First Progress Report

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

Reporting Period: 13 April 1964 - 13 August 1964

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

Project No. 1KO-25601-A-033

Prepared for

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ABSTRACT

This report covers the work conducted during the period 13 April 1964 to 13 August 1964. The natural proteolytic enzyme of beef muscle has been extracted and purified, using procedures analogous to those described in the literature for isolation of proteolytic activity from rabbit muscle.

The activity of the enzyme has been differentiated from that of pepsin and trypsin, and some of its characteristics have been established.

Appropriate assay procedures have been developed based on the hydrolyses of urea denatured hemoglobin as measured by increase in optical absorbance at 280 mμ.

Active purified preparations of the proteolytic enzyme have been subjected to radio-frequency irradiation over a limited range in beginning to determine the correct frequency at which the enzyme is most susceptible to attack <u>in vitro</u>. Differences between the activity of irradiated and nonirradiated aliquots of the enzyme are determined by appropriate statistical procedures.

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1. INTRODUCTION

This is the first progress 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 irradiation. The successful inactivation of proteolytic activity, in conjunction with the sterilizing effects of high-energy ionizing radiation, will furnish a means for the nonrefrigerated preservation of animal tissues over extended periods of time.

The literature has been reviewed, and a method for the extraction and purification of the natural proteolytic activity of beef muscle was selected. Alternative appropriate assay procedures were tried, and it was finally decided that the most reliable method was based on the proteolytic hydrolyses of a urea denatured hemoglobin solution, buffered at pH 4.0 with an acetate buffer. Enzymatic activity is indicated by the increase in optical absorbance of aliquots of the reaction mixture. Optical measurements are made at 280 mμ 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.

The isolation, purification, and assay procedures are described here in some detail. The protocol for the preparation of the enzymatic activity has now been reduced to a reliable routine, but, in the initial stages of this work, some truly frustrating 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 description of the apparatus used in the radio-frequency irradiation of the enzyme is presented, and the results of the first few attempts at irradiation of active enzymatic material are given.

Because the activity of the enzyme is determined by the slope of the curve relating absorbance with time, it is necessary to determine this slope with considerable precision. Individual assays are therefore being run in triplicate, and each slope is determined on the basis of 6-7 aliquots withdrawn from the reaction mixture over a period of approximately one hour.

It has been found that the enzyme can be successfully lyophilized with only minor loss of activity, although it deteriorates quickly in a few days when left in a soluble state.

2. PREPARATION AND HANDLING OF THE ENZYME

2.1 Isolation and Purification of Proteolytic Activity

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 therefore 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 purification of proteolytic enzyme activity from striated rabbit muscle.[6] When assayed using a urea denatured hemoglobin substrate, buffered with 0.1 M 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. It is, therefore, 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 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.

