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

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by

S. NELSON

MELPAR, Inc.
Falls Church, Virginia

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Contract No. DA 19-129-AMC-262(N)

December 1965

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U. S. Army Materiel Command
U. S. ARMY NATICK LABORATORIES
Natick, Massachusetts

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FOREWORD

Preservation of meat foods by ionizing radiation sucessfully sterilizes the products and presents microbial spoilage during prolonged storage. Treatment required to produce sterility, however, is inadequate to inactivate the natural proteolytic enzymes of the meat. As a result, radiation sterilized raw meat foods deteriorate during storage by proteolytic action, becoming progressively more tender and finally mushy. At the same time a bitter flavor characteristic of free amino acids develops. At present the only effective method for inactivating the natural proteases of meat is a heat treatment equivalent to cooking to medium rare. If radiation sterilized raw meat foods are to be provided for military feeding, some means other than heat are required for proteolytic enzyme inactivation.

The work covered by this report, performed by Melpar, Inc. under Contract No. DA 19-129-AMC-262(N), represents a preliminary investigation of the use of radio-frequency irradiation to inactivate the natural proteolytic enzymes of beef muscle tissue. The investigator was S. Nelson.

The U. S. Army Natick Laboratories Project Officer was A.S. Henick of Food Chemistry Branch, and the Alternate Project Officer was E. Wierbicki of Irradiated Food Products Branch, both of Food Division.

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ABSTRACT

The report covers the work conducted during the period 13 April 1964 to 13 April 1965. 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.

Preliminary experiments using purified proteolytic enzymes isolated from beef liver tissue were conducted to determine the effects of various r-f parameters. From 4 design studies, it could not be conclusively determined as to what the levels of the main factors and the interaction factors necessary for maximum inactivation are. It has been shown however that a set condition about 40% inactivation of the proteolytic enzymes can be effected.

R-f radiation experiments on ground beef tissue gave inconclusive results. Chief problem area lies in the fact that the extraction and purification process does not yield consistent results.

1. INTRODUCTION

This is the final report on Contract No. DA 19-129-AMC-262(N) with the U.S. Army Natick Laboratories, Natick, Massachusetts. The objective of this study is to inactivate the natural proteolytic enzymes of beef muscle tissue by radio-frequency energy. The successful inactivation of proteolytic activity, in conjunction with the sterilizing effects of high-energy ionizing radiation, will provide a means for the non-refrigerated preservation of animal tissues over extended periods of time.

The literature has been reviewed, and a method for the extraction, isolation, and purification of the natural proteolytic enzyme of beef liver and muscle tissues was selected and is described in some detail. The protocol for the preparation of the proteolytic enzymes has been reduced to a reliable routine, but, in the initial stages of this work, some difficulties were encountered. Specifically, the effects of slight variations in pH during the isolation procedure are profound, and these were found to make the difference between a highly active preparation and a preparation with no activity whatsoever.

A number of methods and/or variation of the methods used for measuring the activity of the proteolytic enzyme are described in this report. The assay methods have all been tried and it was finally determined that the most reliable

method was based on the proteolytic hydrolyses of a urea denatured hemoglobin solution, buffered at pH 4.2 with a 0.1M acetate buffer. Enzymatic activity is indicated by the increase in optical absorbance of aliquots of the reaction mixture. Optical measurements are made at 280 mu wavelength, in the near ultraviolet region. The slope of the linear curve relating increase in absorbance with time is taken as the measure of the amount of enzymatic activity.

Because the activity of the enzymes is determined by the slopes of the curve relating absorbance with time, it is necessary to determine this slope with considerable precision. Individual assays are therefore being conducted in duplicates, and each slope is determined on the basis of the 6-7 aliquots withdrawn from the reaction mixture over a period of either 25 or 60 minutes.

A detailed description of the schematic of the apparatus used for the radio-frequency radiation experiments is incorporated in this report along with the descriptions of the methods employed for the measuring of the radio-frequency parameters, such as the power, voltages, and the frequency of the radiation.

The preliminary experiments on the program enzymes dealt primarily with the studies on the purified proteolytic enzymes obtained from beef liver tissues. In attempt to obtain some concrete information as to what levels of various r-f

parameters are most suitable for the inactivation of the enzymes, a factorial design involving a systematic arrangement of the values of the variables was devised. Such a design which encompasses as many as five factors affords knowledge of the main effects and the interaction of these effects. Thus 4 designs with as many as 16 runs/design were set up. The parameters studied were frequency, temperature, voltage, power, duty cycle, and the irradiation time. Other parameters which might have an effect on the system were kept constant within the design.

Of the 4 designs completed, we were unable to determine what the effects of the various parameters were and how they were affecting the denaturation process. However, at specified set of conditions inactivation of the enzyme was demonstrated.

2. EXPERIMENTAL PROCEDURE

2.1 Preparation and Handling of the Enzyme

2.1.1 Isolation and Purification of Proteolytic Activity from Beef Tissue

Perhaps the earliest description of an isolated cathepsin or proteolytic enzyme from beef tissue was that of Balls in 1938.[1] His enzyme exhibited a pH optimum of approximately 4.1, and it was thus differentiated from the activity of pepsin (pH optimum approximately 2)[2,3] and trypsin or chymotrypsin (pH optimum approximately 8).[4,5]

In 1950, Snoke and Neurath described in detail the isolation and purification of proteolytic enzymes from striated rabbit muscle.[6] When assayed using a urea denatured hemoglobin substrate, buffered with 0.1M acetate, their enzyme exhibited a pH optimum at approximately 4.0. The enzyme was activated by ferrous ions, and to a lesser extent by magnesium and manganese. Zinc, calcium, cobalt, and cysteine had no clear influence on activity.

The Snoke and Neurath procedure has been used for the preparation of enzyme in this study, and is described here in detail. A schematic flow chart of the isolation and purification procedure is presented in figure 1.

Fresh beef tissue was obtained at a slaughter house and transported to the laboratory in an ice chest. The tissue was then divided into pieces weighing approximately 200 gms and quick frozen with liquid nitrogen. The frozen beef tissue was stored in a deep freeze at -15°C until ready for use, at which time it was chopped into cubes approximately 1/2 inch on a side while still frozen.

It was found that the enzymatic activity is apparently somewhat sensitive to heat during the early stages of purification; in any event, the heat generated in a large, one-quart Waring Blendor by the action of the blades was enough to darken the meat homogenate; no activity was obtained from such preparations. A smaller Osterizer blendor jar of 1/2-pint capacity was used in subsequent experiments with better results.

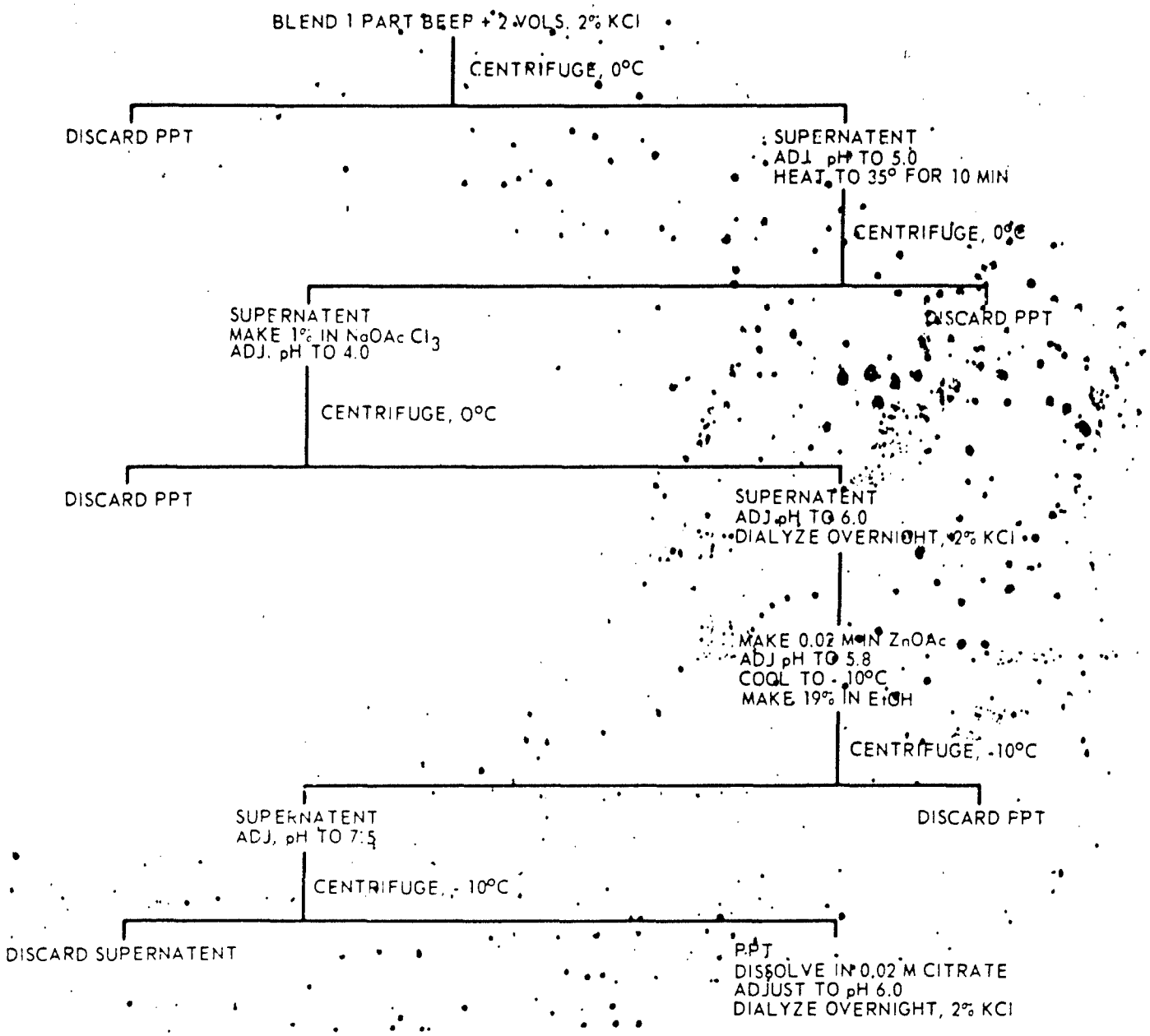


Figure 1. Purification Scheme of Proteolytic Enzyme From Beef Tissue

The 1/2-pint jars conveniently hold 50 gms of partially frozen beef tissue, plus 100 ml 2% KCl chilled to 4°C. The mixture is allowed to blend for no more than 45 seconds. The pink homogenate is then allowed to set at 4°C in a refrigerator overnight.

