ON THE DISAGGREGATION OF ACTOMYOSIN

A thesis presented by Wiktor Heller in partial fulfilment

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"On the disaggregatinn of Actomyosin"

The contractile part of muscle the muscle-fibril contains about 43% of actomyosin. To understand the processes taking place in fibrils during contraction and relaxation it is necessary to know the nature of the forces aperating in actomyosin particles. The muscle fibrils are striated. They are composed of two kinds of bands A-bands containing actomyosin and I-bands containing ATP. Myosin hydrolyses ATP. This is an energy yielding process. ATP disaggregates actomyosin, into actin and myosin. Szent-Györgyi prepared actomyosin threads and investigated their protperties in vitro. He noticed a rapid contraction of threads suspended in muscle extracted or in a solution of KCl and ATP. It can be expected that a similar process takes place in muscle during contraction. The nature of the forces operating between actin and myosin in actomyosin particles is the object of this study. The disaggregation of actomyosin can also be caused by many inorganic salts, some organic reagents and by a high alkalinity of the solution.

It was found that actomyosin at pH 6 or below was precipitated in 0.5 M. KCl solution. In the range 6.4-7.4 the state of aggregation of actomyosin was not affected. Increase of the alkalinity promoted disaggregation of actomyosin until finally at pH 9.75 myosin started to desintegrate.

The effect of neutral salt upon the viscosity of actomyosin was investigated. Conclusions are based mostly on the results observed at low concentrations of salts to avoid the interference of secondary reactions that could be expected at higher concentrations.

The activity of the cations increases in the following order: $Li < Na < K < Cs < NH_4 < Mg < Ca < Ba$.

The activity of the anions increases in series $Cl < Br, NO_3 < J$.

Sulfates, phosphates and fluorides are effective salting out agents.

The effect of urea and some related substances was studied. The number of reagents used in these experiments was very limited due to low solubility of organic reagents in water. The following order of activity was found thioacetamide > thiourea > acetamide > succinimide > methylurea > urea.

These results are not in accord with experiments performed by Habiby (M.A. thesis A.U.B. 1947) with gelatin. It seems that these reagents have specific effect that is dependent on the protein used.

From the above mentioned results it was concluded that between actin and myosin exist two types of bonds :

1) Electrostatic bonds which are affected by hydrogen-ion concentration and presence of salts.

2) Linkages of unidentified nature ruptured to some organic reagents

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I. Introduction;

The muscle proteins were first investigated about ninety years age. The first publication about this subject was written by Kühne (14). It was soon realized, that muscle plasma contains several proteins. To enable the study of the physical and chemical properties of these proteins, it was necessary to separate the mixture into components.

The first attempt in this direction was done by Halliburton (11), who separated the muscle proteins according to the temperature of coagulation:

Paramysoinagene	coagulated	at 47°C.
Myosinogene	coagulated	at 56°C.
Myoglobulin	coagulated	at 63°C.
Albumine	coagulated	at 73°C.
Myoalbumose	non coagulating	at 73°C.

This work forms an example of the tendency prevailing at that time to characterize proteins according to their coagulation temperatures, as it was believed that these coagulation points have the same significance as melting points in ordinary chemistry. For different reasons this is not the case. On the one hand the coagulation temperature depends on external conditions for example on the speed of heating (as a consequence of the kinetics of denaturation). On the other hand, if in a mixture of proteins one component coagulates or precipitates, it may take another component with it, which would otherwise remain soluble under these conditions.

As a consequence of such difficulties, the coagulation temperature is no longer considered as a suitable means to characterize proteins, except perhaps in very extreme cases like certain enzymes resistant against boiling in dilute HCl (Banga).

Later muscle proteins have been studied by a large number of

investigators who applied a varied terminology to the substances they discovered. Separation into components was made by the differential extraction and precipitation with inorganic salts solutions of different concentrations.

Fürth (9) in his investigations gave the name "myosin" to Halliburton's (11) paramyosinogene. The special properties of this protein are the subject of my studies.

Very thorough investigations of the properties of different fractions of muscle proteins were made by Howe (12). He separated the components by extraction in salts solutions of different concentrations.

Edsall (4) and von Muralt and Edsall (21) used the same mehtod of separation as Howe. They were especially interested in physico-chemical properties of myosin as solubility and others. In investigating the properties of this protein they noticed the unusual high viscosity and double refraction of flow. Von Muralt and Edsall paid only moderate attention to the viscosity, a property which at that time was not yet rightly appreciated. They thoroughly investigated the birefringence of flow, a property which attracted their attention because of the supposition that myosin forms the material of the anisotropic A-bonds. In their interpretations they make use of the results of Freundlich and his school with inorganic anisotropic colloids and develop the concept that myosin molecules are long and asymmetric. This conclusions, fundamentally important for the understanding of muscular function, became confirmed by investigations of X-ray diffraction by Bailey (22) and viscosity by Mommaerts, lately also in a most direct way by the electronic microscope (von Ardenne and Weber, Hall, Jakns and Schmitt).

At the same time many biochemists were interested in the energy-liberating reactions in muscles. Meyerhof (15) has published the theory of the energy-cycle in muscles according to which lactic acid is responsible for the contraction. The further investigations have

disproved Meyerhof's theory, but in spite of that our knowledge of the energy liberating reactions in muscles far surpassed theknowledge of the structure of muscles. It was expected that the physico-chemical properties of the isolated muscle proteins will be found intimately related to its functions within the muscle fiber. However, concerning such physico-chemical properties there existed only the vaguest ideas, until through the work of Muralt and Edsall the importance of the fibrous nature of the molecules became more obvious. Also after this insight, the nature of the changes leading to contraction remained entirely unknown but for a vague feeling that some kind of relations between energy yielding metabolic reactions and changes in the protein molecules should exist.

There was no doubt that each chemical reaction taking place in muscles needs a specific enzymes. All enzymes are proteins and therefore the scientists have been investigating the relations between the muscle proteins and the energy liberating reactions.

Engelhardt and Ljubimowa (8) have found the splitting effect of myosin on adenosine triphosphate (ATP). It was already known at that time that hydrolysis of ATP is the energy-liberating reaction in muscles. This discovery has given the stimulus the investigators of muscle proteins to study the properties of myosin.

Earlier investigations on this point, including attempts by Engelhardt did not give any results, as "myosin" was believed to be a pure substance. Certain irregularities in its behaviour seem to have been noticed (as one can see from a careful study of the papers of Edsall or Needham) but no explanation was given.

The first progress started with the discovery of Szent-Györgyi, who differentiated two forms of myosin. Myosin can be extracted from muscles as less active myosin A amd more active myosin B. The myosin prepared by previous investigators was a mixture of myosin A and B in different proportions. Myosin A is transformed into B if it stands in

prolonged contact with muscle particles. It was shown by Szent-Györgyi and his collaborators that myosin B is a stoichiometric compound of myosin A and another protein called by them "Actin". To the myosin-actin complex was given the name "actomyosin". Actin is a part of insoluble muscle residue. Balenovic and Straub (2) have found that actin represents about 12-15% of the muscle proteins. Making some allowance for the connective tissue and the nucleo-proteins of the muscle's nuclei there is not much protein left unaccounted.

As was shown by Banga and Szent-Györgyi (3), myosin A has, dissolved in Weber solution (0.6M. KCl, 0.04M. $NaHCO_3$, 0.01M, Na_2CO_3), a relatively low viscosity which is not influenced by ATP, whereas actomyosin (myosin B) has under the same conditions, a relatively high viscosity which is brought down to the level of the viscosity of myosin A of the same concentration by the addition of small amounts of ATP. This difference in viscosity is even more striking than it seems, if one considers the influence of the velocity gradient during the measurements studied by Mommaerts (16).

When actomyosin dissolved in Weber solution is pressed through a capillary tube into water it precipitates in the form of threads.

Szent-Györgyi (19) suspended an actomyosin thread in a fresh aqueous extract of muscle. He noticed a violent contraction. He was able to perform the same experiment by suspending an actomyosin thread in a solution of ATP and KCl. The rate of constraction was greatly increased by addition of Mg^{++} ions that are present in the interfibrillary fluid in muscles. Through this behaviour towards ATP and ions, the actomyosin thread acts as a model of the fibril and demostrates that myosin has properties which make it a suitable material for a contractile structure. Pure myosin threads do not contract. This is considered by Szent-Györgyi as an indication that only actomyosin is contractile, and

that A-bonds therefore necessarily consist of actomyosin.

Mommaerts and Straub have found that actomyosin and myosin A form a complex with ATP.

Myosin exists therefore in four different forms :1. free myosin (Myosin A), 2. ATP-myosin, 3. actomyosin, 4. ATP-actomyosin.

It has been shown by Banga and Szent-Györgyi (3) that in the absence of ATP the myosin of the muscle is not extracted with Weber solution.

In living muscles, under normal conditions, there is a high concentration of ATP which is kept constant. It has been concluded by Szent-Györgyi (19) that the living as well as the freshly minced muscle contains the highly sensitive ATP-actomyosin complex.

However it has been shown by Caspersson and Thorell (20) that in resting muscle ATP is concentrated in the I-bond of the fibril, and is therefore not in contact with the actomyosin of the A-band. Upon mincing however, this separation is certainly abolished and ATP can react with actomyosin.

Szent-Györgyi concludes from the viscosity measurements that, at the high salt concentration (0.6M. KCl), the complex dissociates into actin and ATP-myosin. This phenomena explains to us the extraction of myosin A and B from muscles. If we suspend the muscle in Weber solution, the ATP-actomyosin dissociates into actin and ATP-myosin. For this reason we always obtain myosin A from fresh muscle containing ATP (30 minutes extraction). On storage the ATP is split and the dissolved ATP-myosin in changed into free myosin which forms actomyosin with actin. The myosin which is already dissolved, will by the formation of this complex, bring the actin into the solution. If we start with muscles free from ATP, the myosin will be present in the form of the stable and insoluble actomyosin, which will not be extracted by the salt solution.