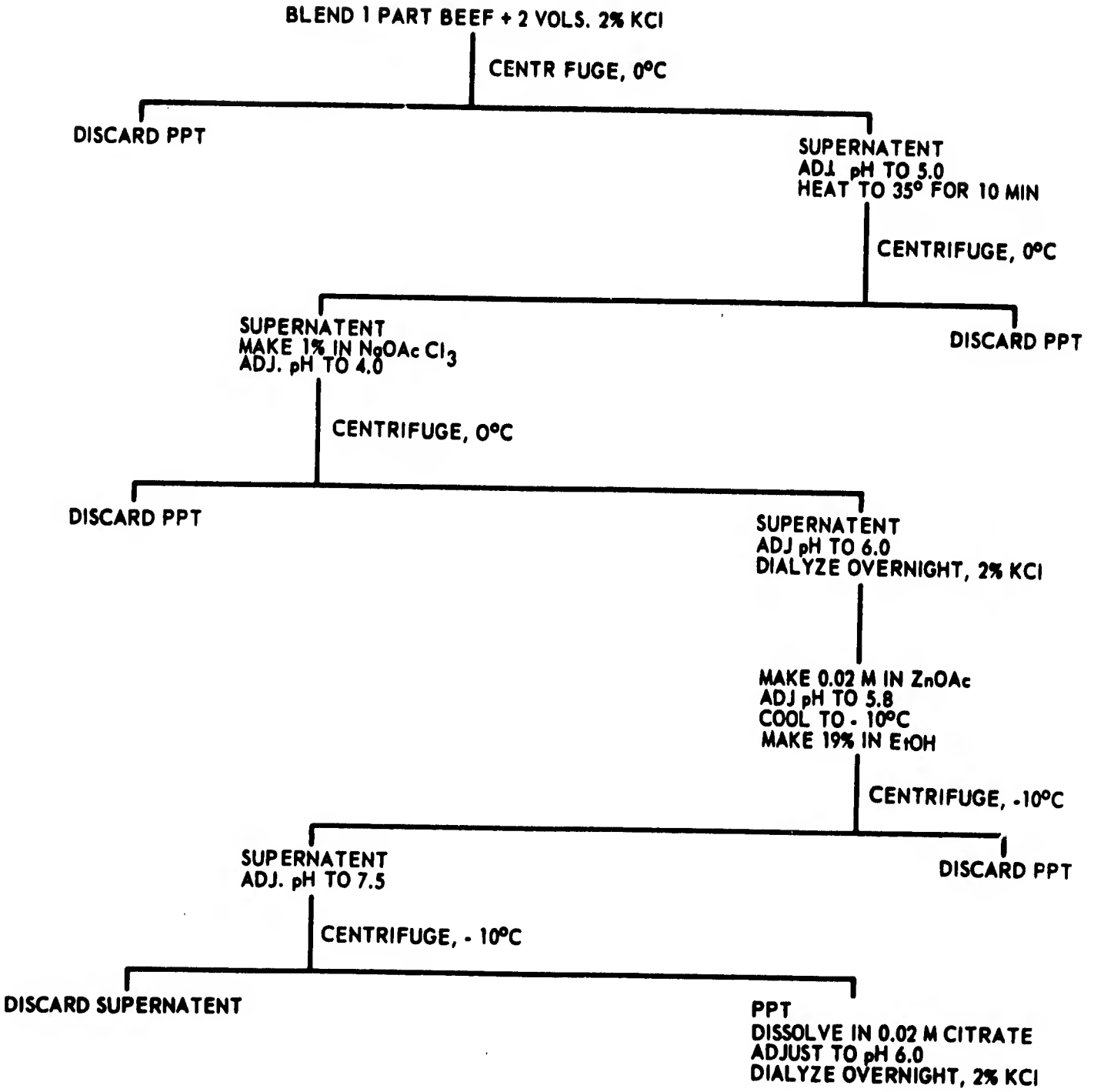

Figure 1. Purification Enzymatic Activity

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 Osteriser blendor jar of 1/2-pint capacity was used in subsequent experiments with better results.

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 reasonably 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 1 N NaOH. The liquid is placed in a dialysis tube approximately 1 inch in diameter and dialyzed in a refrigerator at 4°C overnight against 4-5 changes of 2% KCl.

This first dialyses step presumably removes the sodium trichloro-acetate.

The morning of the third day the solution is removed from the dialyses tubing and sufficient 1.0 M zinc acetate is added to give a final solution which is 0.02 M 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.

Thus, 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 good, it will normally prove soluble in approximately 2 volumes of 0.02 M 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 prove 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.

2.2 Assay Procedures

The assay procedure recommended 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.

The method depends on the hydrolysis of a urea denatured hemoglobin solution. The hydrolysis is performed at 35°C in the presence of 0.033 M acetate buffer at a pH of 4.0. A pH of 4.0 is near the lower limit of the range for acetate buffers, 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 dissolution of the urea is a highly 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 0.1 M acetate. The pH was adjusted electrometrically 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 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 0.5 M ferrous ammonium sulfate solution; the amount of water added was, therefore, 8.7 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 0.5 M solution), giving a final ferrous ion concentration of 0.01 M.

The original paper of Snoke and Neurath[6] contains an error with regard to the optimal concentration of ferrous ion. Their table III shows activation by 0.01 M ferrous ammonium sulfate, and the text contains the statement

"However, maximum activation is obtained at lower concentrations of ferrous ions (0.02M)." Clearly, 0.02M is not lower than 0.01 M, but one is left in doubt as to whether the error involves a replacement of the word lower by the word higher, or whether the stated concentration should have been something like 0.002 M.

One attempt was made to investigate the real optimum concentration of ferrous ion, but this is not as easy as it might first appear. The problem is that ferrous ammonium sulfate is strongly acidic, with a pH only slightly above 2. Any attempts to raise the pH of the concentrated 0.5 M ferrous ammonium sulfate solution to 4.0 result only in the precipitation of hydroxides. The addition of even 0.3 ml of the concentrated ferrous solution to the hemoglobine substrate results in a definite downward shift of the overall pH because of the poor buffering capacity of the acetate solution. Thus, various concentrations of ferrous ammonium sulfate will produce final solutions in which the pH may vary as much as 0.5 unit, and there is always the question of whether differences in activity are due to variations in the amount of ferrous activator or variations in the pH at which the reaction was conducted. Ferrous citrate is certainly a poor alternate choice of activator salts, and there are very few other soluble ferrous salts available.

One cannot help but wonder if Snoke and Neurath were aware of this problem when they specified the optimum concentration of ferrous ion for use as an activator. Rather than spend additional time on this point, we have decided to use the specified 0.3 ml of 0.5 M ferrous ammonium sulfate solution routinely, thereby obtaining a uniform, if not optimal, result.

To summarize, the assay solution consists of a total volume of 15 ml, which contains 1-5 ml enzyme, and is 0.033 M in acetate buffer pH 4.0, 0.01 M in ferrous ammonium sulfate, and has 6.67 mg denatured hemoglobin per ml with 120 mg urea per ml.

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 centrifugal clarification 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 may be read conveniently on any instrument in the visual region at 580, 690, or 750 mμ.

Another alternative is simply to determine the optical density of the clarified TCA solutions at 280 mμ 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