The following morning, the homogenate should still be bright pink in color, and quite fluid unless the original beef tissue contained considerable amounts of tendon. In the latter case, a heavy gelatinous mass is obtained which must be reblended. The extract is transferred to 250 ml polypropylene centrifuge bottles and centrifuged for 1 hour at 5000 rpm and an internal head temperature of approximately 0°C. (We are currently using an IEC refrigerated centrifuge, Model HR-1, equipped with Model 858 head for this purpose.) In a typical preparation, starting with 150 gms beef muscle and 300 ml 2% KCl, the volume of the crude supernatant extract following this first centrifugation amounted to approximately 350 ml.

The lightly colored, opalescent supernatant is adjusted to pH 5.0 using 1 N HCl, and then heated to 35°C in a 40°C water bath with constant agitation. After the solution reaches a temperature of 35°C, it is allowed to set for 10 minutes, and is then recentrifuged as before. The precipitate is a light brown material. Total volume of the supernatant is approximately 97% of that which was heated. The temperature has been reduced to approximately 0°C during centrifugation.

Sufficient 10% sodium trichloroacetate at pH 4.0 is then added to give a final solution which is 1% in NaTCA, and the pH of the whole solution is adjusted to 4.0 with 1 N HCl. The material is centrifuged a third time at the same temperature and speed, and the precipitate discarded.

The pH of the supernatant is now adjusted to 6.0 with 1N NaOH. The liquid is placed in a dialysis tube approximately 3/4 inch in diameter and dialyzed at 4°C overnight against 4-5 changes of 2% KCl.

This first dialyses step presumably removes the sodium trichloroacetate.

The morning of the third day the solution is removed from the dialyses tubing and 1.0M zinc acetate is added to give a final solution which is 0.02M is ZnOAc. The pH is adjusted to 5.8 with 1 N HCl, and the enzyme is returned to 250 ml centrifuge tubes. These are placed in an ice salt bath, and previously chilled ethanol is added slowly as the temperature of the entire solution is chilled to -10°C.

The solution is then centrifuged for 1/2 hour at 5000 rpm, and a head temperature of -10°C. A brown pasty precipitate is obtained which is normally discarded. The pH is raised with 1 N NaOH to 7.5, and the solution is centrifuged a final time, still maintaining a temperature of -10°C.

The second zinc precipitate from the ethanolic solution contains the bulk of the enzymatic activity. It has a bright

pink appearance, much like that of the original beef-KCl homogenate; if the activity is high, it will normally be soluble in approximately 2 volumes of 0.02M citrate buffer, pH 6.0.

The citrate buffer solution of the enzyme is again dialyzed over night against 2% KCl to remove traces of zinc. Some 5-6 changes of KCl solution are normally utilized, and the solution is tested with Dithizone reagent to check removal of zinc.

For every 100 gms of beef tissue one normally obtains approximately 15-20 ml of final enzyme solution containing approximately 3-5 mg protein per ml.

The isolation of proteolytic enzyme from beef liver tissue was done according to the same procedure described for beef muscle tissue.

2.1.2 Assay Procedures

The assay procedure described by Snoke and Neurath[6] is a modification of that described by Anson.[7] More recent descriptions of the same procedures are described by Bergmeyer.[8] Because there are a few minor variations among all of these comparable methods, and because the reasons for some of the adaptations do not seem to have been fully explained in the literature, the complete assay system and procedure are described here.

A method depends on the hydrolysis of a urea denatured hemoglobin solution. The hydrolysis is performed at 35°C

in the presence of 0.033M acetate buffer at a pH of 4.0. A pH of 4.0 is near the lower limit of the range for acetate bufferes, and a citrate buffer solution would seem to be a much more logical choice. However, citrate would undoubtedly sequester the ferrous ions which are added as an activator and, for this reason, an acetate system is about the only readily available buffer system which can be utilized.

Hemoglobin was prepared according to the method of Anson,[7] but it did not prove significantly superior to commercial preparations on the market today. Ten grams of hemoglobin were dissolved in 250 ml distilled water contained in a 500 ml volumetric flask. Some 40 ml 1.0 N NaOH and 180 gms urea were added. The urea dissolved with some difficulty over a period of about 30 minutes with constant stirring. Approximately 50 ml of additional distilled water was added to bring the solution to a total volume of approximately 400 ml. The solution of the urea is an endothermic reaction, and the solution needs to be brought to room temperature with a warm-water bath. The solution was then allowed to set for 1 hour to permit denaturation of the hemoglobin by the urea.

Sufficient sodium acetate was added (6.8 gms/500 ml) to give a final concentration of 0.1M acetate. The pH was adjusted to 4.0 and the solution was diluted to its final volume of 500 ml. Aliquots of the buffered, denatured hemoglobin solution were frozen until ready for use.

Individual assays were conducted by placing 5.0 ml of hemoglobin substrate and 5 ml 0.1M acetate buffer pH 4.2 in a 13 x 150 mm culture tube incubated in a water thermostat at 35°C. Sufficient distilled water was added to give a final volume, after the addition of enzyme and ferrous ion activator of 15 ml. Normally, the amount of enzyme used was 1.0 ml, together with 0.3 ml of 0.5M ferrous ammonium sulfate solution; the amount of water added was, therefore, 3.7 ml. The water and hemoglobin solutions were allowed to equilibrate for at least 1 hour. Ferrous ammonium sulfate was then added (0.3 ml of 0.5M solution), giving a final ferrous ion concentration of 0.01M.

A suitable interval timer is started as soon as the enzyme is added and mixed with the substrate, and two ml aliquots are withdrawn at intervals and quickly mixed with 5 ml of 5% trichloroacetic acid to stop the enzymatic reaction. The tubes are allowed to set at least one-half hour. They are then centrifuged or filtered to give the clear TCA soluble hydrolyses products. Our experience has indicated that clarification by centrifugation gives more consistent results and is, therefore, preferred over filtration. Perhaps a variable portion of the hydrolyses products are adsorbed onto the filter papers.

There are several alternatives for determining the extent of hydrolyses. One of these is the determination of tyrosine with the phenol reagent of Folin and Ciocalteu.[9] Anson's

modification of this method allows the use of a standard curve. The blue color produced may be reaction of the tyrosine and phenol reagent and read conveniently on any instrument in the visual region at 580, 690, or 750 mu.

Another alternative is simply to determine the optical density of the clarified TCA solutions at 280 mu in the near UV. Figure 2 is a UV absorption spectrum of the hemoglobin hydrolyses products which was obtained on a Beckman DK 2 UV recording spectrophotometer. This is a dual-beam instrument, and the curve was obtained with a 0-time sample in the reference beam and a 30-minute sample in the response beam. The curve is, therefore, a true representation of the UV absorption response due to hydrolyses. It is seen from the curve that 280 mu absorption is a valid measure of the extent of hydrolyses.

For routine assay work, the use of the Beckman DU instrument has proven somewhat more convenient. The absorbance of the solutions is determined after setting the instrument to 0 absorbance, using a distilled water blank. The 0-time samples have an absorbance of about 0.350 under these conditions, but this is of little consequence because the important feature is the <u>change</u> in absorbance with time.

Snoke and Neurath[6] expressed activity in terms of an arbitrarily defined "Specific Activity" which they defined as "increase in optical density per hour per mg of muscle protein nitrogen in 1 cc of reaction mixture." Table 1 compares the

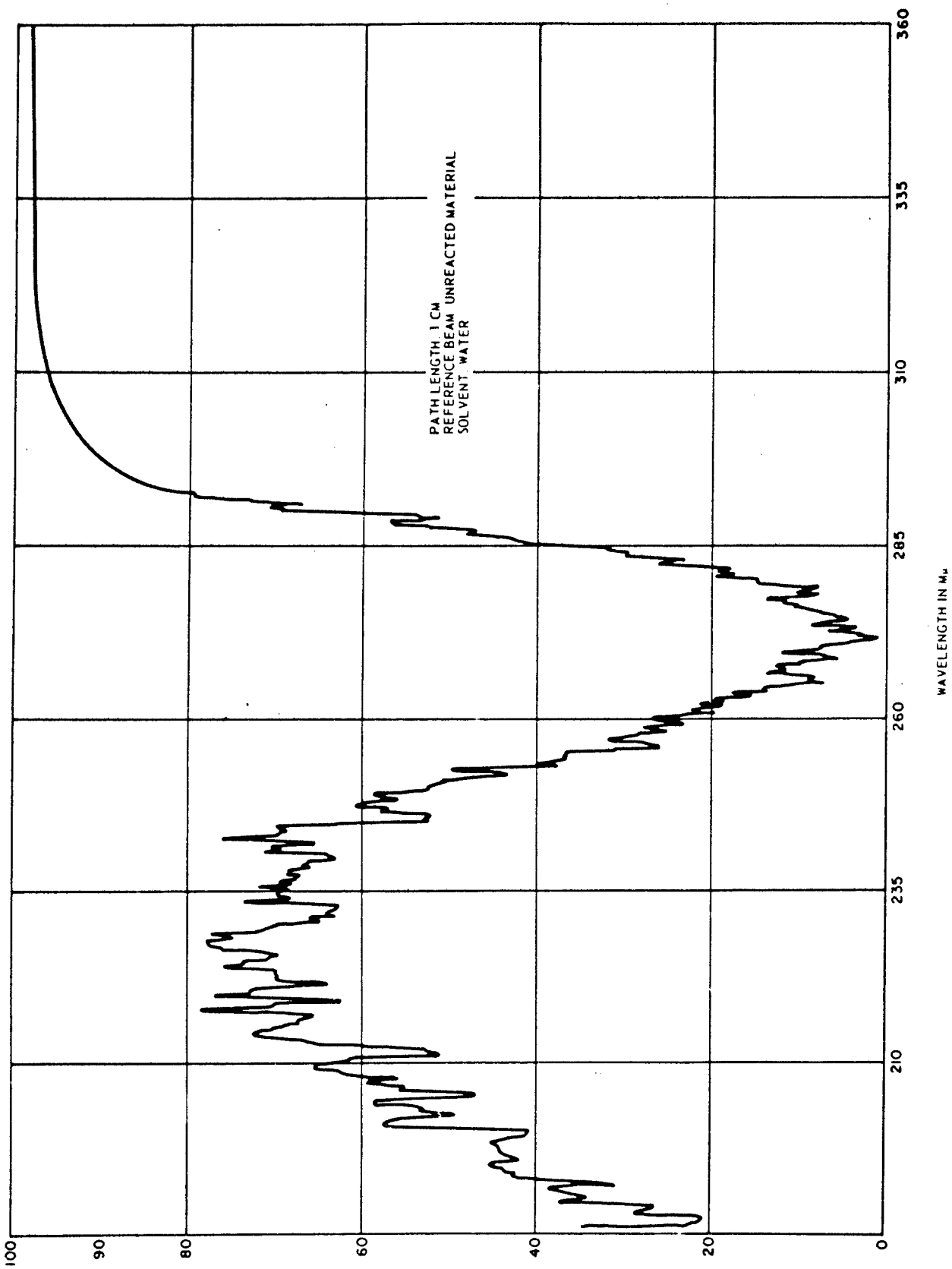


Figure 2. U.V. Absorption Spectra of TCA Soluble Hydrolyzed Product

Table 1

Comparison of Specific Activities of Rabbit and Beef Muscle Tissues[1]

	Rabbit Muscle (Snoke and Neurath)	Beef Muscle
After $NaOAcCl_3$ Precipitation	3.60	19.3
Final Enzyme from Zn Precipitation pH 7.5	447.0	61 7.3

[1]Specific Activity = increase in OD/hr/mg protein N/ml reaction mixture

specific activities of some beef muscle enzymes with the specific activities of the rabbit muscle enzymes as published in the original paper.

