# THE EFFECT OF EXERCISE (TRAINING) ON THE BASAL CONCENTRATION OF ATP IN MUSCLE TISSUE

by

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The problem involved the determination of the base concentration of ATP in the muscle tissue of white rats. These animals had been forced to exercise (training) by swimming. The overload principle was enforced because the swimming was increased daily.

The method used to analyze for ATP involved bioluminescence and spectrophotometry. The luciferin-luciferase system served as the bioluminescence source. The Fisher's "t" test for significance was computed from the data collected. The peak height, obtained from the reaction of ATP and the luciferase system which was recorded, indicated the amount of ATP present in the tissue.

The "t" obtained was not significant at the 5 per cent level of confidence. Based on the findings of this study it was concluded that: (1) there was not a significant change in the base concentration of ATP in the experimental rats as compared to the control rats; (2) there was a definite trend of an increased basal concentration of ATP in the exercised animals.

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# CHAPTER I

## INTRODUCTION

The role of adenosinetriphosphate (ATP) has been investigated by many people regarding the function it plays in muscle contraction, the place it takes in enzymology, and the conversion of chemical energy into mechanical energy. In past years work in the field of enzymology has progressed rapidly with a great deal of this work being done with ATP. Most of this work has involved the determination of the effect of ATP on the hexose and pentose sugars. The role of ATP as a nucleotide has been investigated also. Attempts have been made to determine the qualitative and quantitative concentration of ATP in muscle. Unfortunately most of these studies are of little use to the person in physical education owing to the fact that they were not concerned with the effects of exercise on the concentration of ATP as one of the chemical constituents of muscle.

Donaldson (36) has noted that research from nutritional studies has shown that the internal chemistry of the rat and of man was quite similar; therefore, he said it seemed probable that exercise caused changes in the organs of man similar to the changes shown in rats.

Many studies (14, 36, 37, 38, 56, 57, 78, 79, 80, 102) involving exercise programs have been carried out to see if the training would cause an increase, decrease, or no change in muscle enzymes. None of these studies have dealt with the base ATP concentration.

The base concentration was defined as that concentration of ATP which was attained after twenty-four hours of rest.

As a result of preliminary investigation the need for research in the area of exercise and its effects on the chemical properties of muscle emerged. Consequently the following research was undertaken to determine the effects of exercise (training) on the ATP concentration in the muscle of experimental animals.

The problem was to determine whether or not exercised animals as compared to control animals had a significant increase in the base concentration of ATP. Secondly, a method of measuring the ATP concentration was devised.

The method used to determine the ATP concentration involved measuring the amount of light emitted from the reaction of ATP with the firefly complex, luciferin-luciferase. This light was stoichometrically related to the amount of ATP present in the muscle sample. The Beckman D.U. spectrophotometer measured the amount of light given off. A graphical recorder attached to the spectrophotometer traced the entire reaction.

The animals designated as experimental were put into a forced exercise program. The controls remained inactive. The forced exercise program consisted of swimming the animals. The swimming time was increased daily consequently putting them on a graduated program. This graduated program insured that the overload principle was effective.

The thesis was organized in the following manner: (1) a statement of the problem, (2) a review of the literature pertinent to the study; this contained sections on the history of muscle contraction, methods of determining ATP, and effects of exercise on rats and on the chemicals

found in muscle tissue, (3) the experimental procedures and critical appraisal, (4) presentation and analysis of data, and finally, (4) the summary and conclusions derived from the study.

# CHAPTER II

## STATEMENT OF PROBLEM

The problem involved the determination of the base concentration of ATP in the gastrocnemius muscle of a group of exercised animals which were forced to exercise by swimming. The swimming program was graduated thereby enforcing the overload principle. There were two groups of animals--one control and one experimental. The control group remained inactive while the experimental ones swam daily, six days a week.

Importance of the Study. ATP has been stressed as one of the most important chemicals in the human body. If it is possible for the human body to store ATP in a reserve capacity by increasing the basal concentration then strength and endurance might be increased. In this study an attempt was made to determine whether or not the basal concentration of ATP was increased as a result of an exercise (training) program.

# CHAPTER III

## REVIEW OF LITERATURE

The review of literature regarding effects of exercise on the base concentration of adenosinetriphosphate (ATP) in the gastrocnemius muscle of white rats is organized according to the following areas: (1) historical tracing of muscle contraction theories, (2) experimentation concerned with exercise effects and other effects on rats, (3) methods of analysis for ATP, and (4) related studies concerned with exercise and ATP.

### I. HISTORICAL REVIEW OF THEORIES INVOLVING CHEMICAL CHANGES DURING MUSCULAR CONTRACTION

Theories Prior to 1930. At the close of the nineteenth century Hill's statement seemed to describe the current concept of muscular contraction.

> Muscular energy sprang from a more or less explosive splitting of some molecular complex, which had been built up into some highly unstable form, by the inclusion within it of oxygen taken in by the cell during rest. The breakdown of this hypothetical molecule was supposed to yield both lactic acid and carbon dioxide, these being the two obvious and recognizable products of activity. (4:55)

As far back as 1867 Hermann (47) postulated his inogen theory of contraction which was soon followed by Pfluger's theory (47) of the giant molecule. Inogen was supposed to be the unstable precursor for muscle contraction. This theory developed from the discovery which indicated that contractions can continue and carbon dioxide can be given off

without a free supply of oxygen. During this anaerobic phase of contraction it was thought that acid continues to be produced with no other chemical changes occurring. Hermann and his followers held that inogen was the unstable precursor of both carbon dioxide and lactic acid. They believed that the combination of oxygen and carbon and hydrogen produced the energy for contraction. The oxygen was supplied by the precursor--inogen. After the breakdown needed for contraction the carbon and hydrogen were believed to combine again to form the unstable inogen molecule.

Shortly after the establishment of this concept, the research of Fletcher (45), Fletcher and Hopkins (46), Meyerhof (16), Embden (16), Lohman (67, 68), and others (16, 73) proved the inogen theory to be completely false.

Earlier in 1907, Fletcher and Hopkins had provided some enlightening facts on lactic acid production in muscle. Baldwin (2) has given an excellent summary of their findings.

> (1) Muscle can contract in a perfectly normal manner in complete lack of oxygen.
>
> (2) Lactic acid is produced during anaerobic contraction and piles up with continual stimulation until, in the end, the muscle becomes fatigued.
>
> (3) If the fatigued muscle is then put into oxygen it recovers its ability to contract, and the lactic acid simultaneously disappears.
>
> (4) Less lactic acid is formed in a muscle that is allowed access to oxygen than one which works anaerobically (2:331).

Fletcher and Hopkins' research provided a new direction in muscle contraction theory. In 1913 Fletcher (45) found that lactic acid was always present in large amounts from carbonate displacement which

accounted for the presence of carbon dioxide under anaerobic conditions. Thus Fletcher and Hopkins' research indicated that the inogen theory was false.

Meyerhof lent further support to the new theoretical direction when he discovered that the ultimate source for the formation of lactic acid was glycogen (4). Needham (16) reviewed the Meyerhof cycle and balance sheet as the explanation for muscle contraction.

> The lactic acid was set free on the 'Verkurzungsort'; the contraction was brought about by some consequent surface change, energy, partly of chemical origin (from the glycogen to lactic acid reaction) and partly of physical origin, being liberated and tension developed. The lactic acid then diffused away from the specialized 'Verkurzungsort' to the 'Ermudungsort' which was the whole muscle substance, and was neutralized by the alkaline carbonates, phosphates, and protein salts. This neutralization accompanied relaxation; relaxation, of course, involved the dissipation of the tension as heat, and the neutralization provided energy which restored the physical state of contraction mechanism, so that after relaxation the muscle even in nitrogen was in precisely the same state as before, except for a slight decrease in glycogen, and a slightly higher general potassium lactate concentration. By the energy of physical origin just referred to above, was meant the energy stored in some potential form in the fibrils; this energy of course was derived from the storing up of heat of neutralization of lactic acid during relaxation from the previous contraction. This heat of neutralization, occurring during the relaxation phase, could not supply energy for the contraction which had just taken place; it could, however, be stored to provide energy for the next contraction (16:12).

In 1927 a hitherto unknown compound was isolated from muscle extracts. It was shown to be a member of the phosphate family and when finally isolated was found to be a phosphocreatine. Scientists of that time called it phosphagen.

Before 1927, however, Embden had discovered the importance of phosphate in muscle metabolism. "He pointed out that various early workers had found increased excretion of free phosphate in the urine

after muscular work, and after very great fatigue and also reported increase in the muscle itself." (4:29) Embden formulated a theory based on the presence of lactacidogen as the precursor for lactic acid. He found, while experimenting, that a certain hexosediphosphate--lactacidogen--seemed to break down to form lactic acid and the free phosphate. "After the proof that glycogen disappears in equivalent amounts during lactic acid formation, the glycogen was looked upon as a reservoir for keeping up the supply of lactacidogen." (45:31) After this theoretical suggestion by Embden most workers seemed satisfied to accept the theory that the precursor for lactic acid formation was a hexosediphosphate.

Phosphagen proved to be the product that when broken down provided the energy for contraction. It was so active that it could be broken down under aerobic and anaerobic conditions. During the time of this theory some people began to suspect that since phosphagen breakdown preceded glycogen breakdown, that phosphagen was the immediate source of energy for contraction and that glycogen was utilized to resynthesize the phosphagen.

However, about 1930, Lundsgaard (71, 72, 73) provided the next turning point in the biochemistry of muscle contraction. He found that in muscles poisoned with iodoacetic acid that there was no shift in the acid medium after appearance of rigor mortis. The muscle seemed to become more alkaline and there was no formation of lactic acid. "He also suggested that in the normal muscle the energy derived from the change in glycogen into lactic acid is used to rebuild continually the phosphagen which is breaking down." (16:82) Later Lundsgaard showed that phosphagen

breakdown occurs after a single twitch and therefore could not be the energy source for muscular contraction.

Concerning the changes in H-ion concentration, Wright (27) said that phosphocreatine is broken down into creatine, which is highly basic, and a weak acid, phosphoric acid. Meyerhof found that under anaerobic conditions the muscle first becomes alkaline and then acidic as lactic acid appeared. "These observations prove conclusively that the change in H-ion concentration in muscle cannot be an essential part of the mechanism of contraction." (27:503)

A.V. Hill (4) has done an extreme amount of work concerning muscle contraction and the heat of contraction. His main concern was to look for the cause of the energy needed for contraction. This was done by studying the thermal heat produced before, during, and after contraction under either anaerobic or aerobic conditions. Hill outlined the following steps as being definitely recognizable upon contraction caused by excitation or other stimuli under anaerobic conditions:

(A) Glycogen disappears.

(B) Lactic acid appears in equivalent amounts.

(C) Preformed $CO_2$ is driven off.

(D) Heat is produced proportional to the lactic acid formed.

(E) The hydrogen ion concentration rises.

Hill refused to recognize the lactacidogen theory of Embden as the precursor of lactic acid and he agreed with Meyerhof that phosphagen played a part in the lactic acid cycle.

Steinhaus (19) gave a simple account of the Hill-Meyerhof theory of muscular contraction.

> Following stimulation of a muscle, lactic acid is formed by the splitting of some precursor, probably some form of sugar. This lactic acid in some way causes the muscle fiber to shorten thus producing contraction. The rather immediate neutralization of this acid by muscle buffers permits the fiber to lengthen or relax, and its subsequent oxidation and partial resynthesis to the original precursor form provides the means of recovery to the original state (19:119).

Embden later refuted the Hill-Meyerhof theory on the basis that the muscle was acidic at the time of contraction, and that lactic acid was produced during the contraction and not after it. He discovered adenylic acid and found that this acid was present in muscle at the time of contraction thus supporting his first point that the muscle was acidic at the time of contraction. In support of his second point that lactic acid was produced during contraction and not after it, he found that it continued to be produced even after contraction had ceased.

> Embden's experiments lead him to the view that an alkaline reaction and probably ammonia, is the immediate cause of contraction of the muscle fiber and the subsequent acid reaction chiefly due to the lactic acid is the cause of relaxation (19:120).

Steinhaus summarized the views held and the interrelations believed possible by the workers in muscle contraction at the end of the nineteen twenties.

> When the nerve impulse reaches the muscle, ammonia and perhaps phosphoric acid is split off from the amino group of an 'amino-purinkomplex' (the adenylic acid) changing it to an 'oxypurinkomplex' (Embden). Simultaneously or immediately following this phosphocreatine is split to phosporic acid and creatine (Lundsgaard). Both of these changes induce an alkaline state (Embden) and bring on the contraction which in itself is perhaps the nature of a reversible coagulation of the protein fibrillar structure (the observations of Deuticke on changed solubility of the muscle proteins in fatigue, etc., fit here). Immediately the phosphoric acid combines with a hexose (sugar) to form a more acid hexosephosphate and if the muscle is not poisoned by

> iodoacetic acid, lactic acid is formed from the splitting of this hexosephosphate, also called lactacidogen in the earlier literature. This appearance of acid neutralizes the alkaline condition and brings on relaxation (Embden) (19:120).

This then, in brief, summarizes where the theories of muscle contraction stood prior to the discovery and isolation of adenosinetriphosphate (ATP), adenosinediphosphate (ADP), and adenosinephosphate.

Theories after 1930. The chemical reaction brilliantly discovered by Lohmann and named for him--the Lohmann reaction--led the way for the future developments in the biochemistry of muscle contraction. Lohmann showed that the chemicals used in the process of contraction involved a reversible reaction and that there was very little, if any, energy set free and most certainly no inorganic phosphate (67, 68, 69, 70). The most important point of this reaction is that unless some of the ATP has been broken down into ADP there cannot be any decomposition of phosphagen, that is, "the breakdown of ATP must take place even earlier than that of phosphagen." (2:338) Lohmann (89) held that the hydrolysis of ATP was the immediate source of energy and this compound (ATP) was resynthesized by the transference of a phosphate group from phosphocreatine.

When Englehardt and Ljubimova (39) found that adenosinetriphosphate and myosin were identical compounds it was noted that since myosin was the enzyme which hydrolized ATP and also the contractile element of muscle that ATP must ultimately be bound up with the contractile process. However, little ATP was available in the muscle; therefore, the action of phosphocreatine is of utmost importance according to the Lohmann reaction because it is provided for the resynthesis of ATP from ADP.

At this point mention must be made of one theory that seems to contradict the above theory. Jacob Sacks (89) believed that the fundamental reaction occurring during muscular contraction is an oxidative process not an anaerobic one. He showed that in a steady state the resynthesis cycle explained by Lohmann, of ATP from ADP to PC, is not accomplished in an intact muscle. This is due to the fact that PC is resynthesized ever so slowly and ATP even more slowly than PC and the two processes are independent. The only function of PC is the one originally established by Fiske and Subbarow (92) that it is to buffer the lactic acid formed during anaerobic conditions for contraction. Sacks believed that "during the initial stages of extreme exertion,..., certain secondary anaerobic processes are employed to furnish part of the energy for contraction." (91:159) However, as soon as the initial exertion is over and the circulatory system has adjusted the oxidative process supplies the necessary energy, with any extra energy going to resynthesize lactic acid and hexosephosphate back to glycogen.

Lohmann's reaction has found quite a bit of support. One investigator (1) cited from a study by Ennor and Rosenberg (43) said,

> The continual transfer of phosphoryl groups from ATP to creatine phosphate to ADP in skeletal muscle is evident from the findings of identical turnover of radioactive phosphate in the reactive phosphoryl groups of creatine phosphate and ATP. A sudden loss of ATP during muscular action cannot be immediately restored by oxidative phosphorylation. However, phosphoryl transfer from creatine phosphate immediately buffers the ATP-ADP system at a suitable level (1:78).

This buffer system maintains the optimum level required by the ATP-ADP groups and the phosphoryl reserve to keep them in balance.

A schematic summary of the chemical events in muscle contraction is given in Diagram I. (18:72) Stimulated by a nerve impulse ATP is

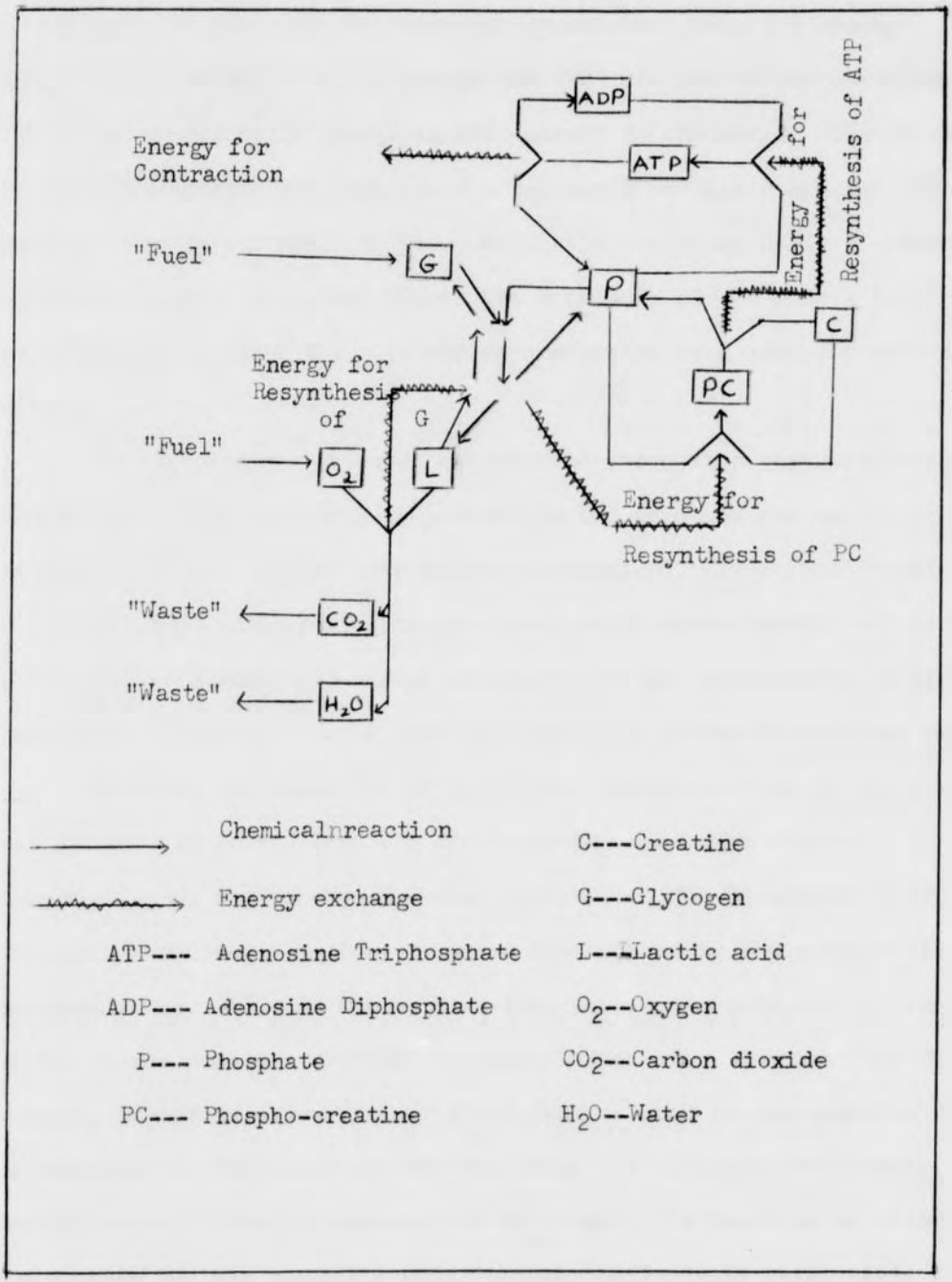


Figure 1. Chemical Events in Muscular Contraction. (From Rasch and Burke, Kinesiology and Applied Anatomy, p. 72.)

broken down yielding ADP and inorganic phosphate. From the energy generated by the splitting of phosphagen (PC) the ADP is converted into ATP and consequently no change in ATP content is exhibited. The PC that was split disappears and creatine and inorganic phosphate appear. If repeated stimuli are applied, the contraction continues until no phosphocreatine remains. As a last resort the myokinase of the muscle is activated and the last trace of ATP decomposes to form adenylic acid and ammonia.

