# Second Progress Report

# EFFECTS OF RADIO-FREQUENCY IRRADIATION ON THE ENZYMES OF BEEF MUSCLE TISSUE

Reporting Period: 13 August 1964-13 December 1964

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ABSTRACT

The report covers the work conducted during the period 13 August through 13 December 1964. The objective of this program is to study the conditions necessary to inactivate the natural proteolytic enzymes of beef muscle tissue by radio-frequency energy.

During this period, three factorial designs covering as many as five variables at two levels have been devised and tested. The designs were analyzed by the conventional Yates Algorithm method. From the three design studies, it cannot be conclusively determined what the main factor and their interaction factors are. However, at specified conditions of an experiment, it was possible to obtain as much as 40% deactivation of the proteolytic enzyme. It is felt that far greater deactivation can be realized by optimizing parameters.

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## 1. INTRODUCTION

This is the second progress report on Contract No. DA 19-129-AMC-262(N) with the U.S. Army Quartermaster Corp of Natick, Massachusetts. The objective of this program is to study the conditions necessary for maximum inactivation of the natural proteolytic enzymes of beef muscle tissue by radio-frequency radiation. The achievement of this objective may contribute to the successful storage of beef under non-refrigerated conditions after its sterilization by high energy ionizing radiation.

During this period, effort was concentrated on the determination of the conditions necessary for the successful inactivation of the enzymes. However, since little is known about the mechanism of inactivation of enzyme by r-f energy, the problem was approached empirically by observing effects at the most probable conditions. Thus, a factorial design involving a systematic arrangement of the values of the variable was most useful for this program. Such a design which encompasses five factors each at two levels, affords knowledge of the main effects and the interactions of these.

Three designs encompassing as many as 16 runs/design were set up. The parameters studied were frequency, temperature, voltage power, duty cycle, and irradiation time. Other parameters which might have an effect on the denaturation process were kept constant within the design. Batch prepara-

tion of enzymes allowed the investigators to maintain constant some of the inherent properties of the solution, such as pH, viscosity, concentration, ionic strength, etc.

In the experiments conducted, we were unable to determine at the present time what the effects of the various parameters were. However, at a specified set of conditions inactivation of enzymes were demonstrated.

## 2. EXPERIMENTAL

### 2.1 Isolation and Purification of Proteolytic Enzyme

The procedure for the isolation and purification of the proteolytic enzymes was described in the previous report. It was found that very large quantities of frozen beef tissue were required for the isolation of sufficient quantities of enzyme, and that the processing of such quantities was too time consuming. Therefore, frozen beef liver was chosen as an alternate source for experimental material. This tissue is rich in proteolytic enzymes and is entirely satisfactory for the required studies.

The enzyme preparations were made in batches that were large enough for 16 runs. After determining a suitable concentration, the preparation was divided into at least 16 aliquots which were then stored in the freezer. In this way uniformity of enzyme sample was assured for an entire set of experiments.

Uniformity of substrate (hemoglobin) was assured in the same way.

### 2.2 R-F Irradiation

The r-f irradiations were carried out with the radio-frequency twice described in the previous report. The irradiation cells were constructed of copper laminated epoxy boards joined with epoxy cement. The copper laminates served as the plates.

Two cells, differing only in dimensions, were used in these experiments. These cells and their nomenclature are:

| Cell | Plate Dimensions | Distance Between Plates | Volume |
|---|---|---|---|
| Cell A | 2 in x 5 in | 0.2 cm | 5 cc |
| Cell B | 2 in x 5 in | 0.4 cm | 8 cc |

In carrying out the irradiation experiments, 5 cc of the enzyme solution were introduced into two identical cells; one cell served as a control for comparison with the experimental cell. Both cells were then placed in a constant temperature water bath. While both were maintained at the same constant temperature, the experimental cell was irradiated. At the completion of irradiation, both cells were removed from the water bath, and their contents were immediately assayed for enzyme activity.

2.3 Measurement of Enzyme Activity

The original procedure was employed for the assay of enzyme activity as follows:

To a 13 x 150 mm test tube was added 5.0 ml of hemoglobin substrate, and 8.7 ml of distilled water. The test tube was then placed in a water bath thermostatted at 35°C; and the solution was allowed to equilibrate to the water bath temperature. 0.3 ml of a 0.5 M ferrous ammonium sulfate solution was then added. Upon addition of 1 cc of enzyme, the solution was rapidly mixed; and 2 ml of the solution was withdrawn and

placed in a test tube containing 5 ml of a 5% trichloroacetate solution. The ester stopped the enzymatic reaction. The removal of similar aliquots was continued five minute intervals for 25 minutes. The solutions were allowed to stand for at least 1/2 hour and were then centrifuged for 30 minutes at 8,000 rpm. The clear TCA soluble hydrolysis product was transferred to a cuvette; and its optical density at 280 mm was measured spectrochemically.

The use of this procedure led to a number of difficulties. In the previous progress report typical enzyme activity curves with absorbance readings of 0.400 units at zero time and 0.60 units after 50 minutes were reported. This type of curve is typical whenever the concentration is extremely high. We have reduced the enzyme concentrations to give absorbance values of 0.2 to 0.3 after 25 minutes of reaction time. However, in a large number of the analyses the linear time-absorbance curves expected at this concentration level were not obtainable. These non-linear curves made it difficult to use the results of the assay since the values of the slopes were used in the analysis of the data.