It is worth going through the calculations of specific activity in some detail because of an ambiguity in that part of the definition which reads "...in 1 cc of reaction mixture." The meaning of that phrase is NOT per 1 cc of reaction mixture, as will be shown.

Consider the authors' precipitate B, obtained at pH 7.5, as listed in their table IV. That solution has a tabulated total activity of 176, protein nitrogen of 0.10 mg/cc, and total volume of 59 cc. The Total Activity is clearly the activity of the total 59 ml of enzyme solution, and activity is defined elsewhere as increase in optical, density per hour.

The activity per ml should, therefore, be changed in optical density per hour per ml of enzyme, or 176/59 = 2.98. The change in optical density per hour per mg protein nitrogen would be 2.98/0.10 = 29.8. This value of 29.8 is what one would normally expect to be the Specific Activity, for its units are expressed in terms of hydrolytic activity under specified conditions per unit time per unit of protein nitrogen. For some reason which is not clear, the authors, then, related Specific Activity back to the total volume of their assay solution, 15 ml. Note especially that their Specific Activity is not expressed per ml of assay mixture, but per 15 ml.

Multiplying the 29. 8 x 15 = 447 gives the tabulated Specific Activity. The original convention has been retained during this work, if for no reason other than to make comparisons of specific activity easier.

Protein nitrogen was determined with a Coleman nitrogen analyzer. This instrument is designed to work well with samples containing 0.1-0.5 mg nitrogen; hence, the protein nitrogen determinations do not require the volume of enzyme solution which would otherwise have to be sacrificed for this purpose. The instrumental method is verified periodically, using samples of chromatographically pure amino acids obtained commercially.

In summary, the assay solution consisted of a total volume of 15 ml containing 1 ml of enzyme, 5 ml of acetate buffer, 5 ml of hemoglobin solution, 0.2 ml ferrous ammonium sulfate and 3.7 ml distilled water. The supernatant was read directly in a Beckman DU spectrophotometer at 380 mu.

2.1.3 Optimum pH for Enzymatic Hydrolyses

The variation of the rate of enzymatic hydrolysis with changes in pH was investigated to help assure that the enzymatic activity was not due to activity of the more common pepsin and trypsin.

Portions of the buffered substrate were adjusted to a series of five different pH values. The initial intention was to select values around the stated optimum pH of 4.0, and vary these by a constant fourfold change in the hydrogen ion

concentration. The values of pH which were chosen were 2.8, 3.4, 4.0, 4.6, and 5.2. Duplicate assays were run at each pH level.

A check of the actual pH of the reaction mixture during the assay indicated that the target values were missed somewhat, presumably because of the effect of the ferrous ammonium sulfate which was mentioned earlier. The actual electrometric values of pH, obtained on the same instrument which had been used just a short time before to standardize the substrates, showed values of 2.7, 3.2, 3.8, 4.3, and 4.9.

From the absorbance readings obtained at 380 mu during the assay, the slopes of the lines of best fit were calculated, and these, in turn, were used to calculate relative activity of the enzymatic reaction at the various actual values of the pH of the reaction mixture. The results are indicated by the curve of figure 3. An optimum pH between 3.8 and 4.3 is indicated.

No additional effort was made to pinpoint the best possible pH because it is clearly difficult to control, and probably not critical for the purposes of this program.

2.1.4 Effect of Temperature on Enzymatic Activity

The effect of incubating the enzyme solution at several temperatures on the rate at which it was able to hydrolyze the hemoglobin substrate was investigated. It should be noted that this is not the same thing as a determination of the optimum

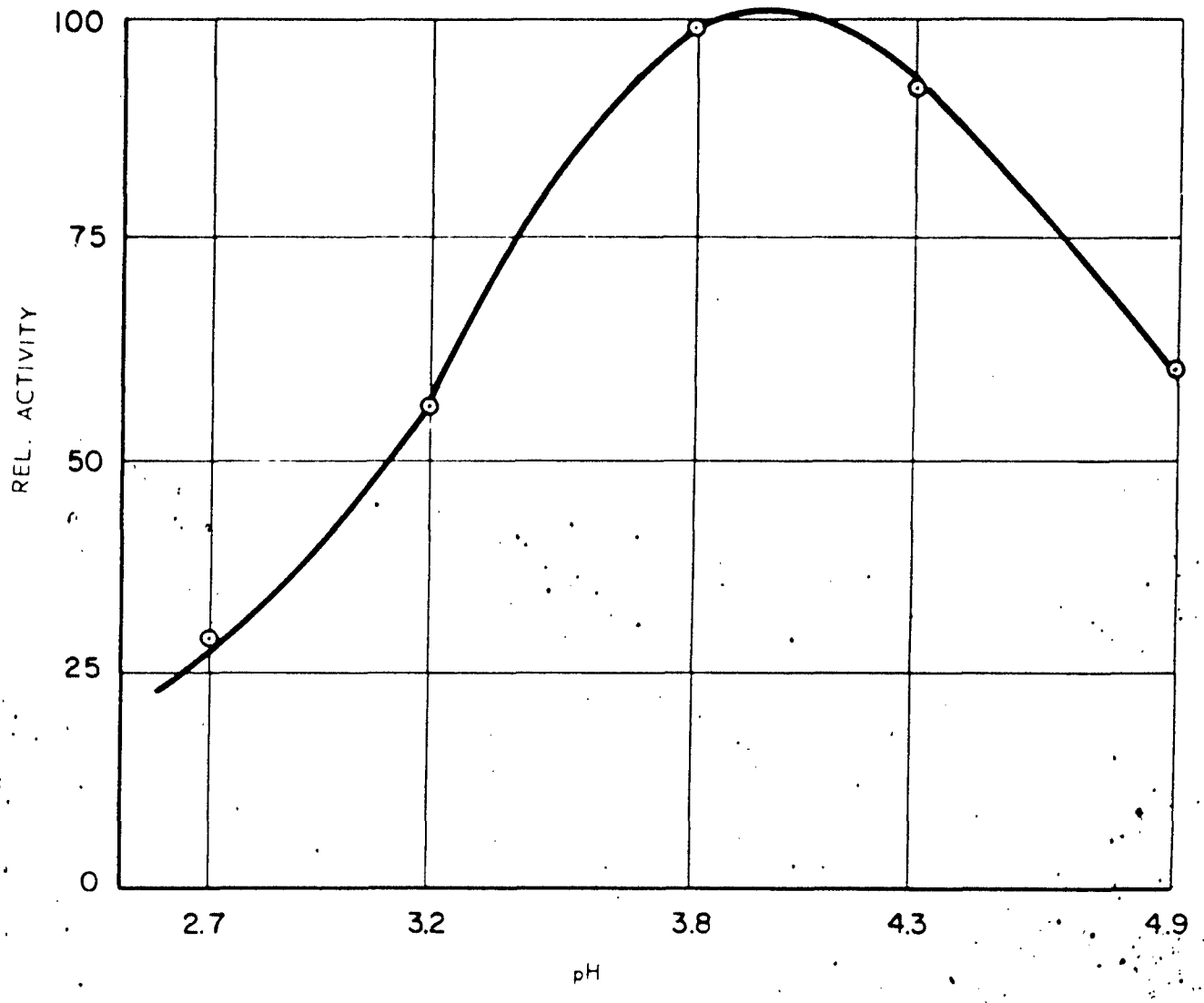


Figure 3. Relative Activities of Proteolytic Enzyme at pH Range 2.7 to 4.9

temperature for the hydrolyses reaction, nor does it indicate anything about the rate at which the enzyme deteriorates at any fixed temperature. It has been our experience that the purified enzyme deteriorates quite rapidly if stored as a solution, and becomes inactive in some four or five days. This may be due to the natural proteolytic activity of the material, which causes it to chew itself into inactive pieces.

Aliquots of an active enzyme preparation were incubated for 24 hours at temperatures of 0°C, 23°C, 40°C, and 60°C. Assays were then run according to the standard procedure described above. The absorbance readings obtained are given in Table 2. The slopes of the lines of best fit were then calculated according to a conventional least squares method, and the rate of hydrolysis per hour calculated by multiplying those slopes (change in optical density per minute) by 60 minutes. The unit activities were then utilized to calculate the specific activities.

In figure 4, the specific activity is plotted as a function of the incubation temperature. There is apparently little difference in activity between 0°C and 20°C, but the activity drops off quickly at higher temperatures. It is quite interesting, however, that even after 24 hours at 60°C appreciable amounts of activity remain.