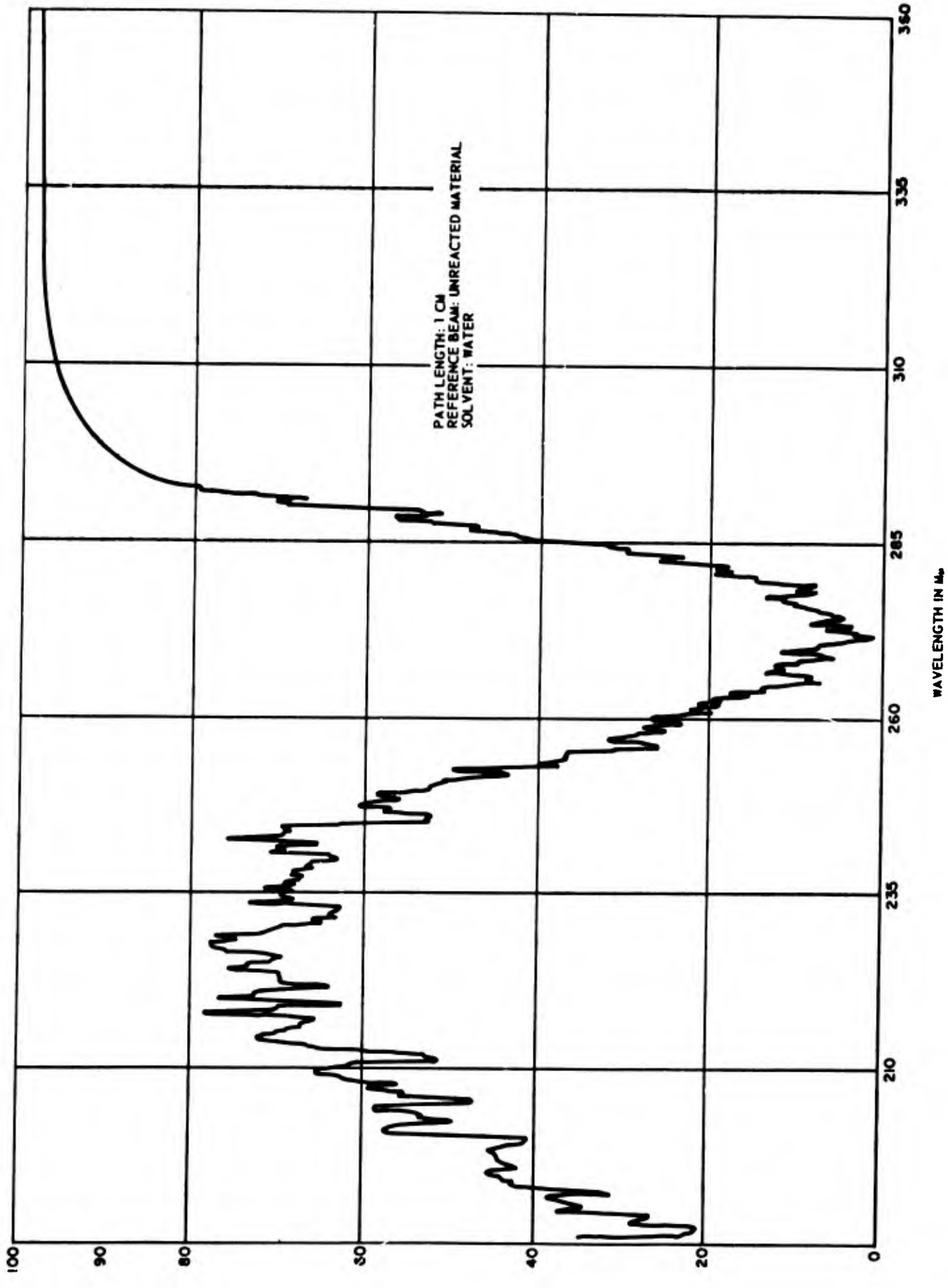

Figure 2. UV Absorption Curve of Reaction Product

representation of the UV absorption response due to hydrolyses. It is seen from the curve that 280 mμ absortion 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 change 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 I compares the 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 1cc 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.

Table I

COMPARISON OF SPECIFIC ACTIVITIES[1]

	Rabbit Muscle (Snoke and Neurath)	Beef Muscle
After NaOAcCl3 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.

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 is being determined using 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.

2.3 Optimum pH for Enzymatic Hydrolyses

The variation of the rate of enzymatic hydrolyses 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 280 mμ 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.

2.4 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 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.