A number of experiments were conducted to determine the conditions necessary to obtain linear response curves. The conditions tested were the effect of buffer, the effect of the activator, ferrous ammonium sulfate, and the effect of the substrate and the source of the substrate on the enzymatic

activity. Only the addition of a larger volume of buffer seems to aid in giving a linear response curve. Varying ferrous ammonium sulfate concentration (values of 0.002, 0.003, 0.007, 0.017, and 0.02 molar) had no significant effect. Variation in the amount of substrate along with the use of commercially prepared hemoglobin instead of our own preparation also showed no effect on the analysis. Upon completion of these experiments, a number of modifications were made on the assay procedures as follows:

a. Substitution of 5 ml of buffer for 5 ml of distilled water; the volume of water was reduced from 8.7 ml to 3.7 ml.

b. Commercially prepared hemoglobin is being used instead of the in-house preparation.

c. In place of 30 minutes centrifuging at 8,000 rpm, the procedure was modified to centrifuging for 5 minutes followed by filtering with Whatman #40 filter paper. The reason for this change is only for convenience.

The procedure now in use includes these changes.

The assay solution consists of a total volume of 15 ml. of solution which contains 1-ml of enzyme. It is 0.033 M with respect to the acetate buffer, (pH of 4.0), 0.01 M in ferrous ammonium sulfate concentration, and has 6.67 mg denatured hemoglobin per ml with 120 mg urea per ml.

## 2.4 Experimental Design

During this period, three statistically designed experiments with at least 8 runs per experiment were conducted along with the replicates of run #3 of Design #1. Design #1 was devised to study five factors involved in the radio-frequency irradiation experiments. These five factors were the irradiation time, target frequency, voltage, temperature and duty cycle at two given levels. The experimental design is given in Table I.

Since the results of Design #1 indicated that the significant factor was the irradiation time along with the interaction of irradiation time and voltage, the second design, Design #2 was devised in which these factors were emphasized. The irradiation time was shortened to 1/4 hour and the power level was substituted for the duty cycle. Also lower levels were designated for both the frequency and the temperature values (See Table II)

In Design #3 only three variables were studied, the frequency of the r-f radiation, the voltage and the power. The irradiation time and the temperature were kept constant at values of 1/2 hour and 16.5°C. (See Table III).

Replicate experiments on run #3 of Design #1 were conducted. These replicates were carried out on three different days using three different batches of enzyme.

TABLE I

Design #1 Experimental Variables and Design

(Cell A)

| Factor Symbol | Factor | Values | |
|---|---|---|---|
| A | Irradiation Time (hours) | 1/2 | 2 |
| B | Target Frequency (mega cycles) | 11.75 | 13.50 |
| C | Temperature (°C) | 16.5 | 19.5 |
| D | Voltage (volts) | 50 | 150 |
| E | Duty Cycle | 0.1 | 0.5 |

| Experiment No. | A Irradiation Time - Hrs. | B Target Frequency-mc | C Temp. °C | D Voltage | E Duty Cycle |
|---|---|---|---|---|---|
| 1 | 1/2 | 11.75 | 16.5 | 50 | 0.5 |
| 2 | 2 | 11.75 | 16.5 | 50 | 0.1 |
| 3 | 1/2 | 13.50 | 16.5 | 50 | 0.1 |
| 4 | 2 | 13.50 | 16.5 | 50 | 0.5 |
| 5 | 1/2 | 11.75 | 19.5 | 50 | 0.1 |
| 6 | 2 | 11.75 | 19.5 | 50 | 0.5 |
| 7 | 1/2 | 13.50 | 19.5 | 50 | 0.5 |
| 8 | 2 | 13.50 | 19.5 | 50 | 0.1 |
| 9 | 1/2 | 11.75 | 16.5 | 150 | 0.1 |
| 10 | 2 | 11.75 | 16.5 | 150 | 0.5 |
| 11 | 1/2 | 13.50 | 16.5 | 150 | 0.5 |
| 12 | 2 | 13.50 | 16.5 | 150 | 0.1 |
| 13 | 1/2 | 11.75 | 19.5 | 150 | 0.5 |
| 14 | 2 | 11.75 | 19.5 | 150 | 0.1 |
| 15 | 1/2 | 13.50 | 19.5 | 150 | 0.1 |
| 16 | 2 | 13.50 | 19.5 | 150 | 0.5 |

Table II

Design #2, Experimental Variables and Design
(Cell A)

| Factor Symbol | Factor | Values | |
|---|---|---|---|
| A | Irradiation time (hours) | 1/4 | 1/2 |
| B | Target Frequency (mc) | 7.0 | 11.85 |
| C | Temperature (°C) | 12.0 | 16.5 |
| D | Voltage (volts) | 25 | 100 |
| E | Power (watts) | 0.1 | 1.0 |

| Experiment No. | A Irradiation Time - Hrs. | B Target Frequency-mc | C Temp. °C | D Voltage | E Power |
|---|---|---|---|---|---|
| 1 | 1/4 | 7.0 | 12.0 | 25 | 0.1 |
| 2 | 1/2 | 7.0 | 16.5 | 25 | 1.0 |
| 3 | 1/4 | 11.85 | 16.5 | 25 | 1.0 |
| 4 | 1/2 | 11.85 | 12.0 | 25 | 0.1 |
| 5 | 1/4 | 7.0 | 12.0 | 100 | 1.0 |
| 6 | 1/2 | 7.0 | 16.5 | 100 | 0.1 |
| 7 | 1/4 | 11.85 | 16.5 | 100 | 0.1 |
| 8 | 1/2 | 11.85 | 12.0 | 100 | 1.0 |

