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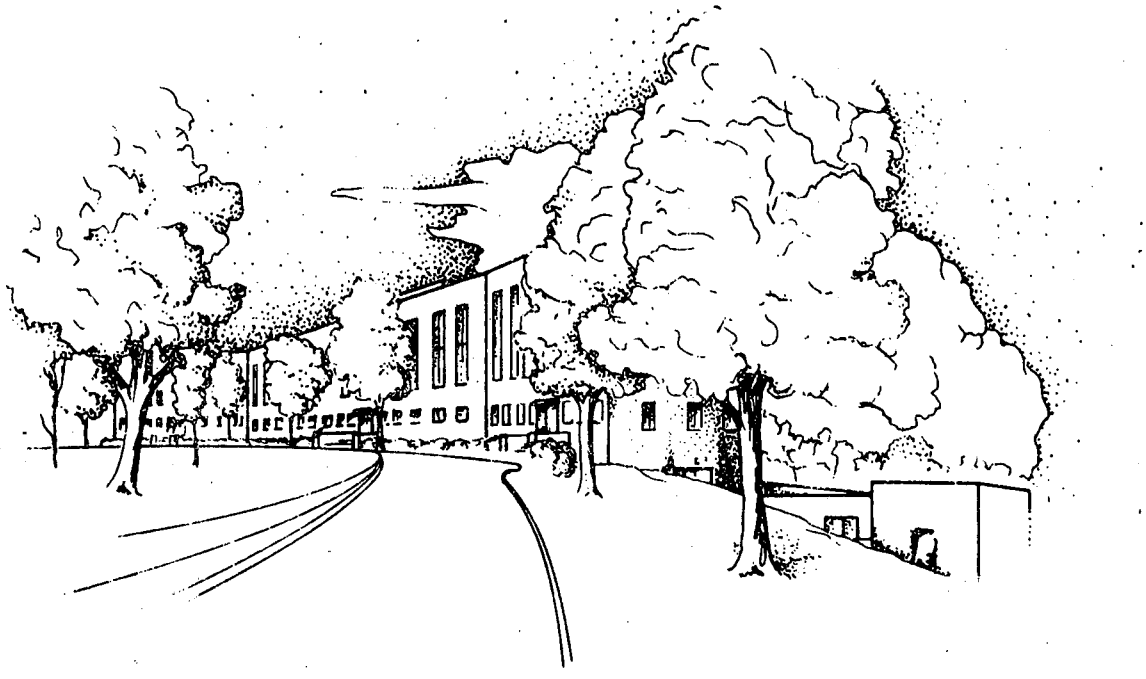

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AND CHYMOTRYPSIN FOLLOWING RADIOFREQUENCY IRRADIATION

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[1]From the Bureau of Medicine and Surgery, Navy Department Research Subtask MF51.524.015-0001BD7X. The opinions and statements contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or of the Naval Service at Large.

ABSTRACT

Protein solutions have been irradiated with pulse modulated radio-frequency energy in the frequency range 10-15 MHz. Frequency-specific structural and functional changes reported by others following such exposures have not been found. No satisfactory explanation for this discrepancy has been determined. However, arguments are presented which suggest that the frequency specificity of effects reported previously is incompatible with the reported experimental parameters.

## INTRODUCTION

There have been many reports of the effect of radiofrequency (rf) and microwave frequency radiation on biologic systems (1,2,3). Effects related to heating are well understood (4); the conversion of electromagnetic energy to heat involves both dielectric absorption and Joule's Law ionic heating. Generally, those effects which can be correlated directly with temperature have been characterized as thermal.

Perhaps the best understood nonthermal effect is "pearl chain formation", an alignment of particles under the influence of an rf field. These phenomena have been termed field-induced-force effects by Schwan et al. (5); according to these authors, such effects cannot be expected to occur in a physiologic milieu in the absence of overt heating phenomena. There have been reported, however, nonthermal effects that occur in the complete absence of any measurable heating. The most intriging example is the phenomenon known as "rf hearing" manifested as a buzzing sensation in humans during exposure to pulse modulated radiation (6).

Structural and/or functional alterations of biologic macromolecules as a consequence of rf exposure have also been reported. The activities of alpha amylase (7,8), cholinesterase (9), chymotrypsin (8), and yeast alchohol dehydrogenase (10) have been studied following exposure to rf fields in the 3 to 30 MHz range. With the exception of the yeast alcohol dehydrogenase, these enzyme activity changes were reported to be independent of temperature.