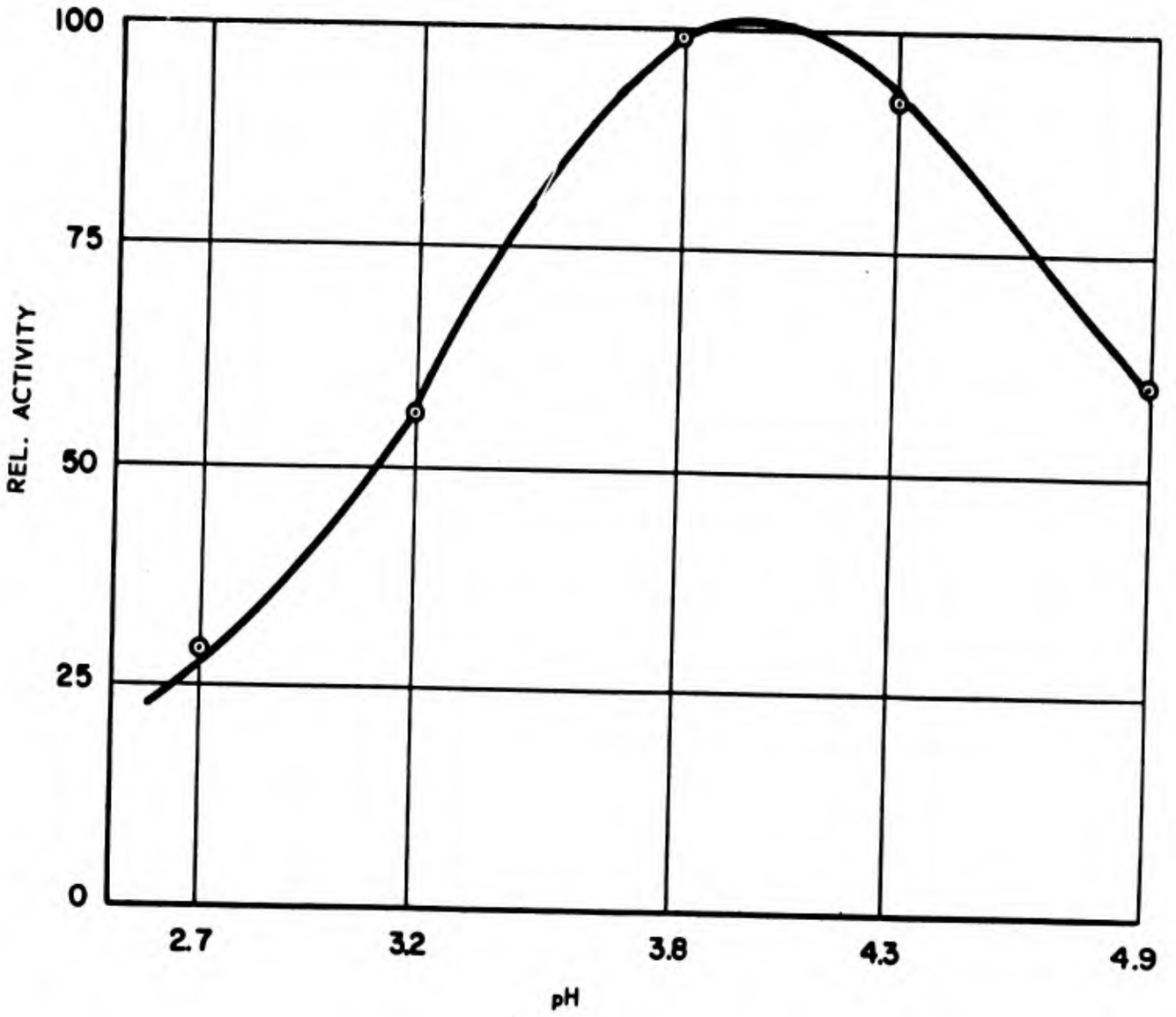

Figure 3. Relative Activity of Enzyme at Various pH Levels

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 II. The slopes of the lines of best fit were then calculated according to a conventional least squares method, and the rate of hydrolyses 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 destroyed during the 24-hour, radio-frequency irradiation by temperature effects alone.

2.5 Lyophilization of the 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.

Table II

EFFECT OF TEMPERATURE ON ENZYMATIC ACTIVITY

	After 24 hours at			
	0°C	23°C	40°C	60°C
Assay: OD at min				
0	0.575	0.552	0.565	0.510
10	0.665	0.632	0.615	0.562
20	0.665	0.648	0.616	0.558
30	0.735	0.735	0.646	0.583
40	0.775	0.775	0.678	0.605
50	0.810	0.804	0.709	0.605
60	0.850	0.822	0.734	0.617
Slope of least-squares line	0.004375	0.004575	0.002704	0.001621
ΔOD/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

