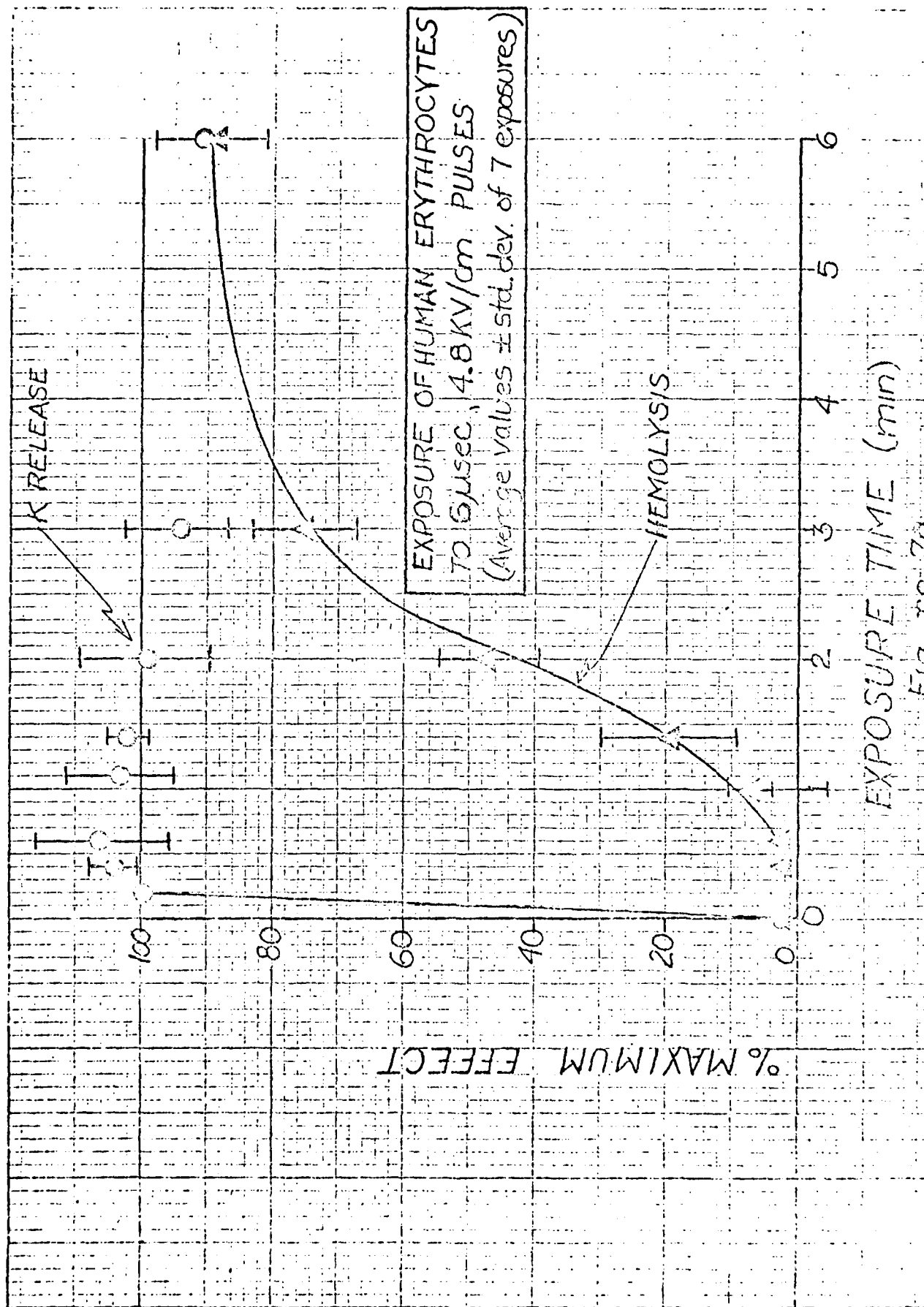
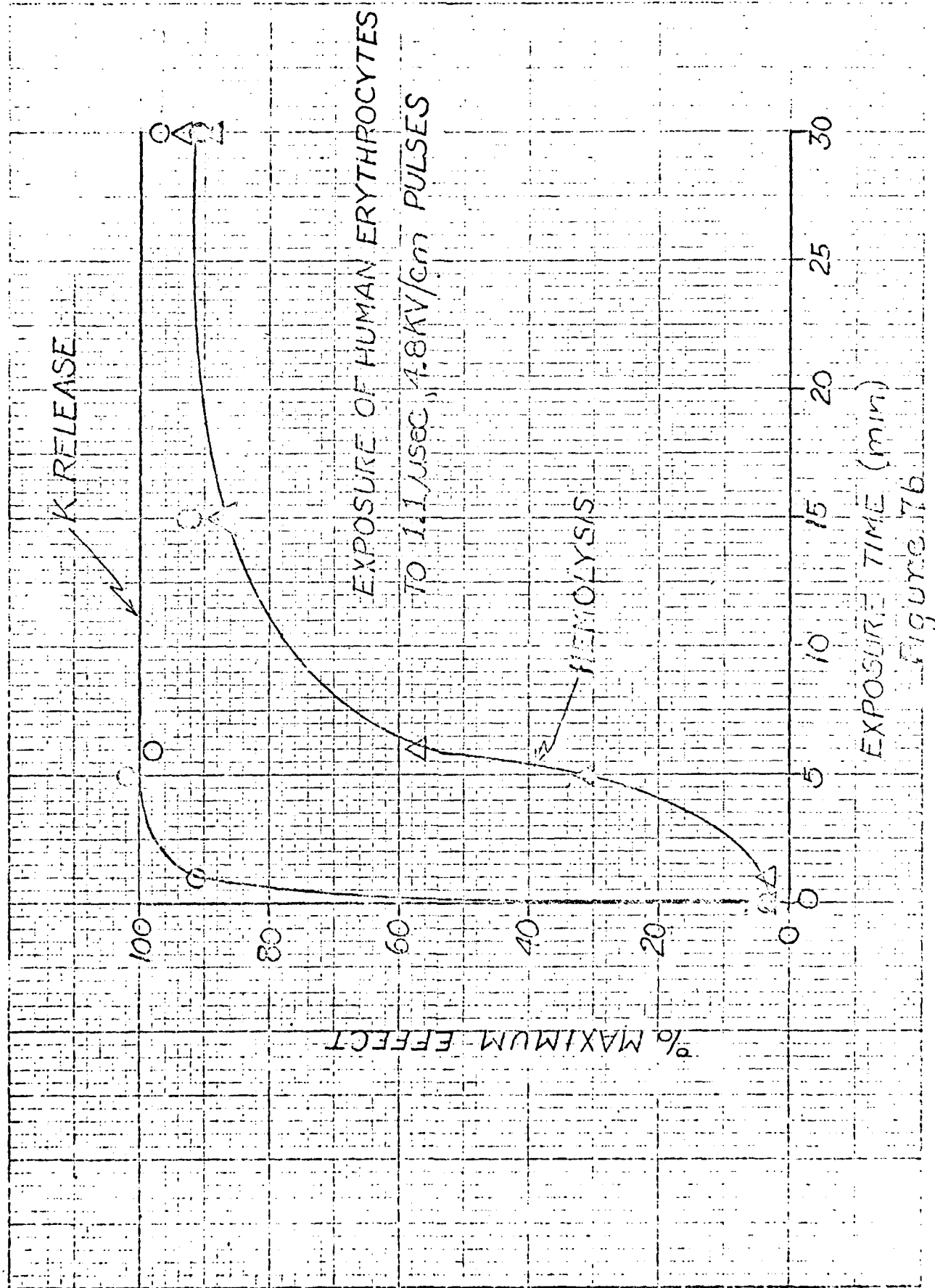
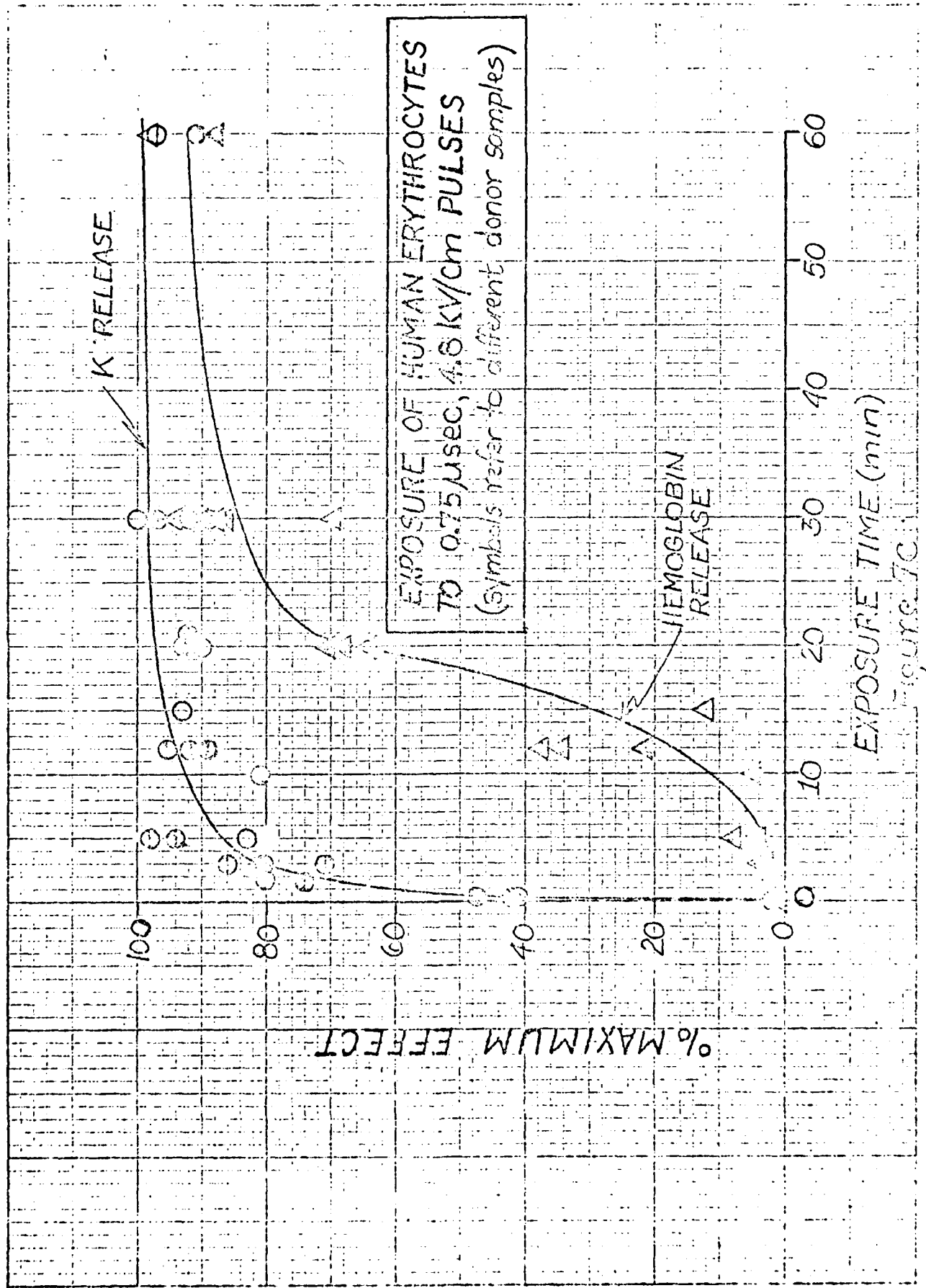