One of the main purposes of this particular study was to be certain that all of the enzymatic activity would not be

Table 2

Effect of Holding Temperature on Enzymatic Activity

	After 24 hours at			
	0°C	23°C	40°C	60°C
Assay: OD at min				
0				
10	0.090	0.080	0.050	0.052
20	0.090	0.096	0.051	0.048
30	0.160	0.183	0.081	0.073
40	0.200	0.223	0.113	0.095
50	0.235	0.252	0.144	0.095
60	0.275	0.280	0.169	0.107
Slope of least-squares line	0.004375	0.004575	0.002704	0.001621
OD/hr (slope x 60)	0.262	0.274	0.162	0.097
Protein N, mg/cc	0.241	0.241	0.241	0.241
Specific Activity	1.63	1.70	1.01	0.60

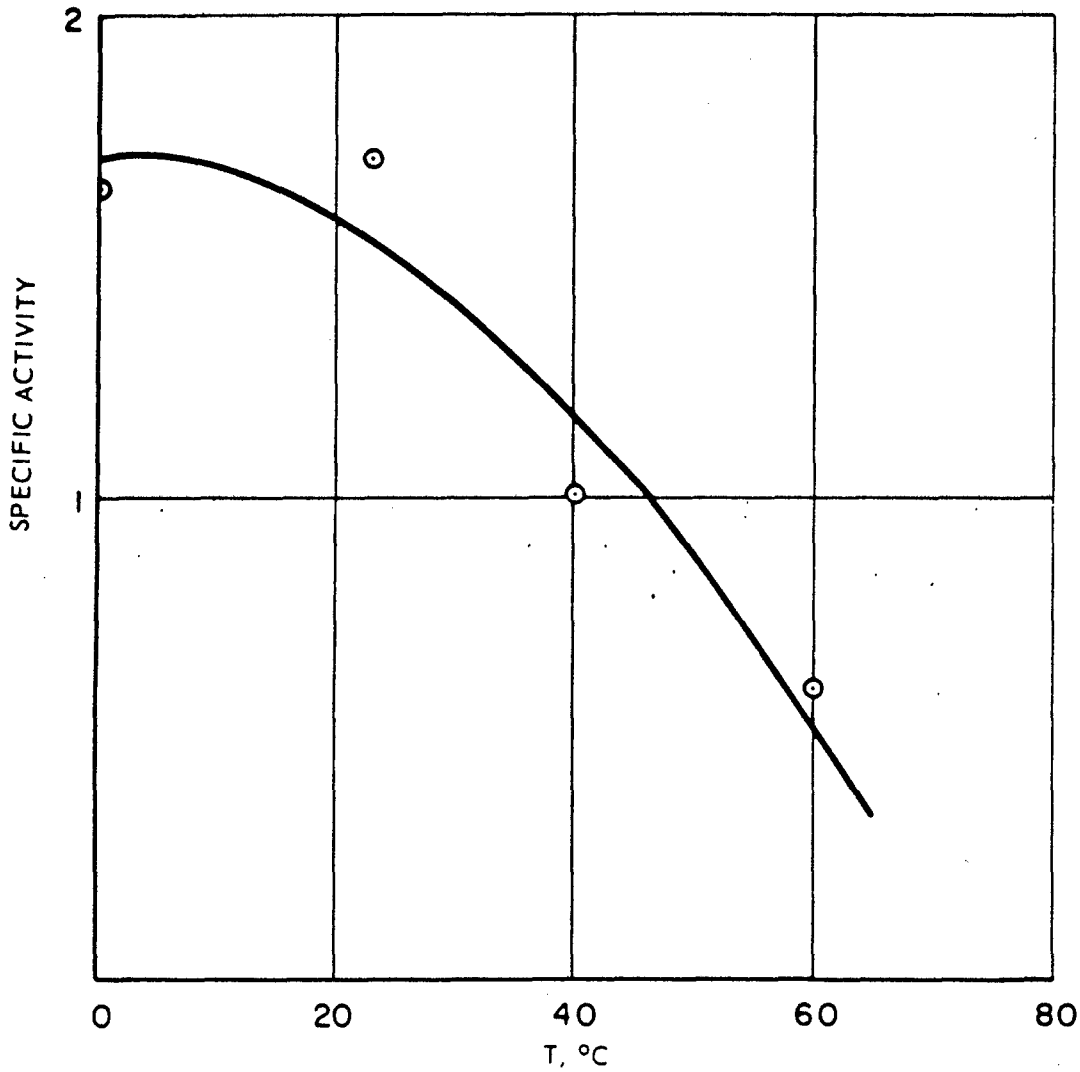


Figure 4. Effect of Holding Temperature on Enzymatic Activity

destroyed during the 24-hour, radio-frequency irradiation by temperature effects alone.

2.1.5 Lyophilization of Enzyme

In general, the stability of the enzyme is so poor that it has not been possible to prepare sufficient material at one time for more than a single r-f experiment. The r-f cells hold approximately 15 ml of enzyme solution each and, with adequate controls, a single experiment consumes all of the preparation at one time. The desirability of being able to salvage as much activity as possible is obvious.

Experiments were, therefore, performed to determine how much of the activity was lost by the process of freeze drying or lyophilizing the enzyme.

Duplicate 5 ml aliquots of an active preparation were placed in identical 50 ml rb flasks and shell frozen with liquid nitrogen. One of the flasks was maintained at -10°C, while the other was connected to a commercial lyophilization apparatus and dried overnight. The dried material was then reconstituted with 5 ml of distilled water; the control was allowed to thaw. Assays were run on both samples. The results are shown in Table 3.

In is obvious that the enzyme can be lyophilized with little or no loss of activity.

2.2 RF Radiation Experiments

2.2.1 Radio-Frequency Radiation Apparatus

A block diagram of the r-f irradiation system is shown in figure 5. It consists of a Textronic Industries

Table 3

Lyophilization of Enzyme

		Control Aliquot	Lyophilized Aliquot
OD,	0 min		
	10 min	0.034	0.035
	20 min	0.080	0.077
	30 min	0.105	0.098
	40 min	0.127	0.121
	50 min	0.136	0.135
	60 min	0.174	0.153
Equation, line of best fit, $y = a + bx$			
	slope, b	0.00276	0.00251
	intercept, a	0.0109	0.0131

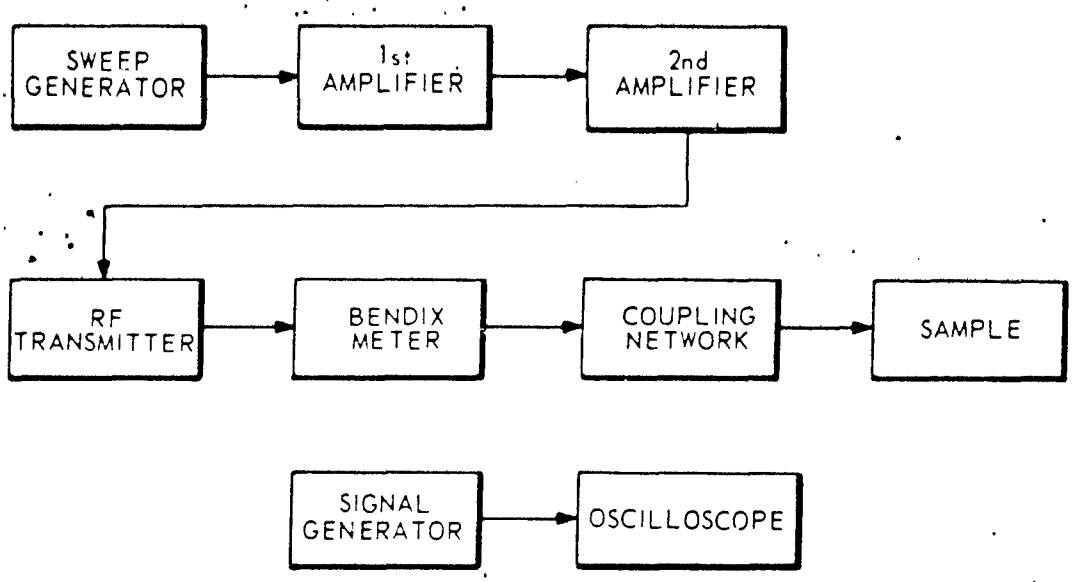


Figure 5. Block Diagram of RF Radiation Apparatus

sweep generator of variable frequency and sweep width. Its output is fed through two Hewlett-Packard wide-band amplifiers (Model 460 A and B). The amplified sweep is then used as the VFO input to a Viking Challenger short-wave transmitter, and the resultant r-f is fed through a Bendix, Model 2633, r-f power and VSWR meter. The irradiation is coupled to the sample cell by an E.F. Johnson variable inductor (Series 229). A Hewlett-Packard signal generator (Model 608A) is used with an oscilloscope to monitor the transmitter frequency.

In operation, the sweep generator frequency and width are first adjusted to give the desired r-f range, e.g., 13.0-13.2 mc. The variable inductor is then adjusted for maximum net power, i.e., forward power less reflected power, as read on the Bendix Power Meter. The net power, frequency and temperature are periodically checked during the irradiation time.

2.2.2 R-F Radiation Studies

During the initial period of the program the source of enzymes chosen was the frozen beef muscle tissue. It was found that a large quantity of frozen beef muscle tissue was required in order to isolate sufficient quantities of enzymes. Since the isolation and the purification process was an extremely tedious and time consuming process, beef liver tissue was selected as the source for the experimental material. Beef liver tissue is known to be rich in the proteolytic enzymes and was found to be much more satisfactory for the required studies.

The enzyme preparations were made in batch quantities that were large enough for at least 20 experiments. The preparations were divided into aliquots which were then stored in the freezer. In this way uniformity of sample was assured for an entire set of experiments.

In carrying out the r-f irradiation experiments, enzyme solution of a quantity sufficient to completely immerse the electrodes is introduced into two identical cells; one cell served as a control for comparison with the experimental cell. Both cells are then placed in a constant temperature water bath. While both are maintained at a constant temperature, the experimental cell is irradiated with r-f energy. At the completion of the irradiation period, both cells are removed from the water bath, and their contents are immediately assayed for enzyme activity.

The cells employed in these studies were fabricated from epoxy boards with the r-f electrodes placed behind a non-conducting coating of epoxy resin. Three type of cells with varying configuration were used in these experiments. These cells and their dimensions are:

Cell	Plate Dimensions	Plate Distance	Volume
Cells A	2" x 5"	0.2 cm	5 cc
Cells B	2" x 5"	0.4 cm	8 cc
Cells C	3" x 10"	0.7 cm	250 cc

Cells A and B were used for the experiments on the purified proteolytic enzymes whereas cells C were used for the experiments on the ground beef muscle tissue.

The program was developed around four statistically designed experiments, each with at least 8 runs. Design #1 was devised to study five general factors affecting the results of r-f irradiation. The five factors were: (1) irradiation time, (2) target frequency, (3) voltage, (4) temperature, and (5) duty cycle. This design is shown in Table 4.

As will be seen in Section 3, the results of design #1 was that the significant factor was the irradiation time and the interaction between the irradiation time with voltage. Consequently design #2 was devised to study these factors. The irradiation time was shortened to 1/4 hours 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 5.)

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 respectively. (See Table 6.)