II. The Effect Of ATP Upon Actomyosin.

II. The Effect Of ATP Upon Actomyosin.

It was shown by Banga and Szent-Györgyi (3) that the viscosity of actomyosin solution is lowered instantenously to the viscosity of myosin by the addtion of ATP. The lowering of the viscosity must be caused by the change of the asymmetry of the particles. Needham (17) tried to explain it by shortening of the particles. If that would be the case, it must cause a high increase of light scattering effect, as was shown by Mommaerts (16). Mommaerts investigated the Tyndall effect of actomyosin solution after addition of ATP. He found the decrease of the light scattering effect to the value of myosin. Obviously ATP does not cause the contraction of actomyosin particles, but disaggregation into actin and myosin only. Straub (18) by mixing in absence of ATP pure actin with myosin in definite proportion obtained actomyosin.

Actomyosin has different physical properties than the myosin solution. It has a much higher viscosity, the double refraction of flow of actomyosin is much stronger as also the mentioned before Tyndall effect. Addition of ATP decreases instantenously the values of these physical characteristics to the values of myosin solution.

Edsall and Mehl (5) without knowing about the difference between myosin A and B were investigating the influence of different inorganic salts on double refraction of flow and the viscosity of myosin. They were working with actomyosin containing some admixture of myosin. This can be concluded from a consideration of their experimental results, notably by comparison of their viscosity curves with those of Mommaerts for actomyosin (16) and from the fact the solution showed double refraction of flow already by merely agitating the solution with a glass rod.

Mommaerts (16) found that urea and some other organic reagents have

a disaggregating effect upon actomyosin. The object of my studies is the quantitative determination of the effect of some organic reagents and different inorganic ions upon the viscosity of actomyosin in the absence of ATP. The purpose of such investigations is to collect information concerning the nature of the bounds or intermalecular forces which are responsible for the association between actin and myosin leading to the formation of large actomyosin particles.

As we know the contractile element of the muscle is the muscle fibril. The fibrils contain about 33% of actomyosin. Obviously the knowledge of the forces operating between actin and myosin will help us to understand the conditions of the fibrils of the resting muscles and their changes during contraction and relaxation.

Some preliminary observations were done by Mommaerts (16) in this field but the work remained very uncomplete. There were indications for the participation of two kinds of forces : some form of electrostatic attraction sensitive towards salts and splitting of certain bounds by urea, guanidine salts and arginine. Such bounds of the latter type seem to be common in proteins and their breaking seems to be one of the essential reactions leading to denaturation. With regard to the general importance of the question of the nature of these bounds, a number of substances has been studied having some structural similarity with urea.

III. The PREPARATION of Actomyosin.

The actomyosin preparations were obtained from the hindleg muscles of rabbits. The rabbits used for these experiments were anesthetized, the muscles were cut out quickly, cooled in ice and minced in a cooled meat grinder after removal of fat. For the preparation of actomyosin the minced muscle was extracted for 6 hours at room temperature or 24 hours at 0°C. with 3 volumes of alkaline 0.6M. KCl solution containing

0.04 M. of $NaHCO_3$ and 0.01M. of Na_2CO_3. After the extraction the residue was removed by centrifugation, the extract filtered through gauze, and the actomyosin precipitated by adding 5 volumes of water redistilled from glass vessel. The solution must be free of traces of copper ions that have a strong denaturating effect upon actomyosin. Acetic acid is added under strong stirring to bring the pH to 6.5 accurately controlled by the glass electrode. The actomyosin precipitate was separated on the centrifuge and dissolved by adding 2M. KCl solution and water till the final salt concentration was 0.5M. and the protein concentration from 0.5 to 1.0%. After three precipitations actomyosin was considered pure with many restrictions, but these are probably of minor importance for the problems investigated. This actomyosin usually contains approximately 16% of actin and is of 100% activity according to the Straub's (18) scale.

It should be mentioned, that if muscles of two different rabbits were extracted in the same way the viscosity of actomyosin obtained was not always equal. Here arises a new problem, which properties of fresh muscle influence the extraction of actin. Nothing is known about this up till now. Irregularities are according to experiences of Szent-Györgyi's and our laboratory especially frequent in summer. The actomyosin is then often of a low activity, a fact which can be prevented by keeping the animals in the cold room during one day before the experiment. The reasons of these and other variations are not yet explained.

It was attempted to use actomyosin of approximately the same viscosity in all experiments.

IV. Determination of Actomyosin Concentration.

The nitrogen content of myosin has been open to some dispute, and for the purpose of assesing myosin concentration it was reinvestigated by Bailey (1). As Greenstein and Edsall pointed out, their own low

figures are probably due to inadequate extraction of fatty material (15.3-15.7%). Bailey confirmed experimentally this view and determined accurately the nitrogen content of myesin to be 16.6%.

Te determine actomyosin concentration of the solution I have determined nitrogen concentration by Kjeldahl's method and multiplied the result by six.

V. Viscosity Measurements;

All substances investigated were dissolved in 0.5M. solution of KCl. The volume was made up with 0.5M. KCl. Therefore the coneentration of KCl was maintained constant during the whole experiment in order not to influence the disaggregation or precipitation of actomyosin. This means that in the experiments on the influence of added salt there was always a basic concentration of 0.5M. KCl in addition to the salt the action of which to be investigated. Since the KCl present may affect the influence of the added ions, certain difficulties in the interpretation of the results can be expected. These complications are unavoidable with the methods employed in this study. It is possible to develop a different system of investigations, in which actomyosin is precipitated by dilution and washed free of salts, after which various amounts of the investigated salt are added to identical samples of the electrolyte free actomyosin. In this case however, not exactly the same thing is investigated. Such measurements which may be of interest, could not be included in this study.

The viscosity was measured in the Ostwald-Neurath viscosimeter disregarding the velocity gradient of the solutions. Why the velocity gradient in the streaming solutions does not influencethe viscosity value in this experiment will be shown later.

As was found by Mommaerts (16) one of the differences between the action of ATP and other reagents upon actomyosin is that ATP disaggregates

actomyosin instantenously while urea for example requires 4 hours time for complete reaction. Considering this point, solutions after preparations were left overnight before measuring the viscosity.

To maintain the temperature constant the viscosimeter was placed in the water bath. The temperature of the water bath was 22.5°C.

VI. Tables of Results

	0	0.2	0.4	0.5	0.6	0.75	0.8	1.0	1.25
KCl				0.94		0.86		0.77	0.66
LiCl	0.94	0.94	0.91		0.63		0.48	0.48	
NaCl	0.94	0.90	0.83		0.75		0.68	0.68	
NH_4Cl	0.94	0.82	0.52		0.375	0.375			
CsCl	0.94	0.87	0.77		0.54		0.32	0.20	
KI	0.94	0.34	0.28		0.25		0.20	0.14	
KBr	0.94	0.65	0.35		0.30		0.27	0.25	
NaF	0.94	1.01	1.09		1.18		1.25	1.34	
KNO_3	0.94	0.76	0.38		0.27		0.25	0.23	
K_2HPO_4	0.94	0.83	0.85		0.93		1.08	1.38	
$CaCl_2$	0.94	0.58	0.52		0.51		0.50	0.48	
$MgCl_2$	0.94	0.62	0.56		0.59		0.64	0.67	
$BaCl_2$	0.94	0.52	0.48		0.47		0.46	0.45	
Na_2SO_4	0.94	0.80	1.04						
KCNS	0.94	0.48	0.44		0.42		0.40	0.38	
$MgSO_4$	0.94	0.71	0.69	0.67					
RbCl	0.94	+	+		+		+	+	
$LaCl_3$	0.94	+	+		+		+	+	

Table 1. (Specific viscosity of actomyosin in salt solutions)
Upper line: the final molar concentration of the salt.
\+ : means precipitation.

The viscosity of each actomyosin solution was tested after adding ATP. By comparison with viscosity of the solution treated with some other reagent it could be concluded whether this substance disaggregates actomyosin into actin and myosin, completely or incompletely, and whether the substance disaggregates myosin particles also.

	0	0.2	0.4	0.6	0.8	1.0	1.2	1.5	1.6	2.0	4.0	6.0
Urea	0.94	0.86	0.79	0.72	0.66	0.57	0.50	0.39	0.38	0.37		
Methyl urea	0.94	0.79	0.72	0.64	0.56	0.54	0.49	0.42	0.40	0.30		
Acetamide	0.94	0.81	0.67	0.52	0.48	0.44	0.40	0.35	0.34	0.17	0.12	0.10
Guanidine HCl	0.94	0.68	0.47	0.46	0.42	0.41						
Semicarbazide HCl	0.94	0.71	0.42	0.39	0.37	0.35						
Thiourea	0.94	0.81	0.66	0.42	0.37	0.27	0.22					
Thioacetamide	0.94	0.50	0.25	0.17	0.17							
Glucose	0.94	0.94	0.94	0.93	0.94	0.94						
Glycine	0.94	0.91	0.91	0.91	0.90	0.90	0.90					
Alanine	0.94	0.81	0.80	0.82	0.88							
Succinimide	0.94	0.80	0.72	0.43	0.39	0.39						

Table 2. (Specific viscosity of actomyosin in organic solvents)
Upper line: The final molar concentration of the solvent.

pH	6.2	6.4	6.6	6.8	7.0	7.4	8.0	8.5	9.0	9.25	9.8	10.3	10.8	11.5	12.0
η_{spec}	0.97	0.94	0.94	0.94	0.94	0.94	0.80	0.68	0.57	0.48	0.40	0.32	0.25	0.25	0.25

Table 3. (Specific viscosity of actomyosin at different hydrogenion concentrations.)