Figure 7a





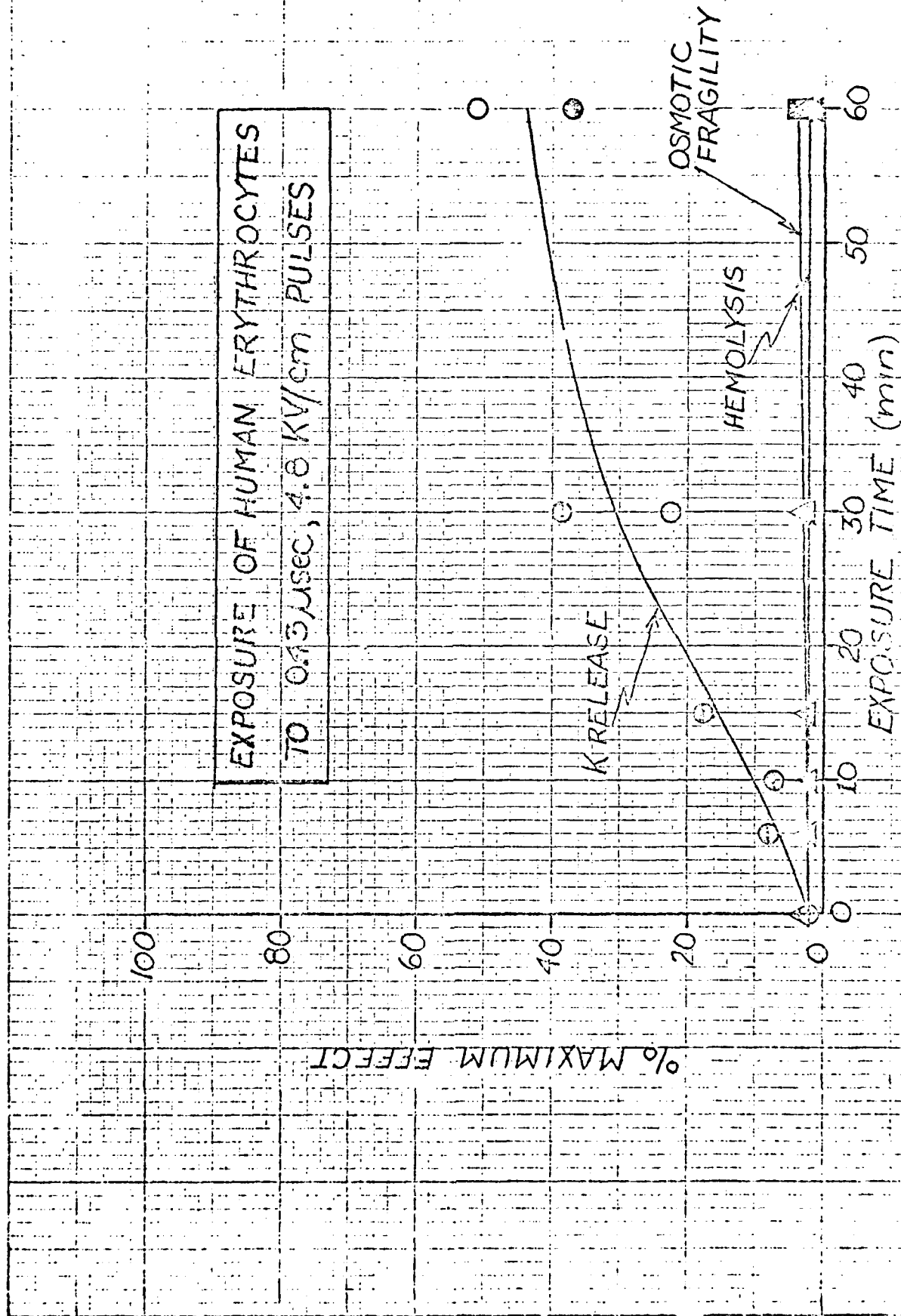


Figure 7d

EFFECT OF EXPOSURE OF HUMAN
ERYTHROCYTES TO $6\mu\text{sec}$ FOR 6 MIN
PULSES

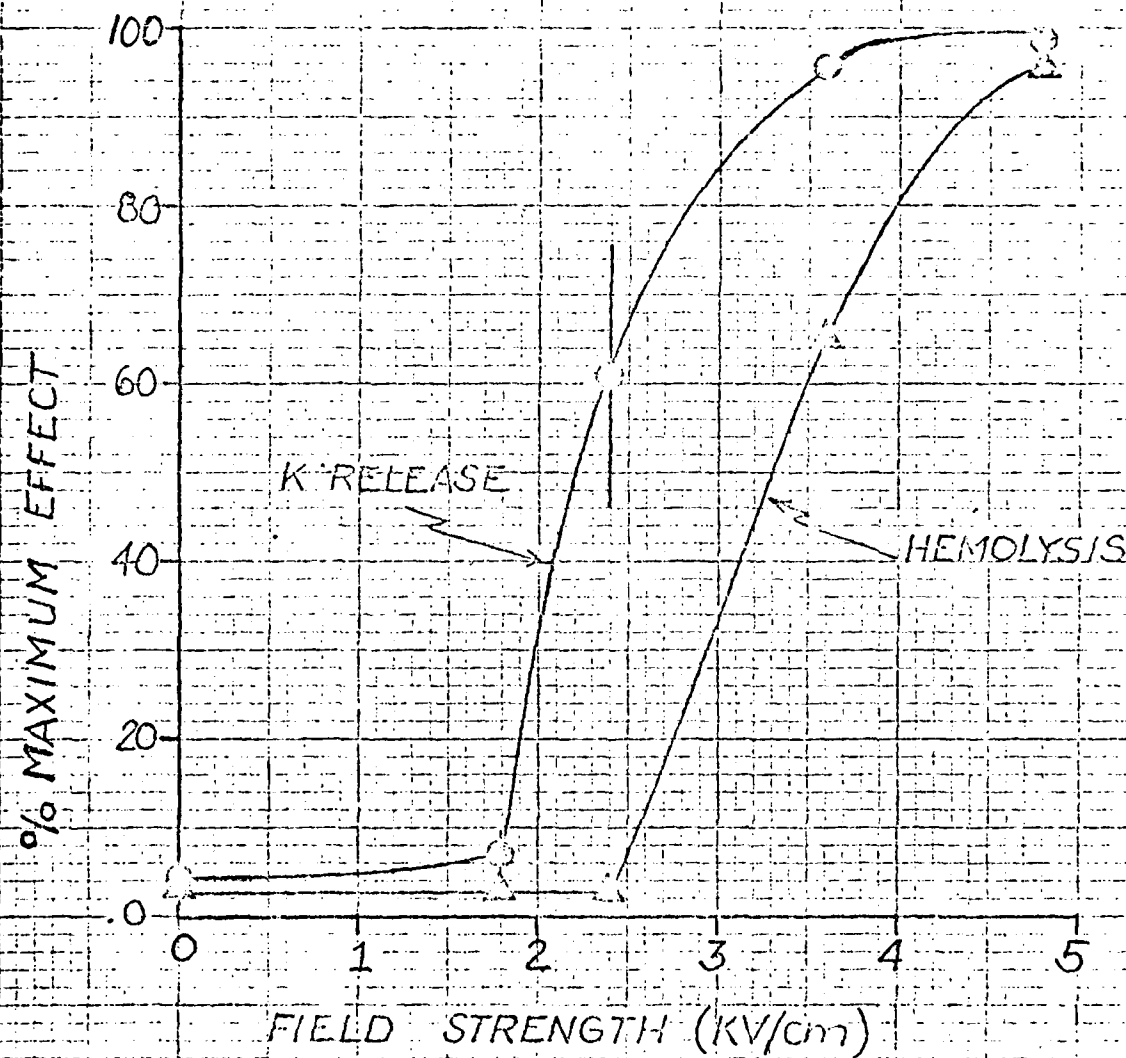