Takashima (10) found no changes in the chromatographic behavior or viscosity of either bovine serum albumin or hemoglobin solutions following exposure to rf radiation between 1 and 60 MHz.

The studies of Bach et al. (11,12) have been widely cited as examples of alterations in protein structure and function resulting from the nonthermal interaction of a protein moiety with an rf electromagnetic field. These reports described the development of a two-component peak in the paper electrophoretic pattern of human gamma globulin following exposure to certain frequencies; the most pronounced changes were reported to occur following irradiation near 13 MHz as shown in Fig. 1.

The present study is an attempt to reproduce the gamma globulin findings of Bach since they appeared to be the most significant in terms of literature citations and possible biologic significance. In addition to the protein gamma globulin, two enzymes, acetylcholinesterase and chymotrypsin, have been reexamined.

## METHODS AND MATERIALS

Irradiation Source. A Navy URC-32 transmitter system was used as a source of radiofrequency energy. This system consists of a stable frequency source, a power amplifier, and an antenna coupler used to match the 50 ohm output of the power amplifier to the irradiation cell which had a resistive impedance of a few ohms; the exact value depended on the nature of the solution being irradiated.

The frequency source utilizes a synthesizer capable of generating frequencies at 1 kHz intervals from 2 to 30 MHz. The system was calibrated against WWV and had a long term stability of one part in $10^6$.

To allow pulsed operation the output of the stable frequency source was fed to a Hewlett Packard double-balanced mixer (HP 10534A) which was used as an rf gate. This gate provided approximately 45 db of carrier isolation and generated an rf pulse the same width as a dc gating pulse which in the present study was supplied by a Tektronics 160 series pulse generator.

Irradiation Cell. In the study conducted by Bach et al. (11,12) the irradiation cells were constructed of plastic with either silver or platinum electrodes ranging from 2 to 5 $cm^2$ in area and spaced 0.3 cm apart.

In the present study an irradiation cell with cylindrical geometry was constructed of lucite with platinum electrodes 3.2 cm in diameter spaced 0.5 cm apart; the electrodes were coated with "platinum black". A small hole in the side of the cell sufficed for filling and washing.

Sample Preparation. Neither the source nor the purity of the human gamma globulin used by Bach et al. was given in their publications. They stated: "Exposures were performed in 2.2% solution in normal saline or saline with phosphate buffer pH 7.6"; the concentration of buffer was not given. Their paper electrophoretic system was used with a barbituric acid-sodium acetate buffer, pH 8.6, ionic strength 0.125 M.

The present study was conducted with 7S human gamma globulin (Calbiochem, A grade). The gamma globulin was only partially soluble at 2.2 per cent concentration and initial irradiations were performed with cloudy solutions; both the soluble and insoluble fractions were examined electrophoretically following irradiation.

Subsequent studies covered systematically all frequencies between 10 and 15 MHz; for these irradiations a completely soluble preparation was examined using a 0.1 M, pH 8.8 tries barbital buffer with a protein concentration of 1 per cent. The same buffer solution was used for the electrophoretic determinations.

The acetylcholinesterase (Worthington Biochemical) was dissolved in phosphate buffer, pH 7.0, 0.02 M to give a protein concentration of 0.010 mg/ml.

The chymotrypsin ( Worthington, 3X crystallized) was dissolved in 0.001 M HCl to give a protein concentration of 1 mg/ml in the irradiation cell.

Irradiation Protocol. Approximately 10 ml of the 1 per cent gamma globulin and enzyme solutions were prepared prior to irradiation. The irradiation cell was filled to capacity (4.0 ml); the remainder of the solution was used as a control sample and was placed into a test tube which was submerged in the oil bath along with the irradiation cell for temperature equilibration prior to irradiation.

Bach et al. reported temperatures within their sample cell to vary from 29.8°C to 38.4°C; in some experiments their cell was air cooled while in others the grounded electrode was water cooled. These authors concluded: "there was no correlation between temperature rise and effect".

In the present study a needle thermistor was used to monitor the temperature inside the cell with an accuracy of 0.1°C; the cell assembly was submerged in a constant temperature oil bath. The thermistor probe was used to determine when the sample had reached temperature equilibrium prior to irradiation and to monitor the sample temperature intermittently during irradiation. The rf heating of the thermistor probe, oriented perpendicular to the electric field within the sample, was negligible.