VII. General Discussion of the measurements.

The increase of viscosity on denaturation in such proteins as egg albumin and edestin is due to the unfolding of the compact undenatured protein into a more extended polypeptide chain. The decrease in the viscosity of myosin, arising from the action of the same denaturing agent that increase of the viscosity of "globular" proteins, is interpreted as due to the breaking of the very elangated native actomyosin particles into smaller and less asymmetrical molecules. There is no contradiction between the two sets of phenomena. Presumably, in either case, certain linkages within the molecule are broken, and these linkages may well be chemically the same in all these different proteins. The changes in viscosity depend, not on the nature of the linkages broken, but on the geometrical configuration of the undenatured molecule and on the changes in that configuration brought about by denaturing agent.

It has become customary to designate substances of the kind of urea as "denaturing agents". This use is natural in the case of globular proteins, with which almost all studies on denaturation were done. The phenomena called "denaturation" in globular proteins do not always have an exact analogy in linear proteins. In globular proteins unfolding may lead to an increased asymmetry of the molecule. Examples of unfolding of lineary proteins are not known. The changes in lineary proteins during denaturation seem tobe splitting of the molecules and usually with the decrease of asymmetry.

In the case of actomyosin one should distinguish two steps of desintegration, first disaggregation of the actomyosin complex to particle having the dimensions of myosin molecules and probably identical with them, (although some actin may be attached to them), and then a further desintegration of the myosin molecules.

Because of its extreme molecular asymmetry myosin gives very viscous solutions. The close relationship between the shape of molecules and the

viscosity of their solutions is now clear. The theoretical expression is :

$$\nu = \frac{\eta_{sp.}}{\Phi} = \frac{1}{\Phi}\left(\frac{\eta}{\eta_0} - 1\right)$$

Φ = volume fraction of the solute

η = viscosity of protein solution

η_0 = viscosity of solvent

$\eta_{sp.}$ = specific viscosity

The magnitude of ν gives information with respect to the asymmetry of the dispersed particles (if certain hydrodynamical conditions are fulfilled).

For a solute made up of incompressible uncharged spherical molecules $\frac{\eta}{\Phi} = 2.5$ from Einstein's theory (6,7); for all non spherical molecules the ratio is greater than 2.5 owing to the additional work which must be done in rotating such molecules under the influence of the velocity gradient in the liquid. This work and therefore the measured apparent viscosity, varies with the magnitude of the velocity gradient. Hence for the interpretation of viscosity measurements in molecules like myosin, the velocity gradient in the viscosimeter must be considered.

According to Krospelin (13) we may describe the measurements in terms of a "mean velocity gradient" ($\overline{\beta}$)

$$\overline{\beta} = \frac{8\,V}{3\pi R^3 t}$$

V = volume of the liquid flowing through the capillary of radius R in time t.

It has been found experimentally that the specific viscosity is a unique function of $\overline{\beta}$ for liquids showing anomalous viscosity, and is independent of the dimensions of the capillary used.

The relation between specific viscosity and mean velocity gradient (fig. 1) was studied by Mommaerts (16). For viscosimeter used in my experiments : V= 3cc. R= 0.05 cms. t= 105 sec. hence

$$\overline{\beta} = \frac{8 \times 3}{3 \times 3.14 \times (0.05)^3 \times 105} \cong 200$$

considering the graph of the relation between specific viscosity and mean velocity gradient it is clearly seen that for the value of $\bar{\beta}$ about 200 and η sp. below one, specific viscosity is almost independent of mean velocity gradient. It is completely justified therefore to treat specific viscosity as an independent variable in these experiments.

VIII. Disaggregating Effect of the Cations.

The disaggregating action of the cations increases in the following order : $Li < Na < K < Cs < NH_4 < Mg < Ca < Ba$. The activity of these ions changes with concentration. In some cases, e.g. Lithium, the course of the curves is not simple, and at higher concentrations a very strong effect is shown. Without neglecting this fact the standpoint will be taken that the lowest concentrations, approaching ideal behaviour, may be the most suitable for interpretation. Therefore low concentrations will be compared. Hence there is the above sequence of the ions, which at higher concentrations would look differently.

Considering the alkali metals, we see that up to the concentration 0.45 M. of the salt the disaggregating properties of the cations are increasing with tha atomic weight. At higher concentrations the order of activities is reversed. There the results are in accord with experiments of Edsall and Mehl (5).

Cesium ions at concentrations above 0.7M. split quite efficiently myosin particles, as shown by the low viscosity, which is far below the viscosity in presence of ATP.

The precipitation of actomyosin by Rubidium was unpredictable as even Cesium that has a much higher atomic weight does not affect the protein in this way. It is quite reasonable to expect that some impurities of heavy metals in RbCl used in these experiments, were present.

The activity of alkaline earth metals is much higher than of GroupI

metals and also increases with atomic weight. Nevertheless it is warth while to notice that Mg^{++} Ca^{++} and Ba^{++} ions are disaggregating very effectively actomyosin into actin and mysoin, but do not split myosin particles. The increase of the concentration of the salt above 0.2M. affects the viscosity slightly and in the case of Mg^{++} it is noticed even an increase of the specific viscosity. The most reasonable explanation of these phenomena would be that alkaline earth metals at higher concentrations promote precipitation. It was found by Straub (18) that Ca^{++} ions are precipitating actin. To dissolve Ca-precipitate of actin 40-50 times as much K^{+} ions should be added as there are Ca^{++} ions present. Therefore at very low concentrations of Ca^{++} the viscosity was not affected by the formation of a precipitate.

The precipitation of actomyosin with Lanthanum chloride as observed in the experiments can be due to the high valency (three) of the cation.

The disaggregating effect of the cations upon actomyosin increases with atomic weight and with valency.

IX. Disaggregating Effect of the Anions.

The disaggregating effect of the anions increases in the following order $Cl < CNS < Br, NO_3 < J$. It is difficult to estimate the disaggregating properties of $SO_4^{=}$, $PO_4^{\equiv}$ and F^{-} ions as there are effective precipitating agents. To ascertain that the increase of the viscosity of actomyosin in presence of sulfates, phosphates and fluorides is due to precipitation ATP was added. The viscosity of the solution was lowered but not so much as of original solution after adding ATP. At low concentrations the disaggregating properties of sulfates and phosphates but not fluorides are more pronounced than their salting out activity.

The strong precipitating effect of fluoride upon actomyosin can be explained as due to small size of the ion. All other halogens have a strong disaggregating effect that increases with atomic weight.

Bromine and iodine split myosin particles.

X. Disaggregating Effect of Some Organic Reagents.

It was shown by Mommaerts (16) that urea has a pronounced disaggregating activity upon actomyosin. Desintegrating activity of urea upon some proteins was studied before the existence of actomyosin was known. Burk and Greenberg (10) determined that hemoglobin dissolved in 6.66M. urea solution has half the molecular weight in water solution.

The activities of sveral organic reagents were investigated to find the groups disaggregating actomyosin. The main obstacle in these studies was the low solubility of many organic compounds in aqueous solutions. Therefore the number of substances studied is so limited.

The effect of urea upon actomyosin is consistent with the results of Mommaerts (16). By substituting one hydrogen with methyl group in urea (methylurea) the activity of the compound increases. The disaggregating effect is much more pronounced when one hydrogen in urea is substituted with amino group (semicarbazide). The activity of guanidine is much stronger than of urea, as was already noticed by Mommaerts (16). The effect of acetamide is also much stronger than of urea. At higher concentrations acetamide (above 1.2M.) splits very strongly myesin particles.

It is difficult to estimate from these experiments the specific activity of differemt groups. Nevertheless by comparison the activities of urea and acetamide with activities of thiourea and thioacetamide there is seen that sulfur is a very strong disaggregating agent, that affects actomyosin and even myosin.

Succinimide in spite of its ring structure is a strong disaggregating compound.

To study the effect of hydroxyl groups glucose was investigated.

As we see from the results glucose does not affect the chemical bonds of actomyosin.

Two amino-acids glycine and alanine were studied. The disaggregating activity of glycine is very weak. The effect of alanine is more pronounced, but it is worth to notice that at higher concentrations (0.4-0.8M.) alanine promotes aggregation. Increase in viscosity was not due precipitation, as by adding ATP to these solutions the value of specific viscosity was decreased to that of mysoin.

XI. Hydrogen-ion concentration.

In all previous experiments the pH of the solutions was controlled to be in range between 6.5 and 7.0, to exlude the influence of acidity upon viscosity. From table 3 follows, that it was justified to consider the change in viscosity at these particular hydrogen-ion concentrations as to be only due to reagent added.

A special set of experiments was devised to investigate the effect of hydrogen-ion concentration upon the state of aggregation of actomyosin. The pH of the solution was changed by adding 0.5M KOH solution. The concentration of the protein was kept constant. No buffer solution was used to avoid the interference of salts.

At pH 6 and below actomyosin precipitated. From pH 6.4 to 7.4 the viscosity did not change. When the alkalinity of the solution increases the viscosity diminishes. At pH approaching 10 the myosin particles split.

XII. Discussion and Interpretation.

A. Myosin and Actomyosin.

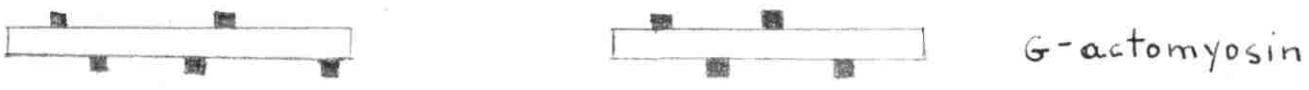
As a basis for the discussion I have to review some facts and problems concerning the actomyosin studied in these experiments. As mentioned before, actomyosin is a complex of two proteins, myosin and actin.