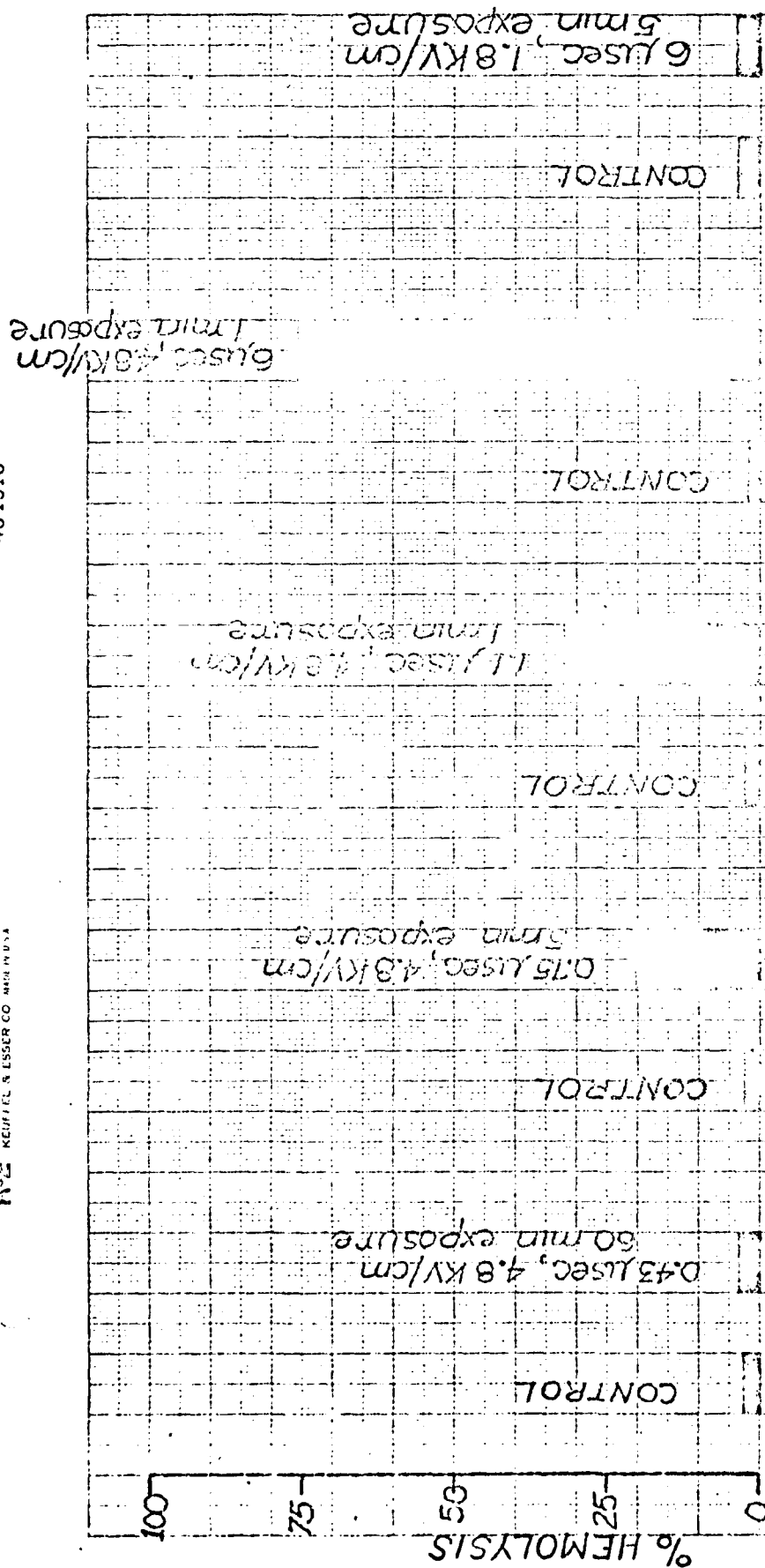
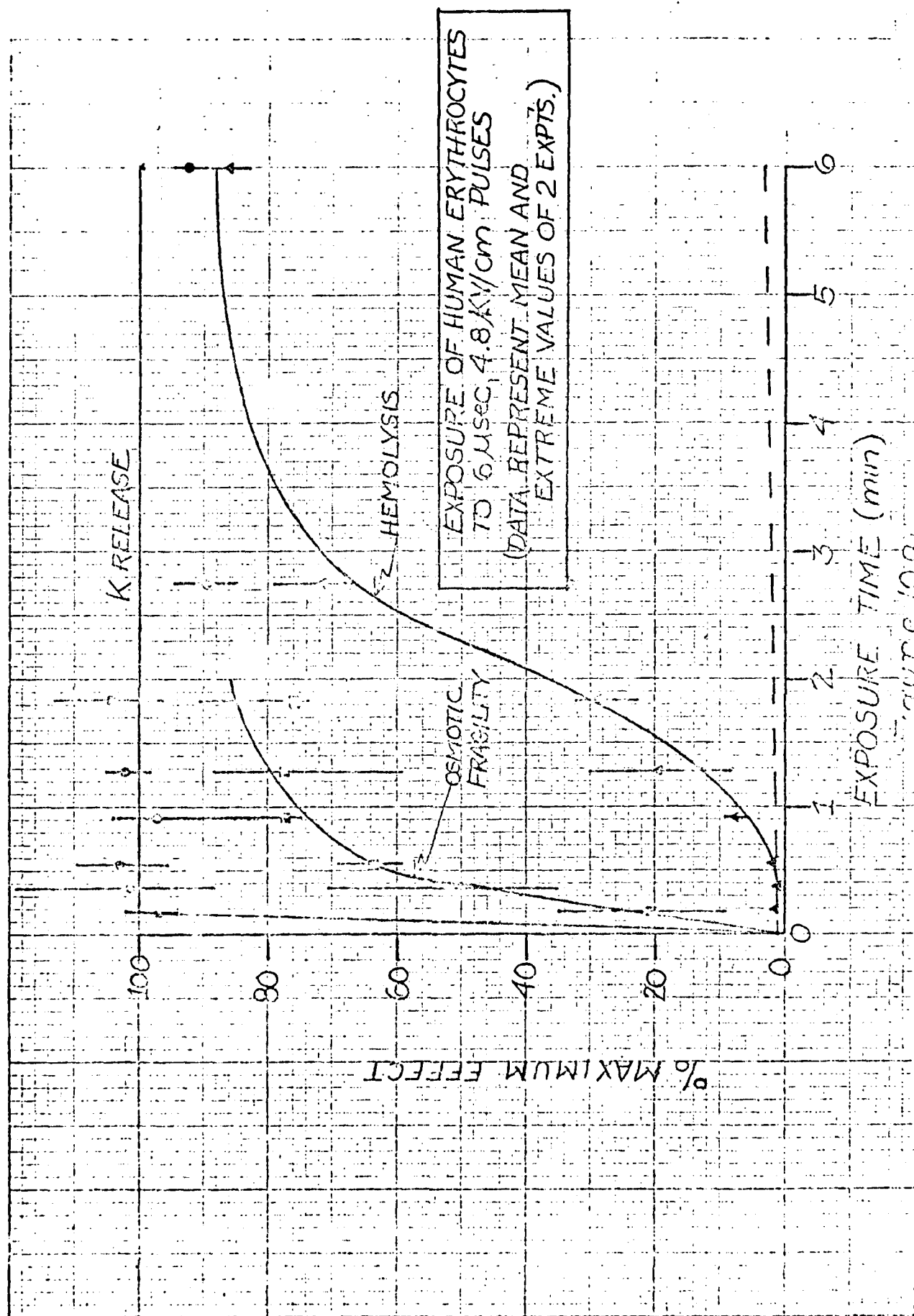


Figure 8



OSMOTIC FRAGILITY OF HUMAN ERYTHROCYTES FOR VARIOUS EXPOSURES
ALIQUOTS OF CELL SUSPENSION INCUBATED IN 0.5% SALINE FOR 30 MIN