The temperature gradients within the sample were less than 0.3°C and the total temperature rise was less than 1°C for all proteins examined in this study. The oil bath temperature was always 32°C; this provided a working temperature within the range of those incurred by Bach et al.

A pulse field strength threshold as well as frequency specificity was reported for the gamma globulin studies conducted by Bach et al. For example, 87 peak to peak volts/cm produced effects at the "critical" frequency (e.g., 13.12 MHz) but a peak to peak field strength of 400 volts/cm produced no effect at "non-critical" frequencies. If the field strength was reduced to 43 peak to peak volts/cm no effect was obtained even at the "critical" frequencies. In the present study the field strength was always 100 peak to peak volts/cm, well above the threshold values reported previously for gamma globulin.

Bach et al. reported gamma globulin effects following irradiation at repetition frequencies ranging from 500 to 2000 Hz and with both 10 and 60 microsecond pulse widths; however, most of his experiments were performed with 10 microsecond pulses. Neither pulse width nor pulse repetition frequency appeared to be critical; therefore a pulse width of 10 microseconds and a repetition frequency of 500 Hz were chosen for the present protein irradiations; this conbination provided the minimum duty cycle consistent with previous studies, including enzyme irradiations, and minimized sample heating.

Following temperature equilibration the sample was irradiated 20 minutes at 10.00 MHz. The frequency was increased by 100 kHz and the sample was irradiated another 20 minutes. This procedure was repeated until the sample had received a total exposure of 100 minutes covering a 500 kHz frequency range.

This multiple irradiation procedure facilitated analysis and was intended to localize any effect to 500 kHz frequency bands. In essence, all frequencies in each band were covered because a pulse modulated rf carrier loses its single frequency characteristic. The rf energy associated with a pulse repetition frequency of 500 Hz and pulse width of 10 μsec is spread over a band of width exceeding 200 kHz; however, the "critical" region for gamma globulin (13.00 to 13.20 MHz) was covered in 10 kHz increments.

Bioassay. Bach et al. used paper electrophoresis to study the effects of rf irradiation on human gamma globulin. Cellulose polyacatate strips (Serophore III) were used for globulin electrophoresis in the present study. The electrophoresis was conducted in a tris-barbital buffer system (pH 8.8, 0.01 M) at 2 ma per strip. The electrophoresis was continued approximately three hours, which was sufficient to move the globulin band over one half the length of the polyacetate strip. The strips were stained with Ponceau S, rinsed and cleared with acetic acid and alcohol, respectively, and scanned with a Gelman optical scanner. Fig. 2 demonstrates the resolution of this system with fresh human serum following one hour of electrophoresis. The albumin, alpha 1, alpha 2, beta, and gamma globulin peaks are well resolved.

The acetylcholinesterase activity was determined after the method of Hertrin (13) which colorimetrically determined the amount of unreacted acetylcholine present as the colored acethydroxamic acid-ferric ion complex. The irradiated and control samples (0.1 ml) were added to 2.0 ml of the substrate solution (0.1 M NaCl, 0.02 M $MgCl_2$, 0.0005% gelatin and $2.5 \times 10^{-3}$ M acetylcholine in 0.02 M sodium phosphate, pH 7.0), incubated at 25°C for two minutes and the reaction was stopped by the addition of 2.0 ml hydroxylamine. The optical density of the solution was measured at 540 mu. The results are reported as the decrease in optical density following incubation.

Chymotrypsin activity was determined after the method of Hummel (14) using benzoyl-L-tyrosine ethyl ester as substrate. The irradiated and control solutions (0.3 ml of a 1:100 dilution of samples) were added to the substrate solutions (0.04 M Tris, .05 M $CaCl_2$, pH 7.8, 0.0005 M benzoyl-L-tyrosine ethyl ester, 25 per cent methanol) and the change in absorbency at 256 mu was monitored every thirty seconds for five minutes. A linear rate of increase was obtained up to an absorbency of 0.100. The results are reported as the change in optical density per minute.