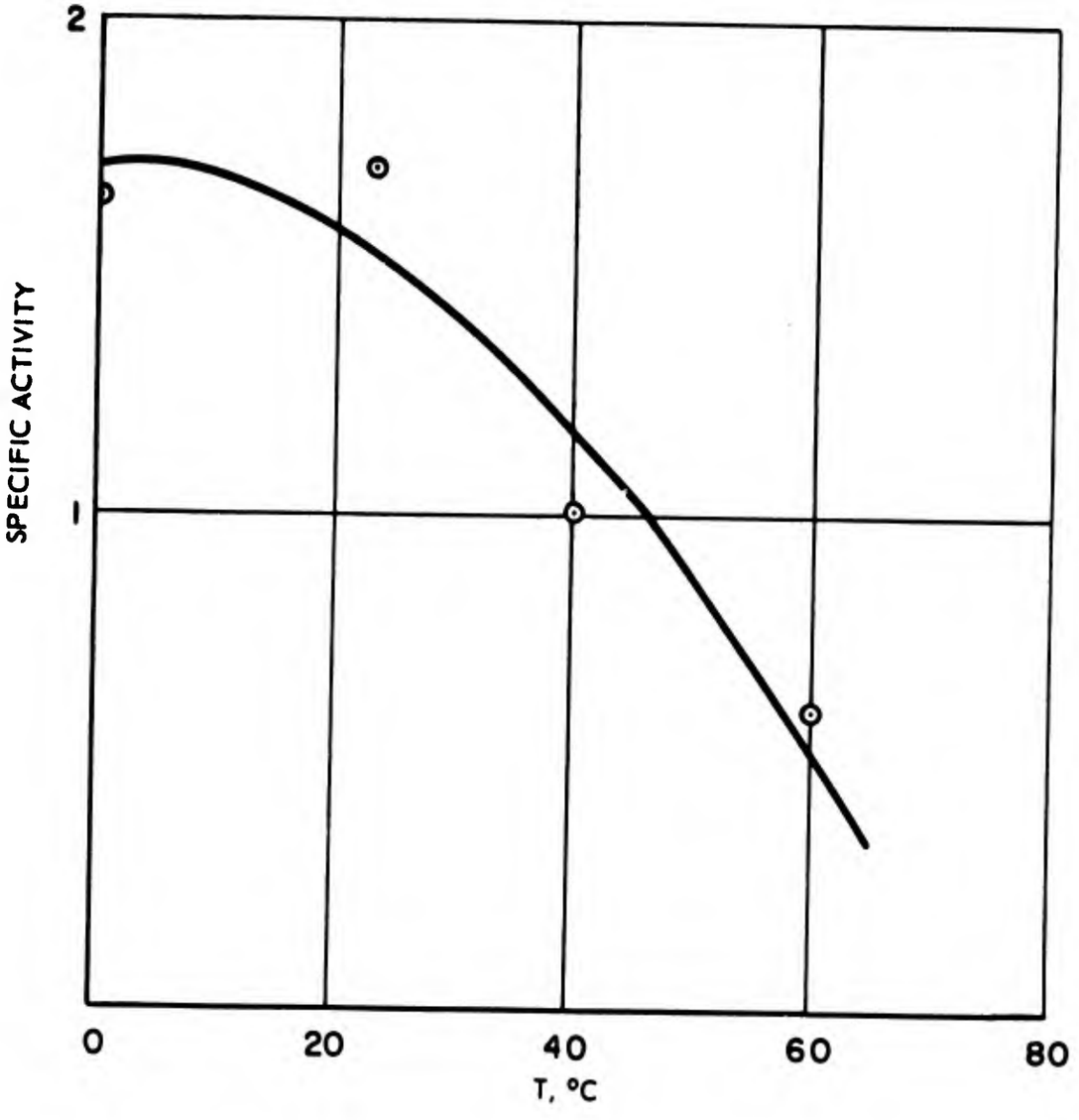

Figure 4. Effect of Holding Temperature on Specific Activity

Duplicate 5 ml aliquots of an active preparation where 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 commerical 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 III.

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

Table III

LYOPHILIZATION OF ENZYME

		Control Aliquot	Lyophilised Aliquot
OD,	0 min	0.329	0.307
	10 min	0.363	0.342
	20 min	0.409	0.384
	30 min	0.434	0.405
	40 min	0.456	0.428
	50 min	0.465	0.442
	60 min	0.503	0.460
Equation, line of best fit, $y = a + bx$			
slope, b		0.00276	0.00251
intercept, a		0.0109	0.0131

3. R-F EXPERIMENTS

3.1 Radio-Frequency Irradiation Apparatus

The apparatus presently being used for the radio-frequency irradiation experiments consists of commercially available compounds. Special modifications are anticipated in the existing system and will be made as the need arises. Thus far, the commercial equipment has served its purpose well. Plans exist to improve the coupling network that feeds the sample cell and to incorporate steady electric and magnetic fields into the system in addition to the r-f field.

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

The sample cells are held in a constant temperature water bath. A control sample is maintained in an identical container in the same bath. It is handled in an identical manner to the sample itself with the exception that the r-f power leads are not connected to it.

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,

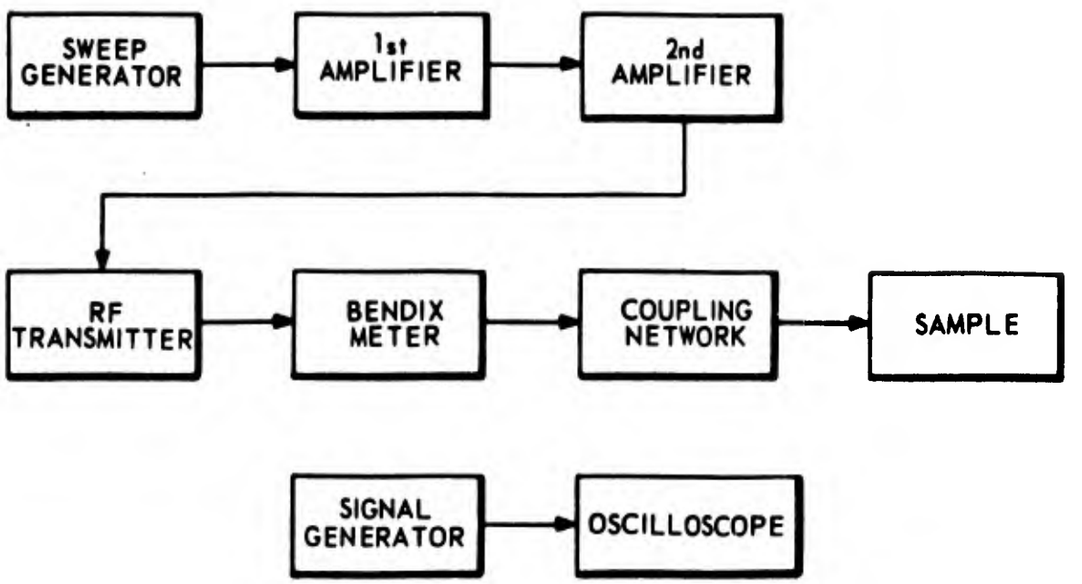

Figure 5. Block Diagram of Electronic System

as read on the Bendix meter. The net power, frequency, and temperature are periodically checked during the irradiation period. A typical set of parameters are 7 watts net, 12.6-12.8 mc, at a temperature of 7.8°C.

3.2 Effects of R-F Irradiation on Enzyme Activity

During the period covered by this report, three successful experiments have been conducted in which samples of the active proteolytic enzyme extracted from beef muscle have been subjected to radio-frequency irradiation. The r-f ranges used have been 13.2-13.0 mc., 13.0-12.8 mc, and 12.8-12.6 mc. A number of additional ranges would have been covered had we not experienced a perplexing problem which was eventually traced to faulty electrometric pH meter. The device was giving readings which were incorrect by nearly one whole pH unit, even though no difficulty was experienced in standardizing it with conventional buffer solutions. The net result was that the final purified enzyme solutions looked good, but they were devoid of activity. Pinpointing the cause in such an involved isolation procedure is a frustrating research problem in itself.