Figure 2



EXPOSURE OF HUMAN ERYTHROCYTES TO 0.75 μ sec, 4.8 kV/cm
 VOLTAGE PULSES

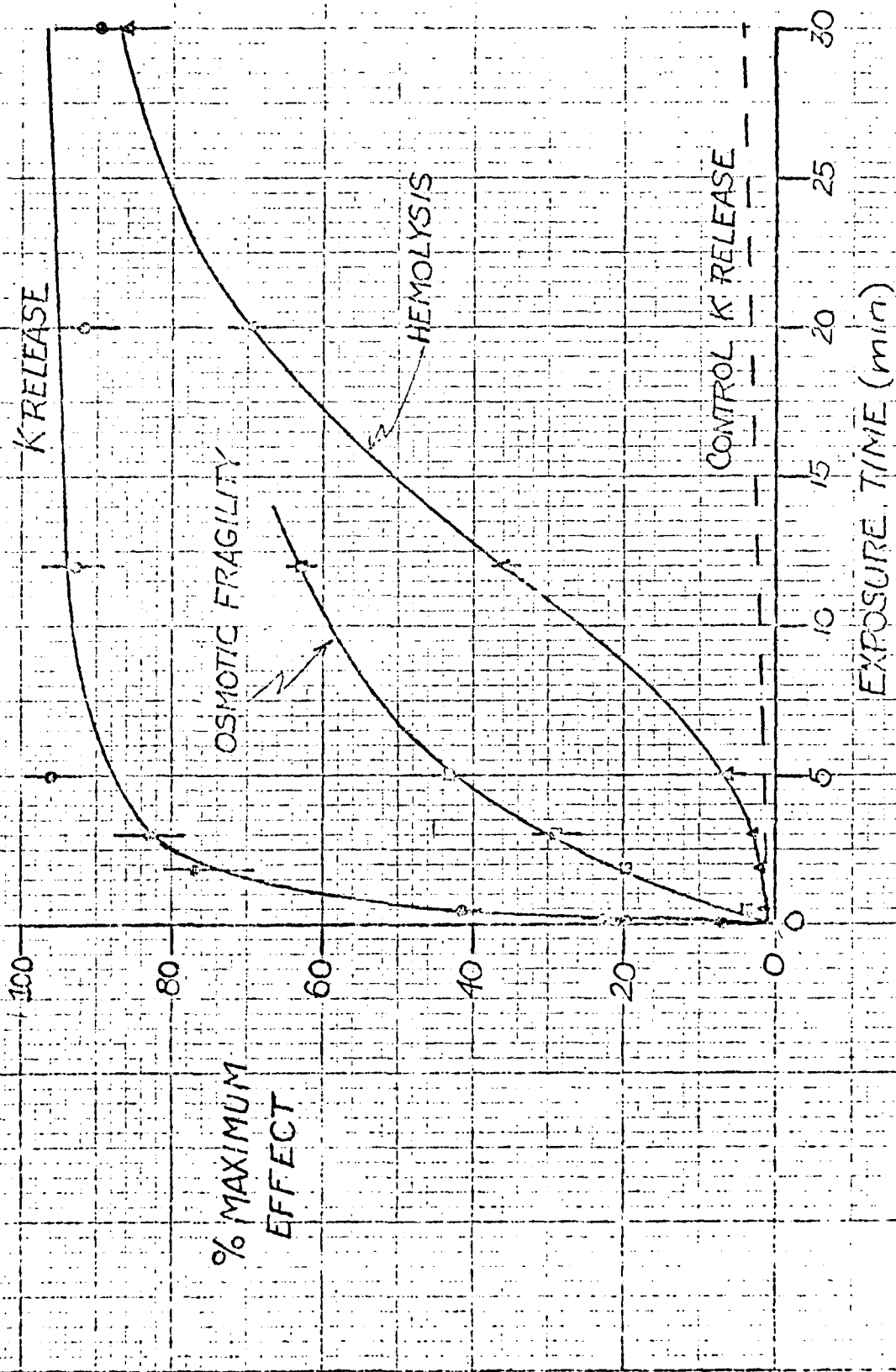
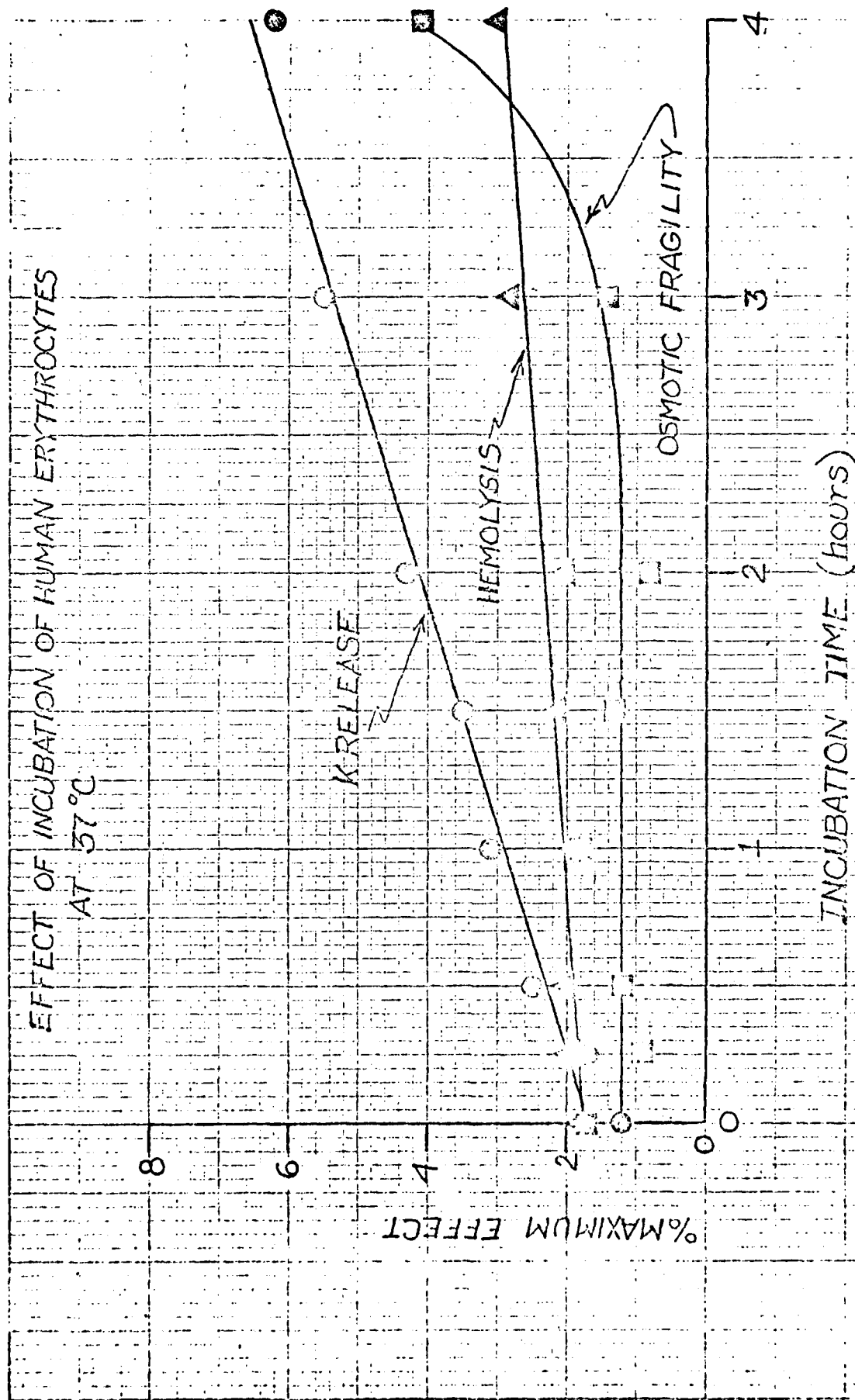


Figure 10b



dependence on pulse time constant has also been noted by Riemann et al. (23), who suggest that the time required to charge the cell membrane capacitance is of the order of 10 μ sec. If this is true, then a reasonable explanation for the dependence of the effects on the pulse time constant, for time constants less than 10 μ sec is that the cell membrane becomes only partially charged and only a fraction of the maximum possible voltage is developed across the membrane. We believe that a more reasonable estimate for the time constant for the charging of the cell membrane capacitance is 0.1 μ sec or less. This time constant can be calculated from either experimental observations or theoretical calculations on the dispersion of cell suspensions. Schwan (37) has shown that the relationship between the characteristic frequency, and the relaxation time constant for a dispersive medium is

$$\tau = (2\pi f_0)^{-1} \quad (3)$$

For a cell suspension, the dispersion and the relaxation process are due to charging of the cell membrane capacitance. Bernhardt and Pauly (38) have calculated the characteristic frequency for various cell shapes. For disk shaped cells the size of erythrocytes in dilute 0.9% NaCl solution, they obtain the following values. With the short semi-axis of the cell parallel to the external field f_0 is 4.8 MHz, for which the corresponding relaxation time is 33 nsec. With the short semi-axis of the cell perpendicular to the external field, f_0 is 2.2 MHz, which corresponds to a relaxation time of 72 nsec. Sale and Hamilton (22) used a spherical model for the erythrocyte and estimated that the membrane charging time constant was less than 0.1 μ s. Bernhardt and Pauly state that the experimental values of relaxation frequencies for suspensions of cells of this type are in the range they calculate. It is shown in Appendix III that if the relaxation time constant is τ_r , and the external electric field applied to the cell suspension is a decaying exponential given by

$$E_e(t) = E_0 \exp(-t/\tau_p) \quad (4)$$

then the voltage across the cell membrane $V_m(t)$, at points on the membrane where this voltage is a maximum, is given by

$$V_m(t) = F_j a_j E_0 \left\{ \frac{\tau_p}{\tau_p - \tau_r} \right\} \cdot \{e^{-t/\tau_p} - e^{-t/\tau_r}\} \quad (5)$$

where F_j is a dimensionless shape factor specific for a given cell and its orientation relative to the field and a_j is the semi-major axis parallel to the external field. In Figure 12 we have plotted this equation for the case of the erythrocyte type cell with the minor semi-axis perpendicular to the applied field ($\tau_r = 72 \text{ nsec}$) and for time constants of $6 \mu\text{sec}$ and $0.43 \mu\text{sec}$ for the applied pulse. These are the longest and shortest pulses used in our experiments. The peak membrane voltage produced by the $0.43 \mu\text{sec}$ pulse is about $3/4$ of that produced by the $6 \mu\text{sec}$ pulse. However, a $6 \mu\text{sec}$ pulse with a peak amplitude of 3.6 kV/cm is much more effective than a $0.43 \mu\text{sec}$ pulse with a peak amplitude of 4.8 kV/cm . For example, comparing Figures 7a and 7d we see that the $6 \mu\text{sec}$ pulse produced a 96% potassium release and 65% hemolysis after a 6 minute exposure, whereas the $0.43 \mu\text{sec}$ pulse produced only an 8% potassium release and no hemolysis above the control after the same exposure time at a field strength of 4.8 kV/cm . Even after a 60 minute exposure, the $0.43 \mu\text{sec}$ pulse produced only about 40-50% potassium release and a negligible amount of hemolysis. Possible causes for the time constant dependence will be discussed later.

A third notable feature of the data is the sigmoid relationship of hemoglobin release to exposure time. That the sigmoid character of the curve is not evident for the exposure of rabbit erythrocytes to $6 \mu\text{sec}$ pulses (Fig. 5b) is probably due to the absence of data at very short exposure times corresponding to 1 or a few pulses. The sigmoid shape of the hemoglobin release versus exposure time curve is evident for human erythrocytes for 6, 1.1, and $0.75 \mu\text{sec}$ pulses (Figs. 7 and 10). No hemolysis was observed for rabbit and human erythrocytes with $0.43 \mu\text{sec}$ pulses. The exposure time dependency appears to indicate a progressive weakening of the cell membrane by the electric field pulses until the membrane is

