THE SOLUBILITY OF MYOSIN IN MAMALIAN MUSCLE AND ITS POSTMORTAL CHANGES

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ABSTRACT

Myosin is the structural protein of the muscle fibrils. It is localized in the A-bands of the striated muscle. It is the contractile element of the fibrils (Engelmann. 8)

Myosin could be extracted from fresh minced muscle by 0.5 Molar KCl solution. Its solubility in the salt solution is decreased if the muscle is stored (Saxl 23, Deuticke 24, Kamp 25).

The extraction of myosin at different time intervals from a suspension of minced muscle in distilled water becomes negligible in about 3 hours. It is somewhat restored on addition of ATP to the muscle pulp.

The yield of the extracted myosin is greater at alkaline reaction.

It is also greater when extraction is affected by 0.5 Mol. NH_4Cl or LiCl solutions e.i. salts possessing disaggregating effect on actomyosin (Heller).

In both cases whether extracted at pH 8 or with NH_4Cl and LiCl solutions the yield of the extracted myosin is greatly diminished in absence of ATP; although it does not become zero as it is the case with extraction done with 0.5 Mol. KCl solution.

Adding ATP to a suspension of minced muscle in distilled water which was kept standing for 4 hours, increases the amount of myosin extracted but does not reach the yield of the myosin extracted from fresh minced muscle.

If the minced muscle is washed first with distilled water, before it is suspended in distilled water, no myosin is extracted by 0.5 Mol. KCl, a minimal amout could be extracted by 0.5 Mol. NH_4Cl. Since washing removes the ATP, it is plansible to suppose that extraction of myosin is in direct relation to the ATP concentration in the muscle pulp.

It was noticed that the yield of the myosin extracted when fresh

minced muscle is suspended in juice of an old muscle suspension, would not give the same yield as when fresh minced muscle is suspended in distilled water although the minced muscle has the same ATP concentration in both cases.

It is believed that in a suspension of fresh minced muscle in distilled water, a sabstance is formed which affects the ATP concentration.

From comparative determination of the breakdown of ATP:

1) in a suspension of minced muscle in distilled water.

2) in a suspension of minced muscle in an old juice suspension.

It is found that that substance accelerates the ATP breakdown. Its nature is thought to be a phosphate acceptor.

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I. Introduction.

The present study was started with the intention to obtain a closer insight into the factors affecting the extraction of myosin, the structure protein of the fibrils, from muscle.

In the course of the experimental work, it soon became obvious that it was necessary to restrict this wide program considerably. This was partly due to difficulties met with during the development of the experimental methods, partly to the results of the experiments showing a more complicated situation than was anticipated.

For these reasons, this investigation does not deal with all problems involved in the extraction of myosin, but is mainly restricted to a first analysis of the post mortal changes going on in muscle after mincing. The choice of this special aspect of the problem is justified on the one hand, on account of its practical significance for the understanding of all preparative and analytical work concerning myosin, on the other hand, because there is all reason to expect close similarity between the process studied here, and certain reactions related to such phenomena as fatigue and rigor mortis, the physiological significance of which does not need to be explained.

The object of this investigation is to study, from the solubility of myosin at a certain period after the animal is killed; and the change of that solubility due to the action of certain specific reagent[5] the state of aggregation of the myosin molecules and the change they undergo during rigor.

Before proceeding to the actual discussion of the experimental work, a short historical introduction will be given.

Kühne in 1864 opened the field for the systematic research on muscle proteins. He was attempting to relate the coagulation of muscle plasma with a change during rigor. The muscle plasma was obtained by

extracting the muscle pulp with a salt solution or, by squizing the muscle pulp in a press. If this extract is left for some time, a coagulum is produced which Kühne gave the name of myosin, which name was also accepted later by Danilewsky(1) and Von Fürth (2). Myosin-free plasma was called muscle serum by analogy to blood plasma and blood serum.

Halliburton (3) in 1887 took up the work of Kühne and tried to find out a precursor to myosin. He distinguished in the muscle plasma five proteins which he characterized by their coagulation temperatures, and the difference in their solubilities. These proteins were called by Halliburton:

Paramyosinogen	Coagulable at 47°C. precipitated by $\frac{1}{2}$ sat.$(NH_4)_2SO_4$ sol.
Myosinogen B	Coagulable at 56°C. precipitated by $\frac{1}{2}$ sat.$(NH_4)_2SO_4$ sol.
Myoglobulin	Coagulable at 63°C. precipitated by sat.$(NH_4)_2SO_4$ sol.
Albumin	Coagulable at 73°C.
Myoalbumose	does not coagulate at 73°C.

The muscle serum contains only the last three substances. Halliburton's deduction was that the first two proteins interact to give the plasma coagulum or the myosin of Kühne, thus concluding to a certain analogy with blood clotting.

Von Fürth (2) would not agree with Halliburton (3) in regard to the properties of paramyosinogen, nor with respect to the number of the protein constituents of muscle. He also modified the nomenclature used by Halliburton (3).

Von Fürth showed that Halliburton's myosin, the coagulation product of paramyosinogen and myosinogen was not composed of two proteins but only one, the paramyosinogen which is the same as Kühne's mysoin.

Howe in 1924 (4) was the first to study the solubility of the

muscle proteins in well buffered solutions of known concentrations and at definite pH values. He found that a mixture of KH_2PO_4 and K_2HPO_4 in the ratio of 1:2 which gives a pH of 7.0, when the salt concentration is between 0.225-0.325 Molar, is the most satisfactory extractant of myosin, the major part of the muscle proteins.

A better extractant was found later by Weber and Meyer 1933 (5) Greenstein and Edsall 1940 (6) independently.

It must be remarked here that, although nowadays partly the same names for the different proteins are used than in these earlier investigations, the meaning of these names in their modern sense does not always coincide with their original significance.

Edsall and Muralt (7) in 1930 have shown that solutions of muscle "globulins" now generally called myosin, do, under certain conditions, possess anisotropic properties. This suggested that the A-bands, which according to Engelmann (8) constitue the contractile parts of the muscle fibres, might be mainly myosin.

This optical anisotropy of certain fragments of the muscle fibrils attracted the attention of several early workers on muscle function and structure, and formed the starting point of much discussion of the submicroscopic structure of the fibril. One of the earlier recognised explanations, now generally adopted, is the insight that the property is an expression of a regular micellar structure. This structure is now understood to a certain extent mainly by through the researches of Weber (9). The main point that in the anisotropic A-bands, we have to do with a regular parallel arrangement of myosin molecules. It is this band which is the site of contractibility.

The understanding of the mechanism of the muscular contraction would then involve a better understanding of myosin.

In that same period between 1924-1939 while the physico-chemical properties and the composition of myosin were being under investigation

by investigators like Edsall, Muralt, Smith and Weber; other workers in the field like Meyerhof were elucidating the chemical reactions involved in the carbohydrate breakdown in muscle, and the energy supplying reactions for the muscle contraction.

Up till 1934 it was generally accepted that muscle contraction is accompanied by appearance of equivalent amounts of inorganic phosphate and free creatine, creatinephosphate disappearing, namely, this process is an enzymic hydrolysis of phosphocreatine with the liberation of the energy required for the muscle contraction. Lohmann (10) in 1934 using muscle extract showed that this process is possible only in presence of adenylic acid (AMP) and adenosinetriphosphatase (ATP-ase), and the energy liberated from ph-creatine breakdown is used only to build up the ATP from AMP:

2 ph-creatine + AMP ------- 2 creatine + ATP

No phosphocreatine breakdown enzymatically is possible in the absence of AMP.