In 0.5M. KCl and at pH values not far from neutrality, myosin is

a well defined protein with a molecular weight of a very definite value and with no pronounced tendency towards intermolecular aggregation (16). Upon addition of actin, the behaviour of myosin changes, the most obvious change being an increase of viscosity and double refraction of flow (DRF). The analysis of these properties showed that these changes are caused by an aggregation of the myosin and actin molecules, mainly or exclusively in longitudinal, end to end arrangement (16) and Mommaerts, Journ of Gen. Physiol. (in press).

A simple explanation of this aggregation would be to assume that myosin and actin fomm a complex system similar to the coacervates studied by Bungenberg de Jong (23). In order to explain the linear character of the aggregation it could be supposed that actin molecules attach themselves at the end of myosin molecules and thus connect them. This explanation is probably not true, as can be concluded from certain experiments of Straub (18). Straub found namely that actin can occur in two forms called by Szent-Györgyi G.- and F-actin. The first form behaves as a globular protein of low viscosity showing no DRF, and is not able to cause the aggregation of myosin although it does actually combine with myosin F-actin, which is formed from G-actin under the influence of salts has an extremely high viscosity and DRF. Apparently it is very anisometric protein, formed from G-actin through longitudinal aggregation. Only this form of actin causes aggregation of myosin.

If actin would form connections between the end surfaces of myosin molecules, there would be no obvious reason why only the F-form is effective. It is more probable that actin associates with myosin molecules in a paralled arrangement and therefore can promote aggregation only in case if its length is of the order of magnitude of myosin. The following schematic drawing illustrates this assumption:



F-actomyosin

This assumption is not sufficiently investigated yet, byt seems at this moment to form the best explanation of the known facts. I do not introduce any arbitrariness into the discussion by the use of above illustrations, since the problem to be analysed is the nature of the forces causing the aggregation. This problem is largely independent of the special type of steric arrangement. The discussion will be restricted to a general nature concerning these aggregative forces.

B. Effect of pH on the Degree of Aggregation of Actomyosin.

In all investigations the degree of aggregation of actomyosin was judged from viscosity measurements. This procedure is based on the insight that the viscosity of the solution containing anisometric particles is a function of the degree of asymmetry of these particles. A lowering in viscosity occurs if disaggregation takes place in such a way that the anisometry of the particles decreases. Apparently this is the case with actomyosin. Orientation leading to DRF depends mainly on the absolute length of the particles. Disaggregation in the case of actomyosin therefore would likewise diminish DRF. This is actually true, but it was not investigated in this work. Observations of this kind with a more restricted number of substances were done by Edsall and Mehl (5), whose results are used for comparison in some cases. In this paper decrease in viscosity is used as a quantitative measure of disaggregation.

As a first application I shall discuss the effect of pH (fig.10) As seem from the graph within a limited range, pH 6.4-7.4, the viscosity does not change. Lower pH values cannot be compared, since the actomyosin begins to precipitate. It is obvious that the state of aggregation is a rather constant property, since it is insensitive

towards a not negligible pH change. If the alkalinity is increased, the actomyosin disaggregates more and more, but the state of disaggregation at every pH is well defined and within not to wide limits the changes in aggregation seem even to be reversible. At still more alkaline reaction disaggreagtion becomes more complete and finally the viscosity may even drop below the level of pure myosin. Such changes due to breakdown of the individual myosin molecules are irreversible and correspond to the irreversible destruction of DRF as found by Muralt and Edsall (2) and Edsall and Mehl (5). Such changes will not be discussed here.

The reproducible decrease of aggregation with increase of pH can be considered as an indication that electrostatic attraction and replusion affect or to a certain extend determine the tendency towards association.

At and above isolectric point (IEP) which pH is 5.3 in case of myosin the carboxyl groups are completely ionized giving negative charges to the molecules. The amino groups are likewise charged at the IEP, but in more alkaline solution the number of positive charges decreases. Apparently a complete pattern of plus and minus charges, existing around the IEP causes strong attraction and thereby insolubility. At higher pH, the number of positive charges and thereby the strength of the electrostatic component of the associating forces is diminished allowing solubility. Increasing pH we pass through a range where the forces of attraction are first still strong enough to keep the actomyosin associated, whereas further this tendency also decreases and disaggregation becomes more and more pronounced. It should be noticed that as positive charges disappear there is not only decrease in attraction by finally also the increasing repulsion between now predominating negative charges.

These considerations show with great probability that electrostatic forces are at least partly responsible for the degree of aggregation

of actomyosin.

C. The Disaggregative Effect of Neutral Salts.

In the experiment[a1] part many examples have been given, showing the disaggregation of actomyosin by neutral salts. It will be discussed now whether these observation support the previous conclusions concerning the significance of aggregative forces.

Whereas in the foregoing introductive remarks it was discussed how electrostatic component of the attraction decreases with increase of pH, by considering the number ofdissociated groups, it is obvious that a more accurate discussion should make use of the activity theory. The effect of added salt upon the activity of inorganic groups shouldbe calculated. In that case the disaggregative effect would be expected to depend on the ionic strength of the added salts. The valency of the ions of the salts used would have a pronounced influence upon the activity.

It is doubtful however whether this procedure is very well applicable to the present case. The concept of ionic strength is useful for the solutions of moderate concentrations, in which the distances between ions are large enough to consider these ions as point-charges without dimensions and specific properties.

That this treatment of the problem is not suitable for my study, is not due to the concentrations of the salts used in this work, because most of the conclusions will preferable be based on the results obtained at high dilutions. The difficulty is rather that the charges of the proteins are not uniformly distributed through the solution but concentrated on the protein molecules. The added ions of the salt will not be homogenously distributed over the available space, but will accumulate around the protein charges. Therefore even at low or moderate concentrations of ions this theory cannot be applied.

It will be preferable therefore to make brief discussion in which the interaction between the ions and the charged groups will be given in a phenomenological way. Considerations of this type were

extensively developed by Bungenberg de Jong (23) and his school. It will be supposed that salts generally act upon charged groups by causing an accumulation of ions of the opposite charge around these groups. Thus their fields are "screened off" from the surrounding so that attractions between them and oppositely charged groups on other protein's molecules are abolished.

First the effect of the alkali halides will be compared. It is found that the efficiency of the alkali metals increases in the order : Li<Na<K<Cs. In this order the ionic radii increases, but the radii of hydrated ions decrease. The effect of water of hydration upon interaction between added ions and ionogenic groups will depend on the affinity of the ion to the ionogenic group and to water. If the polarisability of the ionogenic group is lower than that of water, the ion will retain its water of hydration upon approaching the ionogenic group. If the ionogenic group is more polarisable, the ion may combine with it losing water of hydration.

The carboxyl group in non polar compounds, e.g. fatty acids, is more polarisable than water. However by introduction of polar groups in the neighbourhood of the COO', the polarisability of the latter decreases and becomes less than that of water. This seems to be the case in proteins according to results of Theunissen-van Züp (Ph.D. Thesis, Leiden 1939, oral communication from Dr. Mommaerts). Consequently the cation approaching the COO' group will retain its hydrate water.

It follows from Coulomb's law that the strength of an ionic bound increases, the closer the ions can approach each other. The sizes of hydrated ions should be decisive in the present case and therefore it was predictable that the effect of the alkali metal ions will increase in the order Li<Na<K<Cs, which corresponds to the results of the experiments.

The same regularities are found on comparing the alkaline earth metals. Due to their double valency they are much more active than

the alkali metals.

When we compare the different halides of e.g. potassium, the reasoning is similar, but somewhat more complicated. Obviously these anions interact with the charged amino groups. Here however, not only the size of the hydrated ions is decisive. Anions are much more polarizable than the monovalent or bivalent cations.

Therefore, also the deformability of the anion plays a role and this property changes strongly in the series F Cl Br J. Consequently the differences in the disaggregating action of these anions are expected to be more pronounced than of the cations. This corresponds to the observed results. Actually Fluorine has even an aggregative, precipitating action. The reason for this reversed effect is not clear, but it does not contradict the above argumentation.

The polyvalent ions of sulfates and phosphates have an aggregative, precipitating action. Like in case of fluoride no detailed interpretation will be attempted but these effects may be due to the strong salting out tendency of these ions,.

Summarizing the results obtained with salts, it may be concluded that they are in agreement with predictions that electrostatic attraction is at least partly responsible for the aggregation of actomyosin.

D. The Effect of Urea and other Substances.

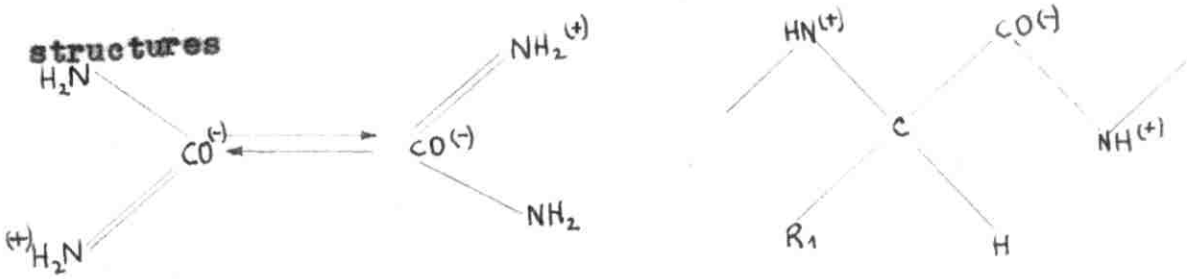
Whereas in the case of electrolytes a detailed explanation was given the results of experiments with organic substances will turn out to be less completely analysable.