## RESULTS

The initial irradiation of insoluble 2.2 per cent solutions at frequencies previously reported to be "critical" (i.e., near 13 MHz) failed to produce any electrophoretically detectable changes in either the soluble or insoluble fractions. The electrophoresis results for the subsequent systematic irradiation of 1 per cent solutions with frequencies between 10.0 and 15.0 MHz is shown in Table I. Each sample was irradiated 20 minutes at each of several frequencies. No evidence of abnormal electrophoretic peaks was obtained for any of the irradiated samples. Normal patterns were also obtained for every control sample. A scan of the cellulose polyacetate strip typical of both the irradiated and control samples is shown in Fig. 3.

The results of acetylcholinesterase irradiation are shown in Table II. A significant difference between the control and irradiated sample was not seen at any frequency. The precision of the techniques was approximately ±4%; all enzymatic assays were performed in triplicate.

The results of the chymotrysin data are presented in Table III. There is no significant difference between control and irradiated samples at any frequency. All assays were performed in triplicate and the precision of the determination is ±3%.

## DISCUSSION

Bach et al. (11,12) reported alpha amylase could be deactivated occasionally to 5 per cent of the original activity. Using pulse modulation parameters similar to those used in the present study, the most effective frequencies were reported to be 11.86, 11.84, and 11.85 MHz. Other investigators (8) concluded that rf irradiation of alpha amylase did not yield reproducible inactivation of enzyme activity. The effects of chymotrypsin irradiation between 10 and 15 MHz have also been studied (8). The results were not reproducible; 30 per cent inactivation was found in some experiments but no inactivation was found in others. These investigators concluded: "an enzyme inactivation of 15% ±15% was found.....inactivation was controlled or limited by some unknown factor".

As shown in Fig. 1, the rf effect on human gamma globulin reported by Bach et al. had an extremely sharp frequency dependence; i.e., a shift of less than 20 kHz from the cirtical frequency would eliminate the effect. However, according to these authors the critical frequency did not remain constant from day to day or sample to sample. They suggested the critical frequency might be dependent on both sample temperature and viscosity. It is conceivable, therefore, that the failure in the present study to demonstrate changes in human gamma globlin following rf irradiation could be attributed to the inability to duplicate exactly some unique experimental condition. This seems rather unlikely for two reasons. The first reason is related to the broad range of frequencies (5000 kHz) covered in the present study. The sample temperature and viscosity in the presnt study almost certainly were not too different from those used by Bach et al. and the shifts in critical frequency reported by them were always quite small, less than 100 kHz.

The second and most important reason stems from the distribution of energy in pulse modulated rf irradiation. If a continuous wave (cw) rf signal is pulse modulated, the resulting signal consists of a broad distribution of frequencies. Fourier analysis shows that the nature of this distribution is determined by the pulse width and the pulse repetition frequency (15). The spectrum is composed of lobes with each lobe containing a number of spectral frequencies. The spectral frequencies, $F_n$, are given by the relationship,

$$F_n = F_o \pm nf$$

where $F_o$ = carrier frequency

$f_r$ = pulse repetition frequency

$n = 0,1,2,3,...$etc.

For a rectangular pulse, the amplitude of the individual spectral frequencies forming the lobes varies as a function of frequency according to the relationship

$$\frac{\mathrm{Sin}(\pi F_o \tau)}{\pi F_o \tau}$$

where $\tau$ is the pulse width. The width of each lobe is determined only by $\tau$. As $\tau$ becomes shorter, the lobes become wider; the central lobe which contains a major portion of the rf energy is always twice as wide as the side lobes. Each side-lobe width is equal to the reciprocal of the pulse width, i.e., equal to $1/\tau$. Figure 4 is a photograph of the spectrum produced by a pulse modulated 13.12 MHz carrier; the pulse width was 10 μsec and the pulse repetition rate was 500 Hz. The photograph was taken from the screen of a Polarad Model 84WA spectrum analyzer. The center lobe alone is 200 kHz wide and the sharp frequency specificity of less than 20 kHz reported by Bach et al. appears to be highly unlikely under these pulse modulation parameters.

## SUMMARY

This investigation has been completely unsuccessful in demonstrating an effect of rf irradiation on enzyme and protein systems previously reported to be altered by such energy. The precision of the present study was within ± 5 per cent which was much less than the magnitude of the changes reported by others to result from the irradiation.

The exact reason for this discrepancy is not known, but the reported effects were not achieved with sufficient regularity and the definition of parameters necessary for their observation was too vague to establish a compelling argument for their existence. Certainly, the frequency dependence of the reported effects is difficult to understand in light of the power distribution expected from the pulsing parameters described.