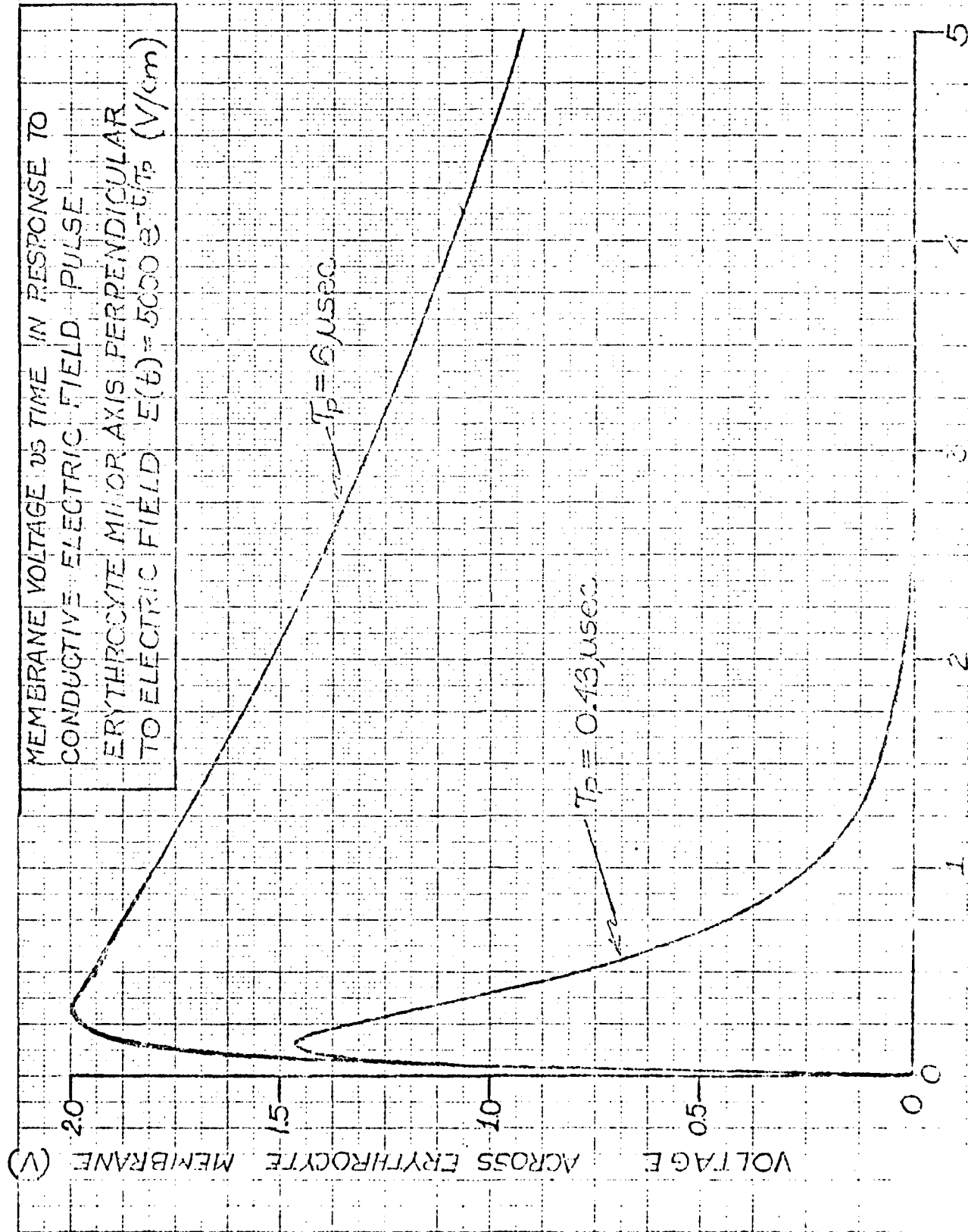


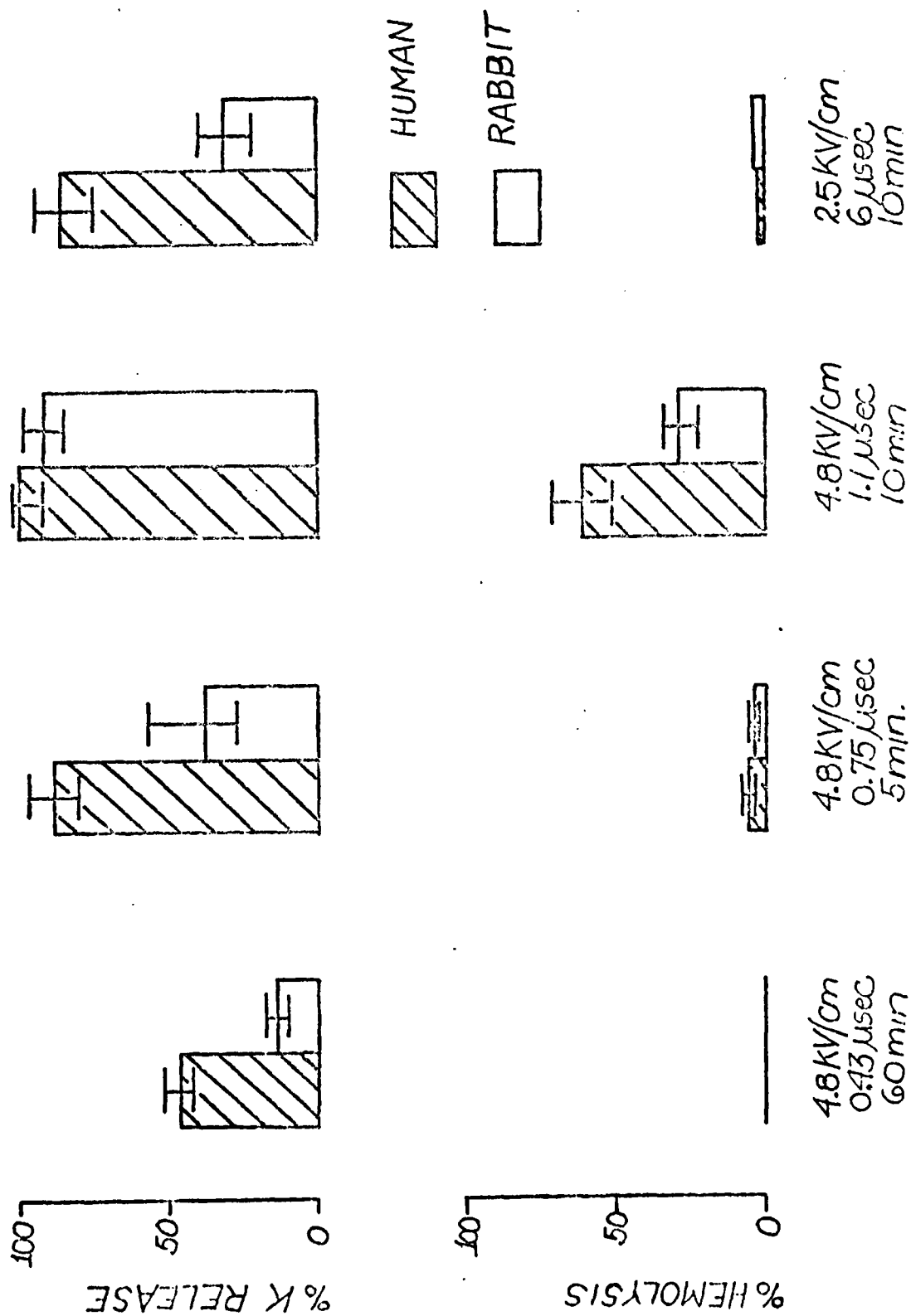
Figure 12

sufficiently altered to allow the release of hemoglobin.

Further evidence that the pulses weaken the cell membrane is provided by the osmotic fragility experiments summarized in Figures 9 and 10. As shown in Fig. 9, the conditions under which there is no effect of the pulses on the osmotic fragility are for time constants of 0.43 μ sec or less and field strengths of 1.8 kV/cm or less. For longer time constants and higher voltages the osmotic fragility of the cells is increased as shown in Fig. 10. A substantial increase in osmotic fragility occurs under conditions where hemolysis is negligible.

We can summarize the data by saying that the first effect of the electric field pulse is to alter the membrane permeability resulting in the efflux of intracellular potassium. Further pulse exposure continues to weaken the membranes such that hemoglobin leaks out when the cells are suspended in hypotonic 0.5% saline. Finally, when a critical number of pulses have been applied to the cells, the membrane is altered to the extent that hemoglobin leaks out of the cells under isotonic conditions. We also note, as indicated in Fig. 13, that human erythrocytes are more sensitive to the effects of the pulses than are rabbit erythrocytes. Although there is insufficient data at present, it appears from preliminary experiments that dog erythrocytes are also more resistant to the effects of pulsed conductive fields than human red cells.

Based on our results, it is useful to consider separate mechanisms for potassium release and for the effect on osmotic fragility and hemoglobin release. Rieman et al. (23) also conclude that hemoglobin release is not necessarily associated with the dielectric breakdown mechanism they propose to account for cellular potassium release. We have not yet determined a mechanism for the effects on osmotic fragility and hemoglobin release. Sale and Hamilton (22) considered the effect of uniform heating of the cell membranes.



COMPARISON OF RABBIT AND HUMAN ERYTHROCYTES UNDER
SELECTED EXPOSURE CONDITIONS

by voltage pulses and found it to be negligible. Tsong et al. (25) have suggested that a thermal gradient which might be produced across the membrane may have a large effect, even if the gradient is of very short duration. Zimmerman et al. (9) considered a theory proposed by Neumann and Rosenheck (32) in which the ion cloud surrounding a cell is displaced by an electric field pulse. They conclude that for cells the size of bovine erythrocytes the time constant for the effect is 820 μ sec, which is much longer than the pulses used in our, and others', experiments on erythrocytes. Thus, ion cloud displacement is not a feasible mechanism for the effects we have detected.

The mechanism that has previously been proposed for potassium release is dielectric breakdown of the cell membrane (23). A difficulty with this theory is related to the fact that Riemann et al. calculate that the time required for the breakdown process is of the order of nanoseconds (23), yet we have found that there is a strong dependence of potassium release on the pulse decay time constant for values less than 10 μ sec. Their suggestion that this is due to a 10 μ sec time constant for charging of the cell membrane does not appear to be valid, as previously discussed. Our data indicate that a minimum time constant of approximately 0.4 μ sec and a minimum field strength of approximately 2 kV/cm are simultaneously required in order to cause cellular potassium release. These facts can be incorporated into a model which suggests that the release of potassium as a result of exposure to 0.75 μ sec and 0.43 μ sec pulses will be an exponential function of the exposure time.

Model for Electric Field-Induced Potassium Release

Assume that an electric field acting upon a cell membrane produces a pore or channel in the membrane through which potassium and other ions diffuse. The dependence of the potassium concentration in the extracellular solution as a function of time may be determined by application of diffusion theory.

If it is assumed that the concentration gradient of potassium in the pore is

linear, then the following relationship is obtained from Fick's law of diffusion
(40)

$$dN/dt = -DA (C_{in} - C_{out}) x^{-1} \quad (6)$$

where N = number of moles of potassium in the cell,

D = pore area or the total area of all pores if there is more than one cell,

X = the length of the pore,

C_{in} = concentration of potassium inside the cell

and C_{out} = concentration of potassium outside the cell.

It will be assumed further that C_{in} and C_{out} are uniform inside and outside of the cell.

Since the total number of potassium ions is conserved, we have

$$dN/dt = V_{in} dC_{in}/dt = -V_{out} dC_{out}/dt \quad (7)$$

where V_{in} and V_{out} are the interior and exterior cell volumes, respectively.

If there is more than one cell, V_{in} is the total interior volume of all cells.