Lohmann showed also that the ATP breakdown is the energy liberating reaction responsible for the muscular contraction. Later in 1939 Engelhardt and Ljubimova (11) discovered that the enzyme responsible for the breakdown of ATP was extracted by the methods used for the preparation of myosin and is always associated with it. They suggested that myosin itself is the ATP-ase, thus catalyzing the splitting of the energy rich phosphate bond.

In the University of Szeged, Szent-Györgyi and his collaborators undertook to find out a solution to many pending problems on myosin.

Myosin was characterized by Edsall and Muralt (12) as the protein fraction of muscle which is extracted by a 0.5 Mol. KCl solution at pH 8.5 - 9 and precipitates at pH 7.0 when the solution is diluted to

0.1 Molar KCl. Szent-Györgyi (13) showed that the myosin of Edsall is really a complex protein of myosin and another protein, actin, (Straub 14). He gave it the name of actomyosin. It approches myosin as a limit when the amount of actin becomes negligible.

According to Szent-Györgyi many acto-myosinscan be prepared, depending on the proportion in which actin and myosin are combined.

He noted the preparation of two actomyosins (13) which may be called actomyosin A and B respectively,.

Actomyosin A is obtained when the muscle particles are extracted only for 20 minutes at 0°C.

Actomyosin B is obtained when the muscle particles remain in contact with the salt solution for 24 hours at 0°C. or 6 hours at room temperature (18-20°C.).

These actomyosins are different with respect to the viscosity, double refraction of flow and, turbidity of their solutions and specially, the viscosity response (15) upon addition of ATP to their solutions.

Szent-Györgyi showed (16) that an actin free myosin could be obtained in crystalline form. Further that actomyosin A is mainly myosin with 1-3% actin while actomyosin B is myosin with about 16% actin.

II. Development of analytical methods.

Before the investigation could be started, it was necessary to work out reproducible procedures for the extraction of myosin from muscle, and for the determination of myosin in the obtained extracts.

The ultimate step in such a procedure would necessarily be the determination of the amount of protein obtained from the extract under such condition that myosin could be considered to be the only protein present. This involves two problems: the separation of the myosin

from other proteins in the extract and the analytical determination of the protein present from its nitrogen fraction.

Since it was expected that rather extensive series of samples, resulting from time taking experiments would have to be analysed, it was firest tried to arrive at a satisfactory method for the determination of protein, not involving chemical digestion.

Colorimetric methods were thought to be the best suited methods, specially if they would not require any tedious chemical treatment before the colorimetric readings.

I tried to adopt the method described by H.W. Robinson and C.G. Hogden (17) "The Biuret reaction in the dtermination of serum proteins". Of course some modifications are necessary so that, it would be used for myosin instead of blodd serum proteins. The difficulty in finding a suitable standard was avoided by the use of the photoelectric colorimeter.

Unfortunately this method has to be abandoned since above certain concentrations the biuret color became turbid, such turbidity hindered the photo-colorimetric readings.

The use of the Folin Phenol reaction was next thought of (18). This method as described in Sahyun (19) make use of a tyrosine standard. Again the use of the photoelectric colorimeter would have made the problem easier except that another difficulty was encountered. To make use of the Photo-colrimeter, it was neeessary to plot a curve of coñentration against % light transmitted (on a semi logarithmic paper). For that purpose crystalline myosin (20) solutions of known concentrations, as determined by the micro-kjeldahl method, were used. It was found later that actomyosin or, any myosin solution with a small actin content could not be determined accuratly, since the color is produced from the reaction of the reagent with the tyrosine groups of

the myosin molecules which was previously denatured by means of a hot NaOH solution, but the tyrosine content of myosin is different from that of actin. It became obvious that except for pure myosin (crystalline myosin) the readings will not be correct.

I should remark that from this difference a method could be found where it is possible to determine the % of actin in any actomyosin solution. The description of this method is out of the scope of this investigation but will be dealt with in a future investigation whenever the opportunities are favorable.

When all these attemps failed to give the desired procedure I had to recur to the digestion method, followed by Nesslerization.

The second problem to be solved was the quantitative separation of the myosin from the other proteins by a single operation. The extracts were always made by strong salt solution, usually 0.5 M. KCl. Such extracts contain, besides myosin, all the other extractable proteins. The method divised was to precipitate the myosin quantitatively and exclusively by diluting the extract with 4 volumes distilled water. The separation of myosin under these circunstances depends however strongly on the pH, and it was necessary to establish the right conditions in order to secure complete separation. It was determined therefore in which pH-range precipitation (isoelectric precipitation) was complete.

The purpose of this determination then, is not to find the exact I.P. of myosin and of actomyosin, which lies outside the scope of our work, but to determine the highest pH at which maximum precipitation of a myosin solution is obtained, when it is diluted from 0.5 Mol. KCl solution to a final salt concentration of 0.1 Mol. The highest possible pH has to be chosen, in order to avoid possible complication due to acidity.

Experimental

2 ml. proteins of a myosin solution containing 0.49 mgm. N per ml. as determined by the micro-kjeldahl method of Koch and Mc Meekin (21), were precipitated by dilution, each at a different pH. The precipitate of each sample was washed by $\frac{1}{20}$ Mol phosphate buffer, of the same pH as at which, precipitation had been carried out, dissolved in 0.5 Mol. KCl solution, and the N-myosin estimated after digestion, by Nesslerization.

Adopting Smith's value (22) of 16.25% for the N-contnet of myosin, table (1) shows the amount of myosin precipitated from 2ml. of the myosin solution containing 0.98 mgm N or 5.98 mgm myosin, at the different pH values.

If we plot the myosin precipitated against the pH, curve (a) of graph (1) is obtained. From the table and the curve we find the highest pH at which complete precipitation of crystalline myosin takes place the value 5.8.

A similar determination was made on 2 ml. of actomyosin B containing 1.49 mgm. N or 8.94 mgm. actomyosin, results expressed in table II and curve (b) of graph I

It is seen from the comparison of the two graphs that for the precipitation of actomyosin a some what more alkaline reaction is permissible . In order to be sure that in experimental cases both actomyosins and also pure myosin will be completely precipitated, pH values around 5.6 were choosen to separate myosin previous to its determination.

III. The time - course of the extractibility of myosin.

The decrease in the amount of protein extractef from fatigued muscle or rigor was reported as early as 1907 by Saxl (23), H.J. Deuticke (24) and Kamp (25) confirming Saxl found that this decrease in the 24 hours stored at 0

stored at 0°C. muscle was in the myosin fraction. While Smith in 1934 (26) and 1937 (27) observed no change in the amount of extractable proteins before and after storing at 0°C. for 24 hours. Szent-Györgyi (16) confirmed Deuticke and Kamp.

The purpose of the performed experiment is to ascertain these observations and to show how the time during which the minced muscle is suspended in distilled water affects the quantity of the myosin extracted.

Experimental

A rabbit was decapitated, skinned and the muscle of the hind legs and back cut off (as soon as possible) and put in ice. They were minced in a cooled meat mincer and suspended in distilled water (2 ml. to each gram of muscle). After 5, 30, 45, 90 and 180 minutes (table 3, graph 3) samples were taken.

To these samples a salt solution (1 ml. per gram) which contained the salt in a three times higher concentration than Weber - Edsall solution is added. After stirring well it was kept standing for 15 minutes at room temperature. Then, the solution was freed from the residue by centrifugation, and the myosin concentration in the supernatent fluid was determined as follows:

An aliquot part is taken and diluted with 4 times its volume of distilled water bringing the salt concentration to 0.1 Mol. The precipitate washed with $\frac{1}{20}$ Mol. phosphate buffer of pH 6.0 and redissolved into a known volume of 0.5 Mol. KCl solution.