Glycolysis plays an important part in preventing that last step. Glycogen is utilized as fuel to produce lactic acid and the energy acquired from this step is used to resynthesize PC. In aerobic conditions, or normally the second phase, oxygen reacts with approximately 4/5 of the lactic acid to produce the energy necessary for the resynthesis of glycogen while 1/5 of the lactic acid is reduced to carbon dioxide and water.

Karpovich (8) suggested an even newer interpretation of the chemical events. He stated that the ATP is broken down into ADP and phosphoric acid; not to the inorganic phosphate. The phosphoric acid combined with glycogen to form fructose diphosphate by the process of phosphorylation. Also PC breaks down into phosphoric acid and creatine and the former combines with ADP to resynthesize ATP. The fructose diphosphate liberates phosphoric acid and this is used to resynthesize PC. The remainder of the reactions are the same. In agreement with Sacks, Karpovich said, "normally contraction of a muscle is based on an oxidative process and the anaerobic phase becomes exclusively responsible for contraction only when the oxygen supply is inadequate." (8:22)

Whatever the interpretation whether schematic or outline, the basic process of conversion of ATP into ADP is generally accepted as the basis for the energy needed for contraction. After the discovery and acceptance of this cycle--usually referred to as the Krebs cycle--the main problem in muscular contraction chemistry became one of trying to decipher when ATP was broken down and how chemical energy was converted into kinetic energy.

Mechanical-Chemical Mechanisms. With Englehardt and Ljubimova's (42) discovery and Bailey's supporting experiments, (29) that myosin and adenosinetriphosphatase (ATPase) were not separate entities, muscle biochemistry began to concentrate on the enzymatic reactions of myosin, actin, and actomyosin. It was discovered in order for any of these to react ATP had to be present. "Direct evidence has been obtained that the contraction of the actomyosin system is dependent on the splitting of ATP. Relaxation always occurs when the splitting of ATP ceases, provided the muscle...is prevented from becoming rigid." (25:189)

The major components of muscle fibrils are myosin, actin, and tropomyosin, just recently discovered. All of these are believed to be involved in the contractile process converting chemical energy into mechanical energy. Perry (17) said,

> The interaction of the myofibrillar proteins actin and myosin is obviously of fundamental importance in the conversion of chemical energy because ATP, which itself undergoes exzymatic change, profoundly modifies this interaction and induces physical changes in the system (17:284).

It is generally accepted now that the energy providing step in the mechanical-chemical process depends upon the hydrolysis of ATP by myosin (ATPase). This ATPase activity of muscle is associated with the

actomyosin component. However, it is still a matter of controversy as to when or where ATP is hydrolyzed ". . . but it is hard to decide whether the transfer of energy to the contractile system occurs during ATP binding or at the moment of dephosphorylation." (17:315)

There are two opposing views as to the state of the proteins which induce contraction. Szent-Gorgyi (23) said that actin and myosin bound to ATP by high energy phosphate are separated from each other and come together to form actomyosin during contraction with the splitting off of the high energy phosphate. Morales and Botts (13) said that contraction occurs on the deformation of the myosin (actomyosin) molecule in the presence of ATP, which is called the binding theory. Both of these views require the presence of high energy phosphate in order for splitting or binding to occur.

Mechanisms of Contraction. "It is reasonable to assume that the conversion of chemical energy to mechanical energy occurs via a change in the structural pattern of the constituent proteins." (30:505) Three structural changes have been postulated:

1. Splitting of ATP from the protein molecule.
2. Binding of ATP to the protein molecule.
3. Polymerization of actin as the basis for contraction. (30)

There are several viewpoints as to the actual mechanical processes occurring during contraction. The two predominant views are (1) folding mechanism, and (2) the sliding mechanism.

Associated with the older theory of the folding mechanism are two contrasting hypotheses. The first hypothesis (13) asserts that myosin exists as thin, long particles kept straight by electrostatic charges

along the molecule. "When actin and myosin-ATP unite in 'excitation' to form actomyosin-ATP and the two proteins discharge each other, the linear configuration becomes improbable and the particle, under the influence of the heat agitation, folds up." (23:115) "Contraction accompanies this binding and the ATP is subsequently dephosphorylated and comes off leaving the element to re-extend." (17:323) In contrast to this, "the other view . . ., requires that the contractile element is phosphorylated and hence a co-valent bond is formed between different points to bring about a shortening." (17:323) A third alternative different from the above is that the actin and myosin molecules are arranged in a helical configuration and stretch and contract like a spring. Shortening within the physiological range is assumed to represent a folding up or a change in the helical arrangement of the sub-units of the myofilaments. (18)

The second and more recent mechanism is called the sliding mechanism. It has displaced the folding mechanism due to the evidence presented from Huxley's (6, 7) observations with the electron microscope. It was first noticed with wide angle or low angle X-ray diffraction that the muscle filaments did not show any change in length upon contraction; thus eliminating the folding theory. However, with the electron microscope, even though it does not provide straight forward proof, "it was found that the length of the A band remains constant and the length of the I band changes with changing muscle length." (7:213) Thus Huxley postulated that contraction is caused by the actin filament sliding past the myosin filament. He gave a hypothetical scheme for the contraction mechanism.

> Observations which have already been described indicate that when a muscle contracts, its actin filaments 'slide'

> past the myosin filaments. The extent of this sliding movement is much greater than the separation of the lateral projections on the myosin filaments, and it therefore follows that there cannot remain attached to the same points on the actin filaments for more than a very small fraction of the total contraction. Thus in contracting muscle it seems likely that repetitive cyclic process goes on at each actin-myosin cross-linkage site, the link being connected for one part of the cycle and disconnected for the rest . . . . The opening and closing of the links corresponds to the binding and dephosphorylation of ATP. Each time one cycle of operation of the links takes place, the actin filaments are forced to slide a short distance past the myosin filaments in the direction of the center of the sacomere. When a load on the muscle opposes this movement, tension is exerted (7:217).

However, "concerning the nature of the interaction between (the) two sets of filaments which actually generate the contraction force, there is at present nothing that deserves to be called evidence." (5:18)

## II. METHODS OF ANALYSIS OF AND PREPARATION OF ATP SAMPLES.

ATP and other purine derivatives have been analyzed by many different methods. Since ATP is found in plants, mitochondria, and muscle tissue, both smooth and straited, each system has to be analyzed differently. The following methods of analysis are those that have been utilized in the analysis of skeletal muscle.

Basically there are two different kinds of analysis--chemical and biological. The chemical analysis employs reactions that can be measured directly by physio-chemical methods. Examples to be discussed in relation to ATP analysis are (1) paper chromatography, (2) ion-exchange, and (3) analytical analysis by seven minute acid hydrolysis. Biological assays, however, are mainly concerned with enzymatic reactions and the determination of the end product by some appropriate chemical method.

Chemical Methods. One of the earliest methods employed to determine the concentration of ATP was to measure the change in the acid-labile phosphorous by the seven minute acid hydrolysis procedure. (10) The ATP was subjected to hydrolysis by a .1 N acid, and the amount of labile phosphorous driven off was determined. It was found that approximately 67 per cent of the labile phosphate is hydrolized at 100°C for seven minutes.

The ion-exchange method of Cohn and Carter (32) was adapted from a method they used to analyze purine and pyrimidine compounds. The method essentially involved the exchange of either and anion or cation on the resin in a column (Dowex 50, Dowex 1). The substance to be analyzed is displaced along the column by solutions of varying chloride content. After separation, the substance is then eluted through the column and analyzed by absorption spectroscopy.

The most widely used process for semi-quantitative analysis is paper chromatography. (31, 32, 41, 49, 61) Hanes and Isherwood (49) gave a good description of the basic method for filter paper chromatography as applied to the phosphate esters. The principle of paper chromatography is similar to an ion-exchange column except filter paper is used to absorb the substance to be separated and identified. The component in an aqueous phase is placed on the paper; it will remain at that spot until further treatment. A partially miscible organic solvent is permitted to flow over the spot. If there is a significant difference in the partion coeffecient of the solute mixture and the organic solvent, separation will ensue. When the coefficient favors the aqueous phase the solute will remain near the spot of application, if not, then it will travel down the chromatogram with the organic solvent.

After careful washing and drying the solute is analyzed by various means. One such means is to elute it from the chromatogram and do quantitative determinations of its respective absorption spectrum. Another analysis is development of the solute with chemicals that produce specific color reactions and then determine how far it has moved down the paper. Also the solute can be further treated by specific enzymes, other chemical methods or by detection of their ultra-violet absorption spectrum.

Biological Methods. Enzymatic determinations depend directly or indirectly upon the concentration of ATP present in the sample. Most of the reactions go to completion and the results are analyzed spectrophotometrically.

As early as 1934 enzymes had been used in various studies concerned with the reduction of ATP to some form or another product which could be analyzed by the change in the extinction coeffecient. Parnas, Osten, and Mann (85) employed a phosphatase which degraded ATP into adenylic acid which could then be acted upon by deaminase to cause the formation of ammonia. This ammonia was measured as the index of ATP produced. In a much later study, Kalckar (55) again used these two enzymes to determine the amount of ATP present in muscle. He found a decrease in the extinction coefficient was equal to the amount of ATP or ADP present. The extinction coefficient, $\epsilon_\lambda$, was determined from the formula: $\epsilon_\lambda = \frac{1}{L} \times \frac{1}{C} \times \log_{10} (I_o/I)_\lambda$. Where L = the depth of the absorbing layer in cm; C = the concentration of absorbing material in moles/1; $I_o$ and I are the light transmission of the sample and reference cell respectively, and $\lambda$ is the wavelength of light in mμ.

At the same time the above procedure was being used Hass (50), followed later by Kornberg (60) and Mommaerts (75) employed a different enzyme to measure ATP. They used hexokinase as their primary enzyme which reduced ATP and glucose to ADP and glucose-6-phosphate. The glucose-6-phosphate, when in the presence of 'Zwischenferment', reacts with TPN (triphosphopyridine nucleotide) to form 6-phosphogluconic acid and $TPNH_2$. The amount of TPN reduced equals the amount of ATP replaced. This is determined by spectrophometric assay or manometrically.

Two of the newer enzymes to be used in ATP assays are phosphoglyceric acid kinase (54) and extracts from firefly lanterns. The former enzyme is derived from peas. The reaction employed in this type of analysis consisted of converting ATP to ADP by means of 3, phosphoglyceric acid which was in turn converted into 1,3-diphosphoglyceric acid. This reaction must be driven to completion by hydroxylamine hydrochloride to trap the 1,3-diphosphoglyceric acid as 1-hydroxamic-3-phosphoglyceric acid. The hydroxamic acid may then be estimated as the colored ferric complex and serves as the measurement of ATP present.

The firefly lantern extract has two chemicals that depend upon the concentration of ATP for activation. They are luciferin and its enzyme, luciferase. William D. McElroy, of Johns Hopkins University, has been working on the firefly system for a number of years. (15, 20, 82, 93) Strehler and Totter (95) must be given credit for the discovery that ATP and ADP concentrations could be determined by the use of luciferin and luciferase.

The principle employed "is based on the linear luminescense response of firefly extracts to added ATP when all other factors are present in

excess." (20:871) According to McElroy and Greene (82:270) "the total light produced is directly proportional to the concentration of these two substrates." In a study on spectral emission and quantum yield it was noted that "one light quantum is emitted for every luciferin molecule oxidized, the yellow-green emission band peking at 562 mμ." (93:136) This method is extremely sensitive and will only detect ATP in the sample. The principle is simple and the equipment and processes for determinations allow for submicro estimations.

Evaluation of Methods. An evaluation of the different types of methods is given in Table I, which is reproduced from a review of methods by Strehler and Totter (21:343).

TABLE I

METHODS OF ATP ANALYSIS

| Method | Sensitivity, μg ~ P (lower limit) | Remarks |
|---|---|---|
| 1. Chemical separation | 10-50 | Loses due to solubility of salts. |
| 2. Ion Exchange | 5-30 | Relatively easy |
| 3. Hexokinase | ca. 20(manometrically)<br>ca. 2(7 min. P) | Intermediate in ease and rapidity. |
| 4. Deaminase | ca. 20 | Limited to clear solutions unless combined with 1 or 2 |
| 5. Firefly | $10^{-1}$ Farrand<br>$10^{-3}$ to $10^{-4}$ quantum counter Beckman D.U. | Rapid, relatively easy |

The five processes given are the more commonly employed ones. Going from top to bottom it is easily seen that the sensitivity of the last process is better than the first. The one not mentioned, phosphoglyceric acid kinase, is specific for ATP but minute quantities cannot be determined. The deaminase cannot be used by itself unless the sample is known to be pure. The hexokinase method is relatively easy but again minute quantities cannot be determined. Of all the methods discussed the luciferase system seems best suited for the detection of small amounts of ATP; it is extremely sensitive to submicro changes; it has the advantages of rapidity and ease, and most important of all it is specific for ATP, that is ADP, phosphocreatine, AMP, acetyl phosphate, inosine triphosphate, uridine triphosphate, guanosine triphosphate and other inorganic phosphates have been found to be completely inactive. On this basis it has been chosen as the method of analysis.

Preparation of Samples. Many different studies have been done on ATP and methods of extraction and the effects of different types of anesthesia. The principle step on which all extractions depend is the deproteinization of the muscle sample. Two acids can be used to do this--perchloric or trichloroacedic acids. (The latter is often referred to as TCA). Most studies have used TCA for the extraction procedure. (11, 20, 39, 58, 59, 83, 90) Dounce (39) gave a detailed description of a procedure for preparing highly pure ATP. LePage (11) summarized the principle thus:

> ATP is present in resting mammalian skeletal muscle to the extent of 350-400 mg. per cent. It is hydrolized to ADP by the enzyme adenosinetriphosphatase when muscle is stimulated. However a good yield of ATP is obtained by inhibiting the

enzyme with Mg. and extracting the muscle tissue with acid. ATP, other phosphate esters and inorganic phosphate are precipitated as Ba salts. The precipitate is dissolved in dilute $HNO_3$ and ATP is precipitated with mercuric nitrate. The precipitate is decomposed with $H_2S$ and the desired product is isolated as the Ba Salt (11:5).

Since TCA is absorbed at the same wave length as adenine these methods of extraction cannot be employed. Perchloric acid can be used instead, but Strehler and McElroy (20) said that boiling the tissue produced an extract which gave results comparable to the other methods.

If the animal is not anesthesized before decapitation and freezing, the muscle is stimulated by the contact with liquid air. Ennor and Rosenberg (43) noted that metabolic reactions are stopped by immersion into liquid air but the fact remained that the ATP concentration was altered by the stimulation of the cold liquid. Consequently several studies have been devoted to the effects of varied types of anesthesia.

Davenport and Davenport (34) and Sacks and Sacks (91) have developed a method whereby the muscle is frozen in situ. This called for leaving the blood and nerve supply intact and freezing the muscle with a $CO_2$ slush. Davenport and Davenport used amytal for an anesthesia while Sacks and Sacks used pento-barbital-sodium. The former was injected intraperitoneally and the latter intravenously. However, the ATP concentration did not remain constant because the muscle contracted during the freezing process.

As mentioned previously it was found that $Mg^{++}$ inhibited the action of adenosinetriphosphatase thus preventing the muscle from contracting. DuBois and co-workers (40) discovered that with an intraperitoneal injection of $MgSO_4 \cdot 7H_2O$ the muscle would not contract when excised and that

freezing was not absolutely necessary. The usual TCA extraction procedure followed.

Summary. Looking back many different methods of analysis and preparation have been discovered. For this study, which shall utilize the luciferin system of analysis by spectroscopy, it was seen that a simple extraction could be used, that is, boiling the sample. Because minute quantities and changes in ATP concentration needed to be detected, it was felt that the bioluminscense reaction of luciferin with ATP which gave a linear response would serve best.

Studies Reporting ATP Concentrations. Since the discovery of ATP, evidence has been presented that ATP is used in many reactions and systems. Many of these systems have been studied and analyzed; in each system ATP seems to have a different function. Necessarily the muscular system will be dealt with herein. It was noted that most studies have dealt with the effects of stimulation upon the ATP and PC concentration.

In two additional studies involving rat brain tissue, done in vivo, the reports indicated a fall in ATP level followed shortly by a return to normal and an increase in PC levels. Albaum et. al. (28) killed his rats by cyanide poisoning and analyzed for ATP, PC, lactic acid, and glycogen levels. He found a decrease in PC, ATP, and glycogen levels with an increase in P, and lactic acid. The average PC concentration for the control group was 65.5 while the cyanide group averaged 39.8. The ATP control was 87.9 and the cyanide group was 35.4. There was a significant "t" for the former of 7.0 and the latter of 5.6.

Dawson and Richter (35) in a later work involving the effect of stimulation on brain phosphate esters found that the rate of synthesis of ATP from PC must be rapid as there was little evidence of considerable ATP breakdown. He showed that the ATP level fell slightly and returned to normal within 25 seconds. From excitation the rats showed a 13 per cent increase in PC. However, there were no significant changes in any other level.

In tumorous muscle tissue LePage (65, 66) has found that the ATP concentration, in one study (66), was 52.0 m gm/100 gm. of tissue. In another study (65) it was found that normal resting tissue contained 542 µm/100 gm. of ATP and 1630 µm/100 gm. of PC. Consequently he showed that tumorous tissue contained less ATP.

LePage has also studied the effect of ischaemia on muscle tissue and the phosphate esters. He found (64) that the effect of hemorrhage on tissue metabolism caused a decrease in the concentration of PC and ATP in muscle. In an earlier work (63) he discovered that muscles when stimulated by shock decrease in PC and ATP concentrations. In a similar study Threlfall and Stoner (96) used ischaemia as a precursor of shock and detected a fall in ATP from 6.1 µm/gm. wet weight to zero.

Lange (62) and Singh and Raju (94) have used chemicals to induce contraction. Lange used acetylcholine and found that there was a fall in the level of ATP after a contraction lasting two seconds taking place at 0°C. Singh and Raju took smooth muscle of frogs and caused contraction by placement into a solution of dinitrophenol. They detected a decrease of 92 per cent between the control and experimental tissue. In the control tissue the ATP level was $1.512 \pm 0.058$ µm/gm. wet weight and in the

experimental tissue the ATP level was $0.114 \pm 0.014$. The experimental group showed a 50 per cent recovery when put back into Ringer's solution.

Electrical stimulation (33, 35, 75, 76, 81) has been the main cause of contraction in muscular studies. It is easy to use and can be regulated so as to cause one twitch or many. Mommaerts (75, 76) took a turtle's muscle because of its slow rate of contraction and studied the breakdown of ATP and PC during one twitch. The usual concentration of ATP was 2- 3 µm/gm. He found no supporting evidence that ATP and PC were broken down during a single twitch.

Munch-Petersen (81) stimulated a muscle and measured the content during the recovery phase of a twitch. He said ATP decreases during short tetanic contractions of approximately 15 seconds duration. He reported the ATP concentration as 1396 µg/gm. - 975 µg/gm. or 463 µg/gm. - 239 µg/gm. at either 0°C or 20°C respectively. Lardy (9) said that there was ten times as much ADP and P in tissue at rest than was to be expected.