Table III

Design #3, Experimental Variables and Design
(Cell B)

| Factor Symbol | Factor | Values | |
|---|---|---|---|
| A | Irradiation time (hours) | 1/2 | |
| B | Target Frequency (mc) | 11.9 | 13.5 |
| C | Temperature (°C) | 16.5 | |
| D | Voltage (volts) | 60 | 100 |
| E | Power (watts) | 0.5 | 1.0 |

| Experiment No. | A Irradiation Time - Hrs. | B Target Frequency-mc | C Temp. °C | D Voltage | E Power |
|---|---|---|---|---|---|
| 1 | 1/2 | 11.9 | 16.5 | 60 | 0.5 |
| 2 | 1/2 | 13.5 | 16.5 | 60 | 0.5 |
| 3 | 1/2 | 11.9 | 16.5 | 100 | 0.5 |
| 4 | 1/2 | 13.5 | 16.5 | 100 | 0.5 |
| 5 | 1/2 | 11.9 | 16.5 | 60 | 1.0 |
| 6 | 1/2 | 13.5 | 16.5 | 60 | 1.0 |
| 7 | 1/2 | 11.9 | 16.5 | 100 | 1.0 |
| 8 | 1/2 | 13.5 | 16.5 | 100 | 1.0 |

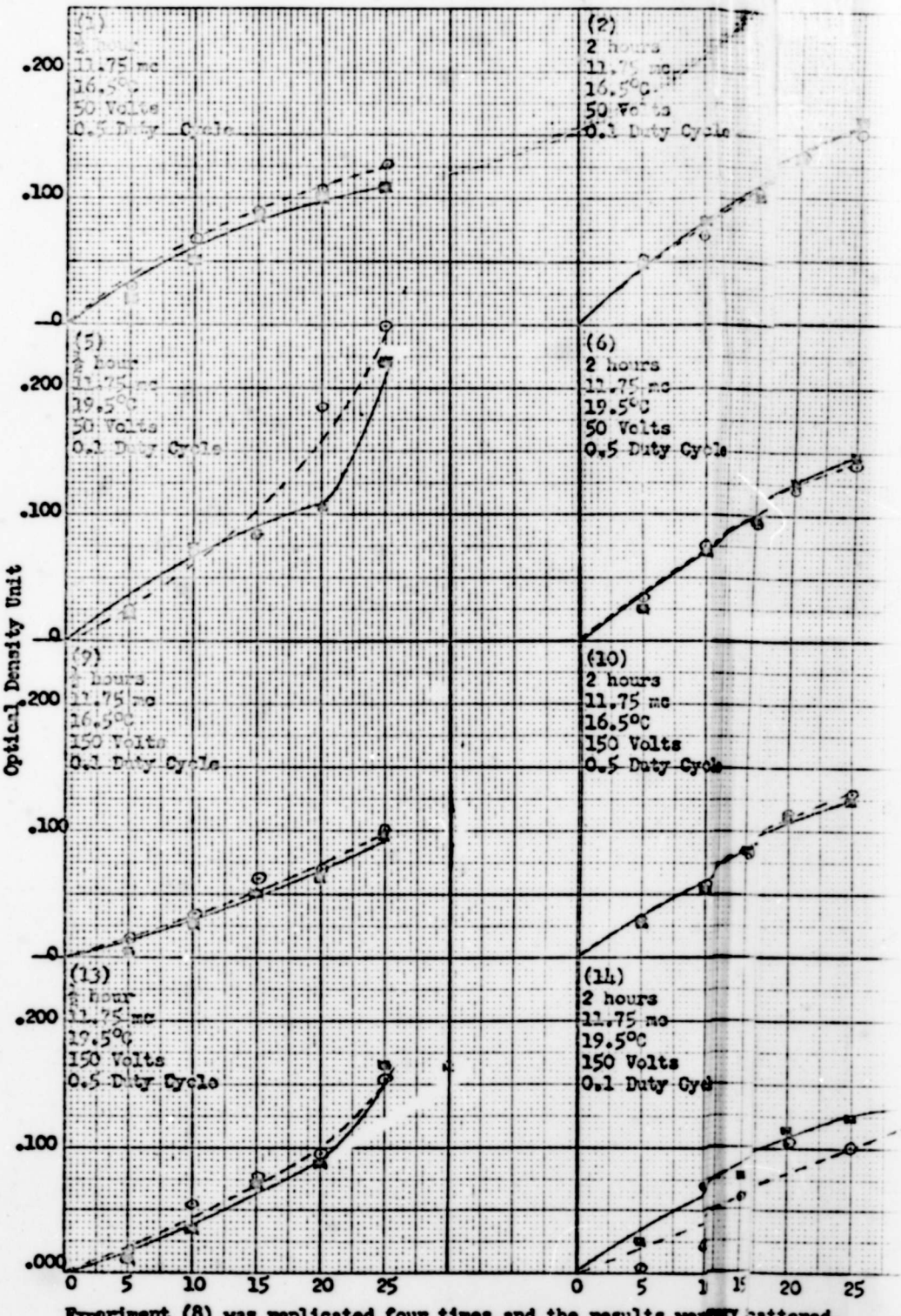
Conditions for the replicates were a frequency of 13.5 mc, irradiation time of 1/2 hour, voltage level of 50 volts, temperature at 16.5°C and a duty cycle of 0.1.

## 3. RESULTS AND DISCUSSION

The results of the experiments of the three designs are shown in graphic form with all the runs of a single design plotted in a single figure. The data were analyzed by the Yates Algorithm method giving the main factor and the interaction of two factors. The complete statistical analysis will be shown in the final report. The inactivation of the enzymes expressed in per cent was determined by comparison of the absorbance of the control and the r-f sample after 25 minutes of reaction time.