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## FIGURE LEGENDS

Fig. 1 Paper electrophoresis results of irradiated human gamma globulin as reported by Bach et al. (7, 11, 12).

Fig. 2 Cellulose Acetate Electrophoresis of human serum obtained.

Fig. 3 Cellulose Acetate Electrophoresis of irradiated bovine serum $\gamma$ globulin.

Fig. 4 Frequency - Power Spectrum of pulse modulated radiofrequency carrier. (pulse width = 10 μsec, center frequency = 13.12 MHz).

13.18 Mc
13.16 Mc
13.14 Mc
13.12 Mc
13.10 Mc
13.08 Mc
13.06 Mc
FREQUENCY

Fig. 1

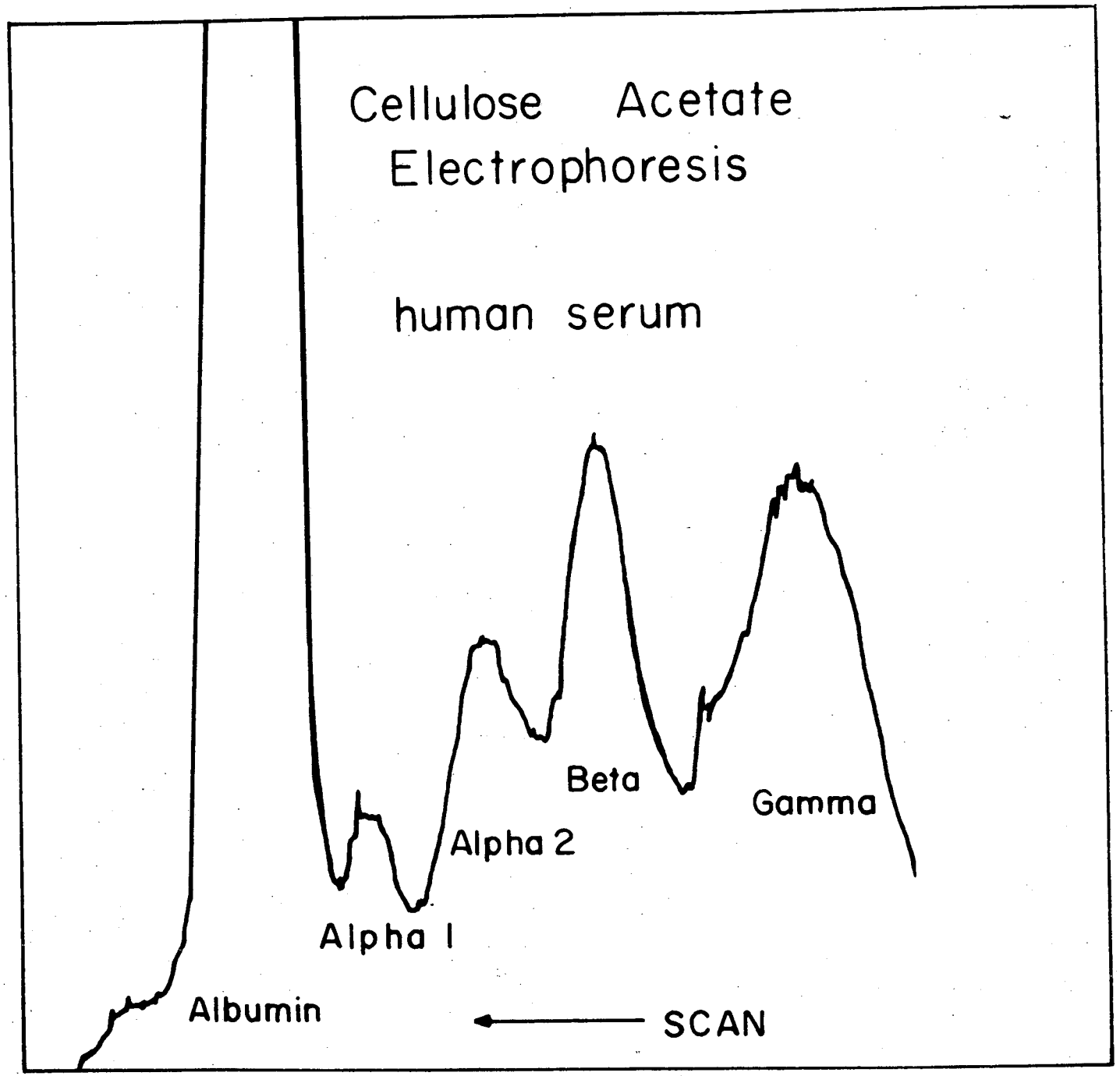
Cellulose Acetate
Electrophoresis
human serum
Albumin
Alpha 1
Alpha 2
Beta
Gamma
SCAN