From Eq (7) we have

$$\frac{dC_{out}}{dt} = \frac{-V_{in}}{V_{out}} \frac{dC_{in}}{dt} \quad (8)$$

Taking the time derivative of both sides of Eq. (6) and using Eq. (7) gives,

$$V_{out} \frac{d^2 C_{out}}{dt^2} = \frac{DA}{X} \left\{ \frac{dC_{in}}{dt} - \frac{dC_{out}}{dt} \right\}$$

Using Eq. (8),

$$V_{out} \frac{d^2 C_{out}}{dt^2} = -\frac{DA}{X} \left\{ \frac{V_{out}}{V_{in}} + 1 \right\} \frac{dC_{out}}{dt}$$

$$\text{or } \frac{XV_{\text{out}}}{DA} \left\{ \frac{V_{\text{in}}}{V_{\text{out}} + V_{\text{in}}} \right\} \frac{d^2 C_{\text{out}}}{dt^2} + \frac{dC_{\text{out}}}{dt} = 0$$

$$\text{or } \tau_D \frac{d^2 C_{\text{out}}}{dt^2} + \frac{dC_{\text{out}}}{dt} = 0 \quad (9)$$

$$\text{where } \tau_D = \frac{XV_{\text{out}}}{DA} \left\{ \frac{V_{\text{in}}}{V_{\text{out}} + V_{\text{in}}} \right\}$$

The solution of Eq. (9) is

$$C_{\text{out}} = C^0 + (C^\infty - C^0) (1 - \exp(-t/\tau_D)), \quad (10)$$

where C^0 and C^∞ are the initial and final concentrations, respectively. If the pore is transient in duration, t in Eq. (10) is replaced by t_e , the effective time during which the channel is open. We can write

$$t_e = Ft,$$

where t is the actual time of exposure to the electric field and F is the fraction of the exposure time that the cell membrane pore is open.

An expression for F may be derived on the basis of the following model.

When the cell membrane potential have been above a threshold voltage V_c for a time t_c , a pore opens in the membrane. The pore persists as long as the membrane voltage remains above V_c and closes when the voltage drops below V_c . Assuming for simplicity, that to a first approximation the voltage across the membrane V_m is exponential in time.

$$V_m = V^0 \exp(-t/\tau_p).$$

V_c and V^0 are related by

$$V_c/V^0 = \exp(-\alpha) = \exp(-t_f/\tau_p) \quad (11)$$

where $t_f = \alpha\tau_p$ is the time during a single exponential pulse that the voltage

across the membrane is greater than or equal to V_c . The time t_{fe} that the pulse is effective in producing a pore is

$$t_{fe} = t_f - t_c.$$

If f is the pulse repetition rate, we have

$$F = f t_{fe}.$$

In Appendix II it is shown that

$$f = (\beta \tau_c)^{-1} = (\beta R_c C)^{-1}$$

Thus,

$$F = (\beta \tau_c)^{-1} (t_f - t_c) = (\beta \tau_c)^{-1} (\alpha \tau_p - t_c)$$

But, $\tau_p = R_s C$ (see Appendix II), so that

$$F = (R_s / \beta R_c) (\alpha - t_c / \tau_p) \quad (12)$$

Equation 12 gives the fraction of the total exposure time during which potassium diffuses from the cell. For every type of ion or molecule that diffuses through the pore, a time dependent concentration will develop according to Eq. (10). Each species of ion or molecule will have a different value of τ_p whereas the value of F will be the same for all ions or molecules.

In summary, we thus assume that after the voltage across the cell membrane has exceeded a critical voltage V_c for a time t_c , a pore or channel is produced in the membrane. The channel persists as long as the membrane voltage remains above V_c and closes when the voltage drops below V_c . During the time the pore is open, ions are assumed to diffuse into and out of the cell through the field-induced pore. The diffusion leads to an exponential change in the ion concentrations which has the time dependence $\exp(-t_e / \tau_p)$ where t_e is the effective time the membrane channel is open and τ_p depends on the diffusion

coefficient and the relative sizes of the channel, the cell, and the external solution. F is the fraction of the exposure time that a pore is open. Thus, F has a dependence on the pulse decay time constant τ_p of the form

$$F = a - b/\tau_p,$$

where a and b are constants as defined in Eq. 12.

For a given amount of potassium release t_e is a constant, C . Thus, if T is the actual exposure time required to produce the potassium release, we have

$$(a - b/\tau_p) \cdot T = C$$

or

$$a - b/\tau_p = C/T$$

A plot of τ_p^{-1} vs T^{-1} should be a straight line if the theory is correct. The present data are not sufficient to determine the validity of this relationship, however. Further work will include exposures to pulses with several different decay time constants less than 0.75 μsec and below 0.4 μsec . The determination of the time T required for a given fractional potassium release for each τ_p will then be plotted as described above to test the theory.

Exposure to 3 GHz Microwaves

For the exposure of erythrocytes to continuous wave microwave radiation, 6 ml of 1:1 suspension of erythrocytes was placed in a plastic tube centered in a section of S band waveguide. The electric field in the waveguide was parallel to the axis of the sample tube. The measured power absorbed by the tube and cell suspension was 245 mW or 41 mW/cm³. This was approximately equivalent to a 40 mW/cm² irradiation in the far field. One control was held at 3°C while a second was kept at room temperature, 25°C. The frequency of the microwave radiation was 3GHz, and the exposure time was 3 hours.

The results of the exposure of rabbit erythrocytes to 3 GHz microwaves are summarized in Table 6. There is no significant differences in the dependent variables between the irradiated sample and the room temperature (25°C) control

TABLE 6 EXPOSURE OF RABBIT ERYTHROCYTES TO 3 GHz MICROWAVES
Mean and Standard Deviation of 4 to 6 determinations for each sample.

Sample	Exposure Time (hrs)	% Potassium Release	Osmotic Fragility (% Hemolysis in 0.5% Saline)	% Hemolysis
25°C Control	0.75	1.8 +0.1	32.0 +3.3	1.8 +0.1
25°C Control	3	3.5 +0.2	31.9 +3.9	2.8 +0.3
27°C Control	3	6.3 +0.3	49.0 +2.6	2.8 +0.3
Exposed to Microwaves Final Temp. 34°C	3	3.1 +0.2	40.2 +1.8	2.1 +0.1

sample. These results are not consistent with those of Baranski et al. (35), who reported that a 3 hour exposure of rabbit erythrocytes to 3GHz microwaves at $1\text{mW}/\text{cm}^2$ produced 10.8 mg% potassium release (equivalent to 19.4% potassium release) and at a far field power density of $10\text{mW}/\text{cm}^2$ an 18.0 mg% potassium release (equivalent to 32.4% release) was reported. This contrasts with our result of 3.1% potassium release using the equivalent power density of approximately $40\text{mW}/\text{cm}^2$ during a 3 hr irradiation. Similar disagreement is found for the hemolysis results. The osmotic fragility results are not directly comparable with those of Baranski et al. (35). Our results are compatible with the results of the pulsed conductive field study which shows that a minimum field of the order of $2\text{kV}/\text{cm}$ is required to produce any effect on the erythrocyte membrane. The maximum electric field in the waveguide in our experiment was on the order of only $10\text{V}/\text{cm}$. Thus one would not expect any microwave effect on the erythrocyte membrane permeability for potassium or hemoglobin if it is assumed that the alterations are dependent only upon the induced field strength in the membrane and the pulse duration. Microwave irradiation resulted in a 26% increase in osmotic fragility but exposure of erythrocytes to a temperature of 37°C for the same duration (ie. 3 hr) led to a 54% increase thus suggesting that the apparent increase in the exposed sample is attributable to microwave-induced heating.

IV SUMMARY AND CONCLUSIONS

The investigation of the effects of pulsed electrical fields on mammalian erythrocytes indicates that such fields produce transient pores or channels in the cell membrane as evidenced by the release of intracellular potassium ions and hemoglobin (and perhaps other intracellular protein molecules). The release has been found to be strongly dependent upon the duration of the electric field pulse as well as the amplitude of the electric field. Significant intracellular potassium release occurs under exposure conditions that do not result in release of protein molecules, suggesting that the size of the induced pore is dependent upon the induced field strength and the duration of the field. The mechanism of

dielectric breakdown of cell membranes does not adequately account for these results. Thus the mechanism for increased membrane permeability is not presently understood and additional studies are required to develop such a mechanism.

✓ The results of studies of the relationship of field strength and pulse duration for the rupture of an artificial bilayer lipid membrane (oxidized cholesterol) indicate a dependency on the pulse duration that is consistent with the effects upon cell membrane permeability. It is not possible, at this time, however, to directly relate these effects since the mechanisms are not well enough understood in either instance. The fact that electrical field-induced alterations in in vitro membrane model systems have been determined to depend upon field strength and pulse duration suggest the need for additional investigations of the involvement of these independent variables in in vivo exposure of mammalian systems.

The in vivo studies ~~we have conducted~~ have involved the exposure to Dutch rabbits to repetitively pulsed electromagnetic fields in an EMP simulator. Such exposure has not been found to result in significant alterations in a number of physiological response variables including the duration of drug induced sleeping time and serum chemistry changes, although there is some suggestion (non-statistically significant) of a post-exposure increase in certain serum enzymes. ← More extensive studies on the effects of EMP exposure on the serum levels of creatine phosphokinase isoenzymes suggest a consistent, albeit, small enzyme elevation. In view of the absence of changes in the other response variables it does not appear that the elevations in CPK isoenzyme levels are due to cell death. An alternative explanation for these enzyme elevations is an increase in cell membrane permeability induced by EMP exposure. Although this mechanism of interaction is consistent with the in vitro results in the model systems investigated, the differences in the exposure conditions in the in vivo and in vitro studies preclude a direct comparison of these results at this time. The conditions for the in vivo EMP exposure are such that the pulse duration cannot be varied

and the field strength cannot be varied independently of the pulse repetition rate. The in vitro investigations of model membrane systems can be used to define the conditions for the induction of transient alterations in membrane permeability but the application of such findings to an assessment of in vivo effects in mammalian systems will require the use of an exposure facility with greater flexibility than that presently available.

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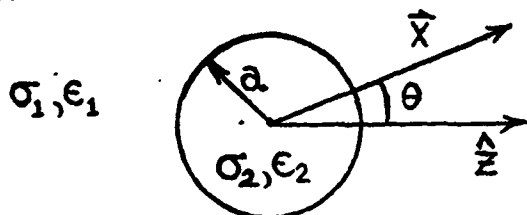
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Appendix I Field Induced in a Sphere by an Electric Field Pulse

As a first step in the calculation we calculate the field induced in a sphere by an A.C. Field. The model is the following, a sphere of conductivity σ_2 and dielectric constant ϵ_2 is imbedded in a medium of conductivity σ_1 and dielectric constant ϵ_1 . A uniform, harmonic electric field of frequency ω is applied. We will find the electric field at all points in the sphere.