The N-content is determined, after digestion, by Nesslerization and the amount of myosin calculated.

Table 3 shows the quantities of myosin extracted at any given time

after mincing the muscle. They are expressed in grams myosin per 100 gr. fresh muscle. Graph (3) is obtained by plotting myosin extracted against time.

There is a decrease of myosin extracted with time. That decrease is not a linear function of time, but its rate increases. The cause of that decrease in the amount of myosin extracted will be discussed in a later section.

Some consideration should be given to the absolute quantities of myosin extracted. The total quantity of myosin in 100 gr. rabbit muscle is of the order of magnitude of 10 gr. As will be seen, the amounts extracted in these experiments are much smaller. This is due to several causes, the main being that only one single extraction with a small volume of solvent was carried out. This was done intentionally, because in case of large quantities of extractants those substances of the muscle determine or influence solubility might become too much diluted. Whereas in case of repeated extractions the conditions during the second and later extraction may be entirely different from those during the first one, through removal of ATP or other factors, or due to changes going on throughout the time required for the manipulations.

In order to reduce as far as possible the inaccuracies which may result from the very incomplete extraction, all the procedures were rigorously standardized. Reproducible results could thus be obtained.

To avoid criticism against this procedure from workers not acquainted with this special problem, it should be stressed that the above source of uncertainty is not as serious as it looks. If it were the purpose to estimate the amount of myosin in muscle, a procedure with only some 20% yield would of course be useless. The object however was to determine how much myosin is set free under certain conditions. This amount does not need to approach a complete yield in a single extraction.

IV. The extraction in its dependance on pH.

In the previous experiment I observed a decrease in the pH of the suspended muscle e.g. shifting towards the acid side, from 6.3 as initial pH it dropped to 5.8.

The pH has of course a great influence on the solubility of myosin. K. Bailey (31) reported, that the solubility of native myosin in presence or absence of salt is a function of the pH. It was decided therefore to control the pH during the extraction, and to test the extractability at different degrees of acidity or alkalinity.

To study the effect of the pH on the solubility of myosin, three buffer phosphate solutions were made, namely:

a) 1 Molar KH_2PO_4, which gave, in combination with the buffering substances of the muscle, a pH around 5.0.

b) 1 Molar K_2HPO_4 which gave in combination with that same buffering substances of the muscle a pH 8.0

c) A mixture of a) and b) in the ratio of 3:1 gave a pH of 6.0-6.2.

Experimental

100 grams fresh minced muscle were suspended in 2 volumes distilled water (2 ml. to each gram muscle). 30 ml. of the suspension (equivalent to 10 grams muscle), were mixed with 10 ml. of each one of the above mentioned buffers respectively, and, with 10 ml. of 1.5 Mol. KCl solution. Thus the ionic strength became equivalent to that of a 0.5 Mol. KCl solution.

The mixtures were extracted for 15 minutes (stirring now and then) at room temperature. The myosin extracted was precipitated by dilution with 4 volumes distilled water, redissolved in 0.5 Mol. KCl. The N-content of solution was determined (by the above described method) and the myosin extracted in each case was calculated in terms of grams per

100 grams muscle.

Note: It was preferable to extract at 0°C. but the cold room convinianees were not yet ready.

In the pH 5.0 extract, I observed a coagulum which appeared few minutes after centrifugation. I recentrifuged to remove this coagulum. It is most probably due to a fraction of the myogen which converts at acid reaction spontaneously into an insoluble coagulum (Bailey, K. 28).

The amount of myosin present if any is negligible. The coagulum can certainly not be myosin, because myosin in 0.5 Mol. KCl is stable even at pH 4.5. Increasing acidity redissolves the myosin if any precipitates. Precipitation of myosin if denatured by acids occurs only after neutralization.

At pH 8 greater amount of myosin is obtained which precipitated completely at pH 7.0. When the final concentration of the salt solution became 0.1 Molar, while the myosin in the solution of pH 6.5 complete precipitation was not observed untill the pH was reduced to 5.9-6.0. This suggests that from pH 8 solution the protein obtained is actomyosin e.g. myosin with greater amount of actin, while from pH 6.5 solution an almost actin free myosin is obtained. How much the actin contents of the myosin obtained by alkaline extraction actually was, has not been determined seperately. The product does not need to be actomyosin B of course.

Table IV shows the results of this performed experiment, the myosin concentration expressed in grams per 100 grams fresh muscle. These results are plotted against time in graph (2), curve (1) for myosin extracted at pH 8, curve (2) for myosin extracted from pH 6.5 solution, whereas at pH 5 practically nothing was dissolved.

It is seen that in both cases the solubility of the myosin decreases with time. But whereas at extraction at pH 6.5 the amount extracted finally drops to zero, there is at pH 8-a not negligible yield even after prolonged standing of the minced muscle before extracting.

Salt solution of pH 6.5 will be then exclusively used for the comparative study on the extraction of myosin which would give a myosin resembling crystalline myosin. When the transformation time of acto-myosin A into actomyosin B is the main subject of the experiment only then an alkaline solution of 0.5 Mol. KCl will be used.

V. The effect of ATP upon the extractability.

Szent-Györgyi (13) showed that the solubility of myosin from fresh muscle in 0.5 Mol. KCl solution is influenced by the presence of ATP., with the disappearance of ATP the myosin extracted is negligible.

Section II of this paper corroborate Szent-Györgyi's observation, because the concentration of ATP in a suspension of minced muscle in distilled water at room temperature becomes negligible 3-5½ hours as shown page (27). Does the restitution of the ATP concentration increase the amount of myosin extracted? To how much?

Experimental

A suspension of minced muscle in 2 volumes distilled water was kept standing at room temperature for 4 hours, after which period no myosin could be extracted by a 0.5 Mol. KCl solution. A control sample was drawn 5 minutes after mixing the muscle with distilled water, extracted as usual with 0.5 Molar KCl solution at pH 6.5, and the myosin was determined and expressed as grams per 100 grams fresh muscle.

After standing 4 hours a sample was taken and ATP added in a concentration equal to its concentration in fresh muscle e.g. (50 mgm/qr. muscle, some allowences were made for its decomposition in the mean time. This sample was then extracted with the salt solution at pH 6.5 (as before) and the amount of myosin extracted was determined and expressed as usual in grams.per100 grams muscle.

Similarly a sample from a 6 hours old suspension was taken

and treated as before.

The results are shown in table VI ~~and Fig. 1~~. It will be seen that whereas the freshly extracted muscle yields the usual amount of myosin, after 4 hours standing almost nothing dissolves. Addition of ATP however restores against the solubility to a considerable extent even after 6 hours.

If we agree with Szent-Györgyi, (21) the extraction of myosin by 0.5 Mol. KCl solution is due to the simultaneous action of ATP and the salt, in other words ATP dissociates the aggregate state in which myosin exists in muscle, (Szent-Györgyi 27). The salt then dissolves the myosin or ATP-myosin. Restoration of the ATP to the same concentration as it is found in fresh muscle, should extract the same amount of myosin as from fresh muscle. Since it is some-what less than the original amount, we may assume:

1) A change in the state of aggregation of the myosin molecules, or some kind of denaturation is involved during standing (rigor) so that the ATP action is hindered.