The results of the experiments in design #1 are given in Figure 1. The analysis of the experimental data was limited because of the inaccuracy of extrapolating from the analytical results a response for use in the mathematical analysis and because of a large experimental error. The one single factor which in itself has an effect on the system is the irradiation time. The shorter irradiation periods of approximately 1/2 hour yield the greatest enzyme inactivation. However, the differences observed among the various experiments at a 1/2 hour radiation indicate quite clearly that other factors contribute to the effect. The probable interaction factors according to the analysis indicate a voltage-power relation.

Of the 16 runs in experimental design #1, only run #3 gave a significant decrease in enzyme activity under the irradiation conditions. The conditions of this experiment




Experiment (8) was replicated four times and the results were very scattered.

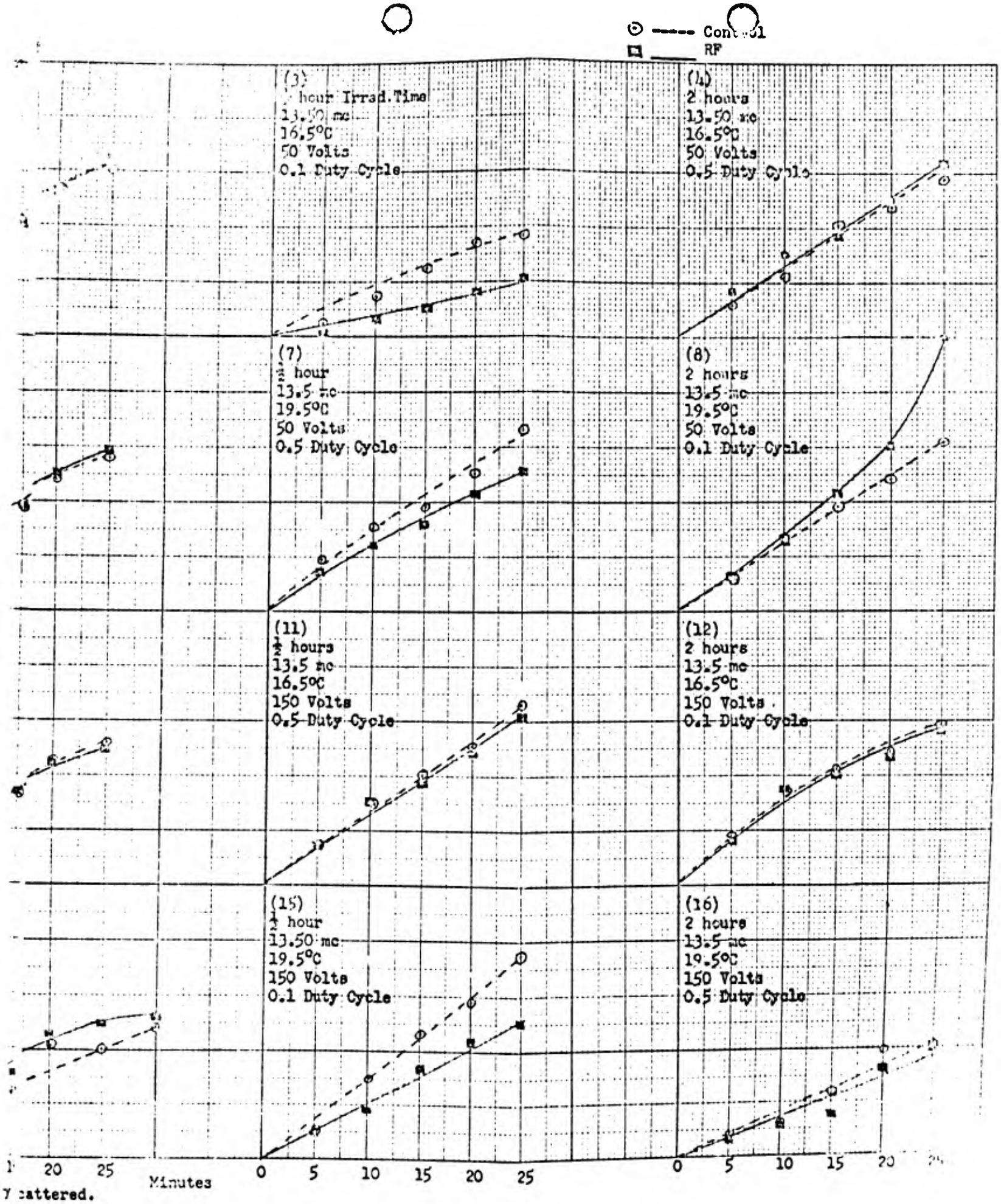



Figure 1. Experimental Results of Design 1

were 13.5 mc, 16.5°C, 50 volts, 0.1 duty cycle and 1/2 hour irradiation time. These conditions resulted in a 45% decrease in enzyme activity. Replicates of this run under identical conditions except for use of a different batch of enzymes gave comparable results with decreases in enzyme activity of about 35 and 43%. These results are shown in figure 2.

Design #2 was set up to study the effects of shorter irradiation times, reduced voltage levels, and lower frequency conditions with power ratings substituted for the duty cycle. The results of these experiments (figure 3) show inactivation of the enzymes at 11.85 mc only. Irradiation of 7.0 mc did not seem to affect enzyme activity. Statistical analysis failed to indicate the main factor and the interaction factors.

Design #3 was set up primarily to study the role of voltage-power relationships. Inactivation of the enzyme was obtained in all the runs. The remaining activity observed in runs 1, 3, 4, were 77, 65, and 78% respectively, of the initial activity. The results of the other five runs may not be considered significant since the observed inactivation falls approximately within the assumed error factor of ±10%. The Yates Algorithm analysis indicates that low power coupled with high voltage levels gave optimum results. (See figure 4).