σ_1 = conductivity in region 1.

ϵ_1 = permittivity in region 1.

a = sphere radius

Far away from the sphere the electric field is $E = E_0 e^{j\omega t} \hat{z}$ or

$$1) \phi(\vec{X}) = -E_0 e^{j\omega t} z = -E_0 e^{j\omega t} r \cos\theta$$

This is one boundary condition.

The second boundary condition is the equality of the normal components of the total current J . $J_{1n} = J_{2n}$ on the sphere boundary, where J_{1n} is the normal component of J in region 1. In terms of σ and E this condition is

2) $\sigma_1 E_{1n} = \sigma_2 E_{2n}$ on the sphere boundary, where E_{1n} = normal component of E in region 1. The last boundary condition is,

3) $E_{1t} = E_{2t}$ on the sphere boundary, where E_{1t} is tangential component of E in region 1. We have assumed no sources of charge or current in the volume of interest so that

$$\nabla \cdot \vec{J}_{\text{total}} = \nabla \cdot \left[\sigma \vec{E} + \epsilon \left(\frac{\partial \vec{E}}{\partial t} \right) \right] = 0$$

Since E is harmonic we have $\left(\frac{\partial \vec{E}}{\partial t} \right) = j\omega \vec{E}$, so that

$$\nabla \cdot \vec{E} = 0$$

In general $\vec{E} = -\nabla \phi - \frac{\partial \vec{A}}{\partial t}$. If we can neglect the vector potential \vec{A} 's

contribution (i.e., $|j \omega A| \ll |\nabla \phi|$) we have

$$4) \nabla^2 \phi = 0,$$

which is Laplace's equation. In spherical coordinates 4) is

$$\nabla^2 \phi = \frac{1}{r^2} \frac{\partial}{\partial r} \left(r^2 \frac{\partial \phi}{\partial r} \right) + \frac{1}{r^2 \sin \theta} \frac{\partial}{\partial \theta} \left(\sin \theta \frac{\partial \phi}{\partial \theta} \right) + \frac{1}{r^2 \sin^2 \theta} \frac{\partial^2 \phi}{\partial \phi^2} = 0$$

where r, θ , and ϕ are the usual spherical coordinates. Separating variables so that $\phi(r, \theta, \phi) = f(r) P(\theta) X(\phi)$ gives the solution in terms of the spherical harmonics.

$$5) \phi(r, \theta, \phi) = \sum_{n=0}^{\infty} \sum_{m=-n}^n (A_{n,m} r^n + B_{n,m} r^{-n-1}) Y_n^m(\theta, \phi)$$

where $A_{n,m}$ and $B_{n,m}$ are constants to be determined, and Y_n^m are the spherical harmonics. Since we have axial symmetry there is no ϕ dependence and only the $m = 0$ terms, $C^{im\phi} = 1$, remain in (5) and $Y_n^0 = P_n(\cos \theta)$, where $P_n(\cos \theta)$ is the Legendre polynomial. Now we write a general solution of ϕ in regions 1 and 2. In region 1 as $r \rightarrow \infty$, $\phi^{(1)} \rightarrow E_0 r \cos \theta = E_0 r P_1(\cos \theta)$.

Thus the most general solution in region 1 is

$$6) \phi^{(1)} = E_0 r \cos \theta + \sum_{n=0}^{\infty} C_n^{(1)} r^{-n-1} P_n(\cos \theta), \text{ where } C_n^{(1)} \text{ are constants}$$

to be determined. In region 2, ϕ must be finite for $r = 0$, thus

$$\phi^{(2)} = \sum_{n=0}^{\infty} C_n^{(2)} r^n P_n(\cos \theta)$$

Now we use the boundary conditions, Eqs. 2) and 3)

$$E_n = (-\nabla \phi)_n = -\left(\frac{\partial \phi}{\partial r} \hat{r} + \frac{1}{r} \frac{\partial \phi}{\partial \theta} \hat{\theta} + \frac{1}{r \sin \theta} \frac{\partial \phi}{\partial \phi} \hat{\phi} \right) \cdot \hat{r}$$

where $\hat{r}, \hat{\theta}$ and $\hat{\phi}$ are the unit vectors in the spherical coordinate system.

Thus $E_n = -\frac{\partial \Phi}{\partial r}$

$$E_t = E_\theta \hat{\theta} + E_\phi \hat{\phi} \quad , \text{ where } E_\theta = -\frac{1}{r} \frac{\partial \Phi}{\partial \theta} \text{ and } E_\phi = \frac{1}{r \sin \theta} \frac{\partial \Phi}{\partial \phi}$$

Since $\frac{\partial \Phi}{\partial \phi} = 0$ we have

$$E_t = -\frac{1}{r} \frac{\partial \Phi}{\partial \theta}$$

Instead of continuity of E_t we can use continuity of Φ as the second boundary condition. Both lead to the result above that at the boundary, where $r = a$,

$$7) -E_0 a + C_1^{(1)} \cdot \frac{1}{a^2} = C_1^{(2)} a$$

$$8) C_0^{(1)} = C_0^{(2)} a$$

$$9) C_n^{(1)} = a^{2n+1} C_n^{(2)}, n \geq 2$$

Since the potential is arbitrary to a constant, we set $C_0^{(1)} = 0, C_0^{(2)} = 0$.

Now using 2) and the fact that P_n 's are orthogonal gives

$$10) -\sigma_1 [E_0 - 2C_1^{(1)} a^{-3}] = -\sigma_2 C_1^{(2)}$$

$$-\sigma_1 [-(n+1)a^{-(n+2)} C_n^{(1)}] = -\sigma_2 [nC_n^{(2)} a^{n-1}]$$

$$11) C_n^{(1)} = -\frac{\sigma_2}{\sigma_1} \left(\frac{n}{n+1} \right) a^{2n+1} C_n^{(2)} \quad n \geq 2$$

Comparing 9) and 11) gives

$$C_n^{(1)} = C_n^{(2)} = 0, n \geq 2$$

We rewrite 7) and 10) as

$$7) C_1^{(1)} - a^3 C_1^{(2)} = +E_0 a^3$$

$$10) \frac{2}{a^3} C_1^{(1)} + \frac{\sigma_2}{\sigma_1} C_1^{(2)} = -E_0$$

From 7'), $C_1^{(1)} = a^3 C_1^{(2)} - E_0 a^3$. Substituting in 10') gives

$$\frac{2}{a^3} [a^3 C_1^{(2)} - E_0 a^3] + C_1^{(2)} (\sigma_2 / \sigma_1) = E_0$$

$$(2 + \sigma_2 / \sigma_1) C_1^{(2)} = 3 E_0$$

$$C_1^{(2)} = -(3 \sigma_1 / (2 \sigma_1 + \sigma_2)) E_0$$

$$C_1^{(1)} = a^3 (C_1^{(2)} + E_0) = a^3 \left(\frac{-3 \sigma_1 + 2 \sigma_1 + \sigma_2}{2 \sigma_1 + \sigma_2} \right) E_0$$

$$C_1^{(1)} = - \left(\frac{\sigma_1 - \sigma_2}{2 \sigma_1 + \sigma_2} \right) a^3 E_0 = \frac{\sigma_2 - \sigma_1}{2 \sigma_1 + \sigma_2} a^3 E_0$$

$$\Phi^{(1)} = (-E_0 r + \frac{\sigma_2 - \sigma_1}{2 \sigma_1 + \sigma_2} \frac{a^3}{r^2} E_0) \cos \theta$$

$$\Phi^{(1)} = - \left[1 - \left(\frac{\sigma_2 - \sigma_1}{2 \sigma_1 + \sigma_2} \right) \left(\frac{a}{r} \right)^3 \right] E_0 r \cos \theta$$

$$\Phi^{(2)} = - \left(\frac{3 \sigma_1}{2 \sigma_1 + \sigma_2} \right) E_0 r \cos \theta$$

Thus inside the sphere

$$E^{(2)} = -\nabla \Phi^{(2)} = \nabla \cdot \left[\left(\frac{3 \sigma_1}{2 \sigma_1 + \sigma_2} \right) E_0 \hat{z} \right]$$

$$\vec{E}^{(2)} = \left(\frac{3 \sigma_1}{2 \sigma_1 + \sigma_2} \right) E_0 \hat{z}$$

In general $\sigma_i = \sigma_{cond} + j\omega\epsilon = \sigma_{cond} + j\omega\epsilon'\epsilon_0$, ϵ' = dielectric constant in MKS units.

So we can make the replacement $\sigma_i = \sigma_i^*$, or by factoring $j\omega\epsilon_0$ we have

$$\sigma_i^* = j\omega\epsilon_0 (\epsilon_i' - \frac{\sigma_{cond}}{\omega\epsilon_0} j) = j\omega\epsilon_0 \epsilon_i^*$$

$$\text{Thus } E^{(2)} = \left(\frac{3 \sigma_1^*}{2 \sigma_1^* + \sigma_2^*} \right) E_0 \hat{z} = \left(\frac{3 \epsilon_1^*}{2 \epsilon_1^* + \epsilon_2^*} \right) E_0 \hat{z}$$

That is, inside a sphere in a uniform harmonic electric field E_0 we have

$$E(\text{sphere}) = \left(\frac{3\epsilon_1^*}{2\epsilon_1^* + \epsilon_2^*} \right) E_0 \hat{z}$$

ϵ_1^* is the complex dielectric constant of the external medium.
 ϵ_2^* is the complex dielectric constant of the sphere.