2) Formation of a substance which favours the splitting of ATP, thus the rate of the disappearance of ATP is greatly increased, allowing no time to its action.

3) Both factors 1 and 2 working together. A decision between these possibilities will be attempted later.

VI. The solubility in different salts.

After it has been shown how the extractability of myosin depends on the pH of the solvent, the effect of the nature of the solvent salt should be discussed.

This is of interest on the one hand, because such studies would allow a correlation with some of the results of Mr. Heller on the disaggregation of actomyosin by salts. On the other hand, because there are certain differences between the investigations of Weber (29) and

Smith (30), on the amount of myosin in muscle, which differences seem to be due, in part at least, to different extractants in use by these authors.

In view of the results of these workers, it was decided to compare the effectiveness of LiCl, KCl and NH_4Cl. A few experiments with NaCl were done which did not show much difference between this salt and KCl.

Experimental

Fresh minced muscle was suspended in 2 volumes distilled water. At different time intervals samples were drawn and extracted respectively with LiCl, NH_4Cl and KCl solutions with final salt concentration of the extracting media equal 0.5 Molar. In each case, the myosin extracted is determined, as before, by digestion and nesslerization.

In these experiments the use of buffer phosphates or other solutions discribed in section (IV) is avoided, in order not to contaminate the medium with other ions. Instead the original pH of the suspension was controlled with glass electrode, and buffer carbonate was added occasionally to neutralize the lactic acid formed.

Table V shows the results of the experiment. Plotting the myosin extracted against time graph (IV) is obtained, curve (2) for LiCl solution, curve (1) for NH_4Cl solution, curve (3) for KCl solution.

NH_4Cl and to a lesser extent also LiCl in a 0.5 Mol. concentration possesses a greater disaggregating power than ATP as shown from comparing their lowering effect on the N_{sp} of an actomyosin solution (see Heller). This reveals itself both in a better extraction at the very beginning of the experiment, and also in a residual solubility after prolonged standing. The curves remind of the solubility at alkaline pH, understandable since also increased alkalinity has a disaggregating effect, as shown by Heller.

If this were sufficient to dissolve myosin from the structure of the fibre without ATP, we would expect the amount of myosin extracted at the beginning and after standing to remain constant. Instead there is a considerable difference between the yields in the presence or absence of ATP.

In the light of these facts we may assume either that ATP has a specification which cannot be replaced by another substance; or that during prolonged standing of the suspension some denaturation or other change take place, preventing the dissolution of the myosin under the disaggregating action of NH_4Cl.

To clarify these points I tried to extract fresh muscle with NH_4Cl solution in absence of ATP.

Experimental

Fresh minced muscle was suspended in 2 volumes distilled water, stirred for few minutes, and divided into two portions.

From the first a sample was drawn extracted with 0.5 Mol. NH_4Cl and the myosin extracted was determined.

The second portion was centrifuged, the supernatent liquid was replaced by an equal volume of distilled water. The mixture mixed well, and two samples were taken; the first extracted with 0.5 Mol. NH_4Cl; the second extracted like the first plus ATP in a concentration equal to that found in fresh muscle.

The experimental results are shown in table VII, they are expressed in grams of myosin extracted from 100 grams fresh minced muscle.

The result is that, although NH_4Cl is able to extract a not inconsiderable quantity of myosin in the absence of ATP. This amount is at any rate much less than the yield obtained in the presence of ATP; also then if care is taken that in both experiments the extraction takes place at the same time after mincing.

It is clear therefore, that the ATP action is specific, probably there is a formation of a complex of myosin, with ATP being a prosthetic group.

VII. Demonstration of the presence of a substance affecting the ATP action.

At the end of section V it was demonstrated that after long standing of minced muscle addition of ATP restores the solubility of the myosin considerable, but not completely. As one possible reason for this difference between "old" and "fresh" minced muscle with equal ATP amount, it was mentioned that the old muscle could perhaps contain a substance which counteracts the dispergating action of the ATP. Such a substance would have been formed during the time of standing, and could in fact be a normal product or intermediate of one of the individual reaction of muscular metabolism.

The action of such a substance could be imagined in two different ways:

The substance could be a direct antagonist against the disaggregating and dissolving action of the ATP or the salt, either by increasing the strength of the bonds to be broken, or by inhibiting the combination between ATP and myosin.

Or, on the other hand, the substance could increase the rate of disappearance of the ATP, either by activating one of the enzymes involved, or perhaps more probably by acting as a phosphate acceptor, furthering the splitting of ATP in that way. Thus, in old muscle pulp the added ATP would disappear faster, and thus not have the chance to act as effectively as in fresh muscle.

It was the purpose of the following experiments to test whether indeed the formation of such an antagonistic factor would be demonstrated, and if so, which, of the mentioned possible explanation of its action, holds true.

Experimental

To prepare that substance, fresh minced muscle was suspended in 2 volumes distilled water and kept standing at room temperature for 6 hours, time during which the specific substance in question would be formed and would accumulate in the juice. The suspension was then centrifuged, the residue discarded and the juice kept for the next experiment.

A second rabbit was killed by dicapitation, skinned rapidly and the muscle were cut off, minced in a cooled meat mincer (special precautions were taken to keep the temperature as near to 0°C. as possible) and the freshly minced muscle was divided into two portions.

The first was suspended in 2 volumes distilled water and served as control.

The second portion was suspended in 2 volumes of the juice obtained above.

Fifteen to twenty minutes are allowed to pass, then samples from both suspensions were drawn and the myosin extracted and determined as Before, suction II

In the meantime the ATP concentration at different time intervals was determined in the following manner:

At 0, 15, 45 and 90 minutes 10 ml. samples were drawn from each of the two suspension (see above) respectively. The proteins were precipitated by 20 ml. of 10% trichloracetic acid solution. The liquid filtered off and its volume measured, then neutralized to phenolphthalein, recording the volume of NaOH used, so that the dilution due to neutralization can be accounted for.

The determination of the ATP concentration requires:

a) determination of the inorganic phosphorus present initially.

b) determination of the total inorganic phosphorus present in a solution which has been hydrolyzed for 15 minutes in 1 N. HCl at 100°C. The ATP-Phosphorus is than estimated by substracting the inorganic P from the hydrolyzable P. e.g. ATP-P= hydrolyzable P-Inorganic P.

Each 2P are produced from one molecule of ATP.

The phosphorus analysis is done according to the method of Fiske and Subbarow (6).

The results of such an experiment are reproduced in table (IX) which shows the time course of the ATP breakdown:

1) in a suspension of minced muscle in distilled water .

2) in the suspension of minced muscle in old juice. See graph (V) curve (a) for the former curve (b) for the latter.

It will be seen that wheres freshly extracted muscle from the distilled water suspension yields the usual amount of myosin : 1.850 gr. per 100 grams muscle. The fresh muscle extracted from an old juice suspension yields only 1.139 grs. per 100 grams muscle.

Also whereas ATP breakdown in the distilled water suspension takes its normal course in the old juice suspension ATP is disappearing at a greater rate; so after 30 minutes, wheras the ATP concentration in the latter case drops to zero, in the former case it is still in a concentration sufficient to promote the extraction of myosin.

From the forgoing experiments one can conclude that ATP has a specific effect on the solubility of myosin, which effect is not possessed by any other substance or at least by substances like LiCl and NH_4Cl which were thought that through their dissociating power would bring myosin into solution.

ATP is constantly disappearing and in its absence no myosin could

be extracted by 0.5 Mol. KCl solution. The breakdown of ATP is accelerated by a substance, formed in the muscle suspension while standing, whose nature is not yet known, probably it is a phosphate acceptor.