The nature of the interactions between the enzyme and the r-f energy is still a matter of conjecture. The results of our experiments indicate that enzyme inactivation can be

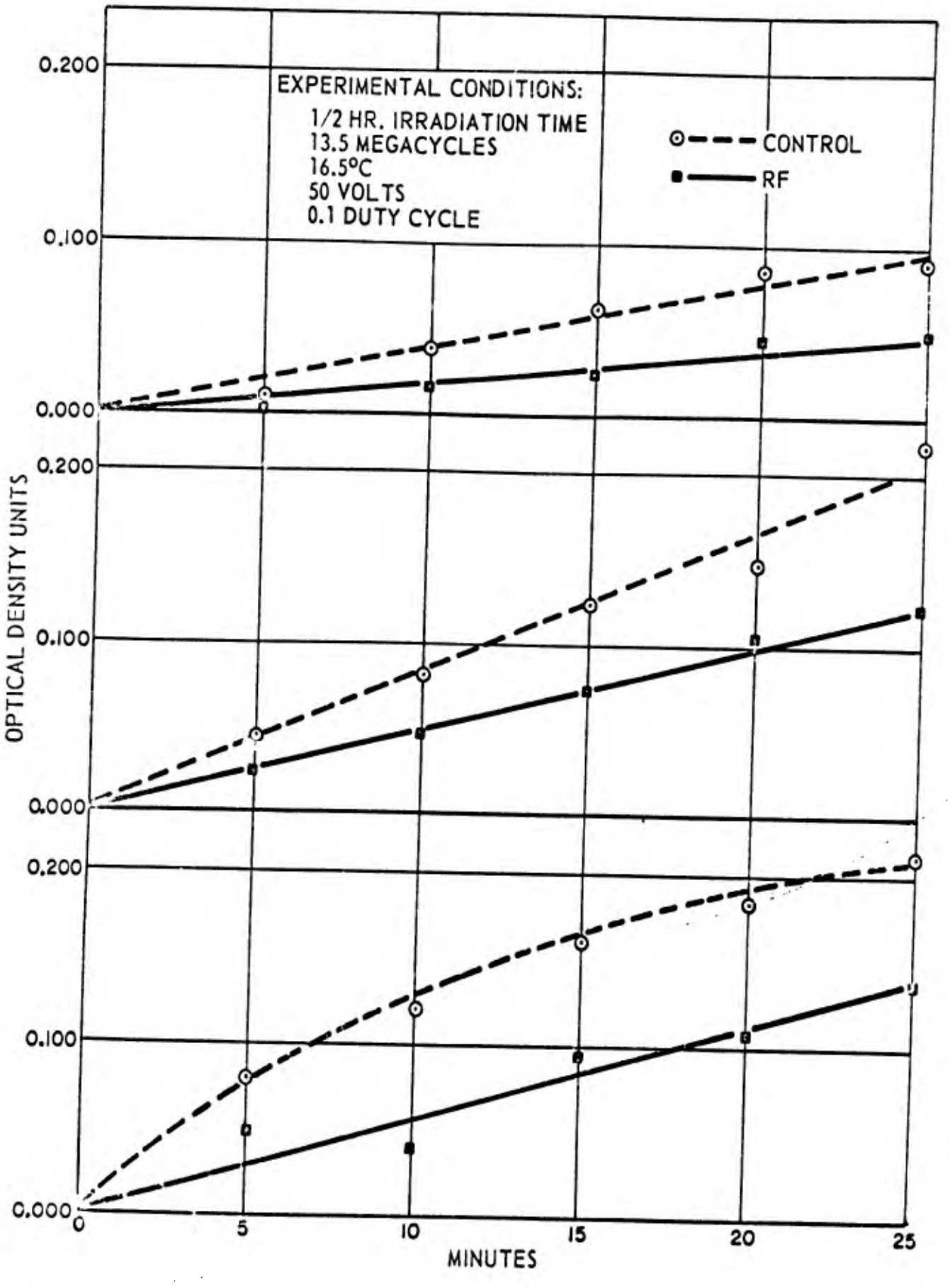
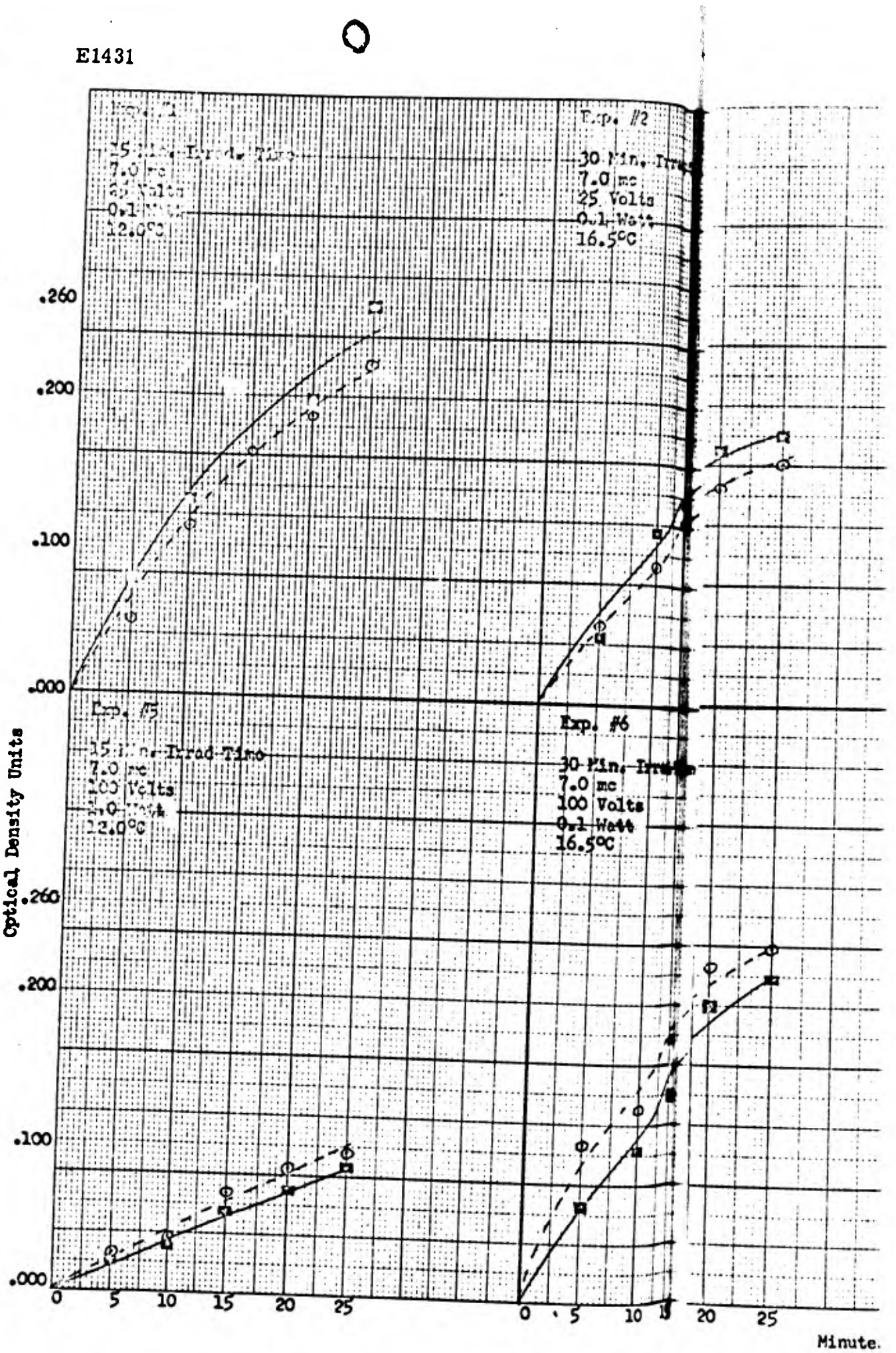