The experiments showed that urea, methylurea, acetamide, thiourea, thioacetamide, guanidine HCl, succinimide and semicarbazide HCl had disaggregating effects.

Habiby (M.A. thesis A.U.B., 1947) studied the effect of similar substances on the intermolecular forces in gelatin. From his analysis it follows that these substances with the exception of strong bases like

guanidine react with gelatin in a different way than salts do. A similar study was not made with actomyosin, since here pH could not be varied. We may perhaps, by analogy, assume that also in case of myosin urea and related compounds react with the protein in a different way than salts, do. What is the exact nature of the bounds, which are split by urea and its analogues cannot be easily decided. Theoretically there is some reason for the assumption that they interfere with the H-bounds between the peptide chains.

It is known that urea and many other compounds form resonating structures due to oscillation of electrons. Urea resonates between two structures



Similar resonance exists between peptide chains and therefore it seems quite probable that urea or its analogue introduced into protein solution will interfere with the H-bounds between these peptide chains. If these were true in this simplest form far all proteins, it should be expected that in comparative investigations of different proteins with different disaggregating substances of the urea-group, the order of activities should be always the same. By comparing the results of these investigation with those of Habiby it turns out that no similarity can be found.

With gelatin at pH 8 we found the following series:
thiourea>thioacetaimde>allylurea>urea>methylurea>acetamide.
With actomyosin at pH 7 the following :
thioacetamide>thiourea>acetamide>succinimide>methylurea>urea.

These series are so much different, that it must be concluded that the theory ascribing the action of these substances to their interaction with the CO-NH groups of the peptide chains by the formation of H-bounds,

is unacceptable. Effects of this kind may be involved, but they are strongly overshadowed by other effects, specific for each protein.

No further analysis of this type of bounds will be attempted at this occasion.

XIII. Appendix.

It was found that actomyosin in vitro is disaggregated by many organic and inorganic substances. Although ATP also disaggregates actomyosin there are many differences in their activities.

Szent-Györgyi (19) tried to explain why myosin cannot be extracted from muscles in the absence of ATP. According to him actomyosin in muscles in abscence of ATP is not disaggregated by salt solution. It is worth to notice that salt solutions, that are not extracting myosin from muscle in the abscence of ATP as was found by Banga and Szent-Györgyi and confirmed in our laboratory, have a high disaggregating effect upon actomyosin in vitro. ATP must have a very specific activity upon actomyosin.

An attempt was made to extract muscle with the solution containing 1.0M. urea and 0.5M. KCl in theabsence of ATP. Actomyosin of low content of actin was found in the solution. The amount of protein extracted could not be measured as some of its was denatured and therefore insoluble due to prolonged contact with urea solution. In order to discuss this unexpected result some more experiments should be done. Anyhow it can be concluded, that there are some differences in the activities of urea and inorganic salts.

XIV. Summary.

1) Solutions of actomyosin and myosin at the same concentrations differ in the viscosity.

2) The viscosity of actomyosin is lowered to that of myosin by addition of ATP- a substance present in muscle.

3) The lowering of the viscosity is due to the disaggregation of

actomyosin particles into actin and myosin.

4) Many salts and organic compounds as well as hygrogen-ion concentration affect the state of aggregation of actomyosin.

5) Actomyosin dissolved in 0.5M. KCl at pH 6 and below is precipitated. In the range of pH 6.4-7.4 the viscosity does not change. By increasing the alkalinity of the solution the viscosity decreases until at pH above 9.75 the particles of myosin are desintegrated.

6) The disaggregating activity of cations upon actomyosin increases with atomic weight and valency according to the following order: $Li < Na < K < Cs < NH_4 < Mg < Ca < Ba$.

7) The disaggregating activity of anions increases with atomic weight: $Cl < Br$, $NO_3 < J$.

8) Phosphates, sulfates and fluorides are precipitating actomyosin.

9) The activity of urea and related compounds was investigated. A pronounced disaggregating effect was noticed.

10) It was concluded that for the stability of the actomyosin complex ionic bounds are partly responsible.

The remainder of the attraction is due to a kind of bounds unidentified as yet.

Acknowledgement.

I am very much indebted to Dr. W.F.H.M. Mommaerts for the suggestion of a problem and for his valuable advice in my work.

I wish to express my thanks to Prof. S.E. Kerr, Head of the Biochemistry Department and to Prof. W.A. West, Head of the Chemistry Department for their assistance in facilitating the completion of this work.

At the end five sheets of graphs are included.

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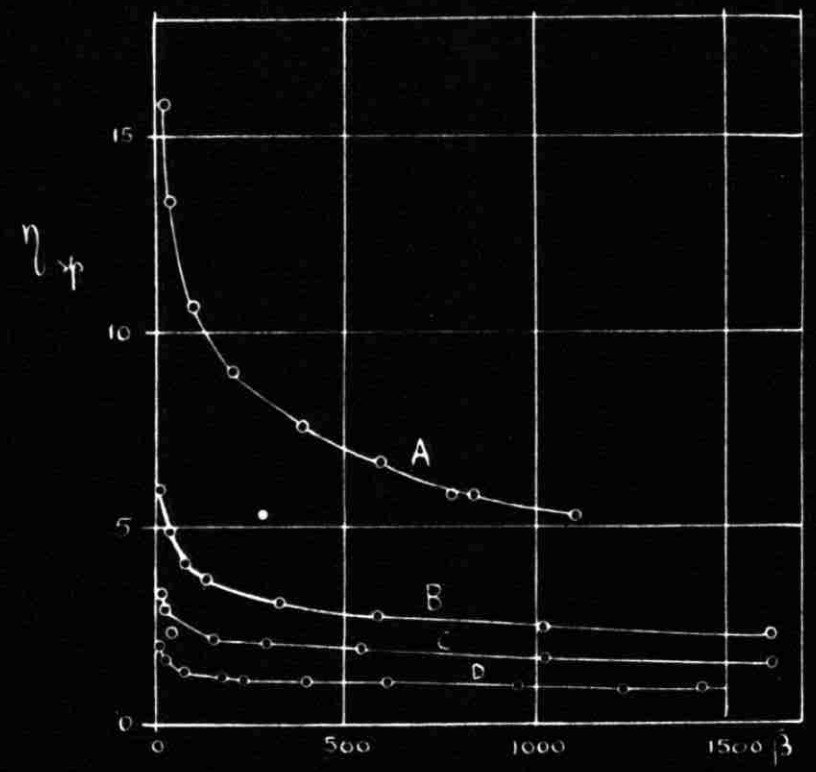
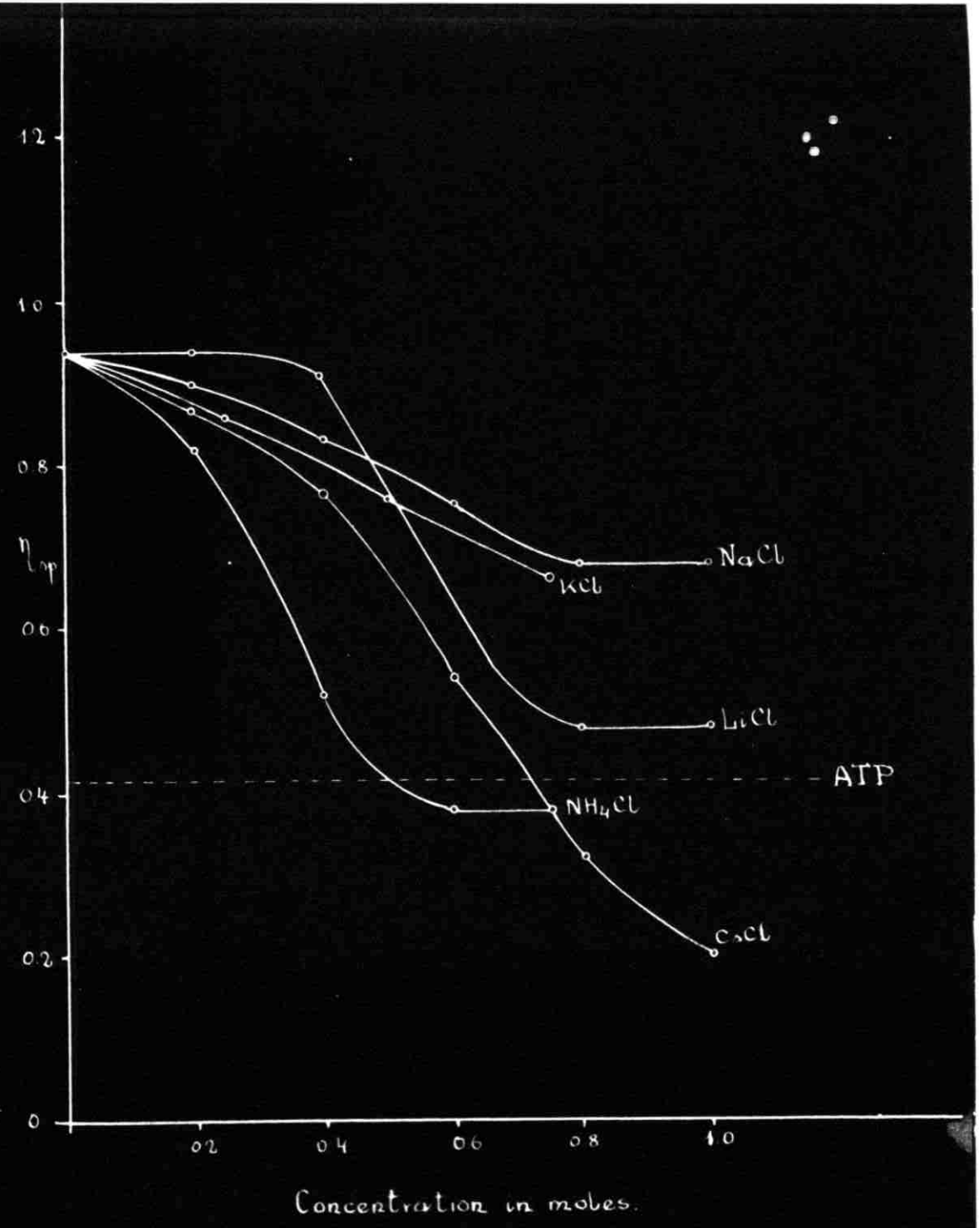


Fig 1. Viscosity of actomyosin solutions as dependent on the velocity gradient. Temperature 21.7°C. Concentrations: curves A,B,C and D:5.1, 3.4, 2.6 and 1.7 mg. per cm³ respectively.