It is hoped that future investigation will reveal the nature of that substance and show the mechanism of the ATP action on the myosin of the muscle fibres.

Discussion of the results.

The purpose of this investigation was to arrive at a closer insight into the conditions determining the extractibility of myosin in striated muscle. This in se of interest first for practical reasons, as related to the problem of preparing myosin from, or determining myosin in muscle. It may be reminded that there are considerable discrepancies between the estimations of the myosin contents of muscle as made by Weber (29) and Smith (30) who used different solvents. Further it had been found already earlier, e.g. by Saxl (23) that the solubility of myosin in the muscle decreases under certain conditions, related to the physiological condition of the muscle, e.g. in fatigue, or post mortem. The analysis of such changes might contribute to a better knowledge of these and other processes in mucle.

It is almost superfluous to mention that not every aspect of this problem could be extensively dealt with. It will be possible however to arrive at some general conclusions regarding the mechanism of the extraction.

One of the solvents most widely used for this purpose is 0.5M. KCl. Other extractants are in use, but also these are salt solutions of a similar concentration, as LiCl or NH_4Cl. The necessity of salt at such a high concentration is emperically understandable, since myosin is insoluble in the absence of salts, unless it is entirely free of actin

which is not the case in muscle.

That however salt alone is not sufficient is convincingly shown by the experiments of section II and VI (confirming Szent-Györgyi) in which minced muscle was kept for different lengths of time in distilled water. After different intervals then salt was added, and the yield of myosin determined. This yield was found to decrease in time . Parallel with this, the amount of ATP in the muscle decreased, so that the hypothesis was proposed already by Szent-Györgyi that myosin can be extracted from the fibril only in the presence of ATP. As was shown at other occasions, ATP is able to dissociate the myosin-actin comlex, apparently the form in which myosin is present in muscle. Addition of ATP to old muscle pulp increases the amount of myosin extracted.

Such experiments were done with different variations, namely at different pH values, and with different salts. In all these cases the decrease in solubility was confirmed. It was found however that in the stronger disaggregating media the extractibility did not drop entirely to zero, but a small residual solubility persisted. This however was much less, so that it seems that even strongly disaggregating media do not extract myosin efficiently in the absence of ATP. At more acid reaction on the other hand, even in the presence of ATP no myosin could be obtained. The parallel between presence or absence of ATP and extractability of myosin in all these cases supports the hypothesis of the necessity of ATP.

Much attention was given to a further analysis of the decrease in solubility occuring in minced muscle on standing. In analogy with the results of Weber and Kamp, with extract of fatigued muscle, it was found that addition of an extract of "old" muscle pulp to freshly minced muscle strongly reduced the solubility of myosin herein. This effect was further analysed, and it was found that upon addition of such "old juice" to

fresh muscle the rate of disappearance of the ATP from the latter was enhanced. The mechanism of the effect of old juice is therefore not to be explained by the presence of a substance which makes myosin insoluble, but of a substance which increases the removal of ATP. It is thought that this substance probable has the nature of a phosphate acceptor, the presence of which opens new or increased possibilities for the breakdown of ATP.

There is all reason to assume that such a substance may make its appearance also in fatigue, or even earlier that that, in muscular activity but this point could not yetbe investigated.

These different lines of evidence all support the view that the presence or absence of ATP decides whether myosin is soluble or not. This would not be understandable if in the muscle myosin would be present as such, since pure myosin dissolves without difficulty in salt solutions. The same is true for actomyosin in an organized form, but Szent-Györgyi has found that actomyosin threads do not dissolve in salt solutions unless ATP is present. It seems therefore that if actomyosin is present in an ordered semicrystalline, fibrous structure, the myosin is held very tightly, and can be removed only after dissociation of the actomyosin complex. Since in all my experiments muscle behaved in the same way, it should be concluded that actomyosin, not myosin, is the structural element of the muscle fibril.

On the basis of this experience it is to be concluded that muscle under the conditions studied here contains F-actomyosin that is myosin combined with polymerized actin, because only such actomyosin would require ATP for its dissolution. The further development of this view point would bring us in disagreement with certain conclusions developped recently by Szent-Györgyi (32), but it seems preferable to postpone discussion of these points till more experiments on muscle under other conditions have been done,.

SUMMARY

A short historical review of the knowledge of the muscle proteins and their physiological significane is given.

A method is described by which the extractibility with 0.5 Mol. KCl solution, of the myosin in rabbit muscle is quantitatively determined.

It is confirmed that the solubility of myosin in minced muscle decreases upon standing as such or in distilled water.

This decrease in solubility is more pronounced when the extraction is done at pH 6.5 than when it takes place at alkaline reaction. In the latter case a certain residual solubility persists after 4 or 6 hours standing, whereas at pH 6.5 the extractibility vanishes completely in such a time. At pH 5.0, nothing can be extracted even from fresh muscle.

If ammonium chloride solution is used as extractant, there remains a residual solubility also at pH 6.5. Ammonium chloride, through its stronger disaggregating action, is a better extractant as far as the yield is concerned.

The decrease in solubility during standing could be ascribed to a disappearance of the ATP from the muscle. If ATP is added, salubility is again restored, even after 6 hours of standing, without ATP, there is no significant extraction of the myosin, even not if NH_4Cl or alkaline KCl is used.

After addition of ATP to old muscle pulp however, the restoration of the solubility is not complete. The difference is due to formation of a substance which acts as an antagonist against the ATP.

The mechanism of the effect of this substance is that it promotes the disappearance of the ATP. probably because it acts towards ATP as a phosphate acceptor.

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George Abu-Haidar

Experimental results

Table I

pH	N-myosin precipitated	Myosin precipitated
5.5	0.90 Mgm.	5.40 Mgm.
5.8	0.90 "	5.40 "
6.0	0.86 "	5.16 "
6.5	0.60 "	3.60 "
7.0	0.40 "	2.40 "

Table II

pH	N-myosin precipitated	Myosin precipitated
5.5	1.42 Mgm.	8.52 Mgm.
6.0	1.42 "	8.52 "
6.3	1.42 "	8.52 "
6.7	1.35 "	8.10 "
7.0	1.20 "	7.20 "
7.5	0.90 "	5.40 "
8.0	0.60 "	3.60 "

Experimental results (Cont'd)

Table III*a

Time after mincing	N-myosin extracted mgm. per 100gr.muscle	myosin extracted gr.per 100gr.muscle
5 minutes	321	1.920
30 "	110	0.660
45 "	37	0.220
90 "	20	0.120
180 "	negligible	negligible

* Extraction of myosin at different time intervals, at room temperature 22°C.

Table III*b

Time after mincing	N-myosin extracted mgm.per 100gr. muscle	myosin extracted gr.per 100 gr.muscle
5 minutes	310	1.860
30 "	107	0.642
2 hours	16	0.096
3.30 "	negligible	negligible

* Extraction of myosin at different time intervals, at room temperature 17°C.

Experimental results (Cont'd)

Table IV*

Time after mincing	pH 8	pH 6.5	pH 5
15 minutes	1.595+	1.305	negligible
45 "	0.960	0.524	"
2 hours	0.474	0.090	"
4 hours	0.390	negligible	"

* Extraction at different pH values, at room temperature : 20°C.

\+ Figures expressed grams of myosin per 100 gr. muscle extracted by 0.5 Mol. KCl solution ; at room temperature : 20°C.