Using frog muscle Mommaerts (12, 77) found a suggestion for the decrease of the ATP level and the increase for ADP and AMP levels. He stated, "in sustained muscular activity . . . a breakdown of PC rather than of ATP is generally detected." (77:957) He had stated this earlier in a treatise on muscular contraction. Perry (86) said about this "after moderate periods of continued activity the ATP level as measurement of acid labile phosphate remains relatively unchanged, whereas the creatine phosphate level falls." Rey (88) concurred with this statement. Perry went on to summarize the role of ATP <u>in vivo</u>,

> 1) there is general agreement that on prolonged tetnus a fall can be detected in PC and to some extent that of ATP. These findings confirm earlier findings and support the

> hypothesis that the energy for contraction is derived from 'energy rich' phosphates. 2) Direct, although not altogether conclusive evidence has been provided for the liberation of inorganic P after a single muscle twitch can take place without measurable changes in the ATP, ADP and creatine phosphate levels. 3) If the splitting of ATP is the source of inorganic phosphate liberated on contraction, the ATP level is maintained instantly either by the glycolic system or the unknown precursor. . . . 4) The possibility remains that the unknown precursor itself is the direct source of 'energy rich' phosphate for contraction. In that event its recharging either directly or indirectly by ATP is slow and apparently a feature of recovery (86:56).

In a classic paper dealing with training effects on creatine content Pallidin and Ferdman (84) said "that creatine content is higher in the trained muscle than in the untrained muscle." (84:174) Again they reported, "the increase in creatine content with training holds less longer than the glycogen content." (84:174) Ferdman and Feinschmidt (44) ascertained that the increase in PC was not stable and disappeared four to six days after suspending training. The training program involved electrical stimulation for nine to twenty-two days. "This training caused no increase in content of pyrophosphate acid of muscle." (51:183)

It is readily seen that the studies on ATP and PC concentrations are concerned with what happens to the concentration levels when affected by some outside stimulus. In no way has an effort been made to see what effect training by an exercise routine would have on the basal concentration of ATP.

## III. GENERAL EFFECTS OF EXERCISE UPON RATS.

Many investigations have dealt with the effects of exercise. However, most of these studies have been done on dogs, or humans. Most studies involving rats were concerned with the growth of the visceral organs.

Donaldson (36, 37, 38) studied the effects of exercise on organ weights in albino rats. He found general improvement in metabolism and circulation, slight increases in the muscular weight, and increases in organ weight. In a study designed to follow the effects of exercise on seven generations he discovered that there was no cumulative effect. In another study he was able to draw the conclusion that even beginning the exercise at different ages produced the same average increase in the organ weight.

Because exercise wheels, even motor driven ones, do not provide continuous activity, Montoye (78, 79, 80) investigated the swimming endurance and organ weights as related to exercise. It was noted in all cases involving swimming as the exercise some percentage of the body weight was attached to the tail. Montoye worked with both mature rats and with young rats. The mature rats, when allowed to exercise spontaneously, showed little effect on their body and organ weights. Montoye discovered,

> in mature rats there is little correlation between an endurance swim and body weight, carcass, specific gravity or the following organ weights: adrenals, liver, testes, kidneys, spleen, and heart (77:482).

An endurance swim has some limitations, that is, some rats seem undisturbed while others were extremely excited. In the case (80) where milk consumption and training were studied Montoye found that replacing part of the diet with milk had no effect on swimming performance, and also, that "endurance swimming time appears to be unrelated to various organ weights." (79:104)

Kenyon (57) exercised one group of white mice on a motor driven treadmill and one group by swimming. The results indicated that training

provided greater endurance in running than in swimming. The average body weights were lower in the exercised mice and the swim group weighed less than the treadmill group.

Another investigation (56) showed that exercise hastened the appearance of the symptoms of vitamin A deficiency and if the animals continued to be deficient in vitamin A their deaths were hastened. A study (48) done for dietary purposes found that rats could be maintained on a normal diet with no increase in allotment for the experimental group over the control group.

Updyke (102) reported that all the rats "suffered from mild to severe forms of chronic murine pneumonia upon which was superimposed an acute respiratory disease." (102:4730) However, he felt that there was no relation between the swimming time and the lung disease.

In two completely different studies, one concerned with blood cholesterol levels (78) and the other which compared female and male rat activity cycles (14), interesting conclusions were reached. The former experiment found that exercise caused a decrease in total and free blood serum cholesterol concentration. The latter, which cited from a study by Hitchcock, said that "females do more muscular work of an endurance type than the males." (14:184) It was noticed that there seemed to be a seasonal variation in activity with more activity taking place in the spring or summer.

## IV. EFFECTS OF EXERCISE ON MUSCULAR CHEMICALS

A survey of the literature showed that little work has been done with the effects of exercise (training) on the chemical constituents of

muscle. Many studies have been done on the effects of training programs upon muscle tissue, fatigue factors, and from the hygenic and educational standpoint. These have been done because human subjects could be used; however, chemical changes and training effects upon muscle systems require careful dissection and analysis of the tissue. It was surmised that little had been done with animals with this viewpoint in mind, and the few studies completed involved mainly the enzymatic systems.

Hearn and Wainio (51, 52), and Hearn and Gollnick (53) explored the effects of a training program upon three different enzyme systems. The program in each case consisted of swimming the animals for thirty minutes from five to eight weeks. An interesting fact was noticed in the report--they were the only ones to inject 15,000 to 60,000 units of penicillin-G for prophylactic measures. In all cases the exercise did not affect the total or unit activities of skeletal muscle, but the unit activity in the heart muscle was greater for the experimental animals than the controls. The enzymes studies were succinic dehydrogenase, aldolase, and ATPase, respectively.

Markarova (74), cited in a study by Hearn and Gollnick (53), swam his rats for one minute per day and increased the swimming time by one minute per day for thirty days. He found that the ATPase activity increased for swims of thirty or sixty but decreased for the 300 minute swim. In the trained group the ATPase activity increased for all swimming times.

Gollnick (101) followed the effects of exercise upon lactic dehydrogenase and ATPase. His experiments differed in that he did not immediately kill his animals but waited until the day after to do so.

He stated that,

> no significant changes were found to have taken place in the lactic dehydrogenase or ATPase activity . . . upon analysis of the activities of skeletal muscle. The training program did, however, produce enzymatic adaptions in the heart ventricles with ATPase and lactic dehydrogenase being significantly greater (101:2345).

Rawlinson and Gould (87) detected an increase, significant at the 5 per cent level, from increasing the exercise, but not significant relative to the total. They found that training did not affect the total activity of ATPase or PCase in muscle.

Palladin and Ferdman (84) and Ferdman and Feinschmidt (44) discovered that the creatine content was higher in a trained muscle than an untrained one, but that the increase disappeared from four to six days after cessation of training. Ferdman and Feinschmidt also found that electrical stimulation (training) caused no increase in the pyrophosphate acid of the muscle. An increase in the glucogen content was noticed by both groups. However, the creatine increase seemed to be less stable than the glycogen increase.

Two Russian workers, Yakolev and Yampolskaya (98, 99, 100), have worked on the biochemical changes that take place during training. They used faradic stimulation (98) or swimming (99) as the types of exercise. In the first case "rabbits, white rats, and mice in training (Faradic stimulation or running) showed enhanced activity with respect to anaerobic and oxidative-reductive processes in muscle activity." (98:10342g) Working together on another project in which either rapid work loads or steady prolonged loads were used, they found that "the levels of phosphagen, glycogen, and phosphorlytic activity are maintained at the level achieved by training most satisfactorily by short rapid loads." (100:10330f)

And finally, Yampolskaya (100) swam white rats and examined them chemically immediately after death. It was found that "trained animals consume much less phosphagen and glycogen than untrained and show more pronounced changes in the enzymatic systems . . . than do the untrained animals. (99:7194e)

Generally it seems that the enzymatic activity is not increased significantly in skeletal muscle. However, in cardiac muscle the activity does increase. The trained animals weigh less than the untrained ones and are the limiting factor in the feeding program. When the training program is stopped the levels of the chemicals decrease to normal within one week.

# CHAPTER IV

## EXPERIMENTAL PROCEDURE

In order to be able to measure the small quantity of ATP present in skeletal muscle tissue an experimental procedure had to be devised which would be sensitive and yet accurate enough for the analysis. The method finally selected involved bioluminescence and spectrophotometry. Other details involved the selection of subjects, the establishment of the variables, the type and length of exercise program and a preliminary experimental process.

### I. SELECTION OF SUBJECTS, VARIABLES, AND TRAINING PROGRAM

Subjects. Twenty-four male, Sprague-Dawley strain, albino rats were selected for use in this study. They were born on December 8, 1964, and were twenty-one days old when they arrived at the laboratory. The subjects were weighed on arrival and again when they were sacrificed. (Table VI, App. A) The rats were placed in metal cages, 9" by 8" by 7", and paired according to weight. (Table VII, App. A) After allowing four days for rest and adjustment to the new environment the training program was started.

Variable Measures. The only known variable was the exercise program. Rats were paired according to weight and designated as experimental or control. Ten pairs were utilized for the experiment with the experimental animal being labeled by a whole number and the control animal given

a subscript "a". Thus the rats were designated as 1 and 1a, 2 and 2a, etc. The experimental animals were exercised by swimming while the controls remained inactive.

The food allowance was the same for all subjects. Fifty grams of ground Purina laboratory chow was placed in their feeders. The amount eaten from day to day was determined according to the following procedure:

(a) The empty food container was weighed and weight recorded on the bottom.

(b) Fifty grams of chow was added and the total weight determined.

(c) The next day the same cup with the remaining food was weighed.

(d) The correct amount of food needed to arrive at the total weight was added to arrive at the original amount.

All animals were fed at the same time every day and in the same order. The containers were removed from the cages, weighed, food was added, and the containers were then returned to the cage. This process started at 8:00 A.M. and all the animals were fed by 8:45 A.M. They were allowed free access to food and water. An example of the difference in food consumption for the days 2-4-65 through 2-13-65 is given in Table VIII (Appendix A). None of the animals were fed or exercised on Sundays during the time of the experiment.

Weights were recorded when the rats arrived, when the training program was started, the fourth week of the experiment, the fifth week of the experiment and when they were killed. The weight differences given in Table IX (Appendix A) were for the above periods.

Control was exacted for (1) room temperature, in both the living area and in the laboratory, which was 72°F., (2) the water temperature was kept between 32 and 37°C. while the subjects were swimming, (3) all weighings of the animals were performed on the same scale and all weighings of tissue and other samples were done on a single pan analytical balance, (4) the pH of the enzyme solution was constant, (5) all samples were boiled for exactly ten minutes, and finally, all samples were analyzed at room temperature of 24-25°C.

Exercise Program. The experimental animals were subjected to an exercise program that created a situation of forced, spontaneous activity. This was done by using swimming as the exercise situation. Each experimental subject was placed in an individual tank of water and forced to swim for two minutes the first day with two minute increments thereafter until a total time of one hour was reached. Each experimental animal was started on the program one day behind the other as seen from Table X (Appendix A). This procedure allowed enough time for the sacrificing and analysis of one pair of animals, experimental and control, per day, twenty-four hours after the experimental animal reached a total swimming time of sixty minutes. In this way there was no pressure of having to kill and devise some way of preserving the dead animal in such a way that the ATP system would not react. All the experimental animals were allowed to rest on Sunday; thus, they swam only six days a week.

Since this study was involved in a training program situation, it was felt that there was no need to attach a certain percentage of the animal's body weight to him in order to produce exercise of a great intensity. Although some of the animals did learn to rest for short

periods of time (10-20 seconds) they had to paddle with their hind legs in order to keep their heads above the water. They seemed to be tired by the time they reached a swimming time of forty minutes.

When the animals finished their swimming time they were removed from the water, dried off and returned to their cages. Unfortunately rats when subjected to swimming are prone to develop upper respiratory diseases. Such was the case with three of the experimental animals. Experimental rat number 1 was lost due to drowning. Although drowning was the primary cause of death it is felt that the pulmonary congestion prevented the animal from breathing properly while swimming and lack of air while swimming caused drowning. Experimental rat number 8 also developed a virus (cold) and lost weight rapidly--after careful attention, he improved and finally recovered. Rat number 9 developed a cold several weeks after he had been training. He did not lose weight nor did he seem to get worse. When he was sacrificed he still had the cold.

When the rats started the program they became excited when placed into the water. Some dived for the bottom in search of a way out. However after the second or third swimming period they seemed calm and resigned to the fact that they had to swim and that there was no escape. Two rats swam at a time in individual tanks; one came out two minutes before the other and a bell rang which designated the end of the time for the second. The rat which swam two minutes longer became conditioned to the bell and would become excited if not removed when it rang.

## II. APPARATUS AND MATERIALS

Selection. After careful consideration of the methods and equipment used in previous studies for analysis of ATP, the apparatus selected was the Beckman D.U. Spectrophotometer. This instrument was selected after deciding that the luciferin-luciferase system was the best and most sensitive analytical method available for detecting minute quantities of ATP. A machine that measured light emission or absorption had to be selected. The D.U. was chosen because there was a procedure of an ATP assay outlined in a bulletin put out by the Worthington Biochemical Corp. (103) which stated that small amounts of ATP from 1 μg and upwards could be measured by the D.U. spectrophotometer. This reaction between luciferin ($LH_2$) and ATP yields luciferyl adenylate ($LH_2$-AMP) and inorganic phospher (P). The LH-AMP in turn reacts immediately with oxygen to give off light and L-AMP. This splitting off of the inorganic P creates enough energy for the reaction to act as a means for high energy source for bioluminescence (fluorescence). This emission of light is what is measured by the spectrophotometer. Very small amounts of ATP can be measured by the luciferin reaction and can be detected by the spectrophotometer. Sub-micro amounts of ($10^{-6}$ grams) ATP can be detected if a photomultiplier attachment is used; however, since muscle tissue is supposed to contain between 2-3 milligrams (mg.) of ATP this adaption was not made.

Description. The Beckman D.U. spectrophotometer is an instrument designed for analytical chemistry procedures. The basic principle on which the machine operates is that light (radiant energy) is passed

through an entrance slit which helps isolate certain wave lengths of light. This radiant energy then enters the monochromator (a quartz dispersing prism) which disperses the light into various wave lengths or narrow bands of radiant energy. "If this spectrum is brought to focus at the exit slit, a narrow portion of the energy can be selected and allowed to pass through into the material being investigated or into a receiver or measuring device." (26:97) The measured amount, whether changing or steady, is read off of a scale located on top of the instrument. The light created by the reaction of luciferin-luciferase is emitted and is not passed through the substance being investigated but is measured directly by the photocell. This light creation is termed bioluminescence. An appropriate wave length is selected for the analysis by another dial on top of the machine. After selecting the proper wave length and the correct slit width, which allows for a greater sensitivity, that is, a broad band or a narrow one, the machine is ready to perform the analysis.

Because of the extreme rapidity of the reaction it was felt that a modification to the D.U. was necessary. This would prevent a large margin of error to be eliminated caused by the time lapse involved in turning the transmittancy scale so that the correct reading could be recorded. This modification was achieved by attaching a Beckman "Linear Potentiometric Recorder" to the D.U. This recorder gave a graphical representation of the reaction taking place. This adaption required one additional piece of equipment to make the modification complete. This was the Beckman "Energy Recording Adapter" (ERA) which when turned on enabled the potentiometric recorder to receive the electrical impulses

created by the light being emitted in the cell. Note should be made of the fact that the sensitivity control and the transmittancy scale are inoperable when the ERA is used.

Another adaption was made to the D.U. This concerned devising a way in which the absorption cell compartment could be made light proof and still allow for the mixing of the constituents necessary for the reaction to occur. This was achieved by placing a piece of black electrical tape over the opening made upon the removal of the screw handle on the compartment lid. This handle was removed to allow the syringe needle to be lowered into the compartment.

Other pieces of apparatus to be described are (1) absorption cells, (2) automatic pipette, (3) swimming tanks and (4) syringes used for measuring the different solutions.

The swimming tanks were large tin cans which had contained feed prior to being cleaned and used as swimming tanks. Their depth was approximately two feet and they were approximately one foot in diameter. The water level in the tanks was maintained at a level whereby the rats could not touch bottom or escape over the side.

The absorption cells were 10 mm. matched quartz, high ultraviolet-near infra red cells. The cells were checked to see if they were equivalent by checking the amount of light transmitted. Both of the cells gave per cent transmittancy readings of 100 per cent thus indicating equivalency. Another minor modification was made. This consisted of placing a cover over the top of the cell to prevent leakage of the solution when the enzyme was added. A polyethylene cover was cut out of a large sheet of the same substance and secured over the top of the cell by a rubber band.

The automatic pipette was a product produced by Clay-Adams, Inc., called the "Aupette". The Aupette automatic syringe is operated by hand, but inside the stainless steel case which surrounds the plunger there is a spring which enables the plunger to be depressed at the same rate each time it is used. This syringe was suspended over the cell compartment and a 1" needle was lowered into the compartment when it was time for the enzyme to be injected into the ATP solution. The needle reached approximately 1/8" into the cell itself. This syringe as well as the ones used to kill the animals, to measure out the $MgSO_4$ and to measure out the ATP solutions was graduated into hundredths of a cubic centimeter. The same syringe was used for all the measurements.

Materials. Materials used in the analysis were (1) ATP, (2) luciferin-luciferase extract, (3) magnesium sulfate, and (4) the boiled muscle extract. The ATP material was obtained from Sigma Chemical Co. as was the firefly extract (luciferin-luciferase). The ATP was 98 per cent pure and contained three moles of water. The pure ATP solutions were made up daily during the "dry" experimental runs. This was done by weighing out varying amounts of ATP (.001, .002, and .003 grams) and dissolving each amount in 1 ml. of distilled water. These solutions of varying concentrations were then analyzed.

The firefly extract contained 50 mg. of dried firefly lanterns. When this lyophilized extract was mixed with 5 ml. of water it then contained 10 mM of luciferin-luciferase, 0.05 M $KH_2AsO_4$ and 0.02 M $MgSO_4$; the pH = 7.4. A fresh bottle of this extract was prepared each time an analysis was done.

The $MgSO_4 \cdot 7H_2O$ solution was prepared by mixing 51 g. in 100 ml. of water. The resulting solution contained 51 per cent $MgSO_4 \cdot 7H_2O$. This substance is commonly recognized as "epsom salts" with one exception, common epsom salts sold in stores is not as pure as the compound used in this study to produce anesthesia and eventually death by cardiac failure.

The muscle extract was formed when the excised gastrocemius muscle was boiled in 10 ml. of distilled water. The muscle was placed in a test tube containing the water and the test tube was placed in a beaker of boiling water and boiled for ten minutes. The muscle was excised daily from the animal scheduled to die. It was found that the solution could not be kept longer than thirty minutes without deterioration of the ATP which was being measured.

Critical Appraisal. The critical limits of the machine were established. It was shown by testing the apparatus that the sensitivity control on the ERA had to be set at its maximum value of 100 per cent in order for the greatest sensitivity to be attained. The ERA sensitivity control was set at 100 per cent and locked in that position by a switch on the 100 per cent adjustment knob. When the recorder is used in conjunction with the D.U. the slit width control is still operative and must be open at its widest limit in order for maximum sensitivity to be attained.