(by Mommaerts) (16)

Graphs



F 2.

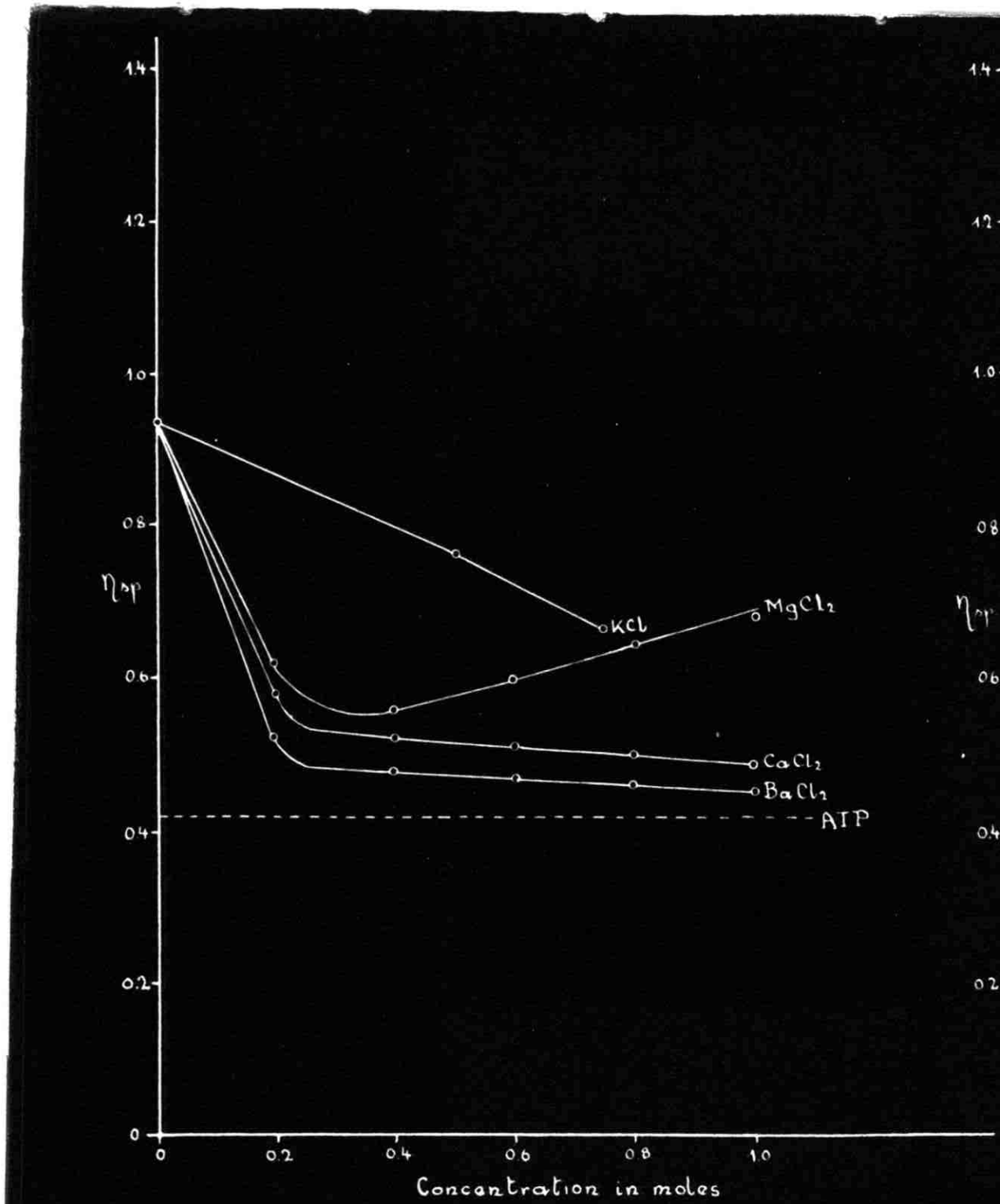

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
η_{sp}
KCl
$MgCl_2$
$CaCl_2$
$BaCl_2$
ATP
0
0.2
0.4
0.6
0.8
1.0
Concantration in moles

Fig. 3.

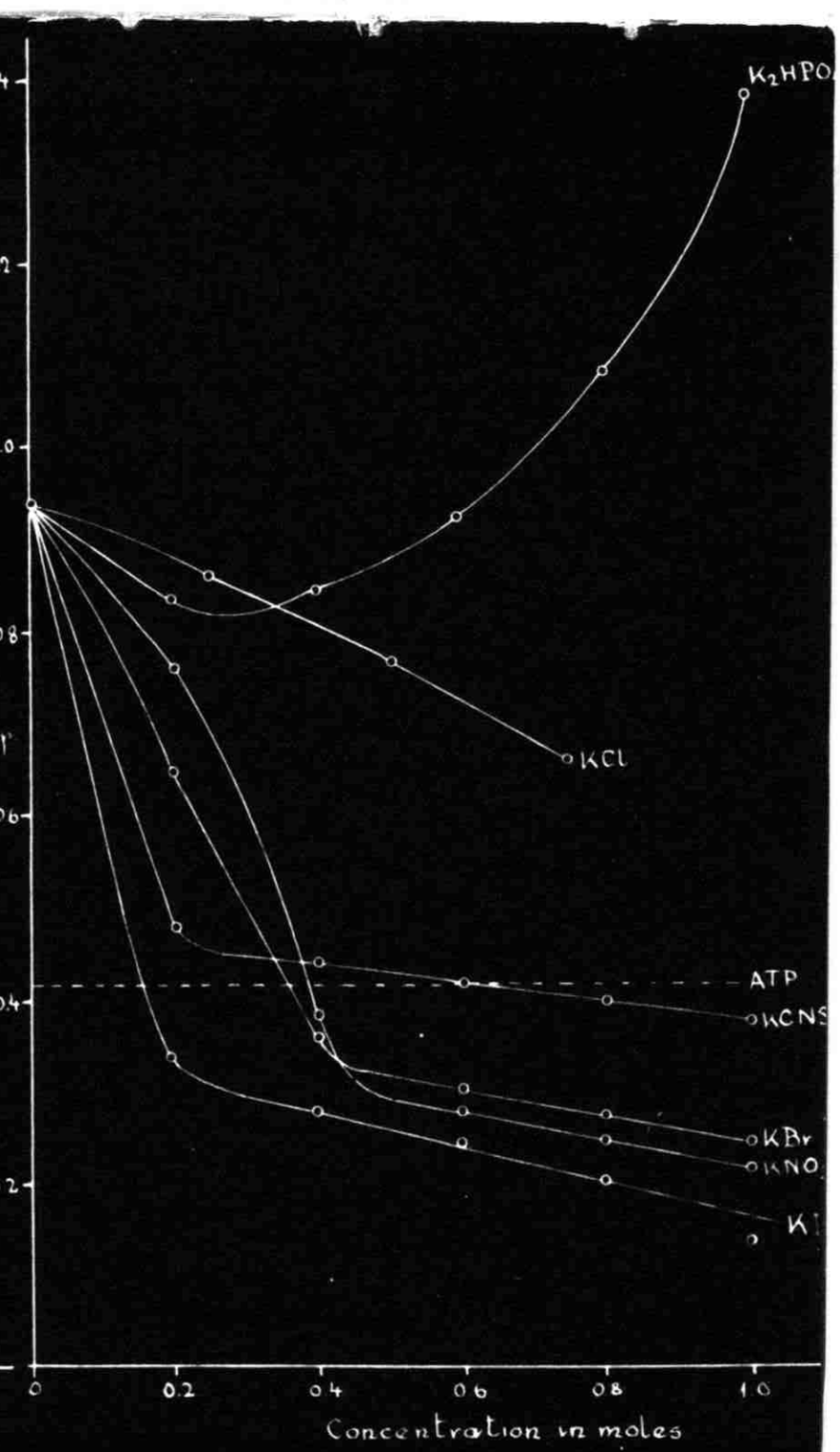

1.4
1.2
1.0
0.8
0.6
0.4
0.2
η_{sp}
K_2HPO_4
KCl
ATP
KCNS
KBr
KNO_3
KI
0
0.2
0.4
0.6
0.8
1.0
Concentration in moles