If the external medium is air

$$\epsilon_1^* \approx 1$$

If the frequency is sufficiently low

$$\epsilon_2^* = \epsilon_2' - j \left(\frac{\sigma_2}{\omega \epsilon_0} \right) \approx -j \frac{\sigma_2}{\omega \epsilon_0}$$

$$\left(\frac{3\epsilon_1^*}{2\epsilon_1^* + \epsilon_2^*} \right) \approx \left(\frac{3}{2 - j \frac{\sigma_2}{\omega \epsilon_0}} \right) \approx \left(\frac{3\omega \epsilon_0}{\sigma_2} \right) E_0 j \hat{z} \quad \text{if } \frac{\omega \epsilon_0}{\sigma_2} \ll \frac{1}{2}$$

ie. the fields inside and outside the sphere are 90° out of phase and

$$11) E(\text{sphere}) = \left(\frac{3\omega \epsilon_0}{\sigma_2} \right) E_0 j \hat{z}$$

For biological tissue $\sigma_2 \approx 10^{-2} \text{ mho} \cdot \text{cm}^{-1} = 1 \text{ mho} \cdot \text{m}^{-1}$

$$\epsilon_0 = (36\pi \times 10^9)^{-1} \text{ farads/m} = 9 \times 10^{-12} \text{ farads/m}$$

$$\frac{3\omega \epsilon_0}{\sigma_2} = \frac{3\omega \cdot 9 \times 10^{-12}}{1} \approx 1.7 \times 10^{-10} \text{ f} = \frac{f}{6 \times 10^{10}}$$

where f is the frequency in Hertz.

Thus for a sphere of biological tissue of conductivity $10^{-2} \text{ mho} \cdot \text{cm}^{-1}$ in a uniform harmonic external field $E_0 e^{j\omega t}$, the field in the sphere is approximately

$$E(\text{sphere}) = \left(\frac{f(\text{Hz})}{6 \times 10^{10}} \right) E_0 j \hat{z} \quad \text{where, } \hat{z} = \text{unit vector in the } z \text{ direction and } j = \sqrt{-1}.$$

This is the result obtained by Schwan (33)

Using the general expression in equation (11) we can derive the field induced by a pulse by using the technique of Fourier analysis. For a harmonic variation in the external field $E_o(\omega, t)$ the field induced in the sphere is

$$E_i(\omega, t) = \frac{\omega}{\omega_o} E_o(\omega, t) j = \frac{\omega}{\omega_o} E_o(\omega) e^{j\omega t} j$$

where $\omega_o = \sigma_2 / 3\epsilon_o$ is a constant dependent on the conductivity of the sphere.

We can consider the $E_i(\omega, t)$ to be the Fourier components of a complex wave - e.g. a pulse, in which case the field induced in the sphere is given by the Fourier integral

$$\begin{aligned} E_i(t) &= \int_0^{\infty} E_i(\omega, t) e^{j\omega t} d\omega \\ &= \int_0^{\infty} \frac{j\omega}{\omega_o} E_o(\omega) e^{j\omega t} d\omega \\ &= \frac{1}{\omega_o} \frac{\partial}{\partial t} \int_0^{\infty} E_o(\omega) e^{j\omega t} d\omega \end{aligned}$$

Thus

$$12) \quad E_i(t) = \omega_o^{-1} \frac{d}{dt} E_o(t)$$

This is the \hat{x} component of a similar expression derived by Guy for an electromagnetic pulse (40).

APPENDIX II Physical Characteristics of Transient Electrical Field Cell Exposure

The physical characteristics of the apparatus used in this study for the exposure of erythrocytes to pulses conductive electrical may be used to determine: 1) the energy E and power P dissipated in the cell sample volume per pulse, 2) maximum temperature rise per pulse $\Delta\theta$, and 3) the time during a given pulse that the voltage to which the cells are exposed is above a given critical value.

The parameters of the exposure system may be designated as follows:

- a) pulse circuit charging time constant $= \tau_c = R_c C$
- b) pulse circuit discharge time constant $= \tau_D = R_s C$
- c) interpulse duration $= T$
- d) pulse repetition rate $= f = T^{-1}$
- e) sample voltage $= V = V_c \exp(-t/\tau_D)$
- f) volume of cell suspension in exposure chamber $= v$

where R_c is the resistance of the charging circuit as shown in Figure 2, C is the capacitance of the energy storage capacitor, R_s is the sample resistance, and V_c is the energy storage capacitor (C) voltage at discharge.

- 1) The total energy delivered to the sample per pulse is given by

$$E = \int_0^{\infty} V(t)^2 R_s^{-1} dt = V_c^2 / R_s \int_0^{\infty} (e^{-t/\tau_D})^2 dt$$

$$= V_c^2 \tau_D / 2 R_s \quad (\text{II-1})$$

Typical values for the parameters in this study are : $V_c = 1.6 \text{KV}$, $\tau_D = 6 \mu\text{sec}$, $R_s = 100 \Omega$, and $v = 0.2 \text{ cm}^3$.

The energy delivered to the sample per pulse is thus $E = 0.38 \text{ J/cm}^3$ or 0.092 cal/cm^3 and the dissipated power $P = E/\tau$, where τ is the pulse duration which may be assumed equal to τ_D . Thus $P = 64 \text{ KW/cm}^3$

- 2) The maximum temperature rise for a $6 \mu\text{sec}$ pulse calculated by assuming a specific heat of the cell suspension of $1 \text{ cal/gm}^\circ\text{C}$ and a density of 1 gm/cm^3

is thus $\Delta\theta = 0.092^\circ\text{C}$. Since the average energy absorbed by the sample is directly proportional to the pulse duration (τ), a pulse of duration less than $6\mu\text{sec}$ will result in a temperature rise of less than 0.092°C .

The time between pulses depends upon the power supply voltage (V_p) and the capacitor discharge voltage (V_c). Assuming that the pulse duration τ is small compared to the interpulse duration T , the following relationship may be used to determine T ,

$$V_c = V_p \left\{ 1 - \exp(-T/\tau_c) \right\},$$

thus

$$T = -\tau_c \ln(1 - V_c/V_p). \quad (\text{II-2})$$

Since $V_c = 1.6 \text{ KV}$ and $V_p = 2 \text{ KV}$, $T = 1.6 \tau_c = 1.6 R_c C$.

The average power dissipated in the sample during T is thus

$$\bar{P} = E/T = V_c^2 / 3.2 R_c. \quad (\text{II-3})$$

The average power thus depends only upon the discharge voltage V_c and the charging resistance R_c . Letting $V_c = 1.6 \text{ KV}$ and $R_c = 10^8 \Omega$, $\bar{P} = 8 \text{ mW/cm}^3$ or if a sample density of 1 g/cm^3 is assumed, the average power is 8 mW/gm .

3) The average time t_0 that the voltage applied to the cell suspension is greater than a given value, V_0 , for the exposure system used in this study may be calculated from a knowledge of the voltage waveform.

$$V_0 = V_c e^{-t_0/\tau_D}$$

Solving for t_0 yields

$$t_0 = \tau_D \ln(V_c/V_0) \quad (\text{II-4})$$

The average time \bar{t} the voltage is above V_0 in the case of repetitive pulsing is given by $\bar{t}_0 = R_s \ln(V_c/V_0)/(1.6 R_c)$ (II-5)

Since R_s , R_c , and V_c are held constant in this study, the average duration the cell sample voltage exceeds a given critical value V_0 is independent of the pulse decay time.

APPENDIX III Calculation of the Voltage across Cell Membranes Produced by an External Electric Field.

As discussed in the text, the charging time constant for the capacitance of a cell membrane is the same as the relaxation time constant τ_R measured in dielectric dispersion studies. Given that the response of the membrane voltage to a step change in the external electric field is an exponential change with time constant τ_R , we can use the concepts of the transfer function and Laplace transform (41) to determine the membrane voltage for an arbitrary applied field pulse. We will first calculate the membrane transfer function and then use the transfer function to calculate the membrane voltage for an applied field which is an exponentially decaying pulse.

The transfer function $H(S)$ is defined in the transformed domain, S , by

$$H(S) = \frac{R(S)}{I(S)}, \quad (\text{III-1})$$

where $I(S)$ is the input and $R(S)$ is the response in the transformed domain.

If $i(t)$ and $r(t)$ are the input and response in the time domain then

$$r(t) = L^{-1} (I(S) H(S)),$$

where L^{-1} is the inverse Laplace transform.

The voltage across the cell membrane at points on the membrane where the voltage is a maximum is related to a uniform D.C. electric field E_0 as follows (38).

$$V_M = F_j a_j E_0, \quad (\text{III-2})$$

where F_j is a dimensionless form factor specific for a given cell shape and orientation relative to the field and a_j is the semi-major axis of the cell parallel to the external field. This result and those to follow are exact only in the case of an infinitely dilute suspension of cells. However, related theories have been shown to be accurate to within a few percent for solid spheres in a concentration of 36% (43) and to give good agreement with

experiment for suspensions of uniform spherical cells near total packing (44). The results should apply quite well to the experiments of Riemann et al. (23) in which the cell concentrations were 1% - 10%.

For a step change of E_0 we have

$$r(t) = V_m(t) = F_j a_j E_0 (1 - \exp(-t/\tau_R)). \quad (\text{III-3})$$

Using a table of Laplace transforms in Ref. 42 we find that for a unit step change $i(t)$ we have

$$I(S) = \frac{1}{S}$$

And

$$R(S) = F_j a_j [S(1 + \tau_R S)]^{-1}$$

$$\text{Thus } H(S) = R(S)/I(S) = F_j a_j (1 + \tau_R S)^{-1} \quad (\text{III-4})$$

If the input is now

$$i(t) = E_0 \exp(-t/\tau_P),$$

then

$$I(S) = E_0 \tau_P (1 + \tau_P S)^{-1} \quad (\text{III-5})$$

Inserting 4) and 5) in Eq. 1) gives the voltage across the membrane

$$V_m(t) = r(t) = F_j a_j E_0 \left\{ \frac{\tau_P}{\tau_P - \tau_R} \right\} \left\{ e^{-t/\tau_P} - e^{-t/\tau_R} \right\}$$

The maximum value of $V_m(t)$ is found to be at a time t_{\max} given by

$$t_{\max} = \tau_P \tau_R \ln(\tau_P/\tau_R) (\tau_P - \tau_R)^{-1} \quad (\text{III-6})$$

The maximum value of $V_m(t)$ is then

$$V_m^{\max} = F_j a_j E_0 (\tau_P/\tau_R)^{-(\tau_R/(\tau_P - \tau_R))} \quad (\text{III-7})$$

Some useful limits are

$$\lim_{T_p \rightarrow \infty} V_m(t) = F_j \bar{a}_j E_o$$

$$\lim_{T_p \rightarrow 0} V_m(t) = F_j \bar{a}_j E_o (T_p/T_R)$$

$$\lim_{T_p \rightarrow T_R} V_m(t) = F_j \bar{a}_j E_o e^{-1}$$