Design #4 consisted of 16 experiments along with 4 simulated r-f experiments, was set up to study the effect of voltage and duty cycle on the inactivation process. (See Table 7.) These factors were studied at three levels for the duty cycle and 4

Table 4
Experimental Variables and Design of Design # 4 (Cell A)

Factor Symbol	Factor	Values	
A	Irradiation Time (hours)	½	2
B	Target Frequency (megacycles)	11.75	13.5
C	Temperature (°C)	16.5	19.5
D	Voltage (volts)	50	150
E	Duty Cycle	0.1	0.5

Experiment Number	A Irradiation Time (hrs.)	B Target Frequency (megacycles)	C Temperature (°C)	D Voltage (volts)	E Duty Cycle	Result
1	½	11.75	16.5	50	0.5	90.5
2	2	11.75	16.5	50	0.1	105.2
3	½	13.5	16.5	50	0.1	58.5
4	2	13.5	16.5	50	0.5	102.5
5	½	11.75	19.5	50	0.1	64.7
6	2	11.75	19.5	50	0.5	106.9
7	½	13.5	19.5	50	0.5	85.2
8	2	13.5	19.5	50	0.1	108.0
9	½	11.75	16.5	150	0.1	97.9
10	2	11.75	16.5	150	0.5	95.3
11	½	13.5	16.5	150	0.5	92.4
12	2	13.5	16.5	150	0.1	95.1
13	½	11.75	19.5	150	0.5	106.4
14	2	11.75	19.5	150	0.1	106.2
15	½	13.5	19.5	150	0.1	79.7
16	2	13.5	19.5	150	0.5	94.7

Table 5

Experimental Variables and Design of Design #2
(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 6

Experimental Variables and Design of Design #3
(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

Table 7

Experimental Variables, Design and Results of Design #4
(Cell B)

Variables	Levels			
	0	1	2	3
Duty Cycle	0.5	.15	.25	
Voltage (volts)	50	100	150	200

Sequence No.	Experiment No.	Duty Cycle	Voltage	Result
4	1	0	0	101.6
10	2	1	0	107.1
21	3	2	0	99.0
12	4	1	0	92.0
2	5	0	1	103.6
13	6	1	1	116.2
17	7	2	1	118.5
15	8	1	1	107.5
18	9	0	2	103.1
19	10	1	2	113.3
7	11	2	2	91.2
20	12	1	2	108.8
8	13	0	3	96.2
5	14	1	3	90.2
3	15	2	3	97.7
6	16	1	3	92.1

Table 7 (Cont'd)

1	A	Simulated RF	96.2
9	B	Simulated RF	95.5
14	C	Simulated RF	102.3
16	D	Simulated RF	117.0

levels for the voltages. The frequency of the r-f radiation was kept at a constant value of 13.5 mc. The power level was kept at 5 watts and the temperature at a constant value of 16.5°C.

R-f irradiation of beef muscle tissue were conducted in similar manner as the r-f irradiation experiments of the purified proteolytic enzymes. However, because of the use of ground beef tissue, some deviation from the procedure is expected and is given.

The beef tissue obtained directly from the slaughter house was kept frozen at -20°C when not in use. About 80 gms of beef muscle tissue, mixed with 80 cc of 2% KCl was blended with a blendor for 10 seconds. The sample was placed in an epoxy cell 8 x 10 x 9/32 (0.7 cm) inches and subjected to r-f energy. A similar sample also placed in a cell but with the absence of r-f energy served as the control sample. Following the irradiation period, both the r-f sample and the control samples were subjected to the extraction procedure for the isolation and purification of enzymes as given in the previous section. The extracted enzymes from both the r-f sample and the control samples were subjected to analysis using the urea-denatured hemoglobin method.

During this period three "simulated r-f" experiments on the ground tissue were conducted to determine the effects of the cell on the enzyme and to determine the effectiveness and

accuracy of the extraction method on two similar samples taken from a common aliquot.

Two r-f irradiation experiments were conducted at the following conditions: temperature, 16.5°C; frequency, 13.5 ±0.3 mc; voltage, 50 volts; duty cycle, 0.1; and watts, 5.0 watts.

3. RESULTS

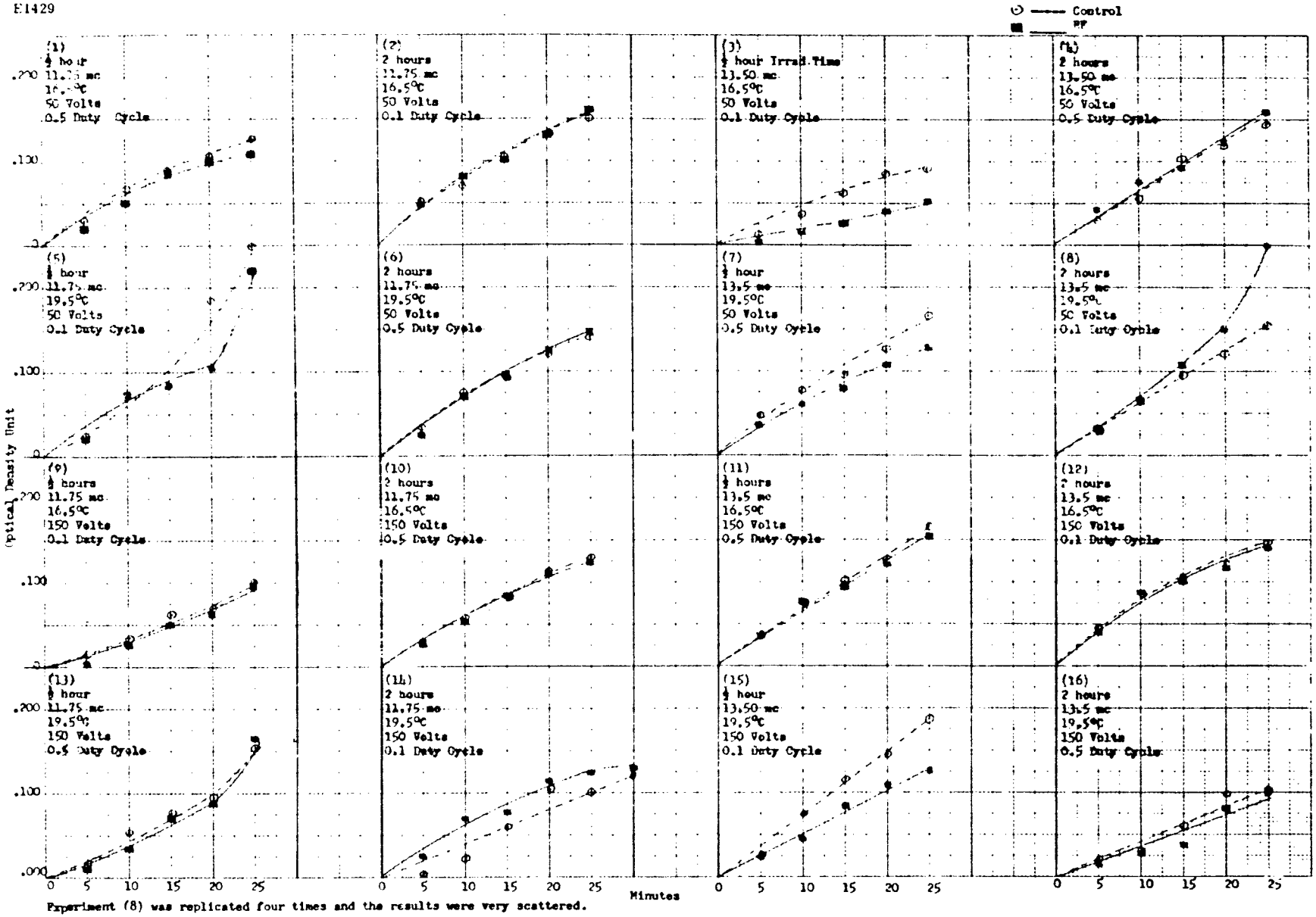
The results of the experiments of the 4 designs are presente in graphic form with all the runs of a design given in a single figure. The inactivation of the enzyme expressed in percent was determined by comparison of the absorbance of the control and the r-f sample after 25 minutes of reaction time. The experimental results of the factorial design were arranged in standard order according to Davies[11] and were analyzed by the Yates algorithm method.[12] In the calculations of the Yates analysis the "Mean Square" values represent the variation of an effect shown as "Effect Estimated" from the expected effect. When the mean square is small (approaching zero) there is no difference between the levels of the referenced variables. Conversely, a large difference between the levels will be reflected by a large mean square. In some cases an additional method is used to determine whether any one factor is significant. The method employed to determine factor significance is the half-normal plot method. The half-normal plots are plots of the ranked absolute values of the effects of a 2^x (factor at 2 levels) experiments on a special grid. If none of the factors or

interaction factors studied has a significant effect on the system, the values will be normally distributable and assume a straight line when plotted on a special probability grid. Factor significance is shown by a large deviation of its effect from the plotted line. The graphical co-ordinates of the effects are determined by the following relation:

$$\text{Prob.} = (i-1/2)/2; \; i = 1, 2, 3.... \text{ etc.}$$

where n is the number of effects estimated. The smallest absolute value corresponds to the probability i = 1, etc.

The results of the experiments in design #1 are given in Figure 6. The analysis of the experimental data were limited because of the inaccuracy of extrapolating from the analytical results a response for use in the mathematical analyses and because of a large experimental error. However, the analysis of data by use of the half-normal plots shows that one factor and possibly two other factors seem to affect the results. (See Figure 7.) 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 yielded the greatest enzyme inactivation. However, the differences observed among the various experiments at a 1/2 hour radiation indicate quite clearly that other factors may contribute to the effect. Inspection of the half-normal plots of this design indicate probable interaction factors of the irradiation time-voltages and the irradiation time-duty cycle effect.