Fig. 2

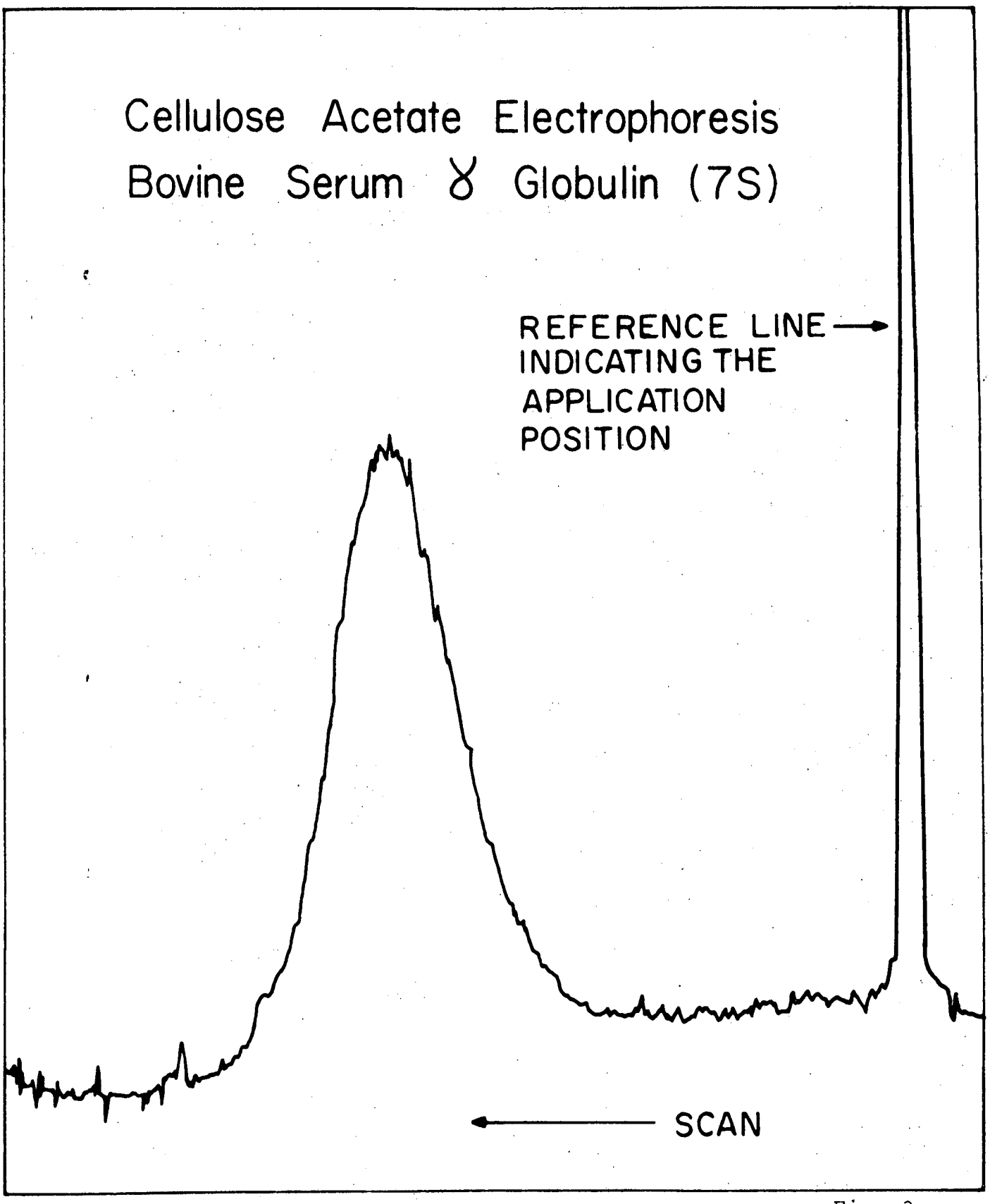
Cellulose Acetate Electrophoresis
Bovine Serum γ Globulin (7S)
REFERENCE LINE
INDICATING THE
APPLICATION
POSITION
SCAN