Table V*

Time after mincing	LiCl+	NH_4Cl+	KCl+
5 minutes	2.680	2.340	1.740
30 "	1.440	1.300	0.650
1 hour	0.860	0.940	0.440
2 hours	0.580	0.780	0.090
4 "	0.560	0.820	negligible

* Extraction in different salt solutions.

\+ Grams of myosin extracted by 0.5 Mol. solutions of LiCl, NH_4Cl and KCl respectively, from 100 grs. fresh minced muscle, at room temperature : 18°C.

Experimental results (Cont'd)

Table VI*

0 minutes	4 hours	4 hours +ATP	6 hours +ATP
1.984 grs. per 100gr.muscle	negligible	1.320 grs. per 100gr.muscle	1.242 gr. per 100 gr. muscle

* Extraction of myosin in presence and absence of ATP by 0.5 Mol. KCl solution at room temperature : 16°C.

Table VII*

5 minutes$_1$	5 minutes$_2$	5 minutes $_3$
2.130 gr.per 100 gr.	0.820 gr. per 100 gr.	1.962 Gr. per 100 gr.

* Extraction of myosin 5 minutes after mincing by 0.5 Mol. NH_4Cl solution at room temperature : 21°C.

1 From fresh muscle in distilled water.

2 " " " whose ATP is washed out.

3 Control : the same as $_2$ + ATP.

Experimental results (Cont'd)

Table VIII*

Experiment No.	Time after mincing	Grams of myosin extracted from 100 gr. muscle	
		1	2
1	15 minutes	1.972	1.032
2	15 "	1.812	1.261
3	15 "	1.767	1.125
Average	- -	1.850	1.139

* Myosin extracted by 0.5 Mol. KCl solution at room temperature : 19°C.

1 The minced muscle was suspended in two volumes distilled water.

2 The minced muscle was suspended in two volumes 6 hours old juice.

Table IX*

TIME after mincing	mgm ATP-P		Mgm ATP	
	1	2	1	2
0 minutes	52	48	480	442
15 "	38	20	350	185
30 "	19	00	175	000
60 "	00	00	000	000

* Disappearance of ATP from:

1 Minced muscle in a distilled water suspension.

2 Minced muscle in an old juice suspension.

Note : Values are mgm. per 100 gr. muscle.