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

Figure 6. Experimental Results of Design 1

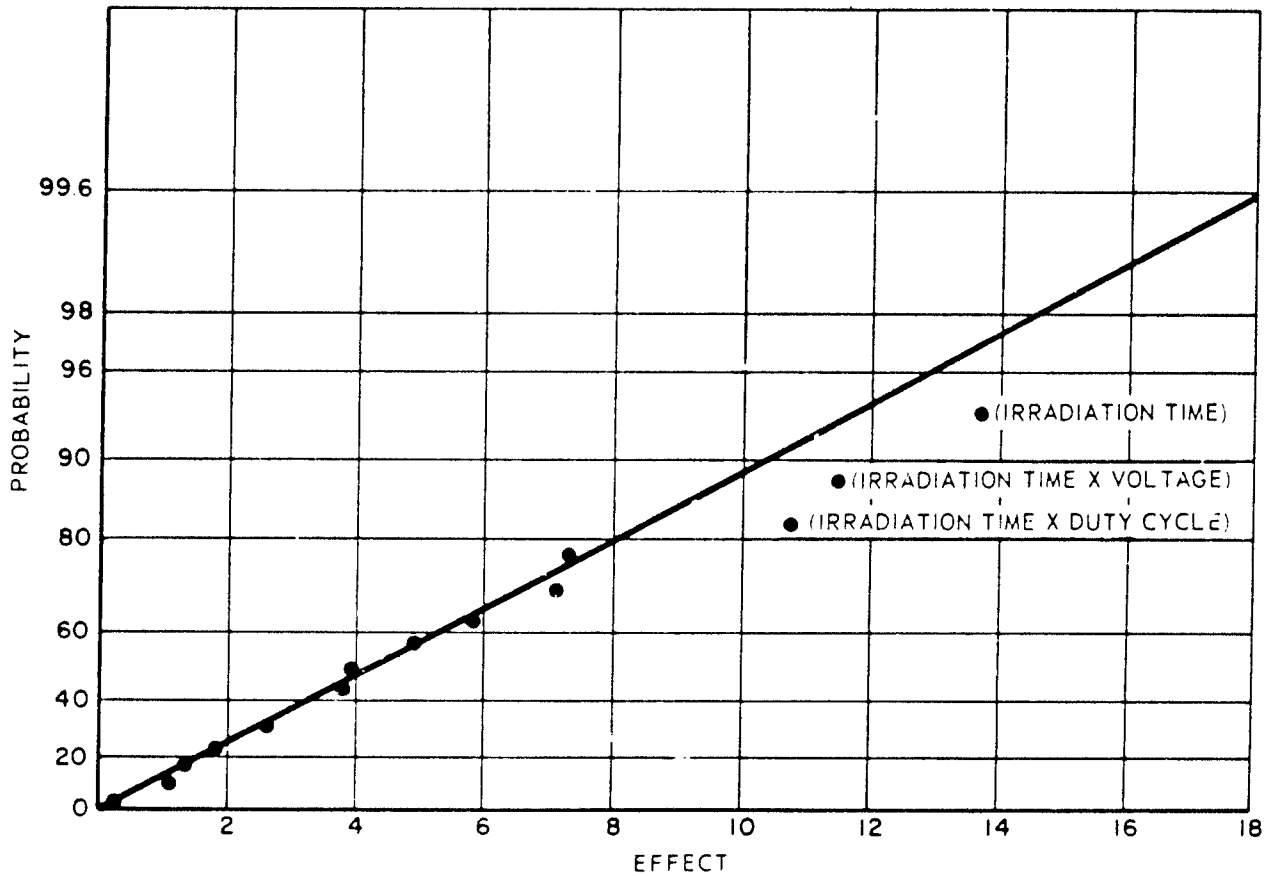


Figure 7. Half-Normal Plots of Experimental Effect of Design 1

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 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 the use of a different batch of enzymes gave comparable results with decrease in enzyme activity of about 35 and 43%. These results are shown in Figure 8.

Design #2 was set up to study the effect of shorter irradiation times, reduced voltage levels, and lower frequency conditions with power rating substituted for the duty cycle. The results of these experiments show inactivation of the enzymes at 11.85 mc only. (See Figure 9.) Irradiation of the enzymes at 7.0 mc did not seem to effect the enzyme activity. Statistical analysis failed to indicate the main facet 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 runs. The remaining activity observed in runs 1, 3, and 4 were 77, 65, and 78% respectively, of the initial activity. (See Figure 10.) The results of the other five runs may not be considered significant since the observed inactivation falls approximately within the assumed error of 10%. The Yates algorithm analyses for this design is given in Table 8.