The automatic pipette, described before, was chosen because a problem of getting duplicate graphs presented itself. The first graphs obtained had different peak heights and slopes. This indicated that the reaction rate was not the same and that the amount of enzyme injected into the cell differed from time to time. The Aupette solved this problem

because the plunger can be locked into position and thus prevent loss of solution; also the movement of the plunger is governed by a spring which enables the plunger to be pushed down at the same rate. Thus the reaction would take place at the same rate if the enzyme was injected at the same time. Practice of this technique was required because the plunger is still operated by hand. When the technique was perfected the graphs were shown to be reproducible as long as all other variables were held constant, that is, the amount of ATP in the solution and the amount of enzyme.

## III. FUNDAMENTAL LAWS

This experiment was based on several laws that are concerned with the measurement of amounts of light energy. This light energy can either be transmitted or be absorbed by the substance being investigated.

The first law states that the energy of a reaction is proportional to the frequency of the radiation. Thus $E=hv$ where E represents the energy in ergs, v represents frequency in cycles per second, and h is a constant with the value $6.6624 \times 10^{-24}$ erg-sec. The h is usually known as Planck's constant. This energy is usually referred to as quanta or photons. Molecules differ in their ability to transmit light quanta; it is this difference which allows for the use of a sensitive phototube that can measure the quanta transmitted as an analytical tool.

The second law that must be followed in all light reactions in which the concentration of an unknown substance is being determined is the Beer-Bouguer Law. Bouguer's law states "that when a beam of parallel monochromatic radiation enters the absorbing medium at right angles to

plane, parallel surfaces of the medium each infinitesimally small layer of the medium decreases the intensity of the beam entering the layer by a constant fraction. Thus, $-dI/I = kdb$ where k is a constant depending on the wavelength, the nature of the medium and the concentration." (26:5) I is used to designate the original intensity of light transmitted to the phototube, dI designates the change from the original, db is the thickness of the absorbing material. The law is usually expressed in the following form: $\log_{10} I_o/I = kb$.

Beer's law states "that the intensity of a beam of parallel monochromatic radiation decreases exponentially as the concentration of the absorbing layer increases." (26:6) This is usually written as the $\log_{10} I_o/I = kc$, where c is the concentration of the absorbing material in any specified unit.

When the two laws are combined, referred to as the Beer-Lambert law, the following equation is used when applied to solutions: $\log_{10} I_{solv}/I_{soln} = a_i bc$ where $a_i$ is a constant dependent upon the wavelength of the light and the nature of the solution.

The relationship between fluorescence and the Beer-Lambert law was given by Boltz. (3) "The Beer-Lambert law cannot be applied directly to fluorescence, since no transmitted light is involved. . . .In the Beer's law equation where $I_o$ is the intensity of radiation entering the cell and I is the intensity of the transmitted radiation, then

$$\frac{I_o}{I} = \text{fraction of intensity not absorbed} = 10^{-ecd}$$

then $I - 10^{-ecd} = \text{fraction absorbed}$

and $I_o (1 - 10^{-ecd}) = \text{intensity absorbed} = I_o - I$

since Fluorescence intensity absorbed

then $F = K I_o (1 - 10^{-ecd})$." (3:97)

Later on Boltz mentioned that "in many cases in the literature where flurometer readings are plotted as a function of the concentration, a straight line results for low concentrations in the order of 1 to 50 micrograms." (3:97) The intensity of fluorescence radiation is very sensitive to changes in pH, temperature, or changing concentrations. The pH was regulated and controlled because of buffering in the enzyme solution. The temperature remained constant during all analyses. However, the concentration did change due to the using up of ATP in the reaction. This fact explains the decay in the amount of light emitted as the reaction proceeded to completion as depicted on the graph by the tapering off in the curve.

From this information analytical procedures can be carried out in which unknown concentrations of substances can be determined. This is done by measuring the intensity of light emitted, %T, for several known concentrations of the substance being analyzed and plotting the results obtained. A graph similar to Figure II on page 46 should be obtained. The %T of the unknown solution is determined by reading the peak height recorded on the graph produced during the reaction of ATP with luciferin-luciferase. The peak height is directly proportional to the amount of ATP in the solution.

## IV. VALIDATION

The standard curve measured the light emitted rather than the light absorbed. Light was produced by the reaction and since no additional

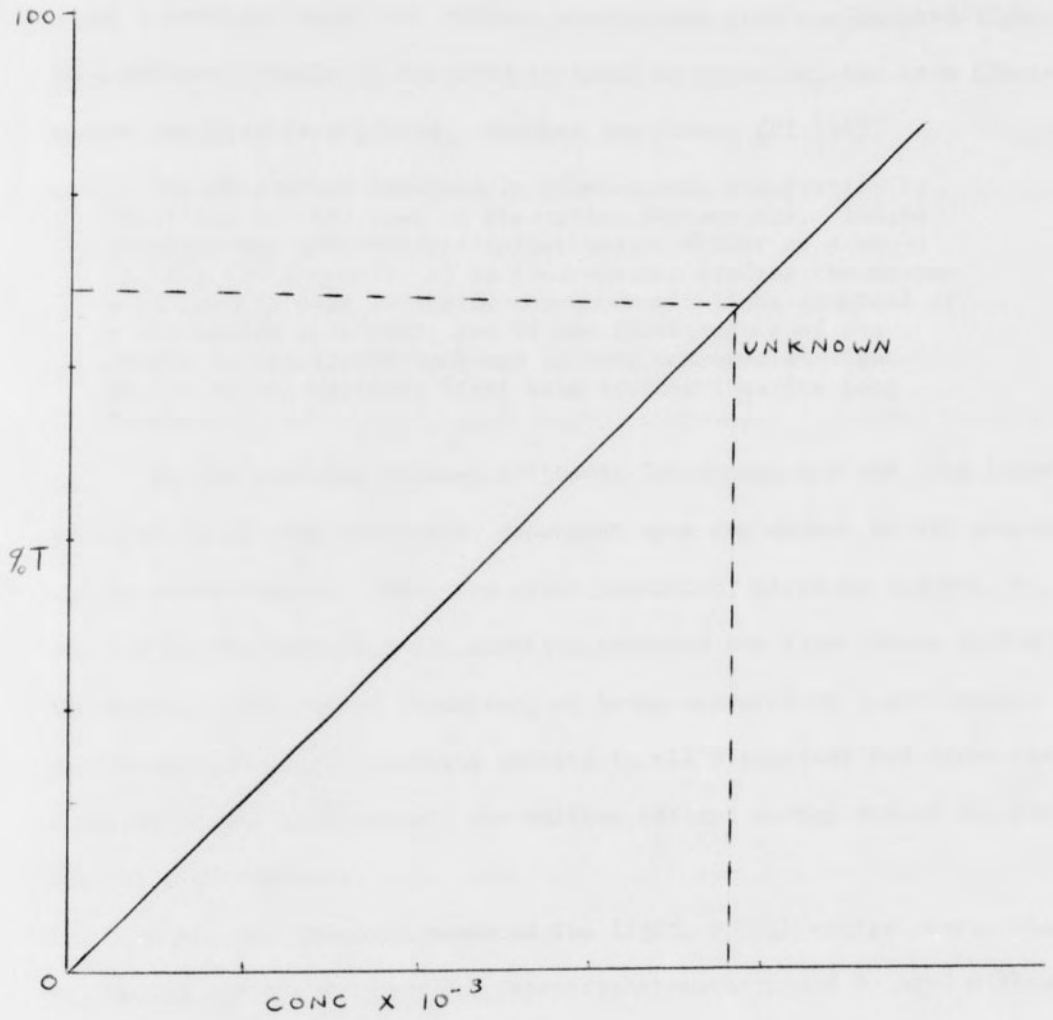


Figure 2. Typical Curve for Known ATP Samples.

light source was used, the radiant energy came from the emitted light. When radiant energy is measured in terms of emission, the term fluorometric analysis is employed. Strobel has stated (22:144),

> The photometry involved in fluorometric observation is identical to that used in absorption photometers. Indeed fluorescence and absorption instruments differ as a whole in only two respects, a) in fluorescence studies the source must furnish high intensity monochromatic light (instead of a continuous spectrum), and b) the fluorescence of the sample is relatively weak and is best measured at right angles to the incident light beam (rather than its long path).

In the reaction between luciferin-luciferase and ATP, the light produced is of high intensity, dependent upon the amount of ATP present, and is monochromatic. Thus the first condition, given by Strobel, is met due to the fact that the reaction produced the light which served as the source. The second condition, of being measured at right angles, is met because the light is being emitted in all directions and since the compartment was light proof, the emitted radiant energy struck the photocell at right angles.

Since the reaction produced the light, a high energy source was not needed and the Beckman D.U. spectrophotometer could be used without the addition of any fluorometric attachments. It was also found that a photomultiplier attachment was not needed to increase the sensitivity of the photoemission tube. With the radiant energy being measured directly by the photoemission tube the wavelength selector did not have to be set at any particular wavelength. The luciferin is excited at 270 mμ and the emission spectrum range is from 400 mμ to 600 mμ. The peak for the emission band is 562 mμ. It was assumed that the emission peak was being

measured because (1) the radiant energy was measured directly and did not pass through the quartz prism, which would select monochromatic bands of light according to what wavelength was selected and (2) was not focused by the exit slit onto the photocell; therefore, the highest intensity of the light had to be measured and the highest intensity occurs at the emission band of 562 mμ. Another reason in support of this concept is, the flash of light is so brief at its highest intensity that it is impossible for the light to go through the entrance slit, hit the prism, and return to the exit slit to be focused. The highest intensity is reached in two seconds.

The standard curve calculated from the peak heights produced by the recorded when known concentrations of ATP were used showed that the light intensity was directly proportional to the concentration of ATP in the solution. The reason that a higher value for the peak height was produced from a small amount of ATP is explained by looking at the following facts. When an outside source of light was passed through the ATP solution before it reacted with the firefly extract a transmittance reading of 100 per cent was effected. It must be remembered that the abscissa is read as 100% T. Upon addition of firefly extract which produced the fluorescence, the percentage of light transmitted decreased as indicated by the peak heights. If the peak height is subtracted from 100 to obtain the light intensity value then it is seen that the intensity is directly proportional to the concentration of ATP in the solution. The peak height actually represents a larger concentration of luciferin which has not reacted to form the luciferly adenylate when small amounts of ATP are present. The energy released from the splitting of the P from the ATP causes the excess luciferin to fluoresce.

## V. PRELIMINARY EXPERIMENTAL PROCESSES

The following experimental procedures had to be done before any actual experimentation with the subjects could be performed: (1) the amount of magnesium sulfate needed to kill the animal slowly had to be established, (2) the experimental techniques had to be perfected, (3) the amount of enzyme to be used, and finally (4) the solutions with known amounts of pure ATP had to be analyzed to ascertain whether the reaction followed the modified Beer's law for fluorescence.

Amount of $MgSO_4$ Used. Four non-experimental rats of varying weights were sacrificed with intraperitoneal injections of magnesium sulfate. The concentration of the magnesium sulfate was 51 per cent, that is, 51 g. per 100 ml. of water were used. It was discovered that .051 g/ 60 g. of body weight was sufficient to cause a slow death with final death attributed to cardiac failure. Table XI (Appendix A) shows the final body weight upon death and the amounts of magnesium sulfate injected intraperitoneally.

Techniques. One problem involving technique needed to be solved. The amount of enzyme injected into the cell had to be consistent. The Aupette syringe solved this problem. The syringe is designed so that the plunger can be locked into one position. Approximately .9 cc's were withdrawn from the vial, the excess enzyme was ejected from the syringe until the needed amount remained. The plunger was locked into this position. Although the plunger was locked to prevent slippage, the syringe could still be used to eject the enzyme into the cell. This was made possible

by the spring mechanism in the barrel of the Aupette. Since the rate of ejection was governed by the action of the spring, practice was necessary in order to be able to inject the enzyme into the cell in less than two seconds. This technique was perfected with the help of a stop watch. The watch was started when the plunger was depressed. It was stopped when the plunger was released. After a lot of practice the time taken to depress the plunger was decreased to the proper time interval.

The only other technique to be perfected was the killing procedure. The lab assistant held the rat's forepaws and hindpaws while the researcher injected the magnesium sulfate into the abdominal region. The injection spot was in the peritoneal region along the mid-line of the abdomen. The rat died in about five minutes.

Amount of Enzyme Used. The enzyme was diluted with 5 ml. of water to achieve the desired pH of 7.4. This pH is close to the pH in vivo. The amount of enzyme chosen was governed by the volume left in the cell after the addition of 1 ml. of ATP solution. One millimeter of enzyme caused an overflow; therefore, .5 ml. was decided upon. As will be shown in the next section this concentration of enzyme extract was sufficient for amounts of ATP varying from 1mg/ml water to 4mg/ml water. Thus the amount and concentration of the firefly extract was established.

Analysis of ATP Solutions. In order to show that the modified Beer's law was followed, that is, the amount of light emitted is proportional to the concentration of the solution which transmitted the light, the procedure which follows was pursued:

A. Initial Steps.

1. Upon arrival the spectrophotometer and recorder were turned on and allowed to warm up for at least one hour.

2. The blue-sensitive tube was selected and positioned by pulling out the phototube knob.

3. The selector switch was set to CHECK.

4. The slit width was set at 2 mm.

5. The transmittancy knob was set to 0% T.

After the proper warm-up period the following steps were followed after the selector switch was turned to .1. All of the above steps were done each day on which an analysis was to be done.

B. Steps for Determining the Concentration.

1. The ATP (pure compound), which had been removed from the freezer during the warm-up time and consequently was at room temperature, was weighed out in the amount desired for the analysis. Amounts of .001 g, .002 g, or .003 g were weighed out. This was done 20 min. before the analysis.

2. The ATP was placed in the absorption cell and 1 ml. of distilled water was added.

3. The cell was cleaned prior to each analysis by rinsing twice with distilled water and twice with acetone. It was then allowed to dry.

4. The bottle of enzyme extract to be used for the analysis had also been removed from the freezer at the time the ATP was removed. To this vial was added 5 ml. of water and the solution was allowed to reach room temperature.

5. The cells with the ATP solution (there were two) were placed in the light proof compartment.

6. The required amount of enzyme, .5 ml., was drawn from the vial as described previously.

7. The syringe was positioned over the cell compartment and lowered into the cell.

8. The NULL-RECORD knob on the ERA was turned to RECORD, thus allowing the recorder to record.

9. The pen control on the recorder was depressed and the pen was gently placed on the paper.

10. The pen was zeroed over the zero line on the paper which represented 100% transmittance. This was accomplished by using the dark current control on the D.U.

11. The record control on the recorder was depressed.

12. Several seconds later after checking to see that the pen had remained zeroed and that no reaction had occurred, the enzyme was injected into the cell.

13. The reaction which was taking place was recorded. A diagram shows this reaction.

14. The tracing was stopped by depressing the control marked standby. The NULL-RECORD knob was returned to NULL, and the shutter switch was returned to OFF.

15. Two runs were made before removing the cells; they were washed and dried as before.

The graphs obtained from the above procedure using .001g, .002 g, or .003 g. of ATP per milliter of water are presented in Appendix B. When the peak heights of these graphs were plotted as peak height versus concentration a straight line was obtained. Thus the modified Beer's law for fluorescence was followed.

## IV. EXPERIMENTAL PROCEDURE

The experiment was conducted on twenty male, albino rats. Ten experimental and ten control animals, which were paired according to weight at the beginning of the experiment, were used. The procedure for warming-up the instrument on the days that experimental data was to be collected followed steps A 1-7 as given in the preceding section.

In order to insure that the experimental animals had twenty-four hours of rest before the time for sacrificing they were swum from eight

in the morning until nine o'clock. The D.U. was turned on and warmed-up until nine. The animals rested from nine until nine the next day at which time they were killed. The lab assistant proceeded with the feeding and began the rest of the animals on their training program. All times were measured by a timer that was developed for use in timing the development of X-rays.

The following steps were done in order to collect the data from the experimental and control animals:

(1) The machine was warmed-up and all necessary chemicals were removed from the freezer.

(2) At 9:00 A.M. the experimental animal to be killed was weighed.

(3) The animal was sacrificed by injecting the proper amount of $MgSO_4$ as determined from the animal's weight.

(4) The animal was returned to his cage and allowed to die.

(5) The gastrocemius muscle was excised.

(6) The muscle was weighed in a pre-weighed tare on the automatic balance.

(7) The muscle was placed in liquid nitrogen--this was done in order to quick freeze the muscle.

(8) The sample was broken into small pieces.

(9) The sample was placed into a test tube containing 10 ml. of water.

(10) The test tube was placed in a beaker containing boiling water and the sample was boiled for exactly ten minutes.

While the experimental animal's muscle tissue was boiling, the killing process was repeated on the control animal.

(11) The hot solution was filtered immediately upon removal from the boiling water.

(12) The flask containing the filtered solution was placed into an $0^{o}$ C. freezer for four minutes until it approximately reached room temperature.

(13) The flask was removed from the freezer and permitted to reach room temperature.

(14) A 1 ml. calibrated syringe was taken and 1 ml. of muscle solution was measured out.

(15) This solution was placed into the absorption cell, a polyethylene cover was placed on, and the cell placed into the light proof compartment.

(16) While the muscle solution was cooling, the enzyme extract was measured out in the Aupette syringe and positioned as described before.

(17) After the absorption cell was placed into the compartment then the Aupette syringe was lowered into the compartment.

At this point in the procedure the process was the same as with the pure ATP samples. Steps B 7-14 were followed. This completed the experimental procedure; however, if the first two graphs did not agree within one-half unit in height and with only a slight deviation in slope the procedure was repeated until two graphs were obtained that met the criteria set forth.

# CHAPTER V

## PRESENTATION AND ANALYSIS OF DATA

The Fisher's "t" test for significance was computed from data collected for each subject. Additional data was recorded concerning weight differences, food consumption, and the training program. The data used for the "t" test was obtained from graphs where the peak height equaled the amount of ATP present in the muscle tissue. A standard curve was also constructed using peak heights obtained from known amounts of ATP.

### I. DATA

Tabular Data. It was very interesting to see how the weights of the experimental rats differed at different stages of the program from the control rats. (Table IX, Appendix A) The animals were paired according to weight (Table VII, Appendix A) and the difference in weight from the beginning to the end was recorded (Table VI, Appendix A). On the average the control animal ate more (Table VIII, Appendix A) and weighed more when sacrificed. Rat #8's weight difference is explained by rapid loss of weight when he developed a cold, and his appetite decreased for several days. Rat #4A's weight cannot be explained--he was never sick and yet the experimental animal ate more and weighed more when sacrificed.

When the animals were sacrificed, the gastrocnemius muscle was excised and weighed (Table II, page 57). This was done so that when the

amount of ATP was ascertained then the weight per gram of tissue for the experimental and control animal could be calculated. This computation was accomplished by dividing the tissue weight into the amount of ATP. The preliminary calculations are shown in Tables IV and V (pages 59 and 60), respectively. The amount of ATP found in each muscle is given in Table III (page 58).

Graphical Data. Graphical data was obtained from each subject. The graphs to be used were selected on the basis of identical peak heights and not more than one-half unit deviation in the slope. All graphs are found in Appendix B. A standard curve (Figure 3, page 61) was constructed from data obtained using known concentrations of ATP in solution. The peak heights were determined by counting the number of deviations from the base line which the pen made before the reaction of ATP with the luciferin-luciferase system. When the peak heights are plotted versus concentration of ATP, a straight line was produced. When an unknown concentration of ATP is in solution the peak height was found on the standard curve; then the ATP concentration was determined by finding the point at which a vertical line drawn from the point found previously on the standare curve met the abscissa. The standard curve obtained in this study had a slope exactly opposite to what would be expected. The reason for this was explained in the last paragraph in section IV of the previous chapter.