Figure 2. Results of Experimental Repetitions

E1431


Exp. #1
15 Min. Irrad-Time
7.0 mc
100 Volts
0.1 Watt
22.0°C
Exp. #2
30 Min. Irrad-Time
7.0 mc
25 Volts
0.1 Watt
16.5°C
Exp. #5
15 Min. Irrad-Time
7.0 mc
100 Volts
0.1 Watt
22.0°C
Exp. #6
30 Min. Irrad-Time
7.0 mc
100 Volts
0.1 Watt
16.5°C
Optical Density Units
.260
.200
.100
.000
0
5
10
15
20
25
Minute.

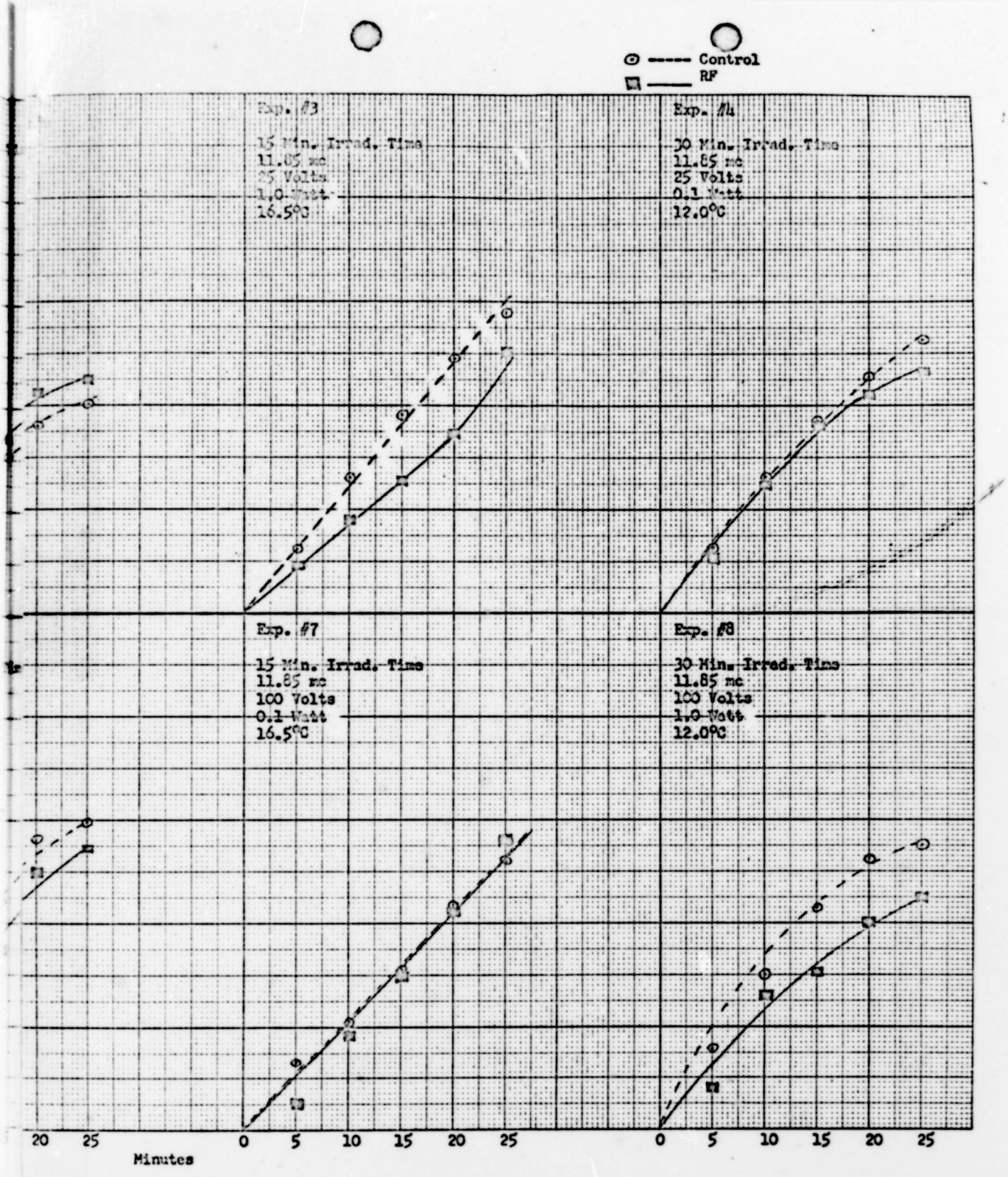
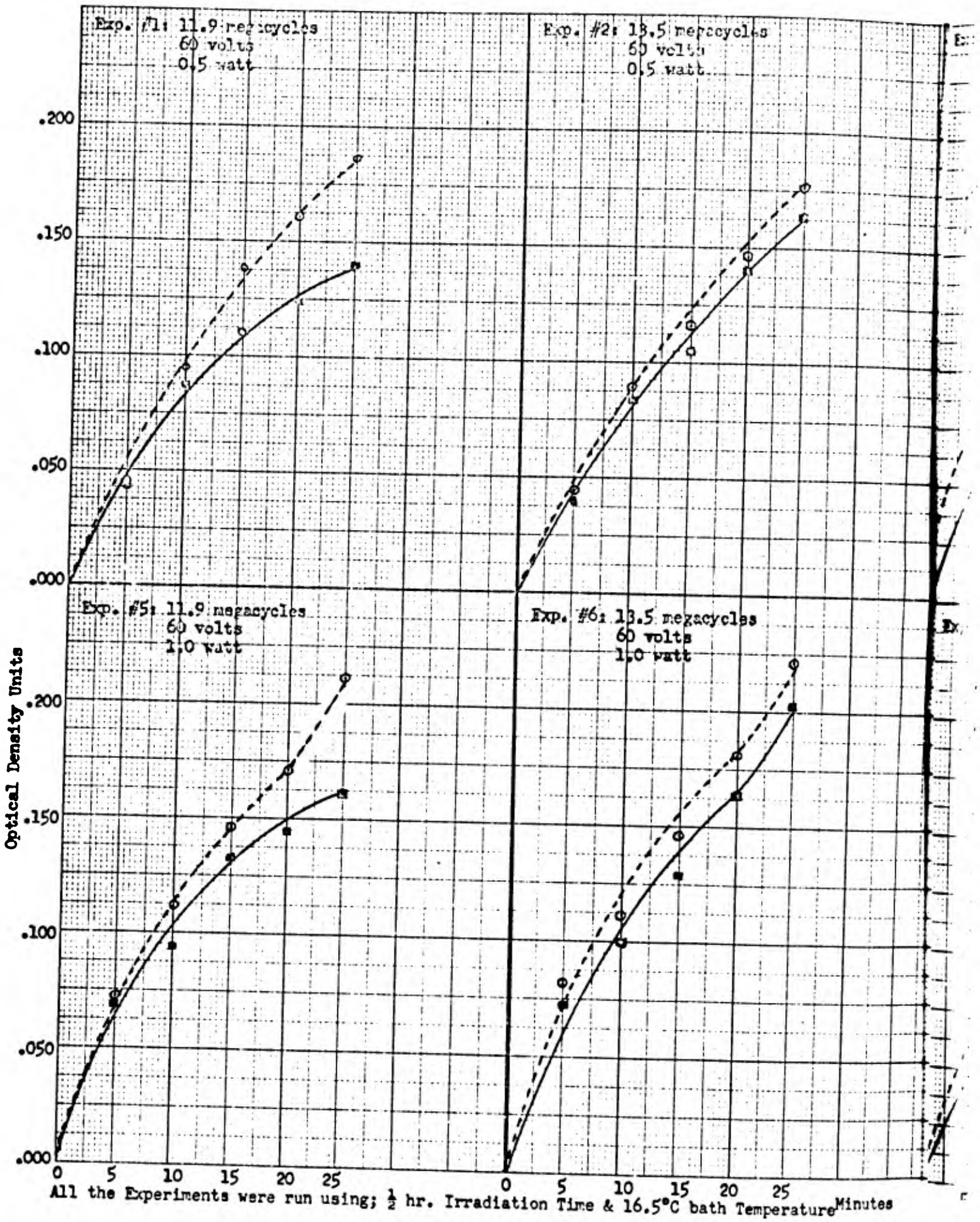



**Figure 3. Experimental Results of Design 2**

E1550




All the Experiments were run using; ½ hr. Irradiation Time & 16.5°C bath Temperature