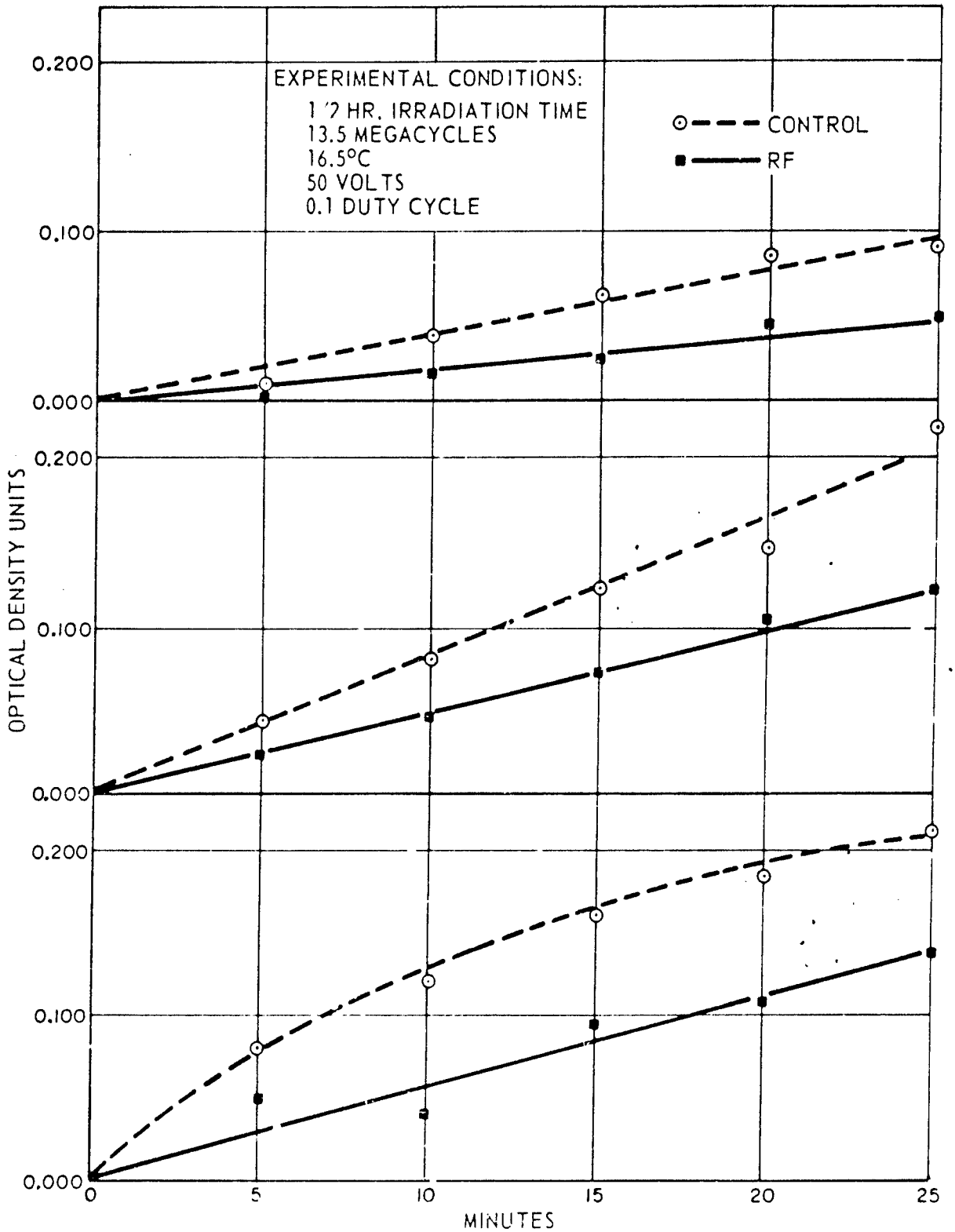


Figure 8. Results of Experimental Repetitions

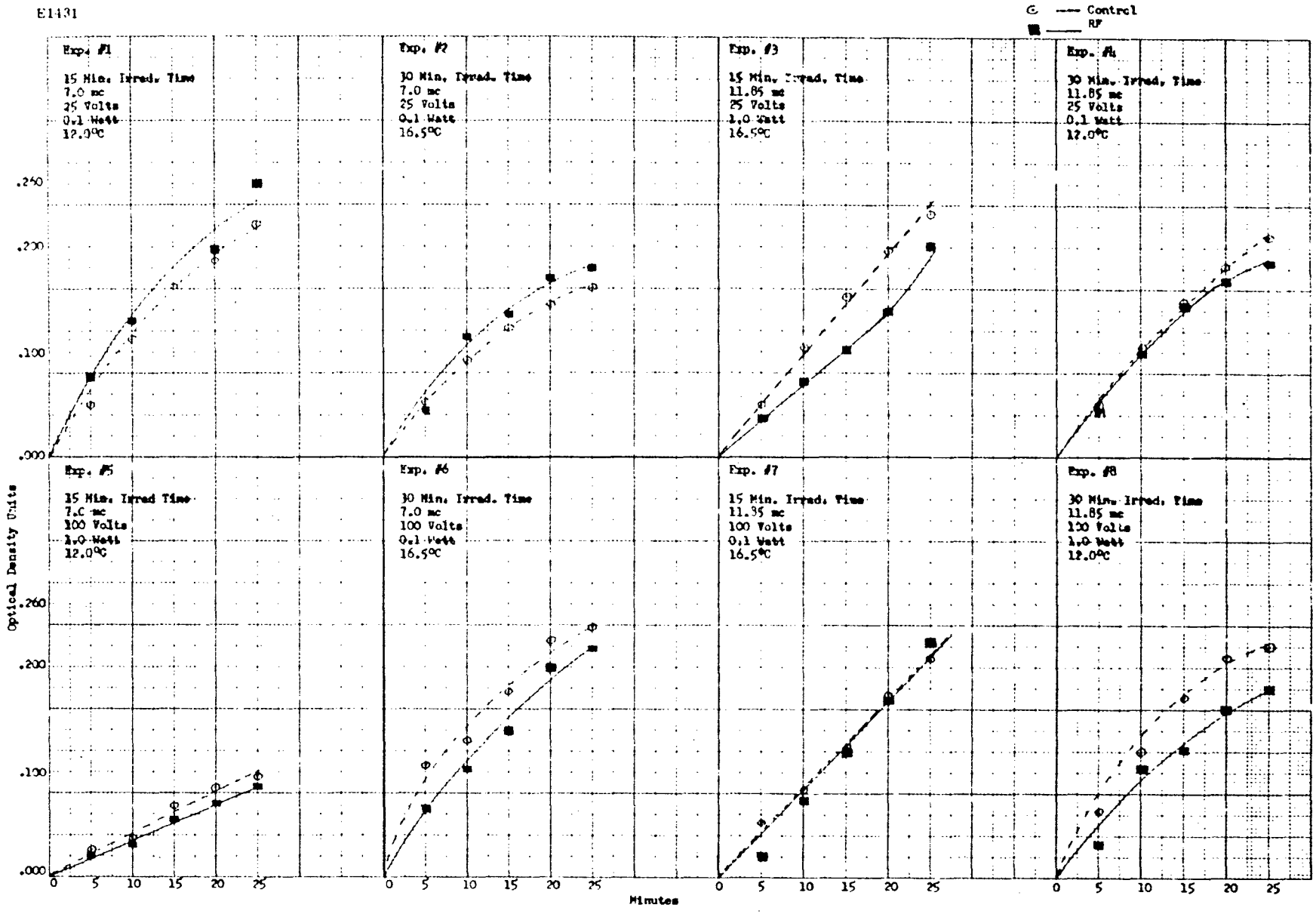


Figure 9. Experimental Results of Design 2

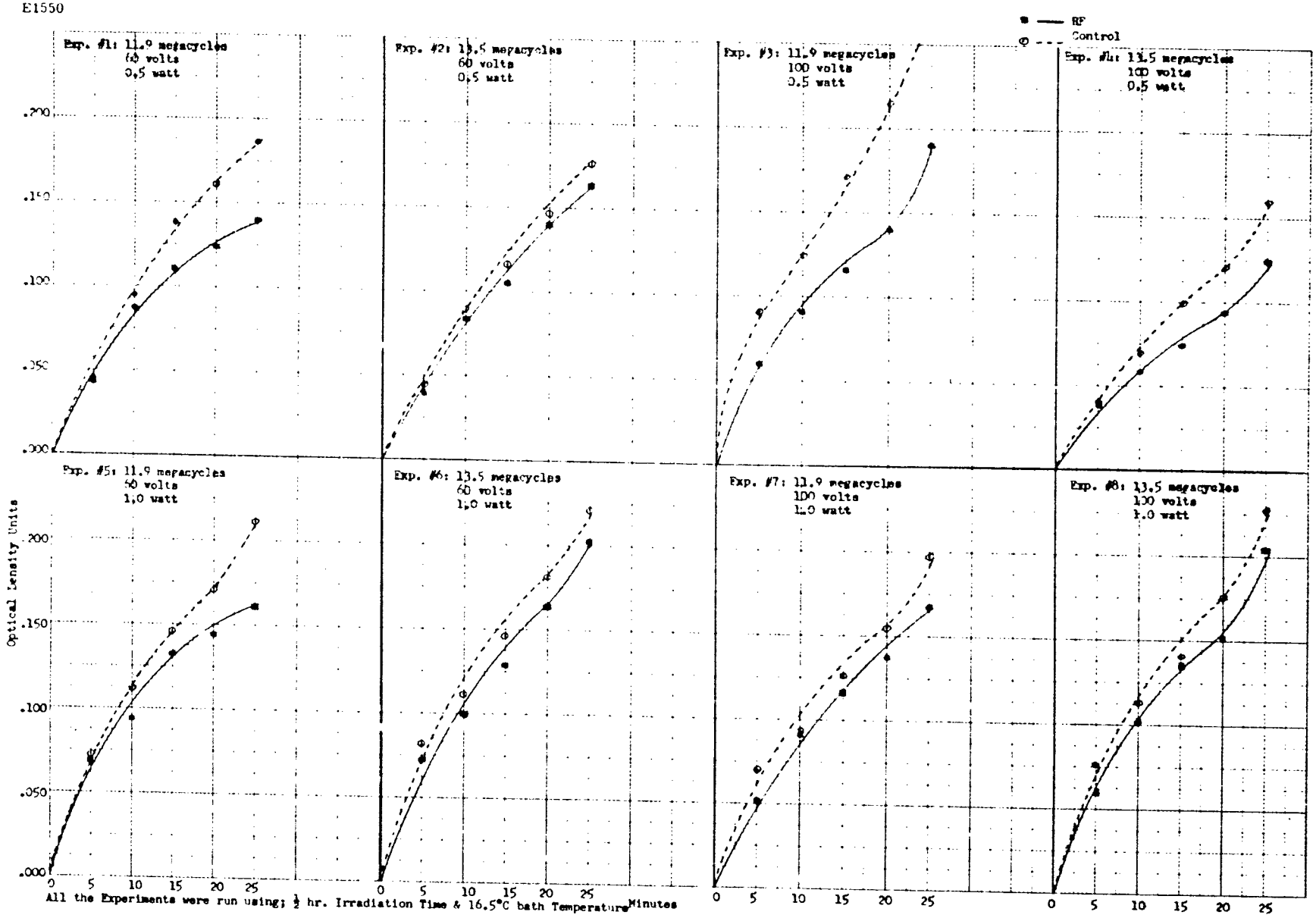


Figure 10. Experimental Results of Design 3

Table 8

Experimental Results and Yates Algorithm Analysis

Experiment No.	Frequency	Voltage	Power	Result
1	11.9	60	0.5	77.0
2	13.5	60	0.5	95.9
3	11.9	100	0.5	64.8
4	13.5	100	0.5	77.7
5	11.9	60	1.0	84.8
6	13.5	60	1.0	90.6
7	11.9	100	1.0	88.4
8	13.5	100	1.0	86.9

Result	(1)	(2)	(3)	Mean Square	Effect Estimated
77.0	172.9	315.4	666.1		
95.9	142.5	350.7	36.1	162.9	A
64.8	175.4	31.8	-30.5	116.3	B
77.7	175.3	4.3	-13.3	22.1	AB
84.8	18.9	-30.4	35.3	155.8	C
90.6	12.9	-0.1	-27.5	94.5	AC
88.4	5.8	-6.0	30.3	114.8	BC
86.9	-1.5	-7.3	-1.3	0.2	ABC

The analysis gives an estimate of the error variance as 57.9 and an estimate of standard deviation of 7.6. Based on the F Test, a test comparing variances, a mean square value greater than 446 is necessary for factor significance. Since none of the factors has a mean square value approaching 446, it can be concluded that none of the factors studied appear to have any effect on the system at the levels studied.

Design #4 was set up to study the effect of the voltage and the duty cycle on the inactivation process. The frequency temperature and the power were kept at a constant level. The experimental results are given in Figure 11. The Yates analyses of the factor significance is given in Table 9. Analyses of the data by half-normal plots shows that the main factors do not seem to affect the results, but however a quadratic effect of the voltage is noted. (See Figure 12).

Results on the r-f irradiation of ground beef muscle tissue were in general rather discouraging. First of all, since the beef tissue contained much less proteolytic enzyme, large samples were required for the irradiation experiments. This necessitated the construction of a larger sample cell which had completely different r-f characteristics as compared to the smaller cells used in the purified proteolytic enzymes experiments. With the use of the larger cells, a maximum of only 5 watts and 50 volts could be obtained because of the larger plate area and plate distance. Secondly, the

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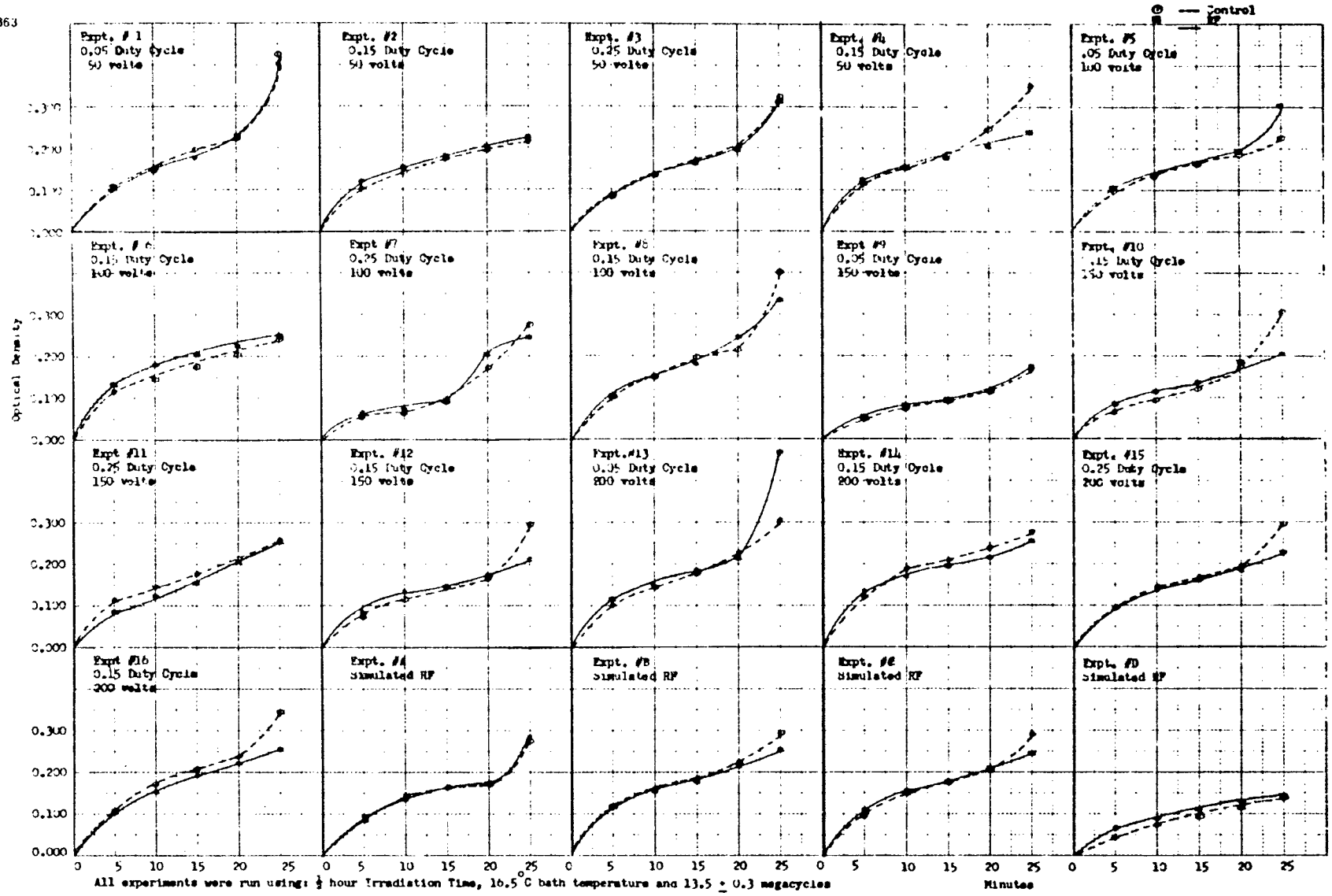

All experiments were run using: ½ hour Irradiation Time, 16.5°C bath temperature and 13.5 ± 0.3 megacycles