The label at the top of the graphs gave the date of the analysis, temperature at which the reaction was run, the amount of enzyme used and the peak height resulting from the reaction. The first three graphs given in Appendix B are the ones from which the standard curve was constructed. These three used known concentrations of ATP and the label

TABLE II

WEIGHT OF TISSUE TAKEN FROM RATS

| Group | Experimental | Control |
|---|---|---|
| 2 | 1.6247 | 1.9365 |
| 3 | 1.7534 | 1.9763 |
| 4 | 1.8252 | 1.7205 |
| 5 | 1.7852 | 2.0177 |
| 6 | 2.0567 | 2.0318 |
| 7 | 1.9702 | 1.8575 |
| 8 | 1.5257 | 2.1057 |
| 9 | 1.6729 | 1.8105 |
| 10 | 1.9954 | 2.3737 |

TABLE III

ATP PER GM OF TISSUE

| Group | Experimental ($X_1$) | Control ($X_2$) |
|---|---|---|
| 2 | .00196 | .00161 |
| 3 | .00171 | .00166 |
| 4 | .00171 | .00181 |
| 5 | .00171 | .00155 |
| 6 | .00129 | .00141 |
| 7 | .00138 | .00136 |
| 8 | .00178 | .00123 |
| 9 | .00163 | .00158 |
| 10 | .00153 | .00134 |

TABLE IV

PRELIMINARY CALCULATIONS FOR EXPERIMENTAL GROUP

| Group E | ATP ·/· tissue wt. | ATP per gm tissue = $X_1$ | $X_1^2$ |
|---|---|---|---|
| 2 | .00319/1.6247 | .00196 | .0000038416 |
| 3 | .00300/1.7534 | .00171 | .0000029241 |
| 4 | .00312/1.8252 | .00171 | .0000029241 |
| 5 | .00306/1.7852 | .00171 | .0000029241 |
| 6 | .00265/2.0567 | .00129 | .0000016641 |
| 7 | .00272/1.9702 | .00138 | .0000019044 |
| 8 | .00272/1.5257 | .00178 | .0000031684 |
| 9 | .00272/1.6729 | .00163 | .0000026569 |
| 10 | .00306/1.9954 | .00153 | .0000032406 |
| | | $X_1$ = 0.01470 | $X_1^2$ = .0000243483 |

$$\text{Mean} = \frac{X_1}{N_1}$$

TABLE V

PRELIMINARY CALCULATIONS FOR CONTROL GROUP

| Group C | ATP /tissue wt. | ATP/gm tissue = $X_2$ | $X_2^2$ |
|---|---|---|---|
| 2a | .00312/1.9365 | .00161 | .0000025921 |
| 3a | .00326/1.9673 | .00166 | .0000027556 |
| 4a | .00312/1.7205 | .00181 | .0000032761 |
| 5a | .00312/2.0177 | .00155 | .0000024025 |
| 6a | .00286/2.0318 | .00141 | .0000019881 |
| 7a | .00252/1.8575 | .00136 | .0000018496 |
| 8a | .00259/2.1057 | .00123 | .0000015129 |
| 9a | .00286/1.8105 | .00158 | .0000024964 |
| 10a | .00319/2.3737 | .00134 | .0000017956 |
| | | $X_2$ = .01355 | $X_2^2$ = .0000206689 |

$$M_2 = \frac{X_2}{N}$$

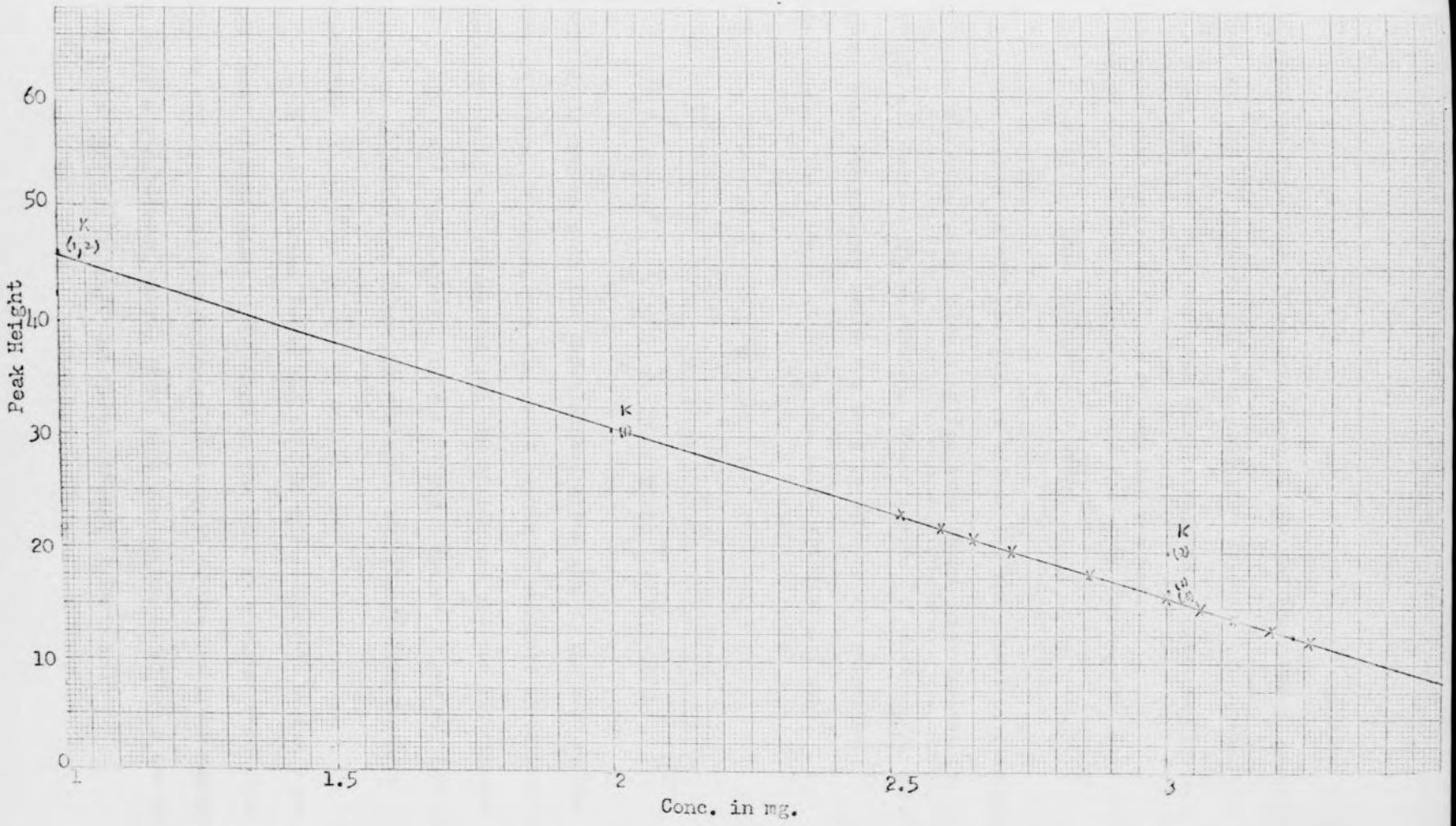


Figure 3. Standard Curve for ATP Knowns.

so indicated this fact. The other graphs are for the unknown concentrations and the ATP concentration is indicated as such. The graphs are read from right to left with the peak heights read in an arbitrary scale. In this case each horizontal line represented one unit.

## II. ANALYSIS OF DATA

The amount of ATP per gram of tissue (X) the square of that amount ($X^2$) and the means were calculated for each group, experimental ($M_1$ = .00163) and control ($M_2$ = .00151). The Fisher's "t" formula for small groups with independent means was used to compute the significant difference. The formula given by Van Dalen (24:318) was used:

$$\frac{M_1 - M_2}{\sqrt{\left[ \left( \sum x_1^2 + \sum x_2^2 \right) \Big/ \left( N_1 + N_2 - 2 \right) \right] \left( 1/N_1 + 1/N_2 \right)}}$$

where $M_1$ is the mean of the experimental group and $M_2$ the mean of the control group. N represents the total number of subjects in each group respectively. $\sum x_1^2$ represents the sum of squares for the experimental group and $\sum x_2^2$ the control. Tables IV and V (pages 59 and 60) show the calculations performed to obtain the means and the $X^2$ from the data collected.

A "t" of 1.32 was found and this is not acceptable at the 5 per cent level of confidence. Although on the average a higher concentration of ATP was found in the exercised animals it was not great enough to be of a significant value. Even with the exclusion of Rat 8 and 8A from the study, the "t" did not change enough to be significant.

Interpretation. On the basis of the data collected and the number of subjects used it is reasonable to assume that training (using an isotonic exercise program) does not produce a significant increase in the base concentration of ATP. Other research (35, 77, 81) has shown that the concentration does decrease momentarily after stimulation by electric shock. There is the possibility that a longer training program and/or one of a different nature would produce significant changes. Maybe just enough ATP is always available when the muscle is called upon to contract. This could be due to the fact that PC is capable of splitting off the P; consequently, regenerating the ATP as fast as it is needed. There is a definite trend for higher ATP concentrations in exercised animals. It is also possible that diet could affect the ATP concentration. If the animals were older when they started on the training program it might develop that growth was the factor necessary for significant increases in the ATP concentration.

# CHAPTER VI

## SUMMARY AND CONCLUSIONS

Summary. The problem undertaken in this study was to ascertain whether the base concentration of ATP in exercised rats significantly increased over the control rats. Secondly an accurate method of measuring the ATP concentration was developed.

Twenty male subjects were selected and paired according to weight from among thirty that were delivered. One subject died as a result of drowning due to pulmonary congestion. The rats were forced to swim daily and each day two more minutes were added to the training program until a length of sixty minutes was attained. The rats started on the program one day behind each other. All rats were fed and treated the same. After reaching the one hour swimming time they were sacrificed. Death ensued from cardiac failure brought on with magnesium sulfate. The essay for ATP was run on the Bechman D.U. spectrophotometer to which a graphical recorder had been attached. Peak heights ranging from 12-21 were recorded. This meant that the ATP concentrations ranged between 2.52 mg per wet tissue weight to 3.26 mg per wet tissue weight.

The instrument used to measure the ATP required a few adaptions. The recorder was attached but in order for it to be operable an "Energy Recording Adapter" had to be attached. This part enabled the recorder to utilize and interpret the electrical impulses coming from the photosensitive cell of the spectrophotometer.

The compartment containing the cell holder and cells was made light proof. This was done by placing black electrical tape over all possible entrances where light could enter. In order to inject the enzyme a screw handle on the top plate was removed and a piece of tape placed over the hole. This allowed the needle of the syringe containing the enzyme to be lowered into the compartment.

The Fisher's "t" test for significance was computed from the data collected. The data used to compute the "t" was secured from the peak height on each graph. This peak height represented the amount of ATP present in the muscle tissue. After finding the amount contained in muscle, calculations were done to find out how much was contained per gram. From this data the means ($M_1$ = 1.63 mg and $M_2$ = 1.51 mg) and the standard error were computed for the "t" test. The t = 1.32 which was not significant at the 5 per cent level of confidence.

Conclusions. Based on the findings of this study, it was concluded that:

1. There was not a significant change in the base concentration of ATP in the experimental rats as compared to the control rats.

2. The control animals on the average ate more and weighed more than the experimental ones.

3. There was a definite trend of an increased basal concentration of ATP in the exercised animals.

4. Since eighty-eight per cent of the exercised rats had a higher ATP concentration per gram of tissue further study would be warranted in this area.

5. The fact that animal tissue was used must be remembered. Therefore these conclusions can only definitely be related to animals.

Suggestions for Further Study. It is recommended that further research be carried out with several factors in mind. The first of these would be to use animals of an older age. It seems that rats of older ages tend to exercise more with the peak activity period coming when they are between 75 and 250 days old (14: 97).

It is further recommended that a larger number of animals be utilized. Since the difference between the means was only .12 mg per gram of tissue for eighteen animals then it is possible that more animals might increase this difference to a significant point.

Additional work could be done by changing the length of the training program and/or increasing the intensity of the amount of exercise.

Another possible study would be to look at the ratio of the concentrations of ATP in muscles used a great deal, e.g., a leg muscle as compared to a muscle used comparatively little, e.g., a back muscle.

It is suggested that this study sustains the thought that phosphocreatine serves as the reserve phosphorous supply since ATP seems to be regenerated very fast (in order to maintain the equilibrium). Consequently there is the possibility that the base phosphocreatine concentration increases. A study utilizing the same methods and procedures could be used with some enzyme such as phosphocreatine kinase being added after the ATP-firefly enzyme reaction. This would cause the regeneration of ATP from phosphocreatine and another light flash should occur. This light

flash would cause another peak to occur on the graph. The height of this peak should be directly proportional to the concentration of ATP regenerated and consequently be proportional to the amount of phosphocreatine used to regenerate the ATP.

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# APPENDIX A

TABLE VI

ORIGINAL AND FINAL WEIGHTS OF RATS

| Rat Number | Original Weight | Final Weight |
|---|---|---|
| 1 | 59 | died |
| 1a | 59 | ---- |
| 2 | 59 | 281 |
| 2a | 59 | 327 |
| 3 | 59 | 307 |
| 3a | 59 | 341 |
| 4 | 58 | 321 |
| 4a | 58 | 280 |
| 5 | 58 | 308 |
| 5a | 58 | 340 |
| 6 | 58 | 337 |
| 6a | 58 | 340 |
| 7 | 60 | 325 |
| 7a | 62 | 356 |
| 8 | 60 | 247 |
| 8a | 60 | 340 |
| 9 | 61 | 290 |
| 9a | 60 | 320 |
| 10 | 57 | 321 |
| 10a | 57 | 368 |

NOTE: All weights in this table and the following ones are given in grams.

TABLE VII

PAIRING OF RATS ACCORDING TO WEIGHT

| Rat Number | Initial Weight |
|---|---|
| 1 | 59 |
| 1a | 59 |
| 2 | 59 |
| 2a | 59 |
| 3 | 59 |
| 3a | 59 |
| 4 | 58 |
| 4a | 58 |
| 5 | 58 |
| 5a | 58 |
| 6 | 58 |
| 6a | 58 |
| 7 | 62 |
| 7a | 60 |
| 8 | 60 |
| 8a | 60 |
| 9 | 61 |
| 9a | 60 |
| 10 | 57 |
| 10a | 57 |

TABLE VIII

DIFFERENCES IN FOOD CONSUMPTION

| Date | Group | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| 2-3 | | 1 | 2e | 5e | 5 | 2 | 2 | 8 | 2 | 4 |
| 2-5 | | 5 | 1 | 3e | 0 | 3e | 0 | 8 | 1 | 3 |
| 2-6 | | 1e* | 0 | 5e | 2 | 4 | 2 | 4 | 1 | 8e |
| 2-9 | | | 1e | 3e | 2 | 4e | 1 | 5 | 3 | 4 |
| 2-10 | | | 0 | 2e | 3 | 1e | 0 | 5 | 3 | 3e |
| 2-11 | | | | | 6 | 1e | 4 | 0 | 4 | 5 |
| 2-12 | | | | | 2 | 3e | 3 | 5 | 7 | 1 |
| 2-13 | | | | | | 4e | 2e | 2 | 2 | 0 |

NOTE: The small letter "e" denotes that the experimental animal ate more on that day.

TABLE IX

WEIGHT DIFFERENCES

| Group | Initial | Beginning | 4th Week | 5th Week | End |
|---|---|---|---|---|---|
| 1 | 0 | 4 | died | | |
| 2 | 0 | 6 | 27 | 46 | 46 |
| 3 | 0 | 4 | 17 | 29 | 34 |
| 4 | 0 | 1e | 36e | 48e | 41e |
| 5 | 0 | 2 | 3e | 9 | 22 |
| 6 | 0 | 9 | 8 | 12 | 3 |
| 7 | 2e* | 5e | 7e | 5 | 30 |
| 8 | 0 | 11 | 91 | 105 | 93 |
| 9 | 1e | 3 | 14 | 29 | 30 |
| 10 | 0 | 3 | 43 | 31 | 47 |

NOTE: The small letter "e" denotes that the experimental animal weighed more.

TABLE X

TRAINING PROGRAM

| | M | T | W | Th | F | S | Sun |
|---|---|---|---|---|---|---|---|
| Date | 1/4 | 1/5 | 1/6 | 1/7 | 1/8 | 1/9 | 1/10 |
| | # 1=2 min. | # 1=4 | # 1=6 | # 1=8 | # 1=10 | # 1=12 | |
| | | 2=2 | 2=4 | 2=6 | 2=8 | 2=10 | |
| | | | 3=2 | 3=4 | 3=6 | 3=8 | All |
| | | | | 4=2 | 4=4 | 4=6 | Rest |
| | | | | | 5=2 | 5=4 | |
| | | | | | | 6=2 | |
| Date | 1/11 | 1/12 | 1/13 | 1/14 | 1/15 | 1/16 | 1/17 |
| | # 1=14 min. | # 1=16 | # 1=18 | # 1=20 | # 1=22 | # 1=24 | |
| | 2=12 | 2=14 | 2=16 | 2=18 | 2=20 | 2=22 | |
| | 3=10 | 3=12 | 3=14 | 3=16 | 3=18 | 3=20 | |
| | 4=8 | 4=10 | 4=12 | 4=14 | 4=16 | 4=18 | All |
| | 5=6 | 5=8 | 5=10 | 5=12 | 5=14 | 5=16 | Rest |
| | 6=4 | 6=6 | 6=8 | 6=10 | 6=12 | 6=14 | |
| | 7=2 | 7=4 | 7=6 | 7=8 | 7=10 | 7=12 | |
| | | 8=2 | 8=4 | 8=6 | 8=8 | 8=10 | |
| | | | 9=2 | 9=4 | 9=6 | 9=8 | |
| | | | | 10=2 | 10=4 | 10=6 | |
| Date | 1/18 | 1/19 | 1/20 | 1/21 | 1/22 | 1/23 | 1/24 |
| | # 1=26 min. | # 1=28 | # 1=30 | # 1=32 | # 1=34 | # 1=36 | |
| | 2=24 | 2=26 | 2=28 | 2=30 | 2=32 | 2=34 | |
| | 3=22 | 3=24 | 3=26 | 3=28 | 3=30 | 3=32 | |
| | 4=20 | 4=22 | 4=24 | 4=26 | 4=28 | 4=30 | All |
| | 5=18 | 5=20 | 5=22 | 5=24 | 5=26 | 5=28 | Rest |
| | 6=16 | 6=18 | 6=20 | 6=22 | 6=24 | 6=26 | |
| | 7=14 | 7=16 | 7=18 | 7=20 | 7=22 | 7=24 | |
| | 8=12 | 8=14 | 8=16 | 8=18 | 8=20 | 8=22 | |
| | 9=10 | 9=12 | 9=14 | 9=16 | 9=18 | 9=20 | |
| | 10=8 | 10=10 | 10=12 | 10=14 | 10=16 | 10=18 | |
| Date | 1/25 | 1/26 | 1/27 | 1/28 | 1/29 | 1/30 | 1/31 |
| | # 1=38 min. | # 1=40 | # 1=42 | # 1=44 | # 1=46 | # 1=48 | |
| | 2=36 | 2=38 | 2=40 | 2=42 | 2=44 | 2=46 | |
| | 3=34 | 3=36 | 3=38 | 3=40 | 3=42 | 3=44 | |
| | 4=32 | 4=34 | 4=36 | 4=38 | 4=40 | 4=42 | All |
| | 5=30 | 5=32 | 5=34 | 5=36 | 5=38 | 5=40 | Rest |
| | 6=28 | 6=30 | 6=32 | 6=34 | 6=36 | 6=38 | |
| | 7=26 | 7=28 | 7=30 | 7=32 | 7=34 | 7=36 | |
| | 8=24 | 8=26 | 8=28 | 8=30 | 8=32 | 8=34 | |
| | 9=22 | 9=24 | 9=26 | 9=28 | 9=30 | 9=32 | |
| | 10=20 | 10=22 | 10=24 | 10=26 | 10=28 | 10=30 | |