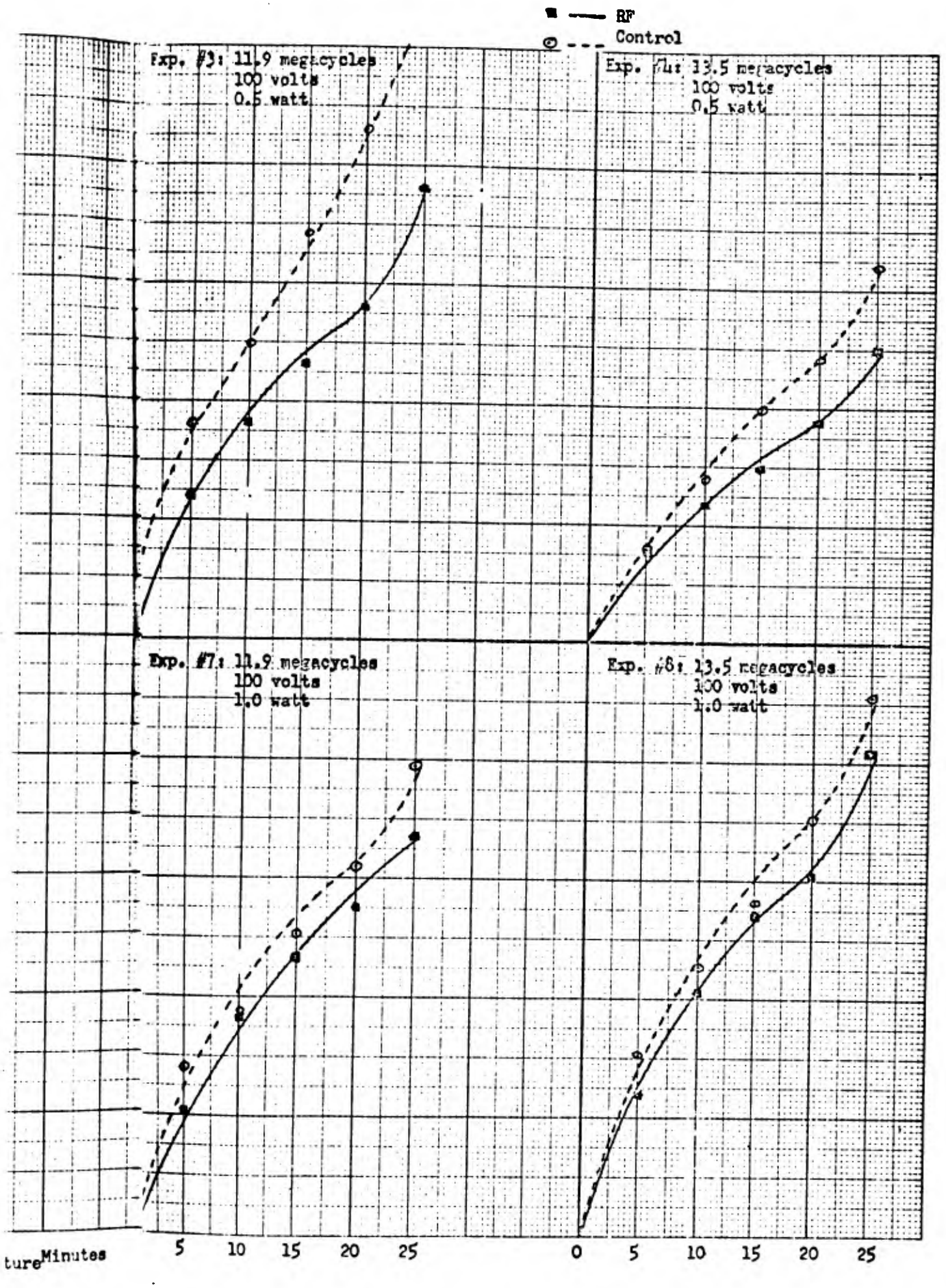



Figure 4. Experimental Results of Design 3

obtained by exposure to an r-f field. However, there is no apparent theory on which a prediction can be based. The variations observed at a constant frequency for example, tend to rule out the hypothesis that the observed effect is frequency specific. On the other hand, the excellent reproducibility obtained under constant conditions leaves no doubt that the effect is real. The reproducibility is especially noteworthy when the natural variations in biological systems is considered. For example, the replicate runs of run #3, design #1, were carried out at different times by different personnel with different enzyme preparations. Yet the agreement among these replicates is well within experimental errors expected.

One aspect of the results that may be considered a problem area is the failure to observe a linear relationship between enzyme activity and time in some few cases. This failure is apparently due to deviations from the Beer-Lambert law. Although the deviations are not serious, it is, nevertheless, worthwhile to look into them. The substitution of another read-out for the present spectrophotometric measurement at 280 mu will not affect the results obtained thus far and may at the same time, permit a better statistical treatment upon completion of the program. One possible approach to this problem is the measurement of absorption in the far ultraviolet region at 185-190 mu. This region has been

extensively studied at Melpar, and the application of this measurement to the determination of enzyme activity has been successful with other enzymes.

## 4. CONCLUSIONS

The results of the irradiation experiments clearly show that the proteolytic enzyme is inactivated upon exposure to r-f energy at 11.9 and 13.5 mc under the specific conditions of our experiments. The analysis of the data indicate that optimal conditions for maximum enzyme inactivation seems to include low power, short time exposure, and high voltage.

It may be concluded that r-f energy does cause enzyme inactivation. However, the conditions under which maximum inactivation is obtained are yet to be determined.

## 5. FUTURE WORK

The research now under way is concerned with the exploration of varying conditions in order to define the conditions that will produce maximum inactivation of the proteolytic enzymes.

Effort will be directed at the determination of changes in structure as indicated by changes in peptide bonds. This will be accomplished by spectrophotometric studies in the far ultraviolet region at 185-190 mu. These measurements are expected to also yield enzyme activity measurements that vary linearly with time.

It is also planned to carry out a complete statistical analysis of all results in order to select the significant factors. Initial studies of proteolytic enzyme inactivation in beef "in situ" will be made.

## 6. ADMINISTRATIVE

The rate of expenditure has parallelled the rate of technical progress fairly closely. The funds yet unexpended are judged sufficient to complete the work required by the contract.