Figure 11. Experimental Results of Design 4

TABLE 9

Yates Analysis of Experimental Data Design No. 4

Sequence Number	Experiment Number	Experimental Results	1	2	3	4	Effect	Effect Estimated
4	1	101.6	208.7	399.7	845.5	1638.1	102.4	Meon
10	2	107.1	191.0	445.8	792.6	16.3	2.04	Quadratic Duty Cycle
21	3	99.0	219.8	416.4	0.1	-24.5	-3.06	Linear Duty Cycle
12	4	92.0	226.0	376.2	16.2	-28.3	-3.54	
2	5	103.6	216.4	-1.5	-11.5	5.9	0.74	Cubic Effect of Voltage
13	6	116.2	200.0	1.6	-13.0	-36.3	-4.54	Quadratic Duty Cycle X Cubic Voltage
17	7	118.5	186.4	27.8	-36.1	43.7	5.46	Linear Duty Cycle X Cubic Voltage
15	8	107.5	189.8	-11.6	7.8	-18.1	-2.26	
18	9	103.1	5.5	-17.7	46.1	-52.9	-6.61	Linear Voltage
19	10	113.3	-7.0	6.2	-40.2	16.1	2.01	Quadratic Duty Cycle X Linear Voltage
7	11	91.2	12.6	-16.4	3.1	-1.5	-0.19	Linear Duty Cycle X Linear Voltage
20	12	108.8	-11.0	3.4	-39.4	43.9	5.49	
8	13	96.2	10.2	-12.5	23.9	-96.3	-12.04	Quadratic Voltage
5	14	90.2	17.6	-23.6	19.8	-42.5	-5.31	Quadratic Duty Cycle X Quadratic Voltage
3	15	97.7	-6.0	7.4	-11.1	-4.1	-0.51	Linear Duty Cycle X Quadratic Voltage
6	16	92.1	-5.6	0.4	-7.0	4.1	0.51	

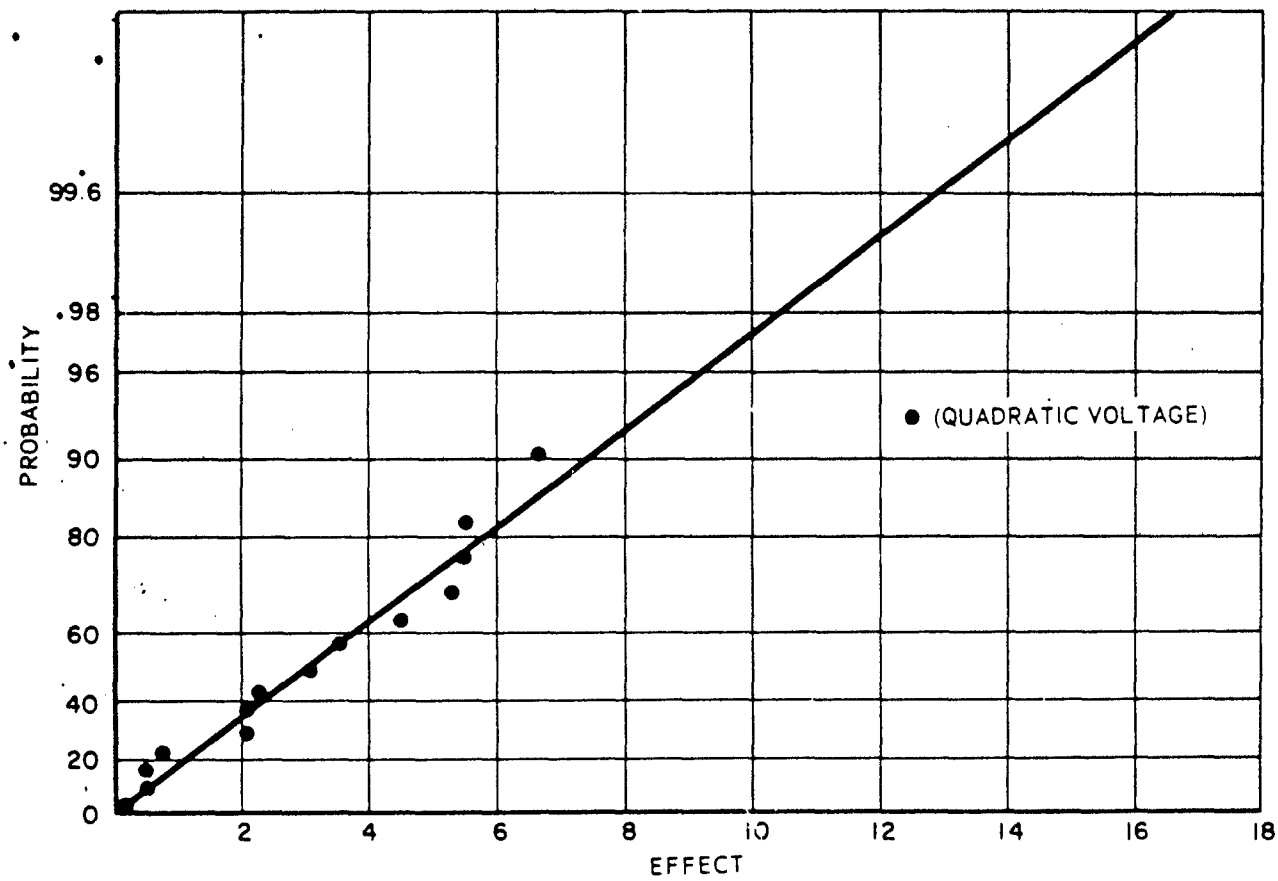

Figure 12. Half-normal Plots of Experimental Effects of Design 4

simulated r-f experiments indicates that a large error is involved in the extraction and the assay process. This large deviation of the activity of extracted enzyme from two samples obtained from an aliquot made analysis of the irradiation experiments extremely difficult.

4. DISCUSSION

The results of the study are ambiguous in a sense, since they do not point clearly to optimum conditions for inactivating proteolytic enzymes in beef. On the other hand, it is perfectly clear that under certain conditions, there is unquestionable inactivation of the enzymes.

The object of seeking conditions for inactivation of proteolytic enzymes is to provide a means of preventing proteolysis in beef that has been sterilized by ionizing radiation for preservation purposes. It is therefore obviously necessary that such enzyme inactivation be accomplished without cooking the meat. This constraint opens the entire question of thermal vs. non-thermal effects of radio-frequency energy on biological macromolecules. Much work has been done on the study of non-thermal effects of r-f on biological materials. Based on this work, it has been contended that r-f at specific frequencies is absorbed by specific molecular species much in the way that ultraviolet, visible, and infrared electromagnetic energy are absorbed by specific atoms, molecules, and chemical groups or bonds. However, a simple theoretical

consideration of the magnitudes of energy that are involved leads immediately to the hypothesis that r-f is not specifically absorbed while the higher frequency regions of the spectrum (e.g., ultraviolet to infrared) are absorbed.

The experimental results obtained in this program, and the reported frequency specific inactivation of cholinesterase, alpha-amylase, and chymotrypsin seem to be at variance with the theoretical considerations cited above. In order to consider this variance from theoretically predicted behavior, it is necessary to disregard any arbitrary distinction between thermal and non-thermal effects. Thus, if a single molecule couples with the alternating electro-magnetic field and begins to vibrate at a frequency influenced by the imposed field, one cannot define the temperature rise since temperature is defined statistically. On the other hand, if 10^{23} molecules couple with the alternating field, the temperature rise can be measured.

The questions of frequency specificity and the thermal or non-thermal nature of the effects of r-f are not academic problems but are directly related to the practical application of enzyme inactivation in radiation-sterilized beef. Whatever the mechanism of inactivation is, there is ample evidence that the phenomenon is real. The possibility of a frequency specific inactivation is especially important in this program. Since beef consists of structural proteins as well as proteins with enzyme activity, the inactivation of the proteolytic enzymes

under conditions which will also seriously denature the structural proteins will lead to cooked beef (such general conditions may also cook other components such as fats, water, etc.) It is therefore worth returning to an examination of reports of specific enzyme inactivations under specific conditions.

It has been demonstrated that field configuration of an r-f irradiation system is a most important factor in any apparent specificity. Thus, a change in cell geometry, distance between electrodes, dielectric constant, temperature, etc. may result in a shift of frequency at which inactivation is observed, or in a loss of inactivation. It is thus reasonable to continue the study from the point of view of defining the conditions resulting in the inactivation of proteolytic enzymes in the presence of other biological materials.

The direction that such a study might take may be based on considerations of relaxation time, and polarity. Thus, it is well established that water absorbs maximally in the microwave region at 2450 mc. It is reasonable to expect that this type of absorption may occur at other frequencies with materials of different molecular weight and of different dipole moment. A study of this kind might be most fruitful if it were conducted on isolated enzyme systems in the presence of selected materials such as other proteins, fats, carbohydrates, and water. The results so obtained might then lend themselves to extrapolation on to complex systems such as beef.

5. SUMMARY AND CONCLUSIONS

A study was carried out to measure the effect of r-f irradiation on the activity of proteolytic enzymes in beef. The enzymes were studied both as isolated systems and in ground beef. The assay procedure was based on a measurement of the hydrolysis of urea denatured hemoglobin. The results of the study indicated that under certain conditions enzyme inactivation of 45% was obtained. These conditions are as follows: 13.5 mc, 16.5°C, 50 volts, 0.1 duty cycle, 1/2 hour radiation time.

Based on the results of the study a recommendation is made that the study be continued from the point of view of inactivating proteolytic enzymes in the presence of other biological materials under controlled conditions.

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13 ABSTRACT

The report covers the work conducted during the period 13 April 1964 to 13 April 1965. 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.

Preliminary experiments using purified proteolytic enzymes isolated from beef liver tissue were conducted to determine the effects of various r-f parameters. From 4 design studies, it could not be conclusively determined as to what the levels of the main factors and the interaction factors necessary for maximum inactivation are. It has been shown however that a set condition about 40% inactivation of the proteolytic enzymes can be effected.

R-f radiation experiments on ground beef tissue gave inconclusive results. Chief problem area lies in the fact that the extraction and purification process does not yield consistent results.

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Beef	9,4		9		5	
Liver	9		9		5	
Muscle tissues	9		9		5	
Radiofrequency	10					
Radiation	10					
Energy	10					
Storage stability	4					
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14. KEY WORDS: Key words are technically meaningful terms or short phrases that characterize a report and may be used as index entries for cataloging the report. Key words must be selected so that no security classification is required. Identifiers, such as equipment model designation, trade name, military project code name, geographic location, may be used as key words but will be followed by an indication of technical context. The assignment of links, rules, and weights is optional.

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