TABLE X (continued)

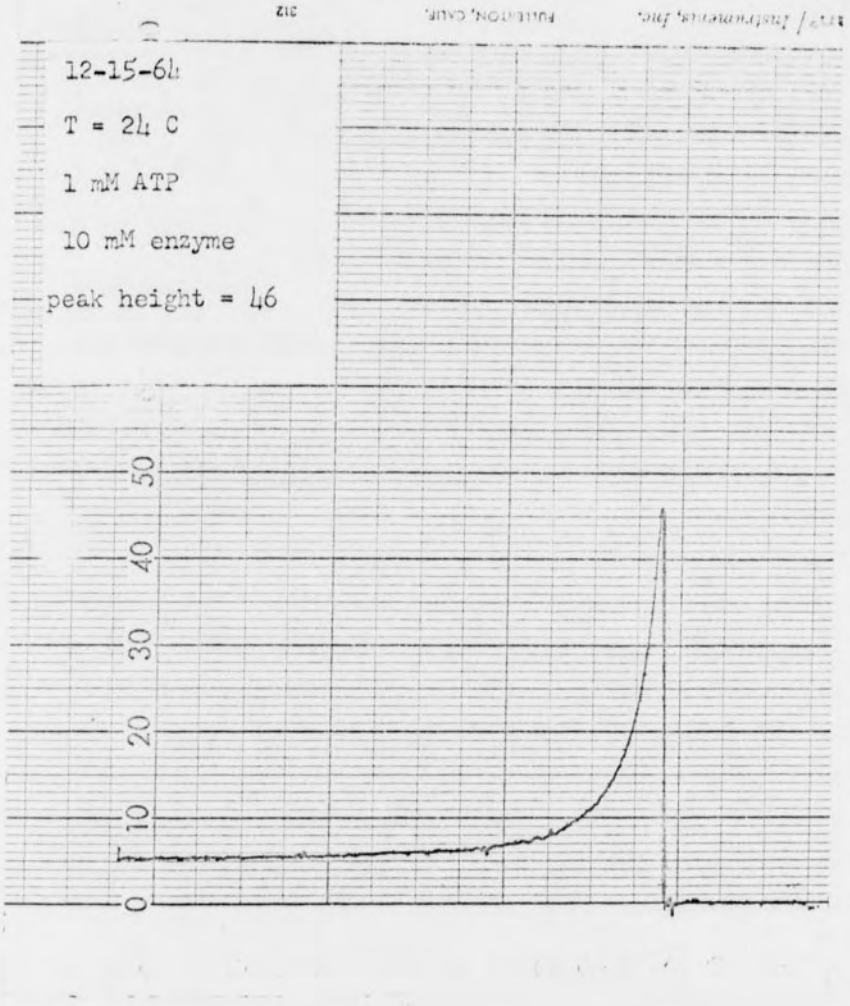
| | M | T | W | Th | F | S | Sun |
|---|---|---|---|---|---|---|---|
| Date | 2/1 | 2/2 | 2/3 | 2/4 | 2/5 | 2/6 | 2/7 |
| | # 1=50 min. | # 1=52 | # 1=54 | # 1=56 | # 1=58 | # 1=60 | Kill #1 |
| | 2=48 | 2=50 | 2=52 | 2=54 | 2=56 | 2=58 | (died |
| | 3=46 | 3=48 | 3=50 | 3=52 | 3=54 | 3=56 | before |
| | 4=44 | 4=46 | 4=48 | 4=50 | 4=52 | 4=54 | time |
| | 5=42 | 5=44 | 5=46 | 5=48 | 5=50 | 5=52 | for |
| | 6=40 | 6=42 | 6=44 | 6=46 | 6=48 | 6=50 | analysis) |
| | 7=38 | 7=40 | 7=42 | 7=44 | 7=46 | 7=48 | |
| | 8=36 | 8=38 | 8=40 | 8=42 | 8=44 | 8=46 | |
| | 9=34 | 9=36 | 9=38 | 9=40 | 9=42 | 9=44 | Rest |
| | 10=32 | 10=34 | 10=36 | 10=38 | 10=40 | 10=42 | |
| Date | 2/8 | 2/9 | 2/10 | 2/11 | 2/12 | 2/13 | 2/14 |
| | --- | Kill #2 | Kill #3 | Kill #4 | Kill #5 | Kill #6 | Kill #7 |
| | # 2=60 | --- | --- | --- | --- | --- | --- |
| | 3=58 | 3=60 | --- | --- | --- | --- | --- |
| | 4=56 | 4=58 | 4=60 | --- | --- | --- | --- |
| | 5=54 | 5=56 | 5=58 | 5=60 | --- | --- | --- |
| | 6=52 | 6=54 | 6=56 | 6=58 | 6=60 | --- | --- |
| | 7=50 | 7=52 | 7=54 | 7=56 | 7=58 | 7=60 | --- |
| | 8=48 | 8=50 | 8=52 | 8=54 | 8=56 | 8=58 | |
| | 9=46 | 9=48 | 9=50 | 9=52 | 9=54 | 9=56 | Rest |
| | 10=44 | 10=46 | 10=48 | 10=50 | 10=52 | 10=54 | |
| Date | 2/15 | 2/16 | 2/17 | 2/18 | | | |
| | --- | Kill #8 | Kill #9 | Kill #10 | | | |
| | --- | --- | --- | --- | | | |
| | --- | --- | --- | --- | | | |
| | --- | --- | --- | --- | | | |
| | --- | --- | --- | --- | | | |
| | --- | --- | --- | --- | | | |
| | --- | --- | --- | --- | | | |
| | # 8=60 | --- | --- | --- | | | |
| | 9=58 | 9=60 | --- | --- | | | |
| | 10=56 | 10=58 | 10=60 | --- | | | |

TABLE XI

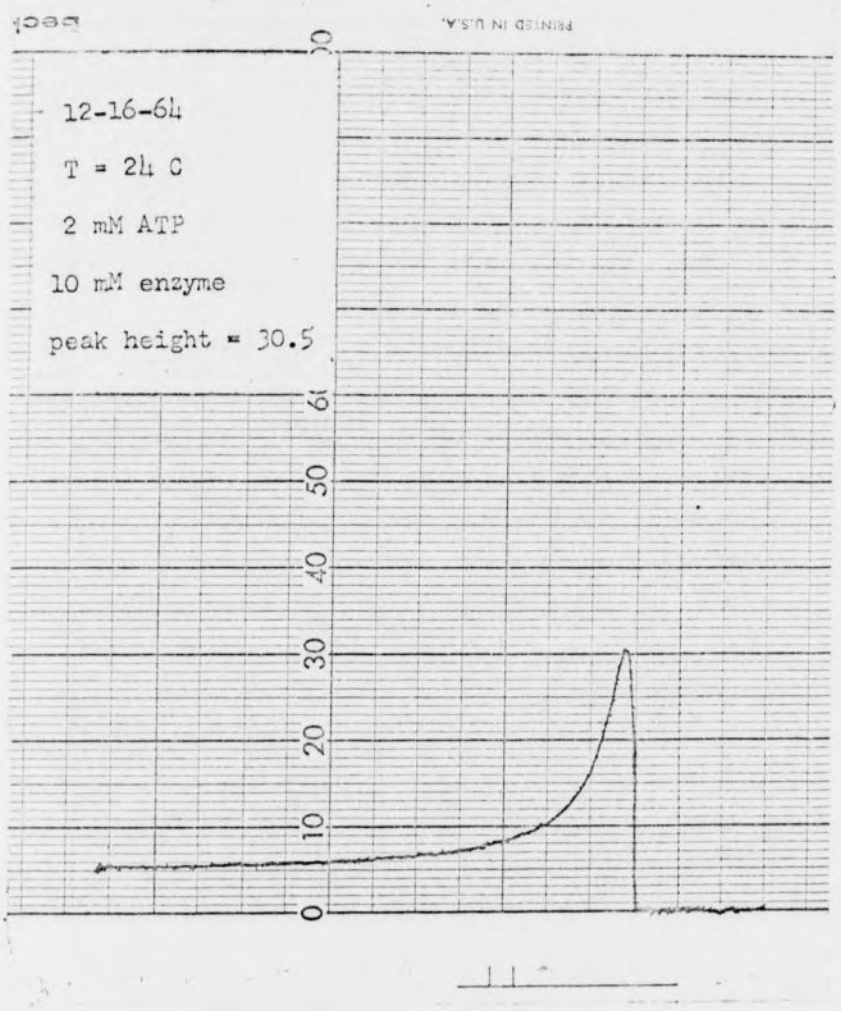
AMOUNT OF $MgSO_4$ USED TO CAUSE DEATH

| Rat Number | $MgSO_4$ in tenths of ml. |
|---|---|
| 2 | .5 |
| 2a | .5 |
| 3 | .5 |
| 3a | .7 |
| 4 | .5 |
| 4a | .5 |
| 5 | .5 |
| 5a | .7 |
| 6 | .7 |
| 6a | .7 |
| 7 | .6 |
| 7a | .6 |
| 8 | .5 |
| 8a | .6 |
| 9 | .5 |
| 9a | .55 |
| 10 | .55 |
| 10a | .6 |

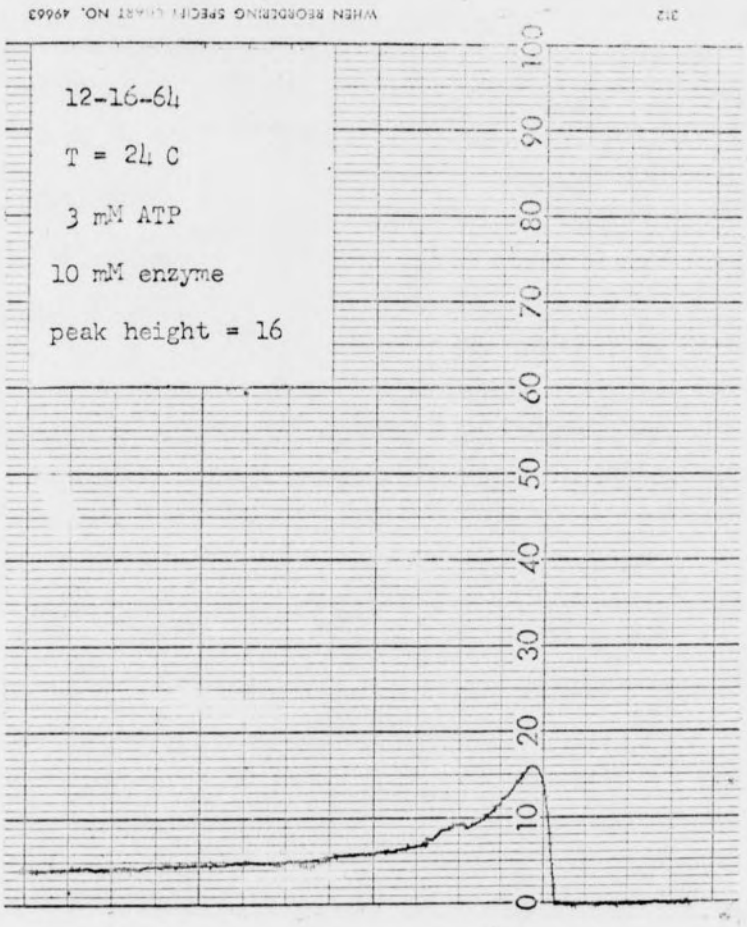
# APPENDIX B



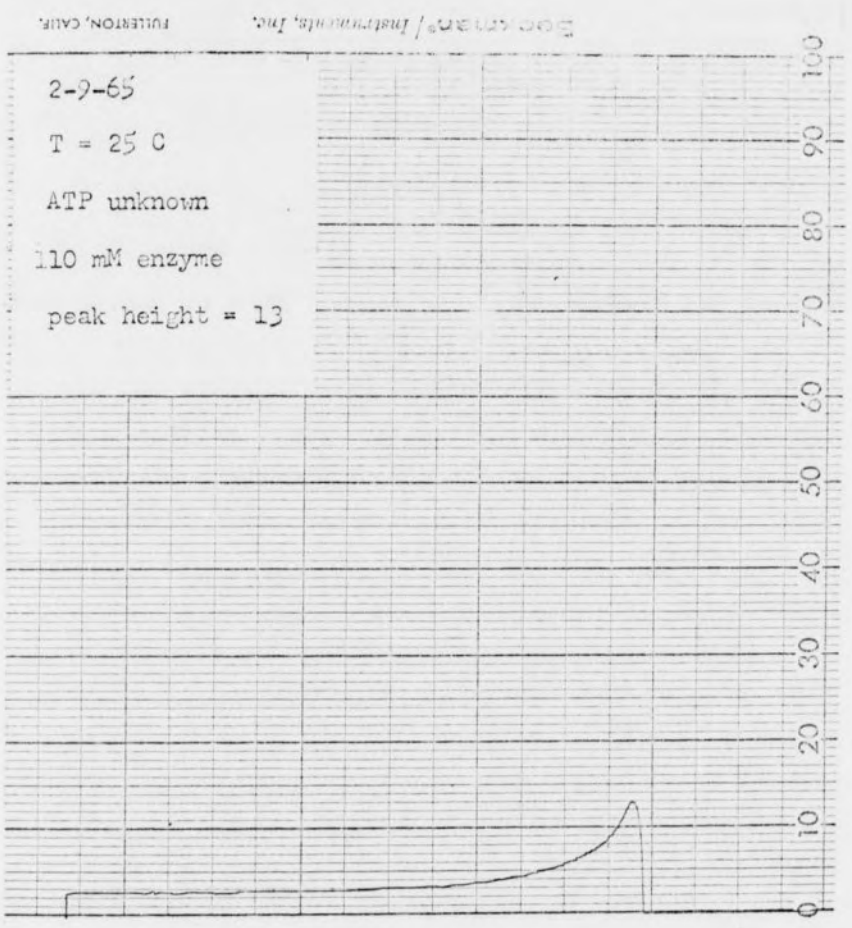

Graph 1. Peak height indicating light intensity of a solution with ATP concentration of 1 mg/ml of water.



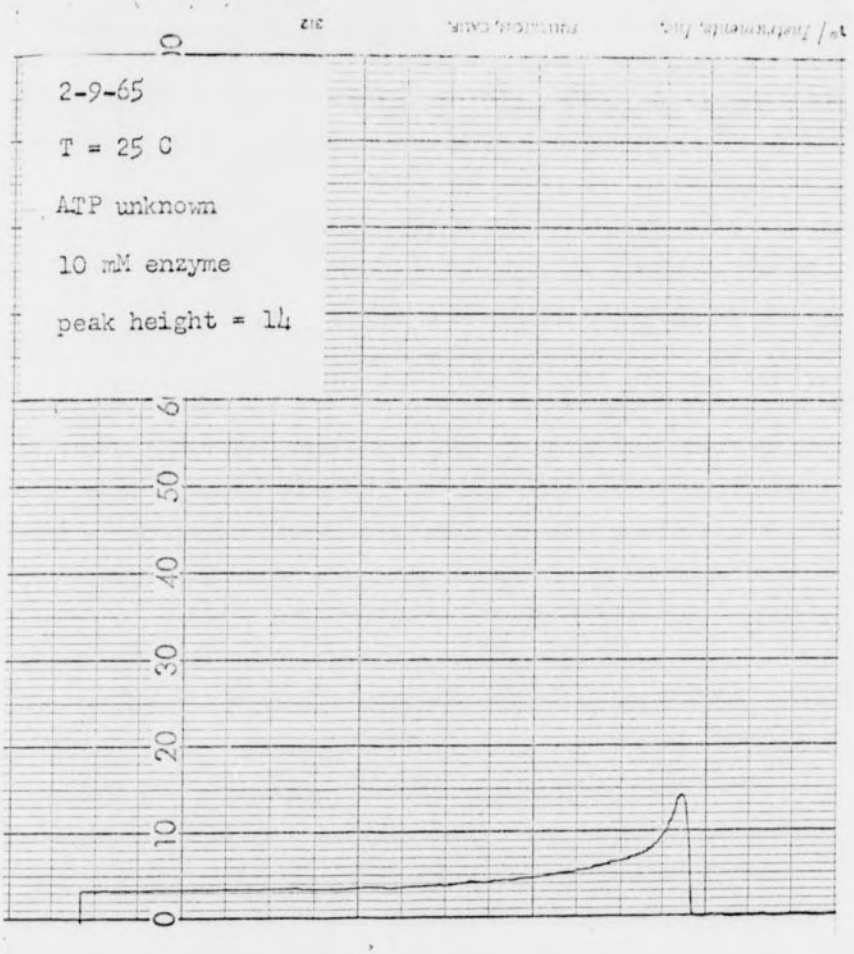

Graph 2. Peak height indicating light intensity of a solution with ATP concentration of 2 mg/ml of water.



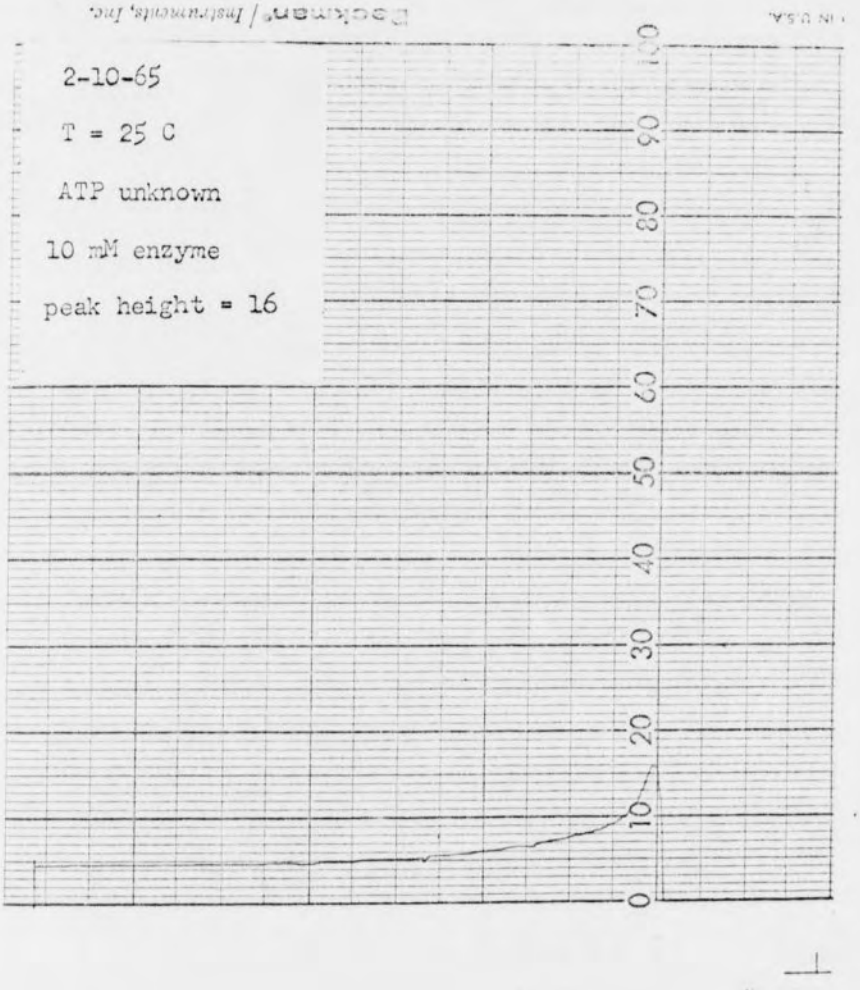

Graph 3. Peak height indicating light intensity of a solution with ATP concentration of 3 mg/ml of water.