Fig. 3

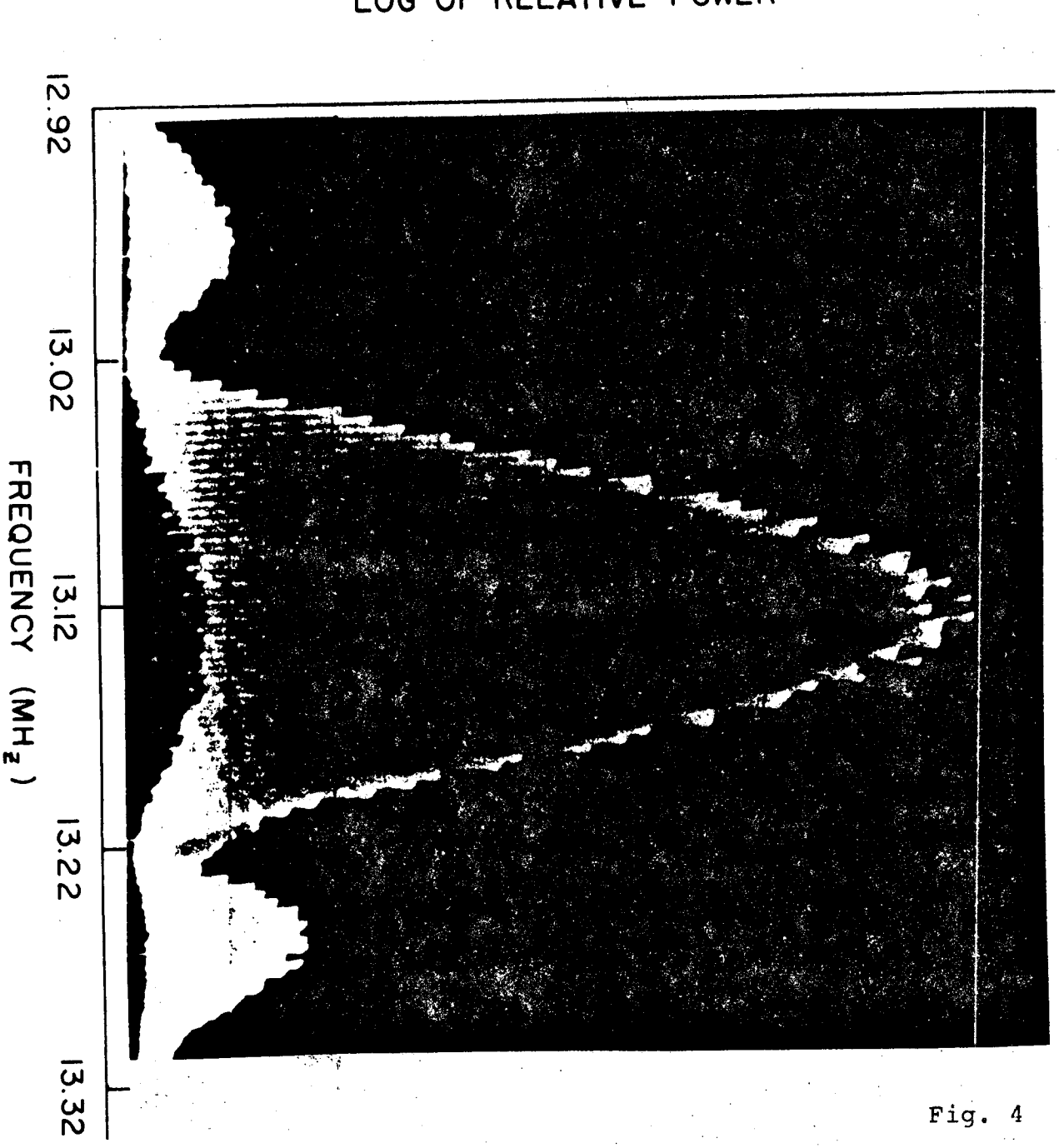
LOG OF RELATIVE POWER
12.92
13.02
13.12
13.22
13.32
FREQUENCY (MHz)