Fig 4

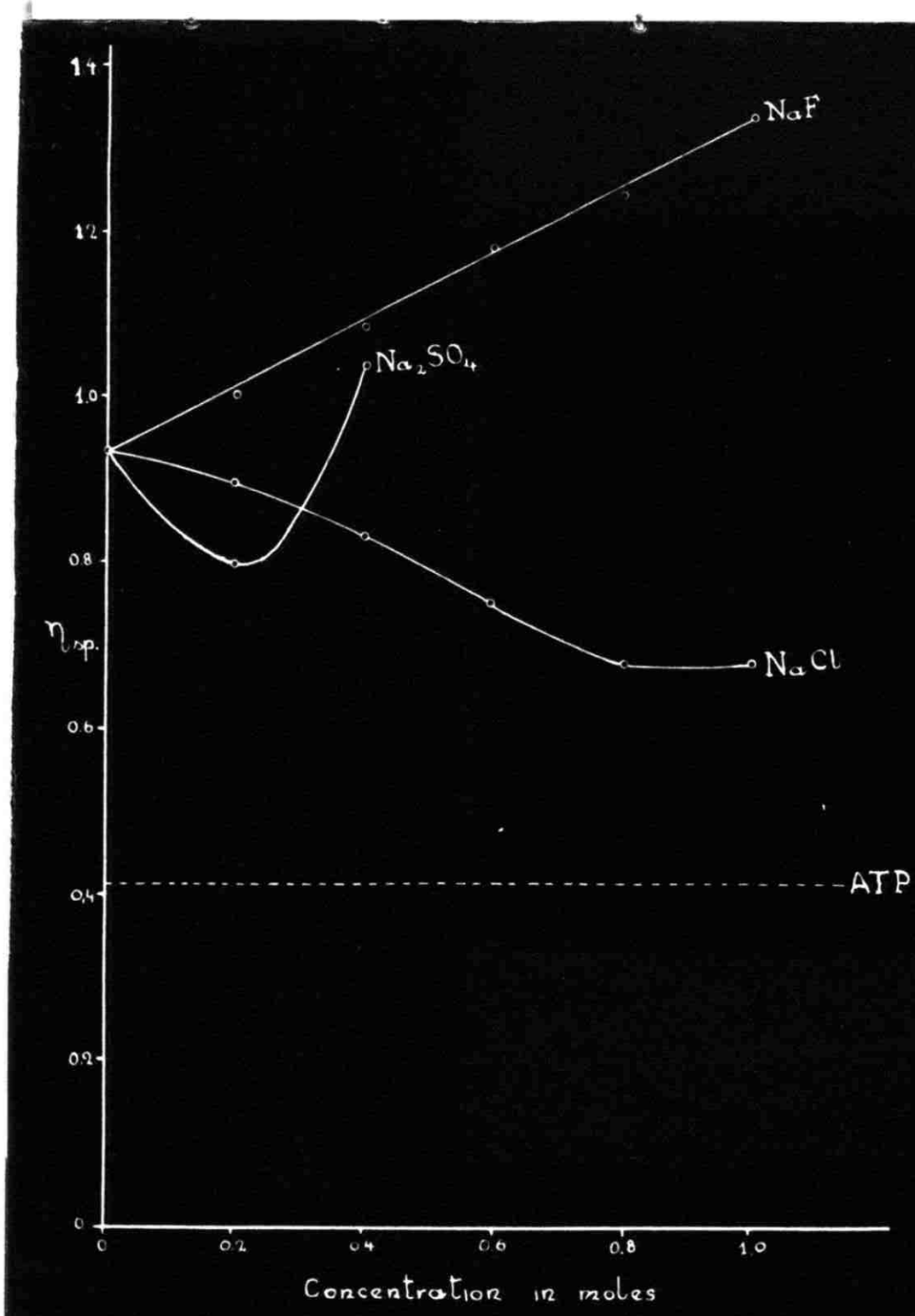

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
$\eta_{sp.}$
NaF
Na_2SO_4
NaCl
ATP
0
0.2
0.4
0.6
0.8
1.0
Concentration in moles

Fig. 5.

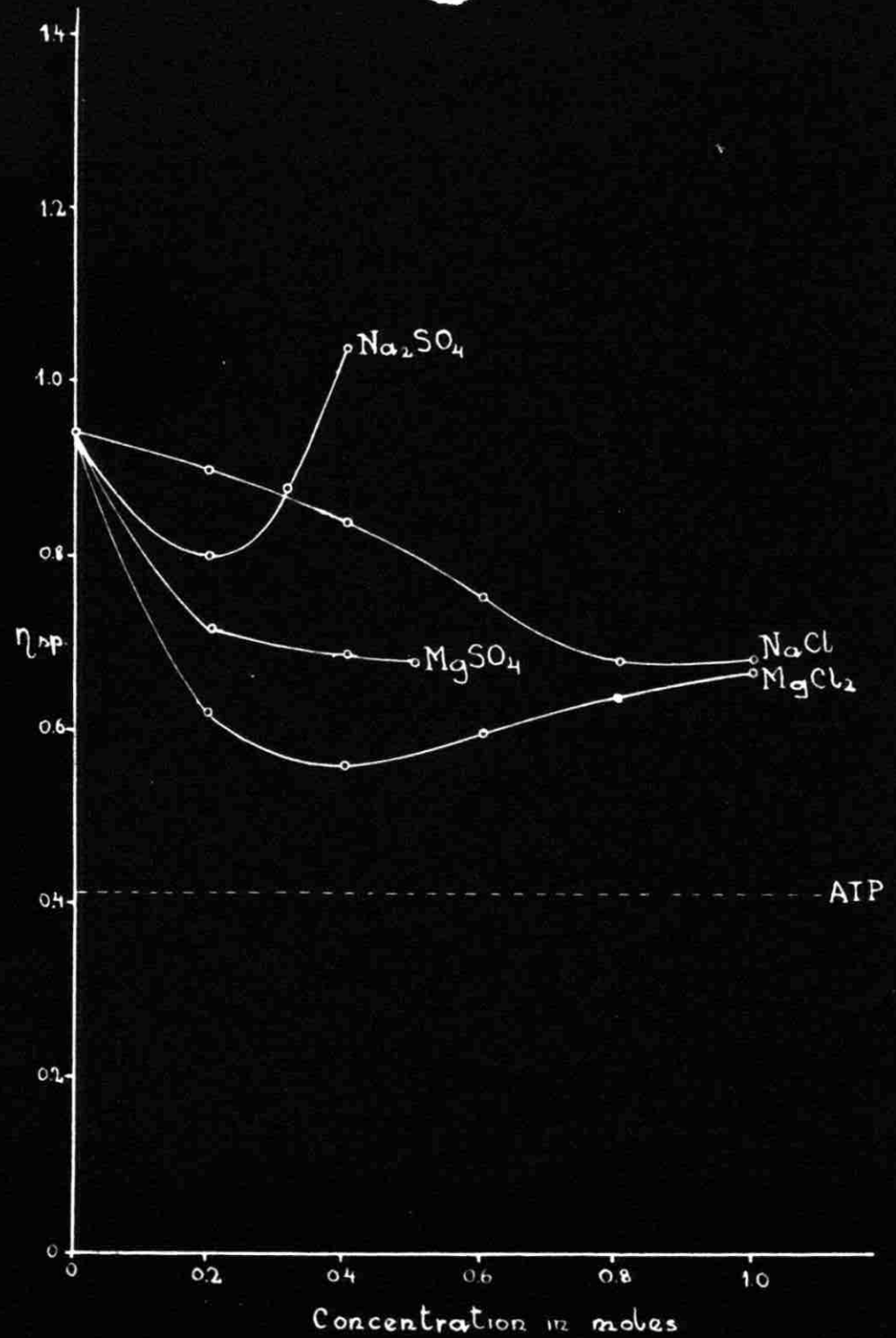

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
$\eta_{sp.}$
Na_2SO_4
$MgSO_4$
NaCl
$MgCl_2$
ATP
0
0.2
0.4
0.6
0.8
1.0
Concentration in moles

Fig. 6.