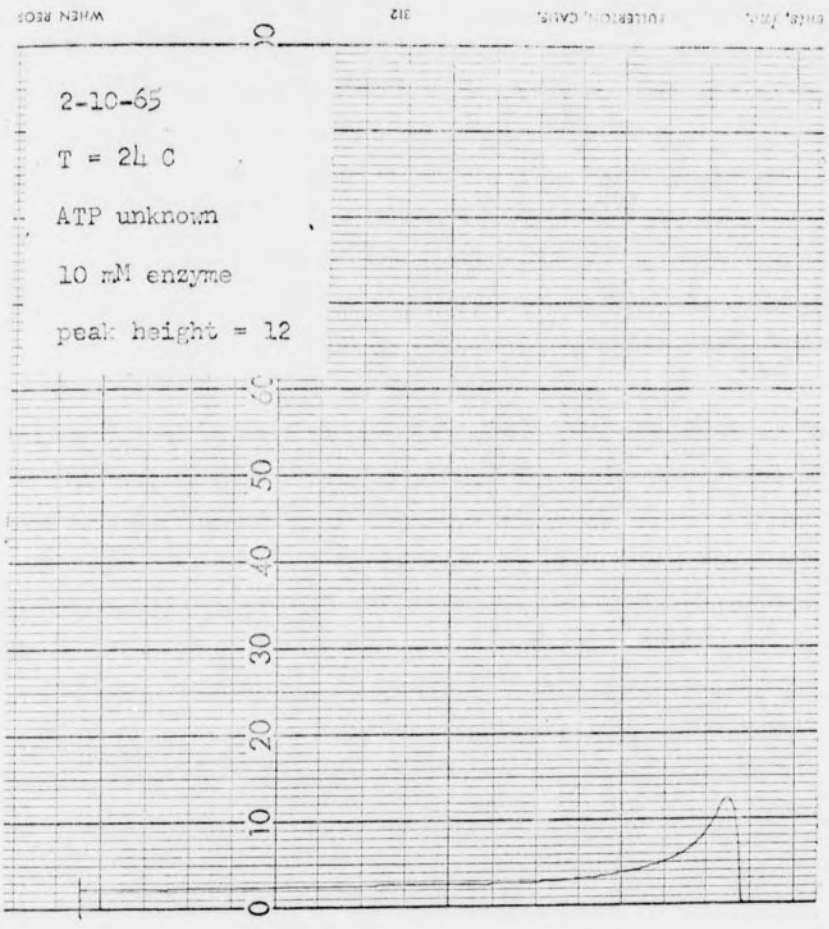

Graph 4. Peak height indicating light intensity which represents the ATP concentration in experimnetal rat 2.



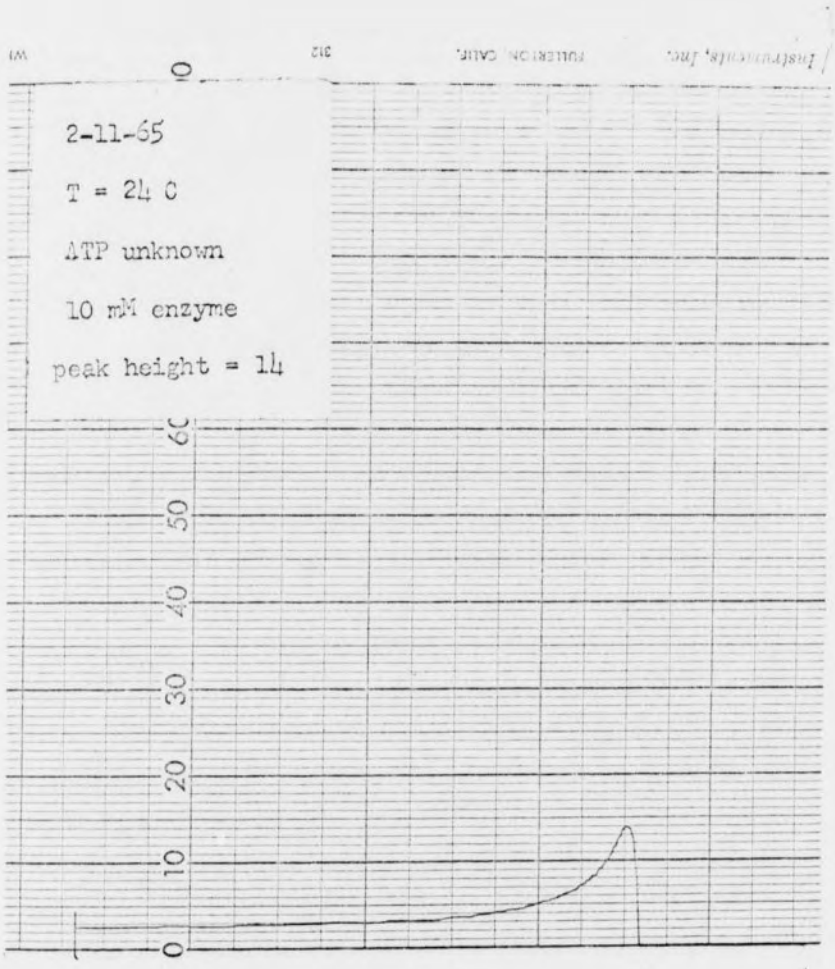

Graph 5. Peak height indicating light intensity which represents the ATP concentration in control rat 2.



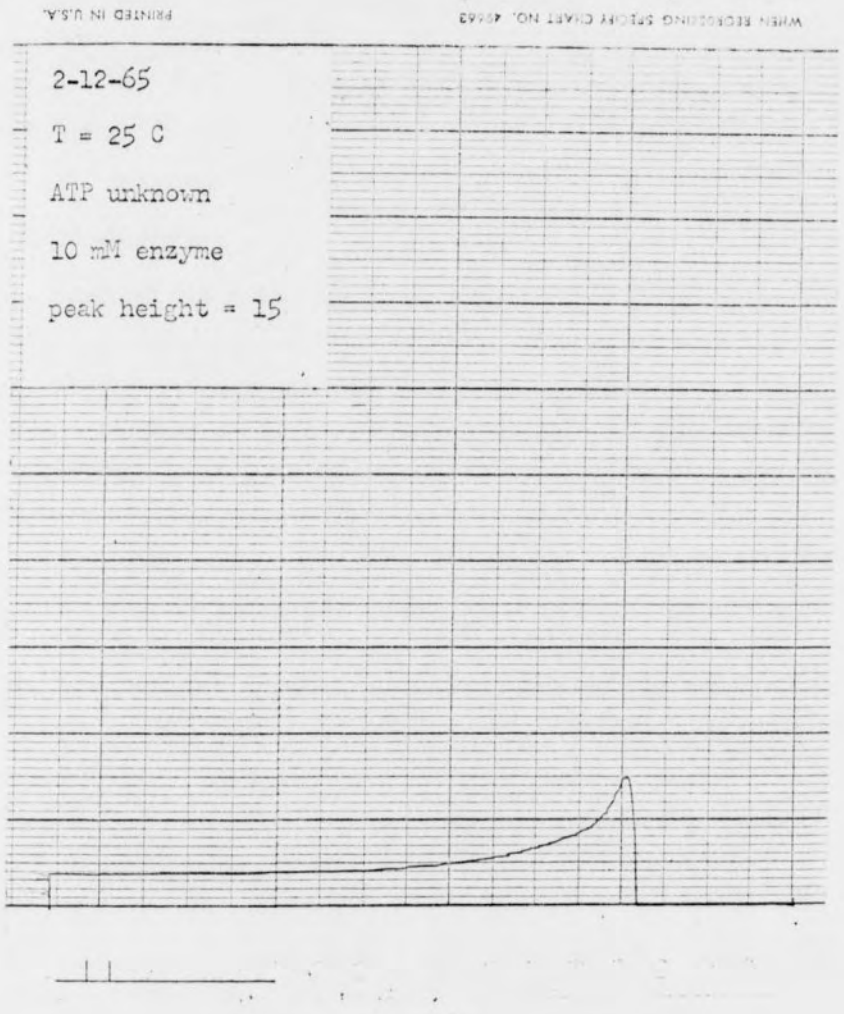

Graph 6. Peak height indicating light intensity which represents the ATP concentration in experimental rat 3.



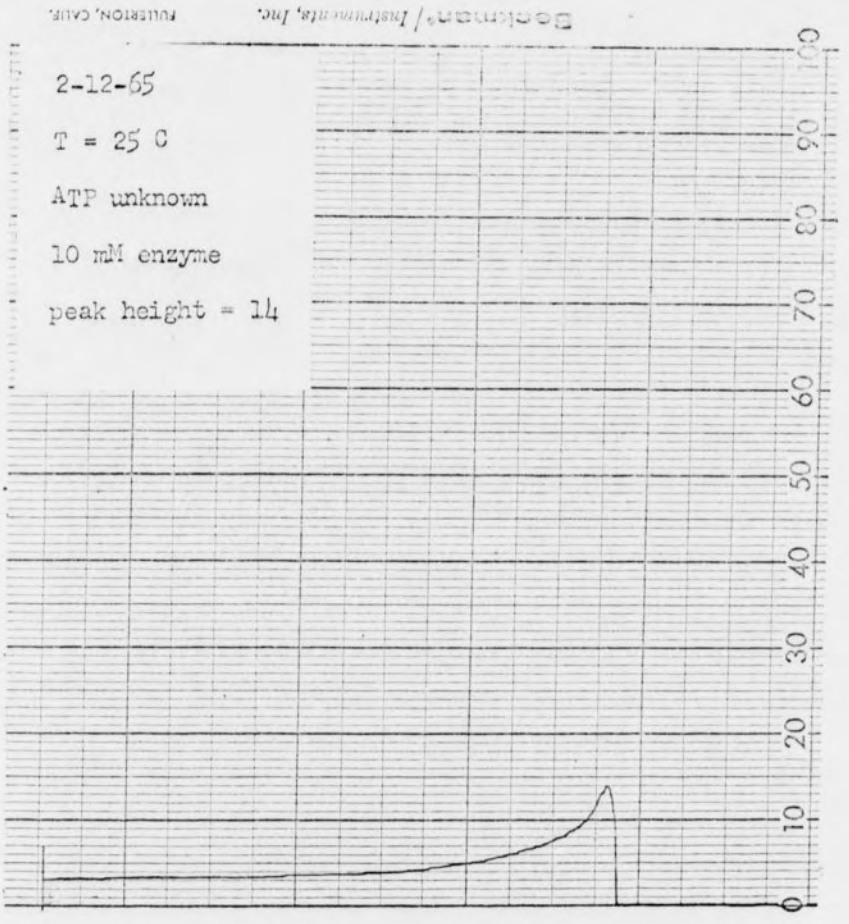

Graph 7. Peak height indicating light intensity which represents the ATP concentration in control rat 3.



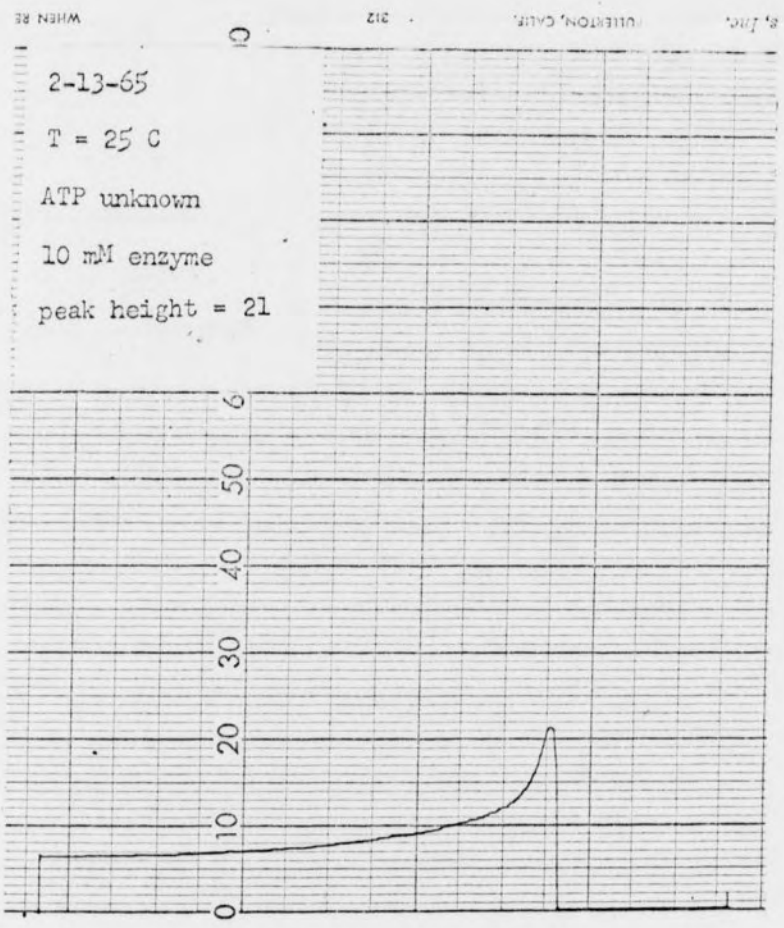

Graph 8. Peak height indicating light intensity which represents the ATP concentration in experimental rat 4.



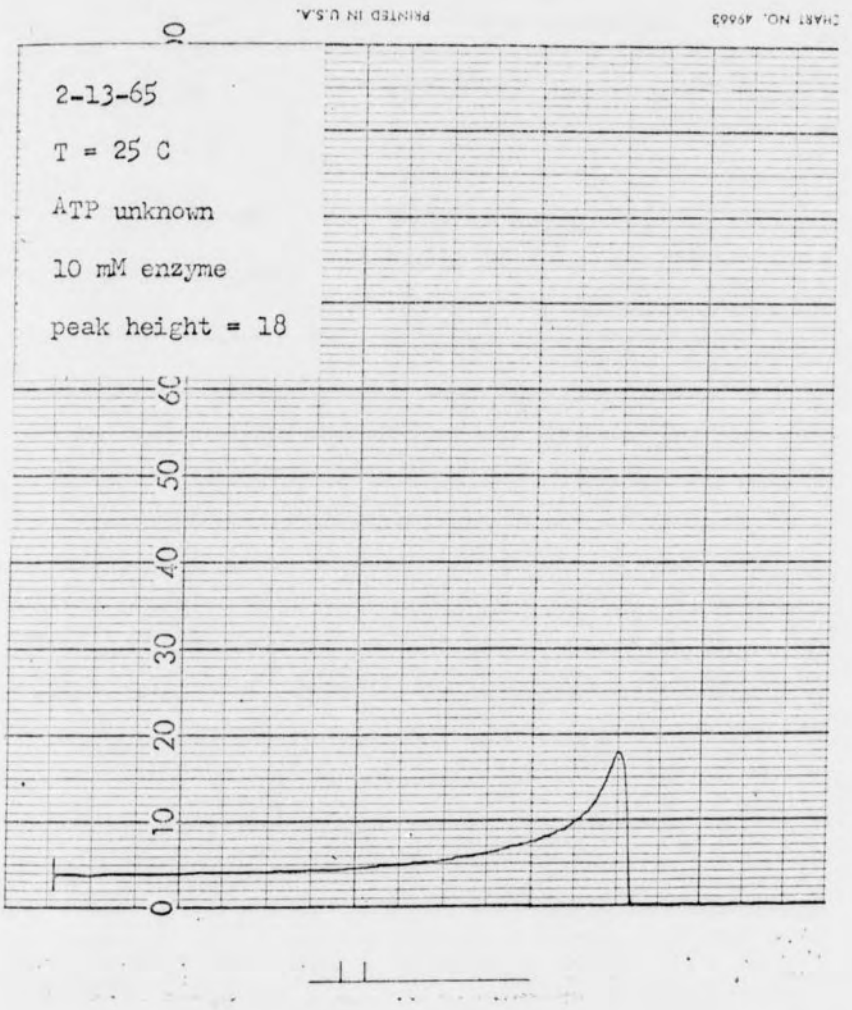

Graph 10. Peak height indicating light intensity which represents the ATP concentration in experimental rat 5.



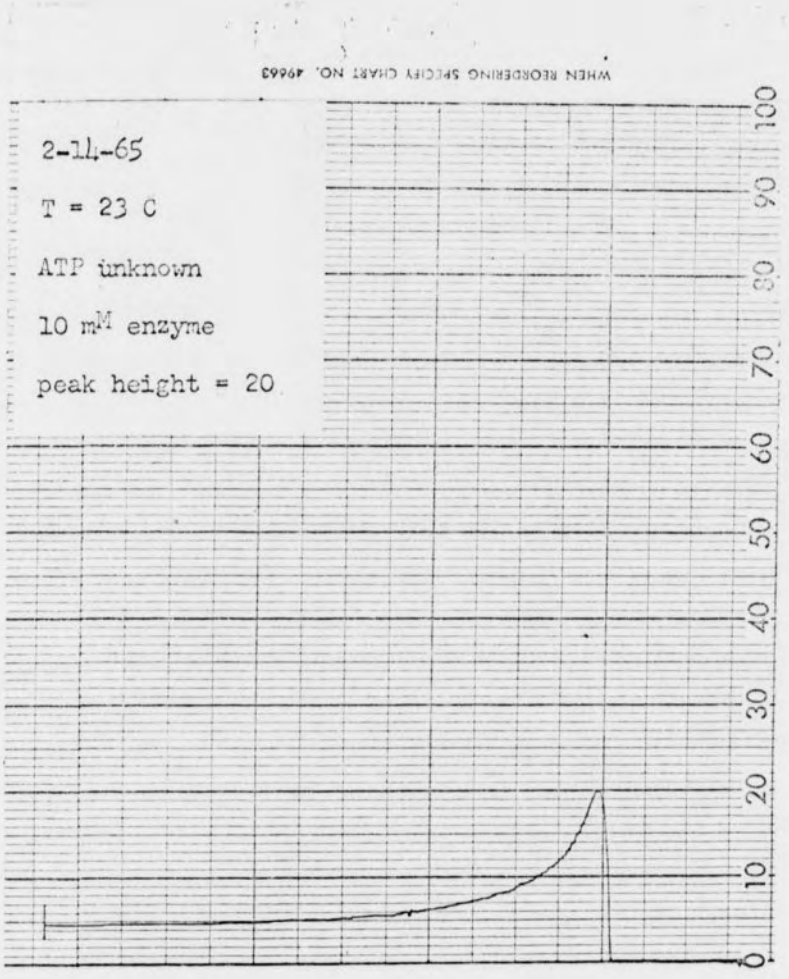

Graph 11. Peak height indicating light intensity which represents the ATP concentration in control rat 5.