Fig. 4

TABLE I: Cellulose Polyacetate Electrophoresis Results for Human Gamma Globulin Solutions Subjected to Radiofrequency Irradiation

| Sample Number | Irradiated at Each of These Frequencies (MHz) for 20 Min | Total Sample Irradiation Time (Min) | Evidence for Multicomponent Electrophoresis Peak |
|---|---|---|---|
| 1 | 10.10,10.20,10.30,10.40,10.50 | 100 | Negative |
| 2 | 10.60,10.70,10.80,10.90,11.00 | 100 | Negative |
| 3 | 11.10,11.20,11.30,11.40,11.50 | 100 | Negative |
| 4 | 11.60,11.70,11.80,11.90,12.00 | 100 | Negative |
| 5 | 12.10,12.20,12.30,12.40,12.50 | 100 | Negative |
| 6 | 12.60,12.70,12.80,12.90,13.00 | 100 | Negative |
| 7 | 13.11,13.12,13.13,13.14,13.15 | 100 | Negative |
| 8 | 13.16,13.17,13.18,13.19,13.20 | 100 | Negative |
| 9 | 13.30,13.40.13.50,13.60,13.70 | 100 | Negative |
| 10 | 13.80,13.90,14.00,14.10,14.20 | 100 | Negative |
| 11 | 14.30,14.40,14.50,14.60,14.70 | 100 | Negative |
| 12 | 14.80,14.90,15.00 | 60 | Negative |

*At least two and usually three electrophoresis strips were obtained for each sample.

TABLE II: Optical Density Determination† of the Activity of Acetylcholinesterase Following Radiofrequency Irradiation

| Sample Number | Irradiated at Each of These Frequencies (MHz) for 20 Min | ΔOD* | |
|---|---|---|---|
| | | Irradiated | Control |
| 1 | 10.0,10.1,10.2,10.3,10.4,10.5, | 0.58±0.02 | 0.59±0.01 |
| 2 | 10.6,10.7,10.8,10.9,11.0 | 0.58±0.03 | 0.58±0.01 |
| 3 | 11.1,11.2,11.3,11.4,11.5 | 0.55±0.02 | 0.56±0.03 |
| 4 | 11.6,11.7,11.8,11.9,12.0 | 0.55±0.03 | 0.55±0.01 |
| 5 | 12.1,12.2,12.3,12.4,12.5 | 0.52±0.01 | 0.54±0.01 |
| 6 | 12.6,12.7,12.8,12.9,13.0 | 0.52±0.02 | 0.54±0.02 |
| 7 | 13.1,13.2,13.3,13.4,13.5 | 0.52±0.02 | 0.53±0.02 |
| 8 | 13.6,13.7,13.8,13.9,14.0 | 0.52±0.02 | 0.52±0.02 |
| 9 | 14.1,14.2,14.3,14.4,14.5 | 0.55±0.01 | 0.53±0.03 |
| 10 | 14.6,14.7,14.8,14.9,15.0 | 0.55±0.02 | 0.52±0.03 |

†Using the method described by: S. Hestrin, J Biol Chem 180:249, 1949.

*Errors represent range.

TABLE III: Optical Density Determination† of the Activity of Chymotrypsin Following Radiofrequency Irradiation

| Sample Number | Irradiated at Each of These Frequencies (MHz) for 20 Min | $\Delta OD \times 10^3 Min^{-1}$ | |
|---|---|---|---|
| | | Irradiated | Control |
| 1 | 10.0,10.1,10.2,10.3,10.4,10.5 | 65±3 | 67±3 |
| 2 | 10.6,10.7,10.8,10.9,11.0 | 70±4 | 70±4 |
| 3 | 11.1,11.2,11.3,11.4,11.5 | 66±2 | 68±2 |
| 4 | 11.6,11.7,11.8,11.9,12.0 | 85±4 | 82±1 |
| 5 | 12.1,12.2,12.3.12.4,12.5 | 65±3 | 65±1 |
| 6 | 12.6,12.7,12.8,12.9,13.0 | 66±2 | 67±1 |
| 7 | 13.1,13.2,13.3,13.4,13.5 | 70±2 | 66±3 |
| 8 | 13.6,13.7,13.8,13.9,14.0 | 66±1 | 68±4 |
| 9 | 14.1,14.2,14.3,14.4,14.5 | 65±4 | 63±1 |
| 10 | 14.6,14.7,14.8,14.9,15.0 | 70±3 | 72±3 |

†Using the method described by: B. C. Hummel, Can J Biochem Physiol 37: 1393, 1959.