The general description of the equipment and procedures used for the irradiation work, and the assay procedures have already been covered in earlier sections of this report. The results obtained for these three successful experiments are presented in tables IV, V, and VI; the curves are shown in corresponding figures 6, 7, and 8.

The data were subjected to a detailed analyses of variance to determine any significant differences between the irradiated and nonirradiated samples, or between either of these and the original enzyme preparation from which aliquots were withdrawn for the irradiation experiment itself. No significant

Table IV

R-F IRRADIATION OF ENZYME, 13.0-13.2 mc

		R-F Control	R-F Irradiation
OD at (280 mμ)	0 min	0.410	0.410
	10 min	0.425	0.420
	20 min	0.510	0.500
	30 min	0.520	0.520
	40 min	0.560	0.560
	50 min	0.560	0.590
line of best fit:	slope, b x 10^3	3.34	3.82
	intercept, a	0.398	0.385

Table V

R-F IRRADIATION OF ENZYME, 12.8-13.0 mc

		R-F Control	R-F Irradiation	Temperature Control
OD (280 mμ)	0 min	0.423	0.398	0.385
	3 min	0.430	0.411	0.412
	6 min	0.425	0.422	0.430
	10 min	0.448	0.424	0.436
	20 min	0.473	0.450	0.464
	35 min	0.499	0.473	0.484
	55 min	0.536	0.524	0.523
line of best fit: slope, b x 10^3		2.09	2.19	2.23

Table VI

R-F IRRADIATION OF ENZYME, 12.6-12.8 mc

		R-F Control	R-F Irradiation	Temperature Control
OD (280 mμ) at	0 min	0.322	0.325	0.329
	10 min	0.363	0.363	0.363
	20 min	0.393	0.397	0.409
	30 min	0.422	0.435	0.434
	40 min	0.442	0.461	0.456
	50 min	0.462	0.473	0.465
	60 min	0.479	0.502	0.503
line of best fit: slope, b x 10^3		2.56	2.91	2.76