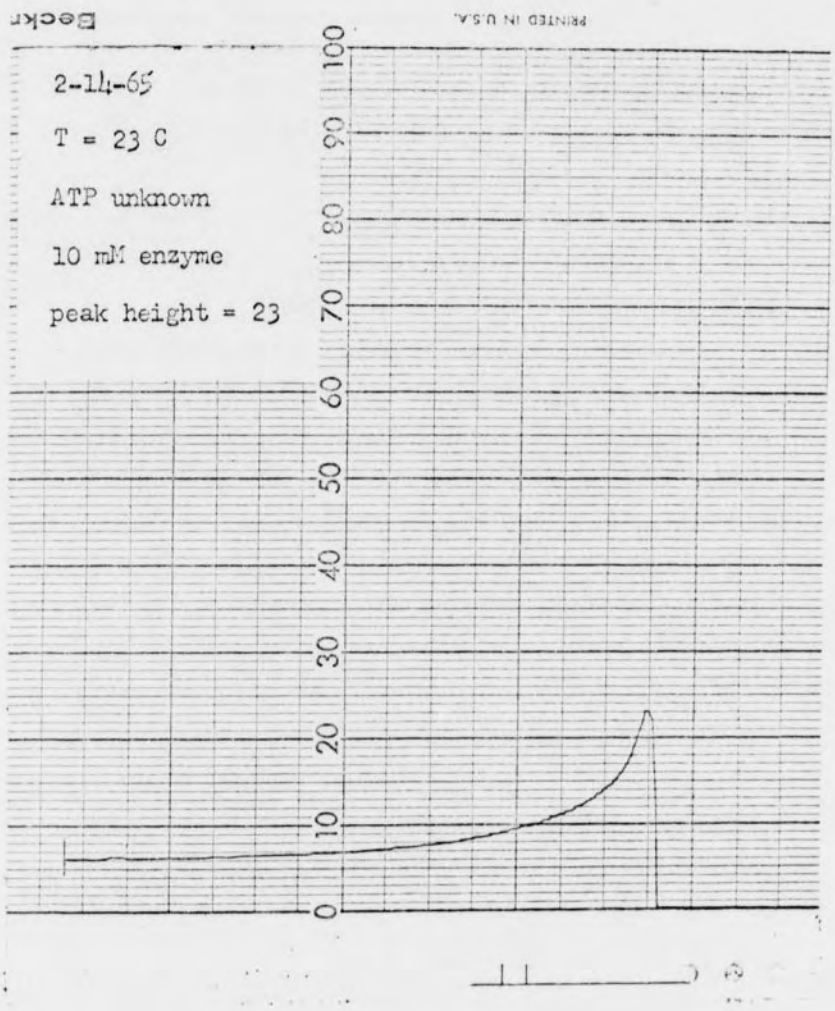

Graph 12. Peak height indicating light intensity which represents the ATP concentration in experimental rat 6.



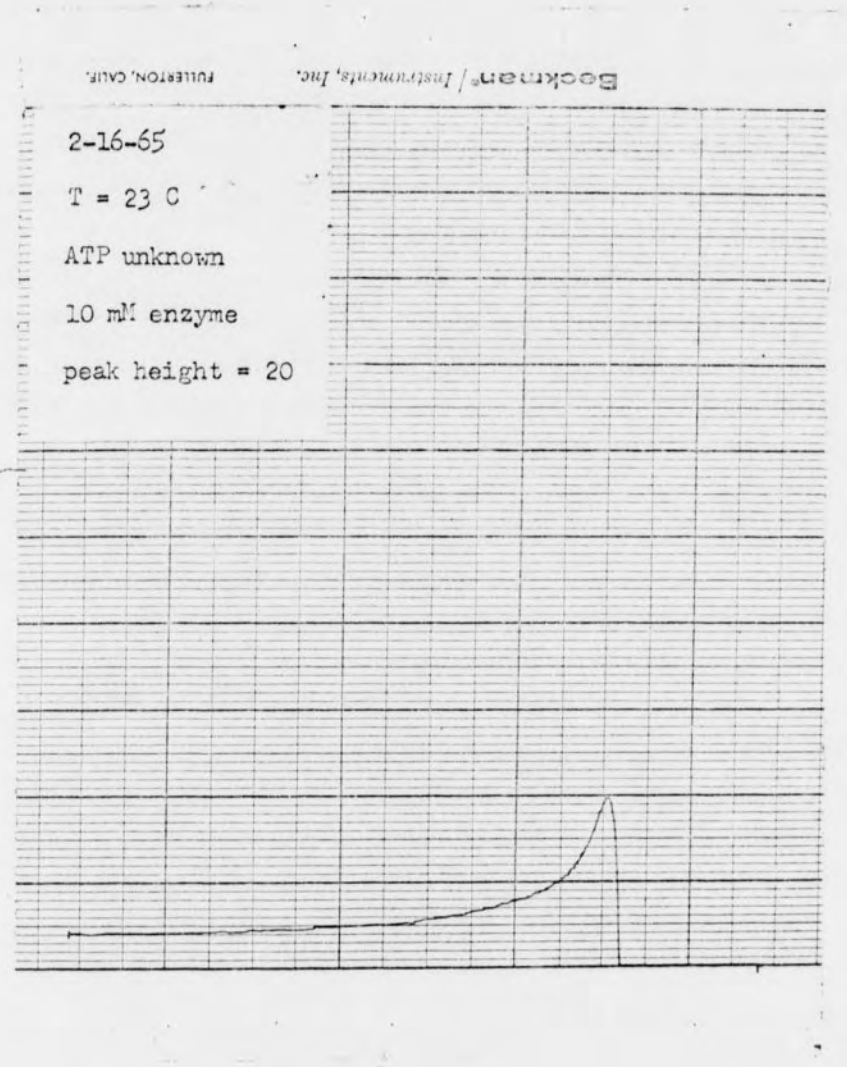

Graph 13. Peak height indicating light intensity which represents the ATP concentration in control rat 6.



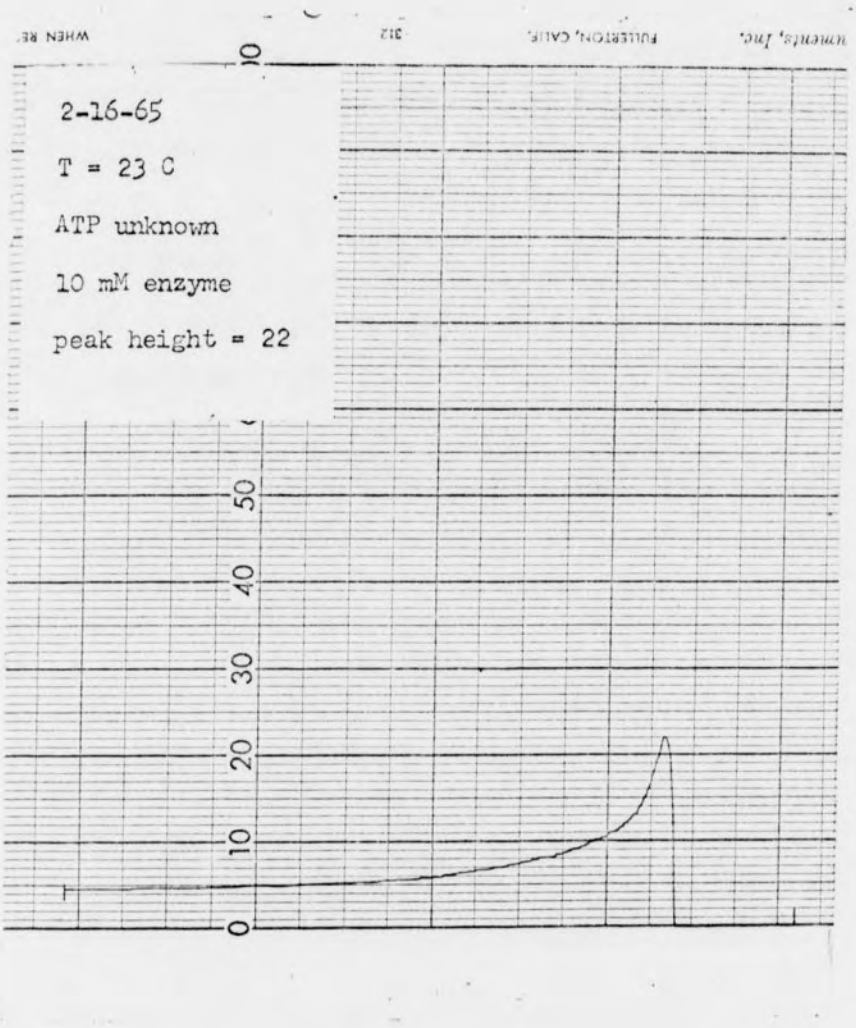

Graph 14. Peak height indicating light intensity which represents the ATP concentration in experimental rat 7.



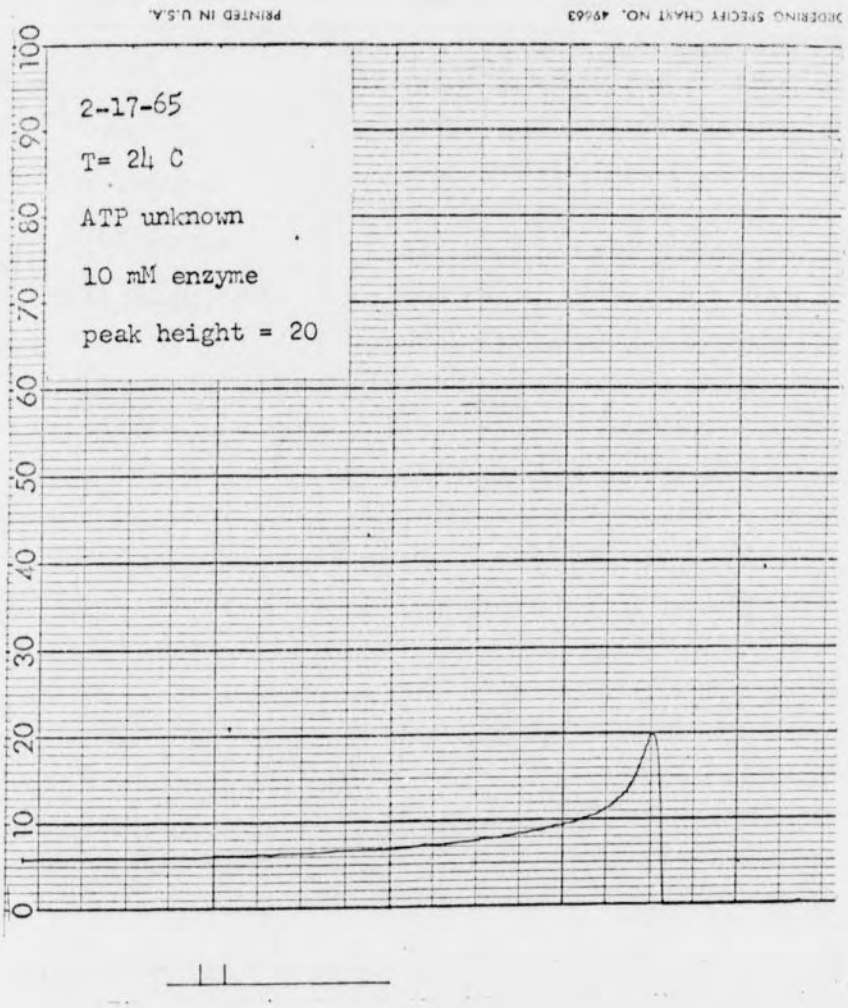

Graph 15. Peak height indicating light intensity which represents the ATP concentration in control rat 7.



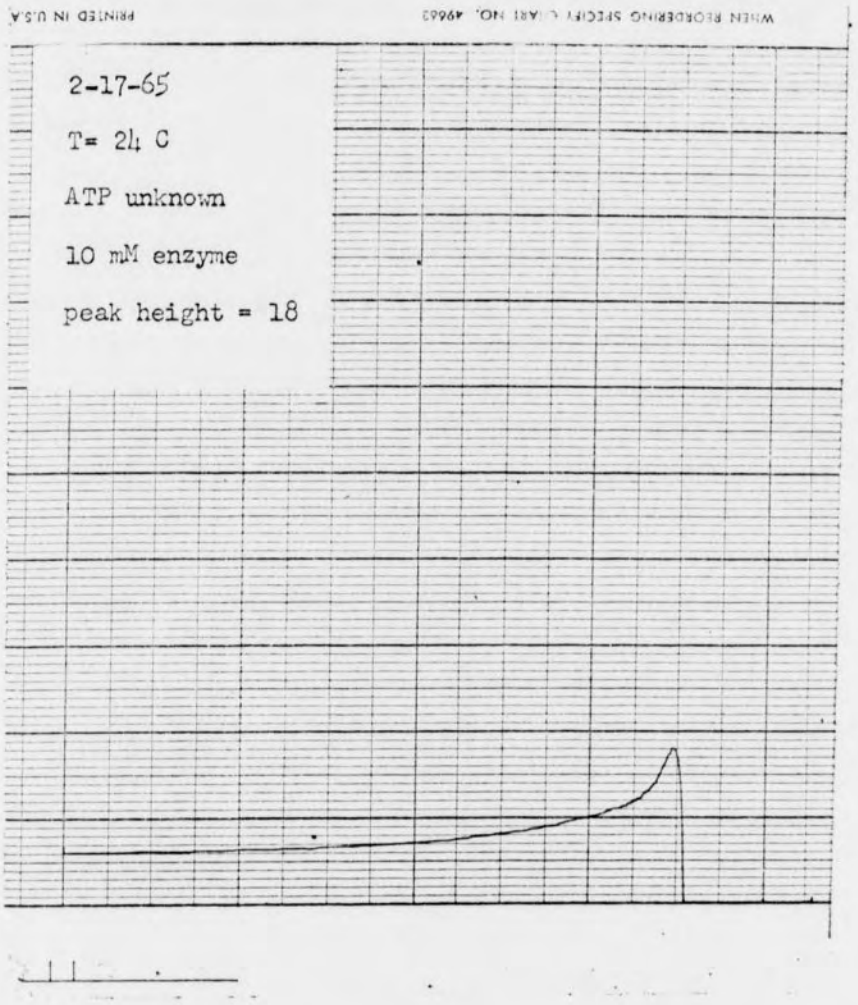

Graph 16. Peak height indicating light intensity which represents the ATP concentration in experimental rat 8.



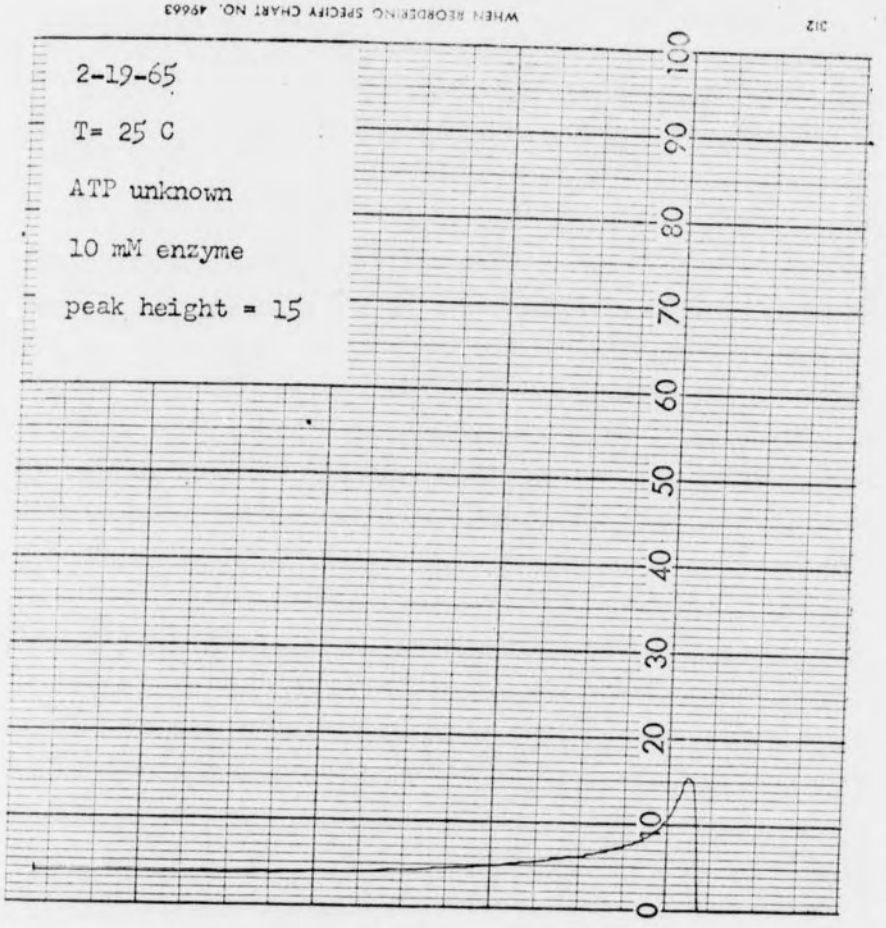

Graph 17. Peak height indicating light intensity which represents the ATP concentration in control rat 8.



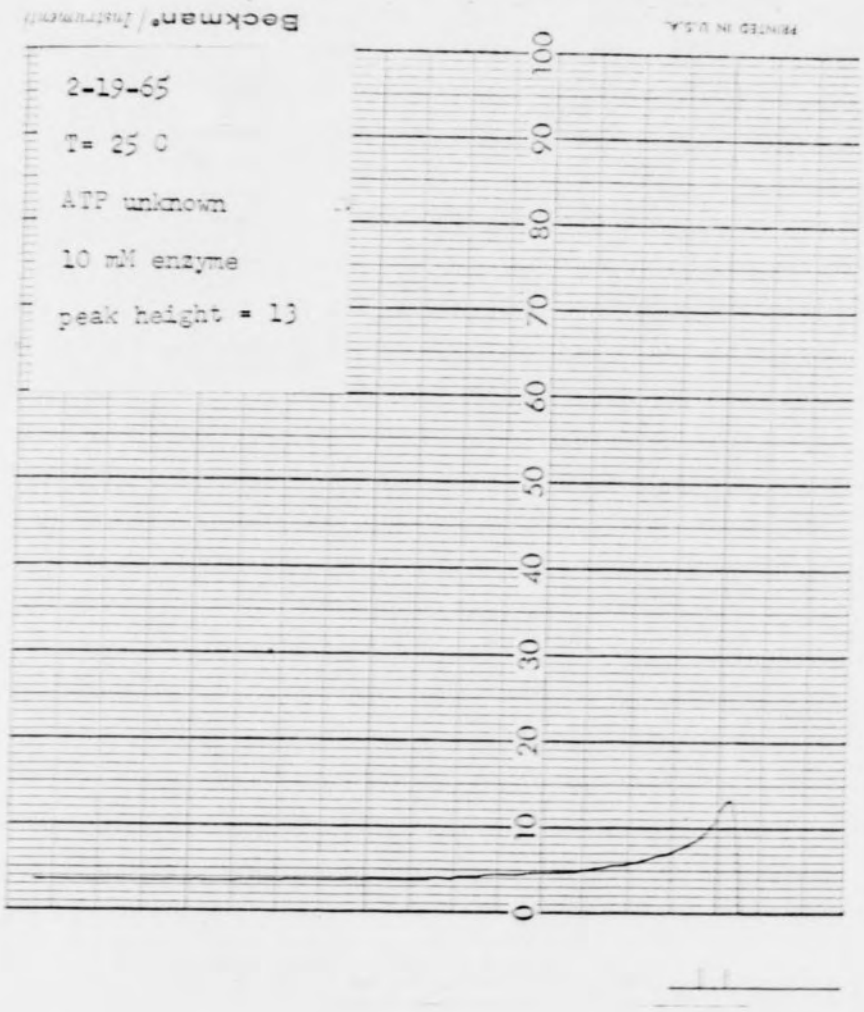

Graph 18. Peak height indicating light intensity which represents the ATP concentration in experimental rat 9.




Graph 19. Peak height indicating light intensity which represents the ATP concentration in control rat 9.




Graph 20. Peak height indicating light intensity which represents the ATP concentration in experimental rat 10.




Graph 21. Peak height indicating light intensity which represents the ATP concentration in control rat 10.