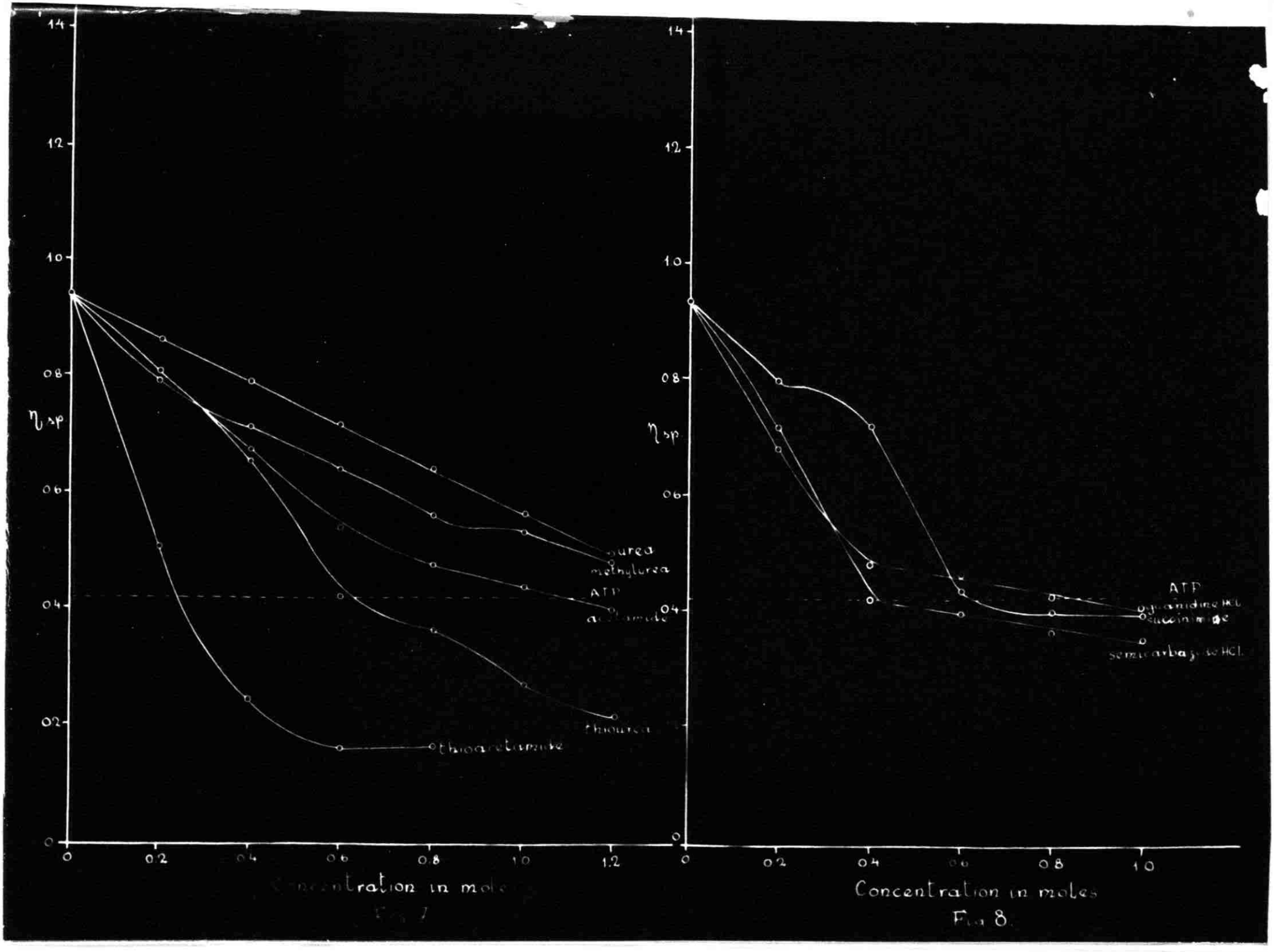
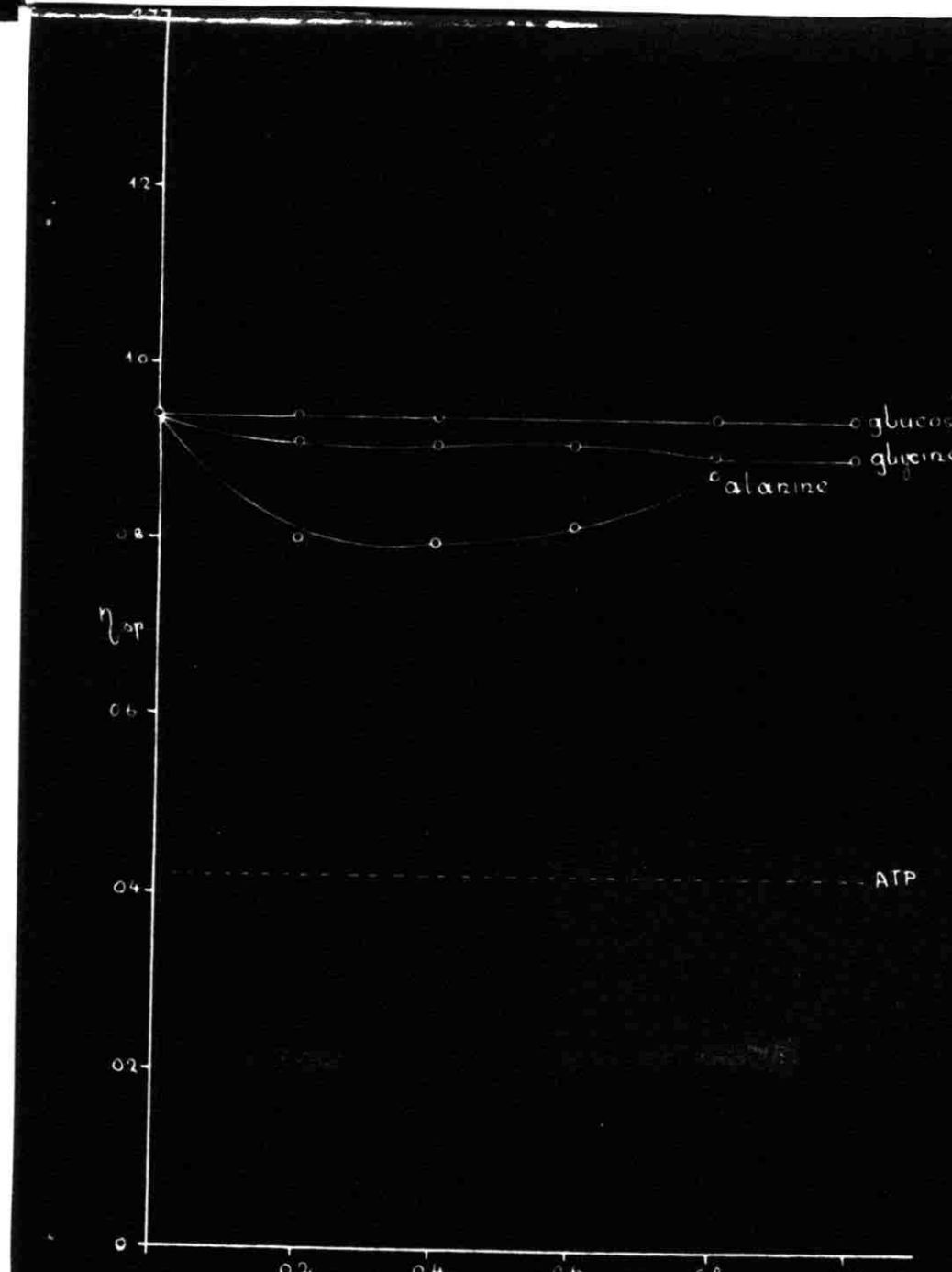

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
η_{sp}
0
0.2
0.4
0.6
0.8
1.0
1.2
Concentration in mole
ATP
thioacetamide
Concentration in moles
ATP
succinimide

Fig 8.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1.0
0.8
0.6
0.4
0.2
0
η_{sp}
glucose
glycine
alanine
ATP
0.2
0.4
0.6
0.8
1.0
Concentration in moles

Fig. 9.

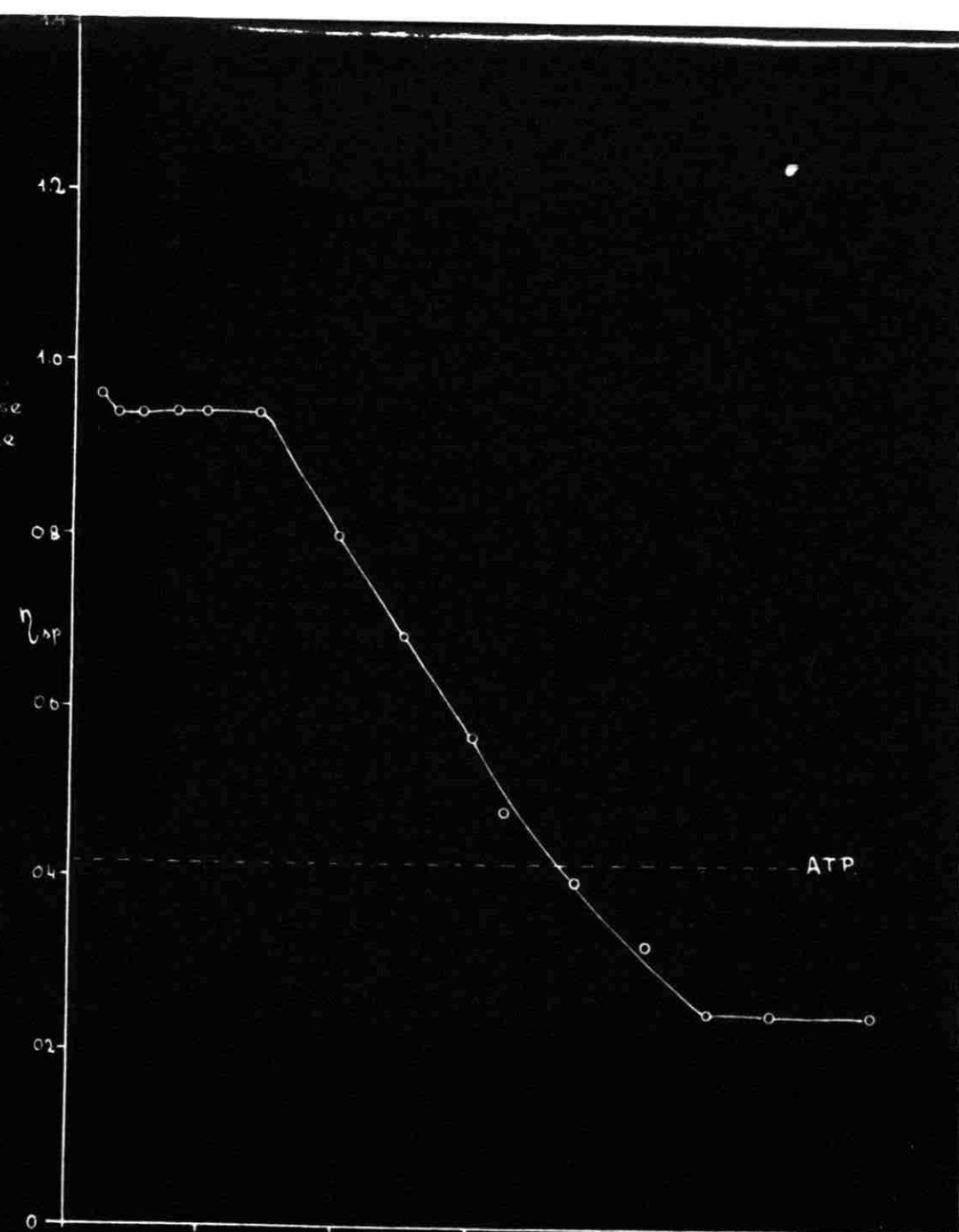
1.2
1.0
0.8
0.6
0.4
0.2
0
η_{sp}
ATP
6.0
7.0
8.0
9.0
10.0
11.0
12.0
pH

Fig 10.