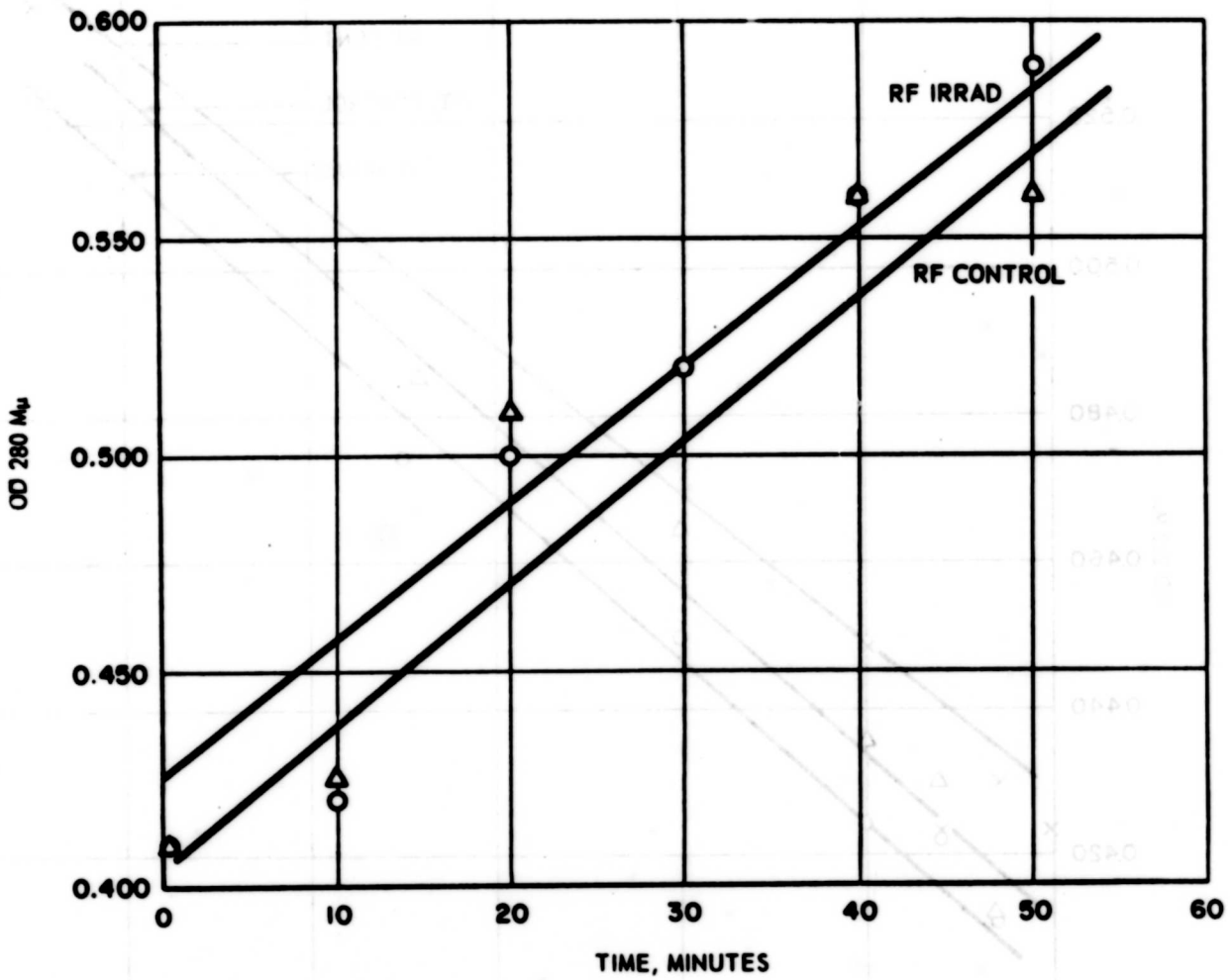

Figure 6. R-F Irradiation of Enzyme, 13.0 - 13.2 mc

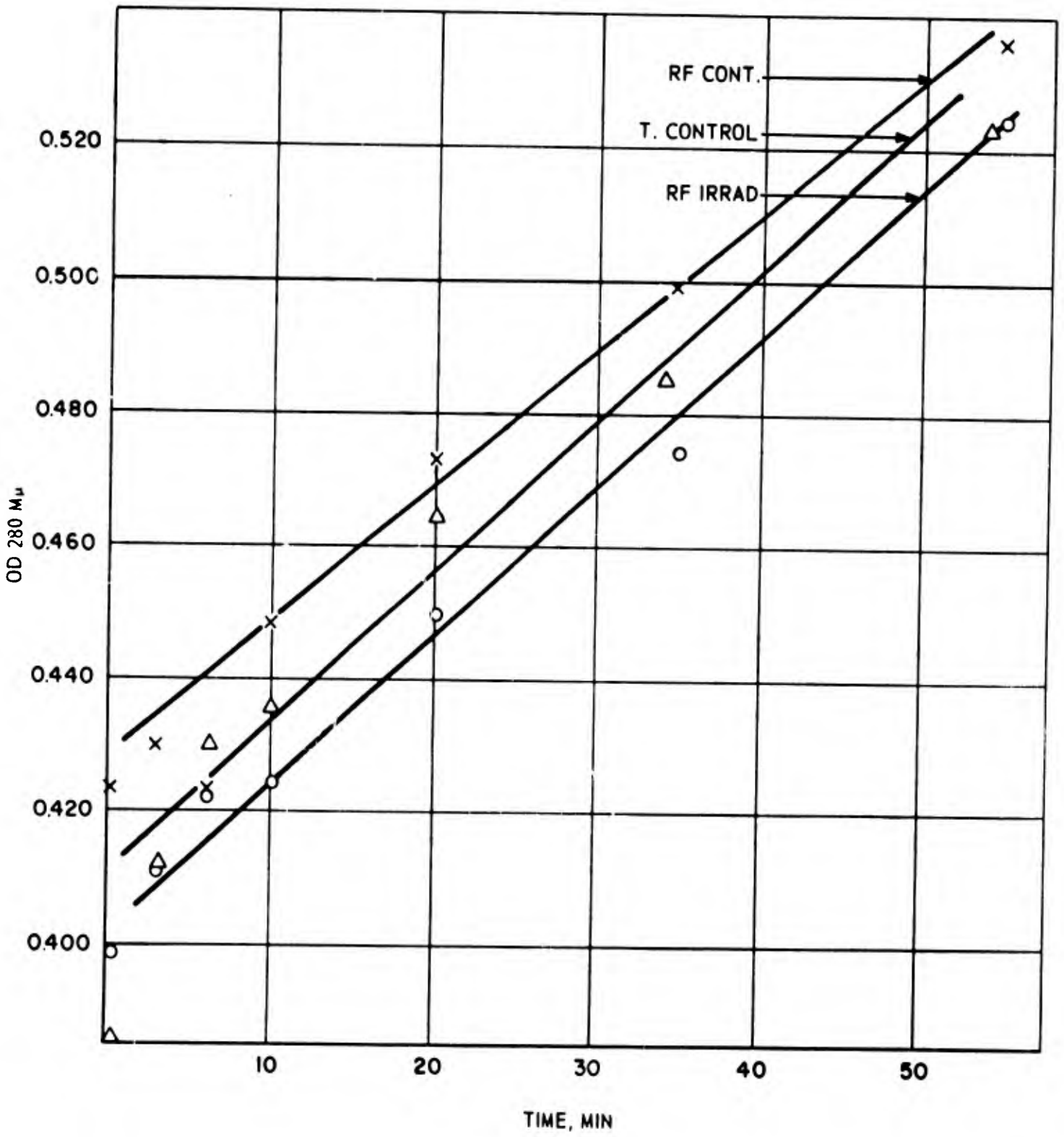

Figure 7. R-F Irradiation of Enzyme, 12.8 - 13.0 mc

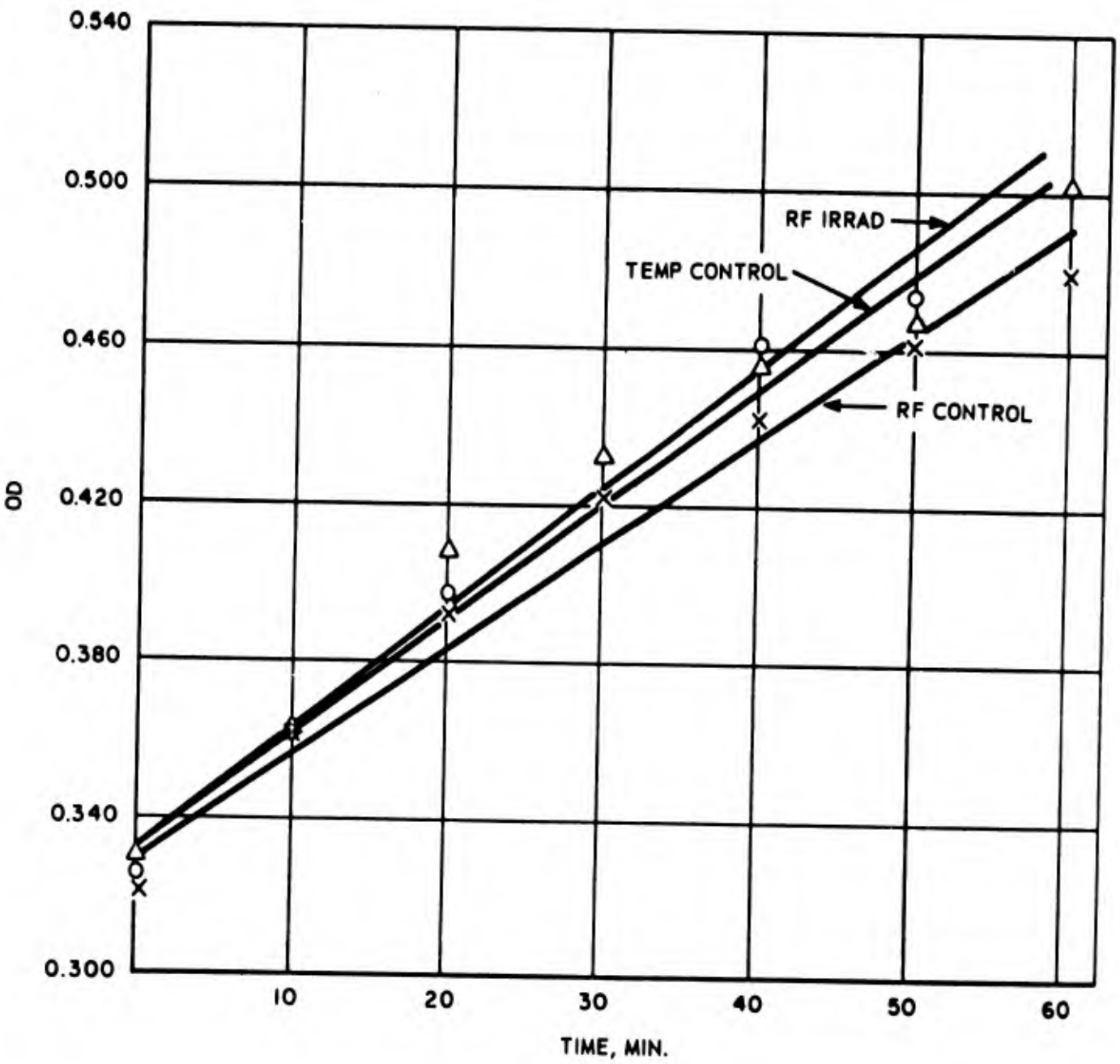

Figure 8. R-F Irradiation of Enzyme, 12.6 - 12.8 mc

effects of r-f irradiation in the range covered have been discovered as yet.

It is to be expected, of course, that the effective resonant frequency or frequencies will be quite sharp. The use of broader ranges during the search phase is not justified because of the danger of subjecting the sample to so little power at the specific resonant frequency that any effect would be missed. It is necessary to keep the total power input to the irradiated sample at a sufficiently low level so that there is no possibility of confusion due to thermal effects.

4. PLANS FOR FUTURE WORK

The major effort at this time has been restricted to developing the techniques for isolation and purification of the proteolytic enzyme from beef muscle, and the study of parameters which are important for the assay of enzymatic activity.

The task of locating the resonant frequency which has greatest effect on activity of the proteolytic enzyme is solely a matter of search. The effectiveness of radio-frequency irradiation is known to depend on such variables as the temperature, viscosity of the solution, amount of power absorbed, voltage of radiant across the cell, and possibly cell geometry. At this time, there is no theoretical basis for predicting a best set of conditions at which resonance will occur.

The range near 13 mc, which has been used in the first few experiments described here, is the range in which Melpar found maximum effects on cholinesterase, and this seemed like a logical starting place for the present effort. There is no particular reason to expect, however, that this should be the resonant frequency for the present enzyme.