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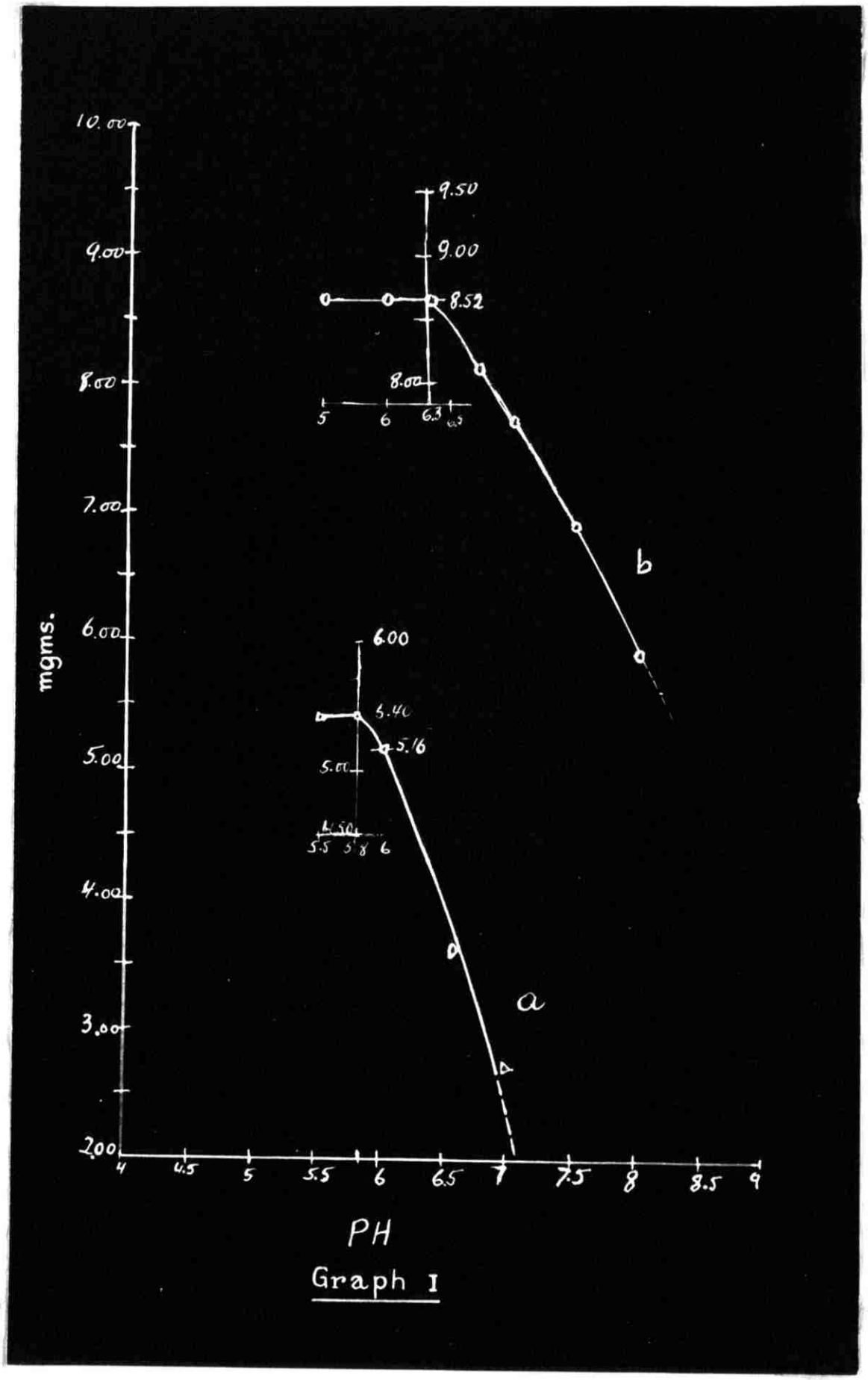
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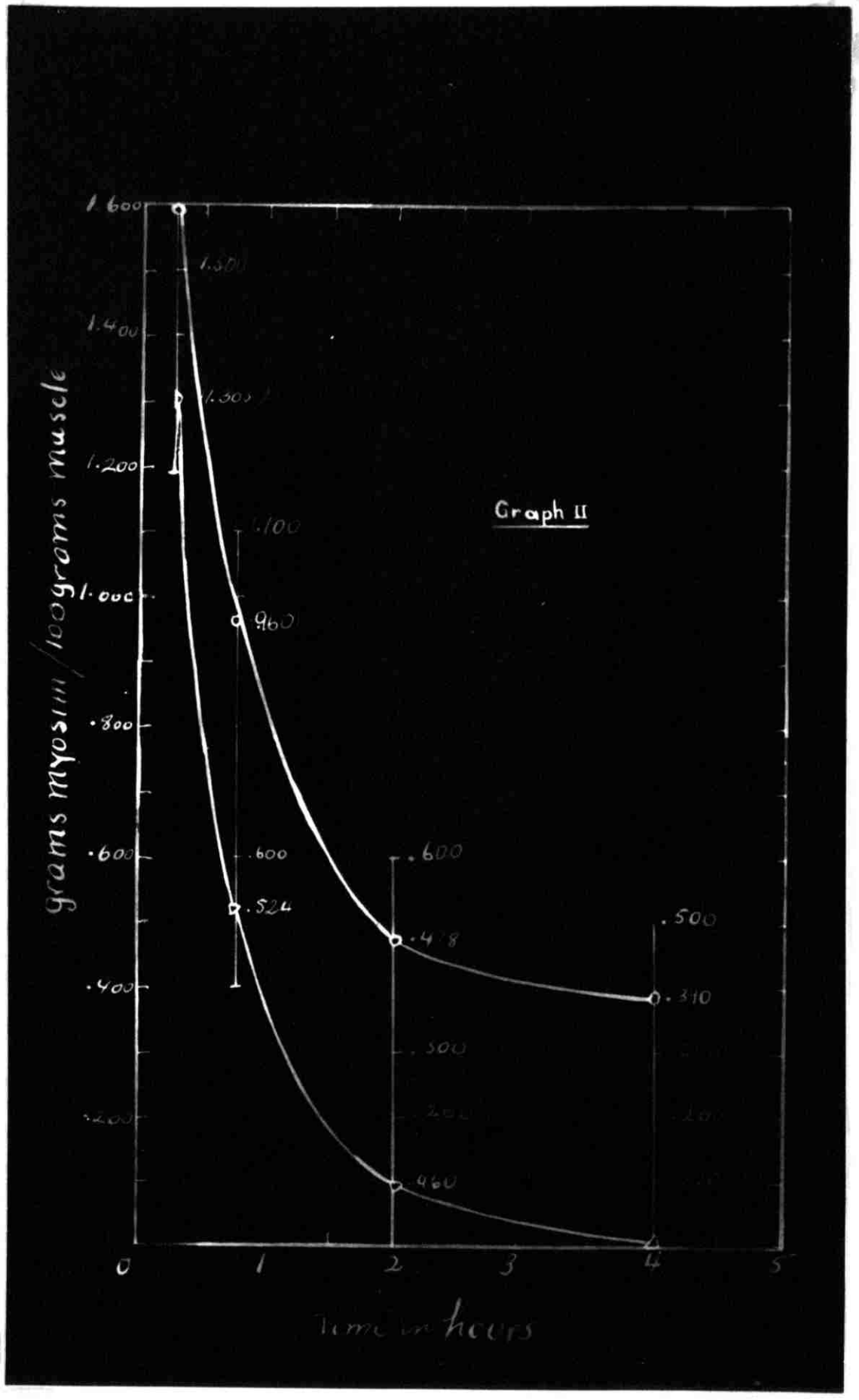
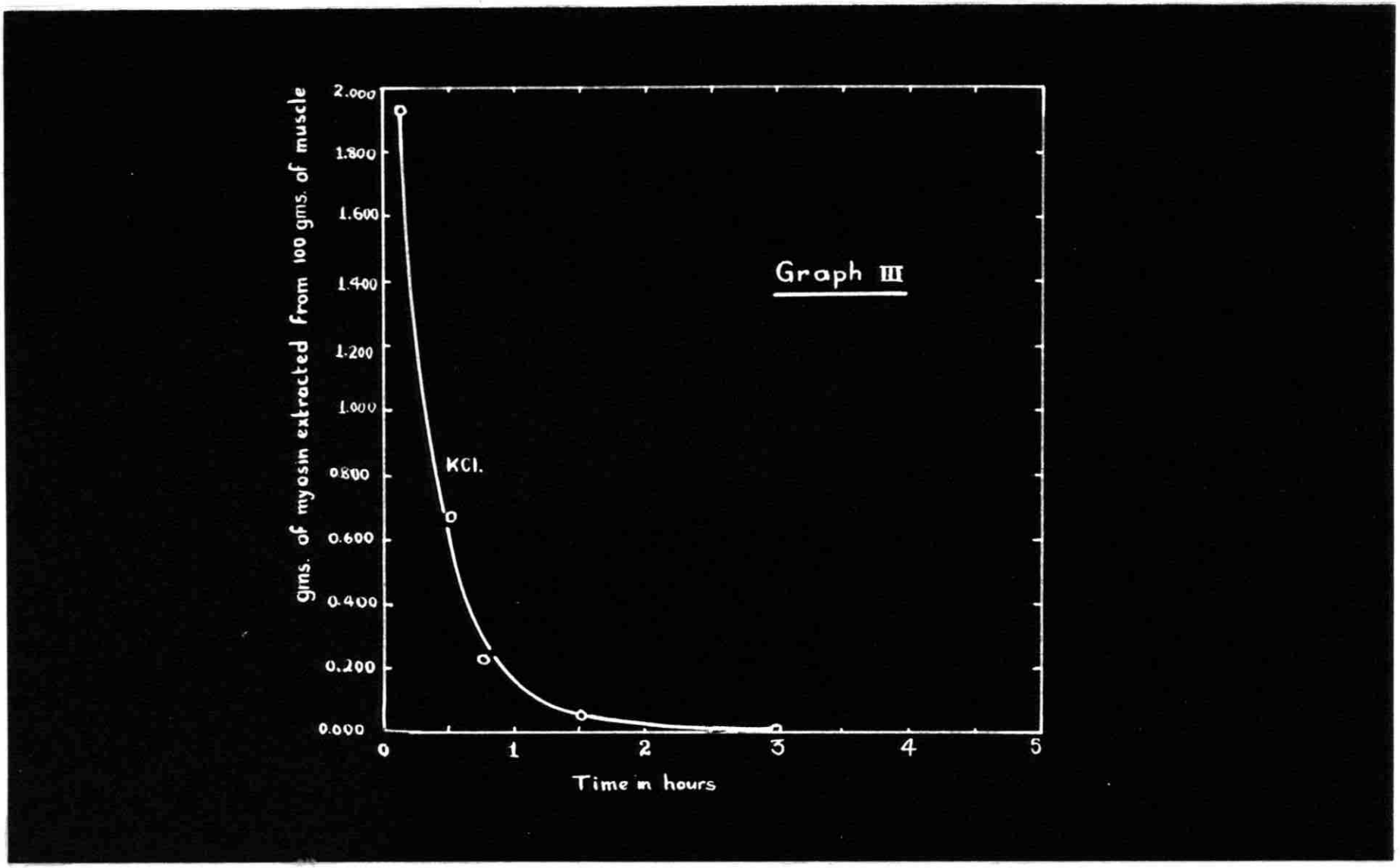
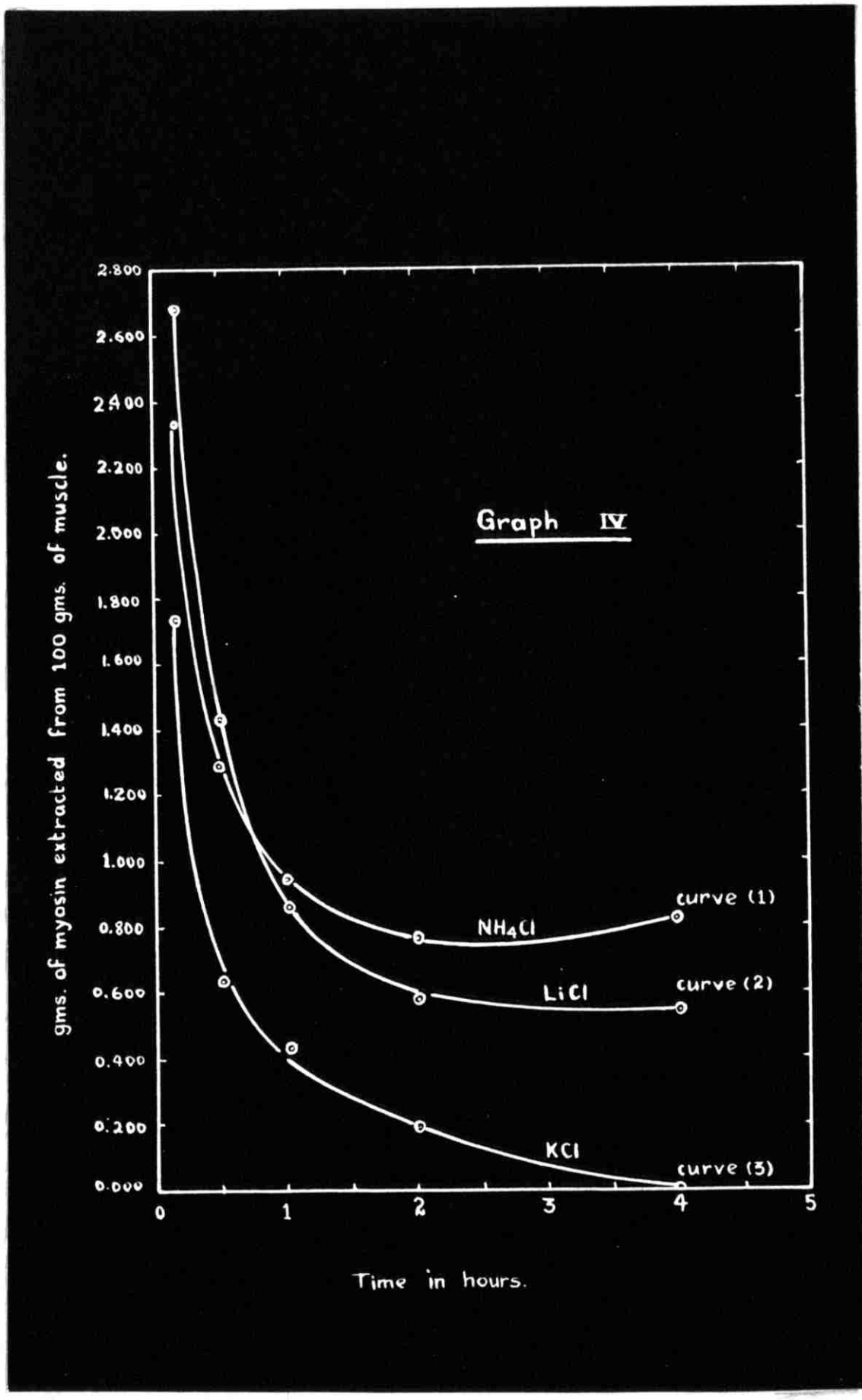
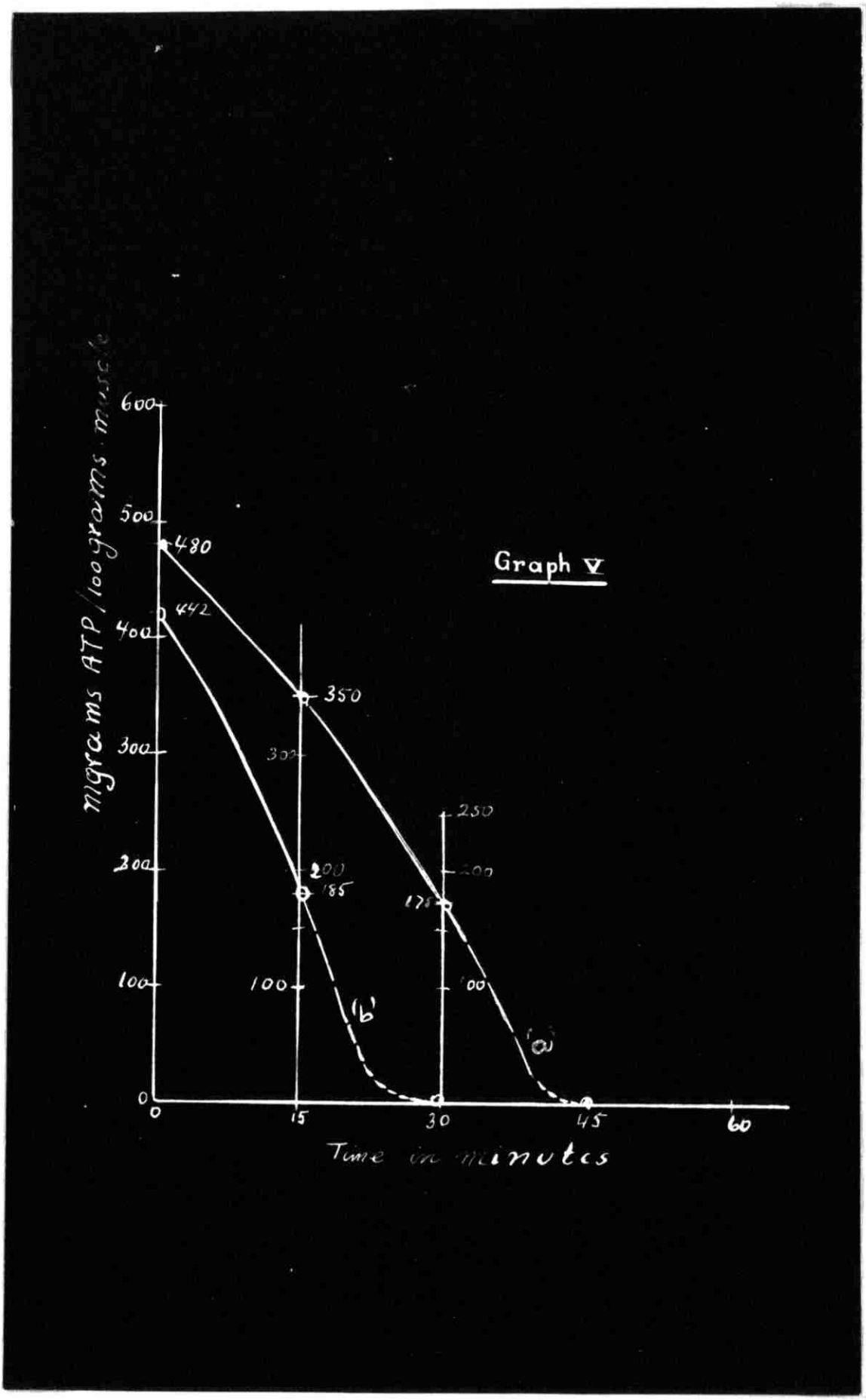
5
6
6.3
b
6.00
5.40
5.16
5.00
4.50
5.5
5
8
6
a
4
4.5
5
5.5
6
6.5
7
7.5
8
8.5
9
PH

Graph I


Graph II
grams myosin / 100 grams muscle
1.600
1.500
1.400
1.300
1.200
1.100
1.000
.900
.800
.700
.600
.500
.400
.300
.200
.100
.960
.600
.524
.600
.418
.500
.310
.500
.460
0
1
2
3
4
5
Time in hours


gms. of myosin extracted from 100 gms. of muscle
2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
KCl.
0
1
2
3
4
5
Time in hours
Graph III


Graph IV
gms. of myosin extracted from 100 gms. of muscle.
2.800
2.600
2.400
2.200
2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
1
2
3
4
5
Time in hours.
curve (1)
NH4Cl
curve (2)
LiCl
KCl
curve (3)


Graph V
milligrams ATP / 100 grams muscle
600
500
400
300
200
100
0
480
442
350
300
250
200
185
178
100
100
(b)
(a)
0
15
30
45
60
Time in minutes