During the coming months, the emphasis will be placed on studying the effect of different irradiation frequencies, and attempting to locate areas of maximum effect. In addition, some effort will be expended on improved methods of assay for the enzymatic activity. Because relatively little work has been done on the natural proteolytic enzymes of beef muscle, there are no known specific synthetic substrates such as are available for other proteolytic enzymes. A simpler and more rapid assay method would do much to speed up the present search.

Other parameters which are known to influence resonance frequency also need to be investigated.

It should be noted that Melpar has, at this time, an active program of research on the effects of radio-frequency irradiation on other biological products and specifically other proteolytic enzymes. The information and techniques which have been found helpful with these other enzymes are being followed carefully for useful clues and information applicable to this contract.

5. REFERENCES

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6. FISCAL AND WORK STATUS

In compliance with the terms of the U.S. Army Natick Laboratories contract with Melpar, the expenditures to date on the contract and the status of the work are as follows:

Fiscal Status

For the period 13 April 1964 to 13 July 1964, -

Total contract dollars (less fee)	$36,880
Actual expenditures and commitments	
Direct labor	4,005
Overhead	5,006
Materials	355
General and administrative	1,031
Other	6
Total expenditures	$10,403
Unexpended	$26,477
Percentage of total funds expended through this period	28%

Work Status

The work performed thus far is estimated to represented approximately 26% of the total effort. The total program is expected to be completed on schedule. Present